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Ex-vivo gene therapy to improve the regenerative features of human mesenchymal stem/stromal cells (MSC) in a model of limb ischemia

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2019

Para ser grande, sê inteiro: nada

Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és

No mínimo que fazes.

Assim em cada lago a lua toda

Brilha, porque alta vive

Ricardo Reis, in "Odes"

Heterónimo de Fernando Pessoa

ABSTRACT

Mesenchymal stem cells (MSC) hold great promise for regenerative medicine and cell therapy due to their unique properties that include paracrine therapeutic activity, immunomodulatory potential and capacity to evade the immune system. However, they have limited engraftment and survival upon *in vivo* administration and, thus, strategies to improve their potential need to be developed. Herein, a new type of non-viral vehicles for MSC genetic engineering – minicircles – were studied in order to efficiently transfect and increase beneficial effects of MSC. Minicircles containing the VEGF therapeutic (MC-VEGF) protein alone or in fusion with the reporter green fluorescent protein (GFP) (MC-VEGF-GFP) were constructed, produced and purified using a recently developed innovative method.

The effect of VEGF-containing minicircles was first confirmed in bone marrow (BM)-derived MSC, since this is the most commonly and widely studied source of these cells. To assess the potential for MSC engineering, minicircles were compared with conventional plasmid constructs (pVAX) with the same genetic cassette (VEGF or VEGF-GFP). BM-MSC were first transfected with VEGF-GFP vectors using microporation to optimize the overall strategy and then vectors containing only VEGF were used. MSC transfection with minicircles led not only to an increased VEGF production, reflected both by analysis of gene expression and protein secretion, but also to an enhancement in MSC angiogenic activity *in vitro* as observed in functional assays with endothelial cells. Alternative sources to BM have also been explored in past years, being the most common adipose tissue (AT) and umbilical cord matrix (UCM). So, VEGF-containing minicircles were tested in these two MSC sources and the angiogenic potential was evaluated in terms of VEGF production and functionality. The three sources showed similar VEGF production levels, as well as *in vitro* angiogenic capacity after transfection with VEGF-encoding minicircles. Hence, the protocol used herein for MSC transfection can be successfully applied to different sources of MSC with similar outcomes.

Finally, MSC from BM genetically modified with VEGF-encoding minicircles were tested in a mouse model of hind limb ischemia in the context of peripheral arterial disease (PAD). The infusion of VEGF-overexpressing MSC promoted significant improvements in muscular function of ischemic mice. The results obtained in present thesis revealed that MSC modified with VEGF-encoding minicircles could be a promising strategy for the treatment of PAD, especially for the no-option patients.

KEYWORDS

Mesenchymal stem/stromal cells

Gene therapy

Minicircles

Angiogenesis

Hind limb ischemia.

RESUMO

As células estaminais mesenquimais (MSC) apresentam um grande potencial na área da medicina regenerativa e terapia celular devido às suas propriedades únicas, incluindo actividade terapêutica através de secreção de factores, potencial imunomodulatório e capacidade de evasão ao sistema imunitário. No entanto, apresentam capacidades de enxerto e sobrevivência limitadas após administração *in vivo*, sendo necessário desenvolver estratégias para aumentar o seu potencial. Um novo tipo de veículos não virais para engenharia genética – minicírculos - foi estudado para transfectar MSC, aumentando os seus efeitos benéficos. Minicírculos com a proteína terapêutica VEGF sozinha (MC-VEGF) ou em fusão com um gene codificante de um repórter fluorescente – GFP – (MC-VEGF-GFP) foram construídos, produzidos e purificados usando um método inovador e recentemente desenvolvido.

O efeito de MC-VEGF foi verificado em MSC da medula óssea (MO), a fonte mais comum e amplamente estudada deste tipo de células. Para determinar o potencial dos minicírculos, estes foram comparados com plasmídeos convencionais (pVAX) contendo a mesma cassete genética (VEGF ou VEGF-GFP). MO-MSC foram inicialmente transfectadas por microporação com vectores VEGF-GFP, de modo a optimizar o processo e depois foram usados vectores apenas com VEGF. A transfeção de MSC com minicírculos levou não só a um aumento na produção de VEGF, reflectido na expressão génica e secreção de proteína, mas também a um melhoramento da actividade angiogénica, observado em ensaios funcionais com células endoteliais. Recentemente, têm vindo a ser exploradas fontes de MSC alternativas à MO, sendo as mais comuns: tecido adiposo (AT) e matriz do cordão umbilical (UCM). Assim, os MC-VEGF foram testados nestas duas fontes e o potencial angiogénico foi avaliado em termos de produção de VEGF e funcionalidade. Após transfecção com minícirculos com VEGF, os níveis de produção de VEGF e a capacidade angiogénica *in vitro* foi semelhante para as três fontes. Assim, demonstrou-se que o protocolo usado para transfecção poderá ser aplicado com sucesso a diferentes fontes de MSC, levando a resultados semelhantes.

Por fim, MO-MSC geneticamente modificadas foram testadas em ratinho num modelo de isquemia de membros inferiores no contexto da doença arterial periférica (DAP). A infusão de MSC sobre-expressando levou a um aumento significativo da função muscular nos ratinhos isquémicos. Os resultados obtidos revelaram que a modificação genética de MSC com MC- VEGF poderá ser uma estratégia promissora para tratamento da DAP, especialmente em doentes sem outra opção terapêutica.

PALAVRAS-CHAVE

Células estaminais mesenquimais (MSC)

Terapia génica

Minicírculos

Angiogénese

Isquemia de membros.

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3-D – Three-dimensional

AAV – Adeno-associated virus

ABI – Ankle brachial index

ADA – Adenosine deaminase

Ang-1 – Angiopoietin 1

Ap2 – adipocyte protein 2

ASC – Adipose stem cells

AT – Adipose tissue

Bcl – B cell lymphoma

BERG – Bioengineering Research Group

bFGF – basic fibroblast growth factor

BM – Bone marrow

BMP-2 – Bone morphogenetic protein 2

CCLs/CXCLs – Chemokine ligands

CD – Cluster of differentiation

CFU-F – Colony forming unit-fibroblastic

CLI – Critical limb ischemia

CLTA4 – cytotoxic T-lymphocyte antigen 4

CMV – Cytomegalovirus

CRISPR – Clustered regularly interspaced short palindromic repeats

CsCl – Cesium chloride

CXCR-4 – Chemokine (C-X-C motif) receptor 4

DMEM – Dulbecco's modified Eagle's medium

DNA – Deoxyribonucleic acid

E. coli – Escherichia coli

ECM – Extra-cellular matrix

EMA – European Medicinal Agency

EPO – Erythropoietin

ESC – Embryonic stem cells

FACS – Fluorescent-activated cell sorting

FBS – Fetal bovine serum

FDA – US Food & Drug Administration

GFP – Green fluorescent protein

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GvHD – Graft-*versus*-host disease

HA – Hydroxyapatite

HCT – Hematopoietic cell transplantation

HGF – Hepatocyte growth factor

HIC – Hydrophobic interaction chromatography

HIF – Hypoxia inducible factor

HIV – Human immunodeficiency virus

HLA-DR – Human Leukocyte Antigen – antigen D Related

HPL – Human platelet lysate

HSC – Hematopoietic stem cells

Hsp – Heat shock protein

HSV – Herpes simplex virus

HO-1 – Heme oxygenase-1

iBB – Institute for Bioengineering and Biosciences

IDO – Indoleamine 2,3-dioxygenase

IFNγ – Interferon γ

Ig – Immunoglobin

IGF-1 – Insulin-like growth factor 1

IL – Interleukin

IMDM – Iscove's modified Dulbecco's medium

iPSC – induced Pluripotent stem cells

ISCT – International Society for Cell Therapy

IST – Instituto Superior Técnico

LIF – Leukemia inhibitory factor

LPL – Lipoprotein lipase

LPLD – LPL deficiency

MACS – Magnetic-activated cell sorting

MC – Minicircle

MHC I/II – Major histocompatibility complex class I or II

MI – Myocardial infarction

MLR – Mixed lymphocyte reaction

MP – Miniplasmid

mRNA – messenger RNA

MRS – Multimer resolution site

MSC – Mesenchymal stem/stromal cells

NLS – Nuclear localization sequence

NO – Nitric oxide

NPC – Nuclear pore complex

OD_{600} – Optical density at 600 nm

PAD – Peripheral arterial disease

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

pDNA – plasmid DNA

PEI – Polyethyleneimine

PGC-1α – peroxisome proliferator-activated receptor-γ coactivator-1 alpha

PGE2 – Prostaglandin E2

PLL – Poly-L-lysine

PP – Parental plasmid

PPARγ – Peroxisome proliferator-activated receptor γ

RNA – Ribonucleic acid

RPE65 - Retinal pigment epithelium-specific protein 65 kDa

runx2 – Runt-related transcription factor 2

SCF – Stem cell factor

SCID – Severe combined immunodeficiency

SDF1α – Stromal-derived factor 1α

SF – Serum-free

sHLA-G – soluble Human leucocyte antigen G

siRNA – small interfering RNA

SSEA-4 – Stage-specific embryonic antigen 4

SVF – Stromal vascular fraction

TALENS – Transcription activator-like effector nucleases

TcO_2 – Transcutaneous oxygen tension

TGF-β – Transforming growth factor

Th – Helper T cell

TLR – Toll-like receptor

TNFα – Transforming necrosis factor α

TRAIL – TNF-related apoptosis inducing ligand

Treg – Regulatory T cell

UCB – Umbilical cord blood

UCM – Umbilical cord matrix

VEGF – Vascular endothelial growth factor

WJ – Wharton Jelly

Xeno – Xenogeneic

XF – Xenogeneic-free

ZFN – Zinc-finger nucleases

αMEM – alfa modified Eagle's medium

AIM OF STUDIES AND THESIS OUTLINE

PAD is a chronic and high burden chronic disease that affects more than 27 million individuals in Europe and USA. The prevalence of PAD in subjects older than 70 ranges between 15-20% and is expecting to increase on the next few years due to population ageing and growing incidence of cardiovascular diseases. In the early stages of the disease, patients may have no or only mild symptoms that can be reversed and/or prevented by a change of lifestyle. However, in more severe cases, as CLI, revascularization may be the only option. This is an extremely invasive procedure with several contra-indications and low effectiveness and some subjects are not eligible, especially those with several co-morbidities. These patients, the so-called no-option patients, are faced with the possibility of limb amputation or even death. So, novel strategies aiming to stimulate collateral blood vessel formation and restoration of blood flow to the ischemic tissues need to be addressed. MSC have emerged as a promising cell type for regeneration of ischemic tissues due to their low immunogenicity, as well as immunomodulatory and regenerative properties. The capacity of MSC to repair and regenerate ischemic tissues has been observed in several studies. The most accepted explanation for this therapeutic activity is that it relies essentially in paracrine secretion of pro-angiogenic soluble factors, such as VEGF. However, the harsh ischemic environments found upon *in vivo* administration may contribute for the low engraftment and survival of MSC. Thus, strategies to improve the potential of MSC-based cell therapies are required. Genetic engineering using pro-angiogenic factors was found to improve their angiogenic activity and induce tissue regeneration, enhancing their overall therapeutic potential for ischemic conditions. Hence, the aim of this thesis was the development of an MSC-based product with improved secretion of VEGF, one of the most important pro-angiogenic factors, for the treatment of PAD. Between the different approaches available for genetic engineering of MSC, a minicircle-based strategy was selected herein, on the basis of several studies that demonstrated its efficacy and safety for gene therapy purposes.

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

Chapter II - DESIGN, PRODUCTION AND PURIFICATION OF NON-VIRAL VECTORS FOR GENE THERAPY

This chapter describes the successful design, production and purification of VEGF-containing vectors suitable for MSC engineering. Minicircles, which are small plasmid derivatives only with transcriptional unit, containing VEGF or VEGF in fusion with GFP (VEGF-GFP) were constructed to be used on next chapters for MSC modification. The benefits of minicircles over conventional plasmids include higher transgene expression and improved safety. To further confirm this assumption, pDNA molecules (pVAX) with similar genetic cassettes (VEGF and VEGF-GFP) were also produced and purified according to standard protocols. One of the major limitations of minicircle application for clinical purposes is the lack of an efficient system for its production and purification. In this chapter we confirmed the efficacy of a recently developed protocol for minicircle production and isolation. The use of a production strain specifically designed for arabinose uptake and recombinase production allowed the obtention of high minicircle yields. On the other hand, the combination of a nicking enzyme digestion step with elution by HIC was able to promote an effective isolation of pure fractions of minicircle. Non-viral vectors (plasmids and minicircles) containing VEGF or VEGF-GFP gene constructs were successfully produced within *E. coli* strains and purified using a commercial kit and/or a HIC approach. After purification procedures, both vectors were pure and ready to be used for human cell transfection.

Chapter III - GENETIC ENGINEERING OF MESENCHYMAL STEM/STROMAL CELLS (MSC) WITH VEGF-GFP-ENCODING VECTORS

This chapter aimed at testing the efficiency of minicircles to be used as vectors for MSC engineering. Gene therapy vectors (plasmids and minicircles) encoding VEGF-GFP fusion gene and obtained as described of previous chapter (II) were used for MSC transfection. It was confirmed the efficacy of a previously established microporation-based protocol for MSC modification. The transfected cells were analyzed in terms of cell viability and recovery, as well as GFP expression and yield of transfection. Microporation of MSC with either pVAX or minicircle does not significantly affect MSC proliferative potential or cell viability. The comparison between GFP expression by MSC after transfection with pVAX-VEGF-GFP or MC-VEGF-GFP, revealed higher GFP^+ cells on the group transfected with pVAX-VEGF-GFP (≈41%) than on cells modified with MC-VEGF-GFP (≈26%). However, those levels were lower than the 67% observed after modification with pVAX-GFP (used as a positive control for transfection). The western blot analysis of cell lysates further confirmed these results. The quantification of VEGF gene

expression by qPCR also showed a higher expression of the transgene on cells modified with pVAX when compared to those engineered with MC-VEGF-GFP. The results from this chapter revealed that the use of a fusion construct might not be the best strategy for monitoring expression of an extracellular protein, such as VEGF, within MSC.

Chapter IV - ENGINEERING OF HUMAN MESENCHYMAL STEM/STROMAÇ CELLS (MSC) WITH VEGF-ENCODING MINICIRCLES FOR ANGIOGENIC *EX VIVO* GENE THERAPY

In Chapter IV it was evaluated the angiogenic potential of MSC genetically engineered with VEGF-encoding minicircles produced and purified as described on Chapter I. MSC were transfected by microporation with either pVAX-VEGF or MC-VEGF. These cells were then analyzed in terms of cell viability and recovery, differentiation potential, immunophenotype, transgene expression and *in vitro* angiogenic capacity. As previously observed on chapter III, the transfection did not significantly affect cell viability or proliferative potential of MSC. Also, the differentiation potential and immunophenotype of these cells were maintained after transfection with pVAX-VEGF or MC-VEGF. The results showed that a higher number of MSC can be recovered from the same initial cell number when using MC-VEGF (≈72%) compared to pVAX-VEGF (≈45%), which represents an advantage of minicircle-based gene therapy over the use of conventional plasmids. The quantification of VEGF expression performed by qPCR and ELISA confirmed the efficient overexpression of this protein after transfection with both pVAX-VEGF and MC-VEGF. The levels of VEGF assessed by these techniques were significantly superior for transfected MSC than for non-modified cells (11.1±3.4 pg/1000 cells day^{-1}). The highest VEGF production was observed two days after transfection with MC-VEGF (644.8±82.5 pg/1,000 cells day^{-1} and 130-fold increase on mRNA copies) compared to the levels for pVAX-VEGF at the same timepoint (508.3±164.0 pg/1,000 cells day^{-1} and 50-fold increased on mRNA copies). Finally, the angiogenic potential of modified cells was confirmed using the following *in vitro* functional assays: cell tube formation and cell migration. The results from this chapter revealed that genetic engineering of MSC with VEGF-encoding vectors is able to improve the *in vitro* angiogenic capacity of these cells as demonstrated by the number of tubes and branch points and percentage of migrated cells. MSC modified with MC-VEGF showed superior angiogenic capacity than those modified with conventional plasmids. The results indicate that MSC engineering with VEGF-encoding minicircles might be a promising strategy for the treatment of ischemic diseases, such as PAD.

Chapter V - COMPARISON OF THE ANGIOGENIC POTENTIAL OF MESENCHYMAL STEM/STROMAL CELLS (MSC) FROM DIFFERENT HUMAN SOURCES AFTER MICROPORATION WITH VEGF-ENCODING MINICIRCLES

In Chapter V it was compared the angiogenic potential of MSC from different sources (BM, AT and UCM) after being transfected with VEGF-encoding minicircles. It was confirmed that microporation with MC-VEGF did not affect proliferative potential, differentiation capacity or immunophenotype of MSC, regardless the source from which the cells were obtained. Also, all the three sources evaluated were successfully and efficiently transfected with MC-VEGF. This was demonstrated both by the higher copies of VEGF gene measured by qPCR or by the increased levels of VEGF protein detected on culture medium by ELISA compared to non-transfected counterparts. The quantification of VEGF secretion to the culture medium by transfected cells on day 2 revealed no significant differences between BM- (543.5±19.9 pg/1000 cells day^{-1}), AT- (462.2±170.8 pg/1000 cells day^{-1}) or UCM-derived cells (612.8±174.9 pg/1000 cells day^{-1}). Similarly, the results from the present chapter showed that the fold-increase on the number VEGF gene copies was identical for the three sources. The *in vitro* angiogenic capacity of these cells was further confirmed using angiogenic functional studies described in Chapter IV. The transfected cells demonstrated superior angiogenic capacity in both tube formation and migration assays, regardless the source. However, no significant differences were observed in terms of functional activity of transfected cells between the three sources. The results from this chapter indicated that MSC from either BM, AT or UCM could be efficiently transfected by microporation using VEGF-encoding minicircles with similar outcomes. The protocol developed in Chapter IV for BM-MSC transfection revealed to be effective for genetic engineering of MSC from other sources.

Chapter VI - EVALUATION OF THE THERAPEUTIC POTENTIAL OF HUMAN MESENCHYMAL STEM/STROMAL CELLS MODIFIED WITH VEGF-ENCODING MINICIRCLES IN AN *IN VIVO* MODEL

The aim of Chapter VI was to evaluate the therapeutic potential of MSC genetically engineered with VEGF-encoding minicircles in a mouse model of hindlimb ischemia in the context of PAD. In this chapter, the angiogenic potential of MSC modified with MC-VEGF was investigated *in vivo*. MSC were modified by nucleofection, which demonstrated to effectively promote transgene expression within these cells. A preliminary analysis with pmaxGFP, a GFP-encoding plasmid, revealed that approximately 65% of nucleofected MSC expressed the transgene. Nucleofection was then applied for engineering BM-MSC with MC-VEGF based on the results from Chapter IV.

To assess the potential of engineered MSC as a treatment for PAD, a previously established mice model of hindlimb ischemia was used. After ischemia induction, mice were treated by intramuscular administration of non-engineered MSC, MSC engineered with MC-VEGF (MSC+MC) or by gene therapy with MC-VEGF followed by electroporation. Every week, the observation of mice limbs was performed to evaluate necrosis and blood flow measurements were performed by Laser Doppler to analyze revascularization. The results from these analyses showed no significant differences between the three treatment groups. Also, the images from histological analysis were similar for the three groups, but all showed decreased fibrosis and reduced adipocyte accumulation when compared to ischemic non-treated mice. The results regarding evaluation of muscle force, however, showed that treatment with MSC+MC was the best option for improving muscular function in ischemic mice limbs. The evaluation of muscle strength was performed 30 days after treatment administration. The highest value was observed for non-ischemic mice (0.36±0.06 N) followed by mice treated with MSC+MC (0.25±0.04 N). Limbs treated with MSC or MC independently showed similar muscle strengths (0.19±0.03 N and 0.20±0.03 N, respectively), but significantly superior to those observed for ischemic, non-treated mice. The results from this chapter demonstrated that MSC engineered with VEGF-encoding minicircles were able to improve functionality on ischemic mice limbs. So, this can be a promising strategy to induce revascularization and promote functional recovery for PAD patients.

Chapter I

INTRODUCTION

I. INTRODUCTION

I.1. Background

The interest over stem cells has been growing over the last decades due to their unique properties, which have great relevance for clinical approaches. These primitive cells have both the capacity to self-renew in an undifferentiated state and the ability to differentiate and give rise to several specialized cell types that are the building blocks of tissues and organs. These distinctive characteristics of stem cells open many clinical opportunities and applications, which can be divided in three major fields: (i) cell therapies and tissue engineering, (ii) disease modelling and (iii) drug development.

Stem cells can be isolated from either embryonic/fetal or adult tissues and, according to their potency, they can be classified as pluripotent or multipotent. Pluripotent stem cells are able to turn into cells from the three germ layers (endoderm, mesoderm and ectoderm), while multipotent cells only can differentiate into a limited number of cell types, all from the same germ layer (Bongso and Lee, 2012). Embryonic stem cells (ESC) isolated from the inner cell mass of blastocyst are very attractive from a clinical point of view and have been extensively studied due to their primitive state and pluripotency, giving them increased versatility (Evans and Kaufman, 1981). Extensive research has also been conducted on adult stem cells, since they have the advantage of not having the ethical constraints associated to ESC obtained from human embryos (Kolios and Moodley, 2013).

To overcome ESC availability and ethical limitations associated with destroying an embryo to obtain the cells, induced pluripotent stem cells (iPSC) were generated by reprogramming of adult somatic cells to an ESC-like state (Takahashi and Yamanaka, 2006). These iPSC have been reprogrammed from adult fibroblasts by addition of four transcription factors, and could be used for development of patient-specific therapies with minimal risk of immune rejection (Yamanaka, 2010). Nevertheless, the clinical use of iPSC for cell replacement strategies have limitations, such as their intrinsic tumorigenic potential (teratoma formation) when injected *in vivo* and the issue in controlling differentiation process which leads to heterogeneous cell populations (Ahmed et al., 2011).

Adult derived multipotent cells have a limited differentiation capacity, only being able to give rise to specialized cells from the same lineage. This can be advantageous in terms of safety for clinical settings, since the differentiation process is much easier to control and there is no risk of

teratoma formation. The role of adult stem cells *in vivo* is to maintain the cell homeostasis, by replacing, with some limitations, cells that die due to injury or disease. Although adult stem cells are not easy to isolate and purify, they have been obtained from different human tissues, such as bone marrow (BM), brain, skin, eyes, heart, kidneys, lungs, gastrointestinal tract, pancreas, liver, breast, ovaries, prostate, and testis (Mimeault and Batra, 2006).

BM is probably the most studied source of adult stem cells, since two types of important stem cells can be isolated from this tissue: (i) mesenchymal stem/stromal cells (MSC) and (ii) hematopoietic stem cells (HSC) (Bonnet, 2003). HSC are responsible for the production and maintenance of all types of blood cells, while MSC give rise to many supporting cells (e.g. stroma). BM is the only known niche where two types of distinct stem cells do not only co-exist but they also functionally cooperate (Bonnet, 2003). In fact, BM transplantation is the most studied and well established stem cell based treatment that started to be applied in late 1950s (Thomas et al., 1957). HSC obtained from a healthy donor can be used to repopulate a damaged BM after myeloablation through irradiation or chemotherapy (Felfly and Haddad, 2014). Although in the past it was believed that the only stem cells present in BM were HSC, in 1970s Friedenstein and colleagues identified a type of BM stem cells that develop into fibroblastic colony forming cells (CFU-F) and are thought to be responsible for the structural and physiological support for hematopoiesis (Friedenstein et al., 1970). Nowadays, it is clear that the functions of MSC are far beyond simply providing support to blood cell formation, and have been extensively studied for different clinical applications. Although BM is probably the most studied niche of MSC, they can be obtained also from neonatal tissues, such as umbilical cord. These tissues may represent promising cell sources with great advantages. Umbilical cord is usually discarded at birth and can be a relevant stem cell source since these cells can be retrieved from both umbilical cord matrix (UCM) and umbilical cord blood (UCB). The clinical use of cells from this tissues, considered as “biological waste”, present many benefits besides the ready availability and ease of collection, since they are more primitive and therefore have a decreased probability of immune rejection and increased proliferation capacity (Hordyjewska et al., 2015).

The effective application of stem cells in clinical practice is limited, mainly due to the low number of cells available in the sources used more frequently, especially from adult tissues. Hence, strategies to expand these cells *ex vivo* in order to obtain clinical meaningful cell numbers need to be established. Also, methods to enhance the stem cell therapeutic potential may be developed. Genetic engineering tools can be used to modify and improve the beneficial properties of these cells, by inducing/inhibiting the production of different factors. Combination of stem cell properties with gene therapy approaches could be a promising strategy to

revolutionize regenerative medicine, by developing new and patient-oriented cell-therapy products.

The work developed throughout this thesis was focused essentially on MSC from BM, but also from UCM and AT, and on gene therapy approaches using non-viral methods to improve the therapeutic properties of this cells, particularly their pro-angiogenic capacity.

The introduction was divided into five chapters: Background (I.1), Mesenchymal Stem/Stromal Cells (I.2), Gene Therapy (I.3), Peripheral Arterial Disease (I.4) and Future perspectives and challenges (I.5).

I.1.1. Mesenchymal Stem/Stromal Cells (MSC)

MSC were first discovered by Friedenstein and co-workers who isolated and identified a group of cells from BM that develop into CFU-F and are associated with hematopoiesis sustenance (Friedenstein et al., 1970). These cells were initially named “marrow stromal cells” because initially their application in laboratory was only to form feeder layers for HSC (Shigematsu et al., 2010). Currently these cells are known as MSC, since they are associated to the normal turnover and preservation of adult cells from mesenchymal lineages (Caplan, 1991).

Although BM was the prevailing source from human MSC, today it is known that these cells can be obtained from other adult sources and also from perinatal tissues. AT (Zuk et al., 2001), dental pulp (Huang et al., 2009) or synovial fluid (Santhagunam et al., 2014) are examples of adult sources alternative to BM. MSC could also be found on perinatal tissues including UCM (Simoes et al., 2013), amniotic fluid (In 't Anker et al., 2003) or placenta (Miao et al., 2006). Throughout this thesis the focus will be on MSC from three sources more relevant in clinical setting: BM, AT and UCM.

MSC hold great promise for numerous clinical applications due to their unique beneficial properties, since they are low immunogenic and have immunomodulatory properties due to the secretion of different soluble factors (Singer and Caplan, 2011, Gebler et al., 2012). These properties open the possibility of using MSC in an allogeneic context, where cells isolated from an individual (donor) are expanded *ex vivo* and used in the patients as an off-the-shelf therapeutic product. This is opposed to the autologous approach, where patient-specific cells are used in a personalized therapy (Elseberg et al., 2017).

I.1.2. MSC characterization

Regardless the tissue from where they are obtained, these cells share some intrinsic properties that allow to identify and classify them as MSC. As all stem cells, they have the ability to self-renew maintaining in an undifferentiated state and are associated with mesenchymal tissue normal renewal and maintenance (Pittenger et al., 1999).

Functionally, adult MSC are characterized by an average doubling time of 33 hours and have a large expansive potential, being able to replicate in culture in an uncommitted state, while retaining their multipotency (Bonnet, 2003). MSC were proven to be able to differentiate into several lineages of mesodermal origin such as cartilage, bone, fat, tendon, muscle, myocardium, and marrow stroma under appropriate culture conditions (Figure I.1).

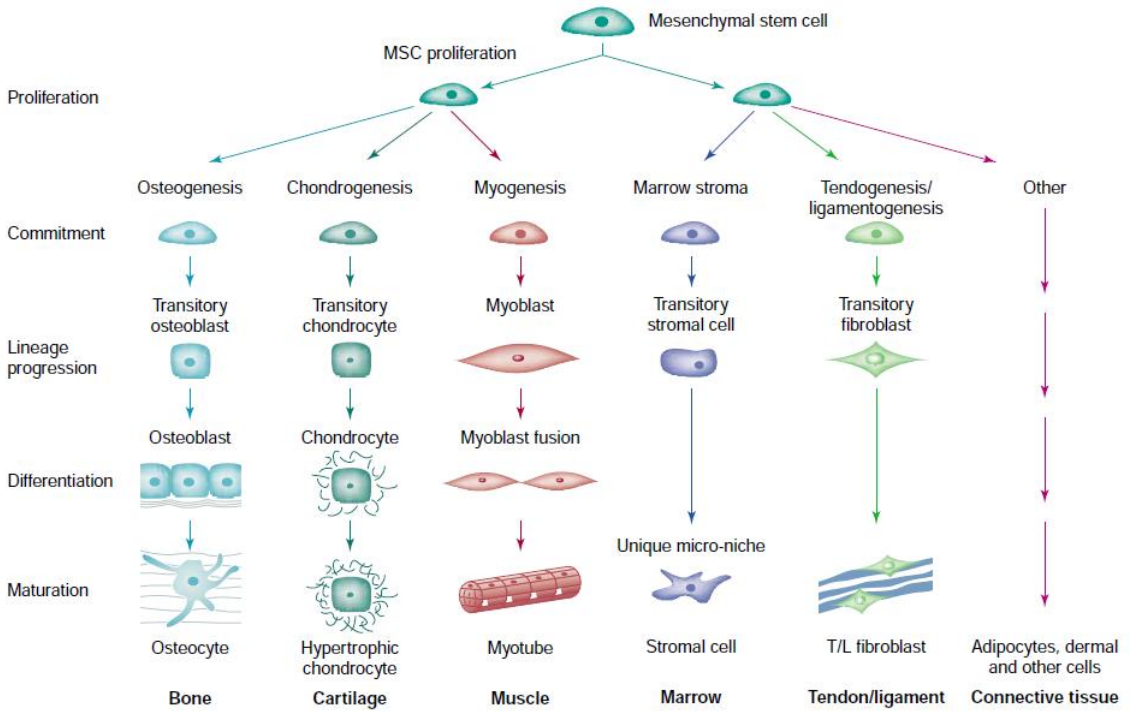

Figure I.1 - Simplified cellular transitions from the MSC to highly differentiated cell phenotypes. MSC are able to undergo extensive cell proliferation prior to differentiate into a range of mesenchymal cell types including bone, cartilage, muscle, stroma, tendon and adipose (Caplan and Bruder, 2001).

Since there is no single and specific biomarker for MSC isolation or characterization and properties of these cells may vary according to the source from where they are obtained, in 2006 the International Society for Cell Therapy (ISCT) proposed three minimal criteria for defining MSC. These criteria are (i) the ability to adhere to plastic under standard culture conditions; (ii) expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR cell surface markers and (iii) the capacity to differentiate into osteoblasts, adipocytes or chondroblasts upon induction *in vitro* (Dominici et al., 2006, Baer and Geiger, 2012). Despite few differences may be observed in MSC properties according to the source, isolation method or expansion conditions, this definition is valid for all MSC.

MSC have been extensively studied on the last years and researchers identified other surface markers besides those proposed by ISCT that can be used to recognize and isolate these cells. A study from Mafi and co-workers summarizes and brings together the available information on MSC cell surface markers. According to this, CD105, CD90, CD73, CD44, CD29 and CD13 are examples of commonly reported positive markers, while CD34, CD14, CD45 and CD11b are the most frequently reported as negative (Mafi et al., 2011, Zuk et al., 2001, Pittenger et al., 1999). This panel of surface markers allows not only to identify and isolate MSC but also to exclude other cell types, likely to be found in MSC cultures as contaminants. CD34 is expressed both on hematopoietic progenitors and endothelial cells (Sidney et al., 2014); CD45 is a pan-leukocyte

marker (Nakano et al., 1990) and CD14 and CD11b are markers of monocytes and macrophages, which are the most likely contaminants of MSC cultures (Pilz et al., 2011). CD79α or CD19 should also be tested since they are expressed on B cells that may also adhere and be maintained on MSC cultures (Wang et al., 2012). Finally, HLA-DR should not be found on MSC, unless they are activated and thus being called "stimulated MSC" (Dominici et al., 2006). According to ISCT criteria, a MSC population is considered "more homogeneous" if it has more than 95% of cells expressing CD73, CD90 and CD105 and has less than 2% of cells expressing the proposed negative markers (Dominici et al., 2006). Although it is not described in ISCT statement, STRO-1 is also a marker commonly used for identification and isolation of MSC (Goncalves et al., 2006, Simmons and Torok-Storb, 1991). However, this is an example of a marker that may be tissue-specific, since STRO-1 has been consensually described as positive for BM derived MSC but his presence in cells isolated from AT is controversial (Gronthos et al., 2001, Zuk et al., 2001).

In contrast with MSC *in vivo*, the proliferation apparatus of cultured MSC is highly activated and the expression of MSC surface markers is increased in culture (Hoogduijn et al., 2014). The impact of culture on the expression of chemokine receptors remains unknown, so cultured and non-cultured MSC may therefore home to different sites *in vivo* and have different interplays with the immune system.

I.1.2.1. Culture conditions

The capacity of MSC to be expanded *in vitro* with relatively great genomic stability and few associated ethical issues marks once again their relevance in cell therapy and regenerative medicine (Stultz et al., 2016, Dominici et al., 2006). To benefit from MSC therapeutic potential there is the need to culture these cells for several passages in order to achieve clinically meaningful cell numbers that cannot be obtained directly from the tissue from where they are isolated. Therefore, development of optimal culture conditions for MSC expansion *in vitro*, able to maximize cell numbers with minimal changes in MSC intrinsic properties, is extremely important. Medium composition, cell seeding density and physical-chemical environment (dissolved O_2 and CO_2, pH and temperature) are examples of culture parameters that may be addressed for optimization (Mushahary et al., 2018). Thus, manipulating culture conditions can also be used to tune MSC properties.

<u>Culture medium formulation</u>

One of the most important parameters in cell culture is the culture medium used. It should not only promote high proliferation but also should not affect cell main characteristics. Medium

formulations are usually composed of a basal medium (with glucose and glutamine) and a supplement rich in growth and adhesion factors that will contribute for cell adhesion and proliferation (Sotiropoulou et al., 2006). Several types of basal media are available, being the most common for MSC culture: Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), Minimum Essential Medium Eagle alpha (αMEM) and Optimem. Although all of these formulations are able (with exception to IMDM) to support MSC growth while maintaining their features, DMEM is the more commonly used (Sotiropoulou et al., 2006). For MSC culture it should be supplemented with 1000 mg/mL glucose (low glucose) and Glutamax instead of L-glutamine, since L-glutamine is chemically unstable and is easily decomposed into ammonia, which may inhibit cell growth (Christie and Butler, 1994).

Culture medium supplementation

Culture media supplemented with fetal bovine serum (FBS) have been routinely used for human MSC expansion at a concentration ranging between 10-20% (Mushahary et al., 2018). Although FBS contains high concentrations of both growth and attachment factors, essential for cell expansion and maintenance, there are many concerns associated to its use. Besides being a complex xeno(geneic) compound with great lot-to-lot variation, it also brings the risk of contamination with prions, virus or mycoplasma and possible immunological reactions (Sundin et al., 2007, Mannello and Tonti, 2007). Another important advantage of replacing animal serum is the decrease on demand of animal supplies (Dimarakis and Levicar, 2006).

To overcome the safety issues related to FBS, human-blood derived supplements, such as human serum, human platelet lysate (HPL) or cord blood serum as well as serum-free formulations with a more defined composition, have been tested for MSC expansion. The use of autologous human serum would be the ideal approach to avoid contaminations or immunological responses, and even though this option should not be completely discarded it is not feasible for an "off-the-shelf" stem cell therapy (Dimarakis and Levicar, 2006). Thus, other xeno-free medium formulations should be established for MSC culture.

Studies with human serum revealed a higher proliferation capacity of MSC compared to the use of FBS while main cell characteristics (morphology, viability, differentiation capacity or immunophenotype) were maintained (Paula et al., 2015). However, human serum has high donor-to-donor variability and presents some other limitations and risks (Karnieli et al., 2017). Although the donor-specific variability could be decreased with pools of serum from a significant number of donors (de Soure et al., 2016, Paula et al., 2015), one of the major limitations is the availability, since there is a limited amount that can be collected. There is also some level of

concern associated with the use of human blood-derived products due to the risk of spreading new and unknown human pathogens.

The supplement choice may also depend on the final therapeutic purpose of the cells. Even though there are few studies using cord blood serum for media supplementation, its use may be advantageous for treatment of bone-related diseases, for example, since MSC expanded with this serum showed not only higher proliferation capacity compared to FBS but also an enhanced osteogenic potential (Jung et al., 2009).

Another extensively studied, and probably the best alternative to FBS for MSC expansion, is HPL (Astori et al., 2016, Burnouf et al., 2016). This lysate can be easily obtained by repeated freeze/thaw cycles and sonication from fresh blood or platelet concentrates and contains a series of bioactive molecules such as chemokines, growth factors and adhesion molecules (Burnouf et al., 2016). Several studies showed that HPL significantly increased the proliferative capacity of MSC from either BM (Capelli et al., 2007), AT (Naaijkens et al., 2012) or UCM (de Soure et al., 2017) without changing their surface markers or differentiation ability. However, contradictory results have been reported regarding MSC immunosuppressive potential. Whereas there are studies reporting that MSC immunomodulatory properties are not affected by culture in HPL (Capelli et al., 2007), a recent study revealed that expansion in HPL may diminish MSC immunosuppressive features (Oikonomopoulos et al., 2015). These discrepancies observed could be due to variability between different HPL batches. Also, HPL or human serum supplements need a prior step of inactivation/irradiation similarly to FBS in order to avoid the risk of viral or pathogen transmission(Viau et al., 2019). In conclusion, the ideal approach is to use a well-defined medium formulation that do not relies in animal or human-derived serum supplementation.

Usually serum-free (SF) media are composed by a basal medium that is chemically defined and by a supplement with high protein content (growth factors, hormones and other proteins) that may be chemically defined or undefined (de Soure et al., 2016). Those undefined formulations are free from serum itself but contain purified proteins and growth factors from human origin. Nevertheless, well-defined SF and xeno-free (XF) formulations with recombinant proteins, instead of human derivatives, are available - TheraPEAK™ MSCGM-CD™ (Lonza) and MSC NutriStem® XF (Biological Industries) - and have been successfully used for MSC expansion (Tan et al., 2015, Wang et al., 2014b), their use is not yet well established for routinely culture of MSC.

There are SF/XF options that despite being not fully defined, have been successfully used for MSC expansion with high quality and reproducibility, reducing the main issues related with use of serum. MesenCult™-XF (Stem Cell Technologies) and StemPro® MSC SFM XF (Thermo Fisher) are examples of such formulations. MesenCult™-XF is able to efficiently promote expansion of MSC from both AT (Al-Saqi et al., 2014) and UCM (Chen et al., 2014), but it was reported to not support BM-MSC expansion beyond passage five (Pal et al., 2009). StemPro® MSC SFM XF on the other hand has been successfully used in our and other groups for expansion of MSC from different sources (Tan et al., 2015, Carmelo et al., 2015, Simoes et al., 2013), leading to high cell numbers without compromising cell surface markers or multilineage differentiation potential. Commonly, the use of these SF media requires an additional substrate to promote cell adhesion, which is not needed for serum-dependent cultures. Still, the major limitation regarding the use of SF formulations is on cell isolation, since none has proven to be as efficient as serum-containing media. Hence, StemPro® MSC SFM XF supplemented with 2.5% of human serum can be efficiently used for isolation of MSC from BM (Chase et al., 2012).

Seeding density

Seeding density is also a critical parameter in MSC culture, it was observed that reducing seeding densities leads to an increase in proliferation. There is an inverse correlation between initial plating density and population doublings, and studies refer an initial plating density of 1000 cells per cm^2 as the optimal condition (Sotiropoulou et al., 2006, Nekanti et al., 2010). It was observed that neither initial seeding density nor passaging density affect MSC phenotype, immunosuppressive capacity or multilineage differentiation potential (Sotiropoulou et al., 2006).

Oxygen tension

Other important factor that may have impact on MSC proliferation is the oxygen tension. In order to mimic the niche of BM-MSC *in vivo*, studies of culturing cells under low oxygen tensions have been performed. It was observed that MSC cultured under hypoxia (2% of O_2) showed higher proliferative and clonogenic potential than when cultured under normoxia (20% of O_2) (dos Santos et al., 2010).

All culture parameters referred to increase MSC proliferation could be easily and rapidly applied to clinical-scale expansion of MSC in fully controlled systems - bioreactors - leading to increased cell yields and therefore reducing culture time and costs. Bioreactors are of special interest in an allogeneic setting, since they can be used to produce multiple doses of MSC-based

therapeutic products that can be available as an off-the-shelf cell therapy, allowing an immediate access for acute interventions.

I.1.2.2. Multilineage differentiation potential

The capacity to differentiate into mesodermal lineages is a determining property of MSC. In fact, the *in vitro* ability to give rise to adipocytes, osteocytes and chondrocytes is one of the minimal criteria to identify MSC according to ISCT (Dominici et al., 2006). This differentiation capacity may be stimulated by proper media supplementations, which activate transcription factors responsible for induction of each cell lineage. The differentiation into adipocytes, osteocytes and chondrocytes can be confirmed by oil droplet production, formation of mineralized matrices and expression of type II collagen, respectively and/or by evaluation of lineage-specific gene expression (Pittenger et al., 1999).

Briefly, the standard adipogenic differentiation protocol consists in culturing MSC in a basal medium containing FBS and supplemented with dexamethasone, insulin, isobutyl methyl xanthine and indomethacin for 2-3 weeks. After that, adipogenic induction is evaluated both by presence of lipid vacuoles, that may be detected with oil red O, and by expression of adipocyte-specific genes, such as peroxisome proliferator-activated receptor γ (PPARγ), adipocyte protein 2 (ap2) and lipoprotein lipase (LPL) (Pittenger et al., 1999, Rosen and MacDougald, 2006).

The classical protocol for osteogenic differentiation requires the incubation of cells for about 2-3 weeks in osteogenic medium, which is a basal medium with FBS complemented with ascorbic acid, β-glycerophosphate and dexamethasone. The osteogenic differentiation is characterized and can be detected by an increase in alkaline phosphatase activity (ALP) and formation of mineralized regions, which are positive for Alizarin Red or von Kossa staining. Expression of osteogenesis-related genes, such as runt-related transcription factor 2 (runx2), osteopontin or bone morphogenetic protein 2 (BMP2), may also be used to evaluate osteogenesis (Neve et al., 2011, Pittenger et al., 1999).

Finally, for chondrocyte differentiation cells are cultured as micromass pellets in a medium containing dexamethasone and transforming growth factor β3 (TGF-β3) without FBS. After 2-3 weeks of culture, production of cartilage-specific sulphated proteoglycans can be observed by staining with Alcian Blue (Mackay et al., 1998). Collagen I and collagen X are examples of chondrogenic genes that may be used to quantify differentiation into this lineage (Pittenger et al., 1999).

Although these are the three main lineages that could be obtained from MSC, they can also differentiate into other cells from mesenchymal tissues upon induction *in vitro*. More than 20 years ago, Wakitani et al. showed that treatment of MSC with 5-azacytidine lead to myotube formation (Wakitani et al., 1995) and later was observed that the use of this compound on MSC lead to a cell population with cardiomyocyte-like properties (Xu et al., 2004). Another report revealed that MSC can give rise to functional muscle fibers able to improve degeneration of muscle tissues (Ninagawa et al., 2013). Even though these reports reveal that MSC may have a myogenic potential *in vitro*, the signaling is not completely understood and further investigation should be performed to comprehend the mechanisms behind myocyte differentiation *in vivo*.

Despite their mesordermal origin, there are reports showing the MSC potential to trans-differentiate into ectodermal lineages, such as neurons (Hofstetter et al., 2002), or endodermal, such as hepatocytes (Hang et al., 2014) or pancreatic β-cells (Tang et al., 2012). However, this plasticity of MSC brings some controversy regarding the functionality of derived cells and the trans-differentiation efficacy and safety is yet to be proved.

I.1.3. MSC isolation and sources

The success in obtaining clinically relevant MSC numbers for therapeutic applications relies not only on culture conditions but also on optimization of efficient isolation procedures. The features of MSC depend on the tissue from where they are obtained. For example, UCM-derived cells are reported to have an increased expansion potential (Simoes et al., 2013, Kern et al., 2006) and AT-MSC to have an improved immunomodulatory capacity (Ribeiro et al., 2013). So, the choice of the ideal MSC source will depend on the therapeutic application. Herein, it will be discussed the isolation procedures and properties of MSC obtained from BM, AT and UCM. Although AT and UCM have advantages over BM, such as ease of collection and higher cell yield, BM is still the most used tissue for MSC isolation. Also, most clinical trials to date with MSC use BM-derived cells.

I.1.3.1. Bone Marrow (BM)

BM is the flexible tissue found in the interior of bones and is the site of hematopoiesis, the process by which all blood cells are produced. This process is supported by the BM stroma which consists of fibroblasts, osteoblasts, adipocytes, endothelial cells, macrophages and extracellular matrix (ECM) (Moore and Lemischka, 2006). MSC were firstly isolated from BM (Friedenstein et al., 1970), but represent only 0.001 to 0.1% of BM cell population and are known to be involved

in hematopoiesis support by providing ECM elements, cytokines and growth factors (Moore and Lemischka, 2006).

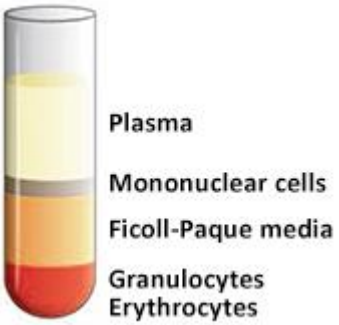

Figure I.2 - Separation of BM components after the density gradient centrifugation step with Ficoll-Paque (Sigma-Aldrich website).

To isolate MSC from BM aspirates, the first step is to perform a density gradient centrifugation step using a polymeric solution (e.g. Ficoll-Paque PLUS or Percoll) to separate the mononuclear cell (MNC) fraction from the other marrow cells, such as erythrocytes, and plasma as schematized in Figure I.2 (Gottipamula et al., 2014). The MNC fractions contain a small number of MSC that can be further isolated by two different methods. The more common and cost-effective is by plastic adherence to tissue culture surfaces (polystyrene), where isolated MNC are plated on culture flasks. Since one of the MSC properties is their ability to adhere to plastic, they remain attached to tissue culture flasks, while contaminant cells are washed away by culture medium changes and passaging (Lennon and Caplan, 2006). The other method to isolate MSC is by immunobased cell sorting, either by fluorescence or magnetic activated cell sorting (FACS or MACS) using antibodies against certain cell markers, which creates a more homogeneous population. Different surface markers have been explored (SSEA-4, CD271 and CD146), but Stro-1 is probably the best well known and studied for BM-MSC isolation (Lv et al., 2014). However, the lack of an MSC specific marker hinders this process since none of the surface markers brought so far is able to be applied to obtain MSC populations from different tissues that met ISCT criteria (Lv et al., 2014). So, most BM-MSC isolation protocols rely on plastic adherence protocols, that also lead to increased cell yields (de Soure et al., 2016).

In order to obtain cell therapy products compliant with good manufacturing practices (GMP), other systems for BM-MSC isolation have been developed. Quantum Cell Expansion System is an example of a hollow fiber bioreactor able to isolate and expand BM-MSC in a functionally closed system, producing the high demanding cell doses required for therapeutic applications with minimal manipulation (Rojewski et al., 2013).

I.1.3.2. Adipose Tissue (AT)

MSC can also be isolated from AT collected from liposuction procedures. The first isolation protocol to obtain stem/stromal cells from AT was described in 2001 and cells obtained share main features with BM-derived MSC (Zuk et al., 2001). A higher number of MSC can be obtained from this source, when compared with BM, since the stromal vascular fraction (SVF) contains up

to 3% of stem/progenitor cells. On the original protocol, lipoaspirates are washed with Phosphate Buffered Saline (PBS) and then subjected to an enzymatic digestion with collagenase to disrupt ECM, followed by a centrifugation step (Zuk et al., 2001). The pellet obtained contains a mixture of different cell populations, including red blood cells, fibroblasts, pericytes, pre-adipocytes and also MSC, and is called SVF. To remove red blood cells a lysis step was added followed by a filtration to remove cell debris. Then, the obtained cell fraction is plated on adherent tissue culture surfaces and non-adherent contaminants may be removed by washing, medium changes and passaging (Gimble et al., 2007). Although this was the first established protocol, slight variations have been implemented by some groups, including the type and concentration of the enzyme used for ECM disruption, digestion times or the size of the pore of the filters used (Mushahary et al., 2018). Additionally, there are protocols with considerable variations, for example avoiding the enzymatic digestion step by using an adherent column (Doi et al., 2014) or purifying MSC directly from SVF using membrane filtration. Still, the enzymatic-based protocol is the most efficient and widely used.

Although the isolation protocol based on plastic adherence is the most commonly used for MSC isolation from AT, attempts to purify subpopulations by either FACS or MACS have also been performed (Busser et al., 2015). In order to have GMP compliant and standardized AT-based cell products, fully closed systems for isolation and processing of AT samples must be developed. In this context, Celution System was developed, which consists in a closed and automated system that is able to reliably and reproducibly isolate and concentrate regenerative cells from AT at patient bedside ready for immediate application (Fraser et al., 2014). Combination of such systems with fully controlled expansion platforms allowing to obtain the higher cell numbers required for an off-the shelf therapeutic product will be a challenging but ideal approach.

I.1.3.3. Umbilical Cord Matrix (UCM)

UCM, also called Wharton's Jelly (WJ) is a valuable source of MSC, since umbilical cord is a tissue considered medical waste which is usually discarded at birth thus not involving painful or invasive procedures. Besides presenting the advantage of having an increased proliferative potential (Simoes et al., 2013), UCM-derived MSC are also more primitive due to expression of some pluripotent markers (Carlin et al., 2006) and exhibit increased degree of multipotency compared to cells obtained from BM or AT (Fong et al., 2011).

The first step to isolate MSC from UCM is the removal of blood and vessels from the tissue followed by mincing in small pieces. After this procedure, two alternative methods can be

applied to further process UCM and isolate MSC populations: explant cultures or enzymatic digestion (Mushahary et al., 2018, de Soure et al., 2016). For explant culture, the small UCM fragments are directly plated on a plastic culture surface with appropriate culture medium and cells are allowed to migrate from the tissue pieces. After several days, the explants can be removed and the derived cells are attached and growing on the tissue culture surface. The enzymatic digestion relies on the use of enzymes (e.g. collagenase or trypsin) to degrade the ECM. The single cells or aggregates released from this step are then plated on plastic culture surfaces with culture medium.

MSC isolated using both procedures have similar morphology and comparable doubling times (Buyl et al., 2015). The use of explant culture has two main advantages: higher cost-effectiveness and minimal cell damage caused by exposure to enzymatic reagents. Still, this method generates higher cell yields in a shorter time-frame, using enzymatic digestion protocols (Han et al., 2013, Buyl et al., 2015). To take advantage of benefits associated with both methods, some authors suggest that a combination procedure may be used to obtain MSC from UCM tissues (Banitalebi Dehkordi et al., 2016).

Protocols for harvesting UCM-derived MSC in an automated and fully-closed system are much more challenging than for BM or AT, since umbilical cord is a side-product of birth and its safety and quality is not a priority. So, there are not available standardized systems for UCM manipulation under fully closed GMP conditions and the process still relies on fully manual procedures.

I.1.4. Potential therapeutic properties of MSC

Besides their multilineage differentiation potential, MSC also have other important features including immunosuppressive/immunomodulatory properties and capacity of homing to injury sites. These features, together with the low immunogenicity of these cells, make them promising candidates for both cell therapy and tissue engineering approaches.

The capacity of systemically infused MSC to migrate to injury sites and promote tissue repair, also called “homing”, is probably associated with the high cytokine concentration present on the damaged tissue, since it is known that these cells have many cytokine receptors (Yagi et al., 2010). On the other hand, the capacity of MSC to be immune evasive can be justified by a reduced expression of immunogenic surface antigens, due to possessing major histo-compatibility complex class I protein (MHC I) and low/absent expression of MHC II (e.g. HLA-DR) (Singer and Caplan, 2011). This characteristic of MSC presents two main advantages for clinical

purposes (i) they can be infused as potential treatment without major risk of rejection and need of immunosuppression and (ii) they can be used to suppress and/or modulate immune system responses in other cell or tissue transplantation approaches.

Although in some cases cell-to-cell contact may be important, it is believed that the main regenerative properties of these cells rely on paracrine secretion of healing soluble factors. In Figure I.3 are summarized the main potential therapeutic actions of these cells and the associated soluble factors (Singer and Caplan, 2011).

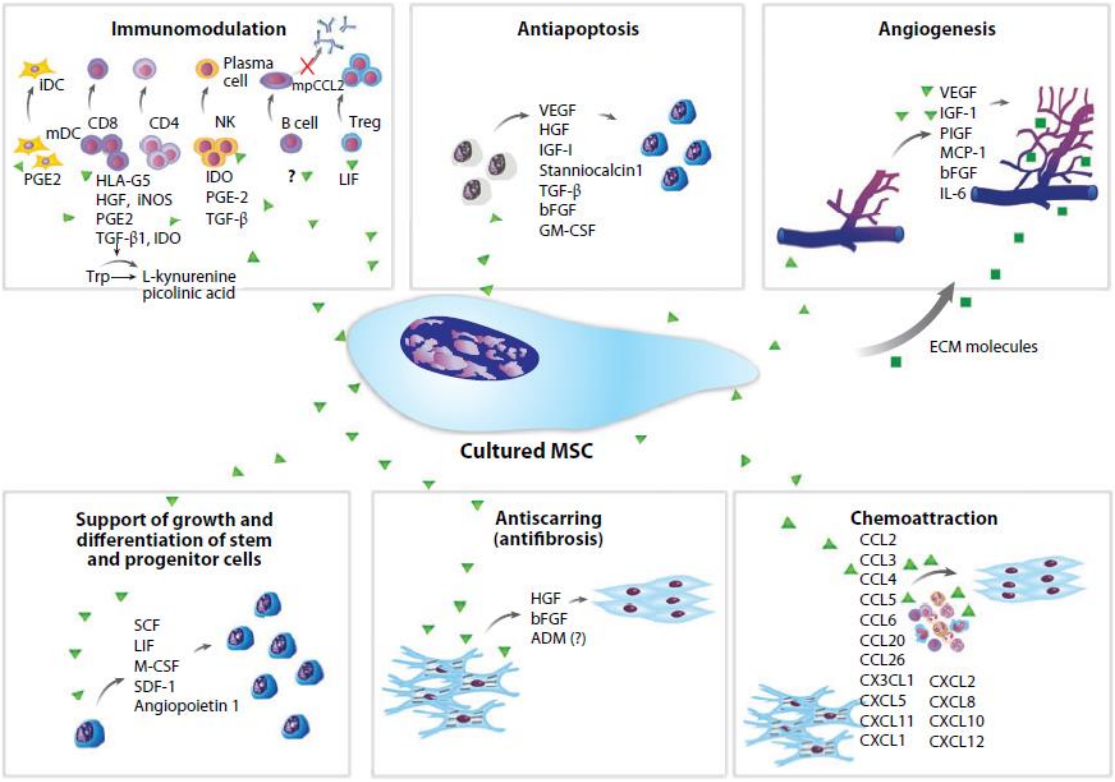

Figure I.3 - Paracrine effects of cultured MSC. These cells are known to secrete many soluble factors which action can be divided into these six mechanisms: immunomodulation, antiapoptosis, angiogenesis, support of cell growth and differentiation, antiscarring and chemoattraction (Singer and Caplan, 2011).

I.1.4.1. Trophic activity

Although the multilineage differentiation potential of MSC was extensively studied for bone or cartilage repair, their trophic activity has been gaining increasing interest in clinical trials in recent years (Caplan and Correa, 2011, Singer and Caplan, 2011, Andrzejewska et al., 2019). This trophic activity, together with the homing ability and low immunogenicity of MSC, has bringing attention for their potential use for wound healing or angiogenesis including on cardiovascular, neural or immunological disorders (Squillaro et al., 2016).

MSC trophic activity was first explored to improve HSC engraftment after hematopoietic cell transplantation (HCT), but when MSC were co-infused with HSC it was observed not only a better HSC engraftment but also a reduction of GvHD symptoms in some patients (Sato et al., 2010, Lazarus et al., 2000). GvHD is a frequent complication and major cause of mortality after HCT and MSC infusion improved overall patient survival even when HLA-mismatch donors were used (Gebler et al., 2012). In fact, the most studied MSC therapeutic application is in the context of GvHD, with many ongoing clinical trials using MSC from different sources (Caplan and Bruder, 2001, Le Blanc et al., 2008, Chullikana et al., 2015)(ClinicalTrials.gov). On the basis of such trials, an MSC cell therapy (Prochymal) has received marketing approval in 2012 in Canada and New Zealand to treat children with acute GvHD. Based on this technology, a similar product (TEMCELL) was developed in Japan and its authorization by Japanese authorities was announced in 2015 (Galipeau and Sensebe, 2018). Since one of the major known limitations of MSC therapy is their poor engraftment after infusion, one can conclude that their beneficial effects are mainly due to their trophic activity.

As described in Figure I.3, MSC-secreted molecules can inhibit apoptosis and scarring, diminish immune system responses, induce angiogenesis and attract and support growth of intrinsic cell populations. Vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), interleukin (IL)-6, stromal-derived factor (SDF)-1α, leukemia-inhibitory factor (LIF), and prostaglandin E2 (PGE2) are between the most important molecules with therapeutic action secreted by MSC. Each of these factors may be associated with more than one therapeutic action with different mechanisms involved. For example, VEGF, which is one of the most potent angiogenic factors, has also anti-apoptotic activity. HGF and LIF are known to be immuno-modulatory molecules, but they are also responsible for apoptosis inhibition or cell support, respectively. SDF-1α, one the other side, has a more specific function, but is one of the most important chemoattractant factors, being responsible for cell migration and support (Singer and Caplan, 2011). In table I.1 are summarized some of the soluble factors secreted by MSC and their function.

Table I.1. Examples of bioactive soluble molecules secreted by MSC and their main actions. Adapted from (Gebler et al., 2012)

Soluble Factor	Function
VEGF	Inhibition of apoptosis; stimulation of angiogenesis
HGF	Inhibition of proliferation and cytotoxicity
IL-6	Regulation of cell migration; Stimulation of mitosis and angiogenesis
SDF1-α	Chemoattraction of lymphocytes and HSC; Support of cell growth/differentiation
LIF	Inhibition of apoptosis

PGE_2	Inhibition of proliferation and cytotoxicity; Stimulation of cell activation; Inhibition of DC and Treg stimulation
IL-10	Inhibition of apoptosis
IDO	Inhibition of proliferation through reduction of tryptophan
NO	Inhibition of cell activation
SCF	Support of growth and differentiation
sHLA-G; TGF-β	Inhibition of proliferation and cytotoxicity; Promotion of Treg generation
IFN-γ; TNF –α; IL-1β	Induction of immunosuppressive factors production
CCLs/CXCLs	Induction of leukocyte migration
Ang-1	Stimulation of angiogenesis

The possibility of the therapeutic action of the bioactive molecules secreted by MSC lead to many studies where culture-derived conditioned media rich in bioactive factors were used in therapeutic approaches instead of the cells (Cunningham et al., 2018, Sagaradze et al., 2019). Studies with cell secretome (i.e. a molecular therapy approach) have the advantage of less ethical issues (e.g. low risk of immune reactions and cancer development (Lee and Hong, 2017)) and reduced need of purification steps required to obtain a homogeneous and consistent cell-based therapy. Despite some positive results, the mechanism of the therapeutic action of MSC are not yet well understood and some groups defend that some benefic mechanisms are cell contact-dependent and thus cells are required (Krampera et al., 2003).

In accordance with these findings, extracellular vesicles (e.g. exossomes) secreted by MSC have been extensively studied in past years. Although purification strategies sill require improvements and the mode of action of this vesicles is far to be well understood, it is known that they have high concentration of a variety of molecules with promising therapeutic potential, including microRNAs, cytokines or lipids (Borger et al., 2017). Thus, exossomes and other secreted vesicles may replace the cells and be used in therapy as immunomodulatory, anti-cancer or pro-angiogenic drugs.

I.1.4.2. Immunomodulation

MSC were firstly identified as able to modulate the immune system after being transplanted into a fetal sheep (Liechty et al., 2000). Long-term engraftment was possible even when the transplant was performed after the development of immunocompetence. This study revealed that MSC may have unique immunologic properties, giving them the ability to evade immunosurveillance of xenogeneic environments.

Several other studies have shown that MSC modulate immunological responses by suppressing T-lymphocyte activity both *in vivo* and *in vitro* (Le Blanc et al., 2003, Bartholomew et al., 2002). MSC administration *in vivo* was able to support and prolong allogeneic skin graft survival (Bartholomew et al., 2002). In an *in vitro* context, mixed lymphocyte reactions (MLR) can be used to evaluate the immunomodulatory potential of MSC. In MLR, T-cells from two different donor populations are co-cultured together with MSC and the effect of the latter on T-cell proliferation is evaluated. MSC are able to diminish T-cell proliferation up to 60% (Di Nicola et al., 2002, Fontaine et al., 2016). In fact, MLR became a standard tool for characterization of MSC and evaluation of their therapeutic potential.

Although the mechanisms behind how T-cell inactivation and immunomodulation are processed by MSC are not totally clear, it is likely that these mechanisms involve not only cell-cell contact, but also secreted soluble factors, since suppression was shown even when cell-cell contact between MSC and lymphocytes was prevented (Di Nicola et al., 2002). Nevertheless, some authors reported a reduction on T-cell inhibition when there is no cell contact (Krampera et al., 2003).

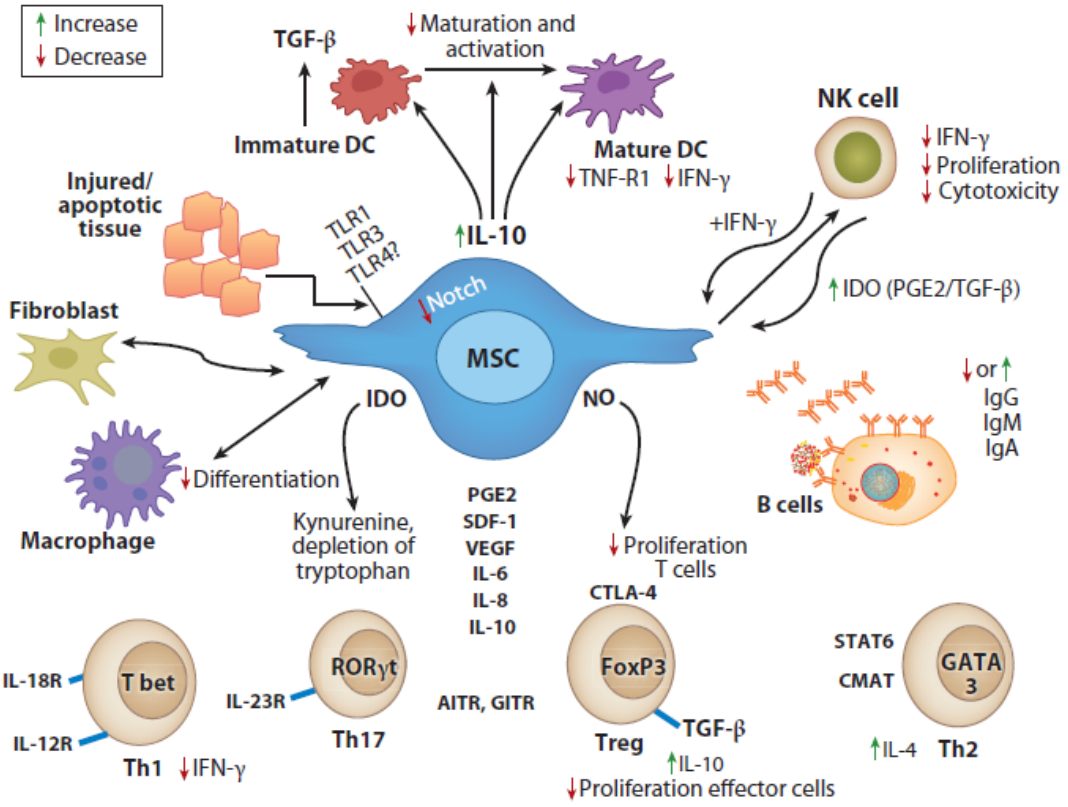

Figure I.4 - Summary of soluble factors and cell-surface proteins involved in interaction between MSC and immune cells. These molecules mediate MSC effects and provide information to MSC about the local environment. CLTA4 - cytotoxic T-lymphocyte antigen 4; Ig – immunoglobin; TLR - Toll-like receptor; Treg - regulatory T cell; Th – helper T cell (Singer and Caplan, 2011).

Among the soluble factors secreted by MSC are growth factors, cytokines, chemokines and hormones that have pleiotropic effects on immune cells. The functions exerted on the immune system by MSC and derived molecules include: inhibition of T-lymphocyte activation and proliferation, blockage of antigen presenting cells (APC) maturation, activation and increase of regulatory cells, modulation of cytokine production by dendritic cells (DC) and suppression of helper T cells and natural killer (NK) by inhibiting secretion of pro-inflammatory cytokines (Interferon (IFN)-γ, IL-1β or tumor necrosis factor (TNF)-α) and increasing anti-inflammatory factors (such as IL-10) (Gebler et al., 2012, Singer and Caplan, 2011). The interactions between MSC and immune system cells, as well as the soluble factors involved, are schematized in Figure I.4.

I.1.4.3. Angiogenesis

The mechanisms involved in blood vessel generation and remodeling can be divided in three categories: (i) vasculogenesis (*de novo* blood vessel formation from endothelial progenitors); (ii) angiogenesis (capillary development from pre-existing vasculature) and (iii) arteriogenesis (expansion of collateral vessels) (Watt et al., 2013, Carmeliet, 2003). Angiogenesis usually occurs in response to stimuli such as hypoxia, mechanical cues or inflammation that induces migration and proliferation of endothelial progenitor cells, forming new sprouting capillaries that will maturate into stable vessel networks (Carmeliet, 2003, Taimeh et al., 2013).

Although the transdifferentiation of MSC into endothelial cells has been already described (Tao et al., 2016) and these have been called vascular stem cells (Lin and Lue, 2013), their capacity to generate endothelial lineages is controversial and it is more likely that their pro-angiogenic potential relies on the paracrine secretion of cytokines that induce blood vessel (re-)generation. One of these is VEGF, one of the most potent pro-angiogenic factors and a key regulator of angiogenesis since the early embryo, being essential in vasculature development (Ferrara, 2001). After birth and throughout the adulthood, VEGF not only induces blood vessel formation through recruitment of endothelial progenitor cells and favors their maintenance and rebuilding, but also promotes restoration of blood supply to ischemic tissues.

Direct infusion of VEGF has been tested for therapeutic angiogenesis, but the short half-life of exogenous VEGF (≈50 minutes) limits this approach (Eppler et al., 2002, Faranesh et al., 2004), emphasizing the need of a more efficient delivery system. Gene therapy approaches with VEGF gene have also been studied to enhance angiogenesis in both pre-clinical (Yasumura et al., 2012, Anderson et al., 2017) and clinical trials (Kusumanto et al., 2006, Deev et al., 2015). However,

although animal studies have revealed promising results, to date, only modest benefits were observed in humans (Taimeh et al., 2013).

The use of MSC able to secrete VEGF, both constitutively and in response to physiological requirements, could overcome the limitations of direct infusion of VEGF (Kagiwada et al., 2008). Furthermore, MSC secrete other pro-angiogenic molecules than VEGF, including IL-6, Ang-1, HGF, basic fibroblast growth factor (bFGF), TGF-β, platelet-derived growth factor (PDGF) that may act alone or together in a synergistic manner to induce blood vessel remodeling and reduce inflammation (Singer and Caplan, 2011, Tao et al., 2016), highlighting MSC potential in angiogenesis.

So, although the poor MSC engraftment usually observed upon infusion of these cells, their trophic activity can compensate, since beneficial effects were observed in some clinical trials regarding cardiovascular diseases (Watt et al., 2013). Of notice, cardiovascular diseases are a leading cause of death worldwide with increasing frequency due to the population ageing and growing incidence of diabetes and obesity. Although those clinical trials are on early phases, essentially safety and feasibility studies, some revealed promising preliminary results. For example, Prochymal, the first stem cell therapy (MSC-based) approved in Canada for treatment of acute GvHD in children, also induced improvements in patients with myocardial infarction (MI) (Hare et al., 2009).

Critical limb ischemia (CLI), the worst stage of peripheral arterial disease (PAD), has also been extensively studied in both pre-clinical and clinical studies using MSC cell therapies (Liew and O'Brien, 2012). Pre-clinical *in vivo* studies revealed encouraging results with improvements in limb reperfusion and decrease in limb loss (Kinnaird et al., 2004, Leroux et al., 2010). Similar results were observed in clinical trials where umbilical cord-derived MSC shown to decrease ischemic rest pain, promote healing of necrotic ulcers and increase capillary density in patients with vasoocclusive Buerger's disease (Kim et al., 2006). A more recent study with BM-MSC revealed comparable results, with improvements in rest pain and increase in ankle pressure for CLI patients (Gupta et al., 2013).

However, concerning the establishment of MSC-based therapy, further studies are required regarding optimization and standardization of parameters such as route of administration, cell dose, origin of cells (tissue source and autologous *versus* allogeneic) and timing of infusion. In animal models intramuscular injections of approximately 1 million cells are commonly administered 24hours after ischemia induction, but there are variations of this protocol not only on the procedures but also on the endpoints assessed (Liew and O'Brien, 2012). There is the

urgent need to have an adjusted and enhanced protocol to obtain an off-the shelf therapy suitable for all PAD patients with no other available option.

Overall, the major limitations regarding the use of MSC in clinical approaches is their low survival and poor engraftment after *in vivo* infusion. To overcome these drawbacks that may hinder the therapeutic benefits of MSC administration, strategies to enhance their healing properties may be developed, including pre-conditioning or genetic engineering of the cells that will be further discussed in next paragraphs.

I.1.5. Strategies to improve the angiogenic potential of MSC

MSC have been pointed as "guardians of inflammation" due to their unique properties as modulators of immune system and mediators of inflammatory responses (Prockop and Youn Oh, 2012), which makes them promising candidates for the treatment of ischemic pathologies. However, the harsh ischemic microenvironments that are found by these cells upon *in vivo* administration lead to reduced survival and poor engraftment essentially due to nutrient deprivation (Potier et al., 2007). Thus, there is the need to develop new strategies to improve the properties of these cells, so their survival can be prolonged or their therapeutic effect can be maximized in their short life under the severe environment found in ischemic regions. Examples of such strategies will be discussed herein and include modulation of cell culture environment, through biochemical and/or mechanical cues or genetic modification with therapeutic or pro-survival genes.

I.1.5.1. Ex-vivo pre-conditioning strategies

By modulating the culture conditions of MSC, it is possible to increase cell survival, enhance their paracrine effects or improve their homing activity to injury sites and, thus increase their overall therapeutic activity (Ferreira et al., 2018, Saparov et al., 2016, Hu and Li, 2018). Different pre-conditioning strategies have been studied to modulate MSC angiogenic capacity which can be divided in two main groups: biochemical (culture media formulation, oxygen tension and addition of soluble factors) and physical (culture configuration (2D *versus* 3D), use of scaffolds or shear stress).

As previously described, medium formulations that are serum-free have been extensively developed during the past years to avoid the commonly used FBS supplementation. Studies demonstrated that serum deprivation can increase MSC angiogenic potential *in vivo* and *in vitro*

by leading to an increase in pro-survival and angiogenic factors, such as VEGF, angiopoietins, IGF-1 and HGF (Oskowitz et al., 2011). Human serum or HPL are promising alternatives to FBS not only in terms of safety but also as having a positive effect on MSC therapeutic properties. Enhanced expression of chemokine receptors, decreased T-cell activation and improved secretion of pro-angiogenic factors are examples of benefits exerted by HPL on MSC activity (Haque et al., 2015, Pérez-Ilzarbe et al., 2009, Schallmoser et al., 2007).

Culturing cells under low oxygen tensions (hypoxia) is one of most used strategies to modulate MSC activity, since this condition closely mimics the environment where these cells are found *in vivo*. MSC are commonly incubated under atmospheric oxygen tensions (≈21%) *in vitro*, usually called normoxia. However, within the human tissues these cells reside in hypoxic microenvironments with low oxygen tensions, between 1-7% (Spencer et al., 2014, Lee et al., 2017, Choi et al., 2014).

Different preconditioning strategies using hypoxic conditions have been tested. Studies where hypoxic culture is alternated with reoxygenation periods revealed to improve not only the proliferation ability of MSC *in vivo,* but also the expression of trophic factors (Kheirandish et al., 2017, Kim et al., 2015). Oxygen tensions that vary between 0.1 to 5% are the most commonly studied and regardless the oxygen concentration tested, the hypoxic pretreatment showed to overall increase cell survival and migration capacity, improve differentiation ability and promote overexpression of angiogenic factors such as VEGF, HGF or bFGF (Sun et al., 2015, Boyette et al., 2014, Liu et al., 2013, Hu and Li, 2018). Besides the augmented production of pro-angiogenic factors, cells pre-treated with hypoxia also showed an increased survival and angiogenic potential *in* vivo, leading to improvements in heart function after being transplanted into rats' hearts (Hu et al., 2008). The mechanisms underlying the beneficial effects of hypoxia on MSC properties are not completely understood, but it is known that hypoxia inducible factor (HIF) 1α plays a central role in maintaining MSC regenerative potential under low oxygen concentration (Haque et al., 2015, Lee et al., 2017). HIF-1α is stabilized under hypoxic conditions and thus regulates the transcription of genes involved in metabolism, angiogenesis and cell migration (Haque et al., 2015).

Though the protective and beneficial effect of hypoxic pretreatment have been observed in several studies, optimal oxygen concentrations and exposure time need yet to be determined and may be dependent on the therapeutic purpose or application of MSC (Hu and Li, 2018).

Cytokines and growth factors may also be used to tune MSC, since the interaction between these molecules and their receptors regulate MSC paracrine activity. The use of inflammatory factors

to mimic the environment found in ischemic regions has been widely used to boost their activity on immune regulation, but some showed that it can also improve their angiogenic potential and survival rate (Ferreira et al., 2018). One of such examples is TNFα, which may be used to accelerate MSC-mediated wound healing by promoting angiogenic activity, proliferation and immune cell infiltration *in vivo* (Heo et al., 2011). In fact, the proteomic analysis of MSC secretome after being stimulated with TNFα and two other pro-inflammatory cytokines (IL-1β and IL-6) revealed an overexpression of several proteins involved in inflammation and angiogenesis (Maffioli et al., 2017). These results show that combination of two or more soluble factors for cell preconditioning may be advantageous to potentiate their effects and thus maximize MSC therapeutic potential. For example, synergistic pretreatment of MSC with IL-1β and TGF-β induced higher VEGF production and increased myocardial recovery than each of the factors alone (Luo et al., 2012). On the other hand, priming with TGF-β also showed to activate MSC pathways related with migration and survival (Li et al., 2016, Dubon et al., 2018), which are essential steps for cell-mediated tissue repair. A similar effect can be observed with SDF1α, since preconditioning MSC with SDF1α increased cell survival and proliferation, as well as enhanced MSC migration and engraftment capacities (Liu et al., 2011, Pasha et al., 2008).

Despite the promising results observed with the use of soluble factors, their short biological half-lives may hinder stable and prolonged beneficial effects on MSC-based cell therapy approaches.

Beyond soluble factors, other compounds, including pharmacological agents or small molecules have been tested to confer some protective effect on MSC and boost their therapeutic properties for cardiovascular applications (Ferreira et al., 2018, Hu and Li, 2018).

Manipulation of the physical environment can be used to modulate MSC activity and tune their properties towards specific applications (Hu and Li, 2018). Culturing these cells in three-dimensional (3-D) microenvironments provide higher cell-cell interactions that will generate more biomechanical and biochemical cues, thus increasing the paracrine activity (Sart et al., 2016). Although MSC are usually cultivated as adherent, different studies showed that culturing them in suspension as 3-D spheroids could improve the secretion of therapeutic molecules (Bartosh et al., 2010, Cheng et al., 2012). In fact, culturing MSC as aggregates lead to an increased production of important pro-angiogenic factors, such as VEGF, bFGF or angiogenin, and also restored the functional expression of chemokine (C-X-C motif) receptor 4 (CXCR4), an important homing factor that regulates adhesion to endothelial cells (Potapova et al., 2007, Potapova et al., 2008).

The use of biocompatible biomaterials, including 3-D scaffolds for cell anchorage and spreading, to closely mimic physiological niches and cell-cell interactions, is also a strategy that may be employed to boost MSC potentiality for angiogenic purposes. For example, the use of scaffolds derived from decellularized cardiac extracellular matrix (ECM) showed to be able to maintain MSC intrinsic differentiation capacity and proliferative potential. Those decellularized scaffolds also enhanced MSC ability to regenerate cardiac tissues through activation of ECM-remodeling factors (Eitan et al., 2010). Other natural biomaterials, including alginate, collagen or fibrin have been effectively used as MSC-delivery scaffolds for cardiac therapies (Russo et al., 2014).

The use of dynamic culture conditions in spinner flasks or bioreactors also affect MSC activity and may be applied to tune their properties. The culture of these cells as 3-D aggregates in stirred spinner flasks can be used to improve MSC properties in terms of differentiation ability, viability and secretion homing factors, such as CXCR4, which plays a key role in cell migration and engraftment towards ischemic regions (Frith et al., 2010). Overall, shear stress seems to positively affect MSC survival and angiogenic capacity, since culturing these cells in stirred conditions (spinner flasks or bioreactors) enhanced their secretory profile for pro-survival and pro-angiogenic molecules, such as VEGF, IL-6 or IGF-1 (Carmelo et al., 2015, Teixeira et al., 2016).

Regardless the huge number of *ex-vivo* strategies available to boost MSC angiogenic potential and improve their survival, making them a more valuable therapeutic option for ischemic diseases, there were not yet found the ideal pre-treatment conditions. Despite the mechanisms underlying MSC tuning trough preconditioning strategies are largely undefined, it is known that different approaches may be followed and combined in an application-dependent manner.

I.1.5.2. Genetic engineering of MSC

The unique characteristics of MSC, including their homing ability, paracrine activity and immunomodulatory potential, suggests that their genetic modification could combine the best of both cell and gene therapy for the treatment of a broad spectrum of diseases. Hence, gene therapy is a relevant and promising approach that may be applied to potentiate the therapeutic activity of MSC and thus extend the range of conditions that they can treat. Though MSC have been successfully used as gene-delivery vehicles in numerous animal studies, these cells failed to demonstrate beneficial effects in clinical trials, since most of them were terminated in an early phase due to the occurrence of adverse events that include toxicity, immunogenicity or oncogenicity (Myers et al., 2010, Bronckaers et al., 2014). The main reason for this failure was the use of viral vectors for cell engineering, and thus other non-viral strategies, which is the main

objective of the present thesis, have to be developed. The different types of vectors, delivery strategies and respective limitations will be further discussed in Chapter I.3. Herein, it will be explored MSC-based gene therapy approaches developed to date towards improving angiogenesis, regardless the methods used.

Different strategies have been used to improve MSC activity for cardiovascular purposes, including engineering with pro-survival genes to increase cell viability on harsh ischemic environment and/or modification with pro-angiogenic genes that accelerate the healing process (Hodgkinson et al., 2010, Tang et al., 2005). Heat shock protein (Hsp)-20, hypoxia-regulated heme oxygenase-1 (HO-1) or B-cell lymphoma (Bcl)-2 are examples of proteins that, when overexpressed on MSC before injection into ischemic regions, have shown to improve survival and reduce apoptosis and thus ameliorated heart function (Wang et al., 2009, Tang et al., 2005, Li et al., 2007). Therefore, Hsp-20, HO-1 and Bcl-2 genes may be used in MSC gene therapy approaches to avoid the usually observed cell death after *in vivo* infusion, one of the major limitations of the clinical use of MSC. As previously described, HIF-1α plays an important role in many cellular processes and its presence maintains MSC survival and proliferation under harsh conditions (Haque et al., 2015). So, overexpression of HIF-1α can also be used as a strategy to reduce cell death by giving some protective effect not only to MSC, but also to surrounding cells (Kiani et al., 2014).

There are some proteins, as angiogenin, that act simultaneously as a protective and angiogenic factors. In fact, angiogenin-engineered MSC showed an increased cell survival and were able to improve vasculogenesis in a rat model of MI (Liu et al., 2008). A similar effect can be observed by overexpression of Akt, a serine-threonine protein kinase involved in survival and anti-apoptotic signaling pathways. MSC overexpressing Akt had not only a higher survival rate after being injected into ischemic hearts, but also demonstrated an improved angiogenic capacity (Mangi et al., 2003, Noiseux et al., 2006), probably due to the secretion of angiogenic soluble factors, such as bFGF, VEGF or HGF, that are known to be upregulated on Akt-overexpressing MSC (Mirotsou et al., 2007).

VEGF is probably one of the most potent and well-studied angiogenic factors, which brings interest for MSC engineering regarding cardiovascular diseases. In fact, MSC overexpressing VEGF (VEGF-MSC) have shown promising results in different animal models and ischemic diseases (Yang et al., 2010, Wang et al., 2006, Matsumoto, 2005). For example the infusion of MSC overexpressing VEGF in a swine model of left ventricular hypertrophy showed to attenuate hypertrophy and induce angiogenesis, improving overall heart function (Wang et al., 2006). On the other hand, in a mouse model for hind limb ischemia, VEGF-MSC enhanced angiogenesis

and avoided limb loss while reducing muscle degeneration and tissue fibrosis (Yang et al., 2010). Besides these two examples, there are many others studies where MSC genetically modified with VEGF showed to improve angiogenesis and ameliorate symptoms on ischemic regions (Hodgkinson et al., 2010, Matsumoto, 2005, Gao et al., 2007). Furthermore, the effect of VEGF on cardiovascular approaches seems to go beyond improving local angiogenesis, being also associated with homing of stem/progenitor cells towards ischemic sites (Tang et al., 2009a).

Other angiogenic proteins have been successfully overexpressed through MSC genetic engineering, alone or in combination, with interesting effects in ischemic conditions. Preliminary results in mice suggest that MSC modification with Ang-1, a molecule known to induce angiogenesis, endothelial survival and vascular stabilization, can significantly ameliorate acute lung injury (Xu et al., 2008) or increase angiogenesis in a model of cerebral ischemia (Onda et al., 2008). Similar improvements were also observed in a porcine model of myocardial ischemia, where Ang1-MSC were able to restore blood flow and promote angiogenesis (Chen et al., 2009). Different authors demonstrated that the angiogenic potential of Ang-1 could be complemented with the protective effect of Akt, by demonstrating that co-expression of Ang-1 and Akt in MSC enhanced cell survival and improved angiogenesis, ameliorating overall heart function after coronary artery occlusion or MI (Jiang et al., 2006, Shujia et al., 2008). HGF is another example of a cardioprotective molecule that may be overexpressed on MSC leading to significant improvements in heart function after MI, including diminish of ischemic area and increase in capillary density (Duan et al., 2003, Deuse et al., 2009).

SDF-1α and its receptor, CXCR-4 are also known to have an important role in MSC activity under ischemic conditions, being involved in cell homing to injured tissues. Infusion of MSC modified with SDF-1α (SDF-1α-MSC) showed to promote cardiac function improvement on a MI mouse model (Zhang et al., 2007). Although the positive effect of SDF-1α-MSC in this study has been pointed to be related with protection and support of resident myocytes, instead of cardiac regeneration by infused cells (Zhang et al., 2007), in other studies SDF-1α overexpression appeared to promote MSC differentiation into endothelial-like cells (Tang et al., 2009b). However, the use of SDF-1α for MSC engineering has some limitations, since its receptor CXCR-4 has low expression on progenitor cells. Thus, alternative strategies using the receptor, CXCR-4, for MSC modification were developed and resulted in increased cell migration and improved cardiac performance (Cheng et al., 2008).

Although the majority studies exploring angiogenic activity of genetically engineered MSC are focused on improving heart function after MI, there also some insights on PAD, which was the motivation for the present thesis. Some of the factors already described, such as HGF or VEGF,

and others, as bFGF or erythropoietin (EPO), were successfully used for modification of MSC in hindlimb ischemia models with significant improvements in angiogenesis (Su et al., 2013, Beegle et al., 2016, Li et al., 2015a, Zhang et al., 2014). While, the results of using MSC engineered with VEGF or EPO showed to increase vascularization and restoration of blood flow, studies with MSC overexpressing HGF or bFGF go beyond, mentioning differentiation into endothelial cells and an augmentation of paracrine secretion of many soluble factors.

Despite the promising and encouraging results found in animal models regarding the use of genetically engineered MSC towards therapeutic angiogenesis, further studies and optimization are required to assure the efficacy and, more importantly, the safety of the cell therapy product.

I.2. Gene Therapy

Gene therapy is the therapeutic delivery of nucleic acids (DNA or RNA) for the treatment or prevention of diseases. Depending on the type of disease, this could be achieved through replacement or removal of a defective gene by a repaired one or by delivery of a therapeutic gene. Although the initial focus of gene therapy was on orphan diseases, for which this was considered the last or unique treatment, currently gene delivery approaches have been applied to a broad spectrum of conditions, including cancer, heart failure and neurodegenerative or metabolic disorders (Kaufmann et al., 2013). Figure I.5 summarizes the main conditions addressed by gene therapy-related clinical trials in August 2018.

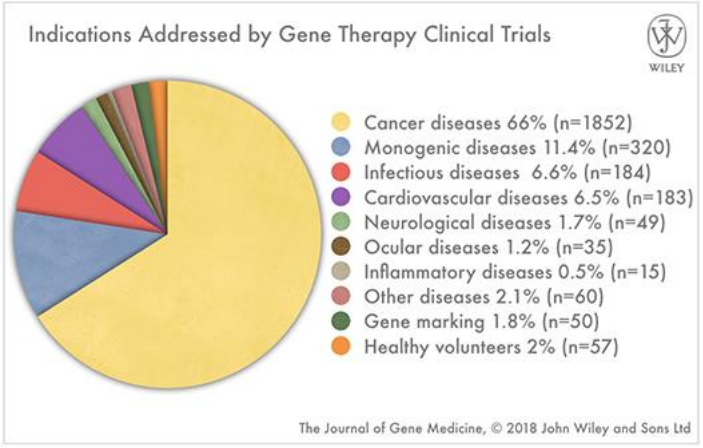

Figure I.5 - Main diseases on which gene therapy clinical trials are focused. (http://www.abedia.com/wiley/indications.php).

The first gene-therapy product, a recombinant human p53 adenovirus, was approved in China on 2003 for the treatment of head and neck cancer (Wilson, 2005). Gendicine®, its commercial name, has been successfully used for 12 years on more than 30,000 patients with no significant associated adverse effects (Zhang et al., 2018). The first gene-based therapeutic approved in Europe, however, has faced a different picture (Yla-Herttuala, 2012). Alipogene tiparvovec, commercially known as Glybera®, consisted of an adeno-associated virus (AVV) that delivers an intact copy of the human LPL gene to muscle cells to treat familial LPL deficiency (LPLD) (Scott, 2015). This product gained market authorization into Europe in 2012 but despite its therapeutic success was withdrawn from the market 5 years later due to commercial failure mainly due to its extremely high cost (i.e. over 1 million euros per patient) (Yu et al., 2018).

More recently, in 2017, US Food & Drug Administration (FDA) approved voretigene neparvovec, commercially available as Luxturna™, for the treatment of Leber's congenital amaurosis, an inherited retinal dystrophy that causes progressive blindness (Ginn et al., 2018). Voretigene neparvovec is an AAV designed to deliver human retinal pigment epithelium-specific protein 65

kDa (RPE65) cDNA to the subretinal space to treat retinal dystrophy. Although it cannot cure the disease, RPE65 replacement improved functional vision in patients that were previously medically untreatable (Russell et al., 2017).

The products described before are classified as *in vivo* gene therapy, since the vectors are directly administered into the body, in contrast with *ex vivo* approaches, where human cells are collected from tissues, genetically engineered outside the body (*ex vivo*) and then re-infused into patients. *Ex vivo* approaches have been gaining attention in the last years since their use can diminish off-target effects, eliminate germ-line transmission and improve efficacy and safety due to the possibility of a tight selection and quality control of engineered cells before administration (Kaufmann et al., 2013).

In fact, the second gene therapy product to be approved in Europe, Strimvelis™, was an *ex vivo* approach that gained market access on 2016 and consisted of a stem cell-based therapy for the treatment of the very rare inherited disorder, adenosine deaminase (ADA)-deficient severe combined immunodeficiency (ADA-SCID)(Yu et al., 2018). This product is an autologous therapy based on CD34+ bone marrow cells transduced with a retrovirus containing ADA gene and revealed to be an effective and safe treatment for ADA-SCID patients (Aiuti et al., 2009, Schimmer and Breazzano, 2016).

Other examples of *ex-vivo* gene therapies are Kymriah® and Yescarta, the first chimeric antigen receptor T-cells (CAR-T) approved by the FDA in 2017 as a first-in-class therapies for the treatment of patients with refractory B-cell precursor acute lymphoblastic leukemia and B-cell lymphoma, respectively (Yu et al., 2018, Ginn et al., 2018). Both products received market authorization by the European Medicines Agency (EMA) in August 2018 (Cuende et al., 2018).

Cuende *et al.* reviewed all the cell/gene therapy products approved worldwide by 2018, being the more important summarized on Table I.2 (Cuende et al., 2018, Ginn et al., 2018).

Though none of the *ex vivo* gene therapy approaches mentioned are MSC-based, the unique properties of these cells make them promising candidates for carrying therapeutic genes, combining the best of both cell and gene therapy fields to treat a wide spectrum of diseases. The use of genetically engineered MSC to date is limited to *in vitro* approaches or few early-stage clinical trials, but the promising preliminary results observed may lead to development of new therapeutics.

Table I.2 Approved gene therapy products, their properties and indication. Adapted from (Ginn et al., 2018).

Tradename	Approval date	Approving agency	Indication	*In vivo* vs. *Ex vivo* (cell vehicle)	Vector (Gene)
Gendicine	October 2003	State Food and Drug Administration of China	Head and neck squamous cell carcinoma	*In vivo*	Adenovirus (*p53*)
Glybera	November 2012	EMA	LPLD	*In vivo*	AAV (*LPL*)
Imlygic	October 2015	FDA/EMA	Melanoma	*In vivo*	Herpes simplex vírus - HSV-1 (*GM-CSF*)
Strimvelis	June 2016	EMA	ADA-SCID	*Ex vivo* (CD34+ bone marrow stem cells)	Retrovirus (*ADA*)
Kymriah	August 2017	FDA/EMA	Acute lymphoblastic leukaemia	*Ex vivo* (T-cells)	Retrovirus-CAR-T cells anti CD19
Yescarta	October 2017	FDA/EMA	B-cell lymphoma	*Ex vivo* (T-cells)	Retrovirus-CAR-T cells anti CD19
Luxturna	December 2017	FDA/EMA	Retinal dystrophy (*RPE65* mutation)	*In vivo*	AAV (*RPE65*)

A vehicle to carry DNA molecule is mandatory for an efficient delivery of genes into human cells *in vivo* or *ex vivo*. The key function of these vehicles or vectors is to deliver and release the therapeutic transgenes into the nucleus of target cells (Ramamoorth and Narvekar, 2015). According to its nature, gene therapy vectors may be divided in two major classes: viral and non-viral systems.

All the examples described above are based on viral systems for gene delivery. However, some clinical trials with viral vectors were prematurely terminated due to the occurrence of severe adverse events (Marshall, 1999, Hacein-Bey-Abina et al., 2003). Thus, other gene delivery strategies have been developed that include not only improved and safer viral vectors but also non-viral systems. These non-viral vectors are not capable of integrating into the genome, having a transient and usually lower expression than viral counterparts, which may be able to integrate into host cells. However, the use of non-viral vectors may be advantageous in many cases where a continuous expression of the transgene is not desirable. However, current approaches with non-viral vectors are mostly limited to *in vitro* studies.

I.2.1. Methodologies for gene delivery into MSC

As stated before, gene therapy methods can be divided into viral and non-viral. However, many different approaches can be pursued within each group according to the specific vehicle used and final application. Although there were several approaches with gene therapy vectors being directly administered into the body, the use of a cellular vehicle with intrinsic therapeutic properties such as MSC could be much more advantageous (Porada and Almeida-Porada, 2010). In fact, genetic engineering of MSC may be used to increase their secretion of specific proteins or to enable their secretion of therapeutic proteins outside their native secretome. These, together with the fact that MSC can be modified with the majority of viral and non-viral vectors clinically used (Oggu et al., 2017), significantly broadens the spectrum of diseases for which MSC can be a therapeutic option. The overexpression of specific transgenes on MSC might have different therapeutic features, including the improvement of their homing ability to increase their overall survival or to deliver specific proteins to injury or cancer sites.

This chapter will discuss the strategies available for gene delivery into MSC, including advantages, drawbacks and main applications.

I.2.1.1. Viral gene delivery

The majority of gene therapy studies have been based on viral vectors, which explore the natural ability of viruses to infect and survive within host cells. Overall, the major reasons for which viral vectors are widely chosen are the high transducing efficiencies achieved and, depending on the type of the vector, the long-term stable expression of the transgene.

The main viral vectors currently used in gene therapy are summarized in Table I.3 together with the respective advantages and drawbacks. The choice of the ideal transduction vector will depend on the aim of therapy as well as on the disease being addressed. Viral transduction of MSC without affecting their differentiation capacity and therapeutic ability could be achieved using retrovirus, lentivirus, adeno-associated virus, adenovirus or baculovirus (Oggu et al., 2017, Park et al., 2015).

The risk of insertional mutagenesis is one of the major concerns regarding the use of viral vectors, especially those that integrate into host genome. To circumvent this limitation, different strategies have been developed to increase viral integration specificity and, thus, the safety of these vectors. Examples of strategies are the inclusion of inducible or tissue-specific promoters or the use of molecular-editing tools such as zinc-finger nucleases (ZFN), transcription activator-

like effector nucleases (TALENS) or clustered regularly interspaced short palindromic repeats (CRISPR). These tools may be used to induce a site-directed integration of the transgene into a "safe harbor" region of the host genome.

Table I.3 - Advantages and disadvantages of the viral vectors available for gene therapy approaches (Sage et al., 2016, Vannucci et al., 2013). ds-double-stranded; ss-single-stranded.

Viral Vector	Structure	Advantages	Disadvantages
Retrovirus	dsRNA	-DNA incorporated into host cell genome -Long-term stable gene expression	-Insertional mutagenesis -Oncogene activation -Infects only diving cells
Lentivirus	dsRNA	-DNA incorporated into host cell genome -Long-term stable gene expression -Infects dividing and quiescent (non-dividing) cells -Replication incompetent -No insertion into oncogene	-Lack of specificity -Safety concerns (HIV vectors) -Insertional mutagenesis
Adenovirus	dsDNA	-DNA incorporated into host cell nucleus -Infects dividing and quiescent cells -Transient gene expression -Lower risk of genotoxicity -Large DNA inserts	-Transient gene expression -Immunogenic -Pre-existing immunity
Adeno-associated Virus (AAV)	ssDNA	-Infects dividing and quiescent cells -Long-term gene expression -Non-cytotoxic -Non-immunogenic	-Small DNA inserts -Need a helper virus to replicate -Need of conversion to dsDNA
Baculovirus	dsDNA	-Non-toxic and non-pathogenic -Large DNA capacity -No replication in mammalian cells	-Glycosylation pattern -Inactivation by human complement
Adeno-associated Virus (AAV)	**ssDNA**	-Infects dividing and quiescent cells -Long-term gene expression -Non-cytotoxic -Non-immunogenic	-Small DNA inserts -Need a helper virus to replicate -Need of conversion to dsDNA
Baculovirus	dsDNA	-Non-toxic and non-pathogenic -Large DNA capacity -No replication in mammalian cells	-Glycosylation pattern -Inactivation by human complement

Retroviruses

Retroviruses are double-stranded RNA viruses that integrate into the genome after viral RNA is reverse transcribed into host DNA. Despite these type of vectors can be efficiently used to integrate proliferating cells, one of the major limitation of retroviruses is their inability to infect post-mitotic cells, as neurons or myocytes, limiting the range of cells targeted for transfection (Dahlberg, 1988). Even so, after integration of viral genome into host cells, a stable and long transgene expression can be achieved using retroviruses. Another limitation of retroviruses is

the possibility of transgene silencing due to methylation of viral promoter during cell differentiation. However, there are some examples where retroviral vectors were efficiently used to genetically engineer MSC *in vitro* in the context of different diseases without significant adverse events (Challita and Kohn, 1994).

After successful pre-clinical studies (Zischek et al., 2009, Niess et al., 2011), a phase I/II clinical trial with MSC retrovirally transduced with a thymidine kinase was designed to test the efficacy and safety of MSC homing into tumor sites in patients with gastrointestinal adenocarcinoma. The thymidine kinase present on the modified cells phosphorylates the prodrug ganciclovir, which is subsequently infused, driving cancer cells to apoptosis (Niess et al., 2015). In a distinct approach, MSC transduced with retroviral vectors encoding for two therapeutic genes/proteins, CXCR4 and receptor activator of nuclear factor-κB, have been effectively used to prevent bone loss in a mouse model (Cho et al., 2009).

Despite these two examples of the beneficial effect of retroviral-transduced MSC, the use of such vectors still raises safety concerns since their random integration into genome could lead to insertional mutagenesis or activation of oncogenes. An example of such risk was observed on a clinical trial where CD34+ bone marrow cells were modified with a retrovirus carrying a cytokine receptor gamma chain for the treatment of X-linked SCID. Although the treatment was able to long term correct immunodeficiency associated with X-SCID, some patients developed leukemia due to insertional oncogenesis (Hacein-Bey-Abina et al., 2003, Hacein-Bey-Abina et al., 2010). Those safety issues have hindered the application of retroviruses for therapeutic gene therapy and that is why other systems have been considered as alternative for MSC transduction.

Lentiviruses

As well as retroviruses, lentiviruses have double-stranded RNA and are able to integrate into host genome, promoting a high and stable long-term expression of transgene. One advantage of lentiviruses is that they are able to infect not only dividing, but also quiescent or slow dividing cells without affecting their viability or differentiation potential (Naldini et al., 1996). Various studies have focused on lentiviral transduction for improving MSC therapeutic properties, for example by increasing their survival on harsh conditions upon administration or by inducing secretion of healing molecules. In fact, in a study where different methods for gene delivery were tested for MSC modification, lentiviruses showed to be the most effective system for MSC transduction with efficiencies up to 95% (McMahon et al., 2006).

The use of a lentiviral vector containing Hsp70 for MSC transduction revealed to increase MSC viability and decrease apoptosis on hypoxic environment in a mouse model of ischemia without affecting cell morphology, viability or differentiation potential (McGinley et al., 2011). A similar protective effect was observed in a rat model of spinal cord injury by transducing MSC with lentivirus containing peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α), which is an important neuronal regulator (Hu et al., 2016). In a hindlimb ischemia mouse model where infused MSC were modified using lentivirus with gremlin1, a novel proangiogenic factor, the benefits were not limited to improvements in cell survival, but an enhanced blood perfusion and angiogenesis were also shown (Xiang et al., 2017).

The potential of lentiviral-transduced MSC has also been extensively explored for cancer therapy. Here the vectors are used to deliver genes coding for specific proteins to MSC that are expressed after cell migration towards tumorigenic sites, thus inducing apoptosis of cancer cells and reducing tumor size (Loebinger et al., 2009, Fei et al., 2012). In fact, there is currently undergoing a clinical trial where MSC genetically engineered with lentiviral vectors are being used as an anti-cancer therapy for lung cancer (Sage et al., 2018). This study was developed based on previous in vivo animal studies where it was demonstrated that MSC transduced with TNF-related apoptosis inducing ligand (TRAIL) are able to home to tumors and induce apoptosis selectively in cancer cells, reducing tumor growth (Lathrop et al., 2015).

Despite no significant adverse events have been observed in the described studies, the integrative nature of lentiviruses brings some safety concerns. Although the probability of aberrant gene activation or disruption is lower than with retroviruses (Sinn et al., 2005), the risk of insertional mutagenesis should still be considered for these systems. To overcome the integration-related concerns, non-integrating lentiviral systems have been designed (Banasik and McCray, 2010). Notwithstanding the fact that those non-integrative systems alone allow only a short-term transgene expression, they may be useful and advantageous for applications where only transient protein production is required. However, these non-integrative systems could be used in combination with novel editing tools to achieve a longer and more stable gene expression. For example, Benabdallah and colleagues used an integration-defective lentivirus combined with ZFN to induce a targeted integration of EPO into a safe harbor locus within MSC genome (Benabdallah et al., 2010).

Another issue regarding the use of lentivirus as gene carriers for human cell delivery is the fact that the majority of lentiviral vectors developed to date are derived from human immunodeficiency virus (HIV), which raises safety and ethical concerns for their application in a clinical setting.

Adenoviruses

Adenoviruses are double-stranded DNA systems without envelope that replicate within the nucleus of infected cells without integrating into host genome (Vannucci et al., 2013). These properties bring two major advantages for adenoviral-based gene therapy approaches: reduced pathogenicity triggered by envelope proteins and no risk of insertional mutagenesis since they are not integrative. So, adenoviral vectors are considered to be safe, but their use is limited to applications where transient expression is required instead of a prolonged and definitive gene correction.

The direct administration of transgenes by adenoviral vectors has been restricted due to the fact that they are known to activate CD4+, CD8+ and antigen presenting cells, which may lead not only to the transgene silencing but also to safety concerns (Yang et al., 1994). The use of a cellular vehicle as MSC may circumvent such limitation, since it was documented that MSC genetically engineered with adenoviruses have no changes in their immunological properties *in vitro* and do not elicit an immune reaction *in vivo* after being administered in mice (Treacy et al., 2012).

The beneficial effects of MSC genetically modified with adenoviral vectors have been observed in different clinical studies. MSC transduced with an adenovirus containing both FGF2 and PDGF genes significantly increased collateral vessel formation and angiogenesis in a mouse model of hindlimb ischemia (Yin et al., 2015). This study highlights another benefit of adenoviral systems which is their high payload capacity, allowing the transduction of MSC with more than one single gene. As all other viral systems previously described, adenoviral transduced MSC have been widely tested as anti-tumor therapies, proving to be able to reduce metastasis in a lung metastatic model or to inhibit tumor growth in a malignant glioma model (Kanehira et al., 2007, Sun et al., 2011).

One major problem regarding MSC transduction with adenoviral systems is that these cells have no adenoviral receptors, being usually resistant to infection. However, this may be overcome by modifying the vectors or by using high vector cell-ratios (Kawabata et al., 2006, Knaan-Shanzer et al., 2005). Thus, although the use a cellular gene delivery vehicle may limit the adenoviral-associated immunogenicity, the high levels or viral particles usually required to achieve MSC transduction may bring some concerns in terms of toxicity and immune reactions (Quaranta et al., 2016). The acute inflammation triggered by adenoviral vectors, however, may be prevented by administration of a second adenovirus containing the anti-inflammatory enzyme HO-1 (McCarter et al., 2003).

Furthermore, while the majority of the studies demonstrated that adenoviral transduction does not affect MSC multipotency (Knaan-Shanzer et al., 2005), it was also reported that the use of adenovirus may influence MSC differentiation (Zaldumbide et al., 2012).

Adeno-associated viruses (AAV)

AAV are small non-enveloped single stranded DNA viruses that need to be co-infected with a helper virus to complete their replication. AAV enters cells by endocytosis and integrate into a specific site of human chromosome 19, staying silent until being rescued by the helper virus (Vannucci et al., 2013). The main advantages of AAV are their reduced immunogenicity and the nonexistent risk of unpredictable integration that may cause insertional mutagenesis. On the other hand, the small AAV genome limits the size of the gene(s) to be transduced (Quaranta et al., 2016).

Several clinical studies have shown the beneficial effects of MSC modified with AAVs without affecting their multipotency (Stender et al., 2007). However, one of the potential applications of AAV as vectors for MSC transduction is to induce cell differentiation towards a specific lineage. In fact, different studies showed that MSC potential for the treatment of articular cartilage defects could be maximized by genetic modification with AAV vectors containing factors that improve their chondrogenic ability, such as IGF-1, TGF-β or SOX9 (Frisch et al., 2014b, Frisch et al., 2014a, Venkatesan et al., 2012). Similarly, the osteogenic potential of MSC can be increased by AAV transduction using BMP-7 gene, which resulted in improved bone formation both *in vitro* and *in vivo* (Kang et al., 2007).

The role of MSC for cardiac repair can also be enhanced by transduction with AAV systems. The AAV-mediated co-expression of Akt1 and Wnt11 molecules by MSC was able to decrease cardiomyocyte apoptosis and increase overall cell survival, while improving cardiac differentiation, being a potential therapeutic strategy for MI patients (Chen et al., 2018). A different approach was followed by Zanotti and colleagues, since they used AVV-transduced MSC to reduce the draining efficiency of lymph nodes by overexpressing tissue inhibitor of metalloproteinase (TIMP)-1, an anti-angiogenic molecule (Zanotti et al., 2016). This effect of TIMP-1 expression has an important impact on inflammatory diseases, since it may be a key modulator of MSC-mediated immunity and inflammation.

Though AAV are able to infect a broad variety of cells, they exhibit some serotype specificity towards the cell type being targeted. On the other hand, the majority of human population has developed antibodies against AAV, which also limits their use to engineer human MSC. To overcome such limitations naturally occurring AAV serotypes other than the most commonly

used AAV-2, and recombinant AAV systems have been developed (Grimm and Kay, 2003). Another issue regarding the use of AAV vectors is the need of converting the single-stranded DNA into a double-stranded molecule before gene expression, which may be a rate-limiting step (Vannucci et al., 2013). Strategies to better understand AAV genome and molecular interactions with cell vehicles need to be addressed to overcome such limitations and take advantage of AAV benefits.

Baculoviruses

Baculoviruses are non-integrating insect viruses with a circular double-stranded DNA with large transgene capacity. This class of virus has been extensively used for recombinant protein production within insect cells for decades (Mansouri and Berger, 2018). In fact, Cervarix, a commercially available vaccine against human papilloma virus approved by EMA and FDA is produced by this system (Harper et al., 2004). Baculoviruses are also able to infect a broad spectrum of mammalian cells, without replicating inside those. This, together with the fact that they are non-pathogenic to humans and the viral DNA degrades over time, makes baculoviruses promising candidates for gene/cell therapy approaches.

Different studies have focused on the capacity of baculovirusess to transduce MSC, being most commonly tested on bone healing approaches. One of such examples is the genetic engineering of MSC using BMP-7-containing baculoviruses to improve their osteogenic potential, which revealed to successfully improve spinal fusion or slow down the progression of disc degeneration in rabbit models (Liao, 2016a, Liao, 2016b). Co-transduction of MSC with BMP-2 and VEGF using baculoviruses also showed to be a promising strategy to augment angiogenesis and accelerate bone repair in critical-sized femoral defects (Lin et al., 2010). However, the applications of baculovirus for MSC genetic engineering go beyond enhancement of their osteogenic potential, being also tested as a systemic gene therapy vehicle. MSC modified with HSV thymidine kinase by baculoviral vectors were able to decrease tumor size and improve mice survival in a model pre-inoculated with glioma cells (Bak et al., 2010). Despite these promising preliminary results, there are some controversial results regarding the effect of baculoviruses on immune activity of MSC and further work is required to confirm the safety and efficacy of those systems (Chuang et al., 2009).

Baculoviral systems may have some other disadvantages, including the possibility of inactivation by human serum complement and the fact that glycosylation pathway of insects is different from the mammalian systems (Kost et al., 2005). This is especially important when baculoviruses are used for overexpression of genes encoding for glyco-proteins. Strategies to overcome those

limitations have been developed and, thus, baculoviral vectors have the potential to be settled as alternatives to other viral systems.

In addition to the viral vectors previously mentioned, other viral vehicles have also been explored although in a less extent, for MSC transduction. HSV, vaccinia virus, Borna disease virus or Sendai virus are such examples that revealed to be advantageous in some applications (Oggu et al., 2017).

Besides the safety issues referred before, one of the key drawbacks associated with the use of viral vectors is the need to establish efficient and cost-effective systems for large-scale production/purification. This, together with the inadequate validation of the viral gene therapy approaches, has increased the interest in alternative gene delivery methods of a non-viral nature.

I.2.1.2. Non-viral gene delivery

Non-viral vectors have been neglected as gene therapy vehicles for years since they have a relatively low transfection efficiency and provide only transient gene expression. However, due to safety concerns associated with the use of viruses, the interest in using non-viral has been growing and significant advances have been made in transfection efficiency and transgene expression duration. In fact, non-viral gene delivery shows important advantages, including less toxicity and pathogenicity, ability to transfer large size transgenes, low cost and straightforwardness of production (Ramamoorth and Narvekar, 2015, Wang et al., 2014a).

While viral systems take advantage of the natural capacity of viruses to infect human cells, non-viral approaches require systems that promote efficient delivery and internalization of the transgenes into specific intracellular compartments (Durymanov and Reineke, 2018). The first barrier for an efficient gene transfer is the cellular membrane, since electrostatic repulsion occurs between nucleic acids and cell membrane, which are both negatively charged (Wang et al., 2014a, Wang et al., 2013). This limits the approaches with use naked DNA and demands for the use of physical or chemical methods to facilitate DNA entry into cell cytoplasm. In physical methods, a physical stimuli is used to trigger temporary holes on the cell membrane, allowing free DNA entry. Chemical methods, on the other hand, rely on the formation of positively charged complexes between DNA and specific carriers that are easily internalized by endocytosis (Medina-Kauwe et al., 2005). In such cases, the release of nucleic acids into the cytoplasm after endocytosis is also an important barrier, since endosomes containing the genetic material will be fused to lysosomes with activated hydrolases and eventually degraded (Durymanov and

Reineke, 2018, Luzio et al., 2001). Strategies to overcome endosomal barrier and successfully deliver nucleic acids have been developed, including the use of lipid components or cationic polymers, such as polyethylenimine (PEI), as nucleic acid carriers (Xu and Szoka, 1996, Boussif et al., 1995).

Once released into the cytosol, nucleic acids may face the possibility of degradation by cytosolic nucleases before being transported to the appropriate sites, due to their slow diffusion within cytoplasm (Lechardeur et al., 2005). Even so, the use of PEI as nucleic acid carrier revealed to decrease DNA degradation by nucleases (Boussif et al., 1995). If gene therapy is performed with mRNA or siRNA molecules, delivery into the cytoplasm is sufficient. DNA, however, must be translocated into the cell nucleus, i.e. there is a need to cross an extra obstacle, the nuclear envelope. The transport through the nuclear envelope is controlled by the nuclear pore complexes (NPC), which are aqueous channels that allow free diffusion of small molecules and restrict the entry of molecules with sizes larger than ≈40 kDa. It is not likely that therapeutic DNA is able to freely cross NPC, since it usually has a considerable length. The uptake of large molecules into the cell nucleus is performed by an energy dependent process through recognition of a specific sequence called nuclear localization sequence (NLS) (Talcott and Moore, 1999). To overcome this limitation and improve nuclear entry, the first approach was to conjugate therapeutic DNA with a NLS peptide. Nevertheless, this strategy lead to controversial results probably due to the complex signaling pathways involved in nuclear transport within different cell types (Zanta et al., 1999, van der Aa et al., 2005). In fact, transfection of MSC using cationic lipids showed to be improved by the inclusion of an NLS in the liposome formulation (Hoare et al., 2010).

Since dividing cells show higher transfection efficiencies than quiescent ones, it is suggested that nuclear uptake of DNA occurs preferentially during or close to mitotic division at the time of nuclear envelope disassembly and reorganization. This is in line with the work from Brunner and co-workers who studied the dependence of transfection efficiency on the cell cycle stage when using non-viral systems. Regardless the type of non-viral carrier used, higher transfection rates were observed when cells were transfected on S ou G2 phase compared to cells on G1 that showed reduced gene expression (Brunner et al., 2000). Additionally, in a study where MSC were transfected with polyplexes, it was observed that low transfection efficiency occurs when cells are cultured under conditions that inhibit cell division (King et al., 2012). Thus, strategies to improve cell division and proliferation may be applied to cell cultures to be transfected, in order to enhance transfection efficiency and maximize transgene expression.

Different chemical and physical methods for gene delivery into MSC have been developed and studied in the last decades (Wang et al., 2014a, Hamann et al., 2019b). A summary of those methods with corresponding advantages and disadvantages is presented on Table I.4 and will be further discussed in the next paragraphs.

Table I.4 - Summary of non-viral gene delivery methods that can be used for genetic modification of MSC. (Wang et al., 2014a, Ramamoorth and Narvekar, 2015)

Method	Reagent(s)/ Strategies	Nucleic acid(s)	Advantages	Disadvantages
Microinjection		DNA	-Simple and non-toxic -Effective and reproducible -Large DNA inserts	-Not suitable for a large number of cells
Electroporation	-Traditional electroporation -Microporation -Nucleofection	DNA mRNA	-Effective and reproducible -Large DNA inserts	-Cell damage -Stability of genomic DNA
Ultra-sound microbubbles		DNA siRNA	-Safety -Flexibility	-Low efficiency -Cell damage
Cationic lipids	-Lipofectamine™ (DOSPA/DOPE) -Lipofectin™ (DOTMA/DOPE) -Escort™ (DOTAP/DOPE) -GenePORTER™ (cationic lipid/DOPE) -Other lipid conjugations	DNA siRNA mRNA	-Easy production -Low-cost -Effective	-Cytotoxicity -Some immunogenicity -Low efficiency in the presence of serumin the culture medium
Cationic polymers	-PEI (alone or modified) -PLL (alone or modified) -Dendrimers -Chitosan -Spermine conjugations -Peptides	DNA siRNA mRNA	-Easy production and functionalization -Low-cost -Effective	-Cytotoxicity -Safety (undegradable polymers)
Inorganic nanoparticles	- Hydroxyapatite (HA) - Magnetic nanoparticles with HA	DNA	-Easy production -Low cytotoxicity -High stability -Functionalization	-Low efficiency

Herein, only gene delivery techniques that were applied to MSC will be discussed, being excluded physical methods such as needle injection, jet injection, gene gun and hydroporation, despite the studies showing their success for other applications (Ramamoorth and Narvekar, 2015). Physical methods are simple, since they do not require any additional step. Physical forces are applied to promote the transient disruption of cellular membrane and deliver nucleic acids

into cytoplasm. Three main physical methods have been studied on MSC and will be further discussed below: microinjection, electroporation and sonoporation.

Microinjection

Gene delivery by microinjection consists in the use of a needle to penetrate cellular membrane and/or nuclear envelope and directly inject genetic material within living cells. One crucial aspect of this method is the needle size, since the use of large needles may irreversibly affect cell integrity. Microinjection using needles with diameters of 200-275 nm showed to efficiently promote transgene delivery (65-75%) into MSC without significantly decreasing cell viability (Han et al., 2008, Tsulaia et al., 2003). Despite its simplicity and efficiency, the method is not feasible for transfection of a large number of cells, since it implies individual manipulation of each cell.

Electroporation

Electroporation is based on the application of transient electric pulses that induce creation of small pores on the cell membrane, allowing free entry of nucleic acids into cytoplasm. This is an economical and reproducible method that can achieve high transfection efficiencies, being a promising alternative to viral systems.

One of the limitations of electroporation is the high cell mortality observed. A system with small-surface area electrodes, where the electroporation is performed at microscale, was developed to overcome such limitation and improve cell survival. This technique, called microporation, allows not only high transgene expression levels but also enhanced cell viability compared to traditional electroporation (Kim et al., 2008). Microporation with the Neon system (Thermo Fisher Scientific) has been effectively used and optimized for MSC transfection with promising results in terms of cell survival and transgene expression and without affecting their intrinsic properties. For BM MSC, the transfection efficiencies reached 40%, with cell viabilities around 90%, whereas for UCM-derived MSC similar viability was observed, but the transgene was expressed in 80% of the cells (Madeira et al., 2011, Lim et al., 2010). The use of microporation for delivery of a minicircle, which is a particular type of DNA molecule that will be further discussed in next sub-chapter, encoding CXCR4 to MSC resulted in higher cell homing towards an injury site *in vivo* (Mun et al., 2016). In fact, the work developed within the scope of present thesis (Chapters IV and V) shows that microporation may be a valuable technique for therapeutic gene delivery into MSC.

Another drawback of electroporation is the need for nuclear uptake of DNA transgenes after being released on cytoplasm. To circumvent this limitation, nucleofection, a new method of

gene deliver that drives DNA molecules directly to cell nuclei by specific combination of electrical parameters and solutions according to cell type, was developed (Gresch et al., 2004). The Nucleofector (Lonza) system has been successfully used for MSC transfection with higher efficiencies than traditional electroporation (Nakashima et al., 2005). Aslan and colleagues showed that nucleofection of MSC lead to transgene expression in up to 88% of cells, albeit with decreased cell viabilities after transfection of around 50%. In the same study, this method revealed to be effective for the delivery of BMP-2 and BMP-6 genes into MSC, which were able to improve calcium deposition and induce bone formation in mice (Aslan et al., 2006). The replacement of DNA molecules by mRNA containing the transgene may also be a promising strategy to increase transfection, since nucleofection of MSC with mRNA showed to achieve significantly higher protein expression levels than when using plasmid DNA (pDNA) (Wiehe et al., 2007). Actually, overexpression of CXCR-4 in MSC by an mRNA nucleofection approach, lead to expression efficiencies up to 93% and cell viabilities higher than 60% (Wiehe et al., 2013). Studies referring MSC nucleofection showed that both immunophenotype and differentiation capacity of the engineered cells are maintained after transfection. The work developed in the Chapter VI of this thesis demonstrates that MSC nucleofection with therapeutic proteins can be a promising strategy for *in vivo* approaches.

While electroporation and its variations are probably the most efficient non-viral methods for gene delivery into MSC, they may bring some safety concerns due to potential influences on genomic DNA stability (Wang et al., 2014a). Another major limitation of electroporation as a transfection technique to be used in a clinical setting is the need to develop novel systems to allow large scale production of genetically engineered cells, since current systems have limited scalability (Hamann et al., 2019b).

Sonoporation

Sonoporation is a non-invasive technique that uses ultrasonic waves to transiently permeabilize cellular membrane and thus allow nucleic acid entry. For sonoporation approaches, genetic material is generally incorporated into gas-filled microbubbles stabilized by biocompatible compounds on the surface (Ramamoorth and Narvekar, 2015). These bubbles are then activated by ultrasound waves and deliver the therapeutic genes into the cell cytoplasm during the temporary permeabilization caused by shock waves released from microbubbles (Wang et al., 2014a).

Despite the safety of this method, its use for gene therapy approaches is limited due to the relative low gene transfer efficiency. Nevertheless, ultrasound-targeted microbubble

destruction under optimized conditions showed to be effective on MSC transfection with VEGF-165 gene, leading to significantly higher VEGF protein yields than non-transfected cells. Although this was only performed *in vitro,* it may be a starting point for MSC genetic engineering using sonoporation techniques (Pu et al., 2011). A different study where MSC were transfected by sonoporation revealed that this may be a useful strategy for RNA interference approaches, since despite the cell damage observed after transfection with the siRNA, knockdown of target mRNA was observed on transfected MSC (Otani et al., 2009).

Notwithstanding the simplicity and efficiency of physical methods for gene delivery into MSC, their major limitation is the reduced scalability, hindering their application for large-scale approaches as allogeneic off-the-shelf cell therapies. On the other side, chemical methods can be easily scaled-up and optimized to improve their gene delivery capacity and targeting. Contrarily to physical methods, where nucleic acids are delivered in their naked form, chemical methods use a carrier to transport and facilitate entry of DNA or RNA molecules into the cell via membrane crossing by endocytosis. The most common carriers that have been tested for gene delivery into MSC are cationic lipids or cationic polymers (synthetic, natural or dendrimers) and, more recently, inorganic molecules.

Cationic lipids

Cationic lipids can self-assemble as liposomes due to their composition: they have hydrophilic heads and hydrophobic tails connected by a linker. Liposomes are positively charged, allowing the formation of stable complexes (i.e. lipoplexes) with negatively charged nucleic acid molecules (Chesnoy and Huang, 2000). The DNA or RNA within lipoplexes could be entrapped in the inner aqueous phase of liposomes or bound to their surface and are thought to be released into the cytosol due to changes in the lipoplexes that destabilize endosomal membranes (Hoekstra et al., 2007). Lipofection is the term used to refer to cell transfection using lipid carriers (Santos et al., 2011).

Although the exact mechanisms involved in lipoplex-based gene delivery are not yet well understood, they have been effectively applied for transfection of MSC with different nucleic acid molecules, including DNA, mRNA or siRNA (Wang et al., 2014a). Indeed, several lipid-based reagents for cell transfection are commercially available nowadays, including Lipofectamine™, Lipofectin®, FuGENE™6, Effectene™, Escort™ or GenePORTER® (Santos et al., 2011, Wang et al., 2014a). Among all these possibilities, Lipofectamine™ is probably the most studied and effective reagent for non-viral MSC engineering.

A study where different non-viral reagents (lipid or polymer-based) were tested for transfection demonstrated that Lipofectamine was actually the best option for MSC genetic engineering. However, the maximum transfection efficiency does not go beyond 20% (Gheisari et al., 2008). Hoelters and colleagues also compared different lipid-based reagents for gene delivery to MSC and, in fact, Lipofectamine 2000 revealed to achieve the highest transfection levels either using DNA (50%) or siRNA (92%) when compared to other lipofection reagents (Hoelters et al., 2005). Another study showed that this reagent is able to transfect MSC from different human sources, including BM, AT and UCM, although with different efficiencies. While the amount of GFP-overexpressing cells reached more than 50% for both BM and UCM, the transfection efficiency for AT-MSC was about 33% (Boura et al., 2013). In both studies, the intrinsic properties of the transfected cells, including immunophenotype and differentiation capacity, were not affected by the lipofection process. As mentioned previously, the use of mRNA for MSC transfection could lead to higher levels of transgene expression than with DNA molecules. This was observed by Rejman and colleagues who used Lipofectamine or a DOTAP/DOPE mixture to deliver mRNA containing CXCR-4 sequence to MSC. The results showed that 80% of cells transfected with liposomes were overexpressing the therapeutic protein, in comparison with the 40% obtained for polymer-based transfection (Rejman et al., 2010). The differences observed in transfection efficiencies between studies regarding MSC transfection may be dependent not only on the type of nucleic acid used (RNA vs. DNA), but also on protocol conditions that were followed and on the MSC origin (human vs animal) or tissue source (BM, AT, UCM or other).

Even though the efficiency of the transfection of MSC using lipid-based reagents is reduced or, in some cases, unobtainable (Hamm et al., 2002), strategies to improve the efficacy of those systems have been developed. The combination of lipid reagents with cationic polymers or the inclusion of peptides containing NLS are only two examples of such strategies that have been applied to enhance lipoplex-mediated gene expression in MSC (Clements et al., 2007, Hoare et al., 2010).

Despite cationic lipid transfection has been widely pointed as safe and low cytotoxic, liposomes can become toxic when a lipid:DNA ratio higher than 3:1 is used (Ramamoorth and Narvekar, 2015). Thus, studies to optimize the lipid:DNA ratio for MSC transfection have been performed in order to maximize transgene expression without significantly affecting cell viability (Madeira et al., 2010, de Carvalho et al., 2018).

Cationic polymers

Cationic polymers are rich in positively charged groups (amines are the most common) that interact with DNA through electrostatic interaction, forming complexes called polyplexes. As lipoplexes, those polyplexes are positively charged and can be easily internalized by cells via endocytosis. Once in the cytosol, the polymers can protect DNA against degradation and facilitate the escape from lysosomal activity (Dang and Leong, 2006). The transfection mediated by cationic polymers is often referred to as polyfection (Santos et al., 2011).

Notwithstanding the first polymer tested as gene therapy vehicle was poly-L-lysine (PLL), it resulted in a poor transgene expression when used on MSC (Santos et al., 2011, Wu and Wu, 1987). The conjugation of PLL with palmitic acid (PA), a naturally occurring lipid, showed to increase five-fold the gene delivery efficiency compared to PLL alone (Incani et al., 2007). Combination of PLL-PA and Lipofectamine 2000 on MSC transfection showed to further improve transgene expression by 2-5% due to an additive effect between the two systems (Clements et al., 2007). Another polymer, polyethylenimine (PEI), revealed to be a better choice for MSC polyfection than PLL. When comparing both polymers, PEI was able to transfect up to 42% MSC whereas the transfection with PLL did not go beyond 11% (Farrell et al., 2007). In fact, presently, PEI is probably the most popular polymer for cell transfection due to its high ability to transfect cells, its reduced cytotoxicity and its capacity to induce the endosome rupture and thereby DNA release in cytoplasm (Boussif et al., 1995). Although PEI exists in branched and linear forms and both have been successfully used for MSC transfection (King et al., 2012), the linear molecule is considered a more efficient gene carrier (Wightman et al., 2001), being commercially available in the form of reagents as ExGen 500 or jetPEI™.

One major advantage of using polymers for gene delivery is the possibility of modifying them to improve transfection, reduce toxicity or increase targeting. Several strategies have been used for PEI modification, including covalent conjugation with hyaluronic acid, which showed not only to enhance transgene expression but also to significantly improve cell viability after transfection (Saraf et al., 2008).

As for lipid-based systems, the amount of polymers used may raise concerns in terms of cytotoxicity. On this behalf, synthetic biodegradable polymers, such as polylactic acid (PLA) or poly(lactic-co-glycolic acid) (PLGA), that are naturally eliminated after DNA release have been investigated for MSC gene delivery. Gwak and co-workers showed that PLGA nanospheres can be a promising approach for gene delivery into MSC, with lower cytotoxicity and higher and longer transgene expression when compared to PEI (Gwak and Kim, 2008).

Other systems besides synthetic polymers have been used for MSC gene delivery, including natural polymers as chitosan or dendrimers as poly(amidoamine) (PAMAM). Despite chitosan has been used for gene delivery into other cell types, its use for MSC transfection is quite limited due to the low transfection efficiency (Corsi et al., 2003). However, its use may be interesting for specific applications when combined with other methodologies. Nie and Wang showed that PLGA/HA scaffolds with encapsulated DNA/chitosan nanoparticles improved cell attachment, cell viability and transfection efficiency, being a promising approach for bone regeneration (Nie and Wang, 2007). Other natural polymers as gelatin or pullulan have been conjugated with spermine for DNA complexation and used for gene delivery approaches into MSC, mainly for differentiation into specific lineages (Hosseinkhani et al., 2006).

PAMAM is a widely studied dendrimer for gene delivery application due to its intrinsic ability to associate, condense and efficiently deliver DNA into several cell types (Santos et al., 2011). In fact, there are two commercially available reagents, SuperFect® and PolyFect® that are PAMAM-derived and were tested for MSC transfection although with low transfection efficiencies (King et al., 2012, Gheisari et al., 2008). The PAMAM dendrimers can also be functionalized with peptides with high affinity to MSC, in order to avoid off-target transfection. This strategy resulted in low cytotoxicity and superior transfection efficiency than using native dendrimers or SuperFect reagent (Santos et al., 2010).

Inorganic particles

Recent interest in using inorganic particles as gene delivery carriers is rooted in several advantages: ease of preparation, wide availability, low cytotoxicity and high stability (Chowdhury and Akaike, 2005). Inorganic particles are able to bind DNA trough adsorption or conjugation before being internalized into cells, and can be used alone or in combination with organic molecules (Wang et al., 2014a).

There are few studies reporting the ability of inorganic particles to transfect MSC, however some revealed advantages over other methods. MSC transfection with BMP-2 using HA nanoparticles lead to increased calcium production with a significantly reduced cytotoxicity than lipid-based reagent, despite the lower transgene expression (Curtin et al., 2012). In a different approach, the addition of gold nanoparticles showed to enhance PEI-mediated MSC transfection by more than 2-fold (Uchimura et al., 2007). A similar strategy was developed by Park and colleagues who demonstrated that the use of silica particles can improve PEI gene delivery capacity into MSC (Park et al., 2010).

Challenges in non-viral gene therapy

Despite the wide range of non-viral methods available for MSC gene delivery, there are still improvements to be made to achieve the high transgene expressions provided by viral vectors. Although scalability remains a major challenge, electroporation still seems to be the best non-viral option for MSC transfection due to the superior efficiencies compared to carrier-dependent methods. One major problem regarding the optimization and definition of an ideal non-viral system is the great variability of transfection effectiveness observed between studies. The heterogeneity of MSC populations, the high donor-to-donor variability and the culture conditions may be the major causes for those inconsistencies. Furthermore, the MSC physiology differs between species, so before a method being considered for clinical application, it should be validated in human cells. Even within human cells, the source is also a point, since using the same method, different transfection levels can be observed according to the source (Boura et al., 2013). Hence, the best combination of cell source, culture conditions and transfection method must be defined for each application and then validated in human cells from several donors.

Although the choice of transfection system is probably the most important step for cell engineering, there are other factors that significantly influence gene delivery and expression. One of those factors is the sequence and structure of nucleic acid molecules. It was already mentioned that the use of RNA molecules instead of DNA could significantly increase gene expression (Wiehe et al., 2007). However, transfection with RNA has a shorter duration, thus replacement of DNA by mRNA could be promising for applications where overexpression of a protein is required within a short time period. For approaches where inhibition of gene expression is required, transfection with siRNA is highly advantageous, since more than 90% knock-down can be obtained (Hoelters et al., 2005). DNA plasmids could also be modified and optimized towards an increased delivery and expression. Replacement of promoters, addition of enhancers or removal of antibiotic resistance markers and CpG regions are examples of modifications that can be performed to pDNA sequences in order to enhance and prolong transgene expression.

Cytomegalovirus (CMV) promoter is the standard promoter used for plasmid transfection, including on MSC. However, due to its viral origin and low and short transgene expression achieved, attempts to replace it by a safe and more efficient mammalian promoter have been performed. Studies demonstrated that cellular promoter for elongation factor-1α (EF-1α) can induce higher and prolonged transgene expression than CMV for a variety of cell types, including MSC (Qin et al., 2010). A study with rat MSC where different promoters have been investigated

for BMP-2 delivery revealed that from the seven promoters tested, four showed better performance than CMV. EF-1a, β-actin, eukaryotic initiation factor 4A1 (eIF-4A1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters lead to higher levels of BMP-2 secretion than CMV 48h after DNA delivery to MSC by electroporation (Ferreira et al., 2012). Despite these results with rat-derived MSC, the replacement of CMV promoter by EF-1α or rous sarcoma virus (RSV) promoter resulted in a significant decrease in transgene expression on human MSC. This study also showed the benefits of adding an enhancer to the plasmid sequence, since removal of the simian virus 40 (SV40) enhancer also diminished gene expression (Hamann et al., 2019a). The discrepancies observed, regarding the use of mammalian promoters, are probably associated with differences on MSC biology between species.

Another plasmid sequence modification developed to improve the potential of non-viral systems consists on the removal of bacterial backbone sequences from pDNA, including origin of replication and antibiotic resistance markers, that can trigger inflammatory responses which may induce transgene silencing (Ahmad-Nejad et al., 2002, Hardee et al., 2017, Häcker et al., 2002). These DNA molecules devoid of bacterial backbone are called minicircles and will be further discussed in the following section.

Besides the strategies described above, other approaches to improve non-viral gene delivery into MSC were developed, including priming of the cells to improve their ability to uptake and express the transgene. MSC priming could be achieved by addition of compounds to culture media such as glucocorticoids, that mitigate toxicity and/or improve transfection or by modification of cell culture surfaces in terms of stiffness, chemical composition or addition of adhesion molecules (Hamann et al., 2019b).

I.2.2. Minicircles as gene therapy vehicles

Despite their lower expression levels compared to viral vectors and transient nature, pDNA are promising molecules for human gene therapy and improvements have been made to enhance their therapeutic potential. DNA plasmids are produced within bacteria, being the most commonly used strain for this purpose *Escherichia coli* (*E. coli*). To be produced in bacteria, pDNA molecules must have a bacterial origin of replication and an antibiotic resistance marker (e.g. Kanamycin – Kan) to allow for the selection of plasmid-harboring bacteria (Hardee et al., 2017). These sequences required for plasmid replication and maintenance within bacteria constitute the bacterial backbone (see Figure I.6). On the other hand, a pDNA vector will also contain a transcription unit (see Figure I.6), which comprises a eukaryotic promoter, the gene of

interest, a polyadenylation (PolyA) sequence to increase transgene expression and reduce degradation by nucleases and any other sequences required for expression in mammalian cells. After delivery, pDNA is maintained within cell nucleus in an episomal form without integrating into host cell genome, and is lost during successive cell divisions (Gill et al., 2009). So, pDNA is particularly interesting for slow or non-dividing cells since it can potentially be maintained for the lifetime of the cell. However, even in such cases, transgene silencing can be observed, which has been associated with the presence of cytosine-guanosine dinucleotide (CpG) motifs (Häcker et al., 2002). These CpG regions are very common in bacterial DNA and were described to trigger immune responses in mammalian cells through activation of Toll-like receptors (TLR)-9 (Walker et al., 2010). The activation of immune system could lead not only to transgene inactivation, but also in more severe cases to inflammatory reactions (Boura et al., 2014, Sawamura et al., 2005).

Another concern regarding the use of pDNA for clinical applications is the presence of antibiotic resistance markers, which led regulatory agencies to discourage its use due to the risk of transference to human microbiome or environment. To overcome such limitations, several modifications of conventional plasmids, including the deletion of unnecessary bacterial sequences, have been performed in an attempt to improve their expression capacity in human cells and reduce safety risks (Hardee et al., 2017). The generation of minicircles, which are small circular DNA molecules that contain only transcriptional unit, is one of such examples (Darquet et al., 1997). The lack of bacterial sequences in minicircles further contributes to improve safety, reduce CpG-mediated immunogenicity and minimize silencing of transgene expression. A number of studies have shown that minicircle-based cell engineering is able to sustain higher and longer transgene expression *in vitro* and *in vivo*, as well as stem cell survival, when compared to conventional plasmid vectors (Darquet et al., 1999, Chen et al., 2003, Dietz et al., 2013, Madeira et al., 2013).

Despite the different methods that have been developed for minicircle production within bacteria (Gaspar et al., 2015), the overall process is similar and schematized on Figure I.6. A conventional plasmid (parental plasmid) that has two recombination sites between bacterial backbone and transcriptional unit regions gives rise to both a miniplasmid and the minicircle, after *in vivo* recombination. While origin of replication, antibiotic resistance marker and other bacterial sequences can be found in the miniplasmid, the minicircle has the transcriptional cassette with promoter, gene of interest and PolyA.

The conversion of parental plasmids into minicircles is a complex process that starts with amplification of precursor plasmids within *E. coli* strains (Prather et al., 2003). After production of high parental plasmid yields there are two crucial steps to generate minicircles. The first is

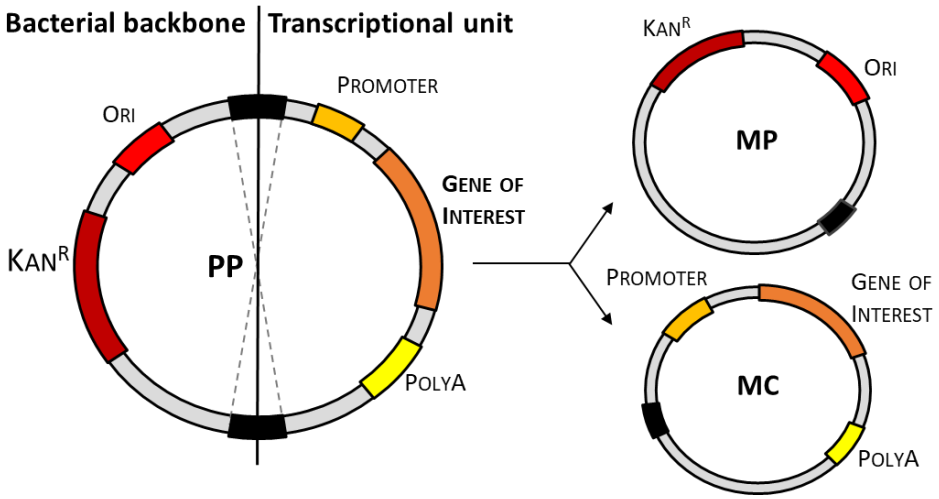

Figure I.6 - Schematic representation of the process for minicircle generation. A conventional plasmid (parental plasmid) with both bacterial backbone and transcriptional unit sequences gives rise to a miniplasmid (bacterial backbone) and a minicircle (transcriptional unit) after recombination. PP – Parental plasmid; MP – Miniplasmid; MC – Minicircle; Ori – Origin of replication; Kan^R - antibiotic resistance marker (kanamycin); PolyA – polyadenylation sequence.

the induction phase, which consists in the addition of a compound, such as L-arabinose, that will activate the expression of genes responsible for mediating the next step, recombination (Kay et al., 2010, Simcikova et al., 2014). The addition of the inducer is a critical stage of minicircle production, since it should be performed at the end of exponential bacterial growth in order to maximize the final yield (Gaspar et al., 2014). After induction, bacterial machinery starts to convert parental plasmids into minicircles and miniplasmids by the action of recombinases that bind to hybrid sites (black regions on Figure I.6), mediating strand exchange and re-assembly (Kay et al., 2010). Despite several phage integrases have been employed as recombinases for minicircle production, including phage λ integrase or phage P1 Cre recombinase (Groth and Calos, 2004, Gaspar et al., 2015), ParA resolvase recombination will be further discussed herein, since this was the system used in the scope of the present thesis.

ParA resolvase is a serine recombinase that mediates site-specific intramolecular recombination between two identical hybrid sites within a supercoiled plasmid. For minicircle production approaches, the ParA gene is commonly inserted in the genome of the producer host under the control of the arabinose inducible pBAD/AraC expression system (Simcikova et al., 2014, Kay et al., 2010, Mayrhofer et al., 2008). In this system, the addition of arabinose will induce the transcription of pBAD, where ParA resolvase is found, whereas in the absence of arabinose, the transcription is repressed by an AraC mediated mechanism. Although residual pBAD activity during bacterial growth has been described, this leaky expression can be prevented by the addition of glucose to the culture medium (Simcikova et al., 2014). So, ParA resolvase expression

on *E. coli* cultures could be dynamically regulated by the addition of arabinose/depletion of glucose. *E. coli* strains with improved capacity for L-arabinose uptake have been developed for this purpose, in order to increase ParA resolvase expression (Alves et al., 2016, Khlebnikov et al., 2001). This system has been used for minicircle generation with recombination efficiencies of up to 99% (Simcikova et al., 2014, Alves et al., 2016, Mayrhofer et al., 2008). After recombination, a decisive step is to isolate minicircle species from miniplasmid and any residual plasmid as required for their use in clinical approaches. This is probably the major bottleneck of the process. However, different studies showed that minicircles could be efficiently separated from the remaining DNA molecules by chromatography methods, either by affinity chromatography, hydrophobic interaction or multimodal chromatography (Alves et al., 2016, Mayrhofer et al., 2008, Silva-Santos et al., 2019).

Another relevant method that greatly improved the procedure for minicircle production is ϕC31-integrase/I-*Sce*I homing endonuclease system. The combination of both an integrase and an endonuclease under the control of the same promotor makes possible, upon induction, not only high-efficient minicircle recombination, but also the degradation of undesired miniplasmid and parental plasmid species (Chen et al., 2005).

The development of systems that allow production and purification of high minicircle yields that meet regulatory requirements is crucial before this system can be applied as large-scale gene therapy vehicle for clinical purposes. Although the removal of other DNA species than minicircle is a key step, this is not the only concern regarding the purification process. There are other bacterial contaminants, such as endotoxins, as well as impurities from the process (solvents or antibiotics) that must be removed due to the risk of generating immune reactions or affecting gene therapy efficiency (Gaspar et al., 2015). On the other hand, the whole process must be in accordance with GMP guidelines for pre-clinical and clinical applications to assure not only the safety, but also the quality, standardization and reproducibility of the system. In fact, there are companies, such as System Biosciences® or Plasmid Factory®, that have commercially available GMP compliant minicircles ready to be used for gene therapy applications.

The biomedical potential of minicircle vectors has been studied in different fields that include not only therapeutic purposes, but also vaccination or cell reprogramming (Schleef et al., 2015, Jia et al., 2010). The direct administration of minicircles has been investigated in animal models for different conditions, including diabetes (Kwon et al., 2012), cancer (Wu et al., 2006) and pulmonary (Munye et al., 2016) or cardiovascular diseases (Chang et al., 2008, Huang et al., 2011). VEGF is an example of the genes tested in this context. In fact, the *in vivo* transfection

with minicircles overexpressing VEGF revealed to promote wound healing in diabetic mice (Kwon et al., 2012) or enhance VEGF amounts in muscle cells and improve endothelial proliferation in a PAD model (Chang et al., 2008).

Despite only few studies investigated the potential of minicircles in the context of *ex vivo* gene therapy of MSC to date, those preliminary reports revealed that this can be a promising strategy for different therapeutic applications. MSC conversion into iPSC can be achieved by transfection of UCM-derived cells with minicircles containing defined reprogramming factors (Lin28, Nanog, Oct4 and Sox2). This transfection process generated ESC-like cells that are able to differentiate into the three germ layers, highlighting the potential of minicircles in adult cell reprogramming (Daneshvar et al., 2015). Park and colleagues showed that MSC engineered with minicircles could be used for delivery of etanercept, a commonly used biologic for the treatment of rheumatoid arthritis, and thus ameliorate collagen-induced arthritis in a mouse model (Park et al., 2017). Other possible outcomes of MSC transfection with minicircles include enhancement of cell survival or improvement of migration capacity. Overexpression of Bcl-2 by AT-derived MSC through minicircle transfection not only showed to reduce apoptosis and improve cell survival but also increased skeletal regeneration in a wound healing mouse model (Hyun et al., 2013). A different approach was followed by Mun and colleagues, who showed that MSC genetically engineered with minicircles encoding CXCR-4 have increased migration ability towards an injury site (Mun et al., 2016). Finally, the angiogenic potential of BM-MSC could also be improved by transfection with minicircles as showed by us and others. Modification of rat MSC using minicircles with eNOS revealed to improve the secretion of angiogenic proteins and also induced cell migration in an *in vitro* wound healing assay (Bandara et al., 2016). It was recently demonstrated that VEGF-engineered MSC using minicircles have not only an enhanced capacity for VEGF production, but also showed an improved angiogenic capacity *in vitro* (Serra et al., 2018).

Although there is still room for improvement and strategies to optimize and standardize both minicircle production and MSC transfection, the encouraging results from these studies are a good starting point. Minicircles can be used to transfect MSC from different sources with higher efficiency than conventional plasmids and with promising *in vivo* and *in vitro* therapeutic activity.

I.3. Peripheral Arterial Disease

PAD, also known as peripheral vascular disease, is a prevalent and high-burden chronic condition caused by the narrowing and obstruction of systemic arteries, which induces a decrease in blood flow to the lower extremities (Conte and Vale, 2018). One of the more severe stages of PAD is CLI, which is associated with high mortality and morbidity (Gresele et al., 2011). PAD symptoms, epidemiology and risk factors, as well as conventional applied therapies will be described further on the next paragraphs. Finally, the last part of this sub-chapter will focus on novel and promising advanced therapies, including gene- and/or cell-based, for PAD treatment.

I.3.1. Disease characterization and current therapies

The prevalence of PAD in the general population ranges between 3-10%, increasing to 15-20% in subjects older than 70 (Gresele and Migliacci, 2009). It is estimated that more than 27 million individuals are affected by this disease in Europe and USA. Subjects with CLI, the most advanced stage of the disease, represent 1% of all PAD patients (Norgren et al., 2007). Due to population ageing and increasing incidence of cardiovascular diseases, and since the highest prevalence of PAD is in elderly people with cardiovascular risk factors, a raise on those numbers is expected for the next decades.

Although other causes have been pointed to cause artery obstruction, the main reason for PAD is the accumulation of atherosclerotic plaques (Conte and Vale, 2018). Those plaques are deposits of fatty acids that accumulate within the arteries, narrowing their diameter and, thus, diminishing blood flow. So, incidence of atherosclerosis in arteries supplying blood to the limbs is the major cause of PAD. This process of plaque accumulation and narrowing of limb arteries is schematized in Figure I.7.

While in the early stages of PAD patients may have no or only mild symptoms, such as a slight leg pain when walking, in more advanced stages subjects may face tissue loss or gangrene due to insufficient blood flow (Aronow, 2012). The limb pain suffered by those subjects is called claudication and may have a variable severity according to disease stage. In some cases there is only a mild and intermittent discomfort that disappears on rest and on most severe cases can be a debilitating pain that hinder exercise or even walking (Dua and Lee, 2016). CLI represents the more advanced stage of PAD and is characterized by a severe artery occlusion that according to ischemia level may cause chronic ischemic rest pain with or without trophic skin changes or

tissue loss (Gresele et al., 2011). Patients with CLI have an increased risk of suffering cardiovascular events.

Since asymptomatic stage is the most prevalent stage of the disease (Fowkes et al., 1991), it is extremely important to identify risk factors that contribute to disease initiation and progression. Age and gender are two major intrinsic risk factors that are associated with PAD development (Dua and Lee, 2016). In fact, there is a linear correlation between aging and onset of the disease, with an increased risk in population over 70 years old. The gender factor is dependent on age, since there is a major incidence on younger males. However, for elderly subjects (>60 years) the gender is no longer relevant (Norgren et al., 2007). Although these are uncontrollable elements, there are some extrinsic risk factors that may be modified to prevent or control disease progression. Smoking is one of such examples, since smokers have a 4-fold increased risk of PAD development with a proportional relationship between the number of cigarettes and the severity of the disease (Dua and Lee, 2016). Many studies showed that there is also a strong association between diabetes mellitus and PAD, being the presence of intermittent claudication 3-fold more common in diabetic patients. Hypertension and dyslipidemia are also two risk factors associated with PAD, however the risk is lower than for smoking or diabetes (Norgren et al., 2007, Aronow, 2012).

For asymptomatic subjects a change of lifestyle, including smoking cessation, weight loss, healthy eating and exercise, may reverse and prevent the progression of PAD. However, for more advanced stages of the disease, as CLI, additional medical treatments are needed. For subjects with intermittent claudication the initial treatment consists in medications for risk factor control, as cholesterol-lowering or hypertension medications, pain-relief narcotics and exercise rehabilitation programs (Kinlay, 2016, Gresele et al., 2011, Aronow, 2012). These strategies could also be associated with other medical therapies, including anti-platelet agents or anticoagulant medication, for control of disease symptoms, ameliorating life quality and reduce the risk of major cardiovascular events, such as stroke or MI, thus improving survival (Gresele et al., 2011, Dua and Lee, 2016). The described strategies are applied not only on early-stage PAD patients, but also on subjects with CLI to whom revascularization is not recommended due to comorbid conditions (Gresele et al., 2011). On more severe cases of CLI, or when failure to respond to pharmacotherapies occurs, limb revascularization should be considered in order to prevent amputations. On CLI patients where successful revascularization is not possible, the risk of limb amputation is over 40% and annual mortality rates within 6 months for such cases reach up to 20% (Dua and Lee, 2016, Norgren et al., 2007). Two major strategies can be used for revascularization: open surgery, which was the gold standard technique for decades, and

endovascular revascularization. Although open surgery has been successfully used with excellent limb salvage rates and clinical durability, endovascular revascularization has been preferred recently due to the reduced morbidity and mortality associated with the procedure (Dattilo and Casserly, 2011). These two procedures were compared in a randomized trial and no significant differences were observed in terms of amputation-free survival or health-related quality of life. However, complications as myocardial infarction (MI) or wound infection were more common on surgical group (bypass) whilst on endovascular (angioplasty) group repeated revascularizations were required (Adam et al., 2005). Despite both techniques have been improved since that study, there is no ideal approach for limb salvage and improve survival on CLI patients and high percentage of those subjects still face the risk of an amputation. Also, the major goal of such procedures is a quick establishment of reperfusion, failing to restore tissue function. In this context, the concept of therapeutic angiogenesis emerged and gene- and/or cell-based therapies to improve blood vessel remodeling and, thus, enhance tissue function in PAD patients have been developed (Grochot-Przeczek et al., 2013).

I.3.2. Cell and/or gene therapy approaches for PAD

Though revascularization may be an effective strategy for limb salvage in some patients, it is far from being the best option since it is an invasive procedure with high associated risks. Furthermore, 50% of CLI patients are not eligible for revascularization, the so-called no-option patients, and from those who are able to perform revascularization the beneficial effects are observed on only 25% (Norgren et al., 2007). Revascularization and pharmacological treatments are usually insufficient to recover blood flow and maintain normal tissue homeostasis. So, novel strategies for PAD treatment have been investigated, aiming to stimulate *in vivo* collateral blood vessel formation and, thus, provide the required blood flow to the ischemic tissues (Shimamura et al., 2013, Grochot-Przeczek et al., 2013, Hassanshahi et al., 2019). These approaches are designated therapeutic angiogenesis.

The first therapeutic angiogenesis strategy pursued to treat PAD involved the direct administration of pro-angiogenic growth factors (protein-based therapy), as VEGF or HGF, among others (Grochot-Przeczek et al., 2013, Hassanshahi et al., 2019). Administration of recombinant VEGF and bFGF alone or in combination showed to increase blood flow in several animal models of limb ischemia (Walder et al., 1996, Yang and Feng, 2000, Asahara et al., 1995). However, the efficacy of these protein-based approaches has been limited probably due to the short half-life of the pro-angiogenic proteins *in vivo* and to some degree of toxicity (Lederman

et al., 2002). Current studies of therapeutic angiogenesis for PAD are more focused on cell- and/or gene-based therapies since they show prolonged and targeted effect when compared to protein-based approaches.

I.3.2.1. Gene therapy approaches

The use of gene therapy strategies that sustain a targeted overexpression of pro-angiogenic genes such as VEGF, FGF or HGF is a promising alternative to treat PAD. VEGF is the most extensively studied factor for such purposes. The first human trial using VEGF-based gene therapies for PAD was initiated in 1994 in the wake of several successful animal studies. Isner and colleagues showed that intra arterially delivery of pDNA containing VEGF by a hydrogel-coated angioplasty catheter was able to improve *in vivo* angiogenesis in a patient with limb ischemia (Isner et al., 1996). Despite this is far from being the ideal approach due to the invasive nature of the procedure, this was the starting point for many other clinical trials using VEGF-base gene therapies for PAD treatment. Two years later, Baumgartner and co-workers demonstrated improvements in rest pain and limb integrity after intramuscular injections of a plasmid with VEGF gene in 10 CLI patients (Baumgartner et al., 1998). Conversely, two clinical trials performed later using adenoviral (Rajagopalan et al., 2003) or plasmid (Kusumanto et al., 2006) vectors for VEGF delivery showed no significant improvements after administration on 11 or 27 CLI patients, respectively. The long-term safety and tolerance of gene-based VEGF therapies was confirmed in a 10-year follow-up where no significant differences were noticed in the number of amputations or causes of death between control and VEGF-treated patients either by adenoviral or plasmid systems (Muona et al., 2012). However, some authors reported the occurrence of adverse events, as edemas, after *in vivo* gene transfer (Rajagopalan et al., 2003). Another limitation of VEGF unregulated overexpression is the possibility of hemangioma formation (Carmeliet, 2000). Thus, the controversial and inconclusive results of such trials hampered the establishment of VEGF gene-based approaches as a viable option for PAD patients to date. Nevertheless, a gene therapy product is commercially available in Russia that is based on a pDNA encoding the VEGF165 gene for the treatment of PAD. This therapy was approved after being showed to improve pain-free walking distance in patients with atherosclerotic lower limb ischemia in a phase III clinical trial (Deev et al., 2015). A 5-year follow up confirmed the safety and efficacy of the product by demonstrating an increased target limb salvage and an improved pain-free walking distance. Also, no significant differences on adverse events were observed between the treated group and control (Deev et al., 2018).

Similar controversial results were observed after FGF based gene therapies on PAD patients. While significant amelioration in pain and reduced risk of amputations was observed in early stage clinical trials (Comerota et al., 2002, Nikol et al., 2008), phase III trials failed to demonstrate the same efficacy and no beneficial effects were observed compared to control group (Belch et al., 2011).

HGF is probably the most relevant factor for gene-based therapeutic angiogenesis since on the opposite of VEGF and FGF, HGF is able to induce angiogenesis by a different pathway associated with less inflammatory changes (Kaga et al., 2012). The angiogenic capacity of HGF was observed in many pre-clinical animal studies (Taniyama et al., 2001, Nakagami et al., 2005), as well as in some clinical trials. The safety and efficacy of HGF-based gene therapies for PAD was first proved on a Phase I/IIa clinical trial where an increase in the ankle-brachial index (ABI) was accompanied by a decrease of the ischemic ulcer size on treated subjects. Also, no peripheral edemas were found, which brings an advantage of HGF over VEGF-based therapies (Morishita et al., 2011). The follow-up results from this study long-established the safety and efficacy of this treatment. After 2 years, all participants treated with HGF have a reduction in rest pain and no major complications or adverse events were detected due to gene therapy (Makino et al., 2012). Powell and colleagues also demonstrated the beneficial effects of HGF gene therapy on patients with CLI. They evaluated limb tissue perfusion as primary endpoint by measuring transcutaneous oxygen tension (TcO_2) after intramuscular injection of different doses of HGF pDNA. Although no differences were observed for secondary endpoints, including ABI, the TcO_2 on high-dose treated group was significantly increased compared with placebo (Powell et al., 2008). The efficacy and safety of plasmid-based HGF gene therapies on CLI was confirmed in a Phase III study with 44 patients. Primary endpoints were the improvement of rest pain in patients without ulcers or reduction of ulcer size in patients in more advanced stages of the disease. This study revealed a significant improvement in primary endpoint in the treated group when compared to placebo (70.4% vs 30.8%). Despite no improvements on ABI or amputation rate have been observed, an overall improvement on quality of life was achieved for the HGF-treated group. Also, no major safety problems have been detected (Shigematsu et al., 2010).

The reasons for the inconclusive results regarding gene therapy clinical trials for PAD remain unknown, but factors such as patient population, severity of disease, dose regimen and selected endpoints, as well as different properties of tested molecules, might influence study outcomes. Regardless the development of gene therapies for ischemic diseases are still ongoing, more effective approaches have been investigated.

I.3.2.2. Cell therapies

The unique properties of stem cells make them promising candidates as an alternative approach for treatment of PAD. Such properties include their ability to secrete a wide range of soluble factors with therapeutic activity and their capacity to differentiate into different cells types, including endothelial cells. In fact, stem cells can improve blood flow in ischemic tissues by four main actions: differentiation into endothelial cells, secretion of pro-angiogenic factors, incorporation into blood vessel walls or supply of appropriate microenvironment for endothelial or other cell types to favor angiogenesis and vascularization processes (Hassanshahi et al., 2019). Different classes of stem or progenitor cells have been investigated in clinical studies for PAD, being the most popular: endothelial progenitor cells (EPC), MSC or MNC, derived from BM or peripheral blood (PB). More than 50 trials have been exploited the potential application of cell therapies on PAD, including either autologous or allogeneic strategies.

EPC were firstly identified in 1997 as putative endothelial progenitors due to their central role in angiogenesis (Asahara et al., 1997). The EPC pro-angiogenic activity is known to be related with the secretion of important soluble factors: VEGF, SDF-1α, PDGF or IGF-1, but the actions of EPC secretome also include improvement of cell proliferation, inhibition of apoptosis as well as recruitment and activation of other stem/progenitor cells (Hassanshahi et al., 2019). Despite the beneficial activity of EPC on ischemic tissues is mainly associated with their paracrine activity over resident endothelial cells, some studies also suggested that EPC can directly integrate into blood vessel walls or differentiate into endothelial cells and thus replace the damaged tissues (Griese et al., 2003). Asahara and colleagues identified EPC by the expression of CD34 and VEGF-receptor 2 (Flk1), however subsequent studies have shown that specific cell markers or functions of EPCs remain controversial (Mayr et al., 2011), since those markers are also expressed by hematopoietic cells (Raval and Losordo, 2013). CD133 has also been used to characterize EPC populations (Gehling et al., 2000). Nevertheless, clinical studies on PAD with EPC populations selected by the expression of CD34 or CD133 markers have been performed with promising preliminary results.

After pre-clinical demonstration of the therapeutic potential of EPC on ischemic diseases (Kalka et al., 2000), trials using these cells on CLI have been developed. Kawamoto and colleagues showed in a Phase I/IIa clinical trial the feasibility and safety of using autologous CD34+ cells (hematopoietic and endothelial progenitor-enriched fraction) for CLI. The treated patients demonstrated a significantly recovery in the primary endpoints and no major amputation occurred and severe adverse events were rare (Kawamoto et al., 2009). Similar beneficial results

were observed by Arici and co-workers, who performed a trial using autologous CD133+ cells in 8 patients suffering from stable CLI. In 75% of the patients, it was observed a complete healing rest pain cessation and walking recovery and no complications have been detected in any of the subjects enrolled (Arici et al., 2015). However, due to the controversy regarding EPC imprecise definition related to its unclear origin, phenotype and properties (Rohde et al., 2006, Mayr et al., 2011), other cell types have been preferred for clinical approaches.

MNC that could be obtained from PB or BM have also been investigated for PAD treatment. Such populations are constituted by different cell types with pro-angiogenic activity, including monocyte and macrophage lineages and also EPC (Hazarika and Annex, 2017). Three different strategies can be used in order to take advantage of MNC properties towards disease treatment: (1) direct intramuscular or intra-arterial administration of BM-MNC; (2) direct administration of cytokine-mobilized and apheresis PB-MNC or (3) mobilization of patient's own MNC to ischemic regions by granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hazarika and Annex, 2017). A meta-analysis of 37 clinical trials showed that both PB- and BM-derived MNC are able to effectively improve ischemia indexes, subjective symptoms and endpoints as ulcer healing and amputation, whereas no significant improvements were observed by GM-CSF monotherapy (Fadini et al., 2010).

Different studies have compared the potential of both BM and PB MNC for ischemic diseases with some controversial results. Dubsky and colleagues demonstrated that patients treated with BM and PB autologous MNC had a significantly higher TcO_2 and lower rate of major amputation by 6 months when compared to the control, but no significant differences were observed between the two treated groups (Dubsky et al., 2013). Similar results were observed in a long-term prognosis study where there were no significant differences in negative prognostic factors (overall survival and amputation-free) between treatments using the two cell sources (Onodera et al., 2011). On the other hand, in Tateishi-Yuyama's study, BM-MNC showed significantly superior capacity to improve ABI and TcO_2 than PB-MNC (Tateishi-Yuyama et al., 2002). A long-term evaluation of this study confirmed the safety and efficacy of the therapy 2 years after initial treatment (Matoba et al., 2008). Despite those controversial outcomes that might be related to differences in study design, both systems showed to demonstrate long-term safety and effectiveness as cell therapies for PAD (Hassanshahi et al., 2019). The choice of the administration route is also an important factor for cell therapy approaches that might affect study outcomes. Although some encouraging results (improved ulcer healing and reduced rest pain) have been obtained on a clinical trial after intra-arterial delivery of BM-MNC (Walter et al.,

2011), intramuscular delivery demonstrated to be better since lower amputation rates have been verified using this method (Xie et al., 2018).

The therapeutic properties of MSC makes those as promising candidates for CLI treatment, which is reflected by the number of pre-clinical studies performed with these cells. The therapeutic activity of MSC on ischemic tissues relies on its capacity to secrete a widely panel of potent soluble factors, as well as on their ability to home to injured tissues and escape ischemia-induced apoptosis (Hassanshahi et al., 2019). The *in vivo* therapeutic angiogenesis of MSC has been confirmed in many animal studies with hindlimb ischemia models (Kinnaird et al., 2004, Leroux et al., 2010, Yan et al., 2013) and in some clinical trials (Lu et al., 2011, Gupta et al., 2013).

In fact, a pilot trial comparing the effect of BM-derived autologous MNC and MSC on diabetic CLI showed that MSC are more effective and well-tolerated. This was demonstrated by a faster and higher ulcer healing and more significant improvements in terms of painless walking time, ABI or TcO_2 on group treated with MSC than on MNC-treated patients. There was no difference on serious adverse events between the two groups (Lu et al., 2011). Another trial exploited the advantage of both cell types and proposed a combined strategy where both MNC and MSC are infused into limb ischemia patients. The assessments performed after 10 months follow-up confirmed the efficacy of such strategy, since improvements in walking time, ABI and quality of life were described, as well as increased perfusion in treated limbs (Lasala et al., 2010).

The majority of the trials described for PAD using cell-based therapies use autologous cells, however, some studies showed PAD patients have not only reduced cells numbers but also may have impaired cell function when compared to healthy donors (Teraa et al., 2013, Kizilay Mancini et al., 2017). Thus, it urges the development of a safety allogenic cell therapy that can be applied as an off-the-shelf treatment for no-option PAD patients. Due to their low-immunogenic and immunomodulatory properties, MSC are promising candidates for such approaches. The safety of using allogeneic BM MSC was tested in a phase I/II clinical trial in patients with established CLI. This trial not only confirmed the safety of this strategy, but also demonstrated the efficacy of MSC on improving rest pain and ABI on CLI patients (Gupta et al., 2013).

One limitation of using BM MSC for cell-therapy application is the higher cells numbers required, in contrast with the reduced amount of MSC that can be obtained from BM. Also, for autologous approaches there are contraindications for repeated BM biopsies, especially on subjects with several co-morbidities as CLI patients. So, other sources of MSC have been investigated for such approaches, including umbilical cord or adipose tissue (see section I.2.2. *MSC isolation and sources*).

Umbilical cords are considered biological waste, usually discarded after birth, so the possibility to use it as reliable source of MSC for therapeutic purposes holds great promise. In fact, Kim and colleagues demonstrated that transplantation of UC-derived MSC into four patients with Buerger's disease with CLI ameliorated their symptoms and improved peripheral circulation. Pain at rest of all treated patients was alleviated and skin lesions of 50% of patients were healed within 120 days. Likewise, angiography showed increased capillary formation and decreased vascular resistance on disease-affected regions. It should be noted that no side effects or allograft rejection signs were observed within the 25 month follow-up (Kim et al., 2006). Despite few clinical trials have been focused on umbilical cord-derived MSC to date, a phase I study confirmed the safety and tolerance of such approach in CLI patients. In this study, UC-derived MSC were intramuscularly injected in eight subjects with CLI that were considered ineligible for revascularization. Although some minor adverse events were observed after cell administration, all were resolved without additional treatment. Furthermore, 75% of ulcerations were completely healed after treatment and angiographic scores were improved in three of the eight patients (Yang et al., 2013).

AT-derived cells can also be an alternative for clinical application, since higher MSC yields can be harvested from this source with less invasive surgical procedures compared to BM. Moreover, AT-MSC have higher proliferation capacity than BM-derived counterparts (Frese et al., 2016). The capacity of AT-MSC to improve hind limb ischemia recovery was observed in several animal models and some clinical trials (Hassanshahi et al., 2019, Zhao et al., 2017). The intramuscular injection of AT-derived MSC revealed to promote clinical improvement on 66.7% of the treated patients on a pilot study where 15 CLI patients were included. After 6 months, the effects were noted not only on pain rating scales and walking distance, but also on angiography, which showed formation of collateral vessel networks on affected regions. No complications have been documented during this period (Lee et al., 2012). Similar results were observed in the first phase I trial evaluating feasibility and safety of AT-derived cells on CLI patients. Intramuscular injection of autologous AT-MSC lead to improvements in ulcer healing and enhancement of TcO_2 with no major associated complications in any of the seven patients enrolled (Bura et al., 2014). Currently there are several ongoing clinical trials to evaluate the potential of AT on ischemic diseases, using either autologous or allogeneic cells (Zhao et al., 2017).

Taken together, even though MSC cell therapies have shown promising results for ischemic diseases as PAD, the reduced cell numbers that can be obtained, the poor *in vivo* survival and relatively weak homing limited their large-scale application (Shi and Li, 2008). While the reduced

numbers retrieved from human sources could be easily overcome by an *in vitro* expansion before the administration, it is important to have standardized methods that allow cell-product consistency and purity. On the other hand, the *in vivo* survival, as well as homing ability, can be improved by the use of the strategies described on chapter I.2.4., including genetic engineering. Despite combined strategies of both cell and gene therapy for PAD have not reached human clinical trials yet, they showed promising results in many animal studies.

VEGF is a strong angiogenic factor with great potential on ischemic diseases, as already described, so its use for MSC engineering towards PAD treatment has been extensively investigated. MSC from umbilical cord or BM engineered with VEGF and administered on hindlimb ischemia mice models showed to promote not only faster blood flow restoration and vascular proliferation but also increased secretion of angiogenic molecules compared to controls (Beegle et al., 2016, Li et al., 2015c). Similar improvements on angiogenesis have been observed for hindlimb ischemia animal models, using MSC modified with HGF (Su et al., 2013), EPO (Li et al., 2015b) and FGF, alone (Zhang et al., 2014) or in combination with PDGF (Yin et al., 2015). Although none of these approaches have been used in humans to date, these pre-clinical studies revealed promising results that may be translated into clinical trials in an early future.

I.4. Conclusions

MSC have unique therapeutic properties, so their application for clinical purposes have emerged on the past decades with very encouraging results in several pre-clinical studies or early stage clinical trials. However, they failed to demonstrate the same efficacy on large human trials, where only modest benefits have been observed. Three main reasons have been pointed for the lack of MSC efficacy on such trials: poor engraftment, reduced tissue survival or insufficient trophic or immunomodulatory effects. Strategies to overcome these limitations and, consequently, improve MSC healing features have been developed. Genetic engineering with therapeutic factors is an example of such strategies.

Different methods have been investigated for stem cell engineering, including viral and non-viral systems. Despite the higher effectiveness of viral systems when compared to conventional plasmid-based gene therapies, they bring some safety concerns. To overcome the risks of using viral vectors, novel and optimized non-viral vectors have been developed. Minicircles are minimalistic pDNA derivatives, designed for an improved transgene expression and reduced toxicity that can be efficiently delivered to MSC.

The use of pro-angiogenic factors for MSC modification to improve their healing capacity is of special interest in context of cardiovascular ischemic diseases, such as PAD. PAD is a chronic high burden disease that affects up to 10% of worldwide population. Some patients with more severe stages of the disease are faced with the risk of an amputation or even dead due to the lack of viable treatments. Some studies reported the beneficial effects of using cell and/or gene therapies for the treatment of PAD. The use of a cell vehicle with intrinsic therapeutic factors, such as MSC, in combination with a potent pro-angiogenic factor, such as VEGF, might be a promising strategy for PAD treatment. Despite few studies to date reported the use of MSC genetic engineered with VEGF for PAD, several studies confirmed the efficacy of using either MSC or VEGF separately.

I.5. References

ADAM, D. J., BEARD, J. D., CLEVELAND, T., BELL, J., BRADBURY, A. W., FORBES, J. F., FOWKES, F. G., GILLEPSIE, I., RUCKLEY, C. V., RAAB, G. & STORKEY, H. 2005. Bypass versus angioplasty in severe ischaemia of the leg (BASIL): multicentre, randomised controlled trial. *Lancet,* 366**,** 1925-34.

AHMAD-NEJAD, P., HACKER, H., RUTZ, M., BAUER, S., VABULAS, R. M. & WAGNER, H. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol,* 32**,** 1958-68.

AHMED, R. P., ASHRAF, M., BUCCINI, S., SHUJIA, J. & HAIDER, H. 2011. Cardiac tumorigenic potential of induced pluripotent stem cells in an immunocompetent host with myocardial infarction. *Regen Med,* 6**,** 171-8.

AIUTI, A., CATTANEO, F., GALIMBERTI, S., BENNINGHOFF, U., CASSANI, B., CALLEGARO, L., SCARAMUZZA, S., ANDOLFI, G., MIROLO, M., BRIGIDA, I., TABUCCHI, A., CARLUCCI, F., EIBL, M., AKER, M., SLAVIN, S., AL-MOUSA, H., AL GHONAIUM, A., FERSTER, A., DUPPENTHALER, A., NOTARANGELO, L., WINTERGERST, U., BUCKLEY, R. H., BREGNI, M., MARKTEL, S., VALSECCHI, M. G., ROSSI, P., CICERI, F., MINIERO, R., BORDIGNON, C. & RONCAROLO, M. G. 2009. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med,* 360**,** 447-58.

AL-SAQI, S. H., SALIEM, M., ASIKAINEN, S., QUEZADA, H. C., EKBLAD, A., HOVATTA, O., LE BLANC, K., JONASSON, A. F. & GOTHERSTROM, C. 2014. Defined serum-free media for in vitro expansion of adipose-derived mesenchymal stem cells. *Cytotherapy,* 16**,** 915-26.

ALLISON, M. 2009. Genzyme backs Osiris, despite Prochymal flop. *Nat Biotech,* 27**,** 966-967.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2016. Development of a nicking endonuclease-assisted method for the purification of minicircles. *Journal of Chromatography A,* 1443**,** 136-144.

ANDERSON, E. M., SILVA, E. A., HAO, Y., MARTINICK, K. D., VERMILLION, S. A., STAFFORD, A. G., DOHERTY, E. G., WANG, L., DOHERTY, E. J., GROSSMAN, P. M. & MOONEY, D. J. 2017. VEGF and IGF Delivered from Alginate Hydrogels Promote Stable Perfusion Recovery in Ischemic Hind Limbs of Aged Mice and Young Rabbits. *J Vasc Res,* 54**,** 288-298.

ANDRZEJEWSKA, A., LUKOMSKA, B. & JANOWSKI, M. 2019. Mesenchymal stem cells: from roots to boost. *Stem Cells*.

ARICI, V., PEROTTI, C., FABRIZIO, C., DEL FANTE, C., RAGNI, F., ALESSANDRINO, F., VIARENGO, G., PAGANI, M., MOIA, A., TINELLI, C. & BOZZANI, A. 2015. Autologous immuno magnetically selected CD133+ stem cells in the treatment of no-option critical limb ischemia: clinical and contrast enhanced ultrasound assessed results in eight patients. *J Transl Med,* 13**,** 342.

ARONOW, W. S. 2012. Peripheral arterial disease of the lower extremities. *Arch Med Sci,* 8**,** 375-88.

ASAHARA, T., BAUTERS, C., ZHENG, L. P., TAKESHITA, S., BUNTING, S., FERRARA, N., SYMES, J. F. & ISNER, J. M. 1995. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation,* 92**,** Ii365-71.

ASAHARA, T., MUROHARA, T., SULLIVAN, A., SILVER, M., VAN DER ZEE, R., LI, T., WITZENBICHLER, B., SCHATTEMAN, G. & ISNER, J. M. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. *Science,* 275**,** 964-7.

ASLAN, H., ZILBERMAN, Y., ARBELI, V., SHEYN, D., MATAN, Y., LIEBERGALL, M., LI, J. Z., HELM, G. A., GAZIT, D. & GAZIT, Z. 2006. Nucleofection-based ex vivo nonviral gene delivery to human stem cells as a platform for tissue regeneration. *Tissue Eng,* 12**,** 877-89.

ASTORI, G., AMATI, E., BAMBI, F., BERNARDI, M., CHIEREGATO, K., SCHAFER, R., SELLA, S. & RODEGHIERO, F. 2016. Platelet lysate as a substitute for animal serum for the ex-vivo expansion of mesenchymal stem/stromal cells: present and future. *Stem Cell Res Ther,* 7**,** 93.

BAER, P. C. & GEIGER, H. 2012. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells Int,* 2012**,** 812693.

BAK, X. Y., YANG, J. & WANG, S. 2010. Baculovirus-transduced bone marrow mesenchymal stem cells for systemic cancer therapy. *Cancer Gene Ther,* 17**,** 721-9.

BANASIK, M. B. & MCCRAY, P. B., JR. 2010. Integrase-defective lentiviral vectors: progress and applications. *Gene Ther,* 17**,** 150-7.

BANDARA, N., GURUSINGHE, S., CHEN, H., CHEN, S., WANG, L. X., LIM, S. Y. & STRAPPE, P. 2016. Minicircle DNA-mediated endothelial nitric oxide synthase gene transfer enhances angiogenic responses of bone marrow-derived mesenchymal stem cells. *Stem Cell Res Ther,* 7**,** 48.

BANITALEBI DEHKORDI, M., MADJD, Z., CHALESHTORI, M. H., MESHKANI, R., NIKFARJAM, L. & KAJBAFZADEH, A. M. 2016. A Simple, Rapid, and Efficient Method for Isolating Mesenchymal Stem Cells From the Entire Umbilical Cord. *Cell Transplant,* 25**,** 1287-97.

BARTHOLOMEW, A., STURGEON, C., SIATSKAS, M., FERRER, K., MCINTOSH, K., PATIL, S., HARDY, W., DEVINE, S., UCKER, D., DEANS, R., MOSELEY, A. & HOFFMAN, R. 2002. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol,* 30**,** 42-8.

BARTOSH, T. J., YLOSTALO, J. H., MOHAMMADIPOOR, A., BAZHANOV, N., COBLE, K., CLAYPOOL, K., LEE, R. H., CHOI, H. & PROCKOP, D. J. 2010. Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proceedings of the National Academy of Sciences,* 107**,** 13724-13729.

BAUMGARTNER, I., PIECZEK, A., MANOR, O., BLAIR, R., KEARNEY, M., WALSH, K. & ISNER, J. M. 1998. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation,* 97**,** 1114-23.

BEEGLE, J. R., MAGNER, N. L., KALOMOIRIS, S., HARDING, A., ZHOU, P., NACEY, C., WHITE, J. L., PEPPER, K., GRUENLOH, W., ANNETT, G., NOLTA, J. A. & FIERRO, F. A. 2016. Preclinical evaluation of mesenchymal stem cells overexpressing VEGF to treat critical limb ischemia. *Molecular Therapy — Methods & Clinical Development,* 3**,** 16053.

BELCH, J., HIATT, W. R., BAUMGARTNER, I., DRIVER, I. V., NIKOL, S., NORGREN, L. & VAN BELLE, E. 2011. Effect of fibroblast growth factor NV1FGF on amputation and death: a randomised placebo-controlled trial of gene therapy in critical limb ischaemia. *Lancet,* 377**,** 1929-37.

BENABDALLAH, B. F., ALLARD, E., YAO, S., FRIEDMAN, G., GREGORY, P. D., ELIOPOULOS, N., FRADETTE, J., SPEES, J. L., HADDAD, E., HOLMES, M. C. & BEAUSEJOUR, C. M. 2010. Targeted gene addition to human mesenchymal stromal cells as a cell-based plasma-soluble protein delivery platform. *Cytotherapy,* 12**,** 394-9.

BONGSO, A. & LEE, E. H. 2012. Stem Cells: Their Definition, Classification and Sources. *Stem Cells.* WORLD SCIENTIFIC.

BONNET, D. 2003. Biology of human bone marrow stem cells. *Clin Exp Med,* 3**,** 140-9.

BORGER, V., BREMER, M., FERRER-TUR, R., GOCKELN, L., STAMBOULI, O., BECIC, A. & GIEBEL, B. 2017. Mesenchymal Stem/Stromal Cell-Derived Extracellular Vesicles and Their Potential as Novel Immunomodulatory Therapeutic Agents. *Int J Mol Sci,* 18.

BOURA, J. S., SANTOS, F. D., GIMBLE, J. M., CARDOSO, C. M. P., MADEIRA, C., CABRAL, J. M. S. & SILVA, C. L. D. 2013. Direct Head-To-Head Comparison of Cationic Liposome-Mediated Gene Delivery to Mesenchymal Stem/Stromal Cells of Different Human Sources: A Comprehensive Study. *Human Gene Therapy Methods,* 24**,** 38-48.

BOURA, J. S., VANCE, M., YIN, W., MADEIRA, C., LOBATO DA SILVA, C., PORADA, C. D. & ALMEIDA-PORADA, G. 2014. Evaluation of gene delivery strategies to efficiently overexpress functional HLA-G on human bone marrow stromal cells. *Mol Ther Methods Clin Dev,* 2014.

BOUSSIF, O., LEZOUALC'H, F., ZANTA, M. A., MERGNY, M. D., SCHERMAN, D., DEMENEIX, B. & BEHR, J. P. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A,* 92**,** 7297-301.

BOYETTE, L. B., CREASEY, O. A., GUZIK, L., LOZITO, T. & TUAN, R. S. 2014. Human bone marrow-derived mesenchymal stem cells display enhanced clonogenicity but impaired differentiation with hypoxic preconditioning. *Stem Cells Transl Med,* 3**,** 241-54.

BRONCKAERS, A., HILKENS, P., MARTENS, W., GERVOIS, P., RATAJCZAK, J., STRUYS, T. & LAMBRICHTS, I. 2014. Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacol Ther,* 143**,** 181-96.

BRUNNER, S., SAUER, T., CAROTTA, S., COTTEN, M., SALTIK, M. & WAGNER, E. 2000. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Ther,* 7**,** 401-7.

BURA, A., PLANAT-BENARD, V., BOURIN, P., SILVESTRE, J. S., GROSS, F., GROLLEAU, J. L., SAINT-LEBESE, B., PEYRAFITTE, J. A., FLEURY, S., GADELORGE, M., TAURAND, M., DUPUIS-CORONAS, S., LEOBON, B. & CASTEILLA, L. 2014. Phase I trial: the use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia. *Cytotherapy,* 16**,** 245-57.

BURNOUF, T., STRUNK, D., KOH, M. B. & SCHALLMOSER, K. 2016. Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation? *Biomaterials,* 76**,** 371-87.

BUSSER, H., NAJAR, M., RAICEVIC, G., PIETERS, K., VELEZ POMBO, R., PHILIPPART, P., MEULEMAN, N., BRON, D. & LAGNEAUX, L. 2015. Isolation and Characterization of Human Mesenchymal Stromal Cell Subpopulations: Comparison of Bone Marrow and Adipose Tissue. *Stem Cells Dev,* 24**,** 2142-57.

BUYL, K., VANHAECKE, T., DESMAE, T., LAGNEAUX, L., ROGIERS, V., NAJAR, M. & DE KOCK, J. 2015. Evaluation of a new standardized enzymatic isolation protocol for human umbilical cord-derived stem cells. *Toxicol In Vitro,* 29**,** 1254-62.

CAPELLI, C., DOMENGHINI, M., BORLERI, G., BELLAVITA, P., POMA, R., CAROBBIO, A., MICO, C., RAMBALDI, A., GOLAY, J. & INTRONA, M. 2007. Human platelet lysate allows expansion and clinical grade production of mesenchymal stromal cells from small samples of bone marrow aspirates or marrow filter washouts. *Bone Marrow Transplant,* 40**,** 785-91.

CAPLAN, A. I. 1991. Mesenchymal stem cells. *J Orthop Res,* 9**,** 641-50.

CAPLAN, A. I. & BRUDER, S. P. 2001. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med,* 7**,** 259-64.

CAPLAN, A. I. & CORREA, D. 2011. THE MSC: AN INJURY DRUGSTORE. *Cell Stem Cell,* 9**,** 11-5.

CARLIN, R., DAVIS, D., WEISS, M., SCHULTZ, B. & TROYER, D. 2006. Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. *Reprod Biol Endocrinol,* 4**,** 8.

CARMELIET, P. 2000. VEGF gene therapy: stimulating angiogenesis or angioma-genesis? *Nat Med,* 6**,** 1102-3.

CARMELIET, P. 2003. Angiogenesis in health and disease. *Nat Med,* 9**,** 653-60.

CARMELO, J. G., FERNANDES-PLATZGUMMER, A., DIOGO, M. M., DA SILVA, C. L. & CABRAL, J. M. 2015. A xeno-free microcarrier-based stirred culture system for the scalable expansion of human mesenchymal stem/stromal cells isolated from bone marrow and adipose tissue. *Biotechnol J,* 10**,** 1235-47.

CHALLITA, P. M. & KOHN, D. B. 1994. Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc Natl Acad Sci U S A,* 91**,** 2567-71.

CHANG, C.-W., CHRISTENSEN, L. V., LEE, M. & KIM, S. W. 2008. Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy. *Journal of Controlled Release,* 125**,** 155-163.

CHASE, L. G., YANG, S., ZACHAR, V., YANG, Z., LAKSHMIPATHY, U., BRADFORD, J., BOUCHER, S. E. & VEMURI, M. C. 2012. Development and Characterization of a Clinically Compliant Xeno-Free Culture Medium in Good Manufacturing Practice for Human Multipotent Mesenchymal Stem Cells. *Stem Cells Transl Med.*

CHEN, B., CHEN, X., LIU, C., LI, J., LIU, F. & HUANG, Y. 2018. Co-expression of Akt1 and Wnt11 promotes the proliferation and cardiac differentiation of mesenchymal stem cells and attenuates hypoxia/reoxygenation-induced cardiomyocyte apoptosis. *Biomed Pharmacother,* 108**,** 508-514.

CHEN, G., YUE, A., RUAN, Z., YIN, Y., WANG, R., REN, Y. & ZHU, L. 2014. Human umbilical cord-derived mesenchymal stem cells do not undergo malignant transformation during long-term culturing in serum-free medium. *PLoS One,* 9**,** e98565.

CHEN, S. L., ZHU, C. C., LIU, Y. Q., TANG, L. J., YI, L., YU, B. J. & WANG, D. J. 2009. Mesenchymal stem cells genetically modified with the angiopoietin-1 gene enhanced arteriogenesis in a porcine model of chronic myocardial ischaemia. *J Int Med Res,* 37**,** 68-78.

CHEN, Z. Y., HE, C. Y., EHRHARDT, A. & KAY, M. A. 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther,* 8**,** 495-500.

CHEN, Z. Y., HE, C. Y. & KAY, M. A. 2005. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. *Hum Gene Ther,* 16**,** 126-31.

CHENG, N.-C., WANG, S. & YOUNG, T.-H. 2012. The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. *Biomaterials,* 33**,** 1748-1758.

CHENG, Z., OU, L., ZHOU, X., LI, F., JIA, X., ZHANG, Y., LIU, X., LI, Y., WARD, C. A., MELO, L. G. & KONG, D. 2008. Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. *Mol Ther,* 16**,** 571-9.

CHESNOY, S. & HUANG, L. 2000. Structure and function of lipid-DNA complexes for gene delivery. *Annu Rev Biophys Biomol Struct,* 29**,** 27-47.

CHO, S. W., SUN, H. J., YANG, J. Y., JUNG, J. Y., AN, J. H., CHO, H. Y., CHOI, H. J., KIM, S. W., KIM, S. Y., KIM, D. & SHIN, C. S. 2009. Transplantation of mesenchymal stem cells overexpressing RANK-Fc or CXCR4 prevents bone loss in ovariectomized mice. *Mol Ther,* 17**,** 1979-87.

CHOI, J. R., PINGGUAN-MURPHY, B., WAN ABAS, W. A., NOOR AZMI, M. A., OMAR, S. Z., CHUA, K. H. & WAN SAFWANI, W. K. 2014. Impact of low oxygen tension on stemness, proliferation and differentiation potential of human adipose-derived stem cells. *Biochem Biophys Res Commun,* 448**,** 218-24.

CHOWDHURY, E. H. & AKAIKE, T. 2005. Bio-functional inorganic materials: an attractive branch of gene-based nano-medicine delivery for 21st century. *Curr Gene Ther,* 5**,** 669-76.

CHRISTIE, A. & BUTLER, M. 1994. Glutamine-based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase. *J Biotechnol,* 37**,** 277-90.

CHUANG, C. K., WONG, T. H., HWANG, S. M., CHANG, Y. H., CHEN, G. Y., CHIU, Y. C., HUANG, S. F. & HU, Y. C. 2009. Baculovirus transduction of mesenchymal stem cells: in vitro responses and in vivo immune responses after cell transplantation. *Mol Ther,* 17**,** 889-96.

CHULLIKANA, A., MAJUMDAR, A. S., GOTTIPAMULA, S., KRISHNAMURTHY, S., KUMAR, A. S., PRAKASH, V. S. & GUPTA, P. K. 2015. Randomized, double-blind, phase I/II study of intravenous allogeneic mesenchymal stromal cells in acute myocardial infarction. *Cytotherapy,* 17**,** 250-61.

CLEMENTS, B. A., INCANI, V., KUCHARSKI, C., LAVASANIFAR, A., RITCHIE, B. & ULUDAG, H. 2007. A comparative evaluation of poly-L-lysine-palmitic acid and Lipofectamine 2000 for plasmid delivery to bone marrow stromal cells. *Biomaterials,* 28**,** 4693-704.

COMEROTA, A. J., THROM, R. C., MILLER, K. A., HENRY, T., CHRONOS, N., LAIRD, J., SEQUEIRA, R., KENT, C. K., BACCHETTA, M., GOLDMAN, C., SALENIUS, J. P., SCHMIEDER, F. A. & PILSUDSKI, R. 2002. Naked plasmid DNA encoding fibroblast growth factor type 1 for the treatment of end-stage

unreconstructible lower extremity ischemia: preliminary results of a phase I trial. *J Vasc Surg,* 35**,** 930-6.

CONTE, S. M. & VALE, P. R. 2018. Peripheral Arterial Disease. *Heart Lung Circ,* 27**,** 427-432.

CORSI, K., CHELLAT, F., YAHIA, L. & FERNANDES, J. C. 2003. Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. *Biomaterials,* 24**,** 1255-64.

CUENDE, N., RASKO, J. E. J., KOH, M. B. C., DOMINICI, M. & IKONOMOU, L. 2018. Cell, tissue and gene products with marketing authorization in 2018 worldwide. *Cytotherapy,* 20**,** 1401-1413.

CUNNINGHAM, C. J., REDONDO-CASTRO, E. & ALLAN, S. M. 2018. The therapeutic potential of the mesenchymal stem cell secretome in ischaemic stroke. *J Cereb Blood Flow Metab***,** 271678x18776802.

CURTIN, C. M., CUNNIFFE, G. M., LYONS, F. G., BESSHO, K., DICKSON, G. R., DUFFY, G. P. & O'BRIEN, F. J. 2012. Innovative collagen nano-hydroxyapatite scaffolds offer a highly efficient non-viral gene delivery platform for stem cell-mediated bone formation. *Adv Mater,* 24**,** 749-54.

DAHLBERG, J. E. 1988. An overview of retrovirus replication and classification. *Adv Vet Sci Comp Med,* 32**,** 1-35.

DANESHVAR, N., RASEDEE, A., SHAMSABADI, F. T., MOEINI, H., MEHRBOUD, P., RAHMAN, H. S., BOROOJERDI, M. H. & VELLASAMY, S. 2015. Induction of pluripotency in human umbilical cord mesenchymal stem cells in feeder layer-free condition. *Tissue Cell,* 47**,** 575-82.

DANG, J. M. & LEONG, K. W. 2006. Natural polymers for gene delivery and tissue engineering. *Adv Drug Deliv Rev,* 58**,** 487-99.

DARQUET, A. M., CAMERON, B., WILS, P., SCHERMAN, D. & CROUZET, J. 1997. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther,* 4**,** 1341-9.

DARQUET, A. M., RANGARA, R., KREISS, P., SCHWARTZ, B., NAIMI, S., DELAERE, P., CROUZET, J. & SCHERMAN, D. 1999. Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer. *Gene Ther,* 6**,** 209-18.

DATTILO, P. B. & CASSERLY, I. P. 2011. Critical limb ischemia: endovascular strategies for limb salvage. *Prog Cardiovasc Dis,* 54**,** 47-60.

DE CARVALHO, T. G., PELLENZ, F. M., LAUREANO, A., DA ROCHA SILLA, L. M., GIUGLIANI, R., BALDO, G. & MATTE, U. 2018. A simple protocol for transfecting human mesenchymal stem cells. *Biotechnol Lett,* 40**,** 617-622.

DE SOURE, A. M., FERNANDES-PLATZGUMMER, A., DA SILVA, C. L. & CABRAL, J. M. 2016. Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells. *J Biotechnol,* 236**,** 88-109.

DE SOURE, A. M., FERNANDES-PLATZGUMMER, A., MOREIRA, F., LILAIA, C., LIU, S. H., KU, C. P., HUANG, Y. F., MILLIGAN, W., CABRAL, J. M. S. & DA SILVA, C. L. 2017. 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,* 11**,** 1630-1640.

DEEV, R., PLAKSA, I., BOZO, I., MZHAVANADZE, N., SUCHKOV, I., CHERVYAKOV, Y., STAROVEROV, I., KALININ, R. & ISAEV, A. 2018. Results of 5-year follow-up study in patients with peripheral artery disease treated with PL-VEGF165 for intermittent claudication. *Ther Adv Cardiovasc Dis,* 12**,** 237-246.

DEEV, R. V., BOZO, I. Y., MZHAVANADZE, N. D., VORONOV, D. A., GAVRILENKO, A. V., CHERVYAKOV, Y. V., STAROVEROV, I. N., KALININ, R. E., SHVALB, P. G. & ISAEV, A. A. 2015. pCMV-vegf165 Intramuscular Gene Transfer is an Effective Method of Treatment for Patients With Chronic Lower Limb Ischemia. *J Cardiovasc Pharmacol Ther,* 20**,** 473-82.

DEUSE, T., PETER, C., FEDAK, P. W., DOYLE, T., REICHENSPURNER, H., ZIMMERMANN, W. H., ESCHENHAGEN, T., STEIN, W., WU, J. C., ROBBINS, R. C. & SCHREPFER, S. 2009. Hepatocyte

growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. *Circulation,* 120**,** S247-54.

DI NICOLA, M., CARLO-STELLA, C., MAGNI, M., MILANESI, M., LONGONI, P. D., MATTEUCCI, P., GRISANTI, S. & GIANNI, A. M. 2002. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood,* 99**,** 3838-43.

DIETZ, W. M., SKINNER, N. E., HAMILTON, S. E., JUND, M. D., HEITFELD, S. M., LITTERMAN, A. J., HWU, P., CHEN, Z. Y., SALAZAR, A. M., OHLFEST, J. R., BLAZAR, B. R., PENNELL, C. A. & OSBORN, M. J. 2013. Minicircle DNA is superior to plasmid DNA in eliciting antigen-specific CD8+ T-cell responses. *Mol Ther,* 21**,** 1526-35.

DIMARAKIS, I. & LEVICAR, N. 2006. Cell culture medium composition and translational adult bone marrow-derived stem cell research. *Stem Cells.* United States.

DOI, K., KUNO, S., KOBAYASHI, A., HAMABUCHI, T., KATO, H., KINOSHITA, K., ETO, H., AOI, N. & YOSHIMURA, K. 2014. Enrichment isolation of adipose-derived stem/stromal cells from the liquid portion of liposuction aspirates with the use of an adherent column. *Cytotherapy,* 16**,** 381-91.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-7.

DOS SANTOS, F., ANDRADE, P. Z., BOURA, J. S., ABECASIS, M. M., DA SILVA, C. U. L. & CABRAL, J. M. S. 2010. Ex vivo expansion of human mesenchymal stem cells: A more effective cell proliferation kinetics and metabolism under hypoxia. *Journal of Cellular Physiology,* 223**,** 27-35.

DUA, A. & LEE, C. J. 2016. Epidemiology of Peripheral Arterial Disease and Critical Limb Ischemia. *Tech Vasc Interv Radiol,* 19**,** 91-5.

DUAN, H. F., WU, C. T., WU, D. L., LU, Y., LIU, H. J., HA, X. Q., ZHANG, Q. W., WANG, H., JIA, X. X. & WANG, L. S. 2003. Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor. *Mol Ther,* 8**,** 467-74.

DUBON, M. J., YU, J., CHOI, S. & PARK, K. S. 2018. Transforming growth factor beta induces bone marrow mesenchymal stem cell migration via noncanonical signals and N-cadherin. *J Cell Physiol,* 233**,** 201-213.

DUBSKY, M., JIRKOVSKA, A., BEM, R., FEJFAROVA, V., PAGACOVA, L., SIXTA, B., VARGA, M., LANGKRAMER, S., SYKOVA, E. & JUDE, E. B. 2013. Both autologous bone marrow mononuclear cell and peripheral blood progenitor cell therapies similarly improve ischaemia in patients with diabetic foot in comparison with control treatment. *Diabetes Metab Res Rev,* 29**,** 369-76.

DURYMANOV, M. & REINEKE, J. 2018. Non-viral Delivery of Nucleic Acids: Insight Into Mechanisms of Overcoming Intracellular Barriers. *Front Pharmacol,* 9**,** 971.

EITAN, Y., SARIG, U., DAHAN, N. & MACHLUF, M. 2010. Acellular cardiac extracellular matrix as a scaffold for tissue engineering: in vitro cell support, remodeling, and biocompatibility. *Tissue Eng Part C Methods,* 16**,** 671-83.

ELSEBERG, C., LEBER, J., WEIDNER, T. & CZERMAK, P. 2017. The Challenge of Human Mesenchymal Stromal Cell Expansion: Current and Prospective Answers. *New Insights into Cell Culture Technology.*

EPPLER, S. M., COMBS, D. L., HENRY, T. D., LOPEZ, J. J., ELLIS, S. G., YI, J. H., ANNEX, B. H., MCCLUSKEY, E. R. & ZIONCHECK, T. F. 2002. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. *Clin Pharmacol Ther,* 72**,** 20-32.

EVANS, M. J. & KAUFMAN, M. H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature,* 292**,** 154-6.

FADINI, G. P., AGOSTINI, C. & AVOGARO, A. 2010. Autologous stem cell therapy for peripheral arterial disease meta-analysis and systematic review of the literature. *Atherosclerosis,* 209**,** 10-7.

FARANESH, A. Z., NASTLEY, M. T., DE LA CRUZ, C. P., HALLER, M. F., LAQUERRIERE, P., LEONG, K. W. & MCVEIGH, E. R. 2004. In Vitro Release of Vascular Endothelial Growth Factor From Gadolinium-Doped Biodegradable Microspheres. *Magn Reson Med,* 51**,** 1265-71.

FARRELL, L. L., PEPIN, J., KUCHARSKI, C., LIN, X., XU, Z. & ULUDAG, H. 2007. A comparison of the effectiveness of cationic polymers poly-L-lysine (PLL) and polyethylenimine (PEI) for non-viral delivery of plasmid DNA to bone marrow stromal cells (BMSC). *Eur J Pharm Biopharm,* 65**,** 388-97.

FEI, S., QI, X., KEDONG, S., GUANGCHUN, J., JIAN, L. & WEI, Q. 2012. The antitumor effect of mesenchymal stem cells transduced with a lentiviral vector expressing cytosine deaminase in a rat glioma model. *J Cancer Res Clin Oncol,* 138**,** 347-57.

FELFLY, H. & HADDAD, G. G. 2014. Hematopoietic stem cells: potential new applications for translational medicine. *J Stem Cells,* 9**,** 163-97.

FERRARA, N. 2001. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol,* 280**,** C1358-66.

FERREIRA, E., POTIER, E., VAUDIN, P., OUDINA, K., BENSIDHOUM, M., LOGEART-AVRAMOGLOU, D., MIR, L. M. & PETITE, H. 2012. Sustained and promoter dependent bone morphogenetic protein expression by rat mesenchymal stem cells after BMP-2 transgene electrotransfer. *Eur Cell Mater,* 24**,** 18-28.

FERREIRA, J. R., TEIXEIRA, G. Q., SANTOS, S. G., BARBOSA, M. A., ALMEIDA-PORADA, G. & GONCALVES, R. M. 2018. Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-conditioning. *Front Immunol,* 9**,** 2837.

FONG, C. Y., CHAK, L. L., BISWAS, A., TAN, J. H., GAUTHAMAN, K., CHAN, W. K. & BONGSO, A. 2011. Human Wharton's jelly stem cells have unique transcriptome profiles compared to human embryonic stem cells and other mesenchymal stem cells. *Stem Cell Rev,* 7**,** 1-16.

FONTAINE, M. J., SHIH, H., SCHAFER, R. & PITTENGER, M. F. 2016. Unraveling the Mesenchymal Stromal Cells' Paracrine Immunomodulatory Effects. *Transfus Med Rev,* 30**,** 37-43.

FOWKES, F. G., HOUSLEY, E., CAWOOD, E. H., MACINTYRE, C. C., RUCKLEY, C. V. & PRESCOTT, R. J. 1991. Edinburgh Artery Study: prevalence of asymptomatic and symptomatic peripheral arterial disease in the general population. *Int J Epidemiol,* 20**,** 384-92.

FRASER, J. K., HICOK, K. C., SHANAHAN, R., ZHU, M., MILLER, S. & ARM, D. M. 2014. The Celution® System: Automated Processing of Adipose-Derived Regenerative Cells in a Functionally Closed System. *Adv Wound Care (New Rochelle).*

FRESE, L., DIJKMAN, P. E. & HOERSTRUP, S. P. 2016. Adipose Tissue-Derived Stem Cells in Regenerative Medicine. *Transfus Med Hemother,* 43**,** 268-274.

FRIEDENSTEIN, A. J., CHAILAKHJAN, R. K. & LALYKINA, K. S. 1970. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet,* 3**,** 393-403.

FRISCH, J., VENKATESAN, J. K., REY-RICO, A., SCHMITT, G., MADRY, H. & CUCCHIARINI, M. 2014a. Determination of the chondrogenic differentiation processes in human bone marrow-derived mesenchymal stem cells genetically modified to overexpress transforming growth factor-beta via recombinant adeno-associated viral vectors. *Hum Gene Ther,* 25**,** 1050-60.

FRISCH, J., VENKATESAN, J. K., REY-RICO, A., SCHMITT, G., MADRY, H. & CUCCHIARINI, M. 2014b. Influence of insulin-like growth factor I overexpression via recombinant adeno-associated vector gene transfer upon the biological activities and differentiation potential of human bone marrow-derived mesenchymal stem cells. *Stem Cell Res Ther,* 5**,** 103.

FRITH, J. E., THOMSON, B. & GENEVER, P. G. 2010. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. *Tissue Eng Part C Methods,* 16**,** 735-49.

GALIPEAU, J. & SENSEBE, L. 2018. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell,* 22**,** 824-833.

GAO, F., HE, T., WANG, H., YU, S., YI, D., LIU, W. & CAI, Z. 2007. A promising strategy for the treatment of ischemic heart disease: Mesenchymal stem cell-mediated vascular endothelial growth factor gene transfer in rats. *Can J Cardiol,* 23**,** 891-8.

GASPAR, V., DE MELO-DIOGO, D., COSTA, E., MOREIRA, A., QUEIROZ, J., PICHON, C., CORREIA, I. & SOUSA, F. 2015. Minicircle DNA vectors for gene therapy: advances and applications. *Expert Opin Biol Ther,* 15**,** 353-79.

GASPAR, V. M., MAIA, C. J., QUEIROZ, J. A., PICHON, C., CORREIA, I. J. & SOUSA, F. 2014. Improved minicircle DNA biosynthesis for gene therapy applications. *Hum Gene Ther Methods,* 25**,** 93-105.

GEBLER, A., ZABEL, O. & SELIGER, B. 2012. The immunomodulatory capacity of mesenchymal stem cells. *Trends in Molecular Medicine,* 18**,** 128-134.

GEHLING, U. M., ERGUN, S., SCHUMACHER, U., WAGENER, C., PANTEL, K., OTTE, M., SCHUCH, G., SCHAFHAUSEN, P., MENDE, T., KILIC, N., KLUGE, K., SCHAFER, B., HOSSFELD, D. K. & FIEDLER, W. 2000. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood,* 95**,** 3106-12.

GHEISARI, Y., SOLEIMANI, M., AZADMANESH, K. & ZEINALI, S. 2008. Multipotent mesenchymal stromal cells: optimization and comparison of five cationic polymer-based gene delivery methods. *Cytotherapy,* 10**,** 815-23.

GILL, D. R., PRINGLE, I. A. & HYDE, S. C. 2009. Progress and prospects: the design and production of plasmid vectors. *Gene Ther,* 16**,** 165-71.

GIMBLE, J. M., KATZ, A. J. & BUNNELL, B. A. 2007. Adipose-derived stem cells for regenerative medicine. *Circ Res,* 100**,** 1249-60.

GINN, S. L., AMAYA, A. K., ALEXANDER, I. E., EDELSTEIN, M. & ABEDI, M. R. 2018. Gene therapy clinical trials worldwide to 2017: An update. *J Gene Med,* 20**,** e3015.

GONCALVES, R., LOBATO DA SILVA, C., CABRAL, J. M., ZANJANI, E. D. & ALMEIDA-PORADA, G. 2006. A Stro-1(+) human universal stromal feeder layer to expand/maintain human bone marrow hematopoietic stem/progenitor cells in a serum-free culture system. *Exp Hematol,* 34**,** 1353-9.

GOTTIPAMULA, S., ASHWIN, K. M., MUTTIGI, M. S., KANNAN, S., KOLKUNDKAR, U. & SEETHARAM, R. N. 2014. Isolation, expansion and characterization of bone marrow-derived mesenchymal stromal cells in serum-free conditions. *Cell Tissue Res,* 356**,** 123-35.

GRESCH, O., ENGEL, F. B., NESIC, D., TRAN, T. T., ENGLAND, H. M., HICKMAN, E. S., KORNER, I., GAN, L., CHEN, S., CASTRO-OBREGON, S., HAMMERMANN, R., WOLF, J., MULLER-HARTMANN, H., NIX, M., SIEBENKOTTEN, G., KRAUS, G. & LUN, K. 2004. New non-viral method for gene transfer into primary cells. *Methods,* 33**,** 151-63.

GRESELE, P., BUSTI, C. & FIERRO, T. 2011. Critical limb ischemia. *Internal and Emergency Medicine,* 6**,** 129-134.

GRESELE, P. & MIGLIACCI, R. 2009. The peripheral arterial disease subgroup in the CHARISMA trial: does it tell us anything new? *Eur Heart J,* 30**,** 131-2.

GRIESE, D. P., EHSAN, A., MELO, L. G., KONG, D., ZHANG, L., MANN, M. J., PRATT, R. E., MULLIGAN, R. C. & DZAU, V. J. 2003. Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. *Circulation,* 108**,** 2710-5.

GRIMM, D. & KAY, M. A. 2003. From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. *Curr Gene Ther,* 3**,** 281-304.

GROCHOT-PRZECZEK, A., DULAK, J. & JOZKOWICZ, A. 2013. Therapeutic angiogenesis for revascularization in peripheral artery disease. *Gene,* 525**,** 220-228.

GRONTHOS, S., FRANKLIN, D. M., LEDDY, H. A., ROBEY, P. G., STORMS, R. W. & GIMBLE, J. M. 2001. Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol,* 189**,** 54-63.

GROTH, A. C. & CALOS, M. P. 2004. Phage integrases: biology and applications. *J Mol Biol,* 335**,** 667-78.

GUPTA, P. K., CHULLIKANA, A., PARAKH, R., DESAI, S., DAS, A., GOTTIPAMULA, S., KRISHNAMURTHY, S., ANTHONY, N., PHERWANI, A. & MAJUMDAR, A. S. 2013. A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. *J Transl Med,* 11**,** 143.

GWAK, S. J. & KIM, B. S. 2008. Poly(lactic-co-glycolic acid) nanosphere as a vehicle for gene delivery to human cord blood-derived mesenchymal stem cells: comparison with polyethylenimine. *Biotechnol Lett,* 30**,** 1177-82.

HACEIN-BEY-ABINA, S., HAUER, J., LIM, A., PICARD, C., WANG, G. P., BERRY, C. C., MARTINACHE, C., RIEUX-LAUCAT, F., LATOUR, S., BELOHRADSKY, B. H., LEIVA, L., SORENSEN, R., DEBRE, M., CASANOVA, J. L., BLANCHE, S., DURANDY, A., BUSHMAN, F. D., FISCHER, A. & CAVAZZANA-CALVO, M. 2010. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med,* 363**,** 355-64.

HACEIN-BEY-ABINA, S., VON KALLE, C., SCHMIDT, M., LE DEIST, F., WULFFRAAT, N., MCINTYRE, E., RADFORD, I., VILLEVAL, J. L., FRASER, C. C., CAVAZZANA-CALVO, M. & FISCHER, A. 2003. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med.* United States.

HÄCKER, G., REDECKE, V. & HÄCKER, H. 2002. Activation of the immune system by bacterial CpG-DNA. *Immunology,* 105**,** 245-51.

HAMANN, A., BROAD, K., NGUYEN, A. & PANNIER, A. K. 2019a. Mechanisms of unprimed and dexamethasone-primed nonviral gene delivery to human mesenchymal stem cells. *Biotechnol Bioeng,* 116**,** 427-443.

HAMANN, A., NGUYEN, A. & PANNIER, A. K. 2019b. Nucleic acid delivery to mesenchymal stem cells: a review of nonviral methods and applications. *J Biol Eng,* 13**,** 7.

HAMM, A., KROTT, N., BREIBACH, I., BLINDT, R. & BOSSERHOFF, A. K. 2002. Efficient transfection method for primary cells. *Tissue Eng,* 8**,** 235-45.

HAN, S. W., NAKAMURA, C., KOTOBUKI, N., OBATAYA, I., OHGUSHI, H., NAGAMUNE, T. & MIYAKE, J. 2008. High-efficiency DNA injection into a single human mesenchymal stem cell using a nanoneedle and atomic force microscopy. *Nanomedicine,* 4**,** 215-25.

HAN, Y. F., TAO, R., SUN, T. J., CHAI, J. K., XU, G. & LIU, J. 2013. Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods. *Cytotechnology,* 65**,** 819-27.

HANG, H., YU, Y., WU, N., HUANG, Q., XIA, Q. & BIAN, J. 2014. Induction of Highly Functional Hepatocytes from Human Umbilical Cord Mesenchymal Stem Cells by HNF4α Transduction. *PLoS One.*

HAQUE, N., KASIM, N. H. & RAHMAN, M. T. 2015. Optimization of pre-transplantation conditions to enhance the efficacy of mesenchymal stem cells. *Int J Biol Sci,* 11**,** 324-34.

HARDEE, C. L., AREVALO-SOLIZ, L. M., HORNSTEIN, B. D. & ZECHIEDRICH, L. 2017. Advances in Non-Viral DNA Vectors for Gene Therapy. *Genes (Basel),* 8.

HARE, J. M., TRAVERSE, J. H., HENRY, T. D., DIB, N., STRUMPF, R. K., SCHULMAN, S. P., GERSTENBLITH, G., DEMARIA, A. N., DENKTAS, A. E., GAMMON, R. S., HERMILLER, J. B., JR., REISMAN, M. A., SCHAER, G. L. & SHERMAN, W. 2009. 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,* 54**,** 2277-86.

HARPER, D. M., FRANCO, E. L., WHEELER, C., FERRIS, D. G., JENKINS, D., SCHUIND, A., ZAHAF, T., INNIS, B., NAUD, P., DE CARVALHO, N. S., ROTELI-MARTINS, C. M., TEIXEIRA, J., BLATTER, M. M., KORN, A. P., QUINT, W. & DUBIN, G. 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet,* 364**,** 1757-65.

HASSANSHAHI, M., KHABBAZI, S., PEYMANFAR, Y., HASSANSHAHI, A., HOSSEINI-KHAH, Z., SU, Y. W. & XIAN, C. J. 2019. Critical limb ischemia: Current and novel therapeutic strategies. *J Cell Physiol*.

HAZARIKA, S. & ANNEX, B. H. 2017. Gene and Cell Therapy for Critical Limb Ischemia. *In:* DIETER, R. S., DIETER, J. R. A., DIETER, I. I. I. R. A. & NANJUNDAPPA, A. (eds.) *Critical Limb Ischemia: Acute and Chronic.* Cham: Springer International Publishing.

HEO, S. C., JEON, E. S., LEE, I. H., KIM, H. S., KIM, M. B. & KIM, J. H. 2011. Tumor necrosis factor-alpha-activated human adipose tissue-derived mesenchymal stem cells accelerate cutaneous wound healing through paracrine mechanisms. *J Invest Dermatol,* 131**,** 1559-67.

HOARE, M., GREISER, U., SCHU, S., MASHAYEKHI, K., AYDOGAN, E., MURPHY, M., BARRY, F., RITTER, T. & O'BRIEN, T. 2010. Enhanced lipoplex-mediated gene expression in mesenchymal stem cells using reiterated nuclear localization sequence peptides. *J Gene Med,* 12**,** 207-18.

HODGKINSON, C. P., GOMEZ, J. A., MIROTSOU, M. & DZAU, V. J. 2010. Genetic engineering of mesenchymal stem cells and its application in human disease therapy. *Hum Gene Ther,* 21**,** 1513-26.

HOEKSTRA, D., REJMAN, J., WASUNGU, L., SHI, F. & ZUHORN, I. 2007. Gene delivery by cationic lipids: in and out of an endosome. *Biochem Soc Trans,* 35**,** 68-71.

HOELTERS, J., CICCARELLA, M., DRECHSEL, M., GEISSLER, C., GULKAN, H., BOCKER, W., SCHIEKER, M., JOCHUM, M. & NETH, P. 2005. Nonviral genetic modification mediates effective transgene expression and functional RNA interference in human mesenchymal stem cells. *J Gene Med,* 7**,** 718-28.

HOFSTETTER, C. P., SCHWARZ, E. J., HESS, D., WIDENFALK, J., EL MANIRA, A., PROCKOP, D. J. & OLSON, L. 2002. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proc Natl Acad Sci U S A,* 99**,** 2199-204.

HOOGDUIJN, M. J., BETJES, M. G. & BAAN, C. C. 2014. Mesenchymal stromal cells for organ transplantation: different sources and unique characteristics? *Curr Opin Organ Transplant,* 19**,** 41-6.

HORDYJEWSKA, A., POPIOŁEK, Ł. & HORECKA, A. 2015. Characteristics of hematopoietic stem cells of umbilical cord blood. *Cytotechnology.*

HOSSEINKHANI, H., YAMAMOTO, M., INATSUGU, Y., HIRAOKA, Y., INOUE, S., SHIMOKAWA, H. & TABATA, Y. 2006. Enhanced ectopic bone formation using a combination of plasmid DNA impregnation into 3-D scaffold and bioreactor perfusion culture. *Biomaterials,* 27**,** 1387-98.

HU, C. & LI, L. 2018. Preconditioning influences mesenchymal stem cell properties in vitro and in vivo. *J Cell Mol Med,* 22**,** 1428-1442.

HU, J., LANG, Y., ZHANG, T., NI, S. & LU, H. 2016. Lentivirus-mediated PGC-1alpha overexpression protects against traumatic spinal cord injury in rats. *Neuroscience,* 328**,** 40-9.

HU, X., YU, S. P., FRASER, J. L., LU, Z., OGLE, M. E., WANG, J. A. & WEI, L. 2008. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J Thorac Cardiovasc Surg,* 135**,** 799-808.

HUANG, G. T., GRONTHOS, S. & SHI, S. 2009. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res,* 88**,** 792-806.

HUANG, M., NGUYEN, P., JIA, F., HU, S., GONG, Y., DE ALMEIDA, P. E., WANG, L., NAG, D., KAY, M. A., GIACCIA, A. J., ROBBINS, R. C. & WU, J. C. 2011. Double knockdown of prolyl hydroxylase and factor-inhibiting hypoxia-inducible factor with nonviral minicircle gene therapy enhances stem cell mobilization and angiogenesis after myocardial infarction. *Circulation,* 124**,** S46-54.

HYUN, J., GROVA, M., NEJADNIK, H., LO, D., MORRISON, S., MONTORO, D., CHUNG, M., ZIMMERMANN, A., WALMSLEY, G. G., LEE, M., DALDRUP-LINK, H., WAN, D. C. & LONGAKER, M. T. 2013. Enhancing in vivo survival of adipose-derived stromal cells through Bcl-2 overexpression using a minicircle vector. *Stem Cells Transl Med,* 2**,** 690-702.

IN 'T ANKER, P. S., SCHERJON, S. A., KLEIJBURG-VAN DER KEUR, C., NOORT, W. A., CLAAS, F. H., WILLEMZE, R., FIBBE, W. E. & KANHAI, H. H. 2003. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood.* United States.

INCANI, V., TUNIS, E., CLEMENTS, B. A., OLSON, C., KUCHARSKI, C., LAVASANIFAR, A. & ULUDAG, H. 2007. Palmitic acid substitution on cationic polymers for effective delivery of plasmid DNA to bone marrow stromal cells. *J Biomed Mater Res A,* 81**,** 493-504.

ISNER, J. M., PIECZEK, A., SCHAINFELD, R., BLAIR, R., HALEY, L., ASAHARA, T., ROSENFIELD, K., RAZVI, S., WALSH, K. & SYMES, J. F. 1996. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet,* 348**,** 370-4.

JIA, F., WILSON, K. D., SUN, N., GUPTA, D. M., HUANG, M., LI, Z., PANETTA, N. J., CHEN, Z. Y., ROBBINS, R. C., KAY, M. A., LONGAKER, M. T. & WU, J. C. 2010. A nonviral minicircle vector for deriving human iPS cells. *Nat Methods,* 7**,** 197-9.

JIANG, S., HAIDER, H., IDRIS, N. M., SALIM, A. & ASHRAF, M. 2006. Supportive interaction between cell survival signaling and angiocompetent factors enhances donor cell survival and promotes angiomyogenesis for cardiac repair. *Circ Res,* 99**,** 776-84.

JUNG, J., MOON, N., AHN, J. Y., OH, E. J., KIM, M., CHO, C. S., SHIN, J. C. & OH, I. H. 2009. Mesenchymal stromal cells expanded in human allogenic cord blood serum display higher self-renewal and enhanced osteogenic potential. *Stem Cells Dev,* 18**,** 559-71.

KAGA, T., KAWANO, H., SAKAGUCHI, M., NAKAZAWA, T., TANIYAMA, Y. & MORISHITA, R. 2012. Hepatocyte growth factor stimulated angiogenesis without inflammation: differential actions between hepatocyte growth factor, vascular endothelial growth factor and basic fibroblast growth factor. *Vascul Pharmacol,* 57**,** 3-9.

KAGIWADA, H., YASHIKI, T., OHSHIMA, A., TADOKORO, M., NAGAYA, N. & OHGUSHI, H. 2008. Human mesenchymal stem cells as a stable source of VEGF-producing cells. *Journal of Tissue Engineering and Regenerative Medicine,* 2**,** 184-189.

KALKA, C., MASUDA, H., TAKAHASHI, T., KALKA-MOLL, W. M., SILVER, M., KEARNEY, M., LI, T., ISNER, J. M. & ASAHARA, T. 2000. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci U S A,* 97**,** 3422-7.

KANEHIRA, M., XIN, H., HOSHINO, K., MAEMONDO, M., MIZUGUCHI, H., HAYAKAWA, T., MATSUMOTO, K., NAKAMURA, T., NUKIWA, T. & SAIJO, Y. 2007. Targeted delivery of NK4 to multiple lung tumors by bone marrow-derived mesenchymal stem cells. *Cancer Gene Ther,* 14**,** 894-903.

KANG, Y., LIAO, W. M., YUAN, Z. H., SHENG, P. Y., ZHANG, L. J., YUAN, X. W. & LEI, L. 2007. In vitro and in vivo induction of bone formation based on adeno-associated virus-mediated BMP-7 gene therapy using human adipose-derived mesenchymal stem cells. *Acta Pharmacol Sin,* 28**,** 839-49.

KARNIELI, O., FRIEDNER, O. M., ALLICKSON, J. G., ZHANG, N., JUNG, S., FIORENTINI, D., ABRAHAM, E., EAKER, S. S., YONG, T. K., CHAN, A., GRIFFITHS, S., WHEN, A. K. & OH, S. 2017. A consensus introduction to serum replacements and serum-free media for cellular therapies. *Cytotherapy,* 19**,** 155-169.

KAUFMANN, K. B., BUNING, H., GALY, A., SCHAMBACH, A. & GREZ, M. 2013. Gene therapy on the move. *EMBO Mol Med,* 5**,** 1642-61.

KAWABATA, K., SAKURAI, F., KOIZUMI, N., HAYAKAWA, T. & MIZUGUCHI, H. 2006. Adenovirus vector-mediated gene transfer into stem cells. *Mol Pharm,* 3**,** 95-103.

KAWAMOTO, A., KATAYAMA, M., HANDA, N., KINOSHITA, M., TAKANO, H., HORII, M., SADAMOTO, K., YOKOYAMA, A., YAMANAKA, T., ONODERA, R., KURODA, A., BABA, R., KANEKO, Y., TSUKIE, T., KURIMOTO, Y., OKADA, Y., KIHARA, Y., MORIOKA, S., FUKUSHIMA, M. & ASAHARA, T. 2009. Intramuscular transplantation of G-CSF-mobilized CD34(+) cells in patients with critical limb ischemia: a phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial. *Stem Cells,* 27**,** 2857-64.

KAY, M. A., HE, C. Y. & CHEN, Z. Y. 2010. A robust system for production of minicircle DNA vectors. *Nat Biotechnol,* 28**,** 1287-9.

KERN, S., EICHLER, H., STOEVE, J., KLUTER, H. & BIEBACK, K. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells,* 24**,** 1294-301.

KHEIRANDISH, M., GAVGANI, S. P. & SAMIEE, S. 2017. The effect of hypoxia preconditioning on the neural and stemness genes expression profiling in human umbilical cord blood mesenchymal stem cells. *Transfus Apher Sci,* 56**,** 392-399.

KHLEBNIKOV, A., DATSENKO, K. A., SKAUG, T., WANNER, B. L. & KEASLING, J. D. 2001. Homogeneous expression of the P(BAD) promoter in Escherichia coli by constitutive expression of the low-affinity high-capacity AraE transporter. *Microbiology,* 147**,** 3241-7.

KIANI, A. A., ABDI, J., HALABIAN, R., ROUDKENAR, M. H., AMIRIZADEH, N., SOLEIMAN SOLTANPOUR, M. & KAZEMI, A. 2014. Over expression of HIF-1alpha in human mesenchymal stem cells increases their supportive functions for hematopoietic stem cells in an experimental co-culture model. *Hematology,* 19**,** 85-98.

KIM, J. A., CHO, K., SHIN, M. S., LEE, W. G., JUNG, N., CHUNG, C. & CHANG, J. K. 2008. A novel electroporation method using a capillary and wire-type electrode. *Biosens Bioelectron,* 23**,** 1353-60.

KIM, S. W., HAN, H., CHAE, G. T., LEE, S. H., BO, S., YOON, J. H., LEE, Y. S., LEE, K. S., PARK, H. K. & KANG, K. S. 2006. Successful stem cell therapy using umbilical cord blood-derived multipotent stem cells for Buerger's disease and ischemic limb disease animal model. *Stem Cells,* 24**,** 1620-6.

KIM, Y. S., NOH, M. Y., CHO, K. A., KIM, H., KWON, M. S., KIM, K. S., KIM, J., KOH, S. H. & KIM, S. H. 2015. Hypoxia/Reoxygenation-Preconditioned Human Bone Marrow-Derived Mesenchymal Stromal Cells Rescue Ischemic Rat Cortical Neurons by Enhancing Trophic Factor Release. *Mol Neurobiol,* 52**,** 792-803.

KING, W. J., KOURIS, N. A., CHOI, S., OGLE, B. M. & MURPHY, W. L. 2012. Environmental parameters influence non-viral transfection of human mesenchymal stem cells for tissue engineering applications. *Cell Tissue Res,* 347**,** 689-99.

KINLAY, S. 2016. Management of Critical Limb Ischemia. *Circ Cardiovasc Interv,* 9**,** e001946.

KINNAIRD, T., STABILE, E., BURNETT, M. S., LEE, C. W., BARR, S., FUCHS, S. & EPSTEIN, S. E. 2004. 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,* 94**,** 678-85.

KIZILAY MANCINI, O., LORA, M., SHUM-TIM, D., NADEAU, S., RODIER, F. & COLMEGNA, I. 2017. A Proinflammatory Secretome Mediates the Impaired Immunopotency of Human Mesenchymal Stromal Cells in Elderly Patients with Atherosclerosis. *Stem Cells Transl Med,* 6**,** 1132-1140.

KNAAN-SHANZER, S., VAN DE WATERING, M. J., VAN DER VELDE, I., GONCALVES, M. A., VALERIO, D. & DE VRIES, A. A. 2005. Endowing human adenovirus serotype 5 vectors with fiber domains of species B greatly enhances gene transfer into human mesenchymal stem cells. *Stem Cells,* 23**,** 1598-607.

KOLIOS, G. & MOODLEY, Y. 2013. Introduction to stem cells and regenerative medicine. *Respiration,* 85**,** 3-10.

KOST, T. A., CONDREAY, J. P. & JARVIS, D. L. 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol,* 23**,** 567-75.

KRAMPERA, M., GLENNIE, S., DYSON, J., SCOTT, D., LAYLOR, R., SIMPSON, E. & DAZZI, F. 2003. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood,* 101**,** 3722-9.

KUSUMANTO, Y. H., VAN WEEL, V., MULDER, N. H., SMIT, A. J., VAN DEN DUNGEN, J. J., HOOYMANS, J. M., SLUITER, W. J., TIO, R. A., QUAX, P. H., GANS, R. O., DULLAART, R. P. & HOSPERS, G. A. 2006. Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. *Hum Gene Ther,* 17**,** 683-91.

KWON, M. J., AN, S., CHOI, S., NAM, K., JUNG, H. S., YOON, C. S., KO, J. H., JUN, H. J., KIM, T. K., JUNG, S. J., PARK, J. H., LEE, Y. & PARK, J.-S. 2012. Effective healing of diabetic skin wounds by using nonviral gene therapy based on minicircle vascular endothelial growth factor DNA and a cationic dendrimer. *The Journal of Gene Medicine,* 14**,** 272-278.

LASALA, G. P., SILVA, J. A., GARDNER, P. A. & MINGUELL, J. J. 2010. Combination stem cell therapy for the treatment of severe limb ischemia: safety and efficacy analysis. *Angiology,* 61**,** 551-6.

LATHROP, M. J., SAGE, E. K., MACURA, S. L., BROOKS, E. M., CRUZ, F., BONENFANT, N. R., SOKOCEVIC, D., MACPHERSON, M. B., BEUSCHEL, S. L., DUNAWAY, C. W., SHUKLA, A., JANES, S. M., STEELE, C., MOSSMAN, B. T. & WEISS, D. J. 2015. Antitumor effects of TRAIL-expressing mesenchymal stromal cells in a mouse xenograft model of human mesothelioma. *Cancer Gene Ther,* 22**,** 44-54.

LAZARUS, H., CURTIN, P. & DEVINE, S. 2000. *Role of mesenchymal stem cells (MSC) in allogeneic transplantation: Early phase I clinical results*.

LE BLANC, K., FRASSONI, F., BALL, L., LOCATELLI, F., ROELOFS, H., LEWIS, I., LANINO, E., SUNDBERG, B., BERNARDO, M. E., REMBERGER, M., DINI, G., EGELER, R. M., BACIGALUPO, A., FIBBE, W. & RINGDEN, O. 2008. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet,* 371**,** 1579-86.

LE BLANC, K., TAMMIK, L., SUNDBERG, B., HAYNESWORTH, S. E. & RINGDEN, O. 2003. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol,* 57**,** 11-20.

LECHARDEUR, D., VERKMAN, A. S. & LUKACS, G. L. 2005. Intracellular routing of plasmid DNA during non-viral gene transfer. *Adv Drug Deliv Rev,* 57**,** 755-67.

LEDERMAN, R. J., MENDELSOHN, F. O., ANDERSON, R. D., SAUCEDO, J. F., TENAGLIA, A. N., HERMILLER, J. B., HILLEGASS, W. B., ROCHA-SINGH, K., MOON, T. E., WHITEHOUSE, M. J. & ANNEX, B. H. 2002. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. *Lancet,* 359**,** 2053-8.

LEE, H. C., AN, S. G., LEE, H. W., PARK, J. S., CHA, K. S., HONG, T. J., PARK, J. H., LEE, S. Y., KIM, S. P., KIM, Y. D., CHUNG, S. W., BAE, Y. C., SHIN, Y. B., KIM, J. I. & JUNG, J. S. 2012. Safety and effect of adipose tissue-derived stem cell implantation in patients with critical limb ischemia: a pilot study. *Circ J,* 76**,** 1750-60.

LEE, H. Y. & HONG, I. S. 2017. Double-edged sword of mesenchymal stem cells: Cancer-promoting versus therapeutic potential. *Cancer Sci,* 108**,** 1939-1946.

LEE, J. H., YOON, Y. M. & LEE, S. H. 2017. Hypoxic Preconditioning Promotes the Bioactivities of Mesenchymal Stem Cells via the HIF-1alpha-GRP78-Akt Axis. *Int J Mol Sci,* 18.

LENNON, D. P. & CAPLAN, A. I. 2006. Isolation of human marrow-derived mesenchymal stem cells. *Exp Hematol,* 34**,** 1604-5.

LEROUX, L., DESCAMPS, B., TOJAIS, N. F., SEGUY, B., OSES, P., MOREAU, C., DARET, D., IVANOVIC, Z., BOIRON, J. M., LAMAZIERE, J. M., DUFOURCQ, P., COUFFINHAL, T. & DUPLAA, C. 2010. Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway. *Mol Ther,* 18**,** 1545-52.

LI, D., LIU, Q., QI, L., DAI, X., LIU, H. & WANG, Y. 2016. Low levels of TGF-beta1 enhance human umbilical cord-derived mesenchymal stem cell fibronectin production and extend survival time in a rat model of lipopolysaccharide-induced acute lung injury. *Mol Med Rep,* 14**,** 1681-92.

LI, J. P., WANG, D. W. & SONG, Q. H. 2015a. Transplantation of erythropoietin gene-transfected umbilical cord mesenchymal stem cells as a treatment for limb ischemia in rats. *Genetics and Molecular Research,* 14**,** 19005-19015.

LI, M., ZHAO, Y., HAO, H., DAI, H., HAN, Q., TONG, C., LIU, J., HAN, W. & FU, X. 2015b. Mesenchymal Stem Cell–Conditioned Medium Improves the Proliferation and Migration of Keratinocytes in a Diabetes-Like Microenvironment. *The International Journal of Lower Extremity Wounds,* 14**,** 73-86.

LI, W., MA, N., ONG, L. L., NESSELMANN, C., KLOPSCH, C., LADILOV, Y., FURLANI, D., PIECHACZEK, C., MOEBIUS, J. M., LUTZOW, K., LENDLEIN, A., STAMM, C., LI, R. K. & STEINHOFF, G. 2007. Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. *Stem Cells,* 25**,** 2118-27.

LI, X., GAN, K., SONG, G. & WANG, C. 2015c. VEGF gene transfected umbilical cord mesenchymal stem cells transplantation improve the lower limb vascular lesions of diabetic rats. *J Diabetes Complications,* 29**,** 872-81.

LIAO, J. C. 2016a. Bone Marrow Mesenchymal Stem Cells Expressing Baculovirus-Engineered Bone Morphogenetic Protein-7 Enhance Rabbit Posterolateral Fusion. *Int J Mol Sci,* 17.

LIAO, J. C. 2016b. Cell Therapy Using Bone Marrow-Derived Stem Cell Overexpressing BMP-7 for Degenerative Discs in a Rat Tail Disc Model. *Int J Mol Sci,* 17.

LIECHTY, K. W., MACKENZIE, T. C., SHAABAN, A. F., RADU, A., MOSELEY, A. M., DEANS, R., MARSHAK, D. R. & FLAKE, A. W. 2000. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat Med,* 6**,** 1282-6.

LIEW, A. & O'BRIEN, T. 2012. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Res Ther,* 3**,** 28.

LIM, J. Y., PARK, S. H., JEONG, C. H., OH, J. H., KIM, S. M., RYU, C. H., PARK, S. A., AHN, J. G., OH, W., JEUN, S. S. & CHANG, J. W. 2010. Microporation is a valuable transfection method for efficient gene delivery into human umbilical cord blood-derived mesenchymal stem cells. *BMC Biotechnol,* 10**,** 38.

LIN, C. S. & LUE, T. F. 2013. Defining vascular stem cells. *Stem Cells Dev,* 22**,** 1018-26.

LIN, C. Y., CHANG, Y. H., LIN, K. J., YEN, T. C., TAI, C. L., CHEN, C. Y., LO, W. H., HSIAO, I. T. & HU, Y. C. 2010. The healing of critical-sized femoral segmental bone defects in rabbits using baculovirus-engineered mesenchymal stem cells. *Biomaterials,* 31**,** 3222-30.

LIU, L., GAO, J., YUAN, Y., CHANG, Q., LIAO, Y. & LU, F. 2013. Hypoxia preconditioned human adipose derived mesenchymal stem cells enhance angiogenic potential via secretion of increased VEGF and bFGF. *Cell Biol Int,* 37**,** 551-60.

LIU, X., DUAN, B., CHENG, Z., JIA, X., MAO, L., FU, H., CHE, Y., OU, L., LIU, L. & KONG, D. 2011. SDF-1/CXCR4 axis modulates bone marrow mesenchymal stem cell apoptosis, migration and cytokine secretion. *Protein Cell,* 2**,** 845-54.

LIU, X. H., BAI, C. G., XU, Z. Y., HUANG, S. D., YUAN, Y., GONG, D. J. & ZHANG, J. R. 2008. Therapeutic potential of angiogenin modified mesenchymal stem cells: angiogenin improves mesenchymal stem cells survival under hypoxia and enhances vasculogenesis in myocardial infarction. *Microvasc Res,* 76**,** 23-30.

LOEBINGER, M. R., EDDAOUDI, A., DAVIES, D. & JANES, S. M. 2009. Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer. *Cancer Res,* 69**,** 4134-42.

LU, D., CHEN, B., LIANG, Z., DENG, W., JIANG, Y., LI, S., XU, J., WU, Q., ZHANG, Z., XIE, B. & CHEN, S. 2011. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabetes Res Clin Pract,* 92**,** 26-36.

LUO, Y., WANG, Y., POYNTER, J. A., MANUKYAN, M. C., HERRMANN, J. L., ABARBANELL, A. M., WEIL, B. R. & MELDRUM, D. R. 2012. Pretreating mesenchymal stem cells with interleukin-1beta and transforming growth factor-beta synergistically increases vascular endothelial growth factor production and improves mesenchymal stem cell-mediated myocardial protection after acute ischemia. *Surgery,* 151**,** 353-63.

LUZIO, J. P., MULLOCK, B. M., PRYOR, P. R., LINDSAY, M. R., JAMES, D. E. & PIPER, R. C. 2001. Relationship between endosomes and lysosomes. *Biochem Soc Trans,* 29**,** 476-80.

LV, F. J., TUAN, R. S., CHEUNG, K. M. & LEUNG, V. Y. 2014. Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells,* 32**,** 1408-19.

MACKAY, A. M., BECK, S. C., MURPHY, J. M., BARRY, F. P., CHICHESTER, C. O. & PITTENGER, M. F. 1998. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng,* 4**,** 415-28.

MADEIRA, C., MENDES, R. D., RIBEIRO, S. C., BOURA, J. S., AIRES-BARROS, M. R., DA SILVA, C. L. & CABRAL, J. M. S. 2010. Nonviral Gene Delivery to Mesenchymal Stem Cells Using Cationic Liposomes for Gene and Cell Therapy. *Journal of Biomedicine and Biotechnology,* 2010**,** 1-12.

MADEIRA, C., RIBEIRO, S. C., PINHEIRO, I. S. M., MARTINS, S. A. M., ANDRADE, P. Z., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Gene delivery to human bone marrow mesenchymal stem cells by microporation. *Journal of Biotechnology,* 151**,** 130-136.

MADEIRA, C., RODRIGUES, C. A. V., REIS, M. S. C., FERREIRA, F. F. C. G., CORREIA, R. E. S. M., DIOGO, M. M. & CABRAL, J. M. S. 2013. Nonviral Gene Delivery to Neural Stem Cells with Minicircles by Microporation. *Biomacromolecules,* 14**,** 1379-1387.

MAFFIOLI, E., NONNIS, S., ANGIONI, R., SANTAGATA, F., CALI, B., ZANOTTI, L., NEGRI, A., VIOLA, A. & TEDESCHI, G. 2017. Proteomic analysis of the secretome of human bone marrow-derived mesenchymal stem cells primed by pro-inflammatory cytokines. *J Proteomics,* 166**,** 115-126.

MAFI, P., HINDOCHA, S., MAFI, R., GRIFFIN, M. & KHAN, W. S. 2011. Adult Mesenchymal Stem Cells and Cell Surface Characterization - A Systematic Review of the Literature. *The Open Orthopaedics Journal,* 5**,** 253-260.

MAKINO, H., AOKI, M., HASHIYA, N., YAMASAKI, K., AZUMA, J., SAWA, Y., KANEDA, Y., OGIHARA, T. & MORISHITA, R. 2012. Long-term follow-up evaluation of results from clinical trial using hepatocyte growth factor gene to treat severe peripheral arterial disease. *Arterioscler Thromb Vasc Biol,* 32**,** 2503-9.

MANGI, A. A., NOISEUX, N., KONG, D., HE, H., REZVANI, M., INGWALL, J. S. & DZAU, V. J. 2003. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med,* 9**,** 1195-201.

MANNELLO, F. & TONTI, G. A. 2007. Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! *Stem Cells,* 25**,** 1603-9.

MANSOURI, M. & BERGER, P. 2018. Baculovirus for gene delivery to mammalian cells: Past, present and future. *Plasmid,* 98**,** 1-7.

MARSHALL, E. 1999. Gene therapy death prompts review of adenovirus vector. *Science,* 286**,** 2244-5.

MATOBA, S., TATSUMI, T., MUROHARA, T., IMAIZUMI, T., KATSUDA, Y., ITO, M., SAITO, Y., UEMURA, S., SUZUKI, H., FUKUMOTO, S., YAMAMOTO, Y., ONODERA, R., TERAMUKAI, S., FUKUSHIMA, M. & MATSUBARA, H. 2008. Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] trial) in patients with chronic limb ischemia. *Am Heart J,* 156**,** 1010-8.

MATSUMOTO, R. 2005. Vascular Endothelial Growth Factor-Expressing Mesenchymal Stem Cell Transplantation for the Treatment of Acute Myocardial Infarction. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 25**,** 1168-1173.

MAYR, M., NIEDERSEER, D. & NIEBAUER, J. 2011. From bench to bedside: what physicians need to know about endothelial progenitor cells. *Am J Med,* 124**,** 489-97.

MAYRHOFER, P., BLAESEN, M., SCHLEEF, M. & JECHLINGER, W. 2008. Minicircle-DNA production by site specific recombination and protein-DNA interaction chromatography. *J Gene Med,* 10**,** 1253-69.

MCCARTER, S. D., SCOTT, J. R., LEE, P. J., ZHANG, X., CHOI, A. M., MCLEAN, C. A., BADHWAR, A., DUNGEY, A. A., BIHARI, A., HARRIS, K. A. & POTTER, R. F. 2003. Cotransfection of heme oxygenase-1 prevents the acute inflammation elicited by a second adenovirus. *Gene Ther,* 10**,** 1629-35.

MCGINLEY, L., MCMAHON, J., STRAPPE, P., BARRY, F., MURPHY, M., O'TOOLE, D. & O'BRIEN, T. 2011. Lentiviral vector mediated modification of mesenchymal stem cells & enhanced survival in an in vitro model of ischaemia. *Stem Cell Res Ther,* 2**,** 12.

MCMAHON, J. M., CONROY, S., LYONS, M., GREISER, U., O'SHEA, C., STRAPPE, P., HOWARD, L., MURPHY, M., BARRY, F. & O'BRIEN, T. 2006. Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors. *Stem Cells Dev,* 15**,** 87-96.

MEDINA-KAUWE, L. K., XIE, J. & HAMM-ALVAREZ, S. 2005. Intracellular trafficking of nonviral vectors. *Gene Ther,* 12**,** 1734-51.

MIAO, Z., JIN, J., CHEN, L., ZHU, J., HUANG, W., ZHAO, J., QIAN, H. & ZHANG, X. 2006. Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. *Cell Biol Int,* 30**,** 681-7.

MIMEAULT, M. & BATRA, S. K. 2006. Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. *Stem Cells,* 24**,** 2319-45.

MIROTSOU, M., ZHANG, Z., DEB, A., ZHANG, L., GNECCHI, M., NOISEUX, N., MU, H., PACHORI, A. & DZAU, V. 2007. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A,* 104**,** 1643-8.

MOORE, K. A. & LEMISCHKA, I. R. 2006. Stem cells and their niches. *Science,* 311**,** 1880-5.

MORISHITA, R., MAKINO, H., AOKI, M., HASHIYA, N., YAMASAKI, K., AZUMA, J., TANIYAMA, Y., SAWA, Y., KANEDA, Y. & OGIHARA, T. 2011. Phase I/IIa clinical trial of therapeutic angiogenesis using hepatocyte growth factor gene transfer to treat critical limb ischemia. *Arterioscler Thromb Vasc Biol,* 31**,** 713-20.

MUN, J.-Y., SHIN, K. K., KWON, O., LIM, Y. T. & OH, D.-B. 2016. Minicircle microporation-based non-viral gene delivery improved the targeting of mesenchymal stem cells to an injury site. *Biomaterials,* 101**,** 310-320.

MUNYE, M. M., TAGALAKIS, A. D., BARNES, J. L., BROWN, R. E., MCANULTY, R. J., HOWE, S. J. & HART, S. L. 2016. Minicircle DNA Provides Enhanced and Prolonged Transgene Expression Following Airway Gene Transfer. *Sci Rep,* 6**,** 23125.

MUONA, K., MAKINEN, K., HEDMAN, M., MANNINEN, H. & YLA-HERTTUALA, S. 2012. 10-year safety follow-up in patients with local VEGF gene transfer to ischemic lower limb. *Gene Ther,* 19**,** 392-5.

MUSHAHARY, D., SPITTLER, A., KASPER, C., WEBER, V. & CHARWAT, V. 2018. Isolation, cultivation, and characterization of human mesenchymal stem cells. *Cytometry A,* 93**,** 19-31.

MYERS, T. J., GRANERO-MOLTO, F., LONGOBARDI, L., LI, T., YAN, Y. & SPAGNOLI, A. 2010. Mesenchymal stem cells at the intersection of cell and gene therapy. *Expert Opinion on Biological Therapy,* 10**,** 1663-1679.

NAAIJKENS, B. A., NIESSEN, H. W., PRINS, H. J., KRIJNEN, P. A., KOKHUIS, T. J., DE JONG, N., VAN HINSBERGH, V. W., KAMP, O., HELDER, M. N., MUSTERS, R. J., VAN DIJK, A. & JUFFERMANS, L. J. 2012. Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications. *Cell Tissue Res,* 348**,** 119-30.

NAKAGAMI, H., KANEDA, Y., OGIHARA, T. & MORISHITA, R. 2005. Hepatocyte growth factor as potential cardiovascular therapy. *Expert Rev Cardiovasc Ther,* 3**,** 513-9.

NAKANO, A., HARADA, T., MORIKAWA, S. & KATO, Y. 1990. Expression of leukocyte common antigen (CD45) on various human leukemia/lymphoma cell lines. *Acta Pathol Jpn,* 40**,** 107-15.

NAKASHIMA, S., MATSUYAMA, Y., NITTA, A., SAKAI, Y. & ISHIGURO, N. 2005. Highly efficient transfection of human marrow stromal cells by nucleofection. *Transplant Proc,* 37**,** 2290-2.

NALDINI, L., BLOMER, U., GALLAY, P., ORY, D., MULLIGAN, R., GAGE, F. H., VERMA, I. M. & TRONO, D. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science,* 272**,** 263-7.

NEKANTI, U., MOHANTY, L., VENUGOPAL, P., BALASUBRAMANIAN, S., TOTEY, S. & TA, M. 2010. Optimization and scale-up of Wharton's jelly-derived mesenchymal stem cells for clinical applications. *Stem Cell Res,* 5**,** 244-54.

NEVE, A., CORRADO, A. & CANTATORE, F. P. 2011. Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res,* 343**,** 289-302.

NIE, H. & WANG, C. H. 2007. Fabrication and characterization of PLGA/HAp composite scaffolds for delivery of BMP-2 plasmid DNA. *J Control Release,* 120**,** 111-21.

NIESS, H., BAO, Q., CONRAD, C., ZISCHEK, C., NOTOHAMIPRODJO, M., SCHWAB, F., SCHWARZ, B., HUSS, R., JAUCH, K. W., NELSON, P. J. & BRUNS, C. J. 2011. Selective targeting of genetically engineered mesenchymal stem cells to tumor stroma microenvironments using tissue-specific suicide gene expression suppresses growth of hepatocellular carcinoma. *Ann Surg,* 254**,** 767-74; discussion 774-5.

NIESS, H., VON EINEM, J. C., THOMAS, M. N., MICHL, M., ANGELE, M. K., HUSS, R., GUNTHER, C., NELSON, P. J., BRUNS, C. J. & HEINEMANN, V. 2015. Treatment of advanced gastrointestinal tumors with genetically modified autologous mesenchymal stromal cells (TREAT-ME1): study protocol of a phase I/II clinical trial. *BMC Cancer,* 15**,** 237.

NIKOL, S., BAUMGARTNER, I., VAN BELLE, E., DIEHM, C., VISONA, A., CAPOGROSSI, M. C., FERREIRA-MALDENT, N., GALLINO, A., GRAHAM WYATT, M., DINESH WIJESINGHE, L., FUSARI, M., STEPHAN, D., EMMERICH, J., POMPILIO, G., VERMASSEN, F., PHAM, E., GREK, V., COLEMAN, M. & MEYER, F. 2008. Therapeutic Angiogenesis With Intramuscular NV1FGF Improves Amputation-free Survival in Patients With Critical Limb Ischemia. *Mol Ther,* 16**,** 972-978.

NINAGAWA, N. T., ISOBE, E., HIRAYAMA, Y., MURAKAMI, R., KOMATSU, K., NAGAI, M., KOBAYASHI, M., KAWABATA, Y. & TORIHASHI, S. 2013. Transplantated mesenchymal stem cells derived from embryonic stem cells promote muscle regeneration and accelerate functional recovery of injured skeletal muscle. *Biores Open Access,* 2**,** 295-306.

NOISEUX, N., GNECCHI, M., LOPEZ-ILASACA, M., ZHANG, L., SOLOMON, S. D., DEB, A., DZAU, V. J. & PRATT, R. E. 2006. Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Mol Ther,* 14**,** 840-50.

NORGREN, L., HIATT, W. R., DORMANDY, J. A., NEHLER, M. R., HARRIS, K. A. & FOWKES, F. G. R. 2007. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *Journal of Vascular Surgery,* 45**,** S5-S67.

OGGU, G. S., SASIKUMAR, S., REDDY, N., ELLA, K. K. R., RAO, C. M. & BOKARA, K. K. 2017. Gene Delivery Approaches for Mesenchymal Stem Cell Therapy: Strategies to Increase Efficiency and Specificity. *Stem Cell Rev,* 13**,** 725-740.

OIKONOMOPOULOS, A., VAN DEEN, W. K., MANANSALA, A.-R., LACEY, P. N., TOMAKILI, T. A., ZIMAN, A. & HOMMES, D. W. 2015. Optimization of human mesenchymal stem cell manufacturing: the effects of animal/xeno-free media. *Scientific Reports,* 5**,** 16570.

ONDA, T., HONMOU, O., HARADA, K., HOUKIN, K., HAMADA, H. & KOCSIS, J. D. 2008. Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. *J Cereb Blood Flow Metab,* 28**,** 329-40.

ONODERA, R., TERAMUKAI, S., TANAKA, S., KOJIMA, S., HORIE, T., MATOBA, S., MUROHARA, T., MATSUBARA, H. & FUKUSHIMA, M. 2011. Bone marrow mononuclear cells versus G-CSF-mobilized peripheral blood mononuclear cells for treatment of lower limb ASO: pooled analysis for long-term prognosis. *Bone Marrow Transplant,* 46**,** 278-84.

OSKOWITZ, A., MCFERRIN, H., GUTSCHOW, M., CARTER, M. L. & POCHAMPALLY, R. 2011. Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. *Stem Cell Res,* 6**,** 215-25.

OTANI, K., YAMAHARA, K., OHNISHI, S., OBATA, H., KITAMURA, S. & NAGAYA, N. 2009. Nonviral delivery of siRNA into mesenchymal stem cells by a combination of ultrasound and microbubbles. *J Control Release,* 133**,** 146-53.

PAL, R., HANWATE, M., JAN, M. & TOTEY, S. 2009. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. *J Tissue Eng Regen Med,* 3**,** 163-74.

PARK, J. S., NA, K., WOO, D. G., YANG, H. N., KIM, J. M., KIM, J. H., CHUNG, H. M. & PARK, K. H. 2010. Non-viral gene delivery of DNA polyplexed with nanoparticles transfected into human mesenchymal stem cells. *Biomaterials,* 31**,** 124-32.

PARK, J. S., SURYAPRAKASH, S., LAO, Y. H. & LEONG, K. W. 2015. Engineering mesenchymal stem cells for regenerative medicine and drug delivery. *Methods,* 84**,** 3-16.

PARK, N., RIM, Y. A., JUNG, H., KIM, J., YI, H., KIM, Y., JANG, Y., JUNG, S. M., LEE, J., KWOK, S. K., PARK, S. H. & JU, J. H. 2017. Etanercept-Synthesising Mesenchymal Stem Cells Efficiently Ameliorate Collagen-Induced Arthritis. *Sci Rep,* 7**,** 39593.

PASHA, Z., WANG, Y., SHEIKH, R., ZHANG, D., ZHAO, T. & ASHRAF, M. 2008. Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium. *Cardiovasc Res,* 77**,** 134-42.

PAULA, A. C., MARTINS, T. M., ZONARI, A., FRADE, S. P., ANGELO, P. C., GOMES, D. A. & GOES, A. M. 2015. 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 Res Ther.*

PÉREZ-ILZARBE, M., DÍEZ-CAMPELO, M., ARANDA, P., TABERA, S., LOPEZ, T., DEL CAÑIZO, C., MERINO, J., MORENO, C., ANDREU, E. J., PRÓSPER, F. & PÉREZ-SIMÓN, J. A. 2009. Comparison of ex vivo expansion culture conditions of mesenchymal stem cells for human cell therapy. *Transfusion,* 49**,** 1901-1910.

PILZ, G. A., BRAUN, J., ULRICH, C., FELKA, T., WARSTAT, K., RUH, M., SCHEWE, B., ABELE, H., LARBI, A. & AICHER, W. K. 2011. Human mesenchymal stromal cells express CD14 cross-reactive epitopes. *Cytometry A,* 79**,** 635-45.

PITTENGER, M. F., MACKAY, A. M., BECK, S. C., JAISWAL, R. K., DOUGLAS, R., MOSCA, J. D., MOORMAN, M. A., SIMONETTI, D. W., CRAIG, S. & MARSHAK, D. R. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science,* 284**,** 143-7.

PORADA, C. D. & ALMEIDA-PORADA, G. 2010. Mesenchymal stem cells as therapeutics and vehicles for gene and drug delivery. *Advanced Drug Delivery Reviews,* 62**,** 1156-1166.

POTAPOVA, I. A., BRINK, P. R., COHEN, I. S. & DORONIN, S. V. 2008. Culturing of Human Mesenchymal Stem Cells as Three-dimensional Aggregates Induces Functional Expression of CXCR4 That Regulates Adhesion to Endothelial Cells. *Journal of Biological Chemistry,* 283**,** 13100-13107.

POTAPOVA, I. A., GAUDETTE, G. R., BRINK, P. R., ROBINSON, R. B., ROSEN, M. R., COHEN, I. S. & DORONIN, S. V. 2007. Mesenchymal Stem Cells Support Migration, Extracellular Matrix Invasion, Proliferation, and Survival of Endothelial Cells In Vitro. *Stem Cells,* 25**,** 1761-1768.

POTIER, E., FERREIRA, E., MEUNIER, A., SEDEL, L., LOGEART-AVRAMOGLOU, D. & PETITE, H. 2007. Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue Eng,* 13**,** 1325-31.

POWELL, R. J., SIMONS, M., MENDELSOHN, F. O., DANIEL, G., HENRY, T. D., KOGA, M., MORISHITA, R. & ANNEX, B. H. 2008. Results of a double-blind, placebo-controlled study to assess the safety of intramuscular injection of hepatocyte growth factor plasmid to improve limb perfusion in patients with critical limb ischemia. *Circulation,* 118**,** 58-65.

PRATHER, K. J., SAGAR, S., MURPHY, J. & CHARTRAIN, M. 2003. Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. *Enzyme and Microbial Technology,* 33**,** 865-883.

PROCKOP, D. J. & YOUN OH, J. 2012. Mesenchymal Stem/Stromal Cells (MSCs): Role as Guardians of Inflammation. *Molecular Therapy,* 20**,** 14-20.

PU, Z., YOU, X., XU, Q., GAO, F., XIE, X., ZHANG, H. & JIAN'AN, W. 2011. Protein expression of mesenchymal stem cells after transfection of pcDNA3.1(-)-hVEGF(1)(6)(5) by ultrasound-targeted microbubble destruction. *J Biomed Biotechnol,* 2011**,** 839653.

QIN, J. Y., ZHANG, L., CLIFT, K. L., HULUR, I., XIANG, A. P., REN, B. Z. & LAHN, B. T. 2010. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One,* 5**,** e10611.

QUARANTA, P., FOCOSI, D., FREER, G. & PISTELLO, M. 2016. Tweaking Mesenchymal Stem/Progenitor Cell Immunomodulatory Properties with Viral Vectors Delivering Cytokines. *Stem Cells Dev,* 25**,** 1321-41.

RAJAGOPALAN, S., MOHLER, E. R., 3RD, LEDERMAN, R. J., MENDELSOHN, F. O., SAUCEDO, J. F., GOLDMAN, C. K., BLEBEA, J., MACKO, J., KESSLER, P. D., RASMUSSEN, H. S. & ANNEX, B. H. 2003. Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation,* 108**,** 1933-8.

RAMAMOORTH, M. & NARVEKAR, A. 2015. Non viral vectors in gene therapy- an overview. *J Clin Diagn Res,* 9**,** Ge01-6.

RAVAL, Z. & LOSORDO, D. W. 2013. Cell Therapy of Peripheral Arterial Disease: From Experimental Findings to Clinical Trials. *Circulation research,* 112**,** 10.1161/CIRCRESAHA.113.300565.

REJMAN, J., TAVERNIER, G., BAVARSAD, N., DEMEESTER, J. & DE SMEDT, S. C. 2010. mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers. *J Control Release,* 147**,** 385-91.

RIBEIRO, A., LARANJEIRA, P., MENDES, S., VELADA, I., LEITE, C., ANDRADE, P., SANTOS, F., HENRIQUES, A., GRÃOS, M., CARDOSO, C. M. P., MARTINHO, A., PAIS, M., DA SILVA, C. L., CABRAL, J., TRINDADE, H. & PAIVA, A. 2013. 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 Res Ther.*

ROHDE, E., MALISCHNIK, C., THALER, D., MAIERHOFER, T., LINKESCH, W., LANZER, G., GUELLY, C. & STRUNK, D. 2006. Blood monocytes mimic endothelial progenitor cells. *Stem Cells,* 24**,** 357-67.

ROJEWSKI, M. T., FEKETE, N., BAILA, S., NGUYEN, K., FURST, D., ANTWILER, D., DAUSEND, J., KREJA, L., IGNATIUS, A., SENSEBE, L. & SCHREZENMEIER, H. 2013. GMP-compliant isolation and expansion of bone marrow-derived MSCs in the closed, automated device quantum cell expansion system. *Cell Transplant,* 22**,** 1981-2000.

ROSEN, E. D. & MACDOUGALD, O. A. 2006. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol,* 7**,** 885-96.

RUSSELL, S., BENNETT, J., WELLMAN, J. A., CHUNG, D. C., YU, Z. F., TILLMAN, A., WITTES, J., PAPPAS, J., ELCI, O., MCCAGUE, S., CROSS, D., MARSHALL, K. A., WALSHIRE, J., KEHOE, T. L., REICHERT, H., DAVIS, M., RAFFINI, L., GEORGE, L. A., HUDSON, F. P., DINGFIELD, L., ZHU, X., HALLER, J. A., SOHN, E. H., MAHAJAN, V. B., PFEIFER, W., WECKMANN, M., JOHNSON, C., GEWAILY, D., DRACK, A., STONE, E., WACHTEL, K., SIMONELLI, F., LEROY, B. P., WRIGHT, J. F., HIGH, K. A. & MAGUIRE, A. M. 2017. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *Lancet,* 390**,** 849-860.

RUSSO, V., YOUNG, S., HAMILTON, A., AMSDEN, B. G. & FLYNN, L. E. 2014. Mesenchymal stem cell delivery strategies to promote cardiac regeneration following ischemic injury. *Biomaterials,* 35**,** 3956-74.

SAGARADZE, G., GRIGORIEVA, O., NIMIRITSKY, P., BASALOVA, N., KALININA, N., AKOPYAN, Z. & EFIMENKO, A. 2019. Conditioned Medium from Human Mesenchymal Stromal Cells: Towards the Clinical Translation. *Int J Mol Sci,* 20.

SAGE, E., DAVIES, A., KOLLURI, K., PATRICK, S., WEIL, B., VITORINO TENDEIRO PEREIRA REGO, R., EDWARDS, A., BAIN, O., SANTILLI, G., THAKRAR, R., CHAMPION, K., DAY, A., POPOVA, B., FULLEN, D., THRASHER, A., KALBER, T., FORSTER, M., LYTHGOE, M., LOWDELL, M. & JANES, S. M. 2018.

Targeted stem cells expressing TRAIL as a therapy for lung Cancer TACTICAL: a phase I/II trial. *Lung Cancer,* 115**,** S87.

SAGE, E. K., THAKRAR, R. M. & JANES, S. M. 2016. Genetically modified mesenchymal stromal cells in cancer therapy. *Cytotherapy,* 18**,** 1435-1445.

SANTHAGUNAM, A., DOS SANTOS, F., MADEIRA, C., SALGUEIRO, J. B. & CABRAL, J. M. 2014. Isolation and ex vivo expansion of synovial mesenchymal stromal cells for cartilage repair. *Cytotherapy,* 16**,** 440-53.

SANTOS, J. L., PANDITA, D., RODRIGUES, J., PEGO, A. P., GRANJA, P. L., BALIAN, G. & TOMAS, H. 2010. Receptor-mediated gene delivery using PAMAM dendrimers conjugated with peptides recognized by mesenchymal stem cells. *Mol Pharm,* 7**,** 763-74.

SANTOS, J. L., PANDITA, D., RODRIGUES, J., PEGO, A. P., GRANJA, P. L. & TOMAS, H. 2011. Non-viral gene delivery to mesenchymal stem cells: methods, strategies and application in bone tissue engineering and regeneration. *Curr Gene Ther,* 11**,** 46-57.

SAPAROV, A., OGAY, V., NURGOZHIN, T., JUMABAY, M. & CHEN, W. C. 2016. Preconditioning of Human Mesenchymal Stem Cells to Enhance Their Regulation of the Immune Response. *Stem Cells Int,* 2016**,** 3924858.

SARAF, A., HACKER, M. C., SITHARAMAN, B., GRANDE-ALLEN, K. J., BARRY, M. A. & MIKOS, A. G. 2008. Synthesis and conformational evaluation of a novel gene delivery vector for human mesenchymal stem cells. *Biomacromolecules,* 9**,** 818-27.

SART, S., AGATHOS, S. N., LI, Y. & MA, T. 2016. Regulation of mesenchymal stem cell 3D microenvironment: From macro to microfluidic bioreactors. *Biotechnol J,* 11**,** 43-57.

SATO, K., OZAKI, K., MORI, M., MUROI, K. & OZAWA, K. 2010. Mesenchymal stromal cells for graft-versus-host disease : basic aspects and clinical outcomes. *J Clin Exp Hematop,* 50**,** 79-89.

SAWAMURA, D., ABE, R., GOTO, M., AKIYAMA, M., HEMMI, H., AKIRA, S. & SHIMIZU, H. 2005. Direct injection of plasmid DNA into the skin induces dermatitis by activation of monocytes through toll-like receptor 9. *J Gene Med,* 7**,** 664-71.

SCHALLMOSER, K., BARTMANN, C., ROHDE, E., REINISCH, A., KASHOFER, K., STADELMEYER, E., DREXLER, C., LANZER, G., LINKESCH, W. & STRUNK, D. 2007. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. *Transfusion,* 47**,** 1436-46.

SCHIMMER, J. & BREAZZANO, S. 2016. Investor Outlook: Rising from the Ashes; GSK's European Approval of Strimvelis for ADA-SCID. *Hum Gene Ther Clin Dev,* 27**,** 57-61.

SCHLEEF, M., SCHIRMBECK, R., REISER, M., MICHEL, M. L. & SCHMEER, M. 2015. Minicircle: Next Generation DNA Vectors for Vaccination. *Methods Mol Biol,* 1317**,** 327-39.

SCOTT, L. J. 2015. Alipogene tiparvovec: a review of its use in adults with familial lipoprotein lipase deficiency. *Drugs,* 75**,** 175-82.

SERRA, J., ALVES, C. P. A., BRITO, L., MONTEIRO, G. A., CABRAL, J. M. S., PRAZERES, D. M. F. & DA SILVA, C. L. 2018. Engineering of Human Mesenchymal Stem/Stromal Cells with Vascular Endothelial Growth Factor-Encoding Minicircles for Angiogenic Ex Vivo Gene Therapy. *Hum Gene Ther*.

SHI, R. Z. & LI, Q. P. 2008. Improving outcome of transplanted mesenchymal stem cells for ischemic heart disease. *Biochem Biophys Res Commun,* 376**,** 247-50.

SHIGEMATSU, H., YASUDA, K., IWAI, T., SASAJIMA, T., ISHIMARU, S., OHASHI, Y., YAMAGUCHI, T., OGIHARA, T. & MORISHITA, R. 2010. Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia. *Gene Ther,* 17**,** 1152-61.

SHIMAMURA, M., NAKAGAMI, H., KORIYAMA, H. & MORISHITA, R. 2013. Gene Therapy and Cell-Based Therapies for Therapeutic Angiogenesis in Peripheral Artery Disease. *BioMed Research International,* 2013**,** 1-8.

SHUJIA, J., HAIDER, H. K., IDRIS, N. M., LU, G. & ASHRAF, M. 2008. Stable therapeutic effects of mesenchymal stem cell-based multiple gene delivery for cardiac repair. *Cardiovasc Res,* 77**,** 525-33.

SIDNEY, L. E., BRANCH, M. J., DUNPHY, S. E., DUA, H. S. & HOPKINSON, A. 2014. Concise Review: Evidence for CD34 as a Common Marker for Diverse Progenitors. *Stem Cells,* 32**,** 1380-9.

SILVA-SANTOS, A. R., ALVES, C. P. A., MONTEIRO, G., AZEVEDO, A. M. & PRAZERES, D. M. F. 2019. Multimodal chromatography of supercoiled minicircles: A closer look into DNA-ligand interactions. *Separation and Purification Technology,* 212**,** 161-170.

SIMCIKOVA, M., PRATHER, K. L. J., PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. On the dual effect of glucose during production of pBAD/AraC-based minicircles. *Vaccine,* 32**,** 2843-2846.

SIMMONS, P. J. & TOROK-STORB, B. 1991. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood,* 78**,** 55-62.

SIMOES, I. N., BOURA, J. S., DOS SANTOS, F., ANDRADE, P. Z., CARDOSO, C. M., GIMBLE, J. M., DA SILVA, C. L. & CABRAL, J. M. 2013. Human mesenchymal stem cells from the umbilical cord matrix: successful isolation and ex vivo expansion using serum-/xeno-free culture media. *Biotechnol J,* 8**,** 448-58.

SINGER, N. G. & CAPLAN, A. I. 2011. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol,* 6**,** 457-78.

SINN, P. L., SAUTER, S. L. & MCCRAY, P. B., JR. 2005. Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors--design, biosafety, and production. *Gene Ther,* 12**,** 1089-98.

SOTIROPOULOU, P. A., PEREZ, S. A., SALAGIANNI, M., BAXEVANIS, C. N. & PAPAMICHAIL, M. 2006. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells,* 24**,** 462-71.

SPENCER, J. A., FERRARO, F., ROUSSAKIS, E., KLEIN, A., WU, J., RUNNELS, J. M., ZAHER, W., MORTENSEN, L. J., ALT, C., TURCOTTE, R., YUSUF, R., COTE, D., VINOGRADOV, S. A., SCADDEN, D. T. & LIN, C. P. 2014. Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature,* 508**,** 269-73.

SQUILLARO, T., PELUSO, G. & GALDERISI, U. 2016. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell Transplant,* 25**,** 829-48.

STENDER, S., MURPHY, M., O'BRIEN, T., STENGAARD, C., ULRICH-VINTHER, M., SOBALLE, K. & BARRY, F. 2007. Adeno-associated viral vector transduction of human mesenchymal stem cells. *Eur Cell Mater,* 13**,** 93-9; discussion 99.

STULTZ, B. G., MCGINNIS, K., THOMPSON, E. E., LO SURDO, J. L., BAUER, S. R. & HURSH, D. A. 2016. Chromosomal Stability of Mesenchymal Stromal Cells During In Vitro Culture. *Cytotherapy,* 18**,** 336-43.

SU, G. H., SUN, Y. F., LU, Y. X., SHUAI, X. X., LIAO, Y. H., LIU, Q. Y., HAN, J. & LUO, P. 2013. Hepatocyte growth factor gene-modified bone marrow-derived mesenchymal stem cells transplantation promotes angiogenesis in a rat model of hindlimb ischemia. *J Huazhong Univ Sci Technolog Med Sci,* 33**,** 511-519.

SUN, J., WEI, Z. Z., GU, X., ZHANG, J. Y., ZHANG, Y., LI, J. & WEI, L. 2015. Intranasal delivery of hypoxia-preconditioned bone marrow-derived mesenchymal stem cells enhanced regenerative effects after intracerebral hemorrhagic stroke in mice. *Exp Neurol,* 272**,** 78-87.

SUN, X. L., XU, Z. M., KE, Y. Q., HU, C. C., WANG, S. Y., LING, G. Q., YAN, Z. J., LIU, Y. J., SONG, Z. H., JIANG, X. D. & XU, R. X. 2011. Molecular targeting of malignant glioma cells with an EphA2-specific immunotoxin delivered by human bone marrow-derived mesenchymal stem cells. *Cancer Lett,* 312**,** 168-77.

SUNDIN, M., RINGDEN, O., SUNDBERG, B., NAVA, S., GOTHERSTROM, C. & LE BLANC, K. 2007. No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum

antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. *Haematologica,* 92**,** 1208-15.

TAIMEH, Z., LOUGHRAN, J., BIRKS, E. J. & BOLLI, R. 2013. Vascular endothelial growth factor in heart failure. *Nat Rev Cardiol,* 10**,** 519-30.

TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126**,** 663-76.

TALCOTT, B. & MOORE, M. S. 1999. Getting across the nuclear pore complex. *Trends Cell Biol,* 9**,** 312-8.

TAN, K. Y., TEO, K. L., LIM, J. F., CHEN, A. K., CHOOLANI, M., REUVENY, S., CHAN, J. & OH, S. K. 2015. Serum-free media formulations are cell line-specific and require optimization for microcarrier culture. *Cytotherapy,* 17**,** 1152-65.

TANG, D. Q., WANG, Q., BURKHARDT, B. R., LITHERLAND, S. A., ATKINSON, M. A. & YANG, L. J. 2012. In vitro generation of functional insulin-producing cells from human bone marrow-derived stem cells, but long-term culture running risk of malignant transformation. *Am J Stem Cells,* 1**,** 114-27.

TANG, J., WANG, J., KONG, X., YANG, J., GUO, L., ZHENG, F., ZHANG, L., HUANG, Y. & WAN, Y. 2009a. Vascular endothelial growth factor promotes cardiac stem cell migration via the PI3K/Akt pathway. *Exp Cell Res,* 315**,** 3521-31.

TANG, J., WANG, J., YANG, J., KONG, X., ZHENG, F., GUO, L., ZHANG, L. & HUANG, Y. 2009b. Mesenchymal stem cells over-expressing SDF-1 promote angiogenesis and improve heart function in experimental myocardial infarction in rats. *Eur J Cardiothorac Surg,* 36**,** 644-50.

TANG, Y. L., TANG, Y., ZHANG, Y. C., QIAN, K., SHEN, L. & PHILLIPS, M. I. 2005. Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. *J Am Coll Cardiol,* 46**,** 1339-50.

TANIYAMA, Y., MORISHITA, R., AOKI, M., NAKAGAMI, H., YAMAMOTO, K., YAMAZAKI, K., MATSUMOTO, K., NAKAMURA, T., KANEDA, Y. & OGIHARA, T. 2001. Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat and rabbit hindlimb ischemia models: preclinical study for treatment of peripheral arterial disease. *Gene Ther,* 8**,** 181-9.

TAO, H., HAN, Z., HAN, Z. C. & LI, Z. 2016. Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. *Stem Cells Int,* 2016**,** 1314709.

TATEISHI-YUYAMA, E., MATSUBARA, H., MUROHARA, T., IKEDA, U., SHINTANI, S., MASAKI, H., AMANO, K., KISHIMOTO, Y., YOSHIMOTO, K., AKASHI, H., SHIMADA, K., IWASAKA, T. & IMAIZUMI, T. 2002. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet,* 360**,** 427-35.

TEIXEIRA, F. G., PANCHALINGAM, K. M., ASSUNCAO-SILVA, R., SERRA, S. C., MENDES-PINHEIRO, B., PATRICIO, P., JUNG, S., ANJO, S. I., MANADAS, B., PINTO, L., SOUSA, N., BEHIE, L. A. & SALGADO, A. J. 2016. Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors: Impact on Neuronal Cell Proliferation, Survival and Differentiation. *Sci Rep,* 6**,** 27791.

TERAA, M., SPRENGERS, R. W., WESTERWEEL, P. E., GREMMELS, H., GOUMANS, M. J., TEERLINK, T., MOLL, F. L. & VERHAAR, M. C. 2013. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS One,* 8**,** e55592.

THOMAS, E. D., LOCHTE, H. L., JR., LU, W. C. & FERREBEE, J. W. 1957. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med,* 257**,** 491-6.

TREACY, O., RYAN, A. E., HEINZL, T., O'FLYNN, L., CREGG, M., WILK, M., ODOARDI, F., LOHAN, P., O'BRIEN, T., NOSOV, M. & RITTER, T. 2012. Adenoviral transduction of mesenchymal stem cells: in vitro responses and in vivo immune responses after cell transplantation. *PLoS One,* 7**,** e42662.

TSULAIA, T. V., PROKOPISHYN, N. L., YAO, A., CARSRUD, N. D., CAROU, M. C., BROWN, D. B., DAVIS, B. R. & YANNARIELLO-BROWN, J. 2003. Glass needle-mediated microinjection of macromolecules and transgenes into primary human mesenchymal stem cells. *J Biomed Sci,* 10**,** 328-36.

UCHIMURA, E., YAMADA, S., UEBERSAX, L., FUJITA, S., MIYAKE, M. & MIYAKE, J. 2007. Method for reverse transfection using gold colloid as a nano-scaffold. *J Biosci Bioeng,* 103**,** 101-3.

VAN DER AA, M. A., KONING, G. A., D'OLIVEIRA, C., OOSTING, R. S., WILSCHUT, K. J., HENNINK, W. E. & CROMMELIN, D. J. 2005. An NLS peptide covalently linked to linear DNA does not enhance transfection efficiency of cationic polymer based gene delivery systems. *J Gene Med,* 7**,** 208-17.

VANNUCCI, L., LAI, M., CHIUPPESI, F., CECCHERINI-NELLI, L. & PISTELLO, M. 2013. Viral vectors: a look back and ahead on gene transfer technology. *New Microbiol,* 36**,** 1-22.

VENKATESAN, J. K., EKICI, M., MADRY, H., SCHMITT, G., KOHN, D. & CUCCHIARINI, M. 2012. SOX9 gene transfer via safe, stable, replication-defective recombinant adeno-associated virus vectors as a novel, powerful tool to enhance the chondrogenic potential of human mesenchymal stem cells. *Stem Cell Res Ther,* 3**,** 22.

VIAU, S., EAP, S., CHABRAND, L., LAGRANGE, A. & DELORME, B. 2019. Viral inactivation of human platelet lysate by gamma irradiation preserves its optimal efficiency in the expansion of human bone marrow mesenchymal stromal cells. *Transfusion,* 59**,** 1069-1079.

WAKITANI, S., SAITO, T. & CAPLAN, A. I. 1995. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve,* 18**,** 1417-26.

WALDER, C. E., ERRETT, C. J., BUNTING, S., LINDQUIST, P., OGEZ, J. R., HEINSOHN, H. G., FERRARA, N. & THOMAS, G. R. 1996. Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hindlimb ischemia. *J Cardiovasc Pharmacol,* 27**,** 91-8.

WALKER, W. E., BOOTH, C. J. & GOLDSTEIN, D. R. 2010. TLR9 and IRF3 Cooperate to Induce a Systemic Inflammatory Response in Mice Injected With Liposome:DNA. *Mol Ther.*

WALTER, D. H., KRANKENBERG, H., BALZER, J. O., KALKA, C., BAUMGARTNER, I., SCHLUTER, M., TONN, T., SEEGER, F., DIMMELER, S., LINDHOFF-LAST, E. & ZEIHER, A. M. 2011. Intraarterial administration of bone marrow mononuclear cells in patients with critical limb ischemia: a randomized-start, placebo-controlled pilot trial (PROVASA). *Circ Cardiovasc Interv,* 4**,** 26-37.

WANG, K., WEI, G. & LIU, D. 2012. CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. *Exp Hematol Oncol,* 1**,** 36.

WANG, W., LI, W., MA, N. & STEINHOFF, G. 2013. Non-viral gene delivery methods. *Curr Pharm Biotechnol,* 14**,** 46-60.

WANG, W., XU, X., LI, Z., LENDLEIN, A. & MA, N. 2014a. Genetic engineering of mesenchymal stem cells by non-viral gene delivery. *Clin Hemorheol Microcirc,* 58**,** 19-48.

WANG, X., HU, Q., MANSOOR, A., LEE, J., WANG, Z., LEE, T., FROM, A. H. & ZHANG, J. 2006. Bioenergetic and functional consequences of stem cell-based VEGF delivery in pressure-overloaded swine hearts. *Am J Physiol Heart Circ Physiol,* 290**,** H1393-405.

WANG, X., ZHAO, T., HUANG, W., WANG, T., QIAN, J., XU, M., KRANIAS, E. G., WANG, Y. & FAN, G. C. 2009. Hsp20-engineered mesenchymal stem cells are resistant to oxidative stress via enhanced activation of Akt and increased secretion of growth factors. *Stem Cells,* 27**,** 3021-31.

WANG, Y., WU, H., YANG, Z., CHI, Y., MENG, L., MAO, A., YAN, S., HU, S., ZHANG, J., ZHANG, Y., YU, W., MA, Y., LI, T., CHENG, Y., WANG, S., LIU, J., HAN, J., LI, C., LIU, L., XU, J., HAN, Z. B. & HAN, Z. C. 2014b. 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,* 5**,** 132.

WATT, S. M., GULLO, F., VAN DER GARDE, M., MARKESON, D., CAMICIA, R., KHOO, C. P. & ZWAGINGA, J. J. 2013. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. *Br Med Bull,* 108**,** 25-53.

WIEHE, J. M., KAYA, Z., HOMANN, J. M., WOHRLE, J., VOGT, K., NGUYEN, T., ROTTBAUER, W., TORZEWSKI, J., FEKETE, N., ROJEWSKI, M., SCHREZENMEIER, H., MOEPPS, B. & ZIMMERMANN, O. 2013. GMP-adapted overexpression of CXCR4 in human mesenchymal stem cells for cardiac repair. *Int J Cardiol,* 167**,** 2073-81.

WIEHE, J. M., PONSAERTS, P., ROJEWSKI, M. T., HOMANN, J. M., GREINER, J., KRONAWITTER, D., SCHREZENMEIER, H., HOMBACH, V., WIESNETH, M., ZIMMERMANN, O. & TORZEWSKI, J. 2007. mRNA-mediated gene delivery into human progenitor cells promotes highly efficient protein expression. *J Cell Mol Med,* 11**,** 521-30.

WIGHTMAN, L., KIRCHEIS, R., ROSSLER, V., CAROTTA, S., RUZICKA, R., KURSA, M. & WAGNER, E. 2001. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J Gene Med,* 3**,** 362-72.

WILSON, J. M. 2005. Gendicine: the first commercial gene therapy product. *Hum Gene Ther,* 16**,** 1014-5.

WU, G. Y. & WU, C. H. 1987. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem,* 262**,** 4429-32.

WU, J., XIAO, X., ZHAO, P., XUE, G., ZHU, Y., ZHU, X., ZHENG, L., ZENG, Y. & HUANG, W. 2006. Minicircle-IFNgamma induces antiproliferative and antitumoral effects in human nasopharyngeal carcinoma. *Clin Cancer Res,* 12**,** 4702-13.

XIANG, Q., HONG, D., LIAO, Y., CAO, Y., LIU, M., PANG, J., ZHOU, J., WANG, G., YANG, R., WANG, M. & XIANG, A. P. 2017. Overexpression of Gremlin1 in Mesenchymal Stem Cells Improves Hindlimb Ischemia in Mice by Enhancing Cell Survival. *J Cell Physiol,* 232**,** 996-1007.

XIE, B., LUO, H., ZHANG, Y., WANG, Q., ZHOU, C. & XU, D. 2018. Autologous Stem Cell Therapy in Critical Limb Ischemia: A Meta-Analysis of Randomized Controlled Trials. *Stem Cells Int,* 2018**,** 7528464.

XU, J., QU, J., CAO, L., SAI, Y., CHEN, C., HE, L. & YU, L. 2008. Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol,* 214**,** 472-81.

XU, W., ZHANG, X., QIAN, H., ZHU, W., SUN, X., HU, J., ZHOU, H. & CHEN, Y. 2004. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. *Exp Biol Med (Maywood),* 229**,** 623-31.

XU, Y. & SZOKA, F. C., JR. 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry,* 35**,** 5616-23.

YAGI, H., SOTO-GUTIERREZ, A., PAREKKADAN, B., KITAGAWA, Y., TOMPKINS, R. G., KOBAYASHI, N. & YARMUSH, M. L. 2010. Mesenchymal stem cells: Mechanisms of immunomodulation and homing. *Cell Transplant,* 19**,** 667-79.

YAMANAKA, S. 2010. Patient-specific pluripotent stem cells become even more accessible. *Cell Stem Cell,* 7**,** 1-2.

YAN, J., TIE, G., XU, T. Y., CECCHINI, K. & MESSINA, L. M. 2013. Mesenchymal stem cells as a treatment for peripheral arterial disease: current status and potential impact of type II diabetes on their therapeutic efficacy. *Stem Cell Rev,* 9**,** 360-72.

YANG, F., CHO, S. W., SON, S. M., BOGATYREV, S. R., SINGH, D., GREEN, J. J., MEI, Y., PARK, S., BHANG, S. H., KIM, B. S., LANGER, R. & ANDERSON, D. G. 2010. Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles. *Proc Natl Acad Sci U S A,* 107**,** 3317-22.

YANG, H. T. & FENG, Y. 2000. bFGF increases collateral blood flow in aged rats with femoral artery ligation. *Am J Physiol Heart Circ Physiol,* 278**,** H85-93.

YANG, S. S., KIM, N. R., PARK, K. B., DO, Y. S., ROH, K., KANG, K. S. & KIM, D. I. 2013. A phase I study of human cord blood-derived mesenchymal stem cell therapy in patients with peripheral arterial occlusive disease. *Int J Stem Cells,* 6**,** 37-44.

YANG, Y., NUNES, F. A., BERENCSI, K., FURTH, E. E., GONCZOL, E. & WILSON, J. M. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A,* 91**,** 4407-11.

YASUMURA, E. G., STILHANO, R. S., SAMOTO, V. Y., MATSUMOTO, P. K., DE CARVALHO, L. P., VALERO LAPCHIK, V. B. & HAN, S. W. 2012. Treatment of Mouse Limb Ischemia with an Integrative

Hypoxia-Responsive Vector Expressing the Vascular Endothelial Growth Factor Gene. *PLOS ONE,* 7**,** e33944.

YIN, T., HE, S., SU, C., CHEN, X., ZHANG, D., WAN, Y., YE, T., SHEN, G., WANG, Y., SHI, H., YANG, L. & WEI, Y. 2015. Genetically modified human placenta-derived mesenchymal stem cells with FGF-2 and PDGF-BB enhance neovascularization in a model of hindlimb ischemia. *Molecular Medicine Reports*.

YLA-HERTTUALA, S. 2012. Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. *Mol Ther.* United States.

YU, T. T. L., GUPTA, P., RONFARD, V., VERTES, A. A. & BAYON, Y. 2018. Recent Progress in European Advanced Therapy Medicinal Products and Beyond. *Front Bioeng Biotechnol,* 6**,** 130.

ZALDUMBIDE, A., CARLOTTI, F., GONCALVES, M. A., KNAAN-SHANZER, S., CRAMER, S. J., ROEP, B. O., WIERTZ, E. J. & HOEBEN, R. C. 2012. Adenoviral vectors stimulate glucagon transcription in human mesenchymal stem cells expressing pancreatic transcription factors. *PLoS One,* 7**,** e48093.

ZANOTTI, L., ANGIONI, R., CALI, B., SOLDANI, C., PLOIA, C., MOALLI, F., GARGESHA, M., D'AMICO, G., ELLIMAN, S., TEDESCHI, G., MAFFIOLI, E., NEGRI, A., ZACCHIGNA, S., SARUKHAN, A., STEIN, J. V. & VIOLA, A. 2016. Mouse mesenchymal stem cells inhibit high endothelial cell activation and lymphocyte homing to lymph nodes by releasing TIMP-1. *Leukemia,* 30**,** 1143-54.

ZANTA, M. A., BELGUISE-VALLADIER, P. & BEHR, J. P. 1999. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci U S A,* 96**,** 91-6.

ZHANG, J. C., ZHENG, G. F., WU, L., OU YANG, L. Y. & LI, W. X. 2014. Bone marrow mesenchymal stem cells overexpressing human basic fibroblast growth factor increase vasculogenesis in ischemic rats. *Braz J Med Biol Res,* 47**,** 886-94.

ZHANG, M., MAL, N., KIEDROWSKI, M., CHACKO, M., ASKARI, A. T., POPOVIC, Z. B., KOC, O. N. & PENN, M. S. 2007. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *Faseb j,* 21**,** 3197-207.

ZHANG, W. W., LI, L., LI, D., LIU, J., LI, X., LI, W., XU, X., ZHANG, M. J., CHANDLER, L. A., LIN, H., HU, A., XU, W. & LAM, D. M. 2018. The First Approved Gene Therapy Product for Cancer Ad-p53 (Gendicine): 12 Years in the Clinic. *Hum Gene Ther,* 29**,** 160-179.

ZHAO, L., JOHNSON, T. & LIU, D. 2017. Therapeutic angiogenesis of adipose-derived stem cells for ischemic diseases. *Stem Cell Res Ther,* 8**,** 125.

ZISCHEK, C., NIESS, H., ISCHENKO, I., CONRAD, C., HUSS, R., JAUCH, K. W., NELSON, P. J. & BRUNS, C. 2009. Targeting tumor stroma using engineered mesenchymal stem cells reduces the growth of pancreatic carcinoma. *Ann Surg,* 250**,** 747-53.

ZUK, P. A., ZHU, M., MIZUNO, H., HUANG, J., FUTRELL, J. W., KATZ, A. J., BENHAIM, P., LORENZ, H. P. & HEDRICK, M. H. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng,* 7**,** 211-28.

Chapter II

DESIGN, PRODUCTION AND PURIFICATION OF NON-VIRAL VECTORS FOR GENE THERAPY

II. DESIGN, PRODUCTION AND PURIFICATION OF NON-VIRAL VECTORS FOR GENE THERAPY

II.1. Summary

Gene therapy approaches have been extensively investigated for the treatment or prevention of several diseases. Despite viral vectors are able to promote high transgene expression, these raise some safety concerns. Non-viral systems, such as plasmids, were developed as safer alternatives. A particular type of non-viral vectors, minicircles (MC), demonstrated to promote high transgene expressions with reduced toxicity. These MC are obtained by recombination of parental plasmids (PP), which generates not only the MC but also a miniplasmid (MP) with bacterial sequences. One of the major limitations of the wide application of MC for clinical purposes is the lack of an efficient system for its production and purification from MP counterparts. In the present work, a novel and effective method for MC production and purification is proposed. Firstly, PP and plasmids conventionally used in transfection approaches (pVAX) containing the vascular endothelial growth factor (VEGF) therapeutic gene alone or in fusion with green fluorescent protein (GFP) were constructed. Then, PP was produced in a recently developed bacteria strain specifically designed for arabinose uptake and recombinase production. Finally, MC were isolate by the combining a digestion step using a nicking enzyme and an elution step-based hydrophobic interaction chromatography (HIC). Using this strategy, a 100% recombination efficiency was achieved, since no PP was observed two hours after recombination was triggered. Also, the changes in DNA topology induced by enzymatic nicking step allowed an efficient separation of different DNA molecules by HIC using elution steps with different salt concentrations. At the end of the process, pure and transfection-graded MC are obtained, ready to be transfected into mammalian cells.

II.2. Background

Gene therapy used nucleic acid molecules (DNA or RNA) to treat diseases through removal or replacement of a defective gene by a repaired one or by delivery of therapeutic gene(s). These strategies have been extensively explored for the treatment of a broad spectrum of conditions (Kaufmann et al., 2013). In fact, currently, there are some gene-based therapeutic products approved by regulatory authorities (i.e. FDA, EMA) and commercially available (Cuende et al., 2018).

For an efficient delivery of the genes into human cells a vector is required to carry the DNA and deliver it into target cells (Ramamoorth and Narvekar, 2015). According to its nature, gene therapy vectors may be classified as viral or non-viral. Despite the majority of approved products are based on viral strategies, their use may raise some safety concerns since some studies with virus were early terminated due to occurrence of adverse events (Marshall, 1999, Hacein-Bey-Abina et al., 2003). To overcome such concerns, non-viral systems, based on the use of DNA plasmids in combination with physical or chemical delivery methods, have been developed (Wang et al., 2013). Even though non-viral vectors are not capable of integrating into the genome, having a transient and usually lower expression than viral counterparts, their use may be advantageous not only in terms of safety, but also in cases where a continuous expression of the transgene is not desirable.

Plasmid DNA (pDNA) molecules used for transfection contain the gene of interest and are produced within bacteria, most commonly using *Escherichia coli (E. coli)* strains. Thus, pDNA molecules must contain a bacterial origin of replication and an antibiotic resistance marker for the selection of plasmid-harboring bacteria (Hardee et al., 2017). These sequences, which are required for plasmid replication and maintenance within bacteria, constitute the bacterial backbone. Additionally, pDNA molecules contain a transcription unit with the gene of interest under the control of a promoter, the polyadenylation sequence and any other sequences required for expression of the transgene in mammalian cells.

Despite the described safety of non-viral vectors, the presence of bacterial-derived sequences may trigger an immune response on transfected cells. The cytosine-guanosine dinucleotide (CpG) motifs are very common on bacterial DNA and were described to trigger immune responses in mammalian cells through activation of Toll-like receptor (TLR)-9 (Walker et al., 2010). Thus, the activation of the immune system by the presence of CpG may cause transgene silencing (Häcker et al., 2002), or, in more severe cases, lead to inflammatory reactions (Boura et al., 2014, Sawamura et al., 2005). Another concern regarding the use of pDNA is the presence

of antibiotic resistance due to the risk of transference to human microbiome or environment (Hardee et al., 2017).

To overcome these limitations, modifications of conventional plasmids have been performed. The generation of minicircles (MC), small circular DNA molecules that contain only transcriptional unit part of a conventional plasmid, is one of such examples (Darquet et al., 1997). The lack of bacterial sequences and the reduced size of MC contribute to enhance transfection, improve safety and reduce CpG-mediated immunogenicity and silencing. Different studies demonstrated that MC-based therapy is able to sustain higher and longer transgene expression *in vitro* and *in vivo*, as well as stem cell survival, when compared to conventional plasmid vectors (Darquet et al., 1999, Chen et al., 2003, Dietz et al., 2013, Madeira et al., 2013).

MC are obtained from parental plasmids (PP) upon excision of the expression cassette via site-specific recombination between two multimer resolution sites (MRS) strategically placed within PP sequence. After this step, two different molecules are generated: MC containing the transcription unit and a miniplasmid (MP) where the bacterial backbone sequences can be found (Darquet et al., 1997, Darquet et al., 1999, Jechlinger et al., 2004, Mayrhofer et al., 2008).

The conversion of PP into MC is a complex process that starts with amplification of precursor plasmids within *E. coli* (Prather et al., 2003). After production of high PP yields, two crucial steps are required: induction and recombination (Kay et al., 2010, Simcikova et al., 2014). Induction is performed by the addition of a compound (e.g. L-arabinose), which activates the expression of genes coding for enzymes (recombinases) that mediate recombination step. In general the addition of inductor must be performed by the end of the exponential phase to maximize MC yield (Gaspar et al., 2014). Recombination consists in the conversion of PP into MC and MP by the action of recombinases, such as ParA resolvase, that bind to hybrid sites and mediate strand exchange and re-assembly (Kay et al., 2010).

The major challenge regarding a wider application of MC-based gene therapies is to achieve high yields of MC and to effectively isolate and purify them from the MP (and also PP) counterparts. This is particularly challenging due to the similarities between MP and MC in terms of size, topology and abundance. The first method developed for MC isolation relied on MP linearization with a restriction enzyme followed by caesium chloride (CsCl) gradient centrifugation (Darquet et al., 1997, Darquet et al., 1999). However, this method is not feasible for large scale MC production nor compatible with regulatory guidelines (Mayrhofer and Iro, 2012, Prazeres, 2011). Most recently, Kay and colleagues developed an improved approach: an inducible I-*Sce*I nuclease was included in bacterial host cells that specifically degrades MP, which was designed

to have an I-*Sce*I recognition site (Kay et al., 2010). Using this system, MP species are degraded in culture and MC can be further purified using common pDNA isolation techniques.

In the present work, an alternative strategy was followed for MC purification. *E.coli* B2WP, a strain with improved arabinose uptake that was constructed from BW27783 through stable insertion of a single copy of parA in the bacterial chromosome under the control of the arabinose inducible pBAD/AraC promoter was used (Jechlinger et al., 2004). After *E. coli* transformation with PP and growth until late exponential phase, the parA expression is induced by addition of arabinose to the culture and recombination of PP occurs. Glucose is a repressor of pBAD/AraC promoter, so inclusion of glucose on culture medium during the initial stages of the culture prevents the leaky expression of parA, inhibiting early recombination (Simcikova et al., 2014). Following recombination, the total pDNA is then purified from bacterial cultures using conventional methods and a solution containing a mixture of MC, MP and some un-recombined PP is obtained. The purification of MC from MP can be achieved by exploring the enzymatic activity of *Nb.B*bvCI, which is an endonuclease that has the capacity of cleaving only one of the DNA strands, introducing a nick instead of a break in the double DNA chain (Heiter et al., 2005). Since a recognition site of *Nb.B*bvCI was strategically placed in MP sequence, this action induces a conformation change in MP from supercoiled into open circular (or relaxed) isoform. On the contrary, the supercoiled MC is maintained intact and so hydrophobic interaction chromatography (HIC) approaches may then be used to isolate MC by exploring the differences in hydrophobicity between the distinct DNA isoforms (supercoiled *versus* open circular) (Bo et al., 2013, Urthaler et al., 2005). This strategy, optimized at Bioengineering Research Group (BERG), iBB – Institute for Bioengineering and Biosciences, can be used to effectively produce pure MC fractions that are free from nucleic acid or other impurities (Alves et al., 2016, Alves et al., 2018).

Therapeutic gene delivery can be performed through direct infusion of the gene of interest (*in vivo*) or by genetically engineering human cells outside the body then re-infused them into patients (Kaufmann et al., 2013). Although there were several approaches with gene therapy vectors being directly administered into the body, the use of a cellular vehicle with intrinsic therapeutic features, such as mesenchymal stem/stromal cells (MSC), could be much more advantageous (Porada and Almeida-Porada, 2010). Vascular endothelial growth factor (VEGF) is one of the most potent and probably the most studied pro-angiogenic factor and is known to be secreted by MSC (Ferrara, 2001, Singer and Caplan, 2011). So, MC encoding for VEGF might be potentially used to achieve high expression of this protein and, thus, enhance the intrinsic angiogenic activity of human MSC.

Herein, a strategy to effectively produce and purify high MC yields free from nucleic acid impurities is described, which can be used to transfect MSC. Construction, production and purification of conventional pDNA vectors (pVAX) will also be approached as they will be used as control on transfection experiments. MC and pVAX containing the VEGF gene alone or in fusion with a reporter gene – green fluorescent protein (GFP) were constructed and purified.

II.3. Materials and Methods

II.3.1. Plasmid construction: pVAX-VEGF-GFP and pMINILi-CVG

The plasmids used throughout the present thesis are represented in Figure II.1. Plasmids containing VEGF-GFP (pVAX-VEGF-GFP, 4563 bp and pMINILi-CVG, 4273 bp) had already been constructed at BERG-iBB, as previously described (Azzoni et al., 2007, Simcikova et al., 2014, Alves et al., 2016). Briefly, the parental plasmid pMINILi-CVG contains the VEGF-GFP gene fusion construct under the control of cytomegalovirus (CMV) immediate early-promoter, two multimer resolution sites (MRS) flanking the expression cassette, pMB1 origin of replication, kanamycin resistance gene and BGH polyadenylation sequence. The pVAX-VEGF-GFP was obtained from the previously constructed pVAX1GFP-BGH, which is derived from the commercial pVAX1LacZ (6050 bp, Invitrogen), by introduction of the VEGF gene in fusion with the GFP reporter (Azzoni et al., 2007, Alves et al., 2016).

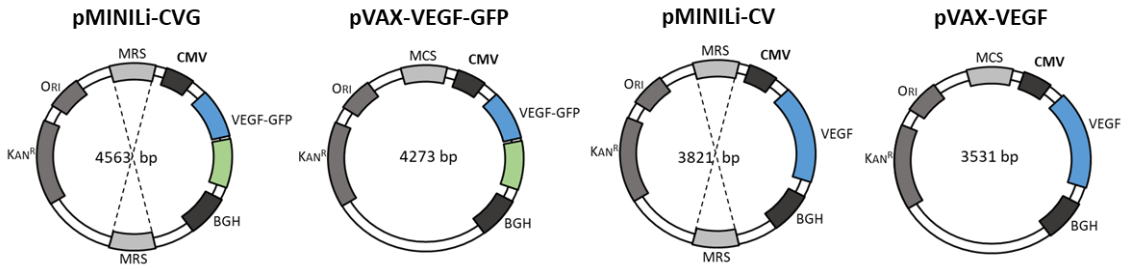

Figure II.1 – Plasmid constructions used throughout this work to transform E. coli strains: pMINILi-CVG, pVAX-VEGF-GFP, pMINILi-CV and pVAX-VEGF. The first two contain the VEGF-GFP fusion gene expression cassette, whilst the last two have only the VEGF gene inserted in the same construction.

II.3.2. GFP removal to obtain pVAX-VEGF and pMINILi-CV

To obtain the constructions (pVAX-VEGF, 3531 bp and pMINILi-CV, 3821 bp) with VEGF gene alone, GFP removal from pVAX-VEGF-GFP and pMINILi-CVG was performed. Firstly, both pVAX-VEGF-GFP and pMINILi-CVG were digested with XpnI and XbaI restriction enzymes (both from Promega) to remove the fusion expression cassette (VEGF-GFP) and an agarose gel electrophoresis was performed. The bands corresponding to empty vectors were cut and directly purified from the gel with QIAquick Gel Extraction Kit (Qiagen) according to manufacturer instructions. The fragment corresponding to VEGF-GFP was also extracted and purified from gel. To obtain the VEGF gene for insertion into empty vectors, polymerase chain reaction (PCR) amplification was performed using this fragment and the following primers synthesized by StabVida: VEGF_fwd (CCCACTGCTTACTGGTTATCG) and VEGF_rev_STOP (ATCTATTCTAGA**TTA**CC GCCTCGGTTGTC). The reverse primer (VEGF_rev_STOP) was specifically

designed to introduce an XbaI restriction site (*underlined*) and a stop codon (*bold*) by directed mutagenesis via PCR amplification. PCR Thermocycler Biometra® TGradient and a KOD Hot Start Master Mix (Novagen®) were used according to the manufacturer instructions for this purpose. The PCR product was then purified using QIAquick PCR Purification Kit (Qiagen) and digested with KpnI and XbaI to obtain an insert compatible with the empty vectors. Finally, the ligation between the empty vectors and insert was performed using a T4 DNA ligase (Thermo Scientific) and a 5:1 insert vector molar ratio. The ligation reaction was accomplished after 1hour incubation at 22˚C. The correct construction of those plasmids was first verified by agarose gel electrophoresis and further confirmed by sequencing using primers for CMV (StabVida). The overall procedure followed for the construction of VEGF-containing plasmids is schematized on Figure II.2.

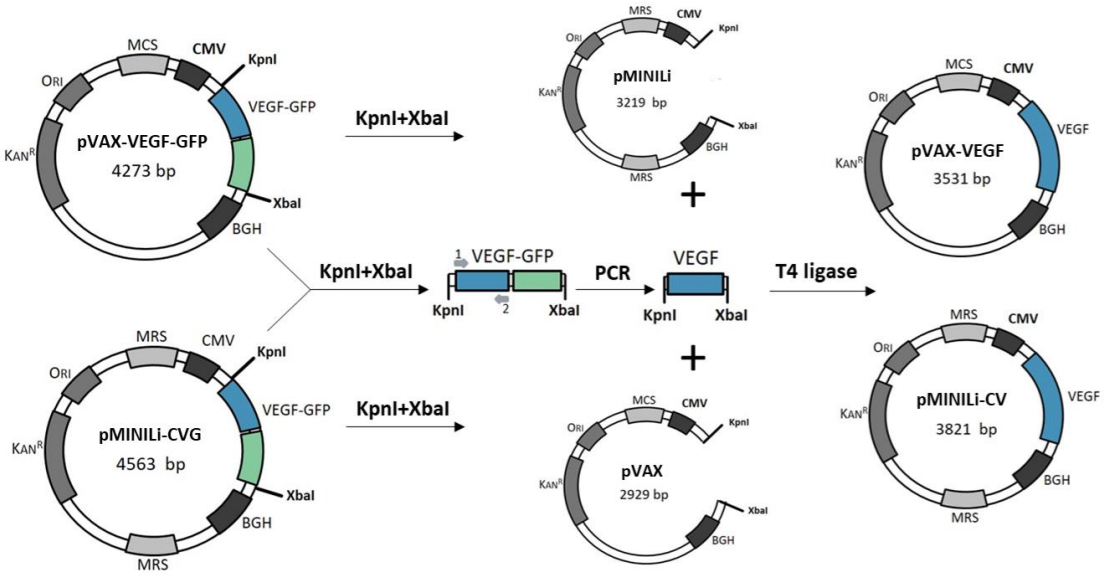

Figure II.2 - Schematization of the overall procedure used for construction of pVAX-VEGF and pMINILi-CV from pVAX-VEGF-GFP and pMINILi-CVG by removal of GFP using restriction enzyme digestion. Site directed mutagenesis and PCR amplification were used for insertion of a recognition site for XbaI and a stop codon into the VEGF insert. The ligation between this insert and empty plasmids was performed by T4 ligase.

II.3.3. Bacterial strain

Escherichia coli (E. coli) strains were transformed with plasmids of interest by heat shock. The pVAX plasmids were produced within the DH5α strain, a common *E. coli* strain used for plasmid production. The pMINILi constructs, on the other hand, were amplified within *E. coli* B2WP. This strain was obtained from BW27783 (The Coli Genetic Stock Center at Yale), an *E. coli* strain improved for arabinose uptake, after disruption of *EndA* and *RecA* genes and insertion of P_{BAD}/araC-parA cassette, which contains the ParA resolvase gene under a P_{BAD} promoter with an

optimized ribosome binding site and the AraC repressor gene in opposite direction (Simcikova et al., 2014, Alves et al., 2016).

II.3.4. Plasmid and minicircle production

The first step for large-scale plasmid production within bacteria was the preparation of pre-inoculum by inoculating a loop of frozen DH5α or BW2P cells harboring the plasmids of interest into 15 mL tubes with 5 mL of LB medium (Sigma) supplemented with 30 µg/mL kanamycin (Amresco). In the case of pMINILi, 0.5% (w/v) of glucose was also added to the pre-inoculum to repress P_{BAD} cassette and prevent leaky expression of ParA. Pre-inoculum for both strains was incubated overnight at 37ºC and 250 rpm. Then, an inoculum with 30 mL of LB supplemented with 30 µg/mL kanamycin and 0.5% glucose (only in the case of BW2P strain) was prepared in 100 mL shake flasks. The pre-inoculum optical density at 600 nm (OD_{600}) was measured and the amount required to adjust the initial OD_{600} of inoculum to 0.1 was used to inoculate the 100 mL flasks. *E. coli* strains were cultured at 37ºC and 250 rpm until an OD_{600} of ≈2.5 is reached. Then, 2 L shake flaks with 500 mL of LB and 30 µg/mL kanamycin were prepared. DH5α from inoculum were used to directly seed the 2 L flasks with an OD_{600} of 0.1 and were cultured at 37ºC and 250 rpm until reach the early stationary phase (≈5 h, OD_{600}≈5). After that, the medium was removed by centrifugation and the DH5α pellet was frozen at -20ºC until further analysis. For the BW2P strain, the volume of inoculum corresponding to an initial OD_{600} of 0.1 in 2 L flasks was centrifuged to remove glucose and the pellet was ressuspended and added to the 500 mL of LB with kanamycin and cultured at 37ºC and 250 rpm until late exponential phase (OD_{600} = 2.5-3). At this point, 0.01% (w/v) of L-(+)Arabinose (Merck) is added to the culture to induce PP recombination and generation of MC and MP. After 2 h of recombination, culture medium was removed by centrifugation and cell pellets were kept at -20ºC until further processing. Samples of the culture were retrieved at 0, 1 or 2 hours after recombination for analysis of recombination efficiency.

II.3.5. Analysis of recombination efficiency

Analysis of recombination efficiency was calculated using ImageJ software, as previously described (Alves et al., 2016). Briefly, the total pDNA from samples collected 2 h after recombination was purified using High Pure Plasmid Isolation Kit (Roche) and the resulting DNA was digested with SacII restriction enzyme, which has only one recognition site in PP (and MP). The digested samples were then run on an agarose gel electrophoresis and the intensity of the

bands, which is proportional to the quantity of linearized PP and MP present on the gel, was used to estimate the recombination efficiency.

II.3.6. Primary purification and nuclease digestion of the miniplasmid

For plasmid isolation from bacterial cultures, the pellets were thawed and purified using an endotoxin-free plasmid DNA purification kit (Macherey-Nagel). After this primary purification, pVAX plasmids are considered purified and ready to be transfected into mammalian cells. However, for minicircles, further purification steps are required. The first of these steps is the digestion of total nucleic acid solution obtained after kit purification with *Nb.B*bvCI (New England Biolabs). This nicking enzyme converts the supercoiled forms of PP and MP into the corresponding open circular forms, leaving supercoiled MC intact. This step is extensively described elsewhere (Alves et al., 2016, Alves et al., 2018).

II.3.7. Minicircle purification by hydrophobic interaction chromatography

After nuclease digestion, the solution containing MC is conditioned with 2.5 M of ammonium sulfate before the purification. This step not only facilitates the correct isoform separation during the chromatographic process, but also promotes the salting-out of *Nb.B*bvCI reducing its content from the solution (Alves et al., 2018). Then, supercoiled MC is isolated from other isoforms in the solution by hydrophobic interaction chromatography as described by Alves and colleagues (Alves et al., 2016, Alves et al., 2018). Briefly, a Tricorn 10/100 column (GE Healthcare) packed with 10 mL of PhenylSepharose 6 Fast Flow resin was used in connection to an ÄKTApurifier100 system (GE Healthcare). To promote elution of different DNA isoforms, different amounts of buffer A (2.2 M Ammonium Sulfate in 10 mM Tris–HCl, 1 mM EDTA, pH 8) and buffer B (10 mM Tris–HCl, 1 mM EDTA pH 8) were used in the mobile phase and a flow rate of 2 mL/min was set. Before the administration of nucleic acid solution, the column was equilibrated with 3 column volumes (CV) of 17% buffer B. Then, the MC-containing solution was injected and the column was washed with 4CV of 17% buffer B to remove the unbound material. Elution steps were then performed starting with an amount of buffer B of 35% (2CV) followed by an increasing to 100% (2CV). The absorbance at 254 nm of eluate was continuously measured with an UV detector positioned at the column outlet and eluate fractions of 1.5 mL were collected during the run.

After chromatographic purification, the peak fractions were then analyzed in an agarose gel electrophoresis. The fractions where a band corresponding to supercoiled MC was found were collected and processed in 2 mL Amicon® Ultra-2 30k (Merk Millipore), according to the respective protocol. This diafiltration step was performed to remove the salt and concentrate the sample. After this stage, MC solution is ready to be used for mammalian cell transfection studies (as described on Chapters III, IV, V and VI). The MC were kept in milli-Q water at 4ºC until further use.

II.4. Results and Discussion

II.4.1. Construction of plasmids enclosing the VEGF gene: pVAX-VEGF and pMINILi-CV

Plasmids with VEGF gene alone were constructed from pVAX-VEGF-GFP and pMINILi-CVG through replacement of complete VEGF-GFP region by a VEGF gene obtained by PCR amplification. To obtain the empty vectors for VEGF cloning, restriction enzyme digestion was performed. The agarose gels presented on Figure II.3 show the digestion of pMINILi-CVG (A) and pVAX-VEGF-GFP (B) with XbaI and KpnI restriction enzymes, as well as the product of PCR amplification. The bands showed in Figure II.3 confirmed the correct digestion or amplification of the constructs, since all have the expected molecular weight. The empty pVAX and pMINILi vectors are supposed to have 2929 bp and 3219 bp, respectively and VEGF gene length after PCR and enzyme digestion is expected to be 687 bp, which is in accordance to the data obtained on the agarose gel. The correct insertion of VEGF gene into empty vectors and, thus, the successful construction of pVAX-VEGF and pMINILi-CV was confirmed by sequencing.

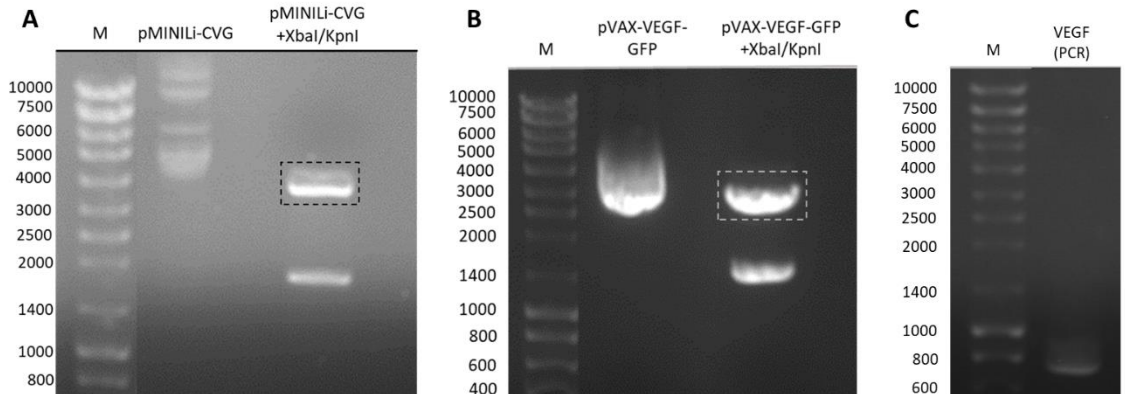

Figure II.3 – Agarose gel electrophoresis analysis of pMINILi-CVG (A) and pVAX-VEGF-GFP (B) before and after digestion with KpnI and XbaI restriction enzymes. The dashed lines show the empty vectors that were cut and extracted from the gel and used to insert VEGF gene represented in C. The VEGF insert was obtained by PCR amplification of VEGF-GFP fusion gene using specific primers for VEGF region. The reverse primer was designed to introduce a stop codon and recognition site for XbaI on the VEGF amplicon. M – Molecular weight marker (NZYDNA Ladder III, Nzytech).

The use of vectors containing fluorescent reporter genes, such as GFP, might be a useful strategy to evaluate *in vitro* or *in vivo* gene expression, since they can be easily detected and quantified by simple assays as fluorescent microscopy or flow cytometry, respectively (Kain et al., 1995).

The use of GFP and other fluorescent proteins as reporters of gene expression has many applications in MSC engineering, including on optimization of transfection/transduction protocols (McMahon et al., 2006, Madeira et al., 2011), selection of best promoters and other regulatory sequences (Qin et al., 2010) or detection of protein localization after gene transfer

(Cao et al., 2016). Also, construction of transgenes where the therapeutic factor is in fusion with a reporter protein has also been a popular strategy used in the past decades for quantification of gene expression by simple imaging techniques. Nevertheless, analysis at mRNA level revealed that in some cases this might not be the best approach (Belancio, 2011). Indeed, the use of an intracellular protein for monitoring the expression of proteins that are supposed to be secreted to the extracellular medium, as is the case of VEGF, is far from being the ideal approach. However, there are some studies demonstrating the effective use of VEGF-GFP constructs to induce VEGF overexpression in mice models for different diseases (Rakoczy et al., 2003, Dall'Era et al., 2008). Despite these studies, it was observed that the use of VEGF-GFP expression cassette may affect VEGF expression and GFP quantification does not reflect the VEGF expression when this gene is transfected alone, as it will be demonstrated in the next chapters of present thesis (Chapters III and IV). Moreover, it is not feasible to use a vector containing a reporter gene in human clinical approaches. Hence, the construction of vectors free from GFP, containing only the therapeutic VEGF gene, was a mandatory step in the scope of this work.

II.4.2. Bacterial growth and plasmid production

After bacterial transformation, the four plasmids were replicated within DH5α (pVAX) or BW2P (pMINILi) *E. coli* strains. Pre-inocula from 15 mL tubes were used to inoculate the 100 mL shake flasks (30 mL of LB medium) with a starting OD_{600}=0.1. Those inocula were grown up to mid-exponential phase (OD_{600}≈3). At this point, they were used to inoculate the 2 L shake flasks (with 500 mL of LB). The initial OD600 for these growths was set as 0.1 and samples of each culture were collected at different timepoints to obtain the growth curves represented in Figure II.4. In Figures II.4 A and C are represented the inoculum and growth of BW2P harboring pMINILi-CVG and pMINILi-CV, respectively. The dashed lines represent the period where L-(+)Arabinose was added to the culture medium to induce PP recombination (OD_{600}≈2.5-3). The growth curves for DH5α transformed with pVAX-VEGF-GFP (B) or pVAX-VEGF (D) are shown on the right side of the Figure II.4 are shown. All the bacteria display the typical *E.coli* growth curve and no significant differences were observed in growth profiles, regardless the strain (BW2P vs. DH5α) or the inserted plasmid (pMINILi vs. pVAX) used.

LB medium supplemented with kanamycin was used to culture both strains, since all the plasmids have the kanamycin resistance gene. Glucose was also added to the medium in BW2P cultures to repress any residual pBAD/AraC activity and leaky expression of ParA resolvase that might promote an early recombination of PP (Mayrhofer et al., 2008, Simcikova et al., 2014,

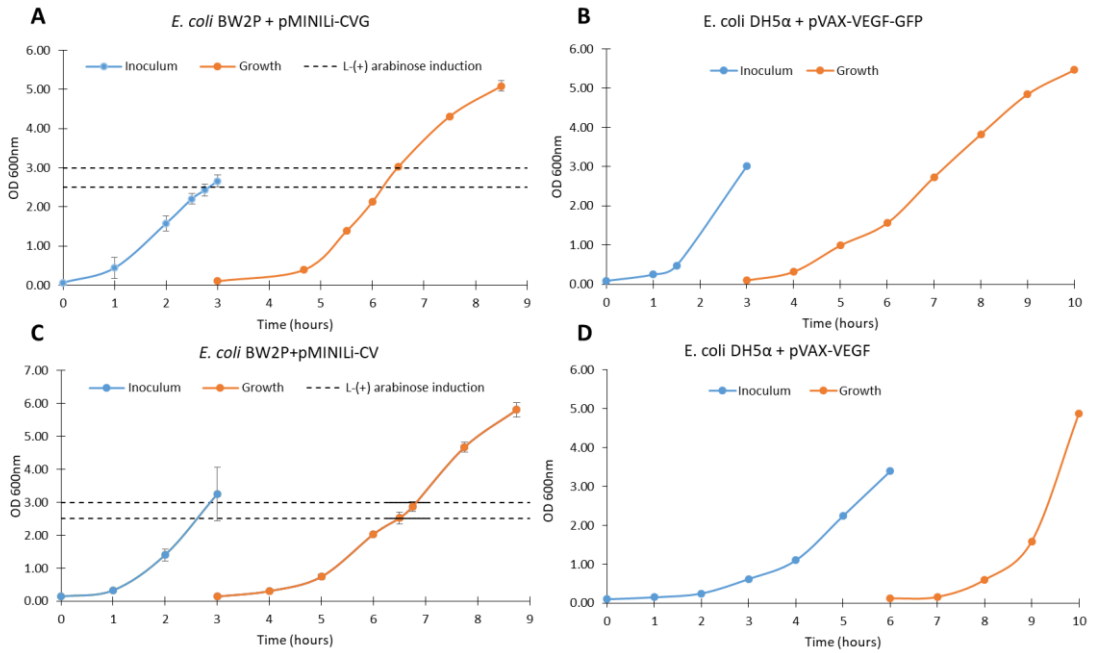

Figure II.4 - Growth curves for *E. coli* strains BW2P (**A** and **C**) and DH5α (**B** and **D**) transformed with pVAX or pMINILi, respectively. On the upper part of the figure (A and B) are represented the growth curves of bacteria transformed with plasmids containing the VEGF-GFP fusion gene, while on the bottom (C and D) are shown the growth curves of E. coli harboring the plasmids with VEGF gene alone. The blue lines show the inoculum phase and the orange ones represent the growth phase. The dashed lines on BW2P culture represent the addition of L-arabinose to induce plasmid recombination that was performed at an OD_{600} between 2.5 and 3.

Guzman et al., 1995). The activation of pBAD cassette was then induced at late exponential phase by addition of L-arabinose to the culture medium. This induces the expression of *ParA* gene and, thus, the production of ParA resolvase. This enzyme will then catalyze the intramolecular recombination between the two MRS of pMINILi. As a result of this process, two distinct DNA molecules are generated, a MC with the expression cassette containing the gene of interest (VEGF-GFP or VEGF), and a MP with prokaryotic backbone sequences. To assure a complete recombination, BW2P were cultured for two additional hours after inducing recombination. Culture samples were collected for total pDNA isolation just before the onset of recombination (0 h) and 1 or 2 h after recombination. The total pDNA obtained from those samples was analyzed by agarose gel electrophoresis (Figure II.5). This Figure shows that before recombination (0h) only the bands corresponding to PP are observed, confirming that no early recombination occurred before the addition of L-arabinose. However, after inducing recombination, MC and MP were produced as demonstrated by the appearance of two bands (1 h and 2 h). The fact that PP is absent after recombination (no band is observed in the PP region) means that all PP was converted into MC and MP. So, a recombination efficiency of 100% was observed for both pMINILi-CVG and pMINILi-CVGN. These results were in accordance with previous works from BERG-iBB and other groups where recombination efficiencies >96% have

been obtained using this recombination system (Mayrhofer et al., 2008, Simcikova et al., 2014, Alves et al., 2016).

It should be noted that in Figure II.5A the MC has a higher molecular weight (2457 bp) than MP (2106 bp), so the MC band appears above. When GFP is removed, the MC molecular weight is reduced (1718 bp) and is lower that the observed for MP. The corresponding band hence appears below the MP band in Figure II.5B.

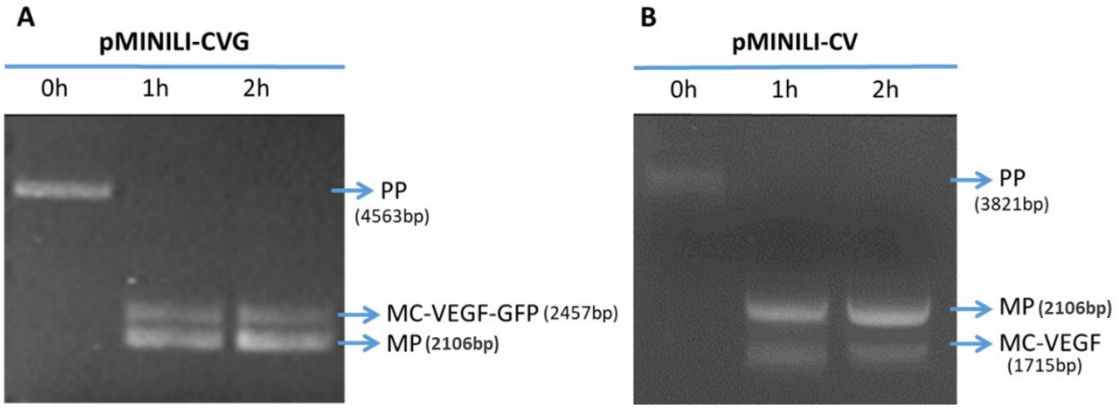

Figure II.5 - Agarose gel electrophoresis analysis of total pDNA obtained from E.coli BW2P cultures before recombination (0 h) or 1 h or 2 h after recombination. At 0 h only one band is observed corresponding to parental plasmid (PP) – pMNILi-CVG (A) or pMINILi-CV (B). 1 or 2 h after L-arabinose addition (recombination) two bands are shown, representing miniplasmid (MP) and minicircle (MC).

II.4.3. Primary purification and hydrophobic interaction chromatography (HIC)

To purify total plasmid from bacterial cultures, a commercial kit that assures an endotoxin-free purification was used. It is known that lipopolyssacharides (LPS) present in the outer membrane of Gram-negative bacteria, also called "endotoxins", may be contaminants of pDNA preparations. The presence of LPS on DNA solutions has been associated with lower transgene expressions after transfection and might also be involved in non-specific activation of the immune system (Weber et al., 1995). So, the use of endotoxin-free approached for DNA purification is extremely important for gene therapy applications.

After this primary purification, pVAX plasmids (pVAX-VEGF-GFP and pVAX-VEGF) are ready to be used for transfection into mammalian cells, as MSC. In case of MC, however, further purification steps are required to remove MP and some remaining, un-recombined PP that might be in the solution. The first step towards MC purification is a digestion step with the *Nb.B*bvCI restriction enzyme, which is responsible for nicking only one of the strands of DNA instead of cleaving it (Heiter et al., 2005). A recognition site for this enzyme was strategically placed in the bacterial backbone sequence of pMINILi that, after the recombination step, stays on MP (Alves et al.,

2016). Thus, the nicking of one of the DNA strands on supercoiled MP and PP induces the conversion of both into the corresponding open circular forms. MC has no recognition sites for this enzyme, so it will remain in the supercoiled state. The analysis of DNA solution on an agarose gel electrophoresis before and after nicking is shown in Figure II.6. This digestion step contributes to increase the differences in hydrophobicity between PP, MP species and MC species, thus, facilitating the chromatographic purification by HIC. In fact, the use of HIC for isolation of supercoiled DNA from other isoforms has already been described by several authors (Bo et al., 2013, Urthaler et al., 2005). An intermediary conditioning step with ammonium sulfate was performed before the chromatographic process to prepare the sample for HIC and reduce the amount of restriction enzyme by salting-out.

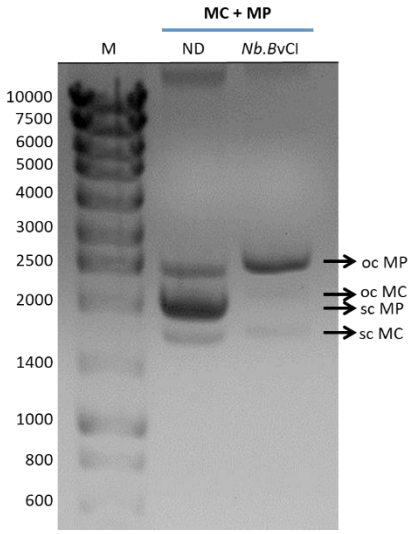

Figure II.6 – Agarose gel electrophoresis analysis of a solution containing a mixture of MC and MP before (ND) and after digestion with Nb.BvCI. ND – Non-digested.

After the pre-conditioning step, the solution containing MC is loaded onto a phenyl-Sepharose column pre-equilibrated with 17% buffer B, according to the procedure described in Materials and Methods. The elution is then performed by steps: an initial washing step with 17% buffer B to remove the unbound material followed by elution of supercoiled MC that is promoted by an increasing the amount of buffer B to 35%. Finally, some RNA and any other bound molecules are eluted in the last step with 100% of buffer B. The resulting chromatograms (A and B) as well as agarose gel electrophoresis of the samples collected during the elution steps (C and D) are shown in Figure II.7 (A/C-pMINILi-CVG, B/D-pMINILi-CV). The chromatograms are characterized by three mains peaks corresponding to different amounts of buffer B. The first, early-eluting (17% of buffer B) fractions (3-9) contain essentially open circular MP, as it can be observed by the band in the gel. Other DNA molecules (e.g. genomic DNA fragments) that did not bind to the resin under the conditions used may also be present. The increase of buffer B to 35% promotes

the elution of supercoiled MC as shown by the second peak of the chromatogram and corresponding bands on the gel (fractions 35-39). Finally, strongly bound material is eluted with 100% of buffer B as a smaller third peak. No corresponding bands are detected in the gel and thus the components in this fraction cannot be correctly identified. However, the faint blur that is seen on the low molecular weight region of fraction 54 from Figure II.7C is typical of RNA. This is also consistent with previous results, which show that RNA is the major constituent of fractions eluting with 100% of buffer B (Alves et al., 2016). So, combination of HIC, a technique that effectively isolates supercoiled DNA from other topoisomers (Bo et al., 2013, Urthaler et al., 2005), with a digestion step using a nicking enzyme (*Nb.B*bvCI), that further contributes to improve differences in hydrophobicity, is able to generate supercoiled MC that are free from other DNA molecules (Figure II.7). These results are in accordance with the data published by Alves *et al.* (Alves et al., 2016). After the chromatographic step and agarose gel analysis, all fractions containing MC were collected and diafiltered to remove the salt and concentrate the samples. After the diafiltration step, the MC are pure and ready to be used in transfection studies with MSC or other mammalian cell types.

The separation of different DNA topoisomers using HIC columns is possible due to the preferential and stronger interaction of the ligands with the bases of supercoiled isoforms than

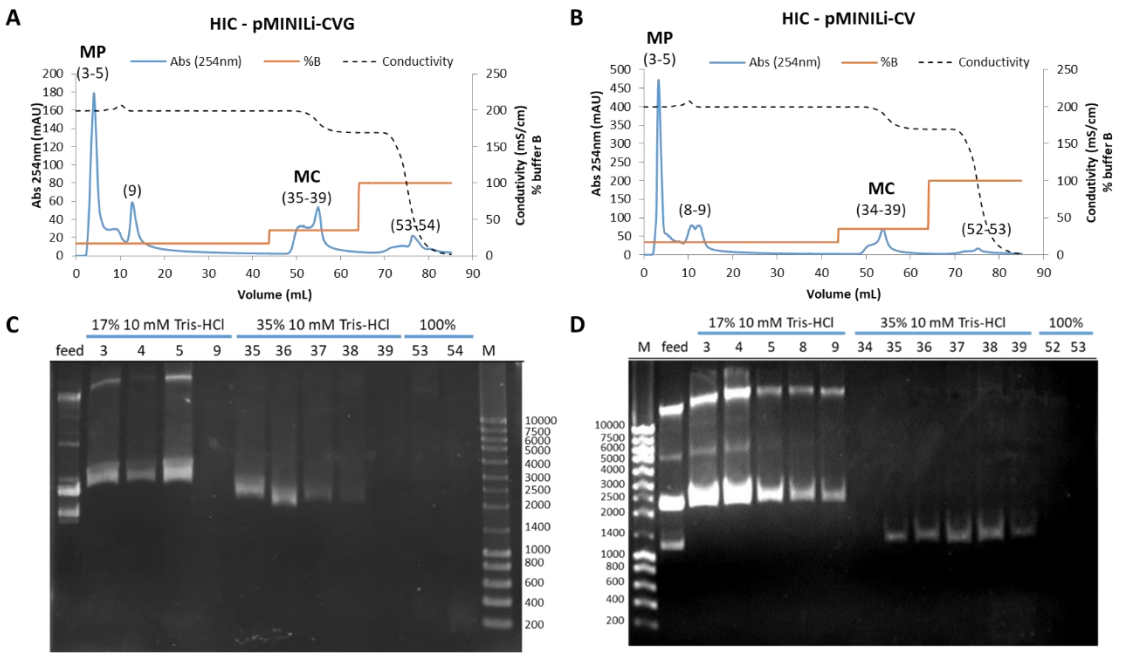

Figure II.7 – A, **B** - Chromatograms (blue lines) of pre-purified samples (MC + MP) from pMINILi-CVG (A) or pMINILi-CV (B) obtained after digestion with Nb.BbvCI loaded onto a phenyl-Sepharose column pre-equilibrated with 1.83 M ammonium sulphate (17% buffer B). Stepwise elution (% buffer B – orange) was performed at 2 mL/min with 4 CV of 17% buffer B, 2 CV of 35% buffer B (1.43 M) and 2 CV of 100% buffer B (0 M). Numbers over peaks correspond to collected fractions. CV – column volume. **C**, **D** - Agarose gel electrophoresis analysis of fractions collected during the chromatographic runs shown in A and B. The numbers above the lanes correspond to collected fractions.

with the bases of open circular ones. The use of high salt concentrations for pre-conditioning and in first elution steps contributes to further expose those bases, increasing the affinity between the ligand and supercoiled MC (Prazeres, 2011).

The use of a chromatographic step with a conventional and commercially available phenyl-Sepharose column, represents an advantage over the method described by Mayrhofer and colleagues for MC isolation, which requires a tailor-made affinity adsorbent (Mayrhofer et al., 2008). Also, their purification method involves the insertion of additional sequences on MC that are not needed for its action. Herein, the changes required (the insertion of a recognition site for *Nb.B*vCI) were performed within the MP instead, which is discarded after the purification. On the other side, Kay and co-workers also took advantage of an endonuclease (I-SceI) restriction site placed on MP for MC purification (Kay et al., 2010). However, the digestion step described takes place *in vivo*, in contrast to the herein described system, where the digestion is performed *in vitro*. Thus, the present method offers two main advantages: *in vitro* digestion is easier to control and scale-up and does not impose and extra metabolic load on producing bacteria. Furthermore, the HIC approach allows also the removal of other impurities than MP, including some open circular MC and residual RNA. It is well described that DNA molecules in supercoiled form are more effective for cell transfection than the other isoforms (Remaut et al., 2006, Sousa et al., 2009). Thus, it is of great interest to have a solution with pure supercoiled MC free from other isoforms.

II.5. Conclusions

In this chapter, the successful construction of two DNA molecules that will be applied further in the context of present thesis was achieved. Despite the use of a GFP reporter might be useful in the initial optimization steps, its removal is mandatory for the clinical application of these vectors. The complete purification of transfection-graded conventional plasmids (pVAX) was also effectively performed using a commercial kit. However, the production and purification of MC vectors is much more challenging.

Herein, a recently developed method for isolation and purification of transfection-graded MC is described (Alves et al., 2016, Alves et al., 2018). The absence of PP after recombination step confirmed the efficiency of the recombination process triggered by parA resolvase, whose expression is induced by L-arabinose addition. In fact, the addition of the inducer in one of the crucial steps of the recombination process. Herein, the induction was performed at the end of exponential growth phase, in order to maximize the final MC yield (Gaspar et al., 2014). The use of the BW2P strain further contributed to the efficiency of the process. Besides the inclusion of the parA gene under the control of pBAD/AraC promoter, this strain was specifically designed towards improved arabinose uptake. Both aspects contributed to the increase in the efficiency of recombination process. The inclusion of a digestion step using a nicking enzyme is a key aspect of the MC isolation method used. *Nb.B*vCI cleavage causes the relaxation of MP but has no effect on MC, which remains in the supercoiled form. Several authors described the separation of different DNA topoisomers using chromatographic strategies (Bo et al., 2013, Urthaler et al., 2005). Thus, the use of a HIC column with different elution steps with decreasing concentrations of ammonium sulphate was able to efficiently separate supercoiled MC from the other DNA topoisomers, including the open circular MP. The herein produced MC are free from other nucleic acid impurities and suitable for transfection of mammalian cells, namely MSC (Serra et al., 2018).

II.6. References

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2016. Development of a nicking endonuclease-assisted method for the purification of minicircles. *Journal of Chromatography A,* 1443**,** 136-144.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2018. Production and Purification of Supercoiled Minicircles by a Combination of *in vitro* Endonuclease Nicking and Hydrophobic Interaction Chromatography. *Human Gene Therapy Methods,* (in press).

AZZONI, A. R., RIBEIRO, S. C., MONTEIRO, G. A. & PRAZERES, D. M. F. 2007. The impact of polyadenylation signals on plasmid nuclease-resistance and transgene expression. *The Journal of Gene Medicine,* 9**,** 392-402.

BELANCIO, V. P. 2011. Importance of RNA analysis in interpretation of reporter gene expression data. *Anal Biochem,* 417**,** 159-61.

BO, H., WANG, J., CHEN, Q., SHEN, H., WU, F., SHAO, H. & HUANG, S. 2013. Using a single hydrophobic-interaction chromatography to purify pharmaceutical-grade supercoiled plasmid DNA from other isoforms. *Pharm Biol,* 51**,** 42-8.

BOURA, J. S., VANCE, M., YIN, W., MADEIRA, C., LOBATO DA SILVA, C., PORADA, C. D. & ALMEIDA-PORADA, G. 2014. Evaluation of gene delivery strategies to efficiently overexpress functional HLA-G on human bone marrow stromal cells. *Mol Ther Methods Clin Dev,* 2014.

CAO, J., HOU, S., DING, H., LIU, Z., SONG, M., QIN, X., WANG, X., YU, M., SUN, Z., LIU, J., SUN, S., XIAO, P., LV, Q. & FAN, H. 2016. In Vivo Tracking of Systemically Administered Allogeneic Bone Marrow Mesenchymal Stem Cells in Normal Rats through Bioluminescence Imaging. *Stem Cells Int,* 2016**,** 3970942.

CHEN, Z. Y., HE, C. Y., EHRHARDT, A. & KAY, M. A. 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther,* 8**,** 495-500.

CUENDE, N., RASKO, J. E. J., KOH, M. B. C., DOMINICI, M. & IKONOMOU, L. 2018. Cell, tissue and gene products with marketing authorization in 2018 worldwide. *Cytotherapy,* 20**,** 1401-1413.

DALL'ERA, J. E., MEACHAM, R. B., MILLS, J. N., KOUL, S., CARLSEN, S. N., MYERS, J. B. & KOUL, H. K. 2008. Vascular endothelial growth factor (VEGF) gene therapy using a nonviral gene delivery system improves erectile function in a diabetic rat model. *Int J Impot Res,* 20**,** 307-14.

DARQUET, A. M., CAMERON, B., WILS, P., SCHERMAN, D. & CROUZET, J. 1997. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther,* 4**,** 1341-9.

DARQUET, A. M., RANGARA, R., KREISS, P., SCHWARTZ, B., NAIMI, S., DELAERE, P., CROUZET, J. & SCHERMAN, D. 1999. Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer. *Gene Ther,* 6**,** 209-18.

DIETZ, W. M., SKINNER, N. E., HAMILTON, S. E., JUND, M. D., HEITFELD, S. M., LITTERMAN, A. J., HWU, P., CHEN, Z. Y., SALAZAR, A. M., OHLFEST, J. R., BLAZAR, B. R., PENNELL, C. A. & OSBORN, M. J. 2013. Minicircle DNA is superior to plasmid DNA in eliciting antigen-specific CD8+ T-cell responses. *Mol Ther,* 21**,** 1526-35.

FERRARA, N. 2001. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol,* 280**,** C1358-66.

GASPAR, V. M., MAIA, C. J., QUEIROZ, J. A., PICHON, C., CORREIA, I. J. & SOUSA, F. 2014. Improved minicircle DNA biosynthesis for gene therapy applications. *Hum Gene Ther Methods,* 25**,** 93-105.

GUZMAN, L. M., BELIN, D., CARSON, M. J. & BECKWITH, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol,* 177**,** 4121-30.

HACEIN-BEY-ABINA, S., VON KALLE, C., SCHMIDT, M., LE DEIST, F., WULFFRAAT, N., MCINTYRE, E., RADFORD, I., VILLEVAL, J. L., FRASER, C. C., CAVAZZANA-CALVO, M. & FISCHER, A. 2003. A serious

adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med.* United States.

HÄCKER, G., REDECKE, V. & HÄCKER, H. 2002. Activation of the immune system by bacterial CpG-DNA. *Immunology,* 105**,** 245-51.

HARDEE, C. L., AREVALO-SOLIZ, L. M., HORNSTEIN, B. D. & ZECHIEDRICH, L. 2017. Advances in Non-Viral DNA Vectors for Gene Therapy. *Genes (Basel),* 8.

HEITER, D. F., LUNNEN, K. D. & WILSON, G. G. 2005. Site-specific DNA-nicking mutants of the heterodimeric restriction endonuclease R.BbvCI. *J Mol Biol,* 348**,** 631-40.

JECHLINGER, W., AZIMPOUR TABRIZI, C., LUBITZ, W. & MAYRHOFER, P. 2004. Minicircle DNA immobilized in bacterial ghosts: in vivo production of safe non-viral DNA delivery vehicles. *J Mol Microbiol Biotechnol,* 8**,** 222-31.

KAIN, S. R., ADAMS, M., KONDEPUDI, A., YANG, T. T., WARD, W. W. & KITTS, P. 1995. Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques,* 19**,** 650-5.

KAUFMANN, K. B., BUNING, H., GALY, A., SCHAMBACH, A. & GREZ, M. 2013. Gene therapy on the move. *EMBO Mol Med,* 5**,** 1642-61.

KAY, M. A., HE, C. Y. & CHEN, Z. Y. 2010. A robust system for production of minicircle DNA vectors. *Nat Biotechnol,* 28**,** 1287-9.

MADEIRA, C., RIBEIRO, S. C., PINHEIRO, I. S. M., MARTINS, S. A. M., ANDRADE, P. Z., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Gene delivery to human bone marrow mesenchymal stem cells by microporation. *Journal of Biotechnology,* 151**,** 130-136.

MADEIRA, C., RODRIGUES, C. A. V., REIS, M. S. C., FERREIRA, F. F. C. G., CORREIA, R. E. S. M., DIOGO, M. M. & CABRAL, J. M. S. 2013. Nonviral Gene Delivery to Neural Stem Cells with Minicircles by Microporation. *Biomacromolecules,* 14**,** 1379-1387.

MARSHALL, E. 1999. Gene therapy death prompts review of adenovirus vector. *Science,* 286**,** 2244-5.

MAYRHOFER, P., BLAESEN, M., SCHLEEF, M. & JECHLINGER, W. 2008. Minicircle-DNA production by site specific recombination and protein-DNA interaction chromatography. *J Gene Med,* 10**,** 1253-69.

MAYRHOFER, P. & IRO, M. 2012. Minicircle-DNA. *In:* THALHAMER, J., WEISS, R. & SCHEIBLHOFER, S. (eds.) *Gene Vaccines.* Springer-Verlag.

MCMAHON, J. M., CONROY, S., LYONS, M., GREISER, U., O'SHEA, C., STRAPPE, P., HOWARD, L., MURPHY, M., BARRY, F. & O'BRIEN, T. 2006. Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors. *Stem Cells Dev,* 15**,** 87-96.

PORADA, C. D. & ALMEIDA-PORADA, G. 2010. Mesenchymal stem cells as therapeutics and vehicles for gene and drug delivery. *Advanced Drug Delivery Reviews,* 62**,** 1156-1166.

PRATHER, K. J., SAGAR, S., MURPHY, J. & CHARTRAIN, M. 2003. Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. *Enzyme and Microbial Technology,* 33**,** 865-883.

PRAZERES, D. 2011. *Plasmid Biopharmaceuticals: Basics, Applications and Manufacturing*.

QIN, J. Y., ZHANG, L., CLIFT, K. L., HULUR, I., XIANG, A. P., REN, B. Z. & LAHN, B. T. 2010. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One,* 5**,** e10611.

RAKOCZY, P. E., BRANKOV, M., FONCECA, A., ZAKNICH, T., RAE, B. C. & LAI, C. M. 2003. Enhanced recombinant adeno-associated virus-mediated vascular endothelial growth factor expression in the adult mouse retina: a potential model for diabetic retinopathy. *Diabetes,* 52**,** 857-63.

RAMAMOORTH, M. & NARVEKAR, A. 2015. Non viral vectors in gene therapy- an overview. *J Clin Diagn Res,* 9**,** Ge01-6.

REMAUT, K., SANDERS, N. N., FAYAZPOUR, F., DEMEESTER, J. & DE SMEDT, S. C. 2006. Influence of plasmid DNA topology on the transfection properties of DOTAP/DOPE lipoplexes. *J Control Release,* 115**,** 335-43.

SAWAMURA, D., ABE, R., GOTO, M., AKIYAMA, M., HEMMI, H., AKIRA, S. & SHIMIZU, H. 2005. Direct injection of plasmid DNA into the skin induces dermatitis by activation of monocytes through toll-like receptor 9. *J Gene Med,* 7**,** 664-71.

SERRA, J., ALVES, C. P. A., BRITO, L., MONTEIRO, G. A., CABRAL, J. M. S., PRAZERES, D. M. F. & DA SILVA, C. L. 2018. Engineering of Human Mesenchymal Stem/Stromal Cells with Vascular Endothelial Growth Factor-Encoding Minicircles for Angiogenic Ex Vivo Gene Therapy. *Hum Gene Ther*.

SIMCIKOVA, M., PRATHER, K. L. J., PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. On the dual effect of glucose during production of pBAD/AraC-based minicircles. *Vaccine,* 32**,** 2843-2846.

SINGER, N. G. & CAPLAN, A. I. 2011. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol,* 6**,** 457-78.

SOUSA, F., PRAZERES, D. M. & QUEIROZ, J. A. 2009. Improvement of transfection efficiency by using supercoiled plasmid DNA purified with arginine affinity chromatography. *J Gene Med,* 11**,** 79-88.

URTHALER, J., BUCHINGER, W. & NECINA, R. 2005. Industrial Scale cGMP Purification of Pharmaceutical Grade Plasmid-DNA. *Chemical Engineering & Technology,* 28**,** 1408-1420.

WALKER, W. E., BOOTH, C. J. & GOLDSTEIN, D. R. 2010. TLR9 and IRF3 Cooperate to Induce a Systemic Inflammatory Response in Mice Injected With Liposome:DNA. *Mol Ther.*

WANG, W., LI, W., MA, N. & STEINHOFF, G. 2013. Non-viral gene delivery methods. *Curr Pharm Biotechnol,* 14**,** 46-60.

WEBER, M., MÖLLER, K., WELZECK, M. & SCHORR, J. 1995. Short technical reports. Effects of lipopolysaccharide on transfection efficiency in eukaryotic cells. *BioTechniques,* 19**,** 930-940.

Chapter III

GENETIC ENGINEERING OF MESENCHYMAL STEM/STROMAL CELLS (MSC) WITH VEGF-GFP-ENCODING VECTORS

III. GENETIC ENGINEERING OF MESENCHYMAL STEM/STROMAL CELLS (MSC) WITH VEGF-GFP-ENCODING VECTORS

III.1. Summary

Mesenchymal stem/stromal cells (MSC) have unique therapeutic features that could be further enhanced by genetic engineering approaches using therapeutic factors. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that may be delivered into MSC in order to improve their healing properties in the context of ischemic diseases. Despite the proven effectiveness of viral vectors in gene therapy approaches, these bring some safety concerns. To overcome this issue, non-viral plasmid-based vectors have been developed on past years. In the present work, MSC were modified with minicircles, a particular type of non-viral vectors optimized towards an improved transfection and reduced cytotoxicity. Transfection-grade minicircles containing the VEGF gene in fusion with green fluorescent protein (GFP) – MC-VEGF-GFP – were produced and purified according to a recently developed protocol and used for MSC transfection. This was performed using an optimized and validated microporation protocol. Conventional plasmids (pVAX) containing the VEGF-GFP fusion gene, or GFP alone, were used as controls. GFP expression, evaluated by flow cytometry, was achieved in 66.8% ± 5.9% of the cells after pVAX-GFP delivery, confirming the efficacy of the transfection protocol. On the other hand, a higher GFP expression was observed in cells microporated with pVAX-VEGF-GFP (12.1% ± 3.1%) compared to cells transfected with MC-VEGF-GFP (3.1% ± 1.2%). These results were further confirmed by western blot analysis of cell lysates. VEGF expression was measured by real time PCR and similar results were observed. MSC transfected with pVAX-VEGF-GFP showed VEGF expression levels 13.5 ± 1.7-fold higher than control MSC (non-transfected), whereas the condition with minicircle resulted in a 9.5 ± 2.0-fold increase in VEGF expression. The plasmid vectors lead to higher production of GFP and VEGF by the transfected cells than minicircles, against the expectations. Although further studies are required to confirm these results, this preliminary analysis revealed that fusion with GFP might not be the best strategy to monitor VEGF expression in transfection studies with MSC. Therefore, in the following chapters, constructs containing only the gene of interest (VEGF) will be studied.

III.2. Background

Human mesenchymal stem/stromal cells (MSC) are multipotent stem cells with unique properties. MSC have the ability to self-renew maintaining an undifferentiated state and are associated with mesenchymal tissue renewal and turnover (Pittenger et al., 1999). These cells are able to differentiate into several lineages of mesodermal origin, such as cartilage, bone, fat, tendon or muscle (Caplan and Bruder, 2001). Nevertheless, the most explored feature of MSC in clinical studies is their ability to secrete a wide panel of soluble molecules with therapeutic activity, including immunomodulation, anti-apoptosis, anti-scarring, chemoattraction, angiogenesis or support of cell growth and differentiation (Singer and Caplan, 2011). This, together with their described low-immunogenicity (Le Blanc et al., 2003a), makes these cells as promising candidates for off-the-shelf allogeneic cell therapy approaches. Although the therapeutic benefit of MSC administration has been observed in several studies, clinical trials have produced conflicting data or shown only modest benefits (Allison, 2009).

Due to their role as modulators of immune system and mediators of inflammatory responses (Prockop and Youn Oh, 2012), MSC have been extensively investigated for the treatment of ischemic diseases. However, the harsh ischemic microenvironments that are faced by the cells upon *in vivo* transplantation lead to reduced survival and poor engraftment (Potier et al., 2007). Thus, strategies to improve MSC therapeutic properties in a robust and reliable way are required. Genetic modification with therapeutic factors is an example of such strategies.

The action of MSC in the context of ischemic diseases has been attributed to the secretion of pro-angiogenic signaling factors such as vascular endothelial growth factor (VEGF), among others. VEGF is a key regulator of physiological angiogenesis, known to promote endothelial cell growth and survival, and one of the most important and powerful promoters of vascular regeneration (Ferrara et al., 2003). Several studies have suggested the use of MSC-based cell therapy to enhance angiogenesis and induce tissue repair in many cardiovascular diseases, including peripheral arterial disease or myocardial infarction (Tang et al., 2005b, Liew and O'Brien, 2012). In that regard, genetic engineering of MSC with VEGF-encoding vectors can improve the intrinsic secretion of VEGF by cultured MSC, thus improving their angiogenic capacity and therapeutic potential for ischemic diseases.

In the last decades, genetic engineering of MSC has been efficiently accomplished by viral-based strategies. However, safety and manufacturing issues related with the use of viral vectors have led to the development of safer and easier to produce non-viral systems. Plasmids are especially

adequate for efficient delivery of genes into MSC due to their reduced immunogenicity, low risk of insertional mutagenesis and ease of manufacturing (Prazeres and Monteiro, 2014).

In order to improve the transgene expression levels achieved by genetic engineering with plasmids, which are usually lower than those obtained by using virus, improved DNA vectors have been developed (Hardee et al., 2017). Minicircles (MC), which are small plasmid derivatives devoid of bacterial backbone, are examples of such vectors (Darquet et al., 1997). Their smaller size when compared to conventional plasmids leads to an improved transfection efficiency. On the other hand, the lack of bacterial sequences that may cause some immunogenicity and/or transgene silencing contributes to enhance gene expression and reduce cytotoxicity (Sawamura et al., 2005, Boura et al., 2014, Walker et al., 2010).

A number of studies have shown that MC-based cell engineering is able to sustain higher and longer transgene expression, as well as stem cell survival (Darquet et al., 1999, Chen et al., 2003, Dietz et al., 2013, Madeira et al., 2013, Munye et al., 2016), when compared to conventional plasmid vectors. Furthermore, MC have been shown to be superior to plasmids when used as vectors for VEGF *in vivo* gene therapy (Chang et al., 2008, Yoon et al., 2009, Ko et al., 2011).

Herein, MC produced and purified by a process recently established at BERG-iBB (Alves et al., 2016, Alves et al., 2018) were used to transfect MSC from bone marrow (BM) in a context of an *ex vivo* gene therapy (genetic engineering of cells outside the body) (Kaufmann et al., 2013). The transfection was performed by microporation, according to a protocol previously optimized (Madeira et al., 2011). MSC were transfected with a plasmid or a MC containing VEGF-GFP, and a plasmid encoding for GFP only was used as control. Microporation demonstrated to be an efficient method for gene delivery into MSC, achieving high GFP expression levels with pVAX-GFP. However, results also showed that fusion with GFP might not be the best strategy to monitor VEGF transgene expression in transfection studies with MSC.

III.3. Materials and Methods

III.3.1. Plasmid construction, production and purification

Plasmid construction, production and purification was performed as described on previous Chapter (Chapter II). The parental plasmid (pMINILi-CVG, 4563 bp) and control plasmids (pVAX-GFP, 3697 bp and pVAX-VEGF-GFP, 4273 bp) used herein were constructed as described elsewhere (Azzoni et al., 2007, Simcikova et al., 2014, Alves et al., 2016). Briefly, the parental plasmid pMINILi-CVG contains the VEGF-GFP gene fusion construct under the control of cytomegalovirus (CMV) immediate early-promoter, two multimer resolution sites (MRS) flanking the expression cassette, pMB1 origin of replication, kanamycin resistance gene and BGH polyadenylation sequence (Figure III.1A). The pVAX-VEGF-GFP (Figure III.1B) was obtained from the previously constructed pVAX1GFP-BGH (referred herein as pVAX-GFP – Figure III.1C), which is derived from the commercial pVAX1LacZ (6050 bp, Invitrogen), by introduction of the VEGF gene in fusion with the GFP reporter (Azzoni et al., 2007, Alves et al., 2016). Plasmids (pVAX-VEGF-GFP and pVAX-GFP) were produced in an *E. coli* DH5α strain and purified using an endotoxin-free plasmid DNA purification kit (Macherey-Nagel), as previously described (Madeira et al., 2011). The concentration of purified pDNA solutions was assayed by spectrophotometry at 260nm (NanoDrop, Thermo Scientific) and DNA integrity was confirmed by DNA agarose gels.

MC were produced in a BW2P *E. coli* strain, previously transformed with the parental plasmid (PP) pMINLI-CVG, according to previously established methods (Simcikova et al., 2014, Alves et al., 2016). *E. coli* BW2P was grown until the late exponential phase and then recombination was induced for 2 h by addition of 0.01% (w/v) L-(+)arabinose (Merck). As a result of intramolecular recombination between the two MRS, a MC with the expression cassette and a miniplasmid (MP) with the prokaryotic backbone sequences are obtained. All plasmid DNA species were recovered and purified from the producer cells using an endotoxin-free plasmid DNA purification kit (Macherey-Nagel). Then, MC was separated from other DNA forms by performing a digestion

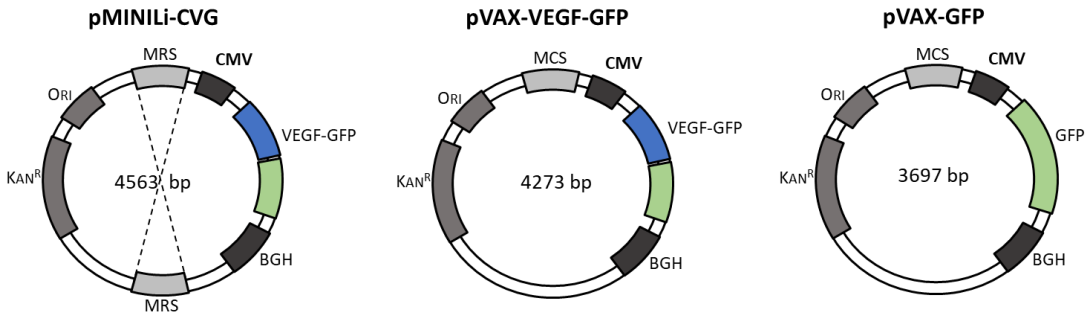

Figure III.1 - Plasmids used for transfection within the present work in order to overexpress the VEGF protein in MSC. The plasmids were produced by *E. coli* strains and then purified before being used in transfection studies with MSC.

with nicking endonuclease (*Nb*.*B*bvCI) followed by hydrophobic interaction chromatography (HIC), as described and optimized at BERG-iBB (Alves et al., 2016, Alves et al., 2018).

III.3.2. Isolation and culture of human bone marrow-derived MSC

Mesenchymal stem/stromal cells (MSC) used in the present work were isolated from bone marrow (BM) of healthy donors after informed consent. After isolation and initial expansion as described elsewhere (dos Santos et al., 2010), cells were maintained cryopreserved until further use. Upon thawing, MSC were expanded until passages 4-7 under xenogeneic(xeno)-free culture conditions, as previously established (dos Santos et al., 2011, dos Santos et al., 2014) At each passage, cells were plated at a cell density between 3,000-6,000 cells/cm^2 on CELLstart™ CTS™ (Invitrogen) pre-coated T-flasks using StemPro® MSC SFM XenoFree (Invitrogen) supplemented with 1% GlutaMAX™-I CTS™ (Invitrogen) and 1% Antibiotic-Antimycotic (Invitrogen). Cells were maintained at 37ºC and 5% CO_2 in a humidified atmosphere and culture medium was exchanged every 3-4 days. At 70% cell confluence, MSC were detached from the flasks by adding TrypLE™ Select CTS™ (Invitrogen) solution 1x in Phosphate Buffered Saline (PBS, Gibco). Cell number and viability were determined using the Trypan Blue (Gibco) exclusion method. BM MSC at passages between 4 and 7 from three independent donors were used.

III.3.3. Microporation with VEGF-GFP (or GFP) encoding vectors

Microporation of BM MSC was performed as previously optimized (Madeira et al., 2011). For each condition, 1.5×10^6 cells were resuspended in 100 µl of resuspension buffer (buffer R, Invitrogen) and incubated with 10 µg of pVAX-GFP or the equivalent number of molecules of pVAX-VEGF-GFP (11 µg) and MC-VEGF-GFP (6.33 µg). Electroporation was performed using the Neon® Transfection System and a Microporator MP-100 (Digital Bio, Invitrogen) using 1 pulse with 1,000 V of voltage and 40 ms of width. After microporation, the cell suspension was incubated with 900 µL of Opti-MEM™ I Reduced Serum Medium (Gibco) for 20-30 min. Next, cells were plated at a density of 7,000-8,000 cells/cm^2 in pre-warmed StemPro® XenoFree culture medium. At each time point (day 2, day 5 and day 7), the number of cells was estimated using the trypan blue dye exclusion method. Two controls were prepared: MSC microporated without DNA (“Micro”) and non-microporated cells, referred herein as “control”, which will be used for comparison in subsequent studies.

III.3.4. Cell viability and recovery, yield of transfection and GFP expression

Cell viability was measured by dividing the number of viable cells by the total cell number, both assessed using trypan blue dye exclusion method. Cell recovery was determined after 48 h, by calculating the ratio between viable cells in each condition and viable cells in the control (non-microporated), as described elsewhere (Madeira et al., 2011). Yield of transfection was calculated according to the equation established by Madeira and colleagues: multiplication of the number of GFP-expressing cells by the number of cells alive and then dividing this value by the total cell number (Madeira et al., 2011). The number of GFP- expressing cells (GFP+ cells) was acquired by flow cytometry using FACScalibur equipment/CellQuest software (BD Biosciences) and analyzed using FlowJo® (LLC).

III.3.5. Analysis of GFP production by Western blot

The production of GFP by MSC transfected with pVAX-GFP, pVAX-VEGF-GFP and MC-VEGF-GFP was investigated by western blot 48 h after microporation. Both culture supernatants and cell lysates were analyzed. Culture supernatants were collected, centrifuged to remove any cell debris and kept at -80ºC until further analysis. To obtain cell lysates, cells pellets previously obtained and maintained at -80ºC were incubated with catenin-lysis buffer containing phosphatase and protease inhibitors. SDS-PAGE gel electrophoresis analysis of supernatant and lysate samples from pVAX-GFP, pVAX-VEGF-GFP and MC-VEGF-GFP was performed for protein separation. Then, the proteins were transferred to a PVDF membrane for 120 min in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell equipped with the Mini Trans-Blot® Module (Bio-Rad) according to manufacturer's protocol. The membrane was then incubated with blocking buffer (TBST + 5% non-fat dry milk) for 1 h and left overnight with the primary antibody GFP Antibody (B-2) (sc-9996, Santa Cruz Biotechnology) diluted 1:500 at 4ºC. Then, the membrane was incubated with HRP-conjugated secondary antibody goat anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology) diluted 1:2000 for 1 h. Finally, the membrane was incubated with visualization solution (3.3-DAB in TBS with hydrogen peroxide 30%) for 20 min and reaction was stopped with tap water. After drying the blot, an image was captured.

III.3.6. Quantification of VEGF expression by qPCR

The levels of VEGF expression by MSC transfected with MC-VEGF-GFP and pVAX-VEGF-GFP were measured by quantitative real-time PCR (qPCR) 2 and 5 days after microporation. For qPCR, at

least 150,000 cells were harvested at day 2 and day 5 and kept at -80 ºC as a dry pellet until further analysis. RNeasy Mini Kit (Qiagen) was used for RNA isolation and iScript cDNA Synthesis Kit (Bio-Rad) was used for cDNA synthesis after quantification by UV spectrophotometry on a NanoDrop. The qPCR analysis was performed in a 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 reaction volume. The following primers (StabVida) were used for VEGF amplification: VEGF_fwd – GGAGGAGGGCAGAATCATCAC and VEGF_rev – GGTCTCGAT TGGATGGCAGT. To determine the fold change in mRNA expression, the 2−ΔΔCT method of relative quantification was applied (Livak and Schmittgen, 2001), using GAPDH as the housekeeping gene and non-microporated MSC as baseline.

III.3.7. Statistical analysis

All data is presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 and significance was determined by Tukey's multiple comparison test and set at a p-value <0.05.

III.4. Results and Discussion

III.4.1. Characterization of MSC after microporation: proliferative potential and viability, cell recovery and yield of transfection

Plasmids containing GFP (pVAX-GFP) and VEGF-GFP (pVAX-VEGF-GFP) were produced within *E. coli* DH5α and purified with a commercial kit, as described in Materials and Methods. The MC-VEGF-GFP was produced in *E. coli* BW2P through recombination of the PP (pMINILi-CVG) and then purified with different steps, including a chromatographic procedure (HIC), according to a recently established protocol (Simcikova et al., 2014, Alves et al., 2016). The three vectors were then used to transfect MSC from BM using microporation, an electroporation-based method with reduced electrode surface area to diminish cell mortality (Kim et al., 2008). MSC microporated with pVAX-GFP, pVAX-VEGF-GFP and MC-VEGF-GFP were analyzed at three different timepoints: day 2, day 5 and day 7.

To evaluate the impact of the microporation with the vectors of interest on cell proliferative potential, cell numbers were determined at each time point (Figure III.2A). The data confirmed that non-transfected cells (control) achieved the highest cell numbers in all time points but the differences were not statistically significant. Although the microporated cells seem to be slightly affected by the microporation procedure right after transfection, at day 7 cells have their growth capacity recovered. This decrease in the proliferative capacity of MSC is most likely related to the microporation process itself and not to the presence of DNA, since a reduction on cell numbers was also observed when MSC were microporated (Micro). This is in accordance with previously published reports that demonstrated a slight decrease on MSC cell numbers or viability after microporation (Madeira et al., 2011, Abdul Halim et al., 2014, Lim et al., 2010). Nevertheless, the comparison of different transfection techniques revealed that microporation is one of the methods that causes less cell damage on MSC (Abdul Halim et al., 2014). In the present work, cell viabilities were also measured and values >90% were obtained for all the conditions and timepoints. It is noteworthy that despite the parameter used to evaluate cell integrity after transfection in most published works is cell viability (Lim et al., 2010, Abdul Halim et al., 2014), high viabilities can be observed even when few cells are recovered from electroporation process. Hence, herein cell recovery was also investigated in addition to cell viability.

Figure III.2B shows cell recoveries and transfection yields calculated for all the microporation conditions 48 h after transfection. No statistically significant differences were found for cell recoveries. Nevertheless, the highest percentage was observed for cells microporated without

DNA (Micro; 55.9±16.8%) when compared to transfected samples (pVAX-GFP: 28.0±6.8%; pVAX-VEGF-GFP: 44.5±11.1%; MC-VEGF-GFP: 28.9±5.4%). These results reveal that DNA may cause some cytotoxicity, which is in accordance with the study from Madeira and co-workers, who observed a decrease in cell recoveries when DNA amounts were increased (Madeira et al., 2011). The cell recoveries observed for DNA-transfected cells were similar to those observed in other studies where plasmid DNA was used (Madeira et al., 2011, Aluigi et al., 2006). However, and considering the reduced size of MC compared to plasmid vectors, a higher cell recovery was expected for cells transfected with MC than for plasmid-transfected cells, as reported by other authors (Boura et al., 2014).

Regarding the yield of transfection, the highest values were observed for transfection with pVAX-GFP (12.3±4.1%) followed by pVAX-VEGF-GFP (10.1±5.2%) and MC-VEGF-GFP (1±0.4%), but the differences were not statistically significant. The high transgene size of the fusion cassette (VEGF-GFP) may be one of the reasons for the low yields observed for VEGF-GFP-encoding molecules. Nevertheless, more replicates of the experiment must be performed in order to confirm this assumption. Even if the highest transfection yields were observed for pVAX-GFP, the values were lower than expected according to other reports, where yields of 35% were achieved using a similar protocol at BERG-iBB (Madeira et al., 2011). The discrepancy between the result in present thesis and the one published previously is probably associated with the reduced cell recoveries (28±6.8%) calculated for this condition. Few authors published results regarding the yield of transfection after microporation, being only focused on the percentage of GFP+ cells (Abdul Halim et al., 2014, Mun et al., 2016, Wang et al., 2009b, Lim et al., 2010). However, it is important to correlate the transgene expression with the number of viable cells, since in specific therapeutic settings a lower expression of the transgene within a

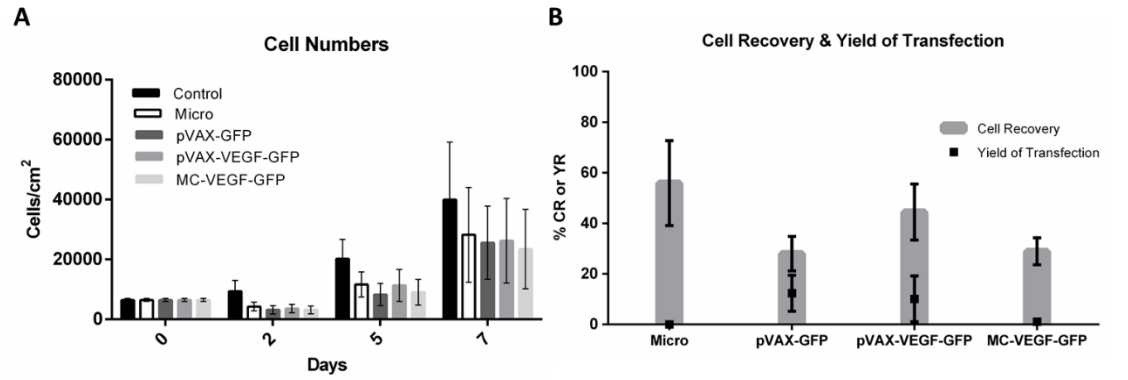

Figure III.2 – Analysis of MSC behavior after transfection with pVAX-GFP, pVAX-VEGF-GFP and MC-VEGF-GFP. (A) Cell numbers observed for all the tested conditions and timepoints. (B) Values for cell recovery and yield of transfection regarding the microporation with no DNA (Micro) and with each of the vectors studied calculated 48 h after transfection. Each bar represents the mean ± SEM, n=3.

high number of cells might be preferred than a higher expression in fewer cells (Santos et al., 2009).

III.4.2. Analysis of GFP expression and production by flow cytometry and Western blot

GFP production by transfected MSC was evaluated both by flow cytometry and western blot techniques and the results are shown in Figure III.3. Flow cytometry analysis (Figure III.3A) demonstrated that the percentage of GFP-expressing cells after 48 h was significantly higher for transfection with pVAX-GFP (66.8±5.9%) compared with pVAX-VEGF-GFP (12.1±3.1%) or MC-VEGF (3.1±3.2%). As already referred, the low expression of GFP on fusion constructs is likely related to its large size.

Different transfection efficiencies (measured by %GFP+ cells) have been reported in literature for studies of MSC microporation using GFP-encoding plasmids, varying between 40-80% (Madeira et al., 2011, Lim et al., 2010, Wang et al., 2009b, Abdul Halim et al., 2014). Those discrepancies between studies are probably related to the different microporation conditions and/or pDNA amounts used, and distinct detection methods or quantification at variable timepoints. Nevertheless, the values observed in the present work are in accordance or superior, in some cases, to those obtained by other authors, revealing that the protocol used herein is able to achieve high and comparable transgene expressions.

Although most studies assume that the percentage of GFP+ cells is equivalent to the transfection efficiency, herein this assumption is only valid for cells transfected with pVAX-GFP as discussed next. The transfection efficiency (GFP+ cells) for pVAX-GFP cannot be directly compared with the values of GFP+ cells observed for pVAX-VEGF-GFP or MC-VEGF-GFP due to the presence of VEGF. It is known that the intracellular accumulation of GFP in transfected cells lead to a time-dependent increase of GFP+ cells (Kobelt et al., 2013). However, for VEGF-GFP constructs, the resulting fusion protein is probably secreted to the medium (Guzman-Hernandez et al., 2014). In fact, the secretion signal in the N-terminus of VEGF was not modified by the fusion with the GFP gene, which was performed at its C-terminus. Also, the smaller size of the GFP transgene compared to VEGF-GFP constructs may also lead to higher expression due to the lower burden imposed to the cell transduction machinery.

Contrary to expectations, increased GFP expression was observed for cells transfected with plasmid DNA when compared to those modified with MC. Other studies confirmed a higher expression of transgenes by MSC modified with MC when compared to conventional plasmids

(Boura et al., 2014, Bandara et al., 2016). The data of Figure III.3A also shows that GFP expression levels decrease with time, as expected due to the transient nature of non-viral transfection. Nevertheless, GFP cell expression levels observed with pVAX-GFP were still detectable 7 days after transfection and significantly higher (25.6±13.5%) than for the other conditions.

To our best knowledge, there is no data available regarding MSC transfection with MC containing VEGF-GFP. Nevertheless, a previous work regarding MSC non-viral engineering using pDNA with VEGF-GFP fusion construct revealed transfection efficiencies of ≈52% measured by assessing GFP expression. However, cell transfection was not performed by microporation but a lipid-based transfection optimized with a sleeping beauty transposon system and stem cell targeting peptides was used instead (Wang et al., 2015).

One of the possibilities for the low number of GFP+ cells driven by the fusion constructs was that the overexpressed protein is being secreted to the culture medium instead of being kept within the cell due to the extracellular nature of the VEGF protein. To further understand the levels of GFP production and to confirm if some of the protein is being secreted to the culture medium, western blot analysis of both cell lysates and culture supernatants was performed (Figure III.3-B). The results from western blot analysis were in accordance to flow cytometry data. Regarding cell lysates, the most intense band was obtained for cells transfected with pVAX-GFP (1), being followed by pVAX-VEGF-GFP (2), indicating a higher expression of GFP for these conditions compared to MC-VEGF-GFP (3). The slight and clear band shown for MC-VEGF-GFP (3) confirms the low GFP expression by MSC transfected with this construct, as previously demonstrated by flow cytometry. On the other hand, the supernatant analysis revealed the presence of a band only for pVAX-GFP, indicating that the GFP was present only in the culture medium retrieved from this condition, contrarily to the initial expectations. No GFP was detected in culture

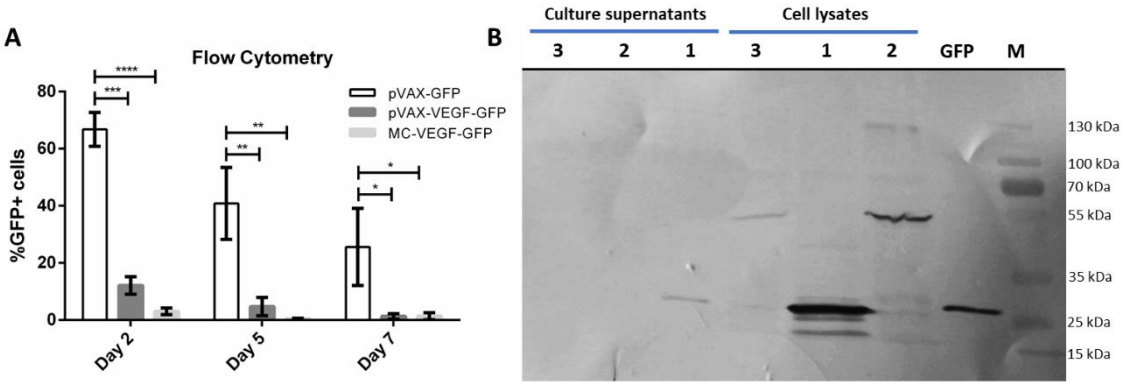

Figure III.3 - Analysis of GFP expression by MSC after transfection using flow cytometry (**A**) and western blot (**B**). **A** -Flow cytometry detection of GFP expressed by MSC transfected with pVAX-GFP, pVAX-VEGF-GFP and MC-VEGF-GFP at different timepoints after transfection (2, 5 or 7 days). Each bar represents the mean ± SEM, n=3, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. **B** – Western blot analysis of culture supernatants and cell lysates of MSC obtained 48 h after transfection with pVAX-GFP (1), pVAX-VEGF-GFP (2) and MC-VEGF-GFP (3). GFP – Green fluorescent protein (positive control). M – Protein molecular weight marker (PageRuler™ Plus Prestained 10-250 kDa, Thermo Scientific).

supernatants from pVAX-VEGF-GFP or MC-VEGF-GFP by western blot analysis. According to previous results, it was expected that VEGF-GFP was detected both on cell surface and in the culture medium. In fact, a report studying the VEGF trafficking demonstrated that VEGF-GFP fusion protein was able to dimerize and be secreted in a biologically active form after being transfected into COS-7 cells (Guzman-Hernandez et al., 2014).

III.4.3. Quantification of VEGF expression

To further confirm the transgene expression levels, VEGF relative expression was measured by qPCR 2 and 5 days after transfection with either pVAX-VEGF-GFP or MC-VEGF-GFP (Figure III.4). The results demonstrated that 2 days after transfection, VEGF expression was significantly

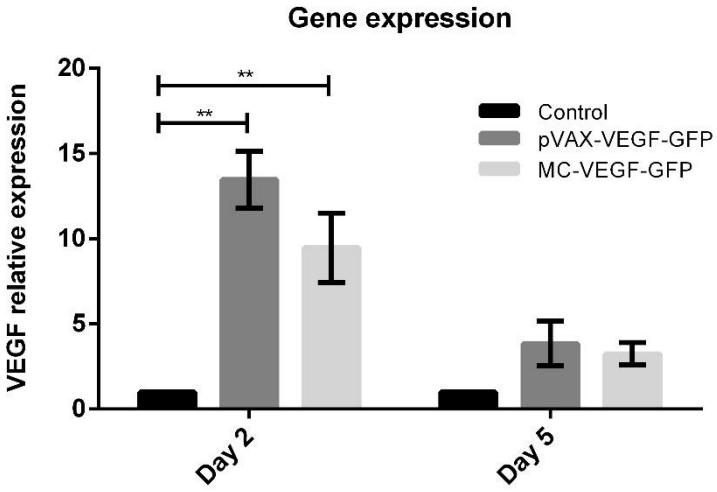

Figure III.4 - Evaluation of gene expression by qPCR 2 and 5 days after MSC microporation with pVAX-VEGF-GFP and MC-VEGF-GFP. Each bar represents the mean ± SEM, n=2, **p<0.01.

increased on transfected samples compared to non-transfected control MSC (pVAX: 13.5±1.7-fold; MC: 9.5±2.0-fold). The increased expression on transfected samples was maintained 5 days after microporation (pVAX: 3.8±1.3-fold; MC: 3.2±0.7-fold), but the differences regarding the control MSC (no transfected) were no longer statistically significant.

The results obtained herein for gene expression 48 h after microporation are in agreement with the data observed on the western blot regarding GFP protein production. On day 2, higher levels of transgene were observed for pVAX-VEGF-GFP than for MC-VEGF-GFP, despite the differences were not statistically significant. On day 5, both vectors lead to similar VEGF expression levels, meaning that MC may have induced a more prolonged transgene expression than conventional plasmids, as described by other authors (Mun et al., 2016).

Several studies regarding *in vivo* (Dall'Era et al., 2008) or *ex vivo* (Luo et al., 2017) gene therapies described the use of VEGF-GFP constructs to monitor the VEGF transgene expression by GFP fluorescence. However, few authors reported genetic engineering of MSC with those fusion constructs. Instead, the studies regarding genetic modification of MSC with VEGF that reported GFP expression employed GFP-encoding vectors separately as proof-of-concept for preliminary assessment of transfection efficiency (Beegle et al., 2016, Locatelli et al., 2013). So, the use of the GFP reporter protein to monitor VEGF expression might not be the best strategy for optimization studies concerning the genetic engineering of MSC.

Also, the expression levels using a plasmid containing only one gene are supposed to be higher that when two genes have to be transcribed and translated, imposing an extra metabolic load to the cell machinery. The results shown in the present work are in accordance with this assumption, since an increased GFP expression was observed for cells transfected with pVAX-GFP compared to pVAX-VEGF-GFP. GFP, usually expressed as a monocistronic mRNA, is herein expressed as a fusion protein with VEGF, which may affect protein structure and consequently its fluorescent activity. This, together with the fact that GFP is an intracellular protein unlike VEGF, which is a secreted protein, may contribute to the reduced GFP expression observed herein when fusion constructs were used to transfect MSC.

III.5. Conclusions

The results presented in this chapter demonstrated the effective transfection of human MSC by microporation of vectors encoding for VEGF, a therapeutic factor relevant for angiogenesis. Despite the results concerning the expression of the fusion protein (VEGF-GFP) were not conclusive, high GFP expression levels were achieved using this protocol for pVAX-GFP transfection. The present work also allowed the validation of three different methodologies for transgene expression evaluation: flow cytometry, western blot and real time PCR. All these techniques were able to show differences between different transfection conditions and control (non-transfected) cells. Flow cytometry analysis was possible due to the presence of the GFP in all constructs and is the fastest and most used method for analysis of transfection efficiency. Herein, flow cytometry monitoring of GFP was used to demonstrate the effectiveness of the present transfection protocol, since VEGF expression could not be precisely assessed using this technique.

The optimization of a western blot protocol for detection of the overexpressed protein within the cells or in the culture medium (*i.e.* supernatant) was also performed. Western blot analysis detected GFP protein in cell lysates from all transfected conditions, but GFP was detected in the culture medium only when pVAX-GFP was used, contrarily to the expected due to the extracellular nature of the VEGF protein.

Finally, to evaluate the expression of the therapeutic gene, VEGF, qPCR was performed and the results were similar to those observed with previous techniques: pVAX vector induced higher transgene expression than MC. Although further studies with more replicates are needed to confirm these results, the use of a fusion protein might also not be the best strategy to evaluate VEGF transfection efficiency on human MSC.

A promising strategy to directly evaluate the MC transfection efficiency by fluorescence-based assays, such as flow cytometry, could be to have a MC construct containing only the GFP gene in a similar genetic cassette than the observed on pVAX-GFP. So, the capacity of these two constructs to transfect MSC could be directly compared by flow cytometry or other fluorescence detection methods. However, this was considered to be out of the scope of the present work, since the main objective is to have a therapeutic vector with the pro-angiogenic molecule, VEGF. Therefore, the next step will be the removal of GFP from the construction and the comparison of the two vectors (pVAX and MC) containing only VEGF. The results from this chapter demonstrated that this comparison can be possible using qPCR.

III.6. References

ABDUL HALIM, N. S., FAKIRUDDIN, K. S., ALI, S. A. & YAHAYA, B. H. 2014. A comparative study of non-viral gene delivery techniques to human adipose-derived mesenchymal stem cell. *Int J Mol Sci,* 15**,** 15044-60.

ALLISON, M. 2009. Genzyme backs Osiris, despite Prochymal flop. *Nat Biotech,* 27**,** 966-967.

ALUIGI, M., FOGLI, M., CURTI, A., ISIDORI, A., GRUPPIONI, E., CHIODONI, C., COLOMBO, M. P., VERSURA, P., D'ERRICO-GRIGIONI, A., FERRI, E., BACCARANI, M. & LEMOLI, R. M. 2006. Nucleofection Is an Efficient Nonviral Transfection Technique for Human Bone Marrow–Derived Mesenchymal Stem Cells. *STEM CELLS,* 24**,** 454-461.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2016. Development of a nicking endonuclease-assisted method for the purification of minicircles. *Journal of Chromatography A,* 1443**,** 136-144.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2018. Production and Purification of Supercoiled Minicircles by a Combination of *in vitro* Endonuclease Nicking and Hydrophobic Interaction Chromatography. *Human Gene Therapy Methods,* (in press).

AZZONI, A. R., RIBEIRO, S. C., MONTEIRO, G. A. & PRAZERES, D. M. F. 2007. The impact of polyadenylation signals on plasmid nuclease-resistance and transgene expression. *The Journal of Gene Medicine,* 9**,** 392-402.

BANDARA, N., GURUSINGHE, S., CHEN, H., CHEN, S., WANG, L. X., LIM, S. Y. & STRAPPE, P. 2016. Minicircle DNA-mediated endothelial nitric oxide synthase gene transfer enhances angiogenic responses of bone marrow-derived mesenchymal stem cells. *Stem Cell Res Ther,* 7**,** 48.

BEEGLE, J. R., MAGNER, N. L., KALOMOIRIS, S., HARDING, A., ZHOU, P., NACEY, C., WHITE, J. L., PEPPER, K., GRUENLOH, W., ANNETT, G., NOLTA, J. A. & FIERRO, F. A. 2016. Preclinical evaluation of mesenchymal stem cells overexpressing VEGF to treat critical limb ischemia. *Molecular Therapy — Methods & Clinical Development,* 3**,** 16053.

BOURA, J. S., VANCE, M., YIN, W., MADEIRA, C., LOBATO DA SILVA, C., PORADA, C. D. & ALMEIDA-PORADA, G. 2014. Evaluation of gene delivery strategies to efficiently overexpress functional HLA-G on human bone marrow stromal cells. *Mol Ther Methods Clin Dev,* 2014.

CAPLAN, A. I. & BRUDER, S. P. 2001. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med,* 7**,** 259-64.

CHANG, C.-W., CHRISTENSEN, L. V., LEE, M. & KIM, S. W. 2008. Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy. *Journal of Controlled Release,* 125**,** 155-163.

CHEN, Z. Y., HE, C. Y., EHRHARDT, A. & KAY, M. A. 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther,* 8**,** 495-500.

DALL'ERA, J. E., MEACHAM, R. B., MILLS, J. N., KOUL, S., CARLSEN, S. N., MYERS, J. B. & KOUL, H. K. 2008. Vascular endothelial growth factor (VEGF) gene therapy using a nonviral gene delivery system improves erectile function in a diabetic rat model. *Int J Impot Res,* 20**,** 307-14.

DARQUET, A. M., CAMERON, B., WILS, P., SCHERMAN, D. & CROUZET, J. 1997. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther,* 4**,** 1341-9.

DARQUET, A. M., RANGARA, R., KREISS, P., SCHWARTZ, B., NAIMI, S., DELAERE, P., CROUZET, J. & SCHERMAN, D. 1999. Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer. *Gene Ther,* 6**,** 209-18.

DIETZ, W. M., SKINNER, N. E., HAMILTON, S. E., JUND, M. D., HEITFELD, S. M., LITTERMAN, A. J., HWU, P., CHEN, Z. Y., SALAZAR, A. M., OHLFEST, J. R., BLAZAR, B. R., PENNELL, C. A. & OSBORN, M. J. 2013. Minicircle DNA is superior to plasmid DNA in eliciting antigen-specific CD8+ T-cell responses. *Mol Ther,* 21**,** 1526-35.

DOS SANTOS, F., ANDRADE, P. Z., ABECASIS, M. M., GIMBLE, J. M., CHASE, L. G., CAMPBELL, A. M., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Toward a Clinical-Grade Expansion of Mesenchymal Stem Cells from Human Sources: A Microcarrier-Based Culture System Under Xeno-Free Conditions. *Tissue Engineering. Part C, Methods,* 17**,** 1201-1210.

DOS SANTOS, F., ANDRADE, P. Z., BOURA, J. S., ABECASIS, M. M., DA SILVA, C. U. L. & CABRAL, J. M. S. 2010. Ex vivo expansion of human mesenchymal stem cells: A more effective cell proliferation kinetics and metabolism under hypoxia. *Journal of Cellular Physiology,* 223**,** 27-35.

DOS SANTOS, F., CAMPBELL, A., FERNANDES-PLATZGUMMER, A., ANDRADE, P. Z., GIMBLE, J. M., WEN, Y., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2014. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. *Biotechnology and Bioengineering,* 111**,** 1116-1127.

FERRARA, N., GERBER, H.-P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9**,** 669-676.

GUZMAN-HERNANDEZ, M. L., POTTER, G., EGERVARI, K., KISS, J. Z. & BALLA, T. 2014. Secretion of VEGF-165 has unique characteristics, including shedding from the plasma membrane. *Mol Biol Cell,* 25**,** 1061-72.

HARDEE, C. L., AREVALO-SOLIZ, L. M., HORNSTEIN, B. D. & ZECHIEDRICH, L. 2017. Advances in Non-Viral DNA Vectors for Gene Therapy. *Genes (Basel),* 8.

KAUFMANN, K. B., BUNING, H., GALY, A., SCHAMBACH, A. & GREZ, M. 2013. Gene therapy on the move. *EMBO Mol Med,* 5**,** 1642-61.

KIM, J. A., CHO, K., SHIN, M. S., LEE, W. G., JUNG, N., CHUNG, C. & CHANG, J. K. 2008. A novel electroporation method using a capillary and wire-type electrode. *Biosens Bioelectron,* 23**,** 1353-60.

KO, J., JUN, H., CHUNG, H., YOON, C., KIM, T., KWON, M., LEE, S., JUNG, S., KIM, M. & PARK, J. H. 2011. Comparison of EGF with VEGF Non-Viral Gene Therapy for Cutaneous Wound Healing of Streptozotocin Diabetic Mice. *Diabetes Metab J,* 35**,** 226-35.

KOBELT, D., SCHLEEF, M., SCHMEER, M., AUMANN, J., SCHLAG, P. M. & WALTHER, W. 2013. Performance of high quality minicircle DNA for in vitro and in vivo gene transfer. *Mol Biotechnol,* 53**,** 80-9.

LE BLANC, K., TAMMIK, C., ROSENDAHL, K., ZETTERBERG, E. & RINGDEN, O. 2003. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol,* 31**,** 890-6.

LIEW, A. & O'BRIEN, T. 2012. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Res Ther,* 3**,** 28.

LIM, J. Y., PARK, S. H., JEONG, C. H., OH, J. H., KIM, S. M., RYU, C. H., PARK, S. A., AHN, J. G., OH, W., JEUN, S. S. & CHANG, J. W. 2010. Microporation is a valuable transfection method for efficient gene delivery into human umbilical cord blood-derived mesenchymal stem cells. *BMC Biotechnol,* 10**,** 38.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LOCATELLI, P., OLEA, F. D., HNATIUK, A., SEPULVEDA, D., PEREZ SAEZ, J. M., ARGUELLO, R. & CROTTOGINI, A. 2013. Efficient plasmid-mediated gene transfection of ovine bone marrow mesenchymal stromal cells. *Cytotherapy,* 15**,** 163-70.

LUO, M., HUANG, H., HOU, L., SHAO, S., HUANG, S. & ZHAO, X. 2017. Construction and expression of a lentivirus expression vector carrying the VEGF165-EGFP fusion gene in breast cancer MCF-7 cells. *Oncol Lett,* 13**,** 1745-1752.

MADEIRA, C., RIBEIRO, S. C., PINHEIRO, I. S. M., MARTINS, S. A. M., ANDRADE, P. Z., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Gene delivery to human bone marrow mesenchymal stem cells by microporation. *Journal of Biotechnology,* 151**,** 130-136.

MADEIRA, C., RODRIGUES, C. A. V., REIS, M. S. C., FERREIRA, F. F. C. G., CORREIA, R. E. S. M., DIOGO, M. M. & CABRAL, J. M. S. 2013. Nonviral Gene Delivery to Neural Stem Cells with Minicircles by Microporation. *Biomacromolecules,* 14**,** 1379-1387.

MUN, J.-Y., SHIN, K. K., KWON, O., LIM, Y. T. & OH, D.-B. 2016. Minicircle microporation-based non-viral gene delivery improved the targeting of mesenchymal stem cells to an injury site. *Biomaterials,* 101**,** 310-320.

MUNYE, M. M., TAGALAKIS, A. D., BARNES, J. L., BROWN, R. E., MCANULTY, R. J., HOWE, S. J. & HART, S. L. 2016. Minicircle DNA Provides Enhanced and Prolonged Transgene Expression Following Airway Gene Transfer. *Sci Rep,* 6**,** 23125.

PITTENGER, M. F., MACKAY, A. M., BECK, S. C., JAISWAL, R. K., DOUGLAS, R., MOSCA, J. D., MOORMAN, M. A., SIMONETTI, D. W., CRAIG, S. & MARSHAK, D. R. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science,* 284**,** 143-7.

POTIER, E., FERREIRA, E., MEUNIER, A., SEDEL, L., LOGEART-AVRAMOGLOU, D. & PETITE, H. 2007. Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue Eng,* 13**,** 1325-31.

PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. Plasmid Biopharmaceuticals. *Microbiology Spectrum,* 2.

PROCKOP, D. J. & YOUN OH, J. 2012. Mesenchymal Stem/Stromal Cells (MSCs): Role as Guardians of Inflammation. *Molecular Therapy,* 20**,** 14-20.

SANTOS, J. L., ORAMAS, E., PEGO, A. P., GRANJA, P. L. & TOMAS, H. 2009. Osteogenic differentiation of mesenchymal stem cells using PAMAM dendrimers as gene delivery vectors. *J Control Release,* 134**,** 141-8.

SAWAMURA, D., ABE, R., GOTO, M., AKIYAMA, M., HEMMI, H., AKIRA, S. & SHIMIZU, H. 2005. Direct injection of plasmid DNA into the skin induces dermatitis by activation of monocytes through toll-like receptor 9. *J Gene Med,* 7**,** 664-71.

SIMCIKOVA, M., PRATHER, K. L. J., PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. On the dual effect of glucose during production of pBAD/AraC-based minicircles. *Vaccine,* 32**,** 2843-2846.

SINGER, N. G. & CAPLAN, A. I. 2011. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol,* 6**,** 457-78.

TANG, Y. L., ZHAO, Q., QIN, X., SHEN, L., CHENG, L., GE, J. & PHILLIPS, M. I. 2005. Paracrine Action Enhances the Effects of Autologous Mesenchymal Stem Cell Transplantation on Vascular Regeneration in Rat Model of Myocardial Infarction. *The Annals of Thoracic Surgery,* 80**,** 229-237.

WALKER, W. E., BOOTH, C. J. & GOLDSTEIN, D. R. 2010. TLR9 and IRF3 Cooperate to Induce a Systemic Inflammatory Response in Mice Injected With Liposome:DNA. *Mol Ther.*

WANG, D. D., YANG, M., ZHU, Y. & MAO, C. 2015. Reiterated Targeting Peptides on the Nanoparticle Surface Significantly Promote Targeted Vascular Endothelial Growth Factor Gene Delivery to Stem Cells. *Biomacromolecules,* 16**,** 3897-903.

WANG, Y. H., HO, M. L., CHANG, J. K., CHU, H. C., LAI, S. C. & WANG, G. J. 2009. Microporation is a valuable transfection method for gene expression in human adipose tissue-derived stem cells. *Mol Ther,* 17**,** 302-8.

YOON, C. S., JUNG, H. S., KWON, M. J., LEE, S. H., KIM, C. W., KIM, M. K., LEE, M. & PARK, J. H. 2009.

Sonoporation of the minicircle-VEGF(165) for wound healing of diabetic mice. *Pharm Res,* 26**,** 794-801.

Chapter IV

ENGINEERING OF HUMAN MESENCHYMAL STEM/STROMAL CELLS (MSC) WITH VEGF-ENCODING MINICIRCLES FOR ANGIOGENIC *EX VIVO* GENE THERAPY

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IV. ENGINEERING OF HUMAN MESENCHYMAL STEM/STROMAL CELLS (MSC) WITH VEGF-ENCODING MINICIRCLES FOR ANGIOGENIC *EX VIVO* GENE THERAPY

IV.1. Summary

Peripheral artery disease (PAD) is a debilitating and prevalent condition characterized by the blockage of arteries, leading to limb amputation in more severe cases. Mesenchymal stem/stromal cells (MSC) are known to have intrinsic regenerative properties that can be potentiated by the introduction of pro-angiogenic genes such as the vascular endothelial growth factor (VEGF). Herein we propose the use of human bone marrow (BM) MSC transiently transfected with minicircles encoding for VEGF as an *ex vivo* gene therapy strategy to enhance angiogenesis in PAD patients. The VEGF gene was cloned in minicircle and conventional plasmid vectors and used to transfect BM- derived MSC *ex vivo*. VEGF expression was evaluated both by qPCR and ELISA. The number of VEGF transcripts following MSC transfection with minicircles increased 130-fold relatively to the expression in non-transfected MSC, whereas for the plasmid (pVAX1)-based transfection the increase was 50-fold. Compared to the VEGF basal levels secreted by MSC (11.1±3.4 pg/1000 cells day^{-1}), significantly higher values were detected by ELISA after both minicircle and pVAX1 transfection (644.8±82.5 and 508.3±164.0 pg/1,000 cells day^{-1}, respectively). The VEGF overexpression improved the angiogenic potential of MSC *in vitro*, as confirmed by endothelial cell tube formation and cell migration assays, without affecting the expansion potential *ex vivo*, as well as multilineage differentiation capacity or immunophenotype of MSC. Although pre-clinical *in vivo* studies are required, these results suggest that minicircle-mediated VEGF gene delivery, combined with the unique properties of human MSC, could represent a promising *ex vivo* gene therapy approach for an improved angiogenesis in the context of PAD.

IV.2. Background

Peripheral arterial disease (PAD) is a chronic disease caused by obstruction of arteries, leading to decreased blood flow to the lower extremities. PAD affects 3-10% of the world population and about 30% of these patients are faced with the possibility of limb amputation. In order to recover tissue oxygenation, those patients are dependent on the adaptation of pre-existing vessels or on the formation of new vessels (Gresele et al., 2011). Therapeutic delivery of angiogenic factors and/or stem/progenitor cells to promote revascularization of the ischemic regions represents a potential therapeutic approach to regenerate damaged tissue and prevent amputations in patients with PAD (Yasumura et al., 2012, Cunha et al., 2013).

Human mesenchymal stem/stromal cells (MSC) are multipotent cells known to set-up a regenerative environment and modulate anti-inflammatory responses (Prockop and Youn Oh, 2012). Though the mechanisms underlying the immunomodulatory capacity of MSC are not totally understood, some studies have suggested that it is not only dependent on cell-cell contact and most likely relies on the secretion of soluble factors that act through a paracrine way on other cells (Di Nicola et al., 2002, Singer and Caplan, 2011, Gebler et al., 2012). These paracrine effects of MSC include: immunomodulation, anti-apoptosis, angiogenesis, anti-scarring, chemoattraction and support of the growth and differentiation of other stem/progenitor cells (Singer and Caplan, 2011). While it is not known if potential rejection of donor MSC (described as immunoevasive) may influence the efficacy of allogeneic MSC therapies (Ankrum et al., 2014), several clinical studies have advocated for an allogeneic setting. This would make it possible to use an off-the-shelf product, allowing an immediate access for acute interventions. Furthermore, a large number of cells from young and healthy donors would be available that could overcome the age-related loss of regenerative capacity of patient cells (Rao and Mattson, 2001).

The action of MSC in the context of an angiogenic therapy has been attributed to the secretion of signalling factors such as vascular endothelial growth factor (VEGF), among others. VEGF is a key regulator of physiological angiogenesis, known to promote endothelial cell growth and survival, and one of the most important and powerful promoters of vascular regeneration (Ferrara et al., 2003). Several studies have suggested the use of MSC-based cell therapy to enhance angiogenesis and induce tissue repair in PAD (Tang et al., 2005b, Liew and O'Brien, 2012). Nevertheless, although the therapeutic benefit of MSC administration has been observed in several studies, clinical trials have produced conflicting data or shown only modest benefits (Allison, 2009). Thus, strategies to improve MSC therapeutic properties in a robust and reliable

way are required. In that regard, genetic engineering of MSC with VEGF-encoding vectors can improve the intrinsic secretion of VEGF by cultured MSC, thus improving the angiogenic potential of these cells in a context of an *ex vivo* gene therapy (Beegle et al., 2016). *Ex vivo* gene therapy involves genetic engineering of cells outside the body and their subsequent transplantation back into patients (Gowing et al., 2017).

The genetic modification of human MSC would require vectors that efficiently and safely transfer the target therapeutic gene. Plasmids are especially adequate for this purpose due to their low immunogenicity, non-integrative nature (low risk of insertional mutagenesis) and ease of manufacturing when compared with the viral vectors (Prazeres and Monteiro, 2014). The key disadvantages of plasmids in the context of *ex vivo* gene therapy of PAD are the low transfection efficiency and the short duration of transgene expression (Raval and Losordo, 2013). However, this can be partially circumvented by using small plasmid derivatives called minicircles that carry only the transgene expression cassette, and thus improve transfection on account of their lower size (Darquet et al., 1997). The lack of bacterial sequences in minicircles (e.g. origin of replication, antibiotic resistant marker) further contributes to improve safety, reduce CpG-mediated immunogenicity and minimize silencing of transgene expression. A number of studies have shown that minicircle-based cell engineering is able to sustain higher and longer transgene expression, as well as stem cell survival (Darquet et al., 1999, Chen et al., 2003, Dietz et al., 2013, Madeira et al., 2013, Munye et al., 2016), when compared to conventional plasmid vectors. Furthermore, minicircles have been shown to be superior to plasmids in what concerns angiogenic VEGF expression (Chang et al., 2008, Yoon et al., 2009, Ko et al., 2011). In spite of their advantages, the technology for minicircle manufacturing in suitable amounts and with adequate purity to run preclinical and clinical trials is still incipient. In this context, a production strain system and a purification process for optimal minicircle manufacture have been recently established in our laboratory (Simcikova et al., 2014, Alves et al., 2016).

Based on our previous studies focused on the optimization of non-viral gene delivery to MSC of different human tissue sources using a reporter fluorescent protein – GFP (Madeira et al., 2011, Boura et al., 2013), herein, we developed a strategy to improve the angiogenic potential of cultured human BM-MSC by transiently modifying the cells with minicircles encoding VEGF. Engineered MSC were characterized to confirm the maintenance of their intrinsic properties and to evaluate transgene expression and *in vitro* angiogenic potential. This represents, to our best knowledge, the first study where MSC were modified with VEGF-encoding minicircles.

IV.3. Materials and Methods

IV.3.1. Plasmid construction, production and purification

The control plasmid (pVAX-VEGF, 3,531 bp) and parental plasmid (pMINILi-CV, 3,821 bp) expressing VEGF (165a) gene were obtained from pVAX-VEGF-GFP and pMINILi-CVG, respectively, by removing the GFP gene. Those vectors were constructed as described elsewhere and transformed by heat shock into *E. coli* strains (Azzoni et al., 2007, Simcikova et al., 2014, Alves et al., 2016). pMINILi-CV contains an expression cassette with VEGF and the human cytomegalovirus (CMV) immediate-early promoter, two multimer resolution sites (MRS) flanking the expression cassette, pMB1 origin of replication, kanamycin resistance gene and BGH polyadenylation sequence (Figure IV.1A). Plasmids coding for VEGF (pVAX-VEGF) were produced in *E. coli* DH5α strain and purified using an endotoxin-free plasmid DNA purification kit (Macherey-Nagel), as previously described (Madeira et al., 2011). The concentration of purified pDNA solutions was assayed by spectrophotometry at 260nm (NanoDrop, Thermo Scientific) and DNA integrity was confirmed by DNA agarose gels stained with ethidium bromide.

Minicircles were produced in a BW2P *E. coli* strain harboring the parental plasmid pMINLI-CV, according to previously established methods (Simcikova et al., 2014, Alves et al., 2016). *E. coli* BW2P was grown until the late exponential phase and then recombination was induced for 2 hours by addition of 0.01% (w/v) L-(+)arabinose (Merck). As a result of intramolecular recombination between the two MRS, both a minicircle with the expression cassette and a miniplasmid (MP) with the prokaryotic backbone sequences are obtained. Next, all plasmid DNA species were recovered and purified from the producer cells using an endotoxin-free plasmid DNA purification kit (Macherey-Nagel). Then, minicircle was separated from other DNA forms by performing a digestion with nicking endonuclease (*Nb.B*bvCI) followed by hydrophobic interaction chromatography (HIC), as described and optimized by our group (Alves et al., 2016, Alves et al., 2018).

IV.3.2. Isolation and culture of human bone marrow-derived MSC

Human bone marrow (BM)-derived MSC were isolated and expanded from healthy donors after informed consent as described elsewhere (dos Santos et al., 2010) and maintained cryopreserved in liquid/vapour phase nitrogen containers. Upon thawing, cells were cultured for 3 to 5 passages under xenogeneic(xeno)-free culture conditions as previously established (dos Santos et al., 2011, dos Santos et al., 2014). Cells were plated at a cell density between

3,000-6,000 cells/cm^2 on CELLstart™ CTS™ (Invitrogen) pre-coated T-flasks using StemPro® MSC SFM XenoFree (Invitrogen) supplemented with 1% GlutaMAX™-I CTS™ (Invitrogen) and 1% Antibiotic-Antimycotic (Invitrogen). Cells were maintained at 37ºC and 5% CO_2 in a humidified atmosphere and culture medium was exchange every 3-4 days. At 70% cell confluence, MSC were detached from the flasks by adding TrypLE™ Select CTS™ (Invitrogen) solution 1x in Phosphate Buffer Saline (PBS, Gibco). Cell number and viability were determined using the Trypan Blue (Gibco) exclusion method. BM MSC at passages between 3 and 5 were used, from four independent donors.

IV.3.3. Microporation of BM MSC with pVAX-VEGF and MC-VEGF

Microporation of BM MSC was performed as previously optimized (Madeira et al., 2011). For each condition, 1.5×10^6 cells were resuspended in 100 µl of resuspension buffer (buffer R, Invitrogen) and incubated with 10 µg of pVAX-VEGF (or the equivalent number of molecules of MC-VEGF). Electroporation was performed using the Neon® Transfection System and a Microporator MP-100 (Digital Bio, Invitrogen) using 1 pulse with 1,000 V of pulse voltage and 40 ms of width. After microporation, the cell suspension was incubated with 900 µL of Opti-MEM™ I Reduced Serum Medium (Gibco) for 20-30 min. Next, cells were plated at a density of 7,000-8,000 cells/cm^2 in pre-warmed StemPro® XenoFree culture medium. At each time point (day 2, day 5 and day 7), the number of cells was estimated using the trypan blue dye exclusion method. Cell recovery for each microporated sample was determined after 48 h, by calculating the ratio between viable cells in the condition where cells were microporated and viable cells in the control condition (non-electroporated), as described elsewhere (Madeira et al., 2011). Two controls were also prepared, a control for microporation process corresponding to MSC microporated without DNA and non-microporated cells, referred herein as 'control', which will be used for comparison in subsequent studies.

IV.3.4. *In vitro* multilineage differentiation potential and immunophenotype characterization of transfected cells

Upon 7 days after transfection, the osteogenic and adipogenic differentiation potential of cells transfected with both pVAX-VEGF and MC-VEGF were assessed as previously described using StemPro® Osteogenesis/Adipogenesis Differentiation Kits (Life Technologies) (dos Santos et al., 2011).

For immunophenotypic characterization of the engineered cells with the two vectors, these were analyzed by flow cytometry, 48 h and 7 days after transfection, using a panel of mouse anti-human monoclonal antibodies (PE-conjugated) against: CD34, CD45, CD90, CD73, CD80, CD14, CD105 and human leukocyte antigen (HLA)-DR (all from Biolegend). Cells were incubated with the monoclonal antibodies for 15 min in the dark at room temperature, then were washed in 2 mL of PBS and finally fixed with 1% paraformaldehyde (PFA, Sigma). Appropriate isotype controls (IgGγ1 and IgGγ2b) were also prepared. A minimum of 10,000 events was collected for each sample and the CellQuest (Becton Dickinson) and FlowJo® (LLC) software were used for acquisition and analysis, respectively.

IV.3.5. Quantification of VEGF expression by the transfected cells using qPCR and ELISA

To quantify the expression of VEGF by the cells transfected with pVAX-VEGF and MC-VEGF, both real time PCR (qPCR) and ELISA were performed. For qPCR, cells were harvested at each time point (day 2, day 5 and day 7), centrifuged and kept at -80 °C until further analysis. Total RNA was isolated using RNeasy Mini Kit (Qiagen). RNA was quantified by UV spectrophotometry (NanoDrop) and cDNA was synthetized with the iScript cDNA Synthesis Kit (Bio-Rad). The qPCR analysis was performed in a 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 reaction volume. The following primers (StabVida) were used for VEGF amplification: VEGF_fwd – GGAGGAGGGCAGAATCATCAC and VEGF_rev – GGTCTCGATTGGATGGCAGT. The $2^{-\Delta\Delta CT}$ method of relative quantification was applied to determine the fold change in mRNA expression (Livak and Schmittgen, 2001). GAPDH was used as the housekeeping gene and non-microporated MSC as a baseline.

For ELISA, culture supernatant was collected at each time point (day 2, day 5 and day 7), centrifuged at 500 g for 10 min and kept at -80 °C until further analysis. A Human VEGF-A ELISA kit (RayBiotech) was used, following the manufacturer's instructions.

IV.3.6. Preparation of conditioned medium for functional assays

To prepare conditioned media from cultured MSC for functional assays, control and MSC engineered with pVAX-VEGF or MC-VEGF were plated at a density of 12,500 cells/cm^2 using StemPro MSC SFM XenoFree for 24 h after microporation. After 24 h, the medium was changed to Endothelial Basal Medium (EBM-2, Lonza) and maintained for 48 h. Conditioned medium was

collected and normalized to cell number 72 h after transfection and kept at -80ºC after centrifugation. Fresh EBM-2 and Endothelial Cell Growth Medium (EGM-2, Lonza) were used as controls.

IV.3.7. Endothelial cell tube formation assay

A functional assay that relies in the capacity of human endothelial vein endothelial cells (HUVEC) to form tube networks when cultured in Matrigel was used to evaluate the angiogenic potential of modified cells (Arnaoutova et al., 2009). Conditioned medium (200 µl) derived from both control cultures and gene modified MSC (pVAX-VEGF and MC-VEGF) was used to cultivate commercially available HUVEC (from BD) on 96-well plates (2×10^4 cells/well) previously coated with Matrigel Basement Membrane Matrix (Corning) for 8 h at 37 ºC and 5% CO_2. After 8 h, tube formation was quantified and both tube length and tube connections were measured using microscope and ImageJ (NIH) software (Arnaoutova et al., 2009, Arutyunyan et al., 2016).

IV.3.8. Endothelial transwell migration assay

The migration of endothelial cells in response to soluble factors, such as VEGF, is one of the crucial events during angiogenesis (Carmeliet, 2000a). Herein conditioned medium collected from MSC transfected cultures was used as a stimulus and HUVEC's capacity to migrate through transwell inserts was quantified by using 8 µm pore transwell inserts (Millipore) previously coated with 10 µg/ml fibronectin (Sigma) for 1 h at 37 ºC (Goodwin, 2007, Chen et al., 2014b). After a wash with PBS, 50,000 HUVEC were added to the transwells using 100 µl of EBM-2, which were then inserted and maintained in a 24-well plate with 600 µl of conditioned medium for 6 h at 37 ºC and 5% CO_2. After 6 h, the transwells were washed with PBS and the cells in the upper chamber were removed with cotton swabs. The cells that migrated to the lower side of the insert were stained with crystal violet 0.5% (Sigma) for 30 min (Kadekar et al., 2015). After washing twice with PBS, each insert was observed under the microscope and the total number of migrated HUVEC per optical field was quantified (100x magnification).

IV.3.9. Statistical analysis

All data is presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 and significance was determined by Tukey's multiple comparison test and set at a p-value <0.05.

IV.4. Results

IV.4.1. Minicircle production and purification

Minicircles were produced in an *E. coli* strain BW2P as described (Simcikova et al., 2014, Alves et al., 2016). This strain contains a copy of the *parA* gene inserted in the bacterial chromosome under the control of the arabinose inducible P_{BAD}/AraC promoter (Jechlinger et al., 2004). Addition of L-arabinose induces expression of ParA resolvase, the enzyme that mediates intramolecular recombination between the two multimer resolution sites (MRS) in the parental plasmid (PP) backbone. This recombination generates a 1,715 bp minicircle (MC) with the expression cassette (MC-VEGF) and a 2,106 bp miniplasmid (MP) with the prokaryotic backbone sequences (Figure IV.1A). Recombination was confirmed by agarose gel electrophoresis analysis of purified DNA before induction (0 h) and 1 or 2 h after induction (Figure IV.1B). To isolate MC-

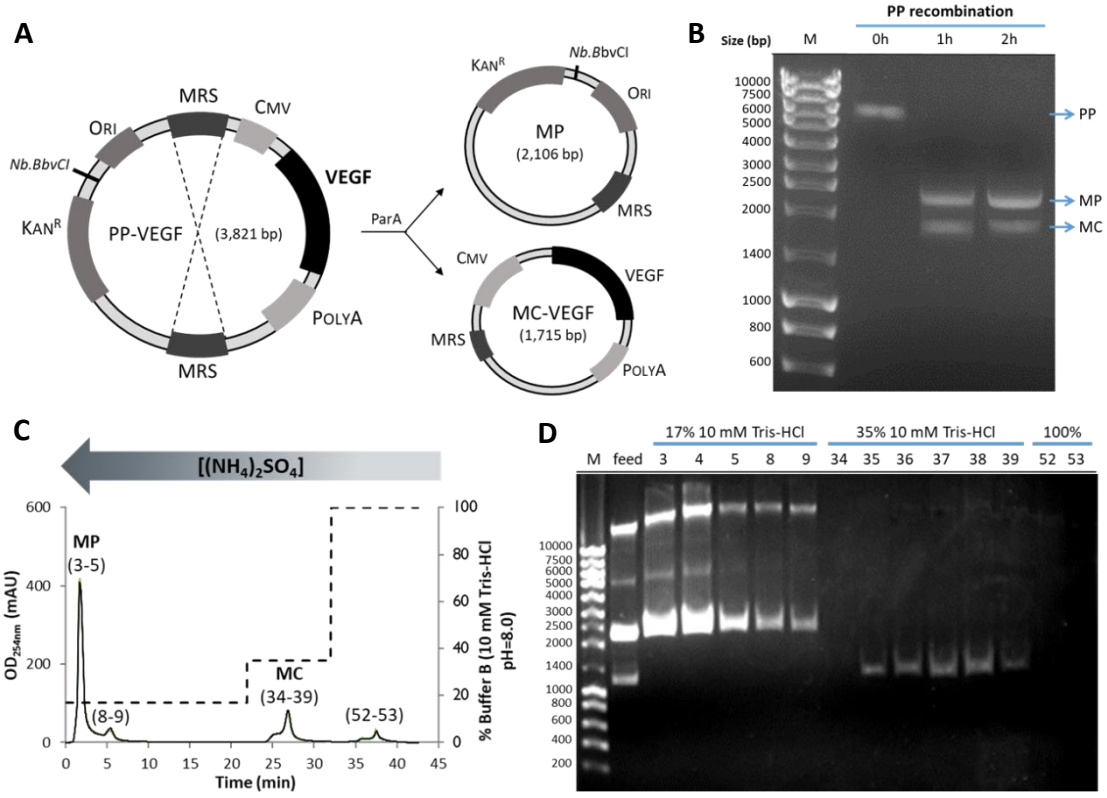

Figure IV.1 - Minicircle production and purification by E. coli BW2P and hydrophobic interaction chromatography (HIC). (**A**) Schematic representation of miniplasmid (MP) and minicircle (MC) formation by intramolecular recombination of the parental plasmid (PP) after induction of ParA resolvase expression with L-Arabinose. (**B**) Agarose gel electrophoresis analysis of pDNA purified from E. coli cells collected before (0 h) and after (1 h and 2 h) induction of recombination by L-arabinose. (**C**) Chromatogram (continuous line) of pre-purified samples (MC + MP) obtained after digestion with Nb.BbvCI loaded onto a phenyl-Sepharose column pre-equilibrated with 1.83 M ammonium sulphate (17% buffer B). Stepwise elution (% buffer B – dashed line) was performed according to a previously established protocol (Alves et al. 2016) at 2 mL/min with 4 CV of 17% buffer B, 2 CV of 35% buffer B (1.43 M) and 2 CV of 100% buffer B (0 M). Numbers over peaks correspond to collected fractions. CV – column volume. (**D**) Agarose gel electrophoresis analysis of fractions (20 µl) collected during the chromatographic run shown in C. The numbers above the lanes correspond to collected fractions.

VEGF from MP and un-recombined PP, a digestion step with the nicking enzyme *Nb.B*bvCI was performed after cell lysis and DNA purification, to convert supercoiled (sc) MP and PP molecules into the corresponding open circular (oc) isoforms. Since the nicking site for *Nb.B*bvCI is located in the prokaryotic backbone, the MC remains unaffected. Next, hydrophobic interaction chromatography was used to separate sc MC from oc DNA by exploring differences in the hydrophobicity of the molecules (Alves et al., 2016). A phenyl-Sepharose column was used and elution was promoted using a stepwise strategy with decreasing salt ($(NH_4)_2SO_4$) concentrations (Figure IV.1C). Agarose gel electrophoresis (Figure IV.1D) of eluted fractions showed that un-bound MP molecules are washed out of the column at 17% buffer B (fractions 3-5 e 8-9), MC-VEGF (fractions 35-39) is eluted by increasing buffer B to 35% and some residual RNA (fraction 52-53) is eluted during the last step by maximizing buffer B to 100%.

IV.4.2. Characterization of human MSC engineered with VEGF-encoding vectors: proliferative capacity, cell recovery, immunophenotype and multilineage differentiation potential

In this study, MSC from bone marrow (BM) cultured under xenogeneic(xeno)-free conditions (dos Santos et al., 2011) were transfected by microporation with two non-viral vectors encoding for VEGF: a plasmid vector (pVAX-VEGF) and a minicircle (MC-VEGF). Non-transfected cells were used as negative control, and cells microporated (without DNA) were also included in the study. The transfected cells were analyzed at days 2, 5 and 7 post transfection, according to a previously established transfection procedure (Boura et al., 2013). To evaluate the impact of microporation-based gene delivery on the proliferative capability of MSC, the number of viable cells was determined (Figure IV.2A). Non-transfected cells (used as a control) displayed the

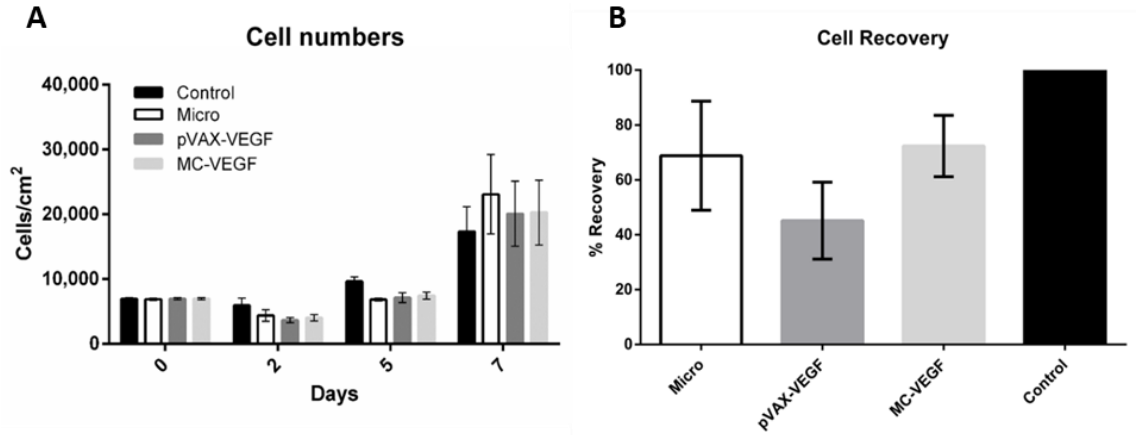

Figure IV.2 - Analysis of the BM MSC behavior after microporation with pVAX-VEGF and MC-VEGF. (A) Number of viable cells before (0) and after (2, 5 or 7 days) microporation. Values are mean±standard error of mean (SEM), n=4. (B) Cell recovery after microporation of MSC with pVAX-VEGF and MC-VEGF. Values are mean±SEM, n=4.

highest cell number at both day 2 and day 5, but at day 7 there are almost no differences between the groups. Cell recovery, which reflects the level of cell death in microporated samples (Figure IV.2B), was calculated at day 2, as described elsewhere (Madeira et al., 2011). Non-transfected cells were considered to have a recovery of 100% and lower values were observed for the conditions where cells were microporated. Cells microporated without DNA (Micro; used as a control for microporation process) or with MC-VEGF showed similar recoveries (68.8±19.8% and 72.3±11.2%, respectively), which were higher when compared to cells transfected with pVAX-VEGF (45.1±14.0%).

The differentiation potential and immunophenotype of microporated cells was assessed based on the minimal criteria proposed to define human MSC (Dominici et al., 2006). To test the effect of microporation on MSC multipotency, differentiation protocols towards osteogenic and adipogenic lineages were accomplished. By using lineage-specific stains, it was observed that cells engineered with the two vectors (pVAX-VEGF e MC-VEGF) were able to give rise to both osteocytes and adipocytes *in vitro* (Figure IV.3A). To evaluate the immnunophenotype of the modified cells, flow cytometry employing a panel of monoclonal antibodies against different surface markers was used. Regardless of the vector used, engineered MSC maintained their phenotypic profile after transfection, i.e. negative (<5%) for CD34, CD45, CD80, CD14 and HLA-DR and positive (>95%) for CD90, CD73 and CD105 (Figure IV.3B).

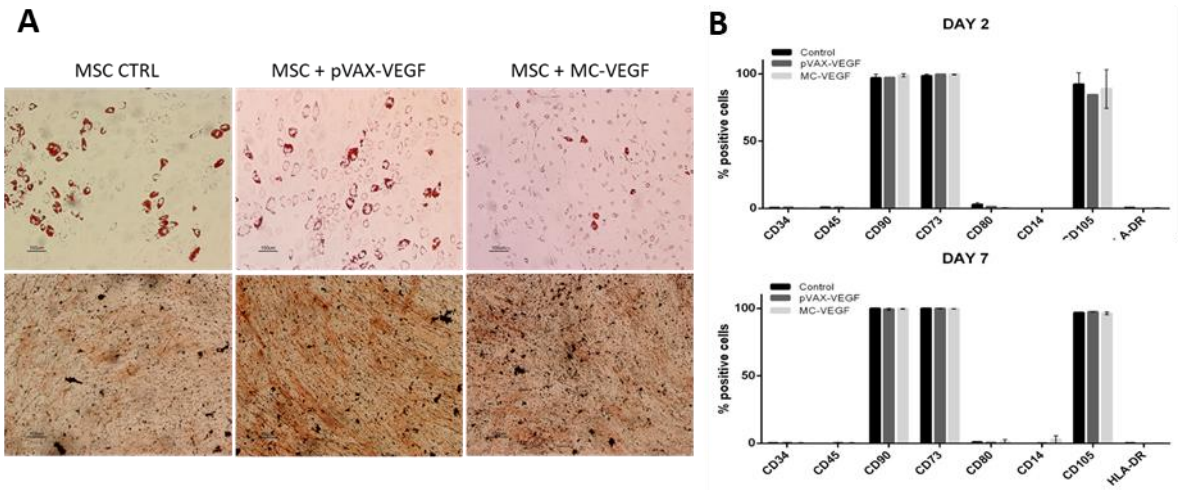

Figure IV.3 - Characterization of BM MSC after transfection with pVAX-VEGF and MC-VEGF. **(A)** Evaluation of MSC differentiation potential into adipocytes (up) and osteocytes (down) after microporation. **(B)** Phenotypic analysis of cells 2 and 7 days after microporation. Assessment of CD34, CD45, CD90, CD73, CD80, CD14, CD105 and HLA-DR surface markers. Values are mean±SEM, n=3.

IV.4.3. Quantification of VEGF expression and secretion by engineered MSC

VEGF production was evaluated at days 2, 5 and 7 both by qPCR (Figure IV.4A) and ELISA (Figure IV.4B). Two days after microporation, MSC transfected with MC-VEGF showed 130-fold higher mRNA copies of VEGF than non-transfected cells (control) ($p<0.01$), while cells transfected with pVAX-VEGF showed just a 50-fold increase on VEGF mRNA copies compared to the control ($p<0.05$). The VEGF expression decreased from day 2 to day 5, remaining higher for transfected cells when compared with the control (though differences were no longer statistically significant). Finally, on day 7 the expression of VEGF was almost the same for transfected and non-transfected cells. Overall, for the three time points tested the highest VEGF expression was observed for cells transfected with MC-VEGF.

The profiles of VEGF secretion into the culture medium were very similar. Highest VEGF secretion rates were observed at day 2 for cells transfected with MC-VEGF (644.8±82.5 pg/1,000 cells day^{-1}), followed by pVAX-VEGF transfected MSC (508.3±164.0 pg/1,000 cells day^{-1}). No significant VEGF secretion (11.1±3.4 pg/1,000 cells day^{-1}) was observed for control MSC (non-transfected). Though the VEGF production rate decreased until day 5, the trend observed was similar to day 2 with highest VEGF secretion for MC-VEGF (212.4±50.9 pg/1,000 cells day^{-1}) supernatant and almost no VEGF on control samples (4.8±1.5 pg/1,000 cells day^{-1}). At day 7 there were only smaller differences on production rates for engineered cells (87.7±27.7 and 33.2±16.5 pg/1,000 cells day^{-1} for MC-VEGF and pVAX-VEGF, respectively) compared to the control (17.4±3.5 pg/1,000 cells day^{-1}).

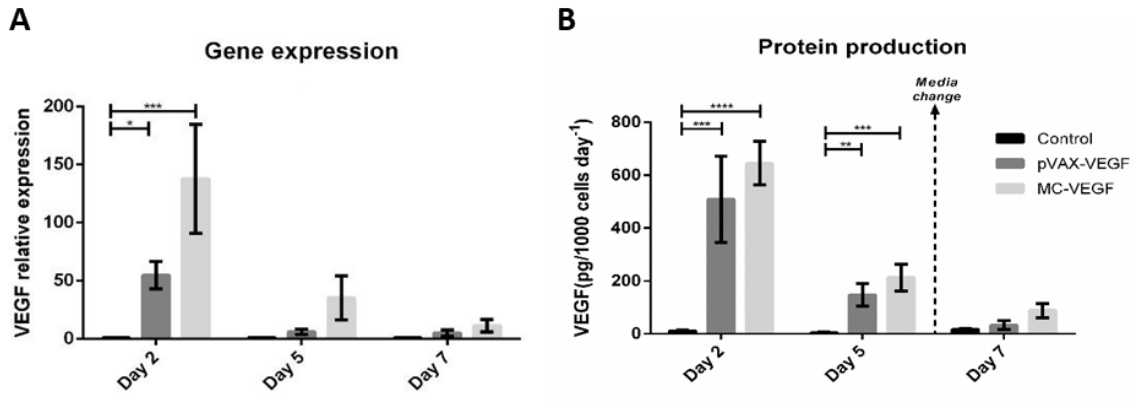

Figure IV.4 - Evaluation of transgene delivery 2, 5 or 7 days after microporation with pVAX-VEGF and MC-VEGF. Analysis of BM MSC VEGF **(A)** gene expression by qPCR and **(B)** protein production by ELISA. Each bar represents the mean ± SEM, n=4, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

IV.4.4. Angiogenic capacity of the engineered MSC assessed by *in vitro* functional assays

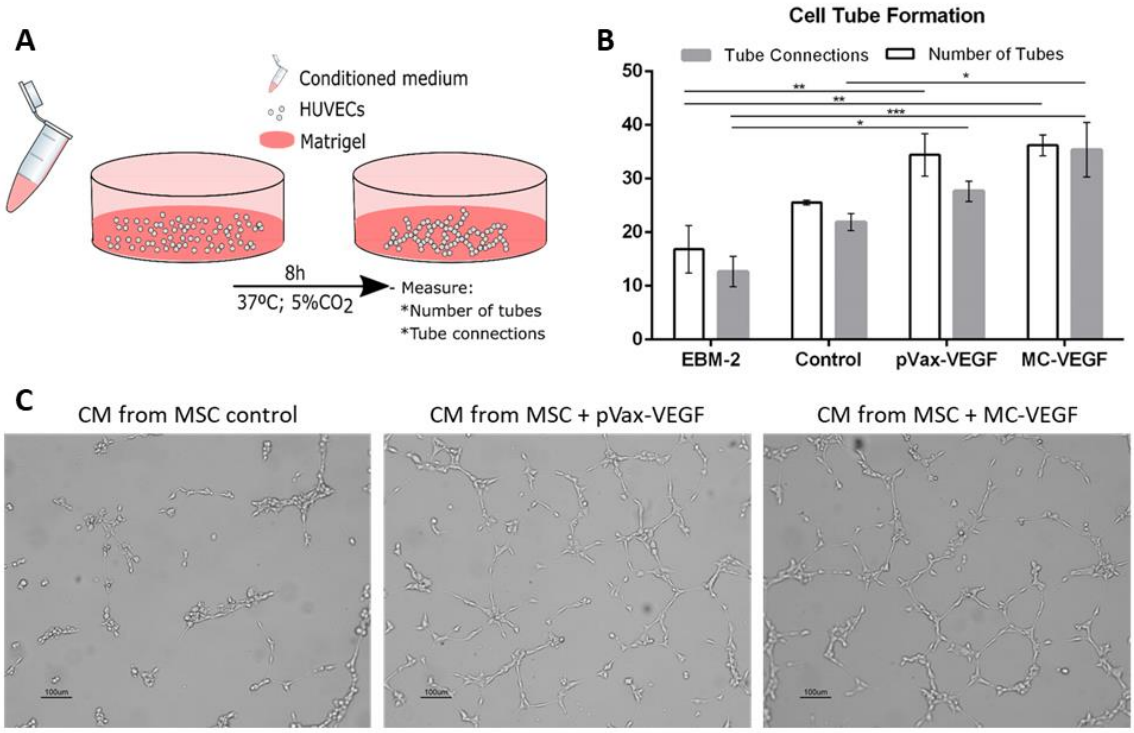

Figure IV.5 - Cell tube formation assay. **(A)** Schematic representation of the assay: HUVEC were cultured in Matrigel with conditioned medium from modified (with pVAX-VEGF and MC-VEGF) and unmodified MSC (control) for 8 h. **(B)** Number of tubes and branch points observed per optical field after 8 h for each condition tested. Values are presented as mean ± SEM, n=4, *p<0.05; **p<0.01; ***p<0.001. **(C)** Images of tube formation by HUVEC after being cultured for 8 h with conditioned medium (CM) from MSC alone, MSC modified with pVAX-VEGF and MSC modified with MC-VEGF.

To evaluate the angiogenic potential of modified cells, conditioned medium retrieved 72 h after microporation (or culture) from cultures of transfected and non-transfected (control) MSC, as described in *Materials and Methods,* was used for the cultivation of HUVEC on Matrigel coated surfaces (Figure IV.5A). After 8 h, cell tube formation by HUVEC was observed and quantified with regard to the number of tubes and branch points (Figures IV.5B and IV.5C). The conditioned media retrieved from cultures of cells modified with pVAX-VEGF and MC-VEGF induced the formation of more tubes (34.4±4.0 and 36.2±1.9, respectively), as well as connections (27.7±1.9 and 35.4±5.1, respectively), compared to the basal medium EBM-2 (negative control) ($p < 0.05$ for pVAX-VEGF and $p<0.01$ for MC-VEGF) or conditioned medium obtained from cultures of non-transfected cells ($p<0.05$ for MC-VEGF). Although the differences between MC-VEGF and pVAX-VEGF were not statistically significant, conditioned medium from cells transfected with MC-VEGF showed an increased potential to induce tube formation by HUVEC, suggesting a higher angiogenic potential compared to MSC transfected with pVAX-VEGF.

The migration ability of HUVEC towards a conditioned medium obtained from cultured MSC was also evaluated, as schematized in Figure IV.6A. HUVEC were cultured for 6 h in the upper part of the transwell, while conditioned medium from transfected (with pVAX-VEGF or MC-VEGF) and non-transfected cells was kept in culture plate wells where transwells were placed. HUVEC cells that migrated through the transwell pores were counted and the values were normalized

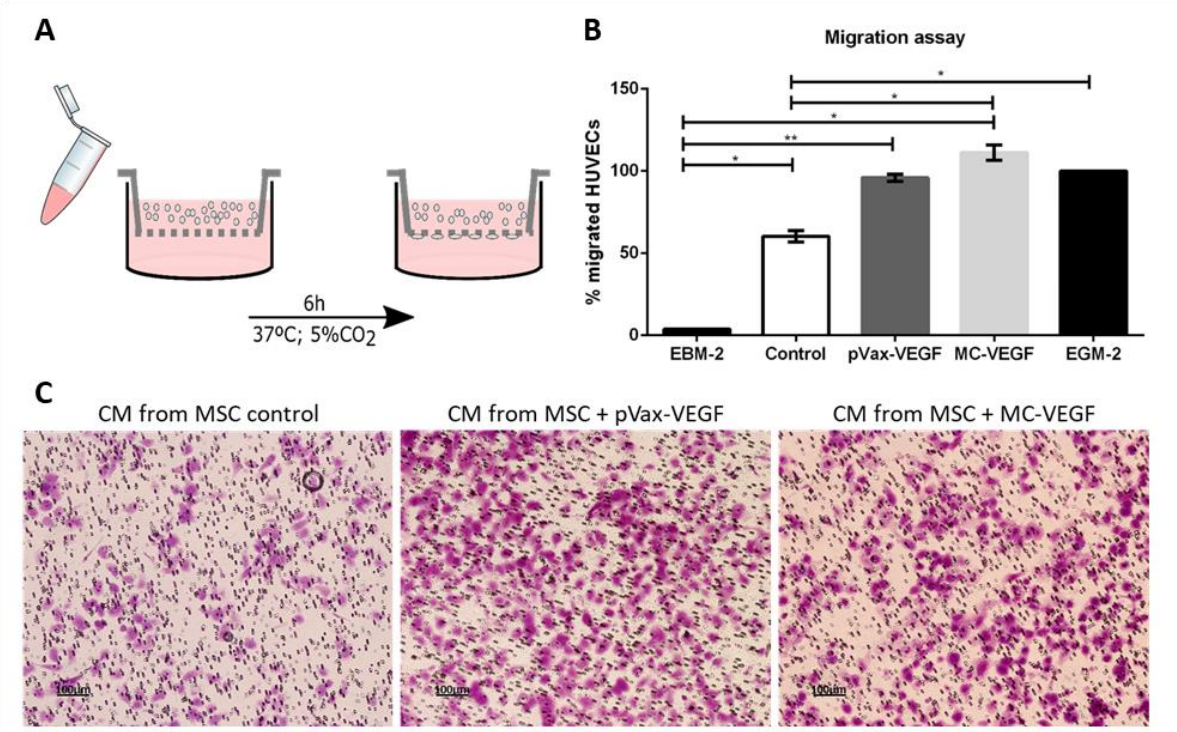

Figure IV.6 - Transwell migration assay. **(A)** Schematic representation of the assay: HUVEC were cultured in a transwell within a plate with conditioned medium from modified (with pVAX-VEGF and MC-VEGF) and unmodified MSC (control) for 6 h. **(B)** Percentage of HUVEC that migrated through the transwell towards conditioned medium samples normalized to a positive control (EGM-2). Values are presented as mean ± SEM, n=3, *p<0.05; **p<0.01 **(C)** HUVEC that migrated trough the transwell towards conditioned medium (CM) from MSC alone, MSC modified with pVAX-VEGF and MSC modified with MC-VEGF stained with crystal violet 0.5%.

relatively to a positive control where complete culture medium for endothelial cell growth was employed (EGM-2) (Figures IV.6A and IV.6B). Conditioned medium retrieved from cultures of MSC engineered with MC-VEGF cultured for 72 h induced the highest HUVEC migration (111.3±4.6%). Furthermore, the difference between this and culture supernatant from control cells (non-transfected) (60.3±3.6%) was statistically significant ($p<0.05$). Conditioned medium obtained from cells transfected with pVAX-VEGF (also after 72 h of culture) also lead to high HUVEC migration rates (96.0±2.2%), with values similar to the positive control (100%).

IV.5. Discussion

MSC have been extensively exploited in experimental clinical studies for the treatment of a wide range of conditions (cardiovascular, neurological or autoimmune disorders (Hare et al., 2009, Liang et al., 2010, Sato et al., 2010, Connick et al., 2011)), due to their unique properties, namely their trophic activity and immunomodulatory potential. In this context, strategies to improve their potential therapeutic effects have been developed, namely genetic engineering for the overexpression of specific therapeutic proteins in a setting of *ex vivo* gene therapy (Myers et al., 2010, Nowakowski et al., 2015). Although MSC are able to efficiently overexpress genes transferred by viral vectors, this strategy has the inherent potential risks of oncogene activation or tumor-suppressor gene inactivation, as well as immunogenic responses (Steven et al., 2003, Collins et al., 2009). Although safer than viral vectors, plasmid DNA vectors can still produce immunological responses and silence transgene expression due to the presence of bacterial regions in the backbone (e.g. bacterial origin of replication, antibiotic resistance marker) (Zhao et al., 2004, Riu et al., 2007, Lu et al., 2012). Minicircles, on the other hand, can be less immunogenic (and thus safer) due to the reduced content of bacterial sequences rich in unmethylated CpG residues that may activate the immune system, and are more efficiently delivered into stem cells by microporation due to their lower size compared to plasmid vectors (Darquet et al., 1997, Gaspar et al., 2015).

The angiogenic factor VEGF has been extensively investigated in the context of ischemia treatment in PAD in both pre-clinical studies (Walder et al., 1996, Becit et al., 2001, Yasumura et al., 2012, Anderson et al., 2017) and clinical trials (Isner et al., 1996, Kusumanto et al., 2006, Deev et al., 2015). In fact, there is already an approved gene therapy product for PAD treatment (Neovasculgen®) which is based on a plasmid DNA encoding the VEGF165 gene under the control of the CMV promoter (Deev et al., 2015).

Efforts have also been made to explore the angiogenic potential of MSC to treat PAD (Yan et al., 2013). Nevertheless, these non-stimulated cells secrete limited levels of VEGF (lower than 20 pg/1,000 cells day^{-1}), as shown by us and others (Singer and Caplan, 2011). In this work, we sought to exploit the inherent regenerative properties of MSC and the angiogenic role of VEGF by transiently engineering these cells to overexpress this factor. VEGF-containing minicircles (MC-VEGF) produced and purified by a recently established protocol (Simcikova et al., 2014, Alves et al., 2016), as well as a conventional plasmid vector (pVAX-VEGF), were efficiently used to transfect BM MSC by microporation, an electroporation-based method with reduced electrode surface area to diminish cell mortality (Kim et al., 2008). The therapeutic features of

the modified cells were verified by VEGF quantification, but also by *in vitro* functional assays that were developed to confirm the angiogenic potential of these cells and predict their effect *in vivo* (Sanz-Nogués and O'Brien, 2016). All the work was developed under xeno(geneic)-free conditions already established for MSC culture at our group, in order to achieve a reproducible, safe and reliable MSC cell therapy product (dos Santos et al., 2011).

In most studies where minicircles have been tested for the genetic modification of human MSC, commercially available molecules (Daneshvar et al., 2015) or minicircles purified with a different method were used (Kay et al., 2010, Hyun et al., 2013, Mun et al., 2016, Park et al., 2017). The purification approach used in the study by Kay and co-workers is based on an inducible-SceI nuclease integrated in the bacterial chromosome that degrades miniplasmid (MP) species *in vivo* (Kay et al., 2010). Our methodology for vector manufacturing has at least two advantages: (1) it takes place *in vitro,* so is easier to control and does not impose an extra metabolic burden on producer cells and (2) the use of HIC allows separation of supercoiled from other MC isoforms (Alves et al., 2016). The method was effective for the purification of MC-VEGF and we obtained isolated fractions of supercoiled MC-VEGF that were ready for the transfection of MSC.

The parameter used to evaluate cell integrity after transfection in most published works is cell viability (Lim et al., 2010, Abdul Halim et al., 2014), but high viabilities can be observed even when few cells are recovered from electroporation process. Herein, we evaluated cell recovery (Figure IV.2B), which was observed to be higher when cells were microporated with MC-VEGF (72.3±11.2%) compared to pVAX-VEGF (45.1±14.0%). The cell recoveries observed for MC-VEGF were greater than those reported by Aluigi and co-workers (≈45%), who used the same cell type but another electroporation method – nucleofection - and a different DNA molecule (Aluigi et al., 2017). Another report using a similar protocol showed comparable cell recoveries for the microporation of BM MSC (Madeira et al., 2011). The high recoveries observed herein for MC-VEGF may represent an advantage over common plasmids from the clinical standpoint, as an increased number of cells can be recovered from the same initial cell number.

Transfection with either MC-VEGF or pVAX-VEGF did not change MSC features, namely *in vitro* differentiation capacity and immunophenotype, proposed as minimal criteria for MSC identity (Dominici et al., 2006), in agreement with other studies using plasmid vectors (Lim et al., 2010, Madeira et al., 2011, Abdul Halim et al., 2014). Of notice, although microporation process seems to slow-down the proliferation rate of the cells in culture even without DNA, microporated MSC have their growth potential completely recovered after 7 days, reaching the same numbers that non-transfected control cells. Furthermore, for MSC microporated with MC-VEGF, the recoveries were higher (72.3±11.2%) and similar to microporation control (68.8±19.8%) when

compared to values obtained for pVAX-VEGF (45.1±14.0%), showing, as expected, that small MC-VEGF molecules causes less cell damage than the conventional plasmids (Boura et al., 2014).

The efficiency of the protocol of MSC microporation with MC-VEGF or pVAX-VEGF was firstly evaluated by quantifying VEGF levels in terms of gene expression (qPCR) and protein production (ELISA). Two days after transfection, cells transfected with minicircles showed higher VEGF production (644.8±82.5 pg/1,000 cells day^{-1}) than cells modified with pVAX (508.3±164.0 pg/1,000 cells day^{-1}), reflected not only by protein amount in culture supernatant but also by VEGF mRNA copies. Analysis of VEGF copies revealed an increase of ≈130-fold and ≈50-fold for BM MSC transfected with MC-VEGF and pVAX-VEGF, respectively, when compared to non-modified cells. The rise observed in protein production rate compared to the control was ≈59-fold for MC-VEGF and ≈46-fold for pVAX-VEGF. In both analyses the same tendency was verified, with higher values observed for MC-VEGF samples. The smaller change observed between the two tested vectors in what concerns protein amount, when compared to mRNA copies, can be due to the short half-life of the VEGF protein, which is approximately 50 minutes (Faranesh et al., 2004).

Even though viral vectors have been proposed as more efficient for cell engineering, our transfection protocol showed an increased VEGF expression compared to some reports where virus were used. Beegle and colleagues reported that using lentivirus to overexpress VEGF in bone marrow MSC for hind limb ischemia led to an increase of 10-fold in VEGF protein production compared to non-transduced cells measured by ELISA 72 h after viral transduction (Beegle et al., 2016). In another study, a combination of adenovirus transduction with microencapsulation was developed to induce VEGF overexpression and promote vascularization of tissue-engineered dermis. Here, maximum VEGF production rates measured by ELISA were observed 8 days after transduction with a 20-fold increase in VEGF production rates relatively to non-transduced cells (Han et al., 2014). Although in the mentioned work VEGF levels were maintained elevated for a longer period, being still detectable after 14 days, our strategy led to higher fold-increase in VEGF production (59-fold) regarding basal levels observed in non-modified cells.

In a recent study reporting the use of minicircles to transfect bone marrow MSC using microporation, the transfection efficiency observed was around 40% (Mun et al., 2016). Although our system cannot be directly compared with this since a reporter gene (luciferase) was used to evaluate transfection and a different therapeutic gene (CXCR4) was tested, we showed similar and promising results using a therapeutic pro-angiogenic protein (without the use of a reporter that may affect VEGF expression) in a completely xeno-free setting. Our

analysis was not focused only on transgene expression, but also in the function of the obtained cell therapy product, so we tested our modified cells with *in vitro* functional studies that mimic *in vivo* angiogenesis processes.

VEGF is known to recruit and promote migration of endothelial cells, two important steps in blood vessel formation and remodeling. In order to evaluate the biological activity of the expressed VEGF in this regard, functional assays were performed to predict and evaluate the angiogenic potential of BM MSC engineered with pVAX-VEGF and MC-VEGF by microporation (Chang et al., 2014). Experiments were thus set up where conditioned media from cultures of gene modified and control cells were used as a stimulus. In particular, endothelial cell tube formation assays were used to evaluate the capacity of HUVEC to form tubular structures and transwell migration assays were used to assess the ability of endothelial cells to migrate towards a stimulus, in this case the high VEGF levels, since this is one of the key mechanisms underlying angiogenesis (Carmeliet, 2000a, Goodwin, 2007).

The results for cell tube formation were in accordance with the results for VEGF expression, since conditioned medium from cells modified with MC-VEGF lead to the formation of the highest number of tubes (36.2±1.9) and branchpoints (35.4±5.1) by HUVEC. Supernatants retrieved from cultures transfected with pVAX-VEGF also contributed to an increased amount of tubes (34.4±3.9) and tube connections (27.7±1.9) compared to non-modified cells (25.6±0.4 tubes and 21.9±1.6 connections). It was expected that high VEGF levels would maximize HUVEC tube formation capacity since, as an angiogenic factor, VEGF increases the potential of endothelial cells to re-organize and form this tube-like structures that resemble blood vessels (Arutyunyan et al., 2016). Similar results were observed for cell migration studies performed with HUVEC using conditioned media retrieved from MSC cultures, where induction with complete endothelial growth medium (EGM-2) was set as 100% migration. For the conditioned medium obtained from cultures of MC-VEGF-transfected cells, the highest migration (111.3±4.6%) of HUVEC throughout the transwell pores was observed, followed by the supernatant from cells transfected with pVAX-VEGF (96.0±2.1%) that led to a migration similar to the set control (100%). Once again, these results are related with VEGF amount in culture medium that is also recognized as recruiter of endothelial cells, thus promoting HUVEC migration (Arutyunyan et al., 2016). These results suggest that modification of MSC using VEGF-containing vectors may contribute to an improvement of the angiogenic potential of these cells, verified by an increase in endothelial cell migration and organization into vessel-like structures.

The *ex vivo* gene therapy strategy developed herein primes an increased VEGF secretion by MSC within a short timeframe (≈2-5 days), which can be advantageous for some critical situations

where a fast intervention is required. In this scenario, upon the administration of the modified MSC, the peak of VEGF production will start by promoting endothelial cell migration and proliferation, thus accelerating angiogenesis at the injury site. Then, the decrease in VEGF secretion will avoid an uncontrolled angiogenic process and, therefore, an abnormal blood vessel growth which, in most severe cases can potentially lead to hemangiomas or even cancer (Folkman, 1971, Lee et al., 2000).

Overall, transfection of MC-VEGF into BM MSC showed not only to induce higher VEGF levels compared to pVAX-VEGF, but also resulted in an increased angiogenic potential of these cells as assessed in *in vitro* functional studies. Although the differences may not be statistically significant, the use of these smaller vectors present other advantages, being safety one of the most important. Minicircles have lower size and have reduced numbers of unmethylated CpG sequences, which are commonly found in bacterial DNA and have been documented to trigger immune response through TLR9 activation (Häcker et al., 2002, Walker et al., 2010, Boura et al., 2014). Further studies are required in this field, such as evaluation of TLR9 expression after transfection, since we recently observed an increase in TLR9 even when small DNA molecules as minicircles are used (Boura et al., 2014).

Even though further studies are required, namely assessment of therapeutic potential *in vivo* on a limb ischemia model to evaluate and predict the efficacy of this approach in PAD, this study showed encouraging results on the efficient non-viral gene modification of BM MSC towards the establishment of an angiogenic *ex vivo* gene therapy strategy to further improve the intrinsic therapeutic features of MSC.

IV.6. References

ABDUL HALIM, N. S., FAKIRUDDIN, K. S., ALI, S. A. & YAHAYA, B. H. 2014. A comparative study of non-viral gene delivery techniques to human adipose-derived mesenchymal stem cell. *Int J Mol Sci,* 15**,** 15044-60.

ALLISON, M. 2009. Genzyme backs Osiris, despite Prochymal flop. *Nat Biotech,* 27**,** 966-967.

ALUIGI, M., FOGLI, M., CURTI, A., ISIDORI, A., GRUPPIONI, E., CHIODONI, C., COLOMBO, M. P., VERSURA, P., D'ERRICO-GRIGIONI, A., FERRI, E., BACCARANI, M. & LEMOLI, R. M. 2017. Nucleofection Is an Efficient Nonviral Transfection Technique for Human Bone Marrow–Derived Mesenchymal Stem Cells. *STEM CELLS,* 24**,** 454-461.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2016. Development of a nicking endonuclease-assisted method for the purification of minicircles. *Journal of Chromatography A,* 1443**,** 136-144.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2018. Production and Purification of Supercoiled Minicircles by a Combination of *in vitro* Endonuclease Nicking and Hydrophobic Interaction Chromatography. *Human Gene Therapy Methods,* (in press).

ANDERSON, E. M., SILVA, E. A., HAO, Y., MARTINICK, K. D., VERMILLION, S. A., STAFFORD, A. G., DOHERTY, E. G., WANG, L., DOHERTY, E. J., GROSSMAN, P. M. & MOONEY, D. J. 2017. VEGF and IGF Delivered from Alginate Hydrogels Promote Stable Perfusion Recovery in Ischemic Hind Limbs of Aged Mice and Young Rabbits. *J Vasc Res,* 54**,** 288-298.

ANKRUM, J. A., ONG, J. F. & KARP, J. M. 2014. Mesenchymal stem cells: immune evasive, not immune privileged. *Nat Biotechnol,* 32**,** 252-60.

ARNAOUTOVA, I., GEORGE, J., KLEINMAN, H. K. & BENTON, G. 2009. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis,* 12**,** 267-274.

ARUTYUNYAN, I., FATKHUDINOV, T., KANANYKHINA, E., USMAN, N., ELCHANINOV, A., MAKAROV, A., BOLSHAKOVA, G., GOLDSHTEIN, D. & SUKHIKH, G. 2016. Role of VEGF-A in angiogenesis promoted by umbilical cord-derived mesenchymal stromal/stem cells: in vitro study. *Stem Cell Research & Therapy,* 7.

AZZONI, A. R., RIBEIRO, S. C., MONTEIRO, G. A. & PRAZERES, D. M. F. 2007. The impact of polyadenylation signals on plasmid nuclease-resistance and transgene expression. *The Journal of Gene Medicine,* 9**,** 392-402.

BECIT, N., CEVIZ, M., KOCAK, H., YEKELER, I., UNLU, Y., CELENK, C. & AKIN, Y. 2001. The effect of vascular endothelial growth factor on angiogenesis: an experimental study. *Eur J Vasc Endovasc Surg,* 22**,** 310-6.

BEEGLE, J. R., MAGNER, N. L., KALOMOIRIS, S., HARDING, A., ZHOU, P., NACEY, C., WHITE, J. L., PEPPER, K., GRUENLOH, W., ANNETT, G., NOLTA, J. A. & FIERRO, F. A. 2016. Preclinical evaluation of mesenchymal stem cells overexpressing VEGF to treat critical limb ischemia. *Molecular Therapy — Methods & Clinical Development,* 3**,** 16053.

BOURA, J. S., SANTOS, F. D., GIMBLE, J. M., CARDOSO, C. M. P., MADEIRA, C., CABRAL, J. M. S. & SILVA, C. L. D. 2013. Direct Head-To-Head Comparison of Cationic Liposome-Mediated Gene Delivery to Mesenchymal Stem/Stromal Cells of Different Human Sources: A Comprehensive Study. *Human Gene Therapy Methods,* 24**,** 38-48.

BOURA, J. S., VANCE, M., YIN, W., MADEIRA, C., LOBATO DA SILVA, C., PORADA, C. D. & ALMEIDA-PORADA, G. 2014. Evaluation of gene delivery strategies to efficiently overexpress functional HLA-G on human bone marrow stromal cells. *Mol Ther Methods Clin Dev,* 2014.

CARMELIET, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med,* 6**,** 389-95.

CHANG, C.-W., CHRISTENSEN, L. V., LEE, M. & KIM, S. W. 2008. Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy. *Journal of Controlled Release,* 125**,** 155-163.

CHANG, M.-C., TSAO, C.-H., HUANG, W.-H., CHIH-HSUEH CHEN, P. & HUNG, S.-C. 2014. Conditioned medium derived from mesenchymal stem cells overexpressing HPV16 E6E7 dramatically improves ischemic limb. *Journal of Molecular and Cellular Cardiology,* 72**,** 339-349.

CHEN, L., XU, Y., ZHAO, J., ZHANG, Z., YANG, R., XIE, J., LIU, X. & QI, S. 2014. Conditioned medium from hypoxic bone marrow-derived mesenchymal stem cells enhances wound healing in mice. *PLoS One,* 9**,** e96161.

CHEN, Z. Y., HE, C. Y., EHRHARDT, A. & KAY, M. A. 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther,* 8**,** 495-500.

COLLINS, M. B., SAM, L. S., SEAN, K., EVELIEN, F. G., IAIN, C. R., YASUHIRO, T. & MARY, K. 2009. Insertional Gene Activation by Lentiviral and Gammaretroviral Vectors.

CONNICK, P., KOLAPPAN, M., PATANI, R., SCOTT, M. A., CRAWLEY, C., HE, X. L., RICHARDSON, K., BARBER, K., WEBBER, D. J., WHEELER-KINGSHOTT, C. A., TOZER, D. J., SAMSON, R. S., THOMAS, D. L., DU, M. Q., LUAN, S. L., MICHELL, A. W., ALTMANN, D. R., THOMPSON, A. J., MILLER, D. H., COMPSTON, A. & CHANDRAN, S. 2011. The mesenchymal stem cells in multiple sclerosis (MSCIMS) trial protocol and baseline cohort characteristics: an open-label pre-test: post-test study with blinded outcome assessments. *Trials,* 12**,** 62.

CUNHA, F. F. D., MARTINS, L., MARTIN, P. K. M., STILHANO, R. S., PAREDES GAMERO, E. J. & HAN, S. W. 2013. Comparison of treatments of peripheral arterial disease with mesenchymal stromal cells and mesenchymal stromal cells modified with granulocyte and macrophage colony-stimulating factor. *Cytotherapy,* 15**,** 820-829.

DANESHVAR, N., RASEDEE, A., SHAMSABADI, F. T., MOEINI, H., MEHRBOUD, P., RAHMAN, H. S., BOROOJERDI, M. H. & VELLASAMY, S. 2015. Induction of pluripotency in human umbilical cord mesenchymal stem cells in feeder layer-free condition. *Tissue Cell,* 47**,** 575-82.

DARQUET, A. M., CAMERON, B., WILS, P., SCHERMAN, D. & CROUZET, J. 1997. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther,* 4**,** 1341-9.

DARQUET, A. M., RANGARA, R., KREISS, P., SCHWARTZ, B., NAIMI, S., DELAERE, P., CROUZET, J. & SCHERMAN, D. 1999. Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer. *Gene Ther,* 6**,** 209-18.

DEEV, R. V., BOZO, I. Y., MZHAVANADZE, N. D., VORONOV, D. A., GAVRILENKO, A. V., CHERVYAKOV, Y. V., STAROVEROV, I. N., KALININ, R. E., SHVALB, P. G. & ISAEV, A. A. 2015. pCMV-vegf165 Intramuscular Gene Transfer is an Effective Method of Treatment for Patients With Chronic Lower Limb Ischemia. *J Cardiovasc Pharmacol Ther,* 20**,** 473-82.

DI NICOLA, M., CARLO-STELLA, C., MAGNI, M., MILANESI, M., LONGONI, P. D., MATTEUCCI, P., GRISANTI, S. & GIANNI, A. M. 2002. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood,* 99**,** 3838-43.

DIETZ, W. M., SKINNER, N. E., HAMILTON, S. E., JUND, M. D., HEITFELD, S. M., LITTERMAN, A. J., HWU, P., CHEN, Z. Y., SALAZAR, A. M., OHLFEST, J. R., BLAZAR, B. R., PENNELL, C. A. & OSBORN, M. J. 2013. Minicircle DNA is superior to plasmid DNA in eliciting antigen-specific CD8+ T-cell responses. *Mol Ther,* 21**,** 1526-35.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-7.

DOS SANTOS, F., ANDRADE, P. Z., ABECASIS, M. M., GIMBLE, J. M., CHASE, L. G., CAMPBELL, A. M., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Toward a Clinical-Grade Expansion of Mesenchymal Stem Cells from Human Sources: A Microcarrier-Based Culture System Under Xeno-Free Conditions. *Tissue Engineering. Part C, Methods,* 17**,** 1201-1210.

DOS SANTOS, F., ANDRADE, P. Z., BOURA, J. S., ABECASIS, M. M., DA SILVA, C. U. L. & CABRAL, J. M. S. 2010. Ex vivo expansion of human mesenchymal stem cells: A more effective cell proliferation kinetics and metabolism under hypoxia. *Journal of Cellular Physiology,* 223**,** 27-35.

DOS SANTOS, F., CAMPBELL, A., FERNANDES-PLATZGUMMER, A., ANDRADE, P. Z., GIMBLE, J. M., WEN, Y., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2014. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. *Biotechnology and Bioengineering,* 111**,** 1116-1127.

FARANESH, A. Z., NASTLEY, M. T., DE LA CRUZ, C. P., HALLER, M. F., LAQUERRIERE, P., LEONG, K. W. & MCVEIGH, E. R. 2004. In Vitro Release of Vascular Endothelial Growth Factor From Gadolinium-Doped Biodegradable Microspheres. *Magn Reson Med,* 51**,** 1265-71.

FERRARA, N., GERBER, H.-P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9**,** 669-676.

FOLKMAN, J. 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med,* 285**,** 1182-6.

GASPAR, V., DE MELO-DIOGO, D., COSTA, E., MOREIRA, A., QUEIROZ, J., PICHON, C., CORREIA, I. & SOUSA, F. 2015. Minicircle DNA vectors for gene therapy: advances and applications. *Expert Opin Biol Ther,* 15**,** 353-79.

GEBLER, A., ZABEL, O. & SELIGER, B. 2012. The immunomodulatory capacity of mesenchymal stem cells. *Trends in Molecular Medicine,* 18**,** 128-134.

GOODWIN, A. M. 2007. In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvasc Res,* 74**,** 172-83.

GOWING, G., SVENDSEN, S. & SVENDSEN, C. N. 2017. Ex vivo gene therapy for the treatment of neurological disorders. *Prog Brain Res,* 230**,** 99-132.

GRESELE, P., BUSTI, C. & FIERRO, T. 2011. Critical limb ischemia. *Internal and Emergency Medicine,* 6**,** 129-134.

HÄCKER, G., REDECKE, V. & HÄCKER, H. 2002. Activation of the immune system by bacterial CpG-DNA. *Immunology,* 105**,** 245-51.

HAN, Y., TAO, R., HAN, Y., SUN, T., CHAI, J., XU, G. & LIU, J. 2014. Microencapsulated VEGF gene–modified umbilical cord mesenchymal stromal cells promote the vascularization of tissue-engineered dermis: an experimental study. *Cytotherapy,* 16**,** 160-169.

HARE, J. M., TRAVERSE, J. H., HENRY, T. D., DIB, N., STRUMPF, R. K., SCHULMAN, S. P., GERSTENBLITH, G., DEMARIA, A. N., DENKTAS, A. E., GAMMON, R. S., HERMILLER, J. B., JR., REISMAN, M. A., SCHAER, G. L. & SHERMAN, W. 2009. 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,* 54**,** 2277-86.

HYUN, J., GROVA, M., NEJADNIK, H., LO, D., MORRISON, S., MONTORO, D., CHUNG, M., ZIMMERMANN, A., WALMSLEY, G. G., LEE, M., DALDRUP-LINK, H., WAN, D. C. & LONGAKER, M. T. 2013. Enhancing in vivo survival of adipose-derived stromal cells through Bcl-2 overexpression using a minicircle vector. *Stem Cells Transl Med,* 2**,** 690-702.

ISNER, J. M., PIECZEK, A., SCHAINFELD, R., BLAIR, R., HALEY, L., ASAHARA, T., ROSENFIELD, K., RAZVI, S., WALSH, K. & SYMES, J. F. 1996. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet,* 348**,** 370-4.

JECHLINGER, W., AZIMPOUR TABRIZI, C., LUBITZ, W. & MAYRHOFER, P. 2004. Minicircle DNA immobilized in bacterial ghosts: in vivo production of safe non-viral DNA delivery vehicles. *J Mol Microbiol Biotechnol,* 8**,** 222-31.

KADEKAR, D., KALE, V. & LIMAYE, L. 2015. Differential ability of MSCs isolated from placenta and cord as feeders for supporting ex vivo expansion of umbilical cord blood derived CD34(+) cells. *Stem Cell Res Ther,* 6**,** 201.

KAY, M. A., HE, C. Y. & CHEN, Z. Y. 2010. A robust system for production of minicircle DNA vectors. *Nat Biotechnol,* 28**,** 1287-9.

KIM, J. A., CHO, K., SHIN, M. S., LEE, W. G., JUNG, N., CHUNG, C. & CHANG, J. K. 2008. A novel electroporation method using a capillary and wire-type electrode. *Biosens Bioelectron,* 23**,** 1353-60.

KO, J., JUN, H., CHUNG, H., YOON, C., KIM, T., KWON, M., LEE, S., JUNG, S., KIM, M. & PARK, J. H. 2011. Comparison of EGF with VEGF Non-Viral Gene Therapy for Cutaneous Wound Healing of Streptozotocin Diabetic Mice. *Diabetes Metab J,* 35**,** 226-35.

KUSUMANTO, Y. H., VAN WEEL, V., MULDER, N. H., SMIT, A. J., VAN DEN DUNGEN, J. J., HOOYMANS, J. M., SLUITER, W. J., TIO, R. A., QUAX, P. H., GANS, R. O., DULLAART, R. P. & HOSPERS, G. A. 2006. Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. *Hum Gene Ther,* 17**,** 683-91.

LEE, R. J., SPRINGER, M. L., BLANCO-BOSE, W. E., SHAW, R., URSELL, P. C. & BLAU, H. M. 2000. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation,* 102**,** 898-901.

LIANG, J., ZHANG, H., HUA, B., WANG, H., LU, L., SHI, S., HOU, Y., ZENG, X., GILKESON, G. S. & SUN, L. 2010. Allogenic mesenchymal stem cells transplantation in refractory systemic lupus erythematosus: a pilot clinical study. *Ann Rheum Dis,* 69**,** 1423-9.

LIEW, A. & O'BRIEN, T. 2012. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Res Ther,* 3**,** 28.

LIM, J. Y., PARK, S. H., JEONG, C. H., OH, J. H., KIM, S. M., RYU, C. H., PARK, S. A., AHN, J. G., OH, W., JEUN, S. S. & CHANG, J. W. 2010. Microporation is a valuable transfection method for efficient gene delivery into human umbilical cord blood-derived mesenchymal stem cells. *BMC Biotechnol,* 10**,** 38.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LU, J., ZHANG, F., XU, S., FIRE, A. Z. & KAY, M. A. 2012. The Extragenic Spacer Length Between the 5′ and 3′ Ends of the Transgene Expression Cassette Affects Transgene Silencing From Plasmid-based Vectors. *Mol Ther.*

MADEIRA, C., RIBEIRO, S. C., PINHEIRO, I. S. M., MARTINS, S. A. M., ANDRADE, P. Z., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Gene delivery to human bone marrow mesenchymal stem cells by microporation. *Journal of Biotechnology,* 151**,** 130-136.

MADEIRA, C., RODRIGUES, C. A. V., REIS, M. S. C., FERREIRA, F. F. C. G., CORREIA, R. E. S. M., DIOGO, M. M. & CABRAL, J. M. S. 2013. Nonviral Gene Delivery to Neural Stem Cells with Minicircles by Microporation. *Biomacromolecules,* 14**,** 1379-1387.

MUN, J.-Y., SHIN, K. K., KWON, O., LIM, Y. T. & OH, D.-B. 2016. Minicircle microporation-based non-viral gene delivery improved the targeting of mesenchymal stem cells to an injury site. *Biomaterials,* 101**,** 310-320.

MUNYE, M. M., TAGALAKIS, A. D., BARNES, J. L., BROWN, R. E., MCANULTY, R. J., HOWE, S. J. & HART, S. L. 2016. Minicircle DNA Provides Enhanced and Prolonged Transgene Expression Following Airway Gene Transfer. *Sci Rep,* 6**,** 23125.

MYERS, T. J., GRANERO-MOLTO, F., LONGOBARDI, L., LI, T., YAN, Y. & SPAGNOLI, A. 2010. Mesenchymal stem cells at the intersection of cell and gene therapy. *Expert Opinion on Biological Therapy,* 10**,** 1663-1679.

NOWAKOWSKI, A., WALCZAK, P., JANOWSKI, M. & LUKOMSKA, B. 2015. Genetic Engineering of Mesenchymal Stem Cells for Regenerative Medicine. *Stem Cells and Development,* 24**,** 2219-2242.

PARK, N., RIM, Y. A., JUNG, H., KIM, J., YI, H., KIM, Y., JANG, Y., JUNG, S. M., LEE, J., KWOK, S. K., PARK, S. H. & JU, J. H. 2017. Etanercept-Synthesising Mesenchymal Stem Cells Efficiently Ameliorate Collagen-Induced Arthritis. *Sci Rep,* 7**,** 39593.

PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. Plasmid Biopharmaceuticals. *Microbiology Spectrum,* 2.

PROCKOP, D. J. & YOUN OH, J. 2012. Mesenchymal Stem/Stromal Cells (MSCs): Role as Guardians of Inflammation. *Molecular Therapy,* 20**,** 14-20.

RAO, M. S. & MATTSON, M. P. 2001. Stem cells and aging: expanding the possibilities. *Mechanisms of Ageing and Development,* 122**,** 713-734.

RAVAL, Z. & LOSORDO, D. W. 2013. Cell Therapy of Peripheral Arterial Disease: From Experimental Findings to Clinical Trials. *Circulation research,* 112**,** 10.1161/CIRCRESAHA.113.300565.

RIU, E., CHEN, Z. Y., XU, H., HE, C. Y. & KAY, M. A. 2007. Histone modifications are associated with the persistence or silencing of vector-mediated transgene expression in vivo. *Mol Ther,* 15**,** 1348-55.

SANZ-NOGUÉS, C. & O'BRIEN, T. 2016. In vitro models for assessing therapeutic angiogenesis. *Drug Discovery Today,* 21**,** 1495-1503.

SATO, K., OZAKI, K., MORI, M., MUROI, K. & OZAWA, K. 2010. Mesenchymal stromal cells for graft-versus-host disease : basic aspects and clinical outcomes. *J Clin Exp Hematop,* 50**,** 79-89.

SIMCIKOVA, M., PRATHER, K. L. J., PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. On the dual effect of glucose during production of pBAD/AraC-based minicircles. *Vaccine,* 32**,** 2843-2846.

SINGER, N. G. & CAPLAN, A. I. 2011. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol,* 6**,** 457-78.

STEVEN, E. R., NARENDRA, C., FRANK, S. L., NELSON, A. W., ADAM, B., GUANG-PING, G., JAMES, M. W. & MARK, L. B. 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Molecular Genetics and Metabolism,* 80**,** 148-158.

TANG, Y. L., ZHAO, Q., QIN, X., SHEN, L., CHENG, L., GE, J. & PHILLIPS, M. I. 2005. Paracrine Action Enhances the Effects of Autologous Mesenchymal Stem Cell Transplantation on Vascular Regeneration in Rat Model of Myocardial Infarction. *The Annals of Thoracic Surgery,* 80**,** 229-237.

WALDER, C. E., ERRETT, C. J., BUNTING, S., LINDQUIST, P., OGEZ, J. R., HEINSOHN, H. G., FERRARA, N. & THOMAS, G. R. 1996. Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hindlimb ischemia. *J Cardiovasc Pharmacol,* 27**,** 91-8.

WALKER, W. E., BOOTH, C. J. & GOLDSTEIN, D. R. 2010. TLR9 and IRF3 Cooperate to Induce a Systemic Inflammatory Response in Mice Injected With Liposome:DNA. *Mol Ther.*

YAN, J., TIE, G., XU, T. Y., CECCHINI, K. & MESSINA, L. M. 2013. Mesenchymal stem cells as a treatment for peripheral arterial disease: current status and potential impact of type II diabetes on their therapeutic efficacy. *Stem Cell Rev,* 9**,** 360-72.

YASUMURA, E. G., STILHANO, R. S., SAMOTO, V. Y., MATSUMOTO, P. K., DE CARVALHO, L. P., VALERO LAPCHIK, V. B. & HAN, S. W. 2012. Treatment of Mouse Limb Ischemia with an Integrative Hypoxia-Responsive Vector Expressing the Vascular Endothelial Growth Factor Gene. *PLOS ONE,* 7**,** e33944.

YOON, C. S., JUNG, H. S., KWON, M. J., LEE, S. H., KIM, C. W., KIM, M. K., LEE, M. & PARK, J. H. 2009. Sonoporation of the minicircle-VEGF(165) for wound healing of diabetic mice. *Pharm Res,* 26**,** 794-801.

ZHAO, H., HEMMI, H., AKIRA, S., CHENG, S. H., SCHEULE, R. K. & YEW, N. S. 2004. Contribution of Toll-like receptor 9 signaling to the acute inflammatory response to nonviral vectors. *Mol Ther,* 9**,** 241-8.

Chapter V

COMPARISON OF THE ANGIOGENIC POTENTIAL OF MESENCHYMAL STEM/STROMAL CELLS (MSC) FROM DIFFERENT HUMAN SOURCES AFTER MICROPORATION WITH VEGF-ENCODING MINICIRCLES

V. COMPARISON OF THE ANGIOGENIC POTENTIAL OF MESENCHYMAL STEM/STROMAL CELLS (MSC) FROM DIFFERENT HUMAN SOURCES AFTER MICROPORATION WITH VEGF-ENCODING MINICIRCLES

V.1. Summary

Mesenchymal stem/stromal cells (MSC) are well-known for their trophic activity, immunomodulation and low-immunogenicity. In particular, and given their angiogenic potential, which relies on the secretion of soluble factors such as vascular endothelial growth factor (VEGF), MSC have been explored in treatment of ischemic diseases. However, their low engraftment and reduced survival has limited a broader application in a clinical setting. Gene transfer of pro-angiogenic factors may be used to enhance MSC therapeutic features for ischemic conditions. Despite the majority of studies regarding MSC clinical application used cells isolated from bone marrow (BM), cells with similar intrinsic properties could be easily isolated from other sources. Adipose tissue (AT) and umbilical cord matrix (UCM) are examples of alternative sources of MSC from which they can be obtained with less invasive procedures than for BM. Herein, we developed a protocol to transiently transfect MSC from these sources using VEGF-encoding minicircles (MC-VEGF), which are a novel class of non-viral vectors with improved transgene expression and reduced toxicity. MSC isolated from BM, AT or UCM showed similar levels of VEGF secretion to the culture medium (543.5±19.9, 462.2±170.8 and 612.8±174.9 pg/1000 cells day^{-1}, respectively) two days after transfection with MC-VEGF. Those values were significantly higher when compared to control, non-transfected cells (BM: 5.79±1.21 pg/1000 cells day^{-1}; AT: 2.28±0.71 pg/1000 cells day^{-1}; UCM: 0.20±0.02 pg/1000 cells day^{-1}), indicating an enhancement on VEGF production for the engineered cell samples. These results were further confirmed by quantitative PCR. Similarly, transfected cells displayed higher angiogenic activity when compared to controls, as demonstrated by *in vitro* functional studies of cell tube formation and migration. However, no significant differences were observed between the three tissue sources. Also, transfection with MC-VEGF did not affect MSC intrinsic properties, such as proliferative potential, differentiation capacity and immunophenotype, for any of the investigated sources. These results suggest not only that minicircles can be successfully used for transfection of BM-, AT- or UCM-MSC with high efficiency, but also that the developed protocol might be effectively applied for human MSC transfection, regardless of the source from which cells were retrieved.

V.2. Background

Mesenchymal stem/stromal cells (MSC) are promising candidates for cell therapy approaches due to their unique therapeutic properties that rely essentially on their trophic activity (Singer and Caplan, 2011), immunomodulation and low-immunogenicity (Le Blanc et al., 2003a). Due to their angiogenic capacity, MSC have been exploited for ischemic conditions, such as myocardial infarction or peripheral arterial disease (Tang et al., 2005b, Liew and O'Brien, 2012, Watt et al., 2013). However, the harsh microenvironments found by these cells upon *in vivo* administration may hinder their survival and engraftment (Potier et al., 2007). Genetic engineering of MSC with therapeutic factors can be performed to prolong their survival and/or their therapeutic effect. Vascular endothelial growth factor (VEGF), which is a key regulator of physiological angiogenesis (Ferrara et al., 2003), is known to be secreted by MSC, playing a central role in their pro-angiogenic activity (Kagiwada et al., 2008). However, the low levels of VEGF secreted constitutively by MSC (>20 pg/1,000 cells day^{-1}) lead to the development of genetic engineering approaches to enhance MSC angiogenic capacity.

Despite the use of viral vectors for genetic modification of MSC have been shown to be the most effective (Stiehler et al., 2006, McMahon et al., 2006), they bring some safety concerns due to the risk of insertional mutagenesis and has limitations in terms of large-scale production and manufacturing (Hacein-Bey-Abina et al., 2003, van der Loo and Wright, 2016). To overcome such limitations, non-viral systems improved to maximize transgene expression and reduce toxicity have been developed. Minicircles are small plasmid derivatives that carry only the transgene expression cassette (Darquet et al., 1997) and due to their lower size and absence of bacterial sequences are expected to induce higher transgene expression with improved safety (Darquet et al., 1999). Minicircle-based cell engineering has been demonstrated to sustain higher and longer transgene expression than conventional plasmids, as well as prolonged stem cell survival (Darquet et al., 1999, Chen et al., 2003, Dietz et al., 2013, Madeira et al., 2013, Munye et al., 2016). In what concerns VEGF transgene expression, minicircles also have shown to be superior to plasmids (Chang et al., 2008, Yoon et al., 2009, Ko et al., 2011). In this context, a production strain system and a purification process for optimal minicircle manufacture have been recently established at BERG-iBB (Simcikova et al., 2014, Alves et al., 2016).

The majority of MSC-based clinical trials performed to date used cells isolated from bone marrow (BM) (Galipeau and Sensebe, 2018). However, other alternative and, in some cases, more advantageous sources are available. MSC could be efficiently isolated from other adult tissues, such as the adipose tissue (AT) (Gimble and Guilak, 2003), as well as from neonatal

sources, such as the umbilical cord matrix (UCM) (Simoes et al., 2013). The use of MSC from those sources has advantages over BM: ease of collection (minimally/non-invasive procedures) and increased proliferative potential (Kern et al., 2006). Also, UCM-derived cells are more primitive and exhibit increased degree of multipotency compared to cells obtained from adult sources (Fong et al., 2011). Although cells from all these sources share many of the main MSC intrinsic features, some differences in terms of angiogenic potential have been documented (Du et al., 2016). Nevertheless, the angiogenic capacity of cells from these three sources can be enhanced by using genetically engineered VEGF-encoding vectors (Beegle et al., 2016, Cho et al., 2017, Shevchenko et al., 2013).

The application of MSC in a clinical setting may involve either autologous (patient-specific cells used in a personalized therapy) or allogeneic (cells isolated from another individual, expanded *ex vivo* and administered in the patient in an off-the-shelf therapy) approaches (Elseberg et al., 2017). Despite the use of autologous MSC might be the ideal approach, is applicable only to adult sources, such as AT or BM, and even for those there are some potential limitations. For BM-MSC the major limitations are the invasive procedures and the decreased biological activity of MSC from elderly donors, including lower proliferative capacity and differentiation potential (Stenderup et al., 2003, Mueller and Glowacki, 2001). Although AT-MSC could be easily obtained, the low numbers retrieved from thinner donors may limit an autologous setting. Also, the isolation of autologous MSC is a time-consuming process, hindering their use to treat acute diseases such as stroke or myocardial infarction. Thus, the use allogeneic MSC from young healthy donors that are readily available and can be administered immediately is probably the most reasonable approach (Zhang et al., 2015).

The work developed herein was based on previous studies performed at iBB, which focused on the optimization of non-viral gene delivery to MSC of different human tissue sources (Madeira et al., 2011, Boura et al., 2013) and on the results presented in Chapter IV regarding the transfection of BM-MSC using VEGF-encoding minicircles (Serra et al., 2018). A strategy to improve the angiogenic potential of cultured MSC isolated from BM, AT or UCM was developed based on the transient modification of MSC using minicircles encoding VEGF, which were produced and purified according to recently developed protocol (Alves et al., 2016, Alves et al., 2018). To date, no systematic side-by-side comparison exists focused on MSC obtained from different human tissue sources that were genetically engineered with VEGF-encoding minicircle vectors. Genetically engineered MSC from BM, AT and UCM were characterized to confirm the maintenance of MSC features and were compared in terms of transgene expression and angiogenic capacity.

V.3. Materials and Methods

V.3.1. Plasmid construction, production and purification

Construction, production and purification of plasmids used in present Chapter were performed as previously described on Chapters III and IV. The parental plasmid (pMINILi-CV, 3,821 bp) expressing VEGF (165a) gene was obtained from pMINILi-CVG, by removing the GFP gene. The vector was constructed and transformed by heat shock into *E. coli* as described elsewhere (Azzoni et al., 2007, Simcikova et al., 2014, Alves et al., 2016). pMINILi-CV contains an expression cassette with VEGF and the human cytomegalovirus (CMV) immediate-early promoter, two multimer resolution sites (MRS) flanking the expression cassette, pMB1 origin of replication, kanamycin resistance gene and BGH polyadenylation sequence (Serra et al., 2018). BW2P *E. coli* strain harboring the parental plasmid pMINLI-CV was used for minicircle production, according to previously established methods (Simcikova et al., 2014, Alves et al., 2016). *E. coli* was grown until the late exponential phase and then recombination was induced for 2 hours by addition of 0.01% (w/v) L-(+)arabinose (Merck). After recombination, both a minicircle with the expression cassette and a miniplasmid (MP) with the prokaryotic backbone sequences are obtained. All plasmid DNA species were recovered and purified from the producer cells using an endotoxin-free plasmid DNA purification kit (Macherey-Nagel). Then, the minicircle was separated from other DNA forms by performing a digestion with nicking endonuclease (*Nb*.*B*bvCI) followed by hydrophobic interaction chromatography (HIC), as described and optimized at BERG-iBB (Alves et al., 2016, Alves et al., 2018). The concentration of purified pDNA solution was assayed by spectrophotometry at 260nm (NanoDrop, Thermo Scientific) and DNA integrity was confirmed by DNA agarose gels stained with ethidium bromide.

V.3.2. Isolation and culture of human MSC

Human MSC from BM, UCM or AT were isolated from healthy donors after informed consent and expanded according to previously established protocols (dos Santos et al., 2010, de Soure et al., 2017, Gimble and Guilak, 2003). MSC derived from the three sources were maintained cryopreserved in liquid/vapour phase nitrogen containers. Upon thawing, cells were cultured for 3 to 5 passages under xenogeneic (xeno)-free culture conditions as described elsewhere (dos Santos et al., 2011, dos Santos et al., 2014). Cells were plated at a cell density between 3,000-6,000 cells/cm^2 on CELLstart™ CTS™ (Invitrogen) pre-coated T-flasks using StemPro® MSC SFM XenoFree (Invitrogen) supplemented with 1% GlutaMAX™-I CTS™ (Invitrogen) and 1% Antibiotic-Antimycotic (Invitrogen). Cells were maintained at 37ºC and 5% CO_2 in a humidified atmosphere

and culture medium was exchanged every 3-4 days. At 70% cell confluence, MSC were detached from the flasks by adding TrypLE™ Select CTS™ (Invitrogen) solution 1x in Phosphate Buffered Saline (PBS, Gibco). Cell number and viability were determined using the Trypan Blue (Gibco) exclusion method. MSC at passages between 3 and 5 were used, from three independent donors for each source.

V.3.3. Microporation of MSC with VEGF-encoding minicircles

Microporation of MSC was performed according to a previously established protocol (Madeira et al., 2011, Serra et al., 2018). Briefly, 1.5×10^6 cells from each source were resuspended in 100 µl of resuspension buffer (buffer R, Invitrogen) and incubated with 4.9 µg of MC-VEGF (Serra et al., 2018). Electroporation was performed using the Neon® Transfection System and a Microporator MP-100 (Digital Bio, Invitrogen) using 1 pulse with 1,000 V of pulse voltage and 40 ms of width. After microporation, the cell suspension was incubated with 900 µL of Opti-MEM™ I Reduced Serum Medium (Gibco) for 20-30 min. Finally, cells were plated at a density of 7,000-8,000 cells/cm^2 in pre-warmed StemPro® XenoFree culture medium. At each time point (day 2 and day 5), cell numbers were estimated using the trypan blue dye exclusion method. Cell recovery for each microporated sample was determined after 48 h, by calculating the ratio between viable cells in the condition where cells were microporated and viable cells in the control condition (non-electroporated), as described elsewhere (Madeira et al., 2011). Two controls were also prepared, a control for microporation process corresponding to MSC microporated without DNA (referred as “micro”) and non-microporated cells, referred herein as “control”, which will be used for comparison in subsequent studies.

V.3.4. *In vitro* multilineage differentiation potential and immunophenotype characterization of transfected cells

The osteogenic, adipogenic and chondrogenic differentiation potential of cells from BM, UCM and AT transfected with MC-VEGF were assessed as previously described using StemPro® Osteogenesis/Adipogenesis/Chondrogenesis Differentiation Kits (Life Technologies) (dos Santos et al., 2011).

The immunophenotypic profile of the engineered cells was analyzed by flow cytometry, 48h and 5 days after transfection, using a panel of mouse anti-human monoclonal antibodies (PE-conjugated) against: CD34, CD45, CD90, CD73, CD80, CD14, CD105 and human leukocyte antigen (HLA)-DR (all from Biolegend). Cells were incubated with the monoclonal antibodies for 15 min

in the dark at room temperature, then were washed in 2 mL of PBS and finally fixed with 1% paraformaldehyde (PFA, Sigma). Appropriate isotype controls (IgGγ1 and IgGγ2b) were also prepared. A minimum of 10,000 events was collected for each sample and the CellQuest (Becton Dickinson) and FlowJo® (LLC) softwares were used for acquisition and analysis, respectively.

V.3.5. Quantification of VEGF expression by qPCR and ELISA

To quantify the gene expression and protein production of VEGF by the MSC transfected with MC-VEGF, real time PCR (qPCR) and ELISA were performed, respectively, according to previously established protocols described on Chapter IV (Serra et al., 2018). For qPCR, cells were harvested at each time point (day 2 and day 5), centrifuged and kept as a dry pellet at -80 ºC until further analysis. Total RNA was isolated using RNeasy Mini Kit (Qiagen) and quantified by UV spectrophotometry (NanoDrop). For cDNA synthetization iScript cDNA Synthesis Kit (Bio-Rad) was used. The qPCR analysis was performed in a 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 reaction volume. The following primers (StabVida) were used for VEGF amplification: VEGF_fwd – GGAGGAGGGCAGAATCATCAC and VEGF_rev – GGTCTCGAT TGGATGGCAGT. The $2^{-\Delta\Delta CT}$ method of relative quantification was applied to determine the fold change in mRNA expression (Livak and Schmittgen, 2001). GAPDH was used as the housekeeping gene and non-microporated MSC as a baseline.

For ELISA, culture supernatants from the three MSC sources were collected at each time point (day 2 and day 5), centrifuged at 500 g for 10 min and kept at -80 ºC until further analysis. A Human VEGF-A ELISA kit (RayBiotech) was used, following the manufacturer's instructions.

V.3.6. Angiogenesis functional studies with endothelial cells: cell tube formation and cell migration assays

Conditioned media retrieved from cultures of transfected and control MSC obtained from the three sources was used for functional assays and prepared as established elsewhere and described on Chapter IV (Serra et al., 2018). Briefly, cells were plated at a density of 12,500 cells/cm^2 using StemPro MSC SFM XenoFree for 24 h after microporation. After 24 h, the medium was changed to Endothelial Basal Medium (EBM-2, Lonza) and maintained for 48 h. Conditioned medium was collected and normalized to cell number 72 h after transfection, centrifuged and kept at -80ºC. Fresh EBM-2 and Endothelial Cell Growth Medium (EGM-2, Lonza) were used as controls.

Cell tube formation consists in a functional assay that relies in the capacity of human endothelial vein endothelial cells (HUVEC) to form tube networks when cultured in Matrigel (Arnaoutova et al., 2009) and was performed as described on Chapter IV. Conditioned medium samples derived from both control and gene modified MSC (BM, AT or UCM) were used to cultivate HUVEC (BD). Then, tube formation was analyzed: tube length and tube connections were measured using microscope and ImageJ (NIH) software (Arnaoutova et al., 2009, Arutyunyan et al., 2016).

The capacity of endothelial cells to migrate in response to soluble factors, such as VEGF, is one of the crucial events during angiogenesis (Carmeliet, 2000a) and can be assessed by endothelial cell migration assay, which was performed according to the procedure described on Chapter IV. Briefly, conditioned medium collected from MSC transfected cultures was used as a stimulus and HUVEC's capacity to migrate was quantified (Goodwin, 2007, Chen et al., 2014b). The cells were stained with crystal violet 0.5% (Sigma), observed under the microscope and the total number of migrated HUVEC per optical field was quantified (100x magnification).

V.3.7. Statistical analysis

All data is presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 and significance was determined by Tukey's multiple comparison test and set at a p-value <0.05.

V.4. Results

V.4.1. Characterization of MSC after microporation: proliferative potential and cell recovery

In the present work, MSC from different sources cultured under xenogeneic(xeno)-free conditions (dos Santos et al., 2011) were transfected by microporation with a minicircle encoding for VEGF (MC-VEGF). Non-transfected cells were used as control and cells microporated (without DNA) were also included (referred as "Micro"). The transfected cells were analyzed at days 2 and 5 post transfection. To evaluate the impact of the microporation-based gene delivery on the proliferative capability of MSC, the number of viable cells was determined (Figure V.1). At day 2, non-transfected cells (control) showed higher cell numbers than microporated samples for BM and AT-derived MSC. For UCM-MSC, all the conditions demonstrated similar cell numbers. Microporated cell samples from the three sources displayed identical cell numbers on day 2, regardless the presence of DNA. On day 5, the cell numbers observed for the three conditions were similar for both UCM and AT-derived cells. For BM-MSC, the highest cell numbers were observed for the control condition, corresponding to non-transfected cells. However, none of the differences observed between the conditions for all timepoints were statistically significant.

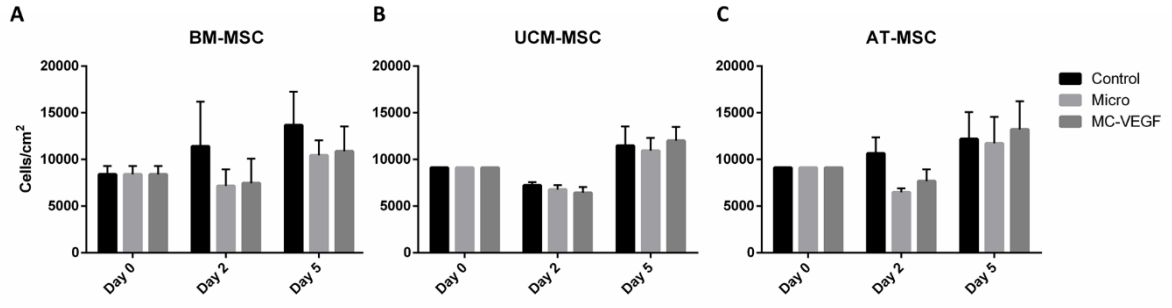

Figure V.1 - Analysis of the proliferative potential of MSC from BM **(A)**, UCM **(B)** or AT **(C)** after microporation with MC-VEGF. Control – non-transfected cells; Micro – cells microporated without DNA. Values are mean ± SEM, n=3.

Cell recovery, which reflects the level of cell death in electroporated samples was calculated at day 2, as described elsewhere (Madeira et al., 2011) and is shown in Figure V.2. Non-transfected cells were considered to have a recovery of 100% for all the sources and, based on that, the values for microporation conditions were determined. For MSC isolated from BM or AT, the cell recoveries of microporated samples were similar, regardless the presence of DNA (BM: micro – 71.3±10.2%; MC-VEGF – 69.3±6.0%; AT: Micro – 62.7±6.2%; MC-VEGF – 72.3±5.0%). For UCM-derived cells, the microporated samples with MC-VEGF (88.7±5.8%) or without DNA (96.3±5.5%) showed similar recoveries than the control sample.

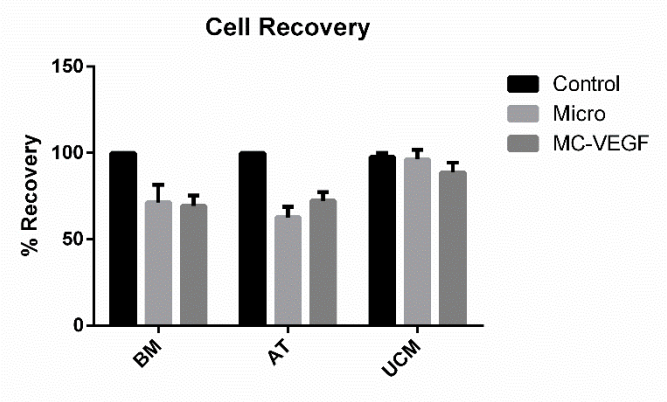

Figure V.2 - Analysis of cell recoveries for MSC from BM, AT or UCM after microporation with MC-VEGF. Control – non-transfected cells; Micro – cells microporated without DNA. Values are mean±SEM, n=3.

V.4.2. Maintenance of the MSC features after microporation: immunophenotype and multilineage differentiation potential

The differentiation potential and immunophenotype of microporated cells was assessed based on the minimal criteria proposed by International Society of Cell Therapy (ISCT) to define human MSC (Dominici et al., 2006, Baer and Geiger, 2012). Differentiation protocols towards osteogenic, adipogenic and chondrogenic lineages were accomplished to evaluate if microporation with MC-VEGF affects the multipotency of MSC from the three sources tested.

The differentiation potential of transfected MSC was confirmed by using lineage-specific stains (Figure V.3). Osteogenic differentiation was confirmed by alkaline phosphatase (ALP) and Von Kossa (dark) staining of early osteocytes and calcium deposits, respectively. The red droplets represent the lipid vacuoles stained with Oil Red-O, demonstrating the ability of MSC to

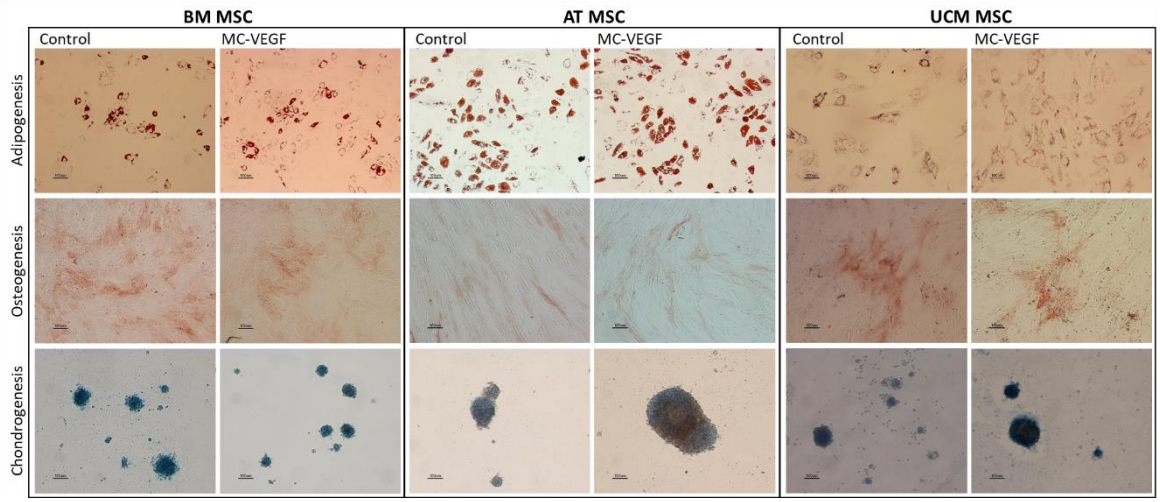

Figure V.3 - Evaluation of the differentiation potential into adipocytes, osteocytes and chondrocytes of MSC from different tissue sources after microporation with VEGF-encoding minicircles. Cell differentiation was induced for 14 days and was assessed by staining for osteogenesis (alkaline phosphatase and von Kossa), adipogenesis (Oil Red-O), and chondrogenesis (Alcian blue).

originate adipocytes. Alcian blue was used to stain proteoglycans and, thus, assess chondrogenic differentiation. Hence, MSC from all sources (BM, UCM and AT) maintained their multilineage differentiation potential after being microporated with VEGF-encoding minicircles similarly to control, non-transfected cells.

Flow cytometry analysis using a panel of monoclonal antibodies against different surface markers selected according to ISCT criteria to define MSC was used to evaluate the maintenance of MSC immunophenotype after microporation with MC-VEGF. Antibodies against the following surface markers were tested: CD34, CD45, CD73, CD80, CD90, CD105, CD14 and HLA-DR. The results presented on Figure V.4 show that MSC immunophenotype was maintained after transfection. The three MSC sources tested herein by modification with MC-VEGF were positive (>90%) for CD73, CD90 and CD105 and negative (<5%) for the remaining surface markers after 2 or 5 days of culture.

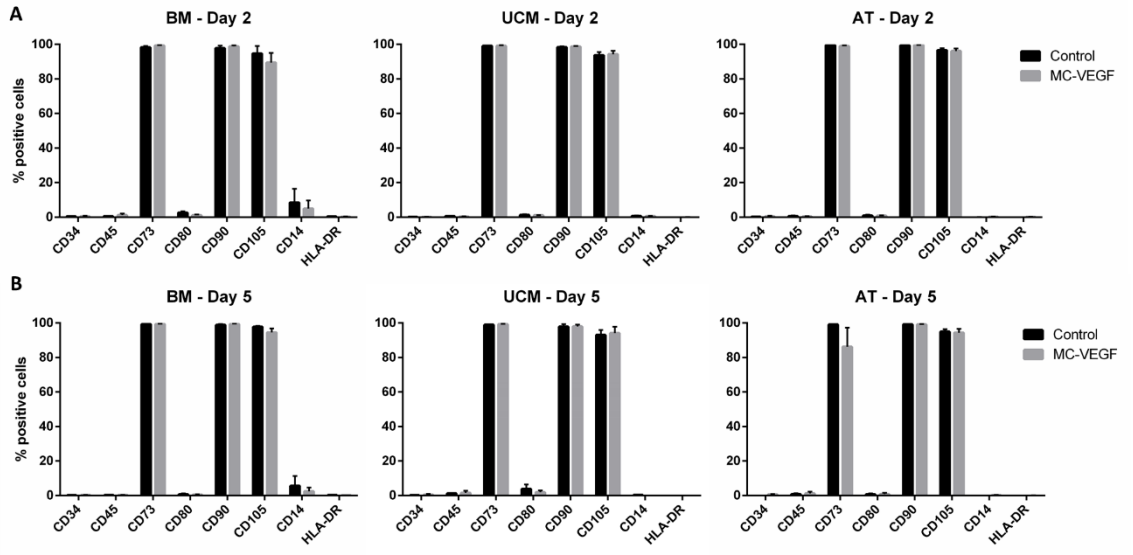

Figure V.4 - Phenotypic analysis of cells 2 **(A)** and 5 **(B)** days after microporation. Assessment of CD34, CD45, CD90, CD73, CD80, CD14, CD105 and HLA-DR surface markers. Values are mean±SEM, n=3.

V.4.3. Quantification of VEGF gene expression and protein production by MSC engineered with MC-VEGF

The efficiency of transfection was assessed by analysis of VEGF expression by qPCR (Figure V.5A) and ELISA (Figure V.5B). Genetically engineered MSC from BM, UCM and AT were evaluated in what concerns VEGF production at two timepoints after transfection: days 2 and 5. Non-transfected cells from each source were used as controls for qPCR or ELISA.

The VEGF expression by cells from each tissue source was measured by assessing the fold increased on mRNA copies of the VEGF gene that were produced compared to the control. Two days after transfection, cells from all the sources modified with MC-VEGF showed significantly higher mRNA copies of VEGF gene than the corresponding control samples (Figure V.5A). The highest increase on VEGF expression was observed for UCM-MSC (154.5±81.0-fold), followed by AT-MSC (104.8±45.1-fold) and BM-MSC (65.4±15.7-fold). However, the differences observed between the three sources were not statistically significant. Despite VEGF mRNA levels significantly dropped on day 5, the highest fold-increase on VEGF expression was also shown for UCM-MSC (45.9±23.8-fold) compared to the low levels detected for BM (8.32±1.2-fold) or AT (4.1±1.1-fold).

The profiles of VEGF secretion into the culture medium after transfection with MC-VEGF were similar for cells from the three sources evaluated and were significantly increased compared to non-transfected controls. The levels of VEGF in the conditioned medium retrieved from cultures of control, non-transfected cells were measured on day 2 and on day 5, showing that BM-MSC have the highest levels of basal VEGF production (Day 2 - 5.79±1.21 pg/1000 cells day^{-1}; Day 5 - 5.80±1.31 pg/1000 cells day^{-1}), followed by AT (Day 2 - 2.28±0.7 pg/1000 cells day^{-1}; Day 5 - 3.51±0.93 pg/1000 cells day^{-1}) and the results for UCM were almost undetectable (Day 2 - 0.20±0.02 pg/1000 cells day^{-1}; Day 5 - 0.16±0.06 pg/1000 cells day^{-1}). On day 2 the values observed for BM-, AT- and UCM-MSC transfected with MC-VEGF were 543.5±19.9, 462.2±170.8 and 612.8±174.9 pg/1000 cells day^{-1}, respectively (Figure V.5-B). These levels decreased to 130.2±21.1 pg/1000 cells day^{-1} for BM, 94.5±17.7 pg/1000 cells day^{-1} for AT and 116.6±21.5 pg/1000 cells day^{-1} for UCM on day 5.

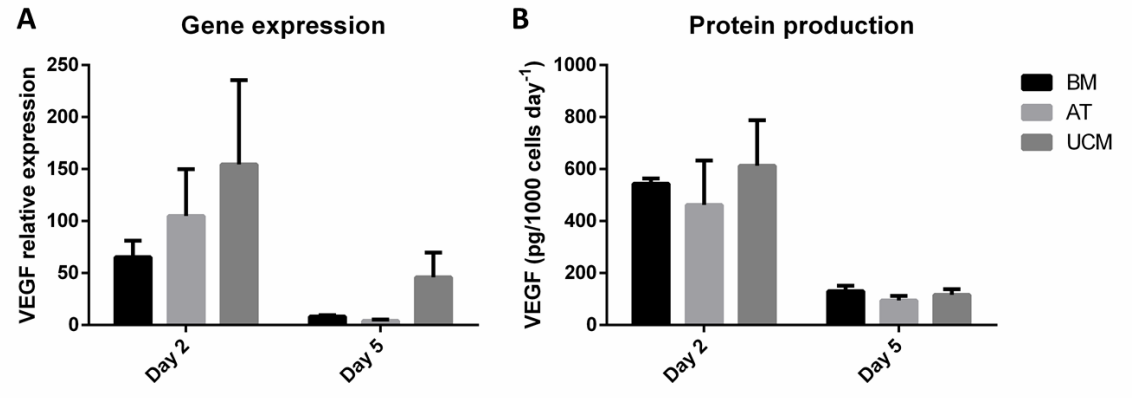

Figure V.5 - Evaluation of transgene delivery into BM, AT and UCM MSC, assessed 2 or 5 days after microporation with MC-VEGF. Analysis of VEGF **(A)** gene expression by qPCR and **(B)** protein production by ELISA. Each bar represents the mean ± SEM, n=3.

V.4.4. Angiogenesis functional studies: cell tube formation and cell migration assays

To evaluate the angiogenic potential of genetic engineered cells from different sources, conditioned medium retrieved 72 h after microporation (or culture) from cultures of transfected and non-transfected (control) MSC was used. For the cell tube formation assay, conditioned medium was used for cultivation of HUVEC on Matrigel coated plates for 8 h. After that, HUVEC capacity to form tube-like structures was evaluated by quantification of number of tubes and branch points (Figure V.6).

No statistically significant differences have been observed for the number of tubes or branch points between all the conditions tested herein. However, transfected cells from all the three sources showed increased capacity to induce tube formation than the corresponding controls, as demonstrated by the higher number of tubes and branch points. The highest tube formation capacity was observed for conditioned medium from transfected AT-MSC (33.17±5.1 tubes and 25.0±3.5 tube connections), followed by transfected BM-MSC (27.6±4.8 tubes and 20.8±3.8 tube connections) and UCM-MSC (24.5±7.5 tubes and 16.0±6.6 tube connections). Thus, the

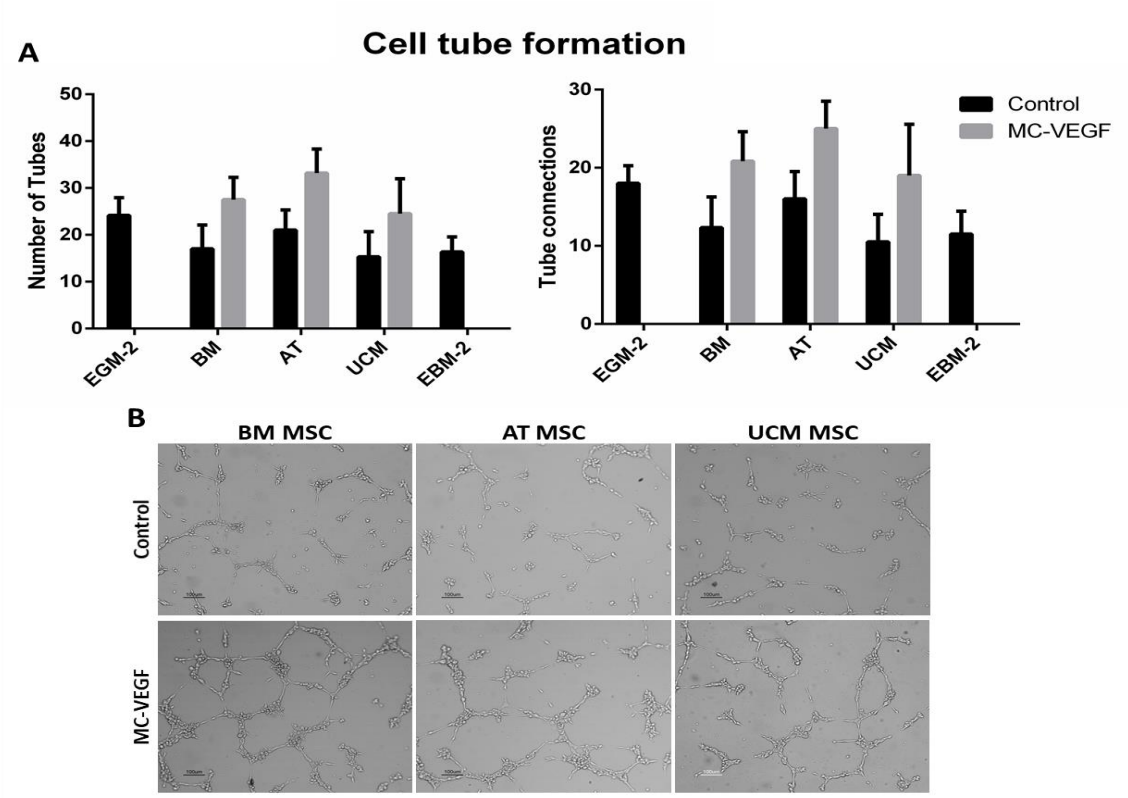

Figure V.6 - Cell tube formation assay using conditioning medium from MSC from different sources after being transfected with VEGF-encoding MC. **(A)** Number of tubes and branch points (connections) observed per optical field after 8 h for each condition tested. Values are presented as mean ± SEM; n=2 **(B)** Images of cell tube formation by HUVEC after being cultured for 8 h with conditioned medium from transfected (MC-VEGF) or non-transfected (control) cells from different sources (BM, AT and UCM).

conditioned medium from genetically engineered cells, regardless the source from MSC were retrieved, also lead to higher number of tubes and connections compared to both negative (EBM-2; 16.3±3.2 tubes and 11.5±2.9 tube connections) and positive (EGM-2; 24.2±3.8 tubes and 18.0±2.3 tube connections) controls. For control, non-transfected cells, the tendency was similar to the observed for transfected MSC: highest tube formation capacity was induced by AT-MSC (21.0±4.3 tubes and 16±3.5 tube connections) followed by BM (17.0±5.1 tubes and 12.3±3.9 connections) and UCM (15.3±5.4 tubes and 10.5±3.5 connections).

The HUVEC migration capacity towards conditioned medium obtained from cultured MSC was also investigated in order to assess the angiogenic potential of cells transfected with MC-VEGF. For this assay, HUVEC were cultured for 6 h in the upper part of the transwell, while conditioned medium from genetically engineered and control (non-transfected) MSC from different sources was kept in the bottom. HUVEC that migrate through the transwell pores were counted and the values were normalized relatively to a positive control where complete culture medium (EGM-2) for endothelial cells was used (Figure V.7).

All the transfected samples, as well as the positive control (EGM-2), showed to promote statistically significantly higher HUVEC migration (p-value<0.01) than basal endothelial medium (EBM-2; 14.0±2.0%), used herein as negative control. Two of the transfected conditions also lead to superior HUVEC migration than the positive control: AT-MSC (105.5±20.5%), which showed the highest percentage, and UCM-MSC (104.0±3.0%). Despite the conditioned medium regarding BM-MSC transfected with MC-VEGF induced a lower HUVEC migration (90.0±12.0%) than these two sources, the differences were not statistically significant. For non-transfected (control) MSC the highest migration was obtained for UCM-MSC (86.0±17.0%), whereas similar percentages were observed for BM- (60.5±10.5%) and AT-derived cells (64.0±1.0%).

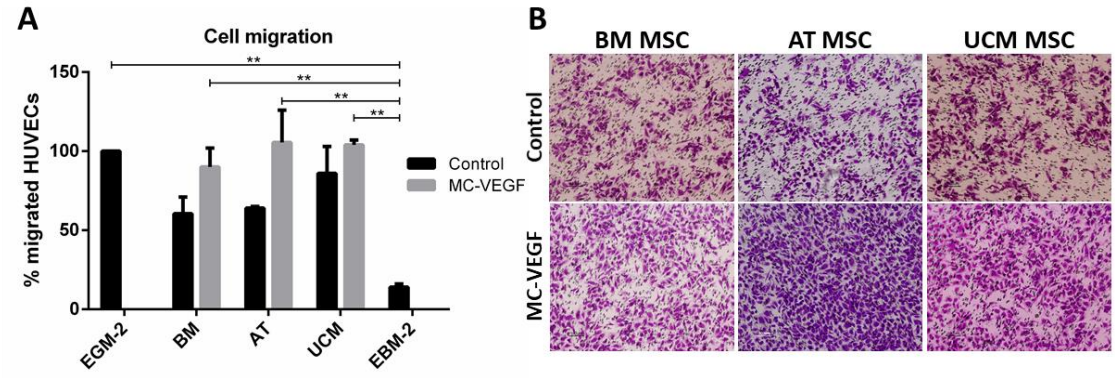

Figure V.7 - Endothelial cell migration assay using conditioning medium from MSC from different sources after being transfected with VEGF-encoding MC. **(A)** Percentage of HUVEC that migrated trough transwell towards conditioned medium samples normalized to a positive control (EGM-2). Values are presented as mean ± SEM, n=2, **p<0.01. **(B)** HUVEC that migrates through the transwell towards conditioned medium from transfected or (MC-VEGF) or non-transfected (control) cells from different sources (BM, AT or UCM).

V.5. Discussion

MSC have been extensively studied in the context of cell therapies due to their interesting therapeutic properties that include immunomodulatory and trophic activity, among others. Although the therapeutic benefit of MSC administration has been documented in several clinical studies (Le Blanc et al., 2008, Hare et al., 2009, Gupta et al., 2013), there is some conflicting data since no or only modest evidences of improvement have been observed in some studies (Allison, 2009, Chullikana et al., 2015, Wang et al., 2017). Thus, strategies to improve their therapeutic benefit have been employed, namely genetic engineering with therapeutic genes.

The application of MSC for the treatment of cardiovascular ischemic diseases, such as myocardial infarction or peripheral arterial disease, has been investigated in many clinical studies with promising results (Hare et al., 2009, Gupta et al., 2013). It is believed that the beneficial effect of MSC in ischemic conditions relies on its capacity to secrete soluble factors with potent pro-angiogenic activity, such as VEGF (Kagiwada et al., 2008). Minicircles, a novel class of non-viral systems specifically designed to maximize gene expression and reduce toxicity (Darquet et al., 1999), containing the VEGF gene were used herein for the genetic engineering of human MSC from different sources.

Despite BM was historically the first source from which MSC were obtained and probably the most studied in clinical approaches, cells with similar properties can be isolated from other sources, presenting several advantages. The procedure for BM-MSC harvesting is highly invasive and the number and proliferation potential of obtained cells is known to decline with increasing age of the donors (Nishida et al., 1999, Stenderup et al., 2003). Therefore, alternative sources from which MSC can be isolated have been extensively studied. AT and UCM are examples of tissues from which high MSC yields could be easily obtained without the need of invasive procedures (Gimble and Guilak, 2003, Simoes et al., 2013). Cells from these sources share the main intrinsic properties of MSC (Hass et al., 2011), but also demonstrated to have specific features that might vary according to the tissue from they were obtained. For example, UCM-derived cells are reported to have an increased expansion potential (Simoes et al., 2013, Kern et al., 2006) and AT-MSC to have an improved immunomodulatory capacity (Ribeiro et al., 2013).

A comparison between MSC from different tissue sources was performed upon transfection with VEGF-encoding minicircles (MC-VEGF) using the protocol previously established on Chapter IV for BM-derived cells (Serra et al., 2018). Minicircles were produced and purified through a recently established protocol (Alves et al., 2016) and used to transfect MSC by microporation using a previously optimized strategy (Madeira et al., 2011). This represents, to our best

knowledge, the first systematic side-by-side comparison of the angiogenic activity of human MSC from different tissue sources genetically engineered to overexpress VEGF through transfection with minicircle vectors.

The results from the present study revealed that, regardless of the source from which cells were retrieved, microporation did not affect MSC main properties, including proliferative potential, differentiation capacity and immunophenotypic potential, as previously reported (Abdul Halim et al., 2014, Madeira et al., 2011, Lim et al., 2010). A subtle decrease in cell numbers was observed for microporated samples on day 2, especially regarding AT- and BM-MSC. This is in accordance with results in Chapter IV (Serra et al., 2018) and may indicate that microporation slightly slows-down MSC growth *in vitro*. However, MSC have their growth potential recovered on day 5, as reflected by the cell numbers observed.

The analysis of cell recoveries after transfection with MC-VEGF revealed a higher recovery of UCM MSC (88.7±5.8%) compared to other sources (BM-69.3±6.0%; AT-72.3±5.0%). These results are superior than those observed in another study regarding the transfection of MSC from these three sources using lipid-based strategies, where the recoveries were below 60% for all the sources (Boura et al., 2013). Therefore, microporation might be considered advantageous compared to lipofection for MSC genetic engineering, especially for umbilical cord derived-MSC, as already documented by other authors (Lim et al., 2010). In fact, the results observed regarding cell numbers and recoveries may indicate that MSC from UCM are the less affected by the microporation procedure.

To evaluate and compare the efficiency of transfection among cells from each tissue source, VEGF gene expression levels were quantified by qPCR and VEGF secretion to the culture medium was analyzed by ELISA. Two days after transfection, UCM-MSC was the source expressing the highest number of mRNA copies of VEGF transgene, but the differences to the other sources were not statistically significant. Despite the differences described for VEGF gene expression (qPCR), the levels of VEGF protein in culture supernatants (ELISA) from transfected cells were similar for all the three sources investigated. These VEGF levels were also comparable to those documented in Chapter IV for BM-MSC (Serra et al., 2018).

The results on protein analysis regarding non-transfected controls on day 2 showed that BM-MSC secreted the highest basal levels of VEGF followed by AT-MSC, whereas almost no VEGF was observed for UCM-MSC. These results were expected according to a previous report where a comparison of angiogenic properties between different MSC sources (non-engineered) revealed that BM-MSC secreted more VEGF than other sources (Du et al., 2016). The reduced or

absent VEGF levels in conditioned medium from cultures of umbilical cord-derived MSC has also been reported by other authors (Kuchroo et al., 2015).

In a previous study comparing the capacity of these three MSC sources to be transfected by lipofection, AT revealed to have significant lowest transgene expression (≈33%), when compared to BM (≈58%) or UCM (≈54%) two days after transfection (Boura et al., 2013). The results by Boura and colleagues could not be directly compared to the present study, since a conventional plasmid containing green fluorescent protein (GFP) was used and transgene expression was monitored by fluorescence analysis using flow cytometry. Herein, microporated AT-MSC also showed the lowest production of VEGF as measured by ELISA, but the differences to other sources were found insignificant. The absence of significant differences on transgene expression between AT-, BM- and UCM-MSC has already been described in another study (Benabdallah et al., 2010). However, these authors used a different transgene and a viral-based protocol with integrative vectors.

The analysis on the angiogenic potential of transfected cells herein was not limited to the quantification of transgene expression levels and the functional angiogenic activity of these cells was also evaluated. *In vitro* functional studies that mimic *in vivo* angiogenesis and which are widely used to demonstrate angiogenic activity were applied (Arutyunyan et al., 2016, Goodwin, 2007, Du et al., 2016). Experiments were set up where conditioned media retrieved from cultures of transfected and control MSC were used as a stimulus as described in Chapter IV. In particular, an endothelial cell tube formation assay was used to evaluate the capacity of HUVEC to form tube-like structures and migration assay was used to assess the ability of endothelial cells to migrate towards the VEGF stimulus.

The results for functional studies were in accordance with the VEGF production levels. Conditions transfected with MC-VEGF showed higher capacity to induce tube formation and endothelial cell migration, as previously demonstrated for BM-MSC on Chapter IV (Serra et al., 2018). Similar angiogenic activities were observed for cells from the three MSC sources. Cell tube formation assay using conditioned medium from transfected samples revealed no significant differences between the three sources neither in terms of number of tubes nor branch points. Also, no significant differences were observed between the three sources for cell migration studies, where conditioned medium from transfected cells induced endothelial cell migrations comparable to the positive control. The angiogenic activity of non-transfected control samples was also similar between the three sources as revealed by the number of tubes and branch points and percentage of migration. All those values were lower than those observed for cells engineered with MC-VEGF, but were identical for the three sources, with exception of

cell migration for UCM-MSC. The conditioned medium from non-transfected UCM-MSC showed to promote higher endothelial cell migration than conditioned medium from other control samples. Also, the percentage of migrated HUVEC for UCM-MSC (86.0±17.0%) almost reached the levels observed for transfected counterpart (104.0±3.0%). This, together with the fact that similar functional angiogenic activities were observed even for the controls were basal VEGF expressions were heterogeneous, demonstrates that VEGF is not the only responsible for angiogenesis. In fact, hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) are examples of two well-known pro-angiogenic factors that were described to be upregulated on umbilical cord-derived MSC compared to BM or AT (Du et al., 2016). Overall, blood vessel formation and remodeling are a complex processes with several soluble factors and cell types involved (Carmeliet, 2000a). However, VEGF plays a central role in this process and enhancement of its secretion by genetic engineering approaches may significantly improve the angiogenic capacity of MSC as documented in the literature (Beegle et al., 2016, Locatelli et al., 2013, Serra et al., 2018).

One of the major concerns regarding VEGF overexpression is the possibility of promoting an abnormal blood flow vessel growth, which in most severe cases can potentially lead to hemangiomas or even cancer (Folkman, 1971, Lee et al., 2000). Thus, a minicircle-based approach has the advantage of being transient, promoting high VEGF production in a limited timeframe. The VEGF peak observed two days after transfection might be able to induce endothelial cell migration and organization, accelerating early angiogenesis at ischemic regions. Then, the decrease on VEGF production will avoid an uncontrolled blood vessel development, limiting the side-effects of VEGF overexpression.

As referred on Chapter IV, the use of minicircles as vectors for gene delivery is also one of the most valuable benefits of the present work. These smaller vectors have been reported to induce higher levels of transgene expression and present superior safety when compared to conventional plasmids. These advantages are related to their lower size and reduced number of unmethylated CpG regions, which may trigger immune responses in human cells through TLR9 activation (Häcker et al., 2002, Walker et al., 2010, Boura et al., 2014). To further confirm the non-immunogenic nature of the minicircle, evaluations of TLR9 expression must be performed in future studies.

The present work shows that combination of microporation with minicircles may represent an alternative and effective approach for MSC transfection towards angiogenic therapies, regardless the cell source. In fact, this is an important advantage in a clinical context since a standard protocol might be applied to different sources of MSC, which can be differentially

selected according to the application, with homogenous outcomes. This protocol can be applied either as an off-the-shelf allogeneic approach or in an autologous setting using patient-derived MSC from BM or AT. However, allogeneic cells have advantages over autologous cells, including the possibility of using more primitive sources of MSC (such as UCM). In addition, an allogeneic approach overcomes limitations related with impaired function of cells from elderly or diseased patients and allows a more quickly intervention (Stenderup et al., 2003, Teraa et al., 2013, Kizilay Mancini et al., 2017).

Although some properties have been described to be shared by all MSC populations, one of the constraints regarding clinical applicability of MSC therapies is the high variability between sources (Du et al., 2016) and between donors within the same source (Kang et al., 2018, Siegel et al., 2013). This might be one of the reasons for the lack of statistically significance in studies where therapeutic activity of MSC is analyzed and limits their employment as an off-the-shelf therapeutic product. This can be partially circumvented by the development of robust, reliable and standard potency assays to consistently characterize MSC therapeutic activity (Ketterl et al., 2015).

V.6. References

ABDUL HALIM, N. S., FAKIRUDDIN, K. S., ALI, S. A. & YAHAYA, B. H. 2014. A comparative study of non-viral gene delivery techniques to human adipose-derived mesenchymal stem cell. *Int J Mol Sci,* 15**,** 15044-60.

ALLISON, M. 2009. Genzyme backs Osiris, despite Prochymal flop. *Nat Biotech,* 27**,** 966-967.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2016. Development of a nicking endonuclease-assisted method for the purification of minicircles. *Journal of Chromatography A,* 1443**,** 136-144.

ALVES, C. P. A., ŠIMČÍKOVÁ, M., BRITO, L., MONTEIRO, G. A. & PRAZERES, D. M. F. 2018. Production and Purification of Supercoiled Minicircles by a Combination of *in vitro* Endonuclease Nicking and Hydrophobic Interaction Chromatography. *Human Gene Therapy Methods,* (in press).

ARNAOUTOVA, I., GEORGE, J., KLEINMAN, H. K. & BENTON, G. 2009. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis,* 12**,** 267-274.

ARUTYUNYAN, I., FATKHUDINOV, T., KANANYKHINA, E., USMAN, N., ELCHANINOV, A., MAKAROV, A., BOLSHAKOVA, G., GOLDSHTEIN, D. & SUKHIKH, G. 2016. Role of VEGF-A in angiogenesis promoted by umbilical cord-derived mesenchymal stromal/stem cells: in vitro study. *Stem Cell Research & Therapy,* 7.

AZZONI, A. R., RIBEIRO, S. C., MONTEIRO, G. A. & PRAZERES, D. M. F. 2007. The impact of polyadenylation signals on plasmid nuclease-resistance and transgene expression. *The Journal of Gene Medicine,* 9**,** 392-402.

BAER, P. C. & GEIGER, H. 2012. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells Int,* 2012**,** 812693.

BEEGLE, J. R., MAGNER, N. L., KALOMOIRIS, S., HARDING, A., ZHOU, P., NACEY, C., WHITE, J. L., PEPPER, K., GRUENLOH, W., ANNETT, G., NOLTA, J. A. & FIERRO, F. A. 2016. Preclinical evaluation of mesenchymal stem cells overexpressing VEGF to treat critical limb ischemia. *Molecular Therapy — Methods & Clinical Development,* 3**,** 16053.

BENABDALLAH, B. F., ALLARD, E., YAO, S., FRIEDMAN, G., GREGORY, P. D., ELIOPOULOS, N., FRADETTE, J., SPEES, J. L., HADDAD, E., HOLMES, M. C. & BEAUSEJOUR, C. M. 2010. Targeted gene addition to human mesenchymal stromal cells as a cell-based plasma-soluble protein delivery platform. *Cytotherapy,* 12**,** 394-9.

BOURA, J. S., SANTOS, F. D., GIMBLE, J. M., CARDOSO, C. M. P., MADEIRA, C., CABRAL, J. M. S. & SILVA, C. L. D. 2013. Direct Head-To-Head Comparison of Cationic Liposome-Mediated Gene Delivery to Mesenchymal Stem/Stromal Cells of Different Human Sources: A Comprehensive Study. *Human Gene Therapy Methods,* 24**,** 38-48.

BOURA, J. S., VANCE, M., YIN, W., MADEIRA, C., LOBATO DA SILVA, C., PORADA, C. D. & ALMEIDA-PORADA, G. 2014. Evaluation of gene delivery strategies to efficiently overexpress functional HLA-G on human bone marrow stromal cells. *Mol Ther Methods Clin Dev,* 2014.

CARMELIET, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med,* 6**,** 389-95.

CHANG, C.-W., CHRISTENSEN, L. V., LEE, M. & KIM, S. W. 2008. Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy. *Journal of Controlled Release,* 125**,** 155-163.

CHEN, L., XU, Y., ZHAO, J., ZHANG, Z., YANG, R., XIE, J., LIU, X. & QI, S. 2014. Conditioned medium from hypoxic bone marrow-derived mesenchymal stem cells enhances wound healing in mice. *PLoS One,* 9**,** e96161.

CHEN, Z. Y., HE, C. Y., EHRHARDT, A. & KAY, M. A. 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther,* 8**,** 495-500.

CHO, H. M., KIM, P. H., CHANG, H. K., SHEN, Y. M., BONSRA, K., KANG, B. J., YUM, S. Y., KIM, J. H., LEE, S. Y., CHOI, M. C., KIM, H. H., JANG, G. & CHO, J. Y. 2017. Targeted Genome Engineering to Control VEGF Expression in Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells: Potential Implications for the Treatment of Myocardial Infarction. *Stem Cells Transl Med,* 6**,** 1040-1051.

CHULLIKANA, A., MAJUMDAR, A. S., GOTTIPAMULA, S., KRISHNAMURTHY, S., KUMAR, A. S., PRAKASH, V. S. & GUPTA, P. K. 2015. Randomized, double-blind, phase I/II study of intravenous allogeneic mesenchymal stromal cells in acute myocardial infarction. *Cytotherapy,* 17**,** 250-61.

DARQUET, A. M., CAMERON, B., WILS, P., SCHERMAN, D. & CROUZET, J. 1997. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther,* 4**,** 1341-9.

DARQUET, A. M., RANGARA, R., KREISS, P., SCHWARTZ, B., NAIMI, S., DELAERE, P., CROUZET, J. & SCHERMAN, D. 1999. Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer. *Gene Ther,* 6**,** 209-18.

DE SOURE, A. M., FERNANDES-PLATZGUMMER, A., MOREIRA, F., LILAIA, C., LIU, S. H., KU, C. P., HUANG, Y. F., MILLIGAN, W., CABRAL, J. M. S. & DA SILVA, C. L. 2017. 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,* 11**,** 1630-1640.

DIETZ, W. M., SKINNER, N. E., HAMILTON, S. E., JUND, M. D., HEITFELD, S. M., LITTERMAN, A. J., HWU, P., CHEN, Z. Y., SALAZAR, A. M., OHLFEST, J. R., BLAZAR, B. R., PENNELL, C. A. & OSBORN, M. J. 2013. Minicircle DNA is superior to plasmid DNA in eliciting antigen-specific CD8+ T-cell responses. *Mol Ther,* 21**,** 1526-35.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-7.

DOS SANTOS, F., ANDRADE, P. Z., ABECASIS, M. M., GIMBLE, J. M., CHASE, L. G., CAMPBELL, A. M., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Toward a Clinical-Grade Expansion of Mesenchymal Stem Cells from Human Sources: A Microcarrier-Based Culture System Under Xeno-Free Conditions. *Tissue Engineering. Part C, Methods,* 17**,** 1201-1210.

DOS SANTOS, F., ANDRADE, P. Z., BOURA, J. S., ABECASIS, M. M., DA SILVA, C. U. L. & CABRAL, J. M. S. 2010. Ex vivo expansion of human mesenchymal stem cells: A more effective cell proliferation kinetics and metabolism under hypoxia. *Journal of Cellular Physiology,* 223**,** 27-35.

DOS SANTOS, F., CAMPBELL, A., FERNANDES-PLATZGUMMER, A., ANDRADE, P. Z., GIMBLE, J. M., WEN, Y., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2014. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. *Biotechnology and Bioengineering,* 111**,** 1116-1127.

DU, W. J., CHI, Y., YANG, Z. X., LI, Z. J., CUI, J. J., SONG, B. Q., LI, X., YANG, S. G., HAN, Z. B. & HAN, Z. C. 2016. Heterogeneity of proangiogenic features in mesenchymal stem cells derived from bone marrow, adipose tissue, umbilical cord, and placenta. *Stem Cell Res Ther,* 7**,** 163.

ELSEBERG, C., LEBER, J., WEIDNER, T. & CZERMAK, P. 2017. The Challenge of Human Mesenchymal Stromal Cell Expansion: Current and Prospective Answers. *New Insights into Cell Culture Technology.*

FERRARA, N., GERBER, H.-P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9**,** 669-676.

FOLKMAN, J. 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med,* 285**,** 1182-6.

FONG, C. Y., CHAK, L. L., BISWAS, A., TAN, J. H., GAUTHAMAN, K., CHAN, W. K. & BONGSO, A. 2011. Human Wharton's jelly stem cells have unique transcriptome profiles compared to human embryonic stem cells and other mesenchymal stem cells. *Stem Cell Rev,* 7**,** 1-16.

GALIPEAU, J. & SENSEBE, L. 2018. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell,* 22**,** 824-833.

GIMBLE, J. & GUILAK, F. 2003. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy,* 5**,** 362-9.

GOODWIN, A. M. 2007. In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvasc Res,* 74**,** 172-83.

GUPTA, P. K., CHULLIKANA, A., PARAKH, R., DESAI, S., DAS, A., GOTTIPAMULA, S., KRISHNAMURTHY, S., ANTHONY, N., PHERWANI, A. & MAJUMDAR, A. S. 2013. A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. *J Transl Med,* 11**,** 143.

HACEIN-BEY-ABINA, S., VON KALLE, C., SCHMIDT, M., LE DEIST, F., WULFFRAAT, N., MCINTYRE, E., RADFORD, I., VILLEVAL, J. L., FRASER, C. C., CAVAZZANA-CALVO, M. & FISCHER, A. 2003. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med.* United States.

HÄCKER, G., REDECKE, V. & HÄCKER, H. 2002. Activation of the immune system by bacterial CpG-DNA. *Immunology,* 105**,** 245-51.

HARE, J. M., TRAVERSE, J. H., HENRY, T. D., DIB, N., STRUMPF, R. K., SCHULMAN, S. P., GERSTENBLITH, G., DEMARIA, A. N., DENKTAS, A. E., GAMMON, R. S., HERMILLER, J. B., JR., REISMAN, M. A., SCHAER, G. L. & SHERMAN, W. 2009. 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,* 54**,** 2277-86.

HASS, R., KASPER, C., BOHM, S. & JACOBS, R. 2011. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal,* 9**,** 12.

KAGIWADA, H., YASHIKI, T., OHSHIMA, A., TADOKORO, M., NAGAYA, N. & OHGUSHI, H. 2008. Human mesenchymal stem cells as a stable source of VEGF-producing cells. *Journal of Tissue Engineering and Regenerative Medicine,* 2**,** 184-189.

KANG, I., LEE, B. C., CHOI, S. W., LEE, J. Y., KIM, J. J., KIM, B. E., KIM, D. H., LEE, S. E., SHIN, N., SEO, Y., KIM, H. S., KIM, D. I. & KANG, K. S. 2018. Donor-dependent variation of human umbilical cord blood mesenchymal stem cells in response to hypoxic preconditioning and amelioration of limb ischemia. *Exp Mol Med,* 50**,** 35.

KERN, S., EICHLER, H., STOEVE, J., KLUTER, H. & BIEBACK, K. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells,* 24**,** 1294-301.

KETTERL, N., BRACHTL, G., SCHUH, C., BIEBACK, K., SCHALLMOSER, K., REINISCH, A. & STRUNK, D. 2015. A robust potency assay highlights significant donor variation of human mesenchymal stem/progenitor cell immune modulatory capacity and extended radio-resistance. *Stem Cell Res Ther,* 6**,** 236.

KIZILAY MANCINI, O., LORA, M., SHUM-TIM, D., NADEAU, S., RODIER, F. & COLMEGNA, I. 2017. A Proinflammatory Secretome Mediates the Impaired Immunopotency of Human Mesenchymal Stromal Cells in Elderly Patients with Atherosclerosis. *Stem Cells Transl Med,* 6**,** 1132-1140.

KO, J., JUN, H., CHUNG, H., YOON, C., KIM, T., KWON, M., LEE, S., JUNG, S., KIM, M. & PARK, J. H. 2011. Comparison of EGF with VEGF Non-Viral Gene Therapy for Cutaneous Wound Healing of Streptozotocin Diabetic Mice. *Diabetes Metab J,* 35**,** 226-35.

KUCHROO, P., DAVE, V., VIJAYAN, A., VISWANATHAN, C. & GHOSH, D. 2015. Paracrine factors secreted by umbilical cord-derived mesenchymal stem cells induce angiogenesis in vitro by a VEGF-independent pathway. *Stem Cells Dev,* 24**,** 437-50.

LE BLANC, K., FRASSONI, F., BALL, L., LOCATELLI, F., ROELOFS, H., LEWIS, I., LANINO, E., SUNDBERG, B., BERNARDO, M. E., REMBERGER, M., DINI, G., EGELER, R. M., BACIGALUPO, A., FIBBE, W. & RINGDEN, O. 2008. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet,* 371**,** 1579-86.

LE BLANC, K., TAMMIK, C., ROSENDAHL, K., ZETTERBERG, E. & RINGDEN, O. 2003. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol,* 31**,** 890-6.

LEE, R. J., SPRINGER, M. L., BLANCO-BOSE, W. E., SHAW, R., URSELL, P. C. & BLAU, H. M. 2000. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation,* 102**,** 898-901.

LIEW, A. & O'BRIEN, T. 2012. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Res Ther,* 3**,** 28.

LIM, J. Y., PARK, S. H., JEONG, C. H., OH, J. H., KIM, S. M., RYU, C. H., PARK, S. A., AHN, J. G., OH, W., JEUN, S. S. & CHANG, J. W. 2010. Microporation is a valuable transfection method for efficient gene delivery into human umbilical cord blood-derived mesenchymal stem cells. *BMC Biotechnol,* 10**,** 38.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LOCATELLI, P., OLEA, F. D., HNATIUK, A., SEPULVEDA, D., PEREZ SAEZ, J. M., ARGUELLO, R. & CROTTOGINI, A. 2013. Efficient plasmid-mediated gene transfection of ovine bone marrow mesenchymal stromal cells. *Cytotherapy,* 15**,** 163-70.

MADEIRA, C., RIBEIRO, S. C., PINHEIRO, I. S. M., MARTINS, S. A. M., ANDRADE, P. Z., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Gene delivery to human bone marrow mesenchymal stem cells by microporation. *Journal of Biotechnology,* 151**,** 130-136.

MADEIRA, C., RODRIGUES, C. A. V., REIS, M. S. C., FERREIRA, F. F. C. G., CORREIA, R. E. S. M., DIOGO, M. M. & CABRAL, J. M. S. 2013. Nonviral Gene Delivery to Neural Stem Cells with Minicircles by Microporation. *Biomacromolecules,* 14**,** 1379-1387.

MCMAHON, J. M., CONROY, S., LYONS, M., GREISER, U., O'SHEA, C., STRAPPE, P., HOWARD, L., MURPHY, M., BARRY, F. & O'BRIEN, T. 2006. Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors. *Stem Cells Dev,* 15**,** 87-96.

MUELLER, S. M. & GLOWACKI, J. 2001. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J Cell Biochem,* 82**,** 583-90.

MUNYE, M. M., TAGALAKIS, A. D., BARNES, J. L., BROWN, R. E., MCANULTY, R. J., HOWE, S. J. & HART, S. L. 2016. Minicircle DNA Provides Enhanced and Prolonged Transgene Expression Following Airway Gene Transfer. *Sci Rep,* 6**,** 23125.

NISHIDA, S., ENDO, N., YAMAGIWA, H., TANIZAWA, T. & TAKAHASHI, H. E. 1999. Number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation. *J Bone Miner Metab,* 17**,** 171-7.

POTIER, E., FERREIRA, E., MEUNIER, A., SEDEL, L., LOGEART-AVRAMOGLOU, D. & PETITE, H. 2007. Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue Eng,* 13**,** 1325-31.

RIBEIRO, A., LARANJEIRA, P., MENDES, S., VELADA, I., LEITE, C., ANDRADE, P., SANTOS, F., HENRIQUES, A., GRÃOS, M., CARDOSO, C. M. P., MARTINHO, A., PAIS, M., DA SILVA, C. L., CABRAL, J., TRINDADE, H. & PAIVA, A. 2013. 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 Res Ther.*

SERRA, J., ALVES, C. P. A., BRITO, L., MONTEIRO, G. A., CABRAL, J. M. S., PRAZERES, D. M. F. & DA SILVA, C. L. 2018. Engineering of Human Mesenchymal Stem/Stromal Cells with Vascular Endothelial Growth Factor-Encoding Minicircles for Angiogenic Ex Vivo Gene Therapy. *Hum Gene Ther*.

SHEVCHENKO, E. K., MAKAREVICH, P. I., TSOKOLAEVA, Z. I., BOLDYREVA, M. A., SYSOEVA, V. Y., TKACHUK, V. A. & PARFYONOVA, Y. V. 2013. Transplantation of modified human adipose derived stromal cells expressing VEGF165 results in more efficient angiogenic response in ischemic skeletal muscle. *J Transl Med,* 11**,** 138.

SIEGEL, G., KLUBA, T., HERMANUTZ-KLEIN, U., BIEBACK, K., NORTHOFF, H. & SCHÄFER, R. 2013. Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Medicine,* 11**,** 146.

SIMCIKOVA, M., PRATHER, K. L. J., PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. On the dual effect of glucose during production of pBAD/AraC-based minicircles. *Vaccine,* 32**,** 2843-2846.

SIMOES, I. N., BOURA, J. S., DOS SANTOS, F., ANDRADE, P. Z., CARDOSO, C. M., GIMBLE, J. M., DA SILVA, C. L. & CABRAL, J. M. 2013. Human mesenchymal stem cells from the umbilical cord matrix: successful isolation and ex vivo expansion using serum-/xeno-free culture media. *Biotechnol J,* 8**,** 448-58.

SINGER, N. G. & CAPLAN, A. I. 2011. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol,* 6**,** 457-78.

STENDERUP, K., JUSTESEN, J., CLAUSEN, C. & KASSEM, M. 2003. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone,* 33**,** 919-26.

STIEHLER, M., DUCH, M., MYGIND, T., LI, H., ULRICH-VINTHER, M., MODIN, C., BAATRUP, A., LIND, M., PEDERSEN, F. S. & BUNGER, C. E. 2006. Optimizing viral and non-viral gene transfer methods for genetic modification of porcine mesenchymal stem cells. *Adv Exp Med Biol,* 585**,** 31-48.

TANG, Y. L., ZHAO, Q., QIN, X., SHEN, L., CHENG, L., GE, J. & PHILLIPS, M. I. 2005. Paracrine Action Enhances the Effects of Autologous Mesenchymal Stem Cell Transplantation on Vascular Regeneration in Rat Model of Myocardial Infarction. *The Annals of Thoracic Surgery,* 80**,** 229-237.

TERAA, M., SPRENGERS, R. W., WESTERWEEL, P. E., GREMMELS, H., GOUMANS, M. J., TEERLINK, T., MOLL, F. L. & VERHAAR, M. C. 2013. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS One,* 8**,** e55592.

VAN DER LOO, J. C. & WRIGHT, J. F. 2016. Progress and challenges in viral vector manufacturing. *Hum Mol Genet,* 25**,** R42-52.

WALKER, W. E., BOOTH, C. J. & GOLDSTEIN, D. R. 2010. TLR9 and IRF3 Cooperate to Induce a Systemic Inflammatory Response in Mice Injected With Liposome:DNA. *Mol Ther.*

WANG, Z., WANG, L., SU, X., PU, J., JIANG, M. & HE, B. 2017. Rational transplant timing and dose of mesenchymal stromal cells in patients with acute myocardial infarction: a meta-analysis of randomized controlled trials. *Stem Cell Res Ther,* 8**,** 21.

WATT, S. M., GULLO, F., VAN DER GARDE, M., MARKESON, D., CAMICIA, R., KHOO, C. P. & ZWAGINGA, J. J. 2013. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. *Br Med Bull,* 108**,** 25-53.

YOON, C. S., JUNG, H. S., KWON, M. J., LEE, S. H., KIM, C. W., KIM, M. K., LEE, M. & PARK, J. H. 2009. Sonoporation of the minicircle-VEGF(165) for wound healing of diabetic mice. *Pharm Res,* 26**,** 794-801.

ZHANG, J., HUANG, X., WANG, H., LIU, X., ZHANG, T., WANG, Y. & HU, D. 2015. The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. *Stem Cell Res Ther,* 6**,** 234.

Chapter VI

EVALUATION OF THE THERAPEUTIC POTENTIAL OF HUMAN MESENCHYMAL STEM/STROMAL CELLS MODIFIED WITH VEGF-ENCODING MINICIRCLES IN AN *IN VIVO* MODEL

VI. EVALUATION OF THE THERAPEUTIC POTENTIAL OF HUMAN MESENCHYMAL STEM/STROMAL CELLS MODIFIED WITH VEGF-ENCODING MINICIRCLES IN AN *IN VIVO* MODEL

VI.1. Summary

Peripheral arterial disease (PAD) is a highly prevalent chronic disease caused by obstruction of arteries in the lower extremities. With disease progression some patients are faced with the possibility of limb amputation or even dead. The current available therapies have limited effectiveness for patients with late stage of the disease. Novel and promising treatments that consist in the administration of cell or gene therapies with pro-angiogenic activity have been developed. Mesenchymal stem/stromal cells (MSC), which are known to have an angiogenic activity through secretion of a broad spectrum of soluble factors, have been extensively exploited in PAD treatment. Vascular endothelial growth factor (VEGF) is one of those soluble factors and is probably the most potent angiogenic molecule. VEGF-based gene therapies have also been investigated in the context of PAD. In the present work, cell-based gene therapy that relies on MSC genetically engineered to overexpress VEGF protein is proposed. To overcome risks associated with viral vectors and the limitation of plasmids in terms of transgene expression, minicircle vectors were used for MSC transfection. Minicircles are small circular DNA molecules, derived from plasmids but with lower size and reduced toxicity. Bone marrow (BM)-derived MSC transfected with VEGF-encoding minicircles (MC-VEGF) were then administered in a mice model of hindlimb ischemia. Treatment with MSC modified with MC-VEGF (MSC+MC) was compared with non-transfected MSC and with direct gene therapy with MC-VEGF. Mice were evaluated for 30 days after treatment by weekly blood flow analysis and visual assessment of necrosis on limbs. No significant differences were observed on blood reperfusion or limb necrosis between the three treated groups, but all showed improved recovery when compared to ischemic non-treated mice. At day 30 after treatment, muscle force of mice ischemic limbs was evaluated. The group treated with MSC+MC demonstrated the highest muscle strength (0.25±0.04 N) when compared to non-transfected MSC (0.19±0.03 N) or MC-VEGF (0.20±0.03 N) alone. Overall, results indicate that treatment with MSC overexpressing VEGF can be a promising strategy to induce recovery of muscular function in PAD patients suffering from limb ischemia.

VI.2. Background

Peripheral arterial disease (PAD) is a prevalent and high burden chronic disease caused by narrowing and obstruction arteries, leading to decreased blood flow to the lower extremities (Conte and Vale, 2018, Norgren et al., 2007). PAD affects 3-10% of the world population and about 30% of such patients are faced with the possibility of limb amputation within 1 year (Norgren et al., 2007). One of the more severe stages of PAD is critical limb ischemia (CLI), which is associated with high mortality and morbidity. The only option for patients suffering from severe CLI is revascularization, but this is an invasive procedure with associated risks, especially for PAD patients who usually have several co-morbidities (Gresele et al., 2011). Also, 50% of CLI patients are not eligible for revascularization, the so-called no-option patients. Those patients are dependent on the adaptation of pre-existing collateral vessels (arteriogenesis) or on the formation of new vessels through vasculogenesis or angiogenesis to recover tissue oxygenation (Gresele et al., 2011, Carmeliet, 2000a). Current treatments for PAD are insufficient and ineffective on promoting those actions, so novel therapies addressing PAD treatment have been developed. The use of stem/progenitor cells and/or angiogenic factors are example of this strategies usually designated as therapeutic angiogenesis (Grochot-Przeczek et al., 2013, Hassanshahi et al., 2019).

Multipotent mesenchymal stem/stromal cells (MSC) are characterized *in vitro* by their plastic adherence, capacity to self-renew and ability to differentiate into osteocytes, adipocytes and chondrocytes (Dominici et al., 2006). Also, they are known to have a pro-angiogenic capacity and thus, have been extensively exploited in ischemic cardiovascular approaches (Watt et al., 2013). Although some studies suggested that MSC differentiate into endothelial cells (Tao et al., 2016, Lin and Lue, 2013), their pro-angiogenic potential most probably relies on their well-characterized trophic activity (Singer and Caplan, 2011). Another important feature of MSC is their reduced immunogenicity, which makes them suitable for allogeneic off-the-self therapies (Le Blanc et al., 2003a). MSC have been extensively exploited in the context of PAD in both pre-clinical (Kinnaird et al., 2004, Leroux et al., 2010, Liew and O'Brien, 2012) and clinical studies (Gupta et al., 2013, Lu et al., 2011). However, it is known that they have a limited survival and engraftment on harsh ischemic tissues (Potier et al., 2007) and some trials regarding MSC application for cardiac ischemic diseases showed conflicting data or only modest benefits (Chullikana et al., 2015 , Wang et al., 2017). Thus, strategies to improve MSC therapeutic properties in the context of ischemic diseases, such as PAD, have been investigated in the last decades, namely genetic engineering with pro-angiogenic factors.

Vascular endothelial growth factor (VEGF) is a key regulator of physiological angiogenesis, known to promote endothelial cell growth and survival, being crucial for vascular regeneration (Ferrara et al., 2003). In fact, several studies reported the beneficial effect of VEGF gene therapy in the context of PAD (Isner et al., 1996, Kusumanto et al., 2006, Deev et al., 2015). The combination of VEGF gene therapy with a cell vehicle that displays intrinsic therapeutic properties might be a promising approach for the treatment of ischemic chronic conditions, such as PAD. Thus, within the scope of this PhD thesis, it is proposed the genetic engineering of MSC using VEGF-encoding vectors as a potential therapy for PAD patients.

In what concerns genetic engineering approaches, vectors for an effective and safely deliver of transgenes into the cells are required. Despite the use of viral vectors has been described to be more efficient than non-viral counterparts, their use has been associated with several adverse events (Hacein-Bey-Abina et al., 2003). Plasmids, on the other hand, are especially adequate for MSC modification due to their low immunogenicity, reduced risk of insertional mutagenesis and ease of manufacturing (Prazeres and Monteiro, 2014). However, there are some limitations related with conventional plasmids, including low transfection efficiency and short duration of transgene expression (Raval and Losordo, 2013). Small plasmid derivatives that carry only the transgene expression cassette - minicircles - have been developed to overcome plasmid-associated issues. Minicircles are free from bacterial sequences that may activate immune system and thus promote transgene silencing (Häcker et al., 2002). Also, their lower size may further contribute to improve transfection and, consequently, transgene expression (Darquet et al., 1997). Several studies confirmed that minicircle-based delivery led to superior and prolonged transgene expression, as well as enhanced stem cell survival than conventional plasmid systems (Darquet et al., 1999, Chen et al., 2003, Dietz et al., 2013, Madeira et al., 2013, Munye et al., 2016. Minicircles also demonstrated to promote an efficient delivery of VEGF in the context of angiogenic therapies (Chang et al., 2008, Yoon et al., 2009, Ko et al., 2011).

The work presented in Chapters IV and V focused on the improvement of MSC angiogenic potential *in vitro* using VEGF-containing minicircles. Herein, this strategy is tested *in vivo* using a mouse model for hindlimb ischemia previously established in context of PAD (Cunha et al., 2013, Martins et al., 2014). Mice were observed for 30 days after treatment and the angiogenic potential of genetic engineered MSC was evaluated by measuring limb necrotic score, blood reperfusion and muscle force and by performing histological analysis. This represents, to our best knowledge, the first report where the angiogenic potential of MSC genetically engineered with VEGF-encoding minicircles was evaluated in an *in vivo* model of hindlimb ischemia.

VI.3. Materials and Methods

VI.3.1. Plasmid construction, production and purification

The parental plasmid (pMINILi-CV, 3,821 bp) expressing VEGF (165a) gene was obtained from pMINILi-CVG, by removing the GFP gene (Chapter II). The vector was constructed and transformed by heat shock into *E. coli* as described elsewhere (Azzoni et al., 2007, Simcikova et al., 2014, Alves et al., 2016). pMINILi-CV contains an expression cassette with VEGF and the human cytomegalovirus (CMV) immediate-early promoter, two multimer resolution sites (MRS) flanking the expression cassette, pMB1 origin of replication, kanamycin resistance gene and BGH polyadenylation sequence (Serra et al., 2018). *E. coli* BW2P harboring the parental plasmid pMINLI-CV were used for minicircle production, according to previously established methods (Simcikova et al., 2014, Alves et al., 2016). *E. coli* was grown until the late exponential phase and then recombination was induced for 2 h (hours) by addition of 0.01% (w/v) L-(+)arabinose (Merck) to obtain the minicircle with the expression cassette and a miniplasmid (MP) with the prokaryotic backbone. All plasmid DNA species were recovered and purified using an endotoxin-free plasmid DNA purification kit (Macherey-Nagel). Minicircle was separated from other DNA forms by digestion with nicking endonuclease (Nb.BbvCI) followed by hydrophobic interaction chromatography (HIC), as described elsewhere (Alves et al., 2016, Alves et al., 2018). The concentration of purified pDNA solution was assayed on NanoDrop (Thermo Scientific) and DNA integrity was confirmed by agarose gel electrophoresis.

VI.3.2. Culture of human MSC from BM

Human MSC from BM were kindly provided by Doctor Kamilla Swiech (Dept. of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo). MSC were maintained frozen at -80ºC until further use. Upon thawing, cells were cultured for 1 to 4 passages under xenogeneic (xeno)-free culture conditions, as described elsewhere (dos Santos et al., 2011, dos Santos et al., 2014). Cells were plated at a cell density between 3,000-6,000 cells/cm^2 on CELLstart™ CTS™ (Invitrogen) pre-coated T-flasks using StemPro® MSC SFM XenoFree (Invitrogen) supplemented with 1% GlutaMAX™-I CTS™ (Invitrogen) and 1% Antibiotic-Antimycotic (Invitrogen). Cells were maintained at 37ºC and 5% CO_2 in a humidified atmosphere and culture medium was exchanged every 3-4 days. At 70% cell confluence, MSC were detached from the flasks by adding TrypLE™ Select CTS™ (Invitrogen) solution 1x in Phosphate Buffered Saline (PBS, Gibco). Cell number and viability were determined using the Trypan Blue (Gibco) exclusion method. BM-MSC at passages between 4 and 6 were used.

VI.3.3. Nucleofection of BM-MSC with VEGF-encoding minicircles (or pMaxGFP)

BM-MSC nucleofection was performed according to the Human MSC Nucleofector Kit (Lonza) protocol. Briefly, 5×10^5 were ressuspended in 100ul of nucleofection buffer with 4 µl of pmaxGFP™ (provided with the kit) or 2 µg of MC-VEGF and nucleofected using the U-23 program of Nucleofector™ 2b Device. After nucleofection, cells were carefully transferred to Opti-MEM™ I Reduced Serum Medium (Gibco) and maintained for 20-30 minutes. Then, cells were plated at a density of 7,000-8,000 cells/cm^2 in pre-warmed StemPro® XenoFree culture medium and maintained at 37°C and 5% CO_2 in a humidified atmosphere for 48 h. After that, cell numbers were estimated using the trypan blue dye exclusion method. Non-nucleofected human BM-MSC were used as control.

VI.3.4. Analysis of nucleofection efficiency *in vitro*

Before transfection with MC-VEGF, the protocol for human MSC nucleofection was evaluated with the positive control vector pmaxGFP™. The cells transfected with this vector were then analyzed by fluorescence microscopy and flow cytometry to assess transfection efficiency by monitoring green fluorescent protein (GFP) expression. To confirm the efficiency of nucleofection with MC-VEGF, the levels of VEGF protein in MSC culture medium were evaluated 24 h and 48 h after nucleofection using Human VEGF DuoSet ELISA kit (R&D systems).

VI.3.5. Hindlimb ischemia induction

All mice used for experiments were obtained from the animal house of the Federal University of Sao Paulo (UNIFESP). All experiments were carried out in accordance with the recommendations for the proper care and use of laboratory animals, as recommended by the Ethic Committee of the UNIFESP, who reviewed and approved the experiments previously to their realization. Surgical induction of ischemia was performed in 10- to 12-week-old Balb/c male mice. First, mice anesthesia was performed by intraperitoneal injection with ketamine (10 mg/kg) and xylazine (10 mg/kg). Then, ischemia was induced in right leg by excision of femoral artery by cauterization from its upper branch near the iliac artery to the bifurcation of the popliteal artery, on the basis of a procedure previously established (Martins et al., 2014, Cunha et al., 2013). Mice were maintained in a warm surface until complete recovery from anesthesia and then placed and maintained in a microisolator with ventilated shelves with food and water supplies. The animals were followed for 30 days.

VI.3.6. Cell-based gene therapy to ischemic mice

Three days after ischemic induction, mice were anesthetized and treated with MSC alone, MSC+MC-VEGF or MC-VEGF. Thus, mice were divided into five groups as follows: non-ischemic mice (N-IS) (n=10), ischemic mice (IS) (n=10), ischemic mice treated with human MSC (MSC) (n=10), ischemic mice treated with human MSC modified with MC-VEGF (MSC+MC) (n=10) and ischemic mice treated with MC-VEGF (MC) (n=8). For mice assigned to groups treated with MSC, 5×10^5 cells transfected with MC-VEGF (MSC+MC group) or non-transfected (MSC) were injected into the middle of the quadriceps in 85 µl of PBS using a 21-gauge needle. The animals treated with MC-VEGF were carefully injected with 50 µg of MSC-VEGF in 85 µl of PBS at the middle of quadriceps. After DNA infusion, electroporation was performed as follows: 6 pulses of 100V/cm and 40 milliseconds were applied with 1 second interval. Mice were maintained under observation in a warm surface until complete recovery from anesthesia

VI.3.7. Visual assessment of ischemic limbs

Visual assessment of ischemic limbs was performed weekly on the basis of the necrosis score described by Martins and colleagues (Martins et al., 2014), which is divided as follows: I - no necrosis; II - blackened nails; III - necrosis of toes and IV - necrosis below the heel.

VI.3.8. Analysis of blood flow by laser Doppler

The restoration of blood flow after ischemia was evaluated weekly by laser Doppler. For blood flow analysis, mice were anesthetized as previously described, placed on the dorsal decubitus and blood flow measurements were performed using moorLDI2-HIR equipment (Moor Instruments). The results obtained were then analyzed and quantified using MoorLDI V6.0 (Moor Instruments) software.

VI.3.9. Evaluation of muscle force

The isometric muscle force of mice was determined 30 days after treatment before the euthanasia of the animals according to a method already standardized (Martins et al., 2014, Sacramento et al., 2009). Briefly, mice were placed on the ventral decubitus after anesthesia and the gastrocnemius muscle was isolated, while the vascular connections and muscle origins were maintained intact. The calcaneal tendon was then isolated and connected to the force transducer MLT 1030/D (ADInstruments). The distal portion of sciatic nerve was placed over a

bipolar electrode connected to a stimulator (Grass S88; Grass Instruments). The muscle function was evaluated by measuring isomeric contraction response of the right gastrocnemius muscle. Pulses of 100 mV and 1 ms with 60 Hz were applied with 1-minute interval. For muscle force measurement it was used the software LabChart Pro (ADInstruments). After muscular strength analysis, the mass of *rectus femoris* and gastrocnemius muscles were determined on an analytical balance.

VI.3.10. Quantification of VEGF *in vivo* by ELISA

The levels of human and mouse VEGF in samples of blood and muscle were measured 30 days after the treatment using the Human VEGF DuoSet ELISA kit or Mouse VEGF DuoSet ELISA kit according to manufacturer's instructions. Blood obtained from mice hearts was centrifuged (2,300 g, 5 min) and plasma was collected for VEGF quantification. For the extraction of proteins from muscles, *rectus femoris* muscles were extracted from euthanized mice and frozen in liquid nitrogen. After addition of lysis buffer containing 0.6 µl of PMSF (Invitrogen) and 0.6 ul of Protease Inhibitor Cocktail (Sigma), the muscle samples were mechanically homogenized in a TissueLyser II (Qiagen) at 10 oscillation/minute for 10 min. The resulting homogenate was then centrifuged for 30 min at 15,200 g and 4°C and the resulting supernatant was collected for VEGF analysis.

VI.3.11. Histological analysis

The animals were euthanized 30 days after treatment and the *rectus femoris* muscles were removed, washed with PBS and fixed with paraformaldehyde (PFA) 4% for 24 h. Then, muscles were preserved with increasing amounts of sucrose (10, 20 and 30%) (Merck) for 72 h (24 h in each solution). Muscle samples were frozen at -20°C with the inclusion matrix Killik-Oct (Easy-Path), indicated for preparation of tissue samples to the cut on cryostat and then kept at -80°C until further analysis. Muscle tissue sections of 8 µm thickness were obtained using the cryostat Leica CM 1950 (Leica Biosystems). Before staining and microscopy analysis, samples were fixed with PFA 4% for 15 min and then washed with PBS. Staining with hematoxylin and eosin (HE) was performed to determine the degree of muscle regeneration and fibrosis or the presence of infiltrated adipocytes. Staining with HE (Easy-Path) was performed according to common routine staining protocol, as described elsewhere (Martins et al., 2014) and 10 random optical fields with a magnification of 10x were selected from each glass slide.

VI.3.12. Statistical analysis

All data is presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 software. Significance was determined by Tukey's multiple comparison test and set at a p-value (p) <0.05.

VI.4. Results

VI.4.1. Analysis of nucleofection efficiency for human MSC engineering

The transfection efficiency of human MSC using nucleofection was first assessed with pmaxGFP™, a plasmid expressing GFP provided by the nucleofector manufacturer. The GFP expression on viable cells was monitored by fluorescence microscopy (Figure V.1A) and flow cytometry (Figure VI.2B). Both analyses confirmed the effectiveness of nucleofection for human MSC transfection, since a high number of green cells was observed either under the microscope or by flow cytometry (64.7% GFP^+ cells). No GFP-expressing cells were detected on control, non-transfected, cells.

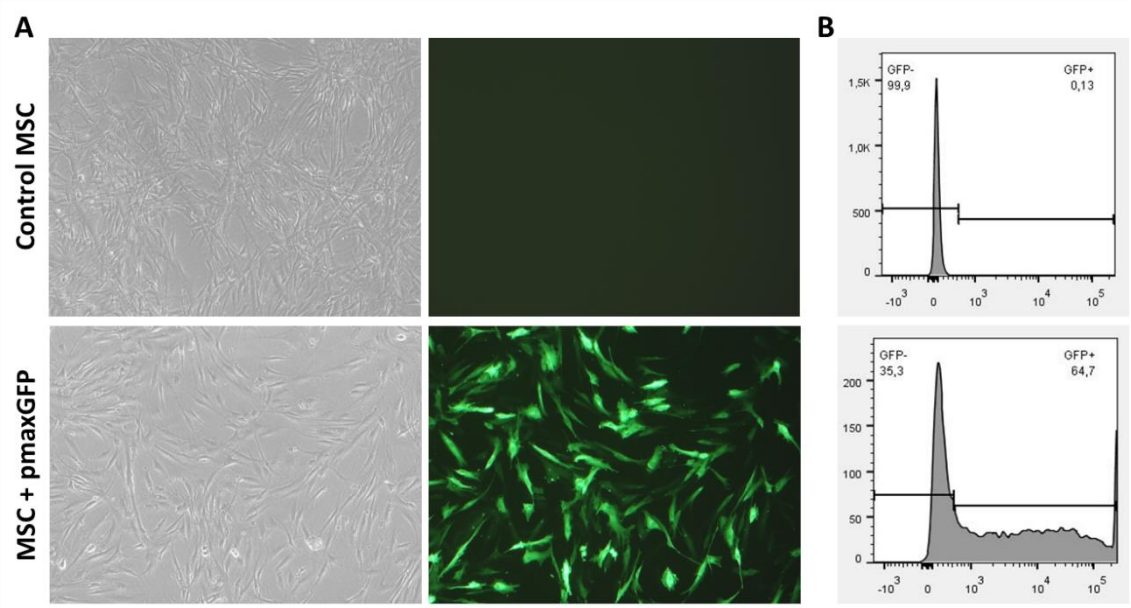

Figure VI.1 - Analysis of nucleofection efficiency for transfection of human MSC with pmaxGFP™ by **(A)** fluorescent microscopy and **(B)** flow cytometry.

After the preliminary confirmation of the nucleofection protocol effectiveness using GFP, human MSC were transfected with VEGF-encoding minicircles (MC-VEGF). The analysis of transgene expression was performed through quantification of VEGF amounts in the culture medium by ELISA (Figure VI.2). Culture medium from control (non-transfected) and nucleofected cells was collected 24 h and 48 h after transfection. The results revealed a significantly higher (p-value<0.01) production of VEGF by transfected cells when compared to non-modified controls, regardless the timepoint. The highest expression was observed 48 h after transfection with MC-VEGF ($4.5\times10^4\pm1.5\times10^4$ pg/mL), but the differences were not statistically significant compared to VEGF levels obtained at 24 h ($3.9\times10^4\pm0.9\times10^4$ pg/mL). These values indicate an increase of ≈90-fold on VEGF secretion after transfection with MC-VEGF.

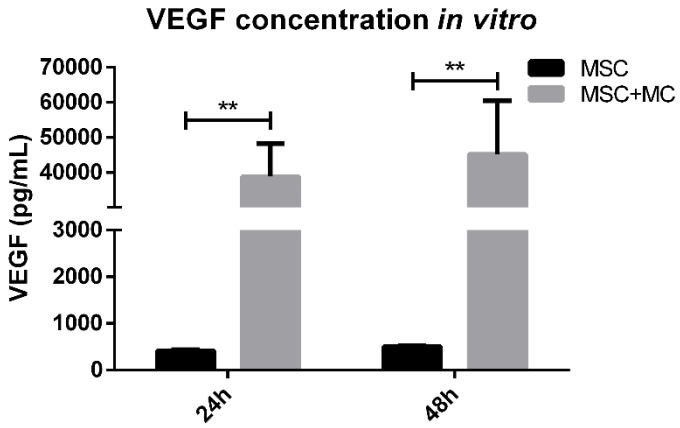

Figure VI.2 - Analysis of VEGF production by ELISA 24 or 48 hours after nucleofection with MC-VEGF (MSC+MC). Non-transfected cells were used as control (MSC). Values are presented as mean ± SEM, n=10 (replicates), **p<0.01

VI.4.2. Visual assessment of limbs

The animals were followed for 30 days after treatment and visual assessment of necrotic tissue in the limbs was performed every week (days 7, 14, 21 and 28). The results of this analysis are presented in Figure VI.3. Images of mice limbs were taken at each timepoint (Figure VI.3A) and necrosis (Figure VI.3B) was evaluated on the basis of the following score: I - no necrosis; II - blackened nails; III - necrosis of toes and IV - necrosis below the heel. None of the mice evaluated showed necrosis below the heel (score IV) and no necrosis (score I) was observed in healthy mice (N-IS).

For mice treated with MSC, 30% showed necrosis of toes (III) and 10% had blackened nails (II) on day 7, but on day 14 there were no mice with necrosis of toes (III) and 40% had blackened nails (II); on day 21, this percentage decreased to 30%. Regarding mice treated with MSC+MC, 50% had some degree of necrosis on day 7 (20% - necrosis of toes (III) and 30% - nail blackening (II)), but this percentage was reduced to 40% on day 14 (10% - score III and 30% - score II) and to 20% (only score II) on day 21. Finally, for the MC treated group 50% had nail blackening (II) on day 7 and the remaining mice had no necrosis (I). This percentage was reduced to 25% (only score II) on day 14 and to 12.5% on day 21. Regardless the type of treatment (MSC, MSC+MC or MC), all the treated mice had a necrosis score of II or lower after 28 days, even those who had a higher score in previous timepoints. In fact, on day 28, only the following mice showed necrosis (all score II): 20% of the mice from the groups treated with MSC or MSC+MC, 12.5% of the MC group and 40% of the ischemic, non-treated group (IS).

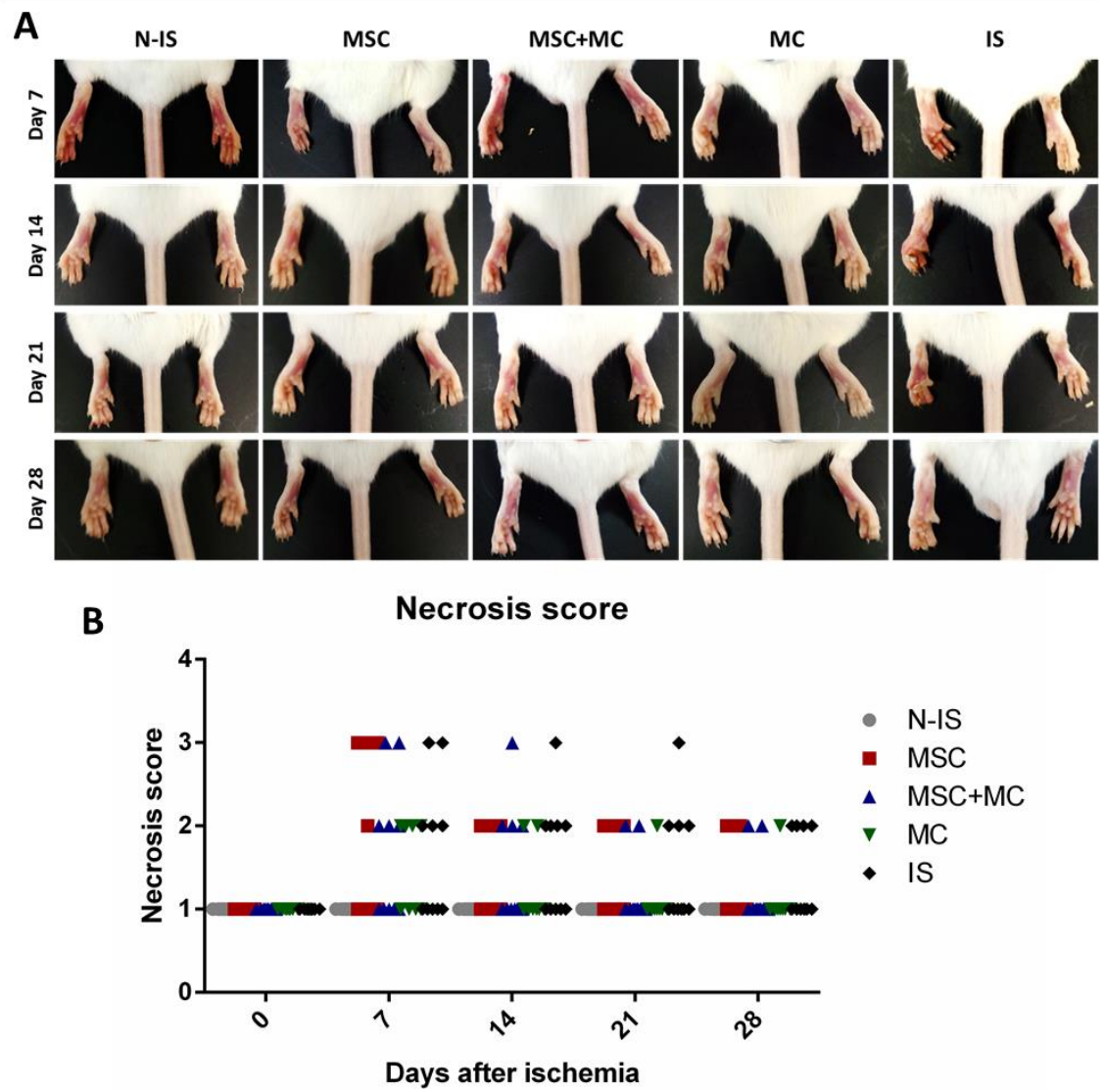

Figure VI.3 - Visual assessment of mice limbs for healthy (N-IS), treated (MSC, MSC+MC and MC) or ischemic (IS) mice performed weekly for 30 days. **(A)** Visual evaluation of limb necrosis and **(B)** quantification of necrosis degree according to the following scale: I - no necrosis; II - blackened nails; III - necrosis of toes and IV - necrosis below the heel. Plot of individual values; n=10 (N-IS, MSC, MSC+MC, IS); n=8 (MC). N-IS - non-ischemic mice; MSC - ischemic mice treated with human MSC; MSC+MC - ischemic mice treated with human MSC modified with MC-VEGF; MC - ischemic mice treated with MC-VEGF; IS - ischemic mice.

VI.4.3. Analysis of blood flow

After ischemia induction, reperfusion of mice limbs was evaluated weekly by laser Doppler (Figure VI.4). To assess the differences in blood flow between the treatments investigated, the levels of blood flow of all ischemic conditions were normalized relatively to the corresponding non-ischemic (N-IS) mice (set as 100%) at each timepoint. The measurements for day 0 were performed immediately after ischemia induction, confirming that all mice that underwent the surgery had no blood flow in right limb and, thus, ischemia was successfully induced. The results demonstrated that blood flow in right limbs of ischemic mice increases with the time, indicating blood reperfusion in ischemic limbs, regardless of the type of treatment. However, the blood

flow observed on ischemic limbs was significantly lower than on control (N-IS) mice for all the timepoints. Also, no significant differences were shown between the groups of ischemic mice (MSC, MSC+MC, MC or IS). So, the reperfusion rates of ischemic limbs seem to be similar for the three tested treatments and for the ischemic, non-treated, mice.

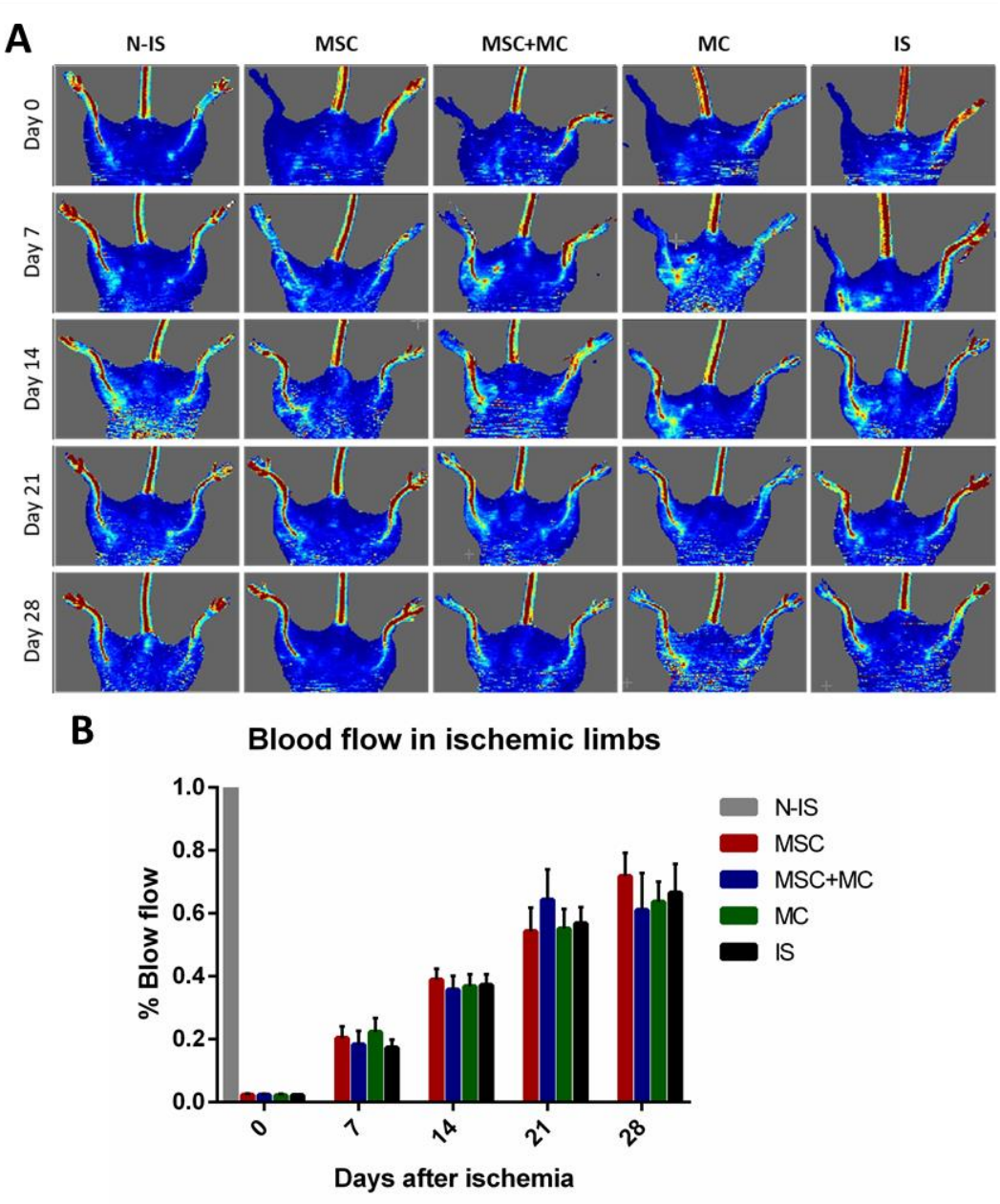

Figure VI.4 - Measurements of blood flow on healthy (N-IS), treated (MSC, MSC+MC and MC) and ischemic (IS) mice performed weekly for 30 days by laser doppler. Images obtained using moorLDI2-HIR equipment **(A)** were used to calculate the percentage of the blood flow for each ischemic group every week **(B)**. The values were normalized to positive control (non-ischemic mice) are presented as mean ± SEM, n=10 (N-IS, MSC, MSC+MC, IS); n=8 (MC). N-IS - non-ischemic mice; MSC - ischemic mice treated with human MSC; MSC+MC - ischemic mice treated with human MSC modified with MC-VEGF; MC - ischemic mice treated with MC-VEGF; IS - ischemic mice.

VI.4.4. Evaluation of muscle force

The assessment of gastrocnemius muscle force was performed 30 days after treatment, before mice euthanasia. Then, both gastrocnemius and *rectus femoris* muscles were weighed. These results are presented in Figure VI.5. Although the highest muscle force was observed for non-ischemic (N-IS) mice (0.36±0.06 N), no statistically significant differences were found between this and all treated groups (MSC, MSC+MC and MC). Also, mice from all the treated groups showed significantly higher muscle force than ischemic, non-treated mice (IS) (0.12±0.03 N) (p-value<0.05). Mice treated with MSC+MC had a muscle strength of 0.25±0.04 N, which was the highest between the three treated groups. MSC- and MC-treated limbs showed similar muscle strengths, of 0.19±0.03 N and 0.20±0.03 N, respectively. Regarding muscle weight, *rectus femoris* muscles from mice from all groups had similar weight. For gastrocnemius muscle, the highest weight was observed on non-ischemic mice and the lowest in non-treated ischemic mice, but no statistically significant differences were verified between any of the groups.

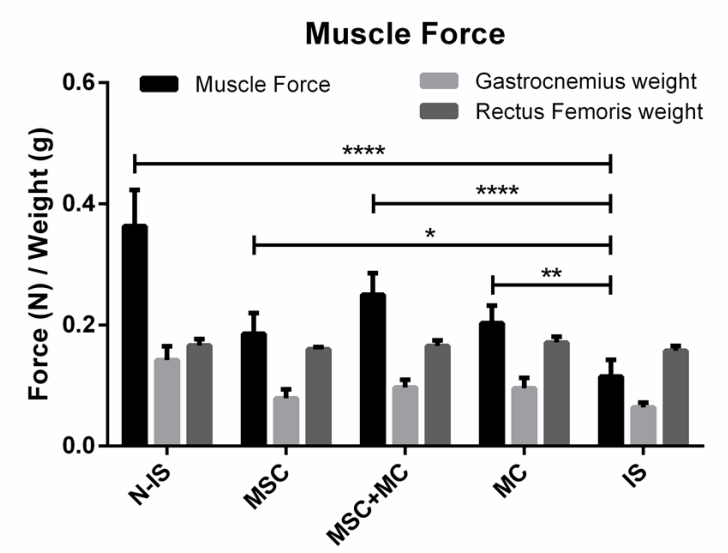

Figure VI.5 - Determination of gastrocnemius muscle force of non-ischemic (N-IS), treated (MSC, MSC+MC and MC) and non-treated ischemic (IS) mice 30 days after treatment. Weight of gastrocnemius and rectus femoris muscles was also included. Values are presented as mean ± SEM, n=10 (N-IS, MSC and MSC+MC), n=8 (MC and IS), *p<0.05; **p<0.01; ****p<0.0001. N-IS - non-ischemic mice; MSC - ischemic mice treated with human MSC; MSC+MC - ischemic mice treated with human MSC modified with MC-VEGF; MC - ischemic mice treated with MC-VEGF; IS - ischemic mice.

VI.4.5. Quantification of VEGF levels *in vivo*

ELISA kits for human and mouse VEGF detection were used to quantify the levels of VEGF protein in mice blood and muscle (*rectus femoris*) 30 days after treatment. No human VEGF was detected in samples of muscle or serum of mice from any of the groups investigated. Also, mouse VEGF was not detected in mice blood 30 days after treatment. The levels of mouse VEGF detected on muscles retrieved from mice from healthy (N-IS), treated (MSC, MSC+MC and MC)

and non-treated ischemic (IS) groups are shown in Figure VI.6. Mice from all the conditions had relatively low and comparable levels of VEGF protein in muscle 30 days after treatment.

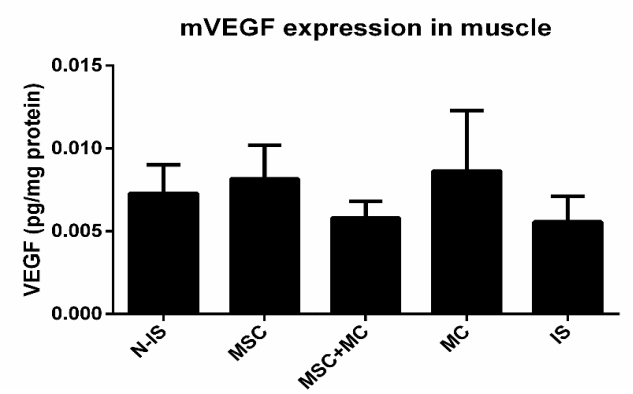

Figure VI.6 - Levels of mouse VEGF detected on muscles (rectus femoris) from healthy (N-IS), treated (MSC, MSC+MC and MC) and ischemic mice 30 days after treatment. Values are presented as mean ± SEM, n=5 (N-IS, MSC, MSC+MC and IS), n=4 (MC).

VI.4.6. Histological analysis

Inflammation is an important process for muscle degeneration and regeneration, so muscle tissues were histologically analyzed by HE staining to evaluate fibrosis and adipocyte infiltration (Figure VI.7). No significant differences were observed between the treated groups in terms of muscle degeneration or regeneration, but all showed lower number of infiltrated adipocytes (black arrows) than ischemic, non-treated mice. Also, the degree of fibrosis was reduced for all the treated mice when compared to ischemic group (stained in purple and marked with *).

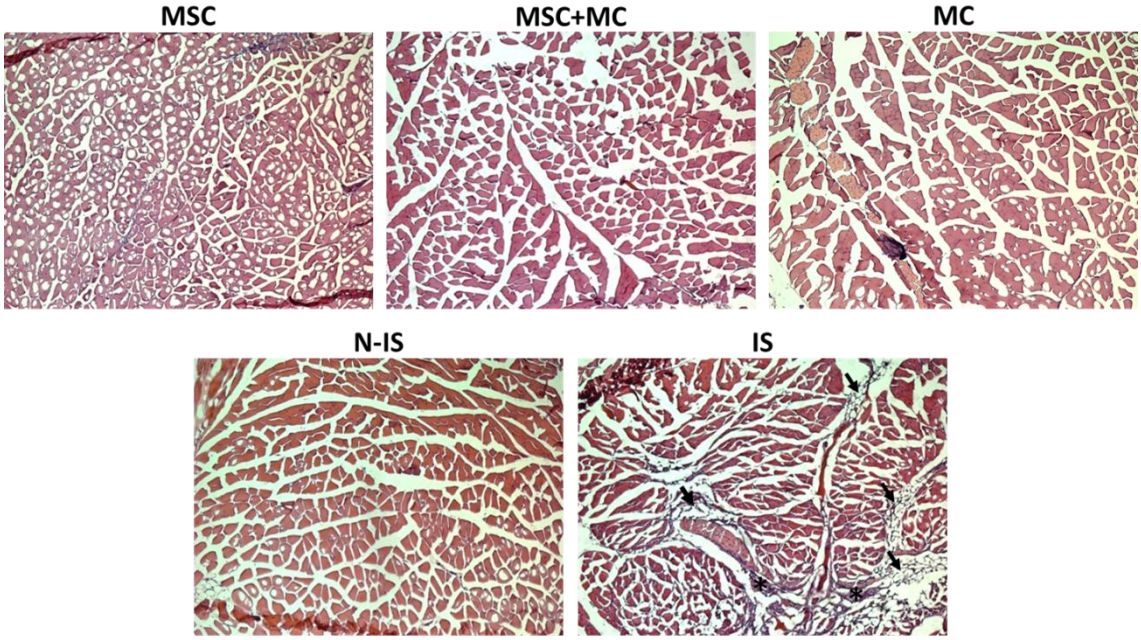

Figure VI.7 - Histological analysis of gastrocnemius muscles collected 30 days after treatment with MSC, MSC+MC and MC. Non-ischemic mice (N-IS) and non-treated, ischemic mice (IS) were also evaluated as controls. Tissue samples were stained with HE to confirm adipocyte infiltration (black arrow) and fibrotic areas (*).

VI.5. Discussion

PAD is a prevalent chronic disease known to affect 3-10% of the world population (Norgren et al., 2007). The current treatments for the more severe stage of the disease, CLI, consist on revascularization, which is a highly invasive procedure that in some cases is insufficient or ineffective, especially for subjects with several associated co-morbidities. Also, half of the PAD patients suffering CLI are not eligible for such procedure (Gresele et al., 2011). The development of novel angiogenic therapeutic approaches based on cell and/or gene therapies might be a promising strategy for PAD treatment, especially for those no-option patients (Hassanshahi et al., 2019, Grochot-Przeczek et al., 2013).

Several reports have demonstrated the potential of MSC administration, alone or genetically engineered with therapeutic genes, in hind limb ischemia mice models of PAD (Martins et al., 2014, Cunha et al., 2013). On this basis, it was hypothesized herein that MSC genetically engineered with VEGF-encoding minicircles could be an efficient therapy for limb ischemia. This work was also based on Chapter IV where genetic engineering of MSC using MC-VEGF demonstrated to significantly improve MSC angiogenic activity *in vitro* without affecting their intrinsic properties (Serra et al., 2018). Minicircles were selected for MSC modification on account of their small size and low toxicity, which, consequently, promotes high transgene expression and reduced risk of adverse reactions (Darquet et al., 1997, Gaspar et al., 2015). In fact, several authors documented the effective transfection of MSC using minicircle vectors (Bandara et al., 2016, Park et al., 2017, Mun et al., 2016). Also, in the current context, a transient transgene expression is desirable to avoid an uncontrolled VEGF expression, which, in more severe cases, may induce hemangioma formation (Lee et al., 2000).

The efficiency of nucleofection as a strategy for MSC modification was firstly confirmed using GFP-encoding plasmids (pmaxGFP®). The results revealed a transfection efficiency of 64.7%, which is in accordance with other reports where nucleofection demonstrated to be an effective method for overexpressing therapeutic proteins to human MSC (Aslan et al., 2006, Mok et al., 2008, Boura et al., 2014). MSC transfection with VEGF-encoding minicircles was then performed by nucleofection. The levels of VEGF secreted were quantified by ELISA at 24 and 48 hours after nucleofection, revealing that the highest VEGF production was achieved 48 hours after transfection. This is in accordance with previous studies where highest transgene expression was observed two days after transfection (Boura et al., 2014, Locatelli et al., 2015, Zhu et al., 2012). An increase of approximately 90-fold was observed on VEGF expression for minicircle-transfected cells compared to non-modified MSC, which confirms the efficient transfection of

MSC. In fact, the fold increase observed herein for VEGF expression was superior than the previously obtained in Chapter IV (Serra et al., 2018) and in other articles in literature using either non-viral or viral methods (Zhu et al., 2012, Beegle et al., 2016). The higher transgene expression achieved herein might be related with the use of nucleofection technique, which was specifically optimized to drive DNA molecules directly to cell nuclei (Gresch et al., 2004) and, thus, improve transgene expression. In fact, several studies demonstrated high transgene expression levels after nucleofection of MSC, confirming the efficacy of this strategy for MSC engineering (Aluigi et al., 2006, Aslan et al., 2006, Boura et al., 2014).

To test the initial hypothesis, a stable and uniform limb ischemia model developed to mimic human PAD was used herein. To obtain this model, femoral artery of mice right limbs was excised together with its branches, as described elsewhere (Cunha et al., 2013, Martins et al., 2014, Sacramento et al., 2009), but artery removal was performed by cauterization to avoid and reduce mice bleeding. Despite this process severely affects limb circulation and may cause limb darkening and even loss, in severe cases, it is important to note that this is not a model for chronic ischemia (Cunha et al., 2013). Models for chronic disease are difficult to establish in mice due to their physiology and small size. So, the mice used herein represents an acute ischemia model instead.

Genetically engineered MSC, as well as control non-transfected cells and minicircles alone, were administered to quadriceps of ischemic mice three days after ischemia induction and 48 hours after transfection. Evaluation of limb necrosis and blood reperfusion were performed weekly by visual assessment and laser doppler, respectively. No significant differences were observed in terms of level of necrosis or blood reperfusion rates between the four ischemic groups investigated. However, 28 days after ischemia, 40% of ischemic non-treated mice (IS) showed some degree of necrosis, whereas this value was <20% for all treated mice (MSC, MSC+MC and MC). The reduced degree of necrosis in treated mice is in accordance with previous reports regarding the administration of non-engineered MSC or genetically engineered with therapeutic factors to mice ischemic limbs (Cunha et al., 2013, Martins et al., 2014). However, contrarily to the referred study, none of the mice analyses herein demonstrated advanced necrosis (stage IV). These differences might be related to the degree of ischemia induced that might be variable according to the protocol followed. It is extremely important to have a standardized protocol and that all the surgeries and treatments are performed by the same hand to avoid operator-related variations.

Similarly, no differences were observed in what concerns limb reperfusion rates measured weekly on ischemic mice. At day 0 (after surgery), all mice from ischemic groups had no blood

flow in the right limb, as expected, confirming that ischemia was successfully induced. However, all the ischemic mice recovered blood flow to the ischemic limb after 28 days, but none reached the blood flow levels of non-ischemic, healthy mice. Despite no significant differences were observed between the groups at any timepoint, the mice treated with MSC+MC seem to have higher level of blood flow in the right limb than other conditions on day 21. This might suggest a faster recovery of blood flow after ischemia when MSC overexpressing VEGF were used as treatment. This is in agreement with previous studies that demonstrated improvements on blood flow in hindlimb ischemia mice models after administration of MSC genetically engineered with VEGF (Beegle et al., 2016, Li et al., 2015c). Also, several authors described benefits on blood reperfusion after treatment with either MSC (Kinnaird et al., 2004, Zhu et al., 2011) or VEGF alone (Anderson et al., 2017, Yasumura et al., 2012).

To confirm the achievement of tissue repair and recovery of function in treated mice, the strength of the gastrocnemius muscle was measured 30 days after treatment. Despite this muscle was not manipulated during surgery for ischemic induction, it is known that ischemia is a complex process that may affect distant tissues (Cunha et al., 2013). Non-ischemic mice (N-IS) and ischemic non-treated mice (IS) showed the highest (0.36±0.06 N) and lowest (0.12±0.03 N) muscle strengths, respectively. All the treated groups (MSC, MSC+MC and MC) displayed significantly higher muscle force than non-treated ischemic mice. Of notice, mice treated with genetically engineered cells (MSC+MC) showed the highest muscle force (0.25±0.04 N; $p<0.0001$). The treatment with MSC and MC alone lead to similar muscle strengths, 0.19±0.03 N ($p<0.01$) and 0.20±0.03 N ($p<0.05$), respectively. The values observed herein for muscle force were lower than those previously obtained by other authors after treatment with MSC (Cunha et al., 2013) or VEGF alone (Yasumura et al., 2012). However, muscle strength reported by those authors for healthy non-ischemic was also superior (>1.0 N) than the observed in the present work. This may indicate that the discrepancies are most probably related to differences in the procedure performance or apparatus assembling than with the effectiveness of the treatment.

The histological qualitative analysis after HE staining confirmed the previously described results. No significant differences were observed between the three treatments described, but the tissues from treated mice had lower fibrosis and adipocytes infiltration than non-treated ischemic mice. The large area occupied by adipocytes in muscle from ischemic mice indicate poor muscle regeneration and is known to affect muscle function (Pagano et al., 2015), as demonstrated by the reduced muscle strength observed for this group. The effect of MSC on fibrosis reduction has been described by other authors (Ortiz et al., 2003) and is probably related

to its extensive trophic activity (Singer and Caplan, 2011). The anti-fibrotic activity of VEGF has also been previously demonstrated in the context of PAD (Yasumura et al., 2012).

Despite no significant differences were observed on limb necrosis, blood reperfusion or histological analysis between the three treated groups, mice treated with MSC+MC showed superior functional improvement, as demonstrated by muscle force measurement. So, it was demonstrated that combination of both pro-angiogenic treatments might be a promising strategy to potentiate the benefits of each treatment. Some studies showed limited engraftment and low survival after *in vivo* MSC infusion, which might limit their beneficial effects (Shi and Li, 2008, Allison, 2009, Hoffmann et al., 2010). On the other side, direct *in vivo* VEGF gene therapy showed not only to promote modest improvements (Muona et al., 2012), but also lead to occurrence of some adverse events upon gene transfer, such as edemas (Rajagopalan et al., 2003).

In conclusion, MSC modification with VEGF in a context of *ex vivo* gene therapy, which consists in cell engineering outside the body and subsequent transplantation into patients (Gowing et al., 2017), might be a promising strategy to improve therapeutic outcomes in PAD patients. Despite several authors have previously demonstrated the angiogenic potential and therapeutic effect of MSC genetically engineered with VEGF in hindlimb ischemia models using either viral (Beegle et al., 2016) or non-viral vectors (Li et al., 2015c), one of the major benefits of the work described herein is the use of novel and innovative vectors, minicircles. Minicircles have been described to display enhanced transgene expression and reduced toxicity (Darquet et al., 1999), being advantageous over the previously investigated systems. Also, the use of a low immunogenic cell vehicle as MSC (Le Blanc et al., 2003a), might allow to have a potential off-the-shelf therapy for PAD patients with no other available option. Despite further studies and standardization of procedures are required before moving to clinical trials, this study might present a step ahead towards the application of cell/gene therapies in the context of regenerative angiogenesis.

VI.6. References

ALLISON, M. 2009. Genzyme backs Osiris, despite Prochymal flop. *Nat Biotech,* 27**,** 966-967.

ALUIGI, M., FOGLI, M., CURTI, A., ISIDORI, A., GRUPPIONI, E., CHIODONI, C., COLOMBO, M. P., VERSURA, P., D'ERRICO-GRIGIONI, A., FERRI, E., BACCARANI, M. & LEMOLI, R. M. 2006. Nucleofection Is an Efficient Nonviral Transfection Technique for Human Bone Marrow–Derived Mesenchymal Stem Cells. *STEM CELLS,* 24**,** 454-461.

ANDERSON, E. M., SILVA, E. A., HAO, Y., MARTINICK, K. D., VERMILLION, S. A., STAFFORD, A. G., DOHERTY, E. G., WANG, L., DOHERTY, E. J., GROSSMAN, P. M. & MOONEY, D. J. 2017. VEGF and IGF Delivered from Alginate Hydrogels Promote Stable Perfusion Recovery in Ischemic Hind Limbs of Aged Mice and Young Rabbits. *J Vasc Res,* 54**,** 288-298.

ASLAN, H., ZILBERMAN, Y., ARBELI, V., SHEYN, D., MATAN, Y., LIEBERGALL, M., LI, J. Z., HELM, G. A., GAZIT, D. & GAZIT, Z. 2006. Nucleofection-based ex vivo nonviral gene delivery to human stem cells as a platform for tissue regeneration. *Tissue Eng,* 12**,** 877-89.

BANDARA, N., GURUSINGHE, S., CHEN, H., CHEN, S., WANG, L. X., LIM, S. Y. & STRAPPE, P. 2016. Minicircle DNA-mediated endothelial nitric oxide synthase gene transfer enhances angiogenic responses of bone marrow-derived mesenchymal stem cells. *Stem Cell Res Ther,* 7**,** 48.

BEEGLE, J. R., MAGNER, N. L., KALOMOIRIS, S., HARDING, A., ZHOU, P., NACEY, C., WHITE, J. L., PEPPER, K., GRUENLOH, W., ANNETT, G., NOLTA, J. A. & FIERRO, F. A. 2016. Preclinical evaluation of mesenchymal stem cells overexpressing VEGF to treat critical limb ischemia. *Molecular Therapy — Methods & Clinical Development,* 3**,** 16053.

BOURA, J. S., VANCE, M., YIN, W., MADEIRA, C., LOBATO DA SILVA, C., PORADA, C. D. & ALMEIDA-PORADA, G. 2014. Evaluation of gene delivery strategies to efficiently overexpress functional HLA-G on human bone marrow stromal cells. *Mol Ther Methods Clin Dev,* 2014.

CARMELIET, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med,* 6**,** 389-95.

CHANG, C.-W., CHRISTENSEN, L. V., LEE, M. & KIM, S. W. 2008. Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy. *Journal of Controlled Release,* 125**,** 155-163.

CHULLIKANA, A., MAJUMDAR, A. S., GOTTIPAMULA, S., KRISHNAMURTHY, S., KUMAR, A. S., PRAKASH, V. S. & GUPTA, P. K. 2015. Randomized, double-blind, phase I/II study of intravenous allogeneic mesenchymal stromal cells in acute myocardial infarction. *Cytotherapy,* 17**,** 250-61.

CONTE, S. M. & VALE, P. R. 2018. Peripheral Arterial Disease. *Heart Lung Circ,* 27**,** 427-432.

CUNHA, F. F. D., MARTINS, L., MARTIN, P. K. M., STILHANO, R. S., PAREDES GAMERO, E. J. & HAN, S. W. 2013. Comparison of treatments of peripheral arterial disease with mesenchymal stromal cells and mesenchymal stromal cells modified with granulocyte and macrophage colony-stimulating factor. *Cytotherapy,* 15**,** 820-829.

DARQUET, A. M., CAMERON, B., WILS, P., SCHERMAN, D. & CROUZET, J. 1997. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther,* 4**,** 1341-9.

DARQUET, A. M., RANGARA, R., KREISS, P., SCHWARTZ, B., NAIMI, S., DELAERE, P., CROUZET, J. & SCHERMAN, D. 1999. Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer. *Gene Ther,* 6**,** 209-18.

DEEV, R. V., BOZO, I. Y., MZHAVANADZE, N. D., VORONOV, D. A., GAVRILENKO, A. V., CHERVYAKOV, Y. V., STAROVEROV, I. N., KALININ, R. E., SHVALB, P. G. & ISAEV, A. A. 2015. pCMV-vegf165 Intramuscular Gene Transfer is an Effective Method of Treatment for Patients With Chronic Lower Limb Ischemia. *J Cardiovasc Pharmacol Ther,* 20**,** 473-82.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent

mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-7.

DOS SANTOS, F., ANDRADE, P. Z., ABECASIS, M. M., GIMBLE, J. M., CHASE, L. G., CAMPBELL, A. M., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2011. Toward a Clinical-Grade Expansion of Mesenchymal Stem Cells from Human Sources: A Microcarrier-Based Culture System Under Xeno-Free Conditions. *Tissue Engineering. Part C, Methods,* 17**,** 1201-1210.

DOS SANTOS, F., CAMPBELL, A., FERNANDES-PLATZGUMMER, A., ANDRADE, P. Z., GIMBLE, J. M., WEN, Y., BOUCHER, S., VEMURI, M. C., DA SILVA, C. L. & CABRAL, J. M. S. 2014. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. *Biotechnology and Bioengineering,* 111**,** 1116-1127.

FERRARA, N., GERBER, H.-P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9**,** 669-676.

GASPAR, V., DE MELO-DIOGO, D., COSTA, E., MOREIRA, A., QUEIROZ, J., PICHON, C., CORREIA, I. & SOUSA, F. 2015. Minicircle DNA vectors for gene therapy: advances and applications. *Expert Opin Biol Ther,* 15**,** 353-79.

GOWING, G., SVENDSEN, S. & SVENDSEN, C. N. 2017. Ex vivo gene therapy for the treatment of neurological disorders. *Prog Brain Res,* 230**,** 99-132.

GRESCH, O., ENGEL, F. B., NESIC, D., TRAN, T. T., ENGLAND, H. M., HICKMAN, E. S., KORNER, I., GAN, L., CHEN, S., CASTRO-OBREGON, S., HAMMERMANN, R., WOLF, J., MULLER-HARTMANN, H., NIX, M., SIEBENKOTTEN, G., KRAUS, G. & LUN, K. 2004. New non-viral method for gene transfer into primary cells. *Methods,* 33**,** 151-63.

GRESELE, P., BUSTI, C. & FIERRO, T. 2011. Critical limb ischemia. *Internal and Emergency Medicine,* 6**,** 129-134.

GROCHOT-PRZECZEK, A., DULAK, J. & JOZKOWICZ, A. 2013. Therapeutic angiogenesis for revascularization in peripheral artery disease. *Gene,* 525**,** 220-228.

GUPTA, P. K., CHULLIKANA, A., PARAKH, R., DESAI, S., DAS, A., GOTTIPAMULA, S., KRISHNAMURTHY, S., ANTHONY, N., PHERWANI, A. & MAJUMDAR, A. S. 2013. A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. *J Transl Med,* 11**,** 143.

HACEIN-BEY-ABINA, S., VON KALLE, C., SCHMIDT, M., LE DEIST, F., WULFFRAAT, N., MCINTYRE, E., RADFORD, I., VILLEVAL, J. L., FRASER, C. C., CAVAZZANA-CALVO, M. & FISCHER, A. 2003. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med.* United States.

HÄCKER, G., REDECKE, V. & HÄCKER, H. 2002. Activation of the immune system by bacterial CpG-DNA. *Immunology,* 105**,** 245-51.

HASSANSHAHI, M., KHABBAZI, S., PEYMANFAR, Y., HASSANSHAHI, A., HOSSEINI-KHAH, Z., SU, Y. W. & XIAN, C. J. 2019. Critical limb ischemia: Current and novel therapeutic strategies. *J Cell Physiol.*

HOFFMANN, J., GLASSFORD, A. J., DOYLE, T. C., ROBBINS, R. C., SCHREPFER, S. & PELLETIER, M. P. 2010. Angiogenic effects despite limited cell survival of bone marrow-derived mesenchymal stem cells under ischemia. *Thorac Cardiovasc Surg,* 58**,** 136-42.

ISNER, J. M., PIECZEK, A., SCHAINFELD, R., BLAIR, R., HALEY, L., ASAHARA, T., ROSENFIELD, K., RAZVI, S., WALSH, K. & SYMES, J. F. 1996. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet,* 348**,** 370-4.

KINNAIRD, T., STABILE, E., BURNETT, M. S., LEE, C. W., BARR, S., FUCHS, S. & EPSTEIN, S. E. 2004. 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,* 94**,** 678-85.

KO, J., JUN, H., CHUNG, H., YOON, C., KIM, T., KWON, M., LEE, S., JUNG, S., KIM, M. & PARK, J. H. 2011. Comparison of EGF with VEGF Non-Viral Gene Therapy for Cutaneous Wound Healing of Streptozotocin Diabetic Mice. *Diabetes Metab J,* 35**,** 226-35.

KUSUMANTO, Y. H., VAN WEEL, V., MULDER, N. H., SMIT, A. J., VAN DEN DUNGEN, J. J., HOOYMANS, J. M., SLUITER, W. J., TIO, R. A., QUAX, P. H., GANS, R. O., DULLAART, R. P. & HOSPERS, G. A. 2006. Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. *Hum Gene Ther,* 17**,** 683-91.

LE BLANC, K., TAMMIK, C., ROSENDAHL, K., ZETTERBERG, E. & RINGDEN, O. 2003. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol,* 31**,** 890-6.

LEE, R. J., SPRINGER, M. L., BLANCO-BOSE, W. E., SHAW, R., URSELL, P. C. & BLAU, H. M. 2000. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation,* 102**,** 898-901.

LEROUX, L., DESCAMPS, B., TOJAIS, N. F., SEGUY, B., OSES, P., MOREAU, C., DARET, D., IVANOVIC, Z., BOIRON, J. M., LAMAZIERE, J. M., DUFOURCQ, P., COUFFINHAL, T. & DUPLAA, C. 2010. Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway. *Mol Ther,* 18**,** 1545-52.

LI, X., GAN, K., SONG, G. & WANG, C. 2015. VEGF gene transfected umbilical cord mesenchymal stem cells transplantation improve the lower limb vascular lesions of diabetic rats. *J Diabetes Complications,* 29**,** 872-81.

LIEW, A. & O'BRIEN, T. 2012. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Res Ther,* 3**,** 28.

LIN, C. S. & LUE, T. F. 2013. Defining vascular stem cells. *Stem Cells Dev,* 22**,** 1018-26.

LOCATELLI, P., OLEA, F. D., HNATIUK, A., DE LORENZI, A., CERDA, M., GIMENEZ, C. S., SEPULVEDA, D., LAGUENS, R. & CROTTOGINI, A. 2015. Mesenchymal stromal cells overexpressing vascular endothelial growth factor in ovine myocardial infarction. *Gene Ther,* 22**,** 449-57.

LU, D., CHEN, B., LIANG, Z., DENG, W., JIANG, Y., LI, S., XU, J., WU, Q., ZHANG, Z., XIE, B. & CHEN, S. 2011. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabetes Res Clin Pract,* 92**,** 26-36.

MARTINS, L., MARTIN, P. K. & HAN, S. W. 2014. Angiogenic properties of mesenchymal stem cells in a mouse model of limb ischemia. *Methods Mol Biol,* 1213**,** 147-69.

MOK, P. L., CHEONG, S. K., LEONG, C. F. & OTHMAN, A. 2008. In vitro expression of erythropoietin by transfected human mesenchymal stromal cells. *Cytotherapy,* 10**,** 116-24.

MUN, J.-Y., SHIN, K. K., KWON, O., LIM, Y. T. & OH, D.-B. 2016. Minicircle microporation-based non-viral gene delivery improved the targeting of mesenchymal stem cells to an injury site. *Biomaterials,* 101**,** 310-320.

MUONA, K., MAKINEN, K., HEDMAN, M., MANNINEN, H. & YLA-HERTTUALA, S. 2012. 10-year safety follow-up in patients with local VEGF gene transfer to ischemic lower limb. *Gene Ther,* 19**,** 392-5.

NORGREN, L., HIATT, W. R., DORMANDY, J. A., NEHLER, M. R., HARRIS, K. A. & FOWKES, F. G. R. 2007. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *Journal of Vascular Surgery,* 45**,** S5-S67.

ORTIZ, L. A., GAMBELLI, F., MCBRIDE, C., GAUPP, D., BADDOO, M., KAMINSKI, N. & PHINNEY, D. G. 2003. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A,* 100**,** 8407-11.

PAGANO, A. F., DEMANGEL, R., BRIOCHE, T., JUBLANC, E., BERTRAND-GADAY, C., CANDAU, R., DECHESNE, C. A., DANI, C., BONNIEU, A., PY, G. & CHOPARD, A. 2015. Muscle Regeneration with Intermuscular Adipose Tissue (IMAT) Accumulation Is Modulated by Mechanical Constraints. *PLoS One,* 10**,** e0144230.

PARK, N., RIM, Y. A., JUNG, H., KIM, J., YI, H., KIM, Y., JANG, Y., JUNG, S. M., LEE, J., KWOK, S. K., PARK, S. H. & JU, J. H. 2017. Etanercept-Synthesising Mesenchymal Stem Cells Efficiently Ameliorate Collagen-Induced Arthritis. *Sci Rep,* 7**,** 39593.

PRAZERES, D. M. F. & MONTEIRO, G. A. 2014. Plasmid Biopharmaceuticals. *Microbiology Spectrum,* 2.

RAJAGOPALAN, S., MOHLER, E. R., 3RD, LEDERMAN, R. J., MENDELSOHN, F. O., SAUCEDO, J. F., GOLDMAN, C. K., BLEBEA, J., MACKO, J., KESSLER, P. D., RASMUSSEN, H. S. & ANNEX, B. H. 2003. Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation,* 108**,** 1933-8.

RAVAL, Z. & LOSORDO, D. W. 2013. Cell Therapy of Peripheral Arterial Disease: From Experimental Findings to Clinical Trials. *Circulation research,* 112**,** 10.1161/CIRCRESAHA.113.300565.

SACRAMENTO, C. B., CANTAGALLI, V. D., GRINGS, M., CARVALHO, L. P., BAPTISTA-SILVA, J. C., BEUTEL, A., BERGAMASCHI, C. T., DE CAMPOS JUNIOR, R. R., DE MORAES, J. Z., TAKIYA, C. M., SAMOTO, V. Y., BOROJEVIC, R., DA SILVA, F. H., NARDI, N. B., DOHMANN, H. F., JUNIOR, H. S., VALERO, V. B. & HAN, S. W. 2009. Granulocyte-macrophage colony-stimulating factor gene based therapy for acute limb ischemia in a mouse model. *J Gene Med,* 11**,** 345-53.

SERRA, J., ALVES, C. P. A., BRITO, L., MONTEIRO, G. A., CABRAL, J. M. S., PRAZERES, D. M. F. & DA SILVA, C. L. 2018. Engineering of Human Mesenchymal Stem/Stromal Cells with Vascular Endothelial Growth Factor-Encoding Minicircles for Angiogenic Ex Vivo Gene Therapy. *Hum Gene Ther*.

SHI, R. Z. & LI, Q. P. 2008. Improving outcome of transplanted mesenchymal stem cells for ischemic heart disease. *Biochem Biophys Res Commun,* 376**,** 247-50.

SINGER, N. G. & CAPLAN, A. I. 2011. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol,* 6**,** 457-78.

TAO, H., HAN, Z., HAN, Z. C. & LI, Z. 2016. Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. *Stem Cells Int,* 2016**,** 1314709.

WANG, Z., WANG, L., SU, X., PU, J., JIANG, M. & HE, B. 2017. Rational transplant timing and dose of mesenchymal stromal cells in patients with acute myocardial infarction: a meta-analysis of randomized controlled trials. *Stem Cell Res Ther,* 8**,** 21.

WATT, S. M., GULLO, F., VAN DER GARDE, M., MARKESON, D., CAMICIA, R., KHOO, C. P. & ZWAGINGA, J. J. 2013. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. *Br Med Bull,* 108**,** 25-53.

YASUMURA, E. G., STILHANO, R. S., SAMOTO, V. Y., MATSUMOTO, P. K., DE CARVALHO, L. P., VALERO LAPCHIK, V. B. & HAN, S. W. 2012. Treatment of Mouse Limb Ischemia with an Integrative Hypoxia-Responsive Vector Expressing the Vascular Endothelial Growth Factor Gene. *PLOS ONE,* 7**,** e33944.

YOON, C. S., JUNG, H. S., KWON, M. J., LEE, S. H., KIM, C. W., KIM, M. K., LEE, M. & PARK, J. H. 2009. Sonoporation of the minicircle-VEGF(165) for wound healing of diabetic mice. *Pharm Res,* 26**,** 794-801.

ZHU, C. J., DONG, J. X., LI, J., ZHANG, M. J., WANG, L. P. & LUO, L. 2011. Preliminary study on the mechanism of acupoint injection of bone marrow mesenchymal stem cells in improving blood flow in the rat of hind limb ischemia. *J Tradit Chin Med,* 31**,** 241-5.

ZHU, K., LAI, H., GUO, C., XU, D. & WANG, C. 2012. Novel vascular endothelial growth factor gene delivery system-manipulated mesenchymal stem cells repair infarcted myocardium. *Exp Biol Med (Maywood),* 237**,** 678-87.

Chapter VII

FINAL REMARKS AND FUTURE PERSPECTIVES

VII. FINAL REMARKS AND FUTURE PERSPECTIVES

In this thesis, it was proposed the use of MSC genetically engineered with VEGF-encoding minicircles for the treatment of PAD. Firstly, it was achieved the successful construction and purification of VEGF-encoding vectors suitable for MSC modification. Those vectors were then successfully delivered into BM-MSC by microporation or nucleofection, significantly enhancing the VEGF levels secreted by these cells. Microporation technique was also effective for engineering of MSC from other sources, including AT or UCM. Transfected MSC from all the three sources demonstrated not only to produce high levels of VEGF protein than non-transfected counterparts, but also showed an improved angiogenic capacity on *in vitro* functional assays. Finally, the potential of engineered MSC was confirmed *in vivo* using a mouse model for hindlimb ischemia. The treatment of ischemic mice with MSC+MC-VEGF lead to significant improvements on muscular function. Despite further studies are required, these preliminary results revealed promising insights for angiogenic therapies. This might be a starting point towards the development of an efficient gene/cell-based therapeutic product to improve angiogenesis and induce revascularization for no-option PAD patients.

One of the major limitations of analyzing clinical trials performed to date using cell/gene therapies for PAD is the lack of consensus on study design. The variation of crucial parameters, like patient type, cell dosing, clinical endpoints and long-term follow up, limits the comparison of outcomes between different trials (Liew and O'Brien, 2012). Despite the fact that atherosclerosis is the major cause of PAD, there are other less common reasons for PAD development, including inflammatory disorders. Studies performed to date with cell therapies have been focused on both types of patients, and there are no evidence of better response to either form of the disease. Cell dosing and administration are also very important parameters that should be standardized, not only regarding the number of administered cells but also in terms of infusion methods (intramuscular vs. intravenous) and regimes (single dose vs. multiple doses). The selection of endpoints, as well as the duration of post-administration follow-up are also major issues where no consensus has been observed (Liew and O'Brien, 2012). To overcome such limitation and allow direct comparison between trials, the Society for Vascular Surgery determined specific objective performance goals (OPGs) to define therapeutic benchmarks for CLI (Conte, 2010). The patient's follow-up on CLI trials may vary from 3 months to one year (Liew and O'Brien, 2012), but according to OPGs the minimal exposure time for relevant clinical

efficacy is one year (Conte, 2010). Despite the absence of unanimity on different trials and the modest effectiveness, all have confirmed the safety of using MSC for PAD treatment.

The selection of the best MSC source for angiogenic therapies is also a matter of controversy, since, the results published to date showed some conflicting data (Fideles et al., 2019, Du et al., 2016). Nevertheless, it is know that MSC from different sources secrete different levels of trophic factors (Melief et al., 2013). Another important choice regarding cell source is the use autologous *versus* allogeneic MSC. Even though the majority of MSC clinical trials performed to date were based on autologous cell therapies, the use of allogeneic sources has a great advantage. Allogeneic sources allow to have an off-the-shelf therapy for PAD patients, even for those to whom MSC harvesting is contra-indicated. Moreover, although there are some conflicting data in this field (Smadja et al., 2011), some studies demonstrated that MSC from patients with ischemic disease may have impaired therapeutic activity (Kizilay Mancini et al., 2017, Teraa et al., 2013).

A recent strategy developed to improve the survival and engraftment of cell therapeutics is the use of biocompatible scaffolds that provide a better microenvironment for the administered cells. For example, the administration of MSC encapsulated within alginate in a mouse model of hindlimb ischemia revealed to promote a higher increase in vascular density (70% vs 22%) and perfusion (21% vs 0%) compared to non-encapsulated cells (Landazuri et al., 2016). Similarly, the delivery of MSC using a PEGylated fibrin gel lead to a greater number of mature blood vessels than MSC delivered alone (Ricles et al., 2016). Platelet lysate hydrogels have also been described to enhance MSC angiogenic activity, improving perfusion after being used as platforms for cell transfer into mice ischemic limbs (Robinson et al., 2016). An important advantage of the development of tissue engineering biocompatible scaffolds is the possibility of using them as gene delivery systems. Indeed, the incorporation of DNA molecules on 3D matrices allow sustained and prolonged gene expression in a manner that more closely resembles the natural extracellular environment (Wang et al., 2014). The interactions between cells and cell-matrix are different on 2D and 3D settings, so the use of 3D platforms for gene delivery to better mimic the MSC behavior *in vivo* is a promising and very interesting strategy. Although the major works on scaffold-based transfection were directed to formation of bone or cartilage (Raisin et al., 2016), there are some recent insights in ischemic diseases. One of these examples is the combination of PEI nanoparticles containing SDF-1α gene with collagen-based scaffolds. Laiva and colleagues demonstrated that MSC cultured on those scaffolds have increased expression of pro-angiogenic factors and enhanced angiogenic potential *in vitro* (Laiva et al., 2018).

The development of novel and precise non-viral genome-editing technologies also opened new possibilities in field of gene therapy. ZFN, TALENS as CRISPR are examples of such approaches that can be used for site-specific gene modification and thus induce a stable long-term transgene expression. While ZFN and TALEN systems are dependent on protein structures for DNA sequence recognition, CRISPR-Cas9 system enables DNA recognition through RNA-defined specificity. Hence, the emergence of this technology allowed the simplification of the whole gene-editing process, since it only requires the design of a single guide RNA, no longer than 20 nucleotides, that is complementary to the target DNA sequence (Rui et al., 2019). Although these technologies are relatively new, they have already been applied for MSC genome editing in different therapeutic areas, including angiogenesis.

Chang and colleagues demonstrated the efficacy of combining the use of biomaterials with those novel genome editing strategies. TALEN system was used to edit UCB-MSC and integrate HGF gene into a safe harbor site under the control of an inducible promoter, resulting on cells with enhanced mobility and anti-apoptotic responses, as well as improved capacity to induce tube-formation. Those cells were then encapsulated within alginate microgels for the controlled expression and secretion of HGF and their potential was investigated *in vivo*. This strategy revealed to promote an enhanced angiogenesis in a mouse hindlimb ischemia model (Chang et al., 2016).

Notwithstanding that further studies are required before therapies with genetically engineered cells can used in a clinical setting, extremely relevant insights in this field have been performed over the last decades.

VII.1. References

CHANG, H. K., KIM, P. H., CHO, H. M., YUM, S. Y., CHOI, Y. J., SON, Y., LEE, D., KANG, I., KANG, K. S., JANG, G. & CHO, J. Y. 2016. Inducible HGF-secreting Human Umbilical Cord Blood-derived MSCs Produced via TALEN-mediated Genome Editing Promoted Angiogenesis. *Mol Ther,* 24**,** 1644-54.

CONTE, M. S. 2010. Understanding objective performance goals for critical limb ischemia trials. *Semin Vasc Surg,* 23**,** 129-37.

DU, W. J., CHI, Y., YANG, Z. X., LI, Z. J., CUI, J. J., SONG, B. Q., LI, X., YANG, S. G., HAN, Z. B. & HAN, Z. C. 2016. Heterogeneity of proangiogenic features in mesenchymal stem cells derived from bone marrow, adipose tissue, umbilical cord, and placenta. *Stem Cell Res Ther,* 7**,** 163.

FIDELES, S. O. M., ORTIZ, A. C., ASSIS, A. F., DUARTE, M. J., OLIVEIRA, F. S., PASSOS, G. A., BELOTI, M. M. & ROSA, A. L. 2019. Effect of cell source and osteoblast differentiation on gene expression profiles of mesenchymal stem cells derived from bone marrow or adipose tissue. *J Cell Biochem*.

KIZILAY MANCINI, O., LORA, M., SHUM-TIM, D., NADEAU, S., RODIER, F. & COLMEGNA, I. 2017. A Proinflammatory Secretome Mediates the Impaired Immunopotency of Human Mesenchymal Stromal Cells in Elderly Patients with Atherosclerosis. *Stem Cells Transl Med,* 6**,** 1132-1140.

LAIVA, A. L., RAFTERY, R. M., KEOGH, M. B. & O'BRIEN, F. J. 2018. Pro-angiogenic impact of SDF-1alpha gene-activated collagen-based scaffolds in stem cell driven angiogenesis. *Int J Pharm,* 544**,** 372-379.

LANDAZURI, N., LEVIT, R. D., JOSEPH, G., ORTEGA-LEGASPI, J. M., FLORES, C. A., WEISS, D., SAMBANIS, A., WEBER, C. J., SAFLEY, S. A. & TAYLOR, W. R. 2016. Alginate microencapsulation of human mesenchymal stem cells as a strategy to enhance paracrine-mediated vascular recovery after hindlimb ischaemia. *J Tissue Eng Regen Med,* 10**,** 222-32.

LIEW, A. & O'BRIEN, T. 2012. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Res Ther,* 3**,** 28.

MELIEF, S. M., ZWAGINGA, J. J., FIBBE, W. E. & ROELOFS, H. 2013. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. *Stem Cells Transl Med,* 2**,** 455-63.

RAISIN, S., BELAMIE, E. & MORILLE, M. 2016. Non-viral gene activated matrices for mesenchymal stem cells based tissue engineering of bone and cartilage. *Biomaterials,* 104**,** 223-37.

RICLES, L. M., HSIEH, P. L., DANA, N., RYBALKO, V., KRAYNAK, C., FARRAR, R. P. & SUGGS, L. J. 2016. Therapeutic assessment of mesenchymal stem cells delivered within a PEGylated fibrin gel following an ischemic injury. *Biomaterials,* 102**,** 9-19.

ROBINSON, S. T., DOUGLAS, A. M., CHADID, T., KUO, K., RAJABALAN, A., LI, H., COPLAND, I. B., BARKER, T. H., GALIPEAU, J. & BREWSTER, L. P. 2016. A novel platelet lysate hydrogel for endothelial cell and mesenchymal stem cell-directed neovascularization. *Acta Biomater,* 36**,** 86-98.

RUI, Y., WILSON, D. R. & GREEN, J. J. 2019. Non-Viral Delivery To Enable Genome Editing. *Trends Biotechnol,* 37**,** 281-293.

SMADJA, D. M., D'AUDIGIER, C., GUERIN, C. L., MAUGE, L., DIZIER, B., SILVESTRE, J. S., CORTIVO, L. D., GAUSSEM, P. & EMMERICH, J. 2011. Angiogenic potential of BM MSCs derived from patients with critical leg ischemia. *Bone Marrow Transplantation,* 47**,** 997.

TERAA, M., SPRENGERS, R. W., WESTERWEEL, P. E., GREMMELS, H., GOUMANS, M. J., TEERLINK, T., MOLL, F. L. & VERHAAR, M. C. 2013. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS One,* 8**,** e55592.

WANG, W., XU, X., LI, Z., LENDLEIN, A. & MA, N. 2014. Genetic engineering of mesenchymal stem cells by non-viral gene delivery. *Clin Hemorheol Microcirc,* 58**,** 19-48.