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MECHANISMS OF DECORIN INHIBITION OF VEGF-STIMULATED HUMAN TROPHOBLAST MIGRATION AND ACQUISITION OF AN ENDOVASCULAR PHENOTYPE

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MECHANISMS OF DECORIN INHIBITION OF VEGF-STIMULATED HUMAN TROPHOBLAST MIGRATION AND ACQUISITION OF AN ENDOVASCULAR PHENOTYPE

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ABSTRACT

The human placenta is a highly invasive tumour-like organ in which fetal-derived trophoblast cells constitute the major cell type. A subpopulation of trophoblast cells, known as extravillous trophoblast, invades the uterine decidua and maternal arteries to establish sufficient fetal-maternal exchange to maintain healthy utero-placental homeostasis. Trophoblast invasion is highly regulated by a variety of factors at the fetal-maternal interface. Decorin is a member of the small leucine-rich proteoglycan family, produced by the decidua, and is a negative regulator of trophoblast invasiveness. I hypothesized that decorin inhibits vascular endothelial growth factor (VEGF)-stimulated endovascular differentiation and migration by interfering with signalling pathways downstream of VEGF Receptor (VEGFR)-2. Using *in vitro* migration and endothelial-like tube formation assays, I determined that decorin inhibits trophoblast migration and endovascular differentiation by interfering with VEGF-induced p38 and p44/p42 mitogen-activated protein kinase (MAPK) activation. These results have implications for the pathobiology of preeclampsia, a hypo-invasive trophoblast disorder in pregnancy.

Key Words: decorin, vascular endothelial growth factor, placenta, extravillous trophoblast, migration, endovascular differentiation, mitogen-activated protein kinases

CO-AUTHORSHIP STATEMENT

Western blot analyses in Figure A3, were done by Dr. Ganareddy V. Girish.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
Cdc42	Cell Division Control Protein 42
CHO	Chinese hamster ovary
CSF-1	Colony Stimulating Factor 1
DCN	Decorin
dNk	Decidual Natural Killer
DPBS	Dulbecco's Phosphate Buffered Saline
E-Cadherin	Epithelial-Cadherin
EDTA	Ethylenediaminetetraacetic acid
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eNOS	Endothelial Nitric Oxide Synthase
EVT	Extravillous Trophoblast
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
Flk-1	Fetal Liver Kinase 1
Flt-1	Fms-Related Tyrosine Kinase 1
GAG	Glycosaminoglycan Chain
GFR	Growth Factor Reduced
hCG	Human Chorionic Gonadotropin
HELLP	Hemolytic Anemia, Elevated Liver Enzymes, Low Platelet Count
HGF	Hepatocyte Growth Factor
HLA-G	Human Leukocyte Antigen G
HRP	Horseradish Peroxidase
HSP27	Heat Shock Protein 27
HSPG	Heparan Sulphate Proteoglycan
HUVEC	Human Umbilical Vein Endothelial Cells

IGFBP-1	Insulin-Like Growth Factor Binding Protein 1
IGF-II	Insulin-Like Growth Factor II
IGFR-1	Insulin-Like Growth Factor Type 1 Receptor
IUGR	Intrauterine Growth Restriction
KDR	Kinase Insert Domain Receptor
LRP-1	Low density lipoprotein receptor-related protein 1
LRR	Leucine Rich Repeat
MAPK	Mitogen-Activated Protein Kinase
MAPKAPK-2/3	Mitogen-Activated Protein Kinase-Activated Protein Kinase-2/3
MEK1/2	Mitogen-Activated Protein Kinase Kinase
MMP	Matrix Metalloproteinases
M-PER	Mammalian Protein Extraction Reagent
mRNA	Messenger Ribonucleic Acid
NaF	Sodium Fluoride
Na_3VO_4	Sodium Orthovanadate
PCR	Polymerase Chain Reaction
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PKB	Protein Kinase B
PlGF	Placental Growth Factor
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SFM	Serum Free Media
SLRP	Small Leucine-Rich Proteoglycan
SV40	Simian Virus 40
TBS-T	Tris-Buffered Saline with Tween-20
TGF-β	Transforming Growth Factor β
TIMP	Tissue Inhibitors of Metalloproteinases
uPA	Urokinase Plasminogen Activator
VCAM-1	Vascular Cell Adhesion Molecule-1
VE-Cadherin	Vascular Endothelial-Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Vascular Endothelial Growth Factor Receptor

CHAPTER ONE: INTRODUCTION

1.1 The Placenta

In Eutherian mammals, a placenta must be established for successful development of the fetus (reviewed by (Herr et al., 2010)). There are three main types of placenta based on the degree of placental invasion of the uterus: the non-invasive "epitheliochorial" placenta, the moderately invasive "endotheliochoriol" placenta and the highly invasive "haemochorial" placenta (Chakraborty et al., 2002; Leiser and Kaufmann, 1994; Mossman, 1987). The human placenta belongs to the third category and acts as a highly invasive tumour-like organ that invades the maternal uterine endometrium and its vasculature in order to establish adequate maternal-fetal exchange (Lala and Hamilton, 1996).

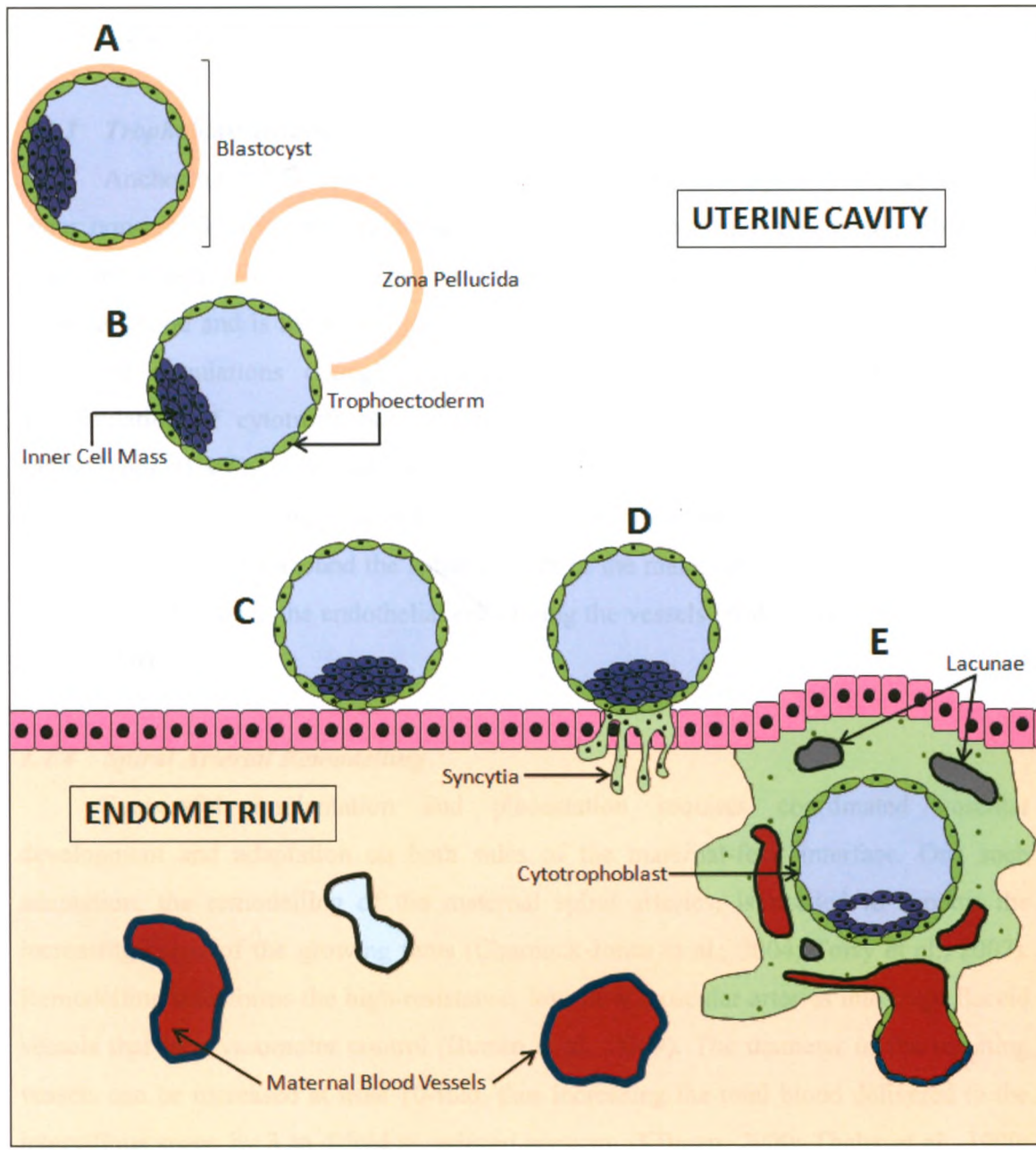
1.1.1 Blastocyst Implantation

The human placenta develops primarily through the proliferation and differentiation of embryonic trophoblast cells derived from the trophoectoderm of the pre-implantation blastocyst (Lala and Hamilton, 1996). The blastocyst consists of an inner cell mass, which gives rise to the fetus, surrounded by a single layer of mononucleated trophoblasts referred to as the trophoectoderm (Huppertz, 2008). The part of the trophoblast that attaches the blastocyst to the uterine epithelium is defined by the localization of the inner cell mass. Only the trophoblasts lining the inner cell mass, known as polar trophoblasts, are able to finally lead to implantation (Aplin, 2000). These trophoblasts breach the uterine epithelium and penetrate the basement membrane and the underlying connective tissue (Lala and Graham, 1990) (*Figure 1*).

1.1.2 Development of Villi

By ten days post-fertilization, the invading polar trophoblasts undergo syncytial fusion to form multinucleated syncytiotrophoblasts (Anin et al., 2004; Jones and Fox, 1991). Within the syncytiotrophoblast, fluid-filled spaces occur and soon coalesce to form larger lacunae which further develop into the intervillous space. The syncytiotrophoblastic masses between the larger lacunae further develop into the chorionic villi of the placenta. The remaining mononucleated trophoblasts act as a source of stem cells and are referred to as cytotrophoblasts (Huppertz, 2008).

Figure 1. Steps in Human Blastocyst Implantation. A) *Transport.* The blastocyst enters the uterine cavity. B) *Hatching and Orientation.* The zona pellucida dissolves and the inner cell mass is oriented towards the uterine epithelium. The trophoectodermal cells surrounding the inner cell mass are referred to as polar trophoblasts. C) *Adhesion.* The blastocyst adheres to the endometrial epithelium. D) *Invasion and syncitialization.* Polar trophoblasts breach the uterine epithelium and penetrate the basement membrane and underlying connective tissue. By ten days post-fertilization, the invading trophoblasts fuse to form multinucleated syncytiotrophoblasts. These cells proliferate and invade the endometrium. E) *Villous formation.* Within the syncytiotrophoblast, fluid-filled spaces occur and coalesce to become larger lacunae which further develop into the intervillous space. The chorionic villi are formed by the mass of syncytiotrophoblasts between the larger lacunae. The former trophectodermal cells act as stem cells and are now called cytotrophoblasts.


A
Blastocyst
UTERINE CAVITY
Zona Pellucida
B
Trophoectoderm
Inner Cell Mass
D
C
E
Lacunae
Syncytia
ENDOMETRIUM
Cytotrophoblast
Maternal Blood Vessels

By weeks 10-12 of pregnancy, the intervillous space has developed and is filled with maternal blood, bathing two types of chorionic villi: the floating villi, which are involved in exchange between the maternal and fetal circulations; and the anchoring villi , which anchor the placenta to the uterus (Bischof and Irminger-Finger, 2005).

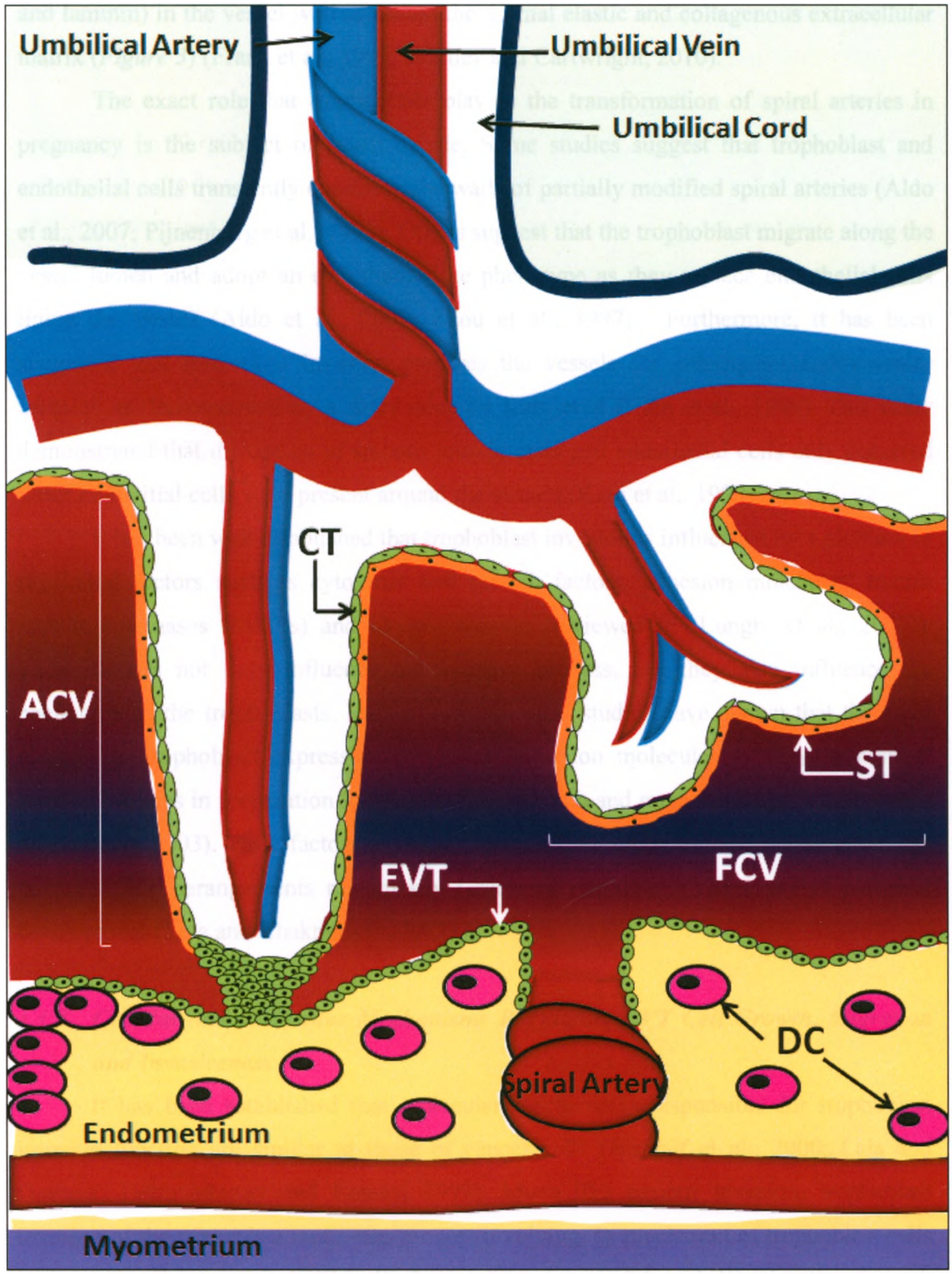
1.1.3 Trophoblast Invasion

Anchoring and Floating chorionic villi are lined by two layers of trophoblasts: the outer non-proliferative, multinucleated syncytiotrophoblasts and the inner proliferative cytotrophoblasts *(Figure 2)*. The syncytiotrophoblast layer is in direct contact with maternal blood and is the site of gas, nutrient and waste exchange between the maternal and fetal circulations (Lunghi et al., 2007; Yamamoto-Tabata et al., 2004). A subpopulation of cytotrophoblasts in the anchoring villi referred to as extravillous trophoblasts (EVT), aggregate into cell columns, proliferate, and migrate into the decidualized endometrium (referred to as interstitial invasion). During endovascular invasion, EVT cells surround the spiral arteries of the maternal vasculature, migrate into the lumen and replace the endothelial cells lining the vessels (Aldo et al., 2007; Espinoza et al., 2006).

1.1.4 Spiral Arterial Remodelling

Successful implantation and placentation requires coordinated vascular development and adaptation on both sides of the maternal-fetal interface. One such adaptation, the remodelling of the maternal spiral arteries, is needed to support the increasing needs of the growing fetus (Charnock-Jones et al., 2004; Torry et al., 2007). Remodelling transforms the high-resistance, low-flow muscular arteries into large flaccid vessels that lack vasomotor control (Burton et al., 2009). The diameter of the resulting vessels can be increased at least 10-fold, thus increasing the total blood delivered to the intervillous space by 3 to 4-fold at reduced pressure (Kliman, 2000; Thaler et al., 1990; Whitley and Cartwright, 2009). These changes occur as a result of loss of smooth muscle and lamina from the vessel wall and temporary replacement of the endothelium with a trophoblast layer. Following trophoblast invasion and replacement of endothelial cells, these trophoblasts deposit a fibrinoid matrix (composed of fibronectin, collagen type IV

Figure 2. First Trimester Placenta. The intervillous space develops by weeks 10-12 of pregnancy and is filled by maternal blood which bathes two types of chorionic villi: the floating villi (FCV) and the anchoring villi (ACV). Both villi are lined by two layers of trophoblasts: the outer non-proliferative, multinucleated syncytiotrophoblast (ST) and the inner proliferative cytotrophoblasts (CT). A subpopulation of cytotrophoblasts referred to as extravillous trophoblasts (EVT) invade out of the cell columns derived from the anchoring villi. These cells proliferate, migrate out of the villi, and invade the decidualized (DC) endometrium and occasionally the adjacent myometrium. Some extravillous trophoblasts also invade and remodel the maternal spiral arteries allowing for adequate placental perfusion with maternal blood.


Umbilical Artery
Umbilical Vein
Umbilical Cord
CT
ACV
ST
FCV
EVT
DC
Spiral Artery
Endometrium
Myometrium

and laminin) in the vessel wall replacing the normal elastic and collagenous extracellular matrix (*Figure 3*) (Frank et al., 1994; Whitley and Cartwright, 2010).

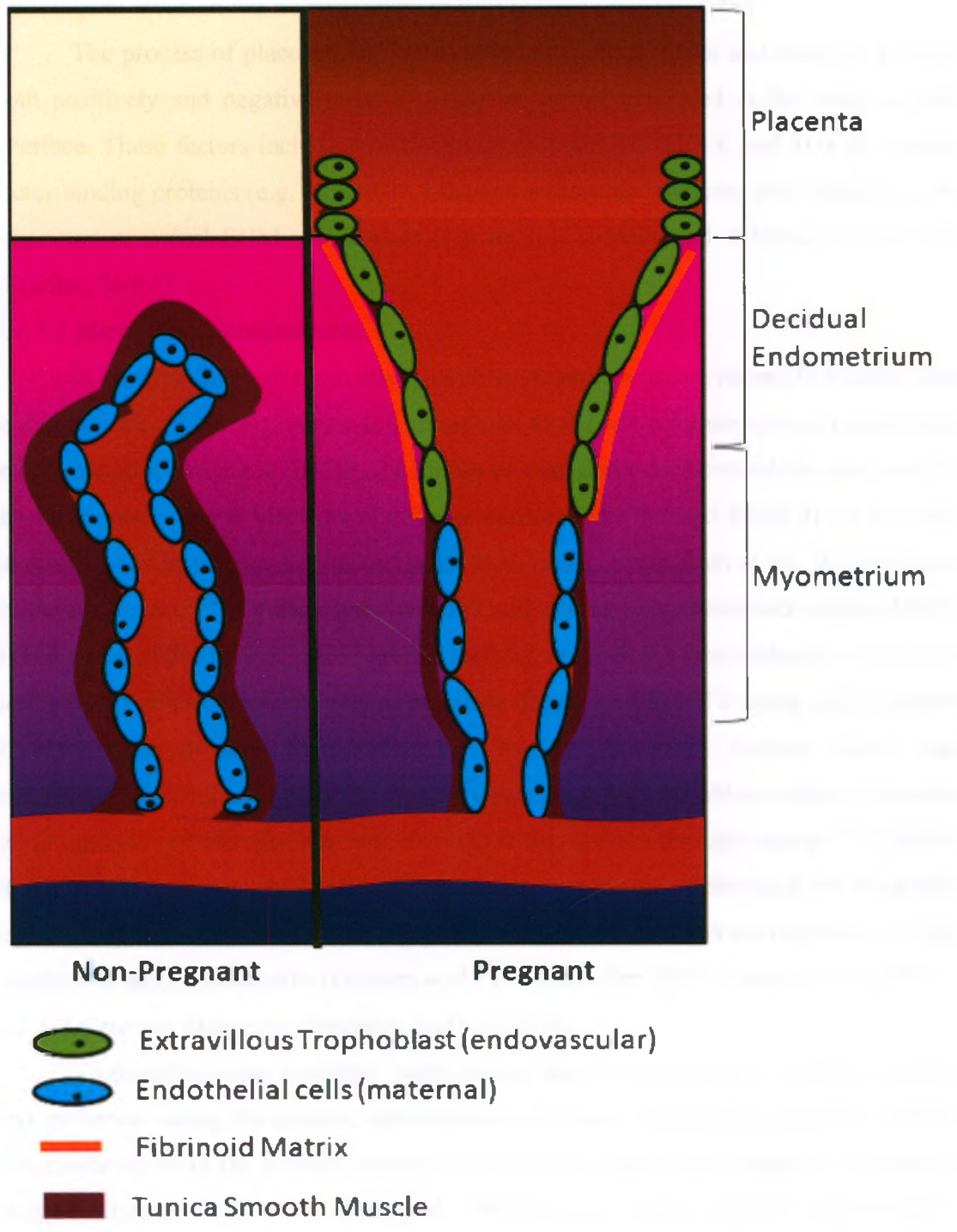
The exact role that trophoblasts play in the transformation of spiral arteries in pregnancy is the subject of much debate. Some studies suggest that trophoblast and endothelial cells transiently coexist in the walls of partially modified spiral arteries (Aldo et al., 2007; Pijnenborg et al., 1998). Others suggest that the trophoblast migrate along the vessel lumen and adopt an endothelial-like phenotype as they replace endothelial cells lining the vessel (Aldo et al., 2007; Zhou et al., 1997). Furthermore, it has been suggested that interstitial invasion prepares the vessels for subsequent endovascular invasion as demonstrated by a study done by Kam et al (Kam et al., 1999). This study demonstrated that disruption of smooth muscle cells and endothelial cells only occurred when interstitial cells were present around the vessels (Kam et al., 1999) .

It has been well established that trophoblast invasion is influenced by a plethora of regulating factors such as cytokines and growth factors, adhesion molecules, matrix metalloproteinases (MMPs) and oxygen tension (reviewed in (Lunghi et al., 2007)). These factors not only influence the invasive process, but they also influence the phenotype of the trophoblasts. Immunohistochemical studies have shown that there are changes in trophoblast expression of various adhesion molecules as they near spiral arteries, perhaps in preparation for their interaction with and replacement of vascular cells (Zhou et al., 2003). Thus, factors regulating normal EVT migration and invasiveness and their possible derangements remain key targets of research in the study of placental development (Lala and Chakraborty, 2003).

1.1.5 Cellular and Molecular Mechanisms Regulating EVT Cell Growth, Migration and Invasiveness

It has been established that molecular mechanisms responsible for trophoblast invasive function are similar to those of cancer cells (Bischof et al., 2000; Lala and Graham, 1990; Murray and Lessey, 1999). Much like tumour invasion, trophoblast invasion of the uterus is a multi-step process involving: I) attachment of trophoblast cells to the extracellular matrix (ECM); II) degradation of the ECM; and III) migration through the ECM. However, unlike tumour invasion, trophoblast invasion is restricted to the

Figure 3. Spiral Arterial Remodelling. Remodelling of the maternal spiral arteries is needed to support the increasing needs of the growing fetus. This adaptation transforms high-resistance, low-flow muscular arteries (non-pregnant) into large flaccid vessels (pregnant). These changes occur as a result of loss of smooth muscle and the temporary replacement of the endothelium with an extravillous trophoblast layer. Transformation of the maternal vessels is necessary for unimpeded placental perfusion that is required for adequate exchange of crucial molecules between the maternal and fetal circulations.


Placenta
Decidual Endometrium
Myometrium
Non-Pregnant
Pregnant
Extravillous Trophoblast (endovascular)
Endothelial cells (maternal)
Fibrinoid Matrix
Tunica Smooth Muscle

decidualized endometrium and the proximal third of the myometrium, whereas pathological invasion has no such boundaries (Lala and Graham, 1990).

The process of placental invasiveness is under strict spatial and temporal control, both positively and negatively, by a variety of factors produced at the fetal-maternal interface. These factors include growth factors (e.g. VEGF, IGF-II, and TGF-β), growth factor binding proteins (e.g. IGFBP-1), adhesion molecules (integrins and cadherins), and other non-structural ECM components (e.g. decorin, MMPs and TIMPS) (reviewed in (Knofler, 2010)).

1.1.5.1 Matrix Metalloproteinases

In general, invasive trophoblasts express various protease systems that allow them to degrade the ECM to promote cell migration. One such protease system includes the matrix metalloproteinases (MMPs), a family of zinc-dependent proteolytic enzymes. Of the 26 members of the MMP family, the gelatinases (MMP-2 and MMP-9) are the most studied MMPs in placental invasion (Isaka et al., 2003; Staun-Ram et al., 2004). During the first trimester, EVT cells express MMP-2 and villous cytotrophoblasts express MMP-9 (Isaka et al., 2003) and it is well established that, *in vitro*, cytotrophoblastic cells secrete both MMP-2 and -9. These secretions primarily degrade collagen IV along with a number of other ECM proteins (collagen I, V, VII, IX, fibronectin, laminin, elastin and vitronectin) (Bischof et al., 1995). MMPs are necessary for trophoblast invasion since the MMP inhibitor, phenanthroline, inhibits trophoblast invasion through matrigel™ (Bischof et al., 1995). Activation of MMPs and, thus, the invasion of trophoblasts are negatively regulated by tissue inhibitors of metalloproteinases (TIMPs), which are expressed by both trophoblast and decidual cells (Graham et al., 1992; Knofler, 2010; Polette et al., 1994).

1.1.5.2 Adhesion Molecules (Integrins and Cadherins)

Trophoblasts also modulate expression of adhesion molecules, such as integrins and cadherins, along the invasive differentiation pathway (reviewed in (Knofler, 2010)). Cytotrophoblasts in the villous basement membrane express α6β4 integrin, a laminin-5 receptor (Damsky et al., 1994; Zhou et al., 1997)). As trophoblasts differentiate along the invasive pathway they begin to down-regulate α6β4 integrin and up-regulate α1β1 integrin (Damsky et al., 1994; Zhou et al., 1993). The latter integrin is thought to promote invasiveness by binding to collagens and laminins, importantly laminin-2, which is

abundantly expressed in the decidua (Church et al., 1996). A similar upregulation of α5β1 (fibronectin receptor) is critical in trophoblast migration (Irving and Lala, 1995).

Expression of cadherins also changes as invasive cytotrophoblast undergo an epithelial-to-endothelial transformation. The adherens junction protein E-cadherin, a marker of polarized epithelium, is expressed in villous cytotrophoblasts, whereas, cytotrophoblasts invading the decidua and its vasculature express VE-cadherin, an adhesion marker characteristic of endothelial cells (Zhou et al., 1997).

1.1.5.3 Growth Factors

Numerous growth factors have been identified at the maternal-fetal interface controlling EVT cell proliferation, migration and invasiveness (Bischof et al., 2001; Lala and Chakraborty, 2003). Members of the epidermal growth factor (EGF) family (EGF, TGFα, amphiregulin), vascular endothelial growth factor 121 ($VEGF_{121}$), placental growth factor (PlGF) and colony stimulating factor 1 (CSF-1) all stimulate EVT cell proliferation (Athanassiades et al., 1998; Athanassiades and Lala, 1998; Hamilton et al., 1998; Lysiak et al., 1995a; Lysiak et al., 1993; Lysiak et al., 1995b). EVT cells express EGFR (receptor for EGF family of ligands), as well as, flt-1 and KDR (receptors for vascular endothelial growth factor and placental growth factor) (Athanassiades et al., 1998; Athanassiades and Lala, 1998; Hamilton et al., 1998; Lysiak et al., 1995a; Lysiak et al., 1994). Thus, it is suggested that these growth factors act in an autocrine and paracrine manner (Knofler, 2010). Of all the growth factors examined, insulin-like growth factor-II (IGF-II) produced by the trophoblast and its binding protein, IGFBP-1, produced by the decidua appear to be the most important molecules at the maternal-fetal interface, positively regulating EVT migration/invasiveness without affecting proliferation (Hamilton et al., 1998; Irving and Lala, 1995).

1.1.5.4 Inhibitory Factors Controlling Trophoblast Cell Functions

Factors inhibiting EVT cell proliferation, migration and invasion include transforming growth factor-β (TGF-β) and a TGF-β-binding proteoglycan, decorin (Graham and Lala, 1991; Graham et al., 1992; Xu et al., 2002). TGF-β is produced primarily by the decidua and to some extent by trophoblast cells and is a key negative regulator of EVT cell functions such as proliferation (Graham et al., 1992), migration (Irving and Lala, 1995), and invasiveness (Graham and Lala, 1991). The anti-invasive

function of TGF-β on the trophoblast is due to up-regulation of TIMP-1 and PAI-1 and down regulation of uPA (Graham and Lala, 1991; Graham, 1997) whereas anti-migratory action is due to up-regulation of integrins making the cells more adhesive to the ECM (Irving and Lala, 1995).

Immunohistochemical analysis revealed that decorin, a member of the small leucine rich proteoglycan (SLRP) family capable of binding TGF-β, is co-localized with TGF-β in the first-trimester decidual ECM *in situ* (Lysiak et al., 1995a). Decorin exerts an inhibitory effect on EVT cell functions, such as cell proliferation, migration and invasiveness, independent of TGF-β. It was also suggested that decorin serves as a storage device for the inactive form of TGF-β, until it is cleaved by plasmin activated by trophoblast-derived urokinase type plasminogen activator (uPA), to prevent over-invasion (Xu et al., 2002).

The discovery of new molecules involved in normal EVT cell migration and invasiveness and the establishment of the molecular mechanisms of known molecules will be important to understanding the pathophysiology of placental disorders such as preeclampsia (Lala and Chakraborty, 2003).

1.1.6 Preeclampsia

Derangements in normal trophoblast functions result in poor placental perfusion which is regarded as the root cause of the maternal syndrome, preeclampsia (Lala and Chakraborty, 2003). Preeclampsia is a pregnancy-associated disorder characterised by the onset of hypertension, proteinuria and edema in the mother (Kanasaki and Kalluri, 2009). This devastating disease occurs in approximately 5% of pregnancies worldwide (Kanasaki and Kalluri, 2009) and is commonly associated with related diseases that affect the fetus (such as intrauterine growth restriction; IUGR) or leads to further complications in the mother (such as Hemolysis, Elevated Liver enzymes, Low Platelet count; HELLP syndrome) (Mohaupt, 2007).

Abnormalities in trophoblast functions, such as inadequate invasion of the uterus and failure to remodel the spiral arteries, are commonly associated with the pathology of preeclampsia (Goldman-Wohl and Yagel, 2002; Lala and Chakraborty, 2003; Zhou et al.,

1997). Failure of normal trophoblast migration/invasiveness may result from defective molecular mechanisms regulating normal placental development.

In preeclampsia, a number of molecular defects are reported. Expression of MMP-9 fails to be up-regulated, thus limiting trophoblast invasion of the decidua (Lim et al., 1997); EVT cells fail to express specific adhesion molecules and fail to acquire an endothelial-like phenotype (Damsky et al., 1994; Zhou et al., 1997). These events may result in shallow invasion and the inability of trophoblasts to replace the endothelial lining of the uterine spiral arteries (Kaufmann et al., 2003).

In preeclampsia, secretion and expression of angiogenic factors and their receptors are often altered. For example there is decreased expression of VEGF receptors and/or an increased secretion of their soluble form (soluble fms-like tyrosine kinase 1; sFlt1) (Maynard et al., 2003). The increase in circulating sFlt1 results in a decrease in the level of circulating VEGF and PlGF, which may be responsible for several of the clinical and pathological signs of preeclampsia (Maynard et al., 2008).

1.2 Decorin

1.2.1 Decorin Structure

Decorin is a member of the small leucine-rich proteoglycan (SLRP) family. It is present in a variety of connective tissues and is composed of a 40-kDa core protein and a glycosaminoglycan (GAG) chain that is tissue-specific (Iozzo, 1997). The core protein of decorin consists of four domains: domain I contains a signal peptide and a propeptide; domain II contains four evenly spaced cysteine residues and the GAG attachment site; domain III contains the leucine-rich repeats (LRR); and domain IV contains a relatively large loop with two cysteine residues (*Figure 4*) (reviewed in (Iozzo, 1998)).

The signal peptide in domain I targets the nascent core protein to the rough endoplasmic reticulum, however, the function of the propeptide is less clear (Iozzo, 1998). Mammalian cells transfected with constructs containing deletions in decorin propeptides secrete proteoglycans with shorter glycosaminoglycan chains, suggesting that the propeptide may function as a recognition site for xylosyltransferase, the first enzyme involved in the biosynthesis of glysoaminoglycans (Sawhney et al., 1991).

Domain II contains the GAG attachment site. This region is negatively charged and carries the sulphated GAGs (Iozzo, 1998). Decorin can occur as a monoglycanated or biglycanated species with potential attachment sites at Ser^{4}, Ser^{5} and Ser^{11} (Neame et al., 1989). The composition of the GAG chain is tissue specific; for example, chondroitin sulphate in bone and dermatan sulphate in cartilage and skin (Hocking et al., 1998).

Domain III consists of ten tandem leucine-rich repeats (LRRs) with the repeating consensus sequence being in the form of LxxLxLxxNxLSxL, where L is leucine, isoleucine or valine, x is any amino acid, N is asparagine and S is serine (Iozzo, 1997). The LRR region of decorin can be substituted with N-linked oligosaccharides at three potential sites. These N-linked oligosaccharides are suggested to impede self-aggregation in order to favour interactions with extracellular matrix components and/or cell surface receptors (Iozzo, 1998).

Finally, the C-terminal end (domain IV) is the least characterized domain (Iozzo, 1998). This domain is approximately 50 amino acids long consisting of two cysteine residues, that form a disulphide bond, spaced by 32 intervening amino acids, thereby forming a large loop of approximately 34 amino acid residues (known as the "ear" repeat) (Iozzo, 1998).

1.2.2 Functions of Decorin

Most of the biological functions of decorin are mediated by the organization of the leucine-rich repeats in the core protein which folds into an arch-shaped structure (Iozzo, 1997). The outer convex surface of the arch is formed by α–helices and the concave surface, which is well-suited to bind both globular and non-globular proteins, is formed by the curved β-sheet (Weber et al., 1996). Although crystal structure analysis of decorin indicated that it is a stable dimer with large interfaces, it has been shown that biologically active decorin is a monomer in solution, and thus acts as a monovalent ligand for various extracellular matrix proteins, growth factors, and cell surface receptors (Goldoni et al., 2004; Scott et al., 2004).

Decorin is a ubiquitous component of the extracellular matrix and serves multiple functions including regulation of collagen fibrillogenesis, maintenance of tissue integrity

and serving as a reservoir for transforming growth factor-β (TGF-β) (Fairlie et al., 1998; Iozzo, 1997; Weber et al., 1996).

Type I collagen binds to LRR4-6 of the decorin core protein with high affinity and to the C-terminus with low affinity (Chen and Birk, 2010; Svensson et al., 1995). This binding has important biological consequences, *in vivo*, as demonstrated by the phenotype of decorin-null mice (Iozzo, 1997). Although these animals grow to adulthood, they reveal a phenotype characterized by skin fragility (Iozzo, 1997). Ultrastructural analysis revealed that dermal collagen showed abnormal collagen fibril organization with irregular packing and great variation in the average diameter of fibrils (Iozzo, 1997). These findings and those from *in vitro* studies, suggest that decorin plays an important role in regulating collagen fibril growth and assembly (Chen and Birk, 2010; Iozzo, 1997; Mochida et al., 2009; Weber et al., 1996).

In addition to its structural roles, decorin also functions in binding growth factors (e.g. TGF-β) and modulating cellular functions (reviewed in (Chen and Birk, 2010; Iozzo, 1997; Schaefer and Iozzo, 2008; Schaefer and Schaefer, 2010). TGF-β binds to decorin between LRR3 and 5 (Schonherr et al., 1998). Decorin inhibits growth of Chinese hamster ovary (CHO) cells due, at least in part, to the decorin-TGFβ interaction (Yamaguchi et al., 1990). Decorin binding to mature TGF-β inactivates TGF-β, in some cases, by hindering TGF-β receptor activation and sequestration into the extracellular matrix (Kresse and Schonherr, 2001) resulting in interference with downstream signalling (e.g. phosphorylation of Smad2 or LRP-1) (Abdel-Waheb et al., 2002; Iozzo, 1997; Schaefer and Schaefer, 2010; Schonherr et al., 1998).

Decorin can negatively regulate a variety of cellular functions, either by binding to extracellular matrix molecules or cell surface receptors. For example, decorin inhibited cellular adhesion and migration in endothelial cells by interacting with fibronectin (Kinsella et al., 2000; Winnemoller et al., 1991) and thrombospondin (Davies et al., 2001; Winnemoller et al., 1992). Decorin also exerts anti-proliferative effects on many cell types including endothelial cells (Santra et al., 1995; Schonherr et al., 2001; Xaus et al., 2001; Xu et al., 2002). It also inhibited angiogenesis, *in vitro,* by blocking migratory function of endothelial cells (Davies et al., 2001; Kinsella et al., 2000). In a study done by Sulochana *et al*, purified GAG-free decorin and its 26-residue leucine-rich repeat, LRR5,

inhibited vascular endothelial growth factor (VEGF)-induced proliferation, migration and tube formation by endothelial cells *in vitro* (Fan H et al., 2008; Sulochana et al., 2005).

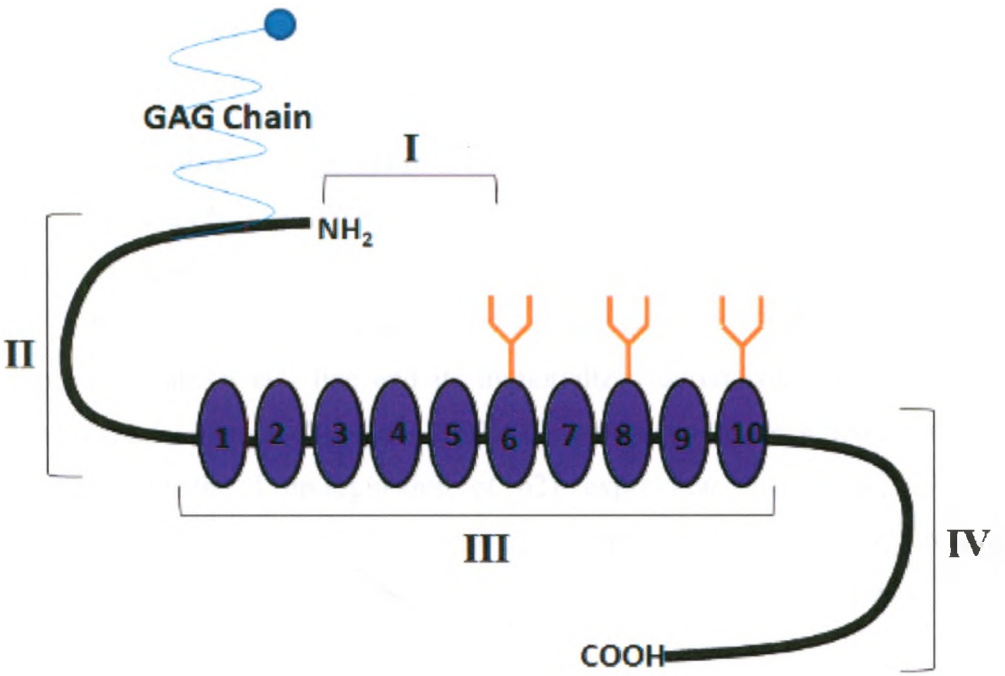
1.2.3 Decorin Receptors

Although biological effects of decorin on many cell types have been well studied, the identity of cell surface receptors responsible for these effects has remained elusive. So far, decorin is implicated in regulating multiple cellular functions due to its ability to bind to several tyrosine kinase receptors (e.g. EGFR, IGF1-R and MET) (reviewed in (Schaefer and Iozzo, 2008)).

Exogenous decorin directly binds to and induces dimerization of epidermal growth factor receptor (EGFR) in A431 squamous carcinoma cells (Iozzo et al., 1999). The binding affinity of decorin for EGFR was approximately 90-fold lower than EGF's affinity for EGFR (Iozzo et al., 1999). The effect of decorin on the phosphorylation status of EGFR depended on the manner in which cells were treated with decorin: exogenous decorin induces transient EGFR phosphorylation (Iozzo et al., 1999), whereas, overexpression of decorin in the same cell line (A431) inhibited phosphorylation of this receptor (Csordas et al., 2000). Decorin-induced activation of EGFR led to dimerization and autophosphorylation of the receptor, triggering a signal cascade including activation of mitogen-activated p rotein kinases (MAPKs), mobilization of intracellular calcium, eventual up-regulation of p21 and a decrease in proliferation (Iozzo, 1998; Patel et al., 1998; Zhu et al., 2005). Exogenous decorin, induced caveolar-dependant endocytosis (Csordas et al., 2000; Zhu et al., 2005) which resulted in reduced cell growth and increased apoptosis (Hu et al., 2009; Seidler et al., 2006).

Decorin also interacts with insulin-like growth factor 1 receptor (IGF1-R) in endothelial cells. This interaction leads to receptor phosphorylation and down-regulation of the receptor resulting in the promotion of cell survival (Schonherr et al., 2005). Furthermore, decorin-mediated activation of this receptor, in association with its interaction with $\alpha 2\beta 1$ integrin promoted endothelial cell adhesion and motility on collagen-1 (Fiedler et al., 2008).

Figure 4. Decorin Structure. Decorin, a member of the small leucine-rich proteoglycan (SLRP) family, is composed of a 40-kDa core protein and a glycosaminoglycan (GAG) chain. The core protein consists of four domains: I) signal peptide and propeptide; II) four evenly spaced cysteine residues and GAG attachment site; III) leucine-rich repeats; and IV) C-terminal end.



N-linked oligosaccharide

O-linked dermatan sulfate

Leucine-rich repeats

Recently, decorin has been shown to bind and interact with MET, the receptor for hepatocyte growth factor (HGF) on HeLa cells. This binding led to receptor phosphorylation, ectodomain shedding and degradation of the endocytosed receptor. Binding also triggered apoptosis by the induction of caspase-3/7 activity. Furthermore, decorin-MET interaction caused down-regulation of β-catenin levels and transcriptional activity (Goldoni et al., 2009).

1.2.4 Role of Decorin in Trophoblast Functions

Immunohistochemical analysis revealed that decorin co-localizes with TGF-β in first trimester decidual extracellular matrix *in situ* (Lysiak et al., 1995a). Decorin inhibited cell proliferation, migration and invasiveness of HTR-8 cells, a primary extravillous trophoblast cell line and its immortalized derivative, HTR-8/SVneo, in a TGF-β-independent manner (Xu et al., 2002). The anti-proliferative action of decorin was due to decorin-mediated up-regulation of p21 expression (Xu et al., 2002). JAR choriocarcinoma cells, derived from a malignant trophoblastic cancer, were resistant to the anti-proliferative, anti-migratory, and anti–invasive effects of decorin (Xu et al., 2002).

Recently, the role of multiple tyrosine kinase receptors in decorin-mediated control of extravillous trophoblast cell functions has been identified. These include an anti-proliferative action by epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR)-2, and an anti-migratory action by insulin-like growth factor 1 receptor (IGF1-R) (Iacob et al., 2008). In the same study, it was also demonstrated that decorin stimulated phosphorylation of IGF1-R and VEGFR-2, which also occurred with their natural ligands (IGF-1 and $VEGF_{121}$, respectively). Phosphorylation of IGF1-R and VEGFR-2 could be blocked effectively with specific receptor kinase inhibitors (PPP and KRN633, respectively) (Iacob et al., 2008).

It has recently been demonstrated that decorin binds to VEGFR-2 (Khan et al., 2009). This binding was shown with Far-Western and co-immunoprecipitation analysis. In the former analysis, the binding was demonstrated with recombinant VEGFR-2/Fc chimera in a cell–free system as well as proteins of cell lysates of EVT cells. Human umbilical vein endothelial cells (HUVEC) and human embryonic kidney (HEK) cells were used as positive and negative controls, respectively. In co-immunoprecipitation

experiments, decorin binding to VEGFR-2 could effectively be abolished by pre-treatment with a VEGFR-2 blocking monoclonal antibody raised against the ligand binding domain of VEGFR-2, indicating an overlap between decorin binding and VEGF binding domains. Co-immunoprecipitation analysis also revealed that the 13 amino-acid sequence QMIVIELGTNPLK in the LRR5 peptide of decorin is essential for decorin interaction with VEGFR-2 on EVT cells. Finally, radio-ligand binding assays were performed to determine the binding affinity of decorin for VEGFR-2 in EVT cells relative to VEGF-E, a VEGFR-2 specific ligand (Shibuya, 2003). Decorin's affinity was found to be 10-fold lower than VEGF-E, demonstrating that decorin is a low affinity ligand for VEGFR-2 (Khan et al., 2009).

1.3 Vascular Endothelial Growth Factors and Receptors

Members of the vascular endothelial growth factor (VEGF) family regulate cellular functions involved in vascular development and angiogenesis under physiological and pathological conditions (including proliferation, migration, survival and permeability) by binding to a number of receptors (*Figure 5*) (Holmes et al., 2007; Kliche S and Waltenberger J, 2001). The mammalian VEGF family comprises five secreted glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) (reviewed in (Otrock et al., 2007)). All of these growth factors are endogenously expressed and are secreted as dimeric glycoproteins that all contain eight regularly spaced cysteine residues (Kliche S and Waltenberger J, 2001). VEGFs exert their biological actions through interaction with one of three receptors: vascular endothelial growth factor receptor-1 (VEGFR-1; Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (*Figure 5*) (Cross et al., 2003). Binding of VEGFs to the receptors induce dimerization and activation, triggering a signal cascade that directs cellular function (Klagsbrun and D'Amore, 1996).

1.3.1 Vascular Endothelial Growth Factor-A Signalling in Migration

VEGF-A (also referred to as VEGF) was first identified as a vascular permeability factor secreted by tumour cells (Senger et al., 1983). However, it is now apparent that this cytokine regulates multiple biological functions, such as cell proliferation, survival and migration (Zachary and Gliki, 2001). There are five different isoforms of VEGF named

according to the number of amino acid residues they consist of: 121, 145, 165, 189 and 206 (Neufeld et al., 1996). These VEGF isoforms result from alternative splicing of human *VEGFA* mRNA from a single gene containing eight exons (Zachary and Gliki, 2001).

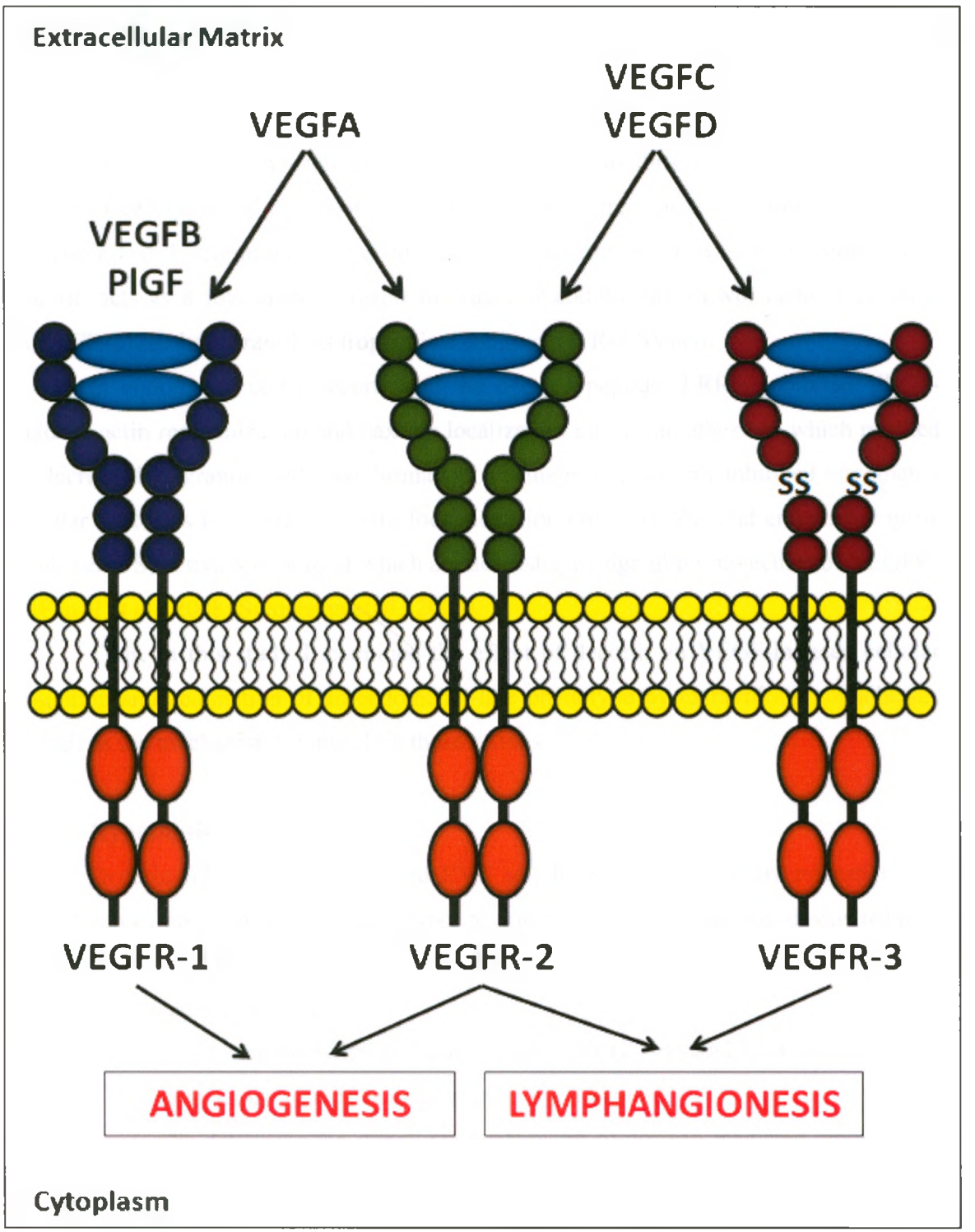
VEGFR-2 is the major mediator of several physiological and pathological effects of VEGF, including cell migration (Holmes et al., 2007). Several molecules are implicated in VEGF-dependent cytoskeleton regulation and cell migration including endothelial nitric oxide synthase (eNOS), focal adhesion kinase (FAK), p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2 or p44/42 MAPK) (*Figure 6*) (Abedi and Zachary, 1997; Feliers et al., 2005; Henriksen et al., 2003; Rousseau et al., 1997).

Nitric oxide release, via eNOS activation, from endothelial cells is central to the pro-angiogenic actions of VEGF (Dimmeler et al., 2000). VEGF signaling to eNOS is mediated by activation of VEGFR-2 leading to protein kinase B (PKB; Akt) dependent phosphorylation of eNOS at Ser1177 (Duval et al., 2007). Phosphorylation of eNOS increases its catalytic activity, releasing NO from endothelial cells which results in increased cell migration (Dimmeler et al., 2000).

Another mechanism of VEGF-stimulated endothelial cell migration is activation of focal adhesion kinase (FAK), which initiates the assembly and rearrangements of focal adhesions (Abedi and Zachary, 1997). FAK also activates other focal adhesion-associated proteins, including paxillin, which is recruited to focal adhesion complexes (Abedi and Zachary, 1997). This in turn leads to an accumulation of actin stress fibers associated with paxillin (Morales-Ruiz et al., 2000).

Finally, the MAP kinases, p38 and ERK1/2, are reported to be involved in VEGF-stimulated cell migration in a variety of cell types, including endothelial cells, osteoclasts and smooth muscle cells (Henriksen et al., 2003; Rousseau et al., 1997; Yao et al., 2004; Yu et al., 2004). In endothelial cells, it was demonstrated that VEGF stimulates p38 MAPK which results in activation of MAP kinase activated protein kinase (MAPKAPK)-2/3 and phosphorylation of heat shock protein 27 (HSP27) (Rousseau et al., 1997). VEGF-induced activation of p38 MAPK is mediated through phosphorylation of VEGFR-2 at Tyr1214, triggering activation of Cdc42 (Lamalice et al., 2004). This leads

Figure 5. The Vascular Endothelial Growth Factor Family. Members of the VEGF family regulate cellular functions involved in vascular development, angiogenesis and lymphangiogenesis. The mammalian VEGF family consists of five secreted, covalently-linked homodimeric glycoproteins (blue): VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). These growth factors exert their biological actions through specific interactions with three receptors: vascular endothelial growth factor receptor-1 (VEGFR-1), VEGFR-2 and VEGFR-3. The receptors consists of: an extracellular region composed of seven (VEGFR-1 and 2) or six (VEGFR-3) immunoglobulin (Ig)-like domains (purple, green and pink); a short transmembrane domain; and an intracellular region containing a tyrosine kinase domain (orange) split by a kinase insert domain.


Extracellular Matrix
VEGFA
VEGFC
VEGFD
VEGFB
PlGF
SS
SS
VEGFR-1
VEGFR-2
VEGFR-3
ANGIOGENESIS
LYMPHANGIONESIS
Cytoplasm

to actin polymerization and reorganization into stress fibres, which results in enhanced cell migration (Rousseau et al., 2000).

1.4 Rationale

The small leucine-rich proteoglycan, decorin, inhibits cellular functions in a variety of cell types. A study done by our lab revealed that decorin inhibits extravillous trophoblast cell migration (Xu et al., 2002). Furthermore, it was demonstrated that decorin acts as a low affinity ligand for vascular endothelial growth factor receptor-2 (VEGFR-2) on the extravillous trophoblast cell line, HTR-8/SVneo.

In endothelial cells, decorin and the decorin peptide, LRR5, inhibited VEGF-induced actin reorganization and paxillin localization into focal adhesions which resulted in decreased migration and tube formation. Furthermore, decorin inhibited endothelial cellular functions by interfering with focal adhesion kinase (FAK) and endothelial nitric oxide (eNOS) activation, both of which are downstream signalling molecules of VEGFR-2 (Fan H et al., 2008; Sulochana et al., 2005).

Thus, in this study I examined the effect of decorin on VEGF-induced cellular migration and acquisition of an endothelial-like phenotype by extravillous trophoblasts, as well as the mechanisms controlling these actions.

1.5 Hypothesis

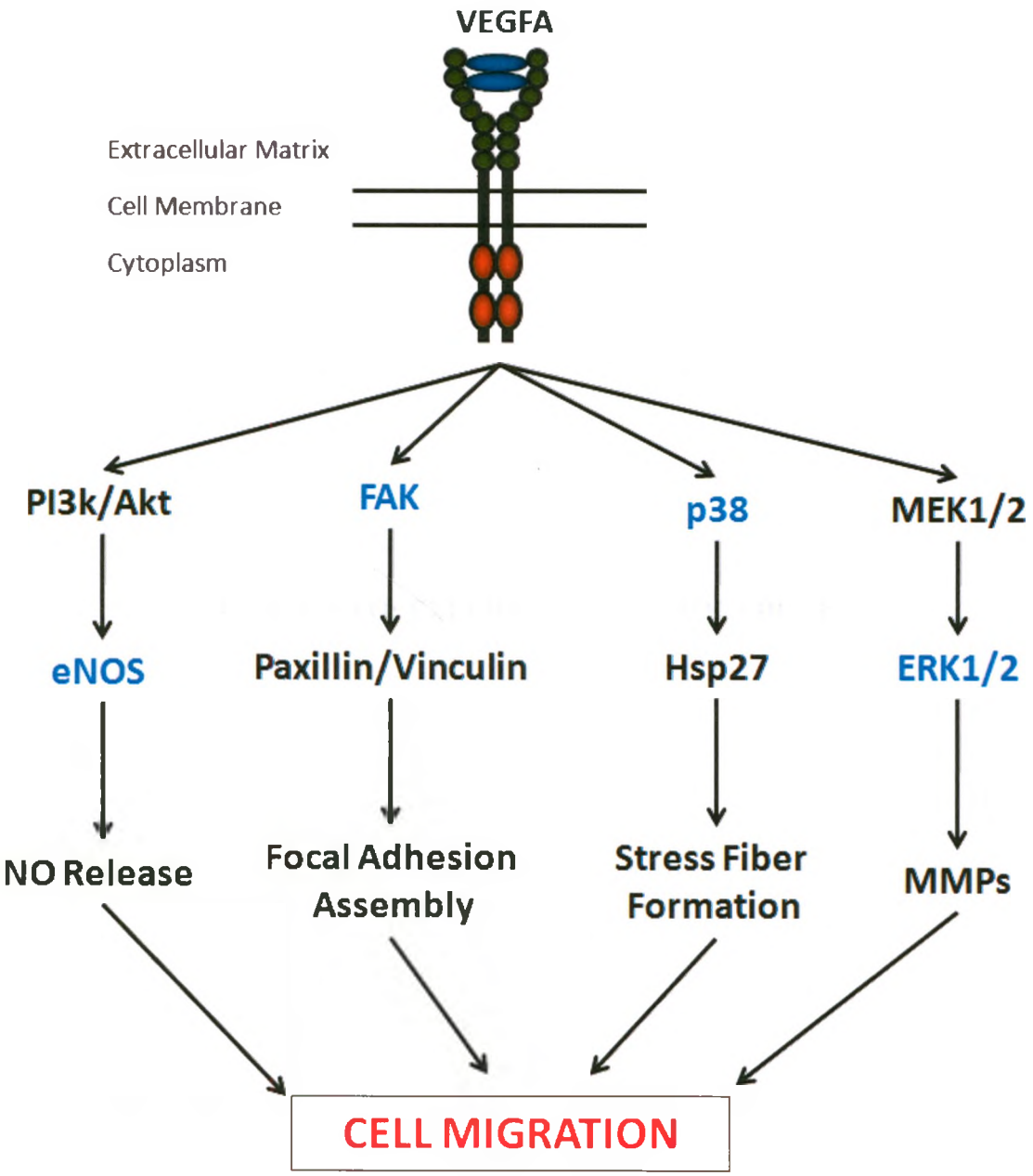
Decorin inhibits VEGF-induced EVT cell functions, specifically migration and acquisition of an endovascular phenotype, by interfering with signalling downstream of VEGFR-2.

The study consists of two **aims**:

Aim One: To determine whether decorin inhibits VEGF-induced EVT cell migration and acquisition of an endovascular phenotype.

Aim Two: To determine the signalling mechanisms of decorin-mediated inhibition of VEGF-induced EVT cell migration

Figure 6. Vascular Endothelial Growth Factor-A Signalling in Cell Migration. VEGF-A exerts the majority of its physiological and pathological effects, including cell migration, through VEGFR-2. Several molecules have been implicated in VEGF-dependent cytoskeleton regulation and cell migration including endothelial nitric oxide synthase (eNOS), focal adhesion kinase (FAK), p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2).


VEGFA
Extracellular Matrix
Cell Membrane
Cytoplasm
PI3k/Akt
FAK
p38
MEK1/2
eNOS
Paxillin/Vinculin
Hsp27
ERK1/2
NO Release
Focal Adhesion Assembly
Stress Fiber Formation
MMPs
CELL MIGRATION

CHAPTER TWO: EXPERIMENTAL PROCEDURES

2.1 Materials

RPMI medium 1640, fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS), 0.25% Tryspin-EDTA and Penicillin/Streptomycin used in cell culture were purchased from Invitrogen. BD Falcon cell culture flasks ($75cm^2$), 6-well plates, 24-well plates, anti-FAK, anti phospho-FAK (pY397), anti phospho-eNOS (pS1177), anti-eNOS/NOS Type III, Transwell inserts (24 well plate, 6.5mm diameter; 8μm pore size) and growth factor reduced (GFR) matrigel were from BD Biosciences. Anti phospho-p44/42 MAPK (Thr202/Tyr204), Anti-p44/42 MAPK, and U0126 were from Cell Signaling. Anti-p38, anti phospho-p38 (Thr180/Tyr182) and anti-β-tubulin (G-8) were from Santa Cruz Biotechnology. M-PER® Mammalian Protein Extraction Reagent, HALT™ Protease Inhibitor Cocktail and Restore™ Plus Western blot stripping buffer were from Pierce. Blotting grade blocker non-fat dry milk, goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies were from Bio-Rad. Bovine articular cartilage-derived decorin and bovine serum albumin (BSA) were purchased from Sigma, decorin peptides (P1-P4) were from GenScript and recombinant human $VEGF_{121}$ was from R&D Systems. Amersham ECL Plus™ Western blotting detection reagent and Amersham Hyperfilm ECL were from GE Healthcare.

2.2 Cell Culture

The EVT cell line, HTR-8/SVneo, was used in all experiments. This cell line was produced in our laboratory by introducing the gene encoding simian virus (SV) 40 large T-antigen into a short-lived first trimester primary EVT cell line, HTR-8, followed by neomycin (Graham et al., 1993). The parental cell line was produced by propagation of EVT cells migrating out of first trimester chorionic villus explants. Both the parental (HTR-8) and immortalized (HTR-8/SVneo) cell lines were morphologically similar (Graham et al., 1993).

Studies have shown that HTR-8/SVneo cells express all the markers of the highly migratory EVT cells *in situ*: cytokeratin 7, 8 and 18, placental type alkaline phosphatase, high affinity urokinase-type plasminogen activator (uPA) receptor, HLA framework antigen w6/32, IGF-II mRNA and protein, integrins α1, α3, α5, αv, β1 and the vitronectin

receptor αvβ3/β5 (Gleeson LM et al., 2001; Irving and Lala, 1995; McKinnon T et al., 2001; Nicola et al., 2005). These cells exhibit phenotypic behaviour of freshly isolated cytotrophoblast cells during Matrigel invasion, including the expression of HLA-G (Appendix; *Figure A1*) (Kilburn et al., 2000). Like primary HTR-8 cells, HTR-8/SVneo cells respond to the migration-stimulating signals of IGFBP-1 (Gleeson LM et al., 2001), IGF-II (Gleeson LM et al., 2001) and uPA (Liu et al., 2003), as well as, to the migration-inhibitory signals of TGF-β (Irving and Lala, 1995) and decorin (Iacob et al., 2008; Xu et al., 2002). Cells (passages 75-90) were grown in RPMI medium 1640 supplemented with 10% FBS, 50 U/ml penicillin and 50ug/ml streptomycin, unless specified otherwise. Cells were incubated at 37°C and 5% CO_2.

2.3 Tube Formation

2.3.1 VEGF- Induced Tube formation

To determine whether $VEGF_{121}$ stimulates the acquisition of an endovascular phenotype by EVT cells, GFR matrigel was thawed overnight at 4°C, diluted in cold basal RPMI 1640 media (1:1 dilution), added to 24-well plates and allowed to solidify at 37°C for 30 minutes. Near confluent cells were trypsinized and re-suspended in SFM to a final concentration of 50,000 cells/ml. Cells (1ml of cell suspension) were seeded on the solidified matrigel and stimulated with varying concentrations of $VEGF_{121}$ (0, 5, 10, 50 and 100ng/ml). The system was incubated at 37°C, 5% CO_2 for 4 to 24 hours. Preliminary experiments established that tube formation was sparse even at 12-24 hours, whereas, in the presence of $VEGF_{121}$ stimulation occurred as early as 4 hours. Pictures were taken of three random fields of view from each triplicate using a Leica EC3 camera.

2.3.2 Decorin Inhibition of VEGF-Induced Tube Formation

To examine whether decorin inhibits VEGF-induced tube formation, twenty-four well plates were coated with diluted GFR matrigel (1:1 dilution in basal RPMI 1640) and allowed to solidify at 37°C. HTR-8/SVneo cells were treated with varying concentrations of decorin from bovine articular cartilage (0, 5, 10, 50 and 100nM) for one hour. After treatment with decorin, 50,000 cells were seeded onto the solidified matrigel and incubated at 37°C, 5% CO_2 for one hour before stimulation with 50ng/ml of $VEGF_{121}$.

Plates were returned to 37°C, 5% CO_2 for 4 hours and pictures were taken of three random fields of view from each triplicate using a Leica EC3 camera.

2.3.3 Tube Quantification

The number of branching points and total tube length were quantified using the NIH ImageJ software. Branching points were considered as a point from which two or more tubes branched.

2.4 Cell Migration

2.4.1 VEGF-Induced Migration

Migration assays were performed using Transwell inserts. First, a time course was performed to determine migration kinetics. HTR-8/SVneo cells were grown to near confluence in complete media. Briefly, cells were serum starved by culturing in SFM overnight, trypsinized, and suspended in SFM to a final concentration of 250,000 cells/ml. Serum-starved cells (200ul of cells suspension) were seeded onto the top chamber of the cell culture inserts. Varying concentrations of $VEGF_{121}$ (0, 5, 20, 50ng/ml) were added to SFM (0.6ml) in the lower chamber. The assembled cell culture insert chamber was then incubated at 37°C, 5% CO_2 for 12h, 24h and 48h. After each time point, the upper surfaces of the membranes were wiped gently with cotton swabs to remove non-migratory cells. The membranes were then fixed and stained with 0.1% crystal violet in methanol, and the absolute number of migrant cells was scored visually using a Leitz Laborlux K light microscope at X400 magnification. The time response was done once with each treatment being tested in triplicate.

Stimulation of EVT cell migration by VEGF was examined using Transwells as previously described for the time course experiment. Serum-starved cells (50,000 cells) were plated onto the top chamber of the cell culture insert and SFM with varying concentrations of $VEGF_{121}$ (0, 5, 20, 50ng/ml) was added to the lower chambers. Cells were allowed to migrate for 24 hours at 37°C, 5% CO_2. Three independent experiments were performed with each treatment tested in triplicate.

2.4.2 Decorin Inhibition of VEGF-Induced Migration

Decorin-mediated effects on VEGF-induced cell migration were examined as previously described in *2.4.1.* Cells were pre-treated with varying concentrations of decorin from bovine articular cartilage (10, 50 and 100nM) or 50nM of decorin peptides (P1-P4) in SFM for one hour at room temperature before being plated onto cell culture inserts. $VEGF_{121}$ (20ng/ml) was added to the lower chamber. Three independent experiments were performed with each condition being tested in triplicate.

2.4.3 MAPK Inhibitors: U0126 and SB203580

Inhibition of VEGF-stimulated EVT cell migration by MAPK inhibitors (U0126 and SB203580 for ERK1/2 and p38, respectively) was examined as previously described in 4.1. Serum-starved cells were pre-treated with varying concentrations of the MEK1/2 inhibitor, U0126 (0, 1, 5, 10uM), or the p38 MAPK inhibitor, SB203580 (0, 1, 5, 25uM) for one hour at room temperature before stimulation with 20ng/ml $VEGF_{121}$. Three independent experiments were performed with each condition being tested in triplicate.

2.5 Western Blot Analysis

To determine whether VEGF stimulates activation of various signalling molecules downstream of VEGFR-2, cells were grown to near confluence in six-well plates and placed in serum free media overnight. Serum-starved cells were treated with 10ng/ml of $VEGF_{121}$ for varying times (0, 5, 10, 15, 30 and 60 minutes). After VEGF treatment, cells were washed with ice-cold DPBS (including 10nM NaF and 1mM Na_3VO_4) and lysed in M-PER® lysis buffer supplemented with HALT™ protease inhibitor cocktail, 10mM NaF and 1mM Na_3VO_4. After five minutes on ice, cells were scraped from the wells and transferred to 1.5ml Eppendorf tubes. Cell lysates were sonicated (five pulses) and centrifuged at 13,000 RCF for 20 minutes at 4°C. The supernatant was collected and protein concentration was measured using the BCA protein assay kit as described by the manufacturer.

To determine whether decorin inhibits VEGF-induced activation of p38 MAPK and ERK1/2, serum-starved cells in six-well plates were treated with varying

concentrations of decorin (0, 1, 5, 10, 100nM) for one hour before stimulation with 10ng/ml $VEGF_{121}$ (5 minutes and 30 minutes for ERK1/2 and p38 MAPK, respectively). Protein extraction followed the protocol described for VEGF-stimulated cells.

2.5.1 Endothelial Nitric Oxide Synthase (eNOS) Activation

50ug of total protein per well was separated by a 1.5 mm 6% SDS-PAGE gel (polyacrylamide gel electrophoresis) at 150V. The proteins were then transferred to a PVDF membrane at 60V for two hours on ice. After transfer, the membrane was blocked in 5% non-fat milk in TBST (20mM tris-base, 0.14M NaCl, 0.05% Tween, pH 7.8) for one hour at room temperature. The blot was incubated in mouse anti-eNOS (pS1177) diluted in blocking buffer (1:1000) overnight shaking at 4°C. The next day, the membrane was washed four times (10 minutes each) in TBST at room temperature followed by a one hour incubation in goat anti-mouse IgG-HRP conjugated secondary antibody diluted in blocking buffer (1:3000). Finally, the membrane was washed four times (10 minutes each) in TBST before detection by ECL reagent.

Following detection of phospho-eNOS, the membrane was washed in TBST for 10 minutes to remove the ECL reagent. The membrane was then stripped using Restore™ Plus Western blot stripping buffer for 15 minutes at room temperature, followed by two washes (10 minutes each) in TBST. The membrane was blocked in 5% non-fat milk in TBST for one hour at room temperature and then incubated in rabbit anti-eNOS diluted in blocking buffer (1:1000) overnight at 4°C. The next day the membrane was washed four times (10 minutes each) and incubated in goat anti-rabbit IgG secondary antibody diluted in 5% non-fat milk in TSBT (1:5000) for one hour at room temperature. Finally, the membrane was washed again four times (10 minutes each) before detection by ECL reagent. VEGF (10ng/ml for 10 minutes) stimulated Human Umbilical Vein Endothelial Cell (HUVEC) lysates were used as a positive control.

2.5.2 Focal Adhesion Kinase Activation (FAK) Activation

25ug of total protein per well was separated by a 1.5mm 10% SDS-PAGE gel (polyacrylamide gel electrophoresis) at 200V. The proteins were then transferred to a PVDF membrane at 60V for 90 minutes on ice. After transfer, the membrane was blocked

in 1% BSA in TBST overnight at 4°C. The next day, the membrane was incubated in mouse anti-human FAK (pY397) diluted in blocking buffer (1:1000) for one hour shaking at room temperature. The next day, the membrane was washed three times (10 minutes each) in TBST at room temperature followed by a one hour incubation in goat anti-mouse IgG secondary antibody diluted in blocking buffer (1:3000). Finally, the membrane was washed three times (10 minutes each) in TBST before detection by ECL reagent.

Following detection of phospho-FAK, the membrane was washed in TBST for 10 minutes to remove the ECL reagent. The membrane was then stripped using Restore™ Plus Western blot stripping buffer for 15 minutes at room temperature, followed by three washes (10 minutes each) in TBST. The detection of total FAK was done in the same manner as the detection of phospho-FAK, however the membrane was probed with a mouse anti-FAK antibody diluted in 1% BSA in TBST (1:1000).

2.5.3 P38 Mitogen-Activated Protein Kinase (MAPK) Activation

25ug of total protein per well was separated by a 1.5mm 12% SDS-PAGE gel (polyacrylamide gel electrophoresis) at 150V. The proteins were then transferred to a PVDF membrane at 100V for 90 minutes on ice. After transfer, the membrane was blocked in 5% non-fat milk in TBST for one hour at room temperature. The membrane was then incubated in rabbit anti-p38 (Thr180/Tyr182) in blocking buffer (1:200) overnight at 4°C. The next day, the membrane was washed three times (5 minutes each) in TBST at room temperature followed by hour incubation in goat anti-rabbit IgG secondary antibody diluted in blocking buffer (1:5000) for one hour at room temperature. Finally, the membrane was washed three times (5 minutes each) in TBST before detection by ECL reagent.

Following detection of phospho-p38, the membrane was washed in TBST for 10 minutes to remove the ECL reagent. The membrane was then stripped using Restore™ Plus Western blot stripping buffer for 10 minutes at room temperature, followed by three washes (10 minutes each) in TBST. The detection of total p38 was done in the same manner as the detection of phospho-p38, however the membrane was probed with a mouse anti-p38 antibody diluted in 5% non-fat milk in TBST (1:200) and a goat anti-mouse IgG secondary antibody was used.

2.5.4 Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) Activation

The detection of phospho-ERK1/2 followed the protocol in 5.3 with the following changes. The membrane was incubated in anti-p44/42 (Thr202/Tyr204) diluted in 5% BSA in TBST (1:1000) overnight at 4°C and a goat anti-rabbit IgG secondary antibody was diluted in 5% non-fat milk in TBST (1:5000) for one hour at room temperature.

Following detection of phospho-ERK1/2, the membrane was washed in TBST for 10 minutes to remove the ECL reagent. The membrane was then stripped using Restore™ Plus Western blot stripping buffer for 15 minutes at room temperature, followed by three washes (10 minutes each) in TBST. The detection of total ERK1/2 was done in the same manner as the detection of phospho-ERK1/2, however, the membrane was probed with a p44/42 MAPK antibody diluted in 5% BSA in TBST (1:1000).

2.5.5 *Densitometry*

NIH ImageJ was used to determine the average density of each band (p38 and ERK1/2 blots only). For each treatment, the band density of phosphorylated protein was normalized to the band density of the corresponding total protein. The results were expressed as the ratio of phosphorylated protein to total protein.

2.6 Statistical Analysis

Tube formation and cell migration data are presented as a grand mean ± standard deviation (SD) for three independent experiments. Data were analyzed with a one-way ANOVA followed by a post-hoc TUKEY's test using GraphPad Prism 5 software.

Densitometry data are presented as a mean ± standard deviation (SD) for three independent experiments. Data were analyzed with a one-way ANOVA followed by a Dunnett's multiple comparison test using GraphPad Prism 5. Differences between two treatment groups were accepted as significant at $P < 0.05$.

CHAPTER THREE: RESULTS

3.1 Effect of $VEGF_{121}$ on Endothelial-Like Tube Formation

Endothelial-like tube formation assays were performed to determine if $VEGF_{121}$ stimulates the acquisition of an endovascular phenotype by EVT cells. Little tube formation was present under serum free conditions in the absence of $VEGF_{121}$, however, tube formation was stimulated at concentrations of $VEGF_{121}$ as low as 10ng/ml as compared to SFM (*Figure 7*). This stimulation increased in a dose-dependent manner up to 50ng/ml of $VEGF_{121}$. At 100ng/ml $VEGF_{121}$, tube formation was significantly increased as compared to SFM, however this stimulation was not weaker than stimulation at 50ng/ml $VEGF_{121}$. These results suggest that HTR-8/SVneo cells have an intrinsic capacity to form capillary-like networks and this capacity can be stimulated with $VEGF_{121}$.

3.2 Effect of Decorin on VEGF-Induced Endothelial-Like Tube Formation

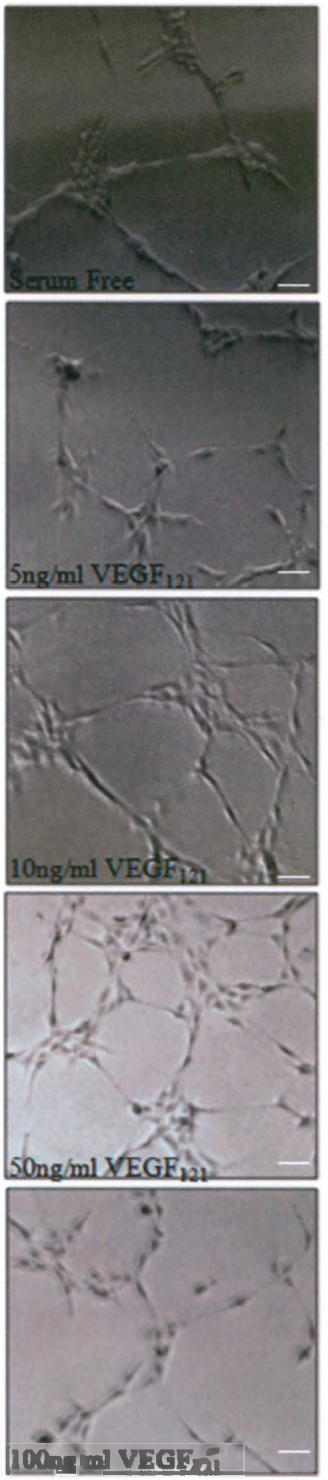
Decorin inhibits trophoblast functions such as proliferation, migration and invasion, *in vitro*, by binding to multiple tyrosine kinase receptors including VEGFR-2 (Iacob et al., 2008; Xu et al., 2002). For this reason, I tested whether decorin could interfere with the ability of EVT cells to adopt an endovascular phenotype. It was found that 50ng/ml $VEGF_{121}$ stimulated capillary-like networks compared to serum free conditions and this stimulation was blocked by decorin (*Figure 8*). Decorin brought VEGF-stimulated tube formation back to control (SFM) levels at a concentration as low as 50nM. Total tube length decreased with increasing decorin concentrations in a dose-dependent manner and total tube length was lower than control levels in the presence of 100nM of decorin. These results suggest that decorin inhibits VEGF-stimulated acquisition of an endothelial-like phenotype by human trophoblast cells.

3.3 Effect of $VEGF_{121}$ on HTR-8/SVneo Migration

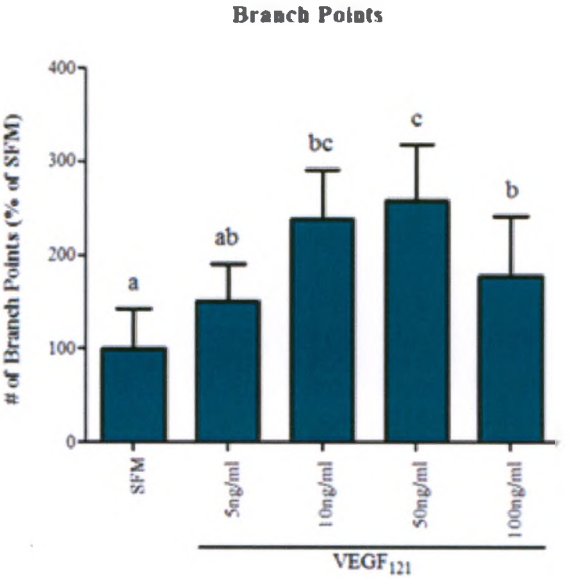
Transwell migration assays were performed to determine whether $VEGF_{121}$ stimulates EVT cell migration, *in vitro*. Initially, a dose and time response to $VEGF_{121}$ was performed to determine when maximum migration occurs (Appendix; *Figure A2*). At 24 hours, a trend towards increasing migration with increasing concentrations of $VEGF_{121}$

Figure 7. $VEGF_{121}$ Stimulates Tube Formation by HTR-8/SVneo. HTR-8/SVneo human extravillous trophoblast cells were seeded on 24-well plates coated with a thin layer of diluted growth factor reduced (GFR) matrigel™. Cells were treated with varying concentrations of human recombinant $VEGF_{121}$ (0, 5, 10, 50, 100ng/ml) and allowed to incubate at 37°C, 5% CO_2. Pictures were taken after four hours of incubation **(A)**. The extent of tube formation increased with increasing doses of VEGF and maximum tube formation occurred in the presence of 50ng/ml $VEGF_{121}$. The extent of tube formation was measured by quantifying **(B)** the number of branching points and **(C)** the total tube length using the NIH ImageJ software. Tube formation data are represented as a mean ± standard deviation (SD) for three independent experiments with each condition being tested in triplicate. Significant differences ($p<0.05$) in the means are indicated by different superscripts.

(A)


Serum Free
5ng/ml VEGF121
10ng/ml VEGF121
50ng/ml VEGF121
100ng/ml VEGF121

(B)


Branch Points
# of Branch Points (% of SFM)
400
300
200
100
0
a
ab
bc
c
b
SFM
5ng/ml
10ng/ml
50ng/ml
100ng/ml
VEGF121

(C)

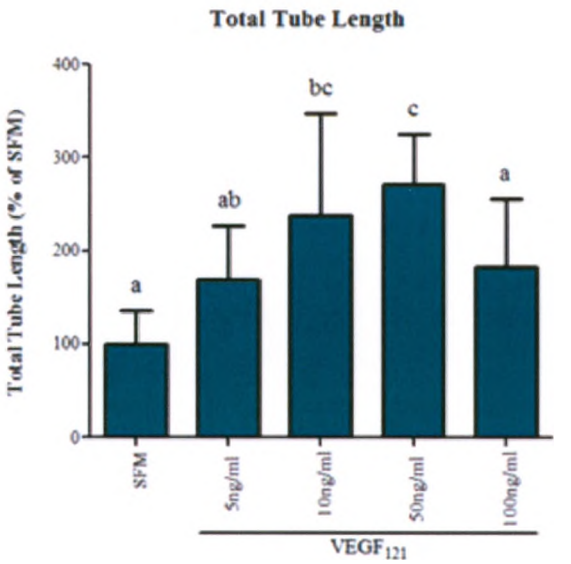
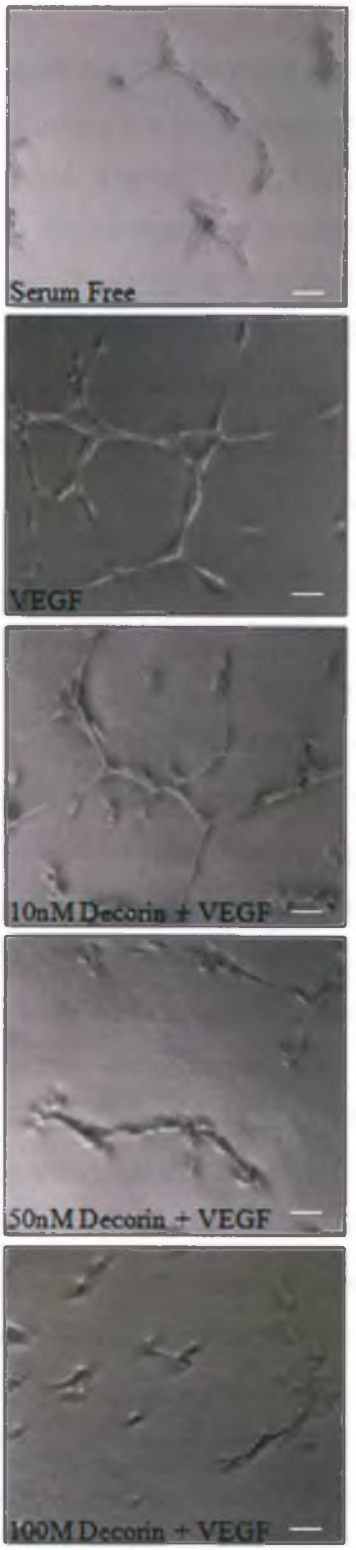
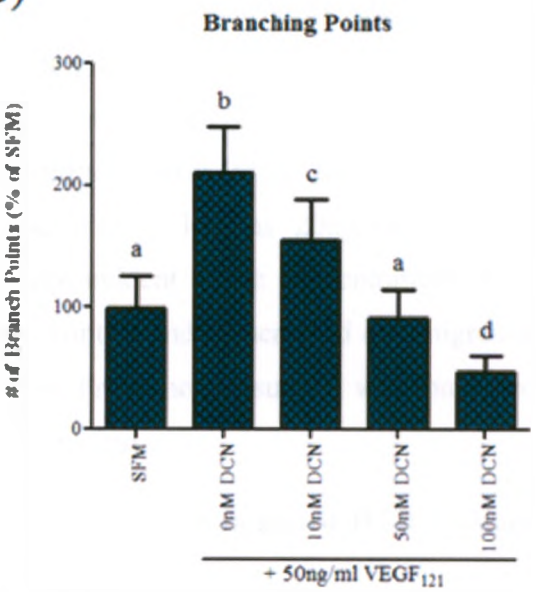

Total Tube Length
Total Tube Length (% of SFM)
400
300
200
100
0
a
ab
bc
c
a
SFM
5ng/ml
10ng/ml
50ng/ml
100ng/ml
VEGF121

Figure 8. Decorin Inhibits VEGF-Induced Tube Formation. HTR-8/SVneo human extravillous trophoblast cells were seeded on 24-well plates coated with a thin layer of diluted growth factor reduced (GFR) matrigel™. Cells were treated with varying concentrations of decorin (0, 10, 50 and 100nM) for one hour before stimulation with 50ng/ml of $VEGF_{121}$. Cells were incubated at 37°C, 5% CO_2 and pictures were taken after four hours **(A)**. Tube formation decreased with increasing doses of decorin with a significant reduction of VEGF-induced tube formation in the presence of 50nM decorin. The extent of tube formation was measured by quantifying **(B)** the number of branching points and **(C)** the total tube length using the NIH ImageJ software. Tube formation data are represented as a mean ± standard deviation (SD) for three independent experiments with each condition being tested in triplicate. Significant differences ($p<0.05$) in the means are indicated by different superscripts.

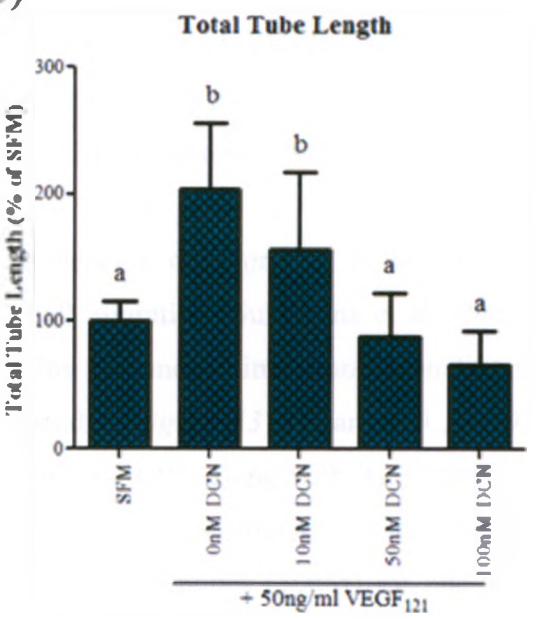
(A)


Serum Free
VEGF
10nM Decorin + VEGF
50nM Decorin + VEGF
100M Decorin + VEGF

(B)


Branching Points
# of Branch Points (% of SFM)
300
200
100
0
a
b
c
a
d
SFM
0nM DCN
10nM DCN
50nM DCN
100nM DCN
+ 50ng/ml VEGF121

(C)


Total Tube Length
Total Tube Length (% of SFM)
300
200
100
0
a
b
b
a
a
SFM
0nM DCN
10nM DCN
50nM DCN
100nM DCN
+ 50ng/ml VEGF121

was observed. However, the number of migratory cells decreased in the presence of $VEGF_{121}$ at 48 hours. This suggests that maximum migration occurs at 24 hours.

Following the time course experiments, the effect of $VEGF_{121}$ on cell migration was examined. The addition of $VEGF_{121}$ to serum-starved cells induced a 2 to 3-fold increase in the number of migratory cells compared to control (SFM) levels (*Figure 9*). Stimulation of migration occurred at concentrations as low as 20ng/ml $VEGF_{121}$. Furthermore, a dose response to $VEGF_{121}$ was not evident at the concentrations used. Fetal bovine serum (FBS) was used as a positive control and it increased cell migration by approximately 3-fold compared to control levels. From these results, it was concluded that $VEGF_{121}$ stimulates HTR-8/SVneo migration, *in vitro*.

3.4 Effect of Decorin and Decorin Peptides on VEGF-Stimulated HTR-8/SVneo Migration

To examine the effect of decorin and decorin leucine-rich repeat 5 (LRR5) peptides (P1-P4) on HTR-8/SVneo migration, serum-starved cells were treated with varying concentrations of decorin for one hour before being seeded on transwell membranes and stimulated with 20ng/ml $VEGF_{121}$. Cells treated with decorin followed by $VEGF_{121}$ stimulation migrated 2-fold less than those treated with $VEGF_{121}$ alone (*Figure 10a*). Migration stimulated by 20ng/ml $VEGF_{121}$ was reduced to control (SFM) levels in the presence of decorin concentrations as low as 10nM. A decorin dose response was not evident for the concentrations used.

The leucine-rich repeat, LRR5, of the decorin core protein is reported to significantly reduce VEGF-induced endothelial cell migration (Sulochana et al., 2005). Furthermore, studies from our lab revealed that this sequence is important in binding to VEGFR-2 on extravillous trophoblast cells (Appendix; *Figure A3*) (Khan et al., 2009). Thus, I set out to determine whether the LRR5 peptide (P1), along with its, C-terminal (P2), middle (P3), and N-terminal (P4) portions could block migration stimulated by VEGF. EVT cells were pre-treated with decorin LRR5 peptides (50nM) for one hour before being placed on Transwell membranes and stimulated with $VEGF_{121}$ (*Figure 10b*). A 3- to 4-fold decrease in the number of migratory cells was observed when cells were treated with decorin LRR5

Figure 9. $VEGF_{121}$ Stimulates HTR-8/SVneo Cell Migration. HTR-8/SVneo human extravillous trophoblast cells were plated on the top chamber of transwell migration inserts and allowed to migrate for 24 hours at 37°C, 5% CO_2. The bottom chamber contained SFM alone or with varying concentrations of $VEGF_{121}$ (5, 20 and 50ng/ml). FBS (10%) was used as a positive control. $VEGF_{121}$ stimulated migration through the membrane at concentrations of 20ng/ml or higher. Migration data are represented as a mean ± standard deviation (SD) for three independent experiments with each condition being tested in triplicates. Significant differences ($p<0.05$) in the means are indicated by different superscripts.

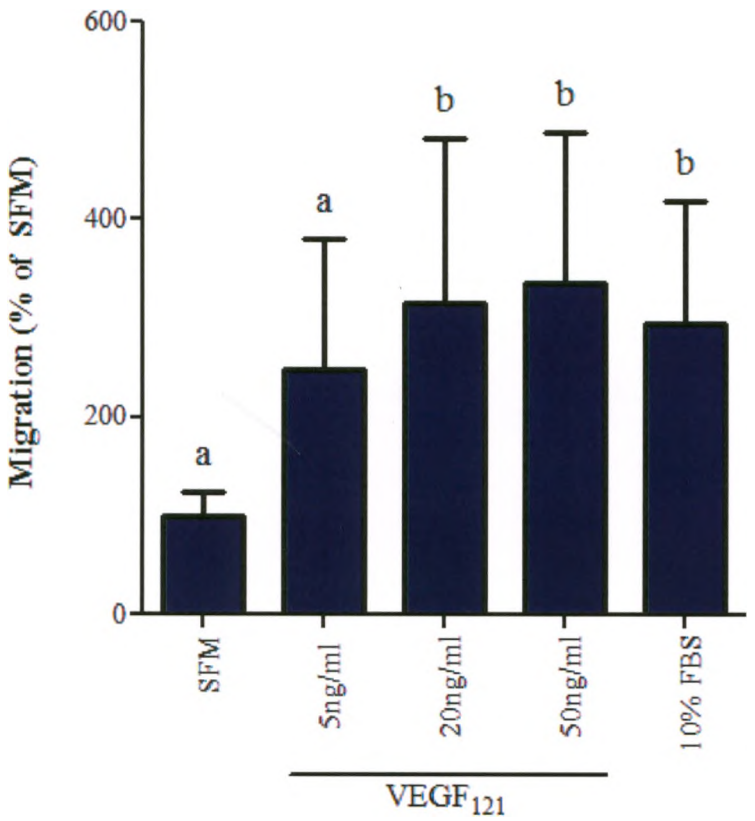
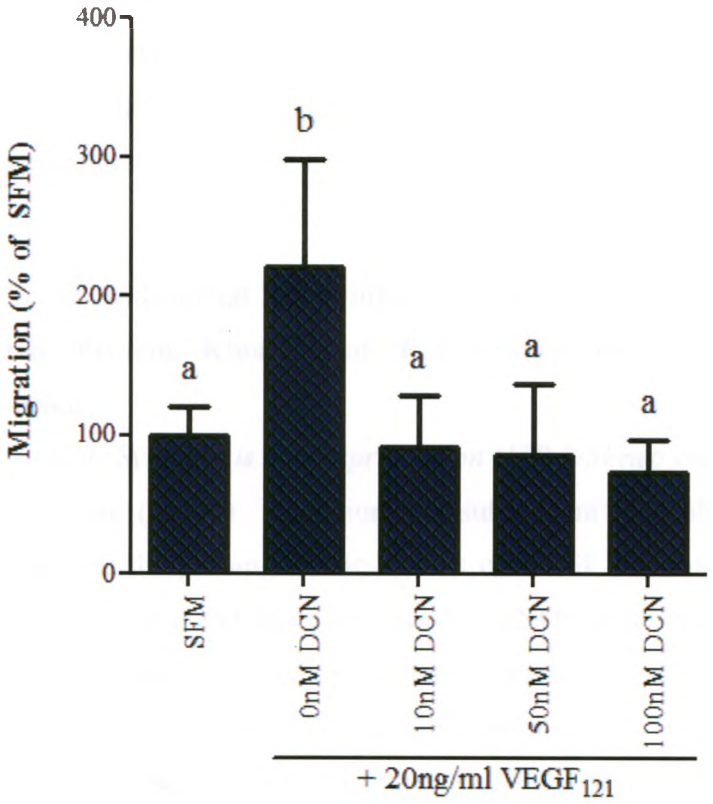
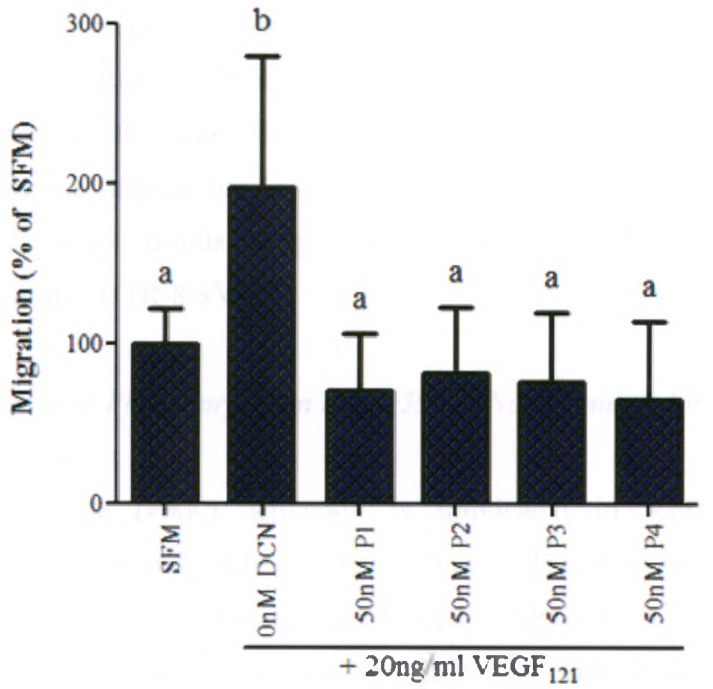

Migration (% of SFM)
600
400
200
0
a
a
b
b
b
SFM
5ng/ml
20ng/ml
50ng/ml
10% FBS
VEGF121

Figure 10. Decorin and Decorin Peptides Interfere with VEGF Stimulation of HTR-8/SVneo Cell Migration. HTR-8/SVneo human extravillous trophoblast cells were pre-treated with varying concentrations of (A) decorin (10, 50, 100nM) or (B) 50nM decorin peptides for one hour. $VEGF_{121}$ (20ng/ml) was placed in the bottom chamber and was used as a chemotactic agent. **(A)** Full-length decorin inhibited VEGF-stimulated migration at a concentration as low as 10nM. This response to decorin was not dose-dependent at the concentrations used. **(B)** All four decorin peptides (P1-P4) inhibited VEGF-stimulated migration to the same degree. Migration data are represented as a mean ± standard deviation (SD) for three independent experiments with each condition being tested in triplicates. Significant differences ($p<0.05$) in the means are indicated by different superscripts.

(A)


Migration (% of SFM)
400
300
200
100
0
a
b
a
a
a
SFM
0nM DCN
10nM DCN
50nM DCN
100nM DCN
+ 20ng/ml VEGF121

(B)


Migration (% of SFM)
300
200
100
0
a
b
a
a
a
a
SFM
0nM DCN
50nM P1
50nM P2
50nM P3
50nM P4
+ 20ng/ml VEGF121

peptides prior to $VEGF_{121}$ stimulation, as compared to cells only stimulated by $VEGF_{121}$. All four peptides blocked VEGF-induced migration to the same degree.

These results, taken together, suggest that decorin and LRR5 peptides are potent inhibiters of VEGF-induced migratory responses of HTR-8/SVneo cells.

3.5 Effect of $VEGF_{121}$ on Endothelial NO Synthase, Focal Adhesion Kinase, p38 Mitogen-Activated Protein Kinase and Extracellular Signal-Regulated Kinase 1/2 Activation

3.5.1 Endothelial Nitric Oxide Synthase is Not Expressed on HTR-8/SVneo cells

Endothelial NO synthase (eNOS) activation and subsequent NO release from endothelial cells is important to the pro-angiogenic actions of VEGF (Dimmeler et al., 2000). Thus, in this study, expression and phosphorylation of eNOS following exposure of HTR-8/SVneo cells to VEGF, was examined by Western blot analysis. Serum-starved EVT cells stimulated with VEGF (10ng/ml) at various times points (0, 5, 10, 15, 30 and 60 minutes) were examined for phosphorylated eNOS at Ser1177 (Appendix; *Figure A4*). No bands were detected in any of the lanes containing HTR-8/SVneo cell lysates, suggesting that eNOS is not phosphorylated by VEGF in HTR-8/SVneo cells. However, phosphorylated eNOS was present in human umbilical vein endothelial cell (HUVEC) lysates (positive control) indicating that the antibody was effective. Membranes were then striped and probed for total eNOS. Again, no bands were present in lanes containing the trophoblast lysates, whereas a stronger band was present in the lane containing HUVEC lysates (positive control). These results suggest that significant eNOS protein is not expressed in the EVT cell line, HTR-8/SVneo.

3.5.2 Focal Adhesion Kinase Phosphorylation at Tyr397 is Not Stimulated by $VEGF_{121}$ in HTR-8/SVneo Cells

Focal adhesion kinase (FAK) activation is implicated in VEGF-induced endothelial cell migration (Abedi and Zachary, 1997). Thus, to determine whether FAK activation is required for VEGF-stimulated EVT cell migration, VEGF-induced phosphorylation and expression of FAK was examined by Western blot analysis. Serum-starved cells were stimulated with VEGF (10ng/ml) at various time points (0, 1, 5, 10, 30

and 60 minutes) and examined for phosphorylated FAK at Tyr397 (Appendix; *Figure A5*). Phosphorylated FAK was present in non-stimulated cell lysates and stimulated cell lysates to the same degree, suggesting that FAK is activated in this highly migratory EVT cell line even under serum-starved conditions, possibly precluding detection of any further stimulation by $VEGF_{121}$. Total FAK was used as a loading control.

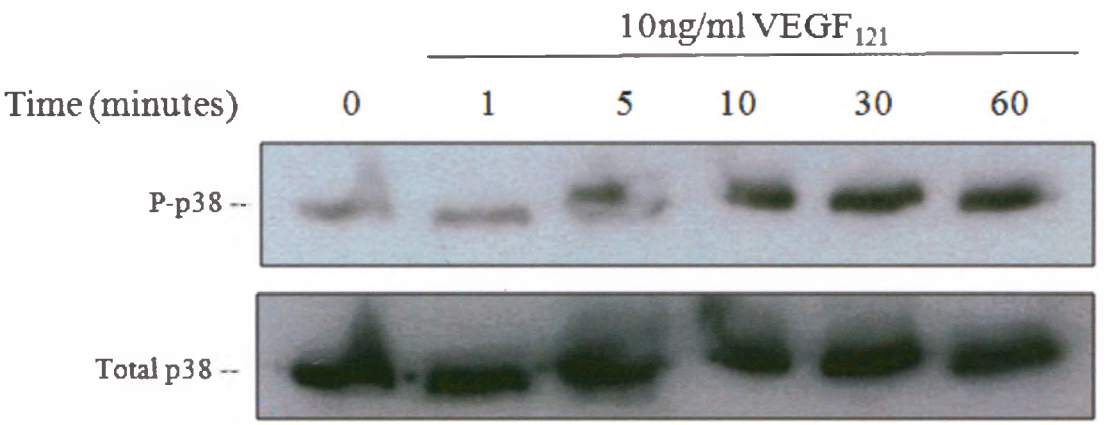
3.5.3 Mitogen-Activated Protein Kinases (p38 and ERK1/2) are Activated by $VEGF_{121}$ in HTR-8/SVneo

Mitogen-activated protein kinases (MAPK) are also implicated in VEGF-stimulated migration in a variety of cell types (Henriksen et al., 2003; Rousseau et al., 1997; Yao et al., 2004; Yu et al., 2004). Western blot analysis was performed to determine whether p38 MAPK and extracellular signal-regulated kinase 1/2 (ERK1/2; p44/p42 MAPK) are involved in VEGF-stimulated HTR-8/SVneo cell migration. Serum-starved cells were stimulated with VEGF (10ng/ml) at various time points (0, 1, 5, 10, 30 and 60 minutes) and analyzed for phosphorylation of p38 at Thr180/Tyr182 and ERK1/2 at Thr202/Tyr204.

When phosphorylation of p38 was examined, a faint band was present in the lane containing cell lysates under serum free conditions (control), suggesting that some p38 is active in the absence of $VEGF_{121}$(*Figure 11*). However, phosphorylation increases after 10 minutes of stimulation with 10ng/ml $VEGF_{121}$, suggesting that $VEGF_{121}$ stimulates phosphorylation of p38 MAPK at Thr180/Tyr182. Densitometry analysis revealed that p38 MAPK reached maximum phosphorylation after 30 minutes of stimulation with $VEGF_{121}$ compared to control levels. Total p38 levels were used to verify equal loading. Serum-starved cells stimulated with 10ng/ml $VEGF_{121}$ for varying amounts of time (0, 1, 5, 10, 30 and 60 minutes) were also examined for phosphorylation of ERK1/2 at Thr202/Tyr204 (*Figure 12*). Very little ERK1/2 is phosphorylated under serum free conditions (control) suggesting that ERK1/2 is not constitutively phosphorylated in HTR-8/SVneo cells. Densitometry analysis revealed that p42 MAPK phosphorylation was significantly greater than serum free levels after 10 minutes and p44 MAPK phosphorylation was significantly greater than serum free levels after 5 minutes of stimulation with $VEGF_{121}$. These results suggest that maximum ERK1/2 phosphorylation

Figure 11. $VEGF_{121}$ Stimulates Phosphorylation of p38 MAPK at Thr180/Tyr182. HTR-8/SVneo human extravillous trophoblast cells were serum starved for 24 hours before stimulation with $VEGF_{121}$ (10ng/ml) for varying amounts of time (0, 1, 5, 10, 30 and 60 minutes). Cell lysates were prepared and analyzed for **(A)** p38 activity by phospho-p38 (Thr180/Tyr182) Western blot and **(B)** densitometry. Phosphorylation of p38 increased significantly at 10 minutes and a maximum activation was noted after 30 minutes of $VEGF_{121}$ stimulation. Densitometry data are represented as a mean ± standard deviation (SD) for three independent experiments with each time point being compared to phosphorylation under serum free conditions (0 minutes). (*) indicates significant differences ($p<0.05$) between treatment and control (0 minutes).

(A)


10ng/ml VEGF121
Time (minutes)
0
1
5
10
30
60
P-p38 --
Total p38 --

(B)

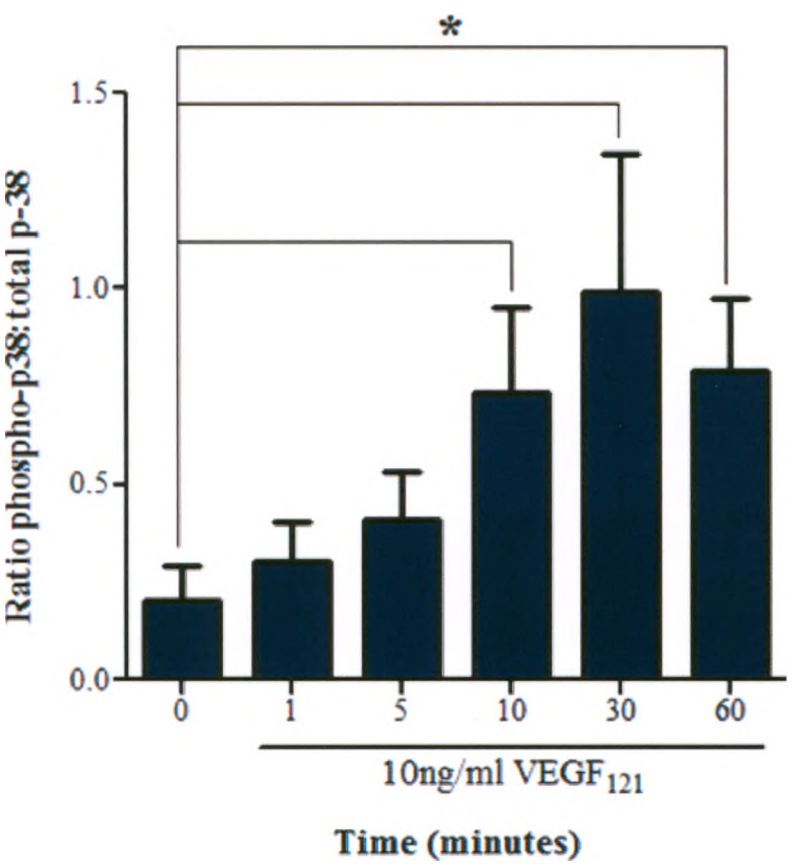
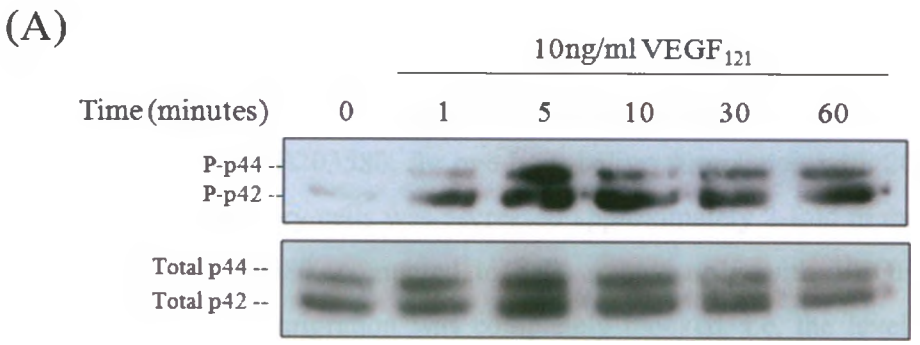
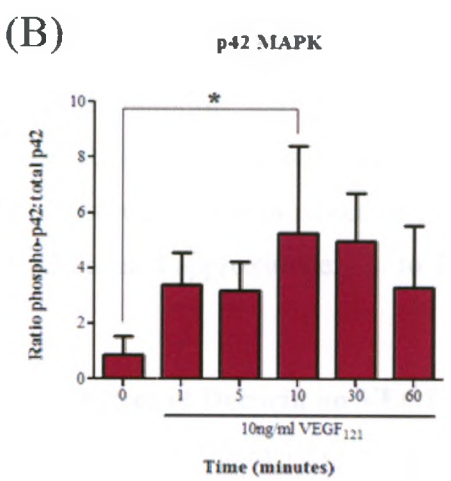
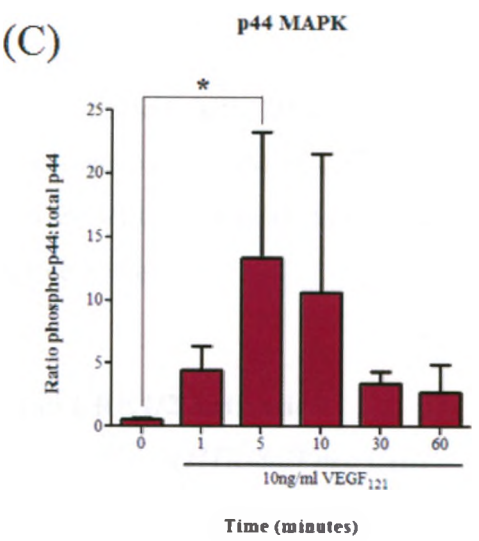

*
1.5
1.0
0.5
0.0
Ratio phospho-p38:total p-38
0
1
5
10
30
60
10ng/ml VEGF121
Time (minutes)

Figure 12. $VEGF_{121}$ Stimulates Phosphorylation of ERK1/2 (p44/p42 MAPK) at Thr202/Tyr204). HTR-8/SVneo cells were serum starved for 24 hours before stimulation with $VEGF_{121}$ (10ng/ml) for varying periods of time (0, 1, 5, 10, 30 and 60 minutes). Cell lysates were prepared and analyzed for p44/42 activity by phospho-p44/42 (Thr202/Tyr204) by **(A)** Western blot and densitometry of **(B)** p42 and **(C)** p44 was performed. Maximum activation of p42 occurred after 10 minutes of $VEGF_{121}$ stimulation, whereas maximum activation of p44 occurred after 5 minutes of stimulation. Densitometry data are represented as a mean ± standard deviation (SD) for three independent experiments with each time point being compared to phosphorylation under serum free conditions (0 minutes). (*) indicates significant differences ($p<0.05$) between treatment and control (0 minutes).


(A)
10ng/ml VEGF121
Time (minutes)
0
1
5
10
30
60
P-p44 --
P-p42 --
Total p44 --
Total p42 --


(B)
p42 MAPK
Ratio phospho-p42:total p42
*
10ng/ml VEGF121
Time (minutes)


(C)
p44 MAPK
Ratio phospho-p44:total p44
*
10ng/ml VEGF121
Time (minutes)

occurs after 5-10 minutes of stimulation with $VEGF_{121}$. This phosphorylation decreased after 10 minutes but did not return to basal levels after 60 minutes of stimulation. Thus, using Western blot analysis I have shown that $VEGF_{121}$ activates both p38 MAPK and ERK1/2 in HTR-8/SVneo EVT cells.

3.6 The Role of p38 MAPK and ERK1/2 in VEGF Stimulation of Cell Migration

3.6.1 p38 MAPK is Required for VEGF-Induced HTR-8/SVneo Migration

Serum-starved cells were pre-treated with varying concentrations (0.5, 5, 25uM) of the p38 inhibitor, SB203580, for one hour before stimulation with 20ng/ml $VEGF_{121}$. The number of migratory cells was decreased approximately 2-fold in the presence of the p38 inhibitor, SB203580, compared to cells treated only with $VEGF_{121}$ (*Figure 13*). VEGF stimulation of migration was completely blocked, i.e. the level was reduced to serum free conditions at SB203580 concentrations as low as 0.5uM. This response was not dose-dependent at the concentrations used.

3.6.2 ERK1/2 is Required for VEGF Stimulation of HTR-8/SVneo Migration

Serum-starved cells were pre-incubated with varying concentrations of the MEK1/2 inhibitor, U0126, for one hour before stimulation with 20ng/ml $VEGF_{121}$. VEGF-stimulated migration was not affected in the presence of 0.5uM of U0126 (*Figure 14*). However, in the presence of at least 5.0uM U0126, VEGF-stimulated cell migration was decreased approximately 2- to 3-fold, to serum free levels.

3.7 Effect of Decorin on VEGF-Induced p38 and ERK1/2 Activation

3.7.1 Decorin Blocks VEGF-Induced p38 MAPK Activation in HTR-8/SVneo Cells

Western blot analysis was performed to determine whether decorin interferes with VEGF-induced p38 MAPK activation. Cells treated with low concentrations of decorin (1nM and 5nM) showed a decrease in phosphorylation at Thr180/Tyr182 (*Figure 15*). However, densitometry analysis revealed that inhibition of VEGF-stimulated p38 activation occurred only with 5nM of decorin. Higher concentrations of decorin (10nM and 100nM) showed inhibition of VEGF-induced phosphorylation to a lesser extent than lower concentrations and this inhibition was not significant.

Figure 13. p38 MAPK is Required for VEGF-Stimulated HTR-8/SVneo migration. HTR-8/SVneo human extravillous trophoblast cells were pre-treated with varying concentrations of the p38 inhibitor, SB203580 (0.5, 5.0, 25uM) for one hour before being placed in the top chamber of the transwell insert. $VEGF_{121}$ (20ng/ml) was placed in the bottom chamber and was used as a chemotactic agent. Inhibiting p38 activity blocked VEGF stimulation of migration, reducing it to serum free levels suggesting that p38 MAPK is required for VEGF stimulation of migration. Migration data are represented as a mean ± standard deviation (SD) for three independent experiments with each condition being tested in triplicates. Significant differences ($p<0.05$) in the means are indicated by different superscripts.

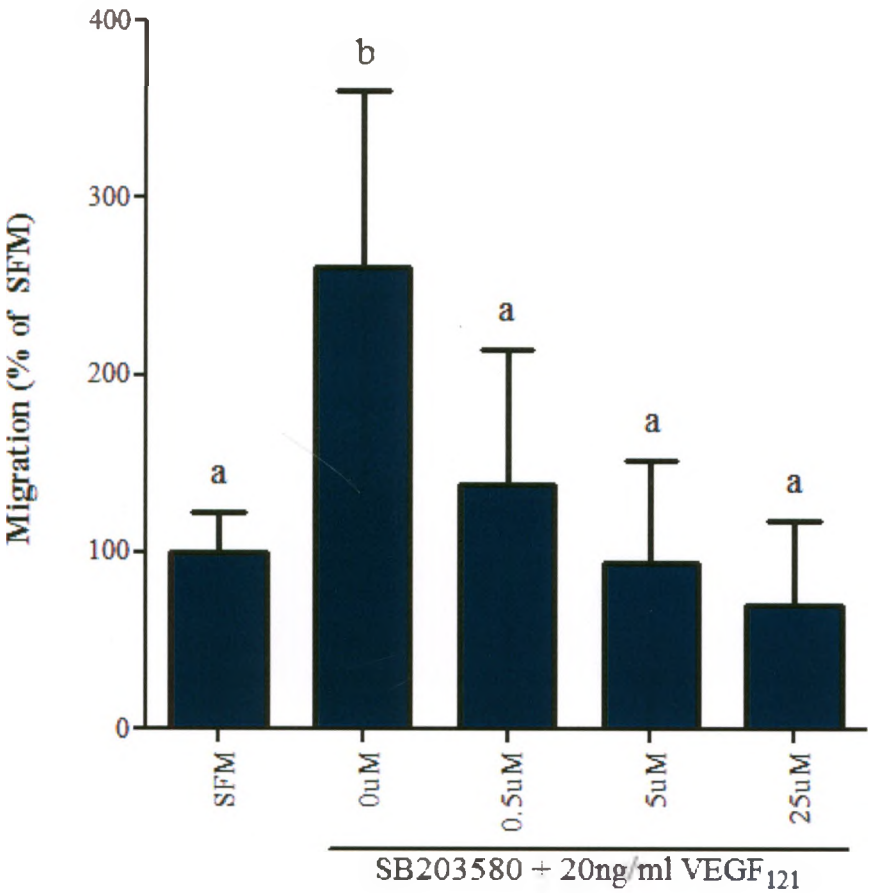
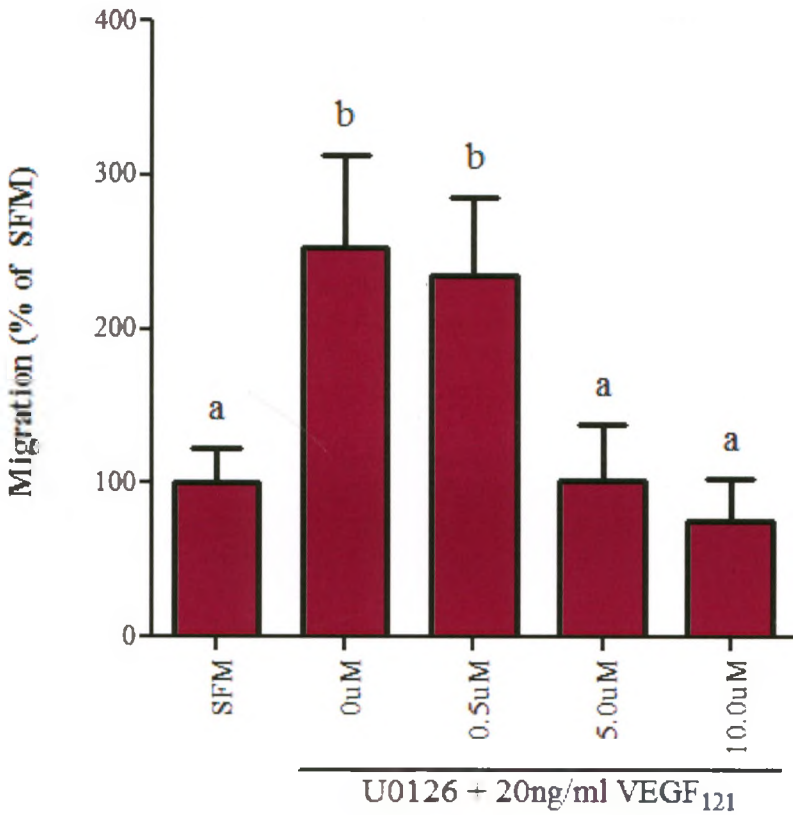

400
300
200
100
0
Migration (% of SFM)
a
b
a
a
a
SFM
0uM
0.5uM
5uM
25uM
SB203580 + 20ng/ml VEGF121

Figure 14. ERK1/2 (p44/p42 MAPK) is Required for VEGF Stimulation of HTR-8/SVneo migration. HTR-8/SVneo human extravillous trophoblast cells were pre-treated with varying concentrations of the MEK1/2 inhibitor, U0126 (0.5, 5.0, 10uM) for one hour before being placed in the top chamber of the transwell insert. $VEGF_{121}$ (20ng/ml) was placed in the bottom chamber and was used as a chemotactic agent. Inhibiting ERK1/2 activity blocked VEGF stimulation of migration, reducing it to serum free levels suggesting that ERK1/2 plays a role in VEGF-stimulated migration. Migration data are represented as a mean ± standard deviation (SD) for three independent experiments with each condition being tested in triplicates. Significant differences ($p<0.05$) in the means are indicated by different superscripts.

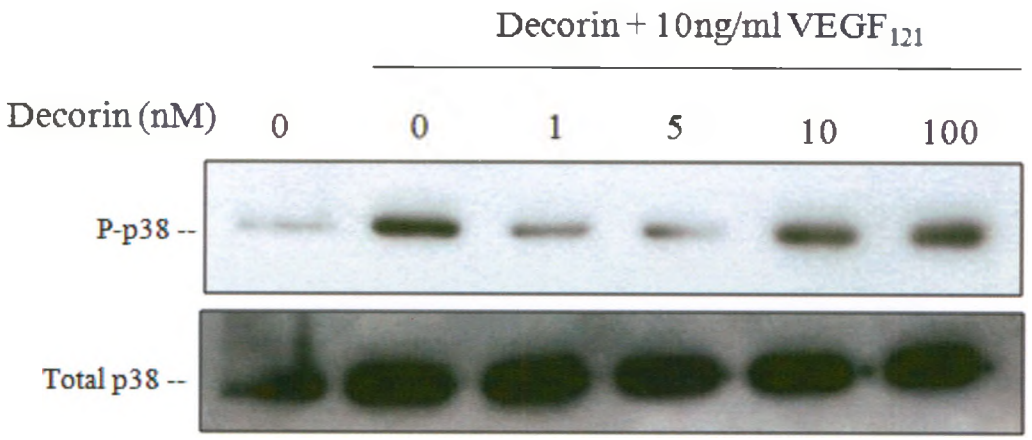

400
300
200
100
0
Migration (% of SFM)
a
b
b
a
a
SFM
0uM
0.5uM
5.0uM
10.0uM
U0126 + 20ng/ml VEGF121

3.7.2 Decorin Blocks VEGF-Stimulated ERK1/2 Activation in HTR-8/SVneo

Western blot analysis was performed to determine whether decorin interferes with VEGF-induced ERK1/2 activation. Cells treated with low concentrations of decorin (1nM and 5nM) showed a decrease in phosphorylation at Thr202/Tyr204, similar to decorin's effect on p38 phosphorylation (*Figure 16*). Densitometry analysis revealed that inhibition of VEGF-induced p44 activation occurred at 1nM and 5nM of decorin. However, no significant inhibition of p42 activation was present. Taken together, these findings suggest that decorin is interfering with VEGF-stimulated signalling, specifically by abrogating p38 and ERK1/2, in HTR-8/SVneo.

Figure 15. Decorin Inhibits VEGF-Induced p38 MAPK Phosphorylation at Thr180/Tyr182. HTR-8/SVneo cells were serum starved for 24 hours followed by treatment with varying concentrations of decorin (0, 1, 5, 10, 100nM). After one hour decorin-treated cells were stimulated with $VEGF_{121}$ (10ng/ml) for 30 minutes. Cell lysates were prepared and analyzed by **(A)** Western blot and **(B)** densitometry. VEGF-induced phosphorylation of Thr180/Tyr182 was inhibited in the presence of 5nM of decorin. Densitometry data are represented as a mean ± standard deviation (SD) for three independent experiments with each time point being compared to phosphorylation under VEGF stimulation only (0nM decorin). (*) indicates significant differences ($p<0.05$) between treatment and control (VEGF only).

(A)


Decorin + 10ng/ml VEGF121
Decorin (nM)
0
0
1
5
10
100
P-p38 --
Total p38 --

(B)

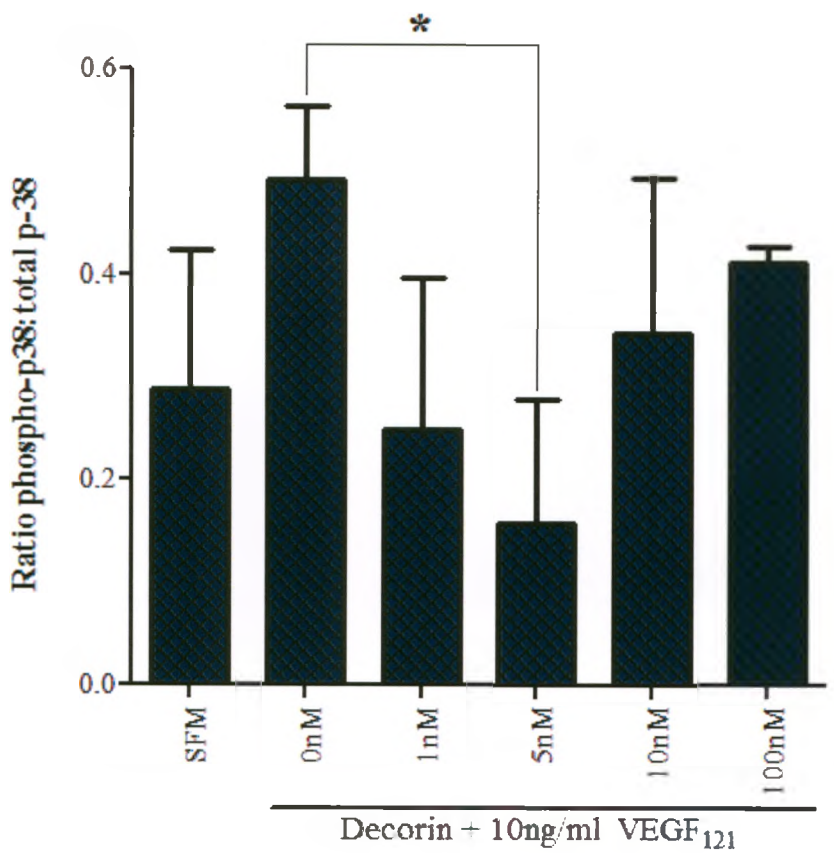
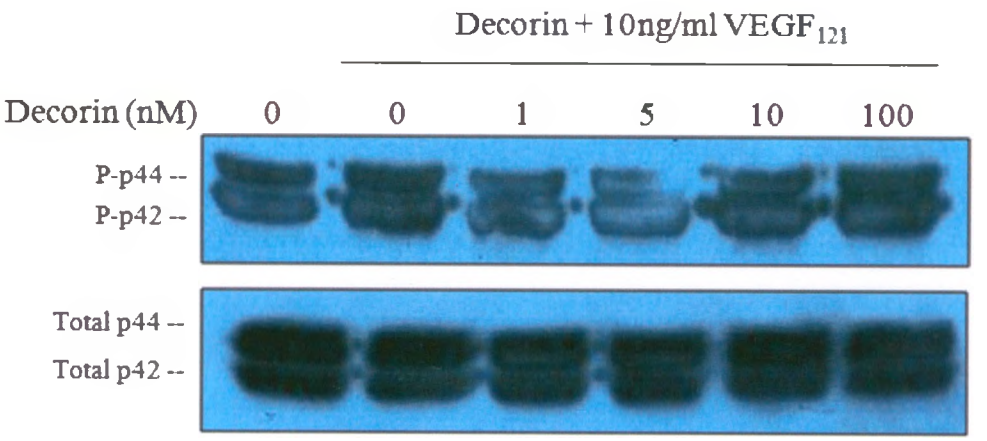
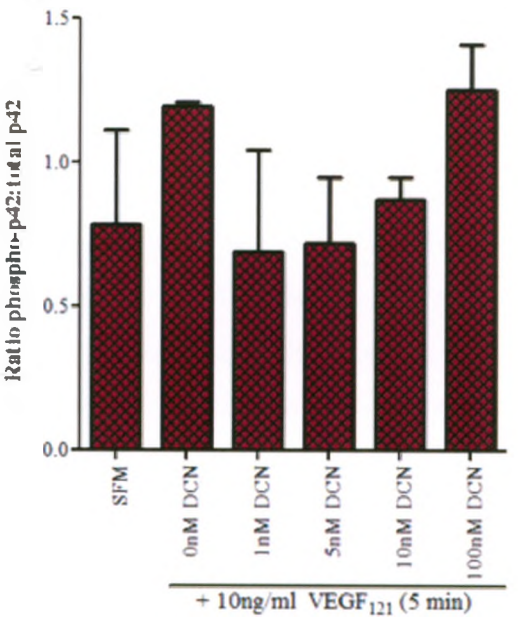

*
Ratio phospho-p38: total p-38
0.6
0.4
0.2
0.0
SFM
0nM
1nM
5nM
10nM
100nM
Decorin + 10ng/ml VEGF121

Figure 16. Decorin Inhibits VEGF-Induced ERK1/2 (p44/p42 MAPK) Phosphorylation at Thr202/Tyr204. HTR-8/SVneo cells were serum starved for 24 hours followed by treatment with varying concentrations of decorin (0, 1, 5, 10, 100nM). After one hour decorin-treated cells were stimulated with $VEGF_{121}$ (10ng/ml) for 5 minutes. Cell lysates were prepared and analyzed by **(A)** Western blot and **(B)** densitometry. VEGF-induced phosphorylation of p44 MAPK was inhibited in the presence of 1nM of decorin. However, decorin did not have a significant effect on VEGF-induced phosphorylation of p42 MAPK. Densitometry data are represented as a mean ± standard deviation (SD) for three independent experiments with each time point being compared to phosphorylation under VEGF stimulation only (0nM decorin). (*) indicates significant differences ($p<0.05$) between treatment and control (VEGF only) and (#) indicates significance ($p<0.05$) between SFM and VEGF only.

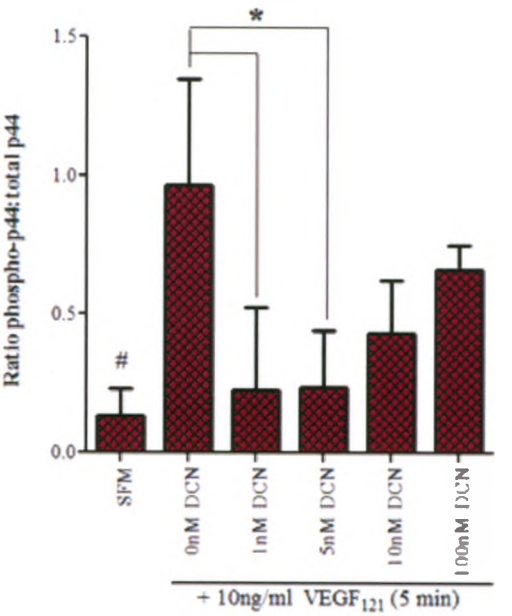
(A)


Decorin + 10ng/ml VEGF121
Decorin (nM) 0 0 1 5 10 100
P-p44 --
P-p42 --
Total p44 --
Total p42 --

(B)


p42 MAPK
Ratio phospho-p42:total p42
1.5
1.0
0.5
0.0
SFM
0nM DCN
1nM DCN
5nM DCN
10nM DCN
100nM DCN
+ 10ng/ml VEGF121 (5 min)

(C)


p44 MAPK
Ratio phospho-p44:total p44
*
#
1.5
1.0
0.5
0.0
SFM
0nM DCN
1nM DCN
5nM DCN
10nM DCN
100nM DCN
+ 10ng/ml VEGF121 (5 min)

CHAPTER FOUR: DISCUSSION

Successful establishment of the placenta is required for a healthy pregnancy (reviewed in (Herr et al., 2010). The human placenta develops primarily from the differentiation and proliferation of trophoblast cells, derived from the trophoectoderm of the blastocyst. These cells must penetrate the uterine epithelium, the underlying connective tissue, as well as the maternal spiral arteries (Huppertz, 2008; Lala and Graham, 1990; Lala and Hamilton, 1996). Much like tumour invasion, trophoblast invasion of the uterus is a multi-step process involving: I) attachment of trophoblast cells to the extracellular matrix (ECM); II) degradation of the ECM; and III) migration through the ECM (Lala and Graham, 1990). However, unlike tumour invasion, trophoblast invasion is strictly regulated, both positively and negatively, by a variety of factors produced at the fetal-maternal interface (reviewed in (Lala and Chakraborty, 2003)). Of the negative regulators, TGF-β and decorin, are important in controlling trophoblast proliferation, migration and invasiveness (Graham and Lala, 1991; Graham et al., 1992; Xu et al., 2002). Furthermore, recent findings in our lab revealed that certain decorin actions can be mediated by binding to VEGFR-2 on trophoblast cells (Iacob et al., 2008; Khan et al., 2009).

For these reasons, in this study I set out to determine: 1) whether decorin inhibits VEGF-induced acquisition of an endovascular phenotype or migratory responses of extravillous trophoblast (EVT) cells; and 2) the signaling mechanisms of decorin-mediated inhibition of VEGF-induced EVT cell migration. By employing the immortalized EVT cell line, HTR-8/SVneo, in tube formation assays, transwell migration assays and Western blot analyses, I demonstrated that VEGF-stimulated EVT cell functions, specifically the acquisition of an endovascular phenotype and cell migration, can be inhibited by the small leucine-rich proteoglycan, decorin. I also found that decorin blocks VEGF-induced activation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2; p42/p44 MAPK).

4.1 Using HTR-8/SVneo Cells as a Model for Trophoblast Invasion

Studies on the biology of trophoblasts have traditionally used primary trophoblast cultures derived after enzymatic dispersion of placental tissue (Kliman et al., 1986) or short-lived primary cell lines created by plating first trimester chorionic villus fragments

(Graham et al., 1992; Irving and Lala, 1995). However, the use of short-term cultures is often not suitable for certain studies due to the heterogeneity of the cell population (Graham et al., 1993). Thus, trophoblast cell lines generated from normal or malignant trophoblast (choriocarcinoma) cells have been employed to further our understanding of trophoblast cell biology and placental development (King et al., 2000).

There are three main types of cell lines derived from the human placenta: primary cell cultures, cells that have been immortalized by *in vitro* transfections and choriocarcinoma cell lines (reviewed in (Sullivan, 2004). Human placental cell lines are positive for cytokeratin-7 (trophoblast marker) and either HLA-negative (villous trophoblast) or HLA-G and CD9 positive (extravillous trophoblast) (King et al., 2000; Sullivan, 2004).

An immortalized first trimester EVT cell line, HTR-8/SVneo, was generated by introducing the gene encoding simian virus 40 (SV40) large T antigen into the short-lived parental EVT cell line, HTR-8 (Graham et al., 1993). HTR-8/SVneo cells share the same epithelial phenotype as the parental cells including their ability to form giant multinucleated cells (Graham et al., 1993). Like the parental cells, these trophoblast cells are positive for human chorionic gonadotropin (hCG) secretion, cytokeratin 7 expression and HLA-G expression when grown on matrigel^{TM}, thus confirming that the immortalized cell line has retained their extravillous trophoblast phenotype (reviewed in (Hannan et al., 2010; King et al., 2000; Sullivan, 2004). These cells have proven to be useful tools for functional studies on proliferation, adhesion, migration and invasion (reviewed in (Hannan et al., 2010). HTR-8/SVneo cells also express cell surface HLA-G and cooperate with vascular endothelial cells, *in vitro*, in forming pseudo-vascular tubes (Kalkunte et al., 2008).

4.2 Acquisition of an Endovascular Phenotype by HTR-8/SVneo: Regulation by $VEGF_{121}$ and Decorin

During placental development, a subpopulation of EVT cells, termed endovascular trophoblasts, invade and replace the endothelium of spiral arteries, transforming these narrow muscular arteries into large flaccid vessels (Chakraborty et al., 2002). Spiral arterial remodeling allows for greater blood flow to the placenta which is

necessary for adequate exchange of crucial molecules between the maternal and fetal circulations (Lala and Chakraborty, 2003).

The exact role that trophoblasts play in the transformation of spiral arteries in pregnancy is still unclear. Some studies suggest that trophoblast and endothelial cells co-exist in the walls of partially modified spiral arteries (Aldo et al., 2007; Pijnenborg et al., 1998), whereas, others suggest that the trophoblast adopt an endothelial-like phenotype as they replace endothelial cells lining the vessel (Aldo et al., 2007; Zhou et al., 1997). However, in both these theories, extravillous trophoblast cells must acquire a phenotype that mimics cells of the vascular system, particularly endothelial cells (Zhou et al., 1997). This change in phenotype includes expression of vascular endothelial markers such as vascular endothelial (VE)-cadherin, platelet endothelial cell adhesion molecule (PECAM)-1, vascular cell adhesion molecule (VCAM)-1 and integrin $\alpha V\beta 3$ (Hu et al., 2010; Zhou et al., 1997) in addition to the ability to mimic the behaviour of endothelial cells in culture on matrigel™ (Aldo et al., 2007; Francis et al., 2008; Kalkunte et al., 2008).

The acquisition of an endovascular phenotype was examined by performing *in vitro* tube formation assays, in which HTR-8/SVneo cells were seeded onto growth factor reduced (GFR) matrigel™. Initially, I set out to determine whether $VEGF_{121}$ could stimulate tube formation and if so, whether decorin could inhibit this stimulation. I found that HTR-8/SVneo cells have an intrinsic ability to form capillary-like structures on GFR matrigel™ that can be stimulated in the presence of $VEGF_{121}$. Maximum stimulation of tube formation occurred in the presence of 50ng/ml $VEGF_{121}$. At 100ng/ml tube formation was decreased compared to the level at 50ng/ml, however, the level of tube formation was still greater than the level under serum free conditions. This decrease in tube formation was likely due to receptor saturation followed by accelerated endocytosis as previously reported in other cell types (Arbiser et al., 2000; Olsson et al., 2006; Valgeirsdottir et al., 1995).

Our finding that HTR-8/SVneo are able to form tube-like structures corresponds with previous findings examining the capacity of trophoblast cells to form capillary-like networks on matrigel™. Fukushima's group found that the human EVT cell line, TCL1, could acquire an endothelial-like phenotype as indicated by its ability to form tube-like

structures on growth factor reduced matrigel™ (Fukushima et al., 2005; Fukushima et al., 2008b; Fukushima et al., 2008a). Another group found that HTR-8/SVneo failed to form tubes on matrigel when cultured alone (Kalkunte et al., 2008). However, in the presence of endothelial cells, these cells migrated towards and synchronized with endothelial cells in capillary-like networks (Aldo et al., 2007; Kalkunte et al., 2008). I, as well as others, have shown that HTR-8/SVneo have an intrinsic capacity to form tubes on matrigel (Hoffman et al., 2009; Hu et al., 2010) and that tube formation is stimulated by various factors released by endothelial cells and decidual natural killer (dNK) cells, including VEGF (Fukushima et al., 2008a; Hoffman et al., 2009; Hu et al., 2010).

Previously, our lab has demonstrated that decorin co-localizes with TGF-β in first trimester decidual extracellular matrix *in situ* (Lysiak et al., 1995a). It was found that decorin acts as a negative regulator of extravillous trophoblast functions by inhibiting cell proliferation, migration and invasiveness of HTR-8 cells, a primary extravillous trophoblast cell line and its immortalized derivative, HTR-8/SVneo, in a TGF-β-independent manner (Xu et al., 2002). In this study, I found that decorin inhibits VEGF-induced acquisition of an endovascular phenotype by HTR-8/SVneo. VEGF-induced tube formation was completely blocked in the presence of 10nM decorin. These results agree with other studies examining the anti-angiogenic function of decorin. One study showed that decorin inhibits tube formation when endothelial cells were grown on decorin-coated surfaces (Davies et al., 2001). Another group revealed that purified decorin and the leucine-rich repeat 5 (LRR5) of decorin core protein act as angiogenesis inhibitors by inhibiting VEGF- induced angiogenesis (Fan H et al., 2008; Sulochana et al., 2005). My results reveal that decorin inhibits VEGF-stimulated acquisition of an endovascular phenotype by extravillous trophoblast cells, *in vitro*.

4.3 HTR-8/SVneo Cell Migration: Regulation by $VEGF_{121}$ and Decorin

Tube formation is a complex process in which cells initially attach to the basement membrane substratum and then migrate towards each other forming capillary-like tubes (Arnaoutova and Kleinman, 2010). Since migration is an integral part of tube

formation, the effect of $VEGF_{121}$ and decorin on HTR-8/SVneo migration was examined by *in vitro* transwell migration assays.

In the present study, EVT cell migration was stimulated with $VEGF_{121}$ at concentrations as low as 20ng/ml. This finding is in apparent contradiction to the study by Athanassiades *et al*, in which they showed $VEGF_{165}$ stimulated proliferation but not migration or invasiveness of the short-lived HTR-8 cells (Athanassiades et al., 1998). In their study, trophoblast cells were suspended in serum reduced media (RPMI 1640 + 2% FBS) and then stimulated with $VEGF_{165}$, whereas in this study, cells were suspended in serum free media (RPMI 1640 + 0.5% BSA) and $VEGF_{121}$ was only added to the bottom chamber of the transwell inserts as a chemotactic agent. Fetal bovine serum (FBS) contains several growth factors and stimulates migration in a variety of cells (Lin et al., 2010; Mishimi and Lotz, 2008). I also showed that cell migration is stimulated in the presence of 10% FBS. Therefore, because the cells were suspended in media containing serum, the growth factors present in the serum likely overshadowed the effects of $VEGF_{165}$ on cell migration. In my study, cells were serum starved to determine the basal level of cell migration.

In this study, $VEGF_{121}$ was used as a chemotactic agent in migration assays because it lacks the heparin binding domain that is present in the other five isoforms of human VEGF. $VEGF_{121}$ is a weak acidic protein that does not bind to heparin and is freely released from producing cells, whereas, the majority of $VEGF_{165}$ is cell or ECM-associated due to interactions with heparan sulphate proteoglycans (HSPGs) (Houck et al., 1992; Park et al., 1993). Thus, stimulation with $VEGF_{121}$ removed any complications that could arise due to heparin binding.

Decorin inhibits migration of EVT cells grown in serum reduced media (RPMI 1640 + 1% FBS) in a dose-dependent manner (Xu et al, 2002). Sulochana *et al*, examined the effect of decorin on VEGF-stimulated endothelial cell migration and determined that decorin as well as its leucine-rich repeat 5 (LRR5) region could potently inhibit cell migration stimulated by VEGF. Thus, I set out to determine whether decorin and the LRR5 peptide could inhibit VEGF stimulation of EVT migration. Stimulation was completely blocked in the presence of as low as 10nM decorin and all four LRR5 peptides (LRR5, LRR5C, LRR5N and LRR5M) to the same degree. These results, for the

first time, reveal that VEGF stimulates HTR-8/SVneo and that stimulation of cell migration can be inhibited by decorin and the decorin LRR5 peptide.

VEGF is primarily produced by the glandular epithelium and decidual macrophages, thus EVT cells expressing VEGF receptors migrate toward the maternal decidua containing the VEGF expressing macrophages and epithelial cells (Cooper et al., 1995).This suggests that *in vivo*, VEGF could be acting as a chemotactic agent for extravillous trophoblast and excessive migration could be regulated by decorin produced in the decidua.

4.4 Signaling Mechanisms of Decorin-Mediated Inhibition of Trophoblast Migration

To gain further information on how decorin inhibits VEGF-stimulated trophoblast cell functions, Western blot analysis was carried out to examine possible signalling pathways. The role of multiple tyrosine kinase receptors have been investigated in decorin-mediated control of EVT cell functions: An anti-proliferative action by epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR)-2, and an anti-migratory action by insulin-like growth factor receptor (IGFR)-1 (Iacob et al., 2008). In the same study, it was also demonstrated that decorin stimulated phosphorylation of IGFR-1 and VEGFR-2, which also occurred with their natural ligands (IGF-1 and $VEGF_{121}$, respectively), and that phosphorylation could be blocked effectively with specific receptor kinase inhibitors (PPP and KRN633, respectively) (Iacob et al., 2008).

Recently, it has been established that decorin binds to VEGFR-2 in EVT cell lysate proteins with an affinity that is 10 fold lower than VEGF-E, a VEGFR-2 specific ligand (Khan et al., 2009). Because decorin binds to VEGFR-2 on HTR-8/SVneo and VEGFR-2 is the principal mediator of VEGF-induced physiological effects, I examined whether decorin may be acting as a negative regulator of trophoblast cell migration by interfering with VEGF signalling downstream of VEGFR-2.

VEGF is reported to stimulate cell migration by activating multiple signalling molecules downstream of VEGFR-2 including, endothelial nitric oxide synthase (eNOS),

focal adhesion kinase (FAK), p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2; p44/p42 MAPK) in a variety of cell types (Abedi and Zachary, 1997; Feliers et al., 2005; Henriksen et al., 2003; Rousseau et al., 1997).

It has been previously reported that Akt-dependent phosphorylation of eNOS at Ser1177 is required for VEGF-induced cell migration (Dimmeler et al., 2000). Furthermore, decorin and its LRR5 peptide inhibited VEGF-induced angiogenic responses (migration) by inhibiting VEGF-induced nitric oxide (NO) production (Fan H et al., 2008). I performed Western blot analysis to determine whether $VEGF_{121}$ stimulates eNOS phosphorylation at Ser1177 on HTR-8/SVneo. My results revealed that eNOS is not expressed in the HTR-8/SVneo cell line and therefore cannot be involved in VEGF-stimulated cell migration.

Another mechanism of VEGF-stimulated endothelial cell migration is through activation of focal adhesion kinase (FAK), which initiates the assembly and rearrangements of focal adhesions (Abedi and Zachary, 1997). Phosphorylation at Tyr397 correlates with increased catalytic activity of FAK and appears to be important for tyrosine phosphorylation of focal-adhesion-associated proteins such as paxillin and vinculin (Parsons et al., 2000; Parsons, 2003). Decorin and its LRR5 peptides inhibited VEGF-induced angiogenic responses (tube-formation and migration) by inhibiting VEGF-induced FAK activation at Tyr397 (Sulochana et al., 2005). The results from my study could neither confirm nor negate those of Sulochana's group. I found that FAK is constitutively active and could not be stimulated further by $VEGF_{121}$ at Tyr397 in HTR-8/SVneo. Thus, this tyrosine residue may not be involved in VEGF-stimulated HTR-8/SVneo migration. However, different phosphorylation sites on FAK have been implicated in VEGF-stimulated cell migration including Tyr407, Tyr861, and Ser732 and must be further examined in trophoblast cell migration (Abu-Ghazaleh et al., 2001; Duval et al., 2007; Le Boeuf et al., 2004; Le Boeuf et al., 2006).

MAPKs are also implicated in VEGF-stimulated migration in a variety of cell types (Henriksen et al., 2003; Rousseau et al., 1997; Rousseau et al., 2000). Thus, I examined whether $VEGF_{121}$ can induce activation of p38 MAPK or ERK1/2. In this study, I found that 10ng/ml $VEGF_{121}$ induced phosphorylation of both p38 MAPK and

ERK1/2 at Thr180/Tyr182 and Thr202/Tyr204, respectively. Next, I examined whether p38 and ERK1/2 were involved in VEGF-stimulated EVT cell migration by blocking ERK1/2 or p38 using the pharmacological inhibitors, U0126 and SB203580, respectively. I found that when p38 and ERK were blocked, migration stimulated by VEGF was also blocked, suggesting that p38 and ERK1/2 play a role in VEGF-induced HTR-8/SVneo migration.

In this study, I demonstrated that VEGF-induced p38 MAPK and ERK1/2 activation can be blocked by very low concentrations of decorin (5nM). However, at higher concentrations of decorin (10 and 100nM), VEGF-stimulated phosphorylation was not compromised. It has been shown that ectopic decorin expression up-regulates VEGF expression through up-regulation and activation of the transcription factors hypoxia inducible factor (HIF1α), signal transducer and activator of transcription 3 (Stat3) and Sp1 in mouse cerebral endothelial cells (Santra et al., 2007). Thus, one mechanism explaining this lack of inhibition in the presence of high concentrations of decorin could be decorin-mediated up-regulation of VEGF expression. The increase of VEGF will compete with decorin for receptor binding sites and with VEGF having a higher affinity for these sites, decorin will not be able to bind the receptors and interfere with VEGF signalling pathways. Further studies examining the effect of decorin on VEGF expression in trophoblast cells must be done in order to obtain a better understanding of our findings.

4.5 Biological and Clinical Implications

The biological significance of decorin antagonism of VEGF-stimulated cellular functions is profound. VEGF is the most important angiogenic factor, under physiological and pathological conditions, that induces cell proliferation, migration and tube formation in endothelial cells (Holmes et al., 2007). Thus, it is likely that the anti-angiogenic action of exogenous decorin in a number of experimental tumour models (Goldoni et al., 2008; Goldoni and Iozzo, 2008; Santra et al., 2000; Seidler et al., 2006) can be explained by decorin antagonism of multiple tyrosine kinase receptors including VEGFR-2.

Decorin inhibition of VEGF-induced cellular functions, notably migration and acquisition of an endovascular phenotype, has strong implications for placental physiology and pathology. Preeclampsia is a multifactorial syndrome that affects

approximately 2-3% of pregnant women and is a major cause of maternal and fetal morbidity and mortality (Lunghi et al., 2007). Poor EVT invasion leads to an inadequate remodeling of the endometrial spiral arteries resulting in poor placental perfusion with maternal blood, which in turn, can cause fetal growth restriction (FGR) and trigger vascular damage in the mother (Lala and Chakraborty, 2003). Increased levels of anti-angiogenic molecules such as soluble VEGFR-1 and soluble endoglin (Erez et al., 2008; Lam et al., 2005; Tjoa et al., 2007) are implicated in the pathogenesis of preeclampsia. Due to the inhibitory role of decorin on trophoblast cell functions, I suggest that decorin over-expression or activity in the decidua may also contribute to this disease.

4.6 Possible Limitations of the Experimental Design

The proposed conclusions in this study rely on results obtained from an *in vitro* model using the immortalized extravillous trophoblast cell line, HTR-8/SVneo. Even though this cell line has been used in many studies examining *in vitro* functional assays such as cell migration, it does not describe the situation *in situ*. This study provides us with an initial understanding of the possible mechanism in which decorin inhibits trophoblast cell migration. The inhibitory action of decorin has been shown in situ on the sprouting of EVT cells in first trimester villous explants cultures (Iacob et al., 2008). Similar explants or explants xenografted under the kidney capsule of immunodeficient mice remain as possible tools for further study.

In this study, I used decorin purified from bovine articular cartilage (Sigma Aldrich) to examine tube formation, migration and signaling pathways. Results presented in this study should be confirmed using recombinant human decorin to see whether the effects on extravillous trophoblast cells are the same. Sulochana's group used both decorin purified from bovine articular cartilage as well as recombinant human decorin and found that both types inhibited human umbilical vascular endothelial cell (HUVEC) tube formation and migration (Sulochana et al., 2005). Also, I compared the binding of decorin purified from bovine articular cartilage and purified crystalline human decorin to VEGFR-2 on HTR-8/SVneo cells (Khan et al., 2009). Both types of decorin as well as human recombinant decorin bind to the receptor. These studies suggest that there are no

functional differences between bovine decorin, crystalline human decorin (from Dr. Paul Scott, University of Alberta) and recombinant human decorin (R&D Systems).

To have complete confidence in our Western blot analysis, more replicates should be done to increase the sample number (n value) and decrease variation between independent experiments. Although, data may appear to be significant, statistical analysis indicates that some treatments are not significant even though there is a clear difference in band intensity. The experiment should be repeated at least three more times to increase the n value to six and thus increase the power of the statistical tests being used.

4.7 Future Directions

Notwithstanding these potential limitations, the work done in the current study provides some understanding on the mechanisms of trophoblast migration through the decidua and the invasion of maternal spiral arteries. However, it has also raised many questions about the role of decorin in trophoblast differentiation and in the process of spiral arterial remodeling.

During invasion of the endothelial lining of maternal arteries, a subpopulation of extravillous trophoblast, called endovascular trophoblast, acquire an endothelial-like phenotype before replacing endothelial cells (Damsky and Fi sher, 1998; Zhou et al., 1997). These cells take on the adhesion receptor phenotype of endothelial cells, including an up-regulation of vascular-endothelial cadherin (VE-Cadherin), vascular cell adhesion molecule (VCAM)-1, platelet-endothelial cell adhesion molecule (PECAM)-1 and integrins $\alpha V\beta 1$ and $\alpha 1\beta 1$ expression (Burrows et al., 1994; Damsky et al., 1992; Zhou et al., 1997). To further confirm that VEGF stimulates the acquisition of an endovascular phenotype, changes in the expression of adhesion molecules, particularly VE-cadherin should be investigated. The effect of decorin on these changes should also be examined by Western blot and quantitative polymerase chain reaction (PCR) analysis.

Extravillous trophoblast cells express VEGFR-1 and VEGFR-2 on their cell surface (Clark et al., 1996; Cooper et al., 1995; Demir et al., 2004; Sugino et al., 2002). Since VEGF binds to both of these receptors, the receptor through which $VEGF_{121}$ is mediating its effect on cell migration remains to be identified. Receptor inhibitors for

VEGFR-1 and VEGFR-2 can be utilized to establish which receptor VEGF is binding to stimulate trophoblast migration.

In future studies, the downstream and upstream signalling molecules involved in p38- and ERK1/2-mediated migration should be examined. In endothelial cells, it was demonstrated that VEGF stimulates p38 MAPK which resulted in activation of MAP kinase activated protein kinase (MAPKAPK)-2/3 and phosphorylation of heat shock protein 27 (HSP27) (Rousseau et al., 1997). VEGF-induced activation of p38 MAPK was mediated through phosphorylation of VEGFR-2 at Tyr1214, triggering activation of Cdc42 (Lamalice et al., 2004). This led to actin polymerization and reorganization into stress fibres, which resulted in enhanced cell migration (Rousseau et al., 2000).

For successful cell migration, multiple steps including focal adhesion and stress fiber assembly and disassembly must occur (reviewed in (Lamalice et al., 2007)). To further analyze the effect of decorin on VEGF-stimulated EVT cell migration, cells treated with $VEGF_{121}$ or decorin plus $VEGF_{121}$ can be fixed and stained for actin and vinculin/paxillin to examine stress fiber formation and focal adhesion assembly, respectively. Confocal microscopy can be employed to visualize proteins and changes in cell morphology.

The findings in this study suggest that decorin is an antagonist to VEGF functions and could thus be a potential marker for the trophoblast hypo-invasive disorder, pre-eclampsia. This antagonistic action of decorin may be due to decorin binding to the VEGF receptors or to VEGF itself. Previous studies have shown that soluble fms-like tyrosine kinase 1 (sFlt-1) is up-regulated in patients suffering from pre-eclampsia and that sFlt-1 acts as a negative regulator of VEGF by binding to VEGF and preventing it from binding to membrane-bound VEGF receptors (VEGFR-1 and VEGFR-2) (Ahmad and Ahmed, 2004; Maynard et al., 2003; Maynard et al., 2008; McKeeman et al., 2004). Thus, excess decorin in the decidua could be removing soluble VEGF from the extracellular matrix and preventing interaction with membrane-bound VEGFR-1 or VEGFR-2 in the same way that sFlt-1 does. To determine whether decorin expression increases in pre-eclamptic placentas, decorin mRNA expression could be assessed by in situ hybridization of healthy and pre-eclamptic placental tissues. Also, blood samples taken from healthy women and those suffering from pre-eclampsia could be tested for differences in decorin

and decorin peptide levels. These experiments may lead to the potential use of decorin as a marker for pre-eclampsia.

CHAPTER FIVE: SUMMARY AND CONCLUSIONS

5.1 Summary

1. Vascular endothelial growth factor 121 ($VEGF_{121}$) stimulates acquisition of an endovascular phenotype by HTR-8/SVneo which can be inhibited by decorin.
2. $VEGF_{121}$ stimulates HTR-8/SVneo migration which can be inhibited by decorin.
3. $VEGF_{121}$ stimulates phosphorylation of p38 mitogen-activated protein kinase (MAPK) at Thr180/Tyr182 in HTR-8/SVneo.
4. $VEGF_{121}$ stimulates phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2; p44/p42 MAPK) at Thr202/Tyr204 in HTR-8/SVneo.
5. Both p38 MAPK and ERK1/2 are required for VEGF-stimulated HTR-8/SVneo migration.
6. Decorin inhibits p38 MAPK and ERK1/2 phosphorylation.

5.2 Conclusions

In this study, I examined whether decorin antagonises VEGF-stimulated EVT cell migration and endothelial-like tube formation on growth factor reduced (GFR) matrigel™ (an *in vitro* model of the endovascular phenotype); and if so, the underlying signalling mechanisms of this inhibition. Decorin was found to inhibit VEGF-stimulated tube formation and migration in a concentration-dependent manner. Western b lot analysis revealed that VEGF stimulated p38 Mitogen-Activated Protein Kinase (MAPK) as well as MAPK p42/p44 (ERK1/2) activation and that these activation events could be blocked by decorin. Pharmacological inhibitors of p38 MAPK and ERK1/2 (SB203580 and U0126, respectively) inhibited VEGF-induced migration. These results taken together, suggest that decorin blocks VEGF stimulation of trophoblast migration by interfering with VEGF-induced p38 and ERK1/2 activation.

Our finding that decorin has the ability to block migration and acquisition of an endovascular phenotype stimulated by VEGF has implications for the pathobiology of preeclampsia, a hypo-invasive trophoblast disorder in pregnancy. Preeclampsia is a multifactorial syndrome that can lead to maternal and fetal morbidity and mortality. Increased levels of anti-angiogenic molecules such as soluble VEGFR-1 and soluble endoglin have been implicated in the pathogenesis of disease. Based on the inhibitory

actions of decorin on trophoblast cell functions, I suggest that decorin over-expression or activity in the decidua may also contribute to this disease.

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APPENDIX: SUPPLEMENTAL FIGURES

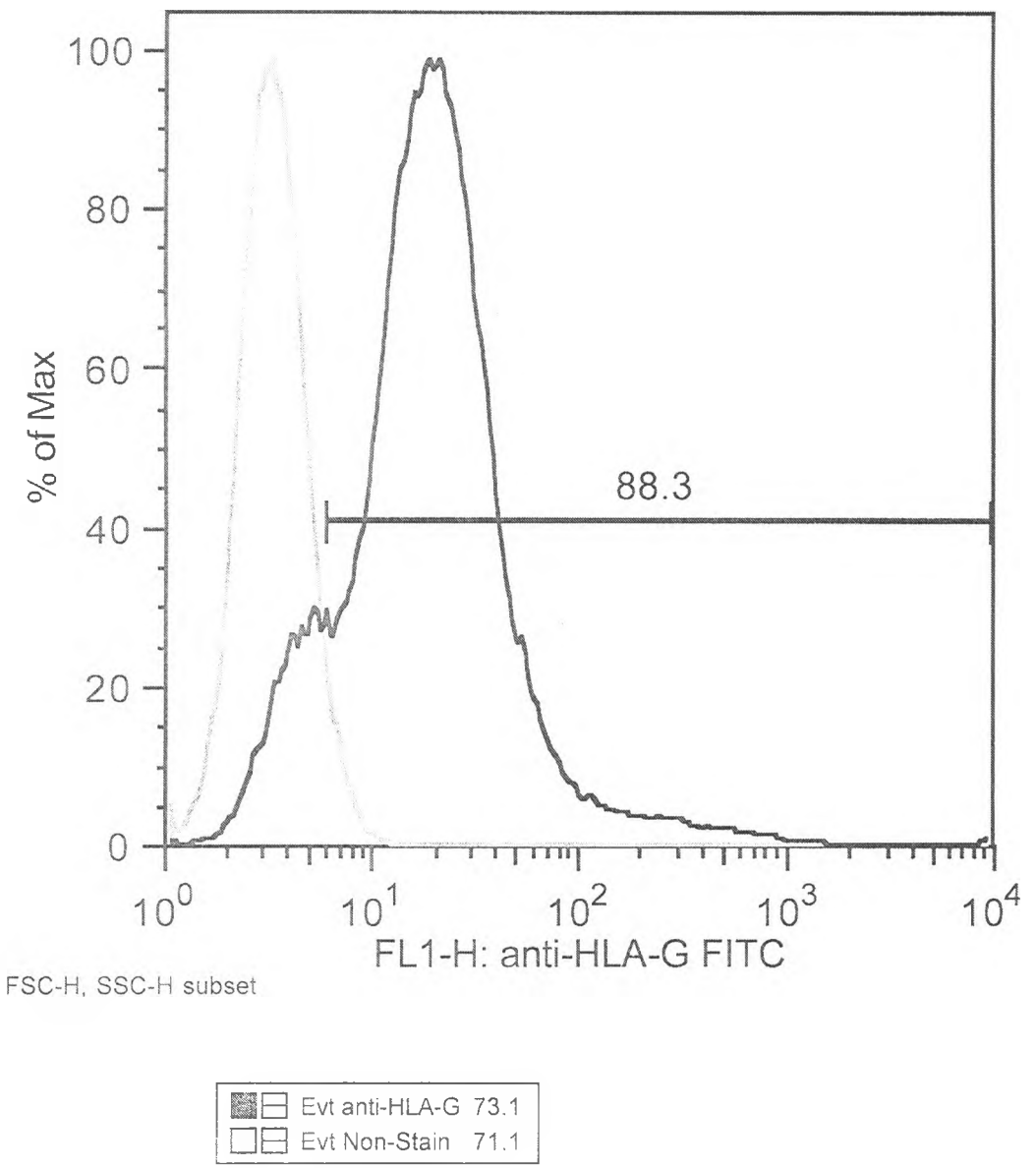


Figure A1. Flow cytometry analysis of HLA-G expression on the cell line, HTR-8/Svneo showing that 88.3% of cells express HLA-G.

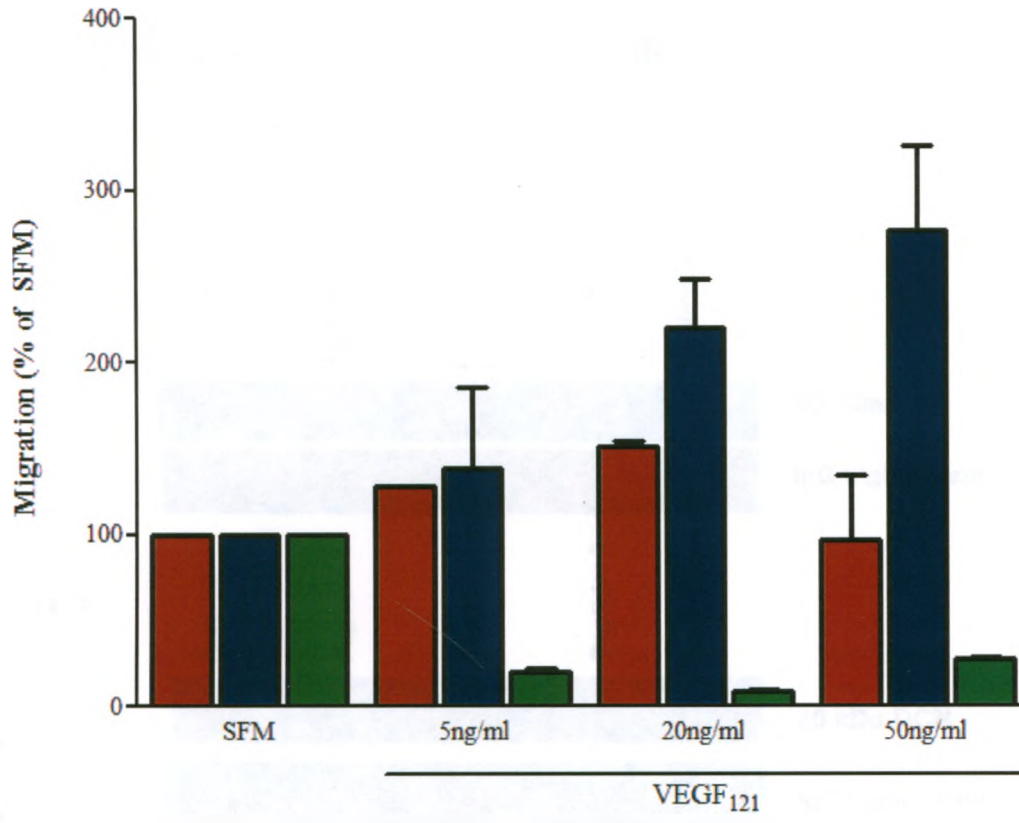
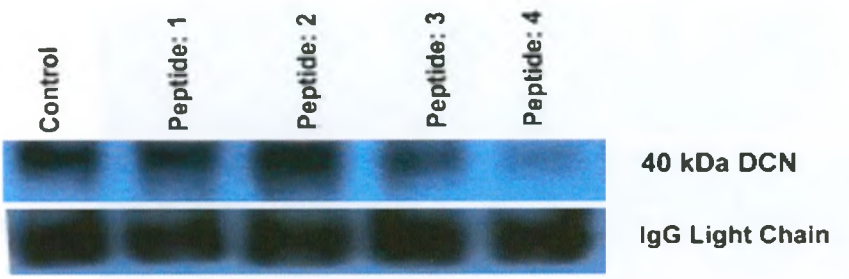


Figure A2. HTR-8/SVneo Migration Peaks at 24 Hours. HTR-8/SVneo cells were plated on the top chamber of transwell migration inserts and allowed to migrate for 12 hours (red), 24 hours (blue) and 48 hours (green). The bottom chamber contained SFM alone or with varying concentrations of $VEGF_{121}$ (5, 20, and 50 ng/ml).

(A)

Name	Amino Acid Sequence	Length
Peptide 1 (P1)	QMIVIELGTNPLKSSGIENGAFQGMK	26
Peptide 2 (P2)	SSGIENGAFQGMK	13
Peptide 3 (P3)	LGTNPLKSSGIE	12
Peptide 4 (P4)	QMIVIELGTNPLK	13

(B)



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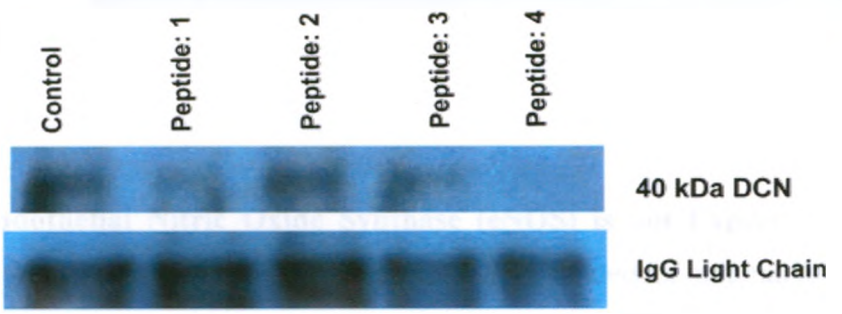

Figure A3. Co-Immunoprecipitation Analysis: Peptide 4 is Required for Decorin Binding to VEGFR-2. (A) Synthetic decorin LRR5 peptide sequences (Genscript). **(B)** Purified VEGFR-2 and **(C)** HTR-8/SVneo cell lysate were incubated with synthetic decorin core peptides (peptide 1-4), which acted as a competitive ligand for VEGFR-2, followed by incubation with decorin core protein. Anti-VEGFR2 antibody was pulled down by A/G sepharose beads and resolved by SDS-PAGE. Decorin core was detected with an anti-decorin antibody. IgG served as a loading control. Peptide 4 bound to VEGFR-2 with the greatest affinity, blocking decorin core protein binding to the receptor. These results suggest that amino acid sequences QMIVIE in peptide 4 are essential for binding of decorin to VEGFR-2. (Work done by Ganareddy V. Girish)

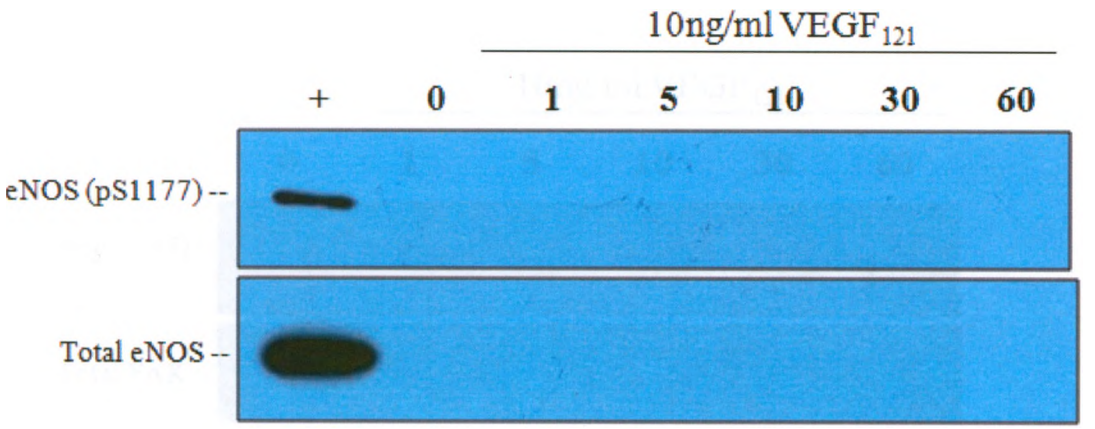

Figure A4. Endothelial Nitric Oxide Synthase (eNOS) is not Expressed in HTR-8/SVneo cells. HTR-8/SVneo cell lysates from VEGF-treated or untreated cells were resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with a anti-phospho-eNOS (pS1177). Then, striped and re-probed with an anti-eNOS antibody. HUVECs were used as a positive control (+). While HUVECs exhibited high levels of eNOS expression and some native phosphorylation of eNOS, no bands were detectable in either case for HTR-8/SVneo cells, suggesting that eNOS is not expressed by these cells.

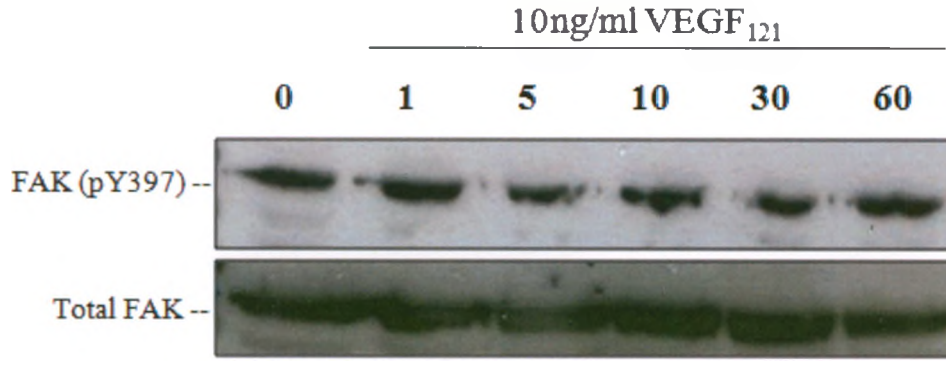

Figure A5. Phosphorylation of Focal Adhesion Kinase (FAK) at Tyr397 is not Stimulated by $VEGF_{121}$ in HTR-8/SVneo cells. HTR-8/SVneo cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with a monoclonal mouse anti-FAK (pY397) antibody. After developing, the membrane was stripped and analyzed for total FAK. Phosphorylation of FAK at Tyr397 did not increase in the presence of $VEGF_{121}$ at the time points examined