Loss of Vascular Homeostasis with Age:
Correlation of Structural Changes in Endothelial Glycosaminoglycans with Endothelial Progenitor Cell Function

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Abstract

The University of Manchester

Abstract of Thesis submitted by Kate Williamson for the degree of PhD, and entitled “Loss of Vascular Homeostasis with Age: Correlation of Structural Changes in Endothelial Glycosaminoglycans with Endothelial Progenitor Cell Function”. August 2012

Ageing poses one of the largest risk factors for the development of cardiovascular disease (CVD). The increased propensity towards vascular pathology with advancing age maybe explained, in part, by a reduction in the ability of circulating endothelial progenitor cells (EPCs) to contribute to vascular repair and regeneration. Among all current putative EPC populations, outgrowth endothelial cells (OECs) display the most features consistent with a human postnatal vasculogenic cell. Cell-surface heparan sulfate (HS) proteoglycans, by virtue of specific sulfated domains within the glycosaminoglycan chain, are able to bind and modulate the activities of a variety of proteins important for EPC mobilisation, homing and function at sites requiring neovascularization. This study aimed to determine if human OEC function is impaired with age, and to ascertain whether this is accompanied by changes in the fine structure of OEC HS.

Using in vitro cell culture methods, OECs were isolated from healthy subjects across an age range and cell phenotype was verified by the demonstration of numerous endothelial, but not hematopoietic, cell characteristics. The functional capacity of peripheral blood derived OECs from young and old subjects, and comparative cord blood derived OECs, was assessed in terms of their susceptibility to apoptosis, proliferative, migratory and tube-forming capabilities. In vitro scratch and transwell migration assays revealed that the migratory capacity of peripheral blood derived OECs isolated from old subjects was impaired in comparison to those from young subjects and cord blood derived OECs. Structural analysis of HS by high performance liquid chromatography (HPLC) demonstrated a significant reduction in the relative percentage of the trisulfated disaccharide, 2-O-sulfated-uronic acid, N, 6-O-sulfated-glucosamine (UA[2S]-GlcNS[6S]), within OEC HS with age (r = -0.847, p=<0.01). Moreover, a decline in the migratory response of OECs towards a gradient of VEGF significantly correlated with the percentage expression of this disaccharide (r = 0.840, p<0.01). Disruption of cell surface HS by pre-treatment with heparinase I and III was found to significantly reduce the VEGF-induced migratory response of peripheral blood derived OECs isolated from young subjects to levels similar to that observed for OECs from older individuals.

Understanding the role of HS in regulating the directional migration of EPCs to sites requiring neovascularization and developing approaches to facilitate EPC migration may aid in the design of more successful strategies to optimise the regenerative capacity of these cells in the ageing vasculature.
Declaration

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Abbreviations

2-CA .............................................. 2-cyanoacetamide
Ac-LDL ........................................ Acetylated low density lipoprotein
AT-III ................................................ Antithrombin-III
AMAC ............................................... 2-aminoacridone
APC .................................................. Allophycocyanin
BCA .................................................. Bicinchoninic acid
BODIPY / BODIPY-FL ......................... 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, hydrazide
BSA .................................................. Bovine Serum Albumin
CAs .................................................. Circulating angiogenic cells
CECs ................................................ Circulating endothelial cells
CFU-Hill cells ................................. Colony forming unit-Hill cells
CHO .................................................. Chinese Hamster Ovary
CVD ................................................ Cardiovascular disease
CL-6B ............................................... Sepharose® CL-6B
CS .................................................. Chondroitin Sulfate
DMSO ............................................... Dimethyl Sulfoxide
dNTPs ............................................... Deoxynucleotide Triphosphates
EDTA ............................................... Ethylenediaminetetraacetic acid
ELISA ............................................... Enzyme-linked immunosorbent assay
eNOS ............................................... Endothelial nitric oxide synthase
EPC .................................................. Endothelial progenitor cell
EPO .................................................. Erythropoietin
ERK ................................................ Extracellular-signal-regulated kinases
EXT ............................................... EXT family of tumour suppressor genes
FCS .................................................. Foetal Calf Serum
FGF ................................................ Fibroblast Growth Factor
FITC ............................................... Fluorescein isothiocyanate
Flt-1 ............................................... Fetal liver kinase-1
GAG ............................................... Glycosaminoglycan
G-CSF ............................................... Granulocyte-colony stimulating factor
GM-CSF……………………Granulocyte-macrophage colony stimulating factor
GFP……………………………………Green Fluorescent Protein
GFR……………………………………Growth Factor Reduced
HBD……………………………………Heparin binding domain
HBSS…………………………………Hank’s Balanced Saline Solution
HCl……………………………………Hydrochloric acid
HGF……………………………………Hepatocyte growth factor
HIF-1α…………………………………Hypoxia inducible factor-1α
HPLC……………………………………High Performance Liquid Chromatography
HPSG…………………………………Heparan Sulfate Proteoglycan
HS……………………………………Heparan Sulfate
HS-2-OST / HS2ST…………………HS 2-O-Sulfotransferase
HS-3-OST……………………………HS 3-O-Sulfotransferase
HS-6-OST……………………………HS 6-O-Sulfotransferase
IL………………………………………Interleukin
IMT……………………………………Intima-media thickness
LU……………………………………Luminescence units
kD……………………………………kiloDalton
kav……………………………………Avidity constant
kd……………………………………Dissociation constant
KDR……………………………………Kinase insert domain receptor
LacZ…………………………………β-galactosidase
MAPK………………………………Mitogen activated protein kinase
MCP-1………………………………Monocyte chemotactic protein-1
MI……………………………………Myocardial infarction
MMP…………………………………Metalloproteinase
MNC……………………………………Mononuclear cell
mRNA………………………………Messenger RNA
MTT…………………………………3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NA……………………………………N-acetylated domain of HS
NaCl……………………………………Sodium chloride
NaOH .................................................. Sodium hydroxide
NDST .................................................. N-deacetylase/N-sulfotransferase
NO ..................................................... Nitric Oxide
NRP-1 .................................................. Neuropilin
NS ..................................................... N-sulfated domain of HS
NA/NS ............................................... Alternating region of HS
OECs .................................................. Outgrowth endothelial cells
P10 ...................................................... Bio-Gel P-10
PAPS ................................................... 3’-Phosphoadenosine-5’-phosphosulfate
PBS ..................................................... Phospho-buffered saline
PCR ..................................................... Polymerase Chain Reaction
PDGF ................................................... Platelet derived growth factor
PE ....................................................... Phycoerythrin
PIGF ................................................... Placental Growth Factor
PFA ...................................................... Paraformaldehyde
PMP ..................................................... Platelet microparticles
PMSF .................................................. Phenylmethanesulfonylfluoride
qPCR ................................................... Quantitative PCR
RNA .................................................... Ribonucleic Acid
ROS ..................................................... Reactive oxygen species
RP-HPLC .............................................. Revered-Phase HPLC
RT-PCR ............................................... Reverse-transcriptase PCR
SAX-HPLC .......................................... Strong Anion Exchange HPLC
SDF-1α ............................................... Stromal derived factor-1 alpha
siRNA ............................................... Small interfering RNA
SOCs .................................................. Smooth muscle outgrowth cells
STS ..................................................... Staurosporine
SULF .................................................. Sulfatase
TAE ..................................................... Tris base, acetic acid and EDTA buffer
TE Buffer ............................................. Tris EDTA buffer
Tris ................................................... tris(hydroxymethyl)aminomethane
UEA ................................................... Ulex Europaeus
UA ..................................................... Uronic Acid
UV............................Ultraviolet
VEGF ..........................Vascular Endothelial Growth Factor
VEGFR-1 ......................VEGF Receptor-1
VEGFR-2 / KDR / flk........VEGF Receptor-2
vWF...........................von Willebrand factor

Monosaccharides

GalNAc  β-D-N-acetylgalactosamine
GlcA    β-D-glucuronic acid
GlcNAc  β-D-N-acetylgalcosamine
GlcNS   α-D-sulfoglucosamine
IdoA    α-L-iduronic acid
UA      Uronic acid
Xyl     β-D-Xylose
NS      N-sulfate group (N-SO₃⁻)
2S      2-O-sulfate group (2-OSO₃⁻)
3S      3-O-sulfate group (3-OSO₃⁻)
6S      6-O-sulfate group (6-OSO₃⁻)
Chapter 1: Introduction
1. Introduction

Despite significant advances in cardiovascular medicine over the past decade, cardiovascular disease (CVD) remains the leading cause of morbidity and mortality in the Western world. A recent update from the World Health Organisation reported that in 2008, an estimated 17.3 million people died from CVDs (Mendis et al., 2011). Age is a significant risk factor for the development and progression of CVD. As the population ages, the incidence of CVD also increases and with it the healthcare costs associated with treatment. A number of changes occur in the cardiovascular system with age that renders the vasculature susceptible to the occurrence of vascular disease and subsequent cardiovascular events. One of the most clinically important of these is the development of vascular endothelial dysfunction. Although the mechanism of age-related impaired endothelial function remains to be fully elucidated, an imbalance between the magnitude of vascular injury and the capacity for repair appears to be involved. Accumulating evidence suggests that bone-marrow derived endothelial progenitor cells (EPCs) play an integral role in the cellular repair mechanisms for endothelial regeneration and maintenance (George et al., 2011). However, EPCs appear to be subject to age-associated changes that diminish their number in circulation and function, culminating in a decreased capacity for neovascularization or insufficient repair of the endothelium following injury (Jie et al., 2009, Heiss et al., 2005, Scheubel et al., 2003, Rauscher et al., 2003, Edelberg et al., 2002).

Gaining an insight into the mechanisms underlying the age-associated reduction in EPC function may provide options for the development of novel therapies in the treatment of vascular disease in the ageing population. One possible candidate for investigation, and the focus of this study, are cell surface heparan sulfate proteoglycans (HSPGs) that are known to regulate the activities of a wide range of protein ligands, some of which play a key role in the mobilisation, homing and reparative capacity of EPCs.
1.1. Ageing and cardiovascular disease

The average lifespan of humans is increasing and with it the percentage of people aged 65 years or over. By 2035, it is projected that approximately 20% of the population in the UK will be aged 65 years and over. Within this age group, CVD is the primary cause of death and disability; accounting for more than 40% of all deaths among those aged over 65 years (Ungvari et al., 2010). Although an inevitable part of life, unfortunately, ageing poses the largest risk factor for the development of CVD. Indeed, the incidence rates of heart disease and stroke increase exponentially with age for both males and females. Furthermore, the cost associated with treatment is an ever increasing economic burden. Thus, it is vital to understand the changes that occur during the ageing process that contribute to the high incidence of cardiovascular disease in this population.

Among the biological structures that are progressively affected by ageing, the endothelium is of crucial importance. The vascular endothelium consists of a monolayer of cells situated at the interface between blood and vascular wall. Once thought to be a static and inert cell layer, the endothelium is, in fact, a dynamic tissue that plays a pivotal role in maintaining cardiovascular homeostasis. The endothelium serves an enormous array of functions including the regulation of vascular tone, vascular growth, vascular permeability, coagulation, platelet adherence and inflammatory responses by controlling leukocyte and monocyte interactions with the vessel wall (Galley and Webster, 2004). As such, maintaining the functional integrity of the endothelial monolayer is of crucial importance for the prevention of vascular diseases, such as atherosclerosis. The term ‘endothelial dysfunction’ refers to a condition in which the endothelium loses its physiological properties and shifts towards a vasoconstrictor, prothrombotic and proinflammatory state. The development of this condition is considered an early and important process which predisposes to many disease processes including atherosclerosis, hypertension, thrombosis and inflammatory syndromes.
Although the development of age-related endothelial dysfunction and CVD is likely to have multifactorial etiologies, a common element appears to be a diminished capacity for vascular repair and regeneration in the ageing host.

1.2. Endothelial progenitor cells (EPCs)

1.2.1. The discovery of circulating EPCs

Vasculogenesis and angiogenesis are two major processes that are responsible for the formation of new blood vessels (neovascularization). Vasculogenesis is defined as the process by which new blood vessels may form de novo through the differentiation of progenitor cells for the endothelial lineage, known as angioblasts, into endothelial cells. In contrast, angiogenesis refers to the growth of new blood vessels from the existing vasculature (Risau, 1997) (Figure 1.1). Traditionally, it was generally accepted that vasculogenesis is restricted to the period of embryonic development and angiogenesis was considered the only means of adult neovascularization. However, this dogma was challenged by several reports describing the re-endothelialization of synthetic vascular grafts by cells circulating in the bloodstream. Pioneering work by Stump et al. described the coverage of porous dacron grafts interposed into the thoracic aortas of young pigs by cells that histologically appeared endothelial. By preventing the vascular grafts from contacting the vascular wall they were able to demonstrate that the monolayer of cells that covered the graft material could only have come from cells contained in the blood (Stump et al., 1963). Similar results were seen with non-porous graft material in dogs (Shi et al., 1998) and left ventricular assist devices in humans (Peichev et al., 2000). In 1997, Asahara and colleagues published a landmark paper in which they demonstrated that CD34+ cells isolated from human blood could be grown in culture under conditions that yielded cells with endothelial characteristics including: the expression of a number of cell surface proteins typical for the endothelial
lineage, such as cluster of differentiation (CD)-31 and TEK tyrosine kinase, endothelial-2 (Tie2); incorporation of acetylated low density lipoprotein (Ac-LDL); and the production of nitric oxide in response to acetylcholine or the endothelial cell specific mitogen vascular endothelial growth factor (VEGF) (Asahara et al., 1997). Importantly, when these putative EPCs, containing a constitutively active β-galactosidase (LacZ) transgene, were injected in vivo into nude mice that had been subject to experimental hind limb ischemic injury, lacZ-positive cells, also CD31+, were evident in newly formed blood vessels around the site of injury, consistent with postnatal vasculogenesis. A series of subsequent experiments by this group utilized a bone marrow transplant model, in which wild type mice were sublethally irradiated and transplanted with bone marrow harvested from transgenic mice constitutively expressing lacZ driven by an endothelial specific promoter. The recipient mice were subject to various kinds of vascular injury such as skin wounding, myocardial infarction (MI) and hindlimb ischemia to serve as a stimulus for neovascularization, after which the number of circulating EPCs were seen to increase and lacZ positive cells were detected in the newly formed vessels. These studies marked a paradigm shift in vascular biology as it became recognised that neovascularization in the adult may depend, at least, in part on a process of postnatal vasculogenesis (Isner and Asahara, 1999, Takahashi et al., 1999).
Figure 1.1. Angiogenesis vs Vasculogenesis. A. Angiogenesis refers to the growth of new vessels via the proliferation and migration of mature endothelial cells (blue). B. Vasculogenesis refers to the *de novo* generation of new blood vessels via the proliferation and differentiation of endothelial progenitor cells (EPCs) (yellow).

### 1.2.2. Approaches to study EPCs

Since the seminal description of a circulating cell population that can contribute to postnatal neovascularization (Asahara et al., 1997), interest in circulating EPCs has soared. However, these reports have often lacked detailed characterization resulting in a host of different cell types being somewhat ambiguously included within the term EPC, thereby generating much debate within the field regarding the nature of an EPC. Putative EPC populations have been identified using two main methodologies; (a) cell sorting by surface phenotype selection or (b) *in vitro* cell culture of the mononuclear cell fraction of peripheral or umbilical cord blood using specific substrates and growth media.
1.2.2.1. Flow cytometric analysis of EPCs

It is possible to isolate circulating EPCs without the need for *in vitro* culture by selecting sub-populations of the mononuclear cell (MNC) fraction based on cell-surface antigen expression. However, to date, no unique or restricted marker that can prospectively identify an EPC from circulating blood has been reported. Given that the field has yet to reach agreement as to the definitive phenotype(s) of an EPC, much debate surrounds the choice of surface markers used in this approach.

According to the initial discovery by Asahara et al. EPCs were defined as cells positive for the hematopoietic stem cell marker CD34 and an endothelial marker fetal liver kinase-1 (Flk-1) (Asahara et al., 1997). However, as CD34 can also be expressed by endothelial cells, Peichev et al. sought to define a panel of cell surface antigens that may better define sloughed circulating endothelial cells (CECs) from circulating EPCs. Given that CD133 (originally termed AC133, prominin-1) was known to be highly expressed on hematopoietic stem cells but is absent from mature endothelial cells, the authors reasoned that any cells which co-express CD34 and CD133 may constitute an immature progenitor population. Furthermore, they proposed that subsets of circulating CD133+ cells that co-express the endothelial marker vascular endothelial growth factor receptor 2 (VEGFR-2) may represent circulating EPCs. To confirm the presence of CD133 and VEGFR-2 expressing cells *in vivo*, Peichev et al. examined the antigenic profiles of the cells that colonised implanted left ventricular assist devices in human subjects. They found that 3% of MNCs on the luminal surface of the left ventricular assist devices co-expressed CD133 and VEGFR-2. Based on these findings, they concluded that EPCs could be defined as circulating CD34+ that co-express CD133 and VEGFR-2 and that quantification of these cells in peripheral circulation may provide important insights into the role of EPCs in different disease states (Peichev et al., 2000). Surprisingly, the authors did not assay for the expression of the universal hematopoietic cell surface antigen, CD45. Nonetheless, the cell surface phenotype CD34,
CD133 and/or VEGFR-2 has gained widespread use as a means to measure circulating EPCs. Indeed, many studies have reported statistically significant correlations between the concentrations of putative EPC populations, expressing this marker set or combinations of these makers, and various disease states, a few of which are listed in Table 1. However, Case et al. demonstrated that CD34+CD133+VEGFR-2+ cells are actually highly enriched for hematopoietic progenitors (>98% of the cells express CD45) and are devoid of cells that display the in vitro and in vivo properties of an EPC (Case et al., 2007). Indeed, it is now clear that many studies claiming to quantify EPCs have actually quantified hematopoietic cells with angiogenic capabilities (Mund and Case, 2011).

Recently, advanced polychromatic (5+/− colour) flow cytometry (PFC) methods have been used to identify circulating progenitor cells involved in neovascularization (Mund et al., 2012, Estes et al., 2010). Such PFC methodologies use multiple fluorescently tagged antibodies to define numerous cell parameters and follow strict criteria for the flow cytometric analysis, including improvements in gating controls via the use of fluorescence minus one (FMO) controls and the use of bioexponential scaling to remove the artifacts created by traditional logarithm scaling. Most recently, using this methodology, circulating EPCs in human cord blood and peripheral blood have been defined by Mund et al. as CD31BrightCD34+CD146+CD105+CD45−CD133-. However, this final population contains both EPCs as well as CECs and the only way to distinguish these two cell types requires subsequent clonogenic assays; EPCs exhibit clonal capability whilst CECs have limited or no clonogenic potential (Mund et al., 2012).

In summary, the lack of consensus definition on the phenotype of an EPC, together with their occurrence at a frequency on the cusp of reproducible detection (0.001-0.1%) and complications associated with antigen promiscuity between the endothelial and hematopoietic lineages make it very difficult to identify EPCs by conventional flow cytometry methods. Advanced PFC methods hold promise but at present, even with
optimal use of the modern flow cytometer, it is not possible to accurately identify and enumerate circulating EPCs by flow cytometry alone. Given the difficulties associated with identifying EPCs by flow cytometry, cell culture methods will be employed in this study as an alternative approach to isolate EPCs from umbilical cord and peripheral blood samples.
Table 1. Human disease states associated with alterations of circulating EPC numbers and/or function.

<table>
<thead>
<tr>
<th>Disease</th>
<th>EPC number</th>
<th>EPC function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery disease</td>
<td>Decreased numbers of circulating EPCs (defined as CD133+ or CD34+/VEGFR-2+) in patients with coronary artery disease.</td>
<td>Decreased CFU-hill colony formation</td>
<td>Werner et al., 2007</td>
</tr>
<tr>
<td></td>
<td>No correlation between circulating EPC numbers (defined as VEGFR-2+/CD34+ and VEGFR-2+/CD133+) and intima-media thickness (IMT).</td>
<td>Significant inverse correlation between IMT, CAC chemotaxis towards VEGF and survival.</td>
<td>Keymel et al., 2008</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Reduced numbers of CFU-hill cells cultured from type I diabetic patients.</td>
<td>Impaired proliferation, adhesion and incorporation into vascular structures of CACs isolated from Type II diabetic patients</td>
<td>Tepper et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Loomans et al., 2004</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td>Reduced in vitro migratory capacity of human CACs in patients with hypertension.</td>
<td>Vasa et al., 2001b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased senescence of CACs in patients with hypertension.</td>
<td>Zhou et al. 2010</td>
</tr>
<tr>
<td>Condition</td>
<td>Decreased numbers of circulating EPCs (defined as CD133+/KDR+) in patients with pulmonary hypertension.</td>
<td>Impaired migration towards SDF-1α, adhesion, incorporation into vascular networks and nitric oxide production by hypoxic murine CACs.</td>
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<tr>
<td>Pulmonary hypertension</td>
<td>Junhui et al., 2008</td>
<td>Marsboom et al., 2008</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis (RA)</td>
<td>Decreased numbers of circulating EPCs (defined as CD34+/KDR+/CD133+) in patients with active disease.</td>
<td>CACs from patients with RA exhibit impaired migratory and adhesive capacities.</td>
<td></td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>Decreased numbers of CFU-Hill cells in patients with cerebrovascular disease than in control subjects.</td>
<td>Ghani et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Acute myocardial infarction (AMI)</td>
<td>CD34+ counts significantly increase in patients with AMI. Patients with AMI had a significantly increased number of circulating EPCs (defined as CD34+CD133+VEGFR-2+) with respect to controls</td>
<td>Shintani et al., 2001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Massa et al., 2005</td>
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1.2.2.2. *In vitro cell culture of EPCs*

In general, 3 methods have been described to isolate and expand EPCs from umbilical cord and peripheral blood mononuclear cells (MNCs) (Figure 1.2).

In the original method developed by Asahara et al. CD34\(^+\) enriched peripheral blood MNCs were plated on fibronectin coated dishes in an endothelial medium and observed to form ‘blood island-like’ clusters of differentiating endothelial cells as early as 3 days after (Asahara et al., 1997). This protocol was subsequently modified and can now be performed using a commercially available kit (Endocult, Stem Cell Technologies, Canada) (Hill et al., 2003). In this method unfractionated MNCs are plated onto fibronectin-coated dishes in the presence of supplemented endothelial growth media and after a 48 hour period of culture, the non-adherent cells are re-plated onto fresh fibronectin coated dishes (Figure 1.2; Method 1). The rationale for the pre-plating step is to deplete the sample of rapidly adhering cells such as macrophages and mature endothelial cells that could contaminate the assay (Shi et al., 1998). Over the next 5-9 days, typical clusters comprised of round cells centrally and sprouts of spindle shaped cells at the periphery emerge. Colonies of this type have been termed colony-forming unit-ECs (CFU-ECs) or colony forming unit-Hill (CFU-Hill) cells (Hill et al., 2003).

Another widely used and methodologically similar approach to isolate EPCs involves plating unfractionated MNCs on fibronectin or gelatin coated dishes in endothelial growth medium. After 4 days of culture, the non-adherent cells are removed, resulting in an adherent target cell population (Figure 1.2; Method 2). The adherent cells which could ingest Ac-LDL and bind the lectin Ulex Europaeus (UEA) were considered to be EPCs. This cell type has been widely referred to as circulating angiogenic cells (CACs) and display morphologically features similar to endothelial cells but, unlike CFU-Hill cells, do not form discrete colonies.
The third method of EPC isolation involves a longer period of culture of unfractionated MNCs on type I collagen coated surfaces in the presence of endothelial specific medium (Figure 1.2; Method 3). In brief, non-adherent cells are regularly discarded during gentle wash steps and after 14-21 days of culture, colonies with a cobblestone morphology typical of endothelial cells emerge from the adherent population of MNCs. This cell type has been termed outgrowth endothelial cells ‘OECs’ (Lin et al., 2000b, Gulati et al., 2003), ‘late EPCs’ (Hur et al., 2004), blood outgrowth endothelial cells (BOECs) (Lin et al., 2002) and endothelial colony forming cells (ECFC) (Ingram et al., 2004).

The nomenclature used to describe the cell types which arise from the three culture methods has been widely varying, contributing to confusion in the field. For clarity, the terms CFU-Hill cells, CACs and OECs will be used herein.
Figure 1.2. Endothelial progenitor cell culture methods. Method 1: Culture of CFU-Hill cells. Non-adherent MNCs are plated on fibronectin-coated tissue surfaces and form colonies after 4-9 days. These colonies have been termed CFU-Hill cells and are comprised of round cells surrounded by spindle-shaped cells. Method 2: Culture of circulating angiogenic cells (CACs). MNCs are plated on fibronectin-coated tissue surfaces for 4 days after which the non-adherent cells are removed. The adherent cells have been termed CACs and do not typically display colony formation. Method 3: Culture of outgrowth endothelial cells (OECs). MNCs plated on collagen I-coated surfaces form colonies, termed OECs, with a cobblestone morphology after 7-21 days of culture. Adapted from Hirschi et al. (2008).
1.2.3. Putative EPC populations

1.2.3.1. CFU-Hill cells

CFU-Hill cells exhibit several endothelial characteristics including the expression of CD31, CD105, CD146, von willebrand factor (vWF) and VEGFR-2, as well as the ability to incorporate Ac-LDL. However, many of these markers and properties are not unique to endothelial cells. Monocytes and macrophages, for example, are known to express ‘endothelial specific’ proteins under certain culture conditions and are able to ingest Ac-LDL (Rohde et al., 2006). Thus, these assays alone do not confirm that this population of putative EPCs has true endothelial cell potential. In fact, CFU-Hill cells have been shown to express a number of monocyte/macrophage markers such as CD14, CD45 and CD115, readily ingest bacteria, display non-specific esterase activity, have a limited proliferative capacity and fail to spontaneously form blood vessels when implanted in vivo (Yoder et al., 2007, Hur et al., 2004). These CFU-Hill colonies actually appear to be composed of a variety of blood cells including monocytes, T-lymphocytes and hematopoietic progenitor cells committed towards the myeloid lineage, that are stimulated under the culture conditions to mimic many features of endothelial cells (Yoder et al., 2007, Rohde et al., 2007, Rohde et al., 2006).

The current evidence suggests that while these cells do not directly incorporate into vascular networks, they are able to augment new vessel formation through the production of angiogenic growth factors, cytokines and chemokines, such as VEGF, interleukin-8 (IL-8), hepatocyte growth factor (HGF) and granulocyte colony stimulating factor (G-CSF) (Critser and Yoder, 2010, Hirschi et al., 2008, Yoder et al., 2007).
1.2.3.2. Circulating angiogenic cells (CACs)

CACs have been shown to promote neovascularization in animal models of MI and limb ischemia (Rehman et al., 2003, Kawamoto et al., 2001, Kalka et al., 2000b). They have several endothelial characteristics including the expression of the endothelial cell surface antigens CD31, CD144, vWF and VEGFR-2 and the ability to bind UEA-1 and ingest Ac-LDL (Vasa et al., 2001b). However, as with the CFU-Hill colonies, these cells do not display all the properties of an EPC, as CACs have been shown to have a low proliferative potential and retain monocytic properties including the expression of CD14, macrophage-1 antigen (Mac-1) and CD11c (Rehman et al., 2003). Recently, it has been demonstrated that this particular method of culture is complicated by the presence of platelets that co-fractionate with the MNCs (Prokopi et al., 2009). In culture, the contaminating platelets disintegrate into smaller platelet microparticles (PMPs) which are taken up by the adherent MNC population resulting in a possible exchange of antigens between cell types. Notably, many of these platelet-derived surface antigens are those also expressed by endothelial cells (CD31, vWF and lectin binding) and convey angiogenic properties to the adherent MNCs in the culture. The PMP-related transfer of endothelial characteristics to MNCs may result in erroneously qualifying the cultured cells as EPCs (Prokopi and Mayr, 2011).

1.2.3.3. Outgrowth endothelial cells (OECs)

Both CFU-Hill cells and CACs are generated from MNC adhesion to particular matrix substrates under a relatively short period of culture. However, a distinctly different population of cells which emerge later in culture were described in a study by Lin et al. Here, peripheral blood MNCs were cultivated from a group of patients that had undergone gender-mismatched bone marrow transplantation. Highly proliferative colonies with mature endothelial cell characteristics emerged 14-21 days after plating the blood cells and were termed OECs. These cells were
capable of more than 1000 fold expansion over a 2 month period in vitro and were of donor origin, suggestive of a marrow origin (Lin et al., 2000b). A number of investigators have since confirmed this seminal article and described the isolation of highly proliferative OECs from umbilical cord blood and peripheral blood. OECs have been shown to express a diverse array of endothelial markers including CD31, VE-CAD, vWF, CD36, CD105, CD146, VEGFR-2 and Tie-2. However, unlike CFU-Hill cells and CACs, this cell type does not express the hematopoietic or monocyte/macrophage cell surface antigens CD45, CD14 and CD115 and is capable of high rates of proliferation in vitro. Furthermore, OECs display functions such in vitro tube formation and in vivo vessel formation with incorporation into the systemic circulation of immunodeficient mice. Thus, among all current putative EPC, OECs display the most features consistent with a human postnatal vasculogenic cell (Medina et al., 2010a, Melero-Martin et al., 2007, Yoder et al., 2007).

It is clear that OECs are clonally unrelated to CFU-Hill cells and CACs however, the exact origin of these cells remains to be elucidated. A common precursor of hematopoietic and endothelial cells, termed the hemangioblast, has often been proposed as the source of OECs (Figure 1.3). Although many studies support the existence of the hemangioblast during embryonic development (Weng et al., 2007, Choi et al., 1998), whether it persists into postnatal life remains a subject of great debate. Furthermore, it has also been speculated that these cells are actually derived from the vessel wall, which, if true, would suggest that these cells are a selection of highly proliferative sloughed endothelial cells rather than EPCs (Fadini et al., 2012, Ingram et al., 2004). However, OECs have been shown to exhibit a greater proliferative potential, release higher levels of angiogenic factors and display enhanced vessel forming ability as compared to mature vessel wall endothelial cells, suggesting that they are in fact a progenitor cell population (Nagano et al., 2007, He et al., 2005, Bompais et al., 2004).
Figure 1.3. Hypothetical model of the origin of endothelial progenitor cells. Mesodermal stem cells reside within the stem cell niche of the bone marrow and can give rise to hemangioblasts that have the ability to differentiate into hematopoietic stem cells and endothelial progenitor cells. Hematopoietic stem cells differentiate into myeloid progenitor cells which give rise to cell such as erythrocytes, monocytes and neutrophils, or lymphoid progenitor cells which give rise to cells such as B- and T-lymphocytes. Endothelial progenitor cells are believed to differentiate into endothelial cells.

1.2.4. The mobilisation and homing of EPCs

In order to participate in postnatal vasculogenesis or vascular repair, EPCs must accomplish four distinct but interrelated steps. They must be able to (i) respond to chemotactic signals that initiate mobilisation from the bone marrow into the circulation; (ii) home to remote sites of vascular injury, ischemia or remodelling; (iii) extravasate from the circulation into such areas and (iv) finally incorporate into the vasculature or exert paracrine support to the endothelium. Some of the major regulators of these steps have now been identified (Figure 1.4).
Figure 1.4 The recruitment and incorporation of EPCs into sites of hypoxia or tissue injury involves a series of distinct, but interrelated steps including mobilisation, chemoattraction, adhesion, endothelial transmigration and invasion/migration into the target site. Here, they can exert their reparative function by in situ differentiation or by paracrine mechanisms. Adapted from Caiado and Diaz (2012).

1.2.4.1. EPC mobilisation

Much work has been dedicated to unravelling the complex process by which physiological and pathophysiological events that require neovascularization induce the mobilisation of EPCs from the bone marrow. The mobilisation of EPCs from the bone marrow, although not completely understood, appears to be regulated by a complex interplay between cytokines, chemokines, growth factors, proteinases and cell adhesion molecules (Figure 1.5). The integrity of the endothelial nitric oxide synthase (eNOS) pathway, including both upstream (e.g. phosphatidylinositol-3-kinase (PI3K), protein kinase B (PKB/Akt)) and downstream effectors appears to be pivotal in the mobilisation of EPCs.

Under steady state conditions, the rate of endothelial cell turnover and frequency of EPCs in the circulation are very low. Within the bone marrow niche, characterised by low oxygen tension and high levels of SDF-1,
EPCs are in a quiescent state in close contact with surrounding stromal cells (Ceradini et al., 2004, Harrison et al., 2002). However, EPCs are mobilised from the bone marrow in response to tissue hypoxia or vascular trauma, which causes the production and release of mobilising factors such as SDF-1, VEGF, granulocyte-monocyte colony stimulating factor (GM-CSF), G-CSF, placenta growth factor (PIGF) and erythropoietin (EPO), to a concentration greater than that in the bone marrow (Urbich and Dimmeler, 2004). These mobilising factors activate stromal endothelial cells in the bone marrow, resulting in the induction of the P13K/Akt/eNOS pathway (Gallagher et al., 2007, Tepper et al., 2002). The activation of this pathway results in the phosphorylation of eNOS, culminating in increased nitric oxide (NO) production. The increase in NO levels elevates the activity of proteinases such as matrix metalloproteinase-9 (MMP-9) which cleave extracellular matrix or cell membrane bound molecules that tether EPCs to bone marrow stromal cells. Bone marrow stromal cells express membrane-bound Kit ligand (mKitL) which is proteolytically cleaved by MMP-9 to produce a soluble Kit ligand (sKitL). sKitL then interacts with the EPC c-Kit receptor to transduce signals that enable the translocation of cKit-positive stem and progenitor cells to the vascular zone of the bone marrow, favouring liberalization of the cells into the circulation (Heissig et al., 2002).
Figure 1.5. Schematic representation of EPC mobilisation from the bone marrow. Tissue hypoxia or vascular trauma results in the production and release of growth factors and chemokines, such as VEGF, SDF-1, GM-CSF, G-CSF and EPO, which activate vascular stromal endothelial cells in the bone marrow, resulting in the induction of the PI3K/Akt/eNOS pathway. Consequently, increased levels of NO production in the bone marrow elevate the activity of MMP-9, which converts mKitL to sKitL. sKitL competes with mKitL for binding to the cKit receptor on EPCs and transduces signals that enable the translocation of EPCs to the vascular zone of the bone marrow. Weakened stromal cell-EPC interactions and the increased circulating levels of SDF-1, result in the release of EPCs from the bone marrow into the peripheral circulation along a gradient of SDF-1. Adapted from Everaert et al. (2010).

There are several lines of evidence to suggest that VEGF is an important mediator of EPC mobilisation. In animal models, exogenous administration of VEGF has been shown to promote EPC mobilisation from the bone marrow, as documented by the increased levels of EPCs (defined as CD34+Flk-1+) in the peripheral circulation (Asahara et al., 1999). In patients with severe angina and no options for percutaneous or surgical revascularization, intramyocardial administration of a plasmid encoding VEGF was seen to increase EPC numbers in the peripheral
circulation (Kalka et al., 2000a). Similarly, VEGF gene transfer in patients with critical limb ischemia was seen to increase the expression of endothelial lineage markers on peripheral blood MNCs by up to 30-fold, which was taken to be indicative of an increase in circulating EPC levels (Kalka et al., 2000a). Consistent with the notion that VEGF is involved in the mobilisation of EPCs, studies in patients who experienced vascular trauma in the form of severe burn injury or coronary artery bypass grafting, have demonstrated that circulating EPCs (defined as VEGFR-2^+CD133^+ cells) peak at 12 hours after injury which closely mirrors the levels of VEGF detected in peripheral circulation (Gill et al., 2001). Likewise, in patients with acute MI, the levels of circulating CD34^+ cells (putative precursors of EPCs) was seen to peak on day 7 after MI, and this rise again mirrors the peak in serum levels of VEGF (Shintani et al., 2001).

A similar effect on the mobilisation of EPCs from the bone marrow has been achieved by the administration of other chemokines, such as SDF-1 and GM-CSF, G-CSF, growth factors, such as platelet derived growth factor (PDGF) and hormones, including oestrogen and EPO. GM-CSF has a known role in stimulating the release of hematopoietic progenitor cells and myeloid lineage cells, and has also been shown to exert a potent stimulatory effect on the mobilisation of EPCs. For example, Takahashi et al. demonstrated that the administration of GM-CSF to mice and rabbits with regional tissue ischemia induced EPC (defined as Sca-1^+ cells) mobilisation with a corresponding improvement in the extent of neovascularization in ischemic tissues (Takahashi et al., 1999). Likewise, the elevation of plasma levels of SDF-1α after intravenous adenovector administration was found to promote the mobilisation of a large number of CD34^+ cells, including both hematopoietic stem cells and EPCs from the murine bone marrow (Hattori et al., 2001, Moore et al., 2001).

The administration of G-CSF has also been shown to increase the number of human CD34^+ cells in circulation which were capable of
enhancing revascularization of regions of ischemic myocardium in an animal model (Kocher et al., 2001). Numerous studies have also highlighted the role of oestrogen in EPC mobilisation from the bone marrow and incorporation at the sites of ischemia (Ruifrok et al., 2009, Hamada et al., 2006, Iwakura et al., 2006, Strehlow et al., 2003). For example, Strehlow et al. demonstrated that ovariectomy of young female mice significantly decreased the levels of EPCs (Sca-1+/Flk-1+) in the peripheral blood and residing bone marrow, but this effect was prevented by treatment with exogenous oestrogen (Strehlow et al., 2003).

1.2.4.2. EPC homing

Once in circulation, EPCs home to sites of vascular injury or ischemia along chemotactic gradients that are formed in these regions. This is a complex process which is dependent upon an intricate interplay between chemokines, chemokine receptors, adhesion molecules and intracellular signalling cascades. The homing of leukocytes to the sites of inflammation has been extensively studied and requires a coordinated sequence of adhesive and signalling events including; selectin-mediated tethering and rolling interactions of leukocytes on the endothelial surface, stimulation by chemokines leading to activation of integrins, integrin-mediated adhesion on endothelial cell monolayers, followed by crawling and transendothelial migration by which leukocytes leave the bloodstream and enter the site of inflammation. The mechanisms of EPC homing to the sites of neovascularization, injury or ischemia are less understood but share at least some common features with the homing of leukocytes (Chavakis and Dimmeler., 2011).

During tissue ischemia, the increased expression and stabilization of the transcription factor, hypoxia inducible factor-1α (HIF-1α), promotes the local production of SDF-1 by hypoxic endothelial cells and is essential for guiding EPCs from the bone marrow towards the sites of ischemia (Ceradini et al., 2004). Numerous studies have demonstrated that SDF-1
and its G-protein coupled receptor CXCR4 play a crucial role in the recruitment of circulating or intravenously infused EPCs to ischemic sites. Indeed, inhibition of SDF-1/CXCR4 axis, using the CXCR4 receptor antagonist AMD3100, has been shown to significantly reduce the homing of stem/progenitor cells to the ischemic myocardium (Abbott et al., 2004). Likewise, inhibition of CXCR4 by neutralizing anti-CXCR4 antibodies was found to significantly reduce SDF-1 induced adhesion of CACs to an endothelial monolayer, in vitro migration and in vivo homing to the ischemic limb in a murine model of hindlimb ischemia (Walter et al., 2005, Ceradini et al., 2004). Moreover, the local administration of SDF-1 has been shown to augment the homing and incorporation of human EPCs at the site of ischemia, resulting in enhanced efficacy of neovascularization after systemic EPC transplantation (Yamaguchi et al., 2003). The importance of the SDF-1/CXCR4 axis for progenitor cell homing is further supported by studies demonstrating that the injection of human CXCR4+ bone marrow-derived MNCs in mice after the induction of hindlimb ischemia improved neovascularization, whereas CXCR4- cells lacked any therapeutic benefit (Seeger et al., 2009).

Additional chemokines involved in EPC homing include the CXC chemokines IL-8, growth-regulated oncogene alpha (Gro-α) and the cellular receptors CXCR2 and CXCR1. IL-8/Gro-α production by the ischemic myocardium has been shown to provide a chemoattractant gradient for the homing of intravenously infused human CD34+ cells to the ischemic heart (Kocher et al., 2006). Furthermore, neutralizing anti-IL-8/Gro-α antibodies or antibodies directed against CXCR2 or CXCR1 were found to reduce the myocardial trafficking of these cells and the associated improvement in neovascularization. Similarly, blocking CXCR2 inhibited homing and incorporation of human EPCs at sites of arterial injury in athymic nude mice (Hristov et al., 2007).

In addition to chemokines, cytokines such as VEGF, produced by damaged and ischemic tissues, are key regulators of progenitor cell
homing. VEGF gene transfer in patients with critical limb ischemia has been shown to augment the homing of EPCs to ischemic foci (Kalka et al., 2000a). Furthermore, conditional induction of VEGF in selected adult organs appears sufficient to instruct the homing of bone-marrow-derived circulating myeloid cells to the organ from which it emanates. In a study by Grunewald et al., VEGF was shown to induce the perivascular expression of SDF-1 that functions, in turn, to position the circulating cells close to the blood vessels from which they can mediate paracrine actions to augment angiogenesis (Grunewald et al., 2006). Beyond VEGF, there is evidence to suggest a role for the insulin-like growth factor (IGF)-2/IGF-2 receptor (IGF2R) system in both the recruitment and incorporation of progenitor cells to ischemic sites. The local delivery of IGF-2 into the ischemic area was shown to promote the homing of intravenously infused human CD34+ cells into the ischemia area following femoral artery ligature in mice. Moreover, a neutralising IGF2 antibody markedly reduced the homing of these cells to the ischemic tissue (Maeng et al., 2009).

1.2.4.3. EPC adherence, transendothelial migration and tissue invasion

Upon interaction with tissue-specific chemokines, EPCs become activated and initiate integrin-mediated adhesion to vascular endothelial cells, followed by transendothelial migration into sites requiring neovascularization. Selectins and specific integrin subunits appear to mediate the homing and adhesion of EPCs to the sites of neovascularization reviewed by (Caiado and Dias, 2012). Following the adherence of EPCs at specific homing sites they need to migrate through the endothelial monolayer and finally migrate and invade the target tissue. At present, little is known regarding the processes of EPC transendothelial migration, migration and tissue invasion. However, there is evidence to suggest that transendothelial migration of EPCs is mediated by β2-integrins and depends on SDF-1, VEGF and monocyte chemotactic protein (MCP)-1 (Chavakis et al., 2005). Subsequently, the
activity of matrix degrading extracellular proteases, such as cathepsins and metalloproteases, appear to be essential for EPC invasion and migration of the target tissue (Chavakis and Dimmeler, 2011).

1.2.5. EPCs in neovascularization and re-endothelialization

There have been conflicting reports regarding the importance of EPCs in neovascularization and restoration of the injured endothelium, mostly because the degree of vascular engraftment by administered putative EPCs has varied among studies. Some groups have reported a major contribution of these cells to the vasculature (Grant et al., 2002), whilst others have estimated their contribution to endothelium to be very low or undetectable (Purhonen et al., 2008, Gothert et al., 2004, Rajantie et al., 2004). These conflicting reports may be due, in part, to a limited analysis of the EPC phenotype in each study, as well as, the lack of definitive methods to distinguish vessel incorporated bone marrow derived endothelial cells from intimately associated perivascular cells. Regardless of the number of cells that physically integrate into the vessel wall, there has been a substantial amount of evidence in both animals and humans to support a role for EPCs in neovascularization and re-endothelialization. Much of what is known regarding the role of EPCs in vascular repair and regeneration arises from the administration of ex vivo expanded EPCs in animal models of experimentally induced ischemia and mechanical vascular injury. Tracking of the transplanted cells has been possible by labelling the cells with tracker dyes (Kaushal et al., 2001) or genetic tags, such as GFP, or by probing for sex chromosomes after sex-mismatched transplantation (Jiang et al., 2004).

CFU-Hill cells, CACs and OECs have all been shown to contribute to neovascularization in animal models of vascular disease, though this maybe through different mechanisms. Within the seminal paper of Asahara it was demonstrated that the delivery of a heterogenous population of CD34+ MNCs, containing putative EPCs, was able to
promote angiogenesis in areas of hind limb ischemia (Asahara et al., 1997). Similarly, studies using various rat myocardial ischemia models have shown that systemically administered or directly injected human CD34+ MNCs (containing both hematopoietic and endothelial progenitors) stimulate angiogenesis, with subsequent improvement in myocardial perfusion and left ventricular function (Kawamoto et al., 2003), (Kocher et al., 2001). Subsequent studies have reported the in vivo efficacy of delivering putative EPCs to augment neovascularization and tissue repair after experimental induced ischemic injuries. The local injection of CACs in an athymic murine model of hindlimb ischemia was shown to improve neovascularization and blood flow recovery, such that limb necrosis and autoamputation was reduced by 50% in comparison to controls; mice receiving human microvascular endothelial cells (HMVECs) or culture media (Kalka et al., 2000b). Histological examination of tissue sections harvested from the ischemic hindlimb revealed that human CACs (labelled with fluorescent carbocyanine Dil dye) homed to the area of new vessel formation and the authors claimed that the CACs were incorporated into neovascular foci (Kalka et al., 2000b). A number of studies have provided evidence for the contribution of OECs to re-establishing endothelial integrity in injured vessels. Griese and colleagues found that OEC transplantation accelerated the re-endothelialization of balloon denuded rabbit carotid arteries, leading to a significant reduction in neointimal formation (Griese et al., 2003). In a study by Hur et al. the abilities of CFU-Hill cells and OECs to improved perfusion and capillary density in a hindlimb ischemic mouse model was comparable (Hur et al., 2004). Further work using a hind limb ischemic mouse model demonstrated that transplantation of CFU-Hill cells combined with OECs resulted in significantly greater vascular regeneration and rescue of limb function following ischemic injury compared to injection of either cell population alone (Yoon et al., 2005). It appears that OECs can structurally contribute to neovessels, while CFU-Hill cells and CACs act in an indirect fashion by paracrine actions which promote vascular repair by stimulating resident endothelial cells and incorporation of OECs.
However, this may be an overly simplistic view, since the delivery of OECs in a rabbit vascular injury model was found to markedly enhance arterial re-endothelialization without long term incorporation, suggesting that these cells may also exert their effects through paracrine mechanisms.

It can be difficult to collectively interpret the data from studies investigating the role of EPCs in vascular repair and regeneration as they use various populations of cells, often assumed to contain putative EPCs. Nonetheless there does appear to be strong evidence for the ability of EPCs to participate in neovascularization and re-endothelialization. However, the reparative actions of EPCs appear to be insufficient to protect against the development of endothelial dysfunction and vascular pathology in older individuals. Some of the reasons for this will be discussed below.

1.2.6. The impact of ageing on EPCs

A loss of vasculoprotection and the progression of CVD with age may, in part, be due to a reduction in EPC numbers in circulation and/or function at the site of injury. Although this remains to be fully elucidated, the link between age and EPC dysfunction is supported by a number of studies. Heiss et al. compared the number and function of CFU-Hill cells isolated from the peripheral blood of healthy young and old (average ages of 25 and 61 years respectively) individuals. Whilst there were no significant differences in the numbers of circulating EPCs (defined as CD34+/KDR+ or CD133+/KDR+) between the two groups, CFU-Hill cells from the old subjects were found to be significantly impaired in terms of proliferation, migration and survival (Heiss et al., 2005). Rauscher et al. demonstrated that bone-marrow derived EPCs (defined as CD31+/CD45−) from young nonatherosclerotic ApoE−/− mice reduced atherosclerotic plaque size in ApoE−/− recipients despite persistent hypercholesterolemia, an effect not detected when using EPCs from old ApoE−/− mice, thus suggesting that
the atheroprotective properties of EPCs are diminished with age (Rauscher et al., 2003). In addition, transplantation of bone marrow-derived EPCs from young, but not old donor mice, was seen to prevent a decline in PDGF-B signalling and cardiac angiogenesis in an ageing murine model (Edelberg et al., 2002). These studies support the idea that age is an important determinant of EPC function, which can contribute to a reduction in cardiovascular repair mechanisms in the ageing host. However, at present, there are limited studies examining the impact of age on the function of human OECs.

It is likely that the age-associated impairment of EPC number and function is due to a variety of environmental changes that impair EPC generation, mobilisation from the bone marrow, homing and function, as well as intracellular alterations within the cells that induce a senescent phenotype (Figure 1.6).
Figure 1.6. EPCs in the ageing vasculature. A wide range of environmental and internal cellular changes occur with advancing age that impair EPC numbers in circulation and function at the site of injury. The generation, mobilisation, homing and survival of circulating EPCs are dependent on the presence of various pro-angiogenic factors, oestrogen and nitric oxide, all of which are known to decline with increasing age. Furthermore, the angiogenic capacity of EPCs is also impaired with age owing to an increase in oxidative stress, inflammation and the induction of a senescent phenotype. The mobilisation and homing of EPCs may also be impaired during ageing due to structural alterations of HSPGs on the cell surface which perturbs the ability of these cells to interact with chemotactic factors that guide their migration.
With advancing age most, if not all, mammalian cell types are subject to internal alterations as well as environmental influences that ultimately cause the cell to enter a state of irreversible growth arrest, termed senescence. Internal changes include a reduction in telomere length which, beyond a critical length leads to genomic instability and ultimately cell cycle arrest. A reduction in telomere length with age has been shown in putative EPCs of healthy, sedentary men and appears to be due to an age-related decline in the expression of telomerase, a telomere elongating reverse transcriptase enzyme (Kushner et al., 2011, Kushner et al., 2009). Indeed, overexpression of the human telomerase reverse transcriptase (hTERT) gene in CACs has been shown to conserve telomerase activity, delay cell senescence and enhance EPC reparative functions in a murine ischemic hind limb model (Murasawa et al., 2002).

An age-related decline in the expression of proangiogenic factors, including growth factors, cytokines and hormones, is also likely to contribute to impaired EPC generation, mobilisation, migration and survival. For example, decreased expression of SDF-1 and VEGF in aged tissues has been shown to impair murine EPC (defined as Flk-1+/CD11b-) trafficking to sites of ischemia, and is related to depressed HIF-1α signalling (Chang et al., 2007). Similarly, the dramatic reduction in levels of the female reproductive hormone oestrogen at the onset of menopause is associated with decreased levels of circulating EPCs (defined as CD34+VEGFR2CD45-) and an increase in CVD in postmenopausal women (Bulut et al., 2007).

The progressive accumulation of oxidative damage in EPCs with age, due to increased production of reactive oxygen species (ROS) and decreased expression of antioxidant proteins, is also likely to diminish progenitor cell function. He et al. demonstrated that CFU-Hill cells derived from old subjects had significantly reduced levels and activity of the antioxidant enzyme glutathione peroxidase-1 (GPx-1) and were more sensitive to oxidative stress-induced apoptosis as compared to EPCs of
younger subjects (He et al., 2009). Ageing is also associated with an upregulation of proatherogenic stimuli including angiotensin II (Ang II) (Wang et al., 2003, Baylis et al., 1997), which has been shown to enhance the production of ROS in CFU-Hill cells and thereby accelerate cellular senescence (Endtmann et al., 2011, Imanishi et al., 2005). Together, these factors may reduce EPC survival capacity and their ability to promote endothelial repair in the ageing host.

In addition, the reduced capacity of the endothelium to generate NO with advancing age is also likely to impair circulating EPC numbers and function at the site of injury. The importance of eNOS expression, and subsequent NO production, for EPC mobilisation, was documented in eNOS null mice by Laufs and colleagues. Here, physical exercise, which increases NO bioavailability, was found to significantly increase EPC numbers (defined as Sca-1+/Flk-1+), while this effect was attenuated in eNOS null mice and wild type mice treated with an eNOS inhibitor (Laufs et al., 2004).

It has been well documented that ageing is associated with the development of chronic low grade inflammation, which renders the vasculature susceptible to the development of CVD (Csiszar et al., 2007, Csiszar et al., 2004). This shift towards a proinflammatory state in the aged vasculature may also be a contributing factor towards EPC dysfunction and a reduced regenerative potential of the ageing host (Zhang et al., 2009).

It is increasingly appreciated that the endothelial glycocalyx, a network of proteoglycans and glycoproteins on the cell surface, plays a complex role in vascular physiology and pathology (Broekhuizen et al., 2009). In the vasculature, heparan sulfate proteoglycans (HSPGs) constitute the predominant type of proteoglycan in the endothelial cell glycocalyx (Figure 1.7). HSPGs are known to regulate a variety of cellular processes including cell adhesion, migration, proliferation and differentiation
However, to date, the involvement of HSPGs as a possible mechanism underlying the age-associated reduction in EPC function has not been explored.

Figure 1.7. The endothelial cell glycocalyx. Electron microscopic visualization of the endothelial glycocalyx of a rat left ventricular myocardial capillary stained with Alcian blue 8GX (A; scale bar represents 1μm. B; scale bar represents 0.5μm). In the vasculature, HSPGs are the predominant proteoglycans present in the glycocalyx and are able to bind a plethora of growth factors, chemokines and cytokines, thereby playing a pivotal role in many biological processes. Taken from van den Berg et al., (2003).

1.3. Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPGs) are expressed by most, if not all, mammalian cell types and are found in abundance in the extracellular matrix and attached to the cell membrane. HSPGs are composed of a core protein to which one or more glycosaminoglycan (GAG) chains are covalently attached. Primarily it is this GAG component, heparan sulfate (HS), which is able to interact with a diverse array of protein ligands, including growth factors and their receptors, chemokines, enzymes, cell adhesion molecules and various extracellular matrix proteins. These interactions can facilitate ligand-receptor binding, alter protein conformation, increase protein stability or modulate growth factor gradient formation (Turnbull et al. 2001). Consequently, HS plays a key role in a network of cellular events such as cell adhesion, proliferation, migration
and differentiation. It is possible that HS is also involved in EPC function by enabling these cells to interact with key growth factors, cytokines and chemokines that are important for cellular responses such as proliferation, migration and survival.

1.3.1. The structure and biosynthesis of heparan sulfate

HS is composed of repeating disaccharide units of uronic acid linked to β-D-glucosamine. The uronic acid may be either D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA), both of which maybe sulfated on the 2-O position (denoted 2S, (GlcA(2S) and IdoA(2S)). The β-D-glucosamine (GlcN) residue can be modified on a number of positions. It may be N-sulfated (GlcNS), N-acetylated (GlcNAc) or N-unsubstituted (although this is rare). GlcNS can be further sulfated on the 6-O position (denoted 6S, GlcNS(6S)), 3-O position (denoted 3S, GlcNS(3S)) or both (GlcNS(3,6S)), while GlcNAc maybe sulfated on the 6-O position (GlcNAc(6S)) and in rare cases on the 3-O position (GlcNAc(3S)) (Figure 1.8) (as reviewed by Sugahara and Kitagawa, 2002). Theoretically, these modifications could produce as many as 48 different disaccharide units, although many of these have yet to be identified in native HS.
Figure 1.8. The structure of a heparin/HS disaccharide unit. Heparin and HS are composed of repeating disaccharide units of uronic acid linked to β-D-glucosamine. The uronic acid may be either D-glucuronic acid (GlcA) (as shown in A) or epimerised at its chiral centre (*) to become α-L-iduronic acid (IdoA) (as shown in B). Both GlcA and IdoA maybe sulfated on the 2-O position (GlcA[2S] and IdoA[2S]). The β-D-glucosamine (GlcN) maybe either N-sulfated (GlcNS) or N-acetylated (GlcNAc), both of which can be sulfated at the 6-O-position (GlcNS[6S] and GlcNAc[6S]). In rare cases, GlcNS and GlcNS[6S] may also be sulfated on the 3-O-position (GlcNS[3S] and GlcNS[3,6S]).

HS chains are synthesised in the Golgi apparatus using nucleotide sugars imported from the cytoplasm. The chains are assembled on the core protein to create a non-sulfated polysaccharide chain which is then modified by an array of enzymes that catalyse epimerisation reactions and sulfation at selective positions (Figure 1.9). HS biosynthesis begins
with the assembly of a tetrasaccharide linkage region, composed of xylose-galactose-galactose-glucuronic acid (Xyl-Gal-Gal-GlcA-), to one or more serine residues in the proteoglycan core protein. An initiating GlcNAc transferase binds to the tetrasaccharide linkage region and catalyses the addition of the first GlcNAc residue of the HS chain. The addition of GlcNAc commits this intermediate to the assembly of HS. Polymerisation of HS then proceeds with the sequential addition of alternating disaccharide units of GlcNAc and GlcA, catalysed by two glycosyl transferases that form a stable heterodimeric complex in the Golgi. These enzymes are encoded by the family of tumour suppressor genes, EXT1 and EXT2. The nascent chain is then modified by a series of interdependent enzymatic reactions. The initial modification is performed by glucosaminyl N-deacetylase/N-sulfotransferases (NDST) which replaces N-acetyl groups with N-sulfate groups on GlcNAc residues. Within these N-sulfated regions, GlcA residues are epimerised to IdoA by glucuronyl C-5 epimerase. After epimerisation, O-sulfation occurs at the C-2 position of the uronic acids by 2-O-sulfotransferase (HS2ST) and at the C-6 position of the glucosamine residues by 6-O-sulfotransferase (HS6ST). On rare occasions, O-sulfation occurs at the C-3 position of GlcNS by 3-O-sulfotransferase (HS3ST). The HSPG is then translocated to the cell membrane where 6-O-endosulfatases (SULFs), sulfatase-1 (Sulf-1) and sulfatase-2 (Sulf-2), expressed at the cell surface and in the extracellular matrix, catalyse the removal of 6-O-sulfate groups from the HS chain to fine tune its structure. HS synthesis is not template driven, and the enzymatic sulfation and epimerisation reactions do not proceed to completion, resulting in considerable heterogeneity within the polysaccharide chain (Morimoto-Tomita et al., 2002).
Figure 1.9. HS Biosynthesis. (1) The first step in HS biosynthesis involves the creation of a tetrasaccharide linkage region to a serine in the core protein. The addition of GlcNAc commits the chain to the HS synthesis pathway. (2) Polymerisation then proceeds by the addition of alternating GlcNAc and GlcA residues to give the HS chain its final length, which is typically between 50-200 disaccharides. Polymerisation is catalysed by two glycosyl transferases, encoded by the family of tumour suppressor genes, EXT1 and EXT2. The chain is then extensively modified by a series of interdependent enzymatic reactions. (3) First, N-deacetylase/N-sulfotransferases (NDST) removes acetyl groups from GlcNAc residues and replaced with N-sulfate groups to form GlcNS. (4) GlcA is then selectively epimerised to IdoA by GlcA C5 epimerase. (5) Subsequently, sulfation occurs at the 2-O-position of GlcA/IdoA and the 6-O- and/or 3-O-positions of GlcNS/GlcNAc, catalysed by 2-O-, 6-O- or 3-O-sulfotransferases respectively. The HSPG is then translocated to the cell membrane where 6-O-endosulfatases can selectively modify the chain by the removal of 6-O-sulfate groups, giving rise to the final polysaccharide chain. HS synthesis is not template driven, and the enzymatic reactions do not proceed to completion, resulting in heterogeneity within the polysaccharide chain.

Not every glucosamine will acquire a sulfate group and because the subsequent modifications occur predominantly in the N-sulfated regions, the HS polysaccharide chain exhibits a domain type arrangement with 3 distinct regions; 1. The unsulfated domains (N-acetyl (NA) domains) consisting largely of GlcA-(1,4)-GlcNAc disaccharides; 2. The sulfated domains (N-sulfated (NS) domains) which consist primarily of IdoA-(1,4)-GlcNS disaccharides which can be O-sulfated as described above; and 3. the alternating domains (NA/NS domains) that intersperse the two other domain classes and consist of both GlcNAc and GlcNS disaccharides. Average HS chain length varies but it is typically 50-200 disaccharide units (25-100kDa). HS chains are very dynamic in structure and can adopt transient helical conformations. Since the size of the HS chains equates too approximately 40-160nm in length, HS chains represent a dominant feature of cell surfaces (Turnbull et al., 2001, Sasisekharan and Venkataraman, 2000). As with the addition of sulfate groups, the introduction of IdoA residues into NS-domains is a crucial modification of the HS chains. This monosaccharide has an unusually flexible pyranose ring conformation which enables it to adopt different conformations,
possibly assisting the binding of HS to its target molecules (Mulloy and Forster, 2000).

The fine structural complexity of HS is caused by both the variation in the disaccharide units and the overall variability created by the domain structure, which together with core protein variability, generates an almost infinite array of structurally diverse HSPGs. The protein binding sites of HS typically reside within the NS and NA/NS domains and in many cases, the density and pattern sulfation within these domains is of special importance in determining HS-protein interactions (Figure 1.10).

![Figure 1.10](image_url)

Figure 1.10. A schematic diagram depicting the domain structure of HS. The HS polysaccharide chain exhibits a domain type structure with differing types and densities of modifications. The NA domains consist primarily of GlcA-GlcNAc residues and can account for up to 50% of the total HS. The NS domains consist primarily of IdoA-GlcNS residues which can be modified by the addition of O-sulfate groups at various positions. NA/NS domains contain a mixture of sulfated and unsulfated residues, and separate the regions of high and low sulfation. The sulfation patterns formed within and by the arrangement of these domains are important for the binding of particular protein ligands receptors such as antithrombin, FGF and VEGF.

HSPGs can be classified into several families based on their core protein structure. Glypicans and syndecans are cell surface HSPGs which are linked to the plasma membrane by a glycosylphosphatidyl inositol (GPI) linkage or a transmembrane domain, respectively. By contrast, perlecan, agrin and collagen XVIII are secreted HSPGs that form part of the extracellular matrix. These core proteins are expressed in a tissue-specific fashion which correlates with their involvement in different signal transduction pathways (Hacker et al., 2005).

The ability of HS to modulate the biological activity of various proteins has been the focus of intense research. As HS is expressed on the surface of
virtually all mammalian cell types the addition of exogenous saccharides can be overcome by endogenous HS. Therefore the removal of HS is often necessary to study its ability to regulate the physiological activity of the protein of interest. This can be achieved by treating the cells with sodium chlorate or heparinase enzymes. Sodium chlorate is a competitive inhibitor of ATP-sulfurylase, a key enzyme in the synthesis of the sulfate donor 3’-phosphoadenosine 5’-phosphosulfate. Consequently, HS reaches the cell surface without sulfate groups, rendering it incapable of binding almost all proteins (Skidmore et al., 2008). Although effective, it should be noted that sodium chlorate treatment inhibits all sulfation reactions, thus the effects are not restricted to HS alone, and it also may have non-specific effects on cells and proteins. By contrast, the heparinase enzymes degrade only HS and thus do not suffer the non-specific effects of sodium chlorate treatment. Alternatively, cell lines that are deficient in HS, due to genetic mutation (Chinese hamster ovary (CHO) cells), or naturally deficient (BaF3 lymphoblastoid cells), can be extremely useful for studying the activities of exogenous HS. However, they often lack many of the signalling systems of interest to HS biologists.

Heparin is sometimes considered to be synonymous with HS, but this is an oversimplification. Heparin is found in cytoplasmic granules of mast cells, whilst HS is present at the cell surface and the extracellular matrix of virtually all mammalian cell types. They also differ in their degree of sulfation, as heparin has higher levels of N- and O- sulfation than HS (Sasisekharan and Venkataraman, 2000).

1.3.2. Heparan sulfate ligand interactions: the importance of specific sulfation patterns

One of the consequences of the immense structural diversity of HS is that these molecules are able to interact with a diverse array of proteins. The binding of proteins by HS was once thought to be a relatively non-specific ionic interaction between negatively charged HS and positively charged
protein. However, it has become increasingly appreciated that the ability of HS to selectively bind certain proteins, and thereby regulate protein activity, is embedded in the fine structure of its polysaccharide chain. Much of our thinking regarding the specificity of HS-ligand interactions was guided by early studies of heparin and its interaction with antithrombin (AT-III). Here, a specific pentasaccharide structure, GlcNAc[6S]-GlcA-GlcNS[3S,6S]-IdoA[2S]-GlcNS[6S], containing a central 3-O-sulfated glucosamine residue, within the polysaccharide chain is essential for high affinity interaction with AT-III (Lindahl et al., 1980). The binding of AT-III to this motif induces a conformational change in AT-III, increasing its anticoagulant activity by orders of magnitude. Following this discovery, it became plausible that the interaction of HS with other proteins may also show similar selectivity. 3-O-sulfated glucosamine is actually a rare component in HS and is not required for the interaction with most ligands. However, the study of the heparin-AT-III interaction stimulated the search for specific arrangements of sulfated sugar residues to achieve selective binding. Selected examples are discussed below.

1.3.2.1. The interaction of HS with FGFs.

The best studied GAG-protein interactions, after heparin and AT-III, are those of HS with fibroblast growth factors (FGFs). The FGF family, composed of 23 members to date, is involved in a wide range of biological processes including cellular proliferation, differentiation, migration, morphogenesis and angiogenesis (Turner and Grose, 2010). FGFs mediate biological responses as extracellular proteins by binding cell surface tyrosine kinase receptors (FGFRs). The structural requirements of heparin and HS to modulate FGF signalling have been intensely studied, and a great deal of information in relation to the best studied basic FGF (FGF-2) and acidic FGF (FGF-1) has emerged. Using cells deficient in HS biosynthesis, it has been demonstrated that these growth factors require HS for their mitogenic activity and turn responsive upon addition of exogenous native heparin (Pye et al., 1998, Ishihara,
Experiments using selectively desulfated heparin preparations and isolated HS NS domains have revealed distinct O-sulfate requirements for interactions with FGF-1 and FGF-2. The minimal binding sequence for FGF-2 within HS consists of a pentasaccharide with the structure GlcA-GlcNS-GlcA-GlcNS-IdoA[2S] (Maccarana et al., 1994). The presence of N-sulfate groups (Turnbull et al., 1992) and 2-O-sulfated IdoA (Maccarana et al., 1994) has been shown to be essential for the ability of HS oligosaccharides to bind FGF-2. Although 6-O-sulfate groups do not lie in the minimal binding sequence for FGF-2, this modification is essential for mediating the biological activity of FGF-2. In fibroblasts deficient in endogenous HS due to chlorate-treatment, exogenous native HS can promote the mitogenic activity of FGF-2. However, selectively 6-O-desulfated heparin failed to induce this activity, although it can compete with native HS for binding to FGF-2 and thus block the heparin-induced mitogenic response (Guimond et al., 1993). Further evidence to support the requirement of 6-O-sulfation of HS for FGF-2 activation comes from studies utilizing libraries of size-defined HS fragments with diverse structures, generated by heparinase digestion, which were screened for their abilities to activate FGF signalling in biological assays (Guimond and Turnbull, 1999, Pye et al., 1998). Several fractions were found to be able to activate FGF-2 signalling in HS deficient cells. Upon analysis of the activating fractions, a significant correlation was demonstrated between increasing 6-O-sulfate content and the ability to promote FGF-2 mitogenic activity (Pye et al., 1998). Activating HS fragments appear to contain the minimal pentasaccharide sequence that mediates binding to FGF-2, flanked by 6-O-sulfate groups which bind to the FGF receptor, thereby allowing functional contacts that promote active signalling (Esko and Selleck, 2002).

The FGF-1 binding domain of HS is distinct from the minimal FGF-2 binding site. The minimal length of oligosaccharide binding to FGF-1, with relatively high affinity, has been reported to be an octasaccharide.
containing a critical IdoA[2S]-GlcNS[6S]-IdoA[2S] trisaccharide motif (Kreuger et al., 1999). 6-O-sulfation of HS was also found to be required for FGF-1 signalling, as chemically 6-O-desulfated heparins were unable to promote FGF-1 mitogenic activity (Guimond et al., 1993). Furthermore, analysis of heparinase digested HS fragments that are capable of activating FGF-1 signalling showed a significant correlation between FGF-1 promoting activity and their 6-O-sulfate content (Pye et al., 2000).

1.3.2.2. The interaction of HS with VEGF

The structural features of HS that are required for binding to and mediating the activity of VEGF is of particular interest given the importance of this factor in processes such as EPC mobilisation and homing. In mammals, the VEGF family consists of several members including, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). In addition proteins that are structurally related to the VEGFs exist in the parapox virus (VEGF-E) and snake venoms (VEGF-F) (Olsson et al., 2006). VEGF-A, frequently referred to as simply VEGF, is a potent mitogen and angiogenic agent. There are several splice variants of VEGF-A, derived from the alternate splicing of exons 6, 7 and 8 of the VEGF gene (Bates et al., 2002). The most abundantly expressed isoforms are VEGF-A\textsubscript{165}, VEGF-A\textsubscript{121}, and VEGF-A\textsubscript{189} (Ruhrberg et al., 2002, Lei et al., 1998, Sugihara et al., 1998) which are secreted by the majority of VEGF expressing cells such as endothelial cells, smooth muscle cells and macrophages (Olsson et al., 2006, Ferrara, 2001b). These isoforms can be distinguished further on the basis of their affinity for heparin, VEGF-A\textsubscript{121} does not bind heparin, VEGF-A\textsubscript{165} has moderate affinity for heparin, and VEGF-A\textsubscript{189} binds heparin strongly (Robinson and Stringer, 2001, Ferrara, 2001a, Neufeld et al., 1994)

Studies aimed at elucidating the structural features of HS that are required for binding of VEGF-A\textsubscript{165} have all implicated N-, 2- and 6-O-sulfate groups, but with different emphasis on their importance. An investigation by Ono et al. (1999) examined the affinity of various heparin derivatives to bind VEGF-A\textsubscript{165}. The authors used an enzyme-linked
immunosorbant assay (ELISA) to determine the binding of VEGF-A\textsubscript{165} upon chemical removal of N-sulfation, 70\% of 2-O-sulfation or 75\% of 6-O-sulfation. The binding of VEGF-A\textsubscript{165} was significantly impaired upon removal of N- and 6-O- sulfate groups, whilst 2-O-desulfated heparin retained the ability to interact with VEGF-A\textsubscript{165}. In an endothelial cell proliferation assay, N-desulfated or 6-O-desulfated heparin were unable to potentiate the mitogenic activity of VEGF-A\textsubscript{165} compared to native heparin, whilst 2-O-desulfated heparin had an intermediate inhibitory effect on cell proliferation. VEGF-A\textsubscript{165} significantly lost its mitogenic activity by the treatment of HUVECs with chlorate. The addition of exogenous native heparin could partially restore the mitogenic activity of VEGF-A, whereas N-, 2-O, and 6-O desulfated heparins failed to do so. Thus, although not required for binding, 2-O-sulfate groups within HS chains maybe required for the mitogenic activity of VEGF-A\textsubscript{165} (Ono et al., 1999).

Consistent with the findings of Ono et al., Robinson et al. found that both N- and 6-O- sulfation within the HS NS-domains are essential for VEGF-A\textsubscript{165} binding, whereas 2-O-sulfate groups contribute to a lesser extent (Robinson et al., 2006). Recently, Zhao et al reported the VEGF-A binding affinities of a small library of heparinase prepared heparin-derived oligosaccharides, varying in length and sulfation patterns. Using surface plasmon resonance, they analysed the interaction of immobilised heparin with VEGF-A\textsubscript{165} and a peptide termed VEGF-A\textsubscript{55}, which corresponded to the heparin binding domain of VEGF-A\textsubscript{165}. Both VEGF proteins demonstrated a strong affinity for heparin. The authors attempted to inhibit this interaction using the heparin-derived oligosaccharides, and demonstrated that an oligosaccharide containing 8 or more sugar residues could significantly decrease VEGF-A interaction with immobilised heparin when used in excess. Furthermore, binding of VEGF-A appeared to depend on the specific structure of the oligosaccharide as trisulfated heparin oligosaccharides were found to bind with the greatest affinity (Zhao et al., 2012).
1.3.2.3. Heparan sulfate ligand interactions: insights from mutants altered in HS biosynthesis

The generation of transgenic animals deficient in enzymes involved in HS biosynthesis has also provided insights into the specificity of HS-protein interactions. The pattern and density of HS sulfation has been shown to be important for HS function in several development processes. For example, gene trap mutation of HS2ST, which catalyzes the transfer of 2-O-sulfate groups, in mice was found to cause renal agenesis, eye and skeletal defects and neonatal lethality. Analysis of kidney development in the HS2ST mutants revealed that the gene was required for mesenchymal condensation around the ureteric bud and initiation of branching morphogenesis. The authors postulated that the HS2ST mutant phenotype could be a consequence of suboptimal presentation of signalling molecules, such as FGFs and Wingless (Wg), to their receptors. The absence of appropriately sulfated HS may also compromise interactions with various extracellular matrix proteins involved in kidney development (Bullock et al., 1998). Kobayashi et al demonstrated that disruption of HS2ST in the developing limb buds of chick embryos, using siRNA, resulted in abnormal limb bud formation and growth, which appeared to be due to insufficient FGF-signalling (Kobayashi et al., 2007). Diminished levels of HS6ST in Drosophila have been shown to result in abnormal tracheal branching (Kamimura et al., 2001). The requirement for 6-O-sulfation of HS for Drosophila tracheogenesis is interesting as it is often regarded as a model for mammalian vasculogenesis. Indeed, both processes share many common molecular pathways controlled by factors such as FGFs, VEGF/PDGF, cadherin, integrins and HIF-1 like factors (Nakato and Kimata, 2002). In addition, modulation of the level of 6-O-sulfation of HS in zebrafish embryos, by morpholino antisense knockdown of HS6STs, was shown to disrupt VEGF-mediated branching morphogenesis of the caudal vein during embryonic development (Chen et al., 2005).
However, the specificity in HS-protein interactions has been questioned due to observations that, in some cases, the deletion of enzymes responsible for certain steps in HS biosynthesis has had only modest effects on developmental processes believed to depend on selective HS-protein interactions. For example mice lacking C5 epimerase (Li et al., 2003), which catalyses the conversion of GlcNS to IdoA residues, or HS2STs (Merry et al., 2001, Bullock et al., 1998), showed no obvious brain phenotype despite the abnormally sulfated HS structures. This is surprising given that cerebral development is known to involve various HS-dependent growth factors, namely FGF-8 (Inatani et al., 2003). Similarly, VEGF signalling within the cardiovascular system of these mutants appeared to be at levels compatible with adequate vasculogenesis and angiogenesis. It could be argued that the mutant HS structures are nonfunctional and compensated for by HS-independent signalling pathways. However, embryos lacking Ext-1, which generates the initial [GlcA-GlcNAc]n polysaccharide chain, die at the gastrula stage (Lin et al., 2000a), indicating the requirement of HS for embryonic growth and morphogenesis. Thus, the aberrant HS species synthesised in the C5 epimerase and HS2ST mutant mice must be able to support some of the activities of the wild-type HS forms to enable these mutants to survive until birth. Biochemical analysis of these mutant forms of HS reveal that the loss of 2-O-sulfation is often associated with a concomitant increase in N- and 6-O- sulfation. Thus, it is possible that this increase in N- and 6-O-sulfation was able to satisfy the requirement for HS in FGF and VEGF signalling events, compensating for the lack of 2-O-sulfation (Kreuger et al., 2006).

It appears that several functionally important HS-protein interactions depend largely on overall charge distribution, whilst others require the presence of specific sulfate modifications within the HS chain. The overall three dimensional arrangement of the polysaccharide chain may also influence HS-ligand interactions.
1.3.3. HSPGs regulate a network of cellular processes

Numerous studies have demonstrated that HSPGs regulate a variety of cellular processes including cell adhesion, migration, proliferation and differentiation (Tumova et al., 2000). Their strategic location at the cell surface and in the extracellular matrix positions them ideally for interactions with many different classes of proteins, ranging from growth factors (FGFs, VEGF, PDGF, HGF), cytokines (interleukins, platelet factor-4), metabolic enzymes (lipoprotein lipase) and structural proteins (fibronectin and laminin), among many others. HS regulates the activities of a wide range of protein ligands through various modes of action such as increasing protein stability, altering protein conformations, enhancing protein-protein interactions, acting as co-receptors to facilitate ligand-receptor binding and sequestering protein ligands at the cell surface or restricting protein motility to form concentration gradients. HS can also protect proteins from degradation, regulate protein transport through basement membranes and localise and/or immobilise various ligands for internalization and degradation (Figure 1.11). These regulatory interactions have been implicated in a range of cellular processes. In the following sections, the involvement of HSPGs in proliferation, migration and differentiation will be discussed given the importance of these processes in the context of EPC biology.
Figure 1.11. HS regulates the activities of a wide range of protein ligands through various modes of action, four of which are illustrated. (1) Conformational activation; the binding of AT-III to HS induces a conformational change in the protein that accelerates its binding and inhibition of the protease Factor Xa. (2) Enhancing protein-protein interactions; the binding of AT-III to HS can also enhance juxtapositioning of AT-III and inactivation of the protease thrombin (which binds to the HS chain proximal to the AT-III binding site). (3) Co-receptor for soluble growth factors; HS can also act as a co-receptor for growth factors such as FGFs by participating in the formation of a stable ternary complex which aids signal transduction. (4) Localisation of proteins at the cell surface. HS may also sequester protein ligands at the cell surface to regulate their distribution or facilitate internalisation. These regulatory interactions have been implicated in a range of cellular processes. Adapted from Turnbull et al. (2001).
1.3.3.1. The involvement of HSPGs in cell proliferation

Cell proliferation is regulated by a broad array of growth factors and cytokines, such as FGFs, hepatocyte growth factor (HGF), PDGF, heparin-binding endothelial growth factor (HB-EGF) and VEGF, which are able to bind to heparin or HS with relatively high affinity. These molecules can bind cell surface receptors but, in many cases, efficient signal transduction is dependent upon the presence of HS, which can localise cytokines in the vicinity of their receptors (McFadden and Kelvin, 1997) or serve as co-receptors for growth factors (Vlodavsky et al., 1996).

It is well known that the FGF family can act as potent mitogens of many cell types (Beenken and Mohammadi, 2009). FGFs mediate their cellular function through binding and activating high-affinity FGFRs. Binding of FGF induces FGFR dimerisation and the activation of the tyrosine kinase domains of the two receptors which, in turn, transphosphorylate one another, activating these domains to phosphorylate downstream signalling pathways including mitogen-activated protein kinases (MAPKs), such as ERK1 and ERK2, which are often associated with the stimulation of cell proliferation (Klint and Claesson-Welsh, 1999). The formation of an extracellular complex involving two FGFRs is therefore crucial to the signalling cascade. Stable binding of FGFs to their FGFR and subsequent signalling requires the presence of HS. In cells that are deficient in, or unable to synthesis HSPGs, FGF-2 alone was unable to activate FGFR-1 and induce mitogenic activity (Delehedde et al., 2000, Pye et al., 1998, Ishihara, 1994, Guimond et al., 1993, Ornitz et al., 1992). The addition of heparin or purified HSPGs can restore the mitogenic response (Delehedde et al., 2000, Ornitz et al., 1992). HS activates signalling by participating in the formation of a stable ternary complex which is made possible by HS-binding motifs on both the FGF ligand and its receptor (Guimond and Turnbull, 1999). Although, FGF-2 can interact with FGFR-1 in the absence of HS, the formation of stable
ternary complexes that display high ligand-receptor affinity and sustained signalling requires the presence of HS.

VEGF-A is a potent mitogen for vascular endothelial cells and elicits a marked angiogenic response in a wide variety of in vivo models (Ferrara, 2001b). VEGF-A exerts its cellular function by binding to tyrosine kinase receptors on the cell surface. Similar to other tyrosine kinase receptors, signalling by VEGFRs is initiated upon binding of a covalently linked dimer to the extracellular receptor domain. This interaction promotes receptor dimerization, which is accompanied by activation of receptor kinase activity, leading to autophosphorylation of the receptors which can induce the activation of downstream signalling pathways (Olsson et al., 2006). The interaction of VEGF-A<sub>165</sub> with HS is essential for proper signalling as demonstrated in a number of cell culture systems. For example, HUVECs treated with sodium chlorate failed to proliferate in response to VEGF-A<sub>165</sub>, however, this was overcome by the addition of heparin to the medium (Ashikari-Hada et al., 2005, Ono et al., 1999, Ishihara, 1994). The binding of VEGF-A<sub>165</sub> by HSPGs appears to enhance the affinity of dimeric VEGF-A<sub>165</sub> binding to its receptors. There are several lines of evidence to support this: firstly, low concentrations of heparin (0.1-10µg/ml) have been shown to strongly potentiate the binding of VEGF-A to VEGFR-2 on endothelial cells (Terman et al., 1994, Gitay-Goren et al., 1992); furthermore, VEGF-A<sub>165</sub>/VEGFR-2 binding is reduced in cells defective in HS synthesis (Dougher et al., 1997), or following treatment with heparinase (Gitay-Goren et al., 1992), but can be overcome by the addition of exogenous heparin. Besides directly enhancing the VEGF-A/VEGFR-2 interaction, HS also influences the interaction of VEGF-A with neuropilin receptor-1 (NRP-1). NRP-1 acts as a co-receptor for VEGF-A<sub>165</sub>, enhancing VEGF-A<sub>165</sub> binding to VEGFR-2, thereby increasing VEGFR-2 signalling and thus the mitogenic and chemotactic activity of VEGF-A. Fuh et al. reported that in the absence of heparin, the interaction of NRP-1 with VEGF-A is fairly weak (Fuh et al., 2000). Thus, HS may assist in the formation VEGF-A/VEGFR-2/NRP-1 complex to ensure effective signal transduction.
1.3.3.2. The involvement of HSPGs in cell migration

The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration. Focal adhesions are specialized zones of tight cell-matrix interactions that provide a linkage between the cells and its external environment. Transmembrane type HSPGs are known to cooperate with integrins to mediate signals that influence cell adhesion, cytoskeletal organisation and motility (Morgan et al., 2007). There are several lines of evidence to suggest that HSPGs can promote focal adhesion formation which retards cell migration. For example, overexpression of Syndecan-4 in CHO cells was shown to result in increased focal adhesion formation, organisation of stress fibers and decreased cell motility (Longley et al., 1999). Conversely, disruption of HSPGs by heparinase treatment was seen to decrease the strength of endothelial cell adhesion, stress fibre formation and the size of focal adhesions, thereby enhancing the speed of endothelial cell migration (Moon et al., 2005).

In addition, HS has been shown to spatially restrict growth factors and chemokines to enable the formation of concentration gradients, important for directed cell migration. Ruhrberg et al. demonstrated that in the absence of VEGF concentration gradients, formed by heparin binding VEGF isoforms, irregular vascular networks are formed. In this study, VEGF-A$^{120/120}$ mouse embryos were engineered to express an isoform of VEGF-A that does not bind heparin. The inability of VEGF-A$_{120}$ to interact with the extracellular matrix on secretion caused changes in the extracellular localisation of VEGF-A, resulting in an altered distribution of endothelial cells within the growing vasculature and impaired directed extension of endothelial cell filopodia. The results suggest that HSPGs are required to shape extracellular gradients of VEGF which guides the sprouting and migration of endothelial cells into vascular networks (Ruhrberg et al., 2002). Consistently, the specific localisation of VEGF was lost and angiogenesis disrupted knockout in zebrafish embryos in which perlecan was knocked out. Again, this supports the notion that HSPGs can sequester VEGF, regulating its positional distribution,
availability and functional activity to promote endothelial cell migration and proliferation during vessel formation (Zoeller et al., 2009). HSPGs also regulate the tissue distribution of chemokines to form chemotactic gradients that recruit neutrophils to the sites of injury. In a study by Wang et al. the biosynthetic enzyme Ndst, which attaches N-sulfate groups to HS chains, was selectively inactivated in endothelial cells or leukocytes. This resulted in the production of only partially sulfated HS by either cell type. The endothelial specific knockout mice were found to have impaired neutrophil infiltration in various models of inflammation (Wang et al., 2005), due in part, to altered chemokine presentation on the luminal surface of the endothelial cells. There is also evidence to suggest that HSPGs are involved in the formation of concentration gradients of the chemokine SDF-1α which can guide the migration of hematopoietic progenitor cells (Albanese et al., 2009, Netelenbos et al., 2002). Furthermore, HSPGs appear to play a similar role in the guiding the migration of EPCs into tissues via the formation of SDF-1α gradients (Prokoph et al., 2012).

1.3.3.3. The involvement of HSPGs in cell differentiation

Cell differentiation is carefully controlled process involving a vast number of factors, some of which are able to bind HS such as Wnts, bone morphogenetic proteins (BMPs), and FGFs. There are several lines of evidence to suggest that HS plays a role in regulating cell differentiation. Firstly, knock out of the HS polymerases Ext-1 or Ext-2 in mice results in early development abnormalities, such as a failure to fully develop extraembryonic structures and lack of any organised mesoderm, implicating a role for HS in mammalian embryogenesis and co-ordination of cell differentiation events (Stickens et al., 2005), (Lin et al., 2000a). To further study the role of HSPGs in cell differentiation, a number of mouse embryonic stem cells with mutations in HS biosynthesis enzymes have been generated. Embryonic stem cells that lack HS because of a targeted deletion of the Ext-1 fail to commit to lineage differentiation upon removal
of leukemia inhibitory factor, apparently due to defect in FGF signalling. However, the addition of heparin or HS from Ext-1+/− embryonic stem cells was found to restore FGF signalling and correlated with exit from self-renewal and differentiation commitment. In this system, HS appears to promote embryonic stem cell differentiation and commitment by facilitating FGF signalling which, in turn, inhibits Nanog expression (Kraushaar et al., 2010). These findings were corroborated in a study by Lanner et al. in which embryonic stem cells lacking Ndst-1 and Ndst-2, two enzymes required for N-sulfation, were shown to be unable to differentiate in response to FGF-4 due to aberrant sulfation of their HS polysaccharide chains (Lanner et al., 2010). The HS produced by Ndst 1/2−/− cells is devoid of both N- and 2-O-sulfate groups and only low levels of 6-O-sulfation remain. Similarly, embryonic stem cells established from the blastocysts of Ndst 1/2−/− mice were found to be unable to respond to VEGF-A preventing their differentiation into blood capillary structures (Jakobsson et al., 2006). Furthermore, embryonic stem cells have been shown to change the structure of their HS as they differentiate along specific cellular lineages, which may cause differential regulation of protein binding (Baldwin et al., 2008, Johnson et al., 2007). Taken together, these findings highlight the importance of HS in cell differentiation.

1.3.4. The impact of age on heparan sulfate structure

Given that different ligands appear to require distinct HS sequences for binding, any change in the fine structure of HS during development, cell differentiation or pathological processes will influence ligand binding abilities and, subsequently, the physiological activities of the ligands. To date, only a small number of studies have examined age-related structural alterations of HSPGs in adults. The first report by Feyzi et al. (1998) demonstrated a change in the fine structure HS from the aortic wall (human abdominal aorta isolated by autopsy) with increasing age. An increase in the amount of 6-O-sulfation,
particularly within NS-domains, resulted in a higher abundance of the trisulfated disaccharide ΔUA[2S]-GlcNS[6S] within the HS chain of older subjects. As a result, HS from older subjects demonstrated a markedly increased affinity for isoforms of PDGF; an important mitogen for vascular smooth muscle cells. This interaction was proposed to affect the tissue localisation of PDGF causing an extracellular accumulation of this growth factor. In atherosclerosis prone individuals, such changes may trigger pathological smooth muscle cell migration and proliferation (Feyzi et al., 1998). Work by Murata et al. also demonstrated an elevated proportion of the trisulfated disaccharide ΔUA[2S]-GlcNS[6S] with ageing in the cerebral arteries (isolated by autopsy) of Japanese women. However decreased levels of this trisulfated disaccharide unit were found in atherosclerotic cerebral arteries compared to unaffected controls (Murata et al., 1997). In another study, ageing was shown to be accompanied with structural changes of HS in the myocardium left ventricle of rats, involving enhanced 6-O-sulfation, reduced N-sulfation and likely reduced 2-O-sulfation (Huynh et al., 2012). Interestingly, in these studies, a change in 6-O-sulfation of the HS chains appears to be a common element during the ageing process.

1.3.5. The role of heparan sulfate in progenitor cell homing and functionality

Hematopoietic development requires the carefully controlled proliferation of hematopoietic stem and progenitor cells and their differentiation along distinct cellular lineages. This process is regulated by a vast number of morphogens, growth factors, cytokines and chemokines, many of which can bind and be functionally modulated by HSPGs. These include; FGFs, BMPs, VEGFs, Wnts, IL-3, GM-CSF, HGF and PDGF (Cool and Nurcombe, 2006).

HSPGs are known to be an essential component of the bone marrow endothelium and appear to have role in regulating hematopoietic progenitor cell fate, retention and proliferation by binding and localizing
growth factors to specific niches within the hematopoietic microenvironment (Cool and Nurcombe, 2006). The microenvironment created by stromal cells has been used to culture hematopoietic progenitors in the absence of exogenously added cytokines. In these studies 50% of long-term-culture-initiating cells (LTC-ICs), so-named because of their ability to produce colony-forming cell (CFC) progeny, can be maintained for up to 8 weeks in the presence of conditioned media from stromal cells. Gupta et al. examined whether LTC-ICs could be maintained in long term bone marrow culture media, supplemented only with a combination of cytokines (IL-6, G-CSF, SCF, LIF, MIP-la, and GM-CSF) at concentrations found in stromal supernatant. Under these conditions they found five times less LTC-ICs were maintained at 5 weeks compared with stromal supernatant, suggesting that besides these cytokines, additional factors are required. In the search for these other factors, the authors purified the glycoproteins and proteoglycans from the supernatant of a supportive murine stromal fibroblast cell line. They found that the addition of HSPGs, along with the supplemented cytokines, could maintain LTC-ICs to the same extent at unfractioned stromal supernatant. Furthermore, following treatment with nitrous acid, which digests HSPGs, LTC-IC maintenance is completely abolished (Gupta et al., 1996). The ability of HS to support the maintenance of LTC-ICs in vitro appears to be due to its ability to modify the activity of several growth factors, cytokines and in particular the chemokine macrophage inflammatory protein-1α (MIP1-α). HS has been shown to influence the biological activity of MIP1- by inducing chemokine oligomerization which can enhance its local concentration at sites of activity and may aid presentations to its receptor (Stringer et al., 2003, Hoogewerf et al., 1997). The HSPG fraction of the bone marrow has also been implicated in the differentiation of the human pro-myelocytic leukemia cell line HL-60 (Luikart et al., 1990). HS polysaccharide chains have also been ascribed a role in the regulation of hematopoietic stem and progenitor cell retention within the bone marrow endothelium niche.
SDF-1 is constitutively expressed by bone marrow stromal cells, and the interaction of this ligand with its receptor, CXCR4, serves as a retention signal for hematopoietic stem and progenitor cells within the bone marrow, preventing their release to the peripheral blood (Papayannopoulou et al., 2003). It is thought that HSPGs present on the bone marrow endothelium bind and present chemokines, such as SDF-1, to their receptors on hematopoietic stem and progenitor cells which can lead to the activation of integrins and thus promote retention of the cells within the bone marrow niche (Netelenbos et al., 2001). Indeed, Sweeney et al. found that treatment of monkeys and mice with the sulfated polysaccharide fucoidan dramatically increased the plasma levels of SDF-1, coinciding with decreased levels in the bone marrow. The authors concluded that this was due to competitive displacement of SDF-1 from its physiologic ‘anchor’ within the bone marrow environment (Sweeney et al., 2002). Netelenbos et al. provided evidence to suggest that HSPGs in the subendothelial matrix can guide migrating hematopoietic progenitor cells into the bone marrow by forming SDF-1 gradients, and also by possibly enabling enhanced signalling of the CXCR4 receptor by the SDF-1/HS complex (Netelenbos et al., 2002). The SDF-1/CXCR4 axis is also known to play a key role in EPC mobilisation and homing and it is plausible that HSPGs are involved in these processes in a similar manner to which they regulate hematopoietic progenitor cell mobilisation and homing.

Another example to illustrate the involvement of HSPGs in the regulation of hematopoietic cells comes from loss of function mutations in the gene encoding the HSPG Glypican-3 (GPC-3) which causes the human X-linked disorder Simpson-Golabi-Behmel syndrome. The loss of GPC-3 results in impaired development of the common hematopoietic lineage from which monocytes/macrophages and peripheral blood MNCs derive; implicating a role for HSPGs in the lineage specification of these two cell types in vivo (Viviano et al., 2005). Given that specific sulfation patterns within HS chains are often required for the binding of particular protein ligands, some of which are involved in
the regulation of hematopoietic progenitor cells, it is conceivable that any change in these sulfated motifs could perturb the ability of HS to regulate progenitor cell proliferation, migration and survival. Indeed, this is illustrated in the case of Hurler syndrome (mucopolysaccharidosis [MPS] type I) in which abnormally sulfated HS results in defective cytokine induced proliferation and survival of progenitor cells.

Given the close developmental association and molecular overlap between EPCs and HPCs, it is possible that HSPGs may play a similar role in the interaction of EPCs with their local microenvironment. By enabling these cells to interact with key factors that drive their mobilisation, homing and processes such as proliferation, survival and differentiation, HSPGs may be important for recruiting and activating EPCs to sites requiring neovascularization.

1.4. Cardiovascular cell based therapies

Numerous clinical trials are ongoing with the aim of utilizing the regenerative capacity of EPCs in both the cardiac and systemic vasculature. These trials have employed autologous transplantation of various populations of cells, believed to consist of or be enriched in EPCs to improve vascular repair and regeneration in a number of disease conditions. These trials have demonstrated the feasibility and safety of using autologous bone marrow MNCs or progenitor cell populations for the treatment of cardiovascular conditions. However, collectively their outcome has been mixed and reported only modest gains in heart function recovery following cell therapy. These results were unlike the dramatic improvements that were predicted from the pre-clinical studies. This may, in part, be due to the heterogeneity in cell populations used in the treatments, and also variations in methodologies relating to methods of cell delivery, patient characteristics, study endpoints and methods of evaluations. Furthermore, a number of studies have suffered from the
lack of appropriate controls, randomization, blinding and a lack of long-term follow up.

There are a number of obstacles to employing these cell-based therapies to improve vascular repair and regeneration in subjects at risk of CVD. Firstly, a critical limitation for the therapeutic application of EPCs is their low number in circulation under homeostatic conditions. This issue may be overcome by inducing the mobilization of these cells from the bone marrow into the circulation by pharmacological or lifestyle interventions. A number of studies have attempted to augment circulating EPC numbers to enhance neovascularization and tissue repair by administration of G-CSF (Engelmann et al., 2006, Ripa et al., 2006, Zohlnhofer et al., 2006). In the randomized double-blind placebo controlled G-CSF ST-Segment Elevation Myocardial Infarction (G-CSF-STEMI) trial, patients suffering from sub-acute MI were randomized to receive either G-CSF at a dose of 10μg/kg body weight/day subcutaneously or placebo. At 3 months, the G-CSF treated patients showed improved myocardial perfusion of the infarct area. However, there were no significant differences between treatment and placebo groups upon examining the improvement of global and local myocardial function from baseline (Engelmann et al., 2006). To date, G-CSF treatment alone does not appear to be an efficient treatment option for patients with acute MI (Engelmann et al., 2010). Aside from the use of exogenously administered growth factors, cytokines or chemokines, HMG-CoA reductase (statins) (Dimmeler et al., 2001, Vasa et al., 2001a), oestrogen (Iwakura et al., 2006), exercise (Laufs et al., 2004) and cessation of smoking (Kondo et al., 2004) have all been shown to augment EPC numbers in circulation.

Additionally, the therapeutic benefit of autologous transfusion of EPCs may be limited in subjects with CVD, owing to a characteristic impairment of the regenerative properties of these cells in such individuals. In such cases, EPCs could possibly be manipulated ex vivo, expanded and reintroduced in vivo to encourage vascular repair. Iwaguro et al. transduced human EPCs with an adenovirus encoding the murine VEGF-A164 gene. These modified EPCs were administrated to athymic nude mice with
hindlimb ischemia and were found to improve neovascularization and blood flow recovery, leading to reduced limb loss and increased limb salvage compared to control animals (administered with EPCs transduced an adenovirus encoding lacZ and non-transduced EPCs). Importantly, the dose of EPCs used for the in vivo transplantation experiments was 30 times less than that reported in previous trials of EPC transplantation to improve ischemic limb salvage (Iwaguro et al., 2002).

A significant challenge associated with the use of these cells as a therapeutic tool to promote neovascularization and vascular repair include problems with the retention and viability of these cells at the site of injection. Thus, greater therapeutic benefits maybe gained using innovative approaches such as the so-called ‘EPC-capture stent’, which is coated with immobilised anti-CD34 antibodies, aimed at guiding circulating stem and progenitor cells to the site of vascular injury (Co et al., 2008). Alternatively, the use of GAG mimetics, which are structural and functional analogs of natural GAGs, holds great promise for regenerative therapies. These compounds have been shown to improve wound healing after topical treatment in various animal models of injury, namely due to their interactions with cytokines (Rouet et al., 2005, Desgranges et al., 1997, Meddahi et al., 1996) and enzymes (Garcia-Filipe et al., 2007, Alexakis et al., 2004) involved in the remodelling process. They also hold promise for optimising the therapeutic properties of stem and progenitor cells. Like HS or heparin these compounds can bind and immobilise and/or activate various regulatory factors to recruit stem and progenitor cells at target sites or alter the microenvironment to promote cell survival, proliferation or differentiation. Albanese et al. demonstrated that intraperitoneal injection of GAG mimetics induces the rapid mobilisation of hematopoietic stem cells into the peripheral circulation, which is associated with an activation of MMP-9 and alteration of the SDF-1 gradient between bone marrow and peripheral blood (Albanese et al., 2009). Recently, Prokoph et al. successfully used a heparin containing hydrogel to generate a localised SDF-1α gradient that
was capable of attracting CACs and subsequently improving neovascularization (Prokoph et al., 2012). In this study, StarPEG-heparin hydrogels loaded with SDF-1α could sustainably release SDF-1α in defined quantities. Furthermore, the hydrogels provided extracellular matrix-like binding of the chemokine, stabilising and protecting it against enzymatic degradation. The transwell migration of CACs towards concentration gradients of SDF-1α formed by the hydrogel was significantly greater than their migratory response towards soluble SDF-1α. Moreover, subcutaneous implantation of the gels in mice combined with the intravenous injection of human CACs was found to augment EPC homing to the site of injection and subsequent neovascularization. Thus, understanding the role of HS modification on OECs with age could help in the design of more successful strategies to optimise the therapeutic properties of these cells.

1.5. Summary

A number of changes occur in the cardiovascular system during the ageing process which renders the vasculature susceptible to the development of vascular pathology. A reduction in the vascular regenerative potential of the ageing host appears to be one such change. Bone-marrow derived circulating EPCs are believed to contribute to vascular repair and regeneration. While there is evidence to suggest that CFU Hill cells and CACs are subject to age-associated changes that impair their function, the impact of ageing on human OEC function has been much less studied. Cell surface HSPGs are known to regulate an array of cellular processes due to their ability to modulate the physiological activities of a wide range of protein ligands, many of which are important factors for EPC mobilisation, homing, survival or in stimulating the reparative functions of these cells. In many cases, the fine structure of the HS chain is a prerequisite for the binding of these protein ligands. Thus, it is conceivable that any modulation of HS structure on the surface of OECs may perturb the binding of growth factors and other
molecules that interact with HS, thereby altering the physiological effects of the ligand and having important functional consequences for the cell. Importantly, ageing has been shown to be accompanied by structural changes of HS, but this has yet to be studied in the context of EPCs. A great deal of research is aimed at harnessing the regenerative capacity of EPCs to treat cardiovascular disease, yet results to date have yielded inconsistent outcomes in terms of therapeutic benefit. Among the challenges associated with the use of these cells as a therapeutic tool to promote neovascularization and vascular repair include problems with the retention and viability of these cells at the site of injection. Understanding the age-associated changes of HS on OECs may reveal new strategies in the design of more successful strategies to optimise the therapeutic properties of these cells.

1.6. Hypothesis

I hypothesise that age-associated functional alterations of OECs may be related, in part, to structural changes of HS on the cell surface.

1.7. Aims and Objectives

The overall aim of this study was to determine if the function of human OECs is impaired with age and to ascertain whether the ageing process is also accompanied by changes in the fine structure of HS on the surface of these progenitor cells. This will be achieved by three key objectives.

The first objective is to isolate and characterise OECs from umbilical cord blood and peripheral blood samples of healthy adult subjects across an age range. OECs will be isolated by the in vitro culture of adherent MNCs in supplemented endothelial growth medium. However, owing to their rarity in circulation under steady-state conditions, OEC are difficult to isolate from adult peripheral blood samples. Therefore, a number culture conditions will be tested in order to determine those that are optimal for OEC isolation in our hands. Subsequently, a number of parameters such
as endothelial- and hematopoietic-lineage cell surface antigen expression, endothelial cell function via Ac-LDL ingestion and \textit{in vitro} tube formation on Matrigel, will be tested to determine that the isolated cells are indeed OECs.

The second objective is to assess the functional capacity of OECs isolated from cord blood samples and peripheral blood samples of young and old healthy subjects. Cell function will be evaluated in a number of ways; (i) the proliferative capacity of OECs will be measured by direct cell counting and MTT assays, (ii) the \textit{in vitro} migratory capacity of OECs will be assessed by their ability to migrate into an area denuded of cells using an \textit{in vitro} scratch assay and secondly by their ability to migrate along a chemotactic gradient of VEGF or SDF-1\(\alpha\) using transwell migration assays, (iii) the survival capacity of OECs under pro-apoptotic conditions will be determined by measuring the levels of active cleaved caspase-3 protein in cell lysates and finally (iv) the ability of the cells to participate in angiogenesis will be assessed by their ability to form capillary-like networks using Matrigel and collagen tube formation assays.

The third objective is to determine whether HS structure changes on the surface of OECs as a consequence of ageing. This will be examined by disaccharide composition analysis of HS extracted from OECs using high performance liquid chromatography (HPLC). Finally, if the ageing process is seen to induce structural changes of HS on the surface of OECs, we will examine if these changes correlate with functional alterations of these progenitor cells.
Chapter 2: Materials and Methods
2. Materials and Methods

Materials

Suppliers of materials and reagents are listed in Appendix I. Recipes for solutions and buffers are detailed in Appendix II. Human umbilical cord derived OECs were obtained from The University of Manchester Biobank (Approval No 08/H1010/55).

Methods

All cell culture techniques were performed under sterile conditions in a class II microbiological laminar flow safety hood. All protocols had been approved by The Institutional Review Board at the University of Manchester, and informed consent was obtained from all volunteers (Ref No 10/H1011.21) (Appendix III).

2.1. Outgrowth endothelial cell (OEC) isolation and in vitro culture

Peripheral venous blood samples (50mls) were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes from healthy subjects; non-smokers, who were not receiving medication for any clinical diagnosis. OECs were isolated as previously described (Mead et al., 2008), with minor modifications and is illustrated in Figure 2.1. Following dilution with two volumes of phosphate buffer saline (PBS) without magnesium and calcium (Sigma-Aldrich, UK), blood was overlaid onto an equivalent volume of Ficoll-paque PLUS (GE Healthcare, UK). Cells were centrifuged at 740 x g for 40 minutes at room temperature. MNCs were isolated from the interface and a viable cell count was obtained using a haemocytometer and 0.2% trypan blue (Sigma-Aldrich, UK). MNCs were seeded at a density of 3-4 x 10^6 cells per well of a 6-well tissue culture
plate, pre-coated with type I rat tail collagen (50μg/ml) (Becton Dickinson Biosciences, UK) using endothelial growth media (EGM-2; Lonza, Switzerland) supplemented with 10% FBS and EGM-2 SingleQuots; basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), ascorbic acid, epidermal growth factor (EGF), heparin, gentamycin sulphate, amphotericin-B (all from Lonza, Switzerland) and 100U/ml Penicillin/Streptomycin (Invitrogen, UK). After 24 hours, non-adherent cells and debris were aspirated and adherent cells were washed once with media after which 4mls of EGM-2 was added to each well. Medium was changed daily for seven days and every other day thereafter. Cultures were examined regularly by light microscopy using an Olympus CKX41 microscope for the emergence of OEC colonies.
Figure 2.1. A schematic diagram depicting the method of OEC isolation from adult peripheral blood. 50mls of adult peripheral blood was collected in EDTA-containing tubes from healthy volunteers. Mononuclear cells (MNCs) were obtained by density gradient centrifugation, resuspended in endothelial medium supplemented with 10% FBS and plated on 6-well culture dishes pre-coated with rat tail collagen type I at a density of 3-4 x 10^6 cells/well. Non-adherent cells were aspirated and fresh medium added every 24 hours for seven days and every other day thereafter until the emergence of OEC colonies, which typically appeared after 14-21 days of culture.
2.1.1. Maintenance of OECs

Propagation was performed when the culture was 75-90% confluent. Briefly, media was removed and discarded from culture flasks. The attached cell layer was washed with Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich, UK). The wash solution was then removed and discarded. Cells were incubated with pre-warmed Trypsin-EDTA solution (Sigma-Aldrich, UK) (approx. 0.05ml/cm²) and observed under an inverted microscope until the cells begun to round and detach from the culture flask. Growth medium was then added to the culture vessel at a volume double to that of the Trypsin/EDTA in order to quench the reaction. The cell suspension was centrifuged at 220 x g for 3 minutes to pellet the cells. The supernatant was then removed and the pellet re-suspended in warm EGM-2. The cells were counted using a haemocytometer and seeded at a density of 10,000 cells per cm² in tissue culture flasks containing an appropriate volume of pre-warm medium. Medium was removed and replaced with an equal volume of medium 24 hours after passage and every 48 hours thereafter until cultures once again approached confluency. Cultures were incubated in a humidified atmosphere at 37°C, 5% CO₂.

2.1.2. Cryogenic storage of OECs

For cryogenic preservation, cells were trypsinized as described above and pelleted by centrifugation (220 x g for 3 minutes). Cell pellets were re-suspended in Cryo-SFM (Promocell, Germany) and transferred into cryogenic vials (1ml/vial) (Nunc, Thermo Scientific, UK) where they were frozen to -80°C by cooling at a rate of 1°C/minute using a Nalgene 5100 Cryo 1°C freezing container (Thermo Scientific, UK). Once frozen, the samples were transferred to liquid nitrogen for long term storage.

Frozen cells were thawed by placing the cryogenic vial in a 37°C water bath and gently agitating until the cell suspension was 90% thawed. The
cell suspension was agitated so that it was completely thawed and immediately transferred to a culture flask containing pre-warmed medium. After 24 hours, culture medium was replaced with an equal volume of fresh medium to remove the cryoprotectant. Cultures were maintained as described previously.

2.2. Phenotypic characterisation of OECs

2.2.1 Phenotypic characterisation by immunocytochemistry

For immunostaining, OECs were seeded into chamber slides (Thermo Scientific, UK) pre-coated with collagen I and allowed to grow to confluence. Growth medium was aspirated and the cells were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich, UK), for 20 minutes. The fixed cells were then washed three times with PBS to remove all traces of PFA and permeabilised by incubating with 0.1% Triton X-100 (Sigma-Aldrich, UK) in PBS for 10 minutes. Cells were then washed with PBS and non-specific binding sites were blocked by incubating the cells with blocking solution (10% goat Serum (Vector Laboratories, UK), 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich, UK), in PBS) for 30 minutes. Subsequently, OECs were incubated with the respective primary antibodies in blocking solution for 1 hour; mouse monoclonal anti-human CD31 (diluted 1:30) (DAKO, Denmark), VE-CAD (diluted 1:50) (AbCam, UK), vWF (diluted 1:50) (AbCam, UK), fluorescein isothiocyanate (FITC)-conjugated Ulex europaeus agglutinin I (UEA) (diluted 1:200) (Vector Laboratories, UK), rabbit anti-human CD14 (diluted 1:20) (Sigma-Aldrich, UK), CD45 (diluted 1:20) (Sigma-Aldrich, UK) and CD34 (diluted 1:100) (Proteintech Group, UK). After washing in excess PBS, OECs were incubated with TRITC phalloidin, a F-actin stain, (1ug/ml, Sigma-Aldrich, UK) and the corresponding secondary antibody in blocking solution for 45 minutes; goat anti-mouse or goat anti-rabbit conjugated to Alexa Fluor® 488 (diluted 1:200) (Molecular Probes, Invitrogen, UK). Finally, cell nuclei were counter-stained with 4ug/ml 4′,6-diamidino-2-phenylindole
dihydrochloride (DAPI; Sigma-Aldrich, UK), and mounted by covering with a drop of VECTASHIELD® mounting medium (Vector Laboratories, UK) over which a coverslip was carefully placed. Fluorescent images were captured using a Leica DM5000B microscope and Leica DFC320 colour camera, running Leica Application Suite software (version 3.7, Leica, UK). All incubations were performed at room temperature.

2.2.2. Assessment of cellular uptake of acetylated low density lipoprotein (Ac-LDL)

OECs were examined for the ability to incorporate 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil)-labelled acetylated low density lipoprotein (Ac-LDL) (Biomedical Technologies, USA) according to a method described by Ingram et al. (2004). OECs were incubated with Dil-Ac-LDL (10 μg/ml) in EGM-2 at 37°C for 4 hours and then fixed with PFA for 20 minutes. After washing three times with PBS, the cells were stained with 4μg/ml of DAPI. Cells were then mounted by covering with a drop of VECTASHIELD® mounting medium (Vector Laboratories, UK) over which a coverslip was carefully placed and examined by fluorescence microscopy (Leica DM5000B).

2.2.3. Phenotypic characterisation by flow cytometric analysis

To analyse cell phenotype using flow cytometry, OECs were trypsinized and resuspended at a density of 1 x 10^5 per sample in 100μl of FACs buffer (2% FBS in PBS). Cells were incubated at 4°C for 10 minutes with an Fc blocking agent (Miltenyi Biotec, Germany) to saturate non-specific antibody binding sites. Subsequently, OECs were incubated with the respective antibodies for 30 minutes at 4°C; FITC-conjugated anti-human CD34 (Miltenyi Biotec, Germany), FITC-conjugated anti human CD146 (Miltenyi biotec, Germany), allophycocyanin (APC)-conjugated anti-human CD31 (Miltenyi biotec, Germany), phycoerythrin (PE)-conjugated anti-human VEGFR-2 (R&D Systems, UK) and vioblue-conjugated anti-
human CD45 (Miltenyi biotec, Germany). Cells were then washed in PBS and incubated with 7-amino-actinomycin D (7-AAD) (Sigma-Aldrich, UK) a DNA intercalater which is excluded by viable cells. Finally, OECs were washed with PBS and resuspended in 500ul of FACs buffer for analysis which was performed using a Beckman Coulter Cyan ADP flow cytometer (Beckman Coulter Inc., USA) with Summit V4.3 software. At least 20,000 events were collected for each sample. Respective isotype controls were used to determine the settings for data analysis. This method was adapted from that described by Ingram et al. (2004).

2.2.4. Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted from cultures of OECs to examine the expression of a number of characteristic endothelial and hematopoietic lineage genes.

2.2.4.1. RNA isolation

To extract total RNA from cultured OECs, cell monolayers were lysed directly in a culture flask by adding 1ml of TRIzol reagent (Invitrogen, UK) and incubating for 5 minutes at room temperature. 0.2ml of 1-bromo-2 chloropropane (BCP) (Sigma-Aldrich, UK) was then added to each sample followed by vigorously shaking for 15 seconds and incubation at room temperature for 3 minutes. Samples were then centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was removed and transferred to a clean microcentrifuge tube. RNA was precipitated from the aqueous phase by mixing with 250μl of isopropanol and incubating at room temperature for 10 minutes. GlycoBlue (2μl) (Ambion, UK) was also added to the samples for use as a visible coprecipitant for nucleic acids. Subsequently, samples were centrifuged at 12,000 x g for 20 minutes at 4°C, after which the supernatant was removed. The RNA pellet was washed with 1ml of 75% ethanol and centrifuged at 12,000 x g for 5 minutes at 4°C. Finally, RNA was air-dried,
resuspended in Tris-EDTA (TE) buffer (Ambion, UK) and the concentration determined by measuring the absorbance at 260nm using a NanoDrop spectrophotometer (ND-1000).

2.2.4.2. DNase treatment

Genomic DNA, contaminating RNA preparations, may serve as a template in PCR to produce false positive signals. To overcome this problem, RNA samples were treated with DNase I, an endonuclease that digests single- and double-stranded DNA, according to the manufacturer’s instructions (Ambion, UK) prior to reverse transcription. Briefly, 10X DNase buffer to a 1X concentration and 4U DNase I enzyme (all from Ambion, UK) were added to the RNA preparations containing up to 10ug nucleic acid per 50μl final volume and incubated at 37°C for 30 minutes. The DNase I was then inactivated by phenol:chloroform extraction. Briefly, RNA preparations were made up to a final volume of 100μl with RNase-free water, before an equal volume of phenol:chloroform:1AA (Ambion, UK) was added to the sample and vortexed to an emulsion. Samples were then centrifuged at 12,000 x g for 5 minutes at 4°C after which the aqueous phase was removed and transferred to a clean microcentrifuge. The RNA was precipitated by adding 10μl of ammonium acetate and 300μl ethanol, plus GlycoBlue as a co-precipitant. The samples were inverted several times to thoroughly mix and then incubated on ice for 30 minutes. Subsequently, the samples were centrifuged at 12,000 x g for 20 minutes at 4°C after which the supernatant was removed. The RNA pellet was washed with 1ml of 75% ethanol and centrifuged at 12,000 x g for 5 minutes at 4°C. Finally, RNA was air-dried, resuspended in TE Buffer and the concentration determined using a NanoDrop spectrophotometer (ND-1000).

2.2.4.3. Reverse transcription of DNase treated RNA

To generate cDNA, DNase-treated RNA (1μg) was reverse transcribed using a Precision nanoScript Reverse transcription kit (PrimerDesign Ltd,
UK) according to the manufacturer’s instructions. Briefly, for the annealing step, RNA samples were incubated with oligo-dT and random nonamer primers at 65°C for 5 minutes after which they were immediately transferred onto ice. For the extension step, nanoScript 10X buffer, dNTP mix (10mM of each), DTT (100mM) and nanoScript reverse transcriptase enzyme, in a total volume of 10μl, was added to the RNA samples. After briefly vortexing, the samples were incubated at 25°C for 5 minutes and then at 55°C for 20 minutes. Finally, the reaction was heat inactivated by incubation at 75°C for 15 minutes and cDNA samples were stored at -20°C until use.

2.2.4.4. RT-PCR

Conventional RT-PCR was performed in a 20μl reaction volume containing: cDNA (50ng), 10pmol/μl forward and reverse primers designed for the particular gene of interest (Eurofins MWG Operon, Germany) and 2x BioMix Red containing; Taq DNA polymerase, dNTPs and an inert red dye for direct gel loading (Bioline, UK). The primers used are listed in Table 2. PCR was performed for 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing for 30 seconds and extension at 72°C for 30 seconds. The PCR products were resolved by electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide (Sigma-Aldrich, UK) staining.
Table 2. Details of primer sequences used for conventional RT-PCR and quantitative real time RT-PCR.

<table>
<thead>
<tr>
<th>Conventional RT-PCR Primers (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
</tr>
<tr>
<td>Sense GACCTGACTGACTACCTCATGA;</td>
</tr>
<tr>
<td>anti-sense AGCATTTGCGGTGGACGATGGAG</td>
</tr>
<tr>
<td>CD14</td>
</tr>
<tr>
<td>Sense CGGCGGTGTCACACCTAGAG;</td>
</tr>
<tr>
<td>anti-sense GCCTACCAGTAGCTGAGCAG</td>
</tr>
<tr>
<td>CD45</td>
</tr>
<tr>
<td>Sense ACAGCCAGCACCCTTCTAC;</td>
</tr>
<tr>
<td>anti-sense GTGCAGGTAAGGCAGCAGA</td>
</tr>
<tr>
<td>VEGFR2</td>
</tr>
<tr>
<td>Sense AGTGATCGGAAATGACACTGGA;</td>
</tr>
<tr>
<td>anti-sense GCACAAAGTGACACGTGAGAT</td>
</tr>
<tr>
<td>vWF</td>
</tr>
<tr>
<td>Sense ACATCACTGCCAGGCTGCAGTA;</td>
</tr>
<tr>
<td>anti-sense CACAAGAGCAGAACATGCAGAG</td>
</tr>
<tr>
<td>VECAD</td>
</tr>
<tr>
<td>Sense ACCGGATGACCAAGTACGC</td>
</tr>
<tr>
<td>anti-sense ACACACTTTGCGCTGGTAGG</td>
</tr>
<tr>
<td>CD34</td>
</tr>
<tr>
<td>Sense CCTCCCAAGTTTTAGGACAA;</td>
</tr>
<tr>
<td>anti-sense CAGCTGTGTGATAAGGGTGG</td>
</tr>
<tr>
<td>CD31</td>
</tr>
<tr>
<td>Sense CAACAGACATGGCAACAAGG</td>
</tr>
<tr>
<td>anti-sense TCCTGGATGGAAGTGGGC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quantitative real time RT-PCR Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4</td>
</tr>
<tr>
<td>Sense CCAAAGAAGGATATAATGAAGTCACT;</td>
</tr>
<tr>
<td>anti-sense GGGCTAAGGGCACAAGAGA</td>
</tr>
<tr>
<td>VEGFR-2</td>
</tr>
<tr>
<td>Sense AAGGAGTCCTGTGGAATCTG;</td>
</tr>
<tr>
<td>anti-sense GTGTCTGTCTCAGGGGAGT</td>
</tr>
<tr>
<td>SULF-1</td>
</tr>
<tr>
<td>Sense AGTTGAGAGGATCTGGTACC</td>
</tr>
<tr>
<td>anti-sense CGTGCTGTGCGAGATAGGA</td>
</tr>
<tr>
<td>SULF-2</td>
</tr>
<tr>
<td>Sense TGAGGACCAAGATGCAACAAGGA</td>
</tr>
<tr>
<td>anti-sense TCTAGGATGTAAGCACCAGATGT</td>
</tr>
</tbody>
</table>
2.2.5. Reverse transcription quantitative real time RT-PCR

Reverse transcription quantitative real-time-PCR (RT-qPCR) was performed to quantify the expression of CXCR4, VEGFR-2, SULF-1 and SULF-2 by OECs.

The PCR products were detected using the fluorescent dye SYBR Green which binds to double stranded DNA to produce a fluorescent signal (Figure 2.2). The intensity of the signal increases with increasing cycle sequences due to the accumulation of the amplified product. The quantitative endpoint for RT-qPCR is the threshold cycle (C_T), which is defined as the PCR cycle at which the fluorescent signal emitted by the reporter dye crosses an arbitrarily placed threshold. The numerical value of the C_T is inversely related to the amount of amplicon in the reaction (i.e. the lower the C_T value, the greater the amount of amplicon).

![SYBR Green-based detection of PCR products in RT-qPCR](image)

Figure 2.2. Schematic diagram illustrating the principle of SYBR-green based detection of PCR products in RT-qPCR. During annealing, PCR primers hybridise to the target to form a region of double-stranded DNA where SYBR green intercalates to produce a fluorescence signal. At the start of the reaction, very little double-stranded DNA is present and thus the fluorescence signal detected by the thermocycler is low. As the reaction proceeds, the amount of double-stranded DNA increases and with it the fluorescence signal as SYBR-green binds all newly synthesised DNA complexes and fluoresces. The fluorescence accumulates as cycling of
PCR continues and is measured at the end of each cycle. The intensity of fluorescence generated by SYBR-green above the threshold value (CT value) is measured and used to quantitate the amount of newly synthesised double-stranded DNA.

RNA was extracted from OECs, DNase treated and converted to cDNA by reverse transcription as described previously. cDNA samples were diluted to a final concentration of 10ng/μl with RNase-free water. RT-qPCR was performed in a 20μl reaction volume containing; cDNA (25ng), primers designed for the particular gene of interest (6pmols forward and reverse) and Precision Mastermix with ROX and SYBR gene (2x reaction buffer, 0.025U/μl Taq polymerase, 5mM MgCl₂) (all PrimerDesign Ltd, UK). The primers used were designed and synthesised by PrimerDesign Ltd (as detailed in Table 2). ATP5B and GAPDH were used as reference genes after validation using a human endogenous control array (Applied Biosystems, UK). The ATP5B and GAPDH primer sequences from PrimerDesign Ltd are not available for publication. Samples were run in triplicate alongside no template controls on a 7900 HT Fast Real-Time PCR system (Applied Biosystems) using the following amplification conditions:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Activation</td>
<td>10min</td>
<td>95°C</td>
<td>x1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>15secs</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60secs</td>
<td>60°C</td>
<td>Cycling x40</td>
</tr>
<tr>
<td>Extension</td>
<td>60secs</td>
<td>60°C</td>
<td></td>
</tr>
</tbody>
</table>

Data were normalized to the endogenous control genes ATP5B and GAPDH, and the fold change in gene expression level was calculated using ΔCT (Cₜ Target - Cₜ Reference).
2.3. Functional Analysis of OECs

For all functional analysis media without supplemented heparin was used.

2.3.1. Cell Proliferation

Two methods were used to assess OEC proliferation;

2.3.1.1. Cell Counting

OECs were trypsinized and a viable cell count determined using a haemocytometer and 0.2% trypan blue. Subsequently, 500µl of cell suspension containing 10,000 cells was added per well of a 24 well plate containing a 13mm coverslip (VWR International, UK) in triplicate and allowed to recover. Following an overnight incubation in 1% FBS to allow the cells to become quiescent, the medium was replaced with EGM-2 10% FBS. The triplicate wells of each sample were immediately fixed in 5% trichloroacetic acetic for 20 minutes at room temperature to give a baseline reading. The remaining cells were fixed after a further 24, 48 and 72 hours of culture. Cells were counterstained with Harris haematoxylin (Sigma-Aldrich, UK) which stains the cell nuclei blue, rinsed with running water and then mounted on glass microscope slides using DPX mounting media (Fischer Scientific, UK). The number of cells was determined by counting three random fields of view per coverslip using a Leica DM5000B microscope (×100 magnification) and Leica DFC320 colour camera, running Leica Application Suite software (version 3.7, Leica, UK). Images were processed using the cell counter tool of Image J software (version 1.43, National Institutes of Health, USA). Cell proliferation was expressed as the fold change in cell number relative to the baseline.

2.3.1.2. MTT assay

Mitochondrial activity was measured as a means of assessing the number of viable cells using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl
tetrazolium bromide (MTT) assay according to the manufacturer’s instructions (Roche Diagnostics, UK). This is a colorimetric assay that measures the reduction of yellow tetrazolium salt (MTT) by mitochondrial succinate dehydrogenase to formazan, an insoluble purple product. The amount of colour produced is directly proportional to the number of viable cells. To measure cell proliferation, OECs were seeded at a density of 3,200 cells per well of a 96 well plate in triplicate and allowed to recover. Following an overnight incubation in 1% FBS, cells were incubated with MTT reagent in 100µl of EGM-2 medium to a final concentration of 0.5mg/ml for 4 hours at 37°C. Subsequently, 100µl of solubilization solution was added to and incubated overnight at 37°C. The released formazan product was measured at a wavelength of 570nm using an ELx800 absorbance microplate reader running Gen5 Software (BioTek Instruments, USA). Background absorbance was also measured at a wavelength of 750nm which was subtracted from the 570nm reading.

2.3.2. Apoptosis assay

2.3.2.1 Detection of cleaved caspase-3 protein

Caspase-3 is a critical initiator of cell apoptosis as it is responsible for the proteolytic cleavage and activation of other caspases, as well as, targets such as poly (ADP-ribose) polymerase (PARP) and DNA fragmentation factor (DFF), resulting in cellular instability, degradation and an irreversible commitment towards apoptotic cell death. Therefore, caspase-3 activation is considered a reliable marker of cells undergoing apoptosis. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments. OEC apoptosis under normal growth conditions (EGM-2 10% FBS), growth factor and serum deprivation (EBM-2 1% FBS) and staurosporine (25nM for 4 hours at 37°C) induced apoptosis, was analysed by the detection of endogenous levels of cleaved caspase-3 protein using a solid phase enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Cell Signaling Technology, USA). To prepare
cell lysates, OECs cultured in 25cm² flasks were washed twice with ice cold PBS and lysed by incubating in 1x cell lysis buffer (Cell Signaling Technology, USA) plus 1mM phenyl-methylsulfonyl fluoride (PMSF) for 5 minutes on ice. Subsequently, cells were removed from the tissue culture flask using a cell scraper and transferred to a microcentrifuge tube. Lysates were briefly sonicated on ice and then centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatant was transferred to a clean microcentrifuge tube and stored at -80°C until use. 100μl of cell lysate, at a concentration of 0.3mg/ml, was mixed with 100μl of sample diluent and incubated overnight at 4°C in caspase-3 antibody coated microwell strips. Following extensive washing with 1x wash buffer (Cell Signaling Technology, USA), 100μl of biotinylated cleaved caspase-3 detection antibody was added to each well and incubated for 1 hour at 37°C. After extensive washing with 1x wash buffer, detection of the bound antibody was achieved by incubating with 100μl of horseradish peroxidase-linked streptavidin antibody for 30 minutes at 37°C. Finally, the wash procedure was repeated and 100μl of 3,3', 5,5"-tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 10 minutes at 37°C. After adding 100μl of STOP solution to each well, the coloured reaction product was measured at 450nm (Elx800 Biotek instruments, USA). The magnitude of optical density for the developed colour is proportional to the quantity of cleaved caspase-3 protein.

2.3.2.2. Determination of protein concentration

Total protein concentration in the lysates was quantified with the use of a detergent-compatible bicinchoninic acid (BCA) kit (PN23225, Thermo Scientific, UK) following the manufacturer’s instructions. This assay is based on the reduction of Cu²⁺ to Cu¹⁺ ions by protein in an alkaline medium. BCA reacts with the reduced cation to produce a purple-coloured reaction product that can be quantified spectrophotometrically. The amount of Cu²⁺ reduced is proportional to the amount of protein present in the solution. A series of BSA standards of known concentration
(2.0mg/ml, 1.5mg/ml, 1.0mg/ml, 0.75mg/ml, 0.5mg/ml, 0.25mg/ml and 0.125mg/ml) were prepared in 1 x cell lysis buffer. Cell lysis buffer alone was used for blank measurements. BSA standards and samples were loaded in triplicate onto a 96 well plate and incubated for 30 minutes at 37°C with BCA working reagent (50:1, BCA Reagent A:B). Absorbance was measured at 570nm on an ELx800 absorbance microplate reader running Gen5 Software (BioTek Instruments, USA). The protein concentration of OEC lysates was determined by comparing their absorbance value to a standard curve created from the 570nm measurements of each BSA standard vs its concentration in µg/ml.

2.3.3. In vitro tube formation assay

The Matrigel assay has been extensively used to study the tube forming potential of endothelial cells. Matrigel (Becton Dickinson, UK) was thawed at 4°C overnight and spread evenly over each well (10µl) of a µ–slide angiogenesis (IBIDI, UK). The plates were incubated at 37°C for 30 minutes to allow the Matrigel to polymerise. OECs were seeded in triplicate at a density of 4,000 cells per well in a total volume of 50µl of EGM-2. Plates were incubated at 37°C for 14 hours and examined for tube-like formation using an inverted phase-contrast light microscope (Olympus CKX41). This method was adapted from that initially described by Mead et al. (2008). Images were captured at x4 magnification and processed using ImageJ software. (http://rsb.info.nih.gov/ij). The main processing steps applied to the images include background subtraction, geometrical filtering and binary thinning procedures to generate a skeleton of the image. From this image a number of morphological features were measured to characterise the capillary-like network including; the total length of the network, the number of closed loops, the number of branching points and the number of junctions.
2.3.4. *in vitro* scratch assay

The migratory capacity of OECs was determined using an *in vitro* scratch assay in which the cells migrate into an area that has been mechanically denuded of cells, according to a method adapted from Liang et al. (2007). OECs were seeded in triplicate at a density of 20,000 cells per well of a 24 well tissue culture plate pre-coated with collagen I and allowed to grow to confluence. Following an overnight incubation in 1% FBS to become quiescent, a scratch wound was inflicted using a p200 pipette tip. The medium was aspirated to remove the debris and cell monolayers were washed twice with PBS, after which 500µl of fresh EGM-2 10% FBS medium was added to each well. The migration of OECs from the wound edge into the cell free area was documented by time-lapse microscopy on an inverted microscope (Leica AS MDW, Leica, UK) at initial wounding and at 2 hour intervals over a 24 hour period. Two fields of view were captured per well during image acquisition. The wound area was calculated by tracing along the border of the wound using ImageJ software analysis. The rate of migration was determined by calculating the percentage of wound closure, as calculated used the following equation:

\[
\left( \frac{\text{Wound area (0hrs)} - \text{Wound area (xhrs)}}{\text{Wound area (0hrs)}} \right) \times 100 = \% \text{ wound closure}
\]

2.3.5 *Oris*™ cell migration assay

OEC migration was also evaluated using the *Oris*™ cell migration assay (CMA) (Platypus Technologies, USA) in which the cells migrate into circular cell-free regions of uniform size. OECs were seeded in triplicate at a density of 10,000 cells per well of an *Oris*™ cell migration collagen I coated 96 well plate containing silicone stoppers and allowed to recover. Following an overnight incubation in 1% FBS to become quiescent, the stoppers were removed and the cells were rinsed twice with PBS, after
which 200µl of fresh EGM-2 10% FBS medium was added to each well. The migration of OECs into the cell free area was documented by time-lapse microscopy on an inverted microscope (Leica AS MDW, Leica, UK) at the pre-migration stage and at 2 hour intervals over a 24 hour period. The rate of migration was determined as described above.

2.3.6. Transwell migration assay

OEC migration towards a chemotactic gradient was carried out in a 24-well, 8µm pore collagen-coated transwell system (Costar® Corning, UK) according to a method adapted from that described by Heiss et al (2008). Prior to seeding in the transwell system, OECs were maintained in EBM-2 containing 1% FBS overnight to become quiescent. OECs were detached from the culture flask using trypsin-EDTA (incubation times were less than 90 seconds) pelleted by centrifugation and seeded at a density of 2×10^4 cells/ml in 100µl of EBM-2 1% FBS in the upper chamber of the transwell assembly. The lower chamber contained 650µl of EBM-2 1% FBS medium with 100ng/ml SDF-1α (Miltenyi Biotech, Germany) or 50ng/ml VEGF-A165 (NIBSC, UK). Wells containing EBM-2 1% FBS alone were used as control samples. After incubation at 37°C and 5% CO₂ for 6 hours, the upper surface of the membrane was gently scraped to remove non-migrating cells and washed with PBS. Migrated cells on the under-side of the membrane were then fixed in ice cold methanol for 15 minutes, washed in PBS, stained in Harris haematoxylin for 5 minutes and finally rinsed clear in water. The number of migrating cells was determined by counting five random fields of view per insert using a Leica DM5000B microscope (×100 magnification) and Leica DFC320 colour camera, running Leica Application Suite software (version 3.7, Leica, UK). Images were processed using the cell counter tool of Image J software. Migration experiments were always performed in duplicate.
2.4. Disaccharide compositional analysis of OEC HS

2.4.1. HS extraction and purification

For disaccharide analysis confluent monolayers of OECs were gently removed from tissue culture flasks with a cell scraper. The cell suspension was then transferred to a 15ml falcon tube and pelleted by centrifugation for 10 min at 16,000 x g. Protease type XIV (final concentration 1mg/ml) (P1547, Sigma-Aldrich, UK) and TritonX-100 (1%) (T8787, Sigma-Aldrich, UK) were added to the cell extract to liberate glycosaminoglycan chains from the core protein. The sample was incubated in a shaking waterbath at 55°C overnight. Following this incubation, the sample was heated to 96°C for 5 minutes to inactivate the protease. To ensure removal of nucleic acids, the sample was incubated with 2mM MgCl$_2$ and benzonase (E-8263, Sigma-Aldrich, UK) (final concentration 125IU) for 2 hours at 37°C. Benzonase was then heat inactivated for 2 minutes at 96°C before the addition of NaCl (Sigma-Aldrich, UK) to a final concentration of 0.1M. The sample was then centrifuged at maximum speed for 10 minutes to pellet any insoluble material. The supernatant was retained and loaded on a Vivapure Mini D spin filter (Diethylaminoethyl-DEAE) (Sartorius AG, Germany), which had been equilibrated in a low salt buffer; 50mM sodium phosphate buffer, 0.15M NaCl, pH6 (loading buffer). Following centrifugation for 1 minute at 500 x g, the eluate was reloaded onto the same filter and centrifuged again. This step was repeated once more, after which the eluate was discarded. The DEAE column was then washed to remove any weakly bound proteins by applying 400µl of loading buffer to the column and centrifuging for 1 minute at 500 x g. The eluate was discarded following this centrifugation step. Highly negatively charged HS was eluted from the column with 400µl of a high salt buffer; 50mM sodium phosphate buffer, 1M NaCl, pH 6 (elution buffer). The eluate was retained and loaded onto an Amicon Ultra 3K centrifugal filter (Millipore, Ireland) which was spun at 14,000 x g until the final retentate was <30µl. 50µl of Milli-Q water was then loaded, in total four times, onto the filter which was spun
to <30μl after each addition. Subsequently, the retentate was removed and the membrane of the filter was washed a total of four times with Milli-Q water to recover any remaining HS. The retentate and washes were pooled and loaded onto a PD-10 desalting column (Amersham Biosciences, UK) equilibrated in Milli-Q water to remove low molecular weight decontaminants. HS was then eluted from the column with 0.5ml fractions of Milli-Q water. After collecting and pooling the fractions (6-8) containing HS, the sample (a total of 1.5ml) was dried in a Christ RVC 2-25 rotational vacuum concentrator (Martin Christ, Germany) maintained at 55°C for approximately 4 hours.

2.4.2. Degradation of HS into constituent disaccharides

HS chains were digested overnight at 32°C with 0.33mIU heparinase I, 0.33mIU heparinase II and 0.33mIU heparinase III (Seikagaku America, USA) in a buffer of 0.1M sodium acetate (Sigma-Aldrich, UK), 10mM calcium acetate (Sigma-Aldrich, UK). This overnight incubation was performed twice to ensure complete digestion of the HS chains.

Following HS extraction from cultured OECs and degradation into constituent disaccharides by heparinase digestion, three fluorophores; 2-cyanoacetamide (2-CA), boron-dipyrromethene (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, BODIPY FL) hydrazide, and 2-Aminoacridone (AMAC) were compared to determine the most sensitive and accurate analytical method for disaccharide compositional analysis of OEC-derived HS.

All buffers and solutions used for high performance liquid chromatography (HPLC) were filtered prior to use.
2.4.3. Strong anion exchange (SAX)-HPLC analysis of 2-CA labelled HS disaccharides.

OEC-derived HS disaccharides were loaded onto a ProPacA1 analytical column (Dionex, USA), equilibrated with Milli-Q water acidified to pH 3.5 with HCl (Buffer A), running on an Agilent 1100 series high performance liquid chromatogram system (Agilent Technologies, UK). Disaccharides were eluted from the column with a gradient of NaCl (HPLC grade, Fluka, UK) in MilliQ water, pH 3.5 (Buffer B), that linearly increased to a final concentration of 1M over a period of 45 minutes at a flow rate of 1ml/min. Separated disaccharides were fluorescently labelled by mixing with equal proportions of 0.5% 2-cyanoacetamide (HPLC grade, Fluka, UK) (Buffer C) and 0.25M NaOH (HPLC grade, Sigma-Aldrich, UK) that were dispensed at a flow rate of 0.45ml/min and reacted at 122°C in a post column reactor (CRX400, Pickering laboratories, USA). Samples were cooled in-line in a cooling tower maintained at approximately 10°C and was detected by in line fluorescence at excitation 346nm and 410nm emission. Disaccharides were identified and calibrated by comparison to 2-CA labelled HS standards of known concentration (0.96nmol) and analysed using ChemStation software (Rev.B.04.01, Agilent Technologies, UK). Following disaccharide elution, the column was washed for 10 minutes in 100% Buffer B and then for 15 minutes in 100% Buffer A.

2.4.4. BODIPY derivatisation and HPLC analysis of HS disaccharides

2.4.4.1. BODIPY derivatisation of HS disaccharides

BODIPY labelling of HS disaccharides was performed as described by (Skidmore et al., 2006). Here, HS samples were re-suspended in 10μl BODIPY® FL hydrazide (D-2371, Invitrogen, UK) in methanol (5mg/ml), and dried by centrifugal evaporation. Subsequently, samples were re-suspended in 10μl 85% DMSO; 15% acetic acid and incubated at room
temperature in the dark for 4 hours. After this time, 10μl of 1M sodium borohydride was added to each of the samples and incubated at room temperature in the dark for 30 minutes. The samples were then flash frozen in liquid nitrogen and lyophilised by freeze drying. Finally, the samples were re-suspended in 20μl 50% DMSO and analysed by strong anion exchange (SAX)-HPLC immediately, or stored at -80°C until analysis.

2.4.4.2. SAX-HPLC analysis of BODIPY labelled HS disaccharides

BODIPY labelled HS disaccharides were analysed by SAX-HPLC according to the method described by (Skidmore et al., 2006). Samples were loaded onto a ProPac PA1 analytical column (Dionex, USA) equilibrated with 150mM NaOH running on an Agilent 1100 series high performance liquid chromatogram system (Agilent Technologies, UK). Excess free BODIPY label was eluted by an 11 minute isocratic wash with 150mM NaOH after which disaccharides were eluted from the column with a linear gradient of NaCl (HPLC grade, Fluka, UK) from 0 to 1M over 30 minutes. HS disaccharides were detected by in line fluorescence at excitation 488nm, 520nm emission. Disaccharides were identified and calibrated by comparison to BODIPY-labelled HS standards of known concentration (0.16nmol) and analysed using ChemStation software (Rev.B.04.01, Agilent Technologies, UK). Following disaccharide elution, the column was washed with 1M NaCl for 3 minutes and then 2M NaCl for 3 minutes, both in the presence of 150mM NaOH.
2.4.5. AMAC derivatisation and HPLC analysis of HS disaccharides

2.4.5.1. Cleanup of AMAC label

Commercial AMAC (Sigma-Aldrich, UK) contains an impurity that can affect HPLC analysis. However, it is possible to remove the majority of this impurity by solid phase extraction chromatography according to the method described by Deakin and Lyon, 2008. Briefly, 1mg AMAC was dissolved in 100µl 85% DMSO; 15% acetic acid and loaded onto a Macro-prep t-butyl HIC column (1ml bed volume, Bio-Rad, UK) equilibrated in Milli-Q water. The column was then washed with 10mls of 20% methanol to remove non-specifically interacting contaminants. Subsequently, the AMAC label was step eluted with 1ml fractions of 100% methanol. Finally, the solution was freeze dried and the lyophilised AMAC was re-suspended to a final concentration of 0.1M in 85%DMSO;15% acetic acid.

2.4.5.2. AMAC derivatisation of HS disaccharides

AMAC derivatisation of HS disaccharides was performed as described by Deakin and Lyon, 2008. Following heparinase digestion, the sample was dried on a centrifugal evaporator, re-suspended in 5µl of 0.1M AMAC in 85% DMSO; 15% acetic acid and incubated at room temperature in the dark for 20 minutes. 5µl of 1M sodium cyanborohydride (Sigma-Aldrich, UK) was then added to the sample and incubated overnight at room temperature in the dark.

2.4.5.3. Reverse Phase (RP)-HPLC analysis of AMAC labelled HS disaccharides.

After labelling the HS disaccharides overnight with AMAC the sample was analysed by reverse phase (RP)-HPLC with modifications to the method described by (Deakin and Lyon, 2008), namely the use of a Kinetex
column coupled with acetonitrile as an eluting solvent (as determined by Hamilton and Stringer unpublished data). AMAC labelled disaccharides were loaded onto a Kinetex C18 HPLC column (100 x 4.6mm; Phenomenex, UK) equilibrated in 0.1M ammonium acetate (Buffer A); 5% acetonitrile (Buffer B) running on an Agilent 1100 series high performance liquid chromatogram system (Agilent Technologies, UK). Disaccharides were eluted from the column at a flow rate of 1ml/min using a gradient of acetonitrile that linearly increased from 5% to 20%, over a period of 15 minutes. Disaccharides were detected in-line at 425nm excitation; 520nm emission. HS disaccharides were identified and calibrated by comparison to AMAC-labelled HS standards of known concentration (0.12nmol) and analysed using ChemStation software (Rev.B.04.01, Agilent Technologies, UK). Following disaccharide elution, the column was washed with 100% Buffer B for 10 minutes and then re-equilibrated in 95% Buffer A; 5% Buffer B for 10 minutes.

2.5. Determination of the molecular size of HS

2.5.1. Metabolic radiolabelling of HS

Sub-confluent (70-80%) cultures of OECs were incubated with EGM-2 10% FBS containing 10µCi/ml tritiated glucosamine hydrochloride d-[6-\(^{3}\)H(N)] (Perkin Elmer, UK) for 24 hours to metabolically label HS. Cells were then scraped from the culture flasks and HS was extracted as previously described.

2.5.2. Sodium borohydride treatment

After HS extraction and following drying of the samples on a rotational vacuum concentrator, HS chains were released from the core protein by alkaline sodium borohydride treatment. Briefly, 1ml of 1M sodium borohydride in the presence of 50mM NaOH was added to the HS sample and incubated at room temperature overnight. 5µl of phenol red
was then added to provide an indication of the pH of the sample. The solution was then neutralised by the drop-wise addition of glacial acetic acid, as determined by comparison to an equivalent volume of Milli-Q water containing 5µl of phenol red. Finally, the sample was desalted on a PD-10 column as previously described. The radioactivity, and thus HS content, was determined by removing 10µl of the sample and mixing with 3ml of Optiphase Hisafe 2 scintillation fluid (Perkin-Elmer, UK) and analysing on a Packard Tri-Carb 2500TR liquid scintillation analyser (Canberra, USA).

2.5.3. CL-6B gel filtration chromatography

To determine the approximate size the metabolically radio-labelled HS fragments, the HS sample (20kdpm) was mixed with 50µl of 100mg/ml dextran blue and 5µl of 50mg/ml phenol red and loaded onto a Sepharose CL-6B column (approximately 70ml bed volume) equilibrated in 10mM Tris; 0.2M NaCl and run at 4ml/hour using a Pharmacia P-1 peristaltic pump (GE Healthcare, UK). 1ml fractions were collected in polypropylene scintillation vials using an Amersham SuperFrac™ fraction collector (GE Healthcare, UK) and the HS content determined by liquid scintillation counting. The avidity constant (K_{av}) of the resolved fragment was calculated using the formula; \( K_{av} = \frac{(\text{peak} - V_o)}{(V_t - V_o)} \), where \( V_o \) is the void volume (as indicated by dextran blue), \( V_t \) is the total column volume (as indicated by phenol red) and \( x \) is the volume which contains the greatest HS content. The antilog of the \( K_{av} \) value was calculated and from this, the molecular weight of the HS fragment was determined by comparison to the calibration curve of Wasteson 1971.

2.6. Heparinase treatment of OECs

A combination of heparinase I and heparinase III was used to cleave HS on the OEC cell surface. Briefly, OECs were treated in suspension with 10mlU/ml of heparinase I and heparinase III for 30mins at 37°C. Cells
were then washed extensively with PBS, counted and seeded into transwell assays as described. Cell migration was expressed as the fold increase relative to untreated cells (in buffer alone).

2.6.1. Analysis of HSPG disruption following Heparinase treatment

To verify the removal of HS from the cell surface, OECs were grown on chamber slides until confluent and then treated with a combination of 10mIU/ml of heparinase I and heparinase III for 30mins at 37°C. Control cells which had not been enzymatically treated (in buffer alone) were run in parallel. The cells were then washed extensively in PBS and fixed in 4% PFA for 20 minutes at room temperature. After blocking non-specific binding sites with 10% goat serum, 1% BSA in PBS for 30 minutes, both untreated and Hep treated OECs were incubated with a HS specific antibody (10E4; dilution 1:100) (Seikagaku America, USA) for 1 hour. After washing in excess PBS, OECs were incubated with TRITC phalloidin (1ug/ml) and a goat anti-mouse Alexa Fluor® 488 (diluted 1:200) for 45 minutes. Finally the cells were washed, and mounted in Vectashield containing DAPI. Fluorescent images were captured using a Leica DM5000B microscope and Leica DFC320 colour camera, running Leica Application Suite software (version 3.7, Leica, UK). The fluorescence intensity of the untreated and treated images were compared to confirm HS removal following heparinase treatment.

2.7. Statistical Analysis

All data are expressed as mean values ± standard error of mean (SEM). The Kruskal-Wallis test was used for an overall comparison between the three groups. Pair-wise comparisons were analysed by Mann Whitney U tests; p=<0.05 (2-tailed) was considered significant. Pearson’s correlation coefficients were used for correlation analysis. All statistical analysis was performed using SPSS 16.0 software (IBM).
Chapter 3:

Results I - Isolation and Characterisation of Outgrowth endothelial cells (OECs)
3. Isolation and characterisation of outgrowth endothelial cells (OECs)

3.1. Introduction

The longstanding view for new blood vessel formation and repair in post-natal life is that it proceeds via the well-recognised and extensively studied processes of angiogenesis and arteriogenesis. However, Asahara et al. presented evidence to consider a new paradigm for the process of neovascularization in the form of post-natal vasculogenesis (Asahara et al., 1997). Asahara and colleagues isolated a population of cells from human peripheral blood by magnetic bead selection on the basis of cell surface antigen expression and demonstrated that these cells could be cultured in vitro to yield cells with endothelial cell characteristics. Using mouse and rabbit models of hindlimb ischemia, they went on to demonstrate that these cells incorporated into the newly formed blood vessels around the site of injury. This discovery radically altered the predominant paradigm for new blood vessel formation in postnatal life to account for the contribution of bone-marrow derived EPCs.

The possibility of inducing new blood vessel formation and/or repairing damaged vessels by delivering/recruiting EPCs is an attractive idea that has stimulated much interest among the scientific community. Indeed, since the seminal article by Asahara et al. interest in circulating EPCs has soared, and one merely has to type the keyword search terms, endothelial progenitor cell, to retrieve more than 11,404 articles including 1,942 review articles in PubMed. However, contradictory findings regarding the origin, identification and contribution of EPCs to the process of neovascularization has generated much debate within the field. The controversy largely appears to be due to differences in the methodology used to isolate and culture EPCs, together with a lack of characterisation of the cells described in many of the reports. Consequently, a host of different cell types have been somewhat ambiguously included within the term EPC and, unsurprisingly, have demonstrated a mixed ability to contribute to blood vessel formation and repair. It is now recognised that
essentially all of the cells originally identified as EPC are in fact hematopoietic lineage cells that display proangiogenic properties. The exception is a rare population of cells, termed outgrowth endothelial cells (OECs), which display functions such as in vitro clonal proliferative potential, in vitro tube formation, in vivo vessel formation with incorporation into the systemic circulation of immunodeficient mice, in vivo chimeric vessel formation into areas of ischemia and an unequivocal endothelial phenotype (Mukai et al., 2008, Melero-Martin et al., 2007, Yoder et al., 2007, Hur et al., 2004). Among all current putative EPC populations, OECs display the most features consistent with a human postnatal vasculogenic cell (Richardson and Yoder, 2011) and are the cell type used in this study.

To date, no unique or restricted marker that can prospectively identify an EPC from circulating blood has been reported which, together with their low frequency in circulation, makes it challenging to identify these cells by flow cytometry. Indeed, many of the clinical trials reporting to quantify circulating EPCs have in fact quantified hematopoietic stem cells (Mund and Case, 2011). Cell culture is an alternative approach to isolate EPCs from umbilical cord or peripheral blood and is based on cell adhesion to specific substrates in endothelial growth medium, enabling expansion of cell numbers. OECs have been isolated from umbilical cord blood and adult peripheral blood by the in vitro culture of adherent mononuclear cells in supplemented endothelial growth medium. However, in the case of peripheral blood-derived OECs, a common barrier to the isolation and subsequent utilization of these cells is their rarity in circulation under steady-state conditions, with approximately 1 colony/10^8 MNCs plated (Richardson and Yoder, 2011).

The first objective of this study was to isolate OECs from peripheral blood samples of healthy adult subjects across an age range. Subsequently, a number of parameters were examined including endothelial- and hematopoietic-lineage cell surface antigen expression, endothelial cell function via Ac-LDL ingestion and in vitro tube formation on Matrigel, to
determine that the isolated cells were indeed OECs. Human umbilical cord blood derived OECs obtained from The University of Manchester Biobank (Approval No 08/H1010/55) were expanded in vitro and characterised alongside peripheral blood derived OECs.

3.2. Isolation of OECs from adult peripheral blood.

Lin et al initially demonstrated that OECs originate from human bone marrow and can be derived from the long-term culture (28 days) of MNCs isolated from ~100mls of adult peripheral blood (Lin et al., 2000b). However, given the large volume of blood required and the fact that the culture methodology is labour intensive and time-consuming, various adaptations of the original protocol have been proposed for the isolation of OECs from umbilical cord blood and adult peripheral blood samples. During the course of this study, much time was spent testing numerous culture conditions which have been reported in the literature in order to determine those that were optimal for OEC isolation in our laboratory. Initially, a novel strategy proposed by Reinisch et al. which was based on a strategy for the large scale clinical grade propagation of mesenchymal stromal cells was attempted (Reinisch et al., 2009). This method appeared ideal as it required a smaller volume of peripheral blood (a maximum of 24mls) from each subject in comparison to previous studies. Furthermore, any manipulation of the freshly obtained cells, such as red blood cell lysis or density gradient centrifugation, was avoided, thereby minimising cell loss and reducing the processing time required for each blood sample. Peripheral blood samples were obtained from 3 healthy subjects from which to isolate OECs according to this published protocol. In all cases, instead of isolating OECs, a distinct population of cells emerged over a 3 week period that exhibited a ‘hill and valley’ (‘hills’ consist of foci of multi-layered cells) morphology typical of smooth muscle rather than endothelial cells. To evaluate cell phenotype, the cells were stained with the endothelial markers vWF and VE-CAD, and the smooth muscle cell marker alpha-smooth muscle actin (α-SMA). The cells were
found to be uniformly negative for all the endothelial markers tested but were shown to be positive for \(\alpha\)-SMA (Figure 3.1). Human aortic endothelial cells (HAoECs) were used as controls to show positive immunoreactivity for the endothelial markers. The cells isolated using this culture method appear to fit the description of smooth muscle outgrowth cells (SOCs), derived from the circulating smooth muscle progenitor cells, as reported by (Simper et al., 2002). Further characterisation would be needed to confirm this, but it was clear that the isolated cells were not OECs which are the cell type of interest to our study. It is unclear as to why, in our hands, the culture method reported by Reinisch et al appeared to enrich for putative SOCs rather than OECs. In light of these findings, an alternative method for the isolation of OECs from peripheral blood was sought.
Figure 3.1. Representative immunofluorescence images of (A) putative SOCs and (B) HAoECs stained with antibodies to VE-CAD, vWF and α-SMA. A phase-contrast image of putative SOCs, derived from cultured MNCs, with a 'hill and valley' morphology is shown in panel A.i, which contrasts with the cobblestone morphology of cultured HAoECs as shown in B.i. Magnification x40, scale bar represents 250µm. SOCs stained universally negative for the endothelial markers; VE-CAD (A.ii) and vWF (A.iii), whilst positive immunoreactivity of these antibodies was evident in cultures of HAoECs (B.ii; VECAD (green) and B.iii; vWF (green)). SOCs stained positive for α-SMA (green) (A.iv), which was not expressed by HAoECs (B.iv). Nuclei were counterstained with DAPI (blue). Actin filaments were stained with phalloidin (red). A.ii-iv; magnification x400, scale bars represent 50µm. B.ii-iv; magnification x600, scale bar represents 20µm.
A simple modification of the protocol reported by Lin et al, which included a passaging step in the early phase of EPC culture, was reported to result in a better efficacy in OEC isolation (Figure 3.2) (Kolbe et al., 2010). To test if this method yielded OEC colonies, buffy coat MNCs were harvested from the peripheral blood of 2 young healthy subjects from which to isolate OECs according to this protocol modification. A single OEC colony emerged from the cultured MNCs of one subject but appeared to be slow growing and could not be expanded further, whilst no OEC colonies were isolated from the second subject. Consequently, we decided not to pursue this method any further.

![Figure 3.2. Schematic diagram of the modified protocol for the isolation of OECs proposed by Kolbe et al. (adapted from Kolbe et al. 2010).](image)

Numerous factors such as the volume of peripheral blood used, type of extracellular matrix coating of culture vessels, density of MNCs seeded, times of first and subsequent media changes and concentration of serum included in the medium were all altered in order to find an isolation method that consistently yielded OECs from peripheral blood.

Of the methods tested, a prolonged culture method reported by Mead et al. appeared to be most successful for the culture of OECs in vitro (Mead et al., 2008). However, the success rate for the isolation and expansion of these cells from peripheral blood remained low at 38%. Upon examining this success rate according to subject age, OEC isolation and expansion was successful in 45%, 44% and 29% of cases from young (20-29 years) (5/11 volunteers), middle-aged (30-49 years) (4/9 volunteers) and old (50-80 years) (4/14 volunteers) subjects respectively. By contrast, OEC
isolation and expansion from umbilical cord blood samples (15mls) of healthy mothers and foetuses had a success rate of 100%, using the same protocol as that used for OEC isolation from adult peripheral blood (Sipos unpublished data) (Figure 3.3). OECs appeared as individual colonies with a cobblestone-like morphology and were enumerated by visual inspection using an inverted microscope. In the successful cases in which OECs emerged from the adherent peripheral blood MNC culture, typically, 1-3 OEC colonies appeared after 21-28 days of cell seeding. There were no differences in the yielded numbers of OEC colonies derived from peripheral blood samples of differently aged adults, i.e. young, middle-aged and old subjects. By contrast, an average of 8 colonies emerged between 14-21 days after seeding MNCs isolated from cord blood samples (Sipos unpublished data). In the case of peripheral blood derived OECs, a further 3-4 weeks of culture was required before first passage, whilst cord blood OECs were typically cultured for 1-2 weeks before first passage. In both cases, OECs could then be expanded through multiple passages, during which time they retained their endothelial morphology, forming cobblestone-like monolayers (Figure 3.4).
Figure 3.3. The success rate of OEC colony isolation from umbilical cord blood (n=5) and peripheral blood of young (n=11), middle-aged (n=9) and old (n=14) subjects.

Figure 3.4. (A) A representative phase-contrast image of an OEC colony derived from peripheral blood and detected at day 21. OECs appeared as individual colonies with a cobblestone morphology (the edges of the colony are highlighted by the dashed lines), which could be expanded whilst retaining their endothelial morphology to (B) form cobblestone monolayers. (A) Magnification x40, scale bar represents 250µm. (B) Magnification x100, scale bar represents 250µm.
3.3. Phenotypic characterisation of OECs

Although the putative OECs isolated from cord and peripheral blood samples grew in monolayers in vitro and exhibited a cobblestone morphology typical of endothelial cells (Figure 3.4.B), further characterisation was needed to verify the OEC identity of the isolated cells. An assessment of multiple parameters including endothelial- and hematopoietic-lineage cell surface antigen expression, endothelial cell function via Ac-LDL ingestion and in vitro tube formation on Matrigel was performed as described below. HAoECs were used as a positive control for the phenotyping assays.

3.3.1. Assessment of cellular uptake of Ac-LDL

Ac-LDL is taken up by endothelial cells via the ‘scavenger cell pathway’ of LDL metabolism. In order to visualise its uptake, this lipoprotein has been labelled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil). The Dil-Ac-LDL uptake assay has been used widely to demonstrate the endothelial cell-like properties of putative EPC populations (Hirschi et al., 2008). The ability of the putative OECs isolated from cord blood and peripheral blood samples to uptake Ac-LDL was examined. The isolated cells were incubated with Dil-Ac-LDL for 4 hours at 37°C and subsequently examined by fluorescence microscopy. Ac-LDL uptake was readily visible in the putative OECs and the fluorescence was predominantly punctuate with a perinuclear distribution. Human dermal fibroblasts, used as a negative control, failed to uptake Ac-LDL (Figure 3.5).
Figure 3.5. Assessment of Ac-LDL uptake and UEA-1 binding by putative OECs using fluorescent microscopy. (A.i.) Ac-LDL uptake was evident in the isolated cells (red), but not by (B.i) human skin fibroblasts. Similarly, (A.ii) the isolated cells were seen to bind the lectin UEA-1 (green), whilst (B.ii) human skin fibroblasts failed to do so. Nuclei were counterstained with DAPI (blue). Actin filaments were stained with phalloidin (red) in panels A.ii and B.ii. Shown are representative examples from 6 independent experiments using different populations of putative OECs isolated from cord blood (n=3) and peripheral blood samples (n=3). Magnification x600, scale bar represents 20µm.
3.3.2. Assessment of cellular binding of UEA-1 lectin

Ulex europaeus I agglutinin (UEA-1) is a lectin that binds to α-L-fructose-containing glycoproteins on the endothelial cell surface. It is possible to visualise this binding by fluorescence microscopy using a fluorophore-conjugated UEA-1. This functional property of endothelial cells has been used widely to examine the endothelial-like properties of isolated cell populations. In this study, lectin binding was evident in the putative OECs isolated from cord and peripheral blood samples, whilst human dermal fibroblasts, used as a negative control, failed to show evidence of lectin binding (Figure 3.5).

Although the isolated cells were shown to bind the lectin UEA-1 and endocytose Ac-LDL, these characteristics have also been reported to be shared by macrophages, epithelial cells and a variety of other cell types, including blood-derived cells (Hirschi et al., 2008). Thus, further assessment of other endothelial marker protein expression was carried out using immunocytochemistry and flow cytometry.

3.3.3. Phenotypic characterisation of isolated OECs by immunocytochemistry

Indirect immunofluorescence staining was performed to examine the expression of a panel of endothelial markers by the isolated cells. CD31, VE-CAD, vWF and CD105 were chosen as representative endothelial markers. CD31 is expressed by platelets, leukocytes and is concentrated at the borders between endothelial cells where it is involved in leukocyte migration, angiogenesis and integrin activation. VE-CAD is located at the junctions between endothelial cells and is vital for the maintenance and control of endothelial cell contacts. vWF, is a glycoprotein that mediates platelet adhesion and is expressed by endothelial cells where is shows a granular pattern of reactivity. CD105 is highly expressed on human vascular endothelial cells and has been suggested to regulate
angiogenesis through interactions with TGF-β superfamily kinase receptors. As noted in section 3.1, there is currently no specific marker to identify human OECs. However, the combined expression of these markers by the isolated cells confirmed an endothelial identity of the cells used in this study. The isolated OECs were found to be uniformly positive for the four endothelial markers examined; CD31 and VE-CAD expression was evident at the cell borders, vWF was detected in a punctate pattern in the cytoplasm, while positive CD105 staining was detected at the cell membrane, particularly at sites of cell-cell contact and in the cytosol (Figure 3.6). Importantly, the cells lacked expression of the hematopoietic markers CD45 and CD14, and α-SMA expression, suggesting that they were not derived from the hematopoietic lineage or from mesenchymal progenitor cells (Figure 3.7). This pattern of staining of OECs is consistent with the results of a number of other studies (Medina et al., 2010a, Khan et al., 2006, Ingram et al., 2004).
Figure 3.6. Representative immunofluorescence micrograph demonstrating the expression of several endothelial markers by putative OECs isolated from cord blood and peripheral blood samples. The isolated OEC populations express (A) CD31 (green), (B) VE-CAD (green), (C) vWF (green) and (D) CD105 (green). Nuclei were counterstained with DAPI (blue). Actin Filaments were stained with phalloidin (red). Shown are representative examples from 6 independent experiments using different populations of putative OECs isolated from cord blood (n=3) and peripheral blood samples (n=3). Magnification x600, scale bar represents 20µm.
Figure 3.7. OEC populations isolated from cord blood and peripheral blood samples do not express CD14 and α-SMA, as shown in A.i and A.ii respectively (B.i) Human CFU-Hill cells were used as a control cell type to demonstrate positive immunoreactivity with CD14 (green), whilst (B.ii) human smooth muscle cells were shown to express α-SMA (green). Nuclei were counterstained with DAPI (blue). Photomicrographs are representative of 6 independent experiments using different donors; cord blood (n=3) and peripheral blood samples (n=3). Ai-ii and Bi-ii; magnification x600, scale bar represents 20µm. Bi; magnification x100, scale bar represents 200µm.
3.3.4. Phenotypic characterisation of isolated OECs by flow cytometry

To further confirm the endothelial phenotype and verify that the cell progeny was not contaminated with other cell types, the isolated cells were examined for the expression of several endothelial and hematopoietic markers by flow cytometry (Figure 3.8). The following cell surface antigens were examined in this next experiment; CD31, VEGFR-2 and CD146, the latter of which is a transmembrane glycoprotein that has been detected in endothelial cells in vascular tissue throughout the body. We also examined the expression of CD34, which is a cell surface protein normally found on hematopoietic stem cells, a subset of mesenchymal stem cells and has also been documented to be expressed by OECs. Flow cytometric analysis revealed that OECs expressed the endothelial cell surface antigens CD31, CD146 and, to a lesser extent CD34 and VEGFR-2, as well as being positive for Dil-Ac-LDL uptake and UEA-1 lectin binding. Importantly, OECs were found to lack expression of the hematopoietic antigens CD45 and CD133. These results are consistent with that reported by other investigators (Medina et al., 2010a).
Figure 3.8. Representative flow cytometry histograms demonstrating the expression of several endothelial markers CD31, VEGFR-2, CD34 and CD146 but not the hematopoietic markers CD133 and CD45 by the OEC populations (red lines). HAoECs were used as a comparator cell type (blue lines). The final panel depicts peripheral blood MNCs (red lines) CD45 and CD133 expression as a comparative control. Red and blue lines represent the fluorescence signal for the indicated markers, grey lines represent the isotypic controls. Plots are depicted with relative cell counts on the y-axes and fluorescence intensity on the x-axes (logarithmic scale).
3.3.5. Phenotypic characterisation of isolated OECs by RT-PCR

Further validation of endothelial gene expression was obtained using RT-PCR. Primers for the endothelial markers CD31, vWF, VECAD, CD34, and VEGFR-2, showed positive amplification in the isolated OECs and in the HAoECs, used as a positive control. By contrast, no amplicons were generated in these two cell types when using primers for the hematopoietic markers CD14 and CD45, while monocytes, used as a positive control, yielded positive bands for the latter (Figure 3.9).

Figure 3.9. RT-PCR confirmed the expression of endothelial, but not hematopoietic, lineage markers by the OEC populations. RT-PCR analysis of RNA harvested from OECs and HAoECs demonstrated amplification of the endothelial markers CD31, vWF, VE-CAD, CD34 and VEGFR-2, but no amplification of the hematopoietic markers CD14 and CD45. In parallel, RNA harvested from human monocytes was used as a positive control for hematopoietic markers and demonstrated amplification of the CD14 and CD45 primers. RT-PCR analysis in the presence of reverse transcriptase is indicated by the + or – at the tops of the columns.
3.3.6. *In vitro tube formation by isolated OECs*

The phenotypic characterisation of the isolated OEC populations was reinforced using a functional tube formation assay. Here, the ability of the isolated cells to form capillary-like structures was analysed by plating on growth factor reduced (GFR) Matrigel coated surfaces and tube formation was examined microscopically. In this assay, the cells appeared elongated and interconnected and had formed adherent cords as early as 2 hours after seeding (Figure 3.10). By 14 hours of incubation, characteristic capillary-like structures had formed and were assembled in a branching network of anatomosing cords, similar to those generated by the control endothelial cells (Figure 3.11). As a negative control, human dermal fibroblasts were seeded under the same conditions on GFR Matrigel and did not form the network of structures that we observed by the isolated OECs (data not shown). In addition, the suspension of the OECs within a gel of rat tail collagen type I was also shown to promote the formation of tube-like structures (Figure 3.12).
Figure 3.10. Representative image of a network of capillary-like structures formed after seeding OECs on Matrigel. The cells migrated throughout the Matrigel surface and formed characteristic tube-like structures assembled in a branching network. Shown are representative examples from 14 independent experiments using different populations of putative OECs isolated from cord blood (n=5) and peripheral blood samples (n=9). Magnification x50, scale bar represents 300µm.
Figure 3.11. The capillary-like structures formed after seeding (A) OECs on Matrigel were similar to those formed by (B) control endothelial cells (HAoECs) under the same conditions. Magnification x100, scale bar represents 250µm.

Figure 3.12. Representative image of the three dimensional network of tube-like structures formed by suspending the OECs within a type I collagen gel and culturing for 48 hours. Magnification x100, scale bar represents 250µm.
3.4. **Discussion**

Since the seminal description of a circulating cell population that can contribute to postnatal neovascularization (Asahara et al., 1997), interest in circulating EPCs has soared. A great deal of this research is aimed at harnessing the regenerative capacity of EPCs to treat cardiovascular disease, which is a primary cause of morbidity and mortality in the ageing population. However, of the putative EPC populations that have been described, much less information is available on peripheral blood derived OECs, the cell type which displays the most features consistent with a human postnatal vasculogenic cell. In many cases this is due to difficulties in isolating the cells from adult peripheral blood given their rarity in circulation.

The first objective of this study was achieved in the isolation and characterisation of OECs from peripheral blood samples of healthy adult subjects across an age range for subsequent functional analysis.

A prolonged culture method described by Mead et al. 2008 proved to be most successful for the isolation and *in vitro* culture of OECs. However, the overall success rate for the isolation of these cells from peripheral blood was found to be considerably lower, at 38%, compared to the 100% success of isolating OECs from umbilical cord blood. The number of OEC colonies isolated from cord blood samples was also higher than that from peripheral blood. These observations are in keeping with previous studies suggesting that umbilical cord blood contains a higher concentration of OECs than adult peripheral blood (Ingram et al., 2004). The success rate for the isolation of peripheral blood derived OECs was much lower from subjects aged over 50 years (29%) in comparison to their younger counterparts (44-45%), suggesting that their numbers in circulation may decline with age. Indeed, a number of studies have suggested that the concentrations of circulating EPCs decline with age (Jie et al., 2009, Scheubel et al., 2003). However, in order to gain quantitative data of putative circulating EPCs, flow cytometric analysis in which EPCs are identified by cell surface phenotype would be necessary.
A major factor which has hampered progress in the field of EPC biology has been a lack of characterisation of the cells referred to as EPCs in many of the investigations. Thus, the cells isolated throughout the course of this study were extensively characterised before being considered to be OECs. Numerous studies have described the OEC immunophenotype as being positive for a diverse array of endothelial markers including CD31, VE-CAD, vWF, CD36, CD105, CD146, VEGFR-2 and Tie-2 whilst negative for the hematopoietic markers CD45 and CD14 (Medina et al., 2010a, Yoder et al., 2007). In addition to exhibiting a cobblestone-like morphology typical of endothelial cells, the OEC phenotype of the isolated cells was illustrated by the expression of multiple endothelial markers, lack of hematopoietic marker expression, ingestion of Ac-LDL, binding of lectin, and the ability to form tube-like structures on Matrigel and within collagen gels. Taken together, the data was consistent with previous reports (Medina et al., 2010a, Yoder et al., 2007, Khan et al., 2006, Ingram et al., 2004) and indicate that the cultures obtained from the subjects in this study conformed to the phenotype of an OEC. The phenotype observed was consistent for OECs isolated from cord and peripheral blood, and in the latter case, was stable across the age groups.

It is important to note that many of the assays used to determine whether the isolated cells were OECs suffer from a number of limitations. For example, the lectin binding and Ac-LDL uptake assays are not characteristics specific for endothelial cells and can also be exhibited by macrophages, epithelial cells and a variety of other cell types (Hirschi et al., 2008). In addition, we used in vitro tube formation assays on Matrigel to demonstrate the endothelial potential of the isolated cells. It should be noted that Matrigel has been shown to induce tube formation from several non-endothelial cells including fibroblasts (Bikfalvi et al., 1991) and CD14+ monocytes (Schmeisser et al., 2001). However, we did not observe the formation of a network of anatomosing cords by human dermal fibroblasts, possibly due to the use of GFR Matrigel in this study, rather than the standard formulation typically used.
Due to the difficulties in OEC isolation, one of the drawbacks of this study is the low numbers of OECs isolated from adult peripheral blood samples for subsequent analysis. Additionally, given the widespread use of medication in the older population, such as statins, and in accordance with the exclusion criteria set out by the ethics approval for this study, the recruitment of old subjects (50-70 years) free of medication proved challenging.

3.4.1. Future directions

In this study, it was recognised that the characterisation of cell types such as OECs cannot be performed by an individual assay used in isolation. However, the multiple parameters which we examined including, the demonstration of an endothelial morphology, the expression of a panel of endothelial markers, Ac-LDL uptake and lectin binding, together with evidence of in vitro tube formation, suggests that the cells isolated in this study can be considered OECs. Nonetheless, a number of issues require further clarification. For example, Matrigel and collagen gels assays were utilized to demonstrate the formation of tube-like structures by OECs. However, tube formation is actually a complex process, thought to be specific to endothelial cells, which requires cells to undergo vacuolization, elongation and coalescence into tube-like structures with lumens. In this study tube formation was examined only in 2-dimension which is unable to demonstrate that the capillary-like structures formed by the isolated cells are bona fide tubes and not just cord-like structures. The distinguishing feature would be to demonstrate the presence of a lumen in a cross section of the cord-like structures formed by OECs. Additionally, a number of studies have shown that OECs form microvessels upon transplantation into immunodeficient mice that functionally connected with host murine vessels and participated in murine blood flow (Yoder et al., 2007). Thus, the most stringent method to assess whether the cells isolated in this study were OECs would be to
implant the cells and examine blood vessel formation in vivo. Consequently, a further extension of this study would be to demonstrate the ability of the isolated cells to function in vivo as postnatal vasculogenic cells.

It is important to note that an aspect of the study which could have influenced the yield of OEC colonies includes a lack of demographic details of the subjects enrolled in the study. Peripheral blood samples were only taken from subjects who were non-smokers and not receiving medication for any clinical diagnosis, and therefore deemed to healthy. However, this is overly simplistic and it possible that some subjects may have medical conditions of which we were unaware. Thus the possibility that factors other than age influenced the isolation of OECs from peripheral blood samples cannot be ruled out. Future studies would need to ensure that only subjects who met strict exclusion criteria were enrolled into the study; subjects should be asymptomatic, normotensive, nondiabetic, normocholesteremic, non-smokers, have no significant medical history and not be taking regular or incidental medication, including hormonal contraception.

In summary, OECs from adult peripheral blood samples of subjects across an age range were successfully isolated and characterised. These cells will be used, along with cord blood derived OECs, for subsequent functional analysis in order to determine whether age is an important determinant of OEC function.
Chapter 4:

Results II - Age-related alterations of OEC function
4. Results II - Age-related alterations of OEC function

4.1. Introduction

Age is a significant risk factor for the development and progression of vascular diseases, such as atherosclerosis. A decline in endogenous cardiovascular repair mechanisms is thought to be among the many aspects of cardiovascular function that change with age and contribute to this increased risk. A number of studies have demonstrated that with age, in both healthy persons and patients with cardiovascular disease, EPCs are subject to changes that diminish their number in circulation and/or function (Xia et al., 2012, Ballard and Edelberg, 2007, Heiss et al., 2005, Rauscher et al., 2003, Scheubel et al., 2003, Edelberg et al., 2002). Such changes are believed to contribute to a decreased capacity for neovascularization or insufficient repair of the endothelium following injury, thereby facilitating the progression of endothelial dysfunction and vascular pathology in the ageing host (Dimmeler and Vasa-Nicotera, 2003). However, it is important to note that these studies used two distinct populations of cells termed CFU-Hill cells and CACs, both of which arise after short term culture. It has been widely criticised to refer to these cells as ‘EPCs’ as it is now recognised that they are in fact hematopoietic lineage cells that display proangiogenic properties (Fadini et al., 2012). These cells, do however, continue to be relevant to the study of cardiovascular disease, as their numbers in circulation has been shown to be inversely correlated with cardiovascular risk factors and to predict cardiovascular events and death independent of both traditional and non-traditional risk factors (Schmidt-Lucke et al., 2005, Werner et al., 2005, Hill et al., 2003). To date, the one cell type encompassed within the term ‘EPC’ that displays a robust proliferative potential, the ability to form vascular networks in implanted Matrigel plugs in vivo and an unequivocal endothelial phenotype is the rare circulating OEC (Medina et al., 2010b, Melero-Martin et al., 2007, Yoder et al., 2007). Yet, while there is
evidence to suggest that the functional capacity of CFU-Hill cells and CACs declines with age, there is a lack of studies that have examined the impact of age on the function of OECs.

To assess whether ageing is accompanied by a decline in OEC function, the proliferative, migratory, tube forming capacity and apoptotic susceptibility of these cells isolated from peripheral blood samples of young (20-30 years) and old (50-70 years) healthy subjects was measured. In parallel, the functional capacity of cord blood derived OECs was also analysed. It has previously been established that OECs from cord blood have a greater proliferative and clonogenic potential than peripheral blood derived OECs (Ingram et al. 2004). Thus, cord blood derived OECs were viewed as representative of a primitive EPC population.

4.2. The proliferative, tube-forming capacity and apoptotic susceptibility of OECs is not significantly impaired with age.

4.2.1. Assessment of OEC proliferation

Quantification of cell proliferation was performed by directly counting OECs from cord blood (n=4) and peripheral blood of young (n=4) and old (n=4) subjects at three time points; baseline, 24 and 48 hours after seeding. Cell proliferation was expressed as the fold change in cell number relative to the baseline. A significant difference was seen in the relative fold increase in cell number between OECs from cord blood (n=4) and those from peripheral blood (n=8) samples at both the 24 and 48 hour time points (p=0.042 and p=0.036 respectively). As depicted in Figure 4.1, cell number was increased an average of 2 fold after 24 hours by OECs from cord blood (n=4) which was significantly higher than the 1.51 and 1.47 fold increase seen by OECs from the peripheral blood of
young (n=4; p=0.047) and old (n=4; p=0.047) subjects respectively. Similarly, 48 hours after seeding, cell number was increased 2.77 fold by OECs from cord blood, whilst proliferation was significantly lower, at a 2.14 and 1.96 fold increase, by peripheral blood OECs of young (n=4; p=0.043) and old (n=4; p=0.042) subjects respectively. However, no statistically significant difference in proliferative capacity was detected between peripheral blood OECs isolated from young and old subjects (Figure 4.1).

![Image of Figure 4.1](image)

**Figure 4.1.** Cell proliferation, as assessed by direct cell counting over a 48 hour period, is significantly different between OECs from cord (n=4) and peripheral blood (n=8) samples. However, no statistically significant difference in proliferative capacity was detected between peripheral blood OECs isolated from young (n=4) and old (n=4) subjects. Columns represent mean values, errors bars are the standard error of the mean. Comparisons between groups were analysed by Mann Whitney U tests. * p<0.05

Although this method is simple and inexpensive, it is unclear whether changes in cell number are due to alterations in proliferation, apoptosis, or both. Thus, to confirm the results obtained from the cell counting experiments the metabolic activity of cultures of OECs was measured and used as a gauge of cell proliferation. Here, OEC populations were
assayed for the production of formazan by the reduction of 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which acts
as an index of the number of metabolically active cells in culture. Since
the reduction of MTT can only occur in metabolically active cells, the
amount of colour produced is directly proportional to the number of viable
cells. In support of the results obtained by cell counting, a significant
difference in proliferation was seen between cord (n=5) and peripheral
blood derived OECs (n=9; p=0.029). As depicted in Figure 4.2, cell
proliferation was significantly higher in cord blood OECs (n=5) compared
to those derived from the peripheral blood of young (n=5; p=0.038) and
old subjects (n=4; p=0.041). Cell proliferation was lower in peripheral
blood derived OECs isolated from old subjects as compared to those
from young subjects, but this was not statistically significant (Figure 4.2).

![Graph showing cell proliferation](image)

**Figure 4.2.** A significant difference in cell proliferation, as assessed using
an MTT assay over a 48hr period, was detected between cord blood OECs
(n=5) compared to peripheral blood derived OECs from both young (n=5)
and old (n=4) subjects. However, no statistically significant difference was
detected between OECs isolated from peripheral blood samples of young
and old subjects. Columns represent mean values, errors bars are the
standard error of the mean. Comparisons between groups were analysed
by Mann Whitney U tests. * p<0.05
4.2.2. Assessment of OEC apoptosis

Recently, ageing has been associated with a proapoptotic phenotype of CACs, characterised by a decreased expression of anti-apoptotic proteins and reduced telomerase activity. It was proposed that these age-related changes contribute to a diminished ability of CACs from older subjects to resist the apoptotic stimulus staurosporine (STS) compared with CACs from young subjects (Kushner et al., 2011). However, the influence of ageing on OEC apoptosis is currently unknown. Whether enhanced susceptibility to STS-induced apoptosis would also be evident in OECs from older subjects was examined in this study.

Caspase-3 activation plays a central role in the execution of cell apoptosis. Activation of intracellular caspase-3 in OECs was induced by incubating the cells with 25nM STS in basal medium for 4 hours at 37°C and detected using a solid phase enzyme-linked immunosorbent assay (ELISA). The concentration was chosen after a preliminary dose-effect study in which STS at a concentration of 25nM induced ~50% of the maximum effect ($p=0.014$) (Figure 4.3).
Figure 4.3. The treatment of OECs with STS stimulates cleavage of caspase-3 in a dose-dependent manner, as detected using a solid-phase sandwich ELISA. The magnitude of optical density is proportional to the quantity of cleaved caspase-3 protein. The experiments were performed three times in duplicate and the results are presented as the mean values, errors bars are the standard error of the mean. * p<0.05 compared with the control (EBM-2 1% FBS).

During STS incubation, culture medium was changed to that containing low serum level (1%) and without additional growth factors. This was in order to maximise the effects of STS treatment on activating caspase-3 as numerous factors within EGM-2, such as VEGF, are known survival factors for endothelial cells (Benjamin et al., 1999, Gerber et al., 1998 Yuan et al., 1996). STS treatment induced apoptosis of OECs as evident by the morphological characteristics of cell shrinkage, cytoplasmic/nuclear condensation and fragmentation (data not shown). Under normal growth conditions (EGM-2 10% FBS), there was no significant difference in baseline concentrations of intracellular active caspase-3 between OECs from cord blood (n=3) and those isolated from peripheral blood samples (n=6), as well as, between peripheral blood OECs isolated from young (n=3) and old (n=3). A modest increase in the concentrations of active caspase-3 was detected under conditions of
serum and growth factor deprivation, but this was not significantly different between the 3 groups. In all three groups STS-induced a significant increase in the levels of intracellular caspase-3 as compared to the basal media control (EBM-2 1% FBS). Upon examining the relative fold increase in the expression of active caspase-3 protein, OECs from cord blood samples (n=3) exhibited an average 2.97 fold increase in intracellular active caspase-3 concentration. Although this fold change was higher than that seen in OECs from peripheral blood of young and old subjects which exhibited a 2.12 (p=0.05) and 2.32 (p=0.05) fold increase respectively, the difference was not statistically significant. Furthermore, no statistically significant difference in caspase-3 concentrations was detected between peripheral blood OECs isolated from young (n=3) and old (n=3) subjects (Figure 4.4).
Figure 4.4. OEC apoptosis, as measured by the levels of endogenous cleaved caspase-3 protein, was analysed under normal growth conditions (EGM-2 10% FBS), serum and growth deprivation (EBM-2 1% FBS) and apoptotic conditions (STS). In all 3 groups, STS treatment induced a significant increase in cleaved caspase-3 levels as compared to basal conditions (EBM-2 1% FBS). (B) The relative fold increase in expression of cleaved caspase-3 relative to the baseline (EBM-2 1% FBS) was not significantly different between OECs from cord blood (n=3) compared to peripheral blood derived OECs from young subjects (n=3) and old subjects (n=3). Columns represent mean values, errors bars are the standard error of the mean. Comparisons between groups were analysed by Mann Whitney U tests.
4.2.3. *In vitro* tube forming capacity

Among the various steps in the process of angiogenesis is the assembly of endothelial cells into tubular structures. This step can be modelled, to some extent, *in vitro* by plating endothelial cells on a supportive extracellular matrix substrate. Matrigel is a gelatinous mixture derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma which is rich in extracellular matrix proteins and stimulates endothelial cells to form capillary-like structures. The Matrigel assay has been extensively used to study *in vitro* angiogenesis as it is relatively simple to perform, rapid and inexpensive. The tube-forming potential of OECs from cord (n=4) and peripheral blood samples of young (n=4) and old (n=4) subjects in such an assay. A growth factor reduced (GFR) form of Matrigel in which the levels of stimulatory cytokines and growth factors are markedly reduced in comparison to standard Matrigel was used. The rationale for this being that the higher levels of angiogenic factors in the standard formulation may mask differences in tube-forming capacity between the OECs under study.

In GFR Matrigel, OECs aligned to form branching, anastomosing tubes with multicentric junctions that created a network of capillary-like structures. Phase-contrast images of the networks were captured and processed using ImageJ software (http://rsb.info.nih.gov/ij). Several morphological features were measured to characterise these capillary-like networks including quantification of the number of closed loops, junctions and branches, as well as, the total length of the network (Figure 4.5). Quantification of the number of closed loops, junctions and branches, as well as, the total length of the network revealed no significant differences in tube forming capacity between the three groups (Figure 4.6).
Figure 4.5. Image analysis of OEC tube formation in GFR Matrigel. (A) Representative phase-contrast image of a network of tube-like structures formed 18hrs after seeding OECs on GFR Matrigel. (B) Image after background subtraction, (C) the image is converted to a binary image and (D) after binary thinning procedures a skeleton of the image is derived (red lines). From this image a number of morphological features can be measured to characterise the capillary-like network. They include; the total length of the network (red lines), the number of closed loops (red areas), the number of branching points (white dots) and the number of junctions. Scale bar represents 250µm.
Figure 4.6. The tube-forming potential of OECs in growth factor reduced Matrigel is not significantly impaired with age. Quantification of the number of (A) closed loops, (B) the number of junctions and (C) the number of branches, as well as, (D) the total length of the network revealed no significant differences in tube forming capacity between OECs from cord blood (n=4) and peripheral blood of young (n=4) and old (n=4) subjects. Columns represent mean values, errors bars are the standard error of the mean. Comparisons between groups were analysed by Mann Whitney U tests.
Despite using the growth factor reduced formulation of Matrigel, it should be noted that this still contains various angiogenic growth factors and HSPGs which may have masked any subtle differences in the tube forming potential of OECs derived from the three sources. Collagen gels have also been widely used in angiogenesis assays and as they lack the endogenous growth factors and HSPGs contained in Matrigel. Whether any differences in OEC tube formation would be evident in this assay was investigated. Peripheral blood derived OECs suspended in a gel containing rat tail collagen I formed tube-like structures within 24 hours which were maintained over several days (Figure 4.7). However, quantification proved difficult as the tubular structures grow in three dimensions within the gel. Fluorescence staining of the tube-like structures and imaging of the gel at multiple focal planes using confocal microscopy was attempted in order to allow quantification. However, unfortunately due to time restraints it was not possible to resolve the technical issues encountered for accurate quantification.

Figure 4.7. Representative image of the three dimensional network of tube-like structures formed by peripheral blood derived OECs suspended within a collagen gel and cultured for 48 hours. Scale bars represent 250µm.
4.3. The migratory response of OECs declines with age.

4.3.1. Assessment of OEC migration using an in vitro scratch assay

The migratory capacity of OECs was initially assessed using an in vitro scratch assay in which cells migrate into an area that has been mechanically denuded of cells. The migration of OECs from the wound edge into the cell free area was documented by time-lapse microscopy at initial wounding and at 2 hour intervals over a 24 hour period (Figure 4.8). The rate of migration was determined by calculating the percentage of area closure over the 24 hour period.

Figure 4.8. Representative phase-contrast image of peripheral blood derived OEC migration in an in vitro scratch assay. A 'scratch' is made on a confluent OEC monolayer to create a cell-free area (the dashed lines define the area lacking cells) into which migration can occur and is recorded at regular time intervals under time-lapse microscopy for 24hrs. Scale bar represents 300µm.

Using this method, a significant reduction in OEC migration was evident with age; at 12 hours 84.87% closure of the wound by cord blood OECs (n=5) was observed compared to 64.95% closure by OECs of young subjects (n=5; p=0.027) and only 39.61% closure from OECs of older subjects (n=3; p=0.034). After 24 hours, OECs from cord blood and peripheral blood samples of young subjects migrated and refilled the cell-free space (100% and 99.3% respectively). By contrast, OECs from older
subjects demonstrated a significant reduction in cell migration with only 87% closure (Figure 4.9).

Figure 4.9. Age-related impairment of OEC migration. (A) The migratory capacity of OECs was determined using an *in vitro* scratch assay in which the cells migrate into an area that has been mechanically denuded of cells. The percentage of area closure was measured at various time points. Alterations in OEC migration were evident after 12hrs, by which time, cord blood OECs had closed the wound by 84.87% (n=4) compared to 64.95% closure by OECs of young subjects (n=5) and only 39.61% closure by OECs of old subjects (n=3). By 24hrs, OECs from cord blood and peripheral blood samples of young subjects migrated and refilled the cell-free space (100% and 99.3% respectively). By contrast, OECs from older subjects demonstrated a significant reduction in cell migration with only 87% closure. Columns represent mean values, errors bars are the standard error. Comparisons between groups were analysed by Mann Whitney U tests. *p ≤ 0.05
The *in vitro* scratch assay is a straightforward, low-cost and well developed method to analyse cell migration *in vitro*. However, a disadvantage of the assay lies in the difficulty in creating scratch wound areas of equal size, thus wounds can be of different widths which can affect the reproducibility and consistency of the results. Therefore, to ensure that the observed differences in OEC migration was not due to a non-uniform nature of the scratch wound areas, cell migration was also evaluated using the Oris™ cell migration assay (CMA). This assay utilizes a 96 well plate format populated with silicone stoppers that serve as barriers to form 2mm central cell-free zones, thereby providing decreased variability over scratch assays (Figure 4.10). In support of the results obtained using the traditional scratch assay, analysis of OEC migration using the Oris™ CMA also revealed significant differences in cell migration between OECs from cord blood (n=3) and peripheral blood of young (n=3) and old (n=3) subjects (Figure 4.11). At the 12 hour time point, cord blood OECs had closed the wound by 93.87%, compared to 70.87% and 46.66% closure by OECs from peripheral blood of young subjects and old subjects respectively.

![Figure 4.10. Representative phase contrast images of peripheral blood derived OEC migration using the Oris™ CMA. OECs are seeded and allowed to adhere in an annular monolayer surrounding the OrisTM cell stoppers. Subsequent removal of the stoppers reveals a 2mm central cell-free zone into which migration can occur and is recorded at regular time intervals under time-lapse microscopy for 24hrs. Scale bar represents 300µm.](image-url)
Figure 4.11. Analysis of OEC migration using the Oris CMA. In support of the results obtained using an in vitro scratch assay, the Oris CMA revealed a significant decline in OEC migration with age; after 12 hours, cord blood OECs (n=3) had closed the cell free area by 93.87%, compared to 70.87% closure by OECs of young subjects (n=3) and 46.66% closure by OECs of old subjects (n=3). Columns represent mean values, errors bars are the standard error. Comparisons between groups were analysed by Mann Whitney U tests. *p ≤ 0.05

4.3.2. Chemotactic response of OECs towards VEGF and SDF-1α.

The in vitro scratch assay provides a measure of cell motility, however as no chemical gradient is established it cannot examine the chemotactic response of cells. A common method to study chemotaxis is the transwell migration assay, also known as the Boyden or modified Boyden chamber assay, in which cells and chemoattractant are separated by a porous membrane. As cells migrate through the membrane toward the chemoattractant, they adhere to the underside of the membrane and can be counted (Figure 4.12).
Using this assay, we evaluated OEC migration towards SDF-1α and VEGF, two factors which have been implicated in the mobilisation and homing of EPCs. Migration of OECs was significantly stimulated by both SDF-1α and VEGF in a concentration dependent manner. The maximal response was observed at a concentration of 100ng/ml of SDF-1α and 50ng/ml of VEGF (Figure 4.13). To ensure that cell migration was due to a chemotaxis effect and not due to random cell movements (chemokinesis) the SDF-1α and VEGF gradients were abolished by the addition of an equal concentration of the chemotactic agent to both the top and bottom chambers. In the absence of an SDF-1α or VEGF gradient, OEC migration was near comparable to the levels observed in the absence of any chemotactic agent.

The migratory response of OECs isolated from cord blood samples (n=4) and from peripheral blood samples of young (n=4) and old subjects (n=4) towards VEGF and SDF-1α are shown in Figure 4.14. Migration towards SDF-1α was similar between cord blood OECs and those isolated from peripheral blood samples of young subjects; SDF-1α induced a 1.93 and 1.92 fold increase in cell migration respectively, but only a 1.5 fold increase in migration of OECs from old subjects (p=0.044). Similarly, in response to VEGF, migration was increased 2.14 and 2.04 fold in OECs from cord blood and peripheral blood of young subjects, while migration
was significantly lower, at only 1.62 fold, in OECs of old subjects ($p=0.029$).

Figure 4.13. SDF-1α and VEGF significantly induced migration of peripheral blood derived OECs from young subjects in a dose dependent manner. The migration of OECs in response to various concentrations of SDF-1α (0-400ng/ml) and VEGF (0-200ng/ml) was examined in a transwell migration assay. An SDF-1α concentration of 100ng/ml and a VEGF concentration of 50ng/ml induced the maximal response. The experiments were performed three times in duplicate and the results are presented as the mean values. Error bars represent the standard error of the mean. * $p \leq 0.05$ compared with the control (no chemoattractant).
The chemotactic response of OECs with age. A significant reduction in SDF-1α (100ng/ml) and VEGF (50ng/ml) induced migration was detected in OECs isolated from old subjects (n=4) as compared to OECs isolated from cord blood samples (n=4) and from the peripheral blood of young subjects (n=4). Columns represent mean values, errors bars are the standard error. Comparisons between groups were analysed by Mann Whitney U tests. *p ≤ 0.05

Figure 4.4. The chemotactic response of OECs with age. A significant reduction in SDF-1α (100ng/ml) and VEGF (50ng/ml) induced migration was detected in OECs isolated from old subjects (n=4) as compared to OECs isolated from cord blood samples (n=4) and from the peripheral blood of young subjects (n=4). Columns represent mean values, errors bars are the standard error. Comparisons between groups were analysed by Mann Whitney U tests. *p ≤ 0.05

4.4. Quantitative analysis of CXCR4 and VEGFR2 expression in OECs during ageing.

A plausible explanation for this reduced migratory response is a decline in the expression of the cell surface receptors for VEGF-A and SDF-1α. The receptor for SDF-1α is CXCR4, whilst VEGFR-2 is the principle mediator of the physiological effects of VEGF-A on endothelial cells. Age-related changes in OEC expression of CXCR4 and VEGFR2 were quantified using reverse transcription quantitative real-time-PCR (RT-qPCR). First, a geNorm kit was used to determine the most suitable reference gene(s) for accurate normalisation in this experimental system. A number of candidate reference genes were compared to determine the most
stably expressed and, therefore representative of the total amount of cDNA, in OECs from cord blood and peripheral blood samples of young and old subjects. The results of the analysis demonstrate that ATP5B or GAPDH were the optimal reference genes to use in this experimental system (Figure 4.15).

![Figure 4.15. The expression stability value (M-value) of candidate reference genes was determined using the geNorm program. The geNorm kit ranks the candidate reference (housekeeping) genes in order of increasing stability from left to right. The gene expression stability value (M) is calculated for each reference gene as the average pairwise variation for that given gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability.](image)

A significant decline in the expression of CXCR4 was detected in OECs isolated from old subjects (n=4) in comparison to OECs from cord blood (n=4) (p=0.037) and peripheral blood of young subjects (n=4; p=0.034), although not between cord blood and young peripheral blood OECs (Figure 4.16). This reduction in CXCR4 expression by OECs from old subjects may contribute to the decline in migration towards SDF-1α observed in the transwell assays. By contrast, the expression levels of
VEGFR-2 did not appear to significantly differ between the three groups of OECs (Figure 4.16). Since the reduction in OEC migration towards VEGF did not appear to be attributable to a reduction in VEGFR-2 expression by OECs with increasing age, alternative underlying mechanisms need to be investigated.
Figure 4.16. Comparison of the expression levels of CXCR4 and VEGFR-2 from OECs isolated from cord blood (n=4) and peripheral blood of young (n=4) and old (n=4) subjects. Real-time quantitative PCR analysis revealed a significant decline in the expression of CXCR4 in OECs isolated from old subjects in comparison to OECs from cord blood and peripheral blood of younger counterparts. By contrast, mRNA levels of VEGFR-2 did not appear to significantly differ between the three groups of OECs. Expression was normalised to ATP5B. Columns represent mean values, errors bars are the standard error. Comparisons between groups were analysed by Mann Whitney U tests. *p ≤ 0.05.
4.5. Discussion

It is well accepted that a characteristic of the ageing process is the development of endothelial cell dysfunction, rendering the vasculature susceptible to the development of atherosclerosis and subsequent cardiovascular events. A deterioration of endogenous EPC function with age is thought to contribute to this increased risk of vascular disease. This study examined whether ageing is accompanied by a decline in OEC function, as measured by the susceptibility to apoptosis, proliferative, migratory and tube forming capacity of these cells isolated from cord blood and peripheral blood samples of young (20-30 years) and old (50-70 years) healthy subjects. These measures are often used as in vitro indicators of EPC function. However, it should be noted that none of these techniques can precisely recapitulate these complex events as they occur in vivo, which is influenced by a complexity of factors and cell types. Nonetheless, the information gained from such techniques can provide useful insights into how cell function is affected by certain conditions, such as ageing, beyond simple number.

The proliferative capacity of OECs, an important functional feature for amplifying the cellular pool, was assessed by direct cell counts and by measuring the metabolic activity of the cell population. A significant difference between the proliferative capacity of OECs from cord blood and those isolated from peripheral blood was evident, which is consistent with observations from previous studies (Ingram et al., 2004). Although the proliferative capacity of peripheral blood derived OECs from old subjects was lower than those isolated from young subjects this did not reach statistical significance. However, this trend could possibly reach significance with increased numbers of subjects within this group. Thus, it is difficult to conclude whether changes in the proliferative response of OECs with human ageing contribute to the impaired vascular repair and regeneration observed in the elderly.

The influence of ageing on OEC susceptibility to STS-induced apoptosis was also examined. Increased apoptotic susceptibility could contribute to
numerical and functional impairments in the endogenous OEC population and, in turn, age-associated CVD risk. Apoptotic susceptibility was assessed by measuring the levels of cleaved caspase-3 protein in OEC lysates. STS was used to activate caspase-3 in OECs as it is a widely used agent for the induction of apoptosis in a variety of cell types. Furthermore, an advantage of using STS as an inducer of apoptosis is that all of the proteins required to execute STS induced cell death are constitutively expressed in the cells (Weil et al., 1996). In response to STS treatment, the relative fold increase in intracellular caspase-3 concentrations was not significantly different in cord blood OECs compared to OECs from peripheral blood OECs. Similarly, no statistically significant difference in apoptotic susceptibility to STS was detected between peripheral blood OECs isolated from young and old subjects. The discrepancy between the report by Kushner et al which suggested that ageing is associated with a diminished ability to resist STS-induced apoptosis and our findings may be due to the differences in the cell types studied i.e. CACs vs OECs (Kushner et al., 2011).

The ability of OECs from the three sources to form capillary-like structures in GFR Matrigel was also determined by measuring a number of morphological features of the tubular networks formed. The tube forming capacities of OECs did not appear to significantly decline with age. However, growth factor reduced preparations of Matrigel are not free from endogenous growth factors and HSPGs which still stimulate high levels of morphogenesis and may have masked any subtle differences between the OEC samples under study. The morphological features examined in this assay were chosen as they appear to be the most widely used and thus would allow us to compare the results with those from other studies. However, it is important to note that these morphological features cannot fully characterise the architecture of the pattern of the tubules. The Matrigel tube formation assay also suffers from a number of limitations including the fact that Matrigel is a proprietary substance whose complete composition is variable and not publicly known. Furthermore, some studies have demonstrated that the tube structures
formed in Matrigel lack a recognisable lumen which may suggest a different mechanism of tube formation than angiogenesis and vasculogenesis (Bikfalvi et al., 1991). Differences in the vessel forming abilities of OECs from cord blood and peripheral blood samples may exist in vivo, where the cells are exposed to a complex environment regulated by interactions with other cells, the surrounding cell matrix and soluble factors that are missing in the in vitro system. Indeed, in a model of in vivo vasculogenesis, Au et al found that umbilical cord blood derived OECs have enhanced blood vessel forming potential compared with peripheral blood OECs which formed vessels that were more unstable and regressed earlier (Au et al., 2008). Furthermore, a recent study in which OECs isolated from young and old rhesus monkeys were implanted in collagen scaffolds and implanted into immunodeficient mice demonstrated that the vessel forming ability of OECs declines with age (Shelley et al., 2012). Thus, it appears that assessing tubule formation in vitro on Matrigel, as in this study, may not have been the most suitable method for assessing the tube forming capacities of OECs.

In order to participate in postnatal vasculogenesis or vascular repair, EPCs must be able to respond to signals that initiate mobilisation from the bone marrow and enable homing to sites requiring neovascularization. Thus, impaired migration of OECs will limit the availability of these reparative cells at the site of injury, hindering repair and neovascularization. Accordingly, the hypothesis that OEC migratory capacity decreases progressively with age was tested, using an in vitro scratch assay and transwell migration system. A diminished migratory capacity of OECs was evident with increasing age using the in vitro scratch assay. Similarly, a significant reduction in OEC migration towards SDF-1α and VEGF-A was evident in OECs isolated from peripheral blood samples of old subjects, although the migratory capacity of OECs from young subjects was not significantly different from OECs of cord blood. These findings support the concept that age has a negative effect on the migratory response of OECs, which has also recently been demonstrated in vivo. Xia and colleagues evaluated the effect of transplantation of
OECs from young and elderly persons on in vivo re-endothelialization capacity in a nude mouse model of carotid injury. The transplantation of OECs from young subjects markedly accelerated re-endothelialization of the injured arteries, an effect which was significantly reduced following the transplantation of OECs from old subjects (Xia et al. 2012). Examination of the injured tissue revealed that far fewer OECs from old subjects were detected within the site of injury as compared to those from young subjects, suggesting that homing capacity of OECs is impaired with age and consequently their re-endothelialization capacity (Xia et al., 2012).

In this study, VEGF and SDF-1α were examined as there are several lines of evidence to suggest that these factors are important for the mobilisation of EPCs from the bone marrow and homing to sites of injury/neovascularization. For example, Yamaguchi et al. demonstrated that the local administration of SDF-1α augments the homing and incorporation of human EPCs at the site of ischemia and thereby enhances neovascularization (Yamaguchi et al., 2003). By contrast, inhibition of SDF-1α/CXCR4 axis, using a CXCR4 receptor antagonist (Abbott et al., 2004) or neutralizing anti-CXCR4 antibodies (Walter et al., 2005, Ceradini et al., 2004), has been shown to significantly reduce progenitor cell homing to the sites of ischemia. Additionally, VEGF gene transfer in patients with critical limb ischemia has been shown to augment the homing of EPCs to ischemic foci (Kalka et al., 2000a). Furthermore, conditional induction of VEGF in selected adult organs has been shown to induce the homing of bone-marrow-derived progenitor cells to the organ from which it emanates (Grunewald et al., 2006). However, a drawback of the transwell migration assays used in this study is the requirement for the preparation of cells in suspension which thereby disrupts cell-cell and cell-ECM interactions. Moreover, in the transwell migration system, cells are exposed to a steep and transient gradient which has been said to be a poor representation of the gradients formed in tissues.
A plausible explanation for this reduced migratory response is a decline in the expression of the cell surface receptors for VEGF and SDF-1α with age which could result in a lesser responsiveness to the ligands. The VEGF receptor family in mammals contains three members; VEGFR-1, VEGFR-2 and VEGFR-3 (also known as Flt-1, Flk-1/KDR and Flt-4 respectively) (Shibuya, 2002). VEGF-A elicits its function primarily by activating two receptor kinases, VEGFR-1 and VEGFR-2. Given that VEGFR-2, not VEGFR-1, is responsible for VEGFA stimulated endothelial cell migration the expression levels of this receptor in OECs during human ageing was examined. No significant difference in the expression levels of VEGFR-2 was detected between OECs isolated from cord blood and peripheral blood of young and old subjects. By contrast, a significant decline in CXCR4 expression was found in OECs from peripheral blood samples of old subjects. These data suggest that a reduction in CXCR4 expression with age could, in part, explain the decline in SDF-1α induced migration observed in OECs from old subjects. However, since the deterioration of VEGF-induced OEC migration with age could not be explained by a reduction in receptor expression, the possible link to an alteration in HS structure on the cell surface was to be next investigated.

4.5.1. Future directions

A number of experimental considerations regarding the present findings should be mentioned. Firstly, numerous pathological, pharmacological and physiological factors have been reported that influence EPC number and function. In an attempt to minimise these potential influences only adults who were free of overt disease, not receiving any medication and who were non-smokers were enrolled in this study. However, no baseline clinical characteristics such as blood pressure, cholesterol levels and body mass index were taken to ensure that all subjects were within the normal limits and not significantly different between groups. Thus, the possibility that factors, other than age, may have influenced the results cannot be ruled out. Similarly, the inherent possibility that genetic and/or
lifestyle behaviours may have influence the results of our study cannot be dismissed. In order to specifically investigate the effect of age on OEC function, future studies should only enroll subjects who meet strict exclusion criteria, as described in Chapter 3.4.1.

There is considerable evidence linking the progressive accumulation of reactive oxygen species (ROS) and oxidative stress to the physiology and pathophysiology of CVD. Interestingly, CFU-Hill cells, CACs and OECs have been shown to express high levels of antioxidant enzymes and as a result, are more resistant to oxidative stress, as compared with human umbilical vein endothelial cells (HUVECs) and adult microvascular endothelial cells (He et al., 2009, Cai et al., 2006, Dernbach et al., 2004, He et al., 2004). Such an enhanced antioxidant system may endow EPCs with improved resistance to oxidative stress and thus the ability to promote vascular regeneration in settings of ischemic injury. However, work has shown that the antioxidant capacity of CACs is impaired with age and as a result, they are more sensitive to oxidative stress-induced apoptosis than CACs from younger subjects (He et al., 2009). Given that oxidative stress is an important feature of age-related CVD risk, it would be interesting to determine whether OECs from older subjects are also more susceptible to apoptosis under induced oxidative stress, due to a decreased expression of antioxidant proteins.

In this study, whether the reduced migratory response of peripheral blood derived OECs from old subjects towards VEGF and SDF-1α may be explained by a reduction in the expression of the cell surface receptors for these two chemotactic agents was addressed using RT-qPCR analysis. No significant age-related differences in the expression levels of VEGFR-2 were observed and therefore it is postulated that other mechanisms may be responsible for the decline in VEGF-induced migration of peripheral blood derived OECs from old subjects. However, further work should measure the levels and activity status of the protein to be sure that there are no significant differences between the 3 sources of OECs used in this study.
This study found that OEC migratory capacity is diminished with age, as determined using an *in vitro* scratch assay and transwell migration system. An extension of this project would be to test the migratory capacity of these cells *in vivo*, such as in animal models of experimentally induced ischemia or injury. A decline in the ability of OECs from old subjects to migrate to an ischemic hindlimb, for example, would strongly support the concept that a decline in the migratory response of OECs with age limits the availability of these reparative cells at the site of injury and thus vascular regeneration and repair in the ageing host.

In summary, these findings suggest that OECs isolated from cord blood or peripheral blood samples from young and old individuals exhibit different characteristics in terms of their migratory capacity. Such changes may modulate the homing capacity of these putative repair cells, thereby contributing to a decreased capacity for neovascularization of ischemic tissues and/or reduced re-endothelialization of vascular lesions in the ageing host. However, the mechanisms underlying this age-related dysfunction are not fully understood.
Chapter 5:

Results III – Age-associated changes of heparan sulfate structure on the surface of OECs
5. Results III – Age-associated changes of heparan sulfate structure on the surface of OECs.

5.1. Introduction

A number of cell processes including cell adhesion, proliferation, migration and differentiation are dependent on factors whose activity is influenced by interactions with HSPGs (Kraushaar et al., 2010, Morgan et al., 2007, Dombrowski et al., 2009, Mitsi et al., 2006). Cell proliferation, migration and differentiation are regulated by a broad array of growth factors and cytokines, many of which bind heparin or HS, such as, the family of FGFs (Rahmoune et al., 1998), VEGF (Robinson et al., 2006), HB-EGF (Higashiyama et al., 1993), HGF (Lyon and Gallagher, 1994), PDGF (Feyzi et al., 1997) and various chemokines. These molecules bind cell surface receptors but, in many cases, are dependent on HS for efficient signal transduction, as an absence of HSPGs in mutant cell lines or cells treated with sodium chlorate or heparinase dramatically diminishes cellular responses (Kang et al., 2011). HSPGs are also known to play several roles in cell adhesion and in the determination of cellular morphology by binding various matrix proteins and integrins (Gopal et al., 2010). Many of the signalling molecules involved in the maintenance of stem cell pluripotency and the differentiation into committed tissue-specific progenitor cells are also regulated by HSPGs (Xu et al., 2005, Sato et al., 2004). For example, embryonic stem cells that are deficient in HS have been found to be incapable of differentiation, upon removal of leukemia inhibitory factor, due to defects in FGF signalling (Kraushaar et al., 2010).

A large amount of evidence suggests that the ability of HS to selectively bind different proteins ligands, and thereby modify their behaviour, is embedded in the fine structure of its polysaccharide chains. For a number of protein ligands, the density and pattern of sulfate substituents along the HS chain appears to be of special importance for both binding
and biological activity. For example, the presence of GlcNS and IdoUA[2S] residues within HS has been shown to be an absolute requirement for the interaction with FGF2 (Faham et al., 1996, Turnbull et al., 1992), whereas 6-O-sulfate groups are required for mitogenic activity of the complex (Pye et al., 1998, Guimond et al., 1993). By contrast, FGF1 requires N-, 2-O- and 6-O-sulfate groups for optimal binding to HS and activation (Pye et al., 2000). Changes in the fine structure of HS during development, differentiation, ageing or pathological processes may perturb these interactions and subsequently the physiological effects of ligand. Understanding the structure-activity relationships of HS is important in order to exploit the potential pharmacological and therapeutic value.

The most common initial approach to characterise HS structure is disaccharide analysis. This type of analysis permits an initial comparison of HS structures, in terms of the proportion of each disaccharide present in the sample, and is a first step towards determining structure-function relationships. Disaccharide analysis is commonly achieved using a combination of bacterial lyase enzymes, heparinase I, II and III, to cleave the HS chains to constituent disaccharides (termed Δ-disaccharides) (Linhardt et al., 1990). Each of the heparinase enzymes has particular cleavage site specificity at the glycosidic linkage within the HS chain. Heparinase I catalyses cleavage between GlcNS-IdoA[2S], and therefore cleaves within the S-domains. By contrast, Heparinase II has a broader specificity and cleaves independently of O- and/or N-sulfation, as well as the type of uronic acid residue. Heparinase III (heparitinase) catalyses cleavage between GlcNAc or GlcNS-GlcA, and thereby acts primarily within the non-sulfated domains (NA domains). When used in combination, these enzymes result in a near-complete depolymerisation of the HS chains to disaccharides.

Quantification of the disaccharide products from enzyme digestion is typically undertaken using high performance liquid chromatography (HPLC) techniques employing ultraviolet (UV) or fluorescence detection
Enzymatic cleavage of HS with heparinase introduces C4-C5 unsaturated double bonds at the non-reducing ends of the uronic acid residues. This enables direct detection of the newly generated constituent disaccharides through UV absorbance at 232nm (Linhardt et al., 1988). However, biological samples, including cells or tissues, are often available in limited amounts and yield very low quantities of purified HS following extraction. In this instance, the use of UV absorbance for detection and quantification of HS often lacks the necessary sensitivity. Furthermore, trace impurities within biological samples, which also absorb at the required UV wavelength, may interfere with such analysis. Increased sensitivity of detection has been achieved by labelling the Δ-disaccharides formed following heparinase digestion with fluorescent tags. Attachment of the fluorophore typically occurs through reductive amination, involving the amine or hydrazide functional group of the fluorophore and the carbonyl group of the reducing end of the disaccharides (Skidmore et al., 2010). Fluorometric analysis provides much greater signal intensities than traditional methods, with reported sensitivities as low as femtomoles. Using the most sensitive method for disaccharide compositional analysis of HS is important to ensure the accuracy of the resulting disaccharide expression profile. In cases where the sample size is small, as in this study, errors in calculating the contribution of each disaccharide to the sample can produce an inaccurate representation of HS structure.

5.2. Comparison of fluorescence detection methods for disaccharide compositional analysis of OEC-derived HS.

Three fluorophores; 2-cyanoacetamide (2-CA) (Toyoda et al., 1997), boron-dipyrromethene (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, BODIPY FL) hydrazide (Skidmore et al., 2010), and 2-Aminoacridone (AMAC) (Deakin and Lyon, 2008), that have been previously coupled to HS disaccharides were compared to
determine the most sensitive and accurate analytical method for disaccharide compositional analysis of OEC-derived HS.

5.2.1. 2-cyanoacetamide (2-CA)

Fluorescence detection using 2-CA has been used extensively for disaccharide compositional analysis of HS from a variety of tissues and cultured cells (Chen et al., 2005, Ledin et al., 2004, Holmborn et al., 2004, Staatz et al., 2001). Toyoda et al determined the limit of detection of 2-CA labelled Δ-disaccharides to be ~3pmol (Toyoda et al., 2000). In this detection method, Δ-disaccharides are separated by strong anion exchange (SAX) chromatography using a linear NaCl gradient which elutes the disaccharide species according to their sulfation density. As the disaccharides elute from the column they are labelled in-line with the HPLC and detected at 346nm excitation; 410nm emission.

Purified OEC-derived HS was degraded into disaccharides with a combination of heparinase I, heparinase II, heparinase III and its composition analysed by SAX-HPLC with detection using 2-CA as a fluorogenic reagent. Eluted disaccharides were quantified by comparison to 2-CA labelled disaccharide standards of known concentration (Figure 5.1). As depicted in Figure 5.2, when using this label to analyse HS extracted from OECs, five of the disaccharide species were identified in the sample, however, this method lacked the sensitivity required to detect the contribution of the rarer disaccharide unit ΔUA[2S]-GlcNS[6S] to the OEC HS sample. However, it is important to note that the 2-CA labelling method was able to detect all six disaccharide units from other HS sources, such as that from cultured HUVECs (Figure 5.3). In addition, post-column derivatization with 2-CA requires a complex set-up for post-column reaction, cooling and detection. Therefore, we tested BODIPY as an alternative fluorescent label for disaccharide compositional analysis of HS isolated from OECs.
Figure 5.1. Representative SAX-HPLC profile of HS disaccharides standards detected in-line using 2-CA as a fluorogenic reagent. Disaccharide standards were analysed by SAX-HPLC on a ProPac PAI column. Disaccharides were eluted with a linear gradient of NaCl and detected fluorometrically (346nm excitation, 410nm emission) by post-column derivatization with 2-CA. The numbers correspond to the HS disaccharides; 1. ΔUA[2S]-GlcNS[6S], 2. ΔUA-GlcNS[6S], 3. ΔUA[2S]-GlcNS, 4. ΔUA-GlcNS, 5. ΔUA-GlcNAC[6S] and 6. ΔUA-GlcNAC. LU denotes luminescence units.
Figure 5.2. Representative SAX-HPLC profile of OEC-derived HS labelled by post-column fluorescent derivatization with 2-CA. HS was isolated from OECs and degraded into constituent disaccharides by enzymatic digestion with heparinase I/II/III. Disaccharides were separated by SAX-HPLC with a NaCl gradient and detected in-line with 2-CA. Five of the disaccharide species were identified in the sample, however it was not possible to detect the less abundant disaccharide ΔUA[2S]-GlcNS[6S]. The numbers correspond to the HS disaccharides; 1. ΔUA[2S]-GlcNS[6S], 2. ΔUA-GlcNS[6S], 3. ΔUA[2S]-GlcNS, 4. ΔUA-GlcNS, 5. ΔUA-GlcNAc[6S] and 6. ΔUA-GlcNAc. The inset depicts an enlargement of the chromatogram in which ΔUA[2S]-GlcNS[6S] is expected to elute but was not detected using the 2-CA method of analysis. LU denotes luminescence units.
Figure 5.3. Representative SAX-HPLC profile of HUVEC-derived HS labelled by post-column fluorescent derivatization with 2-CA. HS was digested with equal proportions of heparinase I/II/III and analysed by SAX-HPLC using 2-CA as a fluorogenic reagent. All six of the HS disaccharides screened for were detected in the sample. The numbers correspond to the HS disaccharides; 1. ΔUA[2S]-GlcNS[6S], 2. ΔUA-GlcNS[6S], 3. ΔUA[2S]-GlcNS, 4. ΔUA-GlcNS, 5. ΔUA-GlcNAc[6S] and 6. ΔUA-GlcNAc. LU denotes luminescence units.

5.2.2. Boron-dipyrromethene hydrazide (BODIPY)

The analysis of HS structures using the fluorophore BODIPY hydrazide, coupled to SAX HPLC, has been reported to have a limit of detection ~100fmol, which provides >1,000 fold increased sensitivity over the use of conventional UV absorbance (Skidmore et al., 2010). In this method of analysis, as with 2-CA labelling, Δ-disaccharides are separated by SAX chromatography using a linear NaCl gradient. However, unlike 2CA labelling, the disaccharides must be derivatised with the fluorophore prior to separation. This derivatisation step increases the time taken for disaccharide analysis and involves the use of toxic reducing agents that require extra precautions when handling and disposing of the samples. HS was purified from OECs, degraded into constituent disaccharides by heparinase digestion and labelled overnight with BODIPY prior to analysis. Disaccharides were separated by application of a NaCl gradient,
detected at 488nm excitation, 520nm emission and quantified by comparison to BODIPY-labelled disaccharide standards of known concentration (Figure 5.4). Despite this fluorophore having a very strong emission signal, it was difficult to detect and integrate peaks corresponding to the disaccharides ΔUA-GlcNAc[6S] and ΔUA[2S]-GlcNS (Figure 5.5). In the case of ΔUA-GlcNAc[6S] it appeared to elute at the time as there is a shift in the background fluorescence during the separation, making detection difficult, whilst the contribution of the ΔUA[2S]-GlcNS disaccharide to the HS sample appeared to be too small to distinguish from the background. Therefore, we attempted labelling and detection using an alternative fluorophore, AMAC.

Figure 5.4. Representative SAX-HPLC profile of BODIPY-labelled HS disaccharides standards. Disaccharide standards were fluorescently labelled with BODIPY and then analysed by SAX-HPLC on a Propac PAI column. Disaccharides were eluted with a linear gradient of NaCl and detected fluorometrically at 488nm excitation, 520nm emission. The numbers correspond to the HS disaccharides; 1. ΔUA[2S]-GlcNS[6S], 2. ΔUA-GlcNS[6S], 3. ΔUA[2S]-GlcNS, 4. ΔUA-GlcNS, 5. ΔUA-GlcNAc[6S] and 6. ΔUA-GlcNAc. LU denotes luminescence units.
Figure 5.5. Representative SAX-HPLC profile of OEC-derived HS derivatised with BODIPY. HS was purified from OECs, digested with heparinases and labelled with BODIPY. Disaccharides were separated with a linear gradient of NaCl. Although all six disaccharides were detected in the sample, it was often difficult to distinguish the disaccharides ΔUA-GlcNS[6S] and ΔUA[2S]-GlcNS (peaks 2 and 3 respectively) from the background, making their integration and measurement difficult. The numbers correspond to the HS disaccharides; 1. ΔUA[2S]-GlcNS[6S], 2. ΔUA-GlcNS[6S], 3. ΔUA[2S]-GlcNS, 4. ΔUA-GlcNS, 5. ΔUA-GlcNAc[6S] and 6. ΔUA-GlcNAc. The inset depicts an enlargement of the chromatogram in which ΔUA-GlcNS[6S] and ΔUA[2S]-GlcNS elute but were difficult to distinguish from background using the BODIPY method. LU denotes luminescence units.

5.2.3. 2-Aminoacridone (AMAC)

The use of AMAC to analyse HS disaccharides has been determined to have a limit of detection of ~200fmol per disaccharide, which is approximately 15-fold more sensitive than 2-CA labelling and similar to that determined for BODIPY labelled Δ-disaccharides (~100fmol) (Deakin and Lyon, 2008). As with BODIPY, the use of AMAC requires disaccharides to be derivatised with the fluorophore prior to separation.
In this detection method, Δ-disaccharides are separated by reverse phase (RP) chromatography using an increasing concentration of organic solvent, which elutes the disaccharide species according to differences in hydrophobicity; trisulfated species first and nonsulfated species last. In the method described by Deakin and Lyon, HS and CS disaccharides derivatised with AMAC were separated on a C18 column by RP-HPLC using methanol as the elution solvent. However, commercial AMAC contains an impurity which, in our experience, would frequently co-elute with the HS disaccharide ΔUA-GlcNAc[6S], even after prior purification of the label. To overcome this problem, work in our laboratory modified the AMAC HPLC method described by Deakin and Lyon to include use of a new type of C18 column and an alternative elution solvent. The use of a Kinetex column coupled with acetonitrile as an eluting solvent was found to give good resolution of ΔUA-GlcNAc[6S] from the free AMAC label (Hamilton & Stringer unpublished data).

OEC-derived HS was purified, degraded into constituent disaccharides by enzymatic digestion with heparinases I, II and III and the resulting disaccharides labelled with AMAC overnight prior to separation. AMAC labelled disaccharides were eluted by an increasing amount of acetonitrile and detected fluorometrically at 425nm excitation, 520nm emission. Individual disaccharides were identified and quantified by comparison to AMAC-labelled disaccharide standards of known concentration (Figure 5.6). The fluorescence profile identified all six Δ-disaccharide species and had a much cleaner baseline in comparison to the profiles of 2-CA and BODIPY labelled OEC HS disaccharides. The peaks were well resolved from the baseline and from each other and distinct from any other impurities and contaminants, thereby enabling accurate quantification of the disaccharide species (Figure 5.7). The prior derivatisation step which extends the time taken for disaccharide analysis and requires the use of toxic reducing agents is a disadvantage associated with the use of the AMAC. Furthermore, the use of acetonitrile as a running buffer for AMAC RP-HPLC requires expensive specialist disposal. However, despite these drawbacks, of the fluorophores tested,
AMAC-labelling provided the most sensitive and accurate detection of OEC-derived HS disaccharides. All subsequent HS disaccharide compositional analysis used pre-column derivatisation with AMAC and fluorescence detection.

Figure 5.6. Representative RP-HPLC profile of AMAC labelled HS disaccharide standards. Disaccharide standards were fluorescently labelled with AMAC and then analysed by RP-HPLC on a Phenomenex Kinetex C18 column. Disaccharides were eluted with an acetonitrile gradient and detected fluorometrically at 425nm excitation, 520nm emission. The numbers correspond to the HS disaccharides (1) ΔUA-(2S)GlcNS(6S), (2) ΔUA-GlcNS(6S), (3) ΔUA-(2S)GlcNS, (4) ΔUA-GlcNS, (5) ΔUA-GlcNAc(6S) and (6) ΔUA-GlcNAc. LU denotes luminescence units.
Figure 5.7. Representative RP-HPLC profile of OEC-derived HS derivatised with AMAC. HS was isolated from OECs, degraded to constituent disaccharides by heparinase digestion and labelled with AMAC. Disaccharides were separated on a reverse phase C18 column by an increasing amount of acetonitrile. All six disaccharide species were identified in the sample and were well resolved from one another and distinct from any other impurities and contaminants. The numbers correspond to the HS disaccharides (1) ΔUA-(2S)GlcNS(6S), (2) ΔUA-GlcNS(6S), (3) ΔUA-(2S)GlcNS, (4) ΔUA-GlcNS, (5) ΔUA-GlcNAc(6S) and (6) ΔUA-GlcNAc. LU denotes luminescence units.

5.3. Age-associated changes of HS structure on OECs.

5.3.1. Disaccharide compositional analysis

To further explore the reduced migratory capacity of OECs detected with increasing age, we investigated the possible link to an alteration in HS structure on the cell surface. Structural changes of HS as a consequence of ageing were examined by disaccharide composition analysis of HS extracted from OECs isolated from cord blood (n=5) and from peripheral blood samples of young (n=5) and old (n=4) healthy subjects. HS was isolated and purified from OECs by proteolytic cleavage and nuclease treatment, followed by reverse phase chromatography. Purified HS from OECs was degraded into disaccharides with a combination of heparinase
I, heparinase II and heparinase III and disaccharide composition analysed by RP-HPLC with detection using AMAC as a fluorogenic reagent. The contribution of each type of disaccharide to the HS sample (expressed as a percentage of the total HS) and the type of sulfate substitution was calculated and is presented in Table 3 and Figure 5.8.

Table 3. Disaccharide composition and sulfate distribution within HS extracted from OECs isolated from cord blood (n=5) and peripheral blood samples of young (n=5) and old (n=4) healthy subjects. The total percentage of N-, 2-O- and 6-O-sulfation was calculated by summing the NS-, 2-O-sulfated, 6-O-sulfated containing disaccharide repeats; N-sulfated: ΔUA-GlcNS, ΔUA-GlcNS(6S), ΔUA-(2S)-GlcNS and ΔUA-(2S)-GlcNS(6S); 2-O-sulfated: ΔUA-(2S)-GlcNS and ΔUA-(2S)-GlcNS(6S); 6-O-sulfated: ΔUA-GlcNAc(6S), ΔUA-GlcNS(6S), ΔUA-(2S)-GlcNS(6S). Values represent mean values, SEM values are given in brackets.

<table>
<thead>
<tr>
<th>Disaccharide composition (% expression (nmol))</th>
<th>Cord Blood OECs</th>
<th>Young OECs</th>
<th>Old OECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA[2S]-GlcNS[6S]</td>
<td>10.85 (+0.11)</td>
<td>8.24 (+1.11)</td>
<td>3.57 (+0.99)</td>
</tr>
<tr>
<td>UA-GlcNS 6S</td>
<td>4.02 (+0.62)</td>
<td>1.79 (+0.46)</td>
<td>1.28 (+0.18)</td>
</tr>
<tr>
<td>UA-2S GlcNS</td>
<td>12.66 (+2.56)</td>
<td>15.36 (+0.97)</td>
<td>22.39 (+4.02)</td>
</tr>
<tr>
<td>UA-GlcNS</td>
<td>17.30 (+2.19)</td>
<td>20.53 (+0.87)</td>
<td>25.22 (+1.14)</td>
</tr>
<tr>
<td>UA-GlcNAc 6S</td>
<td>6.56 (+0.87)</td>
<td>2.84 (+0.25)</td>
<td>3.02 (+0.57)</td>
</tr>
<tr>
<td>UA-GlcNAc</td>
<td>48.61 (+4.24)</td>
<td>51.24 (+2.19)</td>
<td>44.52 (+5.19)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sulfate Composition (% expression (nmol))</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Total 2-O-sulfated</td>
<td>23.52 (+2.57)</td>
<td>23.59 (+1.69)</td>
<td>25.96 (+3.76)</td>
</tr>
<tr>
<td>Total 6-O-sulfated</td>
<td>21.43 (+0.77)</td>
<td>12.87 (+1.37)</td>
<td>7.88 (+1.41)</td>
</tr>
<tr>
<td>Total N-sulfated</td>
<td>44.83 (+4.31)</td>
<td>45.91 (+2.17)</td>
<td>52.46 (+4.27)</td>
</tr>
<tr>
<td>Total Sulfation</td>
<td>51.40 (+4.24)</td>
<td>48.76 (+2.19)</td>
<td>55.48 (+4.50)</td>
</tr>
<tr>
<td>Total Unsulfated</td>
<td>48.61 (+3.96)</td>
<td>55.02 (+3.75)</td>
<td>44.52 (+4.51)</td>
</tr>
</tbody>
</table>
The proportions of specific disaccharide units within OEC HS polysaccharide chains were found to be altered during human ageing. The results of the analysis demonstrate that a major component of OEC HS in all three groups was the disaccharide unit ΔUA-GlcNAc, accounting for 44-54% of the total HS (Figure 5.8). However, a significant reduction in the proportion of the trisulfated disaccharide ΔUA[2S]-GlcNS[6S] was detected within HS samples isolated from peripheral blood of young and old subjects as compared to HS extracted from cord blood OECs (p=0.020). A significant reduction in the abundance of this disaccharide unit was also evident within HS from old subjects in comparison to their younger counterparts (p=0.014). The disaccharide units ΔUA-GlcNS[6S] and ΔUA-GlcNAc[6S] were significantly reduced in OEC-derived HS from peripheral blood of young (p=0.047 and p=0.028 respectively) and old (p=0.014) subjects as compared to the HS samples from cord blood OECs. By contrast, a significant increase in the abundance of the disaccharide units ΔUA[2S]-GlcNS and ΔUA-GlcNS was detected within HS isolated from OECs of old subjects as compared to the HS samples isolated from OECs of cord blood (p=0.05 and p=0.014 respectively) and peripheral blood of young subjects (p=0.027 and p=0.014 respectively). Calculation of the overall extent of 2-O-sulfation and 6-O-sulfation indicated that the age-associated decline in the proportion of the ΔUA[2S]-GlcNS[6S] unit was due to a significant decrease in the levels of 6-O-sulfate substitution of GlcNS residues with increasing age (Pearsons correlation coefficient; -0.830, significant at the p<0.01 level), whilst 2-O-sulfation of GlcA or IdoA residues did not significantly change (Pearsons correlation coefficient; 0.158) (Figure 5.9).
Figure 5.8. Comparative disaccharide composition analysis of HS from OECs isolated from cord blood (green bar) (n=5) and peripheral blood of young (yellow bar) (n=5) and old (blue bar) (n=4) subjects. HS was extracted from OECs, degraded into disaccharides and labelled with AMAC prior to analysis by RP-HPLC. Disaccharides were quantified by calibration against disaccharide standards of known concentration. Data was analysed as (A) the proportion of each type of disaccharide present in the sample (expressed as % of total HS) and (B) grouped by sulfation type. (A) Structural analysis of HS by HPLC demonstrates a significant reduction in the abundance of the trisulfated disaccharide UA[2S]-GlcNS[6S] within the HS of peripheral blood derived OECs from young and old subjects as compared to the HS samples from cord blood OECs. A
significant reduction in the proportion of this disaccharide unit was also detected within HS from peripheral blood derived OECs of old subjects in comparison to younger counterparts. The disaccharide units; UA-GlcNS[6S] and UA-GlcNAc[6S] were significantly reduced within the HS of peripheral blood derived OECs from young and old subjects as compared to the HS isolated from cord blood derived OECs. By contrast, the proportion of the disaccharide units; UA[2S]-GlcNS and UA-GlcNS was significantly elevated within HS isolated from peripheral blood derived OECs of old subjects as compared to the HS samples from OECs of cord blood and peripheral blood of young subjects. (B) Calculation of the overall extent of 2-O-sulfation, 6-O-sulfation, N-sulfation, total sulfation and unsulfated within the various HS samples demonstrated a significant reduction in 6-O-sulfate substitution within HS of peripheral blood derived OECs from young and old subjects in comparison to that from cord blood OECs. The overall extent of 6-O-sulfation was also significantly reduced in HS from peripheral blood derived OECs of old subjects as compared to younger counterparts. Profiles presented are from 3 separate digests of each sample. Bars represent mean values, error bars are the standard error of the mean. Comparisons between groups were analysed by Mann Whitney U tests; * p=<0.05, ** p=<0.01.
Figure 5.9. Age-associated changes in O-sulfate distribution within OEC HS. (A) A significant decrease in the percentage expression of the trisulfated disaccharide unit UA[2S]-GlcNS[6S] within OEC HS chains was detected with increasing age (significant at the 0.01 level (2-tailed)). Calculation of the overall extent of (B) 6-O- and (C) 2-O-sulfate substitution indicated that this decrease was due to a decline in the level of 6-O-sulfation of GlcNS residues (significant at the 0.01 level (2-tailed)), whereas IdoA or GlcA 2-O-sulfation did not significantly change with subject age. Correlations were analysed using Pearson’s correlation coefficients.

5.3.2. Size determination of HS chains from OECs

The length of HS structures is also a critical determinant of the interaction between HS and protein ligands (Osterholm et al., 2009, Yamada et al., 2004). HS chains can vary in size from ~5 to ~70kDa and control over chain length appears to be a regulatory factor in HS-dependent signalling (Iozzo, 2001). For example, HS chain length is substantially reduced, but apparently normally sulfated, in fibroblasts of mice carrying a hypomorph mutation in Ext1. These mutant fibroblasts have been shown to have a decreased capacity to respond to FGF2, which presumably relates to loss of binding of FGF2 to the truncated chain, or an inability of the shorter chains to present FGF2 to its receptor (Osterholm et al., 2009).

Given the importance of HS chain length in a number of HS-dependent growth factor signalling pathways, we investigated whether the size of OEC HS chains are altered during the ageing process. To determine the approximate size of OEC-derived HS, metabolically radio-labelleled HS was analysed by Sepharose CL-6B gel filtration chromatography (Figure 5.10) and sized with reference to published calibration curves (Wasteson, 1971). The avidity constant ($K_{av}$) of the resolved fragment was calculated using the formula; $K_{av} = \frac{\text{peak} - V_0}{V_t - V_0}$. From this, the molecular weight was estimated using the calibration curve of Wasteson 1971. The molecular weight of HS chains on the cell surface was determined for OECs isolated from cord blood (n=3) and peripheral blood of young (n=3)
and old subjects (n=3) (Figure 5.11). The size of the HS chains on the cell surface was within the range of 31.05 - 39.5kDa and was not significantly different between the 3 groups of OECs analysed. Assuming an average molecular mass of 400Da per disaccharide, the chain sizes of OEC-derived HS were within the range of 78-99 disaccharides in length.

**Figure 5.10.** Representative CL-6B profile of OEC-derived HS. 20kdpm of [3H] glucosamine labelled HS extracted from OECs was resolved on a Sepharose CL-6B column to determine the approximate size of the HS fragments. Kav was calculated for the resolved fragment from the elution time of HS and the void (Vo) and total (Vt) markers. From this, the average molecular weight of the fragment was determined by reference to the calibration curve of Wasteson (1971). Vo and Vt are indicated and were determined using Dextran blue and phenol red respectively.
Figure 5.11. Comparison of the molecular weight of HS isolated from OECs of cord blood (n=3) and peripheral blood of young (n=3) and old (n=3) subjects. The size of the HS chains on the cell surface was within the range of 31.05 - 39.5kDa and was not significantly different between the 3 groups of OECs analysed. Bars represent mean values, error bars are the standard error of the mean. The Kruskal-Wallis test was used for an overall comparison between the groups.

5.4. Quantitative analysis of Sulf-1 and Sulf-2 expression in OECs during ageing

The structural changes of HS detected during ageing may reflect an age-dependent alteration of the enzymes regulating the biosynthesis of HS. Structures containing 6-O-sulfate groups in HS are regulated during the process of biosynthesis by sulfotransferases, and also at the post-biosynthesis stage through the action of Sulf-1 and Sulf-2 (Morimoto-Tomita et al., 2002). Given the significant reduction in the abundance of 6-O sulfation, particularly the trisulfated disaccharide ΔUA[2S]-GlcNS[6S], within OEC HS with increasing age, we sought to determine whether this
may be due, in part, to increased levels of Sulf-1 and Sulf-2 expression in OECs from old subjects.

The expression of Sulf-1 and Sulf-2 mRNA in OECs isolated from cord blood (n=4) and peripheral blood samples of young (n=4) and old (n=4) subjects was determined using RT-qPCR. There appeared to be a trend for an increase in expression of both Sulf-1 and Sulf-2 with age, but this did not reach statistical significance, due to the broad range of expression in the samples, particularly for the peripheral blood derived OECs isolated from old subjects (Figure 5.12).
Figure 5.12. Comparison of the expression levels of SULF-1 and SULF-2 from OECs isolated from cord blood (n=4) and peripheral blood of young (n=4) and old (n=4) subjects. Real-time quantitative PCR analysis revealed no statistically significant difference in expression of SULF-1 or SULF-2 between the 3 groups of OECs. Expression was normalised to ATP5B. Median values are presented. Comparisons between groups were analysed by Mann Whitney U tests.
5.5. Discussion

HS is able to interact with a diverse array of regulatory factors, including growth factors and their receptors, cytokines, extracellular matrix molecules and enzymes. These interactions can regulate ligand-receptor binding, conformation, stability, or gradient formation of the corresponding ligands. Consequently, HS plays a key role in a network of cellular events from mediating cell adhesion and migration, to regulating proliferation, and differentiation (Tumova et al., 2000). The ability of HS to interact with specific ligands is thought to be dependent on the pattern and density of sulfate substituents along the chain. Thus, changes in HS structure during ageing may modulate the signalling activity and physiological effects of particular ligands, with important implications for a number of cellular processes. Here, the aim was to determine if human ageing is associated with alterations in the fine structure of HS on the cell surface of OECs.

In order to gain an accurate representation of age-associated changes in OEC HS structure, three fluorophores were compared to determine the most sensitive method for disaccharide compositional analysis. Of the methods tested, AMAC labelling with conditions optimised by work in our laboratory was determined to be the most sensitive method for disaccharide compositional analysis of HS isolated from OECs. The compositional analysis in this study screened for six disaccharide species within HS samples and did not examine the contribution of the rare disaccharide species UA-GlcNAc[3S] and UA-[2S]-GlcNAc. The reasoning for this being that these disaccharide species are so rare and often not detected in biological samples that their exclusion was deemed unlikely to affect our calculations when determining the proportions of the more common disaccharides within the HS samples.

To date, only a small number of studies have examined age-related structural alterations of HSPGs in adults. Feyzi et al. demonstrated a
change in the fine structure of HS from the aortic wall (human abdominal aorta isolated by autopsy) with increasing age (Feyzi et al., 1998). This was suggested to be due to an increase in the amount of 6-O-sulfation, particularly within NS-domains, resulting in a higher abundance of the trisulfated disaccharide ΔUA[2S]-GlcNS[6S]. Work by Murata et al. also demonstrated an elevated proportion of the trisulfated disaccharide ΔUA[2S]-GlcNS[6S] with ageing in the cerebral arteries (isolated by autopsy) of Japanese women (Murata et al., 1997). However decreased levels of this trisulfated disaccharide unit were found in atherosclerotic cerebral arteries compared to unaffected controls. In another study, ageing was shown to be accompanied with structural changes of HS in the myocardium left ventricle of rats, involving enhanced 6-O-sulfation, reduced N-sulfation and likely reduced 2-O-sulfation (Huynh et al., 2012). Interestingly, in these studies, a change in 6-O-sulfation of the HS chains appeared to be a common element during the ageing process.

In this study, age-associated structural changes of HS on the surface of OECs were evident by disaccharide analysis. However, in contrast to previous studies examining age-related changes of HS structure, we found a significant reduction in the percentage expression of the trisulfated ΔUA[2S]-GlcNS[6S] disaccharide unit within OEC HS polysaccharide chains with increasing age. This appears to be due to a significant decrease in the levels of 6-O-sulfate substitution of GlcNS residues with increasing age. Our observations maybe explained by the fact that HS species expressed by different cell types, as well as different tissues are to known to be structurally distinct. It is also apparent that HS structures, particularly with regard to 6-O-sulfation, are modified during processes such as development, ageing and with a number of disease conditions. The change in 6-O-sulfation of OEC HS with age is of interest as 6-O-sulfation of glucosamine has been found to play a crucial role in a number of biological functions. Numerous studies have demonstrated the importance of 6-O-sulfation of HS in the activation of FGF signalling. In chlorate treated fibroblasts, exogenous heparin was found to promote the mitogenic activity of FGF-2, whereas 6-O-desulfated heparin failed to
induce this activity (Guimond et al., 1993). Another study showed that 6-O-desulfated heparin is able to bind to FGF-2 but cannot potentiate the interactions between FGF-2 and FGFR-1 (Rusnati et al., 1994). Crystallographic studies have also supported the involvement of 6-O-sulfation of HS in the cellular response to FGF (Pellegrini et al., 2000). More recently, murine embryonic fibroblasts derived from 6-O-sulfotransferase (HS6ST-1 and HS6ST-2) deficient mice were shown to exhibit a significant reduction in FGF-2 and FGF-4 dependent signalling (Sugaya et al., 2008). A number of studies have also provided evidence that 6-O-sulfated HS is important for the binding of VEGF-A

In HUVECs that are deficient in endogenous HS by chlorate treatment, exogenous heparin can promote the mitogenic activity of VEGF-A. However, selectively N-desulfated and 6-O-desulfated heparin were unable to induce this activity (Ono et al., 1999). The number and arrangement of 6-O-sulfated disaccharide units within HS has been shown to be critical in determining the binding affinity for VEGF-A (Robinson et al., 2006). Furthermore, modulation of the level of 6-O-sulfation of HS in zebrafish embryos, by genetic manipulation of sulfotransferases (HS6STs), was shown to disrupt VEGF-mediated branching morphogenesis of the caudal vein (Chen et al., 2005). The arrangement of 6-O-sulfate groups within HS has also been shown to play an important role in the binding of Endostatin, an endogenous inhibitor of angiogenesis (Blackhall et al., 2003).

The 6-O-sulfation pattern of HS is determined during intracellular biosynthesis and can be further modified at the cell surface by a novel class of extracellular sulfatases, Sulf-1 and Sulf-2. These enzymes exist in a cell surface-associated form and soluble form and hydrolyse the 6-O sulfate groups of HSPGs (Ai et al., 2003, Ohto et al., 2002). The remodelling on 6-O-sulfation of HS by the Sulfs has been shown to regulate HS dependent extracellular signalling including that involved in cell proliferation, migration and differentiation. For example, Sulf-1 mediated 6-O-desulfation of HS has been shown to negatively regulate FGF signalling and FGF-2 dependent angiogenesis by disrupting the
formation of the FGF-HS-FGFR ternary complex (Wang et al., 2004). Numerous studies have demonstrated that Sulf-1 and Sulf-2 act predominately on the trisulfated UA-[2S]GlcNS[6S] (Lamanna et al., 2008, Ai et al., 2006, Ai et al., 2003, Morimoto-Tomita et al., 2002).

This study examined whether the age-associated reduction in the abundance of 6-O sulfation, particularly the trisulfated disaccharide ΔUA[2S]-GlcNS[6S], within OEC HS is due, in part, to an increase in the expression of Sulf-1 and Sulf-2. Although, there was an apparent trend for an increase in expression of both Sulf isoform with age, this did not reach statistical significance. There was a substantial amount of variation in the expression of these enzymes between subjects within each group, particularly within OECs from old subjects. The reasons for this variation are unclear and future work would require a larger number of subjects to be examined to determine whether any significant differences in expression levels do exist. An alternative or additional possibility is that the structural changes of HS detected during ageing may reflect an age-dependent alteration in the expression of the sulfotransferases, which would require further investigation.

5.5.1. Future directions

In addition to biochemical analysis, the structure of HS may also be revealed by the expression patterns of single chain fragment variable (ScFv) antibodies which recognise distinct patterns of sulfation within HS chains. However unlike the disaccharide analysis the antibody epitopes will be dependent on both the sulfation pattern and the conformation this confers to the HS chain. These antibodies have been successfully used to investigate changes in cell surface HS sulfation during the differentiation of embryonic stem cells to neural progenitor cells (Johnson et al., 2007) and during mesodermal differentiation and hemangioblast specification (Baldwin et al., 2008). A useful extension of this project would be to probe OECs from cord blood and peripheral blood of young
and old subjects with a panel of these antibodies. The combination of antibody expression profiles could provide an indication as to the composition of HS on the cell surface to complement the results obtained from disaccharide compositional analysis.

In addition to structural changes of HS, it would also be interesting to determine whether the overall amount of HS on the surface of OECs changes during ageing. This could be achieved by subjecting OECs from the 3 sources used in this study to heparinase treatment and subsequently probing the cells with the monoclonal antibody 3G10, which recognises the ‘stub’ motif remaining on the core protein following heparinase digestion (David et al., 1992). The signal generated by this antibody can be determined by flow cytometric analysis or Western blotting and would be expected to be a function of the amount of core protein and the number of HS attachment sites per molecule. Alternatively, the amount of HS could be determined from disaccharide compositional analysis and standardised to the amount of protein per sample.

Further work is required to determine how the age-associated changes in 6-O-sulfation detected within OEC HS translate into an alteration of the enzymatic machinery regulating HS biosynthesis. In addition to studying the expression of Sulf-1 and Sulf-2 in larger numbers of cord blood OECs and peripheral blood OECs of young and old subjects, it would also be useful to examine the expression of the sulfotransferases. Furthermore, it is also important to bear in mind that the mRNA levels of these enzymes may not vary with age, but rather their translation in functional protein or activity at the cell surface.

In summary, our findings suggest that human ageing is associated with alterations in the fine structure of HS on the cell surface of OECs. These findings raise intriguing questions concerning the functional consequences of such age-related structural changes of HS.
Chapter 6:

Results IV – Correlation between HSPG structure and OEC migration
6. Results IV – Correlation between HSPG structure and OEC migration

6.1. Introduction

In order to participate in postnatal vasculogenesis or vascular repair, EPCs must first respond to signals that initiate mobilisation from the bone marrow and then home to sites requiring neovascularization by following chemotactic gradients that are formed in these regions. After reaching the target site, EPCs must interact with extracellular matrix components in order to arrest and adhere at the site. Here, they must migrate through the endothelium and subendothelial matrix and finally migrate and invade the target tissue, where they can participate in repair and regeneration by either incorporating into the vasculature or exerting paracrine support to the endothelium. The directional migration of EPCs is critical throughout these processes and is mediated by various chemokines, extracellular matrix components, growth factors and membrane receptors. There is a vast amount of evidence to suggest that SDF-1α (Walter et al., 2005, Abbott et al., 2004, Ceradini et al., 2004, Yamaguchi et al., 2003) and VEGF (Grunewald et al., 2006, Kalka et al., 2000a) produced by damaged and ischemic tissues are important chemoattractants for EPCs.

HS is able to bind and regulate the biological activity of various proteins including growth factors, chemokines and their receptors through numerous modes of actions such as facilitating ligand-receptor binding, altering protein conformation, increasing protein stability or modulating growth factor gradient formation. These regulatory interactions play a role in a variety of cellular processes including cell migration (Albanese et al., 2009, Zoeller et al., 2009, Netelenbos et al., 2002, Ruhrberg et al., 2002). Both SDF-1α and VEGF are able to bind HS. In the case of SDF-1α, HS appears to play a role in regulating the activity of this chemokine in several ways, such as, enhancing SDF-1α immobilisation to form gradients of the protein along cell surfaces which guide directional cell migration (Campanella et al., 2006), protecting the enzyme from
enzymatic degradation (Sadir et al., 2004) and possibly by facilitating ligand-receptor binding to initiate signalling (Handel et al., 2005).

Similarly, there are several lines of evidence to suggest that HS is an important modulator of VEGF signalling. VEGF is known to bind with high affinity to HS (Robinson et al., 2006), and cells in which HS has been enzymatically removed have abrogated VEGF signalling (Ashikari-Hada et al., 2005). HS may modulate the activity of VEGF by enhancing the interaction of VEGF with VEGFR-2 directly (Zoeller et al., 2009, Dougher et al., 1997, Gitay-Goren et al., 1992), or by influencing the interaction of VEGF with the co-receptor NRP-1 (Fuh et al., 2000). Alternatively, HS may immobilise VEGF to increase its local concentration at the cell surface, thereby enhancing the probability of receptor binding. The interaction of HS with VEGF has also been shown to shape extracellular gradients of this protein to provide cues for the directional migration of endothelial cells (Ruhrberg et al., 2002).

Numerous studies have investigated the structural features of HS that are required for the binding of VEGF (Zhao et al., 2012, Robinson et al., 2006, Ono et al., 1999). These studies suggest that that both N- and 6-O-sulfate groups within HS are important for the binding of VEGF, whilst 2-O-sulfate groups contribute to a lesser extent for binding, but maybe required for signalling. Much less is known regarding the structural features of HS required for efficient binding of SDF-1α. However, Sadir et al. examined the ability of a range of selectively desulfated heparins to compete with native heparin for binding to SDF-1α. The authors demonstrated that both N- and 2-O-desulfated heparins had a significantly decreased ability to compete for binding, indicating that both N- and 2-O-sulfate groups are important in the HS-SDF-1α interaction. By contrast, the competitive effectiveness of 6-O-desulfated heparin was only slightly reduced compared with native heparin, suggesting that 6-O-sulfation of HS is less important for the binding of SDF-1α (Sadir et al., 2001).

Given that both VEGF and SDF-1α appear to require specific structural features of HS for binding, it is possible that any change in the fine
structure of HS during the ageing process could influence ligand binding abilities and, subsequently, the physiological activities of the ligands.

The final objective of this study was to determine if the structural changes detected within OEC HS during the ageing process correlates with the migratory response of these cells to VEGF and SDF-1α.

6.2. 6-O-sulfation and OEC migration

Chapter 4 describes a significant reduction detected in the migratory response of peripheral blood derived OECs from old subjects towards SDF-1α and VEGF. In these experiments, trypsinization was the most suitable method to detach OECs from culture flasks prior to seeding into transwell migration assays. However, it is important to note that the core protein of HSPGs is sensitive to cleavage by serine proteases such as trypsin, resulting in separation of the region of the proteoglycan bearing the HS side chains from the lipophilic domain, which presumably remains cell-associated (Jalkanen et al., 1987, Rapraeger and Bernfield, 1985). To prevent the loss of HS from the cell surface alternatives to trypsin such as cell scraping and the use of cell dissociation buffers for detaching OECs from the culture flasks prior to transwell migration analysis were tested. In the case of cell scraping it was difficult to obtain a single cell suspension for seeding into the porous transwells without damaging the cells, whilst the cell dissociation buffer was not suitable for detaching the strongly adherent OECs. Consequently trypsin was used, but to minimise the loss of HS incubation times were kept to a minimum (less than 90 seconds). Disaccharide compositional analysis was also performed to detect any changes in HS structure after trypsinization (Figure 6.1). This was in order to ensure that the disaccharide composition analysis of the trypsinized OECs was comparable to the OEC HS described in Chapter 3. Trypsinization of the cells did alter the structure of OEC HS, with significant differences detected in the percentage expression of the disaccharides the UA-[2S]GlcNS, UA-GlcNS and UA-GlcNAc.
Importantly, however, the 6-O-sulfated disaccharides, in particular the UA-[2S]GlcNS[6S], for which we had observed significant age-associated changes in abundance and a significant correlation with migratory response, was not significantly altered following trypsin treatment.

Figure 6.1. Comparative disaccharide composition analysis of HS from OECs isolated from peripheral blood of young (n=3) subjects which had been scraped (yellow bars) or trypsinized (blue bars) from the culture flasks. Structural analysis of HS by HPLC demonstrated a significant difference in the abundance of the UA-[2S]GlcNS, UA-GlcNS and UA-GlcNAc disaccharides following trypsinization of the cells. Importantly, no significant difference in the percentage expression of the disaccharide UA-[2S]GlcNS[6S] was detected between HS extracted from OECs which had been scraped from the culture flasks and those which had been detached by trypsinization. Bars represent mean values, error bars are the standard error of the mean. Comparisons between groups were analysed by Mann Whitney U tests; * p<0.05.

Chapter 5.3 describes the changes detected in O-sulfate distribution within HS chains of OECs during the ageing process. In this part of the study, the aim was to determine whether these age-associated structural changes of HS were associated with the reduced migratory capacity of OECs towards SDF-1α and VEGF that was seen in old subjects. The correlation between variables was analysed using Pearson’s correlation coefficient analysis, denoted r, which provides a measure of the strength
of the association between the two variables. Given the strong, negative correlation observed between subject age and the overall extent of 6-O-sulfation of OEC HS \( (r=-0.830, \ p<0.01) \), initially the association between the total level of 6-O-sulfation within OEC HS and the migratory response of OECs towards VEGF or SDF-1\(\alpha\) was examined. For both SDF-1\(\alpha\) and VEGF induced migration and 6-O-sulfation levels within HS, a strong, positive correlation between these two variables was evident; with higher levels of 6-O-sulfation of HS associated with higher levels of cell migration towards these chemotactic agents (SDF-1; \( r=0.537, \ p=0.048 \) and VEGF; \( r=0.767, \ p<0.01 \)) (Table 4) (Figure 6.2).

Table 4. Pearson's correlation coefficient between measures of 6-O-sulfated disaccharide residues and OEC transwell migration.

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<tr>
<td>SDF-1 induced migration</td>
<td>0.537*</td>
<td>0.682**</td>
<td>0.390</td>
<td>0.096</td>
</tr>
<tr>
<td>VEGF induced migration</td>
<td>0.767*</td>
<td>0.840**</td>
<td>0.485</td>
<td>0.403</td>
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</table>

Values presented are Pearson's correlation coefficient. *\( p<0.05 \) (2-tailed), **\( p<0.01 \) (2-tailed)
Figure 6.2. Correlation between the overall extent of 6-O-sulfation of glucosamine residues within HS chains on the cell surface of OECs and cell migration towards (A) SDF-1α and (B) VEGF. A strong, positive correlation was detected between the total 6-O-sulfate content within OEC HS chains and both SDF-1α \((r=0.537^*)\) and VEGF \((r=0.767^{**})\) induced migration of OECs. Correlations were analysed using Pearsons correlation coefficients. * \(p<0.05\), ** \(p<0.01\).

Next, the question as to whether the changes in OEC migration were associated with changes in the expression of specific 6-O-sulfated disaccharides within the HS chains, rather than simply an overall change in the extent of 6-O-sulfation within HS, was addressed. A strong, positive correlation was found between the percentage expression of the disaccharide UA-[2S]GlcNS[6S] within HS chains on the cell surface and OEC migration towards both SDF-1α and VEGF \((SDF-1\alpha; r=0.682, p=0.007\) and VEGF; \(r =0.840, p<0.01\)). As the levels of expression of this
trisulfated disaccharide increase, so does the relative fold in migration of OECs towards VEGF and SDF-1α (Figure 6.3). There was a moderately strong correlation ($r=0.485$) between the percentage expression of UA-GlcNS[6S] and the relative fold of VEGF-induced cell migration, but this did not reach statistical significance. By contrast, the correlation was weaker between the percentage expression of this disaccharide and SDF-1α-induced cell migration and again did not reach statistical significance (Figure 6.4). Finally, no significant correlations between the expression levels of UA-GlcNAc[6S] within the HS chains with either SDF-1α or VEGF-induced migration of OECs was observed (Figure 6.5).
Figure 6.3. Correlation between the percentage expression of the disaccharide UA-[2S]GlcNS[6S] within HS chains on the cell surface and OEC migration towards (A) SDF-1 and (B) VEGF. A strong, positive correlation was detected between the levels of expression of the UA-[2S]GlcNS[6S] disaccharide and both SDF-1 (r=0.682**) and VEGF (r=0.840**) induced migration of OECs. Correlations were analysed using Pearsons correlation coefficients. **p<0.01.
Figure 6.4. Correlation between the percentage expression of the disaccharide UA-GlcNS[6S] within HS chains on the cell surface and OEC migration towards (A) SDF-1 and (B) VEGF. No significant correlation was detected between the percentage expression of the disaccharide UA-GlcNS[6S] within OEC HS and SDF-1 or VEGF induced cell migration. Correlations were analysed using Pearson's correlation coefficients.
Figure 6.5. Correlation between the percentage expression of the disaccharide UA-GlcNAc[6S] within HS chains on the cell surface and OEC migration towards (A) SDF-1 and (B) VEGF. No significant correlation was detected between the percentage expression of the disaccharide UA-GlcNAc[6S] within OEC HS and SDF-1 (r=0.096, n.s) or VEGF (r=0.403, n.s) induced cell migration. Correlations were analysed using Pearsons correlation coefficients. p<0.05 was considered statistically significant. n.s; non significant.
6.3. HSPG disruption reduces OEC migration.

In order to gain further insight into the role of HS in SDF-1α and VEGF-mediated chemotaxis of OECs, a combination of heparinase I and heparinase III was used to degrade HS on the cell surface and the effect on migration assessed. Heparinase I and III cleave at distinct linkages found in HS; heparinase I cleaves within the highly sulfated regions, whilst heparinase III cleaves at regions of low sulfation. These bacterial lyases have commonly been used to selectively degrade HS on the cell surface and the effect on various cellular processes assessed (Kang et al., 2011, Florian et al., 2003). Several concentrations (1-20mIU/ml) and durations (30-90 minutes) of heparinase I/III treatment, within the range that have previously been reported in the literature, were tested. Longer treatment times appeared to impact upon cell viability, thus a shorter incubation time (30 minutes) using a concentration of heparinase enzymes (10mIU/ml) at the higher end of the range of concentrations was chosen. OECs isolated from the peripheral blood of young subjects (n=3) were pre-treated with a combination of heparinase I and III (10mIU/ml) for 30 minutes at 37°C, prior to seeding in transwell migration assays.

To verify that a substantial fraction of cell surface HS was removed by heparinase treatment, both treated and untreated cells were labelled with an anti-HS antibody (10E4) and visualised with a fluorescently labelled secondary antibody. As shown in Figure 6.6, OECs without enzymatic treatment (incubated in buffer alone) exhibited uniform staining of cell surface HS. By comparison, a substantial reduction of cell surface HS staining was seen following enzymatic treatment, appearing to be proportional to the degradation of HS on the cell surface. It was evident from the immunostaining that not all of the cell surface HS had been removed following heparinase I/III treatment, but longer treatment periods had a detrimental effect on cell viability. The immunostaining results were also confirmed by extracting HS from heparinase I/III treated or untreated (buffer alone) OECs isolated from the peripheral blood of young subjects.
(n=3) and analysing by HPLC; an average reduction of 66.46% in the amount of HS following heparinase I/III treatment.
Figure 6.6. Representative immunofluorescence staining of HS on the surface of OECs. To verify disruption of HSPGs by heparinase treatment (A) cells without enzymatic treatment and (B) treated cells were fixed and stained with an anti-HS (10E4) antibody. Heparinase I/III treated OECs showed decreased cell surface staining with 10E4 (green) as compared to (B) the untreated control cells. Nuclei were counterstained with DAPI (blue), actin filaments were stained with phalloidin (red). Magnification x200, scale bar represents 100µm.
Following exposure to heparinase I/III or buffer alone (control), OECs were immediately seeded in the transwell migration assay and allowed to migrate towards SDF-1α or VEGF for a period of 6 hours. Figure 6.7 shows that heparinase I/III treatment of OECs from young subjects (n=3) significantly reduced their migratory response towards VEGF, indicating that cell surface HS plays a role in VEGF-induced migration of OECs. Of note, the levels of migration of OECs isolated from the peripheral blood of young subjects became similar to those previously observed by OECs isolated from old individuals; VEGF induced a 2.13 fold increase in migration in untreated cells, significantly higher than the 1.62 fold increase in migration of heparinase I/III treated OECs (p=0.046). Although the migratory response of OECs pre-treated with heparinase I/III towards SDF-1α was lower than that seen by the untreated cells, there was no statistically significant difference between the two groups; SDF-1α induced a 1.79 fold increase in migration in untreated cells which was not significantly different to the 1.50 fold increase in migration of heparinase I/III treated OECs.
Figure 6.7. HSPG disruption significantly reduces the migratory response of OECs towards VEGF. Heparinase I/III treatment (blue bars) of OECs isolated from the peripheral blood of young subjects (n=3) was found to significantly reduce their migratory response towards VEGF, but not SDF-1α, in comparison to the untreated control (yellow bars). *p<0.05.

6.5. Discussion

The migration of EPCs is essential for progenitor cell homing to sites of vascular injury or ischemia and both SDF-1α and VEGF are key factors involved in this process. Given that both SDF-1α and VEGF are known to bind HS and, furthermore, appear to require specific structural features of HS for this binding, we hypothesised that a change in the disaccharide composition of OEC HS during ageing correlates with the ability of these factors to mediate cell migration.

A number of investigators have shown that 6-O-sulfate groups within HS are important for the interaction of HS with VEGF (Zhao et al., 2012, Robinson et al., 2006, Ashikari-Hada et al., 2005, Ono et al., 1999). In this study, we found a significant positive correlation between the overall extent of 6-O-sulfation of glucosamine residues within OEC HS
polysaccharide chains and VEGF-induced cell migration. These data indicate that 6-O-sulfate groups within OEC HS may enhance VEGF binding and this interaction could subsequently mediate the physiological activities of VEGF to promote cellular responses including cell migration. (Ashikari-Hada et al., 2005, Chen et al., 2005, Kamimura et al., 2001, Ono et al., 1999). Recently, Zhao et al. demonstrated that heparin oligosaccharides containing the UA-[2S]GlcNS[6S] disaccharide unit possessed the greatest affinity for VEGF-A binding (Zhao et al., 2012). In the correlation analysis performed in this study, higher levels of expression of this disaccharide unit were significantly associated with increased rates of cell migration towards VEGF, suggesting that, in particular, this modification within HS could be important for binding and modulating VEGF activity in relation to OEC migration.

Although much less is known regarding the structural features of HS that are required for SDF-1α binding, both N- and 2-O- sulfate groups have been suggested to be important for this interaction (Sadir et al., 2001). Here, a significant correlation was detected between the overall extent of 6-O-sulfation of OEC HS, again specifically the abundance of UA-[2S]GlcNS[6S] residues, with the levels of cell migration towards SDF-1α; possibly suggesting that 6-O-sulfate groups within HS also play role in the cellular responses to SDF-1α.

To test the importance of HS in SDF-1α and VEGF mediated chemotaxis of OECs HS was removed from the cell surface by heparinase I/III treatment. The disruption of cell surface HSPGs by heparinase I/III treatment was found to significantly reduce the migratory response of OECs towards VEGF compared to the untreated control. The migratory response of OECs towards SDF-1α was reduced following enzymatic degradation of cell surface HS, but this did not reach statistical significance.

It is known that VEGF can bind VEGFR-2 in the absence of HS, but that this interaction is enhanced in the presence of heparin/HS. Ashikari-Hada
et al. examined the mechanism by which heparin enhanced the ability of VEGF-A165 to induce endothelial cell proliferation and tube formation. Ashikari-Hada and colleagues demonstrated that in the presence of heparin, VEGF-induced phosphorylation of VEGFR-2 increased 1.7 fold, which was reduced by 75% if the cells were pre-treated with heparinase. Furthermore, the decrease in VEGF-induced phosphorylation of VEGFR-2 could be rescued by the addition of exogenous heparin. Thus, the authors postulated that HS participates in the formation of a ternary complex with VEGF and VEGFR-2 to promote effective signalling. Interestingly, the authors noted a reduction in the abundance of the trisulfated disaccharide residues within HUVEC HS significantly reduced VEGF-induced VEGFR-2 phosphorylation. In light of these findings, and in support of our correlation analysis, UA-[2S]GlcNS[6S] residues within OEC HS could be important for interaction of VEGF with VEGFR-2 and subsequent signal transduction (Ashikari-Hada et al., 2005). Mechanistically, VEGF/VEGFR-2 signalling is known to promote the migration of endothelial cells, in part, by activating the MAPK/ERK(1/2) and PI3K/Akt signal transduction pathways (Holmes et al., 2007, Ferrara et al., 2003). In addition to modulating the VEGF-VEGFR-2 interaction, the interaction of HS with VEGF may also immobilise the protein at the cell surface to enhance the probability of receptor binding or to spatially restrict the protein to form concentration gradients that guide cell migration (Ruhrberg et al., 2002). In other cases, the HS-VEGF interaction may prolong VEGF activity by sequestration away from degradatory pathways (Gengrinovitch et al., 1999, Soker et al., 1993). It is possible that 6-O-sulfate groups within OEC HS may favour VEGF binding and modulate its biological activity by any of these modes of action to promote OEC migration.

A number of studies provide support for the role of 6-O-sulfated HS in modulating the activity of VEGF to promote cellular responses, such as migration (Ji et al., 2011, Chen et al., 2005). For example overexpression of human Sulf-1, which selectively removes 6-O-sulfate groups within HS, in ovarian and hepatocellular carcinoma cell lines has been shown to
have a negative effect on VEGFR-2 signalling and is associated with anti-angiogenesis (Ji et al., 2011). This is consistent with the concept that 6-O-sulfate groups within HS are important in the binding of VEGF and/or VEGFR-2 and subsequent activity. Chen et al. demonstrated that knockdown of the 6-O-sulfotransferase HS6ST-2 in the zebrafish embryo disrupts branching morphogenesis of the caudal vein; a process involving cellular events such as cell migration, proliferation and differentiation, and appeared to be due to insufficient VEGF signalling (Chen et al., 2005). Furthermore, simultaneous knockdown of VEGF and HS6ST-2 was shown to exacerbate the effects of the reduced 6-O sulfation of HS on the vasculature. A reasonable conclusion from the study by Chen et al. is that the correct levels of 6-O sulfation within HS are required for the interaction with VEGF and consequently the role of this growth factor in the formation of the caudal plexus.

Given the important role of 6-O sulfate groups within HS for VEGF binding and activity, it is possible that the decline in the abundance of these residues within OEC HS during the ageing process perturbs the ability of HS to bind VEGF and, subsequently, the activity of this protein in mediating cell migration.

It is important to note that heparinase pre-treatment did not completely abolish the migratory response of OECs towards VEGF. This may, in part, be due to the fact that not all of the cell surface HS was removed by heparinase I/III treatment. Consequently, the residual levels of HS may have been enough to mediate some VEGF-induced migration of OECs and thereby explain the levels of migration observed by the heparinase treated cells. Furthermore, heparinase enzymes work only on HS that is secreted or present at the cell surface. Thus, once new HS is synthesized and transported to the cell surface it could mediate the chemotactic response of OECs. To overcome this, active heparinase enzymes should be present during the course of the assay but this could have affected OEC viability and is also prohibitive in terms of expense. Furthermore, if digestion is incomplete, the digested fragments could still retain their
activity. Additionally, VEGF is able to bind VEGFR-2 in the absence of HS, and could therefore sub-optimally activate signal transduction pathways, including those that promote cell migration. It is important to note that without the involvement of HS this level of signalling is not as efficient as that in the presence of HS (Ashikari-Hada et al., 2005).

Netelenbos et al. demonstrated that transwell migration filters pre-coated with immobilised HSPGs enhanced hematopoietic progenitor cell migration toward SDF-1α. It was proposed that the stimulating effect of the immobilised HSPGs was due to their ability to form a stable haptotatic gradient of SDF-1α which guided cell migration. Additionally, soluble HS dose-dependently enhanced cell migration towards SDF-1α, which was possibly due to optimised presentation of HS-bound SDF-1α to CXCR4 (Netelenbos et al., 2002). In this study, disruption of HSPGs on the cell surface of OECs reduced OEC migration towards SDF-1α, but this was not statistically significant; possibly due to the low numbers studied. Given the strong correlation detected between the levels of 6-O-sulfation of OEC HS and cell migration towards SDF-1, HS may play a role in modulating SDF-1 activity in relation to cell migration. However, it is possible that HS-independent mechanisms of cell migration are altered with ageing which contribute to a larger extent to the decline in the migratory response of OECs towards SDF-1α. For example, in Chapter 4, we demonstrated a decline in the expression of CXCR4 on the surface of OECs with age. This age-associated reduction in receptor expression could be the major contributor factor to the decline in SDF-1α-induced migration of OECs isolated from old subjects.

An important experimental consideration regarding the present findings relates to the trypsinization of OECs prior to seeding in the transwell migration assays. Detachment of OECs from the culture flasks did alter the structure of OEC HS, with significant differences detected in the percentage expression of the disaccharides the UA-[2S]GlcNS, UA-GlcNS and UA-GlcNAc. However, as the abundance of the 6-O-sulfated disaccharides, namely the UA-[2S]GlcNS[6S] residues, did not
significantly change following trypsin treatment, it was concluded that comparisons could be drawn to the disaccharide compositional analysis of OEC HS described in Chapter 5. Nonetheless, it should be noted that this is an oversimplification and the changes in HS structure on the migrating cells observed following trypsinization may have had an effect on cell migration which was unaccounted for when drawing correlations to previous structural analysis.

6.5.1. Future Directions

Together, these findings suggest a functional significance on VEGF-mediated chemotaxis of OECs as a consequence of HS structural changes during ageing. However, a number of issues remain to be resolved.

An important extension of this study would be to demonstrate that the HS chains of peripheral blood derived OECs isolated from old subjects indeed have a reduced affinity for binding or mediating the signalling of VEGF due to their altered 6-O-sulfate content. This could be determined by assessing the VEGF affinity of HS extracted from OECs of cord blood and peripheral blood from young and old subjects using surface plasmon resonance. Optical biosensors, such as Biacore, utilise surface plasmon resonance technology to provide a measure of binding interactions under controlled conditions. In Biacore, a biological ligand, in this case HS from OECs of cord blood and peripheral blood of young and old subjects, is immobilised onto the surface of a dextran matrix chip. A light source is directed onto the chip surface at an angle that achieves total internal reflection. VEGF or other growth factors of interest are then washed over the surface where they can interact with the immobilised HS. These interactions are measured by detecting changes to the angle at which the light is reflected. These changes are in part related to the amount of material bound to the sensor surface, giving an indication to the extent of interaction.
Additionally, determining whether the HS polysaccharide chains from peripheral blood derived OECs of old subjects have a reduced ability to facilitate the interaction of VEGF with VEGFR-2 would be useful next step. This could be assessed by measuring the levels of VEGF-induced phosphorylation of VEGFR-2 following desulfation of 6-O-sulfate groups within OEC HS. A similar approach could be used to that described by Ashikari-Hada et al. in which HUVECs were treated with low concentrations of sodium chlorate to preferentially reduce 6-O-sulfation of HS at cell surface and the effect on VEGFR-2 phosphorylation following VEGF stimulation examined by Western blotting with an anti-phospho-VEGFR-2 antibody (Ashikari-Hada et al., 2005).

As discussed in 6.2., the cells were trypsinized prior to seeding into the transwell migration assays. We observed no significant changes in the 6-O-sulfated disaccharides of OEC HS following trypsinization and therefore were able to draw comparisons to the disaccharide compositional analysis of OEC HS described in Chapter 5. However, trypsinization did reduce the amount of OEC HS which may have had an effect on VEGF or SDF-1 induced migration which was unaccounted for when drawing correlations to previous structural analysis. Ideally, all OEC samples should have been detached from the culture flasks prior to disaccharide analysis and in parallel their migratory abilities assessed by transwell migration, in order to be sure that the amounts and structure of HS on the surface of the migrating cells was comparable to the disaccharide composition.

Among the challenges associated with the therapeutic use of EPCs to promote neovascularization and vascular repair include problems with the retention, viability and homing of these cells to their target sites. Understanding the role of HSPGs in regulating EPC homing to sites requiring neovascularization and developing approaches to facilitate EPC migration could be of great clinical significance. In this context, greater therapeutic benefits maybe gained using innovative approaches such as
those which exploit the ability of HS to bind and immobilise and/or activate a diverse array of regulatory factors such as VEGF which can modulate EPC homing and retention. Indeed, Prokoph et al illustrated the potential of this therapeutic approach using a StarPEG-heparin containing hydrogel system. This heparin containing biomaterial was shown to bind, protect and cause the sustained release of SDF-1α. It was successfully used to generate a localised SDF-1α gradient that was capable of attracting CACs and subsequently improving neovascularization (Prokoph et al., 2012). There is great interest in this type of approach, as well as in the use of HS mimetics (Frescaline et al., 2012), to create a permissive microenvironment to recruit stem and progenitor cells to the site of injury/ischemia to promote repair and regeneration. One can see the therapeutic potential of this type of approach for enhancing the homing or survival of EPCs to the ischemic heart or to localised areas of damage or injury.
Chapter 7: Conclusions and Perspectives
7. Conclusions and Perspectives

Although pharmacological treatments, including statins and anti-hypertensive drugs, have improved the prognosis for patients with cardiovascular disease, it remains a leading cause of mortality in those aged 65 years and over. Given the increased life expectancy of the population in developed countries, there is a clear need for alternative treatment strategies. In this respect, the relationship between ageing and progenitor cell-mediated repair has gained great interest. Accumulating evidence suggests that age has a negative impact on the reparative functions of circulating EPCs, contributing to a decreased capacity for neovascularization of ischemic tissues and/or reduced re-endothelialization of vascular lesions in the ageing host. Gaining an insight into the involvement of structural alterations of HS as a possible mechanism contributing to the age-associated reduction in EPC function was the focus of this study.

This investigation aimed to determine whether the function of human OECs is impaired with age and to ascertain whether the ageing process is also accompanied by changes in the fine structure of OEC HS polysaccharide chains.

In achievement of the first objective of this study, OECs were isolated and expanded from peripheral blood samples of adult subjects across an age range; non-smokers who were not receiving medication for any clinical diagnosis. Given the controversy within the field owing, in part, to a lack of characterisation of the cells described in many of the reports, multiple parameters were assessed to ensure that the cells which were isolated were in fact OECs. The demonstration of an endothelial morphology, expression of a panel of endothelial markers, Ac-LDL uptake and lectin binding, together with evidence of formation of tube-like structures on Matrigel and within collagen gels, verified that the cells isolated in this study could be considered OECs.
A number of studies support the idea that age is an important determinant of EPC function, which can contribute to a reduction in cardiovascular repair mechanisms in the ageing host (Edelberg et al., 2002, Rauscher et al., 2003, Heiss et al., 2005). While there is evidence to suggest that CFU-Hill cells and CACs are subject to age-associated changes that impair their function, there is, however, a lack of studies examining the impact of ageing on human OECs; the one cell type encompassed within the term ‘EPC’ which displays the most features consistent with a human postnatal vasculogenic cell.

The second objective of this study was to determine the impact of age on OEC function. This was achieved by examining the apoptotic susceptibility, proliferative, migratory and tube forming capacities of OECs isolated from cord blood and from the peripheral blood of young and old subjects. Of the parameters assessed, a diminished migratory capacity towards SDF-1α and VEGF was detected by peripheral blood derived OECs isolated from old subjects using *in vitro* transwell migration assays. Both VEGF and SDF-1α have been widely implicated in the mobilisation of EPCs from the bone marrow and for guiding the homing of these cells to sites of injury or ischemia (Grunewald et al., 2006, Walter et al., 2005, Abbott et al., 2004, Ceradini et al., 2004, Yamaguchi et al., 2003, Kalka et al., 2000a). Although this *in vitro* technique cannot not fully recapitulate what is occurring *in vivo* it provides an indication of the ability of OECs to follow signals involved in homing to sites of requiring neovascularization, an essential step if they are to participate in neovascularization or re-endothelialization. Thus, the impaired migratory response detected in peripheral blood derived OECs from older individuals could limit the availability of these reparative cells at the site of injury, hindering repair and neovascularization. Indeed, support for the concept that age has a negative impact on the migratory capacity of OECs *in vivo* comes from a recently published study by Xia et al. The investigators demonstrated that OECs from elderly subjects had a significantly reduced capacity *in vivo* to promote re-endothelialization of injured arteries after transplantation into nude mice with carotid artery
denudation injury, as compared to OECs from young subjects. Upon examination of the injured tissue significantly fewer OECs were detected within the site of injury, suggesting that OECs from elderly subjects have a reduced capacity to home to the target site and consequently reendothelialization capacity (Xia et al., 2012).

HSPGs are known to participate in an array of cellular processes, such as cell migration, due to their ability to modulate the physiological activities of a wide range of protein ligands including SDF-1α and VEGF. Given that both of these factors appear to require specific structural features of HS for binding, it is possible that any change in the fine structure of HS during the ageing process could alter the binding of these proteins and, subsequently, their ability to promote cell migration.

To date, only a small number of studies have examined age-related structural alterations of HSPGs in adults (Feyzi et al., 1998, Murata et al., 1997), none of which have been in the context of OECs. These studies demonstrated that human ageing is accompanied by structural changes of HS involving enhanced 6-O-sulfation, particularly within NS domains, resulting in a higher abundance of the trisulfated disaccharide ΔUA[2S]-GlcNS[6S]. The third objective of this study was to determine whether HS structure changes on the surface of OECs as a consequence of ageing. This was achieved by disaccharide composition analysis of HS extracted from OECs derived from cord blood and those derived from peripheral blood samples of young and old subjects, using HPLC coupled with AMAC as a fluorogenic agent. A significant reduction in the levels of 6-O-sulfation, and the abundance of the trisulfated disaccharide ΔUA[2S]-GlcNS[6S], was detected within HS isolated from peripheral blood derived OECs as compared to the HS polysaccharide chains of cord blood derived OECs. Similarly, the proportion of this disaccharide residue was also significantly lower within HS from old subjects in comparison to their younger counterparts. Our findings of a reduction in 6-O-sulfation of HS with ageing contrasts with previous studies examining age-related structural changes of HS, but maybe explained by the fact that HS
species expressed by different cell types, as well as different tissues are to known to be structurally distinct.

The final objective of this study was to examine if the structural changes detected within OEC HS correlate with functional alterations of these progenitor cells. Indeed, the levels of SDF-1α and VEGF induced migration of OECs significantly correlated with levels of 6-O-sulfation within HS; with higher levels of 6-O-sulfation of HS, particularly the abundance of ΔUA[2S]-GlcNS[6S] residues, associated with higher levels of cell migration towards these chemotactic agents. This data indicates that 6-O-sulfate groups within OEC HS may be important for SDF-1α and VEGF binding and this interaction could subsequently mediate the physiological activities of these ligands to promote cell migration. Further work is required to determine how the age-associated changes in 6-O-sulfation detected within OEC HS translate into an alteration of the enzymatic machinery regulating HS biosynthesis.

However, care should be taken when interpreting the correlations drawn between HS structure and OEC migration. This is due to the alteration of HS structure on the cell surface following trypsinization of OECs from the culture flasks prior to seeding in transwell migration assays. Trypsinization of the cells was found to significantly alter the abundance of a number of disaccharides within the HS chains. Importantly, the abundance of the 6-O-sulfated disaccharides, UA-[2S]GlcNS[6S], UA-GlcNS[6S] and UA-GlcNAc[6S], did not significantly change following trypsin treatment. Thus, correlations were drawn between the levels of OEC migration and previous analysis of the abundance of these 6-O-sulfated disaccharide units on the cell surface. However, it is possible that trypsinization of the cells may have had effects, such as on HS conformation, which were unaccounted for when drawing correlations to previous structural analysis.
To verify the role of HS in the directional migration of OECs towards SDF-1α and VEGF a substantial amount of cell surface HS was enzymatically removed and the effect on migration assessed. A role for HS in the migratory response of OECs towards VEGF was supported by the significant reduction in cell migration towards this chemotactic agent following HSPG disruption. By contrast, the migratory response of OECs towards SDF-1α was not significantly altered following HSPG disruption. HS may still play a role in modulating SDF-1 activity in relation to cell migration, however, it is possible that HS-independent mechanisms of cell migration are altered with ageing that contribute to a greater extent to the decline in the migratory response of OECs towards SDF-1α.

The binding of VEGF by HS may modulate the biological activity of this protein to promote cell migration in several ways. Firstly, the interaction of HS with VEGF may enhance its interaction with VEGFR-2 to initiate signalling pathways such as MAPK/ERK(1/2) and PI3K/Akt cascades that are known to be involved in endothelial cell migration. Alternatively, the interaction of HS with VEGF may immobilise the protein at the cell surface to enhance the probability of receptor binding (Ruhrberg et al., 2002). In other cases, the HS-VEGF interaction may prolong VEGF activity by sequestration away from degradatory pathways increasing its availability for VEGFR-2 binding (Gengrinovitch et al., 1999, Soker et al., 1993).

Given the important of 6-O sulfate groups within HS for VEGF binding and activity, it is possible that the decline in the abundance of these residues within OEC HS during the ageing process perturbs the ability of HS to bind VEGF and, subsequently, the activity of this protein in mediating cell migration (Figure 7.1). However future work would be needed to determine whether the HS chains of peripheral blood derived OECs isolated from old subjects have a reduced affinity for binding or mediating VEGF signalling due to their altered 6-O-sulfate content.
Figure 7.1. A proposed model for the involvement of HS in OEC migration towards VEGF. 6-O-sulfate groups within OEC HS polysaccharides appear to be important for the binding of VEGF. This interaction could modulate the ability of VEGF to induce cell migration in several ways such as (A) enhancing the interaction of VEGF and VEGFR-2 to facilitate effective signalling which promotes cell migration; (B) the interaction of HS with VEGF may immobilise or spatially restrict the protein at the cell surface to increase the probability of receptor binding; and/or (C) the HS-VEGF interaction may prolong VEGF activity, and thus signalling to promote cell migration, by increasing its stability or protecting the protein from enzymatic degradation. Given the important of 6-O sulfate groups within HS for VEGF binding and activity, it is possible that the decline in the abundance of these residues within OEC HS during the ageing process...
perturbs the ability of HS to bind VEGF which could result in; (A) suboptimal presentation of VEGF to its receptor and subsequent signal transduction pathways involved in cell migration; (B) changes in the abundance of cell surface VEGF could reduce the probability of interaction with its receptor; and/or (C) a reduced ability to protect the protein from enzymatic degradation, thereby reducing its availability for VEGFR-2 binding and subsequent signalling or its extracellular abundance.

Although this study focused on the involvement of HS structural alterations in the age-associated decline of OEC migration, it is important to note a number of environmental changes, as well as intracellular alterations within the cells also occur during the ageing process that contribute this impaired cellular response.

In conclusion, this study demonstrates that the migratory capacity of human OECs significantly declines with increasing age. Furthermore, the reduction in SDF-1α and VEGF-mediated chemotaxis of OECs significantly correlates with structural alterations of HS. This is the first study to show a decline in the functional capacity of OECs during human ageing and a correlation to cell surface glycosaminoglycan structure. Such changes may modulate the homing and engraftment capacity of these repair cells, thereby contributing to the progression of endothelial dysfunction and age-related vascular pathologies. Understanding the role of HSPGs in regulating EPC homing to sites requiring neovascularization and developing approaches to facilitate EPC migration could be of great clinical significance.


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### Appendix I - Materials

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Appendix II – Buffer Recipes

Cell lysis buffer
20mM Tris (pH 7.5)
150mM NaCl
1mM EDTA
1mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA)
1% Triton X-100
2.5mM sodium pyrophosphate
1mM β-glycerophosphate
1mM Na₃V₀₄
1μg/ml leupeptide
1mM PMSF

DEAE elution buffer, pH 6.0
50mM sodium phosphate buffer
1M NaCl

DEAE loading buffer, pH 6.0
50mM sodium phosphate buffer
0.3M NaCl

EBM-2
Endothelial basal media (EBM-2, Lonza, Switzerland) was supplemented with;
1% fetal bovine serum (Sigma-Aldrich, UK)
100U/ml Penicillin/Streptomycin (Invitrogen, UK)
Ascorbic acid (SingleQuots; Lonza, Switzerland)
Gentamycin sulphate (SingleQuots; Lonza, Switzerland)
Amphotericin-B (SingleQuots; Lonza, Switzerland)

EGM-2
Endothelial basal media (EBM-2, Lonza, Switzerland) was supplemented with;
10% fetal bovine serum (Sigma-Aldrich, UK)
Basic fibroblast growth factor (FGF) (SingleQuots; Lonza, Switzerland)
Vascular endothelial growth factor (VEGF) (SingleQuots; Lonza, Switzerland)
Insulin-like growth factor (IGF) (SingleQuots; Lonza, Switzerland)
Epidermal growth factor (EGF) (SingleQuots; Lonza, Switzerland)
Heparin (SingleQuots; Lonza, Switzerland)

TAE Buffer, 50X, pH 8.5
242g Tris base
57.1mls acetic acid
100ml 0.5M EDTA
Made to 1 litre with Milli-Q H₂O
Appendix III – Ethics Forms

Participant Information Sheet

Title of Study:
A study of endothelial progenitor cell function in young and old subjects

1. Why have I been invited?

You are being invited to take part in a research study because you have responded to our request for a blood sample. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

The Patient Advice and Liaison Service (PALS) provide more information about medical research and look at some questions that you may like to ask. This service can be accessed online at http://www.pals.nhs.uk/.

The aim of this study is to examine how the cells that line the blood vessels repair themselves in young and older males and/or females, and to study factors that influence this repair process.

To take part in this study you must:
- Be between 20-80 years of age
- Be a non-smoker
- Not currently be receiving medication for any clinical diagnosis
- Have not undergone treatment with cytotoxic drugs (chemotherapy) within the last six months
- Are not pregnant
- Have not visited a region where blood-borne diseases are prevalent within the last six months
- Fully understand the scope, nature and risks of this study.

2. What is the purpose of the study?

Blood carries certain cells that are involved in helping to repair the lining of the blood vessels. These cells are called endothelial progenitor cells (EPCs) and they are thought to be important in helping the blood vessels to stay healthy. They may also help to protect against the development of hardening of the arteries and heart disease. We wish to take a blood sample and isolate EPCs to study what factors during aging influence...
how they grow and develop. This study will help towards understanding how the blood vessels function in older people and may help us suggest new ways to prevent heart disease.

3. What will happen to me if I take part

We would like to invite you to help us with this study. We will use your blood sample to isolate EPCs for analysis of specific sugar chains on the surface of these cells. We will also evaluate the numbers of these circulating progenitor cells and look at other indicators to help us to determine why these cells behave differently in older people.

The study lasts for four years and will involve 60 subjects. If you agree to take part you would be asked to donate a 60 ml sample of blood. This is a simple procedure carried out by a member of the trained clinical research team should take no more than 15 minutes. We will then separate the blood sample and perform the measurements described above in our laboratory. Your participation in the study is dependant on the fact that you do not have a medical history, are currently not taking any medication and are therefore seen to be a healthy subject.

4. Will my taking part be kept confidential

All this information will be strictly confidential, no personal details will be given to the researchers and none of our samples or forms will identify you personally. Relevant sections of your data collected during the study may be looked at by individuals from the University of Manchester, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

5. What are the possible risks of taking part?

Although, blood donation is a simple procedure, and will be carried by a trained professional, it is important to be aware that there is the potential for bruising and slight discomfort at the site of needle insertion. There is also a small risk of fainting.

Complaints

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If they are unable to resolve your concern or you wish to make a complaint regarding the study, please contact a University Research Practice and Governance Co-ordinator on 0161 2757583 or 0161 2758093 or by email to research-governance@manchester.ac.uk.

Harm

In the event that something does go wrong and you are harmed during the research you may have grounds for a legal action for compensation against The University of Manchester but you may have to pay your legal
costs. The normal National Health Service complaints mechanisms will still be available to you. The University of Manchester has cover for no fault compensation for bodily injury, mental injury or death where the injury resulted from a trial or procedure you received as part of the trial. This would be subject to policy terms and conditions. Any payment would be without legal commitment. (Please ask if you wish more information on this). The University would not be bound to pay this compensation where the injury resulted from a drug or procedure outside the trial protocol or the protocol was not followed."

6. What happens when the research study stops?
All your cells will have been utilised for experiments when the study stops and all of your blood samples will be destroyed when the study stops.

7. Are there any possible benefits?
There will be no direct benefit to you. This study will help us to better understand blood vessel function and repair in the aging population and may suggest new ways to identify those at risk of heart disease and stroke.

8. Do I have to take part?
No, taking part is voluntary. If you would prefer not to take part you do not have to give a reason. If you do decide to take part, we will ask you to sign a consent form and give you a copy of this information sheet and consent form to keep. If you decide to take part, you are still free to withdraw at any time.

Your medical records may be inspected by the research scientists and clinicians organising the study and the data will be kept strictly confidential (no personal details will be given).

9. What will happen to the study results?
We hope to publish our findings in a scientific journal. You will be provided with a copy of the paper should you wish. Otherwise there will be no further interaction with you.

10. What do I do now?
The researcher organising the study will contact you in a few days. She can answer any questions and you can let her know if you are interested in taking part.

Thank you very much for considering taking part in our research.

If you wish to obtain independent advice about this research you may contact:

Patient Advice & Liaison Service
Manchester Royal Infirmary
Oxford Road
Manchester
M13 9WL
Tel: 0161 276 4261

Researchers Contact Details:

Dr. Yvonne Alexander PhD (Scientific advisor)
Dept. of Medicine,
Cardiovascular Research Group
Core Technology Facility, 46 Grafton St.
Manchester. M13 9NT
Tel: 0161 275 1224

Or

Kate Williamson (PhD student enrolled to perform the study)
Dept. of Medicine,
Cardiovascular Research Group
Core Technology Facility, 46 Grafton St. Manchester.
M13 9NT
Tel: 0161 275 1229
Research Participant Consent Form
11-05-10

Title of Study:
A study of endothelial progenitor cell function in young and old subjects

Institution:
Cardiovascular Research Group
The University of Manchester
Core Technology Facility
46 Grafton Street
M13 9NT

Subject’s surname:..............................
Other names:.................................................

1. I confirm that I have read and understand the information sheet dated ________ (Version 2 )

2. I agree to give blood to be used in this study.

3. I have had an opportunity to ask questions and discuss this study.

4. I have received satisfactory answers to all my questions

5. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason.

6. I understand that my samples will be destroyed at the end of the study.

7. I wish to receive a copy of the final report of the study
8. I understand that relevant sections of personal data provided for the purposes of this study may be looked at by individuals from the University of Manchester, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

9. I agree to take part in the study

Name of Participant_________________________________________
Date__________________________
Signature_________________________________________________

Name of Researcher________________________________________
Date___________________________
Signature__________________________________________________

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes
Appendix IV – Melt Curve Analysis

ATP5B

Tm: 82.18

Temperature (°C)
GAPDH

Temperature (°C)
SULF-1

![Graph showing temperature vs. derivative reporter (-Rn)]

Temperature (°C)

Derivative Reporter (-Rn)

Tm: 83.34
SULF-2

Temperature (°C)

Tm: 83.99