THE ISOLATION, CHARACTERISATION AND INVESTIGATION INTO THE IN VITRO BEHAVIOUR OF HUMAN OCULAR VASCULAR ENDOTHELIAL CELLS

ANDREW C BROWNING, BSc

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Browning AC, Gray T, Amoaku WM. Isolation, culture, and characterisation of human macular inner choroidal microvascular endothelial cells. Br J Ophthalmol 2005:89:1343-1347.

Browning AC, Dua HS, Amoaku WM. The effects of growth factors on the proliferation and in vitro angiogenesis of human macular inner choroidal endothelial cells. Br J Ophthalmol 2008:92:1003-1008.

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Abbreviations

ADP	Adenosine diphosphate
AGES	Advanced glycation end products
Akt	Acutely transforming retrovirus AKT8 in rodent T cell lymphoma
AMD	Age related macular degeneration
Apo	Apolipoprotein
BSA	Bovine serum albumin
CD	Cluster differentiation
CFH	Complement factor H
CNV	Choroidal neovascularisation
DABCO	1-4 diazabicyclo[2,2,2] octane
DAG	Diacylglycerol
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylene diamino tetra acetic acid
ERK	Extra cellular signal regulated kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FLK	Foetal liver kinase
FLT	FMS like tyrosine kinase
GTP	Guanosine triphosphate
hCEC	Human choroidal endothelial cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethansulphonic acid
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
ICEC	Inner choroidal endothelial cell
IGF	Insulin-like growth factor
IL	Interleukin
kDa	kilo Dalton
KDR	Kinase insert domain receptor

LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase pathway
MEM	Minimum Essential Medium
MHC	Major Histocompatibility complex
MMP	Matrix metalloproteinases
mRNA	messenger RNA
NGF	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthetase
PDGF	Platelet derived growth factor
PDT	Photodynamic therapy
PECAM	Platelet endothelial cell adhesion molecule
PBS	Phosphate buffered saline
PEDF	Pigment Epithelial Derived Factor
PI	Phosphoinositide
PKC	Protein kinase C
PLGF	Placental growth factor
PDR	Proliferative diabetic retinopathy
RAS	Rat sarcoma
RCS	Royal College of Surgeons
ROP	Retinopathy of prematurity
RPE	Retinal pigment epithelium
SDF	Stromal derived factor

Rationale and Aims

Intraocular angiogenesis is associated with a number of common blinding conditions including wet age-related macular degeneration, proliferative diabetic retinopathy, retinopathy of prematurity and rubeotic glaucoma. The pathogenesis of these disorders is centred on the choroidal, retinal and iris microvascular endothelial cells respectively.

Investigators have attempted to increase the understanding of these disorders by studying vascular endothelial cells in vitro. Due to their availability in large numbers and relative ease of culture, many studies have used ocular cells from non-human sources, or non-ocular human endothelial cells derived from sites such as the umbilical vein (HUVEC).

Workers have therefore inferred and applied conclusions from in vitro studies of macro- and microvascular endothelial cells derived from different species or organs to human eye diseases. It is now widely accepted that endothelial cells derived from different species, vascular organs and from within different vascular beds within those organs display different phenotypical, biochemical and genetic heterogeneity. It follows that the extrapolation of results from these non-human ocular or HUVEC cells may not provide reliable data applicable to human eye disease. Any meaningful insight into the pathogenesis and selective treatment of the diseases mentioned previously would therefore probably only be gained by conducting in vitro studies using microvascular endothelial cells derived from the particular site(s) affected by the disease, i.e. endothelial cells derived from the human retina, choroid and the iris. Within the ocular vascular beds themselves, many diseases appear site specific, such as diabetes predominantly affecting the retinal vasculature and age related macular degeneration affecting the choroidal circulation.

One disease in particular that is the leading cause of blindness in those over 65 is wet age related macular degeneration. The underlying pathophysiological mechanisms responsible for disease progression remain largely unknown. Current opinion suggests an angiogenic response by inner choroidal endothelial cells to a local adverse stimulus driven by a range of cytokines and growth factors. Because of the impact of wet AMD on the quality of life of a large proportion of the elderly population, it was decided to focus attention during the latter part of this research project on the cells thought to be central to the disorder – the inner choroidal endothelial cell. Until now, these critical cells have not been successfully isolated and subjected to in vitro examination.

The aims of this project are therefore:

1) To successfully isolate and culture matched human retinal, choroidal and iris endothelial cells.

2) To acquire and culture unpassaged human umbilical vein endothelial cells (HUVEC).

3) To compare the gene expression profiles of proliferating unpassaged HUVEC and ocular microvascular endothelial cells to determine if HUVEC cells are representative cells to use in ocular angiogenic research.

4) To compare the gene expression profiles of matched, unpassaged human retinal, choroidal and iris endothelial cells to determine whether any differences in gene expression provide insight into the site specificity of ocular vascular disorders and to determine whether any differences would provide potential targets for future anti-angiogenic therapies.

5) To develop a technique for the successful isolation of human macular inner choroidal endothelial cells and to propagate them in culture.

6) To characterise the isolated cells in terms of the surface expression of a range of purported endothelial cell markers.

7) To compare the gene expression profiles of matched unpassaged human macular inner and outer choroidal endothelial cells to determine whether inner choroidal endothelial cells possess any gene expression characteristics that make them susceptible to growth during wet AMD.

8) To compare the gene expression profiles of matched unpassaged human macular and peripheral inner choroidal endothelial cells to determine whether macular inner choroidal endothelial cells possess any gene expression characteristics that make them susceptible to growth during wet AMD.

9) To determine the effect of a range of growth factors on the proliferation and vascular tube formation of human macular inner choroidal endothelial cells.

Overall, the realisation of these objectives will provide an insight as to whether HUVEC cells provide a useful alternative to ocular cells in research into ocular angiogenic diseases given their widespread availability and ease of culture. The comparison of the gene expression profile of ocular endothelial cells will also provide an insight into endothelial cell heterogeneity within the eye and may provide clues as to the reasons for ocular vascular bed disease susceptibility and potential future selective anti-angiogenic treatments.

The in vitro characterisation and gene expression profiling of human inner choroidal endothelial cells will provide an extremely powerful and unique model for the laboratory investigation of the mechanisms underlying age related macular degeneration and may provide an insight into selective treatment of this disease.

Chapter 1

Introduction

Section 1: Vascular Biology

Oxygen is one of the essential requirements for life. The cells of some simple organisms receive oxygen by simple diffusion from the air, while larger organisms such as the fruit fly have developed a system of air filled tubes within cell layers. Because the diffusion limit of oxygen through tissue is 100 to 200µm, vertebrates have developed complex cardiovascular systems lined by endothelium. These systems efficiently maintain oxygen homeostasis in all cells and at the same time facilitate bi-directional exchange of solutes and carbon dioxide; waste product removal; recruitment and diapedesis of leukocytes; antigen presentation; lipoprotein metabolism and temperature regulation. The idea of a vascular system was first proposed by Harvey in 1628 when he suggested that blood circulates around the body. Soon afterwards, Malphigi described the separation of blood and tissues and for the first time visualised blood flow in capillaries. It was not until the nineteenth century (1860) that von Recklinghausen suggested that blood flowed along vessels lined with endothelial cells. Since then, Simionescu has described the interaction of the endothelium with leukocytes and endothelial cell junctions (Simionescu et al, 1976) and many others have subsequently described the numerous “active” functions of the endothelium.

The cardiovascular system is the first organ system to develop in an embryo, the failure of which is universally lethal in early embryogenesis (Risau W, 1995; Risau W, 1997). It evolves by three different, distinct mechanisms: haemovasculogenesis, vasculogenesis and angiogenesis.

Haemovasculogenesis

While it is widely accepted that embryonic haemopoiesis occurs at early stages within the liver before moving to the bone marrow, as long as 100 years ago, it was noted that there was a link between the production of blood cells (erythrocyte precursors) and endothelial cells at the earliest stages of development within the blood islands of the chick yolk sac (Sabin, 1917). Similar findings have subsequently been found in the aorta and umbilical artery (Jaffredo et al, 1998: Hirai et al, 2002). With modern methods of examination, it is now thought that this budding of erythroblasts from endothelial cells is a common event in the embryo and is found to occur in the kidney, brain and skin. The existence of a common precursor for both endothelial and haematopoietic cells (Murray, 1932: Ogawa et al, 2002) led to the term "haemo-vasculogenesis". This is defined as the formation of blood vessels within the embryo during which new blood vessels (vasculogenesis) and blood cells (haematopoiesis) develop simultaneously within the embryo from a common precursor (the haemangioblast) (Sequira-Lopez et al, 2003). Histologically, erythroblasts develop within aggregates of endothelial cell precursors. As the lumen of a vessel forms, the erythroblasts bud from the lining endothelial cells to fill the lumen (Sequira-Lopez et al, 2003). The common precursor cells express βI globin and this has been used as a marker of haemovasculogenesis (Sequira-Lopez et al, 2003). What is not known for certain is the earliest marker of this pluripotent haemangioblast. Various elegant experiments by Yamashita in 2000 and Nishikawa in 1998 using embryonic stem cells show that cells positive for Flk-1 (VEGF receptor 2), which is thought to be an early haemangioblast marker, can develop into either endothelial or haematopoietic cells (and also possibly mural cells) (Yamashita et al, 2000; Nishikawa et al, 1998). The expression of VE-cadherin or not, is an important determinant as to which path the progenitor cell then follows (Nishikawa et al, 1998). Ogawa has subsequently shown that

progenitor cells following haematopoietic lineage then go on to express α 4-integrin. It is suspected that growth factors such as VEGF may be important in determining the route of differentiation taken by the original cells but this is an area where further research is needed. It has now been proposed that the choriocapillaris (part of the choroidal vasculature within the eye) originates via haemovasculogenesis because precursor cells are positive for epsilon globin (an erythroblast marker) and VEGF receptor 2. These cells were seen to aggregate and then differentiate into separate endothelial cells and erythroblasts. (Hasegawa et al, 2007).

Vasculogenesis

Vasculogenesis describes the formation of blood vessels in which endothelial cells differentiate and proliferate in situ within a previously avascular tissue. This primary network includes some of the major vessels such as the aorta and major veins (Yancopoulos et al, 2000; Risau & Flamme, 1995). Vascular progenitor cells either migrate or differentiate locally at sites of vascularisation and coalesce to form a vascular plexus (Hristov M et al, 2003; Ribatti et al, 2009). This process leads to the formation of the major intra-embryonic blood vessels such as the aorta and the primary vascular plexus in the yolk sac. Later, the vascular networks of organs such as the lungs, the myocardium and the liver also form via vasculogenesis (Pardanaud et al, 1989). This initial process appears to be pre-programmed and is independent of the local oxygen concentration. In the yolk sac, the blood islands are composed of haemangioblasts, the precursors of both endothelial and haematopoietic cells. In the peripheral areas of the islands, haemangioblasts differentiate into angioblasts (CD39 or ADPase positive) which later aggregate into vascular networks within the yolk sac and the embryo proper (McLeod et al, 2006). During early *in vitro* vasculogenesis, fibroblast growth factor 2 (FGF-2) appears to be important for angioblast differentiation (Krail et al 1994; Flamme and

Risau 1992; Cox & Pool, 2000). However, *in vivo*, the vasculature of FGF-2 knockout mice models appeared to develop normally (Zhou et al, 1998). Although the reason for this discrepancy is uncertain, it may be due to a high degree of redundancy in the FGF growth factor/receptor system. It is known that at present, there are at least 4 different FGF receptors, 3 of which have 3 subtypes. Each receptor can also bind a number of the 22 structurally related members of the FGF growth factor family. Any knock out model of a particular subtype of FGF molecule would most likely be easily bypassed, thereby suggesting an important role of FGF in embryogenesis. Conversely, vascular endothelial growth factor (VEGF) and VEGF receptor 2 knockout mice die early with vascular maldevelopment suggesting a pivotal role for VEGF and its main receptor in early vasculogenesis (Carmeliet et al, 1996; Shalaby et al, 1995). Other endothelial cytokines/receptors such as the angiopoietins, Tie 1 and 2 and neuropilin are thought to play a more minor role in early vasculogenesis, with animals undergoing normal vessel development but dying of cardiac defects. Once formed, tubes of endothelial cells begin to secrete laminin and collagen IV which in turn forms the basement membrane.

Until recently it was thought that vasculogenesis only occurred in the embryo. It is now known that “post-natal vasculogenesis” occurs via bone marrow-derived endothelial stem and progenitor cells (Hristov M et al, 2003; Asahara et al, 1999; Käßmeyer et al, 2009). Asahara et al isolated mononuclear cells from the blood of patients who had the antigenic characteristics of angioblasts and, after inducing ischaemia in limbs, found bone marrow endothelial progenitor cells within the vessels of the limbs after restoration of blood flow. They state that this is consistent with post natal vasculogenesis. Within the eye, it is thought that the retinal vasculature develops by the process of vasculogenesis (see later) and this is a well studied model of vascular development because of the ease with which it can be studied (McLeod et al, 2006; Saint-Geniez & D’Amore, 2004).

Angiogenesis

Angiogenesis is the term applied to the formation of capillaries from pre-existing vessels. In the embryo it is responsible for expansion of the primary network formed by vasculogenesis. In contrast to vasculogenesis, it appears to be less pre-programmed and is more dependent on tissue hypoxia for its stimulation and regulation (Pugh & Ratcliffe, 2003; Carmeliet, 2003; Semenza, 2007). It occurs by a series of well defined steps which include:

1. Endothelial cell (EC) activation by growth factors such as VEGF and FGF-2 via VEGF receptor 2 and FGF receptors respectively. These receptors are membrane bound tyrosine kinases that lead to the activation of multiple intra-cellular signalling cascades. The principle drive for growth factor up-regulation, especially for VEGF, is tissue hypoxia. There is debate as to whether FGF exerts its pro-angiogenenic effect on endothelial cells by a direct effect on the cellular proliferative mechanism via FGF receptors and intracellular signalling cascades or via up-regulation of the effect of VEGF. Evidence exists for both hypotheses as it is known that specific pro-angiogenic pathways are activated when FGF binds to its receptor (FGFR1) (Cross and Claesson-Welsh), and FGF has also been shown to up-regulate VEGF and VEGF receptor expression on ECs (Murakami and Simons, 2008; Murakami et al 2011; Seghezzi et al, 1998).

2. Degradation of endothelial basement membranes by proteases such as matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA), heparinases, trypases and cathepsins (Kalluri, 2003; Steen et al, 1998). Matrix metalloproteinases are a family of at least 20 different zinc-dependent endopeptidases that are capable of degrading various components of the extracellular matrix (ECM). Under basal conditions, MMPs are capable of remodelling tissue.

However, during periods of angiogenesis, they are able to modify the ECM ahead of proliferating endothelial cells, aiding their passage through tissue. Their activity is regulated by natural inhibitors called Tissue inhibitors of metalloproteinases (TIMPs). MMPs are known to be secreted by proliferating endothelial cells, and in wet AMD, the most important types appear to be MMP2 and MMP9 (gelatinase a and b respectively) (Bandyopadhyay and Rohrer, 2012). They are able to degrade various components of Bruchs membrane such as collagen IV, V and fibronectin (Sethi et al, 2000). Proteinases also play a role in angiogenesis by liberating extra cellular matrix-bound growth factors such as FGF-2, thereby liberating free growth factor which can therefore take part in the angiogenic process.

3. Formation of vascular sprouts and migration of activated endothelial cells through the adjacent extracellular matrix towards the angiogenic stimulus. The tips of the sprouts are formed by specialised endothelial tip cells. Tip cells extend numerous filopodia to sense their microenvironment and lead the direction of the growing sprout. Behind the tip cells, other cells form the stalk of the new vessel. Selection of endothelial cells to become tip cells is affected by Notch expression (Gerhardt et al, 2003; Suchting & Eichmann, 2009). A model of tip cell selection has been proposed that starts with binding of VEGF by VEGF receptor 2 on an endothelial cell which causes the up regulation of the notch ligand delta like ligand 4 (Dll4) on the cell surface. This interacts with notch receptors on surrounding ECs and this in turn leads to down regulation of VEGFR2 on these cells which are thus less sensitive to VEGF signalling. The cells expressing Dll4 and VEGF R2 become non proliferative tip cells, forming filopodia and extending into the surrounding ECM along VEGF gradients (Hellstrom et al, 2007; Gerhardt et al, 2003). Cells expressing notch receptors proliferate and become stalk cells which extend the newly forming blood vessel (Tung et al, 2012). It is not known how specific tip cells are selected

or how their numbers are controlled in relation to the number of stalk cells, but it may be linked to Dll4-notch 1 interactions. This brings about a down regulation of tip cell formation and angiogenesis (Hellstrom et al, 2007; Phng and Gerhardt, 2009) and notch-1/jagged-1 interactions which antagonise the effect of Dll4, leading to pro-angiogenenic signals and increased formation of tip cells (Bernedito, 2009). It is obvious that the ratio of tip to stalk cells needs to be strictly controlled for organised angiogenesis to occur. Growth of the new vessel is affected by integrins which are cell surface receptors for specific ECM components that enable signals from the ECM to be transmitted into the EC (Hynes, 1992). This communication between the activated EC and the ECM is thought to be critical because blocking of integrins signalling significantly inhibits angiogenesis (Umeda et al, 2006; Yasukawa et al, 2004). Microarray analysis of tip cells prepared by laser microdissection demonstrated enrichment for CXCR4 (stromal derived factor receptor) which is a growth factor/receptor combination thought to be important in embryonic vascular development. The tip cells also appeared to be enriched for apelin, VEGF Receptor 2 and angiopoietin 2. The authors conducted a number of elegant experiments and showed that inhibition in CXCR4 signalling resulted in defects in neonatal mice retinal tip cell morphology and function (Strasser et al, 2010).

4. Endothelial cell proliferation.

5. Capillary tube formation. The mechanisms underlying tube formation are largely unknown but probably include changes in cell polarity, cytoskeletal components and interactions with the ECM via integrins (Tung et al, 2012). Two models have been proposed to explain the mechanism of tube formation. The first is cell hollowing. This involves the coalescence of intracellular vesicles to eventually form a lumen. Once the lumen is formed within an individual cell, the ends open and link with adjacent cells to form a primitive vessel

(Folkman and Haudenschild, 1980). An alternative model is known as cord hollowing. In this model, endothelial cells aggregate into a long cord and vesicles form between the cells which eventually form into a lumen (Lueng et al, 1999). While other models probably also exist, the model taking place in an individual setting is probably context driven

6. Recruitment of mural cells to surround and stabilise the newly formed vascular tube. For larger blood vessels (arteries and veins), this involves the recruitment of smooth muscle cells (SMC), while for capillaries, arterioles and venules, pericytes are recruited. It is thought that SMCs and pericytes are derived from a similar lineage and are part of a phenotype continuum. The number of pericytes recruited to a vascular structure per endothelial cell varies within different beds. Within the retina there is 1 pericyte per EC whereas in skeletal muscle there are 100 pericytes per EC (Shepro and Morel, 1993). The reason for this difference is uncertain, but pericytes may be important for the formation of the inner blood retinal barrier or act as receptors for hypoxia or hypoglycaemia; all important factors for normal retinal function. For many years the role of pericytes was ignored as they were thought to be passive cells providing simple mechanical support. It is now known that they are critical for the stability and maintenance of the vessel and the endothelial cells. Abnormalities in pericyte biology are now thought to cause specific diseases in their own right. Proliferating endothelial cells secrete platelet derived growth factor (PDGF) B which attracts PDGFR-β expressing mural cells to the nascent vessel (Lindblom et al, 2003; Lindahl et al, 1997). Mouse knock out models of PDGF-B and PDGFR-β result in a similar lethal phenotype caused by absent pericyte recruitment and major vascular abnormalities (Leveen et al, 1994; Soriano P, 1994). It is thought that localised binding of PDGF-B to the surrounding endothelial cell ECM aides the orderly coverage of the new vessel with pericytes (Armulik et al, 2005). An absence of pericytes on pre-formed vessels, either as a result of

disease (diabetes) or by pharmacological manipulation by the use of PDGF-B antibodies, leads to loss or stripping away of pericytes and vascular disorganisation. Another signalling molecule associated with pericytes is Angiopoietin 1 (Ang 1). It is thought that Ang1 is secreted by pericyte/mural cells and acts in a paracrine fashion on endothelial cells via the Tie 2 receptor to stabilise the vessel. Ang 1 or Tie 2 knockout mice have disorganised basement membranes and poor pericyte coverage.(Sato et al, 1995; Suri et al 1996)

Abnormal or pathological angiogenesis

It was recognised over 100 years ago that blood vessels grow around tumours and that without these new vessels tumours could not grow beyond a certain size or metastasise. In 1968 it was hypothesised that tumours secrete a diffusible angiogenic substance (Greenblatt & Shubi, 1968; Ehrmann & Knoth, 1968) and in 1971 Folkman suggested that inhibiting angiogenesis may stop tumour growth (Folkman, 1971a) and he attempted to isolate this “factor” (Folkman, 1971b). The tumour vessels studied were structurally and functionally abnormal. The vessels were disorganised, dilated and tortuous with excessive branching and there were many openings or gaps both within (fenestrae) and between endothelial cells making them very “leaky”.

In adult normal, non-neoplastic tissue, the majority of vascular endothelial cells are dormant and only approximately 0.01% of endothelial cells are undergoing division at any one time (Engerman et al, 1967). Diseases in which there is excessive angiogenesis are a major cause of disease-related morbidity and mortality. Examples include: psoriasis, atherosclerosis, haemangiomas and endometriosis. Within the eye, a number of common blinding conditions are caused by aberrant angiogenesis including wet age related macular degeneration, proliferative diabetic retinopathy, rubeotic glaucoma and retinopathy of prematurity.

Endothelial Cells

Blood vessels are lined by a monolayer of endothelial cells. The adult human contains approximately 1-6x10^{13} endothelial cells and they cover an approximate surface area of 7m^2 (Cines et al, 1998; Augustin et al, 1994). They are flat, polygonal cells that are elongated in the direction of blood flow and, on cursory examination, appear identical in different parts of the body; but in reality there is significant heterogenic diversity. Their life span is in the region of 100 days (Hobson & Denekamp, 1984). In cross section, the cells are 2-3µm thick at the nucleus but they can be as thin as 0.2µm in the peripheral parts of the cell (Anderson et al, 1995). The thinness of the cell may contribute many of their attributes. Endothelial cells express a number of cell markers that are used to identify them in experimental studies. However, none is entirely endothelial specific, but rather more "endothelial restricted". The intensity of expression is also variable depending on the site of origin of the cells (Langenkamp & Molema, 2009). Endothelial cell restricted markers include: CD31, CD34 and von Willebrand factor (vWf).

Endothelial cell surface markers

CD 31, also known as PECAM 1 (platelet endothelial cell adhesion molecule) is a 130kDa glycoprotein found on endothelial cells and was first cloned in 1990 (Newman et al, 1990). It is also found on platelets and some leukocytes. It is a member of the Ig superfamily and consists of 6 extracellular Ig folds. Its role is as a cell-cell adhesion molecule with its main ligand being other CD31 molecules. Other ligands are thought to include CD38, CD177 and άvβ3 integrin which are found on circulating leukocytes (Deaglio et al, 1998; Sachs et al, 2007). While CD31 is responsible in part for the adhesion of adjacent endothelial cells, its main role appears to be that of the binding of leukocytes to the luminal side of ECs with the specific function of allowing the loosening of the binding of adjacent cells, and to facilitate the passage of the leukocyte between ECs into the extra-vascular compartment (diapedesis). This phenomenon is commonly seen in states of inflammation and allows leukocytes to migrate to sites of trauma and infection. While the mechanisms controlling this event are poorly understood, it is thought that cell binding or the local release of factors by leukocytes leads to activation of the CD31 molecule and the subsequent stimulation of an intracellular signalling cascade. It has been observed that CD31 binding leads to the intracellular phosphorylation of tyrosine and serine/threonine residues on the receptor (Newman and Newman, 2003) which, in turn, can alter the vigour of cell-cell binding. It is not known if or how CD31 interacts with other adhesion molecules important for EC integrity such as ZO-1 and occludin.

CD34 is a 105-120kDa trans-membrane glycoprotein expressed by bone marrow lymphohaematopoietic stem cells, endothelial cell progenitor cells, embryonic fibroblasts and mature vascular endothelial cells (especially tip cells). It was discovered in 1984 during the search for markers of bone marrow progenitor or stem cells (Civin et al, 1984),

labelling approximately 1.5% of bone marrow cells. Despite its wide expression on a range of potentially important precursor cells, its role is poorly understood. Early studies suggested that its expression on endothelial cells plays a role in leukocyte adhesion and homing, particularly in lymphoid tissues via L-selectin expressed by circulating lymphoid cells (Nielsen and McNagny, 2008). Interestingly, CD34 exists as at least two splice variants depending on the maturity of the cells (Krause et al, 1996). The smaller splice variant is missing an exon responsible for coding part of the intracellular domain, with potential sites of phosphorylation which may therefore play a role in intracellular signalling, although the importance of this is currently unknown. Both forms have the same extracellular domains so are therefore not able to be differentiated by antibody labelling of cells.

Another important observation is the loss of vascular endothelial cell expression of CD34 after one or two passages or after exposure to inflammatory mediators such as TNF alpha (which coincidently up-regulates expression of ICAM 1) (Delia et al, 1993). These observations have led to the theory that CD34 is important in cell–cell adhesion, especially during inflammation. Interestingly, CD34 knock out mice appear to have normal bone marrow and endothelial cell function (Cheng et al, 1996), further increasing the mystery of the role of CD34 other than being a useful marker of endothelial cells.

von Willebrand factor (vWf) is a circulating protein important in blood clotting (haemostasis), an absence of which causes the bleeding disorder called von Willibrand's disease. The glycoprotein is critical for the recruitment of platelets to sites of bleeding, thereby providing part of the initial haemostatic plug before the slower activation of blood clotting factors. It is also important for the stabilisation of Factor VIII - clotting factor (thereby enhancing the clotting cascade). It is synthesised and stored within endothelial cells and megakaryocytes (platelet precursors) in electron dense Weibel-Palade (WP) bodies. These electron dense bodies were first recognised using electron microscopy in 1964 in the

cytoplasm of small vessel endothelial cells (Weibel and Palade, 1964). They have a diameter of 0.1-0.3 μm, a length of 1-5μm and they have characteristic longitudinal striations (Valentijn et al, 2008) (see illustration below). In cross section they consist of electron dense tubules which are thought to give them the characteristic striated appearance surrounded by a less dense matrix. For many years, it was thought that vWf was the only secreted constituent of WP bodies. It is now known that tissue plasminogen activator (t-PA), P-selectin, interleukin-8, angiopoietin 2 and endothelin-1 are also stored and secreted (Valentijn et al, 2011). This means that WP bodies contain a number of mediators important in haemostasis, inflammation, angiogenesis and vascular tone. There is a continuous basal secretion of vWf into the blood stream which can be quickly enhanced during times of localised vascular stress by degranulation of WP bodies near sites of vessel (and endothelial) injury by activation of cell surface G protein linked receptors by secretagogues such as thrombin and histamine (Valentijn et al, 2011). Unlike CD31 and CD34, vWf appears to be much more specific for the identification of endothelial cells *in vitro* and *in vivo* as the only other cell known to synthesise vWf, megakaryocytes, are easily identified by their size and morphology and are not found outside of the bone marrow except in certain extremely rare instances of malignancy or systemic organ failure. There is still debate as to whether all endothelial cells process WP bodies and therefore vWf. Much of the confusion appears to derive from species differences. Examination of the pig vascular tree demonstrated a heterogeneous distribution, with vWf being virtually absent in the thoracic and abdominal aorta but present in pulmonary arteries and the vena cava. Interestingly, abnormal WP bodies were found in all ECs (Gebrane-Younes et al, 1991), while Folkman et al in 1979 found that human capillary ECs possessed WB bodies whereas bovine ECs did not. As part of the work of this thesis, it was found that human retinal, choroidal and iris ECs all stained positive for intracellular vWf and expressed mRNA coding for vWf, thereby suggesting that human ocular ECs express vWf and by association possess some form of WP bodies.

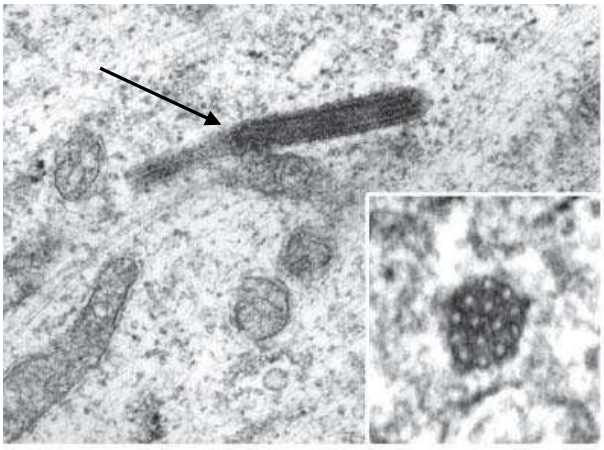


Fig 1.1. Electron micrograph of vascular endothelial cell demonstrating a Weibel-Palade body with its characteristicstriations.The inset shows a cross sectional view demonstrating the intra-organelle tubules which contain vWf. (with permission of Nature publishing)

Role of endothelial cells

Endothelial cells act as gate keepers between the vascular lumen and the extra-vascular environment. Due predominantly to their cell surface receptors and cell-cell junctional molecules they have a range of roles. These include:

1. The regulation of substances and cells passing through cell junctions and through the cells' cytoplasm from the intravascular to the extravascular space. This process is thought to be regulated by a variety of membrane-bound surface receptors for growth factors such as VEGF; pro- and anti-coagulant factors; lipid particles such as LDL; and metabolites such as nitric oxide and hormones (Cines et al, 1998). The degree of "leakiness" of the vasculature is dependent upon the location of the endothelial cells within the body and on its phenotype. The inner choroidal vasculature within the human eye is generally regarded to be semi-permeable due to the presence of fenestrations. As discussed later, these are small, full thickness hole-like structures in endothelial cells, often capped by a semi permeable membrane. They allow the passage of small and

medium sized molecules from the vascular lumen to the extra vascular space. Within the choroid of the eye, they allow the support of retinal pigment epithelial cells and photoreceptors, both of which have very high metabolic demands. Other ECs expressing fenestrations include those found in the glomerulus of the kidney where high rates of solute exchange are required to take place, and in the liver. At the other extreme, some endothelia are resistant to the passage of fluid, solutes, macromolecules and cells from the intra-vascular to the extravascular compartments. Examples are the retinal and brain vasculature. The cells form the inner blood-retinal and blood-brain barriers respectively, and are necessary for the structural integrity and functioning of the retina and brain. The major component of the inner blood-retinal barrier is tight junctions or zonula occludans between ECs (Sagaties et al, 1987). Astrocytes, present in the retina and brain are thought to be important in the formation and maintenance of the retinal and brain endothelial barrier functions by the expression of zonula occludens -1 (ZO-1) (Gardner et al, 1997).

2. The control of blood coagulation by the secretion of factors such as vWf (procoagulant), tissue factor pathway inhibitor (anti-coagulant), thrombomodulin (anti-coagulant), protein S (anti-coagulant) and tissue factor (procoagulant). The coagulation of blood is a complex process. At the site of vascular injury, e.g. a cut, a breach in the endothelium exposes collagen and extracellular matrix molecules which stimulate the clotting cascade, a process characterised by the activation of a large number of circulating intravascular proteins starting with factor XII. In the initial phase of injury, release of factors such as von Willibrand factor by endothelial cells recruits platelets to the site of injury to form the primary haemostatic plug. This is a transient response that aims to stabilise blood loss before the activated clotting cascade permanently closes the defect. In a similar fashion to the control of angiogenesis, blood coagulation is tightly controlled to avoid total coagulation within the vascular

system and relies on a delicate balance of pro- and anti- coagulation factors. Endothelial cells are critical to this balance, secreting factors such as thrombomodulin in order to abrogate the effects of other factors such as vWf.

3. The secretion of substances important in the control of blood flow and pressure such as nitric oxide, prostacyclin and endothelin 1.

   Endothelin-1 (ET-1) was discovered in 1988 and has subsequently been shown to be the most potent vasoconstrictor known to date (Yanagisawa et al, 1988). It also has the capacity to induce vascular remodelling, fibrosis, cell proliferation and apoptosis and is thought to be involved in a number of vascular diseases (Bourque et al, 2011). While three isoforms exist (ET-1 to ET-3), ET-1 is the most important and best studied. It is synthesised predominantly by vascular endothelial cells although it has also been found in vascular smooth muscle cells, the spleen, pancreas and lung. Expression of ET-1 is up-regulated by mediators such as catecholamines, angiotensin II, arginine vasopressin and insulin, and by mechanical factors such as shear stress, hypoxia and bacterial lipopolysaccharide. Expression is inhibited by factors that are important in vasodilatation such as nitric oxide, prostacyclin and atrial natriuretic peptide (KhimjiA and Rockey D, 2010). Secreted ET-1 is functionally inactive, only becoming activated after cleavage by endothelin converting enzymes and matrix metalloproteinases (Bourque et al, 2011). The actions of ET-1 are brought about by two G-protein coupled receptors, ET_A and ET_B, predominantly on vascular smooth muscle cells (thereby bringing about vasoconstriction). The main action of endothelin 1 is vasoconstriction and is thought to be important in the medical condition systemic hypertension. It is now common practice for a range of ET_A antagonists to be used as treatment for hypertension.

Nitric oxide (NO) is a gas that can act as a signalling molecule. One of its main functions on the endothelium is to act as a stimulator of vasodilatation. Its existence was suggested by Furchgott and Zawadzshi in 1980 but it was not until 1987 that its role in vasodilatation was discovered by Rees et al (1991) and confirmed by Ignarro (1989) who demonstrated that NO synthase inhibitors blocked vasodilatation. As NO is a volatile gas and lipophilic, it is freely diffusible across cell membranes and therefore does not need a cell surface receptor to bring about its actions. It is synthesised in response to factors such as shear stress on vessel walls by the action of nitric oxide synthase (NOS) on L-arginine. Within endothelial cells, the NOS isoenzyme is known as endothelial NOS or eNOS. Within neuronal cells which may be juxtaposed in tissues such as the brain and retina, the NOS isoenzyme is neuronal NOS or nNOS. Shear stress (i.e. fast and turbulent flow) within a vessel is detected by endothelial cells and this leads to an increase in intracellular Ca^{2+} which in turn, binds to and activates the regulatory protein calmodulin. Calmodulin in turn activates eNOS, leading to the synthesis of NO. Nitric oxide then diffuses out of the endothelial cell and into juxtaposed vascular smooth muscle cells to stimulate guanylate cyclase. This in turn, inhibits the calcium dependent contraction of the vascular smooth muscle cell i.e. vasodilatation.

Because the up-regulation of NO is both rapid and sensitive to changes in shear stress, it is thought to be the main vasodilatory mechanism maintaining vascular tone. Since its discovery, it has also become apparent that NO has other important vasoprotective roles including protection against apoptosis and other endothelial cell survival functions. In 2001, Brookes et al demonstrated that mice deficient in eNOS, either by gene knockout or by the ingestion of eNOS inhibitors, were protected against oxygen induced vaso-obliteration, thereby suggesting that high levels of NO are pathogenic (Brooks et al, 2001). NO is also thought to play a role in

endothelial cell VEGF signalling and will be discussed in more detail later.

Prostacyclin was discovered in 1977 by Moncada et al although it was initially called prostaglandin I_2. It is produced in endothelial cells by the action of prostacyclin synthase on a derivative of arachidonic acid, released from endothelial cell membranes by phospholipase. As well as acting in an autocrine fashion within the synthesising endothelial cell, it is secreted from the cell and acts in a paracrine fashion on local cells possessing prostacyclin (IP) receptors. Stimulation of this G protein coupled receptor can lead to vasodilatation and the localised inhibition of platelet aggregation. The mechanism of vasodilatation is thought to be due to vascular smooth muscle cell hyperpolarisation. The role of prostacylin in basal vascular tone and systemic hypertension is dubious given the fact that systemic administration of drugs such as aspirin and indomethacin which inhibit the production of prostacyclin have no measureable effect on blood pressure in humans (Parkington et al, 2004).

4. Endothelial cells are sensitive to stretch and shear stress, thereby acting as a measure of blood flow and pressure (Topper et al, 1996, Malek & Izumo, 1995; Korff & Augustin, 1999). The mechanisms underlying the detection of intravascular flow and shear stress and the changes in the cell are poorly understood. Rapid reactions to changes in shear stress include the activation of signalling mechanisms such as the generation of nitric oxide as described previously, the opening of potassium and calcium channels, the activation of focal adhesion kinase, MAPK and PKC (Tzima, 2006). Slower changes include the up-regulation of cell surface ICAM-1, TGF and PDGF, rearrangement of intracellular microfilaments and microtubules (Malek and Izumo, 1996) and their elongation along the direction of flow (Levesque and Nerem, 1985). The cellular mechanisms for sensing flow and stress are poorly understood but

are thought to involve cellular mechano-transducers (Tzima E, 2006). The mechanism is thought to involve the transmission of shear stress from the apical surface of the cell through the cytoskeleton to points of attachment (Davies P, 1995) both within the cell and at its basal surface junction with its basement membrane. Four candidate molecules have been suggested as being possible mechano-transducers involved in this process: surface integrins by virtue of their interactions with the extracellular matrix; ion channels; specialised membrane microdomains and G proteins (Traub and Berk, 1998). Recently, a fifth mechanism was suggested that involves PECAM-1 (CD31) acting via VE-Cadherin and VEGFR2, the latter acting as the signal transduction molecule (Tzima et al, 2005).

5. The secretion of extracellular matrix components such as fibronectin, laminin, collagen and elastin. Endothelial cells do not exist in isolation and require a complex micro-environment around them for cell stability and function. Part of this microenvironment is composed of the extracellular matrix which includes the cell's laminin rich basal anchoring basement membrane and a surrounding, paracellular fibronectin scaffold. The endothelial cells are thought to bind to and interact with the ECM via membrane spanning molecules called integrins. A simplified diagram of the ECM is shown below (Fig 1.2).

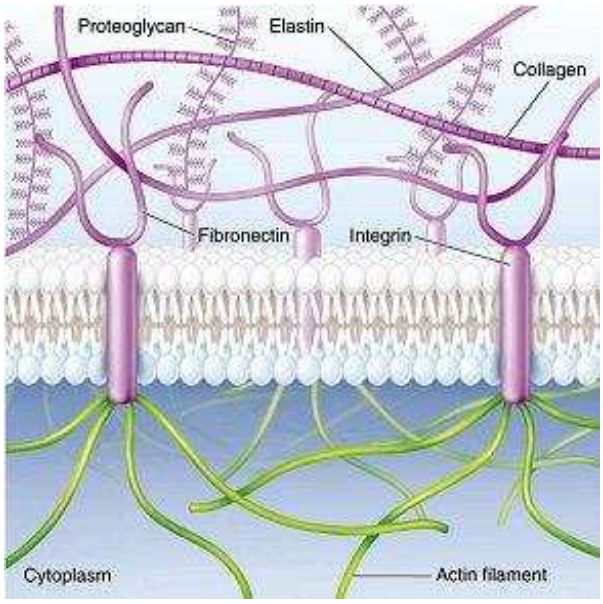


Fig 1.2. Diagrammatic representation of endothelial cell membrane interaction with extracellular matrix (with permission from Springer images)

The base materials of the ECM are proteoglycans, including heparin sulphate, glycosaminoglycans and water. Heparin sulphate is able to bind growth factors such as VEGF and FGF, thereby inactivating them and/or making an extracellular store that can be rapidly activated by enzymatic digestion of the heparin sulphate. Binding of VEGF by the ECM is also thought to be critical in the formation of growth factor gradients, important for both angiogenic sprouting and guidance of tip cells and during embryonic vasculogenesis.

Fibronectin is a high molecular weight (440kDa) proteoglycan dimer within the ECM that binds the extracellular component of integrin molecules. Each molecule has four fibronectin binding domains allowing a fibronectin scaffold to be built up around cells. Additional binding domains allow binding to collagen, fibrin, fibulin 1 and heparin sulphate. *In vitro* studies of isolated vascular endothelial cells have shown that fibronectin is one of the ECM components required for the satisfactory adhesion of ECs to culture plates and

for their subsequent proliferation. This requirement appears particularly important for microvascular ECs.

Integrins are a heterogeneous group of trans-membrane receptors that are composed of two dissimilar chains (α and β chains) .The integrins most commonly associated with endothelial cells are αv, β1 and β3. Their roles are to anchor the cell to the ECM and to provide a signalling pathway from the ECM to the intracellular environment. Matrix-integrin signalling is thought to be critical for embryonic development, cell proliferation, survival and migration. There are many different types of integrin depending on the different combinations of α and β chains used. To date, 18 different α chains and eight β chains have been characterised (Hehlgans and Cordes, 2007). Inside the cell, integrins are attached to microfilaments of the cytoskeleton. At sites of ECM ligand-integrin binding, integrin clustering occurs and complexes form called focal adhesions. It is at these sites that signal transduction is thought to take place. It is thought that integrins themselves do not have any tyrosine kinase activity, but bring about signal transduction by recruitment of accessory molecules such as focal adhesion kinase,(FAK) and integrin linked kinase (ILK)to the intracellular portion of the molecule, thereby activating signalling cascades such as SRC ((Hehlgans and Cordes, 2007).

6. The synthesis of growth factors. Conventional thinking suggests that endothelial cells are acted upon by growth factors secreted by other cells such as retinal pigment epithelial cells and ganglion cells within the eye, or by circulating VEGF derived from platelets (Webb et al, 1998) and neutrophils (Guadry et al, 1997). While the role of growth factors such as VEGF and FGF 2 in vascular development and pathological angiogenesis are well recognised, it has recently been discovered that endothelial cells themselves produce VEGF (Maharaj et al, 2006). It has also recently been demonstrated that genetic deletion of VEGF in the endothelial cells of adult mice led to

progressive endothelial degeneration, microhaemorrhagic events, intravascular thrombosis and death (Lee et al, 2007). Interestingly, circulating levels of VEGF were unaffected. This suggests that the requirement for autocrine VEGF cannot be replaced by circulating or paracrine VEGF, confirming the importance of autocrine VEGF as a cell survival signal. The authors also demonstrated that the actions of autocrine VEGF are mediated via the VEGF Receptor 2.
Endothelial cells have also been demonstrated to produce fibroblast growth factor 2 (FGF2) (Yu et al 1993; Cordon-Cardo et al, 1990). However, the role of FGF2 in auto/paracrine signalling is much less understood than for VEGF. It has recently been reported that increased production of FGF2 by pulmonary endothelial cells contributes to pulmonary smooth muscle hyperplasia seen in the rare condition of primary pulmonary hypertension, although the mechanism has yet to be determined (Tu et al, 2011). As discussed previously, endothelial cells are known to secrete PDGF B which attracts PDGFR-β expressing mural cells to the nascent vessel (Lindblom et al, 2003; Lindahl et al, 1997). Mouse knock out models of PDGF-B and PDGFR-β result in a similar lethal phenotype caused by absent pericyte recruitment and major vascular abnormalities (Leveen et al, 1994; Soriano P, 1994). It is thought that localised binding of PDGF-B to the surrounding endothelial cell ECM aids the orderly coverage of the new vessel with pericytes (Armulik et al, 2005). Basal secretion of PDGF by endothelial cells is required to maintain the mural cell coverage and the function of the vascular structure.

Ultrastructure of endothelial cells

Under electron microscopic examination, the luminal surface of endothelial cells shows fine projections and a coating of glycocalyx (Ryan & Ryan, 1984). The glycocalyx is a mixture of glycoproteins which may be involved in the regulation of solute transport and the mechanical effects of blood flow on the cell and the inhibition of inadvertent activation of the clotting cascade by cell surfaces.

Caveolae

The luminal surface is covered in pits called caveolae (derived from their initial appearance as caves). These structures “bud off” from the surface to form 50-100µvesicles and are seen to migrate through the cytoplasm (transcytosis) and to fuse with the opposite cell surface and release their contents into the extracellular space (Palade and Bruns, 1968). It is thought that caveolae are important for the transcellular passage of solutes and macromolecules such as low-density lipoprotein (LDL), very low density lipoprotein (VLDL), insulin, caeruloplasmin (copper carrying protein), albumin and transferrin (iron carrying protein) from the vascular lumen to the subendothelial space, perhaps by specific macromolecule receptors within individual caveolae (Simionescu and Simionescu, 1991; King and Johnson, 1985).

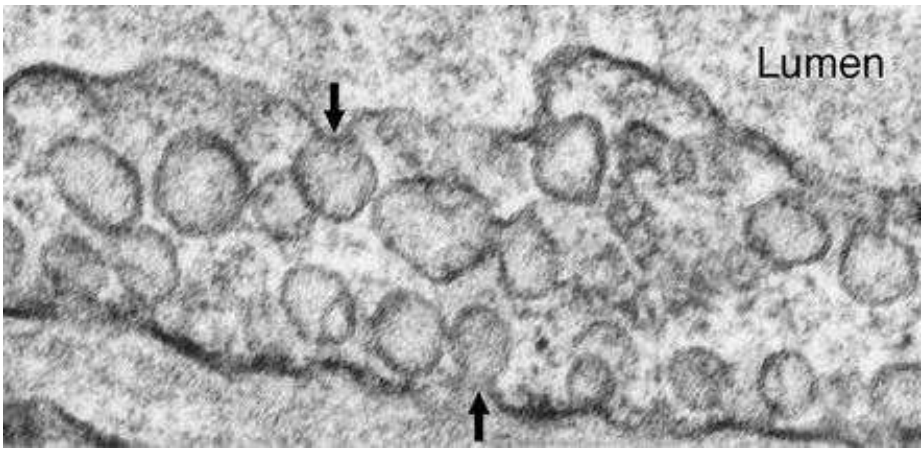


Fig 1.3. The image above is an electron micrograph of an endothelial cell demonstrating numerous caveolae, either budding from the luminal surface (upper arrow), within the cytoplasm or fused with the basal surface of the cell in direct communication with the subcellular space (lower arrow). (with permission from Springer images)

A major structural and critical protein found in caveolae is caveolin-1. This is a 22kDa protein that forms a major structural component of the vesicle membrane and is critical for its function (Rothberg et al, 1992). Without caveolin-1, caveolae do not form as demonstrated in the caveolin null mouse (Razani et al, 2001; Drab et al, 2001). It appears that without caveolae, increased transfer of solutes and macromolecules occurs by an up-regulation of paracellular routes, stimulated by up-regulation of intracellular eNOS activity and subsequent increased NO levels (Schubert et al, 2002). The passage of caveolae through the cell is thought to be along microtubules and is an ATP dependent process (D'Souza et al, 2006).

Fenestrations

Fenestrations are round or oval transcellular holes through the thinnest parts of the endothelial cell cytoplasm. They are found in areas where a high rate of partially selective exchange of components between the intra- and extravascular compartments is required. This exchange is usually limited to water and small solutes with passage of larger

components such as lipids and proteins being inhibited. The fenestrations within the choriocapillaris therefore allow the rapid transfer of nutrients through Bruch's membrane and the RPE, into the highly metabolically active photoreceptors. To date, three types of fenestrations have been described (Satchell and Braet, 2009).

Type 1 fenestrations are 60-70 µm in diameter and are covered by a thin diaphragm. The glycoprotein - plasmalemmal vesicle associated protein 1 (PLVAP-1) is thought to be a major component of this diaphragm. Each fenestration is surrounded by a cytoskeletal lattice. Type I fenestrations are found in the endothelia of endocrine glands, gastrointestinal mucosa and renal tubular capillaries (Fig 1.4). It is thought that the fenestrations found in the human choriocapillaris may be type I by virtue of the expression of PLVAP in gene array experiments (see later) and the possession of a diaphragm.

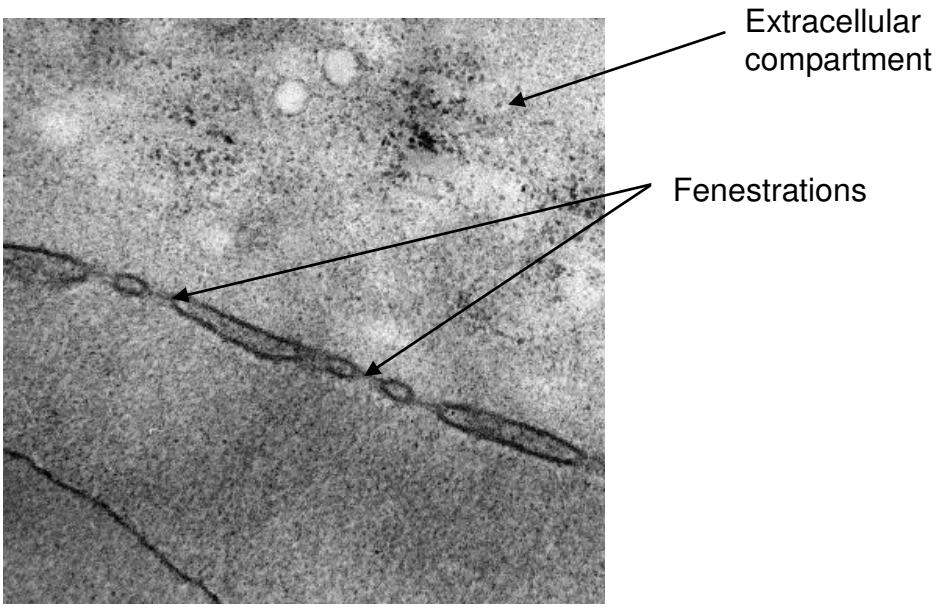


Fig 1.4. Electron micrograph of endothelial cell membrane demonstrating diaphragmed (type 1) fenestrations (with permission of Nature publishing)

Type II fenestrations are found in discontinuous endothelia such as those found in the spleen, liver and bone marrow. These fenestrations are wider (up to 200µm diameter) and do not have diaphragms or express PLVAP.

Type III fenestrations are found in the endothelium of renal glomeruli. They are of a similar size to type I, but like type II, do not express PLVAP or have diaphragms.

Weibel-Palade Bodies

Within the cell, the usual cellular constituents such as mitochondria, endoplasmic reticulum, microfilaments (actin) and intermediate filaments (vimentin) are found. Evidence for the apparatus for cellular excretion of specific molecules is scanty. The characteristic organelle of endothelial cells is the Weibel-Palade body. As previously described, these are intracellular organelles that synthesise and store von Willebrand factor (Factor VIII) which is a glycoprotein that is important in blood clotting and platelet adhesion to extracelluar matrix. The density of these intra-cellular organelles varies with the source or site of the endothelial cells.

Endothelial cell junctional complexes

In order to maintain the integrity of the vascular lumen, endothelial cells adhere to adjacent (endothelial) cells by a range of cell junctional complexes. The range and complexity of the junctions gives a clue to the importance of these junctions in the function of the endothelial barrier in both health and disease. Solutes and macromolecules can pass through the endothelial barrier either by a transcellular route, as discussed above (caveolae and fenestrations) or by a paracellular route, which is controlled by a range of junctional complexes.

These include zonula adherens and zonula occludens. Because of the heterogeneity and variation in function of the endothelial cells in different vascular beds, junctional complexes vary. This has the effect of introducing variability to the barrier function of ECs. As mentioned previously, endothelial cells form the inner blood retinal and blood brain barriers, and these barriers are necessary for the structural integrity and functioning of the retina and brain by excluding toxic solutes and macromolecules from reaching the tissues. In these instances, essential molecules are taken up by active transcellular processes via routes such as caveolae. The major component of the inner blood retinal and the blood brain barriers are tight junctions or zonula occludans between ECs (Sagaties et al, 1987). Astrocytes, present in the retina and brain, are thought to be important in the formation and maintenance of the retinal and brain endothelial barrier functions by modulating the expression of ZO-1 (Gardiner et al, 1997).

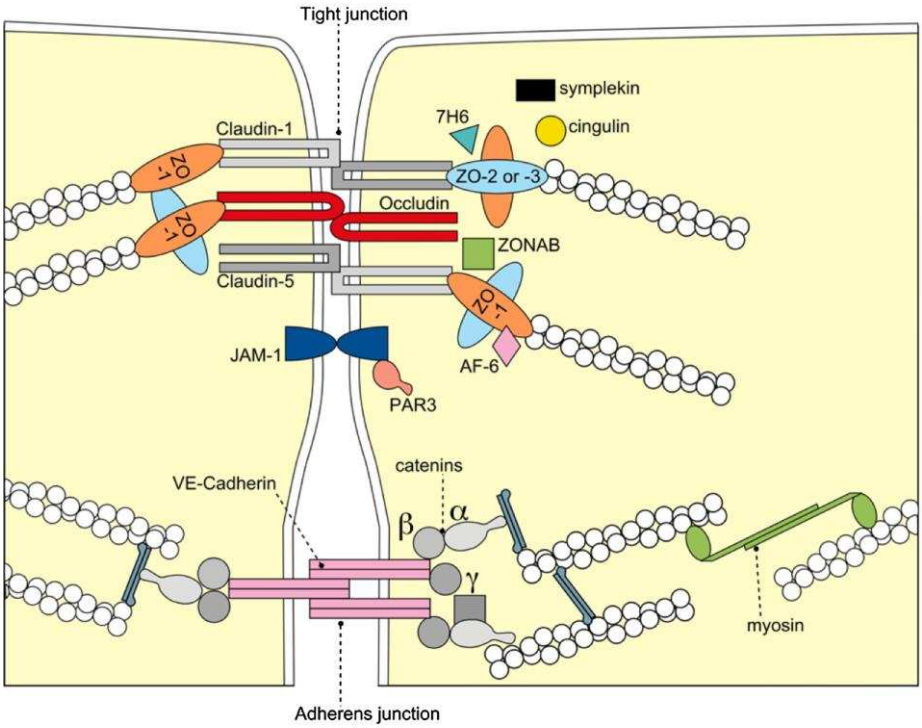


Fig 1.5. A diagram demonstrating the two major types of cell-cell junctions in endothelial cells (zonula occludens (tight junction) and zonula adherens (adherens junction). The diagram demonstrates the orientation of the major components and their interaction with the cytoskeleton of the cells (with permission of Springer images)

Zonula Occludens (ZO) consist of an adhesive belt around cells and are composed of components such as occludin and claudin. They prevent the movement of fluid between cells and are responsible for much of the integrity of the vascular lumen, preventing extravasation of fluid to the interstitial tissue. They also limit the movement of membrane bound proteins from the luminal to the abluminal surface of the cell. The first major protein identified was occludin. This is a 60kDa protein consisting of four transmembrane and two extracellular domains. Each occludin molecule binds to another occludin molecule on an adjacent cell, thereby forming a bond. The intracellular tails of the occludin molecules are linked to the actin filaments of the cytoskeleton by ZO-1 (and ZO-2) molecules. The role of occludin in tight junction formation and function may be complex as occludin deficient cells still demonstrate tight junction structures (Saltou et al, 2000) and

occludin-null mice demonstrated widespread abnormalities such as brain calcification. These findings suggest that structural redundancy exists in tight junction formation and function and that occludin may have wider roles in the body than just tight junction formation. It has been shown however, that the permeability of an endothelial cell barrier is directly related to the expression of occludin (Harhaj et al, 2006). It is widely recognised that increased local levels of VEGF, as seen in diabetic retinopathy, leads to increased vascular permeability. One of the main mechanisms of this increased permeability is the increased phosphorylation of occludin (and probable reduced function), stimulated by VEGF via up-regulation of intracellular PKC (Harhaj et al, 2006).

Another important component of tight junctions is claudin. This is a family of 24 different proteins that have a similar structure to occludin i.e. four transmembrane domains and two extracellular domains. Despite this similarity, claudins and occludins are structurally different. They bind to other claudin molecules of adjacent ECs and are linked to the cytoskeleton, like occludin, by ZO-1. Brain endothelial cells specifically express claudins 1, 2, 3, 5, 11 and 12 (Huber et al, 2001, Sandoval and Witt, 2008). It is thought that claudin 5 is the critical factor in the formation of the blood brain barrier (Tam and Watts, 2010). Junctional adhesion molecules (JAM) are another separate group of proteins involved in the structure of tight junctions. They are members of the immunoglobulin superfamily and three types are found in endothelial cells, JAM A to C (Ballabh et al, 2004). Like occludin and claudins, they attach to the cytoskeleton via ZO-1.

The accessory protein, ZO-1, is a 220kDa phosphoprotein with specific binding sites (PDZ sites) for occludin, claudins and JAM, and binding sites on its C-terminus for actin molecules. Its role is to link these tight junction molecules to the actin cytoskeleton. The molecule also has guanylate cyclase activity and plays a role in signal transduction (Bauer et al, 2010). The importance of ZO-1 in tight junction function is

illustrated by the fact that cells failing to express ZO-1 demonstrate complete disruption of tight junctions (Umeda et al, 2006).

Zonula Adherens (ZA) (cell-cell junctions) usually occur in a more basal location of the cell membrane than zonula occludens. They can take the form of an encircling band around the cell (zonula adherens) or as focal points of adhesion (adhesion plaques). The most important and abundant transmembrane proteins are the Cadherin family. These proteins form homodimers in a calcium dependent manner with other cadherin molecules on adjacent cells. One of the roles of zonula adherens junctions is thought to be the direct connection of the actin filaments of adjacent cells. The intracellular connection of cadherin molecules is via p120 protein and alpha and beta catenin molecules. The cadherin molecules most commonly associated with endothelial cells are VE-Cadherin (Vascular Endothelial), otherwise called cadherin 5, and N-Cadherin **(**Neuronal) which is also found on neuronal and smooth muscle cells. N-Cadherin appears to be present across the endothelial cell membrane and is not localised to adherens junctions. The importance of VE-cadherin in vascular integrity is demonstrated by the fact that injection of anti-VE-cadherin antibodies in mice leads to a marked breakdown in vascular integrity. In contrast, molecules such as VEGF and histamine have a much less dramatic effect and are reversible (Weis and Cheresh, 2005). It is thought that changes in vascular permeability are in part due to effects of molecules such as VEGF on adherens junctions and particular VE-cadherin. Activation of intracellular pathways (SRC) by VEGF can lead to phosphorylation, cleavage and internalisation of VE-cadherin (Esser, 1998).

Effect of growth factors on endothelial cells

The existence of growth factors that stimulate the growth of blood vessels was postulated after the observation of an angiogenic response in transplanted tumours (Folkman et al, 1963, Folkman et al, 1966; Folkman, 1972). Over a hundred years ago, Virchow noted, in a number of German language publications, that tumour growth is accompanied by an increase in vascularity. This was followed in 1927 by an observation by Lewis who described the variability in the vascular structure of tumours in rats and suggested that the tumours were having an influence on the characteristics of the blood vessels (Lewis, 1927). In 1939 Ide used a transparent chamber devised by Sandison, inserted into a rabbit's ear, to study the growth of the vascular supply in a transplanted carcinoma (Ide et al, 1939). It was observed that tumour growth was accompanied by extensive vessel growth, confirming the idea that vascularisation was required to "nourish" the growing tumour. The technique was developed further by Algire et al by quantifying vessel growth with time (Algive et al, 1945). At this point it was noted that vascular growth preceded the rapid growth of the tumour and that vessel growth is an important rate limiting step in tumour growth.

It was hypothesised at the time that a chemical substance may be involved in this process. Potential candidates at the time included the breakdown products of chromatin. However, in 1948, Michaelson suggested the term "Factor X" (Michaelson, 1948). In 1968, a series of experiments showed that vessel growth was stimulated by tumours even if a filter was placed between the tumour and the growing vessels (Greenblatt & Shubi, 1968; Ehrmann & Knoth, 1968). This suggested that a diffusible substance was responsible. A series of landmark experiments starting in 1971 by Judah Folkman reported attempts to isolate "tumour angiogenesis factor" (Folkman, 1971; Klagsbrun et al, 1976; Folkman, 1982). Folkman studied the stimulated growth of vessels in chick chorioallantoic membrane by this factor using cultured

tumour cells and the corneal pocket model (Kenyon et al, 1996). At this time, another pro-angiogenic factor, FGF, was isolated and was thought to be the postulated “tumour angiogenesis factor”. Subsequent work proved that this FGF was not the responsible agent and so the search for the factor continued (Dennis & Rifkin, 1990). In 1983, Senger reported the partial isolation of a protein that stimulated vascular leakage and this was termed “vascular permeability factor” (VPF) (Senger et al, 1983). Soon after, Ferrara and Henzel (1989) isolated a protein from the conditioned medium of bovine pituitary glands which led to a profuse growth of blood vessels in an *in vivo* assay. The fact that the mitogen was secreted and appeared specific for endothelial cells made it unlikely to be FGF (which lacks a secretory component and therefore may act in an autocrine environment). It was therefore termed “vascular endothelial growth factor” (VEGF) (Ferrara & Henzel, 1989). At a similar time, Plouët et al isolated a similar endothelial mitogen and called it “vasculotrophin” (Plouët et al, 1989). It was not long before both VPF and VEGF had been cloned and sequenced and found to be identical (Leung et al, 1989; Keck et al, 1989)

Ten years later, a large number of growth factors had been isolated with pro-angiogenic responses including vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factors (FGF 1 and 2 respectively), insulin-like growth factor 1 (IGF-1), placental growth factor PlGF), hepatocyte growth factor (HGF), tumour necrosis factor alpha (TNF-α) (via up-regulation of VEGF) (Yoshida et al, 1997) and interleukin-8 (Heidemann et al, 2003). To date, VEGF appears to be the most important in bringing about both normal physiological vascular development and pathological angiogenesis. In addition, other factors such as Transforming Growth Factor (TGF), Angiopoietins 1and 2 and Tie 2 are thought to be important in modulating the angiogenic response.

VEGF

As discussed above, vascular endothelial growth factor was originally discovered by Senger, Ferrara and Plouët and was initially called vascular permeability factor because its main effect appeared to be to increase the permeability of blood vessels. VEGF consists of a family of molecules, the most important of which is VEGF A. Other members include VEGF B, C, D and placental growth factor (PlGF), (McColl, 2004). The C and D forms regulate lymphatic angiogenesis. While vascular endothelial cells are the principal target for the actions of VEGF, other cells such as retinal ganglion cells (Nishijima, 2007; Kilic et al, 2006), monocytes (Clauss et al, 1990) and lung alveolar cells (Compernolle, 2002) express receptors to VEGF and appear to play a role in their physiology.

Actions of VEGF on endothelial cells include:

1. Stimulation of cell proliferation

2. Stimulation of cell migration

3. Enhancement of cell survival by the inhibition of cell apoptosis via a PI-3 kinase-Akt pathway and the expression of bcl-2

4. Increasing vascular permeability by alterations in cell-cell adhesion molecules such as the phosphorylation of occludin and VE-cadherin (discussed previously).

5. Vasodilatation by stimulated release of nitric oxide by activation of eNOS.

6. The formation of fenestrae.

In 1992 Breier et al showed that VEGF mRNA was temporally and spatially correlated with angiogenesis in the developing embryo (Breier et al, 1992). The same group later reported that VEGF mRNA was up-regulated in ischaemic areas of aggressive brain tumours, suggesting that it may be stimulated by hypoxia (Plate et al, 1992). It has since been discovered that the stimulation of VEGF is principally under the control of hypoxia inducible factor 1 (HIF-1) which binds to a binding site on the VEGF promoter (Semenza, 2002). In addition, other transcription factors such as the ETS family and reactive oxygen species affect VEGF expression (Randi et al, 2009). Hypoxia inducible factor 1 was discovered in 1992 (Semenza et al, 1992) and is one of the cell's main mechanisms of reacting to varying levels of tissue oxygenation. HIF-1 is a heterodimer protein transcription factor that consists of two proteins, HIF-1α and HIF-1β. HIF-1 activates the transcription of at least 60 genes that are involved in angiogenesis (VEGF), glucose metabolism, and cell proliferation (Semenza G, 2003). Interestingly, the effect of oxygen is not on the synthesis of HIF-1 but on its degradation. Hypoxia leads to an inhibition of its breakdown, thereby leading to elevated levels. Under conditions of normoxia, HIF-1 degradation is regulated by O_2 dependent prolyl hydroxylation which targets the protein for ubiquitination by ubiquitin-protein ligases which include the von Hippel-Lindau tumour suppressor (an important protein mutated in the ocular disease von Hippel-Lindau syndrome) The HIF-1 is therefore rapidly degraded and angiogenic growth factors such as VEGF are not up-regulated. Under conditions of hypoxia, the O_2 dependent hydroxylation does not occur and therefore the HIF-1 is not targeted for degradation. This therefore leads to an up-regulation of VEGF expression.

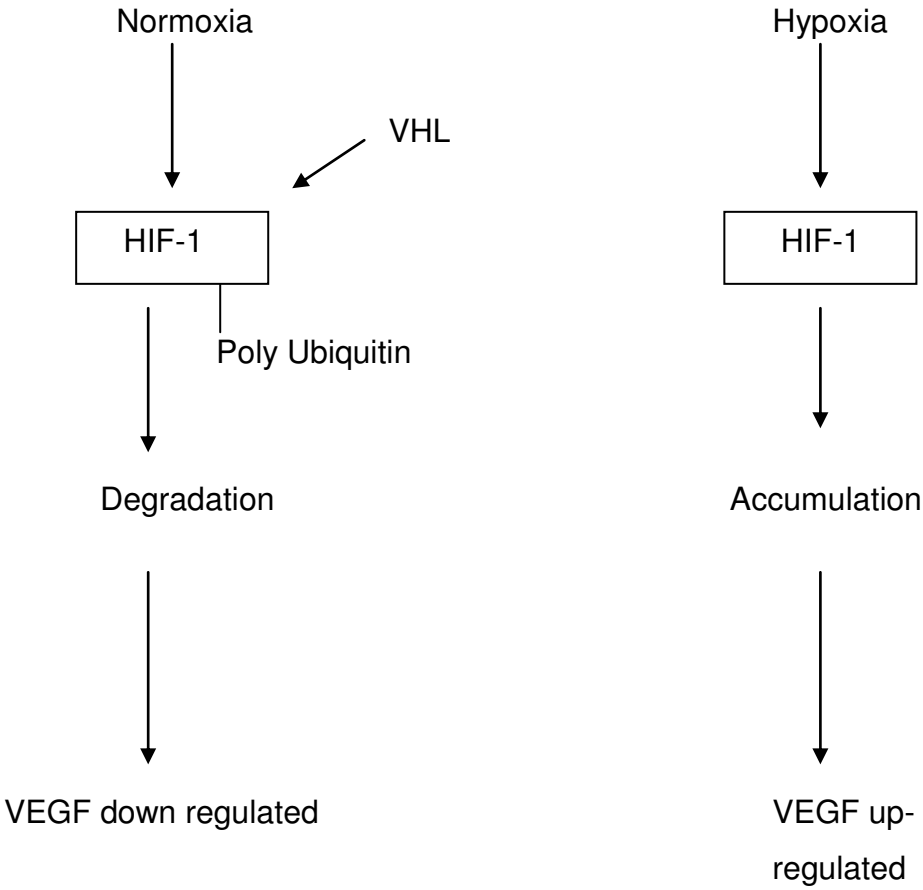


Fig 1.6. A simplified representation of the HIF pathway.

In von Hippel Lindau disease, mutations in the VHL protein lead to inactivation of the ubiquitinisation process thereby inhibiting HIF-1 degradation and up-regulation of local VEGF expression. The disease is characterised by localised vascular tumours in the retina, cerebellum, kidney and pancreas.

Further evidence of VEGF's pro-angiogenic role is provided by results of the *in vivo* use of VEGF blocking antibodies which reduced the growth of glioblastomas (a type of brain tumour) by 80% in mice (Kim et al, 1993) and inhibited iris neovascularisation in a monkey model of retinal ischaemia (Adamis et al, 1996). *In vitro* culture of the glioblastoma cells revealed that the antibodies had no direct action on tumour cell growth but were acting on the tumour blood supply.

The anti-tumour effect of anti-VEGF antibodies has subsequently been demonstrated for a range of animal tumours and has also been shown to have a modest clinical effect in human disease. The humanised anti-VEGF monoclonal antibody referred to as bevacizumab (Avastin™) is licensed for the treatment of bowel tumours and has been shown to prolong life in clinical trials (Vincenzi et al, 2009).

VEGF isoforms

The gene responsible for VEGF A consists of eight exons and seven introns. Alternative splicing of the gene product produces at least four different human isoforms: VEGF $_{121}$, VEGF$_{165}$, VEGF$_{189}$ and VEGF$_{206}$,, consisting of 121, 165, 189 and 206 amino acids respectively. The intensely studied mouse isoforms of VEGF each have one less amino acid than their human equivalent. The main difference between isoforms is the presence of heparin binding domains. While all isoforms have an identical “active” region, VEGF$_{121}$ has no heparin binding domain or tail and appears freely diffusible through the extracellular matrix (ECM), whereas VEGF 206 and 189 both bind heparin avidly and are therefore strongly bound on cell surfaces or within the extracellular matrix. VEGF $_{165}$ has intermediate heparin binding properties. This difference in properties appears to be important in vascular development and in the formation of VEGF concentration gradients (Ruhrberg et al, 2002). It is known that endothelial cells will not form vessels by simply being in a VEGF-rich environment, but need a concentration gradient for the endothelial cells to migrate along, thereby forming a vasculature (Ruhrberg et al, 2002). It is thought that ECM-bound forms of VEGF are released by cleavage by proteases such as plasmin, heparinase and matrix metalloproteases.

Mice expressing a null mutation for VEGF A die in utero with an extremely disorganised and rudimentary vasculature (Carmeliet et al, 1996). Ruhrberg et al in 2002 demonstrated that mice solely expressing

the $VEGF_{120}$ isoform, which is devoid of a heparin binding domain and is thereby unable to associate with the perivascular ECM, develop a vasculature with a marked reduction in branching patterns. They found that this reduction in branching was caused by a redistribution of endothelial cells from the formation of additional branches to being incorporated into the existing vasculature to increase lumen calibre instead. They suggest that variations in the ratio or proportions of VEGF isoforms around the developing vasculature alter the branching patterns by establishing concentration gradients of VEGF along which developing blood vessels grow. They hypothesise that VEGF120 will rapidly diffuse away from the developing vessel while the VEGF 189 isoform will remain juxtaposed to the vessel, neither of which will allow a sufficient concentration gradient of VEGF to be established. It is therefore most likely that a combination of all four isoforms is required to establish the required gradient (Ruhrberg et al, 2002). Although VEGF A is thought to be the most important member of the VEGF family, other members exist such as VEGF B, VEGF C and VEGF D. It is known that the C and D forms are involved in lymphangiogenesis. Other members include placental growth factor (PLGF) and EG-VEGF (endocrine gland VEGF), which is expressed and acts solely on endothelial cells within endocrine glands such as the adrenal gland. Recently, other VEGF “like” molecules have been described including parapox virus open reading frame (VEGFE) (Ogawa et al, 1998) and snake venom derived polypeptide (VEGFF) (Yamazaki et al, 2005). Their roles, if any, in human endothelial biology are unknown. An additional mammalian splice variant of VEGF A is denoted VEGFAxxxb, which binds, but fails to activate VEGF receptors and has therefore been described as anti-angiogenic (Harper and Bates, 2008). It has been found that in the vitreous fluid of diabetic patients, there is a switch from the anti-angiogenic VEGFAxxxb splice variants to the pro-angiogenenic VEGFAxxx variants with increasing retinal ischaemia and neovascularisation (Perrin et al, 2005). This little understood or researched VEGF splice variant may therefore be an important part of

the anti/pro angiogenic balance within tissues, disruption of which can lead to aberrant angiogenesis.

VEGF Receptors

The actions of VEGF on endothelial cells are initiated via two cell-surface receptors. The first of these was discovered in 1992 in the mouse and was termed Flt-1 (de Vries, 1992). Its human equivalent is termed VEGFR1. A second mouse VEGF receptor was discovered soon afterwards and termed Flk1 or KDR (Terman et al, 1992). Its human equivalent was termed VEGFR2. While VEGFR2 is predominantly expressed on vascular endothelial cells and their embryological precursors, it is also found on pancreatic duct cells, retinal progenitor cells and megakaryocytes (Oelrichs et al, 1993), although its function in these cells is unknown. These two receptors are receptor tyrosine kinases with seven extracellular immunoglobulin-type domains, a single transmembrane domain and a single intracellular tyrosine kinase domain (Costa et al, 2004; Neufeld et al, 1999; Ferrara et al, 2003).

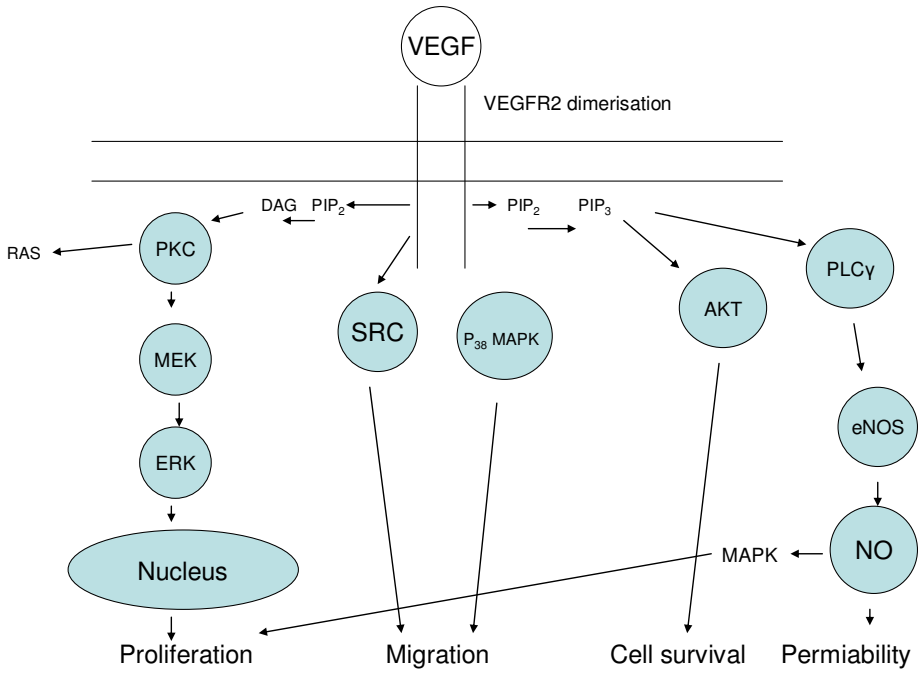
VEGFR2

It is widely accepted that VEGFR2 (Flk 1) is the main receptor involved in endothelial proliferation, survival, migration, permeability and vascular tube formation. VEGFR2 has a binding affinity for VEGF A, ten fold lower than VEGFR1. Binding of VEGF to VEGFR2 leads to receptor dimerisation and conformational changes within the intracellular domain. These changes lead to exposure of the ATP binding site in the kinase domain which in turn leads to autophosphorylation of a number of tyrosine residues (Takahashi et al, 2001). One of the most important residues is Tyr $_{1175}$, phosphorylation of which leads to activation of PLCγ and in turn, IP_3 diacyl glycerol (DAG), PKCα, β and ζ, MER/ERK, phospholipase A_2 and RAS (Wu et

al, 2000; Yu, 1999, Bullard et al, 2003, Koch et al, 2011). This pathway is thought to be important for cell proliferation. Phosphorylation at Tyr_{1214} leads to up-regulation of p38MAPK and this is important for cell migration. The activation of PIP2 to PIP3 at the cytosolic surface of the cell membrane leads to activation of AKT which is thought to be important for cell survival. (Cebe-Suarez et al, 2006). Activation of VEGFR2 also appears to have a role of increasing vascular permeability via the weakening of cell-cell adhesions. Activation of the VEGF receptor leads to eNOS activation via PLCγ with the result that intracellular NO is up-regulated. (as discussed previously). A similar effect is also seen when other pro-angiogenic growth factors such as FGF-2 and TGF beta are added to endothelial cells (Inoue et al, 1995, Wu et al, 1996). In addition to its vasodilatory roles, NO is thought to be an important intermediary in angiogenesis (Hida et al, 2004). Sodium nitroprusside (an exogenous NO donor) stimulates endothelial cell DNA synthesis, proliferation and migration (Zheng et al, 2005). It is thought that NO exerts its effect by stimulation of cyclic guanosine monophosphate (cGMP), a downstream modulator of the VEGF signalling pathways (Ziche et al, 1997, Ignarro et al, 1991). Stimulation of cGMP leads to activation of MAPK, an important mediator on the VEGF signalling pathway. This effect of NO on the up-regulation of cGMP can be inhibited by thalidomide, a drug noted for its anti-angiogenic properties before it was withdrawn from use, by its action on guanylate cyclase Majumder et al, 2009)

Lack of VEGFR2 in the Flk-1 null mouse leads to failure of the development of blood islands and blood vessels and ultimately causes death *in utero* (Shalaby et al, 1995).

Fig 1.7. below is a simplified schematic outline of the actions of VEGF on binding to VEGFR2. Note the receptor has formed an activated dimer. Many of the reported intermediary steps have been omitted for simplicity and clarity.



VEGFR1

The role and actions of VEGFR1 are less well understood and still subject to debate. It is thought that this may be due to variability in receptor function depending on cell type and the developmental stage of the animal. Like VEGFR2, VEGFR1 is highly expressed on vascular endothelial cells and has a binding affinity for VEGF ten fold higher than VEGFR2. It is also expressed on monocytes, macrophages, trophoblasts, smooth muscle cells and renal mesangial cells (Koch et al, 2001). Like VEGFR2, its expression is up-regulated by hypoxia but VEGF binding appears not to stimulate a mitogenic signal. Binding of VEGF to VEGFR1 stimulates very weak intracellular tyrosine kinase activity with different tyrosine residues being activated depending on the ligand i.e. VEGF or placental growth factor (Autiero et al, 2003).

Although weak, activation of tyrosine kinase domains on VEGFR1 does lead to activation of intracellular phospholipase C (PLC) and the generation of inositol 1,4,5 triphosphate (Sawano et al, 1997). One hypothesis for the role of VEGFR1 is that it is a “decoy” for VEGFR2, perhaps mopping up VEGF and thereby limiting its binding and consequent stimulatory actions on VEGFR2 (Park et al, 1994). Other hypotheses include roles as a VEGF reservoir or actin microtubule reorganisation and tube formation (Koch et al, 2011). Recently, actions such as the stimulation of matrix metaloproteinase 9 and the recruitment of endothelial progenitor cells have been ascribed to the receptor (Hristov et al, 2003). Despite the conflicting opinions on the role and importance of VEGFR1, it is clear that Flt-1 (VEGFR1) null mice die *in utero* because endothelial cells develop but fail to form vascular channels (Fong et al 1995). Interestingly, deletion of just the intracellular domain is compatible with normal vascular development (Hiratsuki et al, 1998), giving credence to the hypothesis that it acts in some way as a reservoir or decoy for VEGF. This underlines the important but as yet undefined roles of VEGFR1.

Two other receptors have also been found to bind VEGF. The first is neuropilin 1 which is a receptor found on axons and is thought to play a role in axon guidance (the pathways of blood vessels and nerves often co-exist). It may also augment VEGF binding to VEGFR2 but has no mitogenic actions itself when binding VEGF (Ferrara, 2004). The second is VEGFR3 (or Flt4 in mice). This VEGF receptor is found on lymphatic endothelial cells and is important for lymphangiogenesis and tumour metastasis via lymphatics.

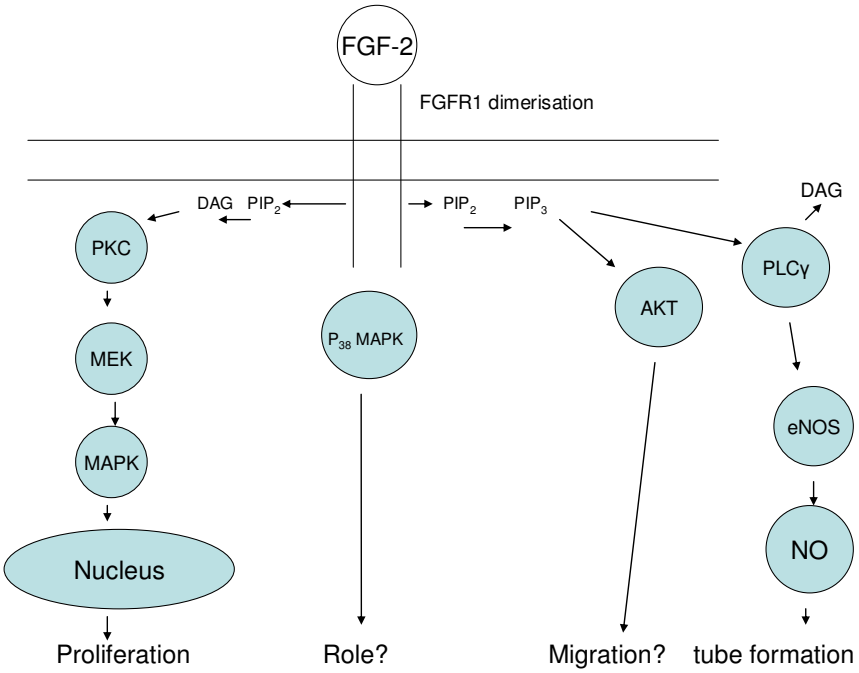
Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are a group of growth factors that have mitogenic actions on a wide range of cells such as fibroblasts and endothelial cells and are also important in embryogenesis (Javerzat, 2002). The mitogenic and migratory action of FGFs on vascular endothelial cells pre-dates the discovery of VEGF, and ironically, for many years was thought to account for the action of VEGF. To date, at least 23 isoforms of FGF have been discovered, acting via 4 tyrosine kinase receptors (Cronauer et al, 2003).

The main FGF receptor on ECs is FGF receptor 1 (FGFR1) and this binds the two most abundant forms, FGF2 (basic FGF) and FGF1 (acidic FGF). Activation leads to stimulation of the mitogen-activated protein kinase pathway (MAPK) (Zubilewicz et al, 2001) and Ca^{2+} channel activation (Rosenthal et al, 2005). An interesting fact about FGF2, the main FGF involved in EC mitogenesis, is that it contains no signal motif after synthesis on the Golgi apparatus. In theory, it cannot be excreted. Interestingly, mice carrying either a null mutation for the FGFR 1 or a transgenic mouse with an inducible defect in ocular FGFR 1 action both demonstrated poor generalised and ocular vascular development (Rousseau et al, 2000; Rousseau et al, 2003). Because of its lack of secretary signal motif, the action of FGF on endothelial cells may either be autocrine in nature or it may leave the cell by an unknown mechanism to produce a paracrine or distant action. Clearly, cells would not express FGF receptors if it was not present in the ECM of cells. Because of the contradictory evidence for FGF having a specific role in angiogenesis (presence of FGF receptors on ECs linked to a proliferative intracellular signalling cascade (Cross and Claesson-Welch, 2001) versus a lack of a secretory signal on the intracellular protein (thereby suggesting an absence of a true stimulatory role), workers have questioned whether FGF 2 acts via a second growth factor such as VEGF (Murakami and Simons, 2008). Masaki et al in

2002 showed that the therapeutic effect of FGF 2 on the improvement of a mouse hind limb ischaemia model could be eliminated by the administration of VEGF neutralising antibodies. This suggested that VEGF was required for the action of FGF 2. Potential mechanisms of action include the stimulation of VEGF secretion by FGF 2 (Seghezzi et al, 1998) or that VEGF is required for FGF receptor function (Pepper and Mandriota, 1998). Welti et al in 2001 demonstrated that FGF 2 still provided pro-angiogenenic signals and stimulated endothelial cell proliferation despite inhibition of the VEGFR2 tyrosine kinase activity using a drug called Sunitinib. This finding has implications for the treatment of angiogenenic diseases such as neovascular age-related macular degeneration or cancer because the endothelial cells may circumvent anti-VEGF treatments by utilising FGF pathways The picture is further confused by the finding that FGF action in the embryo precedes the appearance of VEGF signalling and is also required for VEGF receptor function (Murakami et al, 2011).

Fig 1.8. The diagram below is a simplified schematic outline of the actions of FGF2 on binding to FGFR1. Note that the receptor has formed an activated dimer. Many of the reported intermediary steps have been omitted for simplicity and clarity. Unlike VEGF, many of the roles and pathways of FGF2 action are currently unknown



Insulin like growth Factor 1 (IGF-1)

Insulin like growth factor 1 (IGF-1) is a 7.6 KDA protein important in childhood growth. Its secretion is stimulated by growth hormone and is produced predominantly by the liver and to a lesser extent in a paracrine fashion by target organs. IGF 1 circulates in the blood almost totally bound to at least 6 different IGF binding proteins (Novosyadlyy et al, 2004; Lofqvist et al, 2009), with IGF binding protein 3 being one of the most important (Baxter, 2001). Target cells (which include endothelial cells) possess two different IGF receptors (I and II). Binding of IGF 1 to IGF RI leads to activation of its tyrosine kinase activity which in turn leads to activation of several intracellular pathways such as TOR, Akt, and MAPK (Hellström et al, 2001; Smith et al, 1999) although they are poorly understood. As well as directly stimulating intracellular signalling pathways, IGF 1 is also thought to lead to an up-regulation of VEGF expression by increasing the expression of HIF-1α via PI-3 and MAPK (Fukuda et al, 2002). This would suggest that the effect of IGF-1 on endothelial cells is brought about by direct actions of the IGF receptor and by secondary effects on VEGF. This makes research into the effects of IGF-1 on disease mechanisms difficult to elucidate. This may be the reason why a number of oncology and anti-angiogenesis clinical trials using IGF-1 inhibition have produced disappointing clinical results despite promising *in vitro* experiments.

IGF -1 can also bind to insulin receptors but with much less avidity than that of the IGF receptor.

In specific relation to the eye, IGF 1 is thought to be required for retinal vascular development *in utero*, acting in conjunction with VEGF and its role in retinopathy of prematurity is currently under investigation (Smith et al, 1999; Hellström et al, 2002; Hellström et al, 2001). In adults, it is thought to be one of a range of growth factors involved in choroidal neovascularisation (Lambooij et al, 2003; Rosenthal et al, 2004) and

proliferative diabetic retinopathy (Meyer-Schwickerath et al, 1993; Grant et al, 1986). Interestingly, when given as a subcutaneous injection to diabetic patients, it was found to worsen diabetic retinopathy and is also found in high concentration in the vitreous of eyes with proliferative diabetic retinopathy (PDR) (Grant et al, 1986). However, pituitary dysfunction or surgical removal of the pituitary, with concomitant reduction in IGF-1 levels secondary to low growth hormone levels, can prevent or reverse proliferative diabetic retinopathy (Sharp et al, 1987; Merimee et al, 1970; Wright et al, 1969). Humans with defects in the IGF-1 or IGF-1 receptor gene were found to have reduced retinal vascular branching points (Hellström 2002). Taken together, all of these observations would suggest a critical role for IGF 1 in ocular development and angiogenic diseases.

Placental Growth Factor (PlGF)

PlGF is a member of the VEGF family and was originally cloned from the human placenta as the name suggests, in 1991 (Maglione et al, 1991). Like VEGF, PlGF occurs in at least 4 different isoforms, with isoforms 2 and 4 being strongly bound by heparin. Human PlGF displays 42% sequence homology with human VEGFA, although their 3D structures are almost identical. Despite this similarity in 3D structure, PlGF is almost solely bound by VEGFR1 (Park et al, 1994). However, it may indirectly stimulate VEGFR2 by displacing VEGF from VEGFR1. In addition, PlGF is also to bind to neuropilin 1 and 2. Like VEGF, its expression is up-regulated by hypoxia despite it not having a hypoxia response element in its promoter sequence. Its angiogenenic potential was demonstrated in 1997 (Ziche et al) but knockout models failed to demonstrate any abnormalities in vascular development (Carmeliet et al, 2001) suggesting that its angiogenenic role may be limited to pathological states. While it has been found associated with choroidal neovascular membranes, its role in disease pathogenesis remains uncertain (Rakic et al, 2003).

Angiogenesis modifying factors

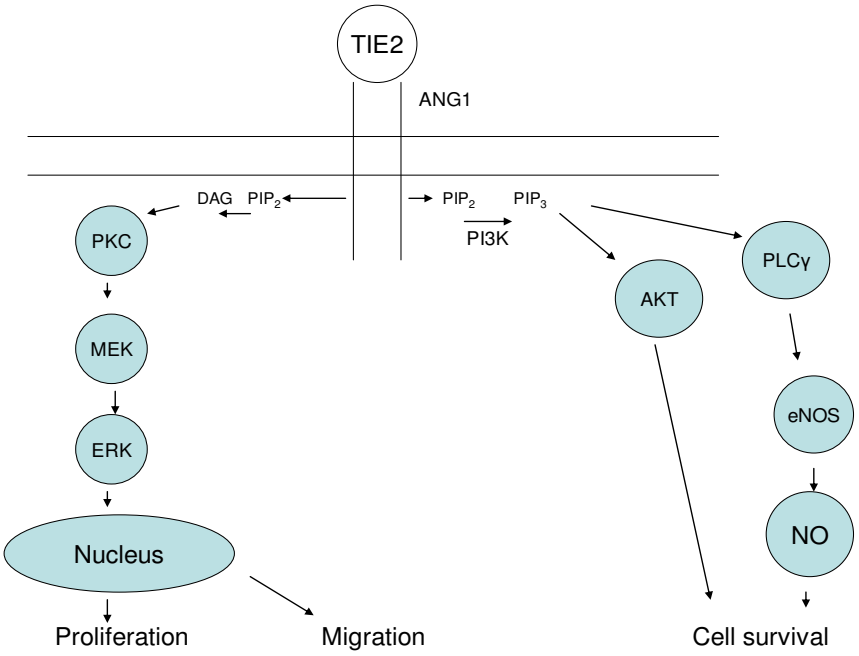
Platelet Derived Growth Factor (PDGF)

Platelet Derived Growth Factor is a growth and chemotactic factor for fibroblasts, smooth muscle cells and glial-derived cells, being found in high concentration as the name suggests, in platelets. It has a dimer structure, being composed of an A and a B chain. This gives rise to three isoforms, PDGF-AA, -BB and -AB. Originally, it was thought that endothelial cells were unresponsive to PDGF but it is now known that endothelial cells express PDGF receptors (Rα and Rβ) and that, in particular, PDGF-BB has some pro-angiogenic action (Risau et al, 1992). It is known that the pericytes of developing blood vessels (in particular, the stlk cells) express PDGF receptors and require endothelial cells to secrete PDGF for their survival (Lindblom et al, 2003). In a PDGF-B knock mouse model animals were found to lack pericytes covering brain microvessels and these tended to form microaneurysms and were prone to rupture (Lindahl et al, 1997). Sprouting capillaries also appeared to fail to attract pericytes. In a mouse model of choroidal neovascularisation; inhibition of both VEGF and PDGF-B signalling was more effective in causing actual new vessel regression than in those animals targeting VEGF alone (Bergers et al, 2003; Jo et al, 2006). In a similar experiment in mice induced to develop proliferative diabetic retinopathy, injection of an anti-PDGF-B aptamer demonstrated significant reduction in disease complications such as retinal detachment compared with those given sham aptamer (Akiyama et al, 2006).

Angiopoietin/Tie system

The human angiopoietin (Ang) / Tie system consists of two Tie tyrosine kinase receptors (Tie 1 and Tie 2) and three secreted ligands (Ang1, Ang2 and Ang4). Angiopoietin 1 functions as a Tie 2 receptor agonist and Ang 2 normally functions as an Ang 1 antagonist (Maisonpierre et al, 1997), except when present in very high *in vitro* concentrations, in which case it can act as a weak agonist (Kim et al, 2000). Little is known about Ang4 but it has been shown to inhibit HUVEC migration towards VEGFA and FGF2 and to also inhibit *in vitro* angiogenesis (Olsen et al, 2006) and so may be important in modulating angiogenesis. The receptor Tie2 is expressed on endothelial cells and binds all angiopoietin ligands whereas Tie 1 has no known ligand but has been shown to bind to Tie 2 and to regulate its activity (Milner et al, 2009). Binding of Ang 1 to Tie2 leads to receptor dimerisation and the resultant auto phosphorylation and activation of intracellular signalling pathways. Signalling molecules activated include: eNOS, SH2 domain containing phosphatase (SHP2) and PI-3K. Many of the signalling components and pathways activated are similar to those seen when VEGF binds to VEGFR2 (see below). However, Tie activation on its own does not cause cell proliferation. This difference in end response may be context driven. It has recently been shown that in quiescent endothelial cells linked by cell-cell junctions, Ang1/Tie2 activation leads to cell survival signals via AKT. Up-regulated “quiescence genes” include kruppel like factor 2 and Dll-4 (Zhang et al, 2011). When endothelial cells are isolated or detached from the effects of surrounding cells, as they may be in angiogenesis, Tie2 is embedded in the cell substratum and Ang1 is associated with ECM, and then the signal switches to a pro-angiogenic pathway (Zhang et al, 2011).

Fig 1.9. The diagram below is a simplified schematic outline of the actions of ANG1 on binding to Tie 2. Note that the receptor has formed an activated dimer.



In quiescent adult vasculature, Ang1 secreted from mural cells induces Tie2 activation in endothelial cells to maintain mature blood vessels by enhancing vascular integrity (barrier function) and endothelial survival (Brindle et al, 2006). It also plays a role in maintaining an effective pericyte covering of vessels. In contrast, the antagonist Ang2 leads to breakdown of cell barrier function. The importance of the Tie/Ang system is revealed when murine gene knock out models for Tie 2 and Ang 1 produce animals with abnormal hearts and abnormal vascular walls (Tachibana et al, 2005; Suri, 1996) while mice overexpressing Ang2 are lethal *in utero* (Maisonpierre et al, 1997), presumably due to competitive inhibition of Ang1.

The role of Tie1 remains elusive but mouse knock down models using siRNA s demonstrate increased Ang1-mediated Tie2 activation, suggesting that it is a negative regulator of Tie 2 activity. Angiopoietin 1 is constitutively expressed in tissues by mural cells and fibroblasts

whereas Ang2 is usually only expressed by endothelial cells at sites of vascular remodelling. At sites of vascular remodelling, expression of Ang2 is up-regulated by VEGF, IGF-1 and PDGF and is stored in Weibel-Palade bodies along with von Willebrand factor. While acting as an Ang1 antagonist at sites of vascular remodelling, Ang2 stimulates the dissociation of pericytes from vessels and increases vascular permeability. Both of these factors are thought to be important for an angiogenic response and so the up-regulation of Ang 2 at sites of angiogenesis may be necessary for vessel growth. Our work shows that Ang2 is expressed by proliferating ocular vascular ECs (see later). Continued Ang2 activity however, after vascular growth has occurred, may prevent appropriate pericyte recruitment and maintenance, thereby making the new vessels unstable and liable to breakdown, particularly if VEGF is withdrawn. As well as maintaining pericyte stability on new vessels, ANG1 also maintains the continuous distribution of CD31 (PECAM1), ZO-1 and VE-cadherin, all of which are important in endothelial cell-cell adhesion (Falcon et al, 2009).

Overall, the data on the Ang/Tie system appears confusing and at times contradictory. While today we know far more about the signalling pathways and interactions within the system, our thoughts on the role of the system in the overall maintenance and evolution of the vasculature still comes back to the original views of the system which suggested that the Ang/Tie system was responsible for vessel “stability”. Several research teams are looking at the potential of the Ang/Tie system in treating angiogenic diseases but at present results are limited. To date, the effect of the system on human disease remains limited to a rare mutation in Tie 2 which leads to a disease characterised by abnormalities of the smooth muscle surrounding small vessels and microaneurysms leading to venous malformations (Vikkula et al, 1996).

Anti-angiogenic factors

It has been suggested for many years that the control of normal (physiological) and pathological angiogenesis is brought about by a fine balance between pro- (VEGF, FGF 2 etc) and anti-angiogenic factors. Soon after Folkman discovered his new angiogenic factor he began to search for a substance that could inhibit it, as this may potentially be used as a treatment for cancer-induced angiogenesis (Folkman, 2004). The first endogenous substance to be discovered was interferon alpha which has been successfully used in the treatment of life threatening haemangiomas in the lung, hand and jaw (White et al, 1989; Marler et al, 2002). This was followed by the discovery of angiostatin (a fragment of plasminogen by O'Reilly et al in 1994)) and endostatin (a fragment of collagen XVIII) (Wen et al, 1999). It had long been recognised that patients with Down's syndrome have a markedly reduced incidence of solid tumours. One explanation for this is the possession of three copies of the collagen XVIII gene with subsequently higher levels of the endostatin collagen fragment (Zorick et al, 2001).

Pigment Epithelial Derived Factor (PEDF)

An angiogenesis inhibitor with specific relevance to the eye is Pigment Epithelial Derived Factor (PEDF). This was first identified in the medium of cultured foetal retinal pigment epithelial (RPE) cells and was found to stimulate the outgrowth of neurites from retinoblastoma cells (Tombran-Tink, 1989). It is a 418 amino acid protein and is a member of the serine protease inhibitor (serpin) family (Barnstable & Tombran-Tink, 2004). Expression studies have revealed that PEDF is secreted by RPE cells into the inter-photoreceptor matrix (Tombran-Tink et al, 1995) and also by cells at the corneal limbus and the ciliary body (Barnstable & Tombran-Tink, 2004). Another major site of PEDF expression is in the central nervous system where it is found in ependymal cells and motor

neurones of the ventral horn (Bilak et al, 1999). Interestingly, PEDF appears to have two major functions: neurotrophic/ neuroprotective and antiangiogenic (Barnstable & Tombran-Tink, 2004).

Neurotrophic/protective functions

In vitro experiments of cultured neurones treated with PEDF show that it stimulates outgrowth from the nerve cells. As it is found in high concentrations in the eye from an early stage of foetal development, it may play a role in retinal development. Support for this role is confirmed by a mouse PEDF knockout that showed abnormal development of the retinal architecture (Doll et al, 2003). Injection of PEDF into the vitreous of mice with mutations in photoreceptor genes significantly reduced photoreceptor degeneration Cayoutte et al, 1999), while injection into the vitreous of rats rescued photoreceptors from light damage (Cao et al, 2001). From these studies, it is thought that PEDF stimulates chemical changes in photoreceptors that make them more resistant to toxic insults. It is not known, however, if PEDF acts directly on the photoreceptors themselves or stimulates adjacent cells such as Muller or RPE cells to produce protective factors.

Antiangiogenic function

It is known that the level of PEDF is reduced in the vitreous of patients with proliferative diabetic retinopathy and in RPE cells cultured under hypoxic conditions and that the degree of neovascularisation appears to be negatively correlated with PEDF levels. PEDF has also been shown to inhibit the migration of endothelial cells in a dose dependent manner (Duh et al, 2002) and is more potent than other naturally occurring angiogenesis inhibitors such as angiostatin, endostatin and thrombospondin 1. It is not known if PEDF exerts its anti-angiogenic effect directly on VEGF expression, on its receptor, or by an intracellular downstream mechanism. However, one action of PEDF on

VEGF receptor 1 was recently described which may explain some of its effect. Cai et al demonstrated that PEDF brings about cleavage and intra cellular translocation of the transmembrane domain of VEGFR1, thereby indirectly down regulating the effect of VEGF (Cai et al, 2006). Evidence for a dedicated PEDF receptor remains elusive. *In vitro*, PEDF has been found to bind to a specific site on retinoblastoma and cerebellar granulosa cells (Alberdi et al, 1999). Purification of the cell membranes bound with PEDF has identified an 80 kDa protein. It has also been found that PEDF binding leads to activation of NF-κB, a transcription factor important in the activation of anti-apoptotic genes, and that it can modify the phosphorylation of ERK1/2 (important in cell proliferation) in cerebellar cells. Evidence exists to show that the two functions of PEDF: neuroprotective and anti-angiogenic are located on two different regions of the molecule (Barnstable & Tombran-Tink, 2004).

Since most angiogenic diseases of the eye lead to neuronal damage as one of the mechanisms of visual loss, PEDF makes a good potential candidate for use in the treatment of ocular neovascular disorders. Recently, PEDF (Mori et al, 2002) and angiostatin (Lai et al, 2001) have been packaged into a viral vector and introduced into rat eyes as a treatment for laser-induced choroidal neovascularisation, and when given systemically to a mouse model of retinal neovascularisation, significant inhibition occurred (Stellmach et al, 2001). Strangely, some workers have demonstrated opposing effects of PEDF on endothelial cells, with low doses (90µg/ml) being inhibitory and high doses (360µg/ml) being stimulatory, with augmentation of choroidal neovascularisation (Apte et al, 2004). Hutchings et al in 2002 also demonstrated that the action of PEDF on endothelial cells also depends on their phenotype, with opposite effects being demonstrated depending on whether they are maintained in VEGF or not. Cells cultured without VEGF which were subsequently exposed to VEGF did not demonstrate proliferation or phosphorylation of ERK1/2 when PEDF

was added, while those cultured/maintained in the presence of VEGF demonstrated ERK1/2 activation and thereby cell proliferation when PEDF was added. The combination of VEGF and PEDF were therefore synergistic. Caution is therefore sounded by the authors in deciding on the dose being contemplated in viral vector studies as opposing effects may be seen to those desired.

Thrombospondin 1

Thrombospondin 1 is a cell attachment factor present in the alpha granules of platelets that is released during the blood clotting process. Its main functions include the enhancement of fibroblast cell adhesion, cancer cell adhesion and prevention of angiogenesis via CD36, the thrombospondin 1 receptor, on endothelial cells. In the eye, it is located between Bruch's membrane and RPE cells and may play a role in inhibiting choroidal neovascularisation from breaking through Bruch's membrane (Miyajima-Uchida et al, 2000) and in inhibiting retinal neovascularisation (Shafiee et al, 2000).

Endothelial cell heterogeneity

It is thought that all endothelial cells are derived from a common precursor, the embryonic mesoderm. During embryogenesis, endothelial precursor cells appear to be co-localised with haematopoietic cells and share a number of cell surface markers (CD34, CD31, CD39, VEGFR-2 and CXCR4) (Hasegawa et al, 2007; Zambidis et al 2005). The production of Hb-ε suggests that they share a common precursor cell, the haemangioblast (Zambidis et al 2005). Although all endothelial cells share a common embryonic precursor, it has been known for many years that there is a wide diversity of endothelial cell phenotypes in both human and mammalian vascular systems (Thorin & Shreeve, 1998). This wide divergence in cell

phenotype may in part be secondary to genetic changes amongst the precursor cells but may also be due to local environmental effects on the developing endothelial cells.

In the human, there are a number of fundamental functional differences between different organ vascular beds (Aird, 2003). Endothelial cells in the glomerulus and the inner choroid allow the passage of fluid and molecules across themselves via small pore-like structures within the cell called fenestrations, whilst brain and retinal endothelial cells maintain very firm inter-cellular junctions to preserve the blood-brain and blood retinal barriers respectively. The liver, spleen and bone marrow sinusoids are different again, being lined by discontinuous endothelium that allows cellular trafficking which is thought to be important in the development of the immune system.

Many human diseases appear to be restricted to specific vascular beds. For instance, many systemic vasculitic disorders appear vessel-specific such as giant cell arteritis, polyarteritis nodosa and idiopathic retinal vasculitis. Tumours also appear to metastasise to selected vascular beds. Diabetes, however, preferentially affects the microvasculature of the retina, kidney and nerves causing extensive vessel closure and subsequent neovascularisation, while the choroid is only mildly affected by a micro-angiopathy that appears to produce few clinical findings apart from some capillary drop-out and leucocyte aggregation within the choriocapillaris microvasculature on histological examination (Cao et al, 1998, Lutty et al, 1997). While the underlying mechanism of many of these processes remains uncertain, it is known that endothelial cells are species (Rhodin,1968; Graier et al, 1996), organ and vascular bed specific (Keegan et al 1982; Thorin & Shreeve, 1998). These differences may in part contribute to the disease site specificity.

At light microscopic level, endothelial cells lining the microvasculature are generally flattened and elongated whereas those lining large vessels (aorta and iliac arteries) are polygonal (Cornhill et al, 1980).

While much of the endothelial cell heterogeneity may be “pre-programmed”, such as the differences between arterial and venous endothelial cells (Adams et al, 1999), it is thought that the cellular microenvironment may also play a role (Aird et al, 1997). The particular characteristics of an individual cell will depend on factors such as the local extracellular matrix, local growth factors, interactions with neighbouring cells, including leukocytes, and local mechanical forces. Examples of this apparent influence of the local environment influencing endothelial cell phenotypes include an animal model (mouse) of atherosclerotic disease in which a segment of vein is inserted into the arterial circulation. On connecting the jugular vein to the carotid artery in mice, the venous endothelial cells change to an arterial phenotype and an expansion in smooth muscle cells in the vessel wall occurs (Kwei et al 2004). Similarly, a model of tumour induced angiogenesis, whereby mouse carcinoma cells are injected subcutaneously, induces blood vessel growth with endothelial cells with a skin phenotype. If the same tumour is implanted into the brain of the mouse, the new vessels are lined by endothelial cells with a brain phenotype (Roberts et al, 1998). Other similar *in vivo* studies demonstrate that endothelial cells can take on host tissue characteristics after transplantation (Moyon et al, 2001; Aird et al, 1997), while removal of ECs from an *in vivo* to an *in vitro* environment can also change cell phenotype (Grau et al, 1996).

The earliest events controlling the differentiation of mesoderm into endothelial cells remain uncertain. Experiments on zebra fish have shown that the arterial and venous phenotype of vascular precursor cells or angioblasts (expression of gridlock) is determined while they are still located in the lateral plate (very early stage of embryogenesis), implying a genetic component to endothelial heterogeneity (Zhong et al, 2001). This finding is reinforced by the finding that in mice, cells destined to be arteriolar and venular ECs express ephrin B2 and the receptor EphB4 respectively before the establishment of a circulation (Adams et al, 1999). In the embryo, venous specification is regulated by the expression of a transcription factor, COUP-TFII, which suppresses

endothelial cell Notch signalling. This leads to a failure in the expression of ephrin-B2, one of the main arterial endothelial determinants. Failure to express ephrin-B2 allows the expression of Eph-B4 on the endothelial cell and the vessel takes on the characteristics of a vein, i.e. thin walled with a sparse muscular coat (You et al, 2005).

The Ephrin family of receptor tyrosine kinases has at least 13 members, while their ligands, the ephrins are divided into two classes. Class A ephrins (ephrin A1-A5) are tethered to the cell membrane and the B class (ephrin B1-B3) which have a transmembrane domain and a small intracellular domain. It is now understood that Ephrin receptors are not specific in the ephrin subtypes that they bind and because both are cell membrane bound, cell–cell contact must be made for interaction to occur (Aitsebaomo et al, 2008). Activation is thought to be bi-directional. While signal transduction via Ephrin receptors is thought to stimulate cell migration, axonal guidance and cell border formation, their precise role in bringing about venous and arterial phenotype differentiation is unknown. While the initial identity of ECs appears to be genetically determined, later, after angioblast migration, local factors such as VEGF gradients can also have an effect on arterio-venous phenotype (Le Bras et al, 2010). It is thought that high local expression of VEGF leads to the activation of members of the FOXC transcription factors which in turn lead to up-regulation of the notch family and an arteriolar phenotype. Experiments have also shown that physiological requirements and haemodynamic influences can alter phenotype. Transplantation of arteriole ECs into veins rapidly changes their phenotype to venular (Moyon et al, 2001). This may be due to local factors such as VEGF secreted by surrounding cells or it may be secondary to differences in flow/shear stress as the placement of venules within a section of high flow artery leads to a rapid change to an arteriolar phenotype.

Inter-organ differences

Craig et al in 1998 showed that endothelial cells derived from the brain, lung, adipose tissue and aorta of sheep differed in terms of ultrastructure and surface molecule expression depending on the site of origin. Müller et al in 2002 also showed that surface expression of ICAM-1, VCAM and E-Selectin on cultured HUVEC and human pulmonary microvascular endothelial cells differed in both the un-stimulated and stimulated states.

There are numerous studies demonstrating differences in *in vitro* behaviour between ECs from different anatomical sites. Examples include:

- Aortic ECs cultured on ECM derived from the lung start to express Lu-ECAM-1 (a lung specific adhesion molecule) whereas the cells develop fenestrations when cultured on ECM derived from kidney (Augustin et al, 1994).

- Differences in the proliferative response of HUVEC and microvascular ECs to VEGF, FGF-2 and placental growth factor. Lang et al in 2001 and 2003 demonstrated that the kinetics of the proliferative response to VEGF and FGF were different for micro- and macrovascular ECs and that placental microvascular ECs responded to placental growth factor whereas HUVECs showed little response (Lang et al, 2001, Lang et al, 2003). These authors also compared the amount of vasoactive substances such as endothelin-1, thromboxane, angiotensin II and prostacyclin released into the medium of HUVEC and placental ECs (microvascular) and found that the latter were more similar to dermal microvascular ECs than macrovascular ECs (Lang et al, 2003).

- Differences in E-selectin expression by human iris and retinal ECs (Silverman et al, 2005).Under unstimulated conditions, the gene expression as measured by gene array of matched iris and retinal ECs were very similar. After inflammatory stimulation by lipopolysaccharide (PLS) or TNF alpha, a marked difference in the expression of E-selectin was noted, with up-regulation in retinal compared with iris ECs.

- Differences in prostaglandin secretion between HUVEC and retinal ECs at different glucose concentrations (Rymaszewski et al, 1992). The authors studied the synthesis of proteins such as prostaglandins and plasminogen activators under normo- and hyperglycaemic conditions. They found that hyperglycaemia stimulated PGE_2 secretion by retinal microvascular ECs but not by HUVEC macrovascular ECs. They also found that hyperglycaemia stimulated the release of plasminogen activator in retinal ECs but not HUVECs. They suggest that these differences may be important for the underlying mechanisms of diabetic retinopathy.

- Differences in the expression of CD34, CD31 and vWf by various macro- and microvascular EC s in culture (Müller et al, 2002). They found that vWf was strongly expressed by HUVECs but less so by pulmonary microvascular ECs (although they were still positive), while both types of EC strongly expressed CD31 and only weakly expressed CD34.

- Differences in retinal and choroidal EC expression of angiopoietin 2 and VEGF receptors under normoxic and hypoxic conditions (Brylla et al, 2003). The authors found that bovine choroidal ECs expressed significant levels of VEGF mRNA compared with bovine retinal ECS under both norm- and hypoxic conditions. They also found that Ang 2 mRNA levels were significantly higher in bovine retinal ECs under both norm- and hypoxic conditions.

- Differences in adenosine receptor (important in vascular tone) expression between HUVEC and dermal microvascular ECs (Feoktistov et al, 2002). The authors found that HUVECs preferentially express A2A adenosine receptors, whereas dermal microvascular ECs express A2B receptors. An adenosine agonist increased expression of IL-8, VEGF and FGF-2 in dermal microvascular ECs but not HUVECs.

- Differences in chemokine receptor (CXCR1-3) and PKC activation between HUVECs and dermal microvascular ECs (Mason et al 1997; Salcedo et al, 2000). Using phorbol esters and more specific PKC activators, the authors demonstrated that activation of PKC in microvascular ECs led to Thy-1 up-regulation compared with HUVECs. They also showed that PKC activation in HUVEC led to up-regulation of E-selctin and VCAM-1 but not dermal microvascular ECS

- Differences in the expression of CD36 between HUVECs and dermal microvascular ECs (Swerlick et al, 1992). CD36 is thought to be a cell surface receptor allowing malaria-infected red blood cells to gain access into endothelial cells. The authors found strong staining for CD36 in dermal microvascular ECs while HUVECs showed no reaction and may be important in certain diseases affecting blood vessels. .

- Lack of expression of ABO blood group antigens by HUVECs but not by other endothelial cells (O'Donnell et al, 2000). The authors studied the HUVECs from 45 different umbilical cords and were unable to detect ABO antigens on any of the isolated HUVECs. This was not due to an absence of the precursor, H substance, but was due to the absence of the enzyme needed to form the blood group antigens. The authors state that HUVECs are the only ECs to lack ABO blood group antigens. This may protect the cord from maternal

antibodies during growth of the foetus. They suggest that care should be taken when extrapolating results from HUVE cells to other adult endothelial cells.

Intra-organ differences

Within the same organ, it has also been shown that ECs from different vascular beds differ (Ghitescu & Robert, 2002). In 1968, Rhodin described ultrastructural differences in mammalian venous ECs that were dependent on whether they were located in veins, venules or venous capillaries (Rhodin, 1968). Müller et al have also shown that in the human lung, ECs from the large and small vessels differed in their staining for CD34, vWf and CD31 (Müller et al, 2002). They found that ECs in the pulmonary veins and arteries stained strongly for vWf and poorly for CD34, while capillary ECs stained strongly for CD34 but poorly for vWf. In 2002, Lang found differences in endothelial cells from within the same placenta (HUVEC and placental venous endothelial cells), noting that placental vein ECs responded to placental growth factor whereas HUVECs did not (Lang I et al, 2003).

Perhaps most importantly, it has also been shown *in vivo* that ECs from larger vessels do not participate in neovascularisation (Klagsbrun and Folkman, 1990) and that VEGF receptors are reportedly inducible on venular and capillary ECs but not arterial ECs (Ferrara et al, 1992). This may explain why neovascularisation in adults appears to originate from post-capillary venules (Gimbrone et al, 1974; Grunt et al, 1986). This observation has recently been questioned however (Shin et al 2001).

Interspecies differences

It has also been recognised for many years that endothelial cells derived from different species are heterogeneous in their phenotype and functional properties. Imegwu et al have described species differences in the growth of human and bovine aortic ECs in co-culture with smooth muscle cells (Imegwu et al, 2001), while Ram and Hiebert demonstrated differences in the response of porcine and bovine ECs to free radical damage (Ram & Hiebert, 2003). Graier et al also found a species difference in the endothelium-dependent relaxation of bovine and porcine coronary artery endothelial cells in the presence of L-Ng nitroarginine and indomethacin (Graier et al, 1996).

It is amazing that much of our knowledge of vascular diseases is based on *in vitro* studies using macrovascular ECs derived from the human umbilical vein (HUVEC), the large scale isolation and culture of which was first described in 1973 by Jaffe (Jaffe et al, 1973). The main reason for the popularity of these cells is that of their wide availability and relative ease of culture. Unfortunately, human umbilical vein cells do not appear to be susceptible to most of the diseases requiring investigation and, *in vitro*, differ significantly from other endothelial cells in their response to important substances such as hyaluronic acid and cytokines (Lokeshwar & Selzer, 2000; Tan et al, 2004). The question needs to be asked as to whether or not HUVEC should be used in experiments investigating human organ selective vascular diseases.

Adult Vascular structure

Arteries

Arteries are usually considered to be high pressure vessels taking blood away from the heart towards the target tissue. They absorb part of the force of systole and help maintain blood flow during diastole. The walls of arteries are composed of three coats. The innermost layer, called the **intima,** consists of a layer of endothelial cells, the basement membrane and an internal elastic lamina. The middle layer, or **media**, consists of one or more layers of smooth muscle cells lying within a network of elastic and collagen fibres; the thickness of this network layer increasing with the size of the artery, being thickest in the aorta where it exceeds the thickness of the smooth muscle cells. Lastly, the **adventitia** is a thin layer of loosely arranged collagen and elastic fibres that wrap around the outside of the vessel. The adventitia is rich in lymphatics and nerves and links the vessel to the surrounding tissue (Gabella G, 1995).

Arterioles

Arterioles are small arteries that have proportionately more smooth muscle cells within their media (up to 6 layers in the retina) and thus act as resistance vessels, controlling the flow of blood within tissues. The smooth muscle cells are often well supplied by autonomic nerve fibres (not so in the retina) and respond to circulating vasoactive substances, to control vessel calibre. Smooth muscle contraction can completely obliterate the lumen of an arteriole, thereby reducing blood flow to the tissue to zero (Gabella G, 1995).

Capillaries

Capillaries are thin walled vessels composed of an inner layer of endothelial cells forming a lumen (5-8µm) that is the approximate diameter of a red blood cell. Surrounding the endothelial cells are pericytes, embedded in a shared basement membrane. Pericytes are thought to be supporting cells, providing both structural and “chemical” support to the juxtaposed endothelial cells. Capillary structure varies depending on the tissue/organ that it serves. The endothelial lining may be continuous as in the retina and muscle; sinusoidal as found in the liver, adrenal medulla and spleen; or fenestrated as found in the renal glomerulus, endocrine glands and the choriocapillaris. Fenestrations are thought to be pores through which substances can transfer between the luminal side of the endothelial cell and the extracellular tissue. They are between 50 and 100µm in diameter and at their edges, the luminal and abluminal surfaces of the endothelial cell in contact. It is now known that fenestrations are not simple holes in the cell membrane but are covered by an electron-dense diaphragm. In continuous capillaries, where a marked barrier to diffusion exists (brain), apart from an absence of fenestrations, the endothelial cells exhibit strong intercellular junctions (zonula occludens) (Gabella G, 1995).

Venules

Venules are formed when at least two capillaries converge. Generally, they do not contain any muscle within their walls. Like capillaries, they are the site of solute transfer and importantly, leukocyte migration, particularly in lymph nodes. In adults, they are thought to be the site at which pathological neovascularisation takes place (Gabella G, 1995).

Veins

Veins are thin-walled low pressure vessels acting as capacitance vessels returning blood to the heart. The intima of veins differs from that of arteries in having no internal elastic lamina. Within the media, the amount of smooth muscle fibres is significantly less, with relatively more collagen and elastic fibres. Certain veins, such as the maternal placental vein, the dural sinus veins of the brain and retinal veins, do not possess any muscular tissue. The adventitia of veins, like arteries, may contain autonomic nerves but these are less abundant. A significant difference between arteries and veins is the presence of valves in many of the latter. These act to prevent reflux of blood, effectively only allowing flow in one direction. Retinal venules do not contain valves (Gabella G, 1995).

Arteries and veins

After the formation of a primitive vascular plexus occurs, remodelling into a more complex network takes place along with the demarcation into arterial and venous territories. Arterial endothelial cells express Ephrin B2 whereas EphB4 is only expressed by veins. This expression continues down to ECs lining capillaries with a definite boundary forming between those cells nearer to arterioles and post capillary venules (Adams et al, 1999).

Section 2: Human ocular vascular structures

The human eye

Before describing the specialised vascular structures of the human eye, it is important to understand the basic anatomy of the eye. The eye ball or globe is made up of two modified spheres, fused together. The smaller modified sphere consists of the cornea; the clear “window” on the front of eye, which allows in light and provides the majority of the focussing power of the eye. The larger, posterior sphere is composed of sclera, the tough, white outer coating of the eye. The globe has an anterior/posterior length of approximately 24mm. The basic structure of the eye is shown below.

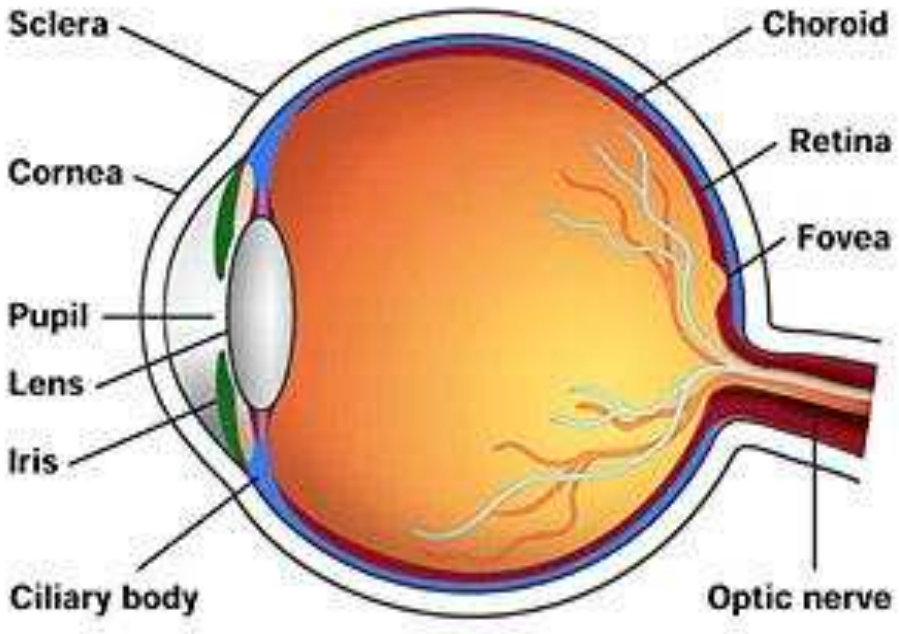


Fig 1.10. Cross section of a human eye showing its basic structural components (Courtesy of University of Utah)

The human eye consists of 3 distinct, concentric layers.

1. The external layer is known as the **corneoscleral envelope**. This layer is tough and inelastic and is composed predominantly of type I collagen. Depending on their orientation, the collagen fibres either give rise to the opaque sclera or the transparent cornea.

2. The middle layer is called the **uvea**. This layer is highly vascular. The anterior part of the uvea is composed of the iris, one of the main functions of which is to control the amount of light entering the eye. The iris is attached to and supported by another part of the uvea, which is known as the ciliary body. The main functions of this structure are to produce aqueous, a clear fluid that fills the anterior part of the eye, and secondly to control the shape of the lens. The third part of the uvea is the choroid. This is a vascular structure and provides nutrients and oxygen for the inner layer of the eye, the retina. The choroid is discussed in more detail later

3. The **retina** is the photosensitive layer of the eye. It is a complex, multi-layered structure composed of neuronal cells and photoreceptors. The inner two thirds of the retina (that part nearest the vitreous) receives its oxygen and nutrients from the retinal vascular circulation. The outer one third of the retina is supplied from the underlying choroidal vasculature. The inside of the eye is not hollow, but is full of vitreous. This is composed of a mixture of collagens, glycoproteins and water and has the consistency of jelly (Sharma and Ehinger, 2003).

The structure of the retina

Within the retina, there are two types of photoreceptors: rods and cones. Rods are a type of neural cell that convert photons of light into electrical signals, and are designed to work at low light levels e.g. at night and at dusk. They have no colour discrimination, only shades of grey from white to black. The other cells, called cones are designed to work in conditions of bright light (i.e. daylight) and can discriminate colour. There are three types of cone: red, green and blue which respond to colours/ wavelengths suggested by their individual names.

By a process of phototransduction, light is converted via chemical reactions into electrical signals. This process takes place in the rods and cones and relies on a plentiful supply of vitamin A as one of the intermediaries, so that the necessary chemical reactions can take place. The resultant electrical signals are transmitted through the layers of the retina where they can be modified and amplified by bipolar cells, until they reach the surface of the retina (nearest the vitreous) From here, the signal is transmitted to the optic nerve by the axons of ganglion cells. Fig 1.11 overleaf demonstrates the basic ultrastructure of the retina (Courtesy of University of Newcastle).

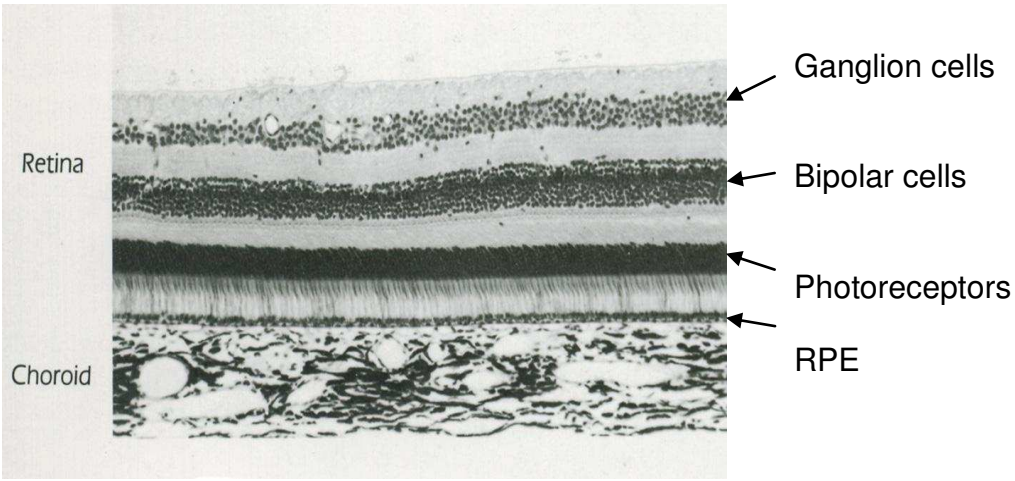


Fig 1.11. The basic histological structure of the human extra macular retina

Underneath the retina, a single layer of cells exists that interdigitates with the outer segments of the photoreceptors. These cells are called retinal pigment epithelial (RPE) cells. The RPE cells are critical for recycling of the visual pigments and chemicals necessary for the photoreceptors to function. The cells are pigmented (contain melanin) and are also important for clearing up the debris from degenerating photoreceptors, pumping fluid out of the retina, secretion of pro- and anti-angiogenic factors and for forming the outer blood-retina barrier (preventing flow of fluid out of choroidal vessels into the retina). Without the supportive role played by the RPE cells, retinal photoreceptors soon die. The RPE cells sit on a thick basement membrane called Bruchs membrane. Bruch's membrane is composed of five layers, rich in collagen IV and is probably is derived from both the overlying RPE cells and the underlying choriocapillaris. It forms a water impermeable membrane between the vascular choroidal compartment and the overlying RPE and retina (Sharma and Ehinger, 2003).

A simplified, close up diagram of the RPE/Bruch's membrane/ Choriocapillaris orientation is shown in Fig 1.12. Appreciation of this anatomical orientation is critical for the understanding of choroidal and retinal diseases.

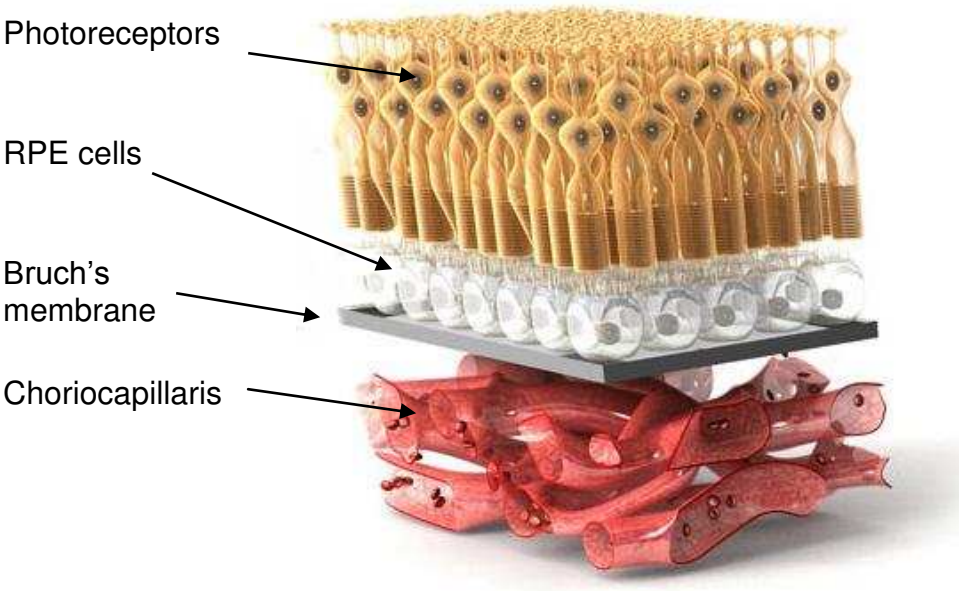


Fig 1.12. Diagrammatic representation of the relationship of outer retinal structures, Bruch's membrane and choroid. (Courtesy of the University of Utah).

The macula

While most of the retina has the structure outlined above, an area diametrically opposite the lens, at the posterior pole of the globe exists called the macula. It has an average diameter of 5-6mm and is the area where most of the light entering the eye is focussed. It is bordered by the upper and lower vascular arcades and the optic disc. Within the macula, there is a disproportionately high density of cones to aid colour discrimination and hyperacuity. In the centre of the macula is an area called the fovea where the only light sensitive cells are cones and this is the area of maximum visual sensitivity. Ultrastructurally, the fovea is thinner than the rest of the retina and is seen in cross section as a dip

(foveal pit). No retinal blood vessels exist in the fovea (thereby avoiding light scattering and aiding resolution of images). The cones in the fovea receive their oxygen supply by diffusion via the choroid (Sharma and Ehinger, 2003a)

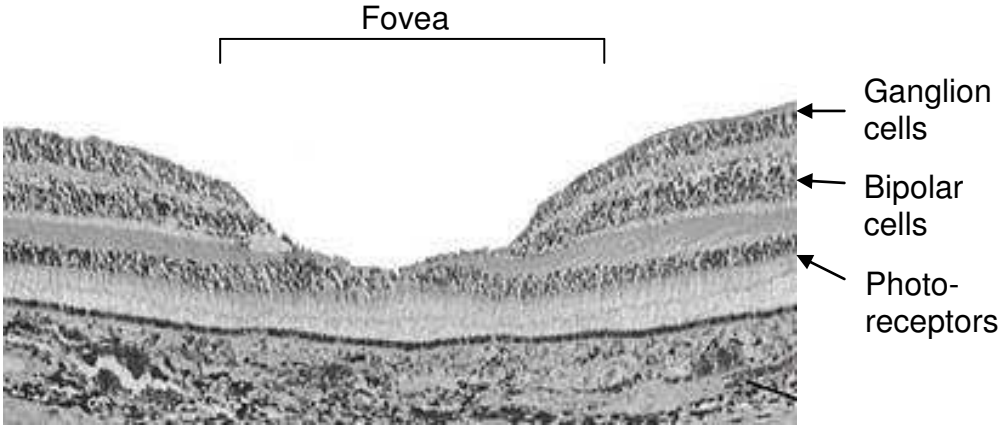


Fig 1.13. The ultra structure of the human macula and fovea (compare this with fig 1.11.). The only structures at the fovea are the fibres of the photoreceptors and the dendrites of the bipolar cells. Together these are known as Henle's layer.

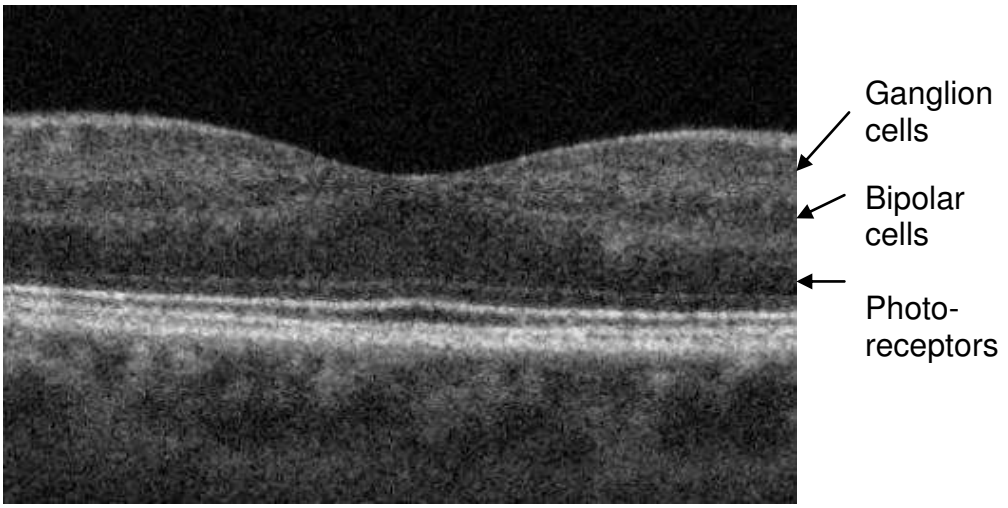


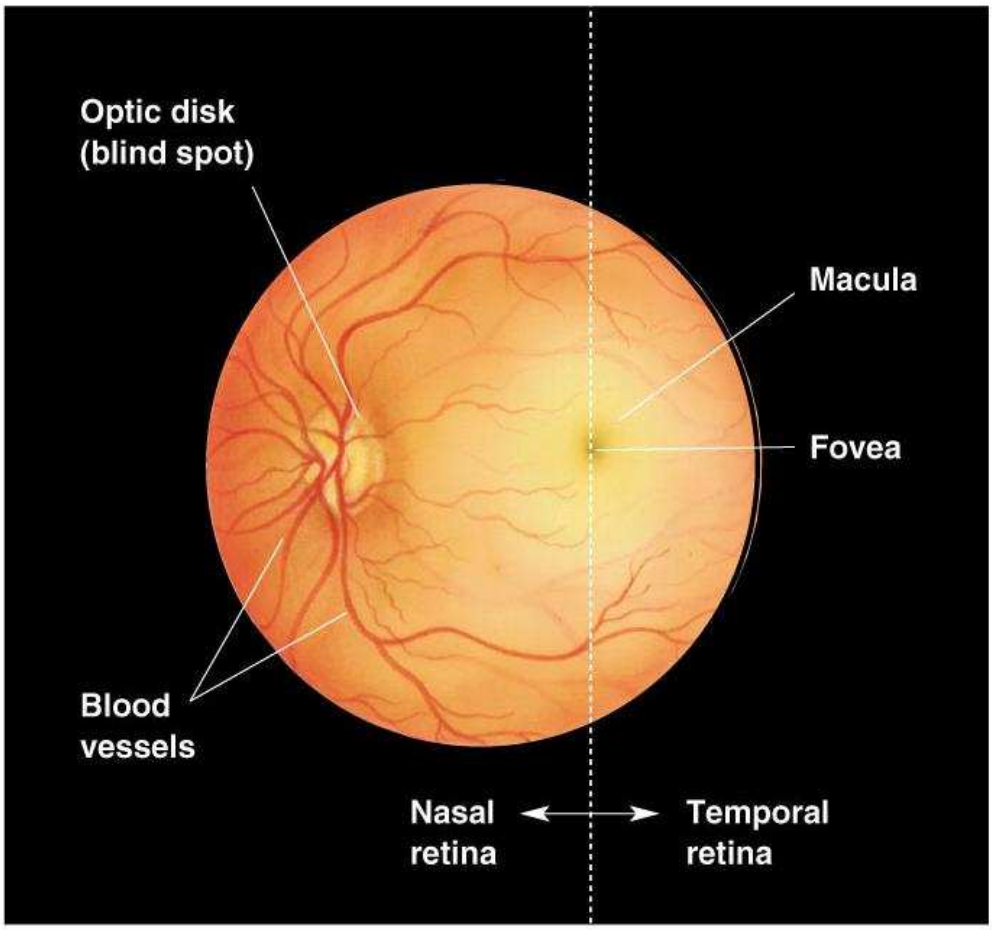
Fig 1.14. A representative Optical Coherence Tomography (OCT) scan through the macula and fovea of a real (normal) patient. The similarities with the histopathological specimen (fig 1.13) are obvious and this technique has revolutionised patient investigation and management of macular diseases (Courtesy of the Royal Victoria Infirmary, Newcastle upon Tyne).

As the main body of this thesis relates to endothelial cell heterogeneity within the human eye, there now follows a description of the different human ocular vascular beds.

Retinal vascular structure

The inner two thirds of the retina are supplied by a network of blood vessels that emanates from the central retinal artery at the optic nerve head. This artery is itself supplied by the ophthalmic artery which ultimately is a branch of the internal carotid artery (the main artery supplying the brain). The central retinal artery usually splits into four arterioles at the optic nerve head which then subdivide to supply each retinal quadrant via a network of capillaries. Each arteriole is also associated with a corresponding venule which carries deoxygenated blood back to the optic nerve head and into the central retinal vein. Because of the multiple sub divisions of each retinal vessel, each person's retinal vasculature is said to be unique and may provide a form of finger print (Cioffi et al, 2003).

Fig 1.15. shows a photograph of a typical retina demonstrating the closely associated arterioles and venules (Courtesy of the University of Newcastle).



Retinal vascular development

In humans, development of the retinal vasculature begins at around week 14 of gestation. It occurs by the process of vasculogenesis from precursors that express CD39 (Fruttiger M, 2002; McLeod et al, 2006). This cell surface molecule is otherwise known as ecto ADPase and is found on endothelial cells in all vascular beds. The molecule is responsible for controlling extracellular ADP concentration and platelet aggregation. These precursor cells also express VEGFR2 but not CD31 or CD34 (McLeod et al 2006), both apparently ubiquitous endothelial markers found in mature endothelial-lined vessels. It is thought that the

retinal vascular precursor cells (angioblasts) may be attracted to the developing retina by stroma derived factor 1 (SDF 1) because the angioblasts also express CXCR4 (SDF 1 receptor). It is thought that the majority of CXCR4-positive precursor cells originate from within the inner retina rather than from distant sites such as bone marrow (McLeod et al 2006). Most mature endothelial cells do not appear to express CXCR4, implying that its main role is in guiding endothelial cells of developing vascular systems. Vasculogenesis begins around the optic nerve head by accumulation of angioblasts. They then aggregate into blood vessels that align themselves along the routes of retinal neurones and an astrocyte template towards the periphery (Dorrell & Friedlander, 2006). In the mouse model of the developing retinal vasculature, it is thought that as the periphery of the developing retina is relatively hypoxic, $VEGF_{188}$ expression by the resident astrocytes and Muller cells acts as a stimulus to the developing vascular network which follows the concentration of $VEGF_{188}$ (Stalmans et al, 2002; Carmeliet and Tessier-Lavigne, 2005). The heparin binding property of VEGF188 is thought to be important because in mice only expressing VEGF120, retinal vascular development is severely impaired (Stalmans et al, 2002).

Retinal vascularisation is not often complete at birth. The retinal vasculature is composed of arterioles, venules and capillaries rather than arteries and veins. The lumen is greatest (100microns) near the disc, where the vessels have neither a continuous muscular coat nor an internal elastic lamina (Hayreh et al, 1989). Retinal blood vessels have no adrenergic vasomotor nerve supply to bring about changes in vascular tone (Steinle & Granger, 2003) and it is thought that changes in vascular calibre and tone are brought about by local mediators secreted, at least in part, by vascular endothelial cells. These factors are vasodilators, such as nitric oxide (NO) synthesised by endothelial nitric oxide synthetase (eNOS) (Bouloumié et al, 1999), adenosine and prostanoids, and vasoconstrictors such as endothelin 1 and angiotensin II.

Blood supply and drainage of the choroid and retina

The globe is supplied by a branch of the internal carotid artery and is drained by the vortex veins which drain into the cavernous sinus as shown in figs 1.16. & 1.17 below.

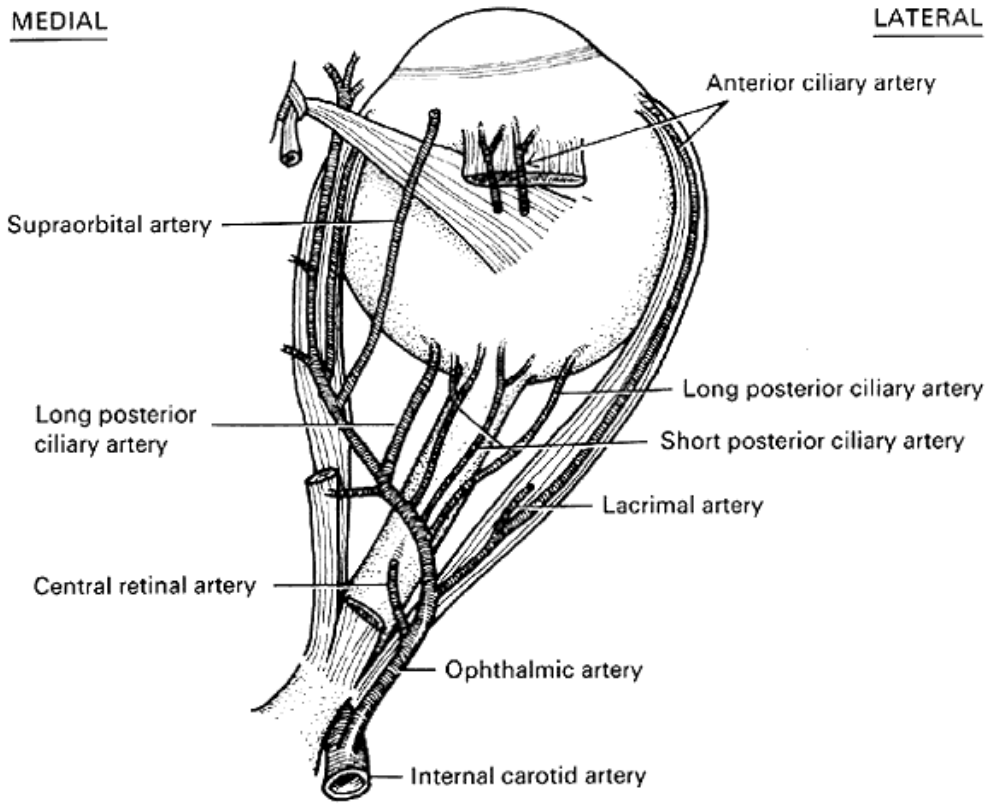


Fig 1.16. The arterial supply of the globe. The eye is supplied by a branch of the internal carotid artery, the ophthalmic artery. This then gives off various branches within the orbit, some of which are called short posterior ciliary arteries. These penetrate the back of the globe to supply segments of the choroid. The retina is supplied by branches of the central retinal artery which enters the globe within the optic nerve before radiating out over the inner retina. Other branches such as the long posterior ciliary arteries supply the front of the eye and the muscles attached to the eye which bring about movement of the globe (non-copyright image).

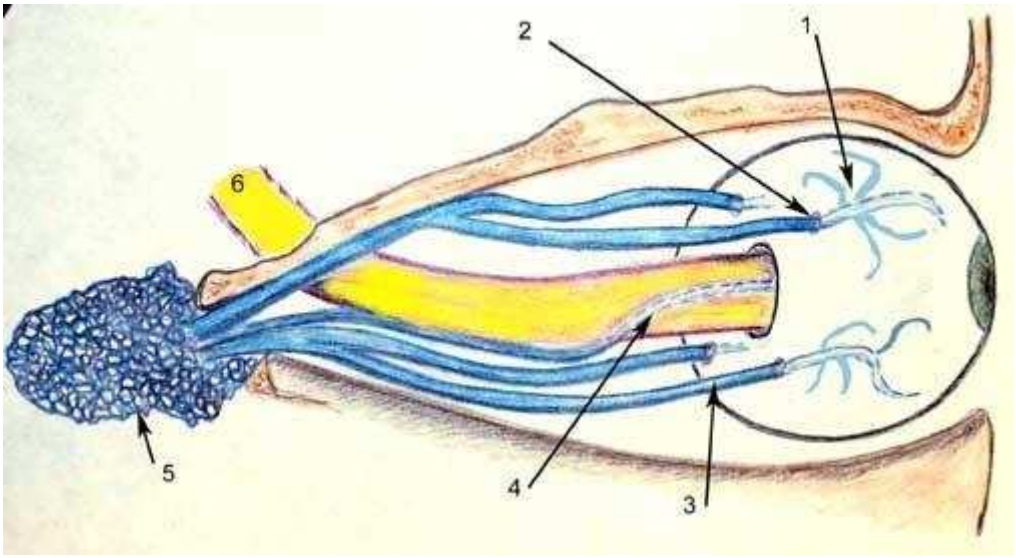


Fig 1.17 The venous drainage of the globe, including the choroid is via a variable number of vortex veins. These drain the choroid and then pass backwards through the scleral coat of the eye. They then pass back into the orbit and congregate in the cavernous sinus which lies beside the pituitary gland. The retina is drained via the central retinal vein, which passes back out of the eye within the optic nerve. Key: 1 = vortex ampullae within choroidal tissue, 2 = superior vortex vein, 3 = inferior vortex vein, 4 = central retinal vein (within optic nerve), 5= cavernous sinus (Courtesy of Medrounds Publishing).

Choroidal vascular structure

The choroid forms part of the uveal tract (choroid, ciliary body and iris) of the eye and lies between the outer sclera (white of the eye) and the inner layer composed of the retina and retinal pigment epithelium (RPE). Its main roles are to supply the photoreceptors with oxygen, to remove waste products from the basal surface of the RPE and to regulate temperature within the photoreceptors. It is approximately 100-220μm thick, depending on site, and is composed almost entirely of blood vessels with some supporting cells such as fibroblasts, macrophages, mast cells, melanocytes and pericytes which surround endothelial capillaries (Bron et al, 2001).

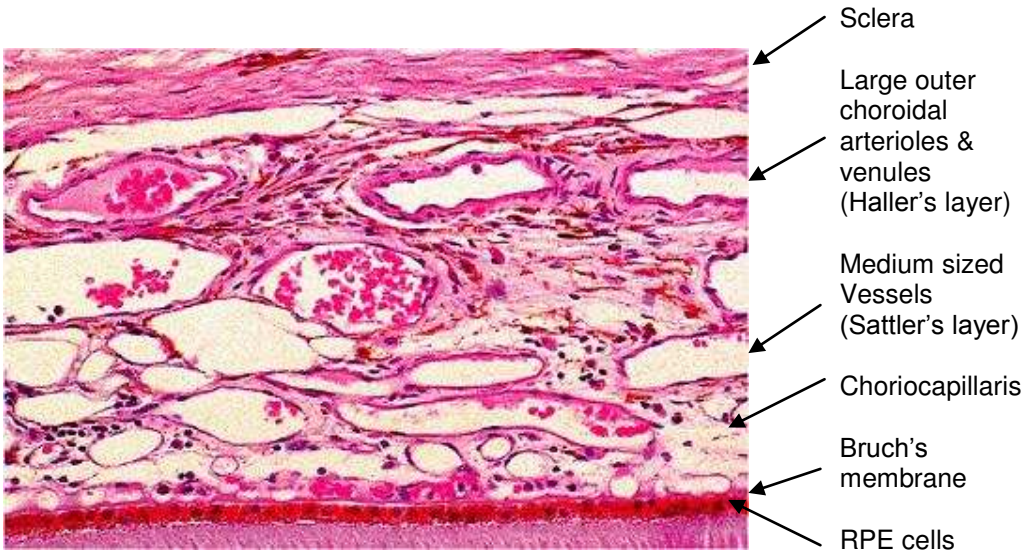


Fig 1.18. Haematoxylin/Eosin section of human choroid (macular) demonstrating the dense vascular network with vessels of decreasing lumen diameter the nearer they are to the RPE layer. The vessels furthest from the RPE represent the feeding arterioles and draining veins. Those nearest the RPE represent the fine network of the choriocapillaris. The cells and tissue between the vessels represent the supporting cells such as melanocytes, fibroblasts and pericytes together with collagens (with permission of Springer images).

Classically, the choroid at the posterior pole has been subdivided into three layers: the outer layer of large vessels (Haller's layer), a middle layer of medium sized vessels (Sattler's layer) and an internal layer adjacent to Bruch's Membrane composed of capillary vessels (choriocapillaris) arranged in a lobular structure (Nuel, 1892; Bron et al, 2001 Olver J, 1990) (see fig 1.9). The large vessels of the outer choroid are supplied by branches of the short posterior ciliary arteries which themselves are supplied by branches of the internal carotid artery (main vessels supplying the brain) (see fig 1.6). Drainage of the choroid is via the vortex veins which drain sectors of the choroid through the scleral coat of the eye and converge on the cavernous sinus (see fig 1.7).

The large vessels of the choroid (branches of the short posterior ciliary arteries) then supply the smaller vessels of Sattler's layer which then sub divide to supply individual lobules of the choriocapillaris. Each choriocapillaris lobule is thought to be composed of a central feeding arteriole and a number of peripheral draining venules (see fig 1.9 and 1.10). Each lobule is not isolated but forms a "honeycombing network" where blood is thought to transfer directly between lobules if required (fig 1.19).

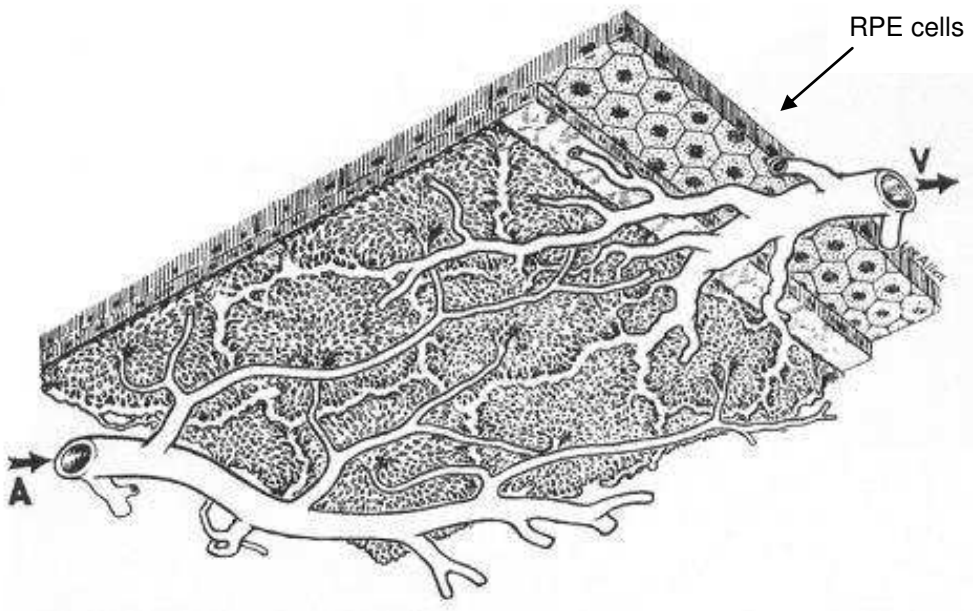
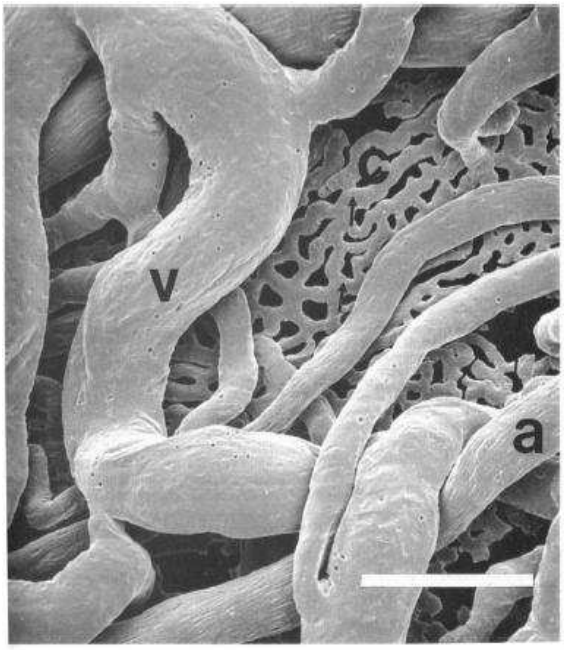
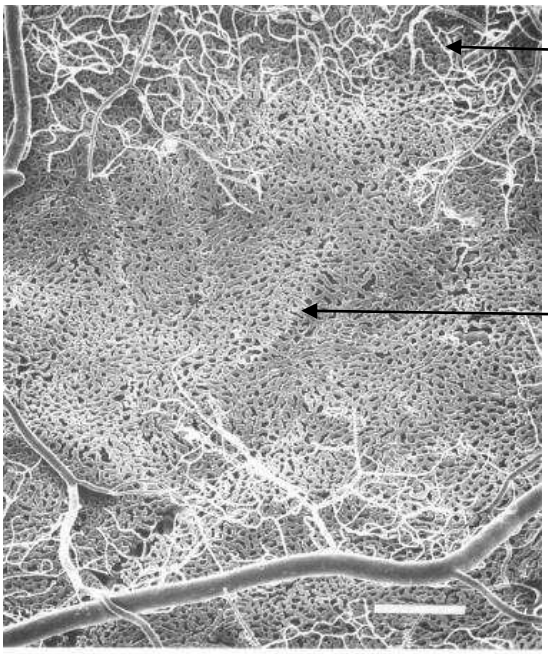


Fig 1.19. A 3-dimensional representation of the pattern of blood supply of choriocapillaris lobules. A = supplying arteriole, V = draining venule. It can be seen that each lobule is supplied by a central arteriole and drained by more than one venule at the edge of each arteriole (from von Graefes Arch Ophthalmol 1974 with permission).



(Bar = 231 microns)

Fig 1.20. Scanning electron micrograph of a cast of human choroidal vasculature viewed from the scleral side, showing draining choroidal veins (v), arterioles (a) and the choriocapillaris (c) visible through the gaps between the large vessels (from Olver J, 1990 Eye, 4:262, with permission).



Retinal capillaries

Choriocapillaris (note the close juxta-position of the retinal capillaries and the choriocapillaris once the RPE cells and Bruch's membrane have been removed)
(Bar = 250 microns)

Fig 1.21. Scanning electron micrograph of a cast of human choriocapillaris viewed from the retinal aspect. Remnants of the retinal vasculature are visible at the top and bottom of the image. The interconnecting nature of the structure of the choriocapillaris is seen. (from Olver J, 1990 Eye, 4:262, with permission).

The endothelial cells of the choriocapillaris appear to send out processes that penetrate the outer level of Bruch's membrane. These processes do not appear to be a harbinger of choroidal neovascularisation, but may stabilise the endothelial cell (Guymer et al, 2004). The peripheral choroid is simpler in structure and is composed of the choriocapillaris and a deeper layer of large vessels (McLeod & Lutty, 1994). The choriocapillaris has a lobular structure and, unlike the retinal circulation, is not an end organ system. A significant difference between the choriocapillaris and most other capillary structures in the body is that the endothelial cells lining the choriocapillaris are fenestrated on the inner or retinal side of the vessels that face the RPE. This corresponds to the polarised expression of VEGF receptors on the endothelial cells facing Bruch's membrane (Blaauwgeers et al, 1999). There is evidence to suggest that the fenestrations within the choriocapillaris are dependent upon factors secreted from the RPE, be they growth factors such as VEGF and FGF or extracellular matrix components. Evidence for this is provided by destroying the RPE either surgically (Hayashi et al, 1999), genetically (May et al, 1996) or chemically with sodium iodate (Korte et al, 1984); the underlying choriocapillaris degenerates, but not the deeper, large choroidal vessels. When the RPE recovers, new areas of choriocapillaris re-establish themselves from the larger choroidal veins by migration of endothelial cells. A similar effect is seen in animals with genetic retinal degenerations where the RPE is primarily involved (RCS rats with hereditary retinal degeneration), although the effect on the choriocapillaris is obviously non-reversible (May et al, 1996). In this model, despite endothelial cell migration and proliferation, choroidal neovascular membranes do not appear to form, perhaps due to Bruch's membrane being intact (c.f. animals genetically programmed to over express VEGF in the RPE, which demonstrate a similar result).

The choroid is innervated by both sympathetic and parasympathetic neurones. Stimulation of the alpha adrenergic sympathetic nerves within the choroid leads to vasoconstriction and a fall in ocular blood volume (the choroid acts as a large capacitance vessel within the eye). Stimulation of the parasympathetic nerves causes choroidal vasodilatation. The arterioles demonstrate a high density of VIPergic neurones (vasodilator). It is also thought that some neurones may release nitric oxide (NO) which acts as a vasodilator for choroidal vessels (Bron et al, 1997a). The choriocapillaris has no innervation.

In contrast to the retina, the choriocapillaris develops at around 6.5 weeks gestation by a process of haemovasculogenesis (see earlier) (Hasegawa et al, 2007; Baba et al, 2009). It is also thought that some of the larger choroidal vessels form by the process of haemo-vasculogenesis as well. However, the density of CD39-positive (angioblasts) and haemoglobin epsilon-positive (a haemo-vasculogenesis marker) cells within the developing choroid at week 12 was insufficient to produce the whole choroid (Hasegawa et al, 2007). Positive staining for proliferation markers at this stage suggests that angiogenesis is also taking place in the developing choroid. It should be noted that the choroid develops far earlier than the retina and both develop by different mechanisms i.e. haemovasculogenesis and vasculogenesis respectively (Hasegawa et al, 2007). This may explain some of the differences between the mature retinal and choroidal vasculature's physiological features, responses and susceptibility to disease (Saint-Geniez & D'Amore, 2004; Allende et al, 2006).

Iris vasculature structure

Traditionally, the vessels of the iris have been regarded as being thick and hyaline in appearance. The adventitia has an abundance of loose connective tissue, perhaps to accommodate the constant “concertina-like” movements of the iris which cause frequent straightening and wrinkling of the vessels. The arteries have a media of circular non-striated smooth muscle and elastic fibres. The iris capillary endothelium is non-fenestrated and has a thick basal lamina, surrounded by pericytes (Bron et al, 1997a). An interesting feature of the iris vasculature is that exposure to histamine renders the vessels permeable to macromolecules, unlike the retinal vasculature which is insensitive (Ashton & Cunha-Vaz, 1965).

Regulation of ocular blood flow

Because the retina has the highest metabolic demand of any tissue and those requirements change between light and dark conditions (Buttery et al, 1991), rregulation of blood flow is therefore paramount and is required to maintain an adequate flow of blood in the face of variations in perfusion pressure. It is also paramount that any vessels supplying blood do so with minimal impact on the light reaching the photoreceptors and on the process occurring in the retina. Interestingly, the regulation of blood flow differs between the retina and choroid. Retinal blood flow increases in response to raised partial pressure of carbon dioxide (pCO_2), while raised partial pressure of oxygen (pO_2) causes vasoconstriction and hence reduced flow. Retinal flow is autoregulated, and under most conditions is not affected by perfusion pressure. Indeed, elevation of intraocular pressure to twice normal levels, resulting in a 36% reduction of perfusion pressure, does not alter retinal blood flow (Riva et al, 1981). The main regulator of blood flow is thought to be the vascular smooth muscle cells of the retinal arterioles.

The depolarisation of the smooth muscle cells can be affected by pCO2 in the blood (high levels cause muscle relaxation and vasodilatation) (Alm and Bill 1972), pH (Hessellund et al, 2006), dark illumination conditions (retinal blood flow increases by 40% in dark conditions)(Feke et al, 1983), and nitric oxide (Buerk et al, 1996). In contrast to the retina, the control of choroidal blood flow is less well understood because of the difficulties in studying the choroidal circulation in detail. However, it is known that choroidal blood flow is increased by raised pCO_2 but is unaffected by raised pO_2. Choroidal blood flow, unlike retinal flow, is not autoregulated, relying on sympathetic (with both noradrenergic and neuropeptide fibres) (Bruun et al, 1984) and parasympathetic (cholinergic) nerves (Bill and Sperber, 1990). Therefore changes in perfusion pressure cause a proportionate change in choroidal blood flow. The regulation of iris blood flow is very poorly understood and little, if any research has been conducted on it.

Section 3: Angiogenic diseases affecting the eye

Wet age related macular degeneration

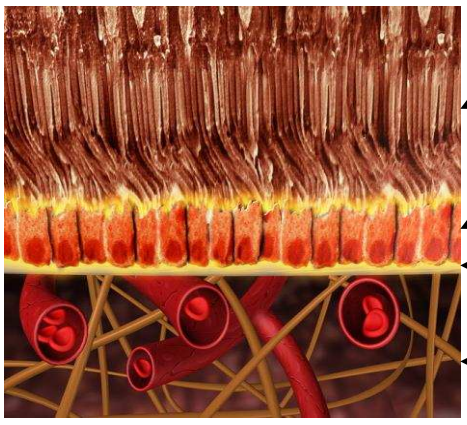
Age related macular degeneration (AMD) is a heterogeneous condition characterised by the deterioration of structure and function of a well defined area at the posterior part of the eye responsible for central and reading vision, i.e. the macula. The disease is the most common cause of irreversible visual loss in the elderly populations of the industrialised world (Klein et al, 1992; Mitchell et al, 1995; Vingerling et al, 1995).

While the cause(s) of AMD are unknown, it is probably polygenic in nature, being affected by multiple genetic and environmental factors such as age, smoking, drusen formation and possession of certain genetic alleles (Hageman et al, 2005). This concept may help to explain the wide range of disease phenotypes. While increasing age is an undoubted risk factor, unfortunately this is not modifiable. However, smoking has been shown to increase the risk of AMD by between 3 and 4 fold (Khan et al, 2006). Smoking cessation regimens therefore offer the prospect of dramatically reducing the incidence of severe visual loss secondary to AMD. While the finding of macular drusen (small yellow sub-retinal deposits) increases dramatically with advancing age, the possession of the large, soft subtype increases the risk of advanced AMD by between 5 and 10 fold (Bird et al, 1995) (see fig 1.23). In addition, the occurrence of advanced AMD (e.g.: a large macular scar secondary to wet AMD) in one eye means that there is a 50% risk of the fellow eye developing a similar lesion over 5 years.

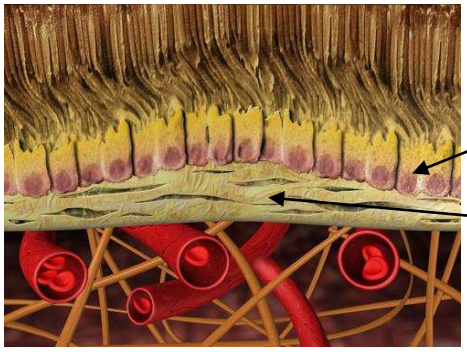
Histologically, the disease is seen to affect all of the major layers of tissue at the macula: the choroid, retinal pigment epithelium (RPE), Bruch's membrane and the retina. Traditionally, the disease is classified into "dry" or atrophic and "wet" or neovascular forms. The dry form of

the disease is characterised by slow, progressive atrophy of the macular retinal pigment epithelium and underlying choroid, leading to secondary retinal photoreceptor death. This form of the disease will not be discussed any further.

The wet form of the disease is characterised by new blood vessel growth (angiogenesis) from the choriocapillaris and inner choroid (Sarks et al 1997) to form a choroidal neovascular membrane (CNV). Growth occurs through physical defects in Bruch's membrane to the potential space underneath the retinal pigment epithelium (RPE) and retina (Kent & Sheridan, 2003; Campochiaro et al, 1999, Kijlstra et al, 2005; Tezel, 2004; Coleman et al, 2008), stimulated by cytokines such as vascular endothelial growth factor (VEGF). These new vessels are associated with massive exudation of fluid and lipid, which subsequently leads to severe damage to the retinal photoreceptors and fibrosis. This form of the disease is often of rapid onset and progression and commonly leads to severe visual loss. A stylised representation of events is shown on below (fig 1.22).



Step 1
Normal retinal, RPE and choroidal anatomy



Step 2
Formation of Drusen

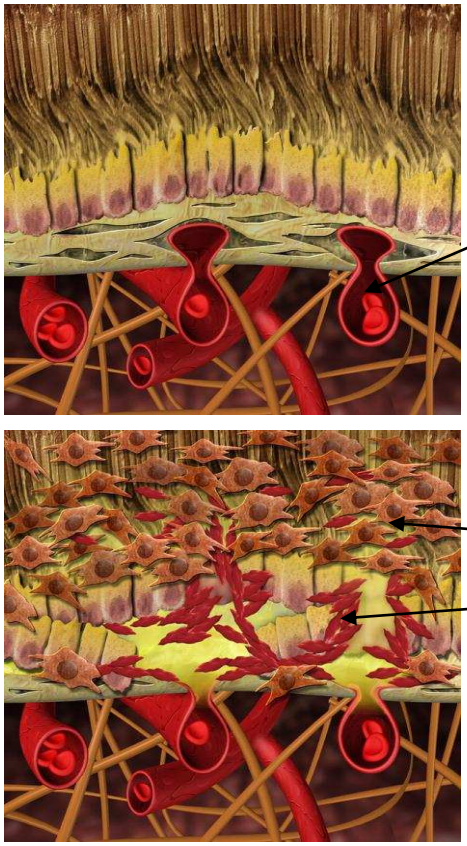


Fig 1.22. A representation of the underlying steps involved in choroidal neovascularisation (courtesy of Netscape, www.Medscape.com).

Most established or experimental treatments for AMD such as external beam radiotherapy, transpupillary thermotherapy and macular surgery aim to bring about closure of the CNV, but do not affect the underlying disease or pathophysiological processes involved. One reason for this is the paucity of knowledge regarding the main cellular key player, the macular inner choroidal endothelial cell. Investigators have studied the disease *in vitro* by studying the phenotype and growth characteristics of bovine choroidal endothelial cells (Liu & Li, 1993; Sakamoto et al, 1995a; Sakamoto et al, 1995b; Morse & Sidikaro, 1990; Hoffman et al, 1998; Liu et al, 1998; Wang et al, 2002; Zubilewicz et al, 2001a; Zubilewicz et al, 2001b ; McLaughlin & de Vries, 2001; Eter & Spitznas, 2002) and heterogeneous mixtures of human choroidal (Penfold et al, 2002; Geisen et al, 2005; Peterson et al, 2007; Sakamoto et al, 1995; Bargagna-Mohan et al, 2006) or macrovascular (umbilical vein)

endothelial cells (Shankar et al, 2008; Stahl et al, 2008). It is known, however, that endothelial cells from different species and anatomical sites vary markedly in their phenotype and growth characteristics (see previous sections). In order to increase understanding of this disease it would seem logical to develop a technique to isolate and study human macular inner choroidal endothelial cells *in vitro*.

Epidemiology

As current demographic changes are skewed towards a more elderly population, the number of people with AMD is likely to increase even further, with marked health economic implications. In the Beaver Dam Eye Study in the USA, the prevalence of advanced AMD increased from 0.1% at age 43 - 54 years to 7.1% among those aged 75 years or older (Klein et al, 1992). In a similar study conducted in Australia, 0 % people under the age of 55 had advanced AMD but this increased to 18.5% in the age 85 and over subgroup (Mitchell et al, 1995). While similar large scale epidemiological studies of the prevalence of AMD have yet to be undertaken in the UK, it is estimated that there are currently 214,000 people visually impaired by the disease and this was expected to rise to 239,000 by 2011 (Owen et al, 2003). It is therefore obvious that AMD is currently a major health burden which is likely to increase substantially in the future due to changes in the demographics of the ageing population. While the cause of the disease is unknown, a number of risk factors have been determined as listed below.

Established risk factors	Relative risk
Age	-
Smoking	3.6
Large drusen	5.7
Soft drusen	9.9
Hypertension	1.2

Clinical features

Early age-related macular degeneration is characterised by thickening and loss of the normal architecture of Bruch's membrane, accumulation of lipofuscin within RPE cells and the formation of Drusen (convex shaped deposits of glycoprotein lipids and inflammation-related molecules) beneath the RPE within Bruch's membrane, predominantly in the macular area. With time, the number of drusen may increase in number and size and take on "soft" characteristics. When this occurs in association with RPE hypo- and hyper-pigmentation, the risk of developing more advanced AMD is high (Klein et al, 2002).

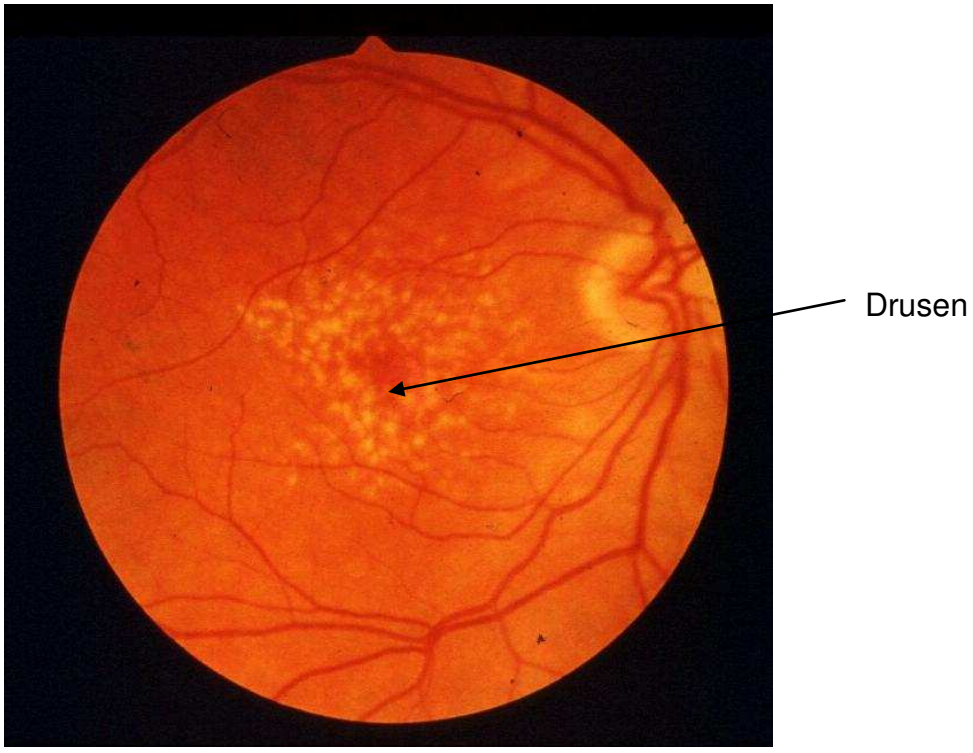


Fig 1.23. A colour fundal photograph of a patient's right eye demonstrating drusen (sub-RPE deposits of amorphous material that increase a patient's risk of developing AMD) (courtesy of the University of Nottingham)

Wet AMD is characterised by serous or haemorrhagic detachment of the RPE by growth of new blood vessels (choroidal neovascular membrane or CNV) (see fig 1.24 and 1.25).

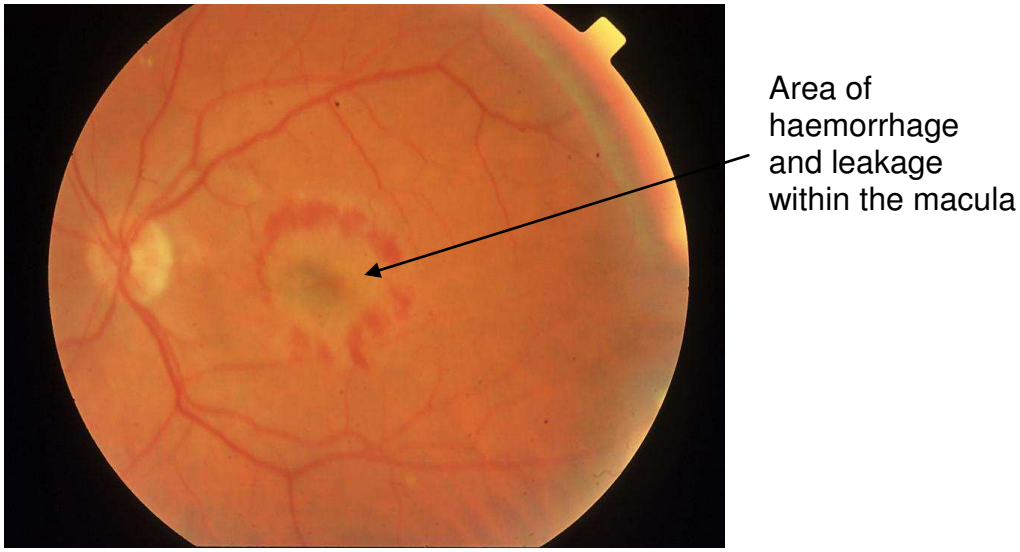


Fig 1.24. Colour fundal photograph of the left eye of a patient with wet AMD. It demonstrates a circular area of haemorrhage in the macula, which encompasses an area of cream and darker pigmentation. These findings would suggest leakage of blood and fluid under the macula. (Courtesy of the University of Nottingham)

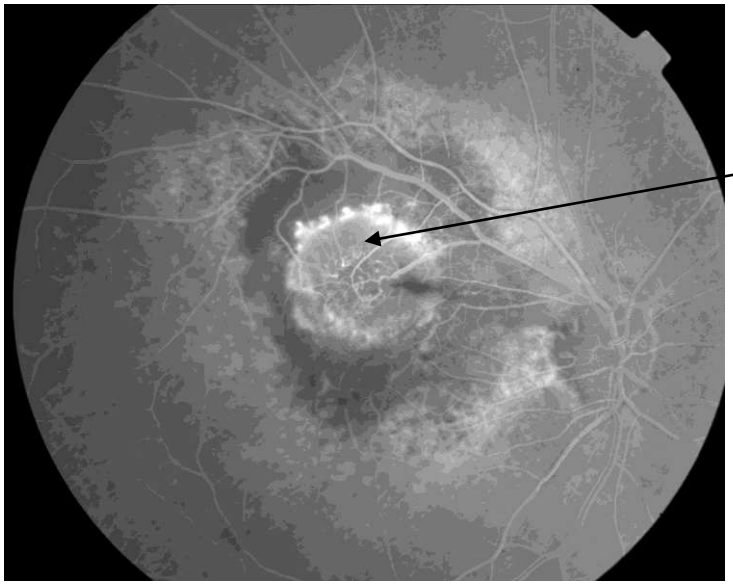


Fig 1.25. Fundal fluorescein angiogram of a patient's right eye demonstrating the leakage of fluorescent dye in the central macular area from growing blood vessels of the choroidal neovascular membrane (Courtesy of the University of Nottingham).

If left untreated, there is eventual death of the overlying photoreceptors and fibrosis, with a significant drop in vision and disruption of the structure by end stage fibrosis. Ultrastructurally, aberrant new vessels are seen to grow from the inner choroidal vessels (predominantly the veins), usually at or near sites of increased deposition of debris within and alongside Bruch's membrane (drusen), through breaks in the overlying membrane, to occupy a position in the potential sub-RPE and sub-retinal spaces (Sarks et al, 1997; Killingsworth, 1995). Along with endothelial cell proliferation within the CNV, mural cells such as pericytes, fibroblasts and smooth muscle cells also proliferate and migrate alongside the ECs. Macrophages are also usually seen associated with the CNV and may in fact pre-date the angiogenic event (Oh et al, 1999; Grossniklaus & Green, 1998; Skeie & Mullins, 2009). In keeping with neovascularisation elsewhere in the body, the choroidal new vessels leak fluid due to a reduction in endothelial cell-cell adhesion.

Pathophysiological mechanisms

As mentioned previously, a number of environmental factors predispose to AMD. Recently, the analysis of drusen showed their composition to include many complement related proteins and the finding of a number of immune related genes that may be important in the pathogenesis of "typical" AMD (Hageman et al, 2005, Klein et al, 2005; Edwards et al, 2005; Haines et al, 2005; Gold et al, 2006) have led researchers to suggest that chronic inflammation is a major contributory factor in the pathogenesis of the disease. The most important gene found to be associated with AMD is that for complement factor H (CFH), with patients harbouring the Y402H mutation having a significantly increased risk (Hageman et al, 2005, Klein et al, 2005; Edwards et al, 2005; Haines et al, 2005). CFH is a component of the alternative complement pathway and is involved in the inhibition of activated complement species. Reduced inhibitory activity caused by the Y402H mutation may lead to uncontrolled complement activation, drusen formation and damage to Bruch's membrane, thereby simulating the early stages of AMD. Recent studies have shown that patients with the Y402H haplotype have a 2.7 to 5.8 fold increased risk of AMD and that possession of the mutation may account for 50% of the total risk of AMD. Patients who are homozygous for the mutation have up to a 7.4 fold increased risk of AMD (Hageman et al, 2005, Klein et al, 2005; Edwards et al, 2005; Haines et al, 2005).

Mutations in other genes such as LOC387715 on chromosome 10q26 (Rivera et al, 2005) and fibulin 5 (Stone et al, 2004) are also thought to confer a higher risk of developing choroidal neovascularisation. However, to date, the risk and possible pathophysiological mechanism is less well understood than that of CFH. During studies of the genetic risk related to CNV, changes in genes coding for complement cascade factor B (BF), complement factor 2 (C2) and Apo E were found to confer relative protection against AMD (Scholl et al, 2007).

Another gene thought to be associated with AMD is SERPING 1 (Ennis et al, 2008).

Further evidence for immune and complement dysregulation in the pathogenesis of AMD is that drusen, which are thought to be central to the early events in the pathogenesis of AMD, contain many complement components and immune related substances. To date, it is the harbouring of mutations in genes such as CFH that confers the highest risk of AMD, implying that some form of immune system dysregulation plays a key role in AMD (Kijlstra et al, 2005; Nussenblatt & Ferris, 2007; Anderson et al 2002; Nussenblatt, 2009; Patel & Chan, 2008). Further evidence is given by the finding that macrophages are found around AMD lesions (Skeie & Mullins, 2009). It is also known that activated macrophages secrete many chemokines (Grossniklaus et al, 2002) that may in turn stimulate the production of VEGF (Oh et al, 1999). Recently, a counter argument has been placed that the macrophages may in fact inhibit angiogenesis, perhaps via Interleukin 10 (Apte et al, 2006). Interestingly, an observation in a patient given Infliximab, an antibody to tumour necrosis factor α (TNFα) given to treat diseases such as rheumatoid arthritis, caused regression of concomitant choroidal neovascularisation (Markomichelakis et al, 2004). This effect is supported by evidence provided by the observation that *in vitro*, TNFα appears to up-regulate the action of Tie2 and VEGF receptors (Hangai et al, 2006), again suggesting that immune related factors are playing a role in the stimulation of wet AMD.

It has been found that VEGF on its own is not sufficient to cause choroidal neovascularisation. Over-expression of VEGF within the retina causes retinal neovascularisation but has no effect on the choroid (Okamoto et al, 1997). Over expression of VEGF by RPE cells did not cause the formation of a true choroidal neovascular membrane except when Bruch's membrane was breached by trauma (in the case of one study, by a needle used to insert the viral vector within the choroid)

(Oshima et al, 2004; Spilsbury et al, 2000; Schwesinger C, 2001). These models suggest that other factors in addition to raised VEGF levels in the choroid are required for wet AMD to occur. Such factors may include iatrogenic or spontaneously occurring breaks in Bruch's membrane. Many growth factors appear to be up-regulated in the area of the choroidal neovascular membrane. It is not known if this is a response to local hypoxia (HIF-1 stimulating VEGF which is classically up-regulated in hypoxia and FGF 2 which can be secreted by macrophages in states of hypoxia) (Ishibashi et al, 1995) or because of local secretion by other cells such as RPE cells or macrophages, stimulated by an unknown non hypoxic stimulus (chronic viral or bacterial infection?). Interestingly, another growth factor, FGF2, again appears to be insufficient to bring about choroidal neovascularisation alone. In a mouse laser model, targeted disruption of the FGF2 gene failed to inhibit development of CNV (Tobe et al, 1998).

The role of growth factors in choroidal neovascularisation

It is well documented that, *in vitro*, endothelial cells from many sites express certain receptors for growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factor β (TGF-β), insulin like growth factor-1 (IGF-1) (Liu et al, 1998; Spraul et al, 2002) and pigment epithelium derived factor (PEDF) (Cai et al, 2006). In addition, immunohistochemical studies of excised choroidal neovascular membranes have shown positive staining for VEGF (Kvanta et al, 1996; Kliffen et al, 1997), FGF (Reddy et al, 1995; Amin et al, 1994; Frank, 1996; Ogata et al, 1996), TGF-ß (Amin et al 1994), angiopoietin 2 (Otani et al, 1999), Tie 2 (Otani et al, 1999), CD 105 endoglin (Grisanti et al, 2004) and PEDF (Matsuoka et al, 2004). Some of these cytokines have a stimulatory effect on human umbilical vein and **bovine** retinal and choriocapillaris endothelial cell proliferation, except TGF-β and PEDF which appear to be inhibitory (Lebrin et al,

2005; Cai et al, 2006; Renno et al, 2002). Some of these cytokines also appear to stimulate the migration of cells towards the source and up-regulate the production of MMPs, the enzymes necessary for the degradation of basement membranes. The role of each of these cytokines in the pathogenesis of AMD in humans can, at best, only be extrapolated from work carried out on different species or from ECs from other sites, as discussed above.

Animal Models

Animal models can provide invaluable insights into the mechanisms and possible treatments of a wide range of diseases. It is not surprising, therefore, that researchers have attempted to study the mechanisms in AMD by using animal models either with genetically induced defects that replicate the human disease susceptibility genes, or by studying animals with phenotypes that appear similar to human AMD. The most commonly used animal in the study of AMD, the mouse, has a major drawback, however; it doesn't have a macula. Interestingly, none of the animal models to date appear to have abnormalities of angiogenesis. It appears that the mechanism of disease is an abnormal insult that leads to a normal angiogenenic response. This would be in agreement with the human disease in which no defects in angiogenesis, e.g. an over-reaction to an angiogenic stimulus, have been found to date.

Genetic mouse models of AMD

CCR2/CCL2 deficiency: This model leads to the deposition of drusen, thickening of Bruch's membrane, geographic atrophy and CNV. The underlying genetic mechanism leads to reduced accumulation of macrophages within tissues such as the RPE/Bruch's complex with reduced clearance of the components that later go on to form drusen (Ambati et al, 2003).

Cx3cr1 deficiency: This mouse model is characterised by retinal degeneration and drusen-like deposits. The underlying mechanism is thought to involve microglial accumulation and damage to photoreceptors (Chan et al, 2008).

CFH deficiency: Mice with null mutations for CFH demonstrate reduced vision and abnormal retinal electrophysiology, increased retinal autofluorescence and accumulation of C3 and outer photoreceptor disorganisation. Fundoscopic examination and the choroidal vasculature were normal however. This model is therefore not a representative model of human disease (Coffey et al, 2007).

Apo E: Mutations in the mouse Apo E4 gene demonstrated many of the features of human AMD when fed a diet high in fat. This is contrary to informed epidemiological opinion which suggests a protective role for Apo E4 in AMD. It also highlights the potential role of diet in AMD pathogenesis (Malek et al, 2005).

Bst: It is reported that Bst/+ mice spontaneously develop sub retinal neovascular membranes. The Bst gene is thought to be important in axonal migration, closure of the optic fissure and involution of the hyaloid system (Smith et al, 2000).

VLDL receptor: The very low density lipoprotein (VLDL) receptor is widely expressed on heart and skeletal muscle, adipose tissue, smooth muscle cells and endothelial cells. It binds a number of ligands including apolipoprotein lipase, thrombospondin 1 and urokinase plasminogen activator. As well as its role in lipid metabolism, it may also play a role in angiogenesis inhibition. Mice lacking the VLDL receptor develop a type of wet AMD characterised by a retinal

angiomatous proliferation (RAP) (Jiang et al, 2009; Hu et al, 2008; Chen et al, 2007). This lesion is also found in some human cases of wet AMD.

SOD1: It is hypothesised that a causative factor in the pathogenesis of wet AMD is oxidative stress on the macula caused by free radicals. Imamura et al demonstrated the formation of drusen, thickening of Bruch's membrane and CNV in mice with a mutation in superoxide dismutase (SOD1) (Imamura et al, 2006).

Laser photocoagulation models of wet AMD

Choroidal neovascularisation can be induced experimentally by rupture of Bruch's membrane by laser photocoagulation. This technique has been conducted in a number of animals such as mice, rats and monkeys and provides an efficient and easily reproducible system for studying some of the underlying mechanisms and provides a useful model to test new agents *in vivo* to determine their effect on the inhibition of CNV growth (Archer & Gardiner, 1981). However, laser induced CNV do not have the relentless natural history as pathologic CNV in AMD The relevance of this model to human disease is unknown as there is probably a major inflammatory mechanism involved in this model because the laser disruption of Bruch's membrane causes major tissue damage. To date, factors such as oestrogens (Tanemura et al, 2004), ICAM 1 and CD44 (Shen et al, 1998), VEGF (Shen et al, 1998; Yi et al, 1997; Baffi et al, 2000), tissue factor (Bora et al, 2003), matrix metalloproteinases (Kvanta et al, 2000) and α_V- integrin (Yasukawa et al, 2004) have all been found to play a role in the growth of these artificially induced neovascular membranes. However, their relevance to human disease remains unknown.

While the initiating event or stimulus for the disease remains unknown, it is clear from immunohistochemical examination of excised choroidal neovascular membranes (CNV) from both humans and animals (Killingsworth et al, 1995; Sarks et al, 1997) that inner choroidal endothelial cells (ICECs) and their supporting pericytes are stimulated to proliferate by paracrine factors generated by juxtapositioned cells such as the retinal pigment epithelium (RPE) or macrophages. Upon stimulation, the ICEC cells degrade local basement membranes by production of matrix metalloproteinases (MMPs), in particular MMP2 and 9 (Kvanta et al, 2000), migrate along cytokine concentration gradients and form tube-like structures that later mature into new blood vessels (angiogenesis).

Treatment

Data from the Macular Photocoagulation Study Group (MPSG) suggests that the natural history of wet AMD is bleak. For many years, the only treatment proven to be effective in arresting the progression of the CNV was laser photocoagulation. However, only a small proportion of patients within a specific disease subgroup have been shown to benefit from this treatment which is also associated with a high rate of disease recurrence (paradoxically often under the fovea) (Macular Photocoagulation Study Group, 1991; Macular Photocoagulation Study Group, 1994). A major advance in treatment, first described in 1999, was the use of Verteporfin photodynamic therapy (PDT). Verteporfin is a photosensitiser, injected intravenously, that is selectively taken up by the abnormal “growing” vessels of the wet AMD lesion. Irradiation of the lesion with a diode laser for 83 seconds leads to vessel thrombosis and lesion regression in many cases, while leaving the foveal/macular photoreceptors unaffected. A study using this treatment showed that after 24 months follow up, the proportion of patients with a particular subtype of wet AMD (predominantly classic) losing fewer than 15 letters on the visual acuity chart was 61% in those given treatment compared

with 46% for those given placebo (TAP Study Group, 2001). Since the occluded vasculature commonly re-canalised, patients required an average of 5.6 treatments in the 24 months of the study. The type of lesion treated in the above trial only constitutes approximately 20% of patients with wet AMD; however this treatment received National Institute of Clinical Excellence and Health (NICE) approval for lesions with a predominantly “classic” appearance on fluorescein angiography. Verteporfin PDT appeared not to be as effective in the type of lesions that constituted the remaining 80% of cases (minimally classic and occult lesions). During the early years of the 21st century, most patients with wet AMD were therefore still effectively untreatable.

Research by our colleagues in oncology has shown that tumours rely on the growth of new blood vessels for their survival and that those blood vessels require VEGF for their growth. This led to the use of a number of anti-VEGF agents in the treatment of solid tumours. Based on those agents, a number of anti-VEGF agents (antibodies and an aptamer) were initially tested in animal models (Krzystolik et al, 2002) and have subsequently been licensed for the intra-ocular treatment of wet AMD. The first licensed drug was pegaptanib (Macugen™). This is an aptamer that binds one subtype of VEGF ($VEGF_{165}$). It is given every 6 weeks in the form of an intra-vitreal injection. Results from two large randomised placebo controlled trials involving around 1200 patients demonstrated visual stabilisation in approximately 70% of patients after receiving the drug for 2 years (VISION Clinical Trial Group, 2006). Very few patients were noted to have improved vision. Approximately 55% of those receiving placebo treatment demonstrated visual stability. Importantly, all wet AMD lesion types (classic and occult) appeared to respond to treatment. Although the drug appeared to be safe, there was a 1.3% incidence of potentially visually devastating intra-ocular infection (endophthalmitis).

More recently, a second agent, Ranibizumab (Lucentis™) has been licensed and approved by NICE. This is a fragment of a monoclonal antibody that binds and inhibits the action of all subtypes of VEGF and was initially shown to be effective in a laser-induced CNV model in monkeys (Krzstolik et al, 2002). It is given every 4 weeks by intra-vitreal injection. The results of a large multicentre placebo controlled randomised trial showed that at 12 months, 95% of patients receiving monthly treatment demonstrated stable vision compared with 62% of those receiving placebo (Brown et al, 2006; Rosenfeld et al, 2006). The latter result highlights the favourable natural history of the disease in some patients. In addition, 34% of those receiving active treatment demonstrated improved vision. These benefits persisted for up to 24months of continuous treatment. As with Macugen™, the major risk of treatment was endophthalmitis.

Before Ranibizumab (Lucentis™) received its license, many ophthalmologists had begun to treat patients with intra-vitreal Bevacizumab (Avastin™) injections. This is an anti-VEGF monoclonal antibody, formulated for the intravenous treatment of bowel cancer (Vincenzi et al, 2009). On a dose for dose basis, it is much cheaper than Ranibizumab (Lucentis™) and may be as effective but it is not licensed for intra-ocular use. At present therefore, only Lucentis™ is approved by NICE for the treatment of wet AMD. Pegaptanib (Macugen™) was not approved for use in the NHS by NICE although clinically effective as it was thought not to be cost-effective, and not as effective as Ranibizumab (Lucentis™).

In vitro study of endothelial cells with relevance to AMD

In an attempt to further our understanding of the complex processes that underlie the proliferation of these inner choroidal endothelial cells, investigators have studied, *in vitro,* the phenotype and growth characteristics of **bovine** choroidal and choriocapillaris endothelial cells (Liu & Li, 1993; Sakamoto et al, 1995a; Sakamoto et al, 1995b; Morse

& Sidikaro, 1990; Hoffman et al, 1998; Liu et al, 1998; Wang et al, 2002; Zubilewicz et al, 2001a; Zubilewicz et al, 2001b; McLaughlin & Vries, 2001; Eter & Spitznas, 2002) and heterogeneous mixtures of **human** choroidal endothelial cells (Penfold et al, 2002; Geisen et al, 2005; Peterson et al, 2007; Sakamoto et al,1995; Bargagna-Mohan et al, 2006) or macrovascular (HUVECs) (Shankar et al, 2008; Stahl et al, 2008).

It is well recognised, however, that endothelial cells from different species and anatomical sites vary widely in their phenotype and growth characteristics. For example, in the eye, retinal endothelial cells resemble those of the brain in having tight junctions but no fenestrations, whereas choroidal endothelial cells express fenestrations. It has also been shown *ex vivo* and *in vitro* that microvascular endothelial cells in the human lung predominantly express CD34, whereas lung macrovascular endothelial cells and HUVECs predominantly express vWf in preference to CD34 (Müller et al, 2002). This means that it may not be appropriate to extrapolate data from more general endothelial cell models to study human choroidal neovascularisation due to the discrete species differences and the unique, anatomical and microvascular environment of the inner choroidal endothelial cell (ICEC). It would therefore seem logical to study **human** inner choroidal endothelial cells in an attempt to further our understanding of the neovascular form of AMD.

In vitro experiments using human choroidal endothelial cells

To date, **human** inner choroidal endothelial cells have not been selectively isolated and studied. However, workers have developed techniques to isolate human choroidal endothelial cells (hCECs) and have conducted a number of studies on them.

Sakamoto et al in 1995a published a method to isolate human CECs using a density gradient to separate choroidal fragments after

collagenase digestion. The growing human CECs were purified by cloning. Using this technique, cells of 95% purity were isolated. Subsequently, Fan et al in 2002 enhanced human CEC purification by utilising anti-CD31 coated paramagnetic beads. They used these cells in a co-culture system with RPE cells stimulated with IL-1 and separated by a semi-permeable membrane to identify disease mechanisms in choroidal neovascularisation (Fan W et al, 2002). Geisen et al (2005) demonstrated increased migration of hCECs across a membrane when exposed to RPE cell conditioned medium and, to a lesser extent, VEGF. Peterson et al in 2007 demonstrated that PI-3K and Akt were important in this process of hCEC migration across a similar membrane. Unusually, VEGF had no effect on cell proliferation in these experiments. This is a very unusual result as, to date, all endothelial cells appear to respond to VEGF. The reason for this atypical result may be due to later passage hCECs being used in their experiments. It is known that endothelial cells, on passaging, may undergo phenotypic change and thus may change their *in vitro* behaviour (Shima et al, 1995; Prasad et al, 2006). Penfold et al in 2002 also used these cells to demonstrate down-regulation of cell surface ICAM 1 and MHC antigens with a type of steroid that has been shown to have a beneficial role in the treatment of wet AMD, thereby hypothesising a mechanism for its actions.

Steinle et al in 2003 demonstrated that sympathetic denervation in animals led to blood vessel growth in both the retina and choroid, and increased levels of local nerve growth factor (NGF) levels. They hypothesised that NGF was responsible for this up-regulation of angiogenesis. Using isolated human retinal and choroidal endothelial cells, they showed a difference in response when the cells were exposed to NGF *in vitro*. Choroidal ECs appeared to respond by increasing proliferation and migration to NGF via ERK 1/2 and Akt, whereas retinal ECs did not respond. An important difference in ocular EC response to external factors was therefore found, reinforcing the heterogenic nature of ECs within a single organ. In a similar vein,

Steinle et al in 2005 also demonstrated that human CECs possess ß adrenoreceptors and that stimulation of the $ß_3$ receptors again resulted in activation of ERK 1/2 and Akt and affected cell migration and invasion. The role of the sympathetic/ parasympathetic nervous systems in choroidal neovascularisation remains unknown, however.

Diabetic retinopathy

Diabetes mellitus currently affects 150 million people worldwide. This number is set to soar by 2025 to reach 300 million, partly because of the epidemic of obesity (King et al, 1998). The majority of patients have type 2 diabetes mellitus where there is resistance to the action of insulin, although it is initially produced in normal amounts. Most patients with this type of diabetes are over the age of 40 years at disease onset. A minority of patients have type 1 or insulin-dependent diabetes which is caused by a lack of insulin due to autoimmune destruction of the pancreatic β cells. Both types of diabetes are characterised by raised blood glucose levels. A major effect of the disease process is the damage caused to the macrovascular and microvascular circulations leading to diseases of large vessels such as stroke and heart attack, and microvascular disorders such as renal glomerular damage, peripheral neuropathy and retinal vascular complications.

Epidemiological studies have shown that after 15 years, almost all patients with type 1 diabetes and around 50% with type 2 diabetes not receiving insulin treatment have some degree of diabetic retinopathy. After 20 years of diabetes, 5.6% of patients will have developed clinically significant macular oedema caused by break down of the inner blood retinal barrier. Also, 7.9% will develop sight threatening retinopathy which includes proliferative diabetic retinopathy (PDR) due to retinal angiogenesis and consequent vitreous haemorrhage and tractional retinal detachment (Klein et al, 1989). More specifically, PDR occurs in 50% of type 1 diabetics and 15% of type 2 patients who have

had the disease for 25 years (Klein et al, 1989), making diabetes the leading cause of blindness in people under 65 years of age.

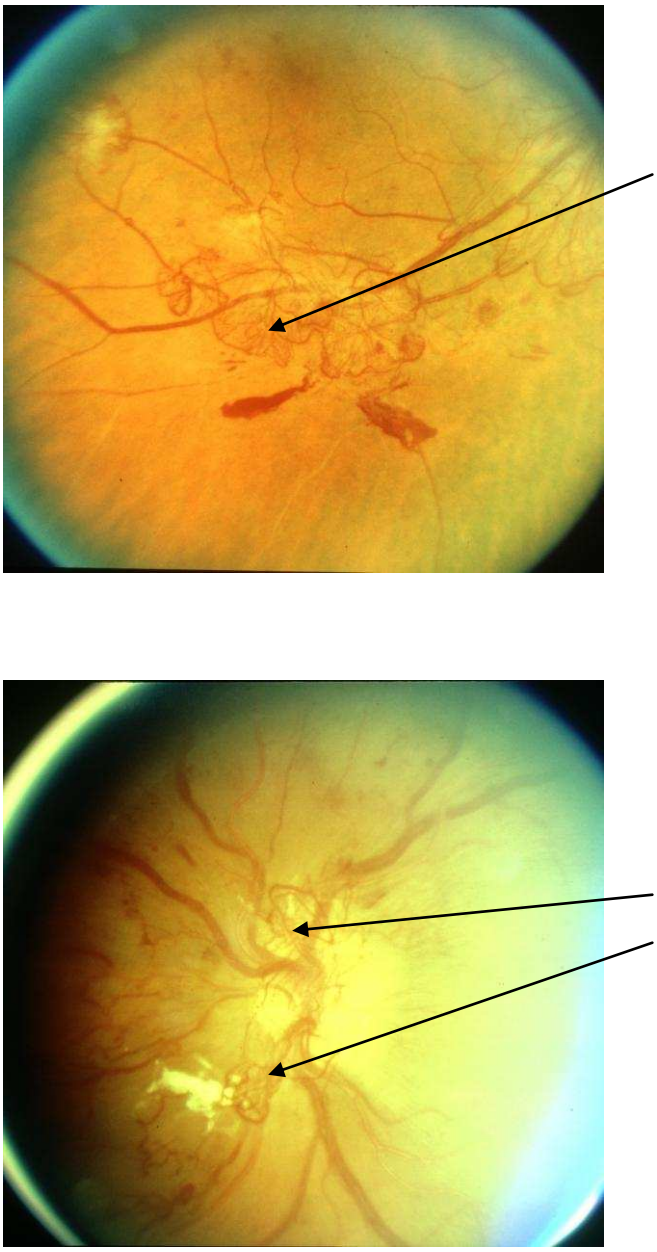


Fig 1.26. Proliferative diabetic retinopathy (neovascularisation of the retina (upper), and optic disc (lower)). (Courtesy of the University of Wisconsin photographic reading centre)

The exact mechanism or pathogenesis of diabetic retinopathy is uncertain but persistent hyperglycaemia eventually leads to microvascular damage to the retina. Possible mechanisms include increased flux of sugars through the polyol or hexosamine pathway with subsequent changes in endothelial cell redox potential. This leads to the accumulation of intracellular sorbitol and cell damage. Another possible important mechanism is excessive activation of the intracellular messenger diacylglycerol (DAG) with subsequent activation of protein kinase (PKC). Lesions include retinal capillary basement membrane thickening, pericyte and vascular smooth muscle cell drop out, capillary microaneurysms and capillary occlusion. Other mechanisms include alterations in blood rheology, accumulation of advanced glycation end products (AGES), free radicals and over-activation of the renin-angiotensin system (Brownlee, 2001; Stitt et al, 2005; Curtis et al, 2009).

Whatever the mechanism, the resulting pathological retinal lesions are caused by progressive inner retinal ischaemia (secondary to microvascular occlusion) and the subsequent secretion of pro-angiogenic growth factors such as VEGF (Campochiaro, 2000). The role of VEGF in the causation of proliferative diabetic retinopathy has been strongly implicated by the finding that repeated intravitreal injections of $VEGF_{165}$ into cynomolgus monkeys produces many of the changes in the retinal vasculature that are seen in diabetes, such as venous dilatation and tortuosity, retinal ischaemia, capillary microaneurysmal dilatation and pre-retinal neovascularisation (Tolentino et al, 1996; Tolentino et al, 2002). It appears that IGF-1 may also play a role in proliferative diabetic retinopathy (Chantelau et al, 1997; Smith et al, 1997; Smith et al, 1999). It was observed many years ago that hypophysectomy (removal of the pituitary gland in patients with acromegaly (who also had diabetic retinopathy) led to a resolution of the PDR (Wright, 1969). It is known that the main stimulus for IGF-1 is growth hormone (GH) produced by the pituitary. Secondly, improved diabetic control by increased levels of insulin leads to higher levels of

IGF 1 (Chantelau et al, 1997) and VEGF (Lu et al, 1999) in the blood. This may explain the commonly occurring worsening of retinopathy when blood sugar levels are dramatically improved. It has also been observed that cardiac events increase when sudden improvement in blood sugars occurs (ACCORDS Group, 2008). Systemic blockade of growth hormone by a GH antagonist also leads to improvement in diabetic retinopathy (Boehm et al, 2000).

Current proven treatment for diabetic retinopathy involves laser photocoagulation to areas of macular oedema (in an effort to stimulate regression of oedema by unknown mechanisms) or the obliteration of areas of retinal ischaemia in an effort to down regulate the production of pro-angiogenic growth factors. As VEGF appears to play a role in both the breakdown of the inner blood retinal barrier (due to increased vascular permeability) and in the growth of new retinal blood vessels (by retinal angiogenesis), researchers have recently investigated the use of intravitreal injections of anti-VEGF antibodies. In clinical trials, these drugs (ranibizumab, bevacizumab and pegaptanib) appear to have a marked effect on the disease. However, they need to be given every 4 to 6 weeks by invasive intra- ocular injections in a similar fashion to the treatment of wet AMD, as mentioned earlier. Other targets for treatment include the interruption of intracellular signalling secondary to up-regulation of PKC activity and inhibition of IGF-1.

It is known that hyperglycaemia leads to an increased synthesis of intracellular diacylglycerol (DAG) in endothelial cells which in turn leads to activation of protein kinase C (PKC) (Donnelly et al, 2004). The effect of increased PKC activation in endothelial cells is to bring about changes in vascular permeability and blood flow and to modify the formation and response to growth factors such as VEGF. The role of PKC in the pathogenesis was therefore thought to be important and has been the subject of much research (Donnelly et al, 2004). There are at least 12 isoforms of PKC with different tissue expression profiles (Moriarty et al, 2000). It emerged that hyperglycaemia leads to a

proportionately larger increase in the isoforms PKC β_I and PKC β_{II} in endothelial cells. Because PKC is widely found in different cells, treatment strategies using non-specific PKC inhibition were likely to lead to widespread tissue dysfunction and toxicity. In 1996, a more selective PKC inhibitor (β_I and β_{II}) became available and was used in a number of diabetic clinical trials The new drug, called LY-333531 (Ruboxistaurin) was found to inhibit the increase in retinal blood flow and reduce macular oedema in diabetic patients (Frank, 2002). In one clinical randomised trial, the drug reduced moderate visual loss by 40% in those given the drug (cf. placebo). There was also a significant reduction in progression of diabetic macular oedema and the requirement of macular laser treatment (Aiello et al 2006). In a second study, the drug appeared to have no effect on the progression of diabetic retinopathy, a disappointing outcome. This study, however, again demonstrated a significant reduction in moderate visual loss (PKC-DRS study group, 2005). Although the preliminary results for the effects of this drug on diabetic eye disease appear encouraging, the drug has yet to be licensed for this use and the FDA in the USA has required further data in the form of further clinical trials before making a decision on its use.

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a proliferative retinopathy seen in “at risk” newborn infants and remains a leading cause of childhood blindness. It was first recognised in 1942 when a link between proliferative retinopathy and neonatal oxygen supplementation was described. Over the next 20 years, as the rate of oxygen supplementation reduced, so did the incidence of ROP. During the 1960’s and 70’s, its incidence increased again due to advances in neonatal care in which lower birth weight children survived. The risk of developing abnormal retinal neovascularisation or ROP appears to be inversely related to birth weight and gestation. During normal

development of the retina, vessels migrate from the optic disc to the periphery of the eye, starting at week 16. Interruption of this process by premature birth leads to areas of avascular peripheral retina being present after birth. If the baby is given high concentrations of inspired oxygen (necessary due to under-developed lungs) this leads to inhibition of normal retinal vascular development by suppressing capillary development and causing vascular obliteration at the interface between normal and avascular retina. If the oxygen tension is then reduced, the areas of avascular retina become hypoxic and secrete growth factors such as VEGF. This in turn leads to uncoordinated retinal neovascularisation at the junctional zone.

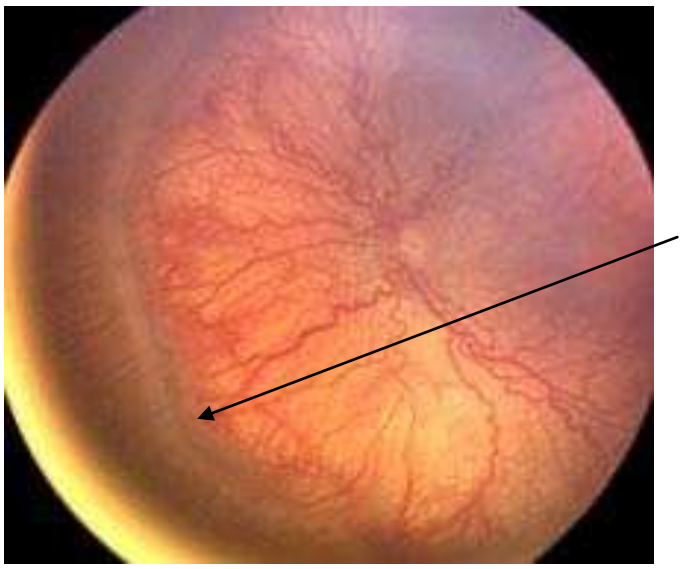


Fig 1.27. Fundus photograph of a premature baby's left eye, demonstrating a ridge of neovascuarisation in the peripheral area of the retina. Also note the dilated and tortuous retinal vessels, indicating marked retinal ischaemia (courtesy of the University of Texas)

The recognition and treatment of ROP involves regular examination of "at risk" eyes for the tell tale signs of retinal neovascularisation forming a ridge at the junctional zone. This would lead to treatment to ablate the areas of avascular retina by laser or cryotherapy. Failure of treatment may lead to retinal detachment by fibrovascular membranes and vitreous haemorrhage, both leading to poor visual outcome.

Much understanding of this disease has come from an animal model in which one week old mice are exposed to 75% oxygen for 5 days and then are returned to room air. Neovascularisation occurred between post natal days 17 and 21, at the junction between vascularised and avascular retina in the mid periphery. In contrast to the human disease, this murine model develops vasoconstriction and ischaemia around the disc at the posterior pole with sparing of the peripheral vessels (in the human disease, the opposite distribution of vascular closure occurs). This distribution is thought to be because of continued perfusion of the murine peripheral retina by remnants of the central hyaloid vessels. The retinal ischaemia induced by the vascular closure causes a reflex over-expression of VEGF which in turn leads to abnormal retinal neovascularisation or angiogenesis (Smith et al, 1994). Nowadays, the use of lower levels of oxygen supplementation would be thought to have led to a marked reduction in the incidence of ROP if the oxygen hypothesis is the unifying hypothesis, but in reality this has not happened. Slidsborg et al in 2008 found that the incidence of ROP doubled in Denmark between 1996 to 2000 and 2001 to 2005, while in Canada, Schiatriti et al in 2008 found an increase between the periods 1992 to 1996 and 1997 to 2001. Conventional treatment of ROP involves the ablation of the ischaemic peripheral retina in the hope of reducing the risk of ultimate retinal detachment. Recently, intravitreal injection of anti-VEGF agents such as bevacizumab (Avastin) has been used experimentally in an attempt to reduce or abolish the neovascularisation (Mintz-Hittner et al, 2011). While the treatment was very successful in treating neovascularisation around the optic disc and macula, it was unsuccessful for disease in the more peripheral retina. This would imply that peripheral disease is not VEGF driven or that other mechanisms are responsible for this stage of disease. Some workers suspect that other factors apart from VEGF may be important in disease pathogenesis and progression. It has been hypothesised that IGF 1 may play a role because its secretion correlates with birth weight and gestational age (Hellström et al, 2002; Hellström et al, 2003). In addition, IGF-1 levels drop in the neonatal period as the main sources

to the infant, the placenta and the amniotic fluid, are lost. Recent studies have shown that IGF-1 is important for retinal vascular development and is an endothelial survival factor (Modanlou et al, 2006; Lofqvist et al, 2009; Hellström et al, 2002) and if IGF-1 levels rise quickly after birth, normal development occurs. It has also been shown that in the presence of low IGF-1 levels, VEGF is insufficient to allow retinal vascular development. It is thought that when IGF-1 levels are low, retinal vessels fail to develop, allowing the retina to become hypoxic and VEGF to accumulate. With the normal increase in IGF 1 with age a threshold is reached that allows VEGF to trigger the massive increase in retinal angiogenesis, implying that IGF 1 is required for maximal VEGF action. It has now been hypothesised that a blood test to measure IGF 1 levels may predict those babies at risk of ROP and that IGF 1 supplementation may restore normal retinal vasculature development in such infants (Hellström et al, 2001).

Iris neovascularisation

Very few disorders appear to specifically affect the iris vasculature with the exception of anterior uveitis, an autoimmune inflammatory condition that targets cells within the iris and the ciliary body. In this condition, leukocytes are attracted to the iris vasculature and pass through gaps between the endothelial cells to reach the tissue interstitium. The underlying pathogenesis of this condition and the target antigens are largely unknown.

Occlusion and subsequent ischaemia of the iris vasculature in conditions such as herpetic uveitis and acute angle closure glaucoma rarely, if ever, give rise to iris neovascularisation. This is perhaps because the level of VEGF stimulated by the occlusion is too low or it is rapidly removed from the anterior segment of the eye by the rapid flow of aqueous. Iris neovascularisation tends to occur in the presence of retinal ischaemia caused by diseases such as diabetes mellitus or by

retinal vascular occlusions such as central retinal vein occlusion. Both of these conditions are thought to generate large amounts of VEGF that diffuse into the anterior chamber and hence come into contact with the iris vasculature. Iris neovascularisation can occur in the absence of retinal neovascularisation. The reason for this is unknown but may include the rapid diffusion of VEGF away from the retina towards the anterior segment and the potential production of pro-angiogenic substances to which the iris but not the retina responds. In a primate model of central retinal vein occlusion with iris neovascularisation, levels of retinal VEGF and VEGF mRNA are significantly raised, while intra-ocular injection of VEGF is sufficient to cause iris new vessel growth (Miller et al, 1994). This effect can be neutralised by injection of anti-VEGF antibodies (Adamis et al, 1996). Iris neovascularisation usually starts at the pupil margin (perhaps due to the flow of VEGF from the posterior segment) and grows across the iris to the drainage angle and the trabecular meshwork. Angiogenesis in the drainage angle impedes the outflow of aqueous and (rubeotic) glaucoma ensues. Histopathological examination reveals that the new vessels arise from pre-existing capillaries, are thin walled and interestingly are fenestrated. Normal iris capillaries are not fenestrated (Bron et al, 1997a).

Conclusion

It is now widely accepted that endothelial cells derived from different species, vascular organs and from within different vascular beds, display phenotypical, biochemical and genetic heterogeneity. It follows that the extrapolation of results from non human ocular or HUVEC cells may not provide reliable data applicable to human eye disease. Any meaningful insight into the pathogenesis and selective treatment of the diseases mentioned previously would therefore probably only be gained by conducting *in vitro* studies using early passage microvascular endothelial cells derived from the particular site(s) affected by specific diseases because within the ocular vascular beds themselves, many

diseases appear site specific. This work therefore aims to develop techniques to isolate human ocular vascular endothelial cells, in particular, those from the inner macular choroid, as these are thought to be crucial to the pathogenesis of the common blinding condition, wet AMD. This work also aims to explore their heterogeneity by comparing their differential gene expression with endothelial cells from other sites, both within the human choroid and at other sites within the eye. I also aim to determine the effects of various growth factors on the macular choroidal endothelial cells in an attempt to more fully understand some of the important mechanisms that may contribute to wet AMD and that may perhaps lead to new targets for disease treatment.

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Chapter 2

The Isolation of Ocular Microvascular Endothelial Cells

Introduction

Previous investigators have utilised a variety of techniques to isolate vascular endothelial cells from a diverse range of human tissues and organs. These include the bone marrow (Rafii et al, 1994; Masek & Sweetenham, 1994), myometrium (Gargett et al, 2000), placenta (Dye et al, 2001), stomach (Hull et al, 1996), saphenous vein (Scoumanne et al, 2002), retinal capillaries (Gitlin and D'Amore, 1983: Su et al, 1992) and iris (Silverman et al, 2001). Early techniques relied on enzyme digestion of the tissue, followed by selective scraping of contaminating cells in culture, followed by preferential growth of endothelial cells in selective media containing a range of agents such as brain extract, retinal extract or heparin. Later, enzyme tissue digestion was first followed by density gradient separation and then "sweeping" of cultured cells. However, more modern techniques rely on cell isolation by endothelial specific/selective antibodies attached to paramagnetic beads such as the "Dynabead™". Endothelial antigens used include CD31 (PECAM1) and lectins such as Ulex europaeus agglutinin 1 (UEA-1). The manufacturer states that binding of CD31 paramagnetic beads has no effect on cell properties or stimulation; however, Tiwari et al in 2003 stated that CD31 coated beads may have an effect on cell proliferation when used in high concentrations (higher than the manufacturers recommendation). A method is described below for the reliable isolation of ocular microvascular endothelial cells from human eyes that may then be used for subsequent analysis.

Methods

Human posterior segments were obtained from UK Transplant within 72 hours of death. The corneas had previously been removed for transplantation and the posterior segments were stored at 4°C in sterile normal saline if consent for their use in research had been obtained. Donors were free of any known ocular disease or systemic infection.

The project had the approval of the Manchester eye bank and the local research ethics committee (Nottingham 2 LREC) (Nottingham Q1060301).

Isolation of human choroidal endothelial cells

Human eyes were dissected on sterile Petri dishes on an open laboratory bench using a binocular dissecting microscope (approximately x10 magnification). It was intended to perform the procedure in a laminar flow hood but this would not accept the dissecting microscope. It was subsequently found that the rate of culture infection using the bench dissection was very low, justifying this approach. Dissection was conducted using disposable equipment or instruments sterilized in 100% alcohol.

The iris was removed and was stored in isolation medium consisting of Minimum Essential Medium (MEM) (Invitrogen Ltd, Paisley, UK) containing 0.25µg/ml amphoterocin B, 100µg/ml streptomycin, 50µg/ml kanamycin and 30µg/ml penicillin for up to 10 minutes at room temperature. Four relieving-incisions were made in the sclera, and the posterior segment was flat mounted. The vitreous was removed and discarded and the neuroretina was teased from the underlying choroid and stored in isolation medium consisting of Minimum Essential Medium (MEM) (Invitrogen Ltd, Paisley, UK) containing 0.25µg/ml amphoterocin B, 100µg/ml streptomycin, 50µg/ml kanamycin and 30µg/ml penicillin. The overlying retinal pigment epithelium (RPE) was removed by gentle brushing with a sterile spatula and irrigated with sterile phosphate buffered saline (PBS) and discarded. The full thickness choroidal sample could now be separated from the sclera with toothed forceps. The bridging vortex veins were cut with sterile scissors, as were the adhesions around the optic disc.

The choroidal samples from each pair of eyes were combined and washed 3 times in isolation medium (composition described above) and cut into 1-2mm pieces. The pieces were incubated in collagenase I (0.1%) in MEM for 60 minutes at 37°C with frequent agitation. After 60 minutes, the collagenase was neutralised with MEM and 10% foetal calf serum (Invitrogen Ltd, Paisley, UK). The mixture was filtered through a sterile 40µm filter (Millipore Ltd, Watford, UK) and the eluate was centrifuged at 75g and washed 3 times in isolation medium. The cells were re-suspended in 1 ml of PBS/ 0.1% BSA and were incubated with 25µl of anti-CD31 coated Dynabeads™ for 15 minutes at 4ºC (Dynal Ltd, Wirral, UK). After binding of the Dynabeads, the complexes were washed 4 times in PBS/ 0.1% BSA using a magnetic particle concentrator (Dynal Ltd, Wirral, UK). The endothelial-bead complexes were re-suspended in Endothelial Growth Medium (EGM2-MV with hydrocortisone omitted) (Cambrex Biosciences, Wokingham, Berks, UK) and seeded onto either 30mm fibronectin-coated culture dishes (Beckton Dickinson, Oxford, UK) or into 25cm^2 fibronectin-coated flasks (Beckton Dickinson, Oxford, UK), depending on the experiment the cells were required for. After overnight incubation at 37ºC in a humidified atmosphere of 5% CO_2, fresh endothelial growth medium was added. After 5-7 days, large areas of confluent cells were present and were removed for experiments with 0.025% trypsin and 0.01% EDTA in sterile PBS, after which the cells were washed 3 times with sterile PBS. All reagents were from Sigma-Aldrich, Poole, Dorset, UK unless otherwise specified.

Isolation of human iris endothelial cells

During the dissection of human donor eyes, the irides were removed and placed into isolation medium as mentioned previously. The irides were removed from the isolation medium and placed onto a sterile petri dish. The posterior aspect of the iris was brushed with a sterile spatula to remove the pigmented epithelium. The irises were then cut up into tiny pieces and washed in isolation medium as described above (for the

choroidal endothelial cells). The tissue was then treated exactly as detailed above to isolate the iris microvascular endothelial cells.

Isolation of human retinal endothelial cells

During the dissection of human donor eyes, the retina was removed and placed into isolation medium as mentioned previously. The retinal samples were transferred onto a sterile petri dish and cut into tiny pieces. The sample was then transferred back into isolation medium and processed as detailed previously to isolate the choroidal microvascular endothelial cells.

Human umbilical vein endothelial cells (HUVEC)

Fresh un-passaged HUVEC cells were purchased from PromoCell GmbH, Heidelberg, Germany. On arrival, the cells were thawed and re-suspended in endothelial growth medium (EGM2-MV with hydrocortisone omitted, Cambrex Biosciences, Wokingham, Berks, UK) and washed 3 times. The cells were purified using Dynabeads and then seeded onto fibronectin-coated 35mm culture dishes (Beckton Dickinson, Oxford, UK) and incubated at 37°C in a humidified atmosphere of 5% CO_2 as previously described.

Endothelial cell characterisation

Prior to use in subsequent experiments, a small sample of trypsinised cells, suspended in EGM-2MV, was placed onto sterile glass cover slips (VWR Ltd, Poole, UK) coated with 1% gelatin (Sigma) (cover slips were immersed in 1% gelatin in sterile PBS for 30 mins at 37°C, then washed gently in sterile PBS and dried in a laminar flow hood). After allowing 3 hours for cells to attach, they were fixed in ice cold methanol at -20°C for 20 minutes. A standard two-stage immunofluoresence technique was applied using primary antibodies to CD31 (murine IgG1) (Dako, Cambridgeshire, UK) at a dilution of 1:20 and vWf (rabbit immunoglobulin fraction) (Dako) at a dilution of 1:200. The primary antibodies were left in contact with the cells for 60 minutes before

washing 4 times with sterile PBS. The secondary antibodies used were: rabbit anti-mouse F(ab')2 fragment fluorescein isothiocyanate (FITC) conjugated (Dako) at a dilution of 1:20 when used to visualise the anti-CD31 primary antibody; and swine anti-rabbit F(ab')2 fragment FITC conjugate (Dako) at a dilution of 1:20 to visualise the anti-vWf primary antibody. The secondary antibodies were left in contact with the cells for 60 minutes before washing 4 times with sterile PBS. The slides were then mounted in glycerol containing 2.5% 1-4 diazabicyclo[2,2,2] octane (DABCO) (Sigma –Aldrich) and observed by confocal fluorescence microscopy (Leica TCS0D, Leica, Milton Keynes, UK). A non-specific anti-rat epitope murine IgG1 antibody was used as a negative control primary antibody for the CD31 (mouse) antibody) and FITC labelled non-specific swine anti-rabbit immunoglobulin fraction (DAKO) as a negative control for the vWf antibody . Negative controls for the secondary antibody (only possible for anti-CD31 as anti-vWf was a labelled primary antibody) was performed by replacing the secondary antibody with PBS In assessing the identity and purity of the endothelial cells, at least 500 nucleated cells were counted after staining with each antibody. For some of the identification experiments, the cell nuclei were also stained with propidium iodide (500nM for 60 seconds) to aid cell detection.

Results

After isolation, HUVEC, choroidal, iris and retinal endothelial cells all grew with a typical cobblestone morphology (figs. 2.1-2.4). By randomly counting at least 500 cells, at least 99.5 % of each cell type demonstrated positive expression of CD31 (fig. 2.5) and vWf (fig. 2.8), thereby confirming their identity as endothelial cells. Negative control samples for both CD31 (primary and secondary antibody) and vWf (primary antibody) failed to show any significant staining, thereby confirming the positive results for the primary antibodies. Each 25cm^2 flask used to grow each sample provided up to 200,000 endothelial cells of extremely high purity for use in subsequent experiments.

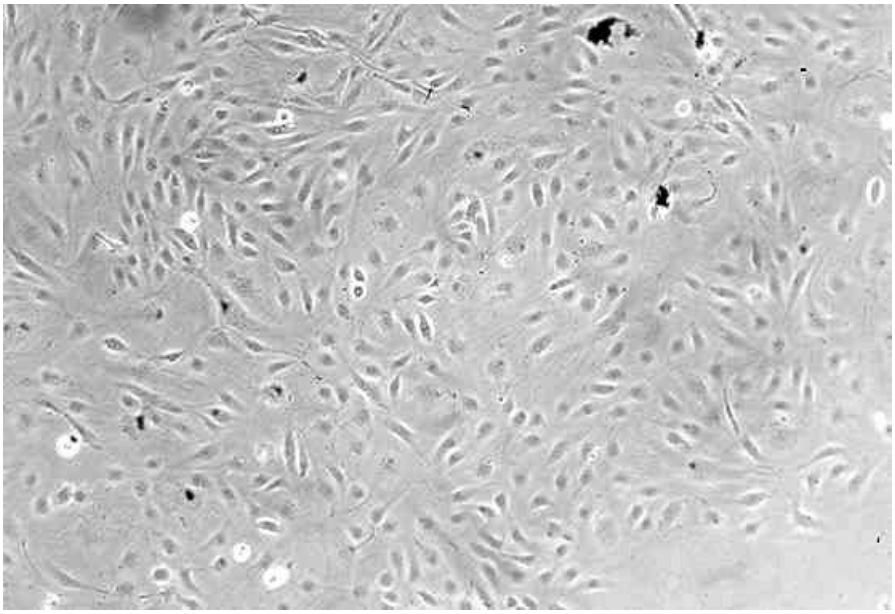

Fig. 2.1. Phase contrast photomicrograph of primary cultures of human umbilical cells (HUVEC) demonstrating a typical cobblestone appearance. The dark clumps are adherent Dynabeads (20x original magnification)

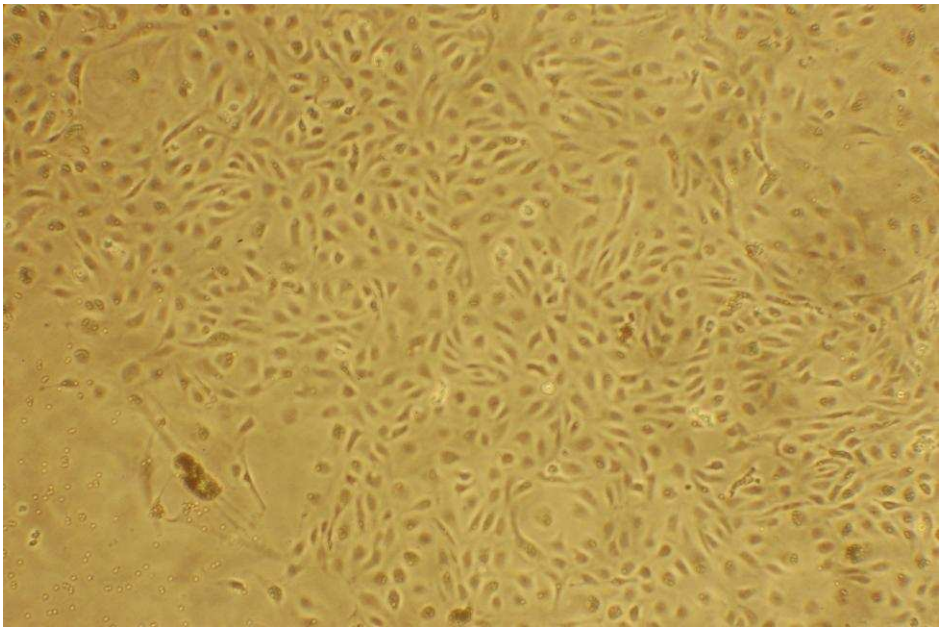

Fig. 2.2. Phase contrast photomicrograph of primary cultures of human choroidal endothelial cells demonstrating a typical cobblestone appearance. The dark clumps are adherent Dynabeads (20x original magnification).

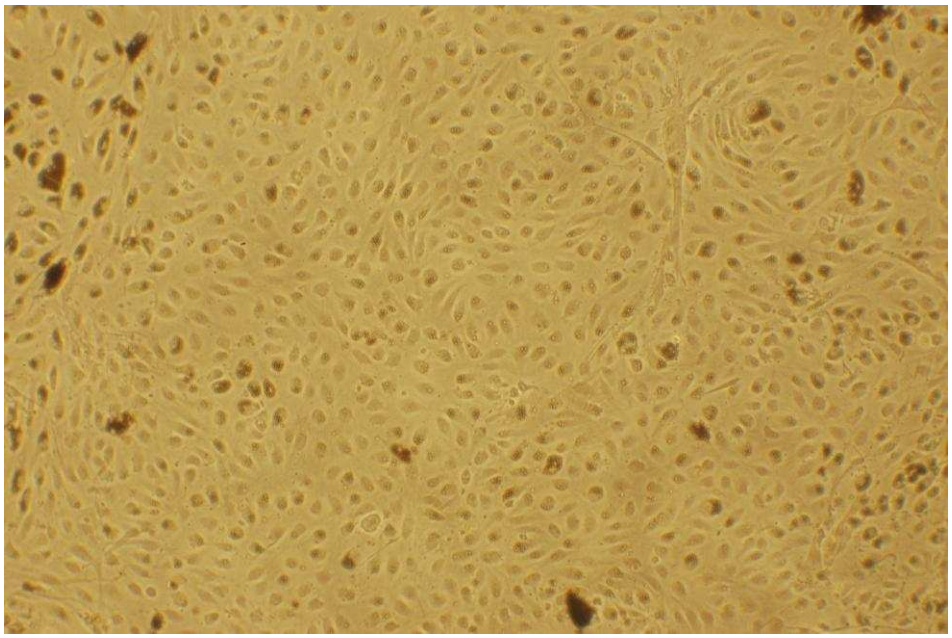

Fig. 2.3. Phase contrast photomicrograph of primary cultures of human retinal endothelial cells demonstrating a typical cobblestone appearance. The dark clumps are adherent Dynabeads (20x original magnification).

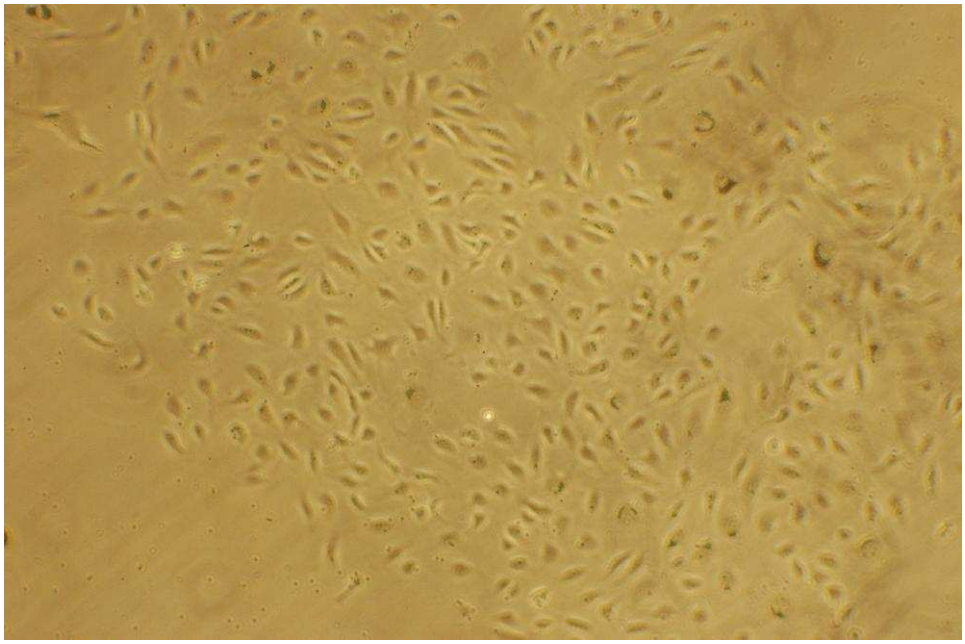

Fig. 2.4. Phase contrast photomicrograph of primary cultures of human iris endothelial cells demonstrating a typical cobblestone appearance. The dark clumps are adherent Dynabeads (20x original magnification).

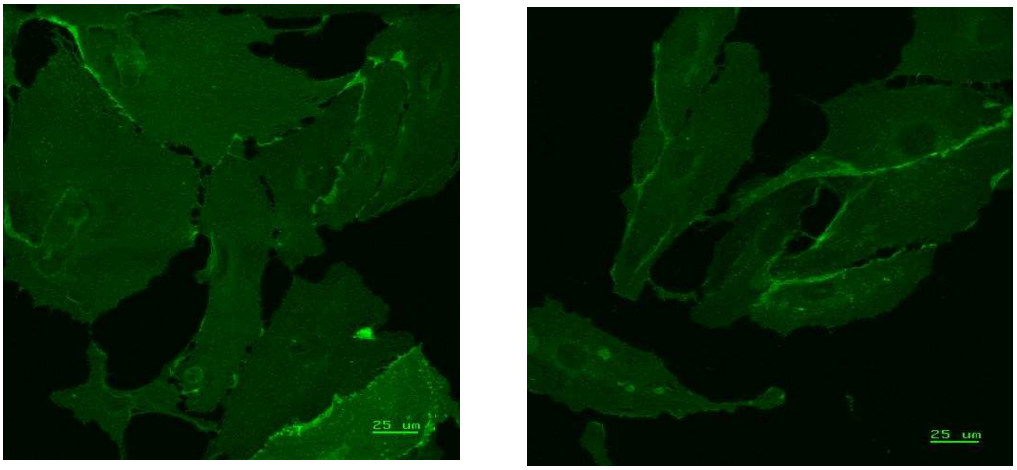

Fig. 2.5a. Immunofluorescent photomicrograph of human retinal (left) and choroidal (right) endothelial cells stained for CD31 (63x original magnification). Note the predominance of staining at edges of cells, the area where the density of the cell adhesion molecule CD 31 is known to be highest.

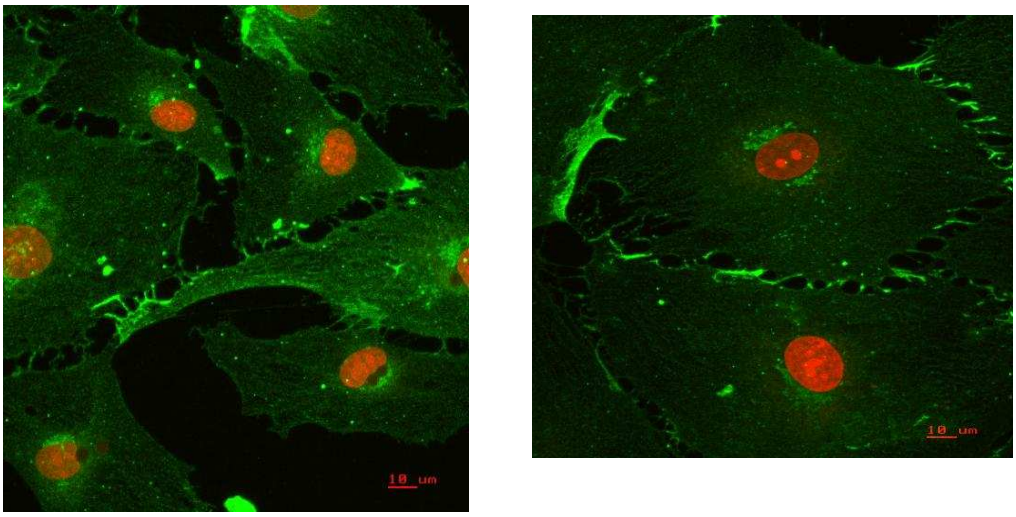

Fig. 2.5b. Immunofluorescent photomicrograph of human iris (left) and umbilical vein (right) endothelial cells stained for CD31 (63x original magnification). Note the predominance of staining at edges of cells, the area where the density of the cell adhesion molecule CD 31 is known to highest. Also note that the nuclei have been stained red with propidium iodide to aid detection.

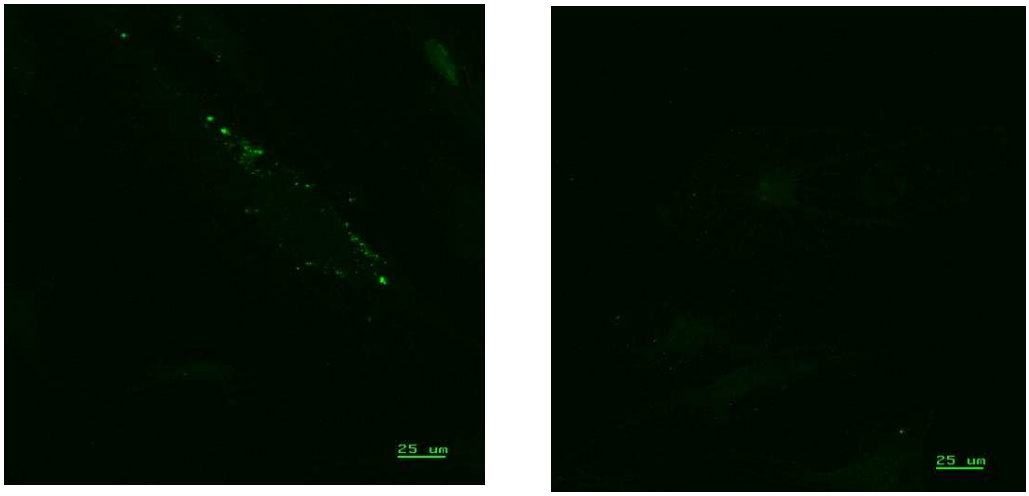

Fig. 2.6a. Immunofluorescent photomicrograph of human retinal (left) and choroidal (right) endothelial cells stained with a non-specific anti-rat epitope murine IgG1 antibody as a negative control of the anti-CD31 primary antibody (63x original magnification).Note the absence of any staining of cells. Some non-specific fluorescence from precipitation of secondary antibody can be seen.

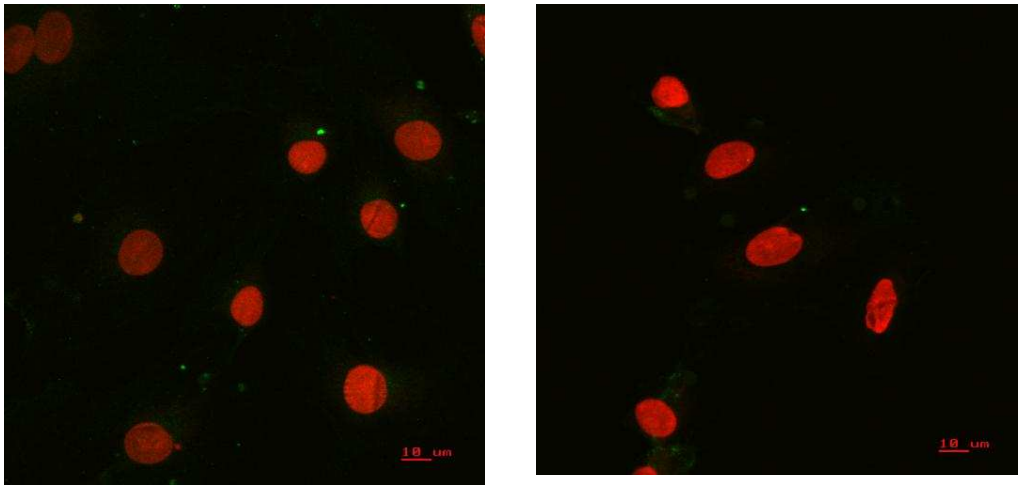

Fig. 2.6b. Immunofluorescent photomicrograph of human iris (left) and umbilical vein (right) endothelial cells stained with a non-specific anti-rat epitope murine IgG1 antibody as a negative control of the anti-CD31 primary antibody (63x original magnification).Note the absence of any staining of cells. Some non-specific fluorescence from precipitation of secondary antibody can be seen. Also note that the nuclei have been stained red with propidium iodide to aid detection.

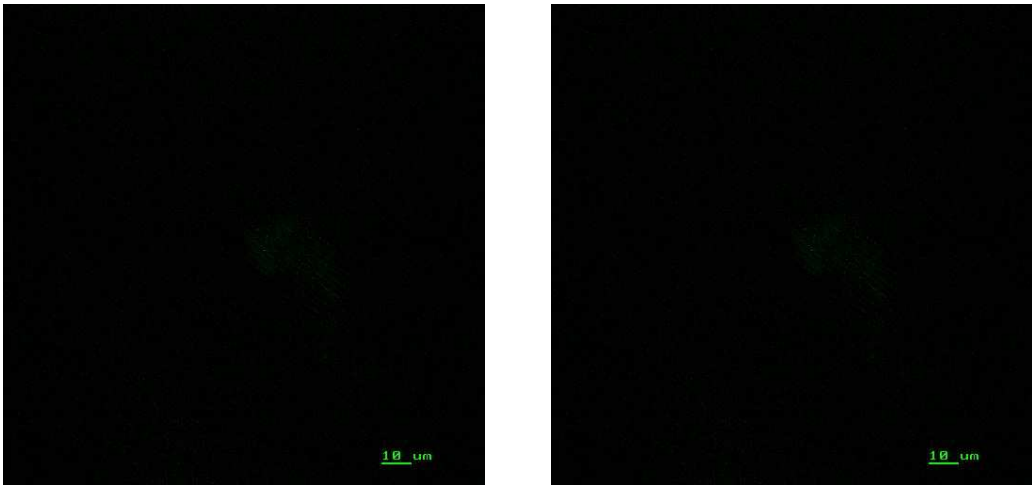

Fig. 2.7. Immunofluorescent photomicrograph of human retinal (left) and choroidal (right) endothelial cells stained with anti-CD31 and the secondary antibody replaced with PBS (63x original magnification).Note the absence of any specific staining of cells. Some very faint background fluorescence from the cells can be seen.

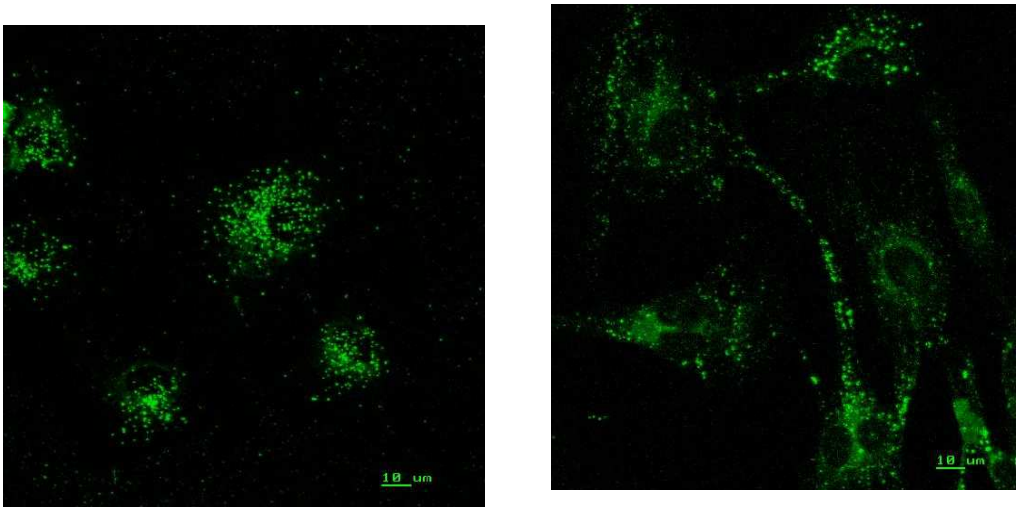

Fig. 2.8a. Immunofluorescent photomicrograph of human retinal (left) and choroidal (right) endothelial cells stained for vWf (63x original magnification). Note the predominance of staining within granules in the cell cytoplasm. While many microvascular ECs are stated not to have electron dense Weibel-Palade bodies, the traditional site of vWf storage, it is now known that vWf can also be stored in alternative intra-cellular vesicles. Analysis of gene expression data from chapter 3 reveals that both choroidal and retinal ECs show a significant expression of vWf.

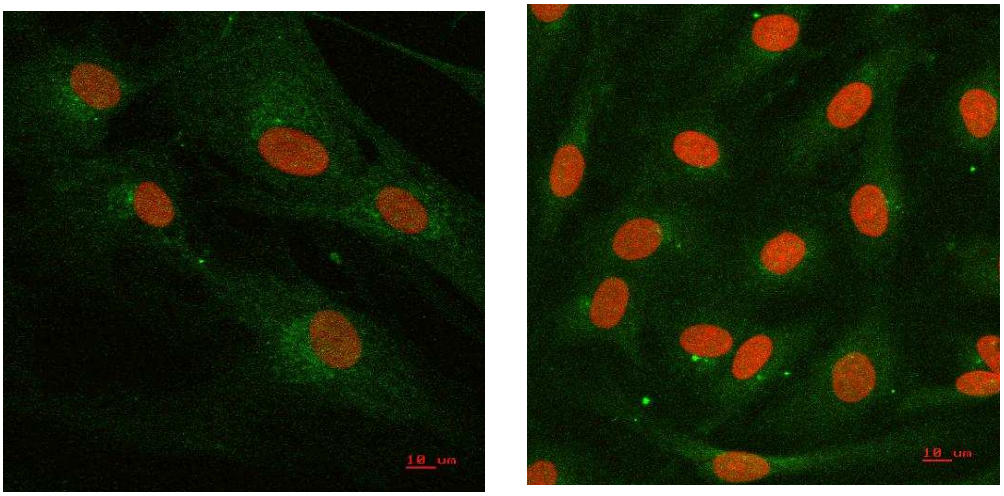

Fig. 2.8b. Immunofluorescent photomicrograph of human iris (left) and umbilical vein (right) endothelial cells stained for vWf (63x original magnification). Note the predominance of cytoplasmic granular staining consistent with the known location of vWf within ECs. Nuclei are stained red with propidium iodide to aim cell localisation.

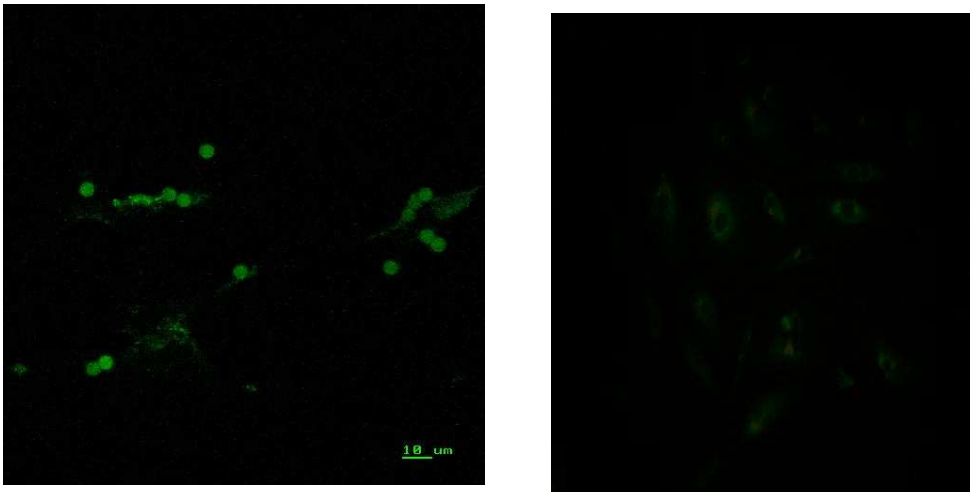

Fig. 2.9a. Immunofluorescent photomicrograph of human retinal (left) and choroidal (right) endothelial cells stained with a FITC labelled non-specific swine anti-rabbit immunoglobulin fraction as a negative control of the anti-vWf primary antibody. Note the absence of any staining of cells. Some very faint fluorescence from non specific binding can be seen as well as the natural fluorescence of Dynabeads (green circles)

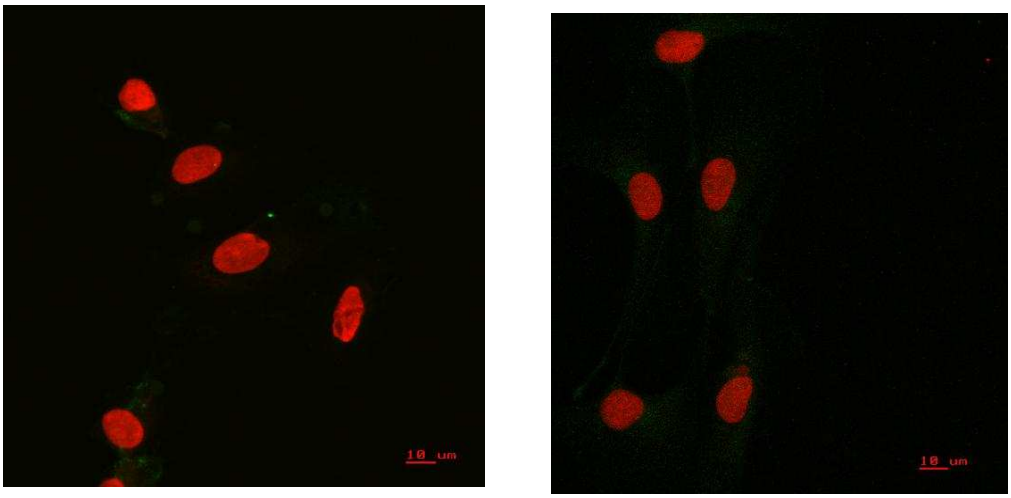

Fig. 2.9b. Immunofluorescent photomicrograph of human iris (left) and HUVEC (right) endothelial cells stained with a FITC labelled non-specific swine anti-rabbit immunoglobulin fraction as a negative control of the anti-vWf primary antibody. Note, the nuclei are stained red with propidium iodide to aid cell localisation.

Discussion

The work described in this chapter demonstrates that it has been possible to reliably isolate microvascular endothelial cells from the choroid, iris, and retina. Interestingly, to the naked eye, they appear similar with typical cobblestone appearances. Visual inspection also failed to show any evidence of Weibel-Palade bodies, often present in macrovascular ECs. These bodies are thought to be the site of vWf storage before release at the cell surface where it plays a role in blood coagulation. It can be seen in Fig 2.8 that numerous points of vWf staining are present in the choroidal EC cytoplasm. While these cells are thought to lack traditional WP bodies, they may store vWf in vesicles that lack the typical electron dense appearance on electron microscopy. It will be shown later that iris, retinal and choroidal ECs all express significant levels of vWf at a gene level when examined by gene microarray analysis, thereby confirming this immunohistochemical result.

The origin and purity of the cells was confirmed and they could therefore be used in any subsequent experiments. The purity of cells matches that from other studies and the reliability of the isolation procedure means that matched iris, retinal and choroidal ECs can be isolated and cultured from the same patients. This will be a major advantage in forthcoming experiments, particularly those utilising gene expression techniques, as it removes much of the variability between samples due to differences in gene expression between different donors e.g. due to their age or genetic “make up”. The technique also allowed sufficient cells from each donor to be cultured. Approximately 200,000 un-passaged endothelial cells could be cultured from a flask seeded with isolated ECs within 5-7 days. As will be seen later, this is enough to perform the experiments necessary to examine both gene expression profiles and to examine their in-vitro behaviour.

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Chapter 3

Comparative Gene Expression Profiling of HUVEC and Ocular Vascular Endothelial Cells

Introduction

Intraocular angiogenesis causes a number of common blinding conditions including wet age-related macular degeneration, proliferative diabetic retinopathy, retinopathy of prematurity and rubeotic glaucoma. The pathogenesis of these disorders is centred upon choroidal, retinal and iris microvascular endothelial cells respectively. Ocular vascular disorders also appear to demonstrate a preference for different microvascular beds and sometimes particular locations within a microvascular bed, e.g. diabetes preferentially affecting the retinal vasculature and age related macular degeneration affecting the choroidal circulation.

Investigators have attempted to increase the understanding of these disorders by studying non-ocular human endothelial cells derived from sites such as the umbilical vein (HUVEC) because of their availability in large numbers and their relative ease of culture. The conclusions drawn from studies using these cells have then been extrapolated to human angiogenic eye diseases. Stahl et al used the proliferation of HUVECs to study the effect of rapamycin on the production of VEGF by RPE cells in co-culture, thereby hypothesising that this drug may have a significant role in the treatment of choroidal neovascularisation (Stahl et al, 2008). Sakamoto et al used human RPE cells, transfected with retroviral vectors in culture with HUVECs, as a model for human ocular angiogenic diseases (Sakamoto et al, 1998), while Kumar (Kumar et al, 2008) also used a co-culture system, this time, of aortic endothelial cells with retinal cells in the investigation of growth factors thought to important in human ocular angiogenic diseases. Sengutpa et al used human lung microvascular ECs to study the effect of stromal-derived factor and extrapolated the results to the study of choroidal neovascularisation (Sengupta et al, 2010), while Hamilton used a co-culture system of HUVE cells and RPE cells, both grown on amniotic

membrane in order to mimic the choriocapillaris/Bruch's membrane/RPE complex found in human eyes (Hamilton et al, 2007).

This *in vitro* system was hypothesised to be a model to investigate the mechanisms of wet AMD. Shankar et al recently used HUVECs to determine the importance of PI3K/AKT pathways in angiogenesis, applying the results to a range of conditions such as proliferative diabetic retinopathy and choroidal neovascularisation (Shankar S, 2008), while Yang et al used HUVECs and RPE conditioned medium to study the pathogenesis of choroidal neovascularisation (Yang et al, 1993). Lastly, Kim et al recently used HUVECs to study the effects of heptanomide (an inhibitor of histone deacetylase) (Kim et al, 2009) and deguelin (Kim et al, 2008) as models for the potential treatment of wet AMD. Because HUVECs are termed macrovascular ECs and are derived from a specialised tissue, it is uncertain if they are representative of ocular microvascular ECs in general and therefore may not be appropriate for investigation of the mechanisms of ocular angiogenic diseases.

HUVECs have also been utilised in the investigation of angiogenic diseases elsewhere in the body. By association, other groups feel that these macrovascular cells offer results that can be applied locally to the blood vessels of the individual tissues of interest. These include melanoma-associated neovascularisation (Boyd et al, 2002; Mangiameli et al, 2007), brain glioblastomas (Chen et al, 2009; Martina et al, 2009), kidney (Ho et al, 2008) and the bone marrow (Cenni et al, 2009). It has been discussed previously in chapter 1, however, that *in vitro* responses of HUVEC cells often differ from those of microvascular origin, such as retinal and dermal ECs, thereby emphasising the degree of scepticism needed when interpreting the results of experiments using HUVECs.

In order to use endothelial cells that are phenotypically closer to those found in the human choroid, other workers have used ocular cells from non-human sources. Examples include the use of bovine choroidal ECs in the investigation of cell signalling pathways (VEGF, PLC $Ca^{2+}$and MAPK) thought to be important in choroidal angiogenesis. Bullard et al used bovine retinal ECs to investigate the importance of MAPK in retinal neovascularisation (Bullard et al, 2003), while Brylla et al used both bovine retinal and choroidal ECs to investigate the effect of hypoxia on angiogenesis (Brylla et al, 2003). The results of these experiments were thought to be applicable to retinal and choroidal neovascularisation in humans, but while the use of site-specific ECs is important, it is not known how close these bovine cells are phenotypically and genotypically to their human counterparts and how much inter species heterogeneity exists in important intracellular signalling pathways.

As detailed previously in chapter 1, it is now widely accepted that endothelial cells derived from different vascular organs and from within different vascular beds within those organs display phenotypic, biochemical and genetic heterogeneity. Any definitive insight into the pathogenesis and selective treatment of human angiogenic eye diseases is therefore likely to be gained only by conducting studies using microvascular endothelial cells derived from the particular ocular site(s) affected by the disease. Recently, Smith et al studied the expression of 8500 genes of human retinal and choroidal endothelial cells using DNA microarrays (Smith et al, 2007). They demonstrated distinct differences in expression profiles between the two cell types, particularly in those genes thought to be important in the immune response and leukocyte trafficking. This may explain why the retina appears to be susceptible to certain inflammatory diseases such as that caused by toxoplasmosis (Smith et al, 2004). Recently, Chi et al used DNA microarrays to explore the diversity of human ECs from different blood vessels (no ocular ECs were examined) (Chi et al, 2003). They found distinct differences in gene expression between macro- and

microvascular endothelial cells and between microvascular ECs from different vascular beds.

The aim of this section of work was to take the previously isolated, unpassaged, proliferating human umbilical vein cells and matched retinal, choroidal and iris endothelial cells and determine the degree of heterogeneity between the macrovascular and ocular microvascular ECs by microarray gene expression profiling. The difference in gene expression profiles was also determined between groups of different matched ocular microvascular ECs. Because of the previously stated interest in ocular angiogenic mechanisms, particular attention was paid to differences in those genes involved in vascular cell signalling.

Methods

Three sets of matched, un-passaged human choroidal, retinal and iris endothelial cells and HUVECs from 3 different donors were cultured to approximately 80% confluence on fibronectin-coated 35mm culture plates as described previously. The age and sex of the donors were: 47yrs male, 58yrs male and 63yrs female. The times from death to endothelial cell isolation were 49, 48 and 52 hours respectively.

RNA extraction

Total RNA was extracted from primary cultures of un-passaged endothelial cells when they had reached approximately 80% confluence, using the Qiagen RNeasy Minikit (Qiagen, Crawley, UK). This method has the advantage that RNA molecules of greater than 200 bases are enriched, but smaller RNA molecules (tRNAs and rRNAs) are selectively excluded.

To each culture plate was added 350µl of Buffer RLT (with β-mercaptoethanol added); this lead to almost instant lysis of the cells with release of RNA. The lysate was immediately pipetted into a microcentrifuge tube and vortexed for 60 seconds to ensure homogenisation. To the homogenised lysate was added an equal volume (350µl) of 70% ethanol and mixed well by pipetting. Each lysate was then added to the top of individual RNeasy columns and centrifuged at >8000g for 15 seconds. The flow-through was discarded and 700µl of Buffer RW1 was added to each column and again was centrifuged at >8000g for 15 seconds. The flow-through was again discarded. Next, 500µl of Buffer RPE was added to the column and centrifuged as before. Again, the flow-through was discarded. This step was repeated as previously, but the tube was centrifuged for 2 minutes to dry the RNeasy silica gel membrane in the column. To elute the RNA, 50µl of RNase-free water was added to the column and centrifuged at >8000g for 1 minute over a clean collection tube.

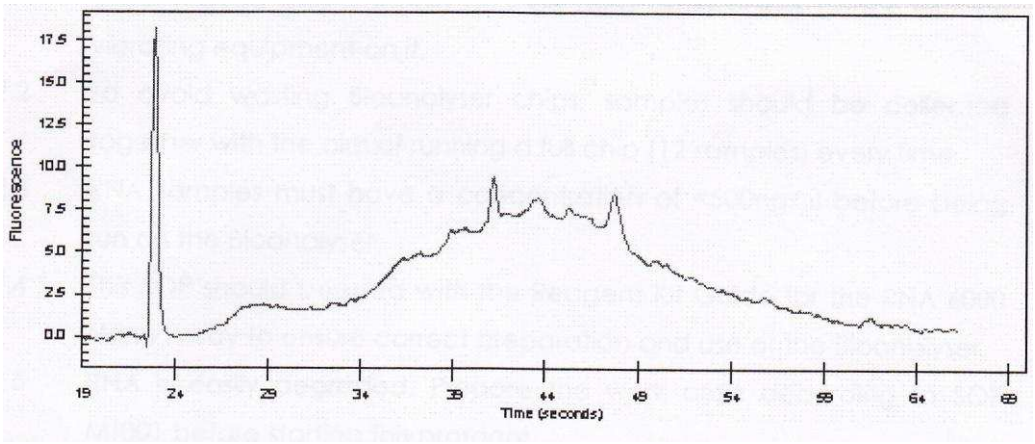
The RNA concentration was measured in each sample using a Nanodrop ND-1000 spectrophotometer before the eluate was transferred into microcentrifuge tubes and stored at -80℃ until used for subsequent analysis. It was found that approximately 5μg of total RNA was obtained from each 35mm culture plate.

Microarray analysis

The previously stored RNA was thawed and the RNA concentration re-checked using the Nanodrop ND-1000 spectrophotometer. The RNA integrity and quality was assessed using an Agilent 2100 Bioanalyser and RNA 6000 Nano kit (Agilent Technologies). Briefly, 1μl aliquots of each RNA sample were taken from the stock samples and diluted, if necessary, to give a concentration of less than 500ng/μl and then stored on ice. The stock sample was immediately returned to the -80℃ freezer. Prior to use, the Agilent 2100 bioanalyser electrode was cleaned with RNasezap by following the manufacturer's instructions. Next, preparation was made for the chip priming station. A new syringe was inserted into the station and 550μl of gel matrix was centrifuged through a spin filter for 10 minutes at 1500g. To a 65μl aliquot of filtered gel was added 1μl of well mixed dye concentrate. After mixing, the gel-dye mixture was centrifuged for 10 minutes at 13000g. A new RNA 6000 nanochip was inserted into the chip priming station and 9μl of the gel-dye mix was pipetted into well "**G"**. Next, the chip priming station was activated and the plunger was released exactly 30 seconds after activation. The plunger was then pulled back to the 1ml position. The chip priming station was then opened and 9μl of gel-dye mix was pipetted into all wells marked "G".

Next, 5μl of the Nano marker was pipetted into the ladder well and the 12 samples wells. Any unused wells had an extra 1μl of marker added so that the volume in all the wells was the same. Next, 1μl of heat-denatured RNA ladder solution was pipetted into the ladder well.

All of the labelled test samples were then heat-denatured at 70°C for 2 minutes using a heat block and 1µl aliquots of each sample were pipetted into the relevant sample well. The chip was then vortex mixed for 1 minute in an IKA vortexer at 2400rpm. The chip was then loaded onto the Agilent 2100 Bioanalyser, the lid closed and the run started (takes 25 minutes). The Agilent 2100 expert software was used to analyse the electropherogram. First, the ladder data was viewed to assess the function of the chip. If this was satisfactory each individual sample was viewed to check for the presence of 2 distinct peaks (rRNA) and a normal distribution curve representing mRNA (see example below).



An Agilant electrophoretogram of biotinylated cRNA probes for microarray analysis

The Affymetrix microarray chips require the use of biotinylated cRNA probes to be generated from the total RNA samples. Briefly, cDNA is synthesised form the total RNA using reverse transcriptase. The cDNA then undergoes second strand synthesis using DNA polymerase and clean up. The dsDNA is then amplified by PCR, to become a template for *in vitro* transcription with RNA polymerase to produce cRNA. This is then labelled with biotin for microarray analysis. During the last stage, amplification of the original RNA template occurs.

First strand cDNA synthesis

First strand cDNA synthesis was conducted using the Roche Applied Sciences Microarray Target Amplification Kit. Total RNA samples (1µg) from samples that had passed Agilent quality control were pipetted into 0.5 ml microfuge tubes and 2µl of T7 oligo Target Amplification Sequence (TAS) primer added. Nuclease-free water was added to bring the final volume up to 10.5µl. After mixing, the samples were incubated for 10 minutes at 70°C in a thermal cycler. To each sample was then added 4µl of reverse transcriptase buffer, 2µl of dNTP mix, 2µl of dithiothreitol (DTT), and 1.5 µl of reverse transcriptase enzyme mix. This mixture was then incubated at 42°C for 1 hour. The samples were then incubated for 5 minutes at 95°C to denature RNA/DNA hybrids. After this, the samples (cDNA) were briefly centrifuged and placed on ice.

Second strand cDNA synthesis

Second strand cDNA synthesis was conducted by adding the following to the cDNA samples: 13.5 µl of nuclease free water, 5µl of Target Amplification Sequence (TAS) – (dN) 10 primer, 5µl of Klenow reaction buffer, 2.5µl of dNTP mix, 4µl of Klenow enzyme (DNA polymerase). After mixing, the samples were incubated for 30 minutes at 37 °C.

Double stranded cDNA purification

The ds-cDNA was then purified using a Roche Microarray Target Purification Kit (Roche). This relies on the binding of nucleic acids to a glass fibre fleece (in filter cartridge) in the presence of ethanol. The filter is then subjected to a number of rapid wash/spin steps to inactivate and wash out RNases and other enzymes. The nucleic acids are then eluted by a low salt solution. Briefly, 1.25 µl of carrier RNA and 50µl of nuclease-free water are added to each sample. Next, 400µl of cDNA

binding buffer (with β-mercaptoethanol added) and 200µl of ethanol were added to the ds-cDNA samples, mixed and pipetted onto the centre of a cDNA filter cartridge placed in a wash tube. This was centrifuged at 6000g for 15 seconds and the eluate discarded. Five hundred microlitres of cDNA wash buffer was added to the top of the cartridge and centrifuged again, as described previously. A further 300µl of wash buffer were added and the cartridge centrifuged at 13000g for 1 minute. The cartridge was then placed into an elution tube and 50µl of elution buffer B added to the centre of the cartridge. This was left at room temperature for 1 minute, and then centrifuged at 6000g for 90 seconds. The eluate was placed on ice.

cDNA amplification by PCR

To 12.5µl of purified cDNA were added: 1µl of TAS primer, 2µl of dNTP mix, 73µl of nuclease free water, 10µl of expand PCR buffer and 1.5µl of expand enzyme mix. The mixture was run on a thermocycler for 27 cycles. The resulting amplified cDNA was purified again using the protocol as described previously. The concentration of DNA was measured using the Nanodrop ND-1000 spectrophotometer. The samples were placed on ice for further use.

Synthesis of cRNA

To synthesise cRNA from the purified ds-cDNA, the Roche Microarray Target RNA Target Synthesis Kit was used (Roche Applied Sciences). Briefly, 200ng of ds-cDNA were mixed with: 4µl of NTP mix, 2.5µl of biotin 16-UTP, 2µl of DTT, 2µl of transcription buffer, 3 µl transcription enzyme blend and the required amount of nuclease-free water to make the entire volume up to 20µl. The mixture was incubated at 37℃ for 3 hours. The final RNA concentration was measured and individual samples were subjected to bioanalysis (Agilent 2100 Bioanalyser RNA 6000 nanoassay) using the protocol described previously to ensure good transcription. The labelled cRNA samples were then fragmented by mixing 20µg (in 32µl) of target cRNA with 8µl of Ambion fragmentation buffer and incubating them at 94℃ for 35 minutes.

Microarray hybridisation

The arrays used in this experiment were Affymetrix GeneChip ® Human Genome U133 Plus 2.0 arrays (Affymetrix, High Wycombe, Bucks, UK). This array offers complete coverage of the human genome plus 6500 additional genes. In reality this offers the potential for analysis of 47,000 different transcripts. The following hybridisation cocktail was prepared for each sample: 15µg of fragmented cRNA, 5µl

of control oligonucleotide B2, 15µl of 20X eukaryotic hybridisation controls, 3µl of Herring sperm DNA, 3µl of acetylated bovine serum albumin, 30µl of DMSO, 150µl 2X hybridisation buffer and sufficient RNase-free water to make a final volume of 300µl. The cocktails were heated to 99°C for 5 minutes then to 45° C for 5 minutes. The arrays were removed from their packaging and filled with 1X hybridisation buffer. The arrays were then incubated for 10 minutes at 45°C while rotating. The buffer solution was removed from the arrays and replaced with 200µl of hybridisation cocktail which was then incubated for 16 hours at 45°C while rotating at 45-60 rpm.

After hybridisation, the cocktails were removed and stored at -80°C in case they were needed later. The Fluidics Station (Affymetrix Fluidics Station 450) required for staining and washing the arrays is prepared as are the SAPE (Strepavidin r-phycoerythrin conjugate) and biotinylated anti-streptavidin antibody solutions. Each individual array is washed and stained and stored in the dark to await reading by array scanner (Affymetrix Scanner 3000). The GCOS software (Affymetrix) was used to monitor scanning and to convert the raw image files into cell intensity files ('.CEL').

Data Analysis

Affymetrix CEL files were imported into GeneSpring GX 11.0.1 and processed with the MAS5 algorithm to generate PMA flags. Probesets were excluded from further analysis if there was not a Present or Marginal flag in 100% of the samples in one of the four cell groups. Affymetrix control probesets were also excluded to leave a probeset list for subsequent analysis. Data were then normalised with GC-RMA to provide expression values. To identify differentially expressed genes between cell groups, ANOVA was performed on this probeset list with Tukey-HSD post-hoc testing and Benjamini-Hochberg false discovery rate control. A difference in expression between probesets with a

corrected p-value of <0.05 and a fold change of greater than 2 were considered to be statistically significant. Data were exported directly from GeneSpring 11.0.1. to Ingenuity Pathway Analysis for these probesets. Heatmaps were drawn using dChip (Harvard University, Boston, USA) (Li and Wong 2001). Pie charts showing the breakdown of up regulated probesets with regard to biological processes were constructed using Panther microarray data analysis software (www.panther.org).

Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. Molecules from the data set that met the cut off criteria and were associated with a canonical pathway in Ingenuity's Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways:

1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed.

2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Real time PCR

Expression data from the microarray experiments were validated by TaqMan real time PCR (ABI). This method exploits the 5' nuclease activity of DNA polymerase to cleave a TaqMan probe during the PCR reaction. The TaqMan probe contains a reporter dye at the 5' end of the probe and a quencher at the 3' end. During the reaction, cleavage of the probe separates the reporter and quencher dye which results in

increased fluorescence of the reporter. Accumulation of the PCR product is detected by monitoring the increase in fluorescence of the reporter dye. Nine transcripts were chosen that demonstrated at least a 2-fold differential expression between ECs from various sources on microarray analysis and that were thought to be relevant to a range of endothelial cellular functions. The chosen transcripts were: Angiopoietin 2, Keratin 18, CD44, CD73 (5 nucleotidase ecto), MAPK 3, Integrin α3, Laminin β2, Decay Accelerating Factor and Carboxypeptidase E. For analysis, cDNA from each of the matched donor samples was generated from total RNA using the Superscript III first strand synthesis system (Invitrogen). Fifty nanograms of RNA from each sample to be assayed were mixed with 1µl of primer and 1µl of dNTP and made up to 10µl with RNase free water. The mixture was incubated at 65°C for 5 minutes then cooled on ice. To each cooled sample, 10µl of synthesis mix was added (composed of 10X RT buffer, $MgCl_2$, DTT, RNaseOUT and Superscript III reverse transcriptase). The mixture was incubated at 25°C for 10 minutes followed by 50 minutes at 50°C. The reaction was terminated by heating the mixtures at 85°C for 5 minutes. After adding 1µl of RNase H and incubating for 20 minutes at 37°C, the samples were stored at -20°C unless they were to be used immediately for further analysis. The resulting cDNA was subjected to real time PCR reaction using the manufacturer's TaqMan Universal Mastermix Kit protocol. Into each well of a 96 well MicroAmp Optical reaction plate, 1µl of cDNA (concentration between 1-100ng/µl) and 19µl of reaction mixture were mixed together. The reaction mixture consisted of the TaqMan probe for the gene of interest or the house keeper gene, nuclease free water and the PCR mastermix. Three replicates were run for each gene of interest. Control samples, consisting of either water or a pooled cDNA mix were used. The 96 well plates were loaded onto the ABI PRISM 7000 sequence detection system and run for 45 cycles. The expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) was chosen for normalisation. Analysis of the relative gene expression data was performed using the $\Delta\Delta$Ct method.

This is represented as follows:

In the TaqMan system, signal (fluorescence) increases with time in direct proportion to the number of starting molecules (mRNA). The accumulation of reaction product (or signal) increases in a sigmoid fashion. The crossing point or threshold (Ct) occurs at the point threshold at which fluorescence can be detected and the Ct is inversely proportional to the initial concentration of the target in the sample. The Ct is usually expressed as a cycle number and can automatically measured by the instrument. The Ct for each target gene can be compared to a house keeper gene for comparative or fold-change analysis.

The calculation of fold change from a Ct value is as follows:

Ct (gene of interest) – Ct (housekeeping gene) = ΔCt

ΔCt (sample of interest i.e. choroid gene) - ΔCt (comparator sample i.e. HUVEC) = ΔΔCt

Fold change = $2^{-\Delta\Delta Ct}$

Results

Confirmation of human endothelial cell identity

Samples of cells from all locations displayed homogenous cobblestone morphology with no evidence of cell contamination. Greater than 99.5% of the endothelial cells from each site demonstrated staining for factor VIII and CD31 prior to their use in the aforementioned experiments, confirming their purity and identity as endothelial cells.

Overview of gene expression patterns

Between 1.5 and 3.4 µg of total RNA were isolated from each 35mm plate. Biotinylated cRNA probes were hybridised to Affymetrix GeneChip ® Human Genome U133 Plus 2.0 arrays containing 47000 transcripts, of which 38500 were well characterised human genes. A total of 26312 probe sets passed quality control testing during analysis.

Gene expression patterns of proliferating HUVEC vs ocular microvascular endothelial cells

When HUVE cells were compared to ocular microvascular cells, 802 probesets demonstrated a significant difference. Within this total, 383 probesets were found to be up-regulated in HUVECs and 419 down-regulated. The probesets for up-regulated genes in HUVECs were enriched with genes important in embryonic development such as the homeobox genes HOX B7, B5, A2, A4, A9 and D8, neuroregulin 1 and osteonectin. Probe sets for ocular microvascular endothelial cells appeared to be enriched in genes important in MHC class I (A – C, F and G) and II (alpha and beta chains), immune responses (Interleukin 6 receptor, CXCL 12 and FAS), signal transduction (EGF receptor, G protein receptors, phospholipase C) and cell response to stimulus (TIMP3, collagen types I and III, laminin beta). Tables 3.1 and 3.2

indicate the 100 most highly expressed probesets in HUVECs and ocular MVECs respectively.

Comparison of HUVECs with different ocular microvascular endothelial cells demonstrated a difference of 2146 probesets (8.9% of probesets that passed quality control) when HUVECs were compared with choroidal ECs (1100 up- and 1046 down-regulated), 1801 probesets (6.8%) when HUVE cells were compared with retinal ECs (797 up- and 1004 down- regulated) and 2325 probesets (8.8%) when HUVE cells were compared with iris ECs (1205 up- and 1120 down-regulated). The raw data is available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nfcplwewowqowpk&acc=GSE20986.

Fig 3.1,shows the heatmap of differential gene expression for those probesets reaching statistical significance. It can be seen that ECs from choroid and iris share similar gene expression profiles, whereas HUVEC and retinal ECs have distinct identities. The positions of important, differentially expressed genes are indicated at the side.

Examination of fig 3.2a and b pie charts shows that HUVE cells demonstrate a greater proportion of up-regulated genes for developmental processes (12% vs 8 %) and metabolic processes (27% vs 16%) compared with ocular MVECs, while ocular MVEC demonstrated a greater proportion of probesets up-regulated for immune function (9% vs 5%) and cell adhesion (5% vs 2%) compared with HUVECs.

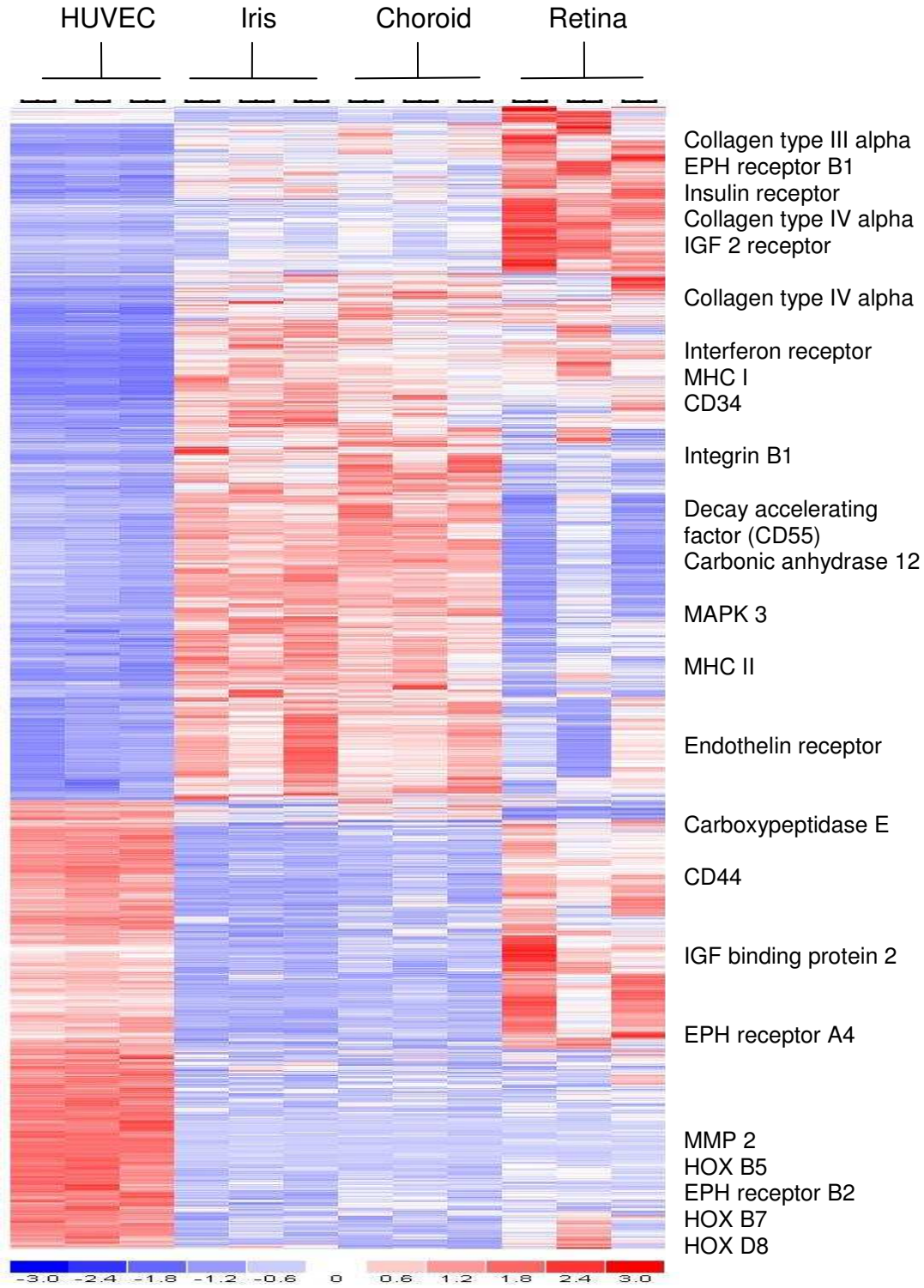


Fig 3.1 Heatmap of ascending differential probeset expression reaching statistical significance with reference to HUVEC and different human ocular microvascular endothelial cells. Upregulated probesets are shown in red, downregulated in blue. A total of 2146 different probesets are represented. The positions of selected probesets thought to be important in endothelial cell biology are shown.

Table 3.1 One hundred most highly expressed probesets in HUVECs compared with ocular microvascular endothelial cells

Gene Title	Fold Change
homeobox A9	384.0
homeobox A9	244.0
regulator of G-protein signalling 5	201.0
homeobox B7	163.0
homeobox B7	145.0
interleukin 1 receptor-like 1	131.0
regulator of G-protein signalling 5	128.0
aldehyde dehydrogenase 1 family, member A2	123.0
cyclin A1	99.0
regulator of G-protein signalling 5	92.0
regulator of G-protein signalling 5	86.0
gamma-aminobutyric acid (GABA) B receptor, 2	80.0
ST2 protein	76.0
serpin peptidase inhibitor, clade D (heparin cofactor), member 1	76.0
chromosome 4 open reading frame 49	66.0
homeobox A5	60.0
hypothetical gene supported by BC013438	48.0
T cell receptor beta constant 1 /// T cell receptor beta constant 2	48.0
growth factor receptor-bound protein 14	35.0
G protein-coupled receptor 37 (endothelin receptor type B-like)	33.0
sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	32.0
kelch-like 13 (Drosophila)	32.0
gamma-aminobutyric acid (GABA) B receptor, 2	32.0
GATA binding protein 3	32.0
family with sequence similarity 101, member A	32.0
WD repeat domain 69	30.0
EPH receptor B2	29.0
erythrocyte membrane protein band 4.1-like 3	29.0
homeobox A7	27.0
heparanase	27.0
fermitin family homolog 3 (Drosophila)	26.0
ALX homeobox 1	26.0
EPH receptor B2	25.0
TRAF2 and NCK interacting kinase	25.0
ST2 protein	24.0
prickle homolog 1 (Drosophila)	24.0
erythrocyte membrane protein band 4.1-like 3	24.0
placenta-specific 8	23.0
gamma-aminobutyric acid (GABA) B receptor, 2	23.0
homeobox B3	23.0

Gene Title	Fold Change
collectin sub-family member 12	22.0
gamma-aminobutyric acid (GABA) B receptor, 2	22.0
homeobox A10	21.0
BMX non-receptor tyrosine kinase	21.0
erythrocyte membrane protein band 4.1-like 3	20.0
globoside alpha-1,3-N-acetylgalactosaminyltransferase 1	20.0
TRAF2 and NCK interacting kinase	19.3
TRAF2 and NCK interacting kinase	18.5
insulin-like growth factor 2 mRNA binding protein 1	18.4
G protein-coupled receptor 143	17.4
EPH receptor B2	17.1
chromosome 6 open reading frame 142	17.0
phosphatidic acid phosphatase type 2 domain containing 1A	16.7
neuroligin 1	16.4
caspase recruitment domain family, member 11	16.3
T cell receptor beta constant 1	15.9
fibrillin 2	15.7
neuregulin 1	15.4
fibronectin type III and SPRY domain containing 1	15.0
insulin-like growth factor 2 mRNA binding protein 1	15.0
neuronatin	14.8
transmembrane protein 163	14.5
Full length insert cDNA clone YW19A06	13.8
E2F transcription factor 8	13.8
thiosulfate sulfurtransferase KAT, putative /// KAT protein	13.7
transmembrane protein 163	13.3
homeobox A3	13.0
paraneoplastic antigen MA2	12.9
protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	12.6
solute carrier family 47, member 1	12.4
prickle homolog 1 (Drosophila)	12.2
keratin 80	12.1
protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	12.0
homeobox D1	11.8
mal, T-cell differentiation protein 2	11.7
follistatin-like 5	11.5
hypothetical LOC401022	11.1
G protein-coupled receptor, family C, group 5, member A	11.0
tumor necrosis factor (ligand) superfamily, member 15	10.8
galanin prepropeptide	10.2
EPH receptor B2	10.2
glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	10.1
Prickle homolog 1 (Drosophila) (PRICKLE1), mRNA	10.1

Gene Title	Fold Change
GATA binding protein 3	9.7
heparanase	9.7
homeobox B2	9.1
protocadherin 7	9.1
neuregulin 1	9.0
family with sequence similarity 174, member B	8.6
runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	8.5
TAF7-like RNA polymerase II,	8.5
PRP31 pre-mRNA processing factor 31 homolog (S. cerevisiae)	8.3
stearoyl-CoA desaturase (delta-9-desaturase)	8.3
FERM domain containing 5	8.2
keratin 15	8.1
homeobox B8	8.1

Table 3.2 One hundred most highly expressed probesets in ocular microvascular endothelial cells compared with HUVE cells

Gene Title	Fold change
collagen, type I, alpha 1	736.0
major histocompatibility complex, class II, DR beta 1	451.0
collagen, type I, alpha 1	422.0
deoxyribonuclease I-like 3	381.0
collagen, type III, alpha 1	222.0
periostin, osteoblast specific factor	212.0
major histocompatibility complex, class II, DR beta 1	202.0
collagen, type VI, alpha 3	182.0
collagen, type I, alpha 2	181.0
cytochrome P450, family 1, subfamily B, polypeptide 1	171.0
collagen, type I, alpha 2	162.0
collagen, type III, alpha 1	156.0
cytochrome P450, family 1, subfamily B, polypeptide 1	156.0
major histocompatibility complex, class II, DP beta 1	149.0
major histocompatibility complex, class II, DP alpha 1	145.0
major histocompatibility complex, class II, DR beta 1	91.0
chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	90.0
paired related homeobox 1	90.0
alpha-2-macroglobulin	85.0
interleukin 13 receptor, alpha 2	80.0
ATP-binding cassette, sub-family B (MDR/TAP), member 1	77.0
selenoprotein P, plasma, 1	71.0
desmoplakin	70.0
TIMP metallopeptidase inhibitor 3	66.0
transgelin	66.0
MHC class II HLA-DRB3 mRNA (HLA-DRB3*01012 allele)	61.0
Rho GTPase activating protein 25	58.0
chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	54.0
SH3 and cysteine rich domain	53.0
major histocompatibility complex, class II, DP alpha 1	47.0
aggrecan	47.0
insulin-like growth factor binding protein 5	45.0
collagen, type XV, alpha 1	44.0
TIMP metallopeptidase inhibitor 3	42.0
hydroxysteroid (17-beta) dehydrogenase 2	42.0
doublecortin-like kinase 1	41.0
periostin, osteoblast specific factor	40.0
cytochrome P450, family 1, subfamily B, polypeptide 1	40.0
synaptopodin 2	38.0
acyl-CoA synthetase long-chain family member 5	38,0
Thy-1 cell surface antigen	37.0
kynureninase (L-kynurenine hydrolase)	35.0

Gene Title	Fold change
Rho GTPase activating protein 25	34.0
hypothetical protein FLJ22662	34.0
suppressor of cytokine signaling 2	33.0
aggrecan	32.0
sushi domain containing 2	31.0
tumor necrosis factor receptor superfamily, member 11b	31.0
GTPase, IMAP family member 5	27.0
filamin A interacting protein 1	27.0
carbonic anhydrase XII	26.0
tenascin C	26.0
crystallin, alpha B	26.0
transgelin	25.0
acyl-CoA synthetase long-chain family member 5	25.0
lymphocyte cytosolic protein 1 (L-plastin)	25.0
insulin-like growth factor binding protein 3	25.0
versican	24.0
aggrecan	24.0
lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	23.0
Ras protein-specific guanine nucleotide-releasing factor 2	23.0
family with sequence similarity 46, member A	23.0
hyaluronan synthase 2	23.0
guanine nucleotide binding protein (G protein), alpha 14	22.0
GTPase, IMAP family member 5	22.0
gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	22.0
fibroblast activation protein, alpha	22.0
mannose receptor, C type 1 /// mannose receptor, C type 1-like 1	20.0
CDNA clone IMAGE:4826696	20.0
phosphotriesterase related	20.0
syndecan 2	20.0
dipeptidyl-peptidase 4	20.0
G protein-coupled receptor 116	19.6
syndecan 2	19.5
chromosome 10 open reading frame 128	19.2
G protein-coupled receptor, family C, group 5, member B	19.0
sprouty homolog 1, antagonist of FGF signaling (Drosophila)	19.0
fibroblast growth factor 13	18.3
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4	18.1
snail homolog 2 (Drosophila)	18.0
NLR family, CARD domain containing 3	17.7
zinc finger protein 415	17.0
CD248 molecule, endosialin	17.0
cysteine-rich protein 1 (intestinal)	16.8

Gene Title	Fold change
ependymin related protein 1 (zebrafish)	16.8
versican	16.8
carbonic anhydrase II	16.4
actin, alpha 2, smooth muscle, aorta	16.3
lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	16.2
suppressor of cytokine signaling 2	16.1

Fig 3.2a. Pie chart of significantly upregulated genes grouped by biological processes in proliferating HUVECs compared with proliferating ocular microvascular endothelial cells

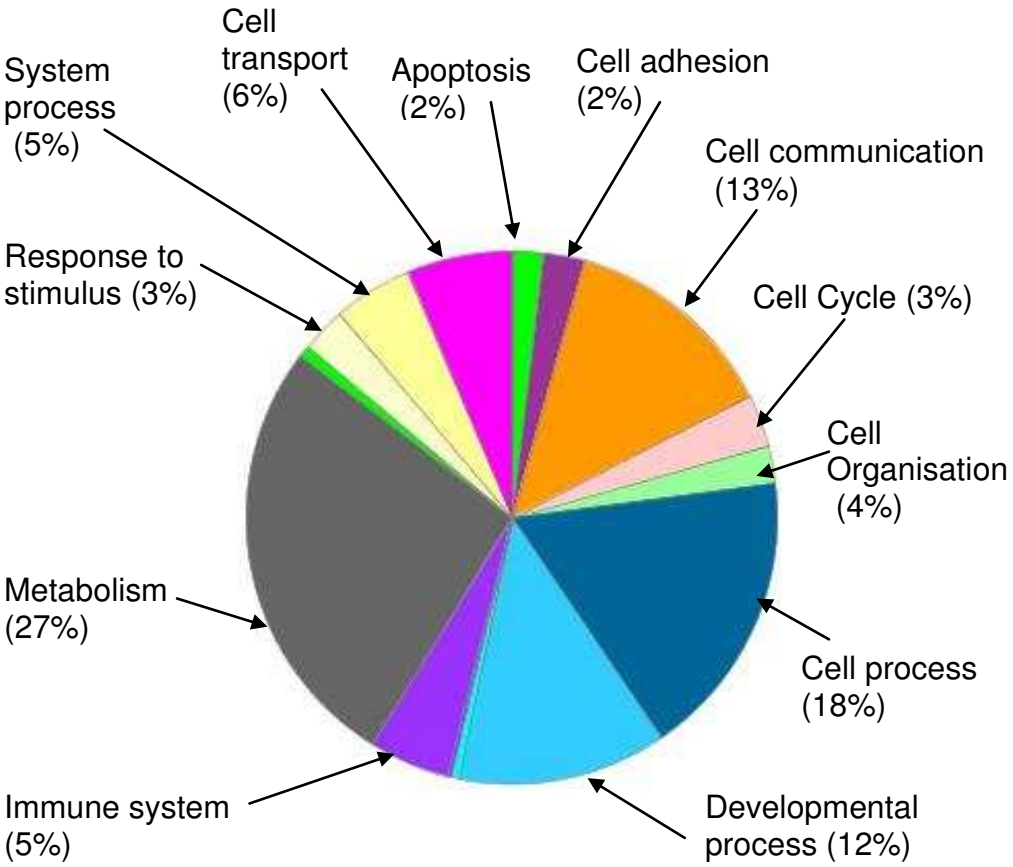
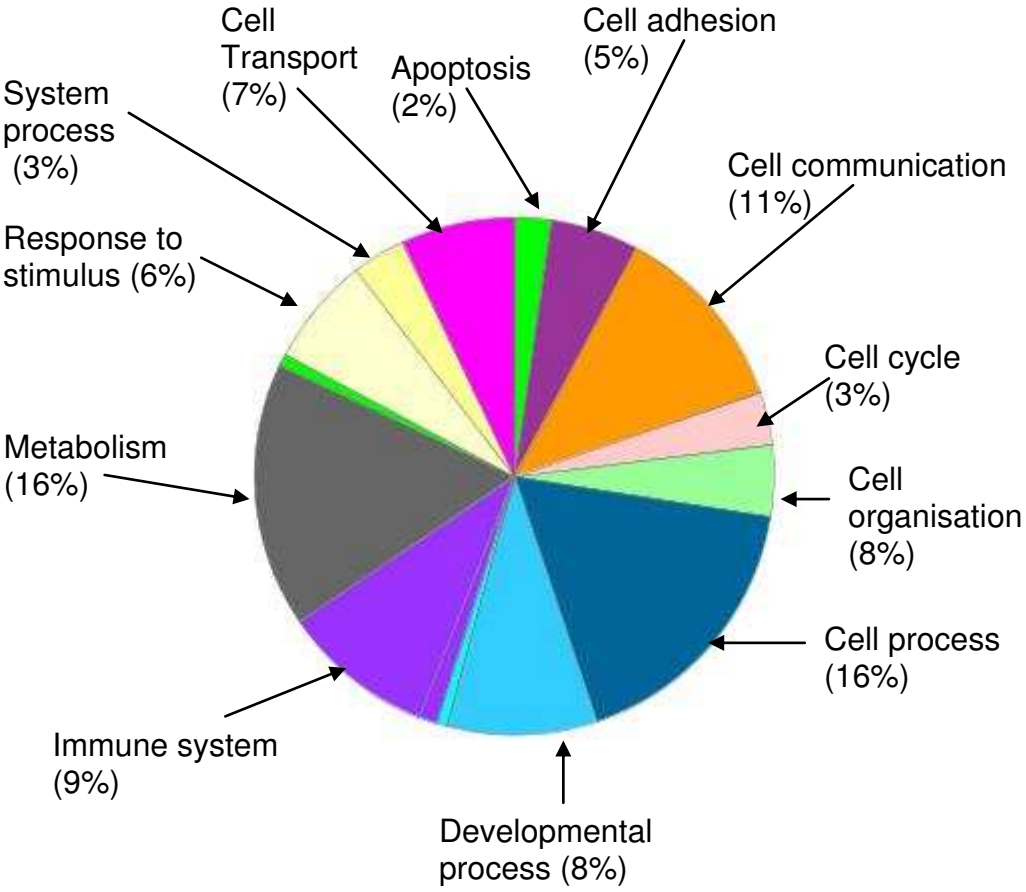


Fig 3.2b. Pie chart of significantly upregulated genes grouped by biological processes in proliferating ocular microvascular endothelial cells compared with proliferating HUVECs.



Proliferating human choroidal versus retinal vascular endothelial cells

Comparison of human choroidal endothelial cells with human retinal endothelial cells demonstrated a difference of 2217 probe sets (8.4% of probesets that passed quality control). Of this total, 1078 were up-regulated in choroidal ECs and 1139 were up-regulated in retinal ECs. Using Ingenuity Pathway Analysis software, differences between retinal and choroidal ECs were shown in the expression of genes involved in a wide range of biological processes such as cell cycle, DNA replication, cell morphology, vascular cell-to-cell interactions, cell movement and gene expression. Because of the enormity of the gene expression data sets, it is not possible to show it in its entirety in this thesis. Tables 3.4 and 3.5 show the top 100 differentially expressed genes for each EC subtype. The complete data set (raw and post analysis) can be obtained from the University of Nottingham, Division of Ophthalmology and Visual Sciences academic secretary. The raw data is also available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nfcplwewowqowpk&acc=GSE20986.

Table 3.3. Differential expression of probe sets involved in cell signalling between matched human choroidal and retinal endothelial cells

Signalling Pathway	Affy ID	Gene Symbol	Gene Name	Fold Change	Direction of regulation (retina vs choroid)
IGF-1 signalling	201163_s_at	IGFBP7	Insulin like growth factor binding protein 7	4.1	Up
	204686_at	IRS1	Insulin receptor substrate 1	6.4	Up
	225330_at	IGF1R	Insulin like growth factor 1 receptor	3.0	Up
VEGF signalling	218488_at	EIF2B3	Eukaryotic translation initiation factor 2B, subunit 3	2.7	Down
	212351_at	EIF2B5	Eukaryotic translation initiation factor 2B, subunit 5	2.0	Down
	200989_at	HIF1A	Hypoxia inducible factor 1 alpha subunit	2.2	Up
	208351_s_at	MAPK1	Mitogen-activated protein kinase 1	2.1	Down
	1553694_a_at	PIK3C2A	Phosphoinositide 3 kinase class 2 alpha	7.9	Down
	210512_s_at	VEGFA	Vascular endothelial growth factor A	8.7	Up
TOR	210949_s_at	EIF3C	Eukaryotic translation initiation factor 3 (subunit C)	2.7	Down
	211937_at	EIF4B	Eukaryotic translation initiation factor 4B	2.0	Down
	208624_s_at	EIF4G1	Eukaryotic translation initiation factor 4 gamma	4.6	Down

Signalling Pathway	Affy ID	Gene Symbol	Gene Name	Fold Change	Direction of regulation (retina vs choroid)
	200989_at	HIF1A	Hypoxia inducible factor 1 alpha subunit	2.2	Up
	204686_at	IRS1	Insulin receptor substrate 1	6.4	Up
	226312_at	RICTOR	RPTOR independent companion of mTOR	2.9	Up
	211578_s_at	RPS6KB1	Ribosomal protein S6 kinase (70kDa)	7.1	Down
PI3K/AKT	211968_s_at	HSP90AA1	Heat shock protein alpha, class A	3.0	Down
	1557910_at	HSP90AB1	Heat shock protein alpha, class B	4.4	Down
	201474_s_at	ITGA3	Integrin alpha 3 (CD49C)	4.4	Up
	1552610_a_at	JAK1	Janus kinase 1	4.7	Down
	212240_s_at	PIK3R1	Phosphoinositide 3 kinase subunit 1	2.2	Down
PI3K/AKT	207749_s_a	PPP2R3A	Protein phosphatase 2 subunit B	2.2	Down
ERK/MAPK	201474_s_at	ITGA3	Integrin alpha 3 (CD49C)	4.4	Up
	201841_s_at	HSPB1	Heat shock protein 1 (27kDa)	3.3	Up
	208351_s_at	MAPK1	Mitogen-activated protein kinase 1	2.1	Down

Signalling Pathway	Affy ID	Gene Symbol	Gene Name	Fold Change	Direction of regulation (retina vs choroid)
ERK/MAPK	1553694_a_at	PIK3C2A	Phosphoinositide-3-kinase, class 2, alpha	7.9	Down
	209785_s_at	PLA2G4C	Phospholipase A2, (group IVC)	3.2	Up
	207821_s_at	PTK2	PTK2 protein tyrosine kinase 2	2.4	Down
	201213_at	PPP1R7	Protein phosphatase 1, subunit 7	2.5	Down
	204284_at	PPP1R3C	Protein phosphatase 1, subunit 3C	3.4	Up
T Cell Receptor	215092_s_at	NFAT5	Nuclear factor of activated T-cells 5	6.7	Down
	201502_s_at	NFKBIA	Nuclear factor of kappa light polypeptide alpha	3.4	Up
	205263_at	BCL10	B-cell CLL/lymphoma 10	3.4	Up
Interferon	209417_s_at	IFI35	Interferon-induced protein 35	2.3	Up
	214022_s_at	IFITM1	Interferon induced transmembrane protein 1	6.3	Up
	201642_at	IFNGR2	Interferon gamma receptor 2	2.9	Up
IL4	203233_at	IL4R	interleukin 4 receptor	2.6	Up

Signalling Pathway	Affy ID	Gene Symbol	Gene Name	Fold Change	Direction of regulation (retina vs choroid)
Wnt	213425_at	WNT5A	Wingless type family	5.2	up
	210220_at	FZD2	Frizzeled homologue 2	10.3	up
	200816_s_at	PAFAH1B	Platelet Activating Factor Acetaldehyde	2.0	up

Fig 3.3 shows the heatmap of differential gene expression for all probesets reaching statistical significance. It can be seen that there is a wide disparity between retinal and choroidal ECs. The positioning of some of the important, differentially expressed genes is indicated at the side.

Figure 3.4a and 3.4b pie charts demonstrate a greater proportion of up-regulated probesets for cell communication processes in retinal ECs (13% vs 8%) when compared with choroidal ECs. All other biological process subsets demonstrated similar proportions of expression in both retinal and choroidal ECs.

Canonical pathway analysis revealed collections of probe sets from a number of signalling pathways that were differentially expressed including: ERK/MAPK, mTOR, VEGF, insulin like growth factor 1 (IGF 1), PI3K/AKT, T cell receptor, and IL4 signalling pathways (Table 3.3). Interestingly, this shows that proliferating retinal ECs demonstrate up-regulation of components of IGF 1 signalling such as IGF binding protein 7 and IGF receptor compared with choroidal ECs. Retinal ECs also demonstrate up-regulation of HIF 1alpha, VEGF and some immune related signalling pathways compared with choroidal ECs.

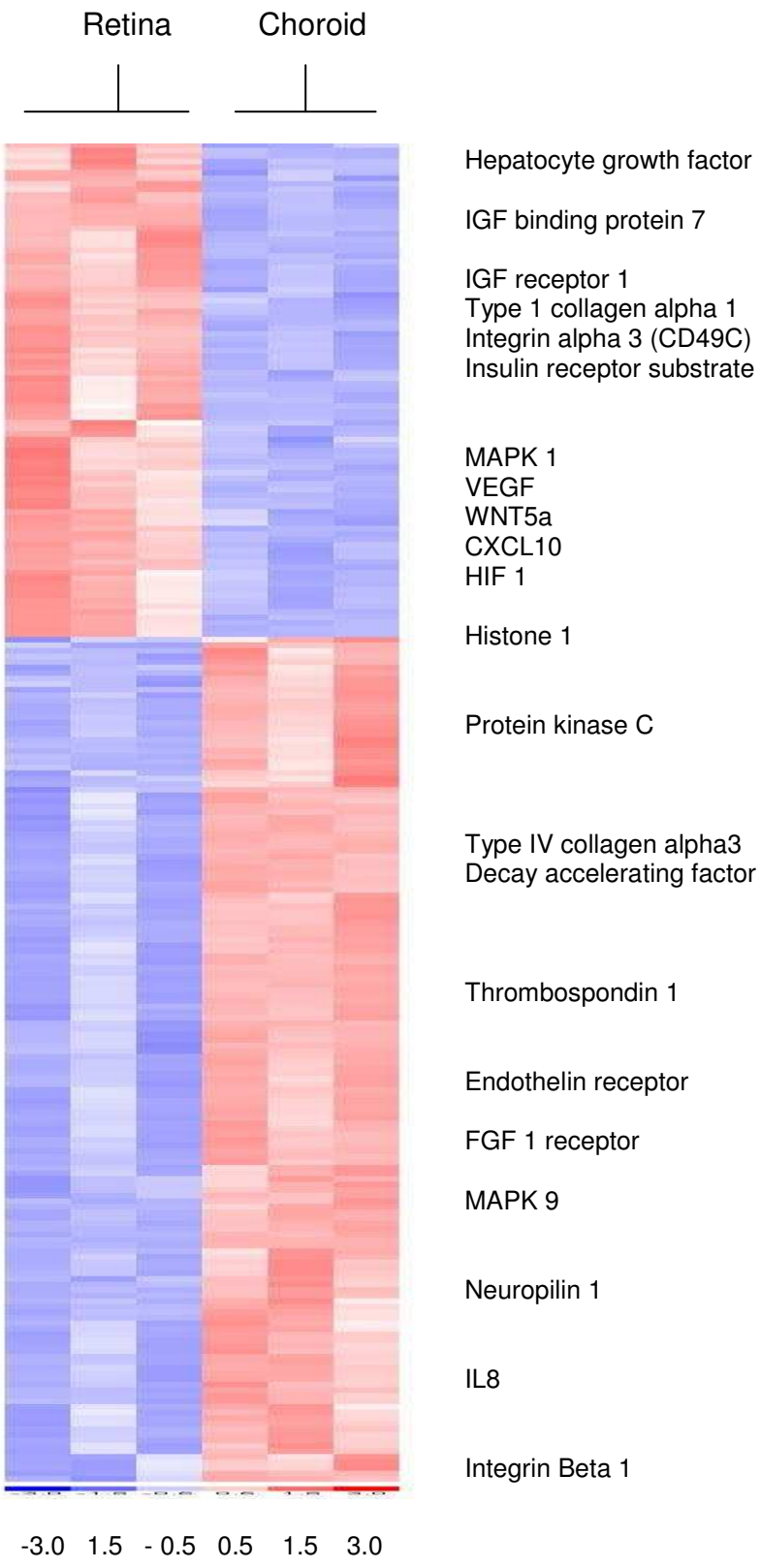


Fig 3.3 Heatmap of ascending differential probeset expression reaching statistical significance with reference to human retinal and choroidal microvascular endothelial cells. Up-regulated probesets are shown in red, down-regulated in blue. A total of 2217 different probesets are represented. The positions of selected probesets thought to be important in endothelial cell biology are shown.

Fig 3.4a. Pie chart of significantly up-regulated genes grouped by biological processes in proliferating human choroidal endothelial cells compared with proliferating human retinal endothelial cells

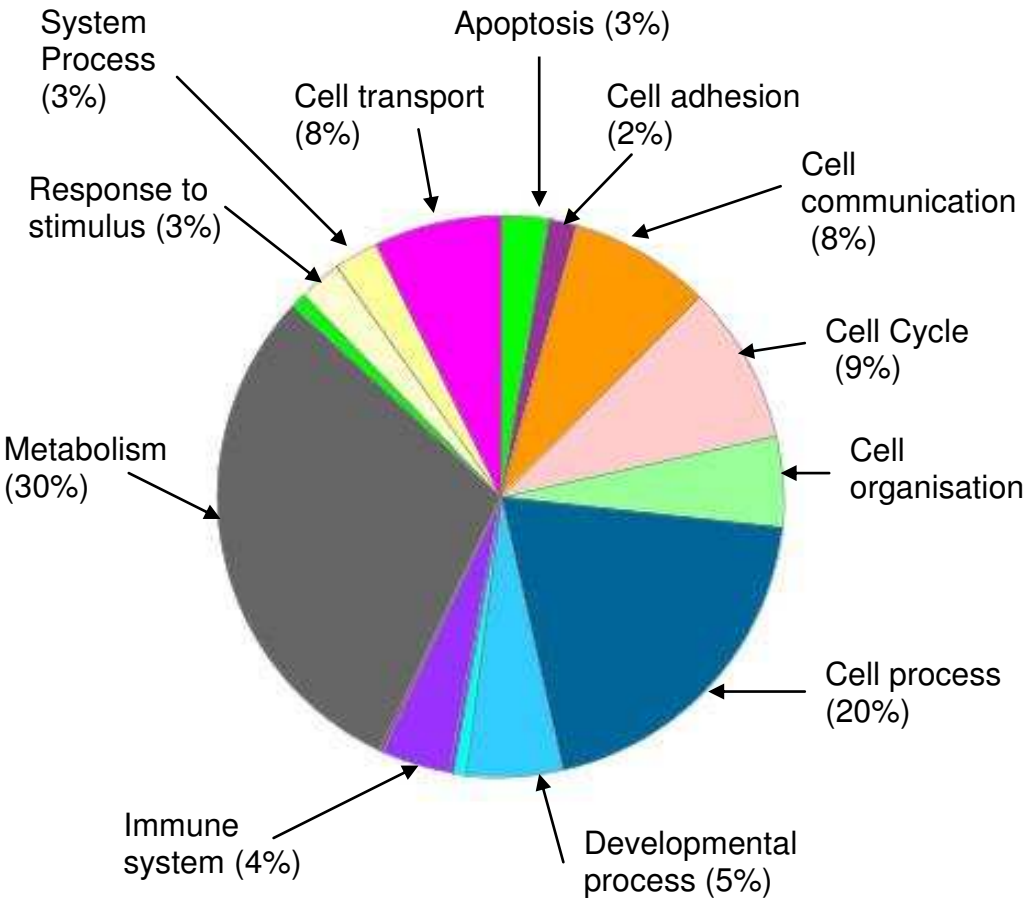


Fig 3.4b. Pie chart of significantly upregulated genes grouped by biological processes in proliferating human retinal endothelial cells compared with proliferating human choroidal endothelia cells

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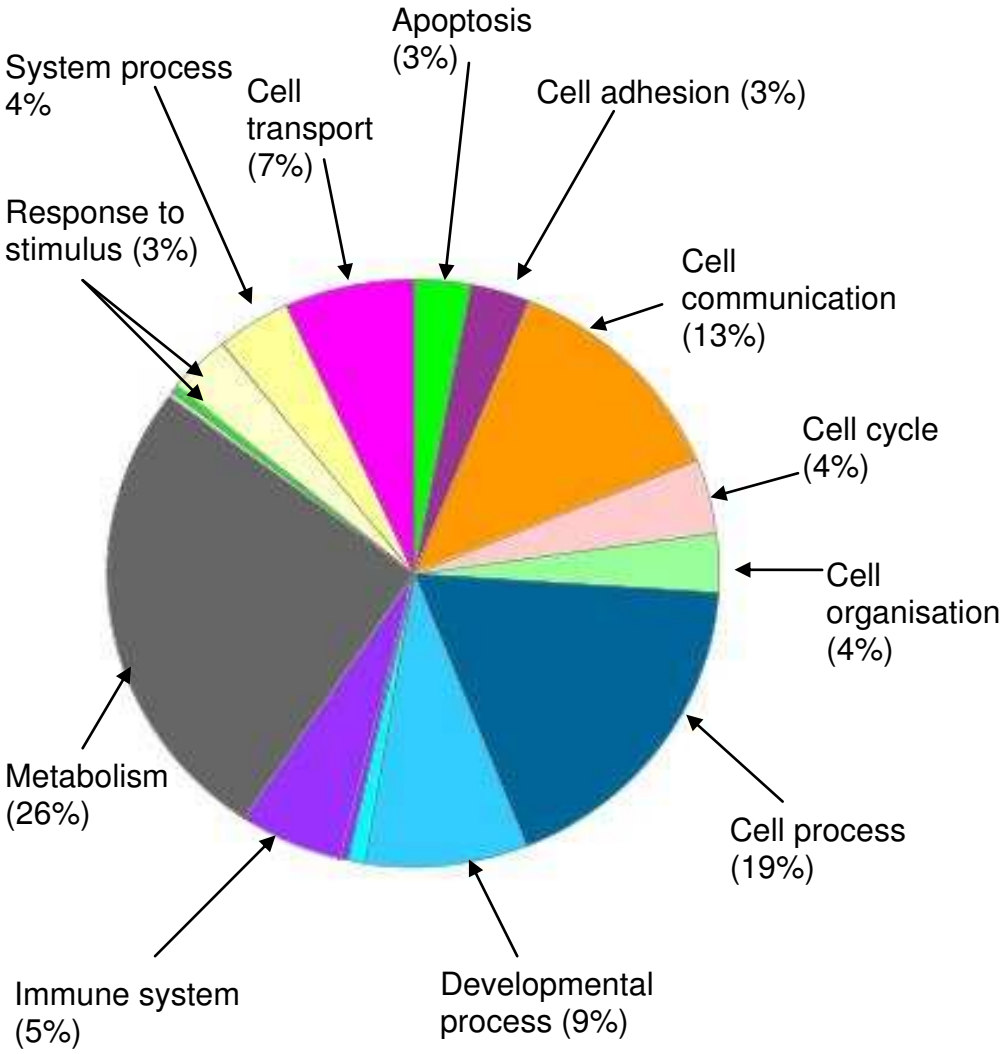

Table 3.4 One hundred most highly expressed probesets in human choroidal microvascular endothelial cells compared with human retinal endothelial cells

Gene Title	Fold Change
histone 1, H1c	9.4
phosphatase and actin regulator 2	8.2
cyclin E2	6.9
Rho-related BTB domain containing 3	6.6
WD repeat domain 4	6.5
epithelial membrane protein 1	6.1
dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein	5.9
NIMA	5.8
cyclin-dependent kinase 2	5.8
TAO kinase 1	5.8
plasminogen activator, urokinase /// plasminogen activator, urokinase	5.5
retinoblastoma 1 (including osteosarcoma)	5.5
HECT domain containing 1	5.5
jumonji domain containing 1C	5.4
KIAA0372	5.3
squalene epoxidase	5.2
protein tyrosine phosphatase, non-receptor type 12	5.1
suppressor of Ty 16 homolog (S. cerevisiae)	5.0
BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	4.9
muscleblind-like 2 (Drosophila)	4.9
chromosome X open reading frame 53	4.9
SLD5 homolog /// SLD5 homolog	4.8
proline-rich nuclear receptor coactivator 2	4.8
thymopoietin	4.8
PTPRF interacting protein, binding protein 1 (liprin beta 1)	4.8
zinc finger protein 146	4.6
PRP4 pre-mRNA processing factor 4	4.5
DEAH (Asp-Glu-Ala-His) box polypeptide 9	4.5
huntingtin interacting protein B	4.5
nuclear factor I/B	4.5
phosphoinositide-3-kinase, class 2, alpha polypeptide	4.4
ATPase family, AAA domain containing 2	4.4
transcription factor Dp-1	4.4
dystonin	4.4
c-Mpl binding protein	4.3
FERM domain containing 4A	4.3
cell division cycle associated 2	4.2
translocase of inner mitochondrial membrane 44 homolog (yeast)	4.1
G protein-coupled receptor kinase 5	4.1

Gene Title	Fold Change
protein BAP28	4.1
hypothetical protein FLJ12735	4.1
UDP-glucose ceramide glucosyltransferase-like 1	4.1
met proto-oncogene (hepatocyte growth factor receptor)	4.0
DnaJ (Hsp40) homolog, subfamily C, member 13	4.0
A kinase (PRKA) anchor protein 1	4.0
septin 10	3.9
RAB6A, member RAS oncogene family	3.9
G elongation factor, mitochondrial 1	3.8
RAS and EF hand domain containing	3.8
RAS and EF hand domain containing	3.8
muted homolog (mouse)	3.8
matrin 3	3.7
MLF1 interacting protein	3.7
lamin B1	3.7
chromodomain protein, Y-like 2	3.7
dUTP pyrophosphatase	3.7
diaphanous homolog 2 (Drosophila)	3.7
sperm specific antigen 2	3.7
RAD21 homolog (S. pombe)	3.7
zinc finger RNA binding protein	3.7
tetratricopeptide repeat domain 3	3.6
M-phase phosphoprotein 9	3.6
vav 3 oncogene	3.6
DEAD (Asp-Glu-Ala-Asp) box polypeptide 42	3.6
cell division cycle 27	3.5
translocated promoter region (to activated MET oncogene)	3.5
cell division cycle 2, G1 to S and G2 to M	3.5
guanine nucleotide binding protein-like 3 (nucleolar)-like	3.5
arrestin, beta 1	3.5
zinc finger RNA binding protein	3.5
replication factor C (activator 1) 2, 40kDa	3.5
nucleolar and spindle associated protein 1	3.4
c-Mpl binding protein	3.4
erythrocyte membrane protein band 4.1-like 3	3.4
cell division cycle 2, G1 to S and G2 to M	3.4
F-box protein 5	3.4
chromosome 6 open reading frame 106	3.3
ankyrin repeat domain 11	3.3
hydroxyacyl-Coenzyme A dehydrogenase	3.3
Zwilch	3.3
chondroitin sulfate proteoglycan 6	3.3
pericentriolar material 1	3.3
amyloid beta (A4) precursor protein	3.3
chromosome condensation 1	3.3
cell division cycle associated 7 /// cell division cycle associated 7	3.2

Gene Title	Fold Change
ATPase family, AAA domain containing 2	3.2
thymidine kinase 1, soluble	3.2
NACHT, leucine rich repeat and PYD (pyrin domain) containing 1	3.2
MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)	3.2
DEP domain containing 1	3.2
hypothetical protein FLJ12973	3.2
erythrocyte membrane protein band 4.1-like 3	3.2
nucleoporin 35kDa	3.2
ets variant gene 4 (E1A enhancer binding protein, E1AF)	3.2
transforming, acidic coiled-coil containing protein 1	3.2

Table 3.5 One hundred most highly expressed probesets in human retinal microvascular endothelial cells compared with human choroidal endothelial cells

Gene Title	Fold Change
hepatocyte growth factor (hepapoietin A; scatter factor)	17.2
gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)	13.2
collagen, type I, alpha 1	11.3
neuronal PAS domain protein 2	7.2
response gene to complement 32	7.2
Tissue factor pathway inhibitor 2	6.4
stanniocalcin 2	6.1
hypothetical gene supported by BC009447	5.8
aldehyde dehydrogenase 1 family, member L2	5.6
collagen, type VI, alpha 1	5.2
lectin, galactoside-binding, soluble, 3 binding protein	5.0
vascular endothelial growth factor	5.0
Notch homolog 3 (Drosophila)	4.9
melanophilin	4.6
Tropomyosin 4	4.3
selenoprotein M	4.2
pecanex-like 2 (Drosophila)	4.2
adrenomedullin	4.2
limitrin	4.1
Hypothetical protein LOC149478	4.1
interferon regulatory factor 7	4.0
Full length insert cDNA YI37C01	3.9
agrin	3.9
Prickle-like 1 (Drosophila)	3.7
CDNA FLJ41321 fis, clone BRAMY2045299	3.7
pleckstrin and Sec7 domain containing 3	3.5
agrin	3.5
Full length insert cDNA YI37C01	3.4
E2F transcription factor 5, p130-binding	3.4
platelet derived growth factor C	3.4
gb:AA805633	3.3
transmembrane anchor protein 1	3.3
integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	3.3
MRNA (clone ICRFp507I1077)	0.2
family with sequence similarity 14, member A	3.2
DNA-damage-inducible transcript 4	3.2
UDP-glucose ceramide glucosyltransferase	3.1
B-cell translocation gene 1, anti-proliferative	3.1
nephronophthisis 3 (adolescent)	3.1
tissue inhibitor of metalloproteinase 2	3.1
follistatin	3.1

Gene Title	Fold Change
zinc and ring finger 1	3.1
RNA binding protein with multiple splicing	3.0
protein phosphatase 1, regulatory (inhibitor) subunit 14B	3.0
Full length insert cDNA YH99G08	3.0
ras homolog gene family, member B	3.0
CDNA FLJ11397 fis, clone HEMBA1000622	3.0
Sine oculis homeobox homolog 1 (Drosophila)	3.0
phosphoinositide-3-kinase, class 2, beta polypeptide	2.9
complement component 1, r subcomponent-like	2.9
yippee-like 2 (Drosophila)	2.9
discoidin domain receptor family, member 1	2.9
Homo sapiens, clone IMAGE:5259272, mRNA	2.9
ADP-ribosylation factor-like 7	2.8
hypothetical protein MGC18216	2.8
Gene 33/Mig-6 (MIG-6)	2.8
ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	2.8
gb:N51405	2.8
Similar to lymphocyte-specific protein 1	2.8
cathepsin C	2.0
glycine receptor, beta	2.8
SNRPN upstream reading frame	2.8
LOC439987	2.8
estrogen-related receptor alpha	2.7
beta-site APP-cleaving enzyme 2	2.7
sin3-associated polypeptide, 30kDa	2.7
pleckstrin homology domain containing, family C (with FERM domain)	2.7
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor	2.7
Hypothetical protein LOC201895	2.7
regulator of G-protein signalling 10	2.7
CDNA clone IMAGE:4797120, partial cds	2.7
LOC440309	2.6
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	2.6
retinol dehydrogenase 11 (all-trans and 9-cis)	2.6
PRKC, apoptosis, WT1, regulator	2.6
ADP-ribosylation factor-like 7	2.6
methionine sulfoxide reductase B2	2.6
uronyl-2-sulfotransferase	2.5
aspartate beta-hydroxylase	2.5
heat shock 27kDa protein 1	2.5
microfibrillar-associated protein 2	2.5
Transcribed locus	2.5
SAR1a gene homolog 1 (S. cerevisiae)	2.5
Hypothetical protein BC001096	2.0

Gene Title	Fold Change
synaptosomal-associated protein, 29kDa	2.5
insulin-like growth factor binding protein 7	2.4
Homo sapiens, clone IMAGE:5261213, mRNA	2.4
Dickkopf homolog 3 (Xenopus laevis)	2.3
Zinc finger protein 302	2.3
zinc finger protein 326	2.4
kinesin family member 22	2.4
erythrocyte membrane protein band 4.1-like 3	2.4
methylthioadenosine phosphorylase	2.5
chromosome 22 open reading frame 18	2.5
histone 1, H4c	2.5
G-2 and S-phase expressed 1	2.8
Protein phosphatase 1F (PP2C domain containing)	2.6
BAT2 domain containing 1	2.6
KIAA1641	2.6
THO complex 3	2.6
interleukin enhancer binding factor 3, 90kDa	2.7

Proliferating human choroidal versus iris vascular endothelial cells

Comparison of human choroidal endothelial cells with human iris endothelial cells demonstrated a difference of 138 probe sets (0.33% of probesets that passed quality control). Of this total, 81 were up-regulated in choroidal ECs and 57 were up-regulated in iris ECs. This study demonstrated a striking homogeneity of gene expression between choroidal and iris tissues which may not be surprising considering that types of endothelial cells are derived from different sites within the uveal tract. Differences in the expression of a small number of genes such as thrombospondin 1, synaptopodin 2, CD74, Carboxypeptidase and IL8 were noted. Canonical pathway analysis failed to reveal any pathways that were significantly represented. A representative heat map is shown in fig 3.5 and a complete list of differentially expressed probesets reaching statistical significance in choroidal and iris ECs are shown in tables 3.6 and 3.7 respectively. Although the relative numbers of differentially up-regulated genes were small, fig 3.6a and 3.6b pie charts demonstrate a greater proportion of up-regulated probesets for cell development in iris ECs (13% vs 8%) when compared with choroidal ECs. Choroidal ECs demonstrated a greater proportion of up-regulated genes for cell metabolism (28% vs 13%) All other biological process subsets demonstrated similar proportions of expression in both iris and choroidal ECs.

Fig 3.5 Heatmap of ascending differential probeset expression reaching statistical significance with reference to human iris and choroidal microvascular endothelial cells. Up-regulated probesets are shown in red, down-regulated in blue. A total of 138 different probesets are represented. The positions of selected probesets thought to be important in endothelial cell biology are shown.

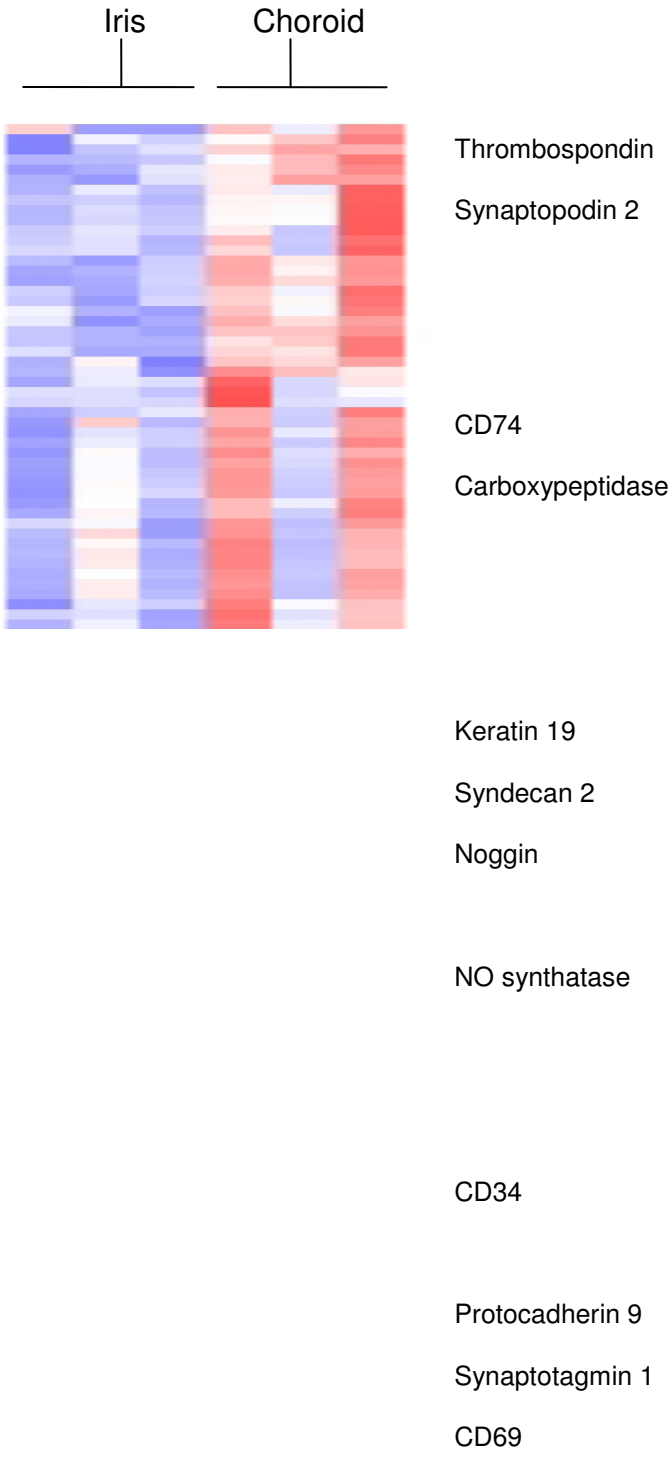

Fig 3.6a. Pie chart of significantly up-regulated genes grouped by biological processes in proliferating human iris endothelial cells compared with proliferating human choroidal endothelial cells

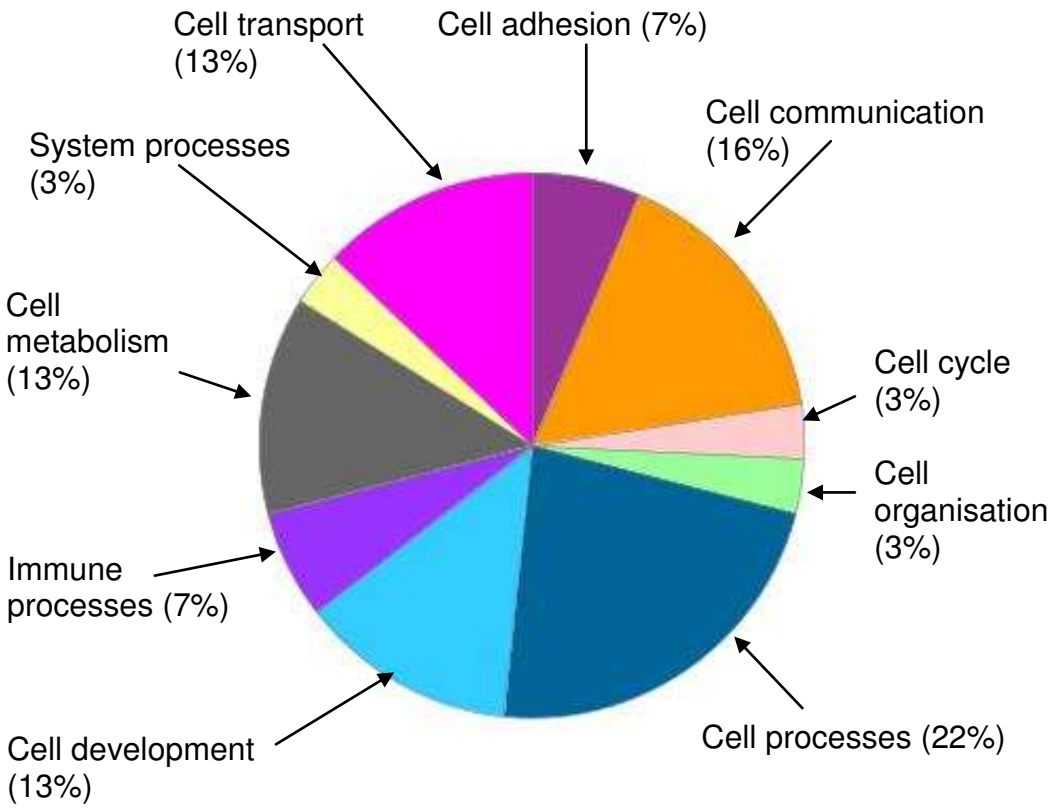

Fig 3.6b. Pie chart of significantly up-regulated genes grouped by biological processes in proliferating human choroidal endothelial cells compared with proliferating human iris endothelial cells

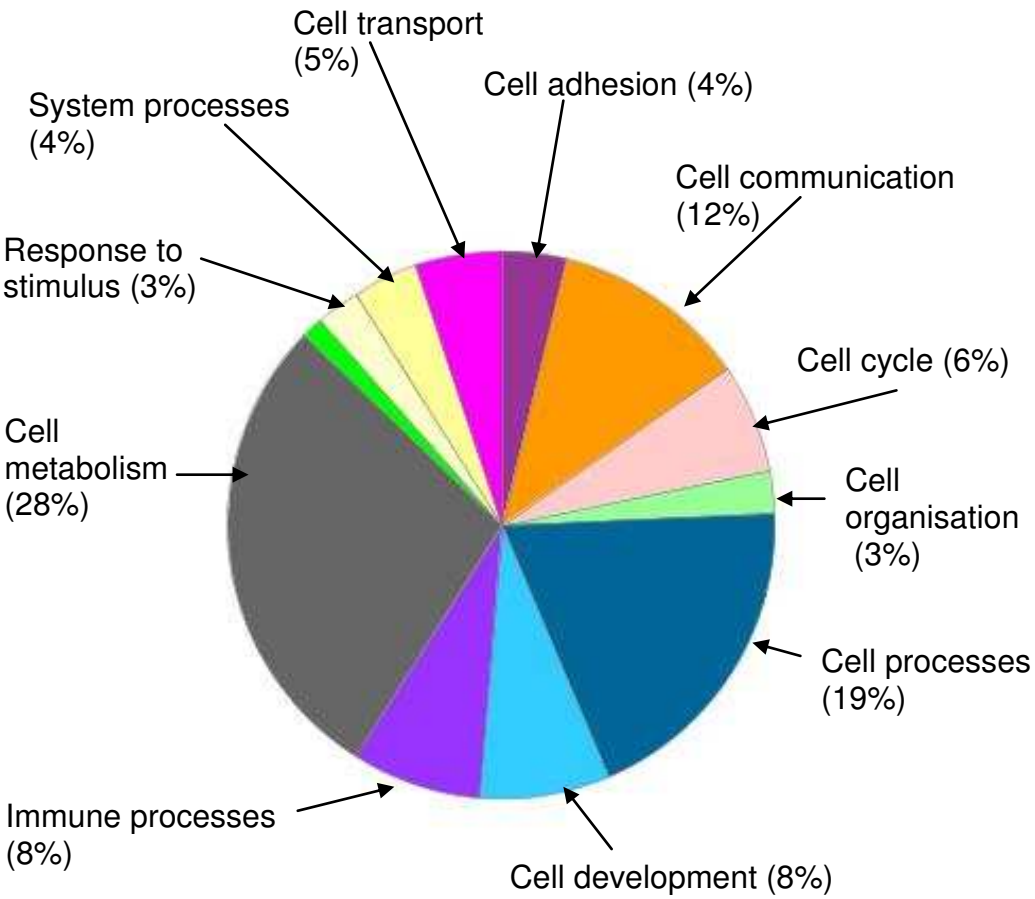

Table 3.6. All probesets reaching differential expression of 2.0 or greater in human choroidal microvascular endothelial cells compared with human iris endothelial cells.

Gene Title	Fold Change
thrombospondin, type I, domain containing 2	6.0
sushi domain containing 2	4.8
synaptopodin 2	4.3
aggrecan 1 (chondroitin sulfate proteoglycan 1,	4.0
aggrecan 1 (chondroitin sulfate proteoglycan 1,	4.0
CD74 antigen	3.9
gb:AB020690.1	3.8
hypothetical protein FLJ22662	3.7
erythrocyte membrane protein band 4.1-like 3	3.5
collagen, type VI, alpha 3	3.4
gb:AI733234	3.3
erythrocyte membrane protein band 4.1-like 3	3.2
succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	3.2
aggrecan 1 (chondroitin sulfate proteoglycan 1,	3.2
insulin-like growth factor binding protein 5	3.2
Protease, serine, 12 (neurotrypsin, motopsin)	3.1
transmembrane protein 46	3.1
carboxypeptidase E	3.1
erythrocyte membrane protein band 4.1-like 3	3.1
interleukin 8	3.0
carboxypeptidase E	3.0
FERM domain containing 3	2.9
steroid sensitive gene 1	2.9
leucine rich repeat containing 15	2.8
GTP binding protein over-expressed in skeletal muscle	2.8
ABI gene family, member 3 (NESH) binding protein	2.8
crystallin, alpha B	2.8
keratin 19	2.8
transketolase (Wernicke-Korsakoff syndrome)	2.8
KIAA1913	2.7
phosphatase and actin regulator 3	2.7
early growth response 1	2.6
periostin, osteoblast specific factor	2.6
inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	2.6
chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	2.6
GLI pathogenesis-related 1 (glioma)	2.6
Inhibin, beta A (activin A, activin AB alpha polypeptide)	2.5
AE binding protein 1	2.5
periostin, osteoblast specific factor	2.5
inhibin, beta A (activin A, activin AB alpha polypeptide)	2.4

Gene Title	Fold Change
UDP-glucose ceramide glucosyltransferase-like 2	2.4
lipopolysaccharide-induced TNF factor	2.4
lipase, endothelial	2.3
prostaglandin-endoperoxide synthase 1	2.3
CDNA clone IMAGE:4079668, partial cds	2.3
GLI pathogenesis-related 1 (glioma)	2.3
syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated	2.3
Hypothetical protein LOC123722	2.3
Transcribed locus	2.3
GAJ protein	2.3
mitochondrial ribosomal protein L43	2.3
Noggin	2.3
transgelin	2.3
syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated	2.3
platelet-derived growth factor alpha polypeptide	2.2
Cadherin 11, type 2, OB-cadherin (osteoblast)	2.2
brain expressed X-linked 2 /// brain expressed X-linked 2	2.2
tenascin C (hexabrachion)	2.2
syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated,	2.2
desmoplakin	2.1
ankyrin repeat domain 1 (cardiac muscle)	2.1
zinc finger protein 643	2.1
GLI pathogenesis-related 1 (glioma)	2.1
KIAA0830 protein	2.1
trefoil factor 3 (intestinal)	2.1
Homo sapiens, clone IMAGE:5285282, mRNA	2.1
deleted in lymphocytic leukaemia, 2 /// BCMS upstream neighbor-like	2.1
deleted in lymphocytic leukemia, 2 /// BCMS upstream neighbor-like	2.1
prostaglandin-endoperoxide synthase 1	2.1
nucleophosmin/nucleoplasmin, 3	2.1
Rap guanine nucleotide exchange factor (GEF) 1	2.1
hypothetical protein LOC144997	2.1
solute carrier family 38, member 5	2.1
cytochrome P450, family 1, subfamily B, polypeptide 1	2.1
cytochrome b5 reductase b5R.2	2.0
follistatin	2.0
heme oxygenase (decycling) 1	2.0
neuron navigator 3	2.0
chromosome 20 open reading frame 35	2.0
prostaglandin-endoperoxide synthase 1	2.0
hypothetical protein LOC286505	2.0
ST3 beta-galactoside alpha-2,3-sialyltransferase 6	2.0

Table 3.7. All probesets reaching diiferential expression of 2.0 or greater in human iris microvascular endothelial cells compared with human choroidal endothelial cells.

Gene Title	Fold Change
cardiomyopathy associated 3	4.2
popeye domain containing 3	3.8
Protocadherin 9	3.8
delta-notch-like EGF repeat-containing transmembrane	3.7
synaptotagmin I	3.6
extracellular link domain containing 1	3.6
CD69 antigen (p60, early T-cell activation antigen)	3.2
interferon-induced protein with tetratricopeptide repeats 1	3.2
family with sequence similarity 38, member B	3.1
extracellular link domain containing 1	2.9
protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	2.9
synaptotagmin I	2.9
interferon-induced protein 44-like	2.8
Homo sapiens, clone IMAGE:5312689, mRNA	2.8
myxovirus (influenza virus) resistance 1, interferon-inducible protein p78	2.7
solute carrier family 6, member 15	2.7
aldo-keto reductase family 1, member C3	2.7
LOC440156	2.7
protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	2.6
KIAA1598	2.6
family with sequence similarity 38, member B	2.6
zinc finger protein 317	2.5
lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	2.5
aldo-keto reductase family 1, member C2	2.5
nitric oxide synthase trafficker	2.4
filamin A interacting protein 1	2.4
neurofilament 3 (150kDa medium)	2.4
myosin VB	2.4
calmegin	2.3
Tissue factor pathway inhibitor 2	2.3
filamin-binding LIM protein-1	2.3
collagen, type XXI, alpha 1 /// collagen, type XXI, alpha 1	2.3
tissue factor pathway inhibitor 2	2.3
BMX non-receptor tyrosine kinase	2.2
Zinc finger-like	2.2
paternally expressed 10	2.2
aldo-keto reductase family 1, member C1	2.2
CD34 antigen	2.2
protocadherin 9	2.3

Gene Title	Fold Change
zona pellucida glycoprotein 3	2.2
chromosome 10 open reading frame 10	2.2
aldo-keto reductase family 1, member C1	2.2
Homo sapiens, clone IMAGE:5261213, mRNA	2.2
KIAA0711 gene product	2.1
KIAA0960 protein	2.1
chemokine (C-C motif) ligand 2	2.1
LOC132671	2.1
hypothetical gene supported by BC009447	2.1
Homo sapiens, clone IMAGE:5261213, mRNA	2.1
filamin A interacting protein 1	2.1
p53 target zinc finger protein	2.1
gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)	2.0
cyclin A1	2.0
lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	2.0
protocadherin 19	2.0

Proliferating human retinal versus iris vascular endothelial cells

Comparison of human retinal endothelial cells with human iris endothelial cells demonstrated a difference of 2041 probe sets (7.8% of probesets that passed quality control). Of this total, 880 were up-regulated in retinal ECs and 1161 were up-regulated in iris ECs. Using Ingenuity Pathway Analysis software, differences between retinal and iris ECs were shown in the expression of genes involved in a wide range of biological processes such as RNA post-translational modification, cell signalling, DNA replication and gene expression. As described previously, there were close similarities between iris and choroidal ECs, meaning that many of the genes differentially expressed between iris and retinal ECs were the same as those differentially expressed between choroidal and retinal ECs. However, canonical pathway analysis revealed a small number of differences in interferon, IL 22 and TGF β signalling pathways (table 3.8). A representative heat map is shown in Fig 3.7 and a list of the top 100 differentially expressed probesets reaching statistical significance in retinal and iris ECs are

shown in tables 3.9 and 3.10 respectively. Figs 3.8a and 3.8b pie charts demonstrate a greater proportion of up-regulated probesets for cell development in iris ECs compared with retinal ECs (19 vs 5%). However, all other biological process subsets demonstrated similar proportions of up-regulation of expression in both iris and ECs. As can be seen from the detailed analysis of differences in gene expression, the pie chart is not a good representation of the more subtle differences in gene expression.

Table 3.8. Differential expression of probe sets involved in cell signalling between matched human iris and retinal endothelial cells

Signalling Pathway	Affy ID	Gene Symbol	Gene Name	Fold Change	Direction of regulation (retina vs Iris)
IL 22	209575_at	IL10RB	Interleukin 10 receptor, beta	2.0	Up
	212046_x_at	MAPK3	Mitogen-activated protein kinase 3	2.1	Up
	208992_s_at	STAT3	Signal transducer and activator of transcription 3	2.1	Up
Interferon	229450_at	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	9.0	Up
	217863_at	PIAS1	Protein inhibitor of activated STAT, 1	2.1	Up
TGF Beta	228121_at	TGFB2	TGF-beta2	7.8	Down
	207334_s_at	TGFBR2	TGF-betaII receptor	2.5	Up
	212666_at	SMURF1	SMAD protein ligase	2.1	Down
	212046_x_at	MAPK 3	Mitogen-activated protein kinase 3	2.1	Up
	210511_s_at	INHBA	Inhibin beta A	14.6	Down

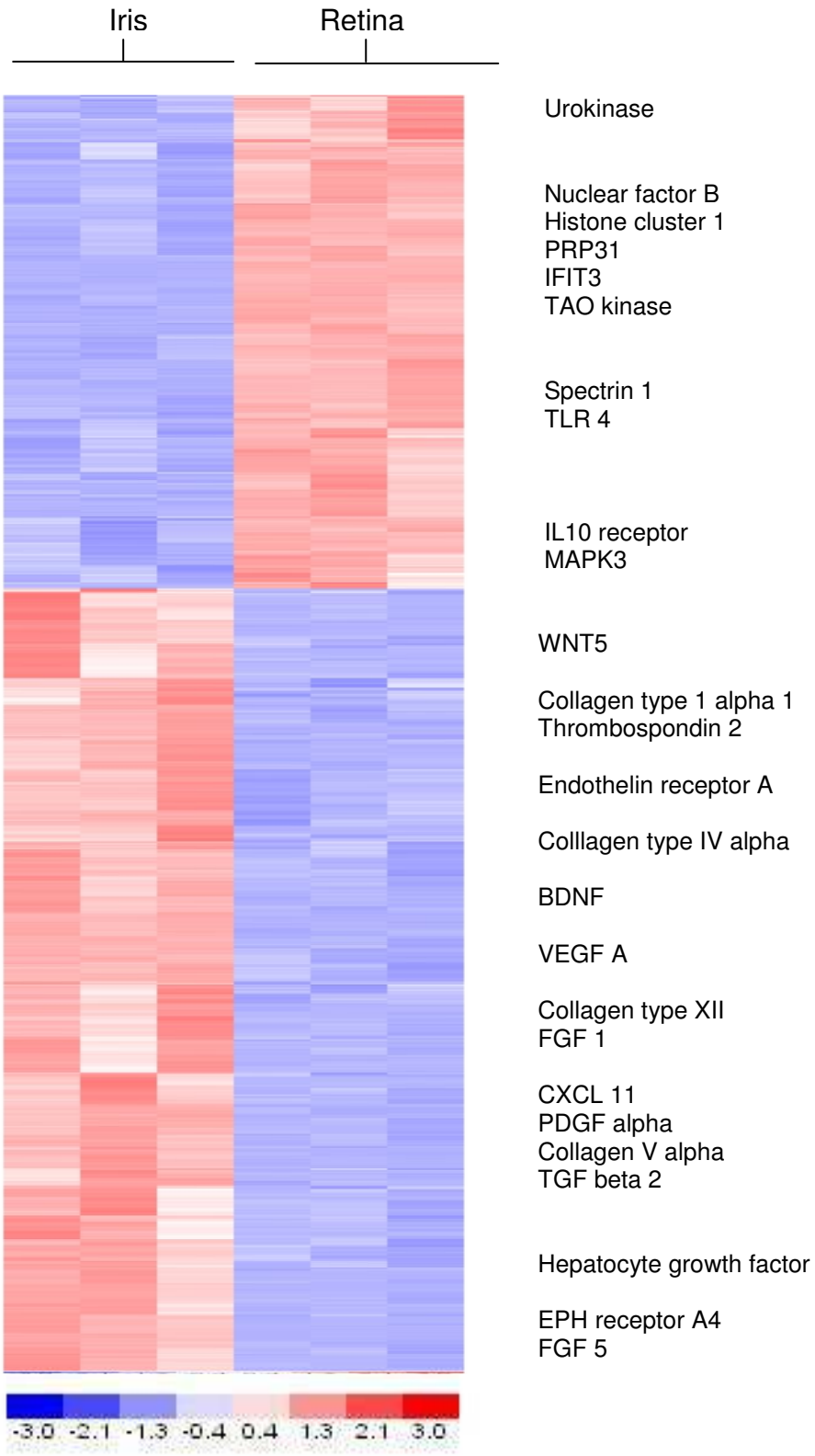

Fig 3.7 Heatmap of ascending differential probeset expression reaching statistical significance with reference to human retinal and iris micro-vascular endothelial cells. Upregulated probesets are shown in red, downregulated in blue. A total of 2041 different probesets are represented. The positions of selected probesets thought to be important in endothelial cell biology are shown.

Fig 3.8a. Pie chart of significantly up-regulated genes grouped by biological processes in proliferating human iris endothelial cells compared with proliferating human retinal endothelial cells

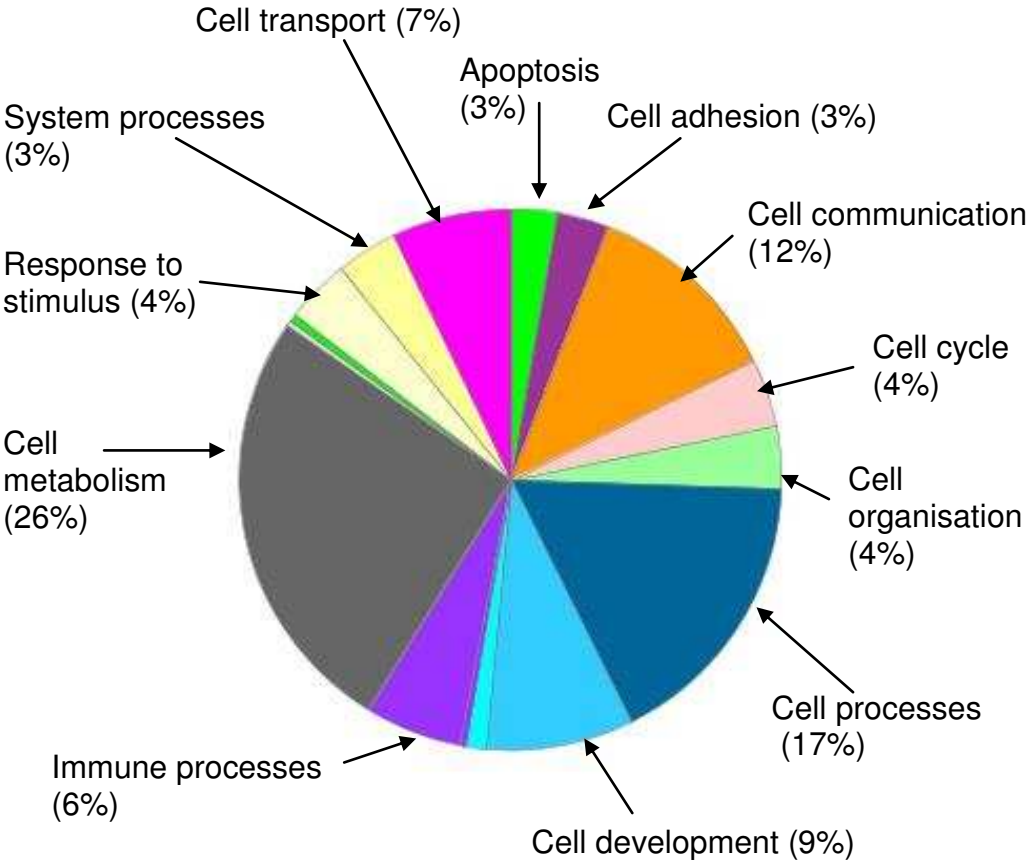

Fig 3.8b. Pie chart of significantly up-regulated genes grouped by biological processes in proliferating human retinal endothelial cells compared with proliferating human iris endothelial cells

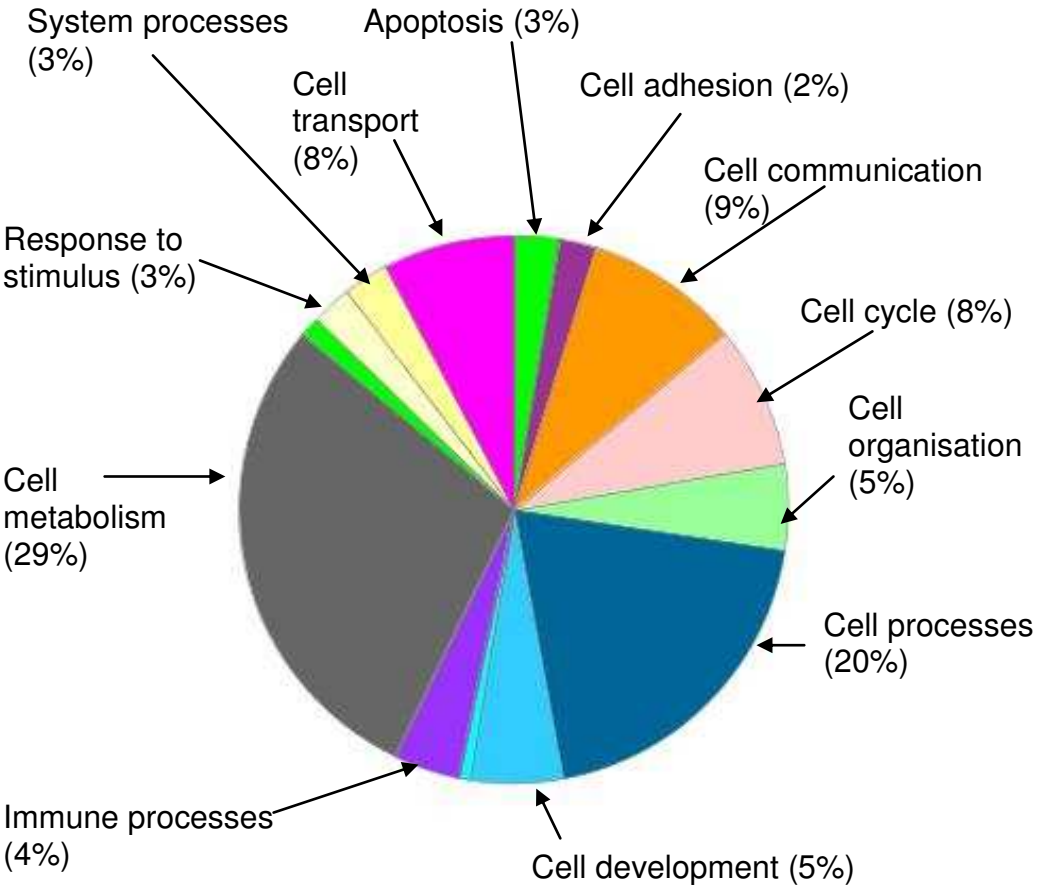

Table 3.9. One hundred most highly expressed probesets in human retinal microvascular endothelial cells compared with human iris endothelial cells.

Gene Title	Fold Change
plasminogen activator, urokinase	20.1
Rho-related BTB domain containing 3	17.5
phosphatase and actin regulator 2	17.0
protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	16.7
SCY1-like 2 (S. cerevisiae)	15.4
protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	15.0
dipeptidyl-peptidase 4	14.8
nuclear factor I/B	14.5
tetratricopeptide repeat domain 37	14.1
dipeptidyl-peptidase 4	13.9
fusion (involved in t(12;16) in malignant liposarcoma)	12.8
histone cluster 1,	12.1
PRP31 pre-mRNA processing factor 31 homolog (S. cerevisiae)	12.0
TAO kinase 1	11.6
squalene epoxidase	11.4
histone cluster 1, H1c	11.3
PRP4 pre-mRNA processing factor 4 homolog B (yeast)	11.0
zinc finger protein 480	10.7
HECT domain containing 1	10.6
epithelial cell transforming sequence 2 oncogene	10.5
zinc finger protein 146	10.2
dystonin	10.0
jumonji domain containing 1C	9.9
G elongation factor, mitochondrial 1	9.9
G protein-coupled receptor kinase 5	9.4
epithelial membrane protein 1	9.3
vacuolar protein sorting 13 homolog C (S. cerevisiae)	9.3
IFIT3	9.3
cyclin E2	9.1
nucleoporin 43kDa	9.1
thymopoietin	8.9
replication factor C (activator 1) 3, 38kDa	8.9
cyclin K	8.8
nuclear factor of activated T-cells 5, tonicity-responsive	8.7
ADAM metallopeptidase domain 15	8.6
M-phase phosphoprotein 9	8.5
septin 10	8.5
baculoviral IAP repeat-containing 5	8.4
family with sequence similarity 115, member A	8.4
mannosidase, alpha, class 1A, member 1	8.3

Gene Title	Fold Change
spectrin, beta, non-erythrocytic 1	8.3
serum deprivation response (phosphatidylserine binding protein)	8.3
phosphoinositide-3-kinase, class 2, alpha polypeptide	8.3
cell division cycle associated 2	8.0
NIMA (never in mitosis gene a)-related kinase 2	7.9
neural precursor cell expressed, developmentally down-regulated 1	7.8
toll-like receptor 4	7.8
matrin 3	7.7
RNA binding motif protein 22	7.7
Rho-guanine nucleotide exchange factor	7.7
ubiquitin specific peptidase 34	7.7
ribonucleotide reductase M1	7.7
SET domain containing 2	7.6
polo-like kinase 4 (Drosophila)	7.6
thrombospondin 1	7.6
BRCA1/BRCA2-containing complex, subunit 3	7.6
twinfilin, actin-binding protein, homolog 1 (Drosophila)	7.4
PCTAIRE protein kinase 2	7.4
cancer susceptibility candidate 5	7.3
sperm associated antigen 9	7.3
AHNAK nucleoprotein	7.3
ribosomal protein S6 kinase, 70kDa, polypeptide 1	7.2
Nipped-B homolog (Drosophila)	7.1
RAS and EF-hand domain containing	7.7
catenin (cadherin-associated protein), alpha 1, 102kDa	7.1
FERM domain containing 4A	7.9
muted homolog (mouse)	7.0
Meis homeobox 1	7.0
kelch repeat and BTB (POZ) domain containing 11	7.0
transcription factor Dp-1	7.0
protein tyrosine phosphatase, non-receptor type 12	6.9
hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A	6.9
RAS and EF-hand domain containing	6.9
Ewing sarcoma breakpoint region 1	6.9
KIAA1826	6.8
phosphoinositide-3-kinase, class 2, alpha polypeptide	6.8
RAB38, member RAS oncogene family	6.7
Fanconi anemia, complementation group M	6.7
Senataxin	6.7
arrestin, beta 1	6.7
FGFR1 oncogene partner 2	6.7
muscleblind-like 2 (Drosophila)	6.6
kinesin family member 22	6.6
budding uninhibited by benzimidazoles 1 homolog (yeast)	6.6

Gene Title	Fold Change
NLR family, pyrin domain containing 1	6.5
suppressor of Ty 16 homolog (S. cerevisiae)	6.5
PTPRF interacting protein, binding protein 1 (liprin beta 1)	6.5
folate hydrolase (prostate-specific membrane antigen) 1	6.5
sema domain, immunoglobulin domain (Ig), short basic domain, secreted	6.3
ADAM metallopeptidase with thrombospondin type 1 motif, 9	6.3
HECT, UBA and WWE domain containing 1	6.3
asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	6.7
zinc finger E-box binding homeobox 1	6.3
sulfatase 2	6.3
DENN/MADD domain containing 3	6.2
nidogen 1	6.2
prion protein	6.2
UEV and lactate/malate dehyrogenase domains	6.2
pericentriolar material 1	6.1

Table 3.10. One hundred most highly expressed probesets in human iris microvascular endothelial cells compared with human retinal endothelial cells.

Gene Title	Fold Change
wnt 5A	36.4
collagen, type I, alpha 1	34.1
interferon, alpha-inducible protein 6	33.8
pleckstrin homology-like domain, family A, member 2	28.8
ADAM metallopeptidase with thrombospondin type 1 motif, 12,	26.0
transmembrane protein 45A	25.7
gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	24.1
Zic family member 2 (odd-paired homolog, Drosophila)	23.6
thrombospondin 2	22.2
CCAAT/enhancer binding protein (C/EBP), beta	21.6
endothelin receptor type A	21.6
gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	19.8
reticulon 1	19.8
cytoglobin	18.9
collagen, type VI, alpha 1	18.8
crystallin, alpha B	18.6
GATA binding protein 6	18.5
brain-derived neurotrophic factor	18.3
potassium voltage-gated channel, Isk-related family, member 4	17.9
lysyl oxidase-like 1	17.4
follistatin	17.3
dermatan sulfate epimerase-like	16.9
glutamyl aminopeptidase (aminopeptidase A)	16.3
coagulation factor II (thrombin) receptor-like 1	16.2
amphiregulin	16.1
nexilin (F actin binding protein)	15.9
inhibin, beta A	15.0
cAMP responsive element binding protein 3-like 1	14.8
collagen, type VI, alpha 3	14.6
scinderin	14.6
EF-hand domain family, member D1	14.5
Full length insert cDNA clone ZA02A01	14.5
chemokine (C-X-C motif) ligand 10	14.4
vascular endothelial growth factor A	14.3
follistatin	14.2
lectin, galactoside-binding, soluble, 3 binding protein	14.2
frizzled homolog 2 (Drosophila)	14.2
regeneration associated muscle protease	14.2
inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	13.8

Gene Title	Fold Change
reticulon 1	13.5
stanniocalcin 2	13.5
solute carrier organic anion transporter family, member 2B1	13.5
collagen, type I, alpha 1	13.5
collagen, type XII, alpha 1	13.4
wingless-type MMTV integration site family, member 5A	13.2
platelet-derived growth factor receptor, beta polypeptide	13.2
adrenergic, beta-1-, receptor	13.1
proline rich 16	13.0
dystrophin	13.0
fibroblast growth factor 1 (acidic)	12.9
insulin receptor substrate 1	12.9
procollagen C-endopeptidase enhancer	12.6
cadherin 6, type 2, K-cadherin (fetal kidney)	12.5
hyaluronan synthase 2	12.5
chemokine (C-X-C motif) ligand 11	12.2
TP53 regulating kinase	12.1
ubiquitin associated and SH3 domain containing, B	12.1
CHD2	11.7
prostaglandin-endoperoxide synthase 2	11.4
chromosome 1 open reading frame 64	11.4
frizzled homolog 7 (Drosophila)	11.2
mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase,	11.2
interferon induced transmembrane protein 1 (9-27)	11.1
hexokinase 2	10.9
forkhead box D1	10.7
methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	10.6
chromosome 1 open reading frame 85	10.6
family with sequence similarity 91, member A2	10.5
jun D proto-oncogene	10.4
chromosome 21 open reading frame 7	10.4
PDZ domain containing ring finger 3	10.2
similar to lymphocyte-specific protein 1	10.2
platelet-derived growth factor alpha polypeptide	9.9
collagen, type V, alpha 3	9.8
neuronal PAS domain protein 2	9.8
hypothetical LOC387763	9.7
chromosome 11 open reading frame 70	9.6
solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	9.4
ring finger protein 182	9.3
interferon-induced protein with tetratricopeptide repeats 3	9.3
spondin 2, extracellular matrix protein	9.2
alpha-kinase 2	9.2

Gene Title	Fold Change
pleckstrin and Sec7 domain containing 3	9.2
synaptotagmin XI	9.1
hepatocyte growth factor (hepapoietin A; scatter factor)	9.0
CKLF-like MARVEL transmembrane domain containing 8	9.0
integrin, alpha 7	8.9
EPH receptor A4	8.8
interferon-induced protein with tetratricopeptide repeats 1	8.6
carboxypeptidase A3 (mast cell)	8.6
fibroblast growth factor 5	8.5
vestigial like 3 (Drosophila)	8.5
translation initiation factor eIF-2B subunit alpha/beta/delta-like protein	8.5
SIX homeobox 1	8.5
neuronal PAS domain protein 2	8.5
cystatin SN	8.4
prostate transmembrane protein, androgen induced 1	8.4
collagen, type V, alpha 3	8.4

Real time PCR

Real time PCR was used to validate the differences in gene expression between HUVEC, iris, retinal and choroidal endothelial cells. Nine transcripts were chosen that demonstrated at least a 2-fold differential expression between ECs from various sources on microarray analysis and that were thought to be relevant to a range of endothelial cellular functions. The chosen transcripts were: Angiopoietin 2, Keratin 18, CD44, CD73 (5 nucleotidase ecto), MAPKK 3, Integrin α3, Laminin β2, Decay Accelerating Factor and Carboxypeptidase M. Fig 3.9 demonstrates examples of real time PCR curves of selected probesets in HUVEC and ocular endothelial cells while table 3.11 shows an example of the output from the ABI PRISM 7000 sequence detection system and demonstrates the reproducibility of the triplicates for each cell type and also shows the figures used in the calculation of the fold change using the ΔCT method.

Table 3.12demonstrates that the differences in expression between the microarray and real time PCR techniques were similar for 8 out of the 9 transcripts evaluated, with only those results from the decay accelerating factor showing a discrepancy. Overall, these results confirm the overall reliability of the results obtained by the Affymetrix microarray technique.

Fig 3.9 demonstrates examples of real time PCR dissociation curves of selected probesets in HUVEC and ocular endothelial cells. The Y axis depicts the fluorescence of the reporter signal normalised to a reference signal (Rn) while the X axis depicts the cycle number. Each probeset under investigation is represented by all 3 curves of the sample triplicate and also includes a probeset for the reference housekeeping gene - hypoxanthine-guanine phosphoribosyltransferase (HPRT). Also represented are the curves of the No Template Control (NTC) (sterile water). Fig 3.9a, demonstrates the dissociation curves for a series of 10 fold dilutions for an Angiopoietin 2 reference sample while Fig 3.9b shows the calculated standard curve derived from Fig 3.9a, demonstrating its linearity of the range tested.
Ct is the crossing point or threshold at which fluorescence can be detected and log C0 is the log of the relative standard concentration (chosen to correspond to the expected relative concentration of probeset in the samples).

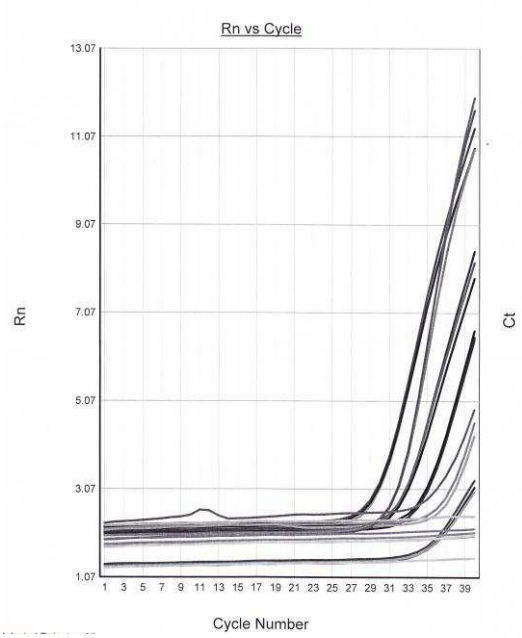

Fig 3.9a

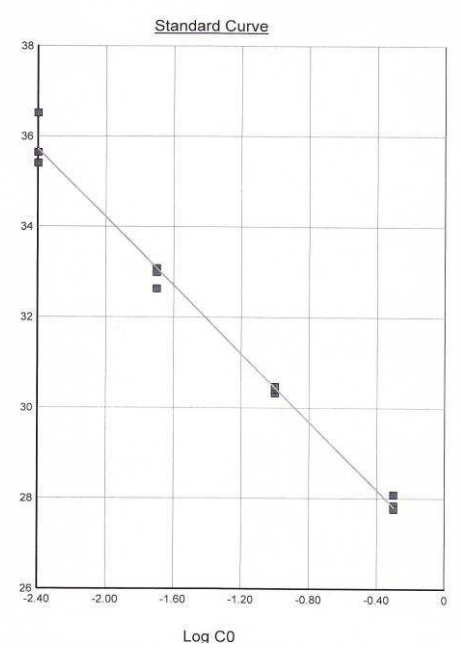

Fig 3.9b

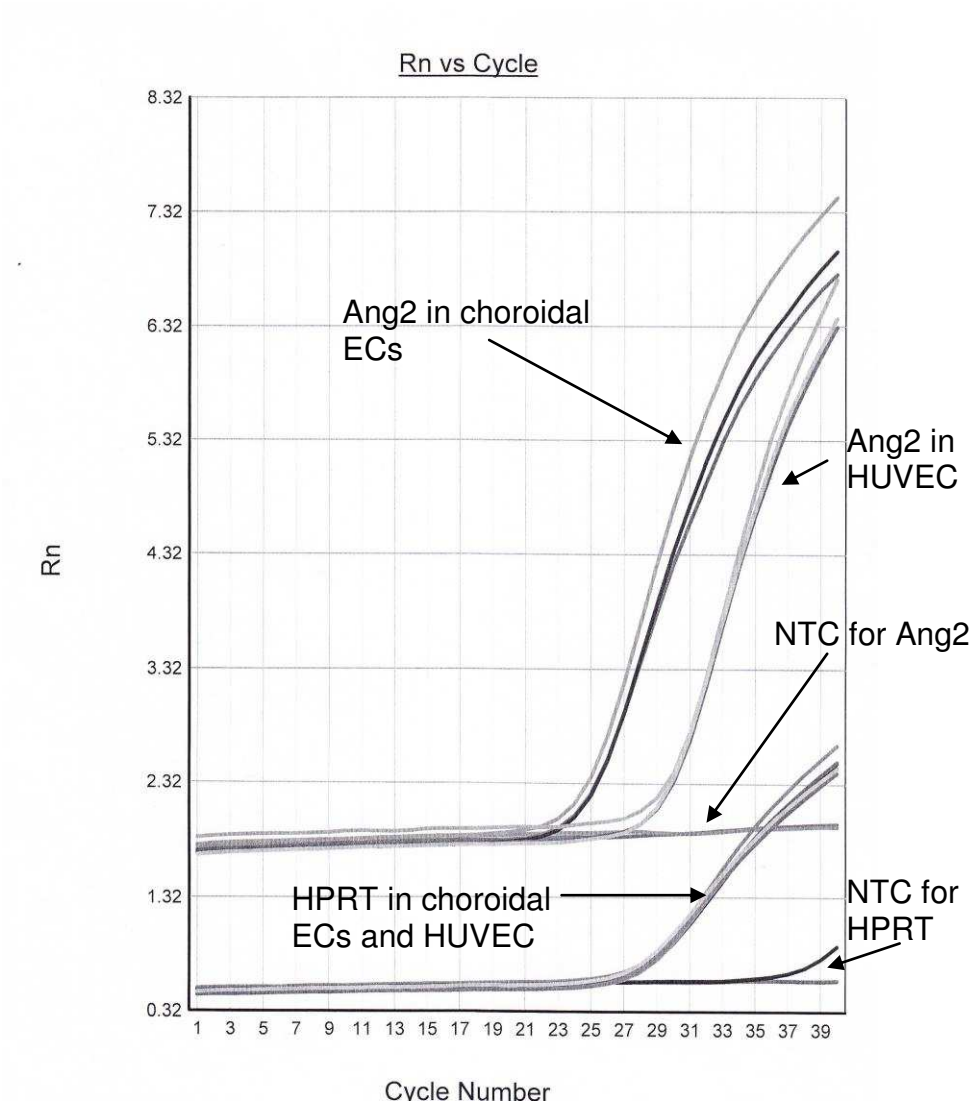

Fig 3.9c demonstrates the QPCR dissociation curves (triplicates) for Angiopoietin 2 expression in HUVEC and human choroidal endothelial cells. The reference housekeeping gene is HPRT.
The No Template Controls are also included

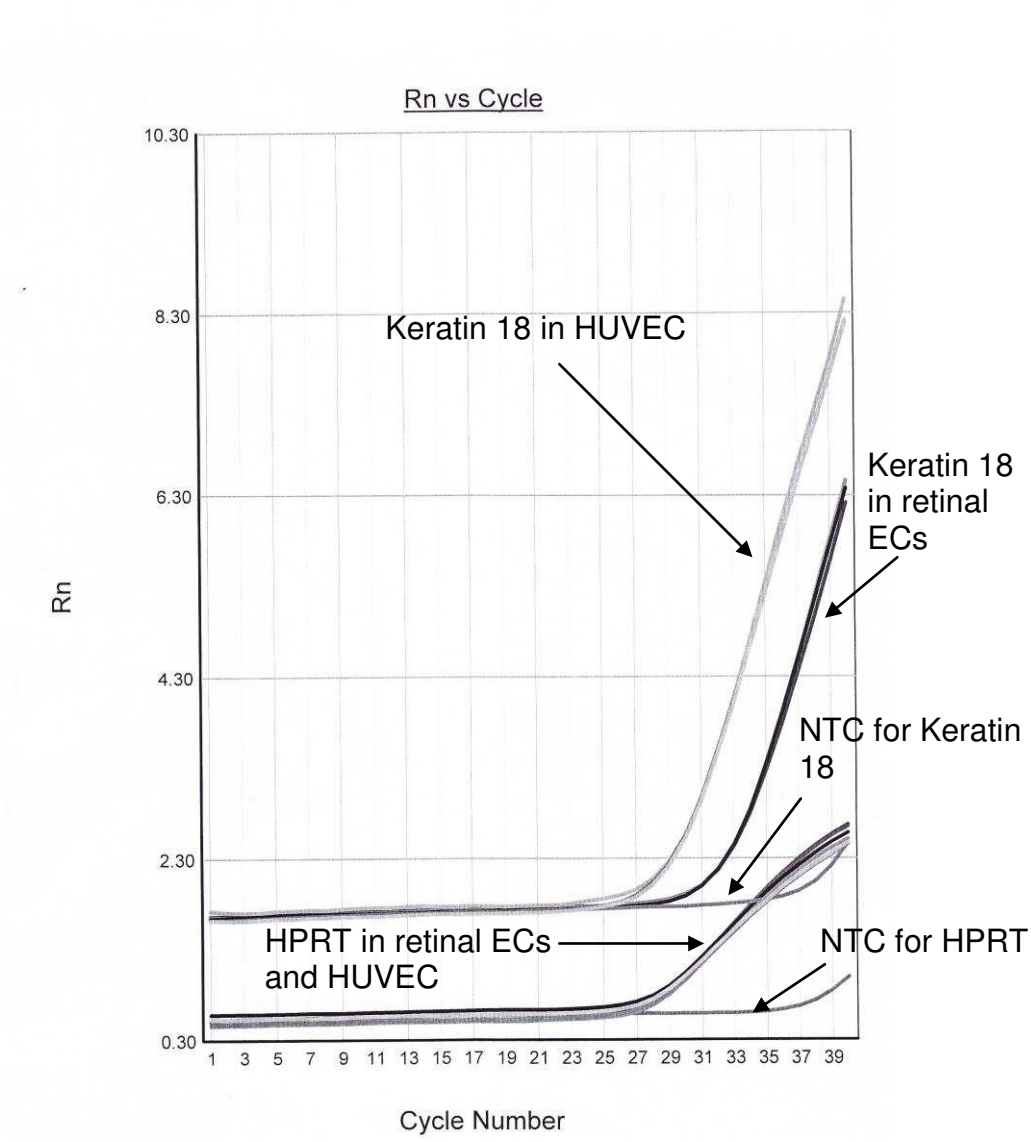

Fig 3.9d demonstrates the QPCR dissociation curves (triplicates) for Keratin 18 expression in HUVEC and human retinal endothelial cells. The reference housekeeping gene is HPRT.
The No Template Controls are also included.

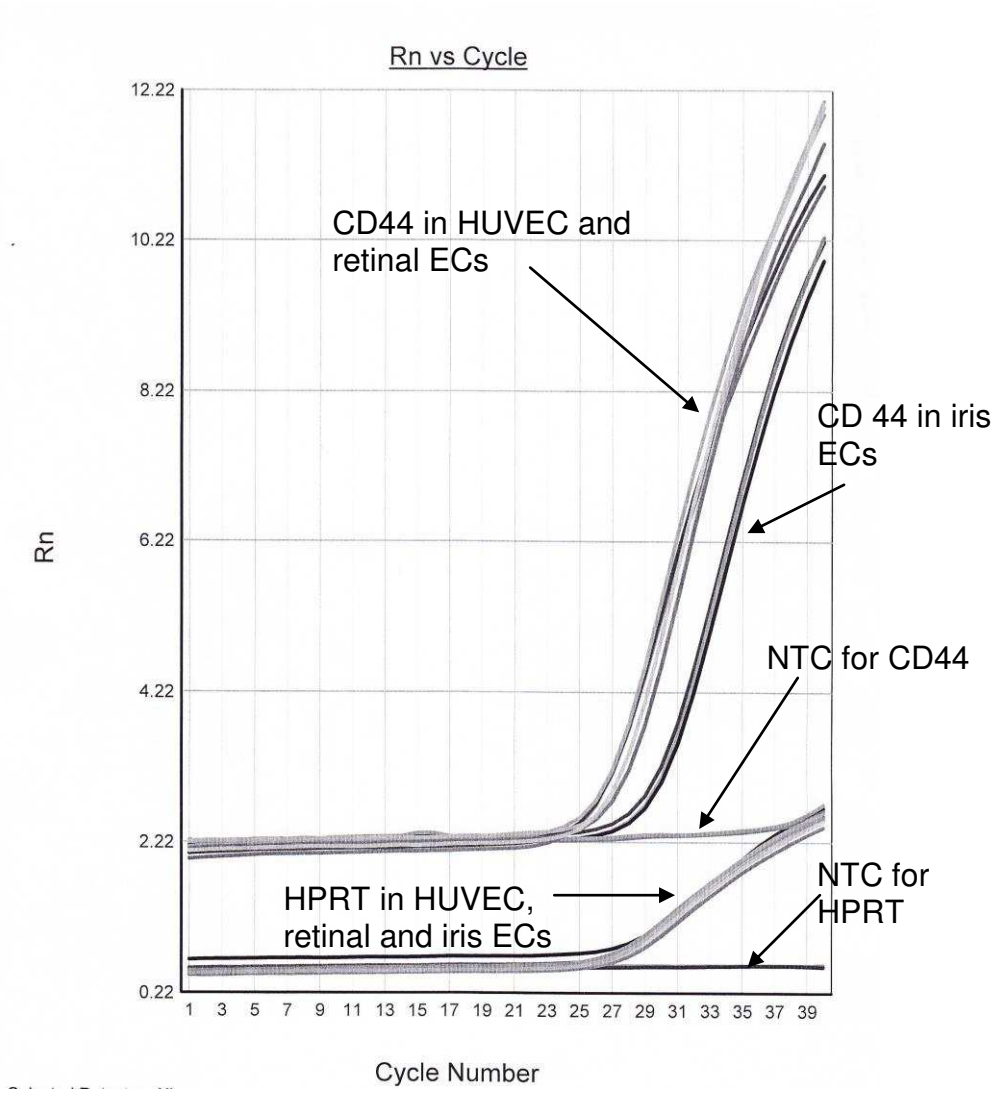

Fig 3.9e demonstrates the QPCR dissociation curves (triplicates) for CD44 expression in HUVEC, retinal and iris human endothelial cells. The reference housekeeping gene is HPRT.
The No Template Controls are also included. Note that the dissociation curves for HUVEC and retinal ECs are almost identical whereas iris ECs shifted to the right (reflecting lower expression of CD44 mRNA).

Table 3.11. A table showing an example of the output of the ABI PRISM 7000 sequence detection system for Angiopoietin 2 QPCR for human iris, retinal, choroidal ECs and HUVEC demonstrating the Ct, average Ct, difference in Ct between the target and reference (HPRT). The last column on the right shows the final fold change expression of Ang2 in individual ocular EC type compared with the HUVEC reference. (See table 3.12 for the complete set of results.

	Well	Detector	Ct (Ang 2)	Average Ct			Ct (HPRT)	Av Ct	DCt	Av DCt	DDCt	Fold Change
IRIS	A1	ab ANG2	24.8		eh HPRT1	Unknown	27.56					
1	A2	ab ANG2	24.59		eh HPRT1	Unknown	27.37					
	A3	ab ANG2	25.05	24.81	eh HPRT1	Unknown	27.31	27.41	-2.6			
IRIS	B1	ab ANG2	24.62		eh HPRT1	Unknown	27.83					
2	B2	ab ANG2	25.58		eh HPRT1	Unknown	27.98					
	B3	ab ANG2	24.61	24.93	eh HPRT1	Unknown	27.99	27.93	-3			
IRIS	C1	ab ANG2	24.32		eh HPRT1	Unknown	27.82					
3	C2	ab ANG2	24.38		eh HPRT1	Unknown	28.03					
	C3	ab ANG2	24.57	24.42	eh HPRT1	Unknown	27.81	27.89	-3.47	-3.02	-3.25	9.5
CHOROID	D1	ab ANG2	25		eh HPRT1	Unknown	27.08					
4	D2	ab ANG2	25.29		eh HPRT1	Unknown	27.18					
	D3	ab ANG2	25.11	25.13	eh HPRT1	Unknown	27.28	27.18	-2.05			
CHOROID	E1	ab ANG2	25.07		eh HPRT1	Unknown	27.05					
5	E2	ab ANG2	25.07		eh HPRT1	Unknown	26.91					
	E3	ab ANG2	25.02	25.05	eh HPRT1	Unknown	27.01	26.99	-1.94			
CHOROID	F1	ab ANG2	25.21		eh HPRT1	Unknown	28.42					
6	F2	ab ANG2	25.46		eh HPRT1	Unknown	28.42					
	F3	ab ANG2	25.95	25.54	eh HPRT1	Unknown	28.58	28.47	-2.93	-2.31	-2.54	5.9
HUVEC	G1	ab ANG2	28.23		eh HPRT1	Unknown	27.9					
7	G2	ab ANG2	28.15		eh HPRT1	Unknown	27.62					
	G3	ab ANG2	28.09	28.16	eh HPRT1	Unknown	27.51	27.68	0.48			
HUVEC	H1	ab ANG2	27.42		eh HPRT1	Unknown	27.65					
8	H2	ab ANG2	27.73		eh HPRT1	Unknown	27.63					

	Well	Detector	Ct (Ang 2)	Average Ct			Ct (HPRT)	Av Ct	DCt	Av DCt	DDCt	Fold Change
	H3	ab ANG2	27.88	27.68	eh HPRT1	Unknown	27.86	27.71	-0.03			
HUVEC	A7	ab ANG2	27.36		eh HPRT1	Unknown	27.18					
9	A8	ab ANG2	27.35		eh HPRT1	Unknown	27.16					
	A9	ab ANG2	27.56	27.42	eh HPRT1	Unknown	27.18	27.17	0.25	0.23	reference	
RETINA	B7	ab ANG2	24.39		eh HPRT1	Unknown	28.95					
11	B8	ab ANG2	24.55		eh HPRT1	Unknown	28.75					
	B9	ab ANG2	24.62	24.52	eh HPRT1	Unknown	28.64	28.78	-4.26			
RETINA	C7	ab ANG2	23.21		eh HPRT1	Unknown	27.02					
12	C8	ab ANG2	23.32		eh HPRT1	Unknown	27.19					
	C9	ab ANG2	23.15	23.22	eh HPRT1	Unknown	26.93	27.05	-3.83			
RETINA	D7	ab ANG2	25.9		eh HPRT1	Unknown	29.53					
20	D8	ab ANG2	26.05		eh HPRT1	Unknown	29					
	D9	ab ANG2	25.78	25.91	eh HPRT1	Unknown	29.16	29.23	-3.32	-3.8	-4.03	16.3

A10	ab ANG2	Undetermined	NTC
A11	ab ANG2	Undetermined	NTC
B10	eh HPRT1	Undetermined	NTC
B11	eh HPRT1	Undetermined	NTC

Table 3.12. Differences in gene expression of selected genes for HUVEC, iris, choroidal and retinal endothelial cells according to microarray (MA) and Real time-PCR (RT-PCR)

		Difference in gene expression (fold change)							
Gene transcript	**Affy ID**	**HUVEC**		**Iris**		**Choroid**		**Retina**	
		MA	**RT-PCR**	**MA**	**RT-PCR**	**MA**	**RT-PCR**	**MA**	**RT-PCR**
Angiopoietin 2	205572_at	0	0	9.2	9.5	7.6	5.9	10.7	16.3
Keratin 18	201596_x_at	0	0	-4.7	-4.2	-4.1	-3.4	-4.7	-2.7
CD44	204489_s_at	0	0	-4.9	-3.5	-4.8	-5.7	<2.0	0.9
CD73	203939_at	0	0	3.9	4.5	3.8	3.0	4.1	4.5
MAPKK 3	201474_s_at	0	0	2.8	3.1	2.3	3.4	4.6	5.3
Integrin α3	201474_s_at	0	0	<2.0	1.6	<2.0	1.4	5.6	4.6
Laminin β2	216264_s_at	0	0	2.9	2.5	2.4	1.4	3.4	2.9
Decay Accelerating Factor (CD55)	201926_s_at	0	0	2.9	1.4	4.0	1.2	<2.0	2.1
Carboxypeptidase M	235706_at	0	0	- 4.4	-5.3	<2.0	-2.5	<2.0	-1.3

Discussion

The well described morphological, biochemical, phenotypical and molecular heterogeneity of endothelial cells raises the question as to whether conclusions drawn from studies using animal or human macrovascular endothelial cells such as HUVECs can be applied to different angiogenic diseases causing blindness in humans, such as wet AMD, proliferative diabetic retinopathy, rubeotic glaucoma and retinopathy of prematurity. Despite their anatomical juxtaposition, the diseases appear to be selective for different ocular vascular beds so any reliable and meaningful insight into their pathophysiological mechanisms and the development of potentially selective treatments would most likely only be gained by conducting studies using microvascular endothelial cells derived from the particular intra-ocular site(s) affected by the condition.

There is a paucity of information as to whether different human ocular microvascular endothelial cells demonstrate similarities in the intracellular mechanisms involved in angiogenesis. Previously, workers have shown a difference between human retinal endothelial and HUVEC cell secretory functions (prostaglandin I_2, and E_2) and their responses to high glucose concentrations in an attempt to understand the underlying mechanisms of proliferative diabetic retinopathy (Rymaszewski et al, 1992). In the investigation of the mechanisms of inflammatory eye disease, Silverman et al in 2005 showed differences in E-Selectin expression using nylon-based gene array by matched human iris and retinal microvascular endothelial cells after stimulation with inflammatory agents (LPS and TNF α). Similarly, Smith et al in 2007 used gene expression profiling to examine differences between matched retinal and choroidal endothelial cells as a means to determine underlying mechanisms of posterior ocular inflammatory diseases. They found an 8.9% difference in transcripts between retinal and choroidal ECs after stimulation with toxoplasma tachyzoites. Many of the

transcripts related to genes important in inflammatory processes, leukocyte trafficking and mediators of immune responses (ICAM 1, E Selectin, chemokines and VCAM 1), suggesting that the retinal vasculature is susceptible to inflammatory processes.

The present study had two main objectives. The first was to compare the gene expression profiles of human umbilical vein ECs with a number of different ocular microvascular endothelial cells to determine whether HUVECs are representative cells to use in ocular vascular research. Secondly, the gene expression profiles, particularly those of canonical cell signalling pathways of matched human choroidal, retinal and iris endothelial cells were compared to determine whether any differences in canonical pathway expression could be identified that could provide insights into our understanding of site-specific mechanisms of ocular angiogenic diseases and perhaps identify areas for future targeted therapies.

In this study, 3% of probesets were differentially expressed between HUVECs and ocular microvascular cells, with 1.4% of probesets being found to be up-regulated in HUVECs. While there was a wide range of different genes that were up-regulated, there was an enrichment of genes involved in embryonic development such as the homeobox genes. Interestingly, Murthi et al in 2007 found a differential expression of homeobox genes between HUVE and placental microvascular endothelial cells, with HLX1 (thought to be important in the development of B cells) being down-regulated in HUVECs (Murthi et al, 2007). This finding is not surprising as the umbilical vein and its endothelial lining is of foetal origin and forms part of the foetoplacental unit and is a high flow vessel with a thick wall containing elastin and muscle fibres, surrounded by Wharton's jelly, carrying oxygenated blood from the placenta to the foetus. It is therefore intrinsically involved in embryonic development. Homeobox (HOX) genes encode a range of transcription factors, found in clusters named A,B,C and D on four different chromosomes and their expression is spatially and temporally

regulated during embryonic development. Under this applicant's guidance, workers in the same laboratory have recently detected expression of HOX B7 and A9 protein in HUVECs by Western blotting, whereas no expression was detected in human retinal or choroidal ECs. Other up-regulated genes included those involved in encoding proteins involved in ECM interactions such as MMP 2, heparinase and fibrillin 2, and cytoskeletal proteins such as cytokeratin 18. These genes may be important given the growth and remodelling of the thick walled umbilical vein required during gestation. Specifically, fibrillins have been shown to be important in the attachment of endothelial cells to the elastin found in the ECM on the abluminal side of vessels, thereby aiding the anchoring of cells to underlying structures (Weber et al, 2002). Interestingly, some cytokeratins, which are intermediate filaments usually found in epithelial cells, have been found to be up-regulated in breast cancer by oestrogens via an oestrogen-response element close to the cytokeratin gene on chromosome 17 (Choi et al, 2000). Its up-regulation in HUVECs may therefore reflect its origin from an oestrogen rich environment. HUVECs also appear to express a distinct pattern of cell membrane components involved in cell signalling, such as the hepatocyte growth factor receptor (c-met). Hepatocyte growth factor (HGF) is a pro-angiogenic factor found in the placenta and is thought to be important in placental and foetal development, although its precise role is unknown (Dash et al, 2005). HGF receptors on HUVE cells may therefore be important in the vascular development of placental tissues. Similarly, ephrin B2 (the transmembrane ligand for the EphB4 receptor) was found to be up-regulated in HUVECs by other workers (Kim et al, 2002) and is one of a group of receptor tyrosine kinases found in abundance in placental tissue (Goldman-Wohl et al, 2004). They are thought to play a role in embryonal vascular and neuronal development and endothelial cell migration. Specifically, the ephrin B2 transmembrane ligand is found in abundance in the placenta, suggesting that the receptor's presence on HUVECs is important and may play a role in vascular development of the foetoplacental unit (Chennakesava et al, 2006). It is interesting to note that ephrin B2 is

usually thought to be an arterial-specific marker. Its presence on HUVECs is surprising until one recognises that the umbilical vein carries highly oxygenated (arterial) blood from the placenta to the foetus and that some think of HUVECs as a specific subtype of arterial ECs (T Gardiner, personal communication).

Approximately 1.6% of probesets were found to be up-regulated in ocular microvascular ECs compared to HUVECs, and again, while this included a diverse range of different genes, there were specific groups which were enriched. These include genes involved in immune responses (MHC class I and II and interleukin receptors), signal transduction and the cellular responses to stimuli. These differences may be explained by the different roles of HUVEC and ocular microvascular ECs. The ocular microvasculature is thought to be important as a barrier to blood borne pathogens where immune cells such as macrophages and lymphocytes, must interact with ECs to traverse this endothelial barrier to enter extravascular tissues. Immune mechanisms are also thought to be important in the pathogenesis of both diabetic retinopathy and wet AMD.

Given that the microvasculature is always undergoing remodelling and is the site of physiological angiogenesis, it is not surprising that a number genes involved in cell signalling (G proteins and phospholipase C) and interactions with the microenvironment such as those involved with the endothelial cell basement membrane (TIMP 3, collagens I and III) are differentially up-regulated. Under this applicant's guidance, workers in the same laboratory have recently detected expression of collagen 1 alpha1 sub unit protein in ocular MVECs by Western blotting, whereas no expression was detected in HUVECs, thereby adding weight to microarray findings. TIMP 3 is thought to modify breakdown of the extracellular matrix and type IV and VI collagen alpha chains, both of which are components of cellular basement membranes. A gene differentially expressed in ocular MVECs is angiopoietin 2, which is thought to modulate vascular stability and remodelling by the

antagonism of Tie 2 receptors and Sprouty which, in turn, is thought to modify the sprouting of growing vessels by interacting with receptor tyrosine kinases which have been stimulated by angiogenic growth factors such as VEGF (Cabrita & Christofori, 2008).

Proliferating ocular MVEC also appear to possess distinct intracellular signalling pathways not utilised significantly by HUVECs. These include pathways utilising components such as MAPKK 3, and PLC alpha 2. The significance of these alternative pathway components is currently unknown but they may provide selective targets for future interventions in modulating cellular function. Ocular microvascular endothelial cells also demonstrate up-regulation of a range of genes encoding cell surface proteins involved in the action of cytokines, cell adhesion and binding to the extracellular matrix. As the microvascular environment may be a site of inflammation, the up-regulation of cell surface proteins involved in leukocyte trafficking such as the mannose receptor 1 and versican may also be important in understanding ocular inflammatory diseases. The mannose receptor has recently been found on dermal MVEC but not HUVEC, reinforcing the significance of the current findings (Groger et al, 2000). All of these findings suggest that ocular MVECs are actively involved in communicating and remodelling their local surroundings and are distinctly different from macrovascular HUVE cells. This was suggested over 20 years ago by Klagsbrun and Folkman who observed that angiogenesis occurs in the microvasculature or capillaries, not in large vessels (Klagsbrun and Folkman, 1990). *In vitro* studies have subsequently shown that microvascular endothelial cells have a higher angiogenic potential than macrovascular ECs, either by their increased expression of matrix metalloproteinases (Jackson and Nguyen, 1997), their response to tumour angiogenesis factor (Keegan et al, 1982), IGF-1 (King et al, 1985) or VEGF (Bian et al, 2006).

This dichotomy in differential gene expression suggests that HUVECs are probably not a suitable substitute for ocular ECs in the study of the underlying mechanisms of ocular vascular diseases.

When matched, proliferating human retinal and choroidal endothelial cells were compared; we found that 8.4% of probesets were differentially expressed. Because of the importance of angiogenesis in a number of common blinding diseases, it was decided to concentrate on those genes important in the angiogenic response, in particular those of canonical signalling pathways,

The selective treatment of diseases relies on the identification of unique, or at least significantly different, attributes in the affected site compared with their surroundings. Finding significant differences between human retinal and choroidal ECs would therefore be advantageous for the selective treatment of either predominantly retinal or choroidal neovascular disorders, while at the same time leaving juxtaposed ECs intact.

The current study shows that retinal ECs demonstrate selective up-regulation of some components of the wnt-5 signalling pathway such as a frizzled homolog and WNT5a. This is a poorly understood pathway that may regulate cell proliferation, apoptosis and branching morphogenesis (Masckauchán et al, 2006; Masckauchán et al, 2005). The importance of this pathway to human retinal vasculogenesis has recently been described (Parmalee & Kitajewski, 2008). Human retinal ECs also demonstrated up-regulated gene expression of a wide range of growth factors such as VEGF, PDGF-C, PDGF-B chain and IL-32. This would imply a local paracrine function for retinal ECs either by stimulating surrounding endothelial cells or by stimulating their surrounding pericytes which are known to be dependent upon PDGF for their maintenance (Jo et al, 2006). It is now known that there is extensive communication between ECs and pericytes and each cell type relies on the other for many of its functions and perhaps even

survival. Pericytes are embedded within the basement membrane of endothelial cells and direct communication may occur via peg-socket junctions which contain gap junctions (Cuevas et al, 1984). What this communication consists of is currently unknown. As mentioned previously, endothelial cells secrete PDGF-B which is thought to promote proliferation and recruitment of pericytes to newly formed vessels. Knockout models show that if this fails to occur, then vascular dysfunction ensues with endothelial hyperplasia and the formation of abnormal endothelial junctions and leads to perinatal death (Hellstrom et al, 2001). It is also thought that TGF-β is secreted by endothelial cells and plays a role in the differentiation of vascular mural cells such as pericytes. It is also thought to act in an autocrine fashion acting via endoglin (a type of TGF receptor) to affect endothelial cell differentiation and proliferation. Finally, there is evidence to suggest that Tie 2-Angiopoietin 1 (Ang-1) interactions are important in EC-pericyte communication. Tie-2 is highly expressed on endothelial cells and its ligand, Ang 1 is expressed by pericytes. Knockout models show that this interaction is essential for vessel maturation and stability (Suri et al, 1996).

Retinal ECs also demonstrate significant expression of growth factor receptors and binding proteins such as the insulin and IGF-2 receptors; stromal-cell-derived-factor receptor (CXCR4); IGF binding proteins 2 and 7; and the interferon gamma and IL-4 receptors. These findings may be important in clinical practice as retinal neovascularisation seen in diabetes appears in part to be related to diabetic control and insulin and IGF levels (Meyer-Schwickerath et al, 1993; Grant et al, 1986). This hypothesis is given additional weight because, when under the guidance of the applicant, workers in Nottingham have recently demonstrated in a functional assay, that IGF 1 stimulates the proliferation of human retinal ECs to a similar degree to VEGF (similar to the findings of Grant et al, 1993a and 1993b), whereas IGF-1 has little effect on matched human choroidal ECs (see later chapter). Other workers have also shown an inhibitory effect on retinal

neovascularisation by agents that block growth hormone or by removal of the patient's pituitary gland (thereby down-regulating circulating IGF 1 levels) (Sharp et al, 1987; Merimee et al, 1970; Wright et al, 1969; Palii et al, 2007). Interestingly, our laboratory found that equimolar concentrations of IGF-1 and VEGF appeared to have synergistic effects on the proliferation of human RECs whereas human CECs did not demonstrate any additional effect above that of VEGF alone. This effect on human RECs may be explained by the findings of Smith et al in 1999, when her group looked at the effects of VEGF and IGF-1 on bovine RECs and found evidence of interactions between the intra cellular signalling pathways for each growth factor, and suggested that IGF 1 is required for maximal neovascularisation to be induced by VEGF. Finally, *in vitro* studies on bovine retinal endothelial cells have demonstrated a significant effect of IGF-1 on cell survival and proliferation (Castellon et al, 2002). IGF-1 is also strongly implicated in the pathogenesis of retinopathy of prematurity (ROP), such that researchers are suggesting manipulation of IGF-1 levels soon after birth as a treatment for this condition.

It is interesting that the proliferation of human CECs does not appear to be dependent on IGF (Browning et al, 2007) and *in vivo*, choroidal neovascularisation does not appear to respond to agents that inhibit IGF (Papadaki et al, 2003), despite a positive effect in an animal model (Bezerra et al, 2005). In contrast, this work has shown that choroidal ECs demonstrate up-regulated expression of the FGF receptor 1. This is important for binding a number of FGF sub-types which have been shown to be important for the development of the choroidal vasculature (Rousseau et al, 2003). Choroidal ECs also appear to differentially express genes involved in intra-cellular signalling such as G-protein-coupled receptor kinase, MAPK 1 and G-protein-coupled receptor 89. This would imply that compared to retinal ECs, proliferating choroidal ECs may be utilising slightly different intra-cellular signalling pathways.

In this study the retinal and choroidal endothelial cells were proliferating under exactly the same conditions, but there were consistent variations in the expression of genes in a number of ubiquitous endothelial cell signalling pathways such as the PI3K/ AKT, ERK/MAPK and mTOR pathways. While both retinal and choroidal endothelial cells appear to utilise all of these pathways, many of the differentially expressed probe sets of the PI3K/AKT pathway were up-regulated in choroidal ECs, suggesting that this is a potentially more important pathway for these cells and perhaps a potential target for inhibitory compounds. Examination of the ERK/MAPK signalling pathways also showed that choroidal ECs demonstrated up-regulation of MAPK 1. At present, the significance of these findings is uncertain but again, it may offer a more selective method of inhibiting retinal or choroidal angiogenesis. This author and co-workers have recently found that the potency of the VEGF 121 and 165 isoforms was greater when used to stimulate proliferation of human retinal endothelial cells compared with matched human choroidal endothelial cells (Stewart et al, 2011). This suggests that components of canonical pathways differentially up-regulated in retinal ECs such as the wnt pathway (WNT5A and Frizzled homologue) and the ERK/MAPK pathway (Integrin alpha 3 and phospholipase A_2 may account for the increased proliferation and may be important drivers for angiogenesis in the retina. This is an area that requires further research as, to date, little is known about the fine details of cell signalling pathways activated in proliferating human choroidal, retinal and iris endothelial cells. Limited data from work on bovine choroidal endothelial cells, stimulated by VEGF, showed activation of MAPK/ERK via PLCγ (Zubilewicz et al, 2001) However, these are very common stimulatory pathways for many different types of cells and so the results are not unsurprising. Again, this is an area where further comparative work is required.

Another specialised feature of the retinal vasculature ECs is the formation of the inner blood retinal barrier (similar to the blood brain barrier). Unlike the choroidal vasculature, which allows small molecules to extravasate from the vascular compartment via fenestrations in choriocapillaris endothelial cells, the retinal vasculature possesses very tight cell junctions between adjacent endothelial cells in order to stop small molecules and cells extravasating unless very specific situations exist. The integrity of this endothelial barrier function is dependent on tight junctions (TJ), composed of molecules such as ZO-1, occludin, cadherins and claudins. The subtypes of TJ molecules found in human retinal ECS are poorly understood. However, Luo et al have recently described some of the molecules found in the murine retinal vasculature. They found that claudins 1,2 and 5 appear to be prominent in retinal ECs whereas claudins 3,4,12,22 and 23 were not detected. In our experiments, compared with human choroidal ECs, it was found that in a similar fashion to Luo et al, human retinal ECs preferentially expressed Claudin 5 (4.2 fold expression), ZO-1 (2.7 fold expression). Unlike Lou et al however, we also found that Claudin 12 was up-regulated (2.4 fold). In 1998, Russ et al demonstrated that human retinal ECs expressed Cadherin 5 (VE Cadherin), suggesting that it was EC specific. In our experiments we did not find it to be preferentially expressed by retinal ECs (it being expressed to the same extent by both retinal and choroidal ECs). We did find however, that the retinal ECs preferentially expressed Cadherin 2. (2.9 fold expression). This is more usually associated with neuronal tissue, hence its other name N-Cadherin and is a new finding. Our results otherwise agree with those published for murine retinal ECs.

Both retinal and choroidal ECs demonstrate up-regulated expression of genes involved in collagen and integrin synthesis. Retinal ECs show up-regulated expression of the alpha chains of collagens XII, VI, V, IV (alpha 1) and III, while choroidal ECs express collagen IV alpha 3 chains. This would suggest that the basement membranes of retinal and choroidal ECs may be different.

Our results also confirm the findings of others that retinal endothelial cells preferentially express genes involved in immune functions (up-regulation of signalling pathways involved in the interferon and interleukin 2 and 4 receptors). There is evidence to suggest that proliferative diabetic retinopathy is a pro-inflammatory condition with lymphocyte infiltration of blood vessels early in the disease (Joussen et al, 2001; Grisanti et al, 1994). There are also elevated levels of inflammatory cytokines such as IL 6 and IL 12 and chemokines CXCL8, CCL2, 4 and 5 in the vitreous of such patients in addition to VEGF (Banerjee et al, 2007; Wakabayashi et al, 2010). Interestingly, Interferon was not detected in the vitreous samples of these patients with retinal neovascularisation which may be of significance as interferon is thought to be anti-angiogenic.

Another striking finding from this study was the similarity between choroidal and iris endothelial cells. The iris and choroid are considered to be different parts of the same tissue, the uveal tract, although the vascular systems in which they belong have entirely different roles. Previous workers looking at the characteristics and role of the iris vasculature in anterior uveitis have shown that human iris ECs constitutively express genes involved in the immune response such as Toll-like receptor 4, ICAM-1 and 2, and demonstrate up-regulation of E-Selectin after LPS stimulation (Silverman et al, 2001; Brito et al, 2004). To our knowledge, however, no comparison has been made with matched choroidal ECs. However, Hageman et al in 1991 did show that choroidal ECs stained positive for carbonic anhydrase IV whereas the iris vasculature did not. In our study, we did not find a significant difference between the expression of any carbonic anhydrase isoenzyme probesets in these two cell types. The small number of differentially expressed probe sets did not show any significant aggregation into known canonical signalling pathways. The down-regulation of genes for SMAD 9, Serpine 1 and hyaluronan synthase suggests that iris ECs interact slightly differently with their surroundings, particularly with respect to the turnover of extracellular matrix and the

level of inhibition of local tissue plasminogen activation and fibrinolysis compared with choroidal ECs. Interestingly, SMAD proteins are intracellular signalling molecules, activated by Bone Morphogenetic Protein (BMP) receptors. These in turn are activated by members of the TGF-beta superfamily (such as BMPs). SMAD 9 has been found to date to be expressed in the heart, lung brain and liver. No ocular expression has been reported (to date). Specific mutations in SMAD 9, leading to a loss in function, have been found in a number of cases of primary pulmonary hypertension (Nasim et al, 2011). This disorder is characterised by the abnormal plexiform growth of endothelial cells which in turn leads to resistance to flow. It appears that a lack of SMAD 9 leads to the abnormal growth of blood vessels. It is also known that Bone Morphogenetic Protein 4 is important for ocular development via the stimulation of SMADs and has also been found to inhibit choroidal angiogenesis in an animal model (Xu et al, 2012). Again, SMADs (including SMAD 9) appear to be acting as an indirect anti-angiogenic factor. Down regulation of SMAD 9 in our experiments would therefore suggest a lack of receptor activation by BMPs in iris ECs which in turn may indirectly be pro-angiogenic. This is an area where future work could be considered.

We found no significant difference in the expression of genes involved in inflammation or immune function as alluded to by other workers. This may be due to a lack of specific stimulation by factors such as LPS.

Because of the marked similarity between iris and choroidal endothelial cells, most of the differences noted between retinal and iris ECs were similar to those seen between retinal and choroidal ECs. There were a few notable exceptions, however, such as in the expression of probesets associated with genes involved in immune or inflammatory signalling pathways. It was shown that proliferating retinal endothelial cells showed up-regulated expression of genes involved in interferon and IL22 signalling compared to iris ECs. This finding adds further weight to the findings of Silverman et al (2005) who found differences in

E-selectin expression using a nylon-based gene array by matched human iris and retinal microvascular endothelial cells after stimulation with inflammatory agents (LPS and TNF α). They found that retinal ECs consistently expressed higher levels of E-selectin mRNA (and protein) after stimulation, compared with matched iris ECs. Similarly, Smith et al (2007) used gene expression profiling to examine differences between matched retinal and choroidal endothelial cells as a means to determine underlying mechanisms of posterior ocular inflammatory diseases. They found an 8.9% difference in transcripts between retinal and choroidal ECs after stimulation with toxoplasma tachyzoites. Many of the transcripts up-regulated in retinal ECs related to genes important in inflammatory processes, leukocyte trafficking and mediators of immune responses (ICAM 1, E selectin, chemokines and VCAM 1). These findings underlie the fact that retinal endothelial cells are probably important in immune function and/or leukocyte trafficking within the retina and may explain why the retinal vasculature may be preferentially susceptible to inflammatory disorders.

The present study has a number of strengths over previous studies. Firstly, the use of purified, unpassaged cells means that the *in vitro* gene expression profiles are as close as possible to the *in vivo* proliferative state. It is known that endothelial cell phenotype changes quickly *in vitro* (Miebach et al, 2006; Kalashnik et al, 2000; Shima et al, 1995) so it would not be unexpected for human ocular vascular endothelial cell gene expression profiles to change in a similar manner. Secondly, the ocular endothelial cells were matched from the same donors, thereby reducing the effect of inter-individual gene expression differences in this study and thirdly, the microarray expression results were validated by RT-PCR (and later by protein expression and functional studies). While the isolation of endothelial cells from cryosections would have allowed the “true” *in vivo* gene expression to be measured, the isolation of cells by techniques such as laser capture is hampered by its small sample size, contamination by non-endothelial

cells and possible post mortem changes in RNA quality. Such a technique also has the disadvantage that the profile of proliferating cells (important in the study of pathological angiogenesis) cannot be easily measured, as most endothelial cells *in vivo* are in a quiescent state.

A criticism of this study is that the expression levels of probesets and the differences between endothelial cells simply reflect different rates of proliferation and cell growth between cells. In answering this criticism, it should be remembered that the cells were matched for donor, growth conditions and confluency. Although not measured directly, all cells reached sub-confluence (approx 80%) at approximately the same times (5-7 days) as described in chapter 2. Any small differences in the rate of growth of cells and therefore reaching the required level of confluence would be unlikely to account for the 10 fold differences in gene expression between iris/choroid and iris/retina. A further feature designed to eliminate low level fluctuation in the differences in gene expression is by only accepting differential expression if all 3 samples for each cell type demonstrate significant results.

In conclusion, this study has demonstrated a significant difference in gene expression between proliferating HUVEC and ocular microvascular ECs. This suggests that extrapolation of *in vitro* results derived from HUVECs to the investigation of ocular diseases should be avoided. Microvascular ECs derived from different sites within human eyes also demonstrated heterogeneity in gene expression. These results may provide insight into different ocular vascular disorders and allow development of new, targeted treatment strategies.

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Chapter 4

The isolation and characterisation of macular inner choroidal endothelial cells

Introduction

The choroid forms part of the uveal tract within the eye and lies between the outer sclera (white of the eye) and the inner layer composed of the retina, and the retinal pigment epithelium (RPE). It is approximately 100-220μm thick, depending on the particular site in the eye, and is composed almost entirely of blood vessels with some supporting cells such as fibroblasts and melanocytes. As previously described, the choroid has historically been sub-divided into 3 layers: the outer layer of large vessels (Haller's layer), a middle layer of medium sized vessels (Sattler's layer) and an internal layer adjacent to Bruch's Membrane composed of capillary vessels (choriocapillaris).

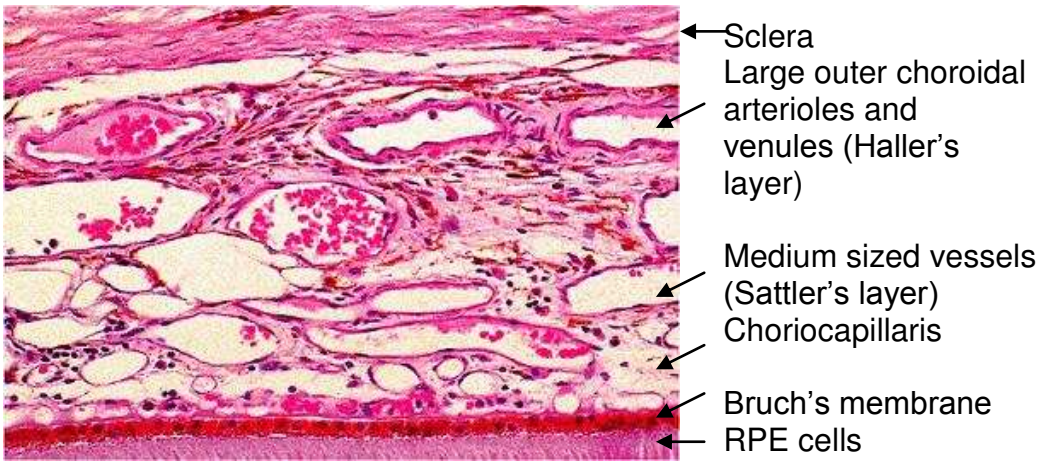

Fig 4.1. A haematoxylin and Eosin stained section of human choroid and RPE demonstrating the different calibres of the choroidal vasculature (with permission of Springer images)

The choroid derives its blood supply from the short posterior ciliary arteries, which are themselves a branch of the ophthalmic artery. This means that the choroid has a very rich blood supply which is effectively derived from a branch of the internal carotid artery (the main function of which is to supply the brain). The choriocapillaris has a honeycomb structure of numerous inter-communicating channels which serve to supply oxygen and nutrients to the overlying RPE cells and photoreceptors. While it appears as a diffuse interconnecting sheet on

electron microscopy (see chapter 1), in reality, it has a lobular structure, with each lobule being supplied by a central arteriole and drained by a peripheral lobular venule. The main “mass” of the choroid is made up of sequential subdivisions of the supplying and draining veins which ensure a rich blood supply to the choriocapillaris (see below). It is thought that the choriocapillaris is important in certain eye disorders as they appear to occur selectively within this vascular bed e.g. wet AMD and certain inflammatory choriocapillopathies such as MEWDS (multiple evanescent white dot syndrome). This suggests that the choriocapillaris is “different” from the rest of the choroidal vasculature.

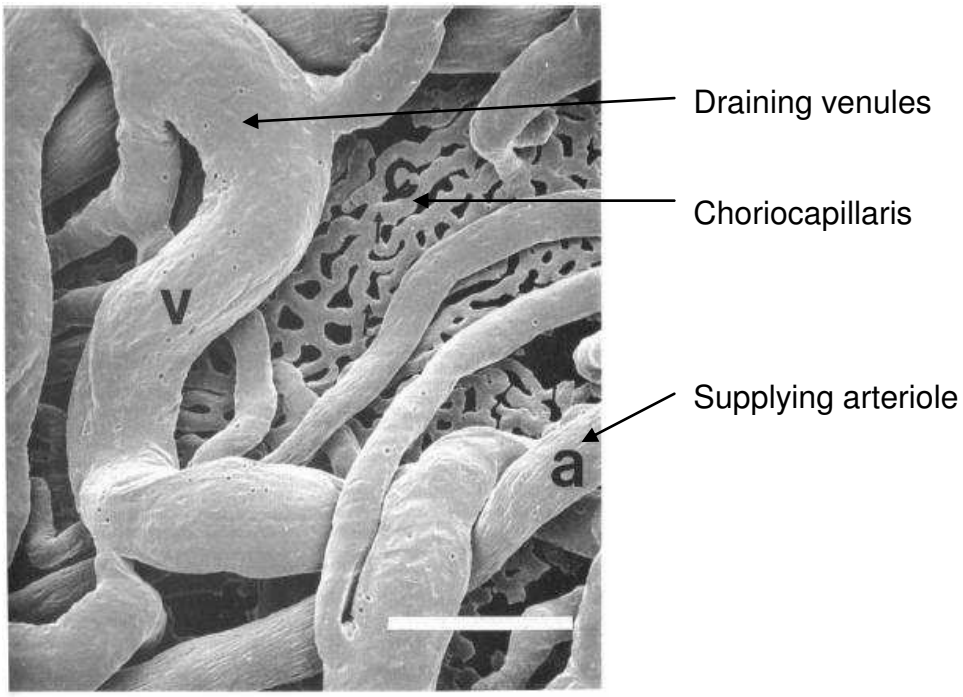

Fig 4.2. Scanning electron micrograph of a cast of human choroidal vasculature viewed from the scleral side showing draining choroidal veins (v), arterioles (a) and the choriocapillaris (c) visible through the gaps between the large vessels (from Olver J, 1990 Eye, 4:262, with permission).

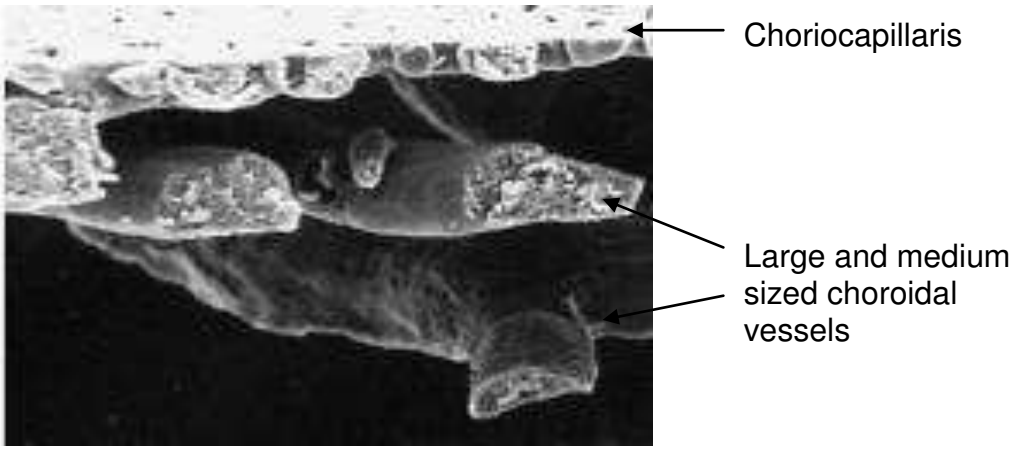

Fig 4.3. Scanning electron micrograph of a cast of human choroidal vasculature viewed from the side showing the large and medium sized choroidal vessels (Haller's and Sattler's layers) and the choriocapillaris visible at the top of the image (From Olver J, 1990 Eye, 4:262, with permission).

The endothelial cells lining the choriocapillaris have an unusual property when compared with the other choroidal, ocular and most other endothelial cells (both micro- and macro-vascular); they are fenestrated on the side facing Bruch's membrane and the RPE cells. Fenestrations are round or oval transcellular holes through the thinnest parts of the endothelial cell cytoplasm. They are found in areas where a high rate of partially selective exchange of components between the intra and extravascular compartments is required. This exchange is usually limited to water and small solutes with passage of larger components such as lipids and proteins being inhibited. The fenestrations within the choriocapillaris therefore allow the rapid transfer of nutrients through Bruch's membrane and the RPE, into the highly metabolically active photoreceptors. To date, three types of fenestrations have been described (Satchell and Braet, 2009):

Type 1 fenestrations are 60-70 µm in diameter and are covered by a thin diaphragm. The glycoprotein - plasmalemmal vesicle associated protein, 1 (PLVAP-1), is thought to be a major component of this diaphragm. Each fenestration is surrounded by a cytoskeletal lattice. Type I fenestrations are found in the endothelia of endocrine glands,

gastrointestinal mucosa and renal tubular capillaries. It is thought that the fenestrations found in the human choriocapillaris may be type I by virtue of the expression of PLVAP in gene array experiments (see later) and the possession of a diaphragm.

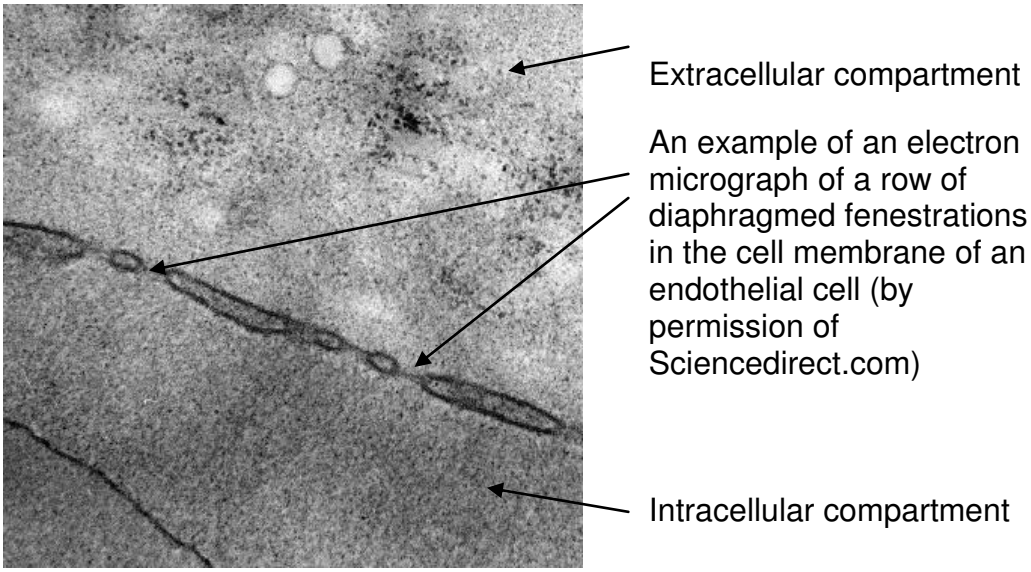

Fig 4.4. An electron micrograph of a renal glomerular endothelial cell membrane demonstrating diaphragmed fenestrations (with permission of Springer images)

Type II fenestrations are found in discontinuous endothelia such as that found in the spleen, liver and bone marrow. These fenestrations are wider (up to 200µm diameter) and do not have diaphragms or express PLVAP.

Type III fenestrations are found in the endothelium of renal glomeruli. They are of a similar size to type I, but like type II, do not express PLVAP or have diaphragms.

While possession of fenestrations is thought to be an endothelial specific marker, of use in the confirmation of EC lineage, the *in vitro* study of fenestrations is somewhat limited by their paucity of expression in many ECs derived from vascular beds known to be fenestrated *in*

vivo. The expression of fenestrations also appears to be lost by prolonged culture and sub-culture. Experiments are therefore best carried out on fresh tissue or unpassaged cells (Satchell and Braet, 2009). Interestingly, fenestrations can also be induced *in vitro* in ECs that do not normally express them. This appears to be easier to perform in microvascular than macrovascular ECs (Esser et al, 1998) and is thought to be dependent on VEGF. Indeed, endothelial cells that are not normally fenestrated such as those found in the cremaster muscle and skin, can be induced to form them by the application of VEGF (Roberts & Palade, 1995). In the eye, VEGF secreted by the RPE is thought to be important for maintaining the state of fenestration of the choriocapillaris ECs as inhibition of intraocular VEGF by bevacizumab (an anti-VEGF antibody) leads to a reduction of choriocapillaris fenestration density (Peters et al, 2007).

To better understand the angiogenic mechanisms important in the development of wet AMD, the leading cause of blindness in those over 65, it would be advantageous to study the behaviour of endothelial cells derived from the site where the disease occurs, that is within the inner choroid of the human macula. This requirement has been reinforced by the previous finding in chapter 3 of significant heterogeneity in different human ocular endothelial cells. What is not known is whether this heterogeneity exists within the choroid at sites known to be predisposed to angiogenic disease (wet AMD).

The isolation of ECs from the choriocapillaris/inner choroid has previously proved difficult. To date, workers have either used complete choroidal enzyme digests which contain all ECs, or have attempted selective enzyme separation of the choriocapillaris away from the underlying tissue (Liu & Li, 1993). This latter technique was conducted in bovine eyes which have a tapetal membrane underlying the choriocapillaris, making isolation simpler than in humans. Selective isolation in humans is also hampered by the increased susceptibility of the choroidal tissues to trypsin/collagenase digestion. This makes

selective isolation extremely difficult to achieve reliably and consistently.

A new technique is therefore required to separate the choriocapillaris/inner choroid from the underlying structures. Interestingly, it has been known for over 100 years that the human choroid is made up of a lamellar structure. In 1892, Nuel described a natural cleavage plane between the layers of Haller (inner choroid) and Sattler (outer choroid) (Nuel, 1892), while in 1912, Saltzmann noted that dissection of the choroid was achieved most easily by teasing apart the layers from the outside (Saltzmann 1912). In order to exploit this phenomenon, two different techniques were tried to reliably separate the layers so that viable ECs could be extracted from the two layers of tissue. The first technique was the use of a Vibratome, which aimed to section the different horizontal layers of adult human choroid. The second technique was an attempt to isolate the desired ECs by manual dissection and cell isolation using paramagnetic beads coated with endothelial selective antibodies after enzyme digestion of the choroid.

After isolation using the latter technique, the macular inner choroidal ECs were characterised in terms of their antigen expression (determined by selective binding of fluorescent labelled antibodies), their ability to form capillary-like tubes in a 3D collagen rich matrix and the presence of fenestrations. After isolation and characterisation, the isolated human inner choroidal endothelial cells could then be used in experiments to aid our understanding of the mechanisms of inner choroidal neovascularisation.

Methods

Vibratome sectioning

The vibratome (Campden Instruments, Loughborough, UK) is a vibrating keratome that enables the sectioning of living tissues into sheets for further physiological experimentation. Living tissue is held within gelatine blocks which is then subject to vibratome sectioning. It has been used successfully to produce functioning sections of the brain (Snyder et al, 2001) and the outer retina (Tezel & Kaplan, 1998; Kaplan et al, 1997; Silverman & Hughes, 1989; Ghosh et al, 1999) which have then been used for subsequent intraocular transplantation. Since retina is of a similar thickness to choroid, it was felt that this technique may be able to produce viable sheets of inner choroid.

Gelatin blocks (10%) (Sigma Alrich, UK) were cast in silver foil and cooled at 4ºC to allow solidification. Flat sections of choroid, approximately 8mm in diameter, were removed from globe flat mounts using an 8mm biopsy punch and layered onto the gelatin block. The choroid was then covered with liquid gelatin (10%) and again allowed to solidify at 4ºC. The gelatin block was then mounted onto the vibratome chuck with superglue and sectioning at various thicknesses (20-50µm) was attempted. Despite varying the gelatin concentration and varying the thickness of the cut section, it was not possible for the blade to enter the choroid to produce a flat sheet of tissue. On all attempts, the blade either passed over the choroid sheet, pressing it further into the gelatin, or simply lifted the whole section out of the gelatin. The reason for this may be that choroid is composed of a dense network of blood vessels held together in a fibrous network which impedes the easy passage of the vibratome blade. This technique was therefore abandoned.

Manual dissection

Human posterior segments were dissected as described in chapter 2 and the vitreous gel removed in order to give access to the posterior segment. A full thickness macular sample, centred on the fovea, consisting of choroidal and scleral tissue was removed with an 8mm sterile punch and transferred to a petri dish. The macula was identified by the presence of yellow macular pigment. The choroid was then gently teased from the attached sclera and turned upside down with the outer choroid facing upwards. With the use of a dissecting microscope (x10 magnification) and fine forceps, a natural cleavage plane was found that allowed the outer choroidal vessels, along with adherent pigmented fibrous tissue, to be peeled off, leaving the relatively non-pigmented inner choroidal vessels and choriocapillaris/Bruch's membrane complex. The tissue samples (inner and outer choroid) were washed 3 times in isolation medium and the vascular endothelial cells were isolated by the method described previously using anti CD31 coated paramagnetic beads (Chapter 2). Matched endothelial cells from the inner and outer macular choroid and peripheral inner choroid were routinely isolated and cultured for further experiments using this technique (described in detail in Chapter 2).

Because of their importance in the pathogenesis of wet age-related macular degeneration, human macular inner choroidal endothelial cells were chosen for further characterisation with a view to further experiments into macular inner choroidal endothelial cell proliferation.

Human inner choroidal endothelial cell characterisation

Immunohistochemistry

A small sample of trypsinised cells, suspended in EGM-2MV, was placed onto sterile glass cover slips (VWR Ltd, Poole, UK) coated with 1% gelatin (Sigma) (cover slips were immersed in 1% gelatin in sterile PBS for 30 mins at 37℃, then washed gently in sterile PBS and dried in a laminar flow hood). After allowing 3 hours for cells to attach, they were fixed in ice cold methanol at -20℃ for 20 minutes. A standard two stage immunofluoresence technique was applied using the primary antibodies listed below in table 4.1. The primary antibodies were left in contact with the cells for 60 minutes before washing 4 times with sterile PBS. The secondary antibodies used were: rabbit anti-mouse F(ab')2 fragment fluorescein isothiocyanate (FITC) conjugated (Dako) at a dilution of 1:20 when used to visualise all primary antibodies except swine anti-rabbit F(ab')2 fragment FITC conjugate (Dako) at a dilution of 1:20 which was used to visualise the anti-vWf primary antibody. The secondary antibodies were left in contact with the cells for 60 minutes before washing 4 times with sterile PBS. The slides were then mounted in glycerol containing 2.5% 1-4 diazabicyclo[2,2,2] octane (DABCO) (Sigma –Aldrich) and observed by confocal fluorescence microscopy (Leica TCS0D, Leica, Milton Keynes, UK). Negative primary antibody controls were conducted by replacing the primary antibody with either a non-specific anti-rat epitope murine IgG1 antibody, rabbit immunoglobulin fraction or PBS. Additional negative controls were conducted using the appropriate primary and secondary antibodies on human RPE cells. In assessing the identity and purity of the endothelial cells, at least 500 nucleated cells were counted after staining with each antibody.

It was felt important to characterise any contamination from other cells found within the choroidal stroma (fibroblasts and RPE cells), therefore human RPE cells and Tenon's capsule fibroblasts were used as controls and staining of samples for αSMA (for RPE cells) and fibroblast surface protein (for fibroblasts) was conducted. E-Selectin expression on endothelial cells was examined after exposure to TNF-α (100pg/ml) for 1 hour.

Table 4.1. Primary antibodies used in the study of human inner choroidal endothelial cells

Epitope	**Class**	**Dilution**	**Source**
CD31	murine IgG1	1:20	Dako
vWf	rabbit Ig fract	1:200	Dako
VEGFR1	murine IgG1	1:100	Sigma-Aldrich
VEGFR2	murine IgG1	1:800	Sigma-Aldrich
αSMA	murine IgG1	1:50	Dako
Fibroblast Surface Protein	murine IgG1	1:200	Dako
E-Selectin	murine IgG1	1:20	Serotec
Negative control	murine IgG1	1:20	Dako
Negative control	Rabbit Ig fraction	1:200	Dako

Transmission electron microscopy

A sample of inner choroidal macular endothelial cells was grown to near confluence on 35mm fibronectin coated culture plates (Becton Dickinson, Oxford, UK) as previously described. The cells were then fixed in situ by immersion in 2.5% glutaraldehyde (in 0.1M cacodylate buffer, pH 7.4) for 16-24 hours and processed for transmission electron microscopy (TEM). The fixed cells, still attached to the plastic culture plate, were cut into 1mm thick slices and washed with cacodylate buffer, followed by secondary fixation in 1% osmium tetroxide for 1 hour. The attached cells were then dehydrated in ascending concentrations of ethanol, followed by infiltration and embedding in Epon resin before polymerization at 60℃ for 16 hours. Suitable areas for TEM were selected from 0.5µm toluidine blue stained sections. After they were trimmed, 80nm sections were cut and mounted on copper grids before double staining with uranyl acetate and lead citrate. A transmission electron microscope (model 1010; JEOL, Welwyn Garden City, UK) was used to observe the prepared sections.
The transverse sections of macular inner choroidal endothelial cells were examined specifically for fenestrations and *in vitro* tube formation

An important property that appears to be limited to vascular endothelial cells is their ability to coalesce into capillary-like tubes in a basement membrane matrix such as Matrigel (BD Biosciences, Oxford, UK). This property can therefore be utilized to identify isolated cells as originating from an endothelial lineage. Matrigel is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma. This is a tumour rich in extracellular matrix proteins such as laminin, collagen IV, heparin sulphate, entactin and nidogen. It also contains TGF-beta, fibroblast growth factor and tissue plasminogen activator. The product has the property of rapidly forming a gel at 22-35℃ and should therefore be used as close as possible to 4℃ until ready to gel, at which point it is brought up to room

temperature. During use, the product should be keep on ice, and used with cooled pipette tips, plates and tubes.

In this experiment, a 1:1 mixture of chilled Matrigel and endothelial growth medium (EGM2-MV) was dispensed into pre-chilled wells of a cooled 96 well plate. The mixture was allowed to solidify at 37℃ for 30 minutes before macular inner choroidal endothelial cells, suspended in endothelial growth medium (EGM2-MV) were seeded at a density of 4.8 x 10^4 per well. The wells were observed hourly for the formation of tubes. For transmission electron microscopy, the growth medium was removed and replaced by 2.5% glutaraldehyde (in 0.1M cacodylate buffer, pH 7.4) for 16-24 hours at 37℃. The fixed Matrigel was then gently removed from the well and processed as described previously.

Results

Human posterior segments from 6 different donors were used. The ages of the donors (years) and the lengths of time from death to endothelial cell isolation (hours) were 45 (52), 76 (42), 70 (43), 37 (60), 83 (60) and 56 (60).

Toludine blue staining of sections of human choroid

After manual dissection of samples of full thickness choroid, light microscopy showed the samples to comprise of Bruch's membrane, along with adherent choriocapillaris and inner choroidal vessels. The large outer choroidal vessels were reliably and reproducibly removed. Examples of the pre and post dissection toluidine stained choroid are shown in 4.5a and 4.5b respectively.

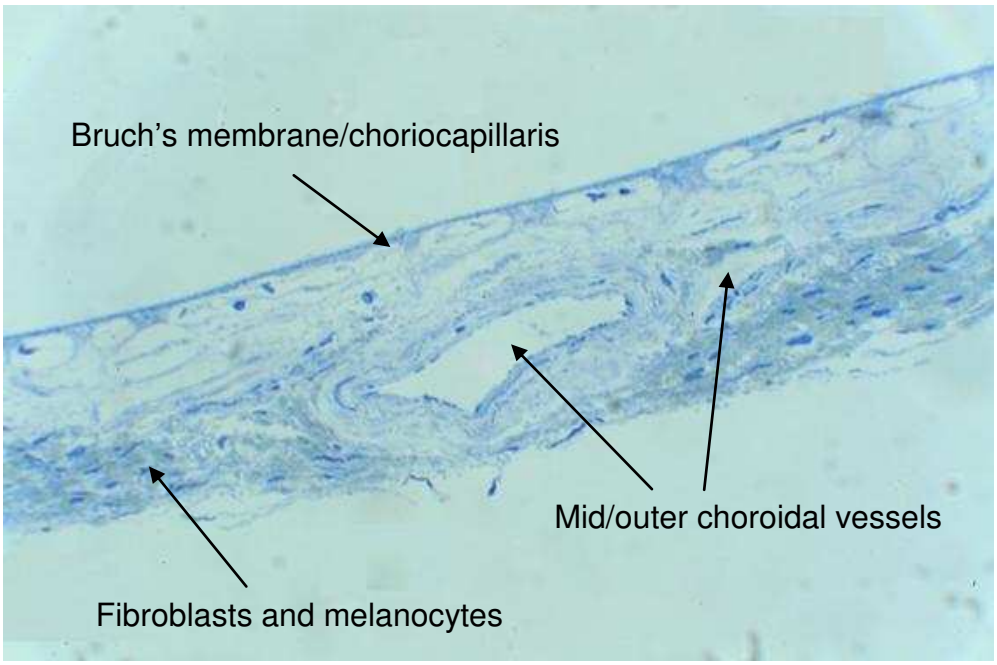

Fig. 4.5a. Histological section of human sub-macular choroid stained with toluidine blue before removal of the outer choroidal vessels showing the dark blue staining of Bruch's membrane with the underlying fine complex of the choriocapillaris and the larger calibre middle and outer choroidal vessels. The nuclei of the supporting choroidal fibroblasts and melanocytes can also be seen (all arrowed.)

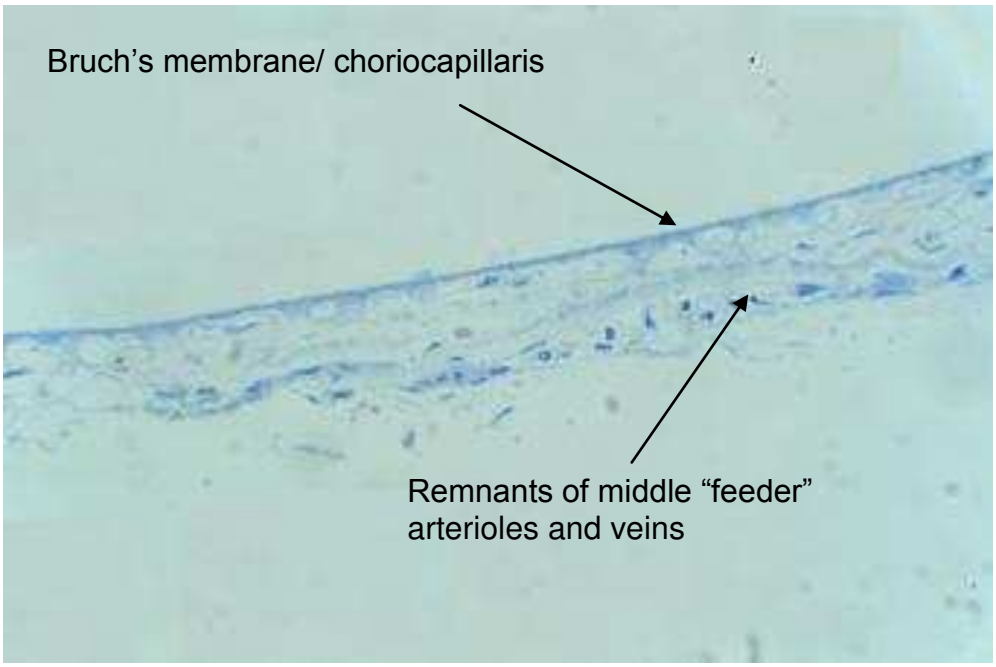

Fig. 4.5b. Histological section of human sub-macular choroid stained with toluidine after removal of the outer choroidal vessels. The residual Bruch's membrane, choriocapillaris and inner choroidal vessels can be seen. Note the absence of large outer choroidal vessels.

Immunohistochemistry

The isolates of macular inner choroidal endothelial cells stained positive for CD31, vWf, VEGFR1, VEGFR2 and E-Selection after stimulation with TNFα. They did not stain with mouse anti-rat negative control, αSmooth muscle actin or fibroblast surface protein. Selected photographs of macular inner choroidal ECs along with the appropriate negative controls are shown in figures 4.6-4.21. As important as the fact that the cells stained positive or negative for a certain antigen, is an understanding of the distribution of the stained antigen and whether the findings are compatible with the knowledge of this protein within endothelial cells.

The macular inner choroidal ECs stained positive for vWf in a granular pattern within the cell cytoplasm. On transmission electron microscopy, no Weibel-Palade bodies were seen. The cells also stained positive for

CD31 (PECAM), with staining heaviest at endothelial cell-cell junctions while VEGF receptors 1 and 2 stained positively across the entire cell, with strong staining particularly around the nucleus (this may represent staining within the Golgi, where proteins are modified after synthesis). The cells were also positive for E-Selectin after stimulation with TNF alpha (the RPE cell control did not show similar up-regulation). The most intense staining was seen around the nucleus, perhaps because of the abrupt up-regulation of protein synthesis and modification within the Golgi. The ECs did **not** demonstrate significant binding of alpha smooth muscle actin (a marker of RPE cells) or fibroblast surface protein (a marker of fibroblasts). Human RPE cells and Tenon's capsule fibroblasts stained positive for alpha smooth muscle actin and fibroblast surface protein respectively.

Matrigel tube formation

Human macular inner choroidal ECs formed capillary-like tubes within 3 hours of suspension (Fig 4.22). By 6 hours the tubes had begun to break up. Control Tenon's capsule fibroblasts did not show any evidence of tube formation. When samples of cells were cultured in Matrigel plugs and processed for transmission EM, numerous lumina were detected when tubes were cut in cross section. In some instances, at least 3 different cells were seen to make up the tube wall (Fig 4.23) and were joined by junctional complexes. This suggests that the tubes were formed by an active process on the part of the cells and had not simply occurred by random cell aggregation.

Fenestrations

Cells were cultured on gelatine coated plastic cover slips in growth medium. They were then fixed and examined by TEM. At no point were electron dense Weibel-Palade bodies found. Scattered throughout the cells, particularly at points where they were at their thinnest were

diaphragmed fenestrations (Fig 4.24). These were seen as gaps within the cell membranes joined by electron dense lines (the diaphragm). Compared to published pictures of fenestrations in freeze fractured ECs, the fenestrations in our cells were much less abundant.

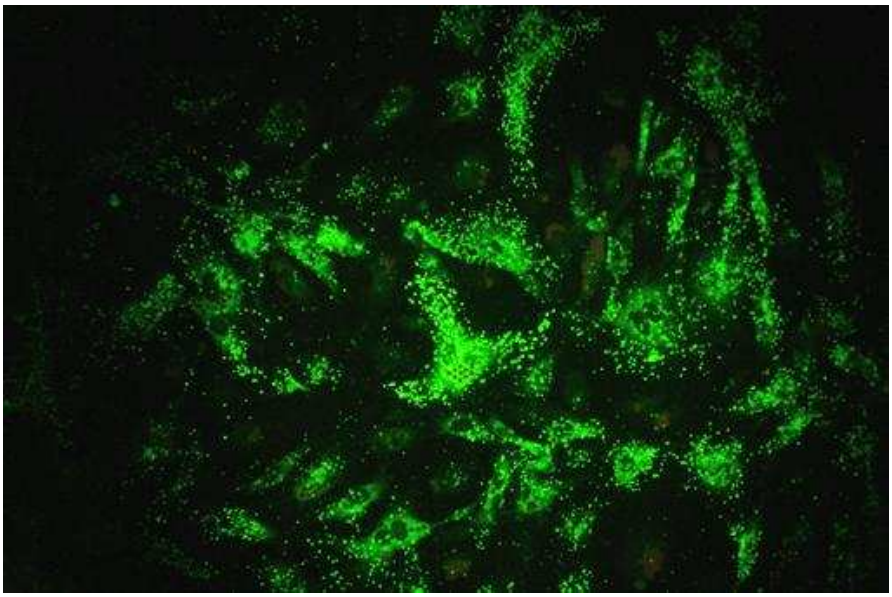

Fig 4.6. Macular inner choroidal endothelial cells stained for vWf (primary antibody - rabbit polyclonal anti-vWf, secondary antibody - swine anti-rabbit F(ab')2 fragment FITC conjugate). This demonstrates cells staining positive in a granular pattern consistent with the known localisation of vWf being within cytoplasmic granules.

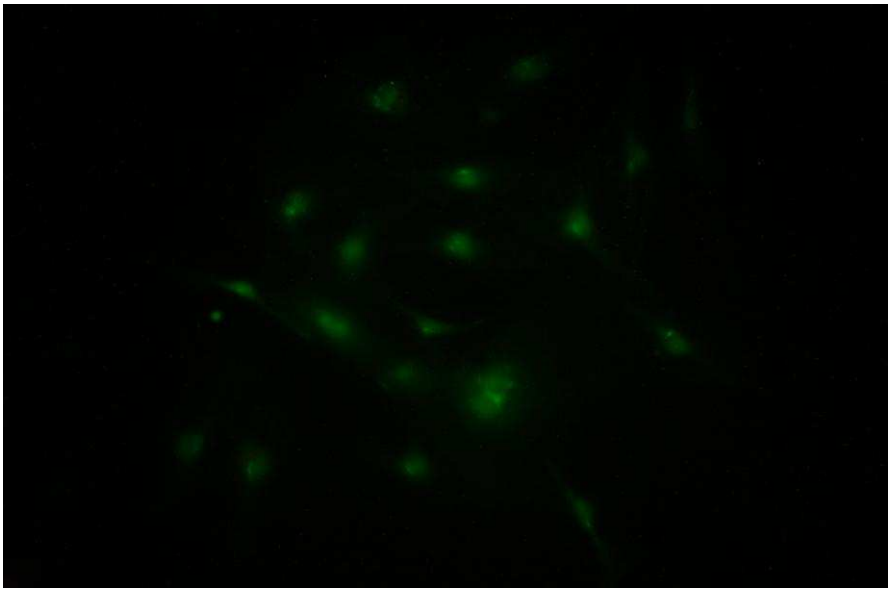

Fig 4.7. Macular inner choroidal endothelial cells negative control, stained with rabbit Ig fraction (primary antibody and secondary antibody - swine anti-rabbit F(ab')2 fragment FITC conjugate). Compared with Fig 4.2 (vWf), there is an absence of granular staining within the cytoplasm indicating that Fig 4.6 (vWf) shows a true positive result There is non-specific staining of cell nuclei.

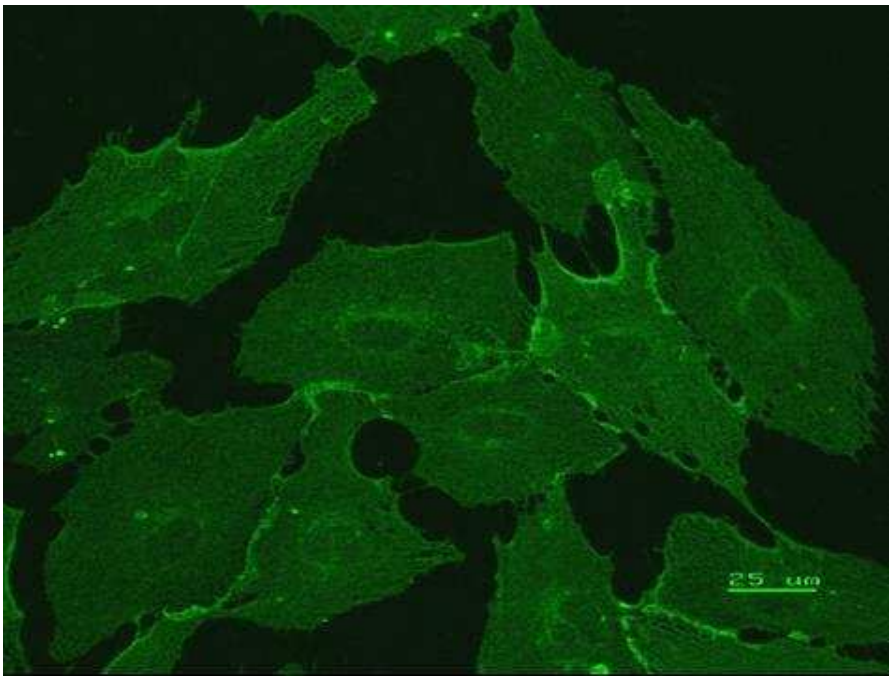

Fig 4.8. Macular inner choroidal endothelial cells stained for CD31. There is a ring of increased staining around the nuclei, perhaps indicating the site of CD31 synthesis. However, maximal staining is located at the cell surface, particularly at points of cell contact. This would be compatible with the known role of CD31 in being an endothelial cell surface adhesion molecule.

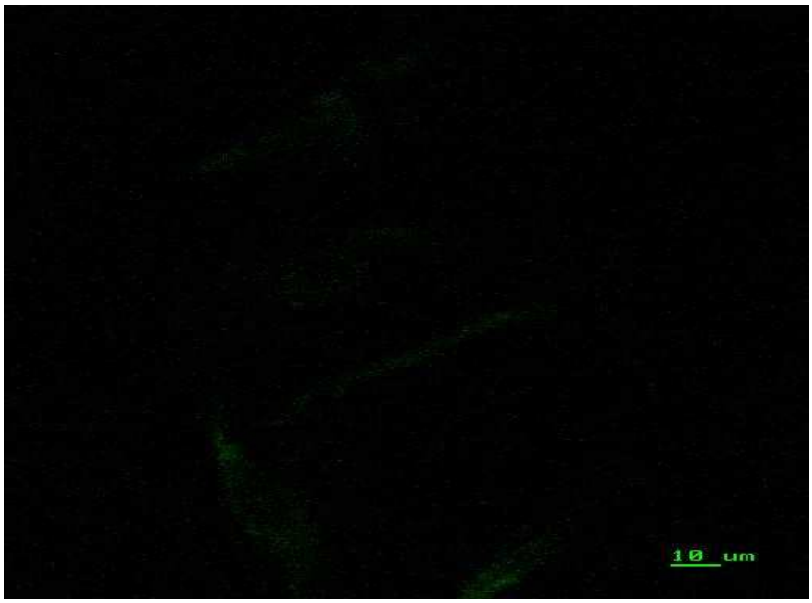

Fig 4.9. Human RPE cells stained for CD31 (negative control). There is a very faint non specific staining of the cells but no paranuclear or cell surface staining.

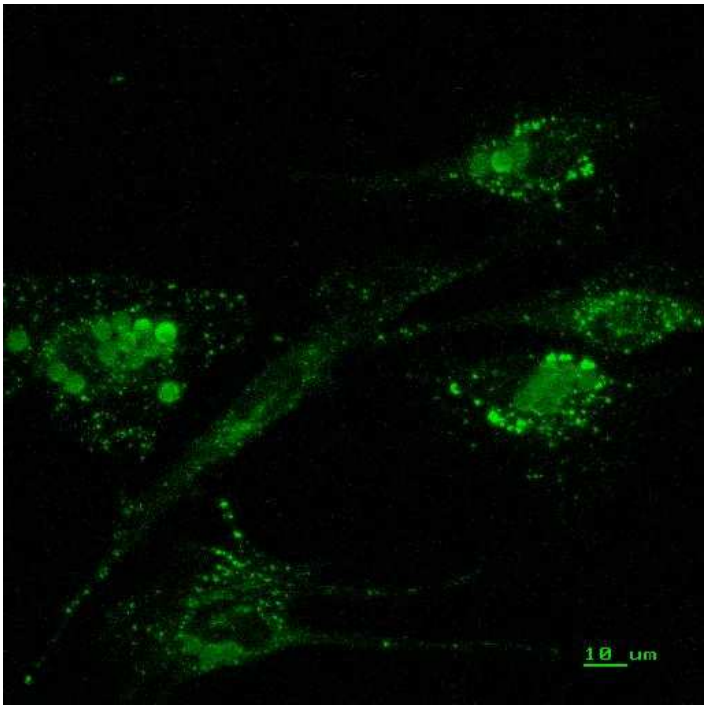

Fig 4.10. Macular inner choroidal endothelial cells stained for VEGF receptor 1. There is homogeneous staining throughout the cell, consistent with the VEGF receptor being abundant on the cell surface. In addition, there appears to be increased staining around the nuclei, perhaps because of the site of protein synthesis and modification. The cell on the left also demonstrates autofluorescence of bound Dynabeads.

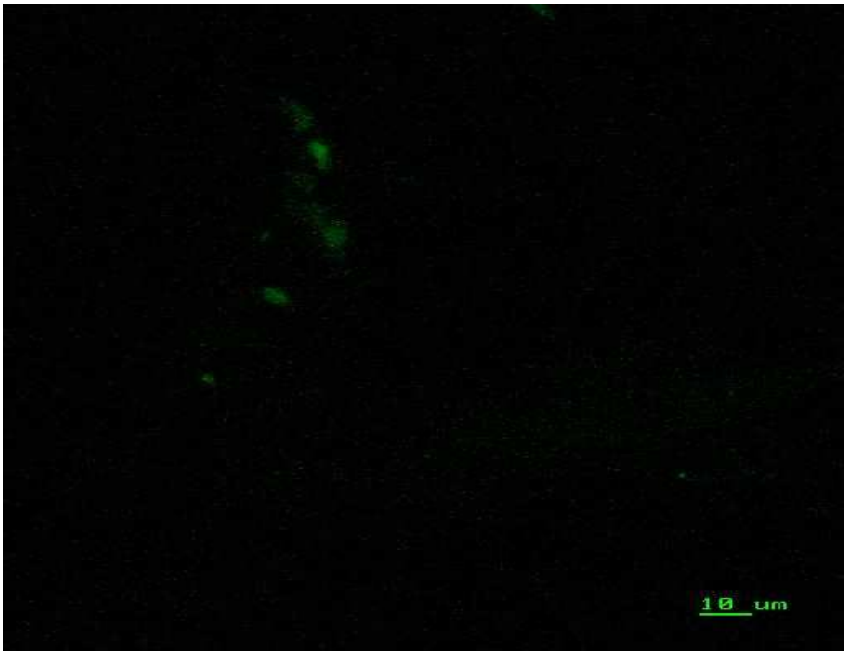

Fig 4.11. Human RPE cells stained for VEGF R1 (negative control). There is a very faint non-specific staining of the cells, but no consistent staining across the cell surface.

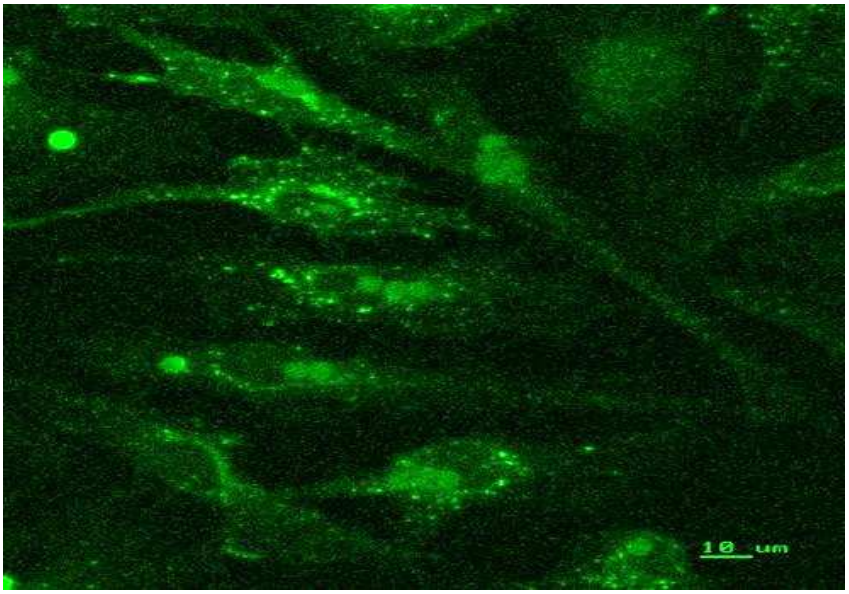

Fig 4.12. Macular inner choroidal endothelial cells stained for VEGF receptor 2 (VEGFR2). There is homogeneous staining throughout the cell, consistent with the VEGF receptor being abundant on the cell surface. In addition, there appears to be increased staining around the nuclei, perhaps because of the site of protein synthesis. This would be consistent with the known distribution of VEGF R2 being abundant across the cell surface.

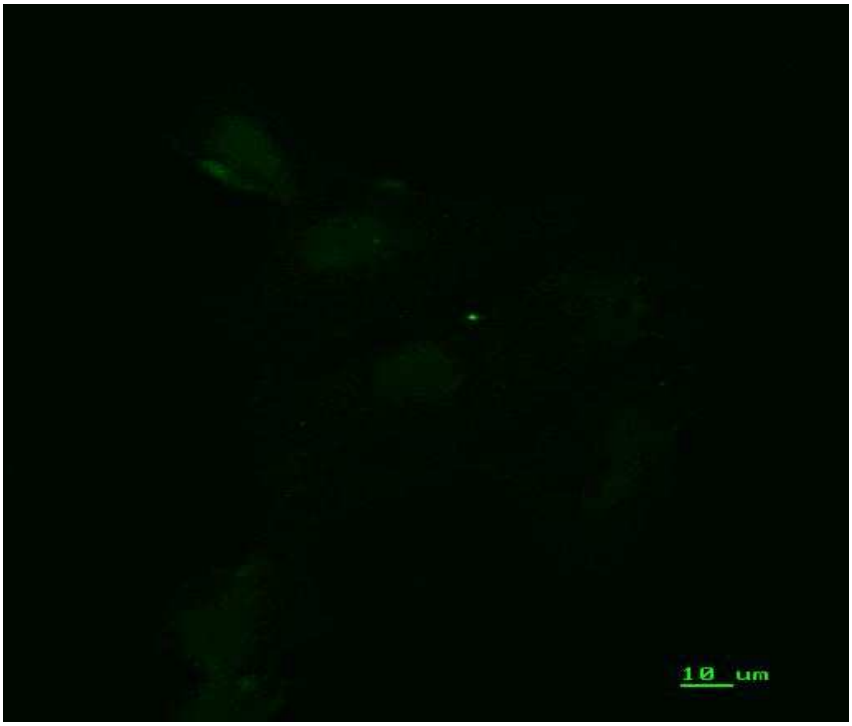

Fig 4.13. Human RPE cells stained for VEGF R2 (negative control). There is a very faint non specific staining of the cells, but no consistent staining across the cell surface.

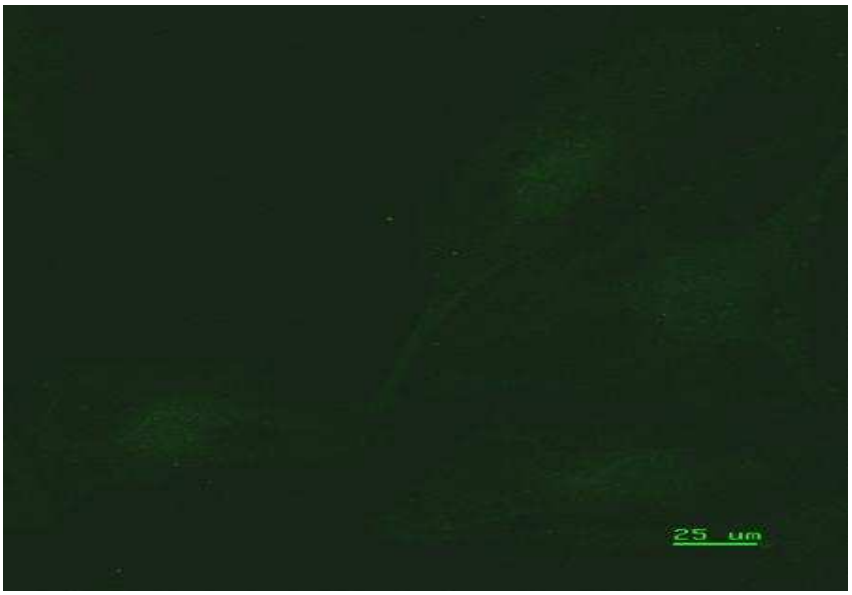

Fig 4.14. Macular inner choroidal endothelial cells stained for E-Selectin (unstimulated). There is a faint non-specific, generalised staining of the cells, perhaps due to background production of E-Selectin by the cells.

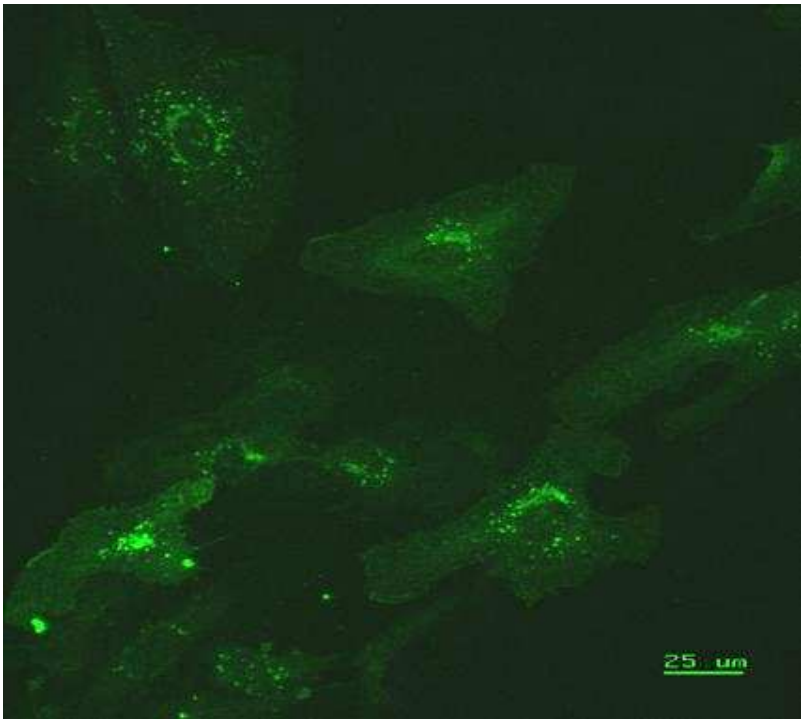

Fig 4.15. Macular inner choroidal endothelial cells stained for E-Selectin (1 hour post TNF-α stimulation). There is increased staining in a granular fashion around the cell nuclei. This may represent the site of E-Selectin protein synthesis or modification e.g. the Golgi apparatus.

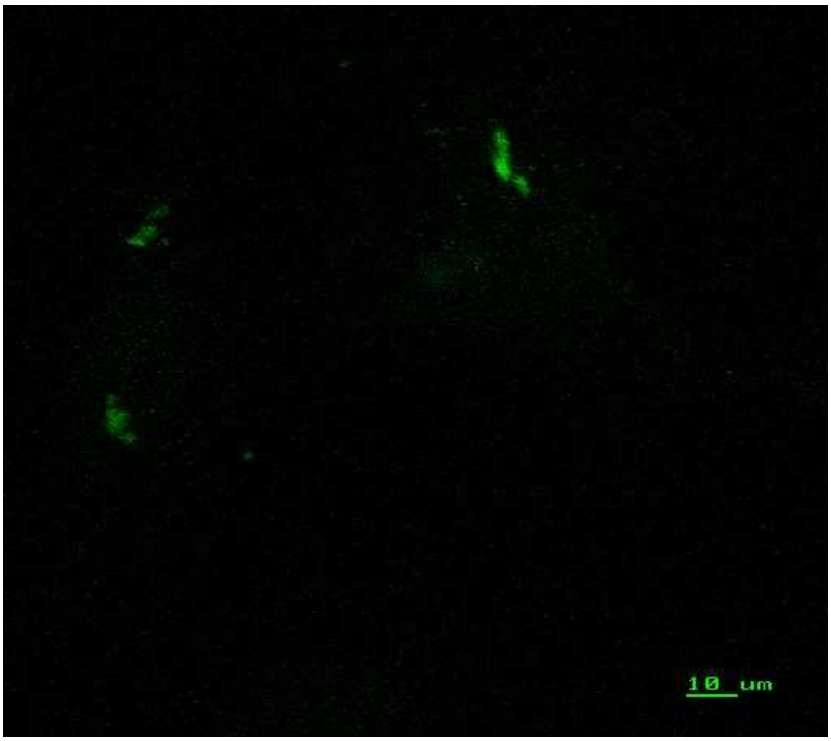

Fig 4.16. Human RPE cells stained for E-Selectin (1 hour post TNF-α stimulation). This is non specific cell staining and possible secondary antibody precipitation, but no evidence of granular paranuclear staining as seen in corresponding endothelial cells.

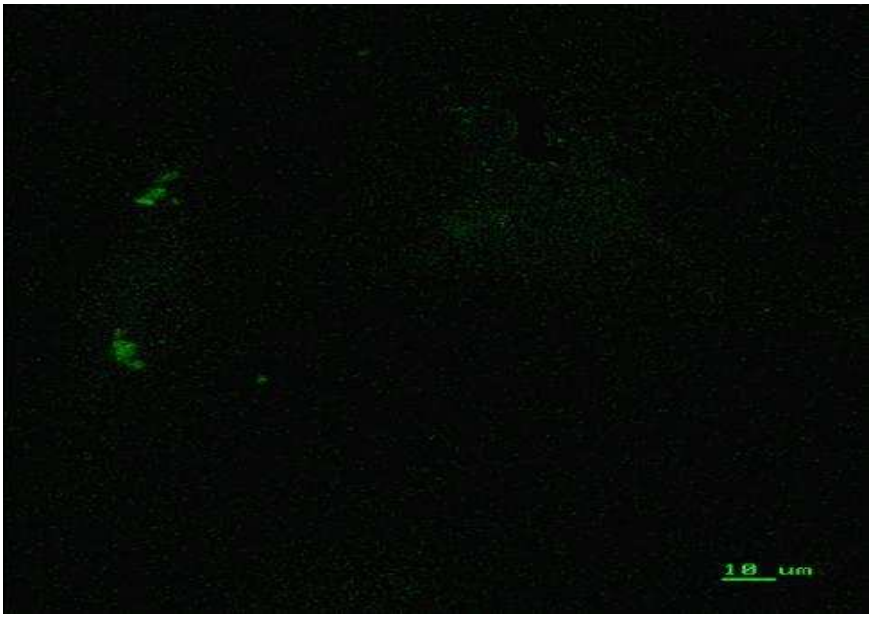

Fig 4.17. Macular inner choroidal endothelial cells stained with mouse anti-rat isotype negative control. There is evidence of faint cell staining and secondary antibody FITC precipitation but no significant or distinct pattern of staining.

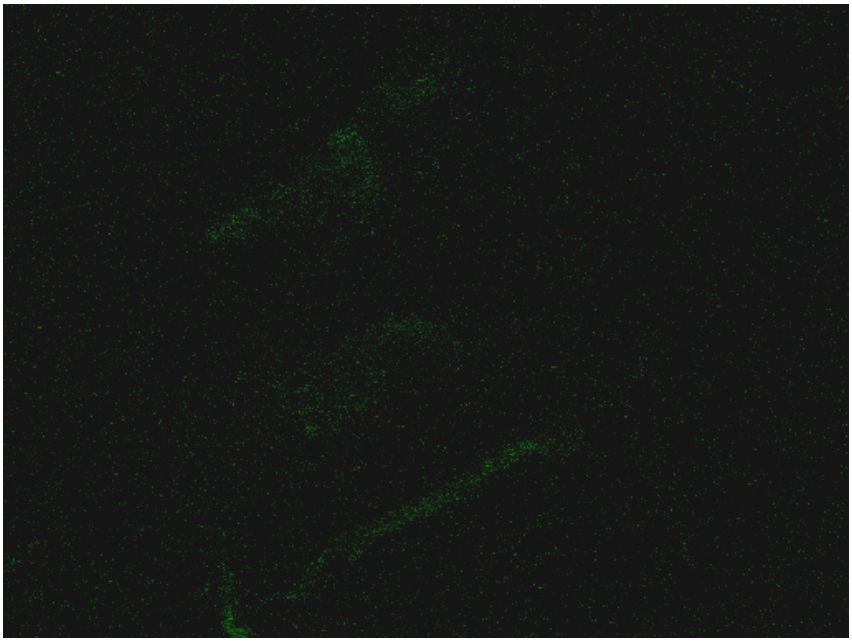

Fig 4.18 Macular inner choroidal endothelial cells stained for alpha-smooth muscle actin (α-SMA). The cells demonstrate no discernable staining. The faint fluorescence is more likely to be due to non specific secondary antibody precipitation.

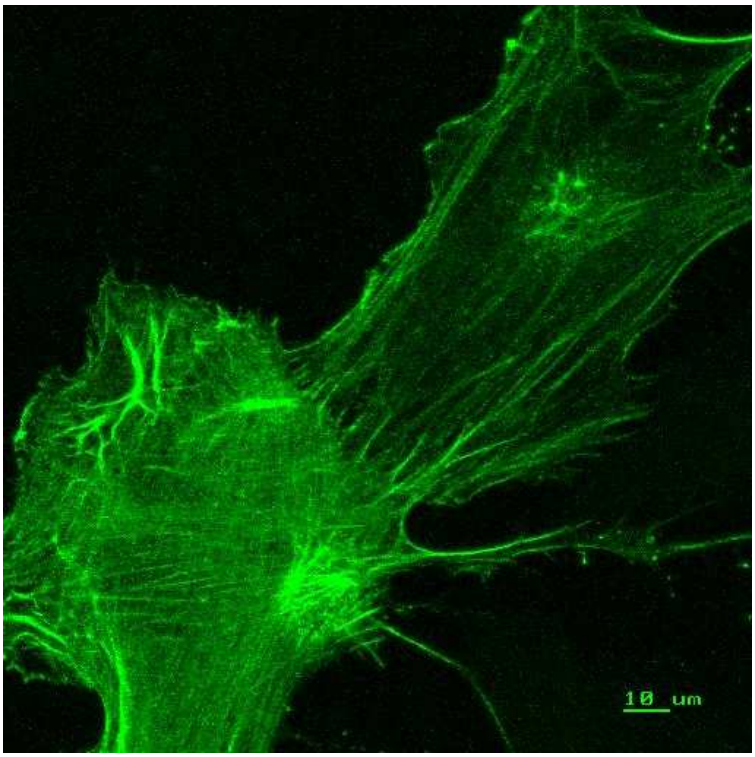

Fig 4.19. Human RPE cell stained for α-smooth muscle actin. There is strong staining indicating that RPE cells express αSMA. This can be used as a marker for cell contamination.

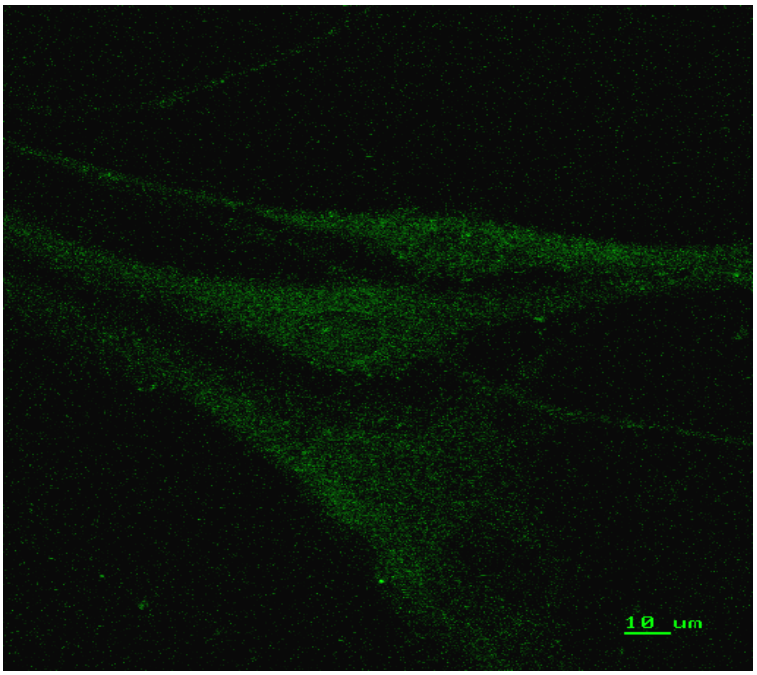

Fig 4.20. Macular inner choroidal endothelial cells stained for Fibroblast surface protein. There is faint and grainy staining across the whole cell which may be non-specific.

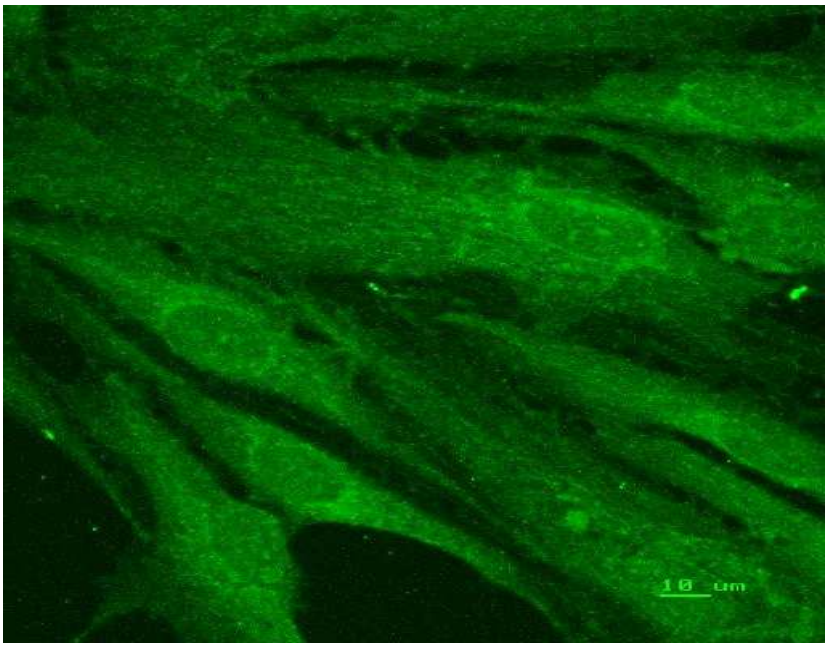

Fig 4.21. Human Tenon's capsule fibroblasts stained for fibroblast surface protein. In comparison with the endothelial cell above, staining is much stronger and homogeneous. This marker can be used as a measure of fibroblast contamination in cell preparations.

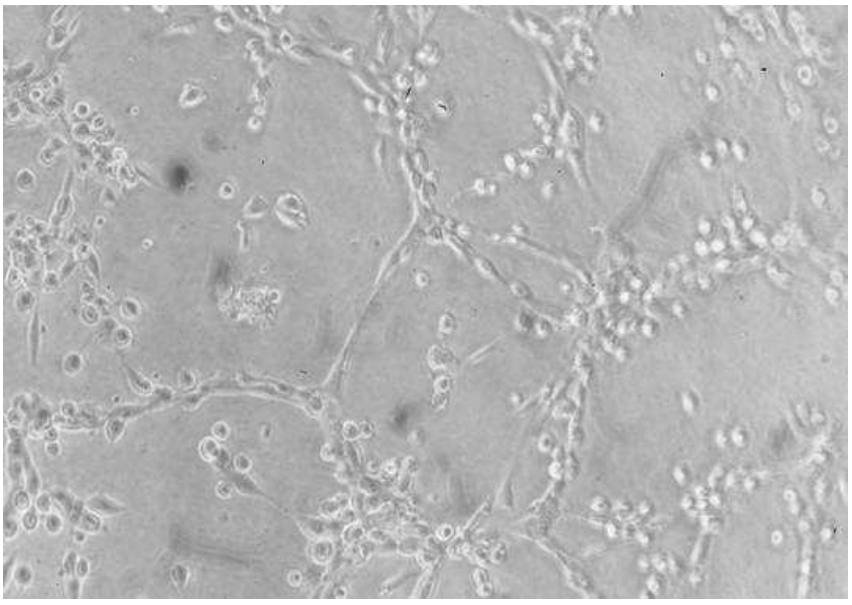

Fig. 4.22. Macular inner choroidal endothelial cells seeded onto Matrigel forming capillary-like structures within 3 hours of seeding.

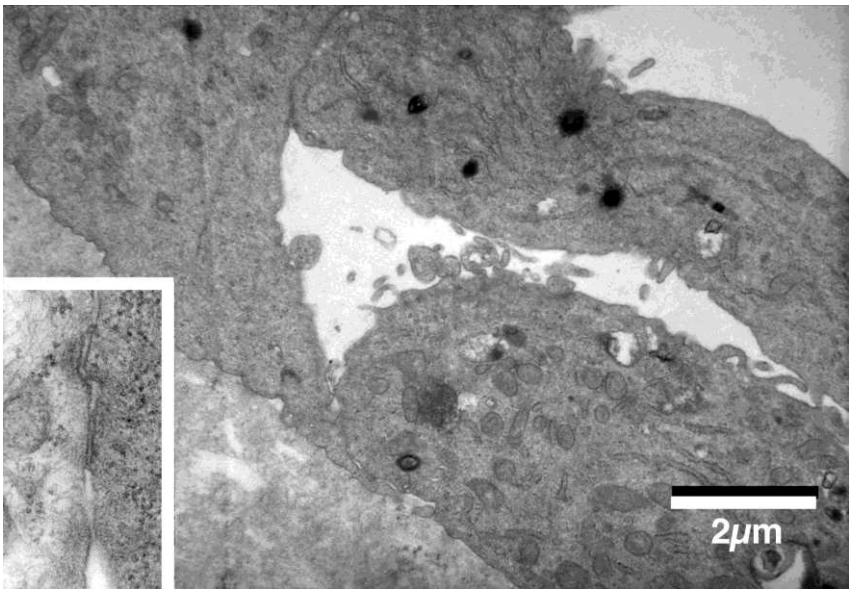

Fig. 4.23. Transmission electron microscopy of macular inner choroidal endothelial cells showed that the tube-like structures possessed a lumen, and each lumen was surrounded by up to 3 cells joined by junctional complexes (inset).

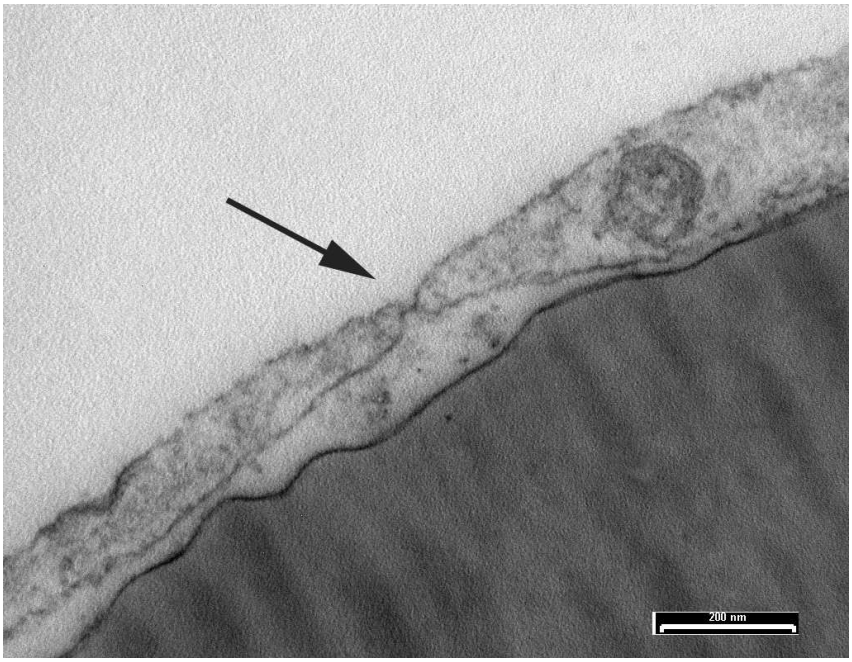

Fig. 4.24. Macular inner choroidal endothelial cell fenestrations (arrowed) were found scattered throughout the cell on T.E.M. The fenestrations had obvious diaphragms suggesting that they were type I fenestrations. The dark homogeneous structure is the gelatin coated plastic cover slip used to culture the cells on.

Discussion

There are certain diseases that appear to selectively affect the human choriocapillaris and inner choroid. The reason for this is currently unknown. They include wet age-related macular degeneration (Sarks et al, 1997) and the primary inflammatory choriocapillopathies such as multiple evanescent white dot syndrome (MEWDS) and acute multi-focal posterior placoid pigment epitheliopathy (AMPPPE) (Cimino et al, 2000). In these latter disorders, because of their rarity and the fact that they tend to affect otherwise young healthy patients, histological evidence is lacking of choriocapillaris involvement. However, analysis of data from indocyanine green angiography points to the inner choroid/choriocapillaris as the site of disease (Cimono et al, 2000, Bouchenaki et al, 2002). In all of these disorders, devastating visual loss can occur and current treatment modalities may not be particularly effective. It is important, therefore to increase our understanding of the underlying pathogenesis of these diseases. As the site of disease lies within the inner choroidal vasculature, the endothelial cells lining these vessels would be obvious candidates for further study.

A major obstacle in trying to correlate endothelial cells with site specific pathological processes is endothelial cell heterogeneity. It has been shown that endothelial cells derived from large vessels (macrovascular ECs) and smaller vessels (microvascular ECs) from different tissues have distinct and characteristic gene expression profiles Chi et al, 2003). Endothelial cell heterogeneity has even been described within the same organ such as the lung (Müller et al, 2002, Ghitescu et al 2002) and the placenta (Lang et al, 2003). These factors and the previously discussed differences in the functional differences in EC derived from different vascular beds suggest that it is probably necessary to use site-specific endothelial cells when studying vascular or angiogenic diseases. This means that it would be useful to describe

a method for the successful and reproducible isolation of human macular inner choroidal endothelial cells

While others have described methods for the isolation of human choroidal endothelial cells that contain a mixture of inner and outer choroidal endothelial cells (Sakamoto et al 1995, Penfold et al, 2002), until now, no method has been described to isolate human macular inner choroidal endothelial cells alone. In 1993, Liu and Li described a technique to isolate choriocapillaris endothelial cells in bovine eyes. This method took advantage of the differences in structure of the choriocapillaris – Bruch's membrane complex to that of the larger choroidal vessels. The method entailed cutting the choroid up into tiny pieces with scissors and digesting the fragments with trypsin. This is designed to dissolve away the large outer choroidal vessels leaving the Bruch's-choriocapillaris complex intact. The resulting fragments were then washed and the free floating choriocapillaris fragments were manually picked out and further digested with collagenase to release individual cells. The cells were then plated and grown in endothelial selective medium. This method has a number of drawbacks in relation to the isolation of the human equivalent cells. Firstly, bovine eyes are known to have a much firmer Bruch's membrane which means that this method is more likely to leave the Bruch's – choriocapillaris complex intact after enzyme digestion. Secondly, the resultant plated cells would have contained a rich mixture of contaminating cells (fibroblasts) which can easily overgrow the cultures. When attempted with human tissue, we failed to produce the necessary cleavage plane, suggesting a stronger bond between the choriocapillaris and the outer choroid in humans,

The aim of this chapter was to determine a new technique for the reliable isolation of human macular inner choroidal ECs. Initially, a manual technique using a Vibratome was attempted. In the past, this machine had been used to cut viable sheets of neuronal tissue including retina for use in functional experiments. It was thought that a

similar technique could be applied to the human choroid. Despite numerous attempts, it was found that the tissue was too firm for the blade to cut reliable sections. In addition, due to the prolonged time taken in attempting to cut the tissue and the severe conditions the cells were exposed to while undergoing sectioning (extreme cold necessary to solidify the gelatine and potential tissue drying), this technique was abandoned.

A different, solution-based approach was taken and in this chapter is described a successful technique for the isolation of human macular inner choroidal ECs that utilises an observation made around 100 years ago. In 1892, Nuel described a natural cleavage plane within the human choroid between the layers of Haller (inner choroid) and Sattler (outer choroid). In 1912 Salzmann noted that dissection of the choroid was easiest when the layers were teased apart from the outside. With the aid of a dissecting microscope and a microdissection technique, it was easy to peel the large outer choroidal vessels from the underlying inner choroid/choriocapillaris complex, hence the adoption of the manual dissection technique. The individual outer choroidal vessels or the inner choroidal complex could then be finely cut into pieces and the tissues gently dissolved in collagenase. In order to increase the purity of cells isolated and therefore to improve the reliability of any downstream experiments, the use of anti-CD31 coated paramagnetic beads replaced the older manual sweeping techniques mentioned previously or the use of endothelial-specific culture medium (which may also support other cells such as fibroblasts). CD 31, otherwise known as PECAM-1 is reported to be a pan-endothelial marker in mature, human endothelial cells (manufacturer's literature). The anti-CD31 paramagnetic bead technique is reported to produce cultures with a purity in excess of 99%.

The isolated, unpassaged cells were found to stain strongly for CD31, vWf, VEGF receptors 1 and 2. The cells were also shown to express E-Selectin (CD62E), an inducible endothelial-leukocyte adhesion molecule, after stimulation with TNF-α, a feature that may be unique to endothelial cells (Bevilacua et al, 1987). The isolated cells were negative for alpha smooth muscle actin and fibroblast surface protein. The finding of positive staining for vWf is an interesting finding as it was originally thought that only macrovascular ECs contained vWf within electron dense Weibel-Palade (WP) bodies (Jaffe et al, 1973; Wagner and Matthews, 1975). These bodies are seen on electron microscopy, as electron dense and contain a defined cytoskeleton. Electron microscopy of human inner choroidal ECs did not reveal any WP bodies. However, vWf staining on immunohistochemistry was granular in pattern within the cell cytoplasm, similar to the known distribution of WP bodies. It may be that in these cells, vWf is contained within different intracellular structures when compared to macrovascular ECs. Interestingly, review of the raw microarray data for macular inner choroidal ECs (see chapter 5) reveals significantly up-regulated gene expression for vWf (as it was for all choroidal ECs and retinal ECs).

The isolation of human macular inner choroidal ECs is further validated by an absence of contamination from RPE cells (negative for α-smooth muscle actin) and fibroblasts (negative for fibroblast surface protein). In this study, the isolated cells also formed fenestrations when grown on fibronectin and formed capillary-like tubes when cultured in Matrigel. The lumen of these tubes was surrounded by two or more cells joined by junctional complexes, suggesting that these cells had not simply aggregated together randomly. Again, the formation of fenestrations and formation of tubes in culture are thought to be specific to endothelial cells.

Considering that the choriocapillaris is heavily fenestrated *in vivo*, I was surprised to find that fenestrations were not found in abundance in this *in vitro* model. This discrepancy in *in vivo* and *in vitro* rates of fenestrations has previously been found in fenestrated kidney ECs (Satchell and Braet 2009). Reasons for this include methods used in tissue preparation, reduced levels of VEGF within the milieu around the ECs (VEGF is thought to be critical for fenestration formation and maintenance, (Peters et al 2007, Esser et al, 1998) or dysregulation of proteins such as PVLAP, known to be important in the assembly of fenestrations. For the purposes of this experiment, no additional VEGF was added to the Matrigel and so may be important as it shows that these cells still express fenestrations under basal conditions, showing that the cells are still reflecting their *in vivo* phenotype even when subjected to *in vitro* conditions. The fenestrations observed had obvious diaphragms thereby suggesting that they are type I fenestrations Their ability to form tubes with junctional complexes also shows that they retain their endothelial phenotype and this observation could be used subsequently as an assay of endothelial cell function.

In summary, a method has been developed to isolate and culture human inner choroidal endothelial cells. During early passages these cells possess the morphological characteristics of vascular endothelial cells; they form fenestrations and capillary tube-like structures and express a number of surface markers consistent with their endothelial cell origin. These cells may therefore be useful in studying the pathophysiological mechanisms of choroidal neovascularisation. This technique is also applicable to the isolation of macular outer choroidal endothelial cells if required.

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Chapter 5

Comparative Gene Expression Profiling of human macular and peripheral inner choroidal endothelial cells and macular outer choroidal endothelial cells

Introduction

The posterior segment of the eye is topographically arranged to provide different functions. Its most widely recognised feature is the variable topographical distribution of rods and cones across the retina which provides predominantly photopic (bright light and colour) and scotopic (low light level and black/white) functions. The area known as the macula has the highest density of photoreceptors (rods and cones), with the central foveal area containing only cones. Although the macula only occupies 1.4% of the retinal area, it contains 8.4% of all retinal cones, 3.4% of rods and 60% of retinal ganglion cells (Curcio et al, 1990). Not surprisingly, comparison of gene expression between the macular and peripheral retina has found that a number of genes are preferentially expressed in the macula. Many of these differences were of axonal origin and included neurofilament 3, brain specific alpha tubulin and light neurofilament (Sharon et al, 2002). Their differential expression probably reflects the increased density of ganglion cells in the macula. The same study found cone-specific genes such as cone alpha transducin, cGMP phosphodiesterase and cone opsins to be more abundant in the macula, but the differences did not reach statistical significance. In contrast, genes up-regulated in the retinal periphery included rod-specific genes such as rhodopsin, transducin and recoverin and non-specific genes such as transferrin (involved in iron transport) and DOPA decarboxylase. While the topographical differences in retinal gene expression can be explained by well documented and understood functions, topographical differences in the distribution in other cells within the posterior segment are less well understood.

Several studies have investigated the variation in RPE gene expression across the posterior segment. It is known that at the light and electron microscope level, RPE cells differ in appearance between those found in the macula and those in the periphery. For example, macular RPE

cells are more columnar and contain more melanin than those in the periphery (Ishibashi et al, 2004). In 2001, Bron stated that at the fovea, each RPE cell measures 12-18µm in width and is 10-14µm in height, while in the periphery they are flatter and are up to 60µm in width (Bron et al, 1997b). However, with increasing age, macular RPE cells become taller and thinner, whilst in the periphery it is vice versa (Watzke et al, 1993). In addition, with increasing age, RPE cells at the macula become less pigmented while those in the periphery become more so. At a gene expression level, Ishibashi et al demonstrated a difference in the expression of a number of genes between peripheral and macular RPE cells prepared by laser microdissection of human eyes (Ishibashi et al, 2004). They identified 11 genes that showed differential expression, each showing reduced expression in the macular area, including: aldehyde dehydrogenase 6, sialic acid synthase, protein kinase C and glutathione S-transferase. Van Soest, using a similar laser capture dissection technique, found 438 genes that were differentially expressed (1-5% of the transcriptome), with 33 showing at least a 4-fold change. There was enrichment of extracellular matrix genes such as collagen types I, II and VI, laminin, TIMP 2 and thrombospondin 4, all being up-regulated in peripheral RPE cells (van Soest et al, 2007). When the van Soest study was compared with the previous study (Ishibashi et al, 2004), only the results for aldehyde dehydrogenase and c-kit were found to be comparable. Reasons for this wide discrepancy between the results of the two studies include sample contamination by photoreceptors (Bowes Rickman et al, 2006), age of the donors and the type of microarray used. The authors of the studies suggest that the up-regulation of genes involved in ECM may represent a role for the RPE in Bruch's membrane turnover. Up-regulation of the aforementioned genes may also contribute to the topographical differences found in the composition and physical properties of Bruch's membrane, part of the basement membrane of the RPE (Chong et al, 2005; Ramrattan et al, 1994; Okubo et al, 1999; Guo et al, 1999). This may in turn lead to disease susceptibility of the macula to conditions such as age-related macular degeneration.

Little is known about the topographical changes within the human choroid. It has been subdivided into three layers: the outer layer of large vessels (Haller's layer), a middle layer of medium sized vessels (Sattler's layer) and an internal layer adjacent to Bruch's Membrane composed of capillary vessels (choriocapillaris), as discussed previously. The choriocapillaris at the macula is supplied by a higher density of pre-capillary arterioles than that found in the periphery, while the peripheral choroid is simpler in structure with the meshwork of the choriocapillaris being much wider than that found in the macular area. In 2006, Mullins et al described differences in the distribution of ICAM 1 across the choriocapillaris and retina, with labelling being stronger in the macular area. They suggested that this may play a role in increased leukocyte trafficking in the macula (Mullins et al, 2006). In 2007, Radeke studied the gene expression of macular and peripheral RPE/choroidal samples and found differences in a number of genes that may have relevance to wet AMD. These include an RPE cell growth factor (TFP12) and a number of inflammation-related genes such as CXCL14, CCL19 and CCL26 (Radeke et al, 2007). Unfortunately, it is not known if these gene differences were solely derived from the RPE or choroid as the samples tested were a mixture of both tissues.

As mentioned previously, choroidal neovascularisation in wet age-related macular degeneration begins within the inner choroid and is almost always within the macular area. Given the fact that the topography of photoreceptors, RPE and Bruch's membrane is different at the macula compared to elsewhere in the eye, it is important to establish whether the choroidal endothelial cells in the sub-macular area are different to those in the periphery, possibly making them more susceptible to certain diseases. It is known that the choroidal vasculature is formed by a process of haemovasculogenesis (Hasegawa et al, 2007). In theory, all choroidal endothelial cells are derived from the same precursor, meaning any differences are probably

due to the local microenvironment that the ECs find themselves in, i.e. the surrounding “different” Bruch’s membrane, photoreceptors or RPE cells. Any differences might also render them differentially responsive to treatment, because selective treatment of disease relies on the identification of unique, or at least significantly different, attributes in the affected site compared with its surroundings.

The aim of this section was to compare the gene expression profiles of matched human sub-macular inner and outer choroidal endothelial cells and matched human sub-macular and peripheral inner choroidal endothelial cells using the gene microarray technique described previously. An additional aim was to determine whether there were, in fact, specific gene expression profiles that were unique to cells within different areas of the human choroid that may help to explain the topographical selectivity of choroidal disease and perhaps lead to the development of site-specific treatments.

Methods

The technique for the isolation of human macular inner choroidal endothelial cells has previously been described in Chapter 4. During dissection of the human choroid, matched 6mm diameter samples of peripheral inner choroid (peripheral area nasal to the optic disc) and macular outer choroidal tissue were collected and treated in the same manner as described for the macular inner choroidal samples.

Briefly, matched human macular inner and outer and peripheral inner choroidal endothelial cells were isolated from anonymised, paired human globes, enucleated within 24 hours of death and free of any known ocular disease. The globes were obtained from UK Transplant after removal of corneas for transplantation. The research had the approval of the local research ethics committee (Nottingham Q1060301). The matched choroidal endothelial cells from the different locations underwent identical isolation procedures using anti CD31 coated Dynabeads (Dynal Ltd, Wirral, UK), were seeded onto fibronectin-coated 35mm culture dishes (Beckton Dickinson, Oxford, UK) and incubated at 37°C in Endothelial Growth Medium (EGM2-MV with hydrocortisone omitted, Cambrex Biosciences, Wokingham, Berks, UK) in a humidified atmosphere of 5% CO_2.

Confirmation of EC purity

The identity and purity of cells used in the microarray assays was confirmed prior to RNA extraction by staining for Factor VIII and CD31 as described in Chapter 4.

RNA extraction

Total RNA was extracted from primary cultures of un-passaged endothelial cells when they had reached approximately 80% confluence, using the Qiagen RNeasy Minikit (Qiagen, Crawley, UK) as described in chapter 3. As before, the isolated RNA was transferred into microcentrifuge tubes and stored at -80℃ until used for subsequent analysis. It was found that approximately 1–3 μg of total RNA was obtained from each 35mm culture plate.

Microarray analysis

The previously stored RNA was processed through the different stages of microarray analysis using Affymetrix GeneChip ® Human Genome U133 Plus 2.0 arrays (Affymetrix, High Wycombe, Bucks, UK) as described in chapter 3.

Data Analysis

Affymetrix CEL files were imported into GeneSpring GX 11.0.1 and processed with the MAS5 algorithm to generate PMA flags. Probesets were excluded from further analysis if there was not a Present or Marginal flag in 100% of the samples in one of the four cell groups. Affymetrix control probesets were also excluded, leaving a probeset list for subsequent analysis. Data was then normalised with GC-RMA to provide expression values. To identify differentially expressed genes between cell groups, ANOVA was performed on this probeset list with Tukey-HSD post-hoc testing and Benjamini-Hochberg false discovery rate control. A difference in expression between probesets with a corrected p-value of <0.05 and a fold-change of greater than 2 were considered to be statistically significant. Data was exported directly from GeneSpring 11.0.1. to Ingenuity Pathway Analysis for these probesets. Biological functions analysis identified areas of the Ingenuity

Functional Analysis library of biological functions that were most significant to the data set. Molecules from the data set that met the cut off criteria and were associated with an area of biological function in Ingenuity's Knowledge Base were considered for the analysis. The significance of the association between the data set and the biological function was measured in two ways:

1) A ratio of the number of molecules from the data set that map to the area, divided by the total number of molecules that map to the functional area is displayed.

2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the functional area is explained by chance alone.

QPCR

The method for QPCR confirmation of microarray probeset expression results has been described previously (Chapter 3).

Results

Nine matched, un-passaged EC samples, representing 3 different intra-choroidal locations from three different donors were propagated under identical conditions. The 3 choroidal areas represented by the matched samples were: macular inner choroidal ECs, macular outer choroidal ECs and peripheral inner choroidal ECs (peripheral area nasal to the optic disc). The age, sex and time from death to the cells being placed in culture were as follows: 58, male, 28 hours; 42, male, 43 hours; 62, female, 36 hours. All eyes were free of ocular disease, in particular the macula, on examination with the dissecting microscope.

Samples of cells from all locations displayed homogeneous cobblestone morphology with no evidence of cell contamination. Greater than 99.5% of the endothelial cells from each site demonstrated staining for factor CD31 prior to their use in the aforementioned experiments, confirming their purity and identity as endothelial cells.

Overview of gene expression patterns

Between 1.5 and 3.4 µg of total RNA were isolated from each 35mm plate. Biotinylated cRNA probes were hybridised to Affymetrix GeneChip ® Human Genome U133 Plus 2.0 arrays containing 47000 transcripts, of which 38500 were well characterised human genes. A total of 23636 probe sets passed quality control testing during analysis.

Proliferating human macular inner choroidal endothelial cells (ECs) versus peripheral inner choroidal ECs

Comparison of matched, un-passaged proliferating human macular inner choroidal endothelial cells (ECs) with peripheral inner choroidal ECs demonstrated a small difference of 35 probe sets (0.15% of probesets that passed quality control) Of this total, 19 were up-regulated in macular inner choroidal ECs and 16 were up-regulated in peripheral inner choroidal ECs. This study demonstrated a striking homogeneity of gene expression between inner choroidal ECs derived from the macula and periphery. Differences in the expression of a small number of genes such as Annexin 10, collagen type XVI and mannose receptor C were noted. Canonical pathway analysis failed to reveal any pathways that were significantly represented. A complete list of differentially expressed probesets reaching statistical significance is shown in tables 5.1 and 5.2.

Table 5.1. All probesets reaching differential expression of 2.0 or greater in human macular inner choroidal endothelial cells compared with matching peripheral inner choroidal ECs.

GENE TITLE	Fold Change
mesoderm specific transcript homolog	2.5
Rho GTPase activating protein 23	3.8
serine (or cysteine) proteinase inhibitor, clade B	2.9
lymphocyte cytosolic protein 1 (L-plastin)	2.2
sema domain, immunoglobulin domain (Ig),(semaphorin)	2.1
eukaryotic translation initiation factor 1A, Y	2.3
annexin A10	4.3
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	2.4
plasmalemma vesicle associated protein	2.2
solute carrier family 6, member 15	2.1
ring finger protein 128	2.7
keratin associated protein 2-1	2.1
SH3-domain GRB2-like 2	2.1
interleukin 13 receptor, alpha 2	2.3
ribosomal protein S4,	2.2
tetraspanin 7	2.5
solute carrier family 4, sodium bicarbonate cotransporter, member 4	2.2
collagen, type XVI, alpha 1	2.1
UDP-N-acetyl-alpha-D-galactosamine:polypeptide	2.2

Table 5.2. All probesets reaching differential expression of 2.0 or greater in human peripheral inner choroidal endothelial cells compared with macular inner choroidal ECs

GENE TITLE	Fold Change
trefoil factor 3 (intestinal)	3.1
Meis1,	2.7
mannose receptor, C type 1	2.2
pregnancy specific beta-1-glycoprotein 5	2.3
fatty acid binding protein 4, adipocyte	2.1
pregnancy specific beta-1-glycoprotein 1	2.1
extracellular link domain containing 1	2.7
ATP-binding cassette, sub-family G	2.1
extracellular link domain containing 1	2.3
pregnancy specific beta-1-glycoprotein 9	2.1
zinc finger protein 659	2.1
coronin, actin binding protein, 1B	2.1
churchill domain containing 1	2.9
tumor-associated calcium signal transducer 2	2.1
periplakin	2.2
heat shock 70kDa protein 6 (HSP70B')	2.8

Proliferating human macular inner choroidal ECs versus macular outer choroidal ECs

Comparison of matched, un-passaged proliferating human macular inner choroidal endothelial cells (ECs) with macular outer choroidal endothelial cells revealed 302 probesets that were differentially expressed between the two cell types (1.3%). Of the 302 differentially expressed probesets, 96 were up-regulated in macular inner choroidal ECs, and 193 were up-regulated in macular outer choroidal ECs. Probe sets for a diverse range of functions including nervous system development (Brain Derived Neurotrophic factor, Neurofilament light and medium polypeptides), cell signalling (MAPK 11, Apelin receptor, Parvin beta, diacylglycerol kinase and MAPK binding protein 1 and WNT5A), cellular growth and proliferation (VEGF, HGF, CXCL12, TGF Beta1 and MMP10), immune response (MHC class II alpha and HLA DR B1 and CD200) and cell morphology (Keratin 19, Collagen 11 alpha subunit and Collagen 15 alpha subunit and PLVAP) were found to be differentially expressed. Lists of the most highly expressed probesets reaching statistical significance are shown in tables 5.3, 5.4 and 5.5. The difference in probeset expression with regards to groupings of biological processes is represented by pie charts in Figs 5.1a and 5.1b. This showed a similar distribution in biological processes. However, macular ICECs demonstrated a slightly greater proportion of probesets involved in cell adhesion, organisation and development

Fig 5.2 demonstrates a heat map of the differential probeset expression reaching statistical significance with reference to human macular inner and outer choroidal microvascular endothelial cells. Selected genes thought to be important in endothelial cell biology are shown on the right of the heat map.

Table 5.3 All probesets reaching differential expression of 2.0 or greater in human macular inner choroidal ECs compared with macular outer choroidal ECs.

GENE TITLE	Fold Change
insulin-like growth factor binding protein 3	11.5
neurofilament, medium polypeptide	10.4
brain-derived neurotrophic factor	8.9
platelet-derived growth factor receptor, alpha polypeptide	8.8
keratin 19	7.3
pleckstrin homology-like domain, family A, member 2	7.1
solute carrier family 6	6.6
popeye domain containing 3	5.9
olfactomedin-like 3	5.6
SIX homeobox 2	5.5
neurofilament, light polypeptide	5.2
mesoderm specific transcript homolog (mouse)	5.1
mannosyl (alpha-1,3-)-glycoprotein beta-1,4-	4.9
vascular endothelial growth factor A	4.7
brain expressed, X-linked 1	4.7
lysophosphatidic acid receptor 1	4.4
collagen, type XI, alpha 1	4.4
pleckstrin and Sec7 domain containing 3	4.3
carbonic anhydrase XII	4.3
procollagen C-endopeptidase enhancer 2	4.2
ABI family, member 3 (NESH) binding protein	4.2
PNMA-like 1	4.1
tissue factor pathway inhibitor 2	3.9
glycine receptor, beta	3.9
wingless-type MMTV integration site family, member 5A	3.9
carbonic anhydrase XII	3.9
forkhead box F2	3.7
MSTP150	3.8
glycine receptor, beta	3.7
TP53 regulating kinase	3.6
calmegin	3.6
carbonic anhydrase XII	3.6
ectonucleotide pyrophosphatase/phosphodiesterase 2	3.5
hepatocyte growth factor (hepapoietin A; scatter factor)	3.4
protein phosphatase 1, regulatory (inhibitor) subunit 14B	3.4
golgi autoantigen, golgin subfamily a, 8A	3.4
lymphoid-restricted membrane protein	3.4
sulfatase 1	3.4
secretogranin II (chromogranin C)	3.4
poliovirus receptor-related 3	3.4
folate receptor 1 (adult)	3.4
transforming growth factor, beta receptor 1	3.3
tissue factor pathway inhibitor 2	3.3

GENE TITLE	Fold Change
emopamil binding protein-like	3.3
aldehyde dehydrogenase 1 family, member A3	3.3
interferon-induced protein with tetratricopeptide repeats 1	3.2
tumor necrosis factor (ligand) superfamily, member 15	3.1
mitogen-activated protein kinase kinase kinase kinase 4	3.1
chromosome 9 open reading frame 40	3.1
neuropilin (NRP) and tolloid (TLL)-like 2	3.1
family with sequence similarity 13, member B	3.1
aspartyl-tRNA synthetase	3.1
paternally expressed 10	3.1
versican	3.1
phosphoglycolate phosphatase	2.9
prostaglandin-endoperoxide synthase 2	2.9
retinol dehydrogenase 14 (all-trans/9-cis/11-cis)	2.9
carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	2.9
proline rich 16	2.9
aryl hydrocarbon receptor	2.9
glycoprotein (transmembrane) nmb	2.9
chromosome 13 open reading frame 15	2.9
B-cell translocation gene 1, anti-proliferative	2.9
phosphatidic acid phosphatase type 2B	2.8
TAF10 RNA polymerase II, TATA box binding protein	2.8
serpin peptidase inhibitor, clade E	2.8
COMM domain containing 2	2.8
transducer of ERBB2, 1	2.8
aldo-keto reductase family 1, member C1	2.8
DnaJ (Hsp40) homolog, subfamily C, member 1	2.8
nebulette	2.8
UDP-N-acetyl-alpha-D-galactosamine:	2.8
histone cluster 1, H2ac	2.8
BAT2 domain containing 1	2.7
lymphoid-restricted membrane protein	2.7
pleiomorphic adenoma gene-like 1	2.7
chromosome 1 open reading frame 63	2.7
insulin-like growth factor binding protein 2, 36kDa	2.7
glioma tumor suppressor candidate region gene 2	2.6
kinectin 1 (kinesin receptor)	2.6
phospholipid scramblase 4	2.6
brain protein I3	2.6
zinc finger E-box binding homeobox 2	2.6
hairy and enhancer of split 1, (Drosophila)	2.6
Cbp/p300-interacting transactivator	2.6
Nedd4 family interacting protein 2	2.6
splicing factor, arginine/serine-rich 18	2.5
endothelin receptor type B	2.5
solute carrier family 25, member 37	2.5
Mdm2 p53 binding protein homolog (mouse)	2.5

GENE TITLE	Fold Change
chondroitin sulfate N-acetylgalactosaminyltransferase 2	2.5
zinc finger protein 623	2.4
plasmalemma vesicle associated protein	2.4
O-linked N-acetylglucosamine (GlcNAc) transferase	2.4
transmembrane protein with EGF-like domain	2.4
phosphoserine phosphatase	2.3
mitogen-activated protein kinase kinase kinase 2	2.3

Table 5.4 Top one hundred probesets reaching differential expression of 2.0 or greater in human macular outer choroidal ECs compared with macular inner choroidal ECs.

Gene Title	Fold Change
histone cluster 1, H3b	14.7
LSM4 homolog, U6	10.2
translocase of inner mitochondrial membrane 44 homolog (yeast)	8.9
chromosome 6 open reading frame 108	8.4
fascin homolog 1, actin-bundling protein	7.9
FXYD domain containing ion transport regulator 6	7.9
leucine rich repeat containing 15	7.6
translocase of inner mitochondrial membrane 44 homolog (yeast)	7.2
kinesin light chain 1	6.9
cyclin K	6.5
polypyrimidine tract binding protein 1	6.1
valyl-tRNA synthetase	6.1
apelin receptor	5.4
fascin homolog 1, actin-bundling protein	5.4
mitogen-activated protein kinase kinase 2	5.1
cleft lip and palate associated transmembrane protein 1	5.1
histone cluster 1, H2bf	4.6
transforming growth factor beta 1 induced transcript 1	4.5
deoxyribonuclease I-like 3	4.5
RAN binding protein 3	4.5
histone deacetylase 5	4.4
cysteine-rich protein 2	4.2
cell division cycle 34 homolog (S. cerevisiae)	4.1
dicarbonyl/L-xylulose reductase	4.1
collagen, type XV, alpha 1	3.9
coronin, actin binding protein, 1B	3.9
mannose receptor, C type 1	3.9
DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	3.9
sema domain, transmembrane domain (TM),	3.9
coronin, actin binding protein, 1B	3.8
GINS complex subunit 4 (Sld5 homolog)	3.7
guanine nucleotide binding protein-like 3 (nucleolar)-like	3.7
protein kinase C and casein kinase substrate in neurons 2	3.6
thimet oligopeptidase 1	3.6
major histocompatibility complex, class II, DR beta 1	3.6
zinc finger protein 688	3.6
histone cluster 1, H1b	3.6
SH3KBP1 binding protein 1	3.6
cleft lip and palate associated transmembrane protein 1	3.5
major histocompatibility complex, class II, DR beta 1	3.5

Gene Title	Fold Change
MAD1 mitotic arrest deficient-like 1 (yeast)	3.4
exosome component 4	3.4
YKT6 v-SNARE homolog (S. cerevisiae)	3.4
zinc finger CCCH-type containing 7B	3.4
ATPase type 13A2	3.3
similar to RABEP2 protein	3.3
hepatoma-derived growth factor-related protein 2	3.3
matrix metallopeptidase 10 (stromelysin 2)	3.2
mitogen-activated protein kinase kinase 2	3.2
endoglin	3.2
F-box and WD repeat domain containing 5	3.2
carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	3.2
lipase, endothelial	3.2
spectrin repeat containing, nuclear envelope 2	3.2
kinesin light chain 1	3.1
homer homolog 3 (Drosophila)	3.1
hypothetical protein LOC286434	3.1
exosome component 4	3.1
glucocorticoid receptor DNA binding factor 1	3.1
chromosome 21 open reading frame 45	3.1
cytochrome P450, family 1, subfamily B, polypeptide 1	3.1
peter pan homolog (Drosophila)	3.1
cytochrome P450, family 1, subfamily B, polypeptide 1	3.1
cyclin D1	3.1
nasal embryonic LHRH factor	3.1
zinc finger protein 160	3.1
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	3.1
small optic lobes homolog (Drosophila)	3.1
splicing factor, arginine/serine-rich 8	3.1
Wolf-Hirschhorn syndrome candidate 1	3.1
dedicator of cytokinesis 6	2.9
lysosomal multispanning membrane protein 5	2.9
ankyrin repeat domain 1 (cardiac muscle)	2.9
ring finger protein 125	2.9
sorbitol dehydrogenase	2.9
sparc/osteonectin,	2.9
Hypothetical protein LOC100129502	2.9
hypothetical protein LOC100132181	2.9
EH-domain containing 1	2.9
hepatocyte growth factor-regulated tyrosine kinase substrate	2.9
thrombospondin 1	2.9
Meis homeobox 2	2.9
mitogen-activated protein kinase 11	2.8
trefoil factor 3 (intestinal)	2.8
IMP (inosine monophosphate) dehydrogenase 1	2.8

Gene Title	Fold Change
ribosomal RNA processing 7 homolog A (S. cerevisiae)	2.8
major histocompatibility complex, class II, DR beta 1	2.8
macrophage erythroblast attacher	2.8
chromosome 7 open reading frame 50	2.8
LSM7 homolog, U6 small nuclear RNA associated	2.8
limb bud and heart development homolog (mouse)	2.8
activin A receptor type II-like 1	2.8
ATG4 autophagy related 4 homolog B (S. cerevisiae)	2.8
guanine nucleotide binding protein (G protein), alpha 11	2.8
major histocompatibility complex, class II, DP alpha 1	2.8
mitogen-activated protein kinase binding protein 1	2.7
major histocompatibility complex, class II, DP beta 1	2.7
translocase of inner mitochondrial membrane 8	2.7
CXCL12	2.4

Fig 5.1a. Pie chart of significantly up-regulated genes grouped by biological processes in proliferating human macular inner choroidal endothelial cells compared with proliferating human macular outer endothelial cells

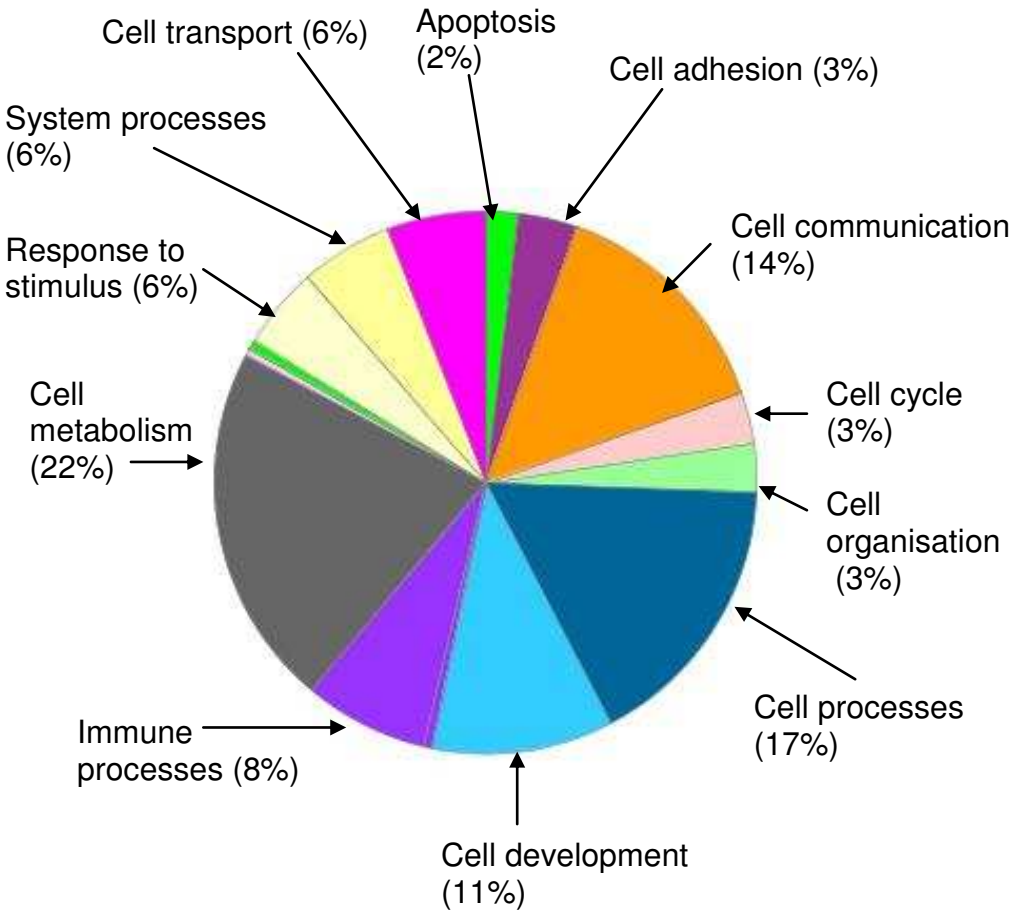


Fig 5.1b. Pie chart of significantly up-regulated genes grouped by biological processes in proliferating human retinal endothelial cells compared with proliferating human iris endothelial cells

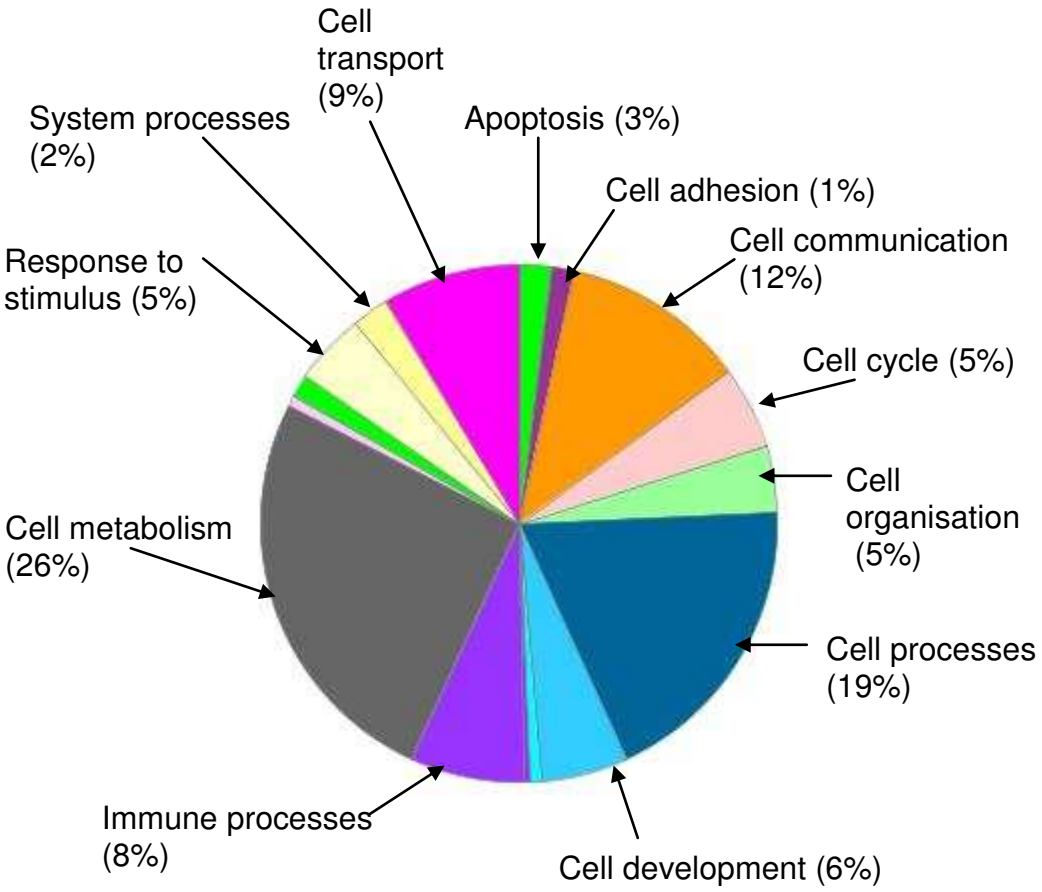


Fig 5.2 Heatmap of the differential probeset expression reaching statistical significance with reference to human macular inner and outer choroidal microvascular endothelial cells. Up-regulated probesets are shown in red, down-regulated in blue. A total of 302 different probesets are represented. The positions of selected probesets thought to be important in endothelial cell biology are shown.

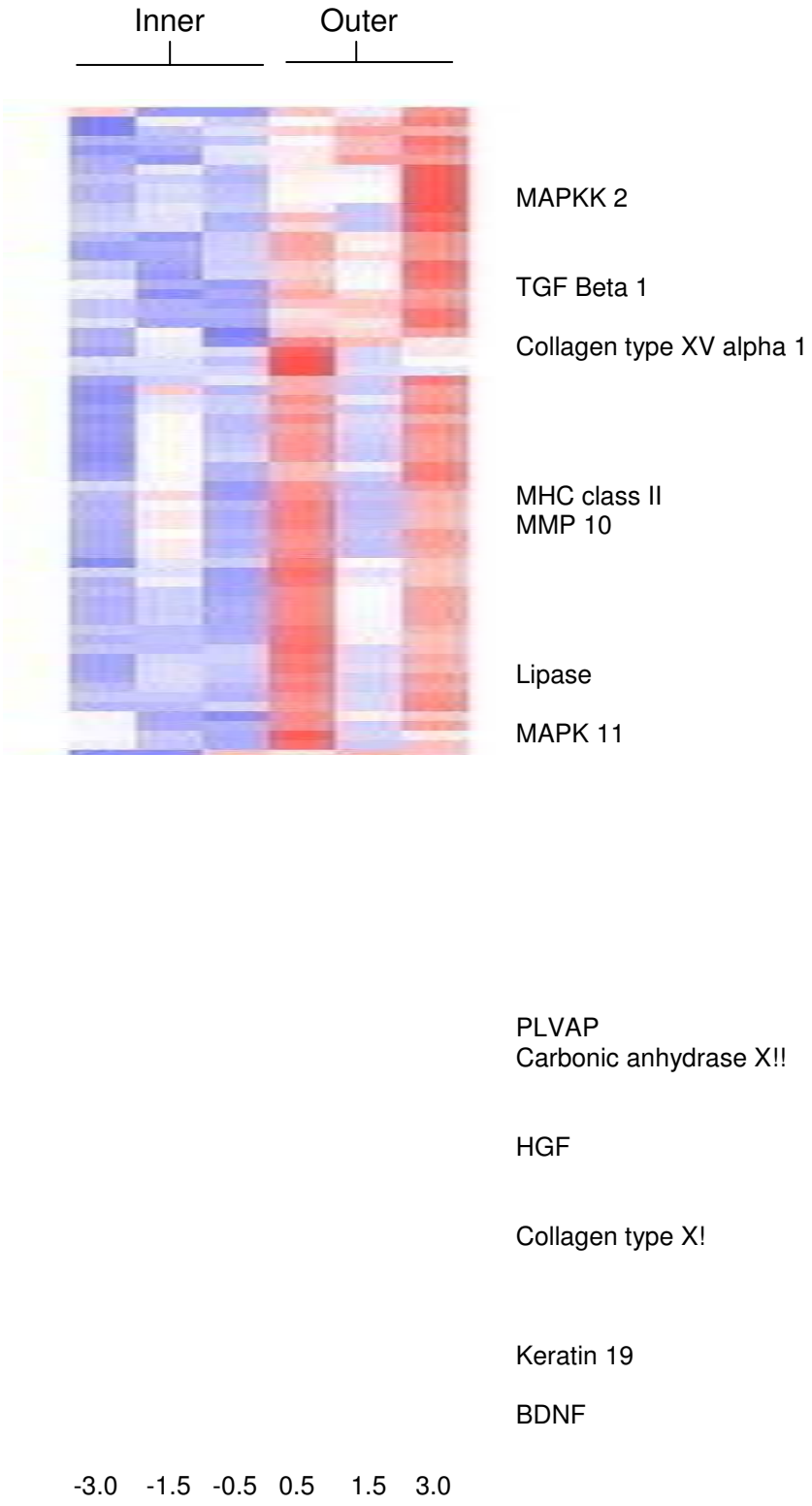


Table 5.5. Comparison of selected probesets up-regulated in human macular inner and outer choroidal endothelial cells

PROBESET	UP-REGULATED IN MACULAR INNER CECS (FOLD CHANGE)	UP-REGULATED IN MACULAR OUTER CECS (FOLD CHANGE)
Nervous system development	Brain derived neurotrophic factor (BDNF) (8.8) Neurofilament light polypeptide (NEFL) (5.2) Neurofilament medium polypeptide (NEFM) (10.4)	
Cell signalling	WNT5A (3.9)	Apelin receptor (5.4) MAPK 11 (2.8) MAPK binding protein 1 (2.7) DAG kinase zeta (2.3) Parvin beta (2.6)
Cell morphology	Collagen XI alpha subunit (4.4) Keratin 19 (7.3)	Collagen XV alpha subunit (4.0)

PROBESET	UP-REGULATED IN MACULAR INNER CECS (FOLD CHANGE)	UP-REGULATED IN MACULAR OUTER CECS (FOLD CHANGE)
Immune function		HLA DR B1 (2.7) MHC class II alpha (2.8) CD200 (2.3)
Cellular growth and proliferation	VEGF (4.7) Hepatocyte growth factor (HGF) (3.4) PDGF receptor alpha (8.8) TGF beta receptor 1 (3.3)	MMP 10 (3.2) CXCL 12 (stromal cell derived factor) (2.4) TGF beta 1 (4.5)
Misc	Plasmalemmal vesicle associated protein-1 (PLVAP) (2.5)	Endothelial lipase (3.2)

Real time PCR

Real time PCR was used to validate the differences in gene expression between proliferating human macular inner and outer choroidal endothelial cells and also human macular inner and peripheral inner choroidal ECs. Five transcripts were chosen that demonstrated at least a 2-fold differential expression between the endothelial cells on microarray analysis and were thought to be relevant to a range of different endothelial cellular functions. The chosen transcripts were: Keratin 19, Brain derived neurotrophic factor, CXCL 12 , Annexin 10 and MAPK 11. The differences in expression between the microarray and real time PCR techniques were similar for all 5 transcripts evaluated (Table 5.6 and 5.7). Fig 5.3 demonstrates examples of real time PCR dissociation curves for Keratin 19 in peripheral inner choroidal ECs, macular inner and macular outer choroidal ECs.

These results confirm the overall reliability of the results obtained by the Affymetrix microarray technique.

Fig 5.3 Examples of the real time PCR dissociation curves for Keratin 19 in peripheral inner choroidal ECs, macular inner and macular outer choroidal ECs. The Y axis depicts the fluorescence of the reporter signal normalised to a reference signal (Rn,) while the X axis depicts the cycle number. The probeset under investigation is represented by all 3 curves of the sample triplicate and also includes a probeset for the reference housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT). Also represented are the curves of the No Template Control (NTC) (sterile water).
Fig 5.3a, demonstrates the dissociation curves for a series of 10 fold dilutions for Keratin 19 reference sample while Fig 5.3b shows the calculated standard curve derived from Fig 5.3a demonstrating its linearity of the range tested.
Ct is the crossing point or threshold at which fluorescence can be detected and log C0 is the log of the relative standard concentration (chosen to correspond to the expected relative concentration of probeset in the samples).

Fig 5.3a

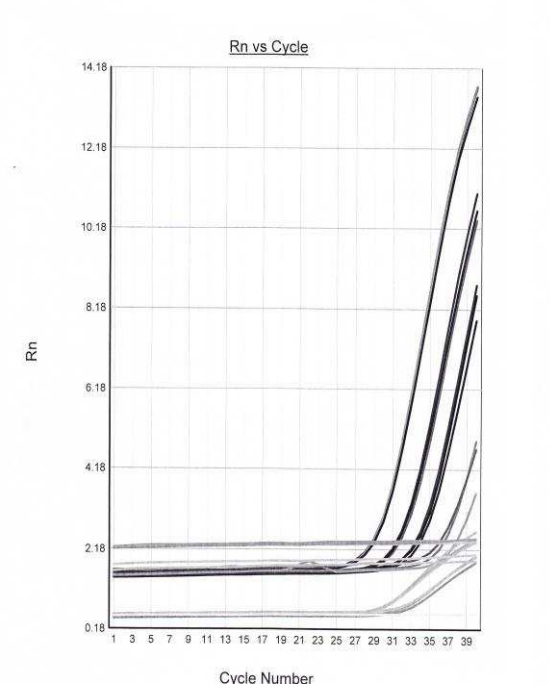
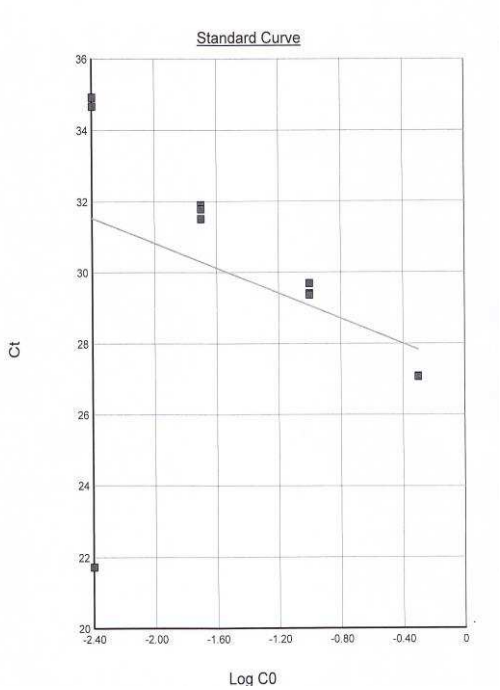


Fig 5.3b



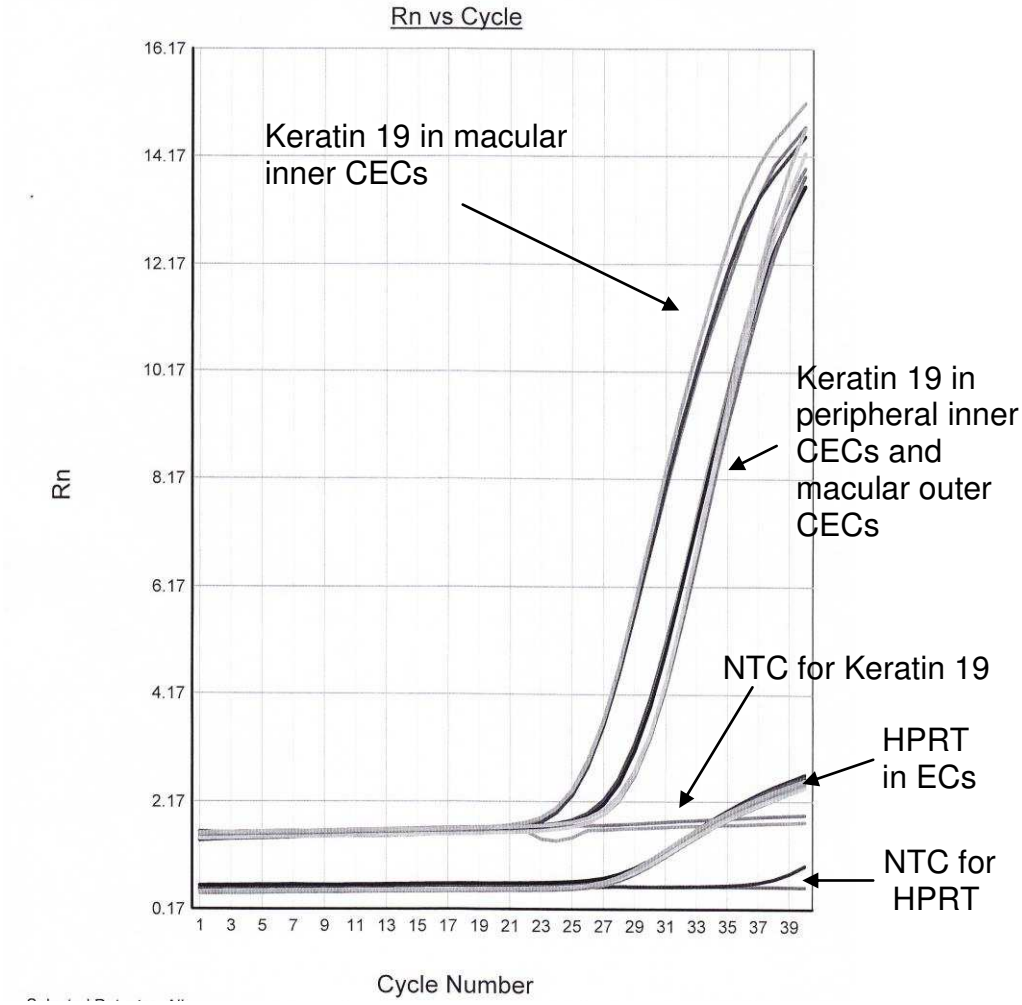


Fig 5.3c demonstrates the QPCR dissociation curves (triplicates) for Keratin 19expression in peripheral inner choroidal ECs, macular inner and macular outer choroidal ECs. The reference housekeeping gene is HPRT. The No Template Controls are also included.

Table 5.6. Differences in gene expression of selected genes for proliferating human macular inner and outer choroidal endothelial cells according to microarray (MA) and Real time-PCR (RT-PCR)

		Difference in gene expression (fold change)	
Gene transcript	**Affy ID**	**Fold change in gene expression relative to human macular inner CECs**	
		Microarray	**RT-PCR**
Keratin 19	201650_at	7.3	10.5
Brain derived Neurotrophic factor	206382_s_at	8.9	7.2
CXCL 12	203666_at	-2.4	-3.6
MAPK 11	206040_s_at	-2.8	-3.3

Table 5.7. Differences in gene expression of a selected gene for proliferating human macular inner and peripheral inner choroidal endothelial cells according to microarray (MA) and Real time-PCR (RT-PCR)

		Difference in gene expression (fold change)	
Gene transcript	**Affy ID**	**Fold change in gene expression relative to human macular inner CECs**	
		Microarray	**RT-PCR**
Annexin 10	210143_at	4.3	3.2

Discussion

One of the mysteries for those investigating and treating diseases of the ocular posterior segment is why different disorders demonstrate exquisite topographic selectivity for the choroidal vascular bed. Another is the quest for treatments that are selective for the cells involved in the pathological process but which leave normal, juxtaposed cells unaffected – the so called magic bullet. In the case of wet AMD, this would involve a treatment that specifically targets the proliferating endothelial cells of the inner choroid of the macula. Choroidal neovascularisation associated with wet age-related macular degeneration appears to have a predilection for the macular area of the posterior pole, unless some other secondary event has affected another area of the posterior segment e.g. damage to Bruch's membrane in the extra-macular area by inflammation or trauma. Interestingly, in an animal model of wet AMD, using focal laser treatment, application of identical burns to the extra-macular areas was relatively ineffective at stimulating a CNV compared with those placed in the macula, suggesting a predilection of the macula for choroidal neovascularisation (Shen et al, 2004).

Disease predilection for different layers of the choroid at similar locations is exemplified by punctate inner choroidopathy (PIC) and multiple evanescent white dot syndrome (MEWDS) which are inflammatory diseases that appear to preferentially affect the inner choroid (Cimono et al, 2000, Bouchenaki et al, 2002), while central serous chorioretinopathy is an idiopathic vasculopathy that also appears to involve the inner choroidal vasculature (based on the results of indocyanine green angiography) (Piccolino et al, 1995; Guyer et al, 1994).

For many years, workers have attempted to determine why the human macula is predisposed to age-related macular degeneration, the leading cause of blindness in those over 65years of age. There are a number of theories including: increased free radical production at the macula caused by the eye's inherent focusing of ultra violet or visible light at this location; a localised immune response at the macula causing secondary angiogenic events; local differences in Bruch's membrane structure; local differences in the RPE; and local differences in the choroid.

The first of these theories is plausible as it is known that visible light generates the production of free radicals which in turn can bring about local tissue damage which, in theory, could predispose to wet AMD. There is animal and epidemiological evidence to suggest that short wave length light (blue/UV) can damage the retina and may increase the risk of AMD (Cruickshanks et al, 2001; Tomany et al 2004; Grimm et al, 2001; Hafezi et al, 1997). However, the majority of large scale epidemiological studies have failed to demonstrate an association (Delcourt et al, 2001; Darzins et al 1997; AREDS Report 19, 2005; McCarty et al, 2001). The lack of a strong association, despite years of work in this area, probably means that the effect, if true, does not account for the majority of the cases and therefore is unlikely to be the major factor in the macular location of the condition.

Chong et al (2005) have shown that the thickness of the elastic layer of Bruch's membrane in the macular area is up to 6 times thinner and up to 5 times less abundant than in the periphery. The integrity of the elastic layer was also significantly lower in eyes with some degree of AMD. They suggest that for these reasons, the macula is predisposed to AMD, perhaps making the in-growth of choroidal blood vessels through Bruch's membrane easier.

The evidence for a local, macular-centred immune response is based on the findings of a predilection for drusen (particularly the soft variety, associated with increased risk of AMD) to form in the macular area (Midena et al, 1994; Abdelsalam et al, 1999) and the recent discovery of drusen containing elements of the immune response such as those involved in the complement cascade. On ultra structural analysis, cells such as macrophages appear to be localised to areas containing drusen, and local production of “active” immune mediators has also been found near to these cells. The localisation of the immune response to the macula is therefore probably based on the topographical distribution of drusen. The reason for their predilection at the macula is unknown but it may be due to the higher metabolic turnover of cells in this area (cones, and RPE cells) and therefore increased waste product formation and accumulation at the macula. More interestingly, it may also be due to the different architectural patterns within the vasculature of the choriocapillaris of the macula compared with peripheral areas (Lengyel et al, 2004), making drusen deposition between choroidal capillaries more likely in this location.

As mentioned previously, workers have studied both the morphological and gene expression differences in human RPE cell topography in an attempt to explain the difference in disease site specificity. However, very little is known regarding differences in gene expression between peripheral and macular choroidal endothelial cells. To date, only one study using microarrays has been conducted and this demonstrated a difference in 76 probesets between macular and peripheral choroid/RPE complexes. Of significance was a number of inflammation related genes such as CXCL14, CCL19 and CCL26 that were significantly up-regulated in the macula (Radeke et al, 2007). Unfortunately, the samples tested were a homogenate of choroid and RPE so any differences could not be ascribed solely to the choroid.

In order to expand our knowledge of the potential endothelial cell diversity within the human choroid, this study compared the differential gene expression of matched human macular and peripheral inner choroidal cells and macular inner and outer choroidal endothelial cells. It would not be surprising if there was heterogeneity within the endothelial cells of the choroid because the inner and outer choroid subserve different functions, with the inner choroid functioning to nourish the outer retina and the RPE layer via its fenestrated endothelium while the outer choroid functions to deliver and regulate blood flow to the inner choroid.

This study failed to demonstrate any significant difference in gene expression between matched human macular and peripheral endothelial cells. This confirms that despite the macular and peripheral choriocapillaris having different ultra-structural appearances, the lining endothelium appears to exhibit the same gene expression. This would suggest that the propensity of the macula to suffer choroidal neovascularisation is not due to topographical differences in the endothelial cells. This would also imply that any treatment designed to target the inner choroidal endothelial cell is just as likely to affect the peripheral cells as it is the macula, with the potential for widespread side effects inside the eye.

Significant differences, however, were found between matched inner and outer macular choroidal endothelial cells with around 300 (1.3%) probesets showing a significant difference. While no major differences in canonical pathways were discovered and many of the probesets remain unclassified, differences in functional areas such as nervous system development, cell signalling, immune functions and cell morphology were represented. In general, inner choroidal ECs demonstrated up-regulation of probesets involved in nervous system development (brain derived neurotrophic factor and neurofilaments), growth factors (VEGF, HGF, PDGF), plasmalemmal vesicle associated protein-1 (PLVAP), collagen XI and keratin 19 while macular outer

choroidal ECs demonstrated up-regulation of probesets involved in immune function (HLA DR B1, MHC class II alpha and CD200), some cell signalling components (DAG, MAPK11 and apelin) and other growth factors (TGF beta and stromal cell derived factor) and collagen XV. These differences are likely to be related to their different functions within the choroidal vascular unit. A hypothesis to explain many of these differences relies on the assumption that the inner macular CECs are the true microvascular ECs where physiological and pathological angiogenesis takes place, whereas the outer macular ECs are lining a type of “macrovascular vessel” whose function is to supply and regulate the supply of blood, including white cells and nutrients, to the choriocapillaris/inner choroid. This would explain why a wide variety of growth factor genes are found to be up-regulated in inner CECs (angiogenesis related). By contrast, up-regulation of immune function genes by the outer choroidal “macrovascular” ECs may control the passage of lymphocytes and macrophages into the inner choroid. It has also been shown that a fragment of collagen XV forms an endostatin, an anti-angiogenic factor thought to be important in inhibition of both physiological and pathological angiogenesis (Sasaki et al, 2002). The finding of the up-regulation of the gene coding collagen XV in outer choroid ECs may explain the absence of pathological angiogenesis in this area.

Perhaps one of the most important probesets found to be differentially expressed is that of plasmalemmal vesicle associated protein-1 (PVLAP). This was found to be up-regulated in macular inner CECs. PVLAP is a major structural protein known to be associated with fenestrations. The macular inner choroidal ECs are known to be fenestrated, whilst the outer choroidal ECs are not, and this is one of the major features differentiating inner choroidal ECs from other ocular endothelial cells. Interestingly, however, not all fenestrated ECs have been shown to express PLVAP, with those of the liver and the glomerulus being negative (Satchell & Braet, 2009).

Brain derived neurotrophic factor (BDNF) is a member of a group of proteins called neurotrophins which promote the growth, survival and differentiation of neurones in the central and peripheral nervous system. Within the eye, it is known that it is secreted by RPE cells, photoreceptors and Muller cells and has been shown experimentally to prevent ischaemic ganglion cell death and to protect photoreceptors from light induced toxicity (Kano et al, 2002). Recently, BDNF has been found to be secreted by vascular endothelial cells and may be responsible for the levels of the growth factor detected in serum. However, its role outside the central and peripheral nervous system remains unknown (Nakahashi et al, 2000). In the current work, BDNF was found to be up-regulated 5-fold in macular inner choroidal ECs compared with matched outer macular ECs. There are a number of hypotheses as to why this should be. It may be involved in the maintenance and function of neurones found within the choroid which are involved in regulation of choriocapillaris blood flow or it may pass across Bruch's membrane and the RPE and be involved in outer photoreceptor function.

Interestingly, inner and outer macular choroidal ECs appear to demonstrate preferential up-regulation of collagen types XI and XV respectively. Collagens are components of cell basement membranes and this difference in collagen expression suggests discrete requirements for these structures within the different layers of the choroid. Collagen XI is a fibrillar collagen, mutations in which have been found in Stickler's syndrome, a condition associated with myopia and abnormalities of the vitreous, retina and posterior segment. Collagen XV is a non-fibrillar type of collagen which is found in some endothelial cell basement membranes and is thought to facilitate its binding to surrounding connective tissue (Amenta et al, 2005). There also appear to be subtle differences in the expression of a number of components involved in cell signalling activation. Inner macular ECs demonstrated up-regulation of the WNT5 pathway, which may be involved in angiogenesis. This is a poorly understood pathway that may regulate

cell proliferation, apoptosis and branching morphogenesis (Masckauchán et al, 2006; Masckauchán et al, 2005). In contrast, outer macular choroidal ECs demonstrated up-regulation of a range of probesets including apelin, MAPK11 and diacyl-glycerol kinase zeta. These peptides are members of a large family of peptides involved in the transduction of cell signalling from cell surface receptors to the nucleus, stimulating cell proliferation. Apelin and its receptor have been found on a variety of endothelial cells (including those in the eye) and activation leads to vasodilatation, proliferation, vasculogenesis and *in vitro* tube formation (Kasai et al, 2004). These findings would suggest that there are subtle differences in the intracellular signalling cascades of proliferating inner and outer choroidal ECs. This finding may be utilised to enable a more selective inhibition of inner choroidal endothelial cell function.

Activation of cell signalling pathways usually requires binding and stimulation of cell surface receptors by ligands (often growth factors, if stimulation of cell proliferation is occurring). Macular inner and outer CECs demonstrated a different pattern of growth factor expression. Stimulation of vascular endothelial cells is usually via growth factors secreted by other cell types such as RPE cells and macrophages which are known to secrete VEGF. The role of similar growth factors expressed by endothelial cells themselves suggests either local paracrine stimulation, perhaps to maintain cell phenotype, or the stimulation of different types of juxtaposed cells such as pericytes or stromal cells. Macular inner choroidal cells demonstrated up-regulation of VEGF and hepatocyte growth factor (HGF). It would be interesting to hypothesise that VEGF secretion is required to maintain the specialised phenotype of endothelial cells within the choriocapillaris (fenestrations). It is known that RPE cells also secrete VEGF which is thought to be important in maintaining choriocapillaris fenestrations.

Hepatocyte growth factor is another potent endothelial mitogen secreted by cells of mesenchymal origin including vascular endothelial cells and macrophages. It has a structure very similar to plasminogen, contains a heparin binding domain and is secreted by cells in an inactive form (Grierson et al, 2000). It relies on the action of serine proteases for activation and activates cells via the c-met receptor (present predominantly on cells of epithelial lineage but includes vascular endothelial cells). In the eye, it is thought to play a role in corneal development and maintenance of normal corneal structure and in the maintenance of trabecular meshwork structure (Grierson et al, 2000). In the posterior segment, the RPE demonstrates a high level of expression of the c-met receptor and this may be necessary for its normal function and secondarily, for photoreceptor health. *In vitro* studies have revealed that HGF protects confluent cultures of RPE cells against apoptosis (Jin et al, 2005), promotes RPE cell proliferation and migration (Miura et al, 2003) and also leads to disassembly of tight and adherens junctions (Jin et al, 2002). Hepatocyte growth factor therefore appears to bring about the same effects on RPE cells as does VEGF on vascular endothelial cells.

There is controversy as to whether RPE cells also secrete HGF, as this would indicate a local paracrine loop (He et al, 1998). However, HGF is known to be required for the maintenance of the RPE barrier function with local over-expression being linked with retinal detachment (Jin et al, 2002; Jin et al, 2004). Elevated levels are also found in the vitreous of diabetics and it has been shown to be a potent angiogenic growth factor (greater than VEGF) and may therefore play a role in proliferative diabetic retinopathy (Nishimura et al, 1999).

While there is a large body of evidence regarding the effect of HGF in retinal neovascularisation, its role in choroidal homeostasis and neovascularisation is less well understood. In a rat laser model of choroidal neovascularisation, HGF was found to be up-regulated early on in the angiogenic process within the choroid (Hu et al, 2009).

To the author's knowledge, no studies on HGF in human eyes, either normal or those with CNV, have been conducted. This study demonstrated that proliferating human macular inner choroidal endothelial cells preferentially express HGF compared with outer choroidal ECs. There are a number of possible reasons for this. Based on the findings from other parts of the eye (RPE), the expression may be part of a paracrine loop required for either the normal functioning of endothelial cells or as an amplification factor for CECs already proliferating (review of raw microarray data shows that the cells also demonstrate background expression of the c-met receptor). However, this does not explain why juxtaposed proliferating outer choroidal ECs do not demonstrate a similar phenomenon. Another reason could be the local (paracrine) secretion of HGF to affect non-endothelial cells such as RPE cells which are known to require HGF for normal cell – cell interactions. These findings may imply that local targeting of HGF in the treatment of choroidal neovascularisation may have inadvertent deleterious effects on the posterior segment of the eye.

In contrast, macular outer choroidal ECs demonstrated up-regulation of probesets for the growth factors CXCL12 (stromal cell derived factor) and TGF-Beta 1. Stromal cell derived factor (SDF-1) is a known mitogen for endothelial cells It is also involved in the attraction of endothelial progenitor cells to areas of neovascularisation and has been found on histological examination of excised choroidal neovascular membranes. Its differential expression by outer macular CECs was previously unknown. However, Bhutto et al (2006) recently demonstrated expression of SDF-1 and its receptor within the choroidal stroma (as well as the RPE) and suggested that they may be involved in the recruitment of leukocytes and other inflammatory cells to the choroidal stroma as well of endothelial progenitor cells during local wound healing responses i.e. angiogenesis (Bhutto et al, 2006).

In summary, this particular part of the study has demonstrated small subtle differences between matched human macular inner and outer choroidal endothelial cells. No significant topographical differences were found between macular and peripheral inner choroidal endothelial cells. This is in contrast to other studies which have shown topographical differences in the structure, function or gene expression of retinal and RPE cells and in Bruch's membrane. Interestingly, when one reviews the results of previous studies which examined the gene expression differences between macular and peripheral tissue using a combination of RPE/choroidal tissue (Radeke et al, 2007; Bowes Rickman et al, 2006), none of the gene differences found in their studies were represented in this present study. This suggests that most of the variation described was probably due to variability within the RPE cells. It is further suggested that the non-significant topographical variability within proliferating inner choroidal endothelial cells is probably not the cause of the site specific selectivity of wet AMD to the macula, and that this phenomenon is more likely, as previously stated, to be due to topographical differences in other ocular cell types or to selective exposure of the macular to a disease causing agent.

Variability was noted, however, in the gene expression of matched macular inner and outer choroidal ECs. This differential gene expression would suggest subtle differences in the roles and microenvironments of the two cell types commensurate with the different structures and functions of the inner and outer choroid. These observed differences may assist us in understanding some of the underlying mechanisms of choroidal neovascularisation and provide potential routes for selective intervention to treat the disease.

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Chapter 6

The effects of growth factors on the proliferation and in-vitro angiogenesis of human macular inner choroidal endothelial cells

Introduction

While wet AMD remains the leading cause of blindness in people over 65 years, the underlying pathophysiological mechanisms responsible for disease initiation and progression remain largely unknown. Current opinion suggests that there is an angiogenic response by inner choroidal endothelial cells to a range of cytokines and growth factors, driven by a local adverse stimulus. An electron microscopic study by Killingsworth in 1995 of eyes with early choroidal neovascularisation demonstrated endothelial sprouts continuous with intra-choroidal vessels penetrating Bruch's membrane. They were also shown to spread between the inner layers of Bruch's membrane in the space normally occupied by drusen. The authors described two phases to the growth of vessels - an early intra-choroidal phase which was of a "low turnover" type, and a later sub-RPE neovascular phase characterised by marked endothelial cell proliferation termed as "high turnover". Interestingly, Guymer et al in 2004 described cellular processes from choroidal capillary endothelial cells that penetrate Bruch's membrane but are not associated with choroidal neovascular membrane formation (Guymer et al, 2004). These processes are thought to stabilise the endothelial cell and to play a structural role in the maintenance of the choriocapillaris. It is not known what factors are important in the differentiation of the endothelial cell processes from physiological to pathological states.

Immunohistochemical examination of surgically excised human choroidal neovascularisation (CNV) and animal models of laser induced CNV implicate several growth factors in the pathogenesis of CNV. Examination of CNV induced in rats by krypton laser photocoagulation has consistently revealed up-regulation of vascular endothelial growth factor (Wada et al, 1999; Yi et al, 1997; Shen et al, 1998) and fibroblast growth factor 2 (Ogata et al, 1996; Frank et al, 1997) by RPE cells, fibroblasts and macrophages within the CNV. Studies conducted on

human CNV secondary to AMD and presumed ocular histoplasmosis syndrome have shown increased expression of VEGF (Grossniklaus et al, 2002; Frank et al 1996; Kliffen et al, 1997; Kvanta et al, 1996), FGF2 (Frank et al, 1996; Amin et al, 1994; Reddy et al, 1995), Insulin- like growth factor 1 (IGF-1) (Rosenthal R et al, 2004; Lambooij et al, 2003), Platelet derived growth factor (PDGF) (Kliffen et al, 1997), connective tissue growth factor (CTGF) (Watanbe et al, 2005; He et al, 2003), Placental growth factor (PlGF) (Rakic et al, 2003), and Interleukin-1β (IL-β) (Zou et al, 2006)

Further evidence for the role of growth factors in the pathogenesis of CNV is supported by the findings that inhibition of VEGF (Kwak et al, 2000), FGF2 (Tobe et al, 1998), IGF-1 (Bezerra et al, 2005), PDGF (Jo et al, 2006) and IL-1 (Zou et al 2006) reduced CNV lesion size and/or fluorescein leakage in animal models. Recent human studies have reported beneficial effects of intra-ocular VEGF inhibition on visual acuity in patients with sub-foveal CNV, further implicating a role for this growth factor in AMD (Rosenfeld et al, 2006). However, evidence from studies of retinal neovascularisation (Watnabe et al, 2005; Dills et al, 1991, Freyberger et al, 2000), in which IGF 1 may play a role, and the observation that inhibition of VEGF does not completely abrogate CNV growth and/or leakage in all cases (Williams & Fekrat, 2006; Gragoudas et all, 2004) makes it likely that other growth factors are also important in choroidal neovascularisation.

In vitro studies using bovine choroidal endothelial cells have shown pro-angiogenic effects for VEGF, FGF2, IGF-1 and PDGF (Sakamoto et al, 1995; Wang et al, 2002; Liu et al, 1998). However, as mentioned previously, it is well recognised that there is a marked heterogeneity amongst endothelial cells from different species and sites, making it difficult to extrapolate these results to human inner macular choroidal disease i.e. wet AMD. It has been previously shown in Chapters 3 and

5 that even within the eye, there is heterogeneity in vascular endothelial cells, even within the layers of the choroid. In this chapter, the in vitro proliferative and angiogenic effects of a range of candidate growth factors on human macular inner choroidal endothelial cells was undertaken in order to increase our understanding of the underlying mechanisms of human choroidal neovascularisation. This research is unique in that, for the first time, it reports the effects of the aforementioned factors on human macular inner choroidal endothelial cells (ICECs).

Methods

Isolation of human macular inner choroidal endothelial cells

The isolation of these cells has been described previously (Chapter 4).

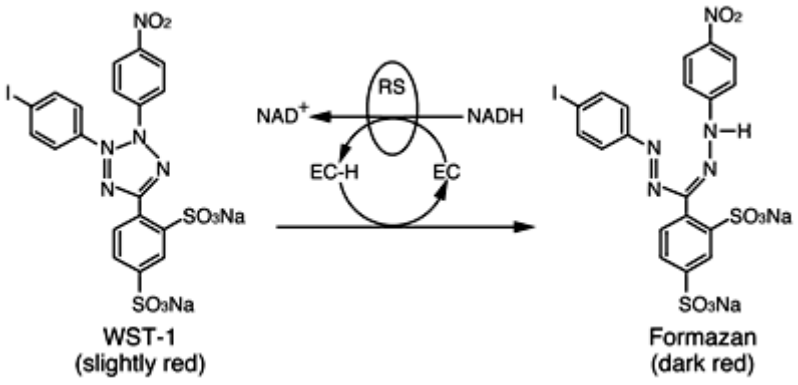
Cell Proliferation assays

Manual Cell counting

Twenty thousand first passage human macular ICECs in EGM2-MV were added to each gelatin (0.1%) coated 35mm cell culture dish, marked with 2mm grids (Nunc, Rochester, NY, USA). The number of culture dishes used allowed testing of each factor at each concentration in triplicate sets. Cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2 for 24 hours to allow cell attachment. The medium was then removed and the cells washed 3 times with sterile PBS. The PBS was then replaced with serum-reduced medium (EGM-2), supplemented with Gentamicin (5µg/ml), Amphoterocin B (2.5µg/ml) and 0.5% foetal calf serum heat inactivated for 4 hours at 56ºC. After 18 hours, the medium was replaced with 2 ml of serum-reduced medium containing selected growth factors ($VEGF^{165}$, FGF2, IGF1, PDGF-AA, PDGF-BB and IL-1β all from R and D systems, Abingdon, UK at 300 and 900pmol/l). Attached cells within 14 squares of each plate were counted 3 times at baseline and 48 hours after addition of growth factor or serum-reduced medium. Cell proliferation (%) was calculated by the mean increase in cell number relative to baseline. For each growth factor investigated, all tests were carried out in triplicate, on human macular ICECs derived from the same donor and repeated on a total of four separate donors.

WST-1 assay

Human macular ICEC proliferation in the presence of different growth factors was also assessed using the WST-1 assay (Roche, Lewes, UK). This is one of a range of rapid, non-radioactive, liquid based assays for assessing cell proliferation by the measurement of metabolic activity. The assays utilise the property of viable and proliferating cells cleaving a tetrazolium salt to formazan by mitochondrial respiratory chain enzymes. This brings about a colour change in the tetrazolium compound that can be measured by a spectrophotometer. The assay is based on the assumption that the more functioning mitochondria that are present, the more intense the colour change that can be measured. Over a specified range of viable or proliferating cells, this colour change is linear. The most common tetrazolium salt used is MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. However it suffers from the disadvantage that the formazan dye produced from MTT is extremely water insoluble, so an additional extraction step is needed before quantification. Instead, WST-1 utilizes a tetrazolium salt [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-terazolium] which produces a water soluble formazan, which makes the assay much easier and user friendly. The change in the structure of the tetrazolium salt is shown below



WST-1
(slightly red)

Formazan
(dark red)

In the assay, two thousand five hundred (2500) first passage ICECs, suspended in EGM2-MV, were added to each well of a fibronectin coated 96 well plate (Beckton Dickinson, Oxford, UK) and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 24 hours to allow cell attachment. The medium was then removed and the cells washed 3 times with PBS and replaced with serum-reduced medium (EGM-2) supplemented with antibiotics and 0.5% heat inactivated serum as described above. After 18 hours, the medium in selected wells was replaced with 150µl of serum reduced medium containing growth factors: $VEGF_{165}$, FGF2, IGF1, PDGF-AA, PDGF-BB and IL-1β, all from R & D systems, Abingdon, UK, at a concentration of 1000 pmol/l. Further alterations in growth factor concentrations were then obtained by a double dilution technique across the plate. The cells were incubated in the presence of the growth factors for 48 hours, after which WST-1 was added to each well and incubated for 4 hours at 37ºC and then the absorbance of each well was recorded at 450nm and 650nm using a Thermamax microplate reader (Molecular Devices, Wokingham, UK) according to the manufacturer's instructions. Cell proliferation was expressed as a percentage increase relative to controls i.e. cells exposed to serum reduced medium without any growth factor added. Each growth factor concentration was examined in triplicate as stated above and the mean values were used in the data analysis.

In vitro Angiogenesis Assay

The three dimensional *in vitro* angiogenesis assay used in this work was a modification of that previously described by Stitt et al in 2005. Briefly, chilled first passage human macular ICECs in EGM2, supplemented with 1.5% heat inactivated foetal calf serum, was mixed with Matrigel (Beckton Dickinson, Oxford, UK) chilled to 4ºC in a ratio of 1:2 to give a final 0.5% foetal calf serum containing ICECs at a concentration of $1x10^7$/ml. Aliquots of the mixture (4µl) were pipetted into the base of the wells of a flat bottom 96 well plate using cooled

pipette tips. The spots of Matrigel mixture were allowed to polymerise at 37ºC in a humidified atmosphere of 5% CO_2 for 1 hour, after which 200µl of serum (0.5%) reduced EGM2 was gently added to each well and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 24 hours. At this stage, ICECs were seen to form a network of tubes within and up to the edge of the Matrigel spot. The medium was gently aspirated and the plate allowed to stand at room temperature in a laminar flow hood for 5 minutes. Fifteen microlitres of a 1:2 mixture of serum (0.5%) reduced EGM2 and chilled Matrigel (4ºC), each supplemented with the same selected growth factors ($VEGF^{165}$, FGF2, IGF-1, PDGF-AA, PDGF-BB and IL-β1 at a final concentration of 1000pmol/l), was carefully pipetted around the edge of the spot to give a duplex culture as previously described (Stitt et al, 2005). After polymerisation at 37ºC for 1 hour, 200µl of serum (0.5%) reduced EGM2 were added to each well. After 48 hours, each spot was observed by phase contrast microscope. As previously described by Stitt et al, a dark line was seen to demarcate the interface between the primary and secondary Matrigel layers. The numbers of endothelial sprouts that were seen to cross the demarcation line were counted around the circumference of each primary Matrigel spot (see Fig 6.1). For each growth factor, the mean number of sprouts counted in 10 wells was compared with the mean number of sprouts counted in control wells exposed to serum reduced (0.5%) EGM2 without any added growth factors.

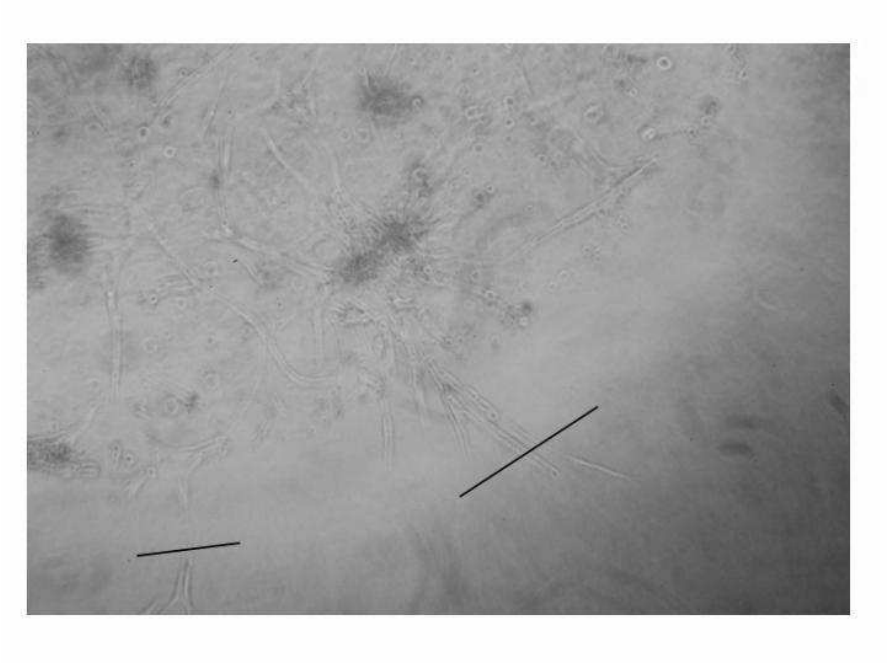


Fig. 6.1. Phase contrast micrograph demonstrating the invasion of preformed microvessels into the secondary Matrigel layer (dark lines represent the demarcation of the two layers of Matrigel).

Statistics

The results are presented as the mean of at least 3 separate experiments ± standard error of the mean (SEM). Differences between growth factors and a serum reduced control were analysed by a Student's t test. Significance was defined as $p < 0.05$.

Results

Human macular ICEC Proliferation

Prior to undertaking the cell proliferation assays, it was shown that first passage human macular ICECs survived without undergoing significant proliferation or phenotype change when maintained for 48 hours in EGM-2 supplemented with 0.5% foetal calf serum, heat inactivated for 4 hours. This provided a baseline against which the effects of various growth factors on endothelial cell proliferation and in-vitro angiogenesis could be determined (Fig 6.2).

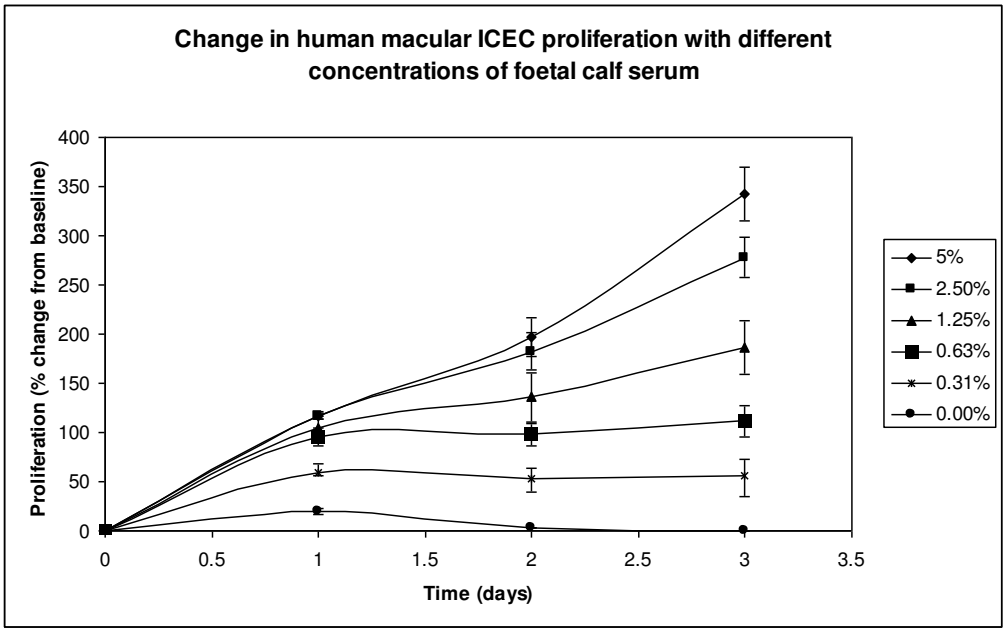


Fig 6.2. Mean proliferation of macular inner choroidal endothelial cells measured using cell counting in the presence of different concentrations of heat inactivated foetal calf serum (error bars indicate ± SEM, n=3.)

Two different techniques were used to determine cell proliferation. The manual cell counting technique allowed determination of absolute cell numbers in response to stimulation with growth factors and monitoring of cell phenotype over time. The other technique was the WST-1 colorimetric assay, which measures cellular mitochondrial activity that can be used as an indicator of cell viability and/or proliferation.

Manual Cell Counting

The mean of 3 experiments carried out in triplicate are summarised in Fig. 6.3 (growth factor concentration of 300pmol/l) and Fig. 6.4 (growth factor concentration of 900 pmol/l). Both $VEGF^{165}$ and FGF2 showed a significant increase in proliferation at both concentrations examined. At 300 pmol/l, $VEGF^{165}$ and FGF2 increased mean proliferation (± SEM) by 40 ± 8% ($p=0.004$) and 42 ± 4% ($p=0.002$) respectively, compared with control serum reduced medium. At 900pmol/l, $VEGF^{165}$ and FGF2 increased mean proliferation (± SEM) by 140 ± 13 % and 107 ± 13 % respectively ($p<0.0004$ for both). Equimolar combinations of $VEGF^{165}$ and FGF2 at 300 and 900 pmol/l increased proliferation (± SEM) by 71 ± 10% and 217 ± 52% respectively (effects approximately additive). There was no significant effect of IGF-1, PDGF-AA, PDGF-BB or IL-1β on hICEC proliferation at either 300 or 900 pmol/l concentrations. Equimolar combinations of $VEGF^{165}$ with any of the other factors, namely IGF-1, PDGF-AA, PDGF-BB and IL-1 β at 900pmol/l did not show a significant increase in proliferation over $VEGF^{165}$ alone.

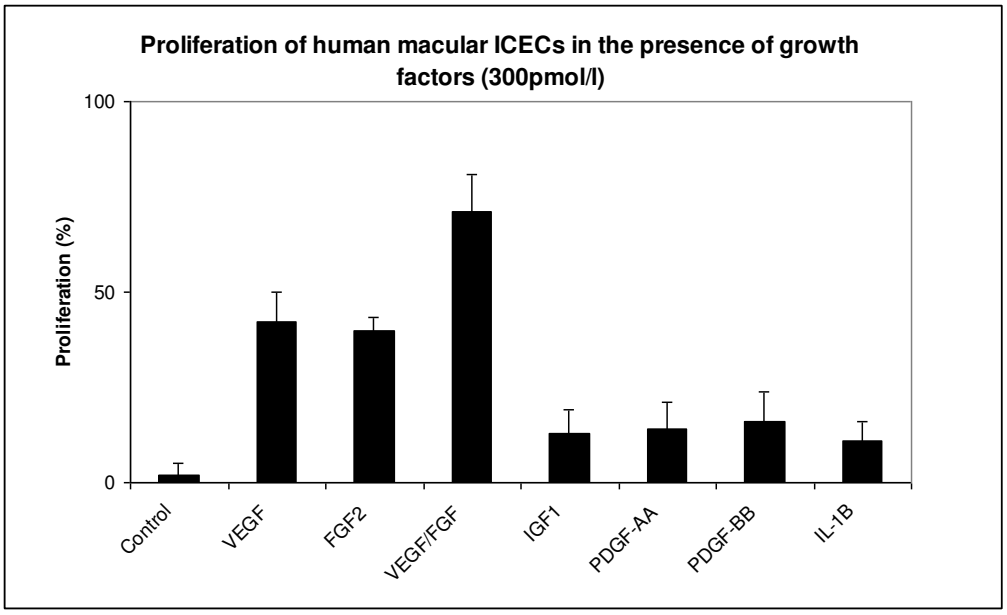


Fig 6.3. Mean proliferation of human macular inner choroidal endothelial cells measured by manual cell counting in the presence of various growth factors at 300 pmol/l (± SEM, n=3)

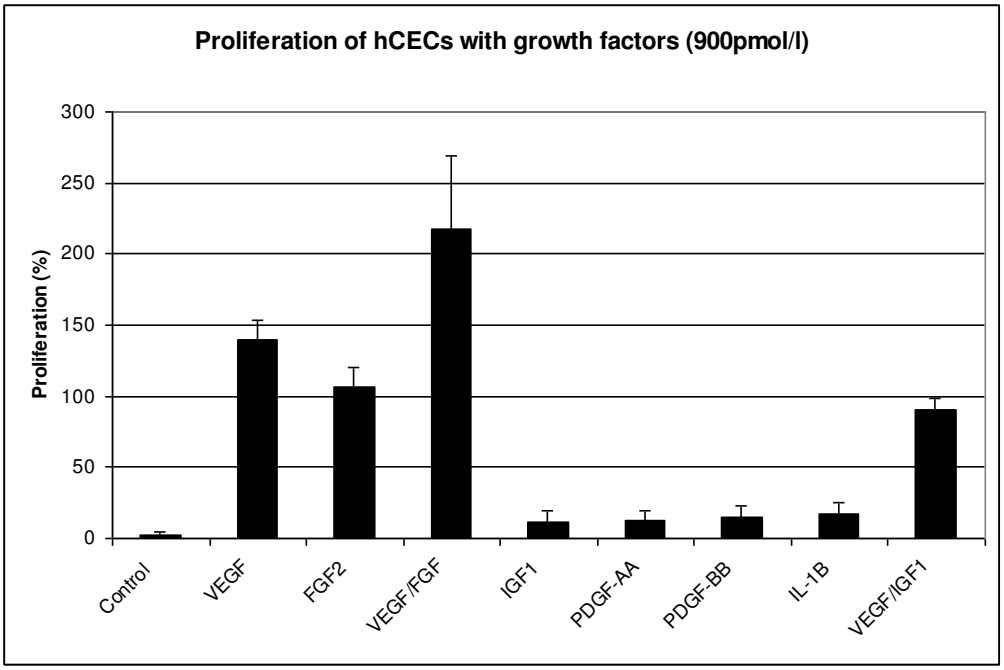


Fig 6.4. Mean proliferation of human macular inner choroidal endothelial cells measured by manual cell counting in the presence of various growth factors at 900 pmol/l (± SEM, n=3)

WST-1 assay

The growth curves representing the mean of 3 different experiments for each growth factor are shown in Fig. 6.5. This shows a significant dose-response effect for $VEGF^{165}$ ($p=0.0002$) and FGF2 ($p=0.02$) between 7.8 and 1000pmol/l. At 1000pmol/l, an equimolar combination of VEGF and FGF2 increased proliferation (± SEM) from 126 ± 9% and 114 ± 23% respectively for the individual growth factors to 193 ± 62% for the combination. There was no significant dose response effect for IGF-1 ($p=0.20$), PDGF-AA ($p=0.53$), PDGF-BB ($p=0.88$) or IL-1β ($p=0.25$) between 7.8 and 1000pmol/l.

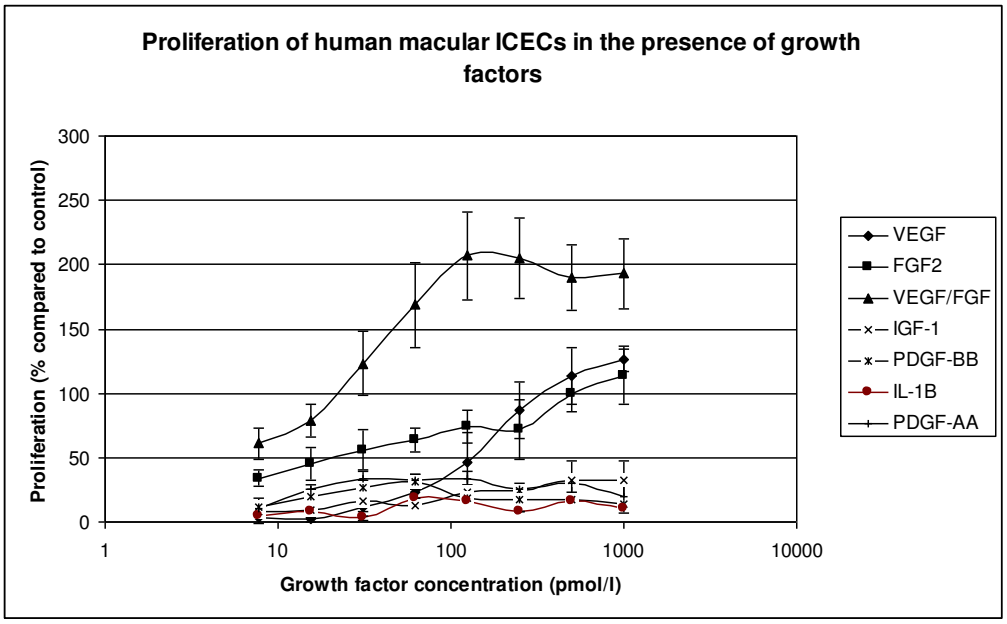


Fig 6.5. Growth curves for human macular inner choroidal endothelial cells measured using the WST-1 assay in the presence of different growth factors. (Error bars indicate ± SEM, n=3)

In vitro Angiogenesis Assay

The mean percentage increase in tubes crossing the junction between the primary and secondary Matrigel layers is shown in Fig 6.6

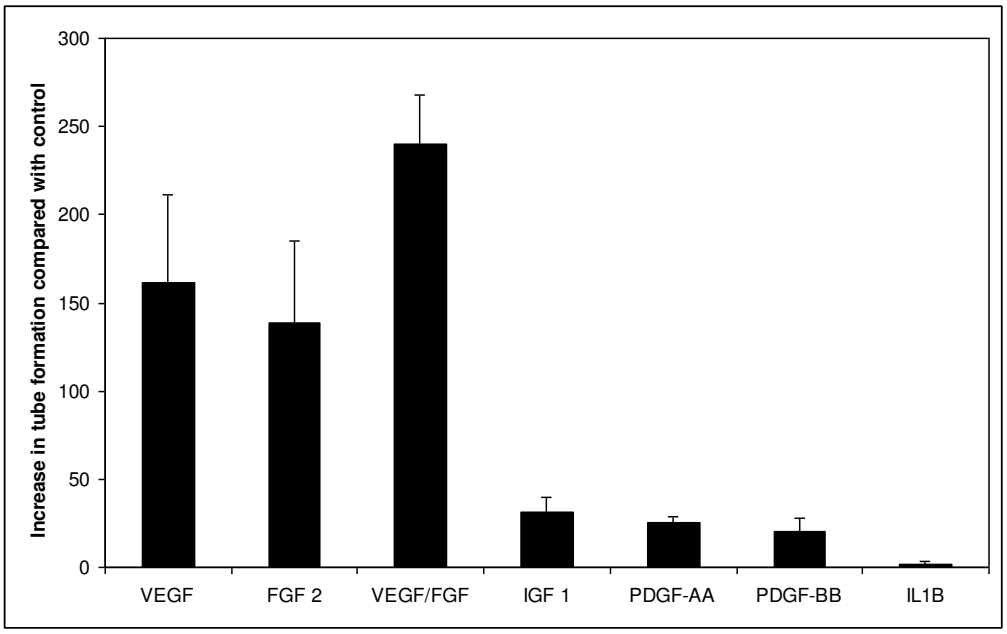


Fig 6.6. Mean increase in tube formation compared with control as a measurement of angiogenesis using 3D double layer Matrigel assay for various growth factors (± SEM, n=3)

Compared with controls (EBM-2 supplemented with 0.5% FCS), there was a significant increase in tube formation (± SEM) for $VEGF^{165}$ and FGF2 at 1000pmol/l by 161± 50 % (p=0.003) and 139 ± 46% (p=0.003) respectively. Equimolar combinations of $VEGF^{165}$ and FGF2 at 1000pmol/l increased tube formation (± SEM) by 240 ± 28%. There was no significant increase in tube formation compared with EBM-2 supplemented with 0.5% FCS for IGF-1 (p=0.21), PDGF-BB (p=0.36), PDGF-AA (p = 0.41) or IL-1β (p=0.83).

Discussion

A number of different growth factors have been found to be associated with CNV tissue removed from human eyes. However, knowledge of their individual roles in disease pathogenesis remains incomplete. Many growth factors have been found to be pro-angiogenic in *in vivo* animal models of CNV and in *in vitro* experiments utilising animal choroidal ECs or human non-choroidal ECs. It is accepted though, that ECs are extremely heterogeneous in nature and may show varying responses to a range of potential mitogens (Thorin & Shreeve, 1998; Rhodin 1968; Graier et al, 1996; Imegwu et al, 2001; Ram & Hiebert, 2003; Craig et al, 1998). To overcome these potential problems in the investigation of the effect of various growth factors in the pathogenesis of choroidal neovascularisation, we have utilised human macular inner choroidal endothelial cells in this work. In addition, all ICECs were used at first passage to reduce potential problems associated with phenotypic change which is known to occur in cultured ECs (Augustin-Voss et al, 1993, Fenyves et al, 1993). With multiple passages these cells may lose their differentiated characteristics which they had *in vivo*, and may be prone to genetic instability. The modified angiogenesis assay used in this work has previously been shown to be an accurate model of the concurrent processes involved in angiogenesis: cell proliferation, BM digestion, migration and tube formation (Stitt et al, 2005). This makes it ideal in the investigation of the factors underlying abnormal choroidal angiogenesis.

This work has shown that both VEGF and FGF2 individually stimulate human ICEC proliferation and angiogenic tube formation with similar potency. This finding is significant because while it is known that the biological effects of VEGF are largely restricted to ECs, the role of FGF2 on CNV pathogenesis has been largely unexplored and remains controversial (Rosenthal et al, 2005; Tobe et al, 1998). Fibroblast growth factor is known to have mitogenic effects on fibroblasts,

chondrocytes, smooth muscle cells and melanocytes in addition to ECs (Burgess & Macaig, 1989). The FGF2 subtype is known to be present in CNV (as discussed previously) and may therefore also promote effects in cells other than ECs in the formation and propagation of CNV. As mentioned in chapter 1, there is still debate as to whether FGF exerts its pro-angiogenenic effect on endothelial cells by its own effect on the cellular proliferative mechanism via FGF receptors and intracellular signalling cascades or via up-regulation of the effect of VEGF. Evidence exists for both hypotheses as it is known that specific pro-angiogenic pathways are activated when FGF binds to its receptor (FGFR1) (Cross and Claesson-Welsh) and FGF has also been shown to up-regulate VEGF and VEGF receptor expression on ECs (Murakami and Simons, 2008; Murakami et al 2011; Seghezzi et al, 1998). This is an area where future research would be beneficial.

When hICECs were exposed to equimolar concentrations of VEGF and FGF2, the effect of the growth factors was additive even within the plateau phase of proliferation of the individual growth factors. This suggests that the growth factors may, at least in part, be acting via different activation pathways, an important factor for future development of anti-angiogenic therapies. This hypothesis is given weight by studies on bovine adrenal ECs which demonstrated synergistic effects of VEGF and FGF2 (Goto et al,1993; Pepper et al, 1992) and on bovine CECs and HUVECs, which showed activation of the intracellular MAPK pathway by VEGF and FGF2 by different mechanisms (Mc Laughlin et al, 2001; Zubilewitz et al, 2001). While little work has previously been conducted on the mechanism of FGF2 stimulation of hCEC, it has recently been found that these proliferating cells preferentially express mRNA for the FGF Receptor 1 compared with retinal endothelial cells (see Chapter 3), again implicating a direct role for FGF-2 in hCEC biology.

It is known that most of the pro-angiogenic actions of FGF 2 are mediated via the receptor tyrosine kinase, FGFR-1. There are at least three other FGF receptors (FGFR2-4) which are present on a variety of different cells, implying a degree of system redundancy as suggested by embryological studies (Cross and Claesson-Welsh, 2001). Activation of the receptor requires dimerisation of two receptors which is followed by autophosphorylation of specific tyrosine residues (Klint and Claesson-Welsh, 1999). The specific sites of phosphorylation have been found to bind molecules such as Crk which leads to MAPK activation, and PLC-γ. Receptor activation is known to lead to activation of a number of intracellular signalling cascades including the Ras pathway, Src receptor kinases, PI3K and the PLC pathway (see Fig 6.7 for a diagrammatic representation).

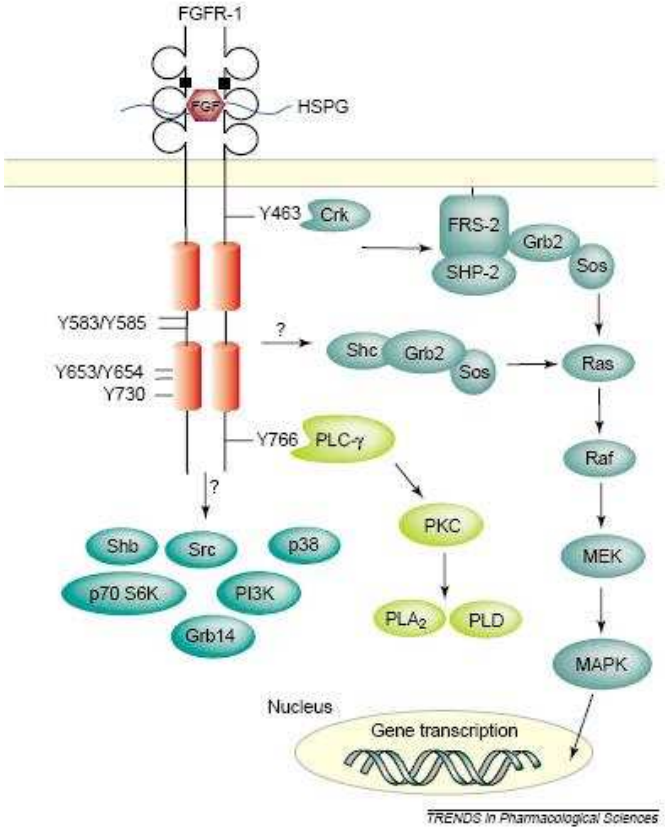


Fig 6.7. An overview of FGFR-1 signalling. As mentioned above, activation leads to the autophosphorylation of several sites of the intracellular molecule which leads to activation of different signalling cascades. The end result appears to be up-regulation of MAPK which is able to enter the nucleus to modify gene expression i.e. cell proliferation and protection from apoptosis. (Reproduced by permission of Elsevier)

In contrast, VEGF-A exerts its pro-angiogenic effects on vascular endothelial cells via two distinct receptors, VEGFR-1 and VEGFR-2. The receptors consist of an extracellular portion composed of seven Ig like domains, a transmembrane domain and an intracellular kinase domain. While VEGFR-1 is a weak tyrosine kinase, but with a ten fold higher affinity for VEGF than R-2, it is VEGFR-2 that is thought to important in angiogenic responses. Binding of VEGF-A to its R-2 receptor activates a number of signalling cascades. Lke FGFR-1, PLCγ is activated and leads to up-regulation of MAPK. In addition, PKC, Akt and PI3K are also activated. These cascades are not dissimilar to the action of FGF (see Fig 6.8 below).

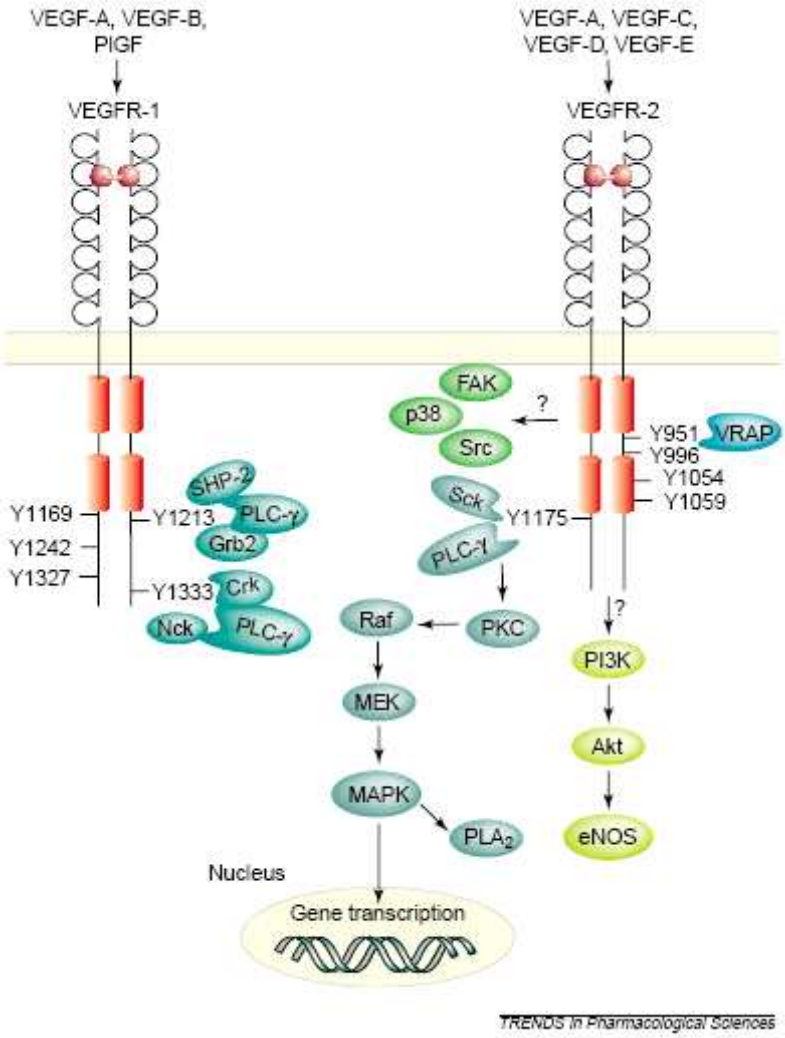


Fig 6.8. An overview of VEGFR-1 and VEGFR-2 signalling. Ligand binding results in receptor dimerisation and the phosphorylation of specific intracellular tyrosine residues. Only the downstream pathways for VEGFR-2 are shown as it is this receptor that is thought to be important for endothelial cell proliferation. In a similar fashion to FGFR-1 activation, MAPK is activated and this can then bring about changes in gene expression within the nucleus. (Reproduced by permission of Elsevier)

Despite the similarities, researchers have attempted to detect differences in the signalling cascades brought about by the actions of VEGF and FGF. In the chick allantoic membrane, FGF 2 mediated angiogenesis was blocked following treatment with a specific MEK inhibitor (thereby reducing MAPK production) (Eliceiri et al, 1998). The same group also demonstrated that the induction of a mouse knockout of Src tyrosine kinase inhibited VEGF but not FGF induced angiogenesis (Eliceiri et al, 1999). As can be seen from the above diagrams, Src is to be found on both cytokine activated pathways. In 2001, Zubilewicz et al demonstrated that FGF 2 induces a marked increase in MEK activity in bovine choroidal EC whereas VEGF did not. This study utilised “specific” inhibitors of intracellular messengers to dissect out important pathways. It is now widely accepted that these inhibitors may not be as specific as once thought and other methods such as proteomics would be more reliable. An example of a once widely used inhibitor is apigenin. It is used as a specific inhibitor of MAPK generation, but can also inhibit Protein Kinase 2 (CK2), nitric oxide synthase-2 (NOS2), Hypoxia-inducible factor 1 alpha (HIF-1α), lipoxygenase and cyclooxygenase-2 (COX-2) (information from Santa Cruz Biotechnology, Ca, USA). This means that any results derived from its use are probably a gross over simplification of the true pathways and interactions involved.

When one considers the questions surrounding whether FGF and VEGF act separately on the stimulation of angiogenesis in endothelial cells and how the intracellular signalling cascades differ it would be interesting to study the effect of anti-VEGF antibodies on the proliferation of CECs when stimulated with FGF and FGF/VEGF combinations. The continued controversy as to whether FGF acts as a pro-angiogenic factor in its own right or acts via effects on VEGF or its receptor is an area where future research is necessary and may lead to future improved treatments for ocular angiogenenic diseases.

The author and co-workers have recently published a study demonstrating the comparative effects of VEGF isoforms on the proliferation of first passage human choroidal ECs and human retinal ECs using the methods described above (Stewart et al, 2011). It was found that both $VEGF_{121}$ and $VEGF_{165}$ were equally potent in stimulating both retinal and choroidal ECs. In addition, retinal ECs demonstrated a higher proliferation than choroidal ECs with equimolar concentrations of both VEGF isoforms. This suggests that retinal and choroidal ECs differ in their response to growth factors (VEGF isoforms) and again validates the results of the gene expression experiments, suggesting significant differences in gene expression (and thereby, possible functional differences) between retinal and choroidal ECs. It would also be interesting to compare the effects of FGF and VEGF/FGF combinations on matched retinal and choroidal endothelial cells as this may give information regarding the possible differential pathogenesis and treatment of retinal and choroidal diseases

Other factors such as PDGF-AA, PDGF-BB, IGF-1 or IL-1β present in excised human CNV tissue did not show significant effects on human ICEC proliferation or angiogenic tube formation in this study. These results are in contradiction to the results found by workers utilising endothelial cells derived from non-human choroidal and animal models as a surrogate to the study of human choroidal disease. While we found no effect of IL-1β on hCECs, Zou et al in 2006 found that inhibition of the effects of IL-1 on HUVEC cells significantly inhibited cell proliferation. Risau et al (1992) found PDGF-BB and to a lesser extent PDGF-AA to be pro-angiogenic in a chick chorioallantoic membrane model and to stimulate chemotaxis of rat brain ECs. Importantly, the preparations of PDGF were derived from recombinant yeast expression systems, so would be unlikely to be contaminated with VEGF. Interestingly, it has recently been suggested that PDGF is important in CNV pathogenesis due to its effect on pericyte recruitment and stabilisation, with absence of PDGF causing regression of new vessels due to loss of supporting pericytes. Agents targeting the intracellular

effects of PDGF have therefore been proposed as a new treatment for CNV. Similarly, whilst a role for IGF-1 in retinal angiogenesis is established (Sharp, 1987; Smith et al, 1999, Hellstrom et al, 2001), its effect on the pathogenesis of CNV is controversial and appears to be dependent on species and/or site under investigation. We found no effect of IGF-1 on the proliferation and angiogenic tube formation of human ICECs. Spraul et al (2002) recently found that IGF-1 stimulated the proliferation of bovine CECs with a similar potency to VEGF, while Grant et al (1993) and Castellon et al (2002) independently found a stimulatory effect of recombinant IGF-1 on human and bovine retinal endothelial cells respectively, suggesting a direct pro-angiogenic effect for IGF-1 in diabetic retinopathy. This differential effect of IGF-1 on different ECs may explain the lack of significant benefit for lanreotide, a potential IGF-1 inhibitor, on the outcome of patients with CNV secondary to AMD (Papadaki et al, 2003). Any effect of IGF-1 on the pathogenesis of CNV may, therefore, be due to indirect effects on other pro-angiogenic cells and pathways.

The author and co-workers recently published a study in which the effect of IGF-1 on unpassaged choroidal and retinal ECs was compared (Browning et al, 2012). This work showed that compared to choroidal ECs, which failed to demonstrate any proliferative response to IGF-1, retinal ECs showed a marked response which also demonstrated an additive response when used in the presence of VEGF. This work supports the gene expression experiments in chapter 3 which found that proliferating retinal ECs preferentially expressed various components of the IGF-1 activation pathways. This has implications for the pathogenesis and future treatments of proliferative diabetic retinopathy. Based on one of the conclusions of the gene expression studies in Chapter 5, it would be interesting to study the effect of Brain Derived Neurotrophic Factor (BDNF) on ICECs.

The potential drawback of this work is that the study of the hICECs took place in an environment far removed from their natural environment and the cells may behave differently *in vitro* to their *in vivo* state. To try and abrogate this effect, only cells of first passage that formed the typical cobblestone morphology in culture on fibronectin plates were used, so that the cells used were as near to the *in vivo* state as possible. However, as hICEC do not naturally exist in isolation *in vivo*, some of the factors tested may be able to influence hICEC indirectly via their effects on other neighbouring cells.

On the other hand, the culture conditions used in this study allowed assessment of individual growth factors without the confounding effects of other factors and other cell types. The biological effect of single growth factors on the hICECs could therefore be tested in isolation.

In summary, this study has found that VEGF and FGF2 stimulate hCEC proliferation and angiogenic tube formation in isolation and produce an additive effect when used in combination. Other growth factors such as IGF-1, PDGF-AA or BB and IL-1β do not have a direct *in vitro* stimulatory effect on hCECs. It is, however, possible that other cytokines, including PDGF-AA or BB, IGF-1 and IL-β 1, may produce an effect by stimulation of other cell types involved in CNV formation. Such effects will require further investigation using co-cultures of different cell types. These results suggest that in addition to VEGF, FGF2 could be a potential therapeutic target for the development of new agents to treat angiogenic diseases such as CNV in humans (Hagedorn et al, 2001).

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Chapter 7

Final Conclusions

Other than glaucoma, angiogenic driven diseases such as proliferative diabetic retinopathy (PDR), wet age-related macular degeneration and retinopathy of prematurity (ROP) remain the most common blinding disorders in the developed world. Unfortunately, many of the underlying mechanisms or causes of these diseases remain unknown and definitive treatment is not usually possible. Treatment modalities therefore rely on tissue destruction in the case of PDR and ROP or on the inhibition of a ubiquitous growth factor (VEGF) in wet AMD. More selective treatment of these conditions would reduce the collateral damage at the site of disease and allow the treatment to be more specific. To allow this more selective treatment to be developed, an increase in the knowledge of site specific differences in tissues is required. In the case of angiogenic diseases, this requires increased knowledge of endothelial cells, particularly those undergoing proliferation in the target tissue of interest.

It has been known for many years that endothelial cells are heterogeneous in nature, even within a single organ such as the lung. It would not be surprising therefore, to speculate that endothelial cells within the eye may differ, even though their different vascular beds may only be separated by a factor of microns. Evidence for this heterogeneity is gleaned from the fact that different diseases appear to affect different ocular vascular beds with marked selectivity, e.g. retinal vasculitis and diabetes selectively affecting the retinal vasculature and wet AMD and inflammatory choriocapillopathies affecting the choroidal vasculature.

For many years, researchers have used HUVECs to study angiogenic processes in normal and pathological states and applied the conclusions to both ocular and non-ocular disorders. A major drawback of this approach is that HUVECs are specialised cells found in a microenvironment not encountered anywhere else in the body and are therefore likely to demonstrate unique responses. The usefulness of any conclusions derived from these cells in the understanding of diseases such as wet AMD and proliferative retinopathy must therefore be questioned. As alluded to previously, there is ample evidence in the literature demonstrating that HUVECs differ in many aspects in terms of their responses to external stimuli compared with other endothelial cells.

In this work, it has been shown that at the level of gene expression, HUVECs are indeed different. They demonstrate significant differences in the expression of at least 800 genes, across many functional groups, when compared with ocular microvascular endothelial cells. Examination of the results obtained from HUVECs revealed that numerous genes involved in embryonic development were up-regulated. This included numerous homeobox transcription factors which have been shown to be crucial to the development of many embryological structures and processes. Reasons for this difference may include the fact that the umbilical vein has the feature of being derived embryologically from the developing foetus. Another reason may be that *in vivo*, angiogenesis tends to occur at the microvascular level of the vascular tree, not in large capacitance vessels (such as the umbilical vein). These findings would seem to confirm earlier opinions, which suggest that HUVECs are different from other endothelial cells, and would lead one to suggest that these cells should not be used for the investigation of complex angiogenic disorders of specialised organs such as the eye. If one is not to use HUVECs in the investigation of human ocular disease, then the question arises as to which cells should be used in *in vitro* models.

Investigation of the gene expression patterns of human ocular endothelial cells revealed that there were significant differences between proliferating human retinal and choroidal endothelial cells. This study revealed that approximately 8% of tested genes showed a significant difference in expression. As the current research is particularly focused on angiogenic processes and diseases, genes thought to be involved in cell signalling pathways and which could be important in cell proliferation were specifically studied. This study found that there were a number of genes encoding proteins of signalling pathways that were differentially expressed between retinal and choroidal endothelial cells. As previously stated, diabetes is the leading cause of blindness in people of working age. From this work, it appears that as well as VEGF playing an important role in the progression and treatment of diabetic retinopathy, IGF-1 also appears to be important. This finding is backed by numerous clinical studies in which IGF1 is manipulated or measured in diabetic subjects. There is also evidence from myself and co-workers that IGF-1 brings about proliferation of human retinal endothelial cells but not human choroidal ECs (Stewart et al, 2011). In addition, the findings from this work have implications for the pathogenesis of ROP, in which the developing retinal vasculature appears to show differential responses to IGF-1 and may be involved in the disease pathogenesis.

Interestingly, comparison of choroidal and iris endothelial cells demonstrated very few significant differences. The reason for this is unknown but may be related to the fact that both the iris and the choroid are part of the same ocular structure – the uveal tract. They may therefore be part of the same embryological precursor.

The research presented in this thesis has a number of advantages compared to previous or similar studies:

1. The work is conducted on human tissue. Some previous work has been conducted on bovine tissue.

2. The endothelial cells are matched in terms of the donor, thereby reducing any variability due to donor differences.

3. The cells are unpassaged. This avoids any differences in gene expression being due to changes related to passage drift.

4. All cells were isolated and grown under exactly the same conditions, thereby avoiding any differences in gene expression being due to external factors.

These rigorous culture and examination conditions allow one to directly compare gene expression and makes any significant differences more meaningful. Also, by studying proliferating cells, cell processes such as signalling pathways important in angiogenesis may be studied. Other work, particularly on ocular RPE cells has been conducted using laser capture microdissection prior to microarray analysis. Unfortunately, this would be unlikely to be suitable for this work, as very few endothelial cells are undergoing proliferation in the normal eye. Secondly, this technique is prone to contamination with other adjacent cells.

In this project, cell purity was maximised by the use of paramagnetic bead separation prior to culture. One drawback of this work is that in all three types of ocular endothelial cells, no separation of venules, arterioles or capillaries was possible. This means that homogeneous mixtures of cells from each anatomical site were used. At present, the limits of microdissection prevent the isolation of individual retinal venules and arterioles to reliably provide enough cells to culture for further analysis. The use of laser capture dissection would provide a

suitable method of isolating samples of the individual vessels (if they could be reliably identified). The previous comments regarding contamination and the quiescent state of the isolated cells mean, however, that the technique in its present state is unlikely to provide additional information than that achieved by the methodology in this project.

A major question that needs to be considered is the origin of the diversity of endothelial cells. Is this diversity due to nature i.e. genetic, or to nurture i.e. the effect of the local environment on the cell? The earliest events controlling the differentiation of mesoderm into endothelial cells remain uncertain. Experiments on zebra fish have shown that the arterial and venous phenotype of vascular precursor cells or angioblasts (expression of gridlock) is determined while they are still located in the lateral plate (very early stage of embryogenesis), implying a genetic component to endothelial heterogeneity (Zhong et al, 2001). This finding is reinforced by the finding that in mice, cells destined to be arteriolar and venular ECs express ephrin B2 and EphB4 respectively before the onset of a circulation (Adams et al, 1999).

While the initial identity of ECs appears to be genetically determined later, after angioblast migration, local factors such as VEGF gradients can also have an effect on arterio-venous phenotype (Le Bras et al, 2010). It is thought that high local expression of VEGF leads to the activation of members of the FOXC transcription factors which in turn leads to up-regulation of notch family and an arteriolar phenotype. Experiments have also shown that physiological requirements and haemodynamic influences can alter phenotype. Transplantation of arteriole ECs into veins rapidly changes their phenotype to venular (Moyon et al, 2001). This may be due to local factors such as VEGF secreted by surrounding cells or it may be secondary to differences in flow/shear stress as the placement of venules within a section of high flow artery leads to a rapid change to an arteriolar phenotype.

Less is known about the variability of EC phenotype between/within organs. It is not known if angioblasts migrate to these different areas or arise *de novo*. Cells arising *de novo* at different sites could obviously demonstrate variable gene expression patterns as they arise in isolation from other endothelial cells. However, it is probable that local factors such as differences in extracellular matrix and cytokines also play a significant part in local cell differentiation. For example, within the brain, the endothelium is highly specialised with avid tight junctions and is non-fenestrated. The cells form the crucial blood-brain barrier. It is thought that juxtaposed cells such as pericytes, astrocytes and neurones are crucial for this EC tight junction formation and thereby the formation of the blood-brain barrier (Hawkins and Davis, 2005; Lee et al, 2003).

It has recently been suggested that the conventional concept of a particular transcription factor binding to the promoter sequence of a gene in order to regulate its transcription does not fully explain some features of endothelial cell gene expression. For instance, how does the endothelial cell environment exert chronic effects on gene expression over weeks or years or when the cell divides and how is this information regarding the environmental effects transmitted to daughter cells? Epigenetics is the term used to try to explain these findings and refers to chromatin based effects on the modification of gene expression. Three distinct mechanisms have been described: DNA methylation, histone post-translational modifications and RNA based mechanisms, all of which modify the structure and accessibility of DNA to transcription factors (Yan et al, 2010).

DNA methylation refers to the addition of a methyl group on DNA cytosine and has the effect of reducing promoter transcription (Meissner et al, 2008) by steric interference of the binding of transcription factors to specific DNA sequences. This mechanism has been described as one method responsible for the modification of HIF-1 alpha gene transcription (Wenger et al, 1998). Within the nucleus, DNA is

packaged by wrapping around histone proteins, forming nucleosomes. Histone proteins can be modified post-translationally by mechanisms such as acetylation which can affect DNA transcription. These modifications can therefore encode regulatory information for cells that reproducibly affect DNA expression and may be passed onto daughter cells. Finally, it has been found that small lengths of RNA (less than 200 nucleotides) can affect DNA expression. These short lengths are called non-coding RNA and many have been found associated with the human genome. How can the environment affect these epigenetic processes? It has been found that shear stress on endothelial cells can modify the histones around which DNA is wrapped (Illi et al, 2003). Shear stress can therefore affect gene expression, particularly at sites of turbulence. This is thought to play a role in the pathogenesis of atherosclerosis, which is characterised by focal pathological changes within blood vessel walls.

It has been shown that endothelial cells of the choroid, iris and retina are all derived from embryonic mesoderm, while supporting pericytes are derived from the neural crest (Gage et al, 2005). Following on from this is the finding that choroidal blood vessels develop by a process of haemovasculogenesis while those of the retina develop by vasculogenesis. The endothelial cells of both the retinal and choroidal vessels themselves are ultimately thought to be derived from CD39 positive angioblasts. What is not known is whether all these angioblasts are the same i.e. from a common precursor which should therefore demonstrate early identical gene expression patterns.

The difference in the gene expression patterns of matched mature human retinal and choroidal endothelial cells may therefore be due in part to this difference in origin (although all are ultimately derived from mesoderm).

The alternative argument is that cues from the local environment around the identical angioblasts/endothelial cells cause them to diversify and differentiate to suit their environment (Janzer and Raff, 1987). Examples include brain and retinal endothelial cells which require strong tight junctions to provide the inner retinal and blood brain barrier or adrenal and inner choroidal endothelial cells which are fenestrated to facilitate passage of fluid and ions across the endothelium. Early experiments using endoderm-derived cells such as cells of developing lung have shown that local cues stimulate the formation and development of angioblasts that go on to form part of the pulmonary vasculature (which is known to be heterogeneous). In contrast, ectoderm-derived cells do not have this effect. This is an example, therefore, of the cell environment influencing angioblast behaviour (probably at the level of the gene).

Further evidence for the effect of the environment on ECs include the finding that HUVECs exposed to TGFβ grow as a rapidly dividing monolayer if cultured on collagen I coated plates, but as tubes when cultured within a 360 degree matrix containing collagen I. Many of the interactions of ECs with their environment are thought to be facilitated by integrins, which are a family of cell surface molecules found on endothelial cells. It is known that the subtypes of these receptors change as the EC develops, thereby suggesting that changes in the development and maturation of the cell are at least in part related to the microenvironment.

It has previously been mentioned that fibroblast growth factor 2 (FGF2) is important for vascular development. However, the effect of VEGF appears to be more dramatic and visual. Culture of mammary gland endothelial cells (Esser et al, 1998) and the *in vivo* exposure of ECs within muscle and skin (Roberts & Palade, 1995) (none of which are normally fenestrated) to VEGF stimulated the formation of fenestrations. This may explain why the retinal pigment epithelium secretes VEGF (and other factors) from its basal surface, across

Bruch's membrane – to maintain fenestrations and cell survival within the inner choroid (Liu et al, 1997). Interestingly, large vessel endothelial cells such as HUVECs can also be stimulated to form fenestrations by exposing them to phorbol ester (Lombardi et al, 1987). Phorbol ester is a tumour promoter which activates intracellular signal transduction cascades via protein kinase C and is commonly used in research to stimulate the cancerous transformation of cells. This finding shows that even cells which *in vivo* have no reason to develop fenestrae maintain the capacity to do so. Gregory Roberts & Palade in 1997 and 1998 found that implantation of tumours containing growing blood vessels took on the phenotype of the tissue the tumour was implanted into (skin and brain), providing further evidence that the local environment influences the phenotype of endothelial cells. Similarly, in a mouse model in which a section of vein was interposed into the arterial circulation, the venous segment soon took on arterial characteristics such as an increase in smooth muscle cells with the vessel wall and the endothelial cells took on arterial characteristics (Kwei et al, 2004). There is substantive proof therefore that the parenchyma can determine the phenotype endothelial cells.

Focussing on the choroidal neovascularisation involved in wet age-related macular degeneration, one could hypothesise that the phenotype of proliferating endothelial cells at the site of disease leads to this devastating angiogenic process which is so frequently located at the macula, an area only a few mm in diameter. Are the unique gene expression profiles previously demonstrated in different proliferating ocular endothelial cells occurring at different sites within the human choroid? If so, does this "local" gene expression profile also provide any clues to future site selective potential treatments? This work has shown that there is no significant difference in gene expression profiles between macular and peripheral human inner choroidal ECs. This result would suggest that the susceptibility of the macula to wet AMD is not due to a fundamental difference in the endothelial cells within the inner choroid. Other reasons could include the previously noted differences in

macular RPE cells or Bruch's membrane. This would also imply that any "magic bullet" treatment would in theory have an effect on peripheral inner CECs as well, giving rise to potential collateral damage and side effects.

When macular inner and outer CECs are compared, however, some interesting differences are found, perhaps giving insight into the different physiological roles of the blood vessels of the two layers of the choroid: the inner as a fenestrated layer supplying the outer retina with oxygen and nutrients and the outer choroid as a layer of larger vessels supplying the inner choroid with a regulated blood supply.

Inner choroidal ECs demonstrated up-regulation of probesets involved in nervous system development, in particular brain derived neurotrophic factor and certain growth factors such as VEGF and HGF. These may act in a local paracrine fashion to maintain local tissue phenotype (maintenance of photoreceptor integrity and RPE polarity) or to provide a local amplification loop to cell proliferation. It was also found that plasmalemmal vesicle associated protein-1 (PLVAP) which is a component of fenestrations, was up-regulated in inner macular endothelial cells (but not outer macular ECs or retinal and iris ECs) [from review of raw microarray data]), confirming the importance of fenestrations to the inner choroidal ECs. In contrast, macular outer choroidal ECs demonstrated up-regulation of probesets involved in immune function, certain cell signalling components and interestingly, different growth factors (TGF-beta and stromal cell derived factor).

It has been demonstrated that human macular inner choroidal ECs have a unique gene expression profile but the next question to be answered is how these cells behave in a microenvironment that aims to mimic wet AMD. While all endothelial cells have been shown to respond positively to VEGF, there are widely reported differences in ECs' responses to other growth factors. Excised choroidal neovascular membranes from humans have been found to contain a wide range of

growth factors, some of which have been found to lead to variable responses with other ECs. Newly isolated and characterised human macular inner choroidal endothelial cells were used to determine their *in vitro* responses to a range of growth factors using an *in vitro* angiogenesis assay. The main aim of this experiment was to determine which growth factors may be important in wet AMD and to perhaps provide other therapeutic targets apart from VEGF for the treatment of the disease. Interestingly, it was found that FGF2 had a similar positive effect on cell proliferation and capillary tube formation and growth to VEGF. This would appear to be a new exciting target for disease treatment. Unfortunately, to date, no drug has been devised to specifically target this growth factor and which could be injected into the human eye. Other factors such as PDGF and IGF-1 appear not to have any direct effect on these ECs. As such, their role in wet AMD must be questioned unless they are acting in a secondary role upon other undetermined cell types (such as the proposed role of PDGF in the maintenance of vascular pericytes).

This work has a number of advantages over previous work conducted on different endothelial cells or other ocular cells in an attempt to understand human ocular diseases. The first advantage is that the cells are isolated from human eyes and in particular, from the areas of the eye affected by various angiogenic diseases. Secondly, unlike techniques using laser capture dissection, contamination from surrounding cells is minimised by using a method to extract endothelial cells using paramagnetic beads. This allows a large number of living cells to be isolated for further investigation, such as microarray or *in vitro* proliferation studies. Another disadvantage of laser capture dissection studies, particularly in studies such as this, which aims to investigate proliferating cells, is that most of the cells isolated are in a quiescent state and are only available in small numbers. Thirdly, cells used in this study were unpassaged. Many studies using endothelial cells, not just those from humans, rely on cells that have undergone multiple passages in an attempt to provide enough cells for large scale

assays. Unfortunately, it is widely accepted that endothelial cells undergo phenotypic changes after multiple passages, thereby making any results derived from these cells questionable. This makes any conclusions derived from my studies more applicable to human disease. In experiments described in this project where cell gene expression was studied, the cells were from matched donors and were cultured under exactly the same conditions, therefore abrogating any differences in gene expression due to differential culture conditions or because of differences between donors.

The main disadvantage of this work is that the endothelial cells are removed from their “normal” surroundings (basement membrane, pericytes etc) which may have an effect on a cell’s *in vitro* performance. In an attempt to circumvent this, in many of the experiments, conditions were made as close as possible to their natural surroundings by using specialised endothelial growth medium and collagen coated plates (an attempt to re-create cell basement membranes). A counter argument in support of studying of cells in isolation is that there is no possibility of their being affected by other cells or extraneous factors. This allows a strict control of the cell environment to occur with small manipulations possible to the experimental conditions, therefore allowing minute changes in cell behaviour to be studied.

In summary, this research work has developed new techniques to isolate ocular endothelial cells from different vascular beds within the human eye and from different levels within the same vascular bed. The study has also demonstrated that HUVECs are not suitable surrogates to be used in the study of human ocular diseases because there are significant gene expression differences between them and ocular microvascular endothelial cells which may affect their *in vitro* behaviour. Within the eye, endothelial cells from different vascular beds show heterogeneity at a gene expression level which may go some way to explaining why different ocular beds appear to be affected by different disorders and respond to different treatments.

A major advantage of this study is that it has allowed one to study endothelial cells from within the human macular inner choroid (the site at which AMD occurs), revealing a unique gene expression profile which may be important in deriving new treatments for wet AMD. Secondly, it has demonstrated that FGF2 may be an important growth factor in the pathogenesis of choroidal neovascularisation and therefore may be a new candidate for therapeutic intervention. It has, furthermore, demonstrated why manipulating IGF may be important in retinal vascular disease, but not in choroidal vascular disease.

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