The Role of Endothelial Progenitor Cell in Therapeutic Neovascularization

Graduate School for Cellular and Biomedical Sciences

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PhD Thesis

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It will have blood, they say; blood will have blood.

- misquoted from

"MACBETH"

William Shakespeare

A PHD THESIS

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ABSTRACT

Atherosclerotic cardiovascular diseases show an increasing prevalence and are a leading cause of mortality and morbidity in the industrialized world. Peripheral arterial disease (PAD) is one of the major manifestations of systemic atherosclerosis affecting the lower extremities. Despite medical advances many patients with PAD are still not eligible for or responsive to traditional therapies and often limb amputation remains to be the only treatment option in severe PAD cases.

Therapeutic neovascularization benefited from stem and progenitor cell therapy is an alternative strategy to promote revascularization of ischemic tissues. The essential role in vessel regeneration has made endothelial progenitor cells (EPC), a precursor of endothelial cells, the most suitable candidate for therapeutic applications aiming at tissue revascularization. Current knowledge suggests that EPC contribute to neovascularization not only by direct participation in tissue homeostasis but mainly via paracrine mechanisms.

In the presented thesis, we exploited the remarkable capacity of EPC to secrete paracrine factors in *ex vivo* controlled hypoxic conditions. The conditioned medium harvested from *in vitro* derived early endothelial progenitor cells (EPC-CM) bears striking angiogenic and tissue regenerative properties. The administration of these factors alone is sufficient to achieve similar therapeutic effect as cell transplantation in a synergistic fashion. The enhanced mobilization and homing of host stem cells from BM through peripheral blood to ischemic region after intramuscular injection of EPC-CM suggest that EPC-CM may promote the endogenous repair systems by both local and systemic actions. Moreover, these trophic factors are able to promote the survival of mature endothelial cells against oxidative stress induced cell death and activate angiogenesis related signal pathways in endothelial cells through the upregulation of specific surface receptors such as PDGF.

The findings reported herein suggest that novel “cell-free” strategies could be developed based on the administration of EPC paracrine factors. The impact of the present study may go beyond the treatments of cardiovascular disorders extending the broad fields of regenerative medicine.

ABBREVIATIONS

ABI	Ankle Branchial Index
acLDL	actylated LDL
AMI	Acute Myocardial Infarction
ANG	Angiopoietin
Apaf	Apoptotic Protease Activating Factor
BH domains	Bcl-2 Homology domains
BM	Bone Marrow
BOP	BH3-Only Protein
CAC	Circulating Angiogenic Cells
CAD	Coronary Artery Disease
CBMC	Cord Blood Mononuclear Cells
CFU	Colony-Forming Unit
CFU-EC	Colony-Forming Unit-Endothelial Cells
CFU-Hill	Colony-Forming Unit-Hill
CLI	Critical Limb Ischemia
CSF	Colony-Stimulating Factor
Cu/ZnSOD	Copper/Zinc SOD
DEL-1	Developmental Endothelial Locus-1
DISC	Death-Inducing Signaling Complex
EC	Endothelial Cell
ECFC	Endothelial Colony-Forming Cells
ECM	Extracelluar Matrix
EGF	Epidermal Growth Factor
eNOS	endothelial NO Sythetase
EPC	Endothelial Progenitor Cell
FADD	Fas-Associated Protein with Death Domain
FGF	Fibrobalst Growth Factor
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GPx	Glutathione Peroxidase
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxia Induced Factor
ICAM-1	Inter-Cellular Adhesion Molecule-1

IGF	Insulin like Growth Factor
iPS	induced Pluripotent Stem Cell
LAD	Left Anterior Descending Coronary Artery
LDL	Low Density Lipoproteins
LVEF	Left Ventricle Ejection Fraction
MCAM	Melanoma Cell Adhesion Molecule
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarcion
MMP	Matrix Metalloproteinase
MnSOD	Manganese SOD
MPO	Myeloperoxidase
MSC	Mesenchymal Stem Cell
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
OEC	Outgrowth Endothelial Cells
PAD	Peripheral Artery Disease
PBMC	Peripheral Blood Mononuclear Cells
PDGF	Platelet Derived Growth Factor
PLG	Poly(Lactide-co-Glycolide)
rhVEGF	recombinant human VEGF
ROS	Reactive Oxygen Speices
SCGF	Stem Cell Growth Factor
SDF-1	Stromal Cell-Derived
siRNA	small interfering RNA
SMC	Smooth Muscle Cell
SOD	Superoxide Dismutase
TGF-β	Transforming Growth Factor-β
TNF-β	Tumor Necrosis Factor-α
UEA-1	Ulex Europaeus Agglutinin-1
VCAM	Vascular Cell Adhesion Molecule-1
VE-cadherin	Vascular Endothelial-cadherin
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
XO	Xanthine Oxidase

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1. INTRODUCTION

1.1 Atherosclerosis and PAD

1.1.1 Atherosclerosis

Atherosclerosis is the main pathology underlying cardiovascular disease in which an artery wall thickens as the result of the development of lipid-rich plaques (such as cholesterol) in the larger arteries.[1] It originates from local endothelium dysfunction due to the oxidative modification of low density lipoproteins (LDL) in the arterial wall by reactive oxygen species (ROS).[2] To react to the damage in the intima, the body's immune system triggers a chronic inflammatory response by simultaneous delivery of white blood cells (mainly macrophages and T cells) and mast cells. However, the recruitment of these white blood cells cannot scavenge completely the growing plaques, resulting in the formation of fatty streaks, progressive atherosclerotic lesion, and ultimately vulnerable plaques.[3] Meanwhile, the plaque is surrounded by smooth muscle cells forming a cap over the affected area. The enlargement of this cap leads to a further narrowing of the vessel and reduction of the blood flow.[4] When advanced plaque eventually ruptures, the shedding of prothrombotic material (i.e. phospholipids, tissue factor) from the core of the plaque into the blood will cause massive platelet aggregation and occlusive thrombosis.[3] For instance, coronary artery disease (CAD), the leading cause of death worldwide, is the end result of the accumulation and activation of atheromatous plaques within coronary arteries.[3]

1.1.2 PAD

Peripheral artery disease (PAD) is caused by a reduction of normal blood flow due to atherosclerosis in the peripheral arteries of the pelvis and limbs, commonly the iliac, femoral, popliteal and tibial arteries, leading to ischemia in the affected lower extremity (**Figure 1**).[5] Similar to CAD, PAD is a major manifestation of systemic atherosclerosis that affects about 8 million Americans and is associated with significant morbidity and mortality.[6] The classification of PAD is based on the Fontaine Staging System introduced in 1954[7]: mild PAD may be asymptomatic (stage I) or cause intermittent claudication (stage II); severe PAD may cause daily rest pain (stage III) with skin atrophy, focal tissue necrosis and gangrene (stage IV).[8] In most cases, patients with PAD also suffer from CAD or other systemic atherosclerotic disorders which associated with similar cardiovascular risk factors as smoking, diabete mellitus, hypertension

and family history.[9, 10] Treatment of mild PAD includes risk factor modification, physical exercise and anti-platelet drugs.[11] Critical limb ischemia (CLI) usually requires percutaneous transluminal angioplasty (PTA) or surgical bypass grafting. However, current pharmacological treatments alone have poor or no efficacy; approximately one-fourth of the patients with CLI are poor candidates for surgical procedure and 30% patients eventually require leg amputation.[12, 13] Therefore, novel strategies for therapeutic neovascularization in terms of gene therapy, cytokine therapy and cell therapy are warranted.

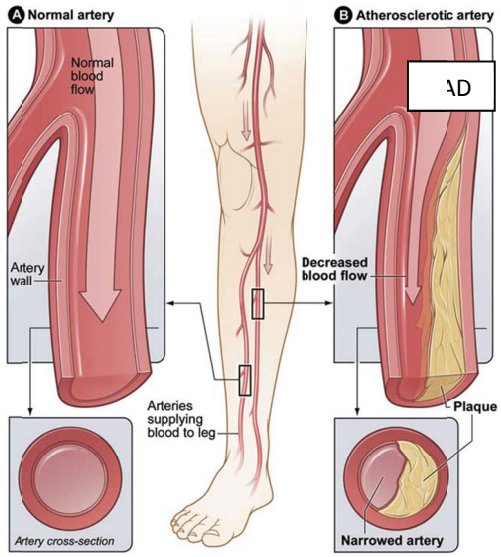

Figure 1. Atherosclerotic artery in peripheral artery disease (PAD). PAD is caused by atherosclerosis in the peripheral arteries of the pelvis and limbs, commonly the iliac, femoral, popliteal and tibial arteries, leading to ischemia in the affected lower extremity. **(A)** A normal artery with normal blood flow. The inset image shows a cross-section. **(B)** An atherosclerotic artery with plaques partially blocking blood flow. The inset image shows a cross-section. Illustration courtesy of NHLBI (www.nhlbi.nih.gov).

1.2 Neovascularization - Arteriogenesis, Angiogenesis and Vasculogenesis

In 1971, Folkman et al. reported that the development of microvasculature is essential for the growth of neoplastic tissue.[14] Therefrom, the concept of angiogenesis was widely accepted over the past years.[15] Three endogenous mechanisms are currently known to be responsible for postnatal neovascularization, namely arteriogenesis, angiogenesis and vasculogenesis (**Figure 2**). Arteriogenesis represents the adaptive remodeling and structural enlargement of preexistent collateral pathways without building new blood vessels; simultaneous sprouting and intussusceptions of neovasculatures from preexistent capillaries was termed as angiogenesis; by recruiting and differentiation of the circulating and resident vascular progenitor cells, *de novo* vasculatures are formed via vasculogenesis.[16] All three mechanisms depend upon a balance of angiogenic factors and angiogenesis inhibitors, including a variety of growth factors, cytokines, chemokines, proteases, adhesion molecules and matrix proteins.[17]

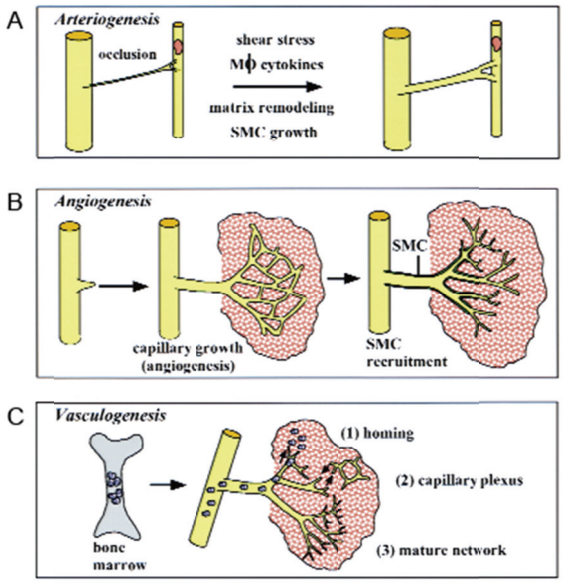

Figure 2. Three mechanisms of postnatal neovascularization. Three endogenous mechanisms are responsible for postnatal neovascularization. **(A)** Arteriogenesis: enlargement and remodeling of preexistent capillaries. **(B)** Angiogenesis: activation and sprouting of preexistent endothelium to form new capillaries. **(C)** Vasculogenesis: Formation of *de novo* capillaries from endothelial progenitor cells. Illustration adapted by permission from Macmillan Publishers Ltd: Nature Medicine. *Carmeliet P, Nature Medicine, **6**, 389 - 395 (2000)*. Copyright© (2000).

1.2.1 Arteriogenesis

Under normal condition for instantaneous regulation, collateral arteries have a small diameter that generates a high vascular resistance in terminal arterioles and precapillary sphincters, a circumstance which precludes a significant flow rate.[18] Their tone is autonomously controlled by the sympathetic nervous system and local products of metabolism, maintaining the blood flow within tolerable limits despite variations in perfusion pressure.[16] However, obstruction of a large conduit arterial segment substantially increases the pressure gradient within the parallel natural bypass collaterals and raises their flow rate.[19, 20] If the flow increases, wall shear stress of the collaterals increases proportionally, inducing an increase in diameter by vasodilatation and remodeling to restore shear stress toward its original level[21]. In most situations, due to the quick fall of the shear stress after vasodilatation and vessel remodeling, arteriogenesis usually stops prematurely and restores 35% to 40% of the maximal conductance of the occluded artery.[22, 23] The obstruction and increased shear stress also activates the endothelium, resulting increased endothelial nitric oxide synthetase (eNOS) and finally triggers a transient nitric oxide (NO)-based vasodilatation.[24] The release of other chemokines and adhesion molecules such as monocyte chemoattractant protein (MCP-1), granulocyte macrophage colony-stimulating factor (GM-CSF), stromal cell-derived factor-1 (SDF-1), transforming growth factor beta (TGF-β), inter-cellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)[25] were also upregulated.[26, 27] Consequently, $CD14^+$ monocytic cells are recruited to the endothelium and make further production of vascular growth factors and cytokines, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF).[27] Molecules involved in cell proliferation and migration are simultaneous upregulated as well, for instance, matrix metalloproteinase-2 and 9 (MMP), and intergrin $\alpha_V\beta_1$ and $\alpha_V\beta_3$.[28-30] A recent study also suggests an essential role of Ephrin-B2 and notch signaling pathway for postnatal arteriogenesis.[31] Moreover, during arteriogenesis, existing vascular smooth muscle cells (SMC) are replaced with new cells. Their proliferation, phenotypic changes from contractile to synthetic, and the occurrence of a neointima are typical.[29, 32, 33] Platelets may also participate in arteriogenesis by releasing SDF-1 and expressing P-selectin to initiate rolling of monocytes and various vascular progenitor cells.[34]

1.2.2 Angiogenesis

Angiogenesis differs from arteriogenesis by occurring in the tissue distal from the arterial occlusion and being stimulated mainly by tissue hypoxia rather than physical forces, resulting in new capillaries.[24] It is a physiological process that new blood vessels sprouts from preexistent vessels and expands the capillary density in tissue parenchyma. The increased diffusive flux of oxygen carried by the new capillaries from the postcapillary venules consequently attenuates the cellular hypoxia.

Several processes are involved in postnatal angiogenesis. The activation of the local endothelium occurs in the first place. Under hypoxic conditions, the transcriptional activator hypoxia inducible factor-1 (HIF-1) is activated as a result of the inhibition of the degradation of its HIF-1α subunit.[35] HIF-1 is involved in expression and activation of over 70 genes relevant to angiogenesis including VEGF, angiopoietin (ANG), TGF and platelet-derived growth factor (PDGF).[36-38] Simultaneously, three different barriers, the sub-endothelial basement membrane between the endothelium and SMC or pericytes, the fibrin gel formed from fibrinogen derived from the vascular bed[39], and the surrounding extracellular matrix (ECM) are degraded by MMPs and urokinase plasminogen activator for the migration of endothelial cells (EC).[40] MMP-2, 3, 7, and 9 degrade the vascular basement membrane composed of type IV, XV, XVIII collagen, laminins, entactin, heparan sulfate proteoglycans, perlecan, and osteonectin. MMP-3, 7, 8, 12 and 13 cleave fibrinogen, making EC invade through a dense fibrin gel.[41, 42] Treating urokinase receptor and MMP-9 with small interfering RNA (siRNA) inhibits the formation of capillaries.[43] The creation of MMP deficient mice also provided important insight into the angiogenic role of MMPs.[44] Finally, EC migrate and proliferate via adhesion molecules towards angiogenic chemotaxis such as VEGF-A, forming nascent capillaries into to the site of angiogenesis. The creation of durable and mature vessels further requires the EC-EC junction, the recruitment of supporting pericytes and SMC, as well as the remodeling of new ECM. Several growth factors participate in these processes. ANG-1 induces stabilization of the immature endothelial cell network by upregulation of CD31, occludin and vascular endothelial-cadherin (VE-cadherin) in EC, as well as promoting pericytes and SMC growth and differentiation.[26] Proliferation and migration of the pericytes onto newly developed capillaries, being responsible for the maturation and stabilization of the neovasculatures[45, 46], requires the secretion of PDGF-BB from neo-endothelium in response to VEGF. The production of VEGF from EC also regulates fibroblast

growth factor -2 (FGF-2) and TGF-β1 expression by several vascular cell types and mediates SMC proliferation and migration.[47]

Another type of angiogenesis exists as intussusception by its mechanism of extending the capillary wall into the lumen to split one vessel into two. By allowing the increase of the number of capillaries without recruiting more endothelial cells, intussusceptive angiogenesis is especially important in embryonic development and under certain pathological circumstances.[48]

1.2.3 Vasculogenesis

Vasculogenesis refers to the *in situ* formation of de novo capillaries from angioblasts or endothelial progenitor cells (EPC) which can further differentiate into endothelial cells.[49, 50] This process particularly relates to the embryonic development of the vascular system, by which the primitive heart and vascular plexus are formed.[49]

Vasculogenesis was at first thought to be restricted to early embryogenesis as the most typical and earliest prenatal vasculogenesis is the formation of blood islands in the yolk sac. In the mouse, blood islands arise between embryonic day 7 (E7) and E7.5, as mesoderm cells adjacent to visceral endoderm proliferate to form mesodermal cell masses/hemanigoblasts[51], which could later give rise to a blood filled vasculature. These hemangioblasts are regarded as the common precursor of both angioblast/endothelial progenitor cells for vasculature outside and hematopoietic stem/progenitor cells for blood inside.[52] After the onset of blood circulation, this network differentiated into an arteriovenous vascular system by selective expression of arterial markers including ephrin-B2, neuropilin-1, notch-3, gridlock[53], and venous markers including neuropilin-2 and EphB4.[54, 55]

Several growth factors are responsible for the prenatal vasculogenesis. VEGF and VEGF receptors (VEGFR) are the first EC-specific signal pathways activated during vasculogenesis and are critical for migration, formation and organization of the primitive vascular structures.[56, 57] Deficiency or interruption of VEGF-VEGFR pathway usually causes embryos die in early days.[56-58] Tie1-ANG1 and Tie2-ANG1 pathways are also essential for embryonic vascular development. Knockout embryos lacking Tie1, Tie2 or ANG-1 die early due to multiple cardiovascular defects.[59-61]

The existence of postnatal vasculogenesis was first observed by Asahara et al. in 1997.[62] *In vitro* culture of $CD34^+$ enriched peripheral blood mononuclear cells (PBMC) of adult species was able to differentiate towards mature EC phenotypes with the expression of classical EC surface markers including VEGFR-2, CD31, eNOS, E-selectin and VE-cadherin, as well as the loss of their stem markers CD34 and CD133.[63] When transplanted into animal models of tissue ischemia, these precursor cells are localized into sites of active neovascularization and function through trophic mechanism and incorporation into nascent endothelium.[64, 65] These findings suggest a mechanism of adult circulating EPC analogue to embryonic angioblasts in their contribution to postnatal neovascularization. Numerous studies now implicate postnatal vasculogenesis in such processes as tissue and organ ischemia, atherosclerosis, thrombosis, wound healing, and tumor development.[66]

The stimuli and regulators of postnatal vasculogenesis are under intensive investigation. GM-CSF can stimulate both hematopoietic progenitor cells[67] and EPC[68] in the circulating blood. VEGF is a major regulator for recruiting circulating EPC into active sites of neovascularization and inducing EC-phenotypic differentiation. Grunewald et al. discovered that the retention of the proangiogenic subpopulation of bone marrow derived circulating precursor cells in peripheral organs requires an additional factor, SDF-1.[69] However, clear evidences of an efficient *in vivo* incorporation of these precursor cells into neo-endothelium are still missing at present. The interrelation between postnatal angiogenesis and vasculogenesis concerning the physiological mechanism and the underlying molecule pathways requires further studies.

1.3 Therapeutic Neovascularization

The approaches of therapeutic neovascularization are to amplify adaptive neovascularization and perfusion in tissues compromised by ischemia.[70, 71] These include administration of angiogenic growth factors to promote endogenous vascularization, augment perfusion and collateral flow (cytokine therapy); delivery of plasmids which encode specific angiogenic proteins *in situ* (gene therapy); and transplantation of autologous or allogeneic vascular precursor cells from the bone marrow (cell therapy).[5, 8, 10, 16, 72]

1.3.1 Cytokine Therapy

Angiogenic cytokines utilized for therapeutic neovascularization may be administered systemically or locally by a precise quantity of recombinant proteins with dose-response relationship. Granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), have been used in clinical practice to stimulate hematopoietic stem cells to proliferate and promote their release from bone marrow (BM) into the blood.[73] It has been demonstrated in recent years that G-CSF and GM-CSF could also act as potent angigogenic factors by stimulating various populations of bone marrow cells and enhancing regenerative vessel formation.[5, 74, 75] MCP-1 is another potent chemotactic factor involved in strategies to enhance therapeutic neovascularization. When infused intra-arterially or injected as plasmid DNA intramuscularly, it leads to the rise in monocyte concentration in the peripheral blood and their accumulation and invasion into the ischemic regions.[76, 77] Moreover, several clinical trials based on systemic administration of recombinant human (rh)VEGF or rhFGF-2 has been conducted on patients with angina, ischemic heart disease, and intermittent claudication.[5, 16] Although the concept of intravascular infusion of these angiogenic growth factors is appealing, their short half-life in circulation, low targeted uptake efficiency and some severe adverse effects have compromised the practical feasibility.[78] Some patients received intravascular infusion of rhVEGF$_{165}$ or rhFGF-2 experienced severe systemic hypotension and increased urinary protein excretion.[16] Novel strategies of cytokine therapies advanced with bioengineering structures are now being developed to achieve targeted and sustained release to amplify the therapeutic outcome. These include the utilization of biodegradable hydrogel, poly(lactide-co-glycolide) (PLG) microspheres, and more recently, nanotechnology based biomaterials.[79-82]

1.3.2 Gene Therapy

A better means to achieve more sustained release and targeted delivery of growth factors is the transfer of genes encoding angiogenic growth factors directly into ischemic tissue. Two types of vector have been widely used: plasmids for non-viral delivery system and viral vectors. Plasmid based gene transfer depends on cellular uptake of genetic materials by chemical transfection.[72] Although compared to viral delivery plasmid usually produces relative low transfer efficiency and short-lived gene expression, they do not generate host inflammatory responses and are generally considered much safer.[16] Viral vectors on the other hand deliver targeted genes by infecting host cells with genetically modified viruses including retroviruses, lentiviruses and adenoviruses.[83] Viral carriers delivery are most appealing for its high efficiency and long durability but they bear a number of disadvantages including risk of toxicity, immunogenicity, insertional mutagenesis and the impracticality for repeated treatments.[84]

The therapeutic gene targets can be sketched out into certain broad categories including cell survival, homing, migration, engraftment, efficiency, deposition of matrix, and the production of factors that recruit endogenous progenitors.[85] The proangiogenic effect of these genes has been documented in various rodent and rabbit models of ischemia. Currently, several clinical trials are under way using the VEGF and FGF families to treat CAD and PAD.[16, 18, 86] A number of phase II clinical gene therapy trials are testing other factors such as HIF-1α, HGF, and developmental endothelial locus-1 (DEL-1).[87, 88] Disappointingly, the majority of the studies failed to meet their primary study endpoint although the use of plasmids is proved to be safe and well tolerated.[16] Only one in 23 published studies demonstrated an improvement in the angiographic indices by intra-arterial infusion of $VEGF_{165}$-adenovirus and VEGF-plasmid/liposome.[89] However, its benefits in improving ankle branchial index (ABI) and peak walking distance were insignificant. Indeed, there is an apparent discrepancy between the outcomes of clinical trials and experimental studies in animal models. This discrepancy may be explained by the differences between animal models and human conditions in several aspects such as age and pathological causes.[18] For instance, animal models are usually based on femoral artery extirpation resulting in acute ischemia, while in humans PAD usually develops chronically over decades in the presence of certain cardiovascular risk factors such as smoking, hypertension and hyperlipidemia.[18, 90] Preliminary clinical results also indicated that the $phVEGF_{165}$ gene transfer was more effective in younger patients with acute vascular inflammation and arterial

thrombosis.[16, 91] The development of novel animal models to closer mimic human clinical pathology is necessary for providing a more precise evaluation of the outcomes in future studies.

1.3.3 Cell Therapy

Few advances in the past decade have generated as much excitement as the discovery of stem cells. The use of embryonic stem cells in clinical trials is compromised by the ethical issues and the potential danger of uncontrollable differentiation into unfavorable cell types.[92] Adult stem or progenitor cells, however, are mostly committed to one particular cell fate with only few cell populations exhibiting pluripotent differentiation capability.[93] These committed stem cells can be found in various adult tissues including bone marrow, circulating blood and most of the organs.[16] Under normal circumstances, these progenitor cells usually maintain undifferentiated and quiescent in microenvironments termed niches[94], while during pathological situations, they are activated by a variety of environmental stimuli to participate into tissue regeneration.[95] Aside from endothelial progenitor cells (see next chapter), several alternative sources of the adult stem or progenitor cells are currently being largely investigated for therapeutic revascularization. These include unfractionated bone marrow aspirates and mesenchymal stem cells (MSC). In the last few years, the successful reprogramming of somatic cells to their pluripotent origins, so-called induced pluripotent stem (iPS) cells, created another revolutionary potential for clinical therapeutics by providing an ample source of stem cells that could be used for transplantation.[96, 97]

1.3.3.1 Bone Marrow Therapy

Most clinical studies on cardiovascular diseases have used unselected bone marrow cells as the delivery product, postulating that hematopoietic stem cells, mesenchymal stem cells, endothelial stem cells and other stem/progenitor cells within this population are the biologically relevant therapeutic agents.[16] This cocktail of differentiated and less differentiated cells may suggest superiority over the use of a selected type of progenitor cell population. The problems, however, are the low specificity of targeted differentiation and unpredictable side effects.[10, 16]

In experimental models of acute myocardial infarction (AMI), functional improvements have been reported after transplantation of unselected BM cell populations.[98, 99] Intracoronary delivery of unselected BM cells was reported to enhance left ventricle ejection fraction (LVEF)

recovery in patients after AMI[100], although the effect does not sustain on long-term follow-up.[101] In a multicenter clinical trial (REPAIR-AMI), 204 patients with AMI received an intracoronary infusion of BM derived progenitor cells into the infarct artery 3 to 7 days after successful reperfusion therapy. The absolute improvement in the LVEF was detected in the BM cell group at 4 months and a reduction in the death, recurrence of myocardial infarction (MI), and any revascularization procedure after one year.[102] However, the results are not convincing to some researchers as another study (ASTAMI) using similar type of cells fails to achieve improvement in global left ventricular function[103] and two major determinants of LVEF recovery—time-to-reperfusion and infarct location—are possible confounders in the REPAIR-AMI study.[104]

As for PAD, the Therapeutic Angiogenesis by Cell Transplantation (TACT) study performed a randomized controlled trial in patients with CLI.[105] At 4 weeks the ABI was significantly improved in limbs injected with BM mononuclear cells, as well as transcutaneous oxygen pressure, pain-free walking time and a reduction of rest pain. These improvements were sustained to the 24-week follow-up.[105] Another study demonstrated an improvement in endothelial function in patients with PAD by intramuscular injection of BM derived mononuclear cells.[106] Interestingly, evidences from different animal studies suggested that BM cells do not only promote vascular development by incorporating into vessel walls, but possibly play a supportive role by either secreting paracrine factors or transdifferentiation into other cell types including fibroblasts, pericytes, and leukocytes.[69, 107]

1.3.3.2 Mesenchymal Stem Cell Therapy

Mesenchymal stem cells (MSC), also referred to as marrow stromal cells or multipotent mesenchymal stromal cells[108], were originally described as adherent cells from bone marrow that form colonies[109] and were found to have multilineage differentiation potential to form connective tissue cell types including osteoblasts, chondrocytes and adipocytes.[110] One guideline to define MSC is tissue-culture plastic adherent cells capable of osteogenesis, adiopogenesis and chondrogenesis that are positive for CD73, CD90 and CD105 but negative for CD11b, CD14, CD34, CD45, CD79a and HLA-DR surface markers.[108] However, a distinct characterization of MSC remains challenging.[111] Bone marrow-derived MSC are extremely heterogeneous and their specific marker expression, proliferation and differentiation potential are tightly dependent on the cell culture condition.[112]

Due to the significant potential of MSC to differentiate into tissue-specific cell types with trophic activity, to promote neovascularization, and to promote potent immunosuppressive effects, studies on bone marrow derived MSC therapy have been carried out for regenerating damaged tissue in cartilage and bone, brain and spinal cord, as well as from cardiovascular disease and myocardial infarction. Furthermore, because of their ability to modulate immune responses, allogeneic transplantation of MSC may be feasible with a low risk of immune rejection.[111, 112]

The applications conceived for MSC therapy focus on both the multilineage differentiation capacity and their paracrine mechanisms.[111, 113] Kinnaird et al. reported that local injection of MSC into the adductor muscles of a mouse model of hindlimb ischemia significantly enhanced perfusion of ischemic tissue and collateral remodeling, lessened tissue damage, and improved limb function through paracrine pathways and without observable MSC incorporation into vessels.[23] MSC are able to express genes encoding a broad spectrum of arteriogenic cytokines and promote both *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms, no labeled MSC were seen engrafted into collaterals.[114] On the other hand, several studies did monitor the physical differentiation of MSC after local cell transplantation. Transplanted MSC are shown to survive well in the ischemic environment of rat limbs and can differentiate into endothelial cells, vascular smooth muscle cells, skeletal muscle cells, and adipocytes.[115]

Recent clinical trials aimed at bone regeneration are dependent on the osteogenic differentiation of MSC while the treatment of cardiovascular diseases has focused almost entirely on the paracrine mechanisms of MSC.[111] For instance, in a total of 19 clinical trials related to cardiovascular disorders using exogenous MSC, 16 were dependent on its proangiogenic, anti-apoptotic or immnomodulating properties.[111] However, the inability to meet primary clinical endpoints in phase III trials may be the result of a low efficiency of engrafted cells that reduces the potential for long-term release of trophic factors.[116] There is novel evidence that the therapeutic effects result in a systemic effect in addition to paracrine signaling and direct cell-cell interactions.[111] Enhanced understanding of the underlying mechanisms of MSC therapeutics would allow novel approaches for higher efficiency and efficacy.

1.3.4 Bioengineering, Nanotechnology and Therapeutic Neovascularization

The development and application of advanced biotechnology holds tremendous potential to revolutionize current therapeutic approaches. Novel nanotechnology based biomaterials,

including particles, fibers, scaffolds and matrices, were synthesized to resemble the native architecture of the cellular microenvironments.[80, 117] These biomaterials can be chemically modified to integrate with drugs, proteins, peptides, genetic materials, and small bio-molecules connecting to cells.[81, 117, 118] Mooney et al. developed a new porous polymer scaffold made from poly(lactide-co-glycolide) (PLG).[119] This bioactive scaffold is made to incorporate one or multiple growth factors using a high-pressure carbon dioxide fabrication process. Importantly, the release kinetics for each growth factor can be controlled individually by altering the polymer formulations and molecular weights.[81, 120] By sustaining the release of growth factors for up to several weeks, this PLG scaffold is able to recruit progenitor cells and effectively promote vascularization in an *in vivo* angiogenesis assay.[81, 119] In another example, Stupp et al. have developed self-assembling nanofibers made from peptide amphiphiles and incorporated with an ariginie-glycine-aspartic acid serine (RGD) sequence from fibronectin for the purpose of binding integrin to promote cell interaction and adhesion.[79] In a mouse model, subcutaneous injection of encapsulated BM mononuclear cells within these nanofibers can clearly lead to enhanced viability, adhesion and proliferation compare to normal cell transplantation.[79] These nanofibers can also bind with papacrine factors secreted from stem cells and induce potent regenerative effect to treat cardiovascular diseases.[121] Other potential applications of bioengineering and nanotechnologies in therapeutic neovascularization include serving as supportive matrix for vascular regeneration, noninvasive tracking of transplanted cells *in vivo* such as magnetic nanoparticles and quantum dots, and intracellular delivery of genes or proteins as a more efficient and effective alternative for naked plasmid injection.[117] Considering the wide spectrum of bioengineering and nanotechnology, it is foreseeable that the utilization of it in current angiogenic therapies will eventually advance our ability in designing novel strategies for better controlled vessel regeneration.

1.3.5 Experimental Models to Study Therapeutic Neovascularization

The use of applicable *in vivo* and *in vitro* models to assay vessel formation is crucial to the search for therapeutic agents that promote neovascularization in the clinical setting.[122] *In vitro* experiments, such as studies of EC, SMC, or progenitor cells, are better suited than *in vivo* research for deducing biological mechanisms of blood vessel formation. With fewer variables and perceptually amplified reactions to subtle causes, results are generally more discernible. For instance, wound healing scratch assay and transwell™ migration assay are well-developed to

measure the EC migration rate and is particularly useful for studies on the effect of cell–cell or cell-cytokine interactions; Matrigel™ based tube formation assay provide a quick assessment of the ability of EC to form 3D structures; *ex vivo* aortic ring assay, having the ability to sprouting native microvascular braches by embedding aortic segments in collagen gel, bridges the gap between *in vivo* and *in vitro* models.[122-124]

In vivo studies, on the other hand, are more informative than *in vitro* studies by providing the complex cellular and molecular activities and relevant pathological condition. The most frequently used models in current practice are the corneal micropocket assay, the chick chorioallantoic membrane assay, and the matrigel™ plug assay.[125] Whole animal models such as embryonic and young, growing zebrafish, and *Xenopus Laevis* tadpole were used for screening small molecules that affect blood vessel formation during development. Adult animal models created by surgical means such as mouse skin flap and dorsal skinfold chamber implantation were also used to study angiogenesis under hypoxia condition.[123, 125]

Another large category of models of angiogenesis are developed to mimicking specific cardiovascular disorders.[126] For example, an acute myocardial infarction model can be established in mouse by ligation of the left anterior descending coronary artery (LAD).[127] Models of peripheral artery diseases are generally applied by unilateral excision of the femoral artery, creating ischemia in the hindlimb.[128] Notably, these surgically created disease-specific animal models usually induced acute ischemia associated with considerable tissue necrosis, altered shear stress and changes in gene expression, generating an immediate, robust intrinsic stimulus for endogenous compensation mechanism such as arteriogenesis and the recruitment of endothelial progenitor cells.[90, 129, 130] These acute syndromes do not occur in real PAD condition in a chronic disease which develops slowly over decades and significantly alters the therapeutic outcomes. In one study, Tang et al. compared gradual to acute arterial occlusion in the hindlimb perfusion in rats and reported that chronic femoral artery occlusion resulted in less recovery of perfusion, as well as a smaller increase in collateral artery diameter.[90] Trying to circumvent this problem, our group has established a model of chronic and rather moderate hindlimb ischemia in athymic nude rat with consistently reduced perfusion levels persisting over a prolonged period of up to 60 days.[130] Being responsive to proangiogenic treatments such as EPC transplantation, such kind of improved animal models with a better simulation of the

pathological environments will allow more precise and standardized therapeutic angiogenesis evaluations in the future.

1.4 Endothelial Progenitor Cell

1.4.1 Discovery of Endothelial Progenitor Cell

The paradigm, that new blood vessel formation in adults is restricted to arteriogenesis and angiogenesis was challenged in 1997. Until then, the work of Folkman et al. since 1971 postulated that new capillaries are formed by the local migration and replication of existent endothelial cells.[14] These transient neovessels are stabilized by the coverage of mural cells such as pericytes and SMC.[131] However, the existence of circulating endothelial progenitor cells (EPC) in adult human discovered by Asahara et al. provided solid evidence for the participation of vasculogenesis during postnatal vessel repairing.[62] In their landmark paper published in Science, they reported that human $CD34^+$ or KDR^+ (Flk-1, VEGFR-2) monocytic cells, mainly derived from bone marrow, can be isolated from peripheral blood, and give rise to differentiated endothelial cells under certain *in vitro* culture conditions.[62] These cells express endothelial-specific markers such as CD31, CD34, KDR, Tie2, ecNOS, and E-selectin, as well as the ability to bind lectin. These cells also displayed limited expression of CD68 and CD45, markers for macrophage and hematopoietic lineages. When injected into a hindlimb ischemia model, EPC appeared integrated into capillary vessel walls, indicating a contribution to neovascularization via vasculogenesis.[64] In the end they suggested potential utility of the EPC as autologous vectors for gene therapy to achieve constitutive expression of angiogenic factors or provisional matrix proteins or both.[62]

The therapeutic potential of EPC therapy was soon investigated by a series of follow-up animal studies. Kalka et al. published their *in vivo* results in Proc. Natl. Acad. Sci. USA in 2000, showing that intracardiac transplantation of *ex vivo* expanded human EPC to athymic nude mice with hindlimb ischemia were able to markedly improve the blood flow recovery and capillary density in the ischemic hindlimb and significantly reduce the rate of limb loss.[64] The exogenous EPC were observed to incorporate into vessels at a low number (range 0.5 - 5 per x10 field) and home specifically into the ischemic area. This study was amongst the first to indicate clearly that *ex vivo* cell therapy, consisting of culture-expanded EPC transplantation, may be used to successfully promote therapeutic neovascularization of ischemic tissues. Such a breakthrough opened up a new era of utilizing adult stem cell therapies on treating cardiovascular disorders and is rapidly being translated from preclinical investigations into clinical practice.

To date, the phenotypic characterization of EPC is still an open issue and matter of scientific debate. At present there is no general consensus about the definition and isolation process of EPC. The term encompasses rather a heterogeneous group of cells that exist in a variety of stages ranging from haemangioblast to fully differentiated EC with distinct function, separate origin, and different protein expression profiles.[16, 132, 133] The generally accepted definition of circulating EPC has used surface markers including CD34, CD133 and KDR.[134] Later studies have suggested that the actual cell population enriched in the $CD34^+$, $CD133^+$, KDR^+ fraction is of hematopoietic lineage and do not form endothelium *in vivo*[135, 136], although the methodology and implication of such studies were soon questioned.[137] However, further studies trying to purify and define "genuine" EPC have been difficult due to the lack of cell surface antigens or markers that distinguish these cells from mature endothelial cells and from subsets of hematopoietic cells.[138, 139] The consensus of a precise definition of EPC is so far still missing.

1.4.2 CFU-EC / CFU-Hill

Various approaches to isolate precursors of endothelial cells have been reported (**Figure 3**). Three most common culture methods have been described in the review by Prater et al.[132] The original method established by Asahara et al. has been further developed and optimized to stimulate the formation of endothelial colonies, namely colony-forming unit (CFU). Gehling et al. introduced the term colony-forming unit endothelial cells (CFU-EC) to scientific readership for the first time in 2000.[140] They described a methylcellulose-based colony assay for EPC. In this assay, $CD133^+$ EPC in semisolid medium supplemented with stem cell growth factor (SCGF) and VEGF could give rise to colonies with a unique morphology. These colonies are composed of small-sized cells that express the endothelial cell antigen von Willebrand factor (vWF) but are negative for CD41a and CD41b, markers for early megakaryocytes, suggesting of endothelial lineage. The $CD133^+$ cells used to generate the CFU-EC can further form new blood vessels *in vivo* supporting tumor angiogenesis. In the meantime, some studies investigating the clonogenic potential of EPC adopted the term CFU-EC despite the fact that different culture systems were used.[141]

However, although the terminological issue was argued by the original authors[142], the colony presented by Hill et al. in 2003 is currently widely referred to as CFU-EC.[132, 143] In this assay, mononuclear cells from venous blood isolated by Ficoll® density gradient centrifugation are

plated on fibronectin-coated 6-well plates in growth medium consisting of Medium199 supplemented with 20% fetal-calf serum for two days to remove mature endothelial cells and monocytes. The non-adherent cells (containing EPC) are then replated on fibronectin-coated 24-well plates. Discrete colonies emerge after 3 days, comprised centrally localized round cells with spindle-shaped cells sprouting at the periphery. These myeloid CFU-Hill colonies[142] exhibit many endothelial characteristics, including expression of CD31, Tie-2, and KDR[62, 144]. Their number correlates to endothelial function and the subjects' cardiovascular risk factors, as determined by Framingham risk factor score, a system to predict the risk of coronary artery disease in persons free of clinical disease.[143] Consistently, the concentration of CFU-EC was lower in the peripheral blood of patients with elevated cholesterol, hypertension, and type II diabetes. Later evidences further unraveled the connection between CFU-Hill colony numbers and different conditions of cardiovascular disorders. For instance, it has been shown that chronic vascular diseases result in reduced CFU-Hill numbers while in the acute phase, the ability to form CFU-Hill colonies is temporarily enhanced.[132] This *in vitro* assay is currently commercial available by STEMCELL Technologies as the 5 Day CFU-Hill Colony Assay and CFU-Hill Medium (formerly Endocult®). In some studies, CFU-Hill/CFU-EC has been transplanted into animal models of ischemia to promote neovascularization.[138, 145]

1.4.3 Early EPC / Circulating Angiogenic Cell

Although the number of CFU-Hill colonies may serve as surrogate biologic marker for vascular function and cumulative cardiovascular risk, the most commonly employed approach to study the therapeutic benefit of EPC is adopted from the work by Kalka et al.[64]. In this method, unfractioned mononuclear cells are plated on culture dishes coated with human fibronectin and maintained in endothelial growth medium supplemented with fetal bovine serum, VEGF-1, FGF-2, epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1), and ascorbic acid. After 4 days non-adherent cells are removed and adherent cells are replated through day 7 - 10. These differentiating cells were shown to have a spindle-shaped morphology, endocytose acetylated-LDL (acLDL), and bind Ulex europaeus agglutinin-1 (UEA-1), consistent with endothelial lineage cells. The majority of the cells express endothelial-specific antigens KDR, CD31, and the monocytic marker CD14. Only less than 20% of the cells express CD34 and integrin $\alpha_V\beta_3$.[64] The fact that CD19, CD3, CD68 are not expressed seemed to exclude a hematopoietic lineage cell population. However this interpretation was soon challenged by later studies.[146, 147] In other

studies adopting similar methodology to isolate EPC, strong expressions of surface antigen Tie2[146] and endoglin[148] were confirmed. These cells, characterized by their time of appearance *in vitro*, have also been referred to as early EPC by Gulati et al. and other researchers.[149, 150]

One major difference of these *ex vivo* expanded early EPC to the progenitor cells first reported by Asahara et al.[62] is the expression of CD45. In Asahara's report, leukocyte common antigen CD45 was identified on 94.1% of freshly isolated cells but disappeared after 7 days of culture.[149] In contrast, the expression of CD45 can be detected in 98% of the early EPC from day 4[146] to day 7[147]. Several other markers of monocyte and macrophage were also expressed in the early EPC, including CD11C and CD163.[146] Since the early EPC do not express typical stem cell markers as CD133 or c-kit[134, 151] and only a minority express the endothelium-specific marker as VE-cadherin and melanoma cell adhesion molecule (MCAM)[147], they have been referred, more appropriately, to as monocyte-derived circulating angiogenic cells (CAC) by Rehman et al.[146] The fact that CD45 is expressed in CAC also suggested the fate of the cells is of hematopoietic and myeloid lineage, in contrast to the initial interpretations from Kalka et al. that a significant contamination by hematopoietic lineage cells was excluded by the low expression of CD3, CD68, and CD19.[64]

Moreover, CAC do not display the colony morphology of CFU-EC though the sprouting spindle-shaped cells from CFU-EC are similar in shape to CAC in culture. Gulati et al. also showed that $CD14^+$ cells was able to produce these spindle-shaped cells but they did not give rise to endothelial outgrowth.[149] Therefore the term EPC should be reserved for a purified cell population directly from the hemangioblast or hematopoietic stem cells[140], and co-express specific endothelial and stem/progenitor markers such as VE-cadherin and CD133.[151]

Prokopi et al. have used proteomic analysis to postulate that the presence of endothelial cell markers (CD31, vWF, lectin binding) in early EPC is a consequence of platelet contamination, rather than endogenous synthesis.[152] In culture monocytic cells readily take up the platelets or platelet-derived microparticles resulting in a possible exchange of antigens between cell types, including those that are conventionally used as endothelial cell markers.[152] Therefore it is important to acknowledge that if platelet specific antigens such as CD41 are not included as controls, those double-positive cells for endothelial and hematopoietic/stem cell markers may not necessarily be "genuine" EPC. The authors also suggested that a significant fraction of the angiogenic activity in the early EPC is due to platelet-derived proteins.[152] However, in our recent

study, a significant up-regulation of KDR mRNA can be detected in early EPC. This endogenous increase of KDR mRNA might in part be interpreted as the result of a "true" endothelial-like phenotypic change occurring in the adherent cells during culture.[147]

1.4.4 Outgrowth Endothelial Cell / Endothelial Colony-Forming Cell

In comparison to early EPC, endothelial colony-forming cells (ECFC) or late outgrowth endothelial cells (OEC) are derived in long term culture of mononuclear cells. Lin et al. generated ECFC from peripheral blood of bone marrow transplant recipients.[153] ECFC colonies, developing after 3 weeks in culture, were of donor karyotype, appeared typically endothelial, and exhibited an extraordinary growth capacity, similar to the circulating bone marrow–derived endothelial cells reported by Shi et al.[154] The culture method of ECFC is similar to early EPC. Adult PBMC or human umbilical cord blood mononuclear cells (CBMC)[155] are collected and plated onto collagen I[153] or gelatin[150]-coated plates in endothelial-specific growth medium. Non-adherent cells are discarded after 24 hours[153] or 6 days[150]. ECFC colonies emerge from the adherent cell population (early EPC) 10 - 21 days after plating (5 - 7 days if human umbilical CBMC are plated) and can be expanded for more than 12 weeks, while early EPC usually show limited proliferation and disappear 3 to 4 weeks later.[150] ECFC show strong proliferative, migrating, and tube-forming capabilities.[132] Others have also reported that ECFC are different from mature EC in terms of caveolae, expression of integrins, resistance to oxidative stress, and angiogenic potency *in vivo*.[156] Therefore, even being phenotypically indistinguishable from cultured EC, ECFC are supposed to be of EPC lineage.

Unlike the spindle-shaped early EPC, ECFC display a cobblestone appearance typical for EC, exhibit stronger expression of VE-cadherin and KDR but are negative for CD14 and CD45, and have significant potential for clonogenic expansion. It is known that upregulation of KDR expression on the endothelial cells causes an increase in VEGF-mediated tube formation on Matrigel.[150] Thus higher expression level of KDR in ECFC may cause better tube formation. VE-cadherin is specifically expressed in adherent junctions of endothelial cells and exerts important functions in cell–cell adhesion.[157] Therefore a higher and longer sustained expression level of VE-cadherin may be the result of a better incorporation capacity of ECFC than early EPC. Moreover, early EPC contribute to neovascularization mainly by paracrine secretion of trophic factors that help recruit resident vascular cells and activate their angiogenic property, whereas late EPC

participate by providing a sufficient number of endothelial cells based on their high proliferation potency.[150, 156] Yoon et al. demonstrated that the transplantation of mixed early EPC and ECFC resulted in synergistic augmentation of angiogenesis in athymic nude mice with hindlimb ischemia, possible through the cross-talk of cytokines and MMPs between the 2 types of cell.[156] Finally, ECFC may also be correlated with the severity of vascular homeostasis and repair.[158]

It is noteworthy that cytokine composition of the culture media determines *in vitro* mononuclear cell differentiation. For instance incubation of similar mononuclear cell cultures with GM-CSF or tumor necrosis factor-α (TNF-α) has been reported to favor isolation of dendritic cells.[159, 160] Other studies suggested that a synergetic progenitor cell population isolated from PBMC is capable of differentiation into angiogenic, myocardial and neural lineages under specific culture condition.[161] Furthermore, it has to be acknowledged that the relationship between *in vitro* cultured progenitor cells and *in vivo* progenitor populations is not well understood and that the differentiation cascade that leads to the formation of endothelial cells either *in vitro* or *in vivo* remains to be elucidated.[162]

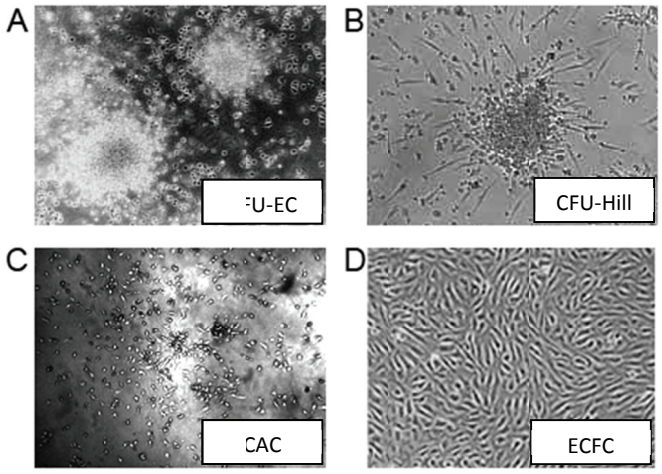

Figure 3. Four types of endothelial progenitor cells (EPC) in different *ex vivo* culture condition. **(A)** Colony-forming unit endothelial cells (CFU-EC) are derived from $CD133^+$ EPC. **(B)** colony-forming unit-Hill cells (CFU-Hill) are generated from non-adhesive peripheral blood mononuclear cells (PBMC) after two days culture. **(C)** Circulating angiogenic cells (CAC) or early EPC appear in early time point from PBMC culture and have limited proliferation and colony forming capacity. **(D)** Clonogenic expansion of endothelial colony-forming cells (ECFC) or late outgrowth endothelial cells (OEC) appears after three weeks of culture and displays potent proliferative, migrating, and tube-forming capabilities.

1.4.5 Mechanism of Homing

In order to exert their the vascular regenerative actions, EPC are mobilized from the bone marrow into the blood stream and are recruited to the sites of nascent vessels. Tissue ischemia is one of the strongest signals that initiate a coordinated sequence of adhesive and signaling events leading to the recruitment and incorporation of EPC.[133] The first step of homing of EPC to ischemic tissue involves adhesion and transmigration occurs in response to a variety of cytokines and integrins activated by hypoxia.[163-165] In a study conducted by Wu et al., ligand/receptor pairs potently involved in mediating EPC recruitment and engraftment to the ischemic myocardium were identified by using a functional genomics approach coupled with real-time PCR analysis, including ICAM-1/CD18, SDF-1/CXCR4, fibronectin-1, VCAM-1/integrin α4, and selectin/selectin-ligand.[166] The involvement of SDF1/CXCR4 and selectin/selectin-ligand in EPC recruitment process were confirmed by other studies.[167-169] Moreover, VEGF level is increased during ischemia and capable to act as a chemoattractive factor to EPC.[170-172] Monocytes may directly respond to VEGF chemotaxis by upregulation of VEGFR-1 expression.[173] Grunewald et al. disclosed in his study a mechanism by which recruited bone marrow-derived circulating cells are retained close to the blood vessels by VEGF induced perivascular SDF-1 expression.[69] Overexpression of SDF-1 has been shown to augment homing and incorporation of stem cells into ischemic tissues.[174, 175] Finally, a variety of different cytokines, chemokines, and proteases upregulated within the ischemic tissue may also be involved in the modulation of EPC trafficking.[176] For instance, MCP-1, interleukins, and MMPs attract and help the invasion of circulating progenitor cells. Transfusion of MCP-1–activated BM progenitor cells prevents intimal formation by transdifferentiation into functional EC-like cells on the injured endothelium[77]; IL-1β, IL-6, IL-8 are reported to increase the mobilization and adhesion by several studies[177-179]; both MMP-2 and MMP-9 are shown to promote EPC mobilization.[180]

1.4.6 Mechanisms of Action on Neovascularization

Since their first description a decade ago the role of EPC in vessel growth and repair has been documented in a number of studies.[64, 143] Preclinical studies and early clinical trials employing progenitor cells for the treatment of ischemic diseases and related sequelae have provided encouraging evidence for safety and efficacy.[162] As a result, the translation into a clinical application has occurred in a remarkably short time. However, the mechanisms of action

underlying the regenerative potential of EPC are not completely understood. It has been generally accepted that two main mechanisms contribute to the functional activity of EPC, namely the physical incorporation and differentiation into matured EC, and the secretion of trophic factors which in turn stimulate neovascularization as well as the recruitment and protection of resident vascular cells and other progenitor cells (**Figure 4**).

Initial reports addressed mainly the capacity of EPC to differentiate into mature endothelial cells and to integrate into newly formed vascular structures.[64] Nevertheless there is lack of consensus concerning the incorporation rate of bone marrow-derived cells. The actual number of incorporating EPC varies from 0% to 90%[133] while the majority of studies show only a rather low number. Some studies only detected bone marrow-derived cells adjacent to vessels.[107, 181] Therefore it has been suggested that the angiogenic activity of EPC does not rely solely on their homing and engraftment but is related to their capacity to secrete growth factors, similar to the role of monocytes/macrophages.[146] This hypothesis is corroborated by the description that EPC are able to elaborate relevant growth factor and cytokines like VEGF, SDF-1, GM-CSF.[146] Furthermore, recent research added new evidence conceiving the central importance of paracrine actions of EPC in the modulation of several vascular functions.[182-184] When human EPC were injected into immunodeficient mice with myocardial infarction, the expression paracrine factors was shown to upregulate endogenous cytokines to stimulate the mobilization and recruitment of BM cells into the infarcted area, resulting in increased capillary density, higher proliferation of myocardial cells, a lower cardiomyocyte apoptosis and reduced infarct size.[184] Condition medium derived from EPC hyperpolarized human coronary artery smooth muscle cells via the secretion of Prostacyclin.[183] A gene analysis revealed that EPC exhibit a high expression of angiogenic growth factors including VEGF, SDF-1, and IGF-1. Neutralization of secreted VEGF and SDF-1 significantly reduced the effect of EPC conditioned medium on the migration of mature endothelial cells and tissue resident cardiac progenitor cells.[182] However, despite the fact that the tissue regenerative capacity driven by EPC-soluble factors is recognized[185, 186], the spectrum of paracrine effectors and their mechanism of action remain largely unexplored. Recently the characterization of multifaceted nature of the EPC secretome has been addressed[146, 147, 187, 188], but further investigations are needed to clarify the activation and the interactions of downstream signals.

1.4.7 Non-Bone-Marrow-Derived EPC

Apart from BM-derived EPC, other non-BM cell populations have been described to give rise to EC. The first evidence came from a study using transplanted grafts, where non-bone-marrow-derived cells replaced EC in transplant arteriosclerosis.[189] A variety of tissues were shown to contain resident stem cells that are capable of differentiating into the endothelial lineage. For example, $Lin^-/c\text{-}kit^+$ cardiac stem cells can give rise to myocytes, smooth muscle, and endothelial cells.[190] EPC existed in the wall of human embryonic aorta can differentiate into mature vascular endothelial cells and form vascular-like structures under *in vitro* condition.[191] Furthermore, EPC derived from peripheral organs such as the liver or spleen contribute to the pool of circulating EPC and therapeutic neovascularization.[192, 193]

Other studies revealed the presence of a complete hierarchy of EPC in the wall of human adult blood vessels.[194] Recently it was shown that human arteries and veins contain vascular wall-resident EPC in the niche located between smooth muscle and adventitial layers of the vascular wall termed vasculogenic zone.[195]

Another rich source of EPC is umbilical cord blood. Cord blood contains higher numbers of $CD133^+$ and $CD34^+$ HSC compared to peripheral blood from adults[155], and is able to differentiate into EC.[196] Cord-blood derived EPC show a higher proliferation capacity and express telomerase, a functional characteristic of stem cell.[155] Taken together, bone marrow, peripheral and cord blood, some organs like liver and intestine as well as the blood vessel wall serve as sources for various populations of EPC.

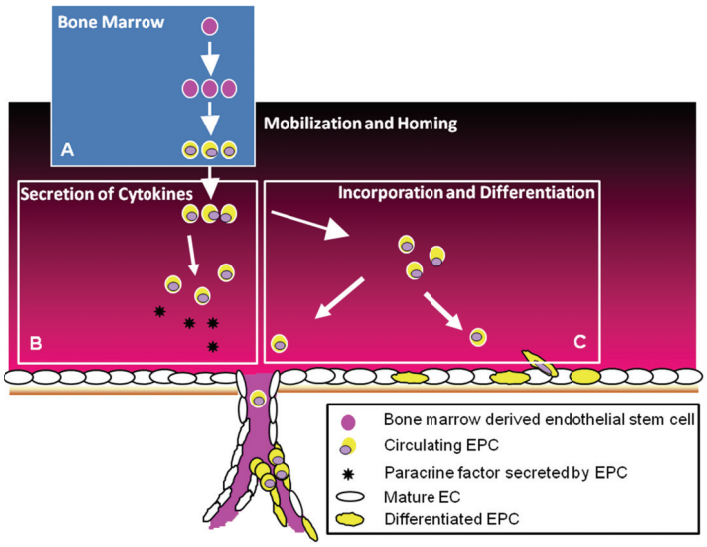

Figure 4. Mechanisms of EPC action on neovascularization. Circulating EPC derived from bone marrow (BM) mobilize into peripheral blood and home to the ischemic site **(A)**. These cells participate to neovascularization via **(B)** secretion of trophic factors, and **(C)** physical incorporation and differentiation into matured EC.

1.5 Apoptosis and Oxidative Stress

1.5.1 Apoptosis

In 1972, Kerr et al. observed in a variety of tissues the phenomenon of apoptosis that small and round membrane encapsulated bodies with condensed chromatin and intact organelles.[197] In contrast to normal necrosis, apoptosis is active, programmed and inherent. It play an essential role in development, cellular turnover and regulation of endocrine-dependent tissues.[198]

Apoptotic cells are characterized by nuclear condensation and fragmentation, cellular shrinkage and membrane blebbing.[198] Unlike necrotic cells which undergo destruction of cell membranes, Cell membranes in apoptotic cells remain intact. Apoptotic cells are cleared from the body through phagocytosis, leaving no trace or inflammation behind.[199]

1.5.2 Apoptosis Pathways

Apoptosis can be divided into two pathways. The extrinsic death receptor pathway can be activated through signals such as toxins, hormones, growth factors, nitric oxide or cytokines. The intrinsic mitochondrial pathway is activated in response to a stress including heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration.[200] Both pathways rely on series of events. For instance, apoptotic signals induce oligomerization of death adaptor proteins such as apoptotic protease activating factor (Apaf)-1 (intrinsic) and Fas-associated protein with death domain (FADD; extrinsic).[201] These complexes then trigger aggregation and autoproteolysis of pro-caspases: the apoptosome facilitates activation of caspase-9 (intrinsic), while caspase-8 is activated by the death-inducing signaling complex (DISC; extrinsic). Finally, these initiator caspases cleave and activate effort caspases (caspase-3, -6, and -7) to amplify the signal and execute cell death.[202, 203]

1.5.3 Caspase Family

One of the hallmarks of apoptosis is activation of a family of serine proteases: cysteinyl aspartate specific proteases (caspases). Caspases are synthesized as inactive pro-caspases. In response to apoptotic signals, caspases are activated by dual proteolytic cleavage which removes the prodomain and separates the large and the small subunit.[204] The activated caspases then cleave an array of cellular substrates and induce the characteristic changes of

apoptotic cell, including inactivation of enzymes involved in DNA repair, breakdown of structural nuclear proteins, and fragmentation of DNA.[205, 206] The family of caspases consists of 14 members and are grouped into three functional classes: inflammatory caspases including caspase-1, -4, -5, -11, -12, -13, and -14; apoptotic initiator caspases including caspase-2, -8, -9, and -10; and apoptotic effector caspases including caspase-3, -6, and -7 which can be activated by the initiator caspases.[199] Furthermore, the substrate proteins cleaved by caspases fall into four major categories: (1) apoptotic proteins such as Bcl-2 and Bid, (2) structural proteins to be degraded to change cells shape and detach cells from the ECM, (3) cellular DNA repair proteins and (4) cell cycle proteins such as p53.[199]

1.5.4 Bcl-2 Family

One of the most important regulators of most forms of apoptotic cell death is the Bcl-2 family which act immediately upstream of the mitochondria. They are characterized by clusters of conserved α-helices called Bcl-2 homology (BH).[207] Anti-apoptotic members (all contain BH domains 1 - 4), such as Bcl-2 and $BclX_L$, can form a hydrophobic pocket in which Bak and Bax are bound and sequestered.[208] Pro-apoptotic members including Bax and Bak (only contain BH1-3), are activated by BH3-only proteins (BOPs) and execute apoptosis.[209] Generally, the intracellular ratio of Bax/Bcl-2 protein have been described as a cellular "rheostat" of apoptosis sensitivity in the sense that it can profoundly influence the ability of a cell to respond to an apoptotic signal.[210, 211] According to this concept, a cell with a high Bax/Bcl-2 ratio will be more sensitive to a given apoptotic stimuli when compared to a similar cell type with a comparatively low Bax/Bcl-2 ratio.

1.5.5 Oxidative Stress Induced Apoptosis

Oxidative stress is a state in which excess reactive oxygen species (ROS) overwhelm endogenous antioxidant systems.[212] It plays a major role in the initiation and progression of cardiovascular dysfunction associated with diseases such as hyperlipidemia, diabetes mellitus, hypertension, and ischemia. One of the most important ROS in the vasculature is superoxide ($\cdot O_2^-$). It is formed by the univalent reduction of oxygen by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase (XO) and other enzyme systems.[213] Although $\cdot O_2^-$ is able to directly affect vascular function, it is also pivotal in generating other reactive species. Reaction of $\cdot O_2^-$ with $\cdot NO^{\cdot}$ can generate peroxynitrite (ONO_2^-), a powerful oxidant. Dismutation of $\cdot O_2^-$ by

superoxide dismutase (SOD) produces a more stable ROS, H_2O_2, which is then converted into harmless H_2O and O_2 by catalase and glutathione peroxidase (GPx; **Figure 5**). H_2O_2 can also be converted to the highly reactive hydroxyl radical (·OH) by reduced transition metals, or be metabolized to HOCl by myeloperoxidase (MPO).[212]

Many functions of the endothelium are affected by ROS. One of the most important is apoptosis. Direct treatment of cells with low doses of oxidants like $\cdot O_2^-$ or H_2O_2 can induce apoptosis of EC, which further leads to EC loss and results in atherogenesis and a procoagulative state.[214] Several groups have suggested that intracellular ROS generation may constitute a conserved apoptotic event and serve as a critical determinant of toxicity.[215] Correspondingly, many anti-apoptotic proteins like Bcl-2 have been accredited an antioxidant function[216, 217], again indicating that ROS generation may be a necessary apoptotic event.

EC apoptosis triggered by increased ROS levels can be inhibited by SOD and catalase.[212] SOD acts as an $\cdot O_2^-$ specific scavenger and is considered to be the first line of defense against oxygen toxicity. SODs exist in two forms in mammalian tissues: copper, zinc (Cu/ZnSOD), and manganese (MnSOD) metalloproteins. MnSOD is found mainly in mitochondria, while Cu/ZnSOD functions in the cytosol as well as in the extracellular space.[218] Interestingly, transfection of cells with MnSOD lacking its mitochondrial matrix signal does not provide protection against radiation. However, insertion of the MnSOD-derived mitochondrial signal sequence into Cu/ZnSOD results in significant radio protection. These data suggest that localization may partially compensate the differences between the SOD species.[219]

Catalase is another ROS scavenger which is located in the peroxisome. It is the major intracellular enzyme responsible for H_2O_2 catabolism.[220] The inverse relationship between the level of catalase and the susceptibility to apoptosis has been revealed by several studies.[221, 222] Catalase is very effective in high-level oxidative stress and protects cells from H_2O_2 produced within the cells or diffused outside the cells. For instance, addition of exogenous catalase usually provides antioxidant protection[223-225], despite that catalase applied to the culture medium cannot penetrate the cell membrane except on some occasions.[220] Conversely, treatment with the catalase inhibitor 3-amino-1,2,4-triazole increased the incidence of apoptosis.[222, 226] Catalase also has peroxidase activity and plays a significant role in the development of tolerance to oxidative stress in the adaptive response of cells.[227]

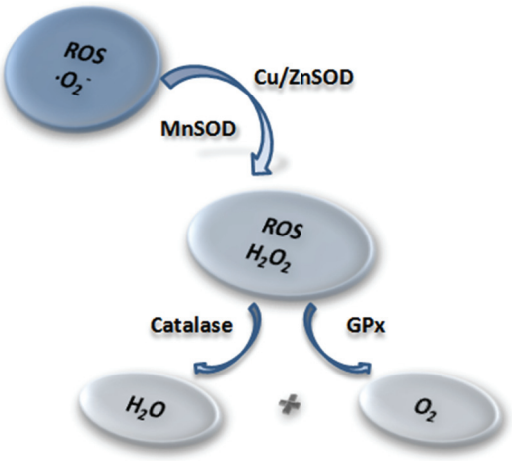

Figure 5. Reactive oxygen species (ROS) and superoxide dismutase (SOD). Dismutation of reactive oxygen free radical $\cdot O_2^-$ by superoxide dismutase (SOD) produces a more stable ROS, H_2O_2. Catalase and glutathione peroxidase (GPx) further convert H_2O_2 into harmless H_2O and O_2.

1.6 Aims of the Thesis

The major aims of this thesis are to:

- Develop a rat model of chronic stable hindlimb ischemia for studies of therapeutic neovascularization. (Experimental work and approaches show in **Paper I**).
- Evaluate the therapeutic effect of the paracrine factors secreted by EPC under *in vitro* culture. (Experimental work and approaches show in **Paper II**).
- Understand the role of paracrine factors in EPC mediated endothelial cell survival and activation. (Experimental work and approaches show in **Paper III** and **Paper IV**).
- Develop a novel "cell-free" strategy based on EPC paracrine factors. (Experimental work and approaches show in **Paper II**).

2. RESULTS

2.1 Paper I – Call for a reference model of chronic hindlimb ischemia to investigate therapeutic angiogenesis

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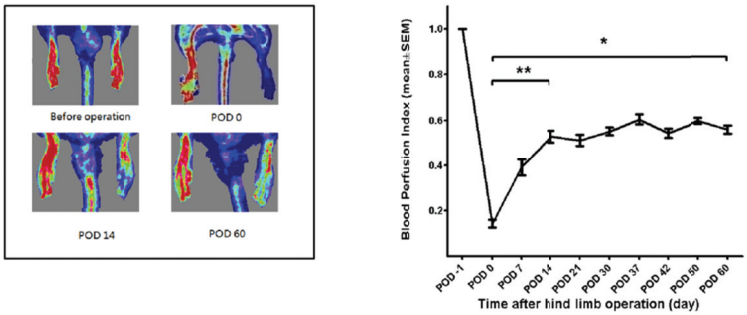
Despite the need of new instruments for understanding the pathogenetic mechanisms of PAD a standardized animal model is long missing. In this study we established a model of moderate ischemia in athymic rnu-rats to mimic chronic stable claudication in humans. Unilateral, hindlimb ischemia was created in rnu-rats by resection of the femoral artery and monitored for 60 days. Laser Doppler measurements of blood flow showed a rather stable perfusion reaching half of the normal perfusion of the non-operated limb. Histopathological assessment (H&E) of the hindlimb musculature on day 60 showed only mild atrophy and essentially no fibrosis or inflammatory cell infiltrate. The mitochondrial activity (MTT assay), physiological activity (swimming assay) and capillary-to-fiber ratio (immunofluorescent staining) of the muscular tissue were substantially reduced. Our data suggested that chronic, moderate hindlimb ischemia with a consistently decreased perfusion can be established in nude rats over a prolonged period and provides a useful, valid and highly reproducible model for studies of pro-angiogenic treatments.



Blood perfusion ratio obtained by laser Doppler imaging system. Laser Doppler imaging is a standard method for non-invasive assessment of the blood flow. Representative Doppler scans showed the level of perfusion before and immediately after surgery as well as on POD 14 and 60 of the study, respectively (**left**). After a significant decrease of perfusion on POD 0, the perfusion ratio increased to 52.9% on POD 14 and remained stable until the end of the observation period (**right**).

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Call for a reference model of chronic hind limb ischemia to investigate therapeutic angiogenesis

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ABSTRACT

A large number of studies utilize animal models to investigate therapeutic angiogenesis. However, the lack of a standardized experimental model leaves the comparison of different studies problematic. To establish a reference model of prolonged moderate tissue ischemia, we created unilateral hind limb ischemia in athymic rnu-rats by surgical excision of the femoral vessels. Blood flow of the limb was monitored for 60 days by laser Doppler imaging. Following a short postoperative period of substantially depressed perfusion, the animals showed a status of moderate hind limb ischemia from day 14 onwards. Thereafter, the perfusion remained at a constant level (55.5% of normal value) until the end of the observation period. Histopathological assessment of the ischemic musculature on postoperative days 28 and 60 showed essentially no inflammatory cell infiltrate or fibrosis. However, the mitochondrial activity and capillary-to-fiber ratio of the muscular tissue was reduced to 52.7% of normal, presenting with a significant weakness of the ischemic limb evidenced by a progressive decline in performance. Intramuscular injection of culture-expanded human endothelial progenitor cells (EPC) resulted in a significant increase in blood flow ($82.0 \pm 3.5\%$ of normal), capillary density (1.60 ± 0.08/muscle fiber) and smooth muscle covered arterioles (8.0 ± 0.6/high power field) in the ischemic hind limb as compared to controls ($55.0 \pm 3.1\%$; 0.99 ± 0.03; 5.0 ± 0.2). In conclusion, chronic, moderate hind limb ischemia with consistently reduced perfusion levels persisting over a prolonged period can be established reliably in rnu athymic nude rats and is responsive to pro-angiogenic treatments such as EPC transplantation. This study provides a detailed protocol of a highly reproducible reference model to test novel therapeutic options for limb ischemia.

1. Introduction

Atherosclerotic cardiovascular diseases have an increasing prevalence and are major causes of mortality and morbidity in the industrialized world (Miller et al., 2007). Despite substantial therapeutic advances in the field over the last decades, the overall clinical development for cardiovascular diseases is still unsatisfactory and the responsiveness of a relevant number of patients to current therapies remains insufficient (Dormandy et al., 1999; Simons et al., 2000). New insights into the complex process of postnatal vessel formation have encouraged the concept of therapeutic angiogenesis and vasculogenesis to overcome this lack of effectiveness in the treatment of cardiovascular diseases (Kalka and Baumgartner, 2008; Syed et al., 2004). Gene-, cell- and cytokine-based therapies are promising strategies to restore blood flow to ischemic tissues as previous studies have shown (Lachmann and Nikol, 2007; Losordo and Dimmeler, 2004a,b; Urbich and Dimmeler, 2004). In order to establish the effectiveness of such novel strategies and to translate in-vitro results into clinical applications, animal models mimicking the disease of interest are being deployed extensively. More specifically, the selection of an appropriate animal model is of major importance in this experimental workflow and may significantly affect the impact of study findings on the further translational process. In addition, the selection of tests performed to assess the development of ischemia and efficacy of therapeutic interventions is critical.

Despite the number of studies addressing new strategies to enhance neovascularization of the ischemic hind limb, an animal model of chronic hind limb ischemia serving as a reference model for therapeutic angiogenesis applications has not been described in detail in the literature to the best of our knowledge. Instead, multiple techniques have been applied to induce more or less profound ischemia that persists over various time frames (Barzelai et al., 2006; Lundberg et al., 2003; Prior et al., 2004). The comparison between different results is thus jeopardized by inconsistent experimental settings. Here, we present a detailed protocol to induce hind limb ischemia in

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athymic nude rats (rnu-rat) by simple vessel excision resulting in persistent but moderate ischemia over a prolonged time period. In addition, we assessed the utility of this model for exploring the therapeutic applications for hind limb ischemia. We suggest the adoption of this highly reproducible model as a reference for future investigations comparing different means of therapeutic angiogenesis including xenogenic cell transplantation.

2. Materials and methods

2.1. Animals

Four week-old male athymic rnu-rats (NIH-Foxn1^{rnu}) were purchased from Charles River Laboratory Inc (Sulzfeld, Germany). The rats were acclimated in our animal facility for 4 weeks prior to the experiments and had unrestricted access to food and water. Anesthesia was obtained using 0.2% isoflurane with oxygen at 2 L/min and was continued during all surgical operations and blood perfusion imaging. All procedures were approved by the Cantonal Ethics Review Board and conducted in accordance with the institutional policies for animal experiments.

2.2. Study design

Eighteen rats were operated at the age of 8 weeks and then examined and monitored over a period of 60 days. Postoperatively, laser Doppler perfusion imaging was acquired at weekly intervals for the full duration of the study. The hind limb functional capacity was assessed at the end of the study protocol by use of a forced swimming test. Animals were sacrificed on postoperative day (POD) 60 for further histopathological analysis to assess the hind limb tissue. For EPC transplantation experiment, eight rats were divided into two groups and received either EPC or control medium administration starting from POD 28.

2.3. Experimental model of chronic, moderate hind limb ischemia

After shaving and disinfection, a 1 cm longitudinal skin incision was made in the groin. The femoral triangle was explored and the major vessels identified and dissected. Prolene 6-0 sutures (Ethicon, Germany) were used for the ligation prior to excision of the arterial vessel bed between the distal end of the external iliac artery (right below the branching of the circumflexa ilium superficialis) and the trifurcation of the femoral artery into the descending genicular, popliteal, and saphenous branches including concomitant profunda femoral and superficial epigastric branches. A schematic diagram of the vessels ligated is illustrated in Fig. 1. The venous structures were disrupted likewise (Goldstein et al., 2006; Goto et al., 2006; Murohara et al., 2000; Westvik et al., 2009). Finally, the overlying skin was closed with a 4-0 vicryl and disinfected. At the end of the procedure, Buprenorphinum (0.1 mg/kg) and Terramycin (60 mg/kg) were injected subcutaneously.

2.4. Laser Doppler perfusion imaging

Blood flow was measured using a laser Doppler imager (Moor, Axminster, UK) and analyzed with the MoorLDI™ Image Review V51 software. The animals were placed on a heating pad in order to maintain constant body temperature during the entire procedure. Two consecutive measurements were made in each rat. The averaged values were then used for the statistical analysis. To achieve maximal experimental uniformity and reduce bias due to ambient temperature or individual basal perfusion rates, the final results were expressed and compared as the ratio of ischemic to normal hind limb.

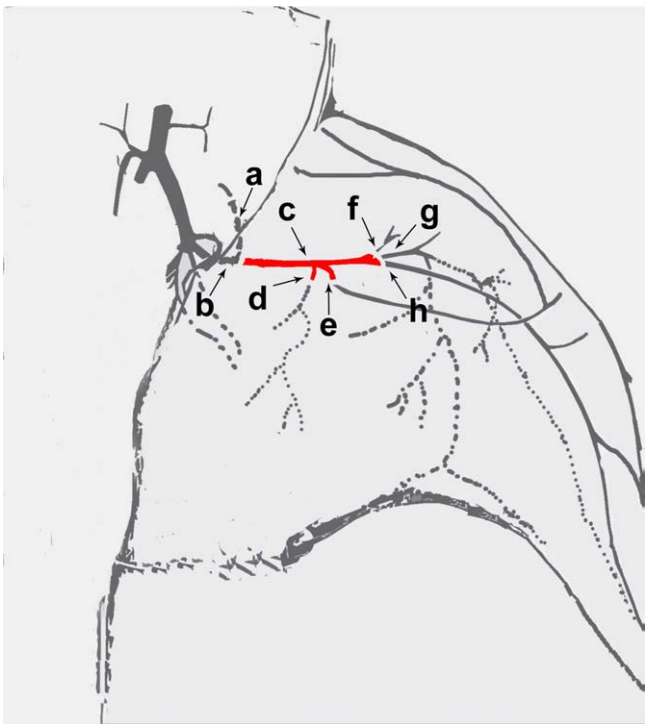

Fig. 1. Schematic diagram illustrating the occlusion sites for unilateral hind limb ischemia. a, Superficial circumflex iliac artery; b, external iliac artery; c, femoral artery; d, profunda femoral artery; e, superficial epigastric artery; f, descending genicular branch; g, popliteal artery; h, saphenous artery. The area highlighted in red indicates the total resected segment. The venous structures were disrupted likewise. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Assessment of hind limb functional capacity

We adopted a modified forced swimming test to estimate the functional capacity of the hind limb under chronic ischemic conditions (Sasaki et al., 2006). For this purpose, operated and non-operated, age-matched healthy control rats ($n=5$) were placed in a water-filled tank at 23 °C to asses their swimming performance at the end of the study (POD 60). The entire test was videotaped to count the number of strokes per minute of each limb individually during 3 consecutive measurement periods (0–1 min, 1–2 min, and 2–3 min). Also, we evaluated the duration of normal swimming capacity defined as the time before occurrence of obvious exhaustion (sparse hind limb activity and near-drowning compared to normal swimming and diving). The ischemic hind limb muscular functional capacity was expressed as the ratio of the ischemic to normal number of hind limb strokes.

2.6. Histology analysis

Mitochondrial activity as a marker of muscular viability was assessed by the MTT reduction test. Briefly, the insoluble formazan salt formed in the gastrocnemius by MTT incubation was extracted in 2-propanol and quantified using a micro-plate reader (Safire Tecan, Switzerland). The results are given as a viability index, which was obtained by calculating the amount of absorbance per gram of dry muscle tissue in accordance with previously described methods (Bonheur et al., 2004; Martou et al., 2006). Gastrocnemius and adductor muscles of the ischemic and contralateral hind limb were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Ten μm thick slices were incubated with hematoxylin and eosin or van Gieson's stain for subsequent histomorphological analysis of tissue integrity and collagen fiber content. Additional slides were stained

with FITC labeled lectin from *Bandeiraea simplicifolia* (BS-1, Sigma-Aldrich, Germany) or with rabbit anti-von Willebrand factor (vWF; AB7356, Chemicon, USA) to determine capillary density. Mouse monoclonal anti-α-smooth muscle actin antibody (α-SMA, Sigma-Aldrich, Germany) was used to detect arterioles in adjacent slides. The number of capillaries and arterioles in each section was counted automatically in 5 random fields by the use of ImageJ software (http://www.rsbweb.nih.gov/ij/). The capillary density was counted in 40× high power fields (HPF). A total of 5 slides taken at 1 mm intervals were assessed from each rat hind limb.

2.7. EPC isolation, culture and transplantation

Human peripheral venous blood samples were obtained from healthy, young volunteers with informed consent. The mononuclear cell population was isolated by gradient centrifugation and cultured for 7 days in complete endothelial cell growth medium (EGM-2-MV, Lonza, Switzerland) containing 5% fetal bovine serum (FBS) to obtain EPC in accordance with previously published method (Kalka et al., 2000).

EPC resuspended in EBM-2 medium (Lonza, Switzerland) without growth factors and FBS were administered four weeks following arterial occlusion by three serial intramuscular injections within 7 days (POD 28, POD 31, POD 34). Each time 1×10^6 EPC or control medium was injected at 5 sites into the ischemic hind limb distal to the arterial occlusion site.

2.8. Statistical analysis

Data are summarized with means ± SEM unless otherwise stated. Proportions were compared by the use of Pearson`s X2-Test and Fisher's exact test, respectively, applying the Bonferroni correction for repetitive testing. Friedman's ANOVA and post-hoc Dunn's test was performed to compare the laser Doppler measurements of limb perfusion at different time points. Differences in the swimming test result between non-operated and operated rats were compared by the Mann-Whitney *U* test. All other quantitative data were compared (operated vs. non-operated hind limb) by means of the Wilcoxon signed rank test. Statistical significance was inferred at a 2-sided value of $P<.05$. Statistics were carried out using the Statistical Package for Social Sciences (SPSS), version 16.

3. Results

3.1. Surgical outcome

Immediately after surgery, all animals developed limb edema and skin paleness which gradually resolved during the first postoperative week. None of the rats showed signs of severe pain, limping, toe necrosis or any other serious complication attributable to the procedure or its impact on hind limb perfusion throughout the duration of the study.

3.2. Creation of chronic hind limb ischemia

As expected, perfusion of the ischemic hind limb was at a minimal level directly after surgery. On the day of surgery (POD 0), the average ischemic to non-ischemic hind limb ratio was at 14.4 ± 0.2% of the pre-operative value ($P<.001$). In the following days, the perfusion ratio increased rapidly to 39.2 ± 0.4% on POD 7 ($P<.001$) and 52.9 ± 0.3% of normal values on POD 14 ($P<.01$). Subsequently, no significant changes in hind limb perfusion were noticed and the level of perfusion remained stable for the remainder of the study. Between POD 14 and POD 60 the average perfusion ratio of the ischemic hind limb ranged between 52.9 ± 0.3% (POD 14) and 55.5 ± 0.2% (POD 60 = end of study; Fig. 2).

3.3. Persistent functional deficiency of the ischemic hind limb

In order to gauge the extent of ischemia imposed on the operated hind limbs by the reduced perfusion level in vivo, we subjected the animals to a forced swimming test. Whereas control rats had a very uniform ratio (left to right hind limb) of strokes per minute, the ratio was significantly decreased in operated rats (Fig. 3). In the first assessment period (minute 0–1) the ratio was 0.83 (vs. 1.03; $P<.01$) and further dwindled to 0.67 (vs. 0.96; $P<.01$) in the second measurement period (minute 1–2). Furthermore, the majority of operated rats showed obvious exhaustion towards the end of second minute and these animals were not able to finish the protocol of the swimming test as like the controls. This finding suggests that ischemia caused a substantial reduction in the overall endurance in the operated animals.

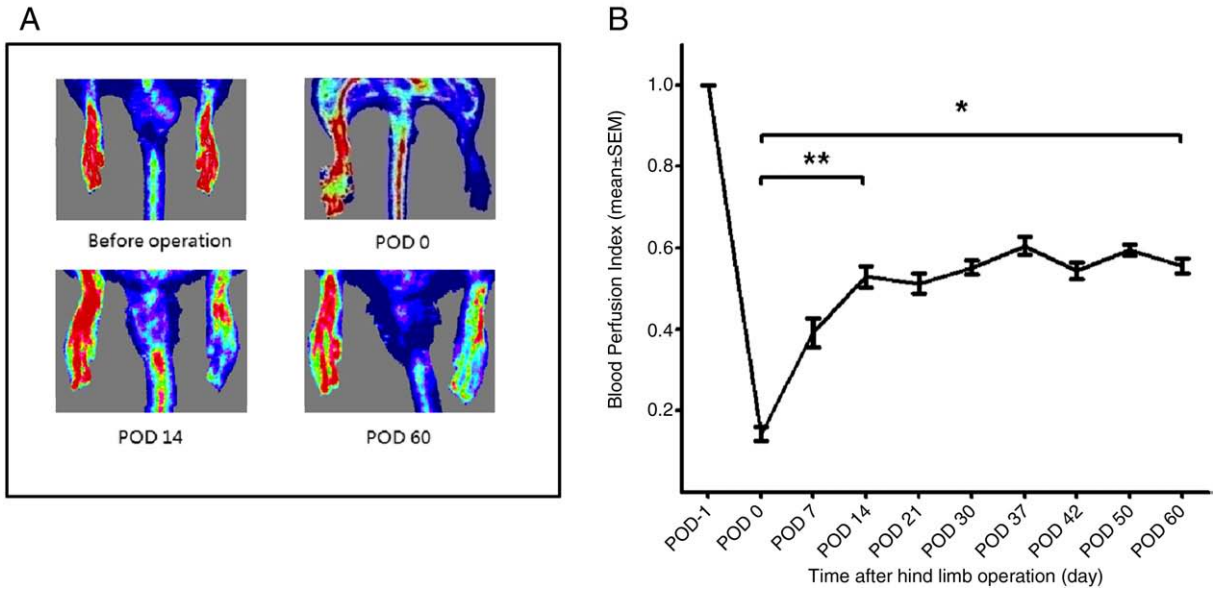

Fig. 2. Blood perfusion ratio obtained by laser Doppler Imaging system. (A) Representative Doppler scans showing the level of perfusion before and immediately after surgery as well as on POD 14 and 60 of the study, respectively. (B) After a significant decrease of perfusion on POD 0, the perfusion ratio increased to 52.9% on POD 14 ($P<.01$; **) and remained stable until the end of the observation period (55.5% on POD 60; $P<.01$; *).

3.4. Prolonged moderate ischemia results in reduced vascularization but not in inflammation or fibrosis

We observed a significant reduction in the capillary network of the chronically underperfused hind limb (Fig. 4). The number of BS-1 lectin-stained capillaries per muscle fiber was reduced greatly (0.96 ± 0.03) compared to the non-ischemic hind limb (1.82 ± 0.06; $P<.0001$). This corresponds to a relative decrease to 52.7% of the capillary numbers in non-ischemic limb. Independent sections stained with anti-vWF antibody confirmed this significant reduction of capillaries in ischemic limb (data not shown). The number of arterioles enveloped with SMA positive perivascular cells, was likewise reduced (4.92 ± 0.19/HPF vs. 8.77 ± 0.59/HPF; $P<.01$; Fig. 5). This reduction in capillary and arterioles density was accompanied by mitochondrial dysfunction in the muscles. The normalized value of the MTT test was clearly lower in the ischemic hind limb (0.59 ± 0.08 vs. 1.00 ± 0.11; $P<.05$) reflecting the depletion of cellular energy sources that occurred in the ischemic musculature (Fig. 6). However, histological analysis of the ischemic muscle revealed no clear evidence of fibrosis, edema, or inflammatory cell infiltration by hematoxylin and eosin or van Gieson's stain (data not shown) on day 28 and 60 after induction of ischemia.

3.5. Intramuscular EPC injection results in increased hind limb perfusion and vascularization

In order to further validate this model and its practical accuracy we decided to perform a pro-angiogenic intervention and study its impact on hind limb ischemia according to previously described techniques (Kalka et al., 2000). Indeed, rats treated with EPC showed a significant improvement in blood flow as early as one week after the last injection. Moreover, perfusion gradually increased to a level of 82.0 ± 3.5% (EPC) after five weeks. In contrast, in the control medium-treated animals blood flow remained constant throughout the study around 50% of that measured in the contralateral, non-ischemic limb (55.0 ± 3.1% at five weeks after treatment; $P<.001$; Fig. 7 A).

The increased blood flow in the EPC-treated ischemic hind limb was associated with a significant increase in capillary density (1.60 ± 0.08 vs. 0.99 ± 0.03 / muscle fiber, Fig. 7 B) and arteriole density (8.0 ± 0.6 vs. 5.0 ± 0.2/ HPF; Fig. 7 C) in comparison control medium-treated hind limbs (both $P<.001$).

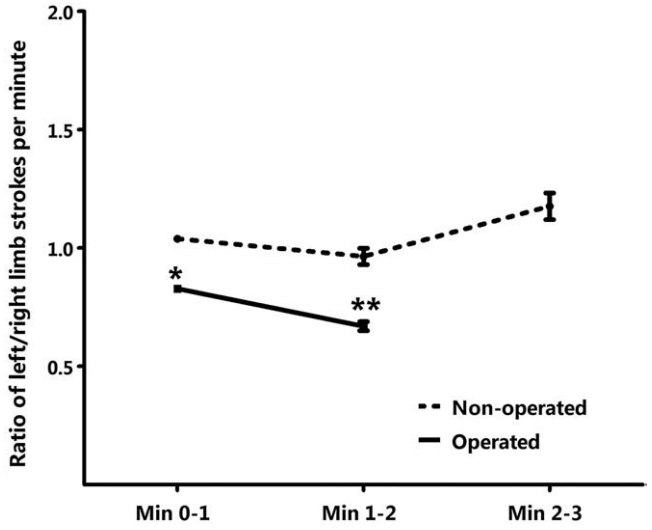

Fig. 3. Hind limb activity assessed by swimming performance. The muscular functional capacity was shown as the ratio of hind limb strokes (left ischemic/right non-ischemic) in operated ischemic group (lower curve) and non-operated healthy control group (upper curve) on POD 60. The non-operated group maintained an overall 1:1 ratio of left to right limb strokes during the 3 min, while the ratio was 0.83 (left/right) in operated animals during the first assessment period (minute 0–1; $P<.01$; *) and further decreased to 0.67 the second assessment period (minute 2–3; $P<.01$; **). The test was terminated in the operated group after 3 min due to obvious exhaustion observed in most of the animals.

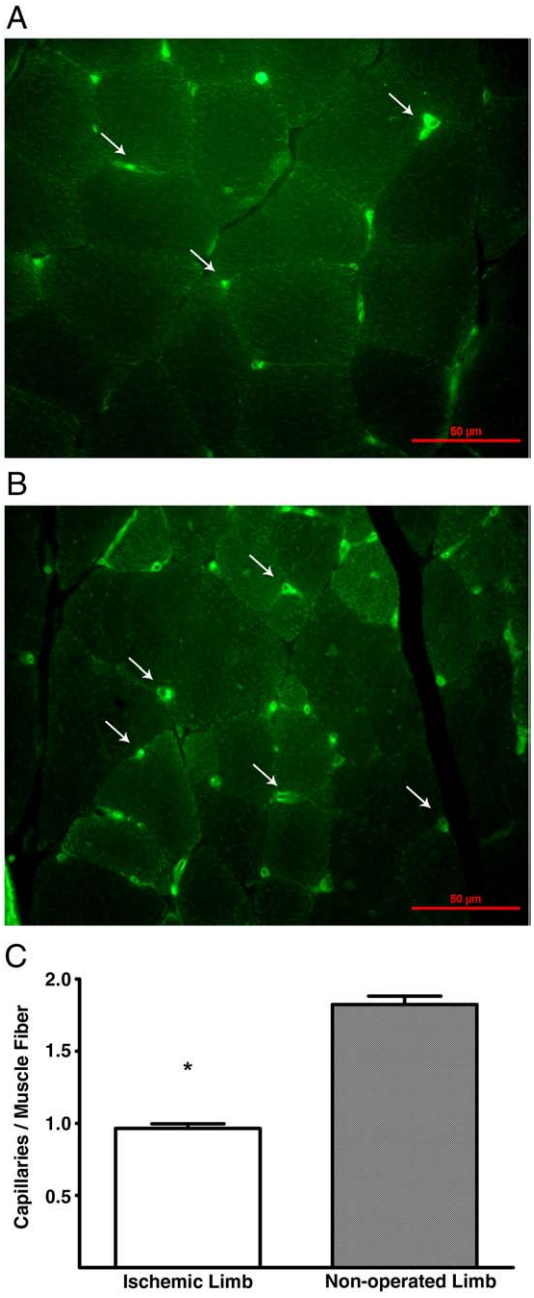

Fig. 4. Decreased capillary density in ischemic tissue. Capillaries were determined by BS-1 lectin staining (green) in ischemic (A) and non-ischemic sample (B). The mean capillary density of ischemic samples (0.96 ± 0.03) significantly decreased compared to the non-ischemic samples (C; 1.82 ± 0.06; $P<.0001$; *). The scale bar represents 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

To date, most studies evaluate in vivo angiogenic therapies in animal models immediately after induction of hind limb ischemia and are limited to a short observation period of approximately 30 days after surgery (Aicher et al., 2006; de Nigris et al., 2007; Duan et al., 2006; Finney et al., 2006; Jiang et al., 2008; Urbich et al., 2005). However, those studies of therapeutic angiogenesis committed to the early postoperative phase are potentially distorted by dramatic endogenous compensation mechanisms in response to acute ischemia

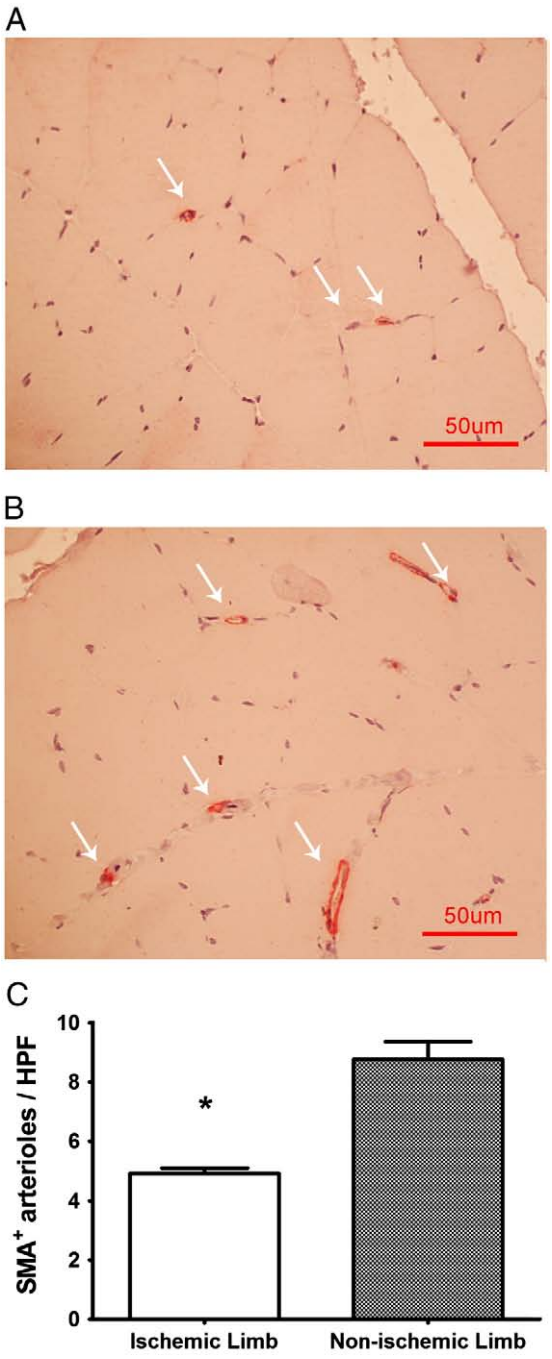

Fig. 5. Smooth muscle covered arterioles in ischemic musculature. Arterioles identified by SMA^+ perivascular cells (red) in ischemic (A) and non-ischemic musculature (B). Their density decreased from 8.77 ± 0.59 / HPF preoperatively to 4.92 ± 0.19 / HPF on POD 60 (C; $P<.01$; *). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which involve triggering of inflammation and induction of vascular remodeling by arteriogenesis and angiogenesis (Couffinhal et al., 1998; Ito et al., 1997; Lloyd et al., 2001; Shireman et al., 2006; Shireman and Quinones, 2005; Tang et al., 2005). Furthermore, this acute phase of ischemia is associated with considerable tissue necrosis, altered shear stress, changes in gene expression and the recruitment of endothelial progenitor cells (Garcia-Cardena et al., 2001; Prior et al., 2004; Topper and Gimbrone, 1999). It is conceivable, that study protocols, which benefit from the profound ischemia occurring immediately after surgery, are subjected to quite ambiguous confounding factors. Therefore, it is reasonable to speculate that the

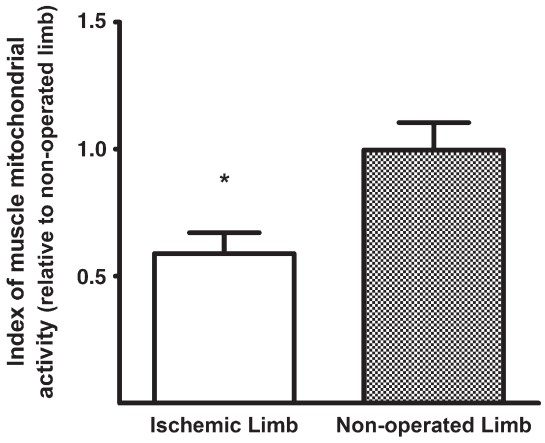

Fig. 6. Decreased muscle viability on POD 60. Muscle mitochondrial activity was assessed by quantification of MTT absorbance per gram of dry muscle sample weight. On POD 60, the normalized muscle viability index was clearly lower in the ischemic hind limb compared to the non-ischemic hind limb (0.59 ± 0.08 vs. 1.00 ± 0.1; $P<.05$).

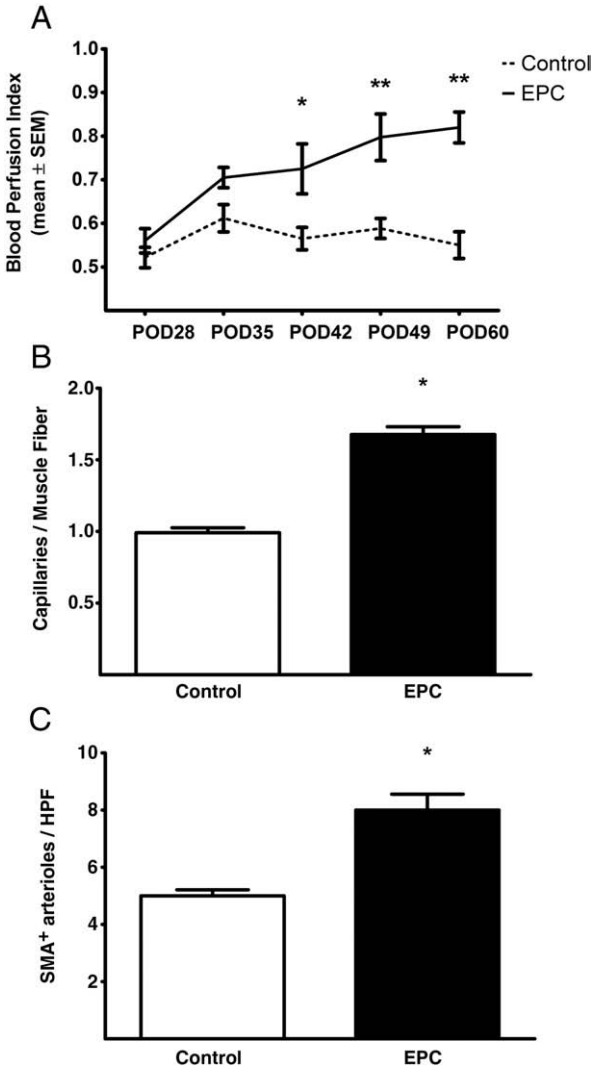

Fig. 7. EPC transplantation improves hind limb perfusion and neovascularization. (A) Quantitative analysis of blood flow expressed as perfusion ratio of the ischemic to the contralateral (non-operated) hind limb upon treatment (iDAY1: first day of EPC injection; iDAY7; iDAY14; iDAY21; iDAY28 and iDAY35 correspond to week 1, 2, 3, 4 and 5 after injection, respectively) *, $P<.01$; **, $P<.001$. (B) Quantitative analysis of capillary density expressed as number of capillaries per muscle fiber. (C) Quantitative analysis of α-SMA^+ vessels per high power field (HPF). *, $P<.001$.

outcomes of experimental therapeutic interventions carried out during the phase of acute ischemia may be significantly altered.

In this study, we present a moderate but consistent hind limb ischemia on athymic rnu-rats by surgical occlusion and excision of femoral vessels, providing a solid reference to increase the reliability and accurateness of comparisons and evaluations of future therapeutic angiogenesis studies. This model does not involve the development of muscle necrosis, fibrosis or inflammation. Also, we document the persistence of ischemia at stable perfusion levels in a chronic animal model that extends well beyond the timeframe used previously. The significant and lasting impairment in the limb blood perfusion along with the decrease of function observed in the animals suggest the validity and practicability of this model.

Two major findings of our study may be of particular importance for investigators using this model for the examination of therapeutic interventions. In accordance with previously published data (Couffinhal et al., 1998; Tang et al., 2005) we observed a significant decrease of blood flow initially, followed by a rapid recovery from ischemia within the first 14 postoperative days. The perfusion of the hind limb reached a moderately depressed level after POD 14 and remained at a constant level for the rest of the study protocol. These data suggest that the instable, acute ischemia phase with a dramatic endogenous compensation process gradually stabilizes after the first 14 days after surgery and reaches a consistent level at POD 28. Thus, we suggest that experiments investigating new strategies aimed to enhance neovascularization and to improve perfusion of chronic ischemic tissue preferably should be carried out in the time period beyond POD 28. This persistent blood flow reduction is in contrast with the progressive flow recovery reported in most of the studies employing model of hind limb ischemia in immuno-competent rats (Enomoto et al., 2006; Lundberg et al., 2003).

The lack of fibrosis and tissues necrosis in the hypoperfused limbs after 4 weeks and even after 9 weeks of ischemia is the other important discovery. It confirms the results of other investigators which suggest that consistent but moderate ischemia in immune-competent rats is likely to be associated with less severe inflammation and necrosis than acute, severe ischemia (Brown et al., 2003; Milkiewicz et al., 2006; Tang et al., 2005). Nonetheless in our model, vascularization and muscle mitochondrial function of the limbs are significantly decreased and the hind limb performance is substantially depressed as shown at the end of our observation period (POD 60). It is reasonable to speculate that the underlying cause of the mitigated development of inflammation, necrosis, and recovery of blood flow and function in the ischemic hind limb relies on the peculiar genetic background of the rnu-nude rats as different pulmonary vascular remodeling capacity has been reported in nude rats compared to euthimic rats (Taraseviciene-Stewart et al., 2007). Eventually, our findings suggest that the combination of the rat strain and the surgical procedure employed in the present study are ideal for investigations of therapeutic angiogenesis.

The efficacy and efficiency of the presented model for studies on therapeutic angiogenesis are illustrated by the Endothelial Progenitor Cells experiments. Intramuscular administration of human peripheral blood-derived EPC significantly promoted revascularization and restoration of peripheral blood flow in the ischemic limbs. These results indicate that the sustained reduction in perfusion and muscle functionality leaves room for therapeutic interventions. More importantly, the investigation of the therapeutic effect of EPC transplantation is not confounded by factors such as inflammation. Thus, we believe this model provides a valuable and stable disease state of moderate ischemia suitable to investigate strategies pursuing the enhancement of neovascularization and functional activity in ischemic tissue.

A limitation of this study important to bear in mind is that the characterization of the acute ischemic period was limited to perfusion measurements by laser Doppler imaging. Since our focus of interest was the investigation of the consequences of prolonged ischemia in the hind limb, we decided to dismiss further analyses of the early changes after induction of ischemia (i.e. between POD 0 to POD 14) as these changes have already been described and discussed in detail before (Couffinhal et al., 1998; Ito et al., 1997; Tang et al., 2005).

5. Conclusions

We present a highly reproducible reference model of moderate chronic hind limb ischemia on athymic rnu-rat as well as a validated experimental protocol for the assessment of the efficacy of different therapeutic strategies aimed to induce neovascularization of the ischemic muscle. Utilizing an immunodeficient animal, simple surgical techniques and accessible experimental methods this model of chronic ischemia will allow standardized therapeutic angiogenesis studies by a broad scientific community.

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2.2 Paper II – Novel cell-free strategy for therapeutic angiogenesis: *In vitro* generated conditioned medium can replace progenitor cell transplantation

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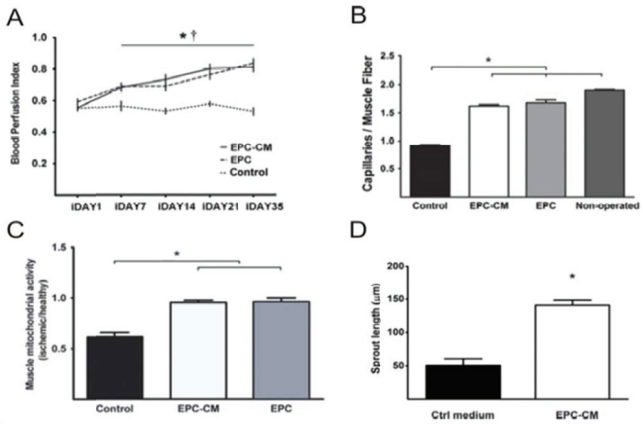
Current evidences suggest that endothelial progenitor cells (EPC) contribute to the complex process of angiogenesis in ischemic tissue by both secretion of paracrine factors and incorporation into developing vessels. We hypothesized that administration of these paracrine factors secreted by cultured EPC alone may be sufficient to achieve a therapeutic angiogenic response *in vivo*. To investigate the benefit of intramuscular injection of EPC-CM, we applied the rat model of chronic hindlimb ischemia. Our data indicated that hindlimb ischemia is highly responsive to conditioned medium. Administration of EPC derived soluble factors alone resulted in equal improvement of hindlimb function by revascularization as cell transplantation. Importantly, EPC-CM could effectively enhance the number of progenitor cells in the bone marrow; promote mobilization into the peripheral blood and their homing to the ischemic limbs, indicating that EPC-CM may promote the endogenous repair systems by both local and systemic actions. Our observations suggested a novel, cell-free treatment for peripheral vascular occlusive disease.



Therapeutic effect of EPC-CM and EPC therapy. Transplantation of EPC-CM and EPC in the ischemic hindlimb promoted significant increase of blood perfusion (Laser Doppler Imaging, **A**) and neovascularization (immunofluorescent staining, **B**). Muscle physiological integrity documented by biochemical activity was restored by treatment with EPC and EPC-CM (MTT assay, **C**). In vitro, EPC-CM pro-angiogenic activity was evidenced by enhanced vascular sprouting in ex-vivo aortic ring assay (Aortic ring assay, **D**).

Novel Cell-Free Strategy for Therapeutic Angiogenesis: *In Vitro* Generated Conditioned Medium Can Replace Progenitor Cell Transplantation

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Abstract

Background: Current evidence suggests that endothelial progenitor cells (EPC) contribute to ischemic tissue repair by both secretion of paracrine factors and incorporation into developing vessels. We tested the hypothesis that cell-free administration of paracrine factors secreted by cultured EPC may achieve an angiogenic effect equivalent to cell therapy.

Methodology/Principal Findings: EPC-derived conditioned medium (EPC-CM) was obtained from culture expanded EPC subjected to 72 hours of hypoxia. *In vitro*, EPC-CM significantly inhibited apoptosis of mature endothelial cells and promoted angiogenesis in a rat aortic ring assay. The therapeutic potential of EPC-CM as compared to EPC transplantation was evaluated in a rat model of chronic hindlimb ischemia. Serial intramuscular injections of EPC-CM and EPC both significantly increased hindlimb blood flow assessed by laser Doppler (81.2±2.9% and 83.7±3.0% vs. 53.5±2.4% of normal, $P<0.01$) and improved muscle performance. A significantly increased capillary density (1.62±0.03 and 1.68±0.05/muscle fiber, $P<0.05$), enhanced vascular maturation (8.6±0.3 and 8.1±0.4/HPF, $P<0.05$) and muscle viability corroborated the findings of improved hindlimb perfusion and muscle function. Furthermore, EPC-CM transplantation stimulated the mobilization of bone marrow (BM)-derived EPC compared to control (678.7±44.1 vs. 340.0±29.1 $CD34^+/CD45^-$ cells/1×10^5 mononuclear cells, $P<0.05$) and their recruitment to the ischemic muscles (5.9±0.7 vs. 2.6±0.4 $CD34^+$ cells/HPF, $P<0.001$) 3 days after the last injection.

Conclusions/Significance: Intramuscular injection of EPC-CM is as effective as cell transplantation for promoting tissue revascularization and functional recovery. Owing to the technical and practical limitations of cell therapy, cell free conditioned media may represent a potent alternative for therapeutic angiogenesis in ischemic cardiovascular diseases.

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Introduction

Cell-based revascularization therapies have recently been tested in clinical trials investigating the therapeutic benefits in patients that suffer from ischemic cardiovascular diseases [1,2]. Most of these studies used autologous cell transplantation given the concern of immune-system reactions. Distinct progenitor and stem cell lines have been described for their outstanding potential to promote tissue revascularization and functional recovery of the affected organ. Thus, a variety of progenitor and stem cell types, isolated from bone marrow and peripheral blood, have been used in patients with myocardial infarction, heart failure and peripheral vascular disease [3]. However, technical and practical limitations due to the invasive methods of harvest and low abundance may hinder the adoption of progenitor cells in clinical applications.

Two predominant mechanisms by which progenitor cells like endothelial progenitor cells (EPC) contribute to postnatal neovascularization have been identified so far [4–7]. *In vivo* animal studies demonstrated that EPC contribute to vessel formation by differentiation into mature endothelial cells and incorporation into the growing vessel wall [8,9]. However, this mechanism seems to play only a marginal role [10–13]. Secondly, circulating EPC isolated from peripheral blood have been shown to release a number of proangiogenic factors [5,14]. As a matter of fact, conditioned medium obtained from EPC cultures contains various proangiogenic growth factors and may therefore support the repair and re-endothelization of injured vessels and thus the regeneration of ischemic tissues [15–17]. We hypothesized that the regenerative potential of paracrine factors secreted by EPC may represent a potent alternative to progenitor cell therapy.

Table 1. Concentration of selected angiogenic growth factors in EPC-CM.

Cytokine/Growth factor	Concentration (pg/ml)	
	Hypoxia	Normoxia
IL-8/CXCL8	29090.7±12279.4	2282.1±406.3
SDF-1/CXCL12	6059.9±654.6	3179.9±488.0
HGF	539.5±141.7	343.4±74.8
Angiogenin	144.6±68.2	72.5±15.8
PDGF-BB	111.6±27.02	19.9±2.2
VEGF-A	25.5±4.8	11.4±5.2

Selected cytokine levels were measured in the conditioned media from culture expanded EPC incubated in hypoxic or normoxic condition for 72 hours.
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Results

Secretion of growth factors by EPC is increased by hypoxia

We have measured the release of different growth factors to determine to what extent hypoxia enhances the paracrine activity of EPC. Indeed, hypoxia (1.5% O_2) induced a significant increase in accumulation of selected factors like Angiogenin, HGF, IL-8, PDGF-BB, SDF-1 and VEGF-A in the EPC conditioned media compared to normoxia ($P<0.05$; Table 1). This effect was due to an augmented secretion since the overall EPC number was not significantly influenced by the oxygen level during culture (data not shown). Therefore, the growth factor enriched EPC-CM from hypoxic cultures was used for subsequent experiments.

EPC-CM enhances endothelial cell-viability *in vitro*

The capacity of EPC-CM to support the viability of nutrient depleted HUVEC was assessed by an assay for survival and for apoptosis. Incubation of EPC-CM resulted in a 45.4±7.0% increase of viable cells compared to control medium ($P<0.001$; Figure 1A). In contrast, the caspase −3/7 activity was reduced to 52.3±2.3% in HUVEC incubated with EPC-CM compared to control medium ($P<0.001$; Figure 1B).

EPC-CM increases vascular sprouting

The *ex vivo* aortic ring assay is commonly used to study the outgrowth of endothelial and surrounding perivascular cells and their organization in tubular, vessel-like structures. EPC-CM showed a substantially higher angiogenic potential to stimulate vessel outgrowth from the aortic ring in comparison to control medium. This was evidenced by a significantly wider (50.69±9.41 vs. 140.90±7.41 μm, $P<0.001$) and clearly denser network of vascular sprouts arising from the aorta (Figure 2).

EPC-CM and EPC transplantation both improve hindlimb perfusion and muscle function

The animal experimental design is illustrated in Figure 3. In the animals receiving control medium, blood flow remained constant throughout the study around 50% of that measured in the non-ischemic limb (53.5±2.4% at five weeks after treatment). In contrast, the rats treated with EPC-CM or EPC showed a significant improvement in blood flow already by one week after the last injection ($P<0.01$). Subsequently, blood flow increased gradually to a level of 81.2±2.9% (EPC-CM) and 83.7±3.0% (EPC) after five weeks (P = n.s. between the two treated groups; Figure 4A, B).

The improved flow recovery in the ischemic hindlimb was associated with a clear restoration of muscle function. Rats treated with control media had severely limited muscle activity with a stroke ratio decreasing from 0.83±0.02 to 0.67±0.02 within the first two minutes of swimming ($P<0.05$). The exercise performance deteriorated further until they were unable to swim in the third minute due to obvious exhaustion (near-drowning). In comparison, EPC-CM and EPC treated animals had a significantly better muscle function as evidenced by a stable hindlimb stroke ratio throughout the exercise (EPC-CM, 0–1 min: 0.89±0.02; 1–2 min: 0.85±0.02; 2–3 min: 0.83±0.03 and EPC, 0–1 min: 0.86±0.01; 1–2 min: 0.82±0.04, 2–3 min: 0.81±0.08). The group of non-operated, healthy control animals demonstrated a uniform one to one stroke ratio during the entire exercise protocol (Figure 5A).

Consistent with functional improvement, viability of the ischemic muscle in control medium treated animals was down to 61.4±4.3% of the healthy hindlimb value (Figure 5B), while in the EPC-CM and EPC group the viability was restored to 95.3±2.2% and 95.9±4.0% of healthy muscle (both $P<0.05$).

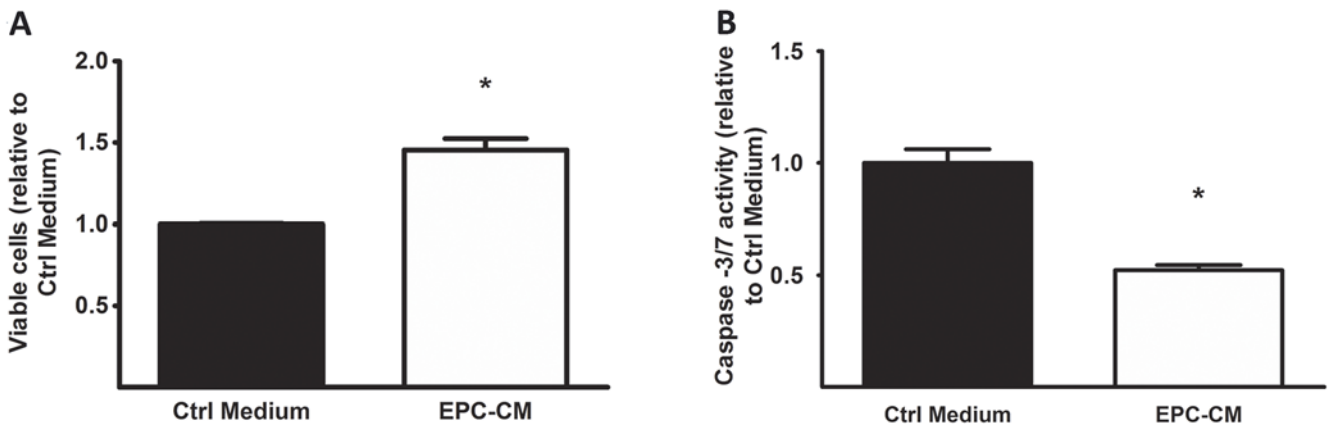

Figure 1. Pro-survival properties of EPC-CM. Serum starved HUVEC were incubated in EPC-CM or control medium for 24 hrs and analyzed for cell survival and extent of apoptosis. **(A)** The number of viable cells was assessed by CyQuant® NF and expressed relative to control. **(B)** Apoptosis was measured by the level of caspase −3/7 activity by Apo-ONE® and expressed relative to control. *, $P<0.001$.
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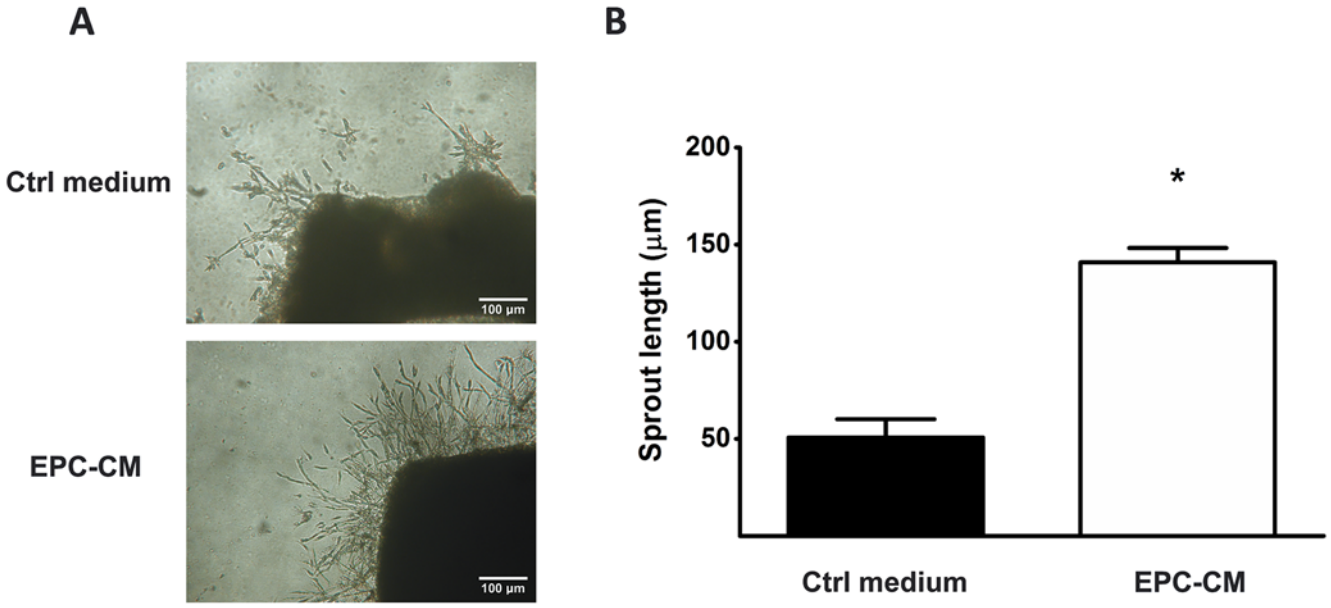

Figure 2. Angiogenic potential of EPC-CM. (A) Representative pictures of vascular outgrowth from 1 mm rat aortic ring embedded in growth factor reduced-Matrigel™ and incubated with EPC-CM or control medium. Incubation with EPC-CM enhanced the formation of capillary outgrowth compared to control medium. **(B)** Quantitative analysis of sprout length induced by incubation with control medium and EPC-CM. *, $P<0.001$.
doi:10.1371/journal.pone.0005643.g002

EPC-CM and EPC transplantation equally induce neovascularization and vascular maturation

Five weeks after treatment, the number of capillaries in hindlimbs treated with control media was 0.92 ± 0.02 per muscle fiber which reflects a more than 40% reduction in capillary density in comparison to non-operated, healthy tissue (1.90 ± 0.02, $P<0.05$). However, treatment with EPC-CM (1.62 ± 0.03, $P<0.05$) and EPC (1.68 ± 0.05, $P<0.05$) induced significant increase in capillary density returning the capillary number to almost 90% of that found in a normal, healthy hindlimb (Figure 6A, B). No evidence of focally enhanced vascularization was detected, as the ratio of capillary density/muscle fiber

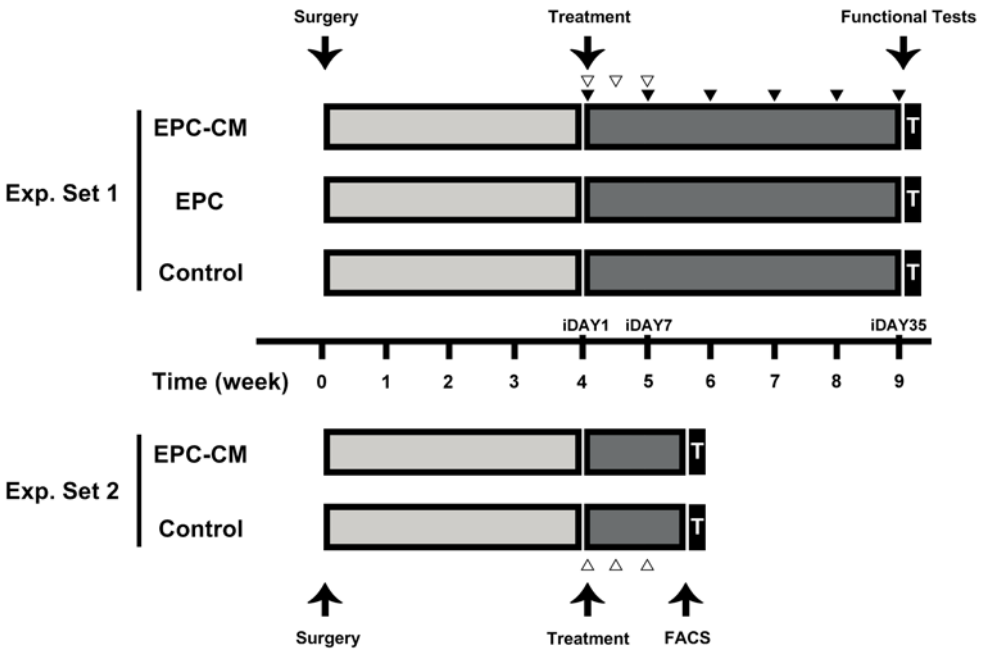

Figure 3. Design of *in vivo* experiments. Two *in vivo* experimental settings were designed to address the effect of the treatment modalities on tissue regeneration and neovascularization (Exp. Set 1) as well as progenitor cells mobilization and recruitment (Exp. Set 2). In both settings, rats were treated by 3 separate intramuscular injections within 7 days (iDAY1- iDAY7), 4 weeks after inducing ischemia as indicated by the white arrowheads (∇). Black arrowheads (▼) indicate blood flow measurements by Laser-Doppler of the hindlimb. T indicates tissue harvest and immunohistochemistry analysis.
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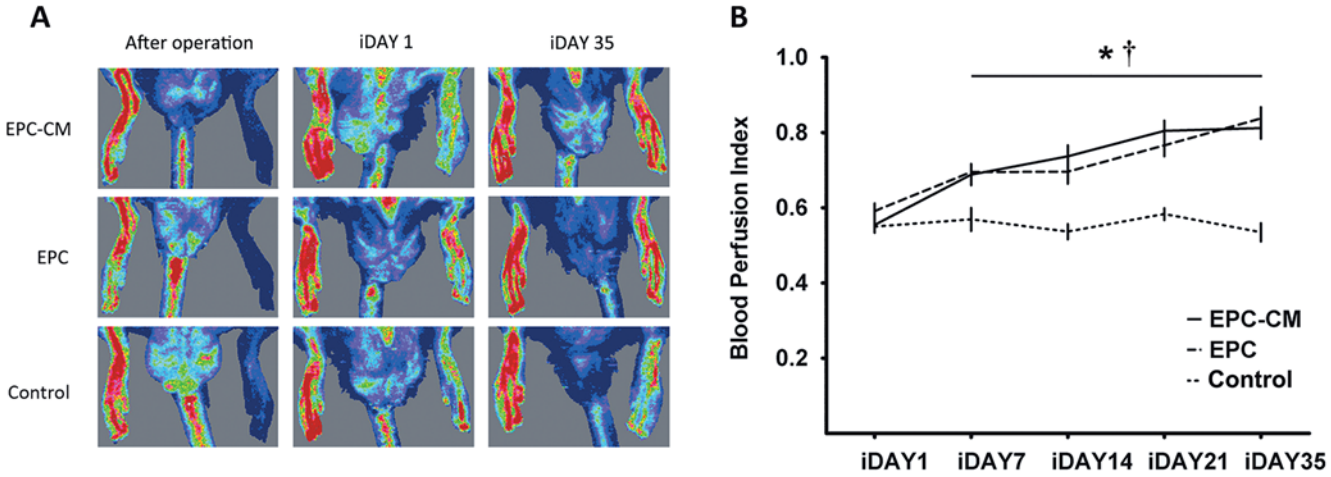

Figure 4. EPC-CM and EPC transplantation improve blood perfusion in the ischemic hindlimb. (A) Representative images of hindlimb blood flow measured by laser Doppler immediately after intramuscular injection of EPC-CM, EPC or control medium (iDAY1, 4 weeks after occlusion of the femoral artery) and the end of the experiment (5 weeks after treatment, iDAY35). **(B)** Quantitative analysis of blood flow expressed as perfusion ratio of the ischemic to the contralateral (non-operated) hindlimb over the observation period (iDAY1: day of EPC-CM or EPC injection; iDAY7; iDAY14; iDAY21; iDAY28 and iDAY35: 1, 2, 3, 4 and 5 weeks after injection, respectively). *, EPC-CM vs. Control, $P<0.01$; †, EPC vs. Control, $P<0.01$.
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obtained from the gastrocnemius muscle at three different anatomic levels showed a relatively uniform and widespread vascularization (from proximal to distal: 1.67 ± 0.06, 1.64 ± 0.04, 1.62 ± 0.04, P = n.s.).

Furthermore, the number of $NG2^+$ pericytes per capillary was significantly higher in animals treated with EPC-CM (0.25 ± 0.02) or EPC (0.24 ± 0.01) as compared to control medium treated rats (0.18 ± 0.01, $P<0.05$; Figure 6C, D). In parallel, the number of vessels coated by smooth muscle cells was also significantly higher in animals treated with EPC-CM or EPC (8.6 ± 0.3/HPF and 8.1 ± 0.4/HPF) as compared to controls (4.9 ± 0.3/HPF, $P<0.05$, Figure 7).

EPC-CM transplantation stimulates mobilization and recruitment of bone marrow-derived EPC to the ischemic hindlimbs

Three days after the last injection, the number of $CD34^+/CD45^-$ progenitor cells/1×10^5 mononuclear cells (MNC) [18] was significantly elevated in the bone marrow (678.7 ± 44.1 vs. 340.0 ± 29.1, $P<0.05$, Figure 8A–C) and the peripheral blood (54.7 ± 10.2 vs. 25.7 ± 1.8, $P<0.05$, Figure 8D–F) of animals treated with EPC-CM as compared to control media. Concomitantly, the number of $CD34^+$ cells within the ischemic muscle tissue of EPC-CM treated limbs were significantly higher than in control media treated (5.9 ± 0.7/HPF vs. 2.6 ± 0.4/HPF in

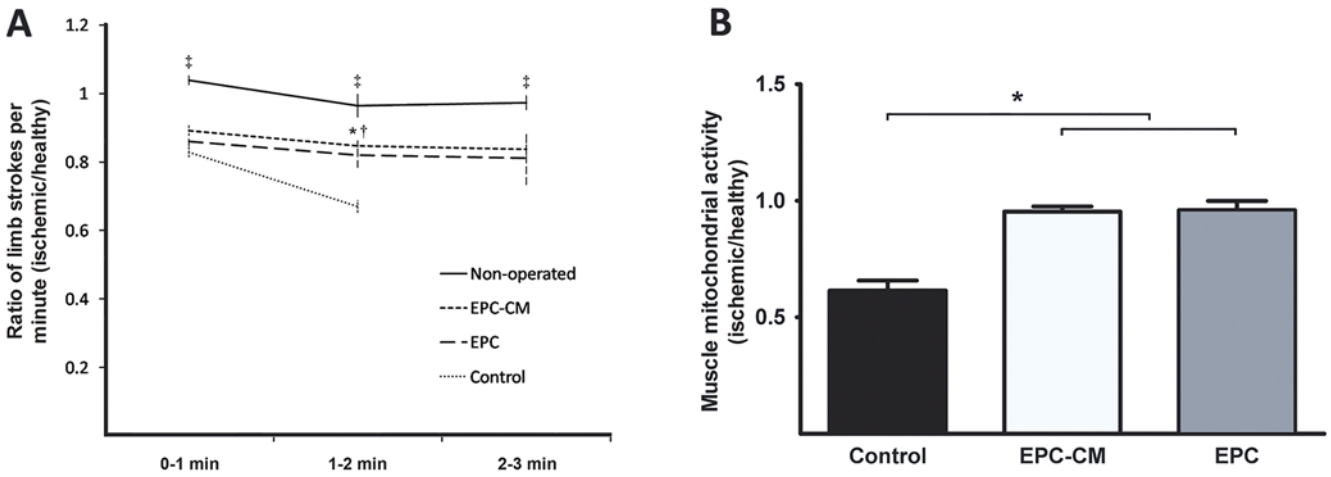

Figure 5. Effect of EPC-CM and EPC transplantation on ischemic muscle function and activity. (A) Muscle function was tested by swimming exercise and expressed as the ratio of ischemic to healthy hindlimb stroke numbers in animals treated with EPC-CM, EPC, control medium or non-operated animals. Swimming activity was monitored for 3 minutes at 1 minute intervals. Rats treated with control medium were not able to complete the exercise due to obvious exhaustion with drowning. *, EPC-CM vs. Control, $P<0.05$; †, EPC vs. Control, $P<0.05$; ‡ Non-operated vs. EPC-CM and EPC, $P<0.05$. **(B)** Muscle mitochondrial activity in animals treated with EPC-CM, EPC or control medium was assessed by MTT reduction in the healthy and ischemic hindlimbs. The activity index is indicated as the ratio ischemic to healthy MTT values per gram of dry tissue. *, $P<0.05$.
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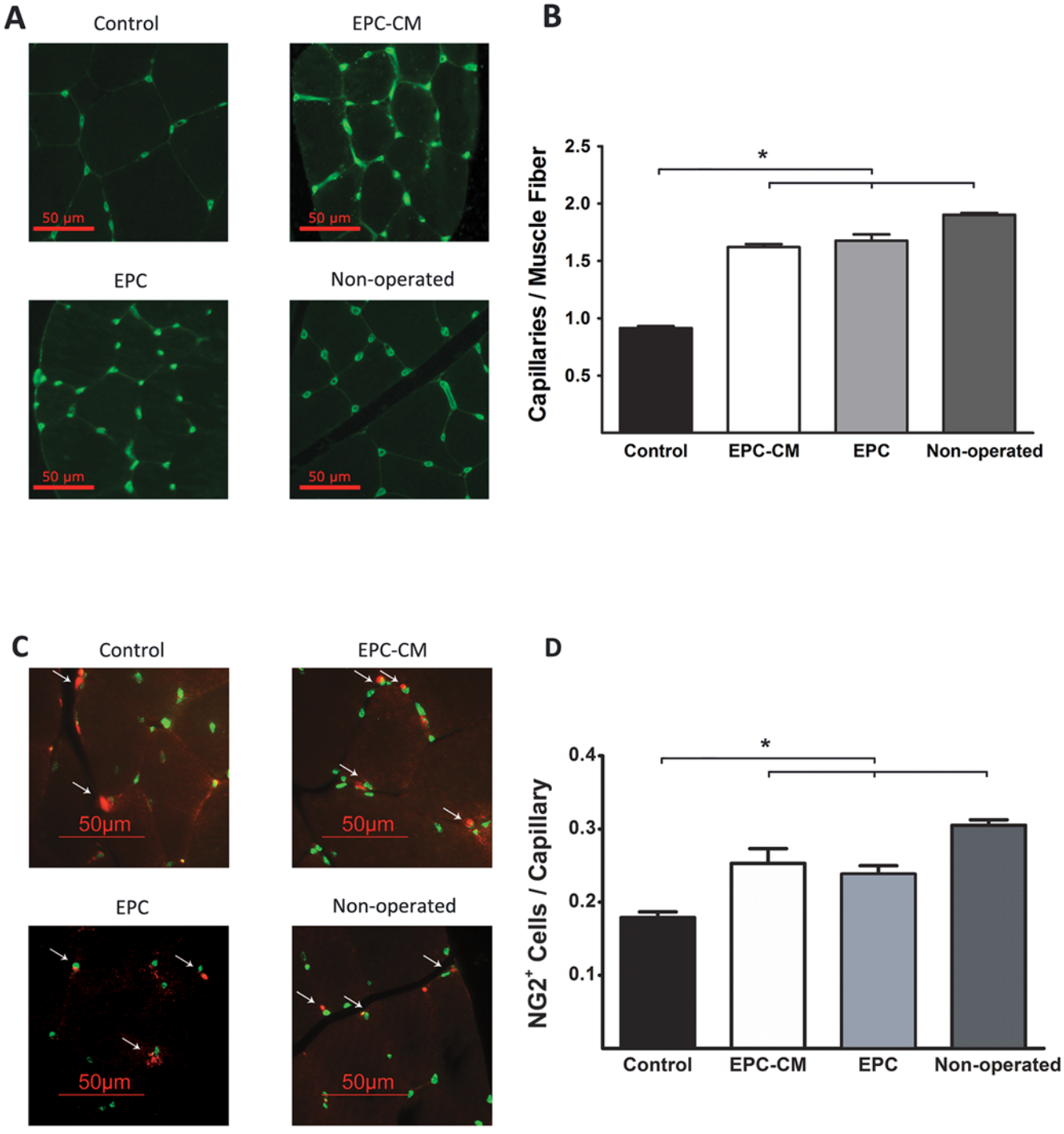

Figure 6. Effect of EPC-CM and EPC transplantation on ischemic muscle neovascularization. (A) Representative images of healthy (non-operated) and ischemic hindlimb muscle of animals treated with EPC-CM, EPC or control medium stained with BS-1 lectin (FITC) to localize capillaries. **(B)** Quantitative analysis of capillary density expressed by the number of capillaries per muscle fiber. *, $P<0.05$. **(C)** $NG2^+$ pericytes (white arrows) were identified (red fluorescence) by being adjacent to endothelial cells stained for von Willebrand Factor (green fluorescence). **(D)** Quantitative analysis of $NG2^+$ cells per capillary in healthy and ischemic hindlimbs treated with EPC-CM, EPC and control medium. *, $P<0.05$.
doi:10.1371/journal.pone.0005643.g006

gasctrocnemius, $P<0.001$, Figure 9A, B). Interestingly, numbers of $CD34^+$ cells were similar in muscles from different anatomic regions (5.3 ± 0.4/HPF vs. 2.2 ± 0.3/HPF in adductor muscle, $P<0.001$), suggesting a widespread recruitment of progenitor cells rather than a localized migration (Figure 9C). Analysis of tissue sections from later time points (iDAY 35) showed a decline of $CD34^+$ cells to levels similar to control media treated animals (Figure 9D–F). These results indicate a temporary but potent systemic effect of EPC-CM on mobilization and homing of progenitor cells to the ischemic muscle.

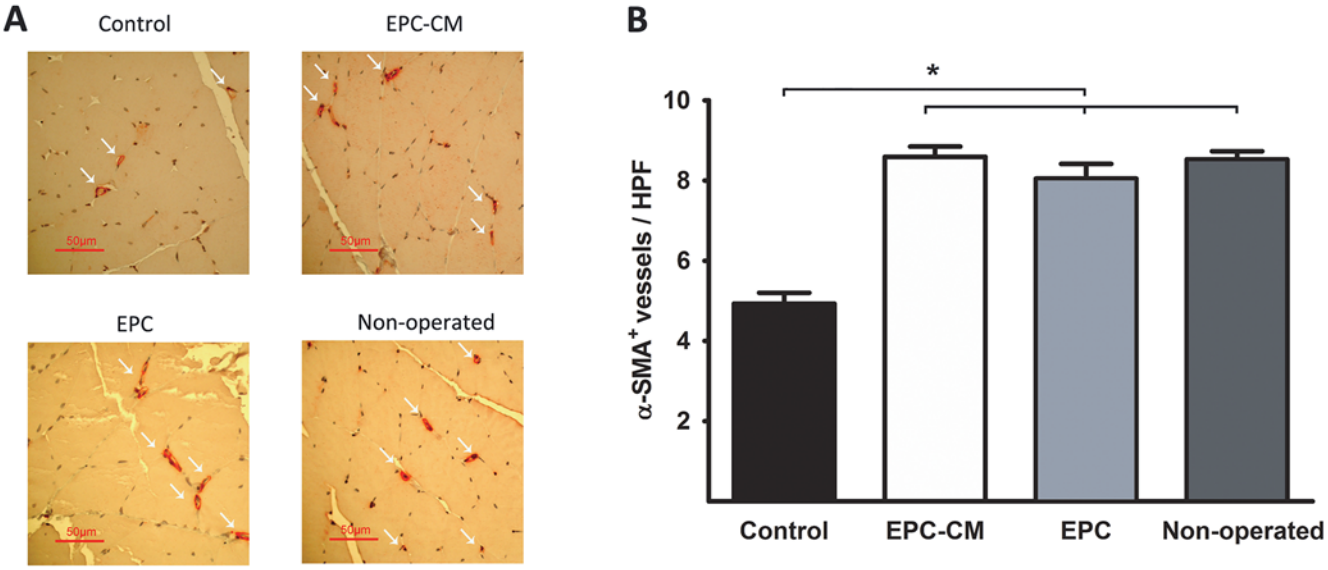

Figure 7. Effect of EPC-CM and EPC transplantation on vascular maturation. (A) Representative images of healthy and ischemic hindlimb muscle of animals treated with EPC-CM, EPC or control medium stained with α-smooth muscle actin (α-SMA) to evidence vascular maturation (red staining, white arrows). **(B)** Quantitative analysis of α-SMA$^+$ vessels per high power field (HPF). *, P<0.05.
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Discussion

In the present study we demonstrate that paracrine factors released by *in vitro* expanded EPC have a potent therapeutic capacity in a rat model of hindlimb ischemia. We present convincing evidence that treatment with EPC-CM leads to a substantial increase in blood flow in the presence of augmented neovascularization, vascular maturation and muscle function in the ischemic hindlimb. Most importantly, the observed regenerative potential after EPC-CM was equivalent to that achieved by EPC transplantation.

Emerging evidence suggests that paracrine signals from stem and progenitor cells are fundamental players in various processes of tissue repair [14,15,19,20] integrating the mechanisms relying on cell differentiation and engraftment. Preclinical studies have described that EPC secretion of factors involved in the regulation of stem cell recruitment and in vascular growth and remodeling (such as SDF-1, VEGF, HGF and MMP-9) [5,17] support the function of mature endothelial cells *in vitro* and tissue regeneration in a variety of animal models [21,22]. However, despite the fact that the regenerative capacity of EPC-secreted factors is recognized [23] the spectrum of paracrine effectors and their mechanisms of action remain largely unexplored. Recently the characterization of multifaceted nature of the EPC secretome has been addressed [16] but further investigations are needed to clarify the activation and the interactions of downstream signals.

Although the aim of this work was not the identification of the molecular effectors responsible for the angiogenic properties of EPC-CM, we have confirmed that EPC release key angiogenic molecules such as Angiogenin, HGF, IL-8, PDGF, SDF-1 and VEGF in culture. Consistent with previous studies reporting the stimulatory activity of hypoxia on differentiated endothelial cells and EPC, we found an increased secretory activity under hypoxic conditions [22,24]. Accordingly, the blend of growth factors contained in the EPC-CM disclosed a strong capacity to sustain fundamental biological functions of mature endothelial cells such as endothelial viability and sprouting. The pro-survival activity of EPC-CM is of utmost relevance in the patho-physiological scenario of chronic muscle tissue ischemia where viability of resident endothelial cells is compromised by the reduced oxygen and nutrient supply [25].

A better understanding of the mechanisms by which cytokines support the functions of resident cells and circulating bone marrow-derived cell populations has led to the development of a number of therapeutic angiogenesis strategies. These include the direct delivery of a variety of recombinant cytokines [26–28] or the gene encoding the desired angiogenic protein [29–32]. However, clinical trials based on the administration of a single factor have shown contrasting results [31,33,34] probably reflecting the fact that the synergic activity of different growth factors is needed to induce formation of stable vascular networks [35,36]. In contrast to findings after VEGF monotherapy, which promotes intense endothelial sprouting, but results in the development of leaky and disorganized conduits [37], EPC-CM induced the formation of a persistent capillary network as clearly evidenced by long-lasting enhanced density of capillaries and mature vessels as detected five weeks after injection. From this one can speculate that the complex process of revascularization in ischemic tissue is improved by the number of soluble factors present in the EPC-CM, which are presumably able to target simultaneously multiple cell types.

The findings reported here potentially have important implications for the development of novel therapeutic strategies. Most importantly, our data suggest a strategy free from the limitations and problems observed with cell transplantation [38]. It has been described that age and other cardiovascular risk factors reduce the availability and function of EPC, thus limiting their therapeutic applicability in affected patients [39–41]. Furthermore, the relative scarcity of circulating EPC and their limited proliferative potential prevent the possibility of expanding these cells in sufficient numbers for some therapeutic applications. Therefore, the use of heterologous cells seems to be the only available option to provide patients suffering from cardiovascular disease with a cell-based therapy. However, immunotolerance concerns and technical as well as practical difficulties may hinder this type of treatment. In contrast, a cell-free medium such as EPC-CM significantly reduces the risk of adverse immunological reactions, simplifies the process

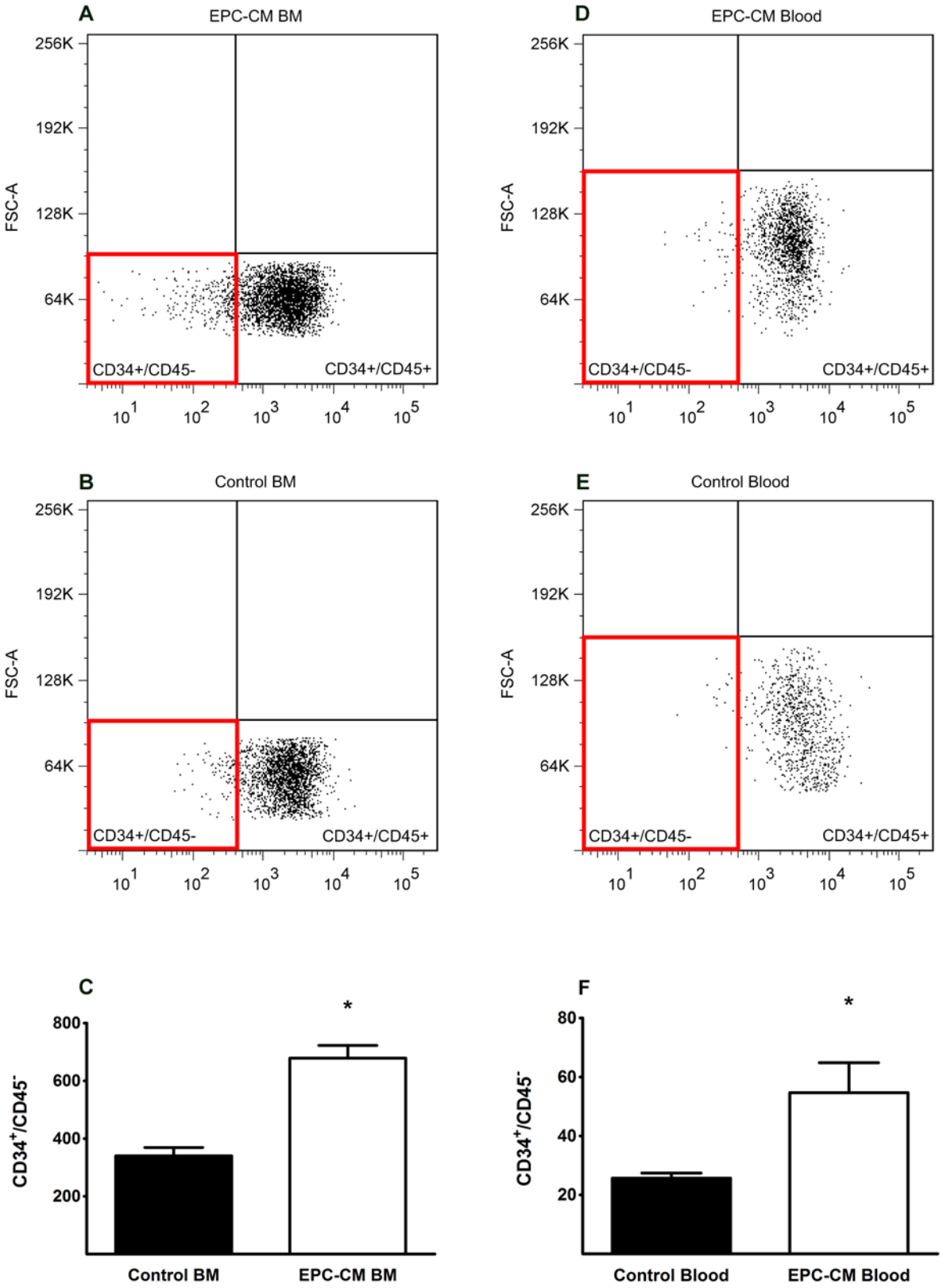

Figure 8. EPC-CM stimulates the mobilization of bone marrow-derived EPC. Representative FACS analysis charts of $CD34^+/CD45^-$ cells isolated from bone marrow **(A** and **B)** and peripheral blood **(D** and **E)** of EPC-CM and control media treated animals 3 days after the last intramuscular injection. Quantitative analyses show significantly increased numbers of $CD34^+/CD45^-$ progenitor cells in the BM **(C)**, and the peripheral blood **(F)** of EPC-CM treated animals. *, $P<0.05$.
doi:10.1371/journal.pone.0005643.g008

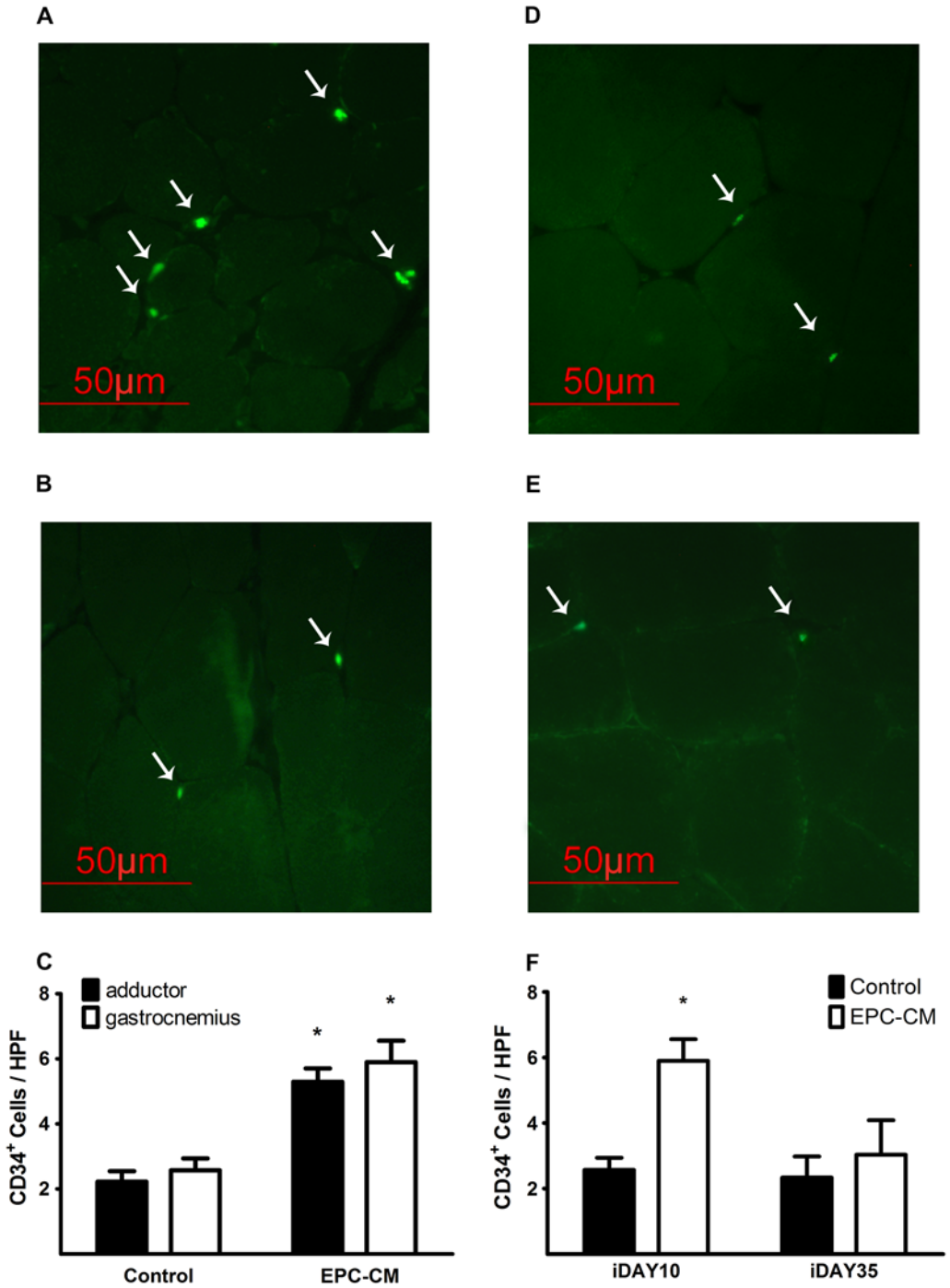

Figure 9. EPC-CM promotes progenitor cells homing to the ischemic tissue. Representative fluoresence pictures of $CD34^+$ immunostaining in ischemic hindlimb tissue 3 days (left panel, iDAY10) and 4 weeks after treatment (right panel, iDAY35). The number of $CD34^+$ cells on iDAY10 was significantly higher in EPC-CM treated limbs **(A)** as compared to control treated animals **(B)** with no evidence for focal recruitment, as $CD34^+$ cells were found to similar extent in different anatomic regions **(C)**. In comparison, 4 weeks after treatment (iDAY35), tissue sections from show decreased numbers of $CD34^+$ cells in EPC-CM treated limbs **(D)** equivalent to numbers found in control **(E)**. Quantitative analysis is depicted reflects the temporary recruitment of $CD34^+$ cells to the ischemic limbs in EPC-CM treated animals **(F)**. *, $P<0.001$.
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of production and thus increases the availability of the therapy. In the present study we have used hypoxic culture conditions to achieve maximum concentrations of growth factors and chemokines in the conditioned medium of EPC [42]. Since the aim of our research was to investigate an alternative therapeutic option to current pre-clinical and clinical EPC transplantation protocols which apply normoxic culture conditions, we have compared the regenerative potential of hypoxic EPC-CM to normoxic EPC cultures. It is of note that the amount of cells required to generate a therapeutic dose of EPC-CM is significantly lower in comparison to the number of EPC needed for transplantation. More precisely, the number of EPC injected was 8-fold higher than the number of

cells required to generate the volume of EPC-CM needed to achieve the same therapeutic benefit. We speculate that a considerable number of EPC undergo cell death during and after transplantation. Additionally, the unfavorable microenvironment present in the ischemic tissue might impair the effectiveness of cell transplantation. In contrast, the administration of a mixture of physiologically relevant cytokines and growth factors as by EPC-CM injection, might induce a permissive milieu for differentiated as well as progenitor cells of the host and thus stimulate the endogenous repair system [43–45]. The prolonged re-vascularization of the ischemic tissue induced by EPC-CM treatment is intriguing given the short half-life of growth factors. In fact, rapid inactivation and degradation are major limitations of the therapeutic approach using intravenous or intramuscular protein applications [46]. The presented data therefore suggest that in our experimental setting the integrity of the proteins in EPC-CM is preserved resulting in a sustained level and activity of growth factors in the tissue with a long-lasting angiogenic effect.VEGF and SDF-1 may be key factors in amplifying the angiogenic signals of EPC-CM as both have be shown to recruit and entrap pro-angiogenic BM-derived cells in the ischemic tissue [47,48]. Indeed, our data provide evidence that intramuscular injection of EPC-CM effectively enhanced the number of EPC in the BM, promoted mobilization into the peripheral blood and their homing to the ischemic limbs. In accordance with previous publications, this effect appears to be temporally limited [49]. Interestingly, this recruitment as well as the induction of neovascularization do not show a focal pattern but appear rather equally distributed throughout the ischemic muscle.

Taken together, our observations support the concept that EPC-CM has the potential to replace cell transplantation. Moreover, this study provides a reference for future investigations which will improve our understanding of the regenerative properties of EPC. In particular, knowledge about the differences in healing capacity between EPC-CM obtained from healthy donors and patients with cardiovascular risk factors in combination with the elucidation of their respective secretomes will give the opportunity to define the paracrine functions of EPC in health and disease. These advances will then serve to set up an effective tool to support the defective paracrine processes in the ischemic tissues. It is, therefore, reasonable to imagine that the development of a synthetic preparation which mimics physiological EPC-CM could provide clinicians with a readily available product of standardized quantity and quality.

In conclusion, we have demonstrated in the present study that the constellation of soluble factors secreted by *in vitro* expanded EPC is able to support revascularization of hindlimb ischemic tissue. These data strongly suggest that interventions based on EPC paracrine factors might effectively replace cell transplantation. Future studies designed to identify these factors and the activation of the respective downstream cellular targets might ultimately provide a more effective and practical therapeutic strategy for the treatment of ischemic diseases.

Materials and Methods

Cell and conditioned medium preparation

Human peripheral venous blood samples were obtained from healthy, young volunteers (n = 7, age range: 24–38 years) with informed consent. The MNC population was isolated by gradient centrifugation and cultured for 7 days in complete endothelial cell growth medium (EGM-2-MV, Lonza, Switzerland) containing 5% fetal bovine serum (FBS) to obtain EPC in accordance with previously published method [11]. To produce human EPC conditioned medium (EPC-CM), EPC were cultured for 72 hours under hypoxic conditions (1.5% O_2, 5% CO_2, 93.5% N_2) in growth factor-free endothelial cell basal medium-2 (EBM-2, Lonza, Switzerland) with 1% FBS. The conditioned medium was then collected and centrifuged to harvest a cell-free solution. EBM-2 containing 1% FBS without supplements served as control medium. Human umbilical vein Endothelial Cells (HUVEC) were isolated from umbilical cords and cultured using a standard protocol [50]. All protocols received full approval from the Cantonal and the Institutional Ethics Review Board and a signed informed consent was obtained from all participants.

ELISA and multiplex assay

The concentration of Angiogenin, Hepatocyte Growth Factor (HGF), Interleukin-8 (IL-8), Platelet Derived Growth Factor B (PDGF-BB), Stromal Cell-Derived Factor -1 (SDF-1) and Vascular Endothelial Growth Factor A (VEGF-A) was assessed in EPC culture supernatants generated under normoxic or hypoxic conditions. Angiogenin levels were determined by ELISA (RayBiotech, Norcross GA, USA) whereas the concentration of the other cytokines was done using a multiplex assay (Bioplex, Bio-Rad, Switzerland) following the manufacturer's instructions. All measurements were performed in duplicates from five different donors.

Cell survival assay

HUVEC were seeded into 96-well plates coated with 1% gelatin and starved in EBM-2 containing 1% FBS for 24 hours. The cells were then exposed to EPC-CM or control medium. After 24 hours the number of viable cells was assessed by use of the CyQuant® NF kit (Molecular Probes, Switzerland). The level of apoptosis was determined measuring the caspase −3 and −7 activity (Apo-ONE® Homogeneous Caspase −3/7 Assay, Promega AG, Switzerland). All experiments were performed in quadruplicates with EPC-CM generated from five different EPC donors.

In vitro angiogenesis assay

Aortas isolated from nude rats were cut into 1 mm thick rings and placed individually in a 24-well plate coated with growth factor-reduced Matrigel™ (Becton Dickinson, Germany) [51] and incubated with EPC-CM or control medium. The experiment was performed in quadruplicates for each culture condition. After 5 days of culture, sprout length was calculated digitally using ImageJ.

In vivo experimental design

The *in vivo* experimental design of the study is schematically depicted in Figure 3. Experimental set 1 was designed to investigate the long term effect of the different treatment strategies on hindlimb perfusion and function. Aim of Experimental Set 2 was to evaluate the stimulation and recruitment of host cells involved in the endogenous repair system in response to EPC-CM treatment.

In vivo angiogenesis model

Chronic hindlimb ischemia was induced by unilateral excision of an arterial segment extending from the external iliac to the femoral vessels (artery and vein) in male athymic nude rats (NIH-Foxn1^{rnu}, Charles River Laboratory Inc, Sulzfeld, Germany) during 0.2% isoflurane anesthesia. Buprenorphinum (Temgesic®, 0.1 mg/kg, Essex Chemie, Switzerland) was injected subcutaneously at the end of the procedure. Rats were then randomly assigned to 3 treatment groups (n = 8 in each group) for 3 serial intramuscular injections of EPC-CM, EPC or control medium within 7 days (Figure 3). Injections were performed four weeks

following arterial occlusion (iDAY1). Each time a total volume of 250 µl EPC-CM or control medium, or 1×10^6 EPC were injected at 5 sites into the ischemic hindlimb distal to the arterial occlusion site. Three ventral injections were placed in the upper limb in proximity to the adductor and semimembranosus muscles. The remaining 2 injections were administered to the ventral lower limb involving the gastrocnemius and flexor digitorum muscles. In order to achieve maximal experimental uniformity, transplanted EPC and EPC-CM were derived from the same donors and used in parallel experiments. All procedures were approved by the Cantonal Ethics Review Board and conducted in accordance with the institutional policies for animal experiments.

Laser Doppler blood perfusion imaging

Blood flow in the ischemic and healthy lower hindlimbs was measured weekly until iDAY 35 using a laser Doppler Imager (Moor, Axminster, UK). The animals were placed on a heating pad in order to maintain a constant body temperature during the entire measurement. In each rat the values of two consecutive measurements were averaged and the perfusion was expressed as ratio of values for the ischemic to normal limb [11].

Assessment of hindlimb function

We adopted a forced swimming test to determine the functional capacity of the ischemic hindlimb after treatment [52]. Animals were placed in a water-filled tank (23°C) to swim. Active strokes per minute of each limb were counted during 3 consecutive periods (0–1 min, 1–2 min, and 2–3 min). Functional muscle activity was calculated as the ratio of number of strokes/min of the ischemic to the healthy hindlimb and compared to non-operated, age-matched healthy rats (n = 5).

Assessment of muscular viability and immunohistochemistry

All animals were euthanized by use of carbon dioxide five weeks after treatment for analysis of mitochondrial activity and immunohistochemistry. The mitochondrial activity, serving as a surrogate marker of muscular viability, was assessed in the hindlimb muscles by the MTT reduction test. Muscle viability was calculated as the ratio of extracted MTT absorbance values per gram of dry tissue of the ischemic and the healthy contralateral limb [53]. The gastrocnemius muscle of the hindlimb was fixed in 4% formaldehyde for 24 hours and embedded in paraffin. Ten µm thick sections were stained with lectin from *Bandeiraea simplicifolia* (BS-1, Sigma-Aldrich, Germany) or with von Willebrand factor (vWF; AB7356, Chemicon, USA) in order to determine capillary density in the muscle tissue. Vascular mural cells were identified as cells immunoreactive for NG2 (N8912, Sigma-Aldrich, Germany) adjacent to endothelial cells stained with vWF. A mouse monoclonal antibody against rat α-smooth muscle actin (α-SMA, Sigma-Aldrich, Germany) and the LSAB^{TM} - alkaline phosphatase kit (Dako, USA) were used to localize vascular maturation [54]. The number of capillaries, NG2$^+$ pericytes and smooth muscle cell-covered vessels was counted in 5 random high power fields (HPF) by use of ImageJ software. Capillary density was expressed as the ratio of capillary numbers per muscle fiber. NG2$^+$ pericyte density was expressed as the ratio of cell numbers per capillary. A total of 10 sections from 3 different muscle levels were obtained per animal and analyzed by a blinded investigator.

Progenitor cells mobilization

To investigate whether the effect of intramuscular applied EPC-CM on tissue regeneration and neovascularization involves the endogenous repair system of bone marrow-derived progenitor cells, we performed experiments according to Experimental Set 2 (Figure 3) to evaluate mobilization, recruitment and homing of progenitor cells [18]. The number of CD34$^+$/CD45$^-$ progenitor cells was measured in the bone marrow and the peripheral blood of EPC-CM and control media treated animals (n = 5) using flow cytometry 3 days after the last treatment. Immediately following isolation, MNC from BM and PB were processed for FACS analysis. The expression of surface markers CD34 (Santa Cruz, USA) and CD45 (Cedarlane Laboratories, Canada) were measured in a LSR II flow cytometer (Becton Dickinson, USA) using the Cell Quest software (Becton Dickinson, USA). Immunofluorescence staining was used to determine the number of CD34$^+$ cells in both adductor and gastrocnemius muscles from ischemic hindlimbs. To compare the recruitment of CD34$^+$ cells at different time points, immunofluorescence staining for CD34$^+$ cells was also performed in tissue sections from gastrocnemius muscles harvested at iDAY35.

Statistical analysis

Data are reported as means±SEM unless otherwise stated. Proportions were compared by use of Pearsons X2-Test and Fisher's exact test, respectively, applying the Bonferroni correction for repetitive testing. Kruskal-Wallis test and post-hoc comparison with Scheffe's test were used to compare means of continuous variables amongst the different study groups. Statistical significance was inferred at a 2-sided value of $P<0.05$. Statistics were carried out using SPSS software package (version 16.0; SPSS Inc, Chicago, IL).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

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Author Contributions

Conceived and designed the experiments: SDS ZY IB CK. Performed the experiments: SDS ZY. Analyzed the data: SDS ZY MWvB ND. Contributed reagents/materials/analysis tools: JV. Wrote the paper: SDS ZY MWvB CK.

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2.3 Paper III – Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells

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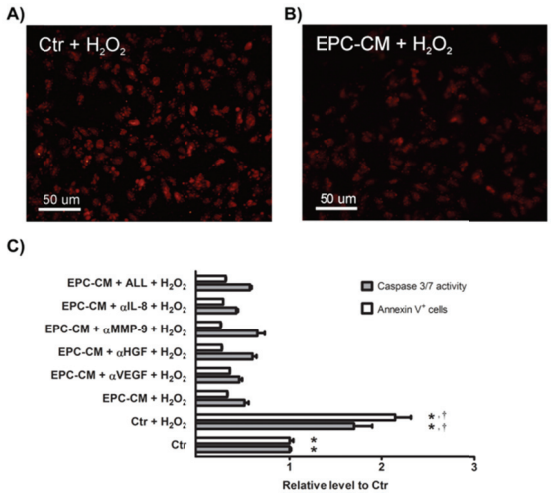
Endothelial progenitor cells (EPC) have been found to be more resistant to oxidative insults than differentiated endothelial cells. In this study we hypothesized whether paracrine factors secreted by EPC (EPC-CM) can have a protective effect against oxidative damage. Endothelial cells were incubated with EPC-CM in presence of hydrogen peroxide as pro-oxidant stimulus. Levels of intracellular ROS measured by DHE and apoptosis by caspase -3/7 activity were found to be significantly lower in the cells incubated with EPC-CM compared to standard culture media as control. This protective effect was associated with increased intracellular level of the antioxidant enzymes catalase, Cu/ZnSOD and MnSOD. Furthermore, the neutralization of these selected factors alone or in combination is not sufficient to attenuate the cyto-protective properties of EPC-CM. These results suggest that other, even unidentified factors or a synergic combination of them might be responsible for the anti-apoptotic effect of EPC-CM.



The cyto-protective properties of EPC-CM via synergistic paracrine secretion. The formation of intracellular ROS was visualized by DHE fluorescence (red). The O^{2-} levels confirmed that ROS formation was significantly reduced in the presence of EPC-CM (**A**) compared to control (**B**) under H_2O_2 induced oxidative stress. The blockage of selected factors did not reverse the apoptotic level of HUVEC under H_2O_2 stress, confirmed by both the caspase 3/7 activity and the number of Annexin V^+ apoptotic cells (**C**), suggesting that soluble factors secreted by EPC exert strong cyto-protective properties via broad synergistic actions.

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Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells

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ABSTRACT

Endothelial progenitor cells (EPC) play a fundamental role in tissue regeneration and vascular repair. Current research suggests that EPC are more resistant to oxidative stress as compared to differentiated endothelial cells. Here we hypothesized that EPC not only possess the ability to protect themselves against oxidative stress but also confer this protection upon differentiated endothelial cells by release of paracrine factors. To test this hypothesis, HUVEC incubated with conditioned medium obtained from early EPC cultures (EPC-CM) were exposed to H_2O_2 to assess the accumulation of intracellular ROS, extent of apoptosis and endothelial cell functionality. Under oxidative stress conditions HUVEC treated with EPC-CM exhibited substantially lower levels of intracellular oxidative stress (0.2 ± 0.02 vs. 0.4 ± 0.03 relative fluorescence units, $p < 0.05$) compared to control medium. Moreover, the incubation with EPC-CM elevated the expression level of antioxidant enzymes in HUVEC (catalase: 2.6 ± 0.4; copper/zinc superoxide dismutase (Cu/ZnSOD): 1.6 ± 0.1; manganese superoxide dismutase (MnSOD): 1.4 ± 0.1-fold increase compared to control, all $p < 0.05$). Furthermore, EPC-CM had the distinct potential to reverse the functional impairment of HUVEC as measured by their capability to form tubular structures in vitro. Finally, incubation of HUVEC with EPC-CM resulted in a significant reduction of apoptosis (0.34 ± 0.01 vs. 1.52 ± 0.12 relative fluorescence units, $p < 0.01$) accompanied by an increased expression ratio of the anti/pro-apoptotic factors Bcl-2/Bax to 2.9 ± 0.7-fold (compared to control, $p < 0.05$). Most importantly, neutralization of selected cytokines such as VEGF, HGF, IL-8 and MMP-9 did not significantly reverse the cyto-protective effect of EPC-CM ($p > 0.05$), suggesting that soluble factors secreted by EPC, possibly via broad synergistic actions, exert strong cyto-protective properties on differentiated endothelium through modulation of intracellular antioxidant defensive mechanisms and pro-survival signals.

1. Introduction

The entire cardiovascular system critically depends on a structurally and functionally intact vascular endothelium. The loss of endothelial integrity and function is known to play a pivotal role in the initiation and progression of vascular dysfunction occurring secondary to diseases such as hypertension or diabetes mellitus ultimately leading to complications including myocardial infarction or stroke [1]. A major initiator of endothelial injury is oxidative stress which results from an imbalanced state of increased reactive oxygen species (ROS) generation and insufficient intracellular antioxidants [2].

As a matter of fact both, oxidative stress due to excessive ROS formation and a defective capacity to detoxify intracellular oxidants are common features of several cardiovascular diseases although the cause–effect relationship between oxidative damage and cardiovascular dysfunction is not completely understood [3].

Growing evidence suggests that EPC contribute to the structural integrity of the vasculature by promoting angiogenesis through the secretion of angiogenic growth factors as well as by their replicative potential and ability to differentiate into mature vascular endothelial cells [4,5]. Previous studies have reported that EPC are

Abbreviations: EPC, Endothelial progenitor cells; HUVEC, Human umbilical vein endothelial cells; EPC-CM, EPC-conditioned medium; HUVEC-CM, HUVEC-conditioned medium; PBMC, Peripheral blood mononuclear cells; DHE, Dihydroethidine; ROS, Reactive oxygen species; PEG-SOD, Polyethylene glycol-superoxide dismutase; H_2O_2, Hydrogen peroxide; O_2^-, Superoxide anion; Cu/ZnSOD, Copper/zinc superoxide dismutase; MnSOD, Manganese superoxide dismutase.

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more resistant to oxidative stress than differentiated endothelial cells due to an elevated expression of the intracellular antioxidant enzymes catalase, glutathione peroxidase and manganese superoxide dismutases (MnSOD) [2]. This resistance against oxidative injury likely allows EPC to survive and exert their angiogenic and vascular repair functions in microenvironments with elevated ROS levels like ischemic or inflamed tissue.

In light of the regenerative properties of EPC we hypothesized that EPC, in particular the subpopulation termed early EPC, may protect not only themselves but also resident endothelial cells from the detrimental effect of oxidative stress through paracrine mechanisms. In the present study we sought to determine, whether soluble factors secreted by EPC support resistance of endothelial cells against ROS toxicity thereby preserving their viability and functional activity under conditions of oxidative stress.

2. Materials and methods

2.1. Cell isolation and culture

HUVEC were isolated from umbilical cord by collagenase digestion [6] and cultivated in complete endothelial cell growth medium (EGM-2-MV, Lonza, Switzerland) containing 5% fetal bovine serum (FBS). All experiments were performed using cells between passages 2 and 6. To harvest endothelial progenitor cells (EPC), peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy human volunteers by density gradient centrifugation with Histopaque®-1077 (Sigma–Aldrich, Switzerland) as described previously [7]. PBMC were plated on culture dishes coated with human fibronectin (Clonetech, Switzerland) and maintained in EGM-2-MV containing 5% FBS. After 4 days in culture, non-adherent cells were removed by a single washing step with phosphate-buffered saline (PBS). Adherent cells were trypsinized, passaged and maintained in culture till day 7. EPC were characterized by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-Ac-LDL, Harbor Bio-products) and BS-1 lectin (Sigma) staining, as well as flow cytometry analysis of the following surface markers: CD34, CD133, CD45, CD14, KDR, CD31, VE-cadherin (CD144) and MCAM (CD146) as published previously [8,9].

2.2. Preparation of conditioned medium

To produce human EPC and HUVEC conditioned medium (EPC-CM and HUVEC-CM, respectively), EPC and HUVEC were cultured for 72 h under hypoxic conditions (1.5% O_2, 5% CO_2, 93.5% N_2) using a humidified gas-sorted anoxic incubator-gloved box (InVivo2 400, Ruskin, UK). A growth factor-free endothelial cell basal medium-2 (EBM-2, Lonza, Switzerland) with 1% FBS was employed in this step and served as control medium throughout the experiments. After incubation, the culture supernatant was centrifuged, sterile filtered with a 0.22 μm filter (TPP, Switzerland) and stored at −80 °C until use.

2.3. Assessment of intracellular ROS

2×10^4 HUVEC were plated per well in an 8-well culture slides (BD, Switzerland) in EGM-2-MV +5% FBS. After overnight starvation, the monolayers were incubated with EPC-CM or control medium and treated with 500 μM H_2O_2 for 8 h. In addition a set of wells were conditioned with 100 U/ml of the superoxide scavenger PEG-SOD (Sigma–Aldrich, Switzerland). To measure the accumulation of intracellular superoxide HUVEC were loaded with 5 μM DHE (Invitrogen, Switzerland) 30 min before the experiment termination. After washing the cells once with PBS, cells were counterstained with DAPI (Invitrogen, Switzerland). Images were acquired with a fluorescent microscope (Nikon Eclipse 800, Japan) at 200-fold magnification and the fluorescence intensity was assessed by Adobe Photoshop CS4. Fluorescent intensity was expressed as the ratio of the DHE fluorescence (red) to the cell number.

2.4. Immunoblot analysis

After exposure to 500 μM H_2O_2 for 24 h HUVEC were lysed in RIPA buffer containing the Protease Inhibitor Cocktail V (Calbiochem, Switzerland). Cell lysates were centrifuged at 4 °C for 20 min and the supernatant was harvested for further analysis. Equal amount of sample proteins were resolved in 12% acrylamide gels as previously described [10], blotted on nitrocellulose and marked using the following primary antibodies: rabbit anti-catalase (219010, Calbiochem), sheep anti-Cu/ZnSOD (574597, Calbiochem), sheep anti-MnSOD (574596, Calbiochem), rabbit anti-Bcl-2 (13-8800, Invitrogen), rabbit anti-Bax (sc-6236, Santa Cruz) and mouse anti-actin (MAB1501, Chemicon) as control.

2.5. In vitro capillary formation

2×10^4 HUVEC/well were plated in Matrigel™-coated 24-well plates (BD, Switzerland). Following 8 h of incubation with EPC-CM or control medium, digital images of the forming capillary-like structures were acquired. In vitro angiogenic activity was assessed measuring the total length and the number of sprouts per high power field with the aid of ImageJ. Calculations were performed in three random high power fields (HPF) and in three independent experiments, respectively.

2.6. Cytokine array

In order to screen the soluble factors contained in EPC-CM, a commercially available antibody array (AAH-CYT-2000, Ray Biotech, USA) for the detection of 174 human growth factors and cytokines was used. HUVEC-CM and the basal medium containing 1% FCS (control medium) served as a reference. The chemiluminescent signal of each factor on the array was acquired by ChemiDoc™ XRS (Bio-Rad AG, Switzerland) and the intensity measured by ImageJ. After background subtraction, the level of the cytokines in EPC-CM was expressed in a semi-quantitative fashion and as fold-increase over HUVEC-CM and basal medium.

2.7. Assessment of apoptotic cell death

5×10^3 HUVEC were plated per well of a 96-well plate. Before the experiment HUVEC were starved overnight in control medium. Thereafter, cells were simultaneously incubated with EPC-CM or control medium and 500 μM H_2O_2 for 18 h.

Induction of apoptosis was assessed by measuring the caspase-3 and caspase-7 activity using the Apo-ONE® homogenous caspase-3/7 kit (Promega, Switzerland). Fluorescence units were measured by the Tecan Safire reader (Tecan, Austria) and values were expressed as percentage relative to the values obtained from control group. To assure that the cyto-protective effect of EPC-CM is specific and dose-dependent, the level of apoptosis was measured in cells incubated with HUVEC-CM and serial dilutions of EPC-CM in control medium to reach a final concentration of 0, 25, 50 and 100% EPC-CM.

2.8. Selected cytokine neutralization

In order to explore whether single soluble factors are responsible for exerting the cyto-protective/anti-apoptotic effect of

EPC-CM, we have blocked the activity of prominent cytokines contained in the EPC-CM. VEGF, HGF, IL-8 and MMP-9 were selected based on the high expression levels and their known anti-apoptotic properties. The activity of these selected factors was blocked using the recommended concentration of the neutralizing antibodies (αVEGF: AF-293-NA, R&D Systems; αHGF: AF-294-NA, R&D Systems; αIL-8: MAB208, R&D Systems; αMMP-9: Clone 6-6B, sc-12759, Santa Cruz Biotechnology). Conditioned medium was incubated with the neutralizing antibodies 30 min prior to the incubation with HUVEC and the level of apoptosis was measured by the caspase-3/7 activity as described above and the number of apoptotic cells by Annexin V/7-AAD staining. Briefly, HUVEC were sequentially incubated with biotinylated Annexin V (1003-100, Biovision), steptavidin-FITC (RPN1232, Amersham) and 7-AAD (51-68981E, BD Biosciences). The Annexin V^+ apoptotic cells were defined as the sum of Annexin V^+/7-AAD^- population (early apoptosis) and Annexin V^+/7-AAD^+ population (late apoptosis) counted by flow cytometry.

2.9. Statistical analysis

All experiments were performed in triplicates. If not otherwise stated, data are presented as mean ± standard error of the mean (SEM). Unpaired Student's *t* test and one-way ANOVA with Scheffe's test for post hoc comparison were used to compare group means. Statistical significance was inferred at a 2-sided $p \leq 0.05$. STATA (Stata Corporation, College Station, TX, version 10.1 for Apple) was used for statistical analyses.

3. Results

3.1. EPC characterization

After 7 days culture, the adherent cells displayed the capacity to uptake Dil-Ac-LDL and bind BS-1 lectin (data not shown). Analysis of the phenotypic pattern by FACS disclosed that a substantial fraction of day 7 adherent cells expressed both endothelial surface markers (CD31: 61.16 ± 6.70%, KDR: 42.48 ± 10.93%) and monocytic markers (CD45: 97.64 ± 1.08%, CD14: 62.51 ± 6.43%), but only few were positive for stem/progenitor markers such as CD34 (2.43 ± 0.49%) and CD133 (1.06 ± 0.20%). Moreover, only a minority of cells expressed the endothelium specific marker VE-cadherin (1.69 ± 0.74%) and MCAM (0.76 ± 0.18%). These data confirmed the presence of early EPC population during in vitro culture and are consistent to the findings reported in previous studies [9,11,12].

3.2. EPC-CM protects endothelial cells against oxidative stress

In order to investigate whether EPC-CM has a protective effect against the formation of intracellular ROS, HUVEC were exposed to the oxidant H_2O_2 in the presence of EPC-CM or control medium and stained with a specific superoxide anion (O_2^-) probe. In the presence of control medium supplemented with H_2O_2, HUVEC displayed a substantial increase O_2^- formation compared to untreated controls (0.4 ± 0.03 vs. 0.18 ± 0.004 fluorescence units, $p < 0.05$; Fig. 1A and B). In contrast, incubation of HUVEC with H_2O_2 in the

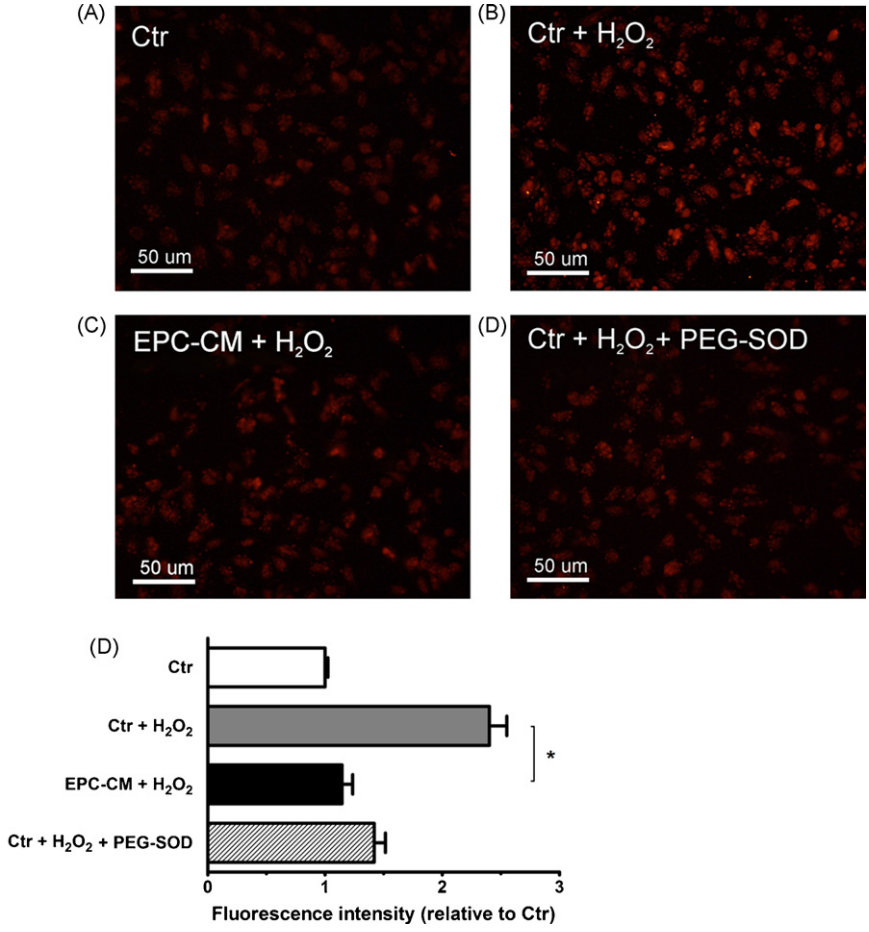

Fig. 1. EPC-CM protects EC from ROS formation. The formation of intracellular ROS under different culture conditions visualized by DHE fluorescence is shown in representative pictures A–D. Quantification of O_2^- levels (E) confirmed that ROS formation was significantly reduced in the presence of EPC-CM (C) compared to control (B) under H_2O_2 induced oxidative stress, to a similar extent as incubation with free-radical scavenger PEG-SOD (D; $^*p < 0.05$).

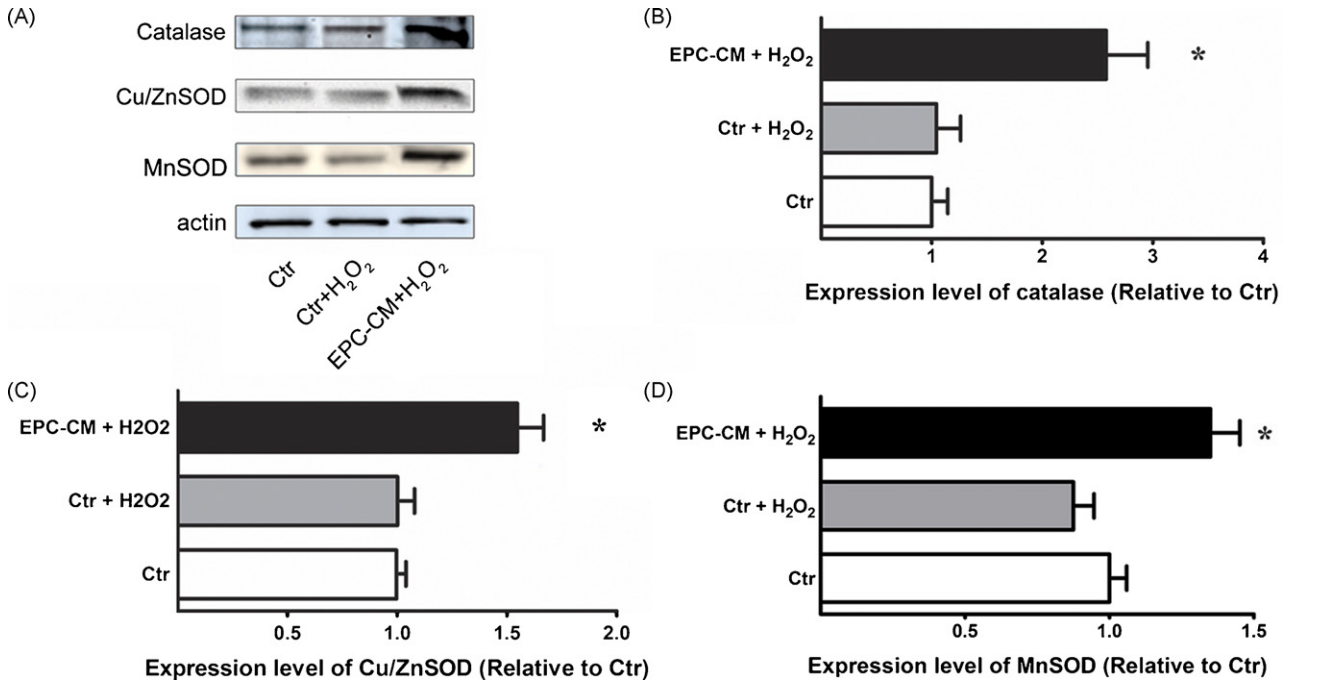

Fig. 2. EPC-CM induces the expression of antioxidant enzymes. The effect of the different culture conditions on the intracellular level of the antioxidant enzymes catalase, Cu/ZnSOD and MnSOD was assessed by Western blot (A). Under oxidative stress conditions (500 μM H_2O_2), HUVEC incubated with EPC-CM displayed an increased level of catalase (B), Cu/ZnSOD (C) and MnSOD expression (D) compared to those cultured in the control medium (*$p < 0.05$ vs. Ctr + H_2O_2).

presence of EPC-CM resulted in significantly lower intracellular O_2^- levels (0.2 ± 0.02, $p < 0.05$; Fig. 1C). The antioxidant effect of the EPC-CM was similar to that of the radical scavenger PEG-SOD (0.26 ± 0.02, $p < 0.05$; Fig. 1D) which kept cellular ROS levels low equivalent to values observed in control cells that were not exposed to H_2O_2.

3.3. EPC-CM increases antioxidant response in endothelial cells

Following the observation that EPC-CM protects HUVEC against O_2^- formation and apoptosis, we investigated whether this effect could be mediated by an increased capacity of the cell to scavenge radicals. Hypothesizing that EPC-CM results in up-regulation of intracellular defense mechanisms against ROS, we assessed the level of the key enzymatic radical scavengers catalase, Cu/ZnSOD and MnSOD. Western blot analysis demonstrated that incubation of HUVEC with H_2O_2 in the presence of EPC-CM significantly up-regulated the expression of catalase (2.6 ± 0.4-fold), Cu/ZnSOD (1.6 ± 0.1-fold) and MnSOD (1.4 ± 0.1-fold) compared to basal medium ($p < 0.05$ vs. Ctr + H_2O_2; Fig. 2A–D).

3.4. EPC-CM maintains endothelial cell function in the presence of increased oxidative stress

To analyze the impact of oxidative stress and EPC-CM on the functional activity of the endothelium, HUVEC were plated on Matrigel™ to induce the formation of tubular-like structures. Exposure to oxidative stress severely impaired the capillary morphogenesis of HUVEC (Fig. 3A and B). Both, total length (105.6 ± 3.5 vs. 21.9 ± 3.4 pixels) and the number of sprouts (36.7 ± 2.7 vs. 2.4 ± 1.4, both $p < 0.01$; Fig. 3D and E) were reduced significantly compared to unexposed controls. In contrast, EPC-CM restored the angiogenic function of HUVEC under oxidative stress and resulted in a much longer and denser network of forming capillaries than control medium + H_2O_2 (length: 98.1 ± 6.5 pixels; sprout number, 30.2 ± 1.5; $p < 0.01$; Fig. 3C–E).

3.5. EPC-CM contains a wide range of cytokines and growth factors

The secretion pattern of 174 human growth factors and cytokines in the EPC-CM was investigated using an antibody array. Amongst all the factors detected in the EPC-CM, 108 were present at a higher concentration compared to conditioned medium generated from HUVEC under identical cell culture conditions (HUVEC-CM). The complete spectrum of the soluble factors found in the EPC-CM and their expression level compared to the control medium and HUVEC-CM is reported in Supplementary Table 1.

3.6. EPC-CM specifically decreases apoptosis in endothelial cells

It is well known that intracellular ROS accumulation rapidly damages the molecular structure of the cell inducing apoptosis. We sought to investigate whether EPC-CM could decrease the level of apoptosis. For this purpose, we assessed the expression of the anti-apoptotic factor Bcl-2 and the pro-apoptotic factor Bax as specific markers of programmed cell death. In addition we measured the activity of caspase-3 and -7. Incubation with H_2O_2 resulted in significantly increased caspase-3 and -7 activity compared to untreated control (1.63 ± 0.10 vs. 1.00 ± 0.13 relative fluorescence units, $p < 0.05$; Fig. 4A) as well as reduced Bcl-2/Bax expression ratio (0.68 ± 0.12-fold compared to control; Fig. 4B and C). This pro-apoptotic effect of H_2O_2 was reversed upon incubation of cells with EPC-CM increasing the expression ratio of Bcl-2/Bax (2.91 ± 0.72-fold compared to untreated controls, $p < 0.05$; Fig. 4B and C). Moreover, the activity of caspase-3 and -7 under oxidative stress conditions reached highest reduction in the presence of undiluted EPC-CM (100% EPC-CM; 0.34 ± 0.01 relative fluorescence units, $p < 0.0001$) and attenuated with the EPC-CM dilutions (0.47 ± 0.01, 1.16 ± 0.03 for 50%, 25% EPC-CM, respectively; Fig. 4A). Importantly, conditioned medium generated from differentiated endothelial cells (HUVEC) did not achieve such anti-apoptotic effect as EPC-CM (2.03 ± 0.11, $p < 0.05$ vs. EPC-CM; Fig. 4A) These results suggest that EPC-CM exerts the anti-apoptotic effect in a dose-

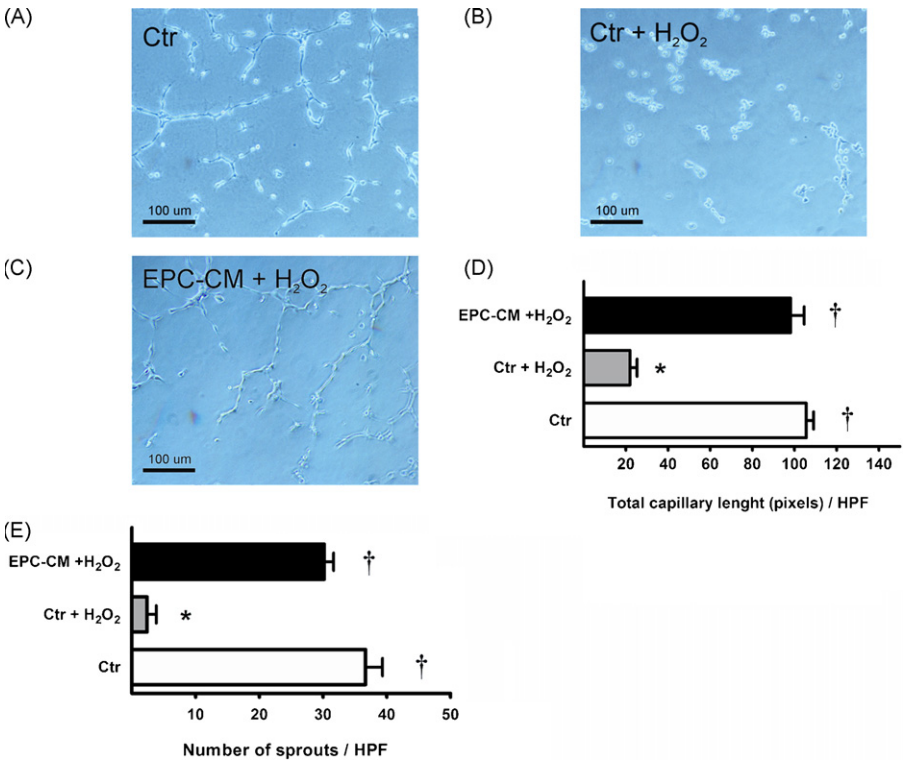

Fig. 3. EPC-CM reverses the inhibitory effect of H_2O_2 on tube formation. The impact of oxidative stress on the functionality of endothelial cells was assessed by culturing HUVEC on Matrigel™. (A) HUVEC cultured for 8 h in basal medium +1% FBS (Ctr) exhibited normal capacity to form capillary-like structures whereas this capability was severely impaired in HUVEC treated with 500 μM H_2O_2 (B). Conversely, incubation with EPC-CM (C) prevented the loss of angiogenic properties of HUVEC with regular formation of endothelial tubes. Quantification of tube formation by total length (D) and the number of capillary-like sprouts per high power field (E) confirmed that EPC-CM abolishes the effect of H_2O_2 on capillary formation ($^{*}p < 0.01$ vs. Ctr; $^{\dagger}p < 0.01$ vs. Ctr + H_2O_2).

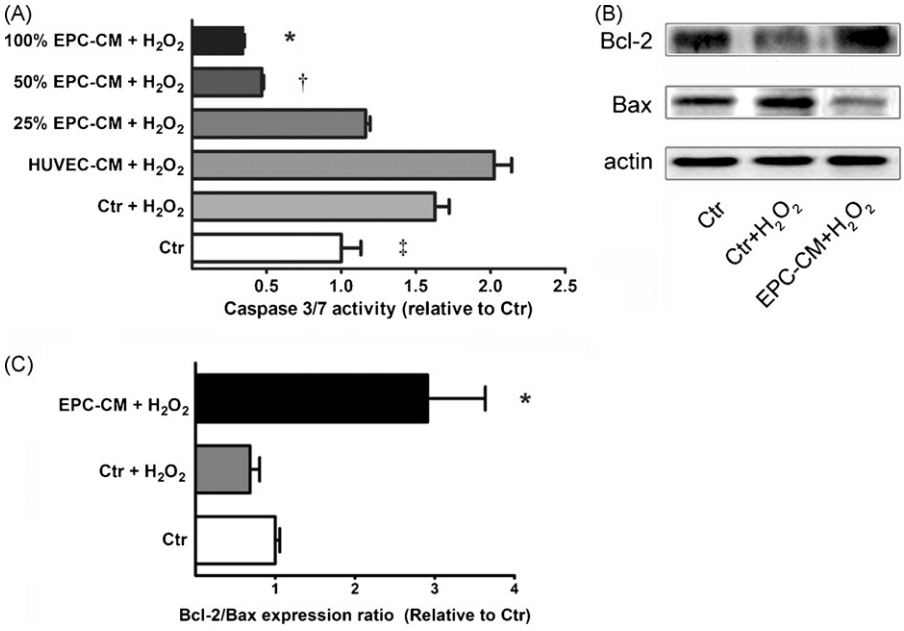

Fig. 4. EPC-CM protects EC from ROS-induced apoptosis. Caspase-3 and -7 activity (A) and Bcl-2/Bax expression (B and C) were measured in HUVEC in order to quantify the extent of apoptotic cell death. Upon oxidative stress conditions (Ctr + H_2O_2), caspase-3/7 activity (normalized to Ctr) increased significantly as compared to cells in control medium (Ctr). HUVEC incubated with undiluted EPC-CM (100% EPC-CM + H_2O_2) showed maximum reduction of caspase-3/7 activation. Dilutions of EPC-CM were accompanied by the gradual increase of caspase-3/7 activity. Replacement of EPC-CM with HUVEC-CM revealed no anti-apoptotic capacity as compared to EPC-CM group, suggesting the specificity of EPC-CM (A; $^{*}p < 0.0001$, $^{\dagger}p < 0.001$ and $^{\ddagger}p < 0.05$ vs. Ctr + H_2O_2). HUVEC incubated with EPC-CM displayed an increased level of Bcl-2 and decreased level of Bax compared to those cultured in the basal medium with 500 μM H_2O_2 (B), resulting in an increased expression ratio of Bcl-2/Bax (C; $^{*}p < 0.05$ vs. Ctr + H_2O_2).

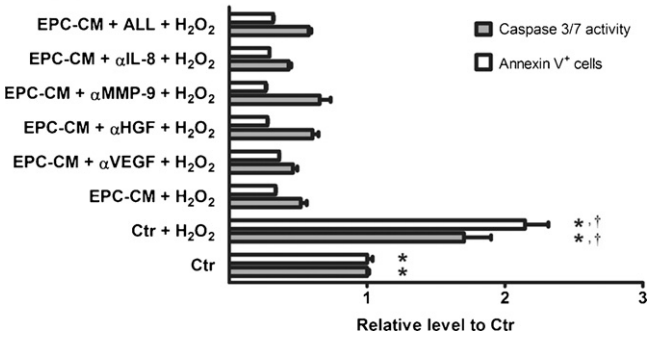

Fig. 5. Selected factor neutralization does not attenuate the anti-apoptotic capacity of EPC-CM. The blockage of selected factors did not reverse the apoptotic level of HUVEC under H_2O_2 stress, confirmed by both the caspase-3/7 activity and the number of Annexin V+ apoptotic cells (*$p < 0.001$ vs. all EPC-CM groups, †$p < 0.001$ vs. Ctr).

dependent manner and the specific composition of the cytokine cocktails in EPC-CM is necessary to elicit cyto-protection.

3.7. The anti-apoptotic effect of EPC-CM is not attenuated by selective cytokine neutralization

Neutralization of selected factors, VEGF, HGF, MMP-9, IL-8 alone or in combination did not result in a significant increase of the caspase-3/7 activity in HUVEC. Likewise, all cytokine neutralization groups did not show rise in number of apoptotic cells binding Annexin V in the presence of H_2O_2 ($p > 0.05$ compared to EPC-CM; Fig. 5).

4. Discussion

In this study we present evidence that paracrine factors secreted by early EPC have the potential to protect differentiated endothelial cells from apoptosis and to preserve their angiogenic capacity under conditions of oxidative stress. Furthermore, our data suggest that the mechanisms responsible for this cyto-protective effect of early EPC-derived soluble factors involve the up-regulation of the antioxidative defenses of endothelial cells.

ROS are important mediators in different signaling pathways of angiogenesis and cell metabolism modulating proliferation, migration and gene expression in mature endothelial cells [13]. Intracellular antioxidant enzymes such as MnSOD, Cu/ZnSOD and catalase maintain the cellular redox homeostasis by preventing excessive ROS formation. However, if the capacity of the endothelium to detoxify ROS is surpassed, the resulting oxidative stress quickly induces severe damages to the DNA, proteins as well as lipids of the various cellular compartments and eventually leads to endothelial dysfunction and apoptosis [14–16]. These events are important triggers of the onset and progression of atherosclerosis and various other cardiovascular diseases [17,18].

There is now compelling evidence that the processes of neovascularization and vessel repair are modulated by EPC in many ways. It is believed that the mechanisms of action of EPC rely on the engraftment into vascular structures upon the differentiation into mature endothelial cells [4] In addition, EPC seem to support the function of resident endothelial cells by the secretion of paracrine factors [9,19]. Recent observations suggest that these two processes are orchestrated by two distinct EPC sub-populations named early and late outgrowth EPC [11,20,21]. These two EPC subtypes present different phenotypic markers which are indicative of their separate lineage derivation. Early EPC are a monocytic type of cells whereas late EPC display surface antigenic markers and morphology of mature endothelial cells. Although hypoxia promotes the secretion of many cytokines in both EPC types [12], early EPC seem to have prevalently secretory functions and promote the integration of late EPC into the growing capillaries [21]. The findings reported in the present study support the concept that early EPC are cells with remarkable secretory capacity. The array of soluble factors secreted by early EPC includes a number of enzymes like matrix proteins, growth factors and cytokines. Amongst the cytokines released by early EPC, IL-6 and IL-11 were reported to have anti-inflammatory and cyto-protective properties rescuing endothelial cells from H_2O_2 induced cell death [22]. Moreover, other studies provided solid evidence that VEGF, HGF and IGF-1 – all secreted by early EPC – exert strong cyto-protective and pro-survival activity inducing the expression of anti-apoptotic [23] and antioxidant [24,25] proteins in endothelial cells.

In agreement with these findings and with the notion of the relatively high resistance of EPC against oxidative injury [2,26], our results suggest that mature endothelial cells challenged by oxidative stress benefit from the capacity of EPC to enhance the endothelial antioxidant defense by paracrine mechanisms. Our data advocate the hypothesis that Bcl-2 is a key modulator of the oxidative state and angiogenic functions of endothelial cells [27]. These results further corroborate the concept that the secretion of survival factors in support of differentiated cells is a pivotal feature of stem and progenitor cells [28], as suggested by the protective capacity of conditioned medium from adipose stem cells on dermal fibroblast exposed to H_2O_2 [29]. Interestingly, we found that the anti-apoptotic effect described in the present work is specific for EPC since conditioned medium for HUVEC was not able to support the viability and functionality of differentiated endothelial cells. To identify key factors mediating the cyto-protective effect of EPC-CM we blocked the activity of some factors contained in the EPC-CM with neutralizing antibodies. As shown in Fig. 5, the neutralization of these selected factors alone or in combination is not sufficient to attenuate the cyto-protective properties of EPC-CM. These results suggest that other, even unidentified factors or a synergic combination of them might be responsible for the anti-apoptotic effect of EPC-CM. Further research is needed to investigate and define these components and paracrine functions of early EPC. Another aspect to be clarified is how the secretory activity of early EPC and the downstream signaling of activated pathways are affected by cardiovascular risk factors associated with oxidative stress. Recent in vitro findings suggest that the physiological changes induced in EPC by an inflammatory environment as in atherosclerosis might contribute to endothelial dysfunction [30].

We have previously described the remarkable properties of EPC-CM to enhance angiogenic functions in endothelial functions in vitro and induce neovascularization in vivo [5]. The present work supplements these findings and provides a mechanistic explanation of the pro-survival properties of EPC-CM under conditions of increased oxidative stress. It hereby supports the hypothesis that the cyto-protective and angiogenic properties of a cocktail of factors obtained from in-vitro cultured EPC offer a valid and valuable alternative to cell transplantation to induce therapeutic angiogenesis.

In conclusion we demonstrate that early EPC not only promote the angiogenic activity of vascular cells but also confer cyto-protection upon endothelial cells by regulation of the antioxidant capacity. We suggest that the induction of pro-survival signals in resident endothelial cells is a key mechanism of early EPC contribution to angiogenesis and regeneration of ischemic tissue.

Author contributions

Design and concept: Z.Y., S.D.S., C.K. Data collection and analysis: Z.Y., S.D.S., J.V., M.W.v.B., D.F., C.K. Manuscript writing: Z.Y., S.D.S., M.W.v.B., C.K. Proofreading: all.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.02.022.

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Supplementary Table

Supplementary Table 1. Cytokine secretion profile of early EPC under 72 hours hypoxic conditioning. The presence and relative levels of a total of 174 cytokines in EPC-CM and HUVEC-CM were screened using a commercially available human cytokine antibody array C series 2000 (AAH-CYT-2000, Ray Biotech, USA). The expression of each cytokine was measured by chemiluminescence intensity by ChemiDoc™ XRS (Bio-Rad AG, Swizerland) and analyzed using ImageJ. The secretion levels were expressed as relative to the positive control in column 1 and 2. The fold-increase over HUVEC-CM was expressed in column 3 (EPC-CM / HUVEC-CM).

Cytokines	EPC-CM	HUVEC-CM	Fold-increase	Cytokines	EPC-CM	HUVEC-CM	Fold-increase
MCP-1	1.32	1.66	0.8	TECK	0.08	0.05	1.6
EGF	0.92	1.33	0.7	LAP	0.08	0.13	0.6
TIMP-2	0.9	0.82	1.1	TNF-β	0.08	0.09	0.9
IL-8	0.75	0.06	13.5	b-NGF	0.07	0.04	2.0
MMP-9	0.69	0.03	27.6	MCP-4	0.07	0.04	1.9
TIMP-1	0.62	0.43	1.4	MIP-3α	0.07	0.08	0.9
CD14	0.6	0.04	16.3	ICAM-2	0.07	0.44	0.2
sTNF RII	0.57	0.13	4.5	Cardiotrophin-1	0.07	0.05	1.4
MIP-1β	0.47	0.13	3.5	IL-13 R alpha 2	0.07	0.05	1.4
uPAR	0.45	0.11	4.0	Eotaxin-3	0.06	0.04	1.7
MDC	0.42	0.14	2.9	Axl	0.06	0.11	0.6
GRO	0.33	0.45	0.7	IGF-II	0.06	0.03	2.1
Angiogenin	0.32	1.24	0.3	SCF R	0.06	0.07	0.9
MIP-1α	0.3	0.34	0.9	IGF-I	0.06	0.04	1.6
IL-1ra	0.29	0.08	3.6	IL-2 R beta	0.06	0.05	1.3
IL-1 R II	0.28	0.12	2.4	IL-16	0.06	0.08	0.8
Fas/TNFRSF6	0.27	0.17	1.6	Activin A	0.06	0.04	1.3
IL-12 p40	0.26	0.02	15.9	HGF	0.06	0.02	3.8
AgRP	0.26	0.18	1.4	SCF	0.06	0.04	1.3
RANTES	0.23	0.13	1.7	IGFBP-6	0.06	0.07	0.8
MIF	0.22	0.07	3.1	GITR-Ligand	0.06	0.03	1.8

NAP-2	0.22	0.14	1.5	VEGF R3	0.06	0.05	1.2
Angiopoietin-2	0.21	0.82	0.3	Fas Ligand	0.06	0.04	1.7
ENA-78	0.21	0.08	2.6	Prolactin	0.06	0.06	1.0
FGF-9	0.2	0.08	2.7	Acrp30	0.06	0.07	0.8
IL-6 R	0.2	0.05	4.0	NT-3	0.06	0.07	0.8
M-CSF	0.2	0.10	2.0	MPIF-1	0.06	0.03	2.2
FGF-4	0.2	0.06	3.3	Tie-1	0.06	0.05	1.2
CXCL- 16	0.19	0.08	2.3	IL-2 R alpha	0.05	0.04	1.5
IL-2 Rα	0.19	0.02	9.3	PDGF AB	0.05	0.11	0.5
Siglec-5	0.18	0.05	3.4	CCL-28	0.05	0.05	1.1
MMP-1	0.18	0.22	0.8	Osteoprotegerin	0.05	0.06	0.9
IGFBP-2	0.17	0.29	0.6	IL-9	0.05	0.04	1.3
ICAM-1	0.17	0.12	1.5	bFGF	0.05	0.13	0.4
MMP-13	0.17	0.05	3.3	BLC	0.05	0.04	1.1
MCP-2	0.16	0.05	3.3	EGF-R	0.05	0.05	1.0
TRAIL R4	0.16	0.10	1.6	IL-1α	0.05	0.07	0.7
VEGF-D	0.15	0.07	2.2	IGFBP-1	0.05	0.05	0.9
GITR	0.15	0.06	2.7	MIP-3β	0.05	0.10	0.5
sTNF RI	0.15	0.09	1.7	IL-18 BP alpha	0.05	0.03	1.6
ALCAM	0.14	0.32	0.4	TGF-alpha	0.05	0.05	0.9
PIGF	0.14	0.16	0.8	SDF-1beta	0.04	0.04	1.0
TGF beta 2	0.14	0.11	1.2	CNTF	0.04	0.04	1.1
Leptin R	0.13	0.08	1.7	MIP-1δ	0.04	0.04	1.2
Oncostatin M	0.13	0.09	1.5	TARC	0.04	0.05	0.8
Eotaxin-2	0.13	0.09	1.4	TNF-α	0.04	0.05	0.9
IGFBP-3	0.13	0.09	1.4	TGF-β3	0.04	0.06	0.7
GDNF	0.12	0.11	1.1	Endoglin	0.04	0.04	1.1
VEGF	0.12	0.06	2.1	FGF-6	0.04	0.04	1.1
sgp130	0.12	0.17	0.7	IL-17	0.04	0.04	0.9
Eotaxin	0.12	0.10	1.3	CK β 8-1	0.04	0.04	1.0
IL-12 p70	0.12	0.15	0.8	PECAM-1	0.04	0.07	0.5
BMP-5	0.12	0.06	1.9	PARC	0.04	0.05	0.8
NT-4	0.12	0.09	1.3	IFN-γ	0.04	0.04	1.0
IP-10	0.12	0.11	1.1	DR6 (TNFRSF21)	0.04	0.04	1.0
BMP-6	0.11	0.08	1.4	IL-10 R beta	0.04	0.02	1.5
LIGHT	0.11	0.10	1.1	BMP-7	0.04	0.03	1.1
TRAIL R3	0.11	0.10	1.0	SDF-1	0.04	0.04	0.8
TIMP-4	0.1	0.10	1.0	ICAM-3	0.04	0.02	1.9
BMP-4	0.1	0.08	1.3	MIG	0.03	0.06	0.6
L-Selectin	0.1	0.05	2.2	Fractalkine	0.03	0.05	0.7
B7-1(CD80)	0.1	0.06	1.8	TGF-β1	0.03	0.07	0.5
IL-6	0.1	0.95	0.1	E-Selectin	0.03	0.03	1.0
Amphiregulin	0.1	0.11	0.9	GM-CSF	0.03	0.02	1.3

HCC-4	0.1	0.03	3.2	GCP-2	0.03	0.04	0.7
IGF-I SR	0.1	0.05	1.9	IL-5 R alpha	0.03	0.03	1.1
MSP-α	0.1	0.08	1.2	IL-21R	0.03	0.03	1.2
Dtk	0.1	0.06	1.6	IL-15	0.03	0.04	0.7
GRO-α	0.09	0.06	1.4	IL-1β	0.03	0.06	0.5
CTACK	0.09	0.06	1.5	I-309	0.02	0.03	0.9
IL-18 R beta	0.09	0.06	1.5	PDGF R beta	0.02	0.02	1.1
BDNF	0.09	0.13	0.7	NGF R	0.02	0.03	0.7
BTC	0.09	0.08	1.2	VEGF R2	0.02	0.03	0.9
IL-1 R4/ST2	0.09	0.06	1.4	Fit-3 Ligand	0.02	0.04	0.7
PDGF-BB	0.09	1.01	0.1	IL-3	0.02	0.03	0.7
Thrombopoietin	0.09	0.06	1.4	PDGF R alpha	0.02	0.02	1.2
PDGF AA	0.09	0.25	0.3	IL-11	0.02	0.04	0.4
M-CSF R	0.08	0.04	2.0	FGF-7	0.02	0.02	0.8
Leptin	0.08	0.08	1.0	IGFBP-4	0.02	0.38	0.0
IL-10	0.08	0.04	1.9	IL-13	0.02	0.02	1.1
I-TAC	0.08	0.08	1.0	IL-2 R gamma	0.02	0.03	0.7
IL-1 RI	0.08	0.08	1.0	VE-Cadherin	0.02	0.01	1.5
LIF	0.08	0.06	1.3	IL-2	0.02	0.04	0.5
Tie-2	0.08	0.08	1.1	IL-5	0.02	0.03	0.5
Lymphotactin	0.08	0.09	0.9	MCP-3	0.02	0.02	0.8
IL-4	0.08	0.04	1.7	GCSF	0.01	0.02	0.6
ErbB3	0.08	0.05	1.6	IL-7	0	0.02	0.2

2.4 Paper IV – Endothelial progenitor cells induce a phenotype shift in differentiated endothelial cells towards PDGF/PDGFR-β axis mediated angiogenesis

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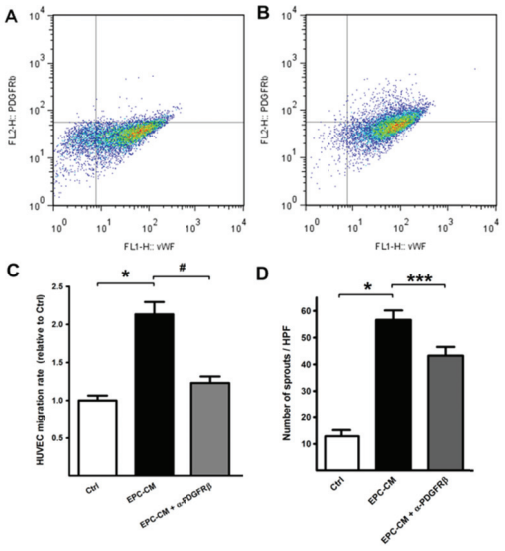
Endothelial Progenitor Cells (EPC) contributes to neovascularization and regeneration of injured endothelium mainly by secretion of multiple angiogenic growth factors. The aim of this study was to investigate the effect of the *in vitro* generated EPC-conditioned meidum (EPC-CM) on activation of the angiogenic function of differentiated endothelial cells and the induction of PDGFR-β expression. Endothelial cells incubated with EPC-CM showed significant increase of proliferation (Cyquant® NF proliferation assay), migration (Transwell® assay), tube formation (Matrigel™ assay), and the expression of PDGFR-β in both total and phosphorylated levels (FACS, Western blot, PCR). Incubation in presence of a PDGFR-β neutralizing antibody significantly attenuated the stimulatory effect of EPC-CM but cannot inhibit the PDGFR-β expression. In contrast addition of recombinant PDGF-BB to the control medium had no effect. These data suggested that the expression of PDGFR-β is critical in response to EPC-CM while the upregulation of its expression by EPC-CM are likely through signal pathways other than PDGF/PDGFR axis.



EPC-CM activated the angiogenic function of differentiated endothelial cells and the induction of PDGFR-β expression. Co-expression of vWF and PDGFR-β on EC was measured using dual color FACS analysis. Compare to control medium (**A**), after EPC-CM exposure the phenotype of EC shifted towards PDGFR-β^+ (**B**). The blockage of the PDGFR-β using neutralizing antibody significantly a attenuated the stimulatory effect of EPC-CM on EC migration (**C**) and tube formation (**D**).

Endothelial Progenitor Cells Induce a Phenotype Shift in Differentiated Endothelial Cells towards PDGF/PDGFRβ Axis-Mediated Angiogenesis

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Non-standard Abbreviations and Acronyms:

EC: Endothelial cell(s)

EPC: Endothelial progenitor cell(s)

EPC-CM: Endothelial progenitor cell conditioned-medium

HUVEC: Human umbilical vein endothelial cells

PDGF: Platelet derived growth factor

PDGFRβ: Platelet derived growth factor-receptor beta

α-PDGFRβ: anti-PDGFRβ antibody

rhPDGF-BB: recombinant human PDGF-BB

Abstract

Background: Endothelial Progenitor Cells (EPC) support neovascularization and regeneration of injured endothelium both by providing a proliferative cell pool capable of differentiation into mature vascular endothelial cells and by secretion of angiogenic growth factors.

Objective: The aim of this study was to investigate the specific role of PDGF-BB secreted by EPC on the angiogenic behavior of differentiated endothelial cells.

Methods & Results: Conditioned medium from human EPC (EPC-CM) cultured in hypoxic conditions contained substantially higher levels of PDGF-BB as compared to normoxic conditions ($P < 0.01$). EPC-CM increased proliferation (1.39-fold; $P < 0.001$) and migration (2.13-fold; $P < 0.001$) of isolated human umbilical vein endothelial cells (HUVEC), as well as sprouting of vascular structures from *ex vivo* cultured aortic rings (2.78-fold increase; $P = 0.01$). The capacity of EPC-CM to modulate the PDGFRβ expression in HUVEC was assessed by western blot and RT-PCR. All the pro-angiogenic effects of EPC-CM on HUVEC could be partially inhibited by use of a neutralizing antibody ($P < 0.01$). EPC-CM triggered a distinct up-regulation of PDGFRβ (2.5 ± 0.5; $P < 0.05$) and its phosphorylation (3.6 ± 0.6; $P < 0.05$) in HUVEC. This was not observed after exposure of HUVEC to recombinant human PDGF-BB alone.

Conclusion: These data indicate that EPC-CM sensitize endothelial cells and induce a pro-angiogenic phenotype including the up-regulation of PDGFRβ, thereby turning the PDGF/PDGFRβ signaling-axis into a critical element of EPC-induced endothelial angiogenesis. This finding may be utilized to enhance EPC-based therapy of ischemic tissue in future.

Keywords: Angiogenesis, cytokines, endothelial progenitor cells, PDGF, revascularization

Introduction

Over the last couple of years, the discovery and characterization of stem and progenitor cells have opened the doors to an exciting new field in biomedicine and potentially new therapeutic options. Given the prevalence of cardiovascular disease and the tremendous interest in re-vascularisation of ischemic tissue, cardiovascular medicine has rapidly become a popular area of investigation with regard to cell-based therapy. In the meantime, many experimental studies have described the enormous regenerative and pro-angiogenic potential of endothelial progenitor cells (EPC). Additionally, clinical studies have consistently linked a low number of circulating EPCs to higher morbidity and mortality in patients with or at risk for cardiovascular diseases [1,2]. This evidence along with the fact that EPCs circulate in the peripheral blood, from which they readily can be isolated, makes them an attractive and promising candidate for cell-based pro-angiogenic therapy of ischemic organs.

The two major mechanisms by which EPC are thought to enhance postnatal angiogenesis is by physical incorporation of the cell into a growing vascular network and by secretion of pro-angiogenic cytokines like VEGF, PDGF and other growth factors in the proximity of sprouting endothelium [3,4,5,6,7,8]. A number of studies have shown promising results of therapeutic angiogenesis in animal models of myocardial infarction, PAOD and other ischemic diseases that were used to study the angiogenic effect of EPC transplantation [6,7,9]. Recently, increasing attention has been given to the capacity of EPC to support the activity and functioning of resident differentiated cells by paracrine mechanisms. Urbich et al. have shown that the combination of soluble growth factors released by EPC, such as VEGF A, stromal cell derived factor-1 (SDF-1), insulin-like growth factors-1 (IGF-1), and hepatocyte growth factor (HGF) are able to promote migration of endothelial cells (EC) and cardiac resident progenitor cells in vitro and in vivo[7].

In previous studies we have demonstrated the significance of the paracrine activity of EPC in neovascularization of various tissues and in models of different cardiovascular conditions, respectively [10,11,12]. Furthermore, we partially characterized the composition of EPC-derived cytokines and its systemic effects after therapeutic administration [10]. Most recently, we also demonstrated how soluble factors secreted by EPC confer strong cyto-protective properties upon differentiated endothelium through modulation of intracellular antioxidant defensive mechanisms and pro-survival signals in differentiated endothelial cells[13]. Yet, the angiogenic cytokine signaling and paracrine function of EPC are still ill-defined, and the exact underlying mechanisms leading to increase angiogenesis and sprouting of resident endothelial cells are poorly understood for the most part.

We and other investigators have shown that EPC, capable of inducing a strong angiogenic response, express and release PDGF-BB in substantial amounts [4,7,10]. The aim of this study was to investigate the role of the PDGF/PDGFRβ axis in the interaction between EPC and differentiated endothelial cells. Specifically, we sought to determine the relation between PDGF-BB secreted by EPC and the expression of PDGFRβ in differentiated endothelium as well as the significance of the PDGFRβ receptor for the angiogenic response. To this end, we performed a comprehensive analysis of the impact of EPC-derived cytokines on the functional and phenotypic properties of differentiated endothelial cells.

Materials & Methods

Ethics Statement

All protocols received full approval from the Cantonal and the Institutional Ethics Review Board at the University of Bern, Switzerland. Written informed consent was obtained from all donors.

Cell culture and conditioned medium preparation

HUVEC were isolated from umbilical cord by collagenase digestion [14] and cultivated in complete endothelial cell growth medium (EGM-2-MV, Lonza, Switzerland) containing 5% of fetal bovine serum (FBS). All experiments were performed using cells between passages 2 to 6. The purity of endothelial cell cultures and endothelial cell characteristics were confirmed by *UEA-1* lectin binding and DiI-Ac-LDL uptake. Peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy human volunteers by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, Switzerland) as described previously [15]. Cells were plated on culture dishes coated with human fibronectin (Sigma-Aldrich, Switzerland) and maintained in endothelial cell basal medium-2 (EBM-2) (Clonetics, San Diego, CA) supplemented with EGM-2 MV SingleQuots containing 5% fetal bovine serum (FBS), human VEGF-1, human fibroblast growth factor-2 (FGF-2), human epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1), and ascorbic acid. After 4 days in culture, non-adherent cells were removed and adherent cells were trypsinized and re-plated at a density of 1 x 10^6 per well through day 7 [15]. To produce human EPC-conditioned medium (EPC-CM), EPC were cultured for 72 hours under hypoxic conditions (1.5% O_2, 5% CO_2, 93% N_2) in a humidified gas-sorted hypoxic incubator using growth facto-free endothelial cell basal medium-2 (EBM-2, Lonza, Switzerland) with 1% FBS. EPC-CM was then centrifuged, sterile filtered with a 0.22 µm filter (TPP, Switzerland) and stored at -80°C until use. Growth factor-free EBM-2 with 1% FBS was used as control medium in all experiments. The concentration of PDGF-BB in EPC-CM was assessed by the Luminex system (Bio-Rad, Switzerland) following the manufacturer's instructions, and as published previously [10].

The effects of five different culture mediums were compared against each other in the in-vitro experiments. This included control medium and EPC-CM (as described above). Furthermore, EPC-CM supplemented with 1 µg / ml of anti-PDGFR β antibody (α-PDGFRβ, AF385, R&D Systems, UK), and control medium containing recombinant human PDGF-BB (rhPDGF-BB) in a final concentration of 100 pg / ml and 100 ng / ml, respectively, were compared.

Survival / proliferation assay

HUVEC were seeded into 96-well plates coated with 1% gelatin at a density of 5 x 10^3 / well and cultured in control medium for 24 hours before experiments. Thereon, culture medium was replaced by the experimental culture mediums and cells were cultured for further 24 hours. Finally, the number of viable cells was assessed by use of the CyQuant® NF kit (Molecular Probes, Switzerland). The proliferation rate was expressed as relative values standardize to the control group.

Migration assay

HUVEC migration was analyzed using Costar® transwell inserts with 8 µm polycarbonate filters (Corning, The Netherlands) in 24-well plates coated overnight and at 4°C with a 1% gelatin solution containing 1 mg / ml fibronectin. One hundred and fifty µL of different medium was placed in the lower chamber of the trans-well system and 5 x 10^4 HUVEC in control medium were placed above the filter and incubated for 12 hours at 37°C. Thereafter non-migrated cells were removed from the system. The migrated cells were fixed in 4% PFA for 20 min at 4°C and stained with crystal violet. The cell number was counted in 4 random high power fields and expressed as values standardized to the control group.

In vitro capillary formation

In vitro formation of capillary-like structures was assessed using cell culture on growth factor reduced Matrigel™ (Becton Dickinson, Switzerland). Forty-thousand cells per well were resuspended in EPC-CM and the other experimental culture mediums, respectively, and seeded on the polymerized Matrigel™ layer. Endothelial cell tube formation was assessed at 8 hours of incubation. Digital microphotographs were taken from three randomly selected high power fields and tube formation was assessed measuring the total length and number of sprouts with the aid of ImageJ (http://rsb.info.nih.gov/ij/).

Ex vivo aortic ring assay

Aortas from 2-month-old Wistar rats were isolated, flushed with PBS solution to remove blood, and freed from adventitial tissue. Aortic rings of 1-mm thickness were placed individually in 24-well plates coated with growth factor reduced Matrigel™ , and incubated at 37 °C for 5 days in the 5 different culture mediums [16]. Quadruplicates were performed for each culture condition. After 5 days of culture, pictures of the aortic rings and the sprouting cells surrounding it were taken at 10× magnification from each of the four quadrants of the aortic ring. The two maximal extensions of the tubular structures sprouting from the aortic ring wall were measured in each quadrant. Average

sprout length was calculated as the mean of the four samples per condition and eight values obtained from each ring.

Assessment of PDGFRβ expression on HUVEC

The expression of PDGFRβ on HUVEC after exposure to EPC-CM or control medium culture was measured by means of western blotting and real-time PCR. Both total (AF385, R&D Systems, UK) and phosphorylated PDGFRβ (07-021, Millipore, Switzerland) levels were measured by use of respective antibodies. The results were normalized to the expression level of α-tubulin (Sigma-Aldrich, Switzerland) and expressed as relative values in comparison to the control group. Total mRNA isolated from HUVEC (RNeasy® Mini Kit, Qiagen, Switzerland) was transcribed to cDNA (SuperScript® VILO™ cDNA Synthesis Kit, Invitrogen, Switzerland). The amount of PDGFRβ mRNA was quantified by real time PCR using Taq primer (Hs00387364, Applied Biosystems, Switzerland). The results were normalized to the mRNA level of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905, Applied Biosystems, Switzerland) and expressed as relative values compared to the control group.

Dual-color Flow cytometric analysis was used to verify the absence of smooth muscle cell contamination in our HUVEC cultures and in order to verify the cell surface expression of PDGFRβ after EPC-CM stimulation for 24 hours. To this end, cells were fixed in 4% PFA for 10 min at 37°C, permeabilized in 90% methanol for 30 min on ice, and stained with both α-PDGFRβ (AF385, R&D Systems, UK) and anti Von Willebrand factor (vWF) antibody (AB7356, Millipore, Switzerland). The percentage of vWF / PDGFRβ double positive fraction of HUVEC was measured individually in cells cultured in control medium, EPC-CM, and control medium containing 100 pg / ml or 100 ng / ml rhPDGF-BB.

Statistical analysis

All experiments were performed in at least triplicates. If not otherwise stated, data are presented as mean ± standard error of the mean (SEM). Unpaired Student's t-test and one-way ANOVA with Scheffe's test for posthoc comparison were used to compare group means, after testing for normality and equal variance of the data. RT-PCR data were analyzed using REST© - Relative Expression Software Tool (http://www.wzw.tum.de/gene-quantification/) to compute relative differences in expression levels, and the Bonferroni correction was applied for multiple testing. All other statistical analyses were carried out in STATA (Stata Corporation, College Station, TX, Version 10.1 for Apple). Statistical significance was inferred at a 2-sided $P \leq 0.05$.

Results

Hypoxia increases the secretion of PDGF-BB from EPC

Previous studies have shown that the expression of paracrine factors released by EPC including VEGF and FGF is significantly induced by hypoxia [17,18]. We determined the release of PDGF-BB by EPC subject to hypoxia in comparison to cells cultured under normoxic conditions. EPC-CM obtained from cultures in hypoxia (111.6 ± 27.0 pg / ml) showed a five-fold increased level of PDGF-BB as compared to normoxic cultures (19.9 ± 2.2 pg / ml, $P < 0.01$).

The PDGFRβ signaling axis is critical for EPC stimulated angiogenic activity of HUVEC

In vitro EPC-CM increased HUVEC proliferation 1.39 ± 0.04 -fold ($P < 0.05$) and EC migration along the cytokine gradient by a factor of 2.13 ± 0.16 ($P < 0.05$) (Figure 1A-1B). When using EPC-CM supplemented with a PDGFRβ neutralizing antibody AF385, the EC failed to migrate along the EPC-CM gradient as sufficient as EPC-CM alone. Furthermore, HUVEC proliferation in presence of EPC-CM was decreased by the neutralizing antibody. In order to test whether PDGF alone is sufficient to elicit this angiogenic activity in differentiated endothelial cells, HUVEC were exposed to control medium supplemented with rhPDGF-BB at a similar concentration as found in EPC-CM (100 pg / ml) or at a 1000-fold higher concentration (100 ng / ml). None of these two concentrations of rhPDGF-BB alone were able to induce an increase in proliferation or migration of HUVEC ($P > 0.05$, Figure 1A-B).

Consistently with the above findings,, exposure of HUVEC to EPC-CM resulted in a significantly increased number and length of capillary-like structures in growth factor reduced Matrigel™ as compared to control medium (56.63 ± 3.56 vs. 12.88 ± 2.36 / HPF in sprout numbers, 5521 ± 268.3 vs. 1636 ± 1448.7 µm / HPF, $P < 0.0001$). Again, blocking of PDGFRβ by AF385 caused partial inhibition of the EPC-CM effect (43.25 ± 3.30 / HPF in sprout numbers, 4338 ± 170.2 µm / HPF; $P < 0.001$ compared to EPC-CM group) whereas rhPDGF-BB supplementation of control medium did not increase the formation of an endothelial network in either the 100 pg / ml or the 100 ng / ml concentration (Figure 2).

Furthermore, in the aortic ring assay, extensive sprouting occurred when the aortic rings were cultured in EPC-CM (145.93 ± 7.69 vs. 52.48 ± 9.76 µm in control, $P < 0.001$; Figure 3B) and this sprouting was lowered in the presence of the PDGFRβ neutralizing antibody AF385 (59.78 ± 7.99 µm, Figure 3C). The high-dose supplementation of control medium with rhPDGF-BB (100ng / ml) showed results comparable to EPC-CM in this assay. (133.95 ± 11.66 µm, $P < 0.001$; Figure 3D-E).

EPC-CM up-regulates PDGFRβ expression on HUVEC

To further investigate the signaling pathway underlying the above findings suggesting that PDGFRβ plays a major role in the EPC-CM stimulated angiogenic response of differentiated EC, we measured the transcriptional and translational levels of PDGFRβ in HUVEC. Upon EPC-CM stimulation, the mRNA level of PDGFRβ was up-regulated 2.8 ± 0.6 -fold compared to control medium ($P < 0.05$, Figure 4A). Western blotting also showed a significantly increased amount of both total PDGFRβ (2.31 ± 0.56 -fold, $P < 0.05$) and its phosphorylated form (3.67 ± 0.66 -fold, $P < 0.05$) expressed by HUVEC when incubated with EPC-CM (Figure 4B-D). Moreover, FACS analyses revealed a clear shift of HUVEC from a vWF^+ / $PDGFR\beta^-$ towards a vWF^+ / $PDGFR\beta^+$ double positive phenotype after EPC-CM incubation (Figure 5A-B, E; $P < 0.001$). Neutralizing the PDGFRβ bioactivity with AF385 had only marginal effect on blocking the stimulation by EPC-CM, and resulted in a similar upregulation of the PDGFRβ (Figure 5C, E; $P < 0.01$). However, such an increased expression of PDGFRβ in HUVEC could not be elicited by adding 100 pg / ml or 100 ng / ml of rhPDGF-BB to the control medium (Figure 5C-D, E).

Discussion

In this study we sought to determine what role PDGF-BB and PDGFRβ play in angiogenesis as part of the interaction between EPC and differentiated endothelial cells. In an analysis of secreted cytokines we found that PDGF-BB is released by EPC in substantial quantities. Moreover, we found that incubation of EPC in reduced oxygen tension leads to a significant, more than fivefold increase in release of PDGF-BB as compared to normoxic conditions. These findings are in agreement with the observations of enhanced release of angiogenic factors by different cell types including hematopoietic stem cells and EPC[10,17,18]. Given the reasonably high levels of PDGF-BB we were interested to elaborate the impact of this specific cytokine on differentiated endothelial cells and its potential implications for angiogenesis.

In multiple tissue culture assays of angiogenesis the exposure of endothelial cells to EPC-CM resulted in increased proliferation, migration and organization of the cells into capillary structures. All of these effects were effectively inhibited by adding a neutralizing anti-PDGFRβ antibody to EPC-CM. Notably, substitution of control medium with even high concentrations of PDGF-BB achieved only minimal angiogenic effects in all experimental settings that we tested. In conjunction with the increased expression of PDGFRβ that we found in EC after incubation with EPC-CM these findings strongly suggest that endothelial cells react in a hitherto undescribed fashion to cytokines released by EPC. Our data suggest that EPC induce a phenotype change of EC towards increased expression of PDGFRβ and sensitivity to PDGF as a pro-angiogenic growth factor. This finding is in line with our previous report that demonstrated a distinct capability of EPC to induce and modulate strong cyto-protective properties in differentiated endothelium through modulation of intracellular antioxidant defensive mechanisms and pro-survival signals[13].

Angiogenesis is a complex process involving multiple cell lines and cytokines that encompasses two major steps, sprouting and proliferation of the endothelium resulting in formation of a primitive vascular plexus and maturation of the latter which includes the incorporation of perivascular cells into the forming vasculature [3]. The role of EPC in postnatal angiogenesis is currently under intense investigation. Multiple lines of evidence suggest that EPC contribute to angiogenesis and vascular repair by proliferation and differentiation into mature EC (structural component) as well as by secretion of multiple cytokines (paracrine component) impacting on the fate and function of various cell types including endothelial cells, perivascular cells and progenitor cells [4,5,6,7,9]. In spite of recent advances in the field the exact composition of factors released by EPC and their relevance in angiogenesis are still poorly understood. However, several investigators have demonstrated the complexity and potency of the EPC secretome that includes many angiogenic factors such as VEGF,

SDF-1 or IGF-1 [7,19]. Our data are in line with these findings and add further evidence that EPC convey critical, angiogenic signals upon differentiated endothelial cells.

PDGF is a well-characterized trophic factor crucial for the survival and functioning of various cells including pericytes and SMC. It is also known that PDGF-BB is expressed by the endothelium nourishing the adjacent perivascular cells [3]. Furthermore, the expression of PDGF receptors has been reported in hemangioblast precursors where it accelerates the differentiation of endothelial cells [20]. However, PDGFRβ has not typically been associated with endothelial cells susceptibility and capability to participate in postnatal angiogenesis. Hence, it is an obvious anticipation that PDGF-BB secreted by EPC increases recruitment and proliferation of perivascular cells in tissues undergoing active angiogenesis. The finding that PDGF-BB is expressed in the EPC secretome, increases endothelial cell migration and capillary formation but not EC proliferation, and the specific inhibition of those processes by blocking of PDGFRβ is intriguing and strongly suggests a critical role of the PDGF / PDGFRβ axis for endothelial cells in the immediate EPC: EC interaction. More specifically, our data indicate that the PDGF / PDGFRβ axis is a critical modulator of the angiogenic response evoked in endothelial cells by EPC. Inhibition of PDGFRβ drastically mitigated the angiogenic response of EC exposed to the EPC secretome but did not extinguish it fully. At the same time, PDGF-BB alone was not a sufficient to promote a angiogenic behavior in endothelial cells. These results suggest that EPC-CM activates multiple pathways while PDGF alone is not sufficient to trigger an effective angiogenic response in EC. These findings could probably best be explained by modulation of classic angiogenesis signaling pathways (e.g. VEGF/VEGFR-2) by the PDGF/PDGFRβ axis. However, this specific, novel hypothesis warrants further investigation.

Although, endothelial cells usually do not express PDGFRβ in high quantities and the relevance of this receptor is unclear in resting endothelium, current literature suggests that under circumstances of increased angiogenesis the expression of PDGFRβ is up-regulated [21,22]. In order to explore this potential explanation for our observations, we determined expression of PDGFRβ in the endothelial cells exposed to EPC-CM. Indeed, we found significantly increased gene and protein expression of PDGFRβ as well as an increased phosphorylation of the receptor in endothelial cells that were exposed to EPC-CM. Also, we show that differentiated cells continue to express EC marker while the expression of PDGFRβ on EC increases. In other words, we suggest that EPC-CM is capable of inducing a change in endothelial phenotype towards increase sensitivity for PDGF, making the latter potentially an effective modulator of endothelial function in the process of angiogenesis. This theory would be consistent with several clinical trials that have shown a significantly increase angiogenic response when cytokines like VEGF are combined with PDGF [23,24], although the impact on perivascular cells certainly is a major reason for the benefits of a combined therapy.

At this point we do not have a definitive answer as to what exactly in the cytokine cocktail from EPC causes this phenotype change in endothelial cells, and further studies will be needed to address this specific question in detail. Also, a limitation of this study is the focus on a single cytokine and its receptor. This must be taken into consideration when interpreting the presented data. We cannot preclude, that other factors released by EPC have similar effects on the endothelium as shown here for PDGF-BB / PDGFRβ. Given the complexity of the EPC secretome and the interaction between EPC paracrine factors and the endothelium, it can be assumed that the PDGF-BB/ PDGFRβ axis is just one of several pathways involved. Thus, we advocate the need for future studies to clarify the EPC:endothelium interactions in more detail.

In summary, we sought to elucidate the role of PDGF and PDGFRβ in the interaction between EPC and resident EC and we found evidence for a change in endothelial PDGFRβ expression after EPC-CM exposure. The phenotypic change was followed by increased susceptibility of the endothelium to PDGF-BB and increased angiogenesis. This new insight into the paracrine activity of EPC and the role of PDGF for the endothelium in our opinion deserves further attention and potentially offers a new pathway that can be manipulated to the end of therapeutic angiogenesis or reduction of tumor vasculature.

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Figure Legends

Figure 1. EPC-CM promotes HUVEC proliferation and migration.

EPC-CM incubation significantly increased proliferation **(A)** and migration **(B)** of HUVEC in comparison to control medium. Blocking PDGFRβ with an antibody offset the chemotactic and proliferative response of HUVEC to EPC-CM in both proliferation and migration. However, addition of recombinant human PDGF-BB at a similar content of EPC-CM (100 pg / ml) or 1000-times higher (100 ng / ml) was not able to promote significant proliferation or migration of HUVEC. *, $P < 0.0001$; #, $P < 0.05$; **, $P < 0.001$.

Figure 2. Angiogenic potential of EPC-CM on HUVEC matrigel tube formation.

EPC-CM incubation for 8 hours strikingly accelerated the formation of capillary-like structures on growth factor reduced matrigel™ **(B)** as compared to control medium incubation **(A)**. Antibody mediated PDGFRβ neutralization partially inhibited the EPC-CM accelerated tube formation **(C)**. rhPDGF-BB conditioning of control medium in either 100 ng / ml **(D)** or 100 pg / ml **(E)** concentration did not have significant effect on promoting the formation of vascular networks. The difference effect between groups was evidenced by both the number of spouts **(F)** and the total capillary length **(G)**. * and **, $P < 0.0001$; # and ##, $P < 0.0001$; ***, $P < 0.05$.

Figure 3. Angiogenic potential of EPC-CM on *ex vivo* aortic ring assays.

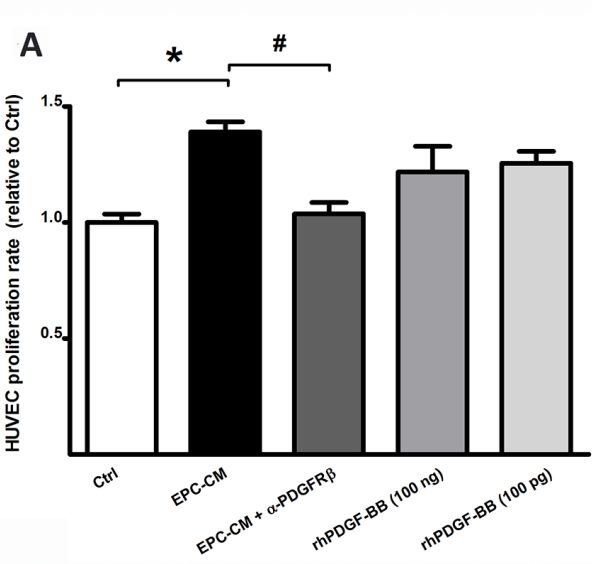
Incubation with EPC-CM **(B)** enhanced the formation of vascular outgrowth from 1 mm rat aortic ring embedded in growth factor reduced-Matrigel™ compared to control medium incubation **(A)**. This enhanced capillary outgrowth could be blocked by the addition of x mg / ml PDGFRβ antibody into EPC-CM **(C)**. Interestingly, a similar vascular sprouting extent could only be observed by stimulation aortic ring with 100 ng / ml rhPDGF-BB (1000-times concentrated than the content in EPC-CM) **(D)**, but not with the concentration at 100 pg / ml **(E)**. The extents of vascular outgrowth were quantitatively analyzed and presented by the length of the sprouts **(F)**. *, # and **, $P < 0.001$.

Figure 4. EPC-CM induces PDGFRβ expression in HUVEC.

mRNA expression of PDGFRβ was determined by real-time PCR of total RNA obtained from HUVEC cultured in EPC-CM or control medium. EPC-CM incubation promoted a 2.8-fold over-expression of PDGFRβ mRNA in HUVEC compared to control medium incubation **(A)**. Protein level of PDGFRβ was analyzed by Western blot **(B)**. The expressions of total PDGFRβ **(C)** as well as its phosphorylated form **(D)** were significantly up-regulated upon incubation with EPC-CM. *, $P < 0.05$.

Figure 5. Co-expression of EC marker and PDGFRβ after EPC-CM incubation.

Co-expression of vWF and PDGFRβ was measured using dual color FACS analysis. HUVEC were kept in control medium containing only 1% FCS or EPC-CM for 24h before the measurement. HUVEC incubated in control medium showed only a fractional amount of vWF^+ / $PDGFR\beta^+$ cells **(A)**. After EPC-CM exposure the proportion of vWF^+ / $PDGFR\beta^+$ double positive population was significantly increased, suggesting a strong phenotype shift of endothelial cells towards $PDGFR\beta^+$ **(B)**. The addition of neutralizing antibody AF385 did not block the upreguation of the PDGFRβ by EPC-CM stimulation **(C)**. However, such enhanced PDGFRβ expression could not be evoked by solely adding 100 ng / ml **(D)** or 100 pg /ml **(E)** rhPDGF-BB to the control medium. *, $P < 0.01$ compared to controls.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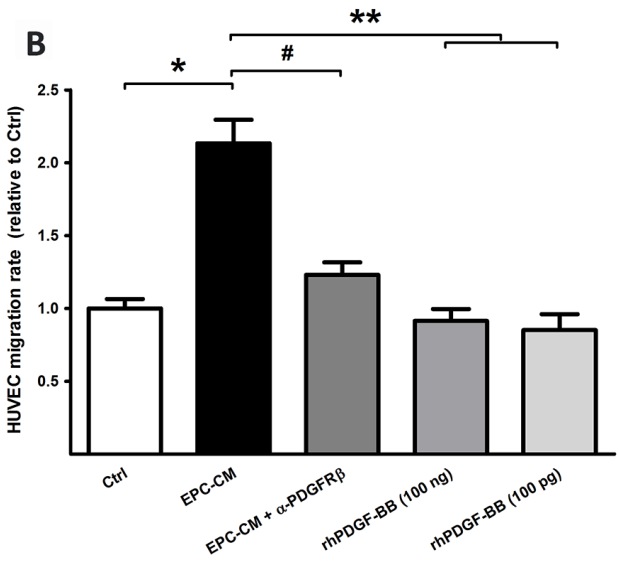
EPC-CM + α-PDGFRβ
rhPDGF-BB (100 ng)
rhPDGF-BB (100 pg)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EPC-CM + α-PDGFRβ
rhPDGF-BB (100 ng)
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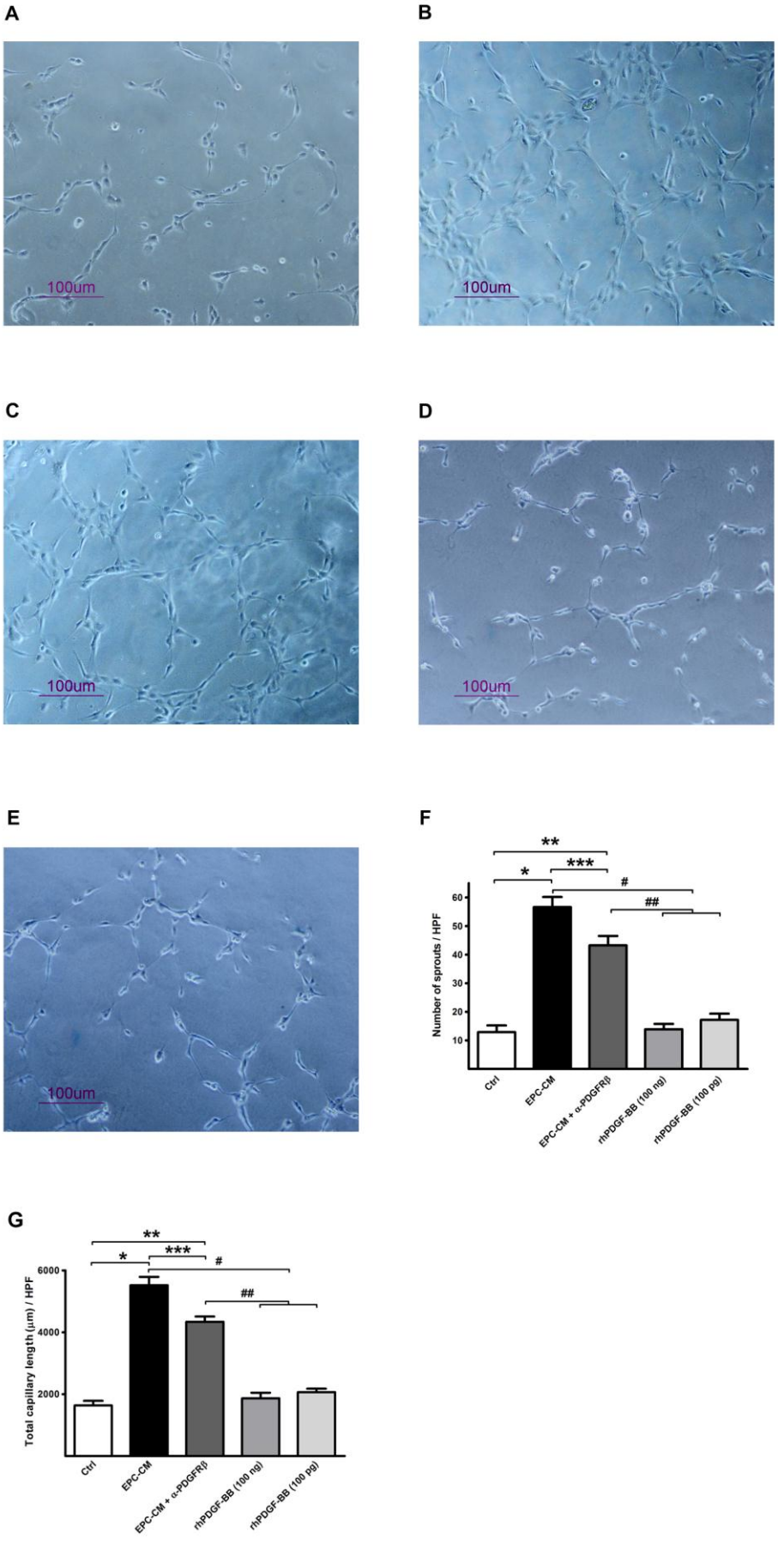
Fig. 1

Fig. 2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C
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D
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E
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rhPDGF-BB (100 ng)
rhPDGF-BB (100 pg)
G
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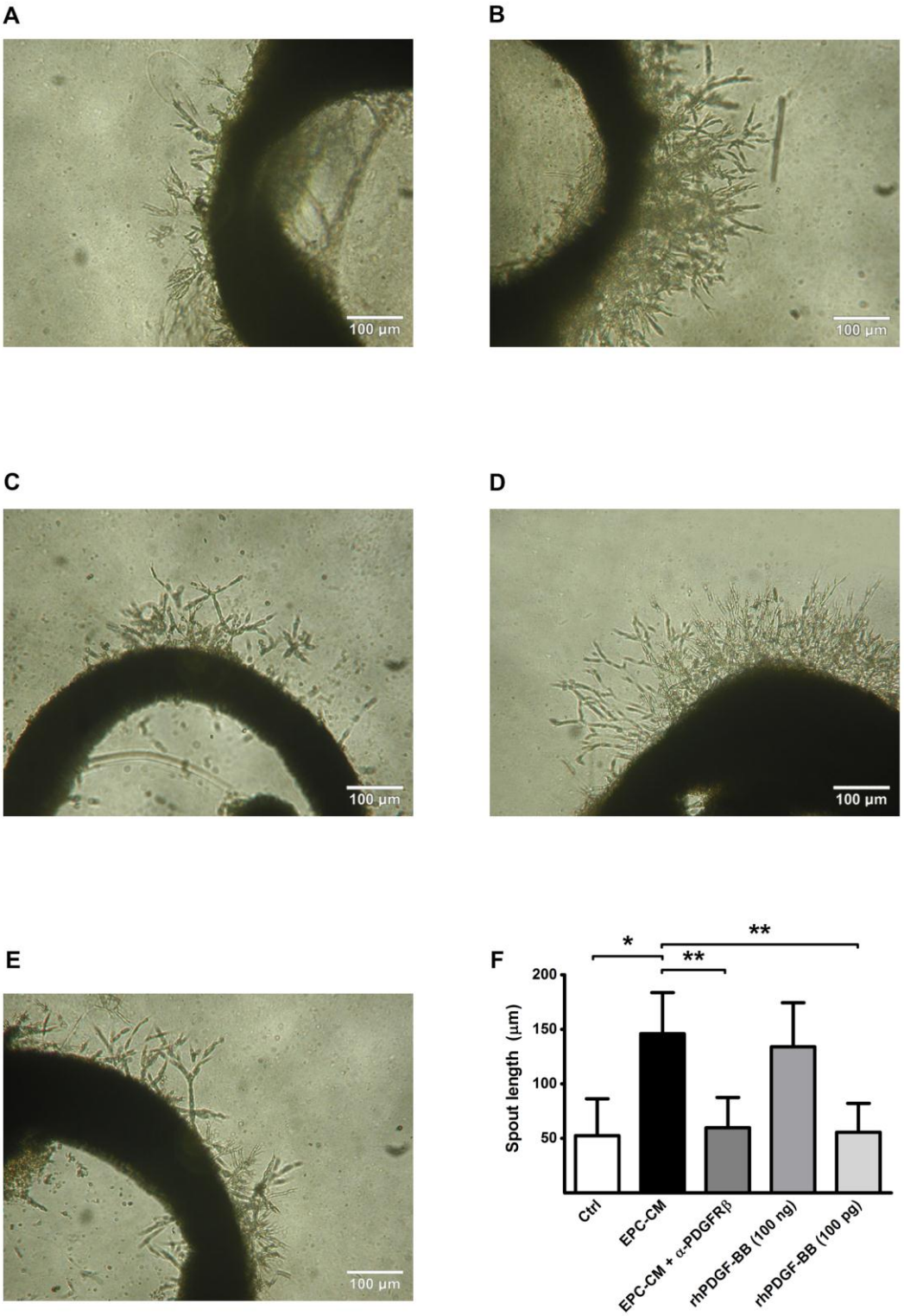
Fig. 3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Spout length (µm)
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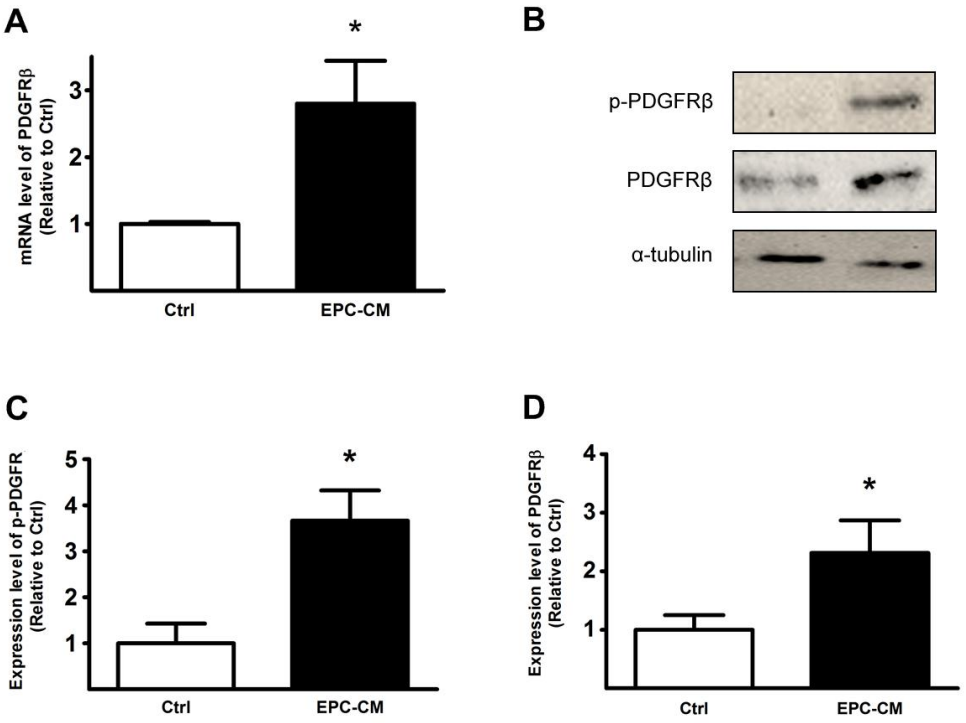
EPC-CM
EPC-CM + α-PDGFRβ
rhPDGF-BB (100 ng)
rhPDGF-BB (100 pg)

Fig. 4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p-PDGFRβ
PDGFRβ
α-tubulin
C
Expression level of p-PDGFR (Relative to Ctrl)
5
4
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D
Expression level of PDGFRβ (Relative to Ctrl)
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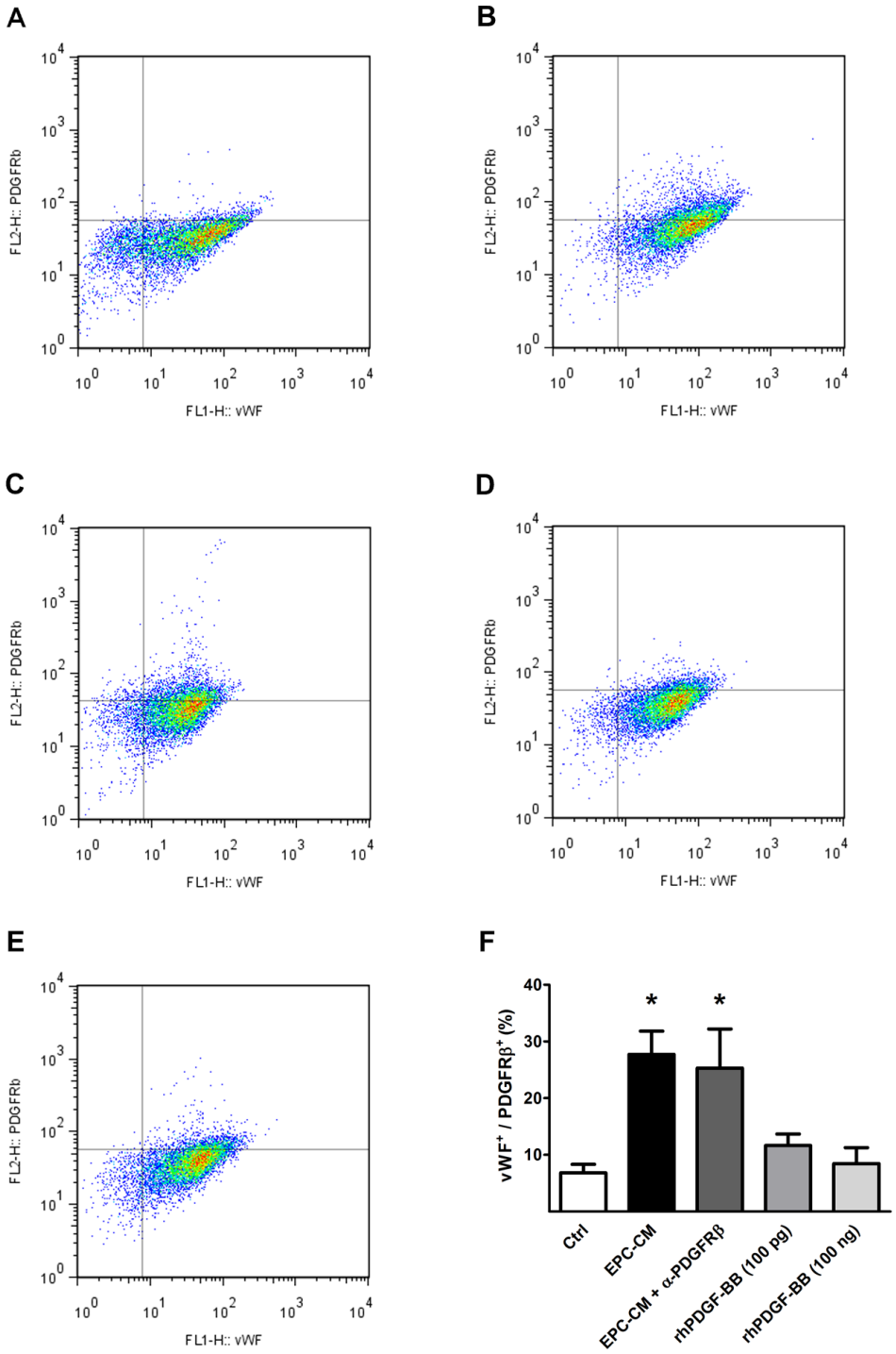
Ctrl
EPC-CM

Fig. 5


A
B
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D
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F
FL2-H:: PDGFRb
FL1-H:: vWF
vWF+ / PDGFRβ+ (%)
Ctrl
EPC-CM
EPC-CM + α-PDGFRβ
rhPDGF-BB (100 pg)
rhPDGF-BB (100 ng)

3. DISCUSSION

3.1 Discussion

Despite medical advances many patients with PAD are still not eligible for or responsive to traditional therapies and often limb amputation remains to be the only treatment option in severe PAD cases. The necessity to overcome this lack of adequate therapeutic options has prompted the development of new therapeutic alternatives. Among these, therapeutic angiogenesis based on adult stem and progenitor cells such as EPC may present an alternative strategy to promote neovascularization in ischemic tissues and appears as a promising tool for regenerative medicine.[162] This strategy has received further substantial impulse from recent advances in molecular and cellular biology like the applications of the recombinant DNA which allows the localized expression of key proteins on targeted cells by means of exogenous plasmids.[228]

In many cases, adult stem cell therapy based on EPC or MSC could exert significant regenerative benefit by both *in situ* cell differentiation and paracrine activity. Disappointingly, in spite of the encouraging results, technical and practical limitations like the invasive methods of harvest, the low abundance and the immune rejection during allogenic transplantation are major hurdles for the translation of cellular transplantation into clinical applications. The number of incorporated EPC into ischemic tissues is probably too low to explain entirely the potent therapeutic outcome. On the other hand, the capacity of EPC to promote revascularization and tissue functional recovery by paracrine mechanisms is consistent with the vascular healing activity of a number of soluble factors released by BM-derived MSC.[229, 230] Extensive research is currently carried out to unravel how the paracrine functions of stem and progenitor cells integrate the modulation of angiogenesis.[65, 146, 147, 187]

Moreover, numerous studies have evidenced the role of paracrine factors from different populations of stem cells when transplanted into the ischemic myocardium and hindlimb.[231] Implantation of bone marrow mononuclear cells into ischemic myocardium was shown to enhance collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines such as FGF, VEGF and ANG-1.[232] Interleukin-10 from transplanted bone marrow mononuclear cells contributed to cardiac protection after myocardial infarction.[233] Local

delivery of MSC augmented collateral perfusion in ischemic hindlimb through the secretion of VEGF, bFGF, PIGF, MCP-1 and a broad spectrum of arteriogenic cytokines.[23, 114] The conditioned medium derived from Akt-modified MSC significantly enhanced the cardiac protection and functional improvement via the secretion of secreted frizzled related protein 2 (Sfrp2).[234-236]

A favorable microenvironment is a prerequisite to the development and maintenance of a stable vascular network.[237] In this context soluble factors supporting the recruitment, survival and differentiation of endogenous cells including tissue resident stem and progenitor cells are of primary importance before the formation of new vascular channels may take place.[65, 113] However, disease related unfavorable pathological microenvironments are associated with upregulation of inflammatory, apoptotic and necrotic signal molecules and are sometimes severe enough to alter/inhibit the beneficial secretion from infused EPC, or even cause cells to die before contribution. Meanwhile it also remains to be determined how environmental factors, EPC tissue progeny or differentiation stage impact the secretory capacity of endothelial precursors. Most notably it has been described that age and other risk factors for cardiovascular disease reduce the availability of EPC and impair their function to varying degrees, thus limiting their therapeutic usefulness in these patient populations.[186, 238, 239] Furthermore, the relative scarcity of circulating EPC and their finite proliferative potential limits the ability to expand these cells in sufficient numbers for some therapeutic applications. Recent findings[194] challenged in part the concept of decreased number of circulating EPC in pathological condition thus underscoring the necessity to investigate in a more exhaustive way how cardiovascular diseases may affect some fundamental functional properties of EPC as mobilization, survival, and capacity to differentiate or secrete paracrine factors. In such scenarios the use of EPC paracrine factors produced by optimized *in vitro* preconditioning would be a convenient and effective alternative.

In the presented studies, we have extensively investigated the regenerative capacity of EPC secreted factors. We have reported that administration of these factors alone may be sufficient to achieve a therapeutic angiogenic response *in vivo* using a rat model of chronic limb ischemia.[65] We also revealed that soluble factors secreted by EPC were able to promote survival of EC against oxidative stress induced cell death by stimulating endogenous protective mechanisms[147] and activate the PDGFR-β expression in EC. Importantly, in these experimental settings the angiogenic effect of the factors secreted by EPC seem to rely not on isolated

cytokines (including VEGF, MMp-9, HGF and IL-8) but rather on a synergistic interaction of multiple factors.[147]

To date, most studies evaluate in vivo angiogenic therapies in animal models immediately after induction of hindlimb ischemia and are limited to a short observation period of approximately 30 days after surgery.[182, 240-244] Those studies of therapeutic angiogenesis committed to the early postoperative phase are potentially distorted by dramatic endogenous compensation mechanisms in response to acute ischemia which involve triggering of inflammation and induction of vascular remodeling by arteriogenesis and angiogenesis.[90, 128, 245-248] Furthermore, this acute phase of ischemia is associated with considerable tissue necrosis, altered shear stress, changes in gene expression and the recruitment of endothelial progenitor cells.[129, 249, 250] It is conceivable, that study protocols, which benefit from the profound ischemia occurring immediately after surgery, are subjected to quite ambiguous confounding factors. Therefore, it is reasonable to speculate that the outcomes of experimental therapeutic interventions carried out during the phase of acute ischemia may be significantly altered.

In order to reliably compare and evaluate the therapeutic effect of EPC and EPC-CM in chronic PAD condition, we established a moderate but consistent hindlimb ischemia on athymic rnu-rats by surgical occlusion and excision of femoral vessels, providing a solid reference to increase the reliability and accurateness of comparisons and evaluations of future therapeutic angiogenesis studies (**Result I**).[130] This model does not involve the development of muscle necrosis, fibrosis or inflammation. Also, we document the persistence of ischemia at stable perfusion levels in a chronic animal model that extends well beyond the timeframe used previously. The significant and lasting impairment in the limb blood perfusion along with the decrease of function observed in the animals suggest the validity and practicability of this model.

By applying this rat model of chronic hindlimb ischemia, we then reported that serial intramuscular injections of factors secreted by EPC in vitro triggered a substantial revascularization of the ischemic muscles accompanied by the recovery of the ischemic muscle tissue activity (**Result II**).[65] Importantly, there is convincing evidence that this tissue revascularization and regeneration capacity is preceded by a systemic effect with a transient increase of progenitor cells ($CD34^+$ cells) in the bone marrow and in the peripheral blood, as well as an augmented recruitment of stem cells within the ischemic muscle. Thus, the enhanced

mobilization and homing of host stem cells suggest that secreted factors might promote the endogenous repair systems by both local and systemic actions. Remarkably, the therapeutic capacity of secreted factors was in general comparable to EPC transplantation. It is of note, however, that the number of cells necessary to generate an equivalent therapeutic dose was much lower for secreted factors production compared to the amount of cells employed for EPC transplantation. This study suggests EPC-CM has the potential to replace cell transplantation.

Paracrine factors secreted by EPC also have the potential to protect differentiated endothelial cells from apoptosis and to preserve their angiogenic capacity under conditions of oxidative stress (**Result III**).[147] The array of soluble factors secreted by EPC includes a number of enzymes like matrix proteins, growth factors and cytokines. Amongst the cytokines released by early EPC, IL-6 and IL-11 were reported to have anti-inflammatory and cyto-protective properties rescuing endothelial cells from H_2O_2 induced cell death [251]. Moreover, other studies provided solid evidence that VEGF, HGF and IGF-1 − all secreted by early EPC − exert strong cyto-protective and pro-survival activity inducing the expression of anti-apoptotic [252] and anti-oxidant [253, 254] proteins in endothelial cells. In agreement with these findings and with the notion of the relatively high resistance of EPC against oxidative injury [255, 256], our study suggests that mature endothelial cells challenged by oxidative stress benefit from the capacity of EPC to enhance the endothelial antioxidant defense by paracrine mechanisms.[147] Our data advocate the hypothesis that Bcl-2 is a key modulator of the oxidative state and angiogenic functions of endothelial cells [257]. These results further corroborate the concept that the secretion of survival factors in support of differentiated cells is a pivotal feature of stem and progenitor cells [258], as suggested by the protective capacity of conditioned medium from adipose stem cells on dermal fibroblast exposed to H_2O_2[259]. Interestingly, the neutralization of several selected factors including VEGF, MMp-9, HGF and IL-8, alone or in combination, is not sufficient to attenuate the cyto-protective properties of EPC-CM. These results suggest that other, even unidentified factors or a synergic combination of them might be responsible for the anti-apoptotic effect of EPC-CM.

PDGF-BB is expressed by the endothelium for the survival and functioning of adjacent perivascular cells.[260] It exists in substantial quantities among numerous growth factors secreted by EPC. Interestingly, when reacting to oxygen deprivation, EPC tend to further upregulate the release of PDGF-BB.[65] These findings are in agreement with several observations of enhanced release of angiogenic factors by different cell types including hematopoietic stem cells [261] and

EPC [262]. On the other hand, the expression of PDGF receptors has been reported in hemangioblast precursors where it accelerates the differentiation of endothelial cells [263]. However, endothelial cells usually do not express PDGFR-β in high quantities and the relevance of this receptor is uncertain in resting endothelium. Current literature suggests that under circumstances of increased angiogenesis the expression of PDGFR-β in matured endothelial cells is upregulated [264, 265].

In our study (**Result IV**), we found increased gene and protein expression of PDGFR-β as well as an increased phosphorylation of the receptor in endothelial cells that were exposed to EPC-CM. In *in vitro* assays of angiogenesis the exposure of endothelial cells to EPC-CM resulted in increased proliferation, migration and organization of the cells into capillary structures. Substitution of control medium with equivalent or even higher concentrations of recombinant human PDGF-BB only achieved minimal angiogenic effects and was not able to initiate the expression of PDGFR-β in EC. Importantly, inhibition of PDGFR-β significantly attenuated the angiogenic response of endothelial cells exposed to the EPC secretome. In other words, our data suggest that EPC-CM is capable of inducing a phenotype shift in endothelial cells by promoting surface expression of PDGFR-β. The upregulation of PDGFR-β might be a critical modulator of the pro-angiogenic response in endothelial cells. This theory would be consistent with several clinical trials that have shown a significant increase in angiogenic response when cytokines like VEGF are combined with PDGF[266, 267], although the impact on perivascular cells certainly is a major reason for the benefits of a combined therapy. Moreover, PDGF-BB alone was not sufficient to promote angiogenic behavior in endothelial cells. The simply incubation with sole recombinant protein rhPDGF-BB failed to reproduce a similar effect of EPC-CM on stimulating PDGFR-β expression. These findings may be explained by the theory that a synergic combination of growth factors within EPC-CM is responsible for the modulation of pro-angiogenic signaling pathways in endothelial cells. Given the complexity of the EPC secretome and the interaction between EPC paracrine factors and the endothelium, it can be assumed that the upregulation of PDGFR-β is just one of several pathways involved. Future studies are needed to clarify the EPC-endothelium interactions in more detail.

Overall, these studies revealed the potent angiogenic capacity of EPC-CM. Our findings suggest the possibility to replace cell transplantation with interventions based solely on soluble factors secreted by EPC. These results might pave the way to the development of novel therapeutic

strategies in regenerative medicine especially for the treatment of cardiovascular disorders. Most importantly, our data propose a strategy free from the limitations and problems observed with cell transplantation.

It has been described that age and other cardiovascular risk factors reduce the availability and function of EPC, thus limiting their therapeutic applicability in diseased patients.[143, 194, 268, 269] Furthermore, the relative scarcity of circulating EPC and their limited proliferative potential prevent the possibility of expanding these cells in sufficient numbers for effective therapeutic applications. Therefore, the use of heterologous cells seems to be the only available option to provide patients suffering from cardiovascular disease with a cell-based therapy. However, immunotolerance concerns and technical as well as practical difficulties may hinder this type of treatment. In contrast, a cell-free medium containing the paracrine factors from EPC significantly reduces the risk of adverse immunological reactions and simplifies the process of production. It is, therefore, reasonable to imagine that secreted factors or synthetic preparation which mimics physiological secreted factors will in future find application in regenerative medicine.

3.2 Limitation

Several limitations may remain in the way to the future applications based on the paracrine mechanism of EPC. Different *in vitro* culture conditions with distinct stimuli (i.e. hypoxia vs. VEGF) and oxygen concentration (i.e. 0.5% O_2 vs. 1.5% O_2) may significantly alter the secretion profile of EPC and therefore require meticulous investigation. Secondly, the EPC used in this study are derived from peripheral blood of healthy donors and may not represent the optimized source to produce effective EPC-CM. It is therefore necessary to further determine the most suitable source of EPC-CM for therapeutic neovascularization and tissue regeneration. For instance, EPC-CM derived from umbilical cord blood derived EPC may have a higher regenerative potential than that from adult EPC culture while severe atherosclerosis burden may impair the therapeutic capacity of EPC-CM.

Thirdly, the cytokines produced *in vitro* may not be able to direct the secretion of angiogenic factors as consistent as *in situ* secretion modulated by the complex communication/interaction of a pathological microenvironment, and may thus attenuate the specificity and potency. The

use of conditioned media extracted from pathological tissues as real “disease-specific” stimuli may therefore be an ultimate solution and result in the most potent regenerative benefit.

Fourthly, the extremely short half-life of those trophic factors after local delivery may interfere negatively with the therapeutic outcome. It is therefore assumable that the utilization of a biomaterial based delivery platform could greatly enhance the therapeutic efficacy and efficiency by prolonging the short half-life of the encapsulated cytokines, programming the release kinetics, and creating a long-term stable microenvironment suitable for tissue regeneration.[81, 120, 270]

Finally, the secretion of some inflammatory factors such as tissue factors, MCP-1, IL-8 may also raise concerns about the potential risks of progenitor cell therapy that EPC transplantation may promote the growth of atherosclerotic plaques.[188] For example, MCP-1 and IL-8 are reported to be key mediators in the growth and formation of atherosclerosis progression and plaque by recruiting circulating inflammatory cells.[271] Future stem cell therapies including the use of stem cell derived paracrine factors need to be proceeded with caution on possible inflammatory activation in the host vasculature to prevent the development of pathological vascular inflammation.

3.3 Future Perspective

In future, therapeutic strategies based on EPC paracrine factors can be used in combination with cell transplantation or serve as an “off-the-shelf” replacement. This strategy presents considerable advantages over conventional cell transplantation like the ready availability, the repeatability and the relative simplification of the process of production. The further enhancement by incorporation of EPC-CM with bioengineered delivery system may also be rapidly translated into the clinic through utilizing materials that have an existing history with the FDA (i.e. PLG). Furthermore, with an enhanced understanding of the protein composition of EPC-CM (i.e. by proteomics), a synthetic product with equivalent composition to physiological EPC-CM can be generated, providing the possibility to achieve sufficient quality, quantity and reproducibility for large-scale manufacture purpose. Finally, this strategy may serve as a paradigm for other cell based therapies with similar rationales depending on trophic mechanisms.

3.4 Personal Contributions

Paper I: Design and concept: **Z.Y.**, S.D.S., C.K. Data collection and analysis: **Z.Y.**, S.D.S., J.V., C.K. Manuscript writing: **Z.Y.**, M.W.v.B., S.D.S., C.K.

Paper II: Conceived and designed the experiments: SDS **ZY** IB CK. Performed the experiments: SDS **ZY**. Analyzed the data: SDS **ZY** MWvB ND. Contributed reagents/materials/analysis tools: JV. Wrote the paper: SDS **ZY** MWvB CK.

Paper III: Design and concept: **Z.Y.**, S.D.S., C.K. Data collection and analysis: **Z.Y.**, S.D.S., J.V., M.W.v.B., D.F., C.K. Manuscript writing: **Z.Y.**, S.D.S., M.W.v.B., C.K.

Paper IV: Conceived and designed the experiments: **ZY** SDS CK. Performed the experiments: **ZY** SDS MWvB. Analyzed the data: **ZY** SDS MWvB CK. Contributed reagents/materials/analysis tools: JV. Wrote the paper: MWvB **ZY** SDS CK.

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6. LIST OF PUBLICATIONS

Original Articles

1. Di Santo S, Diehm N, Ortmann J, Voelzmann J, **Yang Z**, Keo HK, Baumgartner I, Kalka C. Oxidaized low density lipoproteins (oxLDL) impairs endothelial progenitor cell function by downregulation of E-selectin and integrin αvβ5. Biochem Biophys Res Commun. 2008; 373(4):528:532.

2. Di Santo S*, **Yang Z***, Wyler von Ballmoos M, Voelzmann J, Diehm N, Baumgartner I, Kalka C. Novel cell-free strategy for therapeutic angiogenesis: In vitro generated conditioned medium can replace progenitor cell transplantation. PLoS One. 2009 May 21;4(5):e5643.

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

4. **Yang Z**, von Ballmoos MW, Faesselr D, Voelzmann J, Ortmann J, Diehm N, Kalka-Moll W, Baumgartner I, Di Santo S, Kalka C. Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells. Atherosclerosis. 2010 Jul;211(1):103-109.

5. Ortmann J, Veith M, Zingg S, Di Santo S, Traupe T**, Yang Z**, Völzmann J, Dubey R, Christen S, Baumgartner I. Selective activation of estrogen receptor alpha inhibits high glucose induced VSMC proliferation by down regulating ROS and ERK-phospharylation. (Submitted, under revision)

6. von Ballmoos MW*, **Yang Z***, Voelzmann J, Baumgartner I, Kalka C, Di Santo S. Endothelial progenitor cells induce a phenotype shift in differentiated endothelial cells towards PDGF/PDGFR-β axis mediated angiogenesis (Submitted, under revision)

7. Bitterli L, Bühler S, Schmidlin K, Zwahlen M, Voegele J, Di Santo S, Völzmann J, **Yang Z**, Baumgartner I, Diehm N, Kalka C. Number and function of endothelial progenitor cells predict the occurrence and severity of peripheral arterial disease. (Manuscript ready)

Reviews

8. **Yang Z**, Di Santo S, Kalka C. Current developments in the use of stem cell for therapeutic neovascularization: Is the future therapy "cell-free"? (Submitted, under revision)

Published Abstracts

9. von Ballmoos MW*, **Yang Z***, Diehm N, Völzmann J, Baumgartner I, Kalka C, Di Santo S. Endothelial progenitor cells induce a phenotype shift in differentiated endothelial cells towards PDGF-BB sensitivity and increased angiogenesis. J Vasc Res 2009;46:5-60 (DOI: 10.1159/000245770) (Abstract)

7. DECLARATION OF ORIGINALITY

Last name, first name: **Yang, Zijiang**

Matriculation number: **06-129-993**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date	Signature
Bern, 2010.07	
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…each ending is a new beginning…

"Viale Del Giardino", Claude Monet