Dissertation

PhD-Thesis

OXYGEN SENSING ADULT STEM AND PROGENITOR CELLS

(SAUERSTOFF SENSITIVE ADULTE STAMM- UND PROGENITOR ZELLEN)

submitted by

Nicole Anette Hofmann , MSc

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Division of Hematology and Stem Cell Transplantation,

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PD Dr. Dirk Strunk

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Problems are solutions in working cloths

Henry J. Kaiser

Declaration

I, Nicole A. Hofmann, hereby declare that this dissertation is my own original work and that all of the contributing authors to this dissertation have been acknowledged by word in the acknowledgments. All referenced work has been acknowledged in the text. I hereby declare that all work I have done followed the guidelines of “Good Scientific Practice”.

Graz,

Nicole A. Hofmann, MSc

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1. ZUSAMMENFASSUNG

Hypoxie ist einer der Hauptstimuli für die Bildung neuer Blutgefäße (Neo-Vaskulogenese). In einem hypoxischen Umfeld richten sich endotheliale kolonieformende Zellen (ECFCs) in tubulären Strukturen aus, welche sich mit dem bestehenden Blutgefäßsystem verbinden und so funktionelle perfundierte Gefäße bilden. Laut Lehrbuchmeinung hat die Bildung von neuen Gefäßen zur Folge, dass mesenchymale Stamm- und Progenitorzellen (MSPCs) angelockt werden, welche zur Gefäßstabilisierung beitragen. Stammzellen sind ein vielversprechendes Zelltherapeutikum zur Revaskularisierung von ischämischem Gewebe nach verschiedensten Erkrankungen wie Schlaganfall, Herzinfarkt und peripherer, arterieller Verschlusskrankheit (PAVK). Bis jetzt jedoch hat die klinische Applikation von endothelialen Progenitorzellen noch nicht die erwarteten klinischen Effekte erzielt. Ausgehend von vorangehenden Publikationen, die gezeigt haben, dass ECFCs *in vivo* MSPCs brauchen um stabile Gefäße zu bilden, entstand die Hypothese, dass MSPCs eine wichtige Rolle bei der vaskulogenen Reaktion auf hypoxische Stimuli spielen. In unserer Arbeit zeigen wir, dass hypoxische MSPCs *in vivo* in erster Linie die Neo-Vaskulogenese durch einen Mechanismus einleiten der vom hypoxieinduzierbaren Transkriptionsfaktor (HIF) abhängig ist und erst in zweiter Linie zur Gefäßstabilisierung beitragen.

Adulte humane ECFCs und MSPCs wurden aus peripherem Blut beziehungsweise aus dem Knochenmark isoliert und unter humanisierten Bedingungen vermehrt. Wir untersuchten Phänotyp, Langzeitproliferation, HIF-Stabilisierung, Wundheilung, Migration und vaskulogene Funktionen der Progenitorzellen unter reduziertem Sauerstoff (5% O_2), wie wir es in der Vene finden, unter hypoxischen Bedingungen (1% O_2), wie im ischämischen Gewebe, und unter Standardkulturbedingungen (20% O_2). Um ihre Interaktion *in vivo* zu testen, wurden ECFCs und MSPCs zusammen mit verschiedenen extrazellulären Matrices (Matrigel, Collagen/Fibronektin, humanes Thrombozyten-Gel) subkutan in immundefiziente NSG Mäuse (NOD.Cg-Prdc^{scid} Il2rg^{tm1Wjl}/SzJ) implantiert. Um die zellspezifische Rolle der Hypoxiesensorik während der Neo-vaskulogenese *in vivo* zu bestimmen, wurde in ECFCs und MSPCs HIF chemisch und genetisch inhibiert (YC-1 und mRNS). Um zu testen, ob HIF-1α -Zielproteine die

Anwesenheit von MSPCs ersetzen können, wurden ausgewählte Wachstumsfaktoren und Zytokine im Mausmodell erprobt.

Proliferation und –Funktion der Progenitorzellen waren mit sinkendem Sauerstoffgehalt reduziert. HIF-1α wurde von ECFCs nur unter 1% O_2 stabilisiert, während MSPCs HIF-1α schon unter 5% O_2 stabilisierten. ECFCs, die in eine hypoxische Umgebung in NSG Mäuse transplantiert wurden, stabilisierten kein HIF-1α *in vivo*, während MSPCs alleine oder in Kotransplantaten zusammen mit ECFCs schon nach einem Tag *in vivo* ein starkes nukleares HIF-1α-Signal zeigten. Ohne MSPCs gingen ECFCs alleine *in vivo* innerhalb von 24 Stunden in Apoptose. Durch Inhibierung des HIF-1α -Zielproteins VEGF (vaskulärer endothelialer Wachstumsfaktor) konnte die Neo-vaskulogenese blockiert werden. Interessanterweise konnte man durch Substitution von VEGF weder mit ECFCs alleine, noch mit ECFCs plus HIF-depletierten MSPC die Gefäßbildung initiieren. Das Hinzufügen einer komplexen Mixtur von Plättchenfaktoren *in vivo* konnte jedoch zum Teil die vaskulogene Funktion von MSPCs ersetzen.

MSPCs reagieren schneller und sensitiver auf den niedrigen Sauerstoffgehalt durch Stabilisierung von HIF-1α. In der initialen Phase der Neo-vaskulogenese schützen MSPCs die ECFCs vor hypoxieinduzierter Apoptose und leiten unter Anderem auf diese Weise die Gefäßneubildung ein. Überraschenderweise findet die Neo-vaskulogenese unabhängig von endothelialer HIF Stabilisierung statt. Diese Resultate sprechen dafür, dass die Kotransplantation von MSPCs und ECFCs ein vielversprechendes zelluläres Therapeutikum für vaskuläre Regeneration ist. Die Beobachtung, dass VEGF alleine die gefäßinduzierende Funktion von MSPCs *in vivo* nicht ersetzen konnte, unterstreicht die Komplexität der hypoxieinduzierten Signaltransduktion. Die Tatsache, dass die HIF-1α Stabilisierung in MSPCs, jedoch nicht in ECFCs, eine entscheidende Rolle spielt, empfiehlt eine nähere Auseinandersetzung mit MSPCs als therapeutisches Ziel in der Regenerativmedizin und Anti-Tumortherapie.

2. ABSTRACT

Hypoxia is a major stimulus of new vessel formation (neo-vasculogenesis). In a hypoxic environment endothelial colony-forming progenitor cells (ECFCs) arrange into tubular structures which can connect to the pre-existing vasculature and form functional perfused vessels. The current view is that mesenchymal stem and progenitor cells (MSPCs) are recruited subsequently to stabilize vessels. Stem cell therapy to re-vascularize ischemic tissue has been a promising tool for various therapeutic targets including stroke, myocardial infarction and peripheral artery disease. So far, clinical applications of endothelial progenitors have largely failed to meet medical needs. Previous work showing that ECFCs require MSPCs *in vivo* to form patent vessels has provoked the hypothesis that MSPCs have a decisive role in the vasculogenic response to hypoxia. This work demonstrates that ECFCs in hypoxic conditions *in vivo* need the presence of functional MSPCs not only to stabilize but primarily to initiate neo-vasculogenesis by a hypoxia-inducible transcription factor (HIF)-dependent mechanism.

Adult human ECFCs were isolated from peripheral blood and MSPCs from bone marrow aspirates. Both cell types were isolated and expanded under humanized culture conditions. Progenitor cell phenotype, long-term proliferation, HIF stabilization, wound repair, migration and vasculogenic functions were monitored under severe hypoxia (1% O2), venous oxygen levels (5% O2) and standard ambient air culture conditions (20% O2). ECFC and MSPC crosstalk *in vivo* was studied in immune-deficient NSG mice (NOD.Cg-Prdc^{scid} Il2rg^{tm1Wjl}/SzJ) after subcutaneous implantation using various extracellular matrices (Matrigel, collagen/fibronectin, human platelet lysate gel). Chemical and genetic inhibition of HIF (YC-1, shRNA) was used to define the cell type-specific role of hypoxia sensing in MSPCs and ECFCs during vasculogenesis *in vivo*. To determine the capacity of downstream target proteins of HIF-1α to substitute for MSPC presence during vasculogenesis, selected growth factors and cytokines were tested.

Progenitor proliferation and function *in vitro* were reduced with declining oxygen levels. HIF-1α was stabilized by ECFCs only at 1% O2, while MSPCs stabilized HIF-1α already at 5% O2. In an NSG mouse model, ECFCs transplanted into a hypoxic environment did not stabilize HIF-1α, while transplanted MSPCs alone or MSPCs in co-transplants showed strong nuclear HIF-1α stabilization as early as 1

day after transplantation. In the absence of MSPCs, ECFCs injected alone largely underwent apoptosis within 24h *in vivo*. Chemical as well as genetic inhibition of HIF-1α stabilization in MSPCs but not in ECFCs significantly abrogated vessel formation *in vivo*. Blocking the HIF-1α down-stream target VEGF resulted in inhibition of neo-vasculogenesis. Interestingly, substitution of VEGF alone could not restore vessel formation *in vivo*; neither when injected together with ECFCs alone nor in a model where ECFCs were co-transplanted with HIF-depleted MSPCs. Supplementation of a complex mixture of platelet-derived factors *in vivo* could only in part substitute the vasculogenic function of HIF-competent MSPCs.

MSPCs react to a low oxygen environment by stabilizing HIF-1α faster and more sensitively than ECFCs. In the initial phase of vasculogenesis MSPCs promote vessel formation at least in part by rescuing ECFCs from hypoxia-induced apoptosis by a HIF-dependent trophic mechanism. Surprisingly, neo-vasculogenesis can occur independently of endothelial HIF stabilization. These results argue in favor of MSPC/ECFC co-transplantation as a promising cellular therapy for vascular regeneration. The finding that VEGF alone could not compensate for the vasculogenic function of MSPCs *in vivo* highlights the complexity of the hypoxia-induced cytokine network. The fact that HIF stabilization in MSPCs but not in ECFCs is crucial to initiating vascular regeneration supports a shift of focus from endothelial cells to perivascular mesenchymal cells as a therapeutic target in regenerative medicine and anti-angiogenic therapy.

3. Abbreviations

7-AAD	7-Aminoactinomycin D
17-AAD	17-allylaminogeldanamycin
4E-BP1	Eukaryotic initiation factor 4E binding protein
AhR	Aryl hydrocarbon receptor
ALL	Acute lymphatic leukemia
AMP	Adenosine monophosphate
ANG-2	Angiopoietin-2
ARD1	Arrest defective protein 1
ATP	Adenosin 5'-triphosphat
bFGF	Basic fibroblast growth factor
bHLH	Basic-helix-loop-helix
BM	Bone marrow
BMDAC	Bone marrow-derived angiogenic cells
BOECs	Bood outgrowth endothelial cells
CACs	Circulating angiogenic cells
CAR cells	CXCL-12-abundant reticular cells
CBP	CREB binding protein
CFU-EC	Colony-forming unit - endothelial cell
CFU-F	Colony-forming unit - fibroblast
CH1	Cystin-histidin rich
CITED2	CBP/p300-interacting transactivator with glutamic acid (E) and aspartic acid (D)–rich tail 2
CLL	Chronic lymphatic leukemia
CO2	Carbon dioxide
CPDs	Cumulative population doublings
CREB	cAMP response element-binding protein
CVD	Cardiovascular disease
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DFO	deferoxamine
DMOG	Dimethyloxallyl glycine
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
e-	Electron
ECFCs	Endothelial colony forming progenitor cells
ECM	Extracellular matrix
ECs	Endothelial cells

ECV	E3 ubiquitin-protein ligase complex
EGF	Epidermal growth factor
EGM	Endothelial growth medium
eIF-4E	Eukaryotic initiation factor 4E
EPCs	Endothelial progenitor cells
ERK	Extracellular-signal regulated kinase
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorting / scanning
FADH	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FIH	Factor inhibiting hypoxia
FSC	Forward scatter
GvHD	Graft versus host disease
H&E	Hematoxylin and eosin
H^+	Proton
HIF	Hypoxia inducible factor
HPP	High proliferative potential
HRE	Hypoxia response element
HSP90	Heat shock protein 90
HSPCs	Hematopoietic stem and progenitor cells
HUVECs	Human umbilical vein endothelial cells
IGF	Insulin-like growth factor
IκB	Inhibitory κB
IL	Interleukin
LPP	Low proliferative potential
MAPK	Mitogen-activated protein kinase
MEK	MAP/ERK kinase
MEM	Minimum essential medium
MI	Myocardial infarction
MMP	Matrix metallo-proteinase
MNCs	Mononuclear cells
MS	Multiple sclerosis
MSCA-1	Mesenchymal stem cell antigen-1
MSCs	Multipotent mesenchymal stromal / stem cells
MSPCs	Mesenchymal stem / progenitor cells
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NC	Nucleated cells
NED	Nuclear export domain

NF-ĸB	Nuclear factor-ĸB
NLS	Nuclear translocation sequence
NOG mouse	NOD/Shi-scid/IL-2Rγ^{null}; Non-obese diabetic (NOD) severe combined immuno-deficient (Scid, mutation in human homologue *PRKDC* gene) IL-2 receptor γ truncaden (lack T-, B-, NK-, and dendritic cells)
NSG mouse	NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ; Non-obese diabetic (NOD) severe combined immuno-deficient (Scid, mutation in human homologue *PRKDC* gene) IL-2 receptor γ knock-out mouse (lack T-, B-, NK-, and dendritic cells)
O2	Dioxygen
ODD	Oxygen dependent domain
PAI-1	Plasminogen activator inhibitor-1
PAS	Per-ARNT-Sim
PB	Peripheral blood
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDs	Population doublings
PECAM	Platelet endothelial cell adhesion molecule
PHD	Prolyl hydroxylase
pHPL	Pooled human platelet lysate
PI	Propidium iodine
PI3K	Phosphatidylinositol 3-kinase
PlGF	Placental growth factor
Plts	Platelets
RACK1	Receptor of activated protein kinase C
RCC	Renal clear cell carcinoma
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
SC	Stem cell
SDF-1	Stromal derived factor-1
SMA	Smooth muscle actin
SMC	Smooth muscle cells
SSC	Side scatter
TAD	Trans-activation domains
TCA	Tri-carboxylic acid (cycle)
TCR	T cell receptor
TGF	Transforming growth factor
Th1	Type1 helper Tcell
Th2	Type2 helper Tcell

Treg	Regulatory T cell
TNF-α	Tumor necrosis factor- α
UC	Umbilical cord
UCB	Umbilical cord blood
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VHL	Von Hippel Lindau
vWF	Von Willebrand factor
WAT	White adipose tissue
XRE	Xenobiotic response-element

4. Introduction

4.1. Introduction to cellular oxygen sensing

Dependency on oxygen is a double-edged sword for all higher organisms. While oxygen availability enabled primitive protozoans to meet the high energy demands required for the development of complex multicellular organisms, toxic side products of the oxygen metabolism cause aging and cellular death. Additionally, deprivation of oxygen threatens the survival of eukaryotes. Consequently, oxygen-dependent cells have developed mechanisms to sense and adapt to oxygen level changes. If these adaptive mechanisms fail, then a wide variety of diseases can develop. Therefore, the complex process of oxygen sensing is of fundamental biological importance and offers a promising therapeutic target for a diverse range of diseases.

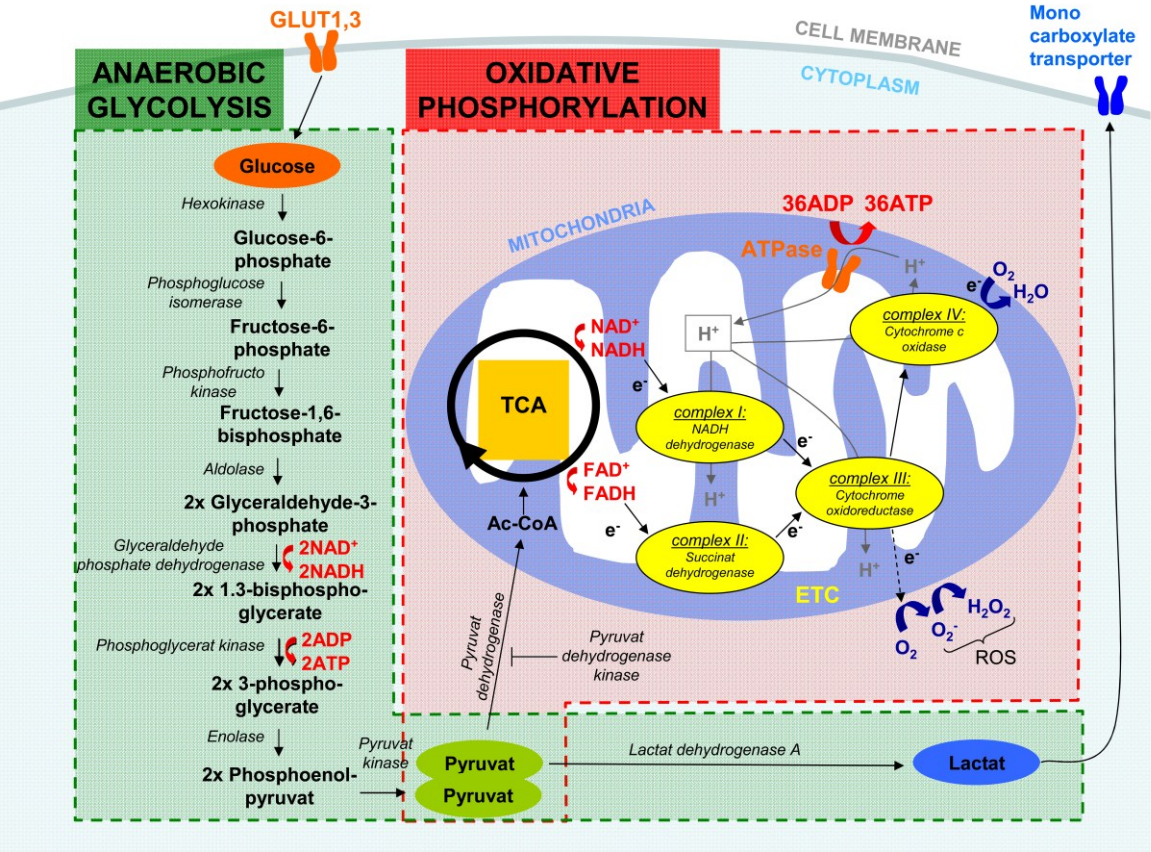


Figure 1. Glucose Metabolism (based on R.A. Gatenby & R.J. Gillies, Nature Reviews Cancer, 2004) (Gatenby, Gillies 2004)

Glucose is transported into the cellular cytoplasm via the glucose transporters 1/3 (GLUT1/3) and subsequently metabolized to pyruvate by producing 2 molecules "adenosin 5'-triphosphate" (ATP). Under normoxic oxygen levels pyruvate is converted to Acetyl Coenzyme A (Ac-CoA). In the mitochondria Ac-CoA enters the tricarboxylic acid (TCA) cycle thereby hydroxylizing "nicotinamide adenine dinucleotide" (NAD^+) and "flavin adenine dinucleotide" (FAD^+) to NADH and FADH, respectively. Subsequently, NADH and FADH are oxidized by complex I (NADH dehydrogenase) and FADH to complex II (succinate dehydrogenase) of the "electron transport chain" (ETC). The respiratory process of the ETC leads to proton (H^+) transfer out of the mitochondrial matrix. An ATPase converts "adenosine monophosphate" (AMP) to ATP by pumping H^+ back into the mitochondria. Thereby ATPases produce 36 ATP per molecule glucose. This process is known as "**oxidative phosphorylation**". Under hypoxic conditions, hypoxia inducible factor-1α (HIF-1α) induces the up-regulation of GLUT1/3, which increases glucose uptake, hexokinase and lactate dehydrogenase A, which catalyze the cleavage of glucose to lactate. Additionally, the hypoxia-induced production of "pyruvate dehydrogenase kinase" inhibits the conversion of pyruvate to Acetyl-CoA by inactivating pyruvate dehydrogenase. This blocks pyruvate from entering the TCA cycle and instead leads to the secretion of the metabolite lactate. The conversion of glucose to lactate is known as "**anaerobic glycolysis**".

Even the simplest anaerobic organism produces energy in the form of "adenosin 5'-triphosphate" (ATP) by converting glucose to lactic acid via a process termed anaerobic glycolysis (Figure 1). About 2.5 million years ago photosynthetic organisms appeared that converted solar energy and carbon dioxide (CO_2) to glucose, releasing dioxygen (O_2) as a side product. As oxygen accumulated in the atmosphere, specialized eukaryotes evolved containing organelles similar to endosomal prokaryotes, known as mitochondria. These mitochondria enabled eukaryotes to metabolize O_2 and glucose to produce 18 times more ATP than through anaerobic glycolysis alone (Gatenby, Gillies 2004, Semenza 2007). In the presence of O_2, cells bypass glycolysis by inhibiting the conversion of pyruvate to lactate and instead use pyruvate dehydrogenase to form Acetyl-Coenzyme A (Ac-CoA) and enter the "tricarboxylic acid cycle" (TCA cycle) in the mitochondria. This metabolic switch known as 'Pasteur effect' was first described in 1857 by Louis Pasteur, who found that oxygen enables yeast to consume more glucose accompanied by decreasing production of lactate (Gatenby, Gillies 2004, Racker 1974). During the TCA pyruvate is metabolized

in the mitochondrial matrix whereby electrons are transferred to the reducing agents 'nicotinamide adenine dinucleotide' (NAD^+) and 'flavin adenine dinucleotide' (FAD^+) thereby producing NADH and FADH, respectively. In the mitochondria NADH passes two electrons (e^-) to complex I (NADH oxidoreductase) and FADH to complex II (succinate oxidoreductase) of the 'electron transport chain' (ETC) (Figure 1). After that the e^-'s are passed on to complex III (cytochrome c oxidoreductase) and finally to complex IV (cytochrome c oxidase) where they react with O_2 and release water (H_2O). While an e^- is transported through the ETC, complex I, III and IV release protons (H^+) to the mitochondrial inter-membrane space, which results in an H^+ gradient. The ATP-synthase (complex V) pumps H^+'s back to the matrix thereby converting adenosine monophosphate (AMP) to ATP. This respiratory process is called 'oxidative phosphorylation' and produces 36 moles of ATP per mole of glucose (Figure 1) (Gatenby, Gillies 2004). Therefore, under sufficient oxygenation, healthy cells produce energy through the highly efficient oxidative phosphorylation. However, under low oxygen concentrations, eukaryotes can switch to anaerobic glycolysis producing lactic acid. The ability to switch between glycolysis and oxidative phosphorylation depending on O_2 availability has enabled eukaryotes to adapt to their environment. The metabolism of O_2 and glucose allowed metazoans about 0.5 billion years ago to meet the high energy demands required to develop complex multicellular organisms (Gatenby, Gillies 2004, Semenza 2007).

A side product of inefficient mitochondrial respiration, due to too high or low oxygen levels, are the highly "reactive oxygen species" (ROS) leading to cellular toxicity and death, but can also activate adaptive mechanisms (Chandel et al. 1998, Guzy et al. 2005, Guzy, Schumacker 2006, Semenza 2007). Excess oxygen or mitochondrial dysfunction causes a fraction of electrons to escape the ETC at complex I and complex III and react with O_2 prematurely, resulting in the formation of superoxide anions. Superoxide dismutase subsequently catalyzes the conversion to hydrogen peroxide (H_2O_2). These so called ROS' can oxidize lipids, enzymatic co-factors, RNA, DNA and proteins leading to their missfolding and loss of function. At the surface of the mitochondria a specialized group of proteins called B-cell lymphoma 2 (Bcl-2) detect the magnitude of damage and in turn initiate apoptosis (Guzy, Schumacker 2006). Paradoxically, production of ROS is

also increased at hypoxic conditions resulting in an increase of hypoxia response (see also Chapter 4.1.3.) (Chandel et al. 1998, Guzy et al. 2005, Guzy, Schumacker 2006, Semenza 2007). ROS production at hypoxic conditions is mediated by complex III by an as of yet unknown mechanism (Guzy et al. 2005). Presumably, the lack of oxygen causes a conformational change of the complex III structure, resulting in a direct electron transfer to O_2 and therefore increased ROS production. In order to maintain oxygen homeostasis, metazoan organisms have developed an intra-cellular oxygen sensor complex.

However, the dependency of metazoans on O_2 and the physical properties of limited diffusion constrained organisms of size. The development of complex respiratory and circulatory systems (trachea, grill, lungs, heart, blood and vasculature) set the essential evolutionary steps to create larger and more compound animals. A labyrinth of blood vessels networking throughout the body evolved enabling blood circulation and therefore allowing O_2, nutrients and immune cells access to all anatomical sites. Oxygen delivery from the highly oxygenated airways via saturated blood to the O_2-consuming mitochondria is driven through diffusion in a gradient dependent manner. While ambient air oxygen of approximately 20% (140 mmHg) at sea level fills our lungs, arteries contain ≈7% O_2 (49 mmHg), veins ≈5% O_2 (35 mmHg) and in specialized niches found in the bone marrow one can find 1% O_2 (7 mmHg) (Ward 2008). At this point it should be noted that in the scientific community standard culture conditions are commonly termed ‘normoxia’ and a wide variety of oxygen concentrations are considered to be ‘hypoxia’ (Forsythe et al. 1996a, Carmeliet et al. 1998, Kelly et al. 2003, Sowter et al. 2003, Tang et al. 2004, Manalo et al. 2005, Kim et al. 2006, Calvani et al. 2006, Mazzone et al. 2009, Fiegl et al. 2009, Decaris et al. 2009, Mazumdar et al. 2010, Tsai et al. 2011, Youn et al. 2011). In contrast to this notion, ambient air should not to be considered a normoxic condition for tissue cells. Therefore, standard culture conditions should instead be called ‘ambient air environment’. Since different cells reside in differently oxygenated regions, the normal physiological oxygen concentration of a specific cell type should be termed ‘physiological normoxia’ or ‘euoxia’ and a low pathological oxygen concentration ‘hypoxia’. In this notion, the physiological normoxic conditions for venous endothelial cells are herein nominated to be 5% O_2 and hypoxia to be < 1% O_2 as implied by Ward et al. (Ward 2008).

4.1.1. Hypoxia inducible transcription factors (HIFs)

The transcription factor HIF plays a key role in the maintenance of oxygen homeostasis in all metazoans (Semenza 2007). Three distinct HIFs have been recognized so far. The first, HIF-1, was initially discovered by Semenza et al. as a DNA-binding protein that activates erythropoietin production in an oxygen-dependent manner (Semenza, Wang 1992). Since its discovery in 1992, hundreds of target genes have been linked to HIF-1 activity including cell-type specific regulation of genes with critical roles in erythropoiesis (formation of red blood cells), angiogenesis (budding of capillaries from existing vessels), glucose metabolism, proliferation, migration and apoptosis (Kelly et al. 2003, Pugh, Ratcliffe 2003, Semenza 2003, Hu et al. 2003, Semenza 2007, Semenza 2011).

HIFs are heterodimers which are composed of an oxygen-regulated α-subunit (HIF-α) and a constitutively expressed β- subunit (HIF-β; also called aryl hydrocarbon receptor nuclear translocator, ARNT). Under normoxia (physiological oxygen levels) HIF-α is continuously synthesized and instantaneously degraded by the proteosome. Under hypoxia HIF-α is stabilized, translocates to the nucleus, binds to HIF-β and acts as a transcriptional activator for target genes containing a hypoxia response element (HRE) (Figure 2) (Semenza, Wang 1992, Semenza 2011).

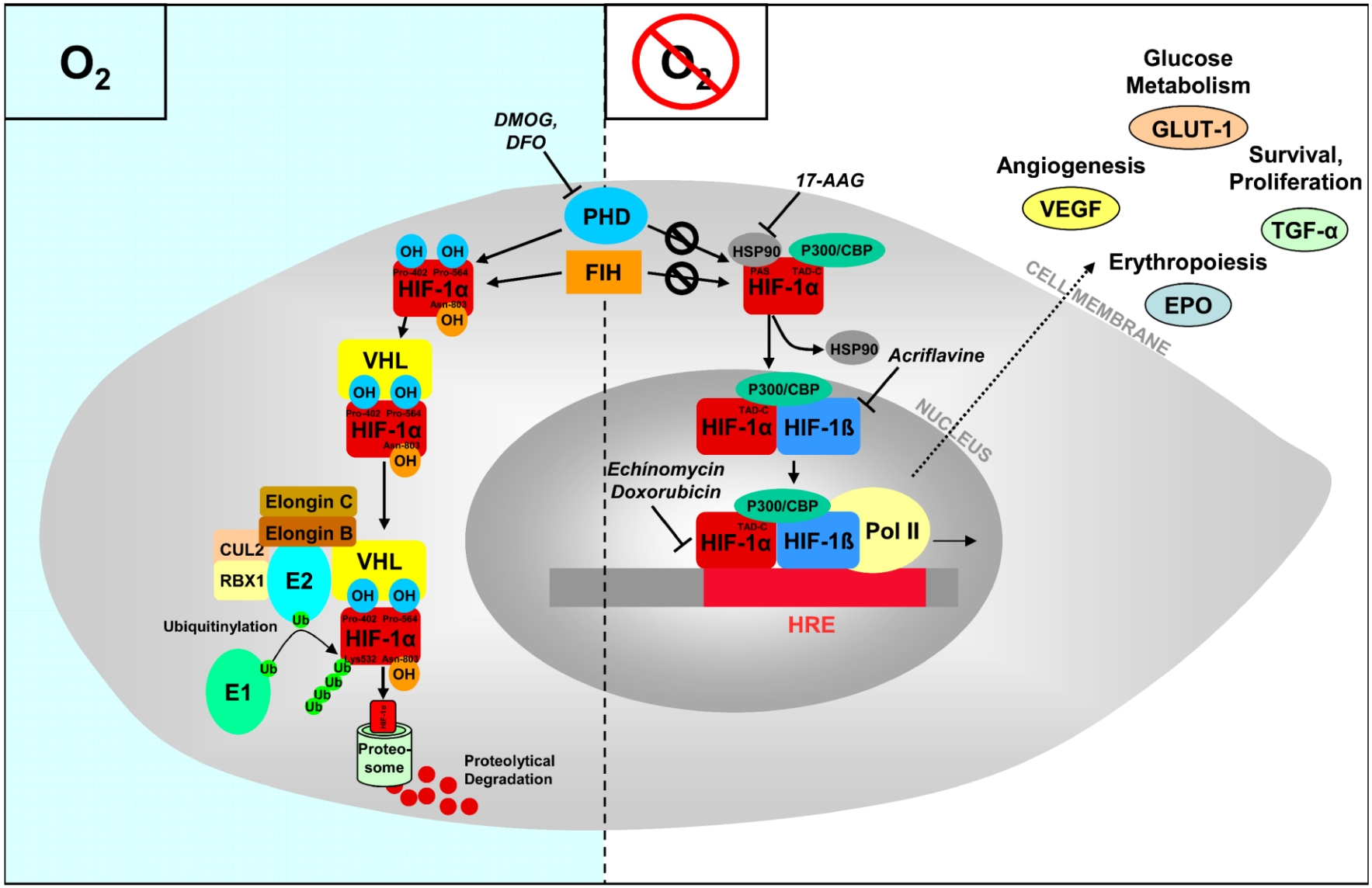

O2
O2
DMOG,
DFO
PHD
FIH
17-AAG
HSP90
P300/CBP
PAS
TAD-C
HIF-1α
Pro-402 Pro-564
Asn-803
OH
VHL
HSP90
Acriflavine
P300/CBP
HIF-1ß
Echínomycin
Doxorubicin
Pol II
HRE
Elongin C
Elongin B
CUL2
RBX1
E2
Ubiquitinylation
Ub
E1
Lys532
Proteo-
some
Proteolytical
Degradation
Glucose
Metabolism
GLUT-1
Angiogenesis
Survival,
Proliferation
VEGF
TGF-α
Erythropoiesis
EPO
CELL MEMBRANE
NUCLEUS

Figure 2. Hypoxia inducible factor-1 (HIF-1) signaling pathway (based on G.L. Semenza, Science, 2007; G.L. Semenza, NEJM, 2011) (Semenza 2007, Semenza 2011)

Under physiological oxygen conditions, prolylhydroxylases 1-3 (PHD1-3) and factor-inhibiting HIF-1 (FIH-1) hydroxylize HIF-1α proline (Pro) and asparagine (Asn) residues, respectively. The hydroxylized amino acids are recognized by the von Hippel Lindau protein (pVHL) which recruits Elongin B, Elongin C, cullin-2 (Cul2), RING-box protein1 (Rbx1) and E2. Together these proteins form the E3 ubiquitin-protein ligase complex (ECV; Elongin B/ cullin-2/ VHL protein). Ubiquitin binds under consumption of adenosine-triphosphate (ATP) to the cystein of an E1 ubiquitin-activating enzyme and is subsequently transferred to a cystein of the E2 ubiquitin-conjugating enzyme of the ECV. Thereafter, HIF-1α is proteolytically degraded.

Under hypoxic conditions HIF-1α escapes hydroxylation and binds to heat shock protein 90 (HSP90), p300 and cyclic adenosine-monophosphate (cAMP) response element-binding protein (CREB) binding protein (CBP). HIF-1α translocates to the nucleus and dimerizes with HIF-1β. The binding of HIF-1α and HIF-1β replaces the stabilizer HSP90 and is enhanced by p300/CBP. Together HIF-1α/HIF-1β act as the HIF-1 transcription factor of genes with a hypoxia response element (HRE); including vascular endothelial growth factor (VEGF), transforming growth factor (TGF-β), erythropoietin (EPO) and glucose transporter (GLUT) 1 and 3 (see also Table 2). The iron chelators dimethyloxaloylglycine (DMOG) or deferoxamine (DFO) inhibit PHD activity and thereby lead to a normoxic stabilization of HIF-1α. HIF-1α stabilization can be inhibited by blocking HSP90 (17-AAG, 17-allyl-aminogel-denamycin). The heterodimerization of HIF-1α/1β can be inhibited by the antiseptic acriflavine. DNA-binding of HIF-1α can be blocked by the antracycline doxorubicin and the antibiotic echinomycin.

The *Hif-1α* gene is encoded on human chromosome 14 (14q23.2). The protein HIF-1α is composed of 826 amino acids and weighs 116 kDa. HIF-1α contains an N-terminal basic-helix-loop-helix (bHLH) domain and two Per-ARNT-Sim homology domains (PAS-A and PAS-B), which are responsible for the binding to HIF-1β and interaction with the HREs (Figure 3). The PAS domains are named after the proteins in which they occur: period circadian protein, aryl hydrocarbon receptor nuclear translocator protein, single-minded protein (Jiang et al. 1996). The middle part of the HIF protein is a very prolyl- and lysine-rich oxygen dependent domain (ODD). On its C-terminus HIF is composed of 2 trans-activation domains (TAD), TAD-N and TAD-C, which are the binding regions of the von Hippel Lindau protein (pVHL) and co-factor p300/cAMP response element-binding protein (CREB) binding protein (CBP) (Wang et al. 1995, Semenza 2003, Semenza 2004) .

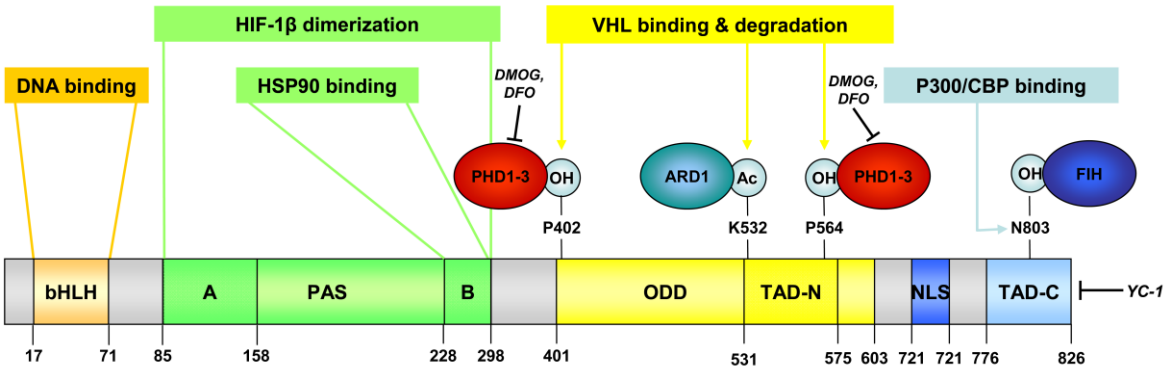


Figure 3. Hypoxia inducible factor-1α (HIF-1α) structure (modified from G.L. Semenza, Nature Reviews Cancer, 2003) (Semenza 2003)

On its N-terminal side HIF-1α is composed of a basic-helix-loop-helix (bHLH) domain and two Per-ARNT-Sim homology domains (PAS-A and PAS-B), which are binding sites for DNA, HIF-1β, and HSP90. In the middle section of HIF-1α a region called the oxygen dependent domain (ODD) contains the prolyl (P402, P564) and lysine (K532) residues that are hydroxylized by prolyl hydroxylases (PHD)1-3 and arrest defective protein 1 (ARD1). The ODD contains the transactivation domain (TAD)-N and is the binding site for the von Hippel Lindau protein (pVHL). On its C-terminal side HIF-1α contains the nuclear translocation sequence (NLS) and the TAD-C, which contains the asparagin (N803) which, under normoxia, can be hydroxylized by factor inhibiting hypoxia (FIH) and under hypoxia bind to the co-factor p300/CBP. HIF-1α activity is inhibited by YC-1 (3-(5'-hydroymethyl-2'furyl)-1-benzyl-indazole) which directly degrades the TAD-C. Dimethyloxaloylglycine (DMOG) or deferoxamine (DFO) act as iron chelators which inhibit PHD activity and thereby lead to a normoxic stabilization of HIF-1α.

The HIF system gains even more complexity when considering the fact that there are three known HIF-α isoforms: HIF-1α, HIF-2α (also called EPAS, endothelial PAS domain protein) and HIF-3α (Semenza 2007). HIF-1α is expressed in virtually all nucleated metazoan cells. HIF-2α is largely restricted to vascular endothelial cells, (Semenza 2011, Hu et al. 2003) but can also be found in kidney fibroblasts, hepatocytes, epithelial and pancreatic interstitial cells, cardiomyocytes and type II pneumocytes, tumor vascular cells, parenchymal cells, and infiltrating macrophages (Hu et al. 2003). Structurally, HIF-1α and HIF-2α share 48% amino acid homology and both can dimerize with HIF-1β in an oxygen dependent manner (Hu et al. 2003). It is an ongoing debate in the field whether HIF-2α can also interact with its own HIF-2β (Sekine et al. 2006). HIF-1α and HIF-2α each have unique cell-specific targets of gene expression (Kelly et al. 2003, Hu et al. 2003).

The precise function of HIF-3α still remains to be resolved, but there is evidence that HIF-3α has a negative regulatory function on HIF-1α and HIF-2α. It has been shown that the transcription of HIF-3α is directly mediated by HIF-1α stabilization. Heihhila et al. have recently demonstrated that HIF-3α acts in a negative feedback loop to inhibit HIF-1α translocation to the nucleus blocking transcriptional activation of the HRE promoters (Manalo et al. 2005, Heikkila et al. 2011).

4.1.2. Oxygen-dependent regulation of HIF: under normoxia

With sufficient oxygenation (normoxia) HIF-1α is hydroxylized by the prolyl-4-hydroxylase (PHD) at the proline residues, P402 and P564, located in the ODD (Figure 3) (Jaakkola et al. 2001, Ivan et al. 2001, Berra et al. 2003). Three isoforms of PHDs have been identified: PHD1, PHD2 and PHD3. Originally, PHDs 1-3 were found to be encoded by the *egl-9* gene (responsible for normal egg laying in *C. elegans*) and are therefore also known as EGLN2, EGLN1 and EGLN3 respectively (Appelhoff et al. 2004). All three PHDs can hydroxylize HIFs, however, there is a strong abundance of PHD2 to hydroxylate HIF-1α and PHD3 to hydroxylate HIF-2α, respectively (Berra et al. 2003, Appelhoff et al. 2004). PHDs are considered to be the cellular oxygen sensors.

PHDs are dioxygenases with an Fe(II) unstably bound to a histidine in its catalytic center. Dioxygenases hydroxylize amino acids by reducing molecular oxygen and therefore oxidizing the cofactor 2-oxoglutarat which releases CO_2 after succination. Different metal salts (e.g. $CoCl_2$) can compete with iron which inactivates the catalytic centre and therefore acts as a ‘hypoxia-mimetic’. Iron chelators, such as dimethyloxaloylglycine (DMOG) or deferoxamine (DFO) have the same effect (see Figure 2 and Figure 3) (Jaakkola et al. 2001, Ivan et al. 2001, Pugh, Ratcliffe 2003, Berra et al. 2003).

The two hydroxylated prolines are recognized by the tumor suppressor protein pVHL, which is the substrate-recognition subunit of the E3 ubiquitin-protein ligase complex (ECV). The pVHL consists of a helix-domain (α-subunit) and a β sandwich-domain (β-subunit). The α-subunit of pVHL recruits the ubiquitin ligases Elongin B, Elongin C, cullin-2 (Cul2), RING-box protein1 (Rbx1) and E2, which together form the ECV (also called VBC-CR complex) (Figure 2) (Stebbins, Kaelin

& Pavletich 1999, Min et al. 2002). The hydroxylated prolines of HIF-1α are bound by the hydrophobic binding pocket of the pVHL β-subunit (Min et al. 2002, Ohh et al. 2000). Additionally, an acetyltransferase ARD1 (arrest defective protein 1) stabilizes the pVHL bondage to HIF-1α by transferring its acetyl (Ac) from an Ac-Coenzyme A (Ac-CoA) to the lysin K532 located in the ODD (Figure 3) (Semenza 2003). The stable connection of pVHL to HIF mediates its ubiquitation. This process is catalyzed in three sequential steps by activating, conjugating, and ligating enzymes. Firstly, ubiquitin binds under consumption of ATP to the cystein of the E1 ubiquitin-activating enzyme and is subsequently transferred to a cystein of the E2 ubiquitin-conjugating enzyme of the ECV. The E3 ubiquitin protein ligase complex then transfers the activated ubiquitin from an E2 ubiquitin-conjugating enzyme to lysine residues (K532, K538, K547) of HIF-1α. Poly-ubiquitation recruits the 26S proteosome, which uses ATP to internalize HIF and degrades it into small peptides (Figure 2) (Hershko et al. 1983, Pickart 2004).

Additionally, under normoxic conditions a second dioxygenase called factor inhibiting HIF (FIH) inhibits HIF trans-activation (Figure 3). FIH hydroxylates the asparaginyl residue N803 in the TAD-C of HIF-1α and, thereby, blocks the binding site of the co-activator p300/CBP. The co-factor p300/CBP is necessary for the dimerization of HIF-1α with 1β, binding to the HRE promoters and the recruitment of RNA polymerase II.

4.1.3. Oxygen-dependent regulation of HIF: under hypoxia

Under hypoxic conditions, the limited oxygen availability reduces PHD and FIH hydroxylation function. Additionally, it has been shown that hypoxia-induced ROS production is involved in the inhibition of PHDs by preventing its redox cycling (Guzy, Schumacker 2006). Lack of prolyl hydroxylation by PHDs blocks pVHL binding and therefore inhibits proteolytic degradation of HIF-1α. Furthermore, the heat shock protein 90 (HSP90) stabilizes HIF-1α by binding to the PAS-B domain and protects it from unspecific degradation (Isaacs et al. 2002, Liu et al. 2007). HIF-1α accumulates in the cytoplasm and independently of HIF-1β translocates to the nucleus via the nuclear localization sequence (NLS) (Kallio et al. 1998, Chilov et al. 1999). NLS motifs are short amino acid moieties that consist of highly conserved basic residues and tag a variety of proteins for nuclear transport.

Integrin 4 and 7 recognize and bind to the HIF-1α NLS sequence and transport it through the nuclear pores into the nucleus. Inside the nucleus, the integrins release HIF-1α in a GTP-dependent manner. The nuclear-located HIF-1β leads to the dissociation of HSP90 from the PAS-B domain of HIF-1α (Chachami et al. 2009). Subsequently, HIF-1β dimerizes with HIF-1α through its bHLH- and PAS domains (Jiang et al. 1996, Chilov et al. 1999). Additionally, under hypoxia FIH can no longer hydroxylize the asparagine N803 of HIF-1α and the co-factor complex p300/CBP can bind to the TAD-C domain. In the nucleus, p300/CBP facilitates trans-activation of the HIF-1α/1β complex. Furthermore, p300/CBP is a histone acetyltransferase which promotes the binding of the HIF-1α TAD-C domain to HREs and thereby mediates the recruitment of RNA polymerase II (Semenza 2003, Semenza 2007, Semenza 2011). Thereby, the HIF-1α/1β complex is a transcriptional activator for genes located in the HREs (Jiang et al. 1996, Semenza 2003).

4.1.4. Oxygen-independent regulation of HIF

The transcriptional modulator called 'CBP/p300-interacting transactivator with glutamic acid (E) and aspartic acid (D)–rich tail 2' (CITED2) is an oxygen-independent negative regulator of HIF-1α with a high affinity for p300. The cytokine-inducible CITED2 and HIF-1α share a conserved sequence located in their trans-activation domains that can bind to the CH1 (cystin-histidin rich) domain of p300/CBP (Freedman et al. 2003). CITED2 competes with HIF-1α for p300 binding and therefore blocks hypoxia-activated gene expression with a high affinity (Bhattacharya et al. 1999, Bhattacharya, Ratcliffe 2003).

Additionally, the half-life of activated HIF-1α is regulated by an O_2-independent mechanism. In the cytoplasm, the receptor of activated protein kinase C (RACK1) competes with HSP90 for the PAS-A domain of HIF-1α (Isaacs et al. 2002, Semenza 2007) . RACK1 is composed of a seven-bladed propeller structure that binds to residues 81-200 of HIF-1α and subsequently recruits Elongin C and Elongin B. These recruited enzymes act as an ECV and lead to the proteolytical degradation of HIF-1α even under hypoxic conditions. This mechanism of HIF-1α is similar to the destruction mediated by the pVHL pathway with the major difference being that it is independent of O_2, PHD2 and pVHL. Therefore, the

HSP90 inhibitor 17- allylaminogeldanamycin (17-AAD) is a promising target for pharmacological induction of HIF-1α destruction (Semenza 2007, Liu et al. 2007).

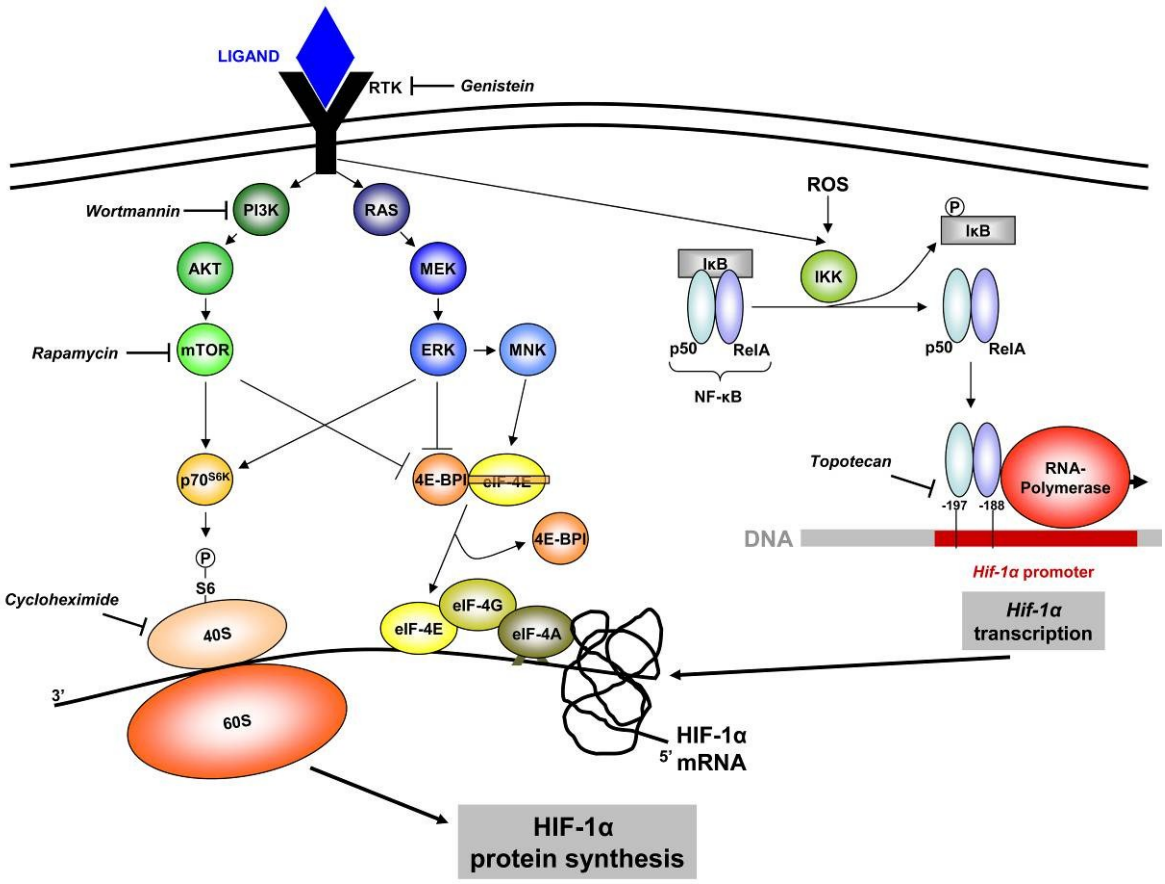


Figure 4. Hypoxia-independent hypoxia inducible factor-1α (HIF-1α) upregulation (modified from G.L. Semenza, Nature Reviews Cancer, 2003; A. Goerlach & S. Bonello, Biochemical Journal, 2008 and H.K. Elzschig & P. Carmeliet, NEJM, 2011) (Semenza 2003, Gorlach, Bonello 2008, Eltzschig, Carmeliet 2011)

Activated receptor tyrosine kinases (RTKs) phosphorylate phosphatidyl inositol-3-kinase (PI3K) and the GTP binding protein rat *s*arcoma (RAS) which both lead to the activation of the protein 70 S6 kinase ($p70^{S6K}$). $p70^{S6K}$ phosphorylates the protein S6 of the small ribosomal subunit (40S) and causes recruitment of the big ribosomal subunit (60S). Additionally, the inhibitory effect of the eukaryotic initiation factor 4E (eIF-4E) binding protein (4E-BP1) is blocked through the activated mammalian target of rapamycin (mTOR) and extracellular-signal regulated kinase (ERK) and causes the release of eIF-4E. eIF-4E together with the helicase eIF-4A and the linker eIF-4G unwind the RNA and facilitate translation by the ribosome. This leads to a hypoxia-independent increase in HIF-1α protein concentration.

Furthermore, RTKs as well as reactive oxygen species (ROS) activate the nuclear factor (NF)-κB inhibitor (IκB) kinase (IKK) which phosphorylates IκB. Phosphorylated IκB releases and therefore

activates NF-κB. The activated NF-κB acts as a transcription factor for a variety of genes including *Hif-1α.* This also mediates a hypoxia independent up-regulation of HIF-1α. HIF-1α synthesis can be inhibited up-stream by blocking the RTK (genistein) or targeting of PI3K (wortmannin) and mTOR (rapamycin), or by inhibition of the topoisomerase I (topotecan).

Furthermore, the translation of HIF-1α can be regulated in an O_2-independent manner by extra-cellular signaling molecules (Fukuda et al. 2002, Semenza 2003, Liu, Simon 2004). Binding of growth factors, cytokines and other signaling molecules to specific receptor tyrosine kinases (RTKs) cause a dimerization and auto-phosphorylation of the receptor (Table 1).

Table 1. Signaling molecules initiating hypioxia-inducible factor-1α (HIF-1α) protein synthesis

Activation of HIF-1α Translation	
Insulin-like growth factor-1 (IGF-1)	(Fukuda et al. 2002)
Angiopoietin-2 (Ang2)	(Richard, Berra & Pouyssegur 2000)
Thrombin	(Richard, Berra & Pouyssegur 2000)
Platelet-derived growth factor (PDGF)	(Richard, Berra & Pouyssegur 2000)
Lipopolysaccharide (LPS)	(Blouin et al. 2004)
Human epidermal growth factor receptor 2 (HER2)	(Laughner et al. 2001)
Epidermal growth factor (EGF)	(Zhong et al. 2000)
Sarcoma (SRC)	(Jiang et al. 1997)
Tumor necrosis factor (TNF)-α	(Haddad, Land 2001)
Stem cell factor (SCF)	(Gibbs et al. 2011)
Transforming growth factor (TGF)-β1	(McMahon et al. 2006)
Interleukin (IL)-1β	(Thornton et al. 2000)
Hepatocyte growth factor (HGF)	(Tacchini et al. 2001)
M1-M2 muscarinic acetylcholine receptors (AchR)	(Hirota et al. 2004)
Reactive oxygen species (ROS)	(Bonello et al. 2007)

An activated RTK phosphorylates the phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Semenza 2003). PI3K phosphorylates the serin/threonin kinase AKT (discovered in an AKR mouse thymoma model (Staal, Hartley & Rowe 1977), which activates the mammalian target of rapamycin (mTOR). This process can be inhibited by the ‘phosphatase

and tensin homologue deleted from chromosome-10' (PTEN). Activated mTOR phosphorylates the eukaryotic initiation factor 4E (eIF-4E) binding protein (4E-BP1). This inactivates its repressor function on eIF-4E. After phosphorylation through mTOR, 4E-BP1 is inhibited and subsequently leads to the activation of eIF-4E. Activated eIF-4E in turn initiates translation by binding to the 5' nucleotides of an mRNA (called "cap structure") and directing it to the ribosome. The translational regulator eIF-4E is part of the eIF-4F complex, which is composed of an ATPas and RNA helicase (eIF-4A) and a linker called eIF-4G which connects eIF-4E and eIF-4A. In the MAPK pathway, the RTK phosphorylates the G-protein RAS, which phosphorylates the downstream targets MAP/ERK kinase (MEK) and thereby regulates the extracellular-signal regulated kinase (ERK). ERK in turn phosphorylates MAPK interacting kinase (MNK), which additionally activates eIF-4E. Both ERK and mTOR activate the p70 S6 kinase ($p70^{S6K}$), which phosphorylates the ribosomal S6 protein of the 40S subunit. Subsequently, the 40S ribosomal subunit binds to mRNA and recruits the 60S ribosomal subunits, which activates translation. Thereby, growth factor signaling leads to increased translation of mRNA, including HIF-1α (Semenza 2003, Fukuda et al. 2002, Liu, Simon 2004). The over-expression of HIF-1α then overcomes the degradation by pVHL and induces transcription of HIF target genes.

Besides translation, the transcription of HIF-1α can also be regulated in a hypoxia-independent manner by the activation of the nuclear factor (NF)-κB signaling pathway (Figure 4). In response to thrombin, hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), angiotensin II and ROS (as H_2O_2), the NF-κB inhibitor (IκB) kinase (IKKα/β) is phosphorylated and in turn phosphorylates IκB. Phosphorylation of IκB in turn leads to the release and activation of the redox-sensitive transcription factor NF-κB (Bonello et al. 2007). The activated NF-κB subunits p50 and p65 (RelA; reticuloendotheliosis viral oncogene homolog A) directly interact with the HIF-1α promoter at -197/-188 bp, thus increasing HIF-1α mRNA expression and protein production (Gorlach, Bonello 2008). Therefore, NF-κB stabilization leads to basal levels of HIF-1α protein in normoxic cells (van Uden, Kenneth & Rocha 2008). Interestingly, *Hif-1α* is not only a target gene of NF-κB. HIF-1α itself also targets NF-κB. The cross-talk between HIF-1α and NF-κB gains even more complexity with the observation that IKKβ can also be hydroxylated by PHD, which suggests that also hypoxia might

activate NF-κB and therefore enhance HIF-1α expression (Cummins et al. 2006). These findings implicate a crucial role of NF-κB for the speed of HIF-1α action and cell-specific sensitivity in response to hypoxic insult. The oxygen-independent activation of HIF-1α through extracellular signals features a promising approach to developing a pro-angiogenic pharmacological therapy.

4.1.5. Competition of HIF and aryl hydrocarbon receptor (AhR) for HIF-1β

The AhR is a ligand-regulated bHLH-PAS transcription factor residing in the cytosol. Upon ligand binding, AhR translocates to the nucleus and dimerizes with HIF-1β (ARNT). Ligands of AhR include polycyclic aromatic carbohydrates (PAH), which appear in gasoline, cigarette smoke, grilled foods (e.g. benoflavone, benzanthracen, benzopyrene) and halogenated aromatic carbohydrates (HAH) including 2,3,7,8-Tetrachloro-dibenzo-*para*-dioxin (TCDD, Dioxin). Dioxin (also known as 'Agent Orange' during the Vietnam War) has a high affinity to the AhR. (Nie, Blankenship & Giesy 2001, Seifert et al. 2008).

AhR is a structural homologue of HIF-1α, which contains a bHLH domain in its N-terminus, followed by two PAS domains (PAS-A and PAS-B) and a C-terminal trans-activation domain. The bHLH domain contains the NLS and the nuclear export domain (NED), which interact with DNA and the chaperone HSP90. The PAS domains participate in hetero-dimerization and DNA binding. The PAS-B domain overlapping sequence 230 to 421 is responsible for the binding of the ligand and a second HSP90 molecule. Besides two molecules of HSP90, AhR forms a complex with the co-chaperone p23, necessary for efficient activation of AhR, and the stabilizing X-associated protein 2 (XAP2).

Following ligand binding (e.g. Dioxin) AhR changes its conformation whereby the NLS mediates the translocation into the nucleus. Inside the nucleus, HIF-1β competes with HSP90 for the interaction with AhR. Dimerization of AhR with HIF-1β leads to transcriptional activation of specific target genes located in XREs (xenobiotic response-elements).

AhR target genes include cytochrome p450 (Jones, Whitlock 1990), UDP-glucuronosyltransferase (Yueh et al. 2003), glutathione S-transferase (Paulson et al. 1990), and erythropoietin (EPO) (Bradfield et al. 1993).

After transcriptional activation, AhR is recycled to the nucleus via the binding of the factor “chromatin region maintenance 1” to the NED. This mediates the export of AhR to the cytosol. Both AhR and HIF-1α share the dimerization partner HIF-1β. It has been shown that activation of AhR sequests the limiting factor HIF-1β, which inhibits its participation in the HIF signaling pathway. AhR is therefore considered a strong HIF competitor able to inhibit HIF-1α function and therefore a possible therapeutic target for anti-tumor therapy (Chan et al. 1999, Nie, Blankenship & Giesy 2001, Seifert et al. 2008).

4.1.6. HIF-induced gene expression

It has been shown that HIF-1α plays a crucial role in the cell-specific transcriptional regulation of a variety of genes involved in cell proliferation and survival, apoptosis, motility, cytoskeletal structure, erythropoiesis, cell adhesion, pH regulation, drug resistance, nucleotide metabolism, glucose metabolism, iron metabolism, extracellular matrix metabolism and angiogenesis (Table 2) (Carmeliet et al. 1998, Semenza 2003, Bracken, Whitelaw & Peet 2003, Liu, Simon 2004). The mechanisms underlying the cell-specific and HIF-1α/2α/3α specific gene expression remain to be resolved (Kelly et al. 2003).

Table 2.: Characterized hypoxia inducible factor-1α (HIF-1α) target genes

Transcriptional regulation	
Basic helix-loop-helix domain containing, class B, 2 (BHLHB2)	(Manalo et al. 2005)
B-cell CLL/lymphoma 6 (BCL6)	(Manalo et al. 2005)
cAMP responsive element binding protein 3-like 2 (CREB3L2)	(Manalo et al. 2005)
Differentiated embryo chrondocyte 1 & 2 (DEC1 & 2)	(Wykoff et al. 2000, Miyazaki et al. 2002)
E26 transformation specific-1 (ETS-1)	(Oikawa et al. 2001)
Finkel-Biskis-Jinkins (FBJ) osteosarcoma viral oncogene homolog (FOS)	(Tsai et al. 2011)
FOS ligand 2 (FOSL2)	(Tsai et al. 2011)
Hairy and enhancer of split 1 (HES1)	(Manalo et al. 2005)
HIV-1 enhancer binding protein 2 (HIVEP2)	(Manalo et al. 2005)
Hypoxia-inducible factor 3, alpha subunit (HIF-3α)	(Manalo et al. 2005)
Jumonji domain containing 1 (JMJD1)	(Manalo et al. 2005)

MAD, mothers against decapentaplegic homolog 6 (MADH6)	(Manalo et al. 2005)
myc-associated factor X interacting protein (MXI1)	(Manalo et al. 2005, Corn et al. 2005)
Myocyte enhancer factor 2A (MEF2A)	(Manalo et al. 2005)
Neuronal PAS domain protein 2 (NPAS2)	(Manalo et al. 2005)
Notch homolog 4 (NOTCH4)	(Manalo et al. 2005)
Nuclear factor of activated T cells 4 (NFATC4)	(Manalo et al. 2005)
Nuclear receptor 77 (NUR77)	(Semenza 2003)
Peroxisome proliferative activated receptor, gamma (PPARG)	(Manalo et al. 2005)
RelA-associated inhibitor (RAI)	(Manalo et al. 2005)
Retinoid-Acid Receptor-related Orphan Receptor gamma t (RORγt)	(Bader, Hsu 2012, Dang et al. 2011)
Short stature homeobox 2 (SHOX2)	(Manalo et al. 2005)
Sirtuin (sir2 homolog) 3 (SIRT3)	(Manalo et al. 2005)
SRY (sex-determining region Y)-box 4 (SOX4)	(Manalo et al. 2005)
Thioredoxin interacting protein (TXNIP)	(Manalo et al. 2005)
Transcription factor (TCF8)	(Manalo et al. 2005)
Transcription factor 7-like 1 (TCF7L1)	(Manalo et al. 2005)
Transducin-like enhancer of split 1 homolog (TLE1)	(Manalo et al. 2005)
TWIST (named for its winding shape)	(Yang, Wu 2008)
V-maf oncogene homolog (MAFF)	(Manalo et al. 2005)
Zinc finger and BTB domain containing 1 (ZBTB1)	(Manalo et al. 2005)
Zinc finger protein 292 (ZNF292)	(Manalo et al. 2005)
Zinc fingers and homeoboxes 2 (ZHX2)	(Manalo et al. 2005)
Other signal transduction	
Adenylate kinase 3 (AK3)	(Manalo et al. 2005)
AMP-activated protein kinase family member 5 (ARK5)	(Manalo et al. 2005)
Calcium/calmodulin-dependent serine protein kinase (CASK)	(Manalo et al. 2005)
CK2 interacting protein 1 (CKIP-1)	(Manalo et al. 2005)
Cyclin-dependent kinase 11 (CDK11)	(Manalo et al. 2005)
Cyclin-dependent kinase inhibitor 1C (CDKN1C)	(Manalo et al. 2005)
Cyclooxygenase-2 (COX-2)	(Bazan, Lukiw 2002)
Dual specificity phosphatase 6 (DUSP6)	(Manalo et al. 2005)
Ectonucleotide pyrophosphatase 1 (ENPP1)	(Manalo et al. 2005)
Factor for adipocyte differentiation 104 (FAD104)	(Manalo et al. 2005)
Tacrolimus (FK506) binding protein 9 (FKBP9)	(Manalo et al. 2005)
Growth arrest and DNA-damage-inducible, beta (GADD45B)	(Manalo et al. 2005)
LIM and senescent cell antigen-like domains 1 (LIMS1)	(Manalo et al. 2005)
Myocytic induction/differentiation originator (MIDOR1)	(Manalo et al. 2005)

PDZ domain containing 3 (PDZK3)	(Manalo et al. 2005)
Pellino homolog 2 (PELI2)	(Manalo et al. 2005)
Phospholipase C, gamma 2 (PLCG2)	(Manalo et al. 2005)
Presenilin-1 (PSEN-1)	(Bazan, Lukiw 2002)
Ras association (RalGDS/AF-6) domain family 2 (RASSF2)	(Manalo et al. 2005)
Regulator of G-protein signaling 3 (RGS3)	(Manalo et al. 2005)
Related RAS (RRAS)	(Manalo et al. 2005)
Rho-related BTB domain containing 1 (RHOBTB1)	(Manalo et al. 2005)
Ribosomal protein S6 kinase (RPS6KA2)	(Manalo et al. 2005)
SPRY domain-containing SOCS box protein (SSB-1)	(Manalo et al. 2005)
Triple functional domain (TRIO)	(Manalo et al. 2005)
WAS protein family, member 2 (WASF2)	(Manalo et al. 2005)
Regulation of HIF-1 activity	
p300-CH1 interacting protein (p35srj)	(Bhattacharya et al. 1999)
Prolyl-4 hydroxylase (PHD)	(Manalo et al. 2005, Takahashi et al. 2000)
Cell proliferation and survival	
Adrenomedullin (ADM)	(Cormier-Regard, Nguyen & Claycomb 1998)
Nuclear growth factor (NGF)	(Nakamura et al. 2011)
Cyclin G2 (CCNG2)	(Manalo et al. 2005, Wykoff et al. 2000)
Erythropoietin (EPO)	(Semenza, Wang 1992)
Interferon (IFN)-γ	(Acosta-Iborra et al. 2009)
Insulin-like growth factor (IGF)-binding protein 1	(Feldser et al. 1999)
IGF-binding protein 2	(Feldser et al. 1999)
IGF-binding protein 3	(Feldser et al. 1999)
IGF 2	(Feldser et al. 1999)
Protein 21 (p21)	(Zaman et al. 1999)
Stem cell factor (SCF)	(Han et al. 2008)
Transforming growth factor (TGF)-α	(Gunaratnam et al. 2003)
Transforming growth factor (TGF)-β2	(Zhang et al. 2003)
Transforming growth factor (TGF)-β3	(Scheid et al. 2002)
Apoptosis	
Necrosis inducing protein (NIP-3)	(Bruick 2000, Sowter et al. 2001)
NIP-3 like protein X (NIX)	(Sowter et al. 2001)
Suppressor of mTOR signaling (RTP801)	(Shoshani et al. 2002)
Cytoskeletal structure and cell migration	
Autocrine motility factor (AMF)	(Funasaka et al. 2005)

Cathepsin D (CATHD)	(Dery, Michaud & Richard 2005)
CXC chemokine receptor 4 (CXCR4)	(Manalo et al. 2005)
Hepatocyte growth factor receptor (HGFR, encoded by c-Met)	(Semenza 2003)
Collagen, type I, alpha 2 (COL1A2)	(Manalo et al. 2005)
Collagen, type IV, alpha 1 & 2 (COL4A1& COL4A2)	(Manalo et al. 2005)
Collagen, type V, alpha 1 (COL5A1)	(Manalo et al. 2005, Wykoff et al. 2000)
Collagen, type IX, alpha 1 (COL9A1)	(Manalo et al. 2005)
Collagen, type XVIII, alpha 1 (COL18A1)	(Manalo et al. 2005)
F-actin	(Vogel et al. 2010)
Fibronectin-1 (FN1)	(Haeberle et al. 2008)
Intestinal trefoil factor (ITF)	(Furuta et al. 2001)
Keratin 14/18/19 (KRT)	(Dery, Michaud & Richard 2005)
Low density lipoprotein receptor-related protein 1 (LRP1)	(Wykoff et al. 2000, Castellano et al. 2011)
Lysyl oxidase (LOX)	(Manalo et al. 2005)
Matrix metallo-proteinase (MMP)-2	(Ahn et al. 2008)
Plasminogen activator inhibitor-1 (PAI1)	(Kietzmann et al. 2003)
Procollagen lysine hydroxylase 2 (PLOD2)	(Manalo et al. 2005)
Procollagen proline 4-hydroxylase, alpha 1 (PRHA1)	(Manalo et al. 2005)
Stromal derived factor (SDF)-1	(Ceradini et al. 2004)
Urokinase plasminogen activator receptor (UPAR)	(Dery, Michaud & Richard 2005)
Vimentin (Vim)	(Wykoff et al. 2000, Higgins et al. 2007)
Cell adhesion	
Monoclonal imperial Cancer Research Fund 2 (MIC2) (CD99)	(Wykoff et al. 2000)
L1 cell adhesion molecule (CAM)	(Wykoff et al. 2000)
pH regulation	
Carboxic anhydrase 9 (CA-9)	(Wykoff et al. 2000)
Iron metabolism	
Ceruloplasmin	(Mukhopadhyay, Mazumder & Fox 2000)
Transferrin	(Rolfs et al. 1997)
Transferrin receptor	(Ponka, Lok 1999)
Nucleotide metabolism	
Adenylate kinase 3 (ADK3)	(O'Rourke et al. 1996)
Ecto-5'-nucleotidase (CD73)	(Synnestvedt et al. 2002)
Tyrosine hydroxylase (TYH)	(Norris, Millhorn 1995)

Glucose metabolism	
Adenylate kinase-3	(O'Rourke et al. 1996)
Aldolase-A	(Semenza et al. 1994)
Aldolase-C	(Semenza et al. 1994)
Carboxic anhydrase- 9 (CA-9)	(Wykoff et al. 2000)
Enolase-1 (ENO1)	(Semenza et al. 1994)
Glucose transporter 1 (GLUT)-1	(Ebert, Firth & Ratcliffe 1995)
Glucose transporter 3 (GLUT)-3	(O'Rourke et al. 1996)
Glucose-6-phosphat isomerase (GPI)	(Funasaka et al. 2005)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	(Graven et al. 1999)
Hexokinase 1 (HK1)	(Mathupala, Rempel & Pedersen 2001)
Hexokinase 2 (HK2)	(Mathupala, Rempel & Pedersen 2001)
Lactase	(Lee et al. 2002)
Lactate dehydrogenase-A (LDHA)	(Semenza et al. 1994)
Phosphofructokinase L (PFKL)	(Semenza et al. 1994)
6-phosphofructo-2-kinase/fructose-2,6-bisphosphate-3 (PFKBF3)	(Minchenko et al. 2002)
Phosphoglycerate kinase 1 (PGK1)	(Semenza et al. 1994)
Pyruvate kinase M (PKM)	(Semenza et al. 1994)
Triosephosphate isomerase (TPI)	(Gess et al. 2004)
Amino-acid metabolism	
Aminopeptidase A (APA)	(Wykoff et al. 2000)
Transglutaminase 1 (TG1)	(Wykoff et al. 2000)
Oxidoreductases	
ERO1-like (ERO1L)	(Manalo et al. 2005)
Lysyl oxidase-like 2 (LOXL2)	(Manalo et al. 2005)
N-myc downstream regulated gene 1 (NDRG1)	(Manalo et al. 2005)
Peptidylglycine alpha-amidating monooxygenase (PAM)	(Manalo et al. 2005)
Prolyl hydroxylase 2 (PHD2, EGLN1)	(Manalo et al. 2005)
Prolyl hydroxylase 3 (PHD3, EGLN3)	(Manalo et al. 2005)
Prostaglandin I 2 (prostacyclin) synthase (PTGIS)	(Manalo et al. 2005)
Prostaglandin-endoperoxide synthase 1 (PTGS1)	(Manalo et al. 2005)
Thioredoxin interacting protein (TXNIP)	(Manalo et al. 2005)
Vascular tone and vascularization	
α_{1B}-adrenergic receptor	(Eckhart et al. 1997)
Adrenomedullin (ADM)	(Cormier-Regard, Nguyen & Claycomb 1998)
Angiopoietin-like 4 (ANGPTL4)	(Manalo et al. 2005)

Basic fibroblast growth factor (bFGF)	(Tamama et al. 2011)
Chemokine ligand 1 (CX3CL1)	(Manalo et al. 2005)
Endothelial nitric oxide synthase (eNOS)-2	(Palmer et al. 1998)
Endothelin-1 (ET-1)	(Minchenko et al. 2002)
Growth differentiation factor 10 (GDF10)	(Manalo et al. 2005)
Heme oxygenase (HO)-1	(Lee et al. 1997)
Hepatocyte growth factor (HGF)	(Tamama et al. 2011)
Inducible nitric oxide synthase (iNOS)-2	(Melillo et al. 1995)
Inhibin, beta A (INHBA)	(Manalo et al. 2005)
Inhibin, beta E (INHBE)	(Manalo et al. 2005)
Leptin (LEP)	(Ambrosini et al. 2002)
Placental growth factor (PlGF)	(Manalo et al. 2005)
Plasminogen activator inhibitor-1 (PAI-1)	(Kietzmann, Roth & Jungermann 1999)
Platelet-derived growth factor (PDGF)	(Manalo et al. 2005, Tamama et al. 2011)
Relaxin 1 (RLN1)	(Manalo et al. 2005)
Stanniocalcin 1 (STC1)	(Manalo et al. 2005)
Stanniocalcin 2 (STC2)	(Manalo et al. 2005)
Transforming growth factor-β (TGF-β)	(Tamama et al. 2011)
Tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2)	(Willam et al. 2000, Schnurch, Risau 1993)
Vascular endothelial growth factor (VEGF)	(Manalo et al. 2005, Tamama et al. 2011, Forsythe et al. 1996b)
VEGF receptor-1 and -2 (Flt1, Flk1/KDR)	(Gerber et al. 1997)
Erythropoiesis	
Erythropoietin (EPO)	(Semenza, Wang 1992)
Epithelial homeostasis	
Immunoglobulin transcription factor (ITF)	(Furuta et al. 2001)
Drug resistance	
Multiple drug resistance (MDR)-1	(Comerford et al. 2002)

To supply hypoxic cells with sufficient energy, HIF-1α mediates a metabolic switch from O_2-dependent oxidative phosphorylation to an anaerobic production of ATP (Pasteur Effect). This requires an increased uptake of glucose, which is regulated by HIF-1α-mediated production of the glucose transporters 1 and 3 (GLUT1/3). Anaerobic energy production involves the fermentation of glucose to lactate without entering the mitochondrial TCA cycle. Therefore, HIF-1α induces the upregulation of hexokinase 1 and 2, which convert glucose to pyruvate, and lactate dehydrogenase A, which catalyzes the cleavage of pyruvate to lactate. Additionally, the hypoxia-induced production of pyruvate dehydrogenase (PDH) kinase inhibits the conversion of pyruvate to Acetyl-CoA by inactivating PDH. This blocks pyruvate from entering the TCA cycle (Figure 1). The metabolite lactic acid is actively transported out of the cell via a mono-carboxylate transporter (Gatenby, Gillies 2004, Semenza 2007).

It is interesting to note that a widely accepted technique of hypoxic cell culture is the use of low oxygen flushed incubators and/or sealed plastic bags accompanied by feeding with (de-oxygenated) cell culture medium in normal oxygenated work stations (Fehrer et al. 2007, Dos Santos et al. 2010, Busletta et al. 2011). These studies describe an increase in cell proliferation which contradicts the findings of this dissertation where a closed hypoxic workstation system (BioSpherix) was used. This provoked the hypothesis that the increase in proliferation observed by other groups is induced by the oxygen fluctuations in the open culture system. One explanation could be that cells cultured at 1% O_2 up-regulate glycolytic proteins (GLUT1/3, hexokinase 1 and 2, lactate dehydrogenase A, PDH kinase) and therefore take up and metabolize more glucose via anaerobic glycolysis. Consequently, when cells are in turn suddenly exposed to high oxygen levels (during feeding) they switch back to oxidative phosphorylation yet still take up more glucose due to the slow degradation of GLUT1/3. Therefore, it is likely that cells exposed to fluctuating oxygen levels generate more energy (ATP) and increase proliferation.

In this dissertation, known targets of HIF-1α (e.g. VEGF) were tested for their pro-vasculogenic function *in vivo*. A deeper understanding of HIF-1α target genes, which are involved in vessel formation, could lead to the development of a cell-free or combined therapy for regenerative medicine and/or anti-tumor angiogenesis.

4.1.7. HIF in homeostasis, disease and therapy

Due to the cell type-specific expression of HIFs, it appears obvious that mutations of the HIF pathway can lead to various disease patterns. Specific *Hif-1α*, *Hif-2α* and *Hif-3α* knockout mouse models have demonstrated the importance of HIFs in embryonic development, especially during the regulation of vessel formation. *Hif-1α* knockout led to fetal lethality between embryonic day (E) 8.5 and E11 with the formation of abnormally enlarged vessels, mesenchymal cell death and neural defects (Iyer et al. 1998, Ryan, Lo & Johnson 1998, Kotch et al. 1999). Interestingly, the embryonic lethality of *Hif-1α* knockout mice did not correlate with VEGF deficiency (Kotch et al. 1999). This finding indicates that VEGF is not the only mediator of vessel formation in this system. Conditional *Hif-1α* knockouts (where floxed *Hif-1α* mice are bread with “Tyrosine kinase with immunoglobulin-like and endothelial growth factor-like domains 2” (Tie-2)-Cre transgenic mice) specifically in endothelial cells resulted in impaired vessel formation as well as reduced tumor size (Tang et al. 2004). 50% of *Hif-2α* knockout mice died intrauterine between E9.5 and E13.5 of cardiac failure and vascular disorganization, while *Hif-2α* deficient neonates died within 2-3 hours of respiratory failure (Peng et al. 2000, Compernolle et al. 2002). A tamoxifen-inducible *Hif-2α* knockout mouse model (Cre protein fused to an estrogen receptor which specifically binds tamoxifen) lead to failure of erythropoiesis, pancytopenia, retinopathy (Gruber et al. 2007, Skuli et al. 2009). Conditional *Hif-2α* knockout specifically in endothelial cells (in a cre-lox model driven by the vascular endothelial cadherin [VE-cadherin] promoter) leads to defective vessel integrity and reduced tumor growth (Skuli et al. 2009). Mice with a *Hif-3α* knockout were viable, but showed an enlargement of the right ventricle and impaired lung remodeling (Yamashita et al. 2008).

These important findings indicate that HIF-1α signaling in ECs might not be as crucial for vessel formation as assumed and support the finding presented in this dissertation that ECs are not the hypoxia sensors initiating neo-vasculogenesis. In this chapter, we will discuss the role of HIF-1α walking the fine line between functional and diseased cells.

4.1.7.1. HIF in innate and adaptive immune response

It has been established that lymphatic organs and inflammatory sites are hypoxic regions. Therefore, cells of the immune system must be able to adapt to O_2 level changes. However, there is a fine line between mounting a sufficient immune response and reaching the levels that would lead to autoimmunity (Gale, Maxwell 2010).

Cells of the innate immune system (neutrophils, macrophages, mast cells, and natural killer cells) react rapidly in response to pathogens by destroying them and activating adaptive immune cells (Eltzschig, Carmeliet 2011). This process is mediated at least in part by HIF-1α up-regulation in the hypoxic environment of the inflammatory site. Increased HIF-1α levels in myeloid cells inhibit apoptosis and stimulate a shift to anaerobic glycolysis. This mediates aggregation, motility, invasiveness, and bactericidal activity of myeloid cells (Eltzschig, Carmeliet 2011).

In adaptive immunity, there have been contradictory findings on the role of HIF-1α for the differentiation and activation of different T cell subtypes. Firstly, HIF-1α has been shown to have an anti-inflammatory role. HIF-1α negatively regulates T cell receptor signaling, which leads to decreased T cell survival, activation and "activation induced cell death" (AICD) in T cells (Neumann et al. 2005). Additionally, HIF-1α induces a shift from type 1 helper T cells (Th1), which stimulate functions of macrophages and cytotoxic T cells, to a type 2 helper T cell (Th2) phenotype, which negatively regulates Th1 cells by producing interleukin-10 (IL-10) and decreasing interferone-γ (IFN-γ) levels (Eltzschig, Carmeliet 2011).

This anti-inflammatory response is contradicted by the recently discovered pro-inflammatory effect of HIF-1α inducing differentiation of CD4+ T cells to Th17-T-cells (Dang et al. 2011). HIF-1α tips the balance from maturation of regulatory T cells (Treg) to Th17-T-cells, a subset of T helper cells producing IL-17. HIF-1α induces differentiation of Th17-T-cells by expressing the Th17 transcription factor RORγt (Bader, Hsu 2012, Dang et al. 2011). Additionally, HIF-1α regulates inflammatory response by mediating the transcription of pro-inflammatory cytokines, including TNF-α, TGF-β and NF-κB (Manalo et al. 2005). Hence, HIF-1α is an important player in the body's inflammation processes.

Additionally, HIF-1α plays an important role in the development of chronic lymphatic leukemia (CLL) (Bader, Hsu 2012). Ghosh et al. found that HIF-1α is

over-expressed in CLL B cells under ambient air conditions (Ghosh et al. 2009). The constitutive expression of HIF-1α in CLL B cells was linked to a defect in pVHL expression by the microRNA miR-92-1 and explains the enhanced VEGF secretion found in CLL cells (Ghosh et al. 2009). Considering the findings presented in this dissertation, the immune regulatory function of mesenchymal cells could also be re-examined focusing on the role of HIF-1α. Perhaps therapies targeting mesenchymal progenitors' HIF-1α expression could also be adapted for auto-immune diseases.

4.1.7.2. HIF in hematopoietic stem and progenitor cell (HSPC) homeostasis

Recently, it has become evident that HIF-1α is necessary for the cell cycle quiescence of HSPCs (Takubo et al. 2010, Bader, Hsu 2012). HSPCs are considered to reside in a hypoxic zone in the bone marrow (endosteal niche) and have been shown to express high levels of HIF-1α maintaining their undifferentiated phenotype and quiescence in a dose dependent manner. HIF-1α deficiency resulted in increased proliferation, differentiation and senescence of HSPCs. In an interferone-inducible *Hif-1α* knockout mouse model (floxed *Hif-1α* mice bred with interferone inducible myxovirus resistance-1 (Mx-1)–Cre mice) it has been shown that *HIF-1α*$^{-/-}$ HSPCs fail to home to the bone marrow after transplantation (Takubo et al. 2010). In contrast, over-expression of HIF-1α by pVHL deletion (floxed *VHL* mice bred with interferone inducible myxovirus resistance-1 (Mx-1)–Cre mice) resulted in increased numbers of non-functional HSPCs and increased quiescence (Takubo et al. 2010, Bader, Hsu 2012). pVHL$^{-/-}$ HSPCs failed to home to the bone marrow and showed increased apoptosis in transplantation experiments. Additionally, it has been shown that loss of HIF-1α leads to a decreased number of hypoxic HSPCs in the bone marrow linking intracellular oxygenation to HIF-1α expression *in vivo*. These findings imply a pivotal role for HIF-1α in HSPC homeostasis and disease. It is not clear whether the role of HIFs in HSPCs is exclusively executed by an O_2-dependent regulation rather than being influenced by the various growth factors and adhesion molecules in the bone marrow niche.

4.1.7.3. HIF in VHL disease

The tumor suppressor pVHL is part of the ECV and leads to the degradation of hydroxylized HIFs (see also chapter 4.1.2.). Patients with a so-called VHL disease carry a heterozygous germ-line mutation of the *VHL* gene on chromosome 3 (3p25-26). One example is the transition of a cysteine (C) > phenylalanine (F) on p162 of chromosome 3 (C162F). A somatic mutation of the second allele then leads to a cell type-related pVHL loss-of-function and inhibits degradation of HIF-1α (Ang et al. 2002). pVHL loss-of-function mediates increase in HIF expression and therefore augmented levels of angiogenesis-related factors including VEGF, EPO, transforming growth factor-β (TGF-β), platelet derived factor (PDGF), CXC-motiv-chemokinrezeptor 4 (CXCR4) and stromal derived factor-1 (SDF-1).

In 1904, the German ophthalmologist Eugen von Hippel was the first to describe the occurrence of little knots of capillaries in the eye, today known as angiomas (Von Hippel E 1904). Almost three decades later, the Swedish pathologist Arvid Lindau discovered comparable angiomas in the brain and spine (Lindau 1927). Since its initial description, so-called VHL disease has been found to be responsible for the regulation of many hyper-vascularized tumors including angiomatosis, hemangioblastomas, pheochromocytoma, pancreatic tumors and renal cell carcinoma (RCC) (Bracken, Whitelaw & Peet 2003, Bader, Hsu 2012). Recently, the contribution of mutations in the *VHL* gene on hematopoietic cells and inflammation has been a field of extensive research. (Gale, Maxwell 2010, Takubo et al. 2010, Bader, Hsu 2012).

A rare homozygous mutation of the *VHL* gene, known as Chuvash mutation (R200W), is responsible for the polycythemia (over-production of erythrocytes) in descendants of the Chuvash Autonomous Republic of Russia. Chuvash patients carry a missense mutation of a Cysteine (C) > Tyrosine (T) on p598 (C598T) of chromosome 3. This transition leads to the replacement of a tryptophan instead of arginine at codon 200 (R200W) of the pVHL (Ang et al. 2002). This mutation affects one target binding site of pVHL, which leads to loss-of-function. Recently, additional missense mutations of pVHL (for example, H191D) have been identified in patients with Chuvash polycythemia in different ethnic groups. Polycythemia in Chuvash polycythemia patients is either a consequence of hypersensitivity of mutant erythrocyte progenitors to EPO (known as primary polycythemia or

polycythemia vera) or over-expression of EPO, not only in the normal EPO-producing kidney fibroblasts but in all R200W carrying cells (known as secondary polycythemia). Recently, the mechanism behind the hypersensitivity of R200W carrying erythrocyte precursors to EPO (primary polycythemia) has been discovered (Russell et al. 2011). In normal cells, binding of EPO to the RTK EPO receptor (EpoR) mediates phosphorylation of the tyrosine kinase JAK2, which subsequently phosphorylates the transcription factor STAT5. Activation of STAT5 mediates its dimerization and translocation to the nucleus where it regulates the expression of target genes responsible for proliferation, survival and differentiation of hematopoietic cells. Additionally, STAT5 triggers a negative feedback loop by activating expression of components of the ECS complex. JAK2 is in turn ubiquitinylated by pVHL, which determines the half-life of the JAK2-STAT5 signaling pathway. In Chuvash patients JAK2 signaling is not decreased due to impaired pVHL function and leads to continuous and hypersensitive signaling upon EPO binding. This results in excessive production of erythrocytes.

In secondary polycythemia pVHL, loss-of-function results in reduced HIF degradation. Consequently, increased HIF-1α levels lead to over-expression of HIF target genes including EPO, which in turn also mediates excessive production of red blood cells (RBCs) (Russell et al. 2011).

However, unlike other VHL mutations, Chuvash mutation carriers do not show increased tumor formation. Vice versa, normal VHL disease patients do not show full-blown polycythemia. The underlying mechanisms distinguishing between pVHL mutations leading to Chuvash polycythemia and tumor formation are still unclear (Ang et al. 2002, Russell et al. 2011, Bader, Hsu 2012).

4.1.7.4. HIF in tumor development

In 1924, Otto Warburg discovered the paradox mechanism that cancer cells uniquely produce ATP by the conversion of glucose to lactic acid even in the presence of O_2. This process of aerobe glycolysis – known as the “Warburg effect” – led him to the hypothesis that cancer cells have impaired mitochondrial function. Even though this hypothesis was proven incorrect, the “Warburg effect” of tumor cells has since been the subject of intensive research (Warburg, Posener & Negelein 1924, Seagroves et al. 2001).

One common feature of many primary tumors is hypoxia due to insufficient blood supply. Hypoxia-induced HIF-1α stabilization is one explanation for increased glucose uptake (GLUT1/3 expression) and the switch to glycolysis (lactate dehydrogenase (LDH) and PDH kinase expression) in rapidly growing tumors. However, specific genetic alterations of the HIF-signaling pathway have been discovered in tumor cells leading to normoxic HIF stabilization or over-expression. Genetic mutations leading to elevated HIF-1α levels were detected in several cancers, including breast, ovarian, uterus (endometrial), esophageal, bladder, colon, skin, prostate, pancreatic and cervical cancer, as well as in RCC carcinomas, non-small-cell lung cancer, Non-Hodgkin lymphomas, cerebral hemangioblastoma, gastro-intestinal tumors and squamous-cell carcinomas of the oropharyngeal region, head and neck. In most cancer patients, increased HIF-1α levels correlate with poor prognostic outcome and increase of mortality (Semenza 2003, Bracken, Whitelaw & Peet 2003).

Genetic alterations include loss-of-function of HIF suppressors (e.g. pVHL), gain-of-function of oncogenes (e.g. ERBB2) or increased signaling through RTKs (e.g. by endothelial growth factor (EGF) or Insulin-like growth factor- 1 (IGF-1)). These mutations result inter alia in HIF-mediated aerobic glycolysis even in the presence of sufficient oxygen. (Warburg, Posener & Negelein 1924, Semenza 2003). Besides metabolic reprogramming, HIF-1α over-expression in cancer cells is also associated with the transcription of genes that play a crucial role in invasion, metastasis, radiation resistance, inflammatory cell recruitment, motility, survival, stem cell maintenance, and angiogenesis (Bertout, Patel & Simon 2008, Semenza 2011, Semenza 2012).

Folkman first described the importance of angiogenesis for the growth of solid tumors (Folkman 1971, Isner, Asahara 1999, Critser, Yoder 2010). HIF-1α over-expression in cancer cells was found to result in increased production of angiogenic factors, including VEGF (Carmeliet, Jain 2011a). The secreted VEGF was shown to bind to the VEGF receptors on the surface of proximal endothelial cells and to activate the RTK-mediated proliferation and migration of endothelial cells. This leads to enhanced angiogenesis, which supplies the tumor with blood, oxygen and nutrients and allows it to grow. Tumor vessels are frequently disorganized and leaky, which leads to increased metastasis. In the past decade, starvation of the tumor by inhibition of tumor angiogenesis has been a promising

strategy of anti-tumor therapy. The application of monoclonal antibodies against VEGF, e.g. Bevacizumab or Sunitinib, in a mouse tumor model *in vivo* resulted in the decrease of tumor size. Unfortunately, the encouraging anti-tumor effects in mouse experiments could not be observed in clinical studies, which implies that VEGF is not the only factor responsible (Carmeliet, Jain 2011a). Additionally, anti-VEGF therapy has even been shown to increase the formation of metastasis.
In the past few years, Carmeliet proposed that inhibiting tumor vessel formation might not be efficient enough to battle solid tumors (Carmeliet, Jain 2011a). Instead, he introduced the new concept of increasing stable and leak-proof (termed vessel normalization) vessels. A high number of stable tumor vessels are considered to enhance susceptibility of tumors to chemotherapeutics and decrease the possibility of tumor cells escaping into the blood system and metastasizing (Carmeliet, Jain 2011a). Both, therapeutic inhibition and induction of tumor angiogenesis requires the elucidation of the complex chemical and cellular key regulators, which initiate new vessel formation.

4.1.7.5. HIF in pulmonary hypertension progression

Chronic lung disease or long-term exposure to high altitudes can result in progressive and fatal hypoxic pulmonary hypertension. When hypoxia occurs in otherwise healthy patients, systemic arteries dilate to increase perfusion and pulmonary arteries constrict to redirect blood to ventilated regions of the lung. In chronic pulmonary hypertension, constriction of the lung arteries occurs permanently throughout the lungs, resulting in progressive tissue hypoxia and right ventricular failure. In patients with pulmonary hypertension, elevated levels of HIF-1α can be found in vascular smooth muscle cells (SMCs) surrounding the endothelial cell layer. Increase in HIF levels in SMCs leads to increased expression of transient receptor–potential calcium channels 1/6 and sodium-hydrogen exchanger 1, and decreased expression of voltage-gated potassium channels. Changes in intracellular potassium, calcium and hydrogen ion concentrations results in SMC hypertrophy, proliferation, depolarization and contraction. Increased proliferation leads to a thicker layer of SMCs surrounding the artery and a decreased arterial lumen, which results in increased pulmonary vascular resistance. Vascular resistance is defined as the arterial pressure against

blood flow, which must be overcome to transport blood in the circulation (Semenza 2011, Semenza 2012). Pulmonary hypertension results in a severe decrease in quality of life and increased mortality.

Interestingly, human populations like Tibetans who live in high altitude regions with only 15% O_2 as compared to 20% O_2 at sea level, have adapted to these conditions without developing pulmonary hypertension. In sea level residents subjected to high altitudes, the hypoxia-induced erythrocytosis is not sufficient to increase oxygen availability, but results in high blood viscosity and polycythemia. However, Tibetans living in high altitudes neither show increased hemoglobin nor enlarged pulmonary arteries and have normal aerobic tissue metabolism. Genetic analysis has revealed hereditary yet uncharacterized mutations of *Hif-2α*, *Fih* and *Phd2* in the Tibetan population (Semenza 2011). The exact mechanisms behind these mutations remain to be resolved.

4.1.7.6. HIF in ischemic cardiovascular disease

Cardiovascular diseases are the number one cause of death worldwide. Thrombosis and arteriosclerosis lead to restricted blood flow and result in insufficient supply of oxygen and nutrients to downstream regions. Depending on the affected region, tissue hypoxia (ischemia) may progress to myocardial infarction, stroke or critical limb ischemia (Bosch-Marce et al. 2007). Normally, ischemia leads to an up-regulation of HIF-1α and HIF-2α in the ischemic cells. Stabilized HIF-1α mediates the expression of angiogenic factors including VEGF, basic fibroblast growth factor (bFGF), SDF-1, placental growth factor (PlGF) and angiopoietin-2 (ANG-2). Secretion of angiogenic factors promotes new vessel formation to restore blood flow. (The different mechanisms of new vessel formation including angiogenesis, intussusceptions and vasculogenesis will be discussed in detail in chapter 4.2.) Additionally, mesenchymal stem and progenitor cells are recruited to the ischemic region and support the newly forming vessels. However, in patients with coronary stenosis a single-nucleotide polymorphism that replaces the HIF-1α proline 582 against a serine has been found to occur five times more frequently than in control volunteers (Semenza 2012). Additionally, age and diabetes are major risk factors that impair adaptive vascular response, due to deficient HIF-1α activity (Bosch-Marce et al. 2007, Semenza 2011).

In retinopathy of prematurity of newborns, the hypo-vascularized eye becomes ischemic (Rey, Semenza 2010). The stabilization of HIF-1α at the site of hypoxia leads to excessive formation of disorganized and leaky new blood vessels. This results in retinal detachment and blindness. Intraocular injections of different HIF-1α and VEGF inhibitors have shown promising results in improving the vision of treated patients.

Taken together HIF-1α is involved in a variety of illnesses and is therefore a promising target for the development of novel therapies.

4.1.7.7. Therapeutics modulating HIF activity

Various therapies have been designed targeting HIF-1α activity. Depending on the disease, therapies have been developed to either increase HIF-1α expression (in limb ischemia, heart attack, and stroke) or inhibit HIF-1α expression (in tumor angiogenesis, VHL disease and pulmonary hypertension).

Therapies inducing HIF-1α activity

In a hind limb ischemia mouse model, the intramuscular administration of a recombinant adenovirus encoding for a constitutively active HIF-1α (AdCA5) increased the secretion of angiogenic factors (e.g. VEGF and PlGF) and was able to restore perfusion (Rey et al. 2009, Semenza 2012). To test the influence of AdCa5 on angiogenic cell homing to the ischemic site, a combinational therapy was designed consisting of intramuscular injection of AdCA5 in female mice and subsequent intravenous injection of 5×10^5 male bone marrow-derived angiogenic cells (BMDACs) one day later. BMDACs isolated from density gradient separated bone marrow aspirates were initially defined as having an endothelial phenotype. However, proper characterization revealed that BMDACs are in fact largely composed of hematopoietic cells (Prokopi et al. 2009). AdCA5 injection increased BMDAC homing to ischemic limb (208 ± 29 copies) in comparison to untreated control mice (51 ± 7 copies). Combinational injection of BMDACs i.v. and intramuscular AdCA5 significantly increased vascular perfusion compared to AdCA5 injection without BMDAC administration (0.80 ± 0.02 vs. 0.69 ± 0.02, respectively). The pre-treatment of BMDACs with DMOG before injection was necessary to ensure their survival in the ischemic tissue and increased their

adherence to the vascular cells. In the clinical setting, a recombinant adenovirus containing a bHLH-PAS domain fused to a herpes simplex virus VP16 was injected into patients with critical limb ischemia. Neither side effects nor beneficial therapeutic effects were registered in these patients after therapy. One idea could be a combinational therapy with AdCA5 treated mesenchymal together with endothelial precursors. Due to the pro-angiogenic properties of these cells, they could more likely yield a clinical effect in comparison to hematopoietic cell administration.

DMOG and DFO are PHD inhibitors and therefore lead to HIF-1α stabilization under normoxia (see Figure 2 and Figure 3). As described earlier, they compete for iron in the active site of PHDs and therefore inactivate the dioxygenases' function. These iron chelators have been used for over 20 years against iron intoxication for patients with chronic anemia who repeatedly receive red blood cell transfusions (Semenza 2012). Recently it has been shown that DFO increases skin wound healing in a diabetic mouse model by increasing HIF-1α function and thereby promoting neo-vasculogenesis (Thangarajah et al. 2010, Semenza 2012). Based on the findings described in this work that HIF-1α stabilizing mesenchymal stem and progenitor cells (MSPCs) initiate neo-vasculogenesis, it could be a promising cell therapeutic strategy to increase HIF-1α stabilization of MSPCs by DMOG or DFO pre-treatment and inject these cells into ischemic tissue to increase vascular repair (Hofmann et al. 2012).

Therapies inhibiting HIF-1α activity

In 2002, Rapisarda et al. introduced a luciferase reporter under the control of three HRE-elements into a stable cell line and performed a mass screening of 2000 chemical compounds to test their ability to act against HIF-1α (Rapisarda et al. 2002). Since then, large numbers of small molecule inhibitors have been developed against different components of the HIF-1α pathway (Park, Chun & Kim 2004).

HIF-1α synthesis can be inhibited up-stream by blocking the RTK (genistein) or targeting of PI3K (wortmannin) and mTOR (rapamycin), or by inhibition of the topoisomerase I (topotecan) (see Figure 4). Chemical compounds influencing HIF-1α stability target HSP90 (17-AAG, 17-allylaminogeldenamycin) (see Figure 2) or directly degrade the COOH-terminus of HIF-1α (YC-1, 3-(5'-hydroymethyl-2'furyl)-

1-benzyl-indazole) (see Figure 3) (Yeo et al. 2003, Semenza 2012,). The heterodimerization of HIF-1α/1β can be inhibited by the antiseptic acriflavine (see Figure 2). DNA-binding of HIF-1α can be blocked by the anthracycline doxorubicin and the antibiotic echinomycin (see Figure 2). HIF-1α signal transduction can be inhibited by imatinib (PDGFR tyrosine kinase inhibitor), erlotinib (EGFR inhibitor) and trastuzumab (ERBB2 receptor tyrosine kinase inhibitor) (Semenza 2012). The underlying inhibitory mechanism for most small-molecule HIF-1α inhibitors is not known.

4. 2. Introduction to vessel formation

During the growth of mammalian embryos, the paraxial and lateral plate mesoderm gives rise to the “hemangioblast” which is the precursor of both hematopoietic stem cells (differentiating into all blood cells) and the angioblast (differentiating into endothelial progenitors) (Risau 1997). Through a process termed “vasculogenesis”, the endothelial progenitor cells (EPCs) of the embryo assemble to form a first capillary plexus, also termed “blood island”. To meet the rush demand of new vessel formation during later embryogenesis and post-natal growth, different modes of vessel generation have evolved (Isner, Asahara 1999). “Angiogenesis” describes the sprouting of new capillaries from pre-existing blood vessels. Existing vessels can divide by “intussusception” into two separate vessels. “Neo-vasculogenesis” defines the postnatal formation of new blood vessels by migration and differentiation of circulating EPCs in response to tissue demands (Figure 5-7). Subsequently, the newly formed vessels undergo maturation by recruiting mesenchymal precursors, which surround and stabilize the EC layer (Risau 1997). The following chapter provides a detailed look at the textbook knowledge of the different modes of new vessel formation and the role of HIF.

4. 2.1. Angiogenesis

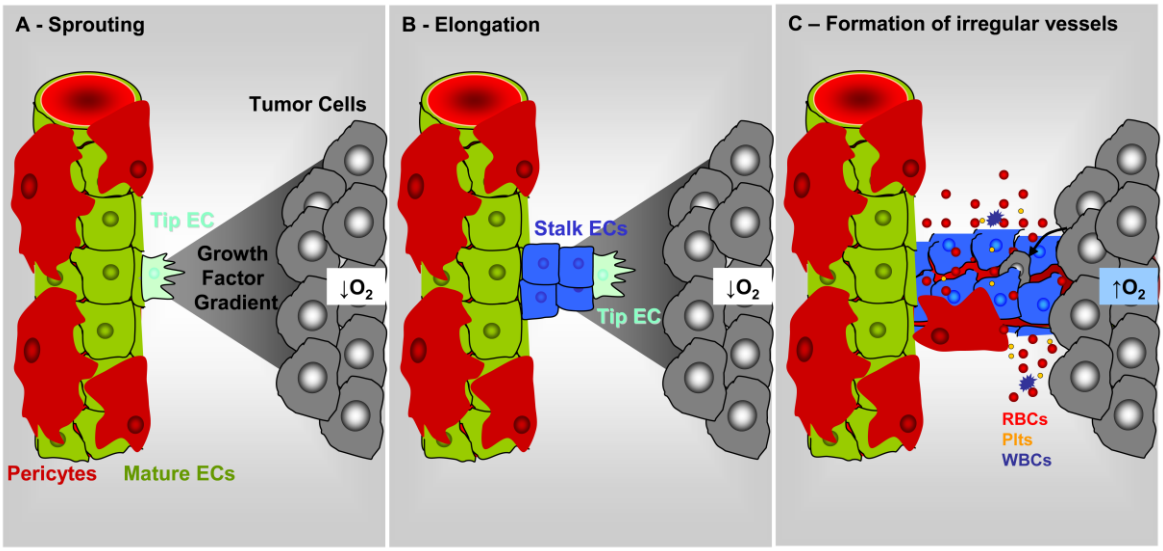


Figure 5. Angiogenesis (modified from P. Carmeliet & R.K. Jain, Nature, 2011)
Angiogenesis (in this case tumor angiogenesis) describes the sprouting of capillaries from a pre-existing vessel. (A) In response to an angiogenic signal (provided from the tumor cells) a tip endothelial cell (EC) is selected that forms philopodia and migrates towards the signal. (B) The neighboring ECs become stalk cells that elongate and follow the tip cell. The newly formed vessel recruits mesenchymal cells to act as stabilizing pericytes. However, tumor vessels are irregular and leaky (with leakage of red blood cells (RBCs), platelets (Plts) and leukocytes (WBCs) and plasma) which allows tumor cells to escape into the blood stream and form metastasis.

Angiogenesis describes the budding of new vessels from the pre-existing vasculature in response to angiogenic stimuli (VEGF, ANG-2, NOTCH ligand, Tie-2) secreted by HIF-1α stabilizing hypoxic or tumor cells (Figure 5) (Risau 1997, Carmeliet 2005, Carmeliet, Jain 2011a). Angiogenesis is a complex process and involves proliferation and elongation of endothelial cells, and also vessel maturation by mesenchymal cell recruitment (Figure 5).
Firstly, ANG-2 leads to pericyte detachment from the existing vessel. Subsequently, matrix-metalloproteinases (MMPs) remodel the basement membrane and VE-cadherin loosens the EC junctions. The vessel becomes more permeable and leaking plasma proteins contribute to the formation of an extracellular matrix (ECM) scaffold (Risau 1997, Carmeliet 2005, Carmeliet, Jain 2011a). Through an as of yet unknown mechanism, one endothelial cell of the

uncovered vessel wall is selected to become a leading tip cell to ensure organized and directed vessel sprouting (Gerhardt et al. 2003). The tip cell forms filopodia, which sense the angiogenic factors (VEGF, neuropilins, NOTCH ligands) and navigate towards the angiogenic signal gradient (Carmeliet, Jain 2011a). Subsequently, the neighboring endothelial cells (ECs), called stalk cells, lengthen the sprouting vessel through proliferation and/or elongation and form a perfused lumen (mediated by NOTCH, NOTCH ligand, PlGF, bFGF). Stalk cells of the perfused lumen resume a phalanx state and the newly formed vessel matures by recruiting stabilizing pericytes (at least in part mediated by secretion of TGF-β, PDGF-BB, ANG-1, NOTCH, bFGF). For additional stabilization against the shear stress of blood flow, tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) are secreted, which mediate the formation of a basement membrane and tight EC junctions. In the event that the newly formed vessel is not perfused, it regresses (Carmeliet, Jain 2011a).

4. 2. 2. Intussusception

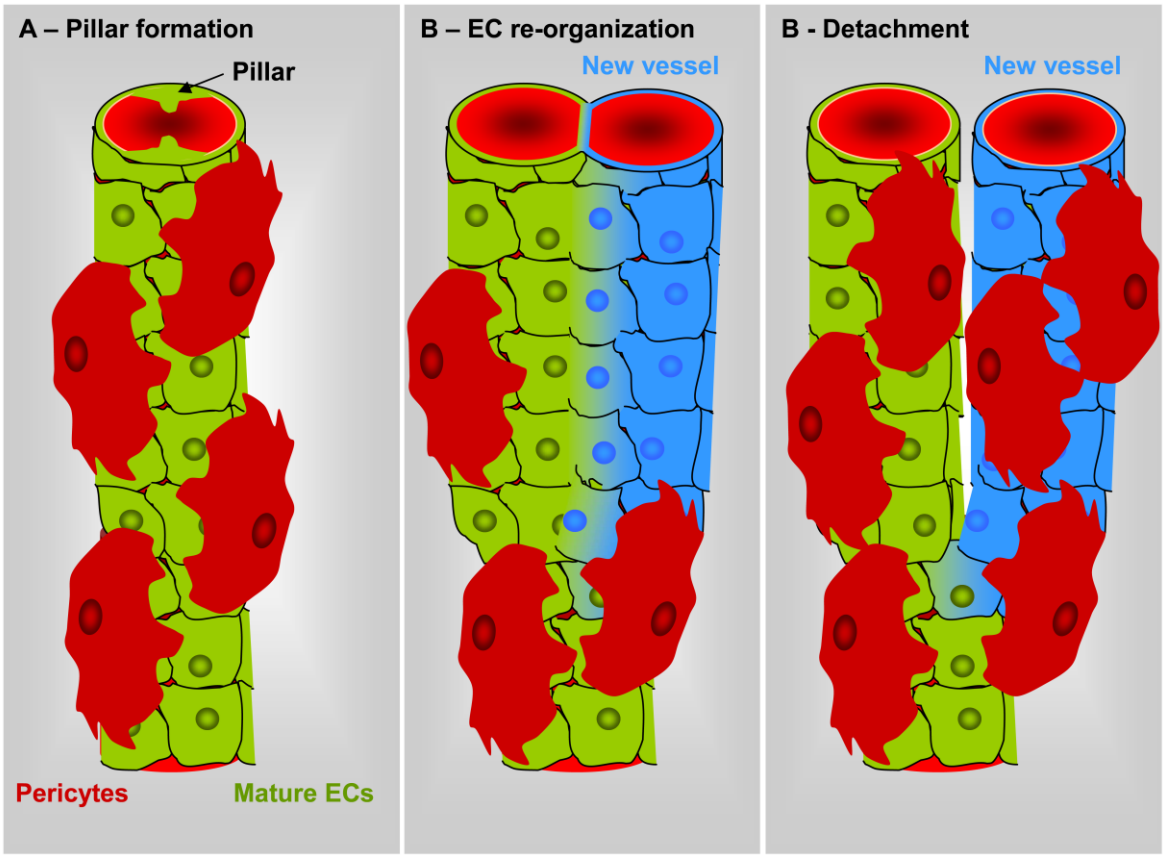


Figure 6. Intussusception (modified from P. Carmeliet & R.K. Jain, Nature, 2011)
Intussusception describes the longitudinal separation of one existing vessel into two. Triggered by an unknown mechanism, pillars invade the vessel lumen and connect the two vessel walls (A). The pillars contract and endothelial cells (EC) re-organize and separate the vessel to form two new vessels (B). Alongside the connected two vessel parts the ECs detach and form two vessels (C).

Intussusception is a mechanism of non-sprouting or splitting angiogenesis, which allows rapid expansion of the vascular network (Figure 6) (Djonov, Baum & Burri 2003). Electron microscopy scanning revealed the formation of tiny EC pillars invading the vascular lumen at sites of corrosion casts. These pillars expanded in four consecutive steps to longitudinally split the vascular lumen and form two new capillaries. Firstly (Phase I), pillars connect the two opposing capillary walls. In Phase II, the ECs loosen their junctions and re-organize along the pillar, which leads to a contraction along the interstitial pillar core. Subsequently (Phase III), pericytes invade this core and cover the newly formed two-lumen parts. Finally (Phase IV), the two lumens detach and form two new vessels (Djonov, Baum &

Burri 2003). As of yet, the underlying mechanism of this process has not been thoroughly described.

4. 2. 3. Neo-Vasculogenesis

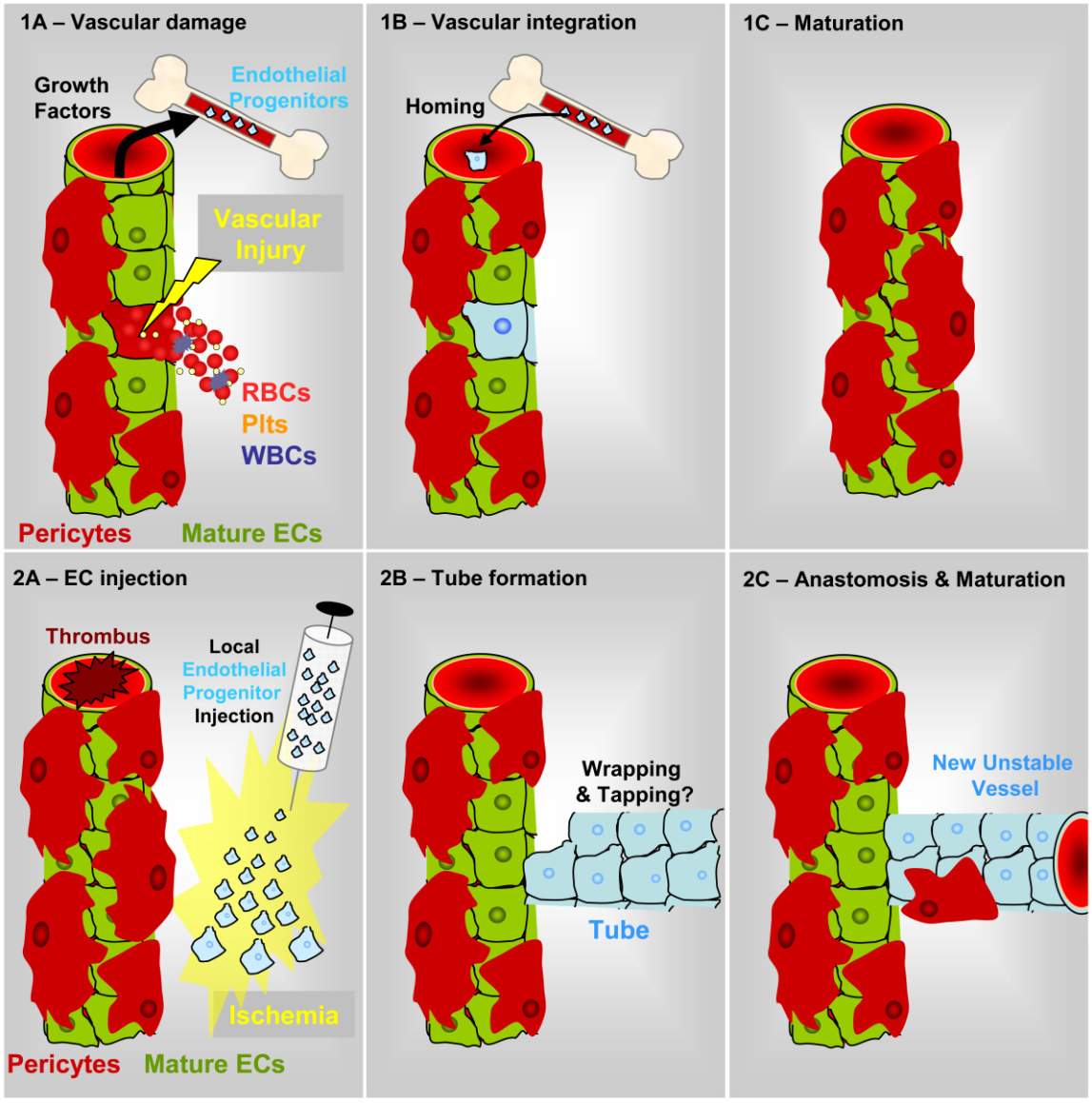


Figure 7. Neo-vasculogenesis and therapeutic neo-vasculogenesis (modified from P. Carmeliet & R.K. Jain, Nature, 2011)

Neo-vasculogenesis is described as occuring after vascular damage (1A-1C) and during therapeutic vasculogenesis (2A-2C). After vascular damage (with leakage of red blood cells (RBCs), platelets (Plts) and leukocytes (WBCs) and plasma) bone marrow-derived or vessel wall-derived somatic endothelial progenitors are recruited (1A). Endothelial progenitors integrate into the vessel wall (1B) and subsequently maturate by recruitment of mesenchymal cells (1C). For therapeutic vasculogenesis after thrombotic (depicted here), ischemic, toxic or metabolic injury,

endothelial cell (EC) progenitors are injected into the site of ischemic tissue damage (2A). ECs arrange, form tube structures (2B) and degrade pericyte coverage. The newly formed tube is considered to connect to the existing vasculature via a mechanism called 'wrapping and tapping'. Following vessel perfusion mesenchymal cells are recruited and act as stabilizing pericytes (2C). Vessels performed by this method are not stable and regress within months.

Neo-vasculogenesis describes the post-natal formation of new vessels and the regeneration of damaged vasculature through migration, proliferation and differentiation of circulating and/or somatic endothelial progenitors (Figure 7). In the search for true endothelial progenitors, different isolation methods have been developed and EPCs have frequently been renamed (Prater et al. 2007, Hirschi, Ingram & Yoder 2008, Critser, Yoder 2010, Strunk 2011).

In 1997, Asahara et al. initially described the isolation of endothelial progenitors from peripheral blood, which could contribute to vascular regeneration (Asahara et al. 1997). These authors used peripheral blood mononuclear cells (MNCs) enriched for $CD34^+$-cells (15.7 ± 3.3% of selected cells expressed CD34), and plated them on fibronectin-coated plates *in vitro* (1000 cells/mm^2). The non-adherent cell fraction was then transferred to new plates and formed cell clusters. These clusters, named endothelial progenitor cells (EPCs) showed a heterogeneous morphological appearance, composed of round cells in the center surrounded by radiating spindle-shaped acetylated low-density lipoprotein (acLDL)-uptaking cells. Phenotype analysis of these cells confirmed expression of typical EC-markers such as CD34, CD31, VEGFR2, Tie-2 and E-selectin, but also the expression of hematopoietic markers like CD14, CD45, and CD115. After injection of $CD34^+$ sorted cells into a hind limb ischemia mouse model, Asahara et al. found that 13.4 ± 5.7% of the injected cells incorporated into the damaged vasculature compared to only 1.6 ± 0.8% of injected $CD34^-$ cells. (Asahara et al. 1997). However, the expression of hematopoietic markers made the true nature of EPCs questionable.

Six years after the discovery of EPCs, Hill et al. proposed a strong correlation between the number of circulating endothelial progenitors and the risk that patients develop cardiovascular diseases (Hill et al. 2003). In a slightly modified assay, they used 1 x 10^6 non-adherent MNCs which were plated on fibronectin-coated 24-well dishes. Colonies formed within 4-9 days, were enumerated and correlated

to the Framingham risk score. Similar to the cells isolated by Asahara, these colonies had the ability to take up acLDL and expressed the typical EC markers CD31, CD105, CD144, CD146, von Willebrand factor (vWF), and VEGF-receptor 2 (VEGF-R2). Cells isolated with the Hill protocol were termed ‘’colony forming unit endothelial cells’’ (CFU-ECs) and were introduced as a promising biological indicator for vascular diseases (Hill et al. 2003). However, phenotypical characterization of CFU-ECs showed that they were composed of a heterogeneous mixture of hematopoietic cells (Case et al. 2007, Timmermans et al. 2007, Yoder et al. 2007). It was shown that CFU-EC resulted from a functional cross-talk between monocytes and T cells, which could mimic ECs *in vitro* due to their ability to uptake acLDL, bind to leptin and express endothelial markers (CD31, CD105, CD144) (Rohde et al. 2006, Rohde et al. 2007). Depletion of monocytes ($CD14^+$) or T cells ($CD2^+$) from MNCs did not allow CFU-EC to form *in vitro*, which argues for the dependency of these colonies on the presence of hematopoietic cells (Rohde et al. 2007). Additionally, it was reported that monocytes could secrete angiogenic growth factors, which explains their pro-angiogenic function *in vitro* (Rohde et al. 2007). Following these findings, CFU-EC were re-defined as CFU-Hill and are no longer considered to be endothelial progenitors (Hirschi, Ingram & Yoder 2008).

Another attempt to identify putative endothelial progenitors entailed culturing peripheral blood mononuclear cells (MNC) under angiogenic differentiation conditions (Vasa et al. 2001a, Vasa et al. 2001b, Vasa et al. 2001c). The whole MNC fraction was cultured on fibronectin-coated plates in a specific endothelial growth medium (EBM-2; Lonza) supplemented with EGF, VEGF, IGF, bFGF, cortisone, ascorbic acid and fetal bovine serum (FBS). Within four days, colonies formed and were named ‘’circulating angiogenic cells’’ (CACs). However, CACs, as EPCs and CFU-Hill, showed only low if any proliferative potential and expressed several hematopoietic cell markers (CD14, CD45, and CD115) again indicating the hematopoietic origin of these proposed endothelial progenitors (Yoder et al. 2007, Rohde et al. 2007). This strengthened the argument that the initial characterization of EPCs based on expression of endothelial cell surface-markers (CD31, CD105, CD144, VEGFR), AcLDL up-take and pro-angiogenic potential was insufficient as ECs and HSPCs have several properties in common (Yoder et al. 2007, Rohde et al. 2007). The need to include evaluation of

hematopoietic markers on isolated cells in order to characterize them as putative endothelial progenitors was evident.

A major breakthrough toward a better understanding of vascular biology relates to the isolation and identification of ECFCs (Ingram et al. 2004, Ingram et al. 2005, Reinisch et al. 2009). Almost ten years after the introduction of adult circulating EPCs, Yoder et al. described a protocol for the isolation of clonally proliferating EPCs from peripheral blood (Yoder et al. 2007). These authors cultured MNCs on collagen I-coated surfaces and non-adherent cells were discarded. Within 7-9 days of culture, colonies formed which were termed ECFCs. These colonies displayed a homogenous and typical EC-like cobble stone morphology. Phenotypic characterization revealed that ECFCs express typical EC surface markers (CD31, CD105, CD144, CD146, vWF, and VEGF-R2), but, most importantly, do not express hematopoietic markers (CD45, CD14 and CD115). Additionally, ECFCs showed robust proliferative potential and were able to be replated to form new clonogenic progeny. When implanted subcutaneously into a mouse model *in vivo* together with an extracellular matrix, ECFCs formed perfused vessels. Using clonogenicity assays, Ingram et al. classified a hierarchy of high proliferative potential (HPP) ECFCs which formed colonies of more than 500 cells within 14 days and low proliferative potential (LPP) ECFCs which formed colonies of less than 500 cells (Ingram et al. 2004, Ingram et al. 2005).

Others later provided evidence for a more robust vessels formation when ECFCs were implanted together with different kinds of stromal cells (Au et al. 2008, Melero-Martin et al. 2008, Reinisch et al. 2009, Traktuev et al. 2009, Koike et al. 2004). We have recently introduced a standardized method of ECFC isolation and large-scale propagation from peripheral and cord blood under serum-free conditions (Reinisch et al. 2009, Hofmann, Reinisch & Strunk 2009, Hofmann, Reinisch & Strunk 2012). ECFCs generated with this protocol formed colonies within 12 days, displayed a typical EC (and not hematopoietic) phenotype, showed robust proliferation potential, full hierarchy and genomic stability. Together with mesenchymal progenitors, ECFCs formed stable perfused vessels in Matrigel *in vivo* within 7 days (Reinisch et al. 2009, Hofmann, Reinisch & Strunk 2009, Hofmann, Reinisch & Strunk 2012).

One of the open questions in therapeutic neo-vasculogenesis is how the newly formed vessels connect to the existing vasculature and become perfused. Recently, Cheng et al. tracked fluorescence-labeled ECs in real time *in vivo* (Cheng et al. 2011). In this study the authors made the surprising observation that the implanted human ECs formed tube structures *in vivo* and subsequently connected to the host vasculature within 4 weeks via a mechanism termed "wrapping and tapping". In comparison to various other groups, initial vessel formation observed by this group was detected at a very late time point (28 days versus 5 days) (Reinisch et al. 2009, Melero-Martin et al. 2008, Traktuev et al. 2009, Hofmann et al. 2012). The authors described that the implanted ECs together with 10T1/2 mural mesenchymal cells initially elongated to form tube-like structures within the ECM. In parallel, host vessels invaded the implanted ECM by means of angiogenesis. Subsequently, the ECs of the newly formed tube wrapped around the invading host vessel and started degrading its basement membrane. As a next step, the implanted ECs tapped into the host vessel thereby opening it up for blood flow. Finally, the perfused new vessel matured by recruiting resident pericytes covering the vessel to ensure stabilization. An explanation for the delayed vessel perfusion could be that the co-transplanted 10T1/2 mural mesenchymal cells are possibly not as potent in their ability to initiate neo-vasculogenesis of human ECs as the human MSPCs used by the other groups (Melero-Martin et al. 2008, Traktuev et al. 2009, Reinisch et al. 2009, Hofmann et al. 2012). Nevertheless, additional questions regarding the exact mechanism of neo-vasculogenesis remain open. There is a lack of data explaining the initial impulse leading endothelial progenitors to realize the need for new vessel. Another unresolved question is the exact role of MSPCs during neo-vasculogenesis.

A better understanding of early signaling events that lead to initiation of neo-vasculogenesis is of great importance for the development of therapeutic strategies in regenerative medicine and anti-tumor therapy. The novel finding that oxygen-sensing MSPCs promote ECFC survival and trigger neo-vasculogenesis is part of the results of this dissertation.

4.2.4. Mesenchymal stem/progenitor cells (MSPCs)

One hallmark of mesenchymal progenitors is that they can participate in immune regulation and differentiate into bone, cartilage, adipose tissue, skeletal muscle and vessel stabilizing pericytes (Prockop 1997, Pittenger et al. 1999). Due to the ability of MSPCs to differentiate into more than three lineages, they are considered multipotent progenitors. This and their inability to form teratomas make them promising candidates for regenerative medicine.

In 1966, Friedenstein et al. were the first to isolate non-hematopoietic cells from the bone marrow, which formed CFU-fibroblasts (CFU-F) *in vitro* (Friedenstein, II & Petrakova 1966). In their pioneering work, the authors demonstrated that these BM-derived stromal cells were capable of forming bone and recruiting hematopoietic host cells when injected subcutaneously into mice (Friedenstein, II & Petrakova 1966, Friedenstein 1968, Friedenstein, Gorskaja & Kulagina 1976). The CFU-Fs were later renamed mesenchymal stem cells or mesenchymal stromal cells (MSCs) (Prockop 1997, Prockop, Sekiya & Colter 2001, Caplan 2008). Due to their tissue specific heterogeneous differentiation capacity (e.g. white adipose tissue (WAT)-mesenchymal cells can not form bone) MSCs can not be considered pure stem cells but are rather composed of both stem and progenitor cells. Therefore, the term used in this dissertation is MSPCs (Hofmann et al. 2012). MSPCs also play a crucial role in vessel maturation. During angiogenesis, the endothelial cells of the newly formed vessels produce signaling molecules such as PDGF, TGF-β and SDF-1, all of which recruit MSPCs and cause them to act as vessel stabilizing pericytes. MSPCs are characterized by their ability to adhere to plastic, differentiate into all three lineages (osteogenic, chondrogenic and adipogenic), and express (to > 95%) the cell-surface markers CD105, CD73, CD90, while lacking the expression of CD14, CD19, CD34, CD45 and HLA-DR (to < 2%) (Dominici et al. 2006).

In the past several decades, MSPCs have been isolated from different tissues, including: umbilical cord blood (UCB) (Erices, Conget & Minguell 2000, Bieback et al. 2004, Kogler et al. 2004, Reinisch et al. 2007), white adipose tissue (Gronthos et al. 2001, Zuk et al. 2002, Kern et al. 2006), umbilical cord (UC) (Romanov, Svintsitskaya & Smirnov 2003, Sarugaser et al. 2005, Lu et al. 2006, Reinisch, Strunk 2009), placenta (Miao et al. 2006) and amniotic fluid (In 't Anker et al.

2003).

While their behavior *in vitro* has been well established, the *in vivo* localization and function of MSPCs are less well defined. Crisan et al. introduced the ongoing debate about whether all mesenchymal cells are pericytes (or related mural cells) and vice versa (Caplan 2008, Crisan et al. 2008). These authors show that sorted and isolated pericytes ($CD146^+$, $CD34^-$, $CD45^-$, $CD56^-$) are multipotent and can differentiate into cartilage, adipose tissue, skeletal muscle and perhaps bone, but do not form teratomas. Additionally, they state that perivascular MSCs express the same cell surface markers as pericytes. However, the perivascular niche also contains hematopoietic cells in pericytic location. Based on these findings, Crisan et al. concluded that not all pericytes are MSCs, but all MSCs are pericytes (Caplan 2008, Crisan et al. 2008). Due to the lack of differing phenotypical markers and differentiation function between pericytes and MSCs, it is very difficult to make a claim for a difference between these isolated cell types.

A specialized subtype of reticular MSPCs is found in the bone marrow residing between the endosteal niche and the bone marrow sinosoids. These reticular cells produce the chemokine SDF-1 (CXCL12) and are therefore called “CXCL12-abundant reticular cells” (CAR cells) (Sugiyama et al. 2006). CAR cells have been shown to generate bone matter and attract a hematopoietic microenvironment when transplanted subcutaneously *in vivo* along with scaffold-like osteoinductive hydroxyapatite/tri-calcium phosphate particles that mimic the 3D *in vivo* structure (Sacchetti et al. 2007). Recently, we developed a novel, extramedullary bone model in NSG mice that generates human bone and bone marrow by using human BM-derived MSPCs and human peripheral blood-derived ECFCs (Chen et al. 2012). In this BM microenvironment model, the authors studied normal hematopoietic cell and leukemic cell engraftment. The authors found that engrafted leukemic cells increased bone marrow hypoxia, and engraftment was able to be blocked by genetic modification of MSPCs (*Hif-1α* knock down). This novel BM niche model could be important for the development of new leukemia therapies.

Due to their differentiation capacity, as well as immune modulatory and hematopoietic support functions, MSPCs have successfully been applied to treat various diseases such as graft-versus-host disease (GvHD) (Le Blanc et al. 2004), osteogenesis imperfecta (Horwitz et al. 1999), Crohn’s disease or multiple

sclerosis (MS) (Uccelli, Mancardi & Chiesa 2008). The enhanced engraftment of HSPCs after the co-transplantation of blood stem cells and MSCs in high dose chemotherapy have been used to treat breast cancer paptients (Koc et al. 2000). Further, MSPCs are considered a promising tool for tissue engineering and suppliers for cytokines and growth factors to enhance angiogenesis in ischemic tissues (Caplan 2008)

4.3. Goal of this thesis

(modified from Hofmann et al. (Hofmann et al. 2012))

The goal of this thesis was to gain insight into whether ECFCs or MSPCs are responsible for O_2 sensing and regulation of hypoxia-induced neo-vasculogenesis. To test for the influence of O_2-level changes on ECFCs and MSPCs, proliferation, colony formation, network formation and wound healing ability should be monitored *in vitro*. To delineate the cellular compartments responsible for hypoxia response during vasculogenesis *in vitro*, a humanized mouse model of endothelial and mesenchymal progenitor co-transplantation (using different extracellular matrices) should be developed and the accumulation of nuclear HIF-1α in MSPCs and ECFCs should be studied. Furthermore, pharmacologic and genetic inhibition of HIF-1α (YC-1 and shRNA) in both cell types should be tested for its effect on neo-vasculogenesis.

5. METHODS:

(The following chapter follows the publication Hofmann et al. 2012 (Hofmann et al. 2012))

Ethics statement

Prior approval was obtained for human cell and tissue sample collection from the Institutional Review Board of the Medical University of Graz (protocols 19-252 ex 07/08, 18-243 ex 06/07, 21.060 ex 09/10). Adult samples were collected after written informed consent from healthy volunteers and cardiovascular disease (CVD) patients and umbilical cord or cord blood samples after written informed consent by the mother after full-term pregnancies in accordance with the Declaration of Helsinki. Animal experiments were approved by the Animal Care and Use Committee at the Veterinary University of Vienna on behalf of the Austrian Ministry of Science and Research according to the criteria published in the Guide for the Care and Use of Laboratory Animals and performed as described (Reinisch et al. 2009). Neonatal fibroblasts were purchased from Lonza (Walkersville, MD).

Human progenitor cell isolation, large scale expansion and long term propagation

ECFCs and MSPCs were isolated and expanded from neonatal UC or UCB, adult peripheral blood (PB) (as depicted in Figure 6 and Figure 7) or BM aspirates following IRB approval as previously described under animal serum-free culture conditions with pooled human platelet lysate (pHPL) replacing FBS (Reinisch et al. 2007, Schallmoser et al. 2007, Schallmoser et al. 2008, Reinisch et al. 2009, Hofmann, Reinisch & Strunk 2012). Additional video protocols covering production of pHPL (www.jove.com/details.php?id=1523) (Schallmoser, Strunk 2009), as well as propagation of blood-derived ECFCs (www.jove.com/details.php?id=1524) (Hofmann, Reinisch & Strunk 2009) and umbilical cord ECFCs and MSPCs (www.jove.com/details.php?id=1525) (Reinisch, Strunk 2009) are available online to support reproducibility. For large scale propagation primary culture-derived ECFCs were seeded in EGM-2 (Lonza) supplemented with 10% pHPL at a density of 100 cells/cm^2 and MSPCs in α-MEM (Sigma-Aldrich, St. Louis, MO), also

supplemented with 10% pHPL at a density of 30 cells/cm^2 in 2,528 cm^2 cell factories (Thermo Fisher Scientific, Freemont, CA). For long term propagation primary culture-derived ECFCs and MSPCs were seeded in triplicate in 75cm^2 flasks at a density of 100 cells/cm^2 (n= 4) and 30 cells/cm^2 (n=2), respectively, as described (Schallmoser et al. 2010). Cells were cryopreserved after expansion until use as described (Strunk et al. 2005).

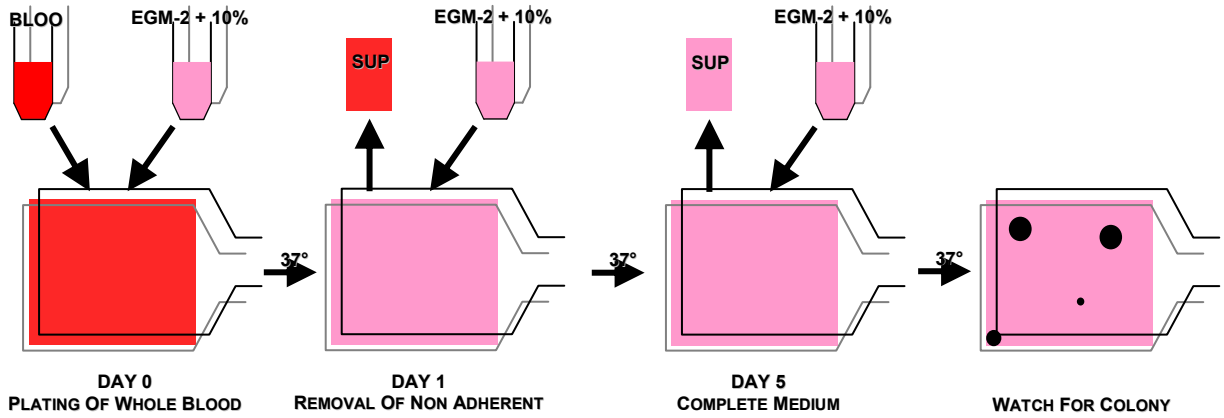


Figure 8. Isolation of endothelial colony forming cells (ECFCs) from human adult PB (modified from Hofmann et a. 2009 (Hofmann, Reinisch & Strunk 2009)**).** This procedure begins with the isolation of ECFCs from adult human peripheral blood. By simply culturing the total blood with animal serum-free optimized endothelial growth medium-2 (EGM-2) in a 75 cm^{2}flask, ECFCs adhere to the flask surface (DAY 0). To remove most of the red and white blood cells, flasks must be washed at DAY 1 and the remaining cells are eliminated by changing the total medium on day 5. Every other day flasks should be screened for outgrowth of colonies. First colonies should be observed around day 12. Subsequently, these cells can be either injected in a mouse model of human vessel regeneration to form functional, perfused vessels *in vivo,* or can be used as a test system to study endothelial biology in vitro.

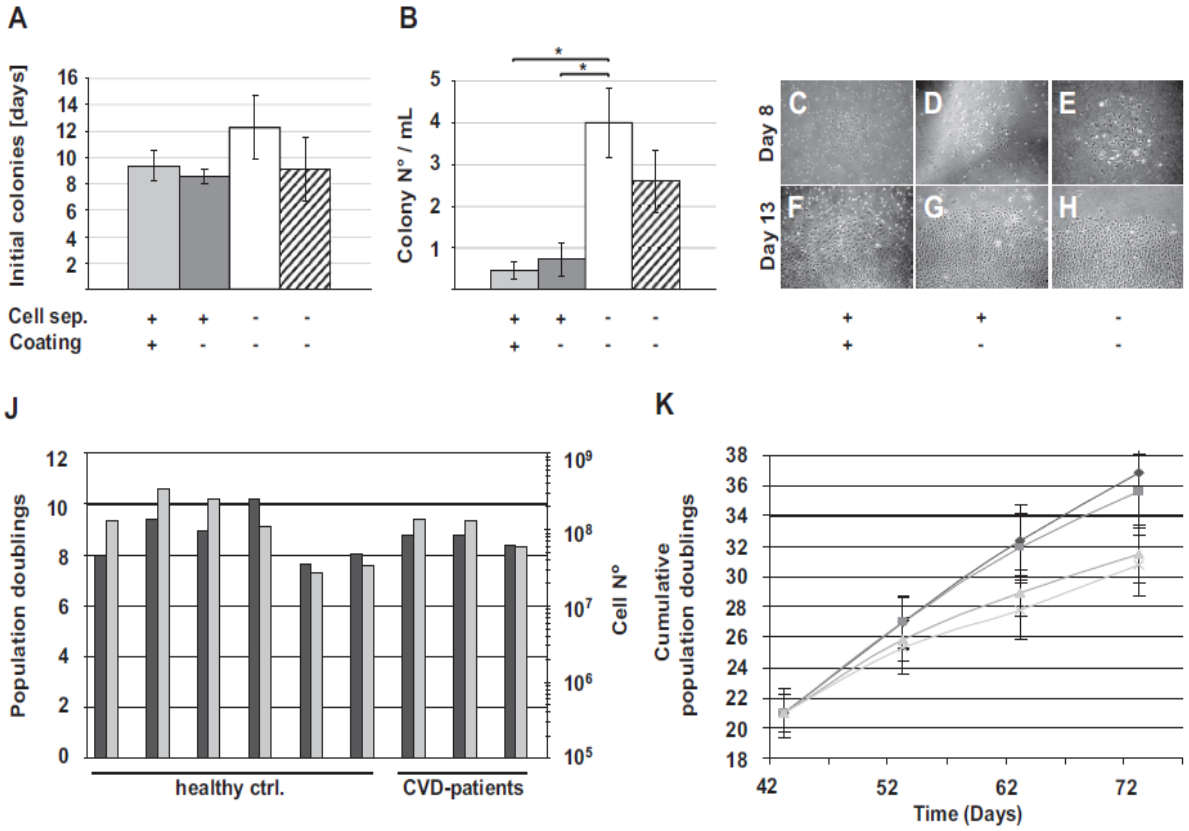


Figure 9. Endothelial colony forming cell (ECFC) recovery from human steady-state PB in an animal serum-free humanized system (modified from Reinisch et al. 2009 (Reinisch et al. 2009)**).** (A, B) Peripheral blood (PB) from healthy volunteers was density gradient-separated (+) to enrich for mononuclear cells (dark gray) or immediately diluted (white) and seeded in endothelial growth medium (EGM)/10% pooled human platelete lysate (pHPL) in 75 cm^2 cell culture flasks. Culture surfaces were coated (light gray) with collagen only when indicated (+). PB from patients with stable cardiovascular disease was also immediately diluted and seeded in EGM/10% pHPL (striped). (A) The initial appearance of visible colonies was determined by daily culture observation. (B) Colony number was counted at the end of the primary 7- to 19-day culture period. Results are shown as mean ±SEM of 6 independent experiments. *p < 0.05. (C-H) Representative early colonies (day 8) and parts of large expanded colonies (day 13) from healthy volunteers are depicted with 4x initial magnification corresponding to different recovery strategies as indicated. Images were captured with a DS-Fi1 camera on a Nikon (Lijnden, Netherlands) Diaphot 300 inverted microscope (original magnification 4_/0.13 NAobjective) with the NIS-Elements D3.0 image acquisition software (Nikon). (J) Population doublings (dark gray) and expanded cell number (light gray) determined after large-scale expansion of ECFCs from 6 healthy volunteers (healthy controls) and 3 CVD patients are shown. (K) Cumulative population doublings (mean ± SD) as obtained during large-scale expansion of ECFCs from 6 healthy volunteers after large-scale expansion are shown. Large-scale expansion-derived cells bear a history of mean 21 population doublings before initiating long-term culture at cell seeding densities of 10 (diamond), 100 (square),

1000 (triangle), and 10,000 cells/cm^2 (x). Cells were reseeded during long-term culture at indicated time points according to their initial seeding density.

Oxygen-dependent cell culture, clonogenicity and re-oxygenation.

ECFCs and MSPCs were grown and manipulated in an XVIVO system hypoxic workstation incubator (BioSpherix, New York, NY) set to 1% or 5% O_2 in 95% humidified atmosphere containing 5% CO_2 at 37°C. Media were equilibrated for 24 hours (h) to the required oxygen level in the hypoxic workstation before use. Conventional control cultures were done in ambient air (20% O_2) in a standard incubator (Heraeus; Thermo Scientific) with 95% humidified atmosphere and 5% CO_2 at 37°C. Oxygen content of media and cell supernatants was verified by blood gas analysis (Cobas B21, Roche, Burgess Hill, West Sussex, UK).

ECFCs and MSPCs cultured at 1% and 5% O_2 for 7 days were harvested in the hypoxic workstation and seeded for proliferation studies in 6-well plates at a density of 100 cells/cm^2 or 30 cells/cm^2, respectively. Clonogenicity of ECFCs and MSPCs was measured after seeding at indicated densities of 1 – 10 cells/cm^2 in 55 cm^2 plates and either cultured in the previous oxygen conditions or re-oxygenated to 20% O_2. After two weeks, cell numbers were measured by hemocytometry and colonies were fixed with paraformaldehyde within the hypoxic workstation, stained with crystal violet (Merck, Darmstadt, Germany) and enumerated as described earlier with ImageJ software (National Institutes of Health, Bethesda, MD) (Reinisch et al. 2009, Schallmoser et al. 2008). Low proliferative potential (LPP) and high proliferative potential (HPP) colonies were defined as colonies comprising of 51 to 500 and more than 500 cells, respectively (Yoder et al. 2007, Reinisch et al. 2009).

Angiogenesis assay and endothelial wound repair *in vitro*.

To test effects of different oxygen concentrations on vascular-like network formation, ECFCs were pre-cultured at 1%, 5% and 20% O_2 for 7 days *in vitro*. After trypsinization 180,000 ECFCs were re-suspended in 2 mL EGM-2/10% pHPL and seeded on 10 cm^2 polymerized Matrigel/well (Angiogenesis assay kit; Millipore, Billerica, MA) according to the manufacturers' instructions. Vascular-like networks (24 h) were documented with a Color View III camera on an Olympus

IX51 microscope with the analysis B acquisition software (all Olympus, Hamburg, Germany). Re-oxygenation to 20% O_2 was done as described above. Tube number and length as determined using ImageJ software (http://rsbweb.nih.gov).
For endothelial wound repair studies ECFCs were seeded at 1 x 10^5 cells per 10 cm^2 (Corning 6-well plate; Sigma) and cultured until confluence (approximately 4 d, 20% O_2). After standardized scratch of the confluent cell layer with a 1,000 µL pipette tip (Gilson, Middleton, WI), cultures were introduced into the O_2-controlled cell observer workspace (Carl Zeiss, Thornwood, NY) and medium was replaced by pre-oxygenized fresh medium (1%, 5% or 20% O_2) as indicated. Cell movement and proliferation were monitored by acquiring video sequences at 20 minute intervals covering a time period of up to 48 hours (Zeiss). Single cell proliferation was evaluated accurately by counting dividing cells on printouts of every video picture. A composite of three time lapse videos covering 16 h of endothelial wound repair video sequences with highlighting cell divisions in red, green and yellow colored numbers under 1%, 5% and 20% O_2, respectively. Area of wound repair was determined using ImageJ software.

Experimental neo-vasculogenesis *in vivo*.

In vivo functionality was analyzed by resuspending 4 x 10^5 bone marrow–derived MSPCs mixed with 1.6 x 10^6 ECFCs per 0.3 mL in either ice-cold Matrigel (Millipore) or pHPL immediately before subcutaneous injection into the flank of immune-deficient NSG mice under general anesthesia as published (Reinisch et al. 2009). Alternatively, ECFCs and MSPCs were mixed with rat collagen and human fibronectin, allowed to coagulate, and were then implanted subcutaneously as a preformed plug in NSG mice as previously described (Mead et al. 2008). Both ECFCs and MSPCs were derived from large scale expansions providing consistent batches of cells after culture on up to 30,000 cm^2 culture area (12 x 4-layered cell factories; Thermo Scientific) as previously published (Reinisch et al. 2009). Mice were sacrificed at days 1, 7 and 14 after injection by cervical dislocation and plugs where explanted. To depict hypoxia, mice received intra-peritoneal injections of pimonidazole (60 mg/kg body weight; Hypoxyprobe, Burlington, MA) 30 minutes before plug explantation. Animal experiments were approved by the Animal Care and Use Committee as specified above.

Immune histochemistry and immune fluorescence.

Sections (1.5 µm) of formalin-fixed (3.7% neutral buffered, over night) paraffin-embedded plugs were de-paraffinized before antigen retrieval by heat (70°C/160 W, 40 min) at either high or low pH depending on the antibody followed by a descending alcohol series (2x xylol, 10 min; 1x ethanol 100%, 5 min; 1x ethanol 90%, 5 min; 1x ethanol 70%, 5 min; 1x ethanol 50%, 5 min; 1x PBS, 5 min). Endogenous peroxidases were blocked with H_2O_2 (10 min) and unspecific antibody binding with Ultra V Block (Thermo Scientific; 5 min) followed by mouse-on-mouse blocking (MOM, 1 h; Vector Laboratories, Burlingame, CA) and serum-free protein block (30 min; Dako, Glostrup, Denmark). Slides were incubated (30 min, RT) with unconjugated monoclonal mouse anti-human antibodies against Vimentin (clone: V9, 0.78 µg/mL, Dako), HIF-1α (clone: 54/HIF-1α, 10 µg/mL; BD Biosciences, San Jose, CA), CD31 (clone: JC70A, 5.15 µg/mL, Dako) or the appropriate amount of IgG1 (BD) control and developed with ultravision LP large volume detection system horseradish peroxydase (HRP) polymer (Thermo Scientific) and diaminobenzidine (DAB) or alkaline phosphatase detection system using fast blue or fast red (Vector) according to manufacturer's instructions. Avidin-biotin blocking (Vector) was used before staining with biotinylated monoclonal rat anti-mouse CD45 (clone: 30-F11, 5 µg/mL; R&D Systems, Minneapolis, MN) and biotinylated monoclonal rat anti-mouse IgG1 (BD) control and detected by streptavidin-HRP conjugate (Dako) and DAB. Pimonidazole was stained with 0.7 µg/mL anti-pimonidazole-FITC (Hypoxyprobe). For permanent staining a secondary HRP-conjugated anti-FITC antibody was applied and developed with DAB. Slides were counterstained with hematoxylin for ≤30 seconds.

For immune fluorescence slides were washed after antigen retrieval (5 min, PBS + 0.01% v/v Triton X-100; Sigma), protein blocked (30 min; Dako) and stained overnight at 4°C with either biotinylated rat anti-mouse Ter119 (clone TER-119, 5 µg/mL, BD), biotinylated rat IgG2b isotype control (5 µg/mL, BD), HIF-1α (10 µg/mL, BD), or rabbit anti-human CD31 (clone: EPR3094, 1:1,000; Abcam, Cambridge, UK), rabbit isotype control (5 µg/mL; Cell Signaling Technology, Danvers, MA), mouse anti-human alpha smooth muscle actin (clone: 1A4, 4.4 µg/mL, Dako), mouse IgG2a isotype control (4.4 µg /mL, Dako) all diluted in stain buffer, washed 3x (PBS + 0.01% Triton X-100) and developed (120 min) with anti-

mouse IgG-Alexa-546 (8 µg/mL, diluted in stain buffer; Invitrogen, Carlsbad, CA) or anti-rabbit IgG-Cy5 (2 µg/mL, Abcam) or streptavidin-conjugated alkaline phosphatase. After embedding in DAPI mounting media (4°C, minimum 60 min; Vector) pictures were taken with a Zeiss LSM510 Meta at 405, 543 and 633 nm excitation wavelengths. Collapsed stacks were reconstructed from z-stacks with Imaris software (Bitplane, Zurich, Switzerland).

To visualize HIF-1α protein in cell cultures at the single cell level, ECFCs and MSPCs were pre-cultured at 20% O_2 to 75% confluence in 8-well glass chamber slides (Thermo Scientific). Medium was replaced by pre-oxygenized 1% or 5% medium in the hypoxic workstation or kept as 20% O_2 and cells were cultured for 5 min - 24 h as specified. Incubation was stopped with ice cold PBS and fixed with ice cold paraformaldehyde (4%; 15 min, on ice) still inside the hypoxic workstation. Immune cytochemistry was performed after H_20_2 block using mouse anti-human HIF-1α antibody (BD) or mouse IgG1 control (10 µg/mL; BD) and visualized with HRP detection system (Thermo Scientific) with DAB according to manufacturers' instructions. Cells were counterstained (15 sec) with hematoxylin and documented with a phase contrast microscope (Olympus).

VEGF measurement in MSPC supernatants.

MSPCs were cultured in α-MEM containing 10% pHPL for 3 days at 1%, 5% or 20% O_2. Supernatants were aspirated and sterile filtered (0.22 µm Steriflip-filter, Thermo Scientific). Supernatant was stored in aliquots at -80°C until measurement. VEGF concentration was determined using Bio-Plex Pro Assay (Bio-Rad, Laboratories, Hercules, CA) and analyzed using the Bio-Plex-200 system (BioRad).

Western blot analysis

ECFCs and MSPCs after 1%, 5% or 20% O_2 culture for 6 h were washed once with ice cold PBS/Proteinase-inhibitor (Sigma) and then scraped in ice cold RIPA buffer (Bio-Rad) supplemented with proteinase and phosphatase-inhibitor (Thermo Scientific) and lysed under constant agitation on ice for 30 min. After sonication (15 sec, on ice; Hielscher, Teltow, Germany) and centrifugation, the protein-containing supernatants were aliquoted and frozen at -80°C. Protein content was determined with a Bradford assay (Bio-Rad) and OD was measured with a

Spectramax (Molecular Devices, Sunnyvale, CA). Proteins (20 µg) were loaded and resolved in a 7.5% SDS-PAGE (Bio-Rad) with in parallel to full-range rainbow™ molecular weight marker (GE Healthcare, Munich, Germany) and transferred onto a nitrocellulose membrane (Bio-Rad). HIF proteins were detected using HIF-1α (clone: 54/HIF1α, 0.5 µg/mL; BD), HIF-1β (clone: 29/HIF1β, 0.125 µg/mL; BD), HIF-2α (clone: A-5, 0.4 µg/mL; Santa Cruz, Santa Cruz, CA) antibodies compared to house-keeping protein control β-actin (clone: C4, 0.2 µg/mL; Santa Cruz) antibodies followed by anti-mouse-HRP antibody (Cell Signaling) and visualized with 'super signal west pico luminol/enhancer developing solution' (Thermo Scientific). Films were exposed to blots in a dark room (10 sec - 5 min). Developed films and blots were scanned overlayed to document precise location of rainbow molecular weight marker bands (Figure 10E and 11).

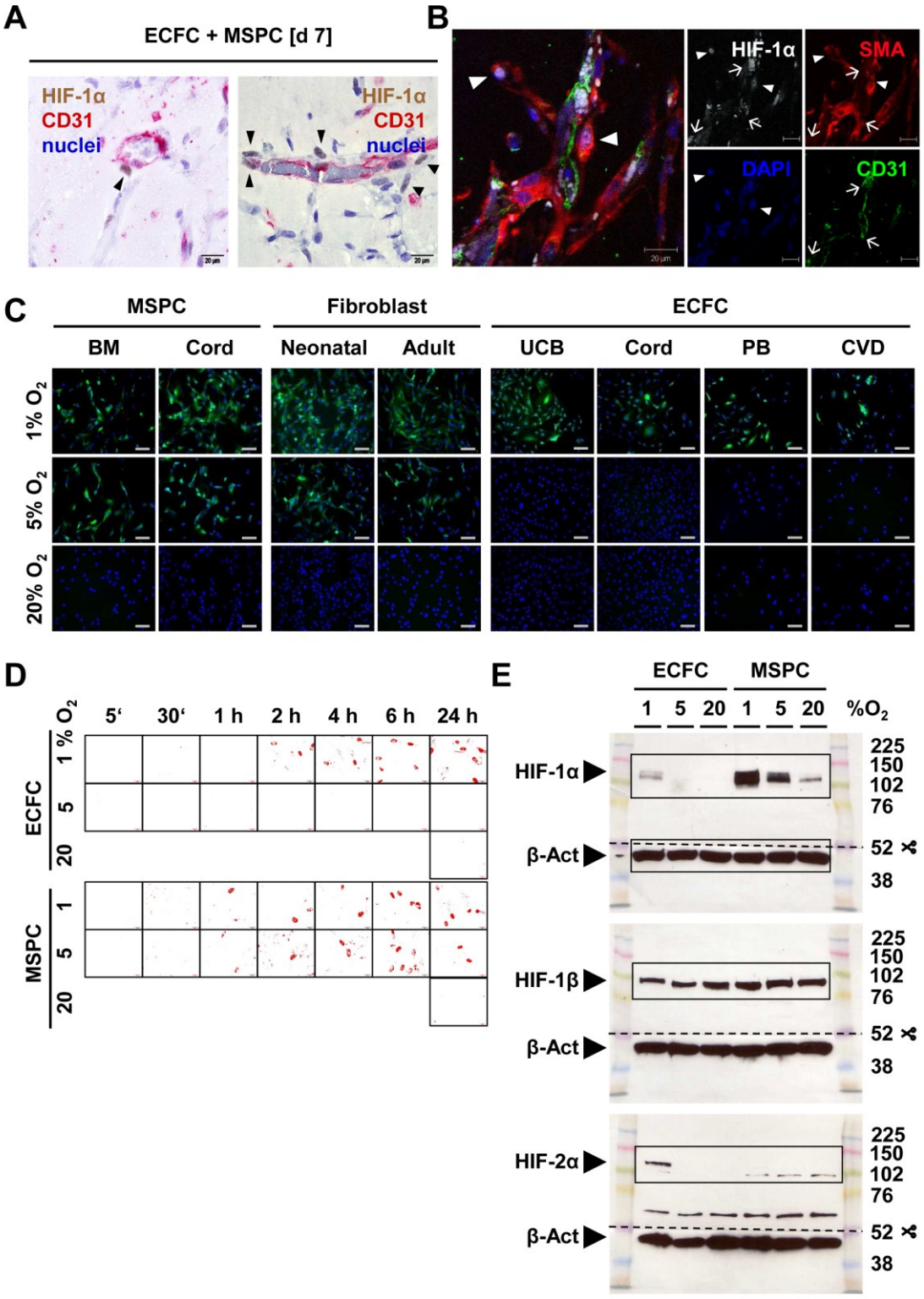


Figure 10. Nuclear hypoxia inducible factor-1α (HIF-1α) signal *in vitro* and *in vivo* (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A**) Immune histochemical staining of Matrigel plugs containing endothelial colony forming cells (ECFCs)/mesenchymal stem and progenitor cells (MSPCs) 7 days (d) after co-transplantation.

Plugs were explanted and sections were stained with anti-HIF-1α (brown; arrow heads), anti-human CD31 (red) and co-stained with hematoxylin (blue).

(**B**) Immune fluorescence staining d7 after co-transplanting ECFCs/MSPCs in Matrigel with anti-HIF-1α (white), anti-human alpha smooth muscle actin (SMA, red), and counterstained with Diamidin-2-phenylindol (DAPI) (blue), anti-human CD31 (green). White arrow heads mark nuclear HIF-1α signals and arrows unspecific background fluorescence of mouse red blood cells.

(**C**) Hypoxyprobe (pimonidazole, green; DAPI nuclear stain in blue) analysis of MSPCs from bone marrow (BM), umbilical cord (Cord), neonatal and adult fibroblasts directly compared to ECFCs from umbilical cord blood (UCB), cord, normal and cardiovascular disease patient-derived (CVD) peripheral blood (PB) cultured at indicated O_2 levels as described in the methods section. Scale bar 100 µm.

(**D**) ECFCs and MSPCs were cultured for indicated intervals at indicated O_2. Fixed cells were stained with anti-HIF-1α. ImageJ (http://rsbweb.nih.gov) processing was used to obtain the transformed red signal. Original data are on file. ECFC start to stabilize HIF-1α in their nucleus after 2 h at 1% O_2 but not at 5 or 20% O_2. MSPCs stabilize HIF-1α after 1h at 1% and 5% O_2.

(**E**) Western blot analysis of ECFC and MSPC total cell lysates after 6 h at indicated O_2. Blots were incubated with HIF-1α, HIF-1β, HIF-2α or β-actin (β-Act) antibodies. Three representative blots scanned in overlay with exposed films. Scissors mark cuts to separately stain β-actin (β-Act) from the same blot. Areas shown in Fig. 11C are boxed.

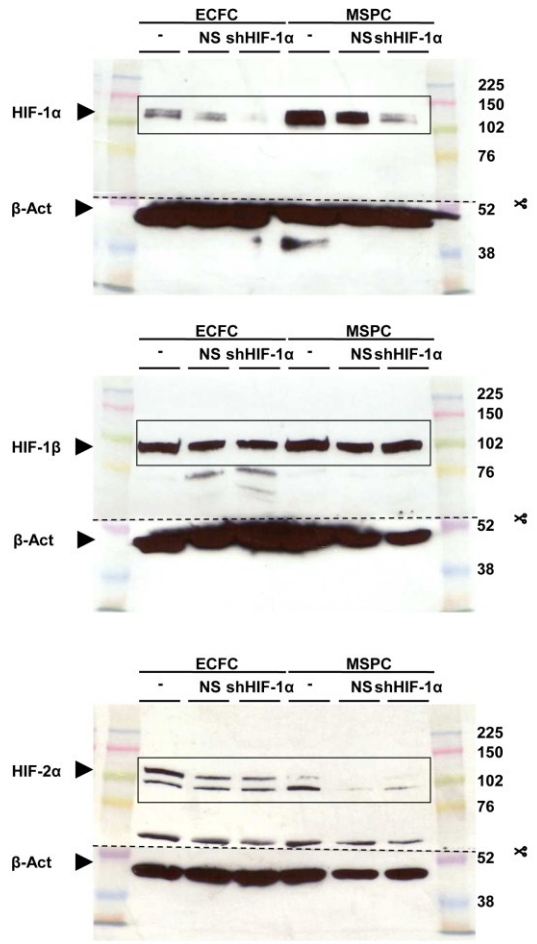


Figure 11. Specific knock-down of hypoxia inducible factor-1α (HIF-1α) in endothelial colony forming cells (ECFCs) and mesenchymal stem and progenitor cells (MSPCs) (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

Total cell lysates of untreated control ECFCs and MSPCs (**-**) or after infection with either pGIPZ-HIF1alpha-shRNA (shHIF-1α) or non-specific pGIPZ-scramble-shRNA (NS) were separated by SDS-PAGE after 6 h of incubation at 1% O_2. Blots were stained with HIF-1α, HIF-2α, HIF-1β or β-actin (β-Act). Three representative blots scanned in overlay with exposed films are shown. Scissors mark cuts to separately stain β-Act from the same blot. Areas shown in Figure 7A are boxed.

Hypoxia response modification *in vitro* and *in vivo*.

The optimal dose of cycloheximide (20 µM, 1 h; Sigma) inhibiting protein synthesis and of YC-1 [3-(5'hydroxymethyl-2'furyl)-1-benzylindazole] (100 µM, 5 min, 37°C; A.G. Scientific, San Diego, CA) blocking HIF-1α and HIF-2α proteins without affecting MSPC and ECFC viability was titrated. Pre-treated MSPCs and ECFCs were washed 3x with PBS and combined with corresponding untreated partner

cells for co-transplantation to study the impact of protein synthesis or HIF inhibition in either cell type on vasculogenesis *in vivo*.

The optimal dose of YC-1 [3-(5'hydroxymethyl-2'furyl)-1-benzylindazole] (100 µM, 5 min, 37°C; A.G. Scientific, San Diego, CA) blocking HIF-1α and HIF-2α proteins was titrated. Pre-treated MSPCs and ECFCs were washed 3x with PBS and combined with corresponding untreated partner cells for co-transplantation to study the impact of HIF inhibition in either cell type on vasculogenesis *in vivo*.
Lenti-viral infections for HIF-1α depletion were carried out according to standard procedures for gene silencing. Briefly, 293T cells were co-transfected with pMD2.G and psPAX2 (Addgene, Cambridge, MA) along with either pGIPZ-HIF1α-shRNA or pGIPZ-scramble-shRNA lent viral constructs (OpenBiosystems, Huntsville, AL) using JetPrime transfection reagent (Polyplus-transfection Inc., New York, NY) according to the manufacturer's protocol. The transfection medium was replaced after 12 h with fresh DMEM/10% FBS (Sigma). After 48 h viral supernatants were collected and concentrated using Centricon Plus-70 filter units (Millipore). Bone marrow-derived MSPCs and peripheral blood-derived ECFCs were each infected in the presence of 8 µg/mL polybrene (Sigma). Two days after infection stably transduced cells were selected with 2 µg/mL puromycin for 2 weeks resulting in a homogeneous population of virtually 100% TurboGFP-positive cells. To study the effect of different cytokines on vessel formation *in vivo* Matrigel (Millipore) was mixed with 4 ng/mL VEGF or a combination of VEGF, EGF, IGF, bFGF-2, hydrocortisone and ascorbic acid (all from Lonza EBM-2 Single Quots) or Matrigel was replaced by pHPL before co-transplantation of either un-manipulated or *Hif-1α* knock down ECFCs and MSPCs. To block VEGF activity *in vivo* mice were injected intraperitoneally every other day during a 7 d or 14 d observation period starting at day zero immediately after co-transplantation of un-manipulated ECFCs and MSPCs as described above with Bevacizumab (Roche; 5 mg/kg per injection). Microvessel density was obtained from 5 high power fields (200 x magnification) of HE stains from at least 2 independent donors and at least 2 independent plugs. Red blood cell filled vessels were quantified by ImageJ software (http://rsbweb.nih.gov) and statistically evaluated by an unpaired student t-test. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Apoptosis assay.

Apoptosis of ECFCs and MSPCs in implants *in vivo* was analyzed with TdT-mediated dUTP nick end labeling (TUNEL; Dead End™ Fluoretric System; Promega, Madison, WI) according to manufacturer's instructions. Nuclei were counterstained with propidium iodide (PI). As a positive control, slides were incubated for 10 min with 1U DNase 1 (Thermo Scientific). As a negative control, incubation buffer was prepared without rTdT. Staining was documented with a confocal microscope (LSM510 Meta, Zeiss).

Statistics.

Values are presented as mean ± standard deviation (SD). Comparisons of tube length in *in vitro* angiogenesis assays and wound repair assays were made by Mann-Whitney U test. Comparisons of microvessel density in the *in vivo* neo-vasculogenesis model were made by unpaired student t-test. Statistical differences were considered significant when the P-value was less than 0.05, very significant when P-value was less than 0.001 and extremely significant when P-value was less or equal 0.0001.

6. RESULTS

(The following chapter follows the publication Hofmann et al. 2012 (Hofmann et al. 2012))

Mesenchymal stem/progenitor cells sense low oxygen more sensitively than endothelial progenitors *in vitro* and *in vivo*

In this study, the subcutaneous co-transplantation of human ECFCs with MSPCs into immune-deficient NSG mice as established previously was used as a vasculogenesis model to create stable perfused human mesenchymal cell-covered vessels (Reinisch et al. 2009). The phenotype of applied cells was shown to be typical for MSPCs and ECFCs (Figure 12).

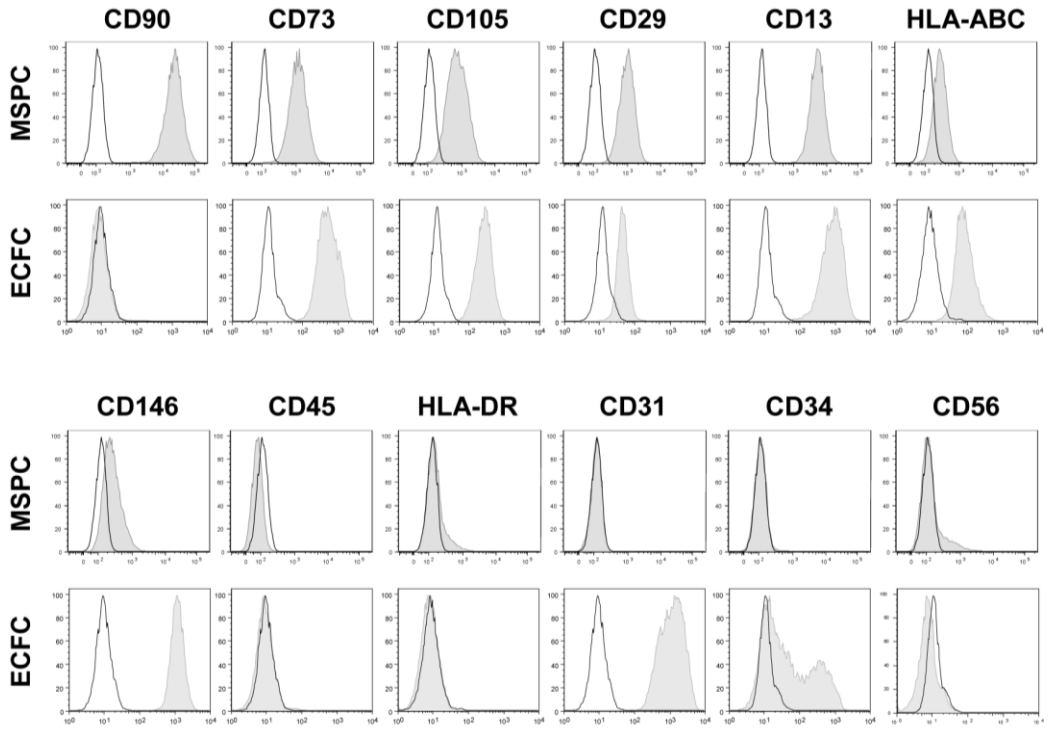


Figure 12. Phenotypic characterization of endothelial colony forming cells (ECFCs) and mesenchymal stem and progenitor cells (MSPCs) (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

The phenotype of MSPCs and ECFCs was characterized by flow cytometry as described previously (n > 5) (Reinisch et al. 2009). MSPCs and ECFCs can be distinguished by their

dissimilar expression of CD90, CD31 and CD34. Both ECFCs and MSPCs lack reactivity with the hematopoietic marker CD45.

Both ECFCs and MSPCs showed no reactivity to the hematopoietic marker CD45. Interestingly, one day after subcutaneous transplantation of ECFCs or MSPCs or co-transplants of ECFCs and MSPCs in an extracellular matrix into NSG mice, plugs containing only ECFCs lacked HIF-1α reactivity while implants containing MSPCs or the mixture of both progenitor cell types showed a strong nuclear HIF-1α signal (Figure 13).

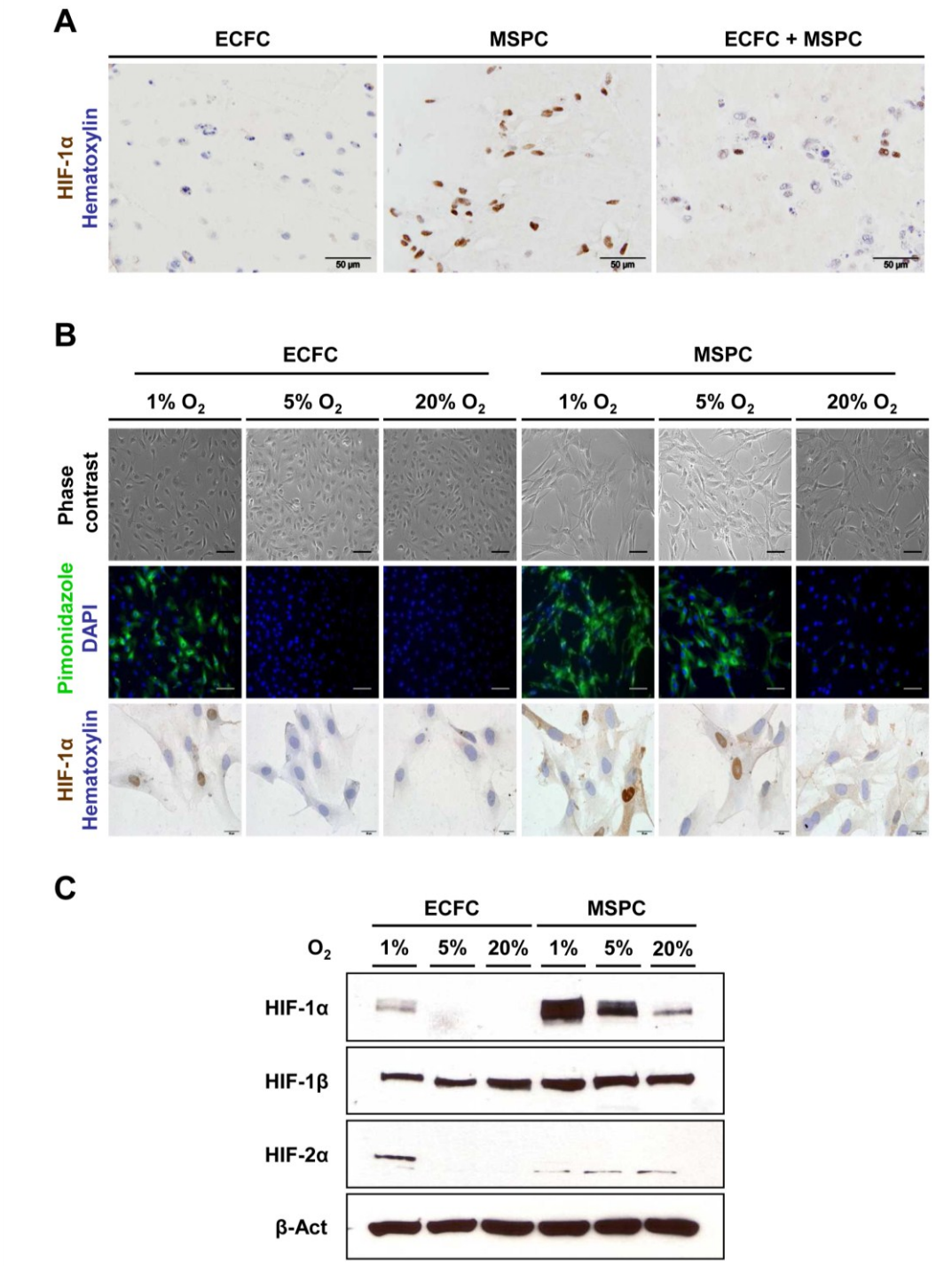


Figure 13. Mural cells are more sensitive to hypoxia than endothelial cells (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A**) Immune histochemical staining of Matrigel plugs 1 day (d) after implantation of either endothelial colony forming cells (ECFCs) only (left picture), mesenchymal stem and progenitor cells (MSPCs) only (middle picture) or ECFCs/MSPCs together (in a 20 : 80 ratio; right picture). Plugs were explanted and sections were stained with anti-hypoxia inducible factor-1α (HIF-1α; diaminobenzidine (DAB), brown) and co-stained with hematoxylin (blue; scale bar 50 µm). Staining

revealed that 100% of MSPCs displayed positive signals for HIF-1α, while ECFCs alone showed no HIF-1α stabilization and in plugs of ECFCs together with MSPCs the number of positive cells corresponded to the 20% MSPCs.
(**B**) To measure the hypoxia response at the single cell level *in vitro*, ECFCs and MSPCs were cultured for 4 h at 1%, 5% and 20% O_2. Phase contrast microphotographs showed an unchanged appearance. Hypoxyprobe (pimonidazole, green; 4′,6-Diamidin-2-phenylindol, DAPI nuclear stain in blue) revealed that MSPCs sensed hypoxia at 5% O_2 and both ECFCs and MSPCs sensed hypoxia at 1% O_2. Accordingly, nuclear HIF-1α protein accumulations were detected in MSPCs at 5% and in both progenitor cell types at 1% O2 (scale bar 100 µm for phase contrast and pimonidazole and 20 µm for HIF-1α pictures).
(**C**) Equal amounts of protein from cell lysates corresponding to the analysis in (B) were tested in western blots showing higher amounts of HIF-1α in MSPCs. MSPCs stabilized HIF-1α at 5% and displayed a minute baseline signal at ambient air levels of 20% O_2 (n = 3; representative blot regions are shown; see Figure 10E for complete blot scans).

When analyzing the human vessels in vascularized plugs after one week we observed that nuclear HIF-1α signals *in situ* were virtually restricted to mural cells (Figure 10A, B). To study the response to low oxygen more precisely at the single cell level over time, human ECFCs and MSPCs of different origins were exposed to reduced oxygen (5% O_2 resembling the human venous oxygen level) and more severe hypoxia (1% O_2) and compared to ambient air standard cell culture conditions (20% O_2). Hypoxia was visualized by pimonidazole binding as described (Chen et al. 2012, Kizaka-Kondoh, Konse-Nagasawa 2009). It is generally accepted that pimonidazole forms aggregates inside of the cell when O_2 levels drop below 1% O_2. The exact mode of action is not yet well understood. ECFCs from all tested sources showed cellular pimonidazole binding indicating hypoxia only at 1% O_2. Surprisingly MSPCs and fibroblasts already reproducibly bound pimonidazole at 5% in addition to 1% O_2 (Figure 10C). Nuclear HIF-1α resembling the pimonidazole results was found in the mesenchymal cells at 5% and 1% but not at 20% O_2 and in ECFCs only at 1% O_2 (Figure 8C and Figure 13B). Kinetic analysis revealed nuclear HIF-1α protein stabilization in MSPCs after 1 h at reduced O_2. ECFCs accumulated HIF-1α starting after 2 h and more prominently after 6 h at 1% O_2 (Figure 10D). Western blotting of cell lysates confirmed single cell results showing markedly increased HIF-1α stabilization in MSPCs when directly compared to ECFCs at 1% O_2. We confirmed a stable HIF-

1β expression in ECFCs and MSPCs and up-regulation of HIF-2α in ECFCs under more severe hypoxic conditions at 1% O_2 (Figure 13C; see also Figure 10E).

ECFCs maintain a quiescent state under hypoxic conditions *in vitro*

It is generally accepted that hypoxia maintains stemness (Rehman 2010). It is not known whether the hypoxic environment during therapeutic vasculogenesis affects progenitor clonogenicity or function. Testing ECFC clonogenicity at 20% O_2 standard conditions confirmed a complete hierarchy of LPP-ECFCs and HPP-ECFCs as previously reported (Yoder et al. 2007, Reinisch et al. 2009).

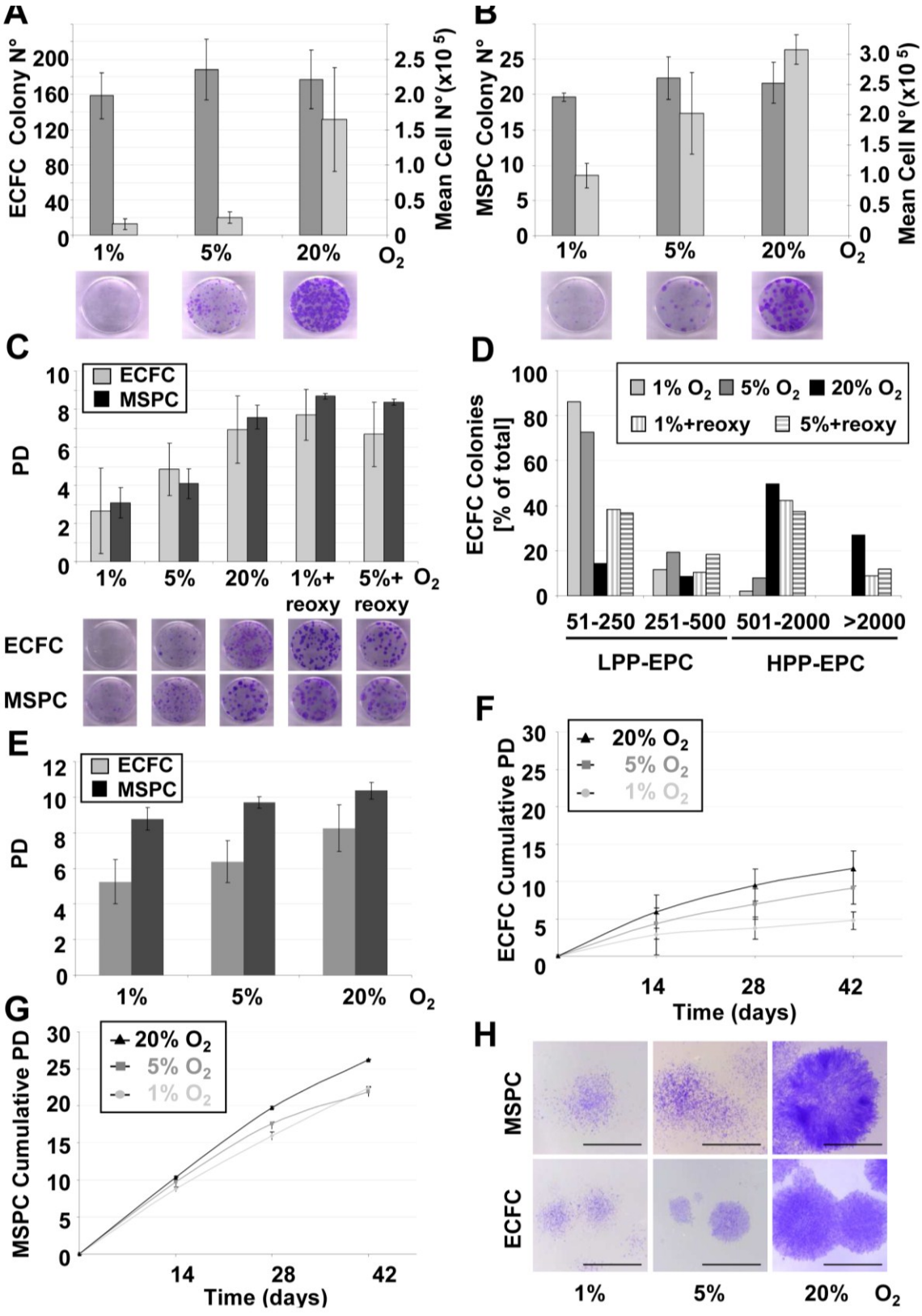


Figure 14. Progenitor clonogenicity and long-term proliferation: proliferative quiescence under reduced O_2 and recapitulation after re-oxygenation (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A, B**) For colony assays, 10 endothelial colony forming cells (ECFCs)/cm^2 (n= 3) or 3 and mesenchymal stem and progenitor cells (MSPCs)/cm^2 (n= 2) were seeded in 55 cm^2 colony plates and grown for 14 days (d) at 1%, 5% or 20% O_2. Colony number and cell number were documented. Culture plates show typical colonies at 1%, 5% and 20% O_2 derived from the same ECFC or MSPC starting population, respectively, and stained with crystal violet as described in the methods section.

(**C**) For re-oxygenation (+ reoxy), population doublings (PD) of ECFCs and MSPCs cultured under 1%, 5% and 20% O_2 for 12 d were compared with PDs of ECFCs and MSPCs pre-cultured at 1 or 5% O_2 for 7 d and then cultured at 20% O_2 for another 12 d (mean ± SD; n = 3). Corresponding representative crystal violet-stained colony plates are shown positioned below their corresponding O_2 conditions.

(**D**) ECFC hierarchy was assessed after 14 d of culture at 1%, 5% and 20% O_2 directly compared to ECFCs pre-cultured for 7 d at 1% or 5% O_2 and subsequently for another 12 d at 20% O_2 (+reoxy) by photo documenting all colonies per plate and semi-automatically counting each cell per scanned colony as described previously using the ImageJ software (http://rsbweb.nih.gov). One representative experiment is shown.

(**E**) To determine population doublings (PD) per passage 100 ECFCs/cm^2 (n= 5) or 30 MSPCs/cm^2 (n=2) were seeded in 75 cm^2 culture flasks and grown for 14 d at 1%, 5% or 20% O_2.

(**F, G)** Cumulative PDs were calculated after long-term culture (3 x 14 d) at 1%, 5% or 20% O_2 for (**F**) ECFCs or (**G**) MSPCs.

(**H**) Representative ECFC and MSPC colonies are shown after 14 d incubation at 1%, 5% or 20% O_2 after crystal violet stain (scale bar 5 mm).

The colony number of ECFCs and MSPCs was stable under all O_2 conditions tested but colony size progressively dropped with decreasing oxygen tension (Figure 14A, B). Progenitors exposed to 1% or 5% O_2 experienced a hierarchy shift towards small colonies but resumed their clonogenic potential after re-oxygenation suggesting that they can maintain a quiescent state in a hypoxic environment and arguing against simple hypoxia-mediated damage (Figure 14C, D). In bulk cultures for large-scale expansion, O_2 reduction diminished proliferation of both progenitor types progressively over time (Figure 14E-H).

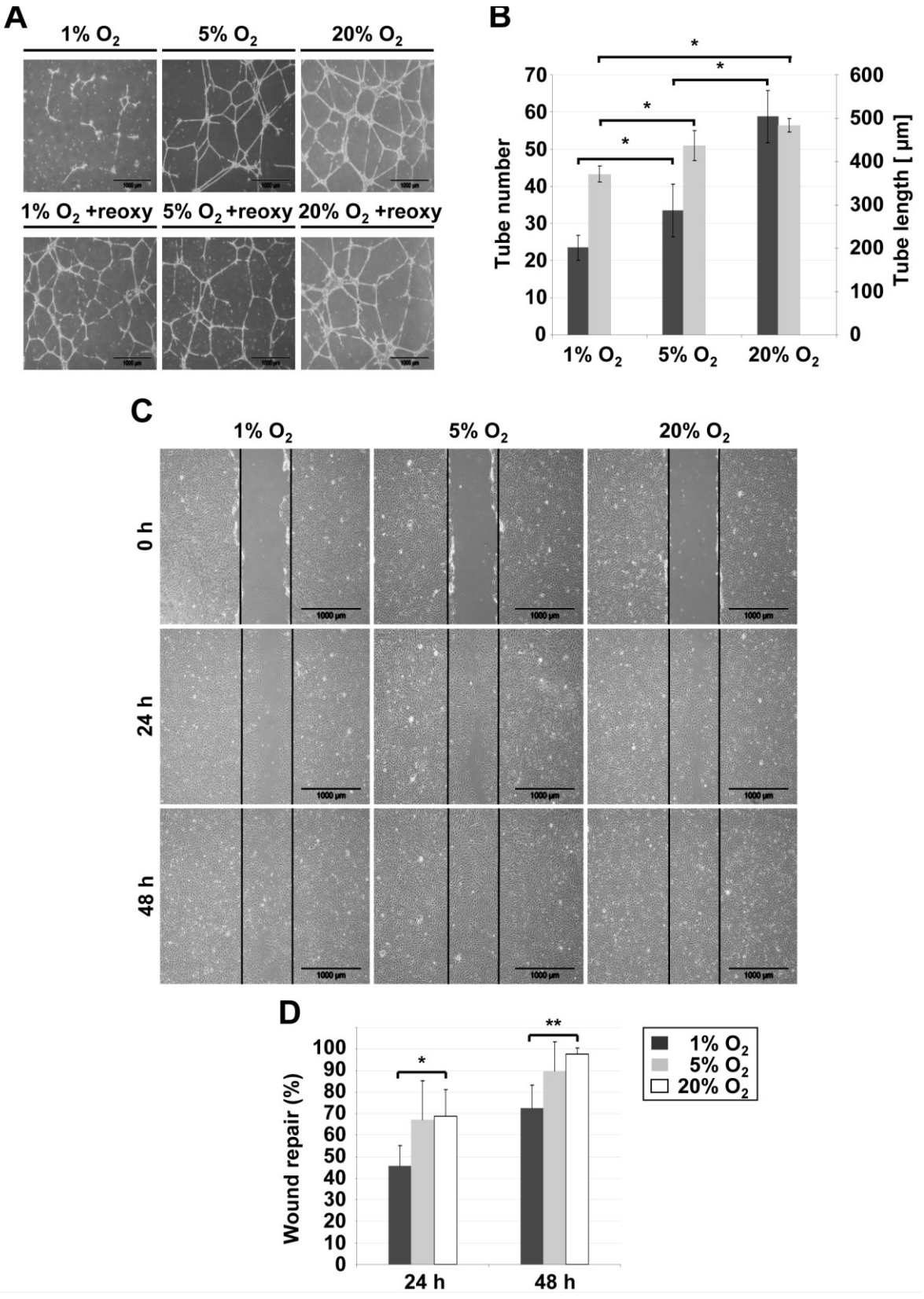

A
1% O2
5% O2
20% O2
1% O2 +reoxy
5% O2 +reoxy
20% O2 +reoxy
B
Tube number
Tube length [µm]
1% O2
5% O2
20% O2
C
1% O2
5% O2
20% O2
0 h
24 h
48 h
1000 µm
D
Wound repair (%)
24 h
48 h
1% O2
5% O2
20% O2

Figure 15. Functional quiescence of progenitor cells under hypoxia *in vitro* (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A**) Vascular-like structures documented 24 h after seeding 1.8 x 10^5 endothelial colony forming cells (ECFCs) per 10 cm^2 on top of a matrix gel in a standard angiogenesis assay showing impaired network formation by ECFCs at 1% more than at 5% O_2 (upper row) and resumed vessel-like structure formation after re-oxygenation back to 20% O_2 standard conditions (+reoxy, lower row; one representative picture series; n = 3).

(**B**) Tube number (black bar) and length (grey bar) as determined using ImageJ software (http://rsbweb.nih.gov) were significantly reduced with decreasing O_2 (mean ± SD; *p < 0.05; n = 4).

(**C**) Wounding an ECFC-derived monolayer in a scratch assay was used to monitor endothelial wound repair under hypoxia (1% O2) as compared to reduced (5% O_2) and ambient air (20% O_2) standard laboratory test conditions (representative examples are shown).

(**D**) A significantly decreased capacity to close an endothelial wound area over time was found at 1% O_2 compared to 20% O_2 (n = 5; * p < 0.05, ** p < 0.001).

To determine the functionality of ECFCs under hypoxia, two standard assays were employed testing angiogenesis and endothelial wound repair *in vitro*. In a Matrigel assay, ECFCs pre-cultured at 5% and 20% O_2 formed complex vascular networks. ECFCs pre-conditioned and tested at 1% O_2 showed significantly reduced number and length of vessel-like structures (Figure 15A, B). When pre-cultured at 1% and subsequently tested at 20% O_2 (re-oxygenation), ECFCs resumed their ability to form complex networks (Figure 15A). In addition, ECFCs almost closed the scratch area in an endothelial wound repair assay at 20% and 5% O_2 within 24 hours. At 1% O_2, the scratch area covered by ECFCs was significantly diminished and was accompanied by reduced proliferation (Figure 15 C, D).

Neo-vasculogenesis *in vivo* depends on MSPC HIF-1α response and is independent of ECFC hypoxia sensing

Neo-vasculogenesis was tested using a progenitor cell co-transplantation model in NSG mice as previously described (Reinisch et al. 2009). Efficiency of experimental vasculogenesis *in vivo* in a previously established ratio of 80% ECFCs admixed with 20% MSPCs (Reinisch et al. 2009) was virtually independent of the carrier matrix protein (Figure 16, 17D).

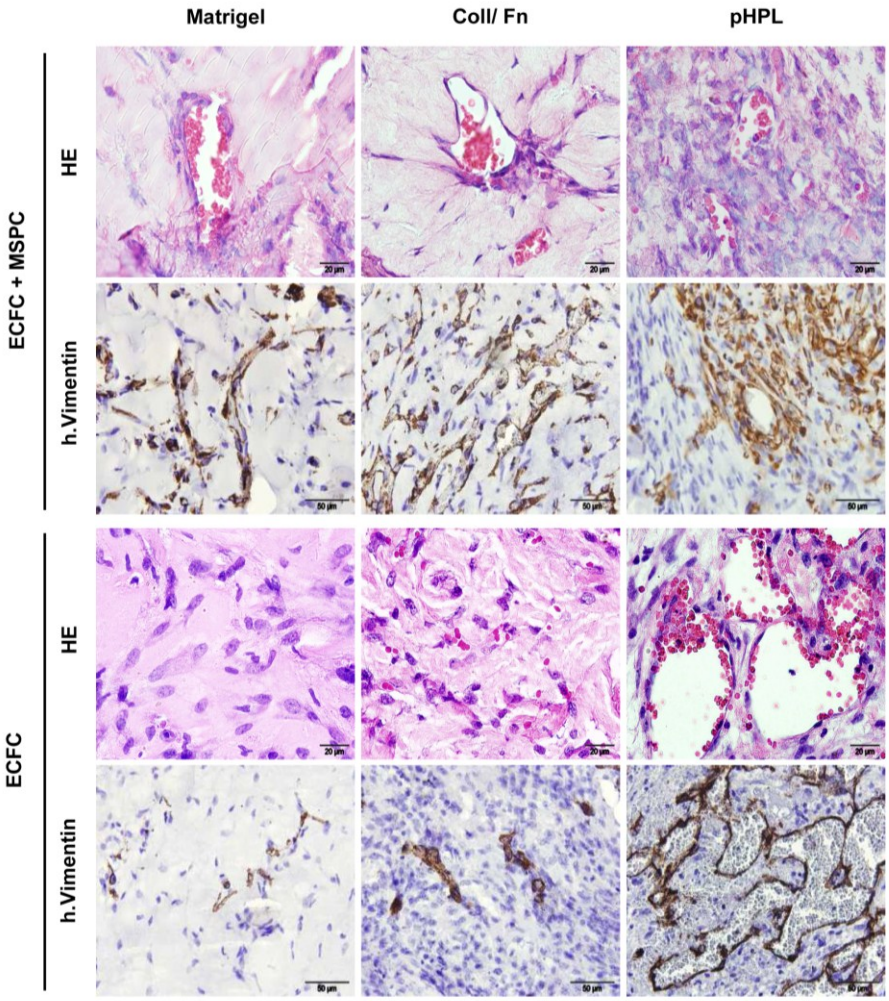


Figure 16. Patent vessel formation depends on functioning mesenchymal stem and progenitor cells (MSPCs) and is virtually matrix- independent (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

Endothelial colony forming cells (ECFCs) (1.6 x 10^6) together with MSPCs (4 x 10^5) for co-transplantation or ECFCs alone (2 x 10^6) were re-suspended in ice cold Matrigel, collagen/fibronectin (Coll/Fn), or pooled human platelet lysate (pHPL), respectively. Aliquots of Matrigel or pHPL were injected and preformed collagen/fibronectin plugs were implanted subcutaneously as described in methods in detail into the flank of mice. Mice were sacrificed on day (d) 7 and 1.5 µm plug sections were either stained with hematoxylin and eosin (HE) or processed for anti-human Vimentin immune histochemistry (h.Vimentin; hematoxylin counterstain, blue; see methods for details and references). Control plugs were also explanted at d 1 (see Figure 12D). The higher cell density despite equal cell input in pHPL implants results from more intense contraction of the matrix *in vivo* compared to Matrigel.

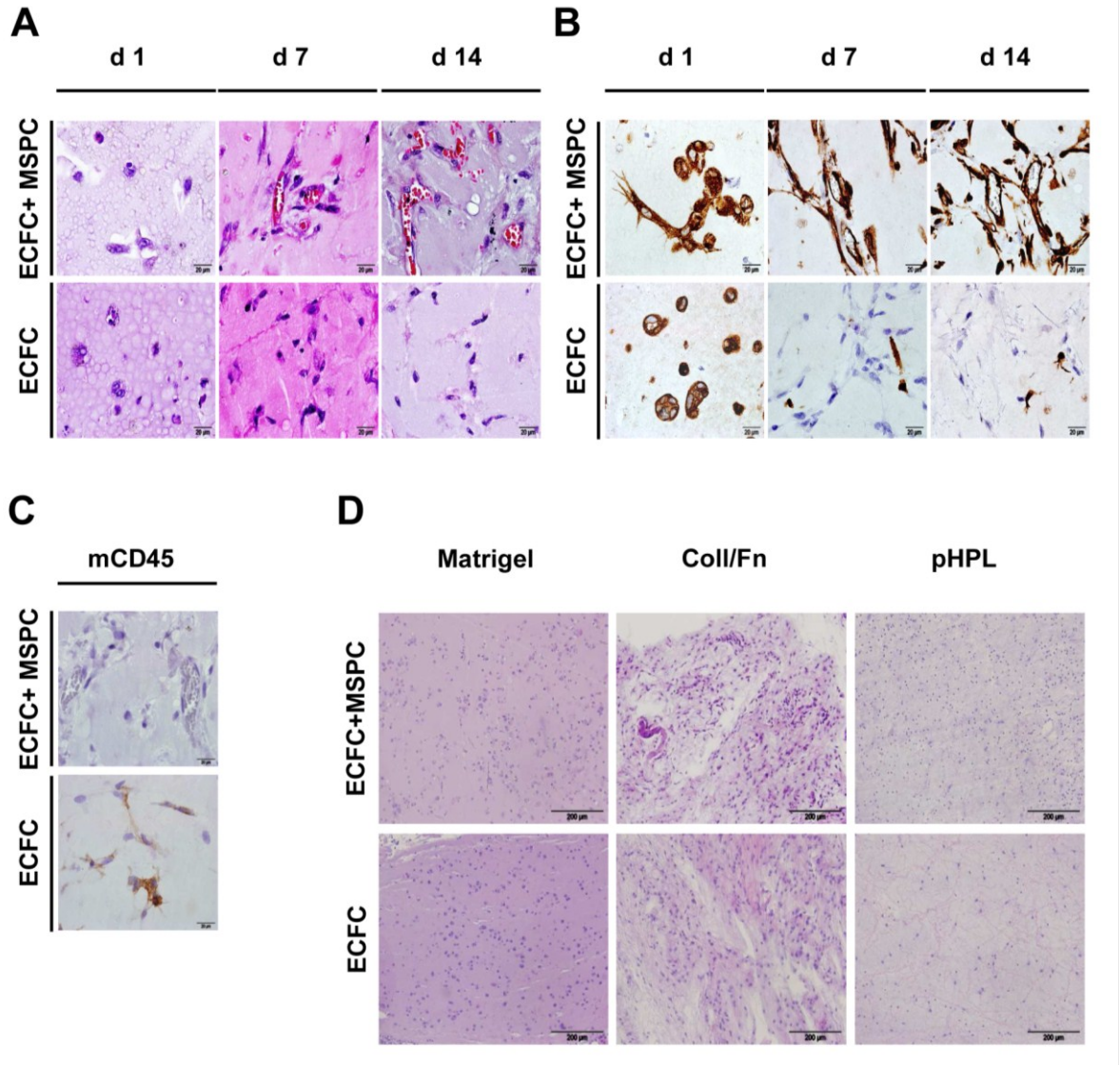


Figure 17. Patent vessel formation depends on mesenchymal stem and progenitor cells (MSPCs) presence and is virtually matrix-independent (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A, B**) Endothelial colony forming cells (ECFCs) alone or MSPCs+ECFCs (ratio 20:80) were re-suspended in Matrigel and injected subcutaneously into immune deficient NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice. Plugs where explanted at days (d) 1, 7 and 14.

(**A**) Hematoxylin and eosin (HE).

(**B**) Mesodermal origin was probed with anti-human Vimentin (brown; nuclei blue, hematoxylin). Implants after ECFC+MSPC co-transplantation showed Vimentin^{+} human vessel formations (d7 & d14) compared to implants of ECFC alone showing not more than rare small vessel-like structures and declining human cell number.

(**C**) Infiltrating Vimentin-negative (non-human) cells in ECFC plugs were mouse CD45^{+} (mouse hematopoietic) cells already 7 d after transplantation.

(**D**) Hematoxylin/eosin staining to visualize cells in different extracellular matrices 1 d after transplantation. MSPCs + ECFCs (top row) or ECFCs only (bottom row) were re-suspended in Matrigel or pooled human platelet lysate (pHPL) and injected subcutaneously (6.6 x 10^6/mL; ratio 20:80; injection volume 300 µL) into immune-deficient NSG mice (n ≥ 3). Preformed collagen/fibronectin plugs containing equal cell compositions were implanted (n = 3).

Interestingly, ECFCs alone largely failed to build complex vessel networks even after 14 days (Figure 17A, B). In the absence of human MSPCs the ECFCs progressively disappeared and were replaced by infiltrating mouse hematopoietic cells over time (Figure 17A- C). For reasons of comparability with existing studies (Reinisch et al. 2009, Melero-Martin et al. 2008, Melero-Martin et al. 2007, Greenberg et al. 2008, Melero-Martin et al. 2010, Frontini et al. 2011, Frontini et al. 2011, Greenberger et al. 2010), Matrigel was chosen for further experiments in this study except when testing for the effect of human platelet-derived growth factors and cytokines present in pHPL. In a hypoxic environment, cells respond by stabilizing HIF1-α or HIF2-α and produce proteins in a cell-type specific manner (Kelly et al. 2003, Manalo et al. 2005). Unexpectedly, the global protein synthesis inhibitor cycloheximide (Croons_ De Meyer_2007) did not affect vessel formation when cycloheximide pre-treated ECFCs where co-transplantated with untreated MSPCs *in vivo* whereas MSPC pre-treatment resulted in a virtually complete abrogation of vessel formation (Figure 18).

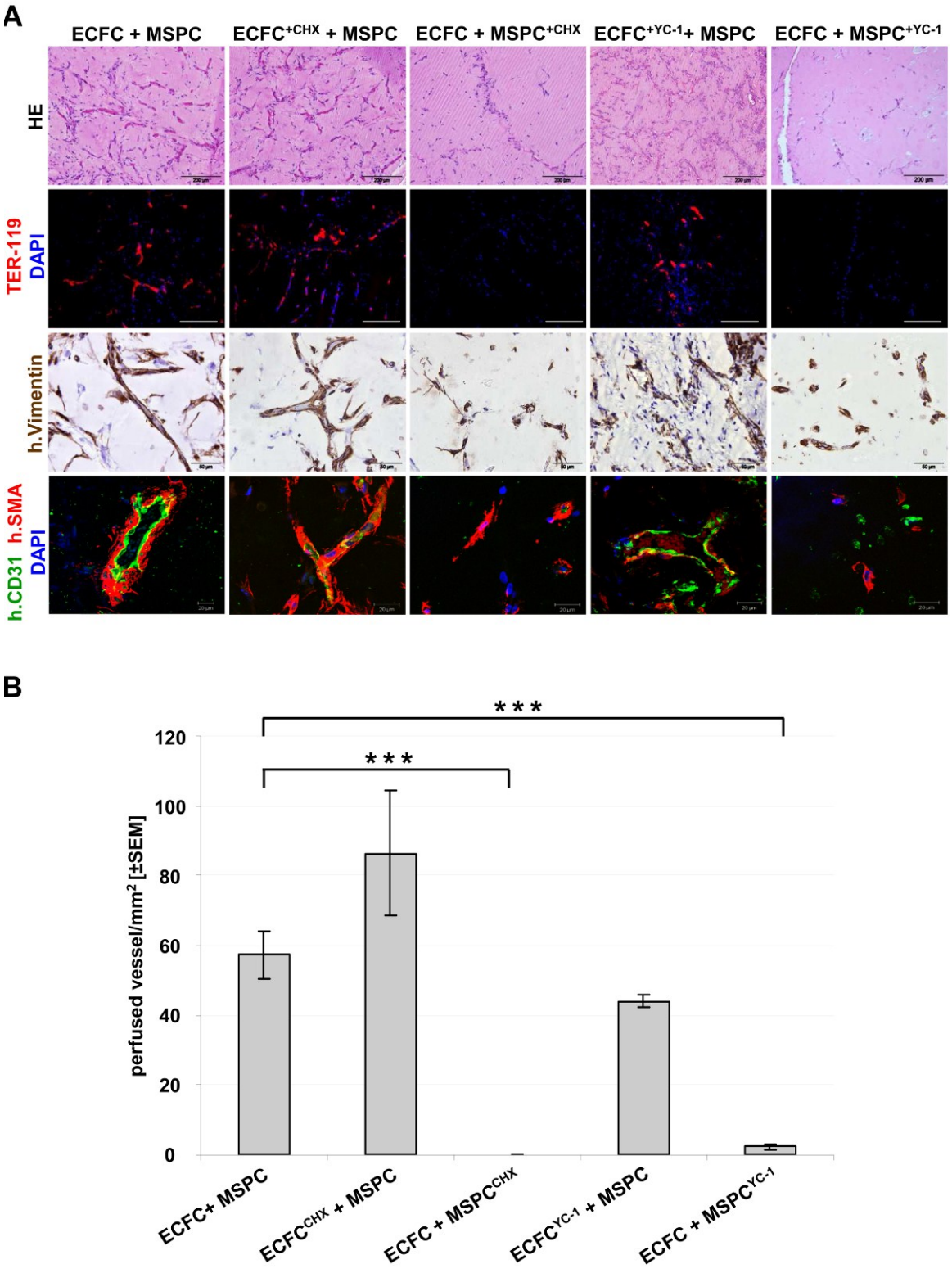


Figure 18. Human vessel formation after progenitor co-transplantation depends on hypoxia-induced factors in mesenchymal stem and progenitor cells (MSPCs) but not endothelial colony forming cells (ECFCs) (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A**) Vessel formation was determined 2 weeks after ECFC + MSPC (2 x 10^6 cells/300µL, ratio of 80:20) co-transplantation. Pre-treatment of one of the two transplanted cell populations with cycloheximide ($ECFC^{+CHX}$, n = 5; $MSPC^{+CHX}$, n = 8) or YC-1 (1 h, 37°C; $ECFC^{+YC1}$ + MSPC, n = 3; ECFC + $MSPC^{+YC1}$, n = 5) compared to untreated transplants (ECFC + MSPC, n = 8) as indicated above each column. Hematoxylin/eosin (HE) staining visualizes morphology (first row; scale bar 200 µm) and the mouse red blood cells (mRBC) resulting from perfusion after connection to the recipients' circulation (Ter119 anti- mouse glycophorin reactivity, red; 4′,6-Diamidin-2-phenylindol, DAPI, blue; second row; scale bar 200 µm). Human mesodermal origin was visualized with anti-human Vimentin monoclonal antibody staining (h.Vimentin, brown; mouse and human nuclei counterstained with hematoxylin, blue; third row; scale bar 50 µm). Plugs contained different numbers of h.Vimentin-negative infiltrating mouse $CD45^+$ hematopoietic cells (see Figure 17C). Fourth row indicating presence or absence of human vessels stained with rabbit anti-human CD31 labeling ECFCs (h.CD31, green), mouse anti-human alpha smooth muscle actin labeling pericytes and mural smooth muscle cells (h.SMA, red) and nuclear counter-stain (DAPI, blue; scale bar 20 µm).
(**B**) Microvessel density was quantified in 200x magnifications of HE-stained Matrigel plug sections by ImageJ and counting red blood cell-filled vessel structures. A significantly decreased capacity to form perfused vessels was found in implants containing cycloheximide (CHX) or YC-1 pre-treated MSPCs compared to untreated co-transplants. (n = 3, 5 high power fields 200 x; *** $p < 0.0001$).

To test which cell type is responsible for the hypoxia sensing during progenitor-derived neo-vasculogenesis *in vivo* and exclude a protein synthesis-independent HIF-mediated anti-vasculogenic pathway, ECFCs and MSPCs were pre-treated with the small molecule hypoxia response inhibitor YC-1 (Li et al. 2008) which blocks both HIF-1α and HIF-2α. Surprisingly, MSPC pre-treatment with YC-1 prior to co-transplantation with untreated ECFCs disabled vessel formation, whereas HIF-1α/HIF-2α inhibition in ECFCs did not significantly affect perfused vessel creation (Figure 18A, B).

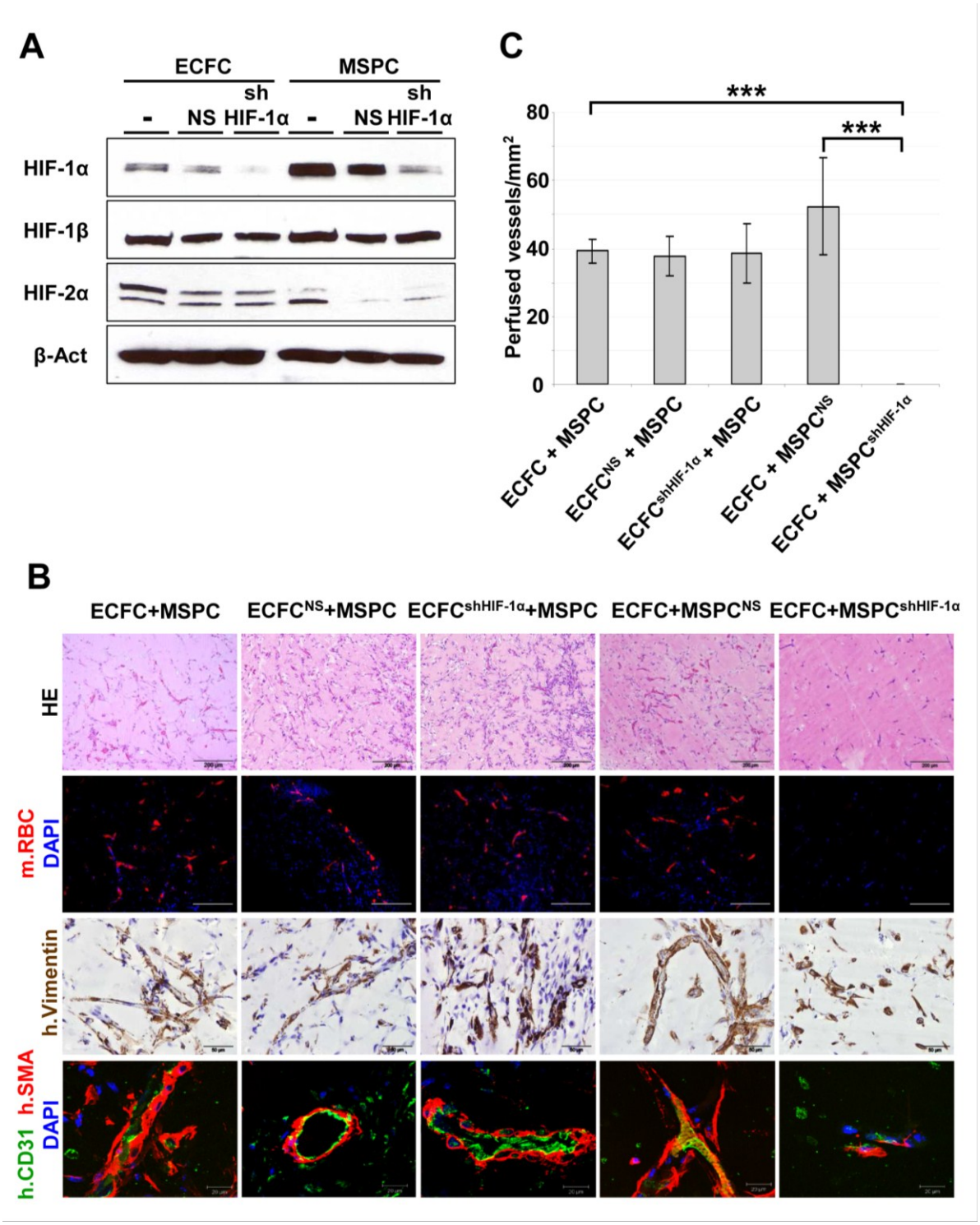


Figure 19. Specific knock-down of hypoxia inducible factor-1α (HIF-1α) in mesenchymal stem and progenitor cells (MSPCs) but not in endothelial colony forming cells (ECFCs) inhibits vessel formation *in vivo* (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A**) Total cell lysates of untreated control (-) ECFCs and MSPCs or after infection with either pGIPZ-HIF1α -shRNA (shHIF-1α) or non-specific pGIPZ-scramble-shRNA (NS) were separated by

SDS-PAGE after 6 h of incubation at 1% O_2. Blots were stained with HIF-1α, HIF-2α, HIF-1β or β-actin (β-Act). Full blots are shown in Figure 11.

(**B**) To delineate the impact of genetic ablation of HIF-1α in either ECFCs or MSPCs before co-transplantation, vessel formation was determined one week after co-transplantation of *Hif-1α*-silenced ($ECFC^{shHIF\text{-}1\alpha}$, n = 3; $MSPC^{shHIF\text{-}1\alpha}$, n = 7) or mock-transfected non-silenced cells ($ECFC^{NS}$, n = 3; $MSPC^{NS}$, n = 7) with genetically un-manipulated corresponding (+ECFC and +MSPC) partner cells as indicated above the columns. Analysis was performed as specified in Figure 18A and is identified on the left side of the picture. Scale bars indicate magnification (first and second row, 200 µm; third row, 50 µm; fourth row, 20 µm).

(**C**) Microvessel density was quantified in 200 x magnifications of hematoxylin and eosin (HE) stained Matrigel plug sections by ImageJ counting red blood cell-filled vessel structures. A significantly decreased capacity to form perfused vessels was found in implants containing shHIF-1α transfected MSPCs compared to untreated co-transplants. (n = 3, 5 high power fields 200 x, mean ± SEM ; *** $p < 0.0001$).

These results were confirmed by specifically silencing *Hif-1α* with small hairpin (sh)-RNA in either cell type before co-transplantation (Figure 19A). *Hif-1α* knock down in MSPCs abolished vessel formation significantly compared to transplants containing mock-transfected MSPCs. In accordance with YC-1 results, genetic *Hif-1α* ablation in ECFCs by sh-RNA did not result in the inhibition of vessel formation (Figure 19B, C).

HIF-competent MSPCs rescue ECFCs from apoptosis

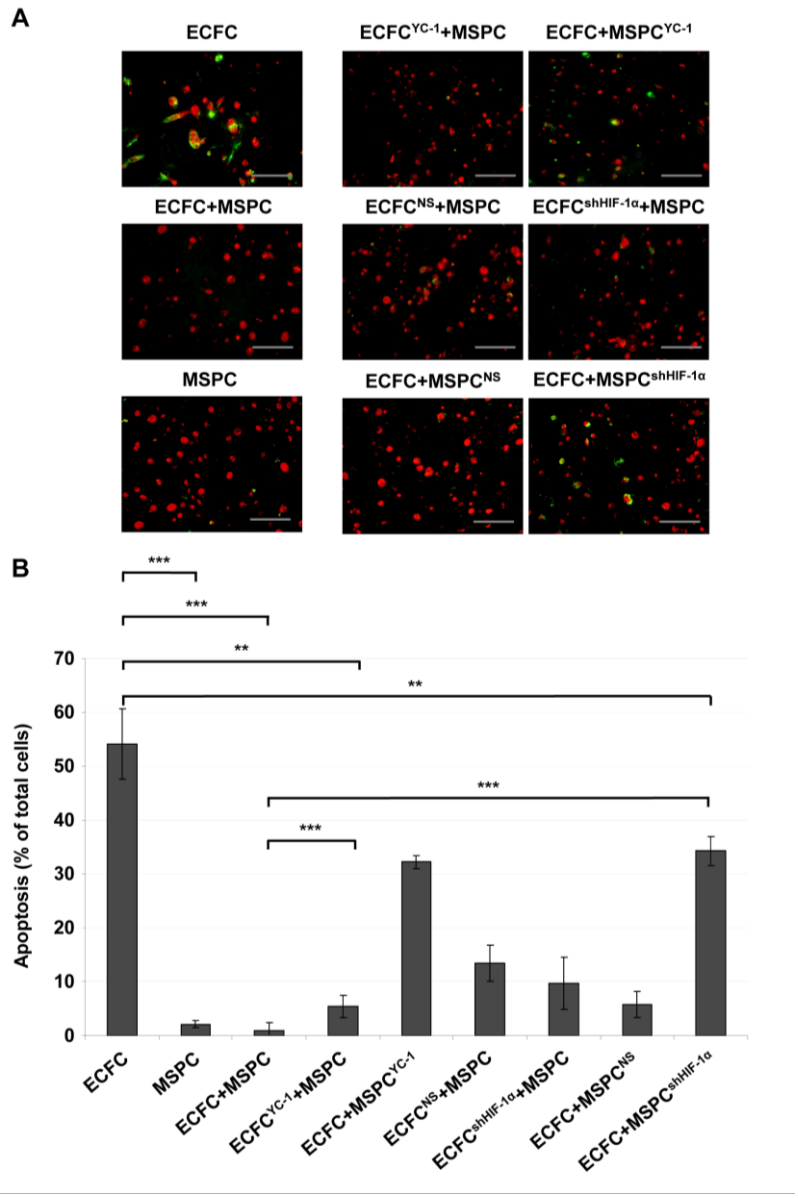


Figure 20. Hypoxia inducible factor (HIF)-competent mesenchymal stem and progenitor cells (MSPCs) are required to avoid premature endothelial colony forming cells (ECFCs) apoptosis *in vivo* (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A**) Apoptosis assay using TdT-mediated dUTP nick end labeling (TUNEL) of ECFC-only or MSPC-only transplants and treated or untreated ECFC+MSPC co-transplants, as indicated above each photograph. All plugs were explanted 24 h after implantation *in vivo*. DNA strand breaks of apoptotic cells were detected by a TUNEL assay kit (Promega) and nuclei were counterstained with propidium iodide (PI, red). Green fluorescence is due to FITC-labeled nucleotide binding to DNA strand breaks of apoptotic cells. Representative pictures from one experiment are shown (scale bar 100 µm) from at least three different donors performed per transplantation type.

(**B**) Apoptotic cells depicted as percentage of total cells ± SD with groups corresponding to the ones in the representative pictures in (A); five high power fields 200 x were counted; ***p < 0.0001, **p < 0.01).

Based on our observation that HIF stabilization in response to hypoxia is an early event in MSPCs that is absent in ECFCs, we asked whether apoptosis also plays a role early in the time course after cell transplantation. We found that the majority of ECFCs had already undergone apoptosis 24 h post transplantation. MSPCs underwent virtually no apoptosis under hypoxic conditions *in vivo* (Figure 20). Co-transplantation with HIF-competent MSPCs rescued ECFCs from apoptosis. Both *Hif-1α* knockdown by shRNA and YC-1-mediated HIF-1α/HIF-2α depletion in MSPCs led to a significant reduction of their anti-apoptotic effect. HIF depletion in ECFCs by either method did not influence early ECFC survival when co-transplanted with HIF-competent MSPCs (Figure 20).

HIF-1α stabilization in response to hypoxia leads to the transcription of more than one hundred target genes including VEGF (more than 170 HIF-1α target genes are summarized in Table 2) (Kelly et al. 2003, Manalo et al. 2005, Ferrara, Gerber & LeCouter 2003). Measuring MSPC cytokine secretion under different O_2 conditions revealed a significant up-regulation of VEGF at hypoxic O_2 levels (Figure 21A). Blocking VEGF *in vivo* by repetitive intraperitoneal injection of bevacicumab largely ablated neo-vasculogenesis after progenitor co-transplantation (Figure 21B).

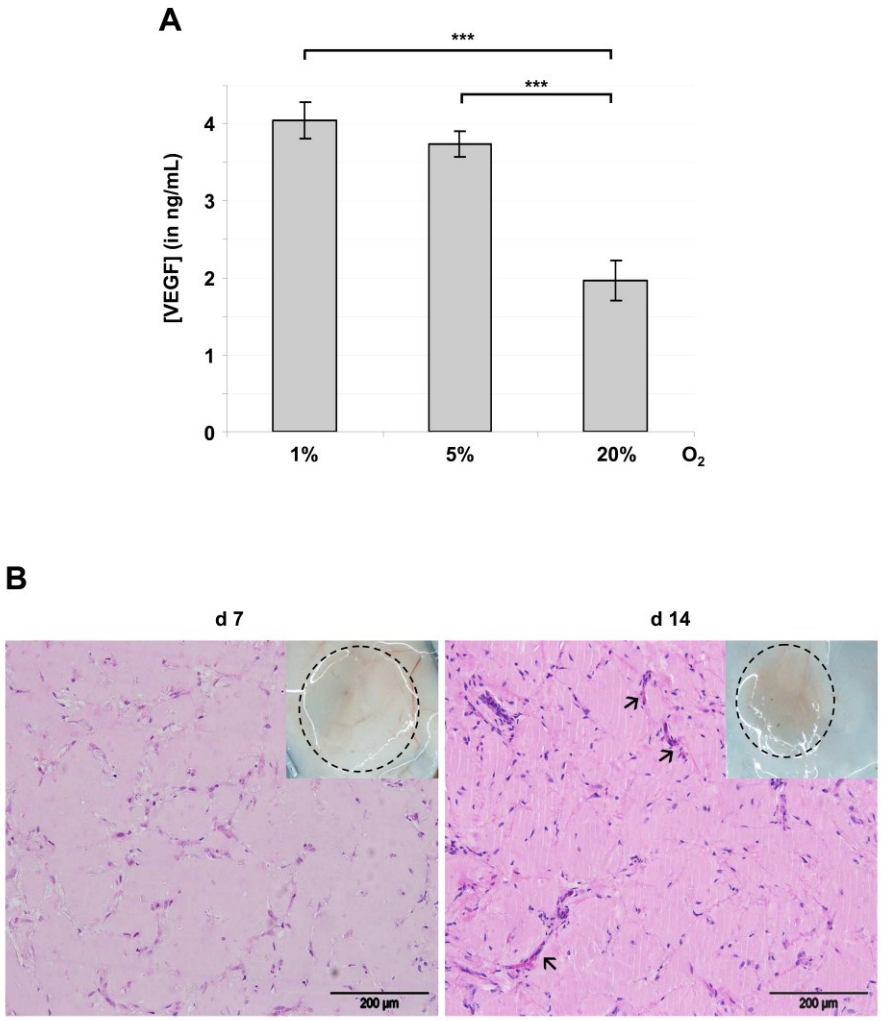


Figure 21. Anti-vascular endothelial growth factor (VEGF) treatment inhibits vessel formation *in vivo* (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(**A**) VEGF concentration in supernatants of endothelial mesenchymal stem and progenitor cells (MSPCs) cultured for 3 days at 1%, 5% and 20% O_2 showed increasing VEGF levels with decreasing oxygen concentration (mean ± SD; n = 3).

(**B**) Anti-VEGF treatment inhibited vessel formation in Matrigel plugs. After subcutaneous co-transplantation of MSPCs and endothelial colony forming cells (ECFCs) (ratio 20:80) into NSG (NOD.Cg-Prdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice recipients were injected i.p. with 5 mg/kg of the therapeutic anti-human VEGF antibody Bevacizumab every other day (d) starting d 1. Hematoxylin/eosin staining showed limited cell arrangement but no human vessel formation after one week (d 7; three doses of antibody). After seven doses of antibody (d 14) some tiny vessels could be observed (arrows). Histology magnification is indicated by scale bar (200 µm). Macrophotography inserts show the freshly explanted pale plugs.

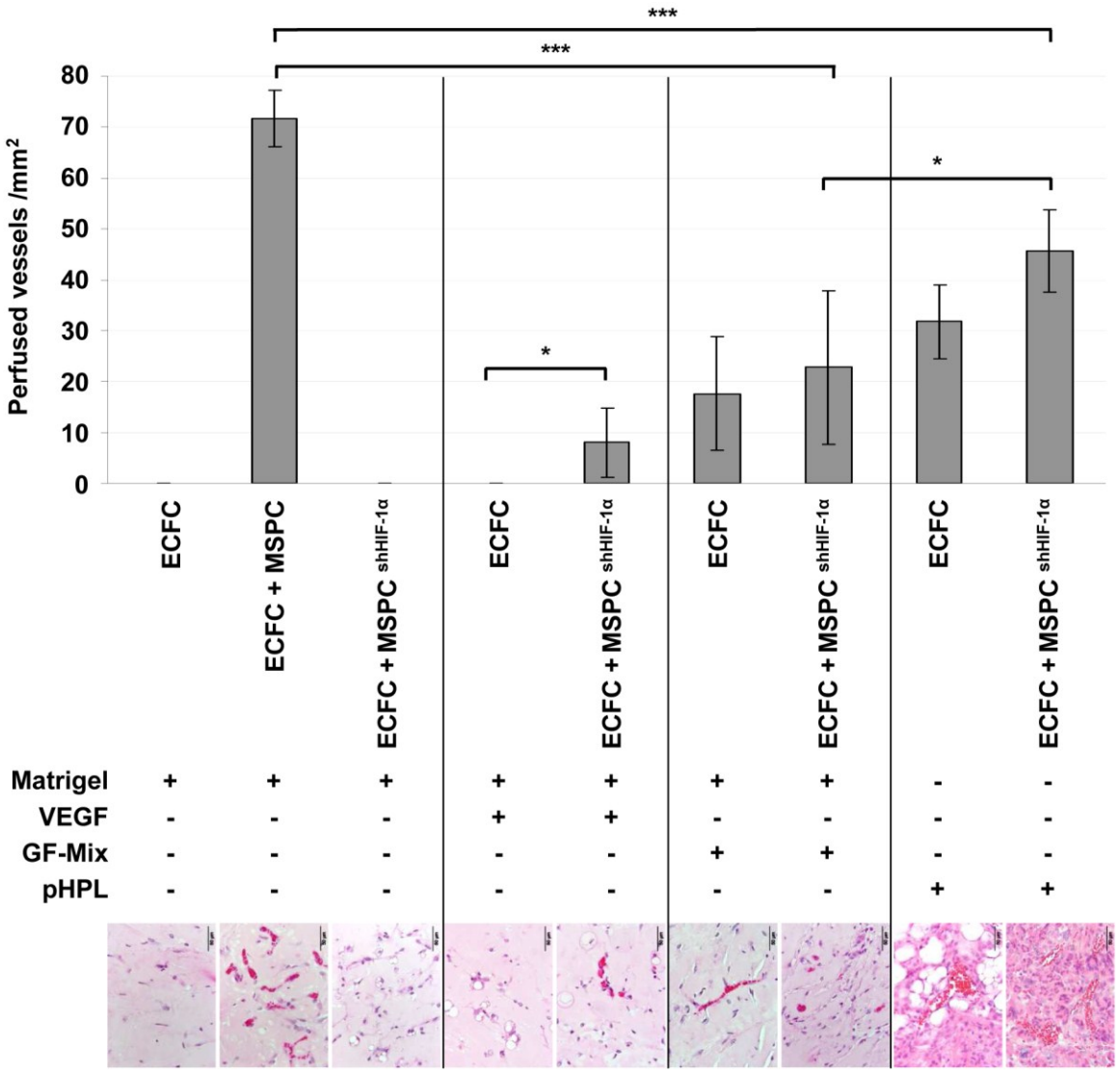


Figure 22. Angiogenic factors can partially substitute mesenchymal stem and progenitor cell (MSPC) hypoxia inducible factor-1α (HIF-1α) deficiency *in vivo* (as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

Endothelial colony forming cells (ECFCs) alone or in combination with MSPCs (either un-manipulated or after shHIF-1α knockdown) were (co)-transplanted in either Matrigel or pooled human platelet lysate (pHPL) gel with or without addition of vascular endothelial growth factor (VEGF) or growth factors (GF)-Mix. GF-Mix was derived from endothelial basal medium supplements and comprised VEGF, endothelial growth factor (EGF), insulin-like growth factor (IGF), basic fibroblast growth factor-2 (bFGF-2), hydrocortisone and ascorbic acid. Mean ± SD results of the number of perfused red blood cell-containing vessels counted in five high power fields (200 X magnification; at least two independent plugs from two independent animals were counted). Microphotographs correspond to the treatment group (scale bar 50 µm; ***$p < 0.0001$, *$p < 0.05$).

To test whether VEGF represents the single dominant MSPC-derived pro-vasculogenic and anti-apoptotic factor in this system, ECFCs alone were transplanted subcutaneously in a matrix supplemented with a saturating concentration of 4 ng/mL VEGF. Whereas VEGF supplementation did not allow ECFCs to form vessels in the absence of HIF-competent MSPCs, it created a small but significant increase in perfused vessels two weeks after co-transplantation of ECFCs with HIF-1α-depleted MSPCs that otherwise reproducibly lacked the potential to contribute to vasculogenesis (Figure 22). To test if a multi-factorial system can rescue ECFCs from apoptosis and initiate vasculogenesis, we supplemented the Matrigel plugs with a defined mixture of vasculogenic growth factors established to support ECFC proliferation and function as standard supplement of the EBM-2 *in vitro* (VEGF, EGF, IGF, bFGF, hydrocortisone and ascorbic acid) (Reinisch et al. 2009). This growth factor (GF)-mix enabled ECFCs alone as well as co-transplants of ECFCs with HIF-1α-depleted MSPCs to form perfused vessels (Figure 22). Vasculogenesis in the presence of the GF-mix was still significantly less efficient than that initiated in the presence of HIF-competent MSPCs.

ECFCs alone injected together with a growth factor-rich pHPL led to the formation of enlarged and lagoon-like vessels which are largely reminiscent of a pathology found in several vascular anomalies including hemangioma (Figure 16 and sample picture in Figure 22). Based on this observation we also tested co-transplants of ECFCs with HIF-1α-depleted MSPCs in pHPL. This resulted in the highest number of perfused vessels formed by ECFCs co-transplanted with HIF-depleted MSPCs, but this combination was still significantly less efficient than co-transplantation of ECFCs with HIF-competent MSPCs (Figure 22).

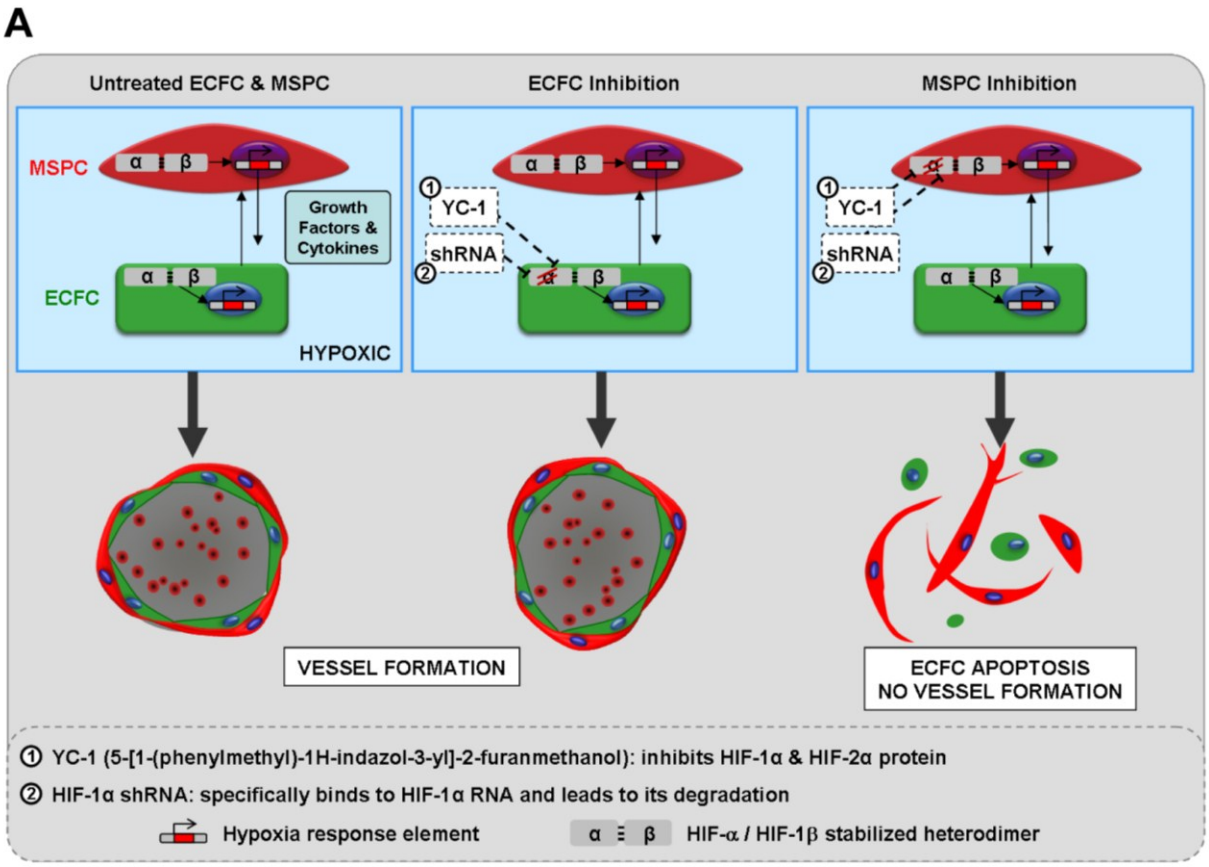
7. Discussion

(The following chapter follows the publication Hofmann et al. 2012 (Hofmann et al. 2012))

Up to now, endothelial cells have been considered the major initiator of neo-vasculogenesis in ischemic tissue (Carmeliet, Jain 2011a). However, clinical trials focusing on endothelial progenitors have been of limited efficiency, both for therapeutic vasculogenesis and for anti-angiogenic therapy. Recently, it has been recognized that endothelial cells require interaction with MSPCs to efficiently build functional and perfused vessels (within different extracellular matrix implants) *in vivo* (Koike et al. 2004, Melero-Martin et al. 2007, Au et al. 2008, Melero-Martin et al. 2008, Traktuev et al. 2009, Reinisch et al. 2009) and regenerate perfusion after hind limb ischemia (Schwarz et al. 2012). In order to develop novel strategies for therapeutic vasculogenesis and against tumor vessel formation, it is of the utmost importance to understand the hypoxia-induced cross-talk of endothelial with mesenchymal cells and to decipher the underlying mechanisms of new vessel formation.

Hypoxia-induced HIF-1α stabilization and transition to the nucleus is a key event initiating the expression of more than 100 cell-specific target genes (Manalo et al. 2005, Semenza 2007). The results presented in this dissertation demonstrate that MSPCs are more susceptible to oxygen level changes and sense hypoxia earlier and more efficiently than vessel wall-lining ECFCs. It has been demonstrated that ECFCs lack detectable HIF-1α one day after transplantation *in vivo* and prior to vessel assembly and plug perfusion. This argues against the simplified view that the endothelial cells are more highly oxygenated due to their vascular location *in vivo*. Based on the finding that MSPCs already stabilize HIF-1α at a base line level at 20% O_2, one could hypothesize that MSPCs are pre-determined to react earlier and/or more sensitively when oxygen levels decrease. This would give MSPCs a natural benefit in responding to hypoxic environments. However, coagulation factors like tissue factor and thrombin are known to interfere with blood vessel development and homeostasis (Mackman, Davis 2011). ANG-2, bacterial LPS and various growth factors and cytokines are non-hypoxic stimuli that can regulate HIF expression (Kuschel, Simon & Tug 2012). Proteases also play a key role in the

later phase of anastomosis of newly formed vessels during a hypothetic process called ‘wrapping and tapping’ which realizes the connection with the existing vasculature (Cheng et al. 2011). Therefore, it remains to be determined whether MSPCs already express HIF-1α at standard culture conditions as a cell culture artifact or whether there is a physiologically rational underlying mechanism.



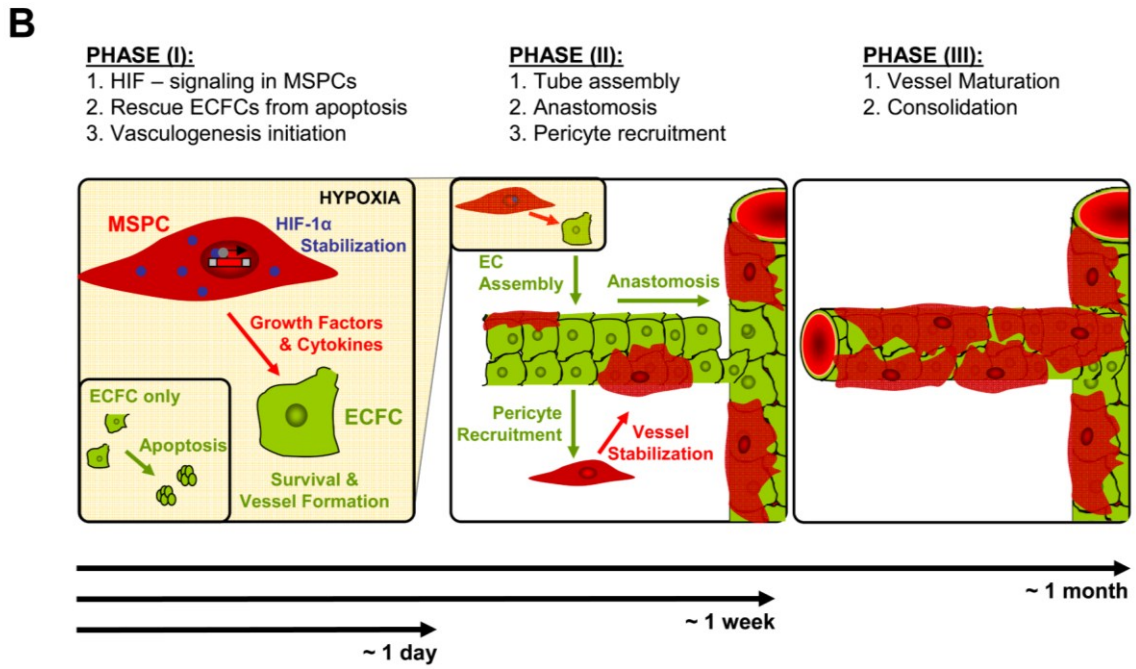


Figure 23. Mesenchymal stem and progenitor cells (MSPCs) are responsible for oxygen sensing and induction of neo-vasculogenesis (cartoon as in Hofmann et al. 2012 (Hofmann et al. 2012)**).**

(A) The experimental strategy of delineating the hypoxia response used pre-treatment of either ECFCs or MSPCs before transplantation with YC-1 (inhibiting both hypoxia inducible factor (HIF)-1α as well as HIF-2α ①) or specific knockdown of HIF-1α RNA (by sh-RNA; ②). The surprising outcome of vessel formation despite the inhibition of ECFC hypoxia response and collapse of neo-vasculogenesis after MSPC inhibition is graphically illustrated.

(B) Based on the current insight, a model is proposed describing three phases of adult vasculogenesis.

Because hypoxia-induced reduction of ECFC and MSPC proliferation was accompanied by unchanged clonogenicity, the initial hypothesis was generated that hypoxia does not damage but rather silences endothelial and mesenchymal progenitors. In accordance to this hypothesis, the phenomenon that hypoxic ECFCs resume their ability to proliferate and form complex vascular networks after re-exposure to 20% O_2 further substantiated the argument that hypoxic ECFCs can maintain a functionally quiescent state which they can resume when re-oxygenated. Therefore, ECFC and MSPC expansion under 5% oxygen would

provide a more physiological culture condition environment; however, this would also limit net cell numbers for clinical application.

The observation that endothelial protein synthesis appeared to be dispensable as indicated by cycloheximide resistance in the herein described co-transplantation model is surprising and demands further investigation. Presumably, the reversible effect of cycloheximide (within first 3 days after implantation) leaves ECFCs in a quiescence state, while the non-treated MSPCs produce angiogenic factors. As soon as the cycloheximide effect is reversed, ECFCs can react to the high quantity of angiogenic factors provided by MSPCs leading to the induction of neo-vasculogenesis. A hypothetic time-dependent accumulation of pro-angiogenic factors secreted by co-transplanted functioning MSPCs in this system could perhaps explain the vessel-forming capacity of CHX-treated ECFCs. The lack of vessel formation when MSPCs are CHX-treated and co-injected with functioning ECFCs could be explained by the finding that ECFCs undergo apoptosis *in vivo* within the first 24 h if they do not receive the survival signals produced by MSPCs.

To delineate which cell-type is responsible for HIF sensing during vasculogenesis, two independent *in vivo* strategies using the HIF inhibitor YC-1 and a shRNA against HIF-1α were designed (Figure 23A). Unexpectedly, neither treatment of ECFCs with the pharmacological HIF-1α / HIF-2α inhibitor YC-1 nor *Hif-1α* knockdown by shRNA in ECFC affected patent vessel formation. In contrast, both *Hif-1α* knockdown as well as YC-1 application in MSPCs completely abrogated neo-vasculogenesis *in vivo*. These findings evidently demonstrated that hypoxia sensing via HIF-1α stabilization in MSPCs but not in ECFCs is a crucial event in therapeutic vasculogenesis. The surprising observation of this dissertation that therapeutic vasculogenesis takes place independently from endothelial HIF functionality *in vivo* might explain the lack of efficacy while using endothelial progenitors only for clinical application. This further strengthens the need to re-examine current concepts in vascular regeneration (Zhou et al. 2011).

It has been previously published that in *Hif-1α* knockout animals, the formation of abnormally enlarged vessels and resulting embryonic lethality were associated with mesenchymal cell death and did not correlate with VEGF deficiency (Kotch et al. 1999). This and the lack of clinical efficiency when using VEGF depletion *in vivo* (Greenberg et al. 2008, Carmeliet, Jain 2011a, Carmeliet, Jain 2011b) provide evidence that VEGF is only one of the pro-vasculogenic factor in this system.

Therefore, the finding described in this dissertation that supplementation of VEGF for injected ECFCs alone could not substitute for the presence of HIF-competent MSPC substantiated the notion that VEGF is not the only mediator during neo-vasculogenesis. This observation implies that a combination with MSPC-targeted therapies could be a promising strategy for the development of an anti-tumor therapy.

Furthermore, it has been reported that HIF-2α is the dominant O_2-responsive transcription factor in endothelial lineage cells (Patel, Simon 2008, Carmeliet, Jain 2011b). The observation (in the study described herein) that YC-1 pre-treatment of ECFCs does not affect vessel formation argues that HIF-2α stabilization in ECs also only plays a subordinate role during neo-vasculogenesis. It has been previously shown that endothelial-specific *Hif-2α* deletion in mice resulted in a virtually normal vascular phenotype except for abnormal vessel permeability, increased vessel formation during vascular regeneration and unstable tumor vascularization (Skuli et al. 2009, Skuli et al. 2012). Therefore, the thin pericyte coverage around some of the vessels derived from YC-1 pre-treated ECFCs in this study (Figure 18A) might be associated with a certain effect of the *Hif-2α* knock-down treatment. Based on these data it seems likely that MSPCs or perhaps other HIF-competent stromal cells can function to rescue vascular regeneration on demand. Hypoxia-induced ECFC apoptosis *in vivo* was prevented by co-transplantation of HIF-competent but not of HIF-deficient MSPCs. This finding is supported by the recent discovery that WAT-derived stem cells rescued the ECs from apoptotic death when co-transplanted with CB-derived endothelial progenitors. This PDGF-independent mechanism thus lead to robust vessel assembly and prevented vessel regression around day 14 post implantation (Traktuev et al. 2009). Recently, it has been shown that MSPCs can also rescue human-induced pluripotent stem (iPS) cells from apoptosis resulting in their long-term engraftment in a myocardial infarct pig model (Templin et al. 2012).

Whether physical contact of ECFCs and MSPCs is necessary to conduct the anti-apoptotic effects of MSPCs remains to be determined. The observation that in the presence of HIF-silenced MSPCs *in vivo* ECFCs were not capable of performing their vasculogenic capacity, argues against the necessity of a stringent physical interaction. In this context, it is interesting to note that part of the effect of HIF-competent MSPCs observed in this study was able to be mimicked by the

utilization of a humanized matrix based on pHPL (Figure 22). These two findings support the hypothesis that the effect of MSPCs is mainly mediated by HIF-mediated protein secretion. Therefore, platelet-derived growth factors in pHPL could be used as a therapeutic agent improving or even substituting cell therapy (Saif et al. 2010, Demidova-Rice et al. 2012). The uncomplicated application of progenitor cells admixed with pHPL solution via a regular syringe may offer a certain technical advantage for clinical application as compared to collagen/fibronectin, which requires solidification before surgical implantation of preformed plugs.

As cardiac regeneration has also been reported as depending on the interaction of endothelial and smooth muscle progenitors in a model of bone marrow-derived cell therapy (Yoon et al. 2010), endothelial-mesenchymal crosstalk may not be limited solely to vascular regeneration. Also the development of a bone and marrow niche involves endothelial and mesenchymal precursor interaction. The developmental program herein depends on the source of MSPCs and the ratio between ECFCs and MSPCs. This system has already been implemented as a model system for a hematopoietic microenvironment in which HIF-1α has been shown to control the engraftment of normal and malignant blood cells (Chen et al. 2012). Furthermore, it has been shown that hematopoietic and tumor cells can also act in concert with endothelial and mesenchymal progenitors to initiate and support vessel formation in hypoxic environments (Pugh, Ratcliffe 2003, Rafii, Lyden 2003, Yoon et al. 2010, Martin et al. 2010, Carmeliet, Jain 2011a, Melero- Carmeliet, Jain 2011b).

Another aspect is the role of mesenchymal cells during tumor angiogenesis. Tumor vessels are known to be leaky and unstable. Carmeliet et al. provoked the discussion surrounding whether enhancing vessel normalization could increase tumor susceptibility for chemotherapeutics and decrease the possibility of cancer cells escaping into the blood stream and forming metastasis (Carmeliet, Jain 2011a). However, this requires a deeper understanding of tumor vessel formation. Considering the findings of this dissertation, a therapeutic strategy specifically increasing mesenchymal HIF-1α expression might offer the trigger to support stable vessel formation for tumors. This could either be done via gene therapy specifically targeting mesenchymal progenitors, though the adequate cell surface markers would firstly have to be determined, or via cellular therapy injecting DMOG-pretreated MSPCs. Even if one could increase tumor vessel normalization

in combination with chemotherapy it would still be doubtful whether this would inhibit rather than increase tumor growth.

The existence of an indirect mesenchymal cell-mediated oxygen sensing pathway which leads to the initiation of vessel formation as observed in this study provoked a proposal of a model which incorporates these new aspects of neo-vasculogenesis (Figure 23B). The findings of this dissertation propose adding a novel MSPC-mediated initiating step to the current knowledge and rearranging the sequence of events that take place during neo-vasculogenesis in three phases (Figure 23B). In contrast to previous findings, this work provides evidence that in an initial phase *in vivo* (Phase I – within 24 hours), hypoxic ECFCs alone undergo apoptosis while MSPCs stabilize HIF-1α and secrete hypoxia-induced proteins that lead to the survival of ECFCs. In Phase II (within 1 week) ECFCs form tubular structures and anastomose with the existing vasculature which leads to the formation of perfused vessels. This process is accompanied by the recruitment of MSPCs and following vessel maturation which leads to vessel stabilization (Phase III – within 1 month). We conclude that ECFC/MSPC co-transplantation represents an important alternative to current endothelial cell therapy strategies for vascular regeneration and tissue engineering. Furthermore, understanding the role stromal cells and their secreted factors play in the orchestrated interplay driving the vascularization machinery will help to critically develop more efficient pro- and anti-angiogenic strategies.

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Oxygen Sensing Mesenchymal Progenitors Promote Neo-Vasculogenesis in a Humanized Mouse Model *In Vivo*

Nicole A. Hofmann[1], Anna Ortner[1], Rodrigo O. Jacamo[2], Andreas Reinisch[1,3], Katharina Schallmoser[1,4], Rokhsareh Rohban[1], Nathalie Etchart[1,3,4], Margareta Fruehwirth[1,3], Christine Beham-Schmid[5], Michael Andreeff[2], Dirk Strunk[1,3]*

1 Stem Cell Research Unit, Medical University of Graz, Graz, Austria, **2** Department of Leukemia, M.D. Anderson Cancer Center, University of Texas, Houston, Texas, United States of America, **3** Department of Hematology and Stem Cell Transplantation, Medical University of Graz, Graz, Austria, **4** Department of Blood Group Serology and Transfusion Medicine, Medical University of Graz, Graz, Austria, **5** Institute of Pathology, Medical University of Graz, Graz, Austria

Abstract

Despite insights into the molecular pathways regulating hypoxia-induced gene expression, it is not known which cell types accomplish oxygen sensing during neo-vasculogenesis. We have developed a humanized mouse model of endothelial and mesenchymal progenitor co-transplantation to delineate the cellular compartments responsible for hypoxia response during vasculogenesis. Mesenchymal stem/progenitor cells (MSPCs) accumulated nuclear hypoxia-inducible transcription factor (HIF)-1α earlier and more sensitively than endothelial colony forming progenitor cells (ECFCs) *in vitro* and *in vivo*. Hypoxic ECFCs showed reduced function *in vitro* and underwent apoptosis within 24h *in vivo* when used without MSPCs. Surprisingly, only in MSPCs did pharmacologic or genetic inhibition of HIF-1α abrogate neo-vasculogenesis. HIF deletion in ECFCs caused no effect. ECFCs could be rescued from hypoxia-induced apoptosis by HIF-competent MSPCs resulting in the formation of patent perfused human vessels. Several angiogenic factors need to act in concert to partially substitute mesenchymal HIF-deficiency. Results demonstrate that ECFCs require HIF-competent vessel wall progenitors to initiate vasculogenesis *in vivo* and to bypass hypoxia-induced apoptosis. We describe a novel mechanistic role of MSPCs as oxygen sensors promoting vasculogenesis thus underscoring their importance for the development of advanced cellular therapies.

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* E-mail: dirk.strunk@medunigraz.at

Introduction

Vascular homeostasis and regeneration play an essential role in development, health and disease [1,2]. Vessel remodeling and repair during postnatal life have long been viewed as occurring exclusively through proliferation and subsequent migration of mature ECs derived from pre-existing vessel walls, a process termed angiogenesis [3]. The isolation of EC progenitors from human blood changed that paradigm and introduced the concept of therapeutic vasculogenesis [4]. The discovery that vessel wall-derived ECs rapidly proliferate because they contain a complete hierarchy of ECFCs supported the concept of the progenitor-dependence of vasculogenesis [5–8]. Cell transplantation to re-vascularize ischemic tissue has thus become a central vision for regenerative medicine [8–10]. Therapeutic targets comprise cardiovascular diseases including stroke, myocardial infarction and peripheral artery disease as well as wound healing and vessel creation as the prerequisite for effective tissue engineering [10].

Initial attempts towards vascular regenerative cell therapy used sole EC or progenitor transplantation. More stable vessel formation during adult neo-vasculogenesis was achieved by the co-transplantation of ECs with stromal cells or more complex progenitor transplants [11–14]. The robust vasculogenic program that endothelial and mesenchymal progenitors can preserve despite *ex vivo* propagation is also evidenced by the fact that the progeny of expanded hemangioma-derived stem cells can still mimic disease pathology after xeno-transplantation [15]. Different types of stromal cells have since been shown to assume mural cell function stabilizing human vessels after co-transplantation in several mouse models [12,14,16–18]. However, the effectors initiating neo-vasculogenesis are as yet unknown.

Most of our current insights into the molecular control of vessel formation have been derived from studying sprouting angiogenesis [10,19]. This process involves HIF-1α which is constitutively expressed and instantaneously degraded at sufficient oxygen tension. Under hypoxia, HIF-1α is stabilized and acts together with HIF-1β as a transcription factor for a multiplicity of target genes carrying hypoxia response elements [20,21]. The current view is that stabilized HIF drives an angiogenic program that results in EC sprouting from existing vessels. The hypoxia-induced production of growth factors is considered to subsequently recruit mesenchymal cells to adapt a pericyte phenotype providing structural stability of the newly formed vessels [1,10,22]. It is not clear whether the same sequence of events is operative during progenitor-derived adult vasculogenesis. Therapeutic vasculogenesis has so far remained an unmet medical need at least in part due

to our limited understanding of the interplay between endothelial and mesenchymal progenitors during re-vascularization of ischemic tissue [9,10]. Here we show for the first time that MSPCs act by sensing low oxygen thereby enabling the initiation of neovasculogenesis in addition to their established function as vessel stabilizing pericytes.

Methods

Ethics Statement

Prior approval was obtained for human cell and tissue sample collection from the Institutional Review Board of the Medical University of Graz (protocols 19-252 ex 07/08, 18-243 ex 06/07, 21.060 ex 09/10). Adult samples were collected after written informed consent from healthy volunteers and cardiovascular disease (CVD) patients, and umbilical cord or cord blood samples after written informed consent by the mother after full-term pregnancies in accordance with the Declaration of Helsinki. Animal experiments were approved by the Animal Care and Use Committee at the Veterinary University of Vienna on behalf of the Austrian Ministry of Science and Research according to the criteria published in the guide for the care and use of laboratory animals and performed as described [17]. Neonatal fibroblasts were purchased from Lonza (Walkersville, MD).

Human Progenitor Cell Isolation, Large-scale Expansion and Long-term Propagation

ECFCs and MSPCs were isolated and expanded from neonatal cord or cord blood (CB), adult peripheral blood (PB) or bone marrow (BM) aspirates following IRB approval as previously described under animal serum-free culture conditions with pooled human platelet lysate (pHPL) replacing fetal bovine serum (FBS) [17,23–25]. Additional video protocols covering production of pHPL (www.jove.com/details.php?id = 1523) [26], as well as propagation of blood-derived ECFCs (www.jove.com/details.php?id = 1524) [27] and umbilical cord ECFCs and MSPCs (www.jove.com/details.php?id = 1525) [28] are available online to support reproducibility. For large-scale propagation, primary culture-derived ECFCs were seeded in EGM-2 (Lonza) supplemented with 10% pHPL at a density of 100 cells/cm^2 and MSPCs in α-MEM (Sigma-Aldrich, St. Louis, MO) also supplemented with 10% pHPL at a density of 30 cells/cm^2 in 2,528 cm^2 cell factories (Thermo Fisher Scientific, Freemont, CA). For long-term propagation primary culture-derived ECFCs and MSPCs were seeded in triplicate in 75cm^2 flasks at a density of 100 cells/cm^2 (n = 4) and 30 cells/cm^2 (n = 2), respectively, as described [29]. Cells were cryopreserved after expansion until use as described [30].

Oxygen-dependent Cell Culture, Clonogenicity and Re-oxygenation

ECFCs and MSPCs were grown and manipulated in an XVIVO system hypoxy workstation incubator (BioSpherix, New York, NY) set to 1% or 5% O_2 in 95% humidified atmosphere containing 5% CO_2 at 37°C. Media were equilibrated for 24 h to the required oxygen level in the hypoxy workstation before use. Conventional control cultures were done in ambient air (20% O_2) in a standard incubator (Heraeus; Thermo Scientific) with 95% humidified atmosphere and 5% CO_2 at 37°C. Oxygen content of media and cell supernatants was verified by blood gas analysis (Cobas B21, Roche, Burgess Hill, West Sussex, UK).

ECFCs and MSPCs cultured at 1% and 5% O_2 for 7 days were harvested in the hypoxy workstation and seeded for proliferation studies in 6-well plates at a density of 100 cells/cm^2 or 30 cells/cm^2, respectively. Clonogenicity of ECFCs and MSPCs was measured after seeding at indicated densities of 1–10 cells/cm^2 in 55cm^2 plates and either cultured in the previous oxygen conditions or re-oxygenated to 20% O_2. After two weeks, cell numbers were measured by hemocytometry and colonies were fixed with paraformaldehyde within the hypoxy workstation, stained with crystal violet (Merck, Darmstadt, Germany) and enumerated as described earlier with ImageJ software (National Institutes of Health, Bethesda, MD) [17,24]. Low proliferative potential (LPP) and high proliferative potential (HPP) colonies were defined as colonies comprising 51 to 500 and more than 500 cells, respectively [7,17].

Angiogenesis Assay and Endothelial Wound Repair *in vitro*

To test the effects of different oxygen concentrations on vascular-like network formation, ECFCs were pre-cultured at 1%, 5% and 20% O_2 for 7 days *in vitro*. After trypsinization, 180,000 ECFCs were re-suspended in 2 mL EGM-2/10% pHPL and seeded on 10 cm^2 polymerized Matrigel/well (Angiogenesis assay kit; Millipore, Billerica, MA) according to the manufacturers' instructions. Vascular-like networks (24 h) were documented with a Color View III camera on an Olympus IX51 microscope with the analySIS B acquisition software (all Olympus, Hamburg, Germany). Re-oxygenation to 20% O_2 was done as described above. Tube number and length were determined using ImageJ software (http://rsbweb.nih.gov).

For endothelial wound repair studies, ECFCs were seeded at 1×10^5 cells per 10 cm^2 (Corning 6-well plate; Sigma) and cultured until confluence (approximately 4 d, 20% O_2). After standardized scratch of the confluent cell layer with a 1,000 µL pipette tip (Gilson, Middleton, WI), cultures were introduced into the O_2-controlled cell observer workspace (Carl Zeiss, Thornwood, NY) and the medium was replaced by pre-oxygenized fresh medium (1%, 5% or 20% O_2) as indicated. Cell movement and proliferation were monitored by acquiring video sequences at 20 minute intervals covering a time period of up to 48 hours (Zeiss). Single cell proliferation was evaluated accurately by counting dividing cells on printouts of every video picture. A composite of three time-lapse videos covering 16 h of endothelial wound repair video sequences with cell divisions identified in red, green and yellow numbers as 1%, 5% and 20% O_2, respectively (see Video S1). The area of wound repair was determined using ImageJ software.

Experimental Neo-vasculogenesis *in vivo*

In vivo functionality was analyzed by resuspending 4×10^5 bone marrow–derived MSPCs mixed with 1.6×10^6 ECFCs per 0.3 mL in either ice-cold Matrigel (Millipore) or pHPL immediately before subcutaneous injection into the flank of immune-deficient NSG (NOD.Cg-Prdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice under general anesthesia as published [17]. Alternatively ECFCs and MSPCs were mixed with rat collagen and human fibronectin, allowed to coagulate, and were then implanted subcutaneously as a preformed plug in NSG mice as previously described [16]. Both ECFCs and MSPCs were derived from large-scale expansions providing consistent batches of cells after culture on up to 30,000 cm^2 of culture area (12x 4-layered cell factories; Thermo Scientific) as previously published [17]. Mice were sacrificed by cervical dislocation at days 1, 7 and 14 after injection and plugs where explanted. To depict hypoxia, mice received intra-peritoneal injections of pimonidazole (60 mg/kg body weight; Hypoxyprobe, Burlington, MA) 30 minutes before plug explantation. Animal experiments were

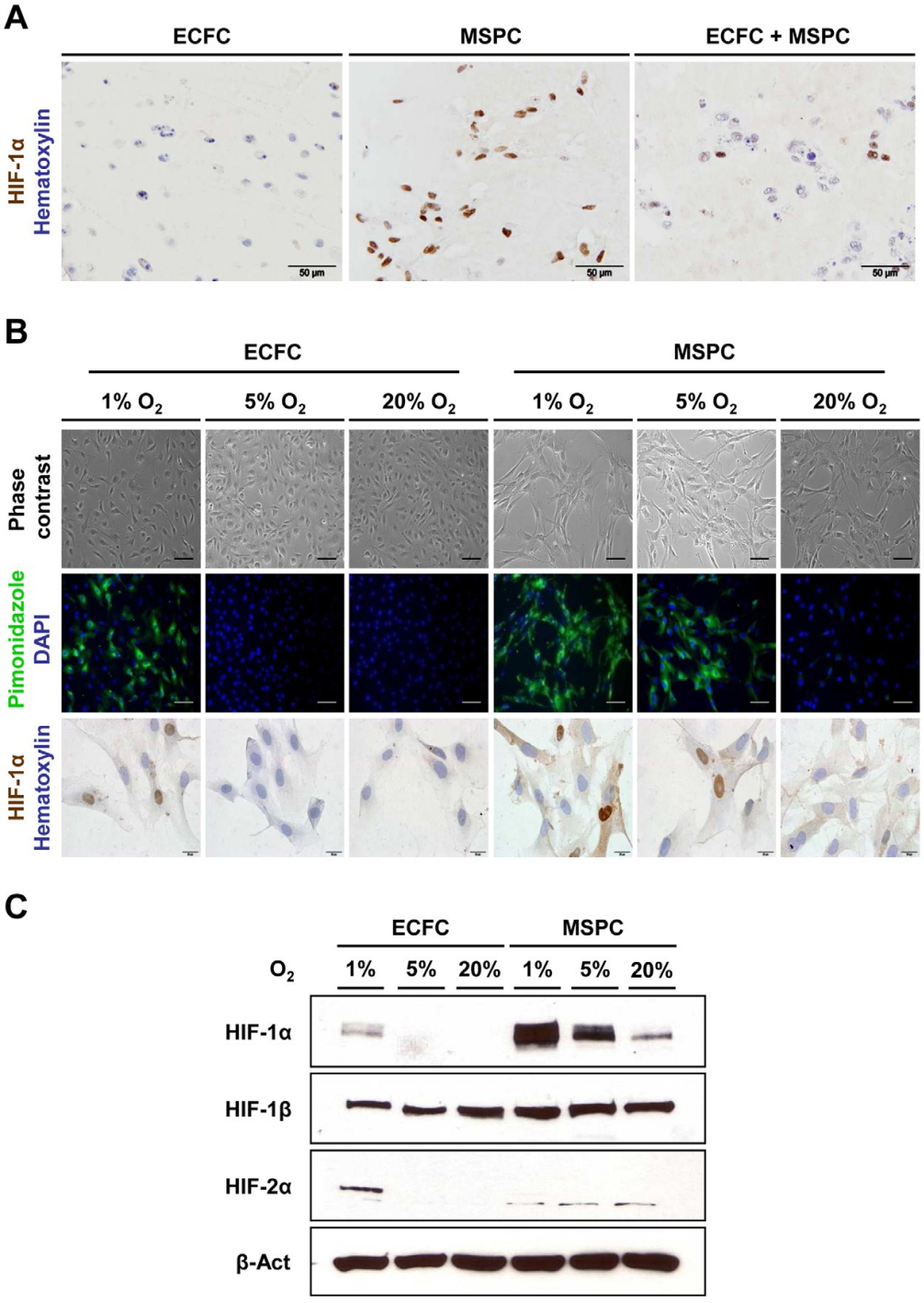

Figure 1. Mural cells are more sensitive to hypoxia than endothelial cells. (**A**) Immune histochemical staining of Matrigel plugs 1 day (d) after implantation of either ECFCs only (left picture), ECFCs/MSPCs together (middle picture) or MSPCs only (right picture). Plugs were explanted and sections were stained with anti-HIF-1α (DAB, brown) and co-stained with hematoxylin (blue; scale bar 50 µm). Staining revealed that 100% of MSPCs

display positive signals for HIF-1α, while ECFCs alone show no HIF-1α stabilization and in plugs of ECFCs together with MSPCs the number of positive cells corresponds to the 20% MSPCs. (**B**) To measure the hypoxia response at the single cell level *in vitro*, ECFCs and MSPCs were cultured for 4 h at 1%, 5% and 20% O_2. Phase contrast microphotographs show an unchanged appearance. Hypoxyprobe (pimonidazole, green; 4′,6-Diamidin-2-phenylindol, DAPI nuclear stain in blue) revealed that MSPCs sense hypoxia at 5% O_2 and both ECFCs and MSPCs sense hypoxia at 1% O_2. Accordingly, nuclear HIF-1α protein accumulation was detected in MSPCs at 5% and in both progenitor cell types at 1% O_2 (scale bar 100 μm for phase contrast and pimonidazole and 20 μm for HIF-1α pictures). (**C**) Equal amounts of protein from cell lysates corresponding to the analysis in (B) were tested in western blots showing higher amounts of HIF-1α in MSPCs. MSPCs stabilize HIF-1α at 5% and display a minute baseline signal at ambient air levels of 20% O_2 (n = 3; representative blot regions are shown; see Figure S1E for complete blot scans).
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approved by the Animal Care and Use Committee as specified above.

Immune Histochemistry and Immune Fluorescence

Sections (1.5 μm) of formalin-fixed (3.7% neutral buffered, overnight) paraffin-embedded plugs were de-paraffinized before antigen retrieval by heat (70°C/160 W, 40 min) at either high or low pH depending on the antibody followed by a descending alcohol series (2x xylol, 10 min; 1x ethanol 100%, 5 min; 1x ethanol 90%, 5 min; 1x ethanol 70%, 5 min; 1x ethanol 50%, 5 min; 1x PBS, 5 min). Endogenous peroxidases were blocked with H_2O_2 (10 min) and unspecific antibody binding with Ultra V Block (Thermo Scientific; 5 min) followed by mouse-on-mouse blocking (MOM, 1 h; Vector Laboratories, Burlingame, CA) and serum-free protein block (30 min; Dako, Glostrup, Denmark). Slides were incubated (30 min, RT) with unconjugated monoclonal mouse anti-human antibodies against Vimentin (clone: V9, 0.78 μg/mL, Dako), HIF-1α (clone: 54/HIF-1α, 10 μg/mL; BD Biosciences, San Jose, CA), CD31 (clone: JC70A, 5.15 μg/mL, Dako) or the appropriate amount of IgG1 (BD) control and developed with ultravision LP large-volume detection system horseradish peroxidase (HRP) polymer (Thermo Scientific) and diaminobenzidine (DAB) or alkaline phosphatase detection system using fast blue or fast red (Vector) according to manufacturer's instructions. Avidin-biotin blocking (Vector) was used before staining with biotinylated monoclonal rat anti-mouse CD45 (clone: 30-F11, 5 μg/mL; R&D Systems, Minneapolis, MN) and biotinylated monoclonal rat anti-mouse IgG1 (BD) control and detected by streptavidin-HRP conjugate (Dako) and DAB. Pimonidazole was stained with 0.7 μg/mL anti-pimonidazole-FITC (Hypoxyprobe). For permanent staining a secondary HRP-conjugated anti-FITC antibody was applied and developed with DAB. Slides were counterstained with hematoxylin for ≤30 seconds.

For immune fluorescence slides were washed after antigen retrieval (5 min, PBS+0.01% v/v Triton X-100; Sigma), protein blocked (30 min; Dako) and stained overnight at 4°C with either biotinylated rat anti-mouse Ter119 (clone TER-119, 5 μg/mL, BD), biotinylated rat IgG2b isotype control (5 μg/mL, BD), HIF-1α (10 μg/mL, BD), or rabbit anti-human CD31 (clone: EPR3094, 1:1,000; Abcam, Cambridge, UK), rabbit isotype control (5 μg/mL; Cell Signaling Technology, Danvers, MA), mouse anti-human alpha smooth muscle actin (clone: 1A4, 4.4 μg/mL, Dako), mouse IgG2a isotype control (4.4 μg/mL, Dako) all diluted in stain buffer, washed 3x (PBS+0.01% Triton X-100) and developed (120 min) with anti-mouse IgG-Alexa-546 (8 μg/mL, diluted in stain buffer; Invitrogen, Carlsbad, CA) or anti-rabbit IgG-Cy5 (2 μg/mL, Abcam) or streptavidin-conjugated alkaline phosphatase. After embedding in DAPI mounting media (4°C, minimum 60 min; Vector) pictures were taken with a Zeiss LSM510 Meta at 405, 543 and 633 nm excitation wavelengths. Collapsed stacks were reconstructed from z-stacks with Imaris software (Bitplane, Zurich, Switzerland).

To visualize HIF-1α protein in cell cultures at the single cell level, ECFCs and MSPCs were pre-cultured at 20% O_2 to 75% confluence in 8-well glass chamber slides (Thermo Scientific). Medium was replaced by pre-oxygenized 1% or 5% medium in the hypoxy workstation or kept as 20% O_2 and cells were cultured for 5 min – 24 h as specified. Incubation was stopped with ice cold PBS and fixed with ice cold paraformaldehyde (4%; 15 min, on ice) while still inside the hypoxy workstation. Immune cytochemistry was performed after H_2O_2 block using mouse anti-human HIF-1α antibody (BD) or mouse IgG1 control (10 μg/mL; BD) and visualized with HRP detection system (Thermo Scientific) with DAB according to manufacturers' instructions. Cells were counterstained (15 sec) with hematoxylin and documented with a phase contrast microscope (Olympus).

Vascular Endothelial Growth Factor (VEGF) Measurement in MSPC Supernatants

MSPCs were cultured in α-MEM containing 10% pHPL for 3 days at 1%, 5% or 20% O_2. Supernatants were aspirated and sterile filtered (0.22 μm Steriflip-filter, Thermo Scientific). Supernatant was stored in aliquots at −80°C until measurement. VEGF concentration was determined using Bio-Plex Pro Assay (Bio-Rad, Laboratories, Hercules, CA) and analysed using the Bio-Plex-200 system (BioRad).

Western Blot Analysis

ECFCs and MSPCs after 1%, 5% or 20% O_2 culture for 6 h were washed once with ice cold PBS/Proteinase-inhibitor (Sigma) and then scraped in ice cold RIPA buffer (Bio-Rad) supplemented with proteinase and phosphatase-inhibitor (Thermo Scientific) and lysed under constant agitation on ice for 30 min. After sonication (15 sec, on ice; Hielscher, Teltow, Germany) and centrifugation, the protein-containing supernatants were aliquoted and frozen at −80°C. Protein content was determined with a Bradford assay (Bio-Rad) and OD was measured with a Spectramax (Molecular Devices, Sunnyvale, CA). Proteins (20 μg) were loaded and resolved in a 7.5% SDS-PAGE (Bio-Rad) in parallel to full-range rainbow™ molecular weight marker (GE Healthcare, Munich, Germany) and transferred onto a nitrocellulose membrane (Bio-Rad). HIF proteins were detected using HIF-1α (clone: 54/HIF1α, 0.5 μg/mL; BD), HIF-1β (clone: 29/HIF1β, 0.125 μg/mL; BD), HIF-2α (clone: A-5, 0.4 μg/mL; Santa Cruz, Santa Cruz, CA) antibodies compared to house-keeping protein control β-actin (clone: C4, 0.2 μg/mL; Santa Cruz) antibodies followed by anti-mouse-HRP antibody (Cell Signaling) and visualized with 'super signal west pico luminol/enhancer developing solution' (Thermo Scientific). Films were exposed to blots in a dark room (10 sec - 5 min). Developed films and blots were scanned overlayed to document the precise location of rainbow molecular weight marker bands (Figure S1E and S2).

Hypoxia Response Modification *in vitro* and *in vivo*

The optimal dose of YC-1 [3-(5′hydroxymethyl-2′furyl)-1-benzylindazole] (100 μM, 5 min, 37°C; A.G. Scientific, San Diego, CA) blocking HIF-1α and HIF-2α proteins was titrated. Pre-treated MSPCs and ECFCs were washed 3x with PBS and

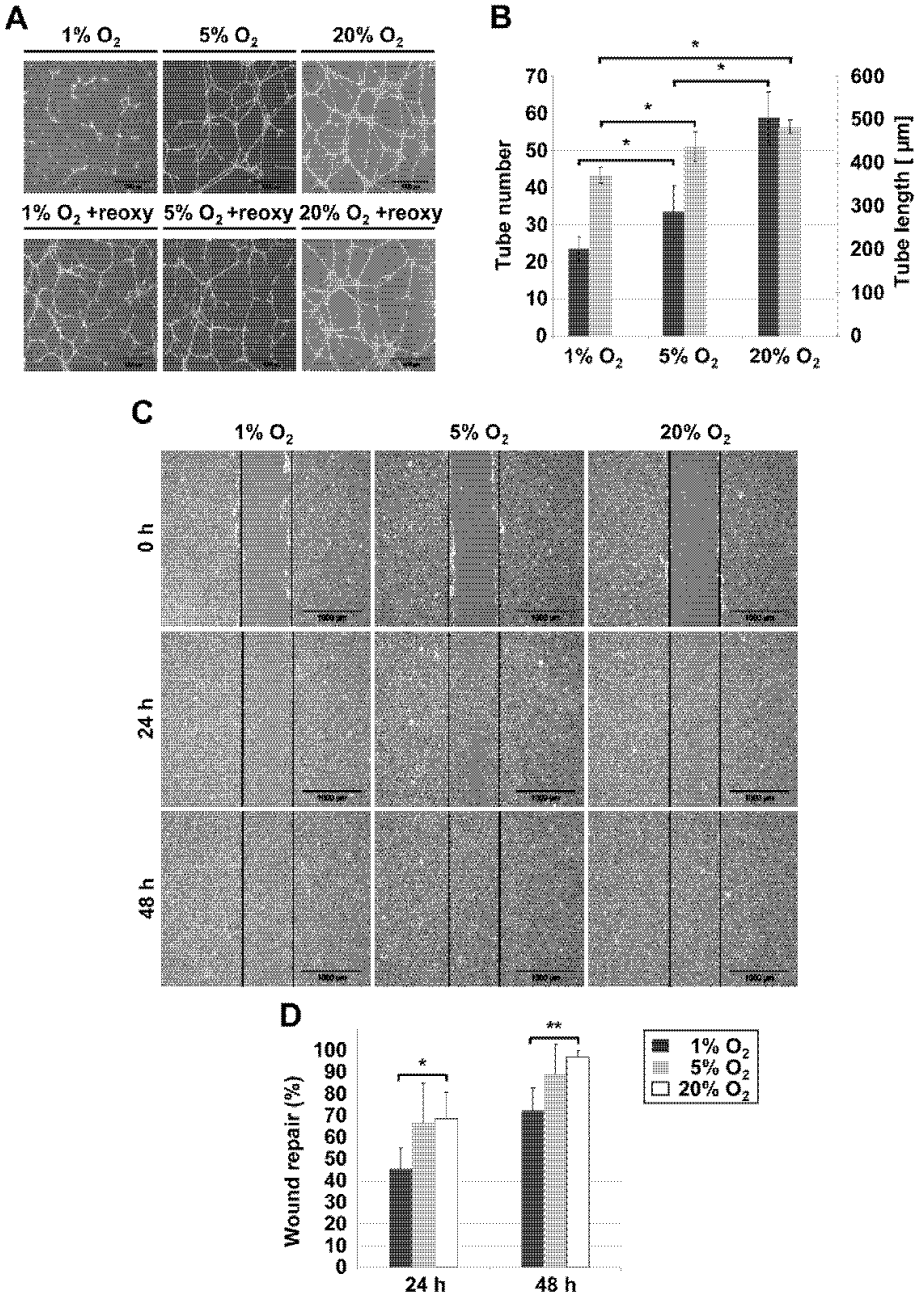

Figure 2. Functional quiescence of progenitor cells under hypoxia *in vitro*. (**A**) Vascular-like structures documented 24 h after seeding 1.8×10^5 ECFCs per 10 cm^2 on top of a matrix gel in a standard angiogenesis assay showing impaired network formation by ECFCs at 1% more than at 5% O_2 (upper row) and resumed vessel-like structure formation after re-oxygenation back to 20% O_2 standard conditions (+reoxy, lower row; one representative picture series; n = 3). (**B**) Tube number (black bar) and length (grey bar) as determined using ImageJ software (http://rsbweb.nih.gov) were significantly reduced with decreasing O_2 (mean ± SD; *$p<0.05$; n = 4). (**C**) Wounding an ECFC-derived monolayer in a scratch assay was used to monitor endothelial wound repair under hypoxia (1% O2) as compared to reduced (5% O_2) and ambient air (20% O_2) standard laboratory test conditions (representative examples are shown; see also Video S1). (**D**) A significantly decreased capacity to close an endothelial wound area over time was found at 1% O_2 compared to 20% O_2 (n = 5; *$p<0.05$, **$p<0.001$).
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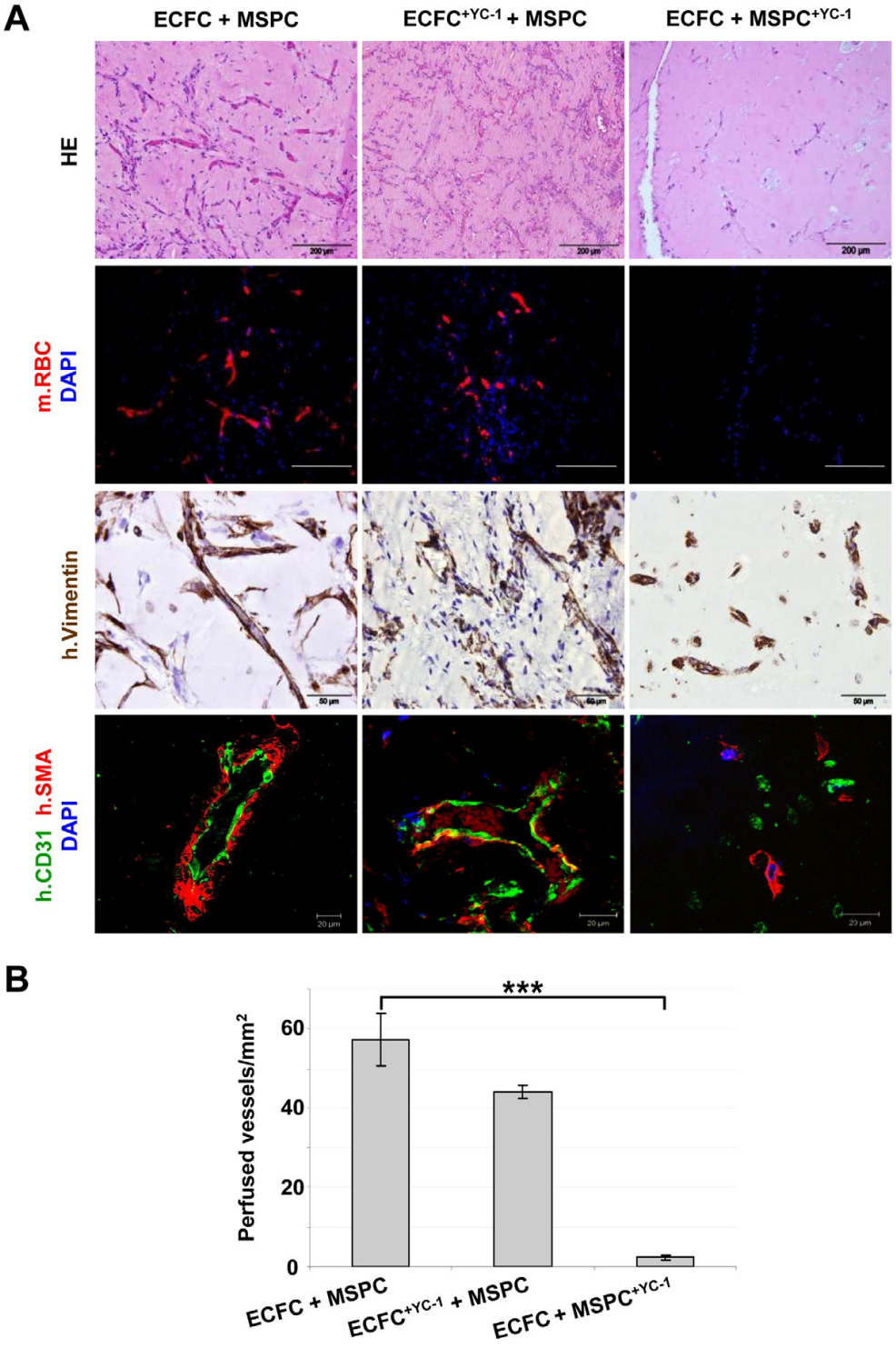
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ECFC + MSPC
ECFC+YC-1 + MSPC
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B
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Perfused vessels/mm2
60
40
20
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ECFC + MSPC
ECFC+YC-1 + MSPC
ECFC + MSPC+YC-1

Figure 3. Human vessel formation after progenitor co-transplantation depends on hypoxia-induced factors in MSPCs but not ECFCs. (**A**) Vessel formation was determined 2 weeks after ECFC+MSPC (2×10^6 cells/300µL, ratio of 80:20) co-transplantation. Pre-treatment of one of the two co-transplanted cell populations with YC-1 (1 h, 37°C; $ECFC^{+YC1}$+MSPC, n = 3; ECFC + $MSPC^{+YC1}$, n = 5) compared to untreated transplants (ECFC+MSPC, n = 8) as indicated above each column. Hematoxylin/eosin (HE) staining visualizes morphology (first row; scale bar 200 µm) and the mouse red blood cells (mRBC) resulting from perfusion after connection to the recipients' circulation (Ter119 anti- mouse glycophorin reactivity, red; 4',6-Diamidin-2-phenylindol, DAPI, blue; second row; scale bar 200 µm). Human mesodermal origin was visualized with anti-human vimentin monoclonal antibody staining (h.Vimentin, brown; mouse and human nuclei counterstained with hematoxylin, blue; third row; scale bar 50 µm). Plugs contain different numbers of h.Vimentin-negative infiltrating mouse $CD45^+$ hematopoietic cells (see Figure S6C). Fourth row indicating presence or absence of human vessels stained with rabbit anti-human CD31 labeling ECFCs (h.CD31, green), mouse anti-human alpha smooth muscle actin labeling pericytes and mural smooth muscle cells (h.SMA, red) and nuclear counter-stain (DAPI, blue; scale bar 20 µm). (**B**) Microvessel density was quantified in 200x magnifications of HE stained Matrigel plug sections by ImageJ and counting red blood cell-filled vessel structures. A significantly decreased capacity to form perfused vessels was found in implants containing YC-1 pre-treated MSPCs compared to untreated co-transplants. (n = 3, 5 high power fields 200x; ***$p<0.0001$).
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combined with corresponding untreated partner cells for co-transplantation to study the impact of HIF inhibition in either cell type on vasculogenesis *in vivo*.

Lentiviral infections for HIF-1α depletion were carried out according to standard procedures for gene silencing. Briefly, 293T cells were co-transfected with pMD2.G and psPAX2 (Addgene, Cambridge, MA) along with either pGIPZ-HIF1α-shRNA or pGIPZ-scramble-shRNA lentiviral constructs (OpenBiosystems, Huntsville, AL) using JetPrime transfection reagent (Polyplus-transfection Inc., New York, NY) according to the manufacturer's protocol. The transfection medium was replaced after 12 h with fresh DMEM/10% FBS (Sigma). After 48 h viral supernatants were collected and concentrated using Centricon Plus-70 filter units (Millipore). Bone marrow-derived MSPCs and peripheral blood-derived ECFCs were each infected in the presence of 8 µg/mL polybrene (Sigma). Two days after infection, stably transduced cells were selected with 2 µg/mL puromycin for 2 weeks resulting in a homogeneous population of virtually 100% TurboGFP-positive cells. To study the effect of different cytokines on vessel formation *in vivo*, Matrigel (Millipore) was mixed with 4 ng/mL of VEGF or a combination of VEGF, epidermal growth factor (EGF), insulin-like growth factor (IGF), basic fibroblast growth factor (FGF-2), hydrocortisone and ascorbic acid (all from Lonza EBM-2 Single Quots). Matrigel was replaced by pHPL before co-transplantation of either un-manipulated or HIF-1α knock down ECFCs and MSPCs wherever indicated. To block VEGF activity *in vivo*, mice were injected intraperitoneally every other day over a 7 or 14 day observation period starting at day zero immediately after co-transplantation of un-manipulated ECFCs and MSPCs as described above with Bevacizumab (Roche; 5 mg/kg per injection). Microvessel density was obtained from 5 high power fields (200x magnification) of HE stains from at least 2 independent donors and at least 2 independent plugs. Red blood cell-filled vessels were quantified by ImageJ software (http://rsbweb.nih.gov) and statistically evaluated by an unpaired student t-test. *$p<0.05$, **$p<0.001$, ***$p<0.0001$.

Apoptosis Assay

Apoptosis of ECFCs and MSPCs in implants *in vivo* was analyzed with TdT-mediated dUTP nick end labeling (TUNEL; Dead End™ Fluoretric System; Promega, Madison, WI) according to manufacturer's instructions. Nuclei were counterstained with propidium iodide (PI). As a positive control, slides were incubated for 10 min with 1U DNase 1 (Thermo Scientific). As a negative control, incubation buffer was prepared without rTdT. Staining was documented with a confocal microscope (LSM510 Meta, Zeiss).

Statistics

Values are presented as mean ± standard deviation (SD). Comparisons of tube length in *in vitro* angiogenesis assays and wound repair assays were made by Mann-Whitney U test. Comparisons of microvessel density in the *in vivo* neo-vasculogenesis model were made by unpaired student t-test. Statistical differences were considered significant when the P-value was less than 0.05, very significant when P-value was less than 0.001 and extremely significant when P-value was less than or equal 0.0001.

Results

Mesenchymal Stem/progenitor Cells Sense Low Oxygen More Sensitively than Endothelial Progenitors *in vitro* and *in vivo*

In this study, the subcutaneous co-transplantation of human ECFCs with MSPCs into immune-deficient NSG mice as established previously was used as a vasculogenesis model to create stable perfused human mesenchymal cell-covered vessels [17]. The phenotype of applied cells was shown to be typical for MSPCs and ECFCs (Figure S3). Both ECFCs and MSPCs showed no reactivity to the hematopoietic marker CD45. Interestingly, one day after progenitor transplantation in advance of any vessel assembly and perfusion, plugs containing only ECFCs lack HIF-1α reactivity while implants containing MSPCs or the mixture of both progenitor cell types showed a strong nuclear HIF-1α signal (Figure 1A). When analyzing the human vessels in vascularized plugs after one week we observed that nuclear HIF-1α signals *in situ* were virtually restricted to mural cells (Figure S1A, B). To study the response to low oxygen more precisely at the single cell level over time, human ECFCs and MSPCs of different origin were exposed to reduced oxygen (5% O_2 resembling the human venous oxygen level) and more severe hypoxia (1% O_2) and compared to ambient air standard cell culture conditions (20% O_2). Hypoxia was visualized by pimonidazole binding as described [31]. ECFCs from all tested sources showed cellular pimonidazole binding indicating hypoxia only at 1% O_2. Surprisingly MSPCs and fibroblasts reproducibly bound pimonidazole already at 5% in addition to 1% O_2 (Figure S1C). Nuclear HIF-1α resembling the pimonidazole results was found in the mesenchymal cells at 5% and 1% but not at 20% O_2 and in ECFCs only at 1% O_2 (Figure 1B). Kinetic analysis revealed nuclear HIF-1α protein stabilization in MSPCs after 1h at reduced O_2. ECFCs accumulated HIF-1α starting after 2h and more prominently after 6h at 1% O_2 (Figure S1D). Western blotting of cell lysates confirmed single cell results showing markedly increased HIF-1α stabilization in MSPCs when directly compared to ECFCs at 1% O_2. We confirmed a stable HIF-1β expression in ECFCs and MSPCs and up-regulation of HIF-2α in ECFCs under more severe hypoxic conditions at 1% O_2 (Figure 1C; see also Figure S1E).

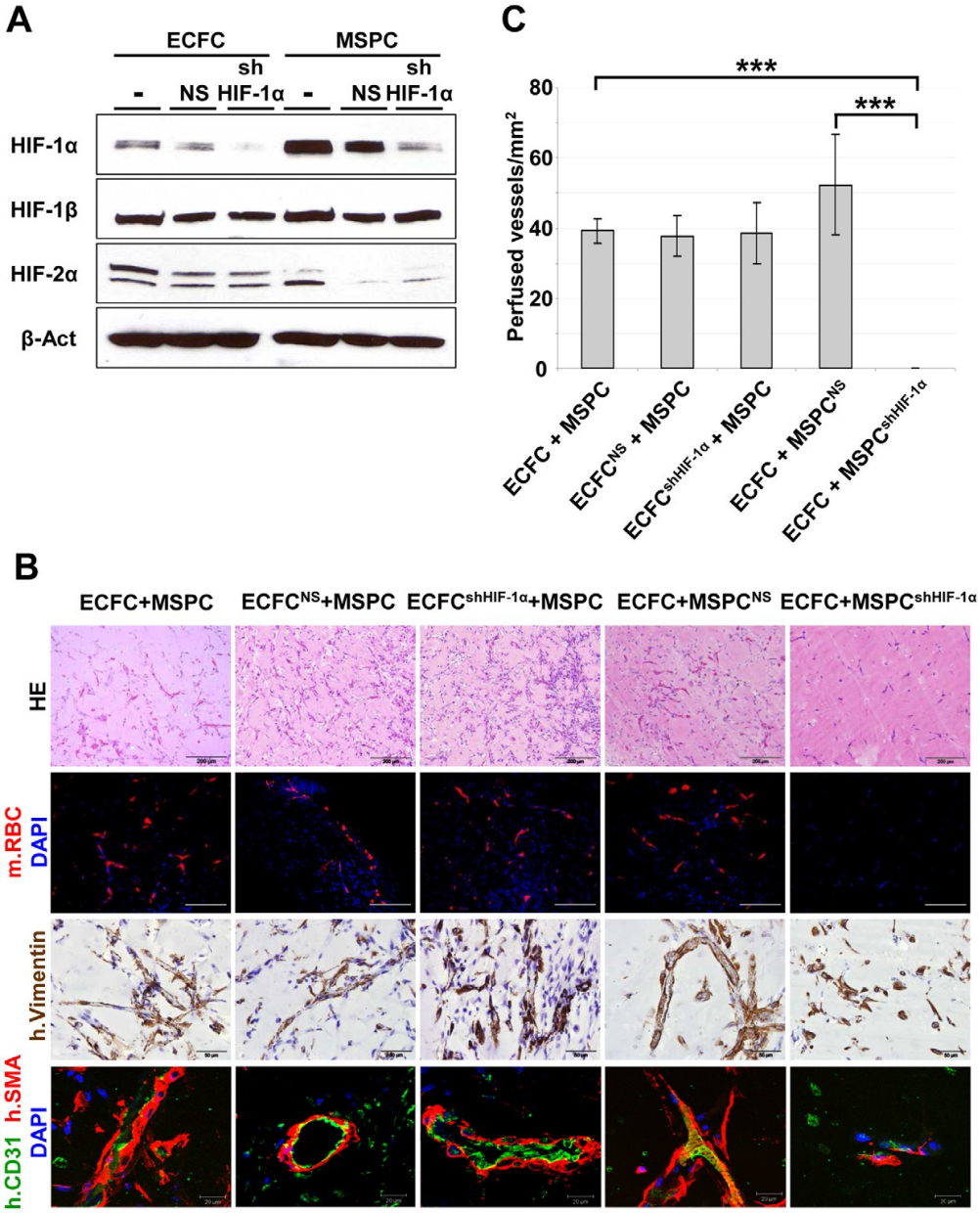

Figure 4. Specific knock-down of HIF-1α in MSPCs but not in ECFCs inhibits vessel formation *in vivo*. (**A**) Total cell lysates of untreated control (−) ECFCs and MSPCs or after infection with either pGIPZ-HIF1α-shRNA (shHIF-1α) or non-specific pGIPZ-scramble-shRNA (NS) were separated by SDS-PAGE after 6 hours of incubation at 1% O_2. Blots were stained with HIF-1α, HIF-2α, HIF-1β or β-actin (β-Act). Full blots are shown in Figure S2. (**B**) To delineate the impact of genetic ablation of HIF-1α in either ECFCs or MSPCs before co-transplantation, vessel formation was determined one week after co-transplantation of HIF-1α-silenced ($ECFC^{shHIF\ 1\alpha}$, n = 3; $MSPC^{shHIF\ 1\alpha}$, n = 7) or mock-transfected non-silenced cells ($ECFC^{NS}$, n = 3; $MSPC^{NS}$, n = 7) with genetically un-manipulated corresponding (+ECFC and +MSPC) partner cells as indicated above the columns. Analysis was performed as specified in Figure 3A and is identified at the left side of the picture. Scale bars indicate magnification (first and second row, 200 µm; third row, 50 µm; fourth row, 20 µm). (**C**) Microvessel density was quantified in 200x magnifications of HE stained Matrigel plug sections by ImageJ counting red blood cell filled vessel structures. A significantly decreased capacity to form perfused vessels was found in implants containing shHIF-1α transfected MSPCs compared to untreated co-transplants. (n = 3, 5 high power fields 200x, mean ± SEM; ***$p<0.0001$).
doi:10.1371/journal.pone.0044468.g004

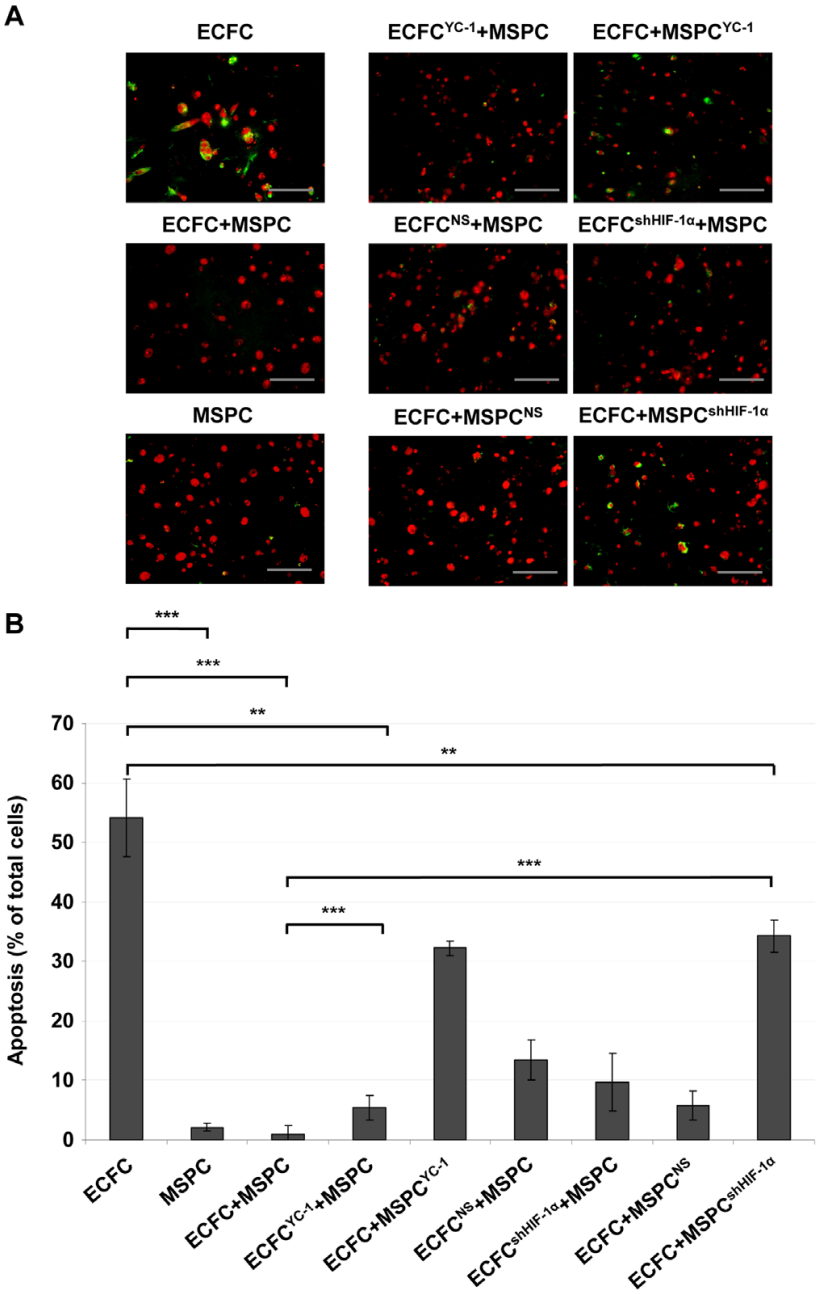

Figure 5. HIF-competent MSPCs are required to avoid premature ECFC apoptosis *in vivo*. (**A**) Apoptosis assay using TdT-mediated dUTP nick end labeling (TUNEL) of ECFC-only or MSPC-only transplants and treated or untreated ECFC+MSPC co-transplants, as indicated above each photograph. All plugs were explanted 24 h after implantation *in vivo*. DNA strand breaks of apoptotic cells were detected by a TUNEL assay kit (Promega) and nuclei were counterstained with propidium iodide (PI, red). Green fluorescence is due to FITC-labeled nucleotide binding to DNA strand breaks of apoptotic cells. Representative pictures from one experiment are shown (scale bar 100 µm, from at least three different donors performed per transplantation type as specified in the legends to Figures 3 and 4). (**B**) Apoptotic cells depicted as percentage of total cells ± SD with groups corresponding to the representative pictures in (A); five high power fields 200x; ***$p<0.0001$, **$p<0.01$).
doi:10.1371/journal.pone.0044468.g005

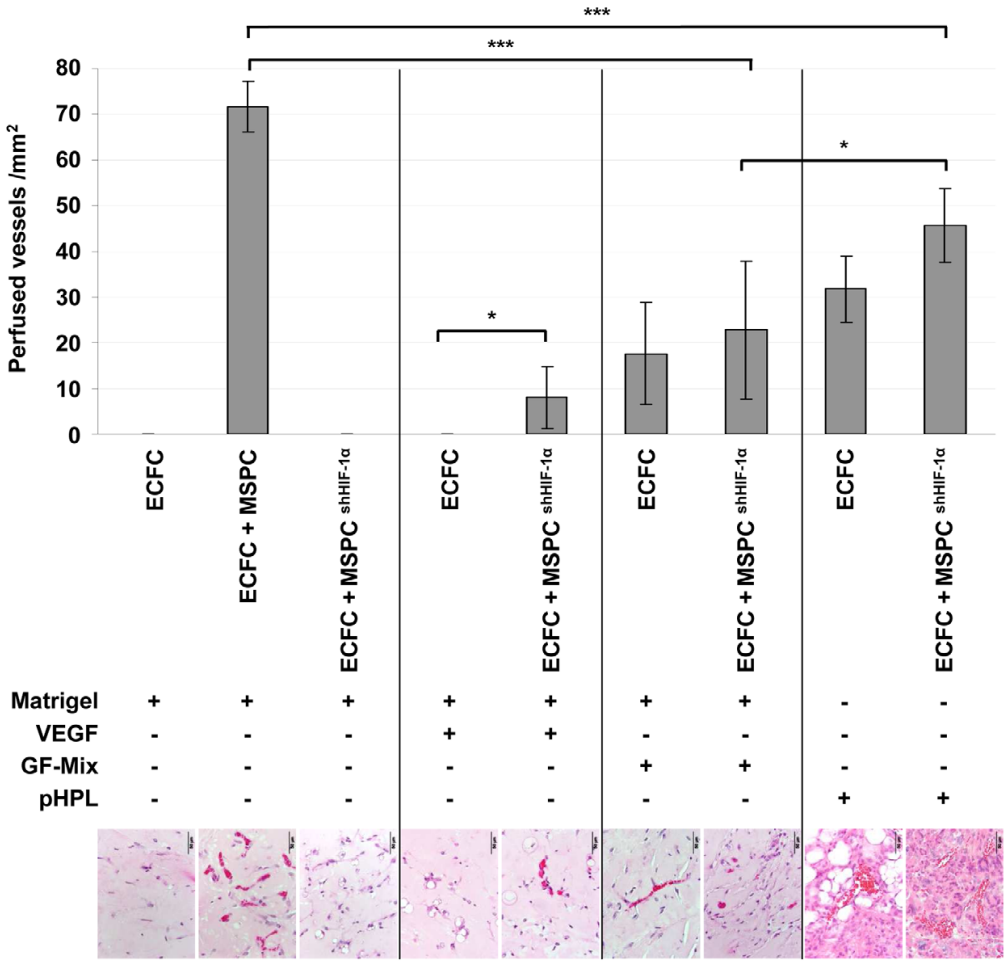

Figure 6. Angiogenic factors can partially substitute MSPC HIF-1α deficiency *in vivo*. ECFCs alone or in combination with MSPCs (either unmanipulated or after shHIF-1α knockdown) were (co)-transplanted in either Matrigel or pooled human platelet lysate (pHPL) gel with or without additional growth factors (GF) as specified. The GF-Mix comprised VEGF, EGF, IGF, FGF-2, hydrocortisone and ascorbic acid. Mean ± SD results of the number of perfused vessels counted in five high power fields (200x, at least two independent plugs and two independent animals). Microphotographs correspond to the treatment group (scale bar 50 µm; ***$p<0.0001$, *$p<0.05$).
doi:10.1371/journal.pone.0044468.g006

ECFCs Maintain a Quiescent State under Hypoxic Conditions *in vitro*

It is generally accepted that hypoxia maintains stemness [32]. It is not known whether the hypoxic environment during therapeutic vasculogenesis affects progenitor clonogenicity or function. Testing ECFC clonogenicity at 20% O_2 standard conditions confirmed a complete hierarchy of LPP-ECFCs and HPP-ECFCs as previously reported [7,17]. The colony number of ECFCs and MSPCs was stable under all O_2 conditions tested but colony size progressively dropped with decreasing oxygen tension (Figure S4A, B). Progenitors exposed to 1% or 5% O_2 experienced a hierarchy shift towards small colonies but resumed their clonogenic potential after re-oxygenation suggesting that they can maintain a quiescent state in a hypoxic environment and arguing against simple hypoxia-mediated damage (Figure S4C, D).

In bulk cultures for large-scale expansion, O_2 reduction diminished proliferation of both progenitor types progressively over time (Figure S4E-H). To determine the functionality of ECFCs under hypoxia, two standard assays were employed testing angiogenesis and endothelial wound repair *in vitro*. In a Matrigel assay, ECFCs pre-cultured at 5% and 20% O_2 formed complex vascular networks. ECFCs pre-conditioned and tested at 1% O_2 showed significantly reduced number and length of vessel-like structures (Figure 2A, B). When pre-cultured at 1% and subsequently tested at 20% O_2 (re-oxygenation), ECFCs resumed their ability to form complex networks (Figure 2A). In addition, ECFCs almost closed the scratch area in an endothelial wound repair assay at 20% and 5% O_2 within 24 hours. At 1% O_2, the scratch area covered by ECFCs was significantly diminished and was accompanied by reduced proliferation (Figure 2C, D; Video S1).

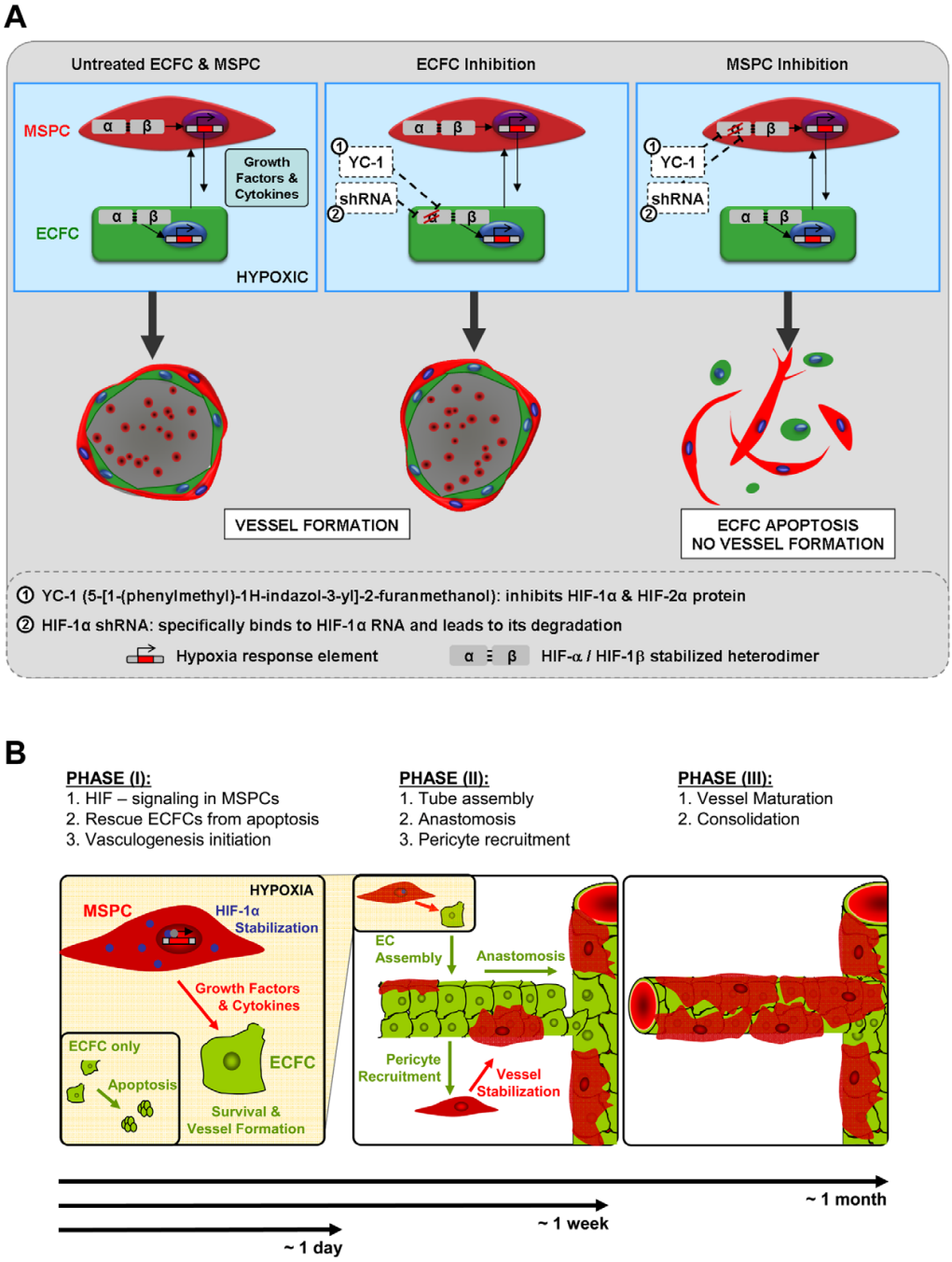

Figure 7. MSPCs are responsible for oxygen sensing and induction of neo-vasculogenesis. (**A**) The experimental strategy to delineate the hypoxia response used pre-treatment of either ECFCs or MSPCs before transplantation with YC-1 (inhibiting both HIF-1α as well as HIF-2α) or specific knockdown of HIF-1α RNA (by sh-RNA;). The surprising outcome of vessel formation despite the inhibition of ECFC hypoxia response and collapse of neo-vasculogenesis after MSPC inhibition is graphically illustrated. (**B**) Based on our current insight a model is proposed describing three temporarily distinct phases of adult vasculogenesis.
doi:10.1371/journal.pone.0044468.g007

Neo-vasculogenesis *in vivo* Depends on MSPC HIF-1α Response and is Independent of ECFC Hypoxia Sensing

Neo-vasculogenesis was tested using a progenitor cell co-transplantation model in NSG mice as previously described [17]. Efficiency of experimental vasculogenesis *in vivo* in a previously established ratio of 80% ECFCs admixed with 20% MSPCs [17] was virtually independent of the carrier matrix protein (Figure S5, S6D). Interestingly, ECFCs alone largely failed to build complex vessel networks even after 14 days (Figure S6A, B). In the absence of human MSPCs the ECFCs progressively disappeared and were replaced by infiltrating mouse hematopoietic cells over time (Figure S6A- C). For reasons of comparability to existing studies [14,15,17,33–36,36] Matrigel was chosen for further experiments in this study except when testing for the effect of human platelet-derived growth factors and cytokines present in pHPL.

In a hypoxic environment, cells respond in a cell-type specific manner by stabilizing HIF1-α or HIF2-α [37,38]. To test which cell type is responsible for the hypoxia sensing during progenitor-derived neo-vasculogenesis *in vivo*, ECFCs and MSPCs were pre-treated with the small molecule hypoxia response inhibitor YC-1 [39] which blocks both HIF-1α and HIF-2α. Surprisingly, MSPC pre-treatment with YC-1 prior to co-transplantation with untreated ECFCs disabled vessel formation, whereas HIF-1α/HIF-2α inhibition in ECFCs did not significantly affect perfused vessel creation (Figure 3A, B). These results were confirmed by specifically silencing HIF-1α with small hairpin (sh)-RNA in either cell type before co-transplantation (Figure 4A). HIF-1α knock down in MSPCs abolished vessel formation significantly compared to transplants containing mock-transfected MSPCs. In accordance with YC-1 results, genetic HIF-1α ablation in ECFCs by sh-RNA did not result in the inhibition of vessel formation (Figure 4B, C).

HIF-competent MSPCs Rescue ECFCs from Apoptosis

Based on our observation that HIF stabilization in response to hypoxia is an early event in MSPCs that is lacking in ECFCs, we asked whether apoptosis also plays a role early in the time course after cell transplantation. We found that the majority of ECFCs had already undergone apoptosis 24h post transplantation. MSPCs underwent virtually no apoptosis under hypoxic conditions *in vitro* or *in vivo* (Figure 5; Figure S7). Co-transplantation with HIF-competent MSPCs rescued ECFCs from apoptosis. Both HIF-1α knockdown by shRNA and YC-1-mediated HIF-1α/HIF-2α depletion in MSPCs led to a significant reduction of their anti-apoptotic effect. HIF depletion in ECFCs by either method did not influence early ECFC survival when co-transplanted with HIF-competent MSPCs (Figure 5).

HIF-1α stabilization in response to hypoxia leads to the transcription of hundreds of target genes including VEGF [37,40]. Measuring MSPC cytokine secretion under different O_2 conditions revealed a significant up-regulation of VEGF at hypoxic O_2 levels. (Figure S8A). Blocking VEGF *in vivo* by repetitive intra peritoneal injection of bevacicumab largely ablated neo-vasculogenesis after progenitor co-transplantation (Figure S8B). To test whether VEGF represents the single dominant MSPC-derived pro-vasculogenic and anti-apoptotic factor in this system, ECFCs alone were transplanted subcutaneously in a matrix supplemented with a saturating concentration of VEGF. Whereas VEGF supplementation did not allow ECFCs to form vessels in the absence of HIF-competent MSPCs, it created a small but significant increase in perfused vessels two weeks after co-transplantation of ECFCs with HIF-1α-depleted MSPCs that otherwise reproducibly lacked the potential to contribute to vasculogenesis (Figure 6). To test if a multi-factorial system can rescue ECFCs from apoptosis and initiate vasculogenesis, we supplemented the Matrigel plugs with a defined mixture of vasculogenic growth factors established to support ECFC proliferation and function *in vitro* (VEGF, EGF, IGF, bFGF, hydrocortisone and ascorbic acid) [17]. This growth factor (GF)-mix enabled ECFCs alone as well as co-transplants of ECFCs with HIF-1α-depleted MSPCs to form perfused vessels (Figure 6). Vasculogenesis in the presence of the GF-mix was still significantly less efficient than that initiated in the presence of HIF-competent MSPCs.

Sole ECFCs injected in a growth factor-rich pHPL led to the formation of enlarged and lagoon-like vessels which is largely reminiscent of a pathology found in several vascular anomalies including hemangioma (Figure S5). Based on this observation we also tested co-transplants of ECFCs with HIF-1α-depleted MSPCs in pHPL. This resulted in the highest number of perfused vessels formed by ECFCs co-transplanted with HIF-depleted MSPCs, but this combination was still significantly less efficient than co-transplantation of ECFCs with HIF-competent MSPCs (Figure 6).

Discussion

Understanding the cross-talk of endothelial and mural cells in response to the hypoxic environment and deciphering the underlying mechanisms of new vessel formation is of great relevance for the development of novel strategies aimed at therapeutic vasculogenesis. Despite promising experimental data, approaches focusing on endothelial progenitors have been of limited efficiency in clinical trials, both for therapeutic vasculogenesis and for anti-angiogenic therapy [10]. It has only recently been recognized that endothelial lineage cells require interaction with MSPCs to efficiently build perfused vessels *in vivo* [11,12,14,17,18,33].

Here we report that perivascular MSPCs sense hypoxia earlier and more efficiently than ECFCs, which build the inner vessel wall. HIF-1α stabilization and transition to the nucleus is a key event initiating gene expression in response to hypoxia [21]. One explanation for the lack of endothelial HIF-1α accumulation in our transplantation model would be its immediate degradation during rising oxygen levels following the initiation of perfusion. Our observation that ECFCs lack detectable HIF-1α as soon as one day after transplantation prior to vessel assembly and plug perfusion, argues against such a simplified view. Two independent strategies were therefore utilized to delineate the role of HIF as a key regulatory element during vasculogenesis (Figure 7A). Unexpectedly, neither HIF-1α knockdown by shRNA nor pharmacologic HIF-1α and HIF-2α deletion by YC-1 in ECFCs affected patent vessel formation. In contrast, HIF-1α knockdown or YC-1-mediated inhibition in MSPCs completely abrogated experimental vasculogenesis. These results clearly demonstrate that HIF-1α stabilization in MSPCs but not in ECFCs is a crucial event in therapeutic vasculogenesis. It will be of great interest to study the impact of this endothelial-mesenchymal crosstalk on the metabolic balance of the two cell types under hypoxic conditions. This may not be limited solely to vascular regeneration, as cardiac regeneration was also found to depend on the functionality of endothelial and smooth muscle precursors in a model of bone marrow-derived cell therapy [41]. Depending on the source of MSPCs and the balance between ECFC and MSPC, developmental programs can be activated that allow the formation of human bone and marrow niche. This system has already enabled us to construct a genetically controlled hematopoietic microenviron-

ment in which HIF-1α controls the engraftment of normal and malignant blood cells [42].

Another aspect of our studies is the role of HIF-2α as an alternative O_2-responsive transcription factor in endothelial lineage cells [43,44]. Our observation that YC-1 pre-treatment of ECFCs does not affect vessel formation in this system does not address the specific function of HIF-2α during vessel normalization [44]. Endothelial-specific *Hif-2α* deletion in mice resulted in a virtually normal vascular phenotype except for abnormal vessel permeability under steady state conditions, while increased vessel formation was observed during vascular regeneration and unstable tumor vascularization [45,46]. Although we did not analyze neovessel stability and/or permeability in this study, the sparse pericyte distribution around some of the vessels derived from YC-1 pre-treated ECFCs (Figure 3A) may be reminiscent of the HIF-2α deficient phenotype. Lack of up-regulation of HIF-2α in MSPCs during HIF-1α knockdown argues against a critical role of HIF-2α in MSPC function.

The diminished expansion of endothelial and mesenchymal progenitor cells as observed in our study under stringently reduced oxygen is of relevance for cell propagation for regenerative purposes. Hypoxic pre-conditioning strategies, despite maintaining clonogenicity, would limit net cell expansion. The phenomenon that ECFCs, after silencing in hypoxia, resume their ability to form complex vascular networks after re-exposure to 20% O_2 further substantiated the argument that ECFCs can maintain a functionally quiescent state in a hypoxic environment capable of being resumed when re-oxygenated. The reduced proliferation and diminished endothelial wound repair as observed under hypoxic conditions *in vitro* support the notion that insufficient ECFC hypoxia response can translate into a reversible functional deficit. Based on our data we speculate that MSPCs or perhaps other HIF-competent stromal cells can function to rescue vascular regeneration on demand. It has been previously demonstrated that adipose tissue-derived stem cells can function as pericytes when co-transplanted with cord blood-derived endothelial progenitors. The mural cells prevent vessel regression around day 14 post implantation by a platelet-derived growth factor (PDGF)-independent mechanism that reduced apoptotic endothelial cell death thus leading to robust vessel assembly [18]. In our *in vivo* model, ECFC apoptosis in the hypoxic environment was prevented by co-transplantation of HIF-competent but not of HIF-deficient MSPCs. Recently, we also found that the anti-apoptotic activity of MSPCs can rescue human-induced pluripotent stem (iPS) cells from apoptosis resulting in their long-term engraftment in a preclinical pig model of myocardial infarction [47].

We conclude that ECFC/MSPC co-transplantation represents a valuable alternative to current endothelial cell therapy strategies for vessel repair and tissue engineering. The ideal carrier for cell application still needs to be determined. A humanized matrix based on pHPL was applied in comparison to a collagen/fibronectin mixture and Matrigel representing an experimental standard. The fact that progenitor cells admixed with pHPL solution can be injected with a regular syringe may offer a certain technical advantage compared to collagen/fibronectin, which requires solidification before surgical implantation of preformed plugs. Injectable type I collagen, fibrin and puramatrix have also been shown to support early vessel formation after two-cell-type implantation [48]. Another recent study showed that co-transplantation of ECFCs with MSPCs in a mouse model was most efficient in regenerating perfusion after hindlimb ischemia [49]. Application of either cell type alone was less efficient but revealed distinct mechanisms by which ECFCs and MSPCs contribute to vascular and tissue regeneration [49]. Whether physical contact of ECFCs and MSPCs is necessary to exert the supportive effects of MSPCs remains to be determined. In the presence of HIF-silenced MSPCs *in vivo*, ECFCs were not capable of performing their vasculogenic capacity. In this context it is interesting to note that part of the effect of HIF-competent MSPCs could be mimicked by pHPL (Figure 6). This supports the hypothesis that the effect of MSPCs is mediated to a reasonable extend by HIF-mediated protein secretion. Whether platelet-derived growth factors in platelet-rich plasma or pHPL could be utilized as an adjuvant improving or even substituting cell therapy remains to be determined [50,51].

The fact that continuous VEGF blockade virtually ablated progenitor-derived neo-vasculogenesis was not unexpected. Because VEGF depletion *in vivo*, despite negatively regulating mural cell function, lacks sustained clinical efficiency [10,34,44], its combination with MSPC-targeted therapies offers an alternative strategy.

Our unexpected observation that therapeutic vasculogenesis can occur independent of endothelial HIF function *in vivo* further strengthens the need to re-examine current concepts in vascular regeneration using innovative tracking strategies at the single cell level [52]. Admitting that EC-targeted deletion of HIF-1α or HIF-2α as published previously [45,53] resulted in a surprisingly unremarkable phenotype, except for its profound disturbance of tumor angiogenesis, may imply that tumor vessel growth as compared to regenerative vasculogenesis (in this study) and angiogenesis [36] follow different developmental programs with a certainly underestimated impact of the perivascular compartment. However, also in the *Hif-1α* knockout animals, the severe vascular defects resulting in embryonic lethality were spatially correlated with perivascular mesenchymal cell death and not associated with VEGF deficiency [54]. It remains to be determined whether MSPCs already express HIF-1α at 20% O_2 as a cell culture artifact or whether there is a physiologically rational pathway activated in MSPCs fitting them with the competence to react to hypoxia earlier and/or more sensitively than other cells. Coagulation factors like tissue factor and thrombin are known to interfere with blood vessel development and homeostasis [55]. Angiotensin II, bacterial lipopolysaccharides (LPS) and various growth factors and cytokines are non-hypoxic stimuli that can regulate HIF expression [56]. Proteases also play a key role in the later phase of anastomosis of newly formed vessels during a process called 'wrapping and tapping' which realizes the connection with the existing vasculature [57]. The existence of an indirect mural cell-mediated oxygen sensing pathway early during the initiation of vasculogenesis as observed in our study prompted us to propose a model which incorporates these new aspects of vasculogenesis (Figure 7B). Further experiments are required to evaluate this model.

The recent discovery that fibroblast growth factor-9 can act on stromal precursors stabilizing vessel outgrowth highlights the role of mural cells during sprouting angiogenesis [36]. Hematopoietic and tumor cells can also act in concert with endothelial and mesenchymal progenitors to initiate, arrange and support vessel formation in hypoxic environments [3,8,10,35,41,44]. Understanding the peculiar role stromal cells can assume in that orchestrated interplay will help to critically develop more efficient pro- and anti-angiogenic strategies.

Supporting Information

Figure S1 Nuclear HIF-1α signal *in vitro* and *in vivo*. (**A**) Immune histochemical staining of matrigel plugs containing ECFCs/MSPCs 7 days (d) after co-transplantation. Plugs were explanted and sections were stained with anti-HIF-1α (brown; arrow heads), anti-human CD31 (red) and co-stained with hematoxylin (blue). (**B**) Immune fluorescence staining d7 after co-transplanting ECFCs/MSPCs in matrigel with anti-HIF-1α (white), anti-human alpha smooth muscle actin (SMA, red), and counterstained with DAPI (blue), anti-human CD31 (green). White arrow heads mark nuclear HIF-1α signals and arrows unspecific background fluorescence of mouse red blood cells. (**C**) Hypoxyprobe (pimonidazole, green; DAPI nuclear stain in blue) analysis of MSPCs from bone marrow (BM), umbilical cord (Cord), neonatal and adult fibroblasts directly compared to ECFCs from umbilical cord blood (UCB), cord, normal and cardiovascular disease patient-derived (CVD) peripheral blood (PB) cultured at indicated O_2 levels as described in the methods section. Scale bar 100 μm. (**D**) ECFCs and MSPCs were cultured for indicated intervals at indicated O_2. Fixed cells were stained with anti-HIF-1α. ImageJ (http://rsbweb.nih.gov) processing was used to obtain the transformed red signal. Original data are on file. ECFC start to stabilize HIF-1α in their nucleus after 2 h at 1% O_2 but not at 5 or 20% O_2. MSPCs stabilize HIF-1α after 1h at 1% and 5% O_2. (**E**) Western blot analysis of ECFC and MSPC total cell lysates after 6h at indicated O_2. Blots were incubated with HIF-1α, HIF-1β, HIF-2α or β-actin (β-Act) antibodies. Three representative blots scanned in overlay with exposed films. Scissors mark cuts to separately stain β-actin (β-Act) from the same blot. Areas shown in Fig. 1B are boxed.
(TIF)

Figure S2 Specific knock-down of HIF-1α in MSPCs and ECFCs. Total cell lysates of untreated control (-) ECFCs and MSPCs or after infection with either pGIPZ-HIF1alpha-shRNA (shHIF-1α) or non-specific pGIPZ-scramble-shRNA (NS) were separated by SDS-PAGE after 6 hours of incubation at 1% O_2. Blots were stained with either HIF-1α, HIF-2α, HIF-1β or β-actin (β-Act). Three representative blots scanned in overlay with exposed films are shown. Scissors mark cuts to separately stain β-Act from the same blot. Areas shown in Figure 5A are boxed.
(TIF)

Figure S3 Phenotypic characterization of MSPCs and ECFCs. The phenotype of MSPCs and ECFCs was characterized by flow cytometry as described previously (n>5) [17]. MSPCs and ECFCs can be distinguished by their dissimilar expression of CD90, CD31 and CD34. Both ECFCs and MSPCs show no reactivity with the hematopoietic marker CD45.
(TIF)

Figure S4 Progenitor clonogenicity and long-term proliferation: proliferative quiescence under reduced O_2 and recapitulation after re-oxygenation. (**A, B**) For colony assays 10 ECFCs/cm^2 (n = 3) or 3 MSPCs/cm^2 (n = 2) were seeded in 55 cm^2 colony plates and grown for 14 days (d) at 1%, 5% or 20% O_2. Colony number and cell number were documented. Culture plates show typical colonies at 1%, 5% and 20% O_2 derived from the same ECFC or MSPC starting population, respectively, and stained with crystal violet as described in the methods section. (**C**) For re-oxygenation (+ reoxy) population doublings (PD) of ECFCs and MSPCs cultured under 1%, 5% and 20% O_2 for 12 d were compared with PDs of ECFCs and MSPCs pre-cultured at 1 or 5% O_2 for 7 d and then cultured at 20% O_2 for another 12 d (mean ± SD; n = 3). Corresponding representative crystal violet-stained colony plates are shown positioned below their corresponding O_2 conditions. (**D**) ECFC hierarchy was assessed after 14 d of culture at 1%, 5% and 20% O_2 directly compared to ECFCs pre-cultured for 7 d at 1% or 5% O_2 and subsequently for another 12 d at 20% O_2 (+reoxy) by photo documenting all colonies per plate and semi-automatically counting every single cell per scanned colony as described previously using the ImageJ software (http://rsbweb.nih.gov). One representative experiment is shown. (**E**) To determine population doublings (PD) per passage 100 ECFCs/cm^2 (n = 5) or 30 MSPCs/cm^2 (n = 2) were seeded in 75 cm^2 culture flasks and grown for 14 d at 1%, 5% or 20% O_2. (**F, G**) Cumulative PDs were calculated after long term culture (3×14 d) at 1%, 5% or 20% O_2 for (**F**) ECFCs or (**G**) MSPCs. (**H**) Representative ECFC and MSPC colonies are shown after 14 d incubation at 1%, 5% or 20% O_2 after crystal violet stain (scale bar 5 mm).
(TIF)

Figure S5 Patent vessel formation depends on functioning MSPCs and is virtually matrix- independent. ECFCs (1.6×10^6) together with MSPCs (4×10^5) for co-transplantation or sole ECFCs (2×10^6) were re-suspended in ice cold matrigel, collagen/fibronectin (Coll/Fn), or pooled human platelet lysate (pHPL), respectively. Aliquots of matrigel or pHPL were injected and preformed collagen/fibronectin plugs were implanted subcutaneously as described in methods in detail into the flank of NSG mice. Mice were sacrificed on day (d) 7 and 1.5 μm plug sections were either stained with hematoxylin and eosin (HE) or processed for anti-human vimentin immune histochemistry (h.Vimentin; hematoxylin counterstain, blue; see methods for details and references). Control plugs were also explanted at d 1 (see Figure S4D). The higher cell density despite equal cell input in pHPL implants results from more intense contraction of the matrix *in vivo* compared to matrigel.
(TIF)

Figure S6 Patent vessel formation depends on MSPC presence and is virtually matrix-independent. (**A, B**) ECFCs alone or MSPCs+ECFCs (ratio 20:80) were re-suspended in matrigel and injected subcutaneously into immune deficient NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice. Plugs where explanted at days (d) 1, 7 and 14. (**A**) Hematoxylin and eosin. (**B**) Mesodermal origin was probed with anti-human vimentin (brown; nuclei blue, hematoxylin). Implants after ECFC+MSPC co-transplantation showed vimentin$^+$ human vessel formations (d7 & d14) compared to implants of ECFC alone showing not more than rare small vessel-like structures and declining human cell number. (**C**) Infiltrating vimentin-negative (non-human) cells in ECFC plugs were mouse CD45$^+$ (mouse hematopoietic) cells already 7 d after transplantation. (**D**) Hematoxylin/eosin staining to visualize cells in different extracellular matrices 1 d after transplantation. MSPCs + ECFCs (top row) or ECFCs only (bottom row) were re-suspended in matrigel or pooled human platelet lysate (pHPL) and injected subcutaneously (6.6×10^6/mL; ratio 20:80; injection volume 300 μL) into immune-deficient NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ; n ≥3). Preformed collagen/fibronectin plugs containing equal cell compositions were implanted (n = 3).
(TIF)

Figure S7 Anti-VEGF treatment inhibits vessel formation *in vivo*. (**A**) VEGF concentration in supernatant of MSPCs cultured for 3 days at 1%, 5% and 20% O_2 showed increasing VEGF levels with decreasing oxygen concentration (mean ± SD; n = 3). (**B**) Anti-VEGF treatment inhibited vessel formation in matrigel plugs. After subcutaneous co-transplantation of MSPCs

and ECFCs (ratio 20:80) into NSG (NOD.Cg-Prdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice recipients were injected i.p. with 5 mg/kg of the therapeutic anti-human VEGF antibody Bevacizumab every other day (d) starting d1. Hematoxylin/eosin staining showed limited cell arrangement but no human vessel formation after one week (d7; three doses of antibody). After seven doses of antibody (d14) some tiny vessels could be observed (arrows). Histology magnification is indicated by scale bar (200 μm). Macro-photography inserts show the freshly explanted pale plugs.
(TIF)

Figure S8 Apoptosis of MSPCs under hypoxia *in vitro*. Representative flow cytometry dot blot showing Annexin V binding to phosphatidylserine of apoptotic MSPCs combined with propidium iodide (PI) labeling after culture under hypoxic conditions (1% oxygen) for 8 days. Annexin V^+/PI^-(Annexin single positive) MSPCs represent apoptotic cells with an intact membrane excluding PI. Annexin V^+/PI^+ (double positive) MSPCs represent terminally dead cells which accumulate PI. (FSC, forward light scatter; SSC, side scatter).
(TIF)

Video S1 Related to Figure 2: Wound healing assay *in vitro*. Wounding an ECFC-derived monolayer in a scratch assay was used to monitor endothelial wound repair under hypoxia (1% O_2) as compared to reduced (5% O_2) and ambient air (20% O_2) standard laboratory test conditions. Running numbers refer to cell divisions and sum up to the total number of single cell divisions for each oxygen concentration.
(7z)

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Author Contributions

Conceived and designed the experiments: NAH DS. Performed the experiments: NAH AO ROJ AR KS RR NE MF. Analyzed the data: NAH AO AR KS RR DS. Contributed reagents/materials/analysis tools: ROJ AR KS CBS MA DS. Wrote the paper: NAH MA DS.

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