# Gene Therapy for Endothelial Progenitor Cell Dysfunction

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# Abstract

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Endothelial progenitor cells (EPCs) have reduced neovascularization capacity in the context of coronary artery disease (CAD) or cardiac risk factors (RFs). Since, endothelial NO synthase (eNOS) is critical to normal EPC function, we hypothesized that bone marrow cells (BMCs) from rats with RFs and EPCs from humans with CAD and/or RFs show dramatically reduced neovascularization capacity in vitro and in vivo, which can be reversed by eNOS overexpression. BMCs were isolated from rat models of type II diabetes and the metabolic syndrome, and we showed a significant reduction in their ability to stimulate neovascularization in vitro and in vivo. In humans, we isolated circulating 'early EPCs' from healthy subjects and patients with CAD and RFs, and transduced them using lentiviral vectors containing either eNOS or GFP (sham). EPCs from patients had reduced in vitro migration in response to SDF-1 or VEGF, which was reversed by eNOS-transduction. In co-culture with human umbilical vein endothelial cells (HUVECs) on Matrigel, eNOS-transduced EPCs contributed to increased and more complex angiogenic tube formation compared to sham-transduced cells. Human EPCs from patients were ineffective in enhancing ischemic hind limb neovascularization and perfusion in a nude mouse, whereas eNOS-transduced EPCs resulted in a significant improvement compared to sham-transduced cells. In a swine model of acute myocardial infarction (MI), eNOS- and non-transfected BMCs both increased left ventricular function compared to sham. However, there was no benefit to eNOS overexpression in this model. Various differences in the models and procedures may explain the incongruous results obtained. Taken together, these results show that

eNOS overexpression significantly improves the neovascularization capacity of EPCs of human subjects with CAD and RFs and could represent an effective adjunctive approach for the improvement of autologous cell therapies for cardiovascular disease.

# Acknowledgements

I would like to thank my supervisors and mentors, Dr. Duncan Stewart and Dr. Michael Kutryk, who encouraged me to take on a very challenging project. I am very grateful for their initial confidence in me, and through their continued support and guidance, I learned a great deal about the various roles of a clinician scientist, and was fortunately involved in many activities beyond the scope of my doctoral project. Their examples of hard work, determination and perseverance greatly inspired and motivated me.

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learning, has been my closest advisor and encouraged me to pursue my doctorate, and now to move on to the next step. To her I dedicate this thesis with gratitude and love.

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# List of Abbreviations

CAD – coronary artery disease

cAMP – cyclic adenine monophosphate

cGMP – cyclic guanosine monophosphate

CXCR-4 - CXC chemokine receptor 4

eNOS – endothelial nitric oxide synthase

EC – endothelial cell

EPC – endothelial progenitor cell

FRS – Framingham risk score

HGP – hepatocyte growth factor

LVEF – left ventricular ejection fraction

LZ – lean Zucker

NO – nitric oxide

NOS – nitric oxide synthase

OZ – obese Zucker (or Zucker FA/FA)

PECAM-1 – platelet endothelial cell adhesion molecule – 1

RF – risk factor

SO – superoxide ($O_2^-$)

VEGF – vascular endothelial growth factors

ZDF – Zucker diabetic fatty

# 1 Background

## 1.1 Endothelial Progenitor Cells

It was previously thought that the repair of damaged endothelium occurred mainly from the migration and proliferation of nearby undamaged endothelial cells (ECs)[1] and that the generation of new vasculature resulted exclusively from the sprouting of neovessels from preexisting blood vessels. This longstanding belief was challenged in 1963, however, when it was shown that Dacron patches were efficiently endothelialized[2], presumably by seeding from circulating cells. It was later shown that these circulating cells were of bone marrow (BM) origin[3,4]. These BM-derived cells, now referred to as endothelial progenitor cells (EPCs), are being studied for their role in the endogenous maintenance and repair of damaged endothelium and their regenerative capacity beyond the endothelium. In particular, EPCs have been tested in cell therapy approaches with the aim of developing novel therapies for cardiovascular conditions currently lacking effective treatment options. The identification, manipulation and transplantation of these cells have generated a great deal of interest in recent years, although significant skepticism and confusion persists.

Circulating BM-derived EPCs were first identified in 1997 as a subset of $CD34^+$ hematopoietic progenitor cells by Asahara and colleagues[3,5]. They reported that peripheral blood mononuclear cells (PB-MNCs) enriched for $CD34^+$ cells could differentiate into endothelial-like cells following culture on fibronectin in the presence of growth factors. After seven days in culture the fraction of cells co-expressing CD34 and VEGFR-2 increased. These cells also expressed other endothelial markers such as CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1), tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2) and nitric oxide

synthase III (NOS III, eNOS), incorporated acetylated low-density lipoprotein (acLDL), bound Ulex (UEA-1) lectin and formed tube-like structures *in vitro*, supporting the contention that they possess the ability to differentiate into ECs. The findings of Asahara and colleagues were corroborated by another group who identified a similar population of cells referring to them as "bone marrow-derived circulating endothelial cells"[4].

The ability to identify and quantify EPCs using immunologic techniques has proven to be a challenge. It is now evident that different subsets of BM or circulating cells can differentiate into endothelial-like cells, which suggests that the original definition of an EPC as CD34+/KDR+ might not represent all EC progenitors. There exist two main approaches to the definition and/or isolation of cells with endothelial characteristics and regenerative capacity: culture selection from mononuclear cells (either BM or peripheral blood-derived) or the use of a set of antibodies to various "identifying" markers of these cells.

*Culture-Modified EPCs*

Culture selection involves the growth of mononuclear cells (MNCs) in selective medium and in fibronectin-coated dishes[6,7]. The medium contains a cocktail of endothelial growth factors, such as VEGF, bFGF, IGF-1, PDGF-BB, as well as ascorbic acid and hydrocortisone. The medium is changed regularly (usually every 24-28 hours) and all non-adherent and dead cells are taken away. All remaining adherent cells are considered responsive to EC growth factors and can bind to basement membrane.

Culture-selected can further be classified into two distinct phenotypes arising in culture[6,8,9], described in table 1.1 and figure 1.1. "Early outgrowth cells", which are the cells most studied to date, are those mainly derived from monocytes, do not proliferate and begin to gradually die after a few weeks in culture[10-13]. In contrast, "late outgrowth cells", which arise from colonies amongst the early outgrowth cells (usually > 2 weeks after isolation), eventually proliferate very rapidly, resemble microvascular ECs (with a cobblestone morphology), and seem to be expandable indefinitely[6,7,12,14,15]. The distinction of these two cell types is reinforced by their expression of different markers. Late outgrowth cells do not express CD1a or CD14 and have a low expression of CD45, the pan-leukocyte marker[8,9].

Most early studies of cell therapy employed early growth cells and thus, there is abundant evidence that transplanted cells can replace damaged endothelium and/or contribute to neovascularization[16-20], although there is much debate about the mechanism of these effects. In contrast, late outgrowth cells have only limitedly been studied in vivo[7,12,15]. It is suggested, however, that their highly replicative potential may be important in neovascularization, when a large supply of regenerative cells is required[21]. It has also been suggested that early and late outgrowth EPCs can act synergistically to stimulate neovascularization[15].

The use of the term "endothelial progenitor cell" to identify these cells is controversial, since they do not proliferate or differentiate into true ECs, and several researchers have proposed it to be replaced with the term "endothelial-like (or angiogenic) culture-modified mononuclear cells". Similarly, late outgrowth cells are much more proliferative than progenitor cells are, and should also be named more appropriately, although their differentiation capacity needs to be further determined prior to the derivation of a new term.

Although this method of selecting regenerative cells can be considered crude and does not use specific markers for selection, several studies have shown that these cells represent a product with regenerative capacity, and their simple isolation procedure makes them particularly amenable to clinical translation.

**a. Early Growth EPCs** **b. Late Outgrowth EPCs**

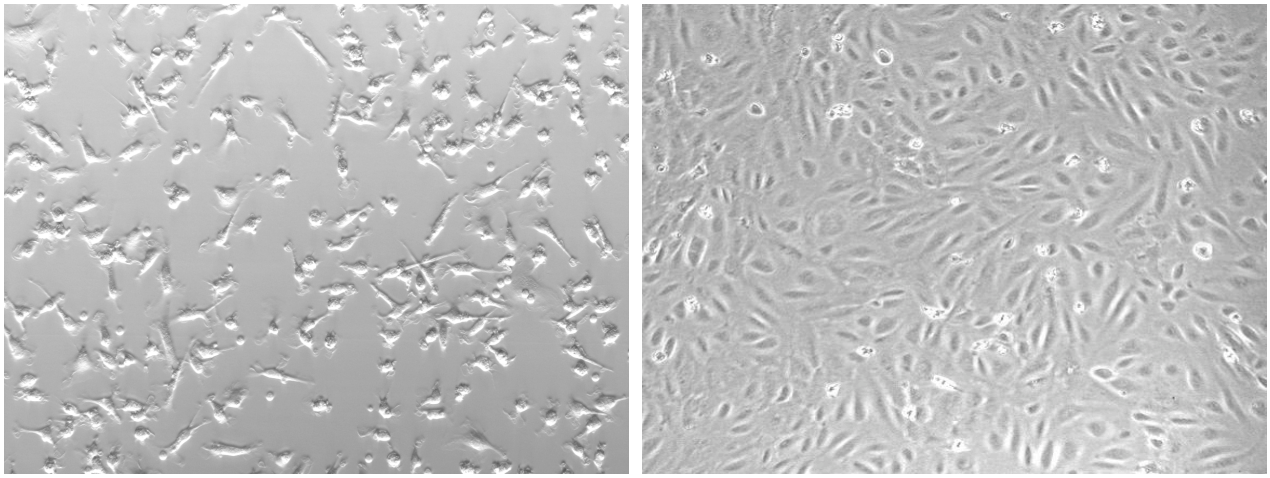

**Figure 1.1: Morphology of Early and Late Outgrowth EPCs.**
Early growth cells (a) exhibit spindle-like morphology and do not proliferate readily, which leads to the typical sub-confluent appearance. Image produced using light microscopy (20X objective and Hofmann contrast). Late outgrowth cells (b) display the typical endothelial cobblestone morphology and proliferate abundantly. Image produced using light microscopy (20X objective).

*Marker Identification of EPCs*

Currently there is no consensus on the description of a "true" EPC. Several groups have attempted to characterize the cells they isolated using a panel of immunological and non-immunological markers. Table 1 illustrates the various descriptions and markers used as well as those co-expressed in related cell types, indicating the degree of overlap between these cell types. The original combination of CD34 and VEGFR-2 remains the most common, but several other markers have been used to refine the identification of this cell type. Some have identified CD133 (or prominin) as an additional marker[8], particularly to identify immature EPCs, but this also has proven to be of limited use as a precise marker. This ambiguity has been underscored by

the demonstration of the endothelial differentiation potential of BM-derived $CD14^+$ MNCs or monocytes[11]. Although several independent groups have shown a clear development of EC phenotype after selective culture, many also found that expression of monocytic antigens persisted[11,22-25]. Several additional studies confirmed the overlap between EC and monocyte phenotypes, suggesting that using traditional EC markers such as acLDL uptake, Ulex binding, CD31, CD105 (endoglin), CD144 (vascular endothelial cadherin), VEGFR-2, CD34 and Tie-2 cannot distinguish between ECs and monocytes, and thus also cannot specifically identify EPCs[3,23,24,26,27]. Similarly, another group showed that cultured $CD34^-CD14^+$ PB-MNCs express Tie-2, VEGFR-2, CD144, von Willebrand factor (vWF), CD146, CD105 and eNOS[22]. Interestingly, another report suggested that monocytes ($CD14^+$) which express low levels of CD34 (undetectable with conventional techniques) may in fact contain the "true" progenitor cell population, with the greatest regenerative capacity[28]. Considering the uncertainty as to the most reliable method to identify circulating EPCs, several studies have employed CD34/VEGFR-2 or CD34/CD133 double positivity to enumerate circulating EPCs and to correlate their concentration to clinical conditions[29-36].

**Table 1.1: Summary of Surface Marker Characterization of EPCs**

| Cell Type Identity | Identifying Markers | Ref. |
|---|---|---|
| *Freshly Isolated CD34$^+$ Cells* | CD34$^+$/flk-1$^+$/CD45$^+$ | 3 |
| | Tie-2$^+$/ AcLDL$^+$ | 37 |
| | CD117$^+$/VEGFR2$^+$/Tie-2$^+$/CD133$^+$ | 38 |
| *Cultured Circulating EPCs* | AcLDL$^+$/Lectin$^+$/VEGFR2$^+$/VE-cadherin$^+$/CD31$^+$/CD14$^+$/CD34$^+$ | 39,40 |
| | AcLDL$^+$/NO$^+$/VEGFR2$^+$/VE-cadherin$^+$/CD31$^+$/vWF$^+$/CD45$^-$ | 41 |
| | AcLDL$^+$/VEGFR2$^+$/Tie-2$^+$/CD31$^+$/vWF$^+$ | 42 |
| | AcLDL$^+$/Lectin$^+$/VEGFR2$^+$/CD105$^+$/vWF$^+$/ CD45$^+$ | 43 |
| *Early Outgrowth EPC* | AcLDL$^+$/Lectin$^+$/VEGFR2$^+$/CD31$^+$/Tie-2$^+$/VE-Cadherin$^-$ /eNOS/CD14$^+$ | 6,7 |
| | VEGFR1$^{weak}$/eNOS$^+$/vWF$^+$/VE-Cadherin$^+$/VEGFR2$^+$ | 12 |
| *Late Outgrowth EPC* | VEGFR2$^+$/CD31$^+$/Tie-2$^+$/VE-Cadherin$^+$/eNOS$^+$/CD14$^-$ | 6,7 |
| | VE-Cadherin$^{strong}$/VEGFR1$^+$/VEGFR2$^+$/eNOS$^+$/vWF$^+$ | 12 |
| *CD14$^+$ MNCs* | CD14$^+$/CD45$^+$/vWF$^{+/-}$/VEGFR1$^+$ | 11,23,44 |
| | CD14$^+$/CD45$^+$/CD34$^+$/VE-Cadherin$^+$/eNOS$^+$/vWF$^+$/Tie2$^+$/ VEGFR1$^+$/VEGFR2$^+$ | 22,24 |

Adapted from: Urbich and Dimmeler[45] and Ward et al.[46]

The failure to reliably characterize EPCs has generated skepticism and confusion within the field of regenerative medicine. However a recent study[44] and an excellent review by Schatteman et al.[21] have suggested that despite phenotypic overlap between various BM-derived or circulating cell types, the endothelial phenotype can best be characterized by three features; eNOS expression, integration into tube-like structures formed by human umbilical vein ECs (HUVECs) and stimulation of tube formation by HUVECs[44]. It is suggested that these criteria might be the best *in vitro* method of judging the endothelial phenotype, regardless of the presence or absence of particular cell surface antigens[21]. This simplified interpretation is important, as it

acknowledges the limitation of surface markers and affirms the importance of the identification of EPCs according to their potential to acquire functional properties of ECs.

## 1.2 EPCs and Neovascularization

There exists a great deal of preclinical evidence for the ability of BM-derived cells to stimulate neovascularization, which includes vasculogenesis, angiogenesis, and arteriogenesis[47]. Aided by the use of genetically tagged BM transplants it has been shown that tissue ischemia releases chemokines such as VEGF and SDF-1, which mobilize EPCs from the marrow and aid in the cells' homing and engraftment into ischemic territory where they participate in the process of neovascularization[5,37-40,48-52]. In small animal models of hind limb ischemia (HLI), direct injection of human circulating EPCs improved neovascularization and subsequent limb perfusion[53]. Similarly, studies using various myocardial ischemia models have shown that systemically administered or BM-recruited circulating EPCs stimulate angiogenesis and arteriogenesis, with subsequent improvement in myocardial perfusion and left ventricular ejection fraction (LVEF)[39,40,54-56]. Direct administration of BM-MNCs has yielded very similar results. In HLI models, intramuscular injection of BM-MNCs increased the number of visible capillaries, limb flow and exercise tolerance compared to controls[57] and augmented small and larger vessel remodeling, with enhanced collateral development (arteriogenesis)[58,59]. Of note, culturing the MNCs under hypoxic conditions improved the effect of cell delivery to the limb[57], suggesting that these cells are particularly responsive to ischemia. In the heart, BMC implantation has been reported to improve myocardial angiogenesis and subsequent perfusion in rat[60] and pig models of myocardial ischemia[61-63], forming the premise of many clinical trials for myocardial ischemia/infarction.

The preclinical evidence for the ability of EPCs to stimulate neovascularization is quite strong, although it is difficult to effectively summarize the data from studies since they employ various disease models, cell types and doses. However, despite any differences in cell characterization, the improvements appear similar[38,39,41,52]. As well, both early and late outgrowth EPCs show comparable in vivo neovascularization capacity[7,12,15]. Fully differentiated mature ECs, however, lack the ability to induce neovascularization[12,38,39] suggesting that the undifferentiated nature of transplanted cells is crucial to their therapeutic function[45].

The ability of EPCs to stimulate neovascularization was initially attributed to their ability to differentiate into ECs and incorporate into neovessels. However, studies allowing the identification of BM-derived cells using reporter genes such as GFP or LacZ have shown greatly varying incorporation rates of administered cells (0-90% of neovessel ECs)[16-20,64]. This variation is likely due to the complex nature of this phenomenon, which requires a multistep process, including mobilization, adhesion, migration, invasion, chemotaxis and differentiation of circulating cells[45]. Any differences in the ischemic model, cell processing, delivery method or endpoint measurement could lead to widely divergent results. Recently, there has been considerable interest in the SDF-1/CXCR4 agonist-receptor system as it is involved with modulating EPC mobilization (with VEGF) from BM[49,65,66] and the homing of EPCs to ischemic tissue[51,65,67]. SDF-1 is also commonly used for the stimulation of EPC chemotaxis in vitro[68,69]. Incubation of EPCs with sphingosine-1-phosphate (S1P), a bioactive lipid, increases in vitro migration of EPCs and in vivo neovascularization through activation of CXCR4[70]. Similarly, the angiogenic capabilities of EPCs are increased by protease-activated receptor-1 (PAR-1) agonism through upregulation of SDF-1 and CXCR4 expression[71] and decreased through impaired CXCR4 signaling[72]. Recently, CXCR4 was shown to be regulated by NO[73] and by caveolin (an

important eNOS binding partner and regulator)[74] in EPCs, affirming that eNOS may also be crucial to their in vivo functional capacity. Finally, EPCs are known to secrete SDF-1[50,71], suggesting that they may also stimulate neighbouring cells in ischemic tissue.

Since the incorporation rate of EPCs is often quite low, their ability to stimulate neovascularization via paracrine effects has gained greater attention recently, as with other regenerative cell types[50,75,76]. This is supported by evidence of their ability to secrete various growth factors such as VEGF, SDF-1, insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF) and NO[50], which could stimulate native endothelium to proliferate and initiate classical angiogenesis[77]. Likely, the overall neovascular response is dependent not only on the ability of cells to localize to the vasculature and incorporate into its structure, it may be predominantly related to their ability to secrete factors which stimulate adjacent cells. In a model of tumor angiogenesis, transplanted cells were not integrated in the tumor vessels *per se* but were found adjacent to the vessels[59,64]. The secretion of paracrine angiogenic agents may not be sufficient, however, as infusion of macrophages, which do secrete growth factors[77,78] and do not incorporate into vessels, caused only a slight increase in collateral flow after ischemia[43]. The ability of EPCs to both incorporate/associate with neovessels and to stimulate native vasculature may be the key to their potent ability to stimulate neovascularization and endothelial repair.

The study of the mechanism of cell therapy is an exciting and expanding field and likely, this process will be better elucidated in coming years. Nevertheless, the exciting results from experiments to date have triggered the use of EPCs for various therapeutic purposes, some of which have already been initiated clinically.

## 1.3 Cell Therapy for Myocardial Repair

In 2001, Orlic et al. first reported regeneration of myocardium by hematopoietic stem cells (HSCs) injected into the infarct border zone of a mouse MI model[79,80]. However, the transdifferentiation of HSCs into cardiomyocytes has been strongly refuted by several studies that have shown the maintenance of hematopoietic characteristics of transplanted cells[81-83]. Evidence for the ability of EPCs to regenerate the myocardium arises from reports that EPCs can differentiate into cardiomyocytes when co-cultured with myocytes[84], as well as a report that they can transdifferentiate into cardiomyocytes, ECs and SMCs[85]. Despite the controversy surrounding the ability of cells to differentiate into cardiomyocytes and a general lack of mechanistic evidence, the ability of cells to improve cardiac function post-MI is strongly supported by preclinical studies[5,38,40,55,79,80,86-91] and basis of a number of clinical trials, as discussed below.

Many theories exist to mechanistically explain the reported benefit in myocardial function following cell therapy. A unified theory is summarized in figure 1.2. First, there is evidence, as presented above, that transplanted cells stimulate neovascularization in the peri-infarct zone, either through direct (via endothelial incorporation) or paracrine means[50], rescuing ischemia and increasing myocardial perfusion[38,92]. The increased perfusion would also lead to greater myocardial regional contractility, thereby increasing myocardial function[92]. Second, transplanted cells may physically transdifferentiate into cardiomyocytes and replenish the dead myocardial tissue, as was suggested by several groups[79,80,85,93-95]. Regenerating lost myocardial tissue would presumably also lead to increased contractility and improved cardiac function. Third, there is some preliminary data suggesting that cells may act by modulating the immune response to

injury and impact on the infarct remodeling process. A recent study showed that an intact immune system was required for the beneficial effect of cell therapy following myocardial ischemia[96]. It was also shown that the ex vivo proliferation of EPCs is dependent on the presence of T cells in culture[97]. An integrated theory of cell therapy post-MI suggests that these three processes occur synergistically. If the myocardial perfusion is augmented, myocyte apoptosis is better prevented and any deposited cells have a greater chance of surviving and regenerating lost myocardium[38,92]. Kocher et al.[38] showed reduction in cardiomyocyte apoptosis and reduced myocardial remodeling following neovascularization due to angioblast administration post-MI. Hiasa et al.[93] showed that BM-MNC administration reduced the infarct size through secretion of VEGF, which was associated with the inhibition of myocyte apoptosis in the peri-infarct zone. Also, it was shown that EPCs stimulate mature ECs and cardiac resident progenitor cells via VEGF, SDF-1, and IGF-1, which may enhance angiogenesis and myogenesis[50,98]. In parallel, the paracrine factors released by donor cells might depress innate and/or acquired immunity and inflammation, which would also reduce the degree of peri-infarct apoptosis and myocardial fibrosis[99]. There is little consensus or definitive mechanistic data for BMC or EPC-mediated myocardial regeneration post-MI, however elucidating such mechanisms is very difficult in animals, and even more so in humans.

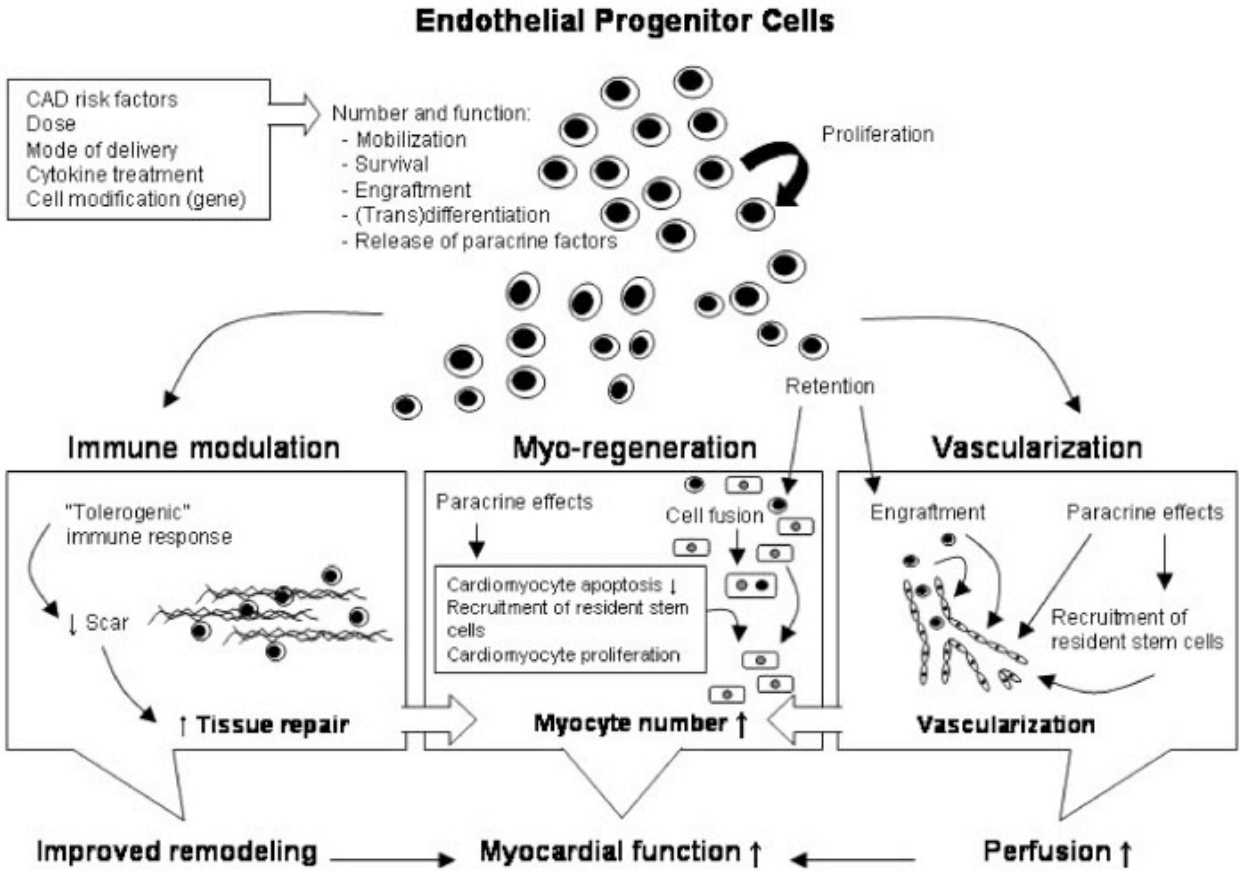


**Figure 1.2: Integrated Mechanistic Theory of Myocardial Regeneration Following Delivery of Regenerative Cells.**

Following administration of EPCs, the cells are thought to promote myocardial function by three main mechanisms: neovascularization, immune modulation and myo-regeneration. EPCs home to and engraft into ischemic myocardium, where they stimulate neovascularization either by incorporation into neovessels or through paracrine stimulation of native vascular cells. EPCs are thought to transdifferentiate into cardiomyocytes, which can replace the lost or dying myocytes and regenerate functional myocardium where scar tissue would normally form. In addition, EPCs may also prevent infarct expansion by preventing native cardiomyocyte apoptosis or that of transplanted cells in the peri-infarct zone. This is accomplished either through paracrine stimulation of myocytes or indirectly due to increased perfusion following vascularization. Thus, vascularization may be necessary for myo-regeneration to take place. Many factors, both clinical and experimental, may impact on the efficiency of these processes, and must be considered when designing cell therapy studies. Furthermore, infarct remodeling may be impacted by the ability of EPCs to modulate the immune response to injury, which might reduce scaring and maintain normal myocardial contractility. The stage of disease and mode of delivery may affect the ability of the myocardium to respond to transplanted cells. CAD RFs, concomitant pharmacological therapy as well as cell manufacturing (including genetic modification) may affect the cells' regenerative capacity. Obtained with permission from Ward et al.[46]

*EPC Mobilization*

An alternative strategy for cell therapy involves the mobilization of EPCs from the BM, thus avoiding the need for cell isolation, processing and delivery. Physiologically, HIF-1α production by ischemic tissue stimulates production of VEGF and SDF-1, which act synergistically to mobilize EPCs from the BM via a MMP-9 dependent mechanism[48,49,66,100]. For therapy, VEGF[101], granulocyte colony-stimulating factor (G-CSF)[29,102,103], stem cell factor (SCF)[52,82] and erythropoietin (Epo)[104,105] have been reported to increase EPC mobilization in preclinical models, with subsequent myocardial neovascularization and improved cardiac function after acute MI[100,102,106-108]. G-CSF has also been shown to have direct myocardial effects, including the inhibition of cardiomyocyte and endothelial apoptosis[109-112] as well as the acceleration of infarct healing by enhancing macrophage infiltration and matrix metalloproteinase (MMP) activation[98,106,113,114]. Epo is a glycoprotein hormone produced in the kidney and liver and acts primarily as a cytokine for erythroid precursors in the bone marrow. However, the receptor for Epo (EpoR) has been found in various cardiovascular cell types (cardiomyocytes, smooth muscle cells and ECs) and is thought to activate downstream signaling through Erk1/2, PI3K and Akt in cardiomyocytes[115-117]. In models of myocardial ischemia or infarction, Epo produces cardioprotection independent of hematocrit[118], through inhibition of apoptosis and inflammation, as well as induction of neovascularization[119]. Apart from its direct effects on the vasculature and the myocardium, Epo is thought to improve myocardial function through the mobilization of regenerative cells from the bone marrow, including mesenchymal stromal cells (MSCs) and EPCs[33,120-122]. Recently, Epo was used in combination with MSC delivery and shown to enhance the angiogenic effect of MSC therapy[120], suggesting a synergistic effect.

## 1.4 Endothelial Nitric Oxide Synthase

*Nitric Oxide*

Nitric oxide (NO) is a free radical gas that has several important physiological roles in the body. Furchgott and Zawadski[123] showed in 1980 that preservation of the endothelium was critical to the vasodilatory capacity of acetylcholine, and concluded that an agent which they termed "endothelium-dependent relaxing factor (EDRF)" was produced by the endothelium to create this effect. In 1987, two separate groups (of Ignarro and of Moncada) were able to show that this agent was in fact NO[124-126]. Moncada's group then showed that NO is biosynthesized from L-arginine and catalyzed by an enzyme, nitric oxide synthase (NOS)[127]. Several additional co-factors are required for the reaction, including Calmodulin/$Ca^{2+}$, nicotine adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH4), haem, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), all of which have binding sites on the enzyme[128].

The overall reaction is:

$$\text{L-arginine} + 2\text{NADPH} + O_2 \rightarrow \text{L-citrulline} + \text{NO} + 2\text{NADP}^+$$

Three isoforms of NOS are known: neuronal (nNOS or NOS I), inducible (iNOS or NOS II) and endothelial (eNOS or NOS III). NOS enzymes are expressed by a variety of cell types. nNOS has been found in neurons, skeletal muscle, the pancreas, and the kidneys. iNOS can be expressed in almost any cell type under cytokine stimulation, but is also constitutively expressed in some tissues such as the bowel wall[129]. eNOS is produced primarily in ECs, but has also been found in cardiomyocytes, platelets, mast cells, bone marrow cells, neurons, erythrocytes, renal epithelium

and leukocytes[130-132]. Table 1.2 shows the gene location and size of each of the three main isoforms. The isoforms are regulated and expressed differently, but are similar in that they all require the formation of dimers to produce NO at full capacity.

**Table 1.2: Nitric Oxide Synthase Isoforms**

| Name | Molecular Weight (kDa) | Gene Locus |
|---|---|---|
| nNOS (NOS I) | 161 | 12q24.2-12Q24.3 |
| iNOS (NOS II) | 131 | 17cen-q11.2 |
| eNOS (NOS III) | 133 | 7q35-7q36 |

Modified from Bruckdorfer et al.[128], Xu et al.[133] and Alderton et al.[134]

The enzyme consists of an oxygenase and a reductase domain, with separate catalytic activities. The oxygenase domain contains binding sites for haem and BH4 and is linked through a binding site for calmodulin (CaM) to the reductase domain, which has binding sites for FAD, FMN and NADPH (Figure 1.2). In order to produce NO from L-arginine this system needs to be fully coupled through BH4. Any deficiency of BH4 will lead to the formation of other products by the reductase, including the superoxide (SO) anion ($O_2^-$), which is a reactive oxygen species (ROS). The capacity of NOS to produce NO or SO is often referred to as a 'double-edged sword'[135], particularly in the endothelium.

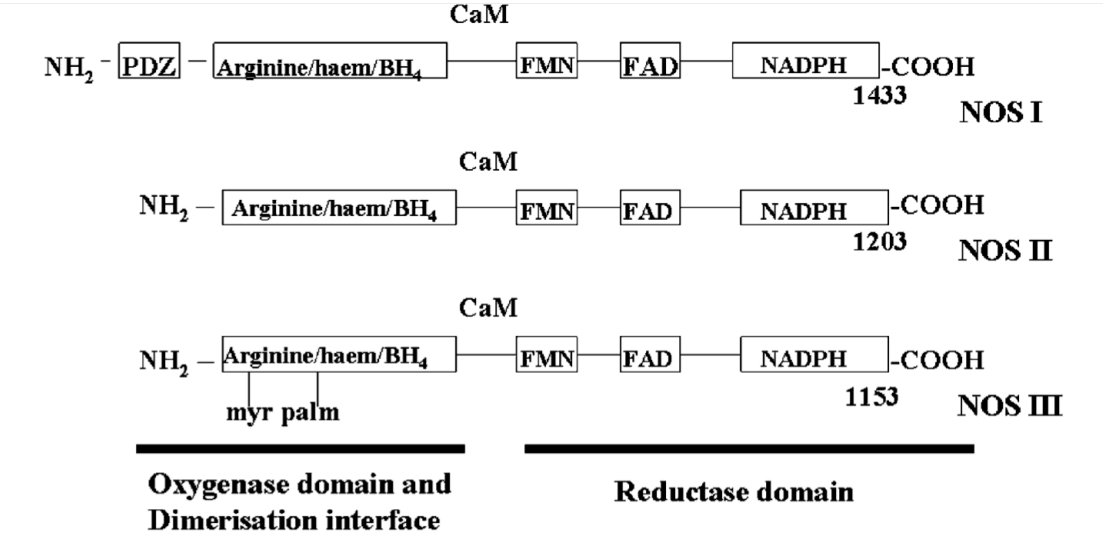


**Figure 1.3: Structure of NOS Isoforms.**
Obtained with permission from Bruckdorfer et al. [128]

The NOS isoforms have different structures that allow them to be located in specific sites, appropriate for the various roles that NO has in the body. The main areas relate to vascular homeostasis (eNOS), neurotransmission (nNOS) and to non-specific immunity (iNOS)[128]. eNOS is docked to the plasma membrane by hydrophobic anchors due to myristoylation and palmitoylation (covalent linkages to the fatty acids, myristic and palmitic acids). nNOS has no anchoring sites to hold it in the plasma membrane but does have a PDZ domain, which targets the enzyme to sites in nerve synapses[134]. Both eNOS and nNOS produce NO at low levels constitutively. iNOS does not produce NO constitutively but in response to external inflammatory stimuli (e.g. bacterial lipopolysaccharide) is transcriptionally and translationally induced to produce NO at levels far greater than eNOS and nNOS (>20x)[136].

As mentioned above, NOS can produce NO and SO, and these products can react with other reactive gases, some of which can lead to oxidative stress in the cell. $O_2^-$ is generated by the one-electron reduction of oxygen by NADPH or NADH oxidases and by respiratory chain enzymes

in the mitochondria[137]. In mitochondria, some of the oxygen (1%) is incompletely reduced to water, leading to the production of superoxide (SO or $O_2^-$). Since this is a potentially damaging ROS, certain antioxidant and enzymatic help to prevent its accumulation. These include the enzyme superoxide dismutase (SOD) that breaks down the SO anion to hydrogen peroxide, which in turn is turned into water by the actions of catalase or glutathione peroxidase.

Superoxide dismutase (SOD) has several forms. CuSOD or ZnSOD (referring to the metals bound to the protein) are found in the cytosol and MnSOD exists in the mitochondria[138,139]. These enzymes allow for rapid dismutation of the SO to hydrogen peroxide ($H_2O_2$). The non-enzymatic reaction between NO and SO has a much greater reaction constant, however, and yields peroxynitrite a powerful oxidant, both a reactive oxygen and a reactive nitrogen species (Figure 1.4)[140].

The chemistry of this reaction can be seen in the following equation.

$$NO + O_2^{\bullet -} \rightarrow ONOO^- + H^+ \rightarrow ONOOH$$

The protonated form of peroxynitrite decomposes to form several reactive products, including nitrogen dioxide and hydroxyl radicals, which are extremely reactive and destructive to cells. Since the production of NO is critical to many physiological processes, and the production of ROS is potentially damaging, the precise regulation of eNOS activity is very important and is impacted on by several factors.

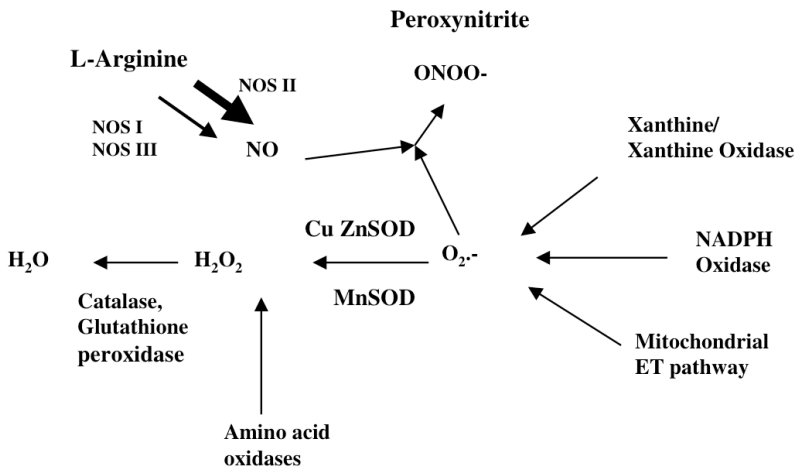


**Figure 1.4: Formation of Nitric Oxide and Other Reactive Oxygen Species**
Obtained with permission from Bruckdorfer et al. [128]

*Endothelial Nitric Oxide Synthase*

In the vasculature, NO, produced primarily by eNOS, regulates vascular tone (i.e. vasodilation), stimulates angiogenesis, is involved with vascular signaling and remodeling (e.g. paracrine endothelial-SMC signaling), inhibits platelet aggregation and inhibits leukocyte-endothelial interactions[141-152] (discussed below). Because NO production must be titrated to respond to various physiological and pathophysiological stimuli, eNOS is subject to strict regulation post-translationally.

*eNOS Regulation*

In ECs, eNOS is located in membrane micro-invaginations called caveolae. Caveolae sequester diverse receptors and signaling proteins from a variety of signal transduction pathways, including G protein coupled receptors (GPCRs), G proteins, growth factor receptors and calcium regulatory proteins. In this way, they position eNOS to receive signals from upstream signaling pathways and facilitate communication with downstream activators[153]. The localization of eNOS to

caveolae is dependent on irreversible, co-translational myristoylation of its N-terminal glycine[141,153]. Myristoylation initially targets eNOS to the cell membrane in general, where reversible post-translational palmitoylation of the Cys15 and Cys26 residues occurs[154]. Following myristoylation and palmitoylation, eNOS has three acyl anchors that anchor it to the caveolar membrane.

Although NO is constitutively produced by eNOS in ECs, eNOS can also be activated and inhibited by various factors. Various cytokine receptors and GPCRs initiate signaling pathways that modulate eNOS activity by two general mechanisms: mobilization of intracellular calcium and direct eNOS phosphorylation (or de-phosphorylation).

Intracellular calcium availability is critical to eNOS activity because maximal catalytic function of eNOS requires $Ca^{2+}$/CaM binding, which facilitates transfer of electrons between the enzyme's reductase and oxygenase domains[155]. GPCR ligands that stimulate intracellular calcium release include bradykinin (B2 receptor), acetylcholine (μ2 muscarinic receptor)[156], histamine, adenosine, ADP/ATP, sphingosine 1-phosphate (S1P), and thrombin[141,157]. In this pathway, phospholipase C (PLC) cleaves the membrane component phosphatidylinositol 4,5-triphosphate into diacylglycerol and inositol 1,4,5-triphosphate (IP3), which binds to IP3 receptors in caveolae and regulate calcium transit through regulation of ion channels[158]. Binding of CaM simultaneously disrupts the inhibitory caveolin–eNOS interaction[159,160]. The ability of CaM to activate eNOS is inhibited by CK2 kinase as phosphorylated CaM dissociates from eNOS.

eNOS activity is regulated by phosphorylation at many residues. Phosphorylation at Ser 1177,

Ser 635, and Ser 617 are stimulatory while phosphorylations at Thr 495 and Ser 116 are inhibitory[161]. Shear stress[162] and various growth factors such as VEGF (through VEGFR-2 agonism) and estrogen (through ER-α and ER-β)[163-165], initiate signaling through phosphoinositide-kinase-3 (PI3K) and protein kinase B (PKB or Akt), which activates eNOS directly by phosphorylation at Ser 1177. Following exposure to VEGF, maximal phosphorylation of Ser 1177 occurs within five minutes[166]. VEGF also induces Ser 116 de-phosphorylation via the phosphatase calcineurin. Interestingly, in contrast to Ser 1177 phosphorylation, Ser 116 de-phosphorylation requires 30 minutes, suggesting a tight temporal regulation of eNOS activity[167]. The pharmacological agents, statins[168] and PPAR-gamma agonists[169], also stimulate eNOS activation via the PI3K/Akt pathway.

The activation of eNOS by Ser 1177 phosphorylation leads to inhibition of CaM dissociation from eNOS and enhancement of eNOS electron transfer[145,170,171]. Apart from Akt, Ser 1177 phosphorylation is catalyzed by numerous kinases, including cyclic AMP-dependent protein kinase (PKA), AMP-activated protein kinase (AMPK), protein kinase G (PKG), and $Ca^{2+}$/CaM dependent protein kinase II (CaM kinase II)[172-175]. Phosphorylation at Ser 617 by either PKA or Akt sensitizes eNOS to CaM binding[176] and possibly modulates phosphorylation at other eNOS sites[161]. Phosphorylation at Ser 635 increases eNOS activity in response to PKA-dependent agonists as well as shear stress[176,177]. Phosphorylation at Thr 495, downstream of protein kinase C (PKC) and AMPK[172], attenuates CaM binding. Likewise, de-phosphorylation of Thr 495, by phosphatase 2A or protein phosphatase 1, enhances the interaction of eNOS and CaM[175,178]. Phosphorylation of eNOS at Ser 116 inhibits enzyme activity, and de-phosphorylation of eNOS at this site is promoted by VEGF[167].

Reversible S-nitrosylation is now considered an important regulator of eNOS activity in ECs[166,179]. eNOS is inhibited by tonic S-nitrosylation at residues Cys 94 and Cys 99[166,180] and de-nitrosylation increases enzyme activity. eNOS itself the source of the NO required for its own S-nitrosylation[166,179]. Due to this tight control, treatment of cultured ECs with eNOS agonists promotes temporary de-nitrosylation and activation of eNOS, which is soon blocked by the re-nitrosylation of eNOS.

Heat shock protein 90 (hsp90), a chaperone involved in protein trafficking and folding, is also involved with eNOS activation. In response to various eNOS agonists, Hsp90 undergoes reversible tyrosine phosphorylation and becomes tightly associated with eNOS[181-183]. Hsp90 enhanced the affinity of eNOS for CaM, favouring NO production rather than SO, and facilitating haem binding[184,185]. Hsp90 is also required for the interaction of Akt with eNOS[182,186] and increases the rate of Akt-dependent phosphorylation of eNOS[186-188], likely by unmasking eNOS phosphorylation sites or increasing the affinity of Akt for eNOS[189]. Also, hsp90 maintains levels of phospho-Akt (the active form of Akt) by the prevention of the proteasomal degradation of PI3K[190] and inhibition of protein phosphatase 2A (which normally inactivates Akt).

eNOS is also regulated by the eNOS Interacting Protein (NOSIP), a protein that binds the carboxy-terminal of the eNOS oxygenase domain and aids in the translocation of eNOS from caveolae to intracellular membranes[191]. Association of eNOS with NOSIP is inhibited by caveolin-1 by competitive binding to the same oxygenase domain. When NOSIP was overexpressed in ECs, NO production by eNOS was reduced, likely by disruption of the interaction of eNOS with caveolar signaling proteins[191].

**Table 1.3: Mechanisms Regulating eNOS Activity**

| Type of Regulation | Factor |
|---|---|
| Substrate Availability | L-Arginine[143] |
| Cofactor Availability | Tetrahydrobiopterin (BH4)[192,193] |
| | NADPH[194] |
| Dimer Formation[195] | |
| Interprotein Interactions | G-Protein Coupled Receptors (GPCR)[196,197] |
| | $Ca^{2+}$/CaM[159,160] |
| | Hsp90[182,184] |
| | Caveolin[159,160,198] |
| | NOS Interacting Protein (NOSIP)[191,199] |
| Phosphorylation[144,175,200] | |
| Subcellular localization[201] | |
| S-Nitrosylation[166,179] | |

## 1.5 eNOS in Cardiovascular Disease

### 1.5.1 eNOS in Normal Endothelial Function

*NO in Vasodilation*

One of the primary roles of vascular NO is to stimulate vasodilation in response to various eNOS agonists. Endothelial-derived NO diffuses across the cell membrane and causes pleiotropic intracellular changes in smooth muscle cells (SMCs), leading to reduced vasoconstriction and vascular tone.

There are no specific receptors for NO but in SMCs the main target protein is the intracellular haem-containing enzyme, soluble (or type 2) guanylyl cyclase (sGC). There is also a membrane bound GC enzyme (type 1) but it is not activated by NO and rather, primarily by anti-natriuretic peptide (ANP). NO has a very high affinity for haem-containing proteins[202] and when NO binds to the sGC protoporphyrin ring, this activates the enzyme. Haem is not required for basal sGC

activity but it is required for sGC to respond to NO[203]. sGC activity leads to the formation of cyclic guanosine monophosphate (cGMP) from the nucleotide GTP, through the reaction:

GTP → 3'5'cyclic GMP + PPi (pyrophosphate)

The sGC enzyme is a heterodimer consisting of isomeric forms α and β subunits; the most prevalent is α1β1[203]. The amino termini of the α1 and β1 subunits are essential for the binding of the haem and the full formation of cGMP[203]. The basal activity of the enzyme can be increased up to 200-fold by the binding of NO. However the half-life of the NO-haem complex is very short (~0.2 seconds), which means that stimulation needs to occur very frequently for a sustained effect[128]. Carbon monoxide (CO) also activates sGC by a similar mechanism but the activation is less efficient[204]. Furthermore, cGMP is only a temporary signaling molecule, as it is rapidly cleaved to the inactive 5'GMP by phosphodiesterases, the most significant of which is phosphodiesterase V (PDE5).

In SMCs, cGMP induces the activation of protein kinase G (PKG; or cGMP-dependent protein kinase I, cGKI), which modifies the activity of other proteins by phosphorylation of Serine or Threonine residues[205,206].

One of the critical roles of NO in the vasculature is to stimulate vasodilation, which is accomplished by the disruption of SMC actin and myosin interactions, thus inhibiting contractions. Vasoconstriction is typically induced by noradrenaline and other vasoconstrictors, which raise SMC calcium levels. These stimuli also activate Rho A kinase, which promotes the phosphorylation of myosin by activation of myosin light chain kinase (MLCK) and inhibition of the myosin light chain phosphatase (MLCP)[207,208]. MLCP normally opposes MLCK. NO-stimulated cyclic GMP activates PKG, which phosphorylates and inactivates Rho A kinase [209].

This allows the activation of MLCP and results in relaxation rather than contraction[209] (Figure 1.6). Prostacyclin, another vasodilator, acts similarly on Rho A kinase via cyclic AMP (cAMP), which is derived from adenylyl cyclase (AC). Therefore, NO acts synergistically with prostacyclin through interactions of the cyclic nucleotides cGMP and cAMP (Figure 1.4).

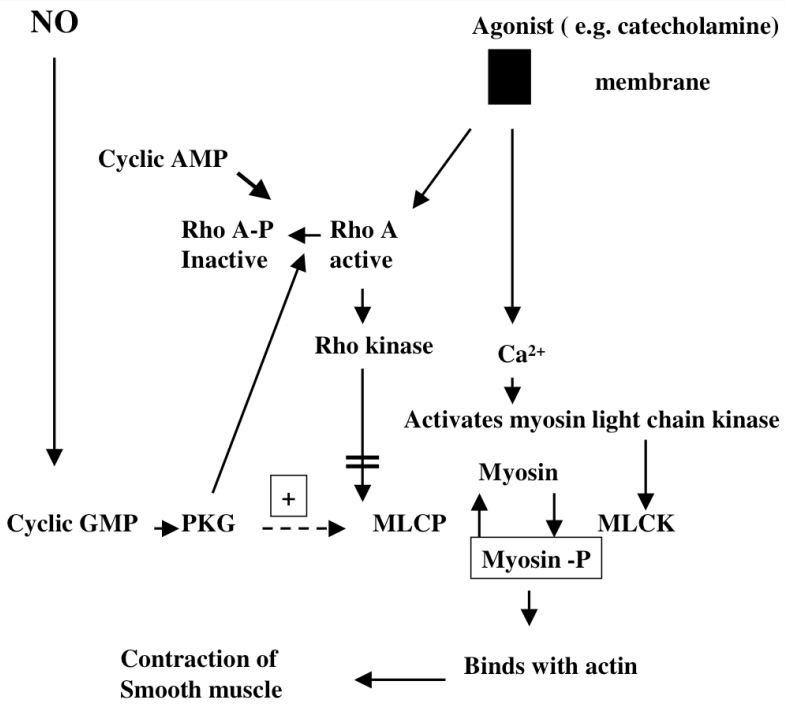


**Figure 1.5: cGMP and Rho A Kinase SMC Relaxation**
Obtained with permission from Bruckdorfer et al. [128]

NO influences SMC relaxation via other mechanisms as well. PKG can phosphorylate potassium ($K^+$) channels ($BK_{Ca}$), increasing $K^+$ efflux and decreasing $Ca^{2+}$ influx[210,211]. PKG also phosphorylates and inhibits the inositol trisphosphate receptor (IP3R) which normally stimulates the release of $Ca^{2+}$ from intracellular stores, leading to MLCK activation and vasoconstriction. PKG can also phosphorylate and inactivate cAMP phosphodiesterase and thereby increase the formation of cAMP by prostacyclin, which also promotes relaxation[212].

*Role of NO in Platelets and Leukocytes*

NO influences vascular homeostasis in many ways beyond modulation of vasomotion, such as inhibition of smooth muscle cell proliferation, platelet aggregation, platelet and monocytes adhesion to the endothelium, LDL oxidation, expression of adhesion molecules and endothelin production (Figure 1.5)[129,213,214].

NO produced in the endothelium can act as a potent inhibitor of platelet adhesion to the subendothelium and their subsequent activation and aggregation[215,216]. During activation, platelets normally release NO (from small amounts of eNOS) and various proteins such as eicosanoids, nucleotides, catecholamines and serotonin[217]. These increase or decrease the activation of the platelets through the involvement of neighbouring platelets to form a platelet plug[218]. NO is thought to act by limiting the spread of the platelet response and thrombus formation[215,217,219]. Platelets express vasodilator sensitive phosphoprotein (VASP), which is involved the polymerization of actin and the formation of lamellipodiae during the initiation of platelet activation. This is done through the interaction with other cytoskeletal proteins, such as zyxin, vinculin and α-actinin. NO has been shown to inhibit VASP, which reduces the polymerization of actin and prevents platelet activation, thereby reducing their aggregation and adhesion to the endothelium[219,220]. Furthermore, the activation of platelets at the sites of vascular injury leads to smooth cell proliferation via the release of proliferative growth factors, including platelet-derived growth factor (PDGF) and vascular endothelial cell growth factor (VEGF)[221]. Thus, by inhibiting platelet activation, NO prevents the underlying smooth muscle cells from being exposed to potent proliferating agents. It has also been proposed that NO also inhibits the expression of the prothrombotic protein tissue factor (TF), which initiates the intrinsic

coagulation pathway, on the endothelial surface, although this is a controversial area[222].

NO inhibits leukocyte adhesion to the endothelium by inhibiting the expression of cell surface adhesion molecules P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1)[223] and preventing the expression of monocyte chemoattractant protein-1 (MCP-1)[224]. Sufficient production of NO and inhibition of leukocyte adhesion is critical as prolonged adhesion of monocytes to the endothelium is pro-atherogenic, since they release IL-1β, causing local inflammation and suppressing the release of biologically active NO in a monocyte concentration-and time-dependent manner[225]. This is supported by observations in apoE/eNOS double knockout mice that have increased endothelial leukocyte adhesion and accelerated development of atherosclerosis[226]. NO also inhibits atherosclerosis and intimal hyperplasia by the direct inhibition of vascular SMC proliferation. In vitro, NO-donors have been shown to inhibit VSMC proliferation by a cGMP-dependent mechanism[227-229]. These actions of NO are critical to disease prevention since the proliferation of vascular SMCs and their over production of extracellular matrix is one of the key events in the progression of atherosclerosis[225,230]. In vivo, the administration of L-arginine or NO-donors to animal models of atherosclerosis has been shown to reduce intimal thickening[231,232].

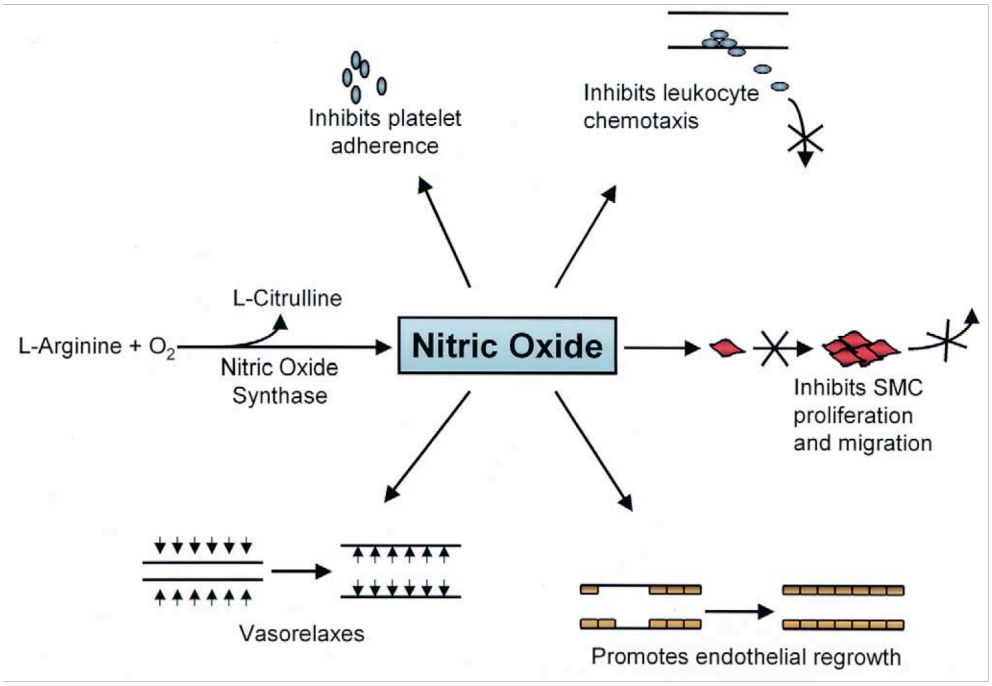


**Figure 1.6: Vascular Protective Effects of Nitric Oxide**
Obtained with permission from Barbato et al. [129]

NO has a number of important vasoprotective roles and is an antiatherogenic, antiproliferative, and antithrombotic factor. Defects in these functions usually result from decreased NO bioavailability and are responsible for diseases such as atherosclerosis, leading to coronary artery disease (CAD), peripheral vascular disease and stroke[233,234].

## 1.5.2 Endothelial Dysfunction

*Cardiovascular Risk Factors and Clinical Manifestations*

There are several RFs for cardiovascular disease, both un-modifiable and modifiable, that may increase cardiovascular risk through endothelial dysfunction. Age is among the most important

un-modifiable RFs as 80% people who die of cardiovascular disease are at least 65 years old and the cardiovascular risk being proportional to age[235]. Ethnicity is another important un-modifiable factor. African-Americans have a greater prevalence of severe hypertension and a higher rate of cardiovascular disease than Caucasians do. In patients with non-ST elevation acute coronary syndrome (positive cardiac markers and/or ischemic ST-segment changes), African-Americans were younger and were more likely to have hypertension, diabetes, heart failure, and renal insufficiency compared to Caucasians[236]. Men have a higher risk of cardiovascular diseases than women do. However, in the 65-year-old or older age group, women who have a heart attack are twice as likely to die from them as men. Under the age of 65, the risk of a heart attack is much higher in men[237-239]. People with a family history of cardiovascular disease are at increased risk themselves. Genetic epidemiological studies have shown that several genetic variants, including those of the genes for eNOS[240-242], angiotensin converting enzyme (ACE)[243] and angiotensin II receptor type 1[244-246], increase the risk for CAD and coronary artery spasm.

Often, cardiovascular disease can be prevented by the treatment and control of modifiable RFs such as hypertension, diabetes mellitus (especially type II), hypercholesterolemia/dyslipidemia, cigarette smoking and obesity[247-249]. Obesity is an important determinant of cardiovascular diseases. Overweight ($25.0 < BMI < 29.9$) or obese ($BMI \geq 30$) individuals experience greatly elevated morbidity and mortality from cardiovascular diseases[250-252]. There is also a very strong association between cigarette smoking and cardiovascular risk[249,253,254], partly due to impaired endothelium-dependent coronary vasodilation[254,255]. Diabetes mellitus is associated with increased rates of morbidity and mortality caused primarily by endothelial dysfunction and the accelerated development of atherosclerotic disease[256].

The overall cardiovascular risk includes the cumulative impact of multiple cardiovascular RFs. Thus multiple sources of mild risk elevation can be more impactful than one area of very high risk. An overall cardiovascular paradigm was developed with data from Framingham Heart Study, which revealed that cardiovascular RFs tend to cluster[248], multiple RFs have a multiplicative impact[257,258], even mild-to-moderate levels of multiple RFs impart substantial risk and multiple areas of slight risk can be more important than one area of very high risk[259,260]. The Framingham Risk Score (FRS) refers to the calculated 10-year risk of coronary heart disease based on a person's age, sex, blood pressure, cholesterol levels (total and HDL) and smoking status. The FRS score can be used to inform modifiable RF management and educate patients about the benefits of lifestyle modification (Figure 1.6).

## Framingham Risk Calculator

**Step 1: Add up risk score**

| Parameters: | Men | Women |
|---|---|---|
| **Age** | | |
| 30-34 | -1 | -9 |
| 35-39 | 0 | -4 |
| 40-44 | 1 | 0 |
| 45-49 | 2 | 3 |
| 50-54 | 3 | 6 |
| 55-59 | 4 | 7 |
| 60-64 | 5 | 8 |
| 65-69 | 6 | 8 |
| 70-74 | 7 | 8 |
| **Total Cholesterol** | | |
| < 4.14 | -3 | -2 |
| 4.15-5.17 | 0 | 0 |
| 5.18-6.21 | 1 | 1 |
| 6.22-7.24 | 2 | 2 |
| > 7.24 | 3 | 3 |
| **HDL-C** | | |
| < 0.90 | 2 | 5 |
| 0.91-1.16 | 1 | 2 |
| 1.17-1.29 | 0 | 1 |
| 1.30-1.55 | 0 | 0 |
| > 1.56 | -2 | -3 |
| **Systolic BP** | | |
| < 120 | 0 | -3 |
| 120-129 | 0 | 0 |
| 130-139 | 1 | 1 |
| 140-149 | 2 | 2 |
| 150-159 | 3 | 3 |
| > 160 | | |
| **Smoker** | | |
| No | 0 | 0 |
| Yes | 2 | 2 |

**Step 2: Estimate 10 yr risk from score**

| | 10 yr Coronary Risk | |
|---|---|---|
| Points total: | Men | Women |
| 1 | 3% | 2% |
| 2 | 4% | 3% |
| 3 | 5% | 3% |
| 4 | 7% | 4% |
| 5 | 8% | 4% |
| 6 | 10% | 5% |
| 7 | 13% | 6% |
| 8 | 16% | 7% |
| 9 | 20% | 8% |
| 10 | 25% | 10% |
| 11 | 31% | 11% |
| 12 | 37% | 13% |
| 13 | 45% | 15% |
| 14 | > 53% | 18% |
| 15 | | 20% |
| 16 | | 24% |
| 17 | | > 27% |

**Step 3: Assign Risk Class and Lipid targets based on 10 yr risk**

| Risk: | Risk Class | LDL | TC/HDL | TG |
|---|---|---|---|---|
| > 30% (**or** CAD, **or** DM) | Very High | < 2.5 | < 4.0 | < 2.0 |
| 20-30% | High | < 3.0 | < 5.0 | < 2.0 |
| 10-20% | Moderate | < 4.0 | < 6.0 | < 2.0 |
| < 10% | Low | < 5.0 | < 7.0 | < 3.0 |

prepared by: Dr. Micheal J. Boldt, MD, FRCP (C)

**Figure 1.7: Framingham Risk Score Calculator**
Obtained from www.medicalrounds.com[261]

Problems in the management of cardiovascular diseases result from their multifactorial etiology.

Most individuals with one cardiovascular RF have others[248,249]. Often, patients with dyslipidemia have hypertension, and patients with diabetes also have hypertension and/or dyslipidemia. Both primary and secondary prevention of cardiovascular diseases require lifestyle changes and the aggressive management of multiple RFs, such as hypertension, dyslipidemia, and diabetes.

Accumulating clinical studies suggest an important pathophysiological role of endothelial dysfunction as assessed by impaired endothelium-dependent vasodilation. There is a proportional relationship between the degree of coronary or peripheral endothelial dysfunction with cardiovascular events[213]. Recently, endothelial dysfunction has been shown to predict future cardiovascular events in patients who have had an acute coronary syndrome[262]. In addition, a prospective study showed that impaired flow-dependent, endothelium mediated vasodilation predicted the occurrence of in-stent restenosis (due to intimal hyperplasia) in patients undergoing percutaneous coronary intervention (PCI)[263].

Bugiardini et al.[139] showed that endothelial dysfunction was associated with the development of coronary disease and persistence of chest pain after a long-term (10-year) follow-up in women with angina but angiographically normal coronary arteries. Furthermore, the number of cardiovascular RFs was correlated with increased carotid intima–media thickening in subjects with impaired endothelium dependent vasodilation but not those with good vasomotion[264], suggesting that efficient endothelial function may abrogate the deleterious impact of RFs on the progression of atherosclerosis.

*eNOS in Endothelial Dysfunction*

Emerging data show that not only is the production of NO crucial in the maintenance of vascular integrity, but the availability of NO and the local redox state of the vessel also have important roles. The clinical relevance of impaired eNOS function can be seen in studies analyzing eNOS gene polymorphisms. The CC type of the 786C/T single-nucleotide polymorphism in the promotor region of eNOS causes impaired shear stress sensitivity of eNOS expression. This type was found to be more frequent in patients with CAD[265], providing evidence that reduced shear stress–dependent eNOS activation may contribute to coronary disease.

Under normal physiological situations there is insufficient $O_2^-$ available to drive the formation of peroxynitrite, but in the context of endothelial dysfunction, the production of $O_2^-$ is increased. ECs and platelets constantly produce low levels of $O_2^-$, which are significantly increased when the cells become activated[266,267]. The enzymic origin of $O_2^-$may vary in different types of disease and could potentially involve NAD(P)H oxidases, xanthine oxidase, lipoxygenase and NOS[268]. While neutrophils (possessing high NADPH oxidase levels) are a potent source of $O_2^-$ as they infiltrate the subendothelial space, significant evidence suggests that NAD(P)H oxidases primarily present in ECs and SMCs represent a major source of $O_2^-$ in dysfunctional endothelium[269]. Accumulation of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) has also been suggested as a major mechanism causing endothelial dysfunction[270,271].

In particular, it has now been recognized that cardiovascular RFs leads to deficiency in endothelial levels of tetrahydrobiopterin (BH4), leading to eNOS uncoupling and production of

$O_2^-$ rather than NO[272]. In hypertensive rats, NO production was shown to be normal, but $O_2^-$ production was elevated and led to decreased vasodilatation[273]. Likely, the $O_2^-$ arose from NAD(P)H oxidases and cyclo-oxygenase, since the activity of both enzymes can be unregulated in hypertension[273,274].

Type I and II diabetes mellitus are associated with a reduced endothelium-dependent relaxation, although the response to exogenous NO-donors is often normal, suggesting a reduced bioavailability of NO[275-278]. Several studies have attempted to address the mechanisms leading to reduced NO bioavailability in diabetes. Vessels in patients with diabetes have an increased concentration of advanced glycation endproducts (AGEs), their corresponding receptor (RAGE), and oxidized low density lipoproteins (LDLs), which can inhibit eNOS and scavenge NO, limiting its bioavailability[279-281]. Diabetes and chronic hyperglycemia also lead to augmented $O_2^-$ production. There is mixed evidence regarding the altered expression or activity of eNOS, however. In studies where human ECs are exposed to hyperglycaemic conditions eNOS mRNA has been shown to both increase and remain unchanged depending on the incubation time[282,283]. Diabetic animal models, however, have demonstrated both increased mRNA and protein for eNOS[192], although other studies have shown that there is reduced L-citrulline formation[284]. There is also reduced accumulation of cGMP in response to acetylcholine in isolated arteries from diabetic models[285]. This suggests that the expression of NOS is slightly enhanced but its activity is compromised. A number of studies have attempted to address NO production by the measurement of the metabolic products of NO, nitrite and nitrate. In animal studies nitrite/nitrate levels are elevated[286], while in humans no differences have been found when compared to controls[287].

*Management of Endothelial Dysfunction*

One approach for management of multiple RFs in cardiovascular disease could be treatment of endothelial dysfunction (enhancing eNOS expression and/or NO bioavailability), since all these RFs are related to endothelial dysfunction. This can be realized by: eNOS overexpression, co-factors (e.g. BH4), substrate (L-arginine), increasing cGMP availability (PDE5 inhibitors) or by increasing NO bioavailability via antioxidants. Both endothelial and cardiomyocyte-specific overexpression of eNOS result in improved left ventricular function following myocardial infarction (MI)[288,289]. Several studies report a beneficial effect of L-arginine supplementation under conditions associated with endothelial dysfunction, such as hypercholesterolemia[290,291], hypertension[292], aging[293] and diabetes[294]. Oral administration of L-arginine reversed the increased monocyte adhesion to ECs in cigarette smokers[295,296] and normalized endothelium-dependent relaxation and atherosclerosis in hypercholesterolemic animals[297]. Furthermore, oral L-arginine had beneficial effects in patients with moderate to severe heart failure, probably due to inhibition of platelet aggregation and reduction in plasma endothelin levels[298]. Administration of BH4[272] and increased BH4 synthesis by targeted transgenic guanosine triphosphate–cyclohydrolase I (GTPCH I) overexpression have been shown to reduce endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice[299]. Lastly, infusion of the antioxidant, vitamin C, reversed the impaired endothelium-dependent relaxation in human hypertensive subjects, again suggestive of an important role for oxygen free radicals[300].

A number of clinical pharmaceutical agents have also been shown to improve endothelial function. The thiazolidinediones, including rosiglitazone, are a class of potent insulin-sensitizing

agents used in the treatment of type II diabetes mellitus. These medications act through peroxisome proliferators–activated receptor-γ (PPAR-γ), which regulates lipid and glucose metabolism. These drugs increase NO availability by decreasing NAD(P)H oxidase activity and thereby decreasing SO production and reducing the quenching effect on NO[301]. Insulin acts through the PI3K/Akt pathway to stimulate eNOS, and this down-regulates endothelial expression of intracellular adhesion molecule-1 (ICAM-1)[302]. HMG-CoA reductase inhibitors (statins) also act trough this pathway to improve NO bioavailability[168] and have been shown to re-couple eNOS in the context of diabetes[303]. Statin treatment improved left ventricular function and survival following MI in wild-type but not in eNOS-deficient mice, further suggesting an important role of eNOS for left ventricular function and survival following MI[304]. Angiotensin II can increase generation of reactive oxygen species and oppose the local action of NO. Angiotensin converting enzyme inhibitors (ACE-I) may be vasoprotective by decreasing this SO production, increasing NO availability and decreasing ADMA concentrations[305,306].

## 1.6 eNOS and Neovascularization

Neovascularization includes vasculogenesis, angiogenesis and arteriogenesis, which often work simulataneously to increase perfusion in a tissue. Blood vessels in the embryo form through vasculogenesis, *in situ* differentiation of undifferentiated precursor cells (angioblasts) to ECs that assemble into a vascular network [307]. The term angiogenesis was first used to describe the growth of endothelial sprouts from preexisting postcapillary venules now generally denotes the growth and remodeling process of the primitive network into a complex network. This involves the enlargement of venules, which sprout or become divided by pillars of periendothelial cells

(intussusception) or by transendothelial cell bridges, which then split into individual capillaries[47]. New vessels in the adult arise mainly through angiogenesis rather than vasculogenesis. Arteriogenesis refers to the expansion and maturation of small vessels, usually collateral branches, into arterioles. This involves SMC recruitment and differentiation, as well as remodeling of the entire vessel wall[47].

In the study of angiogenesis, VEGF has received the most attention as a therapeutic agent as it has long been considered to be the main modulator of angiogenesis. Several pre-clinical and clinical studies have concentrated on the administration of VEGF for increasing tissue perfusion, with several positive results[308-318]. However, NO is involved in VEGF signaling, with actions downstream of VEGF that induce EC proliferation and migration[318-321]. In fact, work with NOS antagonists has shown that eNOS is critical in VEGF-stimulated angiogenesis[321-323]. Despite the presence of ischemia and VEGF induction, $eNOS^{-/-}$ mice have defects in angiogenesis, arteriogenesis and functional blood flow reserve[324,325]. Hypercholesterolemic pigs have a reduced response to exogenous VEGF administration, with reduced eNOS activity and angiogenesis in response to ischemia[326]. The authors suggest that the administration of NO may be necessary to restore the angiogenic response in the setting of hypercholesterolemia. In addition, not only does NO act downstream of VEGF, but when administered exogenously or endogenously, NO induces VEGF expression in vascular smooth muscle cells (SMCs)[327,328]. This implies that the provision of NO may be an ideal means for the promotion of angiogenesis as a signal activating growth factor[327-329]. Advantages of this approach include the direct stimulation of the cGMP-mediated effects of NO and the stimulation of VEGF-mediated pathways.

In order for angiogenesis to occur, proliferating ECs must migrate beneath the basement membrane in order to form capillary sprouts[47,330,331]. EPCs must also migrate through the

endothelium and engraft to participate in new blood vessel formation[45,56,65]. eNOS may be critical for EC motility and migration as NO enhances the expression of $\alpha_v\beta_3$ integrin (CD61)[332] and increased dissolution of the extracellular matrix (ECM) via the basic fibroblast growth factor-induced upregulation of urokinase-type plasminogen activator (uPA)[333]. Kawasaki et al.[149] showed that NO induces EC migration and angiogenesis, mediated by the activation of the PI3K/Akt pathway. Kevil et al.[334] also showed that ICAM-1-regulated EC motility is mediated by a NO-dependent pathway.

## 1.7 Cell Therapy for Acute Myocardial Infarction

### 1.7.1 Acute Coronary Syndrome: Etiology and Classification

Acute Coronary Syndromes (ACS) can be defined as the spectrum of myocardial ischemia through myocardial necrosis, including stable angina, unstable angina (UA) and acute myocardial infarction (AMI). Stable angina pectoris is defined as transient, episodic chest discomfort resulting from myocardial ischemia. UA can be defined using several features: development (i.e., new-onset or recurrent event), duration, provocation, pattern, and magnitude. AMI is defined as myocardial necrosis. The diagnosis of AMI requires two of the following three World Health Organization criteria: clinical history (>20 minutes of chest pain or similar symptoms consistent with ischemia), ECG changes, and/or positive myocardial serum markers. ECG changes could include both ST segment elevation (STEMI) and others, such as T wave inversion and ST segment depression (NSTEMI).

Thrombus formation is considered to be involved in all forms of ACS, including UA, NSTEMI, and STEMI. These syndromes are all initiated by endothelial damage, usually by atherosclerotic

plaque disruption, which leads to platelet aggregation and thrombus formation. Thrombotic vessel obstruction can lead to myocardial ischemia, hypoxia, acidosis, and eventually infarction. The consequences of the occlusion depend on the extent of the thrombotic process, the characteristics of the preexisting plaque, and the availability of collateral circulation.

Another important feature of ACS is vasospasm. After significant thrombotic occlusion, local mediators induce vasospasm, which further compromises blood flow. Central and sympathetic nervous system input increases as α -receptors proliferate within minutes of the occlusion. Unopposed α -sympathetic stimulation can result in more coronary vasospasm. Sympathetic stimulation by endogenous hormones such as epinephrine and serotonin may also result in increased platelet aggregation and neutrophil-mediated vasoconstriction.

Further myocardial injury at the cellular level occurs during the reperfusion phase, either by spontaneous or therapeutically induced thrombolysis. Increased production of reactive oxygen species (ROS), platelet aggregation, complement activation, neutrophil activation, myocardial calcium overload (calcium paradox), and cardiomyocyte apoptosis all contribute to reperfusion injury [335-337]. Neutrophils play an important role in reperfusion injury by occluding capillary lumens, decreasing blood flow, accelerating the inflammatory response, and resulting in the production of chemoattractants, proteolytic enzymes, and ROS.

### 1.7.2 Standard Therapies for AMI

In the US, over 1 million patients per year experience an MI, with an expected mortality of ~25% in three years (2004 NHLBI Morbidity and Mortality Chartbook). Even more disturbing

is the fact that more than 5 million people suffer from heart failure and they face a mortality of ~20% per year. This is despite substantial improvements in pharmacological and interventional therapy of acute MI and other cardiac diseases over the last decades. These statistics underline the need for better therapies.

The fundamentals of the treatment of patients with AMI are: (1) increase myocardial oxygen supply through supplemental inhaled oxygen and reperfusion therapy, which restores coronary blood flow; (2) use β-adrenergic blockade to decrease the force of myocardial contraction and therefore oxygen demand; (3) increase metabolic substrate availability to the myocardium through nitroglycerin, morphine, and reperfusion strategies and; (4) prevent re-occlusion of the coronary artery through inhibition of platelet aggregation and thrombus formation through the use of antiplatelet agents such as aspirin and antithrombins, including heparin.

Both in-hospital and out-of-hospital pharmacologic therapies for ACS continue to evolve through further knowledge obtained from clinical trials, but to date centre around the following agents: (i) aspirin (acetylsalicylic acid); (ii) clopidogrel; (iii) GP IIb/IIIa inhibitors; (iv) unfractionated heparin (UFH) and low molecular weight heparin (LMWH); (v) β-adrenoceptor antagonists (β-blockers); (vii) nitrates; (viii) angiotensin-converting enzyme (ACE) inhibitors; (ix) statins (HMG-CoA-reductase inhibitors); and (x) fibrinolytic agents.

*Reperfusion Therapies*

Several studies have looked at the potential for fibrinolysis as the initial reperfusion therapy for STEMI, in which intravascular clots are dissolved by the action of plasmin on fibrin. Fibrinolytic agents have been compared in a number of head-to-head studies with percutaneous transluminal coronary angioplasty (PTCA). In a substudy of the GUSTO-IIb trial, 1138 patients with STEMI

were randomly assigned to fibrinolysis with tPA versus PTCA. Results from the trial indicate that primary angioplasty was associated with a lower composite outcome of mortality, nonfatal reinfarction rate, and nonfatal stroke in the 30 days after enrollment compared with tPA (9.6% vs 13.6%, respectively; $p = 0.033$)[338].

Keeley et al.[339] performed a meta-analysis of the effects of primary angioplasty versus fibrinolysis for STEMI from 23 randomized trials involving 7739 patients. Eight trials used streptokinase while the remaining 15 trials used tPA. Overall, compared with thrombolytic therapy, patients assigned to primary PTCA were less likely to die (7% vs 9%; $p = 0.0002$), have a nonfatal reinfarction (3% vs 7%; $p<0.0001$), or have a combined endpoint of death, nonfatal reinfarction, and stroke (8% vs 14%; $p < 0.0001$). These outcomes were significantly decreased both in the short-term and long-term. Major bleeding was the only endpoint that was at a higher risk in the PTCA group compared with fibrinolysis. The results seen with primary PTCA remained more favorable than those seen with thrombolytic therapy during long-term follow-up, and were independent of both the type of thrombolytic agent used, and whether or not the patient was transferred for primary PTCA.

The benefit of reperfusion therapy for lowering mortality in patients with ACS has been established, whether it be fibrinolysis or primary PCI. However, there is great variation in the type of reperfusion given and determinations as to which patients are candidates. Furthermore, epidemiologic studies suggest that some patients who appear to be eligible for reperfusion therapy do not receive it. Consequently, Eagle et al.[340]investigated the current practices of reperfusion therapy for STEMI from data extracted retrospectively from GRACE.[341] Of the 9251 patients enrolled, 1763 presented within 12 hours of symptoms of STEMI. Of this group,

approximately 27% did not undergo reperfusion therapy; this group comprised of high-risk patients such as elderly patients (>=75 years of age), patients without complaints of chest pain, those with a history of diabetes mellitus, congestive heart failure, MI, or coronary artery bypass surgery. Even more surprisingly, among patients with congestive heart failure, 60% did not receive reperfusion therapy despite presenting within 12 hours of symptom onset and being diagnosed with STEMI. A similar percentage of patients were also denied reperfusion therapy who presented with anginal equivalent symptoms and STEMI. Clearly, a large gap continues to exist in the number of eligible patients for reperfusion therapy and the treatment modality these patients actually receive.

Both experimental observations and clinical trials support the concept that the beneficial effect of reperfusion is a function of the length of time between symptom onset and treatment. Early patency resulting in myocardial salvage is the key benefit of thrombolytic therapy. The angiographic substudy of 2431 GUSTO (Global Utilization of Streptokinase & t-PA for Occluded Coronary Arteries) showed that preserved left ventricular function and associated significantly lower mortality at both the 24-hour and the 30-day end points are related to angiographic patency at 90 minutes[305,306]. Thrombolytic therapy of patients with AMI has significantly greater benefit for those treated within the first or second hours than for those treated later. The MITI trial demonstrates that the mortality rate among patients treated within 70 minutes is 1.3% compared with 8.7% in those treated later[342]. Conversely, there appears to be a modest but significant benefit for patients treated between 6 and 12 hours after the onset of symptoms than if they were not treated.

It is now well established that substantial delays occur between symptom onset and hospital-based initiation of thrombolytic therapy. From 26% to 44% of patients having an AMI delay

more than 4 hours before seeking medical care. There are often delays associated with transport to the hospital. Further delays occur between the patient's arrival at the hospital and administration of the thrombolytic agent[343-345]. These data support the following conclusions: (1) time is critical to an optimal outcome for patients with AMI; (2) major trials of thrombolytic therapy document that maximum treatment benefit occurs during the first 1 to 2 hours following symptom onset; and (3) a small percentage of patients with AMI receive treatment within 1 hour of onset of symptoms.

The current paradigm in the reperfusion therapy in patients with ST-segment elevation AMI, whether by thrombolysis, primary PCI or rescue PCI, is that the benefit is confined to the first 12 hours after the symptom onset (Figure 1.7)[346]. The benefit of reperfusion therapy beyond 12 h from the onset of symptoms is considered to be minimal and largely not attributable to myocardial salvage. This concept has been echoed by the current guidelines that favour acute reperfusion therapy in patients with ST-segment elevation acute MI within the first 12 h from the symptom onset[346-349]. The current clinical practice however, witnesses that a high proportion of patients with acute MI present beyond this time limit. It has been estimated that from 8.5 to 40% of patients with acute MI present late after symptom onset, being no longer eligible for thrombolysis[347-349]. In order to provide some improvement to these patients, it is important to develop effective adjunctive therapies that are effective when administered later after symptom onset. Since it represents a regenerative therapy rather than a rescue strategy, cell therapy is being investigated as a potential approach to improve healing and restore cardiac function even several days after the acute MI.

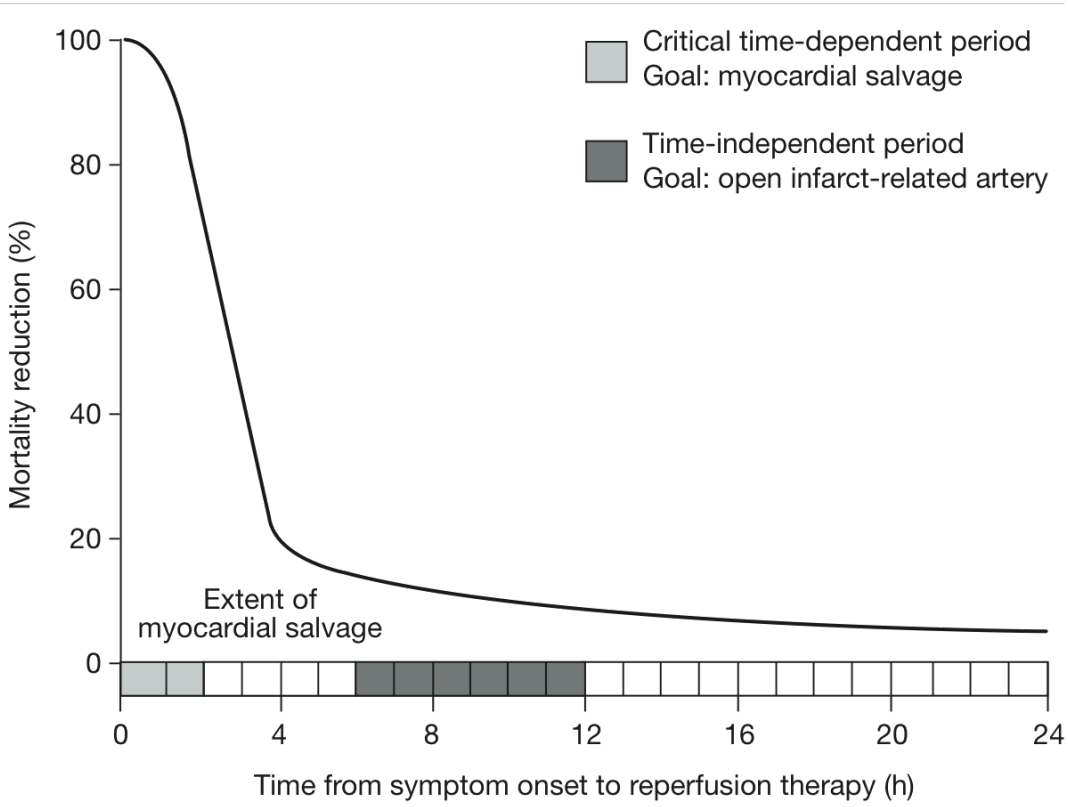


**Figure 1.8: Mortality reduction related to the duration of MI symptoms before reperfusion.** Obtained with permission from Elmariah et al.[346]

## 1.8 Clinical Trials of Cell Therapy Post-Acute Myocardial Infarction

### 1.8.1 Trials Employing Autologous Cell Delivery Alone

To date, 11 completed trials (7 of which were randomized) have been reported, which represents experience with over 600 patients receiving BM-MNCs or circulating EPCs (Table 1.4). These trials have used various cell populations, various cell manufacturing and delivery protocols as well as different endpoint measurements, making direct comparison of these trials very difficult. In most trials, patients underwent angioplasty with stenting to reperfuse the ischemic myocardium, followed by early infusion of cells into the infarct-related coronary artery. Most

studies have used unselected BM-MNCs, although a few studies, such as those of Stamm et al.[350,351] attempt to isolate EPCs using surface markers, CD133 and CD34, respectively. The Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) clinical study also tested the efficacy of EPCs derived from peripheral blood and reported results similar to BM-derived cells[352].

**Table 1.4: Completed Trials Employing BM-MNCs or EPCs following Acute MI**

| Study | Design | n* | Cell Type | Route | Days Post MI | Outcome |
|---|---|---|---|---|---|---|
| ***Non-Randomized Trials*** | | | | | | |
| Strauer et al. [353] | Primarily Safety | 10 | BM-MNCs | IC | 8±2 | Stroke volume↑; infarct size↓; wall motion↑; perfusion↑ |
| TOPCARE-AMI [352,354] | Primarily Safety | 59 | BM-MNCs, PB-EPCs | IC | 4.9±1.5 | EF↑; remodeling↓;Infarct size↓; perfusion↑; |
| Fernandez-Aviles et al. [86] | Primarily Safety | 20 | BM-MNCs | IC | 13.5±5.5 | EF↑; Wall motion↑; ESV↓ |
| Bartunek et al. [355] | Primarily Safety | 19 | $CD133^+$ BM-MNCs | IC | 11.6±1.4 | EF↑; perfusion↑; myocardial viability↑ |
| ***Randomized Controlled Trials (RCTs)*** | | | | | | |
| Ge et al. [356] | Randomized | 10 | BM-MNCs | IC | <1 | EF↑; perfusion↑ |
| Li et al. [357] | Randomized | 35 | PB-EPCs | IC | 7±5 | EF↑; Wall motion↑ |
| BOOST [358,359] | Randomized | 30 | BM-MNCs | IC | 4.8±1.3 | EF↑ (6 months only) |
| Leuven[360] | Randomized, double-blind | 33 | BM-MNCs | IC | <1 | No increase in LV EF; ↓ infarct size; ↑ regional systolic function |
| REPAIR-AMI [361,362] | Randomized, double-blind | 102 | BM-MNCs | IC | 4.3±1.3 | EF↑; greater benefit in <49% EF and >5 days after MI |
| ASTAMI [363] | Randomized | 50 | BM-MNCs | IC | 6.0±1.3 | No benefit |
| FINCELL[364] | Randomized, double-blind | 39 | BM-MNCs | IC | <4 | EF↑ |

* n = number of patients receiving cells.
Abbreviations: IC, intracoronary PB, peripheral blood; BM-MNCs, bone marrow-mononuclear cells, LV EF, left ventricular ejection fraction; ESV, end-systolic volume; MI, myocardial infarction

Although not designed or powered for efficacy, most of the earlier trials suggested that intracoronary (IC) injection of unselected BMCs or EPCs enhanced regional[86,353] or global[352,359] LV function post-MI. In recent years the results of larger, randomized controlled trials have become available. The Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute

Myocardial Infarction (REPAIR-AMI) clinical trial was the first, and still the largest, randomized controlled trial (RCT; double blind) to show efficacy of BM-MNCs in the post-MI setting[361]. This 204 patient study (102 receiving cells) achieved its primary endpoint of improvement in global EF at four months, measured by LV angiography, although the actual improvement was rather modest, with only a 2.5% difference between the cell and placebo groups[361]. Recently, the FINCELL study showed similar results at 6 months[364]. In contrast, two other randomized controlled trials failed to show significant improvement in LV function. The ASTAMI trial was randomized but not blinded and showed no benefit in LVEF measured by MRI[363]. However, this trial used LymphoPrep for cell isolation, as opposed to Ficoll gradient centrifugation that has been mostly used to isolate MNCs in other studies, likely resulting in collection of a different subpopulation of cells. Moreover, the cells were stored overnight in non-buffered saline, which has been shown to adversely affect cell viability[365]. The Leuven study (Belgium), which involved delivery of cells much earlier (within 24 hours) than the other trials, also failed to achieve its primary endpoint but achieved secondary endpoints (i.e. infarct size)[360]. Despite these negative trials, several meta analyses have now been published, all of which support the delivery of EPCs and BM-MNCs into the infarct-related artery, with a 3% mean improvement in global LVEF[366-369]. Figure 1.9 summarizes the results of LVEF improvement from Abdel-Latif et al.[369].

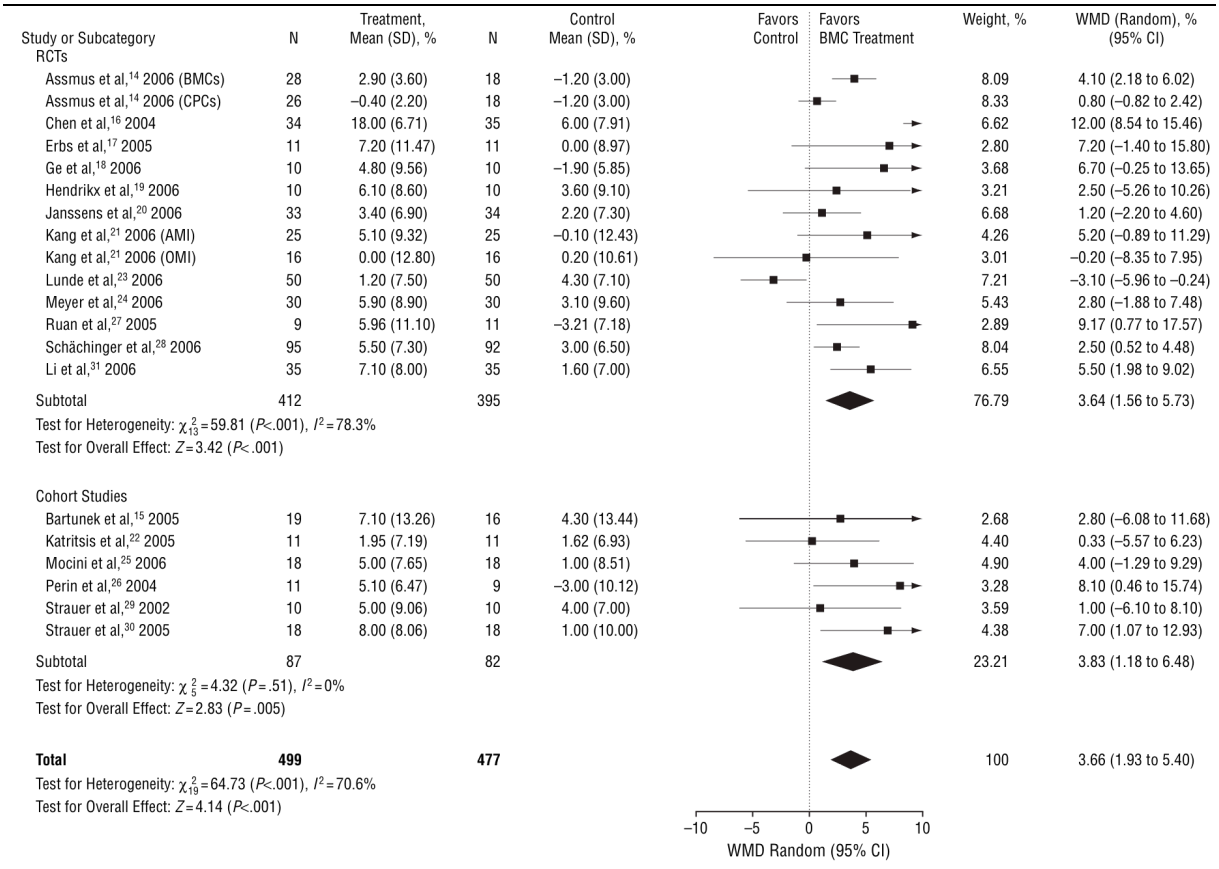


**Figure 1.9: Meta Analysis of Cell Therapy Trials for Acute MI.**
The forest plot shows the adjusted difference in mean (with 95% confidence intervals) of LVEF between cell delivery and control. Delivery of cells resulted in an increase of 3.66% (95% CI 1.93% to 5.40%) favouring cell therapy, which was statistically significant. Obtained with permission from Abdel-Latif et al.[369].

Completed trials, and in particular REPAIR-AMI, have provided important information needed to design more effective trials in the future. Sub-group analysis of REPAIR-AMI revealed that all the improvement could be attributed to patients with EF <49% at randomization, i.e. the patients with more severe LV dysfunction[361]. The finding that patients with smaller infarcts gain less from cell therapy is not surprising, since there is less room for improvement for patients with EF greater than 50% post-MI[370]. This may help explain the relatively modest effects of cell therapy seen in other trials, which have enrolled patients with a mean EF in excess of 50%.

REPAIR-AMI also showed that all the benefit was attributable to patients who received therapy 5 days after infarction, with no improvement seen in patients given cells between 2 and 4 days.

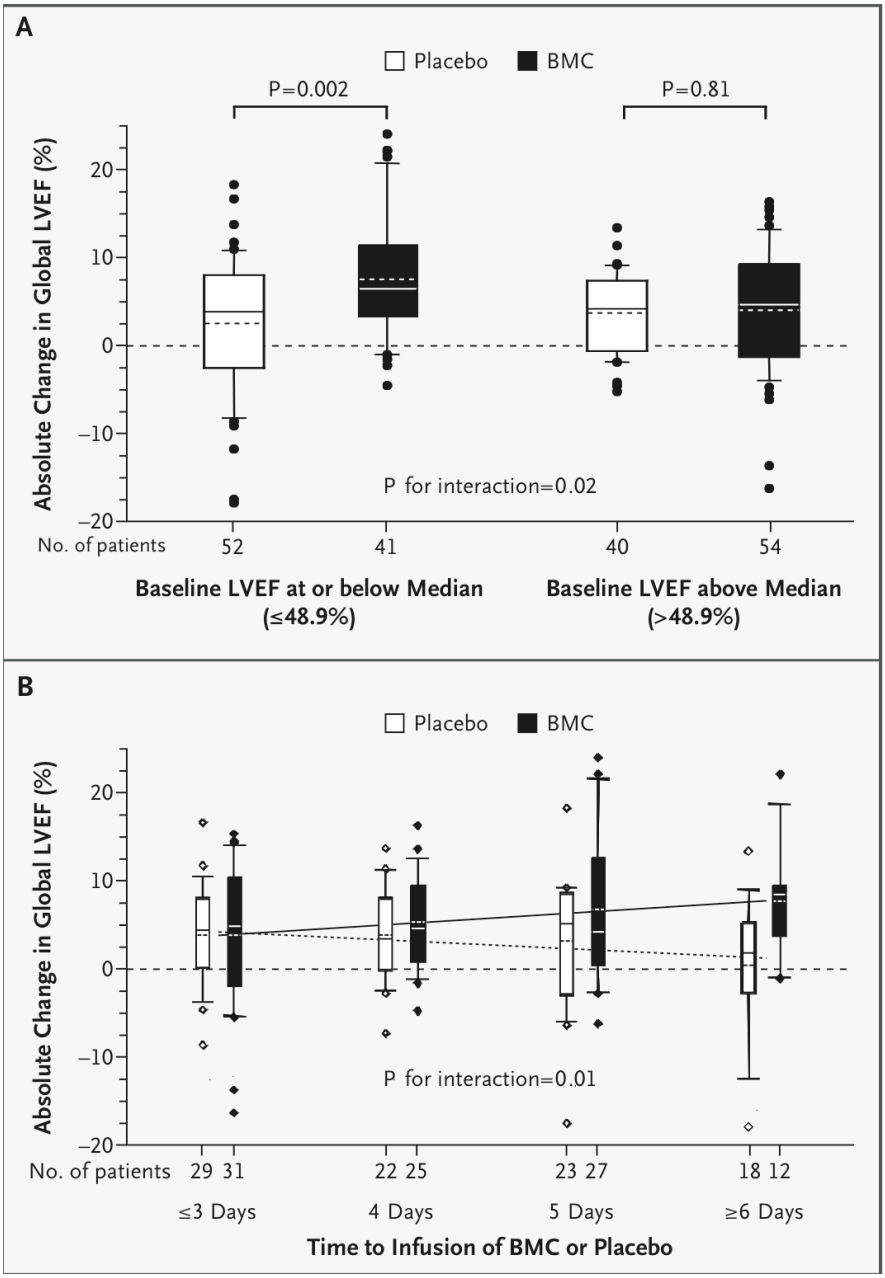


**Figure 1.10: Subgroup Analysis from REPAIR-AMI**
Panel A shows the finding in REPAIR-AMI that in patients with LVEF below 49%, those receiving BMCs had an absolute improvement of 5.0% compared to those receiving placebo. In contrast, for the patients with LVEF >49%, the absoutle diference was only 0.3%. Panel B shows the degree of LVEF improvement seen in patients receiving cells early or late after symptom onset. There was a progressive improvement of the effect of BMC delivery. In fact, no benefit was seen in patients receiving cells ≤ 4 days after symptom onset. Obtained with permission from Schachinger et al.[361]

Timing of cell delivery may have contributed to the lack of benefit seen in the Leuven study, which administered cells within 24 hours of the infarction[360]. This is supported by work in rats, showing a critical dependence of the timing of cell injection post MI, with a peak benefit seen at 7 days[27]. Currently, a large, pivotal trial enrolling over 1400 patients in being planned by the Frankfurt group (Dr. Andreas Zeiher, private communication), which will incorporate these design features (timing and EF at enrolment) and will be able to more reliably answer the question of whether cell therapy post-MI is a feasible strategy to be used routinely in the clinic.

### 1.8.2 Trials Utilizing Pharmacological Cell Mobilization

If effective, administration of mobilizing agents, the most common being G-CSF, could eliminate the need for cell harvest and interventional administration, making it a very attractive approach. Despite these potential advantages, G-CSF also mobilizes inflammatory cells and it is thus possible that mobilization could accelerate atherosclerosis and lead to plaque rupture or other complications[109-111,371]. A number of trials have been completed utilizing G-CSF treatment to mobilize EPCs from the BM, summarized in table 1.5. Although early phase I trials were encouraging, results from recently completed RCTs have generally shown no improvement in myocardial function following G-CSF administration, despite an increase in circulating EPC levels. Moreover, a recent meta analysis of all completed trials shows that, while it is considered safe (which alleviates earlier concerns), G-CSF treatment does not improve myocardial function any more than does placebo administration post-MI[372]. In this way, simple mobilization of EPCs is not sufficient to lead to improvement in myocardial function and it may be necessary to either

inject the cells into the infarct area (either through the vasculature or directly) or increase their homing into this area.

**Table 1.5: Summary of Trials Employing G-CSF Mobilization**

| Trial | Design | Time of Tx (post-MI) | n* | G-CSF Dose (μg/kg) | Tx Duration (days) | Outcome |
|---|---|---|---|---|---|---|
| FIRSTLINE-AMI [114,373] | Randomized, non-blinded | Immed. | 25 | 10 | 6 | ↑ LVEF; ↓ LVED remodeling; ↑ $^{18}$FDG uptake and metabolic activity (PET) in G-CSF group |
| REVIVAL-2 [374,375] | Randomized, double blinded | 5 days | 56 | 10 | 5 | No change in infarct size, EDV, ESV, EF or restenosis rate |
| RECOVER [376] | Randomized, double blinded | < 2 days | 6<br>6 | 5<br>10 | 5 | LVEF greatest in placebo group (no benefit from G-CSF) |
| STEMMI [108] | Randomized, double-blinded | 1-2 days | 39 | 10 | 6 | No difference in LVEF |
| G-CSF STEMI[377] | Randomized, double-blinded | 1-2 days | 23 | 10 | 5 | No difference compared to placebo. No difference in AE. |
| Takano et al.[378] | Randomized, placebo-controlled | <1 day | 18 | 2.5 | 5 | No direct comparison to placebo for myocardial function. No difference in AE. |
| Valgimigli et al. [379] | Randomized placebo-controlled | 2-3 days | 10 | 5 | 4 | No difference in LVEF, coronary late loss. No difference in AE. |

* Denotes number of patients receiving G-CSF. Abbreviations: AE-adverse events, LVEF-left ventricular ejection fraction, LVED-Left ventricular end-diastolic, PET-positron emission tomography, EDV-end-diastolic volume, ESV-end-systolic volume, EF-ejection fraction

### 1.8.3 Conclusion: Cell Therapy Trials

Cell therapy for cardiac regeneration post myocardial infarction is a novel but as yet, an unproven treatment strategy. This innovative strategy holds tremendous promise since, unlike classical pharmacological treatments, the use of regenerative cells could potentially restore functional tissue in regions that otherwise would form only scar. The REPAIR-AMI and FINCELL investigators were able to show a modest but significant improvement in LVEF[361,364]. The other randomized trials were negative, although it has been discussed that their protocols were problematic. The ASTAMI[363] trial used a sub-optimal cell processing protocol[365] and the

Leuven trial[360] likely administered cells too early after infarction. It remains to be seen whether the modest benefits seen in REPAIR-AMI and FINCELL represent reproducible findings and if so, whether a more effective cell therapy strategy could result in more robust improvement. Nevertheless, if the upcoming pivotal trial (Zeiher, personal communication) enrolling 1400 patients confirms a LVEF improvement, this strategy is likely to be adopted clinically. Cell therapy is an adjunctive therapy and, combined with reperfusion and medical management, might represent a clinically significant modality. This is particularly true for patients presenting late after symptom onset, who benefit very little from reperfusion strategies. Moreover, although G-CSF administration alone has been shown to be ineffective in clinical trials, there may be a role for combined use of EPC mobilization and direct delivery, which has shown encouraging results[357,380]. As has been the case with many prominent therapies, some of the best lessons have been learned from negative trials and it is through these lessons that one can select the right patient population, optimal timing of delivery as well as the most useful endpoints.

## 1.9 Endothelial Progenitor Cell Dysfunction

The translation of cell-based approaches developed in otherwise healthy animals to therapeutic strategies is not straightforward as most patients suffering from acute MI have a history of multiple CAD RFs (diabetes, advanced age, smoking, hypertension and hypercholesterolemia) which have been shown to impact on the number and function of circulating EPCs[36]. Clinical reports have also shown that patients with CAD and/or various RFs also show reduced numbers and function of circulating EPCs[34,68,381-384]. A clinical report by Vasa et al. showed that in patients with ischemic heart disease, there is an inverse correlation between the number of

cardiovascular RFs and the number and migratory activity of EPCs[383]. More recently it has been shown that EPCs derived from older individuals[34], and type II diabetics without diagnosed heart disease[381], have impaired survival, proliferation, migration and reduced incorporation into vascular structures. In addition, BM-MNCs harvested from patients with ischemic cardiomyopathy have a profoundly reduced potential for neovascularization[68], suggesting that the dysfunction is not restricted to circulating cells. This impaired ability of EPCs to contribute to neovascularization may reduce the efficacy of autologous cell delivery for therapeutic applications.

Results from experiments with eNOS knockout mice ($eNOS^{-/-}$) have established that the NO pathway plays a critical role in EPC-mediated endothelial maintenance and neovascularization[385-387]. Crucial to the proper function of eNOS is its complex post-translational regulation, involving $Ca^{2+}$-Calmodulin, tetrahydrobyopterin (BH4), Akt (protein kinase B; PKB) and heat shock protein 90 (HSP90), amongst other proteins[144,145,388-392]. The uncoupling of eNOS leads to the production of SO instead of NO, which has deleterious intracellular effects via increased oxidative stress. In ECs, uncoupled eNOS can exacerbate endothelial dysfunction, and improved eNOS coupling has been shown to repair EC dysfunction[388,393,394]. Studies investigating eNOS coupling in EPCs have focused on diabetes. Thum et al. showed that BM-MNCs isolated from diabetic patients have uncoupled eNOS, and produce greater levels of SO than healthy subjects do[395]. Furthermore, organic nitrates (NO donors) such as pentaerythritol-trinitrate (PETriN) or isosorbide dinitrate (ISDN) increase the mobilization and function of EPCs in rats[396]. HMGCoA reductase inhibitors (statins), however, have been shown to improve EPC proliferation, migration and neovascularization capacity[397,398], partly by phosphorylating Akt[398], which itself phosphorylates and activates eNOS. It is evident that the proper activation and

regulation of eNOS is crucial for the production of the angiogenic, vasoprotective and anti-apoptotic NO[135,398,399], rather than the reactive oxygen species (ROS), SO.

Maria Grant's group (University of Florida) has shown that eNOS$^{-/-}$ mice have impaired retinal neovascularization, and that the NO pathway is critical to EPC mediated neovascularization in response to injury and in normal endothelial repair[386]. They also showed that in the context of diabetes, the deformability and resulting migration ability of EPCs is reduced and that exogenous NO treatment can recover some of their function[69]. They further characterized this effect and showed that NO (and carbon monoxide, CO) cause phosphorylation of vasodilator-stimulated phosphoproteins (VASPs), critical actin motor proteins involved in cell migration[400]. NO causes cytoskeletal alterations in the EPCs, but this effect is blunted in the presence of high levels of glucose[400]. In this way, NO was shown to be an important mediator of EPC migration; reduced NO signaling may account for the impaired neovascularization capacity of EPCs obtained from patients with diabetes and potentially, other cardiac RFs.

## 1.10 Enhanced Cell Therapy

There is mounting evidence that, for certain applications, combined cell and gene therapy may be more effective than cell therapy alone. In one study, *ex vivo* transduction of EPCs with vascular endothelial growth factor (VEGF) was shown to improve their capacity to augment new vessel formation in a hind limb ischemia model[401]. It was speculated that increased EPC survival and potential paracrine effects provided by the local secretion of VEGF were responsible. In another study, EPCs transduced *ex vivo* with the human active subunit of the telomerase reverse transcriptase (hTERT) showed a 2- to 4-fold increased capacity to augment blood flow and

capillary density[402]. hTERT antagonizes cellular aging and prevents apoptosis of various cell types. Intriguingly, hTERT may not only directly support EPC survival and proliferation but may also enhance the release of growth factors, which can then act in a paracrine manner. Further support for the effectiveness of cell based gene therapy comes from Mangi et al.[403] who showed that Akt-transduced rat mesenchymal stromal cells (MSCs) showed a dramatic improvement in infarct remodeling post-MI. Suppressing GSK3β, a key regulator in many intracellular signaling pathways, has also been shown to improve the survival and proliferation of EPCs, and lead to improved neovascularization in a mouse model of hindlimb ischemia[404]. In addition, a recent study showed that the age-related impairment in EPC function can be partially reversed by IGF-1 administration, which acts through increased eNOS activation[405].

An alternative approach to gene therapy is the use of small molecules for *ex vivo* pretreatment of cells. Statins, which activate the PI3K/Akt pathway, increase EPC number and function[304,398]. Likewise, the eNOS transcription enhancers, AVE9488, improved capacity of the intravenously infused cells to induce neovascularization and led to increased exercise capacity in a hind-limb ischemia model[406]. Another method of augmenting eNOS expression and activity was established by our group, showing that PPAR-γ agonists prevented the suppression of EPC function by C reactive protein in a NO-dependent manner[384]. As EPCs derived from diabetic patients showed high activity of p38 MAPK, the p38 inhibitor SB203580, was used and found stimulate the proliferation and increase the yield of peripheral blood EPCs. Furthermore, *ex vivo* pretreatment with SB203580 increased the neovascularization capacity of diabetic patient-derived EPC in a hind-limb ischemia model[407]. The same group showed that pharmacologic activation of exchange protein activated by cAMP 1 (Epac 1), a nucleotide exchange protein for

Rap1, which is involved in microtubule assembly and cell migration[408], induced β1- and β2-integrin-dependent migration of EPCs and improved in vivo neovascularization[409].

Improving the rate of targeted recruitment might improve homing and incorporation of cells. SDF-1 stimulates the CXCR4 receptor, which is expressed on EPCs and BMCs and acts as a chemotactic and pro migratory factor[65]. It has also been shown to retain proangiogenic cells in the target area[410] and increase the tissue recruitment of intravenously infused EPCs[51]. Recently, Stefanie Dimmeler's group (Frankfurt) applied low energy 'shock wave' to activate ischemic muscle, which upregulated the expression of SDF-1 and VEGF and increased the recruitment of infused EPCs within the tissue[411].

## 1.11 Rationale and Hypotheses

The field of progenitor cell therapy appears to be poised to transform the management of many cardiovascular diseases. In particular, the use of BM-MNCs and EPCs have shown indications of improved cardiac function post-MI, with a recent meta analysis showing a highly significant mean improvement in global ejection fraction of over 3% compared to control[369]. The overall positive results from these early trials raise two important questions. The first is whether cell therapy really works; in other words whether the results of these smaller trials will be reproducible in larger pivotal studies. A study currently being planned by a German group, led by Dr. Andreas Zeiher, will help answer this question. The second question is whether we can do better, either by selecting a more active subset of highly regenerative progenitor cells, or by enhancing cell activity prior to delivery. In the context of cardiovascular RFs, EPCs have

impaired neovascularization capacity and the bioavailability of NO has been shown to be a critical determinant of these cells' function. Patients being enrolled in these trials invariably have multiple cardiovascular RFs limiting the regenerative capacity of their cells, and improving this capacity may improve the efficacy of their delivery in patients with cardiovascular disease.

Our first hypothesis is that EPCs isolated from animal models of cardiovascular risk and humans with CAD and multiple cardiovascular RFs have severely dysfunctional neovascularization capacity. Although isolated RFs have been shown to partially impair EPC activity, cardiovascular RFs are known to act in an additive manner and most patients with cardiovascular disease have multiple RFs. We wished to assess to what degree EPCs are dysfunctional when these additive factors are present. We isolated bone marrow cells from rat models of the metabolic syndrome (obesity, hypertension, dyslipidemia and insulin resistance or diabetes) and type II diabetes mellitus, and measured their angiogenic capacity using in vitro surrogate assays, as well as in vivo using a nude mouse model of hindlimb ischemia. These results were compared to those of cells isolated from healthy rats of similar genetic background. We also isolated circulating EPCs from human patients with high cardiovascular risk, as assessed by the presence of CAD and high FRS, and compared them to EPCs from healthy subjects using similar in vitro and in vivo assays.

Our second hypothesis was that overexpression of the eNOS gene would improve the angiogenic capacity of the EPCs and would improve their regenerative capacity in models of neovascularization and acute MI. Using the sample population of patients with CAD and multiple RFs, we utilized lentiviral vectors to overexpress either eNOS or green fluorescent protein (GFP) in the EPCs, and their in vitro and in vivo neovascularization capacity was assessed. This approach was translated to a porcine model of acute MI, using the same approach

of intracoronary cell delivery as employed in clinical trials of autologous cell therapy. In this study, eNOS-transfected bone marrow cells were compared to non-transfected cells for their ability to improve LVEF and reduce infarct size, as assessed using cardiac magnetic resonance imaging (MRI).

# 2 Bone Marrow Cell Function in Rat Models of Type II Diabetes and Metabolic Syndrome

## 2.1 Introduction

EPC and bone marrow cell (BMC) dysfunction have been reported in animal models of single cardiovascular risk factors (RF)s. EPCs derived from older individuals[34] and type II diabetics[381] have impaired survival, proliferation, migration and reduced incorporation into vascular structures. Given that patients at the highest cardiovascular risk have the lowest number and poorest migratory and homing capacity of endogenous circulating EPCs[383,412], the use of autologous EPCs for neovascularization in the setting of multiple cardiovascular RFs, may prove much less effective. Although the migration capacity of EPCs in response to VEGF and SDF-1 has been correlated with their ability to stimulate neovascularization in vivo[67,413], what is critical is a thorough assessment of neovascularization capacity of EPCs in the context of multiple cardiovascular RFs, which represents the population of patients being enrolled in cell therapy trials for cardiovascular disease.

We chose to focus on two complex clinical presentations of cardiovascular risk: the metabolic syndrome and type II diabetes mellitus (DM). The World Health Organization (WHO) definition of the metabolic syndrome is the co-occurrence of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance, AND two of the following: hypertension (blood pressure ≥140/90 mmHg), dyslipidemia (elevated triglycerides [TG] and reduced high density lipoprotein cholesterol [HDL-C]), central obesity (by waist circumference or boby mass index [BMI]) and microalbuminuria[414,415]. Type II diabetes mellitus refers to the adult onset form of DM, characterized by high blood glucose due to insulin resistance and relative insulin

deficiency. Many people with Type II DM also present with obesity and dyslipidemia and represent a population of patients with cardiovascular risk that is difficult to manage[414,415].

In this study, we employed rats with metabolic disorders resembling type II diabetes (Zucker Diabetic Fatty - ZDF), or the metabolic syndrome (Obese Zucker - OZ) and hypothesized that these animals would exhibit impaired EPC mobilization and generate BMCs with reduced neovascularization capacity compared to healthy animals (Lean Zucker - LZ). Since hyperglycemia only develops after 10 weeks in the ZDF rats, we compared pre-diabetic (5-6 weeks old) and true diabetic animals (10-12 weeks old) to assess the effect, if any, of true diabetes specifically in this model. The same two age ranges were also used for OZ animals in order to have age-matched comparisons with ZDF animals.

The work in this chapter was done in collaboration with the laboratory of Dr. Howard Leong-Poi at St. Michael's Hospital. I was responsible for the use of in vivo hind limb ischemia model for the assessment of neovascularization capacity of rat-derived cells. I was also involved with the development and optimization of the in vitro migration assay, although the technical work on this and the other in vitro assays was performed by technicians in the laboratories of Dr. Leong-Poi and Dr. Kutryk.

## 2.2 Materials and Methods

*Animal Preparation*

The study protocol was approved by the Animal Care Committee at St. Michael's Hospital Research Institute, University of Toronto. Male obese Zucker (OZ, or Zucker FA/FA), Zucker diabetic fat (ZDF), and lean Zucker (LZ) rats (5-6 weeks and 10-12 weeks, total n=16 per group) were obtained (Charles River Laboratories) for use as BMC-donor animals for in vitro (n=8 per group) and in vivo (n=8 per group) studies. The OZ rat experiences chronic hyperphagia, with the ensuing development of central obesity, insulin resistance, impaired glucose tolerance, hyperlipidemia and hypertension, and thus represents an animal model of the metabolic syndrome. The ZDF rat develops hypercholesterolemia early, and hyperglycemia at 8-10 weeks of age. We chose the Zucker strain of rats due to their multiple cardiovascular RFs, as well as their similar genetic background, making comparisons between groups more feasible. Both the OZ and the ZDF show limited, if any, cardiovascular consequences, and thus represent a model of cardiovascular risk rather cardiovascular disease itself. LZ rats served as age-matched control animals. Prior to sacrifice, peripheral blood was obtained for standard biochemical assays.

*EPC and BMC Isolation and Culture*

Circulating EPCs were isolated via direct cardiac puncture, and purified according to established protocols in our lab. Briefly, cells were centrifuged in a Ficoll-Paque (BD Biosciences) gradient, washed and plated at a density of $1.5 \times 10^6$ cells/mL on fibronectin coated T-25 (Sigma) flasks and grown for 7 days in endothelial cell basal medium 2 (EBM-2; Lonza) supplemented with 5%

FBS, human VEGF-A, human FGF-2, human EGF, IGF-1, hydrocortisone, and ascorbic acid (EGM-2MV Singlequot Media Bullet Kit; Lonza).

BMCs were isolated from the femurs and tibias according to standard techniques. Bones were flushed with PBS (Sigma), and the bone marrow aspirate was washed and plated on fibronectin-coated T-75 flasks. All donor animals were sacrificed via cervical dislocation prior to cell harvesting. As with circulating EPCs, BMCs were cultured for 7 days and maintained in endothelial cell basal medium 2 (EBM-2; Lonza) supplemented with 5% FBS, human VEGF-A, human FGF-2, human EGF, IGF-1, hydrocortisone, and ascorbic acid (EGM-2MV Singlequot Media Bullet Kit; Lonza).

*Freshly Isolated Cell Characterization*

Characterization of circulating EPCs and BMCs was performed at Day 0, immediately after isolation. The number of PB- and BM-EPCs was determined using flow cytometry with EPCs being defined as VEGFR-$2^{+}$/$CD45^{dim}$. Monoclonal anti-rat antibodies against VEGFR-2 (R&D Systems) and CD45 (Beckman Coulter) were conjugated to phycoerythrin (PE) and fluorescein isothiocyanate (FITC), respectively. Corresponding isotype control antibodies were used to establish negative control gating parameters.

*In Vitro BMC Differentiation*

We tested the ability of isolated EPCs to develop an endothelial phenotype in vitro. BM-derived EPCs were cultured on fibronectin-coated dishes and maintained in EC growth factor supplemented medium (EGM-2MV), conditions that drive the cells toward an endothelial phenotype.

*In vitro BMC Apoptosis*

Apoptosis is characterized by a variety of morphological features with one of the earliest being the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane[416]. Once exposed, binding sites on PS become available for Annexin-V. The translocation of PS precedes other apoptotic processes making it an ideal assay for early apoptosis[416]. After 7-days in culture BM-derived EPCs were gently removed from the flask with 0.05% trypsin/EDTA (Cellgro) and assayed for Annexin-V (Roche) staining by flow cytometry. Propidium iodide (PI) was used as a dead cell exclusion.

*In Vitro Migration*

The chemotactic migration capacity of BMCs was measured using a Boyden chamber assay, as previously described[68,417]. Cells were serum-starved for 1 hour, detached from culture dishes, pelleted and resuspended in EBM-2 + 0.5% BSA ($5x10^5$ cells/mL). 500 μL of this cell suspension ($2.5x10^5$ cells) was placed within cell culture inserts (Becton Dickinson, 8 μm pore size) and the inserts placed within 24-well companion plates containing 500 μL of EBM-2+0.5% BSA with $VEGF_{165}$ (50 ng/mL) and SDF-1 (100 ng/mL). $VEGF_{165}$ and SDF-1 concentrations were selected based on prior studies[68,72,417,418] and validated in our laboratory. The 8 μm pore size was selected since the BMCs are approximately 12-18 μm in diameter, and must actively migrate through the smaller pores. After four hours at 37°C, the inserts were removed, and the cells bound to the underside of the membrane were fixed and stained using the DiffQuick staining kit (Sigma). The cells bound to bottom of the membrane were visualized using an inverted light microscope (Nikon Eclipse TS100). Five random fields were photographed per membrane and the number of cells per high-power field (HPF) counted in a blinded manner. We

optimized the assay for a migration time of four hours, although previous studies employed longer incubation times (i.e. 24 hours)[72]. We found the greatest degree of migration toward VEGF and SDF-1 (compared to without chemotactic agents) at four hours, which might reflect low survival when left to incubate for longer without serum (8, 12 and 24 hours).

*In Vitro Angiogenic Tubule Formation*

Cells were cultured for 10 days, trypsinized and plated on BD BioCoat Matrigel Basement Membrane coated 6-well plates (BD BioSciences) at a density of 75,000 cells per well. Cells were suspended in 2mL of medium supplemented with 100ng/mL of recombinant VEGF (R&D Systems) and grown for 24 hours. Tube formation (total length per field) was assessed *in silico* (Image J) and expressed as relative tube formation (relative to cells from 5-6 week LZ animals).

*In Vivo BMC Angiogenic Potential*

The effect of transplanted BMCs on neovascularization was assessed in an ischemic hind limb model in nude BALB/c mice (BALB/cAnNCrl-nuBR, Charles River), as described previously[404]. Briefly, a total of 56 mice underwent left femoral artery ligation and excision. At 3 days post-ligation, $5x10^5$ BMCs were suspended in 100μL of PBS and injected directly into the ischemic left hindlimb muscle over 5 injection sites (n=8 for each of 6 subgroups). Dulbecco's PBS was administered as a non-cell control (n=8). Perfusion of the ischemic and non-ischemic hind limbs was assessed by laser Doppler perfusion imaging (Moor Instruments) at day 0 (immediately post-ligation), and at 3, 7, 14, 21 and 28 days after ligation and stripping.

*Statistical Analyses*

Results of in vitro assays were analyzed using one-way ANOVA with multiple comparisons. In vivo perfusion ratios (at specific time points) were compared using the Mann-Whitney non-parametric test. In vivo time series were analyzed using the repeated measures ANOVA. All data is represented as mean±SEM. Data were considered statistically significant if $p<0.05$.

## 2.3 Results

*Baseline Biochemistry*

Serum biochemistry was normal in LZ rats (Table 2.1). OZ rats displayed elevations in total cholesterol (TC) and triglycerides (TG) at both 5-6 and 10-12 weeks of age, along with elevations in fasting insulin levels at 10-12 weeks, without elevations in FBG. Similarly, fasting FBG and TC were not significantly elevated in 5-6 week old ZDF rats, although TG levels were elevated. As expected, ZDF animals developed diabetes at 10-12 weeks, with elevations in FBG and TC, and even greater levels of TG.

**Table 2.1: Rat Baseline Biochemistry Results**

| | **LZ 5-6 wks** | **LZ 10-12 wks** | **OZ 5-6 wks** | **OZ 10-12 wks** | **ZDF 5-6 wks** | **ZDF 10-12 wks** |
|---|---|---|---|---|---|---|
| Fasting Blood Glucose (mmol/L) | 7.9 ± 1.3 | 7.9 ± 1.8 | 8.0 ± 2.9 | 7.7 ± 2.2 | 8.1 ± 2.6 | 11.2 ± 2.9 |
| Total Cholesterol (mmol/L) | 1.8 ± 0.1 | 1.5 ± 0.1 | 2.3 ± 0.1 | 2.7 ± 1.0 | 2.3 ± 0.4 | 4.6 ± 0.6 |
| Triglycerides (mmol/L) | 0.4 ± 0.2 | 0.4 ± 0.1 | 4.8 ± 0.5 | 6.1 ± 1.2 | 2.95 ± 1.2 | 8.5 ± 1.1 |
| Plasma Insulin (mmol/L) | 3.7 ± 0.6 | 5.9 ± 3.0 | Not available | 13.6 ± 3.6 | Not available | 20.1 ± 13.1 |

Data represented as Mean ± Standard Deviation (n=3 for each)

*EPC Numbers in the Peripheral Blood and Bone Marrow*

There was a marked reduction in circulating EPC number in ZDF and OZ rats, when compared to LZ animals irrespective of age (Figure 2.1A). In contrast, there was no difference in BMC number in all animal and age groups (Figure 2.1B).

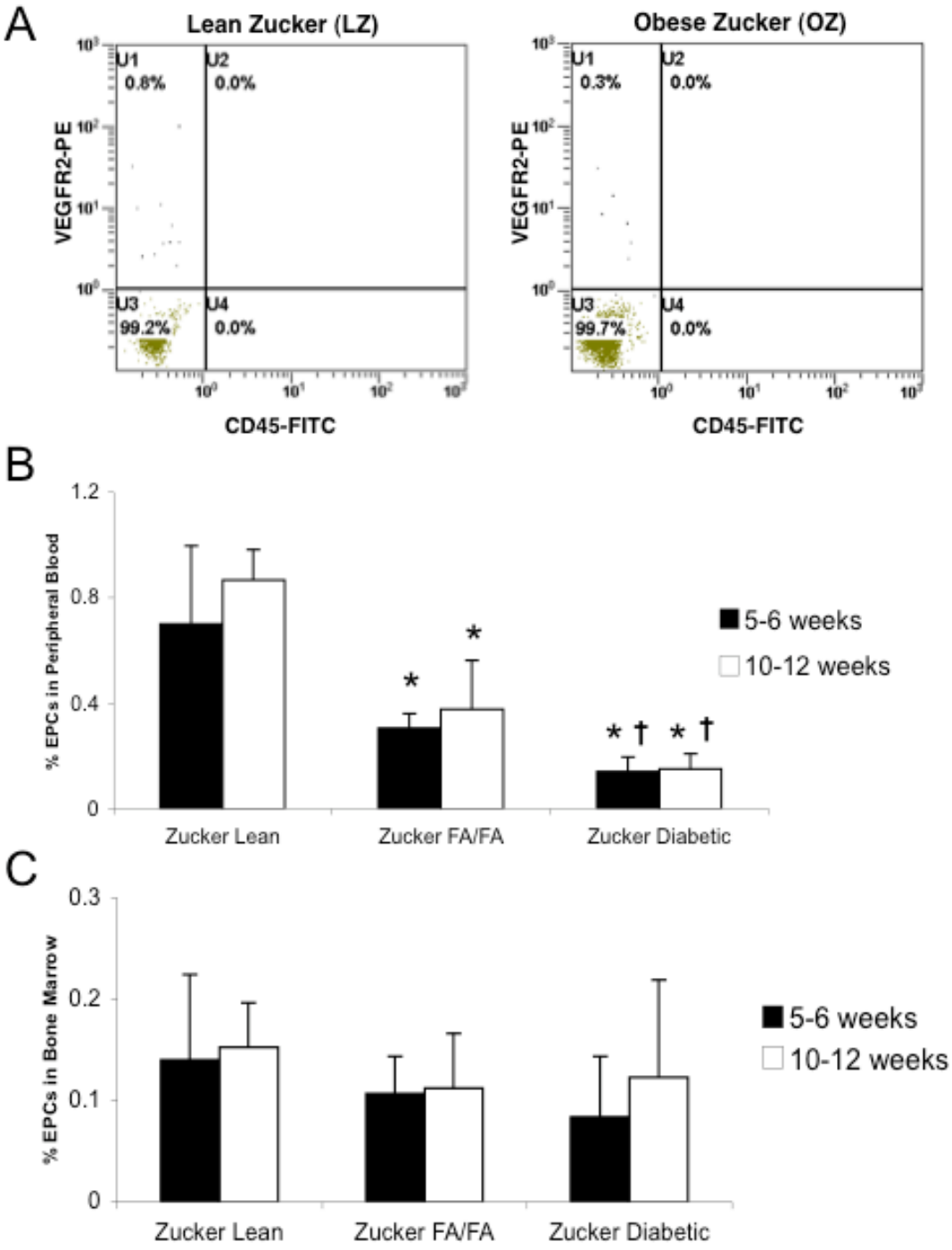


**Figure 2.1: EPC Quantification**
Number of peripheral EPCs (B) and BMCs (C) at Day 0, as determined by flow cytometry ($CD45^{dim}/VEGFR\text{-}2^{+}$). Representative flow cytometry plots (following an initial gating for $CD45^{dim}$ cells) are shown in (A). * represents $p<0.05$ vs. aged-matched LZ rats. † represents $p<0.05$ vs. aged-matched OZ rats. In this figure, "Zucker FA/FA" is used to represent obese Zucker (OZ) rats. n=8 per group.

*In Vitro BMC Basal Apoptosis Rate*

The observed basal apoptotic rate (Annexin-V positive) for BMCs isolated from LZ rats was 1% (Figure 2.2). BMCs isolated from OZ rats showed a trend toward an increased apoptotic rate, which was not significantly different from LZ animals. In comparison, BMCs from ZDF rats showed a significant increase in their apoptotic rate, with a maximal basal apoptotic rate of 8% seen in the older 10-12 week animals. There was a significantly elevated apoptotic rate in BMCs from hyperglycemic (10-12 weeks) ZDF rats compared to 5-6 week old ZDF rats.

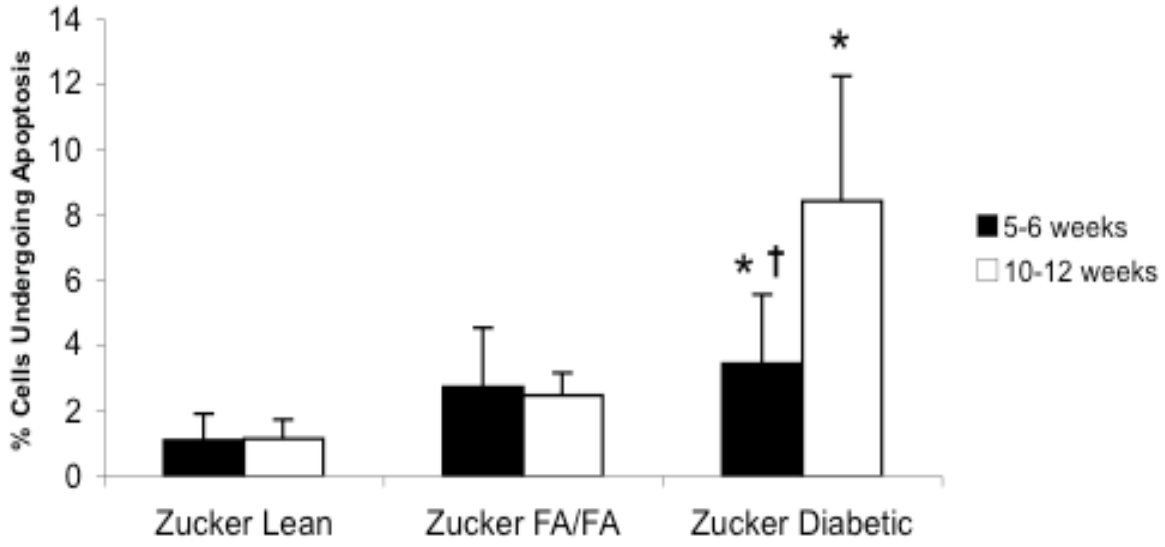


**Figure 2.2: Apoptosis Rate**
The apoptosis rate was elevated in all Zucker diabetic (ZDF) rats, and greatest in fully diabetic 10-12 week animals. * represents $p<0.05$ vs. aged-matched Zucker Lean (LZ). In this figure, "Zucker FA/FA" is used to represent obese Zucker (OZ) rats.† represents $p<0.05$ vs. 10-12 week-old ZDF animals. n=8 per group.

*In Vitro BMC Migration*

Isolated BMCs from both OZ and ZDF animals showed impaired migratory potential in response to VEGF and SDF-1 (Figure 2.3). The impairment in migration was age-independent.

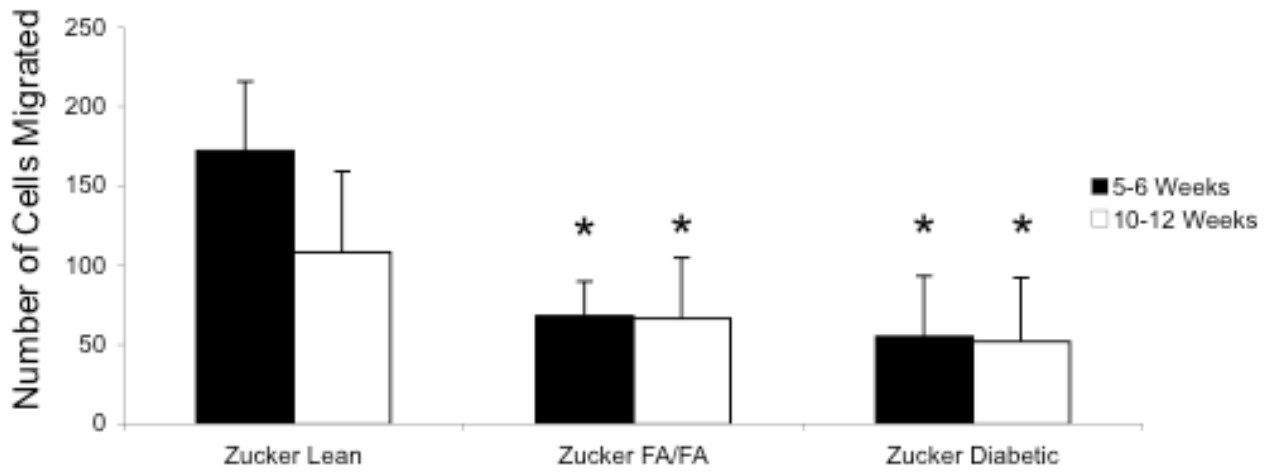


**Figure 2.3: BMC Migration**
Data is expressed as number of cells migrating to the underside of the modified Boyden chamber. * represents $p<0.05$ versus aged-matched Zucker Lean (LZ). In this figure, "Zucker FA/FA" is used to represent obese Zucker (OZ) rats. n=8 per group

*In Vitro Endothelial Networks*

Impaired endothelial network formation was seen with BM-derived BMCs from OZ animals, which was age-independent, with a 4-fold reduction in tubule formation when compare to EPCs from age-matched LZ rats (Figure 2.4). EPCs from ZDF rats also demonstrated significant impairment in network formation, however only from 10-12 week old animals, with no significant impairment in EPCs from 5-6 week old ZDF rats, when compared to LZ rats.

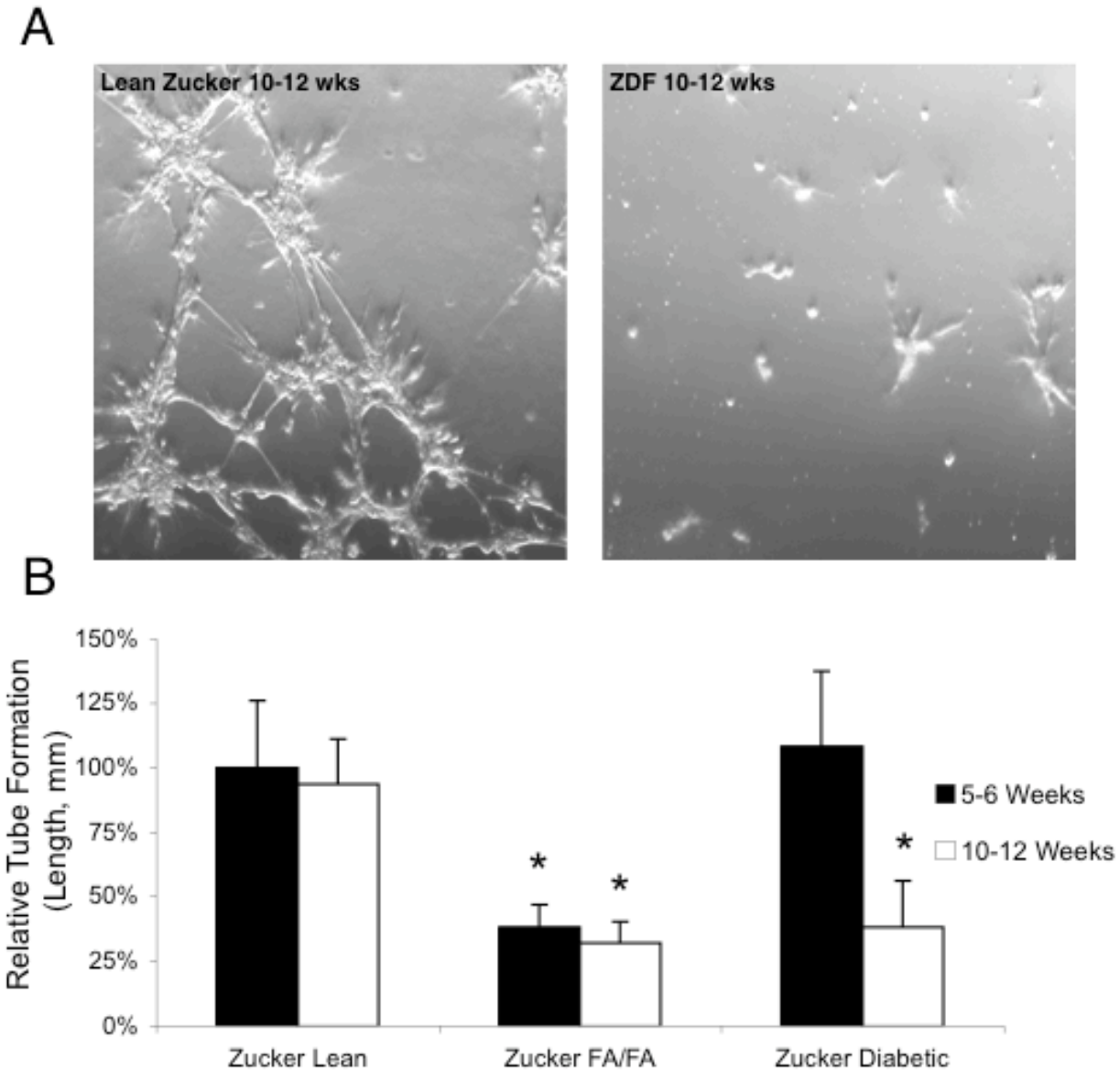


**Figure 2.4: Matrigel Tube Formation Assay**
Panel (A) shows representative images of endothelial network formation on Matrigel. Summary data is shown in panel (B). Data is presented as network length relative to cells isolated from 5-6 week Zucker Lean animals. In this figure, "Zucker FA/FA" is used to represent obese Zucker (OZ) rats. * represents $p<0.05$ vs. age-matched Zucker Lean. n=8 per group

*In Vivo Neovascularization Potential of Injected BMCs*

We tested the angiogenic potential of isolated BM-EPCs in a chronic hind limb ischemia model (Figures 2.5 and 2.6). Prior to BMC injection (3-days post-ligation) perfusion in the distal

ischemic hindlimb was ~10% of the normal contralateral leg, as measured by laser Doppler imaging. As expected, cultured BMCs from control LZ donor animals were able to restore perfusion to the ischemic hindlimb to approximately 70% of normal. There was no difference between the neovascularization response to BMCs from young (5-6 week) and old (10-12 week) LZ animals, so data for LZ rats was pooled (figures 2.6A and B). BMCs from both the 5-6 week old and 10-12 week old ZDF animals had marked impairment in their ability to restore perfusion, and were no different than saline injections (figure 2.6A). Similar results were seen from BMCs isolated from OZ animals irrespective of age (figure 2.6B).

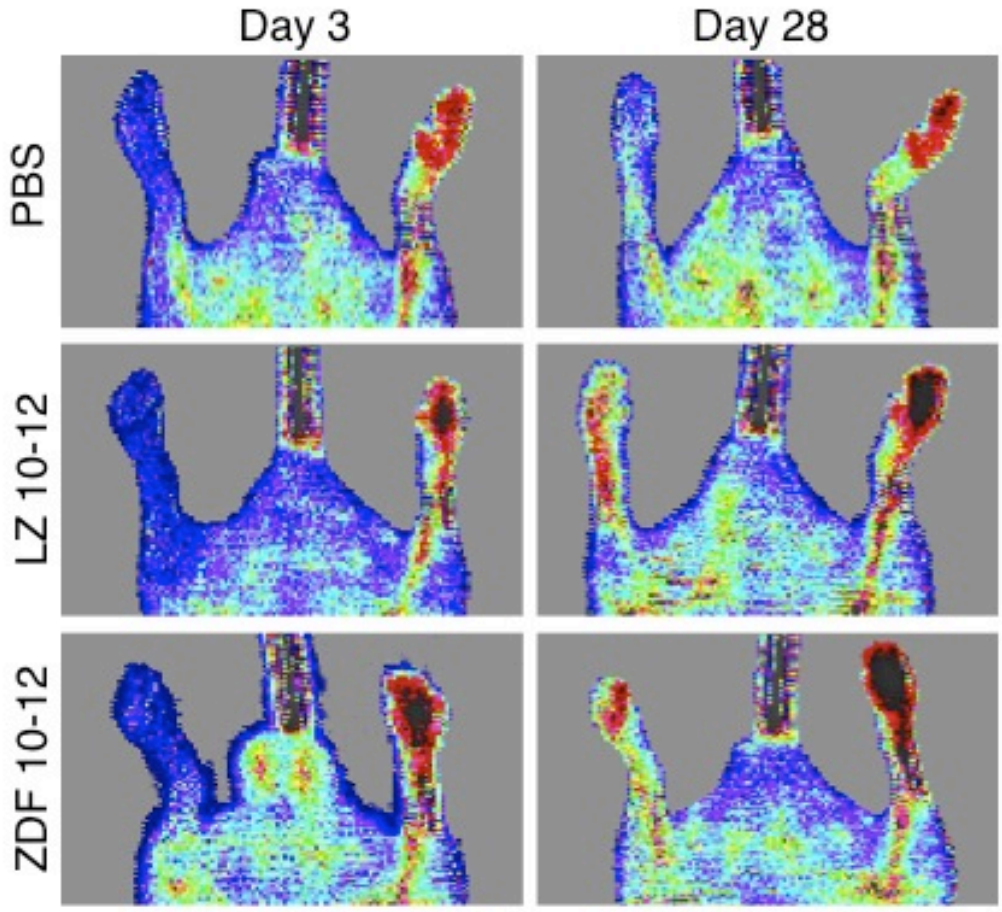


**Figure 2.5: Representative Laser Doppler Perfusion Images**
These images show the progressive reperfusion of the ischemic limb between day 3 (at the time of cell delivery) and day 28 (final time point). Results shown with PBS alone, 10-12 week-old Lean Zucker (LZ) BMCs and 10-12 week old diabetic Zucker (ZDF) BMCs show the degree of reduced benefit conferred by administration of ZDF cells.

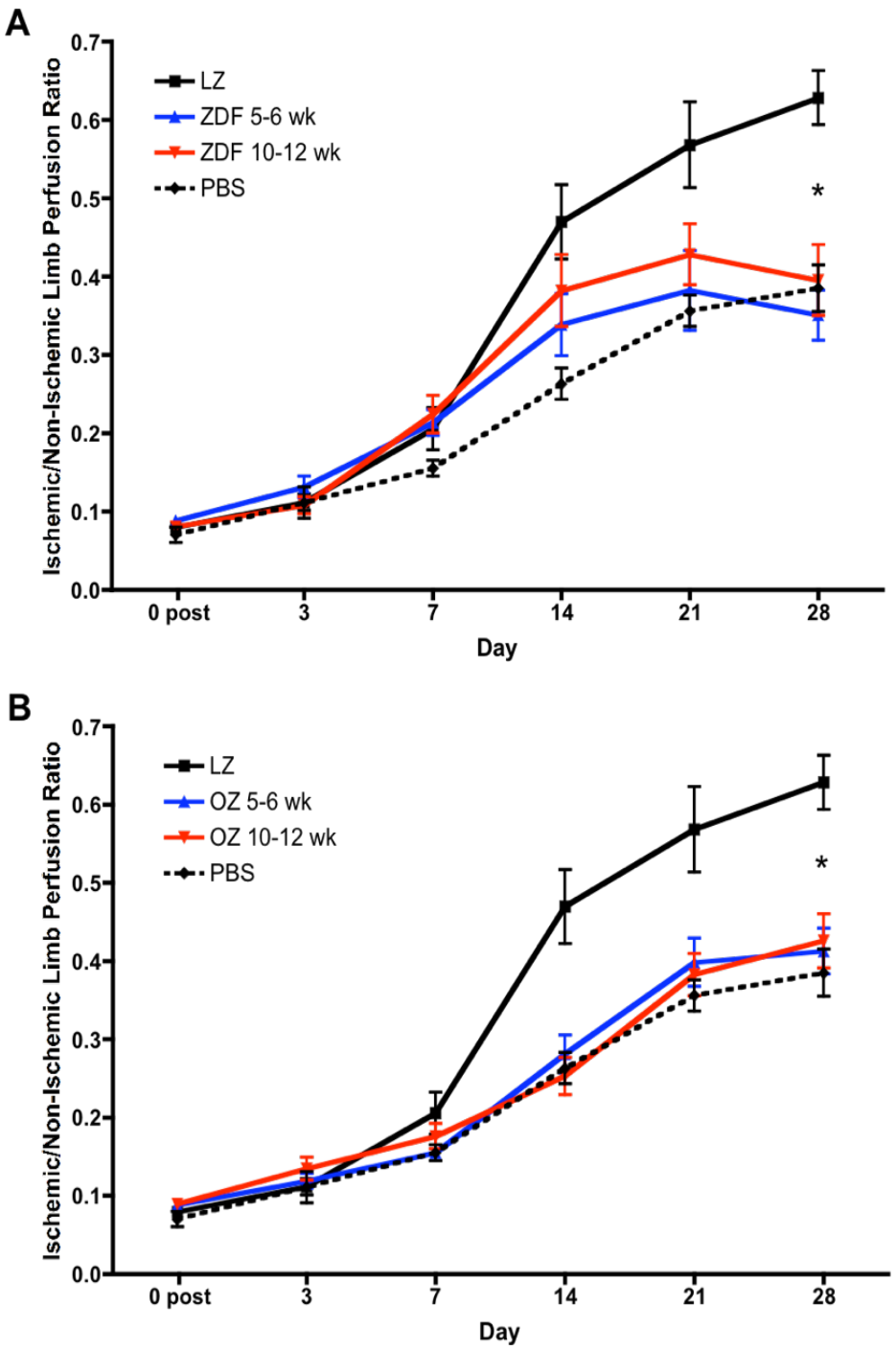


**Figure 2.6: Neovascularization Capacity of Bone Marrow Cells**
The graphs illustrate a significant impairment in the ability of the Zucker Diabetic (A) and Obese Zucker (OZ) (B) BMCs to restore perfusion of the ischemic hind limb, as measured by laser Doppler imaging. [Data from panels A and B were analyzed versus the same age-matched Zucker Lean animals and split for visualization] * represents $p<0.05$ vs. age-matched Zucker Lean (LZ). n=8 per group

## 2.4 Discussion

In the present study, we demonstrate that circulating EPCs are reduced in rat model of diabetes and the metabolic syndrome and that BMCs exhibit a marked impairment in their migratory and proliferative potential. BMCs from animals with diabetes and the metabolic syndrome also have a severely limited capacity to promote neovascularization when injected into ischemic hindlimb skeletal muscle.

There is now abundant evidence demonstrating a link between cardiovascular risk and reduced circulating numbers of EPCs[383,412,419,420]. Thus, diseases that are characterized by a clustering of multiple cardiovascular RFs would be expected to have the most significant impairment of circulating EPCs. For instance, the colony-forming and adhesion capacity of circulating EPCs are significantly lower in diabetic patients with peripheral arterial disease (PAD) versus those without PAD[421]. Furthermore, circulating EPCs isolated from patients with type II diabetes mellitus were found to have reduced proliferation compared to control subjects, and in a Matrigel assay, were 2.5 times less likely to participate in tube formation compared to control non-diabetic EPCs[381]. Similar abnormalities have been described in the metabolic syndrome[422], which is a condition characterized by central obesity, insulin sensitivity, glucose intolerance, dyslipidemia, and hypertension[247]. Recently, Fadini et al.[423] have established a link between metabolic syndrome and EPC number, demonstrating that as more components of the metabolic syndrome are present, the worse the cardiovascular RF profile and the more reduced are circulating EPC numbers.

A novel aspect of this study was the observation of changes in both bone marrow (BM) and circulating EPC numbers. Similar to previous studies, we found that animals with diabetes and the metabolic syndrome have reduced circulating EPC number, with diabetic animals having the

lowest levels. Potential explanations for this include impairment in EPC mobilization from the bone marrow or a reduced pool of marrow resident progenitor cells. Upon examining BM aspirate, we found no differences in the number of VEGFR-$2^{+}$/$CD45^{dim}$ cells between groups. This supports the hypothesis that there is a defect in the mobilization of these cells from the bone marrow into the peripheral circulation, not in BMC production. This is consistent with studies showing impaired Ser 1177 phosphorylation of eNOS in the bone marrow of diabetic mice, which leads to reduced mobilization of EPCs into the circulation[395,424]. Whether similar defects explain the reduced circulating EPC number in the metabolic syndrome is unclear and requires further study.

While many studies have examined the function of circulating EPCs in disease states, only a few have examined BMCs, despite their prevalent use in cell therapy clinical trials[46]. Hirata et al[425] studied BMCs in a rodent model of streptozotocin-induced diabetes and hindlimb ischemia. While they found no difference in the release of angiogenic factors or endothelial differentiation between BMCs from diabetic and non-diabetic rats in vitro, the recovery of perfusion to ischemic hindlimbs was significantly lower in the diabetic compared to non-diabetic animals. Similarly, Li et al[426] studied BMCs in diabetic Zucker rats. BMC characterization did not differ between diabetic and control rats, but further in vitro functional testing, including VEGF production, differentiation, and EC colony-forming potential were significantly lower in diabetic as compared to control rats. They also found that the native recovery of induced limb ischemia in the diabetic rats was also significantly worse than that in control rats.

This is also the first study to directly compare both in vitro and in vivo assessments of the angiogenic potential of BMCs themselves from animals with diabetes and the metabolic syndrome, distinct from local abnormalities in endothelial and vascular function which could

negatively impact of neovascularization in the models. By testing the in vivo neovascularization capacity of all groups of BMCs in young (under 10 weeks old) athymic mice, it was possible to isolate specific donor cell-related defects from confounding host or tissue factors. We found a similar marked impairment in restoration of perfusion recovery using BMCs from both ZDF and OZ rats, irrespective of age. The ZDF BMCs were only able to restore the ischemic/non-ischemic flow ratio to approximately 30% of the non-ischemic limb at day 28, and cells derived from the OZ rats improved the flow ratio to approximately 40%. In comparison, cells from healthy LZ rats were able to improve the flow ratio to about 70%, which was statistically significant compared to all disease groups. At most time points the diabetic and metabolic syndrome cells were no better than PBS injections. This has important clinical relevance, as the incidence of these 2 disease states in patients with CAD and PAD is increasing. Furthermore, many early studies of BMCs or EPCs for therapeutic angiogenesis were performed on a relatively healthy population. For example, in the REPAIR-AMI trial[361], only 16% of patients enrolled had diabetes, while in the BOOST trial[359] the incidence was 10%. With a higher rate of diabetes, the benefit of cell therapy alone might be even lower than the modest results obtained in these trials.

In order to examine the mechanisms behind the lack of efficacy of cell therapy, BMCs from the affected rats were assayed for their propensity to undergo apoptosis, their migration toward VEGF and SDF-1 and their ability to form endothelial networks on Matrigel (basement membrane complex). In the diabetic BMCs there was a significant increase in basal apoptosis rate (5-fold increase in diabetic 10-12 week old cells compared to those from LZ rats), which was not seen in the metabolic syndrome animals. Another novel finding of this study was the pattern of dysfunction of BMCs from normoglycemic 5-6 week old ZDF rats. While their BMCs

showed reduced differentiation and migration similar to fully diabetic hyperglycemic 10-12 week old ZDF animals, they maintained relatively normal rates of apoptosis and tubule formation. Further investigation would be required to elucidate the mechanism responsible for the observed differences between pre-diabetic and diabetic animals, but generally this suggests that hyperglycemia or insulin production may have direct effects on the ability of cells to contribute to neovascularization and reperfusion.

This study has a number of important limitations. While the in vitro work demonstrated important defects in BMC differentiation, apoptosis, endothelial tubule formation and migration responses, the underlying cellular and molecular mechanisms for these deficiencies were not investigated. In addition, the characterization of BMCs and circulating EPCs was not as rigorous as that typically performed with human EPCs, due to the unavailability of rat-specific antibodies to markers such as CD34 and CD133. Since the performance of these assays, further antibodies have become available which have allowed further characterization of the rat circulating and cultured cells.

Taken together these data demonstrate that in the context of metabolic syndrome and type II diabetes, BMCs have a marked functional impairment, which limits their ability to restore perfusion to ischemic tissue. These include a reduced ability to differentiate into mature ECs, a reduced ability to migrate toward VEGF and SDF-1, important mediators of EPC homing, and increased rates of apoptosis. These results should be replicated in human EPCs in order to validate their relevance to the design of future clinical trials of autologous cell therapy for cardiovascular disease.

# 3 Comparison of EPC Function in Low and High Risk Human Subjects

## 3.1 Introduction

Based on promising reports of progenitor cell-mediated neovascularization post-MI in young and otherwise healthy animals, several clinical studies have been performed which have administered autologous BM-MNCs (also referred to as BMCs) or circulating EPCs to patients who had suffered an acute MI. These trials have reported variable and modest improvements in myocardial function[358,359,361]. EPCs from patients with individual CAD RFs have reduced regenerative capacity. Also, chapter 2 describes our experiments in rats showing that in the context of multiple RFs, such as with metabolic syndrome and type II diabetes, EPCs lose nearly all of their ability to stimulate neovascularization. In humans, the function of EPCs obtained from subjects with CAD and multiple cardiac RFs (i.e. advanced age, diabetes, hypercholesterolemia, smoking and hypertension), which are characteristic of the post-MI population being currently enrolled in cell therapy trials, has not been explored rigorously.

This chapter reports on the effect of CAD and multiple cardiac RFs on the regenerative capacity of human EPCs both in vitro and in vivo. We isolated early EPCs from the blood of patients with CAD and multiple cardiac RFs ('high risk') as well as from young, healthy volunteers ('low risk'). As in chapter 2, we compared these cells for their ability to respond to pro-migratory chemotactic agents VEGF and SDF-1. Since EPCs are primarily thought to contribute to neovascularization via paracrine mechanisms, we also measured their production of important pro-angiogenic agents, such as VEGF, HGF, eNOS and PDGF. Finally, we delivered these cells

in the same nude mouse model of hindlimb ischemia to measure their ability to recover perfusion in the ischemic limb.

## 3.2 Material and Methods

*Patient Selection and Recruitment*

Healthy volunteers (low risk subjects, n=42) and patients with RFs with or without diagnosed CAD (high risk subjects, n=46) were enrolled. Inclusion and exclusion criteria were included in table 3.1. Low risk subjects were contacted in person and informed consent was obtained at St. Michael's Hospital, following confirmation of eligibility for inclusion. A registered nurse research coordinator performed the venipuncture from the subject for 80 mL of blood. High risk subjects were recruited from the cardiovascular surgery and cardiology departments, as well as the diabetic clinic at St. Michael's Hospital. Patients scheduled for coronary artery bypass graft (CABG) surgery or percutaneous coronary intervention (PCI) were referred by their physician either at the time of the surgical consult, in the pre-admission facility (with blood work) or pre-operatively. Patients in the diabetic clinic were referred by their physician. An investigator visited each patient and obtained informed consent. A registered nurse research coordinator performed the venipuncture. If the patient was already in the pre-surgical ward or catheterization laboratory, blood was drawn by a registered nurse. All procedures were approved by the St. Michael's Hospital research ethics board.

**Table 3.1: Subject Recruitment Criteria**

*Low Risk Subject Criteria*

Inclusion Criteria:

a) males or females, age 18-80

Exclusion Criteria:

a) pregnancy, lactation
b) Currently taking medication for diabetes or cardiovascular disease (i.e. statins, insulin, ACE inhibitor)
c) History of cardiovascular disease (CHF, AMI, diabetes, ischemic heart disease, hypertension, hypercholesterolemia, metabolic syndrome)
d) Other severe concurrent illness (Chronic renal failure, liver cirrhosis, cancer)

e) Current positive smoking status
f) Inability to provide informed consent

*High Risk Subject Criteria*

Inclusion criteria**:**

a) males or females, 18-80 years of age;
b) Documented coronary artery disease OR one or more of: hypercholesterolemia, type I diabetes, type II diabetes, hypertension, metabolic syndrome, cigarette smoking

Exclusion criteria:

a) Inability to provide informed consent

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*Isolation and Characterization of Peripheral Blood EPCs*

Peripheral blood mononuclear cells (PB-MNCs) were isolated from 80mL of blood by Ficoll gradient centrifugation (CPT Vacutainer Tubes; BD) and plated on human fibronectin-coated dishes ($0.75x10^6$ cells/$cm^2$). Cells were maintained in endothelial basal medium supplemented with 20% FBS and endothelial growth factors (EGM-2MV bullet kit; Lonza), which was replaced every 48 hours. All subsequent experiments were performed following 7 days of culture.

EPCs were characterized by staining with DiI-AcLDL and UEA-1 Lectin[27] and by flow cytometry using monoclonal conjugated antibodies: anti-CD31 (Immunotech), anti-CD14 (Beckman Coulter), pooled anti-CD34 (Beckman Coulter), anti-CD45 (Beckman Coulter) and anti-VEGF-R2/KDR (R&D Systems). Corresponding isotype control antibodies were used to establish negative control gating parameters.

*Boyden Chamber Chemotactic Migration Assay*

The chemotactic migration capacity of EPCs was measured using a Boyden chamber assay, as described previously[68,417] and in chapter 2. EPCs were serum-starved for 1 hour, detached from culture dishes, pelleted and resuspended in EBM-2 + 0.5% BSA ($5x10^5$ cells/mL). 500 μL of this cell suspension ($2.5x10^5$ cells) was placed within cell culture inserts (Becton Dickinson, 8 μm pore size) and the inserts placed within 24-well companion plates containing 500 μL of EBM-2+0.5% BSA with either $VEGF_{165}$ (50 ng/mL) or human SDF-1 (100 ng/mL). $VEGF_{165}$ and SDF-1 concentrations were selected based on prior studies[68,417,418] and validated in our laboratory. The 8 μm pore size was selected since the cells are approximately 10-14 μm in diameter, and must actively migrate through the slightly smaller pores. After four hours at 37°C, the inserts were removed, and the cells bound to the underside of the membrane were fixed and stained using the DiffQuick staining kit (Sigma). The cells bound to bottom of the membrane were visualized using an inverted light microscope (Nikon Eclipse TS100). Five random fields were photographed per membrane and the number of cells per high-power field (HPF) counted in a blinded manner.

*Fibronectin Adhesion Assay*

Cells were serum-starved for 30 minutes, pelleted, resuspended in serum-free medium ($1.2x10^5$/mL) and re-plated on fresh fibronectin-coated 4-well slides ($9x10^4$/well) for 30 minutes at 37°C. Non-adherent cells were removed with PBS and the adherent cells were counted using light microscopy. The data is expressed as the mean number of cells/HPF from 3 fields.

*Gene Expression Analysis*

RNA was isolated using the TRIzol Reagent (Invitrogen) and DNAse treated (Ambion DNA-free) to remove contaminating genomic DNA. cDNA was synthesized from 1 μg of total RNA

using the Omniscript RT (Qiagen) kit. Real-time PCR (ABI-PRISM 7900HT) was then performed using ABI SYBR green or Taqman Gene Expression Assays (for eNOS only). All SYBR green PCR primers were designed using ABI Primer Express Software (Table 1). ACTB (β-Actin) was employed for analysis of relative expression.

**Table 3.2: List of Primers Used for SYBR Green RT-PCR**

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| VEGFA | CTCTACCTCCACCATGCCAAG | AGACATCCATGAACTTCACCACTTC |
| CXCR4 | CTTACTACATTGGGATCAGCATCG | AGTGTTCTCAAACTCACACCCTTG |
| PECAM-1 | CTGCAATGTGCTGTGAATGAAG | TCCTTGTTAGCCTTCTGCTTGG |
| PDGF-B | GATCGAGATTGTGCGGAAGAAG | AGCTGCCACTGTCTCACACTTG |
| HGF | GATTGGATCAGGACCATGTGAG | TCCACGACCAGGAACAATGAC |
| β-Actin | AGCCTCGCCTTTGCCGA | CTGGTGCCTGGGGCG |

*Angiogenic Protein Secretion*

Conditioned medium was obtained following culture in serum-free medium (EBM-2) for 12 hours and stored at -80°C until used for VEGF and HGF protein quantification by ELISA (Human VEGF Quantikine ELISA Kit and Human HGF Quantikine ELISA Kit; R&D Systems). ELISA protocols were exactly as per the manufacturer's instructions.

*In Vivo Neovascularization Model*

Eight to ten-week-old male BALB/c nude mice (BALB/cAnNCrl-nuBR, Charles River) were anaesthetized (200 mg/kg Ketamine and 10 mg/kg Xylazine) and the proximal and distal ends of the left femoral artery, as well as all side branches, were ligated and the femoral artery was cut at two points to induce limb ischemia (day 0). On day 3, $5x10^5$ human EPCs or control (DPBS) were injected into the medial thigh and gastrocnemius muscles of the ischemic limb (total volume of 0.1 mL). Immediately following surgery, and on days 3, 7, 14, 21 and 28,

measurements were made using laser Doppler perfusion imaging (LDPI, Moor Instruments). The ischemic/non-ischemic limb perfusion ratio was used for analysis.

*Statistical Analyses*

Results of in vitro assays and in vivo perfusion ratios (at specific time points) were compared using the Mann-Whitney non-parametric test. In vivo time series were analyzed using the repeated measures ANOVA. All data is represented as mean±SEM. Data were considered statistically significant if $p<0.05$.

# Results

*Subject Characteristics*

Table 3.2 summarizes the demographic and medical history for the high and low risk subjects. The high risk group had a mean age of 66.2±1.2 compared to 31.5±1.6 in the low risk group, and a majority of high risk patients had diagnosed hypercholesterolemia, hypertension, diabetes and CAD. Table 3.3 summarizes the physical exam and laboratory results from all high risk subjects and 20 low risk subjects. Despite a prevalence of diagnosed hypertension and hypercholesterolemia, the systolic and diastolic blood pressures as well as total cholesterol levels were only slightly elevated in the high risk group, which suggests reasonable medical management of these RFs. Subjects in the low risk group had few, if any, cardiovascular RFs. As discussed in the introduction, the Framingham risk score (FRS) is a formula normally used to calculate the 10-year risk of developing coronary heart disease (CHD) in patients without current CHD. Although not clinically relevant for most high-risk subjects enrolled in this study (due to their current CAD), the FRS was employed as a method of correlating the additive effect of the subjects' various cardiovascular RFs with EPC migration (see below).

**Table 3.3: Subject Demographics and Clinical Presentation**

| | **High Risk** | **Low Risk** |
|---|---|---|
| **N** | 46 | 42 |
| **Age** | 66 ± 1 | 32 ± 2 |
| **Gender (% Female)** | 30 | 35 |
| **Clinically Significant CAD (%)** | 78 | 0 |
| **Diabetes Mellitus Type I or II (%)** | 72 | 0 |
| **Diagnosed/Treated Hypertension (%)** | 74 | 0 |
| **Current Smoker (%)** | 28 | 0 |
| **Diagnosed/Treated Hypercholesterolemia (%)** | 76 | 0 |
| **Statin (%)** | 36 | 0 |
| **ACE Inhibitor (%)** | 55 | 0 |
| **Angiotensin Receptor Blocker (%)** | 21 | 0 |

**Table 3.4: Subject Measured Risk Factors and Framingham Risk Score**

| | High Risk | Low Risk |
|---|---|---|
| **n** | 46 | 20 |
| **Systolic Pressure (mmHg)** | 141.6 ± 3.4 | 115.1 ± 1.6 |
| **Diastolic Pressure (mmHg)** | 78.5 ± 1.9 | 72.3 ± 1.2 |
| **Total Cholesterol (mmol/L)** | 4.83 ± 0.19 | 4.63 ± 0.23 |
| **LDL (mmol/L)** | 2.62 ± 0.21 | 2.82 ± 0.26 |
| **HDL (mmol/L)** | 1.04 ± 0.05 | 1.52 ± 0.18 |
| **Framingham Risk Score** | 23.9 ± 1.1 | 2.3 ± 0.2 |

*EPC Characterization*

Greater than 90% of cultured cells were positive for both incorporation of DiI-AcLDL and UEA-1 lectin staining (figure 3.1). Flow cytometric analysis determined that 66% of these cells were positive for KDR, 95% for CD31, 3% for CD34, 72% for CD14 and 99% for CD45 (figure 3.2).

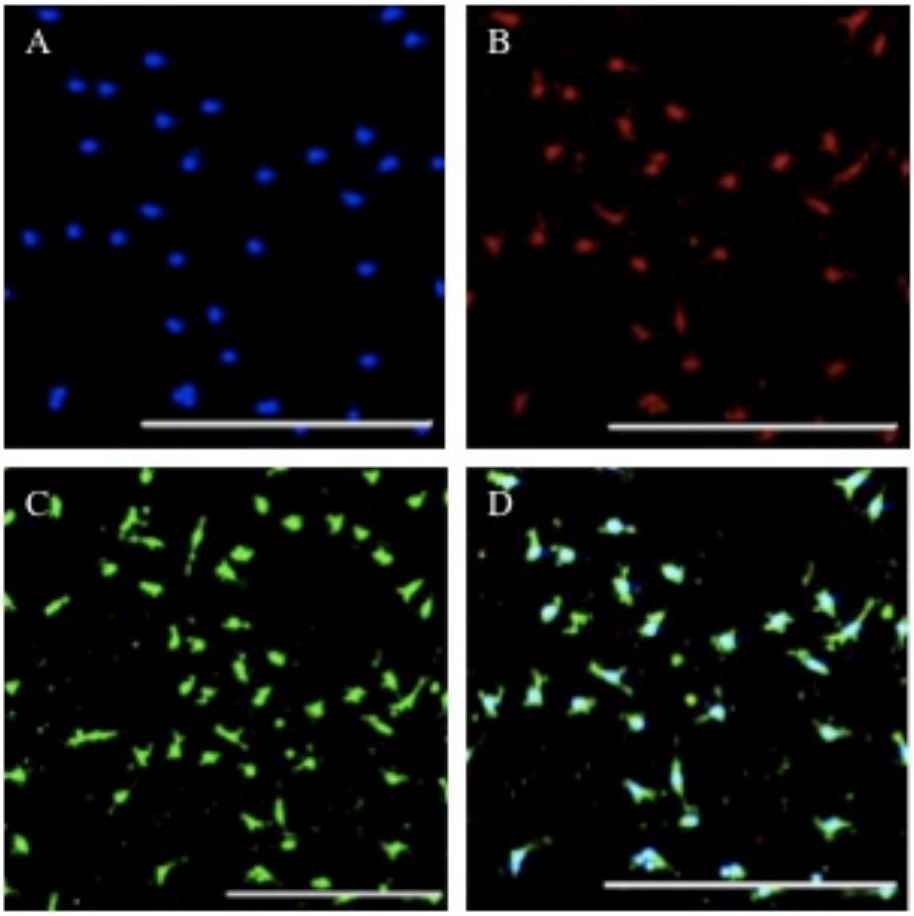


**Figure 3.1: Classical EPC Characterization**
EPC characterization in vitro was performed using confocal microscopy. All cells were identified by staining with (A) ToPro3 nuclear stain and EPCs were identified by positive co-staining with (B) 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate–labeledacetylated low-density lipoprotein (DiI-AcLDL) and (C) FITC-conjugated ulex europaeus-1 (UEA-1) lectin. The merged image (D) shows that over 90% of cells are positive for both markers. Scale bar = 200 μm.

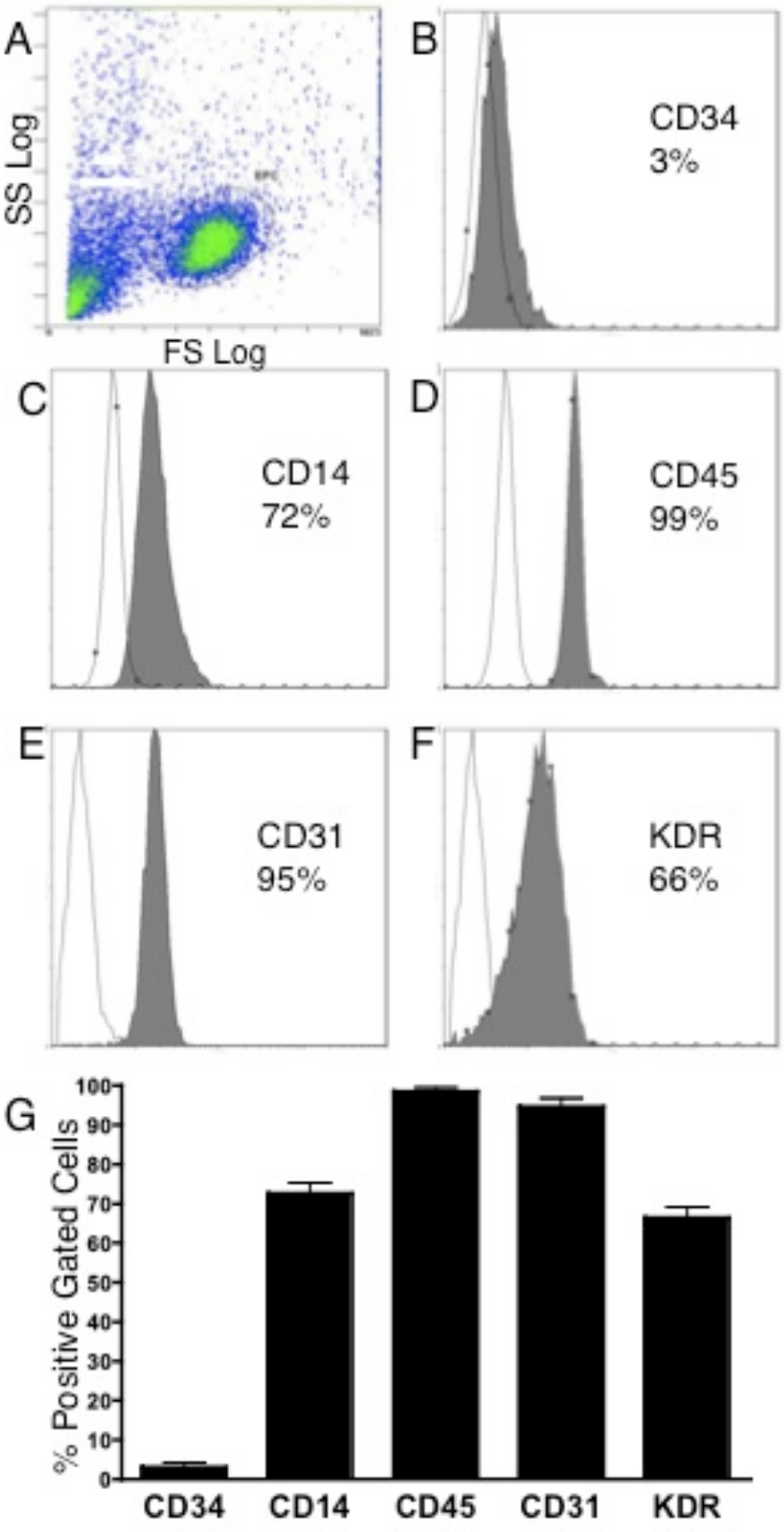


**Figure 3.2: Flow Cytometric Characterization of Cultured EPCs**
Flow cytometry was used to characterize the expression of various surface markers of day 7 cells, gated as shown (A). Monoclonal antibodies to human CD34 (B), CD14 (C), CD45 (D), CD31 (E) and KDR (F) conjugated to either FITC or PE were used; isotype-matched antibodies were used as a negative control. Summary data is also shown (G; n=3).

*Chemotactic Migration and Adhesion Assays*

Compared to low risk subjects, cells from high risk subjects exhibited reduced migration to both SDF-1 (80±11 vs. 119±13 cells/HPF; $p<0.05$) and VEGF-A (74±9 vs. 121±15 cells/HPF; $p<0.05$, figure 3.3A and C). Regression analysis demonstrated a trend toward an inverse relationship between FRS and migratory capacity (Figure 3.3B and D). In contrast, we found no difference in the ability of EPCs from low RISK and high RISK subjects to adhere to fibronectin in vitro (74±8 vs. 64±7 cells/HPF; p=NS; figure 3.4).

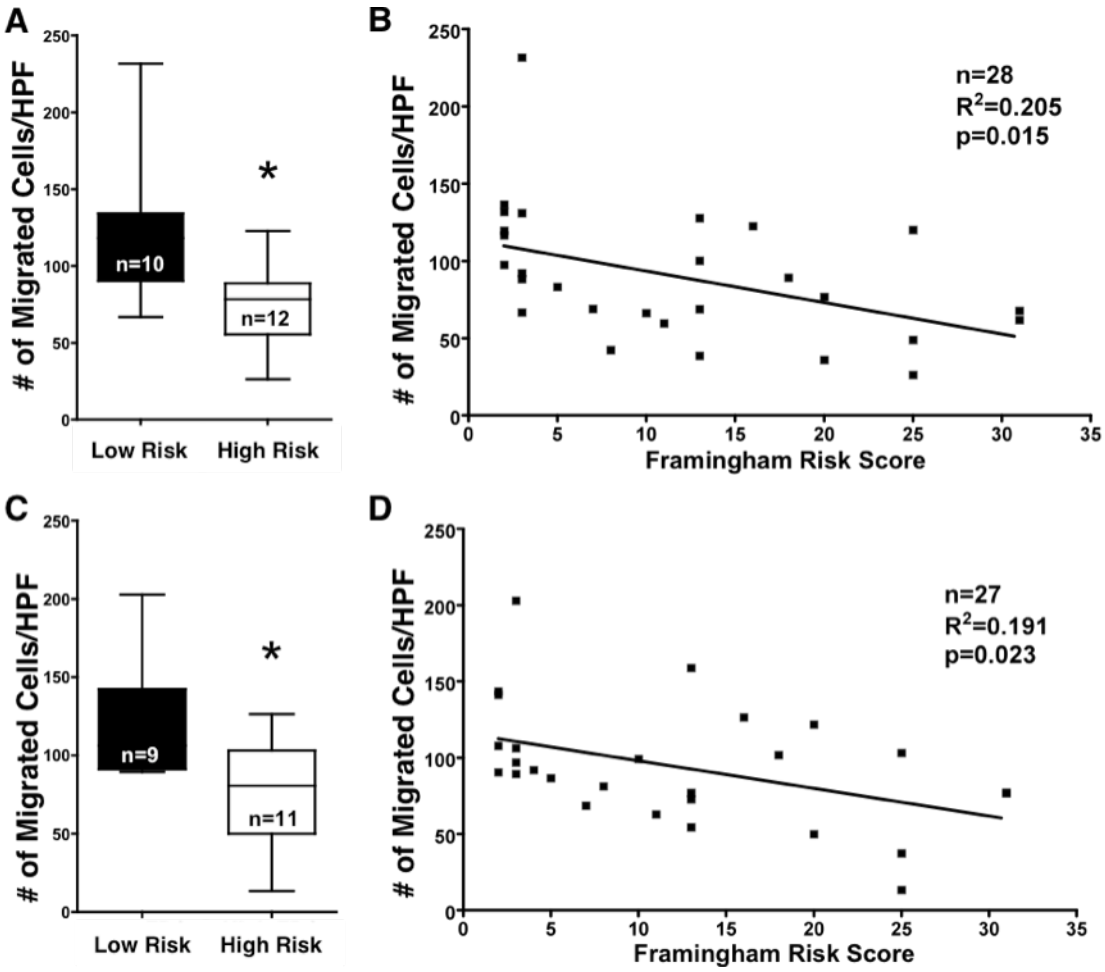


**Figure 3.3: Chemotactic Migration Capacity of Human EPCs**
A modified Boyden chamber assay was used to quantify the ability of EPCs migrate toward VEGF (A and B) or SDF-1 (C and D). EPCs from high risk subjects had reduced migration compared to cells from low RISK subjects (A; C). Using regression analysis, we also observed a significant correlation between FRS and migratory ability toward both chemotactic agents (B; D) * represents $p<0.05$

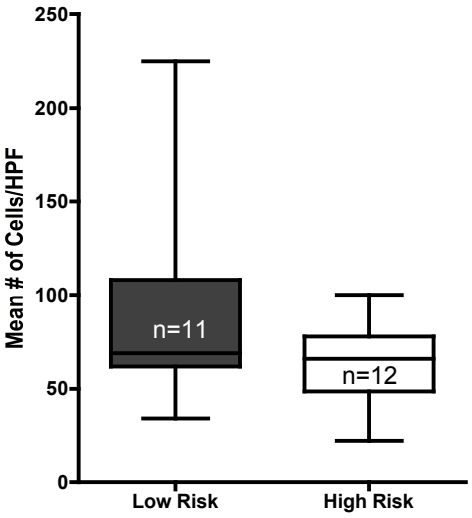


**Figure 3.4: Adhesion to Fibronectin**
EPCs were compared for their ability to adhere to a fibronectin layer. No difference was found in the number of adherent cells between low risk and high risk groups.

*Angiogenic Agent Expression Analysis*

Compared to cells from low risk subjects, we observed a significant reduction in expression of VEGFA mRNA (0.353±0.062 vs. 0.755±0.091; $p<0.01$, Figure 3.5A) and a trend toward reduced expression of eNOS (0.0258±0.0078 vs. 0.0709±0.0214; $p=0.06$) and PDGF-B (10.93±1.47 vs. 16.71±2.69; $p=0.07$) in cells obtained from high risk subjects. However, there was no significant reduction in the expression of HGF (0.108±0.045 vs. 0.281±0.145; $p=0.24$), CXCR4 (41.7±10.9 vs. 103.6±32.4; $p=0.41$) and PECAM-1 (0.53±0.09 vs. 1.09±0.27; $p=0.18$). We also found a reduced protein concentration of VEGF (36.08±4.70 vs. 52.31±4.84 pg/mL; $p<0.05$; Figure 3.5B) and HGF (0.168±0.023 vs. 0.288±0.043 pg/mL; $p<0.05$) in conditioned medium obtained from cells of high risk subjects compared to cells from low risk subjects.

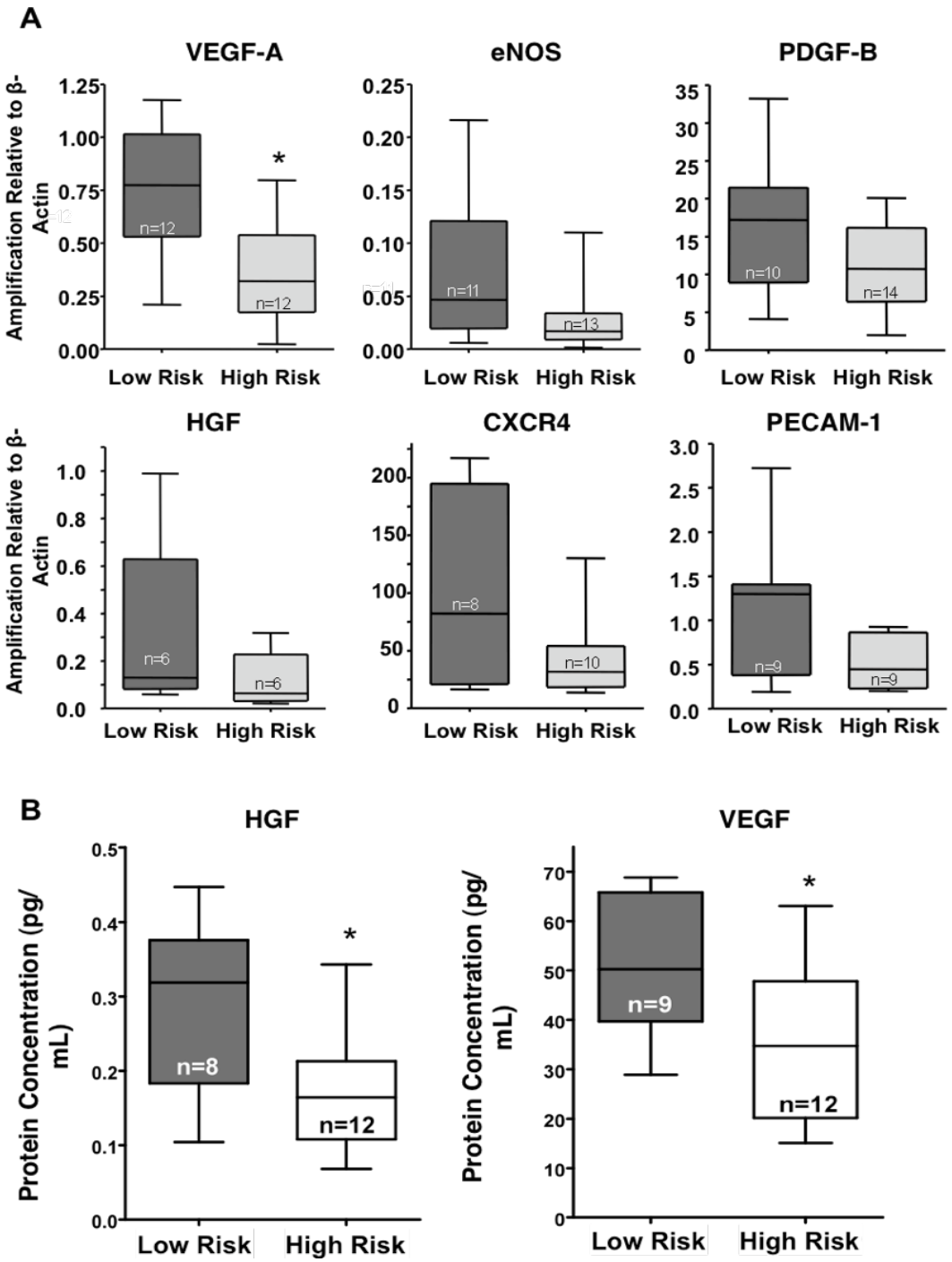


**Figure 3.5: Angiogenic Gene Expression and Protein Secretion**

Quantitative RT-PCR was performed in order to measure the expression of various genes implicated in angiogenesis or endothelial function (A). We observed a significantly lower expression of VEGF mRNA, with trends in reduced expression of eNOS (p=0.06) and PDGF-B (p=0.07). There was no significant reduction in the expression of HGF, CXCR4 and PECAM-1. β-Actin (ACTB) was used to calculate relative expression of each gene (ratio of gene vs. ACTB). Using ELISA assays (B), we also found a significantly reduced amount of VEGF and HGF protein in conditioned medium obtained from culture of high risk EPCs. * represents $p<0.05$

*In Vivo Neovascularization Model*

Compared to animals injected with DPBS (control), animals that received either high or low risk EPCs had increased limb perfusion at day 7 (Figure 3.6). Animals that received cells from low risk subjects exhibited progressive and significant improvement from 7 to 28 days, but those that received EPCs from high risk subjects showed no further resolution of limb perfusion. At day 28, hindlimb perfusion was significantly lower in the group treated with EPCs derived from patients with high vs. low risk (0.402±0.027 vs. 0.541±0.049, respectively; $p<0.05$), and using repeated measures ANOVA, there was a significant difference in the progression of perfusion throughout the time series ($p<0.05$). At day 28, there was no difference in perfusion between animals that received cells from high risk subjects compared with those administered DPBS.

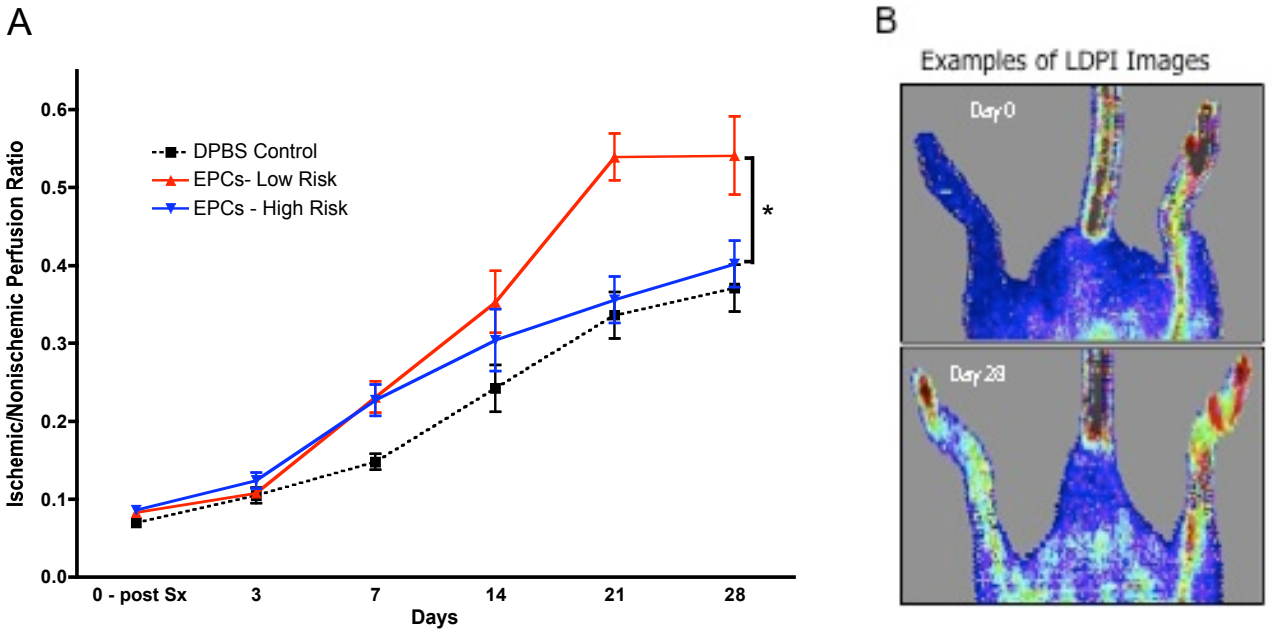


**Figure 3.6: Neovascularization Capacity of Human EPCs**
Using laser Doppler perfusion imaging, we observed a significant reduction in the ability of EPCs from high risk subjects (n=9) to stimulate neovascularization and reperfusion compared to cells from low FRS subjects (n=8). Animals administered DPBS alone (n=8) were used as a control. Statistical comparisons were made at each time point (ANOVA) as well as throughout (repeated measures ANOVA). * represents $p<0.05$ throughout the time series.

## 3.3 Discussion

In this study, EPC function was assessed in a population of patients at high risk for cardiovascular events, with multiple RFs and in most cases, known CAD, compared to a control population with few, if any, RFs. Previously it was shown that patients with CAD RFs exhibited reductions in EPC quantity and function[34,383,427-429]. Three studies have previously assessed the effect of a broad range of factors on EPC function[383,412,430], but these employed single or controversial assays to quantify this effect. Vasa et al[383] correlated subjects' FRS only to EPC number and VEGF-induced migration, and Hill et al.[412] used the colony forming units (EC-CFU) assay exclusively to perform a similar correlation. Liguori et al.[430] used a combination of these techniques. The CFU assay has recently been criticized for its ability to quantify EPC functionality, which limits the relevance of these studies. George et al.[431] showed that CFU numbers did not correlate with CD34/KDR or CD34/CD133/KDR cells. In addition, CFU-ECs and their progeny have been shown to display hematopoietic-restricted and macrophage-specific cellular proteins, possess limited hematopoietic colony forming activity and function as macrophages to ingest bacteria[432]. Our study is the first to include in vitro functional assays, paracrine growth factor quantification as well as assessment of in vivo neovascularization to describe the function of EPCs derived from subjects with advanced age and multiple additional RFs. Since this group of patients is representative of those being included in trials of autologous cell therapy for cardiovascular diseases, such a full and rigorous characterization of EPC dysfunction has direct clinical relevance.

The ability of EPCs to migrate towards chemotactic stimuli has been suggested to be the best surrogate measure of their regenerative capacity in vivo[68]. We observed a significant reduction in the ability of cells from high risk subjects to migrate toward VEGF-A and SDF-1, important

chemotactic agents in EPC homing into ischemic tissues[48,65,101]. In particular, SDF-1 and its receptor, CXCR4, have been shown to be crucial in mediating EPC neovascularization in vivo[51]. However, we observed no significant reduction in expression of CXCR4 in the cells from high risk subjects, and therefore, other mechanisms must account for the reduced migration toward SDF-1 in these cells. Apart from its obvious role in the response to SDF-1, CXCR4 signaling has also been implicated in responses to VEGFR-2[72], suggesting its potential impact on migration toward both SDF-1 and VEGF.

The hind limb ischemia model is probably the best model to assess the efficacy of interventions to promote or inhibit neovascularization in the context of chronic ischemia. Following femoral artery ligation and excision, ischemia triggers positive remodeling of collateral arteries (arteriogenesis) from the internal iliac artery and sprouting of neovessels from existing arterioles (angiogenesis)[47]. The result is a spontaneous progressive improvement in limb perfusion, which is variable depending on the extent of the arterial excision, the species or background strain used and host factors (i.e. age, cardiac RFs, etc.), and the recovery of perfusion can be quantified sequentially using laser Doppler perfusion imaging[41].

Contrary to the greatly improved perfusion obtained with EPCs from healthy subjects, EPCs from high risk subjects did not significantly improve reperfusion in the ischemic limbs of healthy, young mice. Although both cell treatments led to a similar early increase in perfusion, the benefit was not sustained in animals receiving cells from the high risk group, whereas there was progressive improvement in mice receiving low risk cells beyond 14 days, which reached a plateau after day 21. The early increase in perfusion in response to both cell groups may be due to a transient inflammatory response, as previously reported in nude mouse xenotransplantation studies[433,434], with true neovascularization only apparent at later time points. However, our

results clearly show that EPCs from high risk subjects stimulate this process less efficiently than do those from low risk subjects. This is consistent with reports showing reduced angiogenic capacity of EPCs from patients with diabetes[429] or those with advanced age[34]. Therefore, together these results strongly suggest that EPCs from those at risk for cardiovascular events have very little capacity to stimulate neovascularization.

While initially it was thought that EPCs contribute to neovascularization by their incorporation into neovessels, in situ proliferation and transdifferentiation in ECs[16,20,435], it is now thought that much of the benefit of EPC delivery is through paracrine mechanisms, such as the secretion of pro-angiogenic cytokines that stimulate resident EC proliferation and migration, as well as the migration of pericytes and the recruitment of stem and progenitor cells[50,53]. Of note, Iba et al.[53] have shown that the dominant cytokine involved in neovascularization following PB-MNC administration is VEGF, a potent angiogenic agent highly expressed in these cells[13].

We found a significant reduction in VEGF mRNA expression as well as VEGF and HGF protein secretion, consistent with the idea that VEGF is a crucial cytokine for the paracrine activity of EPCs at the site of ischemia, in addition to its role in migration and homing of EPCs. There were also consistent trends toward decreased expression in the other angiogenic cytokines in the high risk subject cells, as well, that of eNOS. These results suggest that reduced paracrine angiogenic signaling may be in part responsible for EPC dysfunction in patients with high risk. NO itself is a potent autocrine and paracrine agent, stimulating angiogenesis and proliferation of ECs[436,437]. NO has also been shown to stimulate VEGF secretion thus stimulating angiogenesis directly and indirectly[327,438]. CXCR4 signaling has also been shown to be regulated by NO in human CD34+ cells[73], suggesting that depressed eNOS activity may reduce homing capacity, which we observed using an in vitro migration assay.

Studies using human early EPCs involve limitations often not experienced with other species or cell types. Since the number of MNCs isolated from each subject is highly variable, as is the survival following 7 days in vitro, we could only perform certain experiments using a subset of the enrolled subjects. For example, in order to conduct migration experiments in triplicate, $1.5x10^6$ cells were needed per subject, which was not always possible. Furthermore, the exact cell count could only be determined immediately prior to the beginning of the migration assay, following detachment from the flasks. Similarly, for gene expression studies, it was often impossible to isolate sufficient high quality mRNA from certain cell samples. Amplification often led to low purity product (as assessed by SYBR green dissociation curves) as well. This type of sampling bias is the cause of the unequal sample size in certain assays. Nevertheless, all assays were carried out with sufficient statistical power.

Taken together, these data indicate that circulating EPCs of patients at risk for cardiovascular events have a reduced ability to stimulate neovascularization, which may in part be due to impaired migration and secretion of angiogenic growth factors. The reduced function of EPCs obtained from patients with multiple RFs is present despite indications that these patients are generally well treated for their cardiac RFs. However, this study does not differentiate the effect of modifiable RFs, such as hypercholesterolemia and smoking, from the effect of advanced age in the function of EPCs, the latter potentially being a strong determinant of the observed dysfunction. Nevertheless, taken together, these data provide valuable insight into the activity of progenitor cells from a population of patients that is representative of those being enrolled in post MI cell therapy trials. These findings strongly suggest that strategies aimed at the improvement of growth factor expression and regenerative capacity of autologous cells ex vivo

are warranted prior to the initiation of large-scale cell therapy trials in the setting of acute MI or other cardiovascular diseases.

# 4 Endothelial NOS Overexpression in EPCs from High Risk Subjects

## 4.1 Introduction

In chapter 3 and previously, it was shown that early growth EPCs obtained from subjects with CAD and/or many cardiac RFs exhibited severely reduced functional capacity, which might compromise their therapeutic use. The regenerative activity of EPCs has been linked to the bioavailability of nitric oxide (NO), which is primarily produced by eNOS in the vasculature. In $eNOS^{-/-}$ mice, EPC mobilization was reduced and the EPCs themselves had impaired ability to stimulate angiogenesis in vivo[385]. Furthermore, EPCs from animals lacking phosphoinositide 3-kinase gamma (PI3K), which activates eNOS, were deficient in proliferation, survival, integration into endothelial networks, and migration[439]. eNOS activity has been shown to be impaired in EPCs from diabetic patients, with reduced NO production and consequent impaired function[395].

In the context of endothelial dysfunction, approaches to improve NO bioavailability have resulted in return to normal endothelial function. Several studies report a beneficial effect of L-arginine supplementation in the context of hypercholesterolemia[290,291], hypertension[292], aging[293] and diabetes[294]. Oral administration of L-arginine reduced monocyte adhesion to ECs in cigarette smokers[295,296] and normalized endothelium-dependent relaxation and atherosclerosis in hypercholesterolemic animals[297]. Both endothelial and cardiomyocyte-specific overexpression of eNOS result in improved left ventricular function following acute MI[288,289]. Administration of BH4[272] and increased BH4 synthesis by targeted transgenic guanosine triphosphate–

cyclohydrolase I (GTPCH I) overexpression have been shown to reduce endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice[299]. Lastly, infusion of the antioxidant, vitamin C, reversed the impaired endothelium-dependent relaxation in human hypertensive subjects, again suggestive of an important role for oxygen free radicals[300]. Therefore, the improvement of NO bioavailability can counteract the atherogenic effects of various deleterious physiological conditions.

Several studies have implicated reduced NO bioavailability in EPC dysfunction. EPCs obtained from eNOS knockout mice ($eNOS^{-/-}$) have impaired ability to maintain endothelium and stimulate neovascularization[385-387]. Thum et al. showed that BM-MNCs isolated from diabetic patients, which have impaired angiogenic capacity, have uncoupled eNOS, and produce greater levels of SO than healthy subjects do[395]. Furthermore, organic nitrates (NO donors) such as pentaerythritol-trinitrate (PETriN) or isosorbide dinitrate (ISDN) increase the mobilization and function of EPCs in rats[396]. Statins have also been shown to improve EPC proliferation, migration and neovascularization capacity[397,398], partly by phosphorylating Akt[398], which itself phosphorylates and activates eNOS. Our own studies in human subjects, discussed in chapter 3, show that EPCs isolated from patients with CAD and multiple RFs have reduced expression of angiogenic growth factors (VEGF and HGF), and show a trend toward reduced eNOS expression as well. These cells have a marked impairment in neovascularization capacity when administered in a nude mouse model of hindlimb ischemia.

Since NO bioavailability is thought to contribute to EPC dysfunction, we hypothesize that eNOS overexpression and increased NO bioavailability in human EPCs will improve their therapeutic capacity, and may reverse the dysfunction previously seen in these cells. If confirmed, overexpression of eNOS may represent an effective strategy to overcome the deleterious

influence of host factors and enhance the efficacy of autologous cell therapy for patients with cardiovascular diseases.

## 4.2 Materials and Methods

*Patient Selection and Recruitment*

We enrolled 130 patients admitted to the cardiology department with CAD and multiple CAD RFs (advanced age, diabetes, hypertension, hypercholesterolemia, smoking). All procedures were approved of by the St. Michael's Hospital research ethics board. Blood samples were obtained by venipuncture or through the arterial sheath at the time of cardiac catheterization.

*Isolation and Characterization of Peripheral Blood Mononuclear Cells*

Peripheral blood mononuclear cells (PB-MNCs) were isolated from 80mL of blood by Ficoll gradient centrifugation (CPT Vacutainer Tubes; BD)[440] and plated at a density of $0.75x10^6$ cells/$cm^2$ on human fibronectin-coated dishes. EPCs were maintained in endothelial cell basal medium (EBM-2) supplemented with 20% FBS and several endothelial growth factors (EGM-2MV SingleQuots; Lonza), which was replaced every 48 hours for 7-8 days. As shown in chapter 3, these "early EPCS" have been characterized by our group and others as being >90% double-positive for incorporation of DiI-AcLDL and staining for UEA-1 lectin, as well as for markers such as CD31, VEGFR-2, CD14, CD45 and a minority CD34[155, 174].

*eNOS Overexpression*

Following 3 days in culture, cells were exposed to lentivirus (MOI=3) containing the coding sequence of eNOS (Lentigen Corporation) or GFP (sham) for 5 hours in regular medium supplemented with 8μg/mL polybrene. An MOI of 3 was chosen based on the optimization of eNOS mRNA expression and cell survival. Cells were maintained in full medium for another 5 days prior to use in experiments (8 days after isolation).

*Quantification of Intracellular Nitric Oxide and Superoxide*

The intracellular content of NO and $O_2^-$ were quantified using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA; Invitrogen) and dihydroethidium (DHE; Invitrogen), respectively. Cells were exposed to NO and $O_2^-$ scavengers 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; 1 μ M; Cayman Chemicals) or polyethylene glycol-superoxide dismutase (PEG-SOD; 100 U/L; Sigma) for 30 minutes prior to exposure to DAF-FM or DHE and maintained in the presence of the scavenger for the whole course of the experiment. In phenol-red free medium (with 1% FBS), cells were exposed to DAF-FM (5 μ M) for 45 minutes or DHE (25 μ M) for 20 minutes, and the regular medium replaced for an additional hour prior to processing for flow cytometry. FL1 and FL2 channels were used for DAF-FM and DHE, respectively, during flow cytometry (Beckman Coulter FC500). Human umbilical vein endothelial cells (HUVECs) and HeLa cells were used as positive and negative controls of eNOS-producing cells, and TNF-α (1 ng/mL for 24 hr) was used to induce SO generation in DHE experiments.

*Quantification of Intracellular cGMP*

A E1A cGMP ELISA kit (Cayman Chemical) was used to measure the amount of cGMP in the cells following transduction. Briefly, pelleted EPCs were frozen at -80°C until needed, then lysed using 0.1M HCl and processed according to the manufacturer's instructions. The Bradford reagent (Sigma) was used to quantify total protein from each sample, to be used to standardize the measurement.

*Western Blot*

100 μ g of total protein was separated by SDS-PAGE and electrically transferred to a nitrocellulose membrane. The membrane was blocked for 1 hr at room temperature with TBS-T buffer (20 mM TrisHCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% skimmed milk, and then incubated overnight at 4ºC with a mouse anti-human eNOS monoclonal antibody (1:5000 diluted in blocking buffer, BD). The second day the membrane was washed 3 times with TBS-T buffer and incubated for 1 hr with a HRP-conjugated anti-mouse secondary antibody (1:5000 diluted in blocking buffer). The membrane was washed 3 times and the specific band was developed using an ECL Western blotting detection kit (Amersham) and visualized with the VersaDoc Imaging System (BioRad).

*Boyden Chamber Chemotactic Migration Assay*

The chemotactic migration capacity of EPCs was measured using a Boyden chamber assay, as previously described[68,417] and described in chapters 2 and 3. EPCs were serum-starved for 1 hour, detached from culture dishes, pelleted and resuspended in EBM-2 + 0.5% BSA ($5x10^5$ cells/mL). 500 μL of this cell suspension ($2.5x10^5$ cells) was placed within cell culture inserts (Becton Dickinson, 8 μm pore size) and the inserts placed within 24-well companion plates containing 500 μL of EBM-2+0.5% BSA with either $VEGF_{165}$ (50 ng/mL) or human SDF-1 (100 ng/mL). $VEGF_{165}$ and SDF-1 concentrations were selected based on prior studies[68,417,418] and validated in our laboratory. The 8 μm pore size was selected since the cells are approximately 10-14 μm in diameter, and must actively migrate through the slightly smaller pores. After four hours at 37°C, the inserts were removed, and the cells bound to the underside of the membrane were fixed and stained using the DiffQuick staining kit (Sigma). The cells bound to bottom of the membrane were visualized using an inverted light microscope (Nikon Eclipse TS100). Five

random fields were photographed per membrane and the number of cells per high-power field (HPF) counted in a blinded manner.

*EPC and HUVEC Co-Culture Tube Formation Assay*

The ability of EPCs to stimulate EC angiogenic tube formation was quantified by co-culture with human umbilical vein ECs (HUVECs) on Matrigel (BD Biosciences). EPCs were pre-labeled using CMTMR fluorescent dye (Invitrogen; 1μM). In 48-well plates coated with Matrigel (200 μL per well), $4x10^4$ HUVECs and $2x10^4$ EPCs were cultured together (in EGM-2MV + 5% FBS) for 16 hours at 37ºC. Confocal microscopy (Leica) was used to capture 5 random fields per well, using both bright field and fluorescence (for CMTMR). In silico, endothelial tubes were skeletonized digitally and various parameters quantified in a blinded manner: total tube length (based on the total pixel % taken by skeletonized tubes), number of nodes (branch points), total number of EPCs, and number of EPCs associated with tubes (directly adjacent to or superimposed over tubes).

*Adhesion Assay*

HUVECs (~90% confluent) were activated by incubation with TNF-α (5 ng/mL) for 4 hours in 4-well chamber slides. CMTMR-labeled EPCs ($1x10^5$) were then cultured over HUVECs in regular EPC medium for 3 hours to allow for adhesion. Following two PBS washes to remove non-adherent EPCs, all remaining cells were fixed with cold 2% paraformaldehyde (PFA) pH 7.4. Three random fields per chamber were visualized by confocal microscopy, using both brightfield and fluorescent channels (for CMTMR detection). The mean number of adherent EPCs was then quantified in a blinded manner.

*Nude Mouse Hind Limb Ischemia Model*

For xenotransplantation experiments using human cells, eight to ten-week-old male BALB/c nude mice were used (BALB/cAnNCrl-nuBR, Charles River). Following anaesthesia with ketamine/xylazine (200/10 mg/kg), the proximal and distal ends of the left femoral artery, as well as all side branches, were ligated and the femoral artery cut at two points to induce limb ischemia. On day 3, $5x10^5$ human EPCs were injected into the medial thigh and gastrocnemius muscles of the ischemic limb (total volume of 0.1 mL). At days 3 (prior to cell injection), 14 and 28, limb flow measurements were made using laser Doppler perfusion imaging (LDPI, Moor Instruments). The ischemic/non-ischemic limb perfusion ratio was used for analysis.

*Statistics*

Results of in vitro assays and in vivo perfusion ratios comparing sham- and eNOS-transduced EPCs were analyzed using the students' paired t-test. In vivo sequential measurements were analyzed using repeated measures ANOVA. All data is represented as mean±SEM. Data were considered statistically significant if $p<0.05$.

## 4.3 Results

*Subject Characteristics*

Table 4.1 summarizes the demographic and medical history information for the enrolled subjects. The mean age was 63±1 and all patients had CAD, and a majority had hypercholesterolemia, hypertension and diabetes mellitus. However, due to ongoing medical management with lipid lowering (i.e. statin) and anti-hypertensive agents, the cholesterol levels and blood pressures were only slightly elevated.

**Table 4.1: Subject Demographics and Cardiovascular Risk Summary**

| | |
|---|---|
| N | 130 |
| Age | 63 ± 1 |
| Gender (% Female) | 26 |
| Clinically Significant CAD (%) | 100.0 |
| Diabetes Mellitus Type I or II (%) | 52 |
| Diagnosed/Treated Hypertension (%) | 75 |
| Systolic Pressure (mmHg) | 133 ± 2 |
| Diastolic Pressure (mmHg) | 76 ± 1 |
| Current Smoker (%) | 31 |
| Diagnosed/Treated Hypercholesterolemia (%) | 79 |
| Total Cholesterol (mmol/L) | 4.74 ± 0.12 |
| LDL (mmol/L) | 2.77 ± 0.08 |
| HDL (mmol/L) | 1.14 ± 0.04 |
| Medications | |
| Statin (%) | 61 |
| ACE Inhibitor (%) | 47 |
| Angiotensin Receptor Blocker (%) | 18 |

*eNOS Overexpression*

Lentiviral eNOS transduction led to a consistent increase in eNOS mRNA expression in EPCs (39.7±13.1 fold increase, figures 4.1A and B), as well as a 5-6 fold increase in eNOS protein, as assessed by Western Blot and densitometry (Figure 4.1C). There was no difference in cell

survival between eNOS and sham-transduced cells, as assessed by the number of remaining adherent cells after 5 days in culture (1.32±0.42 vs. 1.38±0.33 x $10^4$ cells/$cm^2$, respectively).

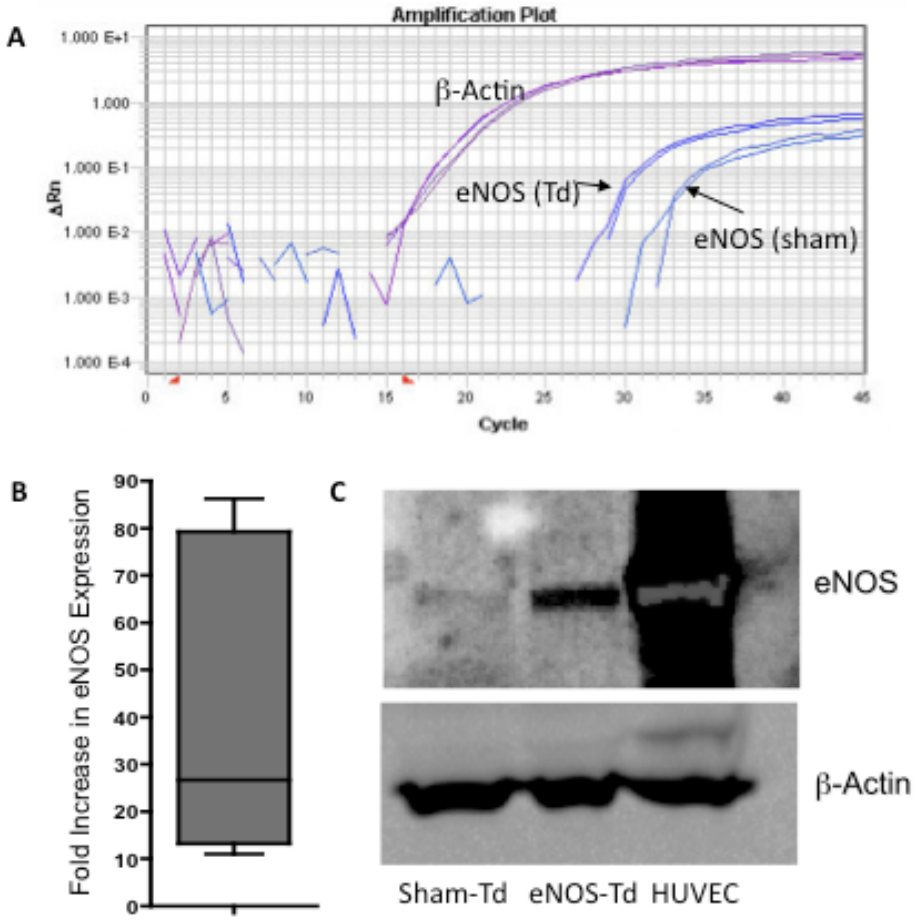


**Figure 4.1: Lentiviral Overexpression of eNOS**
Five days following lentiviral transduction, the expression of eNOS was determined in eNOS- and sham-transduced EPCs using real-time RT-PCR with TaqMan Gene Expression Assays (ABI). Amplification of β-Actin (control) and eNOS for sham- and eNOS-transduced EPC samples are shown (A). The fold-difference in eNOS mRNA expression between eNOS- and sham-transduced cells is variable but consistently elevated following transduction (B; n=6). To determine the degree of eNOS protein overexpression, we performed a Western Blot and calculated the fold-difference using densitometry (C). β-Actin was used as a loading control.

*NO Production and cGMP Upregulation*

Comparison of DAF-FM fluorescence showed a significant increase in NO production by EPCs from CAD patients following eNOS transduction (Figure 4.2A; 2.28±0.29 vs. 1.81±0.17

fluorescence units; n=8, $p<0.05$). Although the relative amount of NO production was low compared with healthy human ECs, the addition of PTIO reduced the DAF-FM signal to comparable levels in both cell groups, confirming the specificity of DAF-FM. Indeed, the PTIO-inhibitable signal was substantially increased in EPCs following eNOS transduction. In addition, eNOS transduction led to consistent and significantly increased cGMP content in eNOS-transduced cells compared to sham-transduced cells (0.578±0.063 vs. 0.475±0.066 pg/mg total protein; n=8, $p<0.001$; Figure 4.2B). Moreover, we used DHE to measure SO production (Figure 4.2C) to assess possible uncoupling of eNOS activity and found no difference between cell treatment groups, whereas treatment with TNF-α resulted in a large increase in SO production by these cells.

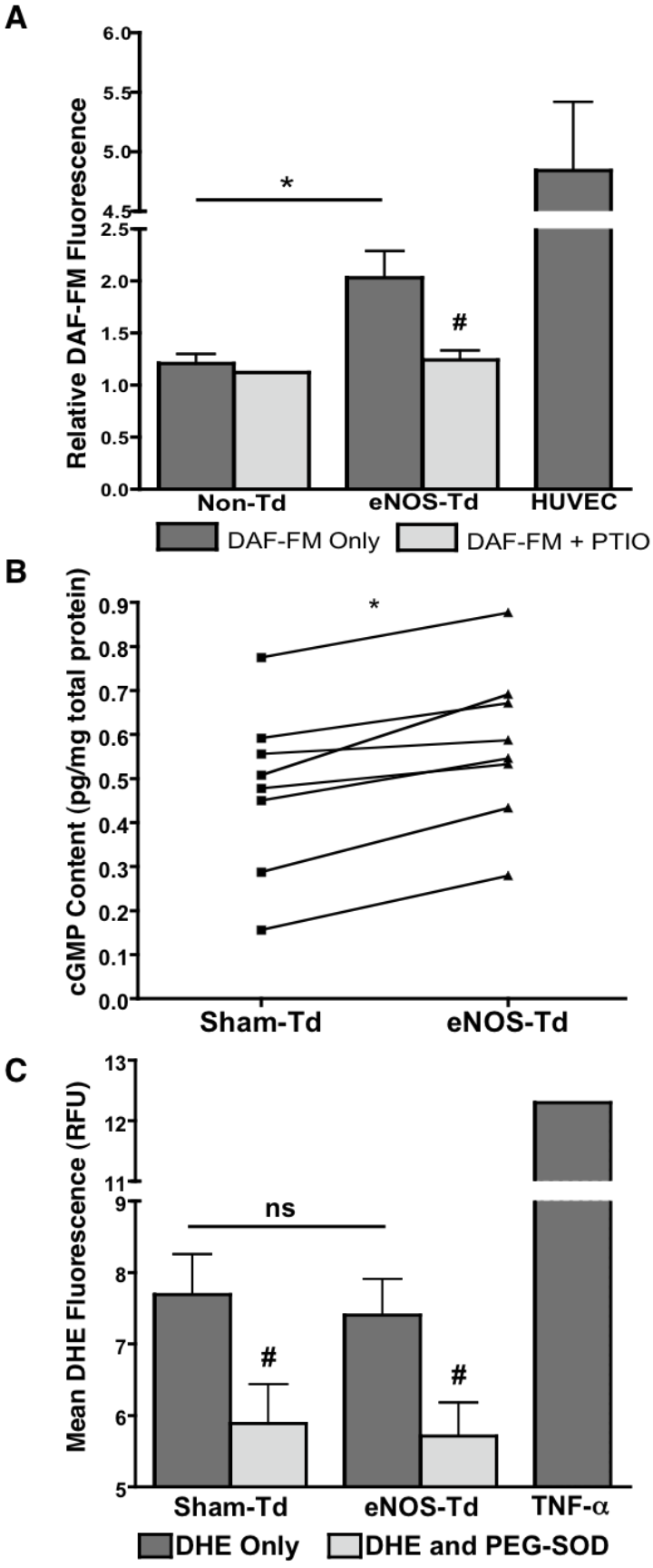


**Figure 4.2: EPC Production of NO or SO**

We quantified NO and $O_2^-$ 5 days following transduction. DAF-FM was used to measure intracellular NO and shown to be higher (approx. 2-fold) in eNOS-transduced cells compared to control (A; n=8; all values relative to DAF-FM +PTIO Non-Td). HUVEC cells were used as positive controls for NO production and a NO scavenger, PTIO, was used to show specificity of the reaction. An ELISA assay was employed to measure cGMP levels, which we also consistently elevated in eNOS-transduced EPCs compared to sham-transduced cells from the same subject (B; n=8). DHE, which indicates intracellular $O_2^-$ was not different between cell groups (C; n=8) but largely elevated following 24-hour exposure to 1 ng/mL TNF-α (positive control; n=2). * represents $p<0.05$ between cell groups (eNOS- vs sham/non-Td); # represents $p<0.05$ ± scavenger.

*Improved Chemotactic Migration*

Compared to sham-transduced cells, eNOS-transduced EPCs had significantly improved migration to both VEGF (48.1±7.0 vs. 33.0±5.0 cells/HPF; $p<0.05$, figure 4.3) and SDF-1 (55.5±9.3 vs. 35.6±4.4 cells/HPF; $p<0.05$).

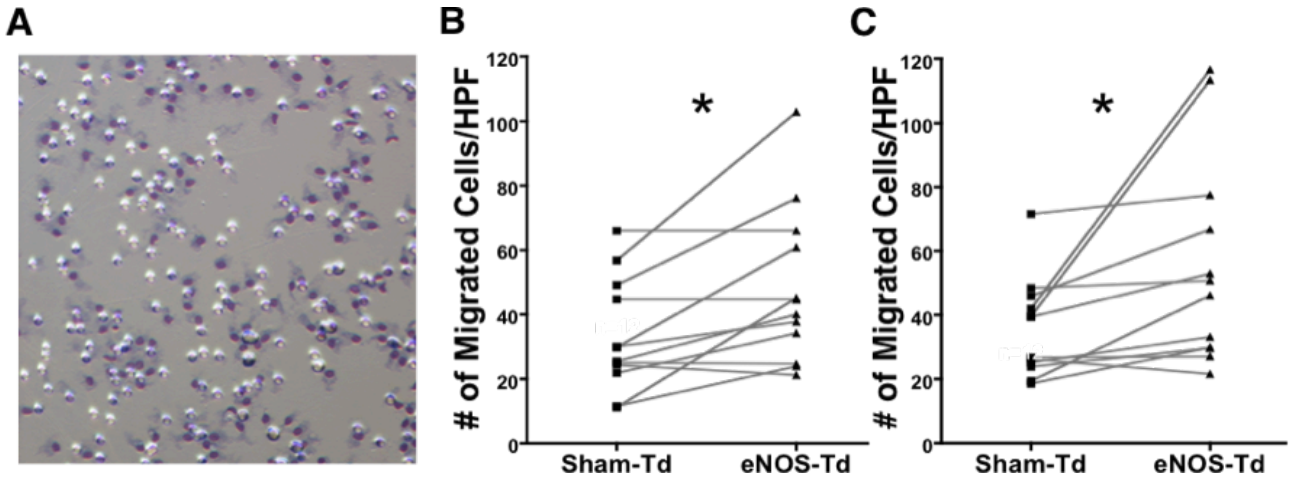


**Figure 4.3: Chemotactic Migration of Transduced EPCs**
Following 5 hour incubation, underside of the membranes were treated with DiffQuick to stain migrated cells, for quantification (A) by light microscopy (20X objective). The number of migrated cells per field were compared between sham- and eNOS-transduced EPCs from the same subject in the presence of either VEGF (B; n=12) or SDF-1 (C; n=12). * represents $p<0.05$.

*Adhesion to Endothelium and Angiogenic Tube Formation*

Adhesion of EPCs to a TNF-activated EC layer was not changed by eNOS overexpression (58.1±6.9 vs. 47.2±4.8 EPCs/HPF; n=10; p=0.119; Figure 4.4). However, we found that eNOS transduction of EPCs from CAD patients enhanced various parameters of angiogenic activity in the EPC/HUVEC Matrigel co-culture assay. Co-culture of HUVEC with eNOS-transduced EPCs (figure 4.5B) lead to greater overall endothelial tube length (4.6±0.6 vs. 3.5±0.6% of total pixels per HPF; $p<0.05$, figure 4.5C) and number of nodes (8.5±1.1 vs. 6.0±1.3 nodes per HPF; $p<0.05$, figure 4.5D) compared to sham-transduced EPCs (figure 4.5A). As well, there was a greater degree of association of eNOS-transduced EPCs with the endothelial tube-like networks in the

eNOS transduced cells, which occurred mainly at the nodal junctions (77.4±3.7 vs. 65.5±3.6 % of total EPCs per HPF; $p<0.01$, figure 4.5E).

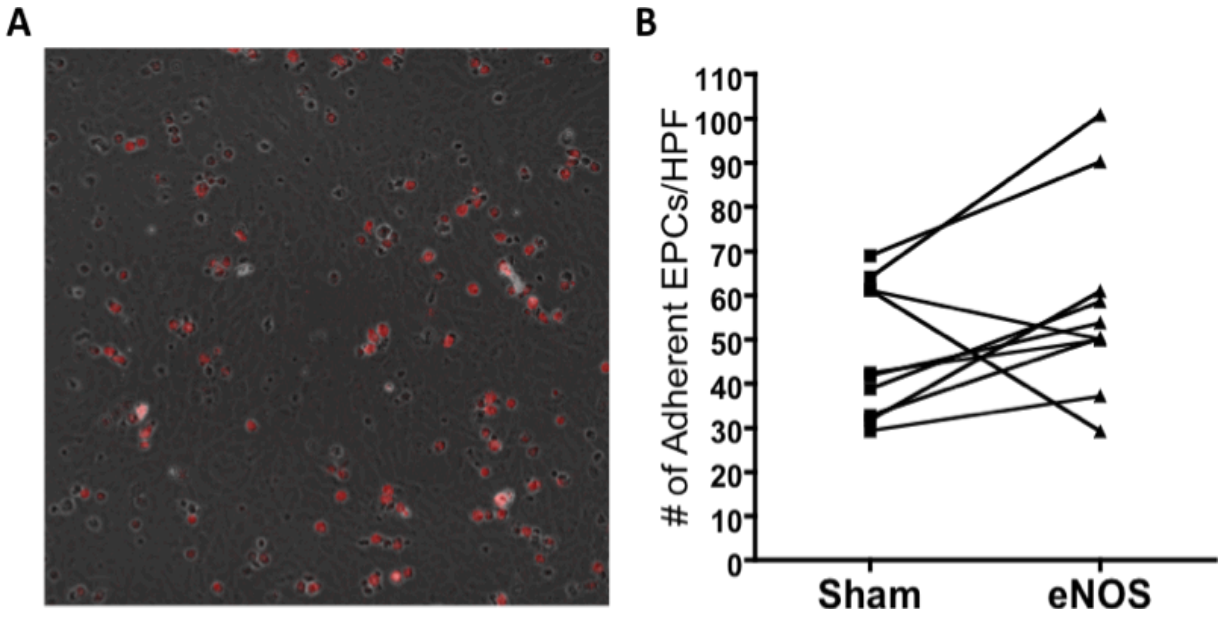


**Figure 4.4: EPC Adhesion to HUVECs**
We measured the ability of EPCs (stained with CMTMR, red) to adhere to TNF-α pre-activated HUVECs. Panel (A) displays a typical adhesion assay result; we did not observe a significant difference in the adhesion of EPCs following eNOS overexpression (B).

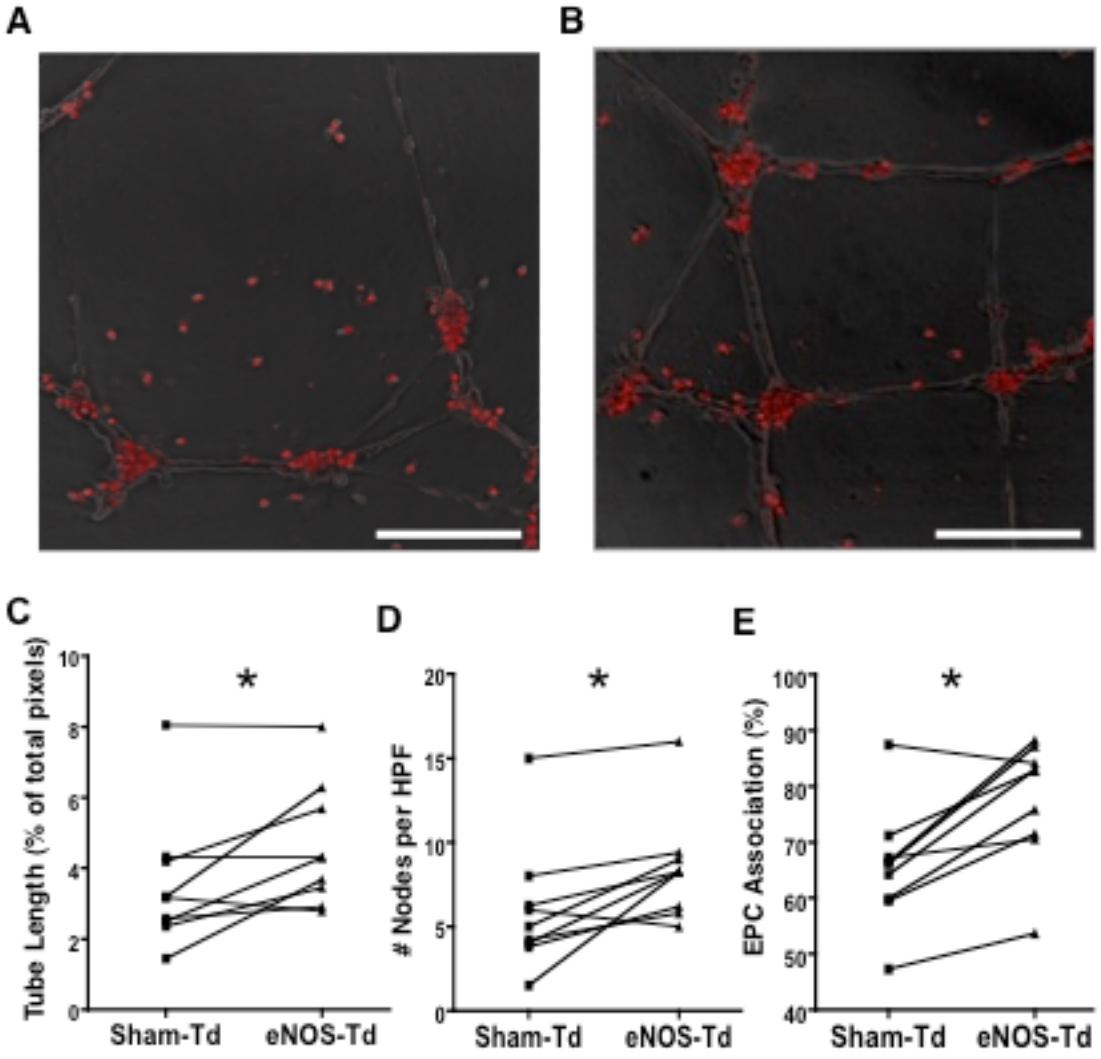


**Figure 4.5: EPC Co-Culture with HUVECs on Matrigel**
We measured the ability of EPCs (stained with CMTMR, red) to associate with activated HUVECs (shown in grey) as well as their ability to stimulate angiogenic tube formation when co-cultured with HUVECs on Matrigel. However, for the tube formation assay, compared to sham-transduced EPCs (A), eNOS-transduced cells (B) were had improved ability to stimulate tube formation, as measured by several parameters: tube length (C), the number of nodes (D) and rate of EPC association with EC tubes (E). * represents $p<0.05$. Scale bars = 150 μm

*In Vivo Neovascularization Model*

Following surgery to induce unilateral hindlimb ischemia, there was an immediate reduction in perfusion to approximately 10% of the nonoperated limb, which remained fairly constant (11-

12%) at the time of cell injection (day 3). Animals receiving eNOS-transduced EPCs obtained from patients with CAD had a greater restoration of hindlimb perfusion at day 14 (0.382±0.026 vs. 0.305±0.026; $p<0.05$) and 28 (0.436±0.030 vs. 0.363±0.032; $p<0.05$; figure 4.6) compared to animals injected with sham-transduced EPCs. Fluorescent microangiography (FMA) confirmed that the gastrocnemius muscle of the ischemic limb demonstrated reduced perfusion and microvessel branching compared to the non-ischemic limb ($p<0.01$, figure 4.7). Furthermore, animals receiving eNOS-transduced EPCs had improved muscle perfusion ($p<0.05$) and vessel branching ($p<0.05$) compared to those receiving sham-transduced EPCs (figure 4.7).

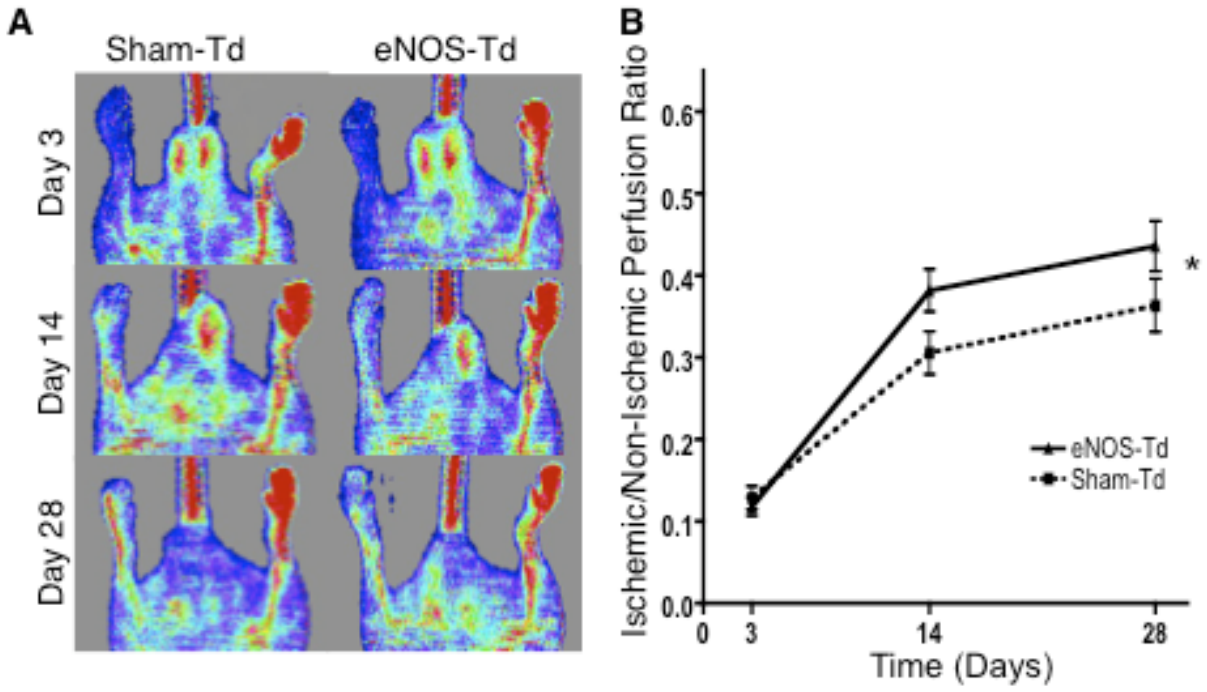


**Figure 4.6: Neovascularization Capacity of Transduced EPCs**
Using laser Doppler perfusion imaging, we observed that eNOS overexpression increased the function of EPCs (n=12) (B), with representative laser Doppler perfusion images shown (A). Data is expressed as the mean ratio of the ischemic/non-ischemic limb perfusion ± SEM. * represents $p<0.05$ using repeated measures ANOVA. # represents $p<0.05$ compared to control using student's t-test at individual time points.

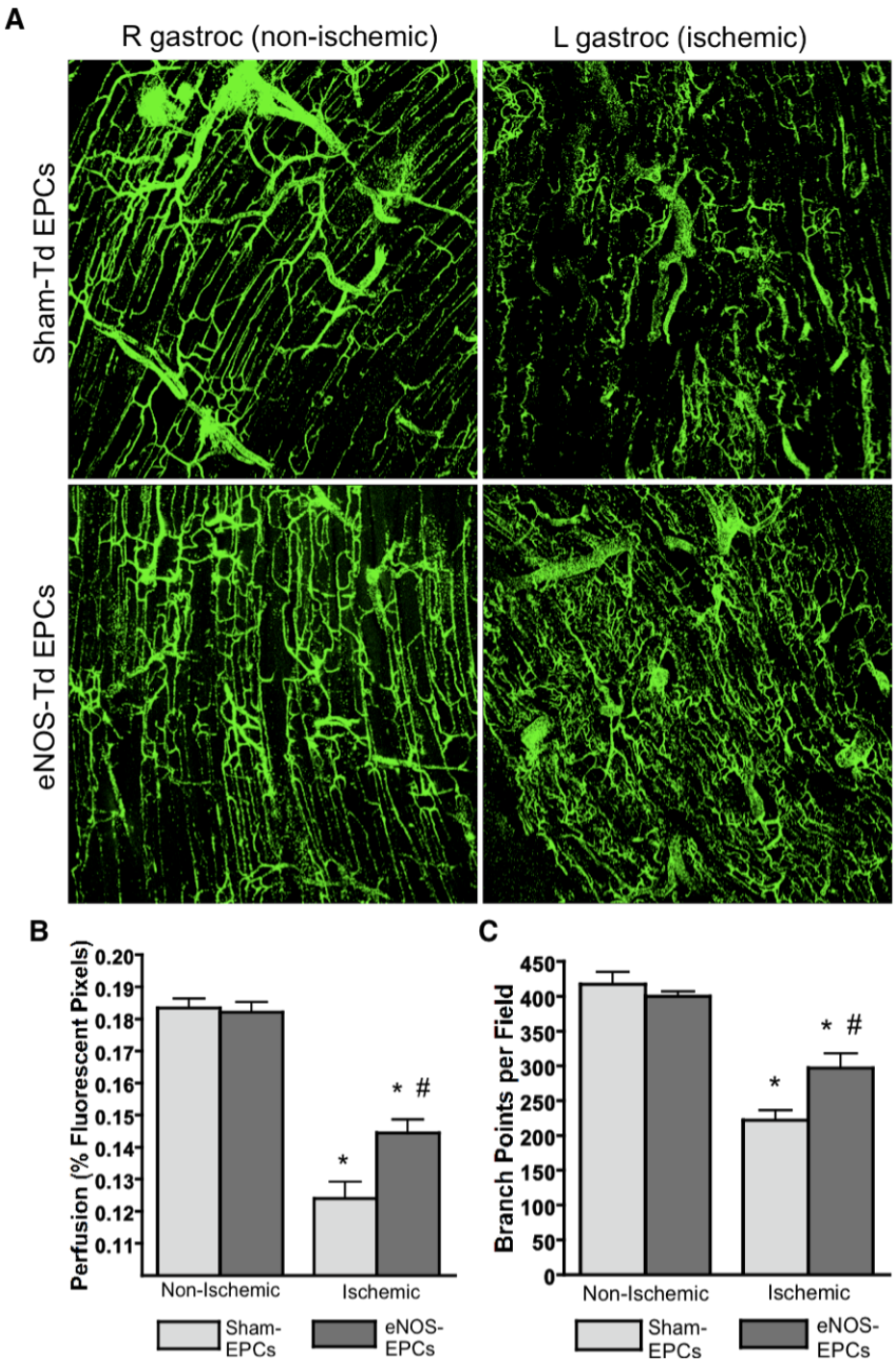


**Figure 4.7: Fluorescent Microangiography**
As shown in representative confocal microscopy stacked sections (A), we observed a highly significant reduction in gastrocnemius muscle perfusion (B) and microvessel branching (C) in ischemic muscles compared to contralateral non-ischemic muscles (n=5, $p<0.01$). Mice receiving eNOS-transduced EPCs showed significant improvement in both parameters compared to those receiving sham-transduced EPCs (n=5, $p<0.05$). * represents $p<0.01$ compared to non-ischemic limb. # represents $p<0.05$ compared to sham-transduced cells

## 4.4 Discussion

In this study, we showed that eNOS gene delivery could substantially overcome the profound EPC dysfunction seen in a relevant patient population for post MI therapy with multiple cardiac RFs and, for the most part, known CAD, and markedly improve the regenerative activity of patient-derived progenitor cells both in vitro and in vivo. Nitric oxide is a potent vasodilator which prevents platelet aggregation, leukocyte extravasation and smooth muscle proliferation[129]. NO has been shown to be involved in downstream VEGF signaling promoting EC proliferation and migration [321] as well as to improved angiogenesis in vivo[323,325]. eNOS knockout mice exhibit defects in angiogenesis, arteriogenesis and functional blood flow reserve[325,385], despite similar levels of ischemia and VEGF induction. As well, it was recently reported that NO was required for EPC mobilization[385] and impaired reperfusion of hindlimb ischemia in $eNOS^{-/-}$ mice could be rescued by wild type EPCs. NO production has also been shown to be critical to the angiogenic activity of EPCs, and cells derived from eNOS-deficient mice exhibit poor in vitro migratory function, impaired ability to stimulate hindlimb neovascularization and production of unbranched and dysfunctional neovessels in the retina[385,386].

Endothelial dysfunction, which is a nearly universal feature of patients with established vascular disease or multiple RFs, is related not only to reduced NO production, but to the balance of NO and SO anion production. In addition to other sources of increased SO anion in vascular disease (i.e. NADPH), eNOS itself can generate reactive oxygen radicals when it is uncoupled. In our experiments, eNOS transduction did not lead to evidence of uncoupled eNOS activity in EPCs from patients with CAD and RFs. Moreover the enhanced eNOS expression in the cells resulted

in increased NO production, without an increase in $O_2^-$. Although the mean increase in NO production was relatively modest following eNOS transduction, there was considerable variability between individual subjects in basal levels and the enhancement following eNOS gene transfer was highly consistent between subjects. As well, this was associated with increased cGMP content, confirming that this level of NO overproduction was biologically significant. Previous studies of EPCs from diabetic patients suggested that PKC-dependent reduction of BH4 uncouples eNOS, leading to elevated $O_2^-$ levels and impaired EPC migration[395]. However, in our study there was no evidence for uncoupled NOS activity following eNOS overexpression despite the high proportion of diabetic patients, perhaps reflecting different the methods for EPC selection, and some recovery during the culture period, since NO and $O_2^-$ quantification were performed after 5 days of culture following transduction, as were all other assays. Further studies are warranted to determine whether and to what degree eNOS would ultimately become uncoupled if exposed to elevated glucose or other sources of oxidative stress, which might reproduce the host environment after autologous cell administration. In any event, our in vivo results suggest that, at least for the purpose of neovascularization measured up to 28 days after transplantation, the eNOS overexpressing human cells from patients with vascular disease or RFs have an improved therapeutic effect when studied in an immunodeficient but otherwise normal host.

The exact mechanism of EPC-mediated neovascularization is still poorly understood. Nevertheless, NO appears to contribute to many angiogenic properties of these cells. Migration toward VEGF and SDF-1 has been suggested to be the best surrogate for EPC in vivo function, due to the important role these agents hold in both the mobilization and homing of EPCs to sites of ischemia[67,413]. eNOS is phosphorylated and activated downstream of VEGFR-2[321], and has

been shown to be critical to the pleiotropic effects of this receptor[441], including directional migration[144]. Recently, NO has been implicated in CXCR4 signaling in $CD34^+$ progenitors, most likely by mediating the oxidation of intracellular protein thiols[73]. This was shown to be independent of cGMP, confirming the dual nature of NO intracellular action. NO has also been implicated in the structural mechanism responsible for cell migration in EPCs. Compared to cells from healthy donors, $CD34^+$ cells from diabetic patients were shown to be more rigid and less motile than cells from healthy donors, which was reversible with exogenous NO administration[69]. The same group has recently suggested that NO is critical in initiating elongation of actin filaments by vasodilator-stimulated phosphoproteins (VASPs) at the leading edge of migrating cells[400]. VASP phosphorylation has also been shown to stimulate actin stress fibers in a cGMP-dependent mechanism and lead to enhanced endothelial tube formation[442]. This study confirms these reports and shows that gene delivery of eNOS is a simple and direct method of increasing the production of NO, and subsequently, the regenerative capacity, of human early growth EPCs.

In addition to direct incorporation into neovessels vessels, there is now considerable evidence showing that EPCs stimulate native endothelium to proliferate and migrate via release of paracrine factors[50,443]. Both direct and paracrine effects can be assessed in the Matrigel co-culture assay, which quantifies the degree of tube formation by HUVECs following addition of EPCs and well as the ability of EPCs to incorporate into the EC capillary-like networks. After 16 hours, there was a greater in vitro angiogenic response with eNOS transduced cells than with sham-transduced cells and eNOS overexpressing cells were more frequently associated with EC tubes. This latter finding was seen despite the lack of effect of eNOS transduction on the adhesion of EPCs to an activated EC monolayer, suggesting that the mechanism of cell

association likely differs between inflammatory and angiogenic assays. Uemura et al.[444] showed that anoxia-pretreated bone marrow stem cells (BMSCs) had increased phosphorylation of Akt and eNOS, accompanied by increased secretion of VEGF, IGF-1, bFGF and SDF-1. When these cells were administered to ischemic myocardium, they improved myocardial function to a greater degree than non-treated cells[444]. In addition to the paracrine release of growth factors, EPCs have recently been shown to release microvesicles (MVs) which contain mRNA of up to 183 transcripts[445]. Of note, eNOS was shown to be a prominent MV transcript, leading to increased eNOS protein expression and angiogenic tube formation in mature ECs exposed to MVs. Other genes regulating the PI3K pathway were also found in the MVs and implicated in this effect since wortmannin, a PI3K inhibitor, abrogated the effect of MVs on ECs. Our results are consistent with these reports of the importance of eNOS in the stimulation of angiogenesis by EPCs. It remains to be determined whether eNOS overexpression improves the secretion of specific angiogenic cytokines or leads to increased eNOS expression in co-cultured ECs.

These results are consistent with reports using a small molecule enhancer of eNOS transcription, AVE9488 (Sanofi Aventis), to improve the function of EPCs from cardiomyopathy patients[406]. As well, treatment of EPCs from elderly individuals with IGF-1 improved differentiation, migratory capacity and the ability to incorporate into forming vascular networks in vitro[405], and increased telomerase activity, eNOS expression, phosphorylation and activity in a PI3K/Akt dependent manner[405]. Similarly, a preclinical study has shown that upregulation of GTP cyclohydrolase I (a key enzyme in the production of tetrahydrobiopterin, BH4, a cofactor for eNOS) through treatment with HMGCoA reductase inhibitors (statins) improves eNOS activity EPC function in this model of diabetes[303]. In this case, statins were able to reverse eNOS uncoupling, which has been shown reduce NO bioavailability in the context of diabetes[395].

We have shown that eNOS gene transfer improves the angiogenic activity of dysfunctional EPCs from patients with coronary disease and/or cardiac RFs, suggesting that such a strategy may improve the efficacy of cell therapy for cardiovascular diseases. In recent trials using autologous cell therapy post-MI, there appears to be some clinical benefit although this is modest[369]. Our results suggest that strategies to restore the angiogenic activity of EPCs from this patient population may be critical in augmenting the degree of clinical benefit and developing robust and truly effective cell therapies for cardiovascular disease.

# 5 Cell Therapy in a Porcine Model of Myocardial Infarction

## 5.1 Introduction

In order to prepare for the possible translation of the results found in the previous in vitro and in vivo experiments into a new clinical therapy, we wished to evaluate the retention of EPCs and the efficacy of eNOS-overexpressing cells in a large animal, porcine model of acute MI that more closely reproduces the challenges that will faced in scaling up for human trails. The pig heart is very similar (in anatomy, cardiomyocyte gene expression and electrical conductance) to that of humans[446,447], and is regularly used as a model to test novel cardiac treatments, including gene therapy[315,448] and cell therapy[63,449]. Cardiac MRI was used to track injected cells as well as to measure the LVEF, an important marker of myocardial function.

Long term cell retention is highest following direct intra-myocardial injection[450]. However, the majority of cell therapy clinical studies have utilized intra-coronary infusion via the infarct related artery (IRA)[369]. Using radio-nuclide labeling and positron emission tomography (PET), it has been shown that cellular retention following intra-coronary delivery is only 1.3-2.6% for unselected BMCs, although this may rise to 39% with CD34-enriched cells[451], which may be more comparable to the culture selected EPCs used in our experiments. PET is a highly sensitive method to follow cell fate in the short term but is limited by poor spatial resolution, cellular toxicity of the radio-tracer and relatively short half-life of the radio-tracer which precludes long-term tracking[452].

MRI has been used to track cells longitudinally following direct intra-myocardial injection[453,454], and acutely following intra-coronary infusion[455]. However, the intracoronary route, by its very nature, results in a more diffuse and less dense distribution of cells, which will be more difficult to visualize. In this study, we tested whether MRI could be used for long-term tracking of iron-labeled bone marrow cells (BMCs) after intracoronary infusion in a porcine model of acute MI.

To track the cells by MRI, paramagnetic iron oxide particles were used. These particles exist as ultrasmall super-paramagnetic iron oxide (USPIO; 40 nm), superparamagnetic iron oxide (SPIO; usually characterized as 100–300 nm) and micrometer-sized particles (MPIO; 1 μm). Bangs particles are magnetite cores encapsulated with styrene/divinyl benzene and conjugated to dragon green fluorescent dye (480-nm excitation, 520-nm emission). It has been shown that MPIOs are efficiently endocytosed by a variety of cells, and that these particles can be used for cellular imaging by MRI[449,453]. Because these particles are polymer-coated and are impregnated with a fluorescent agent, it becomes possible to do both fluorescence microscopy and MRI on cells labeled with such particles. Based on empirical observations, iron oxide particles disrupt the magnetic field for a distance at least 50 times its size, suggesting that cells harboring single, micrometer-sized particles should be detectable by T2*-weighted MRI at resolutions of approximately 50 μm[456,457]. In addition, Bangs particles have been shown to not affect the differentiation, migration and proliferation of mesenchymal stromal cells (MSCs)[453] and $CD34^+$ hematopoietic cells[457].

In addition, BMCs were transfected with eNOS and the efficacy of EPC administration was quantified using cardiac MRI functional endpoints. The work in this chapter was conducted in collaboration with the group of Dr. Alexander Dick, at the Sunnybrook Health Sciences Centre. I was responsible for optimization and implementation of cell processing and transfection, while

the porcine interventions and imaging studies were performed by Dr. Dick's group, including Dr. Warren Foltz, Dr. John Graham and Dr. Ram Vijayaraghavan. However, I was responsible for the data analysis employed and reported herein.

## 5.2 Materials and Methods

*Animal Model*

Studies were conducted in Yorkshire swine (22-28 kg) using institution-approved procedures and protocols. Ten (10) pigs were used to study cell retention (6 received BMCs and 4 received PBS only) and 15 pigs were used to compare the efficacy of BMCs alone (n=4), eNOS-transfected BMCs (n=7) and PBS control (n=4).

*Animal pre-medication*

Premedication consisted of a ketamine/atropine cocktail (35 mg/kg ketamine hydrochloride and 0.05 mg/kg atropine) and masking at 5% halothane in oxygen. Anaesthesia was maintained using 2% isofluorane. To minimize arrhythmias, animals received a pre-operative intravenous bolus followed by constant infusion of both amiodarone (75 mg bolus, 1.5 mg/kg/hour infusion) and lidocaine (20 mg bolus, 3 mg/kg/hour infusion). Animals also received metoprolol during the 5-day interval prior to experimentation (50mg/day, in feed). Peri-procedural anti-coagulation was given in the form of intravenous unfractionated heparin (70-90 units/kg).

*Cell Isolation and Preparation*

Immediately prior to infarct creation, bone marrow (40-50 mL) was aspirated from both iliac crests. The mononuclear fraction was obtained using Ficoll gradient centrifugation (Cell Preparation Tube, BD). This mononuclear fraction was aspirated, washed twice and plated on human fibronectin coated flasks in endothelial basal medium supplemented with endothelial growth medium SingleQuots (EGM-2MV, Lonza) and 20% fetal bovine serum[420] and cultured for 7 days with media changes every 48 hours.

*Iron Labeling*

Prior to cell injection, the cells were incubated for 12-18 hours with super-paramagnetic iron oxide particles (SPIO) conjugated with a fluorescent dye. These iron fluorophores (Bangs Laboratories, Fisher, IN) have an average size of 0.9 μm, contain both magnetite iron oxide as well as a fluorescein-5-isocyothianate analog (Dragon Green) and are readily taken up by dividing cells. Incubation was at a concentration of 10 μL/mL which has previously been shown to have no effect on cellular differentiation, migration and proliferation in several cell lineages[457]. After this incubation period, the cells were washed twice in phosphate buffered saline (PBS) and incubated for 30 minutes with a fluorescent cell viability marker, Cell Tracker Orange (Orange-fluorescent tetramethylrhodamine – CMTMR; Invitrogen Inc, Burlington, ON). Following this second incubation, they were again washed twice in PBS, trypsinized, washed twice in PBS and made up to a final volume of 2 mL in PBS. Cell viability was confirmed using trypan blue exclusion. Animals were randomly assigned to receive either cells or an equivalent volume of the PBS vehicle.

*Infarct Creation*

A percutaneous anterior MI was created as previously described[458]. Briefly, the femoral artery was cannulated and the LAD (distal to the second diagonal branch) occluded for 90 minutes using a conventional angioplasty balloon catheter. Post-procedure angiography confirmed the reperfused nature of the infarct by demonstrating LAD patency.

*Cellular Delivery*

Seven days following MI the animals were re-anaesthetized, instrumented and an over-the-wire (OTW) angioplasty balloon (Guidant Opensail, Abbott Laboratories, Abbott Park, IL) advanced

to the LAD to the previous site of infarct creation. In an attempt to minimize cellular washout, the cells were hand injected through the central lumen of the OTW balloon during two 1-minute periods of low pressure inflation. To minimize further ischemia, the balloon was deflated for one minute between cell injections.

*eNOS Transfection*

One day prior to cell delivery, cells from animals in the eNOS-BMC group were detached from flasks using Trypsin (0.05%), centrifuged and resuspended at 3.0 x $10^7$ cells/mL in nucleofection buffer (Amaxa). 100 μL of suspension ($3.0x10^6$ cells at a time) was loaded into cuvettes and electroporated in the presence of 5 μg of eNOS plasmid (pVAX1-eNOS) using the Amaxa device (Nucleofector I). Cells were then re-plated onto flasks overnight, prior to delivery into the coronary artery.

*RT-PCR*

Two days (48 hours) following transfection, the expression of eNOS was determined in eNOS- and non-transfected BMCs using real-time RT-PCR with TaqMan Gene Expression Assays (ABI). RNA was isolated from cells using the Trizol Reagent and reverse transcribed using Omniscript (Qiagen). eNOS amplification was compared to that of β-Actin (endogenous control).

*Imaging*

All MRI studies were performed on a GE 1.5T Signa Excite system (GE Healthcare, Milwaukee, WI). The animal was placed in a plexiform case to allow scanning in the supine position. A 5-inch surface of general purpose flex coil was placed on the anterior thoracic wall of the animal. Cardiac gating was achieved utilizing vector gating or, if inadequate, through a plethysmography

trace using an Invivo Magnitute™ physiological monitor (Invivo Corporation, Orlando, FL) received from the pig's tail. Respiratory artifacts were reduced by both securing of forelimbs to the base of the plexiform case and signal averaging. Prior to cell injection, all pigs underwent a baseline MRI study that included a steady-state free precession (SSFP) functional study and infarct imaging with delayed hyperenhancement (DE-MRI). The location of the cells' magnetic label was assessed using a T2*-weighted sequence which should show the cellular iron as signal hypointensity. Typical MR imaging parameters were as follows:

SSFP - A total of 10 to 16 slices covering the whole left ventricle from the mitral valve to the apex were acquired with 5 mm slice thickness without spacing. TR ~4.0 ms, TE ~1.4 ms, flip angle 60º, field of view 23 cm, matrix 256x192, number of excitations 4-6, 20 cardiac phases per slice.

DE-MRI (IR-prepared FGRE) - performed 10-20 minutes following 0.2mmol/kg bolus intravenous injection of Gadolinium-DTPA (Magnevist, Berlex, Montville, NJ). The DE-MRI sequence spanned the whole left ventricle. TI varied from 200 ms to 300 ms dependent on myocardial nulling, TR/TE 7.1/3.4 ms, flip angle 30°, slice thickness 5 mm without spacing, matrix 256x192, field of view 23x18 cm, and number of excitations 6. T2*-weighted images - a FGRE sequence was employed. TE = 4.7 - 10ms, TR=9.8, voxels size 0.6x0.5x5mm. Animals were imaged immediately following cell delivery and at 1 and 6 weeks following cell delivery.

*Data Analysis*

SSFP LV function and infarct volume analysis were performed on a GE Advantage workstation (version 4.2) using Mass Plus software (Medis, Leiden, Netherlands). The operators evaluating these were blinded to which treatment group each animal had been assigned.

*Signal Intensity Ratios*

Using T2-weighted imaging sequences, cellular iron should be visible as a hypointense (i.e. dark) signal. Comparing the signal intensity (SI) from areas with labeled cells with the SI from areas of remote (uninfarcted) myocardium gives an indirect indicator of retained cell number. The higher the number of retained labeled cells, the lower the SI ratio. For quantitative SI analysis, two regions of interests (ROIs) were drawn manually: one in the area where cell signal hypointensity was deemed maximal (by visual assessment); and another in a control area unaffected by the infarct (usually the posterior-inferior segments of the LV). SI values were obtained using Xcinema (Stanford University). To evaluate relative cell hypointensity (and thus an indication of cellular retention), the ratio SI(infarct)/SI(control) was calculated at all three time-points.

Sections were counter-stained with DAPI (Sigma, Oakville, ON) and mounted with FluorSave Reagent (Calbiochem, San Diego, CA). Epi-fluorescence microscopy was conducted on an Axiovert 200 fluorescence microscope (Carl Zeiss Canada Ltd, Toronto, ON), followed by image analysis with Axiovision 40 (version 4.5.0.0) software.

*Statistical Analysis*

All data are expressed as mean value ± SEM. Differences in LVEF and infarct volume were evaluated using paired student's t-test or 1-way ANOVA. Sequential differences in LVEF and infarct volume were calculated for each animal and compared between each cohort using a paired student's t-test. Changes were considered significant when $p<0.05$.

## 5.3 Results

*Cellular Yield and Labeling*

In the first set of experiments, each animal received an average of 13.4± 4.4 x $10^6$ BMCs (range 6 – 20 x $10^6$, median 13.75 x $10^6$). Following the dual labeling technique described (Bangs and CMTMR), viability was >85% by trypan blue exclusion and >99% of cells were positive for incorporation of the particles. In the second series of experiments, animals received 2.0x$10^{7}$ - BMCs.

*eNOS Expression*

The fold-difference in eNOS mRNA expression between eNOS- and non-transfected cells was variable but consistently elevated following transfection. Compared to non-transfected BMCs, the overexpression of eNOS mRNA in the BMCs was 1.13±0.34 x$10^3$ fold (n=3).

*Immediate and Longitudinal Tracking*

Immediately following cell delivery, hypointensity characteristic of the magnetic label was evident in all cell-treated animals. Comparison with the corresponding IR-GRE infarct images reveals the cell hypointensity to be more within the infarct-border region rather than within the infarct itself (figure 5.1). Sequential imaging of all cell-injected animals at the three time points (Day 0, 7 and 42) revealed persistence of the cellular label hypointensity (figure 5.1A). At the six-week time point, there was obvious myocardial thinning in the region of infarction. Also at 6 weeks, the cell label hypointensity appeared more within the centre of the infarction rather than around the infarct border zone (figure 5.1A). The ratio SI(infarct)/SI(normal) returned toward unity over the six week time-period, with a significant majority of cell signal hypointensity being

lost in the first week following cell delivery (figure 5.1B). Immediately following cell delivery, the ratio was 0.57±0.14; at 1 week 0.89±0.06; and at 6 weeks 0.84±0.12 (figure 5.1B).

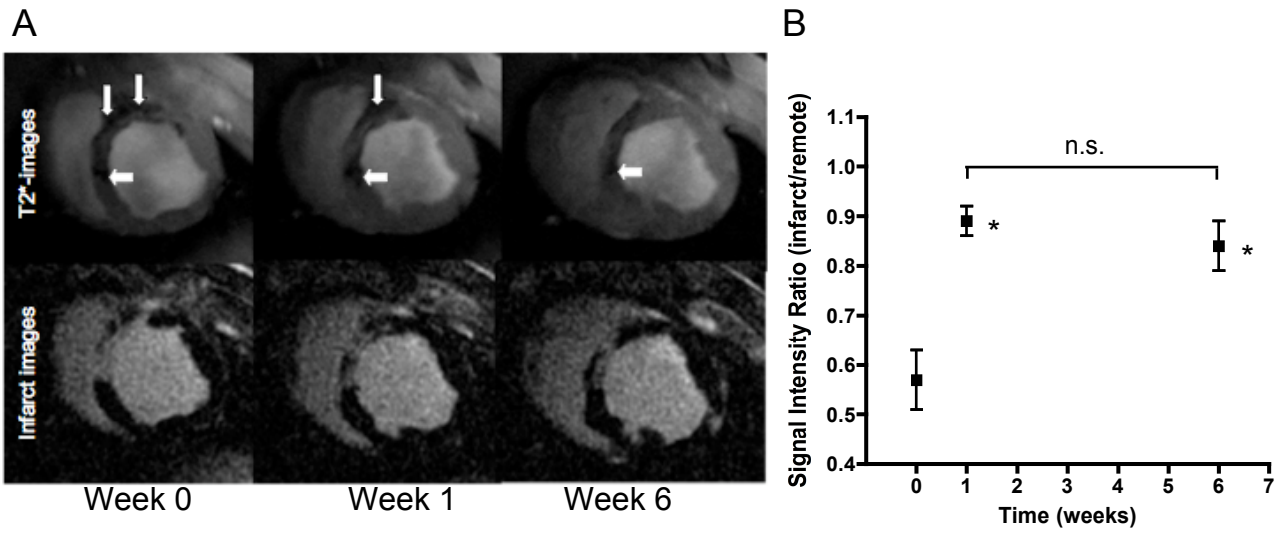


**Figure 5.1: Persistence of BMCs in Myocardium by MRI**
The location of SPIO-labeled cells (hypointensity in T2-weighted images) can be seen in the top images (A). In IR-GRE images (bottom images), signal hypointensity can be seen longitudinally, showing infarct resolution. Signal intensity ratios, indicating persistence of cells, are summarized (panel B). * represents $p<0.05$ relative to week 0.

*Cardiac Function by MRI*

Although baseline measures of LVEF and infarct size were not equivalent between the two groups, we did observe differences in the response following BMC injection versus vehicle alone (PBS). Intra-coronary infusion of the BMCs resulted in significant improvement in LVEF at 6 weeks compared to baseline (29.8±2.4 to 34.5±1.3, $p<0.01$, figure 5.2A) but there was no change in the PBS control group (34.3±2.1 to 31.9±3.0, $p=0.42$). Also, there was a significant decline in infarct volume in the BMC group (7.0±0.7 mL to 4.8±0.5 mL, $p=0.001$, figure 5.2B) but no change in infarct volume in the PBS group (4.6±0.9 mL to 4.4±0.9 mL, $p=0.7$).

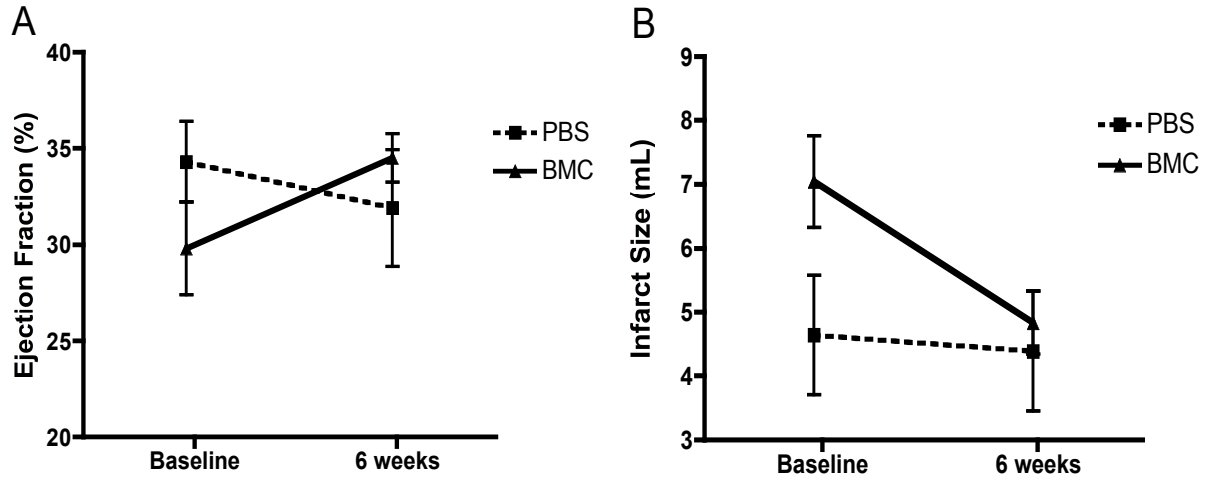


**Figure 5.2: Changes in MRI-Assessed Cardiac Function Following BMC Delivery**

*Efficacy of Genetically-Modified BMCs*

In a separate series using eNOS-transfected BMCs (eNOS-BMCs) or non-transfected BMCs (nt-BMCs), absolute LVEF increased significantly in the nt-BMC group (29.05±2.70 to 36.90±3.27%, n=4, p=0.013) whereas the eNOS-BMC group increased non-significantly (28.86±1.38 to 33.50±2.02%, n=7, p=0.153, figure 5.3A). The LVEF worsened in the PBS control group (35.53±2.21 to 28.65±2.74%, n=4, p=0.034). When the mean absolute improvement in LVEF was compared to PBS control, both cell groups showed significant improvement (nt-BMC: 7.85±1.46 vs. -6.88±1.94, p=0.029); eNOS-BMC: 4.64±2.83 vs. -6.88±1.94, p=0.042) as well as the combined cell therapy group (eNOS- and nt-BMCs groups merged, 5.81±1.88 vs. -6.88±1.94, p=0.016), with no significant difference between eNOS and non-transfected cell groups (p=0.573, figure 5.3B).

Infarct volume did not significantly decrease in the nt-BMC group (8.70±2.95 to 5.79±1.06, n=4, p=0.231) but trended toward a reduction in the PBS group (8.33±0.10 to 6.54±0.73, n=4, p=0.078) and was significantly reduced in the eNOS-BMC group (8.26±0.78 to 6.60±0.47, n=7, p=0.037). When the mean absolute change in infarct volume was analyzed however (figure 5.3D), there were no differences between any of the three groups (control: -1.79±0.68, nt-BMCs: -2.9±1.94, eNOS-BMCs: -1.66±0.63) or the merged "all cells" group (-2.11±0.77).

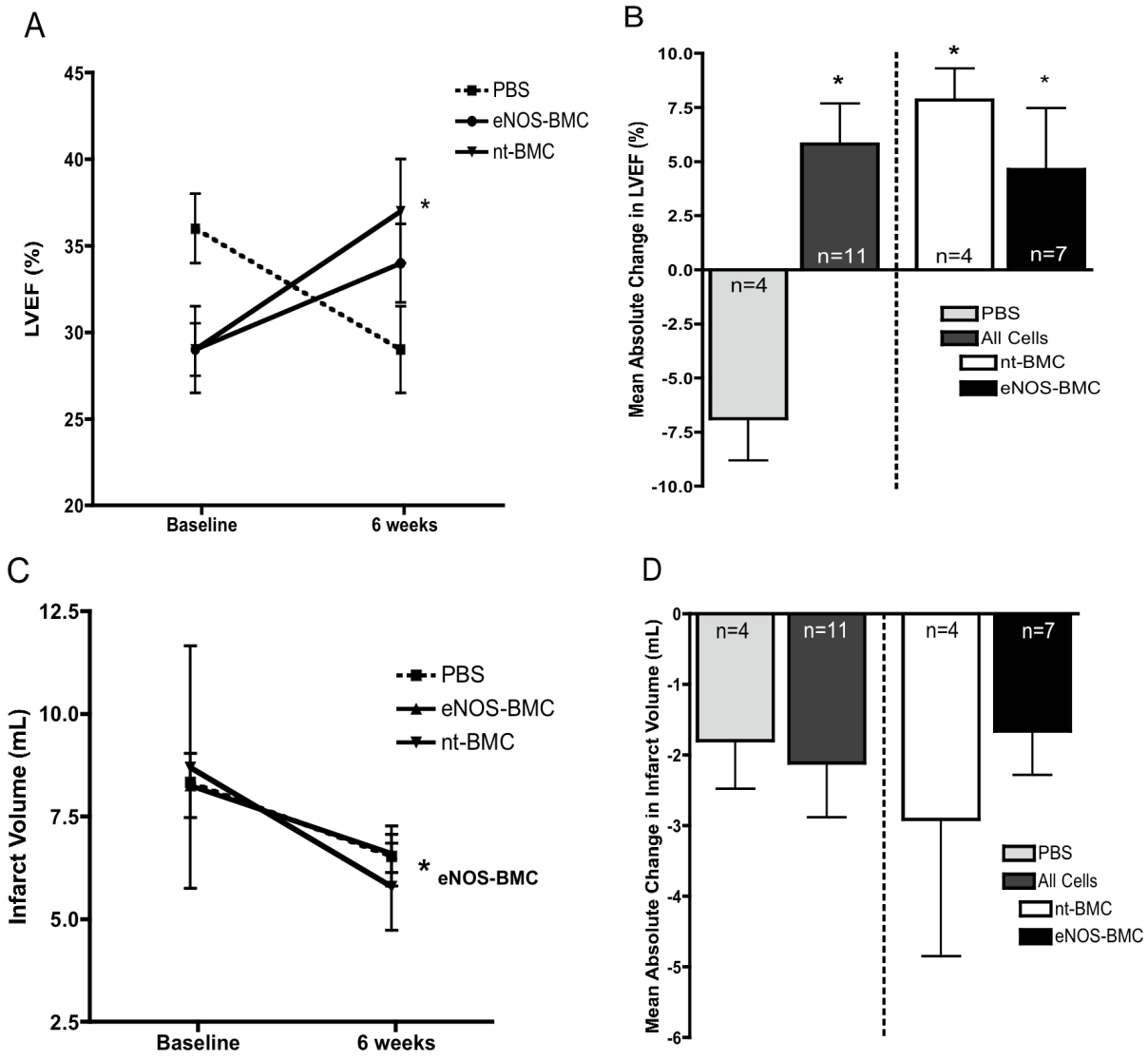


**Figure 5.3: Changes in Cardiac Function Following Delivery of eNOS Transfected BMCs**
The mean LVEF and infarct volume in each group are shown in panels A and C respectively. The mean absolute change in LVEF and infarct volume are shown in panels B and D, respectively. For the latter, the "all cells" groups refers to the combination of the eNOS-BMC and nt-BMC groups, which are also shown individually on the right. * represents $p<0.05$.

## 5.4 Discussion

*Comparison of BMC and PBS Delivery*

These results show that magnetically labeled cells can be tracked as long as 6 weeks using MRI. Immediately following delivery the cells appear in the infarct-border region but over time there is redistribution into the healing scar.

Although it has previously been reported that EPCs preferentially migrate to the infarct-border region[451,459], the rapidity with which the EPCs appeared in this region is consistent with IC delivery, and the lack of labeling of the infarct core likely reflects a relative lack of perfusion of this region or a sub-optimal environment for cell seeding. In contrast, Baklanov et al.[455] showed that feromoxide-labeled cells diffusely distributed within the infarct core itself following intra-coronary infusion in a similar swine model. The model we employed includes a longer occlusion time compared to that of Baklanov et al. (90 vs. 60 minutes), which may explain this apparent discrepancy. The present model is associated with both microvascular obstruction (MVO) and intra-myocardial hemorrhage[458].

Interestingly, six weeks following infusion, the persisting cells, though lower in number, appear within the infarct region itself. Although the exact position of the cells may be affected by infarct remodeling and/or growth of the heart between one and six weeks, this may also be due to migration of the cells into the infarct zone. Migration or homing of cells into infarction zones have been described following intravenous and intra-myocardial delivery[460,461] but this is the first report of this pattern following IC delivery using MRI. The presence of cells at the 6-week time point is noteworthy. In an attempt to maximize cellular retention and efficacy, we waited at least 7 days following MI, to avoid delivering cells into the acutely damaged myocardium. This is

consistent with results of REPAIR-AMI, which showed that only patients receiving cells greater than five days following injury benefit from cell therapy[361]. Although performed in a rat model rather than large animal or humans, previous work from our group has also shown that day 7 is the optimal delivery time to ensure maximal cell retention[462].

In preliminary experiments, we found that passage along the central lumen of the OTW balloon catheter caused an 11% loss of viable cells (by trypan blue exclusion), likely due to shearing within the catheter. Therefore, the MRI scan performed immediately following cell delivery visualizes iron both within viable cells and also extra-cellular iron from lysed cells. Extra-cellular SPIOs are presumably rapidly cleared by phagocytes, such as macrophages, suggesting that the iron signal hypointensity witnessed at the two later time points (1 and 6 weeks) likely represents label only within viable cells. It is possible that persisting iron and fluorescent signals imaged in myocardial sections could represent phagocytosis by tissue macrophages of Bang's particles released from dying cells. Currently, tissue histology is being performed to determine whether the SPIOs are within macrophages or if iron-fluorescence containing cells can be seen as separate and distinct from macrophages. This data will help elucidate the data of signal retention following cell delivery.

In the first set of animals (BMC vs. PBS), we observed a significant improvement in both LVEF and infarct volume over the 6 week study period. However, the initial LVEF and infarct sizes were not equivalent between the experimental and control groups, reflecting ineffective randomization in a very small study. The small sample size is a limitation in making any conclusions on the efficacy of cell therapy in the setting of acute MI, although these results confirm several studies employing BMCs in porcine models of myocardial injury[55,62,87,450]. Long-term persistence of transplanted EPCs raises the possibility that these cells may have contributed

directly to revascularization or repair of the infarct area, although the mechanism of action cannot be determined from these studies, and paracrine effects are also likely.

*Efficacy of Cell Delivery: eNOS-Transfected BMC vs. Non-Transfected BMCs*

Although this experiment was designed to include equal numbers of animals in each group, the final data includes only four (4) animals in the control (PBS) and nt-BMC groups, and seven (7) in the eNOS-BMC group. The main complication was death in the first week post-infarction, prior to cell delivery. Further animals would need to be included in a blinded manner in order to fully evaluate the efficacy of each treatment group.

Nevertheless, we observed a significant improvement in LVEF in animals receiving nt-BMCs, whereas the eNOS-BMC group exhibited a similar increase in LVEF, however, did not reach significance. In contrast, the animals receiving PBS alone again showed a reduction in LVEF between 1 and 6 weeks. However, since the animals receiving PBS alone had a significantly greater LVEF at baseline than those receiving BMCs, making comparisons between the experimental and control groups is problematic. Given the relatively low sample size, it is quite possible that the higher LVEF in the saline treated animals may represent a sampling artifact. However, given that there was a higher mortality in this group, it is possible that this reflects the loss of animals that had lower contractile function post MI. When looking at the overall response of all animals receiving BMCs compared to PBS, however, there was a significant improvement in LVEF. The mechanism of any benefit seen in this study was not explored. Since we have established that the cells show sustained persistence, they may have contributed directly to revascularization or repair of the infarct area. Conversely, it is more likely that this was achieved

by paracrine means[50,75,463] as the majority of cells were still lost in the weeks following transplantation.

The finding that the eNOS-BMC group appeared to improve less than the nt-BMC group did (although there was no significant difference between the two groups) contrast with the results found with human cells in the previous chapter. However, it must be recognized that a major distinction between these studies is that while the latter used cells derived from patients with CAD, which exhibited a profound dysfunction in regenerative capacity, the current experiments were performed using EPCs from young, healthy swine, which exhibited excellent activity as evidenced by their ability to improve healing post MI. The combined effect of advanced age and chronic cardiac RFs, such as with the human patients' EPCs, may make these dysfunctional cells more responsive to eNOS overexpression than the pig BMCs are. Indeed, although not significant, the patient-derived EPCs exhibited a strong trend toward reduced eNOS expression compared to healthy subjects. In addition, the transfection procedure itself might have resulted in stress and subtle changes in pig BMC activity, even though overall viability was reasonably high. Unfortunately, due to the difficulty of performing these experiments, a "null transfected" group could not be included.

There are also several other differences in the model and experimental design that might also have contributed to these differences in results between the experiments with human and porcine EPCs. Firstly, the cell type employed was quite different. Although BMCs and circulating EPCs are often considered equivalent due to similar isolation methods and culture, their morphology and growth dynamics are quite distinct. Early EPCs are non-proliferative until approximately 14 days in culture and remain small (10-12 μm in diameter). In contrast, BMCs are immediately hyper-proliferative and often require passage within the first week in culture. The BMCs are

notably much larger (15-20 μm) and tolerate manipulation much more readily. Of note, human circulating EPCs do not tolerate Amaxa transfection, with over 50% of cells dying (although this can be improved with thorough optimization), while greater than 99% of BMCs survive.

Compared to lentiviral transduction, electroporation induces much greater levels of eNOS overexpression than does lentiviral transduction at MOI=3. This is confirmed as the overexpression of eNOS mRNA in the BMCs was over 1000-fold, compared to only 20-100-fold achieved with lentivirus in human EPCs. At this level of expression, eNOS might be in excess and lead to oxidative stress through SO production rather than NO. Further studies are needed to determine the optimal transfection protocol to optimize NO production. The various differences between the cells employed and the method of gene transfer might explain the discrepancy between the results of eNOS overexpression in human and porcine experiments.

In summary, following MI, MRI can be used to track intracoronary delivered, magnetically labeled BMCs over prolonged time periods, up to 6 weeks. Over this time, these cells appear to migrate from the infarct-border region into the infarct core. Administration of these cells is safe and results in improvement in LV systolic function and infarct volume. Although eNOS-overexpressing BMCs were not found to improve the systolic function compared to non-transfected BMCs, many cellular and experimental variables may explain this result, in particular in contrast to the effect of eNOS transduction in human EPCs. Further experiments are warranted to clarify the difference between the effect of eNOS overexpression in human and swine cells.

# 6 General Discussion

In summary, we hypothesized that bone marrow cells (BMCs) from rats with RFs and EPCs from humans with CAD and/or RFs have dramatically reduced neovascularization capacity in vitro and in vivo, which can be reversed by eNOS overexpression. BMCs were isolated from rat models of type II diabetes and the metabolic syndrome, and we showed a significant reduction in their ability to stimulate neovascularization in vitro and in vivo. In humans, we isolated circulating 'early EPCs' from healthy subjects and patients with CAD and RFs, and transduced them using lentiviral vectors containing either eNOS or GFP (sham). EPCs from patients had reduced in vitro migration in response to SDF-1 or VEGF, which was reversed by eNOS-transduction. In co-culture with human umbilical vein endothelial cells (HUVECs) on Matrigel, eNOS-transduced EPCs contributed to increased and more complex angiogenic tube formation compared to sham-transduced cells. Human EPCs from patients were ineffective in enhancing ischemic hind limb neovascularization and perfusion in a nude mouse, whereas eNOS-transduced EPCs resulted in a significant improvement compared to sham-transduced cells. In a swine model of acute myocardial infarction (MI), BMCs increased left ventricular function compared to sham. However, there was no benefit to eNOS overexpression in this model. Various differences in the models and procedures may explain the incongruous results obtained. Taken together, these results show that eNOS overexpression significantly improves the neovascularization capacity of EPCs of human subjects with CAD and RFs and could represent an effective adjunctive approach for the improvement of autologous cell therapies for cardiovascular disease.

The results presented in this thesis demonstrate, in various models, that cell therapy using BMCs or circulating EPCs is effective in stimulating neovascularization and improving systolic cardiac function. The finding that BMCs and early EPCs are dramatically dysfunctional in the presence of CAD and/or multiple cardiovascular RFs provides a caveat to their use for autologous cell therapy. Through the overexpression of eNOS, we were able to increase the bioavailability of NO in EPCs and partially restore their neovascularization capacity. The successful application of this approach in human trials of autologous cell therapy could potentially provide a novel adjunctive therapy following acute MI.

Since their discovery in 1997[3], EPCs have received a great deal of attention for their reported ability to repair damaged endothelium and stimulate angiogenesis. In various models of cardiovascular injury, such as endothelial injury, hindlimb ischemia and myocardial infarction, BM-derived and circulating EPCs showed benefit over placebo. They were able to prevent smooth muscle proliferation and intimal hyperplasia, improve hindlimb perfusion and increase LVEF. Some studies, although very few, even reported the transdifferentiation of EPCs into cardiomyocytes[464], which led to replacement of lost contractile cells following myocardial necrosis. Early clinical trials enrolling patients post-MI, although not designed or powered for efficacy, reported improved global and regional LVEF, reduced infarct size, abrogated remodeling and fibrosis as well as improved myocardial perfusion[354,359,465].

More recently, due to a lack of consensus of the mechanism of EPC-mediated tissue repair or a firm definition of a "true" EPC, the field of cell therapy has been met with greater skepticism. This sentiment was promoted by the publication of two high profile randomized controlled trials showing no statistically significant benefit of cell therapy[360,363] compared to placebo and the finding that EPCs and BMCs from patients with cardiovascular RFs have reduced therapeutic

benefit compared to healthy subjects[68,383]. To optimists, what has emerged is the idea that the administration of regenerative cells into a disease tissue is a complicated approach, with several parameters that need to be understood and optimized for its practical application.

The largest trial, REPAIR-AMI, led to an overall improvement in LVEF of 2.5%, and subgroup analysis showed a potential benefit of up to 5% if including only patients with large MI or delivery of cells greater than 4 days after symptom onset[361]. REPAIR-AMI also employed the most robust methods of cell processing, and Seeger et al.[365] showed that the methods used by ASTAMI investigators compromised the survival and neovascularization capacity of the BMCs and likely contributed to the negative result of this trial. A recent trial from Finland, FINCELL[466], also showed significant LVEF benefit following BMC injection, confirming the results obtained by the REPAIR-AMI investigators. With several meta analyses showing a mean benefit of cell therapy on LVEF of 3-4%[368,369], despite the inclusion of negative trials, a major question now is whether a larger, pivotal trial will show a similar result. Dr. Andreas Zeiher has recently announced that he has obtained sufficient funding to pursue such a trial, enrolling up to 1400 patients following acute MI across Europe. Thus, a more definitive clinical answer will be provided within 3 years, based on Dr. Zeiher's projections. If this trial is positive, it is speculated that BMC administration could become a therapeutic option for those presenting with large acute MI and requiring an adjunct to conventional reperfusion strategies.

The focus of our work, however, is on the reported EPC dysfunction found in the context of cardiovascular RFs and established coronary disease, which may limit the effectiveness of autologous cell therapy for those who need the therapy the most. If the cells can be modified to improve their regenerative function ex vivo prior to delivery, patients with multiple cardiovascular RFs may still receive the full benefit of cell administration.

Several studies have shown the impact of simple RFs on EPC function[421,427-429], and others have studied surrogate markers of EPC function, such as migration and correlated these to cardiovascular risk[36,383,412,430]. To gain a greater understanding of the degree of EPC dysfunction in the setting of multiple RFs, however, we isolated cells from rats with complex disorders, the metabolic syndrome and type II diabetes mellitus. Although we expected to find reduced neovascularization capacity of rat BMCs based on previous reports, there was complete lack of benefit compared to control (PBS). Only cells isolated from lean Zucker rats (LZ) were able to provide adequate hindlimb reperfusion following ischemic injury in recipient nude mice.

Furthermore, in vitro assays elucidated potential mechanisms of cell dysfunction, particularly the role of diabetes. BMCs isolated from 10-12 week old ZDF rats had significantly increased apoptotic rates and reduced endothelial network formation compared to cells from pre-diabetic rats (5-6 week old ZDF). This suggests a direct deleterious effect of hyperglycemia or insulin on the BMCs, which were dramatically increased in the 10-12 week old ZDF animals. To confirm these findings, BMCs can be cultured in high glucose medium (similar to levels seen in ZDF rats) and their apoptotic rate measured through more robust methods, such as by the measurement of caspase 3 activation or DNA fragmentation (TUNEL staining). Insulin may also be supplemented to the medium to assess the effect of this hormone on the angiogenic properties of the cells. Insulin is known to be protective in ECs and also stimulate eNOS in BMCs. However, eNOS has been shown to be uncoupled in EPCs derived from diabetic rats and humans, and may produce SO rather than NO in response to eNOS agonists. To answer this question, further experiments should be performed to measure NO production in isolated BMCs, either by quantifying its byproducts, nitrates and nitrites, or directly using NO-reactive dyes such as DAF-FM diacetate.

The results obtained using human peripheral blood-derived early EPCs confirm those found with the rat BMCs. Although EPCs from young, healthy subjects were found to be quite effective at restoring hindlimb perfusion in the nude mouse, those from patients with CAD and multiple cardiovascular RFs showed little improvement. Migration in response to VEGF and SDF-1 were also reduced; migration of the cells was shown to be indirectly correlated with the sum of the additive RFs (quantified by the FRS). Again, although these results were expected due to previous reports of reduced EPC migration and neovascularization capacity due to cardiovascular RFs, the severity of dysfunction was notable in this study. In vivo, there was no difference between high risk EPC delivery and PBS alone in the recovery of hindlimb perfusion, indicating a complete loss of regenerative function due to the additive RFs and current CAD. Not only were these cells unable to migrate toward VEGF and SDF-1 in vitro, they also had significantly reduced production of VEGF mRNA and protein as well as HGF protein, indicating a reduced ability to stimulate neighbouring cells in a paracrine manner. Although the precise impact of the reductions in angiogenic protein expression were not assessed in these experiments the paracrine function of EPCs has emerged as the most important mechanism for their neovascularization capacity. It is thus plausible that reduced angiogenic protein secretion may be an important mechanism of EPC dysfunction in these cells. There was also a strong trend toward reduced expression of eNOS mRNA compared to cells obtained from healthy volunteers, although protein levels could not be quantified by Western Blot. Since eNOS activity is known to be a strong contributor to EPC angiogenic function, and may modulate the response and production of other angiogenic agents (i.e. VEGF) this finding is critical. The stimulation of neovascularization via paracrine growth factors is the predominant theory of EPC function, but has yet to be shown definitively. Although proteins such as VEGF, HGF, SDF-1 and PDGF-BB

have been shown to be secreted by these cells, experiments blocking their production (via gene knockdown) or their binding availability in vivo (by injection of soluble receptors in the tissue) are warranted. These experiments might help elucidate the contribution of reduced VEGF and HGF production, if any, on the reduced neovascularization capacity of EPCs seen in this study.

Since eNOS and NO production are considered critical to endothelial and EPC function, including the stimulation of neovascularization, we sought to reverse EPC dysfunction by increasing NO bioavailability in the patients' cells. For human early EPCs we employed lentiviral vectors to overexpress eNOS protein, which led to stable transduction and continuous overexpression of mRNA, to levels 20-100 fold greater than usual. The degree of eNOS protein expression was more moderate (5-6 fold), however, leading to a 2-fold increase in NO production. While this may seem marginal and potentially ineffective, on the contrary, dramatic overproduction of eNOS may in fact be deleterious, especially in the context of cardiovascular RFs. eNOS may become uncoupled and contribute to further oxidative stress, especially if consistently overexpressed. While post-translational modifications inactivating the enzyme might abrogate this ROS overproduction, these mechanisms would likely be ineffective when faced with artificially elevated levels of eNOS protein. Indeed, lentiviral transduction of eNOS consistently increased NO and cGMP levels in patient's EPCs, with no detectable increase in SO production. This suggests that the administered eNOS remained coupled within the cell, at least as long as 5 days following transduction.

Part of this in vivo benefit of transduced EPCs can be explained by our findings that eNOS-transduced EPCs had improved migration and stimulation of EC angiogenesis in vitro. The latter assay is of particular relevance, since it measures the ability of EPCs to stimulate neovascularization of native endothelium, regardless of the cause. EPCs may differentiate into

ECs and contribute to the neoendothelium or they may act to stimulate migration and organization of HUVECs, perhaps by paracrine means. Although EPCs did not appear to differentiate and form into mature ECs, eNOS-transduced EPCs were found to be more strongly associated with the endothelial branch points and tubules. The improved endothelial network formation might then be explained by the greater association of eNOS-transduced cells at critical branch points, aided by their secretion of angiogenic agents. Why eNOS-transduced EPCs were more frequently bound to HUVECs needs to be more fully elucidated. We did not find a difference in the adhesive capacity of EPCs to activated endothelium. While this does not rule out the possibility of improved adhesion of eNOS-transduced EPCs to angiogenic ECs, it suggests other mechanisms of association in the co-culture angiogenesis assay.

Although we found differences in angiogenic gene expression and protein secretion in EPCs from high risk subjects, this thesis does not include an assessment of angiogenic agent expression following eNOS overexpression. Currently, we are performing such studies using RT-PCR arrays (SuperArray Biosciences). Preliminary studies (n=3) show increased mRNA expression of FGF-1, Angiopoietin-1, von Willebrand Factor, VEGFR-2, and many other genes involved in angiogenesis and endothelial function. We also see a modest reduction in superoxide dismutase, which may indicate a change in the oxidative status of the cells following transduction. These results require increased sample size to be interpreted appropriately and validated using protein assays such as ELISAs. If these are confirmed, however, they may shed light on some of the mechanism of eNOS overexpression on the improved neovascularization capacity of transduced cells.

Although gene transfer was not able to completely restore EPC function to levels of healthy subjects, this nevertheless represents a significant result. Expansion of these studies might

include the combination of ex vivo eNOS overexpression with small molecules, which activate eNOS or help maintain its coupled state, such as with statins, PPAR-γ agonists, or BH4. It is also important to consider the state of the recipient tissue, as ischemia releases several ROS and provides a potentially toxic environment for the delivered cells. With eNOS-overexpressing cells, this is particularly relevant, as the benefit of eNOS overexpression might be reduced by local inflammatory mediators acting to suppress or uncouple eNOS activity. Local tissue administration of SDF-1 or VEGF might also improve engraftment of delivered cells, while also activating a NO-mediated pro-migratory phenotype.

From 80 mL of human blood, we could only isolate sufficient cells to perform one assay from each blood sample, which is an important limitation of this project. With more cells, it would be important to correlate the degree of eNOS overexpression in a particular sample to the degree of benefit seen in functional assays. For the same reason, we could not include a "non-transduced" group to control for the potential effect of viral transduction per se on the function of EPCs. Similarly, we could not correlate the amount of VEGF or HGF production with the in vitro or in vivo function of the cells. In future experiments, it would be useful to obtain a much larger sample of MNCs, such as by leukopheresis, and plan to perform multiple assays on each sample, in addition to eNOS transduction.

Although the benefit of eNOS overexpression was not confirmed in a porcine model of acute MI, several cellular and experimental differences might explain the discrepancy with the human overexpression study, as summarized in chapter 5. Of note, these were young healthy pigs, and likely had no dysfunction in eNOS expression of production. In this way, these cells were likely less responsive to eNOS overexpression compared to the human EPCs, which were eNOS-deficient and dysfunctional. Also, the method of transfection, electroporation, is quite damaging

to the cells, and may reduce any benefit of eNOS overexpression, despite the cells surviving the insult. In future experiments, a sham-group (electroporation only) would help elucidate this potential effect. As mentioned above, the 1000-fold overexpression of eNOS mRNA may also be less effective than a more moderate overexpression.

We did, however, confirm the benefit of delivering autologous BMCs through the coronary circulation for the improvement of LVEF. While the PBS and BMC groups did not equal baseline LVEF, this may be in light of reduced survival of pigs with reduced LV function. Since the samples sizes are uneven, further inclusion of animals receiving saline and non-transfected BMCs will be included in this study to further evaluate the effect of BMCs in this model. We also described a method of tracking the cells using SPIOs and MRI. Although most cells do not persist past one week, those that do can be seen up to 6 weeks later, suggesting long-term engraftment. MRI tracking might become a feasible method for developing future trials of cell therapy, with the confirmation of localized delivery being important.

Many questions exist regarding the mechanism of cell therapy following delivery, which are not specifically addressed in this thesis. In the mouse model of hindlimb ischemia, we observed an initial rapid rise in limb perfusion at day 7, with slowed improvement following this time point in most groups. This might reflect a short-term impact of administered cells, either via clearance or death of the cells within this time period. Although the retention of cells is very low following injection into ischemic tissue, it is unknown whether the survival of the cells is necessary for their effect. It has been suggested that dying cells release many cytokines that have a therapeutic role via modulation of the immune system[99]. In contrast, a study using a thymidine kinase system that induces apoptosis following injection in vivo showed that live cells are required for sustained ventricular function following delivery into a model of MI[467]. In our experiments, we

only injected cells once in vivo and followed the progress of healing and remodeling for 4 or 6 weeks, in the mouse and pig models, respectively. It would be informative to deliver cells at multiple time points and determine whether there is any benefit to repeat versus single injections of live cells. Similarly, injecting cells encapsulated within agarose beads containing extracellular matrix proteins such as fibronectin and fibrinogen[468] might improve the cell survival (by the prevention of anoikis) and reduce clearance from tissue. With enhanced engraftment of live cells, it would be interesting to measure whether there is a benefit to survival in the mouse hindlimb or pig MI models. These experiments are underway and will help inform further cell therapy studies.

The evaluation of eNOS-overexpressing EPCs would be best evaluated in clinical trials post acute MI. Using cells that show no benefit over PBS in our experiments, current clinical trials show an improvement in LVEF of 2-4%, which is equivalent to the benefit seen with using pharmacologic reperfusion[469]. Thus, a further benefit of 2-3% using eNOS-transfected cells would be considered a great improvement. The ENOS and Cell Therapy – Acute Myocardial Infarction (ENACT-AMI) trial, which will begin enrolment in 2009-2010, is poised to answer the question of whether eNOS overexpression can contribute to improved myocardial function in a population of patients with large MI. This trial will compare the safety and efficacy of using early EPCs transfected with eNOS, non-transfected EPCs or saline alone in a randomized, double-blind method (n=30 per group). If this trial shows that eNOS transfection is effective clinically, the combination of cell and gene delivery might be considered as a feasible therapeutic strategy for patients with large infarctions, who otherwise would not benefit from conventional treatments. Furthermore, it would show that translational research in regenerative medicine can make a great impact on the health and lives of our citizens.

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