The Mechanism(s) by which Pigment Epithelium-derived Factor Regulate Angiogenesis

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A thesis submitted to Cardiff University in accordance with the requirements for the degree of Doctor of Philosophy in the disciplines of Cell and Molecular Biology

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November 2007

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Acknowledgements

It's been a long way to get to so-far. Without these people's support it would be only a dream.

First of all, I would like to thank my supervisors, Professor Mike Boulton and Dr Jun Cai, for giving me this opportunity, for their support, advice, guidance and encouragement, both during their time in Cardiff and from afar in Texas.

I am also extremely grateful to Dr. Julie Albon who has surpassed herself in her level of support. Drs. Malgorzata Rozanowska and Vanessa Davies who have provided me with the moral support throughout the course.

Thanks to the members of 2.62 and then 2.54, I feel twenty-years younger, in particular to Linda for sharing her experience in research and fluorescence microscope; to Matthew Davies for his sense of humour, moral support and for reading my manuscripts; to Li Bo, Gill, Bablin, Llinos, Miguel for their moral support. Thanks to Steve, Sue, John, Rob and Phil for always being there to help.

To Sarah for her fluorescence microscope expertise. To Dr. Kerenza Njoh for her assistant in using a fluorescent microplate reader for the γ-secretase activity assay.

A special thank-you to Dr. Sophia Gillbert, my former colleague in Bioscience, for always being there to assist or troubleshoot and share her expertise in biochemistry and molecular biology, for reading my manuscripts and for her moral support particular in the most difficult period of my PhD. A big thank-you to Professor Vic Duance and Dr. Emma Blain for their encouragement and moral support and always being there to assist.

A big thank you all to former colleagues in PHL: Dr Sally Cordan, Myfanwy, Joanne and Catherine. The training I received and the experience I gained there made my PhD study much easier.

A special thank-you to Dr. Julie Fox, my very first supervisor in research, for her full support, valuable advice and guidance, enthusiasm and motivation.

A special thank-you to Dr Song Han, an unofficial supervisor, for always being there to assist or troubleshoot and share her expertise in molecular biology, for her friendship and her moral support, particularly during the most difficult period of my PhD.

A special thank-you to my friends: Catherine Charbet, Jie Luan and Shu-Rong Li for their friendship and moral support.

Finally, my very special thanks to my husband for his encouragement and firm faith in me and my son for his forbearance and support.

Abstract

Pigment Epithelium-derived Factor (PEDF), a natural protein possessing both neuroprotective and anti-angiogenic properties, is a very unique and attractive candidate as a therapeutic agent in the management of pathological neovascular diseases, such as tumours, age-related macular degeneration (AMD) and diabetic retinopathy. While it is well-known that PEDF can exert powerful effects on various tissues and cells, the underlying mechanism of PEDF's action is not well understood. This study investigated the relationship between vascular endothelial growth factor (VEGF)/PEDF and VEGFR-1/VEGFR-2 by exploring Presenilin-1(PS-1) dependent regulated intramembrane proteolysis (RIP). Work on this non-classical pathway was initiated by Cai et al., (2006) using *in vitro* models of bovine retinal microvascular endothelial cells (BRMECs). Current study used BRMECs and human retinal pigment epithelial (HRPE) cells.

In this study, BRMECs and HRPE cells were isolated and cultured. BRMECs were used as an angiogenic cell type while HRPE cells were used as an angiogenic regulator cell type. The characteristics of endothelial and epithelial cells and the localisation of VEGFR-1, VEGFR-2 and PS in BRMECs and HRPE cells were determined using immunocytochemistry techniques. The effects of VEGF and PEDF on VEGFR-1, VEGFR-2 and PS were assessed using immunocytochemistry and Western blotting. γ-secretase activity in BRMECs and HRPE cells treated with various growth factors were analysed using a γ-secretase activity kit. The role of VEGF on the production of PEDF and the expression of VEGFR-1, VEGFR-2 and PS in HRPE cells was investigated at both the transcriptional and translational levels. The techniques, VEGF-small interfering ribonucleic acid (VEGF-siRNA), reverse transcription–polymerase chain reaction (RT-PCR), Western blotting and Enzyme-linked immunosorbent assay (ELISA) were used for the investigation.

Results obtained from the project showed that PEDF had a regulatory role in the counterbalance of VEGFR-1 and VEGFR-2 expression in cultured BRMECs. PEDF upregulated γ-secretase activity and PS-1 expression in BRMECs while VEGF acted as an antagonist of the effect of PEDF. In contrast, in HRPE cells, VEGF upregulated γ-secretase activity and PEDF acted as an antagonist of the effect of VEGF. VEGF-siRNA induced a reduction of PEDF at both transcriptional and protein levels and a reduction of VEGFR-1 at the protein level. The effects of VEGF and PEDF on VEGFR-1 and VEGFR-2 may be cell type dependent.

This study strengthens the view that PEDF can exert different regulatory effects on the same molecule (s) in different cell types. PEDF acts either antagonistically to VEGF or synergistically dependent upon the target molecule. Deciphering the cellular and molecular mechanisms underlying these interactions will not only contribute to our understanding of PEDF's action but also provide the foundation to maximise the therapeutic potential of this protein.

Abbreviations

AD	alzheimer's disease
ADAM	a disintegrin and metalloprotease
AICD	app intracellular domain
AIDS	acquired immunodeficiency syndrome
Aph-1	anteriior pharynx defective-1
AMD	age-related macular degeneration
APP	amyloid β-protein precursor
APS	ammonium persulphate
Arg	arginine
Asp	aspartic acid
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BM	bone marrow
BRB	blood-retinal barrier
BRMECs	bovine retinal microvescular endothelial cells
BSA	bovine serum albumin
CAM	chorioallantoic membrane
cDNA	complementary deoxyribonucleic acid
CNS	central nerves system
CNV	choroidal neovascularisation
CORD	cone-rod dystrophy
CSF	cerebrospinal fluid
CSF-1R	colony stimulating factor -1 receptor
CTF	c-terminal fragment
DABCYL	4-(4-dimethylaminophenylazo)benzoic acid
DMSO	dimethylsulphoxide
dH_2O	deionised water
dsRNA	double-stranded RNA
EC	endothelial cell
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ED_{50}	median effective does
EDANS	5-(2-aminorthyl)aminonaphthalene-1-sulfonic acid
EDTA	ethylenediaminetetraacetic acid
EEC	embryonic ectoderm
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial precursor cells
ER	endoplasmic reticulum
ErbB4	v-erb-a erythroblastic leukaemia viral oncogene homolog 4 (avian)
ERK	extracellular-signal-regulated kinase
EXE	extra-embryonic ectoderm
FasL	fas ligand
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate

Flt-1	fms (feline McDonough sarcoma virus)-like tyrosyl kinase-1
Flk-1	fetal liver kinase-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HMW	high molecular weight
HRP	horseradish peroxidase
HRPE	human retinal pigment epithelium
IC_{50}	median inhibitory concentration (check)
IFN-α	interferon
Ig	immunoglobulin
IL-1β	interleukin
IL-8	interleukin – 8
IP-10	interferon-γ-inducible protein 10
KDR	kinase domain-containing receptor
kDa	killo dalton
Lys	lysine
MAPK	mitogen-activated protein kinase
MEC	microvascular endothelial cell
MEM	minimum essential medium
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NCT	nicastrin
NF-κB	nuclear factor κB
$(NH_4)_2SO_4$	ammonium sulphate
NICD	notch intracellular domain
nt	nucleotide
dNTP	deoxynucleotide-triphosphate
NTF	n-terminal fragment
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	polypoidal choroidal vasculopathy
PDR	proliferative diabetic retinopathy
PDGF	platelet-derived growth factor
PEDF	pigment epithelium-derived factor
Pen-2	presenilin enhancer-2
Pl3K	phosphatidylinositol 3-kinase
PlGF	placenta growth factor
PKC	protein kinase C
PLCγ	phosphrolipase C gamma
PPS	posterior primitive streak
PS	presenilin
RCL	reactive central loop
RIP	regulated intramembrane proteolysis
RISC	rna-induced silencing complexes
RNAi	ribonucleic acid interference
ROP	retinopathy of prematurity
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
RT-PCR	reverse transcription–polymerise chain reaction

SEM	standard error of mean
SH2	src homology 2
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Serpin	serine protease inhibitor
shRNA	short hairpin RNA
siRNA	small interfering RNA
S-PEK	subcellular proteome extraction kit
SPP	signal peptide peptidase
SPSS	statistical package for the social sciences
SREBP	sterol regulatory element binding protein
TACE	tumour necrosis factor-alpha-converting enzyme
TBE	tris-borate-edta
TEMED	n,n,n',n'-tetramethylethylendiamine
TGF-β	transforming growth factor beta
TGN	trans-golgi network
TIMP	tissue inhibitor of matrix metalloproteinase
TM	transmembrane
TNF-α	tumour necrosis factor alpha
TSAd	t-cell specific adapter d
Tx-sol	triton x-100 soluble
Tx-insol	triton x-100 insoluble
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VWF	von willebrand factor
γ-secretase	gamma-secretase
WR	working reagent
WS	working solution

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Chapter 1

Introduction

1. Introduction

Neovascularisation, the formation of blood vessels, is one of the most vital biological processes required for the formation and physiological function of virtually all organs in both normal and disease conditions (Visconti, *et al.*, 2002; Schmid and Varner, 2007). In a broad range of pathological conditions including those with an enhanced or insufficient blood supply, neovascularisation is a key event (Distler, *et al.*, 2002). In some human diseases such as in cancer and ocular retinopathies, aberrant neovascularisation can potentially be blocked therapeutically to prevent disease progression (Folkman, 1995). In other diseases, such as in heart ischemia, brain infarction and wound healing, the process of neovasculatisation can be used to restore the vital function of the affected organs (Isner, 2002).

1.1 Neovascularisation

1.1.1 Definition

Neovascularisation is the formation of blood vessels in areas that were previously avascular, and it occurs by both vasculogenesis and angiogenesis (Chang *et al.*, 2001; Schmid and Varner, 2007; Afzal *et al.*, 2007).

Vasculogenesis is the coalescence of new blood vessels from individual endothelial cells or progenitor cells. The formation of the initial vascular tree during embryonic vascular development and the formation of new blood vessels by circulating endothelial progenitor in adult animal are examples of vasculogenesis (Schmid and Varner, 2007). Vasculogenesis was thought to occur only in early embryogenesis. However, it has been found that in adults, vasculogenesis occurs as endothelial precursor cells (EPC) derived from the bone marrow (BM) enter the circulation and to be incorporated into neovascular foci in injured corneas, ischemic hindlimbs and tumour vasculature (Takahashi *et al.*, 1999; Afzal *et al.*, 2007).

Angiogenesis is defined as the sprouting of new blood vessels from the pre-existing microvasculature and the modification of the initial irregularly organized

endothelial tubular network into a structured three-dimensional network of branching vessels, result in a functional circulatory system (Carmeliet, 2000; Yancopoulos, *et al.*, 2000; Distler, *et al.*, 2002; Coultas *et al.*, 2005). Angiogenic sprouting is responsible for vascularising certain structures during normal development, such as the neural tube or the retina, and for most new vessel formation in the adult (Moore, 2002).

Angiogenesis was considered the sole mechanism of blood vessel formation in postnatal life, until recently accumulating evidence suggests that endothelial stem cells are pres ent in adults and participate in new blood vessel formation in normal and pathological states, including tumours.

1.1.2 The process of neovascularisation

During embryogenesis, neovascularisation is initiated from vasculogenesis, i.e. formation of a functional circulation from EPC. It is initiated as the appearance of mesodermal cells with vascular endothelial growth factor receptor 2 (VEGFR-2 or Flk-1) positive in the posterior primitive streak (PPS); VEGFR-2 positive cells in the PPS give rise to both blood and endothelium (haemangioblasts), but are restricted to haematopoietic or angiogenic fate after emigrating into extra-embryonic sites [extra-embryonic ectoderm (EXE), yolk sac and allantois) and intra-embryonic sites [embryonic ectoderm (EEC)]. In the yolk sac, these progenitors aggregate into endothelial-lined blood islands that then fuse to generate a primary capillary plexus. The primary capillary plexus undergoes remodelling along with intra-embryonic vessels to form a mature circulation. Intra-embryonic angioblasts migrate along distinct pathways before aggregating directly into the dorsal aorta or cardinal vein, without a plexus intermediate. The primary vessels (capillary plexus, dorsal aorta and cardinal vein) then remodel, together with the extra-embryonic plexus, to form a mature vasculature, which along with VEGF and Notch involves the angiopoietins and Tie receptors. Mural cells (pericytes and smooth-muscles cells) proliferate and differentiate in response to TGF-β, and are recruited to vessels by PDGF secreted by endothelial

cells (reviewed by Coultas *et al.*, 2005). The formation of a functional circulation from endothelial progenitors is shown in Fig. 1.1.

In adulthood, the EPC mobilised from the bone marrow can enter into the blood circulation and may be recruited and incorporated into sites of active neovascularisation during tissue ischemia, vascular trauma, or tumour growth. Moreover, expansion and mobilisation of EPC may augment the resident population of endothelial cells (ECs) competent to respond to exogenous angiogenic cytokines (Isner and Asahara, 1999; reviewed by Ribatti, 2007) (Fig.1.2).

The process of angiogenesis occurs as an orderly cascade of events (see Fig1.2). It is initiated from the production and release of angiogenic stimulators, such as vascular endothelial growth factor (VEGF), from the tissues by certain physiological or diseased conditions. These angiogenic stimulators diffuse into the nearby tissues and bind to the receptors on the ECs that are located on the nearby pre-existing blood vessels to activate signal transduction pathways. The activated ECs produce new molecules including enzymes, resulting in the dissolving the basement membrane of the existing blood vessels; and increasing the vasodilatation and endothelial permeability. The activated ECs proliferate and migrate towards the diseased tissue, with the help of adhesion molecules/integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$) to pull the sprouting new blood vessel forward. Meanwhile matrix metalloproteinases (MMPs) dissolve the tissue in front of the sprouting vessel tip to accommodate the sprouting process. Sprouting ECs roll up to form a blood vessel tube (lumen formation), and individual blood vessel tubes connect to form blood vessel loops that can circulate blood. Smooth muscle cells and pericytes join in to stabilise vessels and blood flow begins (http://www.angio.org/understanding/content_understanding.html>accessed on: 3rd Feb. 2004; Distler, *et al.*, 2002).

As a dynamic multistep process, angiogenesis can be summarised by the following steps: the degradation of the extracellular matrix (ECM) surrounding

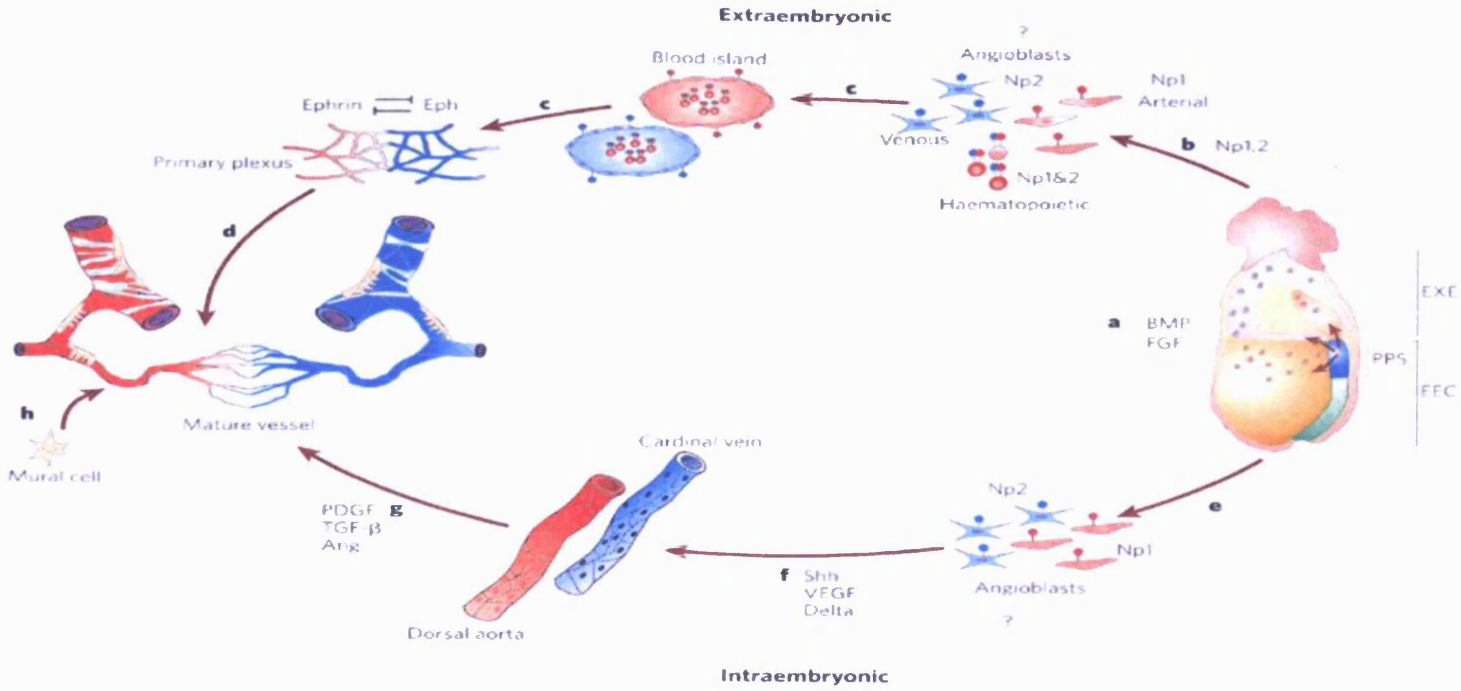


Fig. 1.1 Formation of a functional circulation from endothelial progenitors. **a**, Vascular progenitors appear in response to bFGF and BMP4 in PPS as VEGFR-2/Flk1-positive mesodermal cells. **b**, Flk1-positive cells in the primitive streak give rise to both blood and endothelium (haemangioblasts), but are restricted to haematopoietic or angiogenic fate after emigrating into extra-embryonic sites (extra-embryonic ectoderm (EXE), yolk sac and allantois) and intra-embryonic sites (embryonic ectoderm (EEC)). **c**, In the yolk sac, these progenitors aggregate into endothelial-lined blood islands that then fuse to generate a primary capillary plexus. **d**, The primary capillary plexus undergoes remodelling along with intra-embryonic vessels to form a mature circulation (**g**). **e**, Intra-embryonic angioblasts migrate along distinct pathways before (**f**) aggregating directly into the dorsal aorta or cardinal vein, without a plexus intermediate. **g**, The primary vessels (capillary plexus, dorsal aorta and cardinal vein) then remodel, together with the extra-embryonic plexus, to form a mature vasculature, which along with VEGF and Notch involves the angiopoietins and Tie receptors[4]. **h**, Mural cells (pericytes and smooth-muscle cells) proliferate and differentiate in response to TGF-β signalling, and are recruited to vessels by PDGF secreted by endothelial cells[5, 6]. Ang, angiopoietin; Eph, Eph receptor family; Shh, sonic hedgehog; Np, neuropilin. (Adapted from Coultas *et al.*, 2005).

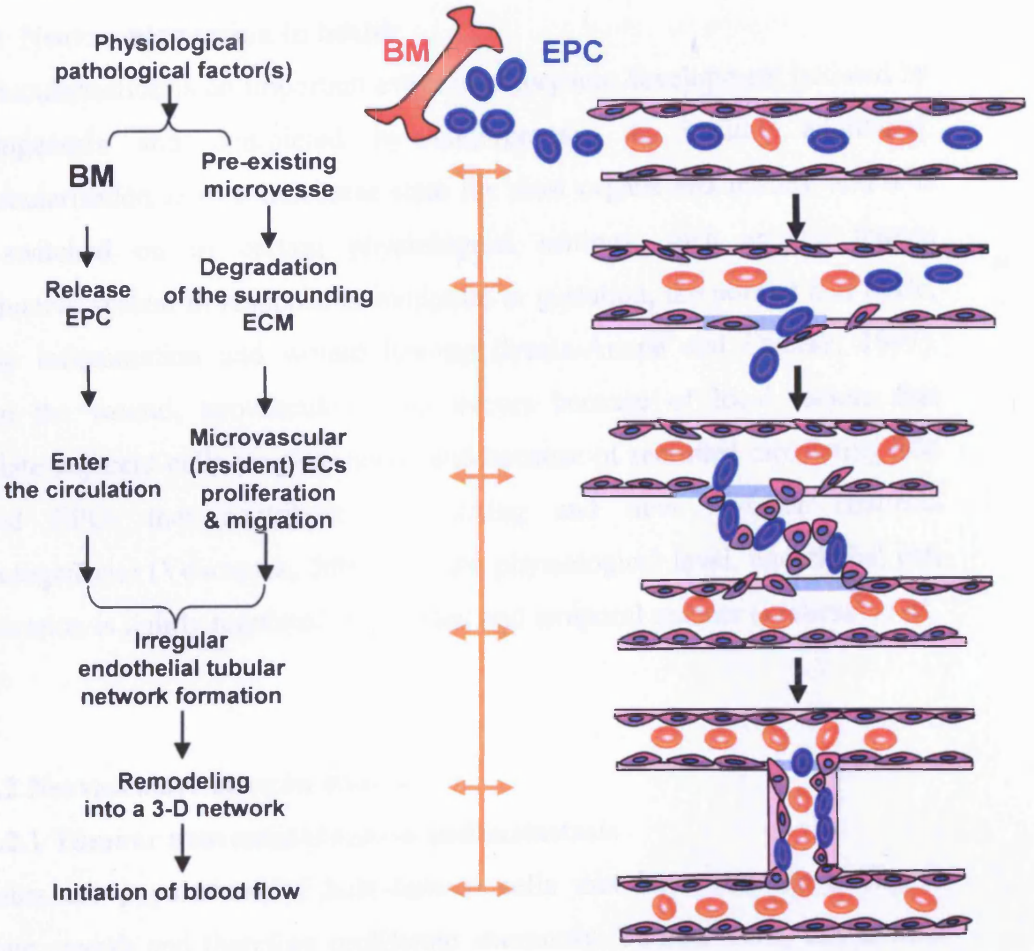


Fig 1.2 Vasculogenesis and angiogenesis cascade of events in adult.
BM:bone marrow; EPC: Endothelial precursor cells.

the pre-existing vessels by proteolytic enzymes, endothelial cells migration toward an angiogenic stimulus and their proliferation, formation of the tube-like structure, fusion of the formed vessel and initiation of blood flow (Cao, 2001)

1.1.3 Neovascularisation in health and disease

1.1.3.1 Neovascularisation in health

Neovascularisation is an important event in embryonic development initiated by vasculogenesis and completed by angiogenesis. In healthy adulthood, neovascularisation is in a quiescent state for most organs and tissues, and it is only switched on in certain physiological settings, such as the female reproductive system in response to ovulation or gestation, the normal hair cycle, chronic inflammation and wound healing (Iruela-Arispe and Dvorak, 1997). Within the wound, neovascularisation occurs because of local factors that stimulate adjacent cells (angiogenesis) and because of recruited circulating BM derived EPCs that contribute to existing and new vascular channels (vasculogenesis) (Velazquez, 2007). At the physiological level, endothelial cell proliferation is tightly regulated in a spatial and temporal manner (Liekens, *et al.*, 2001).

1.1.3.2 Neovascularisation in disease

1.1.3.2.1 Tumour neovascularisation and metastasis

Tumours are populations of host-derived cells that have lost the ability to regulate growth and therefore proliferate aberrantly. Tumour cells, like normal cells, require an adequate supply of oxygen and nutrients and an effective means to remove waste in order for metabolic processes to occur and survival to be maintained. Normal cells and tissues rely on physiological vasculogenesis and angiogenesis to provide them with a vasculature that fulfils their metabolic demands. Tumour cells, on the other hand, can induce their own blood supply from the pre-existing vasculature in a process that mimics normal angiogenesis (Papetti and Herman, 2002). Without angiogenesis, tumour cells can only proliferate to a certain size and remain in a dormant state (Cao, 2001). The 'angiogenic switch' can be turned on by hypoxia and tumour derived growth

factors (Folkman, 1995). Recent studies have also shown that vasculogenesis also occurs during tumour neovascularisation (Lyden *et al.*, 2001; Asahara *et al.*, 1997; Garmy-Susini, 2005; Schmid and Varner, 2007). EPCs may be mobilised from the bone marrow by tumour tissue-derived cytokines, such as VEGF (Asahara and Kawamoto, 2004; Dome *et al.*, 2007). Recruitment of EPC to tumour neovascularisation takes place in five steps: 1) active arrest and homing of the circulating cells within the angiogenic microvasculature; 2) transendothelial extravasation into the interstitial space; 3) extravascular formation of cellular clusters; creation of vascular sprouts and cellular networks and 5) incorporation into a functional microvasculature (Ribatti *et al.*, 2007).

Tumours become cancerous when they metastasise, i.e. the cells migrate from a primary location to a distant secondary location and re-grow into a new tumour, a process mediated by penetration of the blood or lymphatic vessels. Neovascular channels allow tumour cells to metastasise hematogenously (Ribatti *et al.*, 2007).

1.1.3.2.2 Corneal, retinal and choroidal neovascularisation

Ocular neovascularisation is the leading cause of blindness in a variety of clinical conditions (Das and McGuire, 2003; Afzal *et al.* 2007). Corneal neovascularisation is usually associated with inflammatory or infectious disorders of the ocular surface (Chang *et al.*, 2001). Neovascularization arising from the retinal circulation is seen commonly in proliferative diabetic retinopathy (PDR), retinal vein occlusion, retinopathy of prematurity (ROP), and sickle cell retinopathy (Das and McGuire, 2003). All these conditions can result in intraocular haemorrhage and tractional retinal detachment leading to severe visual loss. PDR is the most common cause of early-onset vision loss in young people in developing countries (Aiello, 2003), and retinal neovascularization is a significant contributory factor to this vision loss (Das and McGuire, 2003). In addition, hyperglycaemia indirectly regulates the synthesis of a variety of angiogenic factors in retinopathy (Cai and Boulton, 2002). In elderly people, age-related macular degeneration (AMD) is the most common cause of severe vision loss in the USA, and choroidal neovascularization (CNV) is seen in the majority

of AMD patients with severe vision loss (20/200 or worse) (Das and McGuire, 2003; Afzal *et al.* 2007).

Although retinal neovascularisation is thought to be due to the proliferation of endothelial cells from the existing blood vessels by angiogenesis, recent evidence suggests that adult haematopoietic stem cells can enter the circulation and reach the areas of angiogenesis, and can clonally differentiate into endothelial cells (Grant *et al.*, 2002).

1.1.4 Regulation of neovascularisation

Neovascularisation, a complex process involving extensive interplay between cells, soluble factors, and ECM components (Liekens, *et al.*, 2001), is under stringent regulation by both endogenous activators and inhibitors (Hanahan *et al.*, 1996). A wide range of angiogenic inducers, including growth factors, chemokines, angiogenic enzymes, endothelial specific receptors, and adhesion molecules are involved in the neovascularisation process. When sufficient neovascularization has occurred, angiogenic factors are down-regulated or the local concentration of inhibitors increased. As a result, the endothelial cells become quiescent, and the vessels remain or regress if no longer needed. Therefore, neovascularisation is a highly ordered process under tight control and the normal healthy body retains a perfect balance between the release of angiogenic stimuli and angiogenic inhibitors (Liekens, *et al.*, 2001). When angiogenic stimuli are produced in excess of the inhibitors, the balance is tipped in favour of blood vessel formation and vice versa (Fig 1.3).

Since each step in the angiogenic cascade involves a great variety of enzymes, cytokines, and receptors, neovascularisation presents a range of possible targets for therapeutic intervention (Liekens, *et al.*, 2001). On the other hand, anti-angiogenic therapy, which targets activated endothelial cells, offers several advantages over therapy directed against tumour cells. First, endothelial cells are a genetically stable, diploid, and homogenous target, and spontaneous mutations rarely occur. Also, turnover of tumour endothelial cells may be 50

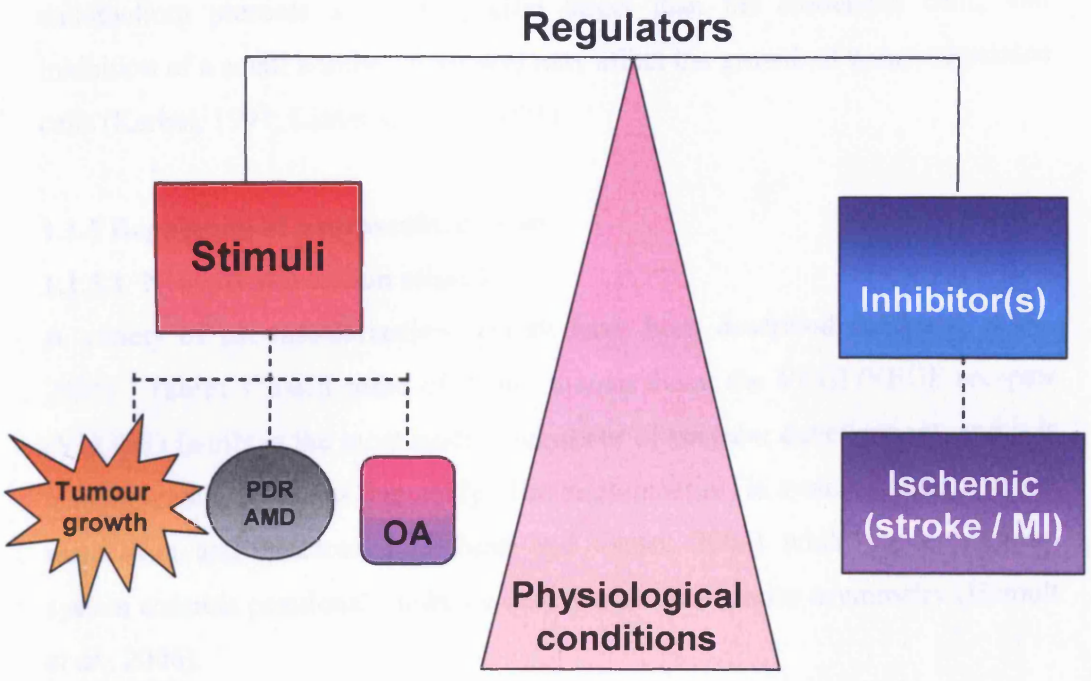


Fig 1.3 Regulation of angiogenesis. PDR: proliferative diabitic retinalpathy; AMD: age-related macular degeneration; OA: Osteoarthritis; MI: myocardial infarction.

times higher than that of endothelium in normal quiescent tissues, and activated blood vessels express specific markers, such as integrin $\alpha_v\beta_3$, Tie, and VEGF receptors. Because anti-angiogenic therapy is directed at activated endothelial cells, its target should be easily accessible by systemic administration. Finally, different tumour cells are sustained by a single capillary, and associated endothelial cells contribute to both endothelial and associated cell growth by releasing autocrine and paracrine factors. Consequently, the activated endothelium presents a more specific target than the associated cells, and inhibition of a small number of vessels may affect the growth of many associated cells (Kerbel, 1991; Liekens, *et al.*, 2001).

1.1.5 Regulators of neovascularisation

1.1.5.1 Neovascularisation stimuli

A variety of neovascularisation stimuli have been described (Liekens, *et al.*, 2001). Table1.1 listed some of them. Among them, the VEGF/VEGF receptor (VEGFR) family is the most studied regulator of vascular development, and it is also the central focus of this study. The angiopoietin/Tie system controls vessel maturation and quiescence (Eklund and Olsen, 2006) while the eph/Ephrin system controls positional guidance cues and arterio-venous asymmetry (Heroult *et al.*, 2006).

1.1.5.2 Neovascularisation inhibitors

A number of endogenous inhibitors have been identified and Table1.2 listed some of them. The most studied negative regulators include angiostatin (O'Reilly *et al.*, 1994), endostatin (O'Reilly *et al.*, 1997), and thrombospondin (Dameron *et al.*, 1994). PEDF in particular will be discussed in detail later.

1.2 Ligands and receptors of VEGF family

1.2.1 Ligands of VEGF family

The ligands of VEGF family currently consist of seven secreted glycoproteins that designated as: VEGF-A (or VEGF), VEGF-B, VEGF-C, VEGF-D, orf virus VEGF (VEGF-E), placental growth factor (PlGF) (Veikkola and Alitalo, 1999;

Table 1.1 Endogenous inducers of angiogenesis (Induction of EC proliferation, migration, and differentiation was measured *in vitro*).

Inducers	EC proliferation	EC migration	EC differentiation	References
Heparin binding peptide growth factors				
VEGF	Yes	Yes	Yes	Veikkola & Alitalo, 1999; Ferrara, 1999.
PlGF	Weak	Yes	?	Bussolino *et al.* 1996.
PDGF	Yes	Yes	Yes	Heldin & Westernmark, 1999
Non- heparin binding peptide growth factors				
TGF-α	Yes	Yes	Yes	Bussolino *et al.* 1996; Jackson *et al.* 1997.
EGF	Yes	Yes	Yes	Bussolino *et al.* 1996; Sato *et al.* 1993.
IGF-I	Yes	Yes	Yes	Sato *et al.* 1993; Bar *et al.* 1988.
Inflammatory mediators				
TNF- α	Inhibition	No	Yes	Jackson *et al.* 1997.
IL-8	Yes	Yes	?	Keane & Strieter, 1999
Enzymes				
COX-2	No	Yes	Yes	Daniel *et al.* 1999.
Angiogenin	No	Yes	Yes	Badet, 1999
Hormones				
Oestrogens	Yes	Yes	Yes	Schnaper *et al.* 1996.
Proliferin	?	Yes	?	Jackson *et al.* 1994.
Cell adhesion molecules				
VCAM-1	No	Yes	?	Koch *et al.* 1995
E-selectin	No	Yes	Yes	Koch *et al.* 1995 ; Nguyen *et al.* 1993
Hematopoietic factors				
Erythropoietin	Yes	?	Yes	Ribatti *et al.* 1999
Others				
Angl	No	Yes	Yes	Hayes *et al.*, 1999 Eklund & Olsen, 2006
EphrinB	Positioning of ECs; ECs interacting with neighboring cells			Heroult *et al.*, 2006

Table 1.2 Endogenous inhibitors of angiogenesis. Downwards arrow (↓) represents inhibition or reduction; upward arrow (↑) represents stimulation or induction.

Inhibitor	Mechanism of action	References
Protein fragments		
Angiostatin (fragment of plasminogen)	↓ EC proliferation, ↑ EC apoptosis	O'Reilly *et al.* 1994; Cao, 1999
Endostatin (fragment of collagen XVIII)	↓ EC proliferation, ↑ EC apoptosis	O'Reilly *et al.* 1997.
aa AT (fragment of antithrombin 3)	↓ EC proliferation, ↑ EC apoptosis	O'Reilly *et al.* 1999.
Prolactin (16 kDa fragment)	↓ EC proliferation ↓ FGF-2-induced angiogenesis	Struman *et al.* 1999.
Soluble mediators		
TSP-1	↓ EC proliferation, ↑ EC apoptosis	Iruela-Arispe and Dvorak, 1997
Troponin I	↓ EC proliferation	Moses *et al.* 1999
IFN-α	↓ EC proliferation, ↑ EC apoptosis ↓ FGF-2-induced angiogenesis	Dinney *et al.* 1998
IFN-γ	↓ EC proliferation, ↑ IP-10	Sato *et al.* 1990
PEDF	↓ EC migration ↓ FGF-2-induced angiogenesis	Dawson *et al.* 1999
IP-10	↓ EC proliferation	Moore *et al.* 1998
	↓ FGF-2 and IL-8 induced migration	
PF-4	↓ EC proliferation ↓ FGF-2 and IL-8 induced migration	Moore *et al.* 1998
IL-12	↑ IFN-γ, ↑ IP-10	Sgadari *et al.* 1996
IL-4	↓ EC migration	Volpert *et al.* 1998
VEGI	↓ EC proliferation	Zhai *et al.* 1999
TIMP-1,-2	↓ MMP activity	Gomez *et al.* 1997
Retinoic acid	↓ EC migration, transcription factor	Diaz *et al.* 2000; Lingen *et al.* 1996
Ang-2	↓ Blood vessel maturation, antagonist of Ang-1	Maisonpierre *et al.* 1997

Liekens, *et al.*, 2001) and VEGF-F (Suto *et al.*, 2005). Each of these proteins contains a signal sequence that is cleaved during biosynthesis. Alternative splicing of their corresponding pre-mRNAs generates multiple isoforms of VEGF, VEGF-B, and PlGF. The VEGF family plays an integral role in angiogenesis, lymphangiogenesis, and vasculogenesis (Roskoski , 2007).

VEGF-A (VEGF)

VEGF was first identified by Senger *et al* (1983) as a vascular permeability factor secreted by tumour cell, and is a 46 kDa homodimeric glycoprotein with both vasopermeability and angiogenic properties (Keck, *et al.,* 1989; Senger *et al.,* 1990; Ferrara *et al.,* 1991a & b).

VEGF is a mitogen and survival factor for vascular ECs (Ferrara and Henzel, 1989; Alon *et al.*, 1995; Gerber *et al.* 1998), and also promoting vascular ECs and monocyte motility (Waltenberger *et al.*, 1994; Barleon *et al.*, 1996). Moreover, VEGF selectively and reversibly permeabilises the endothelium to plasma and plasma proteins without leading to injury (Senger *et al.*, 1990; Dvorak, 2005; Roskoski, 2007). VEGF acts not only on the resident vasculature but also modulates EPC function (Adamis *et al.,* 1994; Ferrara, 2004; Coultas *et al.*, 2005; Afzal *et al.*, 2007). Active VEGF signalling is required for normal development of progenitors of the vascular and haematopoietic system; and plays an important role in shaping the vascular system, organs signal to the vessels that serving them, influencing the endothelial cells to adopt functional specialties, such as the blood-brain barrier and fenestrated endothelium in the kidney glomeruli (Nikolova and Lammert, 2003)

The VEGF gene is located on the short arm of chromosome 6, and is a single gene composed of eight exons and is differentially spliced to yield nine mature isoforms: VEGF121, VEGF165, VEGF189, VEGF206, VEGF145, VEGF183, -165b(an inhibitory isoform), -162, and -148 (Tischer *et al.*, 1991; Neufeld *et al.*, 1999; Robinson and Stringer, 2001). The numeric designation of the isoforms denotes the number of amino acids in the molecule. VEGF165 a secreted

heparin-binding protein being the predominant form followed by the 189 and 121 residue molecules as determined by cDNA analysis of a variety of cell types, tissues, and tumour specimens (Ferrara, *et al.,* 2003; Roskoski, 2007).

VEGF is expressed in different tissues, including brain, kidney, liver, and spleen, and by many cell types including, endothelial cells, macrophages, glial cells, keratinocytes, tumour cells (Rousseau *et al.,* 2000 a & b; Liekens, *et al.*, 2001), and HRPE cells (Ohno-Matsui *et al.*, 2003). Transcription of VEGF mRNA is induced by different growth factors and cytokines, including PDGF, EGF, TNF-α, TGF-β, and IL-1β (Veikkola and Alitalo, 1999; Akagi, *et al.,* 1999; Enholm, *et al.,* 1997; Liekens, *et al.*, 2001). VEGF levels are also regulated by tissue oxygen tension. Exposure to hypoxia induces VEGF expression rapidly and reversibly, through both increased transcription and stabilization of the mRNA (Mukhopadhyay, *et al.* 1995; Ikeda, *et al.,* 1995). In contrast, normoxia down-regulates VEGF production and even causes regression of some newly formed blood vessels.

VEGF binds to two related high affinity cell surface tyrosine kinase receptors: VEGFR-1 and VEGFR-2 (Yancopoulos, *et al.,* 2000). In addition to these receptors, VEGF interacts with a family of co-receptors, the neuropilins (Ferrara *et al.*, 2003).

1.2.2 VEGF receptor family

1.2.2.1 General properties

The VEGF receptor family consists of five members, three receptor protein-tyrosine kinases [VEGFR-1 (Flt-1, fms-like tyrosyl kinase-1, where fms refers to feline McDonough sarcoma virus), VEGFR-2 (KDR in humans, Kinase Domain-containing Receptor /Flk-1 in mice, Fetal liver kinase-1) and VEGFR-3 (Flt-4)] (Galland, *et al.* 1993; Mustonen and Alitalo, 1995; Shibuya, *et al.* 1999; Shibuya and Claesson-Welsh, 2006) and two non-enzymatic receptors (neuropilin-1 and -2) (Ferrara *et al.*, 2003; Roskoski, 2007).

VEGFR-1, VEGFR-2 and VEGFR-3 are highly homologous to each other in overall structure (Shibuya, *et al.* 1990; Alitalo and Carmeliet, 2002), and all three consist of an extracellular ligand-binding domain (a seven Ig-like domain), a transmembrane domain, an intracellular tyrosine kinase domain and downstream carboxyl terminal region (Shibuya, *et al.* 1990; Terman *et al.* 1992 and Millauer *et al.* 1993) (see Fig1.4-Aa). The binding site for VEGF(-A) is located in the second Ig-like domain in VEGFR-1 and the second/third Ig-like domains in VEGFR-2. The downstream structure from the fourth to seventh Ig-like domains in these receptors plays a major role in receptor dimerization and activation (Keyt *et al.*, 1996; Fuh *et al.*, 1998). Among these receptors, VEGFR-1 has highest affinity to VEGF, VEGFR-2 binds with less affinity, and VEGFR-3 shows essentially no binding to VEGF, although it binds VEGF-C and VEGF-D (Veikkola and Alitalo, 1999; Shibuya, 2001a).

Neuropilins are glycoproteins with a molecular weight of 120 -140 kDa (Chen *et al.* 2000). The neuropilins contain a large extracellular component, a transmembrane segment, and short ($\approx$ 40 amino acid residue) intracellular portion (Mamluk *et al.*, 2002). Neuropilins act as co-receptors with large ($\approx$ 250 kDa) transmembrane plexins that transduce semaphoring signalling and as co-receptors with VEGFR-1, VEGFR-2 and VEGFR-3 that transduce VEGF family signalling (Roskoski, 2007).

The VEGF receptor signal transduction pathway remains unclear. Most approaches used for study are based on the classical tyrosine kinase receptor pathway, and have focused on the recognition of the element(s) that are involved in it. Classically, signals from a tyrosine kinase receptor are initiated by binding of a ligand (usually a growth factor) to the receptor, which then forms a dimer. This dimer adds phosphate groups to itself (autophosphorylation), resulting in the creation of docking sites that bind to downstream signal transduction molecules containing Src homology 2 (SH2) domain. Upon ligand binding, a number of signalling pathways can be activated, each one consisting of a chain of

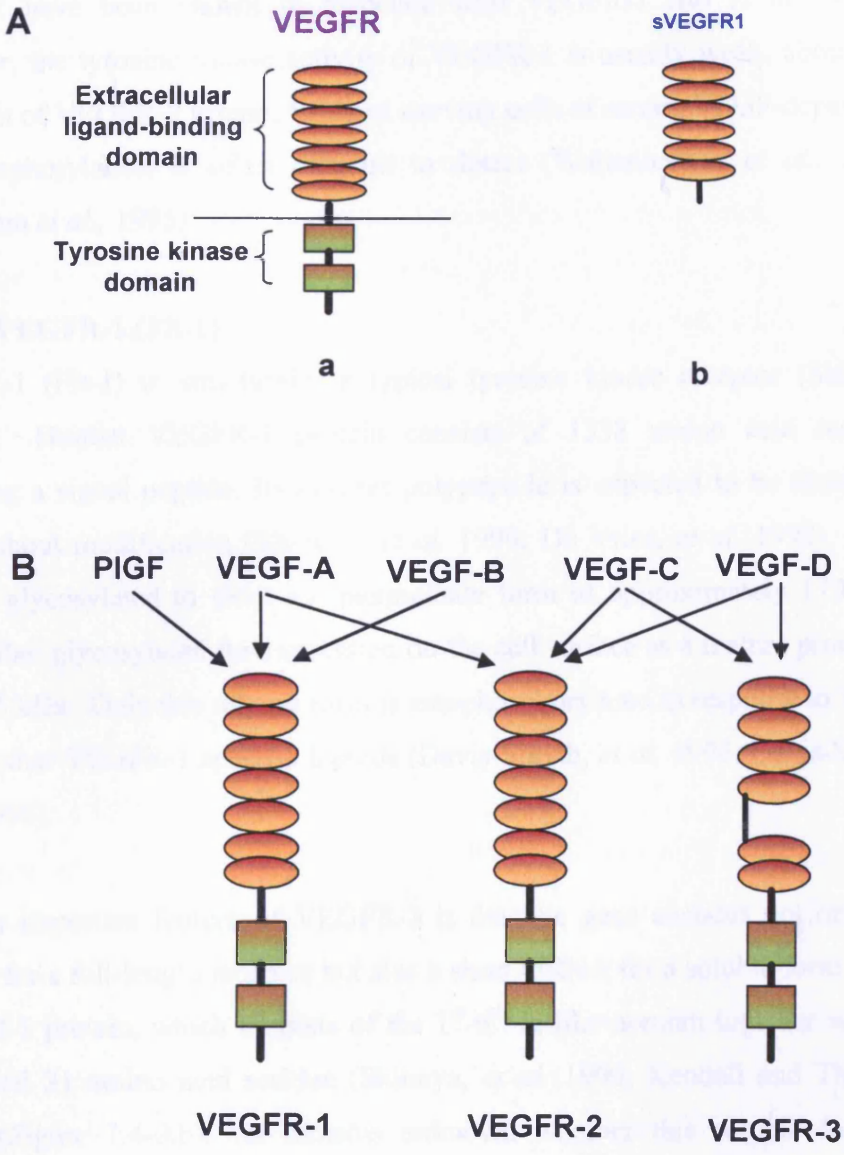


Fig 1.4 A schematic representation of the structure of VEGFRs. (A) the full-length of VEGFR-1(a) and soluble form of VEGFR-1(b); B) shows the interaction between VEGF family and their receptors (Modified from Yancopoulos *et al.*, 2000).

signalling molecules that indirectly alter the expression of target genes in the nucleus (Heldin and Ericsson, 2001). Several adapters such as Nck, Grb2, SHP2 and Crk have been shown to associate with VEGFR-1 (Ito *et al.,* 1998). However, the tyrosine kinase activity of VEGFR-1 is usually weak, about one tenth that of VEGFR-2 kinase. Without starving cells of serum, VEGF-dependent autophosphorylation is often difficult to detect (Waltenberger, *et al.,* 1994; Seetharam *et al.,* 1995).

1.2.2.2 VEGFR-1 (Flt-1)

VEGFR-1 (Flt-1) is structurally a typical tyrosine kinase receptor (Shibuya, 2001b). Human VEGFR-1 protein consists of 1338 amino acid residues including a signal peptide. Its nascent polypeptide is expected to be about 150 kDa without modification (Shibuya, *et al.* 1990; De Vries, *et al.* 1992), but is rapidly glycosylated to form an intermediate form of approximately 170 kDa, and further glycosylated for expression on the cell surface as a mature protein of 180-185 kDa. Only this mature form is autophosphorylated in response to VEGF and to other VEGFR-1 specific ligands (Davis-Smyth, *et al.* 1996; Davis-Smyth, *et al.* 1998).

Another important feature of VEGFR-1 is that the gene encodes not only the mRNA for a full-length receptor but also a short mRNA for a soluble form of the VEGFR-1 protein, which consists of the 1^{st}-6^{th} Ig-like domain together with an additional 31 amino acid residue (Shibuya, *et al.* 1990; Kendall and Thomas, 1993) (Figure 1.4-Ab). It remains unknown whether this soluble form of VEGFR1 is also produced from cleavage. The VEGFR-1 gene in mice consists of 30 exons spanning more than 150 kb (Kondo, *et al.* 1998). Each of the 1^{st} and 2^{nd} Ig-like domains is split into two exons. The soluble form of VEGFR-1 is found to be derived from the first 13 exons and the down-stream 5'-region of intron 13 in this gene (Kondo, *et al.* 1998). Therefore, the VEGFR-1 gene appears to carry two physiologically meaningful 'exon13's, one for the soluble form of VEGFR-1 and the other for the receptor form. The mRNA and the

protein of VEGFR-1 gene are specifically expressed in most vascular ECs (Barleon, *et al.* 1994).

Recently, two groups (Lyden *et al.*, 2001; Kaplan *et al.*, 2005) identified VEGFR-1 positive haematopoietic progenitor cells that multiply in the bone marrow, mobilise to the peripheral blood along with VEGFR-2 positive EPCs, and incorporate into pericapillary connective tissue. More interestingly, these cells seem to home in before the tumour cells arrive, promoting metastatic growth by forming niches where cancer cells can locate and proliferate.

1.2.2.3 VEGFR-2 (Flk-1 or KDR)

VEGFR-2 protein consists of 1338 amino acids in humans, and is expressed mostly on vascular endothelial cells. VEGFR-2 is a marker for the earliest progenitors of the vascular and haematopoietic system, the loss of both vascular and haematopoietic cells in *Flk-1* mutants is one of many pieces of information linking endothelial and haematopoietic development through a possible common progenitor, the haemangioblast (reviewed by Ema and Rossant, 2003; Coultas *et al.*, 2005). VEGFR-2 is also expressed at a significantly lower level in neuronal cells; osteoblasts, pancreatic duct cells, retinal progenitor cells, megakaryocytes (Matsumoto and Claesson-Welsh, 2001), HRPE cells and a fraction of haematopoietic cells which might be the progenitor for endothelial cells (Asahara *et al.*, 1999; Lyden *et al.*, 2001). The biological role of VEGFR-2 in these non-endothelial cells remains to be clarified. In the tumour vasculature VEGFR-2 expression is 3-5-fold higher than in the normal vasculature (Plate *et al.*, 1994). On the other hand, most tumour cells or leukaemia/lymphoma cells do not express VEGFR-2 except for Kaposi sarcoma cells in AIDS patients (Montaldo *et al.*, 2000). Within the tumour tissue, the tumour cells themselves and activated stroma cells express a high level of VEGF but little VEGFR-2, whereas the endothelial cells in the tumour vasculature exhibit an upregulated VEGFR-2 expression, which strongly suggest a paracrine loop of VEGF and VEGFR-2 between tumour cells and vascular endothelial cells for the stimulation of

pathological angiogenesis (Ferrara and Davis-Smyth 1997; Alitalo and Carmeliet, 2002; Shibuya and Claesson-Welsh, 2006; Shibuya, 2006a).

VEGFR-2 is the major positive signal transducer for both physiological and pathological angiogenesis. Upon stimulation with VEGF, VEGFR-2 is autophosphorylated and then PLCγ is tyrosine phosphorylated, resulting in the activation of the downstream PKC–c-Raf-MEK-MAP-kinase pathway (Takahashi *et al.*, 2001); an adaptor molecule, TSAd (T-cell specific adapter) also can be activated, resulting in the stimulation of a migration signal (Zeng *et al.*, 2001; Matsumoto *et al.*, 2005). VEGFR-2 is also known to interact with VE-cadherin and integrins such as αvβ3, and regulates many steps of angiogenesis (Stupack and Cheresh, 2004).

So far it is known that VEGF, PlGF and VEGF-B are the ligands that bind and activate VEGFR-1 (Maglione, *et al.* 1991; Olofsson, *et al.* 1998). The affinity of VEGFR-1 is highest for VEGF (10-30 pM) and much weaker for PlGF (about 200 pM) and VEGF-B. The positive signal of VEGF for endothelial proliferation and vascular permeability from VEGFR-1 contribute about one tenth of the total signal, the major signal is through VEGFR-2. The soluble form of VEGFR-1 has potent negative activity against VEGF-dependent phenomena on ECs because of its strong ligand binding activity. A 10-fold or more excess of soluble VEGFR-1 over VEGF efficiently suppresses the VEGF-induced endothelial cell growth and vascular permeability (Tanaka, *et al.* 1997; Kendall and Thomas, 1993). Thus, the VEGFR-1 gene exerts two opposing biological activities, one positive and the other negative, in angiogenesis. This dual function may be tightly regulated and important for the fine tuning of the formation and maintenance of the blood vessel structure (Shibuya, 2001b). Figure 1.4-B shows the interaction between VEGF family and their receptors.

1.2.2.4 Relationship of VEGFR-1 and VEGFR-2 in angiogenesis

VEGFR-1 and VEGFR-2 are both expressed on vascular endothelial cells (Neufeld *et al.*1999; Ferrara, 2002), and closely involved in the formation of

tube-like structures, blood vessels and lymphatic vessels. VEGFR-1 has been shown to heterodimerise with VEGFR-2 leading to autophosphorylation, activation of VEGFR-2, and angiogenesis (Autiero *et al.* 2003; Rahimi, 2006; Afzal *et al.* 2007). However, the relationship between VEGFR-1 and VEGFR-2 is still unclear. Several studies have indicated that VEGFR-1 and VEGFR-2 have different signal transduction properties (Liekens, *et al.*, 2001). Interaction of VEGF with VEGFR-2 is critical for VEGF induced biological responses. By using chimeric receptors containing the extracellular domain of human CSF-1R/ *c-fms* fused with the entire transmembrane and cytoplasmic domains of murine VEGFR-1 and VEGFR-2, Meyer and Rahimi (2003) dissected the biological importance of VEGFR-1 and VEGFR-2 and their signal transduction relay in angiogenesis. It was demonstrated that selective activation of chimeric VEGFR-2, but not chimeric VEGFR-1, stimulated endothelial cell growth, migration, and differentiation. Stimulation of cells co-expressing chimeric VEGFR-1 and VEGFR-2 suppressed VEGFR-2-mediated endothelial cell growth. Their results also indicate that VEGFR-1, unlike VEGFR-2, is unable to undergo ligand-dependent tyrosine phosphorylation and kinase activation (Rahimi *et al.,* 2000). Using site-directed mutagenesis they demonstrated that tyrosines 799 and 1173 are required for VEGFR-2-mediated endothelial cell growth and activation of PI3 kinase. Further site-directed mutagenesis demonstrated that tyrosine 1212, located in the carboxyl tail of VEGFR-2, is required for the ligand-dependent autophosphorylation of the receptor and its ability to activate signalling proteins. Studies have shown that there is considerable "cross-talk" between VEGFR-1 and VEGFR-2 and that this plays a critical role in regulating VEGFR-2-mediated signalling (Rahimi *et al.,* 2000; Burkhardt and Zacharias, 2001; Zeng *et al.*, 2001), and dependent on conditions, VEGFR-1 can act as a pro- or anti-angiogenic regulator of VEGFR-2.

1.2.2.5 VEGFR-3 (Flt-4)

VEGFR-3 has only six Ig-homology domain (Pajusola *et al.*, 1994). It preferentially binds VEGF-C and VEGF-D. VEGFR-3 shows a more restricted expression pattern when compared with VEGFR-1 or VEGFR-2. It is expressed

in lymphatic ECs, fenestrated capillaries and veins in endocrine organs as well as in monocytes/macrophages (reviewed by Saharinen *et al.*, 2004). VEGFR-3 is involved in the regulation of both blood and lymphatic vessel function (Shibuya and Claesson-Welsh, 2006). It is believed that VEGFR-3 play various roles in cardiovascular development and remodelling of primary vascular networks during embryogenesis and enhancing lymphangiogenesis in adulthood. Inactivation of VEGFR-3 gene results in embryonic death at E9.5, due to abnormal remodelling of the primary vascular plexus (Dumont *et al*, 1998).

VEGFR-3 signal transduction is still relatively unexplored. However, evidence suggests that heterodimerization of VEGFR-2/VEGFR-3 may direct biological signalling such as lymphatic vessel sprouting (reviewed by Shibuya and Claesson-Welsh, 2006). Certain human pathological conditions involve either dysfunction or increased activation of VEGFR-3. Increased expression of VEGF-C and VEGFR-3 plays a role in prostate cancer progression and lymph nodes metastasis (Jennbacken *et al.*, 2005).

1.3 Gamma-secretase (γ-secretase): a role in neovascularisation?

1.3.1 General properties of γ-secretase

γ-secretase is an unusual aspartyl multimeric protease complex responsible for the intramembranous cleavage of a variety of type I integral membrane proteins, including the β-amyloid precursor protein (APP), Notch, ErbB4 (Ni *et al.* 2001; Lee *et al.*, 2002), E-cadherin (Marambaud *et al.* 2002), and CD44 (Lammich, 2002). This protease complex consists of four different integral membrane proteins: **presenilin**, **nicastrin**, **Aph-1** (anterior pharynx defective-1) and **Pen-2** (presenilin enhancer -2) (LaVoie, *et al.* 2003) (Fig.1.5). γ-secretase is involved in development, signal transduction, protease biochemistry and is central to the pathogenesis of Alzheimer's disease (AD) and appears to play a role in vasculogenesis and angiogenesis (Lukiw *et al.*, 2001; Nakajima *et al.*, 2003; Sainson *et al.*, 2005; Serneels *et al.* 2005; Cai *et al.* 2006; Shi and Harris, 2006; Williams *et al.*, 2006).

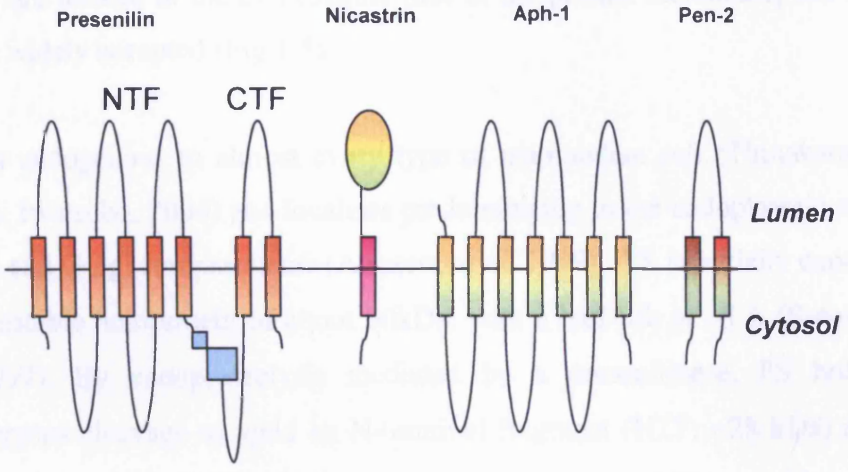


Figure1.5 A schematic representation of the complex of γ-secretase. PS is presented using the 8-TM structural model. (Reproduced from LaVoie *et al.*, 2003).

Presenilin (PS)

Presenilin, the active site of the protease complex, was the first member of the γ-secretase complex to be identified (De Strooper *et al.*, 1998). PS are multipass proteins. Several different topography models for PS have been proposed, either 6- transmembrane (TM), 7-TM, 8-TM or 9-TM domains (Dewji, 2005; Laudon *et al.*, 2005; Oh and Turner, 2005). The 8-TM structural model, where the N-terminal and C-terminal ends as well as the hydrophilic loop between TM6 and TM7 are located at the cytoplasmic face of the plasma membrane, has been the most widely accepted (Fig 1.5).

PS is endogenous to almost every type of mammalian cell (Thinakaran *et al.*, 1996; Iwatsubo, 2004) and localises predominantly in the endoplasmic reticulum (ER) and Golgi compartments (Annaert, *et al.* 1999). PS is initially expressed as an unstable holoprotein of about 50kDa, with a half-life of ~1 h (Ratovitski, *et al.*1997). By endoproteolysis mediated by a presenilinase, PS holoprotein undergoes cleavage to yield an N-terminal fragment (NTF; ~28 kDa) and a C-terminal fragment (CTF; ~22 kDa) that remain associated (Ratovitski, *et al.*1997; Thinakaran, *et al.*1996). This PS heterodimer is significantly more stable than the PS holoprotein, having a half-life of ~30 h, and is thought to be the biologically active form of the protein (Ratovitski, *et al.*1997; Yu, *et al.* 1998 and Iwatsubo, 2004). In the mature active form of γ-secretase complex PS is present as an NTF-CTF heterodimer (Levitan *et al.*, 2001). The mature form of PS (the heterodimer) has been found at the cell surface in complex with the membrane-associated C-terminus of another γ-secretase substrate, Notch (Ray *et al.*, 1999), and with nicastrin (Kaether *et al.*, 2002). However, PS alone is not sufficient for protease activity.

Nicastrin (NCT)

Nicastrin (NCT) is a type I membrane protein that possesses many potential glycosylation sites within its large ectodomain. Three principal forms of NCT exist in cells, the unglycosylated, nascent protein (~80 kDa), an "immature" N-linked glycosylation endoglycosidase H-sensitive (iNCT, ~110 kDa), and a

"mature" N-linked isoform, complex N-linked glycosylation endoglycosidase H-resistant (mNCT ~150 kDa) that is formed after entering the Golgi apparatus (Kaether, *et al.* 2002). This mNCT is associated with active γ-secretase (Kimberly, *et al.* 2002), and importantly, PS is required for the full post-translational generation of this mNCT species (Kaether, *et al.* 2002; Kimberly, *et al.* 2002). Therefore, like PS, NCT undergoes a maturation process that could regulate its association with the γ-secretase complex.

Aph-1 and Pen-2

Accumulating evidences suggest that both Aph-1 and Pen-2 are physical members of the active protease and not transient interactors merely involved in assisting γ-secretase assembly or protein folding (Takasugi, *et al.* 2003).

Aph-1 is conserved from nematodes to humans. It has a predicted molecular weight of ~29 kDa and is predicted to be a 7-TM protein (Goutte *et al.*, 2002). Aph-1 has been found to form a subcomplex with NCT and stabilise the presence of the PS holoprotein within the complex (Takasugi *et al.* 2003; Hu and Fortini, 2003; LaVoie *et al.*, 2003). Down-regulation of Aph-1 (Francis *et al.*, 2002; Lee *et al.*, 2002) by RNA interference (RNAi) is associated with reduced levels of PS NTF/CTF heterodimers and deficient γ-secretase function.

Pen-2 is a membrane protein with two putative TM helices. It consists 101 amino acids with a molecular weight of ~10 kDa (Francis *et al.*, 2002). Down-regulation of Pen-2 (Francis *et al.*, 2002; Steiner *et al.*, 2002) by RNAi is associated with reduced levels of PS NTF/CTF heterodimers and deficient γ-secretase function. Evidence suggests that Pen-2 is required for the endoproteolytic processing of PS (Luo *et al.*, 2003; Takasugi *et al.*, 2003); the stabilisation of the resulting NTF and CTF, and the subsequent activation of the γ-secretase core complex (Takasugi *et al.*, 2003; Kimberly, *et al.* 2003; Hu and Fortini, 2003; Luo *et al.*, 2003; Prokop *et al.*, 2004).

The molecular mass of γ-secretase complex varies depending on different preparations (LaVoie *et al.* 2003). By using Blue Native Polyacrylamide Gel Electrophoresis, Steiner *et al.* (2002) reported the molecular mass of the γ-secretase complex to be ~500 kDa, while LaVoie *et al.* (2003) identified it as ~ 250-~270 kDa using BN-PAGE and Western blotting, corresponding to the apparent combined molecular masses of PS, NCT, Aph-1 and Pen-2 (Kimberly, *et al.* 2003). These two different molecular masses may be from dimeric or monomeric complexes (LaVoie, *et al.* 2003).

By using both biochemical co-immunoprecipitations and confocal immunofluorescence microscopy in Chinese hamster ovary cells, Baulac and colleagues (2003) identified that PS-1, nicastrin, Aph-1, and Pen-2 interact in the Golgi/trans-Golgi network (TGN)-enriched compartments. Using immuno-fluorescence techniques, they provide further evidence for localisation of the γ-secretase components in the Golgi/TGN compartment, rather than in the ER. By using a stepwise experiment, Takasugi *et al.* (2003) demonstrated that Aph-1 stabilises the PS holoprotein in collaboration with NCT, whereas Pen-2 elicits the final maturation of the γ-secretase complex, conferring its activity and inducing endoproteolysis of PS; they also concluded that PS, NCT, Aph-1 and Pen-2 represent the set of proteins that comprise the major framework of γ-secretase.

The mechanism of γ-secretase action is still unclear. By using inhibitor cross-competition kinetics and competition ligand binding, Tian *et al.* (2003) provide evidence that suggests γ-secretase has physical separation of the sites for substrate binding and catalysis. Another unique feature of the γ-secretase cleavage, reported by Gu *et al.* (2001), is the multiplicity of the cutting positions: one in the middle of the transmembrane domain that creates the carboxyl-terminus of Aβ (classical γ-cleavage), and the other close to the inner leaflet of the membrane that liberates Notch intracellular domain (NICD) (notch site-3 cleavage), or APP ICD (AICD; γ'- or ε- cleavage of APP).

1.3.2 γ-secretase and RIP

An emerging model, Regulated Intramembrane Proteolysis (RIP), has been proposed by Kimberly and Wolfe (2003) for the normal biological function of γ-secretase: PS-dependent RIP. In this generalised model, a cell surface receptor protein (such as Notch, APP or ErbB-4) binds to its ligand (hypothetical for APP), which permits a disintegrin metalloprotease - ADAM to cleave the ectodomain just outside of the membrane. The resultant CTF serves as a substrate for γ-secretase cleavage, which cuts within the transmembrane domain. Release of the intracellular domain then permits translocation to the nucleus, interaction with appropriate cellular factors, and alteration of target gene transcription (see Fig.1.6)

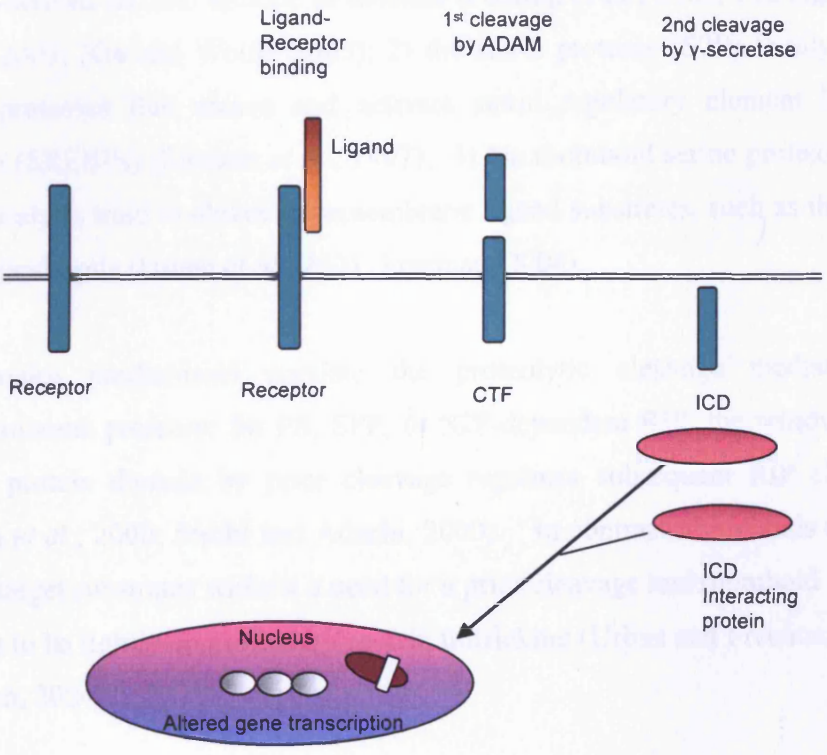


Fig 1.6 A schematic representation of the model of RIP
(Simplified from Kimberly and Wolfe, 2003).

1.3.3 Common Features of RIP

RIP was defined as the process by which transmembrane proteins can be cleaved within the plane of the membrane to liberate cytosolic fragments that enter the nucleus to control gene transcription (Brown, *et al.* 2000). This mechanism influences processes as diverse as cellular differentiation, lipid metabolism, and the response to unfolded proteins (Brown, *et al.* 2000).

RIP is mediated by at least three distinct families of intramembrane proteases, which cleave substrates within their TM domains: 1) the presenilin-type aspartyl proteases, including the PS-dependent γ-secretase (Wolfe *et al.*, 1999a & b) and the signal peptide peptidase (SPP) which is essential for generation of signal peptide-derived HLA-E epitope in humans (Ponting *et al.*, 2002; Martoglio and Golde, 2003; Xia and Wolfe, 2003); 2) the site-2 protease (S2P) family, zinc-metalloproteases that cleave and activate sterol regulatory element binding proteins (SREBPs) (Rawson *et al.*, 1997); 3) the rhomboid serine proteases that use a catalytic triad to cleave transmembrane ligand substrates, such as the main EGF ligand Spitz (Urban *et al.*, 2001; Freeman, 2004).

Two major mechanisms regulate the proteolytic cleavage mediated by intramembrane protease: for PS, SPP, or S2P-dependent RIP, the removal of a certain protein domain by prior cleavage regulates subsequent RIP cleavage (Brown *et al.*, 2000; Struhl and Adachi, 2000). In contrast, rhomboids directly cleave target substrates without a need for a prior cleavage and rhomboid activity appears to be tightly controlled by protein trafficking (Urban and Freeman, 2003; Freeman, 2004)

Table 1.3 lists the reported γ-secretase substrates to date (reviewed by Landman and Kim, 2004; Vetrivel *et al.*, 2006). These substrates share several characteristics: a) they all span the membrane bilayer at least once, though in different directions. b) they can be type I membrane-spanning proteins oriented with their NH_2 termini in the lumen and their COOH termini in the cytosol; or type II membrane protein with its NH_2 terminus in the cytosol;

Table 1.3 Reported γ-secretase substrates

Substrate	Putative proteolysis function	References
APP	Nuclear signaling in complex with Fe65/Tip60	McLoughlin & Miller *et al.*, 1996;
APLP 1/2	Nuclear signaling	Eggert *et al.*, 2004; Scheinfeld *et al.*, 2002
Notch 1-4	Nuclear signaling in complex with transcription factors (e.g. CSL)	Struhl and Greenwald, 1999; Song *et al.*, 1999; Handler *et al.*, 2000; Mizutani *et al.*, 2001; Kimberly *et al.*, 2003
Jagged and Delta-1	Nuclear signaling (via AP-1)	LaVoie and Selkoe, 2003 Bland *et al.*, 2003
ErbB-4	Nuclear signaling in complex with YAP; disassembly of high affinity heteromeric	Ni *et al.*, 2001; Lee *et al.*, 2002
DCC	Nuclear signaling?	Taniguchi *et al.*, 2003
CD44	Nuclear signaling (via CBP supression)	Murakami *et al.*, 2003
LRP	Nuclear signaling in complex with Tip60	May *et al.*, 2003
N-Cadherin	Nuclear signaling (via CBP supression)	Marambaud *et al.*, 2003
NRG-1	Nuclear signaling	Bao *et al.*, 2003
E-Cadherin	Disassembly of adherence junctions; nuclear signaling through liberation of α/ β catenins?	Marambaud *et al.*, 2002
Nectin-1	Disassembly of adherence junctions	Kim *et al.*, 2002
p75NTR	Disassembly of high affinity heteromeric neurotrophin receptor complex	Jung *et al.*, 2003
Syndecan-3	Disassembly of synaptic receptor complexes and / or nuclear signaling via interaction with CASK?	Schulz *et al.*, 2003
GluR subunit 3	Modulation of synaptic receptor function?	Meyer *et al.*, 2003
Na channel β2-subunit	Regulates Na_v1 α-subunit levels and controls cell-surface sodium current densities	Kim *et al.*, 2007
Tyrosinase	Disrupt the melanosomal localisation and melanin synthesis activity	Wang *et al.*, 2006
TNF α	Trigger IL-12 production	Friedmann *et al.*, 2006
VEGFR1	Regulates angiogenesis	Cai *et al.*, 2006
IGF-1 receptor	Nuclear signaling?	McElroy *et al.*, 2007
IL-1 receptor2	?	Kuhn *et al.*, 2007
LRP6	Binding directly with GSK3 and attenuating its activity; Nuclear signaling?	Mi and Johnson, 2007

or two membrane-spanning regions that are inserted in the bilayer in a helical hairpin fashion.

PS-dependent γ-secretase and rhomboids cleave only type-I membrane substrates, while S2P and SPP cleave type-II membrane proteins (Weihofen *et al.*, 2002; Lemberg and Martoglio, 2002).

1.3.4 RIP and Classic Receptor Tyrosine Kinases

Several recent reports suggest that a classic receptor tyrosine kinase, ErbB-4, may also signal through RIP (Ni *et al.,* 2001; Lee *et al.,* 2002). Like the other RIP substrates, ErbB-4 undergoes two cleavage events. The initial cleavage in the extracellular domain is mediated by the metalloprotease TACE (ADAM17) (Rio *et al.,* 2000). This is followed by intramembrane cleavage mediated by γ-secretase /presenilin, which results in translocation of the C-terminal domain to the nucleus. However, it remains to be determined whether endogenous ErbB-4 mediates nuclear signalling. It is still possible that γ-secretase cleavage might facilitate ErbB-4 signalling through activation of protein kinase C, MAP kinase, or PI3 kinase, known targets of ErbB-4 signalling in the cytoplasm. Nevertheless, these findings raise the intriguing possibility that the same receptor may signal through both classic kinase cascades and RIP (Ebinu and Yankner, 2002). Very recently, Cai *et al.* (2006) showed evidence that PEDF inhibits VEGF-induced angiogenesis *in vitro* and this closely correlates to the cleavage of VEGFR-1 via γ-secretase activity. This suggests that VEGFR-1 may be also involved in RIP.

1.3.5 A new non-classical signal pathway for VEGF action?

Considering the evidences reviewed above, a new non-classical signal pathway for VEGF action can be postulated as follows: upon the ligand binding (e.g. VEGF, VEGF-B or PlGF), an enzyme (possibly one of the MMPs) cleaves a portion of the extracellular domain of the VEGFR-1; this 1st cleavage initiates γ-secretase to cleave the cytosolic domain of the VEGFR-1, allowing this fragment

to translocate to the nucleus of the endothelial cells to promote angiogenesis (see Fig 1.7).

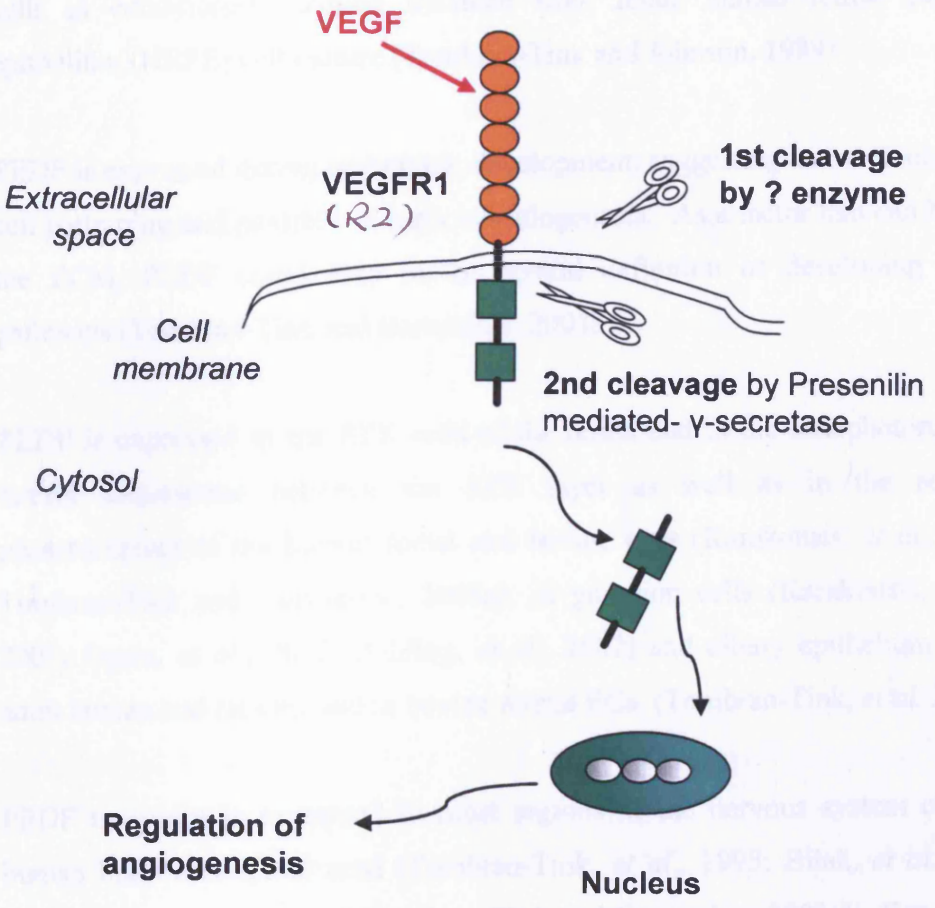


Fig 1.7 A schematic representation of the hypothesis – the effect of VEGF on VEGFR-1/VEGFR-2.

1.4. Pigment Epithelium-Derived Factor (PEDF)

1.4.1 General features of PEDF

Pigment Epithelium-Derived Factor (PEDF), a 50 kDa of glycoprotein with pleiotropic functions, is naturally occurring in the eye and considered as crucial to prevent pathological angiogenesis. PEDF was first identified as a neurotrophic factor possessing neuronal differentiating activity against Y-79 retinoblastoma cells in conditioned medium obtained from foetal human retinal pigment epithelium (HRPE) cell culture (Tombran-Tink and Johnson, 1989)

PEDF is expressed during embryonic development, suggesting an involvement in cell patterning and possibly in early vasculogenesis. As a factor that can bind to the ECM, PEDF could help in the spatial definition of developing vessel pathways (Tombran-Tink and Barnstable, 2003a).

PEDF is expressed in the RPE cells of the retina and in the interphotoreceptor matrix sequestered between the RPE layer as well as in the adjacent photoreceptors of the human foetal and bovine eyes (Karakousis, *et al.*, 2001; Tombran-Tink and Barnstable, 2003a); in ganglion cells (Karakousis, *et al.*, 2001; Ogata, *et al.*, 2002; Behling, *et al.*, 2002) and ciliary epithelium of the adult human and rat eye; and in bovine retinal ECs (Tombran-Tink, *et al.* 2004).

PEDF transcript is expressed in most regions of the nervous system of adult human brain and spinal cord (Tombran-Tink, *et al.,* 1995; Bilak, *et al.* 1999; Tombran-Tink, *et al.* 1996; Tombran-Tink and Barnstable, 2003a). Ependymal cells are likely to be a major source of PEDF in the human CSF, suggesting that, like the retina, much of the brain is bathed in this neurotrophic factor (Bilak, *et al.* 1999; Tombran-Tink and Barnstable, 2003a). It is also expressed in a number of non-neural tissues including skeletal muscle, bone, heart, placenta and liver (Tombran-Tink, *et al.* 1996; Tombran-Tink and Barnstable, 2003a).

The PEDF gene was independently identified as one that is post-transcriptionally and very markedly (>100-fold) up-regulated (Coljee, *et al.* 2000) during G_0

phase in young but not senescent cultured fibroblasts (Pignolo, *et al.* 2003; Tombran-Tink, *et al.* 1995), giving rise to an alternative name of "early population doubling level cDNA", suggesting additional roles in both the cell cycle and senescence.

1.4.2 Biology of PEDF

1.4.2.1 Structure

The human PEDF gene contains 8 exons and a 5' flanking region that is unusual because it contains a dense cluster of *alu* repeats (Tombran-Tink, *et al.* 1996). The PEDF promoter region contains several key regulatory elements including a retinoic acid receptor motif, which may be important in the regulation and cell differentiation function of the gene. The PEDF gene was mapped to human chromosome 17p13 (Tombran-Tink, *et al.* 1996; Tombran-Tink, *et al.* 1994; Goliath, *et al.* 1996), a hot spot for many retinal degenerative diseases that are characterized by loss of photoreceptor function and subsequent visual impairment, e.g. retinitis pigmentosa (RP13), leber's congenital amaurosis and a type of cone-rod dystrophy (CORD5). The PEDF gene encodes a 418 amino acid protein (predicated molecular weight of 46.3 kDa) with a hydrophobic signal characteristic of secreted proteins. PEDF contains a single carbohydrate side chain that raises its apparent molecular weight to a 50kDa single band seen on a silver-stained SDS gel (Dawson, *et al.* 1999) or doublet bands (Tombran-Tink, *et al.*, 1991).

The crystal structure of PEDF was determined using purified recombinant human PEDF expressed in hamster cells (Simonovic, *et al.*, 2001). PEDF has structure and sequence homology to members of the serpin family of proteinase inhibitors and contains a characteristic serpin reactive loop (RCL) (Steele, *et al.*, 1993). PEDF, however, does not exhibit inhibitory activity against any known proteinases, possibly due to differences in the sequence of the RCL (Simonovic, *et al.*, 2001; Steele, *et al.*, 1993). The RCL in PEDF is unusual because it is exposed in the native state so that the central region is largely uncovered, a feature that may allow rapid binding to regulatory proteins. PEDF has a unique

asymmetric charge distribution, that is, the basic residues are concentrated on one surface, and the acidic residues are concentrated on the opposite surface (Simonovic, *et al.* 2001). The PEDF molecule contains two sites for interactions with ECM molecules. By utilising residue-specific chemical modification and site-directed mutagenesis techniques, Yasui, *et al.* (2003) revealed the acidic amino acid residues on PEDF (Asp^{255}, Asp^{257}, and Asp^{299}) are critical for collagen binding whilst the three clustered basic amino acid residues (Arg^{145}, Lys^{146}, and Arg^{148}) are necessary for heparin binding. A 44-amino PEDF peptide, corresponding to position 78-121, was shown to have neuroprotective activity on motor neurons, whereas an adjacent 34 amino acid peptide, corresponding to positions 44-77, did not (Bilak, *et al.*, 2002).

1.4.2.2 Biological functions

1.4.2.2.1 A natural angiogenesis regulator

Dawson, *et al.* (1999) showed that PEDF inhibited angiogenesis process *in vitro* and was more effective than the well-studied angiogenesis inhibitor angiostatin. Biochemically purified recombinant forms of PEDF potently inhibited neovascularization in the rat cornea. In an *in vitro* assay, PEDF inhibited endothelial cell migration in a dose-dependent manner with a median effective dose (ED_{50}) of 0.4 nM, slightly more active than pure angiostatin, thrombospondin-1 (ED_{50} of 0.5 nM), and endostatin (ED_{50} of 3 nM), placing it among the most potent natural inhibitors of angiogenesis. At doses of 1.0 nM or greater, PEDF also inhibited basic fibroblast growth factor (bFGF) induced proliferation of capillary endothelial cells by 40% (Dawson, *et al.* 1999). PEDF inhibited endothelial cell migration toward the angiogenic inducers, such as, PDGF, VEGF, interleukin-8, acidic FGF (aFGF), and lysophosphatidic acid – indicating that PEDF exerts its inhibitory function even in the presence of proangiogenic factors (Dawson, *et al.* 1999).

A recent study has shown that PEDF can inhibit the migration of microvascular cells, and it acts on endothelial cells forming new vessels to induce their apoptosis without harming the pre-existing vasculature (Stellmach, *et al.* 2001).

To an extension, Volpert, *et al.* (2002) demonstrated that PEDF derives this specificity for activated endothelial cells from their dependence on Fas/Fas ligand (FasL) mediated apoptosis. Proliferating endothelial cells are the only targets identified so far in which PEDF promotes cell death. This might be due to a unique type of receptor on endothelial cells or to the regulation of different signal transduction pathways by PEDF (Tombran-Tink and Barnstable, 2003b).

1.4.2.2.2 Neurotrophic and Neuroprotective properties

The first function of PEDF that was identified is its differentiating effect on retinoblastoma cells in the conditioned-medium of foetel human RPE cells (Tombran-Tink and Johnson, 1989; Tombran-Tink, *et al.* 1991). PEDF promotes the transformation of dividing retinoblastoma cells into differentiated neurons. The expression of PEDF in the human retina is seen as early as 7.4 weeks of gestation, a stage when retinal neurons are differentiating (Karakousis, *et al.* 2001).

PEDF is an effective neuroprotective factor in many parts of the nervous system. In the eye, for example, PEDF 1) reduces apoptosis induced by H_2O_2 or light damage in rat photoreceptors (Cao, *et al.* 2001). 2) preserves the spatial organisation, morphology and function of photoreceptors after RPE detachment in a *Xenopus* model of retinal degeneration (Jablonski, *et al.* 2001), and protects retinal neurons from injuries caused by increased intra-ocular pressure from transient ischemic reperfusion (Ogata, *et al.* 2001). Increased pressure within the eye is a major risk factor for glaucoma and its associated ganglion cell death. Experimental pressure-induced ischemia and subsequent reperfusion leads to extensive retinal cell death in the absence of PEDF (Tombran-Tink and Barnstable, 2003a).

Glutamate-mediated excitotoxicity is thought to be an important factor in many acute neurodegenerative conditions such as stroke and epilepsy. In cultured neurons, apoptotic cell death can be induced by micromolar concentrations of glutamate (Otori, *et al.* 1998). PEDF at low nanomolar concentrations

significantly prevents glutamate-induced apoptotic cell death in cultures of cerebella granule cells (Taniwaki, *et al.* 1995; Araki, *et al.* 1998), hippocampal neurons (DeCoster, *et al.* 1999; Houenou, *et al.*1999) and spinal motor neurons (Bilak, *et al.* 1999; Houenou, *et al.*1999). Whether PEDF alters the sensitivity of glutamate receptors, the intracellular movements of calcium or the down-stream response of mitochondria to glutamate is still not known.

1.4.2.2.3 PEDF in non-neuronal cells

Increasing evidence shows that PEDF might also have important effects on non-neuronal cells. PEDF prevents alterations in the morphology of Müller cell (the main glial cell type present in the retina) by attenuating the spatial disorganisation of the inner nuclear layer. PEDF might also mitigate the progression of photoreceptor degeneration by improving biosynthetic activity in Muller cells such as by restoring physiological levels of glutamine synthetase (Jablonski, *et al.* 2001). In cultures of rat brain microglia, PEDF caused changes in morphology and an increase in metabolic activity (Sugita, *et al.* 1997). PEDF also has strong autocrine effects in cells such as the RPE. In neonatal rat cultures, PEDF stimulated RPE cells increased in cytoplasmic area, pigment granule content, and in the development of cell-cell contacts (Malchiodi-Albedi, *et al.* 1998). All of these changes are indicative of normal RPE maturation. Tombran-Tink and Barnstable (2003a) provided evidences suggest that PEDF might not only act on neighbouring cells, but might also be important for maintaining key functional processes in the cells that secrete it.

1.4.2.2.4 In ageing and disease

Recently it has been shown that there is a decrease in PEDF expression with ageing. The levels of PEDF protein decline when primary cells in culture reach senescence (DiPaolo, *et al.* 1995), mirror findings in RPE cells, where PEDF synthesis and secretion declined with time in culture (Tombran-Tink, *et al.* 1995; Hjelmeland, *et al.* 1999). This decline can be reset in cloned animals (Lanza, *et al.* 2000). The exact mechanism that regulates PEDF levels is unclear, but some

evidence supports a post-transcriptional regulation of mRNA stability (Coljee, *et al.* 2000).

In some diseases, PEDF levels increase, such as in the CSF of patients with Amyotrophic Lateral Sclerosis and in rodent retinas after laser-induced retinal injuries (Ogata, *et al.* 2001). These increases might constitute an attempt of the tissue to limit neuronal damage. In contrast, in macular degeneration or diabetic retinopathy, PEDF expression decreased. PEDF also exerts a powerful action on tumours. PEDF promotes differentiation and antiproliferation activity in human retinoblastoma cells (Tombran-Tink and Johnson, 1989; Tombran-Tink, *et al.* 1991). In addition, PEDF treated tumours had less neovascularisation, due to the antiangiogenic action of this factor. Ek *et al.* (2006) reported the multitargeted role of PEDF in the inhibition of growth, angiogenesis and metastasis of two orthotopic models of osteosarcoma (rat UMR 106-01 and human SaOS-2). Through stable plasmid-mediated gene transfer of full-length human PEDF, they show that PEDF overexpression significantly reduced tumour cell proliferation and Matrigel invasion and increased adhesion to collagen type-1 *in vitro*. In vivo, PEDF overexpression dramatically suppressed orthotopic osteosarcoma growth and the development of spontaneous pulmonary metastases. Furthermore, tumours overexpressing PEDF exhibited reduced intratumoral angiogenesis, evidenced by a significant decrease in microvessel density.

On the other hand, other factors such as VEGF that are secreted by the tumour cells promote tumour expansion. A finely tuned balance of mitogenic stimulators and inhibitors might therefore control the progression or regression of such tumours (Tombran-Tink and Barnstable, 2003a & b).

1.4.3 Therapeutic potential of PEDF

From the studies that have been reviewed above, it is clear that PEDF could be used as a therapy to limit ocular angiogenesis that endangers vision. Support comes from the observations in that: 1) PEDF is effective against multiple

inducers of angiogenesis (Dawson, *et al.*, 1999), including the VEGF and IL-8 that are thought to play important roles in inducing ocular angiogenesis (Yoshida, *et al.*, 1998), and it is very effective against ischemia-induced retinal angiogenesis (Dawson, *et al.*, 1999). 2) Changes in ocular concentration of PEDF appear likely to occur and to be important in facilitating human eye diseases (Bouck, 2002). 3) In using PEDF, it is just simply replacing the protein that is naturally present in healthy tissue but has been lost because of disease (Bouck, 2002). A further advantage of these molecules is that they are tolerated in the body and are unlikely to produce the toxic side effects of synthetic inhibitors (Tombran-Tink and Barnstable, 2003b). 4) PEDF has strong neuroprotective effects on neurons from a wide range of brain areas and eye. The protection is effective against many insults including ischemia, excitotoxicity, axotomy and oxidising agents (Tombran-Tink and Barnstable, 2003b). This becomes a desirable side-effect of PEDF.

1.4.4 Mechanism(s) of PEDF action

How does PEDF exert its action? To date, a receptor for PEDF has not yet been identified*, but a putative 80-85 kDa PEDF-binding protein has been identified by two studies using affinity chromatography (Alberdi, *et al.*1999; Aymerich, *et al.* 2001). In one study, radiolabelled [^{125}I] PEDF binds to a single class of binding sites on retinoblastoma and cerebellar granule cells with K_d values of 1.7-3.6 nM and 3.2 nM, respectively, indicating that the activity of PEDF might be mediated by its interaction with a single receptor type (Alberdi, *et al.*1999). In the second study, a single class of binding site was detected with a K_d of 2.5 – 6.5 nM on retinal membranes (Aymerich, *et al.* 2001). Whether this protein represents a receptor for PEDF or is a regulatory protein that is important to its functions, remains unknown.

Three possible signalling pathways have been related to PEDF by examination of the molecules that PEDF activates. First, in cerebellar granule cells, PEDF

* A lipase-linked cell membrane receptor for PEDF has been identified by Notari *et al.* (2006).

stimulates phosphorylation of IκB [inhibitor of nuclear factor κB (NF-κB)], leading to activation and translocation of NF-κB to the nucleus. This results in a sequential chain of events that link the NF-κB pathway to a defined extracellular signal and the transcription of anti-apoptotic and neuroprotective genes (Yabe, *et al.* 2001). Second, under certain growth conditions, PEDF activates the mitogen-activated protein kinase (MAPK) pathway by regulating the phosphorylation of extracellular-signal-regulated kinase (ERK) 1 and 2 in endothelial cells (Hutchings, *et al.* 2002). Third, in the study by Volpert, *et al.* (2002), PEDF generates antiangiogenic signals by activating the Fas-Fas ligand (FasL) death cascade in endothelial cells. However, in mice that are deficient in Fas or FasL, PEDF still inhibits ocular angiogenesis, and hence it must have additional inhibitory actions in endothelial cells, independent of the Fas-FasL cascade (Barreiro, *et al.* 2003). Figure 1.8 summarises the reported (Yabe *et al.*, 2001; Hutchings *et al.*, 2002; Volpert *et al.*, 2002) signalling pathways that PEDF may be involved.

1.5. Retinal pigment epithelium (RPE)

The RPE cells is thought to play a central role in the development of choroidal neovascularisation in AMD, which is the most common cause of severe visual loss in patients over age 60 years in the US alone (Friedman *et al.*, 2004; Brown *et al.*, 2005).

1.5.1 General features of the RPE

The RPE is a monolayer of pigmented cells that lies between the retinal photoreceptors and the choroid, and forming a part of the barrier between the choroidal circulation and retina (Bok, 1993; Marmorstein, 2001; Rizzolo, 1997). The RPE gets its name from the *melanin pigment* that is present within cytoplasmic granules called *melanosomes.* The RPE is derived embryologically from the same neural tube tissue that forms the neurosensory retina, but is differentiated into a secretory epithelium (Marmor, 1998).

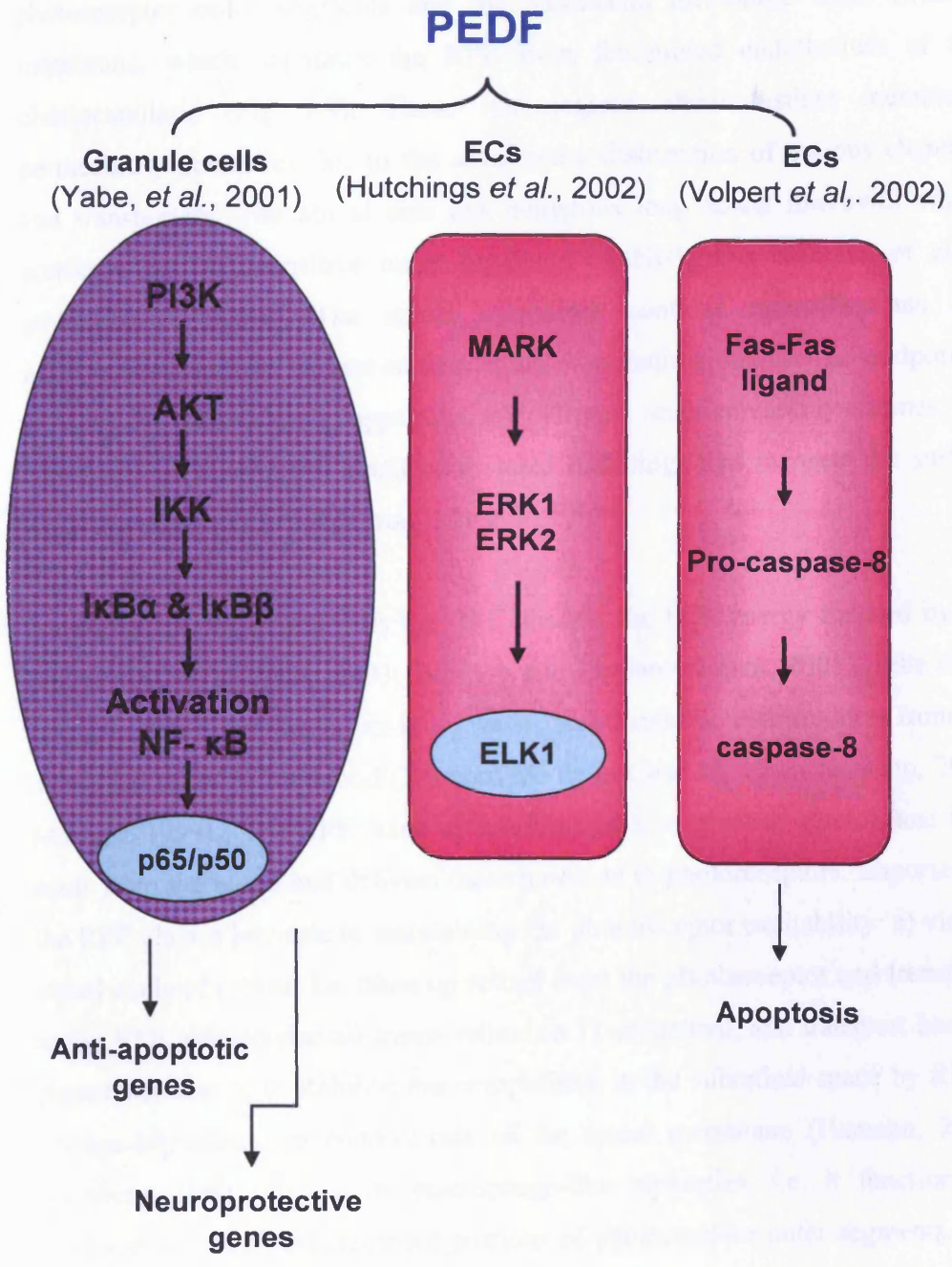


Fig 1.8 A schematic representation of the reported signaling pathways that PEDF involved.

RPE cells are small (about 10-14 μm in diameter) in the macular region, and become flatter and broader with diameter up to 60 μm toward the periphery. The RPE has two distinct membrane regions: the apical membrane that faces the photoreceptor outer segments and the basolateral membrane faces Bruch's membrane, which separates the RPE from fenestrated endothelium of the choriocapillaris (Fig 1.9). These two regions show distinct membrane permeability properties due to the asymmetric distribution of various channels and transporters. The apical side has numerous long apical microvilli which surround the light-sensitive outer segments establishing a complex of close structural interaction. The apical cytoplasm contains microfilaments and microtubules, and the highest concentration of melanin granules. The midportion contains the nucleus, golgi apparatus, endoplasmic reticulum and lysosomes, etc. The basal membrane has small convoluted infoldings that increase the surface area for absorption and secretion (Marmor, 1998).

As a layer of pigmented cells the RPE absorbs the light energy focused by the lens on the retina (Bok, 1993; Boulton and Dayhaw-Barker, 2001). Like other epithelia, RPE also transports ions, water, and metabolic end products from the subretinal space to the blood (Dornonville de la Cour M, 1993; Hamann, 2002; Marmor, 1999). The RPE takes up nutrients such as glucose, retinol, and fatty acids from the blood and delivers these nutrients to photoreceptors. Importantly, the RPE plays a key role in maintaining the photoreceptor excitability: a) via the visual cycle of retinal, i.e. takes up retinal from the photoreceptor and transports to the RPE, reisomerise all-*trans*- retinal to 11-*cis*-retinal, and transport back to photoreceptors; b) to stabilise ion composition in the subretinal space by RPE's voltage-dependent ion conductance of the apical membrane (Hamann, 2002; Steinberg, 1985); c) via its macrophage-like properties, i.e. it functions to phagocytise and digest discarded portions of photoreceptor outer segments, and essential substances such as retinal are recycled and returned to photoreceptors to rebuild light-sensitive outer segments from the base of the photoreceptors (Bok, 1993; Finnemann, 2003). In addition, the RPE is able to secrete a variety of

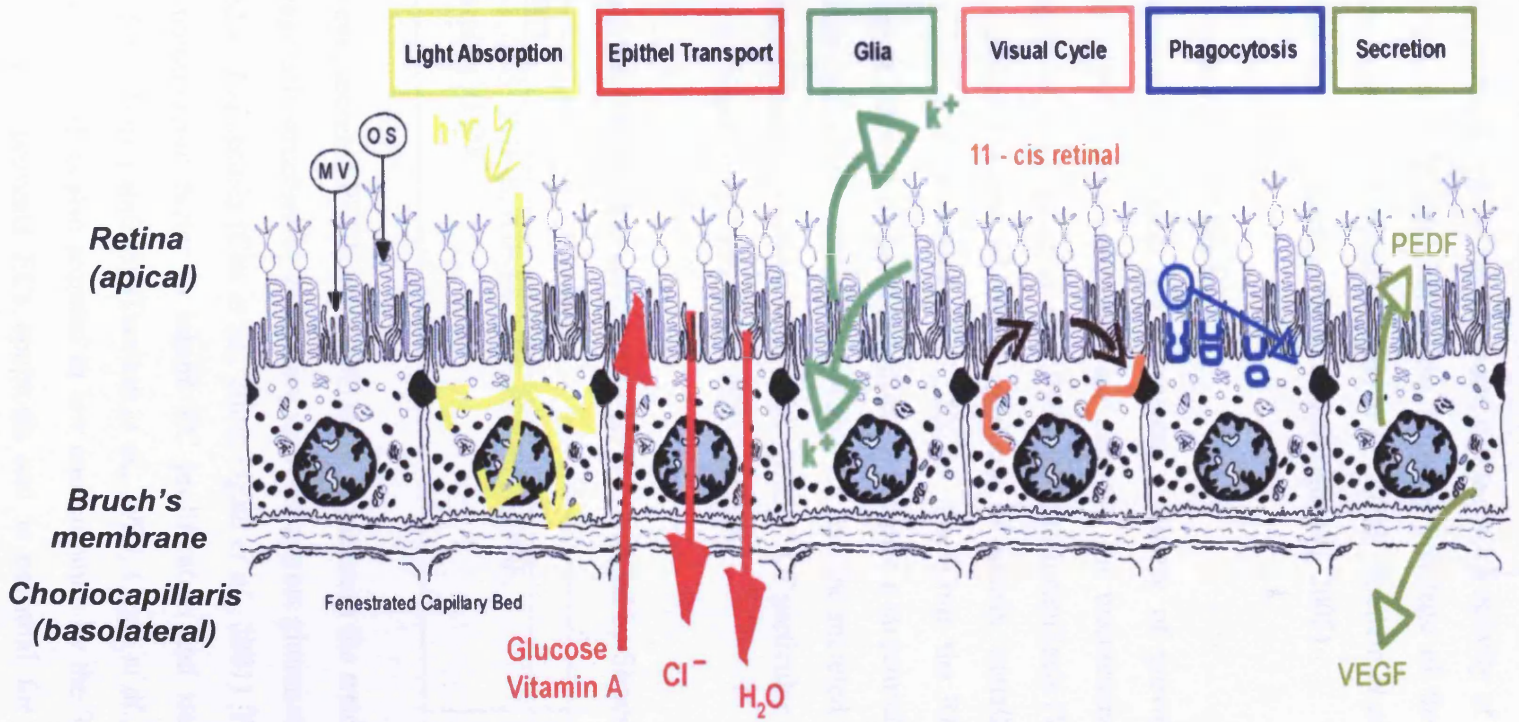


Fig. 1.9 Summary of retinal pigment epithelium (RPE) functions (adapted from Strauss, 2005; doi:10.1152/physrev.00021.2004)

growth factors helping to maintain the structural integrity of choriocapillaris endothelium and photoreceptors. Furthermore, the secretory activity of the RPE plays an important role in establishing the immune privilege of the eye by secreting immunosuppressive factors (Ishida *et al.*, 2003; Streilein *et al.*, 2002). Fig 1.9 summaries the RPE function (adapted from Strauss, 2005).

1.5.2 Secretary function of the RPE

The RPE is known to produce and to secrete a variety of growth factors (Tanihara *et al.*, 1997) and factors that are essential for maintenance of the structural integrity of retina (Cao *et al.*, 1999) and choriocapillaris (Witmer *et al.*2003), e.g., different types of tissue inhibitor of matrix metalloprotease (TIMP) (Alexander *et al.*, 1990; Qi *et al.*, 2002). Therefore, the RPE makes factors that support survival of photoreceptors and ensure a structural basis for optimal circulation and supply of nutrients. Table 1.4 lists the secreted factors by the RPE reported to date (reviewed by Strauss, 2005). Of particular interested for this study is the secretion of VEGF and PEDF by RPE.

Table 1.4 Reported factors that secreted by RPE (reviewed by Strauss, 2005)

Growth factors	Others
VEGF; FGF-1,-2,-5; TGF-β, IGF-I,CNTF, PDGF; LEDGF, Interleukin family; PEDF	MMP-2 MMP-9 TIMPs

In the healthy eye, secreted PEDF from RPE helps to maintain the retinal as well as the choriocapillaris structure by protecting neurons against glutamate-induced or hypoxia–induced apoptosis (Cao *et al.*, 2001; Ogata *et al.*, 2001). PEDF also acts as an antiangiogenic factor to inhibit EC proliferation and stabilise the endothelium of the choriocapillaris (Dawson *et al.*, 1999; Ogata *et al.*, 2002a & b). Interestingly, VEGF is also secreted in low concentration by the RPE in the healthy eye where it prevents ECs apoptosis and is essential for an intact endothelium of the choriocapillaris (Burns and Hartz, 1992). VEGF also acts as a permeability factor stabilising the fenestrations of the endothelium (Roberts and Palade, 1995). In a healthy eye, PEDF and VEGF are secreted at opposite sides

of the RPE: PEDF to the apical side where it acts on neurons and photoreceptors, whereas the majority of VEGF is secreted to the basal side where it acts on the choroidal endothelium (Becerra *et al.*, 2004; Blaaugeers *et al.*, 1999). Growth factor secretion changes in response to damage or injury, which stimulates the RPE to also secrete neuroprotective factors including bFGF or CNTF (Blanco *et al.*, 2000). In the relation between VEGF and PEDF, Ohno-Matsui *et al.* (2003) reported that VEGF upregulates PEDF via VEGFR-1 in HRPE cells in an autocrine manner.

Taken together, the complex functions of the RPE indicate that the RPE is essential for visual function; also an interesting cell model for study the regulation of VEGFR-1/VEGFR-2 by VEGF /PEDF in paracrine/autocrine manners.

1.6. Hypothesis

To date, it is vital to understand the biochemical pathways by which PEDF exerts its actions and how they interact with the pathways that are activated by other factors. From the evidence reviewed above, the action of PEDF may occur through the following steps (Fig 1.10a and Fig 1.10b): PEDF inhibits VEGF induced angiogenesis by inhibiting the enzyme(s) activity of the first or second or both cleavages of VEGFR-1 or VEGFR-2 in the RIP signalling pathway. Consequently, the cleavage of VEGFR-1 (or VEGFR-2) is reduced and less of the cytosolic domain of VEGFR-1 (or VEGFR-2) is translocated into the nuclei, resulting in the inhibition of angiogenesis.

1.7. Project objectives

The overall aim of the project was to define the involvement of a new signalling pathway–PS-dependent RIP – in relation to VEGF/PEDF, VEGFR-1/VEGFR-2 in *in vitro* models of bovine retinal microvascular endothelial cells (BRMECs) and human RPE (HRPE). Specific objectives to achieve this aim include:

1) To investigate the expression profiles of the elements that possibly involved in the RIP pathway in cultured BRMECs and HRPE cells;

2) To define the interactions between the above members in RIP pathway in BRMECs and HRPE cells;
3) To further determine the biological functions of VEGF/PEDF in regulation of angiogenesis through inhibition of VEGF function.

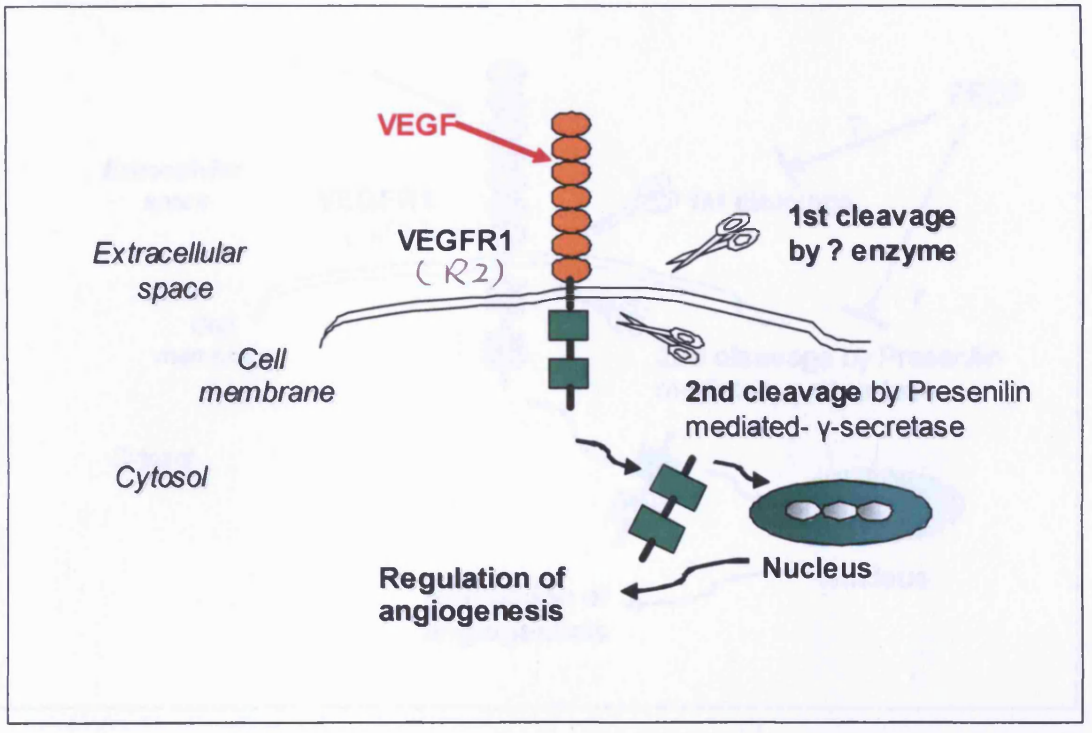


Fig 1.10a A schematic representation of the hypothesis – the effect of VEGF on VEGFR-1 / VEGFR-2. Upon the ligand binding (e.g. VEGF), an enzyme (possibly one of the MMPs) cleaves a portion of the extracellular domain of the VEGFR-1; this 1st cleavage initiates γ-secretase to cleave the cytosolic domain of the VEGFR-1, allowing this fragment to translocate to the nucleus of the endothelial cells to promote angiogenesis.

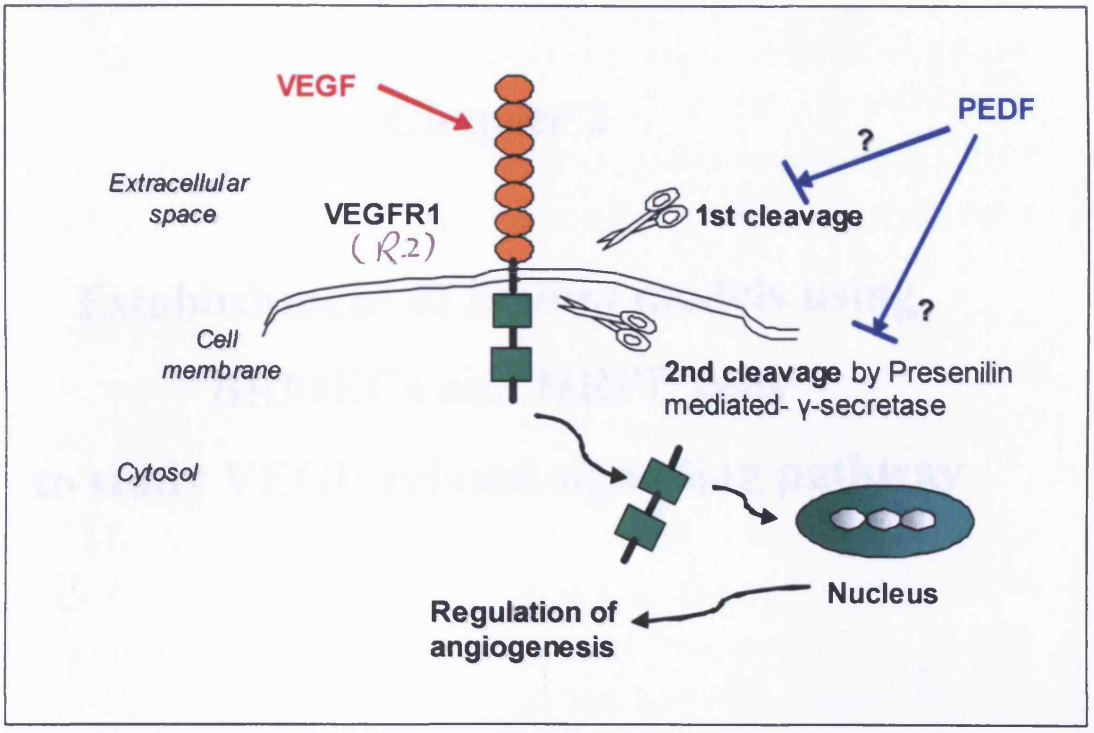


Fig 1.10b. A schematic representation of the hypothesis – the effect of VEGF/PEDF on VEGFR-1/VEGFR-2. PEDF inhibits VEGF induced angiogenesis by inhibiting the enzyme(s) activity of the first or second or both cleavages of VEGFR-1 in the RIP signalling pathway.

Chapter 2

Establishment of *in vitro* models using BRMECs and HRPE cells to study VEGF related signalling pathway

2.1. Introduction

The retina has frequently been used as a source of microvascular endothelial cells (MEC) (Meezan *et al.*, 1974; Frank *et al.*, 1979; Bowman *et al.*, 1982; Rymaszewski *et al.*, 1992), partly because of its extensive microvascular network and partly because of the well-documented involvement of the retinal microvasculature in diabetic retinopathy (reviewed in Afzal *et al.*, 2007). Cultured bovine retinal microvascular endothelial cells (BRMECs) has been used as an *in vitro* model for study of angiogenesis by several groups (Schor, AM and Schor, SL, 1986; Simorre-Pinatel *et al.*1994; Cai *et al.* 2003; Tombrain-Tink *et al.*, 2004). Due to bovine eyes are easy to obtain; eyes are one of the targeted organs from pathological neovascularisation; the MECs are more representative than human umbilical vein endothelial cells (HUVECs) in regard to the functional study in angiogenesis, this project made use of cultured BRMECs as an *in vitro* model. The limitation is due to the variations between the species, the results from BRMECs may not accurately represent human cells.

The retinal pigment epithelium (RPE) is a monolayer of highly specialized cells located between the neural retina and vascular choroids that influences the structure and function of cells in both (Marmor, 1998; Strauss, 2005). The RPE cells may modulate activity of the choriocapillaries through diffusible angiogenesis factors (Henkind and Gartner, 1983; Eichhorn *et al.*, 1996). One of the candidates, vascular endothelial growth factor (VEGF), is secreted by RPE cells in physiological and pathological conditions (Adamis *et al.*, 1993; Kvanta *et al.*, 1996). However, some studies question the importance of VEGF alone in promoting choroidal neovascularisation (Okamoto *et al.*, 1997). More interestingly, pigment epithelial-derived factor (PEDF), a potent antiangiogenic factor, is also secreted by RPE cells. Recent *in vitro* studies have demonstrated that a dynamic balance exits between VEGF and PEDF in physiological conditions that is critical in regulating the behaviour of choroidal endothelial cells (ECs) (Ohno-Matsui *et al.*, 2001). A unique relationship between VEGF and PEDF in HRPE cells has been reported in that VEGF upregulates PEDF via VEGF receptor-1

(VEGFR-1) in an autocrine manner (Ohno-Matsui *et al.*, 2003). Apart from VEGFR-1, HRPE cells also express VEGFR-2. Therefore, cultured HRPE cells would be an ideal *in vitro* model for exploring the relationship further among VEGF, PEDF, VEGFR-1 and VEGFR-2. HRPE can also act as a non-angiogenic cell type to compare with the angiogenic cell type of ECs. It hopes that the differences between endothelial cells and RPE in response to the exogenous VEGF/PEDF would provide new insight about the mechanism of VEGF and PEDF action.

The aim of this study was to set up *in vitro models* using BRMECs, as an angiogenic cell type, and HRPE cells, as a non-angiogenic cell type to compare the interplay among PEDF, VEGF, VEGFR-1, VEGFR-2 and presenilin-1.

The objectives of this study were:

- To establish *in vitro* cultures by isolation of BRMECs from bovine eyes, and isolate HRPE cells from human eyes;
- To characterise the isolated cells by monitoring the purity, cellular markers and cell viability;
- To determine the expression of VEGFR-1 and VEGFR-2.

2.2 Experimental design

This study was designed to be carried out by 1) Isolation of BRMECs from bovine eyes; and isolation of HRPE cells from human donor eyes. 2) Culture both cells using the protocols available in the group. 3) Cell purity monitoring by characterisation of the cells (morphology and specific cellular markers). 4) Cell viability monitoring. 5) Confirmation of expression of the targets of interest (VEGFR-1 and VEGFR-2). A flow chart for the experimental design is shown in Fig 2.1.

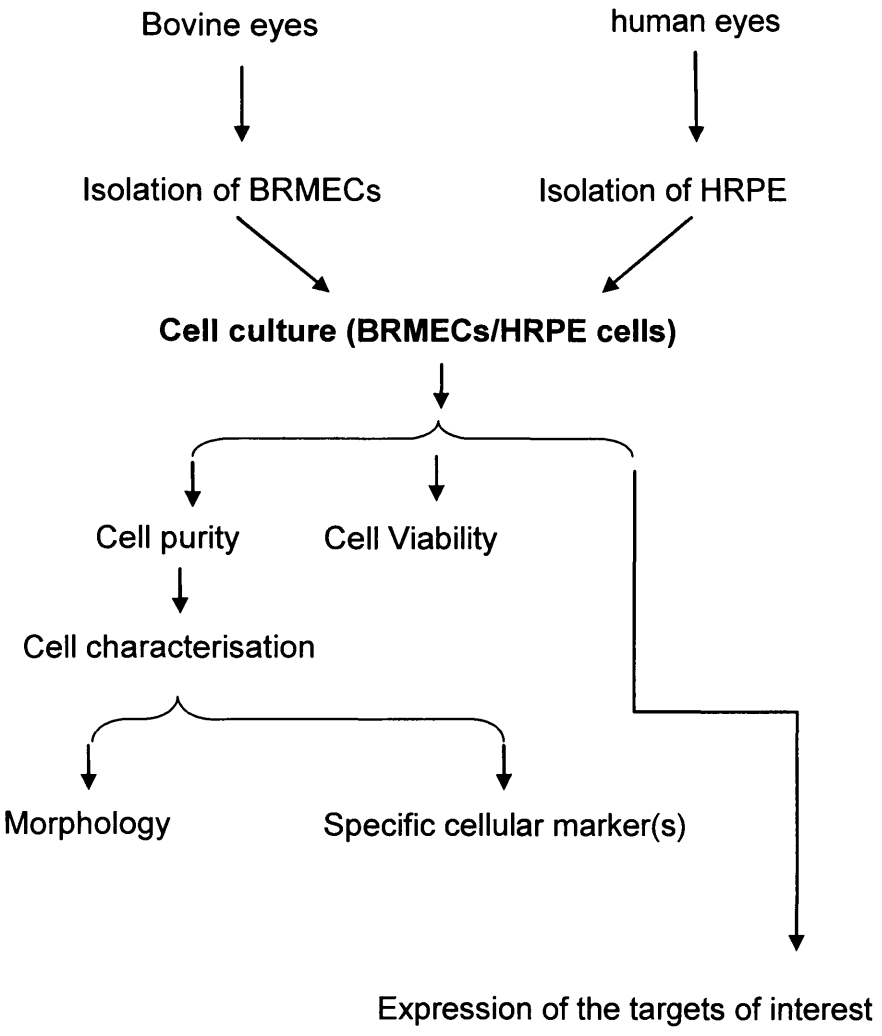


Fig 2.1 Flow chart of experimental design for the chapter 2.

2.3 Materials and methods

2.3.1 Cell culture

All the cell culture procedures were carried out in a Class II laminar flow cabinet.

2.3.1.1 Isolation of bovine retinal microvascular endothelial cells (BRMECs)

BRMECs were isolated from fresh bovine eyes by homogenisation and a series of filtration steps, as described previously (Cai *et al.*, 2003). In brief, bovine eyes were enucleated within 12 hours post-mortem and transported from the local abattoir to the laboratory and processed for ECs within 24 hours. The surrounding tissue of the eyes was cleaned off and the eyeballs were immersed in 5% beta iodine for at least 5 minutes and subsequently washed with 1 x phosphate-buffered saline (PBS). The eyeball was cut open 5 mm posterior to the limbus, the contents removed and the retina separated from the RPE layer and placed in Eagle's Minimum Essential medium (MEM) supplemented with 30 mM hepes buffer at pH 7.4 (EARLE/HEPES) (GIBCO, UK). A pool of bovine retinas was isolated from 10 or 20 bovine eyes for each batch. The retinas were washed with 1×PBS (autoclaved) 3 times then transferred to fresh MEM for homogenisation using a rotary Teflon-glass homogeniser. The resultant microvessels were trapped on an 85 μm nylon mesh and digested by an enzyme cocktail containing 0.5 mg/ml of collagenase (Sigma, USA), 0.2 mg/ml of protease (Sigma, USA) and 0.2 mg/ml deoxyribonuclease (Sigma, USA) in MEM in a standard incubator for 20 - 30 minutes. The ECs from digested microvessels were trapped on a 53 μm mesh, and washed with cold MEM. The ECs were pelleted by centrifugation at 250 g for 10 minutes and the pellets were resuspended using human Microvascular Endothelial Cell Basal Medium with Growth Supplement (see growth medium for BRMECs in Appendix 1) into 2 (from 10 eyes) or 4 (from 20 eyes) T_{25}–flasks (25 cm^2) (Orange Scientific) precoated with the Attachment Factor (ZHS-8949, TCS CellWorks, UK). Cells were incubated at 37°C, with a humidified atmosphere containing 5% CO_2 and 95% air and fed every 2 to 3 days. It was observed that the cell number started increasing within 4 days of initial seeding and reached confluence around 10~14

days. Subculture was carried out at a split ratio of 1:2 or 1:3 when confluence reached 75-80% of the flask surface. The cells were used within 3 passages.

2.3.1.2 Isolation of human retinal pigment epithelial (HRPE) cells

Human donor eyes were obtained from the Bristol Eye bank with specific consent for research use. HRPE cells were isolated as described previously (Boulton *et al.* 1983). In brief, eyes were used within 48 hours post-mortem and cut open at 4mm posterior to the limbus. The vitreous and neural-retina were gently removed. The posterior eyecup was placed on the inverted lid of a universal (sterilised), and the RPE layer was fixed on the edge of the cup if it had detached to reduce the contamination by fibroblast cells. Both the eyecup and the lid were placed into a 60ml sterile pot. 0.25% (w/v) trypsin with 0.05% (w/v) EDTA (see Appendix 1 for more detail) was added to just below the edge of the RPE layer and incubated at 37°C, 5% CO_2 and 95% air for 60 minutes. The digestion was stopped by adding foetal calf serum (FCS). A pellet was collected by centrifugation at 250g for 5 min. The pellet was resuspended in Ham's F-10 medium containing 10% (v/v) FCS (growth medium for HRPE cells, see Appendix 1), and then seeded into one T_{25}-flask per eye. The flasks were kept in an incubator at 37°C with a humidified atmosphere containing 5% CO_2 and 95% air. The cells were fed with Ham's F-10 medium containing 10% FCS every 2 days. It was observed that the cell number started increasing around 7 days after initial plating and reached confluence around 18 days.

2.3.1.3 Subculture and maintenance

Subculture was carried out at a split ratio of 1:2 / or 1:3 when confluence reached 75-80% of the flask surface. In brief, the medium was removed and the cells rinsed in PBS. The addition of trypsin [0.25% (w/v)] · EDTA [0.05% (w/v)] solution was applied to completely cover the monolayer of cells. The flasks were placed in a 37°C incubator for approximately 2 minutes to allow the cells to detach. Proteolysis was stopped by adding the same volume of the medium that designated for the growth of BRMECs or HRPE cells as soon as the cells detached. The detach progress was checked by examination with an inverted

microscope. The cells were pelleted at 250g for 5 minutes and resuspended in the growth medium that specific for BRMECs or HRPE cells, before seeding into new T_{25}-flask for HRPE cells, or the T_{25}-flask that precoated with Attachment Factor for BRMECs at a ratio of 1:2 /or 1:3. Cells were incubated at 37°C, with a humidified atmosphere containing 5% CO_2 and 95% air and fed every 2 to 3 days.

2.3.1.4 Preparation of cell stocks

In order to keep a continuous supply of HRPE cells, some of the HRPE cells were prepared as stocks and stored in liquid nitrogen. HRPE cells were trypsinised from culture vessels at 75 - 85% confluence as described in section 2.3.1.3 and resuspended in FCS containing 10% (v/v) dimethylsulphoxide (DMSO) at a density of 1×10^6 - 1×10^7 cells/ml. Cells were transferred to a cryopreservation vial, frozen at -20°C in a container immersed in isopropanol for 24 hours then transferred to -80°C for another 24 hours. The vials were then transferred to a designated liquid nitrogen tank for long term storage.

2.3.1.5 Recovery of cells from cell stocks

Stock of HRPE cells were recovered from liquid nitrogen storage by rapidly thaw the cryopreservation vial(s) at 37°C in a water bath then seeded into Ham's F-10 medium containing10% FCS. Incubating at 37°C, with a humidified atmosphere containing 5% CO_2 and 95% air for overnight. On the next day, the medium was removed and replaced with fresh Ham's F-10 containing 10% FCS. Then cells were fed every 2-3 days.

2.3.2 Cell viability and purity

Cultures were examined daily using an inverted light microscope to monitor the morphological changes. Photomicrographs were taken at primary and subculture passages using the SPOT Advanced software (Version 3.2.4 for Windows) attached to an OLYMPUS, 1X70 inverted light microscope.

In order to obtain BRMECs of good quality and purity, the morphology and Factor VIII expression of the cultured BRMECs were monitored throughout the whole process of culture. Cytokeratin peptide 18 was monitored as cellular marker for cultured HRPE cells.

Trypan Blue staining was used to monitor cell viability during the subculture. This method is based on the principle that a live (viable) cells do not take up certain dyes, whereas the dead (non-viable) cells do. Cells were trypsinised as described above and resuspended in 1×PBS (0.5 ml). Trypan Blue solution [0.4% (v/v), (0.5 ml)] was added to the cell suspension and mixed thoroughly. With the cover-slip in place, a small amount of Trypan Blue-cell suspension mixture was transferred to both chambers of a haemocytometer. Cells were counted in the 1mm centre square and four 1 mm corner squares. Cell viability was calculated using the equation:

$$cell\ viability = \frac{total\ number\ of\ non-stained\ cells}{total\ number\ of\ non-stained\ cells + total\ number\ of\ stained\ cells} \times 100\%$$

2.3.3 Immunofluorescence staining

2.3.3.1 Procedures for use with polyclonal antibodies

Subconfluent BRMECs/HRPE cells were subcultured on cover slips at a density of 5 x 10^4 cells/well overnight in a 24-well plate. The cells were fixed at room temperature using 4% (w/v) formaldehyde for 10 minutes, and then washed with 1×PBS for 20 minutes with 3 changes. Cells were permeabilised using 0.1% (v/v) Triton X-100 for 5 to10 minutes at room temperature and washed with 1×PBS three times. To suppress non-specific binding of immunoglobulin G (IgG), cells were incubated with 5% blocking serum (derived from the same species in which the secondary antibody is raised, in this case donkey serum was used) for 30 minutes. The blocking buffer was removed and cells were incubated with primary antibody (1:100 dilution, i.e. 0.5–5.0 μg/ml in 1×PBS with 1.5% blocking serum) for 2 hours. Substitution of the primary antibodies with same species serum at the same concentration acted as a negative control. Cells were washed three times with 1×PBS for 5 minutes each, and specific binding was

detected with donkey antibody to rabbit IgG conjugated with Alex Fluor 488 for 60 minutes (1:600 dilution in 1×PBS). For nuclei staining, 3μl/ml of 1% (w/v) Hoechst (No.33342, Sigma-ALDRICH) was added and mixed with the buffer containing the secondary antibody. Cells were washed with three changes of PBS before mounting in Hydromount^{TM} (National diagnostics). Slides were stored in a dark location at 4° C until examination using a fluorescence microscopy (Leica 4000) with FITC/DAPI filters under UV light. Results were recorded using Leica Qfluoro software. Table 2.1 listed the primary antibodies used for the characterisation of BRECs and HRPE cells.

Table 2.1 The list of the primary antibodies used in the immunofluorescence staining

Name	Source	Against a peptide at	Dilution used
VEGFR-1 (Flt-1) (C-17)	Rabbit polyclonal (sc-316)	Carboxy terminus of human origin	1:100
VEGFR-1 (Flt-1) (H-225)	Rabbit polyclonal (sc-9029)	Amino acids 23-247 within the extracellular domain of human origin	1:100
VEGFR-2 (Flk-1) (C-1158)	Rabbit polyclonal (sc-504)	Amino acids 1158-1345 at the carboxy terminus of mouse	1:100
VWF (H-300)	Rabbit polyclonal (sc-14014)	Amino acids 23-247 at the carboxy terminus of human origin	1:100

2.3.3.2 Procedures for use with monoclonal antibody

FITC Monoclonal Anti-Cytokeratin peptide 18 is a purified mouse monoclonal antibody conjugated with fluorescein isothiocyanate (FITC) isomer 1 (Sigma, USA).

Cells were fixed and permeabilised as described in 2.3.3.1. Then cells were incubated with FITC Monoclonal anti-cytokeratin peptide 18 in 1:100 dilution in 1 × PBS for two hours at room temperature (in dark). Substitution of the primary antibody with the serum of same species at the same concentration acted as a negative control. For nuclei staining, 3μl/ml of 1% (w/v) Hoechst was added with the buffer containing the antibody. Cells were washed with 1 × PBS for 15

minutes with three changes, and then mounted with Hydromount™ (National diagnostics). Slides were stored in a dark location at 4° C and examined using a fluorescence microscope with FITC/DAPI filters under UV light. Results were recorded using Leica Qfluoro software.

2.4 Results

2.4.1 Characterisation of cultured BRMECs

The purity of cultured BRMECs was determined by morphology and expression of Von Willebrand Factor (VWF), one of the endothelial phenotype markers. It was observed that cell clusters started appearing approximately 2 to 4 days after the isolation; by day 5-6 an increased cell number and enlarged clusters were observed; by day 8-10 the cells had formed a confluent monolayer (Fig. 2.2 A-C). A typical cobblestone shape of BRMEC morphology was constantly observed in this study [Fig. 2.2(A-C)] which is consistent with the description by Bachetti and Morbidelli (2000). Positive VWF staining (green, Fig 2.3 A) confirming endothelial phenotype and demonstrating that cultures consist of 100% endothelial phenotype by co-immunostaining of the nuclei (blue, Fig 2.3 B); Negative controls confirmed that there was no non-specific staining (Fig.2.3 C&D). Only cells that met the criteria of ECs in morphology and cellular marker were used for this study.

2.4.2 Characterisation of cultured HRPE cells

The character and purity of the cultured HRPE cells were assessed by morphology and immunolocalisation of one of the epithelial markers. A typical epithelioid morphology which is representative of cultured HRPE cells (Fig.2.4) was observed. Cells were observed to progressively depigment and change morphology during the primary culture (Fig.2.4 A-D). The morphology had little change in the subcultures from passage 1 to passage 6 (Figs 2.4 E&F). Fig.2.5 shows phenotype immunostaining of the epithelial marker, cytokeratin peptide 18, in the cultured HRPE cells. Co-immunostaining of the nuclei (Fig 2.5 B) demonstrated that cultures consist of 100% epithelial phenotype. Only cells that met the criteria of epithelial morphology and characters were used for the study.

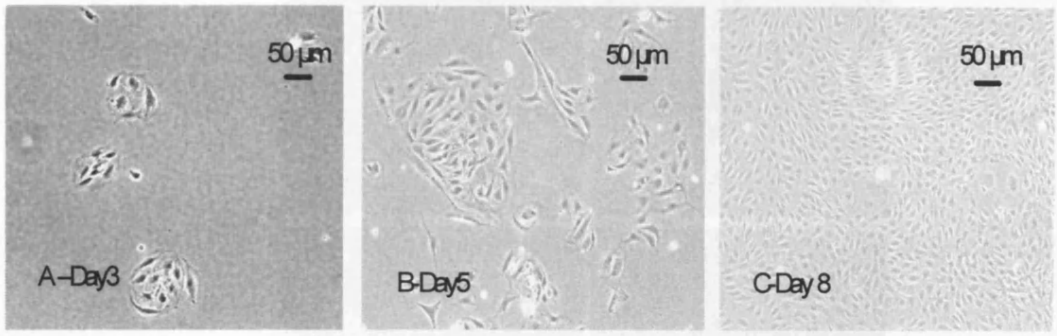


Fig 2.2 Characterisation of the morphology of cultured BRMECs. (A) Approximately 2-4 days after the isolation, cell clusters were observed by Olympus 1×70 inverted microscope. (B) By day 5-6 an increased cell number and enlarged clusters were observed. (C) By day 8-10, the cells had formed a confluent monolayer.

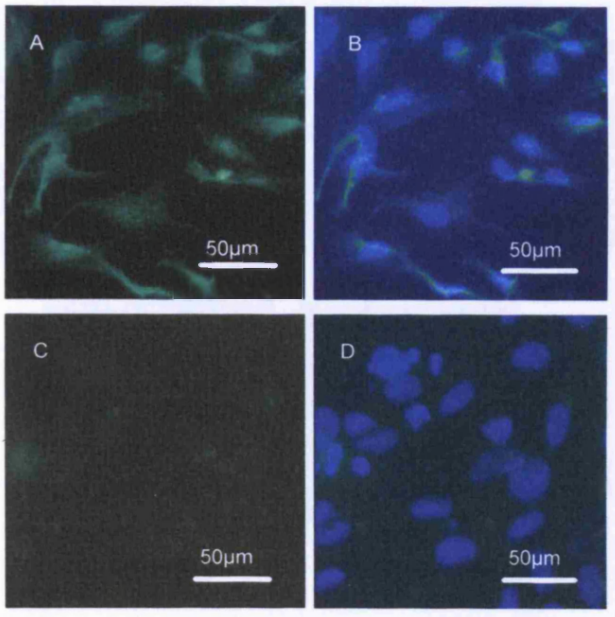


Fig 2.3. Immunolocalisation of VWF in cultured BRMECs. (A) VWF staining in BRMECs (green) (B) Merge of VWF staining with Hoechst nuclei staining (blue). (C) Negative control for VWF. (D) Negative control for VWF combined with Hoechst nuclei staining (blue).

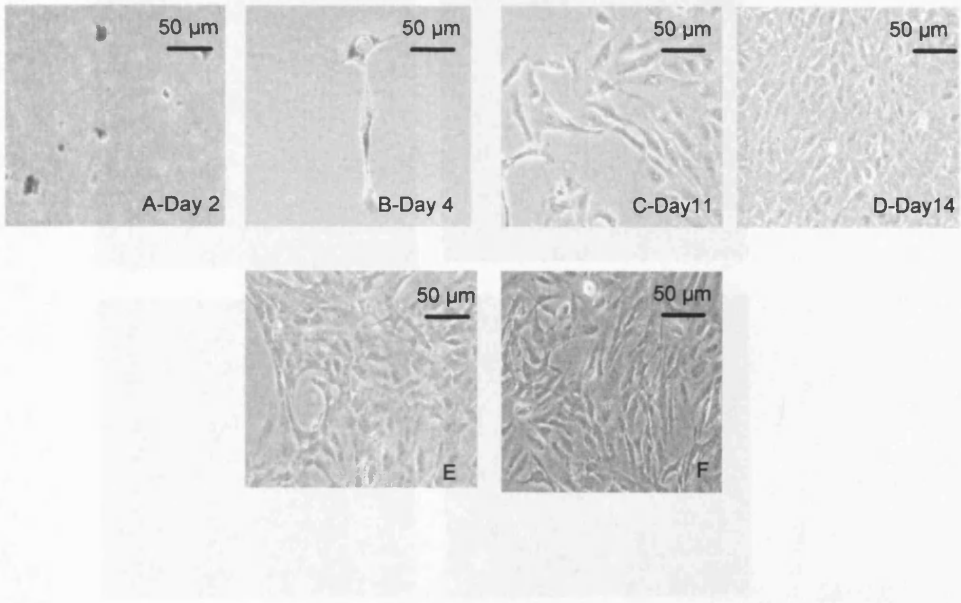


Fig.2.4. Characterization of the morphology of cultured HRPE cells. (A-D) Primary cultures of HRPE cells with a progressive depigmentation and changes in morphology. (E-F) The representatives of passage 2-4 cultures demonstrating little change in the morphology.

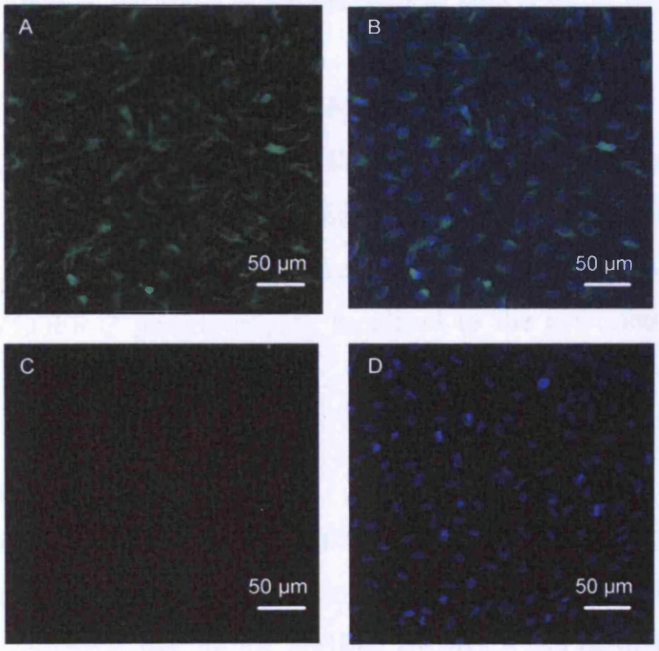


Fig 2.5 Immunolocalisation of Cytokeretin 18 in cultured HRPE cells. (A) Cytokeretin 18 staining in HRPE cells. (B) Merge of Cytokeratin 18 (green) with Hoechst nuclei staining (blue). (C) Negative control. (D) Merge of negative control with Hoechst nuclei staining (blue).

2.4.3 Expression of VEGFR-1 in BRMECs and HRPE cells

Immunostaining for the cytosolic domain and extracellular domain of VEGFR-1 confirmed that VEGFR-1 was expressed in cultured BRMECs (Fig. 2.6). The cytosolic domain of VEGFR-1 localised in both the nuclei and cytoplasm of the BRMECs (Fig 2.6A) while the extracellular domain localised on the membrane (Fig.2.6B). In HRPE cells the immunostaining shows VEGFR-1 cytosolic domain predominantly localised to the perinuclear area (Fig.2.7 A-B).

2.4.4 Expression of VEGFR-2 in BRMECs and HRPE cell

Immunolocalisation showed that VEGFR-2 was expressed in cultured BRMECs (Fig 2.8) and HRPE cells (Fig 2.9). In BRMECs, VEGFR-2 expression was predominantly localised to the perinuclear area (green) (Fig 2.8 A and B). In HRPE cells, VEGFR-2 predominantly localised to the cell membrane and the perinuclear area (Fig 2.9 A and B).

2.5 Discussion

It is essential to obtain highly purified primary cells from animal or human tissue in order to carry out the down-stream functional study using *in vitro* models. The present study confirmed that highly purified BRMECs can be obtained using the combination of homogenisation, digestion by cocktail enzymes and series filtration as previously described (Cai *et al.* 2003). By enzyme digestion, highly purified HRPE also can be obtained as previously reported (Boulton *et al.* 1983).

It has been well documented that VEGFR-1 and VEGFR-2 are co-expressed in a wide range of cells, particularly vascular ECs (Yang *et al.*, 2004). Recently, Ohno-Matsui *et al.* (2003) reported that VEGFR-1 and VEGFR-2 are co-expressed in HRPE cells. However, it was unclear how the VEGFR-1 or VEGFR-2 was distributed in HRPE cells. The result from this study showed that VEGFR-1 is expressed throughout the cell of the cultured BRMECs, which is in agreement with the report by Cai *et al.* (2006); and for the first time this study provides evidence that VEGFR-1 only expressed in the cytoplasm and membrane of cultured HRPE cells in the untreated condition. This study has also confirmed

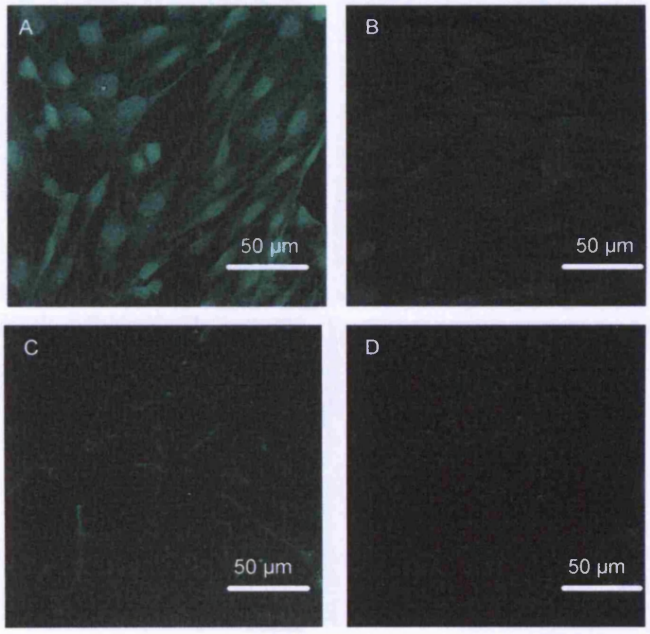


Fig 2.6 Immunolocalisation of VEGFR-1 in cultured BRMECs. (A) The cytosolic domain of VEGFR1 was localised predominantly in the nuclei of BRECs with reduced intensity in cytoplasm, whereas very faint extracellular domain staining was observed in BRMECs by a fluorescence microscopy (B); C) and D) Negative control for the cytosolic domain and extracellular domain of VEGFR-1, respectively.

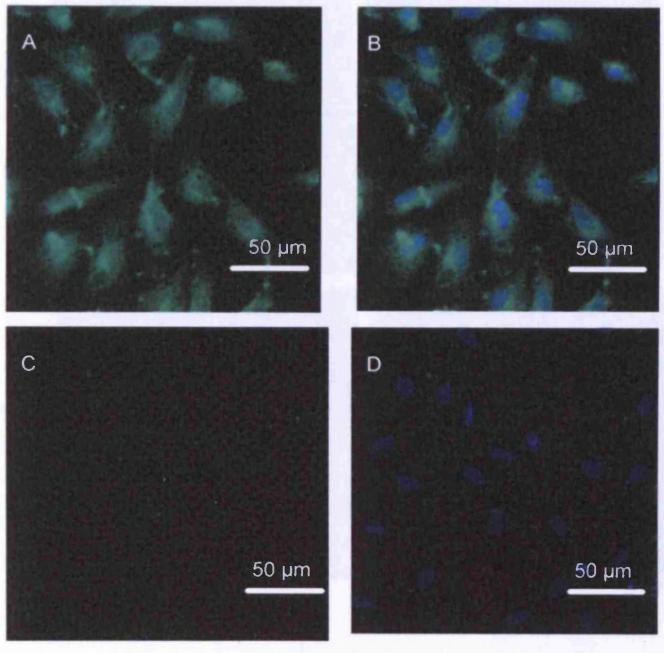


Fig 2.7 Immunolocalisation of the cytosolic domain of VEGFR-1 in cultured HRPE cells. (A) The cytosolic domain of VEGFR-1 was localised mainly to the cytoplasm and membrane of HRPE cells (green). (B) Merge of the cytosolic domain of VEGFR-1 with Hoechst nuclei staining (blue, B). (C) Negative control for the cytosolic domain of VEGFR-1. (D) Merge of the negative staining with Hoechst nuclei staining (blue).

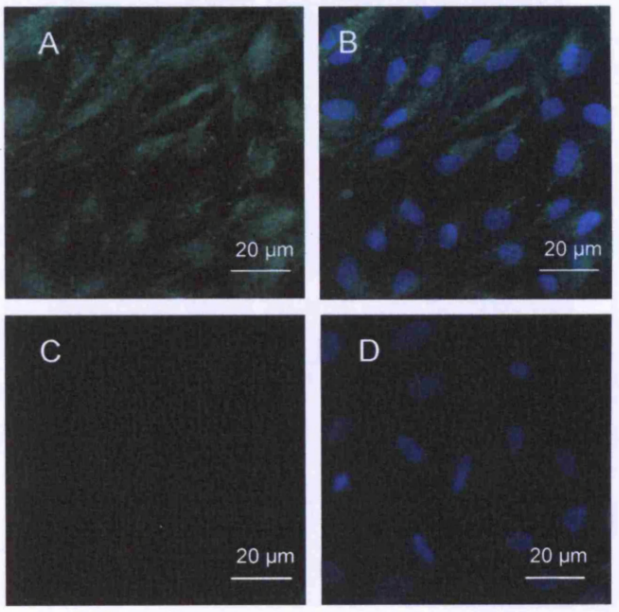


Fig 2.8 Immunolocalisation of VEGFR-2 in cultured BRMECs. (A) VEGFR-2 was predominantly localised to the nuclei and perinuclei area (green). (B) Merge of VEGFR-2 staining with Hoechst nuclei staining. (C) Negative control show no positive staining. (D) Merge of negative control with Hoechst nuclei staining.

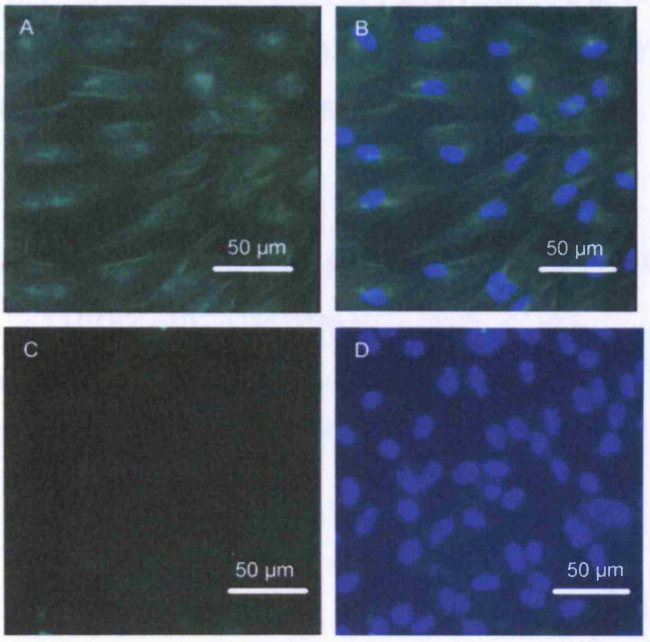


Fig 2.9 Immunolocalisation of VEGFR-2 in cultured HRPE cells. (A) VEGFR-2 expression was predominantly in the prinuclear area and membrane (green). (B) Merge of VEGFR-2 with Hoechst nuclei staining. (C) Negative control shows no positive staining, and combined with Hoechst nuclei staining (D).

the expression of VEGFR-2 in BRMECs and HRPE cells. In BRMECs, VEGFR-2 predominantly localised in the perinuclei, while in HRPE cells VEGFR-2 predominantly expressed on the membrane and perinuclei of the cells. The importance of these differential distributions is yet to be determined.

The confirmation of co-expression of VEGFR-1 and VEGFR-2 in both BRMECs and HRPE cells under normal culture conditions suggests that they may play a role in the orchestration of retinal homeostasis.

2.6 Conclusions

- Highly purified BRMECs and HRPE cells have been isolated. Cultured BRMECs and HRPE cells have been well characterised.
- The interested target molecules, VEGFR-1 and VEGFR-2 are all expressed in the BRMECs and HRPE cells.

Chapter 3

The effects of VEGF and PEDF on the expression of VEGFR-1 and VEGFR-2 in BRMECs and HRPE cells

3.1 Introduction

Terminal differentiation occurs through mechanisms that promote growth arrest and specialisation in cells (Tombran-Tink *et al.* 2004). These two processes are tightly and co-ordinately linked to the control of cell proliferation. Their disruption results in a variety of pathological conditions including cancer and neovascular diseases, which are both characterised by increased cell proliferation. A number of factors regulate these proliferative processes independently or through interdependent regulation of each other.

PEDF was first described as an ocular neurotrophic protein that induced differentiation in human Y79 retinoblastoma cells (Tombran-Tink and Johnson, 1989; Tombran-Tink *et al.,* 1991). It functions as a potent neurotrophic factor that protects neurons in many regions of the central nervous system (CNS) against a wide range of neurodegenerative insults. PEDF is also a natural inhibitor of angiogenesis, targeting the growth of only new vessels (Dawson *et al.*, 1999). Inhibition of endothelial cell growth, blockade of endothelial cell migration, and prevention of new blood vessel are well documented biological features of PEDF in numerous models of retinal neovascular diseases (Mori *et al.*, 2001, 2002; Raisler *et al.*, 2002; Duh *et al.*, 2002). The properties of PEDF make it a strong candidate as a pharmacological tool for slowing the progression of a range of neurodegenerative diseases and those pathologies associated with abnormal vessel growth in the eye and metastatic cancers of various tissues.

Understanding the mechanism of PEDF action is crucial for its clinical applications. There is increasing evidence showing that, at least in the eye, the balance between the pro-angiogenic factor, VEGF, and the anti-angiogenic factor, PEDF, appears to determine the formation of new vessels (Ohno-Matsui *et al.* 2001). An inverse relationship is noted between these two factors in the vitreous of patients with PDR and AMD, suggesting an underlying cooperative relationship between these proteins in maintaining vascular quiescence. The counterbalance of VEGF and PEDF is supported by the previous demonstrations that either inhibition of the VEGF system or over expression of PEDF inhibits choroidal neovascularisation (Krzystolik *et al.*, 2002; Mori *et al.*, 2002).

To further understand the direct and unique relationship between PEDF and VEGF may provide new insight in understanding the mechanism(s) of PEDF action.

To date, three signalling pathways for PEDF action have been proposed and studied intensively: the NF-kB pathway by Yabe *et al.* (2001); the MAPK pathway in ECs by Hutching *et al.* (2002) and the Fas-FasL death cascade in ECs by Volpert *et al.* (2002).

More recently, Cai *et al.* (2006) reported that PEDF inhibits VEGF induced angiogenesis in vitro, and this closely correlates to the cleavage of VEGFR-1 via γ-secretase, suggesting a possible involvement of the **regulated intramembrane proteolysis (RIP) pathway**. It is hypothesised that VEGF, γ-secretase and VEGFR-1 may be the key elements that are involved in this pathway in which PEDF acts as a regulator (see Fig 1.10a and Fig 1.10b for the illustrated hypothesis). Interestingly, three of the above four elements, VEGF, VEGFR-1 and PEDF have been linked together by Ohno-Matsui *et al.* (2003) in HRPE cells, with evidence that VEGF upregulats PEDF in HRPE cells via VEGFR-1 in an autocrine manner.

Based on the studies by Ohno-Matsui *et al.* (2003), Cai *et al.* (2006) and Ni *et al.* (2001), the aim of the project was to investigate the mechanism(s) in which PEDF regulates angiogenesis. This was approached by exploring the involvement of the RIP signalling pathway in relation to VEGF/PEDF and VEGFR-1/VEGFR-2 in *in vitro* models. As the first step, the goal of the current study was to determine whether PEDF functions as an antagonist of VEGF in regulating the expression of VEGFR-1 or VEGFR-2 in BRMECs and HRPE cells.

3.2 Experimental design

To meet the goal of this study, the experiments were carried out from the following aspects: 1) Western blotting for the effect of VEGF/PEDF on the expression of VEGFR-1 and VEGFR-2 in BRMECs and HRPE; 2) Evaluation of the specificity of the antibodies used; 3) Verification of the linearity of the ECL Western blotting; and 4) Immunofluorescence staining for the effect of VEGF/PEDF on the distribution of VEGFR-1 and VEGFR-2 in BRMECs and HRPE. A flow chart for the experimental design is shown in the Fig.3.1.

3.3 Materials and methods

3.3.1 Cell culture

BRMECs and HRPE cells were cultured as described in section 2.3.1. Cells were used within passage 3.

3.3.2 Preparation of whole cell lysates

Cell lysates were obtained essentially as described previously (Cai *et al.*, 2003) with some modifications. Briefly, the culture medium was removed and the cells were washed with ice cold 1 x PBS containing 1x protease inhibitor and phosphatase inhibitor cocktails (Santa Cruz, UK). Ice cold Radioimmunoprecipitation (RIPA) buffer (500 µl) containing 1x protease inhibitor and phosphatase inhibitor cocktails was added to the cell monolayer in a T_{25} flask and gently rocked for 15 minutes at 4°C. The lysed cells were scraped and transferred into a microcentrifuge tube. Further cell disruption was achieved by passing the lysate through a 21-gauge needle to shear the DNA and incubating the lysate on ice for 30 min. The supernatants were obtained by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant representing the total cell lysate was transferred to a new microcentrifuge tube. The protein concentration was estimated using BCA assay (see section 3.3.3) before being subjected to Western blotting.

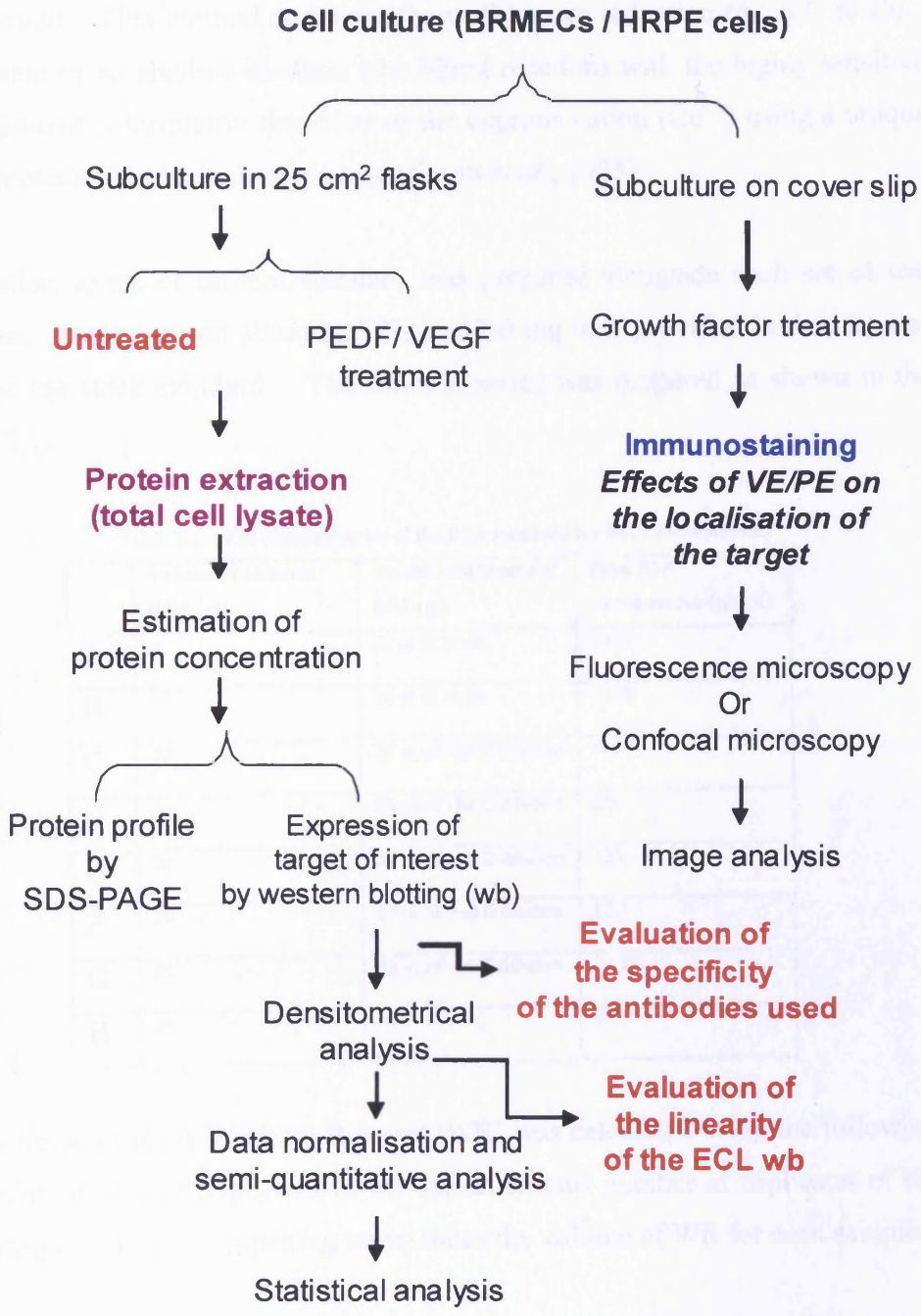


Fig 3.1 Flow chart of experimental design for chapter 3.

3.3.3 Estimation of protein concentration – BCA Protein Assay

The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid (Smith *et al.*, 1985).

A titration series of protein standard was prepared alongside each set of test samples. Bovine serum albumin (BSA) at 2.0 mg /ml (provided in the kit) was used as the stock standard. The dilution series was prepared as shown in the Table 3.1.

Table 3.1. The dilution series of the BSA standard for BCA Protein Assay

	Volume of Diluents (PBS, µl)	Volume and Source of BSA (µl)	Final BSA Concentration (µg/ml)
A	0	20 µl of stock	2,000
B	20	20 µl of stock	1,000
C	20	20 µl of vial B dilution	500
D	20	20 µl of vial C dilution	250
E	20	20 µl of vial D dilution	125
F	20	20 µl of vial E dilution	62.5
G	20	20 µl of vial F dilution	31.25
H	20	0	0

The volume of BCA Working Reagent (WR) was calculated using the following formula: number of duplicates of the standards plus number of triplicates of the test sample plus three (pipetting error) times the volume of WR for each sample.

Total volume of WR = [(# standards) ×2+ (# samples) ×3 + 3] × volume of WR/sample

The WR was prepared by mixing 50 volumes of BCA Reagent A with 1 volume of BCA Reagent B (50:1, Reagent A: B). 10 µl of each standard in duplicate or

test samples in triplicate were added into the wells of a 96-well microplate (Orange Scientific). 200 µl of the WR (sample: WR =1:20) was added to each well and incubated at 37°C for 30 minutes. The plate was cooled to room temperature before measuring the absorbance at 550nm and 570nm on a microplate reader (Multiskan *Ascent* ®, Labsystems, Finland).

The standard curve was prepared by plotting the average measurement of each dilution of BSA standard into a regression of linear fit (Microsoft Excel). Concentration of the total protein in each sample was determined by reference to the standard curve (Microsoft Excel).

3.3.4 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.4.1 Preparation of SDS-PAGE

SDS-PAGE is a dissociating system that is designed to denature proteins into their constituent polypeptides and hence examines the polypeptide composition of samples (Shi and Jackowski, 1998). The anionic detergent SDS denatures the proteins and covers them with an overall negative charge. The sample is then fractionated by electrophoresis through a polyacrylamide gel. The proteins are separated on the basis of their mass, since all of them have an identical charge to mass ratio. The smallest proteins move farthest. SDS-PAGE can be used to estimate the molecular mass of a protein and the number of polypeptide subunits in a protein as well as the degree of sample purity (Hames and Hooper, 2000).

A 10% (w/v) resolving gel with a 5% (w/v) stack gel (Table 3.2) was used to separate the peptides in the samples prepared for the target(s) of interested. Samples were mixed with an equal volume of 2×electrophoresis sample buffer (sc-24945, Bio Rad Laboratories Ltd., Herts, UK) and incubated at 95° C for 5 minutes. Equal amount of protein at a volume of 15~20 µl /well was loaded and the gel was run at 30 mA in 1× laemmli buffer (Bio-Rad Laboratories Ltd., Herts, UK) until the dye approached the bottom of the gel. The gel was analysed by Coomassie staining or silver staining for the protein profile depending on the

protein concentration loaded; or further for western blotting. To ascertain the molecular weights of protein species detected with Coomassie blue staining or silver staining, 5 µl of a prestained molecular weight standard (Precision Plus Protein Standards, range 7.2-250 kDa; BioRad Laboratories, Herts, UK) was applied to the gel.

Table 3.2. Solutions for casting a 10% resolving and 5% stack gel for SDS-PAGE (Cai *et al.*, 2006).

	Resolving Gel (10%) (10ml)	Stacking Gel(5%) (3 ml)
Distilled H_2O	4.0 ml	2.1 ml
30% acrylamide mix	3.3 ml	500 µl
1.5 M Tris/HCl pH 8.8	2.5 ml	-
1.0 M Tris/HCl pH 6.8	-	380 µl
10% SDS	100 µl	30 µl
10% APS	100 µl	30 µl
TEMED	4.0 µl	3.0 µl

3.3.4.2 Visualisation of protein profile by Coomassie brilliant blue staining

Coomassie brilliant blue staining is the most commonly used protein stain to visualise proteins following electrophoresis. After electrophoresis, the gels were immersed in an acidic alcoholic solution of the dye - Coomassie Staining buffer (methanol and acetic acid at a ration of 4.5:1, and Coomassie brilliant Blue R250 2.5 mg/ml). This denatures the proteins, fixes them in the gel and allows the dye to bind to them. Subsequently the gel was distained in 2:1 methanol and acetic acid solution for visualisation of the bands.

3.3.5 Western blotting

Western blotting can be used for detection of one or more antigens in a mixture (Hames and Hooper, 2000). ECL (Enhanced Chemiluminescence) Western blotting from Amersham Biosciences is a light emitting non-radioactive method for detection of immobilised specific antigens, directly or indirectly with Horseradish Peroxidase (HRP) labelled antibodies (Instructions of ECL Western

blotting detection reagents and analysis system, Amersham Biosciences). A schematic representation of the principle of ECL Western Blotting is shown in Fig 3.2.

The procedures of Western blotting can be divided into four major steps: 1) The separation of proteins by SDS-PAGE; 2) the transfer of separated proteins onto a transfer membrane. 3) Immunological probing to detect specific protein antigens of interest. 4) Visualisation of the detection of the specific protein antigen(s) using ECL™.

3.3.5.1 Separation of proteins by SDS-PAGE

Samples were loaded with equal amount of protein and run on a 10% (w/v) SDS-PAGE (5% stacking gel) at 30 mA in duplicate until the samples approached the bottom of the gel, as described in section 3.3.4.1.

3.3.5.2 Transfer of separated proteins onto a transfer membrane

Proteins were transferred from the gel to a nitrocellulose membrane in 1×transfer buffer (Bio-Rad, see Appendix 2) at 005 V for 30 minutes on a semi-dry transfer apparatus (Biometra, Germany). The order of gel and membrane sandwich in the blotting apparatus was as follows: cathode (the top lid) → one layer of saturated filter paper → gel → nitrocellulose membrane → two layers of saturated filter paper → anode (bottom of the apparatus). To check the efficiency of the transfer, membranes were stained with Ponceau S staining buffer (see Appendix 2). Destaining was achieved using dH_2O.

3.3.5.3 Blocking non-specific binding

To minimise non-specific binding, blocking buffer (see the Appendix 2) was applied for 30 minutes at room temperature before incubation with the primary antibody. Alternatively, the membrane may be blocked at 4° C overnight in a container, using the above buffer without Tween-20.

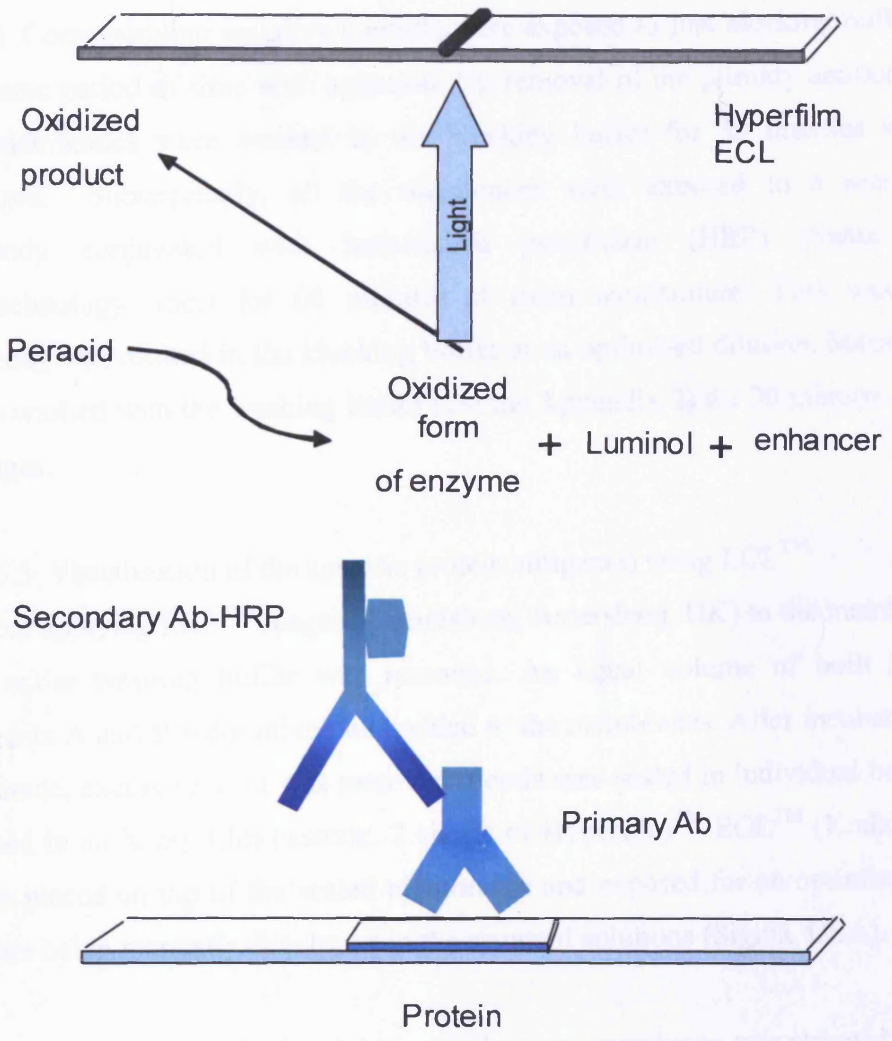


Fig 3.2 Principles of ECL Western blotting. Ab: antibody. HRP: Horseradish Peroxidase. (Reproduced from Instructions of ECL Western blotting detection reagents and analysis system, Amersham Biosciences).

3.3.5.4 Immunological probing to detect specific protein antigens of interest

The blocked membrane was incubated with primary antibody in blocking buffer for 2 hours on a roller at room temperature (an optimised dilution 1:125 was used). Corresponding negative controls were exposed to just blocking buffer for the same period of time with agitation. On removal of the primary antibody, all the membranes were washed in the blocking buffer for 30 minutes with 3 changes. Subsequently, all the membranes were exposed to a secondary antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Inc.) for 60 minutes at room temperature. This secondary antibody was diluted in the blocking buffer at an optimised dilution. Membranes were washed with the washing buffer (see the Appendix 2) for 30 minutes with 3 changes.

3.3.5.5 Visualisation of the specific protein antigen(s) using ECL™

Before applying ECL™ reagent (Amersham, Amersham, UK) to the membranes, the entire washing buffer was removed. An equal volume of both ECL™ reagents A and B were mixed and added to the membranes. After incubation for 1 minute, excess reagent was removed membranes sealed in individual bags and placed in an X ray film cassette. 2 sheets of Hyperfilm™ ECL™ (Kodak, UK) were placed on top of the sealed membranes and exposed for an optimised time before being manually developed in the standard solutions (Sigma, USA).

In order to normalise the band detected, the same membrane was stripped and re-probed with an antibody specific to one of the house-keeping proteins, β-actin (1:200 dilution) (Autogen of Santa Cruze, UK) or GAPDH (1ug/ml) (Ambion, UK). Bands of VEGFR-1 and VEGFR-2 were semi-quantified by densitometry using the Scanimage software and normalised with α-actin or GAPDH level.

3.3.6 Evaluation of linearity of ECL Western blotting detection

It has been demonstrated (Johnstone and Thorpe, 1982) that Hyperfilm ECL exhibits a linear response to the light produced from enhanced chemiluminescence. This relationship can be used for the accurate quantification

of proteins of ECL Western blots, using densitometry (manual book, Amersham Biosciences). The sample containing the protein to be quantified plus a set of standards (known amounts of the same antigen) should be used to prepare a Western blot. However, a standard for the targets of interest, VEGFR-1, VEGFR-2 and Presenilin-1, was not available. This study used semi-quantification instead, i.e. percentage of the protein expression after treatment in comparison with control (set as 100%) and normalised with a house keeping protein, such as α-actin or GAPDH. It is still important that the concentration of the protein to be semi-quantified lies within the linear range, therefore, a dilution series of a sample with highest concentration of protein were prepared for Western blotting.

3.3.7 Evaluation of antibody specificity

To determine the specificity of antibody binding to antigen, VEGFR-1 or VEGFR-2 antibody was blocked by preincubation overnight at 4°C with 5-fold (by weight) excess (recommended by the manufacturer) of VEGFR-1 (Flt-1) or VEGFR-2 (Flk-1) peptide (Santa Cruz) in a small volume (500 μl) of PBS. Following blocking, the antibody/peptide mixture was diluted into appropriate blocking buffer. The immunoreactivity from the pre-incubated antibodies was compared with the immunoreactivity from antibodies incubated overnight in BSA at the same concentration and with membranes or cover-slips incubated with freshly diluted antibodies.

3.3.8 VEGF/PEDF treatment for Western blotting

As described by Cai *et al.* (2006), at passage 2 or 3, 80-90% confluent monolayers (7.5×10^6 cells/flask) in T_{25}-culture flasks were washed with 1×PBS. Then the cells were rendered quiescent in serum free medium for 45 min at 37°C, 5% CO_2. The addition of 100 ng/ml of VEGF, or PEDF in 1% FCS medium respectively in duplicates was followed; flasks without growth factor treatment but 1% FCS medium was included as control. After one hour, 100ng/ml of PEDF was added to one VEGF flask, and 100 ng/ml of VEGF added to one PEDF flask, and further incubated for 24 hours at 37°C, 5% CO_2. The medium was

collected and stored at -20°C. The cells were subjected to the protein extractions (section 3.3.2) and Western blotting (section 3.3.5).

3.3.9 VEGF/PEDF treatment for immunostaining

BRMECs/HRPE cells at 80-90% subconfluence on the cover slip were treated with serum-free medium for 45 minutes. Subsequently 100ng/ml of VEGF or PEDF was added to the wells of 24-well plate containing medium with 1% (v/v) serum respectively. Cells were incubated at 37°C, 5% CO_2 and 95% humidity for 1 hour before addition of 100ng/ml of PEDF (to one of the wells treated with VEGF) or VEGF (to one of the wells treated with PEDF) and further incubated for 24 hours prior to immunostaining. A control without any growth factor treatment was included.

3.3.10 Immunofluorescence staining

Immunofluorescence staining was carried out as previously described in section 2.2.1. Controls, included omission of primary antibodies, substitution of the primary antibodies with the serum from same species and non-treatment wells. Controls for the specificity of the antibodies were also prepared as described in section 3.3.7.

3.3.11 Statistical analysis

The results were given as the mean ± standard error (SEM) from 3 separate experiments. An unpaired Student's t-test was performed to analyse the data with SPSS. A *P* value less than 0.05 ($P<0.05$) considered statistically significant.

3.4 Results

3.4.1 Differential protein profile of whole cell lysates of BRMECs and HRPE cells

Equivalent amounts of protein from samples were separated on 10% SDS-PAGE and stained with Coomassie blue. The pattern of the protein profile in BRMECs was different from the pattern in HRPE cells (Fig 3.3). In BRMECs, several

bands were observed above 250 kDa while only two bands were seen in HRPE cells; a band at 250 kDa was seen in both BRMECs and HRPE cells; two bands between 250 kDa and 150 kDa were observed in BRMECs while no obvious band was observed in the same region in HRPE cells. At 75 kDa and under, the pattern of the bands were very similar in BRMECs and HRPE cells though the intensity of the band varies.

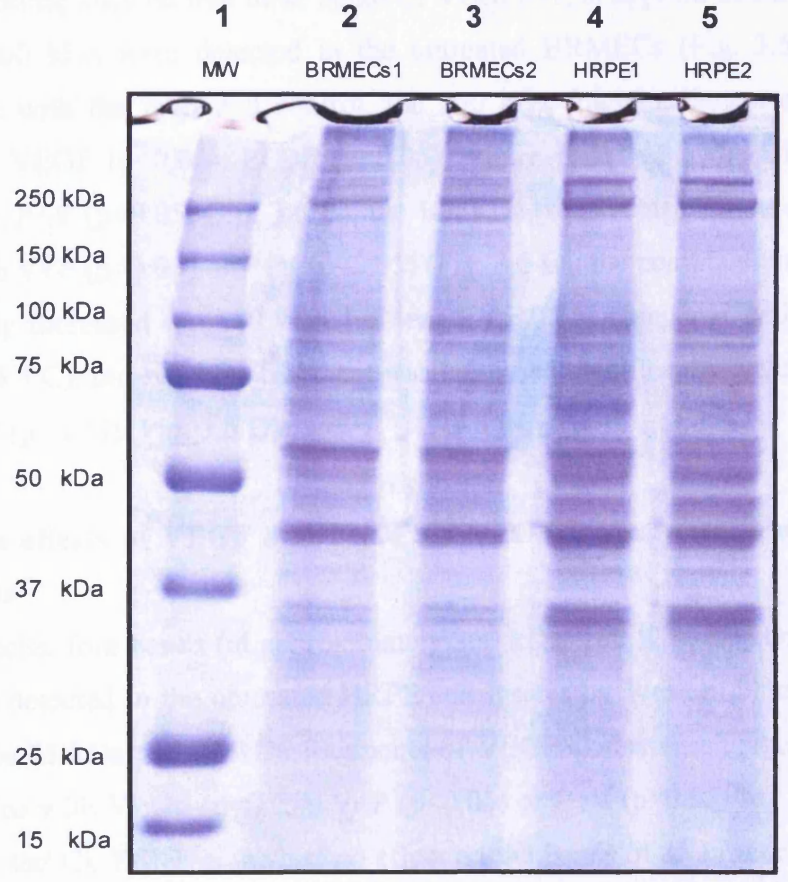


Fig 3.3 Protein profile in whole cell lysates from BRMECs and HRPE cells by Coomassie staining of 10% SDS-PAGE. Whole cell lysates were obtained using RIPA buffer. Lane 1: MW-the molecular weight marker; Lane 2 and 3: whole cell lysates from BRMECs; Lane 3 and 4: whole cell lysates from HRPE cells.

3.4.2 The linearity of ECL western blotting detection

By plotting the peak area [(Optical Density - OD) unit] against quantity of the protein, it was observed that ECL exhibits a linear response (Fig 3.4). Calculated linear regression equation $y = 0.0928X - 0.2418$ where the correlation coefficient $R^2 = 0.9585$.

3.4.3 The effects of VEGF and PEDF on VEGFR-1 expression in BRMECs

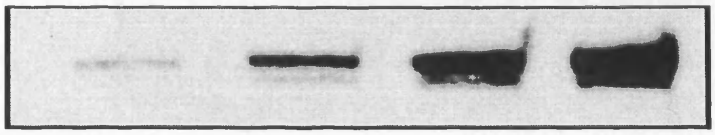
Western blotting showed that three bands of VEGFR-1, at approx. 250 kDa, 180 kDa and 100 kDa were detected in the untreated BRMECs (Fig. 3.5 A). In comparison with the untreated control, the 250 kDa fragment increased when exposed to VEGF ($p<0.05$), PEDF ($p<0.05$), VEGF followed by PEDF (V+P) ($p<0.0001$), P+V ($p<0.05$) (Fig 3.5 B); the 180 kDa (full-length) increased when treated with V+P ($p<0.05$) or P+V ($p<0.05$) (Fig. 3.5 C); the combination of V+P significantly increased the 180 kDa full-length ($p<0.05$) compared with VEGF only (Fig. 3.5 C); the 100 kDa fragment was increased significantly when treated with PEDF ($p<0.05$) (Fig. 3.5 D).

3.4.4 The effects of VEGF and PEDF on VEGFR-1 expression in HRPE cells

In HRPE cells, four bands (at approximately 250 kDa, 180 kDa, 85 kDa and 75 kDa) were detected in the untreated HRPE cell lysates by Western blotting (Fig 3.6 A). The 75 kDa and 85 kDa fragments of VEGFR1 decreased significantly when treated with VEGF ($p<0.05$), V+P ($p<0.05$) or P+V ($p<0.05$) for 24 hours (Fig 3.6 B and C); PEDF alone had no effect on the bands of 85 kDa or 75 kDa, but both fragments significantly decreased when pre-exposed or post-exposed to VEGF ($p<0.05$); VEGF decreased the 75 kDa VEGFR-1 fragment and this effect was enhanced by pre-treatment with PEDF ($p<0.01$).

These bands (either in BRMECs or HRPE cells) were not detected when the primary antibody was omitted or replaced by the IgG from same species. The specificity of the VEGFR-1 antibody was further confirmed by the blockade of the antibody binding with a peptide specific to VEGFR-1 (see the appendix 3).

A



B

Loaded Protein (μg)	Peak area (Optical density unit)
2.8	0.03669
5.6	0.10767
11.2	1.0206
22.4	1.766

C

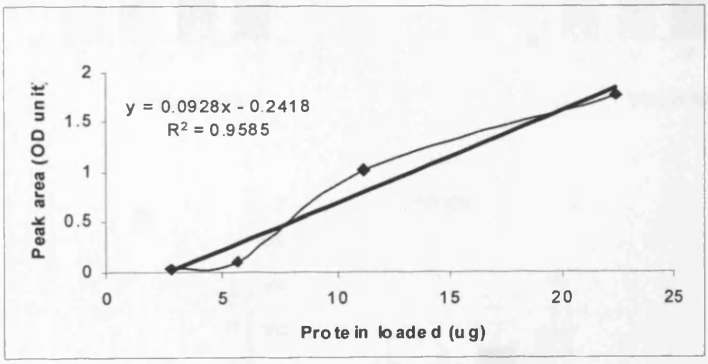


Fig 3.4 The linearity of peak area (OD units) against amount of protein loaded on ECL Western blotting. A: Western blotting of VEGFR-1. B: densitometrical analysis of peak area detected by ECL. C: A linear relation between peak area and amount of protein loaded. Calculated linear regression equation y =0.0928x-0.2418 where the correlation coefficient R2 = 0.9585. The protein loaded ranged from 5–22.4 μg and produced a linear relationship between load (μg) and peak area (OD unit).

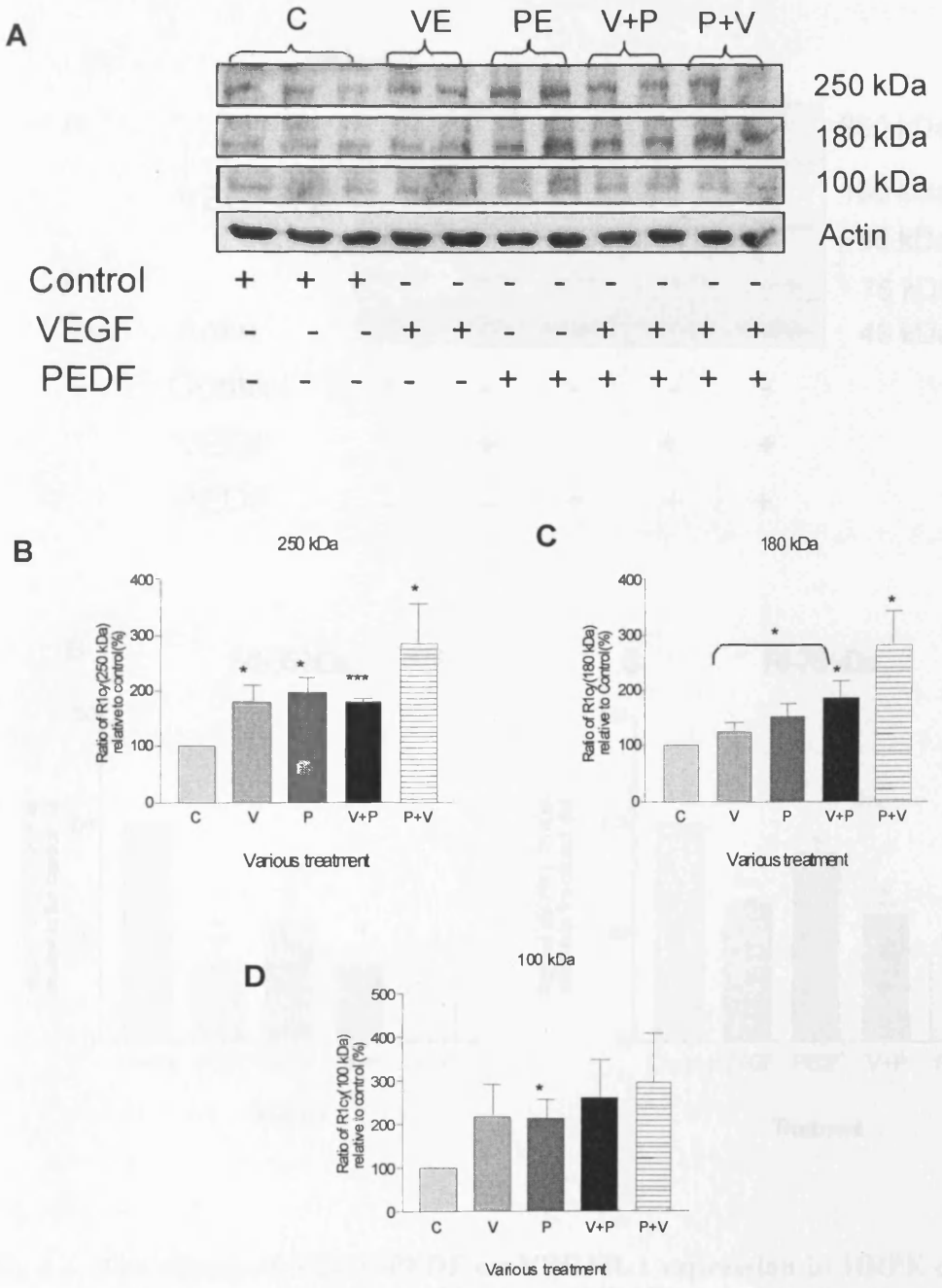


Fig 3.5 The effects of VEGF/PEDF on VEGFR-1 expression in BRMECs by western blotting. (A) Western Blotting of VEGFR-1 in BRMECs; (B-D) Densitometric analysis of immunoblots in (A). Data are normalised to α-actin from 4 separated experiments and presented as the relative ratio of VEGFR-1 to the control. (B) high molecular weight band of 250 kDa, (C) full-length (180 kDa), (D) fragment of 100 kDa. Data are presented as mean ± SEM. *p <0.05; *** p<0.001.

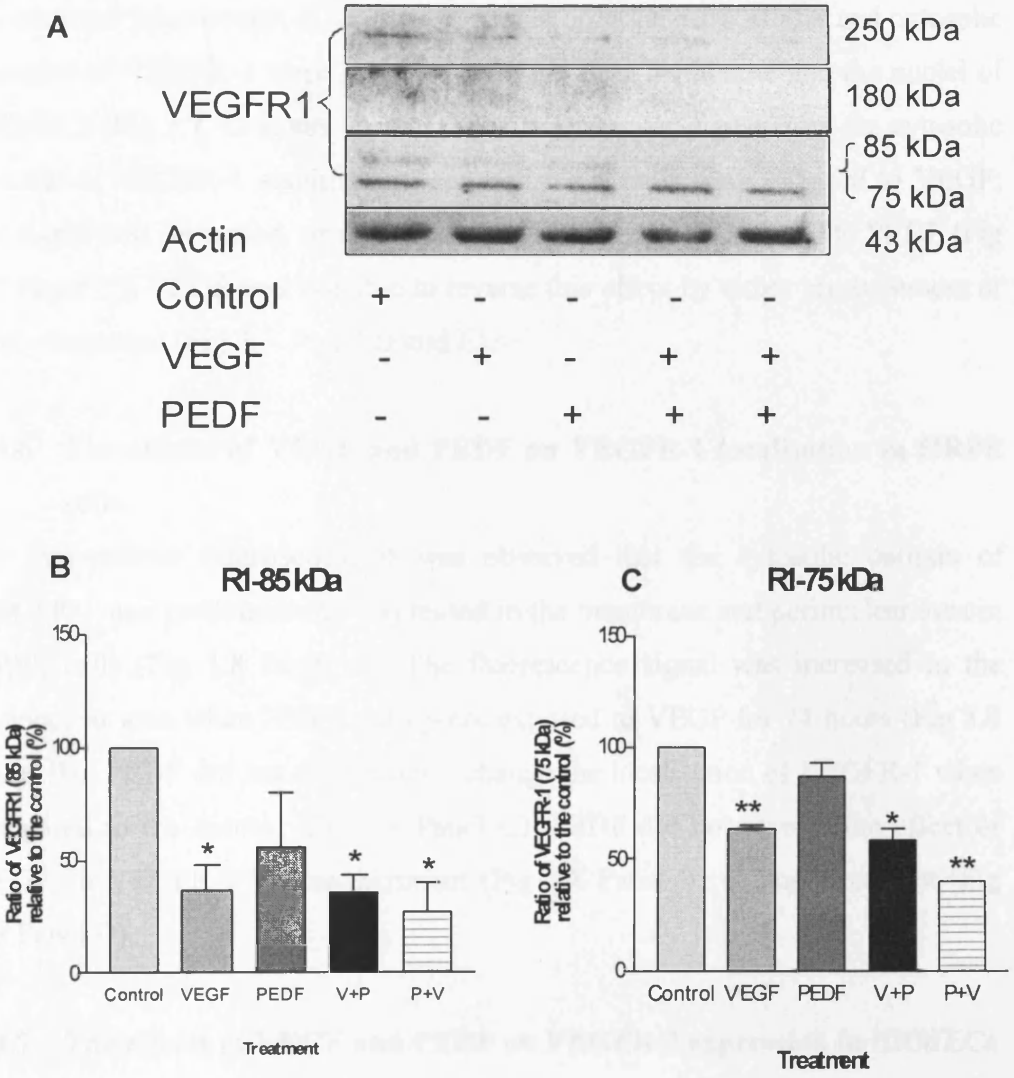


Fig 3.6 The effects of VEGF/PEDF on VEGFR-1 expression in HRPE cells by western blotting. (A) Western blotting of VEGFR-1 in HRPE cells. (B-C) Densitometric analysis of the 85 and 75 kDa bands on immunoblot in (A). Data are normalised to α-actin and presented as the relative ratio of VEGFR-1 to the control from 4 separate experiments. Data are presented as mean ± SEM. *p <0.05; ** p<0.01.

3.4.5 The effects of VEGF and PEDF on VEGFR-1 localisation in BRMECs

By confocal microscopy, it was observed that both the extracellular and cytosolic domains of VEGFR-1 were localised to the plasma membrane and the nuclei of BRMECs (Fig 3.7. Column 1 and 2). The fluorescence signal from the cytosolic domain of VEGFR-1 staining was reduced when cells were exposed to VEGF; the signal was increased, or at least no change when cells exposed to PEDF (Fig 3.7 Panel C); VEGF was not able to reverse this effect by either pre-treatment or post –treatment (Fig 3.7 Panel D and E).

3.4.6 The effects of VEGF and PEDF on VEGFR-1 localisation in HRPE cells

By fluorescence microscopy, it was observed that the cytosolic domain of VEGFR-1 was predominantly expressed in the membrane and perinuclear area in HRPE cells (Fig 3.8 Panel A). The fluorescence signal was increased in the perinuclear area when HRPE cells were exposed to VEGF for 24 hours (Fig 3.8 Panel B). PEDF did not significantly change the localisation of VEGFR-1 when compared to the control (Fig 3.8 Panel C). PEDF did not reverse the effect of VEGF on VEGFR-1 by pre-treatment (Fig 3.8 Panel E) or post-treatment (Fig 3.8 Panel D).

3.4.7 The effects of VEGF and PEDF on VEGFR-2 expression in BRMECs

Western blotting showed that two bands (at approx.180 and 170 kDa) were detected for VEGFR-2 in the untreated BRMECs (Fig.3.9A). Addition of VEGF alone reduced the expression of VEGFR-2 ($p < 0.05$); addition of PEDF also reduced the expression of VEGFR-2 ($p < 0.05$) (Fig 3.9 B). The combination of VEGF and PEDF (either VEGF followed by PEDF or vice versa) had similar effect as VEGF or PEDF alone.

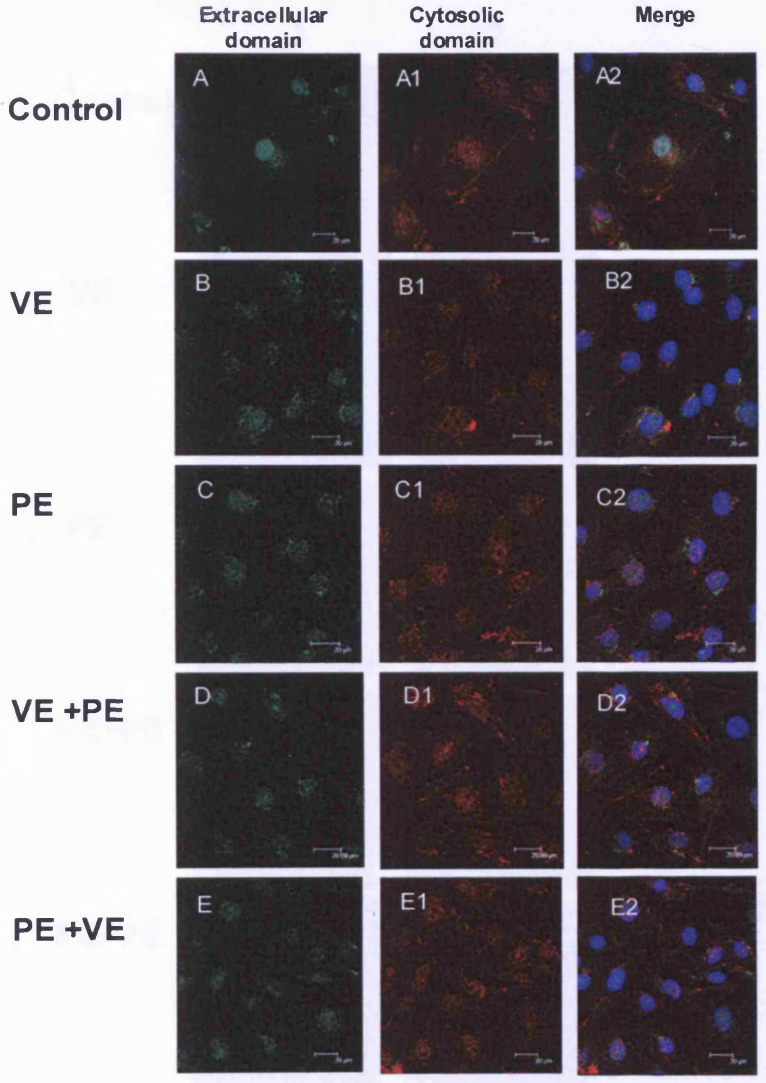


Fig 3.7 The effects of VEGF/PEDF on VEGFR-1 localisation in BRMECs by confocal microscopy. Column 1: VEGFR-1 (extracellular domain) staining (green) in BRMECs. A. control (without treatment); B. VEGF treatment; C. PEDF treatment; D. VEGF followed by PEDF; E. PEDF followed by VEGF. Column 2: VEGFR-1 (cytosolic domain) staining (red). Column 3: Merge of VEGFR-1 cytosolic domain and extracellular domain with nuclei staining.

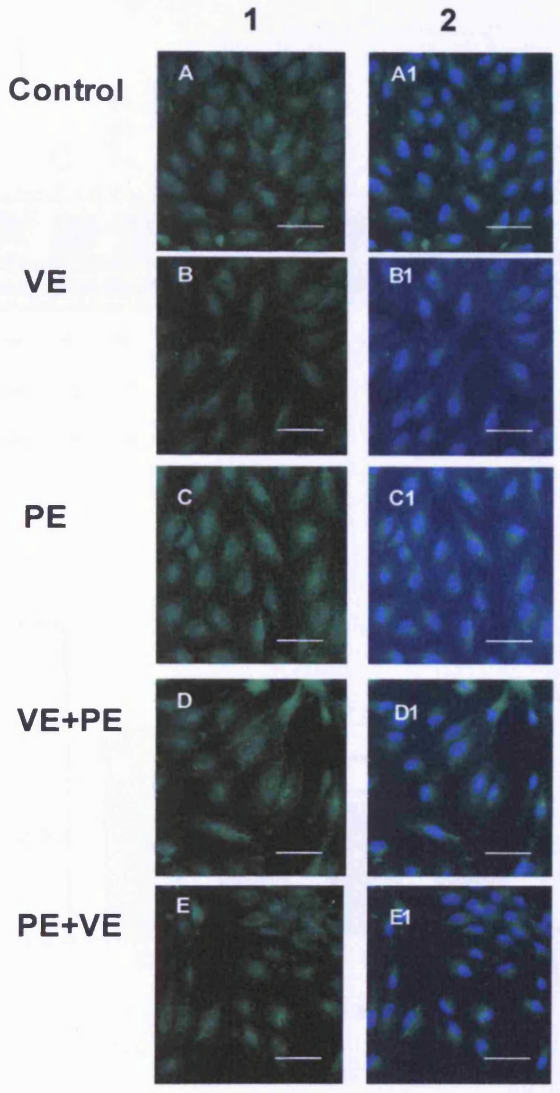


Fig 3.8 The effects of VEGF/PEDF on VEGFR-1 localisation in HRPE cells by fluorescence microscopy. Column 1: VEGFR1 (cytosolic domain) staining (green) in HRPE: A. control (without treatment); B.VEGF treatment; C:PEDF treatment; D: VEGF followed by PEDF; E: PEDF followed by VEGF. Column 2: Merge of VEGFR1 staining with nuclei staining. Scale bar = 50 μm.

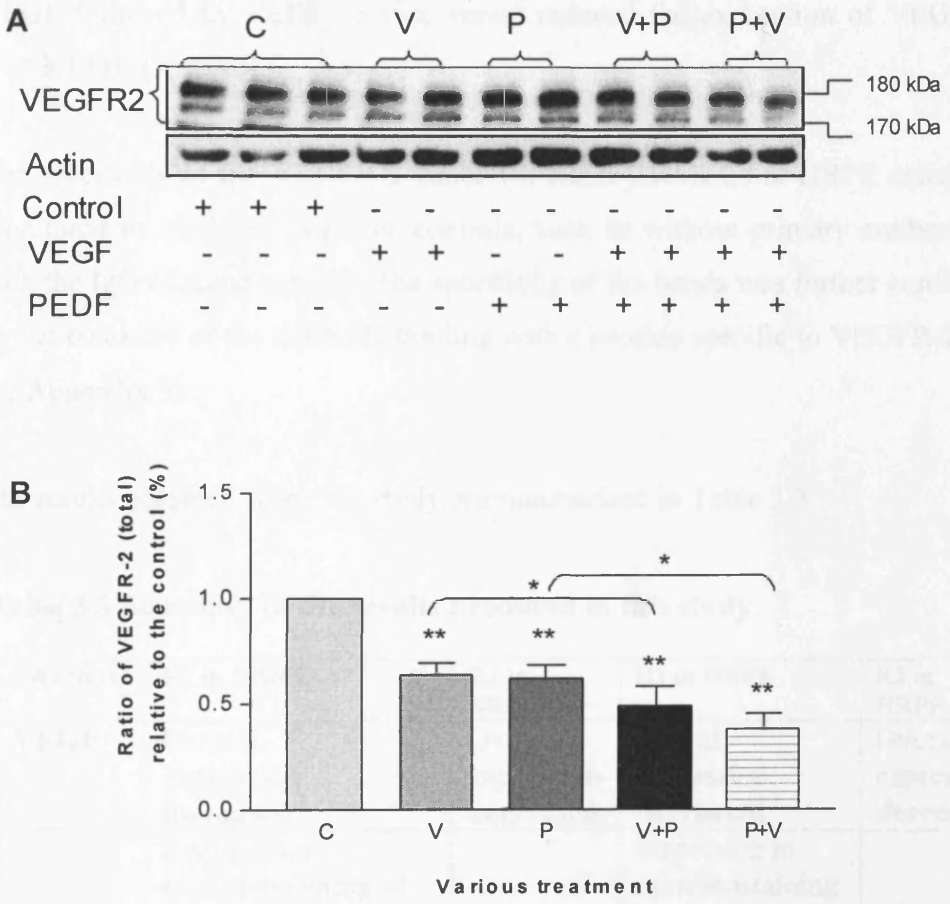


Fig 3.9 The effects of VEGF/PEDF on VEGFR-2 expression in BRMECs by western blotting. (A) Western blotting of VEGFR-2 in BRMECs. (B) Densitometric analysis of immunoblot in (A). Data are normalised to α-actin from 4 separated experiments and presented as the relative ratio of VEGFR-2 to the control. Data are presented as mean ± SEM. *p <0.05, **p <0.01.

3.4.8 The effects of VEGF and PEDF on VEGFR-2 expression in HRPE cells

In HRPE cells, both full length and a fragment of VEGFR-2 were observed in untreated HRPE cells (Fig. 3.10 A). The combination of VEGF and PEDF (either VEGF followed by PEDF or vice versa) reduced the expression of VEGFR-2 (Fig 3.10 B) ($P < 0.01$).

The specificity of the VEGFR-2 bands (in either BRMECs or HRPE cells) was monitored by enclosed negative controls, such as without primary antibody, or with the IgG of same species. The specificity of the bands was further confirmed by the blockade of the antibody binding with a peptide specific to VEGFR-2 (see the Appendix 3).

The results obtained from this study are summarised in Table 3.3.

Table 3.3 Summary of the results produced in this study

Treatment	R1 in BRMECs	R2 in BRMECs	R1 in HRPE	R2 in HRPE
VEGF	Overall expression **increased**	Overall expression **decreased**	Overall expression **decreased**	Overall expression **decreased**
	Reduction in immunostaining of cytosolic domain		Reduction in immunostaining of cytosolic domain	
PEDF	Overall expression **increased**	Overall expression **decreased**	Overall expression **sustained**	Overall expression **reduced**
	No change or increased immunostaining		**No change** or **increased** immunostaining	

3.5 Discussion

Defining the cellular mechanisms responsible for the homeostasis of the retinal vasculature is a prerequisite to achieving a complete understanding of the process of angiogenesis. Despite various attempts aimed at defining the factors that regulate retina microvascular endothelial cells (MECs), the mechanisms involved

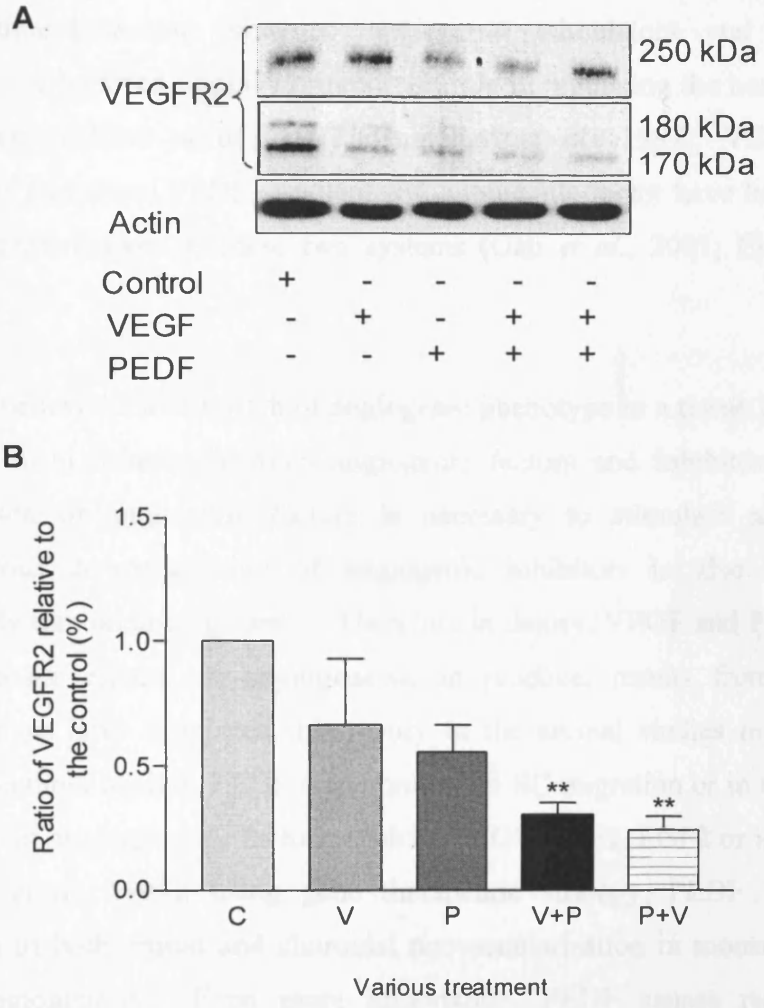


Fig 3.10 The effect of VEGF/PEDF on VEGFR-2 expression in HRPE cells by western blotting. (A) Western blotting of VEGFR-2 in HRPE cells. (B) Densitometric analysis of immunoblots in (A). Data are normalised to α-actin and presented as the relative ratio of VEGFR-2 to the control from 4 separated experiments. Data are presented as mean ± SEM. ** $p<0.01$.

in the determination of MECs state during retinal vasculature homeostasis remain ambiguous.

Two counter-balancing systems: angiogenic stimulators and angiogenic inhibitors are believed to play an important role in regulating the homeostasis of angiogenesis (Miller *et al.*, 1997; Bussolino *et al.*, 1997). VEGF, a pro-angiogenic factor and PEDF, a potent anti-angiogenic factor have been reported as the representatives of these two systems (Gao *et al.*, 2001; Eichler *et al.*, 2006).

It is now believed that a switch of angiogenic phenotype in a tissue is dependent upon the local balance between angiogenic factors and inhibitors. Although upregulation of angiogenic factors is necessary to stimulate angiogenesis, simultaneous downregulation of angiogenic inhibitors is also required to sufficiently turn on angiogenesis. Therefore in theory, VEGF and PEDF should have opposite effects on angiogenesis; in practice, results from numerous investigations have supported this theory in the animal studies or in *in vitro* studies. In those studies, PEDF alone prevented EC migration or in the presence of the potent proangiogenic factors, such as VEGF, FGF1, FGF2 or interleukin-8 (Dawson *et al.* 1999); using gene therapeutic strategy, PEDF inhibits the formation of both retinal and choroidal neovascularisation in mouse models of ocular angiogenesis. Even more importantly, PEDF causes regression of neovascularisation already underway (Mori *et al.* 2001; 2002); recently it also has been shown that PEDF cancels VEGF-induced increases in vascular permeability (Liu *et al.*, 2004). However, some studies observed a dual role of PEDF, in that PEDF can exert opposite effects on EC proliferation dependent upon the maintenance of their phenotype (Hutching *et al.* 2002); Apte *et al.* (2004) observed that low dose (90 μg/ml) of PEDF had significantly inhibitory effect on CNV in mice while a high dose (360 μg/ml) of PEDF can augment the development of the neovascularisation.

Two receptor-tyrosine-kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1 or KDR) that specifically bind to VEGF, have also been studied intensively (Waltenberger *et al.*, 1994). It has been well documented that VEGF provokes neovascularisation by engaging multiple EC surface receptors, including VEGFR-1, VEGFR-2, neuropilin-1, and neuropilin-2, by generating potentially homologous and heterologous signalling networks. The activation of VEGFR-1 and VEGFR-2 plays a critical role in neovascularisation. By using a chimeric system, Meyer and Rahini (2003) demonstrated that selective activation of chimeric VEGFR-2, but not chimeric VEGFR-1, stimulated ECs growth, migration, and differentiation. Stimulation of cells, co-expressing chimeric VEGFR-1 and VEGFR-2, suppressed VEGFR-2-mediated endothelial cell growth. Their results also indicate that VEGFR-1, unlike VEGFR-2, is unable to undergo ligand-dependent tyrosine phosphorylation and kinase activation. In HRPE cells (a non-angiognenic cell type), VEGF upregulates PEDF expression via VEGFR-1 (Ohno-Matsui *et al.* 2003). Very recently, Cai *et al.* (2006) reported that PEDF inhibits angiogenesis via RIP of VEGFR-1 in cultured BRMECs.

Based on the above arguments, the role of VEGF and PEDF in regulating the expression and distribution of VEGFR-1 and VEGFR-2 was examined in BRMECs and HRPE cells, where BRMECs acted as an angiogenic cell type and HRPE cells as a regulatory cell type.

Upon the establishment of the specificity of the antibody of VEGFR-1 and VEGFR-2 and the confirmation of the linearity of ECL Western blotting, this study observed that in BRMECs, VEGF and PEDF (at 100 ng/ml) exert similar positive effects on VEGFR-1 and similar negative effects on VEGFR-2 expression; while in HRPE cells, VEGFR-1 can be downregulated by VEGF but sustained by PEDF while VEGFR-2 can be downregulated by either VEGF or PEDF. As supportive evidence for the involvement of RIP, fragment(s) of the C-terminal of VEGFR-1 were detected in BRMECs (100 kDa) and in HRPE cells (75 kDa and 85 kDa). These fragments responded to the exogenous application

of VEGF or PEDF. In addition, VEGFR-1 was localised to the nuclei of BRMECs and HRPE in the unstimulated states.

MECs have been well documented as a key type of cells in the initiation of angiogenesis (Hudlicka *et al.*, 1992). In adulthood, MECs are quiescence in most organs and only proliferative in certain organs or under pathological conditions; and VEGF is a key growth factor in the induction of ECs proliferation and migration (Hutchings *et al.*, 2002). The upregulation of VEGFR-1 and downregulation of VEGFR-2 expression by VEGF or PEDF in this study suggests that VEGF/PEDF is regulating VEGFR-1 in a paracrine manner (ECs-independent), and VEGFR-2 in an autocrine manner (ECs-dependent). Further more, since VEGF and PEDF can both be produced from BRMECs (Tombran-Tink, *et al.* 2004), and VEGFR-1 and VEGFR-2 are also expressed in BRMECs, the result from the present study suggest that there is an autocrine loop between VEGF, PEDF and VEGFR-2 in BRMECs. The stimulation of VEGFR-1 and inhibition of VEGFR-2 by exogenous VEGF/PEDF suggests that under physiological conditions, VEGFR-1 expression may be in a basal level while VEGFR-2 is in a higher level. If this is the case, it would be interesting in comparison with the autocrine loop among VEGF, PEDF and VEGFR-1 in HRPE cells. Further study by monitoring the production of VEGF/PEDF in BRMECs before the addition of VEGF/PEDF would help to understand better the inter-relationship of VEGF/PEDF and VEGFR-1 and VEGFR-2.

An antagonising relationship between VEGF and PEDF was not observed at the level of VEGFR-1 and VEGFR-2 in the present study. This is in agreement, at least in part, with the reports by Hutching *et al.* (2002) and Apte *et al.* (2004). On the other hand, it is possible that the antagonising relationship of VEGF and PEDF observed by other studies may come from the concert effect of multiple regulators on multiple receptors in vivo or in vitro. Further studies, such as knock down of VEGFR-1/VEGFR-2 in BRMECs and observation of the effects of VEGF/PEDF on ECs proliferation/migration would help to clarify the role of VEGF and PEDF.

HRPE cells have been well documented as a main source of VEGF and PEDF (Ohno-Matsui *et al.*, 2001). In addition, it has been shown that VEGF upregulates PEDF via VEGFR-1 in an autocrine manner (Ohno-Matsui *et al.*, 2003). In the current study, the down regulation of VEGFR-1 fragments in HRPE by exogenous VEGF suggests that VEGFR-1 was in a predominantly active state under physiological conditions. The reduction of the expression of VEGFR-1 fragments by the VEGF is a reflection of the autocrine mechanism in that VEGF production in HRPE is regulated by VEGFR-1; and consequently this regulation leads to the restoration of a normal balance between angiogenic stimulators and inhibitors. This balance might have a key role in maintaining the homeostasis of the retinal vasculature.

To date, there are no reports regarding the state of VEGFR-1/VEGFR-2 in BRMECs/HRPE cells under physiological conditions; and there is no standard marker for indicating if VEGFR-1 or VEGFR-2 is active or inactive. Based on the classical pathway for a tyrosine kinase receptor, ligand-dependent autophosphorylation is considered to be an essential step in receptor activation and recruitment of signalling molecules to the receptor (Heldin and Ericsson, 2001; Meyer and Rahimi, 2003).

However, based on the report by Cai *et al.* (2006), VEGFR-1 may be involved in the RIP pathway, the pathway that has been well documented in ErbB4, Notch signalling pathway and the production of amyloid precursor protein (APP) as reviewed in Chapter 1. As hypothesised in Chapter 1.6, VEGFR-1 can be activated by the sequential cleavage of its ectodomain and cytosolic domain upon ligand binding, and the cleaved cytosolic domain is translocated to the nuclei to regulate genes related to angiogenesis. According to this hypothesis, full-length VEGFR-1 would be an inactive form which should exist only or predominantly in untreated BRMECs or HRPE cells; and the fragment of VEGFR-1 would be the active form and should be present or increased in VEGF-treated BRMECs or HRPE cells. As an antagonist of VEGF in regulation of angiogenesis, PEDF

should exert opposite effects on VEGFR-1 by keeping VEGFR-1 similar to the untreated state, i.e. PEDF has no effect on VEGFR-1 expression when compared with the control. In contrast to the hypothesis, this study demonstrated that PEDF alone significantly upregulated the expression of VEGFR-1 fragment.

The increased full-length and fragment of VEGFR-1 upon the exposure to the exogenous VEGF observed in this study seem not to fit the typical model for RIP, in that the full-length of the receptor should be reduced while the fragment should be increased. However, since other signalling pathways that involved VEGFR-1 have not been eliminated in this study, it is possible that the current observation is the integrated result between the RIP and other signalling pathways, such as autophosphorylation and the activation of the downstream pathways; such a situation has been discussed by Heldin and Ericsson (2001), and a model which reflects the close relation between RIP and classical pathway in tyrosine kinase receptors had been proposed thereafter.

There are more debatable points in regarding the disagreement with the report by Cai *et al.* (2006). In this report an 80 kDa fragment of the C-terminal domain of VEGFR-1 was observed when cells were exposed to VEGF followed by PEDF. This has two implications that 1) instead of VEGF, PEDF may play a role as a ligand of VEGFR-1 and 2) PEDF's ligand role only appears when the phenotype of BRMECs is under VEGF's control and this may be transient when compared with other signalling pathways. However, it is plausible that the homeostasis of angiogenesis is strictly regulated by the dynamic balance between pro-angiogenic factors and anti-angiogenic factors. In the current study, the detection of the 100 kDa fragment in the control and the treatments may reflect the notion of basal expression of VEGFR and VEGF activity. Exogenous PEDF alone seems also play a 'ligand' role based on its effect on the 100 kDa fragment of VEGFR-1. Therefore despite the variations between the experiments, it is possible that these two results may reflect the complex role of PEDF.

By using immunofluorescence staining and confocal microscopy, this study observed that the cytosolic and extracellular domains of VEGFR-1 were localised in the nuclear of untreated BRMECs; though a significant translocation of VEGFR-1 to the nuclei was not seen, the overall distribution of the cytosolic domain of VEGFR-1 was reduced by the addition of VEGF. PEDF alone or in combination with VEGF had no significant effect. The nuclei staining of VEGFR-1 in untreated cells suggests that VEGFR-1 is in a basal activation state. The absence of translocation may be due to the fact that a basal level of VEGFR-1, in untreated conditions, prevents a significant change from being detected by confocal microscopy. Quantitative analysis by live FRET may be a better means to observe the changes caused by the various treatments.

It is worth to noting the observed differences between BRMECs and HRPE cells in their response to exogenous VEGF/PEDF: 1) in response to exogenous VEGF, VEGFR-1 is upregulated in BRMECs while downregulated in HRPE cells; 2) in response to exogenous PEDF, VEGFR-1 is upregulated in BRMECs while sustained or decreased in HRPE; 3) in response to exogenous VEGF or PEDF, VEGFR-2 was downregulated in both BRMECs and HRPE cells. These results thus provide evidence, for the first time, that suggests that VEGF can exert opposite effect on VEGFR-1 depending on cell type; and similarly with PEDF. This may reflect the regulatory role of VEGF/PEDF via VEGFR-1 in angiogenic or no-angiogenic cell types. One limitation of the current study is the use of BRMECs and HRPE cells from different species. Therefore this study only compared the general trend in the hope of providing some new insight for further study. With the availability of human retinal MECs, further study would provide decisive evidence to understand the mechanism of angiogenesis regulation.

Chapter 4

The effects of VEGF and PEDF on γ-secretase activity and PS-1 expression in BRMECs and HRPE cells

4.1 Introduction

The γ-secretase complex is an unusual, multimeric, aspartyl protease responsible for the intramembrane cleavage of a variety of type-I transmembrane proteins, including the amyloid-β (Aβ) precursor protein, Notch, ErbB4, CD44, $p75^{NTR}$ and N-cadherin (Lavoie *et al.*, 2003; Ni *et al.*, 2003). Very recently, VEGFR-1 has been added to this list (Cai *et al.* 2006).

The intramembrane cleavage process and its implication in normal cell signalling have been termed Regulated Intramembrane Proteolysis (RIP) (Brown *et al.* 2000). As one of the three protease families (reviewed in more detail in chapter 1), the main function of Presenilin (PS)-dependent RIP seems to be the regulation of surface to nucleus signalling and gene expression by controlling production of peptides that act either as transcriptional stimulators or repressors (Landman and Kim, 2004). Recent findings for E-cadherin, syndecan-3 and the $p75^{NTR}$ suggest that PS-dependent RIP may also function in non-nuclear signalling pathways. An example of this is its involvement in regulating the formation/disassembly of high-affinity heteromeric receptor complexes and/or adherens junctions, providing additional diversity to PS-dependent RIP-mediated signalling (Landman and Kim, 2004).

γ-Secretase catalyses proteolysis within the transmembrane (TM) domains and has two unique characteristics: 1) There is a lack of requirement for specific amino acid target sequences immediately adjacent to the cleavage site within the TM domains due to a limited homology among the putative cleavage sites of the known γ-secretase substrates; 2) There is a requirement for ectodomain shedding to produce membrane-tethered C-terminal substrate fragments (Struhl and Adachi, 2000; Sisodia *et al.*, 2001; Li, 2001; Kimberly and Wolfe, 2003).

PS and three other multipass membrane proteins, nicastrin, Aph-1 and Pen-2, have been genetically linked to γ-secretase activity (Yu *et al.* 2000a; Goutte *et al.* 2002; Francis *et al.* 2002), and biochemical isolation has provided evidence that these proteins are indeed necessary members of the protease complex (Esler *et*

al. 2002; Steiner *et al.* 2002; Gu *et al.* 2003). Co-expression of these four transmembrane proteins is sufficient to reconstitute γ-secretase activity in yeast, which lacks these mammalian orthologs (Edbauer *et al.* 2003). It has also been observed that the biogenesis, maturation, stability, and the steady state levels of γ-secretase components are co-dependent. Down regulation or targeted gene disruption of any one of these components affects maturation and stability of other subunits, indicating that their assembly into a high molecular weight (HMW) complex is a highly regulated process that occurs during biosynthesis of these polypeptides (Leem *et al.* 2002).

PS is essential for γ-secretase activity and is likely to serve as a catalytic component in the heteromultimeric γ-secretase complex (Sisodia and George-Hyslop, 2002; Xu *et al.* 2002; Haass and Steiner, 2002; Iwatsubo, 2004). In mammals there are two PS homologues, PS-1 and PS-2, both contributing independently to γ-secretase activity (Tandon and Fraser, 2002), and both expressed throughout most adult human tissues and brain regions (Rogaev *et al* 1995; Berezovska *et al.* 1997, 1998).

It is unknown, at the molecular level, whether γ-secretase possesses a homogeneous or a heterogeneous activity. Several factors or processes have been reported to affect the nature and degree of γ-secretase activity; these include γ-secretase complex assembly and activation, the integral regulatory subunit CD147 (Zhou *et al.* 2005), transient or weak binding partners (Takashima *et al.* 1998; Chen *et al.* 2006), inflammatory cytokines (Liao *et al.* 2004; Blasko *et al.* 2000; Gianni *et al.* 2003) and cholesterol and sphingolipid levels (Sparks *et al.* 1990; Sjogren *et al.* 2006), which may affect the proteolytic microenvironment of the γ-secretase complex (reviewed by Zhou *et al.* 2007).

In the study by Cai *et al.* (2006) PEDF was shown to have a positive effect on γ-secretase activity in cultured BRMECs, while VEGF did not. In another study by Yoshida *et al.* (2005), they reported that Aβ accumulation affects the balance between VEGF and PEDF in the RPE, and an accumulation of Aβ reproduces

features characteristic of human AMD. As one of the substrates, Aβ has been tightly linked to γ-secretase (Haass *et al.* 1992; Busciglio *et al.* 1993; Haass and Selkoe 1993).

As HRPE cells express VEGF, PEDF and VEGFR-1, it would be interesting to see 1) if PS-1 and PS-2 are expressed in HRPE cells; 2) if exogenous VEGF and/or PEDF have any effect on PS-1 expression in HRPE; 3) if there is any γ-secretase activity in HRPE cells; and 4) if exogenous VEGF and/or PEDF have any effect on γ-secretase activity in HRPE cells.

The aim of this study was therefore to use BRMECs, as a control cell line, to investigate the association between VEGF and/or PEDF and PS-1 and the potential association between exogenous VEGF and PEDF and the γ-secretase activity in HRPE cells.

4.2 Experimental design

To meet the goal of this study, the experiment was designed to be approached from the following three aspects: 1) Enzyme activity assay for the effects of PEDF/VEGF on γ-secretase activity in BRMECs and HRPE; 2) Immunocytochemistry for the confirmation of PS-1 localisation and the effect of PEDF/VEGF on PS-1 localisation; and 3) Western blotting for the confirmation of PS-1 expression and the effect of PEDF/VEGF on the PS-1 expressions. A flow chart for the experimental design is shown in Fig.4.1.

4.3 Materials and methods

4.3.1 Cell culture

Isolation and growth of BRMECs and HRPE cells were performed as described in section 2.3.1. Cells were used within passage 4.

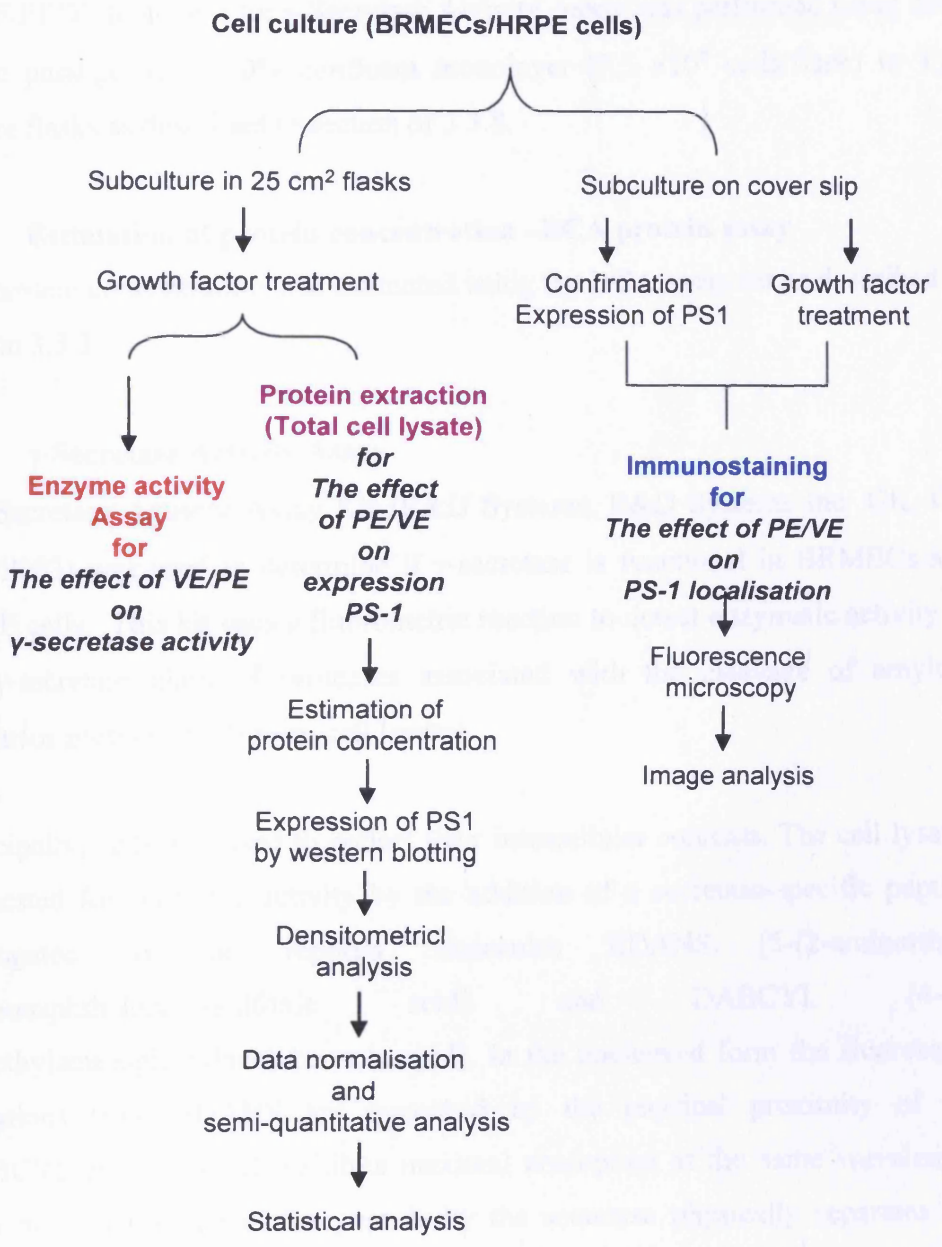


Fig 4.1 Flow chart of experimental design for chapter 4. PE = PEDF, VE=VEGF.

4.3.2 VEGF/PEDF treatment for γ-Secretase Activity Assay and Western blotting

VEGF/PEDF treatment for γ-Secretase Activity Assay was performed using cells within passage 4, 80-90% confluent monolayer (7.5×10^6 cells/flask) in T_{25}-culture flasks as described in section of 3.3.8.

4.3.3 Estimation of protein concentration –BCA protein assay

The protein concentration was estimated using the BCA assay kit as described in section 3.3.3.

4.3.4 γ-Secretase Activity Assay

A γ-Secretase Activity Assay Kit (R&D Systems, R&D Systems Inc. UK. Cat No.FP003) was used to determine if γ-secretase is functional in BRMECs and HRPE cells. This kit uses a fluorometric reaction to detect enzymatic activity of the γ-secretase class of proteases associated with the cleavage of amyloid precursor protein (APP) from cell lysates.

Principally, cells are lysed to collect their intracellular contents. The cell lysates are tested for secretase activity by the addition of a secretase-specific peptide conjugated to the reporter molecules EDANS [5-(2-aminorthyl) aminonaphthalene-1-sulfonic acid] and DABCYL [4-(4-dimethylaminophenylazo) benzoic acid]. In the uncleaved form the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety which exhibits maximal absorption at the same wavelength (495 nm). Cleavage of the peptide by the secretase physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal. The level of secretase enzymatic activity in the cell lysate is proportional to the fluorometric reaction (kit manual). Fig. 4.2 illustrates the principle of the assay.

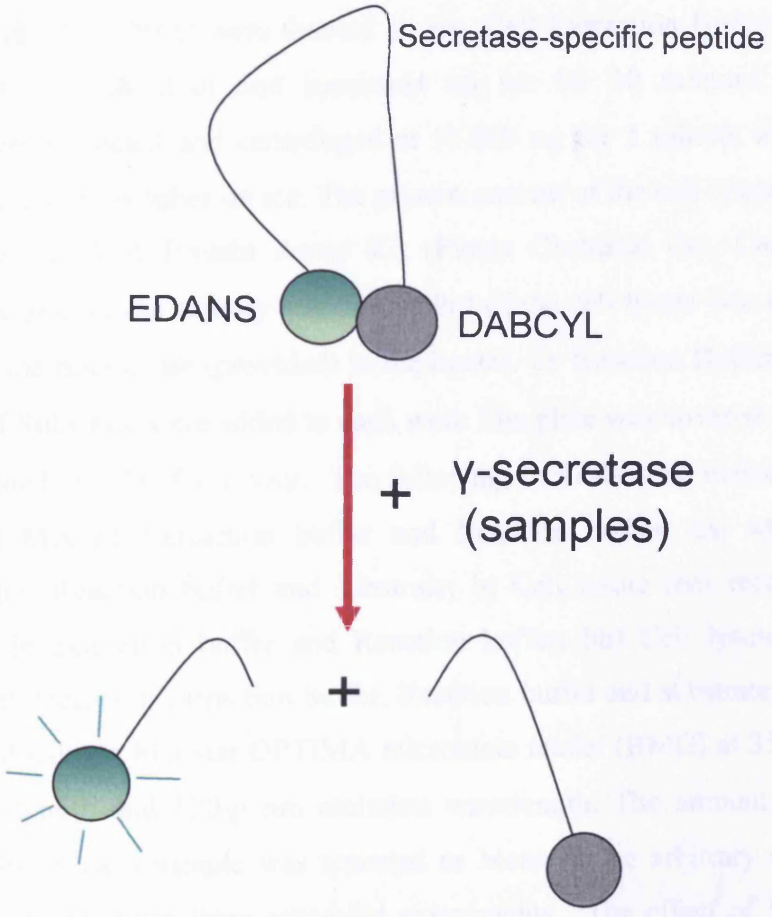


Fig 4.2 Principle of the γ-secretase Activity Kit. A secretase-specific peptide is conjugated to the reporter molecules EDANS and DABCYL. In the uncleaved form the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety which exhibits maximal absorption at the same wavelength (495 nm). Addition of γ-secretase cleaves the peptide and physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal. The level of secretase enzymatic activity in the cell lysate is proportional to the fluorometric reaction.

The γ-secretase activity assay was carried out according to the manufacturer's instruction with some modification. In brief, cells from the T_{25}-culture flasks harvested and stored at -20°C were thawed on ice. Cell Extraction Buffer (0.5 ml) was added to each flask and incubated on ice for 30 minutes. The supernatants were collected and centrifuged at 10,000 ×g for 1 minute at 4°C before transferring to new tubes on ice. The protein content of the cell lysate was estimated using the BCA Protein Assay Kit (Pierce Chemical Co., Cat No. 23225). For the γ-secretase activity reaction, 100μl of the cell lysate was added to each well of the microplate (provided) in duplicates. 2× Reaction Buffer (100 μl) and 10 μl of Substrate were added to each well. The plate was covered in the dark and incubated at 37°C for 1 hour. The following controls were included in each assay: a) Mix of Extraction buffer and Reaction buffer; aa) Mix of Extraction buffer, Reaction buffer and substrate; b) Cell lysate (not receiving growth factor) in extraction buffer and Reaction buffer; bb) Cell lysate (not receiving growth factor) in extraction buffer, Reaction buffer and substrate. The plates were read using a Fluostar OPTIMA microplate reader (BMG) at 355 nm excitation wavelength and 520-p nm emission wavelength. The amount of γ-secretase activity in each sample was reported as Mean of the arbitrary unit ± Standard Error (SEM) from three separated experiments. The effect of VEGF and PEDF on γ-secretase activity in BRMECs and HRPE was expressed as the ratio of the γ-secretase activity relative to the control [bb) Cell lysate (not receiving growth factor) in extraction buffer, Reaction buffer and substrate].

4.3.5 Preparation of a positive control for the γ-secretase activity assay

Mouse brain is a tissue known to contain γ-secretase activity and is recommended as a positive control by the manufacturer. However, this positive control is not included in the kit and is not available commercially. Therefore, a positive control from mouse brain was prepared as following: fresh normal mice brains were obtained from the animal house in the School of Biosciences (Cardiff University), and either stored at -20°C in 1x Cell Extraction Buffer, or homogenised in ice cold 1x Cell Extraction Buffer to yield a final protein concentration of 0.5-2.0 mg/ml.

A dilution series of 20, 30, 40, 50 μg /well of the mouse brain lysate were tested for γ-secretase activity. Mouse brain lysate prepared by different procedures, such as frozen or fresh, homogenised or not, were tested for γ-secretase activity. The preparation with the highest γ-secretase activity was chosen as a positive control for this study.

4.3.6 Immunofluorescence staining

Cell preparation and immunofluorescence staining were performed as described in section 2.2.1. VEGF/PEDF treatment for immunostaining was performed as described in section 3.3.9.

4.3.7 Western blotting

The preparation of whole cell lysate and Western blotting were performed as described in the section of 3.3.5 and 3.3.8.

4.3.8 The primary antibodies used for immunofluorescence staining and western blot

Table 4.1 The list of primary antibodies used in the immunostaining and western blot

Name	Source	Against a peptide at	Dilution used
Presenilin-1 (PS-1) (H-70)	Rabbit polyclonal (sc-7860)	Amino acids 23-247 at the N-terminus of human origin	1:100
Presenilin-2 (PS-2) (C-20)	Goat polyclonal (sc-1456)	A peptide mapping at the C-terminus of PS-2 of human origin	1:100

4.3.9 Statistical analysis

The results were given as the mean ± standard error (SEM) from 3 separate experiments. An unpaired Student's t-test was performed to analyse the data using SPSS program. A *P* value less than 0.05 ($P<0.05$) was considered statistically significant.

4.4 Results

4.4.1 Estimation of the sensitivity of the γ-secretase activity kit

The sensitivity of the γ-secretase activity kit was assessed using the mouse brain preparation. The results showed that the addition of substrate increased the fluorescence signal >1 fold in the background control (dark red) when compared with the background control without substrate [blue] (Fig.4.3). γ-secretase activity was detected above background with statistical significance in the mouse brain preparation when the total protein concentration was>1mg/ml and extracted by fresh homogenisation. This preparation was used as a positive control for the study of γ-secretase activity in BRMECs and HRPE cells.

4.4.2 The effect of VEGF/PEDF on γ-secretase activity in BRMECs and HRPE cells

In BRMECs, the γ-secretase activity assay revealed that cells that were pre-exposed to PEDF had γ-secretase activity increased 13.36 ± 5.4% than the control ($p<0.05$). The fluorescence signal did not significantly increase in the samples that were pre-exposed to VEGF alone, or the combination of VEGF and PEDF (Fig 4.4).

In HRPE cells (Fig 4.5), pre-exposed to VEGF, γ-secretase activity was 14.65 ± 5.8 higher than the control ($p<0.05$); the fluorescence signal did not significantly increase in the samples that were pre-exposed to PEDF alone, or the combination of VEGF and PEDF.

4.4.3 Localisation of PS-1 in BRMECs and HRPE cells

Immunostaining confirmed that PS-1 was expressed in BRMECs (Fig. 4.6) where it was predominantly localised to the cytoplasm and membrane. In HRPE cells, PS-1 was localised in the perinuclear and membrane area (Fig. 4.7).

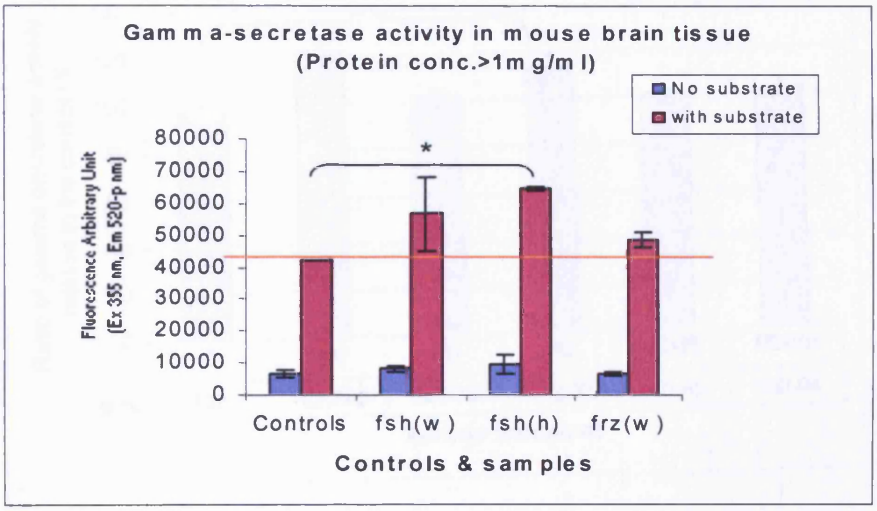


Fig. 4.3 Estimation of the sensitivity of the γ-secretase kit. Mouse brain with total protein concentrations >1mg/ml. γ-secretase activity increased in mouse brain tissue that had been freshly extracted without homogenisation [fsh (w)], with homogenisation [fsh (h)], and extracted from frozen tissue without homogenisation [frz (w)]. The controls for background (blue: without substrate; red: with substrate) were included. The orange line indicates the level of fluorescence taken as baseline. *p <0.05.

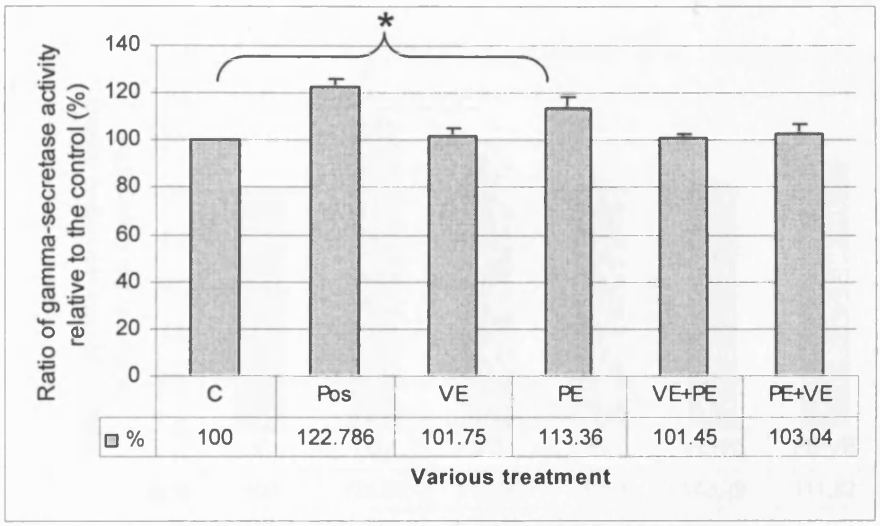


Fig 4.4 The effects of VEGF/PEDF on γ-secretase activity in cultured BRMECs. VE: VEGF, PE: PEDF, VE+PE: VEGF + PEDF, PE+VE: PEDF+VEGF. C: control cell not exposed to growth factors; Pos: positive control made from mouse brain. Data are presented as the ratio of treatment relative to the control. Data are presented as mean ± SEM from 3 independent experiments.. * p <0.05.

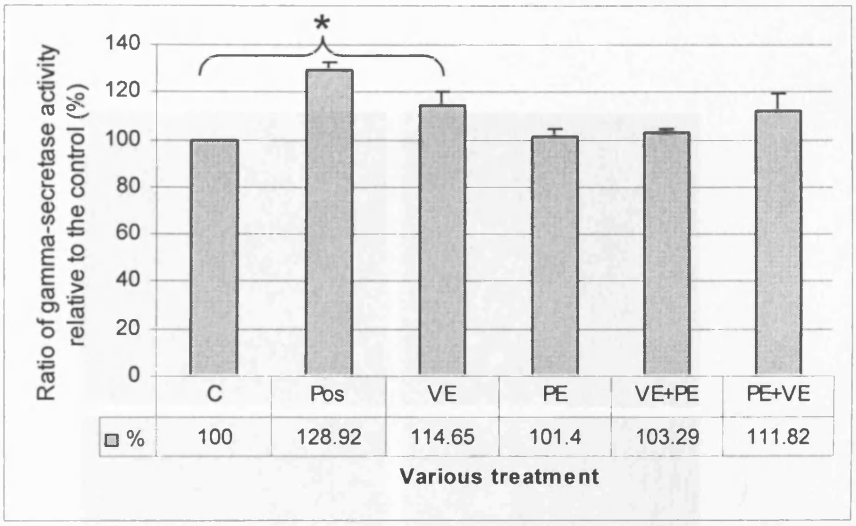


Fig. 4.5 The effects of VEGF/PEDF on γ-secretase activity in cultured HRPE cells. VE: VEGF, PE: PEDF, VE+PE: VEGF + PEDF, PE+VE: PEDF+VEGF. C: control cell not exposed to growth factors; Pos: positive control made from mouse brain. Data are presented as the ratio of treatment relative to the control. Data are presented as mean ± SEM from 3 independent experiments. * p <0.05.

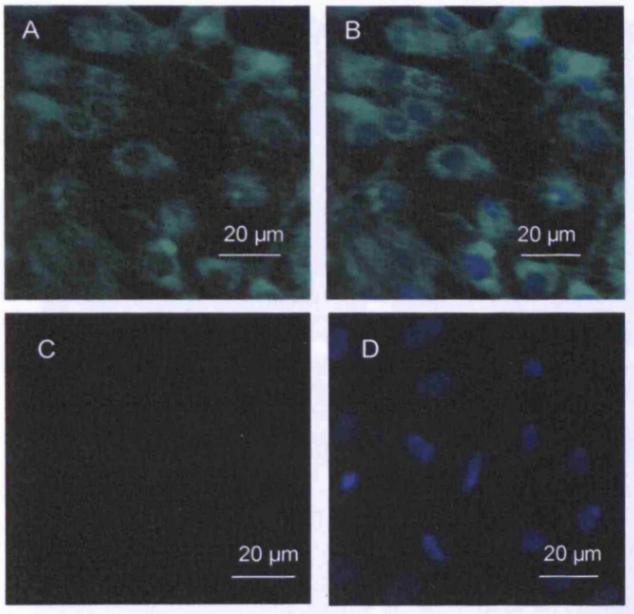


Fig 4.6 Immunolocalisation of Presenilin-1 (PS-1) in cultured BRMECs. A) Presenilin-1 staining was in the cytoplasm (predominantly perinuclear area) and membrane. B)PS-1 staining with Hoechst nuclei stain. C) Negative control shows no positive staining. D) Negative control with Hoechst nuclei stain.

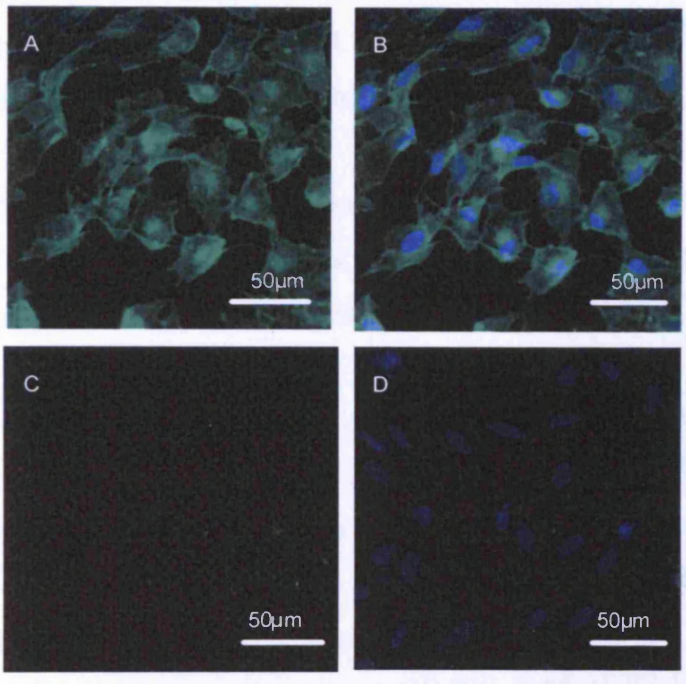


Fig 4.7 Immunolocalisation of PS-1 in cultured HRPE cells. A) Presenilin-1 (PS-1) staining was found predominantly in the cytoplasm (perinuclei) and membrane. B) PS-1 staining with Hoechst nuclei stain. C) Negative control shows no positive stain. D) Negative control with Hoechst nuclei stain.

4.4.4 The effects of VEGF/PEDF on the localisation of PS-1 and PS-2 in HRPE cells

By dual immunolabeling, it was demonstrated that PS-1 and PS-2 were co-expressed in HRPE cells (Fig. 4.8 A2). PS-1 was localised in the perinuclear and membrane area while PS-2 localised predominantly to the perinuclear and nuclear area, and in the nuclei area presented as 'speckles' (Fig 4.8 red).

The addition of VEGF reduced the PS-1 distribution on the membrane of HRPE cells (Fig.4.8B) whereas PEDF alone (Fig. 4.8C) or the combination with VEGF (Fig. 4.8 D+E) had no significant effect on the distribution of PS-1.

The addition of PEDF increased the distribution of PS-2 in the perinuclear and nuclear area (Fig. 4.8 C1). Pre-exposure or post-exposure to VEGF could not reverse the effect of PEDF on PS-2 distribution (Fig. 4.8. D1+E1).

4.4.5 The effects of VEGF/PEDF on the expression of PS-1 in BRMECs

By western blotting, three bands at approx. 120, 100 and 75 kDa were detected in the untreated BRMECs (Fig 4.9 Control), and specific to PS-1 when compared with the negative control (data not shown). A 47 kDa band representing PS-1 was not detected in any of the three experiments. The addition of PEDF increased the 120 kDa high molecular weight (HMW) band of PS-1 ($p<0.01$). The addition of VEGF had no significant effect on all the bands detected (Fig 4.9 A, B, C). The addition of VEGF followed by PEDF (V+P) significantly increased the 75 kDa HMW ($p<0.001$).

4.4.6 The effects of VEGF/PEDF on the expression of PS-1 in HRPE cells

Western blotting of HRPE cell extracts revealed the presence of two bands of 47 kDa and 75 kDa specific to the anti-PS1 antibody (Fig. 4.10A Control). The 75 kDa HMW band significantly decreased in the samples exposed to VEGF followed by PEDF ($p<0.05$) or PEDF followed by VEGF ($p<0.01$). VEGF or PEDF alone had no significant effect on the expression of PS-1 in HRPE cells (Fig 4.10 B, C).

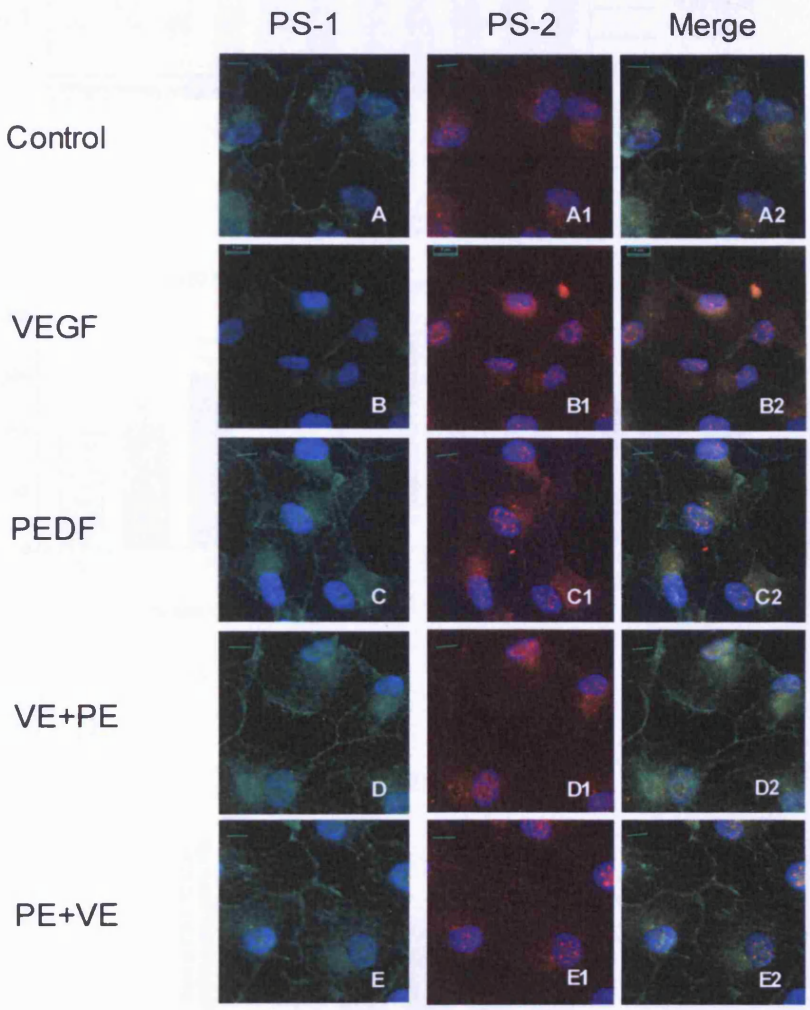


Fig. 4.8. The effects of VEGF and PEDF on PS-1 and PS-2 localisation in cultured HRPE cells by fluorescence microscopy. Column 1: PS-1 (green) with nuclei (blue) staining; A. control; B. VEGF treatment; C. PEDF treatment; D. VEGF followed by PEDF (VE+PE); E:PEDF followed by VEGF (PE+VE). Column 2: PS-2 (red) with nuclei (blue) staining; Column 3: Merge of PS-1 and PS-2 with nuclei staining. Scale bar = 5 μM.

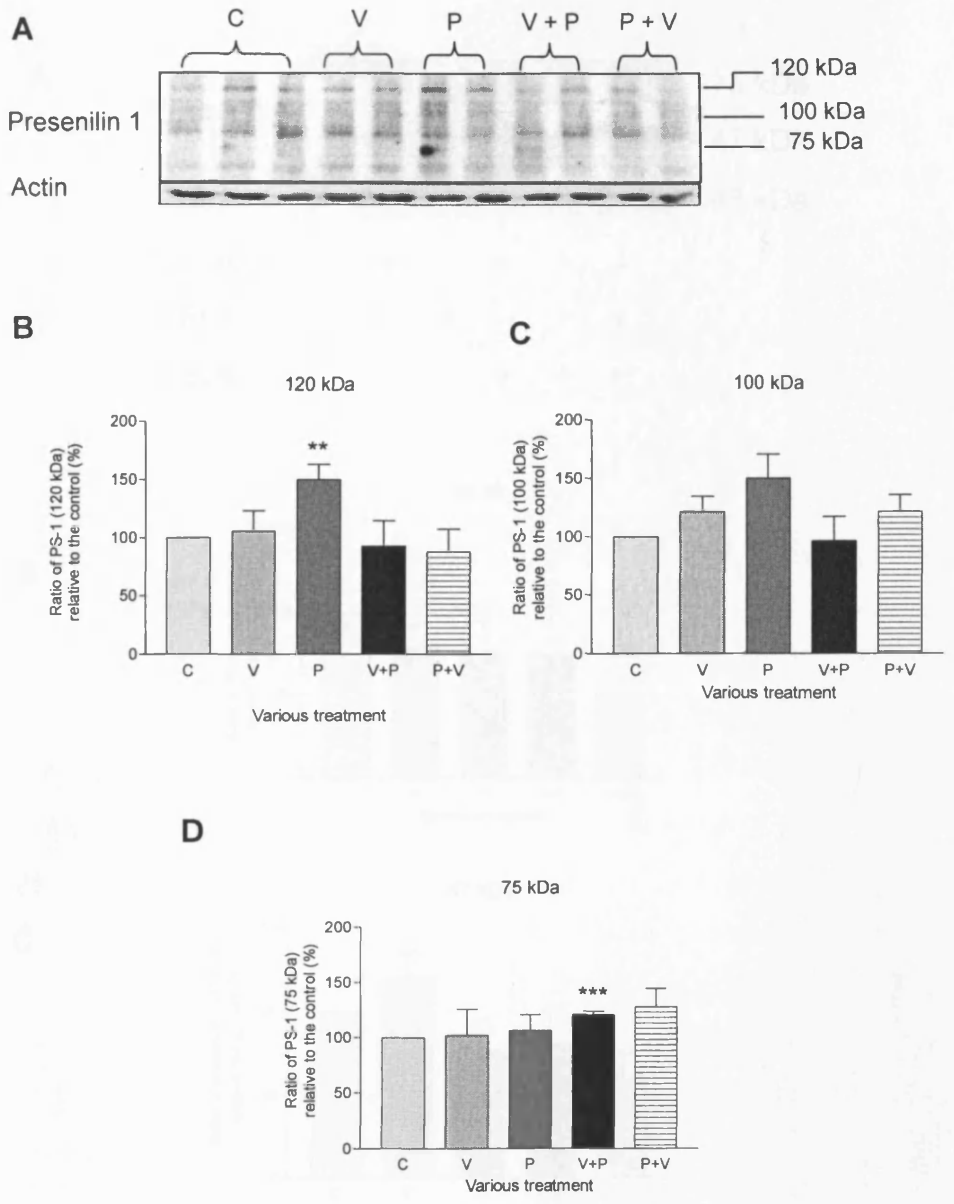


Fig 4.9. The effects of VEGF/PEDF on presenilin-1 (PS-1) expression in BRMECs. (A) Western blotting of BRMECs extracts showing expression of PS-1. (B-D) Densitometric analysis of Western blotting from 4 independant experiments. (B) Data are normalised to α-actin and presented as the relative ratio of the 120 kDa band to the control. (C) Data are normalised to α-actin and presented as the relative ratio of the 100 kDa band to the control. (D) Data are normalised to α-actin and presented as the relative ratio of the 75 kDa band to the control. Data is presented as mean ± SEM. **p <0.01, ***p<0.001. C=control; V=VEGF; P=PEDF; V+P=VEGF+PEDF; P+V= PEDF+VEGF.

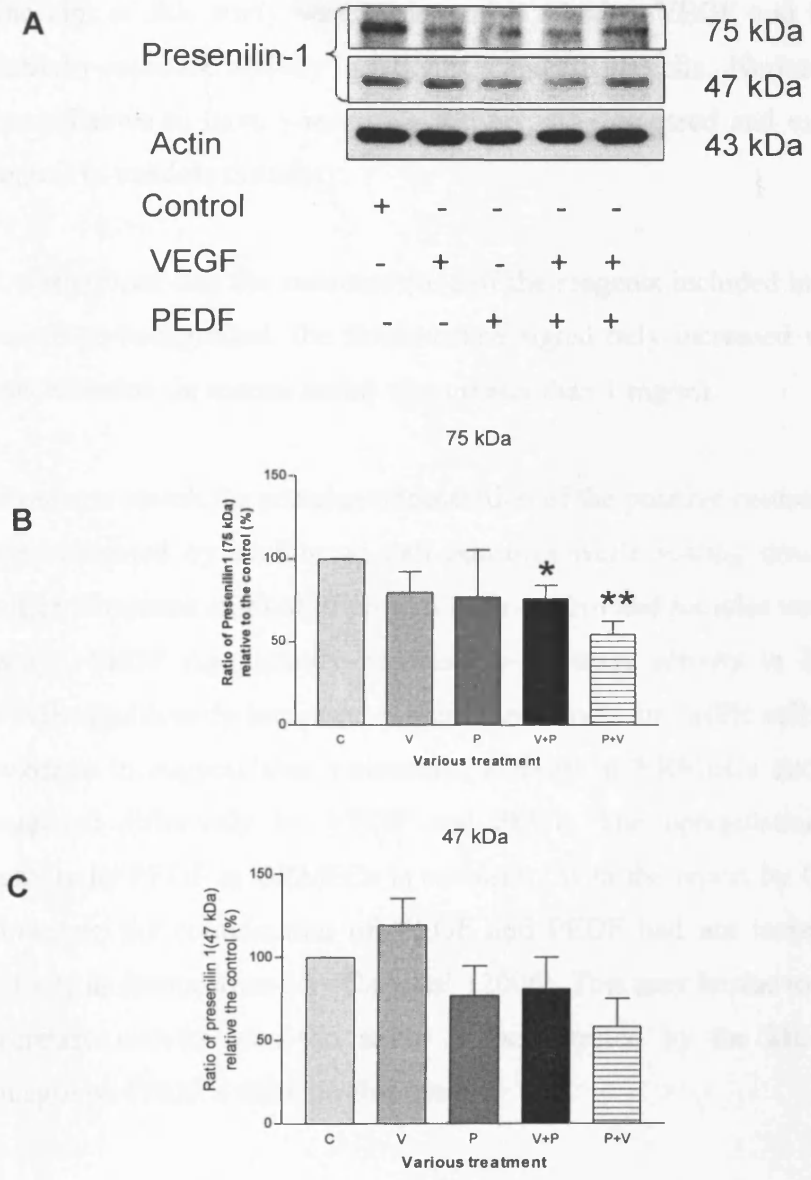


Fig 4.10. The effects of VEGF/PEDF on presenilin-1 (PS-1) expression in HRPE cells. (A) Western blotting of HRPE cell extracts showing the presence of a 75 kDa band as well as the full-length of PS-1 (47 kDa). Blots were stripped and reprobed with an antibody to actin to show equal loading of protein. (B-C) Densitometric analysis of Western blotting from 4 independant experiments. (B) Data are normalised to α-actin and presented as the relative ratio of the 75 kDa band to the control. (C) Data are normalised to α-actin and presented as the relative ratio of the 47 kDa band to the control. Data is presented as mean ± SEM. *p <0.05, **p<0.01. C=control; V=VEGF; P=PEDF; V+P=VEGF+PEDF; P+V= PEDF+VEGF.

4.5 Discussion

The aim of this study was to determine whether VEGF and PEDF treatment altered γ-secretase activity in BRMECs and HRPE cells. Normal mouse brain, a tissue known to have γ-secretase activity, was prepared and used as a positive control to validate the assay.

It was noticed that the substrate (one of the reagents included in the kit) induced very high background, the fluorescence signal only increased when the protein concentration (in mouse brain) was greater than 1 mg/ml.

In order to match the protein concentration of the positive control, the cell lysates were prepared by scaling up cell numbers while scaling down the extraction buffer. The same amount of protein from control and samples was loaded for the assay. PEDF significantly increased γ-secretase activity in BRMECs; whilst VEGF significantly increased γ-secretase activity in HRPE cells. This provides evidence to suggest that γ-secretase activity in BRMECs and HRPE cells is regulated differently by VEGF and PEDF. The upregulation of γ-secretase activity by PEDF in BRMECs is consistent with the report by Cai *et al.* (2006). However, the combination of VEGF and PEDF had not increased γ-secretase activity as demonstrated by Cai *et al.* (2006). This may be due to the change of γ-secretase activity was too small to be detected by the kit. Or VEGF can antagonise PEDF's effect in this case.

The association between γ-secretase and VEGF/PEDF was investigated further by exploring the expression of PS following VEGF and PEDF stimulation, since PS has been regarded as the catalytic centre of γ-secretase (Wolfe *et al.* 1999b; Li *et al.* 2000). It has been well documented that PS, as the core element of γ-secretase, is endogenously expressed in every type of mammalian cell (Thinakaran *et al.*, 1996; Iwatsubo, 2004), localized predominantly to the endoplasmic reticulum (ER) and Golgi compartments (Annaert, *et al.* 1999). A small percentage of PS can be detectable at the cell surface in specific cell types (Ray *et al.* 1999; Schwarzman *et al.* 1999; Kaether *et al.* 2002). This study

confirmed the expression of PS-1 in cultured BRMECs and HRPE cells, and the localisation of PS in both cell types is in agreement with the above reports. Further more, this study revealed, for the first time, that the distribution of PS-1 was differentially regulated by exogenous VEGF and PEDF in HRPE cells, in that VEGF reduced the membrane distribution of PS-1 whilst PEDF sustained the distribution. Pre-exposure or post-exposure of VEGF to HRPE cells did not reverse the effect of PEDF suggesting that VEGF and PEDF act on PS-1 in an antagonising manner. This evidence supports our hypothesis that VEGF and PEDF have oppositing effects on the PS-dependent RIP pathway.

'Speckles' have been considered as storage depots for inactive splicing components, and are less prominent in cells that transcribe RNA at high levels and strikingly prominent when RNA processing is inhibited (Pollard and Earnshaw, 2002). In response to the application of exogenous VEGF, PS-2 distribution increased in the perinuclei and nuclei and decreased in the membrane, suggesting that VEGF promotes the translocation of PS-2 from membrane to perinuclei and nuclei area in HRPE cells. In contrast, PEDF increased the overall distribution of PS-2 in HRPE.

It is not surprising that PS-1 in BRMECs was expressed as three higher molecular weight (HMW) bands of 120, 100 and 75 kDa rather than a 47 kDa band. Such phenomena have been observed by several groups in that PS was presented in HMW aggregates in a range from 100 to 250 kDa, and includes both the PS NTF and CTF (Thinakaran *et al.* 1996; De Strooper *et al.* 1997; Seeger *et al.* 1997). It has also been reported by several groups (Capell *et al.* 1998; Yu *et al.* 1998, 2000a & b) that full-length (FL-PS1) is present in lower molecular weight complexes while the NTF/CTF is present in HMW complexes in cell culture studies. Cai *et al.* (2006) detected FL-PS1 and CTF in BRMECs by immunoprecipitation and Western blotting. Therefore, the HMW bands detected in this study may be just the intermediate complex of γ-secretase that is expressed specifically in the BRMECs. If this is the case, the increase of 120 kDa

by PEDF suggests that PEDF upregulates PS processing into HMW complexes, towards the active form of γ-secretase complex. The upregulation of γ-secretase activity by PEDF from this study supports the above speculation. In contrast, in HRPE cells, the combination of VEGF and PEDF down-regulated the 75 kDa HMW complex but had no significant effect on the band at 47 kDa, suggesting that the effect of PEDF on PS-1 expression in HRPE cells is opposite to the one observed in BRMECs.

Taken together, this study provides evidence for the first time that exogenous VEGF and PEDF have opposite effects on 1) the γ-secretase activity in BRMECs and HRPE cells; and 2) the distribution of PS-1 and PS-2 in HRPE cells. It would be interesting to carry out further work to study the relationship between Aβ, PS and exogenous VEGF/PEDF in HRPE cells, which may contribute to a new therapeutic approach in treatment of AMD by targeting PS or γ-secretase.

Chapter 5

The effects of γ-secretase inhibition on the expression of VEGFR-1 and VEGFR-2 in HRPE cells

5.1 Introduction

γ-Secretase has been implicated in many biological activities, such as the pathogenesis of Alzheimer's disease and developmental signalling by Notch receptor. Other activities involve ErbB4, CD44, VEGFR-1 and E-cadherin as reviewed in Chapters 1 and 4.

Involvement of β-secretase and γ-secretase in producing the β-amyloid component of plaques found in the brains of Alzheimer's patients has led to the design of selective inhibitors of these proteases that might be of therapeutic interest for Alzheimer's disease. The use of selective β and γ-secretase inhibitors might also be important to reveal new functions of these proteases during other physiological processes opening the possibility of new applications for these drugs. Meanwhile, γ-secretase inhibitors have also been used for identifying new substrates that are involved in the PS-dependent RIP pathway by interfering with the molecular interaction between the enzyme (γ-secretase) and its target. For example, Ni *et al.* (2001) used a γ-secretase inhibitor to clarify if the soluble 80 kDa (s80) fragment detected was from γ-secretase cleavage or from contamination.

As discussed previously (Chapters 3 and 4), evidence suggests that there is a relationship between VEGF/PEDF and VEGFR-1/VEGFR-2 (Chapter 3) and between VEGF/PEDF and PS-1 (Chapter 4). However, this evidence is not sufficient to confirm the association of γ-secretase with VEGFR-1. γ-secretase inhibitors provide a mean to examine the association of γ-secretase and VEGFR-1/VEGFR-2.

The aim of this study was to investigate the association between: 1) γ-secretase and VEGFR-1 and 2) γ-secretase and VEGFR-2 in HRPE cells using a γ-secretase inhibitor.

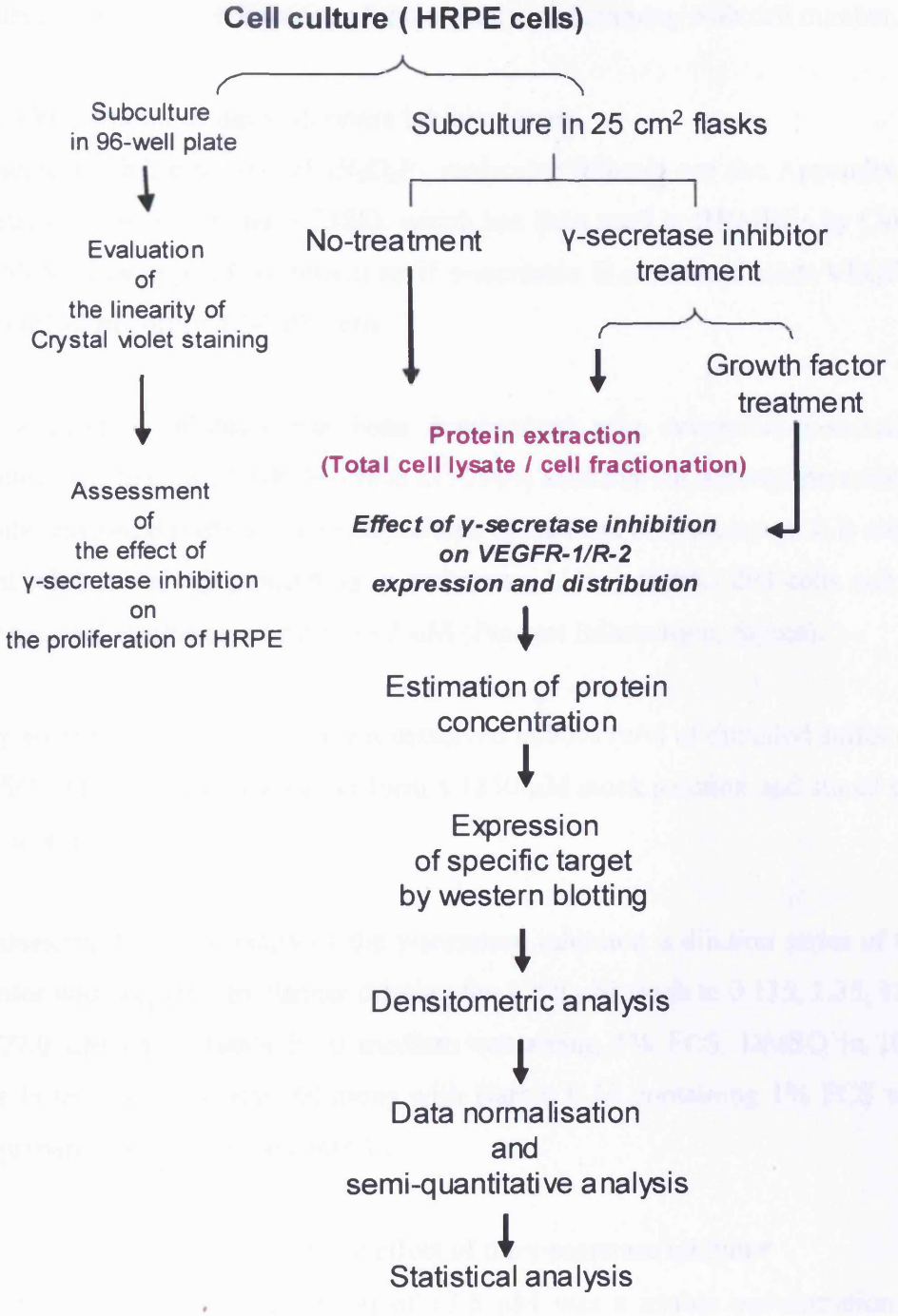


Fig 5.1 Flow chart of experimental design for chapter 5.

plate in replicates of six (from 0/well to 5400/well). A simple regression model was used to analyse the linearity of the crystal violet staining with cell number.

5.3.2.3 Preparation of the γ-secretase inhibitor stock

A γ-secretase inhibitor ($C_{33}H_{57}N_5O_9F_2$, molecular formula see the Appendix 4) (Sigma, Catalogue number S 2188), which has been used in BRMECs by Cai *et al.* (2006), was applied to determine if γ-secretase is associated with VEGFR-1/VEGFR-2 in cultured HRPE cells.

This γ-secretase inhibitor has been documented as a reversible γ-secretase inhibitor (Wolfe *et al.*, 1998; Wolfe *et al.*, 1999) selective for aspartyl protease. It inhibits amyloid β protein (Aβ) biosynthesis at the level of γ-secretase. It is also a potent inhibitor of Aβ production in embryonic kidney (HEK) 293 cells and its IC50 for total inhibition of Aβ is 13.5 μM (Product Information, Sigma).

The γ-secretase inhibitor (1mg) was dissolved in 90% (v/v) of dimethyl sulfoxide (DMSO) with 10% (v/v) water to form a 1350 μM stock solution and stored at -20°C in aliquots.

For assessing the cytotoxcity of the γ-secretase inhibitor, a dilution series of the inhibitor was prepared by further diluting the 1350 μM stock to 0.135, 1.35, 13.5 and 27.0 μM with Ham's F-10 medium containing 1% FCS. DMSO in 10% water in the corresponding dilutions with Ham's F-10 containing 1% FCS was also prepared as the vehicle controls.

5.3.2.4 Evaluation of the cytotoxic effect of the γ-secretase inhibitor

To determine if the concentration of 13.5 μM was a usable concentration in HRPE cells with minimum cytotoxicity, HRPE cells were subcultured in a 96-well plate at 3000 cells/well and maintained in Ham's F-10 medium containing 10% FCS for 24 hours. Prior to the treatment, serum-free medium was replaced and incubated for 45 min. The γ-secretase inhibitor was added at concentrations

ranging from 0.135, 1.35, 13.5 and 27μM in (1000 μl/well of) Ham's F-10 medium containing 1% FCS respectively, so that each well containing a specified concentration of γ-secretase inhibitor and DMSO. Medium without any addition and medium containing DMSO only (in correspondent concentrations) were included as controls. Each treatment and control was prepared in triplicates. Initially, cells were incubated with a given γ-secretase inhibitor concentration for 26 hours at 37 °C. The cytotoxicity of γ-secretase inhibitor was assessed by the Crystal Violet assay.

5.3.3 γ-secretase inhibitor and PEDF / VEGF treatment

Once the optimal concentration of the γ-secretase inhibitor was identified, HRPE cells were subcultured in T_{25} flasks with consistent cell numbers in each flask. At near confluence, the cells were washed with PBS three times, and incubated with serum-free Ham's F-10 medium for 45 min. Cells were treated with 13.5 μM γ-secretase inhibitor in Ham's F-10 medium containing 1% FCS (2 ml/flask) and incubated at 37°C for 2 hours. The addition of PEDF (100 ng/ml) or VEGF (100 ng/ml) was followed respectively. One hour after incubation at 37°C another dose of VEGF (100 ng/ml) was added to one of the flasks that had been treated with PEDF, and same dose of PEDF to one of the flasks that had been treated with VEGF. Cells without any treatment, and cells treated with just the γ-secretase inhibitor were included as controls. Cultures were incubated for a further 24 hours in a 37°C standard incubator. At the end of the time course cells were harvested and subjected to protein extraction.

5.3.4 Preparation of whole cell lysate

The whole cell lysate was prepared as described in section 3.3.2.

5.3.5 Preparation of subcellular fractions

5.3.5.1 Preparation of four subcellular fractions

In order to analyse the localisation of the target of interest, the ProteoExtract™ Subcellular Proteome Extraction Kit (S-PEK) was used in an attempt to obtain four subcellular fractions. The S-PEK uses special reagent mixtures to solubilise

different subcellular compartments while the structural integrity of the subcellular structures is preserved before and during the extraction. It enables the differential extraction of proteins according to their subcellular localisation. The S-PEK procedure yields proteins in their native state (manual of the S-PEK) (Fig 5.2).

Subcellular Proteome Extraction was carried out according to the manufacturer's instruction [ProteoExtract^{TM} Subcellular Proteome Extraction Kit (S-PEK) (Cat. No. 539790, EMD Biosciences, Inc.UK)] with some modification. In brief, the cells in a T_{25}–flask were washed with PBS, and any remaining liquid removed. Ice-cold Extraction buffer I (500µl) containing Protease Inhibitor Cocktail (5µl) was added to each flask and incubated at 4°C for 10 min under gentle agitation. The supernatant (**fraction 1 - cytosolic)** was collected and stored at -20°C in aliquots for further analysis. A mixture of ice-cold Extraction buffer II (500µl) and Protease Inhibitor Cocktail (5µl) was added to each flask and incubated for 30 min at 4°C under gentle agitation. The supernatant (**fraction 2 – organelle / membrane)** was collected and a mixture of ice-cold Extraction buffer III (500µl) with Protease Inhibitor Cocktail (5µl) and Benzonase® (1.5µl) was added to each flask and incubated for 10 min at 4°C under gentle agitation. The supernatant (**fraction 3 -nucleic)** was collected and a mixture of Extraction buffer IV (500µl) with Protease Inhibitor Cocktail (5µl) at room temperature was added to each flask. Cell particles were suspended by vigorously pipetting, and transferred into a new eppendorf (**fraction 4 - cytoskeleton).** The protein concentration of the subcellular protein extracts was determined by BCA Protein Assay as described in section 3.3.3 before being subjected to SDS-PAGE or Western blotting.

5.3.5.2 Preparation of two subcellular fractions

In an attempt to extract subcellular fractions of cells in their native state with a higher protein concentration, extraction of two subcellular fractions was undertaken using Triton X-100 soluble (Tx-sol) (containing the cytosol fraction) and Triton X-100 insoluble (Tx-insol) (containing the cytoskeleton and nuclei

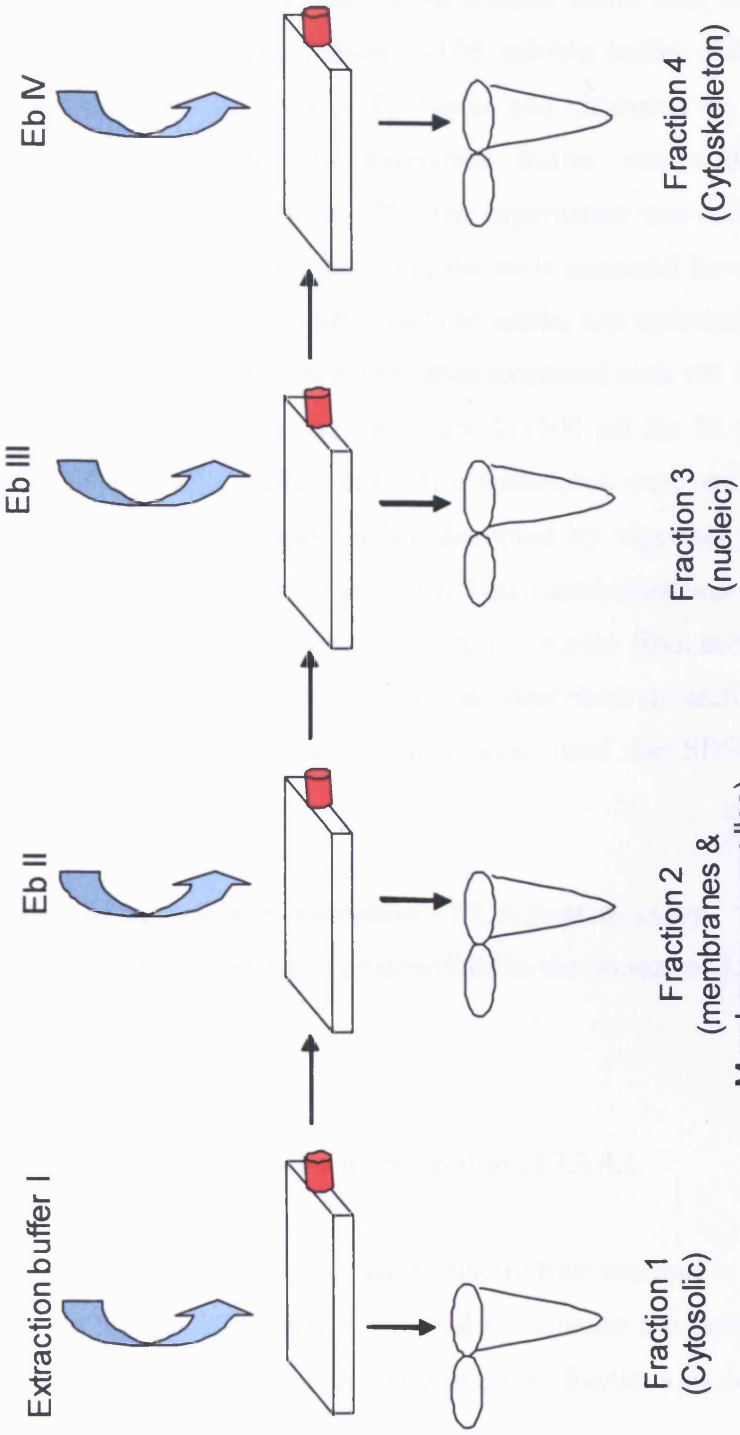


Fig 5.2. Principle of the Subcellular (4 fractions) Proteome Extraction Kit. Eb= Extraction buffer.

fraction) as previously described (Stolz *et al.* 1992; Lampugnani *et al.* 1995; Cai *et al.*1999; Cai *et al.* 2006).

In brief, at near confluence, HRPE cells were washed twice with 1xPBS after removal of Ham's F-10 medium. T riton X-100 soluble buffer (500 µl) (see Appendix 2) was added to the cells in T_{25} flasks and incubated on ice for 30 minutes with gentle agitation. The extraction buffer was collected and centrifuged at 14,000g for 5 minutes at 4°C. The supernatant was defined as the Tx soluble (Tx-sol) fraction. After this step, the cells appeared homogenously adherent to the culture vessel with well preserved nuclei and cytoskeleton fibres under phase contrast microscopy. They were then extracted with 1% SDS buffer containing protease inhibitors (see the Appendix 2) (500 µl) for 10 minutes on ice. The mixture of 1% SDS buffer and cell cytoskeleton were collected into eppendorfs using a cell-scraper and further disrupted by vigorously pipetting before centrifugation at 14,000g for 5 minutes. This supernatant was defined as the Tx-insoluble (Tx-ins) fraction. The protein concentration from both fractions was estimated using the BCA protein assay (as described in section 3.3.3.). Equal amounts of protein from each sample were used for SDS-PAGE or Western blotting.

5.3.6 Estimation of protein concentration – BCA protein assay

The BCA protein assay was performed as described in the section of 3.3.3.

5.3.7 SDS-PAGE

5.3.7.1 Preparation of SDS-PAGE

SDS-PAGE was prepared as described in the section of 3.3.4.1.

5.3.7.2 Visualisation of protein by Coomassie brilliant blue staining

Coomassie brilliant blue staining was performed to visualise the protein profile in the preparation of four subcellular fractions and two fractions as described in section 3.3.4.2.

5.3.7.3 Visualisation of protein by silver staining

All silver staining methods depend on the reduction of ionic to metallic silver to provide metallic silver images (Hames and Hooper, 2000). Silver staining is much more sensitive than Coomassie Blue staining and is usually carried out when the protein concentration is too low to be detected by Coomassie Blue staining.

The procedure of silver staining was undertaken according to the manufacturer's instruction (Pierec, UK) using the GelCode® Colour Silver Stain Kit. On completion of running, SDS –PAGE gels were fixed with the mixture of ethanol and acetic acid (10:1) (v/v) overnight on an agitator. Next day gels were washed with deionised water (dH_2O) for 30 minutes with 4 changes, and incubated with the Silver Working Solution (WS) (Silver Concentrate: Water = 1:14) for 30 minutes at room temperature. Gels were rinsed in dH_2O for 10 seconds and incubated with Reducer WS (equal volumes of Reducer Aldehyde WS and Reducer Base WS, prepared immediately before use) for 5 minutes. Gels were again rinsed with dH_2O very briefly and incubated in Stabilizer WS (1:44 dilution of Stabilizer Concentrate in dH_2O) for up to 2 hours. The bands of each sample were analysed using EPSON scan (EPSON EXPRESSION 1680 Pro).

5.3.8 Western blotting

Western blotting was performed on whole cell lysates (as described in section 3.3.2), two subcellular fractions (section 5.3.5.2) and four subcellular fractions (section 5.3.5.1).

5.3.9 Statistical analysis

The results were given as the mean ± standard error (SEM) from 3 independent experiments. An unpaired Student's t-test was performed to analyse the data (SPSS). A probability value less than 0.05 ($P<0.05$) was considered statistically significant.

5.4 Results

5.4.1 Selection of a usable concentration of γ-secretase inhibitor for treatment with cultured HRPE cells

By crystal violet staining it was observed that the doses of γ-secretase inhibitor applied to the HRPE had no significant effect on HRPE cell growth when compared with the controls as indicated by the orange colour line in Fig. 5.3. Thus, no toxic effect was observed in the concentration range 0.135 μM to 27.0 μM. The 13.5 μM concentration of γ-secretase inhibitor was chosen for the following studies in HRPE cells since it has been previously used in BRMECs by Cai *et al.* (2006).

5.4.2 The crystal violet staining exhibits a linear response to the number of HRPE cells

To confirm the validity of crystal violet staining for monitoring the proliferation of cultured HRPE cells, the staining was performed on cells cultured at different density for 24 hours. By plotting the Optical Density (OD) against the number of the cells used, it was demonstrated that there is a linear relationship between the OD and cell number with a very high coefficient of determination (R^2=0.984) (Fig 5.4). This indicates that the variance in one variable (OD) would predict almost all the variance in the other variable (number of cells). Therefore, the crystal violet staining method is a valid approach to quantify HRPE cell proliferation (Calculated linear regression equation y = 4E-05X + 0.0834 where the correlation coefficient R^2 = 0.984).

5.4.3 Protein profile from 4 subcellular fractions of BRMECs

Coomassie Blue staining of protein showed no visible band from the samples prepared in four subcellular fractions. With silver staining, it was observed that each fraction has its own pattern. Bands were observed above and around 250 kDa position in fraction 1 (F1- the cytosolic) and fraction 2 (F2 - membrane and organelle) whereas in fraction 3 (F3 - nuclei) the bands were weaker and were ambiguous in fraction 4 (F4 - cytoskeleton). See Fig 5.5.

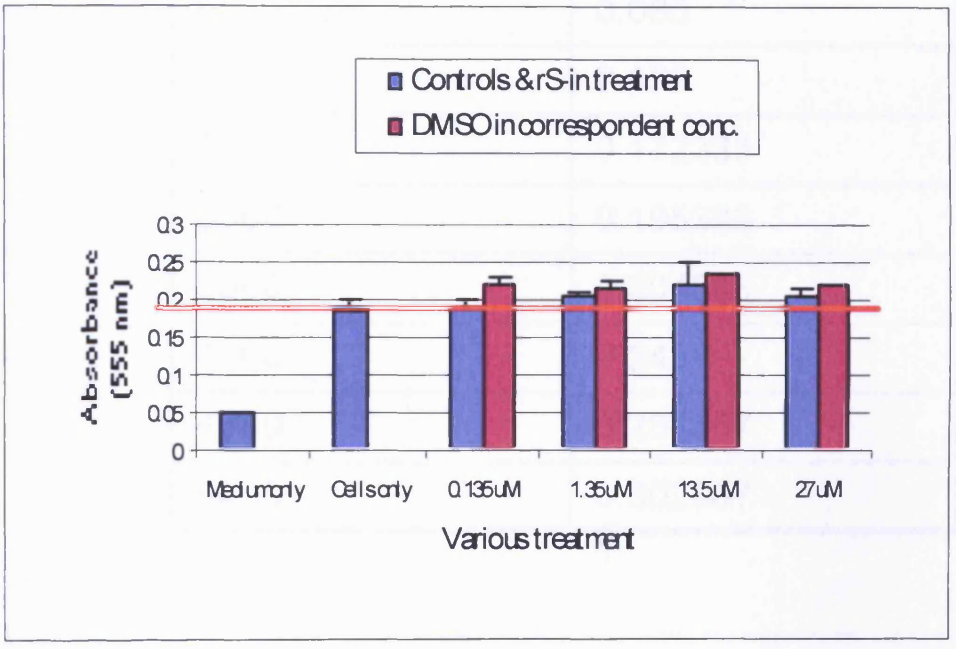


Fig 5.3 Evaluation of a usable concentration of γ-secretase inhibitor in cultured HRPE cells. A dilution series of the γ-secretase inhibitor ranging from 0.135, 1.35, 13.5 and 27 μM were added to the cells and incubated for 26 hours. Medium only and cells without treatment were included as controls; cells exposed to DMSO (vehicle control) in correspondent concentrations (dark red) were also included. Vertical bars are mean ± SEM (n=3). The orange line indicats the level of proliferation taken as baseline. rS-in = γ-secretase inhibitor.

Cell number	Absorbance (555 nM)
0	0.085
1200	0.138
2400	0.172333
3000	0.195333
3600	0.204333
4200	0.248
4800	0.264667
5400	0.302667

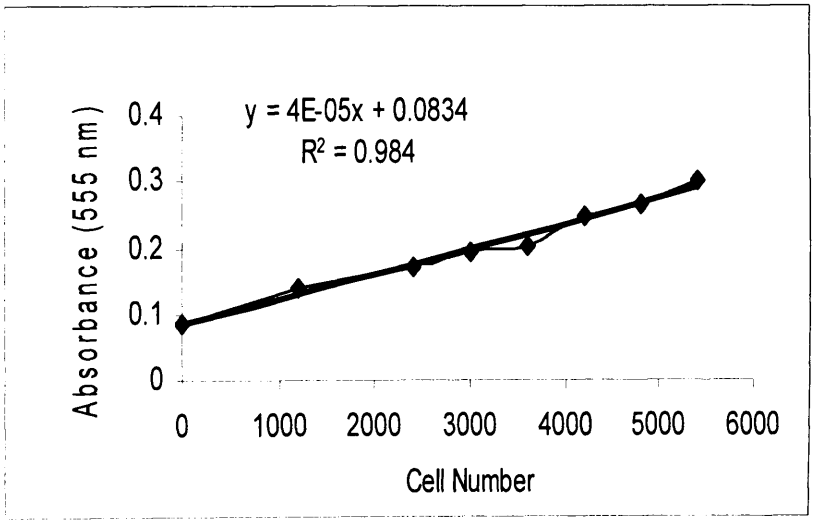


Fig 5.4. The validation of crystal violet staining for monitoring cell proliferation. (A) The absorbance reading (555 nm) corresponding to the cell numbers. (B) Linear relation between the absorbance and cell number.

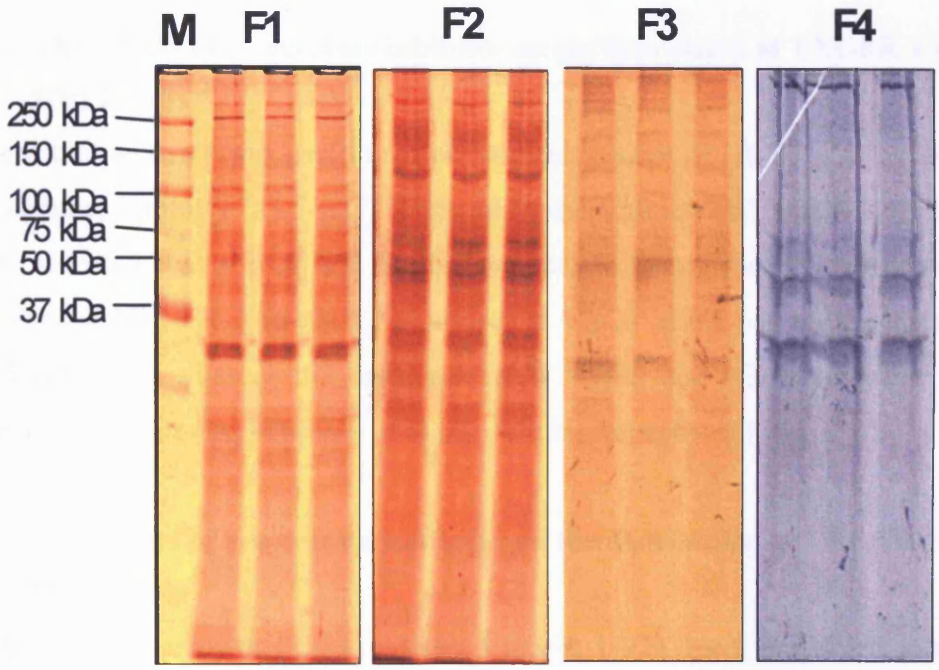


Fig 5.5 Protein profile in four subcellular fractions from BRMECs by silver staining of 10% SDS-PAGE gels. The fractions were obtained using the ProteoExtractTM Subcellular Proteome Extraction Kit. F1: cytosolic fraction. F2: membrane/ organelle fraction. F3: nuclei fraction and F4: cytoskeleton fraction. M: molecular weight marker.

5.4.4 Protein profile from 2 subcellular fractions of HRPE cells

Coomassie Blue staining of 10% SDS-PAGE gels show that the protein profile from the Tx-sol fraction (containing cytosol) differs from the Tx-insol fraction (containing cytoskeleton and nuclei). There are more bands from the range of 75 to 250 kDa in the Tx-sol than in Tx-insol (Fig. 5.6).

5.4.5 The effects of γ-secretase inhibitor on the expression of VEGFR-1 in HRPE cells

Western blot analysis was performed on total cell lysates of HRPE cells exposed to γ-secretase inhibitor (13.5 μM) followed by the addition of VEGF/PEDF. The results revealed that γ-secretase inhibitor alone decreased the 75 and 85 kDa fragments of VEGFR-1 ($p<0.05$). Addition of VEGF alone had no statistically significant effect whereas the combination of VEGF and PEDF sustained the effect of the γ-secretase inhibitor ($p<0.01$, $p<0.05$, respectively) (Fig. 5.7).

5.4.6 The effects of γ-secretase inhibitor on the distribution of VEGFR-1 in HRPE cells

Western blotting analysis failed to detect VEGFR-1/VEGFR-2 expression in any of the four subcellular fractions. In an attempt to increase the concentration of the total protein in each fraction, cells were fractionated into two fractions instead (Tx-sol and Tx-insol). Western blot analysis showed that treatment with the γ-secretase inhibitor increased the 75 kDa and 85 kDa fragments of VEGFR-1 in the insoluble fraction of HRPE cells (containing cytoskeleton and nuclei) ($p<0.05$); addition of VEGF or PEDF or a combination of these did not reverse this effect (Fig 5.8).

5.4.7 The effects of γ-secretase inhibitor on the expression of VEGFR-2 in HRPE cells

Western blot analysis demonstrated that the γ-secretase inhibitor reduced the expression of VEGFR-2 ($p<0.05$), addition of VEGF or PEDF alone, or the combination of these reversed the effect of γ-secretase inhibitor and restored the expression of VEGFR-2 (Fig. 5.9).

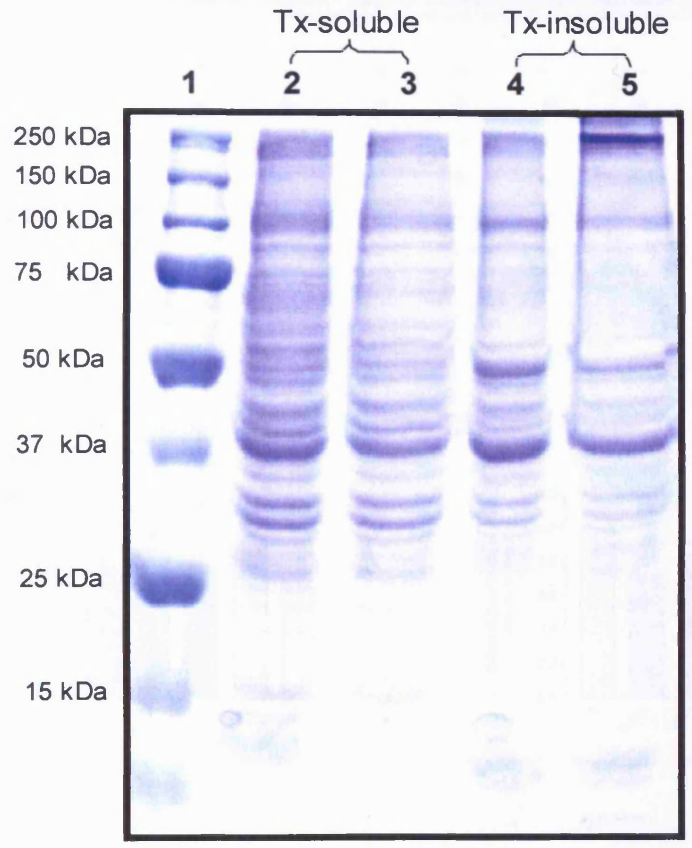


Fig 5.6 Protein profile in two subcellular fractions from HRPE cells by Coomassie staining of 10% SDS-PAGE gels. The two subcellular fractions were obtained using Triton X-100 buffer. Lane1: molecular weight marker; Lane 2 and 3: Triton x-100 soluble fractions. Lane 4 and 5: Triton x -100 insoluble fractions.

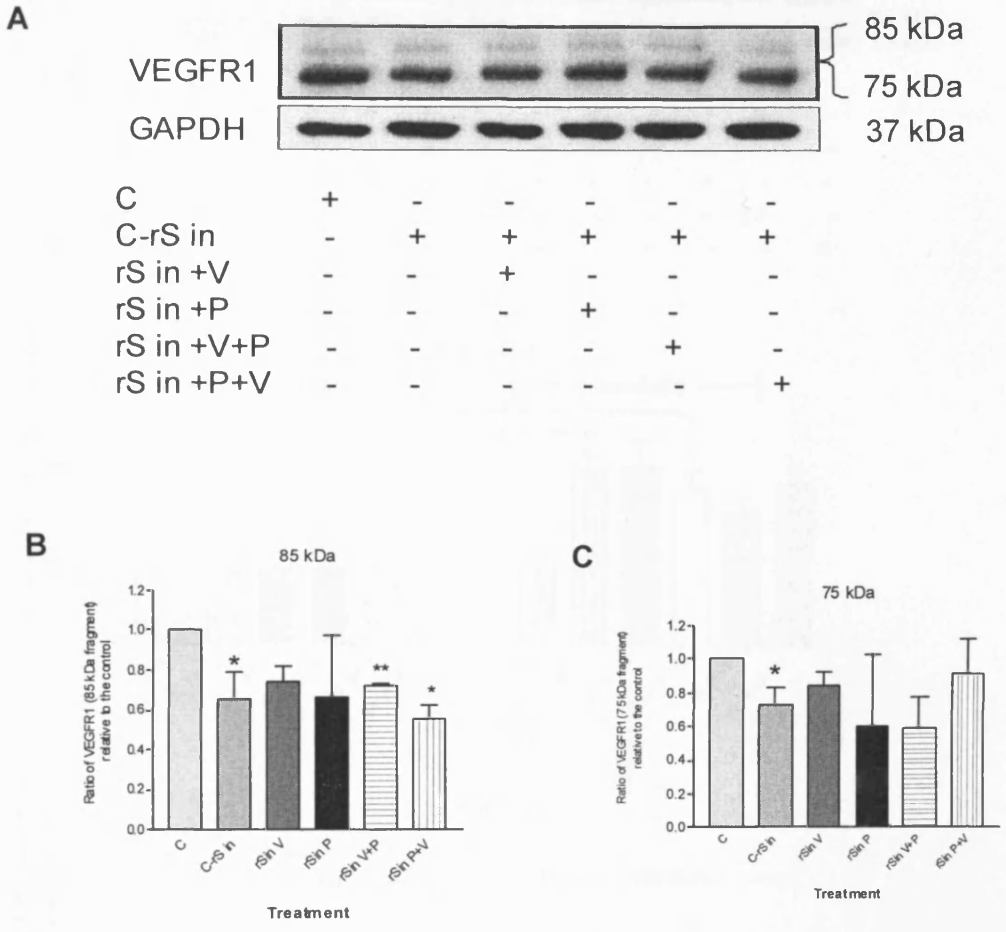


Fig 5.7 The effects of γ-secretase inhibitor on VEGFR-1 expression in HRPE cells by Western blotting. (A) Western blot of VEGFR-1 in HRPE cells. (B&C) Densitometric analysis of immunoblots depicted in (A). Data are normalised with GAPDH from 3 separated experiments and presented as the relative ratio of VEGFR-1 to the control. Data are presented as mean ± SEM. *p <0.05; ** p<0.01. C: non-treatment control. C-rS in: control–γ-secretase inhibitor only. rS-in + V: γ-secretase inhibitor treatment followed by VEGF. rS-in + P: γ-secretase inhibitor treatment followed by PEDF. rS-in + V+P: γ-secretase inhibitor treatment followed by VEGF then PEDF.

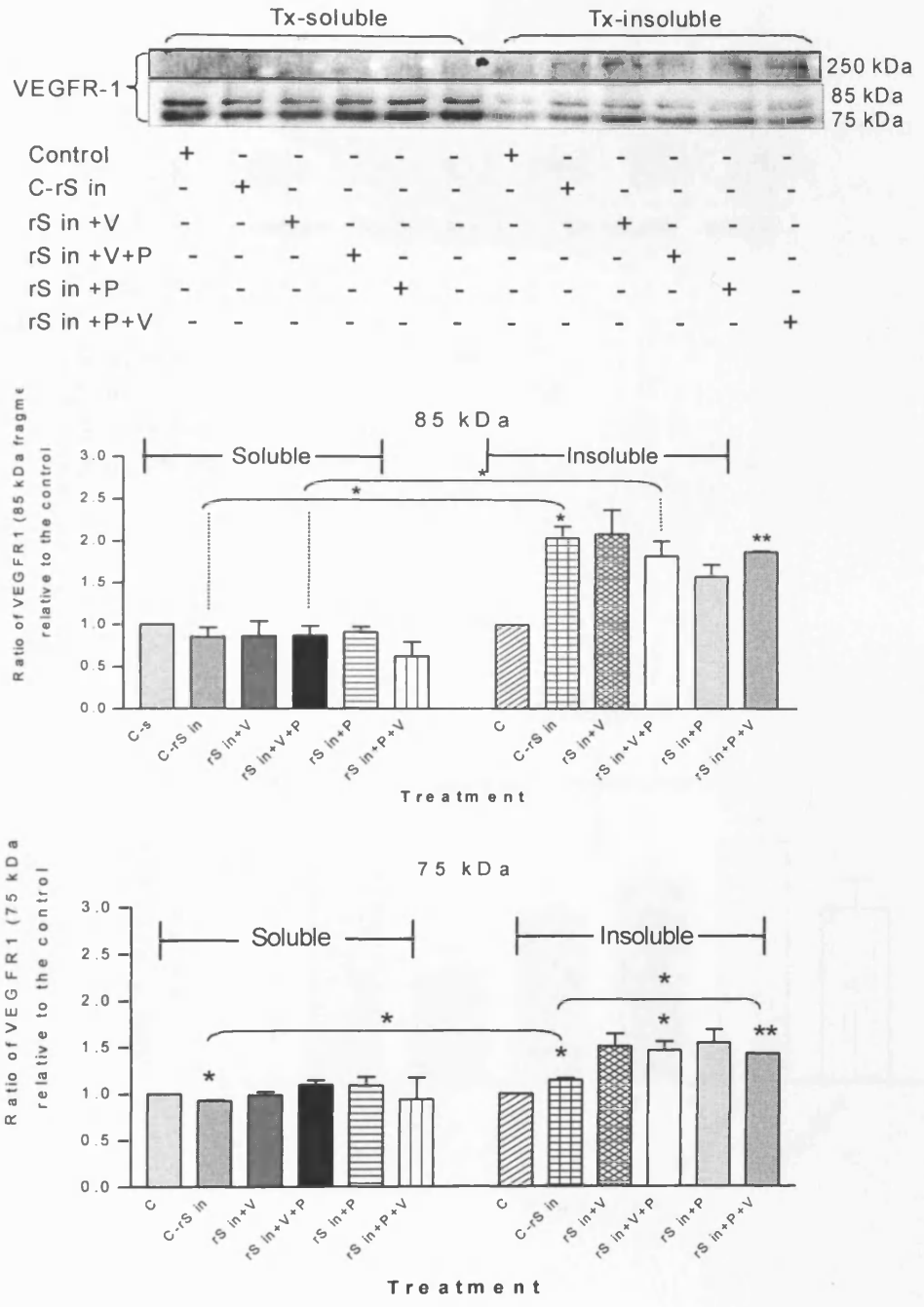
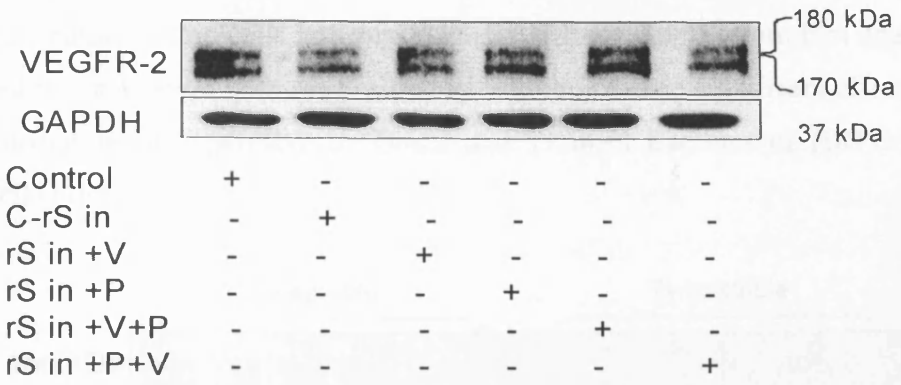


Fig 5.8 The effects of γ-secretase inhibitor on VEGFR-1 distribution in the Tx-s/Tx-ins fractions of HRPE by western blotting. (A) Western blot of VEGFR-1 in Tx-s/Tx-ins fractions. (B & C) Densitometric analysis of immunoblots depicted in (A). Data are presented as the relative ratio of VEGFR-1 to the control from 3 separate experiments. Data are presented as mean ± SEM. *p <0.05; ** p<0.01. C: non-treatment control. C-rS in: control–γ-secretase inhibitor only. rS-in + V: γ-secretase inhibitor treatment followed by VEGF. rS-in + P: γ-secretase inhibitor treatment followed by PEDF. rS-in + V+P: γ-secretase inhibitor treatment followed by VEGF then PEDF.



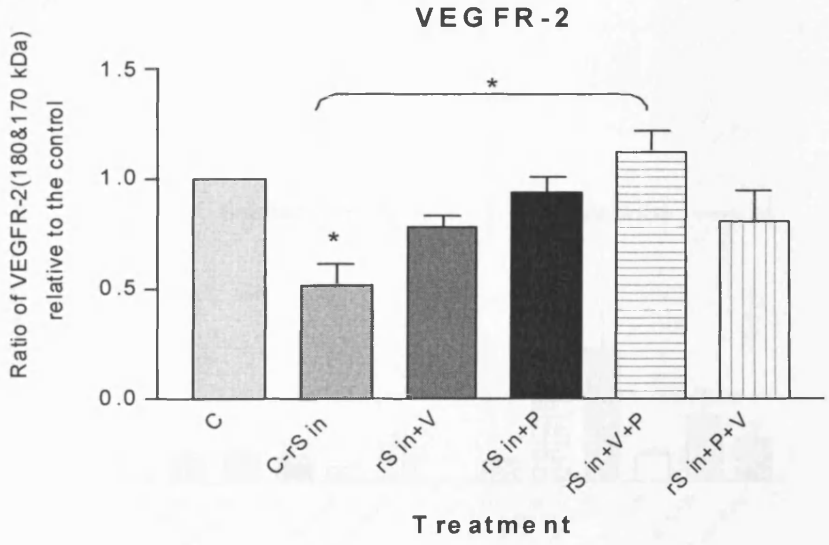


Fig 5.9 The effects of γ-secretase inhibitor on VEGFR-2 expression in HRPE cells by western blotting analysis. (A) Western blot of VEGFR-2 expression in HRPE cells. (B) Densitometric analysis of immunoblots depicted in (A). Data are normalised with GAPDH from 3 separate experiments and presented as the relative ratio of VEGFR-2 to the control. Data are presented as mean ± SEM. *p <0.05. C: non-treatment control. C-rS in: control–γ-secretase inhibitor only. rS-in + V: γ-secretase inhibitor treatment followed by VEGF. rS-in + P: γ-secretase inhibitor treatment followed by PEDF. rS-in + V+P: γ-secretase inhibitor treatment followed by VEGF then PEDF.

5.4.8 The effects of γ-secretase inhibitor on the distribution of VEGFR-2 in HRPE cells

By fractionation of the cells into two subcellular fractions, Western blot analysis revealed that treatment with the γ-secretase inhibitor had no significant effect on the distribution of VEGFR-2 in Tx-sol and Tx-insol fractions of HRPE cells ($p>0.05$) (Fig 5.10).

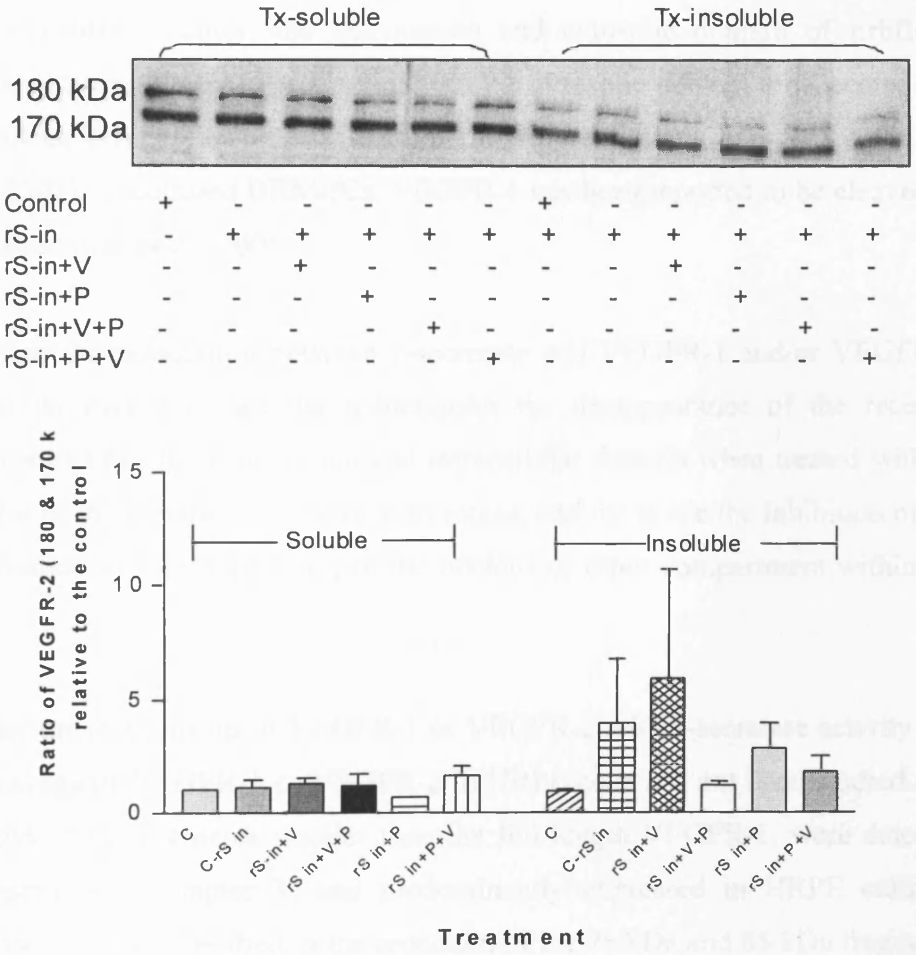


Fig 5.10 The effects of γ-secretase inhibitor on VEGFR-2 distribution in the Tx-s/Tx-ins fractions of HRPE by western blot analysis. (A) Western blot of VEGFR-2 in the Txs/Tx-ins fractions. (B) Densitometric analysis of immunoblots depicted in (A). Data are presented as the relative ratio of VEGFR-2 to the control from 3 separate experiments. Data are presented as mean ± SEM. C: non-treatment control. C-rS in: control–γ-secretase inhibitor only. rS-in + V: γ-secretase inhibitor treatment followed by VEGF. rS-in + P: γ-secretase inhibitor treatment followed by PEDF. rS-in + V+P: γ-secretase inhibitor treatment followed by VEGF then PEDF.

5.5 **Discussion**

A well defined model for the RIP signalling pathway that γ-secretase is involved in has been applied to Notch receptor and ErbB4 as reviewed in chapter 1. In this pathway the signalling is initiated upon the ligand binding. A metalloprotease cleaves the ectodomain of the receptor. Subsequently, a second cleavage mediated by γ-secretase is initiated and results in the release of the intracellular domain from the membrane and facilitates its translocation to the nucleus. In the case of ErbB4 receptor, the ectodomain and cytosolic domain of ErbB4 is cleaved sequentially by two enzymes, and the cytosolic domain translocated into the nucleus to regulate cell proliferation and differentiation (Ni, *et al.*, 2001; Lee *et al.*, 2002). In cultured BRMECs, VEGFR-1 has been reported to be cleaved by γ-secretase (Cai *et al.*, 2006).

To define the association between γ-secretase and VEGFR-1 and/or VEGFR-2, one would expect to see the reduction/or the disappearance of the receptor fragment(s) from the ectodomain and intracellular domain when treated with an inhibitor of the metalloprotease or γ-secretase; and /or to see the inhibition of the translocation of the fragment(s) to the nucleus or other compartment within the cells.

To date, the relationship of VEGFR-1 or VEGFR-2 with γ-secretase activity and the cleavage of VEGFR-1 or VEGFR-2 in HRPE cells has not been studied. In this study, two fragments smaller than the full-length VEGFR-1, were detected (as described in chapter 3) and predominantly expressed in HRPE cells. γ-secretase inhibition resulted in the reduction of the 75 kDa and 85 kDa fragments in the whole cell lysate suggesting that the production of the VEGFR-1 fragments is associated with γ-secretase in cultured HRPE cells. Restoration of the expression of these fragments following the addition of VEGF further supports this relationship. More interestingly is the phenomenon that the inhibitory effect of the γ-secretase inhibitor can be sustained by the combined effect of VEGF and PEDF. These have two implications: 1) VEGF and PEDF

have opposite effects on the γ-secretase inhibitor and 2) PEDF exerts its effect with the presence of VEGF.

Regarding the effects of γ-secretase inhibition on the distribution of VEGFR-1 fragments, the reduction of the fragments in Tx-soluble fraction by the γ-secretase inhibitor suggests that γ-secretase is related to the release of C-terminal fragments of VEGFR-1. It is interesting to note that addition of VEGF or PEDF, or the combination of both cannot reverse the effect of γ-secretase inhibition on the VEGFR-1 distribution. Since the Tx-soluble fraction contains the cytosol whereas the Tx-insoluble fraction contains cytoskeleton and nuclei, these data suggest that γ-secretase regulates VEGFR-1 cleavage and the distribution in HRPE cells.

Similar to the effect on the expression of total VEGFR-1, γ-secretase inhibition resulted in the reduction of VEGFR-2 expression at the total protein level. The addition of VEGF followed by PEDF restored VEGFR-2 expression. This evidence suggest that γ-secretase also regulates VEGFR-2 but the subsequent response to VEGF and PEDF treatment is different from that of VEGFR-1. Another difference is that γ-secretase inhibition had no significant effect on the distribution of VEGFR-2 in Tx-sol/Tx-insol fraction.

Taken together, these results have two implications 1) γ-secretase exists in HRPE cells and is involved in the fragment production of VEGFR-1 and 2) γ-secretase is involved in the VEGFR-2 signalling in a way that is different from VEGFR-1. Further studies are required to clarify the role of soluble fragments of VEGFR-1 in the regulation of angiogenesis.

Chapter 6

The Effects of VEGF-siRNA on the expression of VEGF, PEDF, VEGFR-1, VEGFR-2 and Presenilin-1 in HRPE cells

6.1 Introduction

VEGF plays a central role in vascular homeostasis. It is also a potent angiogenic stimulator and plays an important role in pathological angiogenesis (Shibuya, 2001), such as diabetic retinopathy, choroidal neovascularisation (CNV) (Adamis *et al.*, 1994; Aiello *et al.*1994; Husain *et al,* 2002; Witmer *et al.* 2003) and tumour angiogenesis (Moreira *et al.*, 2007). As reviewed in chapter 1, VEGF increases vascular permeability which might facilitate tumour dissemination via the circulation causing a greater delivery of oxygen and nutrients. It recruits circulating endothelial precursor cells and acts as a survival factor for immature tumour blood vessels. The endotheliotropic activities of VEGF are mediated through the VEGF-specific tyrosine-kinase receptors: VEGFR-1 and VEGFR-2. VEGF and its receptors play a central role in ocular and tumour angiogenesis and, therefore, the blockade of this pathway has been regarded as a promising therapeutic strategy for inhibiting angiogenesis and tumour growth.

Conversely, accumulating evidence has shown that the balance between VEGF and PEDF is crucial for the regulation of vascular permeability and angiogenesis (Zhang *et al.* 2006). Under normal conditions, there is a balance between these two systems which is essential for maintaining the quiescence of retinal vasculature and integrity of the blood-retinal barrier (BRB) (Bussolino *et al.* 1997; Miller *et al.* 1997).

In certain pathological conditions, such as diabetic retinopathy, this balance is disturbed due to the over-production of angiogenic stimulators and the decreased production of angiogenic inhibitors. This will subsequently lead to the BRB breakdown and the over-proliferation of capillary endothelial cells, resulting in retinal neovascularisation (Forsythe *et al.* 1996; Miller *et al.* 1997; Gao *et al.* 2001; Gao & Ma 2002). In animal studies, Gao *et al.* (2001) demonstrated that in rats with oxygen-induced retinopathy, PEDF levels in the retina are significantly decreased in contrast to the increased VEGF levels leading to an increased retinal VEGF/PEDF ratio. This disturbed balance correlates with the formation and progression of neovascularisation. Such evidence suggests an inverse correlation

between VEGF and PEDF levels and that a reciprocal regulation exists between these two major angiogenic regulators (Gao *et al.* 2001; Ohno-Matsui *et al.* 2001; Ohno-Matsui *et al.* 2003; Doll *et al.* 2003).

In contrast, Ogata *et al.* (2002) observed that in rats with experimental CNV, the levels of expression of VEGF and PEDF vary with the severity of CNV. When CNV was active VEGF and PEDF were both strongly expressed in the CNV lesions. However, after the CNV had developed, the expression of both VEGF and PEDF decreased. In another study, Matsuoka *et al.* (2004) reported that VEGF and PEDF were strongly expressed in human active subretinal fibrovascular membranes from age-related macular degeneration (AMD) and polypoidal choroidal vasculopathy (PCV). This evidence suggests that VEGF-induces PEDF gene upregulation and a feedback mechanism may be present in CNV (Tong and Yao, 2006). A similar phenomenon has also been observed in cultured HRPE cells by Ohno-Matsui and colleagues (2003). They demonstrated that VEGF secreted by RPE cells upregulates PEDF expression via VEGFR-1 in an autocrine manner.

Interestingly, it has been demonstrated that VEGF and PEDF both can be produced from the same cells in primary cultures of various cell types, such as cultured HRPE cells (Tombran-Tink *et al.* 1996; Ohno-Matsui *et al.* 2001; Ohno-Matsui *et al.* 2003), cultured primary retinal capillary endothelial cells (Simorre-Pinatel *et al.* 1994; Tombran-Tink *et al.*, 2004), and retinal glial (Muller) cells (Yafai *et al.*, 2007). Understanding the relationship between VEGF and PEDF production in the same cell type may lay the foundation for a different therapeutic approach for the treatment of various angiogenesis related conditions. In chapters 3, 4 and 5, the effect of VEGF and PEDF, on the expression of VEGFR-1, VEGFR-2 and presenilin-1 (PS-1) were investigated in HRPE cells under overlapping of autocrine (HRPE cell dependent) and paracrine (HRPE cell independent) pathways. However, further investigation was needed to define the responsibility of VEGF and PEDF in autocrine and paracrine pathways. Techniques such as neutralising anti-VEGFR-1 or anti-VEGFR-2 antibodies have

been used to study the relationship between VEGF and PEDF in HRPE cells (Ohno-Matsui *et al.* 2003).

RNA interference (RNAi), is the method of choice for studying loss-of-function study and has been applied to silence VEGF expression in various *in vitro* models (Reich *et al.*, 2003; Li *et al.*, 2005; Murata *et al.*, 2006). RNAi has also been used in an attempt to inhibit ocular angiogenesis (Reich *et al.*, 2003; Kim *et al.*, 2004; Murata *et al.*, 2006) or to inhibit the growth of malignant melanoma (Tao *et al.*, 2005) *in vivo*. Despite the above attempts, the effect(s) of the altered expression of specific gene(s) on various downstream signalling and the expression of many other genes remain largely uninvestigated. In the hope of applying small interfering RNA (siRNA) technology to inhibit the expression of VEGF in HRPE cells, this study aimed to dissect the autocrine/paracrine effects of VEGF on PEDF, VEGFR-1, VEGFR-2 and PS-1 expression in HRPE cells.

The principal mechanisms of RNA interference (RNAi)

RNA interference is a normal, endogenous system for regulating gene expression in which short RNA segments, approximately 22 bases in length, form double-stranded structures with an mRNA and either target it for destruction or block its translation. By use of exogenously supplied RNAi sequences, scientists have taken advantage of this system to design new and powerful technologies for gene silencing (Nussbaum *et al.*, 2007; Sandy *et al.* 2005). This enables scientists to analyse the effect of a gene on cellular function in a quick and easy way.

Long double-stranded RNAs (dsRNAs; typically >200 nt) can be used to silence the expression of target genes in many non-mammalian systems, such as worms fruit flies and plants (reviewed in Bantounas *et al.*, 2004). Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNAi pathway. First, an RNase III-like enzyme called Dicer cleaves the dsRNAs into 20-25 nt small interfering RNAs (siRNAs). Next the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing

complexes (RISCs), unwinding in the process. The antisense siRNA strand subsequently guides the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effect step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand, leading to specific gene silencing (Fig 6.1, A).

Upon introduction of long dsRNA (>30 nt), most mammalian cells initiate a potent antiviral response, exemplified by non-specific inhibition of protein synthesis and RNA degradation. To bypass the antiviral response in mammalian cells, RNAi is induced to these systems by either transfecting cells with siRNA (typically 21 bp RNA molecules with 3' dinucleotide overhangs) or by using DNA based vectors to express short hairpin RNA (shRNA) that are processed by Dicer into siRNA molecules (Elbashir *et al.*2001, Caplen *et al.* 2001, Caplen & Mousses, 2003; Hutvagner *et al.* 2001; Hutvagner, 2005) (Fig 6.1, B and C, respectively).

Aims

The aims of this study were to investigate

1) The autocrine effect of VEGF on PEDF, VEGFR-1, VEGFR-2 and PS-1 expression in cultured HRPE cells by knockdown of the VEGF gene using VEGF-siRNA.

2) The paracrine effect of VEGF/PEDF on VEGFR-1, VEGFR-2 and PS-1 expression in cultured HRPE cells by knockdown of the VEGF gene prior to the addition of exogenous VEGF /PEDF.

6.2 Project design

In order to meet the goals of this study, the experiment was designed to be carried out in four stages: 1) preparation of VEGF-siRNA, 2) evaluation of the specificity and efficiency of the VEGF-siRNA, 3) establishment of analytical methods for analysis of the effect of VEGF-siRNA and 4) correlating silencing with biological effect. A flow chart for the experimental design is shown in Fig.6.2.1. & Fig 6.2.2.

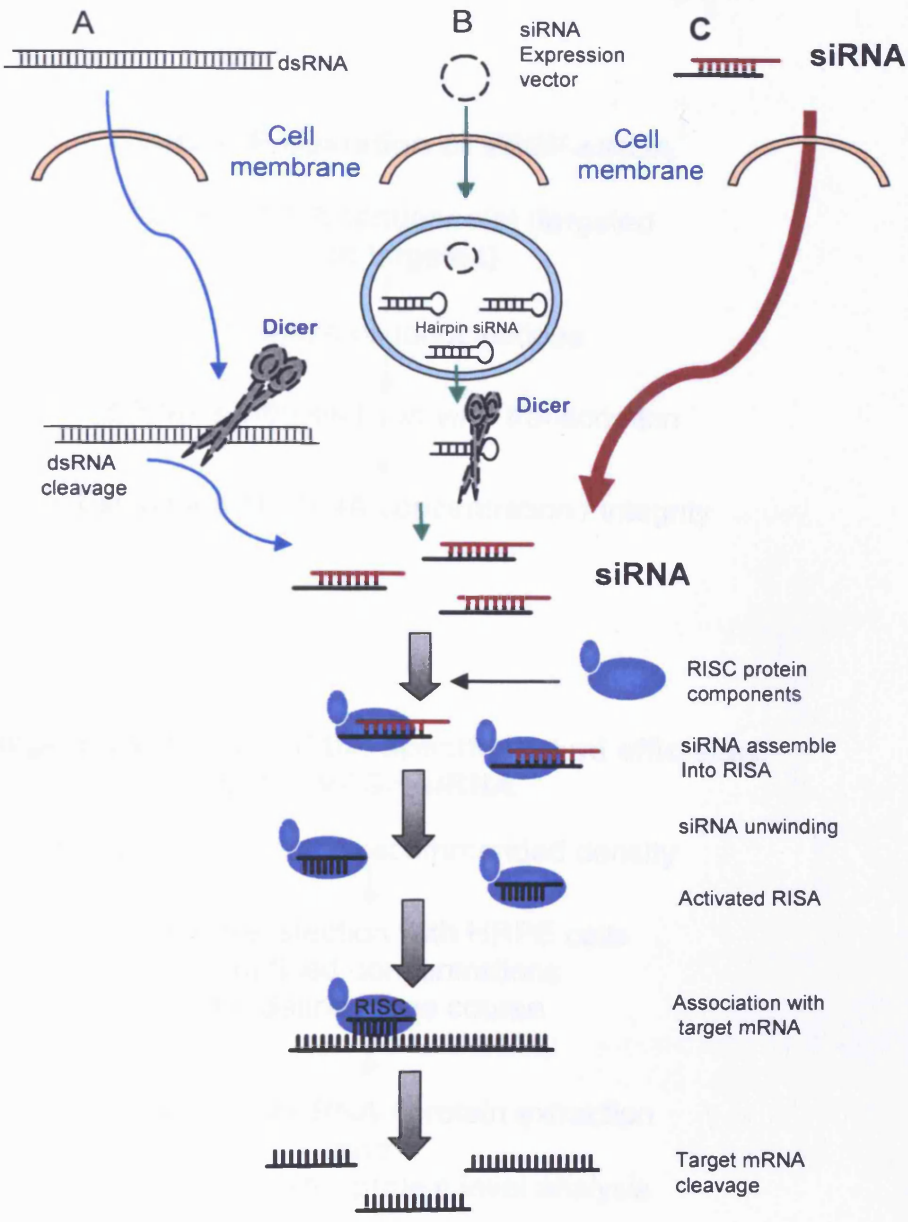


Fig 6.1 The RNAi Pathway. (A) In non-mammalian systems, the RNAi pathway commences when dsRNA (>30bp) is introduced into cells. In mammalian systems RNAi can be triggered by DNA based expression vectors designed to express (B) short hairpin RNA (C) or synthetic siRNA molecules .

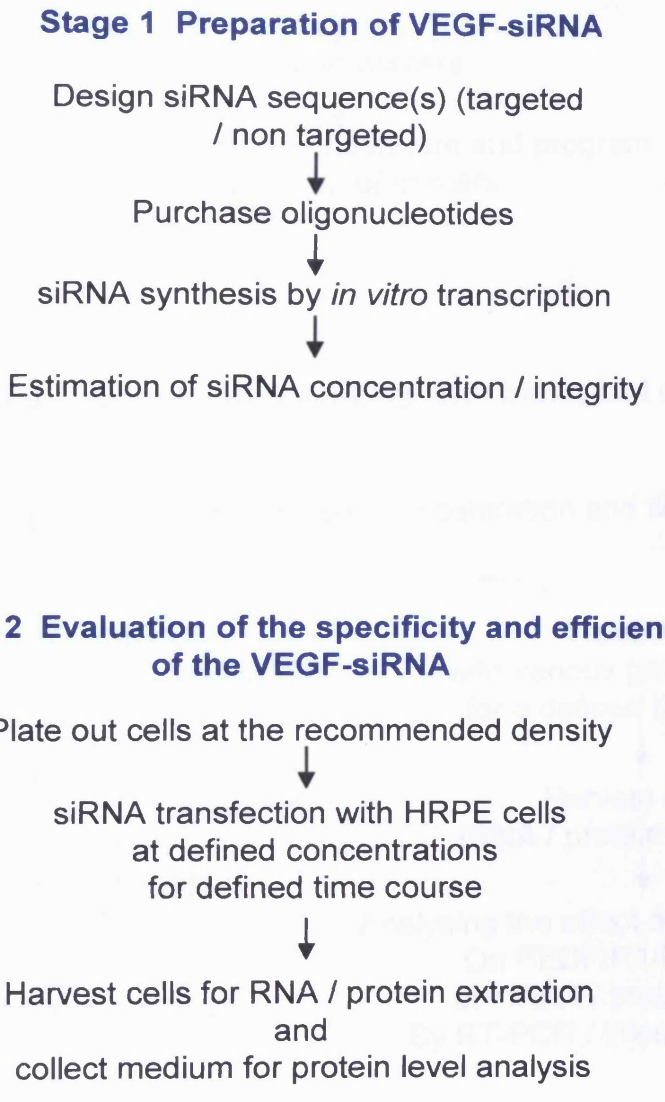


Fig 6.2.1 Flow chart of experimental design for chapter 6.

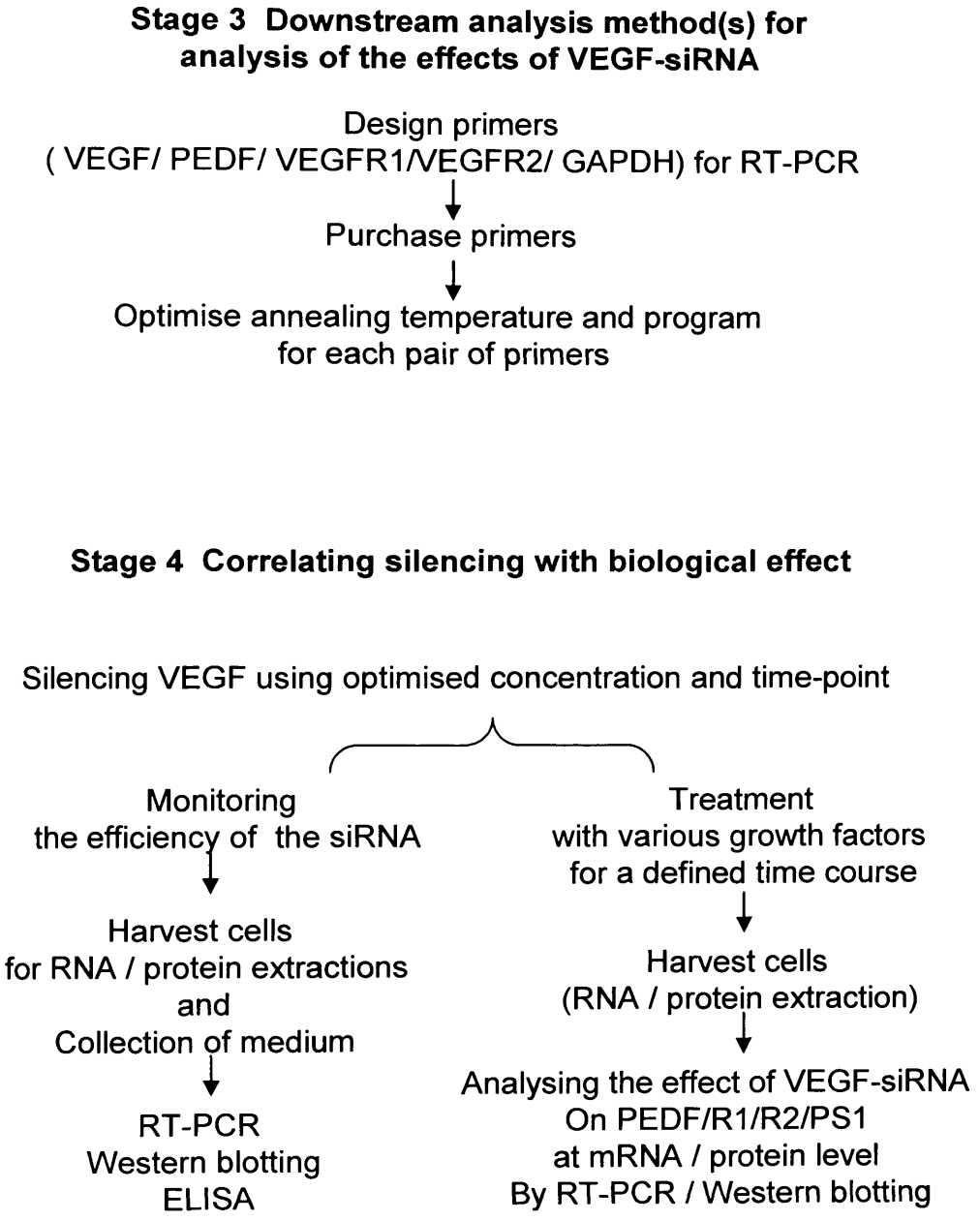


Fig 6.2.2 Flow chart of experimental design for chapter 6.

6.3 Materials & Methods

6.3.1 HRPE cell culture

The isolation, culture and characterisation of HRPE cells were performed as described in 2.2.1. Cells were used within passage 3.

6.3.2 Preparation of siRNA

By *in vitro* transcription, siRNAs were constructed using the *Silencer* siRNA Construction Kit (Ambion, Inc.USA). This kit overcomes the sequence requirements of traditional in vitro transcription strategies by using siRNA template oligonucleotides containing a 'leader' sequence that is complementary to the T7 Promoter Primer included in the kit. The procedures fo r constructing siRNA were as recommended by the manufacture and shown in Fig 6.3.

6.3.2.1 siRNA Design

siRNA specific for human VEGF was designed in two steps according to the instruction of "siRNA Design" that is recommended by Ambion (www.ambion.com/techlib/misc/siRNA_design.html).

The first step was to find 21 nt sequences in the target mRNA that begin with an AA dinucleotide, based on the observation by Elbashir *et al.* (2001) that siRNA with 3' overhanging UU dinucleotides are the most effective. The potential siRNA target sites were selected with 21 nt sequences in the target mRNA that begin with an AA dinucleotide. In order to avoid the cleavage by RNase at single-stranded G residues, the sequence within G residues in the overhang were excluded; the siRNA with 30-50% G/C content was selected rather than those with a higher G/C content.

The second step was to convert the designed siRNA sequence into oligonucleotides based on the following guidelines: 1) The DNA counterpart (antisense template oligonucleotide) of the target mRNA sequence should have the same sequence as the target RNA but U residues are replaced with T's. 2) The sense template oligonucleotide should start with an AA dinucleotide at the 5'

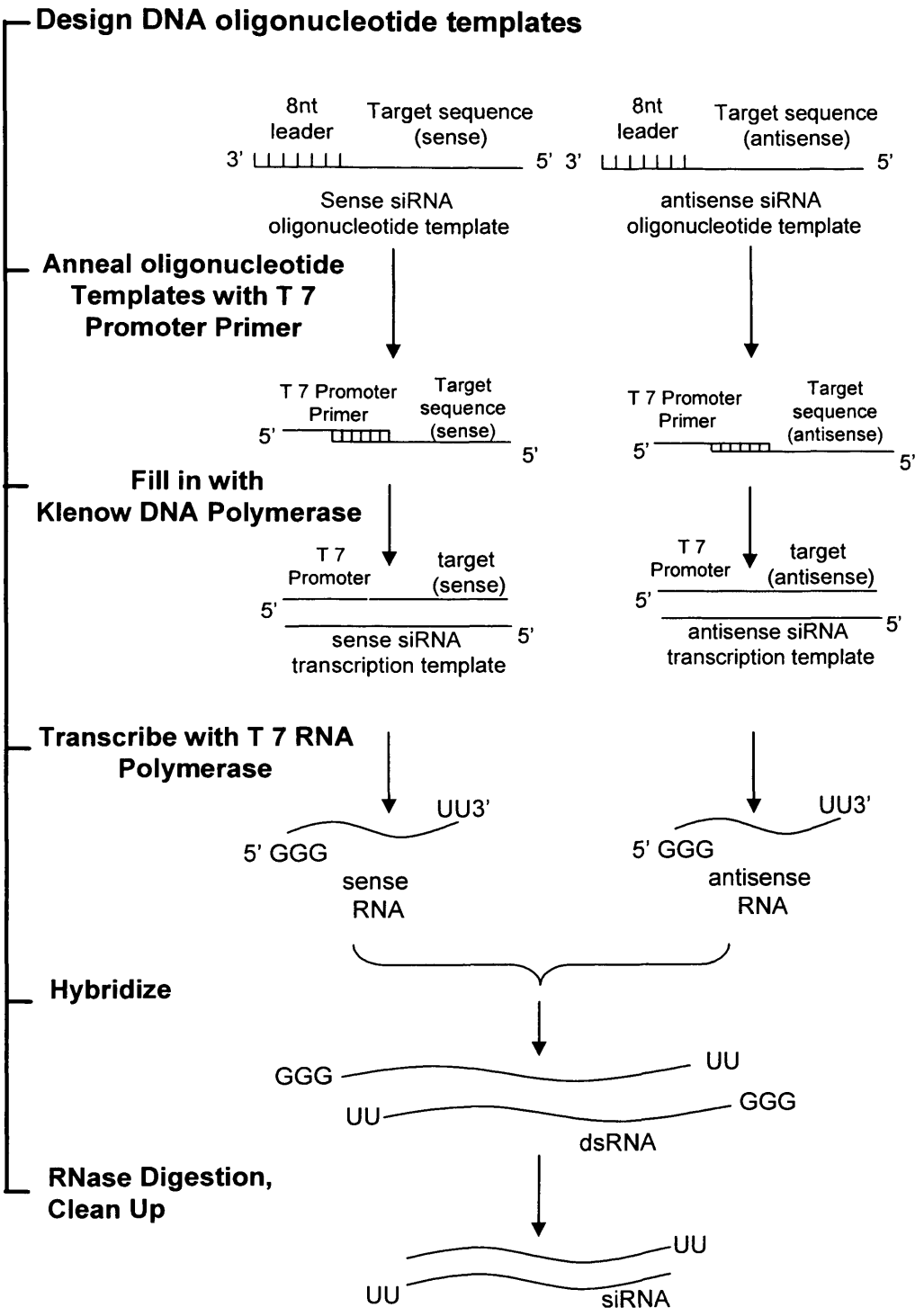


Fig 6.3 Silence siRNA Construction Kit Procedure (Adapted from the *Silencer*® siRNA Construction Kit manual)

end followed by 19 nt that are complementary to the target sequence. 3) The 8 nt at the 3' end of both oligonucleotides should have the following sequence: 5'-CCTGTCTC-3' (which is complementary to the T7 promoter Primer provided with the *Silencer* siRNA Construction Kit). Table 6.1 shows the sequences of the DNA oligonucleotides that were ordered from Sigma-Aldrich.

Table 6.1 Sequences of the oligonucleotides for VEGF-siRNA and scrambled siRNA.

	Sequence (5'-3')
siVEGFant	AAACCTCACCAAGGCCAGCACCCTGTCTC
siVEGFsen	AAGTGCTGGCCTTGGTGAGGTCCTGTCTC
cVEGFant	AAACTACCGTTGTTATAGGTGCCTGTCTC
cVEGFsen	AACACCTATAACAACGGTAGTCCTGTCTC

6.3.2.2 Construction of siRNA

6.3.2.2.1 Preparation of transcription template

The sense and antisense template oligonucleotides (DNA) for each siRNA must be converted to dsDNA with a T7 promoter at the 5' end to make an efficient transcription template. This is accomplished by hybridising the 2 oligonucleotides to the T7 Promoter Primer provided with the *Silencer* siRNA Construction Kit and extending the T7 promoter Primer and template oligonucleotides using a DNA polymerisation reaction.

Purchased DNA-oligonucleotides were resuspended in nuclease-free water to a final concentration of 200 μM.

To hybridise each template oligonucleotide to the T7 Promoter Primer, 2 μl of T7 Promoter Primer, 6 μl of DNA Hyb Buffer and 2 μl of either sense or antisense template oligonucleotide were mixed together in separate tubes and heated to 70°C for 5 minutes, then left at room temperature for 5 minutes. Then the following were added: 2 μl of 10x Klenow Reaction Buffer, 2 μl of 10x dNTP

Mix, 4 µl of Nuclease-free water and 2 µl of Exo-Klenow and incubated at 37°C for 30 minutes.

6.3.2.2.2 Synthesis of dsRNA

Transcription was performed in 20 µl of transcription mix: 2 µl of sense or antisense siRNA template, 4 µl of Nuclease-free water, 10 µl of 2x NTP Mix, 2 µl of 10x T7 Reaction Buffer and 2 µl of T7 Enzyme Mix. This was incubated at 37°C for 2 hours, and then the sense and antisense transcription reaction were combined into a single tube and incubated overnight at 37°C.

6.3.2.2.3 Preparation and Purification of siRNA

The dsRNA made by in vitro transcription has a 5' overhanging leader sequences that must be removed prior to transfection. The leader sequence is digested by a single-strand specific ribonuclease, RNase and DNase. This was achieved by adding 6 µl of Digestion Buffer, 48.5 µl of Nuclease-free water, 3 µl of RNase and 2.5 ul of DNase to the tube of dsRNA from 6.3.2.2.2., and incubating at 37°C for 2 hours. The siRNA was eluted by adding 400 µl siRNA Binding Buffer to the nuclease digestion reaction and incubated at room temperature for 2-5 minutes. The resulting siRNA was purified by glass fiber filter binding and elution which removes excess nucleotides, short oligomers, proteins, and salts in the reaction. The end product was a double-stranded 21-mer siRNA with 3' terminal uridine dimers that can effectively reduce the expression of target mRNA when transfected into mammalian cells.

6.3.2.3 siRNA quantification

The concentration of the siRNA synthesised was measured by diluting a small sample of the siRNA 1:25 into TE (10 mM Tris-HCl pH8, 1mM EDTA) and reading the absorbance at 260 nm in a spectrophotometer.

The concentration of the purified siRNA in µg/ml was determined by multiply the absorbance reading by 1,000 (1,000 = 25-fold dilution x 40 µg siRNA/ml per absorbance unit).

The molar concentration of the siRNA in μM was determined by dividing the μg/ml concentration of the siRNA by 14 (there are 14 μg of RNA in 1 nmole of an average 21-mer dsRNA: 21nt x 2 strands = 42 nt x 0.333 μg/nmol for each nt = 14 μg/nmol).

6.3.2.4 Estimation of siRNA integrity

The integrity of the siRNA was determined by analysis of 4ul of siRNA sample, which was mixed with gel loading buffer at 5:1 (sample: buffer), on a 2 % agarose gel (0.05 ug/ml ethidium bromide) in TBE buffer at 80 volts. Electrophoresis was stopped when the bromophenol blue dye front had migrated two-thirds of the way down the gel.

6.3.3 siRNA Transfection

The choice of siRNA concentration for transfection is critical to the success of gene silencing experiments. Transfecting too much siRNA caused non-specific reductions in gene expression and toxicity to the transfected cells. Transfecting too little siRNA does not change the expression of the target gene.

The choice of a transfection agent for delivery of siRNA is critical for gene silencing experiments. Without efficient transfection siRNA will fail to elicit a cellular response. The *Silencer*® siRNA Transfection II Kit (Ambion, Inc.USA) provides two different transfection agents, siPORT *NeoFX* and siPORT *Amine*. siPORT *NeoFX* is a proprietary mixture of lipids and is compatible with a wide range of cell lines and experimental designs. siPORT *Amine* is a proprietary blend of polyamines that delivers siRNA into mammalian cells with minimal cytotoxicity. Both transfection reagents function by complexing with siRNAs and facilitating their transfer into cells. In this study, siPORT *Amine* was chosen to deliver siRNA into cells, as it has been used successfully in HRPE cells by other users (Li *et al.*, 2005).

In order to provide good gene knockdown while maintaining an acceptable level of cell viability for the particular cell type, optimisation had to be carried out to determine the conditions that produced the best results.

VEGF-siRNA (0, 5, 10, 20, and 30nM) was transfected into HRPE cells for a defined time course of 24, 48 and 72 hours. Scrambled siRNA, at the correspondent concentration to the siRNA, was transfected into HRPE cells as a control (detailed in section 6.3.4).

Approximately 24 hours before transfection, RPE cells were plated in normal growth medium at a cell density of $2x10^5$ ($1x10^5$cell /ml) in 6-well plates, to give 50% to 70% confluency after 24 hours. In sterile polystyrene tubes, siPORT Amine was diluted into Ham's F-10 medium to the concentration recommended by the manufacturer and incubated at room temperature for 30 minutes. Next siRNA was added to the diluted siPORT Amine Transfection Agent and incubated at room temperature for 20 minutes. After renewing the normal growth medium in each well, the Transfection Agent and siRNA complex was overlaid onto the cells. Following incubation of cells under normal cell culture conditions for 24 hours, 1 ml/well of fresh 10% FCS Ham's F-10 was added. The cells were harvested at 24, 48 and 72 hours post transfection for analysis. The medium from each time point also collected for assays.

6.3.4 **siRNA controls** (Whither RNAi? 2003)

In order to monitor the efficiency, non-targeting effect or cytotoxicity effect of the siRNA, the following controls were included in each assay: 1) a negative control siRNA (also called scrambled siRNA or non-targeting siRNA). This was designed by scrambling the nucleotide sequence of the gene-specific siRNA and lacked significant sequence homology to the genome. This scrambled siRNA was included in each assay in order to identify non-specific effects such as non-sequence-specific siRNA effects, cytotoxicity of the transfection agent and/or the siRNA, or suboptimal transfection conditions; 2) a transfection reagent-only control was included for any nontransfection related phenomena; 3) a non-

treatment control which only has normal medium was included. 4) GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was included to monitor the efficiency of the siRNA, and the effect on the targets involved in the down-stream biological function.

Apart from the controls listed above, the efficiency of the siRNA was monitored at the mRNA level using semi-quantitative and protein level using quantitative approaches. In order to reduce the chance of side effects as well as providing grade readout of the effect the siRNA was titrated to the lowest possible level.

6.3.5 RNA extraction

6.3.5.1 Homogenisation of HRPE cells

Cells were lysed directly in a well of a 6-well plate by adding 1 ml of TRIzol® Reagent (Invitrogen) and passing the cell lysate several times through a pipette. The lysate was collected into an eppendorf tube and stored at -80°C until further steps were carried out.

6.3.5.2 Phase separation

The homogenised samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml /sample) was added to the RNA-containing TRIzol, mixed by inverting the tube several times and allowed to stand for 5 minutes at ambient temperature. The whole contents were transferred into a phase lock gel tube and centrifuged at 12,000g for 15 minutes at 4°C. The upper RNA-containing aqueous phase was separated and transferred to a new eppendorf tube. All reagents and plasticware used for RNA extraction were RNase free.

6.3.5.3 RNA precipitation

The RNA in the aqueous phase was precipitated by mixing with an equivalent volume of isopropanol (Sigma, USA). Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000g for 10 minutes at 4°C. The RNA precipitate, a gel-like pellet was located on the side/bottom of the tube.

6.3.5.4 RNA wash

The supernatant was removed and the RNA pellet washed once with 1 volume of 75% ethanol. The sample was mixed by vortexing and centrifuged at 7,500g for 5 minutes at 4°C.

6.3.5.5 Redissolving the RNA

The supernatant was removed and the RNA pellet was briefly air-dried for 5-10 minutes. The RNA pellet was dissolved in 40 ul of RNase free water and incubated at 60°C for 10 minutes.

6.3.5.6 Estimation of RNA concentration and integrity

RNA concentration was determined by spectrophometric analysis at a wavelength of 260 nm and 280 nm (GeneQuant II, Pharmacy); an A_{260}/A_{280} ratio of 1.8-2.0 is expected. The integrity of the RNA was determined by analysis of 1ug on a 1.5% agarose gel (containing 0.05 ug/ml ethidium bromide) in TBE buffer at 100V for 45 -60 minutes. The ribosomal RNA bands (28S and 18S) were visualised by an UV transilluminator (UVIDOC, version 99.01).

6.3.6 RT-PCR (Revrse Transcription –Polymerase Chain Reaction)

6.3.6.1 Primer design and optimisation

Primers were designed, using Primer3, to the mRNA sequences of genes of interest for semi-quantifying cDNA samples and generating amplicons of ≤200bp. The sequences of forward and reverse primer pairs are shown in Table 6.2.

Table 6.2 Oligonucleotide primer sequences.

Gene	Primer sequence	T_{Anneal} (°C)	Product size(bp)
VEGF	F. 5' - GGG CAG AAT CAT CAC GAA GT R. 5' – TGG TGA TGT TGG ACT CCT CA	58	211
PEDF	F.5'-GTG GCA CCT CTG GAA AAG TC R.5'-ACC GAG AAG GAG AAT GCT GA	58	165
VEGFR1	F.5'- TGT CAA TGT GAA ACC CCA GA R.5'- GTC ACA CCT TGC TTC GGA AT	58	175
VEGFR2	F.5'-AGC GAT GGC CTC TTC TGT AA R.5'-ACA CGA CTC CAT GTT GGT CA	58	172
GAPDH	F.5'-TGA TGA CAT CAA CAA GGT GGT GAA R.5'-TCC TTG GAG GCC ATG TGG GCC AT	58	235

The annealing temperature for each primer pair was optimised using the programme shown in the Table 6.3 with a gradient annealing temperature from 50°C to 62°C. The temperature that produced a single band with strongest signal was chosen as the annealing temperature for each pair of the primer. The optimised associated annealing temperature for each pair of primers used in this study is shown in the Table 6.3.

Table 6.3 Program used for PCR reaction.

	Programme	Cycle Numbers
Initial denaturation	94°C, 5 min	1 cycle
Denaturation Annealing Extension	94°C, 30 sec 58°C, 30 sec 72°C, 1 min	35 cycles
Final extension	72°C, 7 min	1 cycle

6.3.6.2 Reverse transcription

cDNA was produced by priming 1ug RNA (~11 µl) with 1 µl (500 ng/µl) of anchored oligo dT (Abgene, UK) at 70°C for 5 minutes to remove any secondary structure then placed on ice immediately. Addition of 4 µl of 5x First Strand Synthesis buffer, 2 µl dNTP mix (5mM each), 1 µl Reverse-iT™ RNase Blend and 1 µl 100 mM DTT were added. Samples were incubated at 47°C for 30 minutes. The reaction was terminated by incubation of the samples at 75°C for 10 minutes. cDNA samples were stored at -20°C. All the reagents were from Reverse-iT™ 1st Strand Synthesis Kit purchased from Abgene (UK).

6.3.6.3 Polymerase chain reaction (PCR)

A standard PCR reaction was used to selectively amplify the gene products of interest. 2 µl of cDNA and 0.5 µl combined forward and reverse primers (mix of 0.25 µl forward and 0.25 µl reverse) were added to 22.5 µl of ReddyMix™ PCR Master Mix (Abgene, UK) resulted in a final reaction volume of 25 µl, containing 0.625 units Thermoprime Plus DNA Polymerase, 75 mM Tris-HCl,

20 mM $(NH_4)_2SO_4$, $MgCl_2$ and 0.01% (v/v) Tween® 20 and dNTPs (0.2mM each).

Reactions were performed using the program shown in Table 6.3. on a PCR mechine (PTC-220 DNA Engine Dyad™ Peltier Thermal Cycler –MJ Research Inc. USA).

6.3.6.4 Detection of RT-PCR product

The RT-PCR products were detected by gel electrophoresis. 1.5% Agarose gel was prepared by dissolving 1.5 g of Agarose powder (Sigma-Aldren, USA) in 100 ml of 1x Tris-Borate-EDTA (TBE) buffer (Sigma-Aldren, USA) containing 0.05 μg/ml of Ethidium Bromide (Sigma).

6.3.7 Quantification of VEGF/PEDF by Enzyme-Linked Immunosorbent Assay (ELISA)

The total amount of VEGF or PEDF in the cell culture medium was quantified using an ELISA kit specific for $VEGF_{165}$ (R&D Systems, Minneapolis, MN, USA) and PEDF (Chemicon Inc.) according to the manufacturers' instructions.

6.3.7.1 Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour develops in proportion to the amount of VEGF bound in the initial step. The colour development is stopped and the intensity of the colour is measured (Fig 6.4).

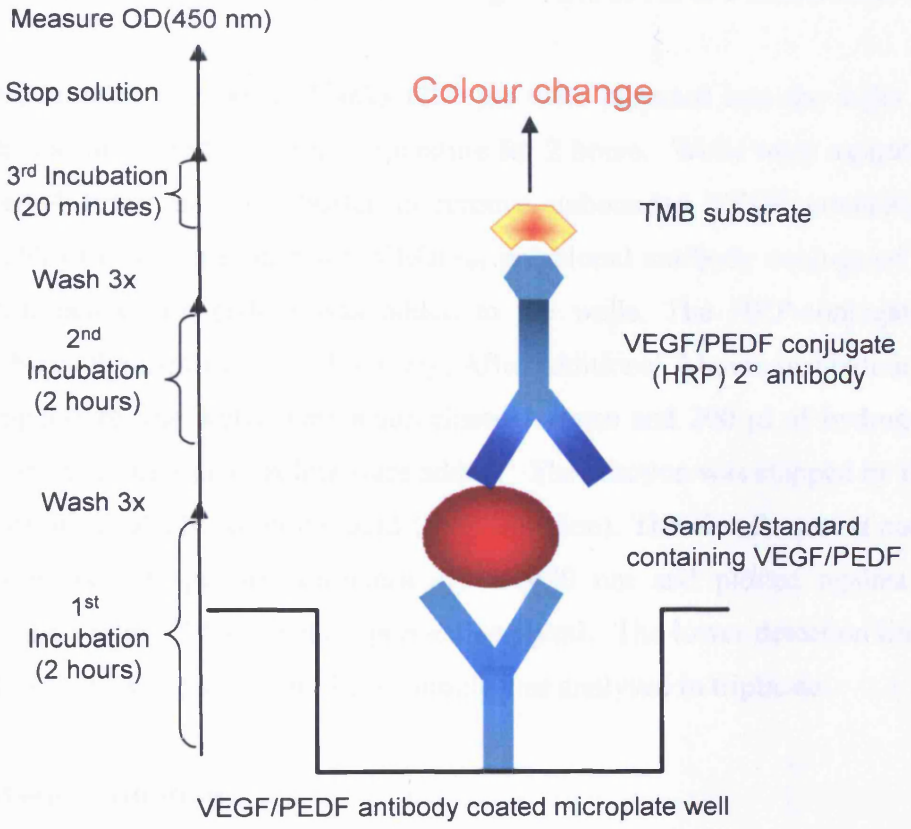


Figure 6.4 Schematic representation of the Enzyme- Linked Immunosorbent Assay (ELISA) (Modified from Emma Blain, PhD Thesis, 2002).

6.3.7.2 Procedures of the assay

The recombinant VEGF standard was reconstituted with 1 ml of Calibrator Diluent RD5K to a stock solution of 2000 pg/ml, and a serial 2-fold dilution was prepared to produce a standard curve ranging from 31.2 pg/ml to 1000 pg/ml. All other reagents were reconstituted according to manufacturer's instructions.

Standards, samples and buffer blanks (200 µl) were pippeted into the wells in triplicates and incubated at room temperature for 2 hours. Wells were aspirated and rinsed 4 times in wash buffer to remove unbounded VEGF containing sample. 200 µl of recombinant anti-$VEGF_{165}$ polyclonal antibody conjugated to horseradish peroxidase (HRP) was added to the wells. The HRP-conjugated antibody binds the captured VEGF moiety. After additional 2 hours incubation at room temperature, the wells were again rinsed 3 times and 200 µl of hydrogen peroxide and tetramethylbenzidine were added. The reaction was stopped by the addition of 50 µl of 2N sulphuric acid (Stop Solution). The absorbance of each well was measured spectrophotometrically at 450 nm and plotted against a standard curve with VEGF levels expressed as ng/ml. The lower detection limit of the ELISA assay is 5.0 pg/ml. Each sample was analysed in triplicate.

6.3.8 Western Blotting

Western blotting was used to analyse the effect of VEGF-siRNA on VEGFR-1, VEGFR-2 and PS-1 expression in HRPE cells with or without additional growth factor treatment. The procedures of western blotting were the same as described in section 4.2.6.

6.3.9 Statistical analysis

The results are given as the mean ± standard error (SEM). Students T-test was performed to analyse the data (SPSS). A p value less than 0.05 ($p<0.05$) was considered statistically significant.

6.4 Results

6.4.1 The efficiency and specificity of the VEGF-siRNA

6.4.1.1 siRNA concentration and integrity

The concentration and purity of the siRNAs were estimated by spectrophotometer, and the result revealed that approximately 100-130 ug (in 100 μl volume) of siRNA was synthesised by in vitro transcription using the kit. The integrity of the siRNA was confirmed by gel electrophoresis (Figure 6.5).

The siRNAs synthesised show similar size and density when compared with the GAPDH-siRNA (Fig.6.5 column 5) which was provided within the Kit.

6.4.1.2 RNA concentration and integrity

The concentration and purity of the RNA was estimated by spectrophotometer. The results revealed that approximately 7-15 μg (in 30 μl of volume) of total RNA was extracted from a 6-well plate when cells were at 50-95% confluence. Mean A_{260}/A_{280} ratios of 1.805 ± 0.156 (n=3) were obtained. The integrity of the RNA was confirmed by gel electrophoresis using 1ug/sample, and the intensity of the band from siRNA treatment /scrambled siRNA / non-treatment were similar: sharp, clear 28S and 18S rRNA bands were observed in the samples with VEGF-siRNA treatment and the samples with the scrambled siRNA or no treatment. The 28S rRNA band is approximately twice as intense as the 18S rRNA band; and this 2:1 ratio (28S:18S) is a good indication that the RNA is intact (Figure 6.6).

6.4.1.3 siRNA effectively and specifically suppressed VEGF mRNA in HRPE cells

In a time course of 24, 48 and 72 hours, the efficacy and potency of the siRNA targeting VEGF was assessed by monitoring VEGF mRNA levels in cells that had received a range of siRNA concentrations (0, 5, 10, 20 and 30 nM), and compared to that in cells treated with a nontargeting negative control siRNA (scrambled siRNA) at the corresponding concentration, and in cells without any treatment (Fig. 6.7)

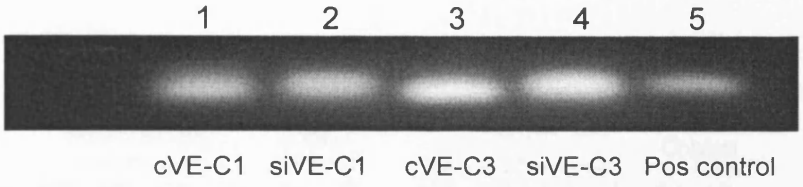


Fig 6.5 The intergrity and quality of VEGF-siRNA synthesed by in vitro transcription (2% Agarose gel). Lane1 and 3 are scrambled siRNA synthesed at separated times (cVE-C1 and cVE-C3); lane 2 and 4 are VEGF-siRNAs (siVE-C1 and siVE-C3); lane 5 is GAPDH-siRNA provided with the Transfection kit (Positive control).

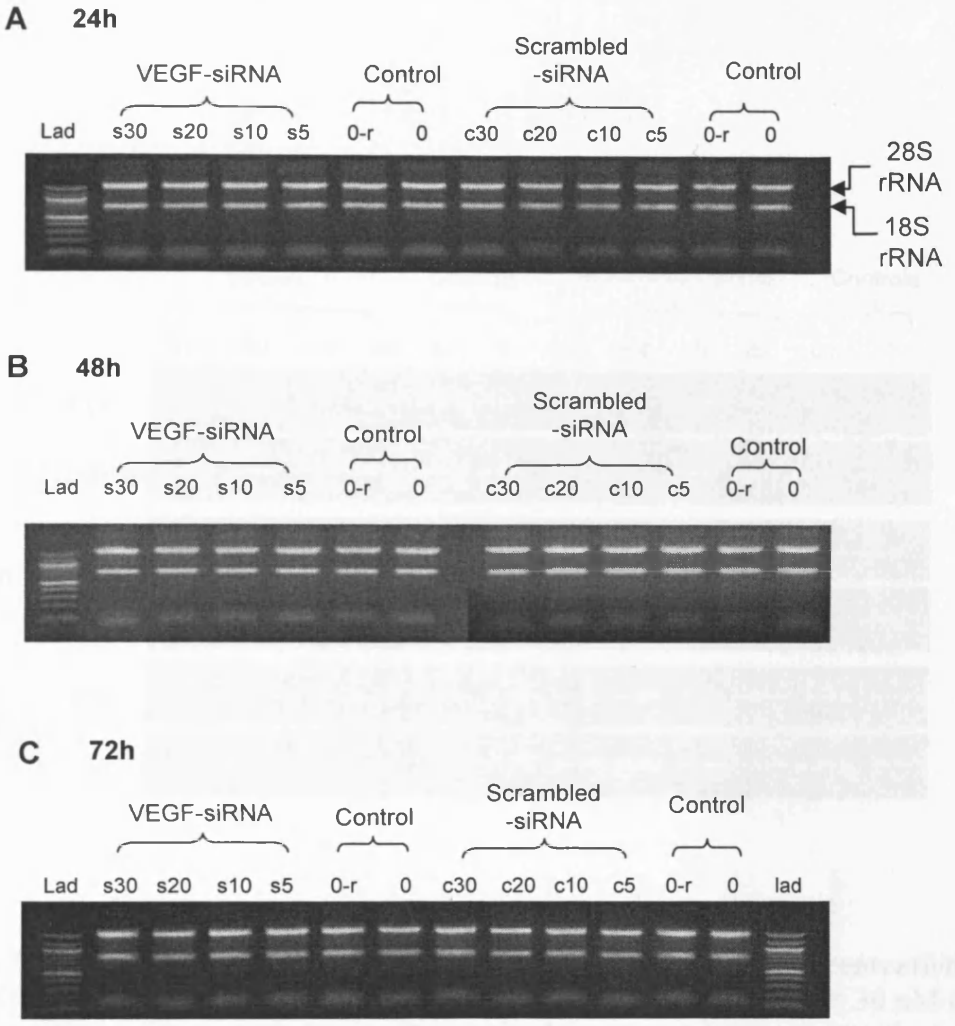


Fig 6.6 The integrity and quality of total RNA extracted by Trizol Reagent and analysed by 1.5% Agarose gel. A: total RNA extracted from cells 24 hours post transfection with various concentration of VEGF-siRNA (5-30 nM) and correspondent scrambled siRNA and controls; B: total RNA extracted from cells 48 hours post transfection; C: total RNA from 72 hours post transfection. Lad = DNA ladder; s30 = 30 nM of VEGF-siRNA; c30 = scrambled siRNA 30 nM; 0-r = control of transfection reagents only and 0 = non-treatment control.

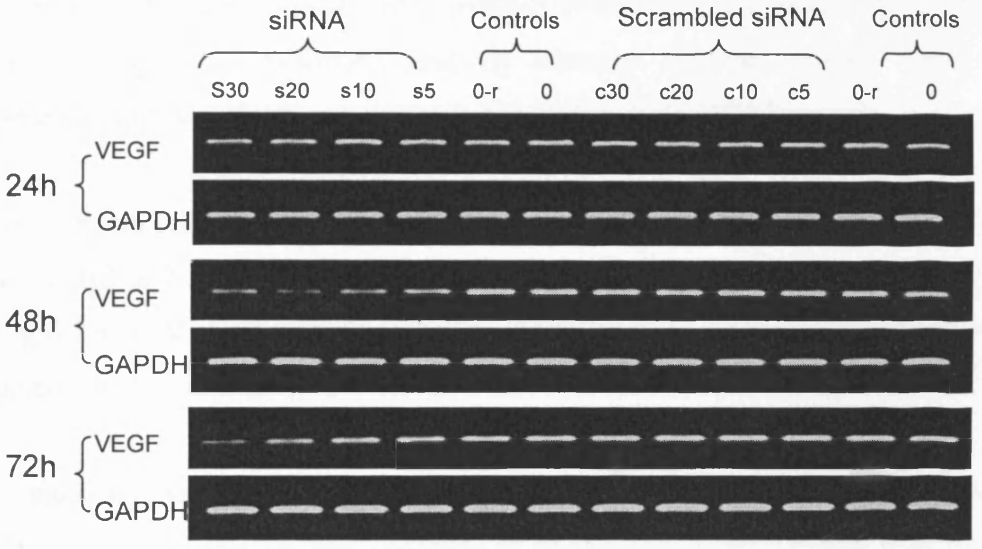


Fig 6.7 Time course of VEGF mRNA reduction by various concentrations of VEGF-siRNA analysed by RT-PCR on 1.5% Agarose gel. s30 = 30 nM of VEGF-siRNA; c30 = scrambled siRNA 30 nM; 0-r = control of transfection reagents only and 0 = non-treatment control.

By densitometric analysis, RT-PCR results showed that at 24 hours post transfection, the maximum silencing effect was 23.5 ± 3.65% of the control (76.41±3.56% remained) (n=3, p=0.0282) with 30 nM of VEGF-siRNA [Fig.6.7.1. A (VEGF) and B], whereas GAPDH mRNA from the corresponding samples had no change [Fig 6.7.1. A (GAPDH) and C].

At 48 hours post transfection, the maximum silencing effect 67.384 ± 2.295% (32.616 ± 2.295% remained) (n=3, p=0.0013) was observed with 30 nM VEGF-siRNA [Fig 6.7.2 A(VEGF) and B] whereas GAPDH mRNA from the corresponding samples had no change [Fig 6.7.2. A (GAPDH) and C].

At 72 hours post transfection, the maximum silencing effect was 67.688 ± 7.59 % (32.312 ± 7.585 % remained) (n=3, p=0.013) with 30 nM of VEGF-siRNA [Fig 6.7.3.A (VEGF) and B], whereas GAPDH mRNA from the corresponding samples had no change [Fig 6.7.3. A (GAPDH) and C].

In summary, the densitometric analysis revealed that the maximum silencing effect on VEGF mRNA was observed at 48 hours post transfection with 30 nM of VEGF-siRNA (Fig 6.7.4).

6.4.1.4 siRNA effectively and specifically suppressed VEGF protein in HRPE cells

The efficacy and potency of the siRNA targeting VEGF were also tested by monitoring VEGF at protein levels in the medium from the cells that had received a range of siRNA concentrations, and compared to that in cells treated with a nontargeting negative control siRNA and in cells without any treatment. To correspond with the time points for mRNA analysis, the medium was collected at 24, 48 and 72hours. VEGF protein was determined by ELISA in triplicates.

The VEGF ELISA result revealed that the standard curve generated using VEGF standards showed a linear regression between the Optical Density (OD) unit and

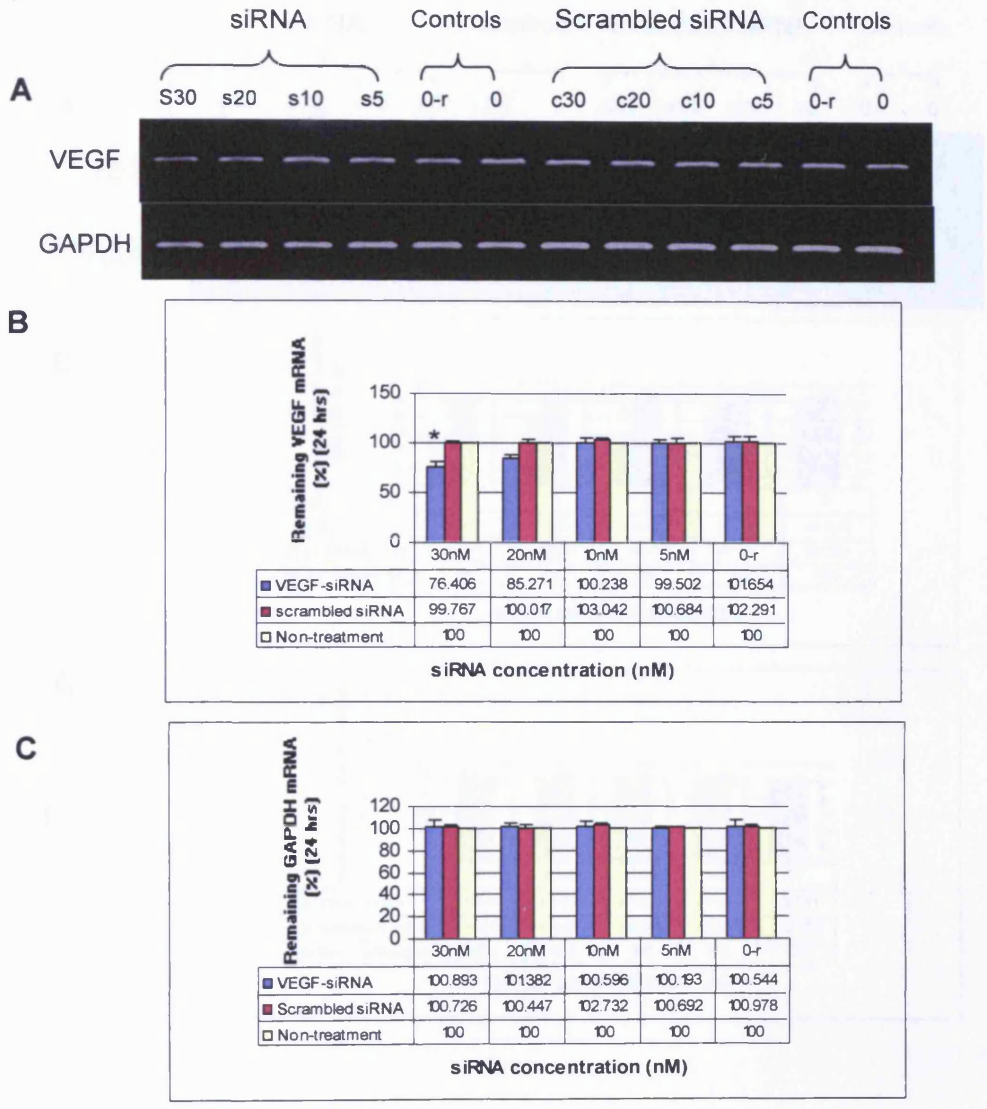
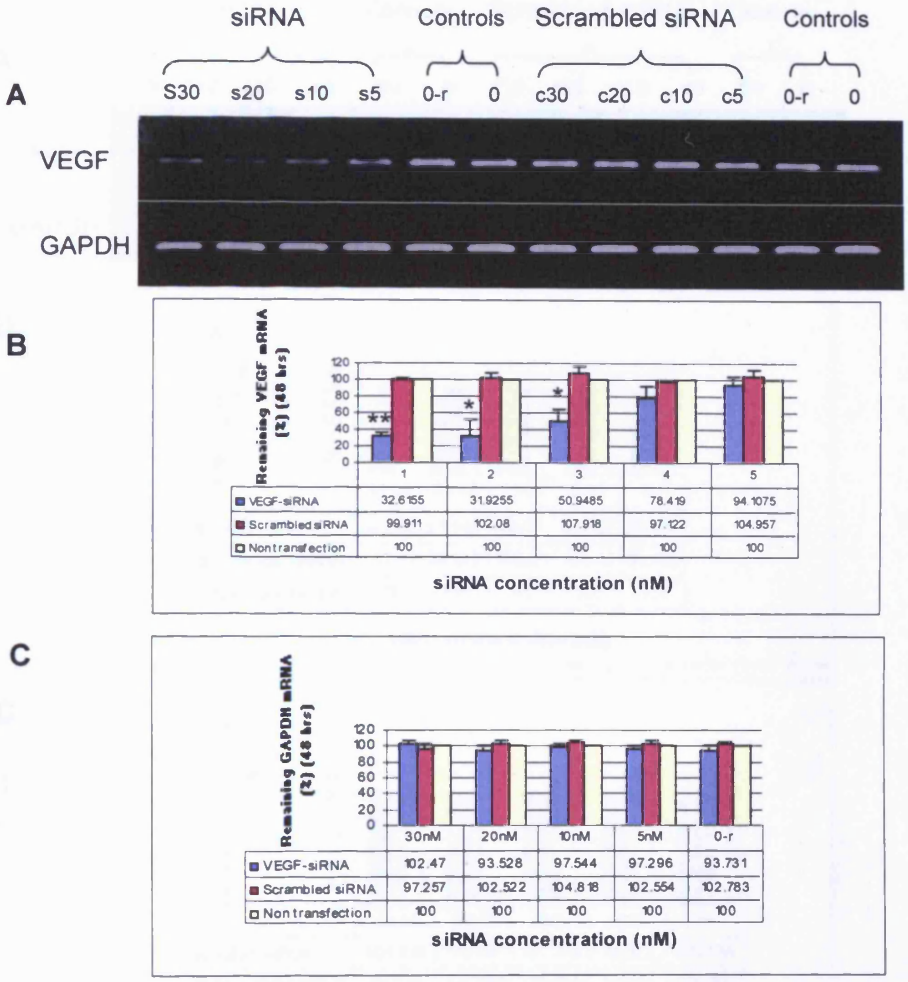


Fig 6.7.1 Efficiency of VEGF-siRNA on VEGF mRNA expression at 24 hrs post-transfection by RT-PCR. (A) RT-PCR analysis of VEGF/GAPDH expression on 1.5% Agarose gel. (B & C) are densitometric analysis of RT-PCR depicted in (A-VEGF) and (A-GAPDH), and are presented as the relative ratio of VEGF or GAPDH to the control from 3 separate experiments, respectively. Vertical bars are mean ± SEM. *p <0.05. At 24 hours post transfection, the maximum silencing effect was 23.5 ± 3.65% (75.4 ± 3.65% remaining) with 30 nM of siRNA-VEGF.



	1	2	3	4	5
VEGF-siRNA	32.6155	31.9255	50.9485	78.419	94.1075
Scrambled siRNA	99.911	102.08	107.918	97.122	104.957
Non transfection	100	100	100	100	100

	30nM	20nM	10nM	5nM	0-r
VEGF-siRNA	102.47	93.528	97.544	97.296	93.731
Scrambled siRNA	97.257	102.522	104.818	102.554	102.783
Non transfection	100	100	100	100	100

Fig 6.7.2 Efficiency of VEGF-siRNA on VEGF mRNA expression at 48 hrs post-transfection by RT-PCR. (A) RT-PCR analysis of VEGF and GAPDH expression on 1.5% Agarose gel. (B & C) are densitometric analysis of RT-PCR depicted in (A-VEGF) and (A-GAPDH), and are presented as the relative ratio of VEGF or GAPDH to the control from 3 separate experiments, respectively. Vertical bars are mean ± SEM. *p <0.05; ** p<0.01. At 48 hours post transfection, the maximum silencing effect was 67.38 ± 2.295% (32.62 ± 2.295% remaining) with 30 nM of siRNA-VEGF.

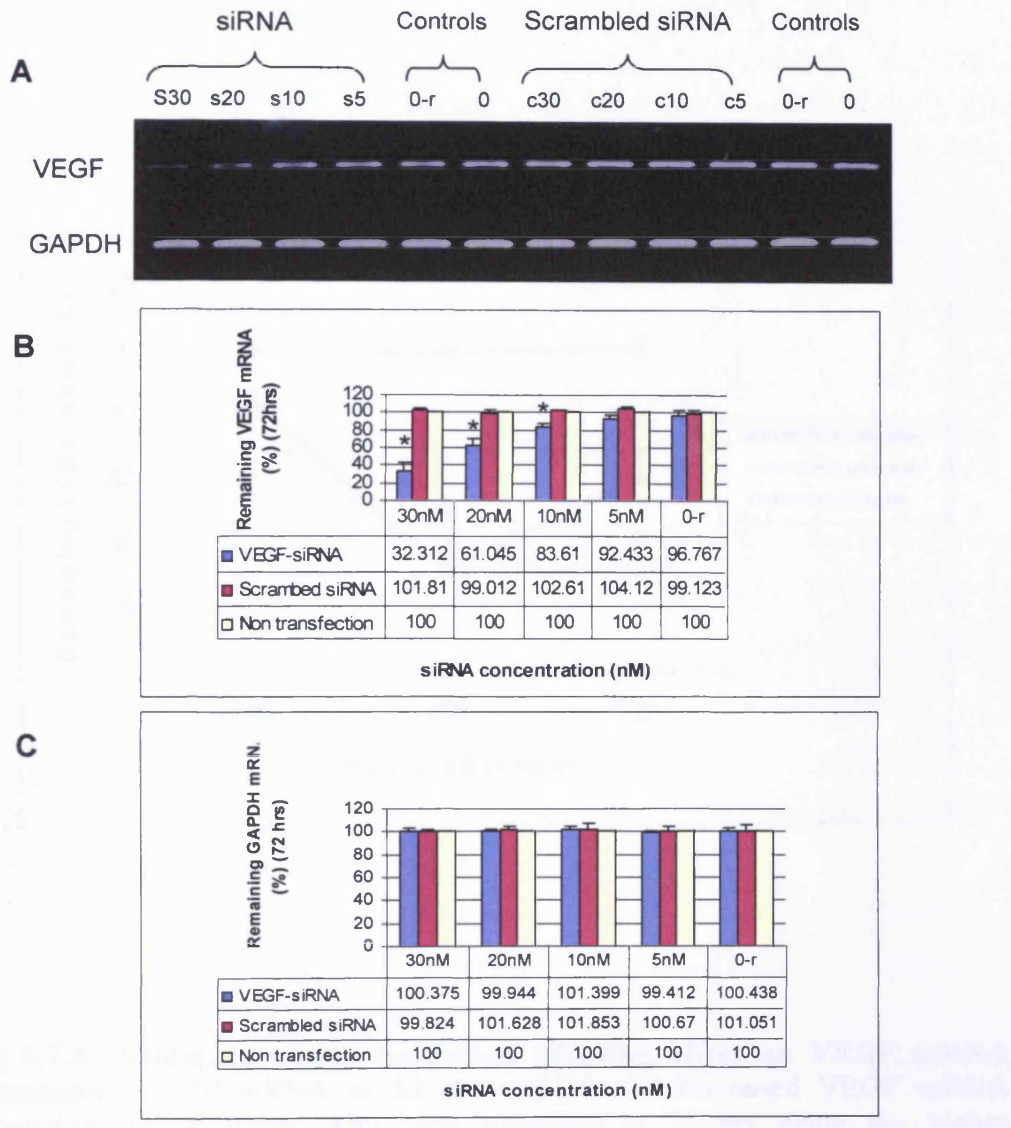


Fig 6.7.3 Efficiency of VEGF-siRNA on VEGF mRNA expression at 72 hrs post-transfection by RT-PCR. (A) RT-PCR analysis of VEGF and GAPDH expression on 1.5% Agarose gel. (B & C) are densitometric analysis of RT-PCR depicted in (A-VEGF) and (A-GAPDH), and are presented as the relative ratio of VEGF or GAPDH to the control from 3 separate experiments, respectively. Vertical bars are mean ± SEM. *p <0.05. At 72 hours post transfection, the maximum silencing effect was 67.688 ± 7.59% (32.31 ± 7.59% remaining) with 30 nM of siRNA-VEGF.

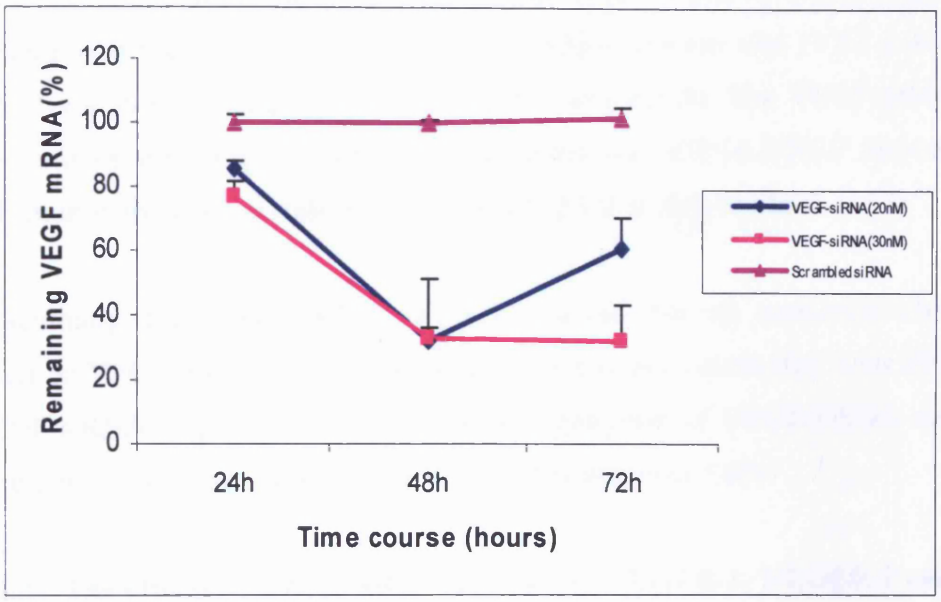


Fig 6.7.4 Summary of the maximum silencing effect on VEGF mRNA expression. VEGF-siRNA at 20 nM and 30 nM decreased VEGF mRNA expression by 48 hours. This was sustained at 72 hrs using the higher concentration of VEGF-siRNA (30 nM).

a series titrations of loaded VEGF (y =0.0015X+0.0211, R2 = 0.9998) (Fig.6.8. A). This allowed the amount of VEGF to be calculated in experimental samples.

At 24 hours post transfection, the reduction of VEGF protein was 11.54 ± 3.05% (88.46 ± 3.05% remained) (n=3, p=0.0307) with 30nM of VEGF-siRNA; at 48 hours post transfection, the reduction of VEGF protein was 43.12 ± 1.952% (56.89 ± 1.952% remaining) (n=3, p=0.0168) with 30 nM of VEGF-siRNA; at 72hours post transfection, the reduction of VEGF protein was 17.51 ± 4.509% (82.49 ± 4.509% remaining)(n=3, p=0.1127) (Fig 6.8.B). The VEGF protein in the samples that were transfected with scrambled siRNA-VEGF showed no reduction in the correspondent time points (Fig 6.8.B dark-red bar).

In summary, the VEGF ELISA analysis revealed that the maximum silencing effect on VEGF protein was observed at 48 hour post-transfection with 30nM of VEGF-siRNA (Fig 6.8.C). The 30nM concentration of VEGF-siRNA and the time-point of 48 hours were chosen for the down-stream study.

6.4.2 The effects of VEGF-siRNA on PEDF, VEGFR-1, VEGFR-2 and PS-1 expression and the autocrine loop

To assess the biological effects of VEGF silencing, it is critical to perform these assays during the time frame in which silencing is most profound. Based on the optimisation results described in section of 6.4.1.3 and 6.4.1.4, the time point of 48 hours post transfection with 30nM VEGF-siRNA was chosen to analyse the effect of VEGF-siRNA on PEDF/VEGFR-1/VEGFR-2 and PS-1.

6.4.2.1 The effects of VEGF-siRNA on PEDF mRNA and protein expression

The effects of VEGF-siRNA on PEDF expression were assessed by monitoring PEDF mRNA levels in cells /or PEDF proteins in the medium of the cells that had received a range of siRNA concentrations for 48 hours compared to that in cells treated with scrambled siRNA or cells without any treatment (Control).

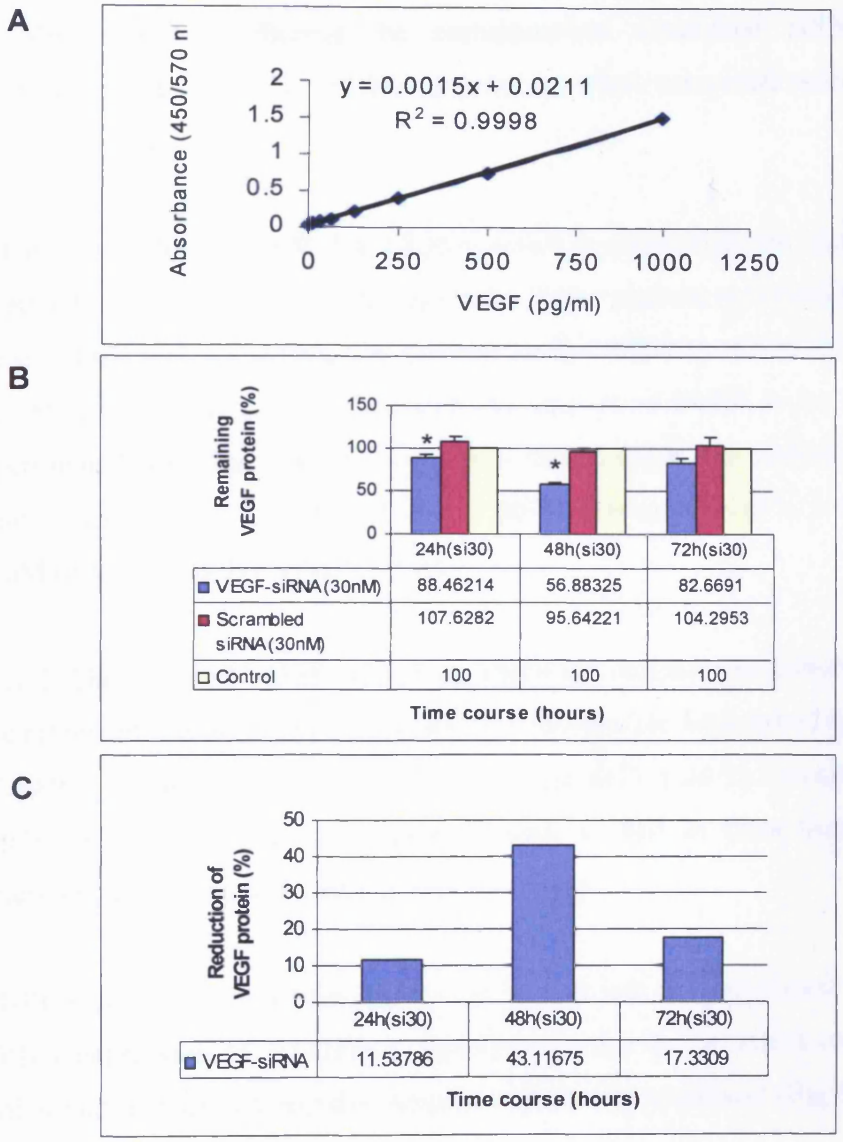


Fig 6.8 The effects of VEGF-siRNAs on VEGF protein. (A) Standard curve for quantifying VEGF protein in the culture media by ELISA. (B) The remaining VEGF protein is presented as the relative ratio of VEGF to the control from 3 separate experiments. Vertical bars are mean ± SEM. *p <0.05. (C) Summary of the silencing effect on VEGF protein with 30 nM of VEGF-siRNA at 24, 48 and 72 hrs post-transfection. The maximum silencing effect was 43.12% at 48 hour post transfection.

RT-PCR results show that at 48 hours post transfection, the reduction of PEDF mRNA was 43.36 ± 5.779% (56.63±5.779% remaining) (n=3, p=0.0166) with 30 nM VEGF-siRNA, whereas the correspondent scrambled siRNA has no significant effect on PEDF mRNA expression when compared with the control (Fig. 6.9 A & B).

At the protein level, the PEDF ELISA result revealed that the standard curve, generated using PEDF standards, showed a linear regression between the Optical Density (OD) unit and a titration series of loaded PEDF ($y = 0.1171X + 0.9946$, $R^2 = 0.9864$) (Fig.6.10. A). This allowed the amount of PEDF to be calculated in experimental samples. At 48 hours post transfection, the reduction of PEDF protein was 55.96 ± 1.607% (44.05 ± 1.607% remaining) (n=3, p=0.0468) with 30 nM of VEGF-siRNA (Fig 6.10 B).

6.4.2.2 The effect of VEGF-siRNA on VEGFR-1 mRNA and protein expression

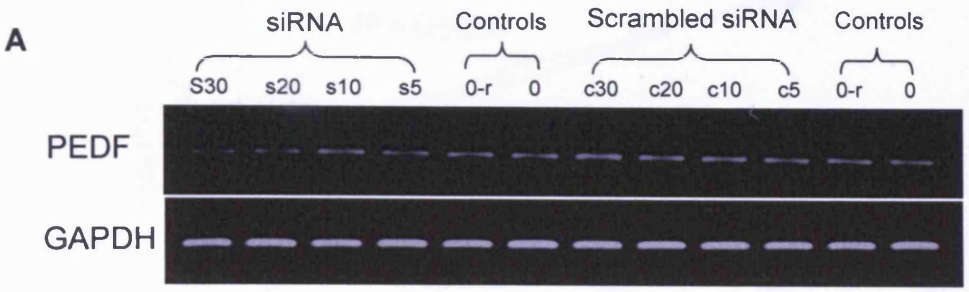
The effects of VEGF-siRNA on VEGFR-1 expression were tested by monitoring VEGFR-1 at mRNA and protein levels in the cells that had received 30nM of VEGF-siRNA for 48 hours, and compared to that in cells treated with the scrambled siRNA or cells without any treatment.

RT-PCR showed that VEGF-siRNA at 30 nM had no significant effect on the mRNA expression of VEGFR-1, which is similar to the effect on the samples with scrambled siRNA and the samples without any treatment (Fig 6.11.A).

Western blot analysis showed that VEGF-siRNA (30 nM) reduced the 75 and 85 kDa fragments (p=0.0453) of VEGFR-1 (n=3) (Fig. 6.11. B&C).

6.4.2.3 The effect of VEGF-siRNA on VEGFR2 mRNA and protein expression

RT-PCR results show that VEGF-siRNA at 30 nM had no significant effect on the mRNA expression of VEGFR-2, which is similar to the effect on the samples with scrambled siRNA and the samples without any treatment (Fig 6.12 A)



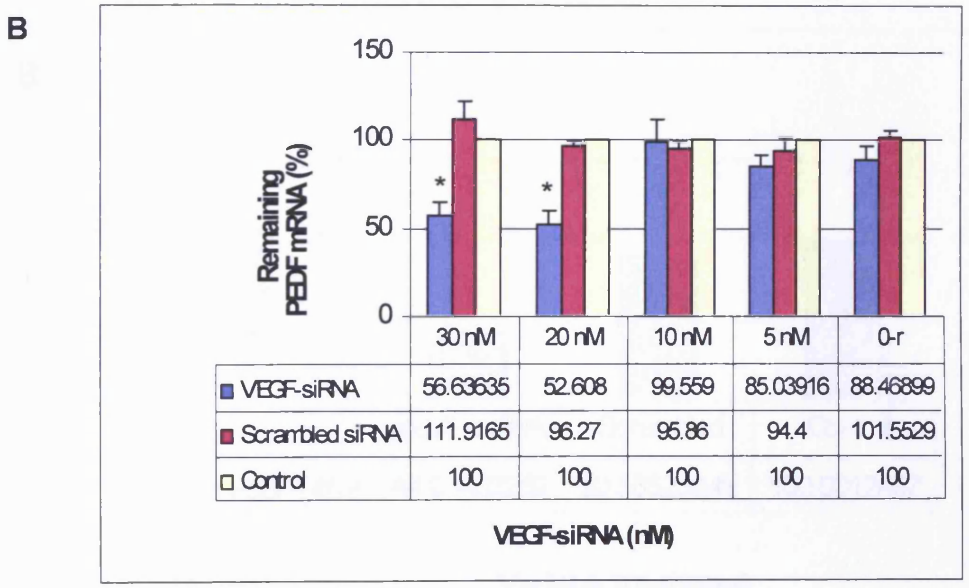
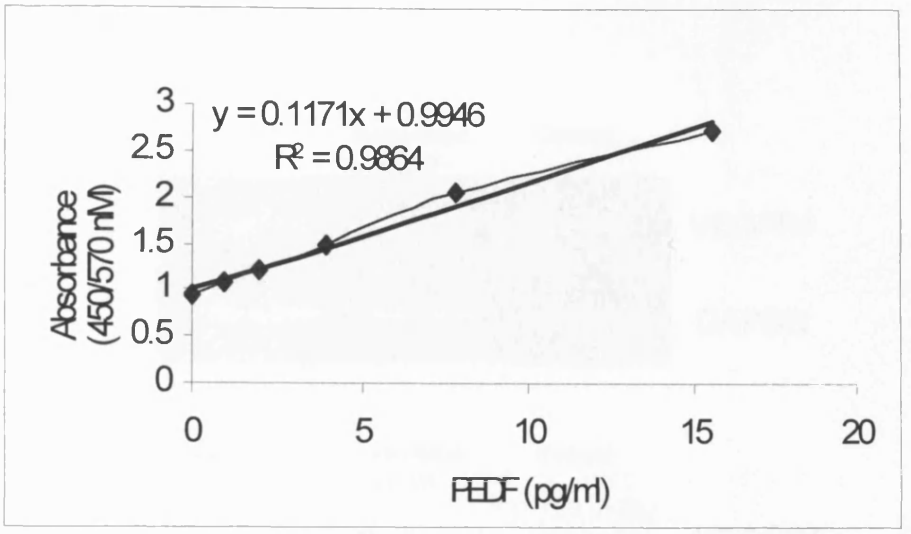


Fig 6.9 The effects of VEGF-siRNA on PEDF mRNA expression in cultured HRPE cells 48 hours post transfection. (A) RT-PCR analysis of PEDF mRNA expression. (B) Densitometric analysis of RT-PCR depicted in (A). Data are presented as the relative ratio of PEDF/GAPDH to the control from 3 separated experiment. *$p<0.05$.

A



B

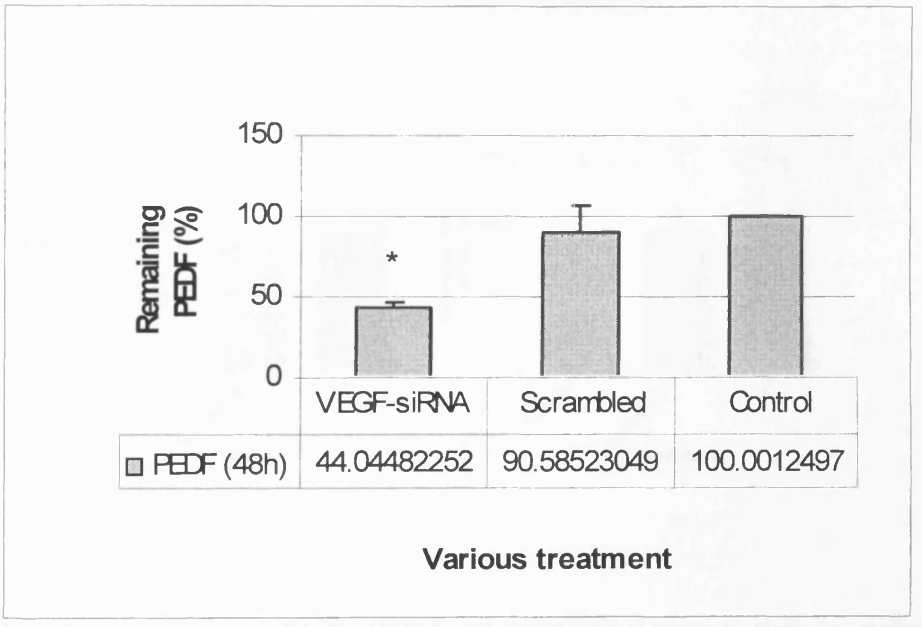


Fig 6.10 The effects of VEGF-siRNA on PEDF protein expression in the medium of cultured HRPE cells at 48 hours post transfection. (A) Standard curve for quantifying VEGF protein in the culture media by ELISA. (B) The remaining VEGF protein is presented as the relative ratio of VEGF to the control from 3 separate experiments. Vertical bars are mean ± SEM. *p <0.05.

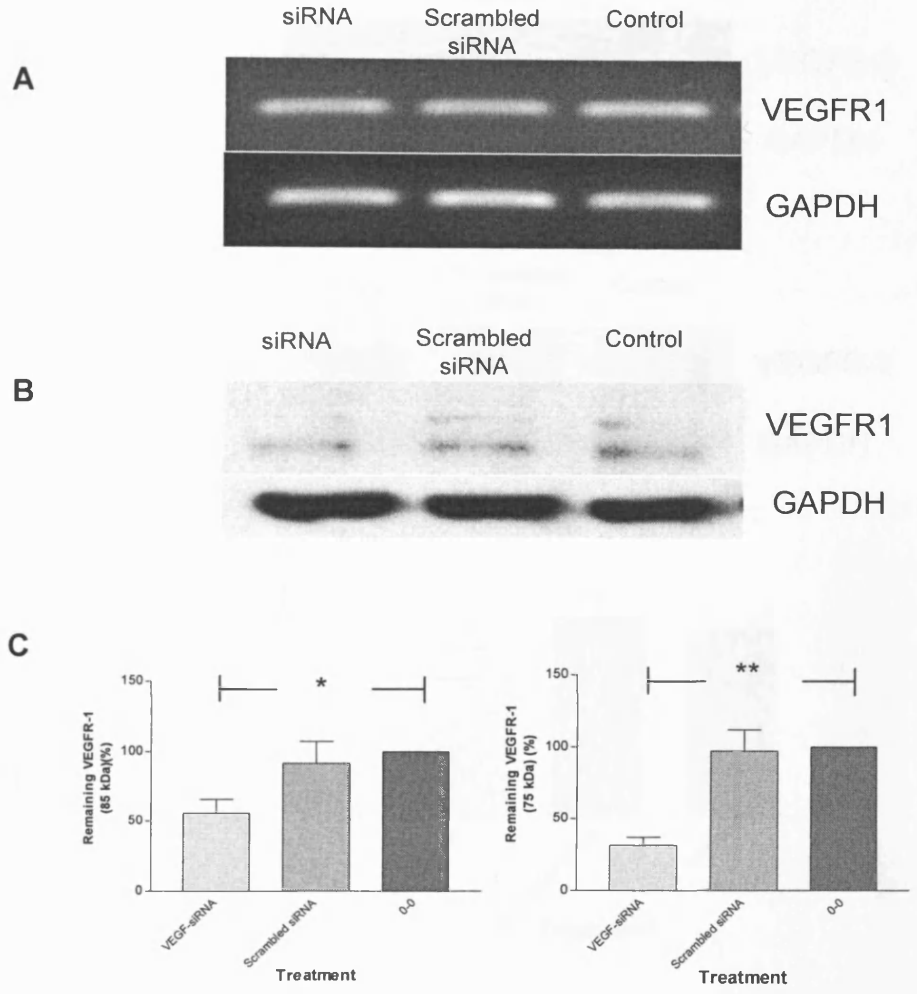


Fig 6.11 The effects of VEGF-siRNA on VEGFR-1 expression in cultured HRPE cells 48 hours post transfection. (A) RT-PCR analysis of VEGFR-1 and GAPDH mRNA expression. Data shown is representative of 3 separate experiments. (B) Western blot of VEGFR-1 protein. Data is representative of 3 separate experiments. Blots were stripped and reprobled for GAPDH. (C) Densitometric analysis of Western blotting depicted in (B). Data is presented as the relative ratio of VEGFR1 fragments/GAPDH to the control from 3 separated experiment. *$p<0.05$. **$p<0.01$.

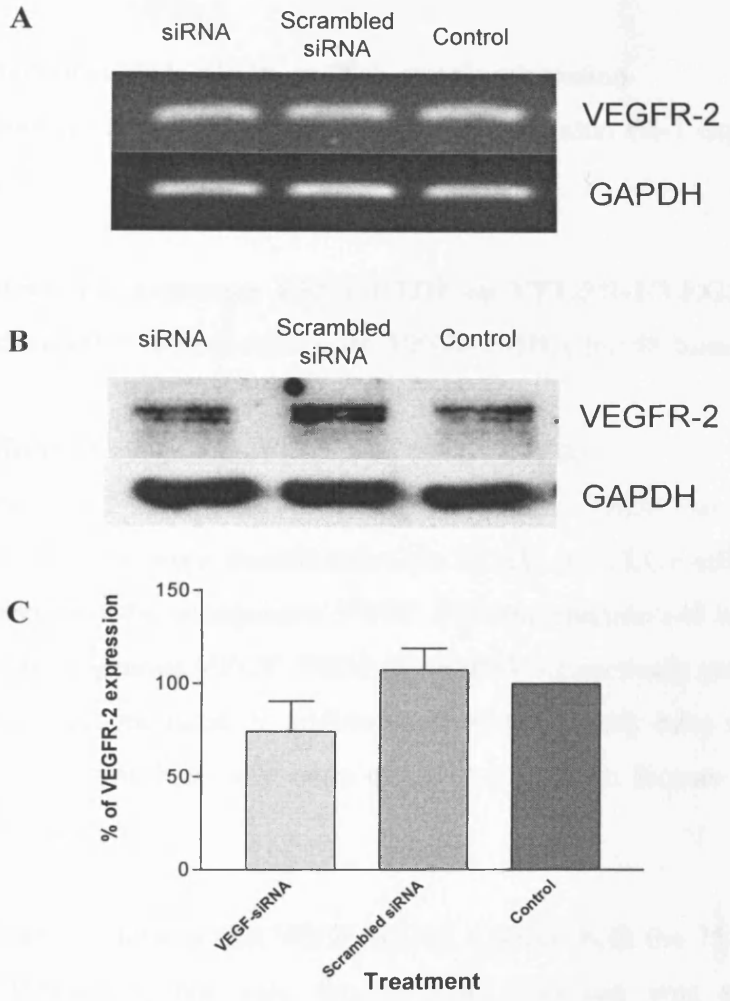


Fig 6.12 The effects of VEGF-siRNA on VEGFR-2 expression in cultured HRPE cells 48 hours post transfection. (A) RT-PCR analysis of VEGFR-2 and GAPDH mRNA expression. Data shown is representative of 3 separate experiments. (B) Western blot of VEGFR-2 protein. Data is representative of 3 separate experiments. Blots were stripped and reprobed for GAPDH. (C) Densitometric analysis of Western blotting depicted in (B). Data is presented as the relative ratio of VEGFR2/GAPDH to the control from 3 separate experiments.

Western blot analysis showed that expression of VEGFR2 was not significantly reduced by VEGF-si RNA treatment. (Fig 6.12 B & C).

6.4.2.4 The effect of siRNA-VEGF on PS-1 protein expression

Western blot analysis showed that VEGF-siRNA did not alter PS-1 expression ($p = 0.0788$) (Fig 6.13).

6.4.3 The effects of exogenous VEGF/PEDF on VEGFR-1/VEGFR-2/PS-1 expression after transfection with VEGF-siRNA for 48 hours

6.4.3.1 The effects of exogenous VEGF/PEDF on VEGFR-1

In order to elucidate the paracrine effect of VEGF/ PEDF on VEGFR-1 expression, HRPE cells were transfected with 30 nM of VEGF-siRNA in an attempt to knockdown the endogenous VEGF. Post transfection (48 hours) cells were treated with exogenous VEGF /PEDF /V+P /P+V respectively and a control without treatment was included. In addition cells that had only been exposed to the siRNA or cells that had only been exposed to growth factors were also included for comparison.

Western blot analysis showed that VEGF-siRNA reduced both the 75 & 85 kDa fragments of VEGFR-1, but only the 75 kDa reduction was statistically significant ($p<0.05$); addition of VEGF (100 ng/ml) overcame the effect of siRNA and increased the fragments to the level of control, and was similar to the one without VEGF-siRNA that received exogenous VEGF treatment. In contrast, addition of PEDF increased both fragments, but only the 75 kDa increased with statistical significance ($p=0.0117$). The combination of VEGF+PEDF /or PEDF+VEGF has a similar effect on the VEGFR-1 fragments to that of PEDF alone ($p <0.05$) (Fig. 6.14)

6.4.3.2 The effects of exogenous VEGF / PEDF on VEGFR-2

The effect of VEGF /PEDF on the expression of VEGFR-2 at post-transfection with VEGF-siRNA was analysed using the aliquots of the same samples for

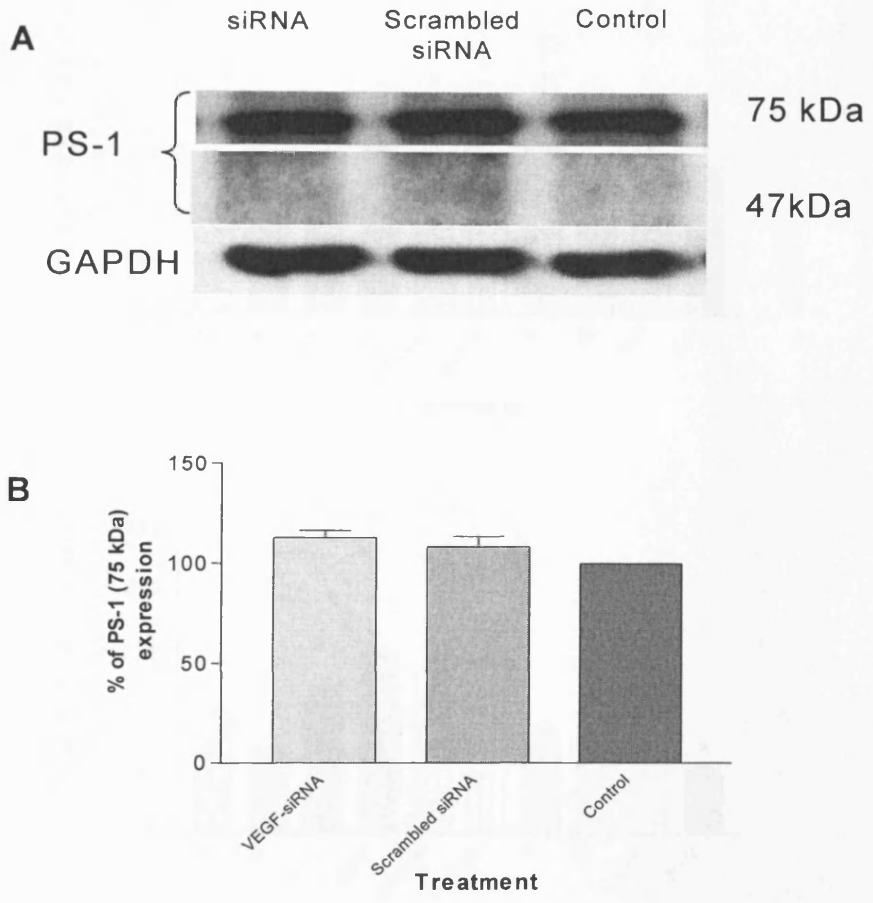


Fig 6.13 The effect of VEGF-siRNA on PS-1 expression in cultured HRPE cells 48 hours post transfection. (A) Western blot of PS-1 protein. Data is representative of 3 separated experiments. Blots were stripped and reprobed for GAPDH. (B) Densitometric analysis of Western blot depicted in (A). Data is presented as the relative ratio of PS1/GAPDH to the control.

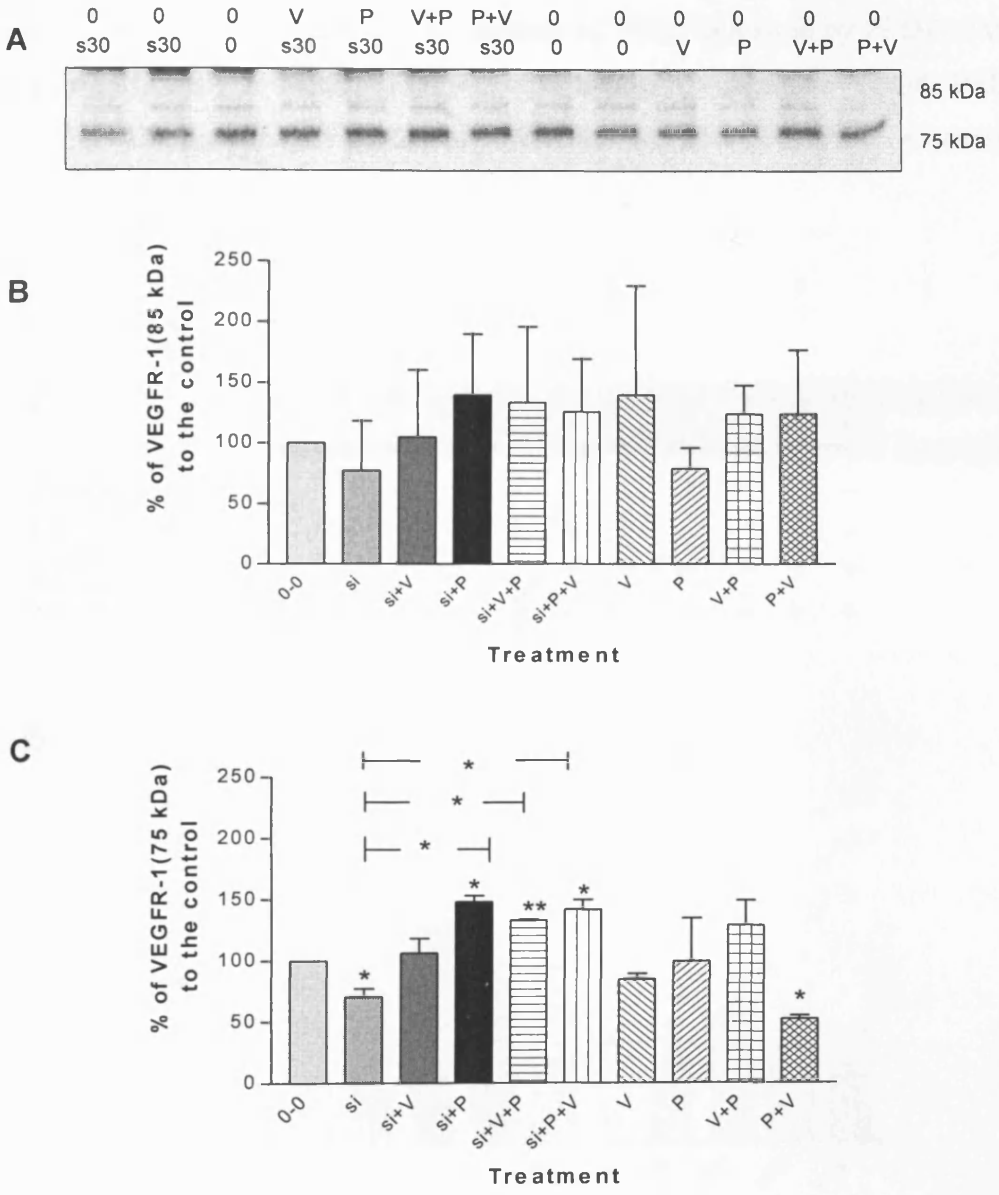


Fig 6.14 The effects of exogenous VEGF/PEDF on VEGFR-1 expression. (A) Representative of western blot of VEGFR-1 expression. (B & C) Densitometric analysis of the 75 and 85 kDa bands in (A). Data are presented as the relative ratio of VEGFR-1/GAPDH to the control from 3 separate experiments. Vertical bars are mean ± SEM. *p <0.05; ** p<0.01 (treatment vs control).

VEGFR-1 analysis. Western blotting showed that VEGF-siRNA did not significantly reduce VEGFR-2. The addition of VEGF followed by PEDF or vice versa significantly reduced VEGFR-2. Interestingly, exogenous VEGF, PEDF, VEGF+PEDF or PEDF+VEGF significantly reduced VEGFR-2 expression ($p<0.05$) (Fig. 6.15).

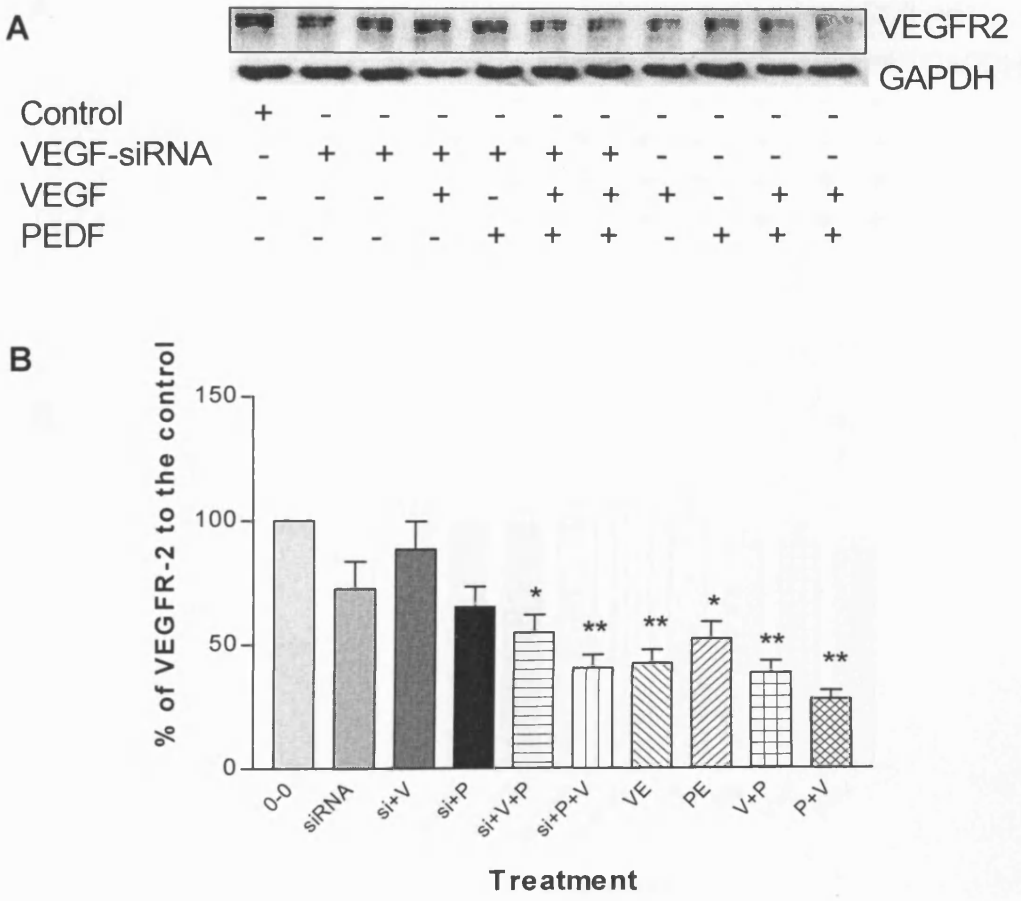


Fig 6.15 The effects of exogenous VEGF/PEDF on VEGFR-2 expression. (A) Representative of western blot of VEGFR-2 expression. (B) Densitometric analysis of VEGFR-2 in (A). Data is presented as the relative ratio of VEGFR-2/GAPDH to the control from 3 separate experiments. Vertical bars are mean ± SEM. *p <0.05; ** p<0.01 (various treatment vs control).

6.4.3.3 The effects of exogenous VEGF / PEDF on PS-1

Western blot analysis showed that VEGF-siRNA (30nM) has no significant effect on PS-1 expression; a similar result was observed following the addition of VEGF, PEDF, VEGF+PEDF or PEDF + VEGF (Fig 6.16).

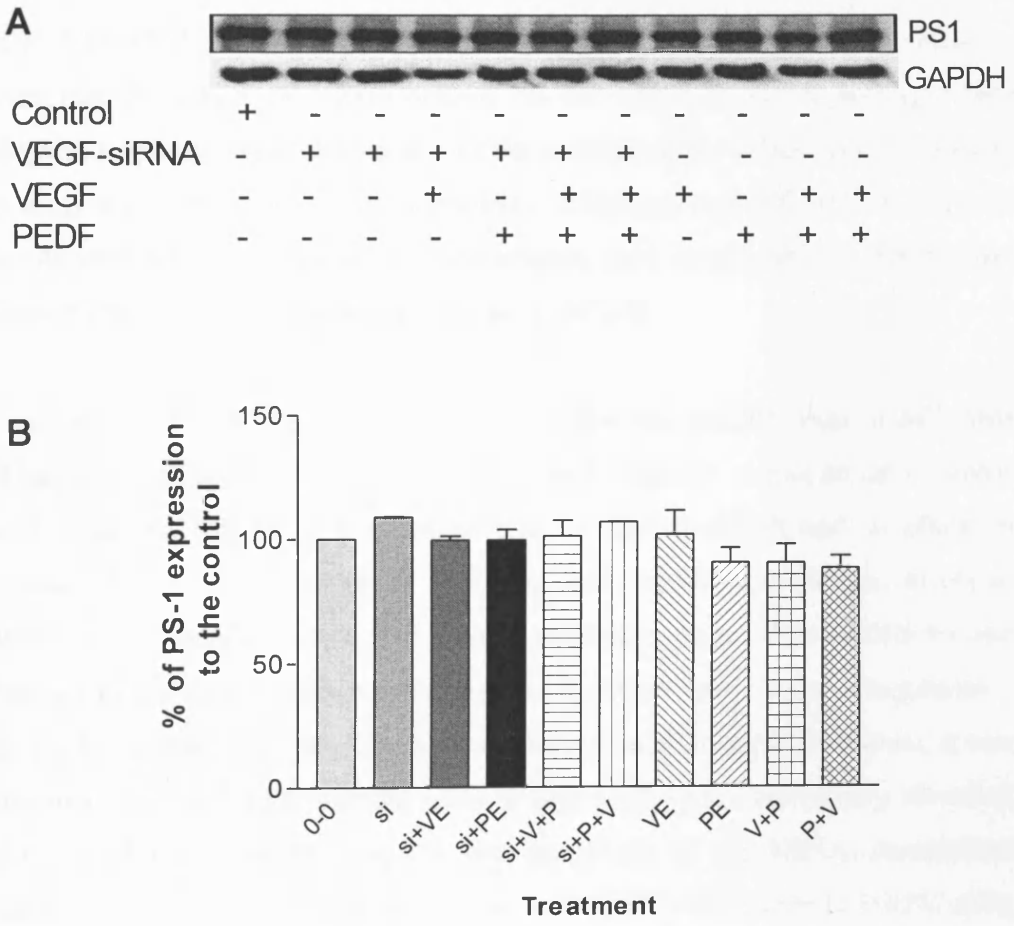


Fig 6.16 The effect of exogenous VEGF/PEDF on PS-1 protein expression. (A) Representative of western blot of PS-1 expression. (B) Densitometric analysis of PS-1 bands in (A). Data is presented as the relative ratio of PS-1/GAPDH to the control from 3 separate experiments. Vertical bars are mean ± SEM.

6.5 Discussion

It has been demonstrated that RNAi mediated by siRNAs can specifically target the sequence(s) of interest in either *in vitro* or *in vivo* models (Bantounas *et al.*, 2004). However, some concern has also arisen due to the observations of non-specific inhibition of protein synthesis and RNA degradation following the use of some siRNA and delivery vector combinations (Jackson *et al.*, 2003).

In this study, VEGF-siRNA was used to address the role of VEGF in relation to PEDF, VEGFR-1, VEGFR-2 and PS-1 in cultured HRPE cells. In order to ensure that the effect of VEGF-siRNA on the down-stream signalling is the biological response rather than non-specific inhibition, the VEGF-siRNA used in this study was first tested for its specificity, efficiency and efficacy. A series of controls were set up alongside the experiments, such as scrambled siRNA, non-treatment controls and house-keeping gene, GAPDH.

By monitoring total RNA quality, VEGF mRNA and protein level in cells that had received a range of siRNA concentrations compared to that in cells treated with a scrambled siRNA, it was shown that the VEGF-siRNA had no effect on the total RNA level but reduced VEGF at both mRNA and protein levels in cultured HRPE cells in a dose and time-dependent manner. The specificity and efficiency of the VEGF-siRNA silencing was further confirmed by comparison with the house-keeping gene GAPDH and non-treatment controls. Hence, it was confirmed that the VEGF-siRNA used in this study was specifically silencing VEGF. In addition, the effectiveness and specificity of the siRNA, constructed by *in vitro* transcription, in the suppression of VEGF expression in HRPE cells, suggests a promising approach towards the treatment of CNV. This adds to a number of existing strategies to inhibit VEGF signal transduction, such as the development of humanised neutralising anti-VEGF monoclonal antibodies, receptor antagonists, soluble receptors, antagonistic VEGF mutants, and inhibitors of VEGF receptor function (Moreira *et al.*, 2007).

Further more, a major finding of this study is that VEGF has a close relationship with VEGFR-1, VEGFR-2 and PEDF in cultured HRPE cells. The time course of siRNA silencing of VEGF mRNA and protein correlated well with decreased PEDF at transcript and protein level. This suggests that VEGF is up-regulating PEDF from the transcriptional level which is in agreement with published reports stating that VEGF up-regulated PEDF through VEGFR-1 in an autocrine manner (Ohno-Matsui *et al.* 2003).

The siRNA silencing of VEGF mRNA and protein also correlated well with decreased VEGFR-1 at the protein level, but not at the mRNA level, suggesting that endogenous VEGF in HRPE cells upregulates VEGFR-1 post-transcriptionally. The consequence of this kind of regulation needs to be further investigated but could involve decreased degradation of the receptors. The VEGF-siRNA has no significant effect on the expression of VEGFR-2 and the 75 kDa high molecular weight (HMW) form of PS-1.

It was observed that the expression of VEGFR-1 fragments in responding to exogenous VEGF/PEDF (when VEGF was inhibited by siRNA) was different from that when VEGF was not inhibited.

In response to exogenous PEDF or PEDF followed by VEGF, after about 68% of endogenous VEGF was silenced, the 75 kDa fragment of VEGFR-1 increased.

This was in contrast to VEGFR-2 expression which was decreased, to a level similar to samples that had been exposed to VEGF and PEDF without inhibition of endogenous VEGF. These observations suggest that the autocrine and paracrine effects of VEGF may need to be considered in the therapeutic strategic designing.

Dias *et al.*, (2000) has reported that certain leukemic cells not only produce VEGF, but also acquire the capacity to express functional VEGFRs, which

results in the generation of an endothelial–independent autocrine loop that supports leukemic survival and migration *in vivo*. Therefore, in VEGF-producing, VEGFR-expressing leukemias, generation of VEGF/VEGFR autocrine (endothelial-independent) and paracrine (endothelial-dependent) loops, may contribute toward leukemic growth. In assessing the relative contribution of paracrine and autocrine VEGF/VEGFR signalling pathways to the growth of human leukaemia *in vivo*, Dias *et al.*, (2001) observed that targeting the paracrine or autocrine VEGF/VEGFR-2 signalling pathway delays leukemic growth, but it is not sufficient to cure inoculated mice. To achieve remission, both autocrine and paracrine pathways were targeted.

Furthermore, the effect of VEGF-siRNA on the expression of PEDF observed in this study not only supports the data showing about the autocrine loop between VEGF and PEDF, but it also challenges the concept of a reciprocal relation between VEGF and PEDF. This suggests that a single-targeted inhibition strategy may need to be replaced by a double- or multi-targeted inhibition.

In summary, this study demonstrates that siRNA, constructed using *in vitro* transcription methods, is efficient to silence the gene of target with minimum of toxicity as reported by Li *et al.*, (2005). This study demonstrates for the first time, the effects of VEGF-siRNA on VEGFR-1, VEGFR-2, PEDF and PS-1 expression in cultured HRPE cells, in that VEGF-siRNA down-regulates PEDF expression at both the transcriptional and protein level, while down-regulation of VEGFR-1 only occurs at the post-transcription level. Future work such as to examine the effects of PEDF-siRNA on the expression of VEGF, VEGFR-1, VEGFR-2 and PS-1 will provide insight into the relationship among these elements in HRPE cells.

Chapter 7

Final Discussion

7.1 Introduction

Neovascularisation is one of the most vital biological processes required for the formation and physiological function of virtually all organs in both normal and disease states (Visconti *et al.*, 2002; Schmid and Varner, 2007).

An underlying common aetiology in pathological conditions, such as tumour growth and neovascular ocular diseases, is the proliferation of aberrant blood vessels. Conversely, an inadequate blood supply can be seen in conditions, such as myocardial infarction and stroke. The importance of neovascularisation in the above conditions has led to intensive research into viable therapies that target new blood vessel formation. Anti-angiogenic therapies, such as an anti-VEGF monoclonal antibody, soluble VEGF receptor chimeric proteins and VEGF antisense oligonucleotides, have been the focus of the management of tumour growth and neovascular ocular disease with reasonable success. However, although reduction in tumour growth has been demonstrated, some side effects were also noted, including the inhibition of local endothelial precursor cell (EPC) function in the retina and choroid. VEGF is a survival factor for all endothelial cells. Loss of VEGF may result in the inability of resident vasculature to proliferate in response to injury and to maintain normal endothelial morphology. Examples of this include the fenestration observed in the choriocapillaris and the kidney glomerulae.

PEDF, a natural inhibitor of angiogenesis with neurotrophic and neuroprotective properties, has been identified in a wide variety of tissues (Tombran-Tink *et al.*, 2003). The finding that PEDF is essential for maintaining avascularity (Dawson *et al.*, 1999) and plays a critical role in preventing aberrant neovascularisation (Stell-mach *et al.*, 2001) suggests that PEDF does not appear to harm the existing vasculature (Bouck, 2002). The inhibitory effect of PEDF on vessel formation appears to be reversible when regulated, transient angiogenesis occurs in situations including tissue repair after injury (Tonnesen *et al.*, 2000). PEDF has been shown to act by inhibition of the angiogenic response to factors such as VEGF and fibroblast growth factor. These features make PEDF an attractive angiogenic inhibitory agent which may avoid the side effect of anti-VEGF

agents. However, controversy exists over the role of PEDF. There is compelling evidence to suggest that PEDF has a dual role in the regulation of neovascularisation as reported by Hutching *et al.*, (2002) and Apte *et al.*, (2004). Depending on the maintenance of endothelial cells (EC)'s phenotype, PEDF can exert opposing effects on EC proliferation (Hutching *et al.* 2002) in cultured BRMECs. In an animal study, Apte *et al.* (2004) observed that low doses (90 μg/ml) of PEDF had a significant inhibitory effect on CNV in mice, while a high dose (360 μg/ml) of PEDF augmented the development of neovascularisation. Therefore an understanding of the mechanism of PEDF's action is central to achieving a therapy with maximal effect.

Three research groups (Yabe *et al.*, 2001; Hutchings *et al.*, 2002; Volpert *et al.*, 2002) have suggested different signalling pathways that PEDF may be involved in, though none of these fully explains the role of PEDF. Recently a fourth signalling pathway has been suggested by Cai *et al.* (2006). They demonstrated that the inhibitory effect of PEDF on VEGF-induced angiogenesis results from the inhibition of VEGFR-2 in BRMECs. This inhibitory effect was achieved by enhancing γ-secretase–dependent cleavage of the C-terminus of VEGFR-1. This suggests that PEDF may be involved in the Regulated Intramembrane Proteolysis (RIP) signalling pathway, which has been well documented to play a very important role in Notch (Struhl and Greenwald, 1999; Handler *et al.*, 2000; Kimberly *et al.*, 2003) and ErbB4 signalling (Ni *et al.*, 2001; Lee *et al.*, 2002) and Aβ production (McLoughlin & Miller *et al.*, 1996). Therefore, understanding the role of VEGF and PEDF in relation to RIP may contribute to a new strategy for treating not only neovascular disease but also a wider range of clinical conditions.

The aim of the project was to investigate the mechanism(s) in which PEDF regulates angiogenesis by exploring the involvement of Presenilin (PS)-dependent RIP in relation to VEGF/PEDF and VEGFR-1/VEGFR-2 in cultured BRMECs and HRPE cells. BRMECs were used as an example of an angiogenic-type cell, whilst HRPE cells act as regulatory cells. The relationship between

VEGF and PEDF in the regulation of VEGFR-1/VEGFR-2 expression was also investigated by the inhibition of VEGF function in HRPE cells.

The hypothesis that VEGF and VEGFR-1 are involved in PS-dependent RIP signalling pathway and PEDF acts as a regulator was examined using immunoblotting techniques and immunocytochemistry. The relationship between VEGF and PEDF in the regulation of VEGFR-1/VEGFR-2 expression was examined using siRNA assays.

Several fundamental objectives were undertaken to address the hypothesis. These included identification of:

1) The role of VEGF and PEDF in the regulation of the expression and localisation of VEGFR-1 and VEGFR-2

2) The role of VEGF/PEDF on the activity of γ-secretase and the expression of PS-1

3) The association between VEGFR-1 and/ or VEGFR-2 and γ-secretase in both BRMECs and HRPE cells. The overall project has led to novel findings and addresses the need for continued research which is detailed in the future work section (section 7.8).

7.2 Summary of findings:

7.2.1 The effects of VEGF and PEDF on VEGFR-1, VEGFR-2 and PS-1 expression and γ-secretase activity in BRMECs and HRPE cells

BRMECs (bovine retina microvascular endothelial cells):

As summarised in Fig 7.1, the results obtained in this thesis indicate that in BRMECs, under normal culture conditions, the expression of VEGFR-1 and VEGFR-2 is counterbalanced. The overall expression of VEGFR-1 can be upregulated by exogenous VEGF or PEDF at 100 ng/ml whereas the overall expression of VEGFR-2 can be downregulated by exogenous VEGF or PEDF at 100 ng/ml. In addition, PEDF can augment VEGF's effect on VEGFR-1 and VEGFR-2 in BRMECs.

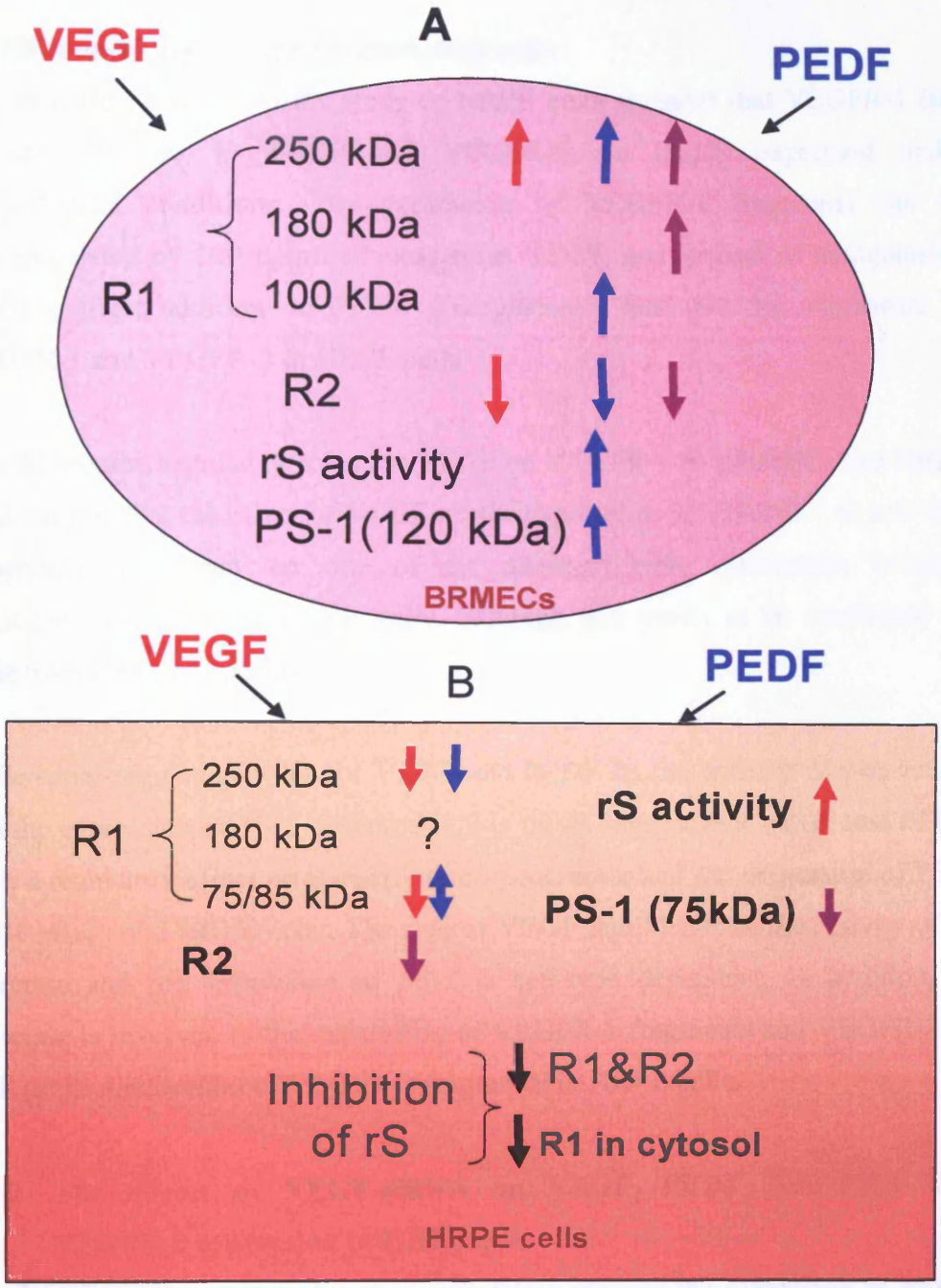


Fig 7.1 Effects of VEGF & PEDF on cell signalling. The effects of VEGF and PEDF on VEGFR-1(R1), VEGFR-2 (R2) and presenilin-1 (PS-1) expression and γ-secretase (rS) activity in (A) BRMECs and (B) HRPE cells. red arrow: VEGF's effect; blue arrow: PEDF's effect and dark red arrow: combined effects of VEGF and PEDF.

HRPE (human retina pigment epithelial) cells:

The evidence obtained in the study of HRPE cells suggests that VEGFR-1 (the 75 and 85 kDa fragments) and VEGFR-2 are highly expressed under physiological conditions. The expression of VEGFR-1 fragments can be downregulated by 100 ng/ml of exogenous VEGF, and instead of antagonising VEGF's effect, addition of PEDF synergistically inhibited the expression of VEGFR-1 and VEGFR-2 in HRPE cells

The differential regulatory roles for VEGF on VEGFR-1 in BRMECs and HRPE cells suggest that the effect of VEGF on the expression of VEGFR-1 is cell-type dependent. This may be one of the distinguishable differences between angiogenic and non-angiogenic cells, although this needs to be confirmed on cells from the same species.

Differential regulatory roles for VEGF and PEDF on the activity of γ-secretase and the expression of PS-1 observed in this thesis suggest that VEGF and PEDF have a regulatory effect on the activity of γ-secretase and the expression of PS-1 in BRMECs and HRPE cells. The role of VEGF and PEDF on the activity of γ-secretase and the expression of PS-1 is cell-type dependent. In addition, γ-secretase is involved in the expression of VEGFR-1 fragments and VEGFR-2 as well as the distribution of VEGFR-1 fragments in HRPE cells.

7.2.2 The effects of VEGF-siRNA on VEGF, PEDF, VEGFR-1 and VEGFR-2 expression in HRPE cells

The VEGF-siRNA used in this study efficiently reduced the expression of VEGF and PEDF at both the transcript and protein level in HRPE cells, as well as VEGFR-1 at the protein level (Fig 7.2). The downregulation of VEGF, as well as PEDF, by VEGF-siRNA supports the existence of an autocrine loop between VEGF and PEDF and indicates that other anti-VEGF agents are likely to have similar effects on PEDF in cells secreting both VEGF and PEDF.

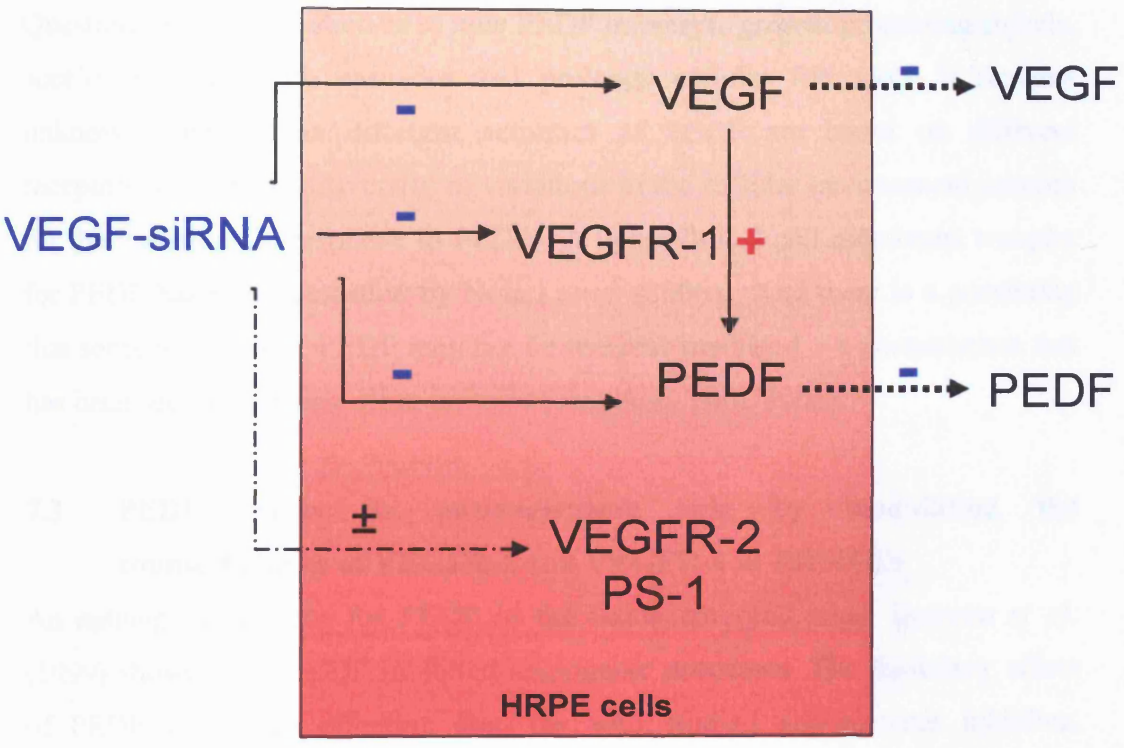


Fig 7.2 Effects of VEGF-siRNA on VEGF cell signalling in HRPE cells. VEGF-siRNA reduced VEGF and PEDF expression at transcript and protein level. VEGF-siRNA reduced VEGFR-1 expression at protein level while had no significant effect on VEGFR-2 and PS-1.

It is likely that the differential role of VEGF and PEDF, on VEGFR-1 and VEGFR-2, in BRMECs and HRPE cells contribute either directly or indirectly to the regulation of neovascularisation. The implications of these roles in the regulation of neovascularisation are discussed below.

Questions have been asked as to how PEDF intercepts growth-promoting signals, accelerates cell death cascades and prolongs cellular life span. It remains unknown whether the different activities of PEDF are based on different receptors or if cellular diversity or variations in the cellular environment account for the variations in response to PEDF. A lipase-linked cell membrane receptor for PEDF has been identified by Notari *et al.* (2006). And there is a possibility that some activities of PEDF may not be receptor mediated – a phenomenon that has been seen with many other proteins (Tombran-Tink, 2005).

7.3 PEDF exerts its antiangiogenic role by modulating the counterbalance of VEGFR-1 and VEGFR-2 in BRMECs

An antiangiogenic role for PEDF in the retina emerged when Dawson *et al.* (1999) showed that PEDF inhibited angiogenic processes. The inhibitory effect of PEDF was more effective than the well studied angiogenesis inhibitor, angiostatin. In those studies, PEDF prevented EC migration alone, or in the presence of the potent proangiogenic factors: FGF-1, FGF-2, VEGF, interleukin-8 and lysophosphatic acid. In animal studies, ocular injection of an adenoviral construct containing the PEDF gene not only inhibited the formation of both retinal and choroidal neovascularisation in mouse models of ocular angiogenesis but also caused the regression of neovascularisation that was already present (Mori *et al.*, 2001; Mori *et al.*, 2002). These studies are convincing and indicate that PEDF establishes specific mechanisms of interference that mitigate vascular growth propelling signals. Furthermore, PEDF was shown to be able to negate the increased vascular permeability induced by VEGF in an animal model (Liu *et al.*, 2004). At the molecular level, Tombran-Tink (2005) has reported that PEDF reduced VEGFR-2 transcription in the adult monkey retina, as well as the human

A-RPE-19 cell line. PEDF also inhibited phosphorylation of VEGFR-2 in HUVECs in the presence of its ligand, VEGF.

VEGFR-2 has been regarded as the major transducer of VEGF signals in ECs, which result in cell proliferation, migration, differentiation, tube formation, increased vascular permeability and maintenance of vascular integrity (Ziche *et al.* 1997). VEGFR-1, on the other hand, has been proposed to play a negative role in the regulation of the levels of endogenous VEGF. This is achieved by absorbing the ligand with its extracellular domain (Shibuya, 2001). A considerable "cross-talk" between VEGFR-1 and VEGFR-2 has been observed by several groups (Rahimi *et al.*, 2000; Burkhardt and Zacharias, 2001; Zeng *et al.*, 2001). They reported that the "cross-talk" between VEGFR-1 and VEGFR-2 played a critical role in regulating VEGFR-2-mediated signalling and that dependent on conditions, VEGFR-1 can act as a pro- or anti-angiogenic regulator of VEGFR-2.

The findings of this study, that PEDF upregulates VEGFR-1 and downregulates VEGFR-2 in BRMECs is in agreement with the above report and supports the proposal that PEDF intercepts growth-promoting signals by modulating the expression of VEGFR-1 and VEGFR-2 in BRMECs, rather than solely downregulating VEGFR-2.

Of most interest is the antagonist effect between VEGF and PEDF was not observed but the synergistic effect on either VEGFR-1 or VEGFR-2 that results from the combined effects of VEGF and PEDF. This suggests that PEDF's maximal effect is dependent upon VEGF. This finding is consistent with previous reports and, furthermore it indicates a regulatory relationship between VEGF and PEDF. Further study is necessary to prove any potential impact on the design of anti-angiogenic strategies.

Based on the accumulating evidence that the role of PEDF is dose-dependent and cell phenotype dependent (Apte *et al.*2004; Hutching *et al.* 2002), caution has been taken when interpreting the data obtained from the present study. The data may represent only part of a mechanism since the data was produced from the treatment of a single dose of VEGF/PEDF. To further our understanding of the relationship between VEGF/PEDF and VEGFR-1/VEGFR-2 future work is required (see section 7.8).

7.4 PEDF exerts its neurotrophic and neuroprotective role by regulating the activity of γ-secretase and the expression of PS-1 in HRPE cells

PEDF was first identified in the conditioned-medium of fetal human RPE cells which had a differentiating effect on retinoblastoma cells (Tombran-Tink and Johnson, 1989; Tombran-Tink, *et al.* 1991). PEDF exerts its neurotrophic and neuroprotective function by protecting various types of cells, including neuronal cells, against oxidative stress or glutamate-induced injury through its anti-oxidative properties (Cao *et al.* 1999; Bilak *et al.* 1999; DeCoster *et al.* 1999; Yamagishi *et al.* 2002; Yamagishi *et al.* 2003; Inagaki *et al.* 2003). Increase in oxidative stress has been related to the hyperactivity of glutamate receptors which results in the disturbance of glutamatergic neurotransmission. Oxidative stress has also been associated with the deposition of Aβ peptides in the brain and therefore implicated in the pathogenesis of Alzheimer's disease (AD) (Butterfield and Pocernich, 2003).

The involvement of γ-secretase in Notch, ErbB4 and VEGFR-1 signalling, and the production of Aβ has been well documented. The implication of γ-secretase in the development, signal transduction, protease biochemistry, neovascularisation and the pathogenesis of AD suggests that the activity of γ-secretase can be beneficial or harmful dependent upon the nature of its targets. A fine line appears to exist between protective and damaging roles.

Several factors or processes have been reported to affect the nature and degree of γ-secretase activity, as reviewed in Chapter 4. PEDF overexpression has been found in cortical neurons and astrocytes in the brains of AD patients (Yamagishi *et al.* 2004), and the distribution of PEDF protein was in concordance with RAGE protein, one of the receptors for Aβ peptide. PEDF overexpression has been suggested as a compensation mechanism in an attempt to prevent neuronal cell injury in AD.

Recently PEDF was also shown to upregulate γ-secretase activity which resulted in the inhibition of angiogenesis in cultured BRMECs (Cai *et al.*, 2006).

Conversely, a product of γ-secretase activity, Aβ, has been reported to have a regulatory role on the endogenous production of VEGF and PEDF in HRPE cells (Yoshida *et al.*, 2005). It was reported that Aβ accumulation induced an increase in VEGF and decrease in PEDF in cultured HRPE cells. Taken together, these studies suggest a regulatory loop between VEGF/PEDF, γ-secretase and the substrate(s) of γ-secretase.

The finding from the present study that PEDF upregulates the activity of γ-secretase in BRMECs is in agreement with Cai *et al.*(2006), in part, and suggests that the activity of γ-secretase in ECs may be beneficial with respect to the regulation of angiogenesis. Furthermore, the upregulation of γ-secretase activity by VEGF and the fact that PEDF can negate VEGF's effect in HRPE cells, increases our understanding of the relationship between VEGF/PEDF and γ-secretase. It also suggests that, in HRPE cells, γ-secretase activity may be harmful rather than beneficial. In addition PEDF may protect HRPE cells by negating the activation of γ-secretase induced by VEGF. Collectively, these findings suggest that the role of VEGF/PEDF on γ-secretase activity is cell-type dependent.

Another finding from the current study that γ-secretase exerts differential regulatory effect on VEGFR-1 and VEGFR-2 in HRPE cells suggests that γ-secretase involved in the VEGFR-1 and VEGFR-2 signalling. Future study is required to further our understanding of the role of γ-secretase on VEGFR-1 and VEGFR-2.

7.5 The role of an autocrine loop between VEGF and PEDF in HRPE cells

A precise balance between stimulators and inhibitors of angiogenesis, such as VEGF and PEDF, respectively, is essential for angiogenic homeostasis, at least, in ocular tissues.

VEGF and PEDF are expressed in a wide variety of tissues and by many cell types as reviewed in the section 1.2.1 and section 1.4.1. More interestingly, VEGF and PEDF can be produced by the same cell, for example HRPE cells (Ohno-Matsui *et al.*, 2001), BRMECs (Tombran-Tink, *et al.* 2004) and glial Muller cells (Eichler, *et al.* 2004). In HRPE cells, VEGF upregulates PEDF expression through VEGFR-1 in an autocrine manner (Ohno-Matsui *et al.*, 2003). On the other hand, VEGF produced by RPE cells may be involved in paracrine signalling between the RPE and choriocapillaris to induce endothelial fenestration (Roberts and Palade, 1995). Therefore, it is possible that VEGF/PEDF regulates endothelial proliferation and migration using both autocrine (EC-dependent) and paracrine (EC-independent) mechanisms. While the roles of VEGF and PEDF in neovascularisation are complicated, VEGF overexpression/and or PEDF under-expression is believed to be a main cause in the initiation of neovascularisation.

Due to its role in tumour neovascularisation, VEGF has been chosen as a therapeutic target to manage pathological neovascularisation. VEGF inhibitors, such as anti-VEGF monoclonal antibody and antisense oligonucleotides have shown partial success and most clinical trials have shown modest effects so far (Afzal *et al.* 2007).

The demonstration that VEGF-siRNA downregulates VEGF, as well as PEDF, in this study suggests that the downregulation of PEDF may be one of the side-effects of anti-VEGF therapy. Such a side-effect has not been considered in any literature. It also challenges the therapeutic approach of targeting a single molecule. This same concern has been raised by Afzal *et al.* (2007).

7.6 Are VEGF, VEGFR-1 and PEDF involved in the RIP signalling pathway?

Regulated Intramembrane Proteolysis (RIP) has been suggested as an non-classical signalling pathway for PEDF's action by Cai *et al.* (2006) who showed that the inhibitory effect of PEDF on VEGF-induced angiogenesis was a result of the inhibition of VEGFR-2 in BRMECs. This inhibitory effect was achieved by enhancing γ-secretase–dependent cleavage of the C-terminus of VEGFR-1.

A model for regulated intramembrane proteolysis of a tyrosine kinase receptor has had been previously suggested by Heldin and Ericsson (2001), when Ni *et al.* (2001) showed that ErbB-4 undergoes proteolysis within its plasma membrane domain. In this model, binding of a ligand to its tyrosine kinase receptor induces activation and autophosphorylation of the receptor and created docking sites for signalling proteins containing SH2 domains. In this way, different signalling pathways, such as those containing mitogen-activated protein (MAP) kinase, are activated. In a separate signalling pathway, PLC-γ activates PKC, which then activates the metalloprotease TACE (tumour necrosis factor-alpha-converting enzyme). This enzyme cleaves off the ectodomain of the receptor and allows intramembrane cleavage of the remaining part by γ-secretase. The cleaved cytoplasmic region of the receptor then moves to the nucleus, where it may affect the transcription of target genes (Heldin and Ericsson (2001) (Fig 7.3).

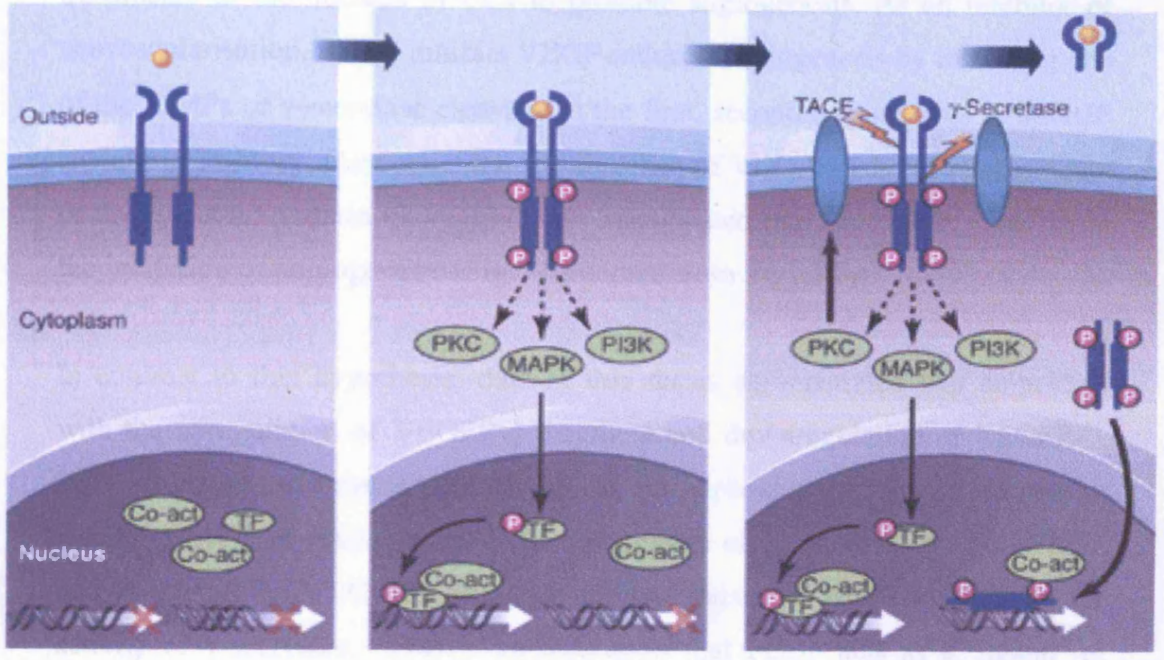


Fig 7.3 Regulated intramembrane proteolysis of a tyrosine kinase receptor. Binding of a ligand to its tyrosine kinase receptor induces activation and autophosphorylation of the receptor and the creation of docking sites for signaling proteins containing SH2 domains. In this way, different signaling pathways, such as that containing mitogen-activated protein (MAP) kinase, are activated. In a separate signaling pathway, PLC-γ activates PKC, which then activates the metalloprotease TACE. This enzyme cleaves off the ectodomain of the receptor and allows intramembrane cleavage of the remaining part by γ-secretase. The cleaved cytoplasmic region of the receptor then moves to the nucleus, where it may affect the transcription of target genes (Copied from Heldin and Ericsson, 2001).

By applying this model to VEGFR-1/VEGFR-2, this thesis hypothesised that upon VEGF binding, an enzyme (possibly one of the MMPs) cleaves a portion of the extracellular domain of the VEGFR-1. This first cleavage triggers γ-secretase to cleave the cytosolic domain of the VEGFR-1, allowing this fragment to translocate to the nucleus of ECs to promote angiogenesis. As an inhibitor of neovascularisation, PEDF inhibits VEGF-induced angiogenesis by inhibiting one of the MMPs or γ-secretase cleavage at the first, second or both sites in the RIP signalling pathway. Consequently, the cleavage of VEGFR-1 is reduced and less of the cytosolic domain of VEGFR-1 is translocated into the nuclei, resulting in the inhibition of angiogenesis (Fig 7.4a copied from Fig1.10b).

In contrast to this hypothesis, data in this thesis demonstrated that coincident with the upregulation of VEGFR-1 fragment and downregulation of VEGFR-2, PEDF upregulated γ-secretase activity and the expression of the high molecular weight (HMW) complex of PS-1. The negligible effect of the combined VEGF and PEDF on the VEGFR-1 fragment is also consistent with the effect on the activity of γ-secretase. This is an indication that PEDF acts as a 'ligand' of VEGFR-1 to initiate the RIP signalling pathway in BRMECs, whilst VEGF seems to act as a regulator (Fig 7.4b).

Increased γ-secretase activity in BRMECs by PEDF at 100 ng/ml has been reported by Cai *et al.* (2006). In their report the release of an 80 kDa fragment of the C-terminal domain of VEGFR-1 due to exposure of BRMECs to VEGF, followed by PEDF, suggests that instead of VEGF, PEDF may play a role as a ligand of VEGFR-1. In addition, PEDF's ligand role only appears when BRMECs are under the influence of VEGF. The result obtained from the current study is in agreement, in part, with the above report. The disagreement on fragment size and the condition of fragment release may be due to the involvement of complicated pathways and interactive effects of growth factors As well as the variables between experimental conditions. The variation of the time for initiation of the treatment may also be an important factor since 'near confluent cells' (Cai *et al.* 2006) and '80-90% of confluency' (current study) can

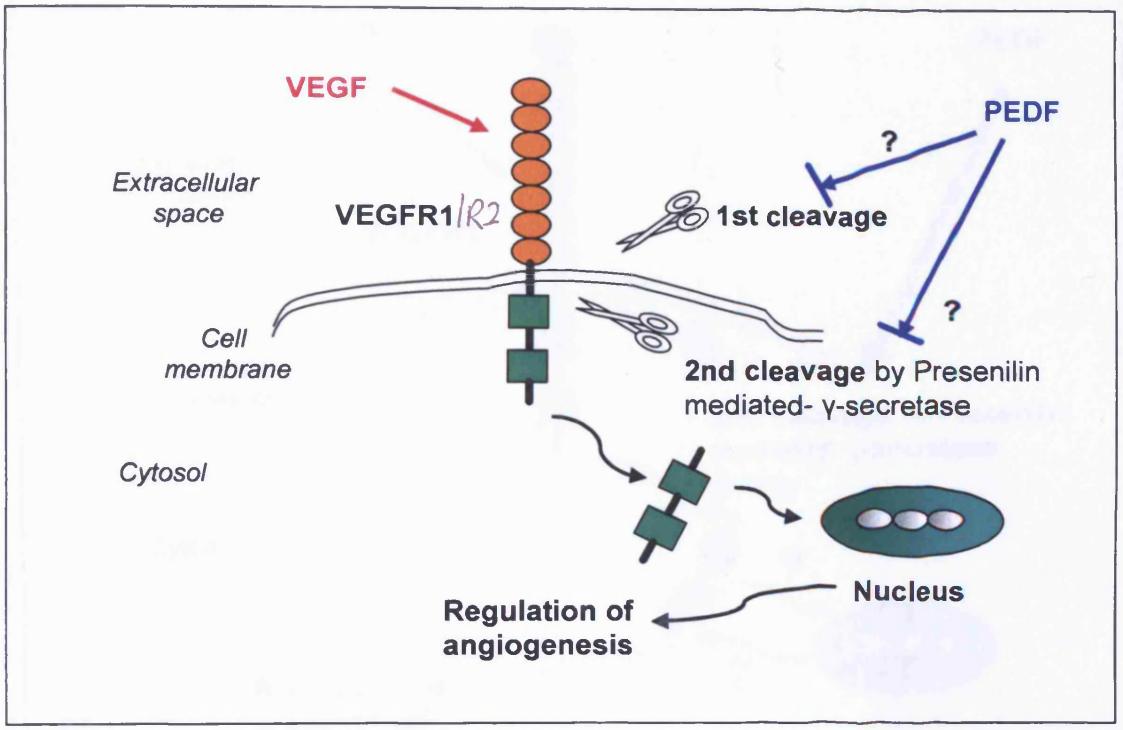


Fig 7.4-1 (= Fig 1.10b). A schematic representation of the hypothesis – the effects of VEGF & PEDF on VEGFR-1/ VEGFR-2. Upon the ligand binding (e.g. VEGF), an enzyme (possibly one of the MMPs) cleaves a portion of the extracellular domain of the VEGFR-1; this 1st cleavage initiates γ-secretase to cleave the cytosolic domain of the VEGFR-1, allowing this fragment to translocate to the nucleus of the endothelial cells to promote angiogenesis. PEDF inhibits VEGF induced angiogenesis by inhibiting the enzyme(s) activity of the first or second or both cleavages of VEGFR-1 in the RIP signalling pathway.

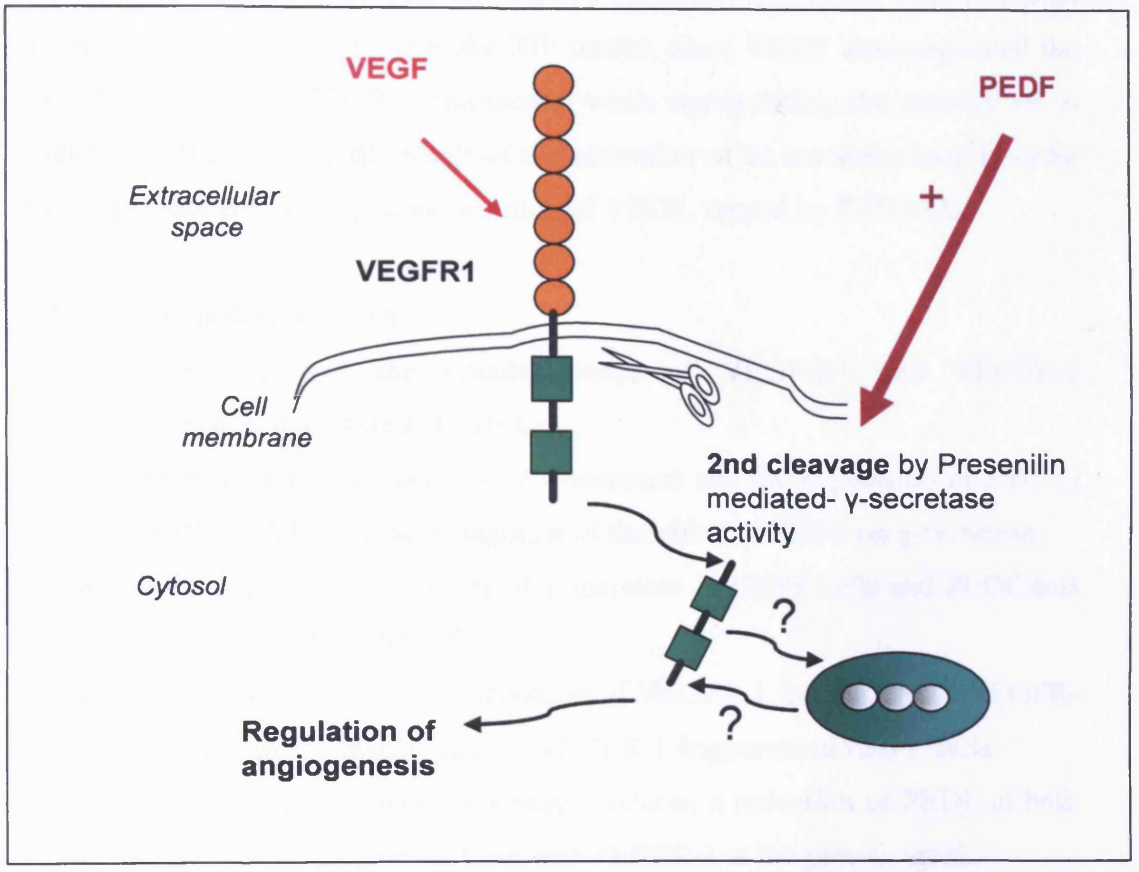


Fig 7.4-2 Amended hypothesis. In contrast to the hypothesis, data in this thesis suggested that exogenous PEDF increased the expression of VEGFR-1 fragment by upregulating γ-secretase activity and the high molecular weight (HMW) complex of PS-1.

vary dependent upon an individual's standard, whereas an elucidated response varies dependent upon the stage of cell growth.

The relationship observed between VEGF, VEGFR-1 and PEDF in HRPE cells seems more difficult to fit into the RIP model, since VEGF downregulated the expression of the VEGFR-1 fragments, while upregulating the activity of γ-secretase. This may be the result of the activation of an autocrine loop in order to compensate for the high concentration of VEGF, sensed by RPE cells.

7.7 Concluding remarks

- PEDF regulates the counterbalance of VEGFR-1 and VEGFR-2 expression in cultured BRMECs.
- PEDF regulates the activity of γ-secretase and the expression of PS-1 in BRMECs. VEGF is an antagonist of the effect of PEDF on γ-secretase.
- VEGF regulates the activity of γ-secretase in HRPE cells and PEDF acts as an antagonist of VEGF.
- γ-secretase regulates the expression of VEGFR-1 fragments and VEGFR-2, as well as the distribution of VEGFR-1 fragments in HRPE cells.
- The VEGF gene silencing strategy induces a reduction of PEDF at both the transcript and protein level and VEGFR-1 at the protein level.
- The effect of VEGF and PEDF on VEGFR-1 and VEGFR-2 is cell type dependent.

7.8 Future work

7.8.1 To examine the effect of VEGF/PEDF on VEGFR-1 and VEGFR-2 expression. A titration series of VEGF concentrations against a single dose of PEDF or vice versa may give a more complete understanding of the relationship between VEGF and PEDF and VEGFR-1/VEGFR-2 expression.

7.8.2 To gain a better understanding about the production of VEGF/PEDF from ECs and RPE cells: measure the production of VEGF/PEDF under various conditions, such as serum free medium versus media containing 1-2% or 10% FCS.

7.8.3 To use the ECs and RPE cells from same species, to compare the difference of VEGFR-1/VEGFR-2 in response to VEGF/PEDF.

7.8.4 To use PEDF-siRNA to determine its effect on the expression of VEGF, VEGFR-1 and VEGFR-2 in HRPE cells.

7.8.5 Sequence analysis of the fragments detected in BRMECs and HRPE cells.

7.8.6 The effects of VEGF/PEDF on VEGFR-1/VEGFR-2 signalling in cultured ECs when co-culturing with RPE cells.

7.8.7 The role of VEGF/PEDF on the progenitors of endothelial and RPE cells.

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APPENDICES

Appendix 1

Reagents for Cell culture

Growth medium for BRECs (TCS CellWorks, UK)
Microvascular Endothelial Cell Growth Supplement (ZHS-8947): 25ml
[containing: heparin,
hydrocortisone,
human epidermal growth factor,
human fibroblast growth factor,
dibutyryl cyclic AMP
5% (v/v) FCS (final concentration)]
Antibiotic Supplement (ZHR-9939): 0.5 ml
[containing: 25 mg/ml gentamicin
50 μg /ml amphotericin B (1000 × concentrate)]
Make up in 500 ml of Microvascular Endothelial Cell Basal Medium (ZHM-2946).

Growth medium for HRPE cells (final concentration):
10% (v/v) FCS
0.06% (w/v) Penicillin-G
0.1% (w/v) Streptomycin
0.1% (w/v) Kanamycin
Make up in Ham's F-10 (Gibco, Life Technologies, Scotland)

Trypsin –EDTA solution
0.25% (m/v) trypsin
0.05% (m/v) EDTA
Disolve in 1 × PBS, mix thoroughly, filtered using 0.2 μm filter, store at -20°C in aliquots.

Antibiotics cocktail
1.0 g Streptomycin Sulphate (Sigma # S-913)
1.0 g Kanamycin (Sigma # K-4000)
0.6 g Penicilin-G (Sigma # PEN–NA)
Disolve in 100 ml of ddH_2O, mix thoroughly, filtered using 0.2 μm filter, store at -20°C in aliquots.

Appendix 2

Reagents for proteomic analysis

Buffers for protein extraction

Extraction buffers for the preparation of two-subcellular fraction (Cai *et al.* 2006)

- **Buffer 1: 1%Triton X-100 soluble buffer**

25 mM	Tris/HCl
150 mM	NaCl
0.25%	Sodium deoxycholate
1 mM	NaF
1 mM	Na_3VO_4
1 mM	EDTA
1 mM	phenylmethylsulfonyl fluoride
1 μg/ml	aprotinin
1 μg/ml	leupeptin
1 μg/ml	pepstatin
1%	Nonidet P-40
1%	Triton X-100

- **Buffer 2: 1% SDS buffer**

25 mM	Tris/HCl
150 mM	NaCl
0.25%	Sodium deoxycholate
1 mM	NaF
1 mM	Na_3VO_4
1 mM	EDTA
1 mM	phenylmethylsulfonyl fluoride
1 μg/ml	aprotinin
1 μg/ml	leupeptin
1 μg/ml	pepstatin
1%	Nonidet P-40
1%	SDS

Extraction buffer for the preparation of whole-cell lysate: RIPA Lysis Buffer (sc-24948, Santa Cruz)

RIPA - containing:	1x	PBS
	1%	Nonidet P-40
	0.5%	sodium deoxycholate
	0.1%	SDS
PMSF	50 μl for 10ml of 1xRIPA	
Protease inhibitor cocktail	50 μl for 10ml of 1xRIPA	

Buffers for the western blotting

Running Buffer -1 ×Tris / Glycine / SDS Buffer

25 mM Tris
192 mM Glycine
0.1% (w/v) SDS
pH 8.3
To make 1 liter of above buffer, add 100 ml of 10 ×Tris / Glycine / SDS Buffer (BIO-RAD, Cat# 161-0732) to 900 ml of dH_2O. Mix thoroughly.

Transfer Buffer - 1×Tris / CAPS Buffer

60 mM Tris
40 mM CAPS
15% (v/v) methonal
pH 9.6
To make 100 ml of above buffer, add 10 ml 10 ×Tris / CAPS Buffer (BIO-RAD, Cat# 161-0778) and 15 ml Methanol to 75 ml of dH_2O just before use. Mix thoroughly.

Ponceau S staining buffer (Sigma,U.S.A.)

2% Ponceau S
30% trichloroacetic acid
30% sulfosalicylic acid
diluted in ddH2O in 1:10 for use

Blocking Buffer

3-5% (w/v) skimmed milk powder (Sigma)
50 mM of Sodium Fluoride (NaF) (1g/500ml PBS)
0.05% (v/v) Tween 20™ (250 ul/500 ml PBS)
Make up in 1 × PBS for use.

Washing Buffer

50 mM of Sodium Fluoride (NaF)
0.05% (v/v) Tween 20™
Make up in 1 × PBS for use.

10 × PBS (1000 ml)

80.0g	NaCl (Sodium Chloride):
2.0g	KCl (Potassium Chloride):
14.4g	Na_2PO_4 (di-Sodium Hydrogen Orthophosphate anhydrous)
2.4g	KH_2PO_4 (Potassium dihydrogen orthophosphate)

Disolves in 800 ml dH_2O, adjusts pH to 7.4 (using HCl), add up to 1000 ml of dH2O, mix well for use. To make 1× PBS: dilute 10× PBS 1:10 in dH_2O, mix well for use.

10 × Lamili Buffer (10L)

303.0g	Tris
1441.4g	Glycine
100.0g	SDS

Dissolves in ddH2O up to the volume of 10 litre.

1.5 M Tris/HCl (100ml)

18.17 g	Tris Base
	HCl to pH8.8
4ml	10% SDS

1.0 M Tris/HCl *(100ml)*

12.10g	Tris Base
	HCl to pH 6.8
4 ml	10% SDS

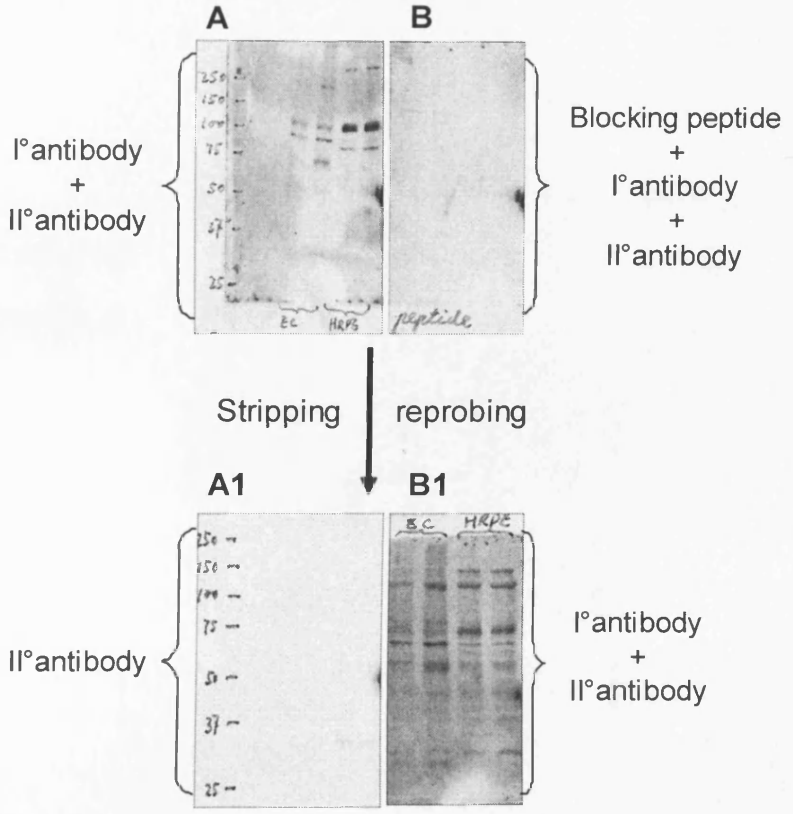
Stripping Buffer *(500 ml)*

3.75 g	Tris Base
	HCl to pH 6.7
3.52 ml	β-mecoptoethanol
10.0 g	SDS

Appendix 3

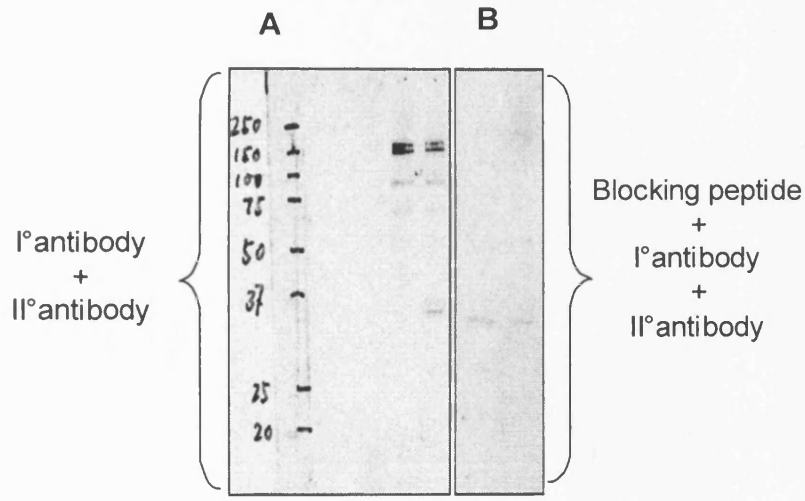
Evaluation of the specificity of the VEGFR-1 and VEGFR-2 antibody

The specificity of the VEGFR-1 antibody – the blockade of the antibody binding with a peptide specific to VEGFR-1.



Neutralisation of the VEGFR-1 antibody binding in BRMECs and HRPE cell lysates by VEGFR-1 blocking peptide by western blotting. (A) one of the duplicates membrane probed with I°and II° antibodies; (B) the 2nd duplicate probed with the blocking piptide prior to the addition of the I°and II° antibodies. The same membranes were stripped then reprobed with II° antibody only (A1) or I°and II° antibodies (B1).

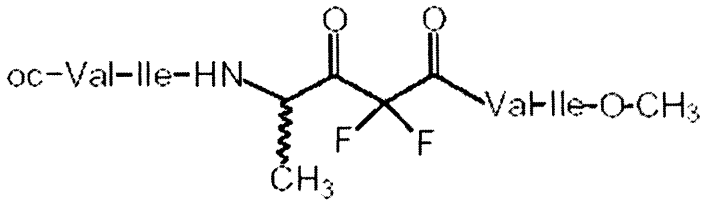
The specificity of the VEGFR-2 antibody – the blockade of the antibody binding with a peptide specific to VEGFR-2.



Neutralisation of the VEGFR-2 antibody binding in HRPE cell lysates with VEGFR-2 blocking peptide by western blotting. A: one of the duplicates membrane probed with I°and II° antibodies; B: another duplicate probed with the blocking piptide prior to the addition of the I°and II° antibodies.

Appendix 4

The molecular formular and structure of the γ-secretase inhibitor (Cat. # S 2188, Sigma): $C_{33}H_{57}N_5O_9F_2$



Appendix 5

RT-PCR relevant

Reverse-iT™ 1st Strand Synthesis –two-stept protocol:

RT1

First strand primer – anchored oligo dT (500 ng/ul)	1 ul
RNA template	1ug/10ul
Sterile H_2O	to 12 ul

RT2

Add the mix of the following components

5x First Strand Synthesis buffer	4 ul
dNTP mix (5 mM each)	2 ul
Reverse-iT™ Rtase Blend	1 ul
DTT (100 mM)	1 ul

Tris-Borate-EDTA (TBE) buffer (Cat.# T4415, Sigma)

Dilution of the TBE stock concentrates (10x) in ddH_2O to a 1 x TBE running buffer results in a buffer containing

89 mM	Tris-borate
2 mM	EDTA
pH 8.3	