UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Towards the Development of a Mesenchymal Stem/Stromal Cell Product with Enhanced Therapeutic Features for Myocardial Infarction

Diogo de Sousa Pinto

Supervisor: Doctor Cláudia Alexandra Martins Lobato da Silva

Co-supervisor: Doctor António José Calhabrês Fiarresga

Thesis approved in public session to obtain the PhD degree in Bioengineering

Jury final classification: Pass with Distinction

2018

UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Towards the Development of a Mesenchymal Stem/Stromal Cell Product with Enhanced Therapeutic Features for Myocardial Infarction

Diogo de Sousa Pinto

Supervisor: Doctor Cláudia Alexandra Martins Lobato da Silva

Co-supervisor: Doctor António José Calhabrês Fiarresga

Thesis approved in public session to obtain the PhD degree in Bioengineering

Jury final classification: Pass with Distinction

Jury

Chairperson: Doctor Joaquim Manuel Sampaio Cabral, Instituto Superior Técnico da Universidade de Lisboa

Members of the Committee:

Doctor Joaquim Manuel Sampaio Cabral, Instituto Superior Técnico da Universidade de Lisboa

Doctor Paula Maria Marques Leal Sanches Alves, Instituto de Tecnologia Química e Biológica António Xavier da Universidade Nova de Lisboa

Doctor Cláudia Alexandra Martins Lobato da Silva, Instituto Superior Técnico da Universidade de Lisboa

Doctor Maria Margarida Fonseca Rodrigues Diogo, Instituto Superior Técnico da Universidade de Lisboa

Doctor Raquel Madeira Gonçalves, Instituto Nacional de Engenharia Biomédica

Funding Institutions

Fundação para a Ciência e Tecnologia

2018

“Faz tudo como se alguém te contemplasse”

Epicuro (341 a.C. – 270 a.C.)

Resumo

Apesar das células estaminais/estromais do mesênquima (CEM) terem já demonstrado o seu potencial na regeneração do miocárdio em numerosos ensaios pré-clínicos e clínicos no contexto do enfarte agudo do miocárdio (EAM), a análise dos mesmos indica resultados moderados, o que demonstra a necessidade de melhorar a eficácia das CEM. O pré-condicionamento de CEM através da modulação do microambiente tem resultado numa melhoria da taxa de sobrevivência destas células, prolongado o seu efeito terapêutico. No contexto das terapias celulares, são necessárias elevadas doses de células para fins clínicos e o sucesso para obtenção de tais doses celulares depende de protocolos de expansão celular eficientes.

Esta tese tem como objetivo o desenvolvimento de um produto celular de CEM com propriedades terapêuticas aumentadas para a regeneração do miocárdio, através da modulação do microambiente. Três tipos diferentes de fontes de CEM (medula óssea (MO), tecido adiposo (TA) e matriz do cordão umbilical (MCU)) foram cultivas em condições livres de componentes do soro e de origem animal e o seu potencial angiogénico foi analisado. Os resultados indicam que as CEM MCU bem como as do TA expressam e secretam maiores níveis de fatores pró-angiogénicos, comparado com células da MO, o que se traduziu num maior potencial angiogénico *in vitro* por parte das CEM MCU e TA. O pré-condicionamento de CEM MO com meio condicionado proveniente de CEM TA e MCU resultou num aumento do potencial angiogénico das CEM MO. Contrariamente, CEM MCU cultivadas com meio condicionado recolhido das CEM MO induziu um decréscimo no potencial angiogénico das CEM MCU. O pré-condicionamento físico de CEM MCU através do seu cultivo em microtransportadores em condições agitadas e baixa concentração de oxigénio (2%) originou um aumento da secreção de fatores pró-angiogénicos, o que se traduziu num potencial angiogénico superior ao de células cultivadas em estático e em concentrações de oxigénio atmosférico (21%). Além disso, os processos de criopreservação e entrega das células não alterou o perfil de expressão dos genes pró-angiogénicos. Para ser possível produzir tal produto, foi estabelecida uma plataforma de expansão de CEM COM e TA, com recurso a microtransportadores e em condições livre de componentes do soro e de origem animal.

Os resultados obtidos contribuem para o desenvolvimento de um produto celular de CEM com propriedades terapêuticas aumentadas para a regeneração do miocárdio.

Palavras-chave: células estaminais/estromais do mesênquima (CEM), angiogénese, enfarte agudo do miocárdio (EAM), pré-condicionamento celular, expansão *ex vivo*

Abstract

Although mesenchymal stem/stromal cells (MSC) have successfully proved to promote myocardial regeneration in numerous experimental and clinical studies, recent analyses of MSC-based clinical trials in the context of acute myocardial infarction (AMI) demonstrated limited benefits, indicating a need to improve its efficacy. Preconditioning MSC through microenvironment modulation was found to improve MSC survival rate and thus prolong their therapeutic effect. In a cell therapy context, large cell doses are required for clinical purposes and the success in obtaining those cell numbers is dependent on efficient *ex vivo* expansion protocols.

This thesis aims at developing an MSC-based product with enhanced therapeutic features for myocardial regeneration, by modulating the cell microenvironment. Three different MSC sources (bone marrow (BM), adipose tissue (AT), and umbilical cord matrix (UCM)) were cultured under xenogeneic-/serum-free (XF) culture conditions and their angiogenic potential was studied. Results indicated that UCM and AT MSC expressed and secreted higher levels of proangiogenic genes, compared with the BM source, which resulted in a higher *in vitro* angiogenic potential of the UCM and AT sources. Preconditioning BM MSC with conditioned medium (CM) harvested from UCM MSC cultures increased BM MSC angiogenic potential. In contrast, UCM MSC cultured with CM derived from BM MSC resulted in reduced cell angiogenic potential. Physically preconditioning UCM MSC through a microcarrier-based culture platform and low oxygen concentration (2% O_2) promoted an increased secretion of several proangiogenic factors, resulting in a superior *in vitro* angiogenic potential of these cells. Additionally, both cryopreservation and delivery processes preserved the angiogenic gene expression profile of preconditioned UCM MSC. To develop such product, a XF microcarrier-based culture system was successfully established for the expansion of UCM and AT MSC.

These results are expected to contribute towards the development of an MSC-based product with enhanced therapeutic features for myocardial regeneration.

Keywords: mesenchymal stem/stromal cells (MSC), angiogenesis, acute myocardial infarction (AMI), cell preconditioning, *ex vivo* expansion

Acknowledgements

First of all, I would like to say that I will write the acknowledgements in Portuguese, my mother language, because words written in our own language mean a lot more than written in any other language. Also, I want that all of my friends and relatives to understand how important they were to me throughout this 4 years of my life and, for some of them, Portuguese is the only language they know. To all the international people that, at some point, were part of my life in the last 4 years, I will acknowledge them in English.

Antes de mais, gostaria de começar por agradecer ao Professor Joaquim Cabral por me ter recebido e integrado tão entusiasticamente no então Stem Cell Bioengineering and Regenerative Medicine Laboratory (SCBL-RM), agora Stem Cell Engineering Research Group (SCERG). A viagem pelo mundo das células estaminais começou há 5 anos, quando integrei o laboratório para desenvolver a minha tese de mestrado. Parece-me que esta "colaboração" correu bastante bem, pois passado 5 anos, estou no mesmo sítio, a escrever a minha tese de doutoramento. Um grande obrigado, não só por me ter acolhido, mas por sempre me ter dado a liberdade para desenvolver as minhas ideias, colaborar com outros alunos e, com certeza, aprender com os meus erros.

Em segundo lugar, queria, e tenho que, agradecer à minha orientadora Cláudia Lobato da Silva. Uma coisa é certa: não estaria a escrever esta tese se não fosses tu. Na realidade, o teu papel começou em 2012, durante as aulas de Engenharia de Células e Tecidos, onde mostravas a tua paixão e dedicação ao mundo das células estaminais. Não me esquecerei do entusiasmo com que transmitias o teu conhecimento aos alunos e como isso foi crucial para eu entrar no mundo das células estaminais. Muito obrigado por todo o conhecimento que me transmitiste, pela orientação do meu trabalho, pelo espírito crítico que me incutiste e, acima de tudo, pelo espírito de responsabilidade e maturidade que me fizeste desenvolver e que tenho a certeza que farão de mim um profissional melhor, onde quer que esteja.

Gostaria também de agradecer ao Doutor António Fiarresga, por ter aceite ser meu co-orientador, mesmo sendo de uma área, à partida, diferente da sua área de formação. Sei como nem sempre é fácil sairmos da nossa zona de conforto e aceitar novos desafios, mas no fundo é isso que nos move e espero que tenha sido uma experiência positiva. Uma vez mais, o meu obrigado pela orientação e pelo feedback que deu ao meu trabalho, quando assim pôde.

De seguida, gostaria de agradecer à Ana Fernandes por todo o apoio e formação que me foi dando ao longo destes anos de trabalho experimental. Foste fundamental para o conhecimento que adquiri, nomeadamente sobre a cultura de células estaminais do mesênquima em reatores, que muitas vezes é aquilo que nos fascina quando pensamos em bioprocesso e células estaminais. Muito obrigado por todo

o conhecimento que me transmitiste. Mais, agradeço-te pela maneira humilde, sincera e humana como sempre me trataste, dentro e fora do laboratório, a mim e a todos os alunos que eu vi passar no laboratório.

Agradeço, também, às pessoas que fizeram diariamente parte da minha vida, os membros do SCERG. No fundo, foram estas as pessoas com quem partilhei a maior parte do meu tempo destes últimos anos, foram eles que estiverem lá quando acordei contente, ou quando acordei triste, quando as coisas correram bem, ou quando nada parecia resultar. Muito obrigado por todos os momentos que partilhámos, pelas horas de almoço, pelas gargalhadas, pelas histórias, pelas conversas, os conselhos, as repreensões. Obrigado por me terem feito crescer e ser uma pessoa melhor, certamente. Obrigado por todos os momentos dentro e fora do laboratório. Não há nada como chegar ao local de trabalho e sentirmo-nos bem. Um especial obrigado à Cláudia Miranda, ao Carlos Rodrigues e ao Tiago Fernandes, por me terem acompanhado durante estes 4 anos. E ao pessoal mais novo, mas que nem por isso significa menos: Carina Manjua, Ana Rita Gomes, Teresa Silva, André Rodrigues, João Cotovio, André Branco, Sara Bucar, Marília Silva, Mariana Branco, Miguel Fuzeta, Mariana Pina, Ana Potes, João Carreira, Diogo Nogueira, Margarida Alves e Geni. Sei que alguns deles serão amigos para a vida.

Um muito obrigado não chega para agradecer aos Santos B: António Soure, Francisco Moreira, Joana Carmelo, Joana Serra, João Silva, Márcia Mata, Marta Carvalho, Marta Costa, Raquel Cunha e Sara Rosa. Sem eles, esta tese não existiria. Porque se estiveram ao meu lado em muitos bons momentos, também estiveram quando as coisas pareciam não fazer sentido e este parecia não ser o caminho certo. Um enorme obrigado pelos grandes momentos que partilhámos, pelas gargalhadas, pelas conversas, pelos conselhos, pelo que me ensinaram quer no laboratório, quer fora dele, pela força e, acima de tudo, pela vossa genuinidade e por não deixarem de ser quem são. Muito obrigado pelos momentos que partilhámos no laboratório, mas acima de tudo, pelos que partilhámos fora dele. Aos grandes amigos que fiz durante estes 4 anos, o meu muito obrigado, e que este tinha sido só o início!

Now, I'll switch to English, so I can acknowledge some of the people that were part of my experience abroad and that really contributed to this thesis. The first person I must acknowledge is Taby. First, thank you for accepting me as a visiting scholar in your lab, even though you like to have a small number of students, so you can guide them on a week basis. But most of all, I want to thank you for everything that you taught me, from the most basic concept of biomechanics, to analyzing data, planning experiments, interpreting results and, above all, to look at the results from a different perspective. All of that knowledge truly contributed to the success of this thesis.

I would also like to thank to Liana, Julia and Alex for being great lab mates, by helping me in the lab, teaching me new techniques and new protocols and discussing the data with me. But most of all, I want

to acknowledge Lina, one of the sweetest persons I have ever met. Thank you so much for everything that you taught me in the lab, all your patience and enthusiasm, but most of all for being a friend, when I really needed one. For listening to me and helping me deciding what to do. Being abroad was really tough, but you and Fede made it a lot easier. A huge thank to Rosa, for being my "mother abroad". Thank you for taking care of me and for always putting a smile on my face, even when it was hard to have one. Finally, I would like to acknowledge Ted, Tim and Michael for being my housemates for a whole year and showing me how life rocks in New Orleans.

A huge thanks to the MIT Portugal colleagues, with whom I had the privilege to share great moments, not only in Lisbon, but also in many other places where we have been to. All those trips, all those subjects and specially you made the first year of the MIT Portugal program an amazing experience that I'll keep in heart for a long time.

Não poderia deixar de agradecer, também, às pessoas que não estão diretamente relacionadas com o laboratório, mas que fazem parte da minha vida, todos os dias, e que foram essenciais durante estes 4 anos.

Um grande obrigado aos MNTs, António Vieira, João Cardoso, André Ferreira, Susana Ferreira, Fábio Viegas, por serem a minha válvula de escape ao fim-de-semana, por fazerem com que, passado todos estes anos, os trampolins ainda façam sentido. Muito obrigado pelos momentos que partilhámos, por tudo o que aprendi com vocês e por me fazerem restaurar energias para a semana de trabalho.

Um grande obrigado também aos meus amigos trampolineiros, Ana Rente, Gustavo Simões, Rúben Esteves, Mafalda Prazeres, Beatriz Martins, Diogo Rodrigues, Susana Morgado, Tiago Sousa, Paula Pimenta, Tomás Fuzil, por me fazerem sentir que frequentar um ginásio trará benefícios (um dia, eventualmente), mas acima de tudo por me fazerem mudar de ares, por, mesmo que não saibam, fazerem-me esquecer as coisas que correram menos bem e perceber que a vida é para ser levada na desportiva. Por serem modelos, cada um à sua maneira, de como ultrapassar obstáculos e adversidades e ainda assim ter sempre um sorriso na cara. Um grande obrigado!

Um grande obrigado, também, à Inês Guimarães, não só por ser uma das minhas melhores e mais antigas amigas, mas por termos caminhado e crescido juntos neste caminho que é a vida. Por sermos pessoas tão diferentes de quando nos conhecemos, mas sem perdermos a nossa essência. E por me mostrar que nem sempre o caminho é feito de coisas tangíveis, concretas, objetivas, que às vezes só precisamos de acreditar. E isso levar-nos-á à felicidade.

Um grande obrigado aos meus amigos mais antigos, os amigos de Leiria: Filipa Santos, João Silva, Mariana Borges, Mariana Sousa, Bruno Faustino, Manuel Sardinha, Catarina Moreno e Inês Miranda,

por fazerem parte da minha vida há muitos anos. Não só pelos últimos 4 anos da minha vida, mas por todos os outros em que crescemos juntos, muitas vezes em sentidos opostos, mas nunca deixando de fazer parte da vida uns dos outros. Muito obrigado pelo vosso apoio, por estarem lá quando precisei, pelas gargalhadas, pelas saídas, pelos passeios, por me ouvirem, pelos conselhos e, acima de tudo, por fazerem estes 4 anos bem mais fáceis do que seria de esperar. Que venham mais etapas, sempre com vocês por perto.

Um obrigado gigante, à Alice e à Marta, por todos os momentos que partilhámos, dentro e fora da faculdade. Não só por todos os momentos de diversão, companhia, festa e boa disposição que fizeram de nós grandes amigos, mas acima de tudo por me terem ajudado nos momentos mais difíceis, por ainda hoje estarem ao meu lado quando preciso, por me ouvirem como poucas pessoas conseguem. Espero ter-vos por perto por muitos e bons anos.

A ti. Que apesar de já não estares, foste uma peça fundamental para o meu crescimento profissional e, acima de tudo, pessoal, durante os últimos quatro anos da minha vida. Um obrigado não chega para agradecer teres estado ao meu lado, por me teres ouvido quando precisei, por todos os momentos bons e menos bons que partilhámos e que me fizerem crescer, e que também fizeram de mim a pessoa que sou hoje. Ter partilhado parte da minha vida contigo foi uma aventura. Obrigado, do fundo do coração.

Agora, às minhas pessoas. Aos meus 4 pilares. Aquelas que, independentemente do que aconteça e dos anos que passarem, serão sempre a minha inspiração, a minha zona de conforto, o sítio onde vou buscar energias para continuar em frente e com quem posso sempre confiar: Joana, Sara, Isaura e Inês. Cada uma de vocês com um papel diferente na minha vida, mas todos eles cruciais para a minha estabilidade. Sem vocês na minha vida, todo o processo de crescimento teria sido bem mais difícil, não só nestes 4 anos, mas sempre. Um enorme obrigado por fazerem parte da minha vida, por me aceitarem, por cuidarem de mim e por serem as melhores pessoas que conheço. Que o continuem a ser nas próximas etapas da minha vida.

E finalmente, à minha casa. Ao meu lar. Às bases de tudo o que sou e do que consegui alcançar. Ao meu porto seguro, nos bons e nos maus momentos. Às pessoas que me viram nascer, crescer e ser o que sou hoje: a minha família. Toda ela., porque todos foram peças fundamentais para o meu crescimento e para a minha formação. Foram modelos nas diferentes fases da minha vida, com tudo o que de bom e menos bom cada um deles, e todos nós, tem. Um grande, grande obrigado por todo o vosso apoio. Em especial, e acima de tudo, às minhas irmãs Margarida e Inês e aos meus pais, José e Fernanda. São um exemplo de perseverança, de luta e de conquista. Se lutei para estar aqui, hoje, profissionalmente e pessoalmente, foi porque sempre me ensinaram que a luta e os princípios são as nossas armas para conseguirmos alcançar os nossos objetivos. E, acima de tudo, ensinaram-me que essa luta tem que sempre feita tendo

por base o respeito por todas as pessoas que passam pela nossa vida. Do fundo do coração, agradeço-vos todo o vosso apoio. Por último, aos meus avós. Por tomarem conta de mim e da minha bonita família durante tantos anos e por também serem um exemplo de luta, cada um deles à sua maneira. À minha avó Mariana e às minhas duas estrelas avó Lili e avô Chico, dedico a minha tese. Um enorme obrigado.

Index

List of Figures

List of Tables

List of Abbreviations

1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
5-aza	5-azacytidine
αMEM	Alpha modified eagle's medium
ACE	Angiotensin converting enzyme
ALCAM	Activated-leukocyte cell adhesion molecule
AMI	Acute myocardial infarction
ANG-2	Angiopoietin-2
ANOVA	Analysis of variance
AOF	Animal origin-free
ASC	Adult stem cells
AT	Adipose tissue
ATMP	Advanced therapy medicinal products
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMMNC	Bone marrow mononuclear cells
BMP	Bone morphogenic protein
CABG	Coronary artery bypass graft
CCR1	C-C motif chemokine receptor 1
CD	Cluster of differentiation
CFU-f	Colony-forming unit-fibroblasts
CM	Conditioned medium
CQA	Critical quality attributes
CS/PC	Cardiac stem/progenitor cells
CTnI	Cardiac troponin-I
CVD	Cardiovascular diseases
CX3CR1	C-X3-C motif chemokine receptor 1
CXCR4	C-X-C motif chemokine receptor 4
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EBM	Endothelial basal medium
EC	Endothelial cells
ECM	Extracellular matrix
EF	Ejection fraction
EGF	Epidermal growth factor
EGM	Endothelial growth medium
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
eNOS	endothelial nitric oxide synthase
EPC	Endothelial progenitor cells

ESC	Embryonic stem cells
EV	Extracellular vesicles
FB/CS	Fetal bovine/calf serum
FC	Flow cytometry
FDA	Food and drug administration
FI	Fold increase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA binding protein 4
GMEM	Glasgow's modified eagle's medium
GM/CP	Good manufacturing/clinical practices
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor-1-alpha
HLA-DR	Human leukocyte antigen D-related
HMVEC	Human microvascular endothelial cells
hPL	Human platelet lysate
HSC	Hematopoietic stem cells
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
iCP	Cardiac-like progenitors
IDO	Indoleamine 2,3Dioxygenase
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Induced nitric oxide synthase
ISCT	International Society for Cellular Therapy
ISF	Integrated shear factor
IST	Instituto Superior Técnico
LFA-1	Leukocyte function-associated antigen-1
LV	Left ventricular
LVAD	Left ventricular assisted device
LVEF	Left ventricular ejection fraction
MCP-1	Monocyte chemotactic protein-1
MHC I/II	Major Histocompatibility Complex class I/II
MMP	Metalloproteinases
MSC	Mesenchymal stem/stromal cells
mRNA	Messenger ribonucleic acid
MYH6	Myosin heavy chain 6
Neu5Gc	N-glycolylneuraminic acid
NK	Natural killer cells
NKX2.5	NK2 homeobox 5
P(3HB-co-4HB)	3-hydroxybutyrate-co-4-hydroxybutyrate
PAT	Process analytical technology
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PD	Population doublings

PDGF	Platelet-derived growth factor
PDGFR-α	Platelet-derived growth factor receptor alpha
PECAM	Platelet/endotelial cell adhesion molecule
PFA	Paraformaldehyde
PGA	Polyglycolic acid
PGE-2	Prostaglandin E2
PGS	Poly(glycerol sebacate)
PKB	Protein kinase B
PLCL	Poly(L-lactide-co-caprolactone)
PlGF	Placental Growth Factor
PPC	Polypropylene carbonate
PRP	Platelet-rich plasma
PSC	Pluripotent stem cells
PU	Polyurethane
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Real-time, reverse transcription polymerase chain reaction
S/XF	Serum-/xenogeneic-free
SCBL-RM	Stem Cell Bioengineering and Regenerative Medicine Laboratory
SCERG	Stem Cell Engineering Research Group
SD	Standard deviation
SDF-1α	Stromal cell derived factor-1alpha
SEM	Standard error of mean
SFM	Serum-free medium
SM	Skeletal myoblasts
SMC	Smooth muscle cells
STR	Stirred tank reactor
TGF-β	Transforming Growth Factor-beta
TNF	Tumor Necrosis Factor
TNNT2	Cardiac troponin T2
UC	Umbilical cords
UCB	Umbilical cord blood
UCM	Umbilical cord matrix
VCAM	Vascular cell adhesion molecule
VEGF	Vascular Endothelial Growth Factor
VW	Vertical-Wheel™
vWF	von Willebrand factor
XF	Xenogeneic-free

I. Introduction

This chapter is based on the chapter: Pinto, DS, da Silva, CL, Cabral, JMS. Scalable Expansion of Mesenchymal Stem/Stromal Cells in Bioreactors: a Focus on Hydrodynamic Characterization. In submission.

I.1 Cardiovascular Diseases

Cardiovascular diseases (CVD) are a group of disorders that affect the heart and blood vessels [1]. Different types of diseases are categorized as CVD, including: i) coronary heart disease, which is a disease of the blood vessels supplying the heart; ii) cerebrovascular disease, a disease of the blood vessels supplying the brain; iii) peripheral vascular disease, referring to a disease of blood vessels supplying the arms and legs; iv) rheumatic heart disease, characterized by damaged heart muscle and/or heart valves due to rheumatic fever, caused by streptococcal bacteria; v) congenital heart disease, resulting from malformations of the heart structure existing at birth; and vi) deep vein thrombosis and pulmonary embolism, which are the results of blood clots in the leg veins that were dislodge and transported to the heart and lungs [2].

Most types of CVD are caused by a process called atherosclerosis, including coronary heart, cerebrovascular and peripheral vascular diseases, while a minority of them are not originated through this biological process, such as congenital heart disease, rheumatic heart disease, cardiomyopathies, and cardiac arrhythmias [1]. Atherosclerosis is a pathological process in the walls of the blood vessels, characterized by the deposition of fatty material and cholesterol (also known as plaques) inside the lumen of medium- and large-sized vessels [1]. As a result, the inner surface of the blood vessels becomes irregular and the lumen becomes narrow, making it harder for the blood to flow through. The formation of plaques also leads to a decreased flexibility of the blood vessels. Eventually, the plaque can rupture, resulting in the formation of a blood clot in the blood stream. If the clot gets trapped in the coronary artery, it can cause a heart attack; if it develops in the brain, it can cause a stroke [1]. However, the cause of heart attacks and strokes are usually associated with a combination of risk factors, such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol, hypertension, diabetes and hyperlipidemia [2].

CVD represent 30% of global mortality and 10% of the global disease burden, making them a global health problem [3]. In fact, CVD are the leading causes of death worldwide, contributing to a total of 17.3 million deaths per year [1] (Figure II.1). Among them, 7.6 million were due to coronary heart disease, where the most common adverse event is the acute myocardial infarction (AMI).

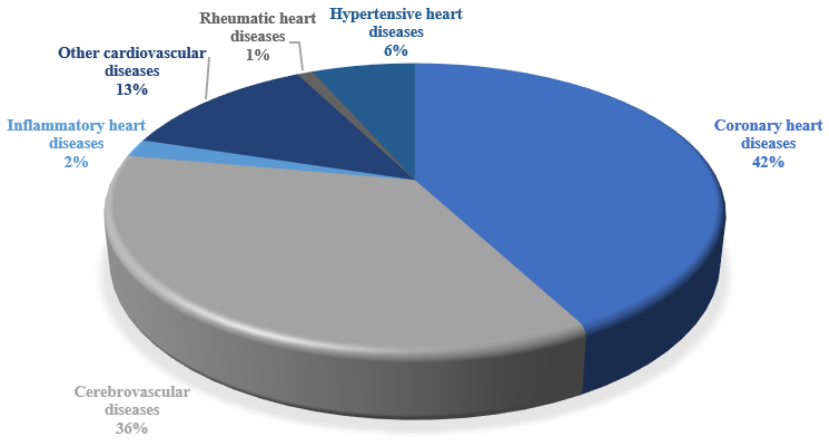

Figure I.1 Distribution of CVD deaths due to coronary heart diseases, cerebrovascular diseases, hypertension heart diseases, rheumatic heart diseases, inflammatory heart diseases, and other CVD [1].

I.1.1 Acute Myocardial Infarction

AMI occurs as a result of myocardial cell necrosis due to sustained and significant ischemia [3]. The ischemic process might be due to either the atherosclerosis process, which involves obstruction to blood flow due to the existence of plaques in the coronary arteries or, much less frequently, to other obstructing agents (e.g. spasm of plaque-free arteries). In a coronary heart disease scenario, the underlying plaques can be stable or unstable. Unstable plaques are related to activated inflammation of the vascular wall at the site of plaques, making them susceptible to erosion, fissuring or even rupture. Platelets, which are activated during inflammatory events, can accumulate at the site of an active plaque, further obstructing blood flow and leading to another coronary heart disease known as unstable angina [3]. Atherosclerotic plaques may expand slowly but more often enlarge with time. Moreover, during the healing process, more layers are added to the plaque, eventually leading to the formation of a stable, fibrotic and calcified plaque [3].

At cellular and molecular levels, AMI triggers numerous events, including apoptosis, necrosis and hypertrophy of cardiomyocytes, tissue fibrosis and inflammation, reduced neovascularization, impaired contractility and pathological remodeling [4]. In fact, scar tissue is formed in the infarct site to maintain the heart's structure while the remaining cardiomyocytes gradually hypertrophy, in an attempt to compensate for those that were lost [5]. The results is the thinning of the myocardial wall and subsequent inadequate ventricular contractions that lead to further dilatation, eventually causing organ failure and death [6].

Various pharmacologic and surgical therapies have proved successful in limiting myocardial remodeling and alleviating some symptoms related to heart failure. A variety of drugs have been shown to reduce

the progression of the disease, including β-blockers, angiotensin converting enzyme (ACE) inhibitors, antiplatelet agents, angiotensin II receptor blockers, statins, among others [7], [8]. Surgery can also be used to revascularize myocardial tissue downstream from a coronary artery occlusion, using a coronary artery bypass graft (CABG). This technique limits the amount of myocardial tissue that is lost during ischemia, statistically improving patients' survival [7]. Despite the demonstrated success of these therapies in improving the damaged heart, severe myocardial infarctions inevitable result in ventricular remodeling, characterized by reduced ejection fraction (EF) and cardiac output. In this scenario, left ventricular assist device (LVAD) or a heart transplant are the only remaining treatment options currently available [7]. LVAD has showed to prolong patients' survival, but problems such as increased risk of thrombosis, gastrointestinal bleeding, infections, and organ failure limit its effectiveness [9]. LVAD are thus being used as an intermediate step before heart transplant, rather than a long-term solution. Consequently, heart transplant is the only therapy that can address tissue loss. However, limited donor supplies, life-lasting use of immunosuppressive drugs, and eventual organ rejection impedes the use of heart transplantation as a viable, widely used cardiac therapy [7].

The main challenge regarding heart failure is its restricted ability for self-regeneration [10]. In this context, cell-based therapies emerged as attractive therapeutic alternatives to repair the damaged heart.

I.2 Cell-Based Therapies in Acute Myocardial Infarction Context

In the last decades, cell-based cardiac therapies have been developed as promising strategies to replace noncontractile fibrous scar tissue after AMI and modulate heart remodeling [11]. In this context, several preclinical and clinical studies have demonstrated the potential of different cell populations in increasing blood flow via neovascularization, modifying scar tissue formation, and eventually improving cardiac function [12], [13]. Although the exact mechanisms of cardiac repair by which transplanted cells exert their therapeutic effect are still poorly understood, two main mechanisms have been hypothesized by researchers: i) direct cardiomyogenic/vasculogenic differentiation, after cell engraftment in the myocardial tissue, and ii) indirect stimulation of the tissue regeneration through paracrine factors, i.e., bioactive factors secreted by transplanted cells that trigger activation of the reparative mechanisms in the damaged tissue [14]. Other mechanisms are now being unveiled and a more detailed discussion of these mechanisms will be performed further.

Several different cell populations have been used to address myocardium damage after AMI, including adult stem cells (ASC), such as skeletal myoblasts (SM), bone marrow mononuclear cells (BMMNC), hematopoietic stem cells (HSC), endothelial progenitor cells (EPC), MSC, cardiopoietic cells, and

cardiac stem/progenitor cells (CS/PC), or pluripotent stem cells (PSC) [11], [15], [16]. The major benefits and limitations of ASC and PSC are represented in Figure I.2.

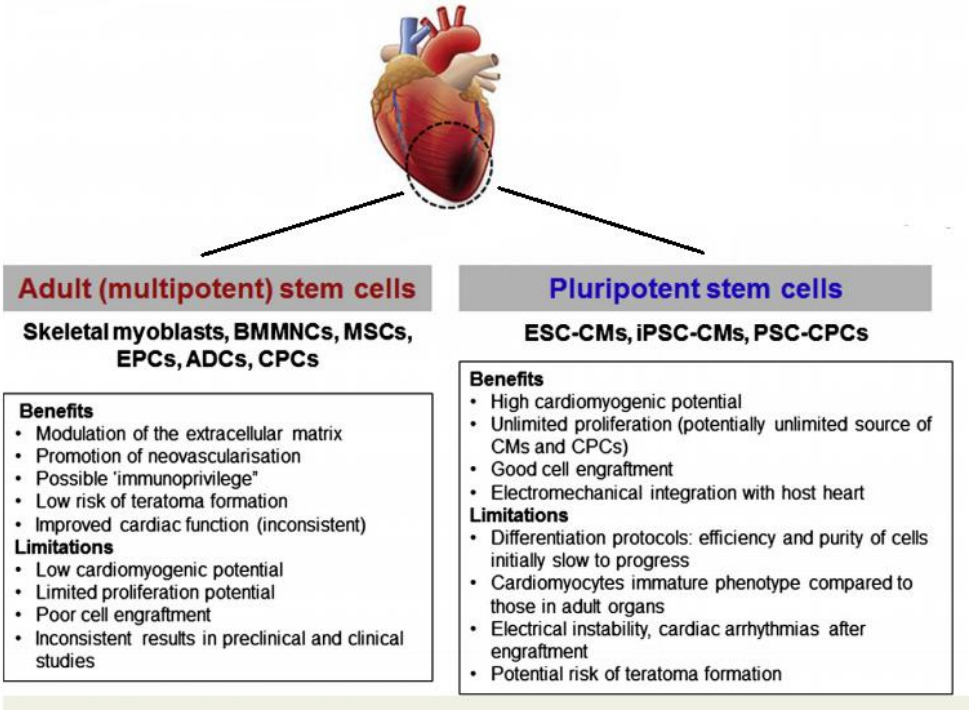

Figure I.2 Major benefits and limitations of using adult stem cells and pluripotent stem cells for heart regeneration and repair (adapted from [17]).

ASC are multipotent cells that were isolated from many fully developed organs. These include populations from both extra-cardiac sources (SM, BMMNC, HSC, EPC, MSC) and from cardiac tissue (CS/PC) [17]. The use of ASC for myocardium repair has demonstrated encouraging (although inconsistent) results in improving cardiac function [18]. Several studies reported ASC ability to modulate the extracellular matrix (ECM) and promote neovascularization through paracrine actions [19]–[21], rather than being from true cardiomyogenic potential. In fact, ASC appear to have little intrinsic cardiomyogenic ability and are unable to generate contractile cardiomyocytes syncytia, despite the positive results regarding cardiomyogenic differentiation *in vitro* [17]. Additionally, inadequate cell survival, retention and homing (i.e. cell migration from the site of delivery to the site of injury) is still observed in ASC therapy.

Unlike ASC, PSC have a tremendous potential to differentiate into cardiomyocytes [22]. Both small and large animal model results show the ability of cardiomyocytes and cardiovascular progenitors derived from PSC to engraft in the infarcted heart [23]–[25]. PSC are capable of limitless proliferation and hence are a potentially unlimited source of cardiomyocytes. Nevertheless, PSC raise some concerns in terms

cardiac differentiation efficiency and teratoma formation. Moreover, some studies have demonstrated electrical instability and cardiac arrhythmias after PSC-derived cardiomyocyte engraftment [17].

MSC, a heterogeneous population of multipotent cells isolated from different human tissues, have been successfully used in the context of AMI, showing promising results under preclinical and clinical studies [26], [27].

I.3 Mesenchymal Stem/Stromal Cells

I.3.1 Mesenchymal Stem/Stromal Cell Definition

Since the discovery of these cells, the definition of MSC has been subject of intense evolution. In 1924, Alexander Maximov used extensive histological findings to identify a singular type of precursor cell within mesenchyme that develops into different types of blood cells [28]. In 1960, scientists McCulloch and Till first revealed the clonal nature of marrow cells [29]. An *ex vivo* assay for examining the clonogenic potential of multipotent marrow cells was later reported in the 1970s by Friedenstein and colleagues [30]. In this assay system, stromal cells were referred to as "colony-forming unit-fibroblasts" (CFU-f) or "marrow stromal fibroblasts". These terms have been gradually abandoned and replaced by diverse, still indistinct denominations like "marrow stromal cells", "mesenchymal stem cells", or "mesenchymal progenitor cells" [31]. To address the inconsistency in nomenclature and account for the biological properties of multipotential, clonogenic, plastic adherent cells derived from various stromal tissues, the committee for the International Society for Cellular Therapy (ISCT) stated that "multipotent mesenchymal stromal cells" (MSC) is the currently recommended designation for multipotent, mesenchymal stromal cells (fibroblast-like, plastic adherent cells, regardless of their tissue of origin) and mesenchymal stem cells (cells that meet specific stem cell criteria) [32]. Chamberlain and co-workers defined mesenchymal stem cells as "nonhematopoietic stromal cells that are capable of differentiating into, and contribute to the regeneration of, mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, and adipose." [33]. Although most denominations reflect a semantic rather than a functional issue, in this work it will be used the term "mesenchymal stem/stromal cells".

It should be noted that clonogenic MSC are a heterogeneous mix of progenitors, in which a subset population is capable of differentiating into cells of mesodermal (adipocytes, osteoblasts, chondrocytes, tenocytes, skeletal myocytes, and visceral stromal cells) [34], as shown in Figure I.3.

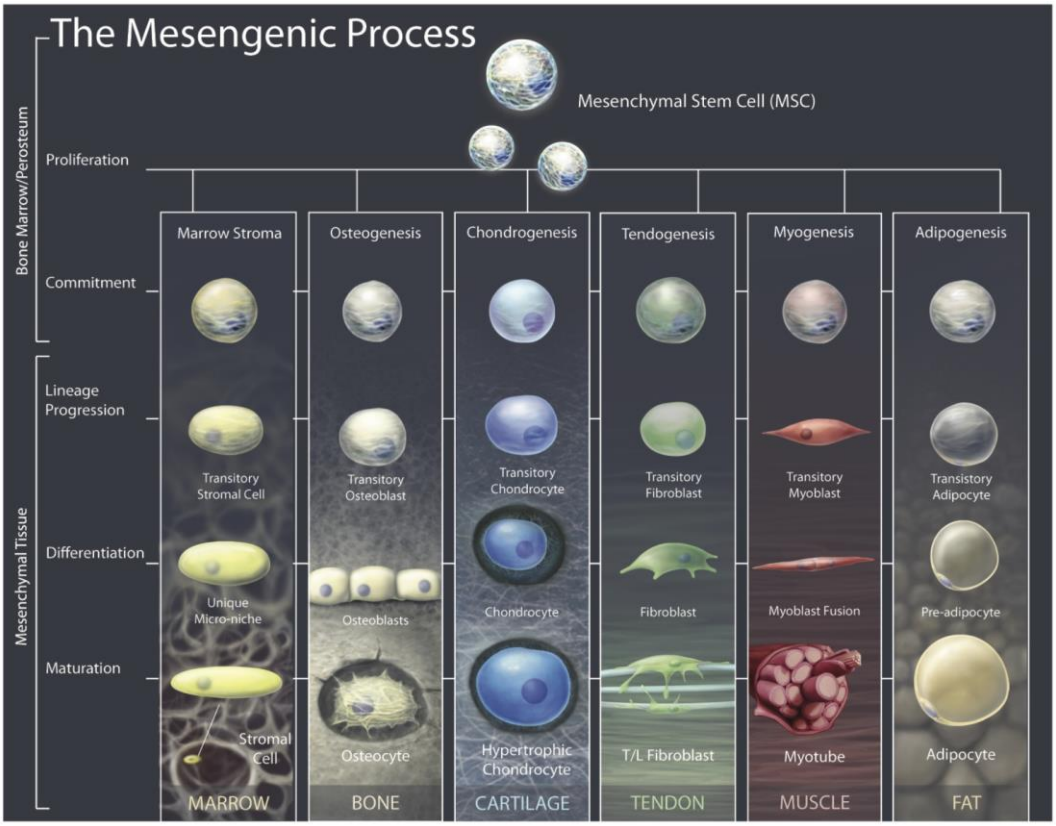

Figure I.3 The mesengenic process. This process depicts mesenchymal progenitor cells entering distinct lineages pathways that contribute to mature tissues [35].

I.3.2 Mesenchymal Stem/Stromal Cell Characteristics

It is first important to mention that the definition of a set of characteristics of MSC is inconsistent among investigators. Many laboratories have developed methods to isolate and expand MSC, which invariably have subtle, and occasionally quite significant, differences. Furthermore, investigators have isolated MSC from a variety of tissues with apparently similar properties [36]. These varied tissue sources and methodologies of cell preparation beg the question about a direct comparison of reported biologic properties and experimental outcomes, especially in the context of cell therapy [32].

To address this problem, the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposes a set of standards to define human MSC for both laboratory-based scientific investigations and for pre-clinical studies. The aim of this position is to define a standard set of criteria to identify MSC, assuming that future research will probably mandate a revision of the criteria as new data emerge. Three parameters are used to describe MSC: 1) adherence to plastic; 2) specific surface antigen expression; 3) multipotent differentiation potential [32].

The first criterion defined by the ISCT is that MSC must present adherence to plastic surfaces when maintained in standard culture conditions using tissue culture flasks. Under these circumstances, most of the cells should acquire a fibroblast-like morphology due to the interactions with the plastic surface [33]. However, cell culture presents a heterogeneous morphology, containing cells ranging from narrow spindle-shaped cells to large polygonal cells and, in confluent conditions, some slightly cuboidal cells [37]. Nevertheless, plastic adherence is a well-described property of MSC, and even unique subsets of MSC that have been described maintain this property [38]. MSC can also be maintained, and possibly expanded, without adherence, although these protocols typically require very specific culture conditions, and these cells, if maintained under standard conditions, would be expected to demonstrate adherence if the cells demonstrate to be a population of MSC [32].

Phenotypically, MSC should express CD105 (endoglin), CD73 (ecto 5' nucleotidase) and CD90 (Thy-1) [32]. MSC are also typically positive for Major Histocompatibility Complex (MHC) class I and Sca-1 [37]. Some other biomarkers have also been reported to be expressed by MSC, but typically their expression is dependent on the tissue source from where the cells were isolated, the method of isolation and culture, and intra-specific variability [37]. In 2007, Chamberlain and co-workers mentioned that MSC do also express CD44 antigen, CD71, CD117, and Stro-1 as well as the adhesion molecules CD106 (vascular cell adhesion molecule [VCAM]-1), CD166 (activated-leukocyte cell adhesion molecule [ALCAM]), intercellular adhesion molecule (ICAM)-1, and CD29 [33].

To avoid MSC misidentification, ISCT recommended that MSC should not express hematopoietic antigens. These markers include CD45 (pan-leukocyte marker), CD34 (primitive hematopoietic progenitors and endothelial cells), CD14 or CD11b (prominently expressed on monocytes and macrophages), and CD79α or CD19 (B cells). HLA-DR (MHC class II surface receptor) molecules should also not be expressed on MSC (unless stimulated), such as CD80 (B7-1), CD86 (B7-2), or CD40 [32]. Other costimulatory molecules not expressed by MSC are the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule [PECAM]-1), CD18 (leukocyte function-associated antigen-1 [LFA-1]), or CD56 (neuronal cell adhesion molecule-1) [33]. The expression of MHC class I and the absence of expression of MHC class II and co-stimulatory molecules by MSC is a prerequisite to possible usage of these cells in clinical applications due to their low or no immunogenicity and no need for immunosuppression before administration [39].

The third criterion defined by ISCT to identify MSC is their ability to trilineage mesenchymal differentiation. Using standard *in vitro* tissue culture-differentiating conditions cells must be shown to differentiate into osteoblasts, adipocytes, and chondroblasts [32].

MSC minimal criteria has been revised to improve the assessment of MSC potency *in vitro* and the clinical outcome of a potential MSC-based therapy. Recently, Samsonraj *et al* purposed additional characterization of MSC identity and potency, including the rate of self-renewal, mRNA expression of *TWIST-1* and *DERMO-1*, and the expression of the cell surface biomarkers STRO-1 and platelet-derived growth factor receptor alpha (PDGFR-α) [40].

Apart from the criteria defined by ISCT and other authors to identify MSC, these cells also present two other interesting characteristics that make them ideal candidates for cell therapy – their immunomodulatory and trophic activities [41]. MSC immunomodulatory properties occur through both cell-cell contact and secretion of a broad range of biologically active molecules, such as growth factors, cytokines and chemokines [41]. When exposed to an inflammatory microenvironment, MSC can regulate many immune effector functions through specific interactions with immune cells that participate in both innate and adaptive responses [42]. The mechanisms of the immunomodulatory effects of MSC include suppression of T cell proliferation, induction of regulatory T cells, influencing dendritic cell maturation and function, suppression of B cell proliferation and terminal differentiation, in addition to immune modulation of other immune cells such as natural killer (NK) cells and macrophages, as shown in Figure I.4. Furthermore, MSC express toll-like receptors, which respond to pathogen-associated molecules to stimulate immune response [43].

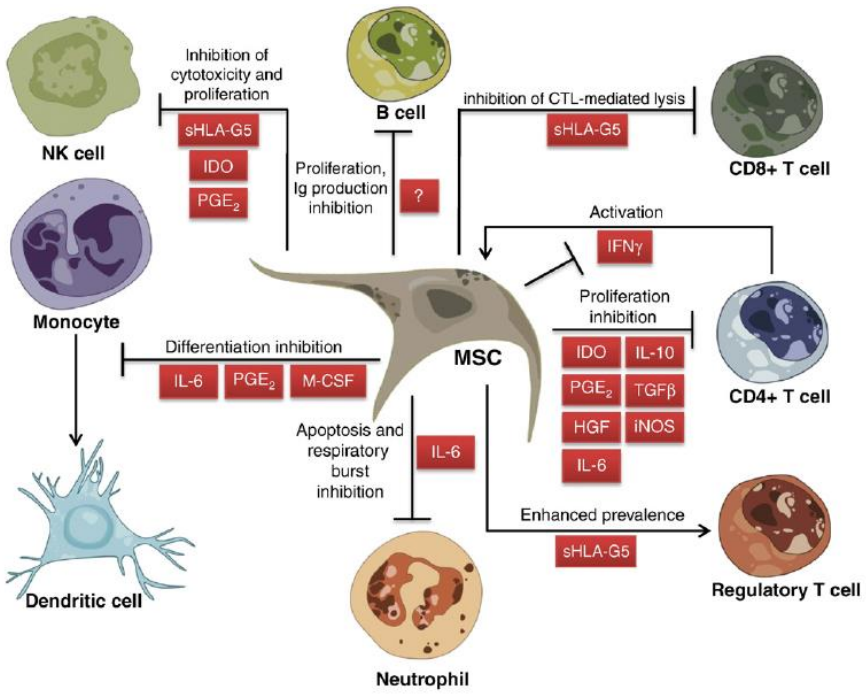

Figure I.4 Possible mechanisms of MSCs immunomodulatory effects on immune cells [39].

The bioactive factors secreted by MSC also mediate trophic mechanisms, whose functions can be divided into four different categories: i) anti-apoptosis; ii) anti-scarring; iii) mitotic (stimulation of

mitosis, proliferation and differentiation of organ-intrinsic precursor or stem cells); and iv) angiogenesis (Figure I.5).

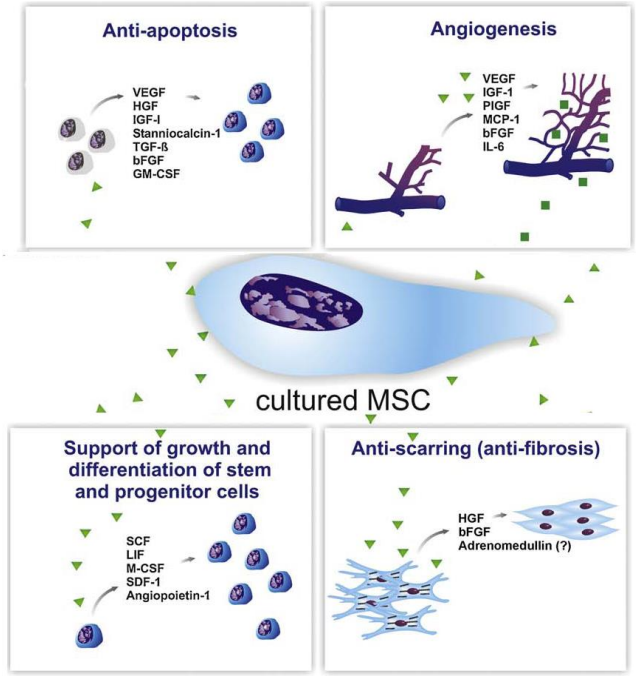

Figure I.5 Trophic activity of cultured MSCs (adapted from [41]).

Some authors also consider the secretion of a variety of chemoattractant molecules, a process called chemoattraction, and the reduction of inflammation by anti-microbial peptides, such as LL-37 [44], as potential therapeutic applications of the paracrine effects of cultured MSC. It is also important to consider that the number of molecules capable of inducing a paracrine action of MSC increases every day and some of them will fit in more than one category [41].

I.3.3 Mesenchymal Stem/Stromal Cell Sources

Using the ISCT minimal criteria, MSC have been isolated from different human adult tissues, including BM (where they were firstly isolated [30]), AT [45], and synovial membrane [46], [47], as well as from neonatal sources, such as UCM [48], umbilical cord blood (UCB) [45], placenta [49], and amniotic fluid [50].

Different MSC sources present different biological features, including cell surface biomarkers, proliferation capacity, differentiation ability, and immunomodulatory and trophic activities [51]. BM MSC are isolated from the MNC fraction of BM aspirates using density-gradient centrifugation. This

harvesting method represents an invasive and painful procedure and it is accompanied by a risk of infection. The MNC fraction contains, among other cell populations, only 0.001 to 0.01% of MSC that can be isolated by two methods: i) plastic adherence or ii) immune-based cell sorting, using different epitopes, such as Stro-1, CD271, SUSD2, MSCA-1, CD13, and CD105 [52], [53]. Plastic adherence is the most common approach to isolate BM MSC, due to its higher cost-effectiveness and cell yields, ease of scaling-up, and reduced risk of contamination [54]. Additionally, cell sorting may cause cell damage and it has not proved to successfully distinguish between multipotent and less potent cells. This method, however, allows the collection of a more homogeneous cell population [54]. BM MSC have proved to differentiate into several types of cells, including chondrocytes, myocytes, osteocytes, adipocytes [55], neurons [56], and hepatocytes [57].

AT MSC are isolated from biological material generated from liposuction, which is a well-tolerated, safer and less invasive procedure than BM aspiration [58]. Moreover, high cell yields can be achieved using a relatively simple isolation protocol consisting of enzymatic digestion of the lipoaspirate with collagenase (about 100,000 MSC per gram of fat) [59]. Despite high similarities with the BM counterpart, some differences were also observed. AT MSC possess higher colony-frequency and a longer culture period without reaching senescence [45]. In addition, MSC derived from the AT do not express CD106 and Stro-1 markers, which are expressed on BM MSC, as well as BMP-2 and dlx5, which are regulators of osteogenic genes. AT MSC were also found to differentiate towards multiple cell lineages, including chondrocytes [60], cardiomyocytes [61], smooth muscle cells [62], endothelial cells [61], and neurons [63]. Nevertheless, the harvesting and isolation methods are also prone to contamination since they consist of open-culture systems [54].

Neonatal sources, such as UCM and UCB, have a significant advantage compare to adult tissues, which is their ready availability since they are considered medical waste and typically discarded after birth, thus avoiding invasive procedures and ethical concerns [51]. UCM MSC also have no accumulated genomic mutations due to disease or aging, compared with adult sources, and a more primitive ontogenic origin, even expressing considerable levels of pluripotency markers [64], [65]. However, the number of MSC isolated from a single UC unit is limited [48].Three different UCM MSC isolation protocols have been described: the enzymatic digestion method, the explant-based culture method and a combination of both protocols [66], [67]. Despite being the most commonly used isolation protocol, the enzymatic digestion presents some drawbacks, such as possible cell damage associated to the exposure to enzymatic agent. The explant-based culture though minimizes cell damage and it is also more cost-effective, compared with the enzymatic digestion [54]. Nonetheless, the overall isolation time is typically higher and associated to lower cell yields [68]. Like the other sources previously mentioned, UCM MSC have the ability to differentiate into adipocytes, osteocytes, chondrocytes, neurons and hepatocytes, although only partially for some lineages [69]–[71].

I.3.4 Mesenchymal Stem/Stromal Cells *Ex Vivo* Expansion

In order to fulfill the potential of MSC as therapeutic agents, large cell numbers and perhaps repeated doses are required, generally in the order of 10^6 to 10^7 cells per kilogram of body weight [72]–[75], depending on the type of disorder. Moreover, human cells that are cultured *ex-vivo* are classed as advanced therapy medicinal products (ATMP) and only 13 MSC-based ATMP are currently approved by the regulatory agencies, partially due to the lack of suitable manufacturing processes [76]. (Table I.1).

Table I.1 Human MSC-based products currently approved by regulatory agencies [77]**.**

Medicinal Product	Company	hMSC source	Indication	Regulatory approval
Allostem	AlloSource	AT MSC	Bone regeneration	FDA
Cartistem	Medipost	UCB MSC	Osteoarthritis	Korea
Grafix	Osiris Therapeutics	BM MSC	Soft tissue defects	FDA
Prochymal	Mesoblast	BM MSC	Graft-versus-host disease	FDA, EMA, Canada and New Zealand
OsteoCel	NuVasive	BM MSC	Spinal bone regeneration	FDA
OvationOS	Osiris Therapeutics	BM MSC	Bone regeneration	FDA
TEMCELL HS	JCR Pharmaceuticals	BM MSC	Graft-versus-host disease	Japan
Trinity Evolution	Orthofix	BM MSC	Bone regeneration	FDA
Trinity ELITE	Orthofix	BM MSC	Bone regeneration	FDA
Hearticellgram-AMI	Pharmicell	BM MSC	Acute myocardial infarction	Korea
Cupistem	Anterogen	AT MSC	Crohn's fistula	Korea
QueenCell	Anterogen	AT MSC	Regeneration of subcutaneous AT	Korea
Ossron	RMS	BM MSC	Bone regeneration	Korea

FDA – Food and Drug Administration; EMA – European Medicines Agency

Therefore, there is need for robust, efficient, and good manufacturing/clinical practice (GM/CP)-compliant processes for the isolation and expansion of MSC, without compromising their therapeutic features. To achieve this, strategies for the clinical grade production of MSC must ensure not only the quality of the final product, but also of the different products at each step and part of the system [78]. In this context, process analytical technology (PAT) is a tool that can be used to control process quality based on online and offline monitoring of different culture parameters [79]. In order to comply with GMP, efforts have been made towards the use of chemically defined, animal-free materials, closed production systems, while performing system validation for each production process, which favors disposable equipment [78]. Scalable culture systems with optimized culture strategies are also prerequisites for the successful transfer of stem cell-based research to clinical settings [80].

I.3.4.1 Critical Culture Parameters and their Impact on Mesenchymal Stem/Stromal Cell Expansion

Appropriate culture conditions are essential for the successful *ex vivo* expansion of MSC. *In vitro* culture conditions aim at mimicking the *in vivo*, highly specialized microenvironment. This microenvironment interacts with several physical and biochemical factors, such as the ECM surrounding the cells and biomolecules affecting growth and differentiation [80]. For the successful *ex vivo* expansion of MSC, these factors have to be modeled by appropriate selection of the biochemical (cell culture medium) and the physicochemical parameters (oxygen concentration, temperature, pH, osmolarity, and culture platform) [80].

I.3.4.1.1 Culture Medium Formulations

Culture medium plays a crucial role on MSC growth and differentiation. Culture medium is typically composed by basal medium comprising salts, glucose (and/or other carbon sources, e.g. glutamine), amino acids and a buffer system [54], [78], and a protein-rich supplement that contains growth and adhesion factors, hormones, and fatty acids required for adhesion and proliferation of MSC [54].

There are different types of basal media commercially available, including Dulbecco's Modified Eagle's Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM), and Minimum Essential Medium Eagle Alpha (αMEM), among others. The only carbon source present in these media is glucose, either at low (1 g/L – αMEM and DMEM-low glucose) or high (4.5 g/L – DMEM-high glucose) concentration. Indeed, high glucose levels have been reported to be either detrimental to MSC [81], inducing senescence by upregulating autophagy mechanisms [82], or having no effect on their proliferative and differentiation abilities [83]. Basal media can be supplemented with L-glutamine, an essential alternative source of energy in cell culture, usually at a concentration of 2 mM [84]. Like glucose, reports on L-glutamine consumption are inconsistent [85]–[87]. Another alternative is the supplementation of culture medium with GlutaMAX™ (containing the di-peptide L-alanyl-L-glutamine), which is more stable and allowed a better MSC proliferation compared with L-glutamine, probably due to a lower ammonia accumulation in culture supernatant [87], [88]. Lactate also plays a critical role on MSC expansion. It is reported that lactate levels above a certain threshold (35.4 mM) inhibit MSC growth by increasing the acidity of the culture medium [87]. Even though not essential, some amino acid are preferentially metabolized by MSC [86], particularly asparagine or tyrosine. Basal media such as Glasgow Minimum Essential Medium (GMEM) are supplemented with non-essential amino acids [89]. Ascorbic acid might also be important for human MSC expansion [90], [91], due to its antioxidant properties, which delay MSC senescence in culture [92]. This vitamin is present in DMEM/F12 and αMEM, but not in DMEM.

The most common type of supplement used for MSC expansion is fetal bovine/calf serum (FB/CS), supplemented in a concentration ranging from 2 – 20% [88]. However, the use of FB/CS in a clinical context carries several disadvantages, including being ill-defined, the wide batch-to-batch variability, and the risk of contamination (with virus and prions) and immunogenicity, which increases the complexity of downstream processing and process standardization [54], [78]. Over the past years, humanized culture medium supplements have been exploited for the isolation and expansion of MSC, such as thrombin-activated platelet rich plasma, pooled human platelet lysate (hPL), and autologous or allogeneic human serum. Despite the positive effects reported for MSC isolation, expansion, and functionality using humanized supplements, extended used for large scale production is discouraged due to limited availability, risk of transmission of human diseases and ill-definition [93]. Replacement of serum- and xenogeneic-derived medium by X/SF formulations is thus of utmost importance. Different groups have successfully promoted the isolation and expansion of MSC using S/XF medium formulations, without compromising cell multilineage differentiation potential [48], [94]–[97]. The ideal candidate should be a chemically defined, serum-free, animal origin-free (AOF, including human) culture medium, which would improve the performance of MSC manufacturing systems both in terms of cell quality and reproducibility [54]. Even though to a lower extent, some groups have reported the efficient expansion of MSC using chemically-defined S/XF formulations [98], [99]. A representative list of the commercially available S/XF formulations used for MSC expansion are reviewed on [78].

Found in supplements or added individually (particularly to chemically-defined S/XF formulations), growth factors are also vital modulators of MSC critical quality attributes (CQA), by regulating their proliferative capacity, differentiation potential, and survival mechanisms [54]. Some factors include basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), PDGF family, transforming growth factor (TGF)-β1, insulin-like growth factor (IGF)-1, and hepatocyte growth factor (HGF) [84], [100]–[102]. In particular, a significant amount of studies have demonstrated that supplementation of culture medium with the potent mitogen bFGF enhances the proliferative and differentiation capacity of BM MSC [102]. However, a more recent study revealed an altered morphology of these cells and increased expression of HLA-DR (i.e. immunogenicity), which may compromise their therapeutic use [88]. The effect of growth factor supplementation on MSC CQA can greatly vary depending on their concentrations and interactions (reviewed in [84]).

I.3.4.1.2 Oxygen Concentration

MSC are traditionally cultured under normoxic oxygen concentration (21% O_2 in ambient air). However, this oxygen concentration might not be the most adequate for the cultivation of MSC, since they are

derived from tissues with a wide range of oxygen tensions (e.g. 1 – 7% in BM and 10 – 15% in AT) [103]. Moreover, researchers found that exposure of MSC to atmospheric oxygen can induce DNA damage, leading to cell senescence and decreased therapeutic efficacy [104]. Therefore, oxygen concentration in culture medium has been studied by comparing standard (21% O_2) to hypoxic (1 – 5% O_2) conditions. Hypoxic conditions have been found to be advantageous for MSC expansion and osteogenic and adipogenic differentiation [105]. Our group has also compared the proliferative and clonogenic potential of BM MSC when cultured at 21% O_2 vs 2% O_2 [106]. We reported that cells expanded under low oxygen concentration present significantly higher proliferative and clonogenic capacities. The same results were reported for AT and UCM MSC cultured under 1 and 5% O_2, respectively [107], [108]. Lavrentieva *et al*. have also demonstrated the improved proliferative potential of different hypoxic MSC populations, compared with normoxic conditions, and the maintenance of their differentiation properties [109]. More important, this group proved that the enhanced growth potential and maintained undifferentiated status of these cells is linked to the oxygen-dependent expression of a particular set of cytokines [110]. Our group has also demonstrated that 2% O_2 leads to an immediate down-regulation of genes involved in DNA repair and damage response pathways, concomitantly with the occurrence of microsatellite instability while maintaining telomere length [111]. In this study, AT MSC were found to react to hypoxic environment more slowly than BM MSC, highlighting the need to optimize oxygen tension according to the cell source. Estrada and co-workers have also verified that MSC expanded under low oxygen concentrations present limited oxidative stress, DNA damage, telomere shortening, and chromosomal abnormalities [104].

Despite the demonstrated enhanced characteristics of MSC cultured under hypoxic conditions, some authors have reported contradictory results [112]. These findings may be due to the duality of cell response facing either short- (also called preconditioning) or long-term exposure to hypoxia [113]. In fact, short-term exposure of MSC to low oxygen concentrations before transplantation is shown to increase their therapeutic capacity [114]–[117]. Nevertheless, oxygen tension plays an important role on MSC metabolism. When MSC are cultivated under hypoxia, they switch their marginal oxidative metabolism towards a complete glycolytic metabolism via the expression of hypoxia-inducible factor-1-alpha (HIF-1-α) [118], [119]. Moreover, Deschepper *et al*. demonstrated that MSC can withstand severe hypoxia, if not combined with glucose depletion [120]. The effects of hypoxic culture on MSC features are well-reviewed by Haque *et al*. [121] and also by Sart *et al* [122], particularly how oxygen tension interacts with glucose levels and its influence on MSC proliferation and differentiation.

I.3.4.1.3 Temperature and pH

Temperature and pH are also known to play an important role on MSC expansion performance. The effect of temperature on MSC cultures have been mainly studied for rodent MSC. Two reports revealed distinct findings regarding the effect of temperature on rat MSC. Stolzing *et al*. observed a lower cell growth rate at a temperature of 32°C, compared with the standard 37°C [123], while Chang and co-workers demonstrated an increased proliferative capacity at a temperature of 33°C [124]. Nonetheless, both studies found that the decreased temperature enhanced MSC differentiation and lowered stress levels (nitric oxide, reactive oxygen species (ROS), and apoptosis).

The optimal range for human MSC expansion has been identified between pH 7.47 and 8.27 [125]. In fact, current strategies to regulate pH in MSC cultures consist in restricting the pH range between 6.8 (lower than this value makes it too acidic for stem cells [126]) and 9 (using a bicarbonate-based buffer [127]). However, depending on the species, alkalinity showed opposite effects on differentiation potential. While a pH over 7.9 inhibited chondro- and osteogenic differentiation in human MSC [125], it enhanced these differentiation potentials in rat MSC [128]. In a different study, human MSC self-renewal capacity and matrix mineralization were found to be affected by pH, in which pH other than the physiological inhibited the deposition of ECM [129]. Contrastingly, pH did not affect MSC osteogenic differentiation since some osteoblast markers were upregulated at both physiological and acidic pH.

More data about the effect of temperature and pH on MSC CQA, including differentiation potential, cytokine release, immune modulation, migration capacity and/or angiogenic potency, as examples [130], is required to establish an efficient and robust protocol for MSC expansion.

I.3.4.1.4 Two-Dimensional vs Three-Dimensional Culture Systems

MSC expansion has traditionally been carried out in planar cultivation systems such as tissue culture flasks or cell factories, for simplicity and easy handling [103]. At a laboratory scale, such systems are cost-effective and easily operable solutions to successfully achieve MSC expansion. However, a considerable high number of flasks would be required to attain clinical-relevant cell numbers. The whole process would be highly time consuming, labor intensive, and would increase the risk of contamination. To overcome these issues, companies have developed static culture system platforms as, for instance, the multi-layered cell factories, as Nunc®'s cell factory and Corning®'s Cell Stack and HYPER-Stack, with increased size and number of polystyrene-treated stacks (1 – 120 layers) [54]. Nonetheless, planar systems are limited by the low surface area-to-volume ratio and lack the ability to monitor and control culture parameters, which most likely results in variability regarding cell numbers and quality [54]. To

overcome these limitations, three-dimensional (3D) culture systems have been developed and optimized for the culture of MSC, including microcarriers, scaffolds or spheroids [54].

MSC cultivation as 3D spheroids emerged from the fact that the 2D culture systems represent an artificial environment, different from the 3D *in vivo* niche. Conversely, 3D culture systems would possibly mimic more accurately the *in vivo* environment of the cells [131]. Thus, efforts have been made towards the development of 3D culture systems using different protocols, including the hanging drop protocol [132] or the AggreWell™ system [133]. Despite their ability to successfully induce MSC aggregation, these platforms are not suitable for the expansion of MSC. To date, there are no reports demonstrating the efficient expansion of MSC cultured as 3D spheroids. However, spheroid culture has been shown to enhance MSC immunomodulatory and regenerative properties [132], [134]–[136], which will be discussed later in detail.

Since there are no protocols reporting the expansion of MSC cultured as spheroids, the development of a more efficient and robust culture platform is of the utmost importance. An efficient approach is based on the use of microcarriers.

I.3.4.1.5 Microcarrier-Based Culture Systems

Microcarriers are small particles made of distinct core materials and presenting different densities, diameters, and surface charges. They are produced from a variety of materials, including polystyrene, glass, gelatin, dextran, alginate or cellulose, and present different physical properties, like stiffness and nanotopography. The majority of the commercially available microcarriers are spherical, but cylindrical (DE53, Whatman) and disc-shaped (Fibra-Cel®, Eppendorf) microcarriers are also available [103]. Depending on their porosity, microcarriers are divided into nonporous, microporous (pore sizes < 1 µm), and macroporous (pore sizes between 10 – 50 µm) types [80]. While for nonporous and microporous microcarriers, the cells attach to and proliferate on the surface of the bead, for macroporous microcarriers the cells grow inside and around the pores. In general, microcarriers were developed as physical support for anchorage-dependent cell culture in suspension systems, providing large surface to volume ratio and facilitating higher density cultures [103]. Additionally, microcarrier-based culture systems overcome some of the issues related to static cultures, such as large volumes of medium required, inefficient gas transfer, existence of oxygen and nutrient concentration gradients, and inadequate monitoring and control [137].

Microcarriers can be functionalized with peptides or ECM proteins, such as fibronectin, collagen, and laminin, to enhance MSC adhesion and proliferation [103]. AOF, synthetic proteins are preferable for

microcarrier functionalization, to avoid contamination with xenogeneic agents and improve process reproducibility. Additionally, components from the culture supplement (i.e. FB/CS and hPL) such as ECM proteins, also nonspecifically bind to the microcarrier surface. This is the reason why an ECM-based protein coating is even more critical when MSC are cultured in SF culture media. The properties of the surface define which ECM proteins will bind to it, and thus the selection of an appropriate microcarrier/protein coating combination is crucial for optimal MSC adhesion and proliferation. For instance, positively charged microcarriers will selectively bind albumin, whereas gelatin-based or coated microcarriers will preferentially bind to fibronectin [138].

MSC from multiple sources have been successfully expanded under S/XF culture conditions using microcarrier-based systems, including cells from BM, AT, UCM, and synovium [47], [94], [139]. However, the source and isolation method by which MSC were obtained can affect their proliferative capacity on the microcarriers. Indeed, distinctive MSC sources express different adhesion molecules and thus the choice of microcarrier/coating solution is highly dependent on the MSC population to be expanded [54].

Despite the demonstrated efficiency, additional data is required on the impact of different microcarrier/coating combinations on MSC CQA. Moreover, cell harvesting from the microcarriers may also raise several challenges regarding MSC properties, especially when MSC are cultured in macro-porous microcarriers. Another key aspect in microcarrier-based culture is the ability of the selected culture systems to support the transfer of the cells from one microcarrier to another, a process known as "bead-to-bead transfer" [140]. This mechanism allows the addition of fresh microcarriers to ongoing cultures and thus providing additional surface area for cells to proliferate [54]. Furthermore, it represents an advantage over conventional planar culture systems since there is no need of cell passaging and sub-culturing to increase the surface area available for cell growth. This reduces culture handling, costs associated with the process, and minimizes the use of enzymatic reagents which can alter cell properties [54].

I.3.4.2 Bioreactor Configurations and Operation

Microcarrier-based culture offers a reproducible, cost-efficient, scalable, and automated platform to successfully achieve the production of MSC in a dynamic 3D system, because they allow controlled inoculation, expansion, and cell harvest in closed bioreactor systems [141]. Such systems provide online monitoring to ensure quality throughout the process, e.g. cell density, medium properties (pH, temperature, dissolved oxygen (DO) and CO_2), and metabolite concentration such as glucose, lactate, ammonia, and glutamine [78]. Some considerations need to be taken into account when operating a

bioreactor system, including: achieving a high surface-volume ratio; sterility must be guaranteed by minimizing the contamination risk, where single-use equipment represent the ideal choice for complying with GMP; low-shear oxygenation by internal or external aeration; and a homogeneous nutrient supply [78]. Important to notice is the difference between the concepts of "scale-up" and "scale-out": while the first one means increasing the size of a single unit, the second means adding more units. The last presents several disadvantages, including increased associated costs and labor and time consuming.

Several bioreactor types have been reported for the expansion of human MSC (Figure I.6). The most commonly used is the stirred tank reactor (STR) [142]–[145]. Other available bioreactor configurations were also used for the efficient expansion of MSC, including wave-mixed bioreactors [146], [147], fixed/packed bed bioreactors [148], [149], hollow fiber bioreactors [150]–[152], parallel plate bioreactors [153], rotating bed bioreactors [154]–[156], and the innovative Vertical-Wheel™ technology [157]. Once the bioreactor configuration is chosen, the cultivation procedure and operation mode must be defined. The operation mode strongly affects the process parameters, which must be adapted to achieve the product specifications [78]. Bioreactors can operate under: batch (*i.e.* medium is not replaced), fed-batch (i.e. with intermittent/continuous addition of fresh medium), or perfusion (i.e. continuous medium replacement). Both fed-batch or perfusion modes can ensure that key nutrients are replenished and metabolic waste products are kept under inhibitory levels [158]. Importantly, paracrine and autocrine growth factors produced by MSC have also been shown to have a determinant role in their successful expansion [159]. Hence, the beneficial paracrine and autocrine factors cannot be completely removed or even excessively diluted, affecting MSC proliferation.

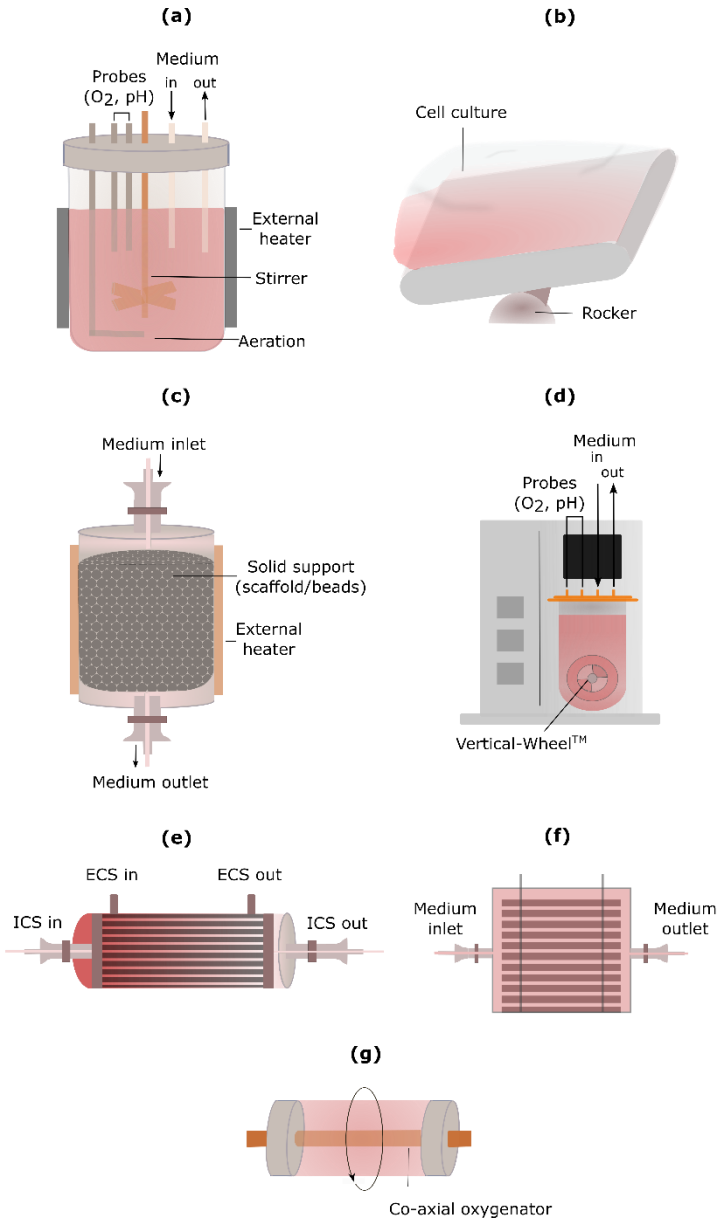

Figure I.6 Different bioreactor configurations tested for mesenchymal stem/stromal cell expansion: (a) stirred tank reactor, (b) wave-mixed bioreactor, (c) fixed/packed bed bioreactor, (d) Vertical-Wheel™ bioreactor, (e) hollow fiber bioreactor, (f) parallel plate bioreactor, (g) rotating bed bioreactor.

I.3.4.2.1 Stirred Tank Reactors

Most studies regarding MSC expansion in stirred conditions have been performed using spinner flasks [94], [95], [139], [160], [161]. These systems are however difficult to automate and control, since the only process control options are pH and oxygen concentration monitoring [78]. Thus, MSC expansion should be performed in fully controlled stirred reactor systems, which allow for a more complete optimization of the culture parameters.

STR consist of a simple vessel that have a centrally located impeller, which agitates the inoculum inside the vessel and provides relatively uniform conditions throughout the medium [158]. There are some STR commercially available at different volumes, such as DASGIP® Parallel Bioreactor Systems, CelliGen® Bioreactors, BIOSTAT® B, BioFlo®, PADReactor®, to name a few. At benchtop scale, our group reported BM and AT MSC expansion in a BioFlo® 1.3 L bioreactor (800 mL working volume), to maximum cell yields of 1.1 x 10^8 and 4.5 x 10^7 cells (1.3 x 10^5 and 0.6 x 10^5 cell/mL), respectively [142]. Both MSC sources were cultured in S/XF conditions, with 25% culture medium renewal every day or every 2 days. Interestingly, BM MSC were also expanded in a perfusion system leading to a higher cell yield of 2 x 10^8 cells (5 x 10^5 cell/mL), compared with the fed-batch regimen with concentrated feeds. These results evidence the importance of providing fresh nutrients/growth factors and removing toxic metabolic products. In two other studies carried out under XF conditions, BM MSC were expanded in 0.25 L BIOSTAT® QPlus and 2 L UniVessel® SU STR to final cell concentrations of 2.8 x 10^5 and 3.4 x 10^5 cell/mL, respectively [143], [157], using MesenCult™-XF culture medium. In MSC production processes with serum-containing media, cells were efficiently expanded in the Mobius® Cell Ready 3 L bioreactor [162], [163], the BIOSTAT® UniVessel 2 L SU [164], [165], and the BIOSTAT® B Plus 5 L bioreactor [144], among others.

The expansion of MSC in stirred bioreactors at an industrial scale was carried out by Schirmaier and colleagues [165], using a BIOSTAT® CultiBag STR 50 L. Culture was performed with 35 L working volume of FBS-supplemented medium, while realizing a partial medium exchange on day 4. Growth on polystyrene microcarriers allowed 3 x 10^8 cells to be harvested on cultivation day 9. Recently, Lawson and colleagues developed an efficient process for MSC expansion in a Mobius® 50 L bioreactor, using hPL-supplemented medium [145]. Different culture parameters were optimized using lab-scale bioreactors, including agitation rate, pH, DO, and medium formulation, resulting in a 43-fold expansion of harvested MSC after 11 days of culture in the 50 L bioreactor.

I.3.4.2.2 Wave-Mixed Bioreactors

Wave-mixed bioreactors consist of a "pillow-like" culture bag, which relies on rocking motion of the platform to create a wave for mixing and bubble-free aeration [103]. The rocking angle and rate must be carefully set up to meet the required culture homogenization and gas transfer through the headspace of the bag. Wave-mixed bioreactors also allow for temperature, pH, and DO monitor and control [80].

The first attempt for MSC expansion using a wave-mixed bioreactor was performed by Akerström [146], in 2009. The author reported the feasibility of expanding MSC in a 2 L Wave Bioreactor™ 2/10 on Cytodex-3 (gelatin-coated dextran) and Cultispher-S (macroporous gelatin) microcarriers. A fold

increase in cell number of around 6 was determined after an 18-day expansion protocol, with carrier feeding on day 11 and 13. More recently, Timmins and co-workers reported the expansion of placental-derived MSC to a fold expansion of around 16, in a Wave Bioreactor™ system using macroporous gelatin microcarriers [147].

I.3.4.2.3 Fixed/Packed Bed Bioreactors

Fixed or packed bed bioreactors consist of an immobilized matrix of particles densely packed enclosed in a cylindrical vessel, where the cells are then seeded [54]. Several types of particles have been used, including macroporous microcarriers, porous ceramic beads, porous glass beads, glass fibers, polyester discs, alginate beads and hydrogels [80]. In these bioreactor configurations, the culture medium is perfused through the bed, providing nutrients to the cells while removing toxic metabolic products. Medium can be supplied through either an external medium reservoir or circulation in an internal loop. However, medium perfusion throughout the bed may generate concentration gradients [166].

Fixed-bed bioreactors have been successfully employed for the expansion of MSC. One such example was reported by Mizukami and colleagues [148] where umbilical cord blood-derived MSC were expanded in a packed-bed bioreactor consisting of immobilized Fibra®-Cel disks in a disposable sterile bottle. Starting with an inoculum of 6 x 10^7 cells, it was possible to produce 4.2 x 10^8 cells, representing a fold increase of 7. However, after using TrypLE™ as dissociation agent, only 18% of the cells were retrieved, highlighting the need to optimize this system in terms of downstream processing. Placental-derived MSC have been cultivated in a scalable fixed bed reactor, consisting of a 13 mL bed encased within a gas-permeable shell for indirect aeration and nutrient supply [149]. A 10-fold expansion in MSC number was achieved after 1 week in culture using serum-supplemented medium. Nevertheless, cell growth in the fixed bed culture was slower than growth in static 2D cultures [149].

Also using immobilized Fibra®-Cel disks, PluriStem Therapeutics is using reusable fixed bed bioreactors (PluriX™ 3D bioreactors) to produce placental MSC (PLX cells). In this system, adherent stromal cells are first isolated from the placenta and then expanded in the PluriX™ bioreactor for several weeks. Cells are harvested based on a proprietary vibration method and stored as ready-to-use products in a cellular therapy context [167], [168]. Fibra®-Cel disks can also be accommodated in a single-use version of the BioBLU® bioreactor, for a vessel volume of up to 5 L [80]. The largest single-use fixed-bed bioreactor is Pall's Integrity™ iCELLis™ bioreactor, with a maximum growth surface of 500 m^2 and a fixed-bed made of polyethylene terephthalate microfibers [169]. To date, there are no reports regarding the expansion of MSC using these two approaches.

I.3.4.2.4 Vertical-Wheel™ Bioreactors

PBS Biotech® has recently developed a novel type of bioreactor system using the innovative Vertical-Wheel™ technology. These systems consist of U-shaped vessels incorporating a vertically rotating wheel, resulting in faster and more efficient mixing at lower agitation rates [157]. The Vertical-Wheel™ bioreactors have proved to be efficient for the expansion of BM MSC, achieving a maximum cell concentration of 3 x 10^5 cells/mL [157]. Despite no significant differences in terms of cell volumetric concentration and expansion factors, Sousa and co-workers reported a higher percentage of proliferative cells in the Vertical-Wheel™ bioreactor, compared with a STR culture. Additionally, this type of bioreactor features easy scalability to industrial volumes, including 15 and 80 L bioreactors.

I.3.4.2.5 Hollow Fiber Bioreactors

Whereas stirred, wave-mixed, fixed-bed, and Vertical Wheel™ bioreactors used for the expansion of MSC are usually operated using microcarriers, hollow fiber and parallel plate reactors run carrier-free.

Hollow fiber bioreactors present high surface area-to-volume (100-200 cm^2/mL) and create 3D environment for cells. They consist of several parallel hollow fibers made of cellulosic, polysulfone, polypropylene, or polyethylene materials, which are encased in a cylindrical cartridge, for instances in polycarbonate, with ports for flow around or inside the fibers. Undesired molecular species are rejected according to the pore size of the semi-permeable hollow fiber membrane [80]. Total cell yields of 10^8 and 10^9 are reported in the literature regarding the use of hollow fiber bioreactors for MSC expansion. Such numbers were achieved using two different commercial systems: FiberCell System (max. growth surface of 2.5 m^2) and Quantum Cell Expansion System (max. growth surface of 2.1 m^2) [80]. Both systems were used for the isolation and propagation of MSC isolated from bone marrow, adipose tissue, periosteum and placenta over few weeks, under FBS-supplemented culture media [150], [170]–[174]. More recently, MSC were also cultivated in the Quantum Cell Expansion System under SF/XF culture conditions, contributing towards the development of GMP-compliant production processes [151], [152]. Nonetheless, hollow fiber bioreactors have limitations regarding nutrient and oxygen gradients in culture, like fixed-bed bioreactors. Thus, these systems are restricted in scalability, specially concerning the length of the fiber and height of the fixed bed. The major challenge, however, for both bioreactor types is posed by cell harvest [80].

I.3.4.2.6 Parallel Plate Bioreactors

In parallel plate bioreactors, the plates are typically made from polystyrene and each plate consists of two compartments separated by a gas-permeable membrane (and impermeable to liquids). Whereas the upper compartment is filled with air, the lower one contains the cells adhered to the surface as monolayer, being continuously supplied with culture medium [80]. Two commercially available examples of parallel plate bioreactors are E-Cube™ System and Integrity™ Xpansion™ Multiplate Bioreactor. Whereas E-Cube™ provides 21,250 cm^2 of maximum growth area and requires an incubator for operation, Integrity™ Xpansion™ bioreactor offers 122,400 cm^2 of maximum growth surface and controls pH and DO with installed sensors and inlets/outlets for gases [80], [103]. Gas exchange is performed in a central column with channels along the stacked circular plates (10-stack planar flasks), where the medium circulates. BM MSC were successfully expanded in the Integrity™ Xpansion™ Multiplate Bioreactor to up to 10^9 cells per batch [153]. The major issue regarding the use of parallel plate bioreactors for MSC expansion is based on the lack of opportunity to monitor cells. However, using holographic microscopy, MSC morphology was monitored on the 10 top plates of the Integrity™ Xpansion™ bioreactor [153].

I.3.4.2.7 Rotating Bed Bioreactors

Rotating bed bioreactors have also been employed for the *ex vivo* expansion of MSC. Diederichs and colleagues firstly reported the cultivation of MSC in a rotating bed bioreactor system (the Zellwerk's rotating bed bioreactor system – ZRP), based on a macroporous zirconium dioxide based ceramic disc called Sponceram [154]. The ZRP system consists of a cylindrical culture vessel with a rotating bed of polycarbonate plates, whose rotation is caused by a non-contact magnetic drive coupled to the culture vessel. Moreover, the system is equipped with ports for sampling, medium exchange, and monitoring and control of culture parameters [80]. The results evidenced a much higher proliferation rate of AT MSC cultured in the ZRP system, compared with static cultivation, measured by glucose and lactate concentrations. Also using a ZRP bioreactor system, Reichardt and colleagues demonstrated a 39-fold increase in the number UC arteries-derived MSC after 9 days [155]. In a different study, culture of UC MSC in a disposable Z 2000 H bioreactor resulted in a reproducible 8-fold expansion of cell numbers after 5 days [156].

I.3.4.3 Hydrodynamic Characterization of Different Bioreactor Configurations and its Impact on the Expansion of Mesenchymal Stem/Stromal Cells

The use of different bioreactor systems to create a highly dynamic culture environment aims at improving the flow of nutrients and oxygen to cells, as well as the removal of waste products away from the cells. However, the hydrodynamic stimuli generated by the dynamic environment interact with the MSC, activating mechanotransduction pathways involved in cell differentiation (*versus* self-renewal) or, ultimately, induction of apoptosis [175]. Thus, a balance must be achieved between culture homogeneity and hydrodynamic forces. In this chapter, different bioreactor configurations will be characterized in terms of fluid dynamics/hydrodynamics, including shear stress and mixing, and oxygen diffusion and the impact of these factors on MSC expansion will be discussed.

I.3.4.3.1 Shear Stress

Doran has defined "fluid" as a substance that undergoes continuous deformation when subjected to a shearing force [176]. Such shearing forces act tangentially to the surfaces over which they are applied. In MSC dynamic cultures, those surfaces can be either planar surfaces, such as the plate surface of parallel plate bioreactors, or 3D surfaces, including scaffolds, 3D spheroids or microcarriers. Thus, MSC growing adherent to a surface are exposed to shear forces from the moving fluid [80]. Shear stress is known to be one of the main culture factors affecting MSC expansion and differentiation [175]. Shear stress strongly depends on the viscosity of the fluid and, in the case of MSC culture medium, the fluids are considered as laminar Newtonian fluids. Under these conditions, the flow regimen in which a bioreactor can operate can be either laminar (viscous forces dominate inertial ones and Reynolds number, *Re* (conceptually the ratio of these two forces) $< 2 \times 10^4$) or turbulent (inertial forces dominate viscous ones, $Re > 2 \times 10^4$) [80], [177]. Examples of bioreactor configurations operating in a laminar flow regimen are packed/fixed-bed bioreactors, parallel plate bioreactors, and hollow fiber bioreactors. Conversely, types of bioreactors operating under turbulent or transiently turbulent regimes for MSC expansion include STR, wave-mixed bioreactors, rotating bed bioreactors, and the Vertical-Wheel™ bioreactors [80].

Parallel plate bioreactors have been well-investigated regarding shear stress, which is low in their case. In 1996, Peng and Palsson studied the influence of the bioreactor geometry on fluid low and the resulting growth and differentiation of BM stem cells [178]. The results indicated a higher cell density and uniformity in the radial-flow bioreactor due to a more uniform environment caused by the hyperbolic velocity and tube-like shear stress contribution, as well as the absence of walls parallel to the flow paths creating slow flowing regions. Flow pattern and shear stress levels were also studied in the Integrity™

Xpansion™ Multiplate Bioreactor, using a computational fluid dynamics (CFD) approach [80]. The analysis revealed the occurrence of gentle laminar flow and a maximum wall shear stress lower than 10 mPa, which was 1000 times lower than in stirred bioreactors. The influence of the superficial velocity of the culture medium on MSC expansion in a fixed-bed bioreactor was carried out by Weber and colleagues [179]. The fact that MSC are exposed to shear stress caused by the medium flow in this type of bioreactors demands the determination of an upper limit for the superficial velocity. Results showed a decrease of more than 50% of the mean growth rate when the superficial velocity was increased from 2.65×10^{-4} m/s to 1.59×10^{-3} m/s, demonstrating the importance of controlling the fluid velocity on laminar flow-operating bioreactors.

Local shear rates in stirred bioreactors vary within the vessel [80]. It is therefore more difficult to associate cellular effects (like cell differentiation or damage) with the magnitude of the prevailing shear rate or the associated shear stress. Several concepts have been proposed to describe the effect of shear stress on cells growing on microcarrier cultures in bioreactor systems [180]. In our lab, dos Santos and colleagues estimated the theoretical values of maximum shear stress under stirred conditions, τ_{max}, as result of flow through Kolmogorov eddies, in a 1.3 L Bioflo® bioreactor system [142]. A maximum shear stress of 1.5 dyn/cm^2 (0.15 N/m^2) was reported for a 0.8 L working volume of BM MSC culture, which was lower than the one determined for a spinner flask culture [95]. Using the same approach, Kaiser and co-workers performed a fluid flow and suspension characterization inside a 125 mL spinner flask [181]. Researchers found that AT MSC cultured in this system tolerate mean and maximum shear stresses in the order of 0.004 to 0.2 N/m^2, respectively. Shear stress levels were also calculated for a BM MSC culture in a Vertical Wheel™ 3 L bioreactor system [157]. Under the culture conditions studied, the shear stress rate was 0.021 N/m^2, which is roughly an order-of-magnitude lower than the levels reported as detrimental for cell growth [181], [182]. Other authors proposed different approaches to estimate the shear stress in stirred bioreactors, including the concept of an "Integrated Shear Factor" (ISF), a measure of strength of the shear field between the impeller and the walls [183]. In wave-mixed bioreactor systems, shear stress is highest at the lowest filling level together with the highest rocking rate and rocking angle [184], [185]. Furthermore, shear stress pattern was more homogeneous in wave-mixed bioreactors with 1D motion than in stirred bioreactors. For this reason, wave-mixed bioreactors are good candidates for the manufacturing of shear sensitive cells.

To summarize, shear effects need to be minimized to successfully expand MSC and prevent undesirable differentiation or cell damage. Controversial results regarding shear stress tolerance of MSC might be due to protective effects of medium components, such as serum, or differences in the type of surface or cell density [80]. For example, cells growing on macroporous microcarriers are better protected against shear than cells growing on non-porous carriers [186]. To minimize shear stress levels in a bioreactor culture, it is important to understand which parameters affect the mixing efficiency.

I.3.4.3.2 Mixing

Mixing is defined as a physical operation that aims at reducing nonuniformities in fluids by eliminating gradients of concentration of solutes, temperature, and other properties [176]. If the system is perfectly mixed, a random, homogeneous distribution of the system components occurs. Mixing efficiency strongly depends on the equipment used, which has a significant effect on agitation efficiency, power input requirements, and operating costs [176].

A crucial factor for the expansion of MSC in microcarrier-based stirred culture systems is the agitation required to maintain a homogeneous suspension of microcarriers. Agitation rate should be set to the minimum value required to maintain the microcarriers in suspension, to reduce shear stress levels on MSC [54]. If the agitation is too low, the microcarriers will settle in the bottom of the culture vessel and will aggregate, impairing cell growth [103]. Conversely, if the agitation is too high, the associated shear stress may induce cell differentiation or cell damage, as previously discussed. To date, the empirical correlation derived by Zwietering in 1958 is still the most widely-used method to characterize a minimum stirring rate to reach a "just suspended" state of the particles, N_{JS} (rev/s) [187]. At this agitation rate value, microcarriers should not remain on the bottom of the vessel for more than 1 or 2 s [187]. At N_{JS}, the concentration of microcarriers decreases with height and could be zero near the surface. As N_{JS} increases, microcarrier distribution becomes homogeneous. When agitation is below N_{JS}, microcarriers fall out of suspension and settle in areas of low flow at the bottom of the vessel [103]. Such conditions may impair cell growth due to the inefficient nutrient supply or the formation of microcarrier aggregates resulting from cell overgrowth [188]. This parameter has been widely accepted as a reliable scale-up factor [165] and depends on the vessel design, the type and concentration of microcarriers used, and those related to the specific process employed [145], [187]. N_{JS1U} has also been employed as suspension criteria and refers to the N_{JS} lower limit, meaning the impeller speed required to locate the particles at the bottom of the vessel with none of them at rest [80]. N_{JS} is strongly related to the specific energy dissipation rate (ε_T), which is defined as the energy lost by the viscous forces in the turbulent flow [176].

Another important factor impacting MSC expansion in dynamic culture systems is the Kolmogorov eddy length scale (λ_K). This parameter is based on Kolmogorov's theory of isotropic turbulence, which claims that mechanical stresses generated on cells growing in dynamic culture systems are originated from the energy transmitted on microcarrier surface by eddies whose size were similar to microcarrier diameter [189], [190]. The Kolmogorov eddy length scale corresponds to the diameter of the smallest eddy generated in the bioreactor, which is formed from bigger eddies that were broken down by the turbulent environment [80]. When the Kolmogorov eddy length scale is 1 to 2/3 of the size of the microcarrier, the flow can cause shear rates that are sufficiently large to damage or even remove cells

from the microcarriers. This parameter is affected by stirrer speed, impeller design, liquid properties, as well as the existence of baffles (protrusions) in the reactor vessel [80].

Other process parameters have been used to characterize different bioreactor systems, including geometric dimensions, volumetric power input, power number, mixing time, Reynolds number, impeller tip speed (u_{tip}), volumetric gas flow, volumetric mass transfer coefficient (k_La), and maximum oxygen demand (OD_{max}) [80]. These values are used to compare process parameters in different bioreactor systems and scales.

N_{JS} and λ_K values were determined for different stirred bioreactor configurations used for MSC expansion, including a 15 mL TAP ambr™ bioreactor, a 250 mL DASGIP bioreactor, and a 5 L Sartorius bioreactor [191]. Depending on the culture conditions and bioreactor geometry, the N_{JS} values ranged from 6.67 s^{-1} in the 15 mL TAP ambr™ bioreactor to 1.25 s^{-1} in the 5 L Sartorius bioreactor, representing a 5-fold decrease in the latter system. λ_K values varied from 52 µm in the 15 mL TAP ambr™ bioreactor to 68 µm in the 250 mL DASGIP bioreactor. Nevertheless, MSC were successfully expanded to cell densities up to 3 x 10^5 cell/mL in each type of bioreactor. Kaiser and colleagues also determined the impeller speed for suspension criteria, including N_{JS} and N_{JS1U}, in 100 mL spinner flask cultures using two different types of microcarriers: Hillex®-II and ProNectin®-F [181]. Results showed that the suspension criteria were fulfilled for ProNectin®-F (N_{JS} = 63 rpm and N_{JS1U} = 49 rpm) at lower stirred speeds than for Hillex®-II (N_{JS} = 105 rpm and N_{JS1U} = 82 rpm) microcarriers, due to their higher density and slightly larger mean particle size of the latter. Moreover, the minimum λ_K value was estimated to be 44 µm at 82 rpm of impeller speed (much lower than the proposed 2/3 microcarrier size), at which no cell death was detected. The highest cell expansion factor was obtained using the ProNectin®-F microcarriers stirred at 49 rpm (31.4 ± 3.6) compared with stirring at 60 rpm (26.4 ± 3.1), demonstrating the importance of optimizing dynamic culture conditions. At a larger scale, Lawson and colleagues defined N_{JS} value as 74 rpm for a 50 L single-use stirred tank reactor culture operating with 15 g/L of collagen coated microcarriers under optimized conditions. The authors also predicted the upper limit for λ_K to be 114 µm and applied to the bioreactor culture. No impact was predicted regarding cell expansion potential and a 43-fold MSC expansion was reached [145].

Mixing efficiency and impact on wave-mixed bioreactors was studied by measuring mixing time, i.e., the time required to achieve 95% homogeneity [184]. Values of mixing time were found to directly depend on the rocking rate and indirectly depend on rocking angle in the BioWave® bioreactor. Mixing times achieved ranged from 10 s to 1,400 s for Newtonian fluids, using 40% to 50% culture volume, respectively. Interestingly, the most inefficient mixing takes place at the lowest rocking rate and rocking angle with 50% culture volume. Regarding cell culture bag size, the most efficient mixing was obtained in a 2 L culture bag, whereas the most inefficient mixing of all bags investigated was found in the 20 L

culture bag [184]. To date, no studies were found regarding the effect of rocking rate, rocking angle and/or culture volume on MSC expansion efficiency.

The novel Vertical-Wheel™ technology features a uniform distribution of the hydrodynamic forces, as demonstrated by Löffelholz and co-workers [192]. Consequently, Sousa and colleagues determined the shear stress rate and the Kolmogorov length scale in a PBS-3 Vertical-Wheel™ (PBS-VW) bioreactor operating at a stirring rate of 17 rpm and compare this with the Biostat Qplus STR agitated at 40-45 rpm [157]. These agitation rate values were set based on previous suspension experiments to determine N_{JS}. Shear stress rate was uniform in the PBS-VW culture vessel (0.021 N/m^2) but proved to be heterogeneous in the STR culture (average: 0.008-0.010 N/m^2; impeller: 0.019-0.024 N/m^2). Also, mixing time is about 3 times lower in the PBS-VW culture (18 s), compared with the STR culture (56 s), as results of the greater degree of fluid dynamic homogeneity observed in the PBS-VW bioreactor. The estimated λ_K values are also above the critical threshold considering the type of microcarriers used in both PBS-VW (151 μm) and STR (average: 220-202 μm; impeller: 157-143 μm) cultures. Despite the hydrodynamic differences, both systems achieved similar BM MSC concentrations (~3 x 10^5 cell/mL) [157].

In non-stirred bioreactors, efficient mixing of the culture medium can be difficult to achieve, particularly when operating at low Reynolds number. Both parallel plate and hollow fiber bioreactors operate in a laminar flow regimen, as previously discussed, which means that the fluid flows in parallel layers and with no disruption of those layers [176]. In these configurations, a low fluid velocity is mandatory, so the fluid flows without lateral mixing and formation of cross-currents perpendicular to the direction of the flow or eddies [193]. At higher fluid velocities, the flow regimen changes from laminar to turbulent and the formation of eddies can induce the activation of pathways involved in cell differentiation and apoptosis and even promote cell removal from the surface of the bioreactor. A low fluid velocity is also required to promote successful MSC expansion in packed/fixed-bed bioreactors. Under these conditions, flow is not disturbed due to the carriers composing the packed-bed and cells remain viable and attached to their surface [176]. At higher velocities, the flow is disturbed due to the presence of the carriers and turbulent eddies are generated behind the particles. Such eddies can be responsible for cell damaging or washout. The influence of the superficial velocity on the growth rate of MSC cultured in a fixed-bed bioreactor system was studied by Weber and co-workers [179], as previously discussed. Yet, no other reports were found studying the impact of fluid velocity and mixing efficiency on MSC expansion in non-stirred bioreactors.

I.3.4.3.3 Oxygen Transfer

Either culturing under normoxic or hypoxic conditions, MSC always require a minimum level of oxygen demand, which must be met and it is related to the cell-specific oxygen uptake rate and maximum cell concentration [177]. The rate of oxygen transfer from gas to liquid is therefore of major importance, particularly in dense cell cultures where the demand of oxygen is high. The rate of oxygen transfer is related to the liquid-phase mass transfer coefficient (k_L, m/s), which links the area available for transfer from the gas phase to the motion of the fluid surrounding that surface [176], [177]. General considerations and calculations regarding oxygen transfer are comprehensively discussed elsewhere [176], [177].

In STR cultures, oxygen has been typically supplied through headspace aeration, since this induces lower shear stress when compared with other aeration systems such as air sparging [54], [142], [176]. Thus, the surface area available is referred to the upper surface of the medium, *A* (surface aeration) and *V* (medium volume). As the scale increases, the *A/V* ratio of the medium decreases. Conversely, k_L increases when the velocity at the surface increases, roughly proportional to the square root of the agitator speed [194]. In 2013, Rafiq and colleagues reported a 5 L stirred bioreactor culture for BM MSC operating without bubbling or headspace aeration, though additional DO entered the bioreactor in the medium during its exchange [144]. After 12 days of culture, DO decreased from 100% to ~50%, which represents the oxygen tension inside the incubator (21% and 10% O_2). During this time, the average specific O_2 uptake of the cells in the 5 L bioreactor was 8.1 fmol/cell.h, which is around one order of magnitude less than with animal cells in free suspension [177]. Due to this very low specific oxygen demand for MSC, it can be achieved at N_{JS} with 100% DO by headspace aeration with air [177]. In other studies, with closer DO control, lower O_2 concentrations (typically 10–30%) led to higher MSC proliferation [143], [157], [164], [165], [195]. When agitation was increased and the surface aeration was substituted by membrane aeration, bubble aeration or microsparging, the k_La values increased from 0.4-1 h^{-1} to 1.9-5.2, 10-15 or 7-50 h^{-1}, respectively [184]. Nevertheless, some of these types of aeration have been shown to be lethal to cells as bubbles burst and a surfactant, typically Pluronic F-68, may be required to prevent it [196].

Volumetric oxygen transfer coefficients measured in wave-mixed bioreactors were comparable to or higher than those published for STR operating with surface or membrane aeration [184]. In the currently available disposable wave-mixed cultivation systems, the oxygen is mainly supplied through surface aeration and k_La lies between 1-15 h^{-1} [184]. Importantly, CELL-tainer® presents superior oxygen mass transfer capacity (up to 700 h^{-1} [197]), compared with the other systems. Oxygen transfer efficiency is directly correlated to rocking rate, rocking angle, and aeration rate. At constant parameters, a decreased culture volume in the bag results in increased specific surface area and power input and thus increases

k_La values. Even minor changes in the rocking rate and/or rocking angle can increase k_La more significantly than raising the aeration rate [184].

Aeration in the PBS-VW bioreactor occurs through two different mechanisms: headspace aeration in the PBS MagDrive reactors and gas sparging introduced into the vessel of PBS AirDrive reactors [198]. In the AirDrive reactors, the air is captured by the cups and then released as the wheel turns, thus driving further wheel rotation. Due to foaming, AirDrive reactors are intended for cultures that can be supplemented with Pluronic F-68 or other antifoaming agents, capable of eliminating the problem of bubble bursting. PBS-VW AirDrive reactors have been characterized and successfully used for the expansion of MSC, as previously mentioned [157]. Since there is no sparging in the MagDrive reactors, they should be used for cultures growing in medium that cannot be supplemented with antifoaming agents [198].

An interesting study developed by Weber and colleagues [199] aimed at modeling a laboratory-scale MSC expansion process in order to transfer a fixed-bed bioreactor system to the production scale – a target cell number of 2 x 10^{10} cells. At a fixed superficial velocity and at 100% DO in the inlet, the maximal fixed bed height is a function mainly of the cell density and the minimal oxygen saturation in the fixed-bed. Thus, the fixed-bed height decreases with increasing outlet oxygen saturation and cell density. This correlation was used to determine the number and volume of parallel operated fixed-bed reactors required for the cultivation of 2 x 10^{10} cells, as function of the thickness ratio, outlet oxygen saturation, and cell density at the end of the cultivation. After 233 h of culture, 2 x10^{10} MSC were obtained using an initial cell density of 1.83 x 10^4 cells/cm^3, a target cell density of 1.83 x 10^6 cells/cm^3, an outlet oxygen saturation of 30%, a fixed-bed height and volume of 13.53 cm and 1946 cm^3, respectively, and 6 fixed-bed reactors operating in parallel [199].

Gas control in hollow fiber bioreactors is performed through a hollow fiber oxygenator [151], [173], which means that oxygen is supplied from a premixed gas tank. Cells can be expanded at their optimal gas concentration by choosing a tank with the desired gas mixture. In 2016, Lambrechts and colleagues developed a monitored hollow fiber bioreactor for large-scale progenitor cell expansion [150]. Here, the bioreactor circuit was modified to able to measure the DO concentration and pH. The results show that DO drops to values close to 10% in the outlet, after 8 days of culture. Although these values are not limiting of cell proliferation, the authors claim that the 10% drop in DO could be counteracted with a feedback control loop. This system would increase the medium circulation rate in the external space between the hollow fibers to increase the homogeneity of oxygen availability over the length of the hollow fibers, without increasing the shear stress on the cells [150].

Internal membrane oxygenators can also be added to parallel plate bioreactor systems to provide oxygen control during the culture [200]. The use of an internal membrane oxygenator can avoid oxygen limitation by appropriate selection of the membrane and thus providing greater control over cell oxygenation.

Several commercially available types of bioreactors allow for an efficient oxygen control using internal oxygenators. An example is the Rotary Cell Culture System, by Synthecon, a rotating bed bioreactor that contains an internal co-axial oxygenator placed in the center that rotates concurrently with outer wall of the vessel. [201]. Here, the gas transfer occurs by diffusion through the silicone oxygenator, thereby avoiding bubble formation and consequent turbulence.

I.3.5 Mesenchymal Stem/Stromal Cells in Acute Myocardial Infarction Context

The immunomodulatory and trophic activities of MSC make them promising candidates in the context of cell-based therapies for the treatment of several different disorders (*e.g.* auto-immune, neurodegenerative, and cardiovascular disease) [202]–[205]. Overall, MSC have been used in preclinical and clinical studies based on two different approaches: i) tissue engineering (*i.e.* regeneration of tissues based on MSC differentiation) and ii) regenerative medicine (*i.e.* therapeutic effects based on the paracrine activity of MSC) [206]. Since MSC have the ability to differentiate into distinctive mesenchymal phenotypes, including bone, cartilage, muscle, fat and tendon [207], they have been used in tissue engineering applications to rebuild damaged or diseased tissues when enclosed in tissue-specific scaffolds and implanted into specific sites. Alternatively, MSC secrete a broad range of bioactive molecules with immunomodulatory, anti-apoptotic, anti-scaring, proangiogenic and promitogenic, as well as chemoattractant properties [41], which can provide a regenerative microenvironment for a variety of injured tissues [206]. In this context, MSC have been exploited (in pre-clinical and clinical studies) for the treatment of AMI [26], [27]. To date (April 2018), a total of 17 ongoing or completed clinical studies involving MSC in AMI context were registered at ClinicalTrials.gov. Most of the studies aim at reporting preliminary data on safety and preliminary efficacy of MSC administration in human patients and, thus, they are phase I, phase I/II, and phase II studies [208].

The clinical use of MSC may involve autologous cells (patient-specific cells that will be used in a personalized therapy) or allogeneic cells (isolated from another individual, expanded *ex vivo* and used in the patient) [78]. In an autologous setting, MSC are collected from the patient, shipped to a facility to be isolated and expanded, shipped back to the clinical center and then infused in the patient [209]. This setting presents two major drawbacks: i) the lag period between the harvesting and infusion of cells,

which may be crucial depending on the condition of the patient; and ii) age- or disease-related morbidities affecting the quality (*i.e.* potency) of the harvested cells, thus reducing the efficacy of the treatment [210], [211].

I.3.5.1 Mechanisms of Action of Mesenchymal Stem/Stromal Cells in Cardiac Regeneration

During the last years, researchers have proposed four potential mechanisms of MSC mediated cardiovascular repair, namely MSC transdifferentiation into cardiomyocytes, MSC fusion with native cells, MSC-induced stimulation of endogenous CS/PC via direct cell-cell interaction, and MSC-paracrine signaling [4], [212] (Figure I.7).

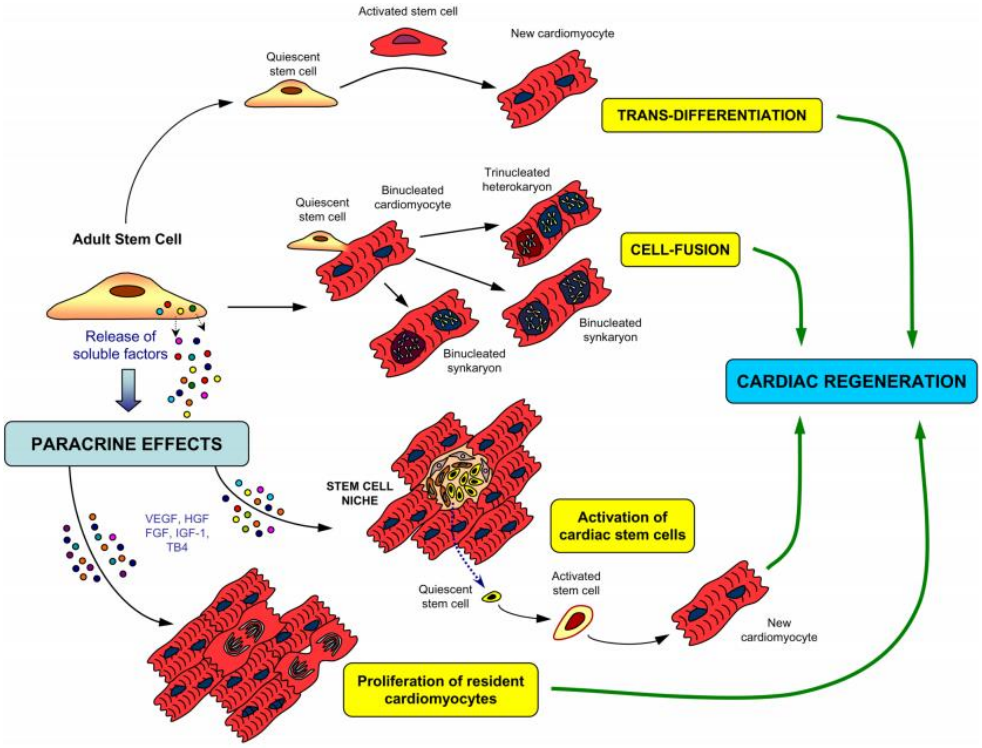

Figure I.7 Mechanisms of action of adult stem cells in cardiac regeneration [20].

MSC *in vivo* transdifferentiation into cardiomyocytes was successfully demonstrated by Shake *et al.* and Toma *et al.*, in studies where injected MSC engrafted into scarred myocardium and expressed cardiomyocyte markers, such as α-actin, desmin, tropomyosin, and myosin heavy chain [213], [214]. However, Shake and colleagues showed that, although injected MSC stained for several muscle markers, their morphology resembled fibroblasts more than cardiomyocytes and no electromechanical junctions

with other graft cells or with host cells were observed [213]. Quevedo and colleagues reported allogeneic MSC engraftment and differentiation into cardiomyocytes, smooth muscle cells, and endothelium in a swine model of chronic ischemic cardiomyopathy [215]. Despite the successful engraftment of MSC, the transdifferentiation process was found to be inefficient at best [214], and occurs only in the presence of native cardiomyocytes [216]–[218]. Moreover, *in vitro* cardiomyocyte transdifferentiation requires the use of inducing factors, such as 5-azacytidine (5-aza), which limits the clinical applicability of such strategy [219].

MSC fusion with native cells was very infrequently observed, eliminating eventual MSC-mediated cardiomyocyte regeneration [217], [218]. Although the stimulation of endogenous CS/PC by MSC via direct cell-cell interaction has been verified *in vitro* and *in vivo* [216], [220], evidences suggest that CS/PC possess only a limited capacity to differentiate into fully mature cardiomyocytes with an adult phenotype [221], [222]. Moreover, the suggested mechanism requires MSC engraftment at the target site of injury, which may be impaired due to the harsh ischemic microenvironment characterized by oxidative stress, inflammation, cytotoxic cytokines and in some instances an absence of ECM for MSC attachment [223], [224].

The most accepted explanation for MSC-mediated cardiovascular repair occurs via paracrine signaling through the secretion of growth factors, cytokines and other signaling molecules. This hypothesis is supported by the work developed by Toma and colleagues, where they reported that after 4 days, only 0.44% of transplanted MSC resided in the myocardium [214]. The secreted factors may induce vascular growth and remodeling, modulate inflammation, attenuate fibrosis, regulate cell survival and differentiation and recruit resident stem or progenitor cells [225], [226].

I.3.5.2 Immunomodulatory Properties of Mesenchymal Stem/Stromal Cells

AMI, as well as other forms of heart failure, is associated with immunological processes in the heart. MSC respond to inflammation by migrating to the site of injury, where the immunological processes is actively suppressed and the inflammation reduced by inhibiting T cell proliferation [227]. MSC-mediated T cell inhibition is determined by the local balance of anti- and pro-inflammatory cytokines [228]. Moreover, Spaggiari and colleagues showed the ability of MSC to inhibit the proliferation of interleukin-2 (IL-2) and IL-15 stimulated natural killer cells [229]. MSC are also capable of altering B cell proliferation, activation, IgG secretion, differentiation, antibody production, and chemotactic behaviors, as well as reducing the expression of MHC II, CD40 and CD86 on dendritic cells following maturation induction [230]. MSC were also found to secrete TSG-6, a powerful anti-inflammatory factor critical for the clinic improvement of myocardial infarct [231]. However, several reports suggested that

the immunomodulatory effects of MSC are transient, dose dependent, require a certain degree of inflammation [232] and might not be strictly suppressive [233], [234].

Two major types of macrophages can be found in the heart after an AMI event: 1) M1 (expressing inducible nitric oxidase synthase (iNOS), MHC class II, CD80, CD86), that cleans the debris and produces pro-inflammatory factors IL-1β, tumor necrosis factor-α (TNF-α), and interferon-ɣ (IFN-ɣ) [235], and after 5 days, 2) M2 (expressing arginase, macrophage mannose receptor CD206), which presents an anti-inflammatory phenotype, reduces the release of pro-inflammatory cytokines and stimulates scar formation and angiogenesis [236]. MSC were shown to boost the differentiation into the M2 subtype [237], whereas the debris-cleaning function remained unchanged [238].

I.3.5.3 Trophic Mechanisms of Mesenchymal Stem/Stromal Cells

Despite low cellular retention and poor cardiomyocyte differentiation, improved heart function is still observed [239], which suggests a putative paracrine mechanism of action for transplanted MSC. MSC secrete a wide range of angiogenic, apoptotic, mitogenic, and homing factors that might be involved in cardiac repair [203]. Among the them are vascular endothelial growth factor (VEGF), bFGF, HGF, IGF-1, TGF-β, stroma-derived factor-1α (SDF-1α), angiopoietin, ILs, and numerous chemoattractants [41]. Tissue concentration of some of these factors is significantly increased in injured hearts treated with MSC [240], [241].

VEGF was found to promote angiogenesis and improve regional blood flow in the surrounding ischemic host myocardium [19], [242], [243], presenting a potent synergism effect with bFGF, another pro-angiogenic factor [244]. Kinnaird and colleagues reported that *in vivo* local delivery of MSC into mice increased distal limb perfusion and vessels number and total cross-sectional area, as well as protein levels of VEGF and bFGF [245]. Hung *et al*. demonstrated that MSC-conditioned medium contains high amounts of angiogenic and anti-apoptotic factors IL-6, VEGF and monocyte chemoattractant protein-1 (MCP-1), which inhibits endothelial cell death and promote the formation of capillary-like structures *in vitro* [246]. Furthermore, Markel and colleagues showed that MSC underexpressing VEGF have significantly less cardioprotective capabilities [247].

HGF is another secreted angiogenic and cardioprotective factor which has been reported to enhance neovascularization, reduce apoptosis and promote cardiomyocyte proliferation [248], [249]. Transplanted MSC were shown to indirectly stimulate endogenous CS/PC through HGF, VEGF and IGF-1 secretion, resulting in activation, proliferation and migration of the target cells [220], leading to partial restoration of cardiac function in ischemic hearts [250]. Several studies demonstrated that SDF-

1α-chemokine receptor 4 (CXCR4) signaling plays an essential role in stem cell recruitment to different tissues, including the heart. CXCR4 is the cell surface receptor for SDF-1 and is expressed on EPC and HSC [251]–[253].

After an AMI event, endogenous SDF-1 secretion declines and returns to normal levels only after 4 to 7 days [254], [255]. Transplanted MSC were shown to express SDF-1, which may facilitate EPC and HSC recruitment and migration to the infarcted tissue. However, culture expanded MSC showed poor homing capacity, probably due to low expression of receptors for chemokines and adhesion ligands, such as CXCR4 and C-C motif chemokine receptor-1 (CCR1) [4]. Furthermore, *in vivo* MSC were reported to alter ECM homeostasis and prevent postinfarction left ventricular (LV) chamber dilation, by attenuating the proliferation of cardiac fibroblasts, downregulating the production of collagen type I and collagen type III and shifting the balance between matrix metalloproteinases (MMP) and their tissue inhibitors toward domination of matrix-degrading effects [256]–[258]. Conversely, some of the secreted factors are also able to modulate MSC activity. VEGF and bFGF appear to stimulate MSC migration, proliferation [259], [260] and induce endothelial differentiation [261], whereas bFGF and TGF-β were shown to induce smooth muscle cell (SMC) differentiation *in vitro* [260], [262]. Several other bioactive factors secreted by MSC have been described as playing a role in cardiac improvement through paracrine mechanisms, as reviewed in [4], [27], [41], [212].

I.3.5.4 Sources of Mesenchymal Stem/Stromal Cells

MSC for cardiac cell therapy can be obtained from different tissues, such as adult (BM, AT, peripheral blood) and neonatal (UCB, UCM, amnion, and placenta) sources.

BM represents the most extensively studied source of MSC, for both pre-clinical data and clinical trials. BM MSC have shown promising potential in cardiac repair due to their high proliferative capacity [263], ability to reduce infarct size [264], *in vitro* capacity to give rise to endothelial cells (EC) and SMC [265] and ability to change the milieu of the damaged cardiac tissue to upregulate VEGF [266]. Cardiomyocyte differentiation proved to be more problematic, as either demethylating agents have been employed [267], or the process has been inefficient and incomplete [268]. Makkar and colleagues verified that, in a porcine model of AMI, direct intramyocardial injection of BM MSC 1 month after the event resulted in preserved LVEF at 60 and 90 days post AMI in comparison to the untreated subjects [269]. In another study, global LVEF improved along with a decrease in infarct size by 40% in sheep treated with intracoronary infusion of allogeneic BM MSC in comparison to untreated sheep [270]. There have also been numerous clinical trials evaluating the effect of BM MSC in humans with cardiovascular diseases. In 2006, Schachinger and colleagues developed a trial aiming at assessing cardiac function after

intracoronary administration of autologous BM MSC 3-7 days post-AMI. After 4 months, the LVEF increased by 5.5% in the treated group vs 3% in the control group [271]. A phase I trial initiated in 2009 evaluated the safety and efficacy of BM MSC (Prochymal) delivered intravenously to patients post-AMI. Results showed better global symptom scores and ejection fraction, confirmed by cardiac MRI, in patients receiving MSC compared with the control group [272]. Despite the promising results obtained with the BM source, it does not qualify as a viable isolation source of cells due to high-grade viral infection and a substantial reduction in the proliferative capacity of these cells with age [273]. Moreover, BM aspiration is an invasive procedure, which causes immense pain to the patients and can also cause infection [51].

The other major source of MSC in the clinical setting is AT. The main advantages of AT MSC are the availability of large number of cells and the ease of acquiring them from liposuction aspirates. On the other hand, it is not clear yet to what extent AT MSC are truly MSC. AT and BM MSC share many biological characteristics; however, there are some differences in their immunophenotype, differentiation potential, transcriptome, proteome and immunomodulatory activity. Some of the differences may be associated with tissue specific features, whereas others are probably related to inherent heterogeneity of both populations [274]. In contrast to BM ones, AT MSC cardiac potential is better documented *in vitro*, showing their capacity to give rise to cardiomyocytes, either by using DMSO [275] or cardiomyocyte extracts [276]. AT MSC were also shown to differentiate into SMC [277], [278] and EC [279], [280], yet the ability of AT MSC to differentiate into these lineages remains controversial. Lee and co-workers not only demonstrated that AT MSC were able to differentiate into vascular EC, but also showed an angiogenic effect through paracrine signaling in an animal model with AMI [281]. Other studies showed that injected AT MSC into rat myocardium post-AMI resulted in an improved LVEF compared with untreated rats [282], as well as reduced fibrosis and wall thinning [283]. More recently, AT MSC were induced to differentiate into cardiac-like progenitors (iCP). At 1-month post-transplant into mice infarcted hearts, iCP and AT MSC showed higher myocardial capillary density, compared with BM MSC. All three samples proved to be efficient in decreasing the infarct size compared with the control or the untreated sample; however, the greatest reduction was seen in those transplanted with iCP derived from AT MSC [284]. Also in 2015, another interesting work developed by Perea-Gil and co-workers was related to the human adult epicardial fat surrounding the heart, which served as a reservoir for mesenchymal-like progenitor cells (cardiac ATDPC) [285]. In addition to the fact that these cells present a cardiac-like phenotype despite their residence in an adipocytic environment, they also have been shown to exert great immunosuppression due to increased T cell proliferation [285]. AT MSC do also secrete various cytokines with different immunomodulatory and trophic effects, which play an important role in cardiac tissue regeneration. A recent study demonstrated that AT MSC showed a better pro-angiogenic profile by secreting higher levels of angiopoietin-1,

angiogenin, VEGF, aFGF and placental growth factor (PlGF) and secreted higher amounts of ECM components and MMP, comparing to BM and UCM MSC [286]. These findings support the use of AT MSC in cardiac cell therapy. The PRECISE Trial uses AT MSC for transendocardial injections in patients with ischemic cardiomyopathy. Preliminary data show improvements in LV mass and motion score index in treated patients after 18 months [287]. Also, in the APOLLO trial, AT MSC were administrated intracoronarily to patients in the acute phase of a large ST-segment elevation AMI (STEMI). The results showed a significant improvement of the perfusion defect and a 50% reduction of myocardial scar formation in treated patients after 6 months [288].

MSC isolated from fetal or neonatal sources have been particularly interesting in the experimental setting. Several studies reported greater plasticity and lower immunogenicity comparing to adult sources, but much of the available data is still controversial [289]. UCB-derived MSC, as well as their proposed multipotent subpopulations, have been extensively studied in models of heart disease, but their isolation from UCB can be difficult [290]. However, it is well accepted that UCB MSC can give rise to cardiac muscles due to their mesodermal origin [27]. Also, a recent study established that co-transplantation of UCB $CD34^+$ and UCM MSC leads to reduction in collagen deposition and improved cardiac function in AMI rabbits [291]. MSC from different compartments of the umbilical cord, such as vein, arteries, Wharton's jelly, umbilical cord lining and so forth, have been observed to accumulate in damaged tissues and support tissue repair [292]. UC MSC are said to have higher proliferative capacity and a lower potential of forming teratomas than BM and AT MSC [70]. Specifically, UCM MSC have been identified to have biological and functional properties of ESC and adult stem cells, thus serving as an alternative source for stem cells with significant barriers of immunorejection, tumorigenesis, teratoma formation and ethical concerns [293], [294]. Swamynathan *et al*. reported that UCM MSC secrete several pro-angiogenic factors, such as VEGF, angiopoietin-1, HGF, TGF-β1, which promotes functional angiogenic potency in the infarcted mammalian heart [295]. However, Amable and colleagues did not reach the same conclusion, showing that UCM MSC secrete significant smaller concentrations of pro-angiogenic factors comparing to BM and AT MSC, but secrete higher concentrations of chemokines, pro-inflammatory proteins and growth factors [286]. Nevertheless, these cells are naturally chemoattracted to the cardiac tissue and they present functional ability to populate the ventricular myocardium [296]. In animal models, UCM MSC effectively induced angiogenesis [297]–[299], showed spontaneous differentiation into cardiomyocytes and EC [297], [298] and induced recruitment of CSC [298]. In a multicenter trial, patients with STEMI received intracoronary infusion of UCM MSC into the infarct artery at five or seven days after successful reperfusion therapy. The results showed a significant increase in the myocardial viability and perfusion within the infarcted territory in the treated group, comparing to the placebo group, as well as an increase in LVEF and decrease in LV end-systolic volumes and end-diastolic volumes after 18 months [300]. Placental tissue

(Pl)- and amniotic fluid (A)-derived MSC have received signification attention due to their surprising cardiomyogenic differentiation potential [228]. Placental MSC have characteristics of both ESC and MSC, as UCM ones, and are available in abundance as medical waste after delivery. However, the main limitation of these cells is the occurrence of high chances of impurity, since the placenta is the common medium of exchange between a mother and the baby [27]. Like UCM and PlMSC, AMSC posse some of the characteristics of ESC and are phenotypically similar to BM MSC, sharing similar immunologic profiles [301]. AMSC are considered a suitable cell source for cellular cardiomyoplasty by both integrating and differentiating into the cardiac tissue [302]. Transplantation of AMSC in a damaged myocardial tissue showed comparable results regarding decreased infarct size, cardiomyocyte-like cell differentiation and improved cardiac function, in comparison with UCB and AT MSC [303]. Also, AMSC present chemotactic characteristics [304], ample availability, lack of ethical concerns and low immune response [303], which makes them potential candidates for cardiac cell therapy.

I.3.5.5 Modulating the Microenvironment to Enhance the Cardiac Regenerative Potential of Mesenchymal Stem/Stromal Cells

Within the last few years, it has become evident the beneficial effect of MSC transplantation on ventricular function and myocardial perfusion. Results obtained by several preclinical and clinical trials led MSC therapy to a new challenging phase. However, these data have far demonstrated moderate and at times inconsistent cardiac regenerative benefits, indicating an urgent need to optimize the therapeutic platform and enhance stem cell potency [305], [306]. Cell potency is one of the major hurdles encountered in MSC therapy, but some other are also described, such as poor cell engraftment and survival and age/disease-related host tissue impairment [307], [308]. Multidisciplinary teams are currently establishing several potentially useful and logistically feasible strategies to achieve more robust clinical efficacy of MSC therapy. Moreover, long-term safety data regarding MSC administration remain lacking and unanticipated side effects may appear much later [308]. Several studies demonstrated that the immunomodulatory and trophic features of MSC can be modulated by the physical microenvironment, such as biochemical (culture media, oxygen concentration, growth factor pre-conditioning) [4], [307], [309] and mechanical (shear stress and material stiffness) [310] factors. Thus, the future success of MSC in cardiovascular therapy will require rational optimization of therapeutic strategies, along with an adequate assessment of benefit and risk factors [308].

I.3.5.5.1 Culture Medium Formulations

MSC-based products used in AMI context are traditionally expanded in serum-containing medium, such as FBS. However, infusion of xenogeneic-expanded MSC might trigger an immune response, leading to immune-mediated cell lysis and failure of MSC regenerative benefits [311], [312]. In fact, MSC expanded in FBS-supplemented media were found to be contaminated with N-glycolylneuraminic acid (Neu5Gc) xenoantigen [313], [314], resulting in an immunological reaction after transplantation with anti-Neu5Gc antibodies present in human serum [315], [316]. Anti-Neu5Gc antibody binding to Neu5Gc xenoantigen may trigger post-transplantation lysis of MSC [307].

Several studies have demonstrated the efficacy of different S/XF formulations in the expansion of MSC from different sources [48], [95], [317], [318]. Moreover, researchers proved that MSC expanded in SFM preserved or even improved their features, such as expression of cell surface markers, differentiation capability and trophic activity [295], [319], [320], comparing to serum-containing media. Specifically, Swamynathan *et al*. reported that serum-deprived human UCM MSC exhibit superior functional angiogenesis in comparison to serum-expanded cells [295], [321]. Similar evidence was found in BM MSC, showing that under the stress of serum deprivation MSC are highly angiogenic and that a population of these cells is able to differentiate into endothelial-like cells [321]. The superior angiogenic potential of serum-deprived MSC might be related to an increased relative expression of VEGF under SFM culture conditions. However, despite a higher relative expression of VEGF in MSC cultured in serum-deprived conditions, the expression of this potent angiogenic factor decreases as the number of population doublings increases, which may impair the potency of a MSC product for myocardial regeneration [322]. Recently, the enhanced angiogenic potential of MSC cultured in SFM was confirmed by Bobis-Wozowicz and colleagues, by culturing UCM MSC under different XF formulations. Despite the variation of the cytokine secretion profile in different types of SFM, UCM MSC showed consistently enhanced cardiomyogenic and angiogenic potential impacting on target cells, possibly due to elevated concentration of several pro-cardiogenic and pro-angiogenic microRNA present in secreted extracellular vesicles [323]. Nonetheless, like FBS-supplemented media, XF formulations are not able to avoid loss of chemokine receptors that are required for cell migration, engraftment and long term regeneration benefits [124].

Other solutions that have emerged as alternatives to FBS as cell culture supplement are humanized media formulations [54], as previously discussed. In a recent study, Amable *et al*. cultured MSC from BM, AT and UCM in 10% platelet-rich plasma (PRP)-supplemented media to assess the impact on cell proliferation, *in vitro* differentiation, expression of cell surface markers and transcription and secretion profiles [324]. Regarding the secretion profile, the authors found that UCM MSC secreted higher concentrations of chemokines and growth factors comparing to other sources, while BM MSC secreted

higher concentrations of pro-inflammatory and pro-angiogenic proteins and AT MSC produced higher amounts of ECM components. Nevertheless, PRP-expanded MSC presented reduced mitogenic and pro-angiogenic profiles when compared with FBS-cultured MSC, in all the cell sources studied [324]. hPL was considered to be the best alternative to FBS due to its superiority in maintaining growth potential, genetic stability, and differentiation potential [325]–[329]. However, contradictory findings have been reported concerning the immunomodulatory activity of MSC expanded in hPL-containing medium [330]. High-throughput proteomic analysis of hPL components identified TGF-β, VEGF, FGF, PDGF and EGF as highly ranked effectors of hPL activity on promoting MSC proliferation [325]–[329], [331]. Schallmoser and co-workers analyzed the mean growth factor concentrations in the supernatants of two different media compositions: α-MEM supplemented with i) 10% FBS and ii) 10% hPL, at days 0 and 13 of culture [331]. Multiplex growth factor analysis revealed that hPL contains significantly higher concentrations of EGF, bFGF, PDGF-AA, PDGF-AB/BB compared with FBS-supplemented media, but VEGF was not detected in any of the formulations. Moreover, the results showed a significantly higher VEGF production by MSC in hPL compared with FBS cultures [331], revealing the greater therapeutic potential of hPL-expanded MSC for myocardial regeneration. However, this evidence is not in accordance to other groups findings. In 2012, Azouna *et al*. demonstrated that FBS-expanded MSC secreted higher amounts of VEGF comparing to MSC expanded in media supplemented with 10% FBS + 5% hPL, 10% hPL and 5% hPL [332]. Recently, Li *et al*. verified that AT MSC cultured under hPL conditions secreted higher concentrations of bFGF, IFN-ɣ and IGF-1, as well as higher immunomodulatory effects, comparing to BM MSC, which secreted greater amounts of SDF-1 and HGF. However, the authors did not find significant differences between AT and BM MSC for the secretion of several factors, such as IL-6, IL-8, TNF-α and VEGF [333]. The *in vitro* tube formation and angiogenic capacity of AT MSC expanded in hPL *vs* FBS was assessed by Trojahn Kølle and colleagues, by culturing unstimulated and VEGF-stimulated cells in FBS- or hPL-supplemented DMEM [329]. No differences were observed between FBS- and hPL-cultured AT MSC with respect to the ability to form ring-like structures[329], which emphasizes the potential of hPL as replacement for FBS in MSC culture. Nonetheless, to produce clinical grade MSC, platelets free of infectious agents are crucial to prevent any possibility of disease transmission [307]. The potential of autologous human serum for the expansion of MSC has gained considerable attention [328], [334]–[336]. Autologous human serum has shown to induce increased MSC proliferation, differentiation potential, gene expression stability and motility compared with MSC cultured in FBS (reviewed in [307]). In addition, autologous serum-expanded MSC significantly decrease the percentage of IFN-ɣ producing activated T cells compared with FBS-expanded cells, which makes them more effective in immunomodulation [328]. Again, the collection of blood from elderly, diseased and inflamed patients could be a limiting factor for serum preparation for the *ex vivo* expansion of MSC for cardiac cell therapy [336]. Allogeneic human serum and human cord blood serum have also been considered as replacement for FBS. While an initial study using allogeneic

human serum for the *in vitro* expansion of MSC indicated overexpression of genes responsible for growth arrest and cell death [335], a more recent work evidenced an increased MSC proliferative profile of allogeneic serum-expanded MSC compared with cells cultured in FBS-containing medium [337]. Moreover, no significant differences were observed, concerning cell morphology, viability, differentiation capacity and immunophenotype [337]. Similar evidences were reported using pooled cord blood serum for the isolation and expansion of MSC [338]–[341]. This success is attributed to the lower level of hemagglutinin in pooled cord blood serum compared with adult serum, and lack of A and B hemagglutinin in pooled allogeneic AB-serum [341]. Despite some *in vitro* evidences of the preservation, or even improvement, of the cardiogenic and angiogenic potential of MSC expanded in serum-free conditions, there is still a lack of evidence of the *in vivo* potential of MSC expanded in such conditions, in AMI context.

I.3.5.5.2 Oxygen Concentration

The causes of cell death in an AMI event are influenced by the ischemic environment, which is devoid of nutrients and oxygen, along with the loss of survival signals for matrix attachment and cell-cell interactions [225]. In a cardiac cell therapy setting, such ischemic environment in the infarcted tissue may cause rapid loss of the transplanted MSC via apoptosis [342], [343]. Thus, preconditioning MSC by hypoxia (1-5% O_2) prior to cell administration may allow the cell to better adapt to the low oxygen concentration in the ischemic tissue and to promote cell engraftment [308]. In addition, tissues where MSC reside are hypoxic in nature, such as the bone marrow, adipose tissue, umbilical cord matrix and so forth [344], and thus hypoxic culture conditions will better resemble the MSC niche microenvironment. The hypoxic environment is characterized by the expression of HIF and especially HIF-1 plays an important role in maintaining the regenerative potential under low oxygen concentrations [307]. In hypoxic conditions, the lack of oxygen causes the suppression of the prolyl-hydroxylation process resulting in stability of HIF-1α, which facilitates its translocation to the nucleus. There, the HIF-1α complex regulates the transcription of genes involved in metabolism, angiogenesis, cell migration and cell fate [307].

Several studies have reported the ability of the *in vitro* hypoxic culture conditions to promote high MSC expansion rate, while maintaining homogeneity, differentiation potential and retard the cellular ageing process [104], [121], [345]. Moreover, MSC subjected to hypoxic conditions are shown to secrete several pro-angiogenic, anti-apoptotic and cardioprotective factors [307]. Kinnaird *et al*. showed a significant increase in the secretion of several arteriogenic factors, including VEGF, bFGF, PlGF and TGF-β, after exposing hMSC to 72 h of hypoxia, comparing to normoxia (20% O_2) conditions [346].

Similar evidences were reported by Ohnishi and colleagues, by analyzing the gene expression of rMSC after 24 h hypoxic pre-conditioning [347]. The results demonstrated that at least 165 genes, including VEGF, EGF and MMP-9 were upregulated >3 fold, comparing to normoxic conditions [347]. These studies are in line with the one recently developed by Paquet and colleagues, where MSC cultured under near anoxia (0.1% O_2) exhibited significantly enhanced chemotactic and pro-angiogenic properties and a significant decrease in the inflammatory profile [348]. Some of these bioactive factors, like VEGF, myocardin and IGF, can not only promote angiogenesis, but also the differentiation of MSC into cardiomyocytes [349], [350]. MSC exert anti-apoptotic effects on terminally ischemic cardiomyocytes through the secretion of several survival factors, such as protein kinase B (PKB or Akt) and endothelial nitric oxide synthase (eNOS) [351]. When cardiomyocytes are exposed to hypoxic conditions, MSC conditioned medium can significantly reduce the number of apoptotic and necrotic cells [225], [352], and MSC-secreted proteins directly influence the function of cardiomyocyte transmembrane ion channels [289]. *In vivo*, hypoxic preconditioning has shown to enhance the anti-apoptotic and remodeling capacity of MSC in rat myocardial infarction [353]. The results evidenced an increased expression of pro-survival and pro-angiogenic factors, as HIF-1, angiopoietin-1, VEGF and its receptors and erythropoietin, resulting in increased angiogenesis/vascularization and paracrine effects [353]. A recent study in nonhuman primates indicated that hypoxic preconditioning improved the effectiveness of MSC transplantation in AMI setting [354]. Hypoxia conditions increased the expression of several pro-survival/pro-angiogenic factors in cultured MSC and significantly improved infarct size and LV function at day 90 after AMI, comparing to normoxic culture conditions[354].

In addition to upregulating many angiogenic and anti-apoptotic factors, hypoxia is known to increase the expression of SDF-1 and its cognate receptor CXCR4, chemokine receptor CX3CR1 and, consequently, enhance MSC engraftment [355]–[358]. In 2010, Song *et al*. reported that ROS diminished BM MSC adherence to the substrate, but when treated with a ROS scavenger (N-acetyl-L-cysteine), engraftment was improved and the increase in fibrosis and infarct size prevented [224]. Other studies demonstrated that combining MSC and pharmacological hyperbaric oxygen led to enhanced cell engraftment and decreased fibrosis at four weeks after transplantation into rat myocardia [359], [360].

Despite the evidences showing the enhanced paracrine effect of MSC cultured under hypoxic conditions, there is a considerable variation of hypoxia exposure time between studies and in the resulting secretion levels of paracrine factors. It is also unclear how long hypoxia preconditioning effects last both *in vitro* and *in vivo* [4]. Moreover, hypoxic conditions unexpectedly attenuated MSC osteogenic potential [361], suggesting that the utility of hypoxia preconditioning may be application specific.

I.3.5.5.3 Mechanical Factors

Several authors proved that mechanical factors, such as fluid shear stress and mechanical strain, can regulate the proliferation, differentiation and paracrine activity of MSC through various signaling pathways, as previously discussed. As in the previous chapter, the response of MSC to mechanical stimuli will be divided into two different mechanisms: i) MSC differentiation towards the vascular and myogenic lineages; and ii) modulation of MSC paracrine activity.

The cells of the cardiovascular system experience numerous mechanical signals, from the fluid shear stress caused by the blood flow to the cyclic mechanical stretch in the myocardium and vessel walls. These signals regulate the functions of EC, SMC, myoblasts and cardiomyocytes [310]. More recently, it was found that such signals also regulate the *in vivo* differentiation of engrafted MSC in the heart. In order to better understand this phenomenon, several *in vitro* studies have been published focusing on the effects of fluid shear stress and strain on MSC differentiation into vascular cells. Fluid shear stress applied to murine C3H/10T1/2 mouse embryonic mesenchymal progenitor cells promoted the expression of EC markers such as CD31, von Willebrand factor (vWF) and VE-cadherin at the mRNA and protein level after 12h of steady shear stress levels of 15 dyn/cm^2 [362]. In 2009, Dong *et al* reported a model of tissue-engineered vascular graft to investigate the effects of shear stress on canine BM MSC differentiation [363]. Fluid shear stress significantly increased the expression of EC markers, such as PECAM-1, VE-cadherin and CD34, at both mRNA and protein levels as compared with static controls. Moreover, protein but not mRNA levels of smooth muscle α-actin and calponin were substantially reduced in shear stress-cultured cells. Such evidences seem to indicate that shear stress promotes endothelial differentiation, while downregulating differentiation towards a SMC phenotype. These results are not corroborated by the work developed by Kobayashi and colleagues, where fluid shear stress has been shown to increase the number of smooth muscle myosin heavy chain positive cells derived from rat BM MSC, after 36 h shear stress (14 dyn/cm^2) stimulation by pulsatile flow [364]. However, the amount of smooth muscle α-actin was not significantly different to static controls. In a comparative study, Kim and co-workers exposed MSC to a shearing stress of 2.5 or 10 dyn/cm^2 for 1 day [365]. The authors reported that MSC that have been exposed to low (2.5 dyn/cm^2) or high (10 dyn/cm^2) shear stress expressed different levels of EC markers such as vWF, calponin and CD31. Similarly, only in high shear stress conditions SMC markers (myocardin, myosin heavy chain and smooth mucle-22α) showed significantly higher expression, indicating that the effect of shear stress on differentiation depends on the stress intensity. Therefore, results concerning MSC differentiation into the vascular lineages remain controversial and more data is required. In addition to BM MSC, other sources have been used to assess MSC differentiation into endothelial-like cells under shear stress, such as AT [366]–[368], amniotic fluid [369] and placental [370] MSC. More recently, fluid shear stress was also found to induce cardiomyogenic differentiation of MSC. In 2010, Huang and colleagues submitted

rat BM MSC to laminar shear stress with a parallel plate-type device, in the presence or absence of 5-aza [371]. Appropriate fluid shear stress treatment alone was able to induce cardiomyogenic differentiation of rat BM MSC, as confirmed by the expression of cardiomyocyte-related markers at both mRNA and protein levels.

Strain was also found to induce vascular differentiation on MSC. Specifically, 2-D cyclic uniaxial strain has been shown to promote MSC differentiation into a vascular SMC phenotype [372], [373]. BM-derived progenitor cells subjected to 10% cyclic uniaxial strain at 60 rpm for 7 days present lower proliferation rate and higher expression of smooth muscle markers, smooth muscle α-actin and h1-calponin [372]. The authors also found that uniaxial cyclic strain causes cells to realign perpendicularly to the axis of strain, which was already reported in SMC [374]. More recently, Park *et al* reported an increase in SM markers (α-actin and smooth muscle 22-α) in MSC subjected to 5% strain at 60 rpm, but the expression returns to basal levels after the cells realign perpendicularly to the axis of strain [310]. Moreover, in contrast to uniaxial strain, the authors found that equiaxial strain decreases expression of both markers, revealing the importance of the type of strain for a particular response. The apparent opposing results may be due to differences in stimulation time, species-specific response or stem cell phenotype [375]. The orientation of the cells in regards to the axis of strain does also play an important role on directing differentiation. In 2006, Kurpinski *et al* demonstrated that cyclic strain with parallel-oriented microgrooves induces global changes in MSC, including an increase in the SM marker calponin-1, decreases in cartilage matrix markers, alterations in cell signaling and an increase in proliferation [376]. However, perpendicular orientation results in a decrease in gene changes and unaffected proliferation. MSC cardiomyocyte differentiation was also achieved by submitting rat BM MSC to cyclic strain, using a stretching device [377]. The results demonstrated that appropriate cyclic strain treatment alone can induce cardiomyogenic differentiation, as confirmed by the expression of cardiomyocyte-related markers at both mRNA and protein levels. Moreover, the authors found that cells exposed to the strain stimulation expressed cardiomyocyte-related markers at a higher level than the shear stimulation. In order to mimic the dynamic physiological environment, well-controlled *in vitro* environments have been assessed using multimodalities of mechanical stimulation. In 2008, O'Cearbhaill *et al* exposed BM MSC to a pulsatile pressure (40-120 mmHg), radial distention of 5% and a shear stress of 10 dyn/cm^2 at a frequency of 60 cycles per minute, for up to 24h [378]. Histological analysis revealed that most of the cells aligned within 20^0 to the direction of flow and adopted a compact cell size, as characteristic of EC. However, gene and protein analysis of both stimulated and static groups showed no EC marker vWF factor expression on either mRNA or protein level. Instead, stimulated cells exhibited greater levels of smooth muscle-associated markers SMA and calponin, in comparison to static samples.

Besides promoting cardiomyocyte differentiation, fluid shear stress and strain are also able to modulate MSC paracrine activity. Shear stress has been found to increase VEGF gene expression while decreasing TGF-β by approximately 50% at the transcriptional level, in a murine embryonic mesenchymal progenitor cell line [362]. This is in line with the results obtained by the same group in 2008, where MSC subjected to 15 dyn/cm^2 express VEGF, but significantly decrease the expression of other factors, such as TGF-β1, PDGF and its receptors – which are known to guide stem cells towards the SMC lineage [379]. In a different study with adult human MSC, shear stress shear stress significantly induced the upregulating of several angiogenic factors, including VEGF and FGF-1, along with vascular SMC markers, including SM-22α and calponin [310]. Unlike the previous study, these authors showed that shear stress promotes an upregulation of TGF-β1, instead of a downregulation. Human AT MSC were also found to secrete higher amounts of VEGF (2 fold *vs* static) when exposed to laminar shear stress of 10 dyn/cm^2 up to 96 h [366]. Recently, Carmelo *et al* reported a priming effect of stirred cultured conditions on BM and AT MSC cytokine production [94]. Both MSC sources secreted higher concentrations of VEGF and IL-6 in a XF microcarrier-based stirred culture system compared with the static conditions, throughout the 7 days of culture. Similar evidences were reported by Teixeira and colleagues, by using computer-controlled suspension bioreactors to enhance human MSC secretory profile [380]. Proteomic analysis revealed that the dynamic culturing of MSC increased the secretion of not only several neuroregulatory molecules, but also pro-angiogenic and pro-survival factors, such as VEGF and IGF-1. Although the previous results confirm the ability of mechanical factors to modulate MSC paracrine activity, more data is required to integrate a robust mechanical platform in the development of an MSC-based product with enhanced therapeutic features for myocardial regeneration.

I.3.5.5.4 Biochemical Factors

MSC preconditioning through the activation of different signaling pathways using biochemical factors has been extensively studied in the context of myocardial regeneration. Such biochemical factors include cytokines, chemokines and growth factors, as well as pharmacological agents, which modulate MSC differentiation and paracrine secretion *in vitro* [4].

Priming MSC with a cocktail of growth factors and cytokines has been found to enhance the expression of cardiac transcription factors and, thus, MSC cardiac therapeutic potential [381]. In this study, MSC were pretreated with a cocktail of growth factors including 50 ng/mL FGF-2, 2 ng/mL IGF-1 and 10 ng/mL bone morphogenic protein (BMP)-2 and its effect on viability and paracrine activity under hypoxic conditions was assessed. The cocktail was found to enhance the expression of cardiac markers and promote cell viability *in vitro* and to decrease the infarct size and improve cardiac function *in vivo*,

compared with untreated MSC. Neuropeptide Y, a neurotransmitter possessing similar efficacy promoting angiogenesis as bFGF and VEGF [382], proved to successfully induce BM MSC cardiomyogenic differentiation, leading to improved angiogenesis and cardiac function along with reduced fibrosis [383].

Several cytokines and growth factors have been used to stimulate MSC paracrine secretion. Stimulation with TNF-α (50 ng/mL) [384], or SDF-1 (50 ng/mL) for 1h [385], or TGF-α (250 ng/mL) and TNF-α (50 ng/mL) for 24h [386] improved VEGF secretion in the conditioned medium, compared with unstimulated MSC. *In vivo* studies revealed that preconditioning MSC with TGF-α + TNF-α not only induced an increase in VEGF expression, but also protected myocardium from ischemic/reperfusion (I/R) injury [386]. More recently, PDGF-BB treatment of MSC was also found to upregulate VEGF, promoting a greater capacity of functional recovery compared with naïve MSC in I/R injured heart [387]. Preconditioning MSC with growth factors has also proved to modulate their engraftment capacity. Incubation of rat MSC with IGF-1 was found to lead to increased MSC CXCR4 expression, increased survival, apoptosis inhibition and stimulation of anti-inflammatory activity [388], [389]. *In vivo*, IGF-1-treated MSC decreased post-infarct LV remodeling and improved cardiac function, in comparison with untreated MSC. Proinflammatory cytokines have also been used in MSC preconditioning in order to better simulate the proinflammatory microenvironment in the ischemic heart. Combining IL-1β and TNF-α, two proinflammatory cytokines, induced an increase in VCAM-1 expression in MSC and in adhesion intensity [390]. This combination of growth factors promoted a decrease in infarct size and improved LV function after MSC-treated intramyocardial transplantation in an AMI rat model. A different combination of cytokines (Flt-3 ligand, SCF, IL-6, HGF and IL-3) was used to stimulate MSC *in vitro*, resulting in a higher level of expression of CXCR4 and improved SDF-1-induced migration capacity (>20 fold *vs* unstimulated MSC) to the BM after 24h after transplantation [391]. Moreover, pretreated MSC showed enhanced homing (>2 fold *vs* unstimulated MSC) to the BM of irradiated mice 2 to 6 months after transplantation.

In addition to MSC *in vitro* preconditioning, Liang and co-workers suggested two other methods to improve the therapeutic efficiency of MSC using cytokines and/or growth factors: i) pretreatment at the site of injury, aiming at attracting more MSC for tissue repair and ii) simultaneous administration of MSC and cytokines/growth factors [392]. Similarly, pharmacological preconditioning has been shown to successfully increase the therapeutic efficacy of MSC in the cardioregenerative context. However, none of these strategies will be discussed here.

I.3.5.5.5 Biomaterials

One of the major limitations of a cardiac cell therapy approach is the very low survival rate after transplantation into the damaged heart tissue [393]. A widely purposed solution is cardiac tissue engineering, in which cells are delivered with or without biomaterials and/or bioactive factors to regenerate the cardiac functions. Adult stem cells, particularly MSC, have been extensively used in cell-based biomaterial approaches for myocardium regenerative purposes. MSC-biomaterial conjugated systems promote cardiac regeneration through two different mechanisms: i) *in vitro* and/or *in vivo* differentiation of MSC towards the cardiomyogenic lineage, and/or ii) secretion of pro-angiogenic bioactive factors.

In vitro/*vivo* differentiation of MSC-derived cardiac tissue has been accomplished using a variety of biomaterial scaffolds. Cutts and colleagues classified these scaffolds into the following categories: i) ECM protein-based biomaterials; ii) decellularized matrices; iii) natural biomaterial scaffolds; and iv) synthetic polymer-based materials [394]. Several studies proved that ECM mechanical and chemical properties significantly affect MSC fate and, thus, ECM protein-based biomaterials have been used as matrices to induce cardiomyocyte differentiation of MSC. Cardiogel, a naturally occurring ECM containing a complex mixture of laminin and fibronectin isolated from cardiac fibroblasts [395], has been used to induce cardiomyocyte differentiation in MSC [396]. Alternatively, several studies reported the use of both purified and recombinant ECM proteins as biomaterials for the generation of cardiomyocytes from MSC, including collagens type I, III, IV, V, laminin and fibronectin. Different types of collagen were found to play a critical role in promoting cardiomyogenic commitment [397]–[399]. *In vivo*, cardiac cells generated on collagen V matrices prevented chamber dilation and improved contractile function in AMI animal models [399]. Decellularized matrices have also been implemented to induce MSC cardiac differentiation. Recently, Sarig *et al*. decellularized full thickness ventricular matrices and repopulated them with MSC and human umbilical vein endothelial cells (HUVEC) to engineer fully vascularized cardiac tissue [400]. The results evidenced the ability of the scaffold to support human cardiomyocytes and to promote new blood vessel formation *ex vivo*. Nevertheless, polymer-based biomaterials (either natural or synthetic) are the most commonly used type of material for cardiac differentiation of adult stem cell populations. Liu *et al* demonstrated that chitosan scaffolds enhanced the cardiomyogenic potential of AT MSC when compared with cells cultured on standard tissue culture polystyrene substrates [401]. In a similar study, chitosan was shown to increase intracellular calcium levels in differentiating AT MSC and to upregulate the expression of the cardiac marker genes GATA-binding protein 4 (GATA4), NK2 homeobox 5 (NKX2.5) and myosin heavy chain-6 (MYH6) [402]. Moreover, Yang *et al* were able to efficiently differentiate MSC into cardiac tissue by culturing them on hybrid substrates consisting of silk fibroin and hyaluronic acid, when compared with cells differentiating only on silk fibroin matrices [403]. Later, the same group reported

that by incorporating chitosan into these silk fibroin/hyaluronic acid scaffolds significantly increased the cardiomyogenic differentiation of MSC [404]. *In vivo*, chitosan-hyaluronan/silk fibroin patches were able to significantly reduce the dilation of the inner diameter of LV of hearts and to promote a wide distribution of blood vessel-like structures in AMI regions. Several synthetic polymer-based materials have been engineered to enhance the differentiation of MSC toward the cardiac lineage. In a recent work, MSC adhesion and cardiac differentiation was assessed in polyurethane (PU), 3-hydroxybutyrate-*co*-4-hydroxybutyrate [P(3HB-*co*-4HB)] and polypropylene carbonate (PPC) scaffolds [405]. The authors found that PU and P(3HB-*co*-4HB) substrates induced optimal cell growth and cardiac differentiation. Polycaprolactone (PCL)-derived scaffolds, such as PCL carbon nanotube composites and PCL-based copolymers, were also shown to enhance cardiomyocyte differentiation of MSC [406], [407]. Moreover, composite scaffolds consisting of polymers and ECM proteins have been used to promote MSC cardiomyogenic differentiation. Poly(glycerol sebacate) (PGS)-collagen hybrid scaffolds proved to differentiate MSC more efficiently than substrates that only contained collagen [408]. MSC biological features, including proliferation, differentiation and immunomodulatory and trophic activities, are strongly modulated by the physicochemical properties of the biomaterial-based matrices/scaffolds, such as mechanical properties, morphology and composition [409]. Recently, Li *et al*. successfully induced cardiac differentiation of MSC *in vitro*, by culturing them in mechanically native-like thermosensitive hydrogels, with different modulus [410]. The results showed that 76% of MSC capsulated in hydrogel with higher modulus (65 kPa) expressed MYH6 and cardiac troponin I (CTnI) proteins, which are essential for cardiomyocytes to contract and relax, indicating the successful cardiomyocyte differentiation of MSC into cardiomyocytes. This modulus-induced method possessed higher cardiomyocyte differentiation efficiency comparing to traditional approaches (MSC co-culture with cardiomyocytes [411] or adding chemical factors, such as 5-aza [412]).

Besides several evidences of MSC cardiomyocyte differentiation and its outcome in heart regeneration, cardiac cell therapy can also be based on the ability of MSC to secrete pro-survival and pro-angiogenic factors responsible for improving heart functions in an AMI context. Wei *et al*. developed a bioengineered cardiac patch composed of sliced porous biological scaffold inserted with multilayered MSC [413]. MSC were seeded and, after 7 days of culture, the tissue construct was implanted *in vivo* using a rat MI model. The LV cavity size was reduced, and the pores were filled by cells with neo-connective tissue fibrils and neo-microvessels. Moreover, expression of angiogenic cytokines (bFGF, vWF and PDGF-B) and cardiac protective factors (IGF-1 and HGF) were also detected in transplanted MSC, revealing a paracrine-mediated effect on the damaged tissue. In a recent study, Abdeen *et al* demonstrated how controlling matrix rigidity and protein composition can influence the secretory profile of MSC [414]. The results showed that MSC cultured on fibronectin-modified hydrogels show stiffness dependence in proangiogenic signaling, with maximum influence on human microvascular endothelial

cells (HMVEC) tubulogenesis observed from 40 kPa. Such evidences are corroborated by a stiffness-dependent expression of multiple factors involved in angiogenesis. In 2015, Xu *et al*. developed a biodegradable hydrogel based on thiolated collagen and multiple acrylate containing oligo (acryloyl carbonate)-b-poly(ethylene glycol)-b-oligo (acryloyl carbonate) copolymers and tested it in a rat infarction model [415]. The results demonstrated that BM MSC-encapsulated gels significantly reduced the infarct size, increased heart wall thickness and the EF 28 days after implantation, although no cytokine analyses were performed. In a different study, alginate encapsulation of BM MSC proved to facilitate paracrine effects, such as increased angiogenesis and decreased scaring and cardiac function [416]. BM MSC also proved to be therapeutically successful in a patch consisting of autologous cells seeded in a collagen scaffold that was engrafted into the epicardial surface of a chronic AMI scar [417]. The treatment led to enhanced angiogenesis and significantly improved cardiac function. AT MSC were also demonstrated as a potential source for biomaterial-based cell therapy in AMI context. Araña and colleagues examined the effect of collagen patches seeded with AT MSC on cardiac function in models of chronic AMI [418]. The delivery of AT MSC in collagen substrates induced increased cell engraftment, reduced fibrosis and improvement in cardiac function, myocardial remodeling and revascularization. Moreover, encapsulation of AT MSC in alginate enhanced retention and survival of cells in a pig model of AMI [419]. However, no significant differences in heart rate and cardiac output were observed between treatment groups. Synthetic polymer-based approaches have also shown promising results on guiding MSC pro-angiogenic signaling. Implementing bioengineered polyglycolic acid (PGA) and poly(L-lactide-co-caprolactone) (PLCL) scaffolds seeded with MSC resulted in improved function of infarcted hearts in animal models, induced angiogenesis and reduced scar size [420], [421].

Direct comparisons between MSC injection alone and combined with biomaterials have been reported by several authors. An interesting work developed by Jin and co-workers compared the effectiveness of direct cell injection *versus* a cardiac patch-based approach [422]. MSC were directly transplanted into a cryoinjury infarct or seeded onto PLCL and implanted over the infarcted area 10 days post-AMI. Results showed MSC differentiation into cardiomyocytes, an increase of 23% in the EF and a decrease in the infarct area of 29% in the polymer + MSC group compared with saline and the acellular scaffold, 4 weeks post-treatment. Similar results were observed in the MSC-only group. In addition, intramyocardial injection of MSC with silanized hydroxypropyl methylcellulose in a rat AMI model showed better performance in cell retention and cardiac function preservation than MSC injection alone [423]. In a swine model of AMI, retention of MSC suspended in 2% alginate before transplantation was approximately 4-fold compared with that in control MSC, 2 weeks after delivery [424]. Similarly, AT MSC co-injection with fibrin glue increased cell survival by about 30% on a rat AMI model [425].

I.3.5.5.6 Two-Dimensional vs Three-Dimensional Culture

As previously mentioned, recent studies proved that culturing MSC as 3D spheroids can improve a range of biological properties, including multi-lineage differentiation potential, secretion of immunomodulatory and trophic factors and resistance against ischemic conditions [426].

Most of the studies exploring MSC spheroid potential for cardiac cell therapy focus on the augmented secretion of bioactive factors involved in paracrine signaling. However, cardiomyogenic differentiation of MSC cultured in 3D spheroids has been already reported [402]. In this study, AT MSC 3D spheroids were formed in chitosan membranes and cardiomyogenic differentiation was induced using 5-aza. After induction, a 4-fold increase in the expression levels of cardiac marker genes GATA4, NKX2.5, cardiac troponin T2 (TNNT2) and MYH6 was observed for AT MSC spheroids on chitosan vs tissue culture polystyrene or polyvinyl alcohol. Moreover, the authors suggest that Wnt11 may be involved in spheroid formation and cardiomyogenic differentiation of MSC on chitosan membranes, since its gene expression was significantly upregulated in such conditions.

The enhanced secretion profile of MSC cultured in 3D spheroids were first reported by Potapova *et al*, where the authors developed a strategy to organize 3D spheroids of varying sizes using a hanging drop protocol, in order to increase the secretion levels of paracrine factors [427]. 3D culture induced high concentrations (5-20 times) of IL-11 as well as the pro-angiogenic factors VEGF, bFGF and angiogenin in the conditioned medium from MSC spheroids compared with conditioned medium from monolayers. The hypoxic conditions observed in the core of the cell aggregates were proposed to be the driving force for the increased secretion levels. One year later, the same group verified that culturing MSC as 3D spheroids restored CXCR4 functional expression, demonstrated by 35% of the cells derived from day 3 spheroids being $CXCR4^+$ [428]. In 2010, the size of spheroid-derived MSC was found to be significantly smaller than MSC from adherent culture, allowing them to more easily avoid lung entrapment *in vivo* following systemic infusion [132]. Moreover, MSC 3D spheroids grown in suspension cultures were reported to secrete 60-fold more TSG-6 protein, compared with adherent monolayer MSC. Recently, MSC aggregation was found to significantly upregulate secretion of anti-inflammatory cytokines via a caspase-dependent mechanism [426]. Tsai *et al* demonstrated that formation of MSC 3D spheroids significantly upregulated caspase 3/7 expression, CXCR4 expression, cell migration, secretion of prostaglandin E2 (PGE-2) and IL-6 and resistance to *in vitro* ischemic stress. In a similar approach, Zimmermann and colleagues showed that culturing human MSC as 3D spheroids increased secretion of the immunomodulatory factors PGE-2, TGF-β1 and IL-6, as well as the intracellular enzyme indoleamine 2,3-dioxygenase (IDO) [136]. However, immunomodulatory factor secretion was found to highly depend on the composition of cell culture medium – serum supplemented medium displayed significantly higher expression of the immunomodulatory factors, comparing to a serum-free

formulation. MSC aggregation process not only induces upregulation of anti-inflammatory proteins, but also pro-angiogenic factors, including VEGF, FGF-2, HGF, CXCR4, EGF, SDF-1 and angiogenin [131]. The potential mechanism for the upregulation of pro-angiogenic factors relies upon the fact that assembly of multicellular aggregates creates spatial gradients of soluble factors such as oxygen, nutrients and regulatory molecules, resulting in a hypoxic microenvironment in the interior of the spheroids [426]. Different MSC sources have been shown to enhance the expression of pro-angiogenic factors when cultured in 3D spheroids. Gingiva-derived MSC spheroids cultured in ultra-low attachment conditions showed an increase of 35% in the $CXCR4^+$ population as well as an increase in the expression of other pro-angiogenic factors, including a 3-fold increase of VEGF and EGF and a 4-fold increase of angiogenin and SDF [429]. AT MSC 3D spheroids cultured in spinner flasks demonstrated a 5- to 10-fold increase in FGF-2, HGF and VEGF expression compared with dissociated cells [430]. Additionally, intramuscular transplantation of AT MSC spheroids to ischemic hindlimbs of athymic mice showed improved cell survival, angiogenic factor secretion, neovascularization and limb survival, comparing to dissociated transplanted cells. Human UCB MSC were also found to upregulate VEGF secretion (3-fold) when cultured in anchorage-deprived suspension 3D spheroids [431]. Moreover, transplantation of UCB MSC 3D spheroids into a rat AMI model resulted in improved LVEF and preventing pathological LV dilatation at 8 weeks post-AMI. The benefits of MSC 3D spheroids have been shown in other preclinical studies, in which intramyocardial transplantation of AT and BM MSC 3D spheroids in a porcine model improved cell retention, survival and integration [432], [433]. Xie and co-workers highlight three aspects responsible for the improved outcomes using 3D spheroid-derived MSC: i) the volume and diameter of cells released from spheroids are about 1/4 and 1/2 of 2-D MSC, respectively, reducing the chance of vascular obstruction, as previously mentioned; ii) the enhanced and long-term survival of spheroid-derived MSC transplanted into the ischemic regions; and iii) the enhanced vascularization and increased functional microvessel density of ischemic tissue compared with 2D-MSC [434]. As in 2D-culture, culture parameters such as culture size and oxygen concentration can modulate the secretory profile of MSC. For example, MSC 3D spheroids with a size of 10,000 cells cultured under 2% O_2 exhibited better production of VEGF than spheroids containing 60,000 cells that were cultured under 20% O_2 [435]. Therefore, careful considerations should be given to culture conditions to achieve optimal and consistent therapeutic results.

I.3.5.5.7 Co-Culture Systems

The combination of different cell populations possessing complementary effects may provide enhanced therapeutic outcomes regarding myocardial regeneration, comparing to any single cell type [436]. In

particular, MSC have been transplanted into ischemic hearts along with other cell populations, including CS/PC [437], [438] and EPC [439], [440], resulting in an enhanced recovery of the infarcted heart.

The ability of MSC to activate c-kit+ resident CSC within the heart [216] was used as the rationale for a study using a mixture of 200 million MSC together with 1 million CSC in a swine model of AMI [438]. MI size reduction was found to be 2-fold greater in the MSC-CSC combination group *vs* either cell group alone. Moreover, LVEF, as well as LV chamber dynamics, was improved in all treatment groups, demonstrating a cardiac recovery to levels near baseline. The same trend was described by Karantalis *et al*, in a study involving the administration of an autologous MSC and CPC combination in a porcine model of chronic ischemic cardiomyopathy [437]. Treatments with either MSC or the combination of MSC and CSC resulted in a significant reduction in scar size, increased viable tissue and improved wall motion. Improved EF, stroke volume, diastolic strain and cardiac output were only observed in the combination-treated animals, which also exhibited increased cardiomyocyte mitotic activity.

Cardioregenerative effect of MSC might be mediated by local paracrine angiogenesis, as previously mentioned, which may trigger resident EPC activation. Thus, transplanting co-culture systems of MSC and EPC into infarcted myocardial tissue may enhance MSC trophic activity, as well as EPC activation. In order to test this hypothesis, Suuronen and colleagues compared the effects of MSC transplantation *vs* EPC *vs* MSC-EPC co-culture in a rat model of myocardial infarction [439]. Postoperative LVEF (EPC – 68.3% ± 9.8%; MSC/EPC – 55.0% ± 11.1%; MSC – 53.0% ± 6.0%) and fractional shortening (EPC – 32.4% ± 5.1%; MSC – 22.5% ± 5.4%) were greater in EPC-treated rats *vs* those receiving other treatments. Arteriolar density within the infarcted border zone was also increased in EPC group, compared with PBS (297%), MSC (205%) and MSC/EPC (101%) groups. In addition, only EPC prevented further contractile deterioration compared with the baseline, whereas the other groups presented continued loss of function after treatment. However, Zhang and colleagues were able to successfully demonstrate the benefits of an EPC/MSC co-culture for myocardial regeneration, in a study where rats receiving both MSC and EPC experienced an increase in the expression levels of angiogenic growth factors, fewer collagen deposition and apoptotic cell number and an improvement in regional myocardial blood flow compared with the groups containing only one cell type [440].

MSC were also found to significantly increase the survival rate, migration and angiogenic potential of mature HUVEC in an *in vitro* co-culture system [441]. This study was carried out under hypoxic conditions to better mimic the microenvironment in the ischemic heart after an AMI event. Under low oxygen tension, MSC expression levels of SDF-1α, VEGF and IL-6 were upregulated and apoptosis was inhibited in HUVEC in the MSC/HUVEC group.

A recent combinatorial stem cell approach used CPC, MSC and EPC together in a cluster, the CardioCluster, to enhance endogenous repair within the heart for long-term improvement [442]. CardioClusters are rationally designed with MSC and CPC in the core and EPC forming the outer layer, since these cells play a vital role in forming neovasculature that will connect the CardioCluster to the living heart tissue, allowing for the revascularization of the damaged myocardium. In contrast, MSC reinforce the 3D structure of the cluster by releasing growth factors that attract and maintain cells within the cluster. *In vitro*, upon induction of an oxidative stress, CardioClusters showed improved cell survival with lower percentage of apoptotic and/or necrotic cell populations compared with the three populations individually. The efficacy of such approach was not yet proved *in vivo*, but small animal model studies are currently underway.

To date, no studies were found reporting the effects of co-culturing MSC with other cell populations regarding MSC differentiation towards the cardiovascular lineages.

I.3.5.6 Translating Mesenchymal Stem/Stromal Cell Microenvironment Modulation to Myocardial Cell Therapy

Several *in vivo* studies using different animal models have demonstrated the beneficial effect of administrating MSC to promote myocardial regeneration after an AMI event. In humans, recent meta-analyzes of randomized clinical trials have confirmed good safety of MSC-based therapies for cardiac regeneration, but have also showed a limited regenerative efficacy, with an increase in the LV systolic function of only 3-10% [355]. In order to increase MSC cardiac regenerative capacity, some clinical trials used cells that were previously preconditioned using different strategies. This chapter will only focus in these specific set of clinical trials.

Oxygen is one of the major plays in modulating the MSC microenvironment. In this context, a clinical trial studied the safety, tolerability and preliminary efficacy of allogeneic low oxygen-cultivated BM MSC (aLoOxMBMC) administrated intravenously to AMI patients. aLoOxMBMC were cultured under low oxygen concentrations and are thus considered ischemic tolerant cells. To date, no reports were found regarding the outcomes of this trial. In an autologous setting, BM MSC were preconditioned with hypoxia and a cocktail of cytokines to induce endothelial commitment and administrated to patients with ischemic hear diseases. The primary endpoint of this study is LVEF measurement 1 year after treatment. Again, no clinical data was found regarding this trial.

Preconditioning MSC with biochemical factors have been studied in the MyStromalCell trial [443], designed to investigate the safety and efficacy of intramyocardial delivery of autologous AT MSC

stimulated with VEGF-A165 to improve myocardial perfusion and exercise capacity in patients with chronic ischemic heart disease. Data collected showed that the treatment proved to be safe, but did not improve exercise capacity compared with placebo [444]. Another example of biochemical modulation of MSC for cardiac cell therapy is the Phase 3 clinical trial C-CURE [445]. Here, MSC were treated *ex vivo* with cytokines to enhance their commitment to cardiopoietic lineage – the BM-derived cardiopoietic cells. Bartunek and co-workers reported significant improvements in LVEF, end-systolic volume, and 6-minute walk distance, compared with the control group [445]. In this context, the CHART-1 trial was designed to validate cardiopoiesis-based therapy in a larger heart failure group [446]. Safety was demonstrated across the group, with exploratory analyses suggesting a benefit of cell treatment in the baseline of the LV end-diastolic volume of the patients.

The first clinical trial using injectable bioabsorbable scaffold (IK-5001), a solution of 1% sodium alginate and 0.3% calcium gluconate, combined with MSC by intracoronary delivery was carried out from 2010 until 2015. Preliminary results show that intracoronary deployment of an IK-5001 scaffold is feasible and well tolerated in patients with STEMI [237]. This first-in-man study provides initial proof of the effectiveness of cardiac tissue engineering on improving cardiac function in AMI patients.

MSC administration in combination with other cell populations has also been tested in clinical trials. In 2005, Katritsis and colleagues reported the administration of a combination of autologous BM MSC and EPC in 11 patients with AMI [447]. The follow-up data demonstrated improved LV function and myocardial perfusion in cell-treated patients, compared with the control population. Scintigraphic imaging indicated cellular repopulation/regeneration of nonviable infarct scares. A different approach combines the use of MSC and c-kit$^+$ CSC in AMI treatment, to further enhance the therapeutic effects of each cell type [438]. This approach will be tested in two different trials: the CONCERT-HF and the TAC-HFT. In the CONCERT-HF trial, ischemic heart failure patients will receive either 150 million MSC, 5 million CSC, a combination of 150 million MSC with 5 million CSC, or a placebo, administrated by transendocardial injection. The clinical trial TAC-HFT will enroll patients with idiopathic dilated cardiomyopathy to receive autologous transplantation of either 200 million MSC, a mixture of 199 million MSC and 1 million CSC, or a placebo. Both studies are in early implementation stages: CONCERT-HF has started recruiting in October 2015, while TAC-HFT is expected to start on March 2020 [436].

Although MSC pharmacological preconditioning has not been discussed in this thesis, some authors reported promising *in vitro* and *in vivo* data on conditioning MSC with different types of drugs, particularly to improve their immunomodulatory and regenerative properties. A Phase 2 trial is being developed to test whether intensive Atorvastatin therapy can improve the outcome of AMI patients who

underwent intracoronary administration of BM MSC. The trial is also in early stage of development and it is not recruiting yet.

Despite the emerging number of clinical trials involving MSC preconditioned through different strategies, there are no current trials making direct comparisons of the effects of preconditioned *vs* nonpreconditioned MSC in the treatment of any cardiac disorder. All the studies mentioned above compared the data obtained to placebo controls, and not to nonpreconditioned cells. In order to fully understand the importance, or not, of preconditioning MSC to cardiac cell therapy, studies performing side-by-side comparison between preconditioned and nonpreconditioned MSC must be carried out.

I.4 Future Directions and Challenges

Current data generated from preclinical and early phase clinical studies have demonstrated the modest contribution of MSC to restore the structural integrity and functionality of the damaged heart. To achieve the best clinical outcome after MSC therapy, several aspects of cell transplantation must be considered, including best MSC source, dose of cells applied, overall patient condition, duration of the disease (acute *vs* chronic), method for cell delivery, age of the patient, and age of the cell donor in case of allogeneic transplantation [448]. Additionally, further studies are required to decipher the fundamental and detailed biological mechanisms associated with MSC cardiac regenerative potential [392]. Here, we will address the scientific-based parameters, namely the best cell source and the MSC mechanisms of action.

The choice of the best MSC source is still a matter of debate, probably due to the lack of data comparing the *in vitro* and *in vivo* cardiac regenerative potential of each of the sources. Several studies have demonstrated that MSC from different sources secrete different levels of trophic factors or propensity toward different lineages, as previously discussed. However, there are many discrepancies among publishing data regarding the properties of each MSC source and thus more detailed studies are needed to obtain consistent results. Such studies will provide a better characterization of the source and may help improve cell preparation methods for specific clinical trials [448]. Conversely, some authors defend that a successful myocardial cell therapy cannot be achieved by just one cell type [438]. Instead, The future of MSC therapy may lie in being the main supporting, trophic and orchestrating cell type in a therapy that will combine different cell types with unique characteristics for myocardial regeneration [449].

Another important challenge regarding the choice of the best cell source is based on the use of autologous *vs* allogeneic cells. Due to MSC low immunogenicity, researchers have been employing both types of cells in clinical studies for myocardial regeneration, where no differences were observed [450].

Nonetheless, further studies are required to fully understand the implications of the two types of therapies. Allogeneic cells have advantages over autologous cells, including the fact that allogeneic cells can be expanded, characterized and prepared more quickly as off-the-shelf products that are ready to be applied when needed. In addition, autologous cells could suffer from age/disease-related host tissue impairment. This risk can be overcame through the application of healthy, young allogeneic cells [448].

MSC cardiac therapy can greatly benefit from the development of sophisticated methods to modulate cell paracrine activity or differentiation ability and thus increasing MSC efficacy. Besides the ones discussed here, pharmacological treatment [27] and genetic engineering [20] also seem to improve MSC therapeutic potential. Genetic engineering, particularly, have demonstrated to greatly improve MSC survival [451], [452], homing [453] and paracrine action [454]–[456]. However, this procedure raises ethical and safety questions and, therefore, application of genetic engineering to cell therapies still seems far on the horizon.

Even if an innovative cell therapy is able to comply with the requirements mentioned above, resulting in a greatly improved potential of an MSC-based product, it is still crucial to understand the molecular mechanisms behind the enhanced therapeutic outcome. Thus, the many effects of MSC in the cardiac context, including transdifferentiation, cell fusion and paracrine secretion, may raise some issues that need to be addressed to obtain maximal benefit. Transdifferentiation and cell fusion, for instance, seem to occur at a very low frequency to account for the meaningful improvements [392]. This fact may be related to the low percentage of MSC engraftment in the damaged tissue. More sophisticated approaches should be developed to specifically target the myocardium tissue and, consequently, improve the success of MSC engraftment. Regarding paracrine actions, some cytokines and chemokines secreted by MSC may be harmful, such as TNF-α and IL-6, which means that a more detailed knowledge of their signaling pathways is mandatory. Nevertheless, the paracrine mechanisms allow the design of a molecular therapy, i.e., applying one trophic factor alone or combined with others as a cocktail therapy [392].

Recently, scientists have been demonstrating particular interest in the potential of extracellular vesicles (EV) secreted by MSC as mediators of their therapeutic potential. EV are a heterogeneous group of nanoparticles composed of a lipid layer enclosing cytoplasmic components, as proteins, nucleic acids and lipids [457]. EV transmit signals to target cells by interacting at the cell surface, by internalization or by fusion with the target cell membrane. The EV cargo can be engineered to enhance their properties or introduce specific effector molecules [457]. These reasons justify the potential use of EV in myocardial regeneration.

In this context, establishing scalable and efficient MSC expansion protocols able to comply with GMP guidelines will greatly contribute towards the development of a robust MSC product or MSC-derived EV product.

I.5 Aim of Study and Thesis Outline

The aim of this thesis was to develop a potential MSC-based product with enhanced therapeutic features for myocardial regeneration. The improvement of the therapeutic potential of the cells was performed through the modulation of their microenvironment, either by biochemical (preconditioning one MSC source with conditioned medium retrieved from other MSC sources) or mechanical (preconditioning MSC through a microcarrier-based culture system and low oxygen concentration (2%)) approaches. Moreover, a S/XF microcarrier-based culture system was successfully established for the expansion of MSC using an innovative disposable bioreactor system, the Vertical-Wheel™ PBS-0.1 MAG system, combined with a commercially available fibrinogen-depleted hPL-based culture supplement.

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

Chapter II – Characterization of Different Sources of Human Mesenchymal Stem/ Stromal Cells

This chapter describes the successful characterization of different sources of human MSC cultured in S/XF conditions, in terms of immunophenotype, proliferative capacity, expression and secretion of growth factors and cytokines and *in vitro* angiogenic capacity. Such characterization if of utmost importance since different MSC sources present different intrinsic cell properties, particularly regarding their proliferative capacity, immunophenotype, differentiation ability, cytokine secretion profile, immunomodulatory and regenerative properties. Therefore, the choice of a specific MSC source for a cell therapy application depends on the characteristics of the disease. Moreover, culturing cells under S/XF conditions is also crucial to comply with GMP guidelines and to guarantee the approval of the regulatory agencies.

In this chapter, human MSC isolated from three different sources (BM, AT and UCM) were expanded in S/XF culture conditions and characterized as mentioned above. All the three sources studied displayed the typical MSC immunophenotypic profile. Under the culture conditions studied, UCM MSC demonstrated a higher proliferative capacity, compared with AT and BM MSC. Gene expression

analysis revealed that VEGF and HGF were overexpressed in UCM MSC, compared with both AT and BM, whereas bFGF was upregulated in AT MSC compared with the other sources. Regarding protein analysis, UCM MSC secreted higher levels of angiogenin and HGF, compared with the other sources, while AT MSC secreted higher concentrations of the proangiogenic factors angiopoietin-2, EGF, bFGF, HB-EGF, leptin, PDGF-BB, PlGF and VEGF. CM retrieved from UCM MSC cultures originated HUVEC tube-like structures with higher number of tubes, while AT MSC CM induced the formation of a higher number of branch points. Conversely, BM MSC CM promoted the migration of a higher percentage of HUVEC, compared with AT and UCM MSC CM.

The results indicate that MSC isolated from different sources present different biological properties, which must be taken in consideration when designing an improved cellular therapy for a specific disease.

Chapter III – Biochemical Modulation of the *In Vitro* Angiogenic Potential of Human Mesenchymal Stem/ Stromal Cells

This chapter aimed at studying the effect of culturing one source of MSC with the CM collected from other MSC sources on their *in vitro* angiogenic properties, in a process named cell preconditioning through indirect cell culture. The modulation of the cell microenvironment has demonstrated to increase the therapeutic properties of the cells, without raising concerns about the approval by the regulatory agencies, such as genetic engineering. While in the biochemical modulation approach, cells are treated with growth factors or chemokines and, in the co-culture cell preconditioning, cells are in direct contact with different cell types, indirect cell preconditioning utilizes the factors secreted by other cell populations (secretome), as well as EV, to improve the therapeutic performance of the target cells.

In this chapter, the three MSC sources (BM, AT and UCM) were cultured and the supernatant generated from each source was collected to precondition the other MSC sources. The effect of this preconditioning strategy was evaluated on MSC immunophenotype, proliferative capacity, expression of proangiogenic factors and *in vitro* HUVEC tube-like structure formation capacity. Results showed that preconditioning MSC with CM retrieved from other MSC sources did not alter the immunophenotypic and proliferative profiles of the cells. However, both the expression of proangiogenic genes and the *in vitro* HUVEC tube-like structure formation capacity are modulated by culturing a MSC source with CM retrieved from other sources. BM MSC cultured with the supernatant collected from AT or UCM MSC increase VEGF and HGF gene expression. In contrast, UCM MSC cultured with BM MSC CM express lower levels of VEGF and bFGF, compared with control conditions. Moreover, BM MSC cultured with CM retrieved from both AT or UCM MSC increase the number of

HUVEC tubes and number of branch points. Conversely, UCM MSC conditioned with BM or AT MSC CM induces a decrease in tube number and number of branch points.

These findings demonstrated that the *in vitro* angiogenic potential of a specific MSC source can be modulated by culturing it with a different MSC source, without compromising cell immunophenotypic and proliferative capacities. These results contributed to the rationale production of a cocktail of factors designed to increase the angiogenic properties of a potential MSC-based product for AMI.

Chapter IV – Bioprocessing Modulation of the *In Vitro* Angiogenic Potential of Human Mesenchymal Stem/ Stromal Cells

Chapter IV aimed at analyzing the effect of dynamic culture conditions and low oxygen concentration on the *in vitro* angiogenic potential of UCM MSC. Moreover, the impact of cell harvesting, storage and delivery on the angiogenic gene profile of these cells was also assessed. Preconditioning cells through the modulation of factors involved in their manufacturing is of utmost interest since they can be easily adapted to the production processes and usually do not require the acquisition of additional material. In this context, dynamic culture and oxygen concentration have raised great interest. Microcarrier-based dynamic culture systems present innumerous advantages and thus these systems are replacing static ones. Additionally, several reports have demonstrated the improved proliferative capacity and metabolism of MSC cultured under low oxygen concentrations. The impact of cell harvesting, cryopreservation and delivery steps is also poorly understood on the therapeutic efficacy of MSC.

Physically preconditioning UCM MSC through a microcarrier-based culture platform and low oxygen concentration (2% O_2) promoted an upregulation of the proangiogenic genes VEGF, bFGF and HGF, compared with MSC cultured under static and normoxic conditions. The increased expression was also observed at the secretion level, where several proangiogenic factors were also upregulated, including not only VEGF, bFGF and HGF, but also EGF, HB-EGF, PlGF, angiogenin, and PDGF-BB. Functional analyses evidenced a superior *in vitro* angiogenic potential of the preconditioned cells. CM retrieved from these cells resulted in an increased number of HUVEC tubes and branch points, as well as increased HUVEC migration through the transwell system. UCM MSC harvested from the microcarriers experienced a downregulation of the proangiogenic genes studied. However, both cryopreservation and delivery processes, at room temperature or 4°C, preserved the angiogenic gene expression profile of preconditioned UCM MSC.

These findings make the preconditioned UCM MSC a powerful candidate for an allogeneic, off-the-shelf product for myocardial regeneration.

Chapter V – Scalable Expansion of Human Mesenchymal Stem/ Stromal Cells in the Single-Use, Vertical-Wheel™ Bioreactor System

The last chapter described the establishment of a S/XF scalable culture platform for the expansion of MSC, using the innovative Vertical-Wheel™ PBS-0.1 MAG culture system combined with a commercially available fibrinogen-depleted human platelet lysate-based culture supplement (UltraGRO™-PURE). Microcarrier-based dynamic culture has proved to enhance the *in vitro* angiogenic potential of UCM MSC. Moreover, these structures have also proved to successfully expand MSC when cultured in dynamic culture systems, such as spinner flasks, stirred tank reactors and wave-mixed bioreactors. Several advantages arise from the use of microcarriers for MSC culture, including large surface-to-volume ratio, reduced culture volume required, and more homogeneous distribution of nutrients and growth factors required for cell metabolism. Microcarrier-base culture systems should comply with GMP guidelines. S/XF materials, as well as disposable products, are prerequisites to reduce batch variability, xenogeneic contamination and risk of bacterial and fungal contamination, which contributes to obtain the approval of the regulatory agencies.

In this chapter, different types of microcarriers and S/XF culture media were screened for the expansion of UCM MSC. After selecting the combinations of microcarriers and culture medium with the highest UCM MSC proliferation potential under static conditions, these combinations were tested under stirred culture conditions, using spinner flasks. Additionally, a different bioreactor configuration, the Vertical-Wheel™ PBS-0.1 MAG system, was tested and optimized for UCM and AT MSC expansion. The impact of the PBS-0.1 MAG culture system on MSC immunophenotype and multilineage differentiation capacity was analyzed. Results show that microcarriers and culture medium screening under static conditions did not promote UCM MSC expansion in spinner flasks. Therefore, a S/XF protocol based on fibrinogen-depleted hPL supplement (UltraGRO™-PURE) was used for further studies, since this protocol has already proved to successfully expand UCM MSC in spinner flasks. By adapting the initial agitation protocol and the feeding regime, cell expansion rate and final cell yield were maximized using an innovative disposable bioreactor system utilizing the Vertical-Wheel™ technology (PBS-0.1 MAG with maximum working volume of 100 mL). UCM MSC were successfully expanded to a maximum cell density of $5.3 \pm 0.4 \times 10^5$ cell/mL, after 7 days of culture (cell viability $\geq 94\%$). Similarly, AT MSC were also efficiently expanded to a maximum cell density of $3.6 \pm 0.7 \times 10^5$ cell/mL, also after 7 days of culture (cell viability $\geq 96\%$). UCM MSC maintained their identity (e.g. immunophenotype and multilineage differentiation capacity), after culture in the PBS-0.1 MAG system.

The establishment of such platform featuring easy scalability to higher volumes represents an important advance in obtaining clinically meaningful MSC numbers with possible enhanced therapeutic features.

I.6 References of Chapter I

[1] S. Mendis, P. Puska, and B. Norrving, *Global Atlas on Cardiovascular Disease Prevention and Control*. World Health Organization in collaboration with the World Heart Federation and the World Stroke Organization, 2011.

[2] W. H. Organization, "WHO | Cardiovascular Diseases (CVDs)," 2017. [Online]. Available: http://www.who.int/mediacentre/factsheets/fs317/en/.

[3] S. Mendis, K. Thygesen, K. Kuulasmaa, S. Giampaoli, M. Mahonen, K. N. Blackett, and L. Lisheng, "World Health Organization definition of myocardial infarction: 2008-09 revision," *Int. J. Epidemiol.*, vol. 40, no. 1, pp. 139–146, 2011.

[4] S. H. Ranganath, O. Levy, M. S. Inamdar, and J. M. Karp, "Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease.," *Cell Stem Cell*, vol. 10, no. 3, pp. 244–58, Mar. 2012.

[5] S. W. M. van den Borne, J. Diez, W. M. Blankesteijn, J. Verjans, L. Hofstra, and J. Narula, "Myocardial remodeling after infarction: the role of myofibroblasts.," *Nat. Rev. Cardiol.*, vol. 7, no. 1, pp. 30–37, Jan. 2010.

[6] H. Wang, J. Zhou, Z. Liu, and C. Wang, "Injectable cardiac tissue engineering for the treatment of myocardial infarction.," *J. Cell. Mol. Med.*, vol. 14, no. 5, pp. 1044–1055, May 2010.

[7] A. Alcon, E. Cagavi Bozkulak, and Y. Qyang, "Regenerating functional heart tissue for myocardial repair.," *Cell. Mol. Life Sci.*, vol. 69, no. 16, pp. 2635–2656, Aug. 2012.

[8] Y.-W. Kwon, H.-M. Yang, and H.-J. Cho, "Cell Therapy for Myocardial Infarction," *International Journal of Stem Cells*, vol. 3, no. 1. pp. 8–15, May-2010.

[9] M. Caccamo, P. Eckman, and R. John, "Current state of ventricular assist devices.," *Curr. Heart Fail. Rep.*, vol. 8, no. 2, pp. 91–98, Jun. 2011.

[10] G. Fonarow, "Heart failure: recent advances in prevention and treatment," *Rev Cardiovasc Med*, vol. 1, no. 1, pp. 25–33, 2000.

[11] L.-C. Hsiao, C. Carr, K.-C. Chang, S.-Z. Lin, and K. Clarke, "Stem cell-based therapy for ischemic heart disease.," *Cell Transplant.*, vol. 22, no. 4, pp. 663–675, 2013.

[12] S. Dimmeler and A. M. Zeiher, "Cell therapy of acute myocardial infarction: open questions.," *Cardiology*, vol. 113, no. 3, pp. 155–160, 2009.

[13] C. E. Murry, L. J. Field, and P. Menasche, "Cell-based cardiac repair: reflections at the 10-year point.," *Circulation*, vol. 112, no. 20, pp. 3174–3183, Nov. 2005.

[14] A. Behfar, R. Crespo-Diaz, A. Terzic, and B. J. Gersh, "Cell therapy for cardiac repair—lessons from clinical trials," *Nat. Rev. Cardiol.*, vol. 11, p. 232, Mar. 2014.

[15] E. Cambria, F. S. Pasqualini, P. Wolint, J. Gunter, J. Steiger, A. Bopp, S. P. Hoerstrup, and M. Y. Emmert, "Translational cardiac stem cell therapy: advancing from first-generation to next-generation cell types.," *NPJ Regen. Med.*, vol. 2, p. 17, 2017.

[16] C. Templin, T. F. Luscher, and U. Landmesser, "Cell-based cardiovascular repair and regeneration in acute myocardial infarction and chronic ischemic cardiomyopathy-current status and future developments.," *Int. J. Dev. Biol.*, vol. 55, no. 4–5, pp. 407–417, 2011.

[17] T. Y. L. Le, S. Thavapalachandran, E. Kizana, and J. J. H. Chong, "New Developments in Cardiac Regeneration," *Hear. Lung Circ.*, vol. 26, no. 4, pp. 316–322, 2017.

[18] S. K. Sanganalmath and R. Bolli, "Cell therapy for heart failure: a comprehensive overview of experimental and clinical studies, current challenges, and future directions.," *Circ. Res.*, vol. 113, no. 6, pp. 810–834, Aug. 2013.

[19] H.-F. Tse, C.-W. Siu, S.-G. Zhu, L. Songyan, Q.-Y. Zhang, W.-H. Lai, Y.-L. Kwong, J. Nicholls, and C.-P. Lau, "Paracrine effects of direct intramyocardial implantation of bone marrow derived cells to enhance neovascularization in chronic ischaemic myocardium.," *Eur. J. Heart Fail.*, vol. 9, no. 8, pp. 747–753, Aug. 2007.

[20] M. Gnecchi, Z. Zhang, A. Ni, and V. J. Dzau, "Paracrine mechanisms in adult stem cell signaling and therapy.," *Circ. Res.*, vol. 103, no. 11, pp. 1204–1219, Nov. 2008.

[21] C. Mias, O. Lairez, E. Trouche, J. Roncalli, D. Calise, M.-H. Seguelas, C. Ordener, M.-D. Piercecchi-Marti, N. Auge, A. N. Salvayre, P. Bourin, A. Parini, and D. Cussac, "Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction.," *Stem Cells*, vol. 27, no. 11, pp. 2734–2743, Nov. 2009.

[22] P. W. Burridge, G. Keller, J. D. Gold, and J. C. Wu, "Production of de novo cardiomyocytes: human

pluripotent stem cell differentiation and direct reprogramming.," *Cell Stem Cell*, vol. 10, no. 1, pp. 16–28, Jan. 2012.

[23] J. J. H. Chong, X. Yang, C. W. Don, E. Minami, Y.-W. Liu, J. J. Weyers, W. M. Mahoney, B. Van Biber, S. M. Cook, N. J. Palpant, J. A. Gantz, J. A. Fugate, V. Muskheli, G. M. Gough, K. W. Vogel, C. A. Astley, C. E. Hotchkiss, A. Baldessari, L. Pabon, H. Reinecke, E. A. Gill, V. Nelson, H.-P. Kiem, M. A. Laflamme, and C. E. Murry, "Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts.," *Nature*, vol. 510, no. 7504, pp. 273–277, Jun. 2014.

[24] J. J. H. Chong and C. E. Murry, "Cardiac Regeneration Using Pluripotent Stem Cells – Progression to Large Animal Models," *Stem cell research*, vol. 13, no. 0. pp. 654–665, Nov-2014.

[25] Y. Shiba, S. Fernandes, W.-Z. Zhu, D. Filice, V. Muskheli, J. Kim, N. J. Palpant, J. Gantz, K. W. Moyes, H. Reinecke, B. Van Biber, T. Dardas, J. L. Mignone, A. Izawa, R. Hanna, M. Viswanathan, J. D. Gold, M. I. Kotlikoff, N. Sarvazyan, M. W. Kay, C. E. Murry, and M. A. Laflamme, "Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts.," *Nature*, vol. 489, no. 7415, pp. 322–325, Sep. 2012.

[26] P. K. Nguyen, J.-W. Rhee, and J. C. Wu, "Adult Stem Cell Therapy and Heart Failure, 2000 to 2016: A Systematic Review.," *JAMA Cardiol.*, vol. 1, no. 7, pp. 831–841, Oct. 2016.

[27] A. Singh, A. Singh, and D. Sen, "Mesenchymal stem cells in cardiac regeneration: a detailed progress report of the last 6 years (2010-2015).," *Stem Cell Res. Ther.*, vol. 7, no. 1, p. 82, Jun. 2016.

[28] A. Maximov, "Relation of blood cells to connective tissues and endothelium," *Physiol. Rev.*, vol. 4, no. 4, pp. 533–563, 1924.

[29] E. A. MCCULLOCH and J. E. TILL, "The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice.," *Radiat. Res.*, vol. 13, pp. 115–125, Jul. 1960.

[30] A. J. Friedenstein, R. K. Chailakhjan, and K. S. Lalykina, "The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells," *Cell Tissue Kinet*, vol. 3, no. 4, pp. 393–403, 1970.

[31] J. J. Minguell, A. Erices, and P. Conget, "Mesenchymal Stem Cells," *Exp. Biol. Med.*, vol. 226, pp. 507–520, 2001.

[32] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, a Keating, D. Prockop, and E. Horwitz, "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.," *Cytotherapy*, vol. 8, no. 4, pp. 315–7, Jan. 2006.

[33] G. Chamberlain, J. Fox, B. Ashton, and J. Middleton, "Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing.," *Stem Cells*, vol. 25, no. 11, pp. 2739–49, Nov. 2007.

[34] D. Menicanin, P. M. Bartold, A. C. W. Zannettino, and S. Gronthos, "Genomic profiling of mesenchymal stem cells.," *Stem Cell Rev.*, vol. 5, no. 1, pp. 36–50, Mar. 2009.

[35] A. I. Caplan and D. Correa, "The MSC: an injury drugstore.," *Cell Stem Cell*, vol. 9, no. 1, pp. 11–5, Jul. 2011.

[36] P. a Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, a J. Katz, P. Benhaim, H. P. Lorenz, and M. H. Hedrick, "Multilineage cells from human adipose tissue: implications for cell-based therapies.," *Tissue Eng.*, vol. 7, no. 2, pp. 211–28, Apr. 2001.

[37] E. H. Javazon, K. J. Beggs, and A. W. Flake, "Mesenchymal stem cells: paradoxes of passaging.," *Exp. Hematol.*, vol. 32, no. 5, pp. 414–25, May 2004.

[38] D. C. Colter, R. Class, C. M. DiGirolamo, and D. J. Prockop, "Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 7, pp. 3213–8, Mar. 2000.

[39] E. Ben-Ami, S. Berrih-Aknin, and A. Miller, "Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases.," *Autoimmun. Rev.*, vol. 10, no. 7, pp. 410–5, May 2011.

[40] R. M. Samsonraj, B. Rai, P. Sathiyanathan, K. J. Puan, O. Rotzschke, J. H. Hui, M. Raghunath, L. W. Stanton, V. Nurcombe, and S. M. Cool, "Establishing criteria for human mesenchymal stem cell potency.," *Stem Cells*, vol. 33, no. 6, pp. 1878–1891, Jun. 2015.

[41] L. D. S. Meirelles, A. M. Fontes, D. T. Covas, and A. I. Caplan, "Mechanisms involved in the therapeutic properties of mesenchymal stem cells.," *Cytokine Growth Factor Rev.*, vol. 20, no. 5–6, pp. 419–27, 2009.

[42] K. English, A. French, and K. J. Wood, "Mesenchymal stromal cells: facilitators of successful transplantation?," *Cell Stem Cell*, vol. 7, no. 4, pp. 431–42, Oct. 2010.

[43] K. Bieback, P. Wuchter, D. Besser, W. Franke, M. Becker, M. Ott, M. Pacher, N. Ma, C. Stamm, H. Klüter, A. Müller, and A. D. Ho, "Mesenchymal stromal cells (MSCs): science and f(r)iction.," *J. Mol. Med. (Berl).*, vol. 90, no. 7, pp. 773–82, Jul. 2012.

[44] A. Krasnodembskaya, Y. Song, X. Fang, N. Gupta, V. Serikov, J.-W. Lee, and M. A. Matthay, "Antibacterial Effect of Human Mesenchymal Stem Cells Is Mediated in Part from Secretion of the Antimicrobial Peptide LL-37," *Stem Cells*, vol. 28, no. 12, pp. 2229–2238, 2010.

[45] S. Kern, H. Eichler, J. Stoeve, H. Klüter, and K. Bieback, "Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue.," *Stem Cells*, vol. 24, no. 5, pp. 1294–301, May 2006.

[46] C. De Bari, F. Dell'Accio, P. Tylzanowski, and F. P. Luyten, "Multipotent mesenchymal stem cells from adult human synovial membrane.," *Arthritis Rheum.*, vol. 44, no. 8, pp. 1928–42, Aug. 2001.

[47] A. Santhagunam, F. Dos Santos, C. Madeira, J. B. Salgueiro, and J. M. S. Cabral, "Isolation and ex vivo expansion of synovial mesenchymal stromal cells for cartilage repair.," *Cytotherapy*, vol. 16, no. 4, pp. 440–453, Apr. 2014.

[48] I. N. Simões, J. S. Boura, F. Dos Santos, P. Z. Andrade, C. M. P. Cardoso, J. M. Gimble, C. L. da Silva, and J. M. S. Cabral, "Human mesenchymal stem cells from the umbilical cord matrix: Successful isolation and ex vivo expansion using serum-/xeno-free culture media.," *Biotechnol. J.*, vol. 8, no. 4, pp. 448–58, Apr. 2013.

[49] K. Igura, X. Zhang, K. Takahashi, A. Mitsuru, S. Yamaguchi, and T. A. Takashi, "Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta.," *Cytotherapy*, vol. 6, no. 6, pp. 543–553, 2004.

[50] P. S. In 't Anker, S. A. Scherjon, C. Kleijburg-van der Keur, W. A. Noort, F. H. J. Claas, R. Willemze, W. E. Fibbe, and H. H. H. Kanhai, "Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation.," *Blood*, vol. 102, no. 4. United States, pp. 1548–1549, Aug-2003.

[51] R. Hass, C. Kasper, S. Bohm, and R. Jacobs, "Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC.," *Cell Commun. Signal.*, vol. 9, p. 12, May 2011.

[52] F.-J. Lv, R. S. Tuan, K. M. C. Cheung, and V. Y. L. Leung, "Concise review: the surface markers and identity of human mesenchymal stem cells.," *Stem Cells*, vol. 32, no. 6, pp. 1408–1419, Jun. 2014.

[53] C. Muniz, C. Teodosio, A. Mayado, A. T. Amaral, S. Matarraz, P. Barcena, M. L. Sanchez, I. Alvarez-Twose, M. Diez-Campelo, A. C. Garcia-Montero, J. F. Blanco, M. C. Del Canizo, J. del Pino Montes, and A. Orfao, "Ex vivo identification and characterization of a population of CD13(high) CD105(+) CD45(-) mesenchymal stem cells in human bone marrow.," *Stem Cell Res. Ther.*, vol. 6, p. 169, Sep. 2015.

[54] A. M. de Soure, A. Fernandes-Platzgummer, C. L. da Silva, and J. M. S. Cabral, "Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells.," *J. Biotechnol.*, vol. 236, pp. 88–109, Oct. 2016.

[55] W. Wagner, R. E. J. Feldmann, A. Seckinger, M. H. Maurer, F. Wein, J. Blake, U. Krause, A. Kalenka, H. F. Burgers, R. Saffrich, P. Wuchter, W. Kuschinsky, and A. D. Ho, "The heterogeneity of human mesenchymal stem cell preparations--evidence from simultaneous analysis of proteomes and transcriptomes.," *Exp. Hematol.*, vol. 34, no. 4, pp. 536–548, Apr. 2006.

[56] Q. Liu, G. Cheng, Z. Wang, S. Zhan, B. Xiong, and X. Zhao, "Bone marrow-derived mesenchymal stem cells differentiate into nerve-like cells in vitro after transfection with brain-derived neurotrophic factor gene," *In Vitro Cellular & Developmental Biology. Animal*, vol. 51, no. 3. Boston, pp. 319–327, 2015.

[57] F. Duenas, V. Becerra, Y. Cortes, S. Vidal, L. Saenz, J. Palomino, M. De Los Reyes, and O. A. Peralta, "Hepatogenic and neurogenic differentiation of bone marrow mesenchymal stem cells from abattoir-derived bovine fetuses.," *BMC Vet. Res.*, vol. 10, p. 154, Jul. 2014.

[58] M. J. Oedayrajsingh-Varma, S. M. van Ham, M. Knippenberg, M. N. Helder, J. Klein-Nulend, T. E. Schouten, M. J. P. F. Ritt, and F. J. van Milligen, "Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure.," *Cytotherapy*, vol. 8, no. 2, pp. 166–177, 2006.

[59] J. C. Ra, S. K. Kang, I. S. Shin, H. G. Park, S. A. Joo, J. G. Kim, B.-C. Kang, Y. S. Lee, K. Nakama, M. Piao, B. Sohl, and A. Kurtz, "Stem cell treatment for patients with autoimmune disease by systemic infusion of culture-expanded autologous adipose tissue derived mesenchymal stem cells.," *J. Transl. Med.*, vol. 9, no. 1, p. 181, Jan. 2011.

[60] H. A. Awad, M. Q. Wickham, H. A. Leddy, J. M. Gimble, and F. Guilak, "Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds.," *Biomaterials*, vol. 25, no. 16, pp. 3211–3222, Jul. 2004.

[61] J. K. Fraser, R. Schreiber, B. Strem, M. Zhu, Z. Alfonso, I. Wulur, and M. H. Hedrick, "Plasticity of human adipose stem cells toward endothelial cells and cardiomyocytes.," *Nat. Clin. Pract. Cardiovasc. Med.*, vol. 3 Suppl 1, pp. S33-7, Mar. 2006.

[62] J. K. Fraser, I. Wulur, Z. Alfonso, and M. H. Hedrick, "Fat tissue: an underappreciated source of stem cells for biotechnology.," *Trends Biotechnol.*, vol. 24, no. 4, pp. 150–154, Apr. 2006.

[63] S. Jang, H.-H. Cho, Y.-B. Cho, J.-S. Park, and H.-S. Jeong, "Functional neural differentiation of human

adipose tissue-derived stem cells using bFGF and forskolin.," *BMC Cell Biol.*, vol. 11, p. 25, Apr. 2010.
[64] H. Klingemann, D. Matzilevich, and J. Marchand, "Mesenchymal Stem Cells - Sources and Clinical Applications.," *Transfus. Med. Hemother.*, vol. 35, no. 4, pp. 272–277, Jan. 2008.
[65] A. Giorgetti, N. Montserrat, T. Aasen, F. Gonzalez, I. Rodriguez-Piza, R. Vassena, A. Raya, S. Boue, M. J. Barrero, B. A. Corbella, M. Torrabadella, A. Veiga, and J. C. Izpisua Belmonte, "Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2.," *Cell stem cell*, vol. 5, no. 4. United States, pp. 353–357, Oct-2009.
[66] K. Buyl, T. Vanhaecke, T. Desmae, L. Lagneaux, V. Rogiers, M. Najar, and J. De Kock, "Evaluation of a new standardized enzymatic isolation protocol for human umbilical cord-derived stem cells.," *Toxicol. In Vitro*, vol. 29, no. 6, pp. 1254–1262, Sep. 2015.
[67] M. B. Dehkordi, Z. Madjd, M. H. Chaleshtori, R. Meshkani, L. Nikfarjam, and A.-M. Kajbafzadeh, "A Simple, Rapid, and Efficient Method for Isolating Mesenchymal Stem Cells from the Entire Umbilical Cord.," *Cell Transplant.*, vol. 25, no. 7, pp. 1287–1297, Jul. 2016.
[68] Y.-F. Han, R. Tao, T.-J. Sun, J.-K. Chai, G. Xu, and J. Liu, "Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods.," *Cytotechnology*, vol. 65, no. 5, pp. 819–827, Oct. 2013.
[69] J.-Y. Hsieh, Y.-S. Fu, S.-J. Chang, Y.-H. Tsuang, and H.-W. Wang, "Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord.," *Stem Cells Dev.*, vol. 19, no. 12, pp. 1895–1910, Dec. 2010.
[70] C.-Y. Fong, L.-L. Chak, A. Biswas, J.-H. Tan, K. Gauthaman, W.-K. Chan, and A. Bongso, "Human Wharton's jelly stem cells have unique transcriptome profiles compared with human embryonic stem cells and other mesenchymal stem cells.," *Stem Cell Rev.*, vol. 7, no. 1, pp. 1–16, Mar. 2011.
[71] D. L. Troyer and M. L. Weiss, "Wharton's jelly-derived cells are a primitive stromal cell population.," *Stem Cells*, vol. 26, no. 3, pp. 591–599, Mar. 2008.
[72] C. van den Bos, R. Keefe, C. Schirmaier, and M. McCaman, "Therapeutic human cells: manufacture for cell therapy/regenerative medicine.," *Adv. Biochem. Eng. Biotechnol.*, vol. 138, pp. 61–97, 2014.
[73] G. Ren, X. Chen, F. Dong, W. Li, X. Ren, Y. Zhang, and Y. Shi, "Concise Review: Mesenchymal Stem Cells and Translational Medicine: Emerging Issues," *Stem Cells Translational Medicine*, vol. 1, no. 1. pp. 51–58, Jan-2012.
[74] F. D. Lublin, J. D. Bowen, J. Huddlestone, M. Kremenchutzky, A. Carpenter, J. R. Corboy, M. S. Freedman, L. Krupp, C. Paulo, R. J. Hariri, and S. A. Fischkoff, "Human placenta-derived cells (PDA-001) for the treatment of adults with multiple sclerosis: a randomized, placebo-controlled, multiple-dose study.," *Mult. Scler. Relat. Disord.*, vol. 3, no. 6, pp. 696–704, Nov. 2014.
[75] I. Molendijk, B. A. Bonsing, H. Roelofs, K. C. M. J. Peeters, M. N. J. M. Wasser, G. Dijkstra, C. J. van der Woude, M. Duijvestein, R. A. Veenendaal, J.-J. Zwaginga, H. W. Verspaget, W. E. Fibbe, A. E. van der Meulen-de Jong, and D. W. Hommes, "Allogeneic Bone Marrow-Derived Mesenchymal Stromal Cells Promote Healing of Refractory Perianal Fistulas in Patients With Crohn's Disease.," *Gastroenterology*, vol. 149, no. 4, p. 918–27.e6, Oct. 2015.
[76] C. Chabannon, C. Faucher, B. Calmels, C. Lemarie, and A. Mahalatchimy, "Accreditation and regulations in cell therapy," *ISBT Sci Ser*, vol. 11, pp. 271–276, 2016.
[77] V. Jossen, C. van den Bos, R. Eibl, and D. Eibl, "Manufacturing human mesenchymal stem cells at clinical scale: process and regulatory challenges.," *Appl. Microbiol. Biotechnol.*, vol. 102, no. 9, pp. 3981–3994, May 2018.
[78] C. Elseberg, J. Leber, T. Weidner, and P. Czermak, "The Challenge of Human Mesenchymal Stromal Cell Expansion: Current and Prospective Answers," in *New Insights into Cell Culture Technology*, S. J. T. Gowder, Ed. Rijeka: InTech, 2017.
[79] C. Justice, A. Brix, D. Freimark, M. Kraume, P. Pfromm, B. Eichenmueller, and P. Czermak, "Process control in cell culture technology using dielectric spectroscopy.," *Biotechnol. Adv.*, vol. 29, no. 4, pp. 391–401, 2011.
[80] V. Jossen, R. Pörtner, S. C. Kaiser, M. Kraume, D. Eibl, and R. Eibl, "Mass Production of Mesenchymal Stem Cells — Impact of Bioreactor Design and Flow Conditions on Proliferation and Differentiation," in *Cells and Biomaterials in Regenerative Medicine*, D. D. Eberli, Ed. InTech, 2014.
[81] C. Cramer, E. Freisinger, R. K. Jones, D. P. Slakey, C. L. Dupin, E. R. Newsome, E. U. Alt, and R. Izadpanah, "Persistent high glucose concentrations alter the regenerative potential of mesenchymal stem cells.," *Stem Cells Dev.*, vol. 19, no. 12, pp. 1875–1884, Dec. 2010.
[82] T.-C. Chang, M.-F. Hsu, and K. K. Wu, "High glucose induces bone marrow-derived mesenchymal stem cell senescence by upregulating autophagy.," *PLoS One*, vol. 10, no. 5, p. e0126537, 2015.
[83] M. Dhanasekaran, S. Indumathi, J. S. Rajkumar, and D. Sudarsanam, "Effect of high glucose on extensive culturing of mesenchymal stem cells derived from subcutaneous fat, omentum fat and bone marrow.," *Cell*

Biochem. Funct., vol. 31, no. 1, pp. 20–29, Jan. 2013.

[84] C. Martin, É. Olmos, M.-L. Collignon, N. De Isla, F. Blanchard, I. Chevalot, A. Marc, and E. Guedon, "Revisiting MSC expansion from critical quality attributes to critical culture process parameters," *Process Biochem.*, vol. 59, pp. 231–243, 2017.

[85] N. Fekete, M. T. Rojewski, R. Lotfi, and H. Schrezenmeier, "Essential components for ex vivo proliferation of mesenchymal stromal cells.," *Tissue Eng. Part C. Methods*, vol. 20, no. 2, pp. 129–139, Feb. 2014.

[86] G. A. Higuera, D. Schop, T. W. G. M. Spitters, R. van Dijkhuizen-Radersma, M. Bracke, J. D. de Bruijn, D. Martens, M. Karperien, A. van Boxtel, and C. A. van Blitterswijk, "Patterns of amino acid metabolism by proliferating human mesenchymal stem cells.," *Tissue Eng. Part A*, vol. 18, no. 5–6, pp. 654–664, Mar. 2012.

[87] D. Schop, F. W. Janssen, L. D. S. van Rijn, H. Fernandes, R. M. Bloem, J. D. de Bruijn, and R. van Dijkhuizen-Radersma, "Growth, metabolism, and growth inhibitors of mesenchymal stem cells.," *Tissue Eng. Part A*, vol. 15, no. 8, pp. 1877–1886, Aug. 2009.

[88] P. a Sotiropoulou, S. a Perez, M. Salagianni, C. N. Baxevanis, and M. Papamichail, "Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells.," *Stem Cells*, vol. 24, no. 2, pp. 462–71, Feb. 2006.

[89] C. Dromard, P. Bourin, M. André, S. De Barros, L. Casteilla, and V. Planat-Benard, "Human adipose derived stroma/stem cells grow in serum-free medium as floating spheres.," *Exp. Cell Res.*, vol. 317, no. 6, pp. 770–80, Apr. 2011.

[90] S. Jung, A. Sen, L. Rosenberg, and L. A. Behie, "Identification of growth and attachment factors for the serum-free isolation and expansion of human mesenchymal stromal cells.," *Cytotherapy*, vol. 12, no. 5, pp. 637–657, Sep. 2010.

[91] S. H. Bae, H. Ryu, K.-J. Rhee, J.-E. Oh, S. K. Baik, K. Y. Shim, J. H. Kong, S. Y. Hyun, H. S. Pack, C. Im, H. C. Shin, Y. M. Kim, H. S. Kim, Y. W. Eom, and J. I. Lee, "L-ascorbic acid 2-phosphate and fibroblast growth factor-2 treatment maintains differentiation potential in bone marrow-derived mesenchymal stem cells through expression of hepatocyte growth factor.," *Growth Factors*, vol. 33, no. 2, pp. 71–78, Apr. 2015.

[92] C. Wei, X. Liu, J. Tao, R. Wu, P. Zhang, Y. Bian, Y. Li, F. Fang, and Y. Zhang, "Effects of vitamin C on characteristics retaining of in vitro-cultured mouse adipose-derived stem cells.," *In Vitro Cell. Dev. Biol. Anim.*, vol. 50, no. 1, pp. 75–86, Jan. 2014.

[93] H. Hemeda, B. Giebel, and W. Wagner, "Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells.," *Cytotherapy*, vol. 16, no. 2, pp. 170–180, Feb. 2014.

[94] J. G. Carmelo, A. Fernandes-Platzgummer, M. M. Diogo, C. L. da Silva, and J. M. S. Cabral, "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.," *Biotechnol. J.*, vol. 10, no. 8, pp. 1235–1247, Aug. 2015.

[95] F. Dos Santos, P. Z. Andrade, M. M. Abecasis, J. M. Gimble, L. G. Chase, A. M. Campbell, S. Boucher, M. C. Vemuri, C. L. Da Silva, and J. M. S. Cabral, "Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions.," *Tissue Eng. Part C. Methods*, vol. 17, no. 12, pp. 1201–10, Dec. 2011.

[96] S. H. Al-Saqi, M. Saliem, S. Asikainen, H. C. Quezada, A. Ekblad, O. Hovatta, K. Le Blanc, A. F. Jonasson, and C. Gotherstrom, "Defined serum-free media for in vitro expansion of adipose-derived mesenchymal stem cells.," *Cytotherapy*, vol. 16, no. 7, pp. 915–926, Jul. 2014.

[97] G. Chen, A. Yue, Z. Ruan, Y. Yin, R. Wang, Y. Ren, and L. Zhu, "Human Umbilical Cord-Derived Mesenchymal Stem Cells Do Not Undergo Malignant Transformation during Long-Term Culturing in Serum-Free Medium," *PLoS ONE*, vol. 9, no. 6. San Francisco, USA, 2014.

[98] K. Y. Tan, K. L. Teo, J. F. Y. Lim, A. K. L. Chen, M. Choolani, S. Reuveny, J. Chan, and S. K. Oh, "Serum-free media formulations are cell line-specific and require optimization for microcarrier culture.," *Cytotherapy*, vol. 17, no. 8, pp. 1152–1165, Aug. 2015.

[99] Y. Wang, H. Wu, Z. Yang, Y. Chi, L. Meng, A. Mao, S. Yan, S. Hu, J. Zhang, Y. Zhang, W. Yu, Y. Ma, T. Li, Y. Cheng, Y. Wang, S. Wang, J. Liu, J. Han, C. Li, L. Liu, J. Xu, Z. B. Han, and Z. C. Han, "Human mesenchymal stem cells possess different biological characteristics but do not change their therapeutic potential when cultured in serum free medium.," *Stem Cell Res. Ther.*, vol. 5, no. 6, p. 132, Dec. 2014.

[100] J. Fiedler, N. Etzel, and R. E. Brenner, "To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF.," *J. Cell. Biochem.*, vol. 93, no. 5, pp. 990–998, Nov. 2004.

[101] K. Tamama, H. Kawasaki, and A. Wells, "Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC.," *J. Biomed. Biotechnol.*, vol. 2010, p. 795385, 2010.

[102] S. Tsutsumi, A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi, and Y. Kato, "Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF.," *Biochem. Biophys. Res. Commun.*, vol. 288, no. 2, pp. 413–419, Oct. 2001.

[103] A. C. Schnitzler, A. Verma, D. E. Kehoe, D. Jing, J. R. Murrell, K. A. Der, M. Aysola, P. J. Rapiejko, S. Punreddy, and M. S. Rook, "Bioprocessing of human mesenchymal stem/stromal cells for therapeutic use: Current technologies and challenges," *Biochem. Eng. J.*, vol. 108, pp. 3–13, 2016.

[104] J. C. Estrada, C. Albo, A. Benguria, A. Dopazo, P. Lopez-Romero, L. Carrera-Quintanar, E. Roche, E. P. Clemente, J. A. Enriquez, A. Bernad, and E. Samper, "Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis.," *Cell Death Differ.*, vol. 19, no. 5, pp. 743–755, May 2012.

[105] M. G. Valorani, E. Montelatici, A. Germani, A. Biddle, D. D'Alessandro, R. Strollo, M. P. Patrizi, L. Lazzari, E. Nye, W. R. Otto, P. Pozzilli, and M. R. Alison, "Pre-culturing human adipose tissue mesenchymal stem cells under hypoxia increases their adipogenic and osteogenic differentiation potentials.," *Cell Prolif.*, vol. 45, no. 3, pp. 225–238, Jun. 2012.

[106] F. Dos Santos, P. Z. Andrade, J. S. Boura, M. M. Abecasis, C. L. da Silva, and J. M. S. Cabral, "Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia.," *J. Cell. Physiol.*, vol. 223, no. 1, pp. 27–35, Apr. 2010.

[107] K. Drela, A. Sarnowska, P. Siedlecka, I. Szablowska-Gadomska, M. Wielgos, M. Jurga, B. Lukomska, and K. Domanska-Janik, "Low oxygen atmosphere facilitates proliferation and maintains undifferentiated state of umbilical cord mesenchymal stem cells in an hypoxia inducible factor-dependent manner.," *Cytotherapy*, vol. 16, no. 7, pp. 881–892, Jul. 2014.

[108] C. Fotia, A. Massa, F. Boriani, N. Baldini, and D. Granchi, "Hypoxia enhances proliferation and stemness of human adipose-derived mesenchymal stem cells.," *Cytotechnology*, vol. 67, no. 6, pp. 1073–1084, Dec. 2015.

[109] A. Lavrentieva, I. Majore, C. Kasper, and R. Hass, "Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells.," *Cell Commun. Signal.*, vol. 8, p. 18, Jul. 2010.

[110] M. Lonne, A. Lavrentieva, J.-G. Walter, and C. Kasper, "Analysis of oxygen-dependent cytokine expression in human mesenchymal stem cells derived from umbilical cord.," *Cell Tissue Res.*, vol. 353, no. 1, pp. 117–122, Jul. 2013.

[111] P. H. Oliveira, J. S. Boura, M. M. Abecasis, J. M. Gimble, C. L. da Silva, and J. M. S. Cabral, "Impact of hypoxia and long-term cultivation on the genomic stability and mitochondrial performance of ex vivo expanded human stem/stromal cells.," *Stem Cell Res.*, vol. 9, no. 3, pp. 225–236, Nov. 2012.

[112] T. G. Graeber, C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe, and A. J. Giaccia, "Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours.," *Nature*, vol. 379, no. 6560, pp. 88–91, Jan. 1996.

[113] L. B. Buravkova, E. R. Andreeva, V. Gogvadze, and B. Zhivotovsky, "Mesenchymal stem cells and hypoxia: where are we?," *Mitochondrion*, vol. 19 Pt A, pp. 105–112, Nov. 2014.

[114] C.-P. Chang, C.-C. Chio, C.-U. Cheong, C.-M. Chao, B.-C. Cheng, and M.-T. Lin, "Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury.," *Clin. Sci. (Lond).*, vol. 124, no. 3, pp. 165–176, Feb. 2013.

[115] I. Rosova, M. Dao, B. Capoccia, D. Link, and J. A. Nolta, "Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells.," *Stem Cells*, vol. 26, no. 8, pp. 2173–2182, Aug. 2008.

[116] K. M. Peterson, A. Aly, A. Lerman, L. O. Lerman, and M. Rodriguez-Porcel, "Improved survival of mesenchymal stromal cell after hypoxia preconditioning: role of oxidative stress.," *Life Sci.*, vol. 88, no. 1–2, pp. 65–73, Jan. 2011.

[117] B. Y. K. Binder, J. E. Sagun, and J. K. Leach, "Reduced serum and hypoxic culture conditions enhance the osteogenic potential of human mesenchymal stem cells.," *Stem Cell Rev.*, vol. 11, no. 3, pp. 387–393, Jun. 2015.

[118] I.-H. Park, K.-H. Kim, H.-K. Choi, J.-S. Shim, S.-Y. Whang, S. J. Hahn, O.-J. Kwon, and I.-H. Oh, "Constitutive stabilization of hypoxia-inducible factor alpha selectively promotes the self-renewal of mesenchymal progenitors and maintains mesenchymal stromal cells in an undifferentiated state.," *Exp. Mol. Med.*, vol. 45, p. e44, Sep. 2013.

[119] N. Munoz, J. Kim, Y. Liu, T. M. Logan, and T. Ma, "Gas chromatography-mass spectrometry analysis of human mesenchymal stem cell metabolism during proliferation and osteogenic differentiation under different oxygen tensions.," *J. Biotechnol.*, vol. 169, pp. 95–102, Jan. 2014.

[120] M. Deschepper, K. Oudina, B. David, V. Myrtil, C. Collet, M. Bensidhoum, D. Logeart-Avramoglou, and H. Petite, "Survival and function of mesenchymal stem cells (MSCs) depend on glucose to overcome

exposure to long-term, severe and continuous hypoxia.," *J. Cell. Mol. Med.*, vol. 15, no. 7, pp. 1505–1514, Jul. 2011.
[121] N. Haque, M. T. Rahman, N. H. Abu Kasim, and A. M. Alabsi, "Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy.," *ScientificWorldJournal.*, vol. 2013, p. 632972, 2013.
[122] S. Sart, S. N. Agathos, and Y. Li, "Process engineering of stem cell metabolism for large scale expansion and differentiation in bioreactors," *Biochem. Eng. J.*, vol. 84, pp. 74–82, 2014.
[123] A. Stolzing and A. Scutt, "Effect of reduced culture temperature on antioxidant defences of mesenchymal stem cells.," *Free Radic. Biol. Med.*, vol. 41, no. 2, pp. 326–338, Jul. 2006.
[124] J. Chang, H. Lei, Q. Liu, S. Qin, K. Ma, S. Luo, X. Zhang, W. Huang, Z. Zuo, H. Fu, and Y. Xia, "Optimization of culture of mesenchymal stem cells: a comparison of conventional plate and microcarrier cultures.," *Cell Prolif.*, vol. 45, no. 5, pp. 430–437, Oct. 2012.
[125] L.-E. Monfoulet, P. Becquart, D. Marchat, K. Vandamme, M. Bourguignon, E. Pacard, V. Viateau, H. Petite, and D. Logeart-Avramoglou, "The pH in the microenvironment of human mesenchymal stem cells is a critical factor for optimal osteogenesis in tissue-engineered constructs.," *Tissue Eng. Part A*, vol. 20, no. 13–14, pp. 1827–1840, Jul. 2014.
[126] C. T. Goudar, R. Matanguihan, E. Long, C. Cruz, C. Zhang, J. M. Piret, and K. B. Konstantinov, "Decreased pCO(2) accumulation by eliminating bicarbonate addition to high cell-density cultures.," *Biotechnol. Bioeng.*, vol. 96, no. 6, pp. 1107–1117, Apr. 2007.
[127] A. N. Brodsky, J. Zhang, R. P. Visconti, and S. W. Harcum, "Expansion of Mesenchymal Stem Cells under Atmospheric Carbon Dioxide," *Biotechnology progress*, vol. 29, no. 5. pp. 1298–1306, Sep-2013.
[128] F. H. Moghadam, T. Tayebi, M. Dehghan, G. Eslami, H. Nadri, A. Moradi, H. Vahedian-Ardakani, and K. Barzegar, "Differentiation of Bone Marrow Mesenchymal Stem Cells into Chondrocytes after Short Term Culture in Alkaline Medium," *International Journal of Hematology-Oncology and Stem Cell Research*, vol. 8, no. 4. pp. 12–19, Oct-2014.
[129] R. Fliefel, C. Popov, M. Troltzsch, J. Kuhnisch, M. Ehrenfeld, and S. Otto, "Mesenchymal stem cell proliferation and mineralization but not osteogenic differentiation are strongly affected by extracellular pH.," *J. Craniomaxillofac. Surg.*, vol. 44, no. 6, pp. 715–724, Jun. 2016.
[130] U. Galderisi and A. Giordano, "The gap between the physiological and therapeutic roles of mesenchymal stem cells.," *Med. Res. Rev.*, vol. 34, no. 5, pp. 1100–1126, Sep. 2014.
[131] S. Sart, A.-C. Tsai, Y. Li, and T. Ma, "Three-dimensional aggregates of mesenchymal stem cells: cellular mechanisms, biological properties, and applications.," *Tissue Eng. Part B. Rev.*, vol. 20, no. 5, pp. 365–380, Oct. 2014.
[132] T. J. Bartosh, J. H. Ylöstalo, A. Mohammadipoor, N. Bazhanov, K. Coble, K. Claypool, R. H. Lee, H. Choi, and D. J. Prockop, "Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 31, pp. 13724–9, 2010.
[133] P. R. Baraniak and T. C. McDevitt, "Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential.," *Cell Tissue Res.*, vol. 347, no. 3, pp. 701–11, Mar. 2012.
[134] N.-C. Cheng, S.-Y. Chen, J.-R. Li, and T.-H. Young, "Short-term spheroid formation enhances the regenerative capacity of adipose-derived stem cells by promoting stemness, angiogenesis, and chemotaxis.," *Stem Cells Transl. Med.*, vol. 2, no. 8, pp. 584–594, Aug. 2013.
[135] J. M. Santos, S. P. Camoes, E. Filipe, M. Cipriano, R. N. Barcia, M. Filipe, M. Teixeira, S. Simoes, M. Gaspar, D. Mosqueira, D. S. Nascimento, P. Pinto-do-O, P. Cruz, H. Cruz, M. Castro, and J. P. Miranda, "Three-dimensional spheroid cell culture of umbilical cord tissue-derived mesenchymal stromal cells leads to enhanced paracrine induction of wound healing.," *Stem Cell Res. Ther.*, vol. 6, p. 90, May 2015.
[136] J. A. Zimmermann and T. C. McDevitt, "Pre-conditioning mesenchymal stromal cell spheroids for immunomodulatory paracrine factor secretion.," *Cytotherapy*, vol. 16, no. 3, pp. 331–345, Mar. 2014.
[137] L.-Y. Sun, S.-Z. Lin, Y.-S. Li, H.-J. Harn, and T.-W. Chiou, "Functional cells cultured on microcarriers for use in regenerative medicine research.," *Cell Transplant.*, vol. 20, no. 1, pp. 49–62, 2011.
[138] A. Mukhopadhyay, S. N. Mukhopadhyay, and G. P. Talwar, "Influence of serum proteins on the kinetics of attachment of Vero cells to cytodex microcarriers.," *J. Chem. Technol. Biotechnol.*, vol. 56, no. 4, pp. 369–374, 1993.
[139] A. M. de Soure, A. Fernandes-Platzgummer, F. Moreira, C. Lilaia, S.-H. Liu, C.-P. Ku, Y.-F. Huang, W. Milligan, J. M. S. Cabral, and C. L. da Silva, "Integrated culture platform based on a human platelet lysate supplement for the isolation and scalable manufacturing of umbilical cord matrix-derived mesenchymal stem/stromal cells.," *J. Tissue Eng. Regen. Med.*, vol. 11, no. 5, pp. 1630–1640, May 2017.
[140] D. Schop, F. W. Janssen, E. Borgart, J. D. de Bruijn, and R. van Dijkhuizen-Radersma, "Expansion of mesenchymal stem cells using a microcarrier-based cultivation system: growth and metabolism.," *J. Tissue*

Eng. Regen. Med., vol. 2, no. 2–3, pp. 126–135, 2008.
[141] L. Sensebe, P. Bourin, and K. Tarte, "Good manufacturing practices production of mesenchymal stem/stromal cells.," *Hum. Gene Ther.*, vol. 22, no. 1, pp. 19–26, Jan. 2011.
[142] F. Dos Santos, A. Campbell, A. Fernandes-Platzgummer, P. Z. Andrade, J. M. Gimble, Y. Wen, S. Boucher, M. C. Vemuri, C. L. da Silva, and J. M. S. Cabral, "A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells.," *Biotechnol. Bioeng.*, vol. 111, no. 6, pp. 1116–27, Jun. 2014.
[143] B. Cunha, T. Aguiar, M. M. Silva, R. J. S. Silva, M. F. Q. Sousa, E. Pineda, C. Peixoto, M. J. T. Carrondo, M. Serra, and P. M. Alves, "Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells.," *J. Biotechnol.*, vol. 213, pp. 97–108, Nov. 2015.
[144] Q. A. Rafiq, K. M. Brosnan, K. Coopman, A. W. Nienow, and C. J. Hewitt, "Culture of human mesenchymal stem cells on microcarriers in a 5 l stirred-tank bioreactor.," *Biotechnol. Lett.*, vol. 35, no. 8, pp. 1233–1245, Aug. 2013.
[145] T. Lawson, D. E. Kehoe, A. C. Schnitzler, P. J. Rapiejko, K. A. Der, K. Philbrick, S. Punreddy, S. Rigby, R. Smith, Q. Feng, J. R. Murrell, and M. S. Rook, "Process development for expansion of human mesenchymal stromal cells in a 50L single-use stirred tank bioreactor," *Biochem. Eng. J.*, vol. 120, pp. 49–62, 2017.
[146] H. Akerstrom, "Expansion of adherent cells for cell therapy," Uppsala University, Sweden, 2009.
[147] N. E. Timmins, M. Kiel, M. Gunther, C. Heazlewood, M. R. Doran, G. Brooke, and K. Atkinson, "Closed system isolation and scalable expansion of human placental mesenchymal stem cells.," *Biotechnol. Bioeng.*, vol. 109, no. 7, pp. 1817–1826, Jul. 2012.
[148] A. Mizukami, M. D. Orellana, S. R. Caruso, K. de Lima Prata, D. T. Covas, and K. Swiech, "Efficient expansion of mesenchymal stromal cells in a disposable fixed bed culture system.," *Biotechnol. Prog.*, vol. 29, no. 2, pp. 568–572, 2013.
[149] M. J. Osiecki, T. D. Michl, B. Kul Babur, M. Kabiri, K. Atkinson, W. B. Lott, H. J. Griesser, and M. R. Doran, "Packed Bed Bioreactor for the Isolation and Expansion of Placental-Derived Mesenchymal Stromal Cells," *PLoS ONE*, vol. 10, no. 12. San Francisco, CA USA, 2015.
[150] T. Lambrechts, I. Papantoniou, B. Rice, J. Schrooten, F. P. Luyten, and J.-M. Aerts, "Large-scale progenitor cell expansion for multiple donors in a monitored hollow fibre bioreactor.," *Cytotherapy*, vol. 18, no. 9, pp. 1219–1233, Sep. 2016.
[151] M. T. Rojewski, N. Fekete, S. Baila, K. Nguyen, D. Furst, D. Antwiler, J. Dausend, L. Kreja, A. Ignatius, L. Sensebe, and H. Schrezenmeier, "GMP-compliant isolation and expansion of bone marrow-derived MSCs in the closed, automated device quantum cell expansion system.," *Cell Transplant.*, vol. 22, no. 11, pp. 1981–2000, 2013.
[152] A. Mizukami, M. S. de Abreu Neto, F. Moreira, A. Fernandes-Platzgummer, Y.-F. Huang, W. Milligan, J. M. S. Cabral, C. L. da Silva, D. T. Covas, and K. Swiech, "A Fully-Closed and Automated Hollow Fiber Bioreactor for Clinical-Grade Manufacturing of Human Mesenchymal Stem/Stromal Cells.," *Stem cell reviews*, vol. 14, no. 1. United States, pp. 141–143, Feb-2018.
[153] F. Collignon, J. Goffinet, J. Cardon, S. Wynants, F. Moncaubeig, M. Egloff, F. Verhoeye, S. Jooris, J. C. Drugmand, C. Homsy, and J. Castillo, "Integrity™ Xpansion™ Multiplate Bioreactor: The Scalable Solution for Adherent Stem Cell Expansion." ATMI LifeSciences, 2012.
[154] S. Diederichs, S. Röker, D. Marten, A. Peterbauer, T. Scheper, M. van Griensven, and C. Kasper, "Dynamic cultivation of human mesenchymal stem cells in a rotating bed bioreactor system based on the Z RP platform.," *Biotechnol. Prog.*, vol. 25, no. 6, pp. 1762–71, 2009.
[155] A. Reichardt, B. Polchow, M. Shakibaei, W. Henrich, R. Hetzer, and C. Lueders, "Large scale expansion of human umbilical cord cells in a rotating bed system bioreactor for cardiovascular tissue engineering applications.," *Open Biomed. Eng. J.*, vol. 7, pp. 50–61, 2013.
[156] A. Neumann, A. Lavrentieva, A. Heilkenbrinker, M. Loenne, and C. Kasper, "Characterization and Application of a Disposable Rotating Bed Bioreactor for Mesenchymal Stem Cell Expansion.," *Bioeng. (Basel, Switzerland)*, vol. 1, no. 4, pp. 231–245, Nov. 2014.
[157] M. F. Q. Sousa, M. M. Silva, D. Giroux, Y. Hashimura, R. Wesselschmidt, B. Lee, A. Roldao, M. J. T. Carrondo, P. M. Alves, and M. Serra, "Production of oncolytic adenovirus and human mesenchymal stem cells in a single-use, Vertical-Wheel bioreactor system: Impact of bioreactor design on performance of microcarrier-based cell culture processes.," *Biotechnol. Prog.*, vol. 31, no. 6, pp. 1600–1612, 2015.
[158] K. M. Panchalingam, S. Jung, L. Rosenberg, and L. A. Behie, "Bioprocessing strategies for the large-scale production of human mesenchymal stem cells: a review," *Stem Cell Research & Therapy*, vol. 6. London, 2015.
[159] D. A. Rider, C. Dombrowski, A. A. Sawyer, G. H. B. Ng, D. Leong, D. W. Hutmacher, V. Nurcombe, and S. M. Cool, "Autocrine fibroblast growth factor 2 increases the multipotentiality of human adipose-derived

mesenchymal stem cells.," *Stem Cells*, vol. 26, no. 6, pp. 1598–1608, Jun. 2008.
[160] T. R. J. Heathman, V. A. M. Glyn, A. Picken, Q. A. Rafiq, K. Coopman, A. W. Nienow, B. Kara, and C. J. Hewitt, "Expansion, harvest and cryopreservation of human mesenchymal stem cells in a serum-free microcarrier process.," *Biotechnol. Bioeng.*, vol. 112, no. 8, pp. 1696–1707, Aug. 2015.
[161] F. Petry, J. R. Smith, J. Leber, D. Salzig, P. Czermak, and M. L. Weiss, "Manufacturing of Human Umbilical Cord Mesenchymal Stromal Cells on Microcarriers in a Dynamic System for Clinical Use," *Stem Cells International*, vol. 2016. 2016.
[162] D. Jing, N. Sunil, S. Punreddy, and M. Aysola, "Growth kinetics of human mesenchymal stem cells in a 3-L single use, stirred tank bioreactor," *BioPharma Int*, vol. 26, pp. 28–38, 2013.
[163] T. A. Grein, J. Leber, M. Blumenstock, F. Petry, T. Weidner, D. Salzig, and P. Czermak, "Multiphase mixing characteristics in a microcarrier-based stirred tank bioreactor suitable for human mesenchymal stem cell expansion," *Process Biochem.*, vol. 51, no. 9, pp. 1109–1119, 2016.
[164] A. K.-L. Chen, Y. K. Chew, H. Y. Tan, S. Reuveny, and S. K. Weng Oh, "Increasing efficiency of human mesenchymal stromal cell culture by optimization of microcarrier concentration and design of medium feed.," *Cytotherapy*, vol. 17, no. 2, pp. 163–173, Feb. 2015.
[165] C. Schirmaier, V. Jossen, S. C. Kaiser, F. Jüngerkes, S. Brill, A. Safavi-Nab, A. Siehoff, C. van den Bos, D. Eibl, and R. Eibl, "Scale-up of adipose tissue-derived mesenchymal stem cell production in stirred single-use bioreactors under low-serum conditions," *Eng. Life Sci.*, vol. 14, no. 3, pp. 292–303, May 2014.
[166] C. Heath and G. Belfort, "Immobilization of suspended mammalian cells: analysis of hollow fiber and microcapsule bioreactors.," *Adv. Biochem. Eng. Biotechnol.*, vol. 34, pp. 1–31, 1987.
[167] W. R. Prather, A. Toren, and M. Meiron, "Placental-derived and expanded mesenchymal stromal cells (PLX-I) to enhance the engraftment of hematopoietic stem cells derived from umbilical cord blood.," *Expert Opin. Biol. Ther.*, vol. 8, no. 8, pp. 1241–1250, Aug. 2008.
[168] A. Lahiani, E. Zahavi, N. Netzer, R. Ofir, L. Pinzur, S. Raveh, H. Arien-Zakay, E. Yavin, and P. Lazarovici, "Human PLacental eXpanded (PLX) mesenchymal-like adherent stromal cells confer neuroprotection to nerve growth factor (NGF)-differentiated PC12 cells exposed to ischemia by secretion of IL-6 and VEGF," *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1853, no. 2, pp. 422–430, 2015.
[169] A. Lennaertz, S. Knowles, J.-C. Drugmand, and J. Castillo, "Viral vector production in the integrity(®)iCELLis(®)single-use fixed-bed bioreactor, from bench-scale to industrial scale," *BMC Proceedings*, vol. 7, no. Suppl 6. p. P59, 2013.
[170] W. Whitford, J. Hardy, and J. Cadwell, "Single-use, continuous processing of primary stem cells," *Cell Ther Process*, vol. 12, no. 3, pp. 27–33, 2014.
[171] M. Jones, M. Varella-Garcia, M. Skokan, S. Bryce, J. Schowinsky, R. Peters, B. Vang, M. Brecheisen, T. Startz, N. Frank, and B. Nankervis, "Genetic stability of bone marrow-derived human mesenchymal stromal cells in the Quantum System.," *Cytotherapy*, vol. 15, no. 11, pp. 1323–1339, Nov. 2013.
[172] P. J. Hanley, Z. Mei, A. G. Durett, M. da G. Cabreira-Hansen, M. Klis, W. Li, Y. Zhao, B. Yang, K. Parsha, O. Mir, F. Vahidy, D. Bloom, R. B. Rice, P. Hematti, S. I. Savitz, and A. P. Gee, "Efficient manufacturing of therapeutic mesenchymal stromal cells with the use of the Quantum Cell Expansion System.," *Cytotherapy*, vol. 16, no. 8, pp. 1048–1058, Aug. 2014.
[173] C. Lechanteur, S. Baila, M. E. Janssens, O. Giet, A. Briquet, E. Baudoux, and Y. Beguin, "Large-Scale Clinical Expansion of Mesenchymal Stem Cells in the GMP-Compliant, Closed Automated Quantum® Cell Expansion System: Comparison with Expansion in Traditional T-Flasks," *Journal of Stem Cell Research & Therapy*, vol. 4, no. 8. OMICS International., 2014.
[174] M. Haack-Sorensen, B. Follin, M. Juhl, S. K. Brorsen, R. H. Sondergaard, J. Kastrup, and A. Ekblond, "Culture expansion of adipose derived stromal cells. A closed automated Quantum Cell Expansion System compared with manual flask-based culture.," *J. Transl. Med.*, vol. 14, no. 1, p. 319, Nov. 2016.
[175] A. B. Yeatts, D. T. Choquette, and J. P. Fisher, "Bioreactors to influence stem cell fate: augmentation of mesenchymal stem cell signaling pathways via dynamic culture systems.," *Biochim. Biophys. Acta*, vol. 1830, no. 2, pp. 2470–80, Feb. 2013.
[176] P. M. Doran, *Bioprocess Engineering Principles*, Second. Academic Press, 1995.
[177] A. W. Nienow, K. Coopman, T. R. J. Heathman, Q. A. Rafiq, and C. J. Hewitt, "Chapter 3 - Bioreactor Engineering Fundamentals for Stem Cell Manufacturing," in *Stem Cell Manufacturing*, J. M. S. Cabral, C. L. de Silva, L. G. Chase, and M. M. Diogo, Eds. Boston: Elsevier, 2016, pp. 43–75.
[178] C. A. Peng and B. O. Palsson, "Cell growth and differentiation on feeder layers is predicted to be influenced by bioreactor geometry.," *Biotechnol. Bioeng.*, vol. 50, no. 5, pp. 479–492, Jun. 1996.
[179] C. Weber, D. Freimark, R. Portner, P. Pino-Grace, S. Pohl, C. Wallrapp, P. Geigle, and P. Czermak, "Expansion of human mesenchymal stem cells in a fixed-bed bioreactor system based on non-porous glass carrier--part A: inoculation, cultivation, and cell harvest procedures.," *Int. J. Artif. Organs*, vol. 33, no. 8, pp. 512–525, Aug. 2010.

[180] R. Eibl, D. Eibl, R. Portner, G. Catapano, and P. Czermak, *Cell and tissue reaction engineering*. Springer-Verlag Berlin Heidelberg, 2009.
[181] S. Kaiser, V. Jossen, C. Schirmaier, D. Eibl, S. Brill, C. van den Bos, and R. Eibl, "Fluid Flow and Cell Proliferation of Mesenchymal Adipose-Derived Stem Cells in Small-Scale, Stirred, Single-Use Bioreactors," *Chemie Ing. Tech.*, vol. 85, no. 1–2, pp. 95–102, 2013.
[182] M. S. Croughan, E. S. Sayre, and D. I. Wang, "Viscous reduction of turbulent damage in animal cell culture.," *Biotechnol. Bioeng.*, vol. 33, no. 7, pp. 862–872, Feb. 1989.
[183] Y. Chisti, "Hydrodynamic damage to animal cells.," *Crit. Rev. Biotechnol.*, vol. 21, no. 2, pp. 67–110, 2001.
[184] R. Eibl, S. Werner, and D. Eibl, "Bag bioreactor based on wave-induced motion: characteristics and applications.," *Adv. Biochem. Eng. Biotechnol.*, vol. 115, pp. 55–87, 2009.
[185] S. Werner, R. Eibl, C. Lettenbauer, M. Roll, D. Eibl, M. De Jesus, X. Zhang, M. Stettler, S. Tissot, C. Burki, G. Broccard, M. Kuhner, R. Tanner, L. Baldi, D. Hacker, and F. M. Wurm, "Innovative, non-stirred bioreactors in scales from milliliters up to 1000 liters for suspension cultures of cells using disposable bags and containers--a Swiss contribution.," *Chimia (Aarau).*, vol. 64, no. 11, pp. 819–823, 2010.
[186] A. Chen, S. Reuveny, and S. Oh, "Application of Human Mesenchymal and Pluripotent Stem Cell Microcarrier Cultures in Cellular Therapy: Achievements and Future Direction.," *Biotechnol. Adv.*, Mar. 2013.
[187] T. N. Zwietering, "Suspending of solid particles in liquid by agitators," *Chem. Eng. Sci.*, vol. 8, no. 3, pp. 244–253, 1958.
[188] J. M. Clark and M. D. Hirtenstein, "Optimizing culture conditions for the production of animal cells in microcarrier culture.," *Ann. N. Y. Acad. Sci.*, vol. 369, pp. 33–46, 1981.
[189] A. N. Kolmogorov, "The local structure of turbulence in incompressible viscous fluid for very large Reynolds numbers," *Proc. USSR Acad. Sci.*, vol. 30, pp. 299–303, 1941.
[190] A. N. Kolmogorov, "Dissipation of Energy in Locally Isotropic Turbulence," *Proc. USSR Acad. Sci.*, vol. 32, pp. 16–18, 1941.
[191] A. W. Nienow, C. J. Hewitt, T. R. J. Heathman, V. A. M. Glyn, G. N. Fonte, M. P. Hanga, K. Coopman, and Q. A. Rafiq, "Agitation conditions for the culture and detachment of hMSCs from microcarriers in multiple bioreactor platforms," *Biochem. Eng. J.*, vol. 108, pp. 24–29, 2016.
[192] C. Löffelholz, S. Kaiser, and S. Werner, "CFD as a tool to characterize single-use bioreactors," in *Single-use technology in biopharmaceutical manufacture*, R. Eibl and D. Eibl, Eds. Hoboken, NJ: John Wiley & Sons, 2010, pp. 263–279.
[193] C. J. Geankoplis, *Transport processes and separation process principles*, Fourth. Prentice Hall Professional Technical Reference, 2003.
[194] M. Lavery and A. W. Nienow, "Oxygen transfer in animal cell culture medium.," *Biotechnol. Bioeng.*, vol. 30, no. 3, pp. 368–373, Aug. 1987.
[195] T. K.-P. Goh, Z.-Y. Zhang, A. K.-L. Chen, S. Reuveny, M. Choolani, J. K. Y. Chan, and S. K.-W. Oh, "Microcarrier culture for efficient expansion and osteogenic differentiation of human fetal mesenchymal stem cells.," *Biores. Open Access*, vol. 2, no. 2, pp. 84–97, Apr. 2013.
[196] A. W. Nienow, "Reactor engineering in large scale animal cell culture.," *Cytotechnology*, vol. 50, no. 1–3, pp. 9–33, Mar. 2006.
[197] N. M. Oosterhuis and P. van der Heiden, "Mass transfer in the CELL-tainer® Disposable Bioreactor. In: Noll T. (eds) Cells and Culture," *ESACT Proc.*, vol. 4, 2010.
[198] PBSBiotech, "The Future of Single Use Bioreactors," 2018. [Online]. Available: https://www.pbsbiotech.com/. [Accessed: 07-Mar-2018].
[199] C. Weber, D. Freimark, R. Portner, P. Pino-Grace, S. Pohl, C. Wallrapp, P. Geigle, and P. Czermak, "Expansion of human mesenchymal stem cells in a fixed-bed bioreactor system based on non-porous glass carrier - Part B: Modeling and scale-up of the system.," *Int. J. Artif. Organs*, vol. 33, no. 11, pp. 782–795, Nov. 2010.
[200] P. Roy, H. Baskaran, A. W. Tilles, M. L. Yarmush, and M. Toner, "Analysis of oxygen transport to hepatocytes in a flat-plate microchannel bioreactor.," *Ann. Biomed. Eng.*, vol. 29, no. 11, pp. 947–955, Nov. 2001.
[201] Synthecon, "How does the RCCS bioreactor work?," 2018. [Online]. Available: http://synthecon.com/faqs.asp?pageid=56&fid=119.
[202] M. E. Bernardo and W. E. Fibbe, "Mesenchymal stromal cells: sensors and switchers of inflammation.," *Cell Stem Cell*, vol. 13, no. 4, pp. 392–402, Oct. 2013.
[203] A. I. Caplan and J. E. Dennis, "Mesenchymal stem cells as trophic mediators.," *J. Cell. Biochem.*, vol. 98, no. 5, pp. 1076–1084, Aug. 2006.
[204] D. M. Patel, J. Shah, and A. S. Srivastava, "Therapeutic potential of mesenchymal stem cells in

regenerative medicine.," *Stem Cells Int.*, vol. 2013, p. 496218, 2013.
[205] R. Rohban and T. R. Pieber, "Mesenchymal Stem and Progenitor Cells in Regeneration: Tissue Specificity and Regenerative Potential.," *Stem Cells Int.*, vol. 2017, p. 5173732, 2017.
[206] A. I. Caplan, "Adult mesenchymal stem cells for tissue engineering versus regenerative medicine.," *J. Cell. Physiol.*, vol. 213, no. 2, pp. 341–347, Nov. 2007.
[207] X. Li, J. Bai, X. Ji, R. Li, Y. Xuan, and Y. Wang, "Comprehensive characterization of four different populations of human mesenchymal stem cells as regards their immune properties, proliferation and differentiation.," *Int. J. Mol. Med.*, vol. 34, no. 3, pp. 695–704, Sep. 2014.
[208] T. Squillaro, G. Peluso, and U. Galderisi, "Clinical Trials With Mesenchymal Stem Cells: An Update.," *Cell Transplant.*, vol. 25, no. 5, pp. 829–848, 2016.
[209] O. Y. Bang, J. S. Lee, P. H. Lee, and G. Lee, "Autologous mesenchymal stem cell transplantation in stroke patients.," *Ann. Neurol.*, vol. 57, no. 6, pp. 874–882, Jun. 2005.
[210] O. Blau, C. D. Baldus, W.-K. Hofmann, G. Thiel, F. Nolte, T. Burmeister, S. Turkmen, O. Benlasfer, E. Schumann, A. Sindram, M. Molkentin, S. Mundlos, U. Keilholz, E. Thiel, and I. W. Blau, "Mesenchymal stromal cells of myelodysplastic syndrome and acute myeloid leukemia patients have distinct genetic abnormalities compared with leukemic blasts.," *Blood*, vol. 118, no. 20, pp. 5583–5592, Nov. 2011.
[211] A. Stolzing, E. Jones, D. McGonagle, and A. Scutt, "Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies.," *Mech. Ageing Dev.*, vol. 129, no. 3, pp. 163–173, Mar. 2008.
[212] C. Gallina, V. Turinetto, and C. Giachino, "A New Paradigm in Cardiac Regeneration: The Mesenchymal Stem Cell Secretome.," *Stem Cells Int.*, vol. 2015, p. 765846, 2015.
[213] J. G. Shake, P. J. Gruber, W. A. Baumgartner, G. Senechal, J. Meyers, J. M. Redmond, M. F. Pittenger, and B. J. Martin, "Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects.," *Ann. Thorac. Surg.*, vol. 73, no. 6, p. 1919–25; discussion 1926, Jun. 2002.
[214] C. Toma, M. F. Pittenger, K. S. Cahill, B. J. Byrne, and P. D. Kessler, "Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart.," *Circulation*, vol. 105, no. 1, pp. 93–98, Jan. 2002.
[215] H. C. Quevedo, K. E. Hatzistergos, B. N. Oskouei, G. S. Feigenbaum, J. E. Rodriguez, D. Valdes, P. M. Pattany, J. P. Zambrano, Q. Hu, I. McNiece, A. W. Heldman, and J. M. Hare, "Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 33, pp. 14022–14027, Aug. 2009.
[216] K. E. Hatzistergos, H. Quevedo, B. N. Oskouei, Q. Hu, G. S. Feigenbaum, I. S. Margitich, R. Mazhari, A. J. Boyle, J. P. Zambrano, J. E. Rodriguez, R. Dulce, P. M. Pattany, D. Valdes, C. Revilla, A. W. Heldman, I. McNiece, and J. M. Hare, "Bone Marrow Mesenchymal Stem Cells Stimulate Cardiac Stem Cell Proliferation and Differentiation," *Circ. Res.*, vol. 107, no. 7, p. 913 LP-922, Sep. 2010.
[217] F. S. Loffredo, M. L. Steinhauser, J. Gannon, and R. T. Lee, "Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair," *Cell Stem Cell*, vol. 8, no. 4, pp. 389–398, 2011.
[218] R. Mazhari and J. M. Hare, "Mechanisms of action of mesenchymal stem cells in cardiac repair: potential influences on the cardiac stem cell niche.," *Nat. Clin. Pract. Cardiovasc. Med.*, vol. 4 Suppl 1, pp. S21-6, Feb. 2007.
[219] M. A. Laflamme and C. E. Murry, "Regenerating the heart," *Nat. Biotechnol.*, vol. 23, no. 7, pp. 845–56, 2005.
[220] C. Nakanishi, M. Yamagishi, K. Yamahara, I. Hagino, H. Mori, Y. Sawa, T. Yagihara, S. Kitamura, and N. Nagaya, "Activation of cardiac progenitor cells through paracrine effects of mesenchymal stem cells.," *Biochem. Biophys. Res. Commun.*, vol. 374, no. 1, pp. 11–16, Sep. 2008.
[221] A. P. Beltrami, L. Barlucchi, D. Torella, M. Baker, F. Limana, S. Chimenti, H. Kasahara, M. Rota, E. Musso, K. Urbanek, A. Leri, J. Kajstura, B. Nadal-Ginard, and P. Anversa, "Adult cardiac stem cells are multipotent and support myocardial regeneration.," *Cell*, vol. 114, no. 6, pp. 763–776, Sep. 2003.
[222] K. Urbanek, M. Rota, S. Cascapera, C. Bearzi, A. Nascimbene, A. De Angelis, T. Hosoda, S. Chimenti, M. Baker, F. Limana, D. Nurzynska, D. Torella, F. Rotatori, R. Rastaldo, E. Musso, F. Quaini, A. Leri, J. Kajstura, and P. Anversa, "Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival.," *Circ. Res.*, vol. 97, no. 7, pp. 663–673, Sep. 2005.
[223] M. Rodrigues, L. G. Griffith, and A. Wells, "Growth factor regulation of proliferation and survival of multipotential stromal cells," *Stem Cell Research & Therapy*, vol. 1, no. 4. p. 32, 2010.
[224] H. Song, M.-J. Cha, B.-W. Song, I.-K. Kim, W. Chang, S. Lim, E. J. Choi, O. Ham, S.-Y. Lee, N. Chung, Y. Jang, and K.-C. Hwang, "Reactive oxygen species inhibit adhesion of mesenchymal stem cells implanted into ischemic myocardium via interference of focal adhesion complex.," *Stem Cells*, vol. 28,

no. 3, pp. 555–563, Mar. 2010.
[225] M. Gnecchi, H. He, O. D. Liang, L. G. Melo, F. Morello, H. Mu, N. Noiseux, L. Zhang, R. E. Pratt, J. S. Ingwall, and V. J. Dzau, "Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells.," *Nature medicine*, vol. 11, no. 4. United States, pp. 367–368, Apr-2005.
[226] A. Stempien-Otero, D. Helterline, T. Plummer, S. Farris, A. Prouse, N. Polissar, D. Stanford, and N. A. Mokadam, "Mechanisms of bone marrow-derived cell therapy in ischemic cardiomyopathy with left ventricular assist device bridge to transplant.," *J. Am. Coll. Cardiol.*, vol. 65, no. 14, pp. 1424–1434, Apr. 2015.
[227] M. Di Nicola, C. Carlo-Stella, M. Magni, M. Milanesi, P. D. Longoni, P. Matteucci, S. Grisanti, and A. M. Gianni, "Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli," *Blood*, vol. 99, no. 10, pp. 3838–3843, May 2002.
[228] Y.-H. Choi, A. Kurtz, and C. Stamm, "Mesenchymal stem cells for cardiac cell therapy.," *Hum. Gene Ther.*, vol. 22, no. 1, pp. 3–17, Jan. 2011.
[229] G. M. Spaggiari, A. Capobianco, H. Abdelrazik, F. Becchetti, M. C. Mingari, and L. Moretta, "Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2.," *Blood*, vol. 111, no. 3, pp. 1327–1333, Feb. 2008.
[230] C. E. Petrie Aronin and R. S. Tuan, "Therapeutic potential of the immunomodulatory activities of adult mesenchymal stem cells.," *Birth Defects Res. C. Embryo Today*, vol. 90, no. 1, pp. 67–74, Mar. 2010.
[231] R. H. Lee, A. a Pulin, M. J. Seo, D. J. Kota, J. Ylostalo, B. L. Larson, L. Semprun-Prieto, P. Delafontaine, and D. J. Prockop, "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*, vol. 5, no. 1, pp. 54–63, Jul. 2009.
[232] P. Renner, E. Eggenhofer, A. Rosenauer, F. C. Popp, J. F. Steinmann, P. Slowik, E. K. Geissler, P. Piso, H. J. Schlitt, and M. H. Dahlke, "Mesenchymal stem cells require a sufficient, ongoing immune response to exert their immunosuppressive function.," *Transplant. Proc.*, vol. 41, no. 6, pp. 2607–2611, 2009.
[233] J. L. Chan, K. C. Tang, A. P. Patel, L. M. Bonilla, N. Pierobon, N. M. Ponzio, and P. Rameshwar, "Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-gamma.," *Blood*, vol. 107, no. 12, pp. 4817–24, Jun. 2006.
[234] K. Le Blanc, L. Tammik, B. Sundberg, S. E. Haynesworth, and O. Ringden, "Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex.," *Scand. J. Immunol.*, vol. 57, no. 1, pp. 11–20, Jan. 2003.
[235] J. M. Lambert, E. F. Lopez, and M. L. Lindsey, "Macrophage roles following myocardial infarction.," *Int. J. Cardiol.*, vol. 130, no. 2, pp. 147–158, Nov. 2008.
[236] J. G. Barin, N. R. Rose, and D. Cihakova, "Macrophage diversity in cardiac inflammation: a review.," *Immunobiology*, vol. 217, no. 5, pp. 468–475, May 2012.
[237] J. Maggini, G. Mirkin, I. Bognanni, J. Holmberg, I. M. Piazzon, I. Nepomnaschy, H. Costa, C. Canones, S. Raiden, M. Vermeulen, and J. R. Geffner, "Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile.," *PLoS One*, vol. 5, no. 2, p. e9252, Feb. 2010.
[238] N. G. Frangogiannis, "Regulation of the inflammatory response in cardiac repair.," *Circ. Res.*, vol. 110, no. 1, pp. 159–173, Jan. 2012.
[239] Y. Zhang, Z. Zhang, F. Gao, H.-F. Tse, V. Tergaonkar, and Q. Lian, "Paracrine regulation in mesenchymal stem cells: the role of Rap1.," *Cell Death Dis.*, vol. 6, p. e1932, Oct. 2015.
[240] N. Nagaya, K. Kangawa, T. Itoh, T. Iwase, S. Murakami, Y. Miyahara, T. Fujii, M. Uematsu, H. Ohgushi, M. Yamagishi, T. Tokudome, H. Mori, K. Miyatake, and S. Kitamura, "Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy.," *Circulation*, vol. 112, no. 8, pp. 1128–1135, Aug. 2005.
[241] Y. Yoon, A. Wecker, L. Heyd, J.-S. Park, T. Tkebuchava, K. Kusano, A. Hanley, H. Scadova, G. Qin, D.-H. Cha, K. L. Johnson, R. Aikawa, T. Asahara, and D. W. Losordo, "Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction.," *J. Clin. Invest.*, vol. 115, no. 2, pp. 326–338, Feb. 2005.
[242] Y. L. Tang, Q. Zhao, Y. C. Zhang, L. Cheng, M. Liu, J. Shi, Y. Z. Yang, C. Pan, J. Ge, and M. I. Phillips, "Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium.," *Regul. Pept.*, vol. 117, no. 1, pp. 3–10, Jan. 2004.
[243] M. Xu, R. Uemura, Y. Dai, Y. Wang, Z. Pasha, and M. Ashraf, "In vitro and in vivo effects of bone marrow stem cells on cardiac structure and function.," *J. Mol. Cell. Cardiol.*, vol. 42, no. 2, pp. 441–448, Feb. 2007.
[244] M. R. Kano, Y. Morishita, C. Iwata, S. Iwasaka, T. Watabe, Y. Ouchi, K. Miyazono, and K. Miyazawa,

"VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling.," *J. Cell Sci.*, vol. 118, no. Pt 16, pp. 3759–3768, Aug. 2005.

[245] T. Kinnaird, E. Stabile, M. S. Burnett, M. Shou, C. W. Lee, S. Barr, S. Fuchs, and S. E. Epstein, "Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms.," *Circulation*, vol. 109, no. 12, pp. 1543–9, Mar. 2004.

[246] S.-C. Hung, R. R. Pochampally, S.-C. Chen, S.-C. Hsu, and D. J. Prockop, "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*, vol. 25, no. 9, pp. 2363–2370, Sep. 2007.

[247] T. A. Markel, Y. Wang, J. L. Herrmann, P. R. Crisostomo, M. Wang, N. M. Novotny, C. M. Herring, J. Tan, T. Lahm, and D. R. Meldrum, "VEGF is critical for stem cell-mediated cardioprotection and a crucial paracrine factor for defining the age threshold in adult and neonatal stem cell function.," *Am. J. Physiol. Heart Circ. Physiol.*, vol. 295, no. 6, pp. H2308-14, Dec. 2008.

[248] J. Rehman, D. Traktuev, J. Li, S. Merfeld-Clauss, C. J. Temm-Grove, J. E. Bovenkerk, C. L. Pell, B. H. Johnstone, R. V Considine, and K. L. March, "Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells.," *Circulation*, vol. 109, no. 10, pp. 1292–8, Mar. 2004.

[249] L. Zhao, X. Liu, Y. Zhang, X. Liang, Y. Ding, Y. Xu, Z. Fang, and F. Zhang, "Enhanced cell survival and paracrine effects of mesenchymal stem cells overexpressing hepatocyte growth factor promote cardioprotection in myocardial infarction.," *Exp. Cell Res.*, vol. 344, no. 1, pp. 30–39, May 2016.

[250] A. Linke, P. Muller, D. Nurzynska, C. Casarsa, D. Torella, A. Nascimbene, C. Castaldo, S. Cascapera, M. Bohm, F. Quaini, K. Urbanek, A. Leri, T. H. Hintze, J. Kajstura, and P. Anversa, "Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 25, pp. 8966–8971, Jun. 2005.

[251] J. Kahn, T. Byk, L. Jansson-Sjostrand, I. Petit, S. Shivtiel, A. Nagler, I. Hardan, V. Deutsch, Z. Gazit, D. Gazit, S. Karlsson, and T. Lapidot, "Overexpression of CXCR4 on human CD34+ progenitors increases their proliferation, migration, and NOD/SCID repopulation.," *Blood*, vol. 103, no. 8, pp. 2942–2949, Apr. 2004.

[252] A. Peled, I. Petit, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, A. Nagler, H. Ben-Hur, A. Many, L. Shultz, O. Lider, R. Alon, D. Zipori, and T. Lapidot, "Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4.," *Science*, vol. 283, no. 5403, pp. 845–848, Feb. 1999.

[253] J. Yamaguchi, K. F. Kusano, O. Masuo, A. Kawamoto, M. Silver, S. Murasawa, M. Bosch-Marce, H. Masuda, D. W. Losordo, J. M. Isner, and T. Asahara, "Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization.," *Circulation*, vol. 107, no. 9, pp. 1322–1328, Mar. 2003.

[254] J. D. Abbott, Y. Huang, D. Liu, R. Hickey, D. S. Krause, and F. J. Giordano, "Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury.," *Circulation*, vol. 110, no. 21, pp. 3300–3305, Nov. 2004.

[255] M. Kucia, J. Ratajczak, R. Reca, A. Janowska-Wieczorek, and M. Z. Ratajczak, "Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury.," *Blood Cells. Mol. Dis.*, vol. 32, no. 1, pp. 52–57, 2004.

[256] S. Ohnishi, H. Sumiyoshi, S. Kitamura, and N. Nagaya, "Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions.," *FEBS Lett.*, vol. 581, no. 21, pp. 3961–3966, Aug. 2007.

[257] X. Xu, Z. Xu, Y. Xu, and G. Cui, "Selective down-regulation of extracellular matrix gene expression by bone marrow derived stem cell transplantation into infarcted myocardium.," *Circ. J.*, vol. 69, no. 10, pp. 1275–1283, Oct. 2005.

[258] E. J. Molina, J. Palma, D. Gupta, D. Torres, J. P. Gaughan, S. Houser, and M. Macha, "Reverse remodeling is associated with changes in extracellular matrix proteases and tissue inhibitors after mesenchymal stem cell (MSC) treatment of pressure overload hypertrophy.," *J. Tissue Eng. Regen. Med.*, vol. 3, no. 2, pp. 85–91, Feb. 2009.

[259] J. Fiedler, F. Leucht, J. Waltenberger, C. Dehio, and R. E. Brenner, "VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells.," *Biochem. Biophys. Res. Commun.*, vol. 334, no. 2, pp. 561–568, Aug. 2005.

[260] A. Schmidt, D. Ladage, T. Schinkothe, U. Klausmann, C. Ulrichs, F.-J. Klinz, K. Brixius, S. Arnhold, B. Desai, U. Mehlhorn, R. H. G. Schwinger, P. Staib, K. Addicks, and W. Bloch, "Basic fibroblast growth factor controls migration in human mesenchymal stem cells.," *Stem Cells*, vol. 24, no. 7, pp. 1750–1758, Jul. 2006.

[261] H. Lin, A. Shabbir, M. Molnar, J. Yang, S. Marion, J. M. J. Canty, and T. Lee, "Adenoviral expression of vascular endothelial growth factor splice variants differentially regulate bone marrow-derived mesenchymal stem cells.," *J. Cell. Physiol.*, vol. 216, no. 2, pp. 458–468, Aug. 2008.

[262] B. Kinner, J. M. Zaleskas, and M. Spector, "Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells.," *Exp. Cell Res.*, vol. 278, no. 1, pp. 72–83, Aug. 2002.

[263] Z. Wen, S. Zheng, C. Zhou, J. Wang, and T. Wang, "Repair mechanisms of bone marrow mesenchymal stem cells in myocardial infarction.," *J. Cell. Mol. Med.*, vol. 15, no. 5, pp. 1032–1043, May 2011.

[264] J.-J. Zhao, X.-C. Liu, F. Kong, T.-G. Qi, G.-H. Cheng, J. Wang, C. Sun, and Y. Luan, "Bone marrow mesenchymal stem cells improve myocardial function in a swine model of acute myocardial infarction.," *Mol. Med. Rep.*, vol. 10, no. 3, pp. 1448–1454, Sep. 2014.

[265] T. P. Lozito, J. M. Taboas, C. K. Kuo, and R. S. Tuan, "Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation.," *J. Cell. Biochem.*, vol. 107, no. 4, pp. 706–713, Jul. 2009.

[266] R. Rahbarghazi, S. M. Nassiri, S. H. Ahmadi, E. Mohammadi, S. Rabbani, A. Araghi, and H. Hosseinkhani, "Dynamic induction of pro-angiogenic milieu after transplantation of marrow-derived mesenchymal stem cells in experimental myocardial infarction.," *Int. J. Cardiol.*, vol. 173, no. 3, pp. 453–466, May 2014.

[267] W. Xu, X. Zhang, H. Qian, W. Zhu, X. Sun, J. Hu, H. Zhou, and Y. Chen, "Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro.," *Exp. Biol. Med. (Maywood).*, vol. 229, no. 7, pp. 623–631, Jul. 2004.

[268] X. Yan, A. Lv, Y. Xing, B. Liu, J. Hou, W. Huang, and Y. Li, "Inhibition of p53-p21 pathway promotes the differentiation of rat bone marrow mesenchymal stem cells into cardiomyocytes.," *Mol. Cell. Biochem.*, vol. 354, no. 1–2, pp. 21–28, Aug. 2011.

[269] R. R. Makkar, M. J. Price, M. Lill, M. Frantzen, K. Takizawa, T. Kleisli, J. Zheng, S. Kar, R. McClelan, T. Miyamota, J. Bick-Forrester, M. C. Fishbein, P. K. Shah, J. S. Forrester, B. Sharifi, P.-S. Chen, and M. Qayyum, "Intramyocardial injection of allogenic bone marrow-derived mesenchymal stem cells without immunosuppression preserves cardiac function in a porcine model of myocardial infarction.," *J. Cardiovasc. Pharmacol. Ther.*, vol. 10, no. 4, pp. 225–233, Dec. 2005.

[270] J. H. Houtgraaf, R. de Jong, K. Kazemi, D. de Groot, T. I. G. van der Spoel, F. Arslan, I. Hoefer, G. Pasterkamp, S. Itescu, F. Zijlstra, M. L. Geleijnse, P. W. Serruys, and H. J. Duckers, "Intracoronary infusion of allogeneic mesenchymal precursor cells directly after experimental acute myocardial infarction reduces infarct size, abrogates adverse remodeling, and improves cardiac function.," *Circ. Res.*, vol. 113, no. 2, pp. 153–166, Jul. 2013.

[271] V. Schachinger, S. Erbs, A. Elsasser, W. Haberbosch, R. Hambrecht, H. Holschermann, J. Yu, R. Corti, D. G. Mathey, C. W. Hamm, T. Suselbeck, B. Assmus, T. Tonn, S. Dimmeler, and A. M. Zeiher, "Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction.," *N. Engl. J. Med.*, vol. 355, no. 12, pp. 1210–1221, Sep. 2006.

[272] J. M. Hare, J. H. Traverse, T. D. Henry, N. Dib, R. K. Strumpf, S. P. Schulman, G. Gerstenblith, A. N. DeMaria, A. E. Denktas, R. S. Gammon, J. J. Hermiller, M. A. Reisman, G. L. Schaer, and W. Sherman, "A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction," *J Am Coll Cardiol*, vol. 54, no. 24, pp. 2277–2286, 2009.

[273] Y. A. Romanov, V. A. Svintsitskaya, and V. N. Smirnov, "Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord.," *Stem Cells*, vol. 21, no. 1, pp. 105–110, 2003.

[274] M. Strioga, S. Viswanathan, A. Darinskas, O. Slaby, and J. Michalek, "Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells.," *Stem Cells Dev.*, vol. 21, no. 14, pp. 2724–2752, Sep. 2012.

[275] A. van Dijk, H. W. M. Niessen, B. Zandieh Doulabi, F. C. Visser, and F. J. van Milligen, "Differentiation of human adipose-derived stem cells towards cardiomyocytes is facilitated by laminin.," *Cell Tissue Res.*, vol. 334, no. 3, pp. 457–467, Dec. 2008.

[276] K. G. Gaustad, A. C. Boquest, B. E. Anderson, A. M. Gerdes, and P. Collas, "Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes.," *Biochem. Biophys. Res. Commun.*, vol. 314, no. 2, pp. 420–427, Feb. 2004.

[277] K. G. Marra, C. A. Brayfield, and J. P. Rubin, "Adipose stem cell differentiation into smooth muscle cells.," *Methods Mol. Biol.*, vol. 702, pp. 261–268, 2011.

[278] S. A. Salem, A. N. M. Hwie, A. Saim, C. H. Chee Kong, I. Sagap, R. Singh, M. R. Yusof, Z. Md Zainuddin, and R. Hj Idrus, "Human adipose tissue derived stem cells as a source of smooth muscle cells in the regeneration of muscular layer of urinary bladder wall.," *Malays. J. Med. Sci.*, vol. 20, no. 4, pp. 80–87, Jul. 2013.

[279] M. Konno, T. S. Hamazaki, S. Fukuda, M. Tokuhara, H. Uchiyama, H. Okazawa, H. Okochi, and M. Asashima, "Efficiently differentiating vascular endothelial cells from adipose tissue-derived mesenchymal stem cells in serum-free culture.," *Biochem. Biophys. Res. Commun.*, vol. 400, no. 4, pp. 461–465, Oct. 2010.

[280] X. Qiu, Y. Zhang, X. Zhao, S. Zhang, J. Wu, H. Guo, and Y. Hu, "Enhancement of endothelial differentiation of adipose derived mesenchymal stem cells by a three-dimensional culture system of microwell.," *Biomaterials*, vol. 53, pp. 600–608, 2015.

[281] H. W. Lee, H. C. Lee, J. H. Park, B. W. Kim, J. Ahn, J. H. Kim, J. S. Park, J.-H. Oh, J. H. Choi, K. S. Cha, T. J. Hong, T. S. Park, S.-P. Kim, S. Song, J. Y. Kim, M. H. Park, and J. S. Jung, "Effects of Intracoronary Administration of Autologous Adipose Tissue-Derived Stem Cells on Acute Myocardial Infarction in a Porcine Model.," *Yonsei Med. J.*, vol. 56, no. 6, pp. 1522–1529, Nov. 2015.

[282] J. Otto Beitnes, E. Oie, A. Shahdadfar, T. Karlsen, R. M. B. Muller, S. Aakhus, F. P. Reinholt, and J. E. Brinchmann, "Intramyocardial injections of human mesenchymal stem cells following acute myocardial infarction modulate scar formation and improve left ventricular function.," *Cell Transplant.*, vol. 21, no. 8, pp. 1697–1709, 2012.

[283] L. Cai, B. H. Johnstone, T. G. Cook, J. Tan, M. C. Fishbein, P.-S. Chen, and K. L. March, "IFATS collection: Human adipose tissue-derived stem cells induce angiogenesis and nerve sprouting following myocardial infarction, in conjunction with potent preservation of cardiac function.," *Stem Cells*, vol. 27, no. 1, pp. 230–237, Jan. 2009.

[284] P. M. C. Davy, K. D. Lye, J. Mathews, J. B. Owens, A. Y. Chow, L. Wong, S. Moisyadi, and R. C. Allsopp, "Human adipose stem cell and ASC-derived cardiac progenitor cellular therapy improves outcomes in a murine model of myocardial infarction," *Stem Cells and Cloning : Advances and Applications*, vol. 8. pp. 135–148, 2015.

[285] I. Perea-Gil, M. Monguio-Tortajada, C. Galvez-Monton, A. Bayes-Genis, F. E. Borras, and S. Roura, "Preclinical evaluation of the immunomodulatory properties of cardiac adipose tissue progenitor cells using umbilical cord blood mesenchymal stem cells: a direct comparative study.," *Biomed Res. Int.*, vol. 2015, p. 439808, 2015.

[286] P. R. Amable, M. V. T. Teixeira, R. B. V. Carias, J. M. Granjeiro, and R. Borojevic, "Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly.," *Stem Cell Res. Ther.*, vol. 5, no. 2, p. 53, Apr. 2014.

[287] E. C. Perin, R. Sanz-Ruiz, P. L. Sanchez, J. Lasso, R. Perez-Cano, J. C. Alonso-Farto, E. Perez-David, M. E. Fernandez-Santos, P. W. Serruys, H. J. Duckers, J. Kastrup, S. Chamuleau, Y. Zheng, G. V Silva, J. T. Willerson, and F. Fernandez-Aviles, "Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial.," *Am. Heart J.*, vol. 168, no. 1, p. 88–95.e2, Jul. 2014.

[288] J. H. Houtgraaf, W. K. den Dekker, B. M. van Dalen, T. Springeling, R. de Jong, R. J. van Geuns, M. L. Geleijnse, F. Fernandez-Aviles, F. Zijlsta, P. W. Serruys, and H. J. Duckers, "First experience in humans using adipose tissue-derived regenerative cells in the treatment of patients with ST-segment elevation myocardial infarction.," *Journal of the American College of Cardiology*, vol. 59, no. 5. United States, pp. 539–540, Jan-2012.

[289] C. Benzhi, Z. Limei, W. Ning, L. Jiaqi, Z. Songling, M. Fanyu, Z. Hongyu, L. Yanjie, A. Jing, and Y. Baofeng, "Bone marrow mesenchymal stem cells upregulate transient outward potassium currents in postnatal rat ventricular myocytes.," *J. Mol. Cell. Cardiol.*, vol. 47, no. 1, pp. 41–48, Jul. 2009.

[290] K. Bieback, S. Kern, H. Kluter, and H. Eichler, "Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood.," *Stem Cells*, vol. 22, no. 4, pp. 625–634, 2004.

[291] T. Li, Q. Ma, M. Ning, Y. Zhao, and Y. Hou, "Cotransplantation of human umbilical cord-derived mesenchymal stem cells and umbilical cord blood-derived CD34(+) cells in a rabbit model of myocardial infarction.," *Mol. Cell. Biochem.*, vol. 387, no. 1–2, pp. 91–100, Feb. 2014.

[292] T. Nagamura-Inoue and H. He, "Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility.," *World J. Stem Cells*, vol. 6, no. 2, pp. 195–202, Apr. 2014.

[293] L. R. Gao, N. K. Zhang, Q. A. Ding, H. Y. Chen, X. Hu, S. Jiang, T. C. Li, Y. Chen, Z. G. Wang, Y. Ye, and Z. M. Zhu, "Common expression of stemness molecular markers and early cardiac transcription factors in human Wharton's jelly-derived mesenchymal stem cells and embryonic stem cells.," *Cell Transplant.*, vol. 22, no. 10, pp. 1883–1900, 2013.

[294] P. Musialek, A. Mazurek, D. Jarocha, L. Tekieli, W. Szot, M. Kostkiewicz, R. P. Banys, M. Urbanczyk, A. Kadzielski, M. Trystula, J. Kijowski, K. Zmudka, P. Podolec, and M. Majka, "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.," *Postep. w Kardiol. interwencyjnej = Adv. Interv. Cardiol.*, vol. 11, no. 2, pp. 100–107, 2015.

[295] P. Swamynathan, P. Venugopal, S. Kannan, C. Thej, U. Kolkundar, S. Bhagwat, M. Ta, A. Sen Majumdar,

and S. Balasubramanian, “Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton’s jelly derived mesenchymal stem cells? A comparative study.,” *Stem Cell Res. Ther.*, vol. 5, no. 4, p. 88, Jul. 2014.

[296] M. Lupu, M. Khalil, E. Andrei, F. Iordache, K. Pfannkuche, K. Neef, A. Georgescu, C. Buzila, K. Brockmeier, H. Maniu, and J. Hescheler, “Integration properties of Wharton’s jelly-derived novel mesenchymal stem cells into ventricular slices of murine hearts.,” *Cell. Physiol. Biochem.*, vol. 28, no. 1, pp. 63–76, 2011.

[297] K. H. Wu, B. Zhou, X. M. Mo, B. Cui, C. T. Yu, S. H. Lu, Z. C. Han, and Y. L. Liu, “Therapeutic potential of human umbilical cord-derived stem cells in ischemic diseases.,” *Transplant. Proc.*, vol. 39, no. 5, pp. 1620–1622, Jun. 2007.

[298] W. Zhang, X.-C. Liu, L. Yang, D.-L. Zhu, Y.-D. Zhang, Y. Chen, and H.-Y. Zhang, “Wharton’s jelly-derived mesenchymal stem cells promote myocardial regeneration and cardiac repair after miniswine acute myocardial infarction.,” *Coron. Artery Dis.*, vol. 24, no. 7, pp. 549–558, Nov. 2013.

[299] D. Santos Nascimento, D. Mosqueira, L. M. Sousa, M. Teixeira, M. Filipe, T. P. Resende, A. F. Araujo, M. Valente, J. Almeida, J. P. Martins, J. M. Santos, R. N. Barcia, P. Cruz, H. Cruz, and P. Pinto-do-O, “Human umbilical cord tissue-derived mesenchymal stromal cells attenuate remodeling after myocardial infarction by proangiogenic, antiapoptotic, and endogenous cell-activation mechanisms.,” *Stem Cell Res. Ther.*, vol. 5, no. 1, p. 5, Jan. 2014.

[300] L. R. Gao, Y. Chen, N. K. Zhang, X. L. Yang, H. L. Liu, Z. G. Wang, X. Y. Yan, Y. Wang, Z. M. Zhu, T. C. Li, L. H. Wang, H. Y. Chen, Y. D. Chen, C. L. Huang, P. Qu, C. Yao, B. Wang, G. H. Chen, Z. M. Wang, Z. Y. Xu, J. Bai, D. Lu, Y. H. Shen, F. Guo, M. Y. Liu, Y. Yang, Y. C. Ding, Y. Yang, H. T. Tian, Q. A. Ding, L. N. Li, X. C. Yang, and X. Hu, “Intracoronary infusion of Wharton’s jelly-derived mesenchymal stem cells in acute myocardial infarction: double-blind, randomized controlled trial.,” *BMC Med.*, vol. 13, p. 162, Jul. 2015.

[301] M. G. Roubelakis, K. I. Pappa, V. Bitsika, D. Zagoura, A. Vlahou, H. A. Papadaki, A. Antsaklis, and N. P. Anagnou, “Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells.,” *Stem Cells Dev.*, vol. 16, no. 6, pp. 931–952, Dec. 2007.

[302] P. Zhao, H. Ise, M. Hongo, M. Ota, I. Konishi, and T. Nikaido, “Human amniotic mesenchymal cells have some characteristics of cardiomyocytes.,” *Transplantation*, vol. 79, no. 5, pp. 528–535, Mar. 2005.

[303] C.-H. Fang, J. Jin, J.-H. Joe, Y.-S. Song, B.-I. So, S. M. Lim, G. J. Cheon, S.-K. Woo, J.-C. Ra, Y.-Y. Lee, and K.-S. Kim, “In vivo differentiation of human amniotic epithelial cells into cardiomyocyte-like cells and cell transplantation effect on myocardial infarction in rats: comparison with cord blood and adipose tissue-derived mesenchymal stem cells.,” *Cell Transplant.*, vol. 21, no. 8, pp. 1687–1696, 2012.

[304] S.-W. Kim, H.-Z. Zhang, C.-E. Kim, J.-M. Kim, and M. H. Kim, “Amniotic mesenchymal stem cells with robust chemotactic properties are effective in the treatment of a myocardial infarction model.,” *Int. J. Cardiol.*, vol. 168, no. 2, pp. 1062–1069, Sep. 2013.

[305] M. Allison, “Genzyme backs Osiris, despite Prochymal flop.,” *Nature biotechnology*, vol. 27, no. 11. United States, pp. 966–967, Nov-2009.

[306] K. Malliaras, M. Kreke, and E. Marban, “The stuttering progress of cell therapy for heart disease.,” *Clin. Pharmacol. Ther.*, vol. 90, no. 4, pp. 532–541, Oct. 2011.

[307] N. Haque, N. H. A. Kasim, and M. T. Rahman, “Optimization of pre-transplantation conditions to enhance the efficacy of mesenchymal stem cells.,” *Int. J. Biol. Sci.*, vol. 11, no. 3, pp. 324–334, 2015.

[308] M. Mastri, H. Lin, and T. Lee, “Enhancing the efficacy of mesenchymal stem cell therapy.,” *World J. Stem Cells*, vol. 6, no. 2, pp. 82–93, 2014.

[309] A. S. Cheng and T. M. Yau, “Paracrine Effects of Cell Transplantation: Strategies to Augment the Efficacy of Cell Therapies,” *Semin. Thorac. Cardiovasc. Surg.*, vol. 20, no. 2, pp. 94–101, 2008.

[310] J. S. Park, N. F. Huang, K. T. Kurpinski, S. Patel, S. Hsu, and S. Li, “Mechanobiology of mesenchymal stem cells and their use in cardiovascular repair.,” *Front. Biosci.*, vol. 12, pp. 5098–5116, Sep. 2007.

[311] J. L. Spees, C. a Gregory, H. Singh, H. A. Tucker, A. Peister, P. J. Lynch, S.-C. Hsu, J. Smith, and D. J. Prockop, “Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy.,” *Mol. Ther.*, vol. 9, no. 5, pp. 747–56, May 2004.

[312] M. Sundin, O. Ringden, B. Sundberg, S. Nava, C. Gotherstrom, and K. Le Blanc, “No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients,” *Haematologica*, vol. 92, no. 9, pp. 1208–1215, Sep. 2007.

[313] A. Heiskanen, T. Satomaa, S. Tiitinen, A. Laitinen, S. Mannelin, U. Impola, M. Mikkola, C. Olsson, H. Miller-Podraza, M. Blomqvist, A. Olonen, H. Salo, P. Lehenkari, T. Tuuri, T. Otonkoski, J. Natunen, J. Saarinen, and J. Laine, “N-glycolylneuraminic acid xenoantigen contamination of human embryonic and

mesenchymal stem cells is substantially reversible.," *Stem Cells*, vol. 25, no. 1, pp. 197–202, Jan. 2007.
[314] H. Komoda, H. Okura, C. M. Lee, N. Sougawa, T. Iwayama, T. Hashikawa, A. Saga, A. Yamamoto-Kakuta, A. Ichinose, S. Murakami, Y. Sawa, and A. Matsuyama, "Reduction of N-glycolylneuraminic acid xenoantigen on human adipose tissue-derived stromal cells/mesenchymal stem cells leads to safer and more useful cell sources for various stem cell therapies.," *Tissue Eng. Part A*, vol. 16, no. 4, pp. 1143–1155, Apr. 2010.
[315] D. Ghaderi, R. E. Taylor, V. Padler-Karavani, S. Diaz, and A. Varki, "Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins.," *Nat. Biotechnol.*, vol. 28, no. 8, pp. 863–867, Aug. 2010.
[316] A. Zhu and R. Hurst, "Anti-N-glycolylneuraminic acid antibodies identified in healthy human serum.," *Xenotransplantation*, vol. 9, no. 6, pp. 376–381, Nov. 2002.
[317] L. G. Chase, U. Lakshmipathy, L. A. Solchaga, M. S. Rao, and M. C. Vemuri, "A novel serum-free medium for the expansion of human mesenchymal stem cells.," *Stem Cell Res. Ther.*, vol. 1, no. 1, p. 8, Apr. 2010.
[318] J. H. An, H. Park, J. A. Song, K. H. Ki, J.-Y. Yang, H. J. Choi, S. W. Cho, S. W. Kim, S. Y. Kim, J. J. Yoo, W.-Y. Baek, J.-E. Kim, S. J. Choi, W. Oh, and C. S. Shin, "Transplantation of human umbilical cord blood-derived mesenchymal stem cells or their conditioned medium prevents bone loss in ovariectomized nude mice.," *Tissue Eng. Part A*, vol. 19, no. 5–6, pp. 685–696, Mar. 2013.
[319] B. Lindroos, S. Boucher, L. Chase, H. Kuokkanen, H. Huhtala, R. Haataja, M. Vemuri, R. Suuronen, and S. Miettinen, "Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro.," *Cytotherapy*, vol. 11, no. 7, pp. 958–72, Jan. 2009.
[320] S. Jung, A. Sen, L. Rosenberg, and L. A. Behie, "Human mesenchymal stem cell culture: rapid and efficient isolation and expansion in a defined serum-free medium.," *J. Tissue Eng. Regen. Med.*, vol. 6, no. 5, pp. 391–403, May 2012.
[321] A. Oskowitz, H. McFerrin, M. Gutschow, M. L. Carter, and R. Pochampally, "Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic.," *Stem Cell Res.*, vol. 6, no. 3, pp. 215–225, May 2011.
[322] T. R. J. Heathman, A. Stolzing, C. Fabian, Q. A. Rafiq, K. Coopman, A. W. Nienow, B. Kara, and C. J. Hewitt, "Serum-free process development: improving the yield and consistency of human mesenchymal stromal cell production.," *Cytotherapy*, vol. 17, no. 11, pp. 1524–1535, Nov. 2015.
[323] S. Bobis-Wozowicz, K. Kmiotek, K. Kania, E. Karnas, A. Labedz-Maslowska, M. Sekula, S. Kedracka-Krok, J. Kolcz, D. Boruczkowski, Z. Madeja, and E. K. Zuba-Surma, "Diverse impact of xeno-free conditions on biological and regenerative properties of hUC-MSCs and their extracellular vesicles.," *J. Mol. Med. (Berl).*, vol. 95, no. 2, pp. 205–220, Feb. 2017.
[324] P. R. Amable, M. V. T. Teixeira, R. B. V. Carias, J. M. Granjeiro, and R. Borojevic, "Mesenchymal stromal cell proliferation, gene expression and protein production in human platelet-rich plasma-supplemented media.," *PLoS One*, vol. 9, no. 8, p. e104662, 2014.
[325] R. Crespo-Diaz, A. Behfar, G. W. Butler, D. J. Padley, M. G. Sarr, J. Bartunek, A. B. Dietz, and A. Terzic, "Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability.," *Cell Transplant.*, vol. 20, no. 6, pp. 797–811, 2011.
[326] S. Griffiths, P. R. Baraniak, I. B. Copland, R. M. Nerem, and T. C. McDevitt, "Human platelet lysate stimulates high-passage and senescent human multipotent mesenchymal stromal cell growth and rejuvenation in vitro.," *Cytotherapy*, vol. 15, no. 12, pp. 1469–1483, Dec. 2013.
[327] S. M. Jonsdottir-Buch, R. Lieder, and O. E. Sigurjonsson, "Platelet lysates produced from expired platelet concentrates support growth and osteogenic differentiation of mesenchymal stem cells.," *PLoS One*, vol. 8, no. 7, p. e68984, 2013.
[328] M. Perez-Ilzarbe, M. Diez-Campelo, P. Aranda, S. Tabera, T. Lopez, C. del Canizo, J. Merino, C. Moreno, E. J. Andreu, F. Prosper, and J. A. Perez-Simon, "Comparison of ex vivo expansion culture conditions of mesenchymal stem cells for human cell therapy.," *Transfusion*, vol. 49, no. 9, pp. 1901–1910, Sep. 2009.
[329] S.-F. Trojahn Kolle, R. S. Oliveri, P. V Glovinski, M. Kirchhoff, A. B. Mathiasen, J. J. Elberg, P. S. Andersen, K. T. Drzewiecki, and A. Fischer-Nielsen, "Pooled human platelet lysate versus fetal bovine serum-investigating the proliferation rate, chromosome stability and angiogenic potential of human adipose tissue-derived stem cells intended for clinical use.," *Cytotherapy*, vol. 15, no. 9, pp. 1086–1097, Sep. 2013.
[330] H. Abdelrazik, G. M. Spaggiari, L. Chiossone, and L. Moretta, "Mesenchymal stem cells expanded in human platelet lysate display a decreased inhibitory capacity on T- and NK-cell proliferation and function.," *Eur. J. Immunol.*, vol. 41, no. 11, pp. 3281–3290, Nov. 2011.
[331] K. Schallmoser, C. Bartmann, E. Rohde, A. Reinisch, K. Kashofer, E. Stadelmeyer, C. Drexler, G. Lanzer, W. Linkesch, and D. Strunk, "Human platelet lysate can replace fetal bovine serum for clinical-scale

expansion of functional mesenchymal stromal cells.," *Transfusion*, vol. 47, no. 8, pp. 1436–1446, Aug. 2007.
[332] N. Ben Azouna, F. Jenhani, Z. Regaya, L. Berraeis, T. Ben Othman, E. Ducrocq, and J. Domenech, "Phenotypical and functional characteristics of mesenchymal stem cells from bone marrow: comparison of culture using different media supplemented with human platelet lysate or fetal bovine serum.," *Stem Cell Res. Ther.*, vol. 3, no. 1, p. 6, Feb. 2012.
[333] C. Li, X. Wu, J. Tong, X. Yang, J. Zhao, Q. Zheng, G. Zhao, and Z. Ma, "Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy.," *Stem Cell Res. Ther.*, vol. 6, p. 55, Apr. 2015.
[334] N. Mizuno, H. Shiba, Y. Ozeki, Y. Mouri, M. Niitani, T. Inui, H. Hayashi, K. Suzuki, S. Tanaka, H. Kawaguchi, and H. Kurihara, "Human autologous serum obtained using a completely closed bag system as a substitute for foetal calf serum in human mesenchymal stem cell cultures.," *Cell Biol. Int.*, vol. 30, no. 6, pp. 521–524, Jun. 2006.
[335] A. Shahdadfar, K. Fronsdal, T. Haug, F. P. Reinholt, and J. E. Brinchmann, "In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability.," *Stem Cells*, vol. 23, no. 9, pp. 1357–1366, Oct. 2005.
[336] N. Stute, K. Holtz, M. Bubenheim, C. Lange, F. Blake, and A. R. Zander, "Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use.," *Exp. Hematol.*, vol. 32, no. 12, pp. 1212–1225, Dec. 2004.
[337] A. C. C. Paula, T. M. M. Martins, A. Zonari, S. P. P. J. Frade, P. C. Angelo, D. A. Gomes, and A. M. Goes, "Human adipose tissue-derived stem cells cultured in xeno-free culture condition enhance c-MYC expression increasing proliferation but bypassing spontaneous cell transformation," *Stem Cell Research & Therapy*, vol. 6, no. 1. London, 2015.
[338] K. Cooper, A. SenMajumdar, and C. Viswanathan, "Derivation, Expansion and Characterization of Clinical Grade Mesenchymal Stem Cells from Umbilical Cord Matrix Using Cord Blood Serum," *International Journal of Stem Cells*, vol. 3, no. 2. pp. 119–128, May-2010.
[339] K. Le Blanc, H. Samuelsson, L. Lonnies, M. Sundin, and O. Ringden, "Generation of immunosuppressive mesenchymal stem cells in allogeneic human serum.," *Transplantation*, vol. 84, no. 8, pp. 1055–1059, Oct. 2007.
[340] S. M. Phadnis, M. V Joglekar, V. Venkateshan, S. M. Ghaskadbi, A. A. Hardikar, and R. R. Bhonde, "Human umbilical cord blood serum promotes growth, proliferation, as well as differentiation of human bone marrow-derived progenitor cells.," *In Vitro Cell. Dev. Biol. Anim.*, vol. 42, no. 10, pp. 283–286, 2006.
[341] K. Turnovcova, K. Ruzickova, V. Vanecek, E. Sykova, and P. Jendelova, "Properties and growth of human bone marrow mesenchymal stromal cells cultivated in different media.," *Cytotherapy*, vol. 11, no. 7, pp. 874–885, 2009.
[342] E. Chavakis, M. Koyanagi, and S. Dimmeler, "Enhancing the outcome of cell therapy for cardiac repair: progress from bench to bedside and back.," *Circulation*, vol. 121, no. 2, pp. 325–335, Jan. 2010.
[343] X.-B. Liu, J.-A. Wang, M. E. Ogle, and L. Wei, "Prolyl hydroxylase inhibitor dimethyloxalylglycine enhances mesenchymal stem cell survival.," *J. Cell. Biochem.*, vol. 106, no. 5, pp. 903–911, Apr. 2009.
[344] A. Mohyeldin, T. Garzon-Muvdi, and A. Quinones-Hinojosa, "Oxygen in stem cell biology: a critical component of the stem cell niche.," *Cell Stem Cell*, vol. 7, no. 2, pp. 150–161, Aug. 2010.
[345] L. Basciano, C. Nemos, B. Foliguet, N. de Isla, M. de Carvalho, N. Tran, and A. Dalloul, "Long term culture of mesenchymal stem cells in hypoxia promotes a genetic program maintaining their undifferentiated and multipotent status.," *BMC Cell Biol.*, vol. 12, p. 12, Mar. 2011.
[346] T. Kinnaird, E. Stabile, M. S. Burnett, C. W. Lee, S. Barr, S. Fuchs, and S. E. Epstein, "Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms," *Circ. Res.*, vol. 94, no. 5, pp. 678–685.
[347] S. Ohnishi, T. Yasuda, S. Kitamura, and N. Nagaya, "Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells.," *Stem Cells*, vol. 25, no. 5, pp. 1166–1177, May 2007.
[348] J. Paquet, M. Deschepper, A. Moya, D. Logeart-Avramoglou, C. Boisson-Vidal, and H. Petite, "Oxygen Tension Regulates Human Mesenchymal Stem Cell Paracrine Functions.," *Stem Cells Transl. Med.*, vol. 4, no. 7, pp. 809–821, Jul. 2015.
[349] P. R. Crisostomo, Y. Wang, T. A. Markel, M. Wang, T. Lahm, and D. R. Meldrum, "Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism.," *Am. J. Physiol. Cell Physiol.*, vol. 294, no. 3, pp. C675-82, Mar. 2008.
[350] J. Yang, W. Zhou, W. Zheng, Y. Ma, L. Lin, T. Tang, J. Liu, J. Yu, X. Zhou, and J. Hu, "Effects of Myocardial Transplantation of Marrow Mesenchymal Stem Cells Transfected with Vascular Endothelial

Growth Factor for the Improvement of Heart Function and Angiogenesis after Myocardial Infarction," *Cardiology*, vol. 107, no. 1, pp. 17–29, 2007.

[351] R. Uemura, M. Xu, N. Ahmad, and M. Ashraf, "Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling.," *Circ. Res.*, vol. 98, no. 11, pp. 1414–1421, Jun. 2006.

[352] A. Cselenyák, E. Pankotai, E. M. Horváth, L. Kiss, and Z. Lacza, "Mesenchymal stem cells rescue cardiomyoblasts from cell death in an in vitro ischemia model via direct cell-to-cell connections," *BMC Cell Biol.*, vol. 11, no. 1, p. 29, 2010.

[353] X. Hu, S. P. Yu, J. L. Fraser, Z. Lu, M. E. Ogle, J.-A. Wang, and L. Wei, "Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis.," *J. Thorac. Cardiovasc. Surg.*, vol. 135, no. 4, pp. 799–808, Apr. 2008.

[354] X. Hu, Y. Xu, Z. Zhong, Y. Wu, J. Zhao, Y. Wang, H. Cheng, M. Kong, F. Zhang, Q. Chen, J. Sun, Q. Li, J. Jin, Q. Li, L. Chen, C. Wang, H. Zhan, Y. Fan, Q. Yang, L. Yu, R. Wu, J. Liang, J. Zhu, Y. Wang, Y. Jin, Y. Lin, F. Yang, L. Jia, W. Zhu, J. Chen, H. Yu, J. Zhang, and J. Wang, "A Large-Scale Investigation of Hypoxia-Preconditioned Allogeneic Mesenchymal Stem Cells for Myocardial Repair in Nonhuman Primates: Paracrine Activity Without Remuscularization.," *Circ. Res.*, vol. 118, no. 6, pp. 970–983, Mar. 2016.

[355] R. J. Phillips, J. Mestas, M. Gharaee-Kermani, M. D. Burdick, A. Sica, J. A. Belperio, M. P. Keane, and R. M. Strieter, "Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha.," *J. Biol. Chem.*, vol. 280, no. 23, pp. 22473–22481, Jun. 2005.

[356] T. Schioppa, B. Uranchimeg, A. Saccani, S. K. Biswas, A. Doni, A. Rapisarda, S. Bernasconi, S. Saccani, M. Nebuloni, L. Vago, A. Mantovani, G. Melillo, and A. Sica, "Regulation of the chemokine receptor CXCR4 by hypoxia.," *J. Exp. Med.*, vol. 198, no. 9, pp. 1391–1402, Nov. 2003.

[357] B. Annabi, Y.-T. Lee, S. Turcotte, E. Naud, R. R. Desrosiers, M. Champagne, N. Eliopoulos, J. Galipeau, and R. Beliveau, "Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation.," *Stem Cells*, vol. 21, no. 3, pp. 337–347, 2003.

[358] S.-C. Hung, R. R. Pochampally, S.-C. Hsu, C. Sanchez, S.-C. Chen, J. Spees, and D. J. Prockop, "Short-Term Exposure of Multipotent Stromal Cells to Low Oxygen Increases Their Expression of CX3CR1 and CXCR4 and Their Engraftment In Vivo," *PLoS ONE*, vol. 2, no. 5. San Francisco, USA, 2007.

[359] M. Khan, S. Meduru, R. Gogna, E. Madan, L. Citro, M. L. Kuppusamy, M. Sayyid, M. Mostafa, R. L. Hamlin, and P. Kuppusamy, "Oxygen cycling in conjunction with stem cell transplantation induces NOS3 expression leading to attenuation of fibrosis and improved cardiac function," *Cardiovascular Research*, vol. 93, no. 1. pp. 89–99, Jan-2012.

[360] M. Khan, S. Meduru, I. K. Mohan, M. L. Kuppusamy, S. Wisel, A. Kulkarni, B. K. Rivera, R. L. Hamlin, and P. Kuppusamy, "Hyperbaric oxygenation enhances transplanted cell graft and functional recovery in the infarct heart.," *J. Mol. Cell. Cardiol.*, vol. 47, no. 2, pp. 275–287, Aug. 2009.

[361] G. Pattappa, S. D. Thorpe, N. C. Jegard, H. K. Heywood, J. D. de Bruijn, and D. A. Lee, "Continuous and uninterrupted oxygen tension influences the colony formation and oxidative metabolism of human mesenchymal stem cells.," *Tissue Eng. Part C. Methods*, vol. 19, no. 1, pp. 68–79, Jan. 2013.

[362] H. Wang, G. M. Riha, S. Yan, M. Li, H. Chai, H. Yang, Q. Yao, and C. Chen, "Shear stress induces endothelial differentiation from a murine embryonic mesenchymal progenitor cell line.," *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 9, pp. 1817–1823, Sep. 2005.

[363] J. Dong, Y. Gu, C. Li, C. Wang, Z. Feng, R. Qiu, B. Chen, J. Li, S. Zhang, Z. Wang, and J. Zhang, "Response of mesenchymal stem cells to shear stress in tissue-engineered vascular grafts.," *Acta Pharmacol. Sin.*, vol. 30, no. 5, pp. 530–536, May 2009.

[364] N. Kobayashi, T. Yasu, H. Ueba, M. Sata, S. Hashimoto, M. Kuroki, M. Saito, and M. Kawakami, "Mechanical stress promotes the expression of smooth muscle-like properties in marrow stromal cells.," *Exp. Hematol.*, vol. 32, no. 12, pp. 1238–1245, Dec. 2004.

[365] D. H. Kim, S.-J. Heo, S.-H. Kim, J. W. Shin, S. H. Park, and J.-W. Shin, "Shear stress magnitude is critical in regulating the differentiation of mesenchymal stem cells even with endothelial growth medium.," *Biotechnol. Lett.*, vol. 33, no. 12, pp. 2351–2359, Dec. 2011.

[366] V. Bassaneze, V. G. Barauna, C. Lavini-Ramos, J. Kalil, I. T. Schettert, A. A. Miyakawa, and J. E. Krieger, "Shear stress induces nitric oxide-mediated vascular endothelial growth factor production in human adipose tissue mesenchymal stem cells.," *Stem Cells Dev.*, vol. 19, no. 3, pp. 371–378, Mar. 2010.

[367] S. Shojaei, M. Tafazzoli-Shahdpour, M. A. Shokrgozar, and N. Haghighipour, "Effects of mechanical and chemical stimuli on differentiation of human adipose-derived stem cells into endothelial cells.," *Int. J. Artif. Organs*, vol. 36, no. 9, pp. 663–673, Oct. 2013.

[368] P. Zhang, N. Moudgill, E. Hager, N. Tarola, C. DiMatteo, S. McIlhenny, T. Tulenko, and P. J. DiMuzio, "Endothelial Differentiation of Adipose-Derived Stem Cells from Elderly Patients with Cardiovascular Disease," *Stem Cells and Development*, vol. 20, no. 6. 140 Huguenot Street, 3rd FloorNew Rochelle, NY 10801USA, pp. 977–988, Jun-2011.

[369] P. Zhang, J. Baxter, K. Vinod, T. N. Tulenko, and P. J. Di Muzio, "Endothelial Differentiation of Amniotic Fluid–Derived Stem Cells: Synergism of Biochemical and Shear Force Stimuli," *Stem Cells and Development*, vol. 18, no. 9. 140 Huguenot Street, 3rd FloorNew Rochelle, NY 10801-5215USA, pp. 1299–1308, Nov-2009.

[370] C.-C. Wu, Y.-C. Chao, C.-N. Chen, S. Chien, Y.-C. Chen, C.-C. Chien, J.-J. Chiu, and B. Linju Yen, "Synergism of biochemical and mechanical stimuli in the differentiation of human placenta-derived multipotent cells into endothelial cells.," *J. Biomech.*, vol. 41, no. 4, pp. 813–821, 2008.

[371] Y. Huang, X. Jia, K. Bai, X. Gong, and Y. Fan, "Effect of fluid shear stress on cardiomyogenic differentiation of rat bone marrow mesenchymal stem cells.," *Arch. Med. Res.*, vol. 41, no. 7, pp. 497–505, Oct. 2010.

[372] D. W. Hamilton, T. M. Maul, and D. A. Vorp, "Characterization of the response of bone marrow-derived progenitor cells to cyclic strain: implications for vascular tissue-engineering applications.," *Tissue Eng.*, vol. 10, no. 3–4, pp. 361–369, 2004.

[373] J. S. Park, J. S. F. Chu, C. Cheng, F. Chen, D. Chen, and S. Li, "Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells.," *Biotechnol. Bioeng.*, vol. 88, no. 3, pp. 359–368, Nov. 2004.

[374] K. Kanda, T. Matsuda, and T. Oka, "Two-dimensional orientational response of smooth muscle cells to cyclic stretching.," *ASAIO J.*, vol. 38, no. 3, pp. M382-5, 1992.

[375] N. F. Huang and S. Li, "Mesenchymal stem cells for vascular regeneration.," *Regen. Med.*, vol. 3, no. 6, pp. 877–892, Nov. 2008.

[376] K. Kurpinski, J. Chu, C. Hashi, and S. Li, "Anisotropic mechanosensing by mesenchymal stem cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 44, pp. 16095–16100, Oct. 2006.

[377] Y. Huang, L. Zheng, X. Gong, X. Jia, W. Song, M. Liu, and Y. Fan, "Effect of Cyclic Strain on Cardiomyogenic Differentiation of Rat Bone Marrow Derived Mesenchymal Stem Cells," *PLoS ONE*, vol. 7, no. 4. San Francisco, USA, 2012.

[378] E. D. O'Cearbhaill, M. A. Punchard, M. Murphy, F. P. Barry, P. E. McHugh, and V. Barron, "Response of mesenchymal stem cells to the biomechanical environment of the endothelium on a flexible tubular silicone substrate.," *Biomaterials*, vol. 29, no. 11, pp. 1610–1619, Apr. 2008.

[379] H. Wang, M. Li, P. H. Lin, Q. Yao, and C. Chen, "Fluid shear stress regulates the expression of TGF-beta1 and its signaling molecules in mouse embryo mesenchymal progenitor cells.," *J. Surg. Res.*, vol. 150, no. 2, pp. 266–270, Dec. 2008.

[380] F. G. Teixeira, K. M. Panchalingam, R. Assunção-Silva, S. C. Serra, B. Mendes-Pinheiro, P. Patrício, S. Jung, S. I. Anjo, B. Manadas, L. Pinto, N. Sousa, L. A. Behie, and A. J. Salgado, "Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors: Impact on Neuronal Cell Proliferation, Survival and Differentiation," *Scientific Reports*, vol. 6. 2016.

[381] J. Y. Hahn, H. J. Cho, H. J. Kang, T. S. Kim, M. H. Kim, J. H. Chung, J. W. Bae, B. H. Oh, Y. B. Park, and H. S. Kim, "Pre-Treatment of Mesenchymal Stem Cells With a Combination of Growth Factors Enhances Gap Junction Formation, Cytoprotective Effect on Cardiomyocytes, and Therapeutic Efficacy for Myocardial Infarction," *J. Am. Coll. Cardiol.*, vol. 51, no. 9, pp. 933–943, 2008.

[382] S. Movafagh, J. Hobson, S. Spiegel, H. Kleinman, and Z. Zukowska, "Neuropeptide Y induces migration, proliferation, and tube formation of endothelial cells bimodally via Y1, Y2, and Y5 receptors," *FASEB J*, vol. 20, no. 11, pp. 1924–6, 2006.

[383] Y. Wang, D. Zhang, M. Ashraf, T. Zhao, W. Huang, A. Ashraf, and A. Balasubramaniam, "Combining neuropeptide Y and mesenchymal stem cells reverses remodeling after myocardial infarction.," *Am. J. Physiol. Heart Circ. Physiol.*, vol. 298, no. 1, pp. H275-86, 2010.

[384] M. Wang, W. Zhang, P. Crisostomo, T. Markel, K. K. Meldrum, X. Y. Fu, and D. R. Meldrum, "STAT3 mediates bone marrow mesenchymal stem cell VEGF production," *J. Mol. Cell. Cardiol.*, vol. 42, no. 6, pp. 1009–1015, 2007.

[385] Z. Pasha, Y. Wang, R. Sheikh, D. Zhang, T. Zhao, and M. Ashraf, "Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium," *Cardiovasc. Res.*, vol. 77, no. 1, pp. 134–142, 2008.

[386] J. Herrmann, Y. Wang, A. Abarbanell, B. Weil, J. Tan, and D. Meldrum, "Preconditioning mesenchymal stem cells with transforming growth factor-alpha improves mesenchymal stem cell-mediated cardioprotection," *Shock*, vol. 33, no. 1, pp. 24–30, 2010.

[387] B. Xu, Y. Luo, Y. Liu, B.-Y. Li, and Y. Wang, "Platelet-derived growth factor-BB enhances MSC-mediated cardioprotection via suppression of miR-320 expression," *Am. J. Physiol. - Hear. Circ. Physiol.*,

vol. 1, p. ajpheart.00737.2014, 2015.
[388] J. Guo, G. Lin, C. Bao, Z. Hu, H. Chu, and M. Hu, "Insulin-like growth factor 1 improves the efficacy of mesenchymal stem cells transplantation in a rat model of myocardial infarction," *J Biomed Sci*, vol. 15, no. 1, pp. 89–97, 2008.
[389] J. Guo, D. Zheng, W. Li, H. Li, A. Zhang, and Z. Li, "Insulin-Like Growth Factor 1 Treatment of MSCs Attenuates Inflammation and Cardiac Dysfunction Following MI," *Inflammation*, vol. 37, no. 6, pp. 2156–2163, 2014.
[390] C. M. Wang, Z. Guo, Y. J. Xie, Y. Y. Hao, J. M. Sun, J. Gu, and A. L. Wang, "Co-treating mesenchymal stem cells with IL-1β and TNF-α increases VCAM-1 expression and improves post-ischemic myocardial function," *Mol. Med. Rep.*, vol. 10, no. 2, pp. 792–798, 2014.
[391] M. Shi, J. Li, L. Liao, B. Chen, B. Li, L. Chen, H. Jia, and R. C. Zhao, "Regulation of CXCR4 expression in human mesenchymal stem cells by cytokin treatment: role in homing efficiency in NOD/SCID mice," *Hematol. J.*, vol. 92, no. 7, pp. 897–904, 2007.
[392] X. Liang, Y. Ding, Y. Zhang, H. F. Tse, and Q. Lian, "Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives," *Cell Transpl.*, vol. 23, no. 9, pp. 1045–1059, 2014.
[393] L. Li, X. Chen, W. E. Wang, and C. Zeng, "How to Improve the Survival of Transplanted Mesenchymal Stem Cell in Ischemic Heart?," *Stem Cells Int.*, vol. 2016, p. 9682757, 2016.
[394] J. Cutts, M. Nikkhah, and D. A. Brafman, "Biomaterial Approaches for Stem Cell-Based Myocardial Tissue Engineering," *Biomarker Insights*, vol. 10, no. Suppl 1. pp. 77–90, 2015.
[395] W. B. VanWinkle, M. B. Snuggs, and L. M. Buja, "Cardiogel: a biosynthetic extracellular matrix for cardiomyocyte culture.," *In Vitro Cell. Dev. Biol. Anim.*, vol. 32, no. 8, pp. 478–485, Sep. 1996.
[396] P. Sreejit and R. S. Verma, "Enhanced cardiomyogenic lineage differentiation of adult bone-marrow-derived stem cells grown on cardiogel.," *Cell Tissue Res.*, vol. 353, no. 3, pp. 443–456, Sep. 2013.
[397] A. Miskon, A. Mahara, H. Uyama, and T. Yamaoka, "A suspension induction for myocardial differentiation of rat mesenchymal stem cells on various extracellular matrix proteins.," *Tissue Eng. Part C. Methods*, vol. 16, no. 5, pp. 979–987, Oct. 2010.
[398] J. A. Santiago, R. Pogemiller, and B. M. Ogle, "Heterogeneous differentiation of human mesenchymal stem cells in response to extended culture in extracellular matrices.," *Tissue Eng. Part A*, vol. 15, no. 12, pp. 3911–3922, Dec. 2009.
[399] G. Tan, W. Shim, Y. Gu, L. Qian, Y. Y. Chung, S. Y. Lim, P. Yong, E. Sim, and P. Wong, "Differential effect of myocardial matrix and integrins on cardiac differentiation of human mesenchymal stem cells.," *Differentiation.*, vol. 79, no. 4–5, pp. 260–271, 2010.
[400] U. Sarig, E. B.-V. Nguyen, Y. Wang, S. Ting, T. Bronshtein, H. Sarig, N. Dahan, M. Gvirtz, S. Reuveny, S. K. W. Oh, T. Scheper, Y. C. F. Boey, S. S. Venkatraman, and M. Machluf, "Pushing the Envelope in Tissue Engineering: Ex Vivo Production of Thick Vascularized Cardiac Extracellular Matrix Constructs," *Tissue Engineering. Part A*, vol. 21, no. 9–10. 140 Huguenot Street, 3rd FloorNew Rochelle, NY 10801USA, pp. 1507–1519, May-2015.
[401] B.-H. Liu, H.-Y. Yeh, Y.-C. Lin, M.-H. Wang, D. C. Chen, B.-H. Lee, and S.-H. Hsu, "Spheroid formation and enhanced cardiomyogenic potential of adipose-derived stem cells grown on chitosan.," *Biores. Open Access*, vol. 2, no. 1, pp. 28–39, Feb. 2013.
[402] H.-Y. Yeh, B.-H. Liu, and S.-H. Hsu, "The calcium-dependent regulation of spheroid formation and cardiomyogenic differentiation for MSCs on chitosan membranes.," *Biomaterials*, vol. 33, no. 35, pp. 8943–8954, Dec. 2012.
[403] M.-C. Yang, N.-H. Chi, N.-K. Chou, Y.-Y. Huang, T.-W. Chung, Y.-L. Chang, H.-C. Liu, M.-J. Shieh, and S.-S. Wang, "The influence of rat mesenchymal stem cell CD44 surface markers on cell growth, fibronectin expression, and cardiomyogenic differentiation on silk fibroin - Hyaluronic acid cardiac patches.," *Biomaterials*, vol. 31, no. 5, pp. 854–862, Feb. 2010.
[404] N.-H. Chi, M.-C. Yang, T.-W. Chung, N.-K. Chou, and S.-S. Wang, "Cardiac repair using chitosan-hyaluronan/silk fibroin patches in a rat heart model with myocardial infarction.," *Carbohydr. Polym.*, vol. 92, no. 1, pp. 591–597, Jan. 2013.
[405] H. Niu, J. Mu, J. Zhang, P. Hu, P. Bo, and Y. Wang, "Comparative study of three types of polymer materials co-cultured with bone marrow mesenchymal stem cells for use as a myocardial patch in cardiomyocyte regeneration.," *J. Mater. Sci. Mater. Med.*, vol. 24, no. 6, pp. 1535–1542, Jun. 2013.
[406] S. W. Crowder, Y. Liang, R. Rath, A. M. Park, S. Maltais, P. N. Pintauro, W. Hofmeister, C. C. Lim, X. Wang, and H.-J. Sung, "Poly(ε-caprolactone)-carbon nanotube composite scaffolds for enhanced cardiac differentiation of human mesenchymal stem cells," *Nanomedicine (London, England)*, vol. 8, no. 11. Nov-2013.
[407] L. Tian, M. P. Prabhakaran, X. Ding, D. Kai, and S. Ramakrishna, "Emulsion electrospun nanofibers as substrates for cardiomyogenic differentiation of mesenchymal stem cells.," *J. Mater. Sci. Mater. Med.*,

vol. 24, no. 11, pp. 2577–2587, Nov. 2013.
[408] R. Ravichandran, J. R. Venugopal, S. Sundarrajan, S. Mukherjee, and S. Ramakrishna, "Cardiogenic differentiation of mesenchymal stem cells on elastomeric poly (glycerol sebacate)/collagen core/shell fibers," *World Journal of Cardiology*, vol. 5, no. 3. pp. 28–41, Mar-2013.
[409] H. J. Anderson, J. K. Sahoo, R. V Ulijn, and M. J. Dalby, "Mesenchymal Stem Cell Fate: Applying Biomaterials for Control of Stem Cell Behavior," *Frontiers in Bioengineering and Biotechnology*, vol. 4. 2016.
[410] Z. Li, X. Guo, A. F. Palmer, H. Das, and J. Guan, "High-efficiency matrix modulus-induced cardiac differentiation of human mesenchymal stem cells inside a thermosensitive hydrogel.," *Acta Biomater.*, vol. 8, no. 10, pp. 3586–3595, Oct. 2012.
[411] D. A. Pijnappels, M. J. Schalij, A. A. Ramkisoensing, J. van Tuyn, A. A. F. de Vries, A. van der Laarse, D. L. Ypey, and D. E. Atsma, "Forced alignment of mesenchymal stem cells undergoing cardiomyogenic differentiation affects functional integration with cardiomyocyte cultures.," *Circ. Res.*, vol. 103, no. 2, pp. 167–176, Jul. 2008.
[412] B. Balana, C. Nicoletti, I. Zahanich, E. M. Graf, T. Christ, S. Boxberger, and U. Ravens, "5-Azacytidine induces changes in electrophysiological properties of human mesenchymal stem cells.," *Cell Res.*, vol. 16, no. 12, pp. 949–960, Dec. 2006.
[413] H.-J. Wei, C.-H. Chen, W.-Y. Lee, I. Chiu, S.-M. Hwang, W.-W. Lin, C.-C. Huang, Y.-C. Yeh, Y. Chang, and H.-W. Sung, "Bioengineered cardiac patch constructed from multilayered mesenchymal stem cells for myocardial repair.," *Biomaterials*, vol. 29, no. 26, pp. 3547–3556, Sep. 2008.
[414] A. A. Abdeen, J. B. Weiss, J. Lee, and K. A. Kilian, "Matrix composition and mechanics direct proangiogenic signaling from mesenchymal stem cells.," *Tissue Eng. Part A*, vol. 20, no. 19–20, pp. 2737–2745, Oct. 2014.
[415] G. Xu, X. Wang, C. Deng, X. Teng, E. J. Suuronen, Z. Shen, and Z. Zhong, "Injectable biodegradable hybrid hydrogels based on thiolated collagen and oligo(acryloyl carbonate)-poly(ethylene glycol)-oligo(acryloyl carbonate) copolymer for functional cardiac regeneration.," *Acta Biomater.*, vol. 15, pp. 55–64, Mar. 2015.
[416] R. D. Levit, N. Landazuri, E. A. Phelps, M. E. Brown, A. J. Garcia, M. E. Davis, G. Joseph, R. Long, S. A. Safley, J. D. Suever, A. N. Lyle, C. J. Weber, and W. R. Taylor, "Cellular encapsulation enhances cardiac repair.," *J. Am. Heart Assoc.*, vol. 2, no. 5, p. e000367, Oct. 2013.
[417] P. Maureira, P.-Y. Marie, F. Yu, S. Poussier, Y. Liu, F. Groubatch, A. Falanga, and N. Tran, "Repairing chronic myocardial infarction with autologous mesenchymal stem cells engineered tissue in rat promotes angiogenesis and limits ventricular remodeling.," *J. Biomed. Sci.*, vol. 19, p. 93, Nov. 2012.
[418] M. Arana, J. J. Gavira, E. Pena, A. Gonzalez, G. Abizanda, M. Cilla, M. M. Perez, E. Albiasu, N. Aguado, M. Casado, B. Lopez, S. Gonzalez, M. Soriano, C. Moreno, J. Merino, J. M. Garcia-Verdugo, J. Diez, M. Doblare, B. Pelacho, and F. Prosper, "Epicardial delivery of collagen patches with adipose-derived stem cells in rat and minipig models of chronic myocardial infarction.," *Biomaterials*, vol. 35, no. 1, pp. 143–151, Jan. 2014.
[419] R. G. Gomez-Mauricio, A. Acarregui, F. M. Sanchez-Margallo, V. Crisostomo, I. Gallo, R. M. Hernandez, J. L. Pedraz, G. Orive, and M. F. Martin-Cancho, "A preliminary approach to the repair of myocardial infarction using adipose tissue-derived stem cells encapsulated in magnetic resonance-labelled alginate microspheres in a porcine model.," *Eur. J. Pharm. Biopharm.*, vol. 84, no. 1, pp. 29–39, May 2013.
[420] S. Fukuhara, S. Tomita, T. Nakatani, T. Fujisato, Y. Ohtsu, M. Ishida, C. Yutani, and S. Kitamura, "Bone marrow cell-seeded biodegradable polymeric scaffold enhances angiogenesis and improves function of the infarcted heart.," *Circ. J.*, vol. 69, no. 7, pp. 850–857, Jul. 2005.
[421] H. Piao, J.-S. Kwon, S. Piao, J.-H. Sohn, Y.-S. Lee, J.-W. Bae, K.-K. Hwang, D.-W. Kim, O. Jeon, B.-S. Kim, Y.-B. Park, and M.-C. Cho, "Effects of cardiac patches engineered with bone marrow-derived mononuclear cells and PGCL scaffolds in a rat myocardial infarction model.," *Biomaterials*, vol. 28, no. 4, pp. 641–649, Feb. 2007.
[422] J. Jin, S. I. Jeong, Y. M. Shin, K. S. Lim, H. soo Shin, Y. M. Lee, H. C. Koh, and K.-S. Kim, "Transplantation of mesenchymal stem cells within a poly(lactide-co-epsilon-caprolactone) scaffold improves cardiac function in a rat myocardial infarction model.," *Eur. J. Heart Fail.*, vol. 11, no. 2, pp. 147–153, Feb. 2009.
[423] E. Mathieu, G. Lamirault, C. Toquet, P. Lhommet, E. Rederstorff, S. Sourice, K. Biteau, P. Hulin, V. Forest, P. Weiss, J. Guicheux, and P. Lemarchand, "Intramyocardial delivery of mesenchymal stem cell-seeded hydrogel preserves cardiac function and attenuates ventricular remodeling after myocardial infarction.," *PLoS One*, vol. 7, no. 12, p. e51991, 2012.
[424] N. C. Panda, S. T. Zuckerman, O. O. Mesubi, D. S. Rosenbaum, M. S. Penn, J. K. Donahue, E. Alsberg, and K. R. Laurita, "Improved conduction and increased cell retention in healed MI using mesenchymal

stem cells suspended in alginate hydrogel.," *J. Interv. Card. Electrophysiol.*, vol. 41, no. 2, pp. 117–127, Nov. 2014.

[425] X. Zhang, H. Wang, X. Ma, A. Adila, B. Wang, F. Liu, B. Chen, C. Wang, and Y. Ma, "Preservation of the cardiac function in infarcted rat hearts by the transplantation of adipose-derived stem cells with injectable fibrin scaffolds.," *Exp. Biol. Med. (Maywood).*, vol. 235, no. 12, pp. 1505–1515, Dec. 2010.

[426] A.-C. Tsai, Y. Liu, X. Yuan, and T. Ma, "Compaction, fusion, and functional activation of three-dimensional human mesenchymal stem cell aggregate.," *Tissue Eng. Part A*, vol. 21, no. 9–10, pp. 1705–1719, May 2015.

[427] I. a Potapova, G. R. Gaudette, P. R. Brink, R. B. Robinson, M. R. Rosen, I. S. Cohen, and S. V Doronin, "Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro.," *Stem Cells*, vol. 25, no. 7, pp. 1761–8, Jul. 2007.

[428] I. a Potapova, P. R. Brink, I. S. Cohen, and S. V Doronin, "Culturing of human mesenchymal stem cells as three-dimensional aggregates induces functional expression of CXCR4 that regulates adhesion to endothelial cells.," *J. Biol. Chem.*, vol. 283, no. 19, pp. 13100–7, May 2008.

[429] Q. Zhang, A. L. Nguyen, S. Shi, C. Hill, P. Wilder-Smith, T. B. Krasieva, and A. D. Le, "Three-dimensional spheroid culture of human gingiva-derived mesenchymal stem cells enhances mitigation of chemotherapy-induced oral mucositis.," *Stem Cells Dev.*, vol. 21, no. 6, pp. 937–947, Apr. 2012.

[430] S. H. Bhang, S.-W. Cho, W.-G. La, T.-J. Lee, H. S. Yang, A.-Y. Sun, S.-H. Baek, J.-W. Rhie, and B.-S. Kim, "Angiogenesis in ischemic tissue produced by spheroid grafting of human adipose-derived stromal cells.," *Biomaterials*, vol. 32, no. 11, pp. 2734–2747, Apr. 2011.

[431] E. J. Lee, S. J. Park, S. K. Kang, G.-H. Kim, H.-J. Kang, S.-W. Lee, H. B. Jeon, and H.-S. Kim, "Spherical bullet formation via E-cadherin promotes therapeutic potency of mesenchymal stem cells derived from human umbilical cord blood for myocardial infarction.," *Mol. Ther.*, vol. 20, no. 7, pp. 1424–1433, Jul. 2012.

[432] M. Y. Emmert, P. Wolint, N. Wickboldt, G. Gemayel, B. Weber, C. E. Brokopp, A. Boni, V. Falk, A. Bosman, M. E. Jaconi, and S. P. Hoerstrup, "Human stem cell-based three-dimensional microtissues for advanced cardiac cell therapies.," *Biomaterials*, vol. 34, no. 27, pp. 6339–6354, Sep. 2013.

[433] M. Y. Emmert, P. Wolint, S. Winklhofer, P. Stolzmann, N. Cesarovic, T. Fleischmann, T. D. L. Nguyen, T. Frauenfelder, R. Boni, J. Scherman, D. Bettex, J. Grunenfelder, R. Schwartlander, V. Vogel, M. Gyongyosi, H. Alkadhi, V. Falk, and S. P. Hoerstrup, "Transcatheter based electromechanical mapping guided intramyocardial transplantation and in vivo tracking of human stem cell based three dimensional microtissues in the porcine heart.," *Biomaterials*, vol. 34, no. 10, pp. 2428–2441, Mar. 2013.

[434] L. Xie, M. Mao, L. Zhou, and B. Jiang, "Spheroid Mesenchymal Stem Cells and Mesenchymal Stem Cell-Derived Microvesicles: Two Potential Therapeutic Strategies.," *Stem Cells Dev.*, vol. 25, no. 3, pp. 203–213, Feb. 2016.

[435] M. L. Skiles, B. Hanna, L. Rucker, A. Tipton, A. Brougham-Cook, E. Jabbarzadeh, and J. O. Blanchette, "ASC spheroid geometry and culture oxygenation differentially impact induction of preangiogenic behaviors in endothelial cells.," *Cell Transplant.*, vol. 24, no. 11, pp. 2323–2335, 2015.

[436] K. M. Broughton and M. A. Sussman, "Empowering Adult Stem Cells for Myocardial Regeneration V2.0," *Circ. Res.*, vol. 118, no. 5, p. 867 LP-880, Mar. 2016.

[437] V. Karantalis, V. Y. Suncion-Loescher, L. Bagno, S. Golpanian, A. Wolf, C. Sanina, C. Premer, A. J. Kanelidis, F. McCall, B. Wang, W. Balkan, J. Rodriguez, M. Rosado, A. Morales, K. Hatzistergos, M. Natsumeda, I. Margitich, I. H. Schulman, S. A. Gomes, M. Mushtaq, D. L. DiFede, J. E. Fishman, P. Pattany, J. P. Zambrano, A. W. Heldman, and J. M. Hare, "Synergistic effects of combined cell therapy for chronic ischemic cardiomyopathy," *J. Am. Coll. Cardiol.*, vol. 66, no. 18, pp. 1990–1999, 2015.

[438] A. R. Williams, K. E. Hatzistergos, B. Addicott, F. Mccall, D. Carvalho, V. Suncion, A. R. Morales, J. Da Silva, M. A. Sussman, A. W. Heldman, and J. M. Hare, "Enhanced Effect of Combining Human Cardiac Stem Cells and Bone Marrow Mesenchymal Stem Cells to Reduce Infarct Size and to Restore Cardiac Function after Myocardial Infarction.," *Circulation*, vol. 127, no. 2, pp. 213–223, 2013.

[439] E. J. Suuronen, J. Price, J. P. Veinot, K. Ascah, V. Kapila, X. W. Guo, S. Wong, T. G. Mesana, and M. Ruel, "Comparative effects of mesenchymal progenitor cells, endothelial progenitor cells, or their combination on myocardial infarct regeneration and cardiac function," *J. Thorac. Cardiovasc. Surg.*, vol. 134, no. 5, pp. 1249–1258, 2007.

[440] X. Zhang, M. Wei, W. Zhu, and B. Han, "Combined transplantation of endothelial progenitor cells and mesenchymal stem cells into a rat model of isoproterenol-induced myocardial injury," *Arch. Cardiovasc. Dis.*, vol. 101, no. 5, pp. 333–342, 2008.

[441] B. Zhang, S. Yang, Y. Zhang, Z. Sun, W. Xu, and S. Ye, "Co-culture of mesenchymal stem cells with umbilical vein endothelial cells under hypoxic condition," *J. Huazhong Univ. Sci. Technol. [Medical Sci.]*, vol. 32, no. 2, pp. 173–180, 2012.

[442] M. Monsanto, S. Mohsin, K. Fisher, and M. A. Sussman, "Enhanced myocardial repair with cardioclusters," *Circ. Res.*, vol. 113, pp. E159–E159, 2013.
[443] A. A. Qayyum, M. Haack-Sorensen, A. B. Mathiasen, E. Jorgensen, A. Ekblond, and J. Kastrup, "Adipose-derived mesenchymal stromal cells for chronic myocardial ischemia (MyStromalCell Trial): study design.," *Regen. Med.*, vol. 7, no. 3, pp. 421–428, May 2012.
[444] A. A. Qayyum, A. B. Mathiasen, N. D. Mygind, J. T. Kühl, E. Jørgensen, S. Helqvist, J. J. Elberg, K. F. Kofoed, N. G. Vejlstrup, A. Fischer-Nielsen, M. Haack-Sørensen, A. Ekblond, and J. Kastrup, "Adipose-Derived Stromal Cells for Treatment of Patients with Chronic Ischemic Heart Disease (MyStromalCell Trial): A Randomized Placebo-Controlled Study," *Stem Cells International*, vol. 2017. 2017.
[445] J. Bartunek, A. Behfar, D. Dolatabadi, M. Vanderheyden, M. Ostojic, J. Dens, B. El Nakadi, M. Banovic, B. Beleslin, M. Vrolix, V. Legrand, C. Vrints, J. L. Vanoverschelde, R. Crespo-Diaz, C. Homsy, M. Tendera, S. Waldman, W. Wijns, and A. Terzic, "Cardiopoietic stem cell therapy in heart failure: the C-CURE (Cardiopoietic stem Cell therapy in heart failURE) multicenter randomized trial with lineage-specified biologics.," *J. Am. Coll. Cardiol.*, vol. 61, no. 23, pp. 2329–2338, Jun. 2013.
[446] J. Bartunek, A. Terzic, B. A. Davison, G. S. Filippatos, S. Radovanovic, B. Beleslin, B. Merkely, P. Musialek, W. Wojakowski, P. Andreka, I. G. Horvath, A. Katz, D. Dolatabadi, B. El Nakadi, A. Arandjelovic, I. Edes, P. M. Seferovic, S. Obradovic, M. Vanderheyden, N. Jagic, I. Petrov, S. Atar, M. Halabi, V. L. Gelev, M. K. Shochat, J. D. Kasprzak, R. Sanz-Ruiz, G. R. Heyndrickx, N. Nyolczas, V. Legrand, A. Guédès, A. Heyse, T. Moccetti, F. Fernandez-Aviles, P. Jimenez-Quevedo, A. Bayes-Genis, J. M. Hernandez-Garcia, F. Ribichini, M. Gruchala, S. A. Waldman, J. R. Teerlink, B. J. Gersh, T. J. Povsic, T. D. Henry, M. Metra, R. J. Hajjar, M. Tendera, A. Behfar, B. Alexandre, A. Seron, W. G. Stough, W. Sherman, G. Cotter, and W. Wijns, "Cardiopoietic cell therapy for advanced ischaemic heart failure: results at 39 weeks of the prospective, randomized, double blind, sham-controlled CHART-1 clinical trial," *European Heart Journal*, vol. 38, no. 9. pp. 648–660, Mar-2017.
[447] D. G. Katritsis, P. A. Sotiropoulou, E. Karvouni, I. Karabinos, S. Korovesis, S. A. Perez, E. M. Voridis, and M. Papamichail, "Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium," *Catheter. Cardiovasc. Interv.*, vol. 65, no. 3, pp. 321–329, 2005.
[448] M. Majka, M. Sulkowski, B. Badyra, and P. Musialek, "Concise Review: Mesenchymal Stem Cells in Cardiovascular Regeneration: Emerging Research Directions and Clinical Applications.," *Stem Cells Transl. Med.*, vol. 6, no. 10, pp. 1859–1867, Oct. 2017.
[449] V. Karantalis and J. M. Hare, "Use of Mesenchymal Stem Cells for Therapy of Cardiac Disease," *Circulation research*, vol. 116, no. 8. pp. 1413–1430, Apr-2015.
[450] J. M. Hare, J. E. Fishman, G. Gerstenblith, D. L. DiFede Velazquez, J. P. Zambrano, V. Y. Suncion, M. Tracy, E. Ghersin, P. V Johnston, J. A. Brinker, E. Breton, J. Davis-Sproul, I. H. Schulman, J. Byrnes, A. M. Mendizabal, M. H. Lowery, D. Rouy, P. Altman, C. Wong Po Foo, P. Ruiz, A. Amador, J. Da Silva, I. K. McNiece, A. W. Heldman, R. George, and A. Lardo, "Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial.," *JAMA*, vol. 308, no. 22, pp. 2369–2379, Dec. 2012.
[451] W. Li, N. Ma, L.-L. Ong, C. Nesselmann, C. Klopsch, Y. Ladilov, D. Furlani, C. Piechaczek, J. M. Moebius, K. Lutzow, A. Lendlein, C. Stamm, R.-K. Li, and G. Steinhoff, "Bcl-2 engineered MSCs inhibited apoptosis and improved heart function.," *Stem Cells*, vol. 25, no. 8, pp. 2118–2127, Aug. 2007.
[452] Y. L. Tang, Y. Tang, Y. C. Zhang, K. Qian, L. Shen, and M. I. Phillips, "Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector.," *J. Am. Coll. Cardiol.*, vol. 46, no. 7, pp. 1339–1350, Oct. 2005.
[453] S. K. Kang, I. S. Shin, M. S. Ko, J. Y. Jo, and J. C. Ra, "Journey of mesenchymal stem cells for homing: strategies to enhance efficacy and safety of stem cell therapy.," *Stem Cells Int.*, vol. 2012, p. 342968, 2012.
[454] H.-F. Duan, C.-T. Wu, D.-L. Wu, Y. Lu, H.-J. Liu, X.-Q. Ha, Q.-W. Zhang, H. Wang, X.-X. Jia, and L.-S. Wang, "Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor.," *Mol. Ther.*, vol. 8, no. 3, pp. 467–474, Sep. 2003.
[455] H. Song, K. Kwon, S. Lim, S.-M. Kang, Y.-G. Ko, Z. Xu, J. H. Chung, B.-S. Kim, H. Lee, B. Joung, S. Park, D. Choi, Y. Jang, N.-S. Chung, K.-J. Yoo, and K.-C. Hwang, "Transfection of mesenchymal stem cells with the FGF-2 gene improves their survival under hypoxic conditions.," *Mol. Cells*, vol. 19, no. 3, pp. 402–407, Jun. 2005.
[456] R. Matsumoto, T. Omura, M. Yoshiyama, T. Hayashi, S. Inamoto, K.-R. Koh, K. Ohta, Y. Izumi, Y. Nakamura, K. Akioka, Y. Kitaura, K. Takeuchi, and J. Yoshikawa, "Vascular endothelial growth factor-expressing mesenchymal stem cell transplantation for the treatment of acute myocardial infarction.," *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 6, pp. 1168–73, Jun. 2005.

[457] S. Baldari, G. Di Rocco, M. Piccoli, M. Pozzobon, M. Muraca, and G. Toietta, “Challenges and Strategies for Improving the Regenerative Effects of Mesenchymal Stromal Cell-Based Therapies.,” *Int. J. Mol. Sci.*, vol. 18, no. 10, Oct. 2017.

II. Characterization of Different Sources of Human Mesenchymal Stem/Stromal Cells

This chapter is based on the article: Pinto, DS, Ahsan, T, Serra, J, Fernandes-Platzgummer, A, Cabral, JMS, da Silva, CL. Bioprocessing Modulation of the *In Vitro* Angiogenic Potential of Human Mesenchymal Stem/Stromal Cells. In preparation.

II.1 Summary

MSC have been used as therapeutic agents for the treatment of several conditions due to their intrinsic immunomodulatory and regenerative properties. Moreover, MSC are low immunogenic and hold the ability to differentiate towards numerous cell types, including osteoblasts, adipocytes and chondrocytes, contributing to their great potential in tissue engineering and regenerative medicine applications. MSC have been isolated from different human sources, including adult sources (BM, adipose AT, synovium, dental pulp) and neonatal sources (UCB, UCM, placenta, amniotic fluid). Years of research have demonstrated that different MSC sources present different intrinsic cell properties, particularly regarding their proliferative capacity, immunophenotype, differentiation ability, cytokine secretion profile, immunomodulatory and regenerative properties. In addition, the successful application of MSC in clinical settings depends on the ability of the isolation and expansion protocols to comply with GMP guidelines.

In this chapter, human MSC isolated from three different sources (BM, AT and UCM) were expanded in S/XF culture conditions and characterized in terms of immunophenotype, proliferative capacity, expression and secretion of growth factors and cytokines and *in vitro* angiogenic capacity. All the three sources studied displayed the typical MSC immunophenotypic profile. Under the culture conditions studied, UCM MSC (PD = 3.76 ± 0.52) demonstrated a higher proliferative capacity, relative to AT (PD = 3.56 ± 0.20) and BM (PD = 2.94 ± 0.23) MSC. Gene expression analysis revealed that VEGF and HGF were overexpressed in UCM MSC, compared with both AT (1.40 ± 0.38 and 6.76 ± 2.30, respectively) and BM (1.30 ± 0.21 and 40.4 ± 9.8, respectively), whereas bFGF was upregulated in AT MSC compared with the other sources (2.58 ± 0.81 *vs* BM MSC and 1.21 ± 0.44 *vs* UCM MSC). Regarding protein analysis, UCM MSC secreted higher levels of angiogenin and HGF, compared with the other sources, while AT MSC secreted higher concentrations of the proangiogenic factors angiopoietin-2, EGF, bFGF, HB-EGF, leptin, PDGF-BB, PlGF and VEGF. Moreover, CM retrieved from UCM MSC cultures originated HUVEC tube-like structures with higher number of tubes (134 ± 49). In contrast, AT MSC CM induced the formation of a higher number of branch points (85 ± 7). Concerning HUVEC migration capacity, BM MSC CM promoted the migration of a higher percentage of HUVEC (87.2 ± 5.7 %), compared with AT (55.0 ± 5.2 %) and UCM (47.3 ± 11.2 %) MSC CM.

The results indicate that MSC isolated from different sources have different properties, including proliferative capacity, expression and secretion of proangiogenic factors and *in vitro* angiogenic potentials. Deciphering the differences among sources contributes to the design of an improved therapy for a specific disease.

II.2 Background

MSC are a heterogeneous population of multipotent progenitor cells that are present in the majority of the human tissues [1]. As multipotent cells, MSC are able to self-renew and give rise to a genetically identical cell or to differentiate towards different cell types. According to the International Society for Cellular Therapy, MSC should be plastic-adherent, express a set of biomarkers (CD73, CD90, CD105) and lack the expression of others (CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR) and have the ability to differentiate into osteocytes, adipocytes and chondrocytes [2]. MSC secrete a broad range of bioactive factors, including growth factors, cytokines and chemokines, that are responsible for their intrinsic immunomodulatory and trophic properties [3]. In this context, MSC have been widely used in preclinical and clinical studies for the potential treatment of several conditions, including graft-versus-host disease, autoimmune diseases (diabetes mellitus type 1, rheumatoid arthritis and multiple sclerosis) and systemic diseases (cardiovascular, neurological and bone disorders) [4]. A total number of 815 ongoing or completed clinical trials involving MSC were registered at ClinicalTrials.gov, until March 2018.

MSC were firstly described by Friedenstein and colleagues as a sub-population of adherent fibroblast-like, clonogenic cells present in the murine bone marrow [5]. These progenitor cells were only termed as "mesenchymal stem cells" in 1991 by Caplan [6], suggesting that adult stem cells would be named MSC if they were able to differentiate into all the cells of the mesoderm. The definition of MSC has been evolving during the 90's and early 00's, until ISCT proposed the minimal criteria to define this population of cells. More recently, the criteria have been revised to improve the assessment of MSC potency *in vitro* and the clinical outcome of a potential MSC-based product [7].

Using the ISCT minimal criteria, MSC have been isolated from different human adult tissues, including BM, AT, synovial membrane, as well as from neonatal sources, such as UCB, UCM, placenta and amniotic fluid. MSC isolated from different sources demonstrated different biological features, including proliferative capacity [8], [9], immunophenotype [10], differentiation ability [11], immunomodulation [12] and regenerative capacity [13]–[15]. These differences are attributed to the impact of the stem cell niche on the cell fate, also known as stem cell niche theory [16], genetic variability and/or epigenetic alterations [13]. Specifically, BM, AT and UCM MSC have proved their distinct regenerative properties [1], [14], [17].

BM aspirates, where BM MSC are isolated from, are rich sources of hematopoietic and nonhematopoietic stem cells, including MSC, which have been shown to participate in hematopoiesis and BM regeneration [18], [19]. MSC have also demonstrated their ability to participate in angiogenesis, by giving rise to pericytes, the perivascular cells on the outer layer of vessels supporting their stability

and directing the blood flow [20]. Additionally, BM MSC have been shown to interact with endothelial colony forming cells to establish perfused micro-vessels *in vivo* [21], [22]. Regarding cardiac regeneration, BM MSC have successfully been used to reduce infarct size [23], modulate the microenvironment of the damaged tissue [24] and stimulate CSC differentiation [25].

Studies reporting the regenerative potential of AT MSC indicated that they successfully contributed to vessel formation [26] and are able to act as pericytes [13]. *In vitro*, this source showed an improved proangiogenic profile and secretion of ECM components and MMP, compared with the BM and UCM counterparts [1]. Moreover, both cardiomyocytes [27], [28] and vascular cells [29], [30] were already obtained from the differentiation of AT MSC. This cell population was also tested for myocardial regeneration *in vivo*, showing an angiogenic effect through paracrine signaling in an animal model of AMI [31].

The potential of UCM MSC in promoting angiogenesis have also been reported [32], [33]. *In vitro* studies demonstrated high secretion levels of several proangiogenic factors, such as VEGF, angiopoietin-1, HGF and TGF-β1, which promoted functional angiogenic potency in the infarcted mammalian heart [34]. In animal models, UCM MSC effectively induced angiogenesis [35]–[37], showed spontaneous differentiation to cardiomyocytes [35], [36] and endothelial cells, and induced recruitment of CSC [36].

Although the immunomodulatory and regenerative properties of MSC diverge between sources, they are also modulated by the cell microenvironment. In fact, several culture parameters affect MSC regenerative properties, including cell culture medium, culture platform, mechanical and biochemical clues and oxygen concentration. Monitoring and controlling these parameters during MSC manufacturing is crucial to obtain not only large cell numbers required for preclinical and clinical studies, but also to achieve a robust and consistent cellular product.

In this context, replacing culture media supplemented with xenogeneic components, such as FB/CS, by S/XF components is of utmost importance to be able to comply with GMP guidelines. Moreover, researchers proved that MSC expanded in S/XF culture conditions preserved or even improved their features, including the expression of cell surface markers, differentiation ability and regenerative properties [34], [38], [39], comparing to serum-containing media.

The aim of this chapter is to characterize three distinct human MSC sources (BM, AT and UCM) expanded in S/XF culture conditions, in terms of immunophenotype, proliferative capacity, expression and secretion of proangiogenic factors and *in vitro* angiogenic potential. To do so, MSC were expanded in a commercially available S/XF culture medium, StemPro® MSC SFM XenoFree, and then

characterized as mentioned above. The results are expected to contribute to the selection of the MSC source with the highest *in vitro* angiogenic potential.

II.3 Materials and Methods

II.3.1 Human Samples

BM MSC were isolated as described by dos Santos *et al* [40]. BM aspirates were obtained from healthy donors after informed consent at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal (Laws nº 97/95, nº 46/2004). AT MSC were isolated and characterized as previously described in the literature [41]. AT aspirates were obtained from healthy donors, upon informed consent, under a protocol reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board. UCM MSC were isolated according to the protocol established by de Soure *et al* [42]. Umbilical cord units were obtained from healthy donors after informed maternal consent at Hospital São Francisco Xavier, Lisboa, Portugal (Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004). All the MSC sources (BM, AT and UCM) were cryopreserved in a liquid/vapor-phase nitrogen container.

II.3.2 *Ex Vivo* Expansion of Human Mesenchymal Stem/Stromal Cells from Different Sources

Upon thawing, MSC from the 3 different sources (BM, AT and UCM) were plated at a cell density between 3000-6000 cell/cm^2 and cultured for 2 passages under S/XF conditions, on CELLstart™ CTS™ (diluted 1:200 in PBS with Ca^{2+} and Mg^{2+}, Life Technologies) precoated T-75 or T-175 flasks (BD Falcon™) using StemPro® MSC SFM XenoFree (Life Technologies). Cells were kept at 37°C and 5% CO_2 in a humidified atmosphere and culture medium was changed every 3-4 days. At 70-80% cell confluence, MSC were detached from the flasks using the XF cell detachment solution TrypLE™ Select CTS™ solution (1X, Life Technologies) for 7 min at 37°C. Cell number and viability were determined using Trypan Blue (Life Technologies) exclusion method.

II.3.3 Human Mesenchymal Stem/Stromal Cell Culture for Characterization of Different Sources

After cell counting, each of the different MSC sources was plated at a cell density between 1000-3000 cell/cm^2 on a T-75 flask and 3 X wells of a tissue culture-treated 6-well plate (Corning Inc.) precoated with CELLstart™ (diluted in 1:200 in PBS with Ca^{2+} and Mg^{2+}) using StemPro® MSC SFM XenoFree. Culture medium was changed at day 3 of culture. At day 7, cells cultured on T-75 flasks were detached using TrypLE™ Select CTS™ solution (1X) and counted using Trypan Blue exclusion method. 3 x 10^5 cells were used for immunophenotypic analysis by flow cytometry (FC) and the remaining cells were collected for gene expression analysis by real-time polymerase chain reaction (RT-PCR). Supernatant was centrifuged at 1500 rpm for 10 min and kept at -80°C for protein quantification by enzyme-linked immunosorbent assay (ELISA). Culture medium of cells cultured on tissue culture-treated 6-well plates was replaced by endothelial cell basal medium (EBM™-2, Lonza). After 48h, cells were detached and counted using Trypan Blue exclusion method and CM was retrieved and centrifuged at 1500 rpm for 10 min and kept at -80°C. MSC CM angiogenic potential was later evaluated through an *in vitro* HUVEC tube-like structure formation assay and a HUVEC migration assay. For each cell source, MSC from at least three independent donors ($n \geq 3$) in passages from P3 to P5 were used.

II.3.4 Characterization of Different Sources of Human Mesenchymal Stem/Stromal Cells

II.3.4.1 Immunophenotypic Profile

The immunophenotypic profile of MSC were analyzed by FC (FACSCalibur equipment, Becton Dickinson), using a panel of mouse anti-human monoclonal antibodies (PE-conjugated, all from BD Biolegend) against: CD73, CD90, CD105, CD34, CD45, CD14, CD19, CD80, and HLA-DR. Cells were incubated with these monoclonal antibodies for 20 min in the dark at room temperature and then cells were washed with PBS (1X) and fixed with 2% paraformaldehyde (PFA, Sigma). Isotype controls were also prepared for every experiment. A minimum of 5000 events was collected for each sample and the CellQuest software (Becton Dickinson) was used for acquisition and analysis.

II.3.4.2 Proliferative Capacity

Cell proliferative capacity was determined through the number of population doublings (PD) of the MSC cultured in the different conditions studied. After cell counting using Trypan Blue exclusion method,

the fold increase (FI) in total cell number was calculated as the ratio of cells obtained at the end of the culture divided by the number of cells plated at day 0. The number of PD was then calculated using the equation PD = log(FI)/log(2).

II.3.4.3 *In Vitro* Angiogenic Potential

The *in vitro* angiogenic potential of the different MSC sources was assessed using 4 different approaches: i) RT-PCR analysis of the expression of the proangiogenic genes VEGF, bFGF, and HGF; ii) quantification of the secretion of the proangiogenic factors: angiogenin, angiopoietin-2 (ANG-2), EGF, bFGF, heparin-binding EGF-like growth factor (HB-EGF), HGF, leptin, PDGF-BB, PlGF and VEGF by ELISA; iii) HUVEC tube-like structure formation assay, using CM retrieved from the different MSC sources; iv) HUVEC migration through a transwell system, using CM retrieved from the different MSC sources.

II.3.4.3.1 Quantitative Real-Time-Polymerase Chain Reaction

Cells harvested at the end of each experiment were centrifuged, lysed and total RNA was isolated using the RNeasy® Mini Kit (Qiagen) according to manufacturer's instruction. RNA was then quantified by UV spectrophotometry (NanoDrop Technologies) at 260 nm. Complementary DNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) with blended oligo (dT), random primers and 500 ng of RNA. For RT-PCR, a two-step PCR run was performed in StepOne Real-Time PCR System (Applied Biosystems™), using Fast SYBR™ Green Master Mix (Applied Biosystems™), 0.5 µM of each primer, and 1 µL of cDNA in 20 µL of final volume. The primers used for VEGF-A, bFGF and HGF gene amplification are presented on Table II.1.

Table II.1 List of PCR primers used to study pro-angiogenic gene expression.

Gene	Primer Sequence	Amplicon size (bp)
Vascular endothelial growth factor-A (VEGF-A)	Fwd: 5'-CGA GGG CCT GGA GTG TGT-3' Rev: 5'-CGC ATA ATC TGC ATG GTG ATG-3'	57
Basic fibroblast growth factor (bFGF)	Fwd: 5'-TGG TAT GTG GCA CTG AAA CGA-3' Rev: 5'-GCC CAG GTC CTG TTT TGG AT	61
Hepatocyte growth factor (HGF)	Fwd: 5'-TCC ACG GAA GAG GAG ATG AGA-3' Rev: 5'-GGC CAT ATA CCA GCT GGG AAA-3'	63

Expression was normalized to the metabolic housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Control assays containing no cDNA templates were also performed.

II.3.4.3.2 Enzyme-Linked Immunosorbent Assay

The secretion of proangiogenic factors by UCM MSC cultured under different conditions was analyzed by a multiplex ELISA. Supernatant from UCM MSC cultured under different culture conditions was kept at -80°C until further analysis. The samples were thawed, diluted 5 X and loaded in the Quantibody® Human Angiogenesis Array ELISA Kit (RayBiotech), according to manufacturer's instructions. Protein concentration was determined by fluorescence measurement provided by RayBiotech.

II.3.4.3.3 HUVEC Tube-Like Structure Formation Assay

The *in vitro* HUVEC tube-like structure formation assay was used to evaluate the ability of HUVEC to form networks of tubes in Matrigel®, when cultured in MSC-derived CM, as previously described [43]. The volume of CM to be used in this assay was normalized to a cell density of approximately 1 x 10^5 cell/mL, by using EBM™-2 for CM dilution (when required). 50 µL of Matrigel® Matrix Basement Membrane (Corning) were plated per well of a 96-well plate and allowed to polymerize for 1h at 37°C. 2 x 10^4 HUVEC resuspended in 200 µL of CM were then added to the Matrigel® layer. After 6h of culture, HUVEC tube-like structures were imaged and number of tubes and number of branch points were quantified using the ImageJ software. HUVEC resuspended in EBM™-2 and endothelial growth media (EGM™-2, Lonza) were used as negative and positive controls, respectively.

II.3.4.3.4 HUVEC Migration Assay

HUVEC migration assay was performed using 6.5 mm, 8 µm pore size transwells (Costar Corp.), coated with 10 µg/mL human fibronectin for 1 hour at 37°C. 3 x 10^4 HUVEC resuspended in 100 µL EBM™-2 were plated in transwell inserts and then placed into 24-well plate containing 700 µL of CM collected from different MSC culture conditions, also normalized to a cell density of approximately 1 x 10^5 cell/mL. Cell migration was measured after 6h of incubation at 37°C. All non-migrated cells were removed from the upper face of the transwell membrane using a cotton swab and migrated cells were fixed with 2% PFA and stained with 0.1% crystal violet in 0.1 M borate, pH 9.0, 2% ethanol. Cell counting in the lower transwell membrane was performed using the ImageJ software. HUVEC placed in EBM™-2 and EGM™-2 were used as negative and positive controls, respectively.

II.3.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Results are presented as mean + standard error of the mean (SEM) of the values obtained for the different MSC donors. Two-way non-parametric analysis of variance (ANOVA) was calculated between three or more different experimental groups, comparing at least two parameters. Two-way ANOVA was followed by Bonferroni test to determine statistically significant differences (*P*-values < 0.05). One-way ANOVA was performed when comparing three or more experimental groups and only one parameter. Kruskal-Wallis test was performed to determine statistically significant differences (*P*-values < 0.05).

II.4 Results

II.4.1 Immunophenotypic and Proliferative Profiles of Different Human Mesenchymal Stem/Stromal Cell Sources

The development of an MSC-based therapeutic product requires GMP-compliant processes. For that reason, human BM, AT and UCM MSC were cultured in a S/XF commercial formulation, StemPro® MSC SFM XenoFree, and their immunophenotypic and proliferative profiles were compared (Figures II.1.A and II.1.B, respectively).

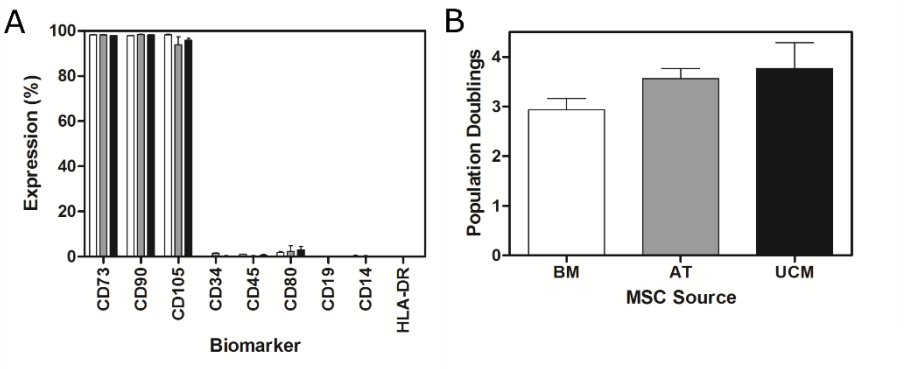

Figure II.1 Immunophenotypic and proliferative profiles of different human mesenchymal stem/stromal cell sources. (A) Cell surface biomarker expression of different human mesenchymal stem/stromal cell (MSC) sources (bone marrow (BM, white); adipose tissue (AT, grey) and umbilical cord matrix (UCM, black)) cultured in StemPro® MSC SFM XenoFree, by flow cytometry analysis. Results are presented as mean + standard error of the mean of the expression of each antigen (%) by each MSC source (*n*=2). (B) Population doublings of different human MSC sources (BM (white), AT (grey) and UCM (black)) cultured in StemPro® MSC SFM XenoFree. Population doublings are calculated as PD = log(FI)/log(2), where FI (fold increase) is calculated as the ratio of cells obtained at the end of the culture divided by the number of cells plated at day 0 and presented as mean + standard error of the mean (*n*=3).

Cells isolated from the BM, AT and UCM display the characteristic human MSC immunophenotype, according to the panel of cell surface markers established by the ISCT to define human MSC [2]. The analysis of Figure II.1.A shows that more than 95% of the cells from the different sources express CD73, CD90 and CD105 markers (except for CD105 marker in AT MSC – 93.9 ± 3.5%), while less than 4% express CD34, CD45, CD80, CD19, CD14 and HLA-DR. CD73 and CD90 expression levels are similar among the sources studied, while CD105 expression is more heterogeneous, ranging from 98.3 ± 0.2% in BM MSC to 95.8 ± 0.9% in UCM MSC and 93.9 ± 3.5% in AT MSC.

Cell proliferative capacity was characterized in terms of PD, for each source of MSC. The results present in Figure II.1.B demonstrate that UCM MSC (PD = 3.76 ± 0.52) present a higher proliferative capacity, compared with AT (PD = 3.56 ± 0.20) and BM (PD = 2.94 ± 0.23) MSC, under the culture conditions studied.

II.4.2 *In Vitro* Angiogenic Potential of Different Human Mesenchymal Stem/Stromal Cell Sources

In addition to the differences regarding immunophenotype and proliferation capacities, MSC from different sources also present significant functional differences, such as the *in vitro* angiogenic potential. These variations are due to the differential expression of pro- and anti-angiogenic factors by different MSC sources. To analyze the *in vitro* angiogenic potential of BM, AT and UCM MSC: i) cells were cultured in StemPro® MSC SFM XenoFree, collected and lysed for VEGF, bFGF and HGF gene expression analysis by RT-PCR; and ii) cells were cultured in endothelial basal medium (EBM™-2) for *in vitro* HUVEC tube-like structure formation and migration assays. Results are shown in Figure II.2.

Different human MSC sources express different levels of proangiogenic factors (Figure II.2.A and II.2.B), resulting in different *in vitro* HUVEC tube-like structure formation and migration capacities (Figure II.2.C-K). Specifically, VEGF and HGF are overexpressed in UCM MSC, compared with both AT (1.40 ± 0.38 and 6.76 ± 2.30, respectively) and BM (1.30 ± 0.21 and 40.4 ± 9.8, respectively), whereas bFGF is upregulated in AT MSC compared with the other sources (2.58 ± 0.81 *vs* BM MSC and 1.21 ± 0.44 *vs* UCM MSC), in the culture conditions studied (Figure II.2.A). In fact, BM MSC present the lowest expression levels of bFGF and HGF of the 3 MSC sources studied.

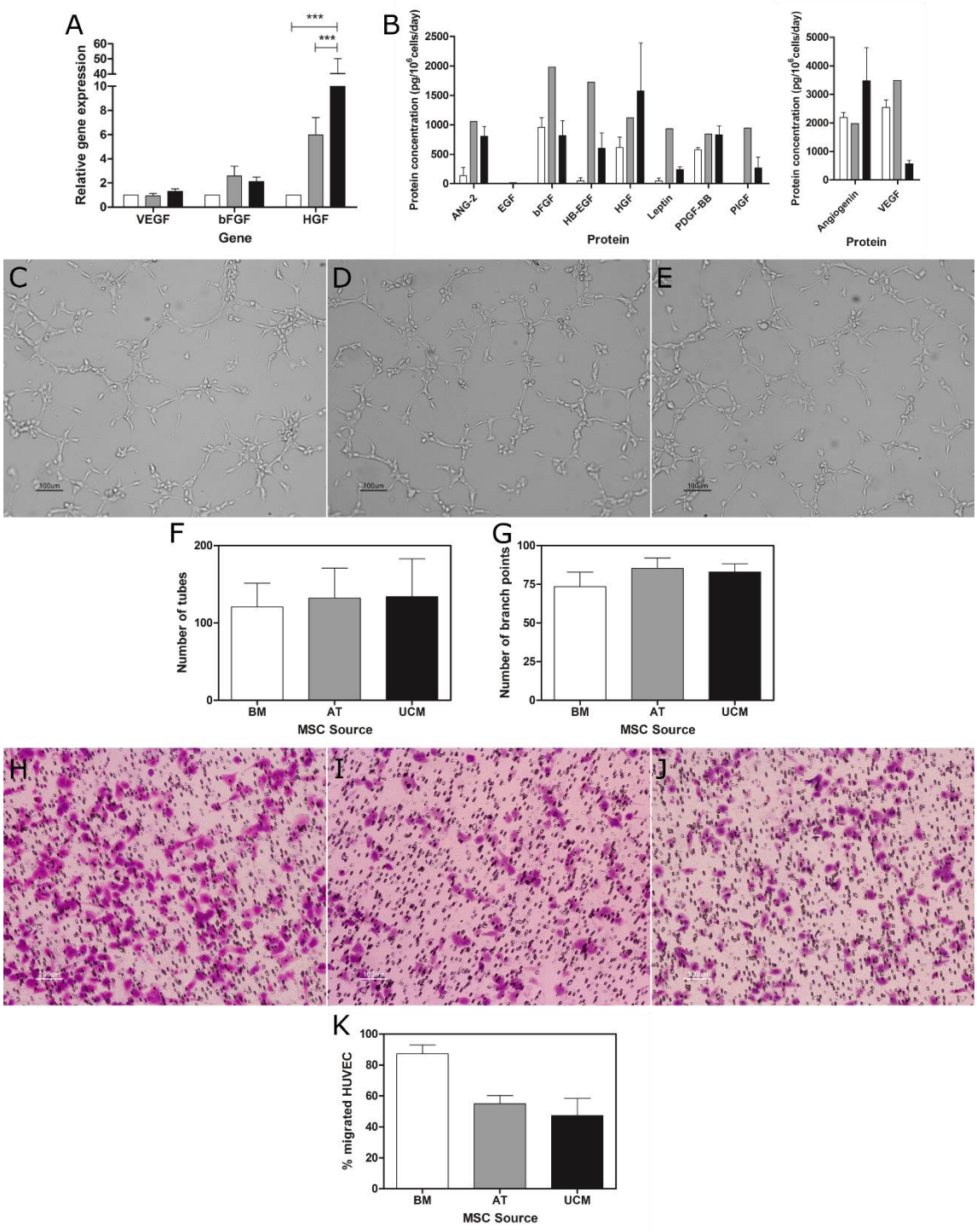

Figure II.2 *In vitro* angiogenic potential of different human mesenchymal stem/stromal cell sources. (A) Relative gene expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) of different human mesenchymal stem/stromal cell (MSC) sources (bone marrow – BM – white, adipose tissue – AT – grey, umbilical cord matrix – UCM – black) cultured in StemPro® MSC SFM XenoFree. Gene expression is normalized to the BM source (white) and values are represented as mean + standard error of mean (SEM, n=3, ***P<0.001). (B) Concentration of pro-angiogenic factors (angiopoietin-2 (ANG-2), epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), HGF, leptin, placental growth factor (PlGF), VEGF, angiogenin, bFGF, and platelet-derived growth factor-BB (PDGF-BB)) in the supernatant of different human MSC sources (BM – white, AT – grey, UCM – black) cultured in StemPro® MSC SFM XenoFree. Values are represented as mean + standard error of mean (SEM, n=2 for BM and UCM MSC, n=1 for AT MSC). Tube-like structures formed by human umbilical vein endothelial cells (HUVEC) resuspended in conditioned

medium (CM) retrieved from different human MSC sources – (C) BM; (D) AT; (E) UCM – cultured in endothelial basal medium (EBM™-2) for 48 h and plated in Matrigel®. (F) Number of tubes and (G) number of branch points were analyzed after 6h. HUVEC cultured in EBM™-2 and EGM™-2 were used as negative and positive controls, respectively. Values are represented as mean + standard error of mean (SEM, n=4). HUVEC migration capacity through the pores of transwell inserts placed into 24-well plates containing CM collected from different MSC sources – (H) BM; (I) AT; (J) UCM. HUVEC migration (K) was measured after 6h of incubation at 37°C, by cell staining with 0.1% crystal violet in 0.1 M borate, pH 9.0, 2% ethanol. HUVEC cultured in EBM™-2 and EGM™-2 were used as negative and positive controls, respectively. Values are represented as mean + standard error of mean (SEM, *n*=3).

Protein analysis revealed that UCM MSC secrete higher levels of HGF (UCM – 1577 ± 812 pg/(10^6cell.day) *vs* BM – 612 ± 179 pg/(10^6cell.day) and AT – 1121 pg/(10^6cell.day)) and angiogenin (UCM – 3474 ± 1165 pg/(10^6cell.day) *vs* BM – 2188 ± 173 pg/(10^6cell.day) and AT – 1982 pg/(10^6cell.day)), while AT MSC secrete higher concentrations of angiopoietin-2 (AT – 1052 pg/(10^6cell.day) *vs* BM – 139 ± 139 pg/(10^6cell.day) and UCM – 805 ± 167 pg/(10^6cell.day)), EGF (AT – 17 pg/(10^6cell.day) *vs* BM – 2 ± 2 pg/(10^6cell.day) and UCM – 3 ± 1 pg/(10^6cell.day)), bFGF (AT – 1982 pg/(10^6cell.day) *vs* BM – 955 ± 166 pg/(10^6cell.day) and UCM – 815 ± 257 pg/(10^6cell.day)), HB-EGF (AT – 1722 pg/(10^6cell.day) *vs* BM – 50 ± 50 pg/(10^6cell.day) and UCM – 600 ± 263 pg/(10^6cell.day)), leptin (AT – 934 pg/(10^6cell.day) *vs* BM – 46 ± 46 pg/(10^6cell.day) and UCM – 242 ± 42 pg/(10^6cell.day)), PDGF-BB (AT – 844 pg/(10^6cell.day) *vs* BM – 572 ± 39 pg/(10^6cell.day) and UCM – 830 ± 151 pg/(10^6cell.day)), PlGF (AT – 944 pg/(10^6cell.day) *vs* BM – 0 ± 0 pg/(10^6cell.day) and UCM – 267 ± 182 pg/(10^6cell.day)), and VEGF (AT – 3486 pg/(10^6cell.day) *vs* BM – 2540 ± 261 pg/(10^6cell.day) and UCM – 568 ± 115 pg/(10^6cell.day)).

CM retrieved from all the MSC sources induces the formation HUVEC tubular structures on Matrigel® (Figure II.2.C-E). However, different sources show different tube formation properties (Figure II.2.F-G). While CM retrieved from BM MSC induces the formation of HUVEC tube-like structures with lower number of tubes (121 ± 31) and number of branch points (73 ± 9), UCM MSC CM originates structures with higher number of tubes (134 ± 49). In contrast, AT MSC CM induces the formation of a higher number of branch points (85 ± 7). CM from different MSC sources induce differential HUVEC migration through the transwell membrane (Figure 2.H-J). BM MSC CM promotes the migration of a higher percentage of HUVEC (87.2 ± 5.7 %), compared with AT (55.0 ± 5.2 %) and UCM (47.3 ± 11.2 %) MSC CM (Figure 2.K).

Despite the differences among sources regarding their capacity to modulate the HUVEC tube formation and migration capacities, such differences were not statistically significant.

II.5 Discussion and Conclusions

In this chapter, immunophenotype, proliferative capacity, expression and secretion of proangiogenic factors and *in vitro* angiogenic potential were characterized for three different MSC sources cultured under XF culture conditions: two adult (BM and AT) and one neonatal (UCM). Neonatal sources, such as UCM, are attractive cell sources since they have no accumulated genomic mutations due to aging or disease, compared with adult sources, and they are isolated from routinely discarded tissues, by non-invasive methods [44]. Additionally, UCM MSC is reported to present higher cell proliferative capacity, followed by AT MSC and finally BM MSC [1], [9], [45], [46], which is in accordance with the results obtained. These reports used α-MEM supplemented with 10% FBS or 10% PL for cell culture, instead of a XF, chemically defined formulation. This may indicate that cell proliferative capacity has a higher dependence on the tissue origin than on the culture media and/or isolation protocols.

Importantly, all the MSC sources studied displayed the MSC immunophenotype. The expression of specific markers, and the lack of expression of some others, is an imperative outcome to define the population of cells obtained at the end of the culture and thus to attain a robust cellular product able to comply with GMP guidelines. Although ISCT have defined the panel of biomarkers required to identify MSC as so [2], researchers have reported some nuances concerning the expression of some biomarkers in different sources. For instance, AT MSC may also express CD34 and CD14 markers [10], [47], although this was not observed in the results. In addition, the expression of CD105 in this source did not reach the minimum required by ISCT for this marker ($\geq 95\%$). This value may be attributed to longer times of exposure to the enzymatic agent for cell detachment [48], due to higher production of ECM components by AT MSC, compared with the other sources.

According to ISCT, MSC should also be able to differentiate towards the osteogenic, adipogenic and chondrogenic lineages [2]. Although the differentiation ability of the three sources studied has not been analyzed in this work, previous studies of our lab have successfully reported the mesodermal differentiation of all the MSC sources used, after expansion in StemPro® MSC SFM XenoFree medium [44], [49].

The different MSC sources studied expressed and secreted different levels of proangiogenic factors. Gene expression analysis demonstrated that UCM MSC expressed higher levels of VEGF and HGF, compared with BM and AT MSC. VEGF and HGF are two of the most important mediators of angiogenesis. VEGF and HGF are known to be involved in endothelial cell migration, mitogenesis, sprouting and tube formation [50], [51]. AT MSC demonstrated the highest expression levels of bFGF, a potent mitogenic factor for endothelial cells [52]. At protein level, these trends were only observed for HGF secretion, and not to VEGF and bFGF. This might be due to the limited sample number for AT

MSC (*n*=1). Moreover, the differences observed for VEGF and bFGF expressions among different sources are not significant, compared with HGF expression, and might not be detected at the protein level. In fact, results obtained for HGF expression and secretion are according to the results published in other works [1], [14]. In accordance are also the results obtained for VEGF secretion, where BM and AT MSC secreted higher levels of these factor, compared with UCM MSC [1]. Conversely, the secretion of the remaining factors studied are contradictory in the literature [1], [14], [53]. In these works, different culture media were used for MSC expansion, which impacts the expression and secretion of several proteins, including the proangiogenic factors. Nevertheless, only a limited percentage of the factors involved in the angiogenic process were measured (anti-angiogenic factors were not quantified) and a more detailed analysis of the MSC secretomes should be carried out.

The *in vitro* angiogenic potential of the three MSC sources was analyzed based on the capacity of the secreted proangiogenic factors to induce the formation of HUVEC tube-like structures and cell migration through transwell membranes. Such assays aim at mimicking the *in vivo* angiogenic process, which is defined as the growth of blood vessels from the existing vasculature [50], and is crucial for cardiac tissue regeneration. Angiogenesis requires the recruitment of endothelial (stem/progenitor) cells, which is analyzed *in vitro* through the HUVEC migration assay, to the place where new blood vessels are being formed from preexisting ones, represented *in vitro* by the HUVEC tube-like formation assay. The results show that different MSC sources present different HUVEC tube-like structure formation and migration abilities. While UCM and AT MSC demonstrated superior HUVEC tube-like structure formation capacity, BM MSC showed higher migration potential. Differences regarding the tube formation capacity of each MSC source may be related with the degree of vascularization of the tissue where they were isolated from, although no literature has been found to support this hypothesis. The same rationale may be used to explain differences in terms of migration capacity. In this case, the BM source should be the one expressing higher levels of chemokines, although the levels of this factors have not been measured. While Li and colleagues demonstrated that BM MSC secrete higher levels of the potent chemokine SDF-1 [54], Amable *et al* reported that AT and UCM MSC secrete higher concentration of other chemoattractants, as RANTES, MCP-1, eotaxin and IP-10 [1]. Testing the different MSC sources *in vivo* would possibly provide more details about the angiogenic capacity of each source. The *in vitro* findings were not in agreement with the ones obtained by Du *et al* [14], reporting higher tube formation capacity for BM MSC.

In this chapter, different human MSC sources were successfully characterized in terms of immunophenotype, proliferative capacity and *in vitro* angiogenic potential. The results show superior proliferative and angiogenic capacity of both AT and UCM MSC, compared with the BM source. The improved angiogenic properties are characterized by increased number of HUVEC tubes and branch points, allowing the formation of a more interconnected network of tubes. Such network is required for

an efficient diffusion of oxygen and nutrients to the ischemic myocardial tissue. Nevertheless, the advantages of the UCM source over AT make them a more attractive source of MSC, from the clinical and regulatory perspectives. Therefore, UCM MSC will be used for further studies in chapter IV.

II.6 References of Chapter II

[1] P. R. Amable, M. V. T. Teixeira, R. B. V. Carias, J. M. Granjeiro, and R. Borojevic, "Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly.," *Stem Cell Res. Ther.*, vol. 5, no. 2, p. 53, Apr. 2014.

[2] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, a Keating, D. Prockop, and E. Horwitz, "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.," *Cytotherapy*, vol. 8, no. 4, pp. 315–7, Jan. 2006.

[3] L. D. S. Meirelles, A. M. Fontes, D. T. Covas, and A. I. Caplan, "Mechanisms involved in the therapeutic properties of mesenchymal stem cells.," *Cytokine Growth Factor Rev.*, vol. 20, no. 5–6, pp. 419–27, 2009.

[4] S. Wang, X. Qu, and R. C. Zhao, "Clinical applications of mesenchymal stem cells," *Journal of Hematology & Oncology*, vol. 5. p. 19, 2012.

[5] A. J. Friedenstein, K. V Petrakova, A. I. Kurolesova, and G. P. Frolova, "Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues.," *Transplantation*, vol. 6, no. 2, pp. 230–247, Mar. 1968.

[6] A. I. Caplan, "Mesenchymal stem cells.," *J. Orthop. Res.*, vol. 9, no. 5, pp. 641–650, Sep. 1991.

[7] R. M. Samsonraj, B. Rai, P. Sathiyanathan, K. J. Puan, O. Rotzschke, J. H. Hui, M. Raghunath, L. W. Stanton, V. Nurcombe, and S. M. Cool, "Establishing criteria for human mesenchymal stem cell potency.," *Stem Cells*, vol. 33, no. 6, pp. 1878–1891, Jun. 2015.

[8] D. Baksh, R. Yao, and R. S. Tuan, "Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow.," *Stem Cells*, vol. 25, no. 6, pp. 1384–1392, Jun. 2007.

[9] P. Stanko, K. Kaiserova, V. Altanerova, and C. Altaner, "Comparison of human mesenchymal stem cells derived from dental pulp, bone marrow, adipose tissue, and umbilical cord tissue by gene expression.," *Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc. Czech. Repub.*, vol. 158, no. 3, pp. 373–377, Sep. 2014.

[10] D. Macrin, J. P. Joseph, A. A. Pillai, and A. Devi, "Eminent Sources of Adult Mesenchymal Stem Cells and Their Therapeutic Imminence.," *Stem Cell Rev.*, vol. 13, no. 6, pp. 741–756, Dec. 2017.

[11] O. V Payushina, E. I. Domaratskaya, and V. I. Starostin, "Mesenchymal stem cells: Sources, phenotype, and differentiation potential," *Biol. Bull.*, vol. 33, no. 1, pp. 2–18, Jan. 2006.

[12] A. Ribeiro, P. Laranjeira, S. Mendes, I. Velada, C. Leite, P. Andrade, F. Santos, A. Henriques, M. Grãos, C. M. P. Cardoso, A. Martinho, Ml. Pais, C. L. da Silva, J. Cabral, H. Trindade, and A. Paiva, "Mesenchymal stem cells from umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood B, natural killer and T cells," *Stem Cell Research & Therapy*, vol. 4, no. 5. p. 125, 2013.

[13] R. Rohban and T. R. Pieber, "Mesenchymal Stem and Progenitor Cells in Regeneration: Tissue Specificity and Regenerative Potential.," *Stem Cells Int.*, vol. 2017, p. 5173732, 2017.

[14] W. J. Du, Y. Chi, Z. X. Yang, Z. J. Li, J. J. Cui, B. Q. Song, X. Li, S. G. Yang, Z. B. Han, and Z. C. Han, "Heterogeneity of proangiogenic features in mesenchymal stem cells derived from bone marrow, adipose tissue, umbilical cord, and placenta," *Stem Cell Research & Therapy*, vol. 7. London, 2016.

[15] A. Assoni, G. Coatti, M. C. Valadares, M. Beccari, J. Gomes, M. Pelatti, M. Mitne-Neto, V. M. Carvalho, and M. Zatz, "Different Donors Mesenchymal Stromal Cells Secretomes Reveal Heterogeneous Profile of Relevance for Therapeutic Use.," *Stem Cells Dev.*, vol. 26, no. 3, pp. 206–214, Feb. 2017.

[16] H. Lin, "The stem-cell niche theory: lessons from flies.," *Nat. Rev. Genet.*, vol. 3, no. 12, pp. 931–940, Dec. 2002.

[17] P. R. Amable, M. V. T. Teixeira, R. B. V. Carias, J. M. Granjeiro, and R. Borojevic, "Mesenchymal stromal cell proliferation, gene expression and protein production in human platelet-rich plasma-supplemented media.," *PLoS One*, vol. 9, no. 8, p. e104662, 2014.

[18] D. Bryder, D. J. Rossi, and I. L. Weissman, "Hematopoietic stem cells: the paradigmatic tissue-specific

stem cell.," *Am. J. Pathol.*, vol. 169, no. 2, pp. 338–346, Aug. 2006.
[19] T. Asahara and A. Kawamoto, "Endothelial progenitor cells for postnatal vasculogenesis.," *Am. J. Physiol. Cell Physiol.*, vol. 287, no. 3, pp. C572-9, Sep. 2004.
[20] G. Bergers and S. Song, "The role of pericytes in blood-vessel formation and maintenance.," *Neuro. Oncol.*, vol. 7, no. 4, pp. 452–464, Oct. 2005.
[21] R. Rohban, N. Etchart, and T. Pieber, "Vasculogenesis Potential of Mesenchymal and Endothelial Stem Cells Isolated from Various Human Tissues.," *bioRxiv*, 2016.
[22] R. Rohban, N. Etchart, and T. Pieber, "Transplantation of Endothelial Progenitor Cells Solely Leads to Development of Functional Neo-vessels in vivo.," *bioRxiv*, 2016.
[23] J.-J. Zhao, X.-C. Liu, F. Kong, T.-G. Qi, G.-H. Cheng, J. Wang, C. Sun, and Y. Luan, "Bone marrow mesenchymal stem cells improve myocardial function in a swine model of acute myocardial infarction.," *Mol. Med. Rep.*, vol. 10, no. 3, pp. 1448–1454, Sep. 2014.
[24] R. Rahbarghazi, S. M. Nassiri, S. H. Ahmadi, E. Mohammadi, S. Rabbani, A. Araghi, and H. Hosseinkhani, "Dynamic induction of pro-angiogenic milieu after transplantation of marrow-derived mesenchymal stem cells in experimental myocardial infarction.," *Int. J. Cardiol.*, vol. 173, no. 3, pp. 453–466, May 2014.
[25] K. E. Hatzistergos, H. Quevedo, B. N. Oskouei, Q. Hu, G. S. Feigenbaum, I. S. Margitich, R. Mazhari, A. J. Boyle, J. P. Zambrano, J. E. Rodriguez, R. Dulce, P. M. Pattany, D. Valdes, C. Revilla, A. W. Heldman, I. McNiece, and J. M. Hare, "Bone Marrow Mesenchymal Stem Cells Stimulate Cardiac Stem Cell Proliferation and Differentiation," *Circ. Res.*, vol. 107, no. 7, p. 913 LP-922, Sep. 2010.
[26] M. H. Moon, S. Y. Kim, Y. J. Kim, S. J. Kim, J. B. Lee, Y. C. Bae, S. M. Sung, and J. S. Jung, "Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia.," *Cell. Physiol. Biochem.*, vol. 17, no. 5–6, pp. 279–290, 2006.
[27] A. van Dijk, H. W. M. Niessen, B. Zandieh Doulabi, F. C. Visser, and F. J. van Milligen, "Differentiation of human adipose-derived stem cells towards cardiomyocytes is facilitated by laminin.," *Cell Tissue Res.*, vol. 334, no. 3, pp. 457–467, Dec. 2008.
[28] K. G. Gaustad, A. C. Boquest, B. E. Anderson, A. M. Gerdes, and P. Collas, "Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes.," *Biochem. Biophys. Res. Commun.*, vol. 314, no. 2, pp. 420–427, Feb. 2004.
[29] K. G. Marra, C. A. Brayfield, and J. P. Rubin, "Adipose stem cell differentiation into smooth muscle cells.," *Methods Mol. Biol.*, vol. 702, pp. 261–268, 2011.
[30] M. Konno, T. S. Hamazaki, S. Fukuda, M. Tokuhara, H. Uchiyama, H. Okazawa, H. Okochi, and M. Asashima, "Efficiently differentiating vascular endothelial cells from adipose tissue-derived mesenchymal stem cells in serum-free culture.," *Biochem. Biophys. Res. Commun.*, vol. 400, no. 4, pp. 461–465, Oct. 2010.
[31] H. W. Lee, H. C. Lee, J. H. Park, B. W. Kim, J. Ahn, J. H. Kim, J. S. Park, J.-H. Oh, J. H. Choi, K. S. Cha, T. J. Hong, T. S. Park, S.-P. Kim, S. Song, J. Y. Kim, M. H. Park, and J. S. Jung, "Effects of Intracoronary Administration of Autologous Adipose Tissue-Derived Stem Cells on Acute Myocardial Infarction in a Porcine Model.," *Yonsei Med. J.*, vol. 56, no. 6, pp. 1522–1529, Nov. 2015.
[32] W. Liao, J. Zhong, J. Yu, J. Xie, Y. Liu, L. Du, S. Yang, P. Liu, J. Xu, J. Wang, Z. Han, and Z. C. Han, "Therapeutic benefit of human umbilical cord derived mesenchymal stromal cells in intracerebral hemorrhage rat: implications of anti-inflammation and angiogenesis.," *Cell. Physiol. Biochem.*, vol. 24, no. 3–4, pp. 307–316, 2009.
[33] W. Liao, J. Xie, J. Zhong, Y. Liu, L. Du, B. Zhou, J. Xu, P. Liu, S. Yang, J. Wang, Z. Han, and Z. C. Han, "Therapeutic effect of human umbilical cord multipotent mesenchymal stromal cells in a rat model of stroke.," *Transplantation*, vol. 87, no. 3, pp. 350–359, Feb. 2009.
[34] P. Swamynathan, P. Venugopal, S. Kannan, C. Thej, U. Kolkundar, S. Bhagwat, M. Ta, A. Sen Majumdar, and S. Balasubramanian, "Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton's jelly derived mesenchymal stem cells? A comparative study.," *Stem Cell Res. Ther.*, vol. 5, no. 4, p. 88, Jul. 2014.
[35] K. H. Wu, B. Zhou, X. M. Mo, B. Cui, C. T. Yu, S. H. Lu, Z. C. Han, and Y. L. Liu, "Therapeutic potential of human umbilical cord-derived stem cells in ischemic diseases.," *Transplant. Proc.*, vol. 39, no. 5, pp. 1620–1622, Jun. 2007.
[36] W. Zhang, X.-C. Liu, L. Yang, D.-L. Zhu, Y.-D. Zhang, Y. Chen, and H.-Y. Zhang, "Wharton's jelly-derived mesenchymal stem cells promote myocardial regeneration and cardiac repair after miniswine acute myocardial infarction.," *Coron. Artery Dis.*, vol. 24, no. 7, pp. 549–558, Nov. 2013.
[37] D. Santos Nascimento, D. Mosqueira, L. M. Sousa, M. Teixeira, M. Filipe, T. P. Resende, A. F. Araujo, M. Valente, J. Almeida, J. P. Martins, J. M. Santos, R. N. Barcia, P. Cruz, H. Cruz, and P. Pinto-do-O, "Human umbilical cord tissue-derived mesenchymal stromal cells attenuate remodeling after myocardial

infarction by proangiogenic, antiapoptotic, and endogenous cell-activation mechanisms.," *Stem Cell Res. Ther.*, vol. 5, no. 1, p. 5, Jan. 2014.

[38] B. Lindroos, S. Boucher, L. Chase, H. Kuokkanen, H. Huhtala, R. Haataja, M. Vemuri, R. Suuronen, and S. Miettinen, "Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro.," *Cytotherapy*, vol. 11, no. 7, pp. 958–72, Jan. 2009.

[39] S. Jung, A. Sen, L. Rosenberg, and L. A. Behie, "Human mesenchymal stem cell culture: rapid and efficient isolation and expansion in a defined serum-free medium.," *J. Tissue Eng. Regen. Med.*, vol. 6, no. 5, pp. 391–403, May 2012.

[40] F. Dos Santos, P. Z. Andrade, J. S. Boura, M. M. Abecasis, C. L. da Silva, and J. M. S. Cabral, "Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia.," *J. Cell. Physiol.*, vol. 223, no. 1, pp. 27–35, Apr. 2010.

[41] J. Gimble and F. Guilak, "Adipose-derived adult stem cells: isolation, characterization, and differentiation potential.," *Cytotherapy*, vol. 5, no. 5, pp. 362–9, Jan. 2003.

[42] A. M. de Soure, A. Fernandes-Platzgummer, F. Moreira, C. Lilaia, S.-H. Liu, C.-P. Ku, Y.-F. Huang, W. Milligan, J. M. S. Cabral, and C. L. da Silva, "Integrated culture platform based on a human platelet lysate supplement for the isolation and scalable manufacturing of umbilical cord matrix-derived mesenchymal stem/stromal cells.," *J. Tissue Eng. Regen. Med.*, vol. 11, no. 5, pp. 1630–1640, May 2017.

[43] I. Arnaoutova and H. K. Kleinman, "In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract.," *Nat. Protoc.*, vol. 5, no. 4, pp. 628–635, Apr. 2010.

[44] I. N. Simões, J. S. Boura, F. Dos Santos, P. Z. Andrade, C. M. P. Cardoso, J. M. Gimble, C. L. da Silva, and J. M. S. Cabral, "Human mesenchymal stem cells from the umbilical cord matrix: Successful isolation and ex vivo expansion using serum-/xeno-free culture media.," *Biotechnol. J.*, vol. 8, no. 4, pp. 448–58, Apr. 2013.

[45] X. Li, J. Bai, X. Ji, R. Li, Y. Xuan, and Y. Wang, "Comprehensive characterization of four different populations of human mesenchymal stem cells as regards their immune properties, proliferation and differentiation.," *Int. J. Mol. Med.*, vol. 34, no. 3, pp. 695–704, Sep. 2014.

[46] R. Fazzina, P. Iudicone, D. Fioravanti, G. Bonanno, P. Totta, I. G. Zizzari, and L. Pierelli, "Potency testing of mesenchymal stromal cell growth expanded in human platelet lysate from different human tissues," *Stem Cell Research & Therapy*, vol. 7, no. 1. London, 2016.

[47] P. C. Baer, "Adipose-derived mesenchymal stromal/stem cells: An update on their phenotype in vivo and in vitro," *World Journal of Stem Cells*, vol. 6, no. 3. pp. 256–265, Jul-2014.

[48] M. A. Brown, C. S. Wallace, C. C. Anamelechi, E. Clermont, W. M. Reichert, and G. A. Truskey, "The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces.," *Biomaterials*, vol. 28, no. 27, pp. 3928–3935, Sep. 2007.

[49] F. Dos Santos, P. Z. Andrade, M. M. Abecasis, J. M. Gimble, L. G. Chase, A. M. Campbell, S. Boucher, M. C. Vemuri, C. L. Da Silva, and J. M. S. Cabral, "Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions.," *Tissue Eng. Part C. Methods*, vol. 17, no. 12, pp. 1201–10, Dec. 2011.

[50] A. A. Ucuzian, A. A. Gassman, A. T. East, and H. P. Greisler, "Molecular Mediators of Angiogenesis," *Journal of burn care & research : official publication of the American Burn Association*, vol. 31, no. 1. p. 158, 2010.

[51] F. Bussolino, M. F. Di Renzo, M. Ziche, E. Bocchietto, M. Olivero, L. Naldini, G. Gaudino, L. Tamagnone, A. Coffer, and P. M. Comoglio, "Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth.," *J. Cell Biol.*, vol. 119, no. 3, pp. 629–641, Nov. 1992.

[52] R. Montesano, J. D. Vassalli, A. Baird, R. Guillemin, and L. Orci, "Basic fibroblast growth factor induces angiogenesis in vitro.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 19. pp. 7297–7301, Oct-1986.

[53] M. Bilal, A. Haseeb, and M. A. Sher Khan, "Intracoronary infusion of Wharton's jelly-derived mesenchymal stem cells: a novel treatment in patients of acute myocardial infarction.," *J. Pak. Med. Assoc.*, vol. 65, no. 12, p. 1369, Dec. 2015.

[54] C. Li, X. Wu, J. Tong, X. Yang, J. Zhao, Q. Zheng, G. Zhao, and Z. Ma, "Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy.," *Stem Cell Res. Ther.*, vol. 6, p. 55, Apr. 2015.

III. Biochemical Modulation of the *In Vitro* Angiogenic Potential of Human Mesenchymal Stem/ Stromal Cells

This chapter is based on the article: Pinto, DS, Ahsan, T, Serra, J, Fernandes-Platzgummer, A, Cabral, JMS, da Silva, CL. Bioprocessing Modulation of the *In Vitro* Angiogenic Potential of Human Mesenchymal Stem/Stromal Cells. In preparation.

III.1 Summary

Although MSC have been proved successful in preclinical and clinical studies for myocardial regeneration, recent analyses of MSC-based clinical trials in the context of AMI demonstrated limited benefits, indicating a need to improve its efficacy. Low cell engraftment and survival, host tissue impairment and lack of efficient and reproducible cell potency assays are known to be major hurdles regarding MSC-based therapy for AMI. Preconditioning MSC through different approaches was found to improve MSC survival rate and thus prolong their therapeutic effect. Particularly, biochemical modulation of the cell microenvironment through the addition of different growth factors or chemokines and the establishment of culture systems with other cell types have showed to increase the regenerative capacity of different sources of MSC.

In this chapter, human MSC isolated from three different sources (BM, AT and UCM) were expanded and cultured in S/XF culture conditions and the supernatant generated from each source was collected to precondition the other MSC sources. The effect of this preconditioning strategy was evaluated on MSC immunophenotype, proliferative capacity, expression of proangiogenic factors and *in vitro* HUVEC tube-like structure formation capacity. Results show that preconditioning MSC with CM retrieved from other MSC sources does not alter the immunophenotypic profile of the cells. Similarly, the proliferative capacity of each MSC source is maintained when using this preconditioning strategy. Both UCM (control – 3.76 ± 0.52, preconditioned with (c/) BM – 3.75 ± 0.52, c/ AT – 3.75 ± 0.54) and AT (control – 3.56 ± 0.20, c/ BM – 3.72 ± 0.23, c/ UCM – 3.89 ± 0.16) MSC still present higher proliferative capacities, compared with BM MSC (control – 2.94 ± 0.23, c/ AT – 2.62 ± 0.36, c/ UCM – 2.79 ± 0.43). However, both the expression of proangiogenic genes and the *in vitro* HUVEC tube-like structure formation capacity are modulated by culturing a MSC source with CM retrieved from other sources. BM MSC cultured with the supernatant collected from AT or UCM MSC increase VEGF (BM c/ AT – 1.23 ± 0.15; BM c/ UCM – 1.45 ± 0.52) and HGF (BM c/ AT – 1.27 ± 0.84; BM c/ UCM – 3.28 ± 1.46) gene expression. In contrast, UCM MSC cultured with BM MSC CM express lower levels of VEGF (0.71 ± 0.13) and bFGF (0.72 ± 0.18), compared with control conditions. Moreover, BM MSC cultured with CM retrieved from both AT or UCM MSC increase the number of HUVEC tubes and branch points. Conversely, UCM MSC conditioned with BM or AT MSC CM induces HUVEC tube decrease in tube number and number of branch points.

These findings demonstrate that the *in vitro* angiogenic potential of an MSC source can be modulated by culturing it with a different MSC source, without compromising cell immunophenotypic and proliferative capacities. Identifying the appropriate growth factors and/or chemokines may lead to the production of a cocktail of factors designed to increase the properties of an MSC-based product for AMI.

III.2 Background

Cardiovascular diseases represent the leading cause of death worldwide, contributing to a total of 7.4 million deaths in 2012 [1]. Among them, the most common adverse event is AMI, occurring as a result of myocardial cell necrosis due to sustained and significant ischemia [2]. Current therapeutic strategies, such as restoration of blood flow or reperfusion, can partially improve the extent and consequences of an infarct, but cardiac transplantation remains the only standard therapy addressing tissue loss [3]. The main challenge regarding cell loss is the restricted ability of the heart for self-regeneration [4]. In this context, cell-based therapies have emerged as an attractive therapeutic alternative to repair damaged myocardium, either by *de novo* cardiomyogenesis or promoting neovascularization [5].

In particular, MSC have emerged as a promising cell type due to their high expansion capacity, ease of isolation, multipotent differentiation ability, low immunogenicity and immunomodulatory and regenerative properties [6], [7]. In fact, the most accepted explanation for MSC-mediated cardiovascular repair occurs via paracrine signaling through the secretion of growth factors, cytokines and other signaling molecules [7], [8]. Such bioactive factors promote neovascularization, activate cytoprotective pathways in reversibly injured cardiomyocytes and stimulate resident CS/PC proliferation/differentiation, which attenuates inflammation, tissue fibrosis and LV remodeling [9], [10]. Some other potential mechanisms of MSC-mediated cardiovascular repair were reported in the literature, such as MSC transdifferentiation into cardiomyocytes, MSC fusion with native cells and MSC-induced stimulation of endogenous CS/PC via direct cell-cell interaction. However, none of these mechanisms have shown as many *in vitro* and *in vivo* evidences as MSC paracrine signaling.

Meta-analysis of 8 relevant MSC-based randomized clinical trials in AMI context revealed a 1.47% increase in LV systolic function after cell transplantation, which highlights the need of improvement of a potential MSC-based product [11]. Poor cell engraftment and survival is one of the major hurdles encountered in MSC-based MI therapy, but others are also described, such as cell potency assessment [12] and age/disease-related host tissue impairment [9]. Multidisciplinary teams are currently establishing several potentially useful strategies to achieve more robust clinical efficacy of MSC therapy. A promising approach is based on the fact that MSC immunomodulatory and trophic features can be augmented through microenvironment modulation, either by biochemical (cell culture media, oxygen concentration, pharmacological agents, cytokines and growth factors, co-culture with other cell types) [13]–[20] and/or mechanical (shear stress, electrical stimuli, material stiffness and topography) [21]–[24] factors. Regarding the biochemical modulation approaches, recent evidences showed that MSC expanded under S/XF culture conditions exhibit superior functional angiogenesis in comparison to serum-expanded cells [16], [17]. Similarly, stimulating MSC with different cocktails of cytokines and growth factors, including TNF-α, TGF-α or IGF-1, led to increased secretion of proangiogenic factors,

cell survival, anti-inflammatory properties and engraftment capacities, resulting in improved cardiac function *in vivo* [25]–[27]. Researchers also found that combining the therapeutic potential of different cell types may also boost the outcomes of a cell-based product for AMI. In this context, MSC have been transplanted into ischemic hearts along with other cell populations, including CSPC [28], [29] and EPC [30], [31], promoting an enhanced recovery of the infarcted heart. Despite these encouraging achievements, more data is required to prove the increased therapeutic potential of these cells.

MSC for cardiac cell therapy can be obtained from both adult (BM, AT, peripheral blood) and neonatal (UCM, UCB, amnion, and placenta) sources. However, MSC isolated from different sources present differential properties and biological functions, such as molecular profile [32]–[34], proliferation capacity [35], [36], differentiation potential [35], immunomodulatory functions [37] and angiogenic properties [38], as demonstrated in Chapter II. Despite these differences, preclinical and clinical data showed an improvement of cardiac function after cell transplantation of different MSC sources [39]–[43].

The present chapter aims at analyzing the effect of culturing a specific MSC source with CM retrieved from other MSC sources on the cell immunophenotype, proliferative capacity, proangiogenic gene expression profile and *in vitro* HUVEC tube-like structure formation ability. Three human MSC sources (BM, AT and UCM) were expansion in a commercially available S/XF culture medium, StemPro® MSC SFM XenoFree. After expansion, the supernatants from the different sources were collected and each source was cultured with the supernatant retrieved from the other sources. Preconditioned cells, along with the respective controls, were characterized as mentioned above. This approach is expected to provide more information about the effect of preconditioning MSC with different concentrations of growth factors on the *in vitro* angiogenic potential of the cells.

III.3 Materials and Methods

III.3.1 Human Samples

BM MSC were isolated as described by dos Santos *et al* [44]. BM aspirates were obtained from healthy donors after informed consent at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal (Laws nº 97/95, nº 46/2004). AT MSC were isolated and characterized as previously described in the literature [45]. AT aspirates were obtained from healthy donors, upon informed consent, under a protocol reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board. UCM MSC were isolated according to the protocol established by de Soure *et al* [46]. Umbilical cord units were obtained from healthy donors after informed maternal consent at Hospital São Francisco

Xavier, Lisboa, Portugal (Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004). All the MSC sources (BM, AT and UCM) were cryopreserved in a liquid/vapor-phase nitrogen container.

III.3.2 *Ex Vivo* Expansion of Human Mesenchymal Stem/Stromal Cells from Different Sources

Upon thawing, MSC from the 3 different sources (BM, AT and UCM) were plated at a cell density between 3000-6000 cell/cm^2 and cultured for 2 passages under S/XF conditions, on CELLstart™ CTS™ (diluted 1:200 in PBS with Ca^{2+} and Mg^{2+}, Life Technologies) precoated T-75 or T-175 flasks (BD Falcon™) using StemPro® MSC SFM XenoFree (Life Technologies). Cells were kept at 37°C and 5% CO_2 in a humidified atmosphere and culture medium was changed every 3-4 days. At 70-80% cell confluence, MSC were detached from the flasks using the XF cell detachment solution TrypLE™ Select CTS™ solution (1X, Life Technologies) for 7 min at 37°C. Cell number and viability were determined using Trypan Blue (Life Technologies) exclusion method.

III.3.3 Human Mesenchymal Stem/Stromal Cell Culture for Preconditioning with Supernatant Retrieved from Other Sources

Each of the different MSC sources was plated at a cell density between 1000-3000 cell/cm^2 on 3X T-75 flasks and 9X wells of tissue culture-treated 6-well plates precoated with CELLstart™ (diluted in 1:200 in PBS with Ca^{2+} and Mg^{2+}) using StemPro® MSC SFM XenoFree. Culture medium was changed at day 3 of culture. For cells cultured on T-75 flasks, at day 5, cell culture supernatant was retrieved and each MSC source was cultured with the supernatant from the other two sources. After 2 days of conditioning, control and preconditioned cells were detached using TrypLE™ Select CTS™ solution (1X) and counted using Trypan Blue exclusion method. 3 x 10^5 cells were used for FC analysis and the remaining cells were collected for RT-PCR analysis. For cells cultured on 6-well plates, culture medium was replaced by EBM™-2, at day 7 of culture. After 24h, CM was retrieved and each MSC source was cultured with CM from the other two sources, for 24 h. Cells were then detached and counted using Trypan Blue exclusion method and CM was retrieved and centrifuged at 1500 rpm for 10 min and kept at -80°C. The angiogenic potential of CM from control and preconditioned cells was later evaluated through a HUVEC tube formation assay. For each cell source, MSC from at least three independent donors ($n \geq 3$) in passages from P3 to P5 were used.

III.3.4 Characterization of Human Mesenchymal Stem/Stromal Cells Preconditioned with Supernatant Retrieved from Other Sources

III.3.4.1 Immunophenotypic Profile

The immunophenotypic profile of MSC were analyzed by FC (FACSCalibur equipment, Becton Dickinson), using a panel of mouse anti-human monoclonal antibodies (PE-conjugated, all from BD Biolegend) against: CD73, CD90, CD105, CD34, CD45, CD14, CD19, CD80, and HLA-DR. Cells were incubated with these monoclonal antibodies for 20 min in the dark at room temperature and then cells were washed with PBS (1X) and fixed with 2% PFA (Sigma). Isotype controls were also prepared for every experiment. A minimum of 5000 events was collected for each sample and the CellQuest software (Becton Dickinson) was used for acquisition and analysis.

III.3.4.2 Proliferative Capacity

Cell proliferative capacity was determined through the number of PD of the MSC cultured in the different conditions studied. After cell counting using Trypan Blue exclusion method, the FI in total cell number was calculated as the ratio of cells obtained at the end of the culture divided by the number of cells plated at day 0. The number of PD was then calculated using the equation PD = log(FI)/log(2).

III.3.4.3 *In Vitro* Angiogenic Potential

The *in vitro* angiogenic potential of the different MSC sources was assessed using two different approaches: i) RT-PCR analysis of the expression of the proangiogenic genes VEGF, bFGF, and HGF; and ii) HUVEC tube-like structure formation assay, using CM retrieved from the different MSC sources.

III.3.4.3.1 Quantitative Real-Time-Polymerase Chain Reaction

Cells harvested at the end of each experiment were centrifuged, lysed and total RNA was isolated using the RNeasy® Mini Kit (Qiagen) according to manufacturer's instruction. RNA was then quantified by UV spectrophotometry (NanoDrop Technologies) at 260 nm. Complementary DNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) with blended oligo (dT), random primers and 500 ng of RNA. For RT-PCR, a two-step PCR run was performed in StepOne Real-Time PCR System (Applied

Biosystems™), using Fast SYBR™ Green Master Mix (Applied Biosystems™), 0.5 µM of each primer, and 1 µL of cDNA in 20 µL of final volume. The primers used for VEGF-A, bFGF and HGF gene amplification are presented on Table III.1.

Table III.1 List of PCR primers used to study pro-angiogenic gene expression.

Gene	Primer Sequence	Amplicon size (bp)
Vascular endothelial growth factor-A (VEGF-A)	Fwd: 5'-CGA GGG CCT GGA GTG TGT-3' Rev: 5'-CGC ATA ATC TGC ATG GTG ATG-3'	57
Basic fibroblast growth factor (bFGF)	Fwd: 5'-TGG TAT GTG GCA CTG AAA CGA-3' Rev: 5'-GCC CAG GTC CTG TTT TGG AT	61
Hepatocyte growth factor (HGF)	Fwd: 5'-TCC ACG GAA GAG GAG ATG AGA-3' Rev: 5'-GGC CAT ATA CCA GCT GGG AAA-3'	63

Expression was normalized to the metabolic housekeeping gene GAPDH. Control assays containing no cDNA templates were also performed.

III.3.4.3.2 HUVEC Tube-Like Structure Formation Assay

The *in vitro* HUVEC tube-like structure formation assay was used to evaluate the ability of HUVEC to form networks of tubes in Matrigel®, when cultured in MSC-derived CM, as previously described [47]. The volume of CM to be used in this assay was normalized to a cell density of approximately 1 x 10^5 cell/mL, by using EBM™-2 for CM dilution (when required). 50 µL of Matrigel® Matrix Basement Membrane (Corning) were plated per well of a 96-well plate and allowed to polymerize for 1h at 37°C. 2 x 10^4 HUVEC resuspended in 200 µL of CM were then added to the Matrigel® layer. After 6h of culture, HUVEC tube-like structures were imaged and number of tubes and number of branch points were quantified using the ImageJ software. HUVEC resuspended in EBM™-2 and EGM™-2 (Lonza) were used as negative and positive controls, respectively.

III.3.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Results are presented as mean + standard error of the mean (SEM) of the values obtained for the different MSC donors. Two-way non-parametric analysis of variance (ANOVA) was calculated between three or more different experimental groups, comparing at least two parameters. Two-way ANOVA was followed by Bonferroni test to determine statistically significant differences (*P*-values < 0.05). One-way ANOVA was performed when

comparing three or more experimental groups and only one parameter. Kruskal-Wallis test was performed to determine statistically significant differences (*P*-values < 0.05).

III.4 Results

III.4.1 Immunophenotypic and Proliferative Profiles of Human Mesenchymal Stem/Stromal Cell Sources Preconditioned with Supernatant Retrieved from Other Sources

Different MSC sources present different *in vitro* angiogenic properties, as previously demonstrated. To better understand the heterogeneity of the features of each MSC source and how the microenvironment modulates these features, cells from the different sources were preconditioned with the CM retrieved from the other sources. The preconditioning effect was evaluated on their immunophenotypic and proliferative profiles, as described in Figure III.1.

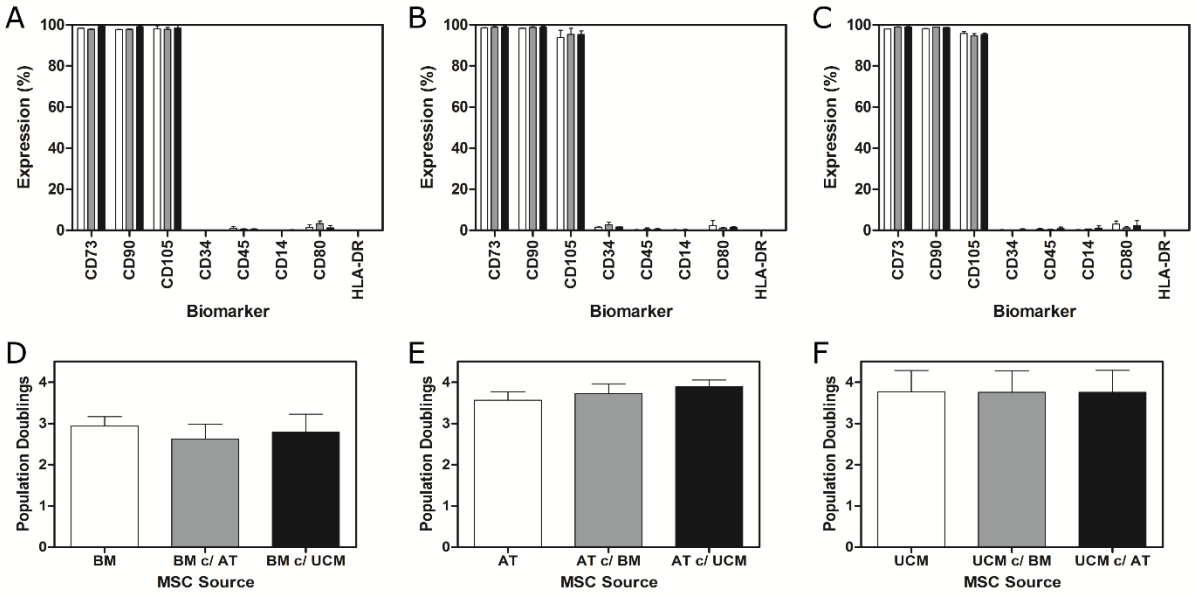

Figure III.1 Immunophenotypic and proliferative profiles of human mesenchymal stem/stromal cells (MSC) preconditioned with supernatant retrieved from other MSC sources. (A-C) Cell surface biomarker expression of nonpreconditioned and preconditioned human mesenchymal stem/stromal cell (MSC) sources (A – bone marrow (BM, white), BM conditioned with adipose tissue (AT)-derived supernatant (grey), BM conditioned with umbilical cord matrix (UCM)-derived supernatant (black); B – AT (white), AT conditioned with BM-derived supernatant (grey), AT conditioned with UCM-derived supernatant (black); C – UCM (white), UCM conditioned with BM-derived supernatant (grey), UCM conditioned with AT-derived supernatant (black)) cultured in StemPro® MSC SFM XenoFree, by flow cytometry analysis. Results are presented as mean + standard error of the mean of the expression of each antigen (%) by each MSC source (*n*=2). (D-F) Population doublings of nonpreconditioned and preconditioned human MSC sources (D – BM (white), BM conditioned with AT-derived supernatant (grey), BM conditioned with UCM-derived supernatant (black); E – AT (white), AT conditioned with BM-derived supernatant (grey), AT conditioned with UCM-derived supernatant (black); F – UCM (white), UCM conditioned with BM-

derived supernatant (grey), UCM conditioned with AT-derived supernatant (black)) cultured in StemPro® MSC SFM XenoFree. Population doublings are calculated as PD = log(FI)/log(2), where FI (fold increase) is calculated as the ratio of cells obtained at the end of the culture divided by the number of cells plated at day 0 and presented as mean + standard error of the mean (*n*=3).

Preconditioning MSC with supernatant retrieved from other MSC sources does not alter the immunophenotypic and proliferative profiles of the cells. Both nonpreconditioned and preconditioned cells present similar expression levels of the characteristic MSC biomarkers CD73, CD90 and CD105 (Figures III.1.A-C). CD105 presents the highest expression heterogeneity between different MSC sources, as described in Chapter II, and those differences are maintained when different sources are cultured with CM retrieved from other sources. While the percentage of CD105+ BM MSC is 98.1 ± 1.5 %, when cells are preconditioned with AT- and UCM-derived supernatants the expression ranges from 97.9 ± 0.9 and 98.5 ± 1.3 %, respectively. Similarly, AT MSC preconditioned with BM- and UCM-derived supernatants express 95.4 ± 3.0 and 95.3 ± 1.7 % CD105, respectively, compared with 93.9 ± 3.5 % of nonpreconditioned cells. UCM MSC cultured with supernatant retrieved from BM and AT express 94.7 ± 1.0 and 95.4 ± 0.6 %, respectively, while UCM MSC express 95.8 ± 0.9 % of CD105.

The proliferative capacity of each MSC source (Figures III.1.D-F) is also maintained when cells are preconditioned with CM retrieved from other sources. Both UCM (control – 3.76 ± 0.52, c/ BM – 3.75 ± 0.52, c/ AT – 3.75 ± 0.54) and AT (control – 3.56 ± 0.20, c/ BM – 3.72 ± 0.23, c/ UCM – 3.89 ± 0.16) MSC still present higher proliferative capacities, compared with BM MSC (control – 2.94 ± 0.23, c/ AT – 2.62 ± 0.36, c/ UCM – 2.79 ± 0.43).

III.4.2 *In Vitro* Angiogenic Profiles of Human Mesenchymal Stem/Stromal Cell Sources Preconditioned with Supernatant Retrieved from Other Sources

As for the immunophenotypic and proliferative profiles, the effect of preconditioning different MSC sources with the supernatant retrieved from other sources was evaluated on their *in vitro* HUVEC tube formation capacity. The results are illustrated on Figure III.2.

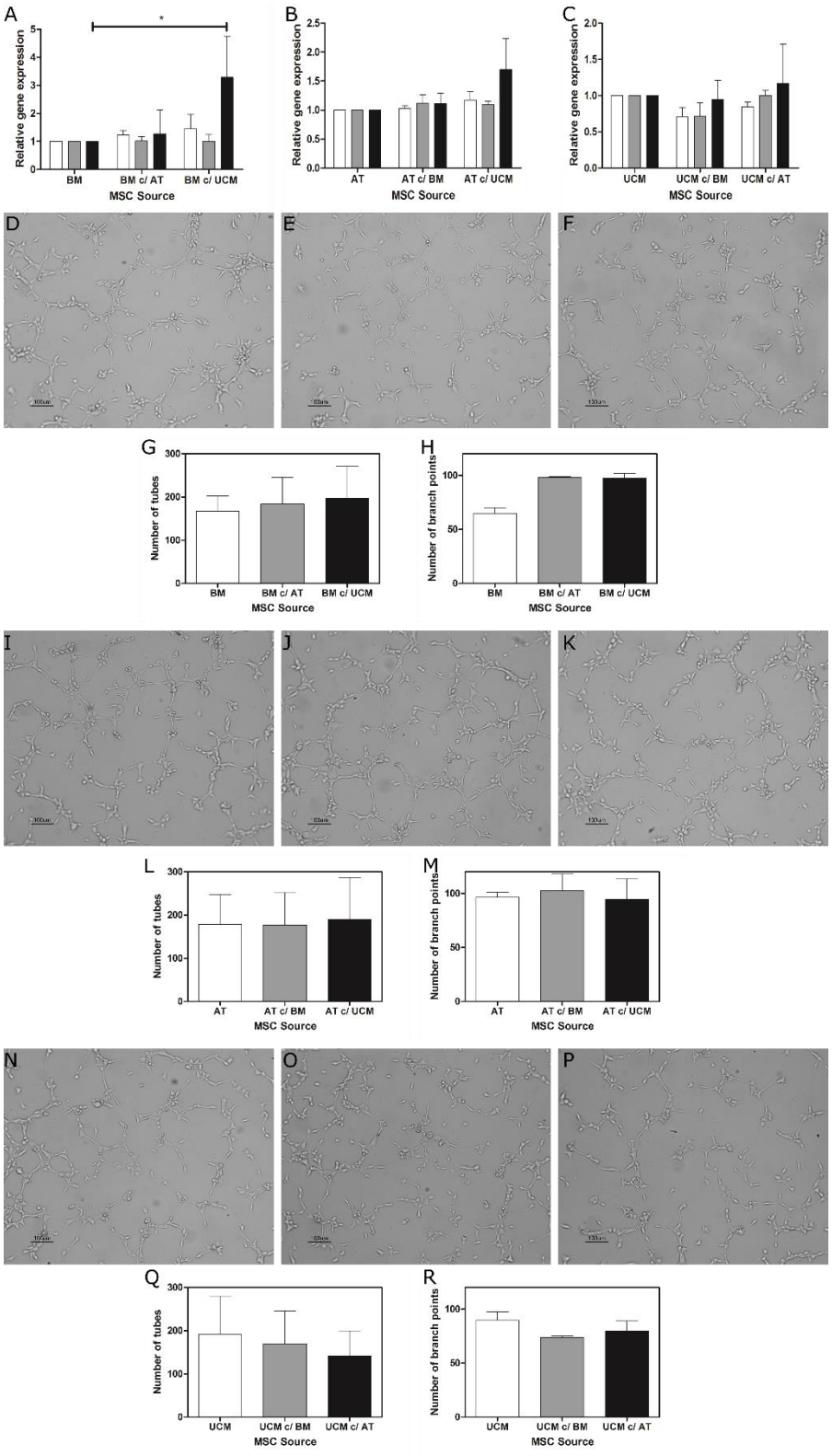

Figure III.2 ***In vitro*** **human umbilical vein endothelial cell tube-like structure formation capacity of human mesenchymal stem/stromal cells (MSC) preconditioned with supernatant retrieved from other MSC sources.** (A-C) Relative gene expression of vascular endothelial growth factor (VEGF, white), basic fibroblast growth factor (bFGF, grey) and hepatocyte growth factor (HGF, black) of nonpreconditioned and preconditioned human mesenchymal stem/stromal cell (MSC) sources (A – bone marrow (BM), BM conditioned with adipose tissue (AT)-derived supernatant, BM conditioned with umbilical cord matrix (UCM)-derived supernatant; B – AT, AT conditioned with BM-derived supernatant, AT conditioned

with UCM-derived supernatant; C – UCM, UCM conditioned with BM-derived supernatant, UCM conditioned with AT-derived supernatant) cultured in StemPro® MSC SFM XenoFree. Gene expression is normalized to the nonpreconditioned BM source and values are represented as mean + standard error of mean (SEM, n=3, *P<0.01). (D-R) Tube-like structures formed by human umbilical vein endothelial cells (HUVEC) resuspended in conditioned medium (CM) retrieved from different nonpreconditioned and preconditioned human MSC sources (D – BM; E – BM conditioned with AT-derived supernatant; F – BM conditioned with UCM-derived supernatant; I – AT; J – AT conditioned with BM-derived supernatant; K – AT conditioned with UCM-derived supernatant; N – UCM; O – UCM conditioned with BM-derived supernatant; P – UCM conditioned with AT-derived supernatant) cultured in endothelial basal medium (EBM™-2) for 48 h and plated in Matrigel®. (G, L, Q) Number of tubes and (H, M, R) number of branch points were analyzed after 6h. HUVEC cultured in EBM™-2 and EGM™-2 were used as negative and positive controls, respectively. Values are represented as mean + standard error of mean (SEM, n=2).

Preconditioning MSC with CM retrieved from other MSC sources modulates both gene expression (Figure III.2.A-C) and cell *in vitro* HUVEC tube formation ability (Figure III.2.D-R). In fact, BM MSC cultured with supernatant collected from AT or UCM MSC increase VEGF (BM c/ AT – 1.23 ± 0.15; BM c/ UCM – 1.45 ± 0.52) and HGF (BM c/ AT – 1.27 ± 0.84; BM c/ UCM – 3.28 ± 1.46) gene expression. No differences are observed at bFGF gene expression level. In contrast, UCM MSC cultured with BM MSC supernatant express lower levels of VEGF (0.71 ± 0.13) and bFGF (0.72 ± 0.18), compared with control conditions. Such pattern is not so evident when UCM MSC are cultured with AT MSC supernatant (VEGF – 0.85 ± 0.07; bFGF – 1.00 ± 0.07; HGF – 1.17 ± 0.54). AT gene expression profile does only display considerable alterations in HGF expression levels when cultured with supernatant collected from UCM (1.70 ± 0.54). In the remaining conditions, the alterations in the AT gene expression profile are negligible (AT c/ BM: VEGF – 1.03 ± 0.05, bFGF – 1.12 ± 0.15, HGF – 1.12 ± 0.18; AT c/ UCM: VEGF – 1.17 ± 0.14, bFGF – 1.09 ± 0.06).

Such alterations at the gene expression levels are translated to the HUVEC tube-like structure formation capacity. BM MSC cultured with CM retrieved from both AT or UCM MSC increase the number of HUVEC tubes (BM – 168 ± 36; BM c/ AT – 184 ± 62; BM c/ UCM – 198 ± 74) and number of branch points (BM – 65 ± 5; BM c/ AT – 98 ± 1; BM c/ UCM – 98 ± 4). Conversely, UCM MSC conditioned with BM or AT MSC CM induces a decrease in HUVEC tube number (UCM – 192 ± 88; UCM c/ BM – 170 ± 76; UCM c/ AT – 142 ± 59) and number of branch points (UCM – 90 ± 8; UCM c/ BM – 74 ± 2; UCM c/ AT – 80 ± 10). Again, AT (number of tubes – 179 ± 70; number of branch points – 97 ± 5) preconditioning with BM (number of tubes – 177 ± 75; number of branch points – 103 ± 16) and UCM (number of tubes – 190 ± 98; number of branch points – 95 ± 19) MSC CM does not follow a specific trend.

III.5 Discussion and Conclusions

The results obtained in this chapter allowed the characterization of three human MSC sources cultured with CM retrieved from other MSC sources, in terms of immunophenotype, proliferative capacity, expression of proangiogenic genes and *in vitro* HUVEC tube-like structure formation capacity. The true nature of this preconditioning strategy can be a matter of debate. The different MSC sources were preconditioned with supernatant retrieved from other sources, and not with a chemically-defined, synthetic cocktail of growth factors specifically designed to improve the angiogenic properties of the cells [25]–[27]. In fact, cell supernatant is a rich source of not only growth factors and chemokines, but also extracellular vesicles, such as exosomes, and nuclei acids, particularly small, noncoding microRNAs [48]. All these components are known to be able to modulate the angiogenic activity of the target cells. For this reason, this strategy should not be entirely classified as a growth factor preconditioning approach. Although the preconditioning effect is the result of cellular products, this approach should also not also be considered a co-culture system, since the different MSC sources are not in direct contact with each other [28]–[31]. The strategy used here is a mixed system between growth factor preconditioning and a co-culture system. From now on, this system will be defined as a biochemical preconditioning strategy or preconditioning through indirect cell culture.

MSC biochemical preconditioning led to the reduction of the differences in the expression of proangiogenic genes among MSC sources. Culturing BM MSC, the source with the lowest expression of the proangiogenic genes, with supernatant collected from sources producing with higher concentrations of those factors, namely UCM and AT MSC, resulted in an increased expression of the proangiogenic genes in the BM MSC population. The opposite trend was observed, when both UCM and AT MSC angiogenic gene expression was reduced by culturing these sources with supernatant retrieved from BM MSC. This preconditioning effect is not only observed at the gene expression level, but also in the HUVEC tube-like structure formation capacity. The results may suggest that the *in vitro* angiogenic potential of a specific MSC source can be modulated by varying the concentration of growth factors and cytokines in the culture medium (such as VEGF, bFGF and HGF), moving towards the rational design of a specific cocktail of factors to maximize MSC potential for cardiac cell therapy. To a better perception of the impact of this preconditioning strategy, protein quantification and the impact of this preconditioning strategy on the HUVEC migration capacity should also be analyzed. Importantly, such strategy does not alter the immunophenotypic and proliferative profile of each MSC source, which reinforces the higher dependence of the MSC immunophenotype and proliferation capacity on tissue origin that on the biochemical factors present in the culture medium, already discussed in Chapter II. Moreover, the maintenance of the immunophenotypic and proliferative profiles characteristics of each source represents a major step towards the establishment of GMP-compliant protocols for the manufacture of preconditioned MSC.

The presenting data suggests that preconditioning MSC through indirect cell culture may be only advantageous for the source with the lowest angiogenic capacity, although only a reduced population of factors have been screened. Further studies should include the analysis of a higher number of pro- and anti-angiogenic factors, as well as *in vivo* testing of the preconditioned cells on myocardial regeneration. Therefore, preconditioning a specific MSC source with supernatant retrieved from other sources can be advantageous when designing a preconditioning protocol to increase the expression of specific factors that are overexpressed in the cell source providing the supernatant. This strategy is particularly suitable for replacing the use of a cocktail containing several growth factors and cytokines, which may not be cost-effective for the large-scale production of an enhanced MSC-based product for AMI.

A possible preconditioning strategy through indirect cell culture can also be envisaged using CM retrieved from different cell types, such as CS/PC, EPC or HSC, to improve the regenerative capacity of a target cell population. In a greater extent than MSC sources, distinct cell types are known to secrete different growth factors and cytokines involved in regeneration. Supernatant from different cell types can be used to increase the expression of those genes in the target regenerative cells, e.g., MSC. Another interesting approach may be the co-administration of different MSC sources to an infarcted myocardium. Providing different MSC sources, and thus different proangiogenic factors, EV and nuclei acids, to a damaged heart would possibly accelerate its recovery.

All the approaches previously discussed may be advantageous to produce an enhanced MSC product comparing to other preconditioning solutions, like incubation with a highly specialized cocktail of growth factors/chemokines and genetic engineer MSC to produce higher amounts of proangiogenic factors. While the first may represent a cost-ineffective solution, the second raises both ethical and regulatory question, since genetic engineering is not yet approved by regulatory agencies, in the context of cellular therapies.

This is the first time that the supernatant collected from different MSC sources is used to modulate the *in vitro* angiogenic properties of other MSC sources.

III.6 References of Chapter III

[1] W. H. Organization, "The top 10 causes of death," 2016. [Online]. Available: http://www.who.int/mediacentre/factsheets/fs310/en/.

[2] S. Mendis, K. Thygesen, K. Kuulasmaa, S. Giampaoli, M. Mahonen, K. N. Blackett, and L. Lisheng, "World Health Organization definition of myocardial infarction: 2008-09 revision," *Int. J. Epidemiol.*, vol. 40, no. 1, pp. 139–146, 2011.

[3] M. A. Laflamme, S. Zbinden, S. E. Epstein, and C. E. Murry, "Cell-Based Therapy for Myocardial Ischemia and Infarction: Pathophysiological Mechanisms," *Annu. Rev. Pathol. Mech. Dis.*, vol. 2, no. 1, pp. 307–339, 2007.

[4] G. Fonarow, “Heart failure: recent advances in prevention and treatment,” *Rev Cardiovasc Med*, vol. 1, no. 1, pp. 25–33, 2000.
[5] K. C. Wollert and H. Drexler, “Cell therapy for the treatment of coronary heart disease: a critical appraisal.,” *Nat. Rev. Cardiol.*, vol. 7, no. 4, pp. 204–15, Apr. 2010.
[6] N. F. Huang and S. Li, “Mesenchymal stem cells for vascular regeneration.,” *Regen. Med.*, vol. 3, no. 6, pp. 877–892, Nov. 2008.
[7] L. D. S. Meirelles, A. M. Fontes, D. T. Covas, and A. I. Caplan, “Mechanisms involved in the therapeutic properties of mesenchymal stem cells.,” *Cytokine Growth Factor Rev.*, vol. 20, no. 5–6, pp. 419–27, 2009.
[8] M. Gnecchi, Z. Zhang, A. Ni, and V. J. Dzau, “Paracrine mechanisms in adult stem cell signaling and therapy.,” *Circ. Res.*, vol. 103, no. 11, pp. 1204–1219, Nov. 2008.
[9] A. A. Karpov, D. V. Udalova, M. G. Pliss, and M. M. Galagudza, “Can the outcomes of mesenchymal stem cell-based therapy for myocardial infarction be improved? Providing weapons and armour to cells,” *Cell Prolif.*, no. August, pp. 1–16, 2016.
[10] M. Mastri, H. Lin, and T. Lee, “Enhancing the efficacy of mesenchymal stem cell therapy.,” *World J. Stem Cells*, vol. 6, no. 2, pp. 82–93, 2014.
[11] Z. Wang, L. Wang, X. Su, J. Pu, M. Jiang, and B. He, “Rational transplant timing and dose of mesenchymal stromal cells in patients with acute myocardial infarction: a meta-analysis of randomized controlled trials,” *Stem Cell Research & Therapy*, vol. 8. London, 2017.
[12] T. Freyman, G. Polin, H. Osman, J. Crary, M. Lu, L. Cheng, M. Palasis, and R. L. Wilensky, “A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction.,” *Eur. Heart J.*, vol. 27, no. 9, pp. 1114–22, May 2006.
[13] S. H. Ranganath, O. Levy, M. S. Inamdar, and J. M. Karp, “Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease.,” *Cell Stem Cell*, vol. 10, no. 3, pp. 244–58, Mar. 2012.
[14] A. S. Cheng and T. M. Yau, “Paracrine Effects of Cell Transplantation: Strategies to Augment the Efficacy of Cell Therapies,” *Semin. Thorac. Cardiovasc. Surg.*, vol. 20, no. 2, pp. 94–101, 2008.
[15] N. Haque, N. H. A. Kasim, and M. T. Rahman, “Optimization of pre-transplantation conditions to enhance the efficacy of mesenchymal stem cells.,” *Int. J. Biol. Sci.*, vol. 11, no. 3, pp. 324–334, 2015.
[16] P. Swamynathan, P. Venugopal, S. Kannan, C. Thej, U. Kolkundar, S. Bhagwat, M. Ta, A. Sen Majumdar, and S. Balasubramanian, “Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton’s jelly derived mesenchymal stem cells? A comparative study.,” *Stem Cell Res. Ther.*, vol. 5, no. 4, p. 88, Jul. 2014.
[17] A. Oskowitz, H. McFerrin, M. Gutschow, M. L. Carter, and R. Pochampally, “Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic.,” *Stem Cell Res.*, vol. 6, no. 3, pp. 215–225, May 2011.
[18] T. Kinnaird, E. Stabile, M. S. Burnett, C. W. Lee, S. Barr, S. Fuchs, and S. E. Epstein, “Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms,” *Circ. Res.*, vol. 94, no. 5, pp. 678–685.
[19] S. Ohnishi, H. Sumiyoshi, S. Kitamura, and N. Nagaya, “Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions.,” *FEBS Lett.*, vol. 581, no. 21, pp. 3961–3966, Aug. 2007.
[20] J. Paquet, M. Deschepper, A. Moya, D. Logeart-Avramoglou, C. Boisson-Vidal, and H. Petite, “Oxygen Tension Regulates Human Mesenchymal Stem Cell Paracrine Functions.,” *Stem Cells Transl. Med.*, vol. 4, no. 7, pp. 809–821, Jul. 2015.
[21] J. S. Park, N. F. Huang, K. T. Kurpinski, S. Patel, S. Hsu, and S. Li, “Mechanobiology of mesenchymal stem cells and their use in cardiovascular repair.,” *Front. Biosci.*, vol. 12, pp. 5098–5116, Sep. 2007.
[22] V. Bassaneze, V. G. Barauna, C. Lavini-Ramos, J. Kalil, I. T. Schettert, A. A. Miyakawa, and J. E. Krieger, “Shear stress induces nitric oxide-mediated vascular endothelial growth factor production in human adipose tissue mesenchymal stem cells.,” *Stem Cells Dev.*, vol. 19, no. 3, pp. 371–378, Mar. 2010.
[23] J. G. Carmelo, A. Fernandes-Platzgummer, M. M. Diogo, C. L. da Silva, and J. M. S. Cabral, “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.,” *Biotechnol. J.*, vol. 10, no. 8, pp. 1235–1247, Aug. 2015.
[24] F. G. Teixeira, K. M. Panchalingam, R. Assunção-Silva, S. C. Serra, B. Mendes-Pinheiro, P. Patrício, S. Jung, S. I. Anjo, B. Manadas, L. Pinto, N. Sousa, L. A. Behie, and A. J. Salgado, “Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors: Impact on Neuronal Cell Proliferation, Survival and Differentiation,” *Scientific Reports*, vol. 6. 2016.
[25] J. Herrmann, Y. Wang, A. Abarbanell, B. Weil, J. Tan, and D. Meldrum, “Preconditioning mesenchymal stem cells with transforming growth factor-alpha improves mesenchymal stem cell-mediated

cardioprotection," *Shock*, vol. 33, no. 1, pp. 24–30, 2010.
[26] J. Guo, G. Lin, C. Bao, Z. Hu, H. Chu, and M. Hu, "Insulin-like growth factor 1 improves the efficacy of mesenchymal stem cells transplantation in a rat model of myocardial infarction," *J Biomed Sci*, vol. 15, no. 1, pp. 89–97, 2008.
[27] J. Guo, D. Zheng, W. Li, H. Li, A. Zhang, and Z. Li, "Insulin-Like Growth Factor 1 Treatment of MSCs Attenuates Inflammation and Cardiac Dysfunction Following MI," *Inflammation*, vol. 37, no. 6, pp. 2156–2163, 2014.
[28] V. Karantalis, V. Y. Suncion-Loescher, L. Bagno, S. Golpanian, A. Wolf, C. Sanina, C. Premer, A. J. Kanelidis, F. McCall, B. Wang, W. Balkan, J. Rodriguez, M. Rosado, A. Morales, K. Hatzistergos, M. Natsumeda, I. Margitich, I. H. Schulman, S. A. Gomes, M. Mushtaq, D. L. DiFede, J. E. Fishman, P. Pattany, J. P. Zambrano, A. W. Heldman, and J. M. Hare, "Synergistic effects of combined cell therapy for chronic ischemic cardiomyopathy," *J. Am. Coll. Cardiol.*, vol. 66, no. 18, pp. 1990–1999, 2015.
[29] A. R. Williams, K. E. Hatzistergos, B. Addicott, F. Mccall, D. Carvalho, V. Suncion, A. R. Morales, J. Da Silva, M. A. Sussman, A. W. Heldman, and J. M. Hare, "Enhanced Effect of Combining Human Cardiac Stem Cells and Bone Marrow Mesenchymal Stem Cells to Reduce Infarct Size and to Restore Cardiac Function after Myocardial Infarction.," *Circulation*, vol. 127, no. 2, pp. 213–223, 2013.
[30] E. J. Suuronen, J. Price, J. P. Veinot, K. Ascah, V. Kapila, X. W. Guo, S. Wong, T. G. Mesana, and M. Ruel, "Comparative effects of mesenchymal progenitor cells, endothelial progenitor cells, or their combination on myocardial infarct regeneration and cardiac function," *J. Thorac. Cardiovasc. Surg.*, vol. 134, no. 5, pp. 1249–1258, 2007.
[31] X. Zhang, M. Wei, W. Zhu, and B. Han, "Combined transplantation of endothelial progenitor cells and mesenchymal stem cells into a rat model of isoproterenol-induced myocardial injury," *Arch. Cardiovasc. Dis.*, vol. 101, no. 5, pp. 333–342, 2008.
[32] J. S. HEO, Y. CHOI, H.-S. KIM, and H. O. K. KIM, "Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue," *International Journal of Molecular Medicine*, vol. 37, no. 1. pp. 115–125, Jan-2016.
[33] A. Assoni, G. Coatti, M. C. Valadares, M. Beccari, J. Gomes, M. Pelatti, M. Mitne-Neto, V. M. Carvalho, and M. Zatz, "Different Donors Mesenchymal Stromal Cells Secretomes Reveal Heterogeneous Profile of Relevance for Therapeutic Use.," *Stem Cells Dev.*, vol. 26, no. 3, pp. 206–214, Feb. 2017.
[34] P. R. Amable, M. V. T. Teixeira, R. B. V. Carias, J. M. Granjeiro, and R. Borojevic, "Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly.," *Stem Cell Res. Ther.*, vol. 5, no. 2, p. 53, Apr. 2014.
[35] D. Baksh, R. Yao, and R. S. Tuan, "Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow.," *Stem Cells*, vol. 25, no. 6, pp. 1384–1392, Jun. 2007.
[36] T. R. J. Heathman, Q. A. Rafiq, A. K. C. Chan, K. Coopman, A. W. Nienow, B. Kara, and C. J. Hewitt, "Characterization of human mesenchymal stem cells from multiple donors and the implications for large scale bioprocess development," *Biochem. Eng. J.*, vol. 108, pp. 14–23, 2016.
[37] J. Peltzer, F. Montespan, C. Thepenier, L. Boutin, G. Uzan, N. Rouas-Freiss, and J.-J. Lataillade, "Heterogeneous functions of perinatal mesenchymal stromal cells require a preselection before their banking for clinical use.," *Stem Cells Dev.*, vol. 24, no. 3, pp. 329–344, Feb. 2015.
[38] W. J. Du, Y. Chi, Z. X. Yang, Z. J. Li, J. J. Cui, B. Q. Song, X. Li, S. G. Yang, Z. B. Han, and Z. C. Han, "Heterogeneity of proangiogenic features in mesenchymal stem cells derived from bone marrow, adipose tissue, umbilical cord, and placenta," *Stem Cell Research & Therapy*, vol. 7. London, 2016.
[39] V. Schachinger, S. Erbs, A. Elsasser, W. Haberbosch, R. Hambrecht, H. Holschermann, J. Yu, R. Corti, D. G. Mathey, C. W. Hamm, T. Suselbeck, B. Assmus, T. Tonn, S. Dimmeler, and A. M. Zeiher, "Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction.," *N. Engl. J. Med.*, vol. 355, no. 12, pp. 1210–1221, Sep. 2006.
[40] J. M. Hare, J. H. Traverse, T. D. Henry, N. Dib, R. K. Strumpf, S. P. Schulman, G. Gerstenblith, A. N. DeMaria, A. E. Denktas, R. S. Gammon, J. J. Hermiller, M. A. Reisman, G. L. Schaer, and W. Sherman, "A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction," *J Am Coll Cardiol*, vol. 54, no. 24, pp. 2277–2286, 2009.
[41] E. C. Perin, R. Sanz-Ruiz, P. L. Sanchez, J. Lasso, R. Perez-Cano, J. C. Alonso-Farto, E. Perez-David, M. E. Fernandez-Santos, P. W. Serruys, H. J. Duckers, J. Kastrup, S. Chamuleau, Y. Zheng, G. V Silva, J. T. Willerson, and F. Fernandez-Aviles, "Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial.," *Am. Heart J.*, vol. 168, no. 1, p. 88–95.e2, Jul. 2014.
[42] J. H. Houtgraaf, W. K. den Dekker, B. M. van Dalen, T. Springeling, R. de Jong, R. J. van Geuns, M. L. Geleijnse, F. Fernandez-Aviles, F. Zijlsta, P. W. Serruys, and H. J. Duckers, "First experience in humans

using adipose tissue-derived regenerative cells in the treatment of patients with ST-segment elevation myocardial infarction.," *Journal of the American College of Cardiology*, vol. 59, no. 5. United States, pp. 539–540, Jan-2012.

[43] L. R. Gao, Y. Chen, N. K. Zhang, X. L. Yang, H. L. Liu, Z. G. Wang, X. Y. Yan, Y. Wang, Z. M. Zhu, T. C. Li, L. H. Wang, H. Y. Chen, Y. D. Chen, C. L. Huang, P. Qu, C. Yao, B. Wang, G. H. Chen, Z. M. Wang, Z. Y. Xu, J. Bai, D. Lu, Y. H. Shen, F. Guo, M. Y. Liu, Y. Yang, Y. C. Ding, Y. Yang, H. T. Tian, Q. A. Ding, L. N. Li, X. C. Yang, and X. Hu, "Intracoronary infusion of Wharton's jelly-derived mesenchymal stem cells in acute myocardial infarction: double-blind, randomized controlled trial.," *BMC Med.*, vol. 13, p. 162, Jul. 2015.

[44] F. Dos Santos, P. Z. Andrade, J. S. Boura, M. M. Abecasis, C. L. da Silva, and J. M. S. Cabral, "Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia.," *J. Cell. Physiol.*, vol. 223, no. 1, pp. 27–35, Apr. 2010.

[45] J. Gimble and F. Guilak, "Adipose-derived adult stem cells: isolation, characterization, and differentiation potential.," *Cytotherapy*, vol. 5, no. 5, pp. 362–9, Jan. 2003.

[46] A. M. de Soure, A. Fernandes-Platzgummer, F. Moreira, C. Lilaia, S.-H. Liu, C.-P. Ku, Y.-F. Huang, W. Milligan, J. M. S. Cabral, and C. L. da Silva, "Integrated culture platform based on a human platelet lysate supplement for the isolation and scalable manufacturing of umbilical cord matrix-derived mesenchymal stem/stromal cells.," *J. Tissue Eng. Regen. Med.*, vol. 11, no. 5, pp. 1630–1640, May 2017.

[47] I. Arnaoutova and H. K. Kleinman, "In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract.," *Nat. Protoc.*, vol. 5, no. 4, pp. 628–635, Apr. 2010.

[48] D. Todorova, S. Simoncini, R. Lacroix, F. Sabatier, and F. Dignat-George, "Extracellular Vesicles in Angiogenesis," *Circulation Research*, vol. 120, no. 10. pp. 1658–1673, May-2017.

IV. Bioprocessing Modulation of the *In Vitro* Angiogenic Potential of Human Mesenchymal Stem/ Stromal Cells

This chapter is based on the article: Pinto, DS, Ahsan, T, Serra, J, Fernandes-Platzgummer, A, Cabral, JMS, da Silva, CL. Bioprocessing Modulation of the *In Vitro* Angiogenic Potential of Human Mesenchymal Stem/Stromal Cells. In preparation.

IV.1 Summary

MSC regenerative potential in AMI context has been augmented using different preconditioning strategies, including biochemical and/or mechanical approaches. Preconditioning cells through the modulation of factors involved in their manufacturing is of utmost interest since they can be easily adapted to the production processes and usually do not require the acquisition of additional material. In this context, dynamic culture and oxygen concentration have raised particular interest. Static MSC cultures have been replaced by dynamic systems, particularly microcarrier-based stirred bioreactors, at both laboratory and industrial scales, due to the innumerous advantages of these systems. Additionally, several reports have demonstrated the improved proliferative capacity and metabolism of MSC cultured under low oxygen concentrations (also known as hypoxia). Another important bioprocessing aspect that is poorly understood is the effect of the harvesting, cryopreservation and delivery steps on the therapeutic efficacy of MSC.

In this chapter, human UCM MSC were cultured under dynamic culture conditions and low oxygen concentrations and the impact of these culture parameters was investigated on the immunophenotypic profile and *in vitro* angiogenic potential of the preconditioned cells. Moreover, the effect of cell harvesting, cryopreservation and delivery processes was evaluated on the proangiogenic gene profile of these cells. Physically preconditioning UCM MSC through a microcarrier-based culture platform and low oxygen concentration (2% O_2) promoted an upregulation of the proangiogenic genes VEGF (5.64 ± 0.55), bFGF (1.87 ± 0.65) and HGF (2.90 ± 1.37), compared with MSC cultured under static and normoxic conditions. The increased expression was also observed at the secretion level, where several proangiogenic factors were also upregulated, including not only VEGF, bFGF and HGF, but also EGF, HB-EGF, PlGF, angiogenin, and PDGF-BB. Functional analyses evidenced a superior *in vitro* angiogenic potential of the preconditioned cells. CM retrieved from these cells resulted in an increased number of HUVEC tubes and branch points, as well as increased HUVEC migration through the transwell system. UCM MSC harvested from the microcarriers experienced a downregulation of 30% of the proangiogenic genes studied. However, both cryopreservation and delivery processes, at room temperature or 4°C, preserved the angiogenic gene expression profile of preconditioned UCM MSC.

The current bioprocessing strategy was able to increase the *in vitro* angiogenic potential of UCM MSC. Moreover, this increased potential does not seem to be greatly affected until reaching its final destination, the patient. These attributes make the preconditioned cells a powerful candidate for an allogeneic, off-the-shelf product for myocardial regeneration.

IV.2 Background

The regenerative capacity of MSC has been modulated through different biochemical and mechanical approaches, or even a mixture of both [1]–[5]. Increasing the paracrine effect of MSC through modulation of bioprocessing parameters associated with cell culture holds great interest since it does not require additional components on the culture system, such as growth factors/cytokines, different cell types, pharmacological agents and genetic engineering, and can be scaled up to industrial scale. The effect of different bioprocessing parameters have been evaluated on MSC paracrine signaling, including cell culture media [6]–[8], oxygen concentration [7], [9], [10], cell expansion platform [11]–[15], and cell storage [16], [17]. The enhanced effect of S/XF culture conditions on MSC angiogenic capacity has already been discussed in Chapter III.

MSC are traditionally cultured under atmospheric oxygen concentration (21% O_2 in ambient air). However, this oxygen concentration might not be the most adequate for the cultivation of MSC, since they are derived from tissues with a wide range of oxygen tensions (e.g. 1 – 7% in BM and 10 – 15% in AT) [18]. Moreover, researchers found that exposure of MSC to atmospheric oxygen can induce DNA damage, leading to cell senescence and decreased therapeutic efficacy [19]. Therefore, oxygen concentration in culture medium has been studied by comparing normoxic (21% O_2) to hypoxic (1 – 5% O_2) conditions. Several studies have demonstrated the positive impact of hypoxic conditions on MSC expansion [19]–[24]. However, oxygen has also showed to play a critical role in the context of AMI. In fact, the causes of cell death in an AMI event are influenced by the ischemic environment, which is devoid of nutrients and oxygen, along with the loss of survival signals for matrix attachment and cell-cell interactions [25]. In a cardiac cell therapy setting, such ischemic environment in the infarcted tissue may cause rapid loss of the transplanted MSC via apoptosis [26], [27]. Thus, preconditioning MSC by hypoxia prior to cell administration may allow the cell to better adapt to the low oxygen concentration in the ischemic tissue and to promote cell engraftment [1]. Researchers have shown that MSC subjected to hypoxic conditions secrete several proangiogenic, chemotactic, antiapoptotic and cardioprotective factors [9], [28], [29].

The culture platform used to expand MSC is also shown to greatly impact the cell biological properties. Since MSC are anchorage-dependent cells, MSC expansion has traditionally been carried out in planar cultivation systems such as tissue culture flasks or cell factories, for simplicity and easy handling [18]. At a laboratory scale, such systems are cost-effective and easily operable to successfully achieve MSC expansion. However, a considerable high number of flasks would be required to attain clinical-relevant cell numbers. The whole process would be highly time consuming, labor intensive, and would increase the risk of contamination. To overcome these issues, 3D culture systems have been developed and optimized for the culture of MSC, including the use of microcarriers, scaffolds or spheroids [30].

Although culturing MSC as spheroids has proved to enhance their immunomodulatory and regenerative properties compared with static culture [14], several studies have demonstrated limited proliferative capacity of MSC cultured in this system [30], making it not suitable for MSC expansion. In contrast, microcarriers have successfully proved their ability to efficiently support MSC expansion [11], [31]–[33]. Microcarriers provide large surface to volume ratio and facilitate higher density cultures [18]. Moreover, microcarrier-based culture systems overcome some of the issues related to static cultures, such as large volumes of medium required, inefficient gas transfer, existence of oxygen and nutrient concentration gradients, and inadequate monitoring and control [34].

To achieve this advantageous scenario, MSC culture on microcarriers is performed in dynamic culture systems named bioreactors. Several types of bioreactor configurations have been employed for the expansion of MSC, including the stirred tank reactor (the most commonly used) [32], [35]–[37], waved-mixed bioreactor [38], [39], fixed/packed bed bioreactors [40], [41], Vertical-Wheel™ bioreactor [42], among others. Bioreactor culture generates shear forces on the surface of the cells adhered to the microcarriers, due to the highly dynamic conditions associated with these culture systems. In fact, shear stress is known to be one of the main culture factors affecting MSC expansion, differentiation and paracrine signaling [13]. Shear stress has been found to induced the upregulating of several angiogenic factors, including VEGF, FGF-1 and IGF-1, in adult human MSC [11], [15], [43]. However, some authors have also reported a downregulation of other important paracrine factors, including TGF-β1, PDGF and its receptors [44], in the presence of shear stress.

All the three bioprocessing parameters described above may be used to increase the angiogenic capacity of MSC. However, little is known about the effects of cell harvesting, storage and delivery on the preservation of the preconditioning effect on the cells. While some authors reported that cryopreservation does not affect MSC potency both *in vitro* and *in vivo* [16], [17], [45], [46], others raise concerns regarding the use of MSC for clinical purposes after cell storage [47], [48].

The work developed in this chapter aims at maximizing the *in vitro* angiogenic capacity of UCM MSC by culturing the cells under dynamic conditions and low oxygen concentration. UCM MSC, one of the sources with the highest *in vitro* angiogenic capacity, as discussed in chapter II, were cultured in spinner flasks, using plastic microcarriers as adhesion surface, under hypoxic conditions (2% O_2). The effect of dynamic culture and hypoxic conditions was evaluated on MSC immunophenotypic profile, expression and secretion of proangiogenic factors and *in vitro* angiogenic potential, and compared with static culture conditions, at 21% O_2. Moreover, the effect of cell harvesting, cryopreservation and delivery processes was evaluated on the proangiogenic gene profile of the preconditioned cells. Understanding the modulation of the cell microenvironment is expected to contribute towards the development of an enhanced MSC-based product for AMI.

IV.3 Materials and Methods

IV.3.1 Human Samples

UCM MSC were isolated according to the protocol established by de Soure *et al* [33]. Umbilical cord units were obtained from healthy donors after informed maternal consent at Hospital São Francisco Xavier, Lisboa, Portugal (Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004). UCM MSC were cryopreserved in a liquid/vapor-phase nitrogen container.

IV.3.2 *Ex Vivo* Expansion of Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells

Upon thawing, UCM MSC were plated at a cell density between 3000-6000 cell/cm^2 and cultured for 2 passages under XF conditions, on CELLstart™ CTS™ (diluted 1:200 in PBS with Ca^{2+} and Mg^{2+}, Life Technologies) precoated T-75 or T-175 flasks (BD Falcon™) using StemPro® MSC SFM XenoFree (Life Technologies). Cells were kept at 37°C and 5% CO_2 in a humidified atmosphere and culture medium was changed every 3-4 days. At 70-80% cell confluence, MSC were detached from the flasks using the xeno-free cell detachment solution TrypLE™ Select CTS™ solution (1X, Life Technologies) for 7 min at 37°C. Cell number and viability were determined using Trypan Blue (Life Technologies) exclusion method.

IV.3.3 Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cell Culture for Preconditioning Through Shear Stress and Oxygen Tension

IV.3.3.1 Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cell Culture Under Static Conditions

UCM MSC were cultured at 5550 cell/cm^2 on 9X wells of 6-well plates precoated with CELLstart™ (diluted in 1:200 in PBS with Ca^{2+} and Mg^{2+}) using StemPro® MSC SFM XenoFree. After 2 days of culture, UCM MSC cultured in 6X wells were detached and collected for FC and RT-PCR analysis. Supernatant was centrifuged at 1500 rpm for 10 min and kept at -80°C for protein quantification by ELISA. In the remaining 3X wells, the supernatant was replaced by EBM™-2 and cells were cultured for 24h. CM was then centrifuged at 1500 rpm for 10 min and stored at -80°C to later assess the angiogenic potential of CM from UCM MSC cultured under static conditions. Cells were detached and

counted using Trypan Blue exclusion method. UCM MSC from three independent donors (*n*=3) in passages from P3 to P5 were used.

IV.3.3.2 Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cell Culture Under Dynamic Conditions

For dynamic culture of UCM MSC, StemSpan™ spinner flasks (STEMCELL™ Technologies) with a working volume of 40 mL of StemPro® MSC SFM XenoFree and equipped with 90° paddles and a magnetic stir bar were used. For each spinner flask culture, 1g of plastic microcarriers (Pall SoloHill®) were prepared according to manufacturer's instructions and then coated with CELLstart™ solution (diluted in 1:100 in PBS with Ca^{2+} and Mg^{2+}) for 1h at 37°C, with intermittent agitation (2 min agitated, 10 min nonagitated) using a Thermomixer® comfort (Eppendorf AG). A total cell number of 4 x 10^6 of previously expanded UCM MSC were added to the precoated microcarriers in the spinner flask, to an initial cell density of 1 x 10^5 cell/mL. The agitation speed was set to 40 rpm during the three days of culture. After 2 days of culture, cell counting on plastic microcarriers was performed as described by dos Santos *et al* [31]. 1 x 10^5 cells were collected for RT-PCR analysis and 3 x 10^5 cells were harvested for FC analysis. UCM MSC that remained in the spinner flask were washed with PBS (1X) and ressuspended in 10 mL of EBM™-2, the minimum volume required to maintain the culture in suspension. After 24h of dynamic conditioning, CM was retrieved, centrifuged at 1500 rpm for 10 min and kept at -80°C. The angiogenic potential of CM from UCM MSC cultured under dynamic conditions was later assessed through HUVEC tube formation and migration assays. UCM MSC from three independent donors (*n*=3) in passages from P3 to P5 were used.

IV.3.3.3 Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cell Culture Under Normoxic and Hypoxic Conditions

UCM MSC cultured in both static and dynamic conditions were also preconditioned to different oxygen concentrations: 21% O_2 (normoxia) and 2% O_2 (hypoxia). Cells were incubated in both normoxic and hypoxic incubators, at 37°C and 5% CO_2 for the 3 days of culture and were then collected for FC, RT-PCR, HUVEC tube formation and migration assays and supernatant collected for ELISA. UCM MSC from three independent donors (*n*=3) in passages from P3 to P5 were used.

IV.3.3.4 Harvesting, Cryopreservation and Delivery of Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells

After culture under dynamic and hypoxic conditions, UCM MSC were collected at different bioprocessing steps for RT-PCR analysis: i) harvesting; ii) delivery (after 4 hours in PBS + 1% human albumin (Octapharma®) at room temperature or 4°C); iii) cryopreservation; and iv) delivery after cryopreservation (after cell thawing and 4 hours in PBS + 1% human albumin at room temperature or 4°C). Cell harvesting was performed using TrypLE Express (1X) incubated at 37°C for 7 min at 750 rpm using Thermomixer comfort. UCM MSC were cryopreserved in 90% StemPro® MSC SFM XenoFree + 10% dimethyl sulfoxide (DMSO Hybri-Max™, Sigma-Aldrich®) at -80°C for 24 h and then transferred to liquid nitrogen for 1 month. UCM MSC from three independent donors (n=3) in passages from P3 to P5 were used.

IV.3.4 Characterization of Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells Cultured under Different Conditions

IV.3.4.1 Immunophenotypic Profile

The immunophenotypic profile of MSC were analyzed by FC (FACSCalibur equipment, Becton Dickinson), using a panel of mouse anti-human monoclonal antibodies (PE-conjugated, all from BD Biolegend) against: CD73, CD90, CD105, CD34, CD45, CD14, CD19, CD80, and HLA-DR. Cells were incubated with these monoclonal antibodies for 20 min in the dark at room temperature and then cells were washed with PBS (1X) and fixed with 2% PFA (Sigma). Isotype controls were also prepared for every experiment. A minimum of 5000 events was collected for each sample and the CellQuest software (Becton Dickinson) was used for acquisition and analysis.

IV.3.4.2 *In Vitro* Angiogenic Potential

The *in vitro* angiogenic potential of the different MSC sources was assessed using 4 different approaches: i) RT-PCR analysis of the expression of the proangiogenic genes VEGF, bFGF, and HGF; ii) quantification of the secretion of the proangiogenic factors: angiogenin, ANG-2, EGF, bFGF, HB-EGF, HGF, leptin, PDGF-BB, PlGF and VEGF by ELISA; iii) HUVEC tube-like structure formation assay, using CM retrieved from the different MSC sources; iv) HUVEC migration through a transwell system, using CM retrieved from the different MSC sources.

IV.3.4.2.1 Quantitative Real-Time-Polymerase Chain Reaction

Cells harvested at the end of each experiment were centrifuged, lysed and total RNA was isolated using the RNeasy® Mini Kit (Qiagen) according to manufacturer's instruction. RNA was then quantified by UV spectrophotometry (NanoDrop Technologies) at 260 nm. Complementary DNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) with blended oligo (dT), random primers and 500 ng of RNA. For RT-PCR, a two-step PCR run was performed in StepOne Real-Time PCR System (Applied Biosystems™), using Fast SYBR™ Green Master Mix (Applied Biosystems™), 0.5 µM of each primer, and 1 µL of cDNA in 20 µL of final volume. The primers used for VEGF-A, bFGF and HGF gene amplification are presented on Table IV.1.

Table IV.1 List of PCR primers used to study pro-angiogenic gene expression.

Gene	Primer Sequence	Amplicon size (bp)
Vascular endothelial growth factor-A (VEGF-A)	Fwd: 5'-CGA GGG CCT GGA GTG TGT-3' Rev: 5'-CGC ATA ATC TGC ATG GTG ATG-3'	57
Basic fibroblast growth factor (bFGF)	Fwd: 5'-TGG TAT GTG GCA CTG AAA CGA-3' Rev: 5'-GCC CAG GTC CTG TTT TGG AT	61
Hepatocyte growth factor (HGF)	Fwd: 5'-TCC ACG GAA GAG GAG ATG AGA-3' Rev: 5'-GGC CAT ATA CCA GCT GGG AAA-3'	63

Expression was normalized to the metabolic housekeeping gene GAPDH. Control assays containing no cDNA templates were also performed.

IV.3.4.2.2 Enzyme-Linked Immunosorbent Assay

The secretion of proangiogenic factors by UCM MSC cultured under different conditions was analyzed by a multiplex ELISA. Supernatant from UCM MSC cultured under different culture conditions was kept at -80°C until further analysis. The samples were thawed, diluted 3 X and loaded in the Quantibody® Human Angiogenesis Array ELISA Kit (RayBiotech), according to manufacturer's instructions. Protein concentration was determined by fluorescence measurement provided by RayBiotech.

IV.3.4.2.3 HUVEC Tube-Like Structure Formation Assay

The *in vitro* HUVEC tube-like structure formation assay was used to evaluate the ability of HUVEC to form networks of tubes in Matrigel®, when cultured in MSC-derived CM, as previously described [49].

The volume of CM to be used in this assay was normalized to a cell density of approximately 1 x 10^5 cell/mL, by using EBM™-2 for CM dilution (when required). 50 µL of Matrigel® Matrix Basement Membrane (Corning) were plated per well of a 96-well plate and allowed to polymerize for 1h at 37°C. 2 x 10^4 HUVEC resuspended in 200 µL of CM were then added to the Matrigel® layer. After 6h of culture, HUVEC tube-like structures were imaged and number of tubes and number of branch points were quantified using the ImageJ software. HUVEC resuspended in EBM™-2 and EGM™-2 (Lonza) were used as negative and positive controls, respectively.

IV.3.4.2.4 HUVEC Migration Assay

HUVEC migration assay was performed using 6.5 mm, 8 µm pore size transwells (Costar Corp.), coated with 10 µg/mL human fibronectin for 1 hour at 37°C. 3 x 10^4 HUVEC resuspended in 100 µL EBM™-2 were plated in transwell inserts and then placed into 24-well plate containing 700 µL of CM collected from different MSC culture conditions, also normalized to a cell density of approximately 1 x 10^5 cell/mL. Cell migration was measured after 6h of incubation at 37°C. All non-migrated cells were removed from the upper face of the transwell membrane using a cotton swab and migrated cells were fixed with 2% PFA and stained with 0.1% crystal violet in 0.1 M borate, pH 9.0, 2% ethanol. Cell counting in the lower transwell membrane was performed using the ImageJ software. HUVEC placed in EBM™-2 and EGM™-2 were used as negative and positive controls, respectively.

IV.3.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Results are presented as mean + standard error of the mean (SEM) of the values obtained for the different MSC donors. Two-way non-parametric analysis of variance (ANOVA) was calculated between three or more different experimental groups, comparing at least two parameters. Two-way ANOVA was followed by Bonferroni test to determine statistically significant differences (*P*-values < 0.05). One-way ANOVA was performed when comparing three or more experimental groups and only one parameter. Kruskal-Wallis test was performed to determine statistically significant differences (*P*-values < 0.05).

IV.4 Results

IV.4.1 Immunophenotypic Profile of Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells Cultured under Dynamic Conditions and Low Oxygen Concentration

Data reported on Chapter II evidenced the different *in vitro* angiogenic properties of the three MSC sources analyzed. UCM MSC, along with AT MSC, proved to express higher levels of the proangiogenic genes VEGF, bFGF and HGF, compared with the BM source, resulting in high number of tubes and branch points. Additionally, UCM MSC presented the highest proliferative capacity, which is advantageous from a bioprocessing perspective. Using these criteria, UCM MSC were selected to be physically preconditioned through a microcarrier-based dynamic culture platform and under hypoxic conditions (2% O_2). The effect of these two bioprocessing parameters was firstly analyzed on UCM MSC immunophenotypic profile (Figure IV.1).

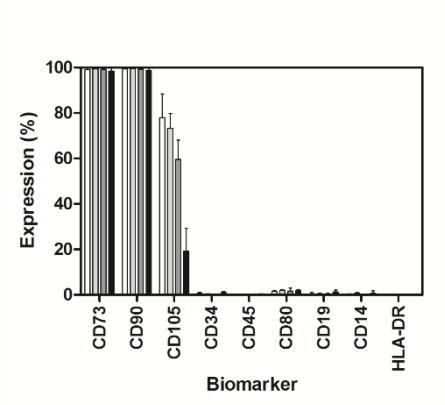

Figure IV.1 Immunophenotypic profile of human umbilical cord matrix-derived mesenchymal stem/stromal cells cultured under static vs dynamic culture and/or normoxic *vs* hypoxic culture conditions. Cell surface biomarker expression of human umbilical cord matrix (UCM)-derived mesenchymal stem/stromal cells (MSC) cultured in: i) static conditions at 21% O_2 (white); ii) static conditions at 2% O_2 (light grey); iii) dynamic conditions at 21% O_2 (dark grey); iv) dynamic conditions at 2% O_2 (black), by flow cytometry analysis. UCM MSC cultured under static conditions were plated on 6-well plates, while cells cultured under dynamic conditions were plated on Pall SoloHill® plastic microcarriers in StemSpan™ spinner flasks. StemPro® MSC SFM XenoFree was used in all culture conditions. Results are presented as mean + standard error of the mean of the expression of each antigen (%) by each MSC source (*n*=3).

UCM MSC cultured under dynamic culture conditions and/or low oxygen concentration exhibit the characteristic MSC immunophenotype (≥ 95% CD73, CD90, CD105 expression), except for CD105 biomarker. Both dynamic culture and hypoxic conditions do not affect CD73 (static normoxia (sn) – 99.2 ± 0.4%; static hypoxia (sh) – 99.4 ± 0.2%; dynamic normoxia (dn) – 99.1 ± 0.2%; dynamic hypoxia (dh) – 98.3 ± 1.1%) and CD90 (sn – 99.3 ± 0.3%; sh – 99.5 ± 0.1%; dn – 99.2 ± 0.2%; dh – 98.8 ± 0.6%) expression, but either individually or in combination these culture conditions affect CD105 expression (sn – 78.0 ± 6.1%; sh – 73.2 ± 3.8%; dn – 59.5 ± 4.9%; dh – 19.3 ± 5.7%).

IV.4.2 *In Vitro* Angiogenic Potential of Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells Cultured under Dynamic Conditions and Low Oxygen Concentration

Modulating shear stress and oxygen concentration does not only affect UCM MSC immunophenotype, but also their *in vitro* angiogenic potential. The effect of dynamic culture and low oxygen concentration was analyzed, and the results are shown on Figure IV.2.

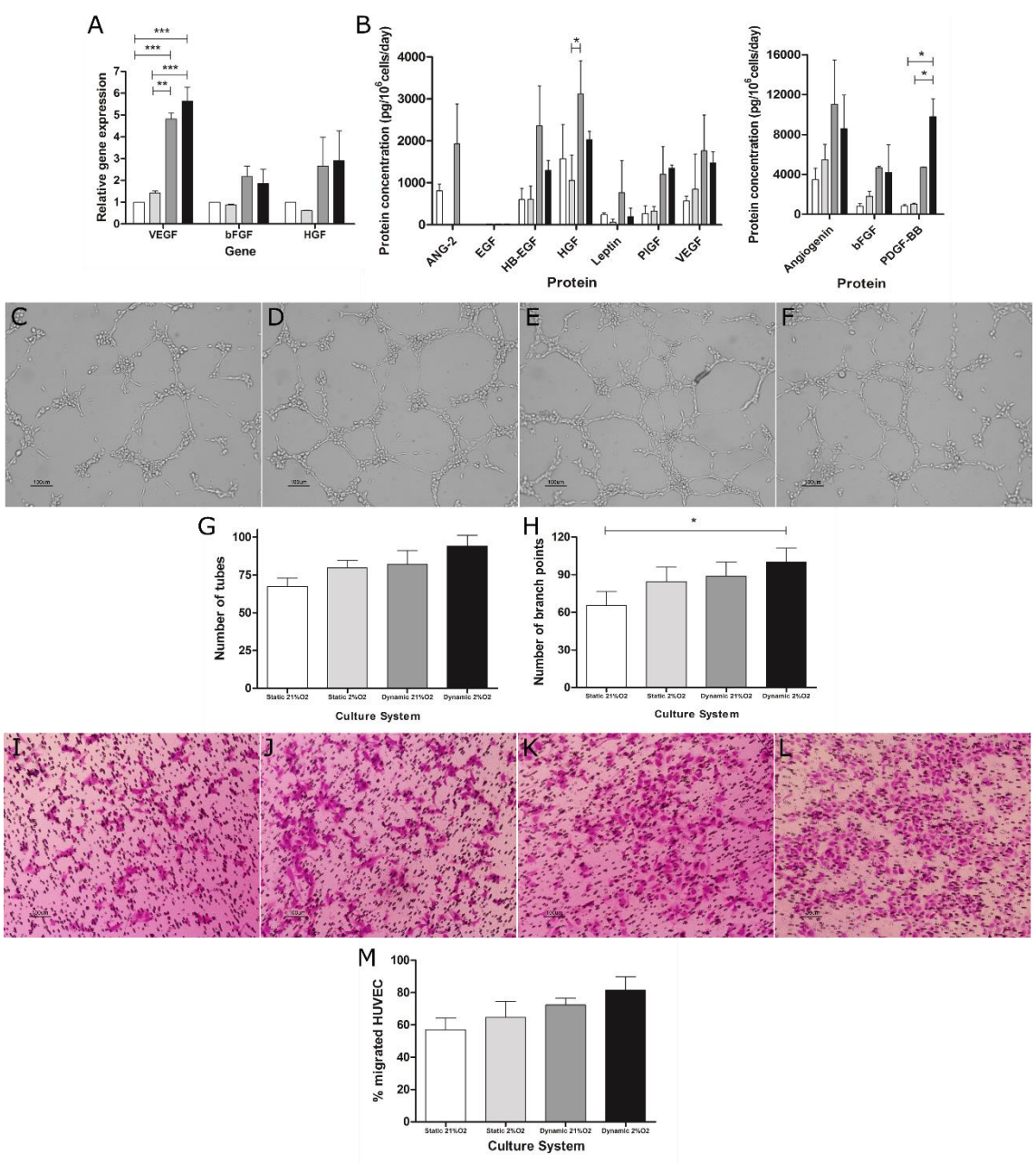

Figure IV.2 *In vitro* angiogenic potential of human umbilical cord matrix-derived mesenchymal stem/stromal cells cultured under static vs dynamic culture and/or normoxic vs hypoxic culture conditions. (A) Relative gene expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) of human umbilical cord matrix (UCM)-derived mesenchymal stem/stromal cells (MSC) cultured in: i) static conditions at 21%

O_2 (white); ii) static conditions at 2% O_2 (light grey); iii) dynamic conditions at 21% O_2 (dark grey); iv) dynamic conditions at 2% O_2 (black). UCM MSC cultured under static conditions were plated on 6-well plates, while cells cultured under dynamic conditions were plated on Pall SoloHill® plastic microcarriers in StemSpan™ spinner flasks. StemPro® MSC SFM XenoFree was used in all culture conditions. Gene expression is normalized to static conditions, 21% O_2 and values are represented as mean + standard error of mean (SEM, n=3, $^{**}P<0.05$; $^{***}P<0.001$). (B) Concentration of pro-angiogenic factors (angiopoietin-2 (ANG-2), epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), HGF, leptin, placental growth factor (PlGF), VEGF, angiogenin, bFGF, and platelet-derived growth factor-BB (PDGF-BB)) in the supernatant of human UCM MSC cultured in static vs dynamic and normoxic vs hypoxic culture conditions, using StemPro® MSC SFM XenoFree. Values are represented as mean + standard error of mean (SEM, n=2, *P<0.01). Tube-like structures formed by human umbilical vein endothelial cells (HUVEC) resuspended in conditioned medium (CM) retrieved from human UCM MSC cultured in static 21% O_2 (C), static 2% O_2 (D), dynamic 21% O_2 (E) and dynamic 2% O_2 (F) culture conditions using endothelial basal medium (EBM™-2) for 24 h and plated in Matrigel®. (G) Number of tubes and (H) number of branch points were analyzed after 6h. HUVEC cultured in EBM™-2 and EGM™-2 were used as negative and positive controls, respectively. Values are represented as mean + standard error of mean (SEM, n=3, $^{*}P<0.05$). HUVEC migration capacity through the pores of transwell inserts placed into 24-well plates containing CM collected from UCM MSC cultured in: i) static conditions at 21% O_2 (I); ii) static conditions at 2% O_2 (J); iii) dynamic conditions at 21% O_2 (K); iv) dynamic conditions at 2% O_2 (L). HUVEC migration (M) was measured after 6h of incubation at 37°C, by cell staining with 0.1% crystal violet in 0.1 M borate, pH 9.0, 2% ethanol. HUVEC cultured in EBM™-2 and EGM™-2 were used as negative and positive controls, respectively. Values are represented as mean + standard error of mean (SEM, n=3).

Gene expression analysis reveals an upregulation of the proangiogenic genes studied when UCM MSC were cultured under dynamic culture conditions (Figure IV.2.A). There is a significant increase in VEGF expression (dn – 4.82 ± 0.24; dh – 5.64 ± 0.55), compared with static conditions, and in bFGF (dn – 2.17 ± 0.49; dh – 1.87 ± 0.65) and HGF (dn – 2.66 ± 1.33; dh – 2.90 ± 1.37) expression, although not statistically significant. These results are confirmed at the protein secretion level (Figure IV.2.B). All the three proangiogenic factors are secreted at higher concentrations when UCM MSC are cultured under dynamic conditions (VEGF: sn – 568 ± 115 pg/(10^6cell.day), dn – 1765 ± 850 pg/(10^6cell.day), dh – 1475 ± 259 pg/(10^6cell.day); bFGF – sn – 815 ± 257 pg/(10^6cell.day), dn – 4662 ± 141 pg/(10^6cell.day), dh – 4176 ± 2792 pg/(10^6cell.day); HGF – sn – 1577 ± 812 pg/(10^6cell.day), dn – 3121 ± 792 pg/(10^6cell.day), dh – 2028 ± 194 pg/(10^6cell.day)). Other proangiogenic factors follow the same trend, such as EGF (sn – 3 ± 1 pg/(10^6cell.day), dn – 17 ± 3 pg/(10^6cell.day), dh – 22 ± 1 pg/(10^6cell.day)), HB-EGF (sn – 600 ± 263 pg/(10^6cell.day), dn – 2358 ± 953 pg/(10^6cell.day), dh – 1294 ± 240 pg/(10^6cell.day)), PlGF (sn – 267 ± 182 pg/(10^6cell.day), dn – 1200 ± 662 pg/(10^6cell.day), dh – 1347 ± 78 pg/(10^6cell.day)), angiogenin (sn – 3474 ± 1165 pg/(10^6cell.day), dn – 11037 ± 4465 pg/(10^6cell.day), dh – 8600 ± 3365 pg/(10^6cell.day)), and PDGF-BB (sn – 830 ± 151 pg/(10^6cell.day), dn – 4723 ± 28 pg/(10^6cell.day), dh – 9813 ± 1797 pg/(10^6cell.day)).

Data also indicates that dynamic culture has a higher impact on UCM MSC angiogenic gene expression than oxygen concentration. In fact, under static conditions, hypoxia induces a moderate VEGF upregulation (1.43 ± 0.09) and secretion (sn – 568 ± 115 pg/(10^6cell.day) *vs* sh – 843 ± 843 pg/(10^6cell.day)) and a downregulation of HGF expression (0.61 ± 0.02) and secretion (sn – 1577 ± 812 pg/(10^6cell.day) *vs* sh – 1053 ± 605 pg/(10^6cell.day)). Leptin secretion shows a similar trend (sn – 242 ± 42 pg/(10^6cell.day) *vs* sh – 67 ± 67 pg/(10^6cell.day)). Results for bFGF suggest higher protein

secretion levels (sn – 815 ± 257 pg/(10^6cell.day) *vs* sh – 1789 ± 541 pg/(10^6cell.day)), but a reduced gene expression profile (0.88 ± 0.03). Angiopoietin-2 is only expressed under 21% O_2 (sn – 805 ± 167 pg/(10^6cell.day), sh – 0 ± 0 pg/(10^6cell.day), dn – 1931 ± 946 pg/(10^6cell.day), dh – 0 ± 0 pg/(10^6cell.day)).

Both dynamic culture and hypoxia, either individually or in combination, modulate HUVEC tube formation (Figure IV.2.C-H) and migration (Figure IV.2.I-M) capacities of UCM MSC CM. Both preconditioning strategies result in increased number of HUVEC tubes (sn – 67 ± 6; sh – 80 ± 5; dn – 82 ± 9) and increased number of branch points (sn – 66 ± 11; sh – 84 ± 12; dn – 89 ± 11), compared with UCM MSC CM collected from static conditions, at 21% O_2. A similar trend is observed at the migration level, where both dynamic culture (72 ± 5 %) and hypoxia (65 ± 10 %) induce the migration of a higher percentage of HUVEC, compared with CM collected from UCM MSC cultured under static and normoxic conditions (57 ± 7 %). Combining dynamic culture and low oxygen concentration promotes a synergistic effect on HUVEC tube formation (number of tubes – 94 ± 7; number of branch points – 100 ± 11) and migration (82 ± 8%) abilities, compared with each effect individually.

IV.4.3 Angiogenic Gene Expression Profile of Physically Preconditioned Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells Throughout the Harvesting, Cryopreservation and Delivery Processes

Preconditioned MSC have demonstrated an improved *in vitro* angiogenic potential compared with nonpreconditioned MSC. However, little is known about the effect of the downstream and delivery processing on the improved potential of preconditioned MSC. In this study, cell viability and VEGF, bFGF and HGF gene expression profile of UCM MSC preconditioned through dynamic culture and low oxygen concentration were analyzed throughout the harvesting, cryopreservation and delivery processes. Results are shown in Figure IV.3.

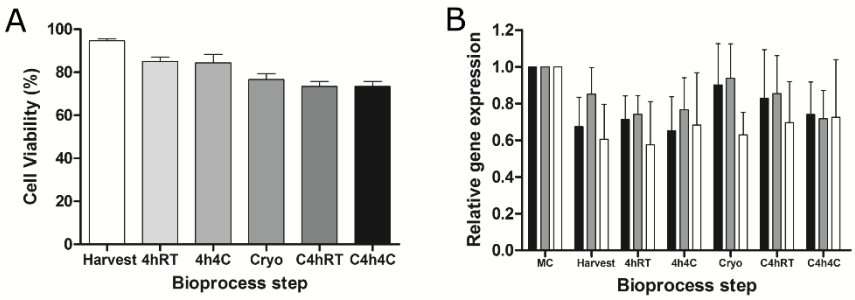

Figure IV.3 Angiogenic gene expression profile of physically preconditioned human umbilical cord matrix-derived mesenchymal stem/stromal cells throughout the harvesting, cryopreservation and delivery processes. (A) Cell viability

(%) of human umbilical cord matrix (UCM)-derived mesenchymal stem/stromal cells (MSC) at different bioprocessing steps: i) harvesting (Harvest), ii) 4 hours after harvesting at room temperature (4hRT), iii) 4 hours after harvesting at 4°C (4h4C), iv) cryopreservation (Cryo), v) 4 hours after thawing at room temperature (C4hRT), and vi) 4 hours after thawing at 4°C (C4h4C). UCM MSC were resuspended in PBS + 1% human albumin after harvesting and cryopreservation. Cell viability was calculated using Trypan Blue exclusion method and values are represented as mean + standard error of mean (SEM, *n*=3). (B) Relative gene expression of vascular endothelial growth factor (VEGF, black), basic fibroblast growth factor (bFGF, grey) and hepatocyte growth factor (HGF, white) of human umbilical cord matrix (UCM)-derived mesenchymal stem/stromal cells (MSC) at different bioprocessing steps: i) microcarriers (MC), ii) harvesting (Harvest), iii) 4 hours after harvesting at room temperature (4hRT), iv) 4 hours after harvesting at 4°C (4h4C), v) cryopreservation (Cryo), vi) 4 hours after thawing at room temperature (C4hRT), and vii) 4 hours after thawing at 4°C (C4h4C). Gene expression is normalized to cells in the microcarriers and values are represented as mean + standard error of mean (SEM, *n*=3).

Results indicate that UCM MSC remain viable after cell harvesting (95 ± 1 %) and after 4 hours resuspended in PBS + 1% human albumin (85 ± 2 %). Moreover, storing cells at 4°C does not affect cell viability (84 ± 4 %), compared with room temperature. After cryopreservation, 77% ± 3 % of UCM MSC are viable and remain so during the delivery process, at room temperature and 4°C (73 ± 2 %).

UCM MSC harvesting from the microcarriers induces a downregulation of VEGF (0.67 ± 0.16), bFGF (0.85 ± 0.15), and HGF (0.61 ± 0.19) expression, although not statistically significant. Such downregulation is maintained throughout the cell delivery process, meaning after 4 hours in PBS + 1% human albumin, at both room temperature (VEGF – 0.71 ± 0.13; bFGF – 0.74 ± 0.10; HGF – 0.58 ± 0.24) and 4°C (VEGF – 0.65 ± 0.18; bFGF – 0.77 ± 0.17; HGF – 0.68 ± 0.29). Cryopreservation for 1 month in liquid nitrogen did not induce alterations in the expression of the proangiogenic genes (VEGF – 0.90 ± 0.22; bFGF – 0.94 ± 0.19; HGF – 0.63 ± 0.12). Cell delivery after cryopreservation of UCM MSC did not also affect the expression of the genes studied (RT: VEGF – 0.83 ± 0.26; bFGF – 0.85 ± 0.21; HGF – 0.70 ± 0.22. 4°C: VEGF – 0.74 ± 0.18; bFGF – 0.72 ± 0.15; HGF – 0.72 ± 0.31).

IV.5 Discussion and Conclusions

Although MSC have emerged as a promising cell source in the myocardial regeneration context, limited and inconsistent preclinical and clinical outcomes have been reported during the last years. Different preconditioning strategies have been found to improve MSC quality in both *in vitro* and *in vivo* settings, including physical, biochemical and/or genetic modulation of the cells or their microenvironment. However, genetic engineering MSC for a therapeutic purpose raises legal and ethical issues, specially concerning product safety. In that context, microenvironment modulation as an alternative to cell modification represents a major advantage towards the development of a GMP-compliant cellular product.

Preconditioning UCM MSC through a microcarrier-based stirred system and low oxygen concentration aims at resembling the environment that transplanted cells face when systemically administrated as an MSC-based product for myocardial infarction: i) the shear forces generated by the bloodstream, and ii) the ischemic environment in the damaged heart. Subjecting cells to such stress conditions may not only increase their chances of survival in the body (out of the scope of this work), but also their *in vitro* angiogenic potential. Therefore, the effect of the two physical preconditioning approaches on their *in vitro* angiogenic potential was analyzed. Both dynamic culture and low oxygen concentration increased the expression and secretion of proangiogenic factors, as previously demonstrated in other works [11], [15], [50], [51], which resulted in an improved *in vitro* angiogenic capacity of the cells. Although hypoxia preconditioning has been associated with improved MSC angiogenic capacity in previous studies [9], [28], [29], this might not always be the case. In fact, results have been controversial concerning the time of exposure to hypoxia: short- (preconditioning) or long-term exposure [52]. Nevertheless, oxygen plays an important role on MSC metabolism. Under normoxic conditions, cells generate energy through ATP production marginally by phosporylative oxidation [53]. When switched to hypoxic conditions, cells rely only on the glycolytic metabolism to produce ATP, via the expression of HIF-1α [54]. Researchers have been demonstrating that MSC withstand severe hypoxia as long as cells do not face glucose starvation [55] and it can even significantly improve cell survival [56]. No reports were found regarding the improved angiogenic capacity of MSC cultured under dynamic conditions.

The improved angiogenic properties of preconditioned UCM MSC are characterized by increased number of HUVEC tubes and branch points, as discussed in Chapter III, allowing the formation of a more interconnected network of tubes. Such network is required for an efficient diffusion of oxygen and nutrients to the ischemic myocardial tissue. Furthermore, preconditioning UCM MSC through dynamic culture and low oxygen concentration also improved the migration ability of HUVEC. Dynamic culture and low oxygen concentration are stress conditions which might be responsible for the upregulation of the proangiogenic (and prosurvival) genes and, thus, for the improved *in vitro* angiogenic potential of these cells. These results are corroborated by *in vivo* studies showing an improvement of the cardiac function using preconditioned MSC, compared with nonpreconditioned cells [57], [58]. From a bioprocessing perspective, culturing MSC in a microcarrier-based stirred tank bioreactor system represents not only a preconditioning strategy to potentially increase cell therapeutic properties, but also enables the development of a controlled, robust and reproducible culture process and a safe and consistent cell product, according to GMP guidelines. Moreover, expanding MSC in a microcarrier-based platform allows a high surface-to-volume ratio, potentiating the total number of cells achieved at the end of the culture. Conversely, clinical-grade production of MSC under controlled oxygen concentrations might not be cost-effective. A careful analysis of the therapeutic benefits of culturing

MSC under low/high oxygen concentrations *vs* atmospheric oxygen concentration is highly recommended. From a careful analysis of the literature, this is the first work reporting the modulation of the MSC *in vitro* angiogenic potential through a bioprocessing approach.

UCM MSC harvested from the microcarriers experience a downregulation of nearly 30% of the proangiogenic genes studied. However, this expression is not altered throughout the cell delivery process, at both room temperature and 4°C. Cell delivery under low temperatures consists of an effective short-term cell preservation method named low temperature cell pausing, which provides time for quality control analysis and transportation, reduced risk of sample contamination and reduced costs [59]. Moreover, cryopreservation of UCM MSC for 1 month in liquid nitrogen does not also affect the proangiogenic gene expression profile. Different findings have been reported concerning the effect of cryopreservation on MSC quality attributes. Some authors have demonstrated that cryopreservation is able to preserve the main characteristics of MSC, including viability, immunomodulating potential, lack of malignant transformation, and cardiac regenerative capacity [16], [17], [45], [46]. Conversely, other teams have reported impaired proliferative and immunomodulatory capacities and cellular organization of cryopreserved MSC, raising concerns regarding the use of MSC for clinical purposes after cell storage [47], [48].

Despite the important contribution of this work to the development of an MSC-based product with enhanced therapeutic features for myocardial regeneration, it still presents some limitations. The first one relies on the marked decrease of CD105 expression in preconditioned MSC. MSC cultured under dynamic culture are described to express lower levels of this biomarker, due to longer times of exposure to the cell detachment reagent and higher agitation rates to dissociate cells from the microcarriers [60]. However, hypoxic conditions were not expected to contribute to a decrease in the CD105 expression. In fact, hypoxia and TGF-β are the main factors cooperating to induce CD105, or endoglin, expression at both the transcriptional and the protein levels [61]. Despite these contradicting results, other authors have already reported a decreased CD105 expression under hypoxic conditions [62]. In this work, Boroujerdi and co-workers surprisingly found hypoxia (8% O_2) induced a reduction in CD105 expression on brain endothelial cells. Further studies will be required to understand if preconditioned cells will reacquire the expression of CD105, which plays an important role on promoting angiogenesis, not only by activation of vascular endothelial cell proliferation but also by induction of the antiapoptotic pathways [61].

An important technical limitation of the study was studying the impact of specific culture conditions (40 rpm of agitation rate, spinner flask culture, 2% O_2) on MSC *in vitro* angiogenic potential. In order to fully maximize the therapeutic potential of these cells, different bioreactor configurations, agitation rates

and oxygen concentrations should be tested and compared. A design of experiment (DoE) approach can be used to find the best combination of these process parameters, using less experimental conditions.

Finally, more UCM MSC donors and *in vivo* testing are required to validate the results obtained here, either statistically and biologically, respectively.

Nevertheless, the results obtained are expected to contribute towards the development of an enhanced, allogeneic, off-the-shelf MSC product for AMI treatment.

IV.6 References of Chapter IV

[1] M. Mastri, H. Lin, and T. Lee, "Enhancing the efficacy of mesenchymal stem cell therapy.," *World J. Stem Cells*, vol. 6, no. 2, pp. 82–93, 2014.

[2] N. Haque, N. H. A. Kasim, and M. T. Rahman, "Optimization of pre-transplantation conditions to enhance the efficacy of mesenchymal stem cells.," *Int. J. Biol. Sci.*, vol. 11, no. 3, pp. 324–334, 2015.

[3] A. S. Cheng and T. M. Yau, "Paracrine Effects of Cell Transplantation: Strategies to Augment the Efficacy of Cell Therapies," *Semin. Thorac. Cardiovasc. Surg.*, vol. 20, no. 2, pp. 94–101, 2008.

[4] A. A. Karpov, D. V. Udalova, M. G. Pliss, and M. M. Galagudza, "Can the outcomes of mesenchymal stem cell-based therapy for myocardial infarction be improved? Providing weapons and armour to cells," *Cell Prolif.*, no. August, pp. 1–16, 2016.

[5] S. Baldari, G. Di Rocco, M. Piccoli, M. Pozzobon, M. Muraca, and G. Toietta, "Challenges and Strategies for Improving the Regenerative Effects of Mesenchymal Stromal Cell-Based Therapies.," *Int. J. Mol. Sci.*, vol. 18, no. 10, Oct. 2017.

[6] P. Swamynathan, P. Venugopal, S. Kannan, C. Thej, U. Kolkundar, S. Bhagwat, M. Ta, A. Sen Majumdar, and S. Balasubramanian, "Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton's jelly derived mesenchymal stem cells? A comparative study.," *Stem Cell Res. Ther.*, vol. 5, no. 4, p. 88, Jul. 2014.

[7] P. Page, J. DeJong, A. Bandstra, and R. A. Boomsma, "Effect of serum and oxygen concentration on gene expression and secretion of paracrine factors by mesenchymal stem cells.," *Int. J. Cell Biol.*, vol. 2014, p. 601063, 2014.

[8] S. Bobis-Wozowicz, K. Kmiotek, K. Kania, E. Karnas, A. Labedz-Maslowska, M. Sekula, S. Kedracka-Krok, J. Kolcz, D. Boruczkowski, Z. Madeja, and E. K. Zuba-Surma, "Diverse impact of xeno-free conditions on biological and regenerative properties of hUC-MSCs and their extracellular vesicles.," *J. Mol. Med. (Berl).*, vol. 95, no. 2, pp. 205–220, Feb. 2017.

[9] J. Paquet, M. Deschepper, A. Moya, D. Logeart-Avramoglou, C. Boisson-Vidal, and H. Petite, "Oxygen Tension Regulates Human Mesenchymal Stem Cell Paracrine Functions.," *Stem Cells Transl. Med.*, vol. 4, no. 7, pp. 809–821, Jul. 2015.

[10] W. L. Grayson, F. Zhao, R. Izadpanah, B. Bunnell, and T. Ma, "Effects of Hypoxia on Human Mesenchymal Stem Cell Expansion and Plasticity in 3D Constructs," *J Cell Physiol*, vol. 207, no. 2, pp. 331–339, 2006.

[11] J. G. Carmelo, A. Fernandes-Platzgummer, M. M. Diogo, C. L. da Silva, and J. M. S. Cabral, "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.," *Biotechnol. J.*, vol. 10, no. 8, pp. 1235–1247, Aug. 2015.

[12] J. Hupfeld, I. H. Gorr, C. Schwald, N. Beaucamp, K. Wiechmann, K. Kuentzer, R. Huss, B. Rieger, M. Neubauer, and H. Wegmeyer, "Modulation of mesenchymal stromal cell characteristics by microcarrier culture in bioreactors.," *Biotechnol. Bioeng.*, vol. 111, no. 11, pp. 2290–302, Nov. 2014.

[13] A. B. Yeatts, D. T. Choquette, and J. P. Fisher, "Bioreactors to influence stem cell fate: augmentation of mesenchymal stem cell signaling pathways via dynamic culture systems.," *Biochim. Biophys. Acta*, vol. 1830, no. 2, pp. 2470–80, Feb. 2013.

[14] T. J. Bartosh, J. H. Ylöstalo, A. Mohammadipoor, N. Bazhanov, K. Coble, K. Claypool, R. H. Lee, H.

Choi, and D. J. Prockop, “Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 31, pp. 13724–9, 2010.

[15] F. G. Teixeira, K. M. Panchalingam, R. Assunção-Silva, S. C. Serra, B. Mendes-Pinheiro, P. Patrício, S. Jung, S. I. Anjo, B. Manadas, L. Pinto, N. Sousa, L. A. Behie, and A. J. Salgado, “Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors: Impact on Neuronal Cell Proliferation, Survival and Differentiation,” *Scientific Reports*, vol. 6. 2016.

[16] S.-P. Chin, A. C. Poey, C.-Y. Wong, S.-K. Chang, W. Teh, T. J. Mohr, and S.-K. Cheong, “Cryopreserved mesenchymal stromal cell treatment is safe and feasible for severe dilated ischemic cardiomyopathy.,” *Cytotherapy*, vol. 12, no. 1, pp. 31–37, 2010.

[17] J. Luetzkendorf, K. Nerger, J. Hering, A. Moegel, K. Hoffmann, C. Hoefers, C. Mueller-Tidow, and L. P. Mueller, “Cryopreservation does not alter main characteristics of Good Manufacturing Process-grade human multipotent mesenchymal stromal cells including immunomodulating potential and lack of malignant transformation.,” *Cytotherapy*, vol. 17, no. 2, pp. 186–198, Feb. 2015.

[18] A. C. Schnitzler, A. Verma, D. E. Kehoe, D. Jing, J. R. Murrell, K. A. Der, M. Aysola, P. J. Rapiejko, S. Punreddy, and M. S. Rook, “Bioprocessing of human mesenchymal stem/stromal cells for therapeutic use: Current technologies and challenges,” *Biochem. Eng. J.*, vol. 108, pp. 3–13, 2016.

[19] J. C. Estrada, C. Albo, A. Benguria, A. Dopazo, P. Lopez-Romero, L. Carrera-Quintanar, E. Roche, E. P. Clemente, J. A. Enriquez, A. Bernad, and E. Samper, “Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis.,” *Cell Death Differ.*, vol. 19, no. 5, pp. 743–755, May 2012.

[20] F. Dos Santos, P. Z. Andrade, J. S. Boura, M. M. Abecasis, C. L. da Silva, and J. M. S. Cabral, “Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia.,” *J. Cell. Physiol.*, vol. 223, no. 1, pp. 27–35, Apr. 2010.

[21] K. Drela, A. Sarnowska, P. Siedlecka, I. Szablowska-Gadomska, M. Wielgos, M. Jurga, B. Lukomska, and K. Domanska-Janik, “Low oxygen atmosphere facilitates proliferation and maintains undifferentiated state of umbilical cord mesenchymal stem cells in an hypoxia inducible factor-dependent manner.,” *Cytotherapy*, vol. 16, no. 7, pp. 881–892, Jul. 2014.

[22] C. Fotia, A. Massa, F. Boriani, N. Baldini, and D. Granchi, “Hypoxia enhances proliferation and stemness of human adipose-derived mesenchymal stem cells.,” *Cytotechnology*, vol. 67, no. 6, pp. 1073–1084, Dec. 2015.

[23] A. Lavrentieva, I. Majore, C. Kasper, and R. Hass, “Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells.,” *Cell Commun. Signal.*, vol. 8, p. 18, Jul. 2010.

[24] P. H. Oliveira, J. S. Boura, M. M. Abecasis, J. M. Gimble, C. L. da Silva, and J. M. S. Cabral, “Impact of hypoxia and long-term cultivation on the genomic stability and mitochondrial performance of ex vivo expanded human stem/stromal cells.,” *Stem Cell Res.*, vol. 9, no. 3, pp. 225–236, Nov. 2012.

[25] M. Gnecchi, H. He, O. D. Liang, L. G. Melo, F. Morello, H. Mu, N. Noiseux, L. Zhang, R. E. Pratt, J. S. Ingwall, and V. J. Dzau, “Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells.,” *Nature medicine*, vol. 11, no. 4. United States, pp. 367–368, Apr-2005.

[26] E. Chavakis, M. Koyanagi, and S. Dimmeler, “Enhancing the outcome of cell therapy for cardiac repair: progress from bench to bedside and back.,” *Circulation*, vol. 121, no. 2, pp. 325–335, Jan. 2010.

[27] X.-B. Liu, J.-A. Wang, M. E. Ogle, and L. Wei, “Prolyl hydroxylase inhibitor dimethyloxalylglycine enhances mesenchymal stem cell survival.,” *J. Cell. Biochem.*, vol. 106, no. 5, pp. 903–911, Apr. 2009.

[28] N. Haque, M. T. Rahman, N. H. Abu Kasim, and A. M. Alabsi, “Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy.,” *ScientificWorldJournal.*, vol. 2013, p. 632972, 2013.

[29] T. Kinnaird, E. Stabile, M. S. Burnett, C. W. Lee, S. Barr, S. Fuchs, and S. E. Epstein, “Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms,” *Circ. Res.*, vol. 94, no. 5, pp. 678–685.

[30] A. M. de Soure, A. Fernandes-Platzgummer, C. L. da Silva, and J. M. S. Cabral, “Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells.,” *J. Biotechnol.*, vol. 236, pp. 88–109, Oct. 2016.

[31] F. Dos Santos, P. Z. Andrade, M. M. Abecasis, J. M. Gimble, L. G. Chase, A. M. Campbell, S. Boucher, M. C. Vemuri, C. L. Da Silva, and J. M. S. Cabral, “Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions.,” *Tissue Eng. Part C. Methods*, vol. 17, no. 12, pp. 1201–10, Dec. 2011.

[32] F. Dos Santos, A. Campbell, A. Fernandes-Platzgummer, P. Z. Andrade, J. M. Gimble, Y. Wen, S. Boucher, M. C. Vemuri, C. L. da Silva, and J. M. S. Cabral, “A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells.,” *Biotechnol. Bioeng.*, vol. 111, no.

6, pp. 1116–27, Jun. 2014.
[33] A. M. de Soure, A. Fernandes-Platzgummer, F. Moreira, C. Lilaia, S.-H. Liu, C.-P. Ku, Y.-F. Huang, W. Milligan, J. M. S. Cabral, and C. L. da Silva, "Integrated culture platform based on a human platelet lysate supplement for the isolation and scalable manufacturing of umbilical cord matrix-derived mesenchymal stem/stromal cells.," *J. Tissue Eng. Regen. Med.*, vol. 11, no. 5, pp. 1630–1640, May 2017.
[34] L.-Y. Sun, S.-Z. Lin, Y.-S. Li, H.-J. Harn, and T.-W. Chiou, "Functional cells cultured on microcarriers for use in regenerative medicine research.," *Cell Transplant.*, vol. 20, no. 1, pp. 49–62, 2011.
[35] B. Cunha, T. Aguiar, M. M. Silva, R. J. S. Silva, M. F. Q. Sousa, E. Pineda, C. Peixoto, M. J. T. Carrondo, M. Serra, and P. M. Alves, "Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells.," *J. Biotechnol.*, vol. 213, pp. 97–108, Nov. 2015.
[36] Q. A. Rafiq, K. M. Brosnan, K. Coopman, A. W. Nienow, and C. J. Hewitt, "Culture of human mesenchymal stem cells on microcarriers in a 5 l stirred-tank bioreactor.," *Biotechnol. Lett.*, vol. 35, no. 8, pp. 1233–1245, Aug. 2013.
[37] T. Lawson, D. E. Kehoe, A. C. Schnitzler, P. J. Rapiejko, K. A. Der, K. Philbrick, S. Punreddy, S. Rigby, R. Smith, Q. Feng, J. R. Murrell, and M. S. Rook, "Process development for expansion of human mesenchymal stromal cells in a 50L single-use stirred tank bioreactor," *Biochem. Eng. J.*, vol. 120, pp. 49–62, 2017.
[38] H. Akerstrom, "Expansion of adherent cells for cell therapy," Uppsala University, Sweden, 2009.
[39] N. E. Timmins, M. Kiel, M. Gunther, C. Heazlewood, M. R. Doran, G. Brooke, and K. Atkinson, "Closed system isolation and scalable expansion of human placental mesenchymal stem cells.," *Biotechnol. Bioeng.*, vol. 109, no. 7, pp. 1817–1826, Jul. 2012.
[40] A. Mizukami, M. D. Orellana, S. R. Caruso, K. de Lima Prata, D. T. Covas, and K. Swiech, "Efficient expansion of mesenchymal stromal cells in a disposable fixed bed culture system.," *Biotechnol. Prog.*, vol. 29, no. 2, pp. 568–572, 2013.
[41] M. J. Osiecki, T. D. Michl, B. Kul Babur, M. Kabiri, K. Atkinson, W. B. Lott, H. J. Griesser, and M. R. Doran, "Packed Bed Bioreactor for the Isolation and Expansion of Placental-Derived Mesenchymal Stromal Cells," *PLoS ONE*, vol. 10, no. 12. San Francisco, CA USA, 2015.
[42] M. F. Q. Sousa, M. M. Silva, D. Giroux, Y. Hashimura, R. Wesselschmidt, B. Lee, A. Roldao, M. J. T. Carrondo, P. M. Alves, and M. Serra, "Production of oncolytic adenovirus and human mesenchymal stem cells in a single-use, Vertical-Wheel bioreactor system: Impact of bioreactor design on performance of microcarrier-based cell culture processes.," *Biotechnol. Prog.*, vol. 31, no. 6, pp. 1600–1612, 2015.
[43] J. S. Park, N. F. Huang, K. T. Kurpinski, S. Patel, S. Hsu, and S. Li, "Mechanobiology of mesenchymal stem cells and their use in cardiovascular repair.," *Front. Biosci.*, vol. 12, pp. 5098–5116, Sep. 2007.
[44] H. Wang, M. Li, P. H. Lin, Q. Yao, and C. Chen, "Fluid shear stress regulates the expression of TGF-beta1 and its signaling molecules in mouse embryo mesenchymal progenitor cells.," *J. Surg. Res.*, vol. 150, no. 2, pp. 266–270, Dec. 2008.
[45] O. W. Gramlich, A. J. Burand, A. J. Brown, R. J. Deutsch, M. H. Kuehn, and J. A. Ankrum, "Cryopreserved Mesenchymal Stromal Cells Maintain Potency in a Retinal Ischemia/Reperfusion Injury Model: Toward an off-the-shelf Therapy," *Scientific Reports*, vol. 6. 2016.
[46] G. Minonzio, M. Corazza, L. Mariotta, M. Gola, M. Zanzi, E. Gandolfi, D. De Fazio, and G. Soldati, "Frozen adipose-derived mesenchymal stem cells maintain high capability to grow and differentiate," *Cryobiology*, vol. 69, no. 2, pp. 211–216, 2014.
[47] J. Galipeau, "Concerns arising from MSC retrieval from cryostorage and effect on immune suppressive function and pharmaceutical usage in clinical trials," *ISBT Sci. Ser.*, vol. 8, no. 1, pp. 100–101, 2013.
[48] X. Xu, Y. Liu, and Z. F. Cui, "Effects of cryopreservation on human mesenchymal stem cells attached to different substrates.," *J. Tissue Eng. Regen. Med.*, vol. 8, no. 8, pp. 664–672, Aug. 2014.
[49] I. Arnaoutova and H. K. Kleinman, "In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract.," *Nat. Protoc.*, vol. 5, no. 4, pp. 628–635, Apr. 2010.
[50] J. G. Rasmussen, O. Frobert, L. Pilgaard, J. Kastrup, U. Simonsen, V. Zachar, and T. Fink, "Prolonged hypoxic culture and trypsinization increase the pro-angiogenic potential of human adipose tissue-derived stem cells.," *Cytotherapy*, vol. 13, no. 3, pp. 318–328, Mar. 2011.
[51] P. R. Crisostomo, Y. Wang, T. A. Markel, M. Wang, T. Lahm, and D. R. Meldrum, "Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism.," *Am. J. Physiol. Cell Physiol.*, vol. 294, no. 3, pp. C675-82, Mar. 2008.
[52] L. B. Buravkova, E. R. Andreeva, V. Gogvadze, and B. Zhivotovsky, "Mesenchymal stem cells and hypoxia: where are we?," *Mitochondrion*, vol. 19 Pt A, pp. 105–112, Nov. 2014.
[53] N. Fillmore, A. Hugi, J. S. Jaswal, J. Mori, R. Paulin, A. Haromy, A. Onay-Besikci, L. Ionescu, B. Thébaud, E. Michelakis, and G. D. Lopaschuk, "Effect of Fatty Acids on Human Bone Marrow Mesenchymal Stem Cell Energy Metabolism and Survival," vol. 10, no. 3. 2015.

[54] I.-H. Park, K.-H. Kim, H.-K. Choi, J.-S. Shim, S.-Y. Whang, S. J. Hahn, O.-J. Kwon, and I.-H. Oh, "Constitutive stabilization of hypoxia-inducible factor alpha selectively promotes the self-renewal of mesenchymal progenitors and maintains mesenchymal stromal cells in an undifferentiated state.," *Exp. Mol. Med.*, vol. 45, p. e44, Sep. 2013.

[55] M. Deschepper, K. Oudina, B. David, V. Myrtil, C. Collet, M. Bensidhoum, D. Logeart-Avramoglou, and H. Petite, "Survival and function of mesenchymal stem cells (MSCs) depend on glucose to overcome exposure to long-term, severe and continuous hypoxia.," *J. Cell. Mol. Med.*, vol. 15, no. 7, pp. 1505–1514, Jul. 2011.

[56] J. Liu, H. Hao, H. Huang, C. Tong, D. Ti, L. Dong, D. Chen, Y. Zhao, H. Liu, W. Han, and X. Fu, "Hypoxia regulates the therapeutic potential of mesenchymal stem cells through enhanced autophagy.," *Int. J. Low. Extrem. Wounds*, vol. 14, no. 1, pp. 63–72, Mar. 2015.

[57] X. Hu, Y. Xu, Z. Zhong, Y. Wu, J. Zhao, Y. Wang, H. Cheng, M. Kong, F. Zhang, Q. Chen, J. Sun, Q. Li, J. Jin, Q. Li, L. Chen, C. Wang, H. Zhan, Y. Fan, Q. Yang, L. Yu, R. Wu, J. Liang, J. Zhu, Y. Wang, Y. Jin, Y. Lin, F. Yang, L. Jia, W. Zhu, J. Chen, H. Yu, J. Zhang, and J. Wang, "A Large-Scale Investigation of Hypoxia-Preconditioned Allogeneic Mesenchymal Stem Cells for Myocardial Repair in Nonhuman Primates: Paracrine Activity Without Remuscularization.," *Circ. Res.*, vol. 118, no. 6, pp. 970–983, Mar. 2016.

[58] X. Hu, S. P. Yu, J. L. Fraser, Z. Lu, M. E. Ogle, J.-A. Wang, and L. Wei, "Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis.," *J. Thorac. Cardiovasc. Surg.*, vol. 135, no. 4, pp. 799–808, Apr. 2008.

[59] N. J. Robinson, A. Picken, and K. Coopman, "Low temperature cell pausing: an alternative short-term preservation method for use in cell therapies including stem cell applications.," *Biotechnol. Lett.*, vol. 36, no. 2, pp. 201–209, Feb. 2014.

[60] M. A. Brown, C. S. Wallace, C. C. Anamelechi, E. Clermont, W. M. Reichert, and G. A. Truskey, "The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces.," *Biomaterials*, vol. 28, no. 27, pp. 3928–3935, Sep. 2007.

[61] E. Kopczyńska and R. Makarewicz, "Endoglin – a marker of vascular endothelial cell proliferation in cancer," *Contemporary Oncology*, vol. 16, no. 1. pp. 68–71, 2012.

[62] A. Boroujerdi, J. V Welser-Alves, U. Tigges, and R. Milner, "Chronic cerebral hypoxia promotes arteriogenic remodeling events that can be identified by reduced endoglin (CD105) expression and a switch in β1 integrins," *Journal of Cerebral Blood Flow & Metabolism*, vol. 32, no. 9. pp. 1820–1830, Sep-2012.

V. Scalable Expansion of Human Mesenchymal Stem/ Stromal Cells in the Single-Use, Vertical-Wheel™ Bioreactor System

This chapter is based on the article: Pinto, DS, Rodrigues, CAV, Jung, SH, Tseng, RJ, Shen, CY, Milligan, W, Lee, B, Lobato da Silva, C, Cabral, JMS. Scalable Expansion of Human Mesenchymal Stem/Stromal Cells in the Single-Use, Vertical-Wheel™ Bioreactor System Using a Human Platelet Lysate Culture Supplement. In preparation.

V.1 Summary

Microcarrier-based dynamic culture has proved to enhance the *in vitro* angiogenic potential of UCM MSC. To design an efficient bioprocess to achieve clinical-relevant number of preconditioned MSC, the desired protocol should also be able to promote MSC expansion. In this context, different bioprocessing parameters should be taken in consideration, including type of microcarriers, cell culture medium and bioreactor configuration and operation mode, when developing a new bioprocess strategy. This process should also be able to comply with GMP guidelines, in order to be approved by regulatory agencies. Serum- and xenogeneic-free materials, as well as disposable products, are prerequisites to reduce batch variability, xenogeneic contamination and risk of bacterial and fungal contamination, which contributes to obtain the approval of the regulatory agencies. Moreover, combining all these parameters will eventually result in a robust platform for the expansion and preconditioning of MSC, leading to high cell numbers and improved cell angiogenic properties.

In this chapter, different types of microcarriers and S/XF culture media were screened for the expansion of UCM MSC. After selecting the combinations of microcarriers and culture medium with the highest UCM MSC proliferation potential under static conditions, these combinations were tested under stirred culture conditions, using spinner flasks. Additionally, a different bioreactor configuration, the Vertical-Wheel™ PBS-0.1 MAG system, was tested and optimized for UCM MSC expansion. Due to the high efficiency achieved, other MSC sources were expanded in this innovative system, including BM and AT MSC. The impact of the PBS-0.1 MAG culture system on MSC immunophenotype and multilineage differentiation capacity was analyzed. Results show that glass microcarriers promoted the highest UCM MSC adhesion values, regardless of the culture medium. However, plastic microcarriers performed better for UCM MSC proliferation in both StemPro® and TheraPEAK™, while glass microcarriers achieved higher proliferation rates in MesenCult™. However, none of these combinations promoted UCM MSC expansion in spinner flasks. Therefore, a S/XF protocol based on fibrinogen-depleted human platelet lysate supplement (UltraGRO™-PURE) was used for further studies, since this protocol has already proved to successfully expand UCM MSC in spinner flasks. By adapting the initial agitation protocol and the feeding regime, cell expansion rate and final cell yield were maximized using an innovative disposable bioreactor system utilizing the Vertical-Wheel™ technology (PBS-0.1 MAG with maximum working volume of 100 mL). UCM MSC were successfully expanded to a maximum cell density of $5.3 \pm 0.4 \times 10^5$ cell/mL, after 7 days of culture (cell viability $\geq$ 94%). Similarly, AT MSC were also efficiently expanded to a maximum cell density of $3.6 \pm 0.7 \times 10^5$ cell/mL, also after 7 days of culture (cell viability $\geq$ 96%). UCM MSC maintained their identity (e.g. immunophenotype and multilineage differentiation potential), after culture in the PBS-0.1 MAG system.

The establishment of such platform featuring easy scalability to higher volumes represents an important advance in obtaining clinically meaningful MSC numbers with possible enhanced therapeutic features.

V.2 Background

Microcarrier-based dynamic culture has proved to enhance the *in vitro* angiogenic potential of UCM MSC, by upregulating the expression and secretion of proangiogenic factors. The enhanced secretion of these factors resulted in higher *in vitro* HUVEC tube-like structure formation capacity and HUVEC migration ability. These findings were reported for UCM MSC cultured in spinner flasks. However, several other bioreactor configurations are commercially available, mainly for the expansion of MSC, as previously discussed on chapter IV. Such bioreactor systems are suitable solutions to achieve clinical relevant MSC numbers. In order to fulfill the potential of MSC as therapeutic agents, large cell numbers and perhaps repeated doses are required, generally in the order of 10^6 to 10^7 cells per kilogram of body weight [1]–[4], depending on the type of disorder. Using bioreactor systems, high cell numbers and improved cell regenerative capacities can be obtained. To maximize MSC expansion and preconditioning, different bioprocessing parameters should be taken in consideration, including type of microcarriers, cell culture medium and bioreactor configuration and operation mode.

Microcarriers are small particles made of distinct core materials and presenting different densities, diameters, and surface charges. They are produced from a variety of materials, including polystyrene, glass, gelatin, dextran, alginate or cellulose, and present different physical properties, like stiffness and nanotopography. The majority of the commercially available microcarriers are spherical, but cylindrical (DE53, Whatman) and disc-shaped (Fibra-Cel®, Eppendorf) microcarriers are also available [5]. Depending on their porosity, microcarriers are divided into nonporous, microporous (pore sizes < 1 μm), and macroporous (pore sizes between 10 – 50 μm) types [6]. Moreover, microcarriers can be functionalized with peptides or ECM proteins, such as fibronectin, collagen, and laminin, to enhance MSC adhesion and proliferation [5]. AOF, synthetic proteins are preferable for microcarrier functionalization, to avoid contamination with xenogeneic agents and improve process reproducibility.

MSC from multiple sources have been successfully expanded using microcarrier-based systems, mainly using basal medium supplemented with FB/CS. Although serum-derived supplements still represent the most commonly used culture medium supplement for MSC expansion, the use of FB/CS in a clinical context carries several disadvantages, including being ill-defined, the wide batch-to-batch variability, and the risk of contamination (with virus and prions) and immunogenicity, which increases complexity of downstream processing and process standardization [7], [8]. Over the past years, humanized products

have been exploited as S/XF culture supplements for the isolation and expansion of MSC, like thrombin-activated PRP [9], autologous [10] and allogeneic [11] human serum, and pooled hPL [12]–[14]. Despite the positive effects reported for MSC isolation, expansion, and functionality using humanized supplements, extended used for large scale production is discouraged due to limited availability, risk of transmission of human diseases and ill-definition [15]. Replacement of serum- and xeno-derived medium by S/XF formulations is thus of utmost importance. Different groups have successfully promoted the isolation and expansion of MSC using S/XF medium formulations, without compromising cell identity [16]–[20]. Some commercially available SF/XF formulations used for MSC expansion include: StemPro® MSC SFM XenoFree (Life Technologies), MesenCult™-ACF (STEMCELL Technologies), MSC NutriStem® XF (Biological Industries) and PRIME-XV® MSC Expansion SFM (Irvine Scientific) [8]. The ideal candidate should be a chemically defined, serum-free, animal origin-free (AOF, including human) culture medium, which would improve the performance of MSC manufacturing systems both in terms of cell quality and reproducibility [7]. An example of a chemically-defined S/XF formulation is the TheraPEAK™ MSCGM-CD™ (Lonza). However, this formulation is not AOF, since it contains human albumin and pasteurized human transferrin [8].

The choice of bioreactor configuration and operation mode is also critical for the successful expansion of MSC. As previously discussed, several different bioreactor configurations have been tested, including stirred tank reactors [21]–[24], waved-mixed bioreactors [25], [26], fixed/packed bed bioreactors [27], [28], the Vertical-Wheel™ bioreactors [29], among others. The innovative Vertical-Wheel™ bioreactors consist of U-shaped vessels incorporating a vertically rotating wheel, resulting in faster and more efficient mixing at lower agitation rates [29]. The Vertical-Wheel™ bioreactors have proved to be efficient for the expansion of BM MSC, achieving a maximum cell concentration of 3×10^5 cells/mL [29]. Additionally, this type of bioreactor features easy scalability to industrial volumes, including 15 and 80 L bioreactors.

Combining all these bioprocessing parameters will eventually result in a robust, GMP-compliant platform for the expansion and preconditioning of MSC, leading to high cell numbers and improved cell angiogenic properties.

The aim of this chapter is to establish a scalable platform for the expansion of human UCM MSC, and potentially other MSC sources, without compromising cell identity. To do so, different types of microcarriers and S/XF formulations were screened for the expansion of UCM MSC. After selecting the combinations of microcarriers and culture medium with the highest UCM MSC proliferation potential under static conditions, these combinations were tested under stirred culture conditions, using spinner flasks. Additionally, UCM MSC were expanded in the Vertical-Wheel™ PBS-0.1 MAG system, by optimizing the culture conditions. The platform was also tested for the expansion of BM and AT MSC.

The impact of the PBS-0.1 MAG culture system on MSC immunophenotype and multilineage differentiation capacity was analyzed. The establishment of such platform featuring easy scalability to higher volumes would represents an important advance in obtaining clinically meaningful MSC numbers with possible enhanced therapeutic features.

V.3 Materials and Methods

V.3.1 Human Samples

BM MSC were isolated as described by dos Santos *et al* [30]. Using this protocol, BM MSC were isolated from the MNC fraction using two different cell culture media: DMEM supplemented with 10% FBS or DMEM supplemented with 5% hPL. BM aspirates were obtained from healthy donors after informed consent at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal (Laws nº 97/95, nº 46/2004). AT MSC were isolated and characterized as previously described in the literature [31]. AT aspirates were obtained from healthy donors, upon informed consent, at Clínica de Todos-os-Santos, Lisboa, Portugal. UCM MSC were isolated according to the protocol established by de Soure *et al* [14]. Umbilical cord units were obtained from healthy donors after informed maternal consent at Hospital São Francisco Xavier, Lisboa, Portugal (Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004). UCM MSC were cryopreserved in a liquid/vapor-phase nitrogen container.

V.3.2 Microcarrier and Culture Medium Screening for the Expansion of Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells under Serum- and Xenogeneic-Free Conditions

V.3.2.1 Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cell Expansion under Static Conditions

Upon thawing, UCM MSC were plated in T-175 flasks (BD Falcon™) at a cell density between 3000-6000 cell/cm^2 and cultured in StemPro® MSC SFM XenoFree (Life Technologies), MesenCult™-ACF (STEMCELL Technologies), or TheraPEAK™ MSCGM-CD™ (Lonza). Cells were kept at 37°C and 5% CO_2 in a humidified atmosphere and culture medium was changed every 3-4 days. At 70-80% cell confluence, MSC were detached from the flasks using the xeno-free cell detachment solution TrypLE™ Select CTS™ solution (1X, Life Technologies) for 7 min at 37°C. Cell number and viability were determined using Trypan Blue (Life Technologies) exclusion method. Before microcarrier/culture

medium screening or cultivation on spinner flasks, UCM MSC were plated at 3000 cell/cm^2 and culture medium was fully renewed every 3-4 days. At 70-80% cell confluence, cells were detached using TrypLE™ Select CTS™ (1X) and counted using Trypan Blue exclusion method. Two independent UCM MSC donors (n = 2) in passages from P3 to P4 were used to perform microcarrier/culture medium screening and spinner flask culture.

V.3.2.2 Microcarrier Screening for the Expansion of Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells

For microcarrier and culture medium screening, different commercially available microcarriers (plastic, plastic Plus, glass, hillex II (Pall SoloHill), pronectin-F and cytodex 3 (Sigma-Aldrich)) and S/XF formulations (StemPro® MSC SFM XenoFree (Life Technologies), MesenCult™-ACF (STEMCELL Technologies) and TheraPEAK™ MSCGM-CD™ (Lonza)) were tested for UCM MSC adhesion (day 1) and proliferation (day 4). 18 cm^2 of each of the microcarriers were prepared according to the manufacturer's instructions and then coated with the recommended coating solution (CELLstart solution (1:100 in PBS) for StemPro® MSC SFM XenoFree and TheraPEAK™ MSCGM-CD™, MesenCult™-SF Attachment Substrate for MesenCult™-ACF) for 1 h at 37°C, with intermittent agitation (2 min at 750 rpm, 10 min nonagitated) using a Thermomixer® comfort (Eppendorf AG). After resuspending in the appropriate culture medium, microcarriers were divided and plated in 6 wells of ultra-low attachment 24-well plates (Corning Inc), in a final area/well of 3 cm^2. 5 x 10^4 previously expanded UCM MSC were plated in each well. From the 6 well plated per culture medium, 2 wells were counted at day 1 of culture (to measure cell adhesion) and 2 wells were counted at day 4 of culture (to measure cell proliferation), using the Trypan Blue exclusion method, as described in [17]. The other 2 wells were stained with 4',6-diamidino-2-phenylindole (DAPI, 1.5 g/mL in PBS), as described in [14], at day 1 and 4 of culture. Two independent UCM MSC donors (n = 2) in passage P4 were used.

V.3.2.2 Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cell Expansion in Spinner Flasks

Bellco® spinner flasks (Bellco Glass, Inc) with a working volume of 40 mL, equipped with 90° paddles and a magnetic stir bar were used. For each spinner flask culture, 1 g of the microcarrier selected in the screening were prepared according to manufacturer's instructions and then coated with the recommended coating solution (CELLstart solution (1:100 in PBS) for StemPro® MSC SFM XenoFree and TheraPEAK™ MSCGM-CD™, MesenCult™-SF Attachment Substrate for MesenCult™-ACF) for

1 h at 37°C, with intermittent agitation (2 min at 750 rpm, 10 min nonagitated) using a Thermomixer® comfort (Eppendorf AG). Inoculation was performed in the 40 mL volume, with an initial cell density of 1 x 10^5 cell/mL. MSC previously expanded under static conditions were added to the microcarrier suspension inside the spinner flask. For the first 24 h, a continuous agitation regimen was set to 30 rpm. At day 1 of culture, the number of cells attached to the microcarriers was determined by Trypan Blue exclusion method, as described in [17], to calculate initial cell adhesion. From day 1 onwards, agitation was set to 40 rpm. Cell counting was performed every day, using the Trypan Blue exclusion method. Cell visualization on microcarriers was performed from day 1 onwards, by staining the cells with 4',6-diamidino-2-phenylindole (DAPI, 1.5 g/mL in PBS), as described in [14].

V.3.3 Mesenchymal Stem/Stromal Cell Expansion in the PBS-0.1 MAG System

V.3.3.1 Mesenchymal Stem/Stromal Cell Expansion under Static Conditions

Upon thawing, MSC from the 3 different sources (BM, AT and UCM) were plated at a cell density between 3000-6000 cell/cm^2 and cultured in low-glucose DMEM (Life Technologies) supplemented with 5% (v/v) UltraGro™-PURE (AventaCell Biomedical) and 1% antibiotic-antimycotic (100X, Gibco™), plated in T-175 flasks (BD Falcon™). Cells were kept at 37°C and 5% CO_2 in a humidified atmosphere and culture medium was changed every 3-4 days. At 70-80% cell confluence, MSC were detached from the flasks using the XF cell detachment solution TrypLE™ Select CTS™ solution (1X, Life Technologies) for 7 min at 37°C. Cell number and viability were determined using Trypan Blue (Life Technologies) exclusion method. Before inoculation in the PBS-0.1 MAG, MSC were plated at 3000 cell/cm^2 and culture medium was fully renewed every 3-4 days. At 70-80% cell confluence, cells were detached using TrypLE™ Select CTS™ (1X) and counted using Trypan Blue exclusion method. For each cell source, MSC from three independent donors (n = 3) in passages from P3 to P4 were used to inoculate the PBS-0.1 MAG system.

V.3.3.2 Mesenchymal Stem/Stromal Cell Expansion under Dynamic Conditions

For the dynamic culture of MSC, PBS-0.1 MAG (PBS Biotech, Inc) with a working volume of 100 mL and equipped with a Vertical-Wheel™ were used. 2 g of plastic microcarriers (Pall SoloHill) per culture were prepared according to manufacturer's instructions and then incubated with DMEM + 50% (v/v) UltraGRO™-PURE for 1 h at 37°C under intermittent agitation (2 min at 750 rpm, 10 min nonagitated), using a Thermomixer comfort (Eppendorf AG). Inoculation in the PBS-0.1 MAG was performed in 60

mL of DMEM + 5% (v/v) UltraGRO™-PURE, with an initial MSC density of 0.83 x 10^5 cell/mL. MSC previously expanded in static conditions were added to the microcarrier suspension inside the PBS-0.1 MAG. During the first 6 h of culture, agitation cycles of 1 min agitating at 25 rpm followed by 20 min with no agitation were used, as reported in [29]. After 6 h, a continuous agitation mode was adopted. At day 1 of culture, the number of cells attached to the microcarriers was determined by Trypan Blue exclusion method, as described in [17], to calculate initial cell adhesion. At day 2 of culture, 40 mL of fresh culture medium with a glucose pulse to a final glucose concentration of 3 g/L were added to the PBS-0.1 MAG (final working volume of 100 mL). For AT MSC culture, agitation was set to 30 rpm, to overcome an increased medium viscosity and excessive cell aggregation. From day 2 onwards, exchange of 25% (v/v) of culture medium was performed every 12 h. Addition of fresh culture medium supplemented with glucose to a final concentration of 3 g/L was performed interspersed with fresh medium with no glucose supplementation. Agitation rate was increased to 35 rpm on AT MSC culture when cell aggregates started to from. Cell growth and viability and nutrient/metabolite analysis were assessed every day, as described in [14]. Cell visualization on microcarriers was performed from day 1 onwards, by staining the cells with DAPI (1.5 g/mL in PBS), as described in [14].

V.3.3.3 Harvesting and Characterization of Expanded Mesenchymal Stem/Stromal Cells

At the end of expansion, UCM and AT MSC were detached from the microcarriers in the PBS-0.1 MAG by transferring the culture to 50 mL tubes (BD Falcon™), washing with PBS (1X), and incubating with TrypLE™ Select CTS™ (1X) for 15 min at 37°C and 750 rpm, in a Thermomixer comfort. Cell suspension was then filtered through a 100 µm cell strainer (BD Biosciences).

Cells before and after PBS-0.1 MAG culture were characterized in terms of immunophenotypic profile and early mesodermal differentiation and proangiogenic gene expression. Multilineage differentiation potential was evaluated after culture on the PBS-0.1 MAG system.

V.3.3.3.1 Immunophenotypic Profile

The immunophenotypic profile of MSC were analyzed by FC (FACSCalibur equipment, Becton Dickinson), using a panel of mouse anti-human monoclonal antibodies (PE-conjugated, all from BD Biolegend) against: CD73, CD90, CD105, CD34, CD45, CD14, CD19, CD80, and HLA-DR. Cells were incubated with these monoclonal antibodies for 20 min in the dark at room temperature and then cells were washed with PBS (1X) and fixed with 2% PFA (Sigma). Isotype controls were also prepared for

every experiment. A minimum of 5000 events was collected for each sample and the CellQuest software (Becton Dickinson) was used for acquisition and analysis.

V.3.3.3.2 Multilineage Differentiation Potential

The multilineage differentiation potential of both UCM and AT MSC harvested at the end of the PBS-0.1 MAG system culture was assessed using the StemPro® Osteogenesis/Adipogenesis/Chondrogenesis Differentiation Kits (Life Technologies), as described in [17].

V.3.3.3.3 Quantitative Real-Time-Polymerase Chain Reaction

Cells were centrifuged, lysed and total RNA was isolated using the RNeasy® Mini Kit (Qiagen) according to manufacturer's instruction. RNA was then quantified by UV spectrophotometry (NanoDrop Technologies) at 260 nm. Complementary DNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) with blended oligo (dT), random primers and 500 ng of RNA. For RT-PCR, a two-step PCR run was performed in StepOne Real-Time PCR System (Applied Biosystems™), using Fast SYBR™ Green Master Mix (Applied Biosystems™), 0.5 µM of each primer, and 1 µL of cDNA in 20 µL of final volume. The primers used to quantify angiogenesis (VEGF-A, bFGF and HGF) are presented on Table V.1.

Table V.1 List of PCR primers used to study early mesodermal differentiation and proangiogenic gene expression.

Gene	Primer Sequence	Amplicon size (bp)
Vascular endothelial growth factor-A (VEGF-A)	Fwd: 5'-CGA GGG CCT GGA GTG TGT-3' Rev: 5'-CGC ATA ATC TGC ATG GTG ATG-3'	57
Basic fibroblast growth factor (bFGF)	Fwd: 5'-TGG TAT GTG GCA CTG AAA CGA-3' Rev: 5'-GCC CAG GTC CTG TTT TGG AT	61
Hepatocyte growth factor (HGF)	Fwd: 5'-TCC ACG GAA GAG GAG ATG AGA-3' Rev: 5'-GGC CAT ATA CCA GCT GGG AAA-3'	63

Expression was normalized to the metabolic housekeeping gene GAPDH. Control assays containing no cDNA templates were also performed.

V.3.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Results are presented as mean + SEM of the values obtained for the different MSC donors. Two-way non-parametric ANOVA was calculated between different experimental groups. One-way ANOVA was performed when comparing unmatched groups. Both analyses were followed by Bonferroni test to determine statistically significant differences (*P*-values < 0.05).

V.4 Results

V.4.1 Microcarrier and Culture Medium Screening for the Expansion of Human Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cells

Dynamic culture systems have been exploited in the context of MSC manufacturing to overcome some of the limitations generated by planar systems, including limited surface area-to-volume ratio, lack of ability to monitor and control culture parameters, cost-effectiveness and handling. To establish an efficient dynamic culture platform for the expansion of MSC, the choice of the best combination of microcarrier type and culture media must be performed. Here, different types of microcarriers and culture media were screened for the adhesion and proliferation of UCM MSC (Figure V.1).

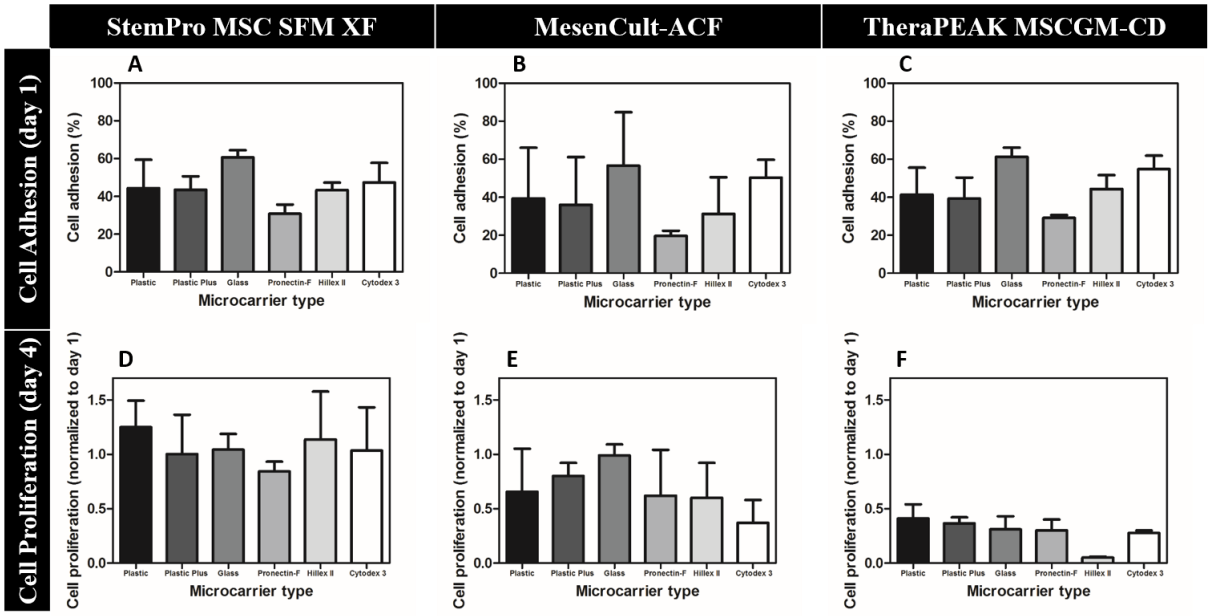

Figure V.1 Microcarrier and culture medium screening for the expansion of umbilical cord matrix (UCM)-derived mesenchymal stem/stromal cells (MSC). Different types of microcarriers (plastic, plastic plus, glass, pronectin-F, hillex II and cytodex 3) and culture media (StemPro® MSC SFM XenoFree, MesenCult™-ACF and TheraPEAK™ MSCGM-CD™) were tested for the expansion of UCM MSC under static conditions. Microcarriers were coated with the recommended coating

solution (CELLstart solution (1:100 in PBS) for StemPro® MSC SFM XenoFree and TheraPEAK™ MSCGM-CD™, MesenCult™-SF Attachment Substrate for MesenCult™-ACF). 5 x 10^4 cells were plated on 3 cm^2 of microcarriers, in ultra-low attachment 24-well plates. Cell counting was performed at day 1 (to calculate cell adhesion) and day 4 (to calculate cell proliferation), using the Trypan Blue exclusion method. Results are presented as mean + standard error of mean (n = 2).

Results show that glass microcarriers promoted the highest UCM MSC adhesion values, regardless of the culture medium (Figure V.1.A-C). In fact, cell adhesion on these microcarriers was around 60% in all the culture media tested (StemPro® – 60 ± 5%, MesenCult™ – 58 ± 26%, TheraPEAK™ - 62 ± 7%). Conversely, pronectin-F microcarriers had the worst performance in terms of UCM MSC adhesion (StemPro® – 33 ± 5%, MesenCult™ – 19 ± 2 %, TheraPEAK™ - 29 ± 1%).

This trend was not observed for UCM MSC proliferation (Figure V.1.D-F). Instead, different types of microcarriers performed better (and worse) for cell proliferation, depending on the culture media. While plastic microcarriers promoted the highest cell proliferation in both StemPro® (1.23 ± 0.25) and TheraPEAK™ (0.42 ± 0.10), glass microcarriers provided the highest proliferation when UCM MSC were cultured in MesenCult™ (0.98 ± 0.12). Pronectin-F microcarriers only performed worst cultured in StemPro® (0.81 ± 0.11). When UCM MSC were cultured in Mesencult™ and TheraPEAK™, the microcarriers promoting the lowest cell proliferation rate were the cytodex 3 (0.37 ± 0.17) and hillex II (0.09 ± 0.01), respectively.

V.4.2 Umbilical Cord Matrix-Derived Mesenchymal Stem/Stromal Cell Expansion in Spinner Flasks

After microcarrier and culture medium screening under static conditions, the best combinations were tested under dynamic culture conditions, using spinner flasks. Specifically, plastic microcarriers coated with CELLstart were selected when UCM MSC were cultured in StemPro® and TheraPEAK™, whereas glass microcarriers coated with MesenCult™-SF Attachment Substrate were chosen for the expansion of UCM MSC in MesenCult™. The results are represented in Figure V.2.

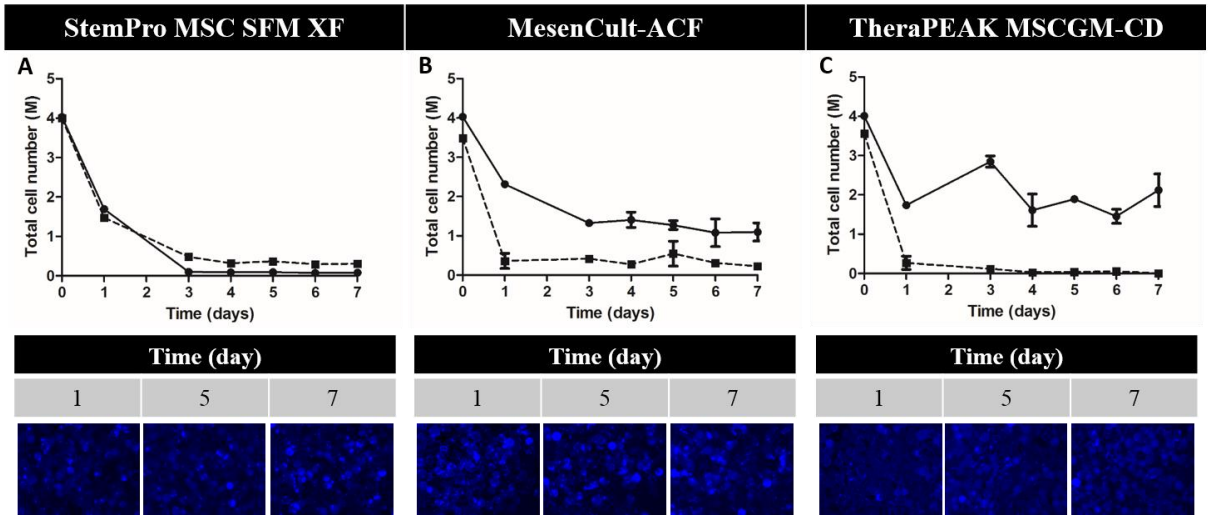

Figure V.2 *Ex-vivo* expansion of umbilical cord matrix (UCM)-derived mesenchymal stem/stromal cells (MSC) in a xeno-free dynamic culture system. Passage 4 UCM MSC were seeded on different combinations of microcarriers and culture media and cultured 7 days, in spinner flasks. Combinations of (A) plastic microcarriers coated with CELLstart (1:100) in StemPro® MSC SFM XenoFree, (B) glass microcarriers coated with MesenCult™-SF Attachment Substrate in MesenCult™-ACF, (C) plastic microcarriers coated with CELLstart (1:100) in TheraPEAK™ MSCGM-CD™, were tested for two different UCM MSC donors (donor 1 – discontinuous line; donor 2 – continuous line). Cell nuclei were stained with 4,6-diamino-2-phenylindole (DAPI) at day 1, 5 and 7 of culture and analyzed using a fluorescent microscope. Results are presented as mean ± standard deviation.

Analysis of Figure V.2 indicates that none of the conditions selected promoted UCM MSC expansion under dynamic culture conditions, for the 7 days of culture. In fact, none of the two donors tested expanded under the culture conditions studied, which is observed by the low cell populating rate of the microcarriers, through DAPI staining.

V.4.3 Mesenchymal Stem/Stromal Cell Expansion in the PBS-0.1 MAG System

Since none of the conditions studied promoted UCM MSC expansion, a successfully established protocol based on culture medium supplemented with hPL [14] was used for further studies. This protocol was established for the expansion of UCM MSC in spinner flasks. In this chapter, an innovative approach developed by PBS Biotech, Inc, the Vertical-Wheel™ PBS system, was tested for the expansion of UCM MSC, under S/XF culture conditions. Due to the high efficiency achieved, other MSC sources were expanded in this system, including AT MSC (Figure V.3).

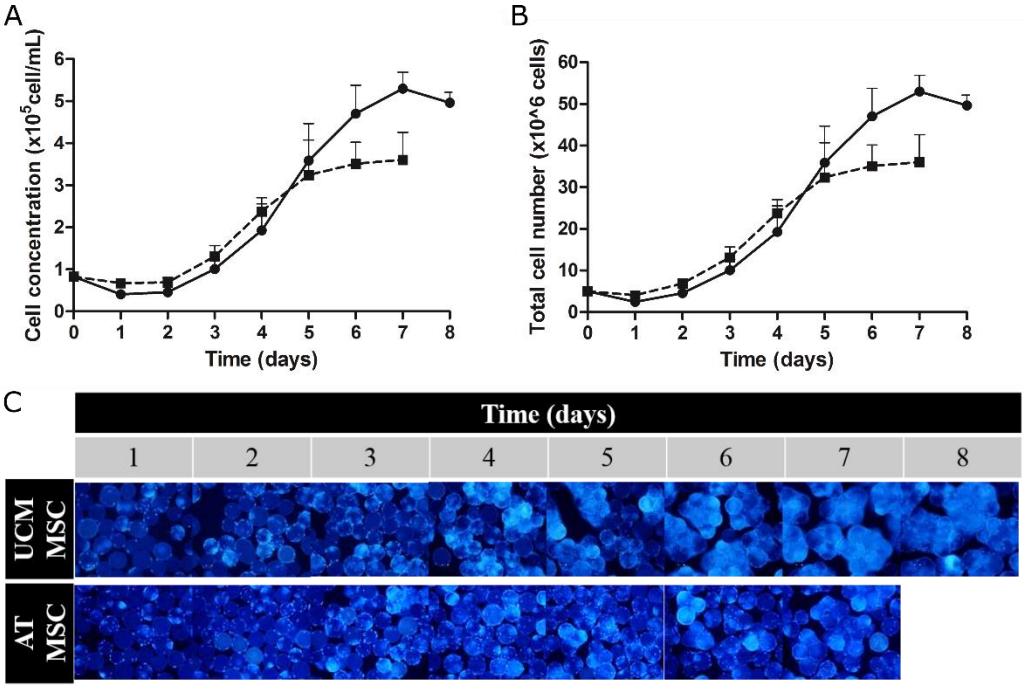

Figure V.3 *Ex-vivo* expansion of umbilical cord matrix (UCM)- and adipose tissue (AT)-derived mesenchymal stem/stromal cells (MSC) in a xeno-free dynamic culture system. Passage 4 UCM and AT MSC were seeded on plastic microcarriers pre-coated with 50% UltraGRO™-PURE and cultured in 5% UltraGRO™-PURE for 8 and 7 days, respectively, in a PBS-0.1 MAG system. (A) UCM (continuous line) and AT (discontinuous line) MSC concentrations (x 10^5 cell/mL) throughout the 7 (UCM MSC) or 8 (AT MSC) days of culture. (B) Total UCM (continuous line) and AT (discontinuous line) MSC number (x 10^6 cells) throughout the 7 (UCM MSC) or 8 (AT MSC) days of culture. (C) Cell nuclei were stained with 4,6-diamino-2-phenylindole (DAPI) every day of the cultures and analyzed using a fluorescent microscope. Results are presented as mean + standard error of mean (n = 3).

By adapting the initial agitation protocol and the feeding regime, both UCM and AT MSC were successfully expanded in the PBS-0.1 MAG VW system, using hPL-supplemented culture medium (Figure V.3.A-B). Although cell adhesion has been higher for AT MSC (81 ± 4%) than for UCM MSC (49 ± 4%), the latest achieved a higher maximum cell concentration (5.3 ± 0.4 x 10^5 cell/mL), compared to AT MSC (3.6 ± 0.7 x 10^5 cell/mL). These cell densities correspond to maximum fold increase values in total cell number (i.e. normalized to the number of cells that successfully adhered to the microcarriers upon 24 h) of 21 ± 1 and 9 ± 1 for UCM and AT MSC, respectively. Cell viability was higher than 94 ± 3% and 96 ± 2% for UCM and AT MSC, respectively, throughout the culture time (data not shown). Maximum cell density was achieved at day 7 of culture for both MSC sources.

The PBS-0.1 MAG system was also used for the expansion of BM MSC. The results obtained are shown in Figure V.4.

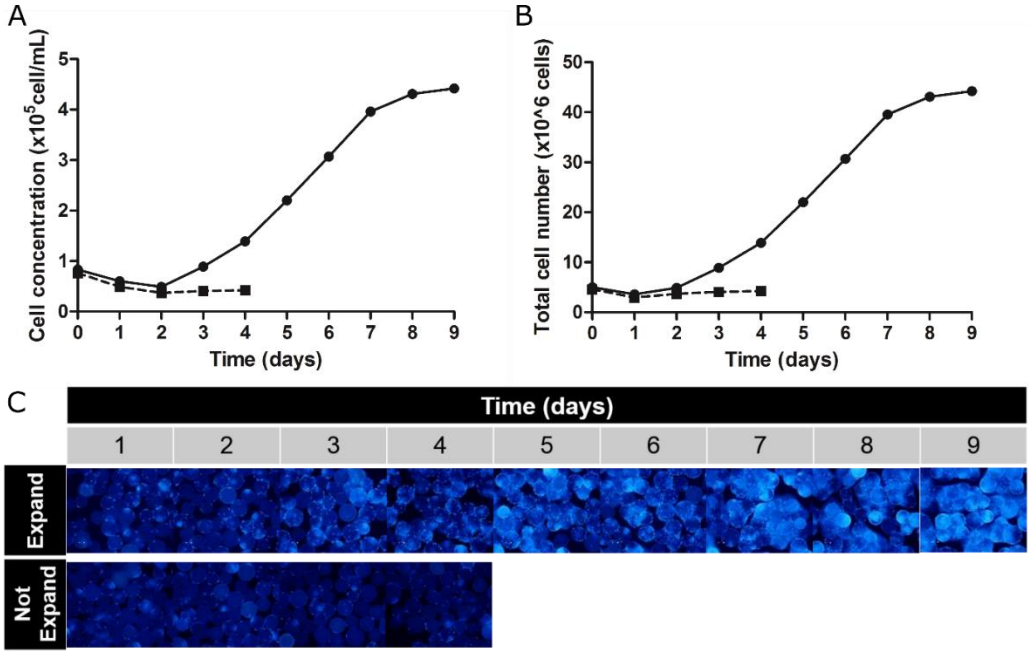

Figure V.4 *Ex-vivo* expansion of bone (BM)-derived mesenchymal stem/stromal cells (MSC) in a xeno-free dynamic culture system. Passage 4 BM MSC were seeded on plastic microcarriers pre-coated with 50% UltraGRO™-PURE and cultured in 5% UltraGRO™-PURE for 9 days, in a PBS-0.1 MAG system. (A) Cell concentrations (x 10^5 cell/mL) of one proliferative (continuous line) and four nonproliferative (discontinuous line) BM MSC donors throughout the 9 days of culture. (B) Total cell numbers (x 10^6 cells) achieved for one proliferative (continuous line) and four nonproliferative (discontinuous line) BM MSC donors throughout the 9 days of culture. (C) Cell nuclei were stained with 4,6-diamino-2-phenylindole (DAPI) every day of the cultures and analyzed using a fluorescent microscope. Results are presented as mean + standard error of mean (n = 4), for the nonproliferative BM MSC donors.

Only one BM MSC donor was successfully expanded in the PBS-0.1 MAG system. Four other donors were tested, but none of them expanded using the dynamic culture platform established (Figure V.4.A-B). Although cell adhesion was similar between the two groups of donors (proliferative – 72% *vs* nonproliferative – 64 ± 7%), the proliferative BM MSC donor achieved a maximum cell density of 4.42 x 10^5 cell/mL, while the maximum cell density for the nonproliferative donors was 0.49 ± 0.07 x 10^5 cell/mL. Such cell densities correspond to maximum fold increase values of 12 and 0.9 ± 0.1 for proliferative and nonproliferative BM MSC, respectively. Cell viability was higher than 93% and 93 ± 5% for proliferative and nonproliferative BM MSC, respectively, throughout the culture time (data not shown). Maximum cell density of the proliferative donor was achieved at day 9 of culture.

V.4.4 Immunophenotype and Multilineage Differentiation Potential of Mesenchymal Stem/Stromal Cells Cultured in the PBS-0.1 MAG System

In order to establish a platform compliant with GMP guidelines for the expansion of MSC, obtaining high cell yields cannot be the only metric used to evaluate the performance of a bioreactor culture system. In fact, the culture system must not compromise the identity of the cells. To evaluate the impact of the PBS-0.1 MAG culture system on MSC identity, the immunophenotypic profile and the multilineage differentiation potential was analyzed after the culture (Figure V.5 and V.6).

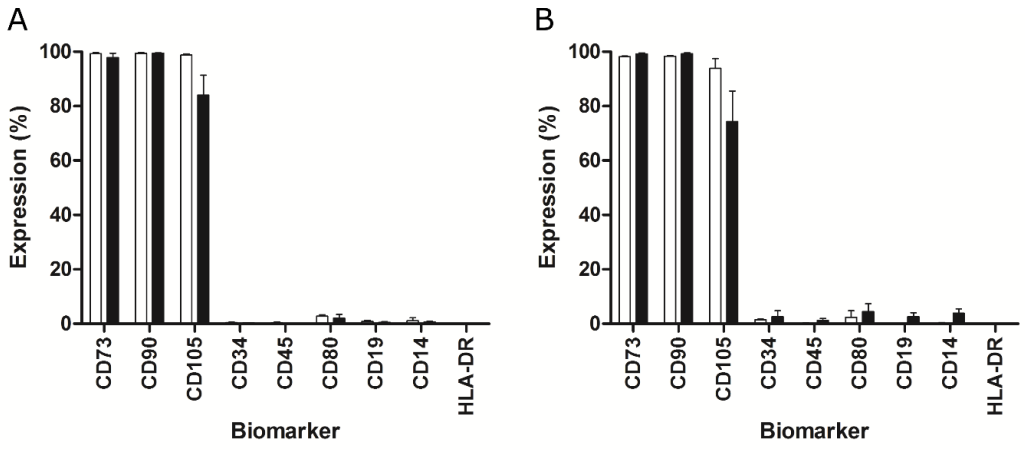

Figure V.5 Immunophenotypic characterization of umbilical cord matrix (UCM)- and adipose tissue (AT)-derived mesenchymal stem/stromal cells (MSC), before and after culture in a xeno-free dynamic culture system. (A) Immunophenotypic characterization of UCM MSC before (white) and after (black) dynamic culture, analyzed by flow cytometry. (B) Immunophenotypic characterization of AT MSC before (white) and after (black) dynamic culture, analyzed by flow cytometry. Results are presented as mean + standard error of mean (n = 2).

MSC from both UCM and AT sources maintain the characteristic MSC immunophenotype after culture in the PBS-0.1 MAG system (Figure V.5.A and V.5.B, respectively). Both CD73 and CD90 biomarkers are expressed in ≥ 95% of the cells. However, CD105 expression is not only heterogeneous among sources (98.8 ± 0.4% in UCM MSC and 93.9 ± 3.5% in AT MSC), but also decreases after culture in the PBS-0.1 MAG system (84.0 ± 7.3% in UCM MSC and 74.3 ± 11.2% in AT MSC).

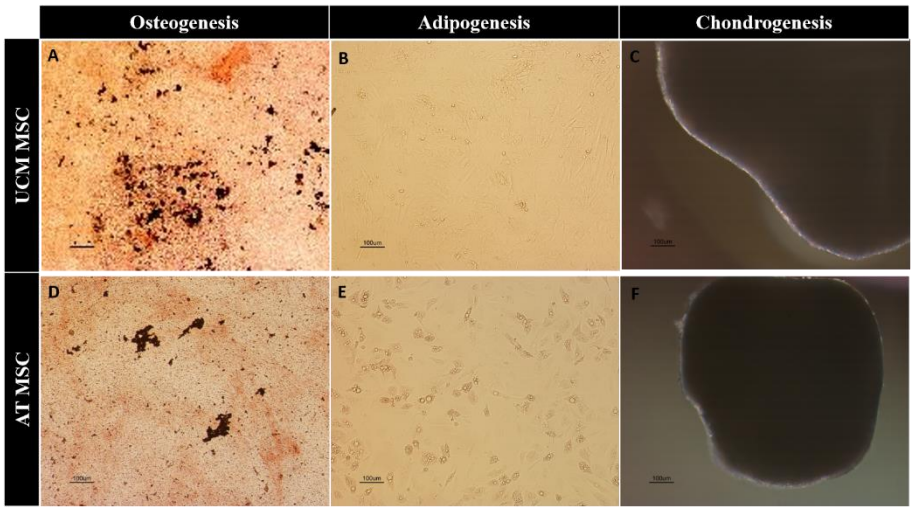

Figure V.6 Multilineage differentiation potential of umbilical cord matrix (UCM)- and adipose tissue (AT)-derived mesenchymal stem/stromal cells (MSC), after culture in a xeno-free dynamic culture system. After dynamic culture, passage 4 UCM and AT MSC were plated and cell differentiation was induced for 14 days and assessed by staining for osteogenesis (ALP and von Kossa, (A) – UCM, (D) – AT), adipogenesis (Oil Red-O, (B) – UCM, (E) – AT) and chondrogenesis (Alcian Blue, (C) – UCM, (F) – AT).

In addition to maintaining the characteristic MSC immunophenotype, cells from both sources maintain their multilineage differentiation ability towards osteocytes, adipocytes and chondrocytes, after culture in the PBS-0.1 MAG system. However, Oil Red-O was not able to stain MSC differentiated into adipocytes (Figure V.6-B and E), despite the existence of lipid vacuoles.

V.4.5 Impact of Mesenchymal Stem/Stromal Cells Cultured in the PBS-0.1 MAG System on the Angiogenic Gene Expression

As studied on Chapter IV, dynamic culture may induce changes in the expression of proangiogenic genes on MSC. After the end of the culture in the PBS-0.1 MAG system, MSC from both sources were collected and the expression of the proangiogenic genes VEGF, bFGF and HGF was analyzed. The results are shown in Figure V.7.

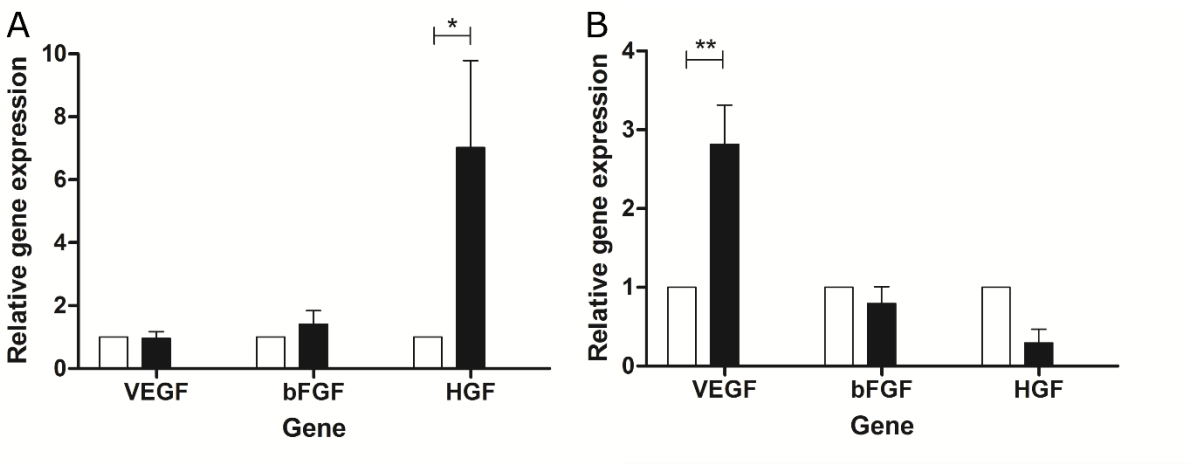

Figure V.*7* Proangiogenic gene expression of umbilical cord matrix (UCM)- and adipose tissue (AT)-derived mesenchymal stem/stromal cells (MSC), after culture in a xeno-free dynamic culture system. Relative gene expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) of human (A) umbilical cord matrix (UCM)- and (B) adipose tissue (AT)-derived mesenchymal stem/stromal cells (MSC), after culture in the PBS-0.1 MAG system, using Dulbecco's modified Eagle medium (DMEM) supplemented with 5% human platelet lysate (hPL). Gene expression is normalized to static conditions and values are presented as mean + standard error of mean (SEM, n=2, *P<0.01; **P<0.05).

Gene expression analyzes indicates a significant upregulation of HGF in UCM MSC (7.02 ± 2.76) and of VEGF in AT MSC (2.82 ± 0.50). Results also show an increased expression of bFGF in UCM MSC (1.41 ± 0.42) and a decreased expression of HGF in AT MSC (0.30 ± 0.17), although both not statistically significant.

V.5 Discussion

In this work, a S/XF, scalable culture platform was established for the expansion of different sources of MSC. Both AT and UCM MSC were successfully expanded to clinical-relevant numbers using a humanized cell culture supplement and an innovative dynamic culture system, which features easily scalability, handling and monitoring. Moreover, MSC maintained their immunophenotype and multilineage differentiation ability. All these achievements are prerequisites for GMP compliance and approval by the regulatory agencies.

Results from previous chapters demonstrated that UCM MSC are a promising cell source for cellular therapies, due to their *in vitro* regenerative capacities. In addition, such features can be improved by culturing the cells and dynamic culture conditions and low oxygen concentrations. These reasons justify the necessity of establishing an efficient culture platform for the expansion of UCM MSC. Moreover, other MSC sources, such as BM and AT MSC, have already been expanded to clinical-relevant numbers,

under S/XF culture conditions [21], [22]. The first step to establish a microcarrier-based culture platform is the rationale selection of the types of microcarriers and culture media. Different types of microcarriers are commercially available in the market, being produced from a variety of materials, including polystyrene, glass, gelatin, dextran, alginate or cellulose, and presenting different physical properties, like stiffness and nanotopography. Moreover, microcarriers can be functionalized with peptides or ECM proteins, such as fibronectin, collagen, and laminin, to promote MSC adhesion and proliferation [5]. In this context, S/XF microcarriers were selected for this study. Collagen-coated plastic, FACTIII and CultiSpher microcarriers were not included in the study since all of them have porcine derivatives on their constitution. In fact, some preliminary screening studies performed with CultiSpher-S microcarriers evidenced their great ability to expand UCM MSC (data not shown). Under dynamic culture conditions, these cells have already been efficiently expanded using Cultispher-S microcarriers in stirred tank bioreactors [32]. Other microcarriers were excluded from the screening, not because of the animal origin of some of their components, but due to difficulties on cell harvesting. Again, preliminary screening showed inefficient cell harvesting on some types of microcarriers, including MicroHex and cytodex 1 microcarriers, even after using high concentrations of the dissociation reagent. Similarly, different types of S/XF formulations were tested for UCM MSC, including a chemically-defined, the TheraPEAK™ MSCGM-CD™, and an animal component-free, the MesenCult™-ACF, formulations. StemPro® MSC SFM was also selected for the screening study since it has already proved to promote UCM MSC expansion in tissue culture flasks [18].

Distinct types of microcarriers/culture media combinations differently promoted UCM MSC adhesion and proliferation. One possible reason to justify these differences is the selective nonspecific adhesion of ECM proteins from the coating solution or culture medium to the microcarriers. It is known that the properties of the microcarrier surface define which ECM proteins will bind to it. Positively charged microcarriers have demonstrated to preferentially bind laminin and collagen IV, whereas gelatin-based or coated microcarriers will specifically bind fibronectin [33]. Glass microcarriers promoted the highest UCM MSC adhesion values, regardless of the culture medium. Glass microcarriers, which are made of silicate glass or silica, acquire a negative surface charge when placed in an aqueous environment [34]. This negative charge will mainly bind fibronectin, which is the major component of both CELLstart™ and MesenCult™-SF Attachment Substrate. Therefore, glass microcarriers might display a higher concentration of coating solution adhered to their surface, compared to the other microcarriers, resulting in higher UCM MSC adhesion values. However, this trend is not observed at the cell proliferation level. In fact, none of the combinations of microcarrier and culture medium promoted the efficient expansion of UCM MSC during the 4 days of culture, under static conditions. The highest expansion rate was observed for cells plated in plastic microcarriers and cultured in StemPro® MSC SF, although far from the values obtained for UCM MSC cultured in tissue culture flasks [18].

Despite the results, the microcarrier type promoting the highest expansion for UCM MSC cultured in each type of culture medium was tested under dynamic culture conditions, using spinner flasks. Unfortunately, none of the conditions assessed promoted the efficient expansion of UCM MSC in spinner flasks. In order to overcome a potential limitation of the coating solution in use, plastic microcarriers were coated with different types of ECM proteins, including fibronectin, laminin and vitronectin, or mixtures of them (data not shown). Coating plastic microcarriers with ECM proteins did not improve cell adhesion or proliferation. In fact, an improvement in cell proliferation was only observed when microcarriers were coated with 50% hPL, a rich source of ECM proteins. These results indicate that the recommended coating solutions are limitative of the proliferative capacity of UCM MSC cultured in plastic microcarriers.

The use of hPL for the efficient expansion of UCM MSC, as culture medium supplement and as coating solution, has already been reported [14], [35], using spinner flasks. In the current work, a different type of culture platform was tested, the Vertical-Wheel™ PBS-0.1 MAG system, with a 100 mL-working volume. The innovative Vertical-Wheel™ systems consist of U-shaped vessels incorporating a vertically rotating wheel, resulting in faster and more efficient mixing at lower agitation rates [29]. Additionally, these types of bioreactors feature easy scalability to industrial volumes, including 15 and 80 L bioreactors.

The first step on the establishment of a scalable culture platform using the PBS-0.1 MAG system was the adaptation of the protocol defined by de Soure et al [14]. The same culture conditions were translated to the VW-PBS system, including the initial cell density, microcarrier type and concentration, coating solution, agitation protocol during cell adhesion and feeding regime. In the end of this experiment, a similar maximum cell density was achieved (approximately 3 x 10^5 cell/mL, data not shown), which proves that the VW-PBS system is not detrimental for the proliferative capacity of the UCM MSC. However, the lag phase of cell growth was considerably long. In order to reduce it, an intermittent agitation protocol during the initial phase of cell adhesion (6 h) was used, as reported by Sousa and co-workers [29]. Such approach allowed the achievement of higher cell adhesion values (49 ± 2% vs 27% under continuous agitation), resulting in similar maximum cell densities in a shorter period of culture time. Additionally, the concentration of microcarriers was doubled, meaning more surface area available for cells to grow. However, metabolite analysis revealed glucose depletion and lactate concentration when the concentration of microcarriers was set to 20 g/L. Therefore, a different feeding regime was adopted, with 25% medium change every 12h and a pulse of glucose in one of the changes. Culture medium exchange with glucose supplementation aimed at avoiding glucose depletion, while maintaining lactate concentration under minimum inhibitory levels. Using this updated protocol, UCM MSC were successfully expanded to a maximum cell density of more than half a million cells per mL. These results are comparable to, or even superior to, those described in the literature for this MSC source

[14], [35]–[38]. Moreover, cells remained viable throughout the culture time and maintained their identity after culture in the PBS-0.1 MAG system. The expression of the CD105 biomarker was affected by the dynamic culture, which is in line with the results obtained in Chapter IV and may be the result of a prolonged harvesting procedure [39].

Due to the high expansion efficiency of this culture platform, other MSC sources were also cultured in the PBS-0.1 MAG system, including AT and BM MSC. Like UCM MSC, AT cells were successfully expanded using the protocol established, until a maximum cell density of $3.6 \pm 0.7 \times 10^5$ cells/mL, after 7 days of culture. These cell numbers are also comparable to, or superior to, those reported by other groups for AT MSC [16], [40]–[42]. Moreover, AT MSC remain viable throughout the culturing time and maintained their immunophenotypic profile, despite the reduced CD105 expression. However, this platform did not work so well for BM MSC. In fact, only one BM MSC donor was able to expand in the PBS-0.1 MAG system, while four other donors did not proliferate. Interestingly, the expanded donor was the only donor being isolated in DMEM + 5% UltraGRO culture medium. All the remaining donors were isolated in DMEM + 10% FBS. These findings show the importance of the isolation step on the efficient expansion of MSC.

The impact of the VWB culture was evaluated on the MSC angiogenic gene expression profile. As demonstrated in Chapter IV, dynamic culture conditions induce an upregulation of proangiogenic genes, as well as in the *in vitro* angiogenic properties of the cells. In this chapter, culture in the PBS-0.1 MAG system resulted in an upregulation of HGF in UCM MSC and VEGF in AT MSC. Such increase in the expression of proangiogenic, and prosurvival, genes is probably related to the stress conditions faced by the cells when cultured under dynamic conditions, as previously discussed. Again, assuming that the shear stress is lower in the VWB system, the improvement of the proangiogenic gene profile of UCM MSC is not as evident as when cells are cultured in spinner flasks. If this hypothesis is true, culturing MSC in spinner flasks may be beneficial rather than culturing them in the VWB system. However, to assess the angiogenic properties of MSC cultured in the VWB system, *in vitro* functional studies should be performed, including the HUVEC tube-like structure and migration assays.

Despite the positive results obtained using hPL as cell culture supplement, it is important to notice that this solution raises some concerns in terms of GMP compliance and process robustness. Despite the positive effects reported for MSC isolation and expansion using humanized supplements, extended used for large scale production is discouraged due to limited availability, risk of transmission of human diseases and ill-definition [15]. In fact, hPL is not chemically defined and presents a high batch-to-batch variability, like FB/CS. Moreover, MSC expanded in hPL-supplemented medium displayed a decreased immunomodulatory activity [43].

More data is required to identify the effects of hPL and the VWB system on MSC biological properties. Particularly, more studies must be performed regarding the early mesodermal differentiation ability, *in vitro* angiogenic potential and immunomodulatory capacity of MSC cultured in these experimental conditions. Even so, the establishment of such platform featuring easy scalability to higher volumes (i.e. PBS-3, PBS-15 and PBS-80, with maximum working volumes of 3L, 15L and 80L, respectively) represents an important advance in obtaining safer and clinically meaningful MSC numbers for clinical translation in a controlled and closed system.

V.6 References of Chapter V

[1] C. van den Bos, R. Keefe, C. Schirmaier, and M. McCaman, "Therapeutic human cells: manufacture for cell therapy/regenerative medicine.," *Adv. Biochem. Eng. Biotechnol.*, vol. 138, pp. 61–97, 2014.

[2] G. Ren, X. Chen, F. Dong, W. Li, X. Ren, Y. Zhang, and Y. Shi, "Concise Review: Mesenchymal Stem Cells and Translational Medicine: Emerging Issues," *Stem Cells Translational Medicine*, vol. 1, no. 1. pp. 51–58, Jan-2012.

[3] F. D. Lublin, J. D. Bowen, J. Huddlestone, M. Kremenchutzky, A. Carpenter, J. R. Corboy, M. S. Freedman, L. Krupp, C. Paulo, R. J. Hariri, and S. A. Fischkoff, "Human placenta-derived cells (PDA-001) for the treatment of adults with multiple sclerosis: a randomized, placebo-controlled, multiple-dose study.," *Mult. Scler. Relat. Disord.*, vol. 3, no. 6, pp. 696–704, Nov. 2014.

[4] I. Molendijk, B. A. Bonsing, H. Roelofs, K. C. M. J. Peeters, M. N. J. M. Wasser, G. Dijkstra, C. J. van der Woude, M. Duijvestein, R. A. Veenendaal, J.-J. Zwaginga, H. W. Verspaget, W. E. Fibbe, A. E. van der Meulen-de Jong, and D. W. Hommes, "Allogeneic Bone Marrow-Derived Mesenchymal Stromal Cells Promote Healing of Refractory Perianal Fistulas in Patients With Crohn's Disease.," *Gastroenterology*, vol. 149, no. 4, p. 918–27.e6, Oct. 2015.

[5] A. C. Schnitzler, A. Verma, D. E. Kehoe, D. Jing, J. R. Murrell, K. A. Der, M. Aysola, P. J. Rapiejko, S. Punreddy, and M. S. Rook, "Bioprocessing of human mesenchymal stem/stromal cells for therapeutic use: Current technologies and challenges," *Biochem. Eng. J.*, vol. 108, pp. 3–13, 2016.

[6] V. Jossen, R. Pörtner, S. C. Kaiser, M. Kraume, D. Eibl, and R. Eibl, "Mass Production of Mesenchymal Stem Cells — Impact of Bioreactor Design and Flow Conditions on Proliferation and Differentiation," in *Cells and Biomaterials in Regenerative Medicine*, D. D. Eberli, Ed. InTech, 2014.

[7] A. M. de Soure, A. Fernandes-Platzgummer, C. L. da Silva, and J. M. S. Cabral, "Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells.," *J. Biotechnol.*, vol. 236, pp. 88–109, Oct. 2016.

[8] C. Elseberg, J. Leber, T. Weidner, and P. Czermak, "The Challenge of Human Mesenchymal Stromal Cell Expansion: Current and Prospective Answers," in *New Insights into Cell Culture Technology*, S. J. T. Gowder, Ed. Rijeka: InTech, 2017.

[9] A. Kocaoemer, S. Kern, H. Kluter, and K. Bieback, "Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue.," *Stem Cells*, vol. 25, no. 5, pp. 1270–1278, May 2007.

[10] N. Stute, K. Holtz, M. Bubenheim, C. Lange, F. Blake, and A. R. Zander, "Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use.," *Exp. Hematol.*, vol. 32, no. 12, pp. 1212–1225, Dec. 2004.

[11] A. Poloni, G. Maurizi, V. Rosini, E. Mondini, S. Mancini, G. Discepoli, S. Biasio, G. Battaglini, S. Felicetti, E. Berardinelli, F. Serrani, and P. Leoni, "Selection of CD271(+) cells and human AB serum allows a large expansion of mesenchymal stromal cells from human bone marrow.," *Cytotherapy*, vol. 11, no. 2, pp. 153–162, 2009.

[12] E. Fernandez-Rebollo, B. Mentrup, R. Ebert, J. Franzen, G. Abagnale, T. Sieben, A. Ostrowska, P. Hoffmann, P.-F. Roux, B. Rath, M. Goodhardt, J.-M. Lemaitre, O. Bischof, F. Jakob, and W. Wagner, "Human Platelet Lysate versus Fetal Calf Serum: These Supplements Do Not Select for Different Mesenchymal Stromal Cells," *Scientific Reports*, vol. 7. London, 2017.

[13] A. A. Antoninus, W. Widowati, L. Wijaya, D. Agustina, S. Puradisastra, S. B. Sumitro, M. A. Widodo, and I. Bachtiar, "Human platelet lysate enhances the proliferation of Wharton's jelly-derived mesenchymal

stem cells," *Biomarkers Genomic Med.*, vol. 7, no. 3, pp. 87–97, 2015.
[14] A. M. de Soure, A. Fernandes-Platzgummer, F. Moreira, C. Lilaia, S.-H. Liu, C.-P. Ku, Y.-F. Huang, W. Milligan, J. M. S. Cabral, and C. L. da Silva, "Integrated culture platform based on a human platelet lysate supplement for the isolation and scalable manufacturing of umbilical cord matrix-derived mesenchymal stem/stromal cells.," *J. Tissue Eng. Regen. Med.*, vol. 11, no. 5, pp. 1630–1640, May 2017.
[15] H. Hemeda, B. Giebel, and W. Wagner, "Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells.," *Cytotherapy*, vol. 16, no. 2, pp. 170–180, Feb. 2014.
[16] J. G. Carmelo, A. Fernandes-Platzgummer, M. M. Diogo, C. L. da Silva, and J. M. S. Cabral, "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.," *Biotechnol. J.*, vol. 10, no. 8, pp. 1235–1247, Aug. 2015.
[17] F. Dos Santos, P. Z. Andrade, M. M. Abecasis, J. M. Gimble, L. G. Chase, A. M. Campbell, S. Boucher, M. C. Vemuri, C. L. Da Silva, and J. M. S. Cabral, "Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions.," *Tissue Eng. Part C. Methods*, vol. 17, no. 12, pp. 1201–10, Dec. 2011.
[18] I. N. Simões, J. S. Boura, F. Dos Santos, P. Z. Andrade, C. M. P. Cardoso, J. M. Gimble, C. L. da Silva, and J. M. S. Cabral, "Human mesenchymal stem cells from the umbilical cord matrix: Successful isolation and ex vivo expansion using serum-/xeno-free culture media.," *Biotechnol. J.*, vol. 8, no. 4, pp. 448–58, Apr. 2013.
[19] S. H. Al-Saqi, M. Saliem, S. Asikainen, H. C. Quezada, A. Ekblad, O. Hovatta, K. Le Blanc, A. F. Jonasson, and C. Gotherstrom, "Defined serum-free media for in vitro expansion of adipose-derived mesenchymal stem cells.," *Cytotherapy*, vol. 16, no. 7, pp. 915–926, Jul. 2014.
[20] G. Chen, A. Yue, Z. Ruan, Y. Yin, R. Wang, Y. Ren, and L. Zhu, "Human Umbilical Cord-Derived Mesenchymal Stem Cells Do Not Undergo Malignant Transformation during Long-Term Culturing in Serum-Free Medium," *PLoS ONE*, vol. 9, no. 6. San Francisco, USA, 2014.
[21] F. Dos Santos, A. Campbell, A. Fernandes-Platzgummer, P. Z. Andrade, J. M. Gimble, Y. Wen, S. Boucher, M. C. Vemuri, C. L. da Silva, and J. M. S. Cabral, "A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells.," *Biotechnol. Bioeng.*, vol. 111, no. 6, pp. 1116–27, Jun. 2014.
[22] B. Cunha, T. Aguiar, M. M. Silva, R. J. S. Silva, M. F. Q. Sousa, E. Pineda, C. Peixoto, M. J. T. Carrondo, M. Serra, and P. M. Alves, "Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells.," *J. Biotechnol.*, vol. 213, pp. 97–108, Nov. 2015.
[23] Q. A. Rafiq, K. M. Brosnan, K. Coopman, A. W. Nienow, and C. J. Hewitt, "Culture of human mesenchymal stem cells on microcarriers in a 5 l stirred-tank bioreactor.," *Biotechnol. Lett.*, vol. 35, no. 8, pp. 1233–1245, Aug. 2013.
[24] T. Lawson, D. E. Kehoe, A. C. Schnitzler, P. J. Rapiejko, K. A. Der, K. Philbrick, S. Punreddy, S. Rigby, R. Smith, Q. Feng, J. R. Murrell, and M. S. Rook, "Process development for expansion of human mesenchymal stromal cells in a 50L single-use stirred tank bioreactor," *Biochem. Eng. J.*, vol. 120, pp. 49–62, 2017.
[25] H. Akerstrom, "Expansion of adherent cells for cell therapy," Uppsala University, Sweden, 2009.
[26] N. E. Timmins, M. Kiel, M. Gunther, C. Heazlewood, M. R. Doran, G. Brooke, and K. Atkinson, "Closed system isolation and scalable expansion of human placental mesenchymal stem cells.," *Biotechnol. Bioeng.*, vol. 109, no. 7, pp. 1817–1826, Jul. 2012.
[27] A. Mizukami, M. D. Orellana, S. R. Caruso, K. de Lima Prata, D. T. Covas, and K. Swiech, "Efficient expansion of mesenchymal stromal cells in a disposable fixed bed culture system.," *Biotechnol. Prog.*, vol. 29, no. 2, pp. 568–572, 2013.
[28] M. J. Osiecki, T. D. Michl, B. Kul Babur, M. Kabiri, K. Atkinson, W. B. Lott, H. J. Griesser, and M. R. Doran, "Packed Bed Bioreactor for the Isolation and Expansion of Placental-Derived Mesenchymal Stromal Cells," *PLoS ONE*, vol. 10, no. 12. San Francisco, CA USA, 2015.
[29] M. F. Q. Sousa, M. M. Silva, D. Giroux, Y. Hashimura, R. Wesselschmidt, B. Lee, A. Roldao, M. J. T. Carrondo, P. M. Alves, and M. Serra, "Production of oncolytic adenovirus and human mesenchymal stem cells in a single-use, Vertical-Wheel bioreactor system: Impact of bioreactor design on performance of microcarrier-based cell culture processes.," *Biotechnol. Prog.*, vol. 31, no. 6, pp. 1600–1612, 2015.
[30] F. Dos Santos, P. Z. Andrade, G. Eibes, C. L. Da Silva, and J. M. S. Cabral, "Ex vivo expansion of human mesenchymal stem cells on microcarriers," in *Methods Mol. Biol.*, vol. 698, L. Springer Science + Business Media, Ed. 2011, pp. 189–198.
[31] A. Ghorbani, S. A. Jalali, and M. Varedi, "Isolation of adipose tissue mesenchymal stem cells without tissue destruction: a non-enzymatic method.," *Tissue Cell*, vol. 46, no. 1, pp. 54–58, Feb. 2014.
[32] A. Mizukami, A. Fernandes-Platzgummer, J. G. Carmelo, K. Swiech, D. T. Covas, J. M. S. Cabral, and C.

L. da Silva, “Stirred tank bioreactor culture combined with serum-/xenogeneic-free culture medium enables an efficient expansion of umbilical cord-derived mesenchymal stem/stromal cells.,” *Biotechnol. J.*, vol. 11, no. 8, pp. 1048–1059, Aug. 2016.

[33] S. Sart, S. N. Agathos, and Y. Li, “Engineering stem cell fate with biochemical and biomechanical properties of microcarriers.,” *Biotechnol. Prog.*, vol. 29, no. 6, pp. 1354–1366, 2013.

[34] S. Behrens and D. Grier, “The charge of glass and silica surfaces,” *J. Chem. Phys.*, vol. 115, no. 6716, 2001.

[35] F. Petry, J. R. Smith, J. Leber, D. Salzig, P. Czermak, and M. L. Weiss, “Manufacturing of Human Umbilical Cord Mesenchymal Stromal Cells on Microcarriers in a Dynamic System for Clinical Use,” *Stem Cells International*, vol. 2016. 2016.

[36] G. Zhao, F. Liu, S. Lan, P. Li, L. Wang, J. Kou, X. Qi, R. Fan, D. Hao, C. Wu, T. Bai, Y. Li, and J. Y. Liu, “Large-scale expansion of Wharton’s jelly-derived mesenchymal stem cells on gelatin microbeads, with retention of self-renewal and multipotency characteristics and the capacity for enhancing skin wound healing,” *Stem Cell Research & Therapy*, vol. 6, no. 1. London, 2015.

[37] F. G. Teixeira, K. M. Panchalingam, S. I. Anjo, B. Manadas, R. Pereira, N. Sousa, A. J. Salgado, and L. A. Behie, “Do hypoxia/normoxia culturing conditions change the neuroregulatory profile of Wharton Jelly mesenchymal stem cell secretome?,” *Stem Cell Res. Ther.*, vol. 6, p. 133, Jul. 2015.

[38] J. Hupfeld, I. H. Gorr, C. Schwald, N. Beaucamp, K. Wiechmann, K. Kuentzer, R. Huss, B. Rieger, M. Neubauer, and H. Wegmeyer, “Modulation of mesenchymal stromal cell characteristics by microcarrier culture in bioreactors.,” *Biotechnol. Bioeng.*, vol. 111, no. 11, pp. 2290–302, Nov. 2014.

[39] M. A. Brown, C. S. Wallace, C. C. Anamelechi, E. Clermont, W. M. Reichert, and G. A. Truskey, “The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces.,” *Biomaterials*, vol. 28, no. 27, pp. 3928–3935, Sep. 2007.

[40] C. Schirmaier, V. Jossen, S. C. Kaiser, F. Jüngerkes, S. Brill, A. Safavi-Nab, A. Siehoff, C. van den Bos, D. Eibl, and R. Eibl, “Scale-up of adipose tissue-derived mesenchymal stem cell production in stirred single-use bioreactors under low-serum conditions,” *Eng. Life Sci.*, vol. 14, no. 3, pp. 292–303, May 2014.

[41] S. Kaiser, V. Jossen, C. Schirmaier, D. Eibl, S. Brill, C. van den Bos, and R. Eibl, “Fluid Flow and Cell Proliferation of Mesenchymal Adipose-Derived Stem Cells in Small-Scale, Stirred, Single-Use Bioreactors,” *Chemie Ing. Tech.*, vol. 85, no. 1–2, pp. 95–102, 2013.

[42] B. Cunha, T. Aguiar, S. B. Carvalho, M. M. Silva, R. A. Gomes, M. J. T. Carrondo, P. Gomes-Alves, C. Peixoto, M. Serra, and P. M. Alves, “Bioprocess integration for human mesenchymal stem cells: From up to downstream processing scale-up to cell proteome characterization.,” *J. Biotechnol.*, vol. 248, pp. 87–98, Apr. 2017.

[43] H. Abdelrazik, G. M. Spaggiari, L. Chiossone, and L. Moretta, “Mesenchymal stem cells expanded in human platelet lysate display a decreased inhibitory capacity on T- and NK-cell proliferation and function.,” *Eur. J. Immunol.*, vol. 41, no. 11, pp. 3281–3290, Nov. 2011.

VI. Conclusions and Future Trends

MSC hold a great potential in the regenerative medicine field. These heterogeneous cell population secretes different types of bioactive molecules, including growth factors, cytokines and chemokines, that are able to induce a regenerative response of the damaged tissue [1]. Such response is mostly accompanied by the modulation of the immune system, where MSC also play an important role, in a process called immunomodulation [2]. In fact, those are the two major reasons for the therapeutic use of MSC in the context of AMI. Some authors also defend that MSC are able to engraft in the host tissue and transdifferentiate into the different cell types present in the myocardial tissue, or even fuse with those cells [3], [4]. However, few evidences corroborate this theory and, therefore, the present work focus on the regeneration ability of MSC through paracrine signaling.

MSC are very heterogeneous in nature, composed by different plastic-adherent cell populations. Efforts have been made to find a specific cell biomarker capable of distinguishing MSC from other cell types, either expressed in the membrane or at intracellular level. To date, no study reported a specific cell marker for this multipotent population. The discovery of such marker would greatly affect the bioprocessing strategies that are currently being used for the isolation and expansion of MSC. The definition of a homogeneous cell population would also facilitate the analysis of the therapeutic potential of those cells, using efficient and robust *in vitro* and *in vivo* assays with enough sensitivity to assess cell potency. In fact, variations in the composition of the cell population is one of the major hurdles to successfully characterize the potency of MSC [5]. Moreover, these cells are not only heterogeneous in their composition, but also present a high variability depending on the source where they were isolated from. MSC isolated from different sources present distinct biomarker expression, differentiation potentials, proliferative capacities, bioactive factors secretion and immunomodulatory and trophic activities [6], [7]. Such differences make scientists wonder if all the cells termed "MSC" should belong to the same designation or if they should rather be divided in different groups, according to some criteria. The generic concept of "stem/stromal cells" may raise some concerns in terms of product robustness and thus regulatory approval. Therefore, the choice of an MSC source is a critical step when envisaging a specific regenerative medicine application. Some of the differences among sources were analyzed in Chapters II and III. Finally, MSC present a high donor-to-donor variability, depending on the age, gender, and health of the individual where cells are being isolated from. All these levels of variability affect the outcomes of a potential MSC-based product, starting from the isolation step until the delivery to the patient.

Distinct aspects of MSC manufacturing were covered in this work, including MSC source, culture platform and oxygen concentration. However, one important topic that was not analyzed was the effect of different types of culture media on MSC *in vitro* angiogenic potential. Researchers have demonstrated that MSC expanded under serum- and xenogeneic-free conditions presented superior functional angiogenesis, compared with cells expanded in serum-containing medium [8]–[10]. A possible

explanation for these findings is the upregulation of prosurvival genes, due to the stress conditions caused by serum deprivation [11]. VEGF, bFGF and HGF are among the list of prosurvival, as well as proangiogenic, factors. In fact, cell survival and angiogenesis are related biological processes since both aim at avoiding death: one at a cellular level (cell survival) and the other at tissue level (angiogenesis) [12]. In this context, preconditioning MSC with basal media might be of great interest to the improved secretion of proangiogenic factors. Basal medium contains water, glucose and salts and this total absence of bioactive factors might induce a marked upregulation of prosurvival and proangiogenic genes. Preliminary data on this topic corroborate this hypothesis. However, further studies must be performed in order to compare the impact of different types of culture media, including serum-containing media, serum-deprived media and basal medium, on MSC *in vitro* angiogenic potential.

In fact, preconditioning MSC with complete medium *vs* basal medium is on the basis of the different angiogenic assays used in this work. While the expression and secretion of proangiogenic genes was analyzed when cells were cultured in complete medium, the *in vitro* angiogenic assays (HUVEC tube-like structure formation and HUVEC migration) were performed with endothelial basal medium conditioned with factors secreted by MSC. The rationale behind the use of basal medium instead of complete medium in the functional assays is to assure that the only bioactive factors contributing to modulate the HUVEC behavior are the factors secreted by the cells, and not the ones present in the culture medium. However, this approach raises some questions concerning the establishment of a comparison between the assays performed with serum-free medium *vs* endothelial basal medium. Certainly, cells do not present the same gene expression and protein secretion profiles in the two conditions where they were cultured. An ideal approach to better mimic the secretion of proangiogenic factors by MSC in the body, as well as their *in vivo* angiogenic potential, would be to culture the cells using the components present in the environment that the cells will face inside the body. In the case of intravenous administration, for instance, cells should be cultured in medium supplemented with human serum, along with other components of the blood, including blood cells and immune cells. This approach would provide the most reliable results regarding the *in vivo* angiogenic potential of MSC.

This strategy aims at preconditioning cells to better adapt them to the microenvironment they will face in the body. However, this approach may not maximize the therapeutic potential of the cells, or the secretion of proangiogenic factors. In fact, it was already discussed that serum deprivation results in improved angiogenic properties of MSC, but one question remains: is this preconditioning effect maintained *in vivo*? Therefore, a careful analysis of the aim of the experiment should be done since the two strategies mentioned above may not lead to the same *in vitro* and *in vivo* outcomes.

Throughout the study, two functional assays were used to analyze the *in vitro* angiogenic potential of MSC: the tube-like structure formation assay and the migration assay. The tube formation assay was

firstly reported by Lawley and Kubota [13] and further optimized by Kanzawa and coworkers [14]. This assay is more representative of the *in vivo* angiogenesis than the 2D assay since ECM is used to recreate the 3D environment. Moreover, some alternative versions of this assay allow the measurement of endothelial cell proliferation and differentiation, as well as testing different pro- and antiangiogenic compounds [15]. The main limitations of the tube formation assays are the lack of consistent lumen formation, homogeneous tubules, sensitivity to uneven matrix coating of wells and cell density, and the time-consuming [15]. Alternatively, the migration assay represents allows a quantitative analysis of endothelial cell migration over time, in a reproducible and short duration approach. Also, this assay is sensitive to small alterations in concentration gradients. Some limitations are associated with this assay, including difficulty to define and maintain transmembrane gradients and to obtain accurate results with low cell counts [15]. Due to the potential limitations of these two *in vitro* assays, further *in vitro* and *ex vivo* studies should be performed, to evaluate the angiogenic capacity of a potential MSC-based product. A commonly used *in vitro* assay is the endothelial proliferation assay [16], [17]. This assay allows the quantification of cell proliferation and apoptosis, in a reproducible and easy way. However, it provided a short windows of analysis due to endothelial cell senescence and it is sensitive to cell density [15]. The wound healing assay also represents a potential *in vitro* assay [18], [19]. This approach involves cell migration in the plate, instead of through the transwell membrane, as in the migration assay. Therefore, the main benefits and limitations are similar to those of the migration assay. *Ex vivo* assays represent a step forward in translating the angiogenic potential of cells to *in vivo* studies. These systems include different types of cells, such as pericytes, smooth muscle cells, among others, in a 3D environment [15]. Examples of available *ex vivo* assays are the chick aortic arch model [20] and the recently developed aortic ring assay [15]. Nevertheless, an ideal angiogenic assay should evaluate endothelial cell migration, proliferation and differentiation into tubular structures; efficiency to promote the formation of functional blood vessels; augmentation or replacement of supporting cell types, including pericytes, smooth muscle cells fibroblasts, in addition to endothelial cells; and processing of ECM and/or basement membrane [15].

The *in vitro* angiogenic assays used in this work allowed the identification of the culture conditions promoting the highest angiogenic properties of UCM MSC: dynamic culture and low oxygen concentration. Here, it is important to distinguish between the overall effect of the dynamic culture and the effect of shear stress. Shear stress is indeed known to increase the secretion of proangiogenic factors [21]–[23], but one should not conclude that the improved angiogenic properties these cells is exclusively due to it. In fact, other culture parameters may be playing an important role on the upregulation of the proangiogenic genes, such as the topography of the microcarriers, the homogeneous availability of metabolites and bioactive factors in the culture medium, among others. The topography of the microcarriers is known to induce alterations in the actin organization and in the cell differentiation

ability [24]. For instance, researchers found that microcarriers favoring round shape and disorganized actin (i.e. cytopore 2) promoted chondrogenesis, while microcarriers favoring cell spreading and the formation of stress fibers inhibited differentiation towards chondrocytes [25]. Similarly, alterations in the cell organization may also induce changes in the expression and secretion of proangiogenic factors. If so, the appropriate selection of a specific type of microcarriers for the modulation of MSC angiogenic potential must be considered. Nevertheless, further studies are required to support this hypothesis. To properly assess the effect of shear stress on the *in vitro* angiogenic potential of MSC, cells should be plated in 2D culture system and subjected to a turbulent shear flow. A possible experimental approach is the design of a microfluidic device to provide a turbulent environment for cell culture. Such approach was already tested by different authors, demonstrating the improved secretion of angiogenic factors [21]–[23].

Another important parameter that regulates the secretion of proangiogenic factors is cell density. The higher the cell density, the higher the number of cell-cell interactions. This type of interactions seems to affect the secretion of bioactive factors. Preliminary data showed that, at higher cell densities, MSC express higher levels of the proangiogenic genes VEGF, bFGF and HGF per cell (data not shown), meaning that each cell expresses higher levels of this proangiogenic genes when cultured at higher cell densities. Such effect might be due to the increased number of cell-cell interactions or to the increased production of EV and consequent increase in paracrine signaling. This augmentation effect was minimized when comparing static *vs* dynamic cultures since UCM MSC density in static conditions (5,500 cell/cm^2) was half of the density of cells in dynamic culture (11,000 cell/cm^2), accounting with 50% adhesion of the cells to the microcarriers and 100% adhesion to tissue culture flasks. Results show that UCM MSC adhesion to microcarriers ranged between 40 to 50% (data not shown), meaning that cell densities were similar in both culture systems.

In general, stress conditions seem to upregulate the expression of proangiogenic genes, including serum-deprived culture (demonstrated in the literature), dynamic culture conditions and low oxygen concentration. Therefore, other stress conditions can also be considered to increase the therapeutic potential of these cells, including other mechanical preconditioning strategies (electrical stimuli and strain), hyperoxia (100% O_2) and pharmacological pretreatment, for instance.

Three other bioprocessing steps can interfere with the angiogenic potential of MSC: cell harvesting, storage and delivery. Cell harvesting demonstrated to induce a downregulation of the proangiogenic genes studied, while cryopreserving cells for up to one month in liquid nitrogen and maintaining the cells for 4 h in PBS + human albumin, at both room temperature of 4 °C, does not seem to impact the expression of those genes. To fully understand the impact of these three bioprocessing steps on the *in vitro* angiogenic potential of UCM MSC, functional assays should have been performed, including the

tube-like structure formation assay and the migration assay. Nevertheless, the results obtained contributed to understand the effect of cell harvesting, storage and delivery on the gene expression profile of three of the most important genes associated with angiogenesis. Moreover, they contribute to the development of an allogeneic, off-the-shelf MSC-based product with enhanced therapeutic features.

Allogeneic cell products are more advantageous than their autologous counterparts. In an allogeneic setting, cells from a specific donor can be selected, based on the ability of cells to proliferate, differentiate or secrete higher concentrations of bioactive factors compared with other donors. Moreover, in an autologous setting, cells derived from the patient may have their biological properties impaired, due to aging or disease [26]. In an allogeneic approach, clinicians can even select the most suitable MSC source for the treatment of a specific disease. Another great advantage based on the results obtained in this work is that an improved allogeneic cell product can be produced, stored and delivered without affecting the preconditioning effect. An allogeneic product represents a major advantage in the context of AMI. After an AMI event, patients should not wait several weeks or even months for a therapeutic solution, due to the extended tissue necrosis and remodeling that occurs after the event. Also, the therapeutic features of MSC from the patients might be impaired due to the disease, in the case of an autologous approach. All these reasons encourage the development of an allogeneic, off-the-shelf enhanced MSC-based product.

To achieve the high cell numbers required for an MSC-based therapy in the AMI context, several millions of cells must be produced and administrated into the patient. Fortunately, the microcarrier-based stirred platform is not only indicated to precondition cells, but also to produce them. In this context, a scalable expansion platform was successfully established to produce UCM and AT MSC, using the PBS-0.1 MAG system under S/XF conditions. In addition, this platform is disposable and easily scalable to industrial scales (PBS-15 and PBS-80, with maximum working volume of 15 L and 80 L, respectively), with the possibility of monitoring and controlling the culture parameters, which greatly contributes to the approval by the regulatory agencies. Nevertheless, such platform was not efficient in promoting BM MSC expansion. Results from Chapter II show that the BM source represents the source with the lowest *in vitro* angiogenic potential, whereas the UCM and AT sources present higher *in vitro* angiogenic capacity, under the culture conditions studied. Therefore, the PBS-0.1 MAG efficiently promotes the expansion of the MSC sources presenting the highest *in vitro* angiogenic potential. The expansion protocol can also be adapted to other types of bioreactors, including the stirred tank reactor or the wave-mixed bioreactor.

Despite the encouraging data generated in this work, the lack of *in vivo* studies still represents its main limitation. *In vivo* studies in AMI animal models would be important to compare the effect of cell preconditioning through the modulation of the bioprocessing parameters, as well as the effect of storage

and delivery on that preconditioning effect. Also, increasing the number of MSC donors in each of the chapters would increase the statistic significance of the results. A nonparametric test was used to analyze the data generated in the different chapters since a normal distribution of the variables can not be assumed without further tests. However, the use of nonparametric tests requires higher number of samples to assure statistical significance, compared with parametric tests.

Another important technical aspect concerning the in vivo studies is the choice of the route of cell administration. Several reports have demonstrated that MSC engraftment in the myocardial tissue occurs only to 0.44% of the cells that are intravenously administrated [27]. In fact, these findings support the regenerative properties of MSC through paracrine signaling. Intravenous administration of the cells will lead to cell washout and probably accumulation in the lungs and spleen [28]. Other will be destroyed by immune cells present in circulation. Therefore, the most appropriate route of administration of MSC should be intramyocardially, either as single cells or cultured in a scaffold. Such approach would increase the chances of cell survival and retention in the damaged myocardium, although being an invasive medical procedure.

A potential approach to overcome the use of cellular products due to the associated technical and regulatory difficulties is molecular therapy. The paracrine action of MSC provides the possibility to administrate one bioactive factor alone or combined with others as a specific cocktail therapy for the treatment of AMI. However, as previously discussed in Chapter II, some bioactive factors may be harmful for the body, such as TNF-α and IL-6 [29], two potent proinflammatory molecules. The main advantage of MSC-based therapy is the sustainable moderate release and concentration of the bioactive factors that might vary according to diverse microenvironment and situations [29]. MSC can adapt to harsh disease conditions, with the aim of restoring physiological status [29].

Recently, another cell-free approach is gaining relevance for the treatment of different diseases, the EV. The clinical use of EV represents a half way solution between molecular and cellular therapy. As previously mentioned in Chapter I, EV are small particles composed of a lipid layer enclosing cytoplasmic components, such as proteins, nucleic acids and lipids, secreted by cells [30]. EV derived from MSC present high therapeutic properties, mainly due to the presence of bioactive factors and small noncoding RNA capable of modulating the angiogenic process. Several preclinical studies in animal models showed that MSC-EV accelerate skin wound healing [31] and reduce infarct area in ischemic cardiac injury [32], [33]. However, several hurdles need to be addressed in the clinical translation of this new therapeutic tool, including the classification of cell secretome as a whole at regulatory levels and the definition of the method of administration [30]. Several advantageous arise from the use of EV under clinical settings. Since EV represent a distinct fraction of the cell secretome, their administration results in a set of signals with more limited and predictable effects. Therefore, the GMP production and release

of EV is less complex compared with living cells, resulting in reduced associated costs [34]. Nevertheless, EV are complex biological structures poorly understood and their isolation procedures are still unsatisfactory [30]. Clinical applications of EV have been limited to a single patient with graft-versus-host disease (GvHD) [35] and a preliminary trial in patients with kidney failure [36]. Therefore, several gaps need to be filled to bring these potential therapeutic tools from bench to bedside.

In conclusion, the main goal of this work was to develop a potential MSC-based product with enhanced therapeutic features for myocardial regeneration. To achieve this goal, the *in vitro* angiogenic potential of different human MSC sources cultured under different serum- and xenogeneic-free culture conditions was characterized. The impact of modulating the cell culture parameters, including culture platform and oxygen concentration, on the *in vitro* angiogenic potential of MSC was evaluated, to maximize their therapeutic capacity. To develop an MSC-based product with enhanced therapeutic capacity, it is not only important to find the culture conditions that would maximize this capacity, but also to establish an efficient, robust and GMP-compliant cell culture platform able to produce clinical-relevant MSC numbers to treat patients with AMI. In this work, all these goals were achieved. The *in vitro* angiogenic properties of MSC were successfully improved by culturing the cells under dynamic conditions and low oxygen concentration and a dynamic culture platform was established for the efficient expansion of MSC with enhanced therapeutic features for myocardial regeneration.

VI.1 References of Conclusion and Future Trends

[1] L. D. S. Meirelles, A. M. Fontes, D. T. Covas, and A. I. Caplan, "Mechanisms involved in the therapeutic properties of mesenchymal stem cells.," *Cytokine Growth Factor Rev.*, vol. 20, no. 5–6, pp. 419–27, 2009.

[2] Y.-H. Choi, A. Kurtz, and C. Stamm, "Mesenchymal stem cells for cardiac cell therapy.," *Hum. Gene Ther.*, vol. 22, no. 1, pp. 3–17, Jan. 2011.

[3] S. H. Ranganath, O. Levy, M. S. Inamdar, and J. M. Karp, "Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease.," *Cell Stem Cell*, vol. 10, no. 3, pp. 244–58, Mar. 2012.

[4] C. Gallina, V. Turinetto, and C. Giachino, "A New Paradigm in Cardiac Regeneration: The Mesenchymal Stem Cell Secretome.," *Stem Cells Int.*, vol. 2015, p. 765846, 2015.

[5] R. M. Samsonraj, B. Rai, P. Sathiyanathan, K. J. Puan, O. Rotzschke, J. H. Hui, M. Raghunath, L. W. Stanton, V. Nurcombe, and S. M. Cool, "Establishing criteria for human mesenchymal stem cell potency.," *Stem Cells*, vol. 33, no. 6, pp. 1878–1891, Jun. 2015.

[6] G. Chamberlain, J. Fox, B. Ashton, and J. Middleton, "Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing.," *Stem Cells*, vol. 25, no. 11, pp. 2739–49, Nov. 2007.

[7] R. Hass, C. Kasper, S. Bohm, and R. Jacobs, "Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC.," *Cell Commun. Signal.*, vol. 9, p. 12, May 2011.

[8] P. Swamynathan, P. Venugopal, S. Kannan, C. Thej, U. Kolkundar, S. Bhagwat, M. Ta, A. Sen Majumdar, and S. Balasubramanian, "Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton's jelly derived mesenchymal stem cells? A comparative study.," *Stem Cell Res. Ther.*, vol. 5, no. 4, p. 88, Jul. 2014.

[9] B. Lindroos, S. Boucher, L. Chase, H. Kuokkanen, H. Huhtala, R. Haataja, M. Vemuri, R. Suuronen, and

S. Miettinen, “Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro.,” *Cytotherapy*, vol. 11, no. 7, pp. 958–72, Jan. 2009.
[10] S. Jung, A. Sen, L. Rosenberg, and L. A. Behie, “Human mesenchymal stem cell culture: rapid and efficient isolation and expansion in a defined serum-free medium.,” *J. Tissue Eng. Regen. Med.*, vol. 6, no. 5, pp. 391–403, May 2012.
[11] H.-D. Xu, D. Wu, J.-H. Gu, J.-B. Ge, J.-C. Wu, R. Han, Z.-Q. Liang, and Z.-H. Qin, “The pro-survival role of autophagy depends on Bcl-2 under nutrition stress conditions.,” *PLoS One*, vol. 8, no. 5, p. e63232, 2013.
[12] E. Chavakis and S. Dimmeler, “Regulation of endothelial cell survival and apoptosis during angiogenesis.,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 22, no. 6, pp. 887–893, Jun. 2002.
[13] T. J. Lawley and Y. Kubota, “Induction of morphologic differentiation of endothelial cells in culture.,” *J. Invest. Dermatol.*, vol. 93, no. 2 Suppl, p. 59S–61S, Aug. 1989.
[14] S. Kanzawa, H. Endo, and N. Shioya, “Improved in vitro angiogenesis model by collagen density reduction and the use of type III collagen.,” *Ann. Plast. Surg.*, vol. 30, no. 3, pp. 244–251, Mar. 1993.
[15] F. Iqbal, P. Szaraz, M. Librach, A. Gauthier-Fisher, and C. L. Librach, “Angiogenic potency evaluation of cell therapy candidates by a novel application of the in vitro aortic ring assay,” *Stem Cell Research & Therapy*, vol. 8. London, 2017.
[16] D. Gomez and N. C. Reich, “Stimulation of primary human endothelial cell proliferation by IFN.,” *J. Immunol.*, vol. 170, no. 11, pp. 5373–5381, Jun. 2003.
[17] C.-H. Yu, J. Wu, Y.-F. Su, P.-Y. Ho, Y.-C. Liang, M.-T. Sheu, and W.-S. Lee, “Anti-proliferation effect of 3-amino-2-imino-3,4-dihydro-2H-1,3-benzothiazin-4-one (BJ-601) on human vascular endothelial cells: G0/G1 p21-associated cell cycle arrest.,” *Biochem. Pharmacol.*, vol. 67, no. 10, pp. 1907–1916, May 2004.
[18] M. Wong and A. Gotlieb, “In vitro reendothelialization of a single-cell wound. Role of microfilament bundles in rapid lamellipodia-mediated wound closure.,” *Lab Invest*, vol. 51, pp. 75–81, 1984.
[19] A. Schor, I. Ellis, and S. Schor, “Chemotaxis and chemokinesis in 3D marcomolecular matrices: relevance to angiogenesis,” *Methods Mol Med*, vol. 46, pp. 163–83, 2001.
[20] C. A. Staton, M. W. R. Reed, and N. J. Brown, “A critical analysis of current in vitro and in vivo angiogenesis assays.,” *Int. J. Exp. Pathol.*, vol. 90, no. 3, pp. 195–221, Jun. 2009.
[21] H. Wang, G. M. Riha, S. Yan, M. Li, H. Chai, H. Yang, Q. Yao, and C. Chen, “Shear stress induces endothelial differentiation from a murine embryonic mesenchymal progenitor cell line.,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 9, pp. 1817–1823, Sep. 2005.
[22] H. Wang, M. Li, P. H. Lin, Q. Yao, and C. Chen, “Fluid shear stress regulates the expression of TGF-beta1 and its signaling molecules in mouse embryo mesenchymal progenitor cells.,” *J. Surg. Res.*, vol. 150, no. 2, pp. 266–270, Dec. 2008.
[23] V. Bassaneze, V. G. Barauna, C. Lavini-Ramos, J. Kalil, I. T. Schettert, A. A. Miyakawa, and J. E. Krieger, “Shear stress induces nitric oxide-mediated vascular endothelial growth factor production in human adipose tissue mesenchymal stem cells.,” *Stem Cells Dev.*, vol. 19, no. 3, pp. 371–378, Mar. 2010.
[24] S. Sart, S. N. Agathos, and Y. Li, “Engineering stem cell fate with biochemical and biomechanical properties of microcarriers.,” *Biotechnol. Prog.*, vol. 29, no. 6, pp. 1354–1366, 2013.
[25] S. Sart, A. Errachid, Y. Schneider, and S. N. Agathos, “Modulation of mesenchymal stem cell actin organization on conventional microcarriers for proliferation and differentiation in stirred bioreactors,” *J. Tissue Eng. Regen. Med.*, vol. 7, pp. 537–551, 2013.
[26] A. M. de Soure, A. Fernandes-Platzgummer, C. L. da Silva, and J. M. S. Cabral, “Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells.,” *J. Biotechnol.*, vol. 236, pp. 88–109, Oct. 2016.
[27] C. Toma, M. F. Pittenger, K. S. Cahill, B. J. Byrne, and P. D. Kessler, “Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart.,” *Circulation*, vol. 105, no. 1, pp. 93–98, Jan. 2002.
[28] J. Leibacher and R. Henschler, “Biodistribution, migration and homing of systemically applied mesenchymal stem/stromal cells,” *Stem Cell Research & Therapy*, vol. 7. London, 2016.
[29] X. Liang, Y. Ding, Y. Zhang, H. F. Tse, and Q. Lian, “Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives,” *Cell Transpl.*, vol. 23, no. 9, pp. 1045–1059, 2014.
[30] S. Baldari, G. Di Rocco, M. Piccoli, M. Pozzobon, M. Muraca, and G. Toietta, “Challenges and Strategies for Improving the Regenerative Effects of Mesenchymal Stromal Cell-Based Therapies.,” *Int. J. Mol. Sci.*, vol. 18, no. 10, Oct. 2017.
[31] B. Zhang, M. Wang, A. Gong, X. Zhang, X. Wu, Y. Zhu, H. Shi, L. Wu, W. Zhu, H. Qian, and W. Xu, “HucMSC-Exosome Mediated-Wnt4 Signaling Is Required for Cutaneous Wound Healing.,” *Stem Cells*, vol. 33, no. 7, pp. 2158–2168, Jul. 2015.
[32] R. C. Lai, F. Arslan, M. M. Lee, N. S. K. Sze, A. Choo, T. S. Chen, M. Salto-Tellez, L. Timmers, C. N.

Lee, R. M. El Oakley, G. Pasterkamp, D. P. V de Kleijn, and S. K. Lim, “Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury.,” *Stem Cell Res.*, vol. 4, no. 3, pp. 214–222, May 2010.

[33] S. Bian, L. Zhang, L. Duan, X. Wang, Y. Min, and H. Yu, “Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model.,” *J. Mol. Med. (Berl).*, vol. 92, no. 4, pp. 387–397, Apr. 2014.

[34] M. Muraca, M. Piccoli, C. Franzin, A. M. Tolomeo, M. Jurga, M. Pozzobon, and G. Perilongo, “Diverging Concepts and Novel Perspectives in Regenerative Medicine.,” *Int. J. Mol. Sci.*, vol. 18, no. 5, May 2017.

[35] L. Kordelas, V. Rebmann, A.-K. Ludwig, S. Radtke, J. Ruesing, T. R. Doeppner, M. Epple, P. A. Horn, D. W. Beelen, and B. Giebel, “MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease.,” *Leukemia*, vol. 28, no. 4. England, pp. 970–973, Apr-2014.

[36] W. Nassar, M. El-Ansary, D. Sabry, M. A. Mostafa, T. Fayad, E. Kotb, M. Temraz, A.-N. Saad, W. Essa, and H. Adel, “Umbilical cord mesenchymal stem cells derived extracellular vesicles can safely ameliorate the progression of chronic kidney diseases,” *Biomaterials Research*, vol. 20. London, 2016.