Characterization of Blood-Derived Human Progenitor Cells for Vascular Regeneration

David Putman, The University of Western Ontario

Supervisor: David Hess, The University of Western Ontario
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology
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CHARACTERIZATION OF BLOOD-DERIVED HUMAN PROGENITOR CELLS FOR VASCULAR REGENERATION

(Thesis format: Integrated Article)

by

David Putman

Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Cardiovascular disease (CVD) remains a leading cause of premature death worldwide. Despite advances in treatment of ischemic diseases including myocardial infarction and peripheral arterial disease (PAD), there remains a need for effective revascularization therapies. Although early cell therapy trials investigated use of mononuclear cells from autologous bone marrow, emerging evidence indicates that purified progenitor cell populations are required to induce optimal vascular regeneration. In addition, autologous cells show reduced efficacy due to CVD-related progenitor dysfunction. This thesis presents preclinical studies characterizing several blood-derived cell types for the development of cellular therapies to treat PAD, focusing on the use of allogeneic umbilical cord blood (UCB) hematopoietic progenitor cells (HPC) and bone marrow multipotent stromal cells (MSC). First, I demonstrated that human UCB cells prospectively purified based on high aldehyde dehydrogenase activity (ALDH\textsuperscript{hi}), promote the recovery of perfusion and vascularization in mice with acute unilateral hindlimb ischemia. Unfortunately, the rarity of ALDH\textsuperscript{hi} cells impedes clinical implementation for patients with PAD. To address this issue, I developed \textit{ex vivo} culture protocols to increase the number of ALDH\textsuperscript{hi} cells available for therapy. I demonstrated that after a 20-fold increase in total cell number, expanded HPC maintained robust vascular regenerative functions. Indeed, expanded UCB promoted endothelial cell survival and endothelial cell tubule formation by paracrine mechanisms. Notably, intramuscular injection of HPC significantly augmented recovery of perfusion and increased vascularization in the injured limb, and increased recovery of limb usage within one week of therapy. MSC have been widely investigated for vascular regenerative cellular therapies, however substantial variability between donor sources indicating a need for better markers to select pro-angiogenic MSC subsets for clinical applications. I demonstrated that MSC retaining high ALDH activity after expansion effected increased endothelial cell proliferation, survival, and tubule formation using \textit{in vitro} systems. Taken together, my work establishes the value of ALDH as a marker of cells with increased vascular regenerative potential. My work also reveals that UCB ALDH\textsuperscript{hi} cells, their \textit{ex vivo} expanded HPC progeny, and MSC retaining high ALDH-activity after expansion represent promising candidates for future development of vascular cell therapies for PAD and CVD.
Keywords

Stem Cell, Progenitor Cell, Cardiovascular Disease, Peripheral Arterial Disease, Ischemia, Regenerative Medicine, Cell Therapy, Umbilical Cord Blood, Aldehyde Dehydrogenase, Angiogenesis, Vasculogenesis, Hematopoietic, Endothelial, Mesenchymal.
Co-Authorship Statement

All studies presented in this thesis were completed by David Putman in the laboratory of Dr. David Hess with assistance from co-authors as listed below. David Hess contributed to conception, design, data analysis, interpretation, and manuscript preparation for all experiments.

Chapter 1:

Some of the sections in the Introduction were previously published in a book chapter co-authored with David Hess and Gillian Bell. Gillian was the primary author of the MSC cell therapy for diabetes sections. Furthermore, some figures presented in Chapter 1 are adapted from a methods paper co-authored with David Hess. Published Manuscripts:


Chapter 2:

Kevin Liu assisted with in vitro assays. Heather Broughton trained me how to do femoral artery ligation surgery and performed surgeries. Gillian Bell assisted with hematopoietic colony forming assays in Chapter 2. Published Manuscript:


Chapter 3:

Ayesh Seneviratne helped develop the ex vivo expansion protocol for HPC with me, and assisted with cell surface marker flow cytometry for HPC characterization. Mark Hewitt assisted with in vitro assays. Manuscript for Submission:

Putman DM, Seneviratne AK, Hewitt MK, Hess DA. Ex vivo expanded hematopoietic progenitors promote endothelial survival after ischemic injury.

iv
Chapter 4:

Erik Leci assisted with endothelial cell proliferation assays and Christine MacCauley assisted with tube forming assays in Chapter 4. Manuscript for Submission:

Putman DM, Leci E, MacCauley C, Hess DA. High aldehyde dehydrogenase activity functionally identifies MSC with increased support of vascular regeneration.

Appendix 1:

Methods paper co-authored with David Hess elaborating on experimental models developed for Chapters 2 and 3.

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To the funding agencies that allowed me to pursue my research and enabled me to share my work internationally, thank you for giving me this wonderful opportunity.

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<tbody>
<tr>
<td>7AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ALDH\textsuperscript{hi}</td>
<td>high aldehyde dehydrogenase activity</td>
</tr>
<tr>
<td>ALDH\textsuperscript{lo}</td>
<td>low aldehyde dehydrogenase activity</td>
</tr>
<tr>
<td>ANG1/2</td>
<td>angiopoietin-1/2</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BFU</td>
<td>blast forming unit</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>c-Kit</td>
<td>Mast/stem cell growth factor receptor, CD117</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CLI</td>
<td>critical limb ischemia</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned media</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>CX3CL</td>
<td>chemokine (C-X3-C motif) ligand</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>chemokine (C-X-C motif) receptor</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3′-diaminobenzidine</td>
</tr>
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<td>DEAB</td>
<td>diethylaminobenzaldehyde</td>
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<tr>
<td>DLL4</td>
<td>delta-like ligand 4</td>
</tr>
<tr>
<td>EBM</td>
<td>endothelial basal media</td>
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<td>endothelial cell</td>
</tr>
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<td>ECFC</td>
<td>endothelial colony forming cell</td>
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<td>Full Form</td>
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<td>-----------</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGM</td>
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<td>ENA-78</td>
<td>epithelial cell-derived neutrophil-activating peptide 78, CXCL5</td>
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<td>EPC</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
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<td>fibroblast growth factor receptor</td>
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<tr>
<td>Flk1</td>
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</tr>
<tr>
<td>FLT3L</td>
<td>fms-related tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>FMO</td>
<td>fluorescence minus one</td>
</tr>
<tr>
<td>GF</td>
<td>growth factor</td>
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<td>growth-regulated alpha protein, CXCL1</td>
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<td>GUSB</td>
<td>β-glucuronidase</td>
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<td>hematopoietic colony forming cell</td>
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<td>human leukocyte antigen</td>
</tr>
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<td>HMVEC</td>
<td>human microvascular endothelial cells</td>
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<tr>
<td>HPC</td>
<td>Hematopoietic progenitor cell</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>I-TAC</td>
<td>interferon-inducible T cell chemoattractant protein, CXCL11</td>
</tr>
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<td>IDO</td>
<td>indoleamine-pyrrole 2,3-dioxygenase</td>
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<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>insulin-like growth factor binding protein 3</td>
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<td>interleukin</td>
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<td>laser Doppler perfusion imaging</td>
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<td>low-affinity nerve growth factor receptor</td>
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<td>matrix metalloproteinase</td>
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<td>MNC</td>
<td>mononuclear cell</td>
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<td>mucopolysaccharidosis type VII</td>
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<td>mRNA</td>
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<td>MSC</td>
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<tr>
<td>MV</td>
<td>microvascular</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<td>ribonuclease/angiogenin inhibitor 1</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal cell-derived factor 1, CXCL12</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cells</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TIE</td>
<td>tyrosine kinase with immunoglobulin-like and EGF-like domains</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
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<td>thrombopoietin</td>
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<tr>
<td>UCB</td>
<td>umbilical cord blood</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>vascular endothelial growth factor-A/B</td>
</tr>
<tr>
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<td>vascular endothelial growth factor receptor 2</td>
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<tr>
<td>vWF</td>
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</table>
Chapter 1

1 Introduction

1.1 Cardiovascular disease

Despite significant progress and advances to improve treatments and management, cardiovascular disease (CVD) remains the leading cause of premature death worldwide and represents a huge burden on the health care systems in North America\(^1,2\). Broadly, CVD includes a diverse array of conditions and affecting the heart and blood vessels. Common classifications of CVD include both chronic conditions such as ischemic heart disease, peripheral vascular disease, cerebrovascular disease and heart failure and acute conditions like myocardial infarction and stroke. CVD accounts for roughly one third of all deaths and it is estimated that CVD and stroke-related healthcare costs for patients are now in excess of $300 billion in the US alone\(^1,3\).

1.1.1 Atherosclerosis

One common risk factor of CVD is atherosclerosis\(^4,5,6\). Atherosclerosis is characterized by the progressive development of complex lesions or plaques that protrude from the vascular lumen effectively narrowing the blood vessel\(^1,3,5,7\). In response to stressors like dyslipidemia, hypertension and inflammation, arterial blood vessel endothelial cells (ECs), which normally do not adhere circulating white blood cells, begin to express adhesion molecules, effectively attracting monocytes to the vessel intima\(^8\). These monocytes differentiate into tissue resident macrophages, which engulf lipoprotein from

\(^1\) Parts of this chapter have been previously published:


the interstitium and become foam cells. This stage is called the fatty streak, named for the appearance of the accumulated lipid filled foam cells in the developing lesion\(^8\). In the developing atheroma, SMC are recruited from the medial layer to the intima where they proliferate with the foam cells. Lipid pools form in the core of the lesion between the ECs and the accumulating foam cells and SMC comprising the pathologic intimal layer\(^9\). The lipid core becomes increasingly apoptotic and necrotic as SMC and foam cells die and release accumulated intracellular lipids and cholesterol. Advancing plaques become increasingly fibrotic and calcified as the lumen of the blood vessel becomes narrowed\(^7\).

As the lesion becomes larger and more fibrotic there is increased risk of developing what is called the vulnerable plaque, characterized by a thin fibrous cap, a large lipid-rich necrotic core, more inflammation and possible neovascularization and haemorrhage. The vulnerable plaque is more prone to initiating thrombosis where a part of the plaque breaks off and enters the circulation\(^10\), or the lesion may become complicated resulting in a hematoma if it becomes vascularized by recruiting new blood vessels\(^8\). Plaque rupture or thrombosis can lead to the acute ischemic events of myocardial infarction or stroke\(^7\).

Chronic reduction in blood flow to the heart or peripheral tissues due to severe narrowing, or stenosis, leads to overt tissue ischemia. Broadly, it has been shown that many factors influence the risk and development of atherosclerotic CVD including physical activity, cigarette smoking, cholesterol levels and blood pressure\(^11,12\).

1.1.2 Chronic vascular diseases

Ischemia is characterized by insufficient blood flow to a given tissue or organ. Chronic ischemia due to atherosclerosis in the limbs or extremities, like the lower leg, is called peripheral arterial disease (PAD)\(^13\). In contrast, coronary artery disease (CAD) presents where there are atheromatous lesions stenosing the coronary arteries feeding the myocardium of the heart which can lead to heart failure\(^14\). The epidemiology of vascular disease is relatively well described and is associated with increased age, and major risk factors for the development and progression of vascular disease include, smoking and diabetes mellitus\(^15,16\). More than half of patients with CAD have type 2 diabetes mellitus associated with insulin resistance and the severity and rate of progression of vascular disease complications is increased in patients with diabetes\(^16\). Furthermore, diabetes is
associated with increased risk for developing PAD. Patients with diabetes have higher rates of PAD and higher rates of intermittent claudication and poor wound healing in the peripheral extremities.\textsuperscript{17,18}

1.1.2.1 Coronary artery disease

CAD is a major component of CVD, contributing to millions of deaths each year due to myocardial infarction and affecting many more people who a debilitated by permanent disability due to progressive heart failure.\textsuperscript{5} The treatment of CAD has improved greatly since the Coronary Artery Surgery Study (CASS) clinical trial first demonstrated the use of coronary artery bypass surgical intervention.\textsuperscript{19} It is estimated that 1% of the Western world is affected by congestive heart failure and the mortality in late stage heart failure is very high, estimated to be above 60% within 10 years in patients above 65.\textsuperscript{14,20} Currently, the best treatment for end stage heart failure is heart transplantation.\textsuperscript{21} Unfortunately, heart transplantation is only an option for a very small subset of patients as there are too few donors to meet the demand for potential recipients. Furthermore many patients do not meet the eligibility criteria for transplantation and do not have any remaining viable medical or surgical options.\textsuperscript{22} In these patients there is a need for alternative therapeutic strategies as well as preventative therapies to reduce progression to end stage heart failure.

1.1.2.2 Peripheral arterial disease

Peripheral vascular or peripheral arterial disease (PAD), like CAD, is generally caused by macrovascular atherosclerotic plaque progression leading to arterial stenosis.\textsuperscript{13,23-25} Other major factors related to PAD incidence are advanced age, smoking and diabetes.\textsuperscript{26,27} PAD of varying severity is extremely widespread and is estimated to affect more than 27 million people in North America and Europe.\textsuperscript{28} The most common symptom in the diagnosis of PAD that presents in patients is intermittent claudication, or limping associated with muscle cramping after moderate exertion and tends to present first in the distal extremity of the calf muscle.\textsuperscript{24} As the arteries of the lower extremities become more severely narrowed the symptoms in the patient worsen. PAD is graded by severity based on degree of tissue ischemia, commonly using the Rutherford scale.\textsuperscript{29} The most
common therapy for PAD has focused on smoking cessation, increasing exercise to induce improved collateralization by arteriogenesis, pharmacological vasodilatation, and surgical revascularization\(^3^0\). Unfortunately, the progression of PAD severity advances aggressively culminating in the most severe form of PAD, critical limb ischemia (CLI)\(^2^4,3^1,3^2\). CLI is characterized by pain at rest and/or tissue loss in the ischemic limb\(^2^4\). Patients with PAD and concomitant diabetes and atherosclerosis often show endothelial dysfunction in the microcirculation in addition to the stenosis further complicating treatment in CLI as vascular regeneration is impaired\(^2^3,2^4\). In CLI there is a significant mismatch in the metabolic demand of the affected area and the ability of the vasculature to adequately perfuse the tissue\(^2^3,3^3\). This perfusion insufficiency causes pain at rest and can lead to persistent non-healing ulcers increasing the risk for co-infection of the wounds\(^2^3\). CLI patients have poor prognosis, leg amputation is required in 30% of patients after 1 year with CLI and mortality at 1 year is greater than 20%\(^2^8\). Notably, CLI patients have very poor psychological quality of life indices, about the same level as terminally-ill cancer patients\(^3^2\). Despite the benefit of surgical revascularization and endovascular catheterization, it is estimated that more than 50% of patients are ineligible for these treatments due to co-morbidity or anatomic complexity\(^3^4\). The lack of effective therapy for many CLI patients indicates a need for improved revascularization strategies.

### 1.2 Vascular biology

Blood vessels are the primary conduits though which blood flows to carry oxygen, carbon dioxide, nutrients, hormones, and blood cells to and from every organ of the body\(^3^5\). The fundamental structural unit of the blood vessel is the EC. The endothelium comprises the single inner layer of the blood vessel surrounding the central lumen through which the blood flows. ECs play an important role in the regulation of blood flow and serve as a barrier between the blood and interstitium. Because of a short diffusion distance (0.1 to 0.2mm) the entire body must be highly vascularised with a branching network of blood vessels\(^3^6,3^7\).

Capillaries are the smallest blood vessels and consist solely of a single layer of ECs surrounded by occasional pericytes. In larger vessels, arterioles and venules, the
endothelium is surrounded by pericytes and connective tissue. Larger and more complex, in arteries and veins the inner layer of ECs is surrounded by a basement membrane and supportive perivascular cells including pericytes and a thick layer of smooth muscle cells (SMC)\textsuperscript{37}. The heart pumps oxygenated blood from the lungs at high pressure through arteries to arterioles and finally to capillaries where nutrient and gas exchange occurs. From the arterial capillaries flow continues into venous circulation and passes from capillaries to venules to larger veins and back to the heart at lower pressure and speed. To accommodate higher pressure of arterial circulation, arteries have thicker layers of SMC compared to veins\textsuperscript{35,38,39}.

ECs help mediate local responses to injury and inflammation by modulating thrombosis and by permitting blood-derived immune cells to pass through to the interstitium\textsuperscript{40-42}. ECs are a heterogeneous cell type and display phenotypic differences between arterial and venous circulation and between organ source and size of vessel\textsuperscript{35,38,39,42}. In the healthy adult, ECs are primarily quiescent and have low turnover within the vessel. They are protected from injury by autocrine survival and maintenance signals, such as vascular endothelial growth factor-A (VEGF-A), angiopoietin-1 (ANG-1) and fibroblast growth factors (FGFs) \textsuperscript{42-44}. Due to their involvement in oxygen supply, blood vessels express oxygen sensors like prolyl hydroxylase domain 2 (PHD2) and hypoxia inducible factors like hypoxia-inducible factors 1α and 2α (HIF-1α and HIF-2α)\textsuperscript{36,45-47}. These permit the ECs to respond appropriately to changes in oxygen levels\textsuperscript{36}. In the perfused vessel, adjacent ECs form a tight monolayer interconnected by junctional molecules like VE-cadherin. The pericytes that surround the vessel share a basement membrane with the ECs and express factors like VEGF-A and ANG-1 that normally maintain homeostatic quiescence in the ECs\textsuperscript{36,48}.

1.2.1 Vascular development

The formation of the vasculature is an important early step in organogenesis during development of the embryo because as an organism grows it needs a more advanced system for oxygen and nutrient exchange and to remove metabolic waste\textsuperscript{37,49}. In the developing embryo the vasculature forms by two primary processes, vasculogenesis and angiogenesis. In vasculogenesis, mesodermal precursor cells migrate, aggregate and
differentiate *de novo* into a hemangioblast, a common precursor of both blood and ECs\textsuperscript{37,49,50}. These hemangioblasts create new blood vessels and form the primary vascular plexus of capillaries. Subsequent expansion and remodeling of the primary capillary plexus occurs by angiogenesis which encompasses many steps including adhesion, migration, proliferation, differentiation and tube formation from the already existing vessel\textsuperscript{36,44,49}.

1.2.1.1 Vasculogenesis in development

Vasculogenesis is the de novo formation of blood vessels from mesodermal progenitor cells. In the mouse the earliest marker of the hemangioblast is Flk1 also known as vascular endothelial growth factor 2 (VEGFR2). VEGFR2 is the primary receptor for VEGF-A. Flk1 marks a subset of Brachyury-positive (mesodermal) cells in the primitive streak which migrate to the extra-embryonic endoderm and form the vascular plexus, part of which will form blood islands. The outer cells in the blood islands are ECs while the inner cells become hematopoietic progenitor cells\textsuperscript{49}. The hemangioblast theory has been supported by findings from human embryonic stem cell *in vitro* differentiation studies\textsuperscript{51} and *in vitro* differentiation of isolated hemangioblasts from mouse embryos\textsuperscript{52}. After the formation of the vascular plexus, angiogenesis becomes the primary mechanism by which blood vessels develop and remodel in the embryo proper.

1.2.2 Angiogenesis

Angiogenesis is defined as the growth of new blood vessels from the preexisting vasculature\textsuperscript{44}. There are two main types of angiogenesis that occur, intussusceptive and sprouting angiogenesis. The primary difference is that sprouting angiogenesis is relatively slow and involves proliferation of ECs and subsequent invasion of vascularized tissues by the growing EC pool\textsuperscript{44}. Intussusceptive angiogenesis involves preexisting, perfused blood vessels which remodel into new vessels and occurs without need for EC proliferation\textsuperscript{53}. Sprouting angiogenesis occurs when ECs become activated by increased levels of angiogenic growth factors like VEGF-A, angiopoietin-2 (ANG-2), and FGFs\textsuperscript{43,54,55}. Chemokines which are released by hypoxic or inflammatory cells will also initiate pro-angiogenic activation in ECs\textsuperscript{56}. Broadly, angiogenesis includes extracellular
matrix and basement membrane remodeling, increased EC migration, and induction of EC proliferation leading to the formation of new blood vessel branches.

1.2.2.1 VEGF and NOTCH signaling in angiogenesis

VEGF signaling causes ECs to reduce VE-cadherin expression and EC-EC junctions loosen, increasing permeability allowing plasma proteins access to the interstitium around the vessel, laying down a scaffold for EC migration\(^57\). Single EC are selected to become the tip cell which lead the growing endothelial tube towards the angiogenic signal\(^58\). In response to a VEGF-A gradient, tip cells upregulate expression of Delta-like ligand 4 (DLL4) which activates NOTCH signaling in adjacent EC cells leading to downregulation of VEGFR-2 and establishment of a stalk cell phenotype\(^58\). This makes stalk cell less responsive to VEGF signaling compared to tip cells and the stalk cells become proliferative, causing the growing vessel sprout to lengthen by cell division. The tip cells develop filopodia enabling them to sense environmental signals and guidance cues to direct the growing vessel branch towards an angiogenic stimulus. Macrophages have been shown to act as myeloid bridge cells that help fuse the sprouting vessels with other growing branches enabling contiguous blood flow in a process known as vascular anastomosis\(^36,59,60\). Although initiated by activated ECs, complete angiogenesis is a complex process requiring the integration of multiple cell types and signaling pathways.

1.2.2.2 Angiogenesis in hypoxia

Hypoxia, or low oxygen tension, is an important mediator of angiogenesis\(^47,61\). The HIFs are transcription factors composed of an oxygen-sensitive \(\alpha\)-subunit (HIF\(\alpha\)) and a stable \(\beta\)-subunit (HIF\(\beta\)), also called the aryl hydrocarbon nuclear translocator (ARNT) which together regulate expression hypoxia-responsive elements\(^47,62\). Under normal oxygen tension, or normoxia, HIF\(\alpha\) is hydroxylated by prolyl-hydroxylase domain (PHD)–containing enzymes and subsequently ubiquitinated, tagging it for proteasomal degradation\(^62\). PHD requires oxygen to hydroxylate HIF\(\alpha\) and is therefore unable to initiate degradation of HIF\(\alpha\) during hypoxia\(^63\). Stable HIF\(\alpha\) forms heterodimers with HIF\(\beta\) and translocates to the nucleus where it regulates expression of hundreds of genes that regulate angiogenesis including VEGF-A\(^45,46,64\). Many other angiogenesis-related
genes have been shown to be regulated by hypoxia, further increasing the complexity of the angiogenic process.

1.2.2.3 Angiopoietin and FGF signaling in angiogenesis

The angiopoietins, ANG1 and ANG2, are important regulators of EC quiescence and angiogenesis. Homeostasis and response to angiogenic activation is regulated by angiopoietin signaling through the TIE receptors TIE-1 and TIE-2. ANG-1 is the primary agonist of TIE-2 and depending on signaling context, ANG-2 is a generally considered a competitive antagonist of ANG-1/TIE-2 signaling axis. Recently ANG-2 has also been shown to be a context-dependent TIE-2 agonist. Quiescence in EC is maintained by ANG-1 signaling through TIE2. ANG1 also promotes mural cell/pericyte coverage and increases deposition of basement membrane, further increasing vessel stability. In response to angiogenic stimuli, the tip cells and sprouting EC release ANG-2. ANG-2 competitively inhibits TIE-2 signaling thereby increasing pericyte detachment, vessel permeability and endothelial branch formation. In response to ANG-2, pericytes initiate proteolytic degradation of the basement membrane by releasing matrix metalloproteinases (MMPs) and detach from the vessel wall. FGF signaling induces angiogenesis directly in ECs or indirectly by activating secretion of other angiogenic factors from neighboring cell types. Furthermore, low levels of active FGF signaling is necessary in quiescent blood vessels as inhibition of FGFR signaling leads to vessel regression.

1.2.2.4 Extracellular matrix modification in angiogenesis

In vascular homeostasis the basement membrane between the ECs and perivascular cells helps maintain EC quiescence. During angiogenesis the basement membrane is degraded and the ECM begins to be remodeled by proteases like the MMPs. Proteolytic degradation of the extracellular matrix (ECM) liberates further VEGF and FGF release from deposits in the ECM as it is remodeled to be more accommodating to migrating and proliferating ECs. Matrix remodeling by MMP activity promotes EC migration and tube formation by creating a pro-angiogenic microenvironment.
1.2.2.5 Chemokine signaling in angiogenesis

Chemokines have been demonstrated to play a role in the regulation of angiogenesis in a number of pathophysiological conditions including ischemia\textsuperscript{36,56}. Chemokines can exert their role in angiogenesis both directly via actions on ECs and indirectly by recruiting leukocytes and modulating growth factor signaling. Chemokines are a family of secreted proteins classified into subgroups based on the spacing and presence of four conserved cysteine residues, C, CC, CXC, and CX3C chemokines\textsuperscript{77}. Several members of the CXCL family have been demonstrated to promote angiogenesis including CXCL1 (GRO-\(\alpha\)), CXCL5 (ENA-78), CXCL8 (IL-8), and CXCL12 (SDF-1)\textsuperscript{78-80}. The mechanism by which CXCL12 promotes angiogenesis is particularly well established\textsuperscript{81,82}. Angiogenic VEGF signaling has been demonstrated to induce EC and perivascular expression of the chemokine SDF-1/CXCL12 which in turn recruits circulating pro-angiogenic myeloid progenitor cells to the vasculature\textsuperscript{83}. These bone marrow (BM) derived angiogenic hematopoietic cells are proposed to provide critical paracrine support enhancing both inflammation and angiogenesis\textsuperscript{81-84}.

Of the CC family cytokines, CC2L (MCP-1, monocyte chemoattractant protein-1)\textsuperscript{85} and CCL5 (RANTES)\textsuperscript{86,87}, have been notably demonstrated to be angiogenic\textsuperscript{85,88}. MCP-1 is highly upregulated in vascular smooth muscle cell after injury to the blood vessel\textsuperscript{89,90}. MCP-1 signaling induces recruitment and transendothelial migration of monocytes and into the intima of the injured vessel\textsuperscript{91}. MCP-1 has been demonstrated to be an important mediator of monocyte-EC adherence which were shown to increase vascular regeneration and revascularization in a balloon-mediated endothelial injury model\textsuperscript{92}. Other studies have also implicated MCP-1 in recruitment of monocytes and macrophages and mediation of angiogenesis during wound repair\textsuperscript{93,94}. MCP-1 has also been implicated in directly promoting angiogenesis by activating chemokine receptors on ECs\textsuperscript{95}. RANTES has been shown to be necessary for angiogenesis to occur in rats with peripheral ischemia through proposed recruitment of myeloid cells to the site of injury\textsuperscript{87}. RANTES, which stands for regulated upon activation, normal T-cell expressed and secreted, also plays an important role in recruitment of monocytes and circulating angiogenic hematopoietic progenitor cells which modulate angiogenesis and vascular repair\textsuperscript{86,96,97}. CX3CL1 has
been demonstrated to promote angiogenesis directly in ECs from skin by recruiting angiogenic lymphocytes. In a similar fashion, other chemokines have been shown to modulate vascular repair and interact differentially with endothelium in various organs further underscoring the phenotypic differences in ECs by tissue residence and location in the vascular network.

1.2.2.6 Vascular remodeling and resolution of angiogenesis

Most of the newly formed blood vessel networks formed during angiogenesis after vascular trauma are chaotic, tortuous and form many dead ends not forming a complete loop from arterial to venous circulation. As such, these vessels are poorly perfused and considered immature, as they lack tight EC-EC VE-cadherin junctions and are consequently leaky. Furthermore, most newly sprouted vessels contain very few pericytes to stabilize them. For the new vessel to become functional and stable it must resume its quiescent state and recruit pericyte support. The recruitment of pericytes to the newly formed vessel is mediated primarily by the platelet derived growth factors (PDGFs), angiopoietins and transforming growth factor-β (TGF-β). In the growing vessel branch ECs secrete PDGF-B to recruit pericytes to stabilize the nascent vessel.

As blood flow is restored to a tissue the hypoxic stress is reduced and consequently, there is a decrease in secretion of angiogenic growth factors and activation of ECs. As the peak of vessel density is reached in the wound bed of ischemic tissue, anti-angiogenic signals begin to outweigh angiogenic stimuli and further sprouting is stopped as the vascular network begins to be pruned back to homeostatic levels. Fluid flow forces or shear stress in newly perfused branches initiates pro-survival mechanisms helping to prune the branching network to ensure optimal blood flow. While only a minority of vessels newly perfused by blood flow will mature and recruit perivascular support and ECM remodeling, the majority of vessel branches which are not adequately perfused will undergo regression. The generally accepted mechanism by which regression and vascular network pruning occurs is through apoptosis, programmed cell death.
During maturation and regression of the vascular network after injury or hypoxia the ECM is concurrently remodeled and new basement membrane components must be deposited surrounding the vessels to be stabilized. MMP-mediated remodeling during this stage can lead to dissociation of EC-ECM contacts and this has been postulated to promote regression of unstable non-perfused vessels. In contrast, tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) promote the deposition of new basement membrane around vessels that undergo subsequent functional maturation.

1.2.3 Arteriogenesis

During arteriogenesis pre-existing arterioles undergo considerable remodeling to form larger, functional collateral blood vessels. Arteriogenesis is stimulated by increased shear stress induced by narrowing in major arteries shunting increased flow into the smaller collateral arterioles. Commonly this occurs as an effect of arterial stenosis during pathological atherosclerotic arterial disease. The process of arteriogenesis is regulated by cytokines like MCP-1 which recruits hematopoietic support cells, and FGF which is mitogen of both ECs and smooth muscle cells. Collectively, the effects modulated during arteriogenesis stimulate extracellular matrix remodeling, and subsequent formation of multilayered arteries, inclusive of the intima (endothelium, basement membrane and pericytes) the media (smooth muscle cells and their ECM) and adventitia (fibroblasts and their extracellular matrix).

Arteriogenesis is mechanically driven by increased pressure which increases stress on the vessel wall; the vessel enlarges until the stress is normalized. The increased shear stress causes the endothelium of the arterioles to increase expression of adhesion molecules leading to increased recruitment of monocytes and macrophages which help drive arteriogenesis. In addition to recruitment of monocytes, circulating BM-derived hematopoietic progenitor cells are recruited to growing arteriole and promote further arteriogenesis. In order to accommodate the growing diameter of the arterioles the ECM must be remodeled in a process similar to that in angiogenesis. Despite an increase in collateral size after arteriogenesis is resolved, the collaterals still do not
recapitulate the full conductance of the artery they’ve replaced allowing for the refinement of endogenous repair by other pro-angiogenic processes.\textsuperscript{114,115,126}

1.2.4 Postnatal vasculogenesis

Vasculogenesis was originally thought to be restricted solely to embryonic development until 1997 when Asahara \textit{et al.} demonstrated evidence for peripheral blood-derived cells which were termed endothelial precursor cells (EPC).\textsuperscript{127} Asahara \textit{et al.} postulated that since angioblasts and hematopoietic cells arise in development nearly simultaneously in extra-embryonic blood islands and share expression of many cell surface antigens that they may share a common progenitor cell (the hemangioblast), which might persist in the adult organism, as is the case with hematopoietic stem cells. They isolated cells from human blood based on CD34 expression and from murine blood by Flk-1 (VEGF-R2) expression and showed some engraftment of transplanted EPC in close association with the injured endothelium. Because integrated EPC co-stained for mature EC marker they postulated that this was evidence of a postnatal vasculogenic process. Many of the findings of this paper became some of the defining and often controversial characteristics of a circulating EPC.\textsuperscript{128} Many subsequent studies have shown that several cell types that have been termed EPC are in fact hematopoietic cells with proangiogenic function and phenotype.\textsuperscript{128-132} Many of these pro-angiogenic hematopoietic cells facilitate robust vascular regeneration without stable integration into the endothelium, in effect supporting the literature on angiogenesis and arteriogenesis, and raising questions on the clinical applicability of postnatal vasculogenesis during vascular repair in the adult.\textsuperscript{81,82,124,133-138} In 2007, Yoder \textit{et al.} defined endothelial colony forming cells (ECFC) as putative EPC, which are rare blood-derived cells that form late-outgrowth adherent colonies of proliferative cells when propagated under strict EC growth conditions, and can be isolated at extremely low frequency from human peripheral blood, BM, and umbilical cord blood (UCB). ECFC are distinguished from pro-angiogenic hematopoietic cell types in human blood as they were exclusively CD45, and represented the only cell type that could integrate directly into perfused vessels \textit{in vivo}.\textsuperscript{128,130-132,139} It has been proposed that pro-angiogenic hematopoietic cells and vessel-resident ECFC cooperatively coordinate the vascular regenerative niche.\textsuperscript{140}
1.3 Cell therapy for vascular regeneration

The identification of molecular mediators, cells and genes involved in angiogenesis and arteriogenesis has presented the possibility of new therapeutic approaches to promote vascular regeneration during CVD\textsuperscript{3,21,23,33,115,141-143}. Many different cell types, including blood-derived angiogenic cells have been previously implicated in vascular repair and have been recently investigated in preclinical animal models and in recent human clinical trials as potential cell therapies for CVD\textsuperscript{21,23}.

1.3.1 Stem cell therapy

A stem cell is defined a cell that can both self renew indefinitely, creating more stem cells, and differentiate into different, more specialized cell types\textsuperscript{144-146}. Stem cells can be further classified by their potency, or more specifically their differentiation potential. A pluripotent cell is capable of differentiating and forming all tissues in the adult organism. These cells are found during development in the inner cell mass of a blastocyst\textsuperscript{147}. Pluripotent cells are absent in postnatal tissues but recently Yamanaka \textit{et al.} demonstrated that terminally differentiated cells like fibroblasts can be transcriptionally reprogramed to a pluripotent state\textsuperscript{148}. These cells, called induced pluripotent stem cells, hold promise in terms of potential ability to make any cell type with one’s one genetics for possible therapeutic applications\textsuperscript{149}.

In the postnatal, adult organism many tissues contain resident cells that demonstrate stem cell characteristics. These multipotent stem or progenitor cells, can form multiple different cell types, but are generally restricted to one tissue type or germ layer from which they are derived. An example of the best-characterized multipotent stem cell is the hematopoietic stem cell which can form all lineages of blood cells found in circulation\textsuperscript{150}. A progenitor cell can form multiple (multipotent) or one (unipotent) type of more specialized cell, but has lost the capacity to self-renew over the life of the organism. An example of a unipotent progenitor cell that has been widely studied in the context of postnatal vasculogenesis is the endothelial progenitor cell (EPC/ECFC) which can differentiate into mature ECs\textsuperscript{129}. 
Bone marrow, mobilized peripheral blood and UCB have been intensely studied as easily accessible and readily-available sources of adult progenitor cells for cell therapy for CVD. Adult or postnatal progenitor cells obtained from human BM or UCB are comprised of a heterogeneous array of regenerative cell subtypes and represent transplantable cells used to reconstitute hematopoiesis in hematological malignancies or to facilitate the repair of damaged or diseased tissues via paracrine effects. However, with the exception of hematopoietic stem cells (HSC) with the phenotype lin-CD34+CD38-CD45RA-Thy1+Rho+CD49f+, prospective purification of infrequent BM- or UCB-derived stem and progenitor cells for use in targeted regenerative therapies is currently underutilized. Indeed, non-hematopoietic stem and progenitor cell subtypes from BM and UCB demonstrate a paucity of specific markers of differentiation in situ, and stem cell surface markers can also vary between species, source, and cell cycle progression. As described in terms of postnatal vasculogenesis, circulating EPC/ECFC which can integrate into repairing vasculature or support angiogenesis show much promise as potential cell therapies for vascular regeneration. Many hematopoietic cell types thought to be EPC in fact represent pro-angiogenic hematopoietic cells.

Asahara et al. originally identified endothelial precursor cells (EPC) as a population of circulating progenitor cells in human peripheral blood that differentiate into mature endothelial cells (EC) in vitro and contribute to vessel formation after transplantation into SCID mice. Later studies showed that these cells expressed the primitive cell markers CD34, CD133, and KDR (VEGFR-2) and can be obtained from other sources such as BM and UCB. However, this cell phenotype was shared by hematopoietic progenitor cells, making discrimination of hematopoietic versus endothelial lineage commitment controversial. Because both myeloid hematopoietic and endothelial progenitor cells have been shown to promote angiogenesis in mouse models, Yoder et al. functionally demonstrated that true EPC are plastic-adherent blood-derived cells propagated in strict EC growth conditions that form proliferative colonies of CD45- ECs capable of forming perfused vessels in gel implants in vivo, termed ECFC. In contrast, the nonadherent CD45+ blood-derived cells that co-expressed typical EC markers (CD31) were not actual
EC precursors but myeloid/macrophage lineage cells, which did not incorporate into newly formed vessels, yet can contribute to angiogenesis through proposed paracrine signaling to activated vessel-derived EC\(^{24,129,134-136,138}\).

With accumulating evidence indicating the promise of multiple BM-derived cell populations for vascular regeneration, clinical trials were initiated investigating the transplantation of heterogeneous BM mononuclear cells (MNCs) to treat limb ischemia\(^{23,161,162}\) and to promote cardiac repair\(^{21,153,163-165}\). However, while many of these trials demonstrated promising results in promoting revascularization, scientists have undertaken studies to take a closer look at the specific progenitor cell fractions that show vascular regenerative promise\(^{166}\).

Using nude mice and femoral artery ligation with complete excision of the femoral artery and vein, López-Holgado et al. demonstrated that treatment with both CD14\(^+\) monocytes and CD133\(^+\) cells from mobilized peripheral blood demonstrate significantly improved perfusion in the surgical limb compared to vehicle treated mice. That research group showed furthermore that these hematopoietic cell population do not transdifferentiate into true endothelial cells or EPC\(^{167}\). Lai et al. demonstrated that various sources of EC support recovery of perfusion in a similar hindlimb ischemia model in SCID mice\(^{168}\). They showed that human umbilical vein EC (HUVEC), EC derived from embryonic stem cells, EC derived from induced pluripotent stem cells, and EC derived from patient BM supported augmented recovery of perfusion compared to vehicle control mice. They derived EC from BM by plating MNC in endothelial growth media (EGM2) and selecting adherent cells similar to ECFC derivation as published by Yoder. Notably they also demonstrated derivation of EC from human BM was more challenging from patients with CVD indicating the value of allogeneic transplantation strategies to treat CVD\(^{168}\).

Recently, using a model of very severe induction of ischemic injury by burning the femoral artery whereby some untreated mice completely lose the surgical limb, Vu et al. demonstrated that treatment with heterogeneous UCB MNC or \textit{ex vivo} expanded CD34\(^+\) CD133\(^+\) cells prevented limb loss and improved recovery of limb function and reduced necrosis\(^{169}\). Vu et al. termed their cells EPC however they did not show that the CD34\(^+\) CD133\(^+\) cells they used were non-hematopoietic or that they could directly integrate into
newly formed vessels making it likely that their “EPC” were in fact a mixture of endothelial and angiogenic hematopoietic cells as shown by others.\textsuperscript{134,167} Notably, there is evidence that there is significant variation in the basal recovery rate of perfusion after femoral artery ligation surgery between different mouse strains, an important caveat for comparing the magnitude of perfusion recovery results between studies\textsuperscript{170,171}.

1.3.1.1 Mesenchymal stromal cells

Mesenchymal stromal/stem cells (MSC), also referred to as multipotent stromal cells, can be isolated from bone marrow and other tissues and have been demonstrated to be a multipotent mesenchymal cell type that differentiate in bone, fat, and cartilage. MSC are defined by their \textit{ex vivo} culture method; they must be plastic adherent, express the markers CD73 (ecto 5’ nucleotidase), CD90 (Thy-1), and CD105 (endoglin) after culture and must not express hematopoietic markers like CD45, CD14\textsuperscript{172}. In addition, MSC need to demonstrate the definitive multipotential differentiation into osteocytes, adipocytes and chondrocytes\textsuperscript{173}. Strictly speaking, MSC should properly be called multipotent mesenchymal progenitor cells as they have not been demonstrated to display full self-renewal capacity to meet the full criteria of a stem cell\textsuperscript{174}.

MSC are widely considered as good candidates for allogeneic cell therapy applications because of their low immunogenicity; they do not express MHC class II (HLA-DR) nor costimulatory molecules like CD40, CD80 and CD86\textsuperscript{175}. Furthermore, MSC have been shown to demonstrate low levels of engraftment and little proliferative capacity after transplantation, reducing risk of transplant malignancy, but also potentially limiting long term therapeutic efficacy\textsuperscript{176}. In light of low levels of engraftment the currently hypothesis regarding MSC contributions towards regenerative therapy is primarily through the modulation of the tissue microenvironment as opposed to cell replacement\textsuperscript{154,177}.

MSC have been shown to augment cardiomyocyte\textsuperscript{178} and EC\textsuperscript{176,179} survival. MSC have also been shown to secrete many angiogenic factors including, VEGF-A, FGF-2, ANG-1, and MCP-1\textsuperscript{176,179-182}. MSC have also been recently identified as a component of vascular pericytes in multiple human organs that express CD146 \textit{in situ} and help stabilize newly formed vessels when co-transplanted with ECs \textit{in vivo}\textsuperscript{183,184}. MSC have furthermore been
shown to play an important role in immunomodulation presenting interesting potential paracrine applications in auto-immune disease and inflammatory conditions\textsuperscript{185}. Taken together these findings show the considerable promise of MSC from human BM or UCB as a potential source for vascular regenerative cell therapies.

1.4 Aldehyde dehydrogenase: a functional marker of stem cells for cell therapy

In order to simultaneously isolate stem and progenitor cells from multiple lineages, BM or UCB mononuclear cells (MNC) can be purified based on a conserved stem cell characteristic, high levels of aldehyde dehydrogenase (ALDH) activity, an intracellular enzyme first reported to be highly expressed in primitive hematopoietic\textsuperscript{186}, and neural progenitor cells\textsuperscript{187}. ALDH\textsubscript{1A1} activity is involved in oxidation of vitamin A to retinoic acid and is predominantly implicated in the protection of long-lived cells from oxidative damage\textsuperscript{186}. Most notably, ALDH activity is downregulated as primitive cells differentiate towards maturity, making ALDH activity a unique function to distinguish essential regenerative precursors from expendable cells. Our group and others have recently shown that high ALDH activity is also a property shared by regenerative progenitors of endothelial and mesenchymal lineages\textsuperscript{134,188}. Purification of BM or UCB cells based on high ALDH-activity can be used to simultaneously isolate adult stem and progenitor cell subtypes for the preclinical development of regenerative therapies inducing tissue repair. Focusing on transplantation studies using human ALDH-expressing progenitor cells for hematopoietic reconstitution, blood vessel formation, and islet regeneration in immunodeficient mice, the Hess laboratory aims to understand how the multiple progenitor subtypes act together to formulate a regenerative niche and to coordinate complex regenerative processes.

1.4.1 FACS purification of multiple human progenitor subtypes using ALDH activity

Intracellular ALDH activity can be quantified using a fluorescent substrate for ALDH, termed Aldefluor reagent\textsuperscript{186}. First synthesized by Clayton Smith’s group in 1999, Aldefluor reagent is a Bodipy fluorochrome conjugated to an aminoacetaldehyde
molecule, an uncharged moiety that can freely cross through the cell membrane. Once inside the cell, cytoplasmic ALDH1A1 converts Aldefluor into a metabolized by ALDH into an anion that becomes trapped in the cell due to its negative charge. Under pharmacological inhibition of ABC transporters contained within Aldefluor buffer, cells with high ALDH activity retain Aldefluor substrate and fluoresce brightly, while cells with lower ALDH activity are more dimly fluorescent. Thus, high-speed fluorescence-activated cell sorting (FACS) can efficiently purify UCB or BM MNC with low side scatter and low versus high ALDH activity. The integrity and function of the isolated cells are not compromised by this procedure since upon removal of the Aldefluor buffer, ATP-binding cassette transporters become reactivated, and Aldefluor is actively effluxed, returning the cell to its original state. Thus, the Aldefluor purification procedure is clinically applicable for the efficient sorting of multiple functional human progenitor cell types based on a highly conserved stem cell function. The amount of ALDH activity in all viable cells falls along a spectrum from low ALDH activity (ALDH\textsubscript{lo}) to high ALDH activity (ALDH\textsubscript{hi}), where ALDH\textsubscript{lo} versus ALDH\textsubscript{hi} cells are distinguished by cluster gating using diethylaminobenzaldehyde or DEAB, a pharmacological inhibitor of ALDH1A1. The basic premise of the Aldefluor assay to assess ALDH activity with FACS is summarised in Figure 1.1.

It has previously been shown that purified ALDH\textsubscript{hi} cells from BM and UCB highly co-expressed stem cell-associated surface markers (CD34, CD133, c-kit) and were enriched for multipotent hematopoietic and mesenchymal stromal progenitors, as well as precursor cells with endothelial colony forming cell (ECFC) capacity in vitro\textsuperscript{134,156,188,189}. In contrast, ALDH\textsubscript{lo} cells were primarily comprised of mature leukocytes (primarily T- and B-cells) and demonstrated little progenitor function in vitro\textsuperscript{134}. Therefore, high ALDH activity simultaneously purifies multiple progenitor cell subtypes ideal for lineage-specific expansion \textit{in vitro}. Subsequently, purified ALDH-purified mixed progenitor cells or their \textit{ex vivo} expanded progeny can be assayed for regenerative functions after xenotransplantation into a variety of immunodeficient models of tissue damage.
Figure 1.1. Schematic overview of FACS isolation of ALDH<sub>hi</sub> cells using Aldefluor.

(A) Aldefluor passively diffuses into cells where is metabolized by ALDH into an anion which can no longer diffuse out of the cell, this leads to accumulation of a fluorescent signal that can be detected and selected by FACS. (B) DEAB inhibition of ALDH activity identifies ALDH<sub>lo</sub> population for gating. (C) Aldefluor labeling allows efficient selection of ALDH<sub>hi</sub> cells from a heterogenous population of UCB MNC by FACS.
Figure 1.1

A

DEAB

Aldefluor

ALDH

No Product

B

Aldefluor + DEAB

Side Scatter vs. Aldefluor

C

Aldefluor

Side Scatter vs. Aldefluor

Progenitor Cell

High ALDH activity

Aldefluor-anion
1.4.2 Cells with high ALDH activity possess hematopoietic repopulating capacity

Hematopoietic stem cells (HSC) and lineage-specific hematopoietic progenitors are responsible for the replenishment and maintenance of blood after BM transplantation\(^{190,191}\). These cells can be isolated from BM, cytokine-mobilized peripheral blood, or UCB based on expression of the cell surface markers CD34 and CD133, and assayed for hematopoietic repopulating function after transplantation into sublethally irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice\(^{192-194}\). Storms et al. first established that the ALDH\(^{hi}\) fraction of human UCB was enriched for primitive hematopoietic progenitors \textit{in vitro} and was depleted of lineage-committed hematopoietic cells\(^{186}\). Subsequently, prospective lineage depletion in combination with the commercially available Aldefluor reagent (Stemcell Technologies, Vancouver, Canada) was used by our group to demonstrate that transplantation of human UCB ALDH\(^{hi}\) cells into NOD/SCID mice resulted in multilineage human hematopoietic engraftment\(^{156}\). Greater than 70\% of the UCB ALDH\(^{hi}\) cell population co-expressed the HSC-associated cell surface markers CD34 and CD133\(^{156}\). ALDH\(^{hi}\) CD34\(^{+}\) cells were highly enriched for short-term myeloid progenitors while ALDH\(^{hi}\) CD34\(^{-}\) cells represented precursors to the CD34\(^{+}\) population that also demonstrated NOD/SCID-repopulating cell (SRC) capacity\(^{195-197}\).

Our laboratory has also demonstrated that while both ALDH\(^{lo}\) CD133\(^{-}\) and ALDH\(^{hi}\) CD133\(^{+}\) cells demonstrated clonogenic hematopoietic progenitor function \textit{in vitro}, only the ALDH\(^{hi}\) CD133\(^{+}\) population was able to engraft the murine BM after intravenous injection\(^{198}\). Furthermore, prospective selection based on both high ALDH activity and CD133 increased the frequency of SRC by tenfold compared to selection by CD133 alone. Notably, ALDH\(^{hi}\) CD133\(^{+}\) cells demonstrated enhanced hematopoietic repopulating function in serial secondary transplants while maintaining primitive hematopoietic phenotypes (CD34\(^{+}\)CD38\(^{-}\))\(^{198}\). In addition to long-term hematopoietic repopulating function, human UCB ALDH\(^{lo}\)CD133\(^{+}\) cells also showed previously unrecognized engraftment in nonhematopoietic tissues such as the liver, lung, heart,
brain, pancreas using the highly sensitive human cell-tracking NOD/SCID MPSVII model\textsuperscript{199}.

Later studies established that hematopoietic engraftment after human UCB transplantation in immunodeficient mice occurs faster with increasing ALDH\textsuperscript{hi} cell doses\textsuperscript{200}. Similar to CD34 expression, clinical reconstitution rates following transplantation of BM or mobilized peripheral blood can be directly correlated with the number of ALDH\textsuperscript{hi} cells infused\textsuperscript{201,202}. As a result of these promising preclinical data and direct potential for clinical translation, recently completed clinical trials designed to assess the safety and efficacy of transplanted allogeneic human UCB ALDH\textsuperscript{hi} cells to enhance the rate of engraftment in the treatment of haematological dysfunction (www.clinicaltrials.gov trial no. NCT00692926) have been reported. In summary, high ALDH activity is now well established as a functional characteristic of repopulating hematopoietic cells, and ALDH activity appears to be a superior indicator of the quality of BM or UCB samples for transplantation compared to standardized CD34\textsuperscript{+} counts\textsuperscript{201}.

1.4.3 Cells with high ALDH activity for vascular regeneration

Although the ALDH\textsuperscript{hi} population from human BM or UCB is more than 90% hematopoietic in origin, it has been shown that the ALDH\textsuperscript{hi} fraction of BM is also significantly enriched for ECFC compared to ALDH\textsuperscript{lo} cells and that high ALDH activity was downregulated as a result of differentiative culture \textit{in vitro}\textsuperscript{134}. Thus, consistent with the classification by Yoder et al., true ECFC initially possesses elevated ALDH activity. Nagano et al. expanded EPC in culture from UCB and subsequently sorted the CD45\textsuperscript{−} EPC progeny based on ALDH activity\textsuperscript{203}. Interestingly, they found that the more differentiated ALDH\textsuperscript{lo} outgrowth population was more proliferative \textit{in vitro} and showed higher expression of hypoxia-inducible factors (HIF1α), VEGF, and the chemokine receptor CXCR4 under hypoxic conditions. They further showed that the cultured ALDH\textsuperscript{lo} outgrowth represented more mature EC that recruited effectively to the site of ischemia and reduced necrosis in a mouse skin flap model of ischemic wound repair\textsuperscript{203}.

Consistent with the idea that high ALDH activity simultaneously purifies stem and progenitor cells from multiple lineages, our group and others have shown that in addition
to HSC and tubule-forming EPC, the BM ALDH\textsuperscript{hi} fraction was highly enriched for MSC that efficiently differentiated into fat, cartilage, and bone in differentiation cultures \textit{in vitro} \textsuperscript{134,189}. Capoccia et al. went on further to describe that transplanted human BM ALDH\textsuperscript{hi} mixed progenitor cells transiently recruited to areas of ischemia and augmented the recovery of blood flow (perfusion ratio of ischemic/control limb of 0.7±0.1) by stimulating the endogenous revascularization of ischemic limbs in immunodeficient NOD/SCID/β2microglobulin null and NOD/SCID/MPSVII mice with acute unilateral hind limb ischemia induced by femoral artery ligation compared to vehicle control treated mice at 21 days after treatment (0.3±0.1)\textsuperscript{134}. Even without permanent engraftment at the site of ischemia, a low dose of ALDH\textsuperscript{hi} cells were more effective at inducing revascularization than transplantation of unsorted BM nucleated cells containing the equivalent of fourfold more ALDH\textsuperscript{hi} cells. Recent work by Sonderaard \textit{et al.} has shown that intravenously transplanted UCB ALDH\textsuperscript{hi} cells recruited specifically to the ischemic myocardium where they augmented vascular density in a murine model of myocardial infarction\textsuperscript{204}.

Although the detailed mechanisms by which ALDH\textsuperscript{hi} cells induced endogenous revascularization remains an active area of investigation in the Hess lab, our working hypothesis describes that high ALDH activity simultaneously depletes for inflammatory immune cells and enriches for multiple proangiogenic progenitor subtypes. After transplantation, ALDH\textsuperscript{hi} progenitor subtypes recruit transiently to areas of regional hypoxic damage and contribute to the generation of a proangiogenic microenvironment by potentially providing both structural and paracrine support.

Despite the paucity of mechanistic details regarding the vascular regenerative potential of ALDH\textsuperscript{hi} cells, clinical trials have been initiated to explore the safety and efficacy of the use of autologous BM ALDH\textsuperscript{hi} cells in many CVD conditions\textsuperscript{205,206}. The FOCUS-Br trial was undertaken to investigate intramyocardial injection of autologous ALDH\textsuperscript{hi} cells for therapeutic angiogenesis (www.clinicaltrials.gov trial no. NCT00314366). The results of the trial showed evidence that transplantation intramyocardial transplantation of ALDH\textsuperscript{hi} cells is safe and supported possible functional benefits in the setting of chronic
myocardial ischemia indicating need for further clinical testing in a larger cohort to fully assess efficacy\textsuperscript{207}.

BM ALDH\textsuperscript{hi} cells have also been investigated in clinical trials for treatment of critical limb ischemia (NCT00392509). Intramuscular transplantation of autologous BM ALDH\textsuperscript{hi} cells were directly compared to unfractionated autologous BM MNC in PAD patients with a Rutherford score of 4 (resting pain) or 5 (ulceration or necrosis)\textsuperscript{208}. Results of the study demonstrated safety and patients that received autologous BM ALDH\textsuperscript{hi} cells showed improved Rutherford category and reduced resting pain. Furthermore patients in the ALDH\textsuperscript{hi} treatment group showed improved ankle to brachial index scores more rapidly than the MNC group indicating improved perfusion of the lower limb. However, patients in both cohorts reported significantly improved quality of life indices.\textsuperscript{208} More recently, a phase 2 placebo-controlled randomized clinical trial has been initiated to investigate transplantation of BM ALDH\textsuperscript{hi} cells to improve symptoms of intermittent claudication (NCT01774097). Another phase 2 clinical trial is currently underway to investigate intracarotid infusion of BM ALDH\textsuperscript{hi} cells for patients after and ischemic stroke (NCT01273337).

1.4.4 ALDH-purified cells stimulating islet revascularization and repair

The Hess laboratory focuses on the development of pre-clinical models to study the potential utility of post-natal stem cells for the treatment of diabetes and its vascular complications. Indeed, the successful induction of angiogenesis or revascularization is a central process in tissue repair. In the context of regenerative therapies for diabetes, the contributions of transplanted stem cells are not limited to the direct replacement of damaged beta cells. As an alternative, the endogenous repair of damaged islets or the generation of new islets \textit{in situ} has also been proposed\textsuperscript{209}. Hess and colleagues were the first to show that transplantation of murine BM-derived MNC or further purified c-kit\textsuperscript{+} progenitor cells stimulate the recovery of streptozotocin (STZ)-damaged islets by inducing proliferation of recipient beta cells and augmenting glycemic control via the endogenous regeneration of beta cell function\textsuperscript{210}. Donor cells with both hematopoietic and EC phenotypes were recruited to ductal regions and surrounded damaged islets, subsequently stimulating beta cell proliferation and insulin production in recipient-
derived beta cells. Several groups have extended these findings to show that islet recovery can be induced by the induction of hematopoietic chimerism in overtly diabetic NOD mice\textsuperscript{211} and that simultaneous infusion of murine BM MNC with allogeneic MSC optimize islet repair and protection against T-cell-mediated beta cell deletion \textsuperscript{212}. Although transplantation of murine BM-derived cells have shown proof of principle that BM-derived progenitor can impact endogenous beta cell regeneration, further purification and transplantation of human progenitor subtypes from multiple sources is underway in our lab to study the actions of specific cellular populations relevant to islet regeneration.

Toward this end, Hess et al. have transplanted human ALDH-purified BM stem cells into STZ-treated hyperglycemic NOD/SCID mice to promote islet regeneration. Transplantation of BM ALDH\textsuperscript{hi} cells led to a significant reduction in blood glucose and increased serum insulin due to an increase in endogenous beta cell proliferation resulting in increased islet size and total beta cell mass\textsuperscript{213}. Notably, transplanted BM ALDH\textsuperscript{hi} cells recruited to damaged islets and stimulated beta cell proliferation associated with functional capillary formation in regenerating islets. Similar to hyperglycemic mice transplanted with human BM cells, mice transplanted with human UCB ALDH\textsuperscript{hi} cells also demonstrated increased islet size and vascularization compared to controls. However, blood glucose reductions were transient, returning to severe hyperglycemia several weeks after transplantation. Furthermore, direct intra-pancreatic delivery of UCB ALDH\textsuperscript{hi} cells increased peri-islet engraftment and stimulated a permanent reduction in hyperglycemia compared to ALDH\textsuperscript{lo} cell controls via the induction of an islet proliferative and revascularization program in recipient islets\textsuperscript{214}. Unlike ALDH-purified BM progenitor populations, ALDH-purified UCB drawn by venipuncture did not consistently establish expandable MSC in culture, indicating a potential requirement for MSC to maintain islet regeneration. Fortunately, adherent MSC can be liberated from UCB after collagenase treatment\textsuperscript{215}, and inclusion of these cells within the transplanted population may further improve islet regeneration.

In the past, clinical applications of UCB-derived stem cells have been limited to transplantation in the fields of hematology or oncology; however, an increasing number of studies support the use of these cells for nonhematopoietic disorders, including
diabetes\textsuperscript{216,217}. UCB MNC transplantation into diabetic mice has shown delayed onset of autoimmunity and insulitis in a model of type 1 diabetes and improved in hyperglycemia and survival rates post-transplantation \textsuperscript{218,219}. A clinical trial using autologous UCB cells is currently underway in children with recently diagnosed type 1 diabetes \textsuperscript{217}. Delayed loss of endogenous insulin production and enhanced glucose control have been reported due to a highly functional population of regulatory T-cells within UCB\textsuperscript{220,221}. However, the functional mechanisms conferring beta cell protection and potential mechanisms for the expansion of beta cell mass and vascularization after UCB transplantation require further preclinical experimentation.

1.4.5 Roles of ALDH-Purified MSC in Islet Regeneration

MSC possess properties beneficial in the repair of tissues damaged by autoimmunity. MSC have been shown to modulate the microenvironment after injury and stimulate a shift from an inflammatory to a regenerative response\textsuperscript{222}, and to aid in tissue repair by exerting antifibrotic and neoangiogenic effects\textsuperscript{223}. MSC have also been shown to migrate toward areas of hypoxia and or tissue damage through their expression of a variety of chemokine receptors and adhesion molecules. Islets attract MSC \textit{in vitro} and \textit{in vivo} by CX3CL1-CX3CR1 and CXCL12-CXCR4 interactions\textsuperscript{224,225}. MSC may also play an important role in modulating the immune response, an important consideration in the treatment of type 1 diabetes. In damaged tissues, MSC stimulate reduced T-cell\textsuperscript{226} and B-cell proliferation\textsuperscript{227}, inhibit maturation as well as differentiation of dendritic cells\textsuperscript{228}, and decrease the production of inflammatory cytokines by immune cells\textsuperscript{229}. MSC can exert these effects by secreting immunosuppressive effectors such as TGF-\(\beta\), IDO, or PGE\(_2\textsuperscript{229}\).

Collectively, these characteristics make transplanted MSC an attractive target for the development of cellular therapies for autoimmune diabetes\textsuperscript{230}. As proof of principle, Lee et al. have shown that multiple high-dose intracardiac infusion of \textit{human} BM-derived MSC into STZ-treated immune-deficient NOD/SCID mice repaired islets and improved hyperglycemia with only minimal engraftment in the pancreas\textsuperscript{231}. Transplantation of ALDH-purified MSC into the tail vein of STZ-treated hyperglycemic mice also stably reduced blood glucose and increased serum insulin. Rather than stimulating increased
islet size and vascularization, MSC transplantation stimulated an increase in the number of small islets present in the pancreas. Newly formed beta cell clusters were small in size with direct proximity to the ductal epithelium. These unique regenerative characteristics suggested the induction of an islet neogenic mechanism, whereby transplanted ALDH-purified MSC stimulated new islet formation\textsuperscript{213}. Similar to findings by Lee et al., transplanted ALDH-purified MSC showed low-level or transient recruitment to the pancreas, but initially lodge in the liver and lung capillaries\textsuperscript{199}, presumably exerting their effects on islet regeneration via the release of unknown paracrine regenerative factors into the bloodstream.

### 1.4.6 Future perspectives for use of ALDH\textsuperscript{hi} cells in cell therapy

BM- or UCB-derived ALDH-purified mixed progenitor cells and ALDH-purified MSC demonstrate a variety of regenerative functions that impact tissue repair and revascularization. However, differences in progenitor frequencies and functions should not be overlooked when considering the optimal progenitor cell populations for regenerative therapies. In addition, the regenerative mechanisms induced by ALDH-expressing progenitors and the paracrine mediators that comprise the regenerative niche require further elucidation. Provided with this foundation of critical information, scientists will be able to design rational regenerative therapies using a combination of regenerative small molecules or directed administration of multiple progenitor cell subtypes to synergize endogenous blood vessel and subsequent tissue repair.

### 1.5 Thesis Overview and Hypotheses

Recently, our lab has shown that human BM ALDH\textsuperscript{hi} mixed progenitor cells transiently recruit to areas of ischemia and augment blood flow by stimulating the endogenous revascularization of ischemic limbs in transplanted immune-deficient mice after induction of unilateral hindlimb ischemia by femoral artery ligation/transection\textsuperscript{134}. However, in patients with CVD, pro-angiogenic cell content or function may be compromised due to chronic diabetes or vascular disease-related pathologies rendering autologous BM transplantation strategies less effective\textsuperscript{129,232-235}. Readily available and early in ontogeny, human umbilical cord blood (UCB) represents a promising allogeneic
source of functional pro-angiogenic progenitor cells. During my thesis work I have investigated the vascular regenerative potential of human UCB and BM-derived hematopoietic and multipotent-stromal progenitor cells for the pre-clinical development of rational allogeneic cell-based therapies for ischemic diseases focusing on PAD. Furthermore, I have focused on global molecular approaches, and functional assays \textit{in vitro} (Figure 1.2) and \textit{in vivo} (Figure 1.3) to determine the paracrine signals elaborated from these pro-angiogenic cell sources that positively impact microvascular endothelial cell activation and function. Our working model for transplanted progenitor cell involvement in vascular regeneration as investigated in this thesis is summarised in Figure 1.4. The central hypothesis of my studies is that high ALDH activity identifies populations of progenitor cells from blood or BM that will demonstrate augmented support of vascular regeneration. Thus, the specific objectives of my studies were as follows.
Figure 1.2. *in vitro* models for determination of progenitor cell contribution to endothelial cell function.

Co-culture assays to assess cell-mediated effects on survival, proliferation, and tube forming capacity of endothelial cells. (A) Schematic overview of hanging transwell co-culture and cell-conditioned medium assays to assess endothelial cell proliferation or survival. (B) Representative photomicrograph of human endothelial cells cultured on plastic. (C) Representative photomicrograph of human endothelial cells growing on a thin layer of Matrigel to assess modulation of tube-like structure formation.

Figure 1.2

A

Cells in Transwell

Endothelial Cells

Cell-conditioned media

B

Endothelial cells on Plastic

200μm

C

Endothelial cells on Matrigel

500μm
Figure 1.3 Transplantation model of acute unilateral hindlimb ischemia.

(A) Diagram of the vasculature of the mouse hindlimb from the inguinal crease to the bifurcation of the femoral artery into the saphenous and popliteal arteries that descend into the calf and lower limb. Ligation sites for femoral artery ligation surgery are indicated. Representative laser Doppler perfusion images of mouse hindquarters before and after ligation and transection of femoral artery and vein. Regions of interest are selected from ankle to toe to quantify relative perfusion in the surgical limb. (B) Outline and timing for tracking recovery of perfusion in the ischemic limb after surgical induction of acute ischemia. Number in lower left quadrant of each laser Doppler perfusion image is the ratio of perfusion in the ischemic limb to the perfusion in the control limb.

Figure 1.3

A  Pre-Surgery LDPI

Ischemic  Control

Perfusion Ratio:
Ischemic/Control = 1.0

B  Femoral nerve
Femoral vein
Superficial epigastric artery
Saphenous artery
Proximal ligation site
Femoral artery
Distal ligation site
Popliteal artery

C  Post-Surgery LDPI

Ischemic  Control

Perfusion Ratio:
Ischemic/Control < 0.1

D

Femoral Artery Ligation Surgery
Cell Transplant

Time (Days)

-1  0  3  7  14  21  28

Laser Doppler Perfusion Imaging (LDPI)
Figure 1.4. Umbilical cord blood and bone marrow-derived stem and progenitor cells support an angiogenic niche.

The ALDH<sup>hi</sup> subpopulation of cells in UCB and BM is enriched for progenitors from the hematopoietic, endothelial, and mesenchymal lineages. Each of these progenitor cell types recruit to and help co-ordinate a pro-angiogenic regenerative microenvironment in ischemic tissues.

Figure 1.4.
1.5.1 Aim 1

- To characterise the capacity of umbilical cord blood-derived ALDH-purified cells to modulate endothelial cell functions \textit{in vitro}

- To assess the effects on vascular regeneration after intra-venous transplantation of UCB ALDH\textsuperscript{hi} cells into immune-deficient mice with unilateral hindlimb ischemia induced by femoral artery ligation surgery.

In Aim 1, I hypothesized that selection of UCB cells based on high ALDH activity will select a population of cells with augmented capacity to promote recovery from ischemic injury.

1.5.2 Aim 2

Due to the relative rarity of ALDH\textsuperscript{hi} cells from UCB or BM, in Aim 2 I sought to determine if it was possible to expand UCB-derived ALDH\textsuperscript{hi} cells \textit{ex vivo} to generate more cells for therapeutic applications without losing beneficial vascular regenerative effect. Therefore the objectives of Aim 2 were two-fold:

- To optimize the \textit{ex vivo} expansion of UCB ALDH\textsuperscript{hi} hematopoietic cells under clinically relevant serum-free conditions and to characterize vascular regenerative functions of the expanded progenitor cells \textit{in vitro}.

- To investigate the paracrine mechanisms by which expanded hematopoietic progenitor cells derived from UCB ALDH\textsuperscript{hi} contribute to revascularization after direct intramuscular injection into the ischemic limb.

In Aim 2, I hypothesized that \textit{ex vivo} expansion of UCB ALDH\textsuperscript{hi} cells would increase the number of pro-angiogenic hematopoietic progenitor cells for application in vascular regeneration without decreasing EC-supportive functions.

1.5.3 Aim 3

BM MSC are a population of progenitor cells that expand readily in \textit{ex vivo} cultures with underutilized potential for vascular regenerative cellular therapies. However, our lab has
demonstrated in our beta cell regeneration model studies that different MSC lines from different donors showed variable efficacy to promote regeneration\textsuperscript{213,236}. Furthermore, it has been previously shown that selection of high ALDH activity in BM cells selected for cells with increased regenerative potential. Therefore the objective in Aim 3 was as follows:

- To characterize the differentiative and vascular regenerative functions of MSC subpopulations selected after expansion for low versus high ALDH activity.

In Aim 3, I hypothesized that selection of MSC subpopulations with high ALDH activity would identify a population of MSC with increased vascular regenerative potential.
1.6 References


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Chapter 2

2 Umbilical Cord Blood-Derived Aldehyde Dehydrogenase-Expressing Progenitor Cells Promote Recovery from Acute Ischemic Injury

This study focused on the characterization of UCB as a source of ALDH\textsuperscript{hi} cells for development of cell therapy for CVD. My objective was to characterize the capacity of umbilical cord blood-derived ALDH-purified cells to modulate endothelial cell functions \textit{in vitro} and to augment revascularisation after intra-venous transplantation into immune-deficient mice with unilateral hindlimb ischemia induced by femoral artery ligation surgery.

\textsuperscript{ii} A version of this chapter has been published: Putman, D. M., Liu, K. Y., Broughton, H. C., Bell, G. I. & Hess, D. A. Umbilical cord blood-derived aldehyde dehydrogenase-expressing progenitor cells promote recovery from acute ischemic injury. Stem Cells 30, 2248–2260 (2012).
2.1 Introduction

Ischemic disease is characterized by the reduction of blood flow to the heart or peripheral tissues and encompasses life-threatening disorders such as ischemic heart disease and critical limb ischemia. Despite advances in pharmacological and surgical management, ischemic disease remains one of the leading causes of morbidity and mortality worldwide\textsuperscript{1,2}. Thus, novel therapies to promote the regeneration of damaged vasculature are under intense pre-clinical investigation\textsuperscript{3-5}.

Angiogenesis, the sprouting of new blood vessels from pre-existing vessels, is a central process for tissue repair, allowing the delivery of circulating pro-angiogenic cells that form a vascular regenerative microenvironment\textsuperscript{6}. Asahara \textit{et al.} first described human bone marrow (BM)-derived cells that promoted the recovery of blood flow, and co-localized with new vessels after transplantation \textit{in vivo}\textsuperscript{6}. Later studies have shown that these rare cells, termed endothelial precursor cells (EPC), express the cell markers CD34, CD133, and VEGFR2\textsuperscript{7}. Because these markers were also expressed on clonally distinct cell types of both hematopoietic and non-hematopoietic lineages, there has been considerable controversy over which cell subtypes best promote vascular regeneration.

Endothelial colony forming cells (ECFC) are rare blood-derived cells that form late-outgrowth adherent colonies of proliferative cells when propagated under strict endothelial cell growth conditions, and can be isolated at increasing frequency from human peripheral blood, BM, and umbilical cord blood (UCB). ECFC are distinguished from pro-angiogenic hematopoietic cell types in human blood as they are exclusively CD45\textsuperscript{–}, and can integrate directly into perfused vessels \textit{in vivo}. Recently, it has been demonstrated that many of the cell types previously referred to as EPC are not endothelial cell precursors \textit{per se}, but represent myeloid/macrophage lineage cells that promote vascular repair through proposed paracrine signaling to vessel-derived EC\textsuperscript{8,9,10-12}. In mouse models, circulating CXCR4\textsuperscript{+}/VEGFR1\textsuperscript{+} hematopoietic cells are recruited to sprouting vessels by secretion of stromal-derived factor (SDF-1) or vascular endothelial growth factor-A (VEGF-A) produced by damaged vasculature\textsuperscript{4,13}. Studies examining
tumor metastasis have demonstrated that even small numbers of these hematopoietic cells play a major role in establishing a permissive niche for tumor vascularization\textsuperscript{14-16}.

Numerous studies have established that a decreased frequency or impaired function of circulating CD133$^+$ or CD34$^+$ cells is associated with increased cardiovascular risk in patients with ischemic heart disease and diabetes\textsuperscript{2,9,17-20}. This, in addition to preclinical data showing the pro-angiogenic potential of BM-derived cells, has led to a number of clinical trials investigating treatment of cardiac\textsuperscript{21-23} and limb ischemia\textsuperscript{24,25} by transplantation of heterogeneous human BM MNC. Although these studies have demonstrated safety in the autologous setting, they have shown only modest improvements in clinical endpoints compared to other approved therapies\textsuperscript{26}. Thus, purification of specific pro-angiogenic cellular sub-types from non-autologous sources, and further characterization of specific sub-type contribution during the coordination of vascular repair is warranted to improve current cell-based therapies for ischemic diseases.

Cord blood represents a promising source of regenerative progenitor cells. Early in ontogeny, UCB has been shown to contain a higher frequency of ECFC\textsuperscript{28}, and UCB-derived ECFC have more robust vessel forming capacity than ECFC from adult blood\textsuperscript{9,29}. Due to recent widespread initiatives to HLA-phenotype and cryopreserve this material, UCB now represents a readily available source of progenitor cells for cell therapy applications. In addition, allogeneic UCB progenitor cell transplantation may have distinct advantages over autologous BM in patients with obesity, chronic diabetes, or vascular disease-related pathologies due to compromised progenitor cell content and function\textsuperscript{30-33}.

Using high aldehyde dehydrogenase (ALDH$^{hi}$) activity, a cytosolic enzyme involved in retinoic acid metabolism and cellular self-protection from oxidative damage\textsuperscript{34-39}, fluorescence activated cell sorting strategies to simultaneously enrich for progenitor cells from hematopoietic, endothelial, mesenchymal lineages\textsuperscript{40}. It has been demonstrated that transplanted ALDH$^{hi}$ progenitors reconstitute hematopoiesis\textsuperscript{34,35}, exhibit widespread tissue distribution\textsuperscript{41}, and recruit to areas of ischemia after transplantation\textsuperscript{40}. Furthermore, BM-derived ALDH$^{hi}$ cells can augment revascularization and blood flow of ischemic limbs in
transplanted mice with acute ischemic injury\textsuperscript{40}. Recently, we proposed a model of
coordinated neovascularization after human progenitor cell transplantation wherein
hematopoietic and non-hematopoietic progenitors act in concert to formulate a pro-
angiogenic niche for vascular regeneration (Appendix 1)\textsuperscript{27}.

Here we show that selection of human UCB cells with high ALDH activity
simultaneously purifies a mixed population of primitive hematopoietic and endothelial
colony-forming cells with potent pro-angiogenic functions. UCB ALDH\textsuperscript{hi} progenitors
demonstrated a pro-angiogenic transcription profile and supported endothelial cell (EC)
survival in liquid culture and tube-like cord formation in growth factor–reduced matrigel.
Transplanted ALDH\textsuperscript{hi} cells improved recovery of blood flow after femoral artery ligation
in NOD/SCID mice. Thus, UCB progenitors purified by high ALDH-activity represents
an allogeneic population of pro-angiogenic cells for the revascularization of ischemic
tissues.

2.2 Methods

2.2.1 Progenitor cell isolation from human umbilical cord blood

UCB was collected after informed consent by venipuncture at the London Health
Sciences Birthing Centre. The human research ethics committee at the University of
Western Ontario approved all studies. After hypaque-ficoll centrifugation, mononuclear
cells (MNC) were depleted of erythrocytes by ammonium chloride lysis and assayed for
ALDH activity using Aldefluor\textsuperscript{TM} reagent (Stemcell Technologies, Vancouver, BC) as
previously described\textsuperscript{40}. FACS was performed to isolate cells with low side scatter, and
high (ALDH\textsuperscript{hi}) vs. low (ALDH\textsuperscript{lo}) ALDH activity to >99% purity. Sorted cells were
washed in PBS to allow efflux of Aldefluor\textsuperscript{TM} substrate via reactivation of inhibited
ABC-transporters.

2.2.2 Cell surface phenotype analysis

UCB MNC, ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} cells were labeled with anti-human antibodies against
pan-leukocyte marker CD45, and co-labeled with CD4 and CD8 (T cells), or CD19 and
CD20 (B cells), or CD33 (myeloid) and CD14 (monocytes), or CD31 (PECAM-1) and
CD144 (VE-cadherin, GeneTex, Irvine, CA), or CD34 and CD38, or CD117 (c-kit) and CD133 (Miltenyi Biotec, Auburn, CA), or CXCR4 and VEGFR2. All antibodies were from BD Biosciences unless otherwise indicated. Cell surface marker expression was measured using a FACSCalibur cytometer (BD Biosciences), and analyzed using FlowJo software.

2.2.3 Colony forming cell assays
UCB MNC, ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} cells were cultured in methylcellulose (Methocult\textsuperscript{®} H4434, Stemcell Technologies) for 14 days and hematopoietic colony formation was enumerated based on cell morphology identifying burst-forming units of erythrocytes (BFU-E), colony-forming units of granulocytes (CFU-G), macrophages (CFU-M), granulocytes and macrophages (CFU-GM), or multilineage mixed colonies (CFU-Mixed) containing cells of all 3 types. ECFC were enumerated after adherent culture for 14 days in complete endothelial growth media (EGM-2 + 2%FBS + EGF, VEGF-A, FGF, IGF-1, Lonza) counting colonies of cells with cobblestone morphology as previously described\textsuperscript{9}.

2.2.4 mRNA isolation and microarray analyses
mRNA was isolated from three sample-matched UCB ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cell cohorts using mRNeasy Mini kits (Qiagen, Mississauga, ON). Microarrays were performed in triplicate using 18 human gene array chips (Affymetrix, Santa Clara, CA) at the London Regional Genomics Facility. Transcripts differentially expressed by UCB ALDH\textsuperscript{hi} versus ALDH\textsuperscript{lo} cells were analyzed using Partek Genomics Suite (Partek Inc., St Louis, MO).

2.2.5 Co-culture assays with human umbilical vein endothelial cells (HUVEC)
To assess paracrine functions of UCB cells on human umbilical vein endothelial cell (HUVEC) survival and proliferation during co-culture \textit{in vitro}, 40x10\textsuperscript{3} HUVEC were cultured in endothelial basal media (EBM2, Lonza) with or without growth factors (EGF, VEGF-A, FGF, IGF-1) and serum (2%) supplementation. Under each condition, 10\textsuperscript{5} ALDH\textsuperscript{hi} or 10\textsuperscript{5} ALDH\textsuperscript{lo} cells were seeded into porous (5\textmu m pore size) hanging transwells suspended above the HUVEC. The number of trypan blue excluding HUVEC present
after 72 hours co-culture was enumerated by blinded hemocytometer counts. For tube-like cord formation assays, 50x10^3 HUVEC, or HUVEC supplemented with 20x10^3 ALDH^{hi} or ALDH^{lo} cells were seeded on growth factor reduced Matrigel (BD Biosciences) and cultured for 72 hours. The number of complete branch points in the cord structures formed by HUVEC was enumerated on an inverted microscope by blinded manual counts of four fields of view at 24, 48, and 72 hours after plating. All co-culture experiments were performed in triplicate.

2.2.6 Murine femoral artery ligation surgery and transplantation of human UCB cells

Right femoral and saphenous artery and vein ligation with complete excision of the femoral artery and vein was performed on anesthetised NOD/SCID (Jackson Laboratory, Bar Harbor, ME) or NOD/SCID/MPSVII mice as previously described. Within 24 hours of surgery, non-irradiated or sublethally irradiated (300cGy) mice were transplanted by tail-vein injection with PBS, 2x10^5 ALDH^{hi} cells, 10x10^6 ALDH^{lo} cells, or 20x10^6 MNC. Cell doses were chosen based on the relative frequency of each cell type within UCB MNC. NOD/SCID mice were used for functional studies to monitor the recovery of perfusion and to quantify blood vessel density as described below. NOD/SCID/MPSVII mice were used to detect human cell engraftment kinetics and localization in muscle sections from ischemic limbs using β-glucuronidase (GUSB) staining as described below.

2.2.7 Quantification of hindlimb perfusion using laser Doppler perfusion imaging

For laser Doppler perfusion imaging (LDPI; MoorLDI-2; Moor Instruments, Devon, UK), anesthetized NOD/SCID mice were placed at 37°C for 5 minutes following shaving of the hindquarters to remove hair. Blood flow was quantified within the hindquarters of live mice after surgery, and at 3, 7, 14, 21, and 28 days post-transplantation. Perfusion ratios (PR) comparing blood flow in the ischemic versus non-ischemic limb were quantified by averaged units of flux from the ankle to toe in each limb using Moorflow software.
2.2.8 Quantification of blood vessel density

Adductor muscles from transplanted mice were embedded and frozen in optimum cutting temperature medium (Sakura Finetek, Torrance, CA), cryosectioned, fixed in 10% formalin (Sigma), and blocked with mouse-on-mouse reagent (Vector Labs, Burlingame, CA). Murine blood vessel density in ischemic and non-ischemic limbs was quantified at day 28 by counting mouse CD31$^+$ capillaries and mouse von Willebrand factor (vWF$^+$) blood vessels using rat-anti mouse CD31 (1:100, BD Biosciences) or rabbit anti-mouse vWF antibody (1:200, Millipore, Temecula, CA) and peroxidase-labelled anti-rat or anti-rabbit immunoglobulin G (IgG) secondary antibodies (Vector) visualised using 3, 3'-diaminobenzidine (DAB) substrate, and counterstained with hematoxylin. Blood vessel density was counted in a blinded fashion from 9 photomicrographic fields using light microscopy.

2.2.9 Quantification of human cell engraftment by flow cytometry

BM and adductor muscles from the ischemic and non-ischemic limbs were analyzed for human cell engraftment at 3, 7, and 28 days after transplantation by flow cytometric detection of human CD45 and HLA-A,B,C in combination with 7-aminoactinomycin D (7-AAD) viability dye (BD Biosciences). Muscle samples were digested with type II collagenase (Worthington Biomedical, Lakewood, NJ) prior to antibody labelling.

2.2.10 Detection of human cells by GUSB-activity

Transplanted NOD/SCID/MPSVII mice with unilateral hindlimb ischemia were sacrificed at 3, 7 and 28 days after transplantation to allow histochemical detection of single human cell engraftment in ischemic muscle sections (GUSB-deficient) based on the detection of ubiquitous GUSB activity in human cells as previously described. Muscle sections were co-stained with vWF to assess the distribution of engrafted human cells with respect to murine vasculature, and with human CD45 to assess the lineage of engrafted cells.
2.2.11 Statistics

Analysis of significance was performed by one-way ANOVA for cell-surface marker and progenitor frequency assays, and by two-way ANOVA with Bonferroni post-hoc tests for all other assays using Graphpad Prism software.

2.3 Results

2.3.1 UCB ALDH$^{hi}$ cells were enriched for myeloid progenitor cell surface phenotypes

Human UCB MNC were collected without prior lineage depletion$^{34}$, and purified based on cells with low side scatter (non-granular) and with low (ALDH$^{lo}$, 41.36±3.6%) vs. high (ALDH$^{hi}$, 0.4±0.1%) ALDH activity compared to ALDH-inhibited (diethylaminobenzaldehyde, DEAB) controls (n=10, Figure 2.1A,B). Compared to unpurified UCB MNC (97.7±0.8% CD45$^{+}$) or ALDH$^{lo}$ cells (99.1±0.5% CD45$^{+}$), ALDH$^{hi}$ cells showed lower expression of the pan-leukocyte marker CD45 (93.3±1.9% CD45$^{+}$, *p<0.05, n=4). Thus, sorted cell populations were gated into hematopoietic (CD45$^{+}$) and non-hematopoietic (CD45$^{-}$) subsets, and further analyzed for primitive vs. mature hematopoietic or endothelial cell surface marker expression.

CD45$^{+}$ ALDH$^{hi}$ cells showed reduced expression lineage-specific markers for T- (CD4 and CD8, Figure 2.1C) and B-lymphocytes (CD19 and CD20, Figure 2.1D) compared to unpurified MNC or ALDH$^{lo}$ cells (**p<0.01, Table 2.1). Over 90% of CD45$^{+}$ ALDH$^{hi}$ cells expressed CD33, an early myeloid lineage marker, but co-expression of the mature monocyte marker CD14 was low (3.3±1.0%, Figure 2.1E; Table 2.1). Similarly, ALDH$^{hi}$ cells showed near homogeneous expression of CD31 or PECAM-1 (96.7±2.9%) but low co-expression of CD144/VE-cadherin (3.2±1.0%, Figure 2.1F; Table 2.1). Notably, the CD45$^{+}$ ALDH$^{hi}$ cells were highly enriched for the co-expression of the progenitor markers CD34 (89.6±5.9%, Figure 2.1G), CD117 (80.2±4.9%), and CD133 (61.5±2.3%, Figure 2.1H) compared to unpurified UCB MNC or ALDH$^{lo}$ cells (*p<0.05, Table 2.1). CD45$^{+}$ ALDH$^{hi}$ cells were enriched for SCID repopulating progenitor phenotype (CD34$^{+}$CD38$^{-}$, Figure 2.1G)$^{33}$, and ALDH$^{lo}$CD34$^{+}$ cells showed co-expression of the
homing marker CXCR4 (14.2±2.9%, Figure 2.1I), but low expression of VEGFR2 (0.5±0.1%, Figure 2.1J).

CD45– cells, representing approximately 7% of the ALDHhi population, also showed enriched expression of CD34 (65.0±14.8%), CD117 (64.9±12.4%), and CD133 (22.5±3.9%) progenitor markers (*p<0.05), but did not show increased expression of the mature EC-associated adhesion molecules (CD31 and CD144) compared to UCB MNC or ALDHlo cells (Table 2.2). Collectively these data indicate that the hematopoietic fraction of ALDHhi cells was depleted of mature lymphocytes and monocytes yet enriched for primitive myeloid progenitor phenotype, and the CD45– portion of the ALDHhi cells similarly retained a primitive non-hematopoietic cell phenotype.

2.3.2 UCB ALDHhi cells demonstrated hematopoietic and endothelial colony formation

UCB MNC, ALDHlo or ALDHhi cells were plated under lineage-specific growth conditions for 14 days to assess hematopoietic and endothelial CFC frequency. After culture in methylcellulose, ALDHhi cells were highly enriched for myeloid-lineage colony forming cells (1 HCFC in 3.6 cells) compared to ALDHlo cells (1 HCFC in 6.6x10³ cells, **p<0.01, n=4). ALDHhi HCFC demonstrated multipotent lineage differentiation with increased production of erythroid, macrophage, granulocyte and mixed morphologies (**p<0.001, Figure 2.1K). To assess ECFC function in vitro, ALDH-purified UCB cells were cultured under stringent conditions established by Yoder and colleagues⁹. Colony enumeration revealed increased ECFC content in ALDHhi cells (1 ECFC in 4.6x10⁴ cells) compared to ALDHhi cells (1 ECFC in 2.2x10⁵ cells, *p<0.05, Figure 2.1L). ECFC progeny were highly proliferative, did not express hematopoietic markers (CD45, CD14), and expressed EC markers (CD31, CD105, CD144). Unlike human BM⁴⁰, MNC or ALDH-sorted cohorts from UCB did not establish plastic adherent multipotent stromal cell colonies in vitro (n=10). Thus, UCB ALDHhi cells represented a mixture of cells with enriched HCFC and ECFC function.
Figure 2.1 Purification of UCB ALDH\textsuperscript{hi} cells enriches for hematopoietic and endothelial colony forming cells.

(A,B) Human UCB MNC were sorted for low side scatter (SSC) and low (ALDH\textsuperscript{lo}=41.2±3.6%) versus high (ALDH\textsuperscript{hi}=0.4±0.1%) ALDH activity with or without DEAB inhibitor. Representative flow cytometry plots show that UCB ALDH\textsuperscript{hi} cells were depleted of cells expressing mature (C) T-lymphocyte (CD4, CD8), (D) B-lymphocyte (CD19, CD20), (E) monocyte (CD14), and (F) endothelial (VE-cadherin, CD144) cell surface markers, were enriched for cells expressing (E) early myeloid (CD33), (F) adhesion (PECAM-1, CD31), and (G,H) primitive progenitor (CD34, CD1117, CD133) markers. (I,J) CD34\textsuperscript{+}ALDH\textsuperscript{hi} cells showed low co-expression of chemokine receptors (CXCR4, VEGFR2). (K) After culture for 14 days in Methylcellulose media (n=7), UCB ALDH\textsuperscript{hi} cells were highly enriched for multipotent HCFC. (L) After culture for 14 days in complete endothelial growth media (EGM-2 + IGF, EGF, bFGF, VEGF-A + 2% serum, n=5), UCB ALDH\textsuperscript{hi} were also enriched for ECFC. Scale bar = 200\textmu m. Data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
Table 2.1 Cell surface marker expression for the hematopoietic (gated CD45+) component of human UCB MNC, ALDH<sup>hi</sup> cells, and ALDH<sup>lo</sup> cells.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Markers (%)</th>
<th>MNC</th>
<th>ALDH&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>ALDH&lt;sup&gt;lo&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Lymphocyte</td>
<td>CD4</td>
<td>47.6±3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5±0.6&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>47.8±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>CD8</td>
<td>24.9±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5±0.3&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>26.1±3.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B-Lymphocyte</td>
<td>CD19</td>
<td>20.3±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6±0.1&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>21.1±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CD20</td>
<td>23.6±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8±0.9&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>21.1±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myeloid/Monocyte</td>
<td>CD33</td>
<td>20.9±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.4±4.8&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>1.4±0.3&lt;sup&gt;***c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>CD14</td>
<td>17.7±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3±1.1&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>0.6±0.2&lt;sup&gt;**b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adhesion</td>
<td>CD31</td>
<td>86.5±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96.7±2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.0±3.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>CD144</td>
<td>21.5±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2±1.0&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>4.9±2.9&lt;sup&gt;**b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Progenitor</td>
<td>CD34</td>
<td>5.1±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.6±5.9&lt;sup&gt;**b&lt;/sup&gt;</td>
<td>0.3±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>CD117/c-kit</td>
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<td>12.5±5.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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</tr>
<tr>
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<td>KDR/VEGFR2</td>
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<td>0.9±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

Data represents mean ± SEM (n=4), different letters denote different expression patterns at *p<0.05, **p<0.01 compared to unpurified MNC.
Table 2.2. Cell surface marker expression for the non-hematopoietic (gated CD45<sup>-</sup>) component of human UCB MNC, ALDH<sup>hi</sup> cells, and ALDH<sup>lo</sup> cells.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Markers (%)</th>
<th>MNC</th>
<th>ALDH&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>ALDH&lt;sup&gt;lo&lt;/sup&gt;</th>
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<td>6.5±3.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Progenitor</td>
<td>CD34</td>
<td>17.9±9.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.0±14.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3±0.6&lt;sup&gt;**c&lt;/sup&gt;</td>
</tr>
<tr>
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<td>CD117/c-kit</td>
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<td>CD133</td>
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<td>KDR/VEGFR2</td>
<td>1.8±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1.4±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

Data represents mean ± SEM (n=4), different letters denote different expression patterns at *p<0.05, **p<0.01 compared to unpurified MNC.
2.3.3 UCB ALDH\textsuperscript{hi} cells demonstrated a pro-angiogenic transcription profile

Affymetrix microarray identified 1253 transcripts with significantly increased (*p<0.05) expression in 3 UCB ALDH\textsuperscript{hi} cell samples compared to sample-matched ALDH\textsuperscript{lo} cells. These transcripts were first filtered to include unique genes translating to plasma membrane bound (196 hits) or extracellular (108 hits) locations, and subsequently filtered for documented angiogenic biological functions (Tables 2.3 and 2.4). As a control for the selection of cells with high ALDH-activity, ALDH1A1 mRNA expression was increased 42-fold in ALDH\textsuperscript{hi} vs ALDH\textsuperscript{lo} cells (p=2.08x10\textsuperscript{-5}). Furthermore, significantly increased CD34, PROM1, and KIT mRNA expression in ALDH\textsuperscript{hi} cells (Table 2.3) correlated with increased CD34, CD133, and c-kit/CD117 cell surface protein expression previously observed by FACS (Figure 2.1, Table 2.1). Transcripts associated with early myeloid progenitor differentiation (FLT3, CSF3R, CD33), integrin-mediated cell adhesion (ITGA9, ITGAV, ITGA2B), and vascular cell signaling (TIE1, TIE2, FGFR1, endoglin) were also highly expressed by UCB ALDH\textsuperscript{hi} cells (Table 2.3). Interestingly, transcripts for secreted cytokines associated with vascular functions (ANGPT1, FGF16, VEGFA), immune cell migration (IL-18, IL-1b, IL-8, CSF1, CXCL2), and modulators of EGF and IGF signaling (EREG, AREG, HBEGF, IGFBP7) were also increased in UCB ALDH\textsuperscript{hi} cells (Table 2.4). Collectively, these data suggested that UCB ALDH\textsuperscript{hi} cells possessed a pro-angiogenic transcription profile compared to ALDH\textsuperscript{lo} cells.
Table 2.3 Angiogenesis-associated transcripts encoding cell surface proteins with increased expression in UCB ALDH\textsuperscript{hi} versus ALDH\textsuperscript{lo} cells.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Common Names</th>
<th>Biological Function</th>
<th>Molecular Function</th>
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<th>p-value vs Alo</th>
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<tbody>
<tr>
<td>PROM1</td>
<td>prominin 1, CD133</td>
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<td>MAPK and Akt signaling</td>
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<td>adherence to BM matrix and cells</td>
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<td>3.7x10^{-5}</td>
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<td>HPC survival, maintenance and migration, HPC marker</td>
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<td>fms-related tyrosine kinase signaling</td>
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<td>1.5x10^{-6}</td>
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<tr>
<td>ITGA9</td>
<td>integrin, alpha 9</td>
<td>cell-cell and cell-matrix adhesion</td>
<td>α9/β1 dimer binds VCAM1</td>
<td>26.8</td>
<td>2.8x10^{-6}</td>
</tr>
<tr>
<td>CSF3R</td>
<td>colony stimulating factor receptor, CD114</td>
<td>granulocyte and neutrophil survival / differentiation</td>
<td>GCSF receptor signaling</td>
<td>20.0</td>
<td>3.4x10^{-3}</td>
</tr>
<tr>
<td>CD109</td>
<td>TGF-beta-1-binding protein</td>
<td>cell division, differentiation, motility, adhesion</td>
<td>heterodimer with TGFβ1 receptor</td>
<td>17.6</td>
<td>9.9x10^{-6}</td>
</tr>
<tr>
<td>CD302</td>
<td>type 1 transmembrane C-type lectin receptor</td>
<td>phagocytosis, cell adhesion and migration</td>
<td>carbohydrate binding / adhesion</td>
<td>17.0</td>
<td>4.1x10^{-4}</td>
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<tr>
<td>EMR2</td>
<td>EGF-module receptor 2 CD312</td>
<td>mucin-like cell adhesion</td>
<td>G-protein coupled receptor signaling</td>
<td>9.2</td>
<td>1.4x10^{-5}</td>
</tr>
<tr>
<td>CD33</td>
<td>myeloid cell surface antigen</td>
<td>adhesion molecule on monomyeloctic cells</td>
<td>tyrosine kinase, binds sialic acid</td>
<td>7.3</td>
<td>3.7x10^{-5}</td>
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<tr>
<td>TIE1</td>
<td>tyrosine kinase with immunoglobulin-like and EGF-like domain 1</td>
<td>regulation of angiogenesis and vasculogenesis</td>
<td>modulates TEK / TIE2 binding to angiopoietins</td>
<td>4.9</td>
<td>3.6x10^{-3}</td>
</tr>
<tr>
<td>ITGAV</td>
<td>integrin, alpha V</td>
<td>cell-cell, cell-matrix adhesion</td>
<td>αV/β3 binds vitronectin</td>
<td>4.6</td>
<td>3.0x10^{-3}</td>
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<tr>
<td>ENG</td>
<td>endoglin, CD105</td>
<td>vascular system development and remodeling, EC migration</td>
<td>complexes with TGF-beta, activin, BMP molecules</td>
<td>2.8</td>
<td>1.8x10^{-3}</td>
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<tr>
<td>FGFR1</td>
<td>fibroblast growth factor receptor 1, CD331</td>
<td>angiogenesis, embryonic development, cell proliferation, differentiation</td>
<td>tyrosine kinase activity, binds FGF molecules</td>
<td>2.8</td>
<td>2.1x10^{-3}</td>
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<tr>
<td>ITGA2B</td>
<td>integrin, alpha 2b</td>
<td>cell-cell and cell-matrix adhesion</td>
<td>α2B/β3 binds fibronectin</td>
<td>2.2</td>
<td>5.9x10^{-4}</td>
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<tr>
<td>TMEFF1</td>
<td>transmembrane protein with EGF-like and follistatin-like domains</td>
<td>unknown function during embryonic development</td>
<td>modulates Nodal and BMP signaling</td>
<td>2.1</td>
<td>2.4x10^{-3}</td>
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<tr>
<td>TEK/TIE2</td>
<td>tyrosine kinase with immunoglobulin-like and EGF-like domain 2</td>
<td>angiogenesis, EC survival, proliferation, migration, adhesion</td>
<td>tyrosine kinase activity, binds angiopoietins</td>
<td>2.0</td>
<td>1.8x10^{-3}</td>
</tr>
</tbody>
</table>

FC, fold change; BMP, bone morphogenenic protein; EC, endothelial cell, EGF, epidermal growth factor; GCSF, granulocyte colony stimulating factor; HPC, hematopoietic progenitor cell; IL-1, interleukin 1; MCSF, macrophage colony stimulating factor; TGF, transforming growth factor; VCAM, vascular cell adhesion molecule.
Table 2.4 Angiogenesis-associated transcripts encoding secreted cytokines with increased expression in UCB ALDH<sup>hi</sup> versus ALDH<sup>lo</sup> cells.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Common Name</th>
<th>Biological Function</th>
<th>Molecular Function</th>
<th>FC vs Alo</th>
<th>p-value vs Alo</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANGPT1</td>
<td>angiopoietin-1</td>
<td>vascular development / remodeling, angiogenesis</td>
<td>TIE2 receptor activation</td>
<td>17.3</td>
<td>3.6x10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL18</td>
<td>interleukin 18 (IFNγ-inducing molecule)</td>
<td>inflammation, EC migration, angiogenesis</td>
<td>IL 18 receptor activation</td>
<td>16.7</td>
<td>7.7x10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL1β</td>
<td>interleukin-1 beta</td>
<td>inflammation, EC differentiation / proliferation</td>
<td>IL-1β receptor activation</td>
<td>16.5</td>
<td>3.8x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>EREG</td>
<td>epiregulin</td>
<td>EC proliferation, angiogenesis</td>
<td>EGF receptor activation</td>
<td>12.1</td>
<td>2.6x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL8 (CXCL8)</td>
<td>interleukin 8</td>
<td>chemoattraction of angiogenic and inflammatory cells</td>
<td>CXCR1 and CXCR2 activation</td>
<td>8.5</td>
<td>3.1x10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>AREG</td>
<td>amphiregulin</td>
<td>EC proliferation, angiogenesis</td>
<td>EGF receptor activation</td>
<td>5.9</td>
<td>1.4x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGFBP7</td>
<td>insulin-like growth factor binding protein 7</td>
<td>implicated in tumor angiogenesis</td>
<td>binds IGF1 and IGF2</td>
<td>4.7</td>
<td>1.5x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP6</td>
<td>bone morphogenic protein 6</td>
<td>EC development, proliferation, angiogenesis</td>
<td>BMP receptor activation</td>
<td>3.9</td>
<td>3.4x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>CSF1</td>
<td>colony stimulating factor 1, M-CSF</td>
<td>macrophage differentiation, migration and function</td>
<td>M-CSF receptor activation</td>
<td>3.4</td>
<td>3.4x10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<tr>
<td>FGF16</td>
<td>fibroblast growth factor 16</td>
<td>angiogenic factor during development</td>
<td>FGFR1 and FGFR2 activation</td>
<td>2.9</td>
<td>3.4x10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
<td>FBLN5</td>
<td>fibrulin 5</td>
<td>EC adhesion during vascular development / remodeling</td>
<td>vascular ligand for integrins</td>
<td>2.5</td>
<td>5.8x10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
<td>chemoattraction of angiogenic and inflammatory cells</td>
<td>CXCR2 activation</td>
<td>2.2</td>
<td>2.4x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>HBEGF</td>
<td>heparin-binding EGF-like growth factor</td>
<td>endothelial cell proliferation, angiogenesis</td>
<td>EGF receptor activation</td>
<td>2.1</td>
<td>8.0x10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
<td>VEGFA</td>
<td>vascular endothelial growth factor A</td>
<td>regulation of angiogenesis and vasculogenesis</td>
<td>VEGFR1 and R2 activation</td>
<td>1.7</td>
<td>4.8x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

FC, fold change; EC, endothelial cell
2.3.4 UCB ALDH\textsuperscript{hi} cells augment the survival of HUVEC \textit{in vitro}

To assess the paracrine functions of ALDH-purified UCB cells on endothelial cell survival and proliferation \textit{in vitro}, 40x10\textsuperscript{3} HUVEC were seeded into transwell co-cultures for 72 hours using a 5μm pore hanging insert containing 10\textsuperscript{5} UCB ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} cells (or basal media control) suspended above HUVEC. Under optimal conditions containing growth factors (GF\textsuperscript{+}) and serum, non-contact co-culture with ALDH\textsuperscript{lo} or ALDH\textsuperscript{hi} cells did not impact the 5-fold expansion of HUVEC observed over 72h (Figure 2.2A,E). Conversely, in GF/serum-free conditions with or without ALDH\textsuperscript{lo} cell supplementation, HUVEC invariably died within 72 hours (Figure 2.2B,E). In contrast, co-culture with ALDH\textsuperscript{hi} cells under GF/serum-free conditions promoted HUVEC viability for 72 hours (*p<0.05, Figure 2.2B,E). Under GF\textsuperscript{+}/serum-free conditions, ALDH\textsuperscript{hi} cell co-culture promoted a modest expansion (1.7-fold) of HUVEC (67.4±7.1x10\textsuperscript{3} cells), compared to >3-fold HUVEC loss with basal media (12.4±2.6x10\textsuperscript{3} cells) or ALDH\textsuperscript{lo} cell supplementation (14.4±2.8x10\textsuperscript{3} cells, *p<0.05, Figure 2.2C,E). Under GF/serum\textsuperscript{+} conditions, ALDH\textsuperscript{lo} or ALDH\textsuperscript{hi} co-culture did not significantly alter HUVEC numbers (Figure 2.2D,E). Thus, non-contact co-culture with human ALDH\textsuperscript{hi} cells promoted HUVEC survival under GF/serum-free conditions. These effects were consistent with the pro-survival stimulus in HUVEC provided by VEGF-A treatment\textsuperscript{44}, a central pro-angiogenic cytokine with increased mRNA-expression in UCB ALDH\textsuperscript{hi} cells (Table 2.4).
Figure 2.2 UCB ALDH<sup>hi</sup> cells promote endothelial cell survival under growth factor and serum-free conditions.

(A-D) Representative photomicrographs of HUVEC density after 72h co-culture with UCB ALDH<sup>lo</sup> or ALDH<sup>hi</sup> cells in hanging transwells with or without growth factor or serum supplementation. Scale bar = 200μm. (E) Non-contact co-culture with human UCB ALDH<sup>hi</sup> cells promoted the survival of HUVEC under growth factor-free, serum-free conditions (n=5). Data are expressed as mean ± SEM, *p<0.05.
Figure 2.2

A
GF+ Serum +

B
GF - Serum -

C
GF + Serum -

D
GF - Serum +

E

<table>
<thead>
<tr>
<th>GFs</th>
<th>Serum</th>
<th>Number of HUVEC (x10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>Control</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>ALDH^hi</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>ALDH^lo</td>
</tr>
</tbody>
</table>

40x10^3 seeded HUVEC
2.3.5 UCB ALDH\textsuperscript{hi} cells augment tube-like cord formation by HUVEC \textit{in vitro}

HUVEC were cultured alone at 50x10\textsuperscript{3} per well, or co-cultured with 20x10\textsuperscript{3} ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} cells on growth factor reduced Matrigel. HUVEC established partially completed cord networks within 24 hours (Figure 2.3A). Although co-culture of HUVEC with UCB ALDH\textsuperscript{lo} cells did not significantly alter cord formation, supplementation with ALDH\textsuperscript{hi} cells increased the number of HUVEC tube-like cord formed at 24 hours after plating (Figure 2.3B and C). Although UCB ALDH\textsuperscript{hi} cells established ECFC in liquid culture (Figure 2.1L, inset), UCB ALDH\textsuperscript{lo} or ALDH\textsuperscript{hi} cells seeded alone on growth factor reduced Matrigel did not spontaneously align to form cord networks (Figure 2.3D), and tube-like cord stability of HUVEC was not enhanced by co-culture with UCB ALDH\textsuperscript{lo} or ALDH\textsuperscript{hi} at 72 hours (Figure 2.3E-H). Enumeration of completed branch points at 24 and 48 hours revealed that co-culture with UCB ALDH\textsuperscript{hi} cells augmented cord formation by HUVEC compared to media controls (n=6, *p<0.05, Figure 2.3H). Therefore, co-culture with UCB ALDH\textsuperscript{hi} cells promoted the cord-forming function of HUVEC \textit{in vitro}. 
Figure 2.3 UCB ALDH$^{hi}$ cells augmented tube-like cord formation by HUVEC.

Representative photomicrographs of tubule formation by HUVEC after 24 (A-C) and 72 (E-G) hours co-culture with ALDH$^{lo}$ or ALDH$^{hi}$ cells or media control. Scale bar = 500μm. (D) ALDH$^{hi}$ cells plated without HUVEC did not form cord structures. (H) Co-culture with ALDH$^{hi}$ cells augmented the formation of complete tubule networks formed by HUVEC at 24 and 48 hours, but did not augment tubule stability after 72 hours co-culture. Data are expressed as mean ± SEM, *p<0.05.
Figure 2.3
2.3.6 Transplanted UCB ALDH^{hi} cells enhanced the recovery of hindlimb perfusion

To determine whether ALDH-purified human UCB cells could support blood vessel regeneration after intravenous transplantation *in vivo*, we induced acute unilateral hindlimb ischemia in NOD/SCID mice by right femoral artery ligation with complete excision of a 0.5cm section of the femoral artery and vein. Blood flow was assessed by LDPI and the perfusion ratio (PR) in the ischemic versus control limb was decreased >10-fold (PR=0.08±0.02) post-surgery confirming consistent induction of unilateral hindlimb ischemia (Figure 2.4). Within 24 hours of surgery mice were tail vein injected with PBS vehicle control, unsorted UCB MNC, ALDH^{hi} cells, or ALDH^{lo} cells. The representative LDPI shown in Figure 2.4 documents the recovery of limb perfusion after transplantation. PBS-injected control mice showed recovery of PR from 0.07±0.01 after surgery to 0.36±0.05 by day 7 post-surgery without subsequent improvement at later time points (Figure 2.4A,E). This baseline recovery of perfusion was sufficient to prevent excess morbidity or limb loss in PBS transplanted mice. Mice transplanted with 20x10^6 MNC or 10x10^6 ALDH^{lo} cells showed equivalent recovery of limb perfusion similar compared to PBS controls (Figure 2.4B,D-E). In contrast, mice transplanted with 50-100-fold fewer ALDH^{hi} cells (2x10^5) showed increased perfusion by day 14 post-transplantation (PR=0.58±0.07) compared to all other treatments and augmented perfusion was maintained for 28 days post-transplantation (PR=0.64±0.06, **p<0.01, Figure 2.4C,E). Thus, intravenous-transplantation of human UCB ALDH^{hi} cells improved the limb perfusion in mice with acute unilateral hindlimb ischemia.
Figure 2.4 Transplanted UCB ALDH_{hi} cells augmented perfusion in ischemic hindlimbs.

Representative LDPI following right femoral artery ligation and tail vein injection of (A) PBS vehicle control (n=7), (B) 2x10^6 unsorted UCB MNC (n=7), (C) 2x10^5 ALDH_{hi} cells (n=7), or (D) 10x10^6 ALDH_{lo} cells (n=7). Numbers in lower left of each image indicate perfusion ratio of the ischemic versus the non-ischemic hindlimb. (E) Compared to all other treatments, transplantation of UCB ALDH_{hi} cells promoted improved recovery of perfusion in ischemic mouse hindlimbs by day 14 and augmented perfusion was maintained to day 28. Data are expressed as mean ± SEM, **p<0.01.
Figure 2.4

A
PBS Vehicle Control

B
20$\times$10$^6$
MNC

C
2$\times$10$^5$
ALDH$^{hi}$

D
10$\times$10$^6$
ALDH$^{lo}$

E

<table>
<thead>
<tr>
<th>Days after Transplantation</th>
<th>PBS control (n=7)</th>
<th>MNC (n=7)</th>
<th>ALDH$^{hi}$ (n=7)</th>
<th>ALDH$^{lo}$ (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.09</td>
<td>0.09</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>3</td>
<td>0.18</td>
<td>0.28</td>
<td>0.56</td>
<td>0.20</td>
</tr>
<tr>
<td>7</td>
<td>0.31</td>
<td>0.37</td>
<td>0.64</td>
<td>0.36</td>
</tr>
<tr>
<td>14</td>
<td>0.28</td>
<td>0.45</td>
<td>0.82</td>
<td>0.37</td>
</tr>
<tr>
<td>21</td>
<td>0.40</td>
<td>0.31</td>
<td>0.78</td>
<td>0.38</td>
</tr>
</tbody>
</table>

E: Mean Perfusion Ratio (Ischemic/Nonischemic)
2.3.7 Transplanted UCB ALDH$^{hi}$ cells increased capillary density in the ischemic limb

To address the recovery of blood vessels in the ischemic hindlimb, sections from the adductor muscle at the site of artery ligation were stained for both murine CD31$^+$ capillary (Figure 2.5A-C) and vWF$^+$ blood vessel (Figure 2.5D-F) density. Compared to the non-ischemic limb at day 28 (65.3±6.1 CD31$^+/\text{mm}^2$, 8.1±1.2 vWF$^+/\text{mm}^2$), PBS-injected mice showed significant reduction (*p<0.05) of both CD31$^+$ capillaries (53.5±4.8/\text{mm}^2, Figure 2.5G) and vWF$^+$ blood vessels (5.5±1.4/\text{mm}^2, Figure 2.5F) within the ischemic hindlimb. However, mice transplanted with UCB ALDH$^{hi}$ cells (73.7±10.5/\text{mm}^2) had significantly more CD31$^+$ capillaries compared to mice transplanted with ALDH$^{lo}$ cells (61.1±12.4/\text{mm}^2, *p<0.05) or PBS (65.3±6.1/\text{mm}^2, **p<0.01, Figure 2.5G). Similarly, mice transplanted with ALDH$^{hi}$ cells (8.8±1.8/\text{mm}^2) showed higher vWF$^+$ blood vessel density than mice injected with ALDH$^{lo}$ cells (5.8±1.2/\text{mm}^2, *p<0.05), or MNC (5.8±0.9/\text{mm}^2, *p<0.05), or PBS-injected controls (8.1±1.2 vWF$^+/\text{mm}^2$, ***p<0.001, Figure 2.5H). Despite only partial recovery of perfusion, CD31$^+$ and vWF$^+$ vessel density in the ischemic limbs of ALDH$^{hi}$ cell-treated mice was equivalent to the vessel density of the non-ischemic control limb at day 28 (Figure 2.5G,H). Thus, UCB ALDH$^{hi}$ cell transplantation increased both CD31$^+$ capillary and vWF$^+$ blood vessel density in the ischemic hindlimb after femoral artery ligation.
Figure 2.5. Transplanted UCB ALDH$_{hi}$ cells augmented blood vessel density within ischemic hindlimbs.

Representative photomicrographs of adductor muscle sections from the ischemic and non-ischemic limbs of mice injected with PBS or UCB ALDH$_{hi}$ cells stained for (A-C) CD31$^+$ capillaries or (D-F) vWF$^+$ blood vessels at day 28 post-transplantation. Scale bar = 100μm. Compared to all other treatments, (G) CD31$^+$ capillary density, and (H) vWF$^+$ blood vessel density was increased in the ischemic limb of mice transplanted with human UCB ALDH$_{hi}$ cells. Data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 as determined by two way ANOVA and Bonferroni post-tests to compare multiple means.
Figure 2.5

A) PBS – Non-ischemic

B) PBS – Ischemic

C) ALDH<sup>hi</sup> – Ischemic

D) vWF

E) vWF

F) vWF

G) CD31<sup>+</sup> Vessels/mm<sup>2</sup>

H) VWF<sup>+</sup> Vessels/mm<sup>2</sup>
2.3.8 Transplanted UCB ALDH\textsuperscript{hi} cells recruited to the ischemic muscle at low frequency

In order to correlate the recovery of perfusion with human cell engraftment, murine BM and adductor muscle were collected at 3, 7, and 28 days after transplantation, and analyzed for the presence of human CD45\textsuperscript{+} or HLA-A,B,C\textsuperscript{+} cells by flow cytometry. Despite consistent human cell hematopoietic chimerism in the BM of mice transplanted with ALDH\textsuperscript{hi} cells (Figure 2.6A,C) or MNC (Figure 2.6C), human cells were not significantly detected in the adductor muscle of ischemic limbs by flow cytometry (Figure 2.6B,D) at day 28. In addition, mice processed for engraftment at earlier time points showed no evidence of human cells in the ischemic limb at 3 (n=3) or 7 days (n=3) post-transplantation by FACS. Therefore, we repeated engraftment experiments using identical cell doses in the GUSB-deficient NOD/SCID/MPSVII recipient to permit the detection of transplanted human cells at single cell resolution. At 3 and 7 days post-transplantation, the detection of GUSB expressing cells confirmed the recruitment of human cells to the ischemic hindlimbs of mice that received MNC (n=4, Figure 2.6E-F) or ALDH\textsuperscript{hi} cells (n=4, Figure 2.6H-I). However, human cells were not detected in the ischemic limbs of mice that received ALDH\textsuperscript{lo} cells (n=4). Although transplanted cells were never detected in the non-ischemic limb, engrafted MNC and ALDH\textsuperscript{hi} cells were observed in ischemic muscle sections surviving out to 28 days (Figure 2.6G, J). Staining for GUSB-activity combined with murine vWF showed engraftment of single human cells between muscle fibres (Figure 2.6K) and in association with the connective tissue surrounding blood vessels (Figure 2.6L). Also, the majority of GUSB\textsuperscript{+} human cells surviving at day 28 co-stained for CD45 (Figure 2.6M). Despite low frequency engraftment in the ischemic region, early recruitment of ALDH\textsuperscript{hi} progenitor cells to the site of ischemic injury was sufficient to augment blood vessel density and improve limb perfusion.
Figure 2.6 Transplanted human UCB ALDH^{hi} cells recruited to the ischemic hindlimb.

Representative flow cytometry plots illustrating the detection of human cells (CD45^{+}/HLA-A,B,C^{+}) cells within (A) the bone marrow and (B) ischemic hindlimbs of mice transplanted with UCB ALDH^{hi} cells. (C,D) Despite engraftment of human cells in the mouse bone marrow, human cells were rarely detected in the ischemic hindlimb by flow cytometry. Representative photomicrographs showing the detection of transplanted human cells (red) in the adductor muscle of the ischemic hindlimb of mice transplanted with (E-G) UCB MNC or (H-J) UCB ALDH^{hi} cells at 3, 7 and 28 days post-transplantation. GUSB^{+} human cells (arrows) were detected in the ischemic limb of mice injected with UCB MNC or ALDH^{hi} cells, but not in mice injected with UCB ALDH^{lo} cells. Co-staining for GUSB-activity with murine vWF at day 28 showed (K) engraftment of single GUSB^{+} cells between muscle fibres, and (L) associated with vWF^{+} blood vessels. (M) GUSB^{+} human cells surviving at day 28 co-stained for CD45. Scale bars = 100μm.
Figure 2.6

A. Bone Marrow
   - Day 7: 0%
   - Day 28: 4.7%

B. Ischemic Muscle
   - Day 7: 0%
   - Day 28: 0%

C. Bone Marrow
   - Engraftment (% CD45/(HLA-A,B,C))
   - D3: 1.3±0.3%
   - D7: 4.2±2.0%

D. Ischemic Muscle
   - Engraftment (% CD45/(HLA-A,B,C))

E. Day 3
   - 20x10^6 MNC
   - GUSB

F. Day 7
   - GUSB

G. Day 28
   - GUSB

H. Day 3
   - 2x10^6 ALDH
   - GUSB

I. Day 7
   - GUSB

J. Day 28
   - GUSB

K. Day 28
   - GUSB/2WF

L. Day 28
   - GUSB/VWF

M. Day 28
   - GUSB/CD45
2.3.9 Sublethal irradiation did not improve perfusion in the ischemic hindlimb

The requirement for preparative irradiation is an important consideration in the design of clinically applicable cell therapies for ischemic diseases. In an attempt to improve human cell engraftment in the ischemic limb, transplantation experiments were repeated after sublethal irradiation (300cGy) administered as a means to reduce xenorejection by residual innate immunity or NK-cell activity in the NOD/SCID model. Surprisingly, in mice receiving UCB ALDH^hi cells, perfusion at early time points (days 3, 7, and 14) was similar for all transplanted groups (Figure 2.7A-E). Although perfusion improved in the ALDH^hi cell transplanted cohort at day 21, perfusion was reduced to baseline by day 28 (Figure 2.7E), indicating that only a transient improvement in limb perfusion was observed. Compared to mice transplanted without preparative irradiation (Figure 2.4), irradiated mice receiving an equal dose of UCB ALDH^hi cells actually showed delayed recovery of perfusion at day 14 (PR_{300cGy} = 0.40±0.05 vs PR_{0cGy} = 0.58±0.07). Nonetheless, at day 28, irradiated mice injected with ALDH^hi cells still showed improved vWF^+ blood vessel density (Figure 2.8) similar to non-irradiated mice.

Irradiated mice also showed enhanced recruitment of ALDH^hi cells to the BM at day 7 (1.3±0.2%, Figure 2.9A,C), resulting in extensive hematopoietic repopulation by day 28 (44.7±2.0%, Figure 2.9A,C). Unexpectedly, human cells in the ischemic limb were not detected by FACS or GUSB staining at day 7 (Figure 2.9B,D,E), and were only rarely detected at day 28 (Figure 2.9F). Thus, irradiation prior to ALDH^hi cells transplantation did not augment engraftment or the recovery of perfusion in the ischemic limb.
Figure 2.7 Transplanted UCB ALDH$^{\text{hi}}$ cells induced transient recovery of perfusion in the ischemic limbs of mice that received preparative irradiation.

Representative LDPI following right femoral artery ligation, 300 cGy sublethal preparative irradiation to suppress immune rejection, and tail vein injection with (A) PBS vehicle control (n=6), (B) 20x10$^6$ unsorted UCB MNC (n=7), (C) 2x10$^5$ UCB ALDH$^{\text{hi}}$ cells (n=8), or (D) 10x10$^6$ ALDH$^{\text{lo}}$ cells (n=8), and monitored for 28 days. Numbers in lower left of each LDPI image indicate perfusion ratio of the ischemic versus the non-ischemic hindlimb from ankle to toe. (E) Summary of mean perfusion ratios in all transplanted mice as described above. Compared to all other treatments, transplantation of UCB ALDH$^{\text{hi}}$ cells promoted a transient recovery of perfusion in ischemic mouse hindlimbs at day 21 post-transplantation. Data are expressed as mean ± SEM, **p<0.01.
Figure 2.7

A  
PBS Vehicle Control

B  
20x10^6 MNC

C  
2x10^5 ALDH^hi

D  
10x10^5 ALDH^lo

E  
Mean Perfusion Ratio (Ischemic/Nonischemic)

Days after Transplantation

- PBS control (n=6)
- UCB MNC (n=6)
- UCB ALDH^hi (n=8)
- UCB ALDH^lo (n=8)
Figure 2.8 Transplanted UCB ALDH<sup>hi</sup> cells augmented blood vessel density in the ischemic hindlimb of irradiated mice.

Representative photomicrographs of adductor muscle sections from the non-ischemic and ischemic hindlimbs of mice injected with (A, B) PBS or (C) UCB ALDH<sup>hi</sup> cells or (D) UCB ALDH<sup>lo</sup> cells stained for vWF<sup>+</sup> blood vessels at day 28 post-transplantation. Scale bar = 100µm. (E) Summary of vWF<sup>+</sup> blood vessel density for each transplanted cohort at 28 days post-transplantation. Compared to all other treatments, blood vessel density was increased in the ischemic limb of mice transplanted with human UCB ALDH<sup>hi</sup> cells. Data are expressed as mean ± SEM, ***p<0.001.
Figure 2.8
Figure 2.9 Transplanted UCB ALDH$^{hi}$ cell showed reduced engraftment in the ischemic limb and increased engraftment in the bone marrow of irradiated mice.

(A,B) Representative flow cytometric detection of human cells in (A) bone marrow or (B) ischemic muscle (B) of mice transplanted with UCB ALDH$^{hi}$ cells after sublethal (300cGy) preparative irradiation. (C-D) Summary of human cell (CD45$^+$/HLA-A,B,C$^+$) engraftment in (C) the bone marrow or (D) ischemic muscle of all transplanted mice at 7 and 28 days–post transplantation. (E, F) Representative photomicrographs showing the detection of transplanted human cells (red) in the adductor muscle of the ischemic hindlimb of mice transplanted with UCB ALDH$^{hi}$ cells at 7 and 28 days post-transplantation. GUSB$^+$ human cells (arrows) were detected in the ischemic limb of mice at 28 days post-transplantation, Scale bar = 100μm.
2.4 Discussion

In the present study we demonstrate that ALDH-expressing cells from human UCB, representing <0.5% of total UCB cells and primarily comprised of primitive myeloid progenitor cells, demonstrated increased expression of mRNA transcripts for several angiogenic cytokines, and stimulated the survival and tubule forming functions of EC during co-culture *in vitro*. Intravenous transplantation of UCB ALDH\textsuperscript{hi} cells also augmented recovery of limb perfusion induced by femoral artery ligation and transection. Although the engraftment of human cells within ischemic muscle was infrequent, UCB ALDH\textsuperscript{hi} cells specifically recruited to the ischemic limb within 3 days of transplantation, and stimulated the recovery of blood vessel and capillary density. Furthermore, ALDH\textsuperscript{hi} cells function within the pancreas to promote islet revascularization after transplantation into streptozotocin-treated mice\textsuperscript{45,46}. Collectively, these data suggest that UCB ALDH\textsuperscript{hi} progenitor cells represent a readily available population of pro-angiogenic cells for the development of cellular therapies to promote endogenous revascularization.

The UCB ALDH\textsuperscript{hi} population was significantly enriched for human hematopoietic progenitor function. Approximately 1 in 4 ALDH\textsuperscript{hi} cells demonstrated multipotent CFU capacity in methylcellulose cultures. UCB ALDH\textsuperscript{hi} cells also showed increased expression of primitive cell surface markers (CD34, c-kit/CD117, and CD133), and >90% of UCB ALDH\textsuperscript{hi} cells expressed the early myeloid marker CD33, indicating a substantial enrichment of early myeloid progenitors. At the same time, UCB ALDH\textsuperscript{hi} cells were depleted of mature T- and B-lymphocytes as well as CD14 expressing monocytes. This is notable as CD14\textsuperscript{+} monocytes have been specifically implicated in the paracrine support of angiogenesis\textsuperscript{47}. However, transplantation of UCB ALDH\textsuperscript{hi} cells, containing only 3% CD14\textsuperscript{+} monocytic cells significantly improved perfusion and blood vessel density in the ischemic limb, whereas transplantation of mice with 100-fold higher doses of unsorted MNC, containing over 17% CD14\textsuperscript{+} monocytes, failed to promote recovery from hindlimb ischemia. Recent studies have demonstrated that murine common myeloid progenitors preferentially differentiate into pro-angiogenic monocytes.
that support neovessel formation in vivo. Although UCB ALDH\textsuperscript{hi} cells may be capable of differentiating into monocytes in vivo, our data indicates that primitive UCB ALDH\textsuperscript{hi} myeloid progenitors play a significant role in support of angiogenesis in vivo.

UCB ALDH\textsuperscript{hi} cells also possessed enriched ECFC capacity in vitro. In contrast to the dominant myeloid progenitor composition, ECFC were infrequent at approximately 1 ECFC in 50,000 ALDH\textsuperscript{hi} cells. Although this represents a significant ECFC enrichment from unpurified UCB MNC\textsuperscript{9}, it is unlikely that the few ECFC within 40x10\textsuperscript{3} ALDH\textsuperscript{hi} cells make a major contribution towards the maintenance of HUVEC survival and the induction of tubule forming function that we observed under growth factor and serum starved conditions in vitro. However, paracrine contributions of the non-hematopoietic component of the ALDH\textsuperscript{hi} population should not be overlooked in vivo. Approximately 7\% of the UCB ALDH\textsuperscript{hi} population were CD45\textsuperscript{-}, and these cells demonstrated high co-expression of primitive endothelial precursor markers (CD34 and CD133). Therefore, we propose that UCB ALDH\textsuperscript{hi} cells represents a heterogeneous mixture of primitive hematopoietic and non-hematopoietic progenitors that may act in synergy to generate a supportive microenvironment to promote EC survival and revascularization function.

After intravenous transplantation, UCB ALDH\textsuperscript{hi} cells induced stable recovery from acute ischemic injury within 2 weeks of transplantation into mice with unilateral hindlimb ischemia. However, permanent engraftment of ALDH\textsuperscript{hi} cells within the ischemic muscle was below the threshold of detection by flow cytometry. Notably, transplantation of 50-100-fold greater cell doses of unpurified UCB MNC or ALDH\textsuperscript{lo} cells also showed little engraftment by FACS. Using NOD/SCID/MPSVII mice, a unique cell tracking model capable of detecting GUSB-expressing human cell at the single cell level, we demonstrate that intravenous-injected UCB ALDH\textsuperscript{hi} cells specifically recruited into the ischemic limb at 3 and 7 days after transplantation, and these cells survived in ischemic muscle tissue, albeit at low frequencies, for up to 28 days. Nonetheless, this low frequency engraftment of ALDH\textsuperscript{hi} cells in the ischemic region at early time points was sufficient to augment the revascularization of ischemic muscle indicating a vascular stabilization was mediated by the ALDH\textsuperscript{hi} cells.
Recent models have proposed a transient role for pro-angiogenic hematopoietic cells in the stabilization of injured vasculature, and in the activation and recruitment of vessel resident endothelial precursors to the sprouting vessel branch. In support of this concept we show that sublethal irradiation prior to transplantation of ALDH$^\text{hi}$ cells reduced recruitment of human cells to the ischemic region, increased engraftment of ALDH$^\text{hi}$ cells in the murine BM, and delayed the functional recovery of perfusion. Therefore, we propose that early recruitment of ALDH$^\text{hi}$ cells to the ischemic region is critical for vascular recovery, allowing ALDH$^\text{hi}$ cells to release pro-angiogenic stimuli that activate an endogenous program for collateral vessel formation resulting in improved limb perfusion. Thus, strategies to improve the efficiency ALDH$^\text{hi}$ progenitor cell delivery, or to prolong survival at or near the site of ischemic injury, is expected to further augment vascular regeneration via paracrine or contact dependent mechanisms.

2.4.1 Conclusion and Summary

Strategies that purify pro-angiogenic cell subtypes from alternate human sources are required to improve the efficacy of human cell therapy trials. Our studies outline several important caveats relevant to the development of cellular therapies to treat ischemic diseases. First, we have identified a mixed population of hematopoietic/myeloid and non-hematopoietic/endothelial ALDH$^\text{hi}$ progenitor cells from human UCB as a readily-available and clinically applicable cell population with potent pro-angiogenic function. Second, the potential use of allogeneic UCB cells will open new avenues towards clinical cell based therapies for ischemic disease, as mounting evidence indicates the potential for progenitor deletion and dysfunction of autologous BM cells in patients with severe diabetes and cardiovascular disease. Third, we have shown that neither high-level nor permanent engraftment of human cells were necessary to mediate augmentation of perfusion and improved vessel density in vivo. In an allogeneic context, such as intramuscular delivery of UCB ALDH$^\text{hi}$ cells in patients with ischemic heart disease or critical limb ischemia, future clinical transplantation strategies employing UCB may require only short-term immune suppression to initiate potent revascularization. Future studies will increase our understanding of the cellular constituents and paracrine
pathways that modulate regenerative angiogenesis, and will lead to novel therapies to improve tissue vascularization during ischemic diseases.
2.5 References


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Chapter 3

3 Ex Vivo Expanded Hematopoietic Progenitor Cells Promote Endothelial Cell Survival and Ischemic Limb Revascularization

Due to the relative rarity of ALDH\textsuperscript{hi} cells from UCB or BM, I aimed in Chapter 3 to determine if it was possible to expand UCB-derived ALDH\textsuperscript{hi} cells \textit{ex vivo} to generate more cells for therapeutic applications without losing beneficial vascular regenerative effect. Therefore the objectives of this chapter 3 were two-fold:

- To optimize the \textit{ex vivo} expansion of UCB ALDH\textsuperscript{hi} hematopoietic cells under clinically relevant serum-free conditions and to characterize vascular regenerative functions of the expanded progenitor cells \textit{in vitro}.

- To investigate the paracrine mechanisms by which expanded hematopoietic progenitor cells derived from UCB ALDH\textsuperscript{hi} contribute to revascularization after direct intramuscular injection into the ischemic limb.

The studies presented in this chapter used HMVEC as the EC source as minor adjustment compared to the HUVEC used in Chapter 2 to more closely model the phenotype of the EC thought be involved in angiogenesis and vascular regeneration. Vascular regeneration tends to affect the capillary microcirculation more so than a large vessel venous EC like HUVEC. The work presented in this chapter build directly upon the findings presented in Chapter 2. Specifically, it demonstrates that UCB ALDH\textsuperscript{hi} cells can be efficiently expanded \textit{ex vivo} without loss of vascular regenerative phenotype. Moreover, it introduces further refinement of the \textit{in vivo} and \textit{in vitro} models of vascular regeneration from those used in Chapter 2. The \textit{in vitro} model used in this chapter introduces further mechanistic characterizing of the effects of HPC on cultured EC in terms of cell survival and death as measured by flow cytometry. Building on the \textit{in vivo} murine hindlimb ischemia model from the previous chapter, here I present techniques to measure limb usage in addition to blood perfusion data. Quantitation of limb usage in addition to simple measurement of perfusion indicates that mice regain functional usage of the limb in addition to recovery of simple blood flow, strengthening the model. Furthermore, the
transplantation method was changed to intramuscular injection to more closely model the transplantation strategy from most PVD and CLI trials as well as to decrease delays in therapeutic benefit due to delayed and inefficient recruitment of cells transplanted intravenously.

3.1 Introduction

Ischemia, caused by insufficient blood flow to a given tissue, represents a widespread burden on human health. Cardiovascular disease (CVD) is the leading cause of death in North America, and accounts for roughly one third of all deaths and related healthcare costs for patients with CVD and stroke is now estimated to be in excess of $300 billion in the US alone1,2. Since Asahara et al. first described a role for circulating bone marrow-derived cells in vascular regeneration, cell therapy has emerged as an important strategy to treat ischemic diseases3-7. Thus, the development of novel cellular therapies for the management of acute cardiovascular diseases such as myocardial infarction and stroke, as well as chronic ischemic disease such as coronary and peripheral arterial disease, remain the focus of intense preclinical research.

Early clinical trials building upon the preclinical promise shown by bone marrow and umbilical cord blood-derived endothelial and hematopoietic progenitor cells showed that transplantation in humans was well-tolerated and safe6-8. Unfortunately, many of these therapies showed mixed results in improving cardiovascular disease outcomes, leading researchers to refocus on better understanding of the role of circulating progenitor cells in vascular repair9. As such, several cell types have been implicated in vascular regeneration including multiple cell types from the hematopoietic lineage10-13.

Recent cell-based approaches for vascular regeneration have focused on better functional characterization of purified cellular subpopulations and the mechanisms by which transplanted progenitor cells mediate vascular regeneration. Several groups have demonstrated that cells of the myeloid lineage recruit to areas of ischemia and support vessel regeneration though paracrine signaling to vessel-resident endothelial cells14-17. Our lab has demonstrated that transplantation of primitive hematopoietic progenitor cells with high aldehyde dehydrogenase (ALDH) activity, derived from human bone marrow
or umbilical cord blood, promote recovery from acute ischemic injury in immune deficient mice with hindlimb ischemia more effectively than unpurified, more heterogeneous populations (Chapter 2)\textsuperscript{18,19}. However, implementation of a cell-based therapy using UCB or BM-derived ALDH\textsuperscript{hi} cells is hindered by the rarity of the ALDH\textsuperscript{hi} cell population as it accounts for less than 1\% of total MNC in BM or UCB samples. However, interest in developing strategies to increase the number of hematopoietic stem and progenitor cells for transplantation into patients with hematological disorders has led to identification of clinically-applicable culture strategies for hematopoietic progenitor expansion using defined, xeno-free conditions supplemented with stem cell factor (SCF), thrombopoietin (TPO) and fms-related tyrosine kinase 3 ligand (FLT3L)\textsuperscript{20-22}.

In this study we characterize serum-free ex vivo expansion of UCB-derived ALDH\textsuperscript{hi} hematopoietic progenitor cells as a transplantable cell population for vascular regenerative cell therapies. We demonstrate that intramuscular transplantation of expanded ALDH\textsuperscript{hi} cells can accelerate the recovery of perfusion and limb use after femoral artery ligation-induced ischemic injury. Even after a 20-fold increase in the total number of myeloid cell progeny available for transplantation, expanded HPC prevented capillary loss as effectively as freshly isolated UCB ALDH\textsuperscript{hi} cells. In addition, the secretion of pro-angiogenic and pro-inflammatory effectors from expanded ALDH\textsuperscript{hi} cells correlated with improved survival and tube formation by human microvascular endothelial cells (HMVEC) during co-culture in vitro. We propose that ex vivo-expanded myeloid progeny derived from the ALDH\textsuperscript{hi} subset of UCB represent a readily available, clinically applicable population for future development of cell therapies to prevent limb loss in patients with severe peripheral artery disease.

3.2 Methods

3.2.1 Purification of human umbilical cord blood cells based on aldehyde dehydrogenase activity

Human umbilical cord blood samples were collected with informed consent immediately after scheduled, full-term Cesarean sections by venipuncture at the London Health Sciences Birthing Centre. The Human Research Ethics Committee at the University of
Western Ontario approved all studies. Cord blood samples were labeled with RosetteSep human cord blood progenitor cell enrichment tetrameric antibody complexes to prospectively deplete cells expressing lineage specific (Lin-) markers CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and glycophorin. After hypaque-ficoll centrifugation (Stem Cell Technologies, Vancouver, Canada) Lin– MNC were further depleted of erythrocytes by ammonium chloride lysis and assayed for ALDH activity using Aldefluor reagent (Stem Cell Technologies) as previously described (Appendix 1, Chapter 2). Fluorescence activated cell sorting was performed to isolate cells with low side scatter and high (ALDHhi) versus low (ALDHlo) ALDH activity. Before use in experiments, sorted cell population of >98% purity were washed in phosphate-buffered saline (PBS) to allow the efflux of Aldefluor substrate via ATP-binding cassette-transporters, leaving the cells unmodified for subsequent functional characterization and transplantation experiments.

3.2.2 Ex vivo expansion of hematopoietic progenitors from UCB ALDHhi cells

After fluorescence activated cell sorting, UCB ALDHhi cells were plated on recombinant human fibronectin coated (1 hour coating with 0.0125 mg/mL fibronectin in PBS followed by sterile water rinse) dishes in X-vivo 15 (Lonza, Basel, Switzerland) supplemented with stem cell factor (SCF, 10ng/mL), Fms-related tyrosine kinase 3 ligand (FLT3LG, 10ng/mL), and thrombopoietin (TPO, 10ng/mL). All growth factors were from Life Technologies, Burlington, ON. Growth media was changed every 3 days and conditioned media was collected after 3 and 6 days of expansion. Ex vivo expanded hematopoietic progenitors (HPC) were harvested and enumerated by Trypan blue viability at day 6.

3.2.3 Assessment of ALDH-activity and cell surface marker expression after ex vivo expansion

After 6 days ex vivo expansion, HPC were first assayed for the retention of high ALDH activity using Aldefluor reagent by flow cytometry to compare fluorescence levels to DEAB-inhibited control samples. Freshly isolated UCB ALDHhi cells and day 6 ex vivo-expanded HPC progeny were labeled with anti-human antibodies for lineage-specific
markers for T lymphocytes (CD3, Biolegend, San Diego, CA), macrophages (CD11b, BD Biosciences), monocytes (CD14, BD Bioscience), B lymphocytes (CD19, BD Bioscience), myeloid cells (CD33, BD Bioscience), and with antibodies for primitive progenitor-specific markers CD34 (Biolegend), CD38 (BD Biosciences), CD133 (Miltenyi Biotech, Auburn CA) and CD117 (BD Bioscience). Viable, 7-amino-actinomycin D (7AAD, BD Bioscience)-excluding cells were assessed for ALDH-activity and cell surface marker expression using an LSRII flow cytometer at the London Regional Flow Cytometry Facility and analyzed using FlowJo software (Treestar, Ashland, OR).

3.2.4 Quantitation of hematopoietic colony forming capacity in vitro

Freshly isolated UCB ALDHhi or 6-day ex vivo-expanded HPC were cultured in semi-solid methylcellulose media (Methocult® H4434, SCT) at limiting dilution. Hematopoietic colony formation at 14 days was enumerated by manual counting under bright field light microscopy, based on cell morphology identifying burst-forming units of erythrocytes (BFU-E), colony-forming units of granulocytes (CFU-G), macrophages (CFU-M), granulocytes and macrophages (CFU-GM), or multilineage mixed colonies (CFU-Mixed) containing cells of all 3 myeloid cell types listed above.

3.2.5 Microarray assessment of gene transcription after ex vivo expansion

mRNA was isolated from 6 day culture expanded HPC or freshly-isolated (uncultured) UCB ALDHhi cells using the mRNeasy Mini kits (n=3, Qiagen, Mississauga, ON). Microarray analysis was performed in triplicate using human array chips (Affymetrix, Santa Carla, CA) at the London Regional Genomics Centre (London, ON). Genes differentially expressed by uncultured UCB ALDHhi cells versus ex vivo-expanded HPC were analyzed using the Partek Genomic Suite and angiogenesis associated-transcripts coding for secreted proteins were identified (Partek, Inc., St. Louis, MO).
3.2.6 HMVEC survival when exposed to HPC-secreted factors

For endothelial cell growth and survival assays, 4x10^4 HMVEC (adult, dermal source) were cultured in endothelial basal media (EBM2-MV, Lonza) with or without growth factors (EGF, VEGF-A, FGF, IGF-1) and serum (5%) supplementation. To assess whether co-culture with expanded HPC could support HMVEC survival under serum-starved, growth factor depleted conditions, 8x10^4 HPC were seeded into porous (1µm) hanging transwells suspended above the HMVEC. The number of trypan blue excluding HMVEC present after 72 hours co-culture was enumerated by blinded hemocytometer counts. To assess HPC-conditioned media could support endothelial cell survival, 3x10^3 HMVEC were plated in endothelial basal media with or without growth factors, or in serum-free HPC-conditioned media collected after 3 or 6 days of expansion. The number of HMVEC present after 72 hours of culture was quantified using the CyQUANT cell proliferation assay (Life Technologies) performed as described by the manufacturers protocol. Briefly, total lysed-cell DNA content was quantified by a fluorescence plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) and cell number was interpolated by a standard curve. To quantify HMVEC apoptosis rates during co-culture or after exposure to HPC-conditioned media, HMVEC were also harvested after 24 hours and labelled with viability dye 7AAD and co-stained with anti-human annexin V and assayed using flow cytometry. Annexin V is expressed on the external cell membrane early during the apoptotic cascade and identified apoptotic cells and 7AAD staining was used to identify live versus dead cells. The frequency of live (7AAD^-/annexin V^-), apoptotic (7AAD^-/annexin V^+), and dead HMVEC (7AAD^+) was quantified using FlowJo analysis software.

3.2.7 HMVEC tubule network formation when exposed to HPC-secreted factors

To assess endothelial cell tubule formation, 1.4x10^4 HMVEC were cultured on growth factor-reduced Matrigel (BD Biosciences) for up to 48 hours. HMVEC Matrigel cultures were bathed with either endothelial basal media (EBM) without growth factors or serum, complete EGM2-MV (EBM + growth factors, +5% serum), or serum-free HPC expansion media, or serum-free HPC-conditioned media collected after 3 or 6 days of
expansion. To assess whether direct cell contact could augment endothelial cell function, HMVEC were also co-cultured on growth factor reduced Matrigel in direct co-culture with $1 \times 10^5$ ex vivo-expanded HPC in EBM. The number of complete branch points in the tubule networks formed by HMVEC was enumerated on an inverted microscope by blinded manual counts of complete branch points using four fields of view in triplicate wells at 6, 24 and 48 hours.

### 3.2.8 Identification of secreted angiogenic proteins during HPC-HMVEC co-culture

Conditioned EBM (without serum or growth factors) was harvested after 72h of culture of $4 \times 10^4$ HMVEC or $8 \times 10^4$ HPC to assess cytokines secreted by each cell type under basal culture conditions. To identify angiogenic proteins differentially secreted when cross-talk between EC and HPC was permitted, HMVEC and HPC were co-cultured together to generate EBM media conditioned by both cell types in direct co-culture simultaneously. Angiogenesis-associated proteins were detected in EC-, HPC-, and EC/HPC co-culture-conditioned media samples using a human angiogenesis antibody array C1000 kit and chemiluminescence imaging of the membranes as per manufacturer instructions (Human Angiogenesis Antibody Array C1000, RayBiotech, Norcross, GA, USA). Mean signal intensities for each protein detected was normalized to signal intensity of negative and positive control spots to adjust for differences in exposures between arrays and between samples as outlined in manufacturer guidelines. Relative fold-changes in protein levels in conditioned media were calculated by densitometry comparing array signal density between 3 different biological samples using Image J software.

### 3.2.9 Murine femoral artery ligation and intramuscular transplantation of expanded HPC

To induce unilateral hindlimb ischemia, surgical ligation and complete resection of the right femoral artery and vein was performed on anesthetised NOD/SCID (Jackson Laboratory, Bar Harbor, ME) or NOD/SCID/MPSVII mice as previously described in detail in Putman et al. in Appendix 1 and elsewhere (Chapter 2, Appendix 1)\textsuperscript{19,24}. Within 24 hours of surgery, mice were transplanted by intramuscular injection at three sites into
the right (surgical/ischemic) adductor muscle with 50μL saline as vehicle control, 20x10^6 unsorted UCB MNC, 1x10^5 UCB ALDH^hi cells, 1x10^5 ALDH^lo cells, or 5x10^5 ex vivo-expanded HPC. Total cell doses injected were selected based on frequency of ALDH^hi cells in unpurified UCB MNC and based on the frequency of cells that retained high ALDH-activity after 6 days of culture.

3.2.10 Quantification of hindlimb perfusion using laser Doppler perfusion imaging

Anesthetized mice were stabilized at 37°C for 5 minutes on a heat pad before blood flow in the lower hindlimbs (ankle to toe) was quantified using laser Doppler perfusion imaging (LDPI, Moor Instruments, Devon, UK). The perfusion ratio of the ischemic/surgical limb compared to the uninjured/control limb was assessed after surgery to confirm induction of acute ischemic injury with a perfusion ratio (PR<0.1) <10% compared to the normal limb. Subsequent LDPI measurements were taken in each mouse at 3, 7, 14, 21, and 28 days after transplantation to track recovery of perfusion temporally.

3.2.11 Gait analysis to assess recovery of limb use after injury

Noldus CatWalk software and equipment (CatWalk 7.1, Noldus, Wageningen, Netherlands) was used to quantify usage of the hindlimb after femoral artery ligation-induced injury. Baseline limb usage was determined by relative print intensity in the right versus left hindlimb, before and after surgery. PBS or expanded HPC-transplanted mice mice were reassessed for limb usage at 3, 7, 14, 21, and 28 days after transplantation by running on the CatWalk system. Relative usage of the injured right hindlimb was quantified by normalizing print intensity of the right hindlimb compared to the contralateral uninjured leg at each time point.

3.2.12 Assessment of hindlimb muscle vascularization

Adductor muscles from transplanted mice were were harvested, embedded in optimum cutting temperature medium (Tissue Tek, Sakura Finetek, Tokyo, Japan), frozen, and cryosectioned. Muscle sections were fixed in 10% formalin (Sigma), and blocked with mouse-on-mouse reagent (Vector Labs, Burlingame, CA). Adductor muscle blood vessel
density in ischemic and nonischemic limbs was quantified at 7 and 28 days after transplantation by quantifying mouse CD31<sup>+</sup> blood vessels using rat anti-mouse CD31 (1:100; BD Biosciences) and peroxidase-labeled anti-rat secondary antibodies (Vector Labs) visualized using 3,3′-diaminobenzidine substrate (Vector), and counterstained with hematoxylin. Blood vessel density was counted in a blinded fashion from nine photomicrographic fields per sample using light microscopy.

3.2.13 Statistics

Analysis of significance was performed by one-way ANOVA with Tukey’s multiple comparison tests for progenitor frequency assays, EC survival/proliferation assays, cytokine array screens, and vessel density histology. Two-way repeated measures ANOVA with Bonferroni post-hoc tests were used for LDPI and Matrigel tube forming time courses. Catwalk limb usage data were analysed using an unpaired Student’s t-test. All statistical analyses were performed using Graphpad Prism software.

3.3 Results

3.3.1 Ex vivo expansion of UCB ALDH<sup>hi</sup> cells

Lineage-depleted (Lin–) human UCB MNC with low side scatter and high (ALDH<sup>hi</sup>, 2.4±0.4% of Lin– cells) or low (ALDH<sup>lo</sup>, 16.2±3.6% of Lin– cells) aldehyde dehydrogenase activity were selected based on diamniobenzaldehyde (DEAB) inhibition gating controls (n=18, Figure 3.1). Next, the FACS-purified UCB ALDH<sup>hi</sup> cells were plated on fibronectin-coated dishes in serum-free X-vivo-15 media supplemented with 10ng/ml of each recombinant human TPO, SCF, and FLT3L. After 6 days in culture, there was a 20.0±5.6 fold increase in total cell number. 13.2±2.4% of expanded cells (Table 3.1, n=4) maintained high ALDH activity amounting to a 2.6-fold expansion of total ALDH<sup>hi</sup> cells over 6 days ex vivo expansion.

After 6 days culture under expansion conditions, resulting hematopoietic cells were assessed for changes in cell surface marker expression compared to freshly isolated UCB ALDH<sup>hi</sup> cells. Expanded progeny showed a decreased frequency of cells expressing the hematopoietic progenitor marker CD34 (29.5±2.7%) compared to freshly isolated
ALDH^{hi} cells (92.5±2.7%, Table 3.1 n=4). Similarly, expanded cells showed a diminished frequency of cells expressing prominin 1 (CD133) from 73.0±1.2% to 10.2±1.8%, and the receptor tyrosine kinase for SCF (c-kit/CD117) from 33.5±8.1% to 2.9±0.6% (Table 3.1, n=4). However, taking into account the 20-fold expansion in total cell number, the total number of CD34^{+} cells was increased 6.4-fold, the total number of CD133^{+} cells was increased 2.8-fold, and the total number of CD117^{+} cells was increased 1.7-fold after 6 days expansion. Culture expanded cells maintained high expression of the early myeloid progenitor marker Siglec-3 (CD33, 96.2±0.07%) compared to parental UCB ALDH^{hi} cells (96.1±0.8%, Table 3.1, n=4). Finally, after 6 days ex vivo culture there was no increase in the expression of mature lineage-specific markers for monocytes (0.3±0.1% CD14^{+}, Table 3.1, n=4), macrophages (0.4±0.2% Mac-1/CD11b^{+}), B cells (0.0±0.0% CD19^{+}, Table 3.1 n=4), or T cells (0.1±0.1% CD3^{+}, Table 3.1 n=4). Collectively, these data indicate that ex vivo culture of UCB ALDH^{hi} cells results in the expansion of the total number of primitive cells that retained early myeloid phenotype without increasing cell surface marker expression associated with mature monocyte or macrophage lineages.
Figure 3.1. Ex vivo expansion of hematopoietic progenitor cells from lineage depleted UCB ALDH$^{hi}$ cells.

Following ficoll-paque centrifugation and bead-labeled antibody negative selection of mature lineage markers (A) human UCB mononuclear cells were selected according to size (forward scatter) and granularity (side scatter). Inhibition of ALDH activity by DEAB prevents accumulation of Aldefluor fluorescence. Cells with low side scatter selected based on low (ALDH$^{lo}$) or high (ALDH$^{hi}$) Aldefluor$^{TM}$ fluorescence (n=18). (B) Schematic overview of ex vivo expansion of hematopoietic progenitor cells (HPC). (C) Representative FACS plots of measures of retention of high ALDH activity in ex vivo-expanded HPC (n=4).
Figure 3.1

A  UCB Mononuclear cells  Aldefluor + DEAB  Aldefluor

Side Scatter  Side Scatter  Side Scatter

Forward Scatter  Aldefluor  Aldefluor

R1  R2  R3

B  6 Days in Serum-free culture:
X-vivo15 + (SCF, TPO, FLT3L)

UCB  ALDH^h

UCB  ALDH^h

Day 3  Day 6  Day 6

HPC  fold expansion

conditioned media (CdM)

20.0±5.6

C  Day 6 HPC  Aldefluor + DEAB  Aldefluor

Side Scatter  Side Scatter  Side Scatter

Forward Scatter  Aldefluor  Aldefluor

ALDH^i  ALDH^h

13.2±2.4% 2.4±0.4%
Table 3.1. Cell surface marker phenotype changes after *ex vivo* expansion of HPC.

<table>
<thead>
<tr>
<th>Lineage Marker</th>
<th>UCB ALDH&lt;sup&gt;hi&lt;/sup&gt; cells (%)</th>
<th><em>ex vivo</em> expanded HPC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematopoietic Progenitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH</td>
<td>100</td>
<td>13.2±2.4</td>
</tr>
<tr>
<td>CD34</td>
<td>92.5±2.7</td>
<td>29.5±4.4</td>
</tr>
<tr>
<td>CD38</td>
<td>75.9±4.9</td>
<td>43.9±7.5</td>
</tr>
<tr>
<td>CD133</td>
<td>73.0±1.2</td>
<td>10.2±1.8</td>
</tr>
<tr>
<td>CD117</td>
<td>33.5±8.1</td>
<td>2.9±0.6</td>
</tr>
<tr>
<td><strong>Myeloid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD33</td>
<td>96.1±0.8</td>
<td>96.2±0.7</td>
</tr>
<tr>
<td><strong>Monocyte</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>2.8±0.9</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td><strong>B cell</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>0.8±0.2</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td><strong>T cell</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>3.0±1.5</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>
3.3.2 Ex vivo expansion of ALDH\textsuperscript{hi} UCB cells increases the total number of hematopoietic progenitor cells.

Fresh UCB ALDH\textsuperscript{hi} cells and 6 day expanded progeny were plated at limiting dilution in semi solid methylcellulose media with hematopoietic growth factors to enumerate clonogenic hematopoietic colony formation before and after 6 days ex vivo expansion. Notably, the ex vivo-expanded HPC retained significant multipotent myeloid colony forming capacity (Figure 3.2A). In freshly isolated UCB ALDH cells, approximately 1 in 3 cells showed hematopoietic colony forming function in methylcellulose. After ex vivo culture, HPC progeny displayed a reduced frequency of colony forming units at approximately 1 in 8 cells. At 20.0±5.6-fold total cell expansion this amounts to a 7.5-fold increase in total hematopoietic colony forming cells after 6 days ex vivo culture. Ex vivo-expanded HPC also showed a significant decrease in the overall frequency of erythroid blast forming units (BFU-E, macrophage (CFU-M) and granulocyte (CFU-G) colony forming units (Figure 3.2A, n=4, p<0.05). Furthermore, ex vivo expansion caused a shift in myeloid lineage specification away from granulocytic differentiation towards erythroid differentiation (Figure 3.2B,C). Taken together, these data illustrate that ex vivo culture of UCB ALDH\textsuperscript{hi} cells effectively increases the total number of hematopoietic progenitor cells (HPC) available for further application.
Figure 3.2. *Ex vivo* expanded HPC retain myeloid multipotency.

(A) Following 6 days of serum free culture expanded HPC retain diminished progenitor cell marker phenotype. (C) HPC plated in semi-solid methylcellulose media show myeloid multipotency at diminished levels compared to (B) freshly isolated ALDH$^{\text{hi}}$ cells. (*p<0.05, n=5).
3.3.3 Expanded HPC retain a vascular regenerative transcription profile

Affymetrix microarray identified multiple transcripts with significantly changed expression levels (>1.5 fold increase, p<0.01) in 3 ex vivo-expanded HPC samples compared to 3 parental, uncultured UCB ALDH^hi^ cell samples. Confirming cell surface phenotype as measured by flow cytometry, ex vivo-expanded HPC expressed significantly less KIT (CD117, p=8.31x10^-6) and PROM1 (CD133, p= 6.88x10^-4) compared to UCB ALDH^hi^ cells. Interestingly, despite significant reduction in CD34 expression at the cell surface there was no significant reduction in CD34 transcript levels in HPC compared to UCB ALDH^hi^ cells. The raw data was next filtered to focus on transcripts coding for secreted proteins with documented biological functions that potentially modulate endothelial cell survival and pro-angiogenic processes that were significantly upregulated after ex vivo expansion (n=3, Table 3.2). Ex vivo-expanded HPC showed upregulation of several transcripts which code for secreted growth factors known to modulate vascular regeneration including epidermal growth factor (EGF), vascular endothelial growth factor A and B (VEGF-A, VEGF-B), and angiopoietin 1 (ANGPT1, Table 3.2). Furthermore, HPC expressed significantly higher levels of transcripts for proteins that modulate vascular growth factor signaling including ribonuclease/angiogenin inhibitor-1 (RNH1, angiogenin regulator) and insulin-like growth factor binding protein 3 (IGFBP3, insulin-like growth factor modulator, Table 3.2). Ex vivo expansion of HPC from UCB ALDH^hi^ cells also increased expression of transcripts associated with matrix modifying proteins, including tissue inhibitor of metalloproteinases 1 and 3 (TIMP-1, TIMP-3, Table 3.2). Collectively, these data suggest that ex vivo-expanded HPC retain a vascular regenerative transcription profile.
Table 3.2. Transcripts for secreted proteins associated with vascular regeneration upregulated in *ex vivo*-expanded HPC

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Common Name</th>
<th>Biological Function</th>
<th>Molecular Function</th>
<th>Fold-Change vs. UCB ALDH&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>P-value vs. UCB ALDH&lt;sup&gt;hi&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
<td>promotes endothelial cell proliferation, survival, differentiation, and migration</td>
<td>EGF receptor activation</td>
<td>6.5</td>
<td>4.6x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>TIMP3&lt;sup&gt;3&lt;/sup&gt;</td>
<td>tissue inhibitor of metallocproteinases</td>
<td>prevents matrix remodeling, stabilizing preexisting vasculature</td>
<td>inhibition of MMP activity</td>
<td>2.9</td>
<td>1.2x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>RNH1</td>
<td>ribonuclease/angiogenin inhibitor 1</td>
<td>promotes cell survival under stress conditions, decreases apoptosis</td>
<td>modulates cellular localization, regulatory activity of angiogenin</td>
<td>2.5</td>
<td>7.7x10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>VEGFA&lt;sup&gt;A&lt;/sup&gt;</td>
<td>vascular endothelial growth factor A</td>
<td>primary regulator and activator of angiogenesis, autocrine signaling promotes vascular homeostasis</td>
<td>VEGFR2 receptor activation</td>
<td>2.5</td>
<td>1.1x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>ANGPT1</td>
<td>angiopoietin 1</td>
<td>stabilizes the blood vasculature by suppressing EC responses during vascular stress</td>
<td>TIE2 receptor activation</td>
<td>2.0</td>
<td>2.4x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>TIMP1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>tissue inhibitor of metallocproteinases 1</td>
<td>MMP-independent functions include inhibition of apoptosis, induction of angiogenesis and cell proliferation</td>
<td>inhibition of MMP activity; stimulating the Akt survival pathway</td>
<td>1.9</td>
<td>5.6x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>VEGFB&lt;sup&gt;B&lt;/sup&gt;</td>
<td>vascular endothelial growth factor B</td>
<td>critical for blood vessel survival under pathological conditions, non-essential role in vessel formation</td>
<td>VEGFR1 receptor activation</td>
<td>1.5</td>
<td>2.3x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>insulin-like growth factor binding protein 3</td>
<td>inhibits endothelial cell apoptosis, modulates IGF signaling</td>
<td></td>
<td>1.5</td>
<td>4.8x10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
3.3.4 Expanded HPC support endothelial cell tubule formation *in vitro*.

To assess whether these expanded HPC supported EC tubule forming function via paracrine actions, human microvascular endothelial cells (HMVEC) were plated on a thin gel of growth factor reduced matrigel bathed in growth factor+/serum+ endothelial growth medium (EGM2-MV, Figure 3.3A), or growth factor-/serum– endothelial basal media (EBM, Figure 3.3B), to serve as positive and negative controls for tubule formation respectively. In addition, HMVEC were exposed to basal HPC expansion media (X-vivo 15 + cytokines) or day 3 or day 6 HPC conditioned media (Figure 3.3D). Finally, HMVEC were co-cultured directly with expanded HPC in EBM (Figure 3.3C). Interestingly, HPC-conditioned media showed no effect on tubule formation compared to basal media controls (n=8). However, after 6 hours of direct co-culture with *ex vivo*-expanded HPC, HMVEC formed significantly more branch points compared to HMVEC grown in EBM or X-vivo 15 vehicle controls (p<0.01, Figure 3.3D, n=4). Furthermore, direct co-culture with HPC supported tubule formation equivalent to HMVEC grown in positive control conditions containing EGM fully supplemented with growth factors and serum. Importantly, HPC plated alone on growth factor reduced matrigel did not result in tubule formation. Together, this data shows that direct co-culture with HPC promotes EC tubule formation under growth factor-free and serum-free conditions.
Figure 3.3. *Ex vivo*-expanded HPC support tubule formation by HMVEC in serum-free, growth factor reduced conditions.

EC were seeded onto growth factor-reduced Matrigel supplemented with endothelial basal media with (A) 5% serum and EC growth factors, (B) basal EC media, (C) \(5 \times 10^3\) expanded HPC in direct co-culture, xvivo15 basal, or day 3 or day 6 HPC conditioned media. Photomicrographs were taken at 6h (A-C), 24h, and 48h and branch points were enumerated by blinded counts (Scale bar = 500 μm). (D) Summary of complete branch points counts per well. (**p<0.01).
3.3.5 Expanded HPC support HMVEC survival under growth factor-free, serum-free conditions

HMVEC were grown on plastic in EGM2 media fully supplemented with serum and growth factors (EGM2-MV, Figure 3.4A), or in basal endothelial cell media (EBM) without serum or growth factors (Figure 3.4B) or in non-contact transwells containing ex vivo-expanded HPC in EBM (Figure 3.4C). Under fully supplemented conditions (EGM2 + serum + cytokines) HMVEC demonstrated a 4-fold increase in cell number in 72 hours, whereas culture in serum and growth factor depleted basal conditions resulted in a 2-fold reduction in cell number. HPC co-culture prevented HMVEC cell loss after 72 hours under basal growth factor-free, serum-free conditions (Figure 3.4D, n=5-8, p<0.05).

To further address the paracrine support of endothelial cell survival under growth factor-free, serum-free conditions, HMVEC were plated in HPC conditioned media and cells were enumerated after 72 hours using the CyQuant system. Similar to the transwell co-culture results, HMVEC grown in day 3 or day 6 conditioned media showed the prevention of cell loss associated under growth factor-free, and serum-free conditions (Figure 3.4E, n=6-7, p<0.05).

Due to the pro-survival responses elicited in HMVEC when exposed to HPC secreted factors, HMVEC were also harvested after 24 hours culture in EGM2-MV, EBM, or in non-contact transwell co-culture with HPC and assessed for apoptosis using 7-aminoactinomycin D (7AAD) combined with annexin V-staining measured by flow cytometry (Figure 3.4F, n=4-6). HPC co-culture significantly reduced the frequency of dead cells (% 7AAD+ cells) compared to EBM-cultured controls (Figure 3.4G, n=4-6, p<0.05). However, HPC co-culture only modestly reduced the frequency of early apoptotic cells (% 7AAD-/Annexin V+). In contrast, HPC conditioned media did not alter total cell death or apoptosis rates compared to EBM or X-vivo 15 vehicle controls (Figure 3.4G). Collectively, these data indicate that HPC co-culture supported the survival of EC in serum-free, growth factor-free conditions.
(A-C) $4 \times 10^4$ HMVEC were cultured for 72 hours in basal media (EBM2) with or without growth factors (EGF, VEGF, FGF, IGF-1) and serum (5%) supplementation. Into each culture a hanging 1μm pore transwell was suspended above the EC and filled with $8 \times 10^4$ day 6 expanded HPC or basal media (Scale bar = 200μm). (D) Non-contact co-culture with D6 expanded HPC promoted the survival of EC under growth factor-free, serum-starved conditions (*p<0.05, n=5-8, in duplicate). (E) $3 \times 10^3$ EC were cultured in HPC expansion basal media (Xvivo 15) with or without EC growth factors (EGF, VEGF, FGF, IGF-1) and serum (5%) supplementation. Day 3 or Day 6 conditioned medium (Xvivo 15 base) collected during HPC expansion supported survival of EC under serum and growth factor-free conditions (n=6-7 in triplicate, *p<0.05). (F) Representative plots showing flow cytometric detection of Annexin V/7AAD survival status of endothelial cells 24 hours after culture. (G) Summary statistics for cell survival status at 24 hours. (different letter denotes *p<0.05).
3.3.6 HMVEC co-culture with HPC increased the secretion of pro-survival signals

HMVEC and HPC were grown in basal endothelial cell media (EBM) alone, or in direct co-culture with both cell types in basal EBM. After 72 hours, the conditioned media from each culture condition was collected and screened for secreted protein content using antibody array. When grown in basal endothelial media without serum or growth factors both HMVEC and *ex vivo*-expanded HPC secreted growth factors, chemokines, matrix modifying proteins and secreted receptor proteins associated with endothelial cell function during angiogenesis (Figures 3.5, 3.6, n=3). Notably, co-culture of HPC with EC significantly increased levels of several growth factors associated with vascular regeneration including epidermal growth factor (EGF, Figure 3.5, n=3, p<0.05) and angiopoietin-2 (Figure 3.6, n=3, p<0.05). Moreover, HPC co-culture increased the amount of angiopoietin-2 secreted into the media significantly more than the additive signals of HMVEC or HPC cultured alone, indicating a potential cellular crosstalk regulating increased angiopoietin 2 secretion (n=3, p<0.05). Many chemokines which have been implicated in the recruitment of inflammatory cell types to areas of ischemia were also significantly increased during HPC co-culture including GROα,β,γ/CXCL1,2,3, IL8/CXCL8, and RANTES/CCL5 (Figure 3.5, n=3, p<0.05). Several matrix modifying proteins were significantly upregulated during HPC co-culture including tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1, TIMP-2, Figure 3.5, n=3, p<0.05) and matrix metalloproteinase-9 (MMP-9, Figure 3.6, n=3, p<0.05). TIMP-2 was significantly upregulated in co-culture compared to the additive signals of HMVEC and HPC alone (n=3, p<0.05). Overall, HPC co-culture with HMVEC in unsupplemented media increased the paracrine and autocrine secretion of several angiogenesis-associated growth factors, chemokines and matrix modifying proteins.
Figure 3.5. HMVEC co-culture with HPC increases secretion of pro-survival signals: Part 1.

(A) Chemiluminescent imaging of RayBio® Membrane-Based Antibody Human Angiogenesis Array C1000 was used to screen and compare expression levels of angiogenesis-associated cytokines, growth factors, proteases, and soluble receptors secreted in basal endothelial media after 72 in culture. (B) HMVEC grown in basal unsupplemented media co-cultured with ex vivo-expanded HPC are exposed to higher levels of pro-survival signals (n=3, *p<0.05).
Figure 3.5

A  EC in EBM  Co-culture  HPC Conditioned Media  Array Map

Array 1

B  Growth Factors

Angiogenin

EGF

PLGF

TGF-β

GRO/CXCL1,2,3

IL-8/CXCL8

Chemokines

MCP-1/CCL2

RANTES/CCL5

Matrix Modifying

TIMP-1

TIMP-2
Figure 3.6. HMVEC co-culture with HPC increases secretion of pro-survival signals: Part 2.

(A) Chemiluminescent imaging of RayBio® Membrane-Based Antibody Human Angiogenesis Array C1000 was used to screen and compare expression levels of angiogenesis-associated cytokines, growth factors, proteases, and soluble receptors secreted in basal endothelial media after 72 in culture. (B) HMVEC grown in basal unsupplemented media co-cultured with ex vivo-expanded HPC are exposed to higher levels of pro-survival signals (n=3, *p<0.05).
Figure 3.6

A  EC in EBM  Co-culture  HPC

Conditioned Media  Array Map

Array 2

B  Growth Factors/Chemokines

Angiopoietin 2  Endostatin  I-TAC/CXCL11

MMP-1  MMP-9

PECAM-1  uPAR  VEGFR2
3.3.7 Expanded HPC enhance recovery of limb perfusion after transplantation into ischemic limbs

Previously in Chapter 2, I demonstrated that freshly isolated UCB ALDH\textsuperscript{hi} cells support recovery of perfusion and revascularization in the ischemic hindlimb of mice after intravenous transplantation into mice with unilateral hindlimb ischemia (Chapter 2)\textsuperscript{19}. To confirm these findings with UCB ALDH\textsuperscript{hi} cells and to assess whether expanded HPC could impact the recovery of perfusion \textit{in vivo}, freshly isolated UCB ALDH\textsuperscript{hi} cells and \textit{ex vivo}-expanded HPC were transplanted intramuscularly into immune deficient NOD/SCID mice with acute unilateral hindlimb ischemia after femoral artery ligation surgery. Hindlimb perfusion was assessed by laser Doppler perfusion imaging (LDPI) and the perfusion ratio of the ischemic limb versus the contralateral control limb was determined weekly for up to 28 days. After femoral artery ligation surgery, the perfusion ratio (PR) of the treatment leg was less than 10\% of the control leg in all transplanted mice (PR≤0.1). Mice treated with intramuscular phosphate buffered saline (PBS, Figure 3.7A) vehicle control established the baseline recovery of perfusion (PR=0.49±0.03 at 28 days after treatment). Mice with ischemic adductor muscle transplanted intramuscularly with freshly isolated 20x10\textsuperscript{6} UCB MNC or 1x10\textsuperscript{5} purified UCB ALDH\textsuperscript{lo} cells showed no significant changes in recovery of perfusion compared to PBS controls (Figure 3.7D). In contrast, mice that were treated by intramuscular transplantation of 1x10\textsuperscript{5} purified UCB ALDH\textsuperscript{hi} cells (Figure 3.7B, Day 7 PR=0.45±0.09) or 5x10\textsuperscript{5} \textit{ex vivo}-expanded HPC (Figure 3.7C, Day 7 PR=0.40±0.02) showed significantly accelerated recovery of perfusion of the ischemic limb compared to PBS vehicle controls (Figure 3.7A, Day 7 PR=0.25±0.02) within 7 days after transplant (Figure 3.7D). At a frequency of 13.2\% of expanded HPC retaining high ALDH activity, mice transplanted with 5x10\textsuperscript{5} HPC receive an equivalent dose of 6.6x10\textsuperscript{4} ALDH\textsuperscript{hi} cells, a lower effective dose of ALDH\textsuperscript{hi} cells. Importantly, improved perfusion at day 7 translated to a significant increase in perfusion at later time points (days 14-28) compared to PBS injected controls. Collectively, these data confirm that transplantation of UCB ALDH\textsuperscript{hi} cells promote recovery or perfusion in the ischemic hindlimb of mice after femoral artery ligation. Furthermore, the data indicates that \textit{ex vivo} expansion of HPC from UCB ALDH\textsuperscript{hi} cells does not diminish vascular regenerative function.
To further assess recovery from acute ischemic injury, limb use by gait analysis of transplanted mice was assessed using Noldus™ Catwalk 7.1 software. Mice treated by intramuscular injection of ex vivo-expanded HPC showed significantly increased usage of the ischemic limb at 7 days after transplant compared to PBS treated control mice (Figure 3.7E). These findings demonstrate that the recovery of perfusion in mice transplanted with UCB ALDH\textsuperscript{hi} cells or ex vivo-expanded HPC is accompanied by augmented recovery of limb usage in the injured limb.
**Figure 3.7. Direct intramuscular injection of UCB ALDH$^{hi}$ cells augment perfusion of ischemic limbs.**

(A-C) Representative LDPI following right femoral artery ligation and tail vein injection of PBS vehicle control, 20x10$^6$ unsorted UCB MNC, 1x10$^5$ ALDH$^{hi}$ cells, 1x10$^5$ ALDH$^{lo}$ cells, or 5x10$^5$ *ex vivo*-expanded HPC monitored for 28 days (n=7-15). Numbers in bottom left of each LDPI image indicate perfusion ratio of the ischemic versus the non-ischemic hindlimb from ankle to toe. (D) Summary of mean perfusion ratio ± SEM as above. Transplantation of UCB ALDH$^{hi}$ cells promoted robust, maintained recovery of perfusion in ischemic mouse hindlimbs by day 7 post-transplantation as compared to saline injected controls (*p<0.05,**p<0.01). (E) Noldus™ catwalk paw print intensity at day 7 post transplantation showed that mice transplanted with HPC regained use of their ischemic leg faster than saline vehicle controls (n=6).
Figure 3.7

A 700 μL PBS Control

B 2×10⁵ UCB ALDH^hi

C 5×10⁵ Day 6 HPC

D

<table>
<thead>
<tr>
<th>Days after Transplantation</th>
<th>Mean Perfusion Ratio (Ischemic/Nonischemic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>PBS control (n=15)</td>
<td>0.08</td>
</tr>
<tr>
<td>UCB MNC (n=7)</td>
<td>0.07</td>
</tr>
<tr>
<td>ALDH^lo (n=7)</td>
<td>0.08</td>
</tr>
<tr>
<td>ALDH^hi (n=7)</td>
<td>0.08</td>
</tr>
<tr>
<td>Expanded HPC (n=8)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

E

Days after Transplantation

<table>
<thead>
<tr>
<th>Days</th>
<th>Normal hind paw</th>
<th>Injured hind paw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>PBS</td>
<td>HPC</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>HPC</td>
</tr>
</tbody>
</table>

Ratio of Proliferative Index (Normal/Injured)

<table>
<thead>
<tr>
<th>PBS</th>
<th>HPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.85</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Indicates statistical significance.
3.3.8 Intramuscular transplantation of HPC prevents loss of endogenous blood vessels after acute ischemic injury

Ischemic and control adductor muscle were cryosectioned and stained for CD31 expression to enumerate the blood vessels present at 7 and 28 days after injury. Within 7 days of femoral artery ligation, saline-treated control mice showed a significant loss of the CD31$^+$ positive vessels in the ischemic limb (61.9±2.1 CD31$^+$ vessels/mm$^2$) compared to the contralateral control limb (69.9±0.8 CD31$^+$ vessels/mm$^2$, $p<0.05$, $n=7$, Figure 3.8C). This loss of vascularization in the ischemic limb (62.5±1.6 CD31$^+$ vessels/mm$^2$, Figure 3.8D) of saline treated mice was consistent at 28 days after induction of ischemic injury compared to normal limb (69.4±0.6 CD31$^+$ vessels/mm$^2$, Figure 3.8D). Mice treated by intramuscular injection of UCB MNC or ALDH$^{lo}$ cells showed no difference in ischemic limb vascularization compared to PBS controls (Figure 3.7). Mice transplanted with ex vivo-expanded HPC showed prevention of CD31$^+$ cell loss at 7 (72.3±0.6 CD31$^+$ vessels/mm$^2$, Figure 3.8C) and 28 days after treatment (68.1±1.8 CD31$^+$ vessels/mm$^2$, Figure 3.8D). Purified ALDH$^{hi}$ cells from fresh UCB similarly showed a reduction in the loss of vascularization in the ischemic muscle at 28 days after treatment (71.8±2.3 CD31$^+$ vessels/mm$^2$, Figure 3.8D). These findings suggested that UCB ALDH$^{hi}$ cells and ex vivo-expanded HPC prevent the loss of CD31$^+$ vessels in the ischemic hindlimb of mice after ischemic injury.
Figure 3.8. Intramuscular transplantation of *ex vivo*-expanded cord blood hematopoietic progenitor cells prevents loss of vascularization of ischemic hindlimb muscle after acute ischemic injury.

(A, B) Representative photomicrographs of CD31 staining in ischemic adductor muscle sections taken 28 days after right femoral artery ligation and intramuscular injection of PBS, ALDH<sup>hi</sup> cells, or HPC. (C, D) Summary of mean vessel density ± SEM in the ischemic or non-ischemic limb at (C) 7 days, or (D) 28 days after transplantation. Mice that were transplanted with fresh UCB ALDH<sup>hi</sup> cells or *ex vivo*-expanded HPC demonstrated increased vascularization of the surgical limb. (*p<0.05).*
Figure 3.8

A

PBS - Normal Muscle
Day 28

HPC - Normal Muscle
Day 28

100 µm

CD31

B

PBS - Ischemic Muscle
Day 28

HPC - Ischemic Muscle
Day 28

100 µm

CD31

C

D

PBS (n=7)
HPC (n=7)

PBS (n=7)
HPC (n=8)
UCB ALDH<sup>lo</sup> (n=7)
UCB ALDH<sup>hi</sup> (n=7)

CD31<sup>+</sup> Vessels/mm<sup>2</sup>

Ischemic
Normal
Day 7 Muscle

CD31<sup>+</sup> Vessels/mm<sup>2</sup>

Ischemic
Normal
Day 28 Muscle
3.4 Discussion

In this study I demonstrate that human UCB-derived cells with high ALDH activity can be readily expanded in serum free conditions without diminishing vascular regenerative function after direct intramuscular transplantation in vivo. After 6 days ex vivo culture in clinically relevant serum free conditions, 20-fold expansion of total hematopoietic cell number was obtained with substantial expansion of total myeloid progenitor cell number. Our findings show that ex vivo-expanded HPC maintained an angiogenic vascular regenerative phenotype by mRNA transcriptional profile. Furthermore, we demonstrate the HPC responded to EC cell signals in culture and increase secretion of angiogenic growth factors, matrix-modifying proteins and chemokines that recruit support cells to the site of injury, inflammation and hypoxia.

This vascular regenerative phenotype of HPC was confirmed by demonstration that HPC supported endothelial cell survival and tubule formation in growth factor-free, serum-free co-culture culture conditions. Importantly, transplantation of ex vivo-expanded HPC into immune-deficient mice with femoral artery ligation–induced hindlimb ischemia promoted recovery of perfusion and prevented loss of vascularization in the hindlimb similar to previously characterized UCB ALDHhi cells (Chapter 2).19 Thus, we propose that ex vivo expansion of UCB ALDHhi cells represents a clinically applicable means of effectively increasing the number myeloid progenitor cells for cardiovascular disease therapy without reducing vascular regenerative function.

After 6 days of serum free expansion culture UCB ALDHhi cells expanded 20-fold in total cell number. Of these expanded HPC, only 13.2±2.4% retained high ALDH activity, amounting to a total 2.6 fold expansion of ALDHhi cells over 6 days ex vivo expansion. Based on other progenitor cell surface marker expression our 6-day expansion protocol also produced a 6.4-fold expansion of CD34+ cells, a 2.8-fold expansion of CD133+ cells and a 1.7-fold expansion in the number of total CD117+ cells, despite a decrease in the relative frequency of cells expressing progenitor markers. The general decrease in progenitor marker expression frequency despite total progenitor cell number is consistent with other published hematopoietic cell expansion protocols20,25,26. Furthermore, it is
possible that the number of CD117 expressing cells is higher than detected by flow cytometry as after prolonged culture in high concentrations of SCF, the ligand for CD117/c-Kit, leads to receptor internalization and recycling as is the case with many receptor tyrosine kinases\(^\text{27}\). Relative decreases in hematopoietic progenitor marker expression were confirmed by microarray results showing significantly lower expression of both CD117/c-Kit (\textit{KIT}) and CD133 (\textit{PROM1}) transcripts. Thus, we demonstrate here that \textit{ex vivo} expansion of UCB ALDH\(^{hi}\) cells effectively increases the number of angiogenic myeloid ALDH\(^{hi}\)/CD34\(^+\)/CD133\(^+\)/CD117\(^+\) progenitors cells available for cell therapy applications.

It has recently been shown that decreasing autocrine inhibitory signaling during HPC expansion by use of a batch-fed system, it is possible to significantly increase the expansion of hematopoietic stem and progenitor cells\(^\text{28,29}\). It is plausible that by applying new, more efficient methods of \textit{ex vivo} HPC expansion, and by the use of novel molecules to prevent progenitor differentiation, it will be possible to further increase the number of hematopoietic cells that retain vascular regenerative function for cell therapy applications outside of transplantation for haematological disorders. This is an important area of development for applications employing UCB-derived cells in an allogeneic setting, because available cell number currently prohibits clinical application.

We also demonstrated that co-culture of HPC with endothelial cells in growth factor-free, serum-free, basal endothelial cell media led to increased survival of endothelial cells as measured by cell counts and by viability dye measures \textit{in vitro}. Furthermore, intramuscular transplantation of HPC into the ischemic hindlimb of mice augmented recovery of perfusion and prevented loss of capillary density within the ischemic muscle tissue. As such, HPC conditioned media contained secreted EGF and co-culture with EC increased the concentrations of EGF significantly compared to EC cultured alone. Moreover, EGF transcript levels were increased 6.5-fold in \textit{ex vivo}-expanded HPC compared to fresh UCB ALDH\(^{hi}\) cells. The observed pro-survival effect on endothelial cells in both \textit{in vitro} and \textit{in vivo} contexts is consistent with potential paracrine stimulatory effects by secreted EGF on endothelial cells. Endothelial cells express epidermal growth
factor receptor (EGFR)\textsuperscript{30-32} and EGFR activation has been shown to activate the PI3K/Akt pathway thereby regulating endothelial cell survival\textsuperscript{33}.

Furthermore, we show that \textit{ex vivo}-expanded HPC retain a vascular regenerative, angiogenic transcriptional and secretory profile beyond EGF expression. After culture, HPC showed upregulation of important regulators of endothelial cell function including angiopoietin 1 (\textit{ANGPT1}) and vascular endothelial growth factors A and B (\textit{VEGF-A, VEGF-B}). During homeostasis \textit{in vivo} EC-EC contacts are stabilized and cell survival is promoted by perivascular cells who secrete angiopoietin 1 and activate the Tie2 receptor on endothelial cells\textsuperscript{34,35}. Vascular endothelial growth factor A (VEGF-A) is the primary regulator of the angiogenic cascade by which endothelial cells become activated to proliferate and migrate in response to hypoxia or injury\textsuperscript{36}. Furthermore, VEGF-A signaling has been shown to be necessary for EC survival signaling in homeostatic quiescence and in hypoxic conditions\textsuperscript{36,37}. Additionally, VEGF-B signaling has been demonstrated to be critical for blood vessel survival under pathological conditions promoting not only EC survival but increasing survival of mural smooth muscle cells or pericytes\textsuperscript{38}. ANG2 is expressed by EC and its expression is increased under hypoxic conditions\textsuperscript{34,39}. ANG2 is stored in endothelial Weibel–Palade bodies which are secreted in response to inflammation and mediate endothelial cell activation\textsuperscript{34,40,41}. However, in the absence of VEGF, angiopoietin 2 induces destabilization and regression of the vasculature. During hypoxia, which promoted both ANG2 and VEGF secretion, neovascularization is induced by a VEGF mediated pro-angiogenic program referred to as the “angiogenic switch”\textsuperscript{34,39}. HPC co-culture increased secreted ANG2 levels in conditioned media significantly indicating a potential role for HPC in increasing activation of endothelial cells during inflammation and hypoxia. Taken together, these data indicate that \textit{ex vivo}-expanded HPC maintain the transcriptional prolife and secretory functions necessary to support vascular stabilization after ischemic injury pushing the EC cell program from regression to survival and stabilization.

HPC co-culture with HMVEC also increased the secretion of TIMP-1 and TIMP-2 \textit{in vitro}. Moreover, \textit{TIMP1} transcript was upregulated in HPC after \textit{ex vivo} expansion from UCB ALDH\textsuperscript{hi} cells. Increased TIMP expression been shown in hematopoietic progenitor
cells and myeloid cells previously, and play a role in modifying the extracellular matrix in response to vascular injury or hypoxia. Broadly, TIMPs inhibit MMP-mediated remodelling of the extracellular matrix and basement membrane, an important step in both vascular regression and angiogenesis. TIMPs promote the stability of blood vessels by preventing breakdown of the supportive extracellular matrix and promoting basement membrane deposition. Beyond the normal activity of TIMPs regulating MMP activity, TIMP-1 has been shown to have MMP-independent functions which include inhibition of apoptosis, induction of angiogenesis and cell proliferation. In particular, TIMP-1 has specifically been shown to prevent TNF-α mediated apoptosis in EC through the Akt pathway. Both the MMP-independent and MMP-mediated effects of TIMP-1 on EC and the vasculature promote endothelial cell survival by supressing cell death are consistent with our *in vitro* and *in vivo* findings showing less cell death in HPC-EC co-culture conditions.

*Ex vivo* HPC expansion led to increases in transcripts associated with modulating vascular growth factor signaling ribonuclease/angiogenin inhibitor 1 (*RNHI*) and insulin-like growth factor binding protein 3 (*IGFBP3*). RNH1 has been shown to promote cell survival in stress conditions by decreasing apoptosis and modulating the cellular localization and activity of angiogenin which is promoter of angiogenesis. IGFBP3 has been demonstrated to inhibit endothelial cell apoptosis by an IGF-independent mechanism. Thus, angiogenin and IGFBP3 may represent important regulators EC function and the effects of these two regulators may also contribute to the pro-survival mechanism consistent with our findings.

Co-culture of HPC with EC increased secreted levels of several chemokines including members of the CXC and CC families, GRO/CXCL1-3, IL-8/CXCL8, and RANTES/CCL5. GRO and CXCL8 are known to play a role in chemokine-mediated regulation of angiogenesis and regulate recruitment of neutrophils, macrophages and lymphocytes to the site of injury. Interestingly, MMP-9 was also secreted by *ex vivo*-expanded HPC. MMP-9 is thought to be important in activating the angiogenic switch, playing a role in increasing VEGF bioavailability by freeing it from deposits within the extracellular matrix. MMP-9 has been further implicated in vascular regeneration by
playing a role in promoting vasculogenesis\textsuperscript{54}. Notably, MMP-9 has been implicated in the mobilization of circulating angiogenic cells that also modulate a pro-angiogenic niche in areas of hypoxia. Collectively, the cumulative effects of these molecular mediators all act to increase the potential recruitment of circulating cells from the host, which can help mediate a vascular regenerative program at the site of injury. These proposed mechanisms could play a plausible role \textit{in vivo} in vascular regeneration seen after transplantation of HPC into ischemic tissues.

Our lab has previously described a subset of hematopoietic progenitor cells with from human BM high ALDH activity that play a role in paracrine support of vascular regeneration (Chapter 2)\textsuperscript{18,24,55}. Perin \textit{et al.} have recently reported phase I/II clinical trials using autologous BM-derived ALDH\textsuperscript{hi} cells for the treatment of critical limb ischemia secondary to peripheral artery disease and ischemic heart failure\textsuperscript{56,57}. Both studies demonstrated the safety of ALDH\textsuperscript{hi} cells in patients with cardiovascular disease. Importantly, in patients with critical limb ischemia, intramuscular injection of autologous BM ALDH\textsuperscript{hi} promoted improved Rutherford category scores and reduced resting pain. Furthermore patients treated by ALDH\textsuperscript{hi} cell transplant showed modest but significantly improved ankle to brachial index scores indicating improved perfusion of the lower limb. Finally, patients who were treated with BM ALDH\textsuperscript{hi} cells reported significantly improved quality of life after treatment\textsuperscript{56}. These data demonstrate that ALDH\textsuperscript{hi} cells are a viable clinical cell population for the treatment of ischemic disease, and our data further suggests that this population may be expanded using clinically applicable conditions to increase the number of pro-vascular progenitor cells available for transplantation.

Notably however, there is emerging evidence that application of autologous bone marrow progenitor cells in patients with diabetes or cardiovascular disease might be problematic. There exists convincing evidence for decreased frequency and reduced regenerative function for circulating and bone marrow progenitor cells in patients with chronic diabetes, cardiovascular disease and increasing age\textsuperscript{58-61}. Unfortunately, the patients who would most benefit from vascular regenerative therapy are generally of increasing age and overwhelming affected by diabetes and cardiovascular disease\textsuperscript{1}, making the development of allogeneic transplantation strategies desirable. As such, human umbilical
cord blood represents an allogeneic source is readily available and may possess distinct advantages over autologous bone marrow as source of cells for vascular regenerative therapy. Most notably, umbilical cord blood is early in ontogeny and free of chronic disease and age related dysfunctions. We have also shown here that ex vivo culture of UCB derived ALDH$^\text{hi}$ cells permits expansion of the number of cells available for therapy. However, it is possible that similar ex vivo expansion strategies could be applied to autologous bone marrow transplants in patients as an alternative in order bypass the need for immune suppression in the allogeneic setting. Nonetheless, we propose that UCB ALDH$^\text{hi}$ cells represent a readily available, and expandable HPC population with robust vascular regenerative properties. Therefore, expanded UCB-derived ALDH$^\text{hi}$ cells represent an excellent candidate population for the development of allogeneic cell therapy for ischemic disease.

Our current work demonstrates that is possible to increase the number of hematopoietic progenitor cells available from UCB ALDH$^\text{hi}$ cells for applications in vascular regeneration without losing regenerative function. We propose that purification of BM or UCB based on high aldehyde dehydrogenase activity followed by ex vivo expansion to increase the progenitor pool poses a promising clinical approach to developing cell therapy for vascular regeneration and the treatment of ischemic disease.
3.5 References


Chapter 4

4  High Aldehyde Dehydrogenase Activity Identifies a Subpopulation of Multipotent Stromal Cells with Increased Vascular Regenerative Function

BM MSC are a population of progenitor cells that expand readily in ex vivo cultures with promising potential for vascular regenerative cellular therapies. However, our lab has demonstrated that different MSC lines from different donors showed variable efficacy to promote regeneration. Furthermore, the Hess lab had previously shown that re-selection of MSC that retained high ALDH activity after culture increased islet regenerative capacity. Therefore the objective of the studies presented in this chapter was as follows:

- To characterize the differentiative and vascular regenerative functions of MSC subpopulations selected after expansion for low versus high ALDH activity.

The studies presented in Chapter 4 use some of the general findings from Chapters 2 and 3 and other work by the Hess laboratory, specifically that selection of cells with high ALDH activity isolates a population of cells with increased vascular regenerative potential, as the basis for an approach to prospectively identify a subset of MSC that might be superior candidates for future development of cellular therapy for CVD. It uses the in vitro models established in Chapters 2 and 3 to establish proof of concept for proposed future in vivo studies with populations of MSC purified by ALDH activity level.
4.1 Introduction

Despite significant progress and advances to improve clinical management, cardiovascular disease (CVD) remains the leading cause of premature death worldwide and represents a huge burden on the health care systems in North America\textsuperscript{1-5}. Ischemia is characterized by insufficient blood flow to a given tissue or organ and includes both acute conditions like myocardial infarction and stroke and chronic ischemic conditions like atherosclerotic arterial disease. Direct and indirect costs of medical management and treatment of CVD is a growing burden on global health care systems, estimated to now cost in excess of $310 billion annually in the United States alone\textsuperscript{3,4,6-8}.

Multipotent stromal cells (MSC) also referred to as mesenchymal stromal stem cells can be isolated from bone marrow (BM) and other tissues. MSC are defined by their \textit{ex vivo} culture method; they must be plastic adherent, express the markers CD73 (ecto 5’ nucleotidase), CD90 (Thy-1), and CD105 (endoglin) after culture and must not express hematopoietic markers like CD45 or CD14\textsuperscript{1,2,7-10}. Additionally, MSC must demonstrate definitive multipotency, by ability to differentiate into osteocytes, adipocytes and chondrocytes\textsuperscript{1-5}.

Broadly, MSC are considered as good candidates for allogeneic cell therapy applications because of their low immunogenicity; they do not express MHC class II (HLA-DR) nor co-stimulatory molecules like CD40, CD80 and CD86\textsuperscript{3,4,6-8}. Furthermore, MSC have been shown to demonstrate multi-tissue engraftment and little proliferative capacity after transplantation, reducing risk of transplantation related malignancies\textsuperscript{1,2,7-10}. In general, MSC contributions towards regenerative therapy is primarily through the modulation of the tissue microenvironment as opposed to cell replacement\textsuperscript{6,8,11}. Indeed MSC have been demonstrated to readily support many repair processes including vascular regeneration\textsuperscript{1,8-10}. MSC have also been shown to augment cardiomyocyte\textsuperscript{1,10-14} and EC\textsuperscript{1,10,15,16} survival, and secrete VEGF-A, FGF-2, ANG-1, and MCP-1\textsuperscript{1,10,12-14,17}. MSC have also been recently identified as a component of vascular pericytes that express CD146 in multiple human organs \textit{in situ} and help stabilize newly formed vessels when co-transplanted with ECs \textit{in vivo}\textsuperscript{15,16,18-20}. MSC have furthermore been shown to play an important role in
immunomodulation presenting interesting potential paracrine applications in auto-
immune disease and inflammatory conditions\textsuperscript{17,18,21-23}. Taken together these unique
properties underscore the considerable promise of MSC from human BM or UCB as a
potential source for vascular regenerative cell therapies.

BM MSC represent a readily available population of progenitor cells that expand
efficiently in \textit{ex vivo} cultures with promising potential for vascular regenerative cellular
therapies. However, an outstanding limitation to the successful implementation of MSC-
mediated regeneration in various clinical therapeutic settings is major inter-donor
variability in MSC function. The Hess laboratory have previously shown that
transplanted BM MSC populations show substantial donor variation in ability to support
regeneration of pancreatic islets in a streptozotocin-induced beta cell deletion model of
diabetes\textsuperscript{19,20}. Other groups have demonstrated donor variation in MSC in terms of
osteogenic differentiative capacity\textsuperscript{21,23,24}. Furthermore, MSC have been shown to undergo
replicative senescence under differentiative growth conditions and subsequent reduction
in regenerative function over prolonged culture in several systems\textsuperscript{19,25}. This decline in
MSC function over extended \textit{ex vivo} culture also demonstrates notable inter-donor
variability\textsuperscript{24}. To improve cell therapy using MSC, it is desirable to identify prospective
markers to identify subpopulation with increased regenerative capacity.

Our laboratory has previously demonstrated that aldehyde dehydrogenase (ALDH), a
cytosolic enzyme involved in retinoic acid synthesis and in cellular self-protection
against oxidative stress is a useful marker of primitive progenitor cells associated with
increased vascular regenerative function (Chapter 2)\textsuperscript{26,27}. Indeed the Hess laboratory have
shown that selection of BM mononuclear cells with high ALDH activity isolates an
enriched starting population of mixed hematopoietic, endothelial and mesenchymal
progenitor cells that supported robust recovery of perfusion and increased vascularization
after femoral artery ligation-induced ischemic injury in an immunodeficient mouse
model\textsuperscript{26}. Furthermore, the Hess laboratory has demonstrated the MSC derived from BM
cells with high ALDH activity support increased regeneration in the diabetic pancreas\textsuperscript{19}.
Thus, we hypothesized that MSC subpopulations with increased ALDH activity will
improve vascular regenerative function via the paracrine support of endothelial cell
activation and neo-vessel forming function. In this chapter, we present preliminary characterization of the differentiation and vascular regenerative properties of MSC subpopulations with high versus low ALDH activity sorted after expansion.

4.2 Methods

4.2.1 BM MSC derivation and culture

BM aspirates were acquired with informed consent during routine pre-screening for BM donation by Dr. Anargyros Xenocostas at the London Health Sciences Centre in London, Ontario, Canada. The mononuclear cell fraction was isolated using ficoll centrifugation. BM mononuclear cells were then depleted of residual red blood cells by lysis in ammonium chloride. Mononuclear cells were plated at 2.7x10^5 cells/cm^2 on tissue culture treated plastic dishes in fully-supplemented Amniomax C-100 media (Gibco, Grand Island, NY). Culture media was changed 48 hours after plating to allow adherent stromal cells to adhere to plastic. Thereafter, growth media was changed 3 times per week for maintenance and cell expansion. Adherent MSC colonies were detectable by light microscopy within 2 weeks of plating. MSC were harvested by trypsin and subsequently passaged at 4.7x10^3 cells/cm^2 for expansion culture. After passage 2-3, MSC were harvested using trypsin and suspended in FBS + 10% DMSO for cryopreservation. Thawed MSC were seeded at 4.7x10^3 cells/cm^2 in Amniomax to reestablish MSC culture and subsequently passaged prior to reaching 80% confluence until sufficient cell numbers were accrued for experiments.

4.2.2 Isolation of MSC subpopulations based on ALDH activity

To assay BM MSC for relative ALDH activity, growth media was aspirated, cells were washed with PBS and cells were collected after trypsin-mediated detachment. After cell counting, MSC were assayed for ALDH activity using Aldefluor reagent at 10x10^6 cells/mL as per manufacturer instructions (Stemcell Technologies, Vancouver, BC). Fluorescence activated cell sorting (FACS) was performed to isolate cells with high (ALDH^hi) or low (ALDH^lo) ALDH activity to >99% purity. ALDH^lo gating was set by using DEAB inhibition of ALDH activity in control samples. Sorted cells were subsequently washed in PBS to allow efflux of Aldefluor substrate via reactivation of
inhibited ABC-transporters prior to plating at 2.7x10^5 cells/cm² on tissue culture treated plastic dishes in fully supplemented Amniomax C-100 media as described above.

4.2.3 Cell surface marker phenotype analysis

BM MSC were harvested with trypsin, collected in PBS+5%FBS and assayed for ALDH activity level by labeling with Aldefluor as above. Subsequently, cells were stained with anti-human antibodies for MSC marker expression CD73, CD90, CD105. To confirm that MSC populations are negative for hematopoietic markers MSC were also labelled with antibodies raised against monocyte marker CD14, and pan-leukocyte marker CD45. Finally, to further characterise MSC sub populations Aldefluor labelled MSC were labelled with antibodies for pericyte marker CD146 and LNGFR (CD271, Miltenyi Biotech, Auburn CA). All other antibodies were obtained from BD Biosciences (Missisauga, ON). Viable, 7-amino-actinomycin D (7AAD, BD Bioscience)-excluding cells were assessed for ALDH-activity and cell surface marker expression using an LSRII flow cytometer at the London Regional Flow Cytometry Facility and analyzed using FlowJo software (Treestar, Ashland, OR). All gating was performed based on fluorescence minus-one controls (FMO.)

4.2.4 in vitro adipogenesis assay

To test differentiation of MSC into the adipose lineage, active ALDH^hi or ALDH^lo MSC cultures were harvested using trypsin and reseeded in 12 well plates at 1x10^4 cells/cm² and grown for 2-4 days to near confluency in Amniomax media. Amniomax media was aspirated and cells were washed with PBS before replacement with adipogenesis differentiation media (Gibco). Adipogenesis media was changed every 3-4 days for 14 days. After 14 days of adipocyte induction, cultures were fixed in formalin and subjected to Oil Red O staining to label intracellular lipid stores. Cells were counterstained with hematoxylin to visualise cell nuclei. Relative induction of adipogenesis was quantified by percentage red pixels of total in photomicrographs measured using ImageJ software.
4.2.5 *in vitro* osteogenesis assay

To assess differentiation of MSC into osteocytes, active ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} MSC cultures were harvested using trypsin and reseeded in 12 well plates at 5x10\textsuperscript{3} cells/cm\textsuperscript{2} and grown for 2-4 days to near confluency in Amniomax. Amniomax media was aspirated and cells were washed with PBS before replacement with osteogenesis differentiation media (Gibco). Osteogenesis media was changed every 3-4 days for 21 days. After 21 days of osteocyte induction, cultures were fixed in formalin and processed for Alizarin Red S staining to detect calcification of osteocytes.

4.2.6 *in vitro* chondrogenesis assay

To assess differentiation of MSC into chondrocytes, active ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} MSC cultures were harvested using trypsin, counted, pelleted and resuspended at 1.6x10\textsuperscript{7} cells/mL of Amniomax. To create MSC micromass cultures 5\mu L aliquots of the concentrated cell solution were carefully seeded in multiwall plates. After establishment and 2 hours of incubation in high humidity conditions at 37°C, chondrogenesis differentiation media (Gibco) was added to the micromass cultures. Chondrogenesis media was changed every 2-3 days for 21 days. After 21 days of chondrocyte induction micromasses were collected and embedded in optimum cutting temperature medium and frozen. Cryosections were prepared at 8\mu m and fixed in formalin prior to Alcian Blue staining to detect proteoglycans produced by chondrocytes. Cryosections were counterstained with Nuclear Fast Red to identify nuclei in chondrocyte micromasses.

4.2.7 Generation of BM ALDH\textsuperscript{lo} and ALDH\textsuperscript{hi} MSC conditioned media

After FACS isolation, ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} MSC were seeded at 4.7x10\textsuperscript{3} cells/cm\textsuperscript{2} and grown in Amniomax for 3 days at 37°C to reestablish culture. After 3 days the Amniomax media was aspirated and replaced with serum-free, growth factor-free, endothelial basal media (EBM, Lonza, Basel, Switzerland). After an additional 48 hours in EBM culture without supplement, ALDH\textsuperscript{hi} MSC- or ALDH\textsuperscript{lo} MSC-conditioned media was collected and filtered at 0.2\mu m and frozen for later use.
4.2.8 HMVEC Culture

Human dermal microvascular endothelial cells (HMVEC) were grown in endothelial basal medium-2 (EBM2, Lonza) supplemented with single-quotos to make endothelial growth medium (EGM2-MV, Lonza) containing fetal bovine serum (5% FBS), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-B), insulin-like growth factor-1 (IGF-1), and ascorbic acid. Cells were seeded at 4.7x10³ cells/cm². For maintenance culture, EGM2-MV was changed every 2-3 days when required.

4.2.9 CyQUANT endothelial cell proliferation assays

HMVEC were seeded at 3x10³ cells/well in 96 well plates using 100μL of fetal bovine serum and growth factor supplemented endothelial growth media (EGM2-MV, GF+/Serum+, Lonza), basal endothelial media with or without growth factors and serum, (GF+/Serum-, GF-/serum+, or GF-/serum-), or in ALDHhi MSC- or ALDHlo MSC-conditioned basal endothelial media. Cultures were maintained for 72 hours at 37°C before each well carefully inverted and blotted to remove media from each well. The whole 96-well microplate was then frozen at -70°C for later use. To quantitate increased DNA content representative of increased cell division, adherent cells were lysed by addition of lysis buffer with CyQUANT GR dye in each well for 2-5 minutes at room temperature, and protected from light as per manufacturer instructions (Molecular Probes, Eugene, OR, USA). Fluorescent signal in each well was assessed by fluorescence microplate reader at 480nm excitation and 520nm emission maxima. Cell number in each well was interpolated from a standard curve generated with known number of HMVEC per well.

4.2.10 Fluorescent cell labeling for co-culture studies

HMVEC were labeled using CellTracker Green CMFDA (Molecular Probes), at a concentration of 2μM in PBS+ PBS+5%FBS. ALDHhi or ALDHlo MSC tagged using CellTracker Red CMPTX (Molecular Probes) at a concentration of 3μM in PBS+5%FBS. Growth medium was aspirated from cell culture flasks and washed with PBS. After washing, cells were incubated in appropriate dye solution for 15-45 min, at 37°C. The
dye solution was then aspirated and replaced with normal growth medium, followed by incubation at 37°C for at least 30 min prior to harvest for co-culture experiments.

4.2.11 HMVEC and MSC co-culture tubule formation assays on Geltrex basement membrane extract

Geltrex reduced growth factor basement membrane extract (Gibco) was coated as a thin gel on a 24-well plate at a volume of 100μL per well, followed by incubation at 37°C for 1-2 hours to allow the gel to set. The appropriate conditioned medium from ALDH\textsuperscript{hi} versus ALDH\textsuperscript{lo} MSC, or from EGM-2 or EBM controls was then added at a volume of 400μL per well. EBM was added to negative control and experimental wells and EGM2-MV was added to positive control wells. CellTracker Green CMFDA tagged HMVEC, with previously established cells under EGM2-MV conditions, were harvested for secondary culture and 4.5x10^4 cells were added to each well. CellTracker Red CMPTX-dyed MSC were harvested and labeled with Aldefluor and sorted into ALDH\textsuperscript{lo} and ALDH\textsuperscript{hi} MSC as described above. CMPTX-tagged ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} MSC populations and were added to experimental wells, at 9x10^3 ALDH\textsuperscript{hi} MSC or ALDH\textsuperscript{lo} MSC per well. Endothelial cell tubule formation was assessed at 6 hours and 24 hours after plating by phase contrast and fluorescent micrographs. Tubule branch points were enumerated using 4 different fields of view by a blinded observer. All co-culture assays were performed in duplicate.

4.2.12 Murine femoral artery ligation and intramuscular transplantation of expanded MSC

To assess the effects of MSC in vascular regeneration \textit{in vivo} we induced acute unilateral hindlimb ischemia by surgical ligation and complete resection of the right femoral artery and vein on anesthetised NOD/SCID (Jackson Laboratory, Bar Harbor, ME) mice as previously described (Chapter 2, Appendix 1)\textsuperscript{27-28}. Within 24 hours of surgery, mice were transplanted by intramuscular injection at three sites into the right (surgical/ischemic) adductor muscle with 50μL saline as vehicle control or 5x10^5 BM MSC.
4.2.13 Quantification of hindlimb perfusion using laser Doppler perfusion imaging

Anesthetized mice were stabilized at 37°C for 5 minutes on a heating pad before blood flow in the lower hindlimbs (ankle to toe) was quantified using laser Doppler perfusion imaging (LDPI, Moor Instruments, Devon, UK). The perfusion ratio of the ischemic/surgical limb compared to the uninjured/control limb was assessed after surgery to confirm induction of acute ischemic injury with a perfusion ratio (PR<0.1) of <10% compared to the normal limb. Subsequent LDPI measurements were taken in each mouse at 3, 7, 14, 21, and 28 days after transplantation to track recovery of perfusion temporally.

4.2.14 Statistics

Data and results are reported as mean values ± SEM. Statistical comparisons were made using a Student’s t-test for adipogenesis induction and one-way ANOVA test followed by Tukey’s Post-Hoc tests for all other assays.

4.3 Results

4.3.1 BM MSC phenotype by ALDH activity level

Human BM-derived MSC cultures were established and assayed for ALDH activity using Aldefluor and FACS. Sorted MSC subpopulations with high (37.4±3.2% ALDH$^{hi}$, Figure 4.1) and low (45±4.2% ALDH$^{lo}$, Figure 4.1) ALDH activity were isolated based upon DEAB-inhibited controls. Both ALDH$^{hi}$ and ALDH$^{lo}$ MSC displayed robust expression of characteristic stromal markers CD73, CD90, CD105 (Figure 4.2, Table 4.1) but did not express hematopoietic marker CD45 (Figure 4.2D, Table 4.1) or monocyte marker CD14 as detected by flow cytometry (Table 4.1). Furthermore, both ALDH$^{hi}$ and ALDH$^{lo}$ MSC highly expressed pericyte marker CD146 and LNGFR/CD271 (Figure 4.2E, Table 4.1). Taken together, these data indicated that both subpopulations BM MSC express MSC cell surface markers, fulfilling an important determinant in the minimal criteria for MSC as set by the International Society for Cellular Therapy$^2$. 
Figure 4.1. Bone marrow MSC purified according to high and low levels of ALDH activity using Fluorescence Activated Cell Sorting.

(A) DEAB inhibits ALDH activity and establishes a negative gate to mark the ALDH$^{lo}$ MSC subset. ALDH$^{lo}$ and ALDH$^{hi}$ MSC subsets sorted by FACS. (B) Representative photomicrographs of MSC FACS sorted into (B) ALDH$^{hi}$ and (C) ALDH$^{lo}$ populations and plated in Amniomax media and grown by plastic adherence (n=14).
Figure 4.1

A

**Aldefluor + DEAB**

**Aldefluor**

Side Scatter

B

ALDH^{+} MSC

C

ALDH^{-} MSC

300um
Figure 4.2. Bone marrow MSC with high and low levels of ALDH activity display MSC markers.

Bone Marrow subpopulations with high and low ALDH activity were isolated by FACS and analyzed for cell surface marker expression associated with MSC phenotype. Both ALDH$^{hi}$ and ALDH$^{lo}$ MSC showed high levels of (A) CD73, (B) CD90, and (C) CD105 expression. Both populations were negative for hematopoietic marker (D) CD45. Both ALDH populations expressed pericyte marker (E) CD146. All gates were set by fluorescence-minus-one controls (FMO).
Figure 4.2

A. CD73

B. CD90

C. CD105

D. CD45

E. CD146

Legend:
- FMO control
- ALDH\textsuperscript{+} MSC
- ALDH\textsuperscript{-} MSC

% max events
Table 4.1. Bone marrow MSC with high and low levels of ALDH activity display MSC markers.

<table>
<thead>
<tr>
<th>Lineage Marker</th>
<th>ALDH\textsuperscript{hi} MSC (+%)</th>
<th>ALDH\textsuperscript{lo} MSC (+%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematopoietic</td>
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</tr>
<tr>
<td>CD45</td>
<td>0.2±0.2</td>
<td>0.3±0.3</td>
</tr>
<tr>
<td>CD14</td>
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<td>2.0±1.8</td>
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<tr>
<td>MSC</td>
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<td></td>
</tr>
<tr>
<td>CD73</td>
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<td>92.4±5.0</td>
</tr>
<tr>
<td>CD90</td>
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<td>80.3±12.1</td>
</tr>
<tr>
<td>CD105</td>
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<td>50.6±15.0</td>
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<tr>
<td>Pericyte</td>
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</tr>
<tr>
<td>CD271</td>
<td>73.3±20.4</td>
<td>69.5±8.5</td>
</tr>
</tbody>
</table>

(n=3-6)
4.3.2 Both BM MSC ALDH$^{hi}$ and ALDH$^{lo}$ subpopulations demonstrate multipotent stromal cell differentiation

Human BM-derived MSC were separated into subpopulations by level of ALDH activity by FACS and were subjected to in vitro differentiation conditions to characterize mesenchymal multipotency. Both ALDH$^{hi}$ and ALDH$^{lo}$ MSC formed Oil Red O positive-staining adipocytes after 14 days culture in adipogenesis induction media (Figure 4.3). Notably, ALDH$^{hi}$ MSC (Figure 4.3A,C) demonstrated significantly increased frequency of adipocyte induction compared to ALDH$^{lo}$ MSC (Figure 4.3B,D) as measured by percent Oil Red O positive staining by culture area (Figure 4.3E, p<0.05, n=4). Both ALDH$^{hi}$ (Figure 4.4A,C) and ALDH$^{lo}$ MSC (Figure 4.4A,C) also demonstrated induction of osteocyte formation as assayed by Alizarin Red S detection of calcium deposition after 21 days in osteogenesis media for 21 days (Figure 4.4). Furthermore, both ALDH$^{hi}$ (Figure 4.5A) and ALDH$^{lo}$ MSC (Figure 4.5B) demonstrated induction of chondrocyte differentiation in micromass culture in chondrogenesis media for 21 as measured by detection of proteoglycan deposition by chondrocytes using Alcian Blue staining in cryosections (Figure 4.5). Taken together, these data indicate that subpopulations of BM MSC with both high and low levels of ALDH activity demonstrate mesenchymal multipotency consistent with the defining minimal criteria$^2$. 
Figure 4.3. Bone marrow-derived ALDH\textsuperscript{\textsubscript{hi}} MSC show increased adipogenesis.

(A,C) ALDH\textsuperscript{lo} MSC and (B,D) ALDH\textsuperscript{hi} MSC were grown for 14 days in adipogenesis differentiation media prior to Oil Red O staining. Representative (A,B) bright field and (C,D) phase contrast photomicrographs of cell cultures stained with Oil Red O and hematoxylin. (E) ALDH\textsuperscript{hi} MSC show greater induction of adipocyte formation as measured by % red pixels indicating positive Oil Red O staining after 14 days in adipogenesis medium compared to ALDH\textsuperscript{lo} MSC (n=4, p<0.05).
Figure 4.4. Bone marrow-derived ALDH$^{hi}$ and ALDH$^{lo}$ MSC differentiate into osteocytes.

(A,C) ALDH$^{lo}$ MSC and (B,D) ALDH$^{hi}$ MSC were grown for 21 days in osteogenesis differentiation media prior to Alizarin Red S staining. Representative (A,B) bright field and (C,D) phase contrast photomicrographs of cell cultures stained with Alizarin Red S. Both ALDH$^{hi}$ and ALDH$^{lo}$ MSC induction of osteocyte formation as measured by % red pixels indicating calcium deposition in osteocytes (n=4).
Figure 4.4.
Figure 4.5 ALDH activity level does not affect chondrocyte differentiation by MSC.

Both bone marrow-derived (A) ALDH\textsuperscript{lo} and (B) ALDH\textsuperscript{hi} MSC showed robust formation of chondrocytes after 21 days micromass culture in chondrogenesis medium. Induction of chondrogenesis in micromass cryosections was confirmed by Alcian blue staining indicating proteoglycan deposition. Nuclear Fast Red counterstaining labels nuclei.
Figure 4.5.
4.3.3 ALDH$^{hi}$ MSC demonstrated increased support of endothelial cell proliferation

To test differences in paracrine support of support of endothelial cell proliferation ALDH-purified ALDH$^{hi}$ or ALDH$^{lo}$ MSC subpopulations were cultured in GF-free serum-free endothelial basal media to generate MSC-conditioned media used for HMVEC growth studies. Dermal-origin HMVEC were cultured on plastic in basal endothelial media with or without GFs (EGF, VEGF-A, FGF, IGF-1) and 5%FBS, or in ALDH$^{hi}$ MSC-conditioned or ALDH$^{lo}$ MSC-conditioned media generated without serum or growth factor supplementation. Conversely, HMVEC grown without either or both serum and growth factors showed no proliferation over 72 hours. HMVEC cultured in GF+/FBS+ endothelial growth media demonstrated approximately 3.5 fold increase in cell number over 72 hours as measured by CyQuant quantification. Notably, ALDH$^{hi}$ MSC-conditioned media significantly increased proliferation of HMVEC compared to ALDH$^{lo}$ MSC-conditioned media and basal endothelial media (p<0.01, n=5). Interestingly, there was notable variation in HMVEC proliferative responses between BM donor source. Pairwise comparisons of ALDH$^{hi}$ vs. ALDH$^{lo}$ MSC-conditioned media within all other donor samples showed significant increases in EC proliferation in ALDH$^{hi}$–treated cultures (p<0.05, n=5). Intriguingly, BM45 MSC conditioned media was most effective at promoting proliferation in both ALDH$^{hi}$ and ALDH$^{lo}$ subpopulations further illustrating inter-donor variability in the induction of HMVEC growth. Taken together these data demonstrate the purification of BM MSC based on high ALDH activity isolated a subpopulation of MSC with increased paracrine secretory support of EC expansion.
Figure 4.6. ALDH<sup>hi</sup> MSC conditioned media supports proliferation of endothelial cells.

Representative photo-micrographs of human micro-vascular endothelial cells (HMVEC) grown in (A) growth factor+/serum+ EGM2 media, (B) basal growth factor-free/serum-free, (C) ALDH<sup>hi</sup> MSC-conditioned media or (D) ALDH<sup>lo</sup> MSC-conditioned media. (E) Compared to ALDH<sup>lo</sup> MSC-conditioned basal media, and basal media, ALDH<sup>Hi</sup> MSC conditioned media increased HMVEC proliferation. (F) Induction of HMVEC proliferation varies according to the individual donor bone marrow source from which the MSC were derived. (E) Different letters denote significant differences in mean (p<0.01, n=5).
Figure 4.6.
4.3.4 ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} MSC augmented HMVEC tubule stability

To assess the potential for MSC-mediated support of endothelial cell tube formation, CellTracker Green CMFDA fluorescent-tagged HMVEC were plated on growth factor reduced Geltrex in basal endothelial media without serum or growth factor supplementation. ALDH sorted MSC subpopulations were fluorescently labelled with CellTracker Red CMPTX and co-cultured with EC at a ratio of 1 MSC:5 EC. Within 6 hours of co-culture, red-labelled MSC were detected in association with newly formed endothelial cell tubules, consistent with perivascular wrapping function of MSC (Figure 4.7G,H). Direct co-culture of HMVECs with either ALDH\textsuperscript{lo} or ALDH\textsuperscript{hi} MSC did not significantly increase tubule branch point formation by HMVECs at 6 hours relative to negative control basal endothelial cell media (Fig. 4.7A-H, n=3). Moreover, the number of branch points formed by HMVEC in MSC co-cultures was reduced compared to the number of branch points formed by HMVEC alone in growth factor- and serum-supplemented media (Figure 4.7Q). However, after 24 hours of co-culture, only ALDH\textsuperscript{hi} MSC supported significantly increased endothelial cell tubule formation compared to basal conditions (Figure 4.7R). Moreover, ALDH\textsuperscript{hi} MSC co-culture promoted tube formation at equivalent levels to HMVEC cultured alone in growth factor and serum supplemented endothelial growth media. ALDH\textsuperscript{lo} MSC co-culture did not significantly alter HMVEC tubule formation compared to controls or ALDH\textsuperscript{hi} MSC co-culture (Figure 4.7R, n=3). These data demonstrated that purification of MSC with high ALDH activity isolates a population of cells with increased support of endothelial cell tubule stabilization.
Figure 4.7. Co-culture with bone marrow-derived ALDH$^{hi}$ and ALDH$^{lo}$ MSC augments HMVEC tubule formation.

Representative photomicrographs of tubule and branch point formation by HMVECs after (A-H) 6 hours or (I-P) 24 hours culture in basal media (EBM, A,E,I,M), positive control media (EGM2-MV, B,F,J,N), EBM with ALDH$^{lo}$ MSC co-culture (C,G,K,O), or EBM with ALDH$^{hi}$ MSC co-culture (D,H,L,P). Scale bar = 300μm. (Q) Co-culture with ALDH$^{hi}$ and ALDH$^{lo}$ MSC significantly augmented tubule branch point formation relative to basal media at 6 hours. (R) ALDH$^{hi}$ MSC-conditioned media supported maintenance of significantly more branch points compared to ALDH$^{lo}$ at 24 hours. Data are expressed as mean ± SEM. (Q,R) Different letters denote significant difference, p<0.05, n=3.
Figure 4.7.
4.3.5 Intramuscular injection of BM MSC promotes accelerated recovery of perfusion after acute ischemic injury.

To establish proof of concept in vivo and to assess the functional role of transplanted MSC in vascular regeneration, NOD/SCID mice were subjected to unilateral femoral artery ligation and transplanted by intramuscular injection of BM MSC (non-sorted) into the injured leg adductor muscle. Mice were monitored for recovery of perfusion using laser Doppler perfusion imaging for 28 days. Seven days after treatment of PBS vehicle-injected control mice had recovered a perfusion ratio (PR, ischemic limb/normal limb) of 0.25±0.03 (Figure 4.8A, n=7). However, mice treated with heterogeneous BM MSC showed significantly augmented rate of recovery of perfusion (PR=0.52±0.07) within 7 days compared to PBS-treated mice (Figure 4.8A, p<0.01, n=6). Although perfusion was increased in MSC transplanted mice at days 7 and 14, significantly improved perfusion was not maintained at later time points (Days 21 and 28). Two different BM MSC donor sources were used. At day 7 post-treatment mice injected with BM20 MSC (Figure 4.8B, PR=0.67±0.06, n=3) showed significantly augmented recovery of perfusion compared to both PBS (Figure 4.8B, PR=0.25±0.03, n=7, p<0.01) and BM18 MSC (Figure 4.8B, PR=0.37±0.03, n=3, p<0.01) treated mice. Notably, mice treated with BM18 MSC showed no significant differences in recovery of perfusion compared to PBS controls.

Taken together, these data indicate that BM MSC support the recovery of perfusion in the ischemic hindlimb of immune deficient mice after acute injury. However, inter-donor variability in the induction of recovered perfusion by different MSC lines was evident. This in vivo model will form the basis for future investigations to determine the in vivo vascular regenerative potential of ALDHlo or ALDHhi MSC sub-populations and to further identify other phenotypes of BM MSC samples associated with increased regenerative function.
Figure 4.8. Bone marrow MSC show differential support of vascular regeneration by donor source.

LDPI was used to quantify perfusion in the hind limb following right femoral artery ligation and intramuscular injection PBS vehicle control (n=7), or 5x10^5 BM MSC (n=6) monitored for 28 days. (A) Perfusion ratio of the ischemic versus the non-ischemic hindlimb from ankle to toe in all transplanted mice as described above. Compared to saline controls, transplantation of BM MSC promotes recovery of perfusion in ischemic mouse hindlimbs by day 7 post-transplantation. (B) Data presented in (A) sub-divided by MSC donor, MSC from BM20 showed increased support of perfusion recovery compared to donor BM18 MSC. Data are expressed as mean ± SEM, *p<0.01 v PBS, #p<0.01 v BM18 MSC.
4.4 Discussion

In this chapter, we demonstrate that BM MSC can be sub-fractionated into populations based upon the level of ALDH enzyme activity. Both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} MSC display the characteristic cell surface marker phenotype typical of mesenchymal stromal cells devoid of hematopoietic cell contamination. Moreover, both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} subpopulations display robust mesenchymal multipotency, demonstrating differentiation capacity to adipocytes, osteocytes and chondrocytes under lineage specific differentiation conditions. We show that the MSC subpopulation with high ALDH activity after culture expansion demonstrates increased paracrine support of endothelial cell proliferation \textit{in vitro}, under serum-free, growth factor free conditions. Furthermore, we demonstrated that ALDH\textsuperscript{hi} MSC also augmented endothelial cell tubule formation, stabilizing tube-like structures possibly through perivascular wrapping. We propose that high ALDH activity provides a clinically relevant, FACS-selectable, and prospective marker to identify MSC subpopulations with increased vessel supportive functions for the development of improved cell therapies for ischemic disease.

The Hess laboratory and others have previously identified cells high levels of ALDH activity as a marker which selects hematopoietic (Chapter 2\textsuperscript{26,27,29-35}), endothelial (Chapter 2\textsuperscript{26,27,26-38}), neural\textsuperscript{39,40}, and mesenchymal\textsuperscript{19,26} cells with primitive, stem/progenitor cell phenotype and function. Our lab has previously demonstrated that initial selection of ALDH\textsuperscript{hi} mixed progenitor cells from BM\textsuperscript{26} or UCB (Chapter 2\textsuperscript{27}) demonstrate increased support of endothelial cell function \textit{in vitro} and enhanced vascular regeneration \textit{in vivo}. The Hess laboratory have shown that while MSC-like cells can be derived from the ALDH\textsuperscript{lo} BM cells, these cells do not demonstrate full multi-lineage differentiation, and are restricted primarily to the adipocytes lineage\textsuperscript{26}. In contrast, MSC grown from ALDH\textsuperscript{hi} BM mononuclear cells represent true MSC, which expand efficiently in \textit{ex vivo} culture and demonstrate full multipotency forming fat, bone and cartilage\textsuperscript{26}. Furthermore, the frequency of mesenchymal colony forming cells is significantly higher in the ALDH\textsuperscript{hi} population of human BM compared to ALDH\textsuperscript{lo} BM\textsuperscript{26}. Together these findings led us to hypothesize that high ALDH activity might be a good prospective marker to further purify an ALDH\textsuperscript{hi} MSC subset with increased capacity for vascular regeneration after
expansion *ex vivo*. Importantly, both the ALDH\textsuperscript{lo} and ALDH\textsuperscript{hi} subsets of expanded MSC demonstrated extensive expression of CD146, a cell surface marker previously associated with perivascular wrapping function for MSC isolated from multiple human tissues *in situ*\textsuperscript{16}.

The findings that BM ALDH\textsuperscript{hi} cell promote recovery of hindlimb ischemia in mice was used as preclinical justification for initiating clinical trials in patients with critical limb ischemia, the most severe form of peripheral arterial disease (PAD)(NCT00392509)\textsuperscript{41}. Intramuscular transplantation of freshly isolated (uncultured) autologous BM ALDH\textsuperscript{hi} cells at multiple sites in the ischemic limb was compared directly compared to unpurified autologous BM MNC in PAD patients with a Rutherford score of 4 (resting pain without significant ulceration) or 5 (resting pain with unresolved ulceration or necrosis)\textsuperscript{41}. Results of this pioneering study demonstrated safety and patients that received autologous BM ALDH\textsuperscript{hi} cells showed improved Rutherford category and reduced resting pain. Furthermore patients in the ALDH\textsuperscript{hi} treatment group showed improved ankle to brachial index scores more rapidly than the MNC group indicating improved perfusion of the lower limb. However, patients in both cohorts reported significantly improved quality of life indices.\textsuperscript{41} More recently, a phase 2 placebo-controlled randomized clinical study has been initiated to investigate transplantation of BM ALDH\textsuperscript{hi} cells to improve symptoms of intermittent claudication (NCT01774097). The Hess laboratory have previously shown that the BM ALDH\textsuperscript{hi} progenitor cells are primarily (>90%) comprised of hematopoietic precursor cells that efficiently form myeloid colonies *in vitro*, and are also highly enriched in CD45\textsuperscript{-} progenitor cells that generate multipotent fibroblast colonies (1 CFU-F in 1500 BM ALDH\textsuperscript{hi} cells) that expand efficiently and adherent to plastic. Furthermore, MSC have been widely investigated in clinical trials for cardiovascular disease. As a readily *ex vivo* expandable population of cells from BM, MSC have been proposed as excellent candidates for cell therapy due to their robust paracrine activities and perivascular wrapping functions. Indeed in diabetic rat models, BM MSC have been demonstrated to promote vascular repair and wound healing\textsuperscript{42-44}. Moreover, a randomized controlled clinical trial was undertaken to compare BM MNC and BM MSC therapy in diabetic patients with critical limb ischemia\textsuperscript{45}. This study
showed that both BM MNC and MSC were safely administered by intramuscular administration and both BM MNC or BM MSC promoted improvements in perfusion, tissue oxygenation, and pain free walking time at 24 weeks post treatment. Notably, greater cell numbers could be generated during the ex vivo expansion of BM MSC compared to the fresh BM MNC, indicating the appeal of ex vivo expansion to increase cell numbers for transplantation.

However, a number of preclinical models have shown that BM MNC and MSC from patients with diabetes or cardiovascular disease show diminished function for vascular regeneration. For this reason, cell therapy approaches using BM MSC combined with ex vivo expansion are appealing as ex vivo expansion and targeted intra-muscular transplantation may restore MSC vascular regenerative function, or alternatively, you could potentially source BM from healthy donors for use in an allogeneic context. In addition, the establishment of national registries to cryopreserve and HLA-phenotype UCB samples may provide a renewable source of vascular regenerative MSC from early ontogeny and devoid of chronic cardiovascular disease co-morbidities. Moving forward towards essential experiments that will establish proof-of-concept, I propose that ALDH activity presents a useful functional marker to identify MSC subpopulations with increased vascular regenerative potential. Furthermore, selection of ALDHhi MSC might also be helpful in screening cell lines to identify sample with increased or diminished vascular regenerative potential. Taken together I propose that BM-derived MSC with high ALDH activity represent a promising candidate for further development of cell therapy for cardiovascular disease broadly and for critical limb ischemia and peripheral arterial disease specifically.
4.5 References


Chapter 5

5 Summary and Discussion

5.1 Summary of Major Findings

The overall objective of this thesis was to expand our understanding of the role of human progenitor cells in vascular regeneration as a means to improve the outcome of potential cell therapy applications in ischemic cardiovascular disease. Towards this end, I have characterized a population of umbilical cord blood derived progenitors purified by selection of cells with high ALDH activity, a mixed stem and progenitor cell population with enhanced regenerative functions. Using complementary \textit{in vitro} and \textit{in vivo} models, I have demonstrated the vascular regenerative effects of UCB ALDH\textsuperscript{hi} cells. Furthermore, I have demonstrated that it is feasible to increase the number of these useful, pro-angiogenic hematopoietic cells for vascular regenerative therapy by clinically applicable, serum-free \textit{ex vivo} expansion without diminishing vascular regenerative activity. Building upon the abundant pre-clinical promise of MSC for vascular regenerative therapies and the concept of high ALDH activity as a marker of cells with increased progenitor cell and vascular regenerative phenotypes, I further demonstrated that MSC with high ALDH activity demonstrate the support of endothelial cell function \textit{in vitro}, further reinforcing the use of ALDH as a prospective marker of multiple progenitor cell lineages with enhanced vascular regenerative functions. Taken together, my studies have demonstrated the viability of high ALDH activity as a useful marker for the isolation and purification of blood and BM-derived cell types with increased efficacy in treatment of ischemic disease (Figure 5.1).
Figure 5.1 Summary of thesis findings.
5.1.1 Chapter 2 – UCB ALDH^{hi} cells induce vascular regeneration

The aims of my studies in chapter 2 were to characterize the capacity of UCB ALDH^{hi} cells to support vascular function in vitro in models of endothelial cell co-culture and in vivo in a murine model of acute unilateral hind limb ischemia. Our lab had previously demonstrated that selection of BM cells with high ALDH activity isolates a subpopulation of cells that reconstitute hematopoiesis^{1,2}, show widespread tissue distribution^{3}, and recruit to areas of ischemia after intravenous transplantation^{4}. Furthermore, the Hess lab have demonstrated that BM ALDH^{hi} cells augment re-vascularization of the ischemic limb in transplanted mice after acute ischemic injury^{4}. Numerous studies have demonstrated that patients affected by CVD and/or diabetes have decreased numbers of circulating pro-angiogenic cells, and furthermore these few circulating have diminished efficacy in supporting vascular regeneration^{5-11}. For this reason my studies focused on the investigation of UCB as an alternate source of ALDH^{hi} pro-angiogenic progenitor cells for allogeneic transplantation approaches to augment the diminished efficacy and frequency of circulating cells for vascular regeneration in patients with CVD where autologous transplantation may be of limited effectiveness.

At the onset of these studies, I hypothesized that transplantation of human UCB ALDH^{hi} HPC would promote vascular regeneration of ischemic limbs in immune-deficient mice with unilateral hind limb ischemia via paracrine mechanisms. I demonstrated that selection of UCB ALDH^{hi} cells isolates a population with increased myeloid HPC phenotype by cell surface marker expression and by colony forming cell capacity in vitro. Furthermore, my studies demonstrated that isolation of cells with high ALDH activity from UCB enriches and selects for rare CD45^{-}ECFC within the more heterogenous MNC population. I showed that the UCB ALDH^{hi} population demonstrated a pro-angiogenic transcription profile compared to UCB cells with low ALDH activity. Indeed, transcription of several important vascular regeneration-associated cytokines including ANG1 and VEGF-A were expressed at significantly higher levels in ALDH^{hi} cells than in UCB ALDH^{lo} cells. In co-culture with EC, UCB ALDH^{hi} cells promoted the survival of EC under growth factor-free, serum-free conditions. Moreover, in co-culture with EC on growth factor-reduced matrigel, UCB ALDH^{hi} cells supported augmented EC tubule
formation. Importantly, intravenous transplantation of UCB ALDH\textsuperscript{hi} cells in an immune-deficient mouse model of acute unilateral hind limb ischemia significantly augmented recovery of perfusion and increased vascularization in the ischemic limb compared to vehicle-transplanted controls. Notably, the value of purification of the ALDH\textsuperscript{hi} progenitor cell subset was shown by increased recovery of perfusion and vascularization compared to transplantation of heterogeneous UCB MNC containing an equivalent dose of ALDH\textsuperscript{hi} cells. After intravenous transplantation of UCB ALDH\textsuperscript{hi} cells mice showed low levels of human cell engraftment specifically in the ischemic muscle. Preparative irradiation to reduce human cell rejection did not enhance recovery of perfusion by enhancing engraftment or hematopoietic reconstitution. Indeed, irradiated mice showed increased recruitment of human cells to the BM consistent with previously demonstrated hematopoietic reconstituting capacity\textsuperscript{1,2}. Transplantation of UCB ALDH\textsuperscript{hi} cells into irradiated mice showed delayed, less robust recovery of perfusion or vascularization in the ischemic hindlimb compared to non-irradiated mice consistent with decreased early recruitment of transplanted ALDH\textsuperscript{hi} cells to the ischemic muscle. Indeed, our lab has previously shown that tail vein injected BM-derived ALDH\textsuperscript{hi} cells recruit specifically to the ischemic hind limb within 6-24 hours after femoral artery ligation surgery indicating the chemotactic nature of ALDH\textsuperscript{hi} cells in response to ischemic damage stimuli\textsuperscript{4}. We concluded that although increased engraftment and survival of ALDH\textsuperscript{hi} cells in the ischemic hindlimb was predicted to improve revascularization, permanent or long-term residence in the ischemic limb was not required to achieve potent recovery of small capillary function.

My work in chapter 2 demonstrated that purification of ALDH\textsuperscript{hi} cells from UCB is a viable, readily available and less invasive alternative source of ALDH\textsuperscript{hi} cells to BM. Due to the early ontogeny of UCB, it will not have any CVD or age-associated morbidities that might impair the most effective applications of cell therapy. The evidence that purified ALDH\textsuperscript{hi} cells were more effective than transplantation of heterogeneous MNC containing as many ALDH\textsuperscript{hi} cells indicates the importance of prospective selection strategies to isolate cell types with increase vascular regenerative function while depleting cells with either no benefit or potentially inhibitory effects. Overall, these
studies indicated that UCB ALDH\textsuperscript{hi} cells are a promising candidate for future development of cell therapies for the treatment of CVD and more specifically for PAD and CLI.

5.1.2 Chapter 3 – \textit{ex vivo} expanded UCB HPC retain pro-angiogenic capacity

Building directly upon the findings of chapter 2, my second aim was to address a primary shortcoming of the use of blood derived ALDH\textsuperscript{hi} cells. Namely, selection of ALDH\textsuperscript{hi} cells for use in a human setting may be hindered by the number of cells available for transplant because the ALDH\textsuperscript{hi} population is very rare accounting for less than 0.5% of total MNC in UCB and less than 1% in total BM MNC\textsuperscript{4,12}. The first goal in chapter 3 was to develop an \textit{ex vivo} expansion protocol which could increase the number of cells available for therapy without untoward differentiation that may decrease the effectiveness of the cells for vascular regeneration. Thus my aim for the studies presented in chapter 3 was to optimize the \textit{ex vivo} expansion of UCB ALDH\textsuperscript{hi} hematopoietic cells under clinically relevant serum-free conditions and to characterize vascular regenerative functions of the expanded progenitor cells \textit{in vitro}. In addition, I wanted to investigate the paracrine mechanisms by which expanded hematopoietic progenitor cells derived from UCB ALDH\textsuperscript{hi} contribute to revascularization after direct intramuscular injection into the ischemic limb. The hypothesis for these studies was that \textit{ex vivo} expansion of UCB ALDH\textsuperscript{hi} cells would increase the number of pro-angiogenic hematopoietic progenitor cells for application in vascular regeneration without decreasing EC-supportive functions.

I demonstrated that \textit{ex vivo} expansion of UCB ALDH\textsuperscript{hi} cells in clinically relevant, serum-free media with recombinant human TPO, FLT3L, and SCF for 6 days imparted a 20-fold expansion of early myeloid cells retaining hematopoietic progenitor phenotype without increasing the number of mature monocytes or macrophages. Moreover, after \textit{ex vivo} expansion from UCB ALDH\textsuperscript{hi} cells, the resultant progeny retained hematopoietic progenitor colony forming function \textit{in vitro} and maintained a pro-angiogenic transcriptional profile similar to freshly isolated UCB ALDH\textsuperscript{hi} parental cells. In fact, the expanded HPC population showed upregulation of several important vascular regeneration-associated transcripts including significant upregulation of EGF, VEGF-A...
and ANG1. In direct co-culture with HMVEC, ex vivo-expanded HPC augmented tubule formation in serum-free growth factor reduced conditions. Furthermore, HPC-conditioned media and co-culture of HPC with HMVEC promoted increased survival of HMVEC in growth factor-free serum-starved conditions. I demonstrated that during co-culture with HMVEC, in basal unsupplemented media, HPC increased secretion of the vascular regenerative growth factors EGF and ANG2. Furthermore HPC co-culture increased secretion of chemokines that have been shown to play a role in modulating recruitment of other support cells that are implicated in vascular regeneration in response to injury or hypoxia including GRO/CXCL1-3, IL-8/CXCL8, and RANTES/CCL5. Finally, HPC co-culture also increased secretion levels of matrix modifying proteins, MMP-9, TIMP-1 and TIMP-2. These pro-angiogenic and matrix-modifying proteins have been implicated in the induction of angiogenic sprouting in vivo and in vascular stabilization and extracellular matrix remodeling respectively.

Using our well established immune-deficient mouse model of acute hindlimb ischemia, I demonstrated that intramuscular injection of ex vivo-expanded HPC augmented recovery of perfusion and increases vascularization of the ischemic muscle. Furthermore, I showed that this improved vascular repair after femoral artery ligation in mice treated with ex vivo expanded UCB HPC also resulted in improved recovery of limb usage compared to vehicle controls. This indicates the usefulness of cell therapy for vascular regeneration reaches beyond improvements in blood flow to ischemic tissue and includes accelerated limb usage during recovery. Adequate perfusion and limb usage in ischemic tissues is an important factor in moderating quality of life improvements in patients afflicted with peripheral artery disease and critical limb ischemia. Taken together, my work in Chapter 3 demonstrated that it is possible to increase the number of pro-angiogenic hematopoietic cells using clinically relevant expansion conditions. Importantly, I demonstrated that this expansion of cell number does not decrease the efficacy for vascular regeneration compared to the parental UCB ALDH^hi cell population. Collectively, the findings from Chapters 2 and 3 demonstrated that UCB is a readily available, expandable source of progenitor cells with vascular regenerative potential, presenting a promising candidate cell population for development of cell therapies for ischemic PAD.
5.1.3 Chapter 4 – MSC with high ALDH activity

Multipotent stromal cells can be isolated from bone marrow (BM) and many other tissues. Broadly, MSC are considered good candidates for allogeneic cell therapy applications because of their established safety after infusion, low immunogenicity and potential immunomodulatory effects. Moreover, MSC are considered to be excellent cellular sources of regenerative cytokines for many tissue repair processes and specifically they have been shown to secrete pro-angiogenic factors\(^{13-17}\). Implementation of MSC therapy has been somewhat hindered by variability in MSC function between donor source and by lack of prospective markers for selection of samples with the optimal regenerative potential for a given application. Our laboratory has previously demonstrated that selection of high ALDH activity isolates an increased frequency of MSC from BM MNC\(^4\) and as MSC differentiate in prolonged \textit{ex vivo} culture, ALDH activity and regenerative capacity is reduced. Furthermore, my earlier studies from Chapters 2 and 3 have shown that high ALDH activity identifies hematopoietic cells with increased vascular regenerative potential. For these reasons I hypothesized that selection of MSC subpopulations with high ALDH activity after expansion culture would identify a population of MSC with enhanced vascular regenerative functions.

In Chapter 4, I demonstrated that after expansion \textit{ex vivo}, both ALDH\(^{hi}\) and ALDH\(^{lo}\) MSC subpopulations expressed the cell surface markers characteristic of MSC, CD73, CD90, and CD105, and were devoid of contaminating hematopoietic cells and monocytes. Moreover, I showed that both MSC sub-populations expressed the pericyte marker CD146\(^{18}\) and the prospective MSC marker low affinity nerve growth factor receptor (LNGFR) also called CD271\(^{19}\). Furthermore, I demonstrated that both ALDH\(^{hi}\) and ALDH\(^{lo}\) subsets of expanded MSC demonstrate mesenchymal multipotency, showing growth factor induced differentiation into bone, fat and cartilage. Thus, both ALDH\(^{hi}\) and ALDH\(^{lo}\) MSC met the strict definition of MSC set forth by the International Society for Cellular Therapy\(^{20}\).

Adapting the \textit{in vitro} co-culture systems I developed in Chapters 2 and 3, I demonstrated that ALDH\(^{hi}\) MSC showed greater support of endothelial cell proliferation in basal conditions compared to ALDH\(^{lo}\) MSC. Moreover, using fluorescent tagging and co-
culture with HMVEC, I demonstrated that ALDH\textsuperscript{hi} MSC demonstrated increased vessel integration/association and provided structural support for the stabilization of EC tubule formation \textit{in vitro} compared to ALDH\textsuperscript{lo} MSC. Based on my findings in Chapter 4, I propose that BM-derived MSC with high ALDH activity represent an excellent candidate for further development of cellular therapies for cardiovascular disease. Moreover, after intramuscular injection of unpurified, culture expanded MSC into immune deficient mice with unilateral hindlimb ischemia I showed evidence for inter-donor sample variability in the capacity of BM MSC to promote vascular regeneration. These findings confirmed the need for further study to identify additional prospective markers to identify MSC with effective vascular regenerative function.

5.2 Clinical Applications

The findings presented in this thesis provide substantial pre-clinical justification for further investigations of potential application of purified UCB ALDH\textsuperscript{hi} cells, \textit{ex vivo}-expanded HPC derived from UCB ALDH\textsuperscript{hi} cells, and MSC subpopulations with high ALDH activity. Indeed, Phase I/II clinical trials headed by Perin and colleagues have demonstrated the safety and efficacy of autologous BM ALDH\textsuperscript{hi} cells by intramyocardial injection in patients with heart failure and by intramuscular injection in patients with critical limb ischemia\textsuperscript{21,22}. My work demonstrates the potential application of UCB-derived ALDH\textsuperscript{hi} cells in allogeneic transplant strategies both by intravenous and intramuscular injection. Specifically in patients with severe PAD, transplantation of BM-derived ALDH\textsuperscript{hi} cell has been demonstrated to support the recovery of perfusion and improved disease outcome, decreasing Rutherford score and resting pain with concomitant increases in self reported quality of life\textsuperscript{21}. These promising findings demonstrate the potential for application of ALDH\textsuperscript{hi} cells broadly for CVD and specifically for patients with PAD and CLI.

Purification by high ALDH activity does not represent the only subpopulation of BM MNC that has been investigated at the clinical level; autologous BM CD34\textsuperscript{+} have been previously assessed in patients with moderate to severe CLI\textsuperscript{23}. These studies also demonstrated safety and suggested a promising trend towards reduced amputation and
improved clinical indices\textsuperscript{23}. However, I postulate that ALDH may represent a superior marker to isolate a subpopulations of cells as it isolates not only the hematopoietic progenitor cell types as would CD34 selection, but also isolates increased frequency of MSC and ECFC\textsuperscript{4,12}. However, to date the majority of cell therapy trials for CVD have investigated heterogenous BM MNC populations. Despite the mixed results in BM MNC therapy for acute myocardial infarction, meta-analyses have shown small benefits in left ventricle ejection fraction\textsuperscript{24}. Furthermore, the clinical trials investigating application of BM MNC for PAD and CLI have shown some promise despite the small sample size of trials completed\textsuperscript{25-27}. As larger trials are completed, higher-powered analyses will be needed clarify disease outcome improvements, and our preclinical data suggests that prospective purification of pro-angiogenic progenitors and concomitant removal of potentially interfering inflammatory cells could potentially help improve trial outcomes.

Interestingly, it has been demonstrated that MSC derived from a type 2 model diabetic (\textit{db/db}) mice showed decreased frequency, recruitment and efficacy in the support of wound healing in diabetic mice compared to wild type controls\textsuperscript{28}. Furthermore, MSC isolated from rats with STZ-induced diabetes showed diminished differentiation, paracrine cytokine secretion, and anti-apoptotic activity compared to healthy controls\textsuperscript{29}. However, studies have shown that using \textit{ex vivo} growth factor (IGF and FGF2) activation of diabetic-derived MSC has been shown to abrogate disease associated dysfunction suggesting possible rejuvenation strategies to augment autologous MSC for CVD patients may be possible\textsuperscript{30}. Moreover, pretreatment of MSC from type 2 (\textit{db/db}) diabetic mice with EGF has been shown to abrogate diabetes-related dysfunction and to support recovery of perfusion in mouse model of hindlimb ischemia\textsuperscript{31}. These finding point towards the potential benefit of a combined cell therapy approach using HPC co-transplantation with HLA-matched MSC. Indeed, our pre-clinical characterization of pro-angiogenic effectors showed that HPC secrete EGF and EGF-family cytokines that are expected improve MSC-mediated vascular regenerative effects \textit{in vivo}. Using a similar approach, clinical trials using co-administration of expanded BM MSC with BM MNC in patients with moderate to severe PAD showed safety and suggested improvements in both hemodynamic and clinical indices\textsuperscript{32}. 
Broadly, most cell therapies investigated for ischemic disease at the clinical level encompass autologous approaches, despite accumulating evidence of disease associated progenitor cell dysfunction in the patients with CVD\textsuperscript{8,9,28,29,33,34}. Thus, the preclinical work presented in this thesis may represent important findings regarding possible alternative allogeneic sources (namely UCB) of purified angiogenic supportive progenitor cell types untouched by chronic CVD co-morbidities. In addition, despite demonstrating safety in multiple applications, arguably many of the clinical trials completed to date have been underpowered to properly assess efficacy in treatment of CVD\textsuperscript{25,26,35,36}. While many have shown promising trends improving disease outcomes, further study is required to properly identify the best candidate and delivery techniques for future cell therapy for CVD\textsuperscript{37}. To date, there have been few significant clinical trials investigating application of allogeneic BM MSC or UCB cells for PAD\textsuperscript{27}. Notwithstanding, the findings presented in this thesis and by other research groups demonstrate the abundant promise shown in preclinical experiments for possible allogeneic cell therapy with BM MSC\textsuperscript{14,38} or UCB-derived cells\textsuperscript{12,39-42} for PAD and CVD broadly. As such, I propose that purified UCB ALDH\textsuperscript{hi} cells, their \textit{ex vivo}-expanded HPC progeny, and the ALDH\textsuperscript{hi} subset of expanded BM MSC all represent promising candidates for future cell therapy applications.

5.2.1 Angiogenic Hematopoietic Cells

It is important to note that UCB ALDH\textsuperscript{hi} cells represent a heterogeneous progenitor cell population, that contained enriched frequency of both hematopoietic and endothelial colony forming cells\textsuperscript{4,12}. Throughout this thesis I have taken great care to delineate the contributions of supportive hematopoietic progenitors from the potential contributions of non-hematopoietic endothelial progenitor cells or EPC. Indeed, confusion in the field still exists because many populations that have been termed “EPC” are in fact hematopoietic cell types that do not give rise to mature EC. Yoder \textit{et al.} have established the phenotype of the true, non-hematopoietic progenitor cells which they termed ECFC, defined by cobblestone morphology, late outgrowth after plating from blood and no expression of hematopoietic markers CD45 or CD14\textsuperscript{6}. Others have called ECFC the late blood outgrowth EPC in contrast to the early outgrowth cells which should be more properly called circulating angiogenic cells (CAC) or colony forming unit-Hill cells (CFU-
The population of cells that Asahara et al. termed EPC in the seminal 1997 manuscript, and early outgrowth CAC in fact more closely resemble heterogeneous populations of VEGF-activated monocytes. Nonetheless, culture-expanded CAC populations derived primarily from CD14+ monocytes have been shown to be an important circulating angiogenic cell type. UCB ALDHhi cells and ex vivo-expanded HPC do not contain notable CD14+ monocytes, an important distinction between UCB ALDHhi cells and CAC broadly. In addition our ALDHhi cell-derived HPC are generated using traditional hematopoietic expansion conditions whereas CAC are expanded using angiogenic growth factors. Both CAC and CFU-Hill cells have been shown to play a role in paracrine support of angiogenesis and vascular regeneration. More generally, ALDHhi cells are different from CAC or CFU-Hill cells in that ALDHhi selection depletes mature macrophages and lymphocytes, which are known to be constituent cells types of those other angiogenic cell populations. However, both CFU-Hill and CAC include hematopoietic progenitor cells, which would have high ALDH activity, indicating some overlap in these pro-angiogenic but non-vessel integrated cellular populations. Since selection of high ALDH activity isolates cells with progenitor cell function, it remains possible that ALDHhi cells or expanded HPC differentiate into mature effectors like monocytes or macrophages in vivo which may contribute to improved revascularization and perfusion after transplantation. However, whether the vascular regenerative effects of UCB ALDHhi cells or expanded HPC is mediated directly by the transplanted cells or by differentiated progeny after transplantation remains to be elucidated after future studies.

5.3 Future Directions

Our screening approach to identify secreted soluble factors that might modulate vascular regeneration was limited by availability of panel candidate factors. To identify a more global profile of blood-derived progenitor cell paracrine factors that modulate vascular regeneration, I propose the development of a proteomic approach to identification of the complete secretome of UCB ALDHhi cells, expanded HPC or ALDHhi MSC. Other groups have done similar proteomic screening to identify the secretome of human embryonic stem cells, EPC, and to identify stem cell-mediated cytokine deposition in extracellular matrix. Identification of a global secretory profile of angiogenic
hematopoietic cells including UCB ALDH\textsuperscript{hi} cells and \textit{ex vivo}-expanded HPC will help determine the mechanisms by which blood cell-mediated vascular regeneration can occur and will help direct future development of rational cell, peptide or drug therapies for CVD.

Beyond identification of the paracrine and juxtacrine factors that regulate angiogenic blood cell-mediated vascular interactions in ischemic disease, I propose that future cell therapy clinical trails should only be undertaken on cell types with better characterized molecular mechanisms of action to prevent unnecessary expenditures on suboptimal cellular populations. For this reason, gain and loss-of-function studies on critical paracrine mediators should be performed on candidate cellular populations to develop a more in depth understanding of cell-mediated vascular regeneration after acute and under chronic ischemic conditions. Better understanding of the molecular mediators regulating effective regeneration could also direct possible future applications of autologous cell therapies with \textit{ex vivo} or correction of CVD-associated angiogenic cell dysfunction.

A limitation of my mouse model for investigating potential cell therapy for CAD or PAD is that femoral artery ligation induces an acute ischemic injury and does not recapitulate the atherosclerotic chronic disease condition. The most common mouse models used to assess atherosclerosis are the apolipoprotein E-deficient (ApoE\textsuperscript{-/-}) and low density lipoprotein receptor knockout (LDLR\textsuperscript{-/-})\textsuperscript{56-58}. The ApoE knockout model is characterized by spontaneous development of atheromatous lesions, the development of which is accelerated on a Western diet\textsuperscript{57}. More recently, investigations have combined the ApoE knockout mice model of atherosclerosis with the acute injury model of femoral artery ligation\textsuperscript{59}. Moreover, one group demonstrated that transplantation of CD34\textsuperscript{+}/M-cadherin\textsuperscript{*} BM-derived progenitor cells promoted increased arteriogenesis in the ischemic hindlimb of ApoE\textsuperscript{-/-} mice with surgically-induced unilateral hindlimb ischemia. These findings indicate that a population of hematopoietic progenitor cells promotes vascular regeneration in a chronic atheromatous model of hindlimb ischemia. I propose investigating application of UCB ALDH\textsuperscript{hi} cells, HPC, and ALDH\textsuperscript{hi} MSC in similar models of chronic ischemic disease to further confirm my findings.
Recently, progress has been made in advancing the effectiveness of *ex vivo* expansion of hematopoietic stem and progenitors cells. A notable example of these novel factors are the aryl hydrocarbon receptor antagonists which have been shown to dramatically increase the expansion of hematopoietic stem cells\(^{60}\). Moreover, the Sauvageau lab has recently identified many new factors involved in HSC regulation\(^{61}\). Beyond new small molecule and protein mediators of HSC/HPC expansion, novel *ex vivo* culture techniques have augmented our ability to increase the number of progenitor cells available after expansion. The Zandstra group demonstrated recently that using a batch-fed culture system which reduces accumulation of autocrine inhibitory factors that dampen expansion significantly increases expansion rates of HPC\(^{62,63}\). Use of these novel expansion strategies will augment our ability in the future to increase the number of cells available for cell therapy in disease conditions beyond simple HSC transplantation for hematological disorders.

As the key cellular mediators of progenitor cell vascular regeneration are better characterized and the molecular mechanisms are elucidated it will become increasingly feasible to consider *ex vivo* bio-engineering approaches to develop *de novo* blood vessels for clinical application. As new scaffolds are identified and characterized such as decellularized blood vessel explants\(^{64}\), our increased understanding of the important cell types in vascular regeneration will help advance possible autologous seeding with BM or blood-derived cells before scaffold implantation. Moreover, it is important to recognize that sufficient vascularization is necessary for long-term successful engraftment of many tissue transplant strategies broadly\(^{65}\). Thus, it will be increasingly important to understand possible autologous or allogeneic cell sources that may augment vascularization of tissue grafts. Indeed, in the practice of reconstructive plastic surgery it is well recognized that proper vascularization is a limiting factor in the implementation of many promising tissue engineering applications\(^{66-68}\). This demonstrates the importance of a better understanding of the cellular mediators of vascular regeneration beyond vascular disease as a key component of regenerative medicine broadly. The work presented in my thesis has advanced our understanding of the particular subpopulations of blood derived cells that help mediate a vascular regenerative niche and demonstrated the effectiveness of UCB
ALDH\textsuperscript{hi} cells, \textit{ex vivo}-expanded HPC and ALDH\textsuperscript{hi} MSC for regenerative medicine applications.

5.4 Conclusions

Overall, the findings presented in this thesis demonstrate that UCB cells with high ALDH activity are a readily available population of pro-angiogenic cells which can be expanded in \textit{ex vivo} culture and present a promising source for cell therapy applications in treatment of CVD. Moreover, I demonstrated that high ALDH activity identifies subpopulations of progenitor cells with increased vascular regenerative functions from UCB HPC and also from BM MSC. My work has advanced the development of further preclinical characterization of candidate cell populations and biomarkers for the future development of allogeneic and autologous cell therapy.
5.5 References


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Appendices


David M. Putman and David A. Hess

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ABSTRACT

This unit describes the isolation and application of human umbilical cord blood progenitors cells to modulate vascular regenerative functions using in vitro co-culture systems and in vivo transplantation models. Using aldehyde dehydrogenase as a marker of stem cell function, blood-derived progenitors can be efficiently purified from human umbilical cord blood using flow cytometry. We describe in vitro approaches to measure cell-mediated effects on the survival, proliferation and tube forming function of endothelial cells using growth rate assays and Matrigel tube forming assays. Additionally, we provide a detailed protocol for inducing acute unilateral hindlimb ischemia in immune-deficient mice to assess progenitor cell modulated effects on vascular regeneration by tracking the recovery of blood flow using non-invasive laser Doppler perfusion imaging. Collectively, we present combined in vitro and in vivo transplantation strategies for the pre-clinical assessment of human progenitor cell-based therapies to treat ischemic disease.

Keywords: aldehyde dehydrogenase • stem cells • umbilical cord blood • critical limb ischemia • angiogenesis
INTRODUCTION

This section describes the isolation and application of pro-angiogenic progenitor cells from human umbilical cord blood (UCB) for the development of cellular therapies to treat ischemic vascular diseases. Angiogenesis, defined as the sprouting of new blood vessels from pre-existing vessels, represents a central process for tissue repair, and allows the delivery of circulating pro-angiogenic progenitor cells from multiple lineages to form a vascular regenerative microenvironment (Rafii and Lyden, 2003).

Due to recent widespread initiatives to HLA-phenotype and cryopreserve human UCB in North America and Europe, UCB now represents a readily available source of progenitor cells for cell therapy applications. Umbilical cord blood represents a rich and renewable source of hematotopoietic (HPC) and endothelial (EPC) progenitor cells that demonstrate a variety of pro-angiogenic functions. Early in ontogeny and untouched by chronic vascular disease-related pathologies, human UCB contains expandable precursor populations of endothelial cells (EC) that represent the building blocks of neovessels (Asahara et al., 1999; Asahara et al., 1997) and early myeloid progenitor cells that provide paracrine support of EC survival and vessel forming functions (Putman et al., 2012).

This unit focuses on the prospective isolation and functional characterization of progenitor cells from human UCB. First, we describe fluorescence activated cell sorting (FACS) of lineage depleted UCB mononuclear cells using high aldehyde dehydrogenase (ALDH^{hi}) activity, a conserved stem cell function that enriches for HPC and EPC frequencies. Second, we outline co-culture strategies to determine the paracrine communication between signal-sending progenitor lineages that support signal-receiving EC survival, proliferation and tube-like structure formation in vitro. Third, we describe in detail, human progenitor cell transplantation methodologies into immunodeficient (NOD/SCID) models of critical limb ischemia induced by unilateral femoral artery ligation and transection. These models have been optimized to permit the survival and function of delivered human progenitor cells in areas of ischemia, and allow the temporal quantification of blood flow recovery in live mice using laser Doppler perfusion imaging,
coupled with detailed characterization of human cell engraftment correlated with the induction of capillary formation *in vivo*. Collectively, these technologies represent valuable platforms to dissect the molecular cross-talk between vascular and hematopoietic progenitor subsets during regenerative angiogenesis. These vascular regenerative processes may also be manipulated to promote the repair of ischemic tissues including the damaged myocardium after infarct, and the recovery of vascular function following stroke. The protocols presented below are detailed descriptions of those previously published by our group (Cappocia et al., 2009, Putman et al., 2012).

*NOTE:* All cell isolation and culture procedures are to be performed in a Class II biological safety cabinet.

*NOTE:* Aseptic technique is required for handling of all the solutions and equipment in contact with living human cells.
BASIC PROTOCOL 1

ISOLATION OF UMBILICAL CORD BLOOD PROGENITOR CELLS WITH HIGH ALDEHYDE DEHYDROGENASE ACTIVITY

Using high aldehyde dehydrogenase (ALDH\textsuperscript{hi}) activity, a cytosolic enzyme involved in retinoic acid metabolism and cellular self-protection from oxidative damage (Hess et al., 2004; Hess et al., 2006; Cai et al., 2004; Pearce et al., 2005; Storms et al., 2005; Corti et al., 2006), and progenitor-associated cell surface markers (CD34, CD133), we have developed fluorescence activated cell sorting (FACS) strategies to enrich for progenitor cells from hematopoietic, endothelial, mesenchymal lineages (Capoccia et al., 2009). This isolation is non-toxic and relies on the principle that ALDH-activity is downregulated during progenitor cell differentiation (Storms et al., 1999). Thus, high ALDH-activity efficiently isolates highly-purified and viable progenitor subtypes for characterization of pro-angiogenic functions using traditional molecular approaches (cell surface marker analyses, Affymetrix microarray, cytokine production by ELISA or western blot; not discussed in this unit). Furthermore, these starting cell populations can be used to establish highly proliferative hematopoietic and endothelial progenitor cultures amenable to co-culture analyses and transplantation into immunodeficient mouse models of limb ischemia described in detail below.

Materials

50mL heparinized syringe (250U) with 16-gauge needle
15mL or 50mL conical tubes
liquid pipettor and sterile 25mL, 10mL, and 5mL pipettes
1-mL and 200-\textmu L pipettors and sterile filtered tips
RosetteSep\textsuperscript{®} human UCB progenitor enrichment cocktail (Stem Cell Technologies (SCT), cat. no. 15026)
peripheral buffered saline (PBS) + 2% fetal bovine serum (FBS HyClone)
Ficoll-Hypaque gradient (density 1.077 g/L, SCT)
Sterile glass pipettes
EasySep\textsuperscript{®} Red Cell Lysis Buffer (SCT, cat. no. 20110)
Beckman GPR centrifuge with horizontal rotor
hemacytometer and inverted light microscope (Zeiss), or automated cell counter

Isolation of UCB Lineage Depleted Mononuclear Cells

1. Using a 50mL heparinized syringe, draw 50mL of human UCB by venipuncture from the umbilical vein of clamped placental and umbilical cord obtained immediately after live birth. UCB can be stored at room temperature for up to 24 hours prior to cell processing without a significant loss of viability.

2. To deplete mature T-lymphocytes, B-lymphocytes, monocytes, NK-cells and red blood cells (optional), transfer UCB sample from syringe into 2-50mL conical tubes and add RosetteSep™ Human UCB Progenitor Enrichment cocktail at 10µL/mL and incubate for 20 min at room temperature.

   *RosetteSep™ is comprised of tetrameric antibody complexes recognizing CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and glycoporphin A. Unwanted mature cells are pelleted with red blood cells after Ficoll-Hypaque centrifugation (see below).*

3. Add an equal volume of room temperature PBS and mix well.

4. Slowly layer 10mL UCB/PBS mixture on the surface of 5mL of Ficoll-Hypaque solution at room temperature. For large volume samples 30mL UCB/PBS mixture can be layered on the surface of 15mL Ficoll Hypaque solution.

   *To maintain the Ficoll-Hypaque / blood interface, hold the receiving tube at a 45° angle and allow the UCB mixture to carefully flow down the side of the tube to settle on the top of the Ficoll-Hypaque without mixing.*

5. Centrifuge for 20-30 min at 450 x g, at 20ºC, with no brake.

   *Human UCB progenitor cells are enriched in the cloudy mononuclear cell layer, above the pelleted RBC and Ficoll-Hypaque and below the clear plasma layer.*
6. Using a sterile pipette, carefully remove the mononuclear cell layer by gently swirling the pipette tip as cells are drawn, and transfer mononuclear cell layer to a fresh 15mL or 50mL conical tube.

7. Wash cells by adding excess PBS + 2% FBS (>3 times the volume of the mononuclear cell layer) and centrifuge for 7 min at 240 x g, at 20°C, with the brake on. After centrifugation, aspirate the supernatant, and resuspend the cell pellet in PBS + 2% FBS, repeat this step 2 times.

The washing step described above is required to reduce platelet contamination and cellular debris from the mononuclear cell layer and wash remaining Ficoll-Hypaque solution from the sample.

8. If visible red cell contamination occurs in the washed mononuclear cell sample (pink tinge in solution), lyse residual RBC by adding 25mL of 1X red cell lysis buffer solution. Allow to stand for 5 min at room temperature, add 25mL of PBS + 2% FBS, and centrifuge for 7 min at 240 x g. After centrifugation, aspirate the supernatant and resuspend the cell pellet in PBS + 2% FBS, repeat this step 2 times. 1-2 rounds of red cell lysis may be required.

Some UCB samples may contain a significant amount of nucleated red blood cells that resist lysis. However, after 2 rounds of lysis and washing the red cell:leukocyte ratio should be <2:1, and manual hemacytometer counts will allow for an accurate quantification of lineage depleted mononuclear cell numbers.

9. After the final wash, resuspend the purified mononuclear cells in 1mL of Aldefluor™ assay buffer. Count viable cell numbers by trypan blue exclusion using a hemocytometer or an automated cell counter.

A typical 50mL UCB sample contains 200-300 x 10⁶ undepleted mononuclear cells. After RosetteSep™ (as above) the total mononuclear cell number is reduced to 5-50x10⁶ lineage depleted cells allowing for a more efficient isolation of ALDH-expressing cells by FACS as described below.
SUPPORT PROTOCOL 1

CELL SEPARATION BASED ON ALDEHYDE DEHYDROGENASE ACTIVITY

The Aldefluor™ reagent kit is used to identify UCB progenitor cells that exhibit high activity levels of the intracellular enzyme ALDH. Activated Aldefluor™ reagent is an aminoacetaldehyde moiety conjugated to a Bodipy fluorochrome (BAAA). BAAA is lipid soluble and freely diffuses into intact and viable mononuclear cells. In the presence of ALDH, BAAA is converted into Bodipy-aminoacetate (BAA⁺) anion, and trapped within the cell due to its negative charge, allowing the accumulation of fluorescent product proportional to the amount of active ALDH within the cell. BAA⁺ can be cleared from the cell acting as a substrate for ATP-binding cassette (ABC) transporters. During the Aldefluor reaction, active efflux of BAA⁺ is minimized by an efflux inhibitor contained within the Aldefluor assay buffer. After cell sorting, this pharmacological inhibition can be removed by washing in PBS. Aldeflour reagent is not cytotoxic. Thus, viable progenitor cells with high ALDH activity can be purified from highly heterogeneous cell populations without affecting progenitor cell functions in subsequent assays. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), is used to control for background fluorescence. The Aldefluor™ separation protocol detailed below has been optimized for human umbilical cord blood, but is also applicable to the isolation of ALDH⁺ cells from human bone marrow.

Materials

Aldefluor™ kit (Stem Cell Technologies, cat. no. 01700) containing:

- Dry Aldefluor™ reagent, 50 μg
- DEAB inhibitor, 1.5 mM, in 95% ethanol, 1 mL
- 2N HCl, 1.5 mL
- DMSO, 1.5 mL
- Aldefluor™ Assay Buffer, 4 x 25 mL
- High-speed fluorescence activated cell sorter, FACS Aria III (Beckton Dickenson)
- 5mL conical tubes with 500μL PBS+5%FBS (for sorted cell collection)

NOTE: Store kit reagents at 4 – 8°C as per manufactures instructions. Do not freeze.
NOTE: The following protocols have been optimized for lineage depleted UCB samples. The protocol has been slightly modified from the manufacturers instructions to conserve reagents.

**Aldefluor Activation**

1. Assemble all kit reagents and warm to room temperature before use.

2. Add 25µL of DMSO to the vial with dry Aldefluor™ reagent, mix well with pipettor, and let stand at room temperature for 1 minute.

   *The dry Aldefluor™ reagent is an orange red powder that changes to a bright yellow green upon addition of DMSO.*

3. Add 25µL of 2N HCl to the Aldefluor™ vial, mix well with pipettor, and incubate the mixture for 15 minutes at room temperature.

4. Add 350µL of Aldefluor™ buffer and mix well. The final concentration of the solubilized Aldefluor reagent is 300µM.

5. Divide the Aldefluor™ reagent into aliquots and store at -20ºC until needed for the Aldefluor™ assay.

**Aldefluor™ Assay**

1. Adjust the cell sample to 5-10x10⁶ cells/mL in Aldefluor™ assay buffer.

2. Add 5µL of DEAB inhibitor to the DEAB control tube. Recap the tube and the DEAB vial immediately.

   *DEAB is provided in 95% ethanol and will evaporate if not capped after use.*

3. Add 5µL of Aldefluor™ per mL of cell suspension. Mix immediately and add 0.5mL of the cell and Aldefluor™ mixture to the DEAB control tube and recap each tube. Incubate each tube for 30 min at 37ºC (do not exceed 60 min).
The ALDH enzymatic reaction begins immediately upon the addition of the activated substrate to the cell suspension. Because the DEAB tube is important in establishing the sort gates for the ALDH\textsuperscript{lo} versus the ALDH\textsuperscript{hi} populations, it is important that the aliquot of Aldefluor\textsuperscript{TM} reacted cells is added to the DEAB control tube without delay.

4. After incubation, centrifuge tubes at 450 x g for 7 min and remove supernatant. Resuspend each tube in 0.5mL of Aldefluor\textsuperscript{TM} assay buffer.

5. Count viable cell numbers using trypan blue exclusion using a hemocytometer or an automated cell counter. For optimal sorting concentration adjust Aldefluor\textsuperscript{TM} treated cell suspension to 30-40x10\textsuperscript{6} cells/mL in Aldefluor\textsuperscript{TM} assay buffer and store on ice or at 4°C until cell sorting.

If cell sorting using ALDH activity in combination with cell surface markers (CD34, CD133, etc) is to be performed, aliquot 0.5 mL of Aldefluor\textsuperscript{TM} treated cells to individual tubes and add 2-5µL of PE- or APC conjugated antibody to cell suspension. To prevent efflux of Aldefluor\textsuperscript{TM} product it is important to perform antibody incubation (20-30 min) in Aldefluor\textsuperscript{TM} assay buffer. Whenever possible keep the cells on ice or at 4°C to minimize fluorescent product efflux.

Cell Sorting Based on ALDH activity

1. Generate a side scatter (SSC) versus forward scatter (FSC) dot plot. In setup mode, place the DEAB control sample on the flow cytometer and adjust the voltages to center the nucleated cell populations to the to the center of the SSC versus FSC plot. Create region (R1) that includes the nucleated cell population and excludes anucleated erythrocytes and cell debris based on the UCB FSC versus SSC profile (Fig. 1A).

Granular cells show high SSC and FSC. Nucleated cells (monocytes, macrophages, T-lymphocytes, B-lymphocytes, and progenitor cells) show low
**SSC with intermediate FSC. Platelets, erythrocytes and cell debris show low SSC and FSC (Fig. 1A).**

2. Create a SSC versus Aldefluor™ fluorescence dot plot (Fig. 1B) gated on R1. In set-up mode, adjust the Aldefluor™ fluorescence photomultiplier tube voltage so that the stained cells in the DEAB control tube are centered in the third log decade on the dot plot (Fig. 1B). Create a region R2 that includes DEAB-inhibited cells with low SSC and low Aldefluor™ fluorescence. Switch the flow cytometer to data acquisition mode and collect 100,000 events in the R1 region.

*Even in the DEAB-inhibited sample, all live nucleated cells will demonstrate Aldefluor™ fluorescence. Thus, the DEAB analyses (Aldefluor™ fluorescence in the presence of DEAB inhibitor) is a necessary control to distinguish background cellular fluorescence (ALDHlo cells) versus cells with high ALDH-activity (ALDHhi cells) using the test sample.*

3. In set-up mode, place the test sample on the flow cytometer. Without adjusting the photomultiplier voltage, create a region (R3) that includes low SSC cells with bright Aldefluor™ fluorescence (ALDHhi cells). Switch the flow cytometer to data acquisition mode and collect 100,000 events in the R1 region.

4. In sorting mode with the sample chiller on, load ALDHlo cell and ALDHhi cell collection tubes each containing 500µL Aldefluor™ assay buffer, and collect the ALDHloSSClo cells (R1 and R2) and ALDHhiSSChi cells (R1 and R3) at 10,000-20,000 events per second (Fig. 1C).

5. After cell sorting, perform a purity check on the collected ALDHlo cell versus ALDHhi cell tubes. Cell populations should be >99% pure.

6. Centrifuge sorted cell tubes at 240 x g for 7 min and aspirate supernatant. Wash cells with 5mL PBS + FBS to remove ABC-transporter inhibition and allow the efflux activated Aldefluor™ from viable cells.
Sorted cell populations are undamaged by cell sorting and ready for use in the in vitro co-culture or in vivo transplantation experiments described below. In addition, UCB ALDHhi cells can be used to establish highly-proliferative cultures of hematopoietic progenitor cells (Fig. 1D) in X-Vivo 15 basal media + 10ng/mL SCF, TPO, Flt-3 ligand (Lonza, cat. no. 04-744Q) on fibronectin coated plates, or endothelial progenitor cells (Fig. 1E) in EGM-2 media + EGF, IGF, FGF, VEGF (Lonza, cat. no. CC-3162) on fibronectin-coated plates.
Appendix 1. Figure 1. Flow cytometric isolation of human UCB mixed progenitor cells using high aldehyde dehydrogenase activity.

(A-C) Representative flow cytometry plots showing the selection of ALDH\textsuperscript{lo} versus ALDH\textsuperscript{hi} mononuclear cells after lineage depletion. (D) Representative photomicrograph of human hematopoietic progenitors from ALDH\textsuperscript{hi} UCB in culture. (E) Representative photomicrograph of endothelial progenitor cells in plastic adherent culture.
Appendix 1, Figure 1

A

B

Aldefluor + DEAB

C

Aldefluor

D

ALDH<sup>+</sup> HPC

ALDH<sup>−</sup> EPC
BASIC PROTOCOL 2

CO-CULTURE OF UCB ALDH\textsuperscript{hi} CELLS TO ASSESS PARACRINE EFFECTS ON ENDOTHELIAL CELL SURVIVAL, PROLIFERATION, AND FUNCTION

This protocol was adapted to characterize paracrine and juxtacrine support provided UCB progenitor cells on mature endothelial cell survival, proliferation and tubule formation \textit{in vitro} (Putman et al., 2012). However, these co-culture systems could easily be adapted to test the potential vascular regenerative paracrine functions of any cell type of interest.

\textit{NOTE: For this protocol human microvascular endothelial cells (HMVEC) are selected to model capillary or small vessel pro-angiogenic responses. Conversely, human umbilical vein endothelial cells (HUVEC) are used to model pro-angiogenic responses by larger vessel-resident endothelial cells.}

Materials

Endothelial cells (HUVEC or HMVEC expanded in adherent culture, ATCC)

EBM-2 Basal Medium (Lonza, cat. no. CC-3156)

EGM-2 MV SingleQuot Kit & Growth Factors (Lonza, cat. no. CC-4147)  
\textit{Contains a cocktail of growth factors (EGF, VEGF, FGF, IGF-1) and fetal bovine serum (5\% FBS) supplements to be added to basal media.}

Tissue culture treated dishes/plates (Corning; 75cm\textsuperscript{2} flasks for expansion, cat. no. 430641, 12 well plate – 3512, 24-well plate – 3524 for co-culture assays)

Phosphate Buffered Saline (PBS, prepared ahead)

Trypsin Solution (Gibco, TrypLE\textsuperscript{TM} Express (1X), no Phenol Red, cat. no. 12604-013)

Phosphate Buffered Saline + 5\% fetal bovine serum (PBS+FBS, prepared ahead)

ALDH-activity purified UCB cells (ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} from Basic Protocol 1)

50mL conical centrifuge tubes

Hanging polycarbonate transwell inserts (3\textmu m pore, Corning, Cat. no. 3402)

Matrigel\textsuperscript{TM} Basement Membrane Matrix - Growth Factor Reduced (BD Biosciences, cat. no. 356231)

Pipettors and 1000\textmu L, 200\textmu L, 20\textmu L sterile tips

Beckman GPR centrifuge with horizontal rotor

hemacytometer and inverted light microscope or automated cell counter
Cell Culture Incubator at 37°C, 5% CO₂

**Endothelial Cell Harvest**

1. Aspirate growth media (EGM2-MV) and gently rinse culture dish with 10mL pure PBS (no FBS).

2. Add 1mL Trypsin solution to culture dish and swirl to cover surface. Incubate at room temperature or 37°C for 2-5 min to dissociate adherent HMVEC.

3. *If cells do not lift easily from the growth surface you can force cell into suspension by hitting the base of the flask against the palm of your hand.*

4. Once cells have lifted from flask (check visually on inverted light microscope) add 10mL PBS + 5% FBS solution and mix by repeated pipetting. Check flask to ensure no cells are left behind uncollected.

5. Collect cells into a 50mL conical tube and centrifuge at 240 x g for 7 minutes.

6. Aspirate supernatant and resuspend cells in 1 mL of EBM2 (Basal EC Media).

7. Count cells using trypan blue exclusion using a hemocytometer and light microscope or automated cell counter.

8. Distribute cells into a 50mL tube for each media condition to be used.

Adjust the ratio of cell suspension added to each tube depending on number of wells needed for assay:

I. **GF+ Serum+** (EGM2-MV; fully supplemented)
   
   *Positive control conditions for optimal EC survival and growth.*

II. **GF- Serum-** (EBM2; unsupplemented basal media)

   *Negative control conditions for baseline EC survival.*

III. **GF+ Serum-** (EBM2 + growth factors)

   *Suboptimal conditions for EC growth without serum.*

IV. **GF- Serum+** (EBM2 + 5% FBS)

   *Suboptimal conditions for EC growth without growth factors.*
9. Spin all tubes at 240 x g for 7 minutes.

10. Aspirate supernatants and resuspend cells in appropriate media at 10^6 cells/mL

**Endothelial Cell Growth Rate Assay**

1. Seed 40x10^3 HMVEC per well in tissue culture-treated 12-well plate.

   *Cell densities have been optimized for a 12 well plate format and should be adjusted accordingly for culture in 6 or 24 well plates.*

2. Under each growth condition, add 10^5 ALDH<sup>hi</sup> or ALDH<sup>lo</sup> cells into porous 3 µm hanging transwells suspended above the HMVEC in the 12 well plate (Fig. 2A).

3. Incubate cell culture dishes at 37°C for 72 hours.

4. At 24, 48, and 72 hours, observe cells under stereomicroscope and record photomicrographs (Fig. 2B).

5. Enumerate trypan blue excluding HMVEC by blinded hemocytometer counts or automated cell counting to determine the total number and frequency of viable versus dead cells.

**Matrigel Tubule Formation Assay**

1. Thaw matrigel overnight in ice bucket in fridge.

2. Plate 200µL into each well of a 24 well plate. Carefully, pipet Matrigel to evenly cover the base of the well.

   *It is important to prechill your plate and pipet tips in the fridge to prevent the Matrigel from solidifying prematurely. Keep plate on an ice block, and Matrigel in ice bucket. Shake the dish if needed to evenly coat well.*

3. Incubate plate at 37°C for at least 30 minutes to allow matrigel layer to set before plating cells.
4. Add appropriate media to each well up to a total volume 400μL.
   
   *EGM2-MV; positive control*
   
   *EBM2; negative control and basal media for cell supplementation*

5. Seed 50x10^3 HMVEC to each Matrigel-coated well.

6. Add 20x10^3 ALDH[^h] or ALDH[^lo] cells.

7. Observe under stereomicroscope and obtain photomicrographs of 3 fields of view per well of matrigel at 24, 48 and 72 hours (Fig. 2C).

8. Enumerate number of branch points per field of view and quantify average tube length using image analysis software such as ImageJ.
SUPPORT PROTOCOL 2

PREPARING CONDITIONED MEDIA TO ASSESS PARACRINE SUPPORT OF ENDOTHELIAL CELL FUNCTION

If desired, basic protocol 2 can be adapted for use with cell-conditioned basal media, prepared in advance, in place of direct co-culture in hanging transwell inserts (Fig. 2A). These assays demonstrate the added benefit of assessing only paracrine effects without the possibility of cell migration through the transwell.

1. Seed standardized number of ALDH<sup>lo</sup> or ALDH<sup>hi</sup> cells into appropriate sized culture dish containing EBM-2, basal media without serum or growth factors.

   *For optimal cell density, seed 400-500x10<sup>3</sup> cells in a T75-flask (10-15mL), 150-200x10<sup>3</sup> cells in a T25 flask (3-5mL), or 50x10<sup>3</sup> cells in a 6 well plate (1mL).*

2. Incubate culture for 24-48 hours at 37°C.

3. Using a pipet collect conditioned media into a 50mL conical centrifuge tube and spin at 450 x g for 5-7 minutes.

4. Collect supernatant and aliquot conditioned media into polystyrene tubes.

5. Store conditioned media at -30°C until use.
Appendix 1. Figure 2. Co-culture assays to assess cell-mediated effect on survival proliferation tube forming capacity of endothelial cells

(A) Schematic overview of hanging transwell co-culture and cell conditioned media assays to assess endothelial cell proliferation. (B) Representative photomicrograph of human endothelial cells cultured on plastic. (C) Representative photomicrograph of human endothelial cells growing on a thin layer of matrigel to assess modulation of tube-like structure formation.
BASIC PROTOCOL 3

MURINE FEMORAL ARTERY LIGATION AND EXCISION AND QUANTIFICATION OF VASCULAR REGENERATION IN VIVO

This protocol outlines a surgical procedure for inducing unilateral acute hindlimb ischemia in immunodeficient mice. This protocol includes a double ligation with complete resection of the femoral artery and vein. This procedure induces more severe ischemia than single ligation or non-resecting models. We have used immune deficient NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME) to allow the survival and function of xenotransplanted human progenitor cells with reduced rejection due to complete knockout of T- and B-lymphocyte function (SCID mutation) and reduced innate immunity (NOD mutation). However, this protocol can be applied to any immunodeficient mouse strain (NUDE, NOD/SCID IL-2R g null, NOD/SCID/MPSVIII mice) noting that there are strain specific differences in the magnitude and rate of recovery of perfusion following human cell transplantation.

NOTE: The procedures described for the transplantation of human progenitor cells into highly immunodeficient mouse models of limb ischemia requires a dedicated barrier facility with an aseptic surgical and monitoring suite and sterile housing using hepa-filtered ventilated racks.

Materials

Ketamine hydrochloride (100mg/mL, 60-70mg/kg)
Xylazine (20mg/mL, 2-4 mg/kg)
Eye lubricant (Allergan Refresh Lacrilube)
Surgical scissors and forceps
Sterile cotton tipped applicator
5-0 Silk Sutures
Nexaband® Liquid Topical Tissue Adhesive (Abbott Animal Health)
Microsurgical inverted microscope (Nikon SMZ2B)
Microinjection Syringes
Mobile Laboratory Animal Anesthesia System (VetEquip, cat. no. 901807)
Electric hair clippers (Wahl)
Hair removal cream (Nair)
Bacteriostatic soap
Isopropyl alcohol
Betadine solution
Sterile cotton tipped applicators
Cautery tool (Alcon Surgical Optemp II)
Bupreorphine (0.1 mg/kg, subcutaneous)
Heat Pad
Laser Doppler imager (Moor LDI2)
OCT tissue embedding medium
Tissue moulds
Liquid nitrogen
Dry ice
Cryostat microtome
Mouse anti-human HLA-A,B,C antibody (1:500, Beckton Dickenson, cat. no. 557347)
Rat anti-mouse CD31 antibody (1:100 Beckton Dickenson, cat. no. 550274)
vWF antibody (1:200 Millipore, cat. no. AB7356)
DAPI reagent
Immunofluorescent Microscope (Zeiss AxioScope™ or similar)

**Anesthesia**

3. Induce initial anesthesia by intraperitoneal injection of ketamine hydrochloride (60-70mg/kg) and xylazine (2-4mg/kg) solution.

   *For ease of use and to reduce the number of injections needed prepare a working solution of ketamine and xylazine in saline or sterile water to use to intraperitoneal injection.*

4. Place mouse into anesthesia induction chamber and fill chamber with 2-5% isoflurane in 100% O₂ at a flow rate of 0.8L/min.
5. Once mouse is unresponsive to external stimuli by foot pinch, place mouse on surgical surface in supine position and immobilize the right hindlimb by taping the foot to the surgical surface.

6. Maintain anesthesia for the duration of the surgical procedure using a nose cone with 1-2% isoflurane in 100% O₂ at a flow rate of 0.8L/minute.

**Surgery**

1. Apply bepanthene ophthalmic ointment to the eyes to prevent drying during surgery.

2. Shave the hair from the lower limb and inguinal region using an electric shaver for bulk hair removal. Completely remove residual hair from lower limb by applying Nair hair removal cream briefly (1 min).

3. Wipe away excess hair removal cream and clean the skin and prepare surgical field with three rounds of bacteriostatic soap wash followed by isopropyl alcohol swab and betadine scrub.

   *From here forward the protocol is most easily completed using a dissecting microscope.*

4. Carefully make a 10-15mm longitudinal incision in the skin following the femoral vessel from the inguinal crease distally down the midpoint of the thigh using surgical scissors and forceps.

5. Use moistened sterile cotton tipped applicator and surgical scissors to gently clear subcutaneous fat away to expose the femoral artery and vein and associated neurovascular bundle (Fig. 3A). Locate the superficial epigastric artery branch point as a landmark.
6. Using the cautery tool, cauterize transversely the subcutaneous fat and the superficial epigastric artery. This will expose the muscle and neurovascular bundle underneath.

7. Carefully separate the femoral nerve from the femoral artery and vein starting near the groin and moving distally towards the knee using forceps and scissors. 

   *Avoid damaging the femoral nerve while separating it from the vessels.*

8. Thread silk suture under the femoral artery and vein and gently slide it proximally toward the groin to maximize the length of the femoral artery and vein to be excised. Ligate the artery and vein using double surgical knots.

9. Thread silk suture under the femoral artery and vein and gently slide it proximally to the bifurcation of the popliteal and saphenous arteries that descend into the calf. Ligate the artery and vein using double surgical knots.

10. Using the cautery tool, cauterize the collateral vessels that emerge from the femoral artery between the ligation sites.

11. Carefully resect and excise the femoral artery and vein bundle between ligation sites with surgical scissors. Typically, it is possible to excise a 5mm segment of the femoral artery and vein between ligation sites. Carefully check the ligation site for full occlusion and for any signs of bleeding.

12. Close skin incision using silk sutures and/or Nexaband tissue glue.

13. For post-operative pain management, administer buprenorphine (0.1mg/kg) by subcutaneous injection.

14. Transfer mouse to recovery cage with direct access to water and food and monitor the mice for 30-60 min ensuring they emerge from anesthesia and retain use of the surgical limb.
It is expected that the mice will show reduced mobility and use of the ischemic limb after surgery. Mice will demonstrate a notable limp for 24-48 hours post-operatively. Gradually, mice will recover full mobility in the surgical limb. Be sure to monitor the incision sites daily for up to 7 days and look for signs of bleeding or excessive grooming that may result in the re-opening of the incision. If signs of incision damage occur, move mouse to a separate cage without littermates to allow healing.

15. Allow mice to recover for 24 hours before administration of the compound or cell type of interest to assess effects on the recovery of blood flow by laser Doppler perfusion imaging of the mouse hindquarters as described below.

Useful administration techniques include intravenous tail vein injection to deliver cells or compounds to the general circulation to study the recruitment of injected cells to the ischemic region. Alternatively, direct intramuscular injection into the ischemic adductor or gastrocnemius muscle can be used to bypass the inefficiencies of cellular recruitment to the ischemic region.

Laser Doppler Perfusion Imaging (LDPI) Analyses

NOTE: LDPI timepoints and a representative example of the recovery of limb perfusion after intravenous injection of 2x10⁵ UCB ALDH<hi> cells is shown in Fig. 3B.

1. Induce anesthesia as described in the surgery section above.

   Depending on the strain of mouse used the injection of xylazine and ketamine to induce anesthesia may not be necessary and induction using inhaled anesthesia may be sufficient.

2. Completely remove any residual hair from lower limb by applying hair removal cream briefly.

   Excess hair re-growth will interfere with the accuracy of the laser Doppler measurements. Typically, residual hair removal will be required every 1-2 weeks depending on mouse strain and fur color.
3. Transfer mouse to 37°C draped heating plate for 5 minutes to stabilize body temperature.

*It is important to ensure stable body temperature in the mouse before LDPI scanning as decreased body will significantly alter blood flow in the hindlimbs.*

4. Maintain anesthesia with 1-2% isoflurane in 100% oxygen at 0.8L/min.

*Carefully maintain anesthetic depth to prevent scan artifact from the mouse moving if too lightly anesthetized or from falsely low measurement of blood flow if too deeply anesthetized.*

5. Place mouse in supine position under laser Doppler perfusion imager and extend hindlimbs. Follow manufacturer instructions for proper calibration of laser and measurement of superficial blood flow using consistent settings between subsequent measures at later time points.

6. Using the LDPI software acquire the perfusion image data. Adjust the background threshold to display LDPI flux measurements on the mouse hindquarters from the waist to the tips of the toes.

7. To quantify relative blood flow in the ischemic versus the control limb set a region of interest on each leg between the toes and heel (Fig. 3A), and measure the units of flux in the ischemic limb versus the non-ischemic limb.

8. To ensure consistent induction of acute unilateral hindlimb ischemia by the surgical procedures described above. Relative perfusion in the ischemic limb of NOD/SCID mice should be <10% of the perfusion flow in the control limb.

9. Repeat measurement of blood flow using the identical protocol at each time point to track the recovery of perfusion over a 28 day time course.

*We recommend performing LDPI at post-transplantation day 0, 3, 7, 14, 21, and 28 (Fig. 3B) in 8-10 mice per cohort to allow for statistical comparison between the recovery of perfusion in control (PBS injected) versus cellular transplanted treatments.*

**Collection of Muscle Samples for Histological Analysis**

1. At the predetermined end point of your experiment, euthanize the mice by cervical dislocation under anesthesia or by CO₂ inhalation.
Depending on the purpose of the experiment we routinely euthanize mice from each transplantation cohort at day 7; to address the recruitment of human cells to the ischemic region and to generate a snapshot of active vascular regenerative processes, at day 14 to address the survival of human cells in the ischemic region at the midpoint of the regenerative process, and at day 28 to document the overall extent of vascular recovery. At each endpoint extensive histochemical analyses can be performed (see below) at the site of surgery in the quadriceps muscle and distally in the gastrocnemius muscle. We routinely monitor human cell engraftment and survival, the recovery of capillary density and small neovessel formation, the induction of EC proliferation and prevention of apoptosis in the ischemic region, in order to fully document the cellular mechanisms of vascular recovery.

2. Remove skin and fasciae from the legs and lower limbs of the mouse. Using dissection scissors remove the superficial thigh muscles by making an incision beginning laterally across the knee and carefully peeling and cutting back the muscle from along the femur up towards the groin.

3. Embed the muscle in OCT compound in tissue mould blocks. Align embedded tissue so that the lateral cut across the knee or ankle is along the base of the block.

4. Dissect the gastrocnemius (calf) muscle by making incisions along the tendons inserting at the ankle and cut proximally along the lower leg bones towards the knee.

5. Embed the calf muscle in OCT compound in tissue mould blocks. Align embedded tissue so that the lateral cut across the knee is along the base of the block.

   Careful placement of each muscle sample in the tissue block allows efficient cross-sectional cuts through the muscle fibers during cryosectioning

6. Quickly freeze muscle sections suspended in OCT using liquid nitrogen flash freeze or by placing trays on a dry ice block.

7. Frozen tissue can be stored at -20°C for up to 7 days. For long-term storage, transfer to a -80°C freezer.

**Human Cell Engraftment and Blood Vessel Density in Hindlimb Muscle Sections**


2. Similarly, immunostain for mouse CD31 expression to visualize murine capillary beds using immunofluorescent microscopy counterstained with DAPI or light microscopy counterstained with hematoxylin to mark nuclei (Fig. 3C).

3. Similarly, immunostain for mouse vWF expression to visualize larger murine arterioles using immunofluorescent microscopy counterstained with DAPI or light microscopy counterstained with hematoxylin to mark nuclei (Fig. 3D). 

   *This labels endothelial cells of larger higher shear stress vessels such as arterioles. Whereas CD31 staining can be inconsistent in larger vessels vWF reliably identifies larger vessels in muscle sections (Pusztaszeri et al. 2006).*

4. Enumerate HLA-A,B,C⁺ human cells, CD31⁺ capillary structures, and vWF⁺ arterioles by blinded manual counts of 3 fields of view in each of 3 sections for each muscle sample.
Appendix 1. Figure 3. Transplantation model of acute unilateral hindlimb ischemia.

(A) Diagram of the vasculature of the mouse hindlimb from the inguinal crease to the bifurcation of the femoral artery into the saphenous and popliteal arteries which descend into the calf and lower limb. Ligation sites for femoral artery ligation surgery are indicated. Representative laser Doppler perfusion images of mouse hindquarters before and after ligation and transection of femoral artery and vein. Regions of interest are selected from ankle to toe to quantify relative perfusion in the surgical limb. (B) Outline and suggested timing for tracking recovery of perfusion in the ischemic limb after surgical induction of acute ischemia. Number in lower left quadrant of each laser Doppler perfusion image is the ratio of perfusion in the ischemic limb to the perfusion in the control limb. (C,D) Representative photomicrographs of ischemic adductor muscle sections stained for (C) CD31 and (D) vWF.
Appendix 1, Figure 3

A. Pre-Surgery LDPI

- Ischemic
- Control

Perfusion Ratio:
Ischemic/Control = 1.0

B. Laser Doppler Perfusion Imaging (LDPI)

- Femoral Artery Ligation Surgery
- Cell Transplant

Time (Days):
-1  0  3  7  14  21  28

Ischemic/Control < 0.1

C. CD31

D. VWF

Scale: 100μm
COMMENTARY

Background Information

Ischemic disease is characterized by the reduction of blood flow to the heart or peripheral tissues and encompasses life-threatening disorders such as ischemic heart disease and critical limb ischemia. Despite advances in the management of these conditions, ischemic disease remains a leading cause of morbidity and mortality worldwide (Dotse nko, 2010; Bozdag-Turan et al., 2011). Thus, exogenous stimulation of blood vessel formation by cell transplantation is under intense investigation for the treatment of ischemic disease.

Numerous studies have established the potential of transplanted bone marrow-derived mononuclear cells implicated in the co-ordination of regenerative angiogenesis, and have generated a number of clinical trials investigating treatment of cardiac (Janssens et al., 2006; Wollert et al., 2004; Schachinger et al., 2006) and limb ischemia (Tateishi-Yuyama et al., 2002; Higashi et al., 2004) by transplantation. Although these studies have demonstrated safety in the autologous setting, they have shown only modest improvements in clinical endpoints (Rosenzweig, 2006). In addition, recent studies in patients with chronic diabetes or vascular disease-related pathologies demonstrate compromised progenitor cell content and function (Werner et al., 2007; Werner et al., 2005; Fadini et al., 2006; Jialal et al.). Thus, the purification of pro-angiogenic cellular sub-types from non-autologous sources, and characterization of specific sub-type contributions during the coordination of vascular repair is warranted to improve current cell-based therapies for ischemic diseases.

Using high aldehyde dehydrogenase-activity, a conserved stem cell function involved in the protection of long-lived cells from oxidative insult, we have identified a purified mix of progenitors from human BM (Capoccia et al., 2009), and from UCB (Putman et al.). We have shown that iv-transplanted ALDH^{hi} mixed progenitor cells can stimulate the recovery of perfusion in mice with femoral artery ligation-induced limb ischemia (Putman et al.). However, few transplanted cells showed permanent integration into newly generated vascular networks. Rather, ALDH^{hi} cells transiently recruited to the ischemic limb and orchestrated murine capillary formation via unidentified secretory
mechanisms. This concept termed “stem cell-mediated vascular regeneration” has become a central process for tissue repair, and provided proof-of-concept for FDA-approval of clinical trials using autologous BM-derived ALDH<sup>hi</sup> cells to treat ischemic heart failure and critical limb ischemia.

**Critical Parameters and Troubleshooting**

**Table 1. Troubleshooting Guide for Common Problems**

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<th>Possible Cause</th>
<th>Potential Solution</th>
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<td><strong>BASIC PROTOCOL 1: Purification of UCB progenitors based on ALDH activity</strong></td>
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<td>Incomplete red blood cell lysis</td>
<td>High frequency of nucleated red blood cells</td>
<td>Perform a second round of lysis in ammonium chloride solution</td>
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<td>Clogging during sort</td>
<td>Too much debris or clumping cells</td>
<td>Keep cells on ice and filter immediately before sorting</td>
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<td><strong>BASIC PROTOCOL 2: Co-culture of UCB progenitors in vitro</strong></td>
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<td>Contact inhibition or overgrowth of HMVEC during co-culture</td>
<td>Initial seeding of cells at too high density</td>
<td>Seed cells at a lowered density. Shorten incubation endpoint</td>
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<td><strong>BASIC PROTOCOL 3: Femoral artery ligation and transplantation in vivo</strong></td>
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<td>Bleeding during surgery</td>
<td>Rupture of blood vessel</td>
<td>Euthanize mouse</td>
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<td>Excessive necrosis in mouse surgical limb</td>
<td>Damage to femoral nerve</td>
<td>Euthanize mouse, take care not to damage the femoral nerve during surgery</td>
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<td>Mouse slow to recover after anesthesia</td>
<td>Excessive anesthesia</td>
<td>Administer intraperitoneal saline, place mouse under heat lamp or on heat pad to keep warm</td>
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**Anticipated Results**

A 40-50mL lineage-depleted UCB sample will typically contain 200-300x10^6 mononucleated white blood cells. Lineage depletion using RosetteSep™ results in the reduction of total cell numbers to <50x10^6 cells for the Aldefluor™ assay and FACS. Approximately 2-3% of lineage depleted cells will demonstrate high ALDH activity (Fig. 1C), and the ALDH\(^{hi}\) population should be separated from the ALDH\(^{lo}\) cells by greater than half a log scale. Depending on cell sorting efficiency a typical UCB sample will yield approximately 2x10^5-1.5x10^6 ALDH\(^{hi}\) cells for functional experimentation. These cell numbers represent ample cell numbers to perform the co-culture experiments in a 12-well format. Furthermore, we routinely inject 2x10^5 cells per mouse during transplantation experiments. Therefore, from a single UCB sample we obtain sufficient purified cell numbers to transplant 4-8 mice plus PBS controls.

In the *in vivo* transplantation model, the surgically-induced reduction in perfusion may differ between individual mouse strains or between surgeons if the surgical technique differs slightly. We recommend setting a maximum perfusion ratio of 0.1 measured immediately after surgery as a criterion to exclude mice with potentially incomplete induction of limb ischemia. In our experience this level of ischemia does not induce loss of limb or excessive morbidity as long as there is no damage to the femoral nerve.

LDPI performed on live anesthetized mice allows the accurate quantification of the recovery of perfusion in the ischemic limb over a pre-determined time course. The recovery of perfusion to the ischemic limb occurs rapidly over the first 7 days. Therefore, several LDPI measurements should be taken in the first week to closely monitor the kinetics of recovery. In PBS-injected control animals, the recovery of perfusion may plateau after 14–28 days at ≈40-50% of the non-ischemic limb depending on the recipient mouse strain. This baseline recovery in perfusion is sufficient to prevent limb loss and avoid significant morbidity and mortality.
Time Considerations

Once familiar with the protocol, the processing of an UCB unit to prepare for Aldefluor sorting can be completed in approximately 3 hours including all incubations. Depending on sorted cell concentration and equipment, a lineage depleted UCB sample can be sorted in 1-2 hours from set-up through shut down. An experienced flow cytometry operator is recommended to properly perform and troubleshoot the important steps of cell sorting. Similarly, an experienced animal technician can complete anesthesia, preparation and surgery on a mouse in 20-30 minutes. Depending on size of LDPI taken, non-invasive perfusion measurement can be completed in 10-15 minutes per mouse including temperature stabilization. Generally, 30 minutes is sufficient time for a mouse to recover from anesthesia and surgery.
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Paulette Goldweber • Associate Manager, Permissions/Global Rights
Appendix 5. Human Research Ethics Approval

Office of Research Ethics
The University of Western Ontario

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D.A. Hess
Review Number: 12934
Review Date: January 22, 2010

Review Level: Expedited
Revision Number: 2
Approved Local # of Participants: 200

Protocol Title: Transplantation of human stem cells for the induction of angiogenesis and the regeneration of beta-cell function

Department and Institution: Vascular Biology, Roberts Research Institute

Sponsor: JUVENILE DIABETES RESEARCH FOUNDATION

Ethics Approval Date: January 26, 2010
Expiry Date: October 31, 2014

Documents Reviewed and Approved: Revised study end date and revised sample size to 200.

Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB’s as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB’s periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;

b) all adverse and unexpected experiences or events that are both serious and unexpected;

c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.
Appendix 6. Animal Use Protocol Ethics Approval

**Animal Use Protocol 2006-126-12**

AUP Number: 2006-126-12

AUP Title: Characterization Of The Angiogenic Potential Of Aldehyde Dehydrogenase Expressing Stem Cells From Human Bone Marrow

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Characterization Of The Angiogenic Potential Of Aldehyde Dehydrogenase Expressing Stem Cells From Human Bone Marrow" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care.

This approval, although valid for four years, and is subject to annual Protocol Renewal.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Curriculum Vitae

David M. Putman

Education

PhD Candidate, Physiology
2009-2014
University of Western Ontario, London, Ontario,
  • Characterization of blood-derived human progenitor cells for vascular regeneration  Supervisor: Dr. David Hess

Honours Bachelor of Medical Sciences, Pharmacology and Toxicology
2005-2009
University of Western Ontario, London, Ontario

Awards, Distinctions and Fellowships

2013-2014  Ontario Graduate Scholarship ($15,000/year)
2013    Graduate Thesis Research Award, Schulich School of Medicine and Dentistry ($500)
2012    Stem Cell Network, Till andMcCulloch Travel Award ($1415)
2012    Graduate Thesis Research Award, Schulich School of Medicine and Dentistry ($400)
2012    International Centre for Genetic Engineering and Biotechnology Travel Award ($390)
2012-2013  Ontario Graduate Scholarship ($15,000/year)
2011-2012  Ontario Graduate Scholarship ($15,000/year)
2011    Graduate Thesis Research Award, Schulich School of Medicine and Dentistry ($300)
2010-2011  CIHR Frederick Banting and Charles Best Canada Graduate Scholarship ($17,500/year)
2010-2011  Ontario Graduate Scholarship ($15,000/year, Declined)
2010    Graduate Thesis Research Award, Schulich School of Medicine and Dentistry ($600)
2009-2010  Heart and Stroke Foundation of Ontario Program Grant in Vascular Biology Graduate Studentship ($13,700)
2009    Best Poster Award, J. Allyn Taylor International Prize in Medicine Symposium ($250)
2009    Heart and Stroke Foundation of Ontario Summer Undergraduate Studentship ($7000)
Publications


Invited Speaker/Oral Abstracts

- **Putman DM**. *Characterization of blood-derived human progenitor cells for vascular regeneration*. Western University, Department of Physiology and Pharmacology. February 24, 2014, London, ON

Poster Abstracts

- **Putman DM**, Seneviratne A, Hess DA. *Serum-free expansion of cord blood hematopoietic progenitors with vascular regenerative functions*. Till and McCulloch Meetings, Stem Cell Network. October 2013, Banff, AB
  o Also presented at Canadian Student Health Research Forum, Canadian Institutes of Health Research National Poster Competition. June 2013, Winnipeg, MB
• Putman DM, Liu KY, Broughton HC, Bell GI, Hess DA. *Human umbilical cord blood progenitors coordinate a regenerative angiogenic microenvironment in ischemic tissues.* Arturo Falaschi Conference Series on Molecular Medicine; Frontiers in Cardiac and Vascular Regeneration. June 2012, Trieste, Italy
• Putman DM, Liu KY, Broughton HC, Bell GI, Hess DA. *Human umbilical cord blood cells with high ALDH activity support endothelial cell functions and augment recovery from acute ischemic injury.* Till and McCulloch Meeting. May 2012, Montreal, QC
• Putman DM, Liu KY, Broughton HC, Bell GI, Hess DA. *Human umbilical cord blood ALDH<sup>hi</sup> cells demonstrate paracrine support of endothelial cell function.* London Health Research Day. March 2012, London, ON
  o Also presented at Charles W. Gowdey Lecture and Research Day, November 2011, London, ON
  o Also presented at J. Allyn Taylor International Prize in Medicine Symposium, November 2011, London, ON
• Putman D, Broughton H, Robson D, Hess D. *Human umbilical cord blood progenitor cells recruit rapidly to areas of ischemic injury and support a pro-angiogenic niche.* Keystone Symposium on Stem Cells in Development, Tissue Homeostasis and Disease Abstracts. January 2011, Santa Fe, NM
  o Also presented at J.A.F. Stevenson Memorial Lecture and Research Day. November 2010, London, ON
• Putman DM, Broughton HC, Robson DL, Bell GI, Hess DA. *Augmented revascularization and recovery from acute limb ischemia after transplantation of human umbilical cord blood progenitor cells with high aldehyde dehydrogenase activity.*
  
  o Also presented at Charles W. Gowdey Lecture and Research Day, November 2009, London, ON
  
  o Also presented at J. Allyn Taylor International Prize in Medicine Symposium, November 2009, London, ON


**Teaching Experience**

**Teaching Assistant: Physiology 4520b**
“Fundamental Concepts in Stem Cell Biology & Regenerative Medicine”
2010-2014
Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario

**Teaching Assistant: Physiology 3130**
“Mammalian Physiology Laboratory”
2009-2010
Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario