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THERAPEUTIC PROPERTIES OF SUPEROXIDE DISMUTASE 3 AND MESENCHYMAL STROMAL CELLS IN PERIPHERAL ISCHEMIA

by

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"Nothing shocks me. I'm a scientist."
Indiana Jones

University of Turku, Faculty of Medicine

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ABSTRACT: The aim of this study was to characterize the cellular mechanisms leading to the beneficial effect of anti-oxidative gene therapy and pro-angiogenic stem cell therapy in acute peripheral ischemia. Post-ischemic events aim to re-establish tissue blood perfusion, to clear cellular debris, and to regenerate lost tissue by differentiation of satellite cells into myoblasts. Although leukocytes have an essential role in clearing cellular debris and promoting angiogenesis, they also contribute to tissue injury through excessive ROS production.

First, we investigated the therapeutic properties of extracellular superoxide dismutase (SOD3) gene transfer. SOD3 was shown to reduce oxidative stress, to normalize glucose metabolism, and to enhance cell proliferation in the ischemic muscle. Analysis of the mitogenic Ras-Erk1/2 pathway showed SOD3 mediated induction offering a plausible explanation for enhanced cell proliferation. In addition, SOD3 reduced NF-κB activity by enhancing IκBα expression thus leading to reduced expression of inflammatory cytokines and adhesion molecules with consequent reduction in macrophage infiltration.

Secondly, we sought to determine the fate and the effect of locally transplanted mesenchymal stem/stromal cells (MSCs) in acute ischemia. We showed that a vast majority of the transplanted cells are cleared from the injury site within 24 hours after local transplantation. Despite rapid clearance, transplantation was able to temporarily promote angiogenesis and cell proliferation in the muscle. Lack of graft-derived growth factor expression suggests other than secretory function to mediate this observed effect.

In conclusion, both SOD3 and MSCs could be utilized to alleviate peripheral ischemia induced tissue injury. We have described a previously unidentified growth regulatory role for SOD3, and suggest a novel mechanism whereby transplanted MSCs enhance the reparative potential of the recipient tissue through physical contacts.

Keywords: SOD3, Mesenchymal stem/stromal cell, Oxidative Stress, Angiogenesis, Inflammation, Cell proliferation, Peripheral Ischemia

Turun yliopisto, Lääketieteellinen tiedekunta

Tekijä:	Juha Petteri Laurila
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TIIVISTELMÄ: Iskemian jälkeiset tapahtumat pyrkivät palauttamaan verenkierron kudokseen, poistamaan kuolleiden solujen jäänteet ja lopuksi korvaamaan menetetyt solut satelliittisolujen erilaistumisen kautta. Tämän tutkimuksen tavoitteena oli määrittää antioksidatiivisen geeniterapian ja verisuonten uudismuodostusta edistävän kantasoluterapian vaikutuksia lihaksen parantumiseen perifeerisessä iskemiassa.

Tutkimme superoksidismutaasi 3 (SOD3)-geeniterapian vaikutusmekanismia iskeemisessä lihaksessa. SOD3 vähensi lihaksen happiradikaalistressiä, normalisoi kudoksen metaboliaa ja lisäsi solujen jakaantumista. SOD3 lisäsi solun jakautumista välittävän Ras-Erk1/2 signalointiverkoston aktiivisuutta, mikä tarjoaa mahdollisen selityksen SOD3:n aiheuttamalle solun jakaantumisen lisäykselle kudoksessa. Tulehdusta välittävien tekijöiden analyysi osoitti SOD3:n vähentävän NF-κB transkriptiotekijän aktiivisuutta, johtaen vähentyneeseen makrofagien kerääntymiseen kudokseen.

Toisena tavoitteenamme oli määrittää mesenkymaalisen kantasolusiiroksen (mesenchymal stromal cells, MSC) vaikutus ja siirrettyjen solujen kohtalo akuutissa alaraajaiskemiassa. Suurin osa kantasoluista tuhoutui vuorokauden kuluessa paikallisesta siirteestä, mistä huolimatta kantasolut kykenivät väliaikaisesti edistämään verisuonten muodostumista ja solujen jakaantumista. Emme kuitenkaan havainneet kantasoluista lähtöisin olevia eritettäviä kasvutekijöitä, mikä viittaisi muun kuin eritystoiminnan olevan tehtyjen havaintojen takana.

Yhteenvetona, tulostemme mukaan sekä SOD3 että MSC:t ovat hyödynnettävissä lieventämään perifeerisen iskemian aiheuttamaa kudosvauriota. Osoitimme aikaisemmin tuntemattoman kasvua edistävän vaikutuksen SOD3:lle ja näytimme MSC:n edistävän vastaanottajakudoksen omaa parantumisprosessia fyysisen kontaktin kautta.

Avainsanat: SOD3, mesenkymaaliset kantasolut, happiradikaalistressi, uudissuonitus, solunjakautuminen, tulehdus,perifeerinen iskemia

TABLE OF CONTENTS

ABBREVIATIONS

Ad	Adenovirus
Akt/PKB	Akt/Protein kinase B
AP-1	Activator protein-1
BLI	Bioluminescent imaging
BM	Basement membrane
	Bone marrow
BMT	Bone marrow transplantation
CAD	Coronary artery disease
CAR	Coxsackie and adenovirus receptor
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CLI	Critical limb ischemia
CREB	cAMP responsive element binding protein
DPI	Diphenyl iodonium
EC	Endothelial cell
	Extracellular
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EPC	Endothelial progenitor cell
Erk	Extracellular signal regulated kinase
FDG	18-Fluorodeoxyglucose
FGF	Fibroblastic growth factor
FSS	Fluid shear stress
GEF	Guanine nucleotide exhange factor
hESC	Human embryonic stem cell
HIF	Hypoxia inducible factor
H_2O_2	Hydrogen peroxide
HSC	Hematopoietic stem cell
ICAM	Intercellular cell adhesion molecule
IFN	Interferon
IkB	Inhibitor of kB
IKK	IkB kinase
IL	Interleukin
IVIS	In vivo imaging system
JNK	c-Jun terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP	Macrophage chemoattractant/chemotactic protein
Mek	MAPK/Erk kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase

MRF	Myogenic regulatory factor
MSC	Mesenchymal stem/stromal cell
NADPH	Nicotinamide dinucleotide phosphate
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	NADPH oxidase
$O_2^{\bullet -}$	Superoxide
ONOO	Peroxynitrite
PAD	Peripheral artery disease
PDGF	Platelet derived growth factor
PDGFR	PDGF receptor
PET	Positron emission tomography
pfu	Plaque forming unit
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidyl inositol 3-kinase
PKC	Protein kinase C
PLC	Protein lipase C
PlGF	Placental growth factor
PMN	Polymorphonuclear neutrophil
PTP	Protein tyrosine phosphatase
Redox	Reduction/oxidation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SMC	Smooth muscle cell
SOD	Superoxide dismutase
TGF-β	Transforming growth factor
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor
XO	Xanthine oxidase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications.

I **Juha P. Laurila***, Maria D. Castellone*, Antonio Curcio, Lilja E. Laatikainen, Merja Haaparanta-Solin, Tove Grönroos, Päivi Marjamäki, Satu Martikainen, Massimo Santoro, and Mikko O. Laukkanen. (2009) Extracellular Superoxide Dismutase is a Growth Regulatory Mediator of Tissue Injury Recovery.
Molecular Therapy, 17: 448-454.

II **Juha P. Laurila**, Lilja E. Laatikainen, Maria D. Castellone, and Mikko O. Laukkanen. (2009) SOD3 Reduces Inflammatory Cell Migration by Regulating Adhesion Molecule and Cytokine Expression.
Plos ONE, 4: e5786.

III **Juha P. Laurila**, Lilja E. Laatikainen, Maria D. Castellone, Parul Trivedi, Jari Heikkilä, Ari Hinkkanen, Peiman Hematti, and Mikko O. Laukkanen. (2009) Human embryonic stem cell-derived mesenchymal stromal cell transplantation in rat hind limb injury model.
Cytotherapy, 11: 726-737.

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INTRODUCTION

Assuming similar increase in life expectancy as has been experienced in past decades, children born today in Finland are expected to reach 83 years of age if a girl, and 76 years if a boy. As the incidence of peripheral artery disease (PAD) is 12-20% in people over 65, a tremendous increase in the number of PAD patients is to be expected in the future. Although usually asymptomatic, PAD has prognostic value for coronary artery disease, and may lead to critical limb ischemia that may leave amputation as the only treatment option.

PAD may be caused by any condition causing stenosis of major conducting arteries in the limbs. Insufficient blood flow (i.e. ischemia) initiates a cascade of events aiming to re-establish blood flow and to ensure recovery and proper function of the affected tissues. Most notable events in post-ischemic tissues are disturbance of tissue redox balance by increased production of reactive oxygen species (ROS), angiogenesis after activation of vascular endothelial cells, massive infiltration of inflammatory leukocytes, and regeneration of lost tissue by proliferating stem and progenitor cells.

Extracellular superoxide dismutase (SOD3) and mesenchymal stromal cells (MSCs) have both been utilized for therapeutic purposes in varying pre-clinical disease models, albeit based on different mechanisms of action. SOD3 is an antioxidative enzyme converting superoxide into hydrogen peroxide whereas MSCs are though to mediate beneficial effects through e.g. promotion of vascular growth. Both have also been proposed to possess anti-inflammatory activity. Several hypotheses have been suggested to contribute to MSC-mediated tissue recovery but the field is riddled with controversial reports thus hindering advancement into clinical trials.

The aim of this study was to characterize the cellular mechanisms mediating the therapeutic properties of SOD3 gene transfer. Furthermore, our goal was to delineate the early events after MSC transplantation in order to decipher the current controversy on the mode of their therapeutic action. To avoid restrictions of autologous cell transplantation, we compared primary bone marrow derived MSCs to a novel MSC population derived from embryonic stem cells that could potentially provide an unlimited source of material for cell therapy.

REVIEW OF LITERATURE

1 Skeletal Muscles and the Blood Vessels that Feed Them

Muscle is a tissue specialized for contractile function, thus being responsible for producing body movements. Muscle contraction is dependent on the energy carried by ATP which is produced either by oxidative phosphorylation or anaerobic glycolysis. Due to high metabolic rate muscle is highly dependent on abundant blood supply.

1.1 Skeletal muscle

Skeletal muscle is composed of terminally differentiated multinucleated myofibers held together by connective tissue that continues as tendons that attach the muscle to bone. Myofibers are surrounded by thin layer of connective tissue, the endomysium. Bundles of myofibers are surrounded by a thicker layer of connective tissue named the perimysium and finally, thick epimysium surrounds the muscle. The perimysium contains small arteries and veins that branch into capillaries within the endomysium. Myofibers form by fusion of myoblasts that originate from self-renewing population of myogenic stem cells. Muscles of the trunk and the limbs originate from presomitic mesoderm while muscles of the head are derived from several sources, e.g. paraxial head mesoderm and the prechordal mesoderm (Sambasivan and Tajbakhsh, 2007). Stem cells giving rise to limb muscles are highly dependent on function of a paired-box transcription factor Pax3. Pax3 mediates migration of muscle stem cells to the limb and subsequently up-regulates expression of the myogenic regulatory factors (MRFs) together with homeodomain transcription factor Six1 (Giordani et al., 2007; Grifone et al., 2005; Relaix et al., 2004). Myogenesis is controlled by distinct transcriptional regulators depending on the location of the stem cells. However, regardless of the location of the cells myogenic signals converge on the MRFs: Myf5, MyoD, Mrf4, and Myogenin. Mice triple mutant for Myf5, MyoD, and Mrf4 completely lack skeletal muscle and myoblasts as myogenesis is halted at the progenitor cell stage (Kablar et al., 2003; Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). In contrast, Myogenin plays an essential role in fusion of myoblasts to form myofibers (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1995; Venuti et al., 1995).

Terminally differentiated myofibers are not capable of further proliferation, yet skeletal muscle has remarkable regenerative potential. This is due to satellite cells located between the plasmamembrane of myofibers and their basement membrane. Normally quiescent satellite cells are activated to proliferate and fuse to make myofibers in case of injury. The capacity of muscle to regenerate after repeated injuries can be attributed to satellite cell self-renewal (Collins et al., 2005; Zammit et al., 2006). The microenvironment maintaining the satellite cells, i.e. the satellite cell niche, is limited to myofiber plasmamembrane and the surrounding basement membrane. Live myofibers reduce satellite cell proliferation in response to mitogen exposure *in vitro* suggesting that myofibers function to maintain satellite cell quiescence (Bischoff, 1990). The developmental origin of satellite cells is yet

to be convincingly resolved. Two embryonic sources have been suggested, either the myogenic lineages derived from somites (Armand et al., 1983) or an endothelial/myogenic precursor derived from embryonic vasculature (Cossu and Mavilio, 2000; De Angelis et al., 1999).

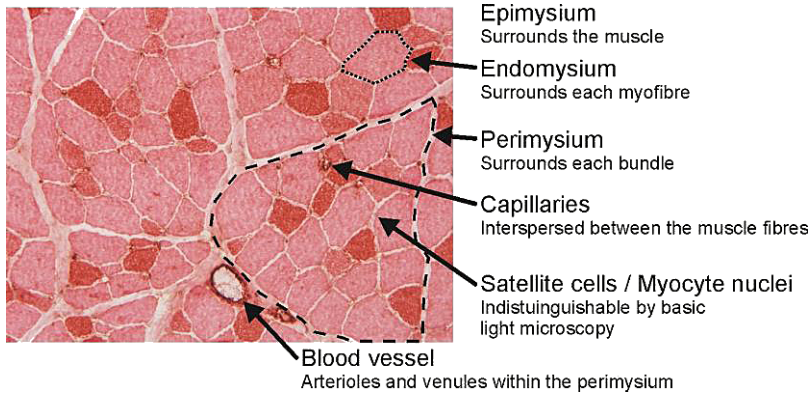

Figure 1. Structure of skeletal muscle. Individual myofibers (myocytes) are surrounded by a layer of connective tissue called the endomysium. Perimysium encloses bundles of myofibers, and finally, the muscle is enveloped by the epimysium. Blood vessels lie within the connective tissue. Nuclei of the multinuclear myocytes cannot be distinguished from satellite cell nuclei based on basic light microscopy. Staining for specific markers such as Pax3 or Pax7 is required to recognize satellite cells.

1.2 Circulation and Pre-Natal Formation of Blood Vessels

1.2.1 Vascular Structure

Healthy living tissue requires constitutive transport of gases, nutrients, hormones, and circulating cells by the cardiovascular system to maintain tissue homeostasis. The vascular network traverses the body beginning form the aorta and branching successively to smaller arteries finally reaching the size of arterioles, terminal arterioles (metarterioles), and capillaries. From capillaries blood is collected into post-capillary venules that converge to larger veins finally draining into the right atrium of heart. Exchange of gases and nutrients takes place predominantly at the level of capillaries while arterioles are mainly responsible for regulating tissue perfusion.

Blood vessels share the same basic structure comprised of three layers: tunica intima, tunica media, and tunica adventitia. The tunica intima is formed by a layer of endothelial cells covering the internal surface of blood vessels and surrounded by a basement membrane with pericytes embedded within it. The tunica media is mainly responsible for the thickness of arterial walls; it is made up of several layers of smooth muscle cells alternating with layers of elastic fibers. In veins, large proportion of the tunica media is composed of connective tissue with abundance of elastic fibers. Finally, the tunica adventitia is the outermost layer comprised of connective tissue and nerves. In large blood vessels tunica adventitia also contains capillaries supplying the smooth muscle cell layer. Due to abundance

of collagen fibers tunica adventitia anchors the blood vessel to the surrounding tissue. In contrast to larger vessels, capillaries are formed only of the tunica intima, i.e. a single layer of endothelial cells surrounded by pericytes within the basement membrane.

1.2.2 Vasculogenesis

The blood vasculature is the first organ to develop during embryogenesis. *De novo* formation of blood vessels is called vasculogenesis encompassing endothelial cell differentiation and subsequent formation of the vascular plexus. Endothelial cells and hematopoietic cells originate from common mesodermal ancestor, the hemangioblast (Jaffredo et al., 2005). Hemangioblasts form blood islands in the yolk sac giving rise to angioblasts at the periphery of the blood islands, and to hematopoietic stem cells at the centre (Choi et al., 1998). Angioblasts proliferate and differentiate to endothelial cells resulting in lumen formation that allows blood flow. Similar process occurs within the embryo, and the primitive vasculatures of the yolk sac and the embryo interconnect. Further development of the vascular tree happens mainly by angiogenesis, sprouting of new blood vessels from pre-existing ones. Finally, the vasculature matures by recruiting pericytes and smooth muscle cells, both of which are indispensable for proper vascular function (Abraham et al., 2008; Armulik et al., 2005).

Vascular endothelial growth factor (VEGF) is a crucial regulator of vasculogenesis and angiogenesis, mutation of single *vegf* allele leads to embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996). In addition, a number of studies in knock-out animals have demonstrated the importance of platelet derived growth factor (PDGF) (Hellstrom et al., 1999; Lindahl et al., 1997), transforming growth factor (TGF)-β (Dickson et al., 1995; Oshima et al., 1996), and angiopoietins (Suri et al., 1996) in vascular development. Although these studies have provided important insight into embryonic vascular development in mouse, the formation of limb vasculature has remained poorly described since most of the models lead to embryonic lethality before limb development has occurred. Interestingly, it has been suggested that Pax3 expressing precursors of the limb skeletal muscle also give rise to the limb endothelium, and the differentiation fate is controlled by extrinsic signals (Kardon et al., 2002).

1.2.3 Vascular Cells

Endothelial cells, pericytes, and smooth muscle cells each exhibit distinct morphological features depending on their location due to functional heterogeneity of blood vessels. However, ECs, Pericytes and VSMCs also demonstrate remarkable phenotypic plasticity depending on the surrounding microenvironment.

Endothelial cells are most commonly identified by surface markers CD31 (PECAM), CD34, von Willebrand factor, Tie-2 and PAL-E (Pathologische Anatomie Leiden-endothelium). However, aortic, venous, and lymphatic endothelia can be distinguished by additional markers, including alkaline phosphatase and Ephrin B2 for arteries, dipeptidylpeptidase, and Eph B4 (receptor for Ephrin B2) for veins, and 5´nucleotidase or Podoplanin for lymphatic

endothelia. A recent study by Hong indicates opposing phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways in arterial specification (Hong et al., 2006; Lamont and Childs, 2006). In skeletal muscle and most other tissues, the vascular endothelium is continuous layer where ECs adhere together through tight junctions and adherens junctions (Aird, 2007). The endothelium is more than merely a passive wall. By regulating leukocyte traffic and initiation of angiogenesis endothelium is in central role in many pathogenic processes such as tumor growth, atherosclerosis, and diabetes. The integrity of vascular endothelium is maintained by contribution of vascular mural cells, but also by autocrine VEGF signaling (Lee et al., 2007). Thus, VEGF not only mediates blood vessel formation but is also crucial factor in maintaining vascular homeostasis.

Pericytes are found at varying densities around pre-capillary arterioles, capillaries, post-capillary venules, and collecting venules (Allt and Lawrenson, 2001; Armulik et al., 2005). Embedded within the basement membrane (BM), pericytes make contacts with the endothelial cells through gaps in the BM, named peg-socket contacts (Cuevas et al., 1984; Gerhardt and Betsholtz, 2003; Gerhardt et al., 2000; Tilton et al., 1979). Apart from their unique position, pericytes can be identified by several molecular markers, yet none of them is completely specific for pericytes or recognizes all pericytes regardless of the location (Armulik et al., 2005). The pericyte coverage varies between 10-50% in vessel abluminal area and between 1:100 and 1:1 in pericyte:EC ratio (Shepro and Morel, 1993), with the lowest pericyte to EC frequency found in skeletal muscle. Pericytes are recruited to developing blood vessels by endothelial expression of PDGF-B (Hellstrom et al., 1999; Lindahl et al., 1997). Subsequently, pericytes are thought to mediate microvascular stabilization and permeability (Murakami and Simons, 2009; Sato and Rifkin, 1989), and contractibility and tone (Rucker et al., 2000).

Smooth muscle cells are responsible for the involuntary rhythmic contractions of e.g. the respiratory and gastrointestinal tracts, and of course the vascular wall. The principal function of the vascular smooth muscle cells is to regulate blood vessel diameter, blood pressure, and distribution of blood flow. VSMCs do not exhibit cross striatation like skeletal or cardiac muscle since myosin and actin filaments are scattered diffusely throughout the VSMC cytoplasm. The actin filaments are anchored to dense bodies connecting to a network of intermediate filaments desmin and vimentin. Most common markers used for identification of SMCs are the myosin heavy chain isoforms SM-1 and SM-2, and smooth muscle actin (SMA)α despite its transient expression during skeletal and cardiac muscle differentiation (Ruzicka and Schwartz, 1988; Sawtell and Lessard, 1989). Smooth muscle cells are predominantly of mesodermal origin although a sub-population of neural crest cells contributes to vascular endothelium of the aortic arches and portions of heart (Kirby, 1988; Kirby and Waldo, 1995; Le Lievre and Le Douarin, 1975). Within respiratory and gastrointestinal tract, SMC origin has been traced to the mesenchyme surrounding the airway or gut epithelium, respectively (Sparrow and Lamb, 2003; Young, 2008).

2 Tissue Response to Ischemia

2.1 Ischemic Diseases

In order to survive cells must reside within 100-200 μm of blood vessel depending on local oxygen consumption and delivery (Carmeliet and Jain, 2000). Blood flow in resting skeletal muscle is in the range of 3-4 mL min^{-1} $100g^{-1}$, relatively slow compared to heart, brain, kidney or liver (McNulty et al., 1995; Nair et al., 1995; Wahren, 1966). However, skeletal muscle has a tremendous capacity to increase blood flow in response to exercise or certain regulators of muscle metabolism, such as insulin or growth hormone (Baron et al., 1993; Fryburg et al., 1991). During intense exercise blood circulation is not able to supply skeletal muscle with sufficient oxygen. Thus, skeletal muscle resorts to anaerobic glycolysis for energy production and is regularly subjected to physiological hypoxia (Richardson et al., 1995). However, skeletal and cardiac muscles are also vulnerable to pathological ischemia, i.e. insufficient blood supply.

The most common cause for ischemia is atherosclerosis, a progressive disease characterized by accumulation of lipids and fibrous elements into arterial walls (plaque formation). Thickening of the vascular wall due to plaque formation is compensated by artery enlargement, thus significant narrowing of the artery lumen (stenosis) is generated only after several cycles of plaque rupture and healing (Kiechl and Willeit, 1999). Atherosclerosis remains asymptomatic for decades, and the first manifestation of the disease is often either myocardial infarction (heart attack) or a stroke. Atherosclerotic coronary artery disease (CAD) is the leading cause of death in western world (Grech, 2003). When stenosis of coronary artery exceeds 50% in diameter, blood flow is reduced so much that angina may be experienced during stress (Grech, 2003). Furthermore, acute complications such as myocardial infarction arise when plaque rupture exposes lipids and initiates coagulation cascade leading to thrombus formation. The primary choices for treatment of chronic stable angina are coronary artery bypass or percutaneous transluminal angioplasty (O'Toole and Grech, 2003).

Atherosclerosis is a systemic disease that may affect any artery. Atherosclerotic stenosis of a conducting artery of a limb leads to peripheral artery disease (PAD). Two classification systems based on severity of the symptoms have been developed for lower extremity PAD, Fontaine and Rutherford (Aslam et al., 2009). PAD symptoms progress from pain during walking that resolves at rest (claudication), to pain that also occurs at rest, and finally to ulceration and tissue loss. PAD affects 15-20% of Americans aged 70 or older. Majority of the patients are asymptomatic or express atypical leg pain, with only 10-35% exhibiting classic claudication (Hirsch et al., 2006). Critical limb ischemia (CLI), characterized by persistent rest-pain and tissue loss, affects 1-2% of patients (Norgren et al., 2007). Due to high prevalence of atypical and asymptomatic cases, PAD is highly under-diagnosed. Early diagnosis would not only allow early intervention, but progression of PAD is a reliable predictor of CAD mortality and morbidity (Criqui

et al., 2008; Dunbar and Mohler, 2005). Progression of early PAD can be affected by life style choices such as exercise and cessation of smoking or by various pharmacological medicines (e.g. aspirin). The most effective treatment for CLI is revascularization of the affected tissue by percutaneous transluminal angioplasty or surgical treatment with endovascular or open vascular surgery using vein grafts or synthetic prostheses. However, 20-30% of patients with chronic CLI cannot be treated by any conventional approach leaving amputation as the only remaining option (Egorova et al., 2009; Norgren et al., 2007).

2.2 Overview of Muscles Response to Ischemia

Acute occlusion of e.g. femoral artery leads to rapid cessation of blood flow followed by oxygen depletion in the distal tissues. The subsequent events in the affected tissues aim to re-establish tissue reductive/oxidative (redox) balance and tissue blood perfusion, to clear cellular debris, and to regenerate lost tissue. The blood vasculature takes a central role in regulating these events by sensing perturbations in blood flow and by mediating subsequent angiogenic and inflammatory processes. Transcription factor hypoxia inducible factor (HIF)-1α has been titled the master regulator of hypoxic response (Wang and Semenza, 1993). Activated HIF-1α induces genes associated with e.g. angiogenesis, glucose uptake, and energy metabolism (Semenza, 2000; Wenger, 2000). However, transcriptional profiles show distinct response to ischemia/reperfusion and hypoxia/reoxygenation in rat despite similar hypoxic burden (Aravindan et al., 2005). Together with previous *in vitro* studies this suggests that mechanosensing of vascular shear stress is an important mediator of the transcriptional response to ischemia (Manevich et al., 2001; Wei et al., 1999).

The most notable gene clusters up-regulated in response to ischemia are the inflammatory mediators, angiogenic factors, and stress-related transcripts (Lee et al., 2004; Paoni et al., 2002). Although pre-conditioning with repetitive short-term ischemia may afford some protection against ischemia by up-regulating antioxidative enzymes such as catalase and superoxide dismutase (Badhwar et al., 2004), endogenous anti-oxidative defense may not be sufficient to overcome the oxidative insult after ischemia/reperfusion injury (Bolcal et al., 2007). Twenty-four hours after experimental femoral artery ligation in mouse, the soleus muscle in the calf exhibits cellular swelling, focal necrosis, and interstitial edema. Two days later the edema has reduced, and muscle has been infiltrated by high numbers of leukocytes, mostly macrophages. Macrophage infiltration coincides with marked up-regulation of inflammatory cytokines and chemokines. At this point satellite cells have also begun to proliferate in order to regenerate myocytes later on. Transcriptional profile would suggest that satellite cells are proliferating during the first week after ischemic occlusion, and differentiate to myoblasts to regenerate myocytes during the second week (Paoni et al., 2002). At the early stages of injury, skeletal muscle adjusts to reduced oxygen supply by down-regulating proteins associated with mitochondrial energy production and up-regulating proteins involved in glycolytic pathway (Lee et al., 2004; Paoni et al., 2002).

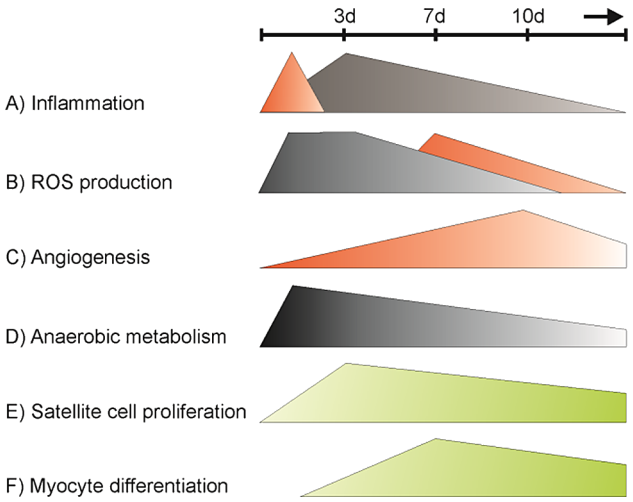

Figure 2. Tissue response to ischemic injury. (A) Following ischemic injury the muscle is rapidly infiltrated by polymorphonuclear neutrophils. Macrophages peak three days after injury and dispose dead neutrophils. (B) Neutrophils and macrophages produce an oxidative burst that is responsible for majority of ROS production in early timepoints. Production of ROS from endothelial cells is however later required for proper angiogenic response. (C) Pro-angiogenic genes are quickly up-regulated leading to steadily increasing capillary density within the muscle. (D) Due to reduced oxygen supply the muscle adjusts to anaerobic metabolism by up-regulating glucose transporters and enzymes involved in anaerobic glycolysis. (E-F) Satellite cells begin to proliferate and differentiate into myoblasts and myocytes to renew dead muscle tissue.

2.3 Tissue Redox Balance

2.3.1 Dedicated Production of ROS

Perhaps paradoxically, ischemia or hypoxia leads to rapid production of reactive oxygen species (ROS) from the vascular endothelium (Gorlach et al., 2000; Zweier et al., 1988), myocytes (Jackson, 2009), and infiltrating leukocytes (Cambria et al., 1991). ROS include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). Excessive production of ROS leads to cytotoxic effects by promoting e.g. DNA breaks and oxidative modification of proteins with subsequent depletion of cellular NAD and ATP pools (Ragu et al., 2007; Schraufstatter et al., 1987; Zweier et al., 1988). Indeed, ROS production has been suggested to contribute to muscle degeneration in muscular dystrophy and ischemia/reperfusion injury (Cambria et al., 1991; Haycock et al., 1996). Superoxide is produced by the mitochondrial electron-transport chain (Baudry et al., 2008), cytochrome P450 (Gottlieb, 2003), Xanthine oxidase (XO)(Baudry et al., 2008) and uncoupled nitric oxide synthase (NOS)(Heinzel et al., 1992; Sun et al.) but also by dedicated enzymes of the NADPH oxidase (Nox) family (Cross and Segal, 2004).

The Nox complexes were first identified in phagocytes generating respiratory burst to kill pathogens. The Nox family has now grown to encompass seven members: Nox1, Nox2 (also known as $gp91^{phox}$), Nox3, Nox4, Nox5, and dual function oxidases DUOX1 and DUOX2 (Leto and Geiszt, 2006). The best characterized member is the phagocytic Nox2 complex comprising enzymatically

inactive membrane-associated heterodimer composed of $gp91^{phox}$ and $p22^{phox}$ subunits. Cytosolic regulatory subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1 or Rac2 are recruited to the complex during enzyme activation. NoxO1 and NoxA1, homologues of $p47^{phox}$ and $p67^{phox}$ are required for Nox1 activation whereas Nox4 is constitutively active (Cheng et al., 2006; Martyn et al., 2006). Nox2 produces $O_2\bullet^-$ by transporting electrons from NADPH through FAD (flavin adenine dinucleotide) and heme to oxygen at the opposite side of the lipid bilayer. However, the dual oxidases contain an N-terminal peroxidase domain which may release H_2O_2 rather than $O_2\bullet^-$ as the primary product (Ameziane-El-Hassani et al., 2005).

In addition to the role of Nox2 in phagocytic oxidative burst, several studies have suggested the Nox enzymes as a major source of ROS in the vascular wall. Flow cessation induces rapid Nox-dependent superoxide production in endothelial cells leading to enhanced proliferation (Manevich et al., 2001; Wei et al., 1999). Vascular endothelial cells express low level of Nox2 that has been shown to be functional and inducible with PMA (Gorlach et al., 2000). Nox1 is also present in ECs but the main enzyme complex appears to be Nox4 (Ago et al., 2004; Lassegue et al., 2001). While Nox1 and Nox2 are structurally and functionally very similar needing cytosolic subunits for activation, no cellular regulatory factors have been recognized for Nox4. Importantly, Nox4 is localized to endoplasmic reticulum and the nucleus, thus it has been suggested to play a role in regulation of gene expression (Kuroda et al., 2005; Van Buul et al., 2005). The source of ROS may however vary in distinct conditions. For example, Al-Mehdi and others have shown pulmonary artery endothelium to rely on XO during hypoxia and Nox during ischemia (Al-Mehdi et al., 1998). However, recent study showed that hydrogen peroxide is in fact the primary product of XO in physiological conditions (Kelley et al.).

2.3.2 Antioxidative Enzymes and Tissue Redox Balance

Heme containing proteins, such as myoglobin of the muscle, are known to avidly convert H_2O_2 into more reactive hydroxyl radical (George and Irvine, 1951). Thus, skeletal muscle may be especially susceptible to free radical mediated cellular injury. Skeletal muscle compensates for continuous oxidative stress by upregulating antioxidative enzymes, for example Mn superoxide dismutase (Ji et al., 2006).

The first line of defense against ROS, and especially superoxide, is comprised of three compartmentalized superoxide dismutase (SOD) isoforms. SODs catalyze reaction:

$$2O_2\bullet^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

Cu/ZnSOD (SOD1) is located in the cytoplasm, nucleus, and the lysosomes while MnSOD (SOD2) is found from mitochondria. SOD3 is the only extracellular isoform, binding to heparin sulphate, collagen, fibulin-5, and hyaluronan of the extracellular matrix due to its C-terminal cluster of positively charged arginine residues (Folz and Crapo, 1994; Gao et al., 2008; Hjalmarsson et al., 1987;

Nguyen et al., 2004; Petersen et al., 2004b). SOD1 is a homodimer whereas SOD2 and SOD3 form tetramers. SOD2 deficiency leads to perinatal lethality due to neurodegeneration and myocardial injury (Lebovitz et al., 1996). In contrast, SOD1 and SOD3 knock-out mice show relatively mild phenotypes. SOD1 deficiency has been associated with female infertility and axon degeneration (Ho et al., 1998; Reaume et al., 1996), while mice lacking SOD3 do not display any spontaneous phenotype (Carlsson et al., 1995). SOD3 null mice are however more susceptible to ischemia/reperfusion injury and lung hyperoxia (Carlsson et al., 1995; Park et al., 2005) and a recent study suggests that SOD3 protects the heart from oxidant-induced fibrosis and leukocyte infiltration (Kliment et al., 2009; van Deel et al., 2008).

SOD3 was last of the three superoxide isoforms to be discovered (Marklund et al., 1982). It exists as a tetramer composed of two covalently linked dimers (Carlsson et al., 1996; Oury et al., 1996). Each SOD3 subunit contains four domains, the C-terminal domain being responsible for binding to the extracellular matrix (Hjalmarsson et al., 1987; Petersen et al., 2004b). However, rat SOD3 differs from its murine, human, and rabbit counterparts as it has low affinity to heparin in physiological conditions (Carlsson et al., 1996). Furthermore, human SOD3 has an additional cysteine residue as compared to rabbit, mouse or rat, enabling alternative folding that results in enzymatically inactive form (Petersen et al., 2004a). Interspecies heterogeneity makes it difficult to extrapolate results from animal models to humans. The main SOD3 expression sites *in vivo* are the vascular wall, lung, kidney, thyroid gland, and epidymis (Marklund, 1984; Perry et al., 1993; Stralin et al., 1995) with minor expression also in liver, heart, brain, spleen, skeletal muscle, and intestine (Folz and Crapo, 1994; Folz et al., 1997). SOD3 represents only a minor part of total SOD activity in most of the tissues. However, SOD3 is highly expressed in VSMCs, and is responsible for approximately 50% of SOD activity in human aorta (Luoma et al., 1998).

Once superoxide is converted to hydrogen peroxide by the superoxide dismutases, it is further processed into water by catalase and a large family of peroxidases. Peroxidases dispose H_2O_2 by oxidizing a secondary reductant such as glutathione (glutathione peroxidases). Catalases are able to utilize H_2O_2 itself as a reductant, oxidizing H_2O_2 to molecular oxygen. Because H_2O_2 is membrane permeable, intracellular peroxidases and catalase may participate in neutralization of extracellular H_2O_2. Physiological concentrations of intracellular H_2O_2 have been shown to range from 0.001 to 1 μM which corresponds to extracellular concentration of 0.01 to 10μM (Antunes and Cadenas, 2000; Stone and Yang, 2006).

Table 1. Redox enxymes.

Enzyme	Reaction	Cellular location
Nox1	$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2\bullet^- + H^+$	Plasmamembrane
Nox2	$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2\bullet^- + H^+$	Plasmamembrane, endosomes
Nox4	$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2\bullet^- + H^+$	Nuclear membrane
Xanthine Oxidase	hypoxanthine + $H_2O + O_2 \rightarrow$ xanthine + H_2O_2 xanthine + $H_2O + O_2 \rightarrow$ uric acid + H_2O_2	Cytoplasm
Myeloperoxidase	$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$	Lysosomes, PMN azurophilic granules
Superoxide dismutase 1	$SOD\text{-}Cu^{2+} + O_2\bullet^- \rightarrow SOD\text{-}Cu^{1+} + O_2$ $SOD\text{-}Cu^{1+} + O_2\bullet^- + 2H^+ \rightarrow SOD\text{-}Cu^{2+} + H_2O_2$	Cytoplasm
Superoxide dismutase 2	$SOD\text{-}Mn^{3+} + O_2\bullet^- \rightarrow SOD\text{-}Mn^{2+} + O_2$ $SOD\text{-}Mn^{2+} + O_2\bullet^- + 2H^+ \rightarrow SOD\text{-}Mn^{3+} + H_2O_2$	Mitochondria
Superoxide dismutase 3	$SOD\text{-}Cu^{2+} + O_2\bullet^- \rightarrow SOD\text{-}Cu^{1+} + O_2$ $SOD\text{-}Cu^{1+} + O_2\bullet^- + 2H^+ \rightarrow SOD\text{-}Cu^{2+} + H_2O_2$	Extracellular
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$	Peroxisome
Glutathione peroxidase (GPx)	$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$	Cytoplasm, Extracellular (GPx2 and 3)
Glutathione reductase	$GS\text{-}SG + NADPH + H^+ \rightarrow 2GSH + NADP^+$	Cytoplasm

2.3.3 ROS Mediated Signaling and Cell Survival

Hydrogen peroxide is able to permeate cell membranes at a rate with permeability coefficients ranging from 0.01 to 0.7 cm/min (Makino et al., 2004). However, in contrast to previous assumptions recent evidence suggests this to happen through aquaporin channels instead of direct permeability (Bienert et al., 2007). Among ROS, H_2O_2 is the best candidate for use as a second messenger due to its ability to permeate cell membranes and its relatively long half-life. However, a major caveat in determining the role of H_2O_2 as a signaling molecule is the frequent use of supraphysiological concentrations in *in vitro* studies. Reassuringly, recent studies with lower concentrations have confirmed many of the previous findings (Fisher, 2009; Forman, 2007). Despite its short half-life, superoxide does have specific effects on some settings that cannot be attributed to H_2O_2 (Madesh et al., 2005). Increasing evidence shows that controlled perturbation of ROS balance is utilized in various signaling cascades including but not limited to activation of PI3K/Akt, NF-κB, and Ras-Erk1/2 pathways. At least in the context of angiotensin II-induced ROS formation in the vascular wall, the Nox complexes appear to be a major source for ROS production (Lassegue et al., 2001).

Transmission of extracellular signals by receptor tyrosine kinases (RTKs) through Ras to the MAPKs (mitogen-activated protein kinase) represents an archetypal example of cellular signaling cascade. ROS may activate this pathway at multiple steps. RTKs such as endothelial growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) regulate a wide range of cellular functions extending from cell proliferation and differentiation to cellular migration and survival. Ligand binding induces RTK dimerization leading to activation of the intracellular C-terminal tyrosine kinase domain. Autophosphorylation of C-terminal tyrosine residues recruits various proteins, most notably including c-Src kinase, phospholipase C (PLC), phosphatidyl inositol-3-kinase (PI3K), and adaptors Grb2 and Shc. The recruited proteins initiate a repertoire of subsequent effector cascades required for a specific response.

The Ras GTPase is the critical link between RTK activation and extracellular-signal regulated kinase (Erk). RTK activation stimulates Ras through Sos (Son-of-Sevenless), a guanine nucleotide exchange factor (GEF) recruited by Grb2 to the activated RTK. Sos induces an exchange of GDP for GTP on Ras which allows Ras to directly interact with its target effectors such as Raf. The classical view of Ras function places the RTK and Ras on the plasma membrane. However, activated Ras proteins can be detected from various intracellular sites including the Golgi, endoplasmic reticulum, and the endosomes (Berthiaume, 2002; Casar et al., 2009). Compartmentalization may determine the strength, duration and targets of Ras signaling thus influencing the ultimate outcome (Casar et al., 2009). Raf family kinases comprise crucial downstream effectors of Ras activation. Raf kinases exist in an inactive state in the cytoplasm wherein the N-terminal domain inhibits the C-terminal kinase domain. Raf activation follows complex process of protein interactions, membrane localization, phosphorylation of activating sites, and dephosphorylation of inhibitory sites. In contrast to the complexity of Raf activation, the subsequent Mek and Erk kinases are activated by simple phosphorylation of activation segments in their kinase domains. The kinase components of the MAPK pathway are brought together by scaffold proteins such as Paxillin which directs Erk activation at sites of focal adhesions (Ishibe et al., 2004). Indeed, the scaffold proteins serve to insulate the MAPK module from irrelevant stimuli and to regulate its subcellular localization. The final effects of Ras-Erk pathways are mediated by transcription factors activated by Erk subsequent to dissociation from the scaffold protein and nuclear translocation.

ROS may lead to Erk activation by interfering with multiple steps in the upstream signaling cascade. EGFRs and PDGFRs are known to be subjected to ligand-independent activation by ROS (Knebel et al., 1996). Furthermore, receptor activation by ROS is not necessary for ROS mediated Ras activation (Lander et al., 1996), nor is Ras expression needed for activation of Erk by ROS (Zou et al., 1996). However, Mek inhibitors U0126 and PD98059 do prevent ROS mediated Erk1/2 activation indicating that ROS do not activate Erk1/2 directly (Lee et al., 2006). Possible pathways leading to Erk1/2 activation include route via c-Src which leads to increased intracellular calcium levels activating protein kinase C (PKC) (Wang et al., 2001). PKC is known to target both Ras and Raf (Buhl et al.,

1995). Finally, thiolate-dependent protein phosphatases utilize a deprotonated cysteine thiolate moiety in their reactive group making them vulnerable to reversible oxidative inactivation that may lead to up-regulation of kinase activity and subsequent Erk1/2 activation (Whisler et al., 1995).

Mammals have at least a dozen MAPK genes, of which Erk 1 and 2, c-Jun terminal kinases (JNK1-3), and p38(α, β, γ, and δ) are the best known. The JNKs and p38 MAPKs are activated in response to different stressors such as inflammatory cytokines, ionizing radiation, DNA damage, and oxidative stress. Erk1/2 on the other hand mediates mitogenic and pro-survival effects in response to growth factors and cell stress. Consequently, aberrant regulation of Erk activity due to e.g. Ras mutations is frequently associated with cancerous growth. Some examples for transcription factors activated by Erk1/2 are the activator protein (AP)-1, c-Myc, and cyclic AMP responsive element binding protein (CREB). Erk may exert its effect on these factors either by direct phosphorylation or through intermediate kinases such as the RSKs (Gelain et al., 2006; Shaywitz and Greenberg, 1999; Sun et al., 2006; Turjanski et al., 2007). Transcription factors of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra2) families make up the AP-1 by forming homo or heterodimers. While c-Jun is under stringent regulation by JNK, Erk1/2 acts synergistically with JNK to activate AP-1 by stimulating c-Fos expression. Transcription factors CREB and AP-1 stimulate prosurvival effects by activating genes such as VEGF-A and Cyclin D1 (Shen et al., 2008; Wu et al., 2007a). VEGF is well known to promote survival of myoblasts (Germani et al., 2003) and endothelial cells (Gerber et al., 1998; Lee et al., 2007), while Cyclin D1 is a crucial cell cycle regulator guarding the cell cycle G1/S transition (Takuwa and Takuwa, 2001).

The mitogenic Ras-Erk pathway is closely intertwined with survival promoting PI3K/Akt pathway. As previously noted, activated RTKs may recruit PI3K. This leads to generation of 3'phosphorylated phosphoinositides that in turn recruit protein kinase B, also known as Akt. Activation of Akt in response to oxidants is dependent on tyrosine kinases; EGFR in HeLa cells (Takuwa and Takuwa, 2001), PDGFR in primary fibroblasts (Klotz et al., 2000), and Syk non-receptor tyrosine kinase in B-cells (Ding et al., 2000). Akt is known to suppress apoptosis by phosphorylation-dependent inhibition of pro-apoptotic factors such as forkhead transcription factors, caspase 9, and BAD (Datta et al., 1999; Kandel and Hay, 1999).

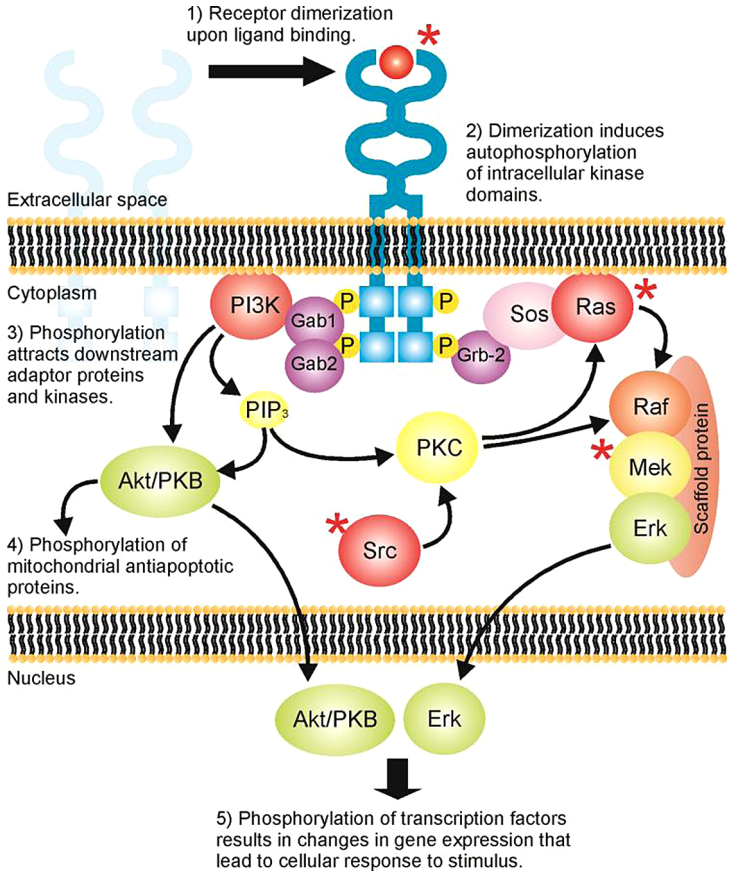

Figure 3. Signaling downstream of receptor tyrosine kinases. 1) Ligand binding induces dimerization of the receptor tyrosine kinases. 2) This in turn enables autophosphorylation of the RTK intracellular domains that 3) are recognized by adaptor proteins such as Grb-2, and Gab1 and 2. Sos is a guanine nucleotide exchange factor that activates Ras by inducing Ras GTP-binding. Activation of Ras leads to phosphorylation of Erk, whereas recruitment of PI3K by the Gab-proteins leads to activation of Akt. 4) Akt promotes cell survival by inhibiting mitochondrial pro-apoptotic proteins. Furthermore, 5) Akt and Erk induce changes in gene expression by direct or indirect modulation of nuclear transcription factor activity. (*) Potential points for ROS-mediated activation: ROS are known to induce ligand-independent RTK activation but also activate several downstream kinases leading to Erk activation.

2.4 Inflammation

2.4.1 Role and Initiation of Inflammation

Accumulation of polymorphonuclear neutrophils (PMNs) to ischemic muscle occurs only hours after acute reduction in arterial perfusion. PMNs are then largely replaced by circulating monocytes that differentiate into macrophages in target tissue. Macrophages reach peak numbers few days after arterial occlusion. The main role of the infiltrating phagocytes is to clear cellular debris from cells that have died from necrosis. Macrophages are also required for tissue regeneration as they promote revascularization of the tissue and enhance satellite cell proliferation (Merly et al., 1999; Tidball and Wehling-Henricks, 2007). Upon activation phagocytic PMNs and macrophages produce potentially cytolytic Nox2 mediated oxidative burst (Entman et al., 1992; Nguyen and Tidball, 2003). Furthermore, degranulation of PMNs releases active myeloperoxidase that may compete with catalase for H_2O_2. MPO produces hypochlorous acid further enhancing PMN mediated cytotoxicity (Winterbourn, 1986). Prolonged or overly aggressive inflammation may prove to be detrimental to the host. Therefore, inflammatory process is tightly regulated by a coordinated program of resolution that is initiated already at few hours after beginning of the inflammatory reaction (Serhan et al., 2008).

Several overlapping mechanisms serve to initiate inflammatory response within ischemic tissue, these include cellular constituents released by necrotic cell death, cytokines, ROS, the complement system (Arumugam et al., 2004), and Toll-like receptor (TLR)-mediated pathways (Arumugam et al., 2009). All of these inflammatory triggers are able to activate transcription factor Nuclear factor (NF)-κB which is considered a central regulator of inflammatory response. Indeed, NF-κB is required for EC activation (Wrighton et al., 1996). NF-κB promotes expression of several inflammatory mediators such as cytokines (IL-1, IL-6, IL-8, TNFα), chemokines (MCP-1, MIP2), and cell adhesion molecules (E-selectin, ICAM-1, VCAM-1) (Collart et al., 1990; De Martin et al., 2000; Denk et al., 2001; Libermann and Baltimore, 1990; Mori and Prager, 1996; Shakhov et al., 1990; Shimizu et al., 1990; Ueda et al., 1997; Widmer et al., 1993). NF-κB comprises homo- or heterodimers formed by family of transcription factors including members RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) (Karin and Ben-Neriah, 2000). Under basal conditions NF-κB is retained in the cytoplasm by inhibitory subunits IκBα, IκBβ or IκBε. The best characterized form of NF-κB activation follows phosphorylation of IκBα Ser 32 and 36 residues by the IKKs 1/α and 2/β (IκB-kinase). Phosphorylation of IκBα directs it to proteasomal degradation thus revealing the NF-κB nuclear localization signal and allowing nuclear translocation and subsequent binding to target promoters (Karin and Ben-Neriah, 2000). Rapid activation of NF-κB by degradation of IκBs is largely dependent on IKKβ, and is referred to as the canonical or classical NF-κB pathway. In what is named the alternative pathway, the IKKα mediates slower NF-κB activation by promoting synthesis of NF-κB2 from its precursor p100

(Senftleben et al., 2001). The classical NF-κB activation has been suggested to be the main mediator of pro-inflammatory gene expression. Indeed, TNFα-induced NF-κB-dependent gene expression of MCP-1, IL-8, ICAM-1, VCAM-1, and E-selectin in endothelial cells has been suggested to be dependent on IKK2 (Denk et al., 2001).

As previously mentioned, NF-κB is known to be regulated by ROS. ROS may affect NF-κB activity both positively and negatively, and the response is cell-type specific relying on several mechanisms (Gloire et al., 2006). However, many aspects of ROS-mediated NF-κB activation are not thoroughly understood. It is known that IL-1, and LPS (lipopolysaccharide) induce superoxide production through Rac1 which is involved in the assembly of the Nox complex (Abo et al., 1991). Rac-induced ROS production is required for IL-1 and LPS-mediated NF-κB activation (Bonizzi et al., 1999; Sanlioglu et al., 2001). Miller et al. showed recently that Nox1 located in signaling endosomes is required for TNF and IL-1 induced NF-κB activation in smooth muscle cells (Miller et al., 2007). Furthermore, PI3K and PKC mediated signaling induce Nox2-dependent NF-κB activation in endothelial cells (Frey et al., 2006). ROS regulate NF-κB activity by e.g. promoting IκBα phosphorylation through both canonical and non-canonical pathways. Src-kinase seems to arise as a major contributor in this process as it has been shown to be able to directly phosphorylate Tyr42 residue on IκBα (Fan et al., 2003) while also promoting canonical IKK mediated phosphorylation (Funakoshi-Tago et al., 2005).

Although most studies concentrate on cytoplasmic activation of NF-κB, NF-κB is also subject to direct oxidation that abrogates its DNA-binding activity in the nucleus (Toledano and Leonard, 1991). Inactivation of NF-κB in oxidative conditions is due to oxidation of critical Cys62 residue (Matthews et al., 1993). The same residue can be modulated by nitrosylation thus making NF-κB susceptible to NO mediated inactivation (Matthews et al., 1996). In addition, NO reduces NF-κB activation by inhibiting IκBα phosphorylation (Katsuyama et al., 1998). As NF-κB has been shown to up-regulate inducible NOS (iNOS), it is thought to function as negative regulator of NF-κB through NO production (Xie et al., 1994).

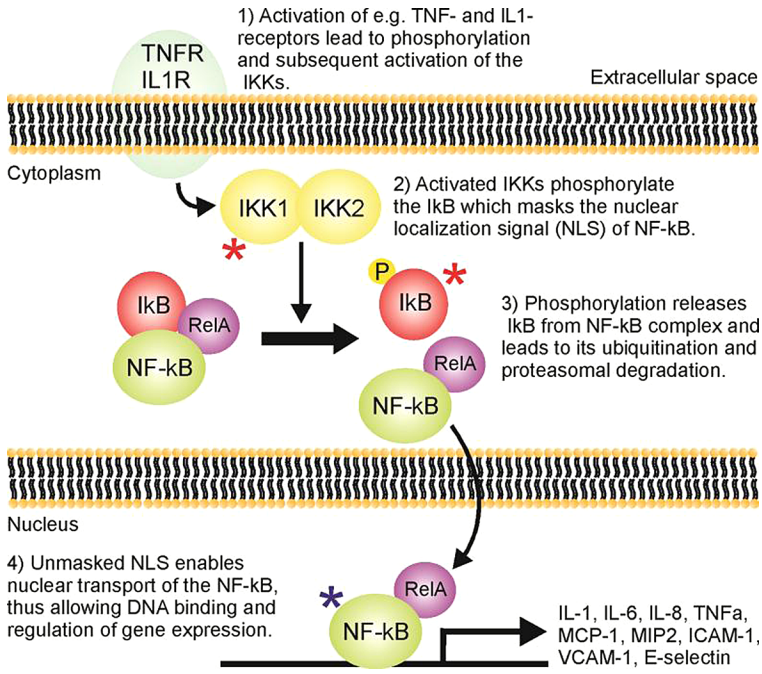

Figure 4. NF-κB signaling. NF-κB may be activated by various stimuli, among them TNF or IL-1. 1) TNF- and IL-1-receptors mediate NF-κB activation by inducing phosphorylation of the IKKs. 2) Subsequently, IKKs phosphorylate IκB within the NF-κB complex 3) leading to ubiquitination and proteasomal degradation of the IκB subunit. 4) Dissociation of IκB from the NF-κB complex reveals nuclear localization signal allowing nuclear transport and regulation of gene expression. (⋆) ROS-mediated activation of NF-κB: ROS activate NF-κB both through the canonical IKK 2/β pathway and by activation of Src kinase, which is able to directly phosphorylate IκB. (⋆) ROS-mediated inhibition of NF-κB: ROS may also suppress NF-κB activity by oxidizing the Cys62 residue that is critical for the DNA-binding of NF-κB.

2.4.2 Leukocyte Extravasation

Upon activation, endothelial cells upregulate adhesion molecules and chemokines initiating a complex cascade of cellular interactions leading to extravasation of leukocytes from the blood vessel lumen to the surrounding tissue. The main site for leukocyte trafficking in muscle is the post-capillary venules (Aird, 2007). Leukocytes penetrate the vascular wall either directly through the ECs (transcellular route) or by squeezing through the EC-EC junctions (paracellular route). Leukocyte transmigration can be divided into several steps including: rolling, activation, arrest, adhesion strengthening, crawling, transmigration, and migration through the basement membrane (Ley et al., 2007).

The process of leukocyte extravasation is initiated when transient molecular interactions induce rolling of the leukocyte along the endothelium (Zarbock and Ley, 2009). Rolling is mainly mediated by L-selectin (CD62L), E-selectin (CD62E), and P-selectin (CD62P). While L-selectin is expressed in most

leukocytes, P- and E-selectin are found in inflamed endothelium. ECs store P-selectin in the Weibel-Palade bodies which release P-selectin on the cell surface upon stimulation, such as oxidative stress (Akgur et al., 2000). In contrast, E-selectin is mainly regulated at transcriptional level by e.g. NF-κB (Collins et al., 1995). P-selectin glycoprotein ligand (PSGL)-1 is the dominant ligand for all of the three selectins. What enables selectins to effectively mediate rolling is their ability to strengthen the ligand-selectin bond when shear stress is applied (Marshall et al., 2003). In fact, selectins require shear stress to function properly, as rolling leukocytes stop and detach when flow is stopped (Finger et al., 1996). Although P- and E-selectin are the most important rolling molecules, integrins also take part in the process. Leukocytes can utilize $\alpha_4\beta_7$ and $\alpha_4\beta_1$ (Very late antigen 4, VLA4) to roll on mucosal vascular addressin cell adhesion molecule (MADCAM)-1 or on vascular cell adhesion molecule (VCAM)-1, respectively (Berlin et al., 1995). VLA4-dependent rolling has been detected on monocytes and T-cells (Chan et al., 2001; Singbartl et al., 2001).

Rolling brings the leukocyte to close proximity with the endothelium and the secreted chemokines bound on glycosaminoglycans of the endothelial cell surface (Johnson et al., 2005). Chemokines act as chemoattractants directing leukocyte traffic and they are subdivided into C, CC, CXC, and CX3C families according to their structure. Fractalkine/CX3CL1 and lymphotactin/XCL1 are the only known members of the CX3C and C chemokine families, respectively. CC and CXC families are more diverse and far better characterized. Homeostatic chemokines such as CCL21 and CCL17 (L for ligand) and their receptor CCR7 (R for receptor) are constitutively expressed in lymphatic organs and mediate physiological leukocyte traffic (Bromley et al., 2008). Inducible chemokines on the other hand are expressed by various cell types and are strongly upregulated by e.g. NF-κB in response to inflammatory stimuli. A significant role has been suggested for MCP-1/CCL2 in ischemic injuries. MCP-1 is a potent attractant for monocytes, NK cells and T cells and its expression peaks early after femoral artery excision or myocardial infarction in mice (Kumar et al., 1997; Lee et al., 2004). Neutralizing antibody to MCP-1 reduces myocardial infarct size and infiltration of macrophages (Ono et al., 1999). In addition to its chemotactic properties, MCP-1 may modulate cytokine expression by the macrophages. MCP-1 has been shown to induce IL-1 and IL-6 expression in monocytes (Jiang et al., 1992).

The leukocyte arrest and adhesion strengthening are mediated by the integrins, a large family of receptors that bind endothelial immunoglobulin superfamily members VCAM-1 and ICAM-1. Integrins are heterodimeric receptors composed of 18 α and 8 β subunits. Integrins of subfamilies β2 and β1 are most commonly involved in mediating tight adhesion of leukocytes to the endothelium. Integrins are maintained in an inactive bent conformation in circulating leukocytes. Chemokine-induced GPCR activation induces conformational changes that enable tight interaction with the ligand and thus leukocyte arrest. Integrin avidity is however also regulated by valency, i.e. the density of integrin heterodimers per area of plasmamembrane involved in cell adhesion.

Before transmigration through the vessel wall, leukocytes crawl along the endothelial surface in search of suitable migration site. Leukocytes usually migrate through the paracellular route in places where there are gaps between pericytes and thus lower extracellular matrix deposition (Wang et al., 2006). Leukocytes may be able to home to these sites because they are more permissive to chemokines thus creating a chemotactic gradient. Crawling behavior has been described for monocytes and neutrophils *in vitro* and *in vivo*, and the process requires Mac-1 (Macrophage receptor1/CD11b/CD18/$\alpha_M\beta_2$ integrin) integrin and its endothelial counterpart ICAM-1 (Phillipson et al., 2006; Schenkel et al., 2004). In Mac-1 knock-out mice the crawling is disabled and promotes leukocyte extravasation through the transcellular route. Transcellular transmigration is poorly understood but appears to be substantially slower than the transcellular route (Phillipson et al., 2006).

Transmigration via the paracellular route is permitted due to redistribution of intercellular junctional molecules. Molecules that might hinder transmigration, such as vascular endothelial (VE)-cadherin, may be redistributed away from junctional regions (Shaw et al., 2001). On the other hand, molecules that mediate EC-leukocyte interactions are brought into the junctional area. For example, platelet/endothelial cell adhesion molecule (PECAM-1/CD31) is stored in intracellular vesicular compartment, and is released to EC junctions to mediate transmigration (Mamdouh et al., 2003). Modulation of the EC cell junctions to allow leukocyte transmigration can be triggered by e.g. integrin binding to VCAM-1 and ICAM-1. Integrin binding to VCAM-1 activates endothelial Nox complex leading to oxidation dependent PKCα activation and to localized actin structural changes that are required for lymphocyte migration (Abdala-Valencia and Cook-Mills, 2006; Matheny et al., 2000). In contrast, XO-derived Nox-independent superoxide production has been suggested to mediate migration of monocytes through the blood-brain barrier due to disruption of tight junctions (Van der Goes et al., 2001). Either way, accumulating evidence suggest ROS to function in opening gaps between endothelial cells to enable leukocyte traffic (Cook-Mills, 2002). Specificity of leukocyte subtype migration may be achieved by distinct expression pattern of chemokines, adhesion molecules, and their ligands. Furthermore, ligand affinity and expression level of the chemokine receptors may contribute to the specificity of leukocyte adhesion (D'Ambrosio et al., 2002).

The final obstacle between migrating leukocyte and the target tissue is formed by the basement membrane mainly composed of collagen IV and laminins 8 and 10 (Hallmann et al., 2005). Leukocytes prefer to extravasate through gaps in the pericyte coverage where expression of ECM proteins is lower than average. Neutrophils express cell surface proteases that facilitate migration. Indeed, the "holes" in basement membrane that leukocytes prefer for transmigration are transiently enlarged after neutrophil migration (Wang et al., 2006). The extracellular matrix may also be degraded by endothelium associated matrix metalloproteinases (MMPs) which are activated by 1μM H_2O_2 derived from Nox produced superoxide (Cook-Mills, 2006; Deem and Cook-Mills, 2004).

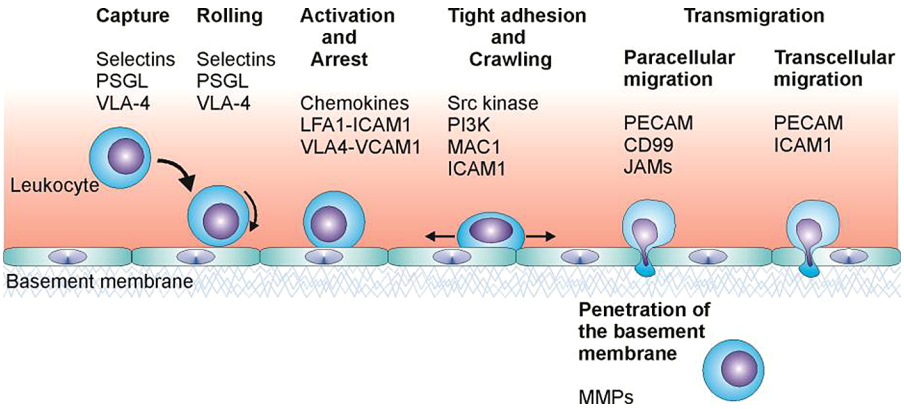

Figure 5. Leukocyte adhesion and transmigration cascade. Leukocyte extravasation is characterized by a cascade of molecular interactions between the leukocyte and the endothelium. The process is understood in increasing precision and some of the key molecules involved in each step are listed. Notably, selectins are crucial for the capture and rolling of leukocytes whereas integrins are best described in mediating tight adhesion. Finally, matrix metalloproteinases enable permeation of the vascular basement membrane (Modified from (Ley et al. 2007)).

2.4.3 Resolution of Inflammation

Due to potentially detrimental effects, inflammatory reaction must be limited both in duration and in area. Contrary to early hypotheses of resolution through passive depletion of inflammatory mediators, a complex, actively driven process has begun to emerge. One of the mechanisms promoting resolution is the propensity of PMNs to succumb to spontaneous apoptosis and phagocytosis-induced cell death (Kennedy and DeLeo, 2009). Apoptotic neutrophils are subsequently ingested by macrophages, thus neutrophils are disposed without releasing cytotoxic molecules that would damage the surrounding tissue. Uptake of apoptotic bodies triggers exit of the macrophage from the inflamed tissue through the draining lymphatics (Bellingan et al., 1996). Macrophages also promote resolution by secreting anti-inflammatory cytokines such as transforming growth factor (TGF)-β (Byrne and Reen, 2002).

In addition to transcriptional regulation of pro-inflammatory cytokines, NF-κB also plays a crucial role in inflammatory resolution. For example, NF-κB mediates expression of IκBα which can effectively inhibit EC activation (Wrighton et al., 1996). Furthermore, cytokines and chemokines are under post-transcriptional regulation by modulation of mRNA stability (IL-1, TNF, IL-6, CCL2, CXCL3, etc.) or by regulation of initiation of translation (TNF, CCL22, CCR3, etc) (Anderson). Finally, glucocorticoids comprise a group of anti-inflammatory mediators (such as prednisolone, dexamethasone, beclomethasone, and of course cortisol and corticosterone) that have been successfully utilized for therapeutic purposes. The use of glucocorticoid drugs is however limited by complications of the cardiovascular system, more specifically atherosclerosis and hypertension (Roy et al., 2009). Glucocorticoids bind cytoplasmic glucocorticoid receptor that

translocates to the nucleus upon ligand binding, and subsequently modulate gene expression to the extent that ~1% of the genome has been suggested to be regulated by these drugs (Rhen and Cidlowski, 2005). One of the described mechanisms is transrepression of NF-κB by activating histone deacetylase 2 that in turn reduces access of NF-κB to promoter region of its target genes (Ito et al., 2006).

2.5 Formation of New Blood Vessels

Facing ischemia, the long-term survival of the affected tissue is critically dependent on the revascularization of the tissue. Blood vasculature is relatively quiescent during adulthood, formation of new blood vessels is mainly restricted to female reproductive cycle, wound healing, and pathological conditions such as atherosclerosis and cancer. In response to ischemia blood vasculature may extend either through *de novo* formation by differentiation of vascular stem cells (post-natal vasculogenesis), or through remodeling of pre-existing vessels by sprouting (angiogenesis) or collateral artery growth (arteriogenesis).

2.5.1 Post-natal Vasculogenesis

Vasculogenesis has traditionally been considered to be restricted to the embryonic generation of the vascular tree. In 1997 Asahara was the first to provide evidence for circulating progenitor cells that contributed to neovascularization (Asahara et al., 1997). Subsequently it was shown that these bone marrow derived cells, endothelial progenitors (EPCs), were able to incorporate to the vessel wall and to differentiate not only into endothelial cells but also periendothelial cells expressing pericyte markers SMA, NG2 and desmin (Rajantie et al., 2004). This led to coining the term "post-natal vasculogenesis" which refers to homing of BM-derived EPCs into sites of neovascularization to differentiate into vascular cells.

Mobilization of EPCs has been found to be significantly increased in response to myocardial or peripheral ischemia (Asahara et al., 1999). The true contribution of EPCs on neovascularization is nevertheless very controversial as the extent of EPC contribution on vessels of ischemic tissue has varied from non existent (Zentilin et al., 2006) to almost 90% of the vascular endothelium (Crosby et al., 2000). Controversy may partly arise from the difficulty to reliably define EPCs as they share many surface markers (e.g. CD34, CD133 and VEGFR2) with the hematopoietic stem cells (HSCs). Indeed, it has been suggested that EPCs and HSCs are derived from a common precursor, the hemangioblast (Flamme and Risau, 1992). EPCs are thought to lose CD133 expression and gain endothelial markers in culture (Peichev et al., 2000); however, Rehman et al. showed that most of these cells express pan-leukocyte marker CD45 and monocytic marker CD14 (Rehman et al., 2003). Further evidence for traditionally defined EPCs actually being angiogenic macrophages has started to emerge. For example, EPCs co-stimulate T-cells as efficiently as monocytes (Piaggio et al., 2009) and the observed upregulation of endothelial markers in culture may actually be due to platelet contamination (Prokopi et al., 2009). Despite the emerging consensus that EPCs are not true endothelial progenitors, it is clear that they do enhance vascular

repair. However, this may be mediated by growth factor secretion rather than differentiation into vascular endothelium (Pearson).

2.5.2 Collateral Growth (Arteriogenesis)

Collateral is a vessel that provides a natural bypass around the site of occlusion. Collaterals form by arteriogenesis, a process wherein small vessels connecting branches of major arteries gradually enlarge to increase bulk blood flow to the ischemic tissue. Collateral growth is rarely limited to a single vessel, instead many small collaterals develop. However, smaller collaterals tend to regress while few larger ones increase in diameter to provide more efficient blood supply (Heil and Schaper, 2004). Despite dramatic increase in the size of the collateral vessels, functional conductance only reaches 40% of normal in the vascular periphery before collateral development is halted due to reduced wall stress (Ito et al., 1997). Exogenous addition of growth factors such as FGF-2 has failed to increase maximal conductance (Lazarous et al., 1995), this is believed to be caused by reduced wall stress due to collateral enlargement, and self-limiting vessel tortuousity due to vessel lengthening (Heil and Schaper, 2004). However, full conductance was reached one week after femoral artery ligation in rabbits by increasing fluid shear stress (FSS) (Eitenmuller et al., 2006). Indeed, arteriogenesis is not induced directly by ischemia as collateral development begins proximal to the occlusion site. Instead, physical forces seem to play a major role in intiating arteriogenesis (Heil and Schaper, 2004).

Vascular wall is affected by circumferential wall stress that mainly affects the SMCs, and FSS which only affects the endothelium. Compelling evidence suggests endothelial cells as the main instigators of arteriogenesis, namely, endothelial denudation and inhibition of endothelial NOS (eNOS) and iNOS by L-NAME (NOS inhibitor) prevent arteriogenesis (Guzman et al., 1997). Further evidence is provided by SMC behavior, SMCs lose their contractile phenotype in order to proliferate but migrate toward the endothelium before they can divide. If SMCs were to initiate collateral development, they would be expected to proliferate within the outer adventitial vessel layers (Schaper, 2009). The mitogenic signal promoting SMC proliferation is however not yet known.

Perivascular leukocyte accumulation has been shown to be important for collateral development. Endothelial cells attract macrophages to collaterals through chemokines such as MCP-1. Local MCP-1 delivery improves arteriogenesis (Ito et al., 1997) while MCP-1 deficiency leads to reduced perivascular macrophage infiltrate and impaired blood flow recovery (Voskuil et al., 2004). However, lymphocytes have also been shown to support arteriogenesis, although this too appears to be mediated by macrophage recruitment. CD4 knock-out mice exhibit 25% reduction in blood flow recovery after femoral artery occlusion at least in part due to reduced macrophage accumulation (Stabile et al., 2003). Studies on CD8 deficient animals have suggested that $CD8^+$ cells recruit $CD4^+$ cells by expressing IL-16 (Stabile et al., 2006). In both these cases, deficient arteriogenesis was rescued by reconstitution of the absent cell population.

2.5.3 Angiogenesis

Angiogenesis is responsible for extending capillary networks thus fulfilling the need for cells to be within 100-200 μm of the closest blood vessel. In addition to sprouting, vascular network can be extended by division of parent vessels by transendothelial cells (bridging) or by periendothelial cells (intussuception). However, little is known about these processes and their physiological roles (Adams and Alitalo, 2007) whereas vascular sprouting is understood in increasing precision and is known to play an important role in for example wound healing and the menstrual cycle. Furthermore, Judah Folkmans hypothesis for angiogenesis-dependent tumor growth has been confirmed, showcasing the importance of angiogenesis also in pathological conditions (Folkman, 1971; Semenza, 2008).

The most important stimulus for angiogenic vascular growth is hypoxia. Endothelial cells lie in prime position to sense perturbations in local oxygen levels, and are equipped with several mechanisms to do so. Oxygen sensing mechanisms include not only mitochondrial mechanisms, but also prolyl hydroxylase domain proteins (PHD1-3) and factor inhibiting HIFs (FIH) (Fraisl et al., 2009). PHDs and FIHs inhibit HIF-1α function through O_2•-dependent hydroxylation of specific proline or asparagyl residues, respectively, thus leading either to proteasomal degradation or to prevention of interaction between HIF-1α and its transcriptional coactivator p300 (Kaelin and Ratcliffe, 2008). During lowered oxygen tension HIF-1α is stabilized enabling transcription of its downstream targets such as VEGF, and inducible nitric oxide synthase (iNOS) (Liao and Johnson, 2007).

HIF-1α is ubiquitously expressed and virtually all cells can therefore up-regulate VEGF under ischemic conditions. VEGF family is comprised of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral), VEGF-F (snake venom), and the Placental growth factor (PlGF) (Roy et al., 2006). VEGFs have distinct biological properties but VEGF-A is the most studied member of the family and is in fact often simply referred to as VEGF. Among the VEGFs, VEGF-A, and VEGF-D seem to have the most profound angiogenic effects (Rissanen et al., 2003). VEGF-A is expressed as at least six different splice variants leading to products with different affinities to ECM components such as heparin (Houck et al., 1991; Lange et al., 2003; Poltorak et al., 1997; Tischer et al., 1991). The shortest isoform $VEGF_{121}$ does not bind the ECM whereas $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ have increasing binding activity. Mice expressing only $VEGF_{120}$ (mice VEGFs are one amino acid shorter than their human counterparts) do not survive to adulthood indicating that binding of VEGF to the ECM is required for proper function (Ruhrberg et al., 2002). Two VEGF-B isoforms have been found whereas VEGF-C and VEGF-D are expressed as single proteolytically activated forms. VEGF-B has a weak angiogenic effect while VEGF-C promotes lymphangiogenesis. VEGF-D appears to be the most versatile member of the family as it is potent inducer of both angiogenesis and lymphangiogenesis (Rissanen et al., 2003). PlGF may promote angiogenesis by e.g. up-regulating VEGF-A (Bottomley et al., 2000), but results concerning PlGF-mediated angiogenesis are controversial.

VEGF family members prompt cellular responses by binding to the dimeric receptor tyrosine kinases VEGFR-1 (Flt-1), VEGFR-2 (Flk-1, KDR), and VEGFR-3 (Flt-4). VEGF receptors have seven extracellular immunoglobulin-like domains, a single transmembrane domain, and an intracellular tyrosine kinase domain. Knockout mice for any of the three receptors die at early embryonic stage (Dumont et al., 1998; Fong et al., 1995; Shalaby et al., 1995). Upon ligand binding, VEGFRs form hetero- or homodimers leading to subsequent activation of signaling events such as the PI3K-Akt pathway that promotes cell survival and migration (Gerber et al., 1998; Yang et al., 2004) or the PLC-PKC-MAPK pathway that induces cell proliferation (Kroll and Waltenberger, 1997; Takahashi et al., 1999). Nuclear mediators of VEGF-induced cellular actions include the CREB transcription factor which may be activated by e.g. PKC (Mayo et al., 2001). VEGFR-2 binds VEGF-A, VEGF-C and VEGF-D, and takes a central role in mediating VEGF function in ECs (Zachary and Gliki, 2001). In contrast, VEGFR-1 is only weakly mitogenic and actually seems to restrain angiogenesis induced by VEGFR-2 (Fong et al., 1995). This inhibitory role may be potentiated by the presence of soluble VEGFR-1 (Kendall and Thomas, 1993), and its affinity to VEGF-A that is about an order of magnitude higher than that of VEGFR-2 (Waltenberger et al., 1994). Finally, as opposed to modulation of angiogenesis, VEGFR-3 mediates lymphangiogenesis by inducing proliferation, migration and survival of lymphatic ECs after binding VEGF-C and VEGF-D (Makinen et al., 2001; Veikkola et al., 2001).

Angiogenesis is initiated by NO-induced vascular dilation and VEGF-induced vascular leakage. Vascular leakage involves redistribution of cell junctional molecules such as PECAM-1 or vascular endothelial (VE)-cadherin. VEGF induces phosphorylation of VE-cadherin through Src, consequently disrupting VE-cadherin stability at adherens junctions (Dejana et al., 2008). Subsequently, matrix metalloproteinases degrade the ECM allowing migration of proliferating EC and releasing growth factors sequestered within. Disruption of the vascular integrity permits extension of vascular sprouts formed by proliferating ECs which are led by tip cells, endothelial cells that express high level of VEGFR-2 to sense concentration gradient of VEGF-A (Adams and Alitalo, 2007). Migration of ECs can be guided by mechanical forces such as fluid shear stress (mechanotaxis), and by both soluble (chemotaxis) and matrix bound (haptotaxis) growth factors (Lamalice et al., 2007; Li et al., 2005). Subsequently, growing vascular sprouts interconnect with either pre-existing vessels or with other sprouts and blood flow is established by lumen formation. Rather little is known about the lumen formation but studies in zebrafish have suggested that lumen forms by intra- and intercellular fusion of large vacuoles (Kamei et al., 2006).

Newly formed blood vessels are prone to regress in absence of stabilizing signals. The tip cells secrete platelet derived growth factor (PDGF)-B which attracts pericytes and smooth muscle cells that are required for stabilization of the newly formed blood vessel (Hellstrom et al., 1999). The pericytes maintain EC quiescence by secreting TGF-β which inhibits endothelial proliferation and migration, and reduces VEGFR-2 expression (Mandriota et al., 1996).

Furthermore, pericytes produce angiopoietin-1, a growth factor that binds receptor Tie-2. Tie-2 is predominantly expressed in endothelial cells, and promotes EC survival and mural cell attachment (Patan, 1998). However, impaired pericyte recruitment in Tie-2 knockout mice may be secondary effect following EC death (Jones et al., 2001a; Puri et al., 1999). Like arteriogenesis, angiogenesis is not only dependent on endothelial cells, pericytes and SMCs, but leukocytes have an important role in supporting angiogenesis. Especially macrophages have an important role as they secrete large amounts of angiogenic factors such as VEGF, PDGF, IL-8, and prostaglandins (Sunderkotter et al., 1994). Macrophages also digest the ECM enabling EC migration (Anghelina et al., 2004). Macrophage recruitment is driven by MCP-1 (CCL2) which is upregulated in ECs in response to both VEGF and ischemia (Yamada et al., 2003).

The role of reactive oxygen species in RTK signaling and inflammation has already been described above demonstrating the importance of ROS in wide variety of cellular processes. Akin to EGFR or PDGFR, VEGFRs induce ROS production upon activation through Rac-dependent stimulation of the associated Nox2 complex (Ushio-Fukai et al., 2002). VEGFR autophosphorylation is subsequently enhanced by PTP inactivation. Nox2 deficient ($gp91^{phox-/-}$) mice exhibit impaired angiogenesis in hindlimb ischemia (Tojo et al., 2005). Intriguingly, Nox2 produced superoxide appeared to originate from inflammatory cells at earlier 3 day time point while EC-produced ROS dominated at 7 days. Furthermore, Tojo et al. showed that H_2O_2 instead of superoxide was responsible for the angiogenic effect. The role of H_2O_2 in angiogenesis is supported by numerous *in vitro* studies wherein low H_2O_2 concentrations promote EC proliferation and tube formation (Yasuda et al., 1999). Deciphering the role of ROS on vascular function is complicated by tight connection with reactive nitrogen species (RNS). Superoxide is eager to react with NO to produce peroxynitrite (ONOO). SOD3 competes with NO for superoxide and has been suggested to be important modulator of NO bioavailability (Jung et al., 2003). Therefore some of the effects seen after interfering with ROS homeostasis may in fact be due to resulting changes in NO/ONOO balance.

2.6 Stem Cell Contribution

2.6.1 Tissue Resident Muscle Stem Cells

Stem cells can be defined as self-renewing cells capable of differentiation into multiple cell types. According to the most stringent classification fertilized egg and embryonic stem cells capable of producing any cell type of the body are the only true stem cells. However, multipotent and unipotent cells with more restricted differentiation potential are often referred to as somatic stem cells. Production of induced pluripotent stem cells with transient exposure to appropriate transcription factors (Stadtfeld et al., 2008; Zhou et al., 2009), and demonstrations of multipotential differentiation capacity in numerous cell populations have caused further confusion in nomenclature (Tsai et al., 2002).

Although necessary, angiogenesis and inflammation are not alone sufficient to restore muscle function after injury. Injury provokes muscle resident stem cells, the satellite cells, to proliferate in order to later differentiate into myoblasts that fuse either with existing muscle or together to form new myotubes. Proven myogenic potential (Konigsberg et al., 1975; Kuang et al., 2008) and self-renewal capacity (Collins et al., 2005; Montarras et al., 2005) have warranted the definition of "stem cell" for satellite cells. According to general consensus satellite cells are recognized by expression of Pax7, a paired box transcription factor highly redundant with its homolog Pax3 (Bosnakovski et al., 2008; Relaix et al., 2004). However, additional markers such as CD34 and Myf5 show heterogeneity of the satellite cell pool (Beauchamp et al., 2000; Kuang et al., 2007). Interestingly, $Pax7^{+}Myf5^{+}$ cells (~90% of $Pax7^{+}$ cells) seem to be committed myogenic progenitors whereas $Pax7^{+}Myf5^{-}$ cells (the remaining ~10%) represent a putative stem cell population that can give rise to $Pax7^{+}Myf5^{+}$ cells (Kuang et al., 2007).

Satellite cells reside in a well defined niche that controls their proliferation and differentiation (Kuang et al., 2008). Upon activation, satellite cells coexpress Pax7 and MyoD, subsequently up-regulating the myogenic regulatory factors (MRFs) and down-regulating Pax7 leading to myogenic differentiation. A recent study by Lepper et al. showed that, contrary to earlier assumption, Pax7 is not required for muscle regeneration in adult tissue although it is essential up to the juvenile period (Lepper et al., 2009). Gene expression studies suggest that cell proliferation in post-ischemic muscle peaks at around 3 days after injury. At this point MyoD and Myf5 also appear remaining up-regulated until 14 days have passed (Paoni et al., 2002). Satellite cell proliferation coincides with maximal macrophage infiltration. In fact, myoblasts derived from satellite cells secrete MCP-1 and CX3CL1 to attract macrophages that subsequently support further myoblast proliferation (Chazaud et al., 2003).

2.6.2 Alternative Origins of Skeletal Muscle

Satellite cells are thought to be solely responsible for muscle growth and regeneration in physiological conditions (Sherwood et al., 2004). However, cells residing within the vascular wall or circulating in the blood stream may contribute to muscle regeneration in pathological conditions. Stem and progenitor cell populations reported to possess myogenic potential include e.g. pericytes and mesenchymal stromal/stem cells whereas EPCs have been suggested to improve tissue healing mainly through enhanced vascular growth.

In 1982, pericytes were for the first time suggested to serve as progenitors for the surrounding tissue during injury (Richardson et al., 1982). Subsequently, evidence for myogenic potential of skeletal muscle derived pericytes has been obtained *in vitro* both by co-culture with myotubes and in presence of myogenic media (Dellavalle et al., 2007). It is notable that pericytes isolated from mouse retinas only fuse with existing myotubes but do not appear capable of myogenic differentiation (Kirillova et al., 2007). Pericyte-like cells have been suggested to form a continuous subendothelial network that could be responsible for multilineage potential found in adipose tissue and skeletal muscle (Andreeva et al.,

1998; Farrington-Rock et al., 2004). Although pericytes are now believed to display osteogenic, chondrogenic and adipogenic potential (Doherty et al., 1998; Farrington-Rock et al., 2004) the results should be viewed with care due to lack of definitive markers for this cell population (Armulik et al., 2005). However, even in the absence of myogenic differentiation, pericytes do play an important role in muscle regeneration by supporting stabilization of newly formed blood vessels.

Several studies have demonstrated incorporation of bone marrow derived cells into diverse tissues including not only skeletal muscle but also heart, liver, and brain (Bittner et al., 1999; Brazelton et al., 2000; Ferrari et al., 1998; Gussoni et al., 1999; Krause et al., 2001; Lagasse et al., 2000; Mezey et al., 2000; Orlic et al., 2001). A possible explanation for myogenic differentiation potential within the bone marrow is the presence of mesenchymal stromal cells (MSCs). MSCs were first discovered in the bone marrow by Friedenstein who named the cells as colony forming units-fibroblastic (CFU-F) (Friedenstein et al., 1974). Almost twenty years later Caplan renamed the cell population as mesenchymal stem cells due to their self-renewal capacity and differentiation potential (Caplan, 1991; Pittenger et al., 1999). Recently, the international society for cellular therapy proposed mesenchymal stromal cell as a more accurate term leaving the stem cell designation to cells that duly deserve it (Horwitz et al., 2005). Notably, the acronym MSC can be used in either case. The minimal criteria defining mesenchymal stromal cells include: plastic adherence in standard culture conditions, expression of cell surface markers CD105, CD73 and CD90, and lack of surface markers CD45, CD34, CD14 (CD11b), CD79α (CD19) and HLA-DR. Furthermore, MSCs are required to exhibit adipogenic, chondrogenic, and osteogenic differentiation potential *in vitro* (Dominici et al., 2006). First discovered in the BM, MSCs have been subsequently isolated from numerous tissues (da Silva Meirelles et al., 2006), this has led to suggestion that MSCs might have perivascular localization and might actually represent pericytes (da Silva Meirelles et al., 2008; da Silva Meirelles et al., 2006). Saito et al. were the first to demonstrate that injection of MSCs into dystrophic muscle is able cure affected myotubes by providing newly synthetized dystrophin to the cell (Saito et al., 1995). More recent studies have provided highly controversial results regarding the engraftment efficiency of transplanted MSCs (Gang et al., 2009; Muller-Ehmsen et al., 2006).

Table 2. Characteristics of the mesenchymal stromal/stem cells.

Mesenchymal Stromal/Stem Cells	
Plastic adherent	
Surface markers	
+	CD105, CD73, CD90
-	CD45, CD34, CD14 (CD11b), CD79α (CD19), HLA-DR
Multipotential differentiation	
	Adipogenic
	Chondrogenic
	Osteogenic

3 Gene and Cell Therapy

3.1 Gene Therapy

In theory, gene and cell therapy have tremendous therapeutic potential in wide variety of pathological conditions. Both fields have faced huge expectations in the past which however have so far been largely unmet. Gene therapy can be defined as transfer of nucleic acids to somatic cells of an individual with a resulting therapeutic effect. Several strategies can be utilized for gene therapy (Fischer and Cavazzana-Calvo, 2008). First, loss-of-function mutations can be repaired by addition of a normal copy of the mutated gene. This approach is the most feasible with current techniques, and has achieved most success including the first gene transfer in man in 1989 (Pahwa et al., 1989) and the recent successes for treatment of adrenoleukodystrophy in two young boys (Cartier et al., 2009), and correction of colorblindess in squirrel monkeys (Mancuso et al., 2009). Second, small interfering RNAs can be utilized to inhibit expression of overly active gene due to gain-of-function mutation (Pelletier et al., 2006). And finally, mutation can be reverted although this is technically difficult, has only been achieved *in vitro*, and clinical application is long way ahead (Porteus and Carroll, 2005).

3.1.1 Gene Transfer Vectors

Optimal gene therapy vector would provide long term stable and regulated expression of the transgene in the target tissue without toxic side effects or activation of immune response. Gene transfer vectors can be divided into viral and non-viral vectors. Non-viral vectors may rely on naked plasmid DNA or different carrier molecules such as liposomes (cationic lipid bilayers) whose advantage is low immunogenicity, ease of production and high DNA-carrying capacity. The disadvantage of liposomes and other non-viral vectors is their low transduction efficiency and transient expression of the transgene (Pathak et al., 2009). Due to their natural propensity to carry genetic material into cells, viral vectors have dominated gene therapy field. Most common viral vectors are adenoviruses, retroviruses, lentiviruses, herpesviruses, and adeno-associated viruses. In viral vectors, sequences essential for replication are replaced by treatment and regulatory sequences rendering the virus replication deficient.

Adenoviruses are non-enveloped viruses with linear double-stranded DNA genome approximately 36 kilobases in length (Cao et al., 2004). The major advantages of adenovirus for gene therapy are high *in vivo* transduction efficiency, ease of production, and ability to transduce terminally differentiated non-dividing cells. On the other hand, the biggest disadvantage is high immunogenicity which prevents repeated administration due to formation of neutralizing antibodies (St George, 2003). As wild type human adenoviruses exist in at least 47 different serotypes, immunogenicity can be avoided to some extent by using vector based on different serotype. The first generation adenoviral vectors are based on serotypes 2 and 5 with deletions in regions E1, E2, and E3 of the viral genome (Cao et al., 2004). Adenovirus provides transient transgene expression which declines due to loss of vector DNA within 1-2 weeks of the gene transfer.

Coxackie-adenovirus receptor (CAR) and α_v integrins serve as receptors for most adenoviruses. However, as these are down-regulated during muscle maturation adenoviral transduction efficiency of skeletal muscle is relatively weak. In contrast, satellite cells and myoblasts are more susceptible to adenoviral infection due to more prominent CAR expression (Nalbantoglu et al., 1999).

RNA viruses of the retrovirus family have been extensively utilized for gene transfer due to their ability to integrate their genome into the host cell leading to stable long term expression of the transgene. Use of retroviruses *in vivo* is restricted by their inability to transduce non-dividing cells such as terminally differentiated myocytes. Therefore, retroviruses are mainly used in *ex vivo* applications such as the much publicized X-SCID (X-linked severe combined immunodeficiency) trials that led to leukemia in 3 out of 20 patients and caused a major setback for the whole gene therapy field (Cavazzana-Calvo et al., 2000; Fischer and Cavazzana-Calvo, 2008; Gaspar et al., 2004; Hacein-Bey-Abina et al., 2003). A separate genus of the retrovirus family, lentiviruses, seem to constitute a safer option as they display substantially weaker genotoxicity (Montini et al., 2009). Further advantage for lentiviruses is provided by their ability to transduce non-dividing cells, although transduction of skeletal muscle and myocardium seems to remain low (Kang et al., 2002).

Table 3. Gene therapy vectors.

Vector	Advantages	Disadvantages
Naked plasmid, liposomes, etc.	Low immunogenicity	Low transduction efficiency
	Ease of production	Transient expression
Adenovirus	High transduction efficiency	High immunogenicity
	Ease of production	Transient expression (<2 weeks)
	Ability to transduce non-dividing cells	Low transduction efficiency in muscle
Adeno-associated virus	Long expression	Limited DNA capacity
	Ability to transduce non-dividing cells	Difficult production
	Tropism for skeletal muscle and myocardium	
	Low immunogenicity	
Retrovirus	Stable gene expression	Unable to transduce non-dividing cells
		Genotoxicity
		Limited capacity
		Low titers
Lentivirus	Ability to transduce non-dividing cells	Low efficacy except in hematopoietic cells
	Low immunogenicity	Relatively difficult production
	Stable gene expression	Relatively low titers
	Low genotoxicity	
	High DNA-capacity	
Herpes simplex-virus	High efficacy	Unable to transduce non-dividing cells
	High DNA capacity	Cytotoxicity
	Ease of production	

3.1.2 Antioxidative Gene Therapy – Extracellular Superoxide Dismutase

The role of reactive oxygen species has long been appreciated in numerous pathological conditions including diseases of the central nervous system (Halliwell, 1992), vascular diseases (Palinski et al., 1989), pulmonary diseases (Ryrfeldt et al., 1993), ischemic conditions (Darley-Usmar et al., 1989), and cancer (Cerutti, 1985). Among SOD isoforms, SOD3 could be best suited for therapeutic applications due to its inherent stability in the extracellular space. SOD3 has been shown to have a half-life of 15-20 hours within vasculature (Karlsson et al., 1993), and approximately 85 hours in muscle or subcutaneous tissue (Karlsson et al., 1994). Studies utilizing both transgenic mice and SOD3 gene transfer have attempted to resolve the therapeutic potential of SOD3 over-expression. While most of the studies concentrate on atherosclerotic, ischemic or pulmonary disease models, the studied conditions range from paracetamol-induced liver injury to antigen induced arthritis (Dai et al., 2003; Laukkanen et al., 2001b), and from erectile function to cancer (Bivalacqua et al., 2003; Chaiswing et al., 2008; Tanaka et al., 2001).

The cardioprotective effect of SOD3 has been reported in several studies starting from the early 1990's. Recombinant human SOD3 was first shown to reduce oxygen radicals in isolated rat hearts (Johansson et al., 1990) with subsequent recovery (83% alone and 103% when administered together with cardioplegic solution) in left ventricular developed pressure. Furthermore, SOD3 therapy led to reduced creatine kinase release marking alleviated myocardial damage (Hatori et al., 1992; Sjoquist et al., 1991). More recently, reduced infarct area and improved contractile function were achieved by systemic injection of SOD3-adenovirus (Li et al., 2001; Li et al., 1998). In addition to the acute damage caused by myocardial ischemia/reperfusion injury, ROS are strongly implicated in atherogenesis, the most common cause for occlusion of both myocardial and peripheral arteries.

Atherogenesis is initiated by subendothelial accumulation of oxidized low density lipoprotein (LDL) particles. SOD3 is highly expressed by macrophages of the atherosclerotic lesions both in WHHL rabbit and human (Luoma et al., 1998). Despite high SOD3 expression, markers for oxidized lipoproteins (malondialdehyde-lysine and hydroxynonenal-lysine) and peroxynitrite-modified proteins (nitrotyrosine residues) were detected in lesions with abundant macrophage infiltrate suggesting that disturbed redox balance plays an important role in atherogenesis. This view is supported by observations of reduced SOD3 content in plasma of patients who have had myocardial infarction (76 ng/ml vs. 110 ng/ml in healthy people) (Wang et al., 1998), and in coronary arteries of patients with CAD (63 U/mg protein vs. 126 U/mg in healthy controls) (Landmesser et al., 2000). However, neither transient adenovirus mediated over-expression nor knock-out studies have managed to provide support for the role of SOD3 in atherogenesis (Laukkanen et al., 2001a; Laukkanen et al., 2001b).

Although SOD3 appears to have little role in atherogenesis, management of restenosis and neointima formation has been more successful (Durand et al., 2005; Ozumi et al., 2005). Local catheter-mediated gene transfer was able to reduce

neointima formation in balloon-denuded rabbits 2 and 4 weeks after gene transfer (Laukkanen et al., 2002). Further studies showed that AdSOD3 gene transfer simultaneously with endothelial denudation and stenting resulted in accelerated endothelial proliferation and enhanced endothelial recovery (67.4% vs. 10.8% at 6 days and 89.3% vs. 45.1% at 28 days)(Brasen et al., 2007).

Alongside the vascular compartment, the lung is clearly an interesting tissue to study redox-mediated cellular events. Lung is also one the tissues with highest endogenous SOD3 expression. In fact, JF1/Msf mice possess a single nucleotide polymorphism in SOD3 promoter resulting in decreased pulmonary SOD3 content and reduced ventilation efficiency (dead space volume/total lung capacity)(Ganguly et al., 2009). Folz et al. have constructed mice with targeted over-expression of SOD3 in alveolar type II and nonciliated bronchial epithelial cells (Folz et al., 1999). Exposure of these mice to >99% oxygen for up to 84 hours resulted in significantly reduced wet/dry weight ratio and approximately 50% reduction in PMN infiltration into lungs of SOD3 transgenic animals. PMN infiltration was suggested to be repressed by inhibition TNFα, MIP-2, and ICAM-1 expression (Folz et al., 1999). Following the report by Folz, additional studies have supported the anti-inflammatory role of SOD3 in lung injuries. SOD3 transgenic mice show reduced lung pathology in response to influenza- or hemorrhage-induced lung injury (Bowler et al., 2001; Suliman et al., 2001). Notably, hemorrhage-induced pulmonary NF-κB and myeloperoxidase activity were reduced by roughly 50% (Bowler et al., 2001). Furthermore, LPS-induced lung inflammation is exaggerated in SOD3 knock-out animals but ameliorated in SOD3 transgenic animals. SOD3 overexpression diminished expression of LPS-induced TNFα, MIP-2, ICAM-1, VCAM-1, E-selectin, and P-selectin suggesting that SOD3 is able to attenuate inflammatory response through inhibition of proinflammatory cytokine and adhesion molecule expression (Bowler et al., 2004).

3.2 Cell Therapy

Bone marrow transplantation (BMT) represents a quintessential cell therapy approach wherein healthy stem cells are used to reconstitute diseased tissue. Long-term success with BMT was first achieved in 1968 when infants with X-linked lymphopenic immune deficiency and Wiscott-Aldrich syndrome were treated with allogeneic transplantation (Albertini and Bach, 1968; Gatti et al., 1968). With advances in histocompatibility testing and donor registries, BMT continues to develop into technique with better survival rate and less toxic effects (such as graft-versus-host-disease). Beside hematopoietic stem cells that form the basis for BMT, other cell populations with varying differentiation states have been studied as potential cell therapeutics. In the context of skeletal muscle muscle, satellite cells, mesenchymal stromal cells, and endothelial progenitor cells are the most notable examples. However, the field is plagued by controversial reports and failure to convincingly demonstrate the mechanism by which different cell populations are able to mediate tissue healing (Boyle et al., 2006; Oettgen et al., 2006).

3.2.1 Mesenchymal Stromal Cell-Mediated Therapy

Because of ethical problems and possible teratogenicity associated with embryonic stem cells (Hyun, ; Knoepfler, 2009), mesenchymal stem/stromal cells have surpassed them as the most interesting cell population for cellular therapy. The use of MSCs for cell therapy usually relies on their ability to home to injured tissue after systemic delivery. Although MSCs preferentially home to site of injury (Li et al., 2002), they are known to spread to various tissues in mice and baboons with highest cell numbers found in lungs, gastrointestinal tissues, kidneys, liver and skin (Devine et al., 2003; Francois et al., 2006). In mouse myocardial infarction, injury homing can be enhanced by left ventricular cavity infusion leading to reduced lung uptake (Barbash et al., 2003). Homing efficiency can also be affected by *in vitro* culture conditions as 1-day exposure to 1% oxygen improved engraftment efficiency in chick embryos (Hung et al., 2007b). Furthermore, drastic (90%) reduction in MSC homing to BM has been reported after only 24 hours in standard culture (Rombouts and Ploemacher, 2003). Requirement for specific tissue homing can be circumvented in tissues that are accessible for direct *in situ* administration. This however does not affect other changes in cellular behavior due to extensive *in vitro* expansion, such as potential genomic instability (Rubio et al., 2005; Shahdadfar et al., 2005; Wang et al., 2005).

Knowledge about the therapeutic application of MSCs to enhance healing of ischemic tissue is largely based on studies of myocardial infarction. Bone marrow derived MSCs are able to induce functional improvement in pre-clinical studies of myocardial infarction with modest success also in clinical trials (Giordano et al., 2007). Therapeutic efficacy has also been achieved in critical limb ischemia using diverse populations of bone marrow mononuclear cells (Miyamoto et al., 2006; Tateishi-Yuyama et al., 2002) but more importantly, MSC transplantation was recently shown to increase blood flow in murine hind limb ischemia model 24 hours after transplantation (Rosova et al., 2008). Furthermore, therapeutic success in pre-clinical models spans diverse disease models such as experimental autoimmune encephalomyelitis (EAE), Diabetes, and wound healing (Fiorina et al., 2009; Hanson et al., ; Rafei et al., 2009). The efficacy of MSC transplantation in these diseases appears to be mediated, at least partly, by immunomodulatory function (Uccelli et al., 2008). However, multipotential differentiation capacity *in vitro* suggested that MSCs could be used to promote tissue regeneration through tissue engraftment and differentiation (Prockop, 1997). Although this may indeed be the case in some circumstances (Sasaki et al., 2008; Wu et al., 2007b), it has become evident that functional improvements are achieved even in absence of efficient engraftment (Iso et al., 2007; Kinnaird et al., 2004b).

A heated discussion about differentiation potential of stem cells arose when hematopoietic and neural stem cells were shown to differentiate into multiple cell lineages representing all three germ cell layers: ectoderm, mesoderm, and endoderm (Clarke et al., 2000). Concomitantly, several reports suggested multilineage differentiation potential for MSCs bringing up high hopes for their therapeutic use (Majumdar et al., 1998; Muraglia et al., 2000; Prockop, 1997).

Studies *in vivo* suggested that MSCs could ameliorate myocardial infarction through differentiation into new myocytes and vascular cells (Orlic et al., 2001; Tang et al., 2006; Tomita et al., 1999). Despite improvements in heart function, observed stem cell engraftment has generally been rather modest (Iso et al., 2007; Noiseux et al., 2006). Together with reports suggesting cell fusion to be responsible for the observed "transdifferentiation" (Hung et al., 2007b) this raised serious doubts about differentiation as the mediator of stem cell induced therapeutic effect.

In the midst on controversy, it has become clear that MSCs are able to promote vascularization of ischemic tissue (Sanz et al., 2008). Re-vascularization is an integral part of the post-ischemic healing process and considerable amount of evidence now suggests that MSCs secrete a large number of soluble factors that not only impede adaptive immune reaction (Uccelli et al., 2008) but also promote angiogenesis and arteriogenesis (Al-Khaldi et al., 2003; Gnecchi et al., 2006; Kinnaird et al., 2004b). More specifically, MSCs secrete growth factors such as VEGF, FGF, IGF, angiogenin, IL-6, and MCP-1 that activate endothelial PI3K/Akt pathway to promote cell survival, proliferation, and migration thus leading to enhanced angiogenic vascular growth (Hung et al., 2007a; Kinnaird et al., 2004a; Potapova et al., 2007; Sadat et al., 2007). MSC secretory function is enhanced by hypoxia which leads to up-regulation of e.g. VEGF-D, PlGF, and MMP-9 (Kinnaird et al., 2004a; Ohnishi et al., 2007). Indeed, it is likely that transplanted MSCs are activated by local microenvironment to produce growth factors that promote tissue healing.

Although beneficial in their own right, the therapeutic applications of MSCs can be further enhanced or expanded by *ex vivo* gene transfer. Recent report showed enhanced perfusion recovery in peripheral ischemia after MSC mediated prostacyclin synthase gene transfer as compared to MSCs or adenoviral gene transfer alone (Ishii et al., 2009). In addition, attempts have been made towards improving MSC bone forming capacity *in vivo* or supporting long term HSC growth *in vitro* by gene transfer of bone morphogenic protein (BMP) or hTERT, respectively (Kawano et al., 2003; Tsuda et al., 2003). Perhaps the most intriguing application is however the use of MSCs as carriers for oncolytic genes into tumors. These studies are warranted by observations that MSCs are able to home to sites of active tumorigenesis after intravenous administration (Nakamizo et al., 2005; Nakamura et al., 2004). MSC-mediated tumor delivery of pro-inflammatory cytokines (such as IFN-α, IFN-β, IL-2, IL-12, or CX3CL1) (Chen et al., 2008; Elzaouk et al., 2006; Nakamizo et al., 2005; Nakamura et al., 2004; Ren et al., 2008a; Ren et al., 2008b; Studeny et al., 2002; Studeny et al., 2004; Xin et al., 2007), suicide genes (thymidine kinase, cytosine deaminase, and TRAIL) (Loebinger et al., 2009; Matuskova et al., ; Miletic et al., 2007), and oncolytic viruses (Komarova et al., 2006; Stoff-Khalili et al., 2007) have led to significantly reduced tumor burden. However, there is evidence that MSCs could in fact support tumor growth and metastasis through immunesuppression and pro-angiogenic action (Djouad et al., 2003; Karnoub et al., 2007; Nauta et al., 2006).

3.2.2 Stem Cell Source

Stem cell therapy requires high numbers of cells and therefore extensive *in vitro* expansion is usually needed. For autologous therapy, the most feasible sources for MSCs are the bone marrow and the adipose tissue. Although MSCs are relatively easily expanded *in vitro*, successful application of autologous stem cell therapy in clinical setting can be limited by ability to quickly produce enough cells. Nevertheless, a major advantage for MSCs is their apparent immunomodulatory action which could potentiate their use in allogeneic transplantation in addition to potential use as direct immunomodulatory therapeutics (Aggarwal and Pittenger, 2005; Uccelli et al., 2008). Although human MSCs express intermediate levels of major histocompatibility complex (MHC)-I and can be induced to express MHC-II, they probably do not stimulate alloreactive T-cells due to lack of co-stimulatory molecules (Di Nicola et al., 2002; Majumdar et al., 2003). In addition, MSCs have been shown to reduce expression of MHC-II and costimulatory molecules CD40 and CD86 in dendritic cells consequently leading to reduced T-cell activation (Djouad et al., 2003; Nauta and Fibbe, 2007). MSCs also arrest proliferation of $CD8^+$ and $CD4^+$ T-cells (Benvenuto et al., 2007; Krampera et al., 2006), as well as natural killer cells (Krampera et al., 2006; Sotiropoulou et al., 2006; Spaggiari et al., 2006), and diminish PMN mediated oxidative burst through IL-6-dependent mechanism (Raffaghello et al., 2008).

Despite ethical dilemmas associated with them, embryonic stem cells could offer virtually unlimited source of material for cell therapy. Therapeutic application of ESCs requires reproducible and safe methods to generate desired cell types suitable for transplantation. MSCs have recently been successfully derived from embryonic stem cells (Barberi et al., 2005; Olivier et al., 2006; Trivedi and Hematti, 2007; Trivedi and Hematti, 2008), and their hypoimmunogenic nature could enable use in allogeneic "from-the-shelf" applications. This approach is however complicated by difficulty to choose optimal *in vitro* culture conditions as they may have significant effect on MSC phenotype (Mannello and Tonti, 2007). It is known that MSCs from different mouse strains have different media requirements for optimal expansion (Peister et al., 2004). Mouse MSCs are also prone to genomic instability and subsequent tumorigenicity in culture (Tolar et al., 2007). Although human MSCs are genetically more stable than their mouse counterparts, spontaneous transformation has been reported also in human cells during long term culture (Rubio et al., 2008; Rubio et al., 2005; Wang et al., 2005). Clinical trials have however shown relatively little adverse effects and no *in vivo* tumorigenicity related to stem cell therapy suggesting that therapeutic use of MSCs is safe and feasible (Boyle et al., 2006).

AIMS OF THE STUDY

Ischemic skeletal muscle goes through a complex sequence of events aimed to re-establish sufficient blood supply and to preserve tissue functionality. However, endogenous processes may prove to be insufficient to retain tissue function. The aim of this thesis was to study gene and stem cell therapy, and their therapeutic mechanisms in the context of acute peripheral artery occlusion. More specifically, we utilized superoxide dismutase 3 (SOD3) and mesenchymal stem/stromal cells (MSCs) to restore tissue redox balance and to promote revascularization, respectively.

The specific aims of the study were divided as follows:

I Characterize the effect of adenovirus mediated SOD3 over-expression on post-ischemic skeletal muscle healing, including tissue metabolism, redox balance, angiogenesis, inflammation and cell proliferation.

II Clarify signal transduction events responsible for regulation of endogenous SOD3 expression.

III Enlighten the signaling cascades mediating therapeutic effects downstream of SOD3 induction.

IV Study the therapeutic potential of a novel hESC-derived MSC population in acute peripheral ischemia.

V Determine the fate of locally transplanted MSCs, and study the mechanism by which they mediate their therapeutic effect.

MATERIALS & METHODS

Materials and methods are described in more detail in the original publications.

1.1 Antibodies

Antigen	Ig class	Supplier	Application	Used in
vWF	rabbit polyclonal	Abcam	IHC	I, III
Ki67	mouse IgG	DakoCytomation	IHC	I, III
3-nitrotyrosine	rabbit IgG	Millipore	IHC	I
CD68	mouse IgG	Serotec	IHC	II, III
CD3	rabbit polyclonal	Serotec	IHC	II, III
Mek1/2	rabbit polyclonal	Cell Signaling	WB	I
pMek1/2	rabbit IgG	Cell Signaling	WB	I
44/42 MAPK	rabbit polyclonal	Cell Signaling	WB	I, III
p44/42 MAPK	rabbit IgG	Cell Signaling	WB	I, III
Akt	rabbit polyclonal	Cell Signaling	WB	I, III
pAkt	rabbit IgG	Cell Signaling	WB	I, III
IkBa	mouse IgG	Santa Cruz	WB	II
PDGFBR	rabbit IgG	Santa Cruz	WB	III
a-tubulin	mouse IgG	Sigma	WB	III
Ras	mouse IgG	Millipore	WB	I

* Abbreviations: WB=Western blotting, IHC=Immunohistochemistry

1.2 Animals, Cells, and Viruses

Animals	Description	Source / Reference	used in
Mouse, Balb/c		Local colony	II
Rat, Fischer 344		Harlan	I, II, III
Cells			
HEK 293T	Human embryonic kidney cells		I, II
hESC-derived MSC	Human embryonic stem cell derived mesenchymal stromal cells	Dr. Peiman Hematti (Trivedi et al. 2007)	III
BM-MSC	Human primary bone marrow mesenchymal stromal cells	Dr. Peiman Hematti	III
Viruses			
Adeno SOD3	Replication deficient E1-partially-E3-deleted AdBglII	Uni. Turku Biotechnology Centre (Laukkanen et al. 2000)	I, II
Adeno LacZ	Replication deficient E1-partially-E3-deleted AdBglII	Uni. Turku Biotechnology Centre	I, II
Lenti Luc	produced using pWPXLd, psPAX2, and pMD2G plasmids	Addgene	III
Lenti GFP	produced using pWPXLd, psPAX2, and pMD2G plasmids	Addgene	III

1.3 Miscellaneous Reagents

Reagent	Application	Supplier	Used in
Dihydroethidium bromide (DHE)	ROS detection	Invitrogen	I
X-Gal	β-Galactosidase staining	Promega	I
Dexamethasone (Oradexon)	Anti-inflammatory treatment	Organon	II
Cyclosporine A	Immunosuppression	Fluka Biochemica	III
Senescence staining kit	Senescence staining	Cell Signaling	III
Fentanyl fluanisone (Hypnorm)	Anesthesia	Janssen Pharmaceutica	I, II, III
Midazolame (Dormicum)	Anesthesia	Roche	I, II, III
Narcanti	Anesthesia	Bristol-Myers Squibb	I, II, III
Proteose peptone	Induction of peritonitis	Difco	II
IL-1β	Induction of peritonitis	R&D Systems	II
Heparin	Peritoneal lavage	Løvens Kemiske Fabrik	II
D-luciferin	Bioluminescnet imaging (IVIS)	Synchem	III
Hexadimethrine bromide	Lentiviral transduction	Sigma	III

1.4 Primers

Target:	Forward:	Reverse:	T_m	used in
huSOD3	CTT CGC CTC TGC TGA AGT CT	GGG TGT TTC GGT ACA AAT GG	60	I
huVEGF-A	TCC GGG TTT TAT CCC TCT TC	TCT GCT GGT TTC CAA AAT CC	55	I, III
huVEGF-D	CGG CAT ACG TTG GAG AGA TT	ATC TTA GGG GTG GGG AGA GA	58	III
huPDGFBR	GTG AAC GCA GTG CAG ACT GT	AGG TGT AGG TCC CCG AGT CT	55	III
huAlu 1	CAT GGT GAA ACC CCG TCT CTA	GCC TCA GCC TCC CGA GTA G	60	III
huB-Act	TGC GTG ACA TTA AGG AGA AG	GCT CGT AGC TCT TCT CCA	*	I, III
rSOD3	GAC CTG GAG ATC TGG ATG GA	GTG GTT GGA GGT GTT CTG CT	60	I
rCyc D1	AAC GTC ACA CGG ACT ACA GG	TGT TCC ATG GCT GGG GCT CTT	55	I
rVEGF-A	CAA TGA TGA AGC CCT GGA GT	TTT CTT GCG CTT TCG TTT TT	50	I, III
rVEGF-D	ATT ATT TGT GCA GCG GGA AA	GGC ATT CTC CAG AAG CAA AG.	55	III
rPDGFBR	GGA GCT TCA CAG AGG ACT GG	GAT CTG GGT GCC ATG AGA GT	55	III
rTNFa	AGA TGT GGA ACT GGC AGA GG	CCC ATT TGG GAA CTT CTC CT	60	II
rIL1a	TCG GGA GGA GAC GAC TCT AA	GAA AGC TGC GGA TGT GAA GT	58	II
rIL6	CCG GAG AGG AGA CTT CAC AG	ACA GTG CAT CAT CGC TGT TC	55	II
rMIP-2	ATC CAG AGC TTG ACG GTG AC	GGA CTT GCC GCT CTT CAG TA	55	II
rMCP1	CTC ACC TGC TGC TAC TCA TTC ACT	TGC TGC TGG TGA TTC TCT TGT AGT	55	II
rICAM	AGG TAT CCA TCC ATC CCA CA	GCC ACA GTT CTC AAA GCA CA	55	II
rVCAM	TGA CAT CTC CCC TGG ATC TC	CTC CAG TTT CCT TCG CTG AC	55	II
rE-Selectin	TTT TTG GCA CGG TAT GTG AA	AGG TTG CTG CCA CAG AGA GT	57	II
rP-Selectin	TTC CCA CAC TTC CTT CTG CT	CAC GCT GTA GTC GGG GTA TT	57	II
rB-Act	TCG TGC GTG ACA TTA AGG AG	GTC AGG CAG CTC GTA GCT CT	*	I, II, III
AdSOD3	GTT GCG TGA GCG GAA AGA TG	GTG AGC GCC TGC CAG ATC TC	60	I
common VEGF-A	GGA CAT CTT CCA GGA GTA	TGC AAC GCG AGT CTG TGT	55	III
common VEGF-D	GTT GCA ATG AAG AGA GCC TT	TCC CAT AGC ATG TCA ATA GG	55	III

* Used T_m varied between 55-60°C

1.5 Methodology

In vitro	Used in
Adenovirus production	I, II
Lentivirus production	III
Cell culture	I, II, III
Transfection	I, II
Lentivirus transduction	III
Senescence staining	III
Luciferase reporter assay	I, II
Ras pull-down assay	I
Sonication and Fixation of cells	III
SOD3 protein activity assay	I
Concavalin A sepharose purification	I
PET imaging	I
Immunohistochemistry, DHE and X-Gal staining	I, II, III
Western blotting	I, II, III
Quantitative RT-PCR	I, II, III
Genomic PCR	III
In vivo	
Rat hindlimb Injury	I, II, III
Intramuscular injection	I, II, III
Mouse peritonitis model	II
IVIS, In vivo bioluminescent imaging (BLI)	III

1.6 Rat Hind Limb Ischemia Model

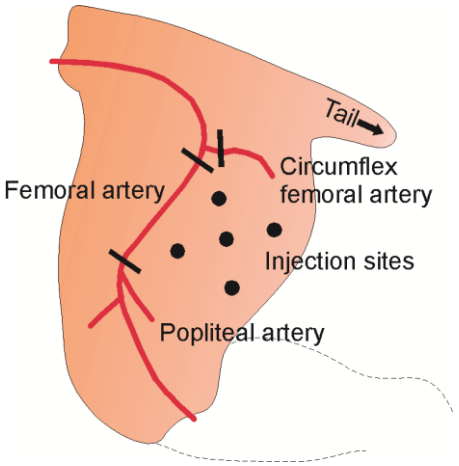

Figure 3. Peripheral ischemia. Ischemia of the hind limb was induced by surgical ligation of the proximal and distal femoral artery, and the lateral circumflex femoral artery. Adenoviruses or cells were injected directly into the muscle either after the surgical operation during same anesthesia, or on the following day, respectively.

RESULTS

1.1 Therapeutic Properties of SOD3 Gene Transfer

1.1.1 Adenovirus-mediated SOD3 gene transfer (I)

Previous studies utilizing both gene transfer and knock-out techniques have established the protective role of SOD3 in ROS-mediated injuries such as brain edema, lung hyperoxia, and oxidant-induced myocardial injury (Carlsson et al., 1995; Oury et al., 1993; van Deel et al., 2008). Although SOD3 constitutes only a minor part of total tissue SOD activity in most tissues, it is the main $O_2\bullet^-$ scavenging enzyme in the extracellular space. Despite its evident role in the upkeep of extracellular redox balance, the cellular mechanisms mediating SOD3 derived therapeutic effects have remained elusive. Peripheral limb ischemia is characterized by rapid post-ischemic ROS production, and thus represents an optimal target for antioxidative SOD3 gene therapy. In our studies we utilized a rat hind limb ischemia model wherein the femoral artery is surgically ligated during anesthesia. To achieve high temporal expression of the transgene we injected $0.5x10^9$ pfu of Adenovirus carrying either rabbit SOD3 (Laukkanen et al., 2000) or LacZ directly into ischemic muscle. Histological β-galactosidase staining showed transduction efficiency between 0.8 and 5% at different time points in the LacZ control group. SOD3 transgene expression was verified by RT-PCR analysis in the SOD3 group. Gene transfer increased muscle SOD3 activity from 1.5±0.31 U/mg (LacZ) to 2.6±0.09 U/mg (SOD3). Increased SOD activity was accompanied by reduced dihydroethidium and nitrotyrosine staining. Consequently, gene transfer resulted in functional up-regulation of SOD3 within the ischemic muscle.

1.1.2 SOD3 gene transfer normalized metabolic performance of ischemic muscle (I)

Decreased oxygen supply and subsequent oxidative stress up-regulate expression of glucose transporters and glycolytic enzymes in skeletal muscle (Semenza, 2000; Wenger, 2000). To analyze the metabolic state of SOD3 treated ischemic muscle we utilized *ex vivo* radiographic PET imaging 10 days after vessel ligation. FDG (18-fluorodeoxyglucose) was administered to the animals 30 minutes before euthanasia, after which muscle samples were frozen, cut into sections, and imaged by radiography. FDG accumulation was assessed by an internal comparison between the healthy leg and the injured leg of each animal. Cells import FDG as they do normal glucose but they are unable to utilize it after phosphorylation by hexokinase resulting in accumulation of phosphorylated FDG within the cells. Accumulation of FDG was increased in the ischemic muscle as compared to the healthy leg in both experimental groups. However, compared to the LacZ control animals (134±0.5% higher signal in injured leg vs. healthy leg) SOD3 gene transfer resulted in significant reduction in FDG accumulation in the injured leg (41±0.1% higher signal as compared to healthy leg) indicating that SOD3 treatment was able to improve the metabolic performance of ischemic muscle 10 days after injury.

1.1.3 SOD3 does not promote angiogenesis but increases cell proliferation and alleviates inflammation (I, II)

Next, histological analyses were performed to assess the tissue responses responsible for SOD3 mediated improvement in tissue metabolism. Histological staining for von Willebrand factor revealed similar angiogenic profile in SOD3 treated animals and the LacZ control group. Capillary density increased from 3 days until the 10 day time point but had already declined at 14 day time point. Staining for Ki67, a marker for actively proliferating cells, showed significant ($p<0.05$) increase in the number of proliferating cells in the SOD3 transduced muscle 3 and 7 days after vessel ligation. This suggests that SOD3 mediates tissue recovery by enhancing cell proliferation rather than by increasing angiogenesis.

Although the difference in Ki67 staining was statistically significant, overall level of cell proliferation was rather low and thus unlikely to alone mediate tissue recovery. Post-ischemic muscle is rapidly infiltrated by polymorphonuclear neutrophils which are subsequently replaced by macrophages accompanied by low number of T-cells. Since previous studies have suggested an anti-inflammatory role for SOD3 in pulmonary injuries (Bowler et al., 2001; Folz et al., 1999), we analyzed the infiltration of $CD68^+$ macrophages and $CD3^+$ T-cells into the ischemic muscle. CD68 staining showed approximately 3-fold reduction in the area of macrophage infiltrate at 3 day time point in the SOD3 animals as compared to control group. However, even more dramatic reduction was apparent at later time points, reaching 12-fold difference 10 days post-ischemia. Further analysis showed 3-5 fold difference in the number of macrophages at all time points analyzed. It is of note that throughout the experiment macrophage numbers in LacZ animals remained higher than what was maximally observed in SOD3 animals. SOD3 had a significant effect also on $CD3^+$ leukocyte population but this was only evident at 10 day time point. In conclusion, SOD3 gene transfer enhances tissue recovery after ischemic insult through combined action of reduced macrophage infiltration and enhanced cell proliferation.

1.1.4 Confirmation of the anti-inflammatory effect of SOD3 in mouse peritonitis (II)

Analysis of the $CD68^+$ and $CD3^+$ leukocyte subsets in rat hind limb ischemia suggested a selective reduction in macrophage infiltration. Therefore, we wanted to further characterize the anti-inflammatory function of SOD3. Mouse peritonitis model was chosen due to its relatively simple execution and efficient analysis of accumulation of different leukocyte subtypes into site of inflammation. First, $0.5x10^9$ pfu of AdSOD3 or AdLacZ was injected into the peritoneal cavity of Balb/c mice. Approximately 72 hours later, peritoneal inflammation was induced by intra-peritoneal injection of 5% proteose peptone supplemented with IL-1β (total volume 1 ml). Finally, the animals were euthanized 18 hours after induction of inflammation, the peritoneal cavity was lavaged, and leukocyte subtypes were analyzed by cytochemical staining. AdSOD3 was administered 3 days prior to induction of inflammation to achieve high transgene expression at the time of acute inflammatory reaction. Indeed, SOD3 treatment significantly reduced total

leukocyte numbers in the peritoneal infiltrate by 39% ($p<0.01$) as compared to LacZ control animals. The observed effect was mostly due to reduced monocyte migration (67% reduction, $p<0.01$). However, infiltration of lymphocytes was also significantly reduced (33%, $p<0.05$) while there was no effect on neutrophils.

To assess the true efficacy of SOD3 as an anti-inflammatory molecule, we tested Dexamethasone treatment as compared to PBS mock treated animals. Dexamethasone belongs to a group of very potent anti-inflammatory glucocorticoid drugs that in addition to their efficacy may cause severe side effects especially in long term use (Bulkley and Roberts, 1974; El-Helou et al., 2008; Kirton et al., 2006; Weinstein et al., 1998). Dexamethasone treatment half an hour before induction of peritonitis caused significant reduction (29%, $p<0.05$) in leukocyte infiltration into peritoneum. Similar to AdSOD3 treatment, the observed reduction was mostly due to lower monocyte migration (59%, $p<0.05$). The effect of dexamethasone on lymphocyte subset failed to reach statistical significance despite 53% reduction whereas there was clearly no effect on neutrophil migration. Leukocyte numbers found in peritoneum after adenoviral LacZ transfer were roughly double when compared to PBS control group. Despite this viral induction of inflammation, SOD3 mediated effect reduced monocyte and lymphocyte numbers to similar levels as dexamethasone. This would strongly suggest that SOD3 is potent inhibitor of acute inflammation.

1.2 Redox Signaling – Cellular Responses to SOD3

1.2.1 SOD3 activates the Ras-Erk and Akt signalling pathways (I)

Reactive oxygen species were long considered merely as harmful side-products of cellular metabolism. However, we are now beginning to understand the extent to which controlled ROS production is involved in cellular processes. For example, low hydrogen peroxide concentrations are known to induce cellular proliferation through activation of small GTPase Ras (Aikawa et al., 1997). Since we found SOD3 to be able to mediate cell proliferation *in vivo*, we were prompted to investigate its role in mitogenic signaling cascades. Ras is known to be important mediator of mitogenic signaling often having a role in e.g. malignant tumor growth. Due to its crucial role in regulating cell proliferation we analyzed the effect of SOD3 on activity of Ras both *in vivo* and *in vitro*. Ras pull-down assay showed marked increase in the amount of GTP-bound active Ras in muscle samples from SOD3 treated animals at 7 day time point. Ras activation was confirmed by *in vitro* analysis in HEK293T cells. Activation of Ras was strongly reduced by diphenyliodonium sulphate (DPI) treatment which inhibits the Nox complex thus diminishing superoxide production and depleting SOD3 from its substrate. In contrast, catalase decomposes H_2O_2 into molecular oxygen and water removing the end-product of SOD3-catalyzed reaction. However, catalase appeared to have no effect on SOD3 mediated activation of Ras. Since H_2O_2 is most likely mediating SOD3 derived activation of Ras, we wanted to verify activation of Ras by H_2O_2. Indeed, exposing cells to 500μM H_2O_2 caused activation of Ras to similar extent as SOD3 transfection.

Interaction of active Ras with its downstream partner Raf leads to consequent activation of the MAPK pathway constituting Raf (MAPKKK), Mek1/2 (MAPKK), and Erk1/2 (MAPK). MAP kinases control gene expression by modulating phosphorylation of nuclear transcription factors such as AP-1 and CREB (Gelain et al., 2006; Shaywitz and Greenberg, 1999; Turjanski et al., 2007). However, the canonical signaling cascades display extensive cross-talk. Accordingly, Ras is known to activate not only the MAPK pathway, but also the PI3K/Akt pathway which is known to promote angiogenesis and cell survival (Wang et al., 1999). Due to their reported role in cell survival and growth, we analyzed the activation status of the MAPK and PI3K/Akt pathways both *in vitro* and *in vivo*. SOD3 was shown to increase phosphorylation of Mek1/2 and Erk1/2 in ischemic muscle 7 days after injury. In *in vitro* studies SOD3 and H_2O_2 activated Mek1/2 and Erk1/2, and SOD3 mediated activation was repressed by DPI. Intriguingly, SOD3 gene transfer had no effect on Akt phosphorylation *in vivo* but activated Akt *in vitro*. Conflicting data on Akt shows that *in vitro* culture conditions may significantly differ from *in vivo* tissue environment. However, as Akt is known to promote angiogenesis the *in vivo* data supports our observation that SOD3 had no effect on new vessel formation.

Phosphorylation by protein kinases represents a central theme in regulation of signal transduction. Protein kinases form complex networks eventually leading to phosphorylation of nuclear transcription factors, including e.g. AP-1, CREB, and NF-κB. Notably, Ras-MAPK pathway promotes activation of AP-1, and CREB subsequently leading to up-regulation of e.g. Cyclin D1 and VEGF-A (Shen et al., 2008; Wu et al., 2007a). We used *in vitro* luciferase assay to determine the transcriptional activity of AP-1/c-Jun, and CREB target promoters after SOD3 transfection. Activity of each of the analyzed transcription factors was induced 2-3 fold in response to SOD3 transfection. Intriguingly, activation of AP-1, and c-Jun were reproduced by H_2O_2 whereas activation of CREB was not. In addition, SOD3 mediated activation of each transcription factor was prevented by DPI treatment but was unaffected by Catalase. In conclusion, activation of CREB and AP-1 through Ras-MAPK pathway could explain SOD3 mediated increased cell proliferation. To further strengthen this view, we performed PCR analysis both *in vivo* and *in vitro* to assess the expression level of AP-1/CREB target genes Cyclin D1 and VEGF-A. Both were significantly increased in SOD3 treated animals as compared to either uninjured or LacZ treated control animals. Finally, *in vitro* analysis verified induction of VEGF-A and Cyclin D1 by members of the Ras signaling cascade: Ras, Braf, Mek1/2 and Erk1/2, as well as by SOD3.

1.2.2 Autostimulatory feedback regulation is driving SOD3 expression (I)

Due to the destructive potential of excessive ROS production, a strict control of redox enzyme expression is required for the maintenance of extracellular redox balance. Although SOD3 removes $O_2^{\bullet -}$ from the extracellular space, the end-product of SOD3 catalyzed reaction is H_2O_2 which in itself is moderately reactive and potentially hazardous in high concentrations. Consequently, previous studies have suggested tight regulation of SOD3 activity at both pre- and post-

transcriptional levels leading to inability to achieve high expression of exogenous SOD3 *in vivo* (Laukkanen et al., 2002; Laukkanen et al., 2001b). Since SOD3 was able to mediate activation Ras and Akt we wanted to test the possibility of feedback regulation. *In vitro* quantitative PCR analysis showed dramatic increase in SOD3 mRNA expression in response to Ras, Braf, Mek, and Erk transfection. This led to modest 2-3 fold increase in SOD3 enzymatic activity. The role of Ras-MAPK in controlling SOD3 expression was verified by U0126, a specific MEK inhibitor which significantly reduced SOD3 expression. Akt had no effect on either SOD3 mRNA expression or enzymatic activity. However, SOD3 was activated by H_2O_2 and NADPH, a substrate for the NOX complexes, whereas DPI treatment reduced SOD3 expression. Taken together, these observations suggest that SOD3 is under autostimulatory feedback regulation driving Ras activation and subsequent cellular proliferation.

1.2.3 SOD3 suppresses NF-κB activity and down-regulates inflammatory cytokine and adhesion molecule expression (II)

NF-κB is a key regulator of the inflammatory response having been shown to be both necessary and sufficient for endothelial up-regulation of adhesion molecules and cytokines (Denk et al., 2001). NF-κB mediates cellular responses to e.g. oxidative stress, cytokines, and bacterial or viral pathogens. In an inactive state NF-κB is confined to cytoplasm due to IκBα binding, and ectopic expression of IκBα effectively abrogates cellular expression of VCAM, IL-1, and IL-6 (Wrighton et al., 1996). To test the effect of SOD3 on NF-κB activity, we performed i*n vitro* luciferase assay in HEK 293T cells wherein the cells were transfected with SOD3 expression plasmid together with plasmid containing NF-κB-responsive promoter upstream of luciferase reporter gene. Luciferase assay revealed a significant 50% ($p<0.01$) reduction in NF-κB activity due to SOD3 transfection. Observed increase in expression of IκBα *in vivo* suggests that reduced NF-κB activity could indeed be responsible for diminished inflammatory leukocyte accumulation in the ischemic muscle.

Due to its central role in regulating inflammatory signaling, cytoplasmic detention of NF-κB by increased IκBα expression could lead to reduced expression of inflammatory cytokines and chemokines. Therefore, we performed quantitative RT-PCR analysis of the *in vivo* expression level of some cytokines in ischemic muscle at 3 day time point. Quantitative RT-PCR showed 70-90% reduction in expression of TNFα, IL1α, IL6, MIP2, and MCP1 in SOD3 animals indicating diminished expression of several important inflammatory mediators. Furthermore, since TNFα, IL1α, and IL6 are important regulators of endothelial adhesion molecule expression we analyzed expression of ICAM, VCAM, E-selectin, and P-selectin from the tissue. Expression of ICAM and VCAM was reduced by roughly 70% while E- and P-selectins were reduced by 90%. In conclusion, SOD3 is likely to inhibit leukocyte migration through inactivation of NF-κB which leads to diminished cytokine expression and subsequent inhibition of endothelial adhesion molecule up-regulation.

1.3 Mesenchymal Stromal Cells Mediating Recovery of Ischemic Muscle

1.3.1 The graft is rapidly cleared after local transplantation (III)

Peripheral ischemic muscle injury is characterized by early focal necrosis, followed by marked monocyte migration, satellite cell proliferation, and angiogenesis (Paoni et al., 2002). Gene therapy with AdSOD3 was able to alleviate inflammation and to enhance cell proliferation in the ischemic muscle, but had no observable effect on capillary density of the tissue. Since re-establishment of blood perfusion is crucial for full recovery of ischemic tissue, we aimed to improve muscle angiogenesis by transplantation of mesenchymal stromal cells. We compared primary human BM-MSCs and a novel MSC population derived from human ESCs (Trivedi and Hematti, 2007; Trivedi and Hematti, 2008). Despite reported hypoimmunogenicity and ability to avoid allograft rejection (Aggarwal and Pittenger, 2005; Uccelli et al., 2008), Cyclosporine A was given to the treated animals to avoid rejection of the MSC xenograft. Furthermore, we decided to concentrate on the early events after cell transplantation to determine the fate of the transplanted cells. Specifically, we wanted to see whether the transplanted MSCs were able to take part in vessel stabilization and maturation by engraftment and formation of pericyte coverage.

In order to track the transplanted hESC-derived MSCs in ischemic rat muscle, the cells were labeled by lentiviral transduction with either GFP or Luciferase at passage 8. Cell morphology was assessed *in vitro* to ensure health of the cells after lentiviral labeling. In addition, cells were stained with β-galactosidase based staining kit to determine the degree of senescence in the cell culture. Human ESC-derived MSCs sustained normal growth for several passages after viral labeling reaching passage 16 before becoming senescent. At 16 passages 56% of the cells were β-galactosidase positive as compared to only 3% at the earlier passage 8.

GFP or Luciferase labeled hESC-derived MSCs were injected locally into the ischemic muscle 1 day after surgical ligation of the femoral artery. The transplanted cells were tracked with bioluminescent imaging (IVIS 50, Xenogen) immediately after transplantation, and 6 and 24 hours post-transplantation. Bioluminescent imaging (BLI) showed 71% decrease in signal intensity after only 6 hours as compared to signal intensity right after transplantation. After 24 hours only 1.5% of signal intensity remained indicating that the transplanted cells are quickly lost after local transplantation. To assess possible engraftment and tissue distribution of the transplanted cells, GFP labeled cells were tracked 3 days after transplantation. To minimize tissue autofluorescence muscle samples were snap-frozen in liquid nitrogen, cut into 10 μm tissue sections, and imaged with fluorescent microscope immediately after animal euthanasia. Interestingly, only few cells were found scattered throughout the tissue at this later time point. However, the presence of transplanted cells was verified by genomic and RT-PCR analysis to detect amplification of human Alu I sequence. While genomic PCR confirmed the presence of transplanted human cells in the ischemic rat muscle, RT-PCR showed that the cells were viable and still transcriptionally active. Overall, bioluminescent imaging together with fluorescent microscopy and PCR

analyses showed the cells to be rapidly lost from the transplanted site. However, lack of BLI signal elsewhere from the body would suggest that the cells do not accumulate in other sites, at least not in high enough numbers to generate detectable BLI signal. Due to ongoing acute inflammatory reaction at the time of transplantation the cells are most likely killed by the inhospitable environment, however, no experimental evidence was pursued to confirm this.

1.3.2 MSCs promote tissue healing despite rapid clearance of the graft (III)

Although tracking of transplanted cells was only done for the hESC-derived MSCs, for further studies animals were divided into five groups with different treatments: untreated controls, hESC-MSCs lysed by sonication, hESC-MSCs fixed in paraformaldehyde, live hESC-MSCs, and live primary human BM-MSCs. To evaluate the tissue response to MSC transplantation after acute ischemia, we performed histological analyses by immunostaining for vWF, CD68, CD3, and Ki67. Staining for von Willebrand factor at 3 day time point showed significant increase in capillary density in animals treated with either BM-MSCs (309±22 capillaries/mm^2), live hESC-MSCs (311±30 capillaries/mm^2), or fixed hESC-MSCs (288±12 capillaries/mm^2) vs. untreated (210±28 capillaries/mm^2) and lysed cell groups (237±43 capillaries/mm^2). However, the effect was only transient as all experimental groups reached similar capillary densities by 9 day time point (approximately 400 capillaries/mm^2).

Since inflammation and angiogenesis are closely intertwined processes, and because MSCs have been suggested to have immunosuppressive function, we wanted to assess infiltration of $CD68^+$ macrophages and $CD3^+$ T-cells into the ischemic muscle. Macrophage accumulation was significantly increased in all groups as compared to untreated animals. More specifically, macrophage infiltration was significantly higher in animals treated with live or lysed hESC-MSCs as compared to animals that received fixed hESC-MSCs or BM-MSCs. Analysis of $CD3^+$ T-cells showed similar trend with highest cell numbers in Lysis group and hESC-MSC group. However, T-cell levels were rather low and none of the inter-group differences reached statistical significance.

To further characterize the tissue response to transplantation we analyzed cell proliferation by immunohistological staining for Ki67. Untreated controls had low number of proliferating cells (4.7±0.6 cells/section). Treatment with lysed cells increased cell proliferation to 15±4.1 cells/section which however failed to reach statistical significance. In contrast, cell proliferation in the rest of the groups was significantly enhanced: fixed cells (21±4.2 cells/section, $p<0.05$), BM-MSCs (21±3.4 cells/section, $p<0.05$), and hESC-MSCs (38±2.6 cells/section, $p<0.01$). In line with analysis of angiogenesis, no significant differences were observed at 9 day time point. Transient effect seen in angiogenesis and cell proliferation suggests that live or fixed MSCs are able to accelerate the endogenous recovery process early after ischemic injury. Intriguingly, tissue response to paraformaldehyde fixed cells was remarkably similar to live hESC-MSCs or BM-MSCs.

1.3.3 Graft secretory function does not sufficiently explain enhanced tissue recovery (III)

Assessment of MSC fate in ischemic tissue showed vast majority of the transplanted cells to be cleared from the site of transplantation within 24 hours. Furthermore, 3 days after transplantation the few cells found from the injured tissue were randomly dispersed and did not appear to be part of any particular tissue infrastructure, e.g. vasculature. Thus, to uncover a mechanism by which MSCs were able to mediate angiogenesis we investigated graft-mediated expression of VEGF-A and -D. Trophic factors, i.e. secretory function, has been suggested to be responsible for MSC mediated angiogenesis (Gnecchi et al., 2006; Kinnaird et al., 2004b). As VEGFs are key regulators of tissue angiogenesis their elevated expression could explain increased capillary density, as well as increased cell proliferation. We used species specific primers to qualitatively and quantitatively distinguish graft vs. host derived VEGF expression at 6 and 72 hour time points. Qualitative RT-PCR analysis revealed faint expression of human VEGF-A 6 hours after hESC-MSC transplantation whereas VEGF-D was only seen with rat specific primers. Graft-derived VEGF expression was not detected at the later 72 hour time point. We next performed quantitative RT-PCR analysis to further characterize graft vs. recipient derived VEGF expression profile. Analysis at 6 hour time point showed markedly higher expression of rat VEGF-A as compared to graft-derived VEGF-A. Because no human mRNA expression was observed in conventional RT-PCR analysis at 72 hour time point, general primers common for both human and rat sequences were used to analyze expression of VEGF-A and VEGF-D at this time point. The data showed 12-fold increase in VEGF-D expression in hESC-MSC treated muscle whereas VEGF-A was not up-regulated. The expression data was further confirmed with assessment of human PDGFR-β expression by RT-PCR and western blotting. Analysis of PDGFR-β expression failed to detect cells of human origin, supporting rapid clearance of the graft.

Finally, activation status of cellular mediators of angiogenic and proliferative responses, the Akt and Ras-MAPK signaling pathways, were analyzed. Both hESC-derived MSC and BM-MSC transplantation increased phosphorylation of Akt and Erk1/2 thus supporting the data that MSC transplantation is able to promote angiogenic and mitogenic responses in the recipient tissue.

DISCUSSION

1.1 SOD3 and MSC-Mediated Therapy – Seeking the Transgene and the Stem Cell Graft

Despite their high immunogenicity adenoviruses have claimed the throne of the most studied gene therapy vector due to their easy production and high transduction efficiency in different tissues, including many non-dividing terminally differentiated cells (Cao et al., 2004). Adenoviruses generally use coxackie adenovirus receptor for entry into their target cells. Because skeletal myocytes down-regulate CAR upon differentiation they make a relatively poor target for adenoviral transduction (Nalbantoglu et al., 1999). On the other hand, myogenic progenitors such as the satellite cells can be effectively transduced by adenovirus. Based on X-Gal staining we observed an overall transduction efficiency of skeletal myotubes between 0.8 and 5% at different time points after local injection of $0.5x10^9$ pfu AdLacZ (I, Figure 1A). No further analysis was conducted on other cell types within the skeletal muscle. This transduction efficiency is in line with previous attempts to transduce skeletal muscle by adenovirus (Huard et al., 1995; Kimura et al., 2004). Importantly, efficacy of SOD3 gene transfer is enhanced by binding of the secreted SOD3 on the extracellular matrix thus bringing non-infected cells under the influence of exogenous SOD3. The situation is however complicated by post-translational regulation of SOD3 by truncation of the C-terminal domain (Enghild et al., 1999) and by production of an enzymatically inactive form (Petersen et al., 2003). In our case, AdSOD3 gene transfer resulted in roughly two-fold increase in SOD3 enzyme activity in Concanavalin A-purified muscle homogenates which is within the range that is usually achieved by exogenous SOD3 over-expression. Notably, 2-3 fold increase in SOD3 concentrations has been shown to be sufficient to mediate therapeutic response in liver damages and neointima formation (Laukkanen et al., 2002; Laukkanen et al., 2001b).

Cell therapy applications using MSCs have now proceeded into clinical trials, some say this may be too soon considering our lacking knowledge of the mechanism by which MSCs mediate their therapeutic effect (Boyle et al., 2006; Oettgen et al., 2006; Uccelli et al., 2008). However, so far only few pathologic complications have been reported suggesting that MSC therapy is safe. A central question regarding MSC-mediated therapeutic effect is the fate of the transplanted cells. Systemically delivered cells accumulate into the lungs but also travel to many other tissues including gastrointestinal tissues, liver, and skin (Devine et al., 2003; Francois et al., 2006). Although MSCs show increased homing into sites of injury (Li et al., 2002) local delivery may be more appropriate, when applicable, because it enables more precise control over the number and functional phenotype of the transplanted cells. We utilized MSCs stably transduced with luciferase to determine their biodistribution after local intramuscular delivery. Bioluminescent imaging (BLI) showed 98.5% reduction in signal intensity within the muscle in only 24 hours whereas no signal was detected elsewhere in the body (III, Figure 1G-J). Consequently, the cells did not appear to accumulate to any other location,

at least not in high enough numbers to generate BLI signal suggesting that the graft was destroyed on site. Despite rapid decrease in BLI signal, small number of transplanted cells was found three days after transplantation when GFP-transduced cells were followed by fluorescent microcopy (III, Figure 1K). Presence of the cells at this later time point was verified by PCR analysis (III, Figure 1L). Since transplantation provoked a significant increase in infiltration of $CD68^+$ macrophages into the muscle (III, Figure 2A-B) despite Cyclosporine A treatment and alleged ability to avoid immunedefense (Aggarwal and Pittenger, 2005; Uccelli et al., 2008), we cannot rule out the possibility of local clearance of the graft by the infiltrating leukocytes. Previous reports on persistence of MSC graft in different injury models have varied extensively (Gang et al., 2009; Muller-Ehmsen et al., 2006; Orlic et al., 2001; Rosova et al., 2008; Tomita et al., 1999), our results fall within the lower range showing no functional engraftment within the target tissue. Thus, our results on MSC fate support the current consensus that engraftment is not the main mediator of MSC-induced therapeutic effect.

1.2 Oxidative Imbalance and Skeletal Muscle Metabolism – Intervention by SOD3

Ischemia/reperfusion injury induces considerable production of reactive oxygen species within the affected tissue. Controversial evidence exists regarding the timing of ROS production. While some studies have indicated ROS production only after re-introduction of oxygen into tissue due to reperfusion (Bertuglia and Giusti, 2003; Kurose et al., 1997), others have suggested this to occur already during ischemia (Baudry et al., 2008; Becker et al., 1999). Our model consisted of chronic ischemia where SOD3 gene transfer was done simultaneously with femoral artery ligation. Therefore this approach could not interfere with the immediate early ROS production from vascular endothelium (Baudry et al., 2008). In contrast, in our studies SOD3 was likely to blunt the leukocyte-derived oxidative burst and to reduce ROS production from ECs of the neovasculature at later time points (Tojo et al., 2005). We assessed oxidative stress in the AdLacZ and AdSOD3 transduced muscles by dihydroethidium staining demonstrating oxidative stress, and by nitrotyrosine staining indicating nitrosative stress. Both markers were reduced in the AdSOD3 group showing that the gene transfer was able to alleviate tissue oxidative stress (I, Figure 1B). As leukocyte-derived ROS have been shown to promote tissue damage in ischemic injury (Ragu et al., 2007; Schraufstatter et al., 1987; Zweier et al., 1988), SOD3 may directly protect the tissue from excessive oxidative burst from the leukocytes.

Hypoxia and ischemia are distinct conditions evoking partly different gene expression patterns in affected tissue (Aravindan et al., 2005). One of the responses common for both is the stabilization of cellular HIF-1α. HIF-1α is an oxygen sensing transcription factor that promotes angiogenesis by activating VEGF expression. Other HIF-1α target genes include hexokinases 1 and 2, enolase 1, lactate hydrogenase A, and pyruvate kinase, all of which play key roles in energy supply by anaerobic glycolysis (Lundby et al., 2009; Semenza, 2000; Wenger, 2000). A subset of skeletal muscle fibers express HIF-1α even in

normoxic conditions (Pisani and Dechesne, 2005) but HIF-1α is further up-regulated in response to ischemia (Milkiewicz et al., 2004). Since the rate-limiting step for glucose use in skeletal muscle is glucose transport, it is important to note that HIF-1α also activates glucose transporter 1 (Ebert et al., 1995). HIF-1α-induced shift towards anaerobic glycolysis and increased glucose transport might explain the observed increase in FDG accumulation within the ischemic legs as compared to healthy legs (I, Figure 1C). This shift was prevented by SOD3 gene transfer suggesting that SOD3 is able to normalize tissue metabolism. We did not analyze the relationship between SOD3 and HIF-1α but inhibition of HIF-1α could explain the effects seen in glucose metabolism. Although superoxide is known to activate HIF-1α in hypoxia-independent manner (Shi et al., 2009) only a couple of studies have shown SOD3 to affect HIF-1α activation (Suliman et al., 2004; Zelko and Folz, 2005). Both these studies concentrated on kidney cells but interestingly showed completely opposite relationship between HIF-1α activity and SOD3. Finally, it is possible that part of the radiographic signal in LacZ animals is caused by infiltrating leukocytes, especially since SOD3 was shown to diminish the leukocyte infiltrate (II, Figures 1 and 2).

1.3 Effect of SOD3 on Leukocyte Infiltration

Polymorphonuclear neutrophils are generally the first leukocyte subset to infiltrate inflamed tissue. Already at 3 days after ischemic injury the neutrophils have largely been replaced by macrophages that clean off the remains of dead cells (Paoni et al., 2002). In addition to their role in clearance of cellular debris, macrophages secrete growth factors and matrix modifying enzymes that together promote EC activation and subsequent angiogenesis (Anghelina et al., 2004; Sunderkotter et al., 1994). Although these functions make macrophages essential for the healing process, they also produce ROS and secrete inflammatory cytokines and chemokines that may lead to excessive inflammatory reaction and tissue damage (Kaul and Forman, 1996; Koj, 1996; Nguyen and Tidball, 2003). ROS are not only involved in leukocyte-mediated oxidative burst but also take part in many steps within the cascade of cellular events culminating in leukocyte extravasation. Therefore it is not surprising that SOD3 was suggested to have anti-inflammatory effect in 1999 (Folz et al., 1999) with further evidence accumulating over the years (Bowler et al., 2001; Bowler et al., 2004; Suliman et al., 2001).

Due to the reported anti-inflammatory effect, we determined the effect of SOD3 gene transfer on accumulation of $CD68^+$ macrophages and $CD3^+$ T-cells within ischemic muscle. Neutrophils were omitted from the analysis because their migration peaks too early for the transgene to affect them in our study setting. Histological analysis showed 3-fold reduction in the inflammatory area 3 days after vessel ligation in SOD3 vs. LacZ control animals as determined by the area occupied by $CD68^+$ macrophages. The difference became even more prominent in the later time points and similar effect was seen in the number of macrophages (II, Figure 1). In contrast, we did not observe any difference in the number of T-cells between the groups in the 3 day time point. The role of T-cells in recovery of ischemic injury has remained uncertain as compared to macrophages and

neutrophils. Low numbers of T-cells have been suggested to attract neutrophils and macrophages to site of myocardial or peripheral ischemia/reperfusion injury (Stabile et al., 2003; Stabile et al., 2006; Yang et al., 2006). Whereas T-cell migration increased in LacZ animals until 10 days after vessel ligation, no such increase was detected in SOD3 treated animals (II, Figure 2). Due to late effect on T-cell accumulation, our results suggest that SOD3 has an indirect effect on them perhaps through inhibition of macrophage infiltration. Although these results confirm the previous suggestions for anti-inflammatory effect of SOD3, it seems to predominantly affect the macrophage subset. Inhibition of macrophage infiltration could lead to general reduction in inflammatory reaction due to reduced cytokine and chemokine secretion.

Implication of macrophage specific inhibition of leukocyte migration prompted us to use a mouse peritonitis model to confirm the effect. This model supported SOD3-induced reduction in total number of infiltrating leukocytes (II, Figure 3A) which was predominantly due to reduced monocyte/macrophage numbers (II, Figure 3B). To compare the efficacy of SOD3 as an anti-inflammatory mediator to existing medication we studied the effect of Dexamethasone treatment in peritonitis. Dexamethasone treatment (50mg/kg) reduced total leukocyte migration to similar extent as SOD3 treatment (II, Figure 4A). Inhibition of leukocyte migration was due to diminished macrophage and lymphocyte recruitment whereas no effect was seen in the neutrophil subset. Weak effect on neutrophils might be due to late time point analyzed (18h after induction of inflammation) as neutrophil numbers have been shown to peak as early as 4 hours after zymosan induced peritonitis (Getting et al., 1997). Determination of which leukocytes will eventually infiltrate target tissue can be affected by any step of the leukocyte extravasation process although transmigration forms the final obstacle. A report by Van der Goes showed that superoxide treatment of rat cerebral endothelial cells increases monocyte adhesion and transmigration by disrupting EC-EC junctions (Van der Goes et al., 2001). As H_2O_2 did not replicate the observed effects but they were abrogated by superoxide scavengers this suggests superoxide as mediator of monocyte migration. However, superoxide has also been implicated in mediating structural changes in EC actin cytoskeleton required for lymphocyte migration (Cook-Mills, 2002; Van der Goes et al., 2001). Therefore inhibition of superoxide-mediated alterations in EC junctions may be unlikely to explain the specific effect SOD3 seemed to have on macrophages.

NF-κB is generally recognized as a central regulator of inflammatory gene expression up-regulating cytokines such as TNFα and IL-1α, and adhesion molecules ICAM-1 and VCAM-1 (Collart et al., 1990; De Martin et al., 2000; Denk et al., 2001; Mori and Prager, 1996; Shakhov et al., 1990). NF-κB can be transcriptionally activated by several stimuli including oxidative stress (Gloire et al., 2006). Therefore it represents an attractive candidate for mediating SOD3-induced reduction in leukocyte traffic. Analysis by *in vitro* luciferase assay showed reduced NF-κB activity after SOD3 transfection. Increased IκBα expression *in vivo* in the SOD3 treated animals would support the role of NF-κB inhibition in mediating anti-inflammatory role of SOD3 (II, Figure 5A). This data

is well in line with previous findings of Nox1-dependent activation of NF-κB in ECs, highlighted by inhibition of NF-κB activation by DPI or SOD treatment (Miller et al., 2007). Furthermore, even earlier studies have previously reported that increased NF-κB activity correlates with Nox-derived superoxide production and is abrogated by SOD3 overexpression (Azevedo et al., 2000; Bowler et al., 2001). SOD3-mediated inhibition of NF-κB may partly result from increased availability of NO (Jung et al., 2003) which is known to inhibit IκBα phosphorylation and subsequent degradation (Katsuyama et al., 1998).

Since genes encoding cytokines TNFα, IL-1α, IL-6, MIP2, and MCP-1 contain NF-κB binding site in their promoters (Collart et al., 1990; De Martin et al., 2000; Denk et al., 2001; Libermann and Baltimore, 1990; Mori and Prager, 1996; Shakhov et al., 1990; Shimizu et al., 1990; Ueda et al., 1997; Widmer et al., 1993) we analyzed their expression in rat muscle by quantitative RT-PCR. All of the analyzed cytokines and chemokines were down-regulated in SOD3 animals (II, Figure 5B). MCP-1 is of special interest in this context because it is a potent attractant for monocytes (Kumar et al., 1997; Lee et al., 2004). Neutralizing antibody for MCP-1 has been shown to reduce myocardial infarct size and infiltration of macrophages (Ono et al., 1999). Consequently, significant reduction in MCP-1 expression in SOD3 treated animals could explain strong inhibition of macrophage infiltration. TNFα, IL-1α and IL-6 up-regulate endothelial expression of ICAM-1, VCAM-1, P-selectin, and E-selectin. In our studies expression of these adhesion molecules was reduced in SOD3 animals as compared to LacZ group (II, Figure 5C). The importance of these adhesion molecules for monocyte/macrophage migration has been clearly demonstrated both *in vivo* and *ex vivo* (Patel et al., 1998; Ramos et al., 1999). Nevertheless, ICAM-1, VCAM-1, P-selectin and E-selectin represent very general mediators of leukocyte extravasation (Ley et al., 2007) and therefore cannot explain such a strong specific effect on macrophage migration. Instead, this data could suggest a general reduction in endothelial activation which might be due to reduced expression of inflammatory cytokines and chemokines. Reduction in cytokine and chemokine expression may conversely be, at least partly, a secondary effect due to diminished macrophage infiltration. Notably, MCP-1 does not only enhance monocyte/macrophage migration but also induces expression of IL-1 and IL-6 in them (Jiang et al., 1992). Therefore, MCP-1 might have a central role in coordinating post-ischemic inflammatory reaction by modulating macrophage function.

1.4 Angiogenesis

1.4.1 SOD3 is Not Sufficient to Promote Angiogensis

Numerous *in vitro* studies have shown that application of low H_2O_2 concentration on endothelial cells promotes EC proliferation and tube formation (Ushio-Fukai, 2006; Yasuda et al., 1999). The role of ROS in angiogenesis has garnered further support from *in vivo* studies wherein $gp91^{phox}$ knock out mice exhibit impaired neovascularization in hind limb ischemia as determined by laser Doppler imaging, capillary density and microsphere measurements (Tojo et al., 2005). Scavenging of H_2O_2 by glutathione peroxidase mimetic ebselen also led to reduced neovascularization suggesting that it indeed is H_2O_2 that mediates neovascularization in vivo. In light of these experiments it would be tempting to draw a conclusion that SOD3 could promote angiogenesis. This view is supported by reduced blood flow in ischemic cremaster muscle of SOD3 knock out mice as compared to wild type animals (Park et al., 2005). We assessed vascular density in the ischemic tissues by immunohistochemical vWF staining. Vascular density increased in both LacZ and SOD3 groups without significant differences between the groups (I, Figure 2A) suggesting that, although necessary for neovascularization, SOD3 is not a major angiogenic factor. It is not yet understood how exactly H_2O_2 is able to promote neovascularization but currently the strongest candidate is mediation of endothelial VEGF signaling (Ushio-Fukai, 2007). However, since $O_2\bullet^-$ reacts eagerly with NO to produce peroxynitrite this interaction cannot be disregarded either, especially as SOD3 lies directly in the path of NO diffusion from ECs to its target VSMCs. Regardless of the mechanism, endogenous SOD3 appears to be sufficient to maintain normal vascular growth in ischemic muscle without added advantage by exogenous SOD3 expression.

1.4.2 A Novel Mechanism for MSC-Mediated Vascular Growth

In a recent study it was shown that human MSCs increased blood flow in murine hind limb ischemia 24 hours after transplantation (Rosova et al., 2008). The amount of transplanted cells that survived for 2 weeks was only 0.2%. Accordingly, the current consensus on the mechanism mediating MSC-derived therapeutic effect has shifted from engraftment and transdifferentiation towards secretory function. MSCs secrete large numbers of growth factors *in vitro*, many of which are further up-regulated by hypoxia (Kinnaird et al., 2004a). Evidence for this trophic effect is provided by efficacy of MSC conditioned media in supporting EC survival, proliferation and tube formation *in vitro* (Hung et al., 2007a; Potapova et al., 2007), and by functional improvement in peripheral and myocardial ischemia that occurs far too quickly to be attributed to MSC differentiation (<72 h) (Gnecchi et al., 2006; Rosova et al., 2008). In agreement with these studies, we saw significant increase in vascular density 3 days after transplantation of either primary human BM-derived MSCs or hESC-derived MSCs (III, Figure 3B). However, the effect was only transient as there was no difference between the groups at 9 day time point (III, Figure 3C). Furthermore,

hESC-derived MSCs that were fixed in 3.7% formaldehyde prior to transplantation elicited similar angiogenic response as live cells whereas cells lysed by sonication did not. This would suggest that secretion of growth factors is not required for MSC-mediated angiogenesis. Inflammatory cells, especially macrophages, are essential mediators of tissue response to injury indicating that enhanced angiogenesis could have been due to enhanced leukocyte infiltration. This does not seem to be the case as all treated groups exhibited increased macrophage migration as compared to untreated controls without any correlation with the observed vascular densities. No differences were observed in T-cell accumulation between the groups providing further evidence that inflammatory reaction could not be solely responsible for increased angiogenesis (III, Figure 2C-D).

To further analyze the secretory function of the graft as compared to endogenous growth factor production, we used species specific quantitative and qualitative detection of VEGF expression. VEGF-A is an essential regulator of angiogenesis (Carmeliet et al., 1996; Ferrara et al., 1996), it is secreted by the MSCs who up-regulate it by 2.5-fold in response to hypoxia (Kinnaird et al., 2004a). Hypoxic MSC conditioned media reduces hypoxia induced apoptosis, and induces survival and *in vitro* tube formation in human aortic endothelial cells by activating PI3K/Akt pathway (Hung et al., 2007a). The effects were only partly inhibited by anti-VEGF, -IL-6, or –FGF antibodies suggesting other factors to be responsible for the observed PI3K/Akt activation (Hung et al., 2007a). Although we did see increased activation of Akt and Erk1/2 (II, Figure 5), our data shows the graft to be only a minor source for VEGF-A 6 hours after transplantation with subsequent reduction to undetectable levels by the 72 hour time point. However, VEGF-D has been suggested to be the strongest angiogenic effector among VEGFs in skeletal muscle (Rissanen et al., 2003). Our expression analyses showed VEGF-D to be solely derived from the recipient tissue in the analyzed time points. As opposed to VEGF-A, host VEGF-D expression was significantly up-regulated 72 hours after transplantation suggesting that transplanted cells are able to boost the tissues own endogenous healing process. Rapid clearance of the graft, lack of graft-derived growth factors, and the ability of formaldehyde-fixed cells to promote angiogenesis suggest a novel mechanism wherein physical intercellular interactions between the MSCs and the recipient tissue mediates enhanced recovery.

1.5 Cell Proliferation

1.5.1 SOD3 in Mitogenic Ras Signaling

Skeletal muscle is maintained and repaired by activation, proliferation, and differentiation of tissue resident satellite cells (Sherwood et al., 2004). Satellite cells reside in quiescent state between the myocyte plasmamembrane and the surrounding basement membrane. Disruption of this cellular niche provides cues for activation of the satellite cells resulting in sequential up-regulation of myogenic regulatory factors (MRFs) and subsequent differentiation into myoblasts that fuse to form myotubes (Cooper et al., 1999; Cornelison and Wold, 1997;

Yablonka-Reuveni and Paterson, 2001). Induction of genes associated with cell proliferation (such as cyclin A, cyclin B1, cyclin D1, and cyclin dependent kinase 2) occurs on days 1-7 after acute hind limb ischemia in mouse (Paoni et al., 2002). Peak in satellite cell proliferation appears to coincide with the highest macrophage infiltration at 3 day time point. Analysis of Ki67 staining 3 and 7 days after ischemia showed increased cell proliferation in SOD3 animals as compared to the LacZ control group. At 10 day time point cell proliferation in SOD3 group had already been reduced to the level of LacZ animals (I, Figure 2B). Our studies concentrated on the muscles of the thigh, not the calf where ischemia is more severe after femoral artery ligation due to lack of collateral anastomoses that could provide supporting blood flow. This could explain why we did not see severe necrosis in the muscle (data not shown) and why the number of proliferating cells in the muscle was relatively low. However, the observation is supported by previous report that SOD3 gene transfer promotes endothelial recovery in stented arteries by accelerating EC proliferation (Brasen et al., 2007).

To explain the mechanism causing the increased cell proliferation, we analyzed the activation status of Ras. The Ras-Erk1/2 pathway represents a quintessential mitogenic signaling cascade that has also been implicated in myoblast proliferation (Jones et al., 2001b; Shefer et al., 2001). SOD3 increased GTP-binding active form of Ras both after AdSOD3 transduction *in vivo*, and after transient *sod3* transfection *in vitro* (I, Figure 3A). SOD3-mediated Ras activation was attenuated by DPI and reproduced with H_2O_2 treatment suggesting that Ras activation is dependent on the end product of SOD3 catalyzed dismutasion reaction, and is regulated by Nox-derived superoxide production. The effect of SOD3 on Ras is most likely mediated by joint function of cell surface receptor tyrosine kinases and protein tyrosine phosphatases, known targets for oxidant mediated activation and inactivation, respectively (Whisler et al., 1995). To trace the signal transduction from Ras to changes in pro-survival gene expression, we showed activation Mek1/2 and Erk1/2, and their target nuclear transcription factors AP-1/c-jun and CREB in response to *sod3* transfection (I, Figure 3B-C). As was true for Ras, activation of these Ras downstream targets was inhibited by DPI whereas all but CRE were activated by H_2O_2 treatment. Inability of H_2O_2 to activate CRE is intriguing since only reported functions for SOD3 are catalysis of the dismutasion reaction and inhibition of ECM degradation by blocking hyaluronan fragmentation (Gao et al., 2008). SOD3 induced signaling events were not affected by transfection of catalase, suggesting that as an intracellular enzyme catalase is not able to interfere with SOD3-derived H_2O_2 signaling. Transcriptional activation of AP-1/c-jun and CRE is known to promote expression of many pro-survival genes, such as VEGF-A and Cyclin D1 which we showed to be upregulated by SOD3 both *in vivo* and *in vitro* (I, igure 4A-D). Activation of VEGF-A expression was likely mediated by Ras-Erk pathway because we did not see SOD3-mediated activation of Akt *in vivo*. *In vitro* Akt activation on the other hand shows that significance of findings obtained in cell culture models must be verified *in vivo*. Finally, discrepancy between increased VEGF-A expression and lack of angiogenic effect in SOD3 treated animals suggests an alternative function

for VEGF-A. Indeed, VEGF-A promotes new myoblast formation, enhances myoblast migration, and decreases apoptosis, suggesting a more general pro-survival function (Germani et al., 2003).

Since SOD3 was shown to promote cell proliferation through activation of the Ras-Erk signaling pathway, we next wanted to determine the possibility of feedback regulation of SOD3. Relatively little is known about the signaling cascades regulating SOD3 expression. SOD3 expression is stimulated by NO, angiotensin, and inflammatory cytokines such as interferon (IFN)-γ and interleukins 1 and 4. In contrast, SOD3 expression is repressed by TNFα, FGF, EGF, and PDGF (Fattman et al., 2003; Zelko et al., 2002). Suppression of SOD3 expression by growth factors would suggest inhibitory role also for Ras pathway. However, transfection of *RasV12*, *Braf V600E*, *Mek1*, and *Erk1* into HEK293T cells showed increased SOD3 production (I, Figure 4E-F). SOD3 expression was also inhibited by specific Mek-inhibitor U0126 further confirming the role of the Ras-Erk cascade in regulation of SOD3. We also tested the effect of NADPH, a substrate for Nox-derived superoxide production, and H_2O_2 itself on SOD3 activation, both stimulated SOD3 expression whereas DPI had an inhibitory effect. In conclusion, this data suggests that SOD3 is regulated by positive feedback loop through H_2O_2 mediated Ras activation.

1.5.2 Mesenchymal Stromal Cells and Endogenous Cell Proliferation

Although angiogenesis represents the central mechanism by which MSCs enhance tissue recovery, satellite cell proliferation is crucial for the final regeneration of the tissue. Much like angiogenesis, cell proliferation in ischemic muscle was significantly increased 3 days after transplantation by formaldehyde-fixed hESC-MSCs and live MSCs. Cell proliferation was diminished by the 9 day time point with some residual proliferation remaining in animals that received live cells. Since single GFP^+ cells were scattered around the injury region instead of the cells occurring in groups, proliferation of the transplanted cells is unlikely to contribute to the analysis of cell proliferation in the tissue.

Table 4. Therapeutic effects of SOD3 gene transfer and mesenchymal Stromal cell transplantation.

Tissue response	AdLacZ / Injury control	AdSOD3	hESC-derived MSC	BM-MSC	Lysed MSCs	Fixed MSCs
Oxidative stress	100 %	reduced	-	-	-	-
Glucose metabolism	100 %	reduced	-	-	-	-
$CD68^+$ macrophage infilration	100 %	reduced	increased	increased	highly increased	increased
$CD3^+$ T-cell infiltration	100 %	reduced	not affected	not affected	not affected	not affected
Angiogenesis	100 %	not affected	increased	increased	not affected	increased
Cell proliferation	100 %	increased	increased	increased	not affected	increased

1.6 hESC-Derived MSCs and Their In Vitro Characteristics

One of the goals of our study was assess the therapeutic potential of a novel hESC-derived MSC population (Trivedi and Hematti, 2007; Trivedi and Hematti, 2008). These cells have multipotential differentiation capacity *in vitro* and express cell surface markers CD105, CD73 and CD90, and lack surface markers CD45 and CD34, thus far complying with the minimal requirements for MSCs (Dominici et al., 2006). Furthermore, these cells express similar levels of MHCs and co-stimulatory molecules as human BM-derived MSCs and do not provoke a T-cell response in co-culture experiments (Trivedi and Hematti, 2008). The hESC-derived MSCs were readily transducible at passage 8 by lentiviral vectors carrying luciferase or GFP reporter genes. Importantly, the cells sustained normal growth for several passages after transduction until reaching senescence at passage 16. We were the first to compare these hESC-derived MSCs to BM-MSCs *in vivo*. Human ESC-derived MSCs elicited very similar therapeutic effect as BM-MSCs in our hind limb injury model. As utilization of MSCs does not require HLA-matching of donor and recipient tissues this suggests that hESCs may provide a potentially unlimited source of cells for cell therapy thus enabling quick treatment without the need for time consuming preparation of autologous cell transplant.

IN CONCLUSION,

Proper function of any given tissue is undeniably dependent on sufficient blood supply. Blood perfusion in resting skeletal muscle is relatively low but muscle has dramatic capacity to increase blood flow when needed (e.g. during exercise). If blood flow in skeletal muscle is obstructed, following ischemia leads to activation of endothelial cells, infiltration of leukocytes, and proliferation of satellite cells. Both endothelial cells and inflammatory leukocytes contribute to production of reactive oxygen species that may accentuate the injury.

Superoxide dismutase 3 (SOD3) is an anti-oxidative enzyme converting superoxide into hydrogen peroxide. SOD3 is secreted to the extracellular space but is bound to cellular surfaces through interaction with heparan sulphate, collagen and fibulin-5. The therapeutic potential of SOD3 has already been studied in many injury models but the exact molecular mechanism mediating observed effects is yet to be presented. Likewise, the mechanism by which mesenchymal stromal cells contribute to tissue recovery is unclear. Currently the weight has shifted from engraftment and differentiation towards trophic function through secretion of growth factors. The goal of our study was to shed light on the mechanisms responsible for SOD3 and MSC-mediated therapeutic effects.

This study demonstrated that SOD3 and MSCs enhance post-ischemic tissue recovery by contributing to several important steps of the healing process. The main findings of the study are: i) SOD3 promotes cell proliferation by activating the mitogenic Ras/Erk pathway. ii) Ras/Erk pathway activates SOD3 expression putting SOD3 under autostimulatory regulation. iii) SOD3 has strong anti-inflammatory effect mediated by reduced NF-κB activity, specifically against monocyte/macrophage lineage. iv) MSCs do not engraft within ischemic muscle nor do they secrete significant amount of growth factors, instead, MSCs promote endogenous potential for tissue recovery through physical contacts with the recipient tissue.

To conclude, we have presented a novel function for SOD3 in mediating mitogenic cell signaling. Furthermore, we show evidence that engraftment and secretory function are not sufficient to explain MSC-mediated therapeutic effect.

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