SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

2037.

LAKATOS KINGA

Szív- és érrendszeri betegségek élettana és klinikuma

című program

Programvezető: Dr. Merkely Béla, egyetemi tanár

Témavezető: Dr. Merkely Béla, egyetemi tanár

The effect of hypoxia and hypoxic preconditioning on human bone marrow mesenchymal stem cells

Kinga Lakatos MD.

Basic Medicine Doctoral School

Semmelweis University

Supervisor: Prof. Dr. Béla Merkely MD. DSc.

Opponents: Dr. Anita Zádori PhD.

Dr. László Cervenak PhD.

Head of the Final Examination Committee: Prof. Dr. Gábor Varga DSc.

Members of the Final Examination Committee: Dr. Katalin Német PhD.

Dr. Levente Kiss PhD.

Budapest

2017

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List of Abbreviations

ALP	alkaline phosphatase
AMI	acute myocardial infarction
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
ANOVA	analysis of variance
ARS	Alizarin Red S
bFGF	basic fibroblast growth factor
BM	bone marrow
BMC	bone marrow cells
BMNC	bone marrow mononuclear cells
BM-MSC	bone marrow mesenchymal stem cells
bsp	bone sialoprotein
Ca	calcium
CABG	coronary artery bypass grafting
CAR	CXCL-12-abundant reticular cells
CD	cluster of differentiation
CFU-F	colony forming unit fibroblast
CM	conditioned media
$CoCl_2$	cobalt-chloride
CSC	cardiac stem cells

CXCL 12	C-X-C-motif chemokine 12
CXCR-4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
DG	diacylglycerols
DGAT	diacylglycerol acyltransferase
DNA	deoxyribonucleic acid
ECM	extracellular matrix
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cells
ESC	embryonic stem cells
EthD- III	ethidium-homodimer III
FBS	fetal bovine serum
FFA	free fatty acid
F-FDG	18F- fluoro-deoxy-D-glucose
FGF-1	fibroblast growth factor 1
FGF-2	fibroblast growth factor 2
FGF-4	fibroblast growth factor 4
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glut-1	glucose transporter 1
GPCR	G protein coupled receptor
HE	heamatopoietic microenvironment
HIF-1	hypoxia inducible factor 1
HGF	hepatocyte growth factor

HLA	human leukocyte antigen
MHC-I	major histocompatibility antigen I
HP	hypoxic preconditioning
HSL	hormone sensitive lipase
HSPC	haematopoietic stem- and progenitor cells
HUVEC	human umbilical vein endothelial cell
IL-3	interleukin 3
IL-6	interleukin 6
IL-8	interleukin 8
IP_3	inositol triphosphate
iPSC	induced pluripotent stem cells
ISCT	International Society of Stem Cell Therapy
IVIS	in vivo imaging system
LDH-A	lactate dehydrogenase A
LepR	Leptin Receptor
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LVEF	left ventricular ejection fraction
LVESV	left ventricular end systolic volume
MACE	major adverse cardiac event
MCAM	melanoma cell adhesion molecule

MCP-1	monocyte chemoattractant protein 1
MEM α	minimal essential medium alpha
MGAT	monoacylglycerol acyltransferase
MI	myocardial infarction
miRNA	micro ribonucleic acids
mRNA	messenger ribonucleic acid
MSC	Mesenchymal Stem Cells
mTIC	total ion chromatogram/current
NaCl	sodium-chloride
NCT	national clinical trial
NF-kB	nuclear factor κB
NG-2	neural/glycan antigen 2
NO	nitric oxide
NOD/SCID-IL2Rγ$^{-/-}$ (NSG)	non-obese diabetic/ severe combined immune deficiency – interleukin 2 receptor gamma chain null
PBS	phosphate buffer saline
PC	phosphatidyl choline
PCNA	proliferating cell nuclear antigen
PC-PLC	phosphatidylcholine-specific phospholipase C
PDGF	platelet derived growth factor

PDGF-Rβ	platelet derived growth factor receptor beta
PE	phosphatidyl ethanolamine
PECAM-1	platelet/endothelial cell adhesion molecule 1
PI	phosphatidyl-inositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinase C
PLA_2	phospholipase A_2
PlGF	placental growth factor
PPH	phosphatide phosphatase
PS	phosphatidyl-serine
PVDF	polyvinylidene fluoride
QC	quality control
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
SCF-1	stem cell factor 1
SDF-1	stromal cell derived factor 1
SEM	standard error of the mean
siRNA	small interfering RNA
SMS	sphingomyelin synthase
STEMI	ST segment elevation myocardial infarction
STRO-1	stromal cell precursor antigen 1

TG	triglycerides
TGL	triacylglycerol lipase
TGF-β	transforming growth factor β
TMCAO	transient medial cerebral artery occlusion
UHPLC – QTOF MS/MS	ultrahigh pressure liquid chromatography – quadrupole time of flight tandem mass spectrometry
VE - cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

1. Introduction

1.1 Stem cells

Stem cells are defined by the ability to give rise to various mature cell types, meanwhile maintaining the capacity to divide and create another stem cell. Stem cells have long been regarded as undifferentiated and capable of indefinite proliferation, self-renewal, and provision of a large number of differentiated cells (Blau et al., 2001, Ramalho-Santos and Willenbring, 2007). Both the development of the embryo and the regeneration of adult tissues throughout the lifetime are possible because of stem cells (Fuchs et al., 2000).

It is difficult to strictly define categories of stem cells, but the most widely used groups are embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and adult/somatic stem cells (Genetic Science Learning Center). Embryonic stem cells only exist at the beginning of development, and are isolated from the inner cell mass of the blastocyst of the pre-implantation mammalian embryo, i.e. the cells contributing to the embryo proper. A. J. Thomson and colleagues were the first to successfully isolate and maintain human embryonic stem cells in culture (Thomson et al., 1998). Embryonic stem cells have the capacity to proliferate extensively in culture, while maintaining pluripotency. Unique properties compared to other cell types include the lack of contact inhibition in culture, specific cell cycle regulation and the ability to form teratomas (tumors that contain cells from all three germ layers) after injection *in vivo*. Embryonic stem cells can contribute to virtually all cell types in the chimeric offspring, including germ line cells, when injected into a host embryo at the blastocyst stage (Rao, 2013). In culture, ESC can be differentiated into various cell types, most commonly through the embryoid body formation method. Generally, 10-20% of cells in the culture can be navigated toward the intended cell type, though differentiated cells are often not the same as adult cells, as they are not fully mature in phenotype and function, but rather fetal or embryonic (Rao, 2013, Avior et al., 2016).

Induced pluripotent stem cells were generated from adult mouse, then human skin fibroblasts (Takahashi and Yamanaka, 2006, Takahashi et al., 2007, Yu et al., 2007). The factors that could induce pluripotency in fully differentiated somatic cells were Oct-3/4,

Sox-2, Klf-4 and c-Myc (Takahashi and Yamanaka, 2006), or Lin28 and Nanog instead of Klf-4 and c-Myc (Yu et al., 2007). Besides the original pluripotency factors, several other transcription factors, small molecules, micro ribonucleic acids (miRNA) and proteins have been used to effectively reprogram somatic cells (Brouwer et al., 2016). Genome-integrative (retrovirus, lentivirus, transposon, bacteriophages and Zinc-finger nucleases) and non-integrative (adenovirus, Sendai virus, minicircle deoxyribonucleic acid (DNA), messenger RNA (mRNA), episomal vectors and proteins) methods are now used to induce pluripotency. Integrative techniques carry the risk of insertional mutagenesis, ineffective silencing of somatic genes and transgene reactivation later on, also, retroviruses require cell division for integration and are highly cell-type specific. Non-integrating techniques are generally less effective or can require multiple transfections to achieve successful reprogramming (Brouwer et al., 2016). The iPSC can be remarkably useful in disease modeling, and in the understanding of disease pathogenesis: patient- derived iPSC can be differentiated into mature cell types demonstrating disease- specific phenotype. Fibroblasts (Huangfu et al., 2008), keratinocytes (Aasen et al., 2008), neural stem cells (Kim et al., 2009), peripheral blood cells (Loh et al., 2009) and cord blood cells (Giorgetti et al., 2010) have been reported as sources of human iPSC. The iPSC can also be a valuable tool for drug screening, replacing animal models (Avior et al., 2016).

Given the almost indefinite capacity to proliferate and self-renew, and the ability to form almost any mature cell type of mammalian organs, embryonic and induced pluripotent stem cells have become incredibly promising candidates for regenerative cell therapy (Fuchs et al., 2000). However, there are major hurdles that have prevented the wide-spread clinical application of both ESC and iPSC for regenerative purposes. The most common issue is the risk of teratoma formation after *in vivo* transplantation of pluripotent cells (Thomson et al., 1998). Other issues include variability in differentiation potentials (Huangfu et al., 2008), the epigenetic memory of the original somatic cell (Kim et al., 2010), iPSC-specific genetic abnormalities (Hussein et al., 2011, Gore et al 2011., Lister et al., 2011), and that the efficiency of reprogramming somatic cells into iPSC is typically less than 1% (Yamanaka, 2009). In a non-human primate model of myocardial infarction (MI),

the transplantation of ESC- or iPSC-derived cardiomyocytes significantly increased the incidence of sustained ventricular arrhythmias in cell treated animals compared to controls (Chong et al., Nature 2014, Shiba et al., Nature 2016).

Adult stem cells originate in the developing embryo and are characterized by the loss of pluripotency during development and tissue- specific differentiation capacity (Kfoury and Scadden, 2015). Adult or tissue stem cells are far from pluripotent embryonic stem cells, but can be found in the adult organism in an undifferentiated, quiescent state (Rao, 2013, Morrison and Spradling, 2008). Certain cells in the tissues retain their ability to divide and self-renew, and have been categorized based on their differentiation capacity: hematopoietic, bone, epithelial muscle-, neural-, and hepatic stem cells, and so on. Adult stem cells are able to give rise to the cells of their tissue of residence. Progenitor cells are committed to a particular lineage, are the immediate precursors of a mature, specialized cell, with their differentiation capacities being limited to just one cell type and having a defined amount of lifetime before programmed senescence and cell death (Young and Black 2013).

1.2 Mesenchymal stem cells

Mesenchymal stem cells (MSC) were first found in the rodent bone marrow (BM), as non-hematopoietic, multipotent progenitors. MSC are adherent to plastic in culture, capable of colony formation and differentiation into osteoblasts, adipocytes and chondrocytes *in vitro* (Friedenstein et al., 1970). A single colony forming unit fibroblast (CFU-F), when transplanted ectopically (subcutaneously), can differentiate into all the cell types of a miniature bone-organ (ossicle), is able to recreate the hematopoietic microenvironment (HME) and capable of self-renewal, during serial transplantation (Sacchetti et al., 2007). The International Society for Cell Therapy (ISCT) definition states that, MSC are adherent to plastic, capable of trilineage differentiation *in vitro*, express CD105, CD73 and CD90 on the cell surface and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen (HLA) class II (Dominici et al., 2006).

1.2.1 Developmental origins of MSC

MSC in the developing organism can be found around the changing sites of hematopoiesis (Kfoury and Scadden, 2015). Accordingly, the earliest site of MSC isolation was the aorta-gonad-mesonephros near the dorsal aorta of the mouse embryo (Mendes et al., 2005). Sox-1^{+} neuroepithelial cells have also been identified as the source of the earliest batch of MSC (Takashima et al., 2007). In a mouse tooth model system, peripheral nerve-associated glial cells contributed to a significant population of MSC during the development, self-renewal and repair of a tooth. Schwann cells gave rise to MSC in the adult mouse incisor, and these MSC produced odontoblasts and dental pulp cells (Kaukua et al., 2014). However, in the postnatal development, MSC from neural origin are replaced by MSC from other sources: based on the analysis of typical mesenchymal markers and the unique Hox genes (that define positional identity during embryonic development and point out anatomical origin of cells in postnatal life) in murine MSC from various tissues, it was concluded that MSC in different organs originate from the post-segmentation mesoderm and develop in a parallel manner in different body segments (Ackema and Charité, 2008, Sági et al., 2012). In conclusion, it is now widely accepted that mesenchymal stem cells present in the adult organism derive from the mesoderm (Bianco et al., 2008), probably colonize tissues along invading blood vessels, and occupy their mural position in response to signals from endothelial cells as it was demonstrated in human MSC transplants (Hellström et al., 1999, Bianco et al 2013, Armulik et al., 2005).

1.2.2 In vivo position of MSC

Stromal cells with colony forming and multipotent differentiation capacity were isolated from a perivascular position in many postnatal organs. Crisan and colleagues prospectively isolated perivascular cells from various tissues of human fetal and adult tissue, which uniformly expressed neural/glycan antigen-2 (NG- 2), CD 146/ MCAM (melanoma cell adhesion molecule) and platelet derived growth factor receptor beta (PDGF-Rβ), and did not express the endothelial markers CD 144 (vascular endothelial (VE) - cadherin), von Willebrand factor (vWF), CD 34 (hematopoietic progenitor cell antigen) and CD 31 (PECAM-1, platelet/endothelial cell adhesion molecule 1). The same

phenotype was found in the perivascular region in skeletal muscle, pancreas, placenta, white adipose tissue, skin, lung, brain, bone marrow sections and was exclusive for perivascular cells. CD 146^{high}/ CD 34 $^{-}$ / CD 45 $^{-}$ / CD 56 $^{-}$ cells were then isolated from muscle and non- muscle tissues, were found to form myotubules in vitro, and generated human spectrin- and dystrophin- expressing myofibers *in vivo*. Perivascular cells after long term culture (up to 11-14 passages) retained their original phenotypes. They also showed strong chemotaxis towards digested extracellular matrix (ECM) components. Most importantly, long-term cultured perivascular cells prospectively isolated based on typical MSC marker expression in their native arrangement, were positive for CD 73, CD 90, CD 105, and negative for CD 34 and HLA- DR among others, and differentiated into adipocytes, osteoblasts and chondrocytes in the respective differentiation media. This study provided strong evidence for the existence of a uniform perivascular cell type in a wide range of human fetal and adult tissues which show MSC features (Crisan et al 2008).

Two main concepts regarding the identity of MSC have emerged, termed ‘fundamental’ and ‘revisionist’ by Bianco in 2013 (Bianco et al., 2013). The ‘fundamental’ concept relies on the original work of Friedenstein et al., where the existence of a committed multipotent skeletal progenitor in the bone marrow has been proven solidly, transplantable locally, but not necessarily systematically. According to the ‘revisionist’ concept, a common progenitor exists not just for skeletal, but for all non-epithelial, non-hematopoietic tissue derived from mesoderm, not only residing in the bone marrow, but also in many other postnatal organs (da Silva Meirelles et al., 2006). The ‘revisionist’ concept was established mainly by Caplan (Caplan, 2008), who first used the term ‘mesenchymal stem cell’. The terms mesenchymal stem cell or mesenchymal stromal cell are both used in the literature (since MSC are not truly multipotent). For the sake of consistency, I use mesenchymal stem cell in this work.

1.2.3 The putative roles of MSC

1.2.3.1 Hematopoietic niche cells

The main role of MSC is to maintain stem cell niches (Kfoury and Scadden, 2015). The most well described is the hematopoietic stem cell niche. Stromal cells that were selected from bone marrow and enriched for CFU-Fs, supported the generation of mature hematopoietic cells from $CD34^+$ cells *in vitro* (Simmons and Torok-Storb, 1991). The *in vivo* support of hematopoiesis by a bone marrow stromal cell- line was first demonstrated in mice following total body irradiation (Anklesaria et al., 1989). There are functionally distinct subpopulations of MSC in the bone marrow, with different cell surface marker expression (nestin, leptin receptor (LepR), and CXCL-12), different spatial distribution, and the capacity to differentiate into cell types of the skeletal system (Kudlik et al., 2015). LepR, C-X-C-motif chemokine 12/ stromal cell derived factor 1 (CXCL-12/ SDF-1), stem cell factor 1 (SCF-1) and Angiopoietin 1 (Ang-1) all play an indispensable role in hematopoietic stem and progenitor cell (HSPC) maintenance (Mendelson and Frenette, 2014). Nestin-expressing cells were identified in mouse bone marrow stroma, were exclusively perivascular in localization and expressed high levels of CXCL-12 and Ang-1 (Méndez-Ferrer et al., 2010). In this regard, they are similar to the previously described CXCL-12-abundant reticular (CAR) cells, also playing a crucial role in quiescent HSPC maintenance (Sugiyama et al., 2006, Omatsu et al., 2010). $Nestin^+$ cells were proven to be bona fide MSC, could form 'mesenspheres' (indicating neuroectodermal origin), which had multilineage differentiation potential *in vitro* and were able to self-renew in serial transplantation experiments of heterotopic ossicle formation. $Nestin^+$ MSC also contributed to skeletal tissue turnover *in vivo* by differentiation into osteoblasts, osteocytes and chondrocytes. The $nestin^+$ MSC were physically associated with HSC, expressed high levels of HSC maintenance genes, and the depletion of $nestin^+$ cells significantly reduced HSC numbers in bone marrow, while HSC rapidly homed near $nestin^+$ MSC in lethally irradiated mice (Méndez-Ferrer et al., 2010). Based on the level of nestin expression, the distribution of nestin-bright and nestin-dim cells was further analyzed. Nestin-bright cells were rare in the BM, localized exclusively around arterioles, were responsible for most of

the CFU-F activity, while nestin-dim cells were more abundant, localized around sinusoids and overlapped with $LepR^+$ cells (Kunisaki et al., 2013). LepR labels a population of cells that produce a large proportion of SCF-1 and CXCL-12 in the bone marrow, have trilineage differentiation ability and expresses low levels of nestin. The crucial role of MSC in HSPC maintenance was also demonstrated by Raaijmakers et al. (2010). Cytokine-treated MSC homing to the BM of irradiated NOD/SCID (non-obese diabetic/ severe combined immune deficiency) mice and hematologic recovery was shown, as well as the crucial role of the SDF-1/ C-X-C chemokine receptor type 4 (CXCR-4) axis in the stem cell niche reconstitution (Shi et al., 2007). MSC are also able to transfer and recreate the hematopoietic environment, through the formation of heterotopic ossicles (Sacchetti et al., 2007). Further studies will hopefully shed light on the the cell surface expression, spatial distribution and functional role of the different MSC subpopulations in the bone marrow.

1.2.3.2 Other stem cell niches

MSC provide stem cell niches other than the hematopoietic niche, where tissue resident stem cells exist and proliferate throughout the lifespan of the adult organism (Kfoury and Scadden, 2015). Mesenchymal stem cells have been isolated from tissues with slow turnover, such as skeletal muscle, where they contributed little to normal tissue homeostasis, but proliferated after toxin-induced muscle injury and upregulated myogenic differentiation-inducing gene expression (Joe et al., 2010). MSC have been also isolated from hair follicle (Wang et al., 2015), and it has been shown in mice that a specific subset of adipose-forming mesenchymal cells is necessary and sufficient for skin stem cell activation, and mice that lack adipocyte precursors showed defects in follicular stem cell activation (Festa et al., 2011).

1.2.3.3 Angiogenesis support

MSC have also been proposed to have a key role in the support of blood vessel formation and regeneration, given their perivascular position and the still growing body of evidence (Watt et al., 2013). MSC have been shown to secrete a wide range of pro-angiogenic factors: Ang-1, Angiopoietin-2 (Ang-2), Endothelin-1, fibroblast growth factor 1 (FGF-1), 2 (FGF-2) and 4 (FGF-4), HGF, interleukin 8 (IL-8), members of the platelet

derived growth factor (PDGF) family, placental growth factor (PlGF), and vascular endothelial growth factor (VEGF), to name a few, as assessed by angiogenic antibody arrays (Watt et al., 2013), and demonstrated by numerous other investigators (Williams et al., 2011). MSC have been shown to contribute to vasculogenesis, the *de novo* assembly of new and stable vasculature from endothelial colony forming cells in *in vitro* and *in vivo* models (Kachgal and Putnam, 2011, Au et al., 2008). MSC also promote endothelial and vascular smooth muscle cell proliferation *in vitro* (Kinnaird et al., 2004). When transplanted along with human endothelial progenitor cells (EPC) into immune deficient mice, they rapidly formed extensive networks of erythrocyte-containing human blood vessels (Melero-Martin et al., 2008), and in a similar study, when co-implanted with human umbilical vein endothelial cells (HUVECs) into mice, MSC formed blood vessels and retained a perivascular position (Au et al., 2008). Of note, when MSC were implanted alone (without HUVECs), functional vasculature was not formed. In addition, murine MSC from four different tissue sources (bone marrow, white adipose tissue, myocardium and skeletal muscle) promoted endothelial cell proliferation, migration and tube formation *in vitro*, and when co-transplanted with endothelial colony- forming cells, formed vascular networks and occupied perivascular position *in vivo*, regardless of the tissue source (Lin et al., 2012). MSC also support arteriogenesis, during which collateral vessels grow in diameter and lead to the revascularization of ischemic tissues (Potente et al., 2011). In this regard, MSC have been injected into mouse hind limb ischemia models and were shown to significantly increase blood flow, collateral number and total collateral cross- sectional area of blood vessels (Kinnaird et al., 2004), and also improved blood flow and enhanced muscle regeneration (Lian et al., 2010).

1.3 The modulation of tissue response to injury by MSC

Besides the support of angiogenesis, cultured MSC were shown to modulate the response to injury by numerous studies (Williams and Hare, 2011). Besides the hind limb ischemia model, regenerative capacity of MSC has been assessed in animal models of MI, cardiomyopathies and stroke (Ranganath et al., 2012). For example, membrane- and nuclear- labeled allogeneic bone marrow MSC were injected after surgically induced MI in

swine, and MSC were still detectable after 8 weeks, expressed muscle specific proteins and were present in vascular structures, expressing vWF and VEGF, while extracardiac engraftment and ectopic tissue formation was not observed (Amado et al., 2005). In a canine model of chronic myocardial ischemia, allogenic MSC injection increased left ventricular ejection fraction (LVEF) and vascular density, and there was a trend towards reduced fibrosis in treatment group compared to controls. Also, MSC labeling co-localized with endothelial cells and smooth muscle cells but not with myocytes (Silva et al., 2005). In a swine model, high dose autologous MSC treatment 3 months after MI caused significant scar size reduction, increased infarct region wall thickness, myocardial blood flow and LVEF (Schuleri et al., 2009). In another swine model of chronic myocardial ischemia, male swine MSC administration into female animals resulted in cell engraftment and co-localization of Y-chromosome with cardiac transcription factors and structural myocyte proteins, while cell engraftment correlated most with improved regional contractility. Scar size reduction, infarct region wall thickening and improved myocardial blood flow was also observed (Quevedo et al., 2009).

Given the low rate of MSC engraftment after cell therapy (reported to be 0.44% of intramyocardially injected cells by Toma et al. in 2002), several other mechanisms have been proposed and demonstrated to be involved in tissue repair by MSC: enhancement of vasculogenesis, cardioprotection (pro-survival effect on cardiomyocytes as well as enhancement of endogenous repair) and attenuation of fibrosis. It seems most likely that through differentiation into vascular cell types as well as the secretion of potent angiogenic factors, MSC promote angiogenesis/vasculogenesis *in vitro* and *in vivo* in various ischemic and non-ischemic settings (Williams and Hare, 2011). Indeed, conditioned medium from MSC overexpressing the pro-survival gene Akt, protected cardiomyocytes from apoptosis during hypoxia *in vitro* and reduced infarct size and improved cardiac function compared to controls after MI in rat hearts (Mangi et al., 2003). MSC have been shown to secrete VEGF, basic fibroblast growth factor (bFGF), PlGF, and monocyte chemoattractant protein-1 (MCP-1) *in vitro* and improved limb perfusion, increased the number and total cross sectional area of collaterals *in vivo*, while higher VEGF and bFGF levels were shown

in the vicinity of injected MSC compared to controls in a hind limb ischemia model in mice (Kinnaird et al., 2004).

Cardioprotection by MSC was shown when Akt-modified MSC enhanced survival of cardiomyocytes after acute myocardial infarction (AMI) through Wnt-signaling (Gnecchi et al., 2006). MSC also promote the proliferation and differentiation of endogenous c-kit$^+$ cardiac stem cells (CSC) in the heart: in a swine model of AMI, there was a 20-fold increase in the number of c-kit$^+$ CSC, 6-fold in GATA-4$^+$ CSC and 4-fold in the number of mitotic myocytes after MSC injection compared to untreated animals. The MSC feeder layer enhanced CSC proliferation in an organotypic culture from the heart, and resulted in Nkx2.5 and troponin I positive cardioblast appearance *in vitro* (Hatzistergos et al., 2010). Human bone marrow MSC and CSC have also been implanted together following MI in immunosuppressed swine by Williams et al. (2013), which resulted in a 2-fold greater reduction in infarct size than with either cell type alone, while all cell therapy groups decreased infarct size compared to placebo group. In cell-treated pigs, LVEF was restored to baseline, while placebo group had decreased left ventricular function. Co-transplantation of the two types of stem cells also led to a 7-fold enhanced engraftment of stem cells compared to either cell type alone. After a MI, the process of remodeling starts in the injured heart, where necrotic myocardium is replaced by scar tissue, while reduction of fibrosis enhances endogenous myogenesis (Serrano et al., 2011). MSC can influence the existing amount of extracellular matrix by secreting proteinases and tilting the balance towards matrix degradation (Molina et al., 2009), and also attenuating fibrosis by suppression of fibroblast proliferation after MI (Mias et al., 2009).

MSC have been shown to contribute to cardiovascular repair via mitochondrial transfer, exosomes, and connexin-43 on the cellular level. Exosomes are secreted by cells, can contain mRNA, miRNA and other non-coding RNAs, and have important roles in programmed cell death, angiogenesis (Deregibus et al., 2007), inflammation and coagulation (Théry et al., 2002, Mittelbrunn et al., 2011). Purified exosomes derived from MSC reduced infarct size in a mouse model of myocardial ischemia/reperfusion injury (Lai et al., 2010). Gentamicin-induced kidney injury in rats was treated by injections of bone

marrow MSC (BM-MSC), conditioned media (CM), CM treated with trypsin or ribonuclease (RNase), or exosome-like microvesicles extracted from the CM. BM-MSC transplantation, its CM or exosome treatment prevented the increase of serum creatinine and urea and also had beneficial effects on necrosis, apoptosis and cell proliferation. If the CM or the microvesicles were treated with RNase, the protective effects were diminished (Reis et al., 2012). MSC released miR-133b-containing microvesicles and increased the level of miR-133b in astrocytes and neurons isolated from mouse brains after transient medial cerebral artery occlusion (TMCAO), and exosome-enriched fractions from MSC exposed to 72 hours to post-TMCAO brain extracts significantly increase the neurite branch number and total neurite length of cultured neurons (Xin et al., 2012). Mitochondrial transfer have been demonstrated by Spees et al., when A549ρ° cells (devoid of mitochondrial DNA), were co-cultured with MSC or skin fibroblasts and some of the A549ρ° cells acquired functional mitochondria. Rescued A549ρ° cells were able to proliferate rapidly, mitochondrial network was restored and 97% of the clones contained mitochondrial DNA from the donor cells, while genomic DNA was from the original cells (Spees et al., 2006). Recently, the comprehensive proteomic analysis of MSC exosomes revealed the presence of various angiogenic proteins within, and nuclear factor κB (NF-kB) signaling was identified as a key mediator of MSC exosome induced angiogenesis in endothelial cells in an *in vitro* model (Anderson et al., 2016). MSC also contribute to the reconstitution of the endogenous stem cell niche in the heart (Mazhari and Hare, 2007)

1.4 Adult stem cells for cardiovascular repair – translational aspects

Although MSC have made one of the fastest transitions from the bench to the clinic, MSC therapy has yielded modest results in terms of efficacy in the cardiovascular field (Karantalis and Hare, 2015). Various approaches have been used: exogenous cell transplants (skeletal myoblasts, bone marrow cells (BMC), mesenchymal stem cells and cardiac progenitor cells), the activation of endogenous cardiac cells, direct reprograming and enhancement of proliferation (Chen et al., 2015). The rationale behind skeletal myoblast therapy was the well-known capacity of skeletal muscle to regenerate through the activation of satellite cells (Brack and Rando, 2012). Two clinical trials, MAGIC

(Menasché et al., 2008) and MARVEL (Povsic et al., 2011) have been conducted, where autologous myoblast transplantation in chronic ischemic heart disease failed to show efficacy, but ventricular arrhythmias were induced, probably due to the lack of electromechanical coupling between cardiomyocytes and transplanted myoblasts, forestalling further studies with this cell type.

1.4.1 Bone marrow cells

Despite evidence that differentiation into cardiomyocyte lineage does not happen in an unselected BMC population (Murry et al 2004), both unselected and selected bone marrow cells have been injected in acute or chronic myocardial ischemia. CD 34 has been the most widely used marker for selection, for example, in the TOP-CARE AMI study, where autologous CD 34^+ cells were injected into the infarct-related artery at 1.5- 5 days post-AMI, and showed limited improvement in LVEF and reduction in left ventricular end systolic volume (LVESV), although no control group was created (Schächinger et al., 2004). Due to the considerable heterogeneity in cell type and study design in BMC transplantation clinical trials, meaningful benefit could not be shown in myocardial ischemia (Simari et al., 2014).

1.4.2 Mesenchymal stem cells

Mesenchymal stem cells were selected from bone marrow cells and were quickly translated into clinical trials. The main advantages of MSC over other types of stem cells are their ready isolation from patients, the easy expansion in culture with maintenance of differentiation potential, their genetic stability (Bernardo et al., 2007), the low risk of immune rejection by host organism, and the lack of ethical concerns plaguing the field of embryonic stem cell research. Of note, MSC isolated from different tissue sources and injected in various scenarios are most likely a heterogeneous population of progenitor cells, where the differentiation potentials may differ (Nombela-Arrieta et al., 2011). In a phase I randomized study, allogeneic MSC were injected 7-10 days after AMI, and were found to be safe: adverse events were similar in the cell treatment and placebo group, no ectopic tissue growth was found with whole body computed tomography (CT), and in addition, a 4-fold decrease in arrhythmias in the MSC group was observed compared to placebo (Hare et

al., 2009). In the APOLLO trial, freshly isolated, adipose-derived MSC were used in 14 patients in the setting of AMI. Intracoronary injection of MSC did not cause microvascular obstruction or alteration in coronary flow, and resulted in perfusion defect improvement and scar size reduction by 50% (Houtgraaf et al., 2012). MSC are most convincing as promoters of functional recovery in chronic ischemic cardiac disease, where the mechanisms of action defined in preclinical models seem to be at work: antifibrosis and neoangiogenesis, with the most prominent result being a 30-50% reduction in scar size (Karantalis et al., 2014, Heldman et al., 2014). PRECISE was the first clinical trial to use adipose-derived MSC for ischemic heart failure, where left ventricular infarcted muscle mass remained the same during follow-up in the cell therapy group, but increased in the placebo group, and perfusion and contractility increased in the cell group (Perin et al., 2014). The strongest effect was observed at the site of MSC injection in the POSEIDON study, where autologous vs allogeneic MSC from BM were compared in ischemic cardiomyopathy patients (Suncion et al., 2014). There was no immunologic reaction following allogeneic stem cell injection and no ventricular arrhythmias or new ischemia occurred during the first 30 days after cell injection, also, ectopic tissue formation did not occur in any of the study patients. Scar size reduction and reverse remodeling was apparent as assessed by CT scan. In the C-CURE trial, autologous MSC were harvested from chronic ischemic cardiomyopathy patients, culture expanded and treated with a cardiogenic cocktail. Following intramyocardial injection, patients were followed for 2 years, during which time there was no evidence of cell- therapy induced toxicity and cell therapy resulted in a favorable response regarding overall clinical parameters, such as quality of life, 6-minute walk test and New York Heart Association stage (Bartunek et al., 2013). The TAC-HFT study examined the safety and efficacy of transendocardial injection of MSC vs placebo and bone marrow mononuclear cells (BMNC) vs placebo (Heldman et al., 2014). No procedure-related serious adverse events were observed at 30 days after cell injection. Interestingly, infarct size was reduced and regional contractility at the site of injection improved in the MSC group, but not in the BMNC or placebo groups, but LVEF did not change. Several other studies using MSC for heart failure are ongoing or awaiting the publication of final results: the AMICI (Safety Study of Allogeneic Mesenchymal

Precursor Cell Infusion in MyoCardial Infarction), the MyStromalCell Trial (MesenchYmal STROMAL CELL Therapy in Patients With Chronic Myocardial Ischemia; Qayyum et al 2012), CHART-1 (Safety and Efficacy of Autologous Cardiopoietic Cells for Treatment of Ischemic Heart Failure; Bartunek et al., 2016) and NCT 02032004, examining allogeneic mesenchymal progenitor cells for the treatment of ischemic heart failure.

1.4.3 Cardiac progenitor and cardiosphere derived cells

Other candidates for cardiac cell therapy are cardiac progenitor cells and cardiosphere-derived cells (CPC and CDC respectively): c-kit$^+$, able to self-renew, and differentiate into cardiomyocytes, endothelial and smooth muscle cells (Beltrami et al 2003 and Messina et al 2004). In SCIPIO, the first- in- human trial using autologous c-kit$^+$ CPCs, cells were injected after 3 months following coronary artery bypass grafting (CABG). At the one year follow- up, LVEF increased by 12% and infarct size was significantly reduced (Bolli et al., 2011). CDC were infused into the infarct-related artery of AMI patients. By the end of the 6-month follow up, no patient suffered a major adverse cardiac event (MACE), and the CDC group showed reductions in scar mass, increases in viable heart mass and regional contractility compared to controls. However, changes in LVEF did not differ between groups (Makkar et al., 2012).

Comparing the modest results of clinical trials to preclinical results, the discrepancy in efficacy is apparent. Several factors are to be mentioned: cell populations used in clinical trials are highly heterogeneous, from BMNC through CD 34$^+$ BM stem cells to adipose derived MSC. There is still no consensus on the optimal timing, delivery method and dosing of cells. It has also been shown, that the proportion of MSC in the bone marrow decreases with age (Sethe et al., 2006), which is particularly important in the case of autologous cell therapy for the elderly heart failure patients. One of the biggest hurdles seems to be the limited retention and survival of cells at the site of interest as demonstrated in animal models. For example, after intravenous infusion, a huge proportion of cells is entrapped in the lung, spleen or liver (Barbash et al., 2003), and even after intramyocardial or intracoronary delivery, cells are taken up into the pulmonary circulation (Hou et al., 2005). Similarly, radiolabeled MSC were found primarily in the lungs after injection into

tail vein of mice, but 24 h after infusion, MSC were lost from the lungs and redistributed to other organs, mainly the kidneys, spleen and liver (Liu et al 2012). Survival of cells in the hostile environment is also an issue, as it was demonstrated in ST- elevation myocardial infarction (STEMI) patients, where only 1.3-2.6% of 18F- fluoro-deoxy-D-glucose- (F-FDG) labeled unselected bone marrow cells remained in the infarct region 50-75 minutes after cell injection into the affected coronary artery (Hofmann et al 2005).

1.5 Improving the efficacy of stem cell therapy: focus on hypoxia and hypoxic preconditioning

Various strategies have been investigated in order to improve the survival and retention of stem cells after injection: more effective ways of delivery (intramyocardial seems to retain more cells then intracoronary or intravenous infusion; Hou et al., 2005), MSC implantation in natural or synthetic polymer scaffolds (scaffolds seeded with MSC were superior to MSC injection alone; Jin et al., 2009), genetic engineering of MSC, preconditioning strategies of MSC before transplantation: hypoxic, hyperoxic and pharmacological, and combined administration of MSC and other types of stem cells (Li et al., 2016). For example, BM-MSC overexpressing the pro-survival gene Akt 1, limited infarct size and improved ventricular function more efficiently than non-modified MSC in a rat model of MI (Gnecchi et al., 2006).

MSC often reside in tissues which exhibit low oxygen tension, such as the BM (1-7% or 4-20 mmHg; (Brahimi-Horn and Pouysségur, 2007, Chow et al., 2001), but are usually expanded in the laboratory under normoxic conditions (20.8% atmospheric oxygen, 160 mmHg oxygen in solution). In many of the clinical or experimental settings MSC are used to promote revascularization at areas with poor circulation, and hence, are injected into an ischemic tissue environment, where they are exposed to a lower oxygen concentration than in their usual *ex vivo* culture conditions: oxygen tension in tissues is 4-20 mmHg, while arterial oxygen is 104 mmHg (Brahimi-Horn and Pouysségur, 2007). Hypoxia exerts strong effects on MSC, affecting their proliferation, differentiation and migration (Das et al., 2010). However, studies have yielded controversial results, which might be caused by the variation in the conditions that have been tested, including time-

span (ranging from minutes to days) and level of hypoxia: from 0.1 to 10% O_2 (Das et al., 2010, Tsai et al., 2012). In addition, it has also been shown that MSC respond to hypoxia by altering their secretome, increasing the amount of secreted proangiogenic factors such as VEGF (Potier et al., 2007). In a study using a mouse hind limb ischemia model, MSC survival after injection into the ischemic limb was greatly enhanced by the hypoxic preconditioning of the cells, and hypoxic preconditioned-MSC increased perfusion, necrosis salvation and capillary density to a greater extent than non-preconditioned cells (Zhu et al., 2014). It has been demonstrated previously that hypoxic preconditioning enhances the therapeutic potential of MSC in applications such as the treatment of cardiac ischemia (Hu et al., 2008, He et al., 2009), critical limb ischemia (Rosová et al., 2008, Huang et al., 2014, Leroux et al., 2010), traumatic brain injury (Chang et al., 2013), and in liver regeneration (Yu et al., 2013). One of the key factors in the hypoxic preconditioning-induced enhancement of tissue repair seems to be the increased retention of MSC in tissues (Hu et al., 2008, Huang et al., 2014 Leroux et al., 2010), but the underlying mechanisms remain elusive. Thus, the study of MSC in low oxygen tensions is of great importance.

The further understanding of MSC biology and response to hypoxia, especially in terms of the secretion of angiogenic factors could be remarkably useful for enhancing the efficacy of MSC-based therapy (Bianco et al., 2013). It has been long established that lipids (fatty acids and their derivatives and substances related biosynthetically or functionally to these compounds) are not only essential building blocks of biological membranes and responsible for energy storage in the cell, but also have important regulatory and signaling functions in almost all cellular processes (Hannun and Obeid, 2008). Changes in lipid levels that result in functional consequences eventually gave rise to the idea of 'bioactive lipids' (Hannun and Obeid, 2008). For example, inositol phospholipids have been shown to modulate acetyl-choline induced intracellular signaling (Hokin and Hokin, 1953), and eicosanoids play a crucial role in inflammatory signaling (Serhan and Savill, 2005). Following G protein coupled receptor (GPCR) activation, the enzymatic cleavage of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) produces two smaller lipids: inositol triphosphate (IP_3), directly activating calcium (Ca) channels, which results in

increased intracellular Ca concentration; and diacylglycerol (DG), directly activating several isoforms of the protein kinase C (PKC) enzyme, which phosphorylates a number of target proteins (Nishizuka, 1992). Accordingly, it has become evident that lipids are crucial first and second messenger molecules (van Meer et al., 2008). It has been also shown that lipids can be involved in hypoxic signaling. Ceramides for example, are increased in renal tubular epithelial cells under hypoxia (Ueda et al., 1998). It has also been shown that the cell lines Hela and 293T increase DG levels when exposed to hypoxia, affecting the activity of the transcription factor hypoxia inducible factor 1 (HIF-1; Temes et al., 2004).

With the evolution of mass spectrometry (MS) techniques and the consequent advance in the identification of lipids, lipidomics 'joined the omics evolution' (cited from Dennis, 2009). The lipidome is the complete lipid composition of a cell, tissue, or organism; lipidomics is the global analysis of the lipidome with a comprehensive mass spectrometry approach, and functional lipidomics is the study of the role played by membrane lipids in biological functions (Dennis, 2009, Shevchenko and Simons 2010). The lipidome of stem cells has not yet been widely studied. Meissen and colleagues compared the lipidome of human embryonic stem cells, induced pluripotent stem cells and the parental fibroblasts of iPSC (Meissen et al., 2012). They found that iPSC undergo a remarkable shift metabolically from the parental fibroblasts toward ESC, suggesting an almost complete metabolic reprogramming. There were no differences between the pluripotent cell types (iPSC and ESC) in free fatty acid (FFA) metabolism, but for example, significant differences in the phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) lipid structures could be detected: in both types of pluripotent stem cells, the amount of the poly-unsaturated (three or more double bonds in the acyl chain) PC species was significantly higher than in fibroblasts. The increase in unsaturation levels of polyunsaturated PC species may alter membrane fluidity, since PC are the most abundant lipid species in the mammalian cell membrane. A study by Fuchs et al. was reporting on the lipidomics of ovine MSC (Fuchs et al., 2008). Very little is known about the overall lipidomics of human MSC, or changes in lipid composition in response to stimuli such as hypoxia.

2 Aims of the study

Hypoxia exerts strong effects on MSC, affecting their proliferation, differentiation and migration (Das et al., 2010), thus, the study of MSC in low oxygen tensions is of great importance. The aims of the first set of experiments were (1) to examine the effects of various levels of hypoxia on MSC proliferation and differentiation, and (2) to study the effects of hypoxic exposure prior to their administration to a hypoxic site, which we refer to as hypoxic pre- conditioning (HP).We sought (3) to determine the optimal hypoxia levels and incubation times to promote survival of MSC in an environment of low oxygen and nutrients *in vitro*, and to enhance MSC retention after *in vivo* administration into mice. In addition, (4) we sought to evaluate the underlying mechanism(s) that promote the survival of MSC and hypothesized, that hypoxic pre- conditioning promotes metabolic adaptations in MSC that allow increased survival under conditions of limited nutrients and oxygen.

Further understanding MSC biology and MSC response to hypoxia, especially in terms of the secretion of angiogenic factors could be remarkably useful for enhancing the efficacy of MSC-based therapy (Bianco et al., 2013). Very little is known about the overall lipidomics of human MSC, or changes in lipid composition in response to stimuli such as hypoxia. In consequence, our aims were (5) to determine the lipid composition of human bone marrow derived MSC, (6) to identify changes induced by exposure to hypoxia and (7) to evaluate whether these changes have a role in the angiogenic potential of MSC.

3 Methods

3.1.1 Isolation and culture of mesenchymal stem cells

Bone marrow aspirates from human donors were purchased from Lonza (Walkersville, USA) or Stem Express (Placerville, USA) and mesenchymal stem cells were isolated as follows (Fierro et al 2011). BM aspirates were passed through 90 μm pore strainers for isolation of bone spicules. Then, strained BM aspirates were diluted with an equal volume of phosphate-buffered saline (PBS) and centrifuged over Ficoll (GE Healthcare, Waukesha, USA) for 30 minutes at 700 x g. Next, mononuclear cells and bone spicules were plated in plastic culture flasks using standard culture medium: Minimum Essential Medium α ((MEM-α;), HyClone, Thermo Scientific, South Logan, USA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals Lawrenceville, USA). After 2 days, non-adherent cells were removed by washing twice with PBS. MSC from passages 3–6 were used for experimentation for hypoxia and hypoxic preconditioning experiments, and passages 4-7 for lipidomics analysis and D609 experiments (see later), where each passage implies 5-7 days in culture (i.e. approximately 3 population doublings).

3.1.2 Immunophenotypic characterization of mesenchymal stem cells

For the immune characterization of MSC by flow cytometry, cells were lifted by trypsin treatment and incubated for 45 minutes with the fluorophore- conjugated antibodies (diluted 1:100) as follows: CD73 (5'-nucleotidase), CD90 (Thy-1) and CD105 (endoglin), CD11b (integrin alpha M), CD14 (monocyte/macrophage marker), CD34 (hematopoietic stem cell marker) and CD45 (lymphocyte common antigen). Cells isolated with our method fulfilled the immunophenotypic criteria of the ISCT (Figure 1., Dominici et al., 2006).

3.1.3 Studies with hypoxia

For studies with hypoxia, cell cultures were performed in incubators (MCO-18M Sanyo) at 37°C with 5% CO_2, humidified atmosphere and dedicated oxygen level (20 (atmospheric), 10, 5 or 1% O_2), as established by replacement with nitrogen injections. In order to establish exact oxygen levels that the cells are exposed to during experimentation; we monitored oxygen diffusion in culture media. An OxyLite monitor and probe were used

in 12-well culture plates containing 1ml of media (αMEM) per well to determine dissolved oxygen concentration (pO_2) over 2 hours, in incubators set for 20%, 10%, 5% and 1% O_2. After placing cell cultures in the respective incubator, full hypoxic levels in the media were reached within one hour and did not change thereafter, when plates were undisturbed (Figure 2).

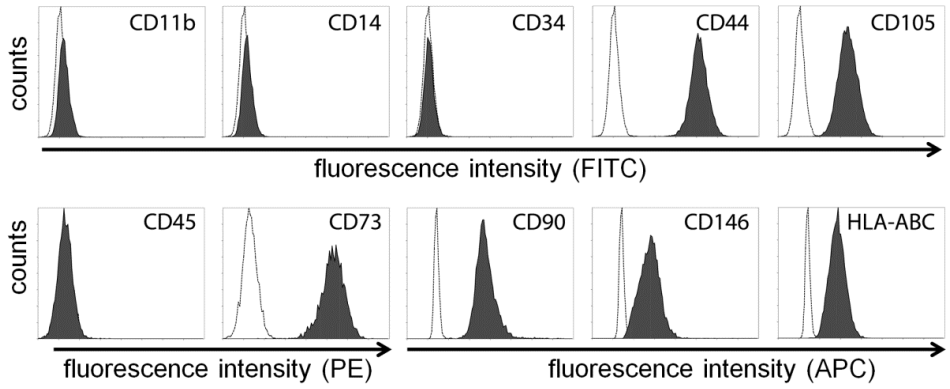

Figure 1. MSC isolated from healthy individuals exhibit a common immune phenotype. Specific surface antigen expressions were determined by flow cytometry based on the ISCT minimal criteria for multipotent mesenchymal stromal cells: CD105, CD73, CD 90 ≥ 95% positive; and CD45, CD34, CD14, CD11b ≤ 2% positive. MSC also expressed HLA-ABC, CD 146, and CD 44.

3.1.4 Cell proliferation analysis

For proliferation assays, MSC were seeded into 12 well plates at 1000 cells/cm^2 seeding density. At every time point, (2-3 days) cells were lifted with trypsin treatment and counted using Trypan blue exclusion dye (Gibco, Thermo Fisher Scientific, South Logan, USA) and a hemocytometer.

3.1.5 Cell cycle analysis

To determine the status in cell cycle, MSC were seeded into 75 cm^2 culture flasks at 500 cells/cm^2 seeding density, and incubated in standard culture medium with varying levels of hypoxia for 4 days, with media change at day 2. Then, cells were lifted by trypsin treatment and permeabilized with ice cold 70% ethanol overnight. After 45 minutes of

incubation with 50µg/ml propidium iodide solution (Roche, Indianapolis, IN, USA) containing 20ng/ml RNAse A (Qiagen, Valencia, CA, USA), followed by a washing step with PBS, samples were evaluated by flow cytometry and analyzed using ModFit LT software (Verity Software House). With this method, the amount of propidium iodide taken up by cells is proportionate to their DNA content.

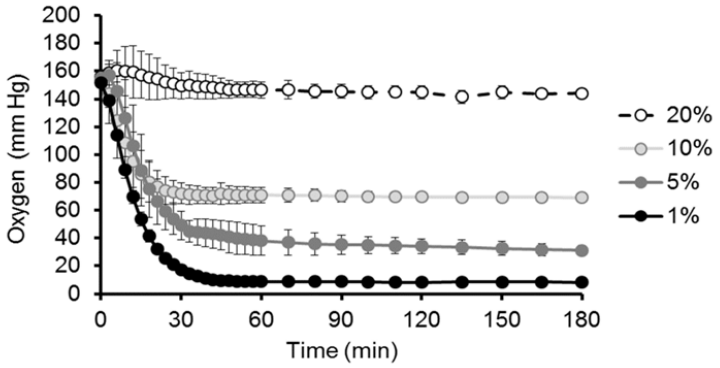

Figure 2. Oxygen partial pressure in culture media placed in incubators with different levels of hypoxia. We monitored oxygen partial pressure in culture media after placement in the respective incubators in order to establish exact level and onset of hypoxia. Note that respective oxygen pressure is reached in the culture medium within one hour of placement in the respective incubator. Data represent the average of 3 measurements.

3.1.6 Osteogenic and adipogenic differentiation assays

For both osteogenesis and adipogenesis, 10,000 MSC/cm^2 were cultured for 14 days in incubators with varying hypoxia levels (20, 10, 5 and 1% O_2), with media changes every three days. Osteogenic medium consisted of standard culture medium supplemented with 0.2 mM ascorbic acid, 0.1 µM dexamethasone, and 20 mM β-glycerolphosphate. Adipogenic medium contained standard culture medium with 0.5 mM isobutilmethylxantine, 50 µM indomethacin, and 0.5 µM dexamethasone. To quantify osteogenesis, alkaline phosphatase (ALP) activity was measured by lifting the cells with trypsin and lysing the cells for protein extraction using 1.5mM Tris-HCl solution containing 1.0 mM $ZnCl_2$, 1.0mM $MgCl_2$, and 1% Triton X-100 for 15 minutes. Lysates were then centrifuged at 12,000*g* for 10 minutes and incubated with p-

nitrophenylphosphate liquid substrate solution (Sigma-Aldrich, St. Louis, USA) for 30 minutes. Released p-nitrophenolate was determined spectrophotometrically at 405 nm. Calcium precipitation was determined using Alizarin Red S indicator (ARS; Ricca Chemicals, USA). Cells were fixed with 10% v/v formalin solution for 15 minutes, washed once with PBS, and stained for 20 minutes with 1% w/v ARS over gentle shaking. Samples were then photographed for visual documentation and then incubated with 10% v/v acetic acid for 30 minutes, the cell layer scraped, and vortexed for 30 seconds, then centrifuged at 12,000 x g for 10 minutes. Optic density of the supernatants was measured at 405 nm. For both ALP and ARS, total protein concentration was determined with Coomassie staining and measured at 595 nm. For adipogenesis, cells were fixed with 10% v/v formalin for 15 minutes, rinsed once with PBS, and stained for 30 minutes with Oil Red O (Electron Microscopy Sciences, Hatfield, PA, USA). Cells were then washed three times with PBS, photographed and incubated with 4% Tween 20 (Affymetrix, Santa Clara, USA) in isopropanol for 5 minutes, in order to release the dye. Optic density of supernatants was then measured at 490 nm.

3.1.7 In vitro cell survival and apoptosis detection

To evaluate survival of MSC in hypoxia and serum deprivation, we first incubated MSC (10,000 cells/cm^2) in 12-well plates in standard culture medium and 1% O_2, for varying amounts of time (0, 16, 48 or 96 hours). Then, culture media was changed to MEMα alone (without FBS) and all plates were transferred to 1% O_2 for up to 12 days with no additional media changes. Every three days, cells were detached by trypsin treatment and counted using Trypan blue exclusion dye and a hemocytometer. To quantify the percentage of dead cells, MSC were cultured on glass cover slips (10,000 cells/cm^2) and incubated for 48 hours in standard culture medium in 20% O_2 (control) or 1% O_2 (HP). Then, medium was changed to serum free medium and plates were transferred to 1% O_2 for 9 days with no additional media changes. Cover slips were then incubated in 4 μM Ethidium- homodimer III (EthD- III; Biotium, Hayward, USA) in PBS for 30 minutes and then mounted on slides with Vectashield Mounting Medium with 4',6-diamidino-2-Phenylindole dihydrochloride (DAPI, Vector Laboratories, Burlingame, USA). Images

were acquired and processed for large-field merge using a BZ-9000 (BIOREVO) fluorescence microscope (Keyence, Itasca, USA) and analyzed for automated counting with NIS-Elements BR software (Nikon). To quantify the percentage of apoptotic cells, MSC were cultured as described above for EthD-III, but in 6-well plates. At day 9, cells were lifted using trypsin and stained with phycoerythrin- Annexin V- Apoptosis Detection Kit I (BD Pharmingen, San Diego, USA) following manufacturer's instructions and measured by flow cytometry.

3.1.8 In vivo retention study

In order to generate luciferase-expressing MSC, we used a third generation lentiviral vector with the general form pCCLc-MNDU3-Luciferase-PGK-eGFP-WPRE. We transduced the cells using protamine sulfate (20 μg/ml) and a quantity of virus equivalent to a multiplicity of infection of 20. Using this protocol, over 90% of cells were enhanced green fluorescent protein (eGFP) positive. For cell administration, immune compromised NOD/SCID-IL2Rγ-/- (NSG) mice were anesthetized using inhaled isoflurane and then injected in the medial hamstring muscles with the luciferase-expressing MSC in 20 μL of HyStem C (BioTime, Alameda, USA). To generate the standard curve, increasing number of cells were used (from 12,500 to 100,000 cells), while, for the retention studies, 200,000 cells were injected. For imaging, 100 μL of 20 mg/mL D-Luciferin Firefly (Perkin Elmer) were injected intraperitoneally into the animals, 10 minutes before bioluminescence detection via In Vivo Imaging System (IVIS) Spectrum imaging for 5 minutes exposure time. LivingImage software was used to quantify MSC's bioluminescence (Perkin Elmer). All animal procedures were performed as approved by the Institutional Animal Care and Use Committee.

3.1.9 Glucose and lactate measurements

Supernatants were collected every three days from MSC cultured under identical conditions to the *in vitro* survival assay (in 12-well plates) described above. Supernatants for glucose measurement were stored at -20°C and those for lactate at -80°C. Glucose and lactate concentrations of supernatants were determined using a Glucose

Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, USA) and a Lactate Colorimetric Assay Kit (BioVision), respectively, following their provided protocols.

3.1.10 Ultrahigh pressure liquid chromatography- quadrupole time-of-flight tandem mass spectrometry (UHPLC- QTOF-MS/MS)

After culture in either 20% or 1% O_2 for 48 hours, MSC were lifted by trypsin treatment (Thermo Scientific) and stored at -80°C. Then, samples were processed for total lipid extraction and mass spectrometry analysis as described by Fiehn and Kind (2007). After quenching the cells, 1 x 10^6 dried cells were added to a 1.5 mL Eppendorf tube, placed on dry ice for 20 minutes to completely freeze and then thawed on ice. The freeze-thaw cycle was repeated twice. Then, 1 mL of pre-chilled (-20˚C) extraction solvent was added to the cells (acetonitrile: isopropanol: water 3: 3: 2 v/v/v) and the freeze-thaw cycle repeated two more times. Samples were then vortexed for 10 seconds, shaken for 5 minutes at 4˚C and centrifuged for 2 minutes at 14,000 x g. 500 µl of the supernatant was evaporated in a cold trap concentrator (Labconco Centrivap, Kansas City, USA) to complete dryness. Lipidomics data were acquired using UHPLC – QTOF MS/MS (Waters Acquity UPLC CSH C18 column). Chromatographic separation was followed by electrospray ionization (ESI) in both positive (Agilent 6530 QTOF MS, Agilent Technologies, Santa Clara, USA) and negative mode (Agilent 6550 QTOF MS); and QTOF-MS/MS. Data were analyzed in a four-stage process. First, raw data were processed in an untargeted (qualitative) manner by MassHunter Qual (v. B.05.00, Agilent Technologies) to find peaks in up to 300 chromatograms. Peak features were then imported into Mass Profiler Professional (Agilent Technologies) for peak alignments to seek which peaks are present in multiple chromatograms, using exclusion criteria by the minimum percentage of chromatograms (30%) in which these peaks are positively detected. Peaks were then manually collated and constrained within the MassHunter quantification software (v. B.05.01 Agilent Technologies) on the accurate mass precursor ion level, using the MS/MS information and the LipidBlast library to identify lipids with manual confirmation of adduct ions and spectral scoring accuracy. The following normalization steps were performed: ‘vector normalization’ in which the sum of all peak heights was calculated for

all identified metabolites (but not the unknowns) for each sample. Such peak-sums were called 'mTIC' ('total ion chromatogram/current'). Subsequently it was determined, whether mTIC averages were significantly different between treatment groups. Because the average mTIC will be different between series of analyses (due to differences in machine sensitivity, tuning, maintenance status and other parameters), additional normalizations were performed. For this purpose, identical samples ('quality control (QC) samples') were analyzed multiple times in all series of data acquisitions (a suitable QC sample was used for every 11th injection). Internal standards were used for absolute quantifications, with a standardized equation for peak height normalizations. Presented data are 'absolute quantifications', meaning they are normalized to the best suited internal standard for which the absolute concentration used in the spiking process is known. The best suited internal standard is defined as the internal standard that belongs to the same lipid class as the metabolite that needs to be normalized. Annotated lipids are abbreviated as: [Lipid class] [total carbon number in the chains]:[total number of double bonds in the chains].

3.1.11 Measurement of DG levels by enzyme-linked immunosorbent assay (ELISA)

To measure DG levels in MSC, we used the Human Diacylglycerol ELISA Kit (Biotrend Chemicals LLC, Destin, USA), following manufacturer's instructions. MSC (10,000 cells/cm^2) were cultured in 6-well plates in normoxia (20% O_2), hypoxia (1% O_2) and with or without O-(octahydro-4,7-methano-1H-inden-5-yl) carbon potassium dithioate (D609; (50 µM)). In addition, MSC in normoxia were also tested in the presence of the hypoxia-mimicking agent Cobalt Chloride ($CoCl_2$; 100 µM). After 48 hours, cells were lifted by trypsin treatment and stored at -80 ˚C as dry cell pellets. Total lipids were extracted as described by Bligh and Dyer, (1959) and Petkovic et al. (2005). Briefly, cell pellets were re-suspended in PBS (100,000 cells/100 µl). Lipids were extracted from cell suspension by addition of 375 µl chloroform: methanol mixture (1:2 v/v), and vortexed for 2 minutes. Then, 125 µl of chloroform was added, and extracts were vortexed for 30 seconds. Finally, samples were washed with 125 µl of 1.5 M sodium-chloride (NaCl), vortexed for 30 seconds, and centrifuged at 500 x g for 10 minutes. Organic phase (lower phase) was used for analysis and measured in triplicate.

3.1.12 Detection of angiogenic factors

In order to detect secretion levels of VEGF, IL- 8/CXCL-8 and Ang-2, MSC (13,000 cells/cm^2) were cultured for 48 hours in 12-well plates in either normoxia or hypoxia and in either presence or absence of 50 μM D609. Then, supernatants (i.e. conditioned media) were collected and stored at -80 ˚C. Quantification of angiogenic factors was performed by ELISA using the respective DuoSet ELISA kits (R&D Systems), following manufacturer's instructions.

To measure mRNA levels of VEGF, IL-8 and Ang-2, cells were cultured as above, but for 12 hours. Total RNA was extracted using Quick-RNA MicroPrep kit (Zymo Research, Irvine, CA). Reverse transcription was performed with 1 μg RNA using TaqMan Reverse Transcription Reagents kit (Life Technologies). For semi-quantitative detection of mRNA levels of VEGF, IL-8, Ang-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control) real time PCR was performed using TaqMan primers/probes: Hs00900055_m1, Hs00174103_m1, Hs01048042_m1 and Hs99999905_m1 respectively (Life Technologies). Special attention was placed on having only minimal variation on GAPDH mRNA levels, in between samples.

3.1.13 Wound/scratch assay

Conditioned media of MSC was prepared and stored as described above: MSC (13,000 cells/cm^2) were cultured for 48 hours in 12-well plates in either normoxia or hypoxia and in either presence or absence of 50 μM D609. Then, supernatants were collected and stored at -80 ˚C. HUVEC- derived VeraVecs (Angiocrine Bioscience, New York, USA; Seandel et al., 2008) were cultured in EndoGro medium (Millipore, Billerica, USA). For wound/scratch assays, Cytoselect 24-well wound healing assay (Cell Biolabs, San Diego, USA) was used as follows: VeraVecs were plated at 79,000 cells / cm^2 in 24-well plates, containing plastic inserts that leave a homogeneous 500 μm gap in between confluent monolayers of cells (Lauffenburger and Horwitz 1996). The next day, inserts were removed and media was changed to the conditioned media from MSC. Regular MSC culture medium (MEM α + 10% FBS) was used as negative control. Pictures were taken immediately, and 10 hours after adding conditioned media. Pictures were then analyzed

using TScratch software (ETH Zürich; Gebäck et al., 2009). Wound closure was determined as [open image area after 10h / initial open image area] x 100. Values represent the average of 3 independent experiments.

3.1.14 Statistical analysis and presentation of data

All experiments were performed at least three times with MSC derived from different donors. The specific number of biological replicates for each experiment is indicated in the respective figure legend as n. In accordance, results are shown as average with the standard error of the mean (SEM) as error bars. When only two conditions were tested, a Student's t-test was used, where significant differences are denoted as: * $p < 0.05$ and ** $p < 0.005$. In contrast, in experiments where four conditions were tested, analysis of variance (ANOVA) or a Student's t-test with Bonferroni correction were applied, with significant differences from control indicated as * $p < 0.0125$. In lipidomics experiments, values are given as mean +/- SEM. A paired Student t-test was used to determine statistically significant differences established as $p < 0.05$ (see in the respective results sections).

4 Results

4.1 Proliferation of MSC is inhibited by hypoxia in a dose-dependent manner

First, we tested the effect of varying levels of hypoxia (1%, 5%, 10% and 20% O_2) on proliferation and differentiation of human BM-MSC. In order to detect exact oxygen levels that the cells were exposed to during experimentation, we monitored oxygen in culture media (Figure 2.) We observed that hypoxia caused a decrease in the proliferation of MSC, and this decrease was proportional to the degree of hypoxia. Culture under 1% O_2 had the strongest effect on MSC, while 10% O_2 seemed to only have a mild effect on the proliferation of MSC. We assessed this by both cell cycle analysis and proliferation assays (Figures 3A and 3B). Culture of MSC in 1% O_2 significantly reduced the amount of cells in S-phase as compared to normoxic controls (Figure 3B).

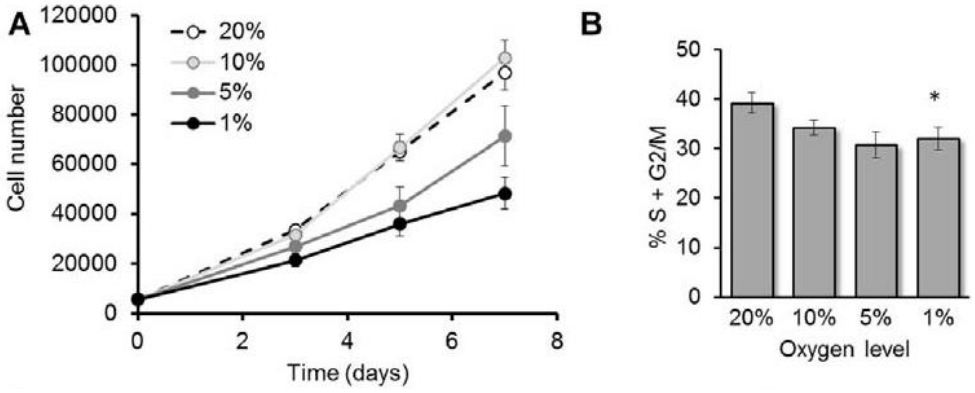

Figure 3. Hypoxia inhibits proliferation of MSC. (A) MSC were cultured in incubators with the indicated oxygen levels and at each time point, cell numbers were determined using a hemocytometer and Trypan blue exclusion dye (n = 3). (B) Similarly, MSC were cultured for three days in the respective incubators prior to cell cycle analysis using propidium iodide staining and flow cytometry (n = 5). Statistical analysis was performed by paired Student's t-test for (B); *: $p < 0.05$

4.2 Differentiation of MSC is inhibited by hypoxia in a dose-dependent manner

Next, we tested whether incubation in varying degrees of hypoxia affected osteogenic or adipogenic differentiation of MSC. Adipogenesis (as assessed by measuring

triglyceride (TG) content of cells using Oil Red O staining) was inhibited to a similar degree with 5% and 1% O_2 (Figures 4A and 4D). Osteogenesis was measured by both ALP activity and calcium precipitation (Alizarin Red S staining). We found that both adipogenic and osteogenic differentiation of MSC are reduced by hypoxia in a dose dependent manner: (Figures 4A, 4B and 4C).

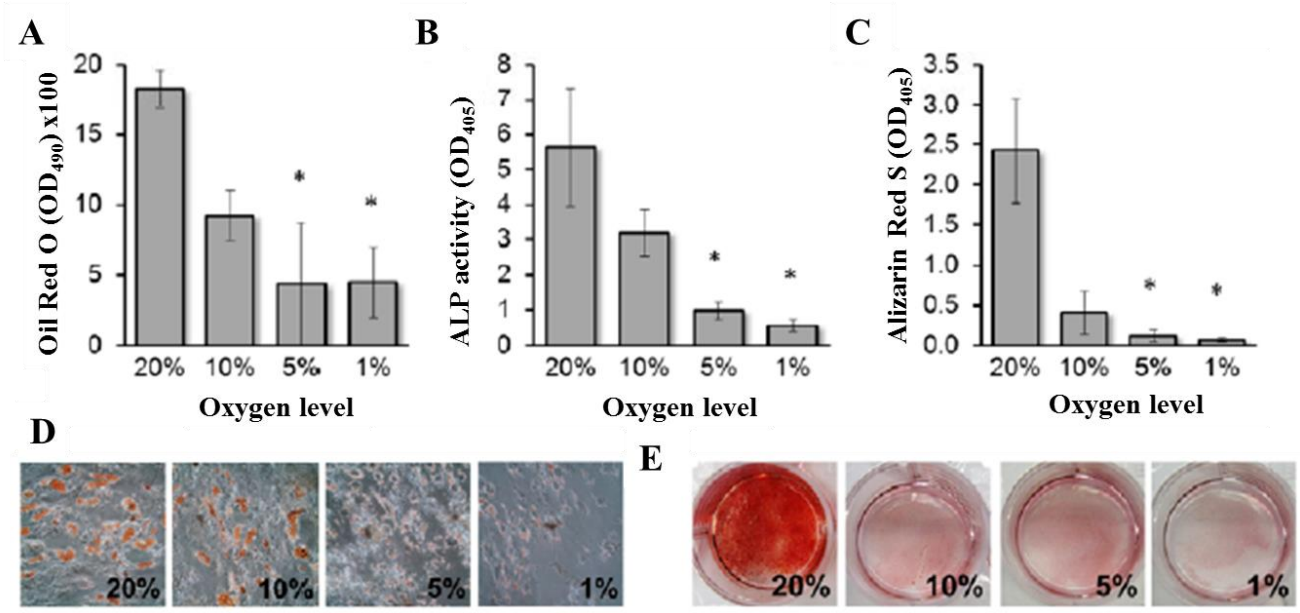

Figure 4. Hypoxia inhibits the differentiation of MSC. (A) After 14 days in incubators with the respective oxygen levels, in the presence of adipogenic media, Oil Red O staining was performed to quantify triglyceride content (n = 3). After 14 days in osteogenic media, alkaline phosphatase activity (B; n = 5) and calcium precipitation (C; Alizarin Red S staining, n = 5) were measured. (D) Representative images of Oil Red O staining after 14 days in adipogenic media (original magnification x100). (E) Selected images of culture wells after Alizarin Red S staining for osteogenesis. Statistical analysis was performed by ANOVA for (A, B, C); *: $p < 0.05$

4.3 Hypoxic pre-conditioning increases survival of MSC *in vitro*

To simulate an environment poor in nutrients and oxygen, we pre-incubated MSC in standard culture media in either 20% O_2 (control) or 1% O_2 (HP), for 16, 48 or 96 hours. Then medium was changed to serum free medium (i.e. MEM-α) and all cells were

transferred to an incubator with 1% O_2 (Figure 5A). Since media were not changed afterwards, MSC experienced starvation due to nutrient deficiency (Mylotte et al 2008). We observed that for all experimental conditions, no living cells were present after 12 days under serum deprivation and hypoxia. However, MSC that received hypoxic preconditioning for 48 or 96 hours showed an approximately 2-fold increase in survival at 6 days under serum deprivation and hypoxia as compared to control MSC (Figure 5B), while 16 hours of hypoxic preconditioning had no effect on survival as compared to controls. These results suggest that hypoxic preconditioning for 48 hours or more transitorily promotes cell survival in environments with limited oxygen and nutrients.

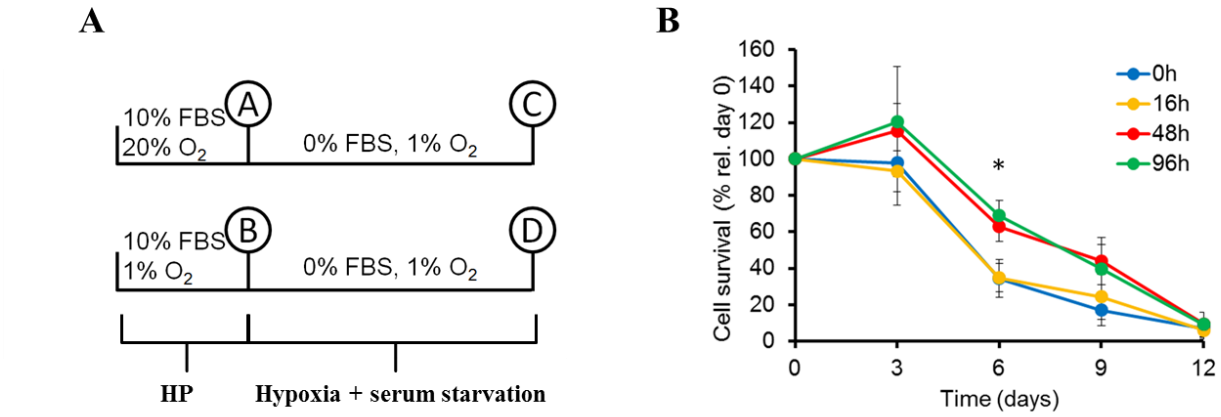

Figure 5. Hypoxic pre-conditioning increases cell survival *in vitro*. (A) Schematic overview of experimental design: MSC were cultured in complete culture media (supplemented with 10% FBS) in normoxia or, for varying times, in hypoxia (1% O_2). Then (time points A and B), culture media was changed to MEM- alpha without supplements (0% FBS), with no further media changes, and incubated in 1% O_2 for up to 12 days. (B) After pre-incubation of MSC for 0, 16, 48, or 96 hours in hypoxia, cells were exposed to the nutrient and oxygen deprivation. Cells were counted using a hemocytometer and Trypan blue exclusion dye every three days. Cell survival is expressed as a percentage of remaining cells relative to day 0, after HP (n = 6). Statistical analysis was performed by ANOVA; *: $p < 0.05$. (HP=hypoxic preconditioning)

To further support these results, MSC were stained with EthD-III. We found that after 9 days in hypoxia and serum deprivation, 48 hour- HP-MSC showed over a 2-fold reduction in dead cells as compared to controls (Figures 6A and 6B). We then measured apoptotic cells by Annexin V staining. We found that after 9 days under hypoxia and serum deprivation, there were significantly less apoptotic cells in the HP-MSC group, than in the control MSC group (Figure 6C). In summary, significant increase in survival was apparent at day 6, but no living cells could be detected after 12 days under serum deprivation and hypoxia.

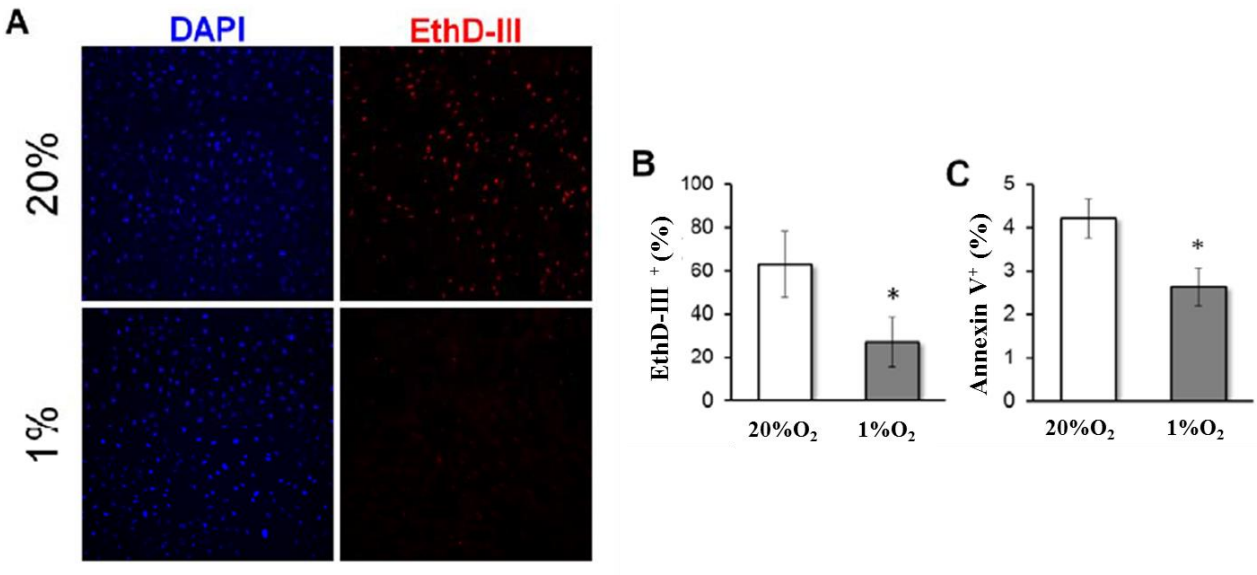

Figure 6. Hypoxic pre-conditioning reduces apoptosis of MSC *in vitro*. After hypoxic pre-conditioning for 48 hours followed by hypoxic culture for 9 further days, as described in Figure 6, MSC were stained with EthD-III (dead cells) and DAPI (total nuclei). (A) Representative images of EthD-III and DAPI staining. (B) Quantification of A (n = 5). (C) Also after 9 days in culture, cells were stained with Annexin V and measured by flow cytometry (n = 5). Statistical analysis was performed by paired Student's t-test; *: $p < 0.05$.

4.4 Hypoxic preconditioning enhances retention of MSC *in vivo*

We wanted to explore whether hypoxic preconditioning could increase the survival of MSC when injected into living tissue, so we injected luciferase- expressing MSC intramuscularly into immune deficient mice and followed their retention over time.

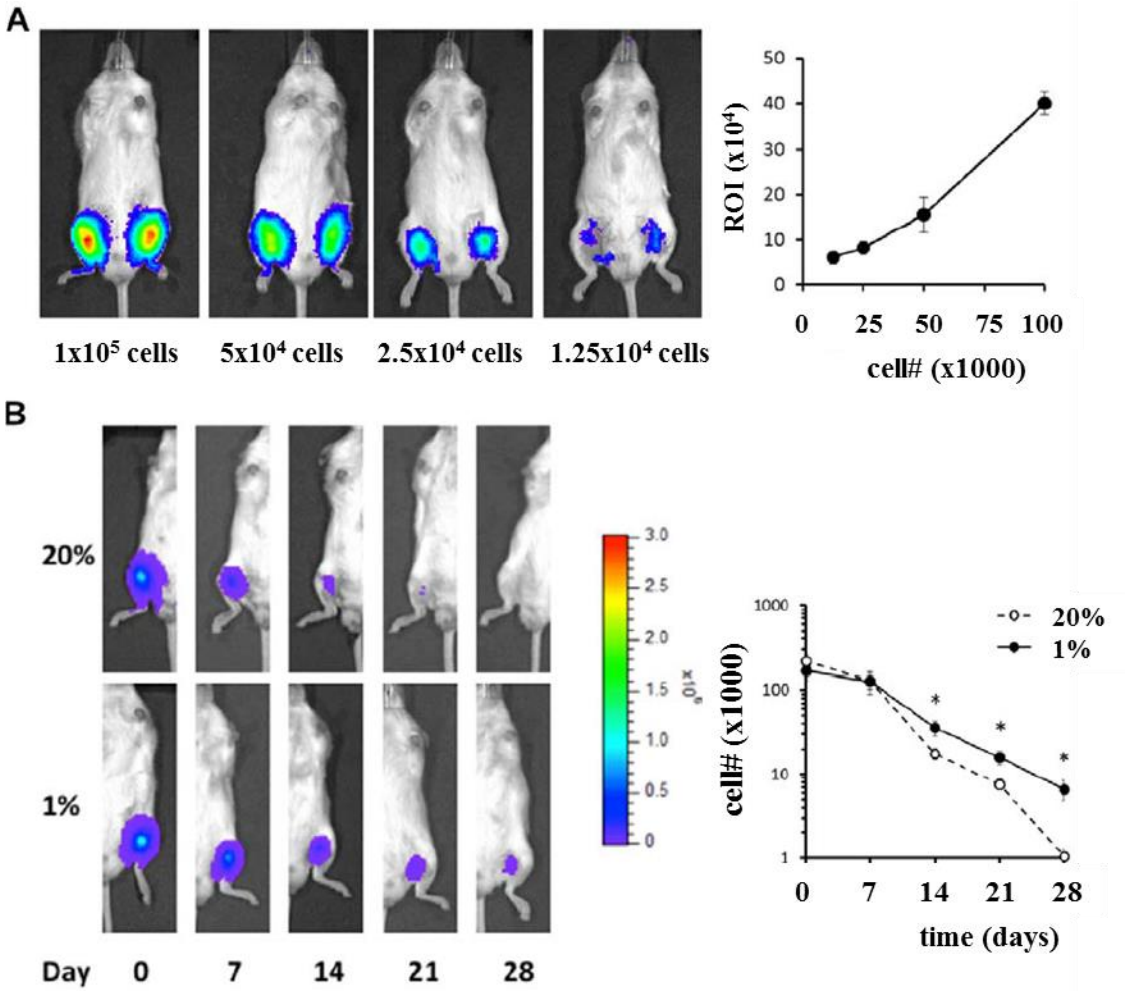

Figure 7. Hypoxic preconditioning enhances the retention of MSC *in vivo*. (A) To establish a correlation between cell numbers and luminescent signal intensity, we injected increasing numbers of luciferase expressing MSC into the medial hamstring muscle of NOD/SCID-IL2Rγ-/- (NSG) mice and measured fluorescent signal by IVIS immediately after. (B) After incubation for 48 hours in either 1% or 20% oxygen, 2 x 10^5 MSC were injected into NSG mice and cell retention was followed for 28 days, with luminescence measured weekly. (n = 14 animals/group, MSC derived from three different donors). For both A and B, panels on the left show representative images, while average of total experiments is shown on the right. Statistical analysis was performed by paired Student's t-test for each individual time point; *: $p < 0.05$

First, to establish a correlation between IVIS- detected luminescence and cell number, we generated a standard curve by injecting increasing numbers of MSC into the medial hamstring muscles of immune deficient NOD/SCID-IL2Rγ $^{-/-}$ mice and measured luminescence immediately thereafter (Figure 7A). We also confirmed the detection limit of this method and found that injection of 5,000 MSC or more emit a robust signal, while the signal of 1,000 luciferase- expressing MSC was barely detectable above background. We then addressed cellular retention with and without hypoxic preconditioning using the standard curve to determine the number of retained cells after injection over time. We found that MSC cultured under 20% O_2 prior to injection, i.e. without HP (controls) are retained for one week after transplantation, but start to decrease rapidly thereafter and are undetectable at 28 days after cell injection (Figure 7B). In contrast, we could detect MSC with 48-hour hypoxic preconditioning after 28 days. Cell retention of HP- MSC was also significantly better at day 14 and day 21 after administration as compared to control MSC. In conclusion, hypoxic preconditioning of MSC in 1% O_2 for 48 hours significantly enhanced their retention *in vivo*.

4.5 Hypoxic pre-conditioned MSC have more glucose available during serum deprivation *in vitro*

We first cultured cells under 1% O_2 for 0, 16, 48, and 96 hours, and found that glucose levels of supernatant media are significantly higher in MSC cultured in hypoxia for 96 hours (Figure 8A). We also measured lactate content in the collected supernatant media, and found, that lactate levels were increased in MSC cultured in hypoxia as compared to controls (Figure 8B). We next preconditioned MSC in 1% O_2 for 16, 48 or 96 hours (controls were cultured in 20% O_2), followed by placement of all cells into 1% O_2 in serum-free medium. We then measured glucose and lactate under serum deprivation and hypoxia at 3, 6, 9 and 12 days (see Figure 6A for experimental design). After 3 days in serum deprivation and hypoxia, glucose levels were over 2-fold higher in supernatant media of 48 and 96 hours hypoxic preconditioned MSC, as compared to controls (without hypoxic preconditioning (Figure 8C).

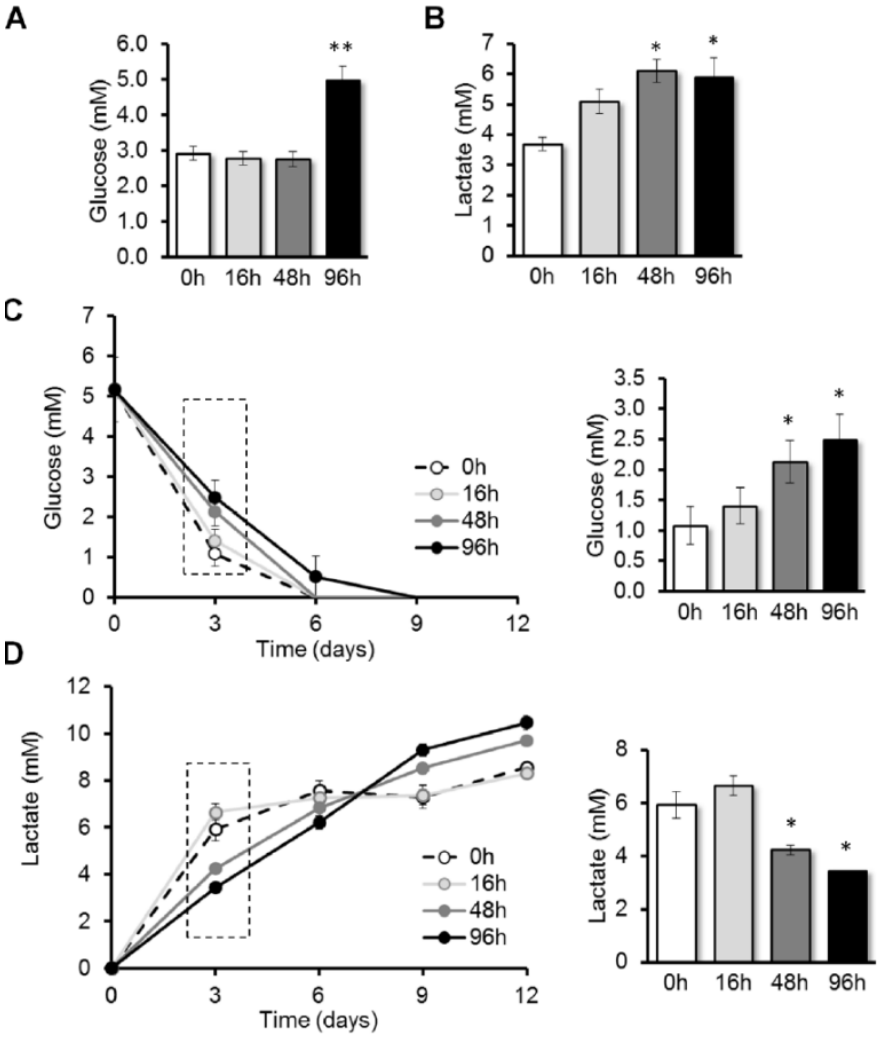

Figure 8. Hypoxic pre-conditioning reduces glucose consumption and lactate secretion. MSC were cultured for 96 hours with a single medium change after 48 hours in 1% O_2 for the indicated time periods. Then, supernatant media were collected to measure glucose (A; n = 5) and lactate (B; n = 4) as described in Methods. Then, MSC were cultured as shown in the scheme of Figure 5A. After HP, MSC were cultured under serum deprivation and 1% O_2 for up to 12 days with no further medium changes. Every 3 days, supernatants were collected to determine glucose (C; n = 3) and lactate (D; n = 3). Histograms to the right of C and D highlight glucose and lactate levels at day 3 of oxygen- and serum-deprivation. Statistical analysis was performed by paired Student's t-test with Bonferroni correction; *: $p< 0.05$ **: $p<0.005$.

Lactate levels were increasing until day 9, and at that time point most of the MSC have died (Figure 8D). We also found that at day 3, lactate levels were significantly lower in 48 and 96 hours HP-MSC as compared to controls.

4.6 Lipid composition of MSC

We isolated MSC from 5 healthy human donors and cultured cells for 48 hours in either normoxia (20% O_2) or hypoxia (1% O_2), and processed cells for total lipid analysis by mass spectrometry. Using a HPLC- ESI- QTOF MS/MS lipid analysis method, a total of 1,965 different molecular ions was detected; 1,444 by positive ion mode and 521 by negative ion mode. Out of these, 1,841 molecular ions (93.7%) were detected in all 5 donors of MSC. Most of these molecular ions are not annotated and are only referred to by an identification number. However, 390 ions (21.2%) could be identified as putative lipid species using the most comprehensive lipid database, LipidBLAST ((Kind et al, 2013): Figure 9A).

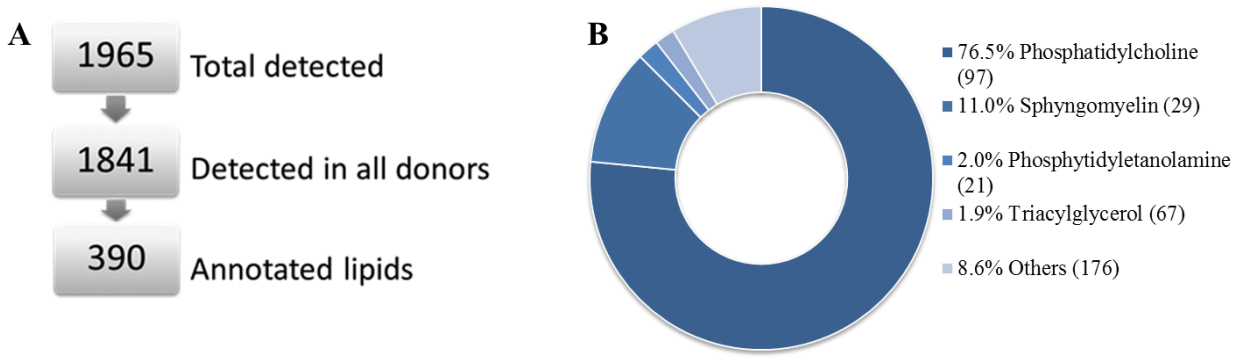

Figure 9. The lipid composition of human mesenchymal bone marrow cells were analyzed by ESI- QTOF MS/MS method. MSC isolated from the bone marrow of 5 healthy human donors were cultured for 48 hours in either 21% or 1% oxygen, and sent for lipidomics analysis. (A) 1965 molecular ions were detected, 1841 consistently in all 5 donors, 390 were identified as putative lipid species. (B) Pie chart of identified lipid species: phosphatidyl- choline, sphyngomyelin, phosphatidil-ethanolamine and triacyglycerol species accounted for over 90% of all identified lipids. (See abbreviations in main text).

From these, phosphatidylcholine species accounted for 76.5% of all identified lipids, followed by sphyngomyelin (11%), phosphatidylethanolamine (2%) and triglyceride (2%) species. Altogether, PC, SM, PE and TG accounted for over 90% of all annotated lipids detected in MSC (Figure 9B). The lipid detected most abundantly was PC (18:1/16:0) accounting for 13.1% of all annotated lipids measured.

4.7 Hypoxia induces changes in the lipid composition of MSC and significantly increases diacylglycerols levels

In order to identify changes in lipid composition of MSC induced by hypoxia, we performed two types of analysis. First, we grouped the detected species by lipid class and performed a Student's t-test to identify significant differences by class between normoxia and hypoxia. We found that TG, DG and fatty acids were significantly increased in MSC exposed to hypoxia by 4.8, 1.8 and 1.3-fold respectively, as compared to MSC in normoxia (Figures 10A and 10B). We then compared differences in the individual lipid species. From the 390 annotated lipids, only 2 (0.5%) were found to be significantly lower in hypoxia (namely cholesteryl ester 20:5 and 5.62_858.59 molecule). In contrast, 41 lipid species (10.5%) were significantly increased in hypoxic MSC as compared to controls. Remarkably, we found that each of the 7 detected DG species was significantly increased in MSC under hypoxia (Figure 11A). In summary, our lipidomic analysis revealed a strong up-regulation of many lipids in MSC exposed for 48 hours to hypoxia and especially noteworthy was the increase of all detected DG species.

To confirm this increase of DG levels in hypoxic MSC, we cultured cells in hypoxia or normoxia for 48 hours and collected cell pellets. After, we performed ELISA, measuring overall DG levels in the cell, and found concurrent results with the mass spectrometry analysis: hypoxia significantly increased overall DG levels in MSC (Figure 11B). To further support these results, we cultured MSC in the presence of Cobalt Chloride ($CoCl_2$), a hypoxia mimicking agent (Goldberg et al 1987; Schuster et al 1989). We found that exposure for 48 hours to $CoCl_2$ exerted a similar effect, inducing an increase of DG levels in MSC (Figure 11B).

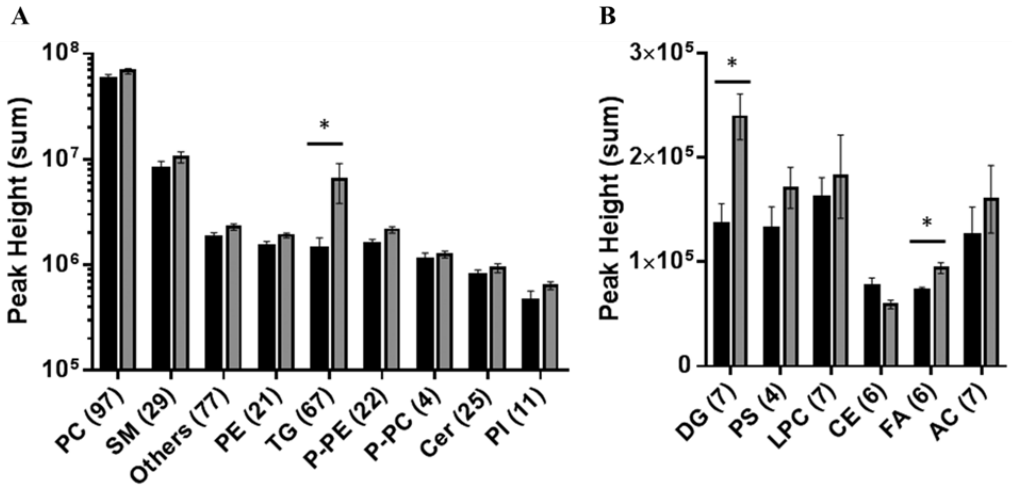

Figure 10. Hypoxia increases the amount of several lipids in MSC. MSC isolated from the bone marrow of 5 healthy human donors were cultured for 48 hours in either 21% or 1% oxygen, and sent for lipidomics analysis. The amount of triglycerides, diacylglycerols and fatty acids was significantly increased in hypoxia. (A) shows the average amount of the most abundant lipids in normoxia (black bars) and hypoxia (grey bars) in logarithmic scale and (B) shows lipids that are present in a lesser amount. Statistical analysis was performed using Student's t-test to compare lipids in normoxia vs hypoxia. * $p<0.05$

To evaluate the possible effects of increased DG levels in MSC in hypoxia, we used D609, an inhibitor of PC-PLC and SMS. We found that addition of D609 (50 μM) reduced DG levels in MSC cultured under hypoxia. Interestingly, D609 did not reduce DG levels in MSC under normoxia, although the differences between DG levels in normoxic and hypoxic MSC treated with D609 were not statistically significant (Figure 11 B). Altogether, D609 treatment decreased overall DG levels in the cells.

4.8 Reduction of diacylglycerol levels with D609 affects secretion of angiogenic proteins in MSC

After incubation of MSC for 48 hours in normoxia or hypoxia, with or without D609 (50 μM), supernatant conditioned media were collected and the amount of secreted VEGF, CXCL8/IL-8 and Ang-2 were measured by ELISA.

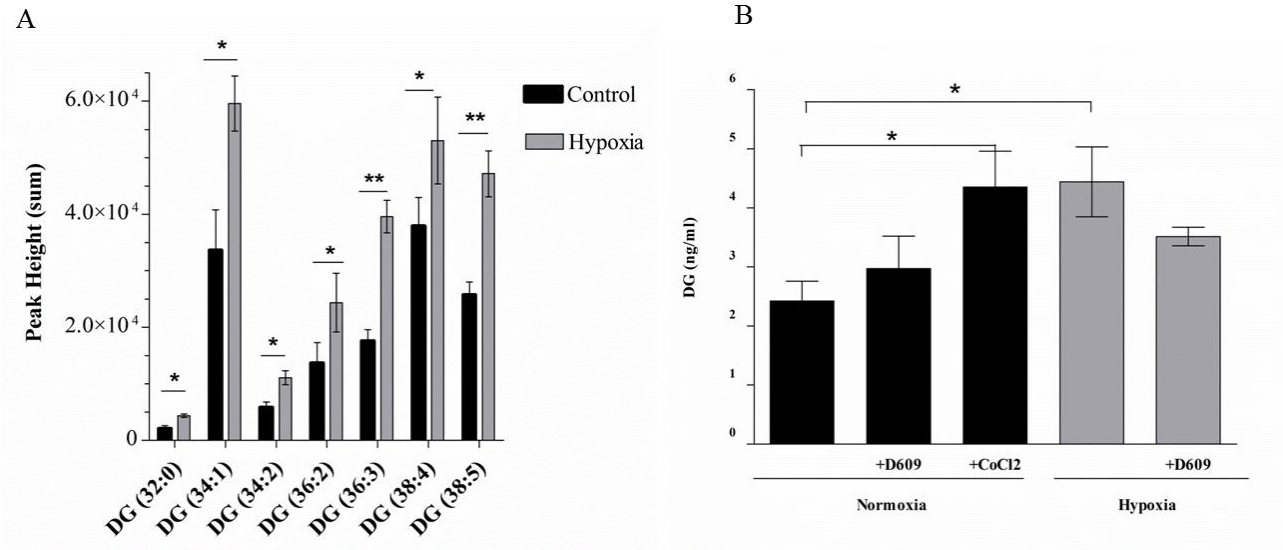

Figure 11. Hypoxia significantly increases the amount of DG species in MSC. MSC isolated from the bone marrow of 5 healthy human donors were cultured for 48 hours in either 21% or 1% oxygen, and sent for lipidomics analysis. (A) All DG species detected by mass spectometry analysis were significantly increased in MSC under hypoxia (grey bars) compared to normoxia (black bars). (B) MSC were cultured for 48 hours in either normoxia, hypoxia or with $CoCl_2$ (100 μM), in the presence or absence of D609 (50 uM). The level of DG was measured by ELISA method. Treatment of the cells decreased the amount of DG in MSC under hypoxia, but not in normoxia. Statistical analysis was performed using Student's t-test comparing individual lipid species (average of 5 donors (A)) and experimental groups (avg. of 3 independent experiments (B)). *$p<0.05$ **$p<0.005$

We observed a significant increase in VEGF secretion in MSC exposed to hypoxia, while addition of D609 (i.e. the reduction of DG levels) caused a decrease in VEGF levels in MSC in both normoxia and hypoxia (Figure 12 A). In contrast, secreted IL-8 levels were reduced by hypoxia, while addition of the enzyme inhibitor D609 increased IL-8 secretion in MSC in both normoxia and hypoxia (Figure 12B). Secreted levels of Ang-2 by MSC were overall very low. However, we detected a slight decrease in Ang-2 secretion in MSC exposed to hypoxia, as compared to controls, while addition of D609 reduced Ang-2 secretion even further in MSC in both normoxia and hypoxia (Figure 12C).

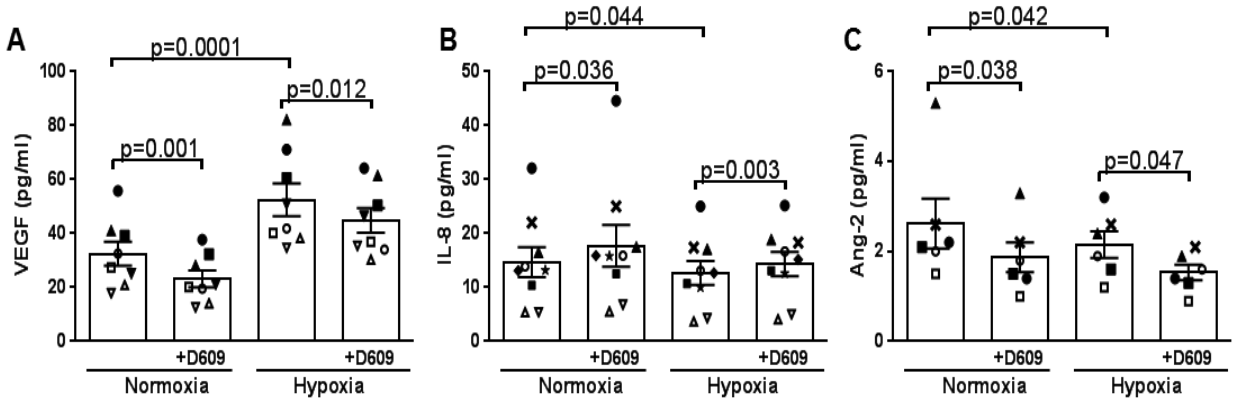

Figure 12. D609 and hypoxia affect the secretion of angiogenic proteins in MSC. After culture of MSC for 48 hours in either normoxia or hypoxia and in the presence or absence of 50 μM D609, cells were counted, supernatants collected and levels of angiogenic factors measured by ELISA. Values represent the amount of angiogenic factor (VEGF, IL-8 or Ang-2) secreted by 1,000 cells over 48 hours. Individual symbols represent single experiments performed with MSC derived from a different donor while bars represent the overall averages. Statistical differences were determined by paired Student's t-tests comparing the indicated conditions, with p values over respective brackets.

Overall, these trends are very similar at mRNA levels, measured after incubation for 12 hours in hypoxia (Figure 13). However, only mRNA levels of VEGF and Ang-2 showed a similar trend to the respective secreted protein levels (Figure 13). Our results show that VEGF and Ang-2 are both regulated transcriptionally by hypoxia, however all other differences in between conditions were not significant. These results suggest that some of the changes in VEGF, IL-8 and Ang-2 levels might be attributed to action on a transcriptional level, but most likely post-transcriptional mechanisms (of either protein synthesis or protein secretion) are also involved. Altogether, our results suggest that DG are involved in the secretion of VEGF, IL-8 and Ang-2 in MSC in both normoxic and hypoxic conditions.

4.9 Reduction of DG in MSC limits their potential to induce migration of endothelial cells

To further investigate whether the increase of DG in MSC under hypoxia could exert a functional effect, we prepared conditioned media from MSC cultured under either normoxia or hypoxia and in the presence or absence of D609.

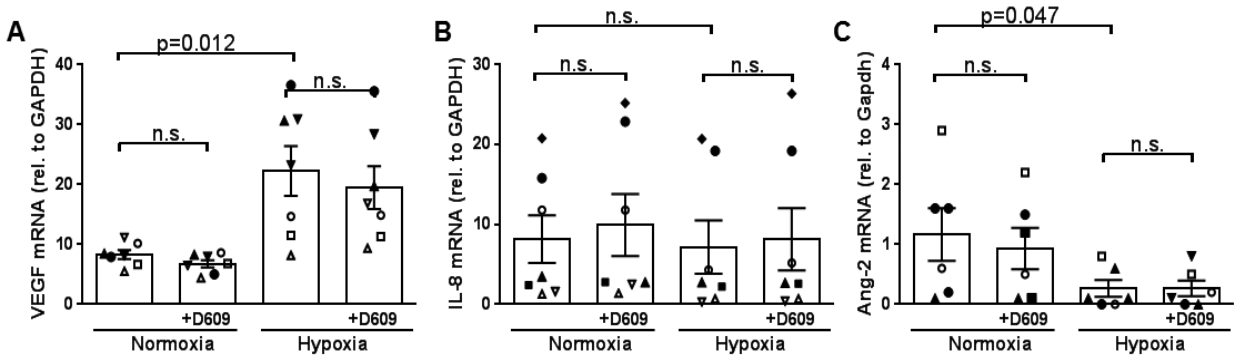

Figure 13. Hypoxia, but not D609 regulates the transcription of VEGF and Ang-2, and IL-8 is unaffected. After culture of MSC for 12 hours in either normoxia or hypoxia and in the presence or absence of 50 μM D609, cells were collected for gene expression analysis. Values represent the amount of mRNA of VEGF, IL-8 and Ang-2 respectively relative to GAPDH. Individual symbols represent single experiments performed with MSC derived from a different donor while bars represent the overall averages. Statistical differences were determined by paired Student's t-tests comparing the indicated conditions, with p values over respective brackets.

We then tested whether these conditioned media affected the migration of human endothelial cells in vitro, using a wound healing assay. We found that open image area was 50,36 ± 7,03% for normoxic (20% O_2) conditioned media, and 57,58 ± 6,2% for normoxic + D609 conditioned media. Whereas open image are was 51,3 ± 3,7% for hypoxic (1% O_2) conditioned media and 70,4 ± 3,1% for hypoxic + D609 conditioned media. Notice that greater open image area represents lower level of migration of the endothelial cells.

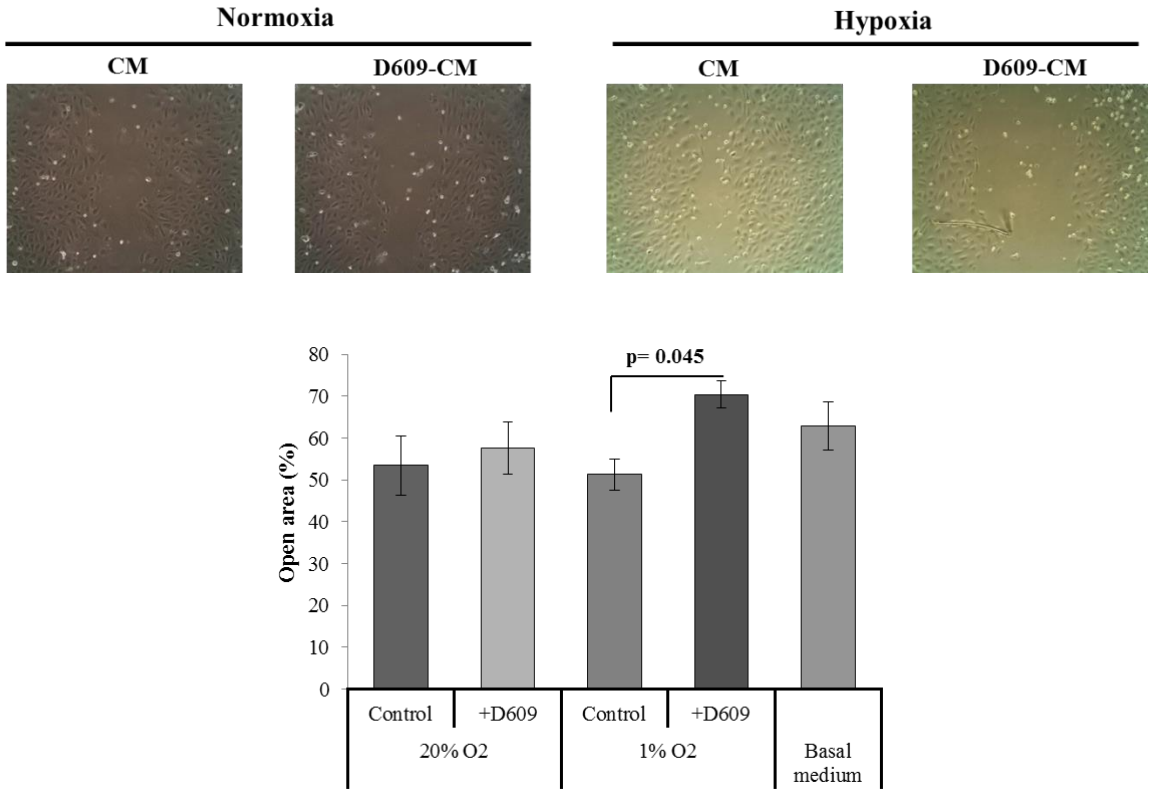

Figure 14. Inhibition of DG synthesis by D609 reduces the endothel migration-inducing potential of MSC. MSC were cultured for 48 hours in either normoxia or hypoxia and in the presence or absence of 50 μM D609, and supernatant media were collected, and the effect of preconditioned media form MSC was tested on the migration of endothelial cells (VeraVecs) in a wound/scratch assay. Pictures were taken immediately and 10 hours after the addition of preconditioned media to the endothelial cells. Upper pictures show representative images of the migration of endothelial cells in a wound/scratch assay, 10 hours after exposure to supernatant of MSC. The bars in the lower panel represent the averages of three independent experiments. Notice that a greater open area represents a lower level of migration of the endothelial cells. Statistical differences were determined by a paired Students t-test.

5 Discussion

5.1 The effect of hypoxia and hypoxic preconditioning on MSC

For our experiments, human mesenchymal stem cells were isolated from bone marrow aspirates of healthy volunteers. Though loosely determined and criticized by many, the ISCT minimal criteria for mesenchymal stem cells has not been replaced yet with a more stringent definition, accordingly, the cells we used in all experiments fulfilled the criteria.

The effect of hypoxia on MSC has been widely studied, usually comparing a single level of hypoxia to normoxia (21% O_2), leading to varying results, mainly due to differences in the level of hypoxia, species or tissue source of MSC, seeding density, and duration of culture (Ma et al., 2009, Tsai et al., 2012). It is important to note that human bone-marrow isolated mesenchymal stem cells reside in an environment *in vivo*, where oxygen concentration is around 1-7% (or 4-20 mmHg, (Brahimi-Horn and Pouysségur, 2007, Chow et al., 2001). When isolated and cultured under standard culture conditions, the cells are exposed to higher oxygen levels than in their native surroundings, therefore do not replicate precisely the *in vivo* conditions (Csete et al., 2005). It has been shown in hematopoietic stem cells (which reside in the bone marrow in a niche created by MSC), that normoxia (up to 18% of air) favors the proliferation of more committed colony forming cells, and hypoxia (1% O_2) concentrates progenitor cells on the highest level of hematopoietic hierarchy (Cipolleschi et al., 1993). Several studies have demonstrated that MSC survive under hypoxic or even anoxic conditions in culture: rates of cell death and hypoxia- induced gene transcription was unaffected during culture in 3% O_2 (Fehrer et al., 2007), and cell viability did not change when MSC were cultured in 0.02% O_2 for 24 hours (Salim et al., 2004). Conversely, serum depletion seems to have a more detrimental effect on MSC than low oxygen concentrations (Mylotte et al., 2008). For example, in a series of experiments in hypoxia with or without glucose and serum deprivation, MSC, when placed in 0.5% O_2 showed no signs of visible cell death, or caspase-3 activity and stained negative for Annexin V up to 72 hours. Whereas, when cells were cultured under 0.5% O_2 in serum- and glucose- free medium, around 30% of the cells were apoptotic after 72 hours in culture.

Indeed, in our experiments, cell numbers do not decrease until day three of culture in 1% O_2 in serum-free media with glucose, neither in the preconditioned, nor in the control group (Figure 5).

The effect of hypoxia on the proliferation and expansion capacity of MSC in short term culture is dependent on the species and tissue source, seeding density and oxygen tensions, but in long term culture, hypoxia seems to be more consistently beneficial for the cells (Tsai et al., 2011 and 2012). Constitutive expression of HIF-1 did not have a profound effect on the mass population of MSC, but increased the number and proliferative capacity of colony forming mesenchymal progenitors (Park et al., 2013). Differentiation of MSC into the osteoblast and adipocyte lineage was shown to be suppressed by hypoxia, but seemed to enhance chondrogenic differentiation, as indicated by increased Sox 5, 6, 9 and type-II collagen gene- and protein expression (Kanichai et al., 2008, Khan et al., 2007). Hypoxia has also been shown to enhance osteo- and chondrogenesis *in vitro* and *in vivo* (Lennon et al., 2001) in rat MSC. Controversy regarding the effect of hypoxia on MSC differentiation can also arise from studies like the one where hypoxia increased lipid droplet deposition in human MSC, thereby mimicking adipocyte phenotype, without upregulation of adipocytic gene expression and true adipogenic conversion (Fink et al., 2004). The increase in the amount of lipid droplets during hypoxia in MSC concurs with our results, where 48 hours of 1% O_2 caused a marked increase in the amount of triglycerides, the main constituents of lipid droplets, stained by Oil Red O. Hypoxic long term culture or hypoxic preconditioning seems to provide a method for maintaining expansion capacity and self-renewal and differentiation capacity for MSC (Tsai et al., 2011, Tsai et al., 2012).

In our experiments, we compared increasing levels of hypoxia to normoxia, culturing cells under 20, 10, 5 and 1% O_2 as established by replacement with nitrogen injections. We found that hypoxia inhibited both the proliferation and differentiation of human MSC proportionally to the degree of hypoxia. The increase in cell number over time was diminished by hypoxia, and the number of cells in G2/M and S phase was significantly lower in 1% O_2 compared to normoxia. Importantly, in terms of osteogenesis, our results

support the notion that MSC- mediated bone repair is strongly dependent on oxygen supply (Ma et al., 2009, D'Ippolito et al., 2006).

Since hypoxic signaling is considered transient (Park et al., 2013), it is critical to address survival of MSC after hypoxic preconditioning followed by additional exposure to hypoxia. Chacko et al showed that hypoxic preconditioning (culture in 0.5% O_2 for 24 hours) significantly reduced the number of apoptotic cells *in vitro* upon new exposure to a near-anoxic environment (0.1% O_2) for 72 hours compared to non-preconditioned cells (Chacko et al., 2010). In a study using a mouse hind limb ischemia model, MSC survival after injection into the ischemic limb was greatly enhanced by the hypoxic preconditioning of the cells, and HP- MSC enhanced perfusion, necrosis salvation and capillary density to a greater extent than non-preconditioned cells, while the small interfering (siRNA) mediated silencing of the glycogen synthase enzyme greatly inhibited the perfusion and necrosis salvation effects of hypoxic preconditioning (Zhu et al., 2014). *In vitro* studies showed, that HP lead to glycogen accumulation in a PI3K/Akt- dependent manner, directly and indirectly through HIF-1 and glycogen synthase kinase 3. As proposed by the authors, accumulated glycogen served as source for glucose and consequently energy production through glycolysis.

In our *in vitro* cell survival assay, we used Trypan- blue exclusion dye for dead cells and counted the cells with a haemocytometer, thereby directly quantifying the living cells over time, as a percentage of the original cell number. Since EthD-III undergoes an enhancement of fluorescence upon binding to nucleic acids and only enters cells with damaged membranes, while giving virtually no background signal; its incorporation directly measured the number of dead cells at day 9 of hypoxia and serum deprivation. We also assessed the number of apoptotic cells and found that the percentage of Annexin V positive cells was substantially lower than the number of dead cells. We speculate that this is partly because apoptosis can happen relatively quickly in a system, in fact the time from initiation to completion can be as short as 2-3 hours, therefore the frequency of apoptotic cells can be low at a given time point or can be limited to specific areas in the cell culture

(Elmore, 2007). Also, Annexin V only represents an early event in the apoptotic cascade, while, under starvation, apoptosis is gradually triggered in the cells.

Next, we investigated, whether hypoxic preconditioning increases cell retention *in vivo*. We found that hypoxic preconditioning significantly increased the retention of MSC *in vivo* up until day 28 following injection compared to non-preconditioned cells, which were virtually undetectable by day 28. Of note, the method used for following cell retention detects living cells only (cells with luciferase activity), implying that hypoxic preconditioning supports cell survival *in vivo*. This result is in line with previous studies where hypoxic preconditioning (0.5% O_2 for 24 hours) increased the survival of green fluorescent protein (GFP)- labeled BM-MSC injected into the infarcted hearts of wild-type mice, and reduced infarct size significantly (Hu et al., 2008). Huang et al showed, that hypoxic preconditioning of MSC attenuated the response of host natural killer cells against transplanted cells, thereby increasing retention and survival of allogenic MSC in a mouse model of hind limb ischemia (Huang et al., 2014). In our system, the immune component was minimized by the use of NSG mice, one of the most immune deficient strains available. It was also shown, that loading 3D constructs with glucose increased the survival of MSC 4-5-fold at day 14 after transplantation compared to cells in non-glucose loaded scaffolds, which suggests that glucose is crucial for the survival of transplanted cells (Deschepper et al., 2013). Another approach was used by Jin et al., where retention of 1^{st} passage MSC were significantly higher compared to 5^{th} passage MSC, when injected intramyocardially after induction of MI in mice, and proliferating cell nuclear antigen (PCNA) positivity in myocardium sections was highest in 1st passage MSC group compared to 5^{th} passage MSC and no cell treatment groups (Jin et al., 2011).

Different mechanisms have been proposed in explanation for the hypoxic-preconditioning- mediated increase in cell retention *in vivo*. Hypoxic preconditioning (3% O_2 for 24 h) improved the migration potential of MSC *in vitro* and increased the uptake of radio-labeled MSC into ischemic kidneys in an ischemia/reperfusion acute kidney injury model through the SDF-1- CXCR-4 axis (Liu et al., 2012). Pre-incubation of cells in hypoxia increased the expression of pro-survival protein Akt (Rosová et al., 2008).

Increased glycogen storage was observed during hypoxic preconditioning in MSC, and glycogen content remained significantly higher during prolonged hypoxia, and promoted survival in a mouse hind limb ischemia model (Zhu et al., 2014). Our results support the notion that cell survival is highly dependent on glucose availability. MSC can survive long term hypoxia when glucose is available (Deschepper et al., 2011), but when cultured in glucose- free medium with glycolysis inhibition, MSC show strong caspase activation and apoptosis induction (Mylotte et al., 2008). We measured glucose content in supernatant media of MSC incubated in identical conditions as our survival assays and found a strong correlation between the depletion of glucose and cell death in time. In hypoxia, MSC switch their metabolic pathway from oxygen- dependent tricarboxylic acid cycle and oxidative phosphorylation to anaerobic glycolysis (Mylotte et al., 2008, Folmes et al., 2012, Dos Santos et al., 2010, Papandreou et al., 2006). Notably, many genes related to glucose metabolism are regulated by HIF-1 during hypoxia, for example, expression of cell membrane glucose transporters (Glut- 1) and lactate dehydrogenase A (LDH-A) increase (Brahimi-Horn and Pouysségur 2007). Our results suggest, that a short period of incubation in hypoxia does not have a profound effect on glucose consumption (up to 48 hours), but induces lactate secretion from MSC (Figure 8). However, long exposure to hypoxia induces MSC to adapt, by decreasing their glucose consumption, and consequently, they secrete less lactate into supernatant media (Figure 8). In our experiments, cell starvation was induced under hypoxic conditions such that, over time, hypoxic preconditioning for 48 or 96 hours promoted the reduction of glucose consumption, thereby allowing for the availability of more glucose over a longer period of time in prolonged hypoxia.

5.2 Study of the lipid composition of MSC in normoxia and hypoxia

Using a mass spectrometry lipid analysis, nearly two thousand molecular ions were detected in MSC isolated from 5 different human donors. From these, 390 molecular ions could be identified as annotated lipid species using the most comprehensive lipid library, LipidBLAST (Kind et al 2013). The overall lipid composition of mesenchymal stem cells has been rarely studied. A study compared phospholipids by mass spectrometry and fatty acids by gas chromatography of MSC derived from five young and five old donors,

focusing on glycerophospholipids, showing that the ratio of PI to PS was increased towards late- passage samples. Late passage MSC also had diminished capacity to suppress T cell proliferation *in vitro*, showing that donor age and cell passaging had an effect on the lipid composition of MSC, which resulted in impaired capacity of the cells to suppress T cell proliferation (Kilpinen et al 2013). Lee et al measured metabolic changes (including several lipids) in clonally isolated human MSC during cellular senescence using UHPLC-QTOF MS/MS and found that lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) levels are increased in senescent MSC (passage 20) compared to non-senescent MSC (passage 7 or 15. Lee et al., 2014). Bergante et al studied changes in the glycosphingolipid content of human MSC undergoing osteogenesis (Bergante et al., 2014).

We observed differences in lipid composition between the 5 different donors tested: 6.3% of molecular ions were identified in some, but not all 5 donors. Given the complexity of lipids, it is possible that some ions/lipids were detectable in some samples, but not others (Hannun and Obeid, 2008). In all cases, these inconsistently detected lipids were only found at very low levels. Our lipidomic analysis revealed a strong up-regulation of many lipids in MSC exposed for 48 hours to hypoxia, similarly to other cell types (Briggs and Glenn, 1976, Gordon et al., 1977). We chose this time point based on our previous experiments, which showed that hypoxic preconditioning of MSC for at least 48 hours induces significant changes and promotes cell survival *in vitro* and *in vivo* (Figure 6 and 7.). Notably, triacylglycerols and fatty acids were significantly increased under hypoxia, a phenomenon that could be explained by the inability of hypoxic cells to support the oxygen-dependent fatty acid oxidation (Bhatnagar et al., 2003). Indeed, during hypoxia, MSC switch to anaerobic glycolysis instead, which results in lactate production and secretion as demonstrated in our experiments (Figure 8). In addition, here we show, that hypoxia consistently induced an increase of all DG detected. DG production in the cell can occur through several different mechanisms (Wattenberg et al., 2006). DG is formed during both the biosynthesis and catabolism of TG, or through phosphatide phosphatase (PPH) enzyme activity: this uses phosphatidic acid to form DG, as a key step in the formation of PC, PE and phosphatidylserines (PS). DG is also produced by the enzymatic cleavage of PI

or PC in the cell membrane, by either PI- or PC- specific phospholipase C, which enzymes are usually activated by receptors in response to agonists at the cell surface (Berstein et al., 1992, Rebecchi and Pentyala, 2000). The enzyme sphingomyelin synthase (SMS) uses ceramide and the phosphocholine head group of PC to produce DG and SM (Figure 15).

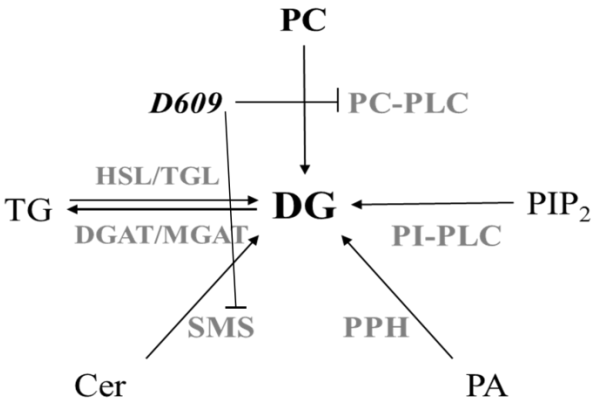

Figure 15. Diacylglycerol production in the cell can occur during the biosynthesis and catabolism of TG, or through phosphatide phosphatase enzyme activity. DG is also produced by the enzymatic cleavage of PI or PC in the cell membrane. SMS uses ceramide and the phosphocholine head group of PC to produce DG and SM. DGAT: diacylglycerol acyltransferase, HSL: hormone sensitive lipase, MGAT: monoacylglycerol acyltransferase, TGL: triacylglycerol lipase. See additional abbreviations in main text.

The increase in DG content during hypoxia has been reported previously, in HeLa and 293-T cells (Temes et al., 2004). It was also observed and in rat smooth muscle cells from intrapulmonary arteries (Weissmann et al., 2006) and in endothelial cells from porcine pulmonary artery: in response to hypoxia, triacylglyerol, diacylglycerol, lysophospholipid and fatty acid ester levels were elevated (Bhat and Block, 1992). In a series of experiments with rabbit hearts, sustained coronary artery occlusion caused an increase in DG content in the control group 2 minutes after the onset of ischaemia, but, brief preconditioning ischemia (5 minutes of coronary occlusion preceding the sustained occlusion), prevented the diacylglycerol response to a second ischemic episode. An enhanced participation of PC to the molecular species composition of diacylglycerol in preconditioned rabbit heart was

also observed (Gysembergh et al., 2000). Altogether, in line with previous publications, our findings suggest that in addition to the accumulation of TG and FA, also DG are commonly increased in cells or tissues exposed to hypoxia. In our study, we did not investigate the specific mechanism(s) responsible for the accumulation of DG under hypoxia in MSC. Several mechanisms are possible however: the excess TG under hypoxia could lead to enhanced formation of DG (Randle et al., 1963, Yu et al., 2002, Szendroedi et al., 2014). Also, DG levels could be increased as a result of the hypoxia-induced PC-PLC activity. Experimental work by Bhat et al. supports this notion, where activity of phospholipase A_2 (PLA_2) and PLC in porcine pulmonary endothelial cells was found to be increased by hypoxia (Bhat and Block, 1992). PC-PLC may be activated by reactive oxygen species (Liu et al., 2007), shown to be excessively generated under hypoxia (Chandel et al., 2000, Duranteau et al., 1998). The increase in DG content upon exposure to hypoxia in the HeLa and 293-T cell lines was mainly dependent on PC-PLC/SMS activity, since the addition of D609 abrogated this effect (Temes et al., 2004). It has been demonstrated, that the transient and the sustained phase of hypoxic pulmonary vasoconstriction appears to be dependent on PC-PLC enzyme activity to a considerable degree (Strielkov et al., 2013). The addition of D609 to isolated rat pulmonary arteries diminished the acute phase and abolished the sustained phase of vasoconstriction induced by hypoxia, while imipramine, a sphingomyelinase inhibitor had no significant effect, indicating, that the effect of D609 in hypoxia relies mainly on PC-PLC inhibition in this setting. The intravenous administration of D609 in rats 30 minutes before the onset of hypoxia completely prevented the development of pulmonary hypertension. As these results by independent groups suggest, PC-PLC activity takes part in hypoxic pulmonary vasoconstriction, by providing DGs, thereby playing a key role in hypoxia-induced cellular processes. Another possible mechanism for DG accumulation in hypoxia is the decreased activity of the enzyme diacylglycerol kinase (DGK), which converts DG into phosphatidic acid, thus, terminating DG signaling. It has been observed in isolated rat hepatocytes, that ischemic preconditioning (10 minutes 0% O2 before prolonged hypoxia) significantly reduced DGK activity and induced hepatocyte tolerance to prolonged hypoxia, and that this

downregulation of DGK activity was essential for the accumulation of DGs (Baldanzi et al., 2010).

In order to determine possible consequences of having increased DG levels in MSC under hypoxia we used the enzyme inhibitor D609. D609 has been shown to decrease DG levels, by blocking two enzymes: PC-PLC and SMS (Antony et al., 2001, (Bettaïeb et al., 1999, Hillemeier et al., 1996, Walter et al., 1996, Zhang et al., 2001). We found that the addition of 50 μM D609 to the culture medium resulted in reduced DG levels in MSC under hypoxia for 48 hours, suggesting that the increase of DG in hypoxia may relate to the activity of enzymes PC-PLC or SMS. Interestingly, D609 did not affect the amount of DGs in MSC in normoxia, although, DG levels did not differ significantly in normoxic and hypoxic MSC treated with D609. MSC promote angiogenesis *in vivo* through secretion of angiogenic signals such as VEGF (Kinnaird et al., 2004; Williams and Hare., 2011). Indeed, MSC usually reside around blood vessels as pericytes (Crisan et al., 2008) promoting blood vessel maturation (Pedersen et al., 2014), so next, we sought to determine that whether the changes in DG levels have a signaling role in the altered expression of angiogenic factors in MSC under hypoxia. Overall VEGF, IL-8 and Ang-2 levels strongly varied among MSC from the individual donors however, our results were consistent among all 6-8 individual experiments. We found, that for both VEGF and IL-8, D609 induced the opposite effect than hypoxia, while secretion of Ang-2 was decreased by both hypoxia and D609. Of note, D609 affected VEGF, IL-8 and Ang-2 secretion in both normoxia and hypoxia, suggesting that DG play a general role in the secretion of these proteins. In addition, the effect of D609 was often milder in hypoxia (in the case of VEGF and Ang-2), possibly due to enhanced production of DG.

The downstream signaling from DG can happen through different pathways. The hypoxia inducible factor HIF-1 might play a role in this process, as a general transcription factor in hypoxia-induced changes in gene expression (Brahimi- Horn and Pouysségur, 2007). This notion could be supported by previous results, proposing a role for DG in the regulation of HIF-1α activity, since the inhibition of PC-PLC/SMS enzymes abrogated both DG increase and HIF-1 activation in hypoxia, and VEGF is a downstream target gene of

HIF-1 (Temes et al., 2004). Notably, this effect of DG on HIF-1 was independent of PKC activity. IL-8 and Ang-2 could be regulated by DG-activated PKC isoforms, similarly to other cell types. Indeed, PKC- mediated IL-8 release and its role in the interaction with acute myeloid leukemia blast cells was shown in human bone marrow MSC (Abdul-Aziz et al., 2015). Interestingly, in mouse mesangial cell lines, hypoxia upregulated Ang-2 mRNA and protein expression (as opposed to what we observed in MSC), while an inhibitor of PKC abrogated this effect, showing, that Ang-2 expression is probably regulated by PKC in hypoxia (Yuan et al., 2000). In HUVECs, lipopolysaccharide enhanced the activity of PC-PLC and the production of IL-8, and addition of D609 inhibited the PC-PLC mediated IL-8 secretion, which results provide another link between PC-PLC, IL-8 and D609 (Zhang et al, 2011).

Angiogenesis can be induced by local tissue ischemia, when, as a consequence of low oxygen levels, HIF-1 is stabilized and promotes the expression of many genes involved in neovascularization such as VEGF, FGF and IGF. In response to the increase in local levels of these cytokines, endothelial cells sprout, migrate and form new thin- walled vessels (Kinnaird et al., 2004). Endothelial cell migration is a critical step during the formation of new blood vessels (Ridley et al., 2003), and multiple factors secreted by MSC are possibly involved in this process, such as VEGF, which promotes endothelial cell migration, and induces endothelial cell proliferation (Al-Khaldi et al., 2003, Williams and Hare 2011). Scratch or wound healing assay is a well-developed method to assess cell migration *in vitro* (Liang et al., 2007). Despite its limitations (one of them is that a chemical gradient is not established in the system), it mimics to some extent the migration of cell sheets (such as endothelial cells) *in vivo*. In our experimental setting, we were examining the possibility, that the reduction in DG levels (i.e. incubation with D609) affects the EC-migration-inducing capacity of MSC. We used VeraVecs endothelial cells and conditioned media from MSC cultured under normoxia or hypoxia and in the presence or absence of D609, and basal media (without serum) as negative control. We used 10 hours as our final time point to minimize the effect of cell proliferation. Cell migration also occurred in the basal media only group, but to a lesser extent than in the conditioned media

from MSC. Since in our experiments we used conditioned media from MSC treated with D609, it is possible, that endothelial cells were directly exposed to D609 lingering in the medium samples. However, the concentration of D609 was rather low at this point, because the half-life of D609 is 1.5 days in culture medium, and we collected conditioned media after 48-hour incubation. Moreover, interestingly, D609 only affected the proangiogenic effect of the conditioned media from MSC cultured in hypoxia, reducing the migration of endothelial cells.

6 Conclusions

The main function of MSC in the bone marrow is to create and maintain the hematopoietic niche through the support of hematopoietic stem cell self-renewal and differentiation (Méndez-Ferrer et al., 2010). Through a rigorous series of experimentation over the years with transplantation of whole bone marrow (Tavassoli and Crosby, 1968), then CFU-F- derived cell populations and finally, a single colony-forming unit fibroblast to ectopic sites into host animals; it has become evident, that the bone marrow stromal cell is responsible for the recreation of the hematopoietic microenvironment. The skeletogenic potential and self-renewal capacity of the BM stromal cell has also become apparent (Sacchetti et al., 2007). Perivascular cells, that are very similar in phenotype and differentiation capacity to the bone marrow stromal cell, have been isolated from many fetal and adult organs and tissues with adipo- osteo- chondro- and myogenic potential (Crisan et al., 2008), which, along with other studies (da Silva Meirelles et al., 2006) eventually gave rise to the idea that in their native perivascular environment, mesenchymal stem cells are actually pericytes (Caplan, 2008). In fact, it seems to be, that MSC comprise a subset from a larger pool of perivascular stem cells. MSC isolated from different body segments share a set of common markers and also express unique or tissue-specific markers pointing to their origin (Ságі et al., 2012, Takashima et al., 2007, Sugiyama et al., 2007 Alliot et al., 1999). Based on prospective isolation studies (Crisan et al., 2008), in my opinion, besides the set of phenotypic markers defined by the ISCT (Dominici et al., 2006), CD 146 is an important, if not specific marker for MSC (Figure 2).

The function of MSC outside the bone marrow has been investigated after extensive *ex vivo* propagation, and among *in vitro* circumstances, which leaves the question of their true *in vivo* function open in my opinion. However, the perivascular (or actually subendothelial, in the bone marrow) position of MSC is solidly proven and a large sum of experimental and clinical data exists, showing that MSC are able to promote endothelial cell proliferation and survival, and to enhance angiogenesis *in vitro*, e.g. in various extracellular-matrix mimicking biomaterials; and *in vivo*, even in ischemic tissue environment such as the mouse hind limb ischemia model. There is promise for MSC to be

used as and 'off-the-shelf' product, either containing cells or only the secretome of MSC, since conditioned media from MSC could exert the same beneficial effect as the cells themselves. Some even refer to MSC as an 'injury drugstore', suggesting, that in the event of tissue injury, perivascular MSC are 'activated' and secrete a wide arrange of anti-apoptotic, anti-inflammatory and proangiogenic factors, creating a regenerative microenvironment *in vivo* (Caplan and Correa, 2011). It is also proving to be an efficient strategy to co-transplant MSC with other cell types, since tissue regeneration cannot be achieved without the aid of more than one cell type (Williams et al., 2013). In the future, MSC may become the main supporting cell type in cell therapy and tissue engineering, where neoangiogenesis and vasculogenesis is essential and could be aided by MSC.

Despite the paramount of data about paracrine factor secretion and beneficial effects on tissue repair in preclinical models, most clinical trials have failed to show robust recovery in cardiovascular disease patients, with the regenerative capacity of MSC somehow 'lost in translation'. Indeed, as discussed above, various factors are possibly responsible for this phenomenon, which highlights the utmost importance of conducting well-designed experiments focused on the nature, basic biology and differentiation potential of MSC. On the other hand, efforts must be aimed at improving the survival and engraftment rates of the injected cells. In my opinion, the combined application of stem cell therapy and various biomaterials could lead to a more successful path in the future.

It has been previously demonstrated that hypoxic preconditioning enhances the therapeutic potential of MSC in applications such as treatment of cardiac ischemia, critical limb ischemia, traumatic brain injury, and in liver regeneration. One of the key factors in the hypoxic preconditioning- induced enhancement of tissue repair seems to be the increased retention of MSC in tissues, but the underlying mechanisms remain elusive. We tested the effects of varying levels of hypoxia on proliferation and differentiation of human MSC, and observed that hypoxia caused a decrease in the proliferation of MSC, and inhibited their osteogenic and adipogenic differentiation proportionally to the degree of hypoxia. Our conclusion is that the proliferation and differentiation of MSC are inhibited by hypoxia in a dose-dependent manner, and that the lowest level of oxygen used, 1% O_2

(equivalent to 10 mmHg O2 in the culture media) exerted the strongest inhibitory effect on MSC, especially during osteogenic differentiation.

We also addressed the effect of HP on the survival of cells upon new exposure to hypoxia, and found 1% oxygen is an optimal level for preconditioning, and established an optimal duration of preconditioning: in our experiments, 16 hours of HP did not have a profound effect on survival, while 48 and 96 hours of HP transitorily promoted cell survival to a similar degree in an *in vitro* environment with limited oxygen and nutrients. Importantly, 48 hours of HP significantly increased cell retention after *in vivo* transplantation. Overall, our results strongly encourage, at both the clinical and research level, pre-incubation of MSC in hypoxia prior to transplantation to enhance their retention. We also investigated the underlying mechanism(s) that promote the survival of hypoxic preconditioned MSC and found that glucose levels are significantly higher in MSC cultured in hypoxia for 96 hours, suggesting that under this condition MSC use up less glucose as compared to normoxic controls. After 3 days in serum deprivation and hypoxia, glucose levels were over 2-fold higher in supernatant media of 48 and 96 hours hypoxic preconditioned MSC, as compared to controls (without hypoxic preconditioning). We propose that, under limited glucose availability, hypoxic preconditioned MSC survive for a longer period of time due to a lower rate of glucose consumption, thereby allowing more glucose availability over a longer period of time.

Further understanding MSC biology and response to hypoxia, especially in terms of the secretion of angiogenic factors could be remarkably useful for enhancing the efficacy of MSC-based therapy. It has long been established that lipids are crucial first and second messenger molecules (van Meer et al., 2008) and it has also been shown that lipids are involved in hypoxic signaling. Analyzing the lipid composition of MSC using HPLC-QTOF-MS/MS and the LipidBLAST library, we found a strong up-regulation of many lipids in MSC exposed for 48 hours to hypoxia and especially noteworthy was the increase of all detected DG species. To study the effects of increased DG levels in hypoxia, we used D609, to reduce overall DG levels, and evaluated the effect on the angiogenic factors secreted by MSC. Altogether, our results suggest that DGs are involved in the secretion of

VEGF, IL-8 and Ang-2 in MSC in both normoxic and hypoxic conditions. We also tested whether conditioned media from MSC cultured under either normoxia or hypoxia and in the presence or absence of D609 affect the migration of human endothelial cells in vitro, using a wound healing assay. Our results indicate that conditioned media from MSC cultured under hypoxia promote endothelial cell migration to a similar extent than MSC under normoxia. However, under hypoxia, addition of D609 reduced the migration of endothelial cells, suggesting that the increase of DG in MSC under hypoxia is an important mechanism to alter the angiogenic secretome of MSC. This suggests that the specific signals of MSC promoting migration of endothelial cells are distinct under normoxia and hypoxia, where under hypoxia signals are more dependent on DG levels. The results of our experiments measuring DG levels in response to hypoxia and D609 also strengthen this argument, since D609 treatment for 48 hours inhibited DG levels in MSC only under hypoxia. Although these are *in vitro* results, cautioning their interpretation, it is interesting to note, that blocking the increase in DG levels in hypoxia seems to have functional consequences in the migration-inducing capacity of MSC.

The changes in lipid composition presented here offer new insight into the changes that MSC undergo under hypoxia, which are important rather from the perspective of the bench, but could impact the therapeutic application at the bedside.

7 Summary

Stem cells with extensive proliferation and differentiation capacity are in the focus of experimental and clinical regenerative therapies. Mesenchymal stem cells (MSC) are self-renewing multipotent progenitors found in a perivascular position, mainly responsible for the support of hematopoiesis, and formation of new blood vessels. In search of factors that could improve the efficacy of MSC therapy, hypoxic preconditioning and the effect of hypoxia on MSC were of great interest to us. We found that hypoxia reduces the proliferation and differentiation of human bone marrow MSC in a dose-dependent manner. Hypoxic preconditioning in 1% O_2 for 48 hours enhanced the survival of cells in an *in vitro* environment poor in nutrients and oxygen, and significantly increased the retention of MSC after intramuscular injection into mice for 21 days. We also found that significantly more glucose was available in the media of MSC cultured in 1% O2 for 96 hours compared to controls, and that hypoxic preconditioning decreased lactate production and glucose consumption upon new exposure to hypoxia. Using a mass spectrometry lipid analysis, we found that many lipids, including all diacylglycerol (DG) species, have increased levels in hypoxia. For further investigation, we used the enzyme inhibitor D609, reducing DG levels. D609 induced the opposite effect than hypoxia regarding angiogenic protein secretion, for both vascular endothelial growth factor (VEGF) and interleukin- 8 (IL-8), while angiopoietin-2 (Ang-2) was decreased by both hypoxia and D609. D609 affected VEGF, IL-8 and Ang-2 secretion in both normoxia and hypoxia, suggesting that DG play a general role in the secretion of these proteins. We examined the possibility, that the changes in DG levels affects the endothelial cell migration-inducing capacity of MSC in a scratch assay, and found that pre-incubation of MSC with D609 decreased their capacity to enhance the migration of endothelial cells. In conclusion, our results strongly encourage the hypoxic preconditioning of MSC before cell implantation in both the clinical and experimental settings. The changes in lipid composition presented here offer new insight into the changes that MSC undergo under hypoxia, which are interesting rather from the perspective of basic science, but could have implications in translational studies.

8 Összefoglalás

Az őssejtek korlátlan proliferációs és differenciációs képességüknek köszönhetően a regeneratív terápiák fókuszába kerültek. A mesenchymalis őssejtek (MSC) önmegújításra képes multipotens progenitorok, melyek perivascularis pozícióban helyezkednek el. Fő funkciójuk a hematopoézis fenntartása és új vérerek kialakulásának támogatása. Kutatásainkban vizsgáltuk, hogy hogyan hat rájuk az oxigén- és tápanyagszegény környezet és hogyan fokozható túlélésük. A hypoxia dózisfüggően csökkentette a MSC proliferációját, illetve osteogen és adipogen differenciációjukat. A 48 órán át tartó hypoxiás prekondicionálás (HP, 1% O_2) növelte a sejtek túlélését oxigén- és tápanyagszegény (iszkémiás) környezetben *in vitro*, valamint fokozta a sejtek retencióját intramuscularis beadást követően 21 napon keresztül egerekben. Azt találtuk, hogy a 96 órán át 1% O_2-ben tenyésztett sejtek felülúszójában szignifikánsan magasabb volt a glükóz tartalom, mint a kontrollcsoportokban, valamint, hogy a HP hatására a sejtek csökkentették glükózfelhasználásukat és laktáttermelésüket új iszkémia-expozíció esetén. Az MSC-k lipidösszetételéz vizsgálva tömegspektrometriával megfigyeltük, hogy számos lipid, köztük a digliceridek (DG) szintje megemelkedik hypoxiában. A D609 enzimgátlóval csökkentettük a DG szinteket, és azt tapasztaltuk, hogy a D609 a hypoxiával ellentétes hatást váltott ki a vaszkuláris endoteliális növekedési faktor (VEGF) és az interleukin- 8 tekintetében, míg az angiopoietin-2 szintjét mind a hypoxia, mind a D609 csökkentette. Mivel a D609 mind normoxiában, mind hypoxiában módosította ezen fehérjék szintjét, feltételezzük, hogy a DG-k általános szerepet játszanak szekréciójukban. *In vitro* „sebgyógyulási modellben" végzett vizsgálattal kimutattuk, hogy a D609 kezelés (és a DG szint csökkenés) mérsékelte az MSC felülúszó endotélsejt migrációt fokozó hatását. Eredményeink alapján javasoljuk a MSC-k hypoxiás prekondicionálását a kísérleti és a klinikai sejtterápiák során. Az általunk leírt lipidösszetétel és a lipidszint változások új oldalról közelítik meg a hypoxia MSC-kre kifejtett hatását, és bár elsősorban az alapkutatás szempontjából érdekesek, az angiogenikus fehérjék szekrécióját moduláló hatásuk miatt később klinikai vizsgálatokban is lehet jelentőségük.

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10 List of Publications

10.1 Publications related to the dissertation:

1. Beegle J, Lakatos K, Kalomoiris S, Stewart H, Isseroff RR, Nolta JA, Fierro FA. (2015) 'Hypoxic preconditioning of mesenchymal stromal cells induces metabolic changes, enhances survival, and promotes cell retention in vivo'. *Stem Cells,* 33(6) pp. 1818-28.

2. Lakatos K, Kalomoiris S, Merkely B, Nolta JA, Fierro FA. (2016) 'Mesenchymal Stem Cells Respond to Hypoxia by Increasing Diacylglycerols', *J Cell Biochem,* 117(2), pp. 300-307.

10.2 Publications not related to the dissertation:

3. Kalomoiris S, Cicchetto AC, Lakatos K, Nolta JA, Fierro FA. (2016) Fibroblast Growth Factor 2 Regulates High Mobility Group A2 Expression in Human Bone Marrow-Derived Mesenchymal Stem Cells. *J Cell Biochem*, 117(9), pp. 2128-37.

4. Nardai S, Dobolyi A, Skopál J, Lakatos K, Merkely B, Nagy Z. (2016) 'Delayed Gelatinase Inhibition Induces Reticulon 4 Receptor Expression in the Peri-Infarct Cortex', *J Neuropathol Exp Neurol.* 75(4), pp. 379-85.

5. Nardai S, Dobolyi A, Pál G, Skopál J, Pintér N, Lakatos K, Merkely B, Nagy Z. (2015) 'Selegiline promotes NOTCH-JAGGED signaling in astrocytes of the peri-infarct region and improves the functional integrity of the neurovascular unit in a rat model of focal ischemia.', *Restor Neurol Neurosci,* 33(1), pp 1-14.

6. Lakatos K, Dékány G, Lendvai Zs, Berta B, Molnár L, Becker D, Nagy Z, Merkely B, Skopál J. (2012) 'Adrenaline induced platelet aggregation in patients with coronary artery disease undergoing stent implantation.' *Cardiologia Hungarica* 42, pp. 106–111.

11 Acknowledgements

First and foremost, I wish to thank Prof. Béla Merkely DSc. for granting me with the opportunity to be a PhD student at the Semmelweis University Heart and Vascular Center, and for his continuous support and the unparalleled possibilities available at the Heart Center during my research.

I wish to thank Dr. Judit Skopál PhD. for introducing me into the world research, and for teaching me the essentials of platelet physiology and cell culture. I want to thank her continuous support and invaluable help, and for being there for me always. I wish to thank Dr. Sándor Nardai PhD. for the encouragement and support. I wish to thank Éva Szigetfű her assistance, Zsuzsanna Lendvai her friendship, and Dr. Edit Gara PhD. her important insight.

I would like to express my deepest and heartfelt gratitude to Dr. Fernando Fierro PhD. at the UC Davis Institute of Regenerative Cures for his guidance and counseling in MSC research, and the results we have achieved during my unforgettable year as a research intern in the Stem Cell Program. I would also like to thank to Prof. Jan Nolta, for providing me with the opportunity to work in her laboratory. I want to thank Dr. Julie Beegle PhD. and Dr. Stefanos Kalomoiris PhD. for the work we have done together, and for being the most amazing and supportive team members and friends that I could have asked for.