MATRIX METALLOPROTEINASE

EXPRESSION, REGULATION AND LOCALISATION

IN MEDIATING TROPHOBLAST INVASION

A dissertation submitted for the

degree of Ph.D.

by

Maria Morgan B. Sc.

Under the supervision of Dr. Susan McDonnell

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School of Biological Sciences, Dublin City University, Dublin 9, Ireland.

<u>Declaration</u>

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed : _____ I.D. No. : 94971153

       Maria Morgan

Date : _____

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Abstract

During early human pregnancy fetal trophoblasts rapidly invade the uterus. The extent and timing of this invasion is precisely regulated. A family of matrix degrading enzymes, called the matrix metalloproteinases (MMPs) have been implicated as major role players in this process. The aims of the research presented in this thesis were to (i) establish a model system using trophoblast continuous cell lines to examine the role of MMPs in trophoblast invasion; (ii) investigate the factors which regulate expression of MMPs during this invasive process; (iii) study the expression of MMPs *in vivo* and examine the role of MMPs in pre-eclampsia; and (iv) isolate and characterise an antibody fragment to MMP-9 using phage display technology.

A model system using trophoblast continuous cell lines was established. These cell lines were characterised extensively with regard to a number of properties including cytokeratin and vimentin expression, hCG, hPL, invasive ability, MMP and TIMP production. Using this system two members of the MMP family, MMP-2 and MMP-9 were identified as important mediators of trophoblast invasion *in vitro*.

The modulation of MMP expression in trophoblast cell lines by cytokines, ECM components, hormones and hypoxic conditions was investigated. Expression of the MMP-2 gene could not be modulated by any of the factors investigated in this study. MMP-9 expression was found to be regulated by certain cytokines and ECM components. In particular IL-1-β was found to upregulate MMP-9 expression at the mRNA and protein level, and to increase the invasive ability of the cells *in vitro*.

A pilot scale study of 9 pre-eclamptic and 10 normal placental biopsies demonstrated no correlation of MMP expression with disease state. Immunohistochemistry demonstrated the expression of MMP-2, MMP-7 and MMP-3 protein in both normal and pre-eclamptic placental sections.

Using a phage display library several clones producing soluble single chain antibody fragments to MMP-9 were isolated following four rounds of panning against MMP-9 protein, however the antibody fragments were found to have low binding affinities.

Abbreviations

AB	Antibody
Abs	Absorbance
AP	Alkaline phosphatase
AP	Activator protein
BCA	Bicinchoninic acid assay
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
CM	Conditioned medium
CMV	Cytomegalovirus
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate
ECM	Extracellular matrix
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
Ets	E-twentysix
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
HBS	HEPES buffered saline
hCG	Human chorionic gonadotrophin
HEPES	N-[2-Hydroxyethl]piperazine-N'-[2-ethanesulfonic acid]

HRP	Horseradish peroxidase
ICM	Inner cell mass
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl β-D-thiogalactopyranoside
LB	Luria-Bertrani broth
MMLV-RT	Moloney murine leukaemia virus-reverse transcritpase
MMP	Matrix metalloproteinase
MOPS	3-[N-Morpholino]propanesulfonic acid
MPBS	Marval PBS
mRNA	Messenger RNA
MW	Molecular weight
NBT	Nitroblue tetrazolium
NP-40	Nonylphenoxy polyethoxy ethanol
OD	Optical density
OPDA	O-phenylene diamine
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline plus Tween
PCR	Polymerase chain reaction
PEA	Polyoma virus enhancer
hPL	Human placental lactogen
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-PCR
SAS	Saturated ammonium sulphate
SDS	Sodium dodecyl sulphate
SV40	Simian virus 40

TBE	Tris borate EDTA
TBST	Tris buffered saline plus Tween
TE	Tris EDTA
TGF-β	Transforming growth factor-β
TIE	TGFβ inhibitory element
TIMP	Tissue inhibitor of metalloproteinases
TRE	TPA responsive element
TPA	12-O-tetradecanoyl-phorbal-13-acetate
UV	Ultraviolet
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Units

bp	Base pairs
Kb	Kilobases
Kda	KiloDaltons
µg	Microgram
µl	Microlitre
µm	Micrometre
C	Degrees celsius
cm	Centimetre
g	Grams
hrs	Hours
Kg	Kilogram
L	Litre
M	Molar
mA	Milliamps
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
ng	Nanogram
nm	Nanometre
V	Volts
w/v	Weight per volume

Publications and Presentations

Publications

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Introduction to the Matrix Metalloproteinases

1. INTRODUCTION

1.1 The Extracellular Matrix

The extracellular matrix (ECM) is a complex and dynamic meshwork of specialized glycoproteins and proteoglycans that are secreted and assembled outside cells. As well as providing structural support in the form of bone, cartilage, and tendon, the ECM influences many cellular and biological processes. It plays a dynamic role in metabolic processes, influencing cellular proliferation, differentiation, apoptosis, cell adhesion and migration, and tissue morphogenesis as well as serving as a repository for biologically active growth factors. The ECM can be divided into two categories : the basement membrane and the interstitial connective tissue. The major components of basement membranes are types IV and V collagens, laminin, entactin, and several proteoglycans. The major protein of connective tissue matrix is collagen (Mayne and Burgeson, 1987). In addition to the collagens, interstitial connective tissue also contains fibronectin, elastin, chondroitin sulfate proteoglycans, heperan sulfate proteoglycans, tenascin, and hyaluronic acid. Laminin and entactin are also found in certain interstitial connective tissues and are not exclusively basement membrane components (Hunter *et al.*, 1989).

Both basement membranes and interstitial connective tissue are important structural features of embryonic and adult tissues and defects in their synthesis and assembly can have profound effects on normal development. The quality and quantity of the ECM matrix depends not only on structural components such as collagen, laminin, and proteoglycan, but also on the regulated expression of matrix-degrading proteases and their inhibitors. These proteinases and inhibitors have, for the most part, been studied in the context of their possible role in tumour cell invasion and metastasis. However, it is likely that the abnormal expression of proteinases by tumour cells reflects some normally transient and well-regulated expression by a subpopulation of precursor cells in the same tissue.

The degradation of ECM proteins can be effected by a variety of enzymatic activities of which there are four main classes (Shi *et al.*, 1993) : (i) serine proteinases e.g. plasminogen activators; (ii) cysteine proteinases e.g. cathepsins B and L; (iii) aspartyl proteinases e.g. cathepsin D and (iv) matrix metalloproteinases (MMP) e.g. MMP-2 and MMP-9. The MMPs, which are the

focus of this thesis, are believed to be the primary contributors to this process as a result of several key features: they are secreted into the extracellular space and function under physiological conditions; they are highly regulated and frequently induced in areas of active matrix remodeling; and members of this family are the only enzymes capable of denaturing fibrillar collagens. The condition of the ECM at any time and place is influenced by the balance between the levels of structural proteins and matrix-degrading proteinases, their inhibitors and activators.

1.2 The Matrix Metalloproteinase Family

The matrix metalloproteinases (MMP) are members of a unique family of zinc-binding endopeptidases that are responsible for degradation of components of the ECM. They are a continually growing group of enzymes that have been implicated in both normal cellular processes e.g. tissue remodeling and repair (Brenner *et al.*, 1989), and in many destructive processes including tumour invasion and metastasis (Powell and Matrisian, 1996) and rheumatoid arthritis (Dean *et al.*, 1989). The proteinases that comprise the MMP family have several distinguishing characteristics: The proteins have a characteristic pattern of conserved domains. Proteolytic activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs). The enzymes are either secreted or exist as transmembrane pro-enzymes that require activation to exert their matrix degrading activity. The active site contains a zinc ion and requires a second metal cofactor such as calcium. Enzyme activity is optimal in the physiological pH range. The properties of the human MMPs which have been cloned and sequenced to date are summarised in Table 1.1.

Table 1.1 Properties of the human matrix metalloproteinases.

MMP	ENZYME NAME	Mol. Weight kD(latent)	Mol. Weight kD(active)	SUBSTRATES	ACTIVATION of other MMPs.
MINIMAL DOMAIN MMP					
MMP-7	Matrilysin, Pump-1	28	19	Proteoglycans, laminin, fibronectin, gelatins, collagen IV, elastin, entactin, tenascin.	MMP-1, MMP-2.
HEMOPEXIN DOMAIN MMPs					
MMP-1	Interstitial collagenase	55	45	Fibrillar collagens (types I,II,III,VII,X), gelatin, proteoglycans.	Unknown
MMP-8	Neutrophil collagenase	75	58	Collagens I, II, III.	Unknown
MMP-13	Collagenase-3	65	55	Collagen II.	Unknown
MMP-12	Metalloelastase	53	45/22	Elastin, fibronectin, collagen IV.	Unknown
MMP-3	Stromelysin-1	57	45	Proteoglycans, laminin, fibronectin, collagen III,IV, V, IX, gelatins.	MMP-1, MMP-8, MMP-9.
MMP-10	Stromelysin-2	57	44	Proteoglycans, fibronectin, collagen III, IV, V, gelatins.	MMP-8
MMP-11	Stromelysin-3	51	44	Laminin, fibronectin (very weakly).	Unknown
FIBRONECTIN DOMAIN MMPs					
MMP-2	Gelatinase A	72	66	Gelatins, collagens IV, V, VII, X, elastin, fibronectin.	Unknown
MMP-9	Gelatinase B	92	86	Gelatins, collagens IV, V, elastin.	Unknown
TRANSMEMBRANE DOMAIN MMPs					
MMP-14	MT1-MMP	63	-	Unknown	MMP-2
MMP-15	MT2-MMP	-	-	Unknown	Unknown
MMP-16	MT3-MMP	-	-	Unknown	MMP-2
MMP-17	MT4-MMP	-	-	Unknown	Unknown
MISCELLANEOUS					
MMP-19	RASI-1	-	-	Gelatins	Unknown
MMP-20	Enamelysin	54	-	Amelogenin	Unknown

1.2.1 Domain Structure of MMPs

The MMP family had previously been divided into subclasses with respect to their substrate specificity. This classification scheme has become somewhat redundant as new MMPs have been cloned and new substrates identified for the enzymes. The latest approach has been to classify the MMPs based primarily on the protein domain structure (Powell and Matrisian, 1996). All MMPs contain three fundamental domains which make up the structural basis for the entire family (Figure 1.1 A). At the amino terminus is the **pre** domain that directs the protein for cellular export and is rapidly removed prior to secretion. The **pro** domain contains a highly conserved segment of eight amino acids, PRCGVPDV, which must be removed by cleavage to activate the enzymes. Latent MMPs are activated by removal of this domain, in turn disengaging the cysteine in the conserved prodomain from the zinc in the adjacent active site. This model of activation termed the 'cysteine switch' mechanism (Van Wart *et al.*, 1990), was based on the ability of various compounds that activate MMPs to disrupt the interaction between the zinc and the conserved cysteine either directly or by altering the protein conformation of the pro domain. This event allows a water molecule to become a forth zinc ligand thus displacing the cysteine. The release of the pro domain from the active site zinc leads to the removal of the pro domain via an autocatalytic mechanism to produce the mature enzymatic form (Figure. 1.1 B). The initial cleavage is performed *in vivo* by other proteases or MMPs, or in some cases plasmin (Powell and Matrisian, 1996). *In vitro*, this activation can be achieved by a variety of agents including: conformational perturbants such as sodium dodecyl sulphate (SDS) and the organomercurials, oxidants such as sulfhydral alkylating agents, and in some cases proteolytic cleavage by trypsin (Grant *et al.*, 1992). The **catalytic** site is the third fundamental domain, which contains the active site whose amino acid consensus sequence is HEXGHXXGXXHS. The active site holds a zinc ion by co-ordinate bonding to the three histidines and a water molecule (Windsor *et al.*, 1994). Studies have shown that the metalloproteinases contain a second zinc binding site which appears to have a major role in stabilizing tertiary structure. Also present are two calcium ions, however these do not appear essential for activity but may play a role in inhibitor binding (Yuan *et al.*, 1994). MMP-7 is the smallest MMP consisting of these three domains and it is tempting to speculate that it is the present-day representative of the primordial gene.
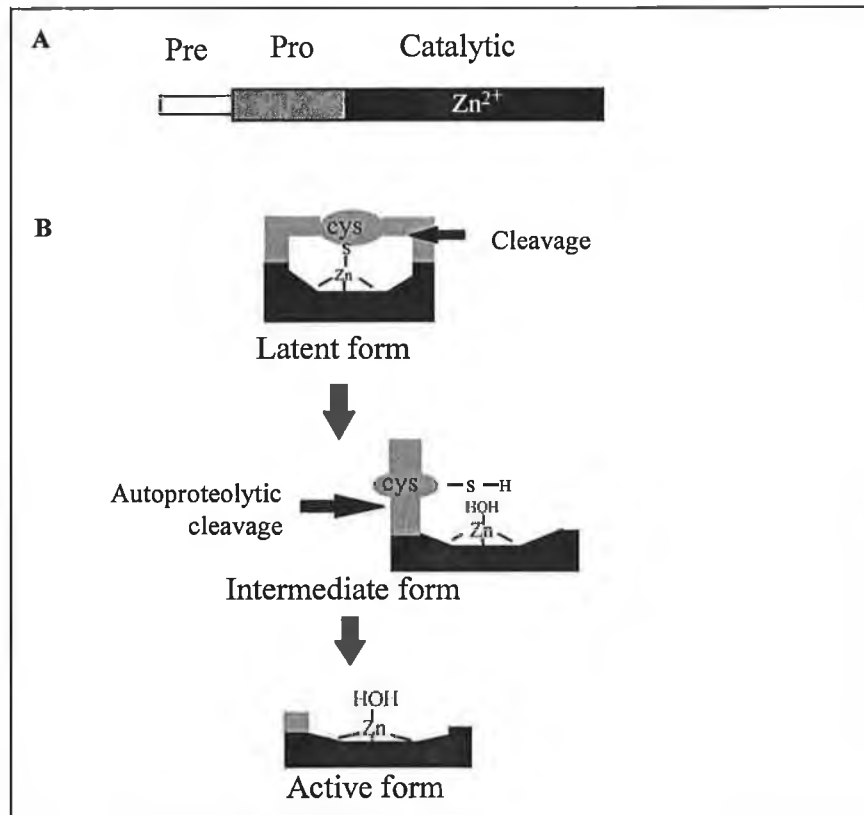
Figure 1.1. A : The fundamental domain structure for MMPs. B : Cysteine switch mechanism for MMP activation. The pro domain of the latent molecule is folded around so that the cysteine residue can form a complex with the zinc molecule. An initial cleavage causes a conformation change in the molecule that disrupts the cysteine-zinc interaction and frees the zinc to participate in the proteolytic cleavage. Thus when the cysteine is 'on' the zinc, the activity of the enzyme is 'off', hence referred to as 'the cysteine switch mechanism' (Van Wart *et al.*, 1990).

In addition to these three MMP domains, the MMP family members have domains that generate diversity in their association with substrates and with cellular and matrix components. MMP-7 is the only family member to date that does not contain a COOH-terminal domain that has homology to a heme binding protein (hemopexin) and the ECM component vitronectin, known as the hemopexin domain. This domain has been shown to be involved in mediating associations with ECM components and inhibitors but has differing functions in individual family members. The hemopexin domains in pro MMP-2 and -9 have been associated with interactions with the inhibitors TIMP-2 and TIMP-1, respectively (Baragi *et al.*, 1994, Murphy *et al.*, 1992). In MMP-1 and -8, this domain is associated with substrate and inhibitor binding (Bigg *et al.*, 1994). The

hemopexin domain of MMP-2 is required for cell surface activation (Ward *et al.*, 1994). The hemopexin domain is linked to the catalytic domain through a short but variable hinge region; its presence in MMP-1 and MMP-8 enables them to cleave fibrillar type I collagen (Hirose *et al.*, 1993). The type IV collagenases MMP-2 and MMP-9 posses fibronectin-like sequences which have been shown to be required for gelatinase activity. MMP-9 is the only MMP to contain a small region similar to the α_2 chain of type V collagen in the hinge region but the functional significance of this insertion is unclear.

The most recently described members of the MMP family contain a domain that is common to many proteins but is newly characterised in the MMPs. The transmembrane-type (MT-) MMPs contain a transmembrane domain near their carboxyl termini which localizes the enzymes to the plasma membrane. This domain contains a hydrophobic amino acid stretch which can pass through the plasma membrane.

In addition to the transmembrane domains, the MT-MMPs (MMPs 14-17) and MMP-11 contain a short region between their pro domain and catalytic domain that fits the consensus cleavage site for furins, which are protein-processing enzymes and have been shown to function in a number of protein maturation pathways (Bresnahan *et al.*, 1990). In MMP-11 this sequence allows for internal cleavage of this MMP, resulting in secretion of the enzyme in an activated form (Pei and Weiss, 1995). These enzymes cannot apparently be activated by agents such as organomercurials but require proteolytic activation by molecules such as furin (Pei and Weiss, 1995). MT1-MMP and MMP-11 have another unique similarity in that neither enzyme appears to efficiently degrade ECM proteins. This observation suggests the possibility that there may be other functions associated with MMPs other than degrading ECM proteins directly.
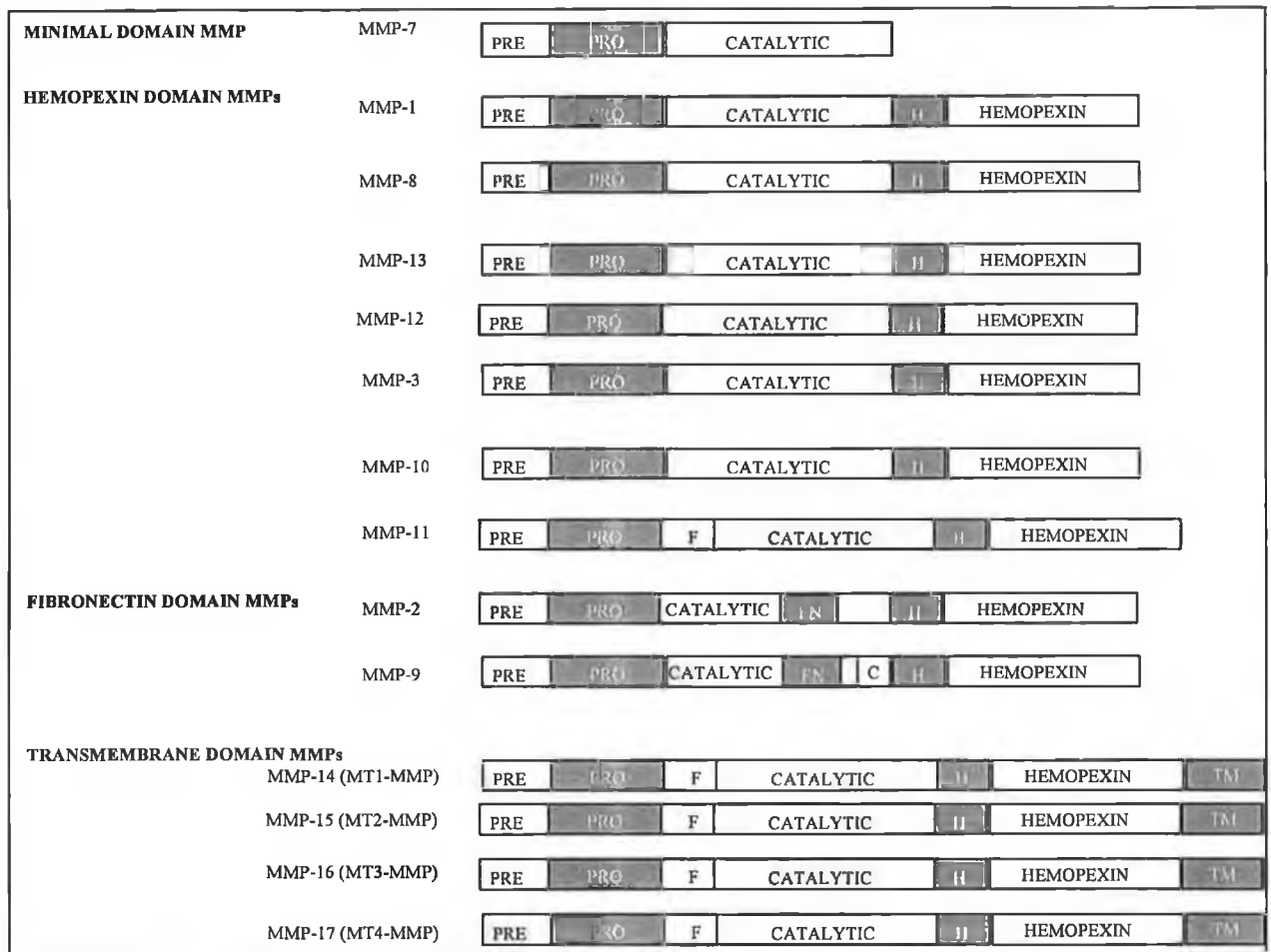
Figure 1.2 Domain Structure of MMP Family. [H = hinge domain; F = furin consensus site; FN = fibronectin-like domain; C = collagen-like domain; TM = transmembrane-like domain].

1.2.2 Properties of the MMPs

It is generally accepted that degradation of the ECM is the end result of a proteolytic cascade involving members of the MMP family. Each MMP has a unique yet slightly overlapping substrate specificity (Table 1.1). In the past the MMP family had been classified into three subclasses with respect to their substrate specificity: (1) the type I collagenases which degrade fibrillar collagens; (2) the type IV collagenases which degrade denatured and basement membrane collagens, and (3) the stromelysins which degrade proteoglycans and glycoproteins.

The collagenases, comprising of MMP-1, MMP-8, and, MMP-13, target primarily fibrillar, but also non fibrillar collagens. Interstitial collagenase (MMP-1) is probably the best characterised of the MMPs. The gene encoding MMP-1 was isolated from a lamda phage human DNA library by Collier *et al.* (1988a). The activated collagenase specifically degrades the native, triple helix of type I collagen, as well as types II and III [collagens types I-III are known as interstitial collagens, hence the name], by making a single, sequence specific cleavage (Miller *et al.*, 1976). The action of MMP-1 on collagen results in 'unwinding' of the helical structure to produce a molecule that is now susceptible to proteolytic cleavage by other proteinases and gelatinases. MMP-8 or neutrophil collagenase was identified and sequenced from a cDNA library derived from peripheral leukocytes of a patient with chronic granulocytic leukemia (Hasty *et al.*, 1990). It is capable of cleaving all three alpha-chains of types I, II and III collagens and has been implicated in the pathology associated with rheumatoid and osteo-arthritis (Ho *et al.*, 1994). MMP-13 also known as collagenase-3 was cloned from a cDNA library derived from a breast tumour (Freije *et al.*, 1994). Increased expression of MMP-13 in breast carcinoma and the absence of detectable expression in most normal tissues has been shown and a possible role in tumorigenesis is proposed (Freije *et al.*, 1994). Analysis of substrate specificity revealed that type II collagen is preferentially hydrolyzed by MMP-13 (Knauper *et al.*, 1996).

The MMPs stromelysin -1, -2, -3, (MMPs -3, -10, -11, respectively), matrilysin (MMP-7) and metalloelastase (MMP-3) are all members of what has been called the stromelysin subclass. They can cleave many ECM components including proteoglycans, fibronectin, collagens, and gelatins but have no proteolytic activity for native type I collagen, with the exception of MMP-11 which as stated already is a weak protease and has thus far been shown to cleave laminin and fibronectin (Murphy *et al.*, 1993). Stromelysin-1 (MMP-3) was identified by Werb and colleagues (Chin *et al.*, 1985) as a secreted MMP. The molecule was originally cloned by virtue of its inducibilty by oncogenes and growth factors and was referred to as transin (Matrisian *et al.*, 1985, McDonnell *et al.*, 1990). Transin was later shown to be the rat homolog of human stromelysin (Muller *et al.*, 1988). Stromelysin-2 (MMP-10) has been identified both in rat and in humans and has similar amino acid sequence (75-80% identity) and substrate specificity to stromelysin-1 (Nicholson *et al.*, 1989). However, the two genes are regulated differently by growth factors. Stromelysin-3 (MMP-11) was isolated from stromal cells surrounding breast carcinomas and its

expression has been associated with the invasive stages of this disease (Basset *et al.*, 1990). Matrilysin (MMP-7) is the smallest member of the MMP family and was first isolated by Muller *et al.* (1988) from a mixed tumour library in an effort to clone stromelysin-related genes. MMP-7 has also been shown to degrade entactin, a basement membrane protein which bridges laminin and type IV collagen (Sires *et al.*, 1993). The fifth member of this subclass, metalloelastase (MMP-12), was originally isolated and cloned from murine macrophages (Shapiro *et al.*, 1992). The human homologue called human macrophage metalloelastase was subsequently cloned by the same group (Shapiro *et al.*, 1993). MMP-12 is specifically associated with mononuclear phagocytes and, as its name suggests, is a potent degrader of elastin (Shapiro *et al.*, 1993).

The type IV collagenases consisting of MMP-2 and MMP-9, also known as the gelatinases, are potent in their ability to cleave denatured collagens and intact type IV collagen. Human MMP-2 was cloned by Collier *et al.* (1988b). It is a 72-kDa proenzyme and when activated degrades the following substrates in order of preference ; gelatin, types IV, V, VII collagen, and fibronectin. Expression of MMP-2 is widespread and is frequently elevated in malignant tumours. MMP-9 was traditionally thought of as the macrophage gelatinase but its expression has now been described in many other cell types including malignant cells, neutrophils, trophoblasts and keratinocytes. MMP-9 was cloned from SV40-transformed human lung fibroblasts (Wilhelm *et al.*, 1986). It is secreted as a 92 kDa proenzyme and when activated degrades gelatins, collagens types IV and V, and elastin.

There are currently 4 known MT-MMPs, all of which have been cloned from human cDNA libraries (Takino *et al.*, 1995a, Takino *et al.*, 1995b, Puente *et al.*, 1996). Membrane localisation is required for the only known activity of MT1-MMP, MT2-MMP, and MT3-MMP; activating MMP-2 on cell membranes surfaces (Sato *et al.*, 1994; Puente *et al.*, 1996). The expression of MT1-MMP also induced binding of MMP-2 to the cell surface by functioning as a receptor, giving the advantage of surface localization of proteinases over pericellular proteolysis (Sato and Seiki, 1996). MT1-MMP has been found to be overexpressed in malignant tumour tissues, including lung and stomach carcinomas, and has been associated with the activation of pro-MMP-2 in these tissues (Sato *et al.*, 1994).

Recently three novel members of the MMP family have been reported, namely MMP-18, MMP-19 and MMP-20. MMP-18 is a novel collagenase isolated from metamorphosing Xenopus

laevis tadpoles, and has essentially identical enzymatic activity towards a collagen substrate as human interstitial collagenase (Stolow *et al.*, 1996). MMP-19 was cloned from a liver cDNA library. This protein exhibits the domain structure characteristic of previously described MMPs but lacks a series of structural features distinctive of the diverse MMP subclasses and has been proposed to represent the first member of a new MMP subfamily (Pandas *et al.*, 1997). MMP-19 is mainly expressed in placenta, lung, pancreas, ovary, spleen and intestine, suggesting it may have a specialised role in these tissues. Sedlacek *et al.* (1998) very recently reported the identification of a gene RASI-1 which encodes a protein showing sequence identity with MMP-19 and proposed that MMP-19 is a novel autoantigen with a role in rheumatoid arthritis. MMP-20 also known as human enamelysin was cloned from odontoblastis cells and has been shown to degrade amelogenin, the major protein component of the enamel matrix. Expression of MMP-20 is highly restricted to dental tissues (Llano *et al.*, 1997).

In addition to ECM components, MMPs can cleave precursor forms of other MMPs, the serine proteinase urokinase-type plasminogen activator (PA), and protease inhibitor α-1-antitrypsin. MMPs have also been implicated in the activation of growth factors [e.g. tumour necrosis factor (TNF)-α (Gearing *et al.*, 1994)] and growth factor receptors [e.g. IL-6 receptor (Chambers and Matrisian, 1997)]. A recent report also describes angiostatin-converting enzyme activity of MMP-7 and MMP-9 by hydrolysing plasminogen to generate angiostatin fragments which inhibit angiogenesis (Patterson and Sang, 1997). Novel functions attributed to the MMPs, such as those mentioned above, are beginning to challenge the traditional views of the roles of MMPs in biology such that it is no longer sufficient to consider each MMP member only in terms of its substrate specificity, but also consider the effects it may have on the cell phenotype.

1.3 Regulation of MMP Expression

The activity of the MMPs is highly regulated, lending support to the idea that they have important biological functions. The regulation of MMP activity is complex and occurs at several different levels : (a) at the transcriptional level; (b) at the activity level by processing of the latent

precursor to an active enzyme; and (c) total proteinase activity is modulated by the presence of TIMPs.

At the transcriptional level the expression of the genes for metalloproteinases and TIMPs is regulated by a diverse range of extracellular stimuli, including growth factors, phorbal esters, hormones, steroids, and adhesion molecules (Birkedal-Hansen *et al.*, 1993). Their expression is also regulated by oncogenes and proto-oncogenes (Crawford and Matrisian, 1996). The effect of these biologically active molecules seems to be influenced by the cell type, species of origin, and conditions of cell growth, because not all cells respond to these stimuli similarly. Among the members of the MMP gene family there are many enzymes with very similar substrate specificity, however the differences in transcriptional regulation between family members goes some way in explaining this apparent overlap. Studies over the past several years have shown that MMPs are regulated both positively and negatively at the transcriptional level. The regulation of MMP at the transcriptional level is considered in detail in the introduction to chapter four. In addition to control at the transcriptional level, MMPs can be regulated post-transcriptionally by changes in mRNA stability (Delany and Brinckerhoff, 1992).

The conversion of latent metalloproteinases to active enzymes is an additional step at which MMP activity is regulated and may involve activation of a proteinase cascade. Using a co-culture system with human keratinocytes and dermal fibroblasts, He *et al.* (1989) have suggested that the endogenous activator of metalloproteinases is plasmin, produced from the plasminogen precursor by the action of plasminogen activators. Plasmin can convert both proMMP-1 and proMMP-3 to their active forms. MMP-7, MMP-3, MMP-10, and the MT1-MMP can activate other MMPs by proteolytic cleavage. Coordinate regulation of the MMPs and plasminogen activators could therefore have synergistic effects. This cascade of proteolytic events, reminiscent of the clotting cascade, may serve as a controlled but powerful mechanism to coordinate complete degradation of multiple components of the ECM.

Finally, MMP activity on ECM substrates is dependent on the balance between the enzymes and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which are reviewed below. The MMPs are components of a system of 'balanced proteolysis' wherein a finely tuned equilibrium exists between the amount of active enzyme and its inhibitors.

1.4 TIMPs

The TIMPs are secreted multifunctional proteins that also play pivotal roles in the regulation of ECM metabolism. Their most widely recognised action is as inhibitors of the MMPs. TIMPs constitute a family of proteins that in humans is currently composed of 4 members, TIMP-1, TIMP-2, TIMP-3 and TIMP-4. These proteins bind non-covalently to MMPs and specifically inhibit their enzymatic activity. TIMPs consist of two distinct structural and functional domains. The N-terminal domain alone is an efficient inhibitor of the MMPs through interaction with the enzymatic catalytic domain. The C-terminal domain has enzyme binding sites which by interaction with the MMP increase the rate of inhibition of the enzyme due to the anchoring effect (Slawomir *et al.*, 1997).

TIMP-1 is a glycoprotein with an apparent molecular size of 28.5 kDa which forms a complex of 1:1 stoichiometry with the activated form of multiple MMPs and is also found associated specifically with the latent form of MMP-9 (Docherty *et al.*, 1985). The gene coding for TIMP-1 has been cloned and sequenced (Mahtani and Willard, 1988). The secreted protein has 184 amino acids and six intramolecular disulfide bonds. TIMP-1 is ubiquitous among tissues and species and is considered a major regulatory inhibitor in the extracellular milieu. In the early mouse embryo, tissue localisation data shows that high levels of TIMP-1 transcripts are found at the sites of active remodeling, such as developing bone, where its expression overlaps significantly with that of members of the transforming growth factor-β (TGF-β) family (Flenniken and Williams, 1990). Early studies investigating the link between TIMP-1 expression and invasive ability in cell lines showed that TIMP-1 levels were decreased 10-20 fold in highly invasive as compared to the normal or poorly invasive cells (Hicks *et al.*, 1984). Later studies linked an increase in invasive properties in poorly invasive cells with a reduction in TIMP-1 expression, using antisense TIMP-1 transfected into Swiss 3T3 cells (Khokha *et al.*, 1989). Human TIMP-1 is known to be identical to a glycoprotein isolated as a factor stimulating colony formation by erythroid precursor cells and referred to as erythroid potentiating activity (EPA). Other evidence suggests that TIMP-1, in particular is a serum mitogen for a wide array of cultured cells (Hayakawa *et al.*, 1992), raising the possibility that TIMP-1 may have a more general growth-stimulatory effect on cells. Thus, TIMP-1 may be functioning simultaneously as a protease inhibitor and growth-promoting factor; a

multifunctional protein regulating turnover of ECM on the one hand and modulating growth properties of cells on the other.

TIMP-2 is a 21 kDa protein which displays similar inhibitory activities against active MMPs as TIMP-1, and in addition TIMP-2 selectively forms a complex with the latent proenzyme form of MMP-2. The secreted protein has 192 amino acid residues and is not glycosylated. TIMP-2 shows an overall 71% similarity to TIMP-1 at the amino acid sequence level. The homology appears closer at the protein level than at the nucleotide level, suggesting that these inhibitors diverged early in the evolution of this gene family (Stetler-Stevenson *et al.*, 1989). The inhibitory effects of TIMP-2 on cell invasion has been demonstrated in a number of studies. DeClerck *et al.* (1992), demonstrated the inhibitory activity of recombinant TIMP-2 (rTIMP-2) on invasion of living smooth muscle cell multilayers by HT1080 cells and by a ras-transformed rat embryo fibroblast line, 4R. rTIMP-2 also inhibited degradation of smooth muscle cell matrixes by these cells. Albini *et al.* (1991), using a reconstituted basement membrane invasion assay, reported that HT1080 tumour cell invasion decreased approximately 85% when exogenous, purified TIMP-2 was added to the system.

TIMP-3 was cloned in 1994 by Uria *et al.* and was found to be expressed in placenta and uterus but not in liver and ovary. TIMP-3 showed low sequence homology with the previously known human TIMPs but showed a high degree of homology with chicken inhibitor of MMPs -3 (ChIMP-3), which was originally isolated as a transformation-induced protein in chicken fibroblasts (Staskus *et al.*, 1991). TIMP-3 appears to be expressed in normal placental and ovarian tissues and in all breast carcinomas examined (Uria *et al.*, 1994). MouseTIMP-3 (mTIMP-3) has been shown to have a distinctive pattern of expression in adult mouse with abundant transcripts detected in kidney, lung, and brain but only low levels detected in bone, a prominent location of mTIMP-1 transcripts (Leco *et al.*, 1994) leading the authors to propose that mTIMP-3 functions in a tissue-specific fashion as part of an acute response to remodeling stimuli.

TIMP-4 was recently cloned and characterised by Greene *et al.* (1996) from a human heart cDNA library. The putative protein shows 57% similarity to TIMP-1 and 70% similarity to TIMP-2 and -3. Tissue expression of TIMP-4 in adult human appears limited. Although large amounts of transcript were detected in the heart, much lower levels of expression were detected in the kidney, pancreas, colon and testes; no TIMP-4 transcripts were detected in other tissues, again suggesting

TIMP-4 may function in a tissue-specific fashion. A recent study by Wang *et al.* (1997) demonstrated the inhibition of tumour growth and metastasis of human breast cancer cells following transfection with TIMP-4. TIMP-4 has also been shown to bind to pro MMP-2 in a similar manner to TIMP-2, which may have physiological significance in modulating the cell surface activation of pro MMP-2 (Bigg *et al.*, 1997).

The existence of a family of inhibitors raises several questions as to potential differences in the physiological roles of the inhibitors. The MMPs make up a gene family themselves, and there appears to be both specificity and overlap in their degradation of ECM substrates. The proMMP-2-TIMP-2 and proMMP-9-TIMP-1 complexes may reflect an additional function for TIMPs in controlling the activation of specific latent MMPs. The notion that individual TIMP family members may have specific physiological roles is supported by gene expression studies. The TIMP-1 gene is highly inducible at the transcriptional level in response to many cytokines, hormones, and the tumour promoting agent 12-O-tetradecanoyl-phorbal-13-acetate (TPA), and is also sensitive to transformation by the *ras* oncogene (Leco *et al.*, 1994). Likewise TIMP-3 expression is not only induced in response to mitogenic stimulation but also is subject to cell cycle regulation (Greene *et al.*, 1996). In contrast, TIMP-2 expression, like that of MMP-2 with which it interacts, is largely constitutive (Stetler-Stevenson *et al.*, 1990b). Where responses of TIMP-2 to stimuli have been observed, the outcomes are opposite to those seen for TIMP-1 (Stetler-Stevenson *et al.*, 1990). This offers a selective means for the regulation of metalloproteinases before and after activation. Thus the net proteolysis will depend on a critical ratio between the TIMP family inhibitors and the metalloproteinases produced in the same micro environment.

MMPs can also be inhibited by α-2-macroglobulin and α1-antitrypsin present in plasma (Barret and Starkey, 1973). α-2-macroglobulin is a non-specific inhibitor of all four classes of proteinases, produced by the liver and detectable in normal serum. The large size (750 kDa) of the inhibitor may decrease its tissue penetration and limits its effectiveness as an inhibitor outside the intravascular compartment. A number of new synthetic agents blocking MMP activity have recently been developed as anti-cancer therapies, including Batimastat and Marimastat which are currently in Phase I/II trials in US, Europe and Canada. Other new agents, currently in clinical trials include Bryostatins, which do not directly affect the activity of MMPs, but can inhibit their production by inhibiting protein kinase C (Slawomir *et al.*, 1997).

1.5 Expression Patterns of MMPs

The expression of MMPs by a variety of cell types has been extensively studied and implicated as important in numerous pathological processes involving tissue destruction. In fact the majority of MMP family members were first isolated and cloned from tumour-associated tissues. These proteinases' roles in normal tissue formation and remodeling has been less frequently studied but initial reports point to complex and highly individualized patterns of expression for the various members of the MMP family. The complexity of expression patterns in normal tissues is demonstrated by examples of cell type- and tissue-specific regulation, inducible and constitutive expression, and discrepancies between *in vitro* and *in vivo* studies. While the whole area of 'normal' MMP expression and function is still in its infancy, there are some general expression patterns emerging.

MMP expression in normal tissues appears to be restricted to certain tissues at specific times, with the possible exception of MMP-2 expression, which in general appears to be more widespread. In developing mouse embryos, MMP-2 is expressed in most mesenchymal-derived tissues and highly expressed in newborn lung, heart, and kidney (Reponen *et al.*, 1992). The enzyme is frequently detected in normal adult tissues and is elevated in cancerous lesions of these tissues (Stetler-Stevenson, 1990a). Although MMP-2 is expressed in many normal tissues, the mRNA is generally restricted to connective tissue cells and rarely observed in epithelial cells *in vivo*. Stromelysin-3 (MMP-11) mRNA is also expressed in connective tissue, but its expression is more restricted and has been observed in specific areas such as in developing digits (Basset *et al.*, 1990) and involuting mammary glands (Lefebvre *et al.*, 1992). In contrast, the mRNA for MMP-7 has been observed in glandular epithelial cells of normal mouse small intestine (Matrisian, 1994) and the cycling human endometrium, and is not seen in stromal cells of the same tissue (Rodgers *et al.*, 1993). MMP-7 is secreted primarily by cells of epithelial origin and may function in glands and ducts as an 'enzymatic pipe cleaner'(Wilson and Matrisian, 1996).

Hematopoietic cells also show distinct and highly specific patterns of expression of MMPs. Neutrophils contain a collagenase distinct from MMP-1; neutophil collagenase (MMP-8). The expression of MMPs in cells of the monocyte/macrophage lineage demonstrates stage-specific expression patterns. Promonocytes produce MMP-7 mRNA, whereas activated macrophages produce MMP-3 and MMP-1 mRNA (Shapiro *et al.*, 1991). MMP-9 is also expressed in

hematopoietic cells. Interestingly, other cell types, such as fibroblast and epithelial cells, have been shown to produce MMP-9 in culture. *In situ* hybridisation studies of developing mouse embryos, however, suggest that the mRNA for this enzyme is restricted to osteoclasts and bone marrow cells *in vivo* (Reponen *et al.*, 1994). Such studies suggest that culture conditions may alter the normal expression patterns of MMPs. Studies of MMP transcriptional regulation in normal and neoplastic cells offers an intriguing opportunity to understand the convergence of these pathways and their pathological and physiological roles.

1.6 Pathological Roles of MMPs

Over expression of MMPs has been associated with tumour progression for many years. Both MMP-3 and MMP-2 cDNA were originally cloned from transformed cells that exhibited increased proteolytic activity relative to parental untransformed lines (Matrisian *et al.*, 1985; Collier *et al.*, 1988b). A functional role was assigned to MMPs in tumour invasion mainly by the use of TIMP as an inhibitor of *in vitro* and *in vivo* invasion (MacDougall and Matrisian, 1996, for review), demonstrating that tumour MMP expression is required for tumour invasion and metastasis. Many oncogenes have been shown to regulate MMP levels, and most MMP s have an activator protein-1 (AP-1) transcription element in their promoter, which may mediate this response (Gaire *et al.*, 1994). The evidence of MMP involvement in invasion and metastasis comes from three main sources: correlative evidence demonstrating the expression of MMPs in advanced-stage tumours (Powell and Matrisian, 1996 for review), *in vitro* models of invasion, and *in vivo* models of invasion and metastasis (DeClerck *et al.*, 1992, Schultz *et al.*, 1988).

Colon cancer has been one of the most studied neoplasms and MMP overexpression has been linked to increased invasiveness and metastasis. Witty *et al.* (1995) have provided evidence that MMP-7 may have an effect on colon cancer cell growth in an orthotopic model of colon cancer. SW480 human colon cancer cells, originally isolated from a primary tumour, were transfected with MMP-7 cDNA under the control of a constitutive promoter and when implanted into the cecum of nude mice the tumours that arose were faster growing than the nontransfected SW480 cells. In the matched cell line SW620, (SW620 cell line was isolated from the same patient as SW480, but from a lymph node metastasis) which constitutively secretes MMP-7, antisense

ablation of the MMP-7 mRNA reduced the number of tumours (Witty *et al.*, 1994). Similar studies on MMP expression in other cancer models have correlated MMP overexpression to invasive potential of tumour cells (McElligott *et al.*, 1997, Benhard *et al.*, 1994)).

1.7 Physiological Roles of MMPs

The best defined correlations of MMP expression with normal functions are found during trophoblast invasion (Cross *et al.*, 1994); endometrial remodeling (Salamonsen, 1994); ovulation (Russell and Findlay, 1995); bone remodeling (Heath *et al.*, 1984); angiogenesis (Fisher *et al*, 1994) and development (Matrisian and Hogan, 1990). In the following sections, examples of metalloproteinase expression in some of these normal cellular events are described and the role of MMPs in mediating physiological functions is explored. Although the metalloproteinases are not the only proteinases which have been implicated in any or all of the following processes, it is beyond the scope of this review to examine the contributions of other classes of proteinases.

1.7.1 Implantation and Placentation

Development of the human fetus depends primarily on the embryo rapidly gaining access to the maternal circulation. Trophoblasts cells which are derived from the outer layer of the blastocyst have developed mechanisms by which they can invade the uterus and tap into the maternal circulation (Figure 1.3) (Cross *et al.*, 1994). In contrast to tumour cell invasion, trophoblast invasion is precisely regulated, being confined spatially to the uterus and temporally to early pregnancy. A number of investigators have shown that various MMPs are important mediators of trophoblast invasion. Fisher *et al.* (1989) were the first group to show that freshly isolated trophoblasts secrete a number of MMPs, some of which varied with the stage of gestation. The two type IV collagenases, MMP-2 and MMP-9 which specifically degrade type IV collagen and gelatins have been of particular interest in this respect. Several investigators have shown that MMP-9 is expressed in trophoblasts isolated from first and second trimesters and that this expression decreases by third trimester. Librach *et al.* (1991) subsequently demonstrated that a function-perturbing antibody specific for MMP-9 completely inhibited trophoblast invasion implying that MMP-9 was critical for invasion. The role of the MMPs in trophoblast invasion will

be dealt with in more detail in chapter 3. Trophoblast invasion, because of its eloquently regulated nature both spatially and temporally presents a unique contrast to the unregulated invasion seen in metastatic cancer and provides an excellent model to study the regulation of MMP expression.
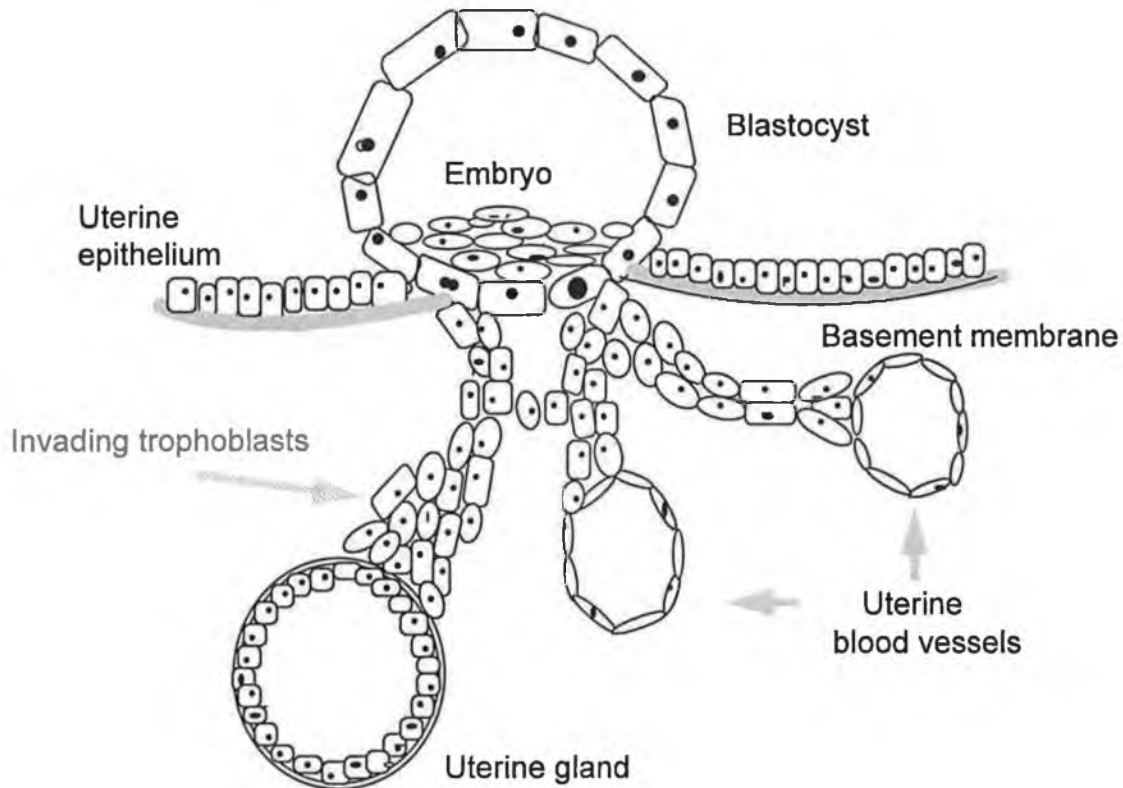
Figure 1.3 Diagram illustrating trophoblast invasiveness during implantation and placental development. Trophoblasts derived from the blastocyst breach the basement membrane and uterine epithelium and tap into maternal circulation.

1.7.2 MMP Expression in the Cycling Human Endometrium

Perhaps the most dramatic expression of MMPs in a normal tissue is seen in the cycling human endometrium. The uterine endometrium, is regularly shed and regenerated throughout reproductive life, implying active and continuous tissue remodeling. This rapid and extensive degree of steroid-mediated tissue remodeling, rivals that of many neoplasias. Rodgers *et al.* (1994) showed that the mRNAs for MMP-1, -3, -10, -11, -2 and -9 were all expressed during the menstrual phase in the human endometrium, but displayed differences in cellular distributions. MMP-7 was expressed in epithelial cells throughout the tissue. MMP-1 and MMP-9 were expressed in stromal cells but were concentrated in the luminal region of the tissue. The transcripts

for MMP-11 and MMP-2 were localised throughout the stromal component of the tissue. This widespread expression of MMPs in all compartments of this tissue suggested that the concerted effort of several MMP family members may play an important role in the breakdown and release of endometrial tissue during menstruation.

In addition to the expression of MMP-2 during the proliferative phase, MMP-7 and MMP-11 transcripts were detected which may be attributed to the remodeling that occurs during the expansion of the tissue. MMP-2 was the only MMP transcript observed during all phases of the cycle including the secretory phase when progesterone levels were elevated. The mRNA for all other MMPs was absent suggesting an inhibitory effect of progesterone on the expression of each of these genes and differences between the MMP-2 promoter and that of the other family members. *In vitro* studies by Bruner *et al.* (1995) confirmed that TGF-β mediated the progesterone suppression of MMP-7 by adjacent stroma. This expression of the MMP family members in a globally coordinated but individually distinct manner mediates localised changes in endometrial extracellular matrix.

1.7.3 Angiogenesis

Angiogenesis is not only important in embryonic development, but also in a variety of normal and pathological conditions in the adult, including ovulation, bone formation, inflammation, wound repair, and tumour growth. The composition of the ECM affects the angiogenic response. In order to form a new capillary sprout in response to an angiogenic stimulus, endothelial cells must first degrade the basement membrane that surrounds the preexisting capillary. The cells then migrate from the vascular wall, penetrating barriers imposed by the ECM, towards the angiogenic stimulus. The subsequent events of proliferation of the cells behind the leading front, formation of a vascular lumen, and further capillary maturation are also effected by ECM composition. Angiogensis is regulated by the presence of angiogenic factors, including fibroblast growth factor (FGF), angiotropin, angiogenin, TNF-α, epidermal growth factor (EGF), and TGF-β (Fisher *et al.*, 1994). The penetration of local ECM barriers by endothelial cells requires spatially and temporally controlled elaboration of an array of proteolytic activities (Moscatelli and Rifkin, 1988). Cultured capillary endothelial cells synthesise increased amounts of

MMP-1 in response to preparations known to contain basic-FGF-like angiogenic activities, TPA also induces MMP-1 and MMP-3 in cultured endothelial cells (Moscatelli and Rifkin, 1988). Mignatti et al. (1989) showed that FGF-induced invasion of cultured bovine capillary endothelial cells was inhibited by inhibitors of both plasmin and metalloproteinase activity, demonstrating that both the plasminogen-activator-plasmin system and specific metalloproteinases were involved in the FGF induced invasion associated with angiogenesis. Fisher et al. (1994) using human umbilical vein endothelial cells (HUVEC) treated with phorbal esters detected a prominent induction of MMP-1, also induced were MMP-9, and MMP-2 production. Two inhibitors of MMPs, the naturally occurring TIMP-1 and the synthetic, peptide inhibitor BB-94 were both effective at blocking HUVEC-mediated collagen degradation, invasion and tubule formation. The authors showed that the inhibitors were acting directly upon MMP-1, demonstrating that MMP-1 was required for angiogensis in vitro (Fisher et al., 1994). Further investigations into the mechanisms by which MMPs modulate the ECM such that angiogensis can occur in a precisely regulated fashion will continue to clarify the specifics of the angiogenic process.

1.7.4. MMPs in Cellular Processes

Tissue remodeling in any form can involve a variety of cellular events, including proliferation, migration, differentiation, and apoptosis. MMPs can participate in these cellular activities in a variety of ways. Through their actions, they can alter cell-matrix and cell-cell interactions, change cell shape, and release or activate growth factors. These are events which in turn can alter cellular processes.

The potential roles for MMPs in cellular proliferation are many. As discussed earlier, MMPs are induced by growth factors and oncogenes that also increase the proliferative index of cells in vitro. The induction of MMPs in proliferating tissues could facilitate the clearing of basement membrane and/or connective tissue matrix components to make room for the multiplying cells as they expand. MMP induction may be part of a programme of gene expression designed to prepare the tissue for growth. Proteolysis of the ECM could result in release of matrix bound growth factors and their receptors, such as FGF and heparin binding EGF (Vlodavsky et al., 1990; Whitelock et al., 1996). Another possibility is that MMPs can directly activate growth factors by processing them from precursor molecules. MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9

activate proTNF-α in this manner (Gearing *et al.*, 1994). Similarly MMP-7 causes the release of the receptor-binding amino-terminal fragment from high molecular weight uPA, which has been shown to be mitogenic and to increase cell motility (Anichini *et al.*, 1994). MMPs may thus affect cellular proliferation by a variety of indirect mechanisms.

The nature of the ECM can influence the apoptotic programme in mammalian cells and this has lead to the association of MMPs with apoptosis. The prevailing hypothesis is that the basement membrane is a survival factor for epithelial cells, and their loss of contact with the basement membrane results in programmed cell death. In the breast, a basement membrane rich in laminin is required for normal mammary gland development and gene expression (Lin and Bissell, 1993). When mammary epithelial cells are placed in culture without critical basement membrane components, they undergo apoptosis (Pullan *et al.*, 1996). *In vivo*, overexpression of the activated form of MMP-3 in the mammary gland of transgenic mice results in loss of basement membrane components, and a marked disruption of ECM structure (Witty *et al.*, 1995). Consistent with a role for MMPs in modulating programmed cell death via alterations in the ECM, these mice show a significant increase in the apoptotic index in mammary tissue (Witty *et al.*, 1995; Boudreau *et al.*, 1995). Studies by Boudreau *et al.* (1995) suggested that basement membrane ligations inhibited IL-1β converting enzyme (ICE; the mammalian cell death-inducing gene) expression, and the loss of this contact resulted in elevated ICE and induction of the apoptotic pathway. MMPs may also activate or release factors involved in the apoptotic process. Fas interactions with its ligand initiate apoptosis in T lymphocyte (Ju *et al.*, 1995). Fas is a member of the TNF/nerve growth factor receptor family and MMPs have the ability to convert proTNF-α and Fas ligand to active, soluble forms, possibly inducing apoptosis.

The MMP family continues to expand with the cloning of the MT-MMPs and the recent cloning of MMP-19 and MMP-20. These new additions add more complexity to the field, while at the same time opening new avenues of research. The classification of the MMPs by protein structure rather than substrate specificity may free the MMPs from the view that they are enzymes that only degrade ECM and highlight the increasing complexity of the structure and function of the MMPs. This complexity is paralleled in the elaborate yet precise manner in which the MMPs are

regulated *in vivo*. The examples given of the physiological roles of MMP are by no means exhaustive but serve in demonstrating the subtle yet critical roles the metalloproteinases play in many different normal cellular processes.

1. 8 Thesis Overview

The research presented in this thesis examines the role of MMPs in trophoblast invasion and has been divided into four main sections;

- The establishment of a model system using continuous trophoblast cell lines to examine the role of MMPs in trophoblast invasion. The trophoblast cell lines were characterised extensively and examined for expression of MMPs and TIMPs (Chapter 3).
- An investigation of the effects of cytokines, ECM components, hormones and hypoxic conditions on MMP mRNA and protein expression in trophoblast cell lines. The effect of cytokines on the invasive ability of the cell lines was also examined (Chapter 4).
- Localisation of MMP protein expression *in vivo* by immunohistochemistry of placental biopsies. Also, a preliminary investigation of the role of MMPs in pre-eclampsia, correlating MMP expression with disease state in 10 pre-eclamptic and 10 normal placental biopsies (Chapter 5).
- The isolation and characterisation of soluble single chain antibody fragments to MMP-9 using phage display technology (Chapter 6).

As the research described above covers a number of distinct but related topics, this thesis has been divided into eight chapters which, it is hoped, will clarify the results. Chapter 1 serves as a general introduction to the MMPs. There is a common Materials and Methods section (Chapter 2) and Bibliography (Chapter 8). Chapters 3, 4, 5 and 6 each have their own introduction, results and discussion sections. Chapter 7 provides an overall summary.

Chapter 2

Materials and Methods

2.1 MATERIALS

All general purpose chemicals and reagents used in experimental work were of analytical grade and were purchased from Sigma Chemical Company, Poole, Dorset, England; BDH Chemicals Ltd., Poole, Dorset, England and Riedal De Haen AG, Seelze, Hannover, Germany.

Cell culture medium, transfection reagent LipofectAMINE and chamber slides were obtained from Gibco BRL, Paisley, Scotland.

Foetal calf serum was supplied by Biowhittaker, Verviers, Belgium.

BRI clone was obtained from the NCTCC, DCU, Dublin 9.

Disposable plastics for animal cell and microbiological culture, and 96-well plates were obtained from Costar, Cambridge, MA02140, USA.

ED_{27}, ED_{31} and ED_{77} cell lines were kindly donated by Douglas Kniss Ph.D., Department of Obstetrics and Gynecology, Ohio State University College of Medicine, Ohio, USA.

BeWo cell line and 9E10 hybridoma cell line were obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury, Wiltshire, England.

BHK cells, transfected BHK cells, riboprobes for MMP-9, and TIMP-1 and -2 standards were obtained from Dylan Edwards Ph.D., University of Calgary, Canada.

HT1080 cell line was a gift from Hugh McGlynn Ph.D., University of Ulster at Coleraine, Coleraine, Northern Ireland.

Microbiological media components and PBS tablets were purchased from Oxoid Ltd., Basingstoke, Hampshire, England.

The VECTASTAIN ABC kits were obtained from Vector laboratories, Burlingame, CA94010, USA.

Enzyme labeled anti-mouse and anti-rabbit antibodies, restriction endonucleases, Taq polymerase and other components of RT-PCR reactions were purchased from Promega Corp., Southampton, Hampshire, England.

Anti-MMP and-TIMP antibodies were purchased from Oncogene Research Products, Calbiochem, 84 Rogers Street, Cambridge, MA 02142, USA.

X-ray film, film developer and fixative were purchased from Kodak, Dublin , Ireland.

The anti-fd bacteriophage antibody, DAB, OPDA and NBT/BCIP tablets, L-glutamine, trypsin, penicillin/streptomycin and gentamycin were purchased from Sigma Chemical Company.

Cytokines, 92kDa gelatinase protein and *in situ* hybridisation reagents were purchased from Boehringer Mannheim, Hannover, Germany.

Biocoat Matrigel invasion chambers were obtained from Collaborative Biomedical Products, Becton Dickinson Labware, 2 Oak Park, Bedford, MA 01730, USA.

RNA ISOLATOR was obtained from Genosys Biotechnologies, Cambridge, UK

The BCA reagent for protein determination and the FITC labeled anti-mouse antibody were obtained from Pierce Chemicals, Rockford, IL 61105, USA.

Specific primers for PCR were made to specifications by Genosys, England.

Qiagen kit for preparation of DNA purchased from Qiagen Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex, UK

Nissim library was obtained from Cambridge Centre for Protein Engineering, Medical Research Council Centre, Hills Road, Cambridge, CB2 2QH, England.

Helper phage VCS-M13 was obtained from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037, USA.

Equipment used is outlined in the relevant methods section

2.2 METHODS

2.2.1 CELL CULTURE METHODS

All cell culture techniques were performed in a sterile environment using a Holten HB255 laminar air flow cabinet. Cells were visualised with an Olympus CK2 inverted phase contrast microscope.

2.2.1.1 Culture of adherent cell lines

ED_{27}*, ED_{31}*, ED_{77}*, BHK, and HT1080 cell lines were maintained in Dublecco's modification of Eagles medium (DMEM) supplemented with 5% (v/v) [DMEM S_5] foetal bovine serum (FBS), 2 mM L-glutamine, 1 mM N-[2-Hydroxyethl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), gentamycin 50µg/ml. BeWo cells were maintained in Hams F-12 nutrient mix supplemented with 10% (v/v) FBS, 1mM L-glutamine, 1mM HEPES, gentamycin 50µg/ml. All cultures were seeded into 25cm^2 and 75 cm^2 tissue culture flasks. As these were all strongly adherent cell lines, trypsinisation was required for harvesting cells prior to subculturing. For trypsinisation, the growth medium was decanted and the flask rinsed with 3 ml of phosphate buffered saline (PBS) to remove any residual FBS which contains trypsin-inhibitory activity (α_2-macroglobulin). 2 ml of fresh trypsin- ethylenediamine tetracetic acid (EDTA) (0.025% (w/v) trypsin with 0.02% (w/v) EDTA in 0.15 M PBS, pH 7.4) was then placed in each flask and the flask incubated at 37°C for 5-10 min or until all the cells were detached from the surface. The cell suspension was decanted into a sterile universal container containing 5 ml growth medium and centrifuged at 2000 rpm for 5 min. Cells were resuspended in culture medium at 2×10^5 - 1×10^6 cells/ml, using 20 ml of medium per 75cm^2 culture flask and 10 ml per 25cm^2 flask. All cell lines were incubated in a humid, 5% (v/v) CO_2 atmosphere at 37°C in an Heraeus cell culture incubator. [*Trophoblast cell lines were established as previously described by Diss *et al.*, (1992). Briefly, cytotrophoblasts were isolated from first trimester chorionic villi samples (CVS) obtained from patients undergoing cytogenetic testing at 9 to 12 weeks gestation. The villi were dissected free from surrounding nonvillous tissue, incubated with trypsin for 1hr at 37°C, followed by collagenase digestion for 2hr at 37°C with agitation to dissociate the cells of the mesenchymal core of the villi. The cells were used after several serial passages and exhibited an epitheloid phenotype.]

2.2.1.2 Culture of cells in suspension

The anti *c-myc* hybridoma 9E10 was maintained in RPMI medium supplemented with 10% (v/v) FBS, 1 mM HEPES, 2 mM L-glutamine, 1 unit/ml penicillin, 1µg/ml streptomycin and 5% BRIclone (BRIclone is a medium supplement for hybridoma cell lines). Cultures were maintained at densities of 2.5×10^5 cells/ml, in 75 cm² flasks. Cultures were passaged by diluting the cells with media. The cells were incubated in a humid 5% CO_2 atmosphere at 37°C.

2.2.1.3 Cell counts

Cell counts were performed using a Neubauer haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viabilities. 20µl trypan blue was added to 100µl cell suspension, and the mixture left to incubate for 2 min. A sample of this mixture was added to the counting chamber of the haemocytometer and the cells visualised by light microscopy. Viable cells excluded the dye and remained clear while dead cells stained blue.

2.2.1.4 Recovery and storage of cells

Long term storage of cells was achieved by storing the cells frozen in liquid nitrogen and maintaining them in a cryofreezer (supplied and serviced by Cooper Cryoservice Ltd.). Cells to be stored were centrifuged and the resulting cell pellet resuspended at a concentration of 1×10^6 cells/ml in FBS containing the cryopreservative dimethylsulphoxide (DMSO) at a final concentration of 10 % (v/v). 1 ml aliquots were transferred to sterile cryotubes, and frozen first at -20°C for 30 min, then overnight at -80°C and then immersed in the liquid nitrogen. Cells were recovered from liquid nitrogen by thawing rapidly at 37°C and transferring to a sterile universal tube containing 5 ml growth media. The cells were centrifuged at 2000 rpm for 5 min, resuspended in fresh medium, transferred to culture flasks and incubated at 37°C in 5% CO_2.

2.2.1.5 Cytokine/TPA/hormone treatment of cells in culture

Cells were routinely cultured for at least 2 passages prior to cytokine,12-O-tetradecanoyl-phorbal-13-acetate (TPA), or hormone treatment. For these experiments, cells were grown until approximately 70% confluent after which the growth medium was decanted and the cells rinsed with sterile PBS. 8 ml of fresh serum free medium was then added to each 75cm² flask. The cells were returned to the incubator

overnight. The following day fresh serum free medium was added and the flasks were supplemented with one of the following cytokines or TPA (final concentrations in brackets) : interleukin-1β (IL-1β) (5 ng/ml), epidermal growth factor (EGF) (5 ng/ml), transforming growth factor-β (TGF-β) (5 ng/ml) and TPA (100 ng/ml). Hormones were added in the following concentrations progesterone (10^{-6} M) and β-estradiol (10^{-8} M). The cells were then returned to the incubator for 8 hr, after which the cells were harvested for total ribonucleic acid (RNA), or for 24 hr after which the conditioned medium was collected.

2.2.1.6 Isolation of placental fibroblast cell line

Forty to eighty grams of tissue were removed from freshly delivered placentae and washed in DMEM S_{10} prior to trypsinisation. The tissue was then finely minced and subjected to serial trypsin/DNase digestion at 37°C. Cellular supernatants were pelleted and pooled and the trypsinisation stopped by cooling to 4°C and by the addition of FBS. The red cells were then removed by the addition of lysis buffer. Following serial washes in PBS the cellular pellet was resuspended in growth medium and the cell suspension placed in a T75cm^2 flask. The cells were cultured over a period of approximately 4 weeks, at which time a homogenous population of fibroblast cells had overgrown all other cell types.

2.2.1.7 Culturing cells under hypoxic conditions

Cells were cultured until 70% confluent in T75cm^2 flasks and serum starved overnight. The flasks (with loosened lids) were then placed in a hypoxic chamber. The chamber was sparged with nitrogen gas for 10 min to expel air and then sealed. An indicator paper was placed in the chamber also, which indicates if hypoxic conditions were reached. Standard tissue culture conditions are 5% CO_2/95% air, and hypoxic conditions are approximately 2% O_2 /93% N_2/5% CO_2. The cells were then left for 8 hr at 37°C. The conditioned medium was collected and RNA isolated.

2.2.2 IMMUNOCYTOCHEMICAL STAINING

Trophoblast cell lines were characterised by immunocytochemical staining for numerous markers, identified by specific antibodies. These antibodies were against cytokeratins 8 and 18 (Dako) used at a 1:50 dilution, vimentin (Sigma Immunochemicals) at 1:50, human chorionic gonadotropin (hCG)

(Dako) at 1: 200, human placental lactogen (hPL) (Dako) at 1: 350. Cells were grown overnight on chamber slides (Nunc) in standard growth medium. The following day cells were rinsed with PBS and fixed for 7 min in a 1: 1 (v/v) mixture of acetone /methanol at -20°C. Primary antisera were diluted in PBS and applied for 1hr at room temperature (RT). Control slides were incubated with nonimmune serum. The α-cytokeratin 8 and 18 antibodies were detected by immunofluorescence using an FITC-labeled anti mouse antibody (Pierce). The other antibodies were visualised using an avidin-biotin peroxidase detection method with kits purchased from Vector Laboratories (Burlingame, CA).

2.2.3 BCA PROTEIN MICROASSAY

In this assay, Cu^{++} reacts with the protein under alkaline conditions to give Cu^{+}, which in turn reacts with BCA to give a coloured product. Two separate reagents are supplied in the commercially available assay kit : A, an alkaline bicarbonate solution and B, a copper sulphate solution. Working solution is prepared by mixing 1 part reagent B with 50 parts reagent A. 200µl of this solution was added to 10µl test sample or protein standard in wells of a microtitre plate. The plate was incubated at 37°C for 30 min. The absorbance of each well was read at 560 nm using a Titertek Twinreader Plus microtitre plate reader. Protein concentrations were determined from a standard curve in the range 0 - 1 mg/ml.

2.2.4 PROTEIN ELECTROPHORESIS

2.2.4.1 SDS Polyacrylamide Gel Electrophoresis (PAGE)

PAGE, in the presence of sodium dodecyl sulphate (SDS) was performed using the discontinuous system described by Laemmli (1970) using 10% or 15% (w/v) polacrylamide gels as necessary. The 10% [volumes for 15% gel in brackets] resolving gels and 3% stacking gels were prepared as follows :

Resolving Gel :   3.3 ml [5 ml] 30%(w/v) acrylamide containing 0.8% (w/v) bisacrylamide,

4 ml [2.3 ml] distilled water,

2.5 ml [2.5 ml] 1.5M Tris, pH 8.8 containing 0.4% (w/v) SDS

0.1 ml [0.1 ml] 10% (w/v) SDS,

0.1 ml [0.1 ml] 10% (w/v) ammonium persulphate (freshly prepared)

	0.005 ml [0.005 ml] TEMED.
Stacking Gel :	0.33 ml 30% acrylamide solution,
	1.4 ml distilled water,
	0.25 ml 0.5M Tris, pH 6.8 containing 0.4% (w/v) SDS,
	0.02 ml 10% SDS,
	0.02 ml 10% ammonium persulphate (freshly prepared)
	0.002 ml TEMED.

Samples were mixed with sample buffer (2% (w/v) SDS; 0.08M Tris, pH 6.8; 10% (w/v) glycerol; 0.2% (w/v) Coomassie Brilliant Blue). The gel was electrophoresed in running buffer, pH 8.3 containing 0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS at 20 mA per gel using an Atto vertical mini-electrophoresis system until the blue dye front reached the bottom of the gel.

2.2.4.2 Gel Staining

2.2.4.2.1 Staining with Coomassie Brilliant Blue

Gels were stained for 2 hr in 0.5% (w/v) Coomassie Brilliant Blue in acetic acid : water : methanol (1 : 10 : 8, v/v/v), and destained overnight in the same solvent system.

2.2.4.2.2 Silver Staining

Following electrophoresis the gel was fixed by incubating for 8 hrs in 5 gel volumes of ethanol:acetic acid:water (30:10:60). The following day the gel was soaked twice in 5 gel volumes of 30% ethanol for 30 min with gentle shaking, then washed 3 times in 10 gel volumes of deionised water for 10 min. The gel was then placed in 5 gel volumes of 0.1% solution of $AgNO_3$ (freshly diluted from a 20% stock), for 30 min with gentle shaking. The gel was washed on both sides under a stream of deionised water, taking care not to let the gel surface go dry. Next 5 gel volumes of 2.5% sodium carbonate, 0.02% formaldehyde was incubated with the gel until stained bands appeared. The reaction was quenched by washing in 1% acetic acid for 5 min and washed in deionised water.

2.2.4.3 Zymography

SDS substrate gels were used to localise enzyme activity by molecular weight. The gel was made by incorporating the protein substrate of interest (gelatin or casein) within the polymerized acrylamide matrix. 10 % or 15% acrylamide gels were used and the amounts for one gel are given below, volumes for 15% gels are in brackets.

Resolving gel :	2.5 ml [2.5 ml] Buffer A (1.5 M Tris-HCl, pH 8.8; 0.4% (w/v) SDS)

2.5 ml [2.5 ml] 3 mg/ml gelatin or casein stock

3.3 ml [5 ml] 30% acrylamide stock

1.7 ml [0 ml] distilled water

33 μl [33μl] 10% ammonium persulphate (freshly prepared)

5 μl [5μl] TEMED

Stacking gel :	0.8 ml Buffer B (0.5 M Tris-HCl, pH 6.8; 0.4% SDS)

0.5 ml 30% acrylamide stock

2 ml distilled water

33μl 10% ammonium persulphate (freshly prepared)

5 μl TEMED

Samples were mixed 3:1 with 4X sample buffer (10% sucrose; 0.25 M Tris-HCl, pH 6.8; 0.1% (w/v) bromophenol blue) and loaded. The gels were run at 20 mA per gel in running buffer (0.025 M Tris; 0.19M glycine; 0.1% SDS) until the dye front reaches the bottom of the gel. Following electrophoresis the gel was soaked in 2.5% Triton-X-100 with gentle shaking for 30 min at RT with one change. The gel was then rinsed in substrate buffer (50 mM Tris-HCl, pH 8.0; 5 mM $CaCl_2$) and incubated for 24 hr in substrate buffer at 37°C. The gel was then stained with Coomassie blue for 2 hr with shaking and destained in water until clear bands were visible.

To confirm bands as metalloproteinases, identical gels were run as described above except the substrate buffer contained one of the following protease inhibitors: 10mM EDTA (MMP inhibitor), 0.3 mM 1,10-phenanthroline (MMP inhibitor), 1 mM PMSF (serine proteinase inhibitor), or 1 mM pepstatin A (aspartic proteinase inhibitor).

2.2.4.4 Reverse Zymography

For reverse zymography to detect the tissue inhibitors of metalloproteinases (TIMPs), SDS-PAGE gels were prepared with the incorporation of matrix metalloproteinases and gelatin into the acrylamide matrix of the gel. 15% gels (10 ml volume) were prepared as in section 2.2.3.3 above except that the water component of the gel was reduced by 1 ml to adjust for the addition of 1 ml of conditioned medium from BHK h92 cells (containing MMPs). Gels were loaded and ran as above, TIMP standard (gift from Dylan Edwards, University of Calgary,Canada) was loaded as a positive control. After electrophoresis, gels were incubated in a solution of 2.5 % Triton-X-100; 50 mM Tris-HCl, pH 7.5; 5 mM CaCl$_2$ once for 15 min, then again overnight at RT with gentle shaking. Next day gels, were rinsed three times with water, then incubated in 50 mM Tris-HCl pH 7.5 and 5 mM CaCl$_2$ for 24 hr at 37°C to allow digestion of the gelatin substrate. Gels were then stained in Coomassie blue for 2 hr and destained until the desired contrast was achieved. The majority of the gel does not stain as the gelatin has been degraded. Dark bands represent inhibition of gelatin degradation by TIMPs within the sample.

2.2.5 WESTERN BLOTTING

Following electrophoresis as outlined in section 2.2.4.1, the gel was soaked for 30 min in cold transfer buffer (0.25 M Tris, pH 8.3; 0.192 M glycine; 20% (v/v) methanol). Nitrocellulose and eight sheets of Whatman filter paper were cut to the same size as the gel and soaked in transfer buffer. The proteins were transferred from the gel to the nitrocellulose using a LKB 2051 Midget Multiblot Electrophoretic Transfer Unit with water cooling for 1 hr at 30 V followed by 1 hr at 60 V. After transfer, the blot was blocked for 1 hr in blocking solution [2.5% (w/v) dried milk dissolved in Tris Buffered Saline plus Tween [TBST] (10 mM Tris pH 8.0; 150mM NaCl; 0.05% (v/v) Tween 20)] and then incubated overnight at 4°C with primary antibody at a suitable dilution in blocking solution (anti-Fd used at 1/750 dilution, 9E10 used at 1/400 dilution). The following day, blots were washed three times for 10 min each with TBST and then incubated for 3 hr gently shaking at RT with a suitable enzyme-labeled secondary antibody diluted according to the manufacturers recommendations in TBST (anti-rabbit-HRP used at 1/5000 dilution, anti-mouse-HRP used at 1/2000 dilution). Following 3 x 10 min washes, the blots were developed without shaking at RT with the appropriate substrate solution. For alkaline phosphate (AP) labeled antibodies, this was BCIP (175 µg/ml) and NBT (500 µg/ml) in AP buffer (0.1

M Tris, pH 9.5; 0.1M NaCl; 5 mM MgCl$_2$). For horseradish peroxidase (HRP) labeled antibodies, the substrate diaminobenzidine (DAB) was prepared by dissolving one 5 mg tablet in 15 ml TBS and adding fresh hydrogen peroxide (H$_2$O$_2$) to a final concentration of 0.024 % (v/v).

2.2.6 *IN VITRO* INVASION ASSAYS

Biocoat Matrigel invasion chambers provided the cells with conditions that allowed assessment of their invasive property *in vitro*. The cell culture inserts contained an 8µm pore size membrane which has been covered with a layer of matrigel which serves as a reconstituted basement membrane *in vitro*. This layer occludes the pores of the membrane blocking non-invasive cells from migrating through the membrane. By contrast, invasive cells are able to detach themselves from and migrate through the Matrigel treated membrane.

The 6-well plate invasion chambers were removed from 4°C storage and allowed to adjust to room temperature and warm (37°C) serum-free culture medium was added to the interior of the inserts and allowed to rehydrate for 2 hr at RT. After rehydration the media was removed from the inserts and replaced with 2 ml cell suspension in serum-free medium prepared at 3.5x10^5 cells/ml. Chemoattractant 2.5 ml (media containing 20% FBS) was added to the bottom wells of the plate. The plates were then incubated for 72 hr at 37°C in 5% CO$_2$ incubator. After incubation, the non-invading cells were removed from the upper surface of the membrane by scrubbing with a cotton tipped swab. The cells on the lower surface of the membrane were then fixed in methanol for 2 min, stained for 1 min in Mayers Haematoxylin and rinsed in tap water several times. The cells and membrane was then dehydrated by incubation for 2 min each in a series of ethanol solutions (30, 50, 70, 90, 100% ethanol). The membranes were then removed from the insert housing with a scalpel and mounted on slides using DPX mounting medium. Invading cells were then viewed under the microscope at 40 X magnification and counted. The percentage invasion for each cell line was calculated by counting all of the stained cells on the filter underside and dividing by the total number of cells plated.

For experiments investigating the effect of cytokines on invasion, cytokines were added to the media used to rehydrate the inserts, the cell suspension, and to the media containing the chemoattractant at the concentrations given in section 2.2.1.5.

2.2.7 ISOLATION AND ANALYSIS OF RNA

RNA is easily degraded by the ubiquitous RNase enzymes. These enzymes are resistant to autoclaving but they can be inactivated by treatment with the chemical diethylpyro-carbonate (DEPC) when it is added to solutions at a final concentration of 0.1 %. Solutions containing amines such as Tris cannot be DEPC-treated directly as the DEPC is inactivated by these chemicals. These solutions were prepared in DEPC-treated water. All other solutions for RNA work were DEPC-treated, and gloves and disposable sterile plastics used at all times. Any glassware used was baked overnight at 200°C. Manipulations with RNA must be carried out quickly and on ice to help prevent degradation of RNA by endogenous RNases.

2.2.7.1 RNA extraction from cultured cells

Total RNA was isolated from equal numbers of cells using RNA ISOLATOR (Genosys Biotechnologies), which is a modified version of the single step method of acid guanidinium thiocyanate-phenol-chloroform extraction. The cultured cells were lysed directly on the culture flask by adding 2 ml of reagent per 75 cm^2 flask and incubating for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. Phase separation was then achieved by adding 0.2 ml of chloroform per 1 ml of reagent, shaken for 15 seconds and incubated at RT for 2-15 min. The resulting mixture was centrifuged at 12,000 rpm for 15 min. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and the colour-less upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins partition to the interphase and organic phase. The aqueous phase was transferred to a fresh tube and the RNA precipitated by adding 0.5 ml of isopropanol per ml of reagent used. Samples were incubated at RT for 10 min and centrifuged at 12,000 rpm for 10 min. The RNA pellet was then washed once with 75% ethanol, allowed to air dry and dissolved in sterile Tris-EDTA (TE), pH 8.0 (10 mM Tris-HCl; 1 mM EDTA). RNA samples were stored at -80°C.

2.2.7.2 Spectrophotometric analysis of RNA

RNA concentration was determined by measuring the absorbance at 260 nm, the wavelength at which nucleic acids absorb maximally ($\lambda_{max.}$). A 40 µg/ml preparation of pure RNA has an absorbance reading of 1 unit at 260nm. The purity of an RNA preparation was determined by reading absorbances at 260 nm, the $\lambda_{max.}$ for nucleic acids, and at 280nm, the $\lambda_{max.}$ for proteins and obtaining the ratio of these absorbances. Pure RNA with no protein contamination has an Abs_{260}/Abs_{280} ratio of 2.0. Lower ratios indicate the presence of protein while higher ratios often indicate residues of organic reagents.

2.2.7.3 RNA analysis by gel electrophoresis

To check that the RNA isolated was intact and had not been degraded, samples were run on 1% agarose gels. The gels were prepared by boiling the agarose in 1X tris borate EDTA (TBE). Once cooled to hand-hot, the gel was cast into the Hybaid Horizontal Gel Electrophoresis system. The RNA samples (5µl) were prepared for electrophoresis by mixing with 15µl sample buffer (50% (v/v) formamide; 8.3 % (v/v) formaldehyde; 0.027 M 3-[N-Morpholino]propanesulfonic acid (MOPS), pH 7; 6.7 mM sodium acetate; 0.67 mM EDTA) and 3µl loading buffer (50 % (v/v) glycerol; 1 mM EDTA; 0.4 % (w/v) bromophenol blue; 1µg/µl ethidium bromide) and the sample heated for 10 min at 65°C prior to loading on the gel. The gel was run at 100V in 1X TBE. As ethidium bromide was included in the loading buffer, there was no need for further staining and the gel could be visualised directly on a UV transilluminator. The presence of 2 strongly staining bands, representing the 28 S and 18 S ribosomal subunits, signified intact RNA. Degradation could be seen as a smear running down the length of the gel.

2.2.8 REVERSE TRANSCRIPTION PCR (RT-PCR)

The polymerase chain reaction (PCR) has emerged as a powerful tool for amplifying small quantities of deoxyribonucleic (DNA) for analysis. RT-PCR is a modification of the technique which allows analysis of small quantities of specific messanger RNA (mRNA). Total RNA is first converted to complementary DNA (cDNA) using a reverse transcriptase enzyme. Specific cDNAs are then amplified in the PCR by inclusion of the appropriate primers. Details of the primers used are shown in Table 2.1. The PCR used was semi-quantitative as a constitutively expressed gene, β-actin, was always amplified

in the same tube with the MMP target. This acted as an internal control and, by calculating ratios of MMP to β-actin, relative amounts of the targets could be determined from reaction to reaction.

2.2.8.1 Reverse transcription

1μg of total RNA (prepared as outlined in section 2.2.7.1) was mixed with 0.5μg oligo dT primers and the mixture brought to a final volume of 5μl with sterile water. This priming reaction was incubated at 70°C for 10 min. To this was added 4μl 5X transcription buffer (supplied with the reverse transcription enzyme by the manufacturer), 2μl 100 mM dithiothretiol, 1μl RNasin, 1μl of a mix of dATP, dCTP, dGTP and dTTP each at a concentration of 10mM, 6μl sterile water, and 1μl of Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). The reaction mixture was incubated at 37°C for one hr before being heated to 95°C for 2 min to inactivate the enzyme. The resulting cDNA was stored at 4°C until required for PCR.

2.2.8.2 Polymerase Chain Reaction

A 45μl PCR mix was prepared by adding 5μl of 10X reaction buffer supplied with the Taq polymerase enzyme, 1μl each of dATP, dCTP, dGTP, and dTTP each at a concentration of 10mM, 1μl (50ng/μl)of each of the forward and reverse primers required in the reaction, 0.5μl Taq DNA polymerase and 38.5μl sterile water in a sterile tube. To this reaction mixture was added 5μl of cDNA prepared as in section 2.2.8.1 and the mixture overlaid with 50μl mineral oil. The tube was placed on a Hybaid thermocycling machine programmed with an initial incubation of 93°C for 3 min, followed by 30 cycles consisting of the following sequential steps : 93°C for 90 seconds, anealing temperature as in Table 2.1 below for 90 seconds and 72°C for 3 min. The PCR products were then removed from under the oil and placed in fresh tubes. 15μl amounts were run on 2% agarose gels as described in section 2.2.8.3 below.

Table 2.1 Sequence of primers used for RT-PCR

Target	Primer sequence	Fragment size	Optimum anealing temp. (°C)	Reference
MMP-9	5 'GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC 3' 5' GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT 3'	640bp	54	Onisto *et al.*, 1993
MMP-2	5' TGA CAT CAA GGG CAT TTC AGG AGC3' 5' GTC CGC CAA ATG AAC GGT CCT TG 3'	180bp	53	Shimonovitz *et al.*, 1994
MMP-7	5' TGT ATC AAA CCT ATG GAA ATG 3' 5' CGC GCA TCT ACA GTT ATT TAC3'	341bp	47	Witty *et al.*, 1994
MMP-3	5' CTT TCC AGG GAT TGA CTC 3' 5' CGG CCA GTA AAA ATA CAA3'	324bp	48	Matrisian *et al.*, 1986
TIMP-1	5'TGC ACC TGT GTC CCA CCC CAC CCA CAG ACG 3' 3'GGC TAT CTG GGA CCG CAG GGA CTG CCA GGT 3'	551bp	53	Onisto *et al.*, 1993
TIMP-3	5'CTG TGC AAC TTC GTG GAG 3' 5' TCG GTA CCA GCT GCA GTA 3'	250bp	55	Apte *et al.*, 1994
β- actin	5' TCA GGA GGA GCA ATG ATC TTG A 3' 5' GAA ATC GTG CGT GAC ATT AAG GAG AAG CT3'	383bp	47-55	Nakajima-Iijima *et al.*, 1985

The primers were chosen so that they amplified sequences specific to the particular gene. Also the β-actin primers were chosen to span an intron. This meant that any amplification of genomic rather than cDNA would result in larger products than those expected.

2.2.8.3 Agarose gel electrophoresis

A 2% (w/v) agarose gel was prepared by boiling the appropriate quantity of agarose in 100 ml of 1X TBE buffer, pH 8.2 (0.08 M Tris; 0.04 M boric acid; 1 mM EDTA). Once cooled to hand-hot, the gel was cast into the Hybaid Horizontal Gel Electrophoresis system.

Samples for electrophoresis were prepared by mixing with a suitable quantity of 6X gel loading buffer (40% w/v sucrose; 0.25% w/v bromophenol blue). Typically 12.5μl of the PCR reaction mixture was mixed with 3 μl of loading buffer and the samples loaded into the wells of the set gel. The gel was run at 100V in 1X TBE. The electrophoresis was completed when the blue loading dye had run to within 0.5cm of the bottom of the gel. The gel was then stained in a solution of 0.5 μg/ml ethidium bromide

for 20 min, briefly destained in distilled water, visualised by placing on a UV transilluminator. The gels were saved on disc using a UHP Gel Documentation System.

2.2.9 Densitometry

Densitometry of protein and RT-PCR gels was performed using a BioRad model GS670 imaging densitometer with BioRad software Molecular Analyst, version 1.3.

2.2.10 IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis of MMP-9, MMP-2, MMP-7 and MMP-3 was preformed on sections obtained from pre-eclamptic and normal placental biopsies using a Vectastain ABC kit from Vector Laboratories. The antibodies used are shown in Table 2.2. 5μm formalin-fixed, paraffin-embedded tissue sections were provided by Prof. P. Kelehan, National Maternity Hospital, Holles Street, Dublin. Prior to immunohistochemistry, the sections were dewaxed and rehydrated by placing in xylene baths for four 5 min incubations followed by serial washes in ethanol baths of decreasing concentration (100, 80, 60, and 30% (v/v)) for 2 min in each. Endogenous peroxidase activity present in the tissue was quenched by incubating the sections for 30 min in 0.3% (v/v) H_2O_2 prepared in methanol. The sections were then washed for 5 min in PBS and blocked by incubation for 20 min with non-immune serum. After removing excess serum, the sections were then incubated for 1 hr at room temperature with the primary antibody diluted in PBS at the appropriate concentration (see Table 2.2). Following a 5 min wash in PBS, the sections were incubated with biotinylated anti mouse solution (kit component) for 30 min. The slides were again washed for 5 min in PBS. They were then incubated for 30 min in Vectastain ABC reagent (kit component) before again being washed for 5 min in PBS. The slides then were developed with peroxidase substrate solution (DAB) prepared as outlined in section 2.2.5. After suitable colour development, the sections were rinsed in tap water and counterstained with haematoxylin. The sections were then dehydrated by passing through a series of ethanol baths of increasing concentration (30, 50, 70, 90, 100% (v/v)), mounted in DPX mounting medium, and viewed using a light microscope.

2.2.10.1 Microwave method for antigen retrival

After dewaxing and rehydrating, slides were immersed in 10mM citrate buffer, pH 6.0. The tissue sections were microwaved in the citrate buffer for 20 min at high power, ensuring slides are covered in buffer at all times. The slides were then allowed to cool in the citrate buffer before continuing with the IHC as in section 2.2.10 above.

Table 2.2 Antibodies used for immunohistochemistry

Antibody Against	Supplier	Source	Optimum dilution for IHC
MMP-2 (GL8)	Celltech Therapeutics	mouse monoclonal Ab	1/40 (0.019 mg/ml)
MMP-9 (MAC96)	Celltech Therapeutics	mouse monoclonal Ab	N/S
MMP-9 (Ab-1)	Oncogene Research	mouse monoclonal Ab	N/S
MMP-9 (Ab-2)	Oncogene Research	mouse monoclonal Ab	N/S
MMP-9 (Ab-3)	Oncogene Research	mouse monoclonal Ab	N/S
MMP-7	Gift from L.Matrisian	rat monoclonal Ab	1/200
MMP-3	Gift from Syntax	rabbit polyclonal Ab	5μg/ml
TIMP-1 (Ab-1)	Oncogene Research	mouse monoclonal Ab	N/S
TIMP-2 (Ab-1)	Oncogene Research	mouse monoclonal Ab	N/S

N/S - Antibodies were tested but found to be unsuitable for immunohistochemistry (IHC) on paraffin embedded tissues.

2.2.11 PHAGE DISPLAY TECHNIQUES

2.2.11.1 Protocol for use of the Library

Precautions were taken throughout the protocol to avoid carry over of phage from flasks and centrifuge bottles, as autoclaving alone is not sufficient to remove all phage contamination. Wherever possible devoted pipettes and disposable plastics were used. All non-disposable plasticware was soaked for 1 hr in 0.1 M NaOH; 1% SDS followed by extensive washing with distilled water, rinsed in ethanol and washed again in distilled water before autoclaving. Glassware used was baked at 200°C for 4 or more hours. Only polypropylene tubes were used during centrifugation steps as phage may absorb non-specifically to other plastics.

Two bacterial strains were used:

1. *E. coli* TG1 which is a suppressor strain (K12, D(lac-pro), supE, thi, hsdD5/F'traD36,proA$^+$B$^+$, lacI^q,lacZDM15) for propagation of phage particles.

2. *E.coli* HB2151 which is a non-suppressor strain (K12, ara,D(lac-pro),thi/F'proA$^+$B$^+$, lacI^{q}ZDM15) for expression of antibody fragments.

2.2.11.2 To prepare an exponentially growing culture

Phage/phagemid infect F$^+$ *E. coli* via the sex pili. For sex pilli production and efficient infection *E. coli* were grown at 37°C and used at log phase (O.D. at 600nm of 0.4-0.5). A bacterial colony from a TYE plate (1.5% agar; 0.136M NaCl; 1% tryptone; 0.5 % yeast extract) was transferred into 2xTY (1.6 % w/v tryptone; 1% w/v yeast extract; 0.085 M NaCl) medium and grown shaking overnight at 37°C. Next day, the culture was subcultured by diluting 1 : 100 into fresh 2xTY medium, grown shaking at 37°C until OD at 600nm was 0.4-0.5 (approximately 2 hr) and then infected with phage.

2.2.11.3 Large scale culture of helper phage

Large quantities of helper phage VCS M13 for rescue of phagemid libraries were prepared by infecting 200µl *E. coli* TG1 at OD$_{600nm}$ 0.2 with 10µl serial dilution's of helper phage (in order to get well separated plaques) at 37°C in waterbath without shaking for 30 min. 3 ml top agar (1% tryptone; 0.5%

yeast extract; 0.17 M NaCl; 0.7% agar) was added and poured onto warm TYE plates. Plates were allowed to set and incubated overnight at 37°C. The following day a small plaque was picked into 3 ml of exponentially growing culture of TG1 (prepared as in section 2.2.14.2) and grown for about 2 hr shaking at 37°C. The 3 ml was then inoculated into 500 ml 2xTY in a 2 litre flask and grown as before for 1 hr and then kanamycin added to a final concentration of 50µg/ml. The culture was grown for a further 16 hr after which the bacteria were spun down at 10,000 rpm, 4°C for 15 min. 1/4 volume of PEG/NaCl solution (20 % polyethylene glycol 6000; 2.5M NaCl) was added to the phage supernatant and incubated for a minimum of 30 min prior to centrifugation at 10,000 rpm, 4°C for 15 min. The pellet was then resuspended in 2 ml TE and filter sterilised through a 0.45µm filter. The stock was then titered (section 2.2.14.4), diluted to 1 x 10^{12} p.f.u./ml and stored in aliquots at -20°C.

2.2.11.4 Titering of phage and phagemid numbers

The numbers of phage in a preparation can be quantified using plaque forming units (pfu). This relies on the ability of the phage to form plaques of slow growing colonies in a lawn of *E.coli*. This is due to the slower growth of bacteria infected with phage particles. The number of phagmids was estimated using pfu's.

100µl of 10 fold serial dilution's of the phage to be quantified were made (down to 10^{-15}), and mixed with 400µl TG1 in late log phase. The TG1 were then mixed with 3 ml top agar and poured onto 2xTY agar plates. Plates were grown overnight at 37°C, plaques appeared as small clearings in the bacterial lawn. pfu/ml was determined by counting the number of plaques on the plate and determining the number per ml of undiluted phage solution.

2.2.11.5 Growth of the Library

50 µl of the bacterial library stock (about $5x10^8$ clones) was inoculated into 50 ml 2xTY containing 100µg/ml ampicillin and 1 % glucose, grown with shaking at 37°C until the OD_{600nm} was 0.5 (about 2 hr). 40 ml of this culture was used to make a secondary stock of the library as in section 2.2.14.13. The remaining 10 ml of the culture was infected with VCS M13 helper phage in a ratio of 1 : 20 (number of bacterial cells : helper phage particles) taking into account that 1 OD bacteria at 600nm = around 8 x

10^8 bacteria/ml) by incubating without shaking in a 37°C waterbath for 30 min. The infected cells were centrifuged at 4,500 rpm for 10 min and the pellet gently resuspended in 30 ml of 2xTY containing 100μg/ml ampicillin and 25μg/ml kanamycin. 270 ml of prewarmed 2xTY containing 100μg/ml ampicillin and 25μg/ml kanamycin was added and incubated shaking at 30°C overnight.

2.2.11.6 Preparing pHEN1 phagmid particles

The phage were concentrated and any soluble antibodies removed (as in TG1 suppression of the amber stop codon encoded at the junction of the antibody gene and gIII is never complete) by precipitating with PEG 6000. The overnight culture from section 2.2.14.5 above was spun at 8,250 rpm for 10 min and 1/5 volume PEG/NaCl added to the supernatant, mixed well and left for 1 hr or more at 4°C. The solution was then spun at 8,250 rpm for 30 min and the supernatant aspirated off. The pellet was briefly respun and any remaining PEG/NaCl aspirated off. After resuspending the pellet in 2 ml PBS, it was spun at 4,500 rpm for 10 min to remove most of the remaining bacterial debris. The phage supernatant was stored at 4°C for short term storage or in PBS, 15 % glycerol for longer term storage at -80°C. This procedure usually yielded 1-5 x 10^{13} pfu.

2.2.11.7 Selection of phage library

The phage library was selected using sterile 96-well plates coated with MMP-9 protein. The wells (10 wells were used for each round of panning) were coated overnight with 100 μl/well of MMP-9 at 50μg/ml in sterile 50mM sodium hydrogen carbonate, pH 9.6 at RT. Next day the wells were washed 3 times with PBS and then blocked with 200μl 2% Marvel in PBS (MPBS) for 2 hr at 37°C. Wells were washed 3 times with PBS and 10^{12} to 10^{13} pfu phage in 2% MPBS added, incubated for 30 min at RT shaking gently and then left to stand for at least a further 90 min at RT. Unbound phage were then thrown away and the wells washed 20 times with PBST (PBS containing 0.1% Tween-20), then 20 times with PBS to remove the detergent. Each washing step was performed by pouring buffer in and immediately out. The bound phage were eluted by adding 100μl 0.1 M glycine-HCL, pH 2.5 per well and incubating for 10 min. Following incubation the contents of the wells were added to a universal containing 200μl 1 M Tris, pH 8.0, for quick neutralisation. The eluted phage were then added to 10 ml

of exponentially growing TG1, incubated for 30 min at 37° C in waterbath without shaking to allow for infection. 100μl of the infected culture was then taken to make 4-5 100-fold serial dilution's and plated onto TYE plates containing 100μg/ml ampicillin and 1% glucose and grown overnight at 37°C. The remaining infected TG1 culture was spun at 4,000 rpm for 12 min and the pelleted bacteria resuspended in 0.5 ml of 2xTY and plated on 2 TYE plates containing 100μg/ml ampicillin and 1% glucose. Plates were grown at 30°C overnight or until colonies were visible.

2.2.11.8 Further rounds of selection

The infected TG1 culture growing on TYE plates were loosened in 1 ml of 2xTY with a glass spreader and 50μl of the scraped bacteria inoculated into 50 ml of 2xTY containing 100μg/ml ampicillin and 1% glucose. The remaining bacteria were stored at -80°C. The bacteria were grown with shaking at 37°C until the OD_{600nm} is 0.5 and then used for further selection by : rescuing the phagmid as in section 2.2.14.5 and PEG precipitating as in section 2.2.14.6 (resuspending the second phage pellet in 2 ml PBS). 1 ml of this phage was stored at 4°C and the other 1 ml aliquot used for the next round of selection as in section 2.2.14.7 and 2.2.14.8. The selection was repeated for another 3 rounds (4 rounds in total).

2.2.11.9 Growth of a secondary source of the library

By keeping some of the material as a secondary stock there is an endless source of the library. 40 ml of exponentially growing bacterial library stock was spun down at 4,500 rpm for 10 min and the cells resuspended in as small a volume as possible of 2xTY (about 500μl). The cells were spread on 2 petri dishes of TYE containing 100μg/ml ampicillin and 1% glucose and grown overnight at 37°C. Next day 0.5 ml of 2xTY, 15% glycerol was added to each dish and the bacteria loosened with a glass spreader. This secondary stock of around 1×10^8 library bacteria per 50μl was stored in aliquots at -80°C.

2.2.11.10 Screening phage particles by ELISA

Populations of phage produced at each round of selection were screened for binding to MMP-9 by ELISA, to identify "polyclonal" phage antibodies. Phage from single infected bacterial colonies from these populations were then screened by ELISA to identify "monoclonal" phage antibodies. Monoclonal phage which were found to have binding activity to MMP-9 had their pHEN phage particles rescued and infected into HB2151 to give monoclonal soluble antibody fragments.

2.2.11.11 Polyclonal Phage ELISA

96-well ELISA plates were coated with 100μl per well of 15μg/ml MMP-9 protein in 50mM sodium hydrogen carbonate, pH 9.6 overnight at RT. The wells were rinsed 3 times with PBS and blocked with 200μl per well of 2% MPBS for 2 hr at 37°C. Wells were washed 3 times with PBS and 10μl PEG precipitated phage from the stored aliquot of phage from the end of each round of selection (about 10^{10}pfu) made up to 100μl with 2% MPBS. This was incubated for 90 min at RT, the test solution then discarded and wells washed 3 times with PBS-0.05 % Tween-20, then 3 times with PBS for 30 min. A 1/750 dilution of anti-fd bacteriophage antibody in 2% MPBS was added for 90 min at RT and again washed 3 times with PBS-0.05% Tween-20, then 3 times with PBS. A 1/5000 dilution of HRP labeled anti-rabbit antibody was then added for 90 min at RT in 2 % MPBS, again washed 3 times with PBS-0.05% Tween-20, then 3 times with PBS. The reaction was developed with substrate solution [one 5 mg OPDA tablet dissolved in 12.5 ml OPDA buffer (0.15M citrate buffer, pH 5.0) and 5μl 30% H_2O_2 (added immediately before use)] for 30 min at 37°C and the reaction stopped by adding 50μl 1M sulphuric acid. The OD was then read at 495nm.

2.2.11.12 Monoclonal phage ELISA

To identify monoclonal phage antibodies the pHEN phage particles were rescued. Individual colonies from the plates after the selection procedure of round 4 were inoculated into 100μl 2xTY containing 100μg/ml ampicillin and 1% glucose in 96-well plates and grown with shaking (250 rpm) overnight at 37°C. A small inoculum, (about 2μl) from this plate was transferred to a second 96-well plate containing 200 μl of 2xTY containing 100μg/ml ampicillin and 1% glucose per well and grown shaking

at 37°C for 3 hr. Glycerol stocks were made of the original 96-well plate, by adding glycerol to a final concentration of 15%, and then storing the plates at -80°C. To each well (of the second plate) 25μl 2xTY containing 100μg/ml ampicillin, 1% glucose and 10^9 pfu VCS M13 helper phage were added. The plate was incubated for 30 min at 37°C and then shaken for 1 hr at 37°C. The plate was spun at 3000 rpm for 10 min and the supernatant aspirated off. The pellets were resuspended in 200μl 2xTY containing 100μg/ml ampicillin and 50μg/ml kanamycin and grown overnight at 30°C. Next day the plate was spun at 3000rpm for 15 min and 100μl of the supernatant used in phage ELISA as described in section 2.2.11.11.

2.2.11.13 Production of soluble antibody fragments

The selected pHENs were then infected into HB2151 and induced to give soluble expression of antibody fragments. From each chosen clone 10μl of phage supernatant was infected into 200μl of exponentially growing HB2151 bacteria for 30 min at 37°C in waterbath. 1, 10, 100 μl, and 1:10 dilution of the culture was then plated on TYE containing 100μg/ml ampicillin and 1% glucose, incubated overnight at 37°C. Individual colonies from these plates were picked into 100μl 2xTY containing 100μg/ml ampicillin and 1% glucose in 96-well plates, grown with shaking (300 rpm) overnight at 37°C. (A glycerol stock was made of this plate, once it had been used to inoculate another plate, by adding glycerol to a final concentration of 15% and stored at -80°C). A small inocula (about 2 μl) was transferred from this plate to a second 96-well plate containing 200μl fresh 2xTY containing 100μg/ml ampicillin and 0.1% glucose per well. Plates were grown at 37°C, shaking until the OD_{600nm} is approximately 0.9, then 25μl 2xTY containing 100μg/ml ampicillin and 9 mM isopropyl β-D-thiogalactopyranoside (IPTG) (final concentration 1 mM IPTG) was added and cultures grown shaking at 30°C for a further 4 - 24 hr (depending on whether periplasmic or secreted antibody fragment desired). Next day the plates were spun at 3000 rpm for 15 min and 100μl of the supernatant (containing soluble scFv) used in ELISA following the ELISA protocol detailed in section 2.2.11.11, except using purified 9E10 antibody (which detects *c-myc*-tagged antibody fragments) at 1/400 dilution, followed by 1/2000 dilution of HRP-anti-mouse antibody.

2.2.11.14 Preparation of anti-*c-myc* antibody (9E10)

The hybridoma cell line 9E10 (ECACC No. 85102202) was passaged in culture medium until 500 ml of spent medium had been collected. The media was centrifuged at 2000 rpm for 10 min to remove any cell debris and stored at 4°C. After noting the volume of supernatant, it was placed on ice and stirred gently. Slowly and with stirring saturated ammonium sulfate (SAS) was added to final concentration of 40% and left stirring for 30 min. After centrifuging at 8000 rpm for 20 min the pellet was resuspended in 20 ml PBS and the 40% SAS cut repeated. The second pellet was then resuspended in 2 ml PBS, dialysised against PBS at 4°C for 48 hr with several changes of PBS. The dialysate was stored at 4°C and titered to find the optimum dilution for use of the antibody.

2.2.11.15 Midi-scale induction of soluble antibody fragments

Single colony of positive clone was grown in 100µl 2xTY containing 100µg/ml ampicillin and 1% glucose in 96-well plates at 37°C overnight with shaking. 50 µl was then inoculated into 5 ml 2xTY containing 100µg/ml ampicillin and 0.1% glucose and grown at 37°C, shaking until OD_{600nm} was 0.9. The cultures were then spun down at 3000 rpm for 10 min. The pellets were washed with sterile PBS and resuspended in 5 ml 2xTY containing 1 mM IPTG and 100 µg/ml ampicillin (no glucose). Cultured were grown at 30°C, shaking for 4 - 24 hr, spun at 8000 rpm for 15 min to remove bacteria and debris. After overnight induction's antibody fragments could be found secreted into supernatant, while shorter incubations would produce antibody in the periplasmic space.

2.2.11.16 Dot blot to detect soluble Ab in supernatants from clones positive by ELISA

Using Bio-Rad bio-dot blot apparatus 400µl of supernatant was blotted under vacuum onto nitrocellulose for 1 hr. The blot was then blocked for 1 hr in 2% MTBS at RT. The anti-*c-myc* antibody was then applied at 1/400 dilution in MTBST overnight at 4°C. The blot was washed 3 times for 10 min in TBST and then incubated with HRP-anti-mouse at 1/2000 dilution in TBST for 2 hr at RT. The blot was then washed 3 x 10 min in TBST and washed once in TBS. The blot was developed in DAB substrate (one 10mg tablet DAB dissolved in 15 ml of TBS plus 12µl H_2O_2, added immediately before use).

2.2.11.17 Titering of antibody fragments

96-well plate was coated at 10μg/ml MMP-9 in 50mM sodium hydrogen carbonate, pH 9.6 overnight at RT (100μl per well). Next day the plate was washed with PBS and blocked in 2% MPBS for 2 hr at 37°C. Plate was washed with PBS and 100μl two fold serial dilution's of bacterial supernatants were added to wells in final concentration of 2% MPBS for 2 hr at RT. Identical samples were analysed, except wells had not been coated with MMP-9. ELISA then continued as in section 2.2.11.11 except using purified 9E10 antibody (which detects *c-myc*-tagged antibody fragments) at 1/400 dilution, followed by 1/2000 dilution of HRP-anti-mouse antibody.

Chapter 3

Establishment of a Model System to Investigate the Role of MMPs in Trophoblast Invasion.

3. Introduction

The establishment of the morphologically and physiologically intimate contact between two genetically different individuals, mother and embryo, which takes place during implantation, has always exerted a fascination on researchers in biology and medicine. Historically, we owe it to Graf von Spcc who in 1883 introduced the concept that trophoblasts may play an active and cytolytic role in attachment and invasion. This was based on studies using histological analysis of implantation sites in guinea pigs (reviewed in Denker, 1978). At the turn of the century the possibility of the involvement of proteolytic enzymes of the trophoblast or endometrium was already under discussion. However, it wasn't until the early 1970s which saw a revival of interest in this area of research, that proteolytic activity was shown to be associated with trophoblasts (Denker, 1978). Developments during the 1970's and 80's in cell biology, particularly the introduction of *in vitro* cell culturing, lead to huge advances in this field. The mid 70's also saw an interest in the development of new concepts in contraception leading to renewed enthusiasm and funding for this area of research.

3. 1 Introduction to implantation and placentation

In a remarkable series of events, implantation and placental development physically connect the mammalian embryo to its mother. Trophoblast differentiation, which results in formation of the placenta, is one of the first critical hurdles the mammalian embyro faces. In mammals, the initial developmental decisions set aside three unique extraembryonic linages that are the precursors of the placenta. The first differentiation event gives rise to trophoblasts, the specialised epithelial cells of the placenta that physically connect the embryo and the uterus. The remaining cells segregate at one pole of the embryo to form the inner cell mass (ICM). Endodermal and mesodermal components of the placenta are later derivatives of the ICM. The differentiation of ICM cells that give rise to the embryo proper does not begin until the first placental structure has formed. Establishing this connection is the embryos first priority, which is essential for its subsequent development. Failures in implantation and placental development are clinically important. About one third of normal human pregnancies end in spontaneous abortion; 22% of such abortions occur before pregnancy is detected clinically (Wilcox *et al.*, 1988). Similarly,

failures in development during the peri-implantation period account for almost 80% of the embryonic loss that occurs in farm animal species (Cross *et al.*, 1991). Even seemingly minor defects in placentation can have severe negative consequences and errors in its regulation can result in pathological conditions which involve placental overinvasion, e.g. placental accreta (pre-malignant) and choriocarcinoma (malignant), or underinvasion e.g. pre-eclampsia. It is essential therefore to gain an understanding of the factors involved in regulating this process.

Placental development starts with the process of implantation which constitutes a series of events. The first stage involves the establishment of position of the blastocyst within the uterus, or attachment. This includes appositional and adhesional events in which the blastocyst first 'finds' its implantation site and then anchors itself to the surface of the epithelium. Once the blastocyst adheres to the uterus, the fetal trophoblast cells, derived from the outer cells of the blastocyst rapidly penetrate the endometrium. Soon thereafter, trophoblasts are found mingled with maternal decidual cells throughout much of the placental bed, a condition that persists during the remainder of the pregnancy. In addition groups of specialized trophoblasts migrate through the decidua, invade the walls of the spiral arterioles, and replace the endothelial lining of these vessels. This invasive activity peaks during the twelfth week of pregnancy and declines rapidly thereafter. The result is the formation of the human hemochorial placenta, in which blood from the maternal circulation constantly bathes the fetal chorionic villi.

Human fetal development depends on the embryo rapidly gaining access to the maternal circulation. This obstacle is overcome by trophoblast cells, which form the fetal portion of the human placenta, by transiently exhibiting an invasive phenotype. In humans, two differentiated trophoblast populations exist, syncytiotrophoblasts and cytotrophoblasts, giving rise to villus- and extravillus- trophoblast populations that are morphologically and functionally distinct (Cross *et al.*, 1994). Proliferative cytotrophoblast stem cells are anchored to basement membranes surrounding the stromal core of chorionic villi. There are two types of chorionic villi found at the maternal-fetal interface, floating and anchoring villi (Figure 3.1). The freely floating villi are constantly bathed by maternal blood, mediating nutrient, waste and gas exchange functions for the developing embryo, and are not directly connected to the uterus. The bulk of the placenta consists of floating chorionic villi. In floating villi the cytotrophoblast stem cells lie beneath the syncytiotrophoblastic covering and are separated from the underlying connective tissue stroma of the villus core, containing fetal

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blood vessels, by a basement membrane (Figure 3.1 A). Cytotrophoblasts in these villi exist exclusively as polarized epithelial monolayers. These cytotrophoblasts, which are highly proliferative in the first trimester of gestation, differentiate by fusing to form the syncytial layer that covers the villus and divide to maintain the complete cytotrophoblast layer present in first trimester chorionic villi. As pregnancy continues, fusing of these cells to form the trophoblast syncytium gradually depletcs this population of cytotrophoblasts cells, possibly because of a decrease in their rate of division. Thus, this layer is incomplete by the second trimester of pregnancy and composed of isolated, single cells at term.
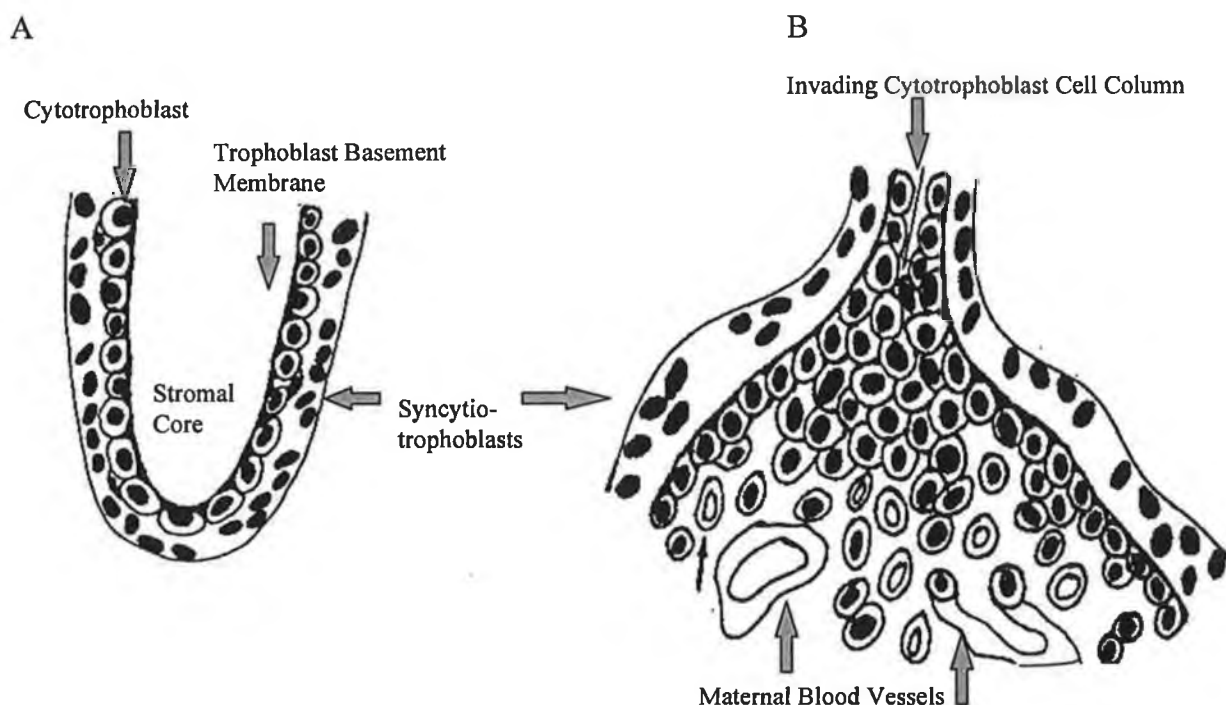
Figure 3.1 (A) Floating villi, cytotrophoblasts located in floating villi lie beneath the trophoblast syncytium and are not directly exposed to maternal blood. **(B)** Anchoring villi, cytotrophoblasts of the anchoring villi lie beneath the syncytium and extend through the endometrium to line the spiral arterioles, thus attaching the fetus to the uterus.

In contrast, anchoring villi are embedded in the uterus and physically attach the fetus to the mother. In anchoring villi, cytotrophoblasts also differentiate into a syncytium that covers most of their surface, but at discrete sites subpopulations of cytotrophoblasts leave the basement membrane, break through the syncytium and form columns of nonpolarized cells (Figure 3.1 B).

These columns give rise to the invasive subpopulation of cytotrophoblast that attaches to and invades the uterus and its arterial system. These cells which are also referred to as intermediate trophoblasts or extravillus trophoblasts, are found in the pregnant endometrium and the first third of the myometrium (collectively called the placental bed). The process of anchoring villus formation, and associated cytotrophoblast invasion of the uterine wall, is extremely active during first trimester, resulting in rapid placental expansion. Thus, human cytotrophoblast differentiation along the invasive pathway is highly unusual in that successful penetration of the uterine wall by cytotrophoblast requires that they transiently express an invasive phenotype similar to that displayed by malignant tumour cells.

Many elements of trophoblast invasion are similar to events that occur during tumour cell invasion. The phenotypic change from carcinoma *in situ* to invasive carcinoma occurs when tumour cells acquire the ability to penetrate an epithelial basement membrane and invade the underlying stroma (Liotta *et al.*, 1986). Likewise after a brief adherent stage, cytotrophoblast cells penetrate the basement membrane of the uterine epithelial cells and invade the stroma and its associated arterioles. These similarities suggest that the two invasive processes may share certain common enzymatic and cellular mechanisms. However, unlike tumour invasion, trophoblast invasion is precisely regulated, confined spatially to the endometrial aspect of the myometrium and continues only until midgestation.

At the interface of the villus and the cell column, the organisation of the cytotrophoblast changes, from a polarized cell layer anchored on a basement membrane to a multilayered column of coherent, nonpolarised cells (Figure 3.2). Where columns contact the uterine wall, they spread laterally and penetrate the endometrium. The column structure is then lost, and the cytotrophoblasts are present as clusters of irregularly shaped cells interspersed among maternal leukocytes and decidual cells of the endometrium. With deeper penetration the cytotrophoblast clusters consist of fewer cells, and single cells are frequently seen within the myometrium. A sub population of these extravillus trophoblasts erode the muscular coat and endothelial lining of uterine arterioles and line these vascular channels as endovascular trophoblast. This process prevents uterine arterioles from responding to maternal vasoactive substances, ensuring a steady supply of maternal blood to the placenta (Loke, 1990). Thus invasive cytotrophoblasts contribute to the hybrid vascular network that carries maternal blood to the floating villi.

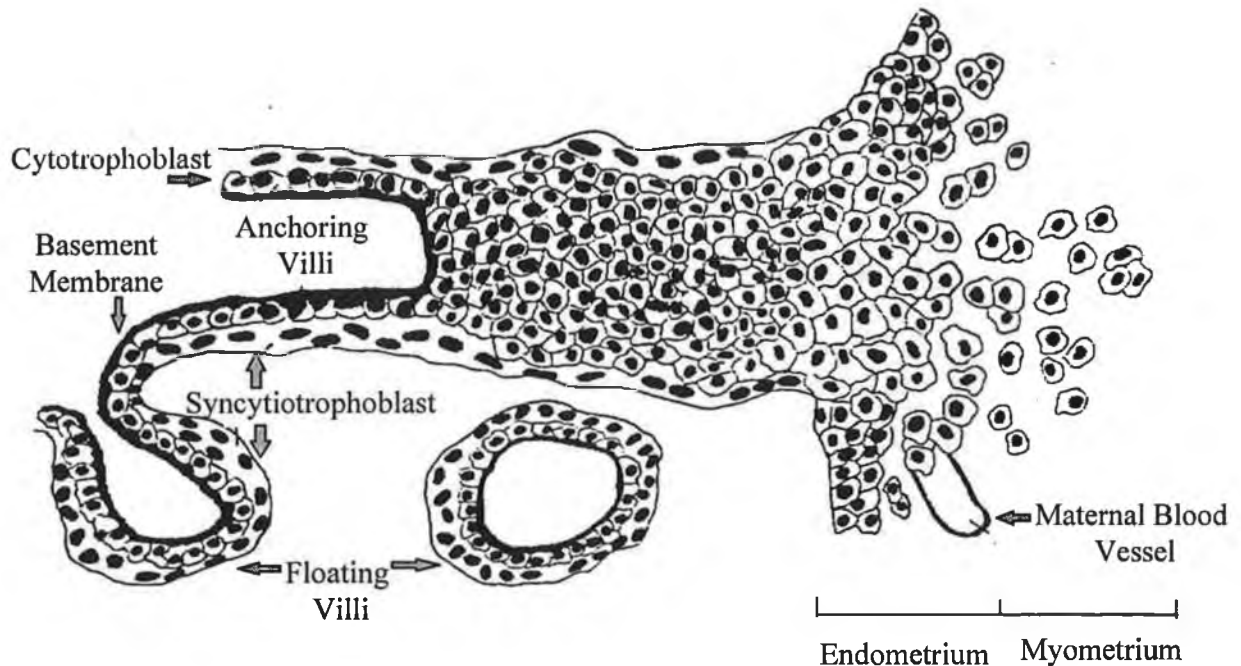Villus Trohpoblast   Extravillus Invasive Trophoblast

Cytotrophoblast

Basement
Membrane   Anchoring
                  Villi

Syncytiotrophoblast

Floating
Villi

Maternal Blood
Vessel

Endometrium   Myometrium

Figure 3.2 Diagram of the fetal-maternal interface in an early gestation placenta showing floating villi and an anchoring villus with an associated cell column, and the uterine wall. Extravillus cytotrophoblasts are invading maternal circulation.

The extent of placental invasion of the uterus differs in various mammalian orders. For instance, the epitheliochorial placenta of herbivorous mammals (e.g. horse, sheep) is a relatively non-invasive placenta in which the chorionic trophoblast remains closely apposed to the intact uterine epithelium. In contrast to this hemochorial placenta of humans and rodents is highly invasive. Trophoblast cells as described above breach the uterine epithelium and eventually invade maternal vessels to come into contact with maternal blood. Nevertheless, at least a single layer of trophoblast cells remains intervening between the maternal blood and the fetal blood vessels. There are important features of hemochorial placentas that are unique to human placentation. First there is generalised mixing of maternal and fetal cells in the placental bed such that no definitive boundary exists between the fetal trophoblast cells and the decidua. Second, human trophoblast invasion is extensive, reaching the first third of the myometrium (Ramsey *et al.*, 1976). These

differences exemplify the complexity and interspecies variability in solving the central problems of placental formation.

3.2 Culturing trophoblast cells *in vitro*

Many aspects of the invasive process cannot be studied by the examination of tissue biopsies as this approach does not allow access to the dynamic process, a prerequisite for studies designed to shed light on the functional consequences of the differential expression of certain molecules during invasion. Differences in placentation between species means that animal studies are of limited value in the interpretation of human placental function and ethical considerations severely limit *in vivo* studies in women. Hence the need to develop alternative *in vitro* methods. Cell culture models allow direct observation of the time-dependent processes of cellular motility, and their experimental manipulation. Cell culture methods also offer a reproducible experimental system able to sustain long terms studies and provide as many replicate samples as may be required. It is unfortunate that recovery of normal extravillous cytotrophoblast in quantities sufficient for experimental study poses technical problems. The availability of tissue from early gestation placentas was in the past very limited, however this has changed somewhat with the introduction and increase of legal abortions in most of Europe and the United States. Attempts to establish replicating cultures of normal placental cells in the past met with limited success due in part to short survival time of the trophoblastic cells in culture, overgrowth by stromal fibroblasts or failure of the cells to replicate. Most of these problems have been overcome with improvements in cell culture techniques.

A variety of techniques have been used to grow and maintain human first-trimester and term placental trophoblasts in culture (Morgan *et al.*, 1985; Kliman *et al.*, 1986; Yagel *et al.*, 1989a; Dodeur *et al.*, 1990). Most methods employ an enzymatic digestion of the placenta, usually undertaken before culturing of mixed cell populations. Alternatively outgrowths from plated chorionic villi fragments without enzymatic treatment have also been used. Kliman *et al.* (1986) reported contamination-free functional cytotrophoblast cells obtained by Percoll gradient centrifugation of enzymatically dispersed term placenta by sequential trypsin and DNase incubations. However, they did not attempt long-term maintenance of these cells by repeated

passage. The method developed by Klimen *et al.* (1986) is still the basis for most isolation methods from term or early gestation placentas. Some cultures have been reported to retain trophoblastic markers even after several passages *in vitro* (Yagel *et al.*, 1989a). Various modifications to the method of Kliman *et al.* (1986) have been made, including additional immunopurification steps to eliminate cells of lymphomyleoid origin which may contaminate the cultures (Bischof *et al.*, 1991). In studies investigating protease production, immunopurification is important as bone marrow derived cells are known to release proteinases. This prompted the development of a technique that unequivocally eliminates known sources of extratrophoblastic proteinases, such as monocytes, macrophages and other lymphomyleloid cells, using an antibody to leukocyte common antigen (LCA) (Bischof *et al.*, 1991, Librach *et al.*, 1991).

The most commonly used replicating human trophoblast cell lines have been derived from malignant trophoblast tumours (choriocarcinomas). Examples of choriocarcinoma cell lines include BeWo, JEG-3, and JAR. These cell lines have some similarity to trophoblasts, but because they are derived from tumours there are also critical differences. Normal trophoblast proliferation and invasion of the uterus are strictly regulated whereas choriocarcinomas are not.

There are a number of reports in the literature of long-term trophoblast cultures which have been immortalized by viral infection (Logan *et al.*, 1992, Graham *et al.*, 1993, Lewis *et al.*, 1996). In an attempt to produce cell lines derived from normal trophoblasts Logan *et al.* (1992) used a temperature-sensitive form of the SV-40 virus to transform primary early-gestation trophoblasts. The resulting cell lines were immortalised and mimicked the phenotype of invasive trophoblasts at both permissive and nonpermissive temperatures. Graham *et al.* (1993) reported comparable results when they carried out a similar experiment. While these transformed cell lines are useful, it is clear that none of the cell lines adequately mimics the phenotype of the normal invading human trophoblast cell.

For this purpose, we have characterised an *in vitro* model in which early-gestation human trophoblasts are growing as continuous cell lines. These cell lines were gifts from Dr. Kniss, Department of Obstetrics and Gynecology, Ohio State University College of Medicine, Ohio. While other investigators have used first-trimester placental tissue obtained from elective pregnancy termination for preparation of trophoblast cultures, Dr. Kniss's laboratory was the first to report the use of first-trimester tissue isolated from transabdominal chorionic villus sampling

(CVS) (Diss *et al.*, 1992). The impetus for establishing this culture model was the current moratorium on the use of any abortus material for research purposes in the state of Ohio. Since many CVS procedures for early cytogenetic analysis are performed at Ohio State University College of Medicine, it was possible to obtain a large bank of cultures for establishing continuous cell lines. All patients gave informed consent for the procedure. First-trimester chorionic villi were obtained from patients for medically indicated cytogenetic studies by CVS at 9 to 12 weeks gestation under ultrasonographic guidance. Chorionic villi were dissected free from surrounding maternal and nonvillus tissues, dissociated with collagenase and trypsin and transferred to tissue culture medium. Kniss *et al.* developed these continuous trophoblast cell lines as a model system to study the regulation of glucose and amino acid transport by insulinlike growth factors in the placenta (Diss *et al.*, 1992, Kniss *et al.*, 1994). Using immunocytochemical and biochemical criteria, they had shown that one representative cell line, ED_{77}, exhibited a trophoblastic phenotype. However, complete characterisation of all the cell lines had not been carried out. Because these cell lines were continuous cell lines and had been passaged many times we were keen to establish that the trophoblastic phenotype had been retained in continuous culture and the cells were suitable as a model system to study the mechanisms of trophoblast invasion *in vitro*. The dissimilarities between development *in utero* and in culture cannot be ignored - the hormonal and anatomic milieu provided by the maternal organism is absent in culture. Nevertheless we believe the study of trophoblast cells in continuous culture provides a useful model system for trophoblast invasion.

3.3 Mechanisms used by trophoblast cells to invade the uterus

Penetration of one or more basement membranes and the underlying connective tissue stroma is a prerequisite for local invasion as well as for metastasis by tumour cells. Similarly in pregnancy, erosion of the basement membrane of the uterine epithelium is essential for implantation. Invasion of the underlying stroma and uterine blood vessels, inclusive of their basement membranes, is also required for establishment of feto-maternal exchange in the hemochorial placenta. Thus, the ability to penetrate basement membranes serves as a reliable marker for cellular invasiveness.

A variety of *in vitro* invasion assays for cancer cells have been designed. These have employed numerous natural basement membrane-containing substrates as well as artificially-reconstituted basement membranes (Liotta *et al.*, 1986, Mignatti *et al.*, 1986). Invasion of the basement membrane depends on two simultaneous events: breakdown of the various basement membrane components and migration of the invasive cell (Liotta *et al.*, 1986).

3.3.1 Matrix Metalloproteinases

The hypothesis that trophoblast invasiveness is associated with proteolytic activity was first proposed more than 50 years ago. Experimental evidence was provided much later by Blandau, who showed that guinea pig trophoblasts were capable of digesting a dried gelatin film (Denker, 1978). A similar observation was also made for the rabbit blastocyst (Denker, 1978). Evidence for human trophoblasts did not come until 1985 when Fisher *et al.* observed morphological evidence of ECM degradation by human cytotrophoblast outgrowths from explants of chorionic villi isolated from first trimester but not second trimester placentas. This finding was later confirmed by Fisher *et al.* (1989) in a follow-up study using short-term cultures of fractionated enzyme-dispersed cytotrophoblast cells from first, second and third trimester human placentas. They showed that isolated first trimester but not second or third trimester human trophoblasts were able to degrade an ECM produced by a teratocarcinoma cell line. Degradation was assessed by the ability of the cells to form circular areas clear of matrix and by the spontaneous release of ^{3}H-labeled matrix components. They also showed, using substrate gels co-polymerised with gelatin, that cell extracts from first trimester trophoblasts presented a more complex pattern of gelatin-degrading enzymes than second or third trimester trophoblasts. Since all the enzymes could be inhibited by 1,10-phenanthroline and not by other protease inhibitors, this indicated that they were MMPs. These studies suggested that the invasive ability of human trophoblast is temporally regulated during pregnancy. Surprisingly, human choriocarcinoma cell lines BeWo and JAR exhibited no ECM-degrading activity in this study. Similar results were reported by Bischof *et al.* (1991) who showed that expression of gelatin-degrading enzymes was developmentally regulated using immunopurified cytotrophoblast cultures from early and late gestation placentas. The MMPs

described by Bischof corresponded to some of those described by Fisher, four MMPs of molecular weight 97, 94, 64, and 59 kDa. However neither report attempted to identify the enzymes. A more recent report by Fisher's research group (Librach *et al.*, 1991) reported the characterisation of the gelatin degrading bands by immunoblotting and identified MMP-2 and MMP-9 as the principle players. Non-invasive placental fibroblasts were also shown to produce MMP-2. This study demonstrated that MMP-9 is regulated in accordance with the invasive properties of the cells; i.e. its expression was highest in early gestation and greatly reduced at term. Such developmental regulation was not seen for MMP-2, with similar expression levels of MMP-2 throughout gestation. MMP inhibitors and a function perturbing antibody for MMP-9 completely inhibited cytotrophoblast invasion *in vitro* (Librach *et al.*, 1991) leading the authors to suggest that MMP-9 plays an integral role in trophoblast invasion.

However there have been some conflicting reports published. Autio-Harmainen *et al.* (1992) and Polette *et al.* (1994) have shown that *in vivo* invasive extravillus cytotrophoblasts express MMP-2 whereas neither the mRNA nor the enzyme was detectable in villous trophoblasts. This would suggest that MMP-2 may be contributing to the invasive phenotype. Another study by Shimonovitz *et al.* (1994) reported the *in vitro* developmental regulation of both MMP-2 and MMP-9 in human trophoblasts isolated from first and third trimester placentas. A larger decrease in MMP-9 activity from early to late gestation was reported but developmental regulation of MMP-2 was also seen, prompting the authors to suggest that both type IV collagenases are important in trophoblast invasion. These studies suggest that MMP-2 may also have a critical role in trophoblast invasion. A recent study by Hurskainen *et al.* (1998) localised MT1-MMP mRNA and protein to invasive extravillous trophoblasts in placental biopsies leading the authors to propose that MT1-MMP may have general importance during trophoblast invasion, acting alone or as a cell-surface activator of other proteinases, in particular MMP-2.

The question of what protects the uterus from destruction by an invasive placenta is intriguing. Current theories all point to the decidual tissue of the pregnant uterus as assuming the protective role. Graham and Lala (1991 and 1992) demonstrated that first trimester human decidual cells produce molecules that inhibit trophoblast invasiveness. The molecules responsible for this anti-invasive effect were identified as TGF-β and TIMP-1, since neutralizing antibodies against either of these molecules could prevent the anti-invasive activity. They also showed that the

mechanism by which TGF-β inhibits trophoblast invasiveness was by induction of TIMP in both the decidua and the trophoblasts (Graham and Lala, 1991 and 1992). The presence of TGF-β in first trimester trophoblast cultures also resulted in a decrease in MMP-9 activity. This TFG-β-mediated decrease in activity was accompanied by a significant increase in TIMP-1 mRNA levels (Graham and Lala, 1991). Thus the proteinases potentially responsible for invasion may also engender the restriction of implantation, creating an elegant self-regulating process analogous to those found in other complex biological systems.

3.3.2 Plasminogen activators

Another class of proteinase, urokinase-type plasminogen activator (uPA), has also been shown to be synthesised and secreted by human cytotrophoblasts (Queenan *et al.*, 1987). However, inhibition of uPA activity does not limit trophoblast invasion *in vitro*. Librach *et al.* (1991) tested the effect of exogenous plasminogen activator inhibitor (PAI) -1, PAI-2, and a polyclonal inhibitory antibody to uPA on cytotrophoblast invasion and showed invasion was decreased by no more than 40%. Moreover, embryos that lack either a functional uPA gene or the low-density lipoprotein receptor-related protein, which is required for uPA receptor internalization, implant normally (Carmeliet *et al.*, 1994). These results suggest that u-PA and plasmin play a role in cytotrophoblast invasion, but cannot totally account for this process. Since u-PA may have a role in the activation of MMPs, this suggests a link between the two classes of proteinases.

3.3.3 Integrin switching during trophoblast invasion

In addition to producing proteinases that degrade the ECM, trophoblasts also change their adhesive properties during invasion. The cells recognise their microenvironment through specific membrane receptors known as integrins, which mediate the interactions between cells and the glycoproteins of the extracellular matrix. Integrins are heterodimeric transmembrane glycoproteins composed of an α and a β subunit. Depending on the αβ combination, integrins will bind to one or another glycoprotein of the ECM. So far eight β and fourteen α subunits have been identified, forming a family of about 20 receptors e.g. α5/β1 binds to fibronectin, α6β1 to laminin. Integrin

receptors can be promiscuous, binding several ligands, and in addition, individual ligands may be recognised by more than one integrin (Burrows *et al.*, 1995).

Human trophoblasts undergo three major transitions in their expression of integrins and ECM components. This occurs *in vivo* as the cells leave their basement membrane. Human cytotrophoblast stem cells within the villi express α6β4 integrin, a receptor for epithelial laminin. As they leave the basement membrane, they down-regulate the α6β4 integrin and begin to express the α5β1 integrin, a fibronectin receptor, along with a fibronectin-rich pericellular ECM. Within the uterine wall, they produce α1β1 integrin, a receptor for laminin and type IV collagen (Damsky *et al.*, 1992a). The integrin switching that occurs *in vivo* is recapitulated when villous stem cells are cultured. Under these conditions, antibodies to laminin, type IV collagen, or α1β1 integrin inhibit cytotrophoblast invasiveness, which suggests that integrin interactions with these ligands promote uterine invasion. Conversely, antibodies to the α5β1 integrin strongly enhance invasion, and addition of its ligand, fibronectin, inhibits invasion. These results suggest that the interaction between α5β1 integrin and fibronectin primarily restrains cytotrophoblast invasiveness. The regulation of adhesion molecule expression therefore contributes to acquisition of an invasive phenotype by cytotrophoblasts.

The aim of the work presented in this chapter was to establish a model system to investigate the role of MMPs in early gestation trophoblast invasion. Unique continuous cell lines from first trimester trophoblasts are first characterised extensively with respect to their cellular origin and phenotype. Then, using this system we examine MMP and TIMP expression and identify the key role players in this process.

3.4 RESULTS

Characterisation of trophoblast cell lines

The trophoblast cell lines ED_{77}, ED_{31} and ED_{27} were established from CVS taken at 9-12 weeks gestation as outlined in section 2.2.1.1. ED_{77} had previously been characterised using limited epithelial and trophoblast specific markers (Diss *et al.*, 1992). The cell line has been in continuous culture since 1992 and it was not known if it remained positive for the same markers since then. The other two trophoblast cell lines ED_{31} and ED_{27} have been in continuous culture in this laboratory for the past 2 years. We have characterised these two cell lines, ED_{27} and ED_{31} and also a choriocarcinoma cell line BeWo and compared them to the ED_{77} cell line. These cell lines were characterised extensively with regard to a number of properties including cytokeratin and vimentin expression, hCG, hPL, invasive ability, as well as MMP and TIMP production. Figure 3.3(A) shows a photograph of the ED_{77} cell line in culture. A placental fibroblast cell line was also established and characterised.

3.4.1 Isolation of placental fibroblasts

Placental fibroblasts were isolated from a term placenta and cultured *in vitro* as described in section 2.2.1.6. The cells were cultured for approximately 10 passages before senescing. Figure 3.3(B) shows a photograph of the cells in culture, displaying the typical fibroblastic phenotype. The fibroblast cell line was a useful control as it was of placental, but not of trophoblastic origin.
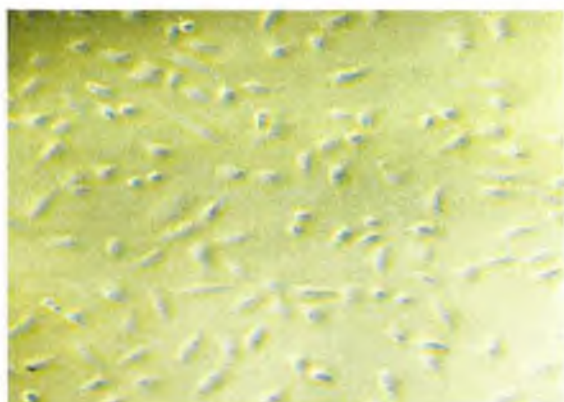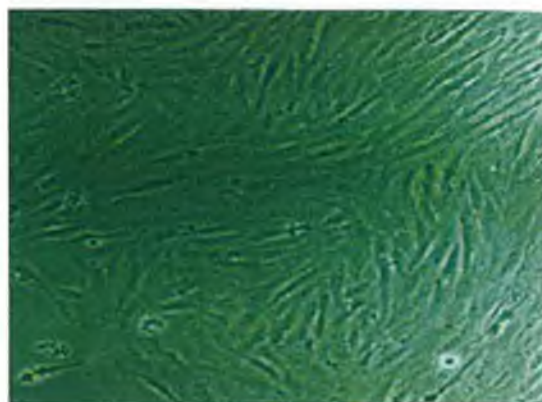
A   B

Figure 3.3 (A) Photograph of trophoblast cell line ED_{77}. **(B)** Photograph of placental fibroblasts isolated from term placenta.

3.4.2 Characterisation of trophoblast cell lines by immunocytochemical staining

The cells were characterised by immunocytochemical staining with numerous specific markers as outlined in section 2.2.2. These markers included antibodies to cytokeratin 8 and 18, vimentin, hCG, and hPL. All of the trophoblast cell lines stained positive for the low molecular weight cytokeratins 8 and 18. Figure. 3.4 shows a photograph of ED_{27} cells which is representative of the staining seen for cytokeratin 8 by all the trophoblast cells, indicating their epithelial phenotype. Cytokeratins are expressed by the epithelial, but not the stromal or vascular elements of tissues. Decidual cells also do not express cytokeratins. Thus cytokeratin staining can be used as a marker for cytotrophoblasts. All of the cell lines, with the exception of the placental fibroblasts were negative for vimentin, one of a class of intermediate filament proteins known to be present in endothelial cells and fibroblasts. This confirms the lack of contamination of the cell lines with these cell types.

Human trophoblast in the placenta elaborates at least two major protein hormones, hCG and hPL. hCG consists of two nonidentical subunits (α and β), and hPL is a single chain polypeptide, which shares greater than 90% homology with human growth hormone. Invasive trophoblasts have been shown to produce hCG (Librach *et al.*, 1991). Intermediate trophoblasts of the uterus stain brightly with anti-hPL antibodies (Pijnenborg, 1996). They are commonly used markers for trophoblast cells. All of the trophoblast cell lines were positive for these two markers indicating their trophoblast origin (Figure 3.5). The results for the characterisation of the trophoblast cell lines by immunocytochemistry are summarized in Table 3.1.

Table 3.I Characterization of trophoblast cell lines by immunocytochemistry.

| Marker	Specificity	ED$_{77}$	ED$_{31}$	ED$_{27}$	BeWo	Placental Fibroblasts
Cytokeratin 8	Epithelial cell marker	positive	positive	positive	positive	negative
Cytokeratin 18	Epithelial cell marker	positive	positive	positive	positive	negative
Vimentin	Mesenchymally-derived cell marker	negative	negative	negative	negative	positive
hCG	trophoblast	positive	positive	positive	positive	negative
hPL	trophoblast	positive	positive	positive	positive	negative
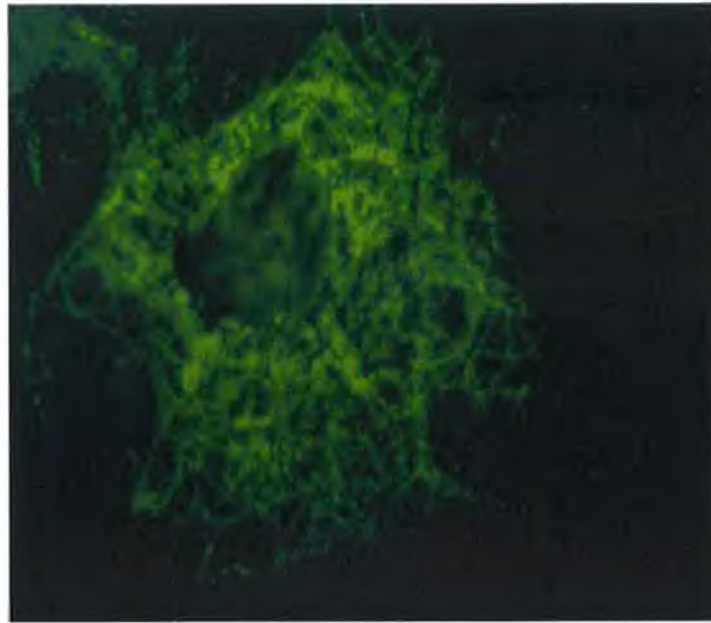
Figure 3.4. Characterisation of trophoblast cells. Immunofluorescence staining of ED_{27} cells with a cytokeratin 8 specific monoclonal antibody (100 X magnification).
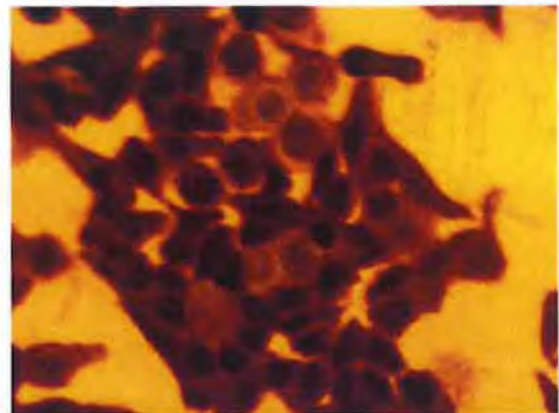
A
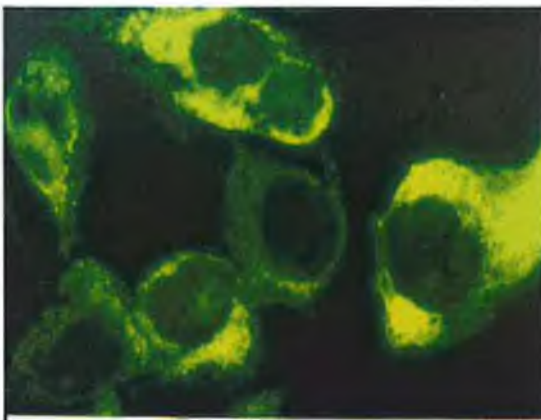
B

Figure 3.5 (A) BeWo cells stained positive for cytokeratin 8 (40X magnification). **(B)** ED_{77} cells stained positive for hCG (40X magnification).

3.4.3 *In vitro* invasion assay

An important biological property of trophoblasts is their ability to invade through extracellular matrices upon which they are plated. To examine this, we used an *in vitro* invasion assay in which the ability of trophoblasts to invade artificial extracellular matrix (Matrigel)-coated filters and emerge through 8μm pores on the filter underside was accessed (section 2.2.6). Following invasion of the matrix, the cells which normally exhibit an epithelial appearance became highly elongated with long processes that stretched through the pores and attached to the underside of the membrane (Figure 3.6). The ED_{77} cell line was found to be the most invasive of the three trophoblast cell lines examined. The ED_{31} and ED_{27} cell lines were also invasive. In contrast the BeWo cells did not invade to the underside of the membrane to any extent under the conditions used in this assay. The fibroblast cell line was also noninvasive. Figure 3.7 shows the percentage *in vitro* invasion for each of the cell lines.
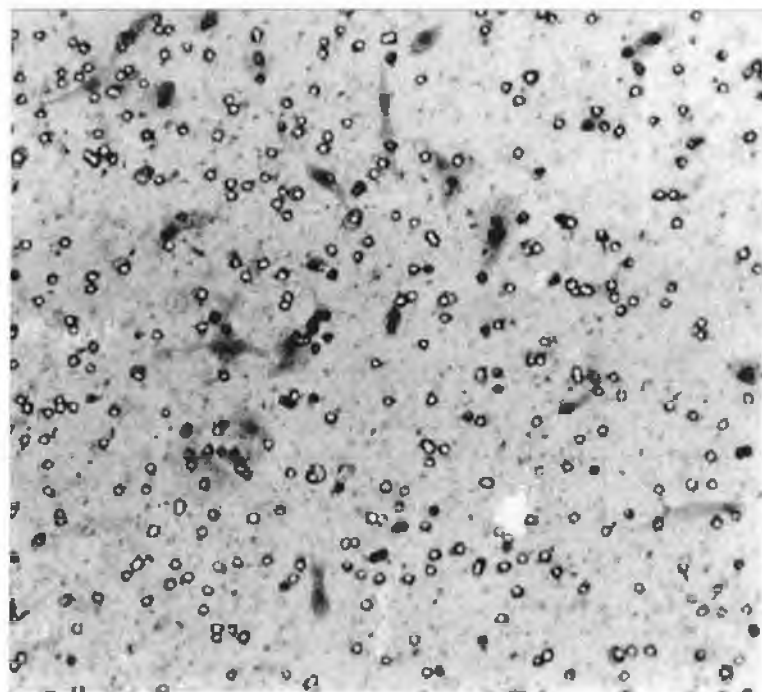

Figure 3.6 ED_{77} cells on underside of membrane following invasion down through MATRIGEL layer. Cells are elongated with processes that stretch through pores(40 X magnification).
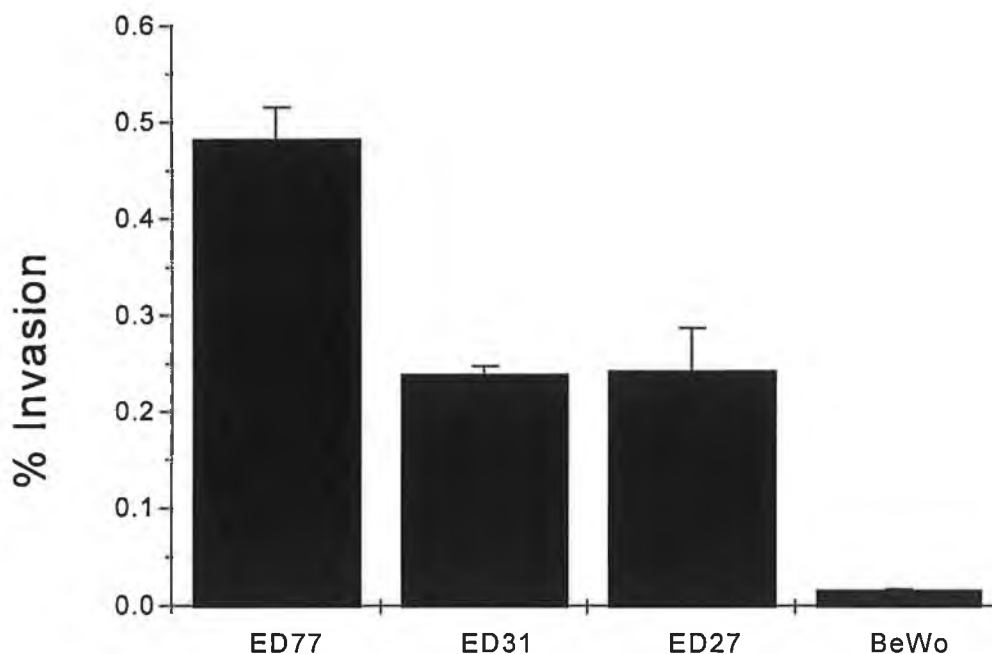
Figure 3.7 *In vitro* invasive behaviour of ED_{77}, ED_{31}, ED_{27} and BeWo cell lines. Values are means of three determinations; bars show standard errors.

3.4.4 Analysis of MMP expression by trophoblast cell lines

Following characterisation of the cell lines we decided to look at the expression of MMPs by substrate zymography in polyacrylamide gels containing either gelatin or casein (section 2.2.4.3). A representative zymogram for gelatinase activity is shown in Figure 3.8. This figure shows the pattern of MMP expression in the trophoblast cell lines, BeWo and, a cell line BHK92 which has been stably transfected with the human MMP-9 gene and constitutively expresses MMP-2. The trophoblast cell lines all show a similar pattern of MMP expression and appear to produce both MMP-2 and MMP-9. It appears that the ED_{77} cell line produced slightly more than the other two cell lines. In contrast the BeWo cell line produced large quantities of latent and active MMP-2 but negligible amounts of MMP-9. In all of the cell lines there appears to be a higher molecular weight band at approximately, 200 kDa, which is thought to be complexes of MMPs. These bands were confirmed as metalloproteinases by incubation of the gels in EDTA which inhibited their activity (Figure 3.9). The activity was not inhibited by either PMSF or pepstatin A, inhibitors of serine and aspartic proteinases, further demonstrating the bands as metalloproteinase (data not shown). RT-

PCR using specific MMP-2 and MMP-9 primers was also carried out (section 2.2.8) and confirmed the results from zymography as shown in Figures 3.10 and 3.11. As casein is specific for members of the stromelysin subgroup, zymography was also carried out using casein as the substrate in polyacrylamide gels (section 2.2.4.3), however no additional bands were seen. RT-PCR, which is a more sensitive detection method was also negative for MMP-7 (Figure 3.9) and MMP-3 (data not shown) confirming the results from casein zymography.

Figure 3.8 Zymographic analysis of serum-free culture medium from trophoblast cell lines ED_{77}, ED_{31}, ED_{27}, BeWo, and BHK 92 and standard molecular weight markers. MMP-2 and MMP-9 are indicated by arrows. Graph shows the relative amounts of MMP-2 and MMP-9 as determined by densitometry.
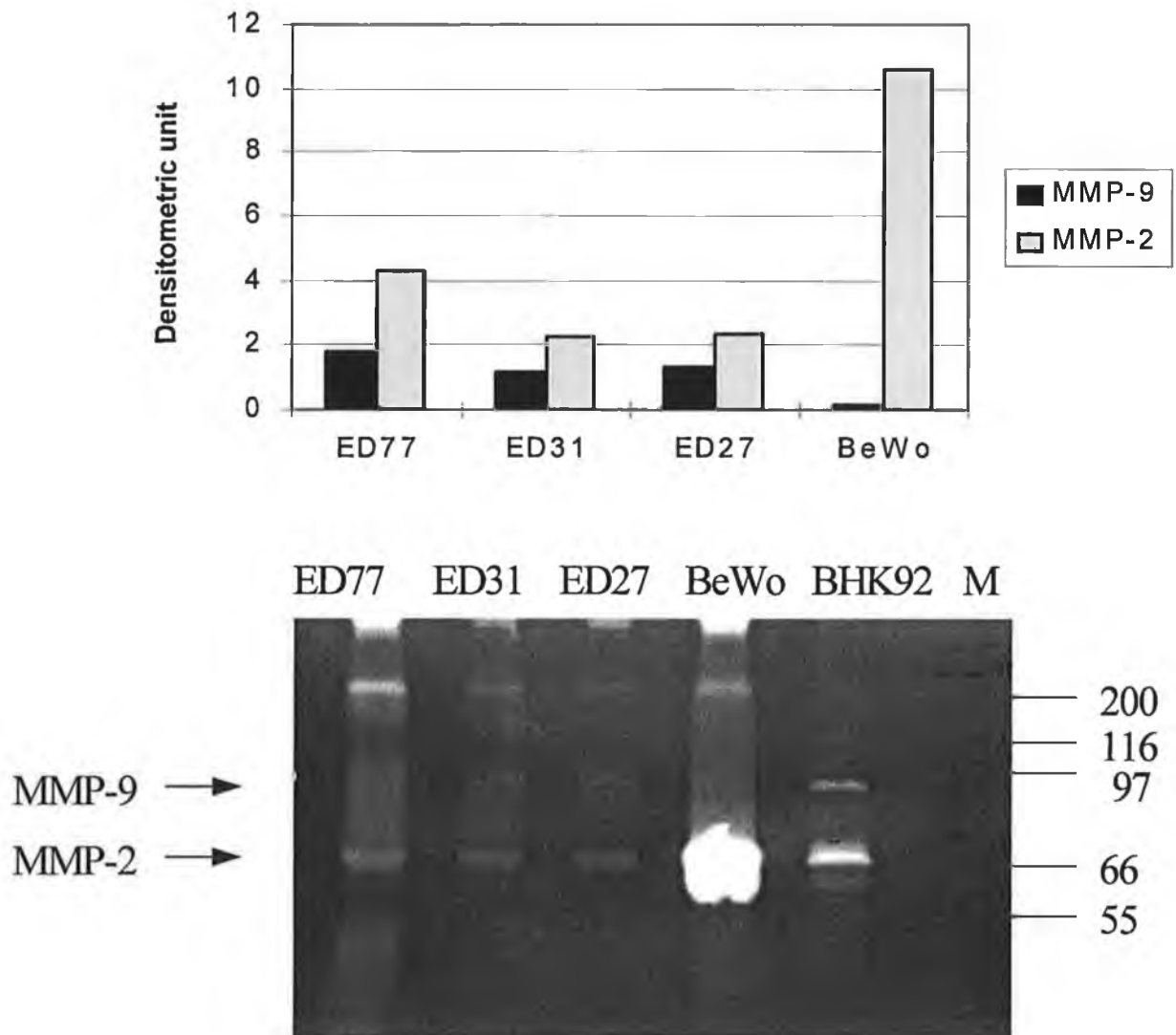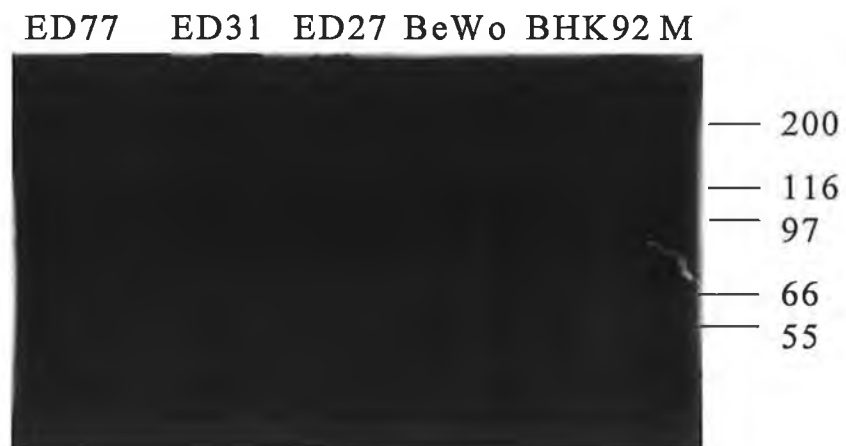
Figure 3.9 Zymographic analysis of serum-free culture medium from trophoblast cell lines ED_{77}, ED_{31}, ED_{27}, BeWo, and BHK 92 and standard molecular weight markers, incubated in EDTA.
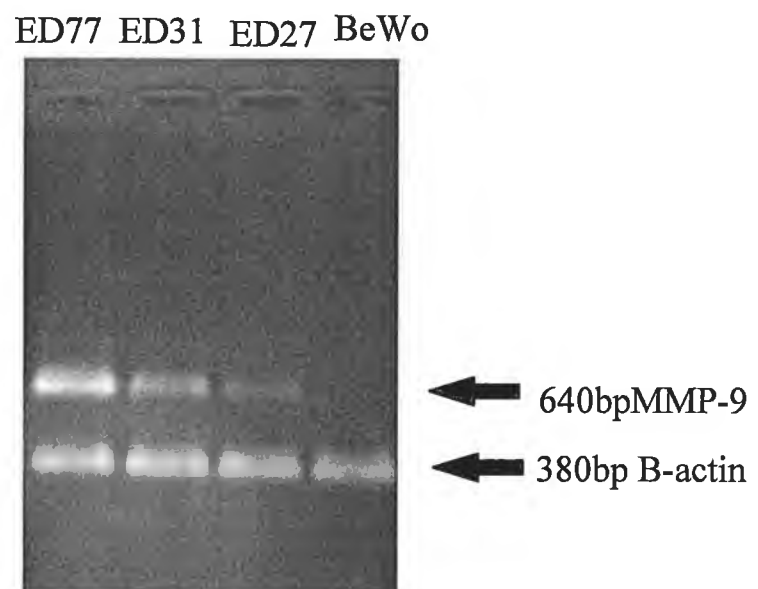
Figure 3.10 RT-PCR analysis of mRNA from trophoblast and BeWo cell lines using β-actin and MMP-9 specific primers

ED77 ED31 ED27 BeWo

⟵ 180bp MMP-2
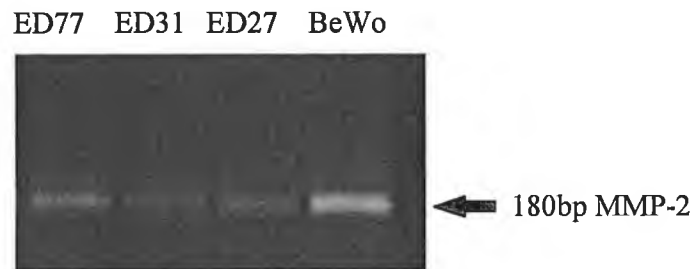
Figure 3.11 RT-PCR analysis of mRNA from trophoblast and BeWo cell lines using specific MMP-2 primers

1   2   3   4   5   6   7   8

⟵ 321bp MMP-7
⟵ 250bp B-actin

Figure 3.12 RT-PCR* analysis of mRNA from trophoblast and BeWo cell lines using β-actin and MMP-7 specific primers. Lanes as follows (1) ED_{77}, (2) ED_{31}, (3) ED_{27}, (4) BeWo, (5) 100bp ladder, (500bp most intense band), (6) positive control, RNA from SW620 cell line known to express MMP-7, (7) negative control, (RNA replaced with water), (8) plasmid containing cDNA for MMP-7 (pCMVMtlAct). *β-actin primers used in this experiment only were as follows: 5' CAACTTCATCCACGTTCACC 3', 3' GAAGAGCCAAGGACAGGTAC 5', giving a product size of 250bp (Takeshita *et al.*, 1994).

3.4.5 Analysis of TIMP expression by trophoblast cell lines

Having established that the trophoblast cell lines produced MMPs we decided to look at expression of their natural inhibitors, the TIMPs. The activity of the MMPs is physiologically regulated by the TIMPs and so the balance between the two is important. Metalloproteinase inhibitor activity in conditioned medium from trophoblast cells was examined by reverse zymography as outlined in section 2.2.4.4. The presence of TIMPs were seen as dark bands where inhibition of gelatin degradation had taken place, the majority of the gel does not stain as the gelatin has been degraded (Figure 3.13). TIMP-1 and TIMP-2 standards were also ran on the gel for reference. Trophoblast cell lines were found to express both TIMP-1 and TIMP-2 with the ED_{27} cell line producing the largest quantities. Interestingly, the BeWo cells were negative for TIMP-1 and -2 by this method. This was confirmed at the mRNA level by RT-PCR using primers for TIMP-1 (Figure 3.14). We also examined mRNA for TIMP-3 expression by RT-PCR as it is a much more sensitive method than reverse zymography. RT-PCR results confirmed that TIMP-3 is not expressed by any of the cell lines (data not shown).
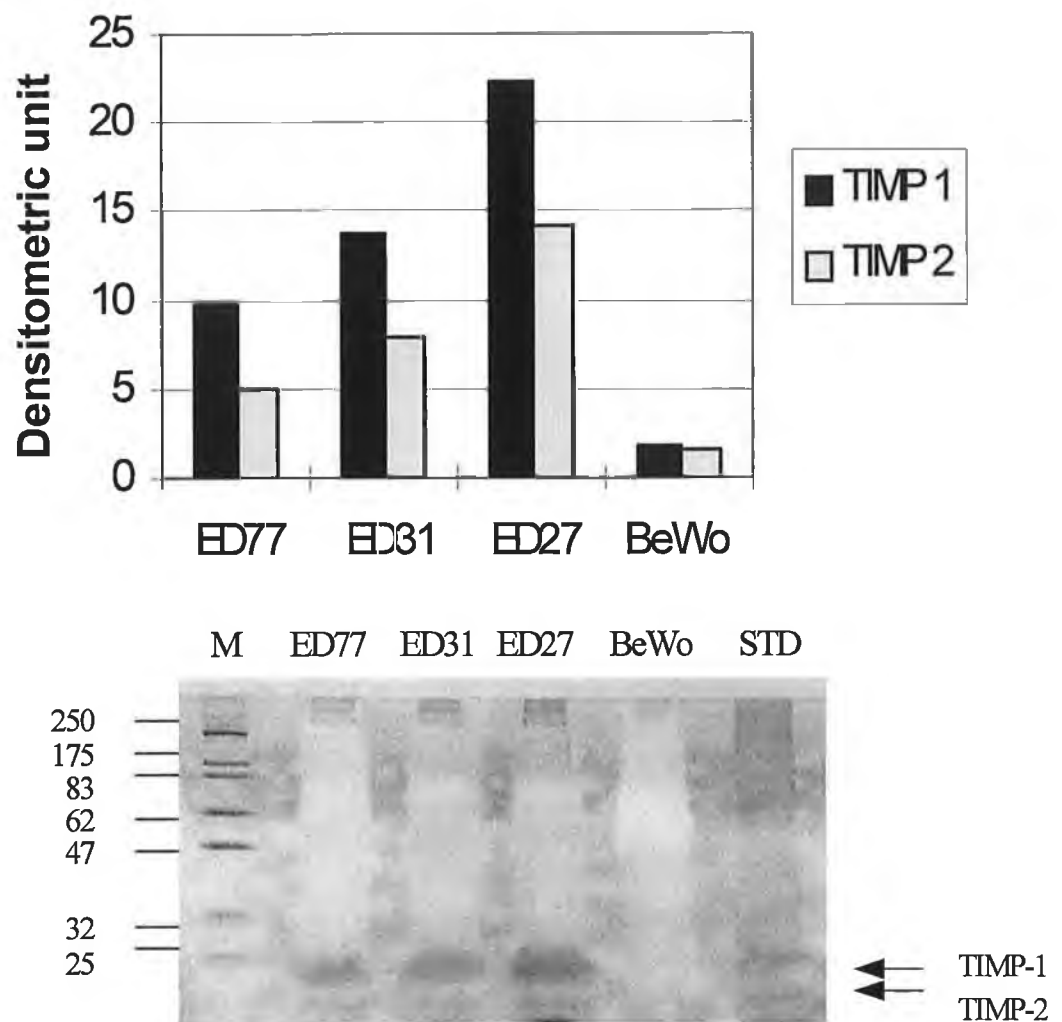
Figure 3.13 Reverse zymogram of serum free culture medium from trophoblast cell lines ED$_{77}$, ED$_{31}$, ED$_{27}$ and BeWo showing the presence of TIMP as dark bands. Gel also shows standard molecular weight markers and TIMP-1 and TIMP-2 standards. Graph shows the relative amounts of TIMP-1 and TIMP-2 as determined by densitometry.
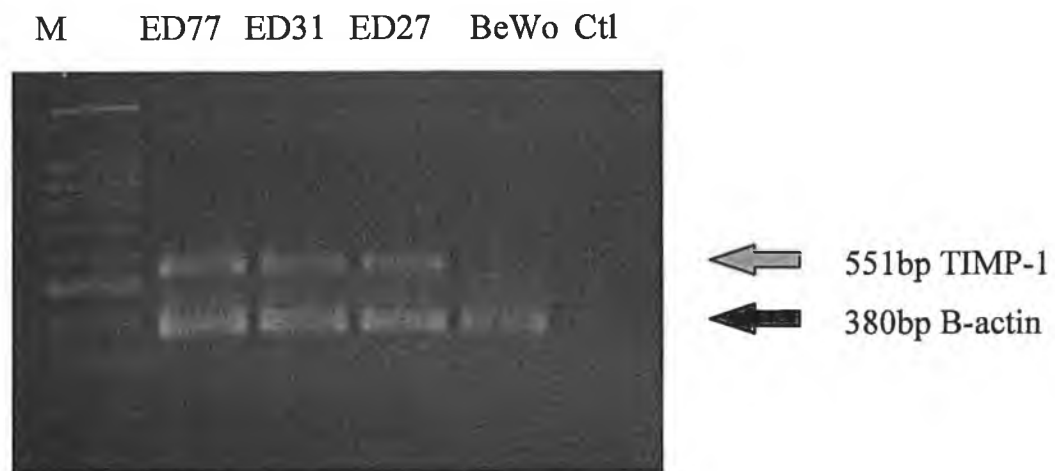
Figure 3.14 RT-PCR analysis of mRNA from trophoblast and BeWo cell lines using β-actin and TIMP-1 specific primers. Lane labeled M contains 100bp ladder, with intense band at 500bp. Lane labeled Ctl was negative control for RT-PCR reaction (RNA replaced with water).

Table 3.2 Summary of MMP and TIMP expression by the cell lines.

MMP/TIMP	ED_{77}	ED_{31}	ED_{27}	BeWo	Placental Fibroblasts
MMP-9	positive	positive	positive	negative	negative
MMP-2	positive	positive	positive	positive	positive
MMP-7	negative	negative	negative	negative	negative
MMP-3	negative	negative	negative	negative	negative
TIMP-1	positive	positive	positive	negative	ND
TIMP-2	positive	positive	positive	negative	ND
TIMP-3	negative	negative	negative	negative	ND

ND = not determined.

3.5 DISCUSSION

In this study we characterised three continuous trophoblast cell lines extensively and examined the expression of MMPs and their natural inhibitors the TIMPs in these unique cell lines. Kniss *et al.* had previously established these continuous cell lines of trophoblast cells derived from first trimester human chorionic villi but had not characterised them. Since these cells were a continuous cell line and had been passaged many times we were keen to establish that this trophoblast phenotype had been retained in continuous culture and were still suitable as a model system to study trophoblasts *in vitro*. We characterised the ED_{77}, ED_{31}, ED_{27} and BeWo cell lines. We found that the cell lines maintained their trophoblastic integrity even after many passages in culture, as indicated by the presence of cytoplasmic hCG and low molecular weight cytokeratins. Also none of the cell lines were positive for vimentin, thus excluding fibroblastic and endothelial contamination. The cells in culture were functionally active as demonstrated by hCG production and their *in vitro* invasive ability.

Using these cells which provided a reproducible *in vitro* system for investigating the biological basis of invasion of trophoblasts, our aim was to identify the specific members of the MMP and TIMP families which play a role in trophoblast invasion. We found that two members of the MMP family, the type IV collagenases - MMP-2 and MMP-9 were specifically expressed by the trophoblast cell lines. Casein zymography, which is specific for members of the stromelysin subgroup, showed no additional bands. RT-PCR, which is a more sensitive detection method was also negative for MMP-7 and MMP-3 confirming the results from zymography. The choriocarcinoma cell line, BeWo produced MMP-2 in larger quantities than the trophoblasts but negligible amounts of MMP-9. We also found the BeWo cells to be non invasive using the culture conditions and assay described. This was surprising, but is in agreement with a previous study where the cells ability to degrade matrix substrates was tested and it was found that the degradative ability of the choriocarcinoma cell lines was comparable with that of first trimester placental fibroblasts. No profile of MMP expression was shown for the choriocarcinomas in this paper but the placental fibroblasts were shown to produce only MMP-2 (Fisher *et al.*, 1989). Since the BeWo cell line was derived from a choriocarcinoma it may give rise to the expectation that its invasive behaviour would be more pronounced than that of normal cytotrophoblasts; despite this, no invasion occurred *in vitro*. Similar findings have been reported for other choriocarcinoma cell lines

(Schor, 1980, Fisher *et al.*, 1989). It is possible that factors required for invasion by the BeWo cells from the hormonal milieu provided by maternal cells are not present *in vitro,* or perhaps long term culture of the BeWo cell line has selected a non-invasive phenotype. Similarly the placental fibroblasts described earlier were found to produce only MMP-2 and were non-invasive. The trophoblast cell lines were capable of invasion and it is likely that the expression of MMP-9 is critical for this invasiveness. ED_{77} was shown to produce the highest levels of MMP-9 and was also found to be the most invasive cell line. This was in agreement with previous studies that have shown MMP-9 to be produced by trophoblasts in a developmentally regulated manner and paralleling invasive ability (Librach *et al.*, 1991, Fisher *et al.*, 1989).

The absence of other members of the MMP family besides the two type IV collagens is interesting but perhaps not surprising since type IV collagen is abundant in pre-implantation endometrium stroma, and is present in the walls of blood vessels (Aplin *et al.*, 1988). Type IV collagen microfibrils have been suggested to play a role in the cross-linking banded fibrils of the major collagens and Aplin *et al.* (1988) speculated that its selective loss might allow local expansion of intercellular spaces while retaining the tissue stability afforded by the major collagens. This might in turn give rise to a matrix environment conducive to interstitial migration of trophoblast.

The activity of the MMPs is physiologically regulated by the TIMPs which bind non-covalently to active metalloproteinase and inhibit their activity. Little is known about their role in trophoblast invasion and there are only two other reports of TIMP expression by trophoblast cells in culture (Graham and Lala, 1992, Bass *et al.*, 1997). Graham and Lala (1992) showed indirectly that trophoblasts produced TIMP-1 since a neutralising antibody to TIMP-1 slightly stimulated invasion. However they did not attempt to characterise the inhibitors produced by the cells. A recent study by Bass *et al.* (1997) reported the expression of TIMP-3 by trophoblasts in culture, and showed the trophoblasts secreted negligible amounts of TIMP-1 and TIMP-2. Graham and Lala (1991) demonstrated that first trimester human decidual cells produced TIMP-1 and TGF-β which inhibited trophoblast invasiveness since neutralising antibodies against either of these molecules could prevent the anti-invasive activity. Another study showed that addition of recombinant TIMP-1 and TIMP-2 could completely inhibit cytotrophoblast invasion *in vitro* (Librach *et al.*, 1991). For the most part, it has previously been assumed that most metalloproteinase inhibitors that function

in the placental bed were probably of maternal origin. However, based on the results presented here we propose that TIMP produced by the trophoblast cells themselves may have a role in local regulation of ECM degradation. In this study the trophoblast lines were found to express TIMP-1 and TIMP-2, but not TIMP-3. Given that TIMP-1 has been shown to form a complex with the inactive proform of MMP-9 (Docherty *et al.*, 1985), while TIMP-2 forms a complex specifically with the proform of MMP-2 (Stetler-Stevenson *et al.*, 1990b), the expression of these two inhibitors is perhaps an in-built auto-regulatory system by trophoblasts. Supporting our hypothesis, several recent *in vivo* immunohistochemical studies of the distribution of TIMP in the human placenta reported staining for TIMP-1 and TIMP-2 in extravillous trophoblasts only during early gestation (Polette *et al.*, 1994; Ruck *et al.*, 1996). Another possibility is that TIMP may be acting as an autocrine growth-promoting factor for the trophoblasts, as has been shown in other systems (Hayakawa *et al.*, 1992). The BeWo cell line did not express any TIMPs. A previous study examining TIMP expression in choriocarcinoma cell lines JEG-3 and JAR, also reported very low levels of TIMP-1 message (Graham and Lala, 1991). Low level TIMP expression may possibly be a feature associated with the loss of regulation inherent with choriocarcinoma. The low level of TIMP expression by the BeWo cells conferred no advantage for invasive potential as the cells were unable to invade the matrigel. This again suggests that MMP-2 expression alone, even in the absence of inhibitors, is not sufficient for an invasive phenotype using the conditions described.

3.6 SUMMARY

Establishment of long term cultures of pure first-trimester human trophoblast cells with functional integrity should provide reproducible *in vitro* systems for investigating the biological basis of invasive behaviour by cells. This study was designed to achieve this objective. We have shown that long term replicating trophoblast cultures were free of contamination from other cell types, as judged by numerous cell-specific markers. Moreover, these long term monolayer cultures were proven to be functionally active since, they produced a significant amount of hCG and hPL, and were shown to be invasive in an *in vitro* invasion assay. These cell lines are unique as they are the first reported trophoblast cell lines to be established from CVS and we have demonstrated that cell lines established from CVS do provide a useful *in vitro* system for studies of placental function. This opens up new avenues for researchers in countries where abortions are illegal or the use of abortus material is prohibited.

We found that two members of the MMP family, the type IV collagenases - MMP-2 and MMP-9 were expressed by the trophoblast cell lines. The choriocarcinoma cell line, BeWo produced MMP-2 in larger quantities but little MMP-9. We also found that the BeWo cells were non-invasive under the culture conditions and assay used. The trophoblast cell lines were capable of invasion and it is likely that the expression of MMP-9 is critical for this invasiveness. We also reported the expression of TIMP-1 and TIMP-2 by the trophoblast cell lines and no TIMP expression by the BeWo cell line.

We propose that in the model system described here MMP-9 is critical for invasion. Identifying the autocrine and/or paracrine mechanisms that control the expression of MMP-9 will be the next key step in advancing our understanding of the unusual differentiation pathway that results in cytotrophoblast invasion of specific portions of the uterus during the critical early phases of pregnancy.

Chapter 4

Modulation of MMP Expression in Trophoblast Cell Lines.

4.1 Introduction

For a specific protein to be produced by a cell, the gene that encodes it must first be transcribed from DNA to mRNA. This mRNA then travels from the cell nucleus to the cytoplasm where it acts as a template from which the protein can be generated. The act of transcription is subject to regulation by factors within the nucleus. These transcription factors are in turn controlled by second messengers, for example cAMP or protein kinase C, changes in the activity of which result from cell signaling events which may occur when a cytokine/hormone/integrin binds to its receptor on the cell surface. To understand how any particular gene is regulated one must first examine its promoter.

4.2 The MMP Promoters

The promoters which control the transcription of protein-coding genes are composed of discrete functional modules, each consisting of approximately 7-20 bp DNA and containing one or more recognition sites for transcriptional activator proteins (Dynan, 1989). The MMP promoters show remarkable similarities with respect to oncoprotein-binding sites (a comparison of the promoter sequences of some MMP genes is shown in Figure 4.1). For example, the promoters of the human MMP genes, with the exception of MMP-2 and MMP-11, each contain a TPA responsive element (TRE), which is a binding site for the Fos/Jun-containing complex, also known as AP-1 (Crawford and Matrisian, 1996). The AP-1 site is critical for responsiveness to a wide array of signals. Mutagenesis studies have shown that the AP-1 from many MMP promoters is required for transcription in response to IL-1 (Fini *et al.*, 1994), TNF-α and TPA (Sato and Seiki, 1993), and fibronectin induction (Tremble *et al.*, 1995), most of which have also been shown to induce AP-1 activity (Angel *et al.*, 1991). Antisense *c-fos* RNA and oligonucleotides inhibit EGF stimulation of MMP-3 transcription (McDonnell *et al.*, 1990) and non-nuclear oncoprotein induction of MMP-1 (Schonthal *et al.*, 1988). Furthermore, a dominant negative *c-jun* mutant, which should activate all AP-1 complexes, inhibited TPA-induced MMP-3 expression in keratinocyte cell lines (Domann *et al.*, 1994). Therefore AP-1 activity appears to be necessary for MMP transcription under a variety of conditions.

All of the MMP promoters aside from that for MMP-2 also contain multiple polyoma virus enhancer (PEA3) sites which bind members of the E-twenty-six (Ets) family of nuclear proto-oncogenes. The PEA3 sites of MMP-1 and MMP-3 promoters have been shown to be important for oncogene and TPA-induced transcription (Wasylyk *et al.*, 1991). The existence of a TGF-β inhibitory element (TIE) has been demonstrated for a number of MMP promoters (Kerr *et al.*, 1990). Binding of the TIE appears to repress stimulatory signals supplied by other transcription factors for MMP-1 and MMP-3. The exact function of the TIE in MMP-9 is not clear (Sato and Seiki, 1993). The c-Fos protein has been demonstrated to be one of the components binding to the DNA binding element associated with the TIE.

All of the MMP promoters except MMP-2 contain a conserved sequence known as the TATA box (consensus sequence TATAAAA), located 20-30 bp upstream of the transcription start site (+1). Genes without TATA boxes can have variable transcription start sites. MMP-2 although lacking a TATA box appears to have two such transcription start sites, 9 bp apart (Huhtala *et al.*, 1990). The lack of a TATA box is often associated with so-called 'housekeeping' or constitutively-expressed genes and the MMP-2 protein is, perhaps, the most widely expressed of the MMPs (Crawford and Matrisian, 1995). The MMP-2 promoter does have some regulatory elements; there are two GC (consensus sequence GGGCGG) boxes (Huhtala *et al.*, 1990). The AP-2 protein appears to mediate transcriptional activation through the second messengers protein kinase C and cAMP-dependent protein kinase A (Imagawa *et al.*, 1987).

Despite these general similarities, the MMP promoters differ in their overall arrangement of onco-protein-binding elements. Human MMP-9, MMP-1, MMP-3 and MMP-10 promoters each contain two AP-1-binding sites, the first AP-1 is approximately 30 bp upstream of the TATA box and a second is located at various points upstream (reviewed in Crawford and Matrisian, 1996). Within the MMP-9 promoter, the upstream AP-1 site is the consensus sequence (TGAGTCA), whereas in the MMP-3 and MMP-1 promoters, the upstream AP-1 site is the nonconsensus sequence, TTAATCA (Sato and Seiki, 1993). The MMP-9 promoter also contains three nuclear factor-κB (NFκB) binding sites.

The MMP-9 promoter also differs in that the AP-1, which can be sufficiently activated by c-Jun/c-Fos heterodimer, is not itself sufficient for induction of the gene by TPA or TNF-α. Further upstream elements, e.g. NFκB, in addition to the AP-1 are required for induction of MMP-9 (Sato

and Seiki, 1993). Thus the transcription of MMP-9 is regulated by a combination of AP-1 proteins with NFκB-like factors, the binding sites for which are not present in the promoter regions of the other MMP genes examined to date. This regulatory mechanism is thus thought to control the expression of MMP-9 gene differently from the mechanism which controls the other MMP genes which share common stimulants for expression.
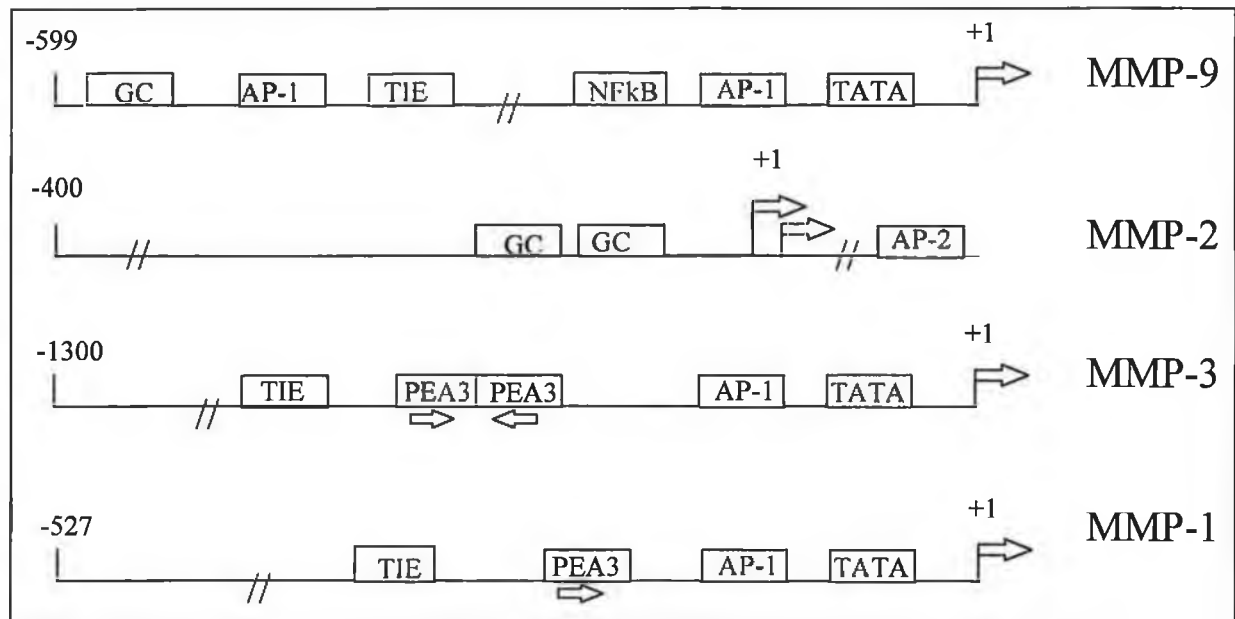
Figure 4.1 A comparison of regulatory elements found in the promoters of MMP genes. Transcription start sites are indicated by +1. TATA boxes, AP-1 binding sites, PEA3 elements, AP-2 recognition sites, GC boxes, NFκB-binding site, and TIEs have been indicated. The orientation of the PEA3 elements relative to transcription are indicated by arrows.

Transcription of MMP genes is associated with sites of tissue remodeling and cellular invasion in both normal and neoplastic tissues. The binding sites for the nuclear proto-oncoprotein families, Fos, Jun, and Ets, found in most MMP promoters, offers a logical connection between MMP transcription and tumour expression. However these cis-elements and trans-acting factors also have been shown to be important for MMP expression in normal tissues in response to a number of stimuli. For example, c-fos transcripts have been examined during mouse development and found in osteogenic tissues at a time coincident with MMP-1 expression (McCabe et al.,

1995). Also, *c-fos, jun B* and *jun D* are induced at wound sites, and in luminal and glandular epithelia of ovariectomized rats after treatment with estrogen (reviewed in Crawford and Matrisian, 1996). In short, nuclear proto-oncoprotein expression coincides with known sites of normal MMP expression.

Most of the potent stimulators of MMP-1 and MMP-3 gene expression, for example TPA, IL-1, or TNF-α, induce the expression of members of the Jun and Fos families (Schonthal *et al.*, 1988, Brenner *et al.*, 1989). The induction of MMP gene expression by these cytokines is prevented by treatment of the cells with cyclohexamide, demonstrating the requirement for ongoing protein synthesis. Furthermore, the induction is down-regulated by glucocorticoids and retinoic acid, which have been shown to block binding to the AP-1 binding site by as yet unclear mechanisms (Nicholson *et al.*, 1990).

4.3 Regulation of MMPs by Cytokines

In the adult body, MMPs are expressed at low levels in the absence of inflammation, wounding or other pathological processes. Transcription of MMP genes is induced or upregulated by stimuli such as IL-1, TNF-α, TGF-α/EGF and PDGF, and with some exceptions repressed by TGF-β, IFN-γ and glucocorticoids. The transcriptional effects of cytokines are mediated through complex signaling pathways. Many cytokine effects converge at the AP-1 site through the transcription factors *c-jun* and *c-fos* (Birkedal-Hansen, 1993). Several cytokine transcriptional effects appear to be mediated in part through protein kinase C-dependent signaling pathways as was described by McDonnell *et al.* (1990) for EGF stimulation of MMP-3 mRNA in rat fibroblasts, but the detailed mechanism is still not completely understood. Induction of the transcription factors *c-jun* and *c-fos* is a relatively early event and maximal transcript levels are observed within half an hour after stimulation. By comparison, emergence of MMP transcripts requires considerably longer, in the range of 3-8 hours. Even longer (14-20 hours) is required for emergence of the protein (Birkedal-Hansen, 1993).

Different cytokines induce overlapping yet distinct repertoires of MMP and TIMPs in cells, and different cell types respond to the same cytokines by expression of unique and distinct combinations of MMP and TIMP genes. For example, IL-1β induces expression of MMP-1 and

MMP-3 in human fibroblasts but not in foreskin keratinocytes (MacNaul *et al.*, 1990, Petersen *et al.*, 1987); MMP-9 is repressed by TGF-β in fibroblasts (Kerr *et al.*, 1988) but stimulated in keratinocytes (Salo *et al.*, 1991); human rheumatoid synovial fibroblasts respond to IL-1β by induction of MMP-3, whereas TNF-α induces primarily MMP-1 (MacNaul *et al.*, 1990); TNF-α, TGF-α and EGF as well as TPA induce high level expression of MMP-3 in fibroblasts but MMP-10 in keratinocytes (Birkedal-Hansen *et al.*, 1993). This demonstrates that the response to stimuli depends on multiple factors including, cell type, species of origin and conditions of growth, and although some general observations can be made, the potential effect of a cytokine on MMP expression in a particular cell type cannot be confidently predicted.

Mackay *et al.* (1992) investigated the effects of IL-1 and TNF-α as well as TPA on MMP expression in a wide range of cell lines. MMP-3, MMP-9 and TIMP-1 stimulation in response to IL-1 or TNF-α treatment was observed in many human tumour cell lines and in normal HUVECs. TPA produced the greatest stimulation, as expected. In a human lung carcinoma cell line TNF-α stimulated MMP-9 but not TIMP-1 (Mackay *et al.*, 1992). Also treatment of human fibroblasts with TGF-β resulted in an elevation in TIMP mRNA and protein and a selective reduction in pro-MMP-1 protein (Overall *et al.*, 1989). This type of disco-ordinate regulation is thought to cause the imbalance in proteinase and inhibitor activities which favours ECM degradation by invasive cells. The MMP-2 gene did not appear to respond to cytokine or tumour promoter stimulation, even in cell lines that responded to tumour promoters with increased production of MMP-1 and MMP-3 (Collier *et al.*, 1988b). The effect of EGF on MMPs has been studied quite extensively and has been shown to be stimulatory for a number of MMPs in various cell types. Oesphageal cell lines have been shown to respond to EGF treatment by upregulating expression of MMP-1, MMP-3, MMP-2 and MMP-9 (Shima *et al.*, 1993)

Negative regulators of MMP gene expression have also been described. TGF-β has an inhibitory effect on growth factor- and oncogene- induced rat MMP-3, which is mediated at the transcriptional level (Matrisian *et al.*, 1986, Kerr *et al.*, 1988). TGF-β also represses proMMP-1 levels (Overall *et al.*, 1989) and growth factor-induced MMP-1 and MMP-3 mRNA while in the same cells it also interacts co-operatively with EGF and bFGF to superinduce TIMP expression (Edwards *et al.*, 1987). Although Chua *et al.* (1985) reported that TGF-β induced increases in MMP-1 secreted by human foreskin fibroblasts. Thus these studies show that the MMP genes can

be modulated by cytokines, although this regulation differs between cell types. However, little research has focused on the effects of cytokines on MMP expression in trophoblast cells.

The following is a brief description of the cytokines used in the course of the investigations of trophoblast cells described later in this chapter. These cytokines were chosen for study because they have been reported to be involved in placental development, however their effects on MMP expression in trophoblast cell lines has not been established.

4.3.1. Interleukin-1β

Interleukin-1 (IL-1) is the term for two polypeptides (IL-1α and IL-1β) that possess a wide spectrum of inflammatory, metabolic, physiological, haematopoietic and immunological activities. Although the two forms of IL-1 are distinct gene products, they recognise the same receptor and share biological properties. IL-1 belongs to a group of cytokines with overlapping biological properties, including TNF-α and IL-6. Known as the pro inflammatory cytokines, they share the ability to stimulate T and B lymphocytes, to augment cell proliferation and to initiate or suppress gene expression for several proteins. The two forms of IL-1 are initially synthesized as 31 kDa precursors, which are cleaved by proteinases to mature IL-1 which is the biologically active form. This cleavage is achieved intracellularly by interleukin-converting enzyme (ICE) or extracellularly by plasmin or elastase (Sedlacek and Möröy, 1995). Although initially described as a product of activated phagocyte, IL-1 has since been shown to be produced by a wide variety of cell types including synovial fibroblasts, keratinocyte, astrocytes, decidual and trophoblast cells (Dinarello, 1988, Librach *et al.*, 1994). This truly pleiotropic molecule has a myriad of effects both in specific cells *in vitro*, and systematically *in vivo*. A number of functions have been described for IL-1 including fever, sleep, anorexia, and hypertension and it can also stimulate host defence properties. The effects of IL-1β on trophoblast function have also been studied, particularly its impact on hormone production. IL-1β stimulates secretion of hCG by cytotrophoblasts by activating IL-6 and the IL-6 receptor system (Masuhiro *et al.*, 1991). In addition, it stimulates trophoblast aromatase activity, as well as the release of corticotropin-releasing factor and adrenocorticotropin hormone from the placenta (Petraglia *et al.*, 1990). Since IL-1 and corticosteroids are present in high

concentrations at the maternal-fetal interface, they are ideal candidate molecules for regulating trophoblast production of MMP-9. Finally placental bed immune interactions are probably also extremely important in controlling cytotrophoblast invasion. It is likely that IL-1β is one of many different cytokines that participate in the cross-talk between maternal and fetal cells.

4.3.2. Epidermal growth factor

Epidermal growth factor (EGF; also termed urogastrone) is a 6 kDa, single-chain polypeptide that is present in many body tissues and fluids. It is capable of modulating the proliferation and differentiated functions of a variety of cell types (Blay and Hollenberg, 1989). There are large numbers of EGF receptors in placental tissues. The distribution of the EGF receptor population in placenta has been studied by both histological and autoradiographical techniques. Chegini and Rao (1985) used light-microscopic autoradiography to identify the sites of ^{125}I-EGF binding in placental tissues, and found high levels of specific binding in the syncytiotrophoblast layer; relatively few grains were observed in association with cytotrophoblasts. Using an immunohistochemical approach, Maruo *et al.* (1987) similarly localised EGF receptors in placental tissue to the syncytiotrophoblasts. Again cytotrophoblasts, did not show any appreciable staining. The prevailing view is that EGF plays a part in the control of trophoblast differentiation (Maruo *et al.*, 1987). Another possible role for EGF within gestation is in the regulation of fetal development. In this context, the syncytiotrophoblast receptor could serve a transport function, transferring EGF from the maternal to the fetal side. Bass *et al.* (1994) reported experiments showing that neither EGF, TGF-α nor any other ligands for the EGF receptor were produced by cytotrophoblasts in culture. Therefore, the source of EGF receptor ligand is likely maternal, suggesting that EGF may be involved in paracrine regulation of trophoblast differentiation.

43.3 Transforming growth factor β

The transforming growth factors (TGFs) were originally named because of their effects on cell phenotype; principally, their ability to promote cell colony formation in suspension culture within agarose (Massague, 1985). It is an operational definition rather than a description of their likely

action *in vivo*. TGF-β is a disulphide-linked dimer with a molecular weight of 25 kDa. There are five isoforms of TGF-β, three of which are found in mammalian tissue (TGF-β-1, 2, 3) (Sedlacek and Möröy, 1995). Most of the currently published reports on the activities of TGF-β have dealt only with TGF-β1. In general, TGF-β1, TGF-β2 and TGF-β3 appear to be functionally equivalent in biological activity *in vitro*. TGF-β1 is relatively abundant in blood platelets and is produced in most tissues, including the placenta (Frolik *et al.*, 1983). It has complex effects on cell growth and differentiation. Thus far, however, its participation in placental physiology remains unclear. The action of TGF-β is strictly dependent upon both the physiological state of the responding cell and the presence of other growth factors. In some cases therefore, TGF-β induces cell proliferation and in other cases cell proliferation is inhibited. The biological function of TGF-β may accordingly be complex in the whole organism and highly dependent upon the exact context in which it acts. TGF-β is an important mediator of the formation of ECM generally, with activities that stimulate formation of ECM and inhibit degradation of ECM. TGF-β has been reported to inhibit MMP-1 and also upregulate TIMP (Edwards *et al.*, 1987).

4.4 Effect of Extracellular Matrix Components and Adhesion Receptors on MMPs

In most biological systems, cells require a substrate of ECM proteins for anchorage and attachment and, to derive traction for migration (Turner, 1992). For example, invasion of host tissues by cancer cells is dependent on the specific interactions of tumour cell surface receptors to ECM or components of basement membranes such as laminin. The migration of neural crest cells *in vivo* and of epidermal cells at a wound site also requires cell-matrix interactions (reviewed by Burrows *et al.*, 1995). It is interesting therefore that during the first trimester of pregnancy, the ECM proteins laminin and fibronectin are particularly abundant in the uterus. These proteins are distributed pericellularly around each individual decidual stromal cell (Loke *et al.*, 1989b) and are also produced by the trophoblast cells (Yagel *et al.*, 1988).

Besides acting as a substrate for adhesion, the ECM can also transmit signals to cells, and these signals are now believed to play a pivotal role in the control of cellular behaviour (Hynes, 1992). It has become clear that integrins can function as true receptors capable of transducing signals to the cells interior (Damsky and Werb, 1992b; Burrows *et al.*, 1995). Integrin molecules

themselves do not have characteristics of signal generating receptors. They lack intrinsic kinase or phosphatase domains or sequences suitable for G protein interactions (Juliano and Haskill, 1993). Two hypotheses for the mechanisms of integrin-mediated signal transduction exist (Juliano and Haskill, 1993), which may be complementary rather than mutually exclusive. Firstly, signaling may be generated by integrins binding directly to the cytoskeleton thus promoting reorganisation of the cellular architecture. The cytoskeletal frameworks formed may influence adhesiveness, cell shape and motility and may also determine how integrin-mediated regulatory signals are propagated throughout the cell. Secondly, integrin-ligation may trigger specific biochemical signals within the cell.

During matrix ligation, integrins cluster at focal adhesion structures and this event may be crucial to integrin-mediated signal transduction. Engagement of integrin-matrix receptors with their cognate ligands or cross linking of integrins with specific antibodies induces a number of physiological changes in the cell, including changes in intracellular pH (Schwartz *et al.*, 1993), intracellular calcium (Pelletier *et al.*, 1992), increases in tyrosine phosphorylation (Guan *et al.*, 1991) and changes in gene expression (Werb *et al.*, 1989). Ligation of integrins alters the cellular pattern of tyrosine phosphorylation (Guan *et al.*, 1991) and this is thought to be the initial event in integrin-mediated signal transduction. An increase in tyrosine phosphorylation in tumour cells has been found to be associated with an increase in their invasion of the surrounding ECM (Mueller *et al.*, 1992). It is therefore possible that similar signaling pathways may be used by trophoblasts during migration through the ECM-rich environment of the decidua.

Villous and nonvillus trophoblasts exist in different microenvironments. Villous trophoblasts, which are non-invasive, are bound to a basement membrane composed of collagen type IV, fibronectin and laminin whereas invasive intermediate trophoblasts, depending on their location (proximal or distal), are surrounded by a matrix composed either of modified laminin or fibronectin and collagen type IV respectively (Damsky *et al.*, 1992a). As described in chapter 3, (section 3.3.3.) trophoblast cells modulate their expression patterns of integrins and ECM components during trophoblast differentiation along the invasive pathway (Damsky *et al.*, 1992). Werb *et al.* (1989) elegantly demonstrated that signal transduction through the fibronectin receptor induced MMP-1 and MMP-3 gene expression in rabbit synovial fibroblasts. Integrins have also been shown to regulate MMP-1 expression in human osteosarcoma cell lines (Riikonen *et al.*,

1995). Bischof *et al.* (1991) reported that trophoblasts grown on Matrigel expressed MMP-9 more intensely, whereas MMP-2 remained unchanged. They also reported induction of MMP-3 when the trophoblast cells were grown on rat tail collagen. The importance of trophoblast-ECM interactions, is also supported by Kliman and Feinberg's report (1990) which demonstrated that ECM thickness modulates morphology and proteolytic activity of trophoblasts. Therefore the regulation of MMP by matrix receptors may be of great importance for cellular invasiveness.

4.5 Regulation of MMPs by Hormones

Hormonal regulation of MMP expression is perhaps best studied in the cycling endometrium (as described in section 1.7.2). In particular, progesterone has been shown to be a potent repressor of MMP concentrations both *in vivo* and *in vitro*. For example, the MMP genes are expressed during the times in the cycle when progesterone concentrations are low i.e. during the proliferative and menstrual stages of the cycle (Rodgers *et al.*, 1994). The mechanism of inhibition is not well understood. Progesterone regulation of gene expression has been shown to be mediated by direct binding of the receptor to negative hormone-response elements in the promoters of many steroid-responsive genes (Gronemeyer, 1991). An attractive hypothesis is that the MMPs are regulated in this manner. While putative hormone-response elements have been identified in the promoters of MMP-1, MMP-3 and MMP-7 (Matrisian *et al.*, 1985, Angel *et al.*, 1987, Gaire *et al.*, 1994), there is no direct evidence that these elements respond to progesterone. At least in the case of MMP-7, there appears to be an indirect effect mediated through the paracrine activity of TGF-β, which then represses MMP-7 via a TIE element in the MMP-7 promoter (Bruner *et al.*, 1995).

The effects of estrogen on MMP expression are not well understood. In the rat, removal of all endogenous steroid hormones by ovariectomy and adrenalectomy resulted in complete loss of collagenase activity and synthesis in the rat uterus (Anuradha and Thampan, 1993). Implantation of estradiol wax pellets into these rats brought about a recovery in synthesis of the enzyme to pre-ovariectomized concentrations, suggesting that uterine collagenase is indeed under the regulatory influence of estradiol. Estrogen has little effect on MMP expression *in vitro*, although in some

cases estrogen enhanced the inhibitory action of progesterone on MMPs, presumably through its ability to upregulate progesterone receptor production (Hulboy *et al.*, 1997).

4.6 Effect of Hypoxia on MMPs

In mammalian cells that have been studied in this context (e.g. endothelial cells, tumour spheroids) hypoxia upregulates the expression of stress (oxygen-regulated proteins), heat shock, and glucose-regulated proteins, as well as cytokines and growth factors. These include erythropoietin (Bondurant *et al.*, 1986), PDGF B chain (Kourembanas *et al.*, 1990), endothelin (Kourembanas *et al.*, 1991), IL-8 (Shreeniwas *et al.*, 1992), vascular endothelial growth factor (Shweiki *et al.*, 1992) and glyceraldehyde-3-phosphate dehydrogenase (Graven *et al.*, 1994). The *cis*-acting DNA sequences and the trans-acting transcription factors that regulate the expression of these hypoxia-induced proteins are beginning to be elucidated. For example, hypoxia-inducible factor 1, a nuclear protein DNA-binding activity induced in hypoxia, upregulates erythropoietin production by binding to an enhancer element in the 3' region of this gene (Wang *et al.*, 1993). Hypoxia inducible factor 1 also induces transcription of glycolytic enzymes, which are vital for the cells ability to switch from aerobic respiration to glycolysis for energy production (Semenza *et al.*, 1994). The expression and/or activity of several other transcription factors including Fos and Jun, NFκB, and the heat shock transcription factor, also increase in hypoxia (Genbacev *et al.*, 1996).

The studies mentioned above suggest that hypoxia can specifically alter a cells gene expression and protein repertoire. Dramatic changes in oxygen content of the placental environment normally occur during early gestation. Namely, before the tenth week of pregnancy there is little blood flow to the intervillus space, due to the fact that cytotrophoblast invasion is largely interstitial, rather than endovascular. As a result the placenta proper is relatively hypoxic (Rodesch *et al.*, 1992). By weeks 12-13, cytotrophoblasts begin to substantially remodel the spiral arterioles and blood flow to the intervillus space increases dramatically (Rodesch *et al.*, 1992). Pre-eclampsia, is a disease of pregnancy which affects 7-10% of all first-time pregnancies. Compared with normal pregnancy, in pre-eclampsia cytotrophoblast invasion is shallow and fewer arterioles are breached, an important factor with regard to the reduction in uteroplacental blood flow

observed in this syndrome. It has been hypothesized that the events that normally take place during the first trimester of pregnancy, which convert the relatively hypoxic environment to one that is relatively well oxygenated, fail to occur in pre-eclampsia. Whether this could affect the cells ability to differentiate and invade remains unknown.

The research presented in the following section attempts to address many of the issues raised above. It is primarily concerned with identifying the biological factors and conditions that may function in regulating trophoblast MMP expression and, thus, could play a hierarchical role in controlling trophoblast invasion.

4.7 RESULTS

4.7.1 Isolation of total RNA from cell lines

To investigate the effects of various biological modifiers on MMP and TIMP expression we examined mRNA levels of target genes and compared them with levels in control untreated cells using RT-PCR. This was achieved by treating all the cell lines with the appropriate biological modifier for 8 hours (section 2.2.1.5), and isolating total RNA was from all cell lines as described in section 2.2.7.1. RNA was quantified as described in section 2.2.7.2. The integrity of the RNA was analyzed by gel electrophoresis as outlined in section 2.2.7.3, and the RNA was later used as template in the RT-PCR reaction (section 2.2.8). Figure 4.2 is a representative gel showing the 28, 18 and 6 S ribosomal subunits demonstrating successful RNA isolation with some degradation. This gel shows the RNA isolated following treatment with cytokines to investigate their effect on MMP-9 mRNA levels in the ED_{77} cell line.
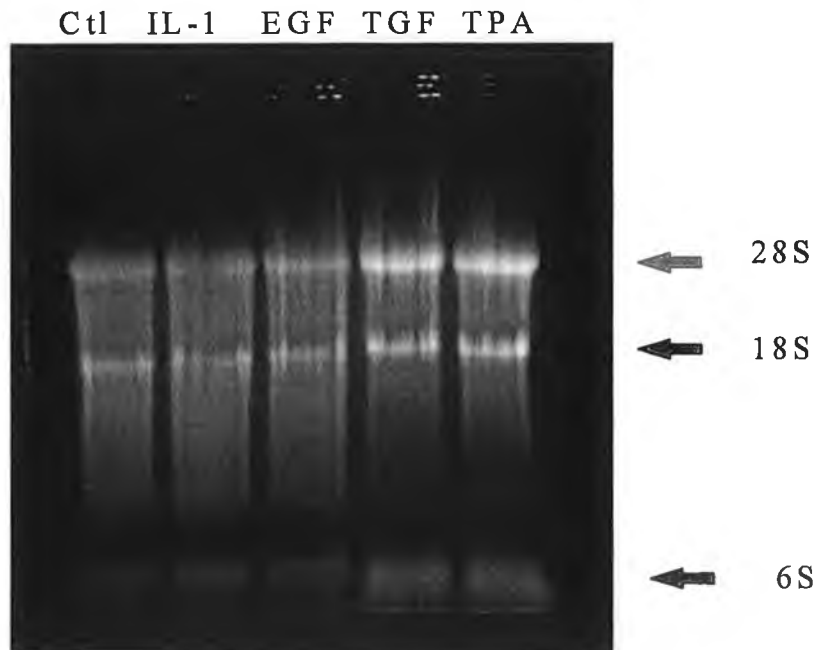

Figure 4.2. Agarose gel electrophoresis showing intact RNA isolated from ED_{77} cells (5 µg RNA loaded). The bands representing the 28S, 18S, and 6S ribosomal subunits can be clearly seen.

4.7.2 Optimisation of PCR reactions.

Each PCR reaction was performed with 2 sets of primers; one set for the gene of interest and the other for β-actin. The inclusion of primers for β-actin, a constitutively expressed gene acts as an internal control and allows for comparisons to be made between samples. The β-actin primers chosen also span an intron and so have the added benefit that any contaminating DNA, which could also be amplified, can be identified as it yields a larger product size. The temperature at which both sets of primers would anneal during the PCR reaction was determined empirically. The results of the optimum annealing temperature during PCR for each set of primers is shown in Table 4.1.

Table 4.1 Optimum annealing temperatures for primers used.

Target	Primer sequence	Fragment size	Optimum annealing temp. (°C)
MMP-9	5 'GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC 3' 5' GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT 3'	640bp	54
MMP-2	5' TGA CAT CAA GGG CAT TTC AGG AGC3' 5' GTC CGC CAA ATG AAC GGT CCT TG 3'	180bp	53
MMP-7	5' TGT ATC CAA CCT ATG GAA ATG 3' 5' CGC GCA TCT ACA GTT ATT TAC3'	341bp	47
MMP-3	5' CTT TCC AGG GAT TGA CTC 3' 5' CGG CCA GTA AAA ATA CAA3'	324bp	48
TIMP-1	5'TGC ACC TGT GTC CCA CCC CAC CCA CAG ACG 3' 3'GGC TAT CTG GGA CCG CAG GGA CTG CCA GGT 3'	551bp	53
TIMP-3	5'CTG TGC AAC TTC GTG GAG 3' 5' TCG GTA CCA GCT GCA GTA 3'	250bp	55
β- actin	5' TCA GGA GGA GCA ATG ATC TTG A 3' 5' GAA ATC GTG CGT GAC ATT AAG GAG AAG CT3'	383bp	47-55

4.7.3 Effects of cytokines on MMP-9 mRNA.

The MMPs are known to be regulated transcriptionally by a variety of cytokines and oncogenes in other systems (McDonnell *et al.*, 1991, Abbas *et al.*, 1991). We decided to investigate the effect of a number of cytokines on MMP-2 and MMP-9 expression in these trophoblast cell lines. The cytokines chosen had been implicated by a number of other investigators in regulating developments at the feto-maternal interface *in vivo* and the cytokines and/or their receptors are known to be present in placental tissue. We looked at the effect of IL-1β, EGF and TGFβ, and also the synthetic tumour promoter TPA on MMP mRNA and protein levels.

The effect of cytokines on MMP mRNA expression levels was investigated using reverse transcription polymerase chain reaction (RT-PCR) using specific primers for MMP-2 and MMP-9, we also amplified a constitutively expressed gene β-actin. Following treatment of the cells with the various cytokines for 8 hours (section 2.2.1.5), the RNA was extracted (2.2.7.1), reverse transcribed to cDNA and amplified for β-actin and either MMP-2 or MMP-9 (section 2.2.8). Figure 4.3 shows a representative RT-PCR gel for ED_{77} showing PCR products for simultaneously amplified MMP-9 and β-actin. Gels were then scanned by densitometry as in section 2.2.9. Results were then graphed using densitometric readings normalised for β-actin expression. This means that any changes detected can be specifically attributed to changes in steady state MMP-9 mRNA levels. The three trophoblast cell lines showed a similar pattern of results with IL-1β and TPA having a stimulatory effect on MMP-9 mRNA production (Figure 4.4) EGF caused a slight upregulation of MMP-9 in all three cell lines. However, TGF-β had no effect in the case of ED_{31} and caused a slight inhibition of MMP-9 mRNA expression in ED_{77} and ED_{27}.

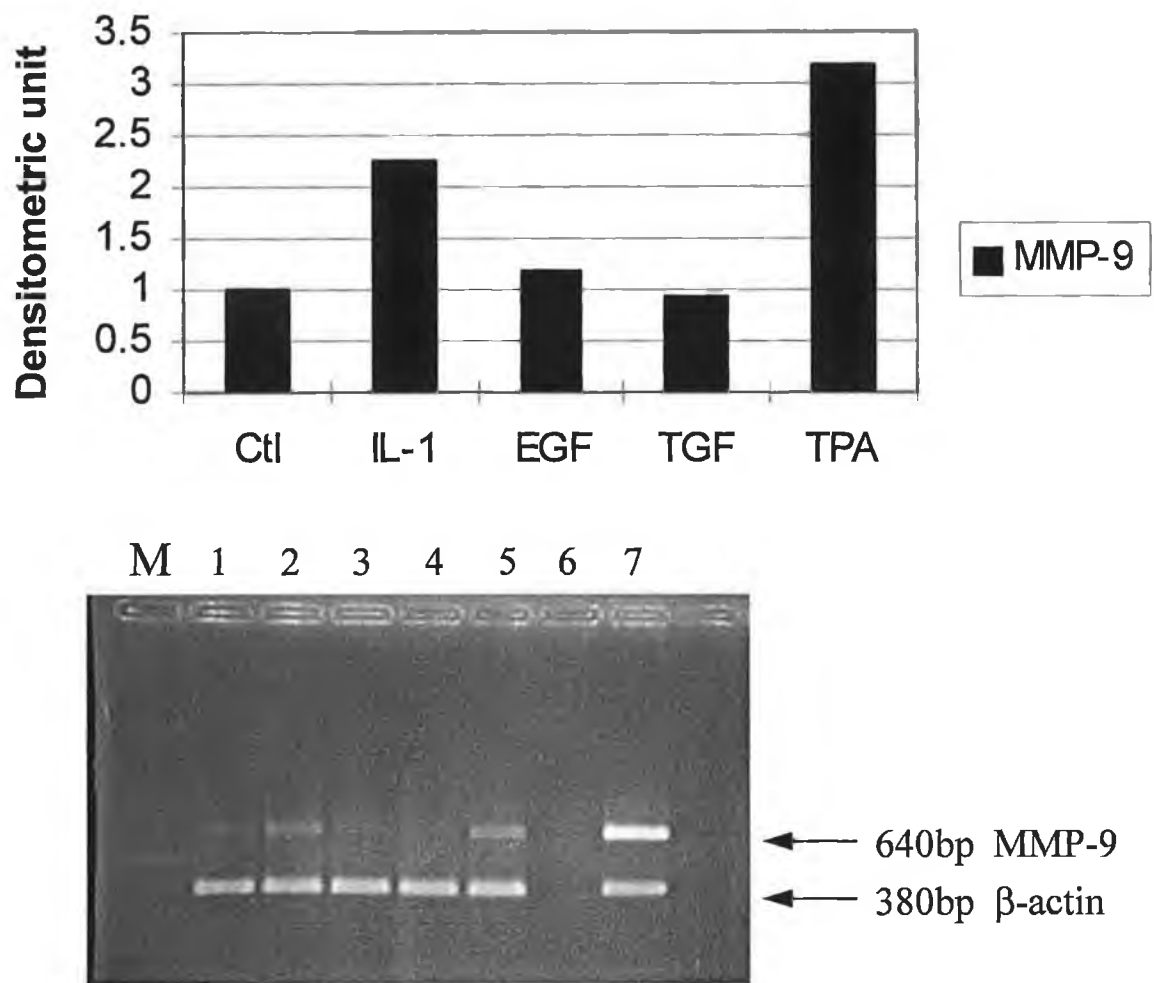**Figure 4.3** RT-PCR analysis of mRNA from ED_{77} trophoblast cells using β-actin and MMP-9 specific primers following treatment with cytokines. Lanes (1) untreated, (2) IL-1β, (3) EGF, (4) TGFβ, (5) TPA, (6) negative control, (7) BHK92 RNA used as positive control. Up-regulation of MMP-9 by IL-1β and TPA (a known inducer of MMP-9) can be seen. Graph shows densitometric analysis normalised for β-actin.
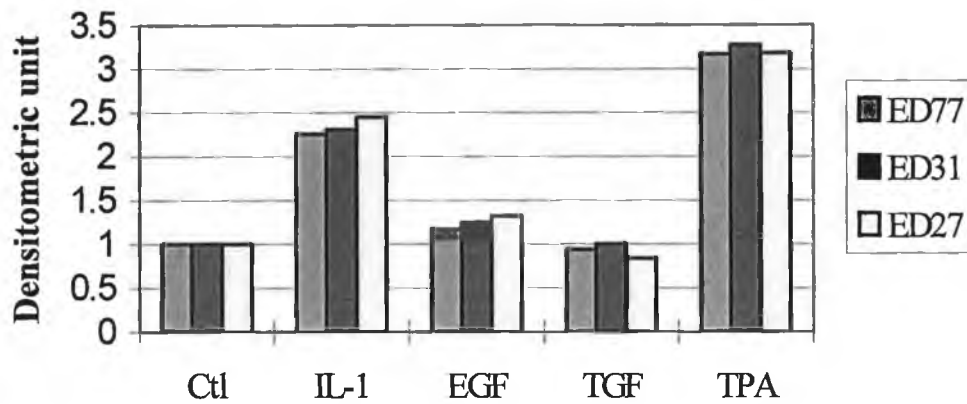
Figure 4.4 Comparison of the effects of cytokines on MMP-9 mRNA expression levels in trophoblast cell lines ED_{77}, ED_{31}, and ED_{27}. Graph shows densitometric analysis of gels with RT-PCR products for MMP-9 normalised for β-actin

4.7.4 Time and Dose response of IL-1β on MMP-9 mRNA levels

Having established that IL-1β had a stimulatory effect on MMP-9 mRNA expression we decided to characterise this response further and investigate the dose response and time course of IL-1β stimulation. To investigate the response of IL-1β at various time points, the cells were treated with 5 ng/ml IL-1β as outlined in section 2.2.1.5, the RNA extracted (2.2.7.1) at 4, 8, 16 and 24 hours post treatment, and mRNA examined by RT-PCR (2.2.8). As shown in Figure 4.5 (A), maximal induction of MMP-9 mRNA levels was seen at 8 hours post treatment with IL-1β. It can also be seen that mRNA levels were approaching control untreated levels by 24 hours post treatment. Increasing concentrations of IL-1β were also tested to see if the response was dose dependent. Figure 4.5 (B) shows that treatment with 1X (5 ng/ml) IL-1β upregulates MMP-9 and this effect is increased when the concentration of IL-1β is increased to 5X (25 ng/ml) but plateaus above 5X concentration of IL-1β. No effect is obvious at concentrations lower than 1X IL-1β.
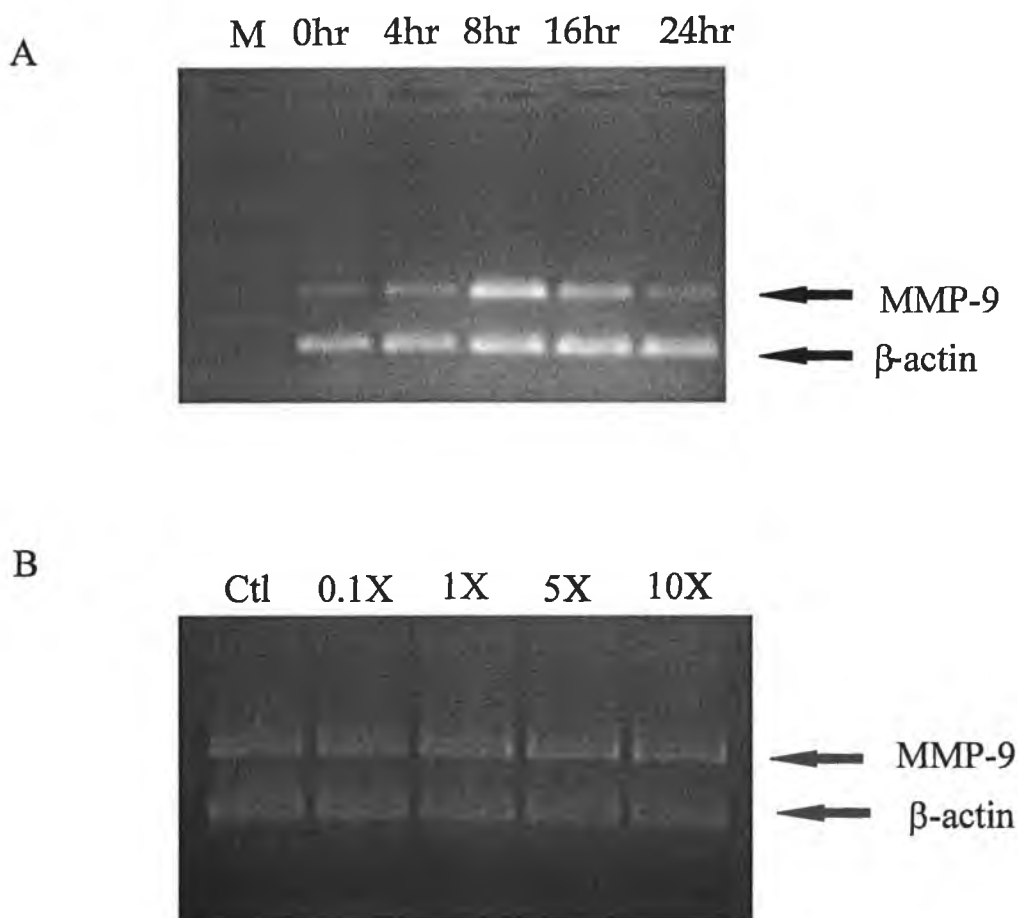
Figure 4.5 (A) RT-PCR analysis of the time taken after treatment with IL-1β until effect can be seen in MMP-9 mRNA expression levels in ED_{77} cells. **(B)** Analysis by RT-PCR of the effect of various concentrations of IL-1β on MMP-9 mRNA levels in ED_{77} cells. Concentrations of IL-1β examined were 0.1X (0.5 ng/ml), 1X (5ng/ml), 5X (25ng/ml), 10X (50ng/ml).

4.7.5 Effects of cytokines on MMP-9 activity levels

The upregulation of MMP-9 at mRNA level was investigated further to see if there was a corresponding increase in MMP-9 protein levels when exposed to IL-1β. Cells were treated with the various cytokines for 24 hours (section 2.2.1.5), the conditioned medium collected, and analysed by zymography (section 2.2.4.3). Figure 4.6 shows a representative zymogram which

shows stimulation of MMP-9 protein following treatment with IL-1β in the ED_{77} cells. This figure also shows that MMP-2 protein expression was not regulated by any of these factors. All three trophoblast cell lines show similar results (Figure 4.7), with IL-1β increasing the amount of protein produced. EGF also caused a small increase in MMP-9 protein levels and TGF-β had little effect on MMP-9 protein levels in the trophoblast cell lines (Figure 4.7). With the exception of TGF-β, the effect of cytokines on MMP-9 at the protein level appear similar to the effects seen at the mRNA level.

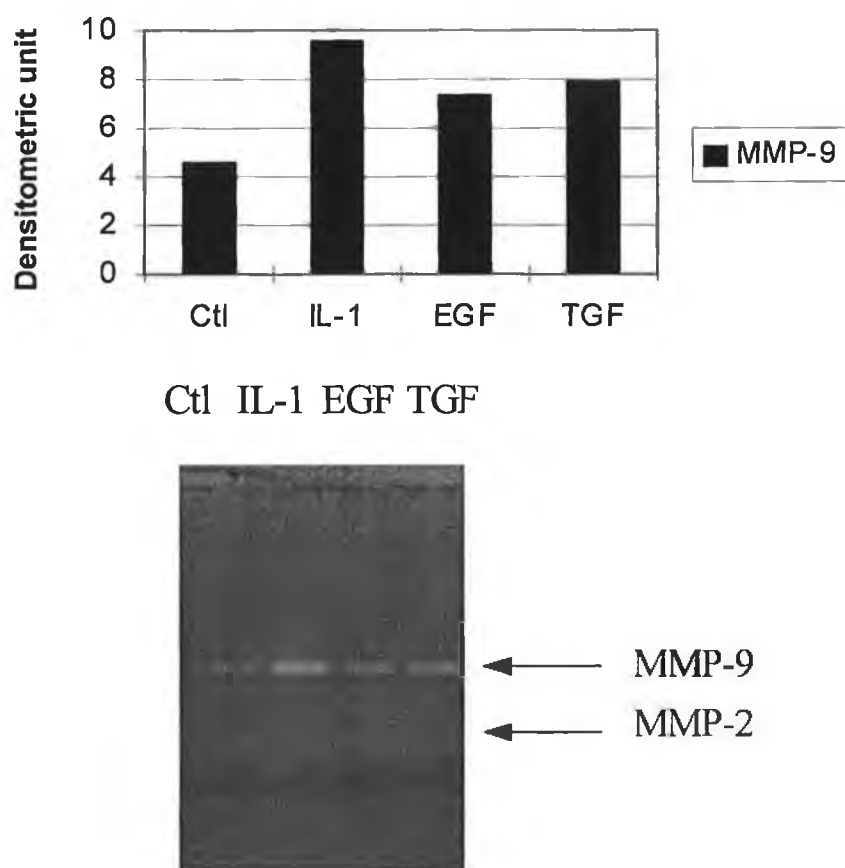Figure 4.6 .Zymogram showing the effect of cytokines on MMP activity in conditioned medium from ED_{77} cells. Lanes : (1) untreated, (2) IL-1β, (3) EGF, and (4) TGFβ. Upregulation of MMP-9 by IL-1β can be seen in lane 2.
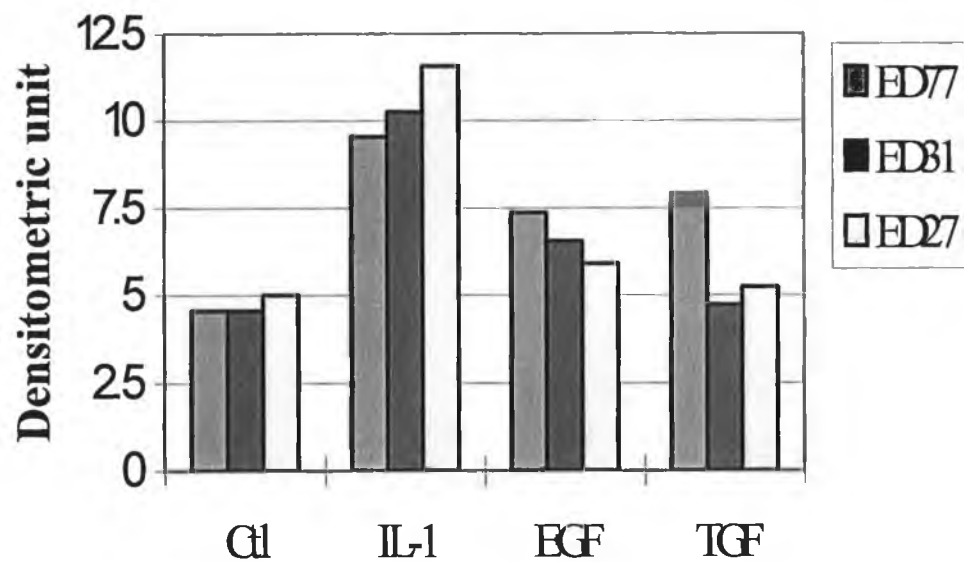
Figure 4.7 Comparison of the effect of cytokines on MMP-9 protein levels in trophoblast cell lines. Graph shows densitometric measurement of MMP-9 bands from zymograms.

4.7.6 Effects of cytokines on *in vitro* invasive ability of trophoblast cell lines

Having established that cytokines could modulate MMP-9 mRNA levels and MMP-9 activity, we investigated the effects of these cytokines on the *in vitro* invasive ability of the cell lines. To determine this, we used an *in vitro* assay of invasive potential in which the ability of trophoblasts to invade extracellular matrix (Matrigel)-coated filters and emerge through 8μm pores on the filter underside was accessed (section 2.2.6). As shown in Figure 4.8 the effect of the cytokines on invasive potential reflects the results seen for MMP-9 at the mRNA and protein level. TPA produced the greatest increase in invasive ability with approximately a 1.2 fold increase over control, followed by IL-1β with approximately a 0.9 fold increase over control untreated cells. EGF was found to have only a slight inducive effect on invasive ability (0.2 fold increase over control). The effects of TGF-β were not so clear, with no apparent effect seen in ED_{77} and ED_{31} cells but in ED_{27} cells a slight inhibitory effect on invasion was seen.
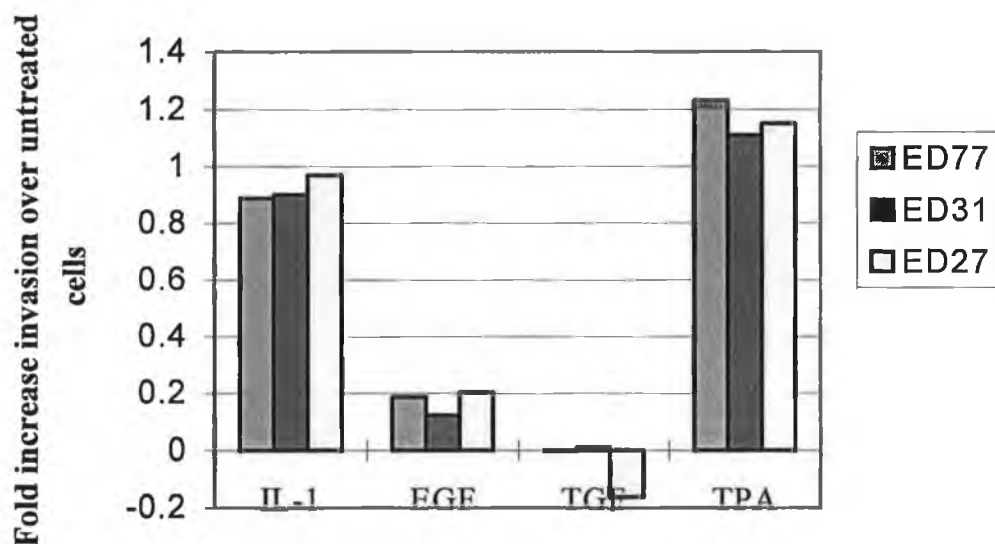
Figure 4.8 Effects of cytokine treatment on *in vitro* invasive ability of trophoblast cell lines, ED_{77}, ED_{31}, and ED_{27}.

4.7.7 Effects of cytokines on MMP-9 mRNA levels in BeWo cells

Although no MMP-9 expression could be detected in the BeWo cells (section 3.4.4) we investigated whether MMP-9 expression could be induced by any of the cytokines tested on the trophoblast cell lines. Interestingly, we found that IL-1β and TPA induced low levels of expression of MMP-9 mRNA (Figure 4.9). EGF and TGF-β had no effect. This slight induction however was not seen at the protein level (data not shown).

Figure 4.9 RT-PCR analysis of mRNA from BeWo cells using β-actin and MMP-9 specific primers following treatment with cytokines. Lanes (1) untreated , (2) IL-1β, (3) EGF, (4) TGFβ, (5) TPA, (6) negative control, (7) BHK92 RNA used as positive control.

4.7.8 Effects of cytokines on MMP-2 expression

We also examined the effect of cytokines on MMP-2 mRNA expression levels in the trophoblast and BeWo cell lines. Unlike MMP-9, MMP-2 mRNA expression levels could not be modulated by treatment with any of the cytokines used in this study, regardless of the cell line. Figure 4.10(A) shows the BeWo cell line stimulated with a variety of cytokines and TPA and shows no stimulation of MMP-2. Likewise Figure 4.10(B) shows ED_{77} with similar results, demonstrating the constituitive nature of MMP-2 expression.
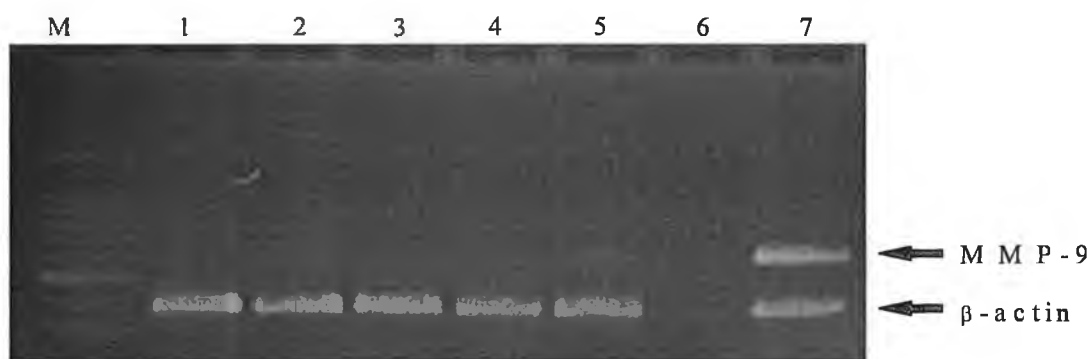

Figure 4.10 (A) RT-PCR analysis of mRNA from BeWo cell line using β-actin and MMP-2 specific primers. Lanes as follows: (M) contains 100 bp ladder, intense band at 500 bp, (1) Untreated BeWo, (2) IL-1β, (3) EGF, (4) TGFβ, (5) TPA, (6) negative control (RNA replaced with water), (7) positive cont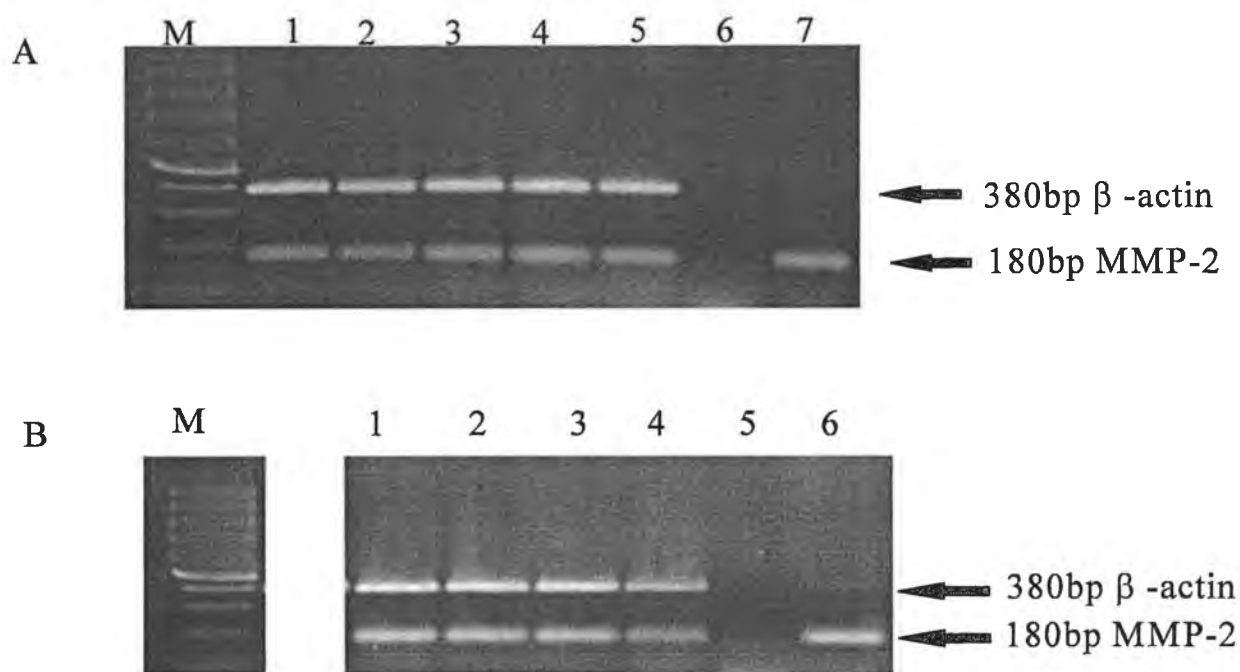rol, plasmid contain cDNA for MMP-2 used as template in PCR. **(B)** RT-PCR analysis of mRNA from ED_{77} cell line using β-actin and MMP-2 specific primers. Lanes as follows: (M) contains 100 bp ladder, intense band at 500 bp, (1) Untreated ED_{77}, (2) IL-1β, (3) EGF, (4) TGFβ, (5) negative control (RNA replaced with water), (6) positive control, plasmid contain cDNA for MMP-2 used in PCR.

4.7.9 Effects of Cytokines on TIMP-1 Expression

TIMP-1 has been shown to be inducible in other systems, however little has been published on TIMPs in trophoblast cells. We found that TIMP-1 and TIMP-2 were produced by the trophoblast cell lines (section 3.4.5) and decided to investigate the effect of cytokines on the expression of TIMP-1 in the trophoblasts. Cells were treated with the cytokines (section 2.2.1.5), RNA extracted (2.2.7) and analysed by RT-PCR (2.2.8). None of the cytokines used in this study stimulated TIMP-1 mRNA levels, as shown in Figure 4.11. However, TPA appeared to repress TIMP-1 mRNA expression.
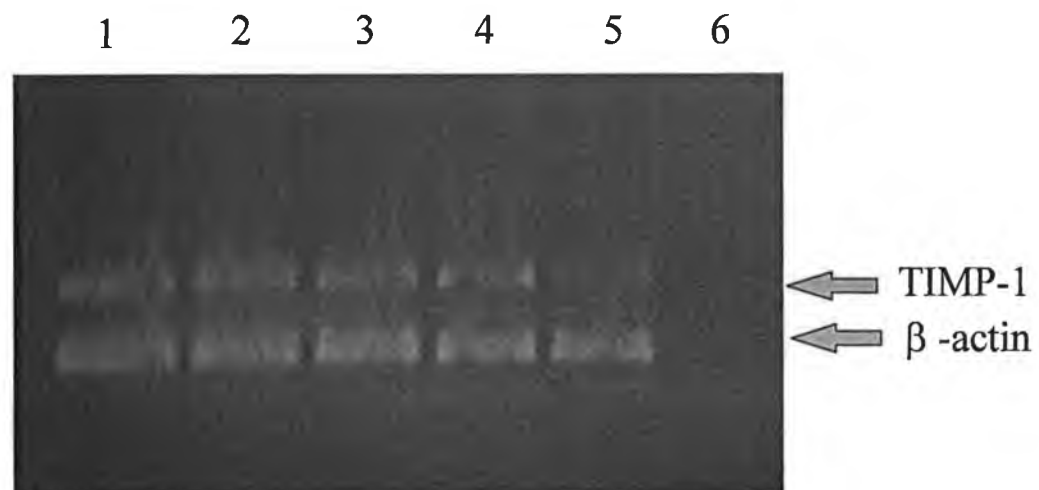
Figure 4.11 RT-PCR analysis of mRNA from ED77 cell line using β-actin and TIMP-1 specific primers. Lanes as follows: (1) Untreated ED77, (2) IL-1β, (3) EGF, (4) TGFβ, (5) TPA, (6) negative control (RNA replaced with water).

4.7.10 Effects of ECM components on MMP Expression

The interactions of cells with components of the ECM, such as fibronectin, laminin, tenascin, and collagens of many types, has been shown to play an important role in tissue remodeling (Liotta *et al.*, 1986). We investigated the effect of culturing the trophoblast cell lines on various different matrices to determine if specific ECM components may have a role in regulating MMP expression. The ECM component laminin was shown to be a strong inducer of MMP-9 mRNA transcripts as shown in Figure 4.12. Other ECM components investigated, fibronectin, type IV collagen and type I collagen had no obvious effect. ECM components had no effect on MMP-2 mRNA levels (data not shown).
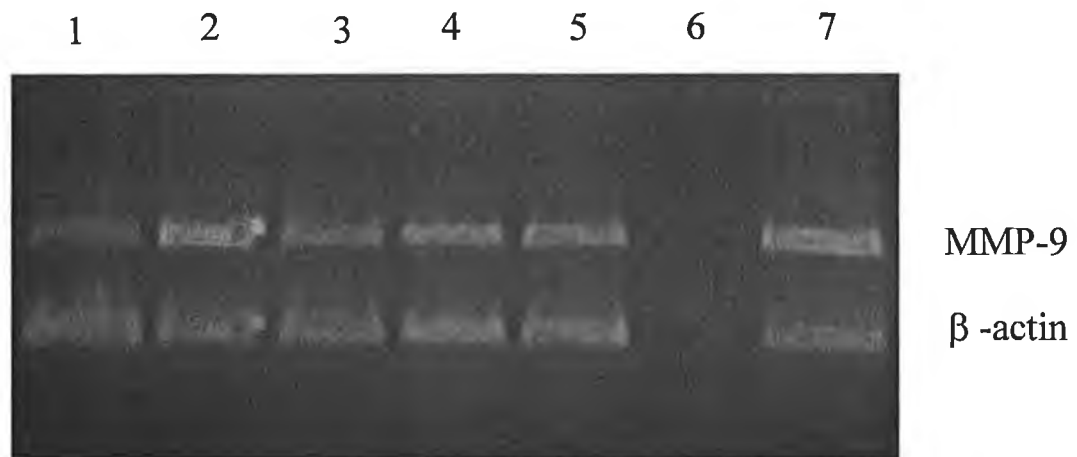
Figure 4.12 Effects of various ECM components on expression of MMP-9 mRNA in ED_{77} cells. Lanes contain RT-PCR products from mRNA isolated from cells grown on (1) plastic, (2) laminin, (3) fibronectin, (4) type IV collagen, (5) type I collagen, (6) negative control, (7) positive control, mRNA from cell line known to express MMP-9.

4.7.11 Effects of progesterone and β-estradiol on MMP expression

As steroid hormone regulation of MMPs has been seen in other systems and because pregnancy is innately hormone driven we decided to examine the possibility that progesterone and β-estradiol regulate MMP expression levels in the trophoblast cell lines. The cells were treated with the hormones as described in section 2.2.1.5, and the RNA extracted and analysed by RT-PCR. No effect was seen on MMP-9 or MMP-2 mRNA expression levels when either progesterone or β-estradiol were added to the cultures, as shown in Figure 4.13 for MMP-9 and data not shown for MMP-2.
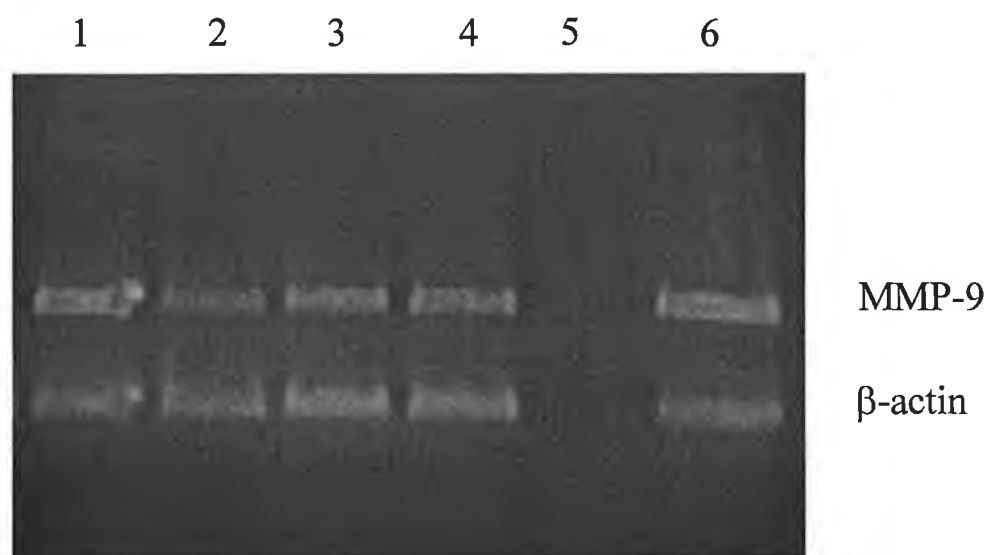
Figure 4.13 RT-PCR analysis of the effect exogenously added hormones; progesterone and estradiol. Lanes as follows: (1) Untreated, (2) progesterone, (3) β-estradiol, (4) progesterone and β-estradiol, (5) negative control, (6) positive control, mRNA from cell line known to express MMP-9.

4.7.12 Effect of Hypoxia on MMP activity

It had been suggested that oxygen tension could regulate trophoblasts ability to differentiate and as a consequence, to express proteins that are critical to the invasive process (Genbacev *et al.*, 1996). We were interested in determining whether a relatively hypoxic environment could affect the trophoblast cells ability to express MMPs. Cells were cultured under hypoxic conditions for 24 hours as described in section 2.2.1.7, conditioned medium collected and analysed by zymography (2.2.4.3). Our findings suggest that hypoxia does not affect MMP expression. No difference was found between MMP protein expression by trophoblast cells cultured for 24 hours under hypoxic or normal oxygen conditions as shown in Figure 4.14. Similar results were seen at the mRNA level (data not shown).
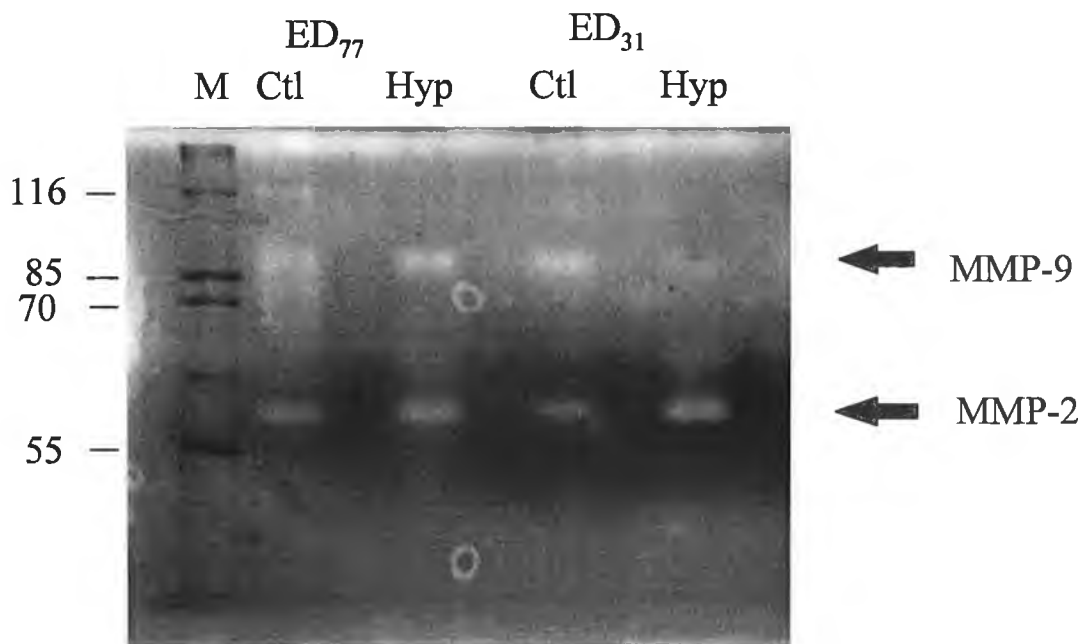
Figure 4.14 Effect of hypoxic growth conditions on MMP activity in ED_{77} and ED_{31} cell lines.

4.8 DISCUSSION

4.8.1 Effects of cytokines on MMP expression

MMP-9 has been shown to be produced by trophoblasts in a developmentally regulated manner, trophoblast cells from the first trimester are highly invasive and this invasiveness decreases by the third trimester (Librach *et al.*, 1991, Bischof *et al.*, 1991). In chapter 3 the critical role played by MMP-9 was also identified in the trophoblast cell lines described in this thesis. Thus studies of the regulatory mechanisms controlling expression of this gene are important for understanding the complex process of invasion which takes place during pregnancy. It is highly likely that trophoblast differentiation along the invasive pathway is a result of the balanced action of growth factors and cytokines on trophoblasts and their surrounding environment. Induction of MMP-9 has previously been reported to be associated with invasiveness in many other systems. For example, the increased invasiveness of osteosarcoma-derived OST cells implanted in nude mice, TNF-α was identified as the inducer for MMP-9 expression in the cells at the mRNA level (Okada *et al.*, 1990). Depending on the cell types, expression of MMP-9 is also stimulated by inducers such as TPA, EGF, IL-1, and oncogene products (Sato and Seiki, 1993). Malignant tumour cells frequently overexpress MMP-9 and much research has been focused on understanding its role in cancer cells (Sato *et al.*, 1992), however little information is available on factors regulating expression of MMP-9 during trophoblast invasion. Since they lie at the maternal-fetal interface, trophoblasts could be influenced by factors from either or both compartments. With regard to autocrine regulators, trophoblast expression of several cytokine-receptor pairs has been described, suggesting that these cells can both produce and respond to EGF, IL-1β and IL-6 (Librach *et al.*, 1994, Shimonovitz *et al.*, 1994).

In the experiments described here, we tested three cytokines, IL-1β, EGF and TGFβ, known to play a role in controlling the growth and differentiation of cells to see whether they affect trophoblast expression of MMPs *in vitro*. Several lines of evidence now support a possible role for IL-1 in human trophoblast physiology. First, measurable amounts of IL-1 were found in the medium of trophoblast cultures (Masuhiro *et al.*, 1991, Simon *et al.*, 1994). Second, human decidua has also been shown to produce IL-1 (Romero, *et al.*, 1989). Third, multiple *in vitro*

studies have shown potent regulatory effects of IL-1 on trophoblast hCG, IL-6, and prostaglandin E₂ production (Masuhiro *et al.*, 1991, Yagel *et al.*, 1989b, Shimonovitz *et al.*, 1995).

In this study we clearly showed that under basal conditions, trophoblasts cells express MMP-9. Furthermore, treatment with IL-1β caused approximately a 1.5-fold increase in the steady state mRNA level of MMP-9, accompanied by a significant increase in the gelatinolytic activity of these cells. This increase in MMP-9 protein levels also lead to a slight increase in the cells *in vitro* invasive ability over untreated cells. These results are in agreement with current theories that MMP-9 expression is directly linked to invasive potential (Sehgal *et al.*, 1998, Bernhard *et al.*, 1994). The stimulatory effect of IL-1β on MMP-9 in trophoblast cultures corroborates previous findings in rat ovary, keratinocytes, and synovial cells (reviewed in Shimonovitz *et al.*, 1996). Recently Librach *et al* (1994) reported similar results of the effect of IL-1β on MMP-9 in trophoblast cells, however only at the protein level. Paulesu *et al.* (1991), using immunohistochemistry, showed that the syncytiotrophoblast and invasive cytotrophoblasts, but not villus cytotrophoblast stem cells, contain immunoreactive IL-1β. Furthermore, staining for this cytokine was strongest during the first trimester. *In vitro* production of IL-1β by trophoblasts was also reported by Librach *et al.* (1994). They reported that release of IL-1β by trophoblasts was developmentally regulated, with highest levels produced by trophoblasts isolated from first trimester placentas and lowest levels from term placentas, paralleling expression of MMP-9. Our results indicate that IL-1β may have an important *in vivo* role in regulating local MMP-9 production and thus the extent of trophoblast invasion which takes place.

To further characterise the modulation of MMP-9 by IL-1β, time course and dose response experiments were carried out by analysing mRNA levels using RT-PCR. Every gene displays a characteristic time course of induction following exposure of the cell to the cytokine. These can roughly be divided into early-response genes which are rapidly induced (within minutes), intermediate-response genes (induced within a few hours), and late-response genes (induced after several hours). The timing of genetic responses reflects the period required for functional transcriptional activators to be produced and act on their intermediate targets. We examined the time course of the effect of IL-1β on MMP-9 mRNA levels. The MMPs are generally accepted as being intermediate response genes and this was shown for MMP-9, with a time period of 4 hours post addition of IL-1β before a change in the mRNA level was seen, which reaches maximal level

at 8 hours. By 24 hours post treatment the cells mRNA levels were again similar to untreated cells. The effect of IL-1β on MMP-9 transcript levels was also found to be dose dependent. A concentration of 5 ng/ml IL-1β was found to be sufficient to upregulate expression compared to untreated cells, with mRNA levels reaching plateau for concentrations greater than 25 ng/ml.

EGF has previously been shown to be a potent upregulator of MMPs particularly MMP-3 and MMP-1 in other cell types (McDonnell *et al.*, 1990, Shima *et al.*, 1993). In this study we found EGF only had a slight effect on trophoblast MMP-9 mRNA or protein expression. We also saw no significant increase in the cells invasive potential when treated with EGF. In placental tissue *in vivo* the EGF receptor population appears to be largely confined to the syncytiotrophoblast cells which are essentially non-proliferative and noninvasive (Chegini and Rao, 1985, Maruo *et al.*, 1987). Since the trophoblasts used here have the phenotype of nonvillus cytotrophoblasts, we would therefore expect that they express few EGF receptors and hence it is no surprise that EGF had only minimal effect. Although the MMP-9 promoter may contain the elements necessary for EGF stimulation of the gene, the level of EGF-receptor expression by the cells will determine the response. Another study by Bass *et al.* (1994) found that EGF increased trophoblast invasion but they observed no effects on proteinase production which is in agreement with our findings where only a slight up-regulation of mRNA levels was seen.

The three trophoblast cell lines showed almost no change in mRNA/protein levels of MMP-9 or in invasive potential over untreated cells when treated with TGF-β, although the ED_{27} cells invasive ability was decreased slightly compared to control. Studies by Sato and Seiki, (1993) on the promoter of MMP-9, reported the presence of the TIE element but the inhibitory effect of TGF-β on MMP-9 expression could not be demonstrated. This was in contrast to the MMP-3 gene where TIE is the element through which TGF-β inhibits the growth factor or oncogene-induced expression of MMP-3 (Kerr *et al.*, 1990). Graham *et al.* (1992) demonstrated that TGF-β blocked trophoblast invasion *in vitro* and attenuated MMP-9 activity by activation of the TIMPs. Studies in other cell types have found that TGF-β increased MMP-9 activity (Shimonovitz *et al.*, 1996, Overall *et al.*, 1991). TGF-β, on the other hand, decreased the expression of IL-1-induced MMP-9 enzyme in the ovary (Hurwitz *et al.*, 1993). It seems that the activity of TGF-β is not solely dependent on the presence of the known TGF-β-responsive elements in the gene's promoter, and it might have alternative signaling pathways in different systems.

In chapter 3 we found that the BeWo cell line did not express MMP-9 (section 3.4.4) and was noninvasive (section 3.4.3). As the BeWo cell line was originally derived from a choriocarcinoma, which is a metastatic tumour, we hypothesized that perhaps a factor necessary for the promotion of invasion was absent *in vitro*. To test this hypothesis we investigated whether MMP-9 expression could be induced by a variety of cytokines in the BeWo cell line. We found that IL-1β induced low level expression of MMP-9 mRNA, though this slight induction was not seen at the protein level. However, it demonstrates that it is possible to stimulate expression of the MMP-9 gene in the BeWos and we speculate that a process similar to this may also occur *in vivo*.

Interestingly, IL-1β, EGF, TGF-β and TPA had no effect on either mRNA levels or the activity of MMP-2. Although both type IV collagenases show almost identical substrate specificity, with the ability to cleave collagen type IV and degrade denatured collagen (gelatin), they have significant dissimilarities with respect to the sequence of their promoter regions, suggesting different transcriptional control. The fact that IL-1β has an effect only on MMP-9 is not surprising. Sequencing of the promoter region reveals two AP-1 sequences and three motifs homologous to the binding site for NFκB, that is known to mediate IL-1β action (Shirakawa *et al.*, 1989). Interestingly, these binding sites could not be found in the promoter region of MMP-2. This may explain the discrepancies in the differential regulation of the two type IV collagenases.

It is important to remember that the actions of cytokines can be profoundly influenced by the milieu in which they act and especially by the presence or absence of other biologically active factors. Under natural conditions a cell rarely, if ever, encounters only one cytokine at a time. Rather, a cell is likely to be exposed to a cocktail of several cytokines and other biologically active agents, the resulting biological action reflecting various synergistic and antagonistic interactions among the agents present. TPA was included in this study because its effect on MMP-9 has been well documented, however in the placenta it would be physiologically irrelevant. TPA is a known inducer of MMP-9 and has been associated with increased invasiveness in many cell types and this study shows trophoblasts are no exception, as it was found to be a potent inducer of MMP-9 mRNA and protein.

4.8.2 Effects of ECM components on MMP expression

The interactions of cells with components of the ECM, such as fibronectin, laminin, tenascin, and collagens of many types, play an important role in tissue remodeling (Liotta *et al.*, 1986). Temporal, spatial, and cell type-specific regulation of the expression of this large variety of ECM molecules and their receptors provides a powerful set of mechanisms for generating diversity required for the proper orchestration of cell behaviour during invasion and tissue remodeling *in vivo*. Cellular invasion of the basement membrane is a multistep process in which cells recognise integral components of this structure and attach to them. Attachment to the basement membrane triggers expression of genes necessary for the invasive cells to detach from the basement membrane, degrade it and migrate through it. Many integrins are known to be capable of signal transduction, mediating between the ECM and the cell interior via its receptor (Shimizu *et al.*, 1990). We decided to investigate the effect of culturing the trophoblast cell lines on various different matrices on MMP expression. The matrices tested included plastic, laminin, fibronectin, type IV collagen and type I collagen.

Although the repertoire of MMPs expressed by the trophoblast cells was not influenced by adhesion of the cells to the various substrates, depending on the nature of the substrate, regulatory effects were seen. MMP-9 expression was upregulated by laminin but there was no effect on MMP-2. Similar results were reported by Emonard *et al.* (1990), who found that when laminin binds to normal as well as malignant trophoblast cells, it stimulates type IV collagenase activity. Laminin has also been shown to increase the release of MMP-9 from malignant cells (Turpeenniemi-Hujanen *et al.*, 1986). Binding to laminin, a basement membrane component, may be important in cellular attachment to the basement membrane (Terranova *et al.*, 1983). The role of laminin in the attachment of trophoblasts cells to basement membranes has not been fully determined. However, trophoblast cells attach better to laminin-coated substrates than to plastic surfaces (Loke *et al.*, 1989a). Interestingly, trophoblast cells (Yagel *et al.*, 1988) as well as tumour cells (Kleinman *et al.*, 1987), have also been shown to produce laminin, indicating an autocrine form of MMP regulation. The results from this study suggests that trophoblasts binding to laminin, which comprises the first step of basement membrane invasion, can induce the second step, namely dissolution of the ECM by MMPs.

4.8.3 Effects of progesterone and β-estradiol on MMP expression

Hormonal regulation of MMPs is an area of research which has been the focus of much interest recently, particularly in the reproductive system. The reproductive organs are distinctive in their requirement for dramatic alterations in structural and functional properties throughout adult life, pregnancy being just one such event. As described in the introduction (section 4.5) it is generally believed that these changes, which are orchestrated by hormones, are frequently mediated by local cytokines and growth factors. Progesterone has been demonstrated to inhibit MMP-9 secretion from endometrial cells, myometrium, and cervical fibroblasts (Hulboy et al., 1997). We found that progesterone, even in the presence of β-estradiol, had no effect on MMP-9 mRNA levels in the trophoblast cell lines. However, this is in contrast with a recently published paper which reports the down-regulation of MMP-9 expression by progesterone in a dose-dependent fashion by first trimester trophoblasts in short term culture (Shimonovitz et al., 1998). This difference in results may be due to differences in trophoblast populations between our continuous cell lines and the primary trophoblast cells used in that study. It is possible that the continuous cell lines have become refractory to the effect of progesterone, due to the length of time they have been in culture. β-estradiol was also found to have no effect on MMP expression. β-estradiol has been shown to stimulate transcription of *c-jun* and *junB*, components of AP-1 (Hulboy et al., 1997). Since AP-1 is an important factor in expression of MMPs, the apparent failure of β-estradiol to strongly affect MMP transcription emphasizes the complexity of hormonal regulation of MMP gene expression.

4.8.4 Effect of Hypoxia on MMP expression

Previous studies have suggested that hypoxia can specifically alter a cell's gene expression and protein repertoire (Krtolica et al., 1996, Genbacev et al., 1996). Here, we report the result of an experiment in which we analysed the effects of varying the oxygen content of the culture atmosphere on MMP expression of trophoblasts *in vitro*. The impetus for this experiment was the dramatic changes in oxygen content of the placental environment that occur during early gestation. Rodesch et al., (1992) used a polarographic oxygen electrode to show that the mean oxygen pressure of the intervillus space was 17.9+/-6.9 mm Hg at 8-10 weeks gestation as compared with 39.6+/-12.3 in the endometrium. By 12-13 weeks the oxygen pressure in the intervillus space was

60.7+/-8.5 mm Hg, a reflection of the fact that trophoblasts were beginning to substantially remodel the spiral arterioles. Our results showed that hypoxia did not alter the cells expression of MMPs. No difference was observed between cells cultured under normal or hypoxic conditions. A report by Genbacev *et al.* (1996) reported that hypoxia decreased trophoblast invasion *in vitro,* however they did not examine MMP expression. It is possible that hypoxia alters some other factor that is critical to the balance between the forces that promote or inhibit invasion other than MMPs.

4.8.5 Effects of cytokines on TIMP-1 expression

The TIMP-1 gene has been reported to be inducible at the transcriptional level in response to cytokines and TPA in many tumour cell lines (Leco *et al.,* 1994, Stetler-Stevenson *et al.,* 1990b). Its regulation in normal cell lines have not been examined to date. We investigated the effect of IL-1β, EGF, TGF-β and TPA on TIMP-1 expression at the mRNA level in trophoblast cell lines. All three cytokines had no effect on TIMP-1 levels, however TPA down-regulated TIMP-1 expression. Although the effect of TPA is irrelevant *in vivo,* this demonstrates that TIMP-1 expression can be modulated. It is also interesting that IL-1β which caused an upregulation of MMP-9 has no effect on TIMP-1, since it is this type of discoordinate regulation *in vivo* which can tip the balance between proteinase and inhibitor activities to favour degradation of the ECM.

4.9 SUMMARY

The results presented in this chapter focused on studies of the regulatory mechanisms controlling expression of MMP-9, which is important for understanding the complex process of invasion which takes place during pregnancy. We have examined the effects of three cytokines on trophoblast MMP production as possible regulators of the invasive process. Of those tested, only IL-1β had a significant effect, upregulating expression of MMP-9 at both the mRNA and protein level, and increasing the invasive ability of the cells. The trophoblasts were also grown on various ECM matrices, and laminin was found to upregulate MMP-9 mRNA expression. Other biological modifiers including progesterone, β-estradiol and hypoxic culture conditions were shown to have no effect on MMP expression in the trophoblast cell lines.

Considering that the type IV collagenases are developmentally regulated during placentation and also the differential response of these two proteinases to cytokine regulation, it is tempting to speculate that cytokines and ECM components, in particular IL-1β and laminin are involved in controlling MMP-9 activity throughout placentation. Thus, we hypothesized that IL-1β and laminin, produced by the trophoblast cells or the neighbouring decidua, may constitute mediators of trophoblast invasion by regulation of the type IV collagenase system. Given the well established activity of IL-1β system at the feto-maternal interface (Yagel *et al.*, 1989b, Librach *et al.*, 1994), demonstration of regulatory effects of IL-1β on MMP-9 provides additional indirect support to the hypothesis that IL-1β may play a meaningful intermediary role in the trophoblast invasion process.

Chapter 5

Localisation of placental MMP expression *in vivo* and investigation of the role of MMPs in pre-eclampsia.

5.1 Structure of The Placenta

During human pregnancy, the uterus and placenta undergo extensive growth and remodeling which requires both the degradation and synthesis of ECM components. A description of the events surrounding placental formation has already been given in chapter 3 (section 3.1 and 3.3). As described earlier the trophoblast gives rise to two layers, an inner cytotrophoblastic layer of mononuclear cells and an outer syncytiotrophoblastic layer derived by fusion of cytotrophoblastic cells to form a continuous multinucleate syncytium in which there is no internal cytoplasmic demarcation by plasma membranes. Within a short time, a sponge-like network of spaces called inter-villus spaces develops surrounding the syncytiotrophoblasts, initially filled with tissue fluid and uterine secretions. Soon afterwards, invasion by the trophoblasts causes disintegration of endometrial capillaries expanding the inter-villus spaces and establishing an arterial supply and venous drainage system. Primary chorionic villi grow out to the periphery and spread out over the interface between the trophoblast and endometrium forming the cytotrophoblastic shell. Side branches grow out into the inter-villus spaces, progressively forming the complex villus structure characteristic of the mature placenta. By about two weeks after implantation, primitive blood vessels begin to develop in the chorionic mesoderm simultaneously with development of the primitive embryonic circulatory system, the embryo now being too large to rely on mere diffusion for its growth and metabolic requirements. Each villus contains a mesenchymal core containing capillaries severed by afferent and efferent fetal blood vessels. Between the villous capillaries and the maternal blood is a continuous layer of syncytiotrophoblast cells supported by a layer of proliferating cytotrophoblast cells. From the fourth month onwards, the cytotrophoblast layer becomes less active and atrophic so that by fetal maturity it is present in very little of the villous surface. As more and more branches are added to the villous tree, the villi become smaller and smaller and the tissue barrier between fetal capillaries and maternal blood is greatly diminished. As the placenta develops, the form of the placenta is essentially established by the end of the fourth month after which the placenta grows in diameter, complementing growth in the size of the uterus.

5.2 Pre-eclampsia

Pre-eclampsia/eclampsia, a disorder unique to pregnancy, is characterised by hypertension, proteinuria (protein in urine), generalised edema (clinically evident swelling), and convulsions. It occurs in 5% to 10% of all pregnancies, primarily in primigravidas (first pregnancy), after the 20th gestional week and most frequently near term (Smith, 1993). Pre-eclampsia may progress rapidly without warning to the convulsive phase termed eclampsia, one of the most dramatic and life-threatening complications of pregnancy. In addition to the effects on the mother, there is a profound impact on the fetus, resulting in increased perinatal mortality and frequent intrauterine growth retardation. Factors associated with an increased risk for this disorder include nulliparity, diabetes, chronic hypertension, multiple gestation, and extremes of age (Barron, 1992). A familial tendency to pre-eclampsia has been suggested, and studies have provided evidence that the disease is heritable (Chesley and Cooper, 1986; Cooper et al., 1988). The specific contribution of environmental versus genetic factors is, at present unknown. Immunological mechanisms have also been implicated, supported by evidence that pre-eclampsia is associated with the primigavid state, pregnancies with a different partner, and women with a history of using barrier contraception, suggesting that previous antigen exposure protects against the disease (Smith, 1993). Pre-eclampsia is characterised by derangements in the structure and function of many organs. Histopathologic studies of placental bed biopsies from patients with pre-eclampsia have revealed diminished trophoblastic invasion of the uterine spiral arteries (Redman, 1991).

Despite the widespread occurrence of pre-eclampsia, the underlying cause or causes of this disease remain elusive. Recently, several theories have implicated humoral factors, particularly those of placental origin, as initiators of the disease process. In normal pregnancy, implantation of the embryo is followed by migration of trophoblastic cells into the walls of the uterine spiral arteries. These vessels undergo marked remodeling, thereby losing their muscular media in order to serve as passive conduits to accommodate a 10-fold increase in uterine blood flow to the growing uterus. Invading trophoblasts in the first half of pregnancy are believed to convert these muscular, narrow-lumen arteries into distended, thin-walled vessels by replacing their endothelia and digesting their subjacent musculoelastic lamina (Robertson et al., 1986). It is thought that this process, which normally occurs between the 10th and 20th gestational weeks, fails to occur in pre-eclampsia and as such is one of the earliest pathophysiologic events in the development of the

disease. In women with pre-eclampsia the trophoblast migration is inhibited and the spiral arteries retain their non-pregnant morphology (musculoelastic structure and endothelium) which predisposes the spiral arteries to vascular spasm and subsequently restricted blood flow to the placental-fetal unit (Serra-Serra *et al.*, 1992). This failure in women who develop pre-eclampsia to undergo the normal pregnancy-induced remodeling that converts these vessels to high volume-low resistance conduits leads to vascular spasm, restricted blood flow, placental ischaemia and the release of toxic substances that enter the maternal circulation, resulting in multi-organ disease. It is unclear which factors regulate trophoblastic migration. However, it is our hypothesis that the MMPs are expressed abnormally in pre-eclampsia, leading to reduced trophoblast invasion, consequently resulting in decreased placental perfusion and the onset of pre-eclampsia.

For many years the placenta has been recognised as an essential component of the disease process, underscored by the fact that only after delivery of the placenta do the signs and symptoms of pre-eclampsia begin to abate (De Groot *et al.*, 1993). In addition, the disease can also complicate some cases of hydatidiform mole where the uterus contains only disordered placental tissue (Redman, 1991). Thus, a fetus is not necessary, only the placenta. Since pre-eclampsia is clinically manifested as a multiple organ disorder, it has been proposed that reduced placental perfusion results in liberation of cytotoxic factors, leading to a generalized activation of maternal vascular endothelial cells which, in turn, result in a systemic maternal syndrome. The nature of the substances toxic to endothelial cells in pre-eclampsia remains speculative. Because of the critical position and actions of endothelial cells throughout the entire vascular tree, dysfunction of these cells can lead to multi-organ failure. Figure 5.1 outlines our proposed pathophysiology of pre-eclampsia.

Since our knowledge of the precise causes of pre-eclampsia is severely limited, therapy up to now has been oriented purely toward alleviating symptoms. Several approaches to the lowering of blood pressure and improvement of blood flow properties have been proposed, including calcium or magnesium supplementation and, most promisingly low-dose aspirin. The premise is that small amounts of aspirin produce a greater inhibition of thromboxane production than that of prostacyclin in both pregnant women and their fetuses thus preventing or reversing the vasodilator-vasoconstrictor eicosanoid imbalance that may underlie much of the pathophysiology of endothelial cell injury (Benigni *et al.*, 1989). Delivery is the only definitive therapy for pre-

eclampsia, all else is palliation. If pre-eclampsia or severe hypertension occurs beyond the 36th week of gestation, a point at which fetal pulmonary maturity has generally occurred, then delivery is clearly in the best interest of both mother and fetus. Delivery is also indicated regardless of gestational age if there is evidence of advanced maternal disease or impending eclampsia, because progression is virtually inevitable unless the uterus is evacuated.

The first stage of the disease lies in the placenta at the time of placentation. The second stage, the clinically overt illness, is a sequel of placental ischaemia, secondary to spiral artery insufficiently. The stage is set during the establishment and development of placentation in the first and second trimesters. Currently the final proof that this model is correct is lacking. However, it is clear that studying the placenta and in particular the trophoblasts will reward those who want to understand pre-eclampsia better.

Primary cause of pre-eclampsia ?

Decreased MMP expression

Inadequate trophoblast invasion of maternal spiral arteries

Decreased placental perfusion

Placental production of endothelial toxin(s)

Endothelial cell injury/activation

Signs and symptoms of pre-eclampsia/eclampsia.
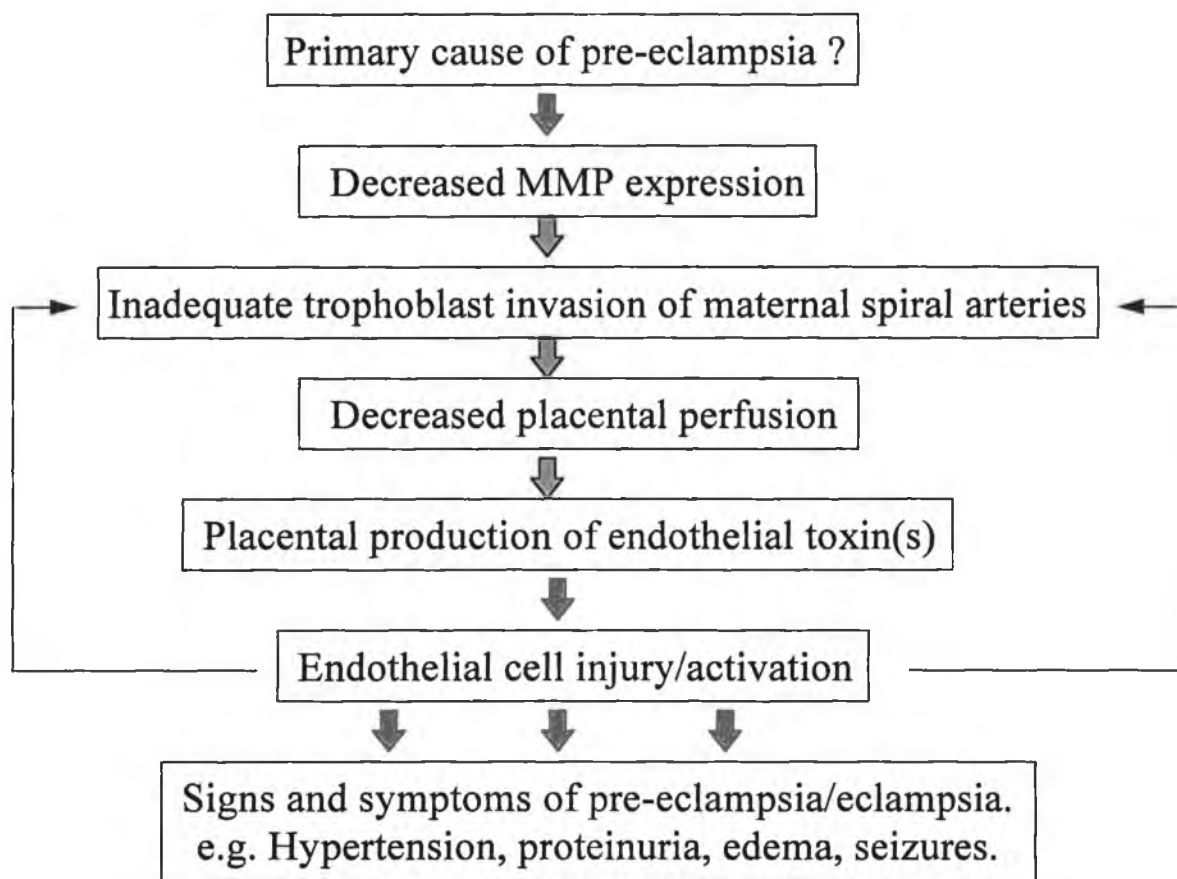e.g. Hypertension, proteinuria, edema, seizures.

Figure 5.1 Proposed pathophysiology of pre-eclampsia.

5.3 MMPs and Pre-eclampsia

It is only recently that interest has focused on the role of MMPs in the etiology of pre-eclampsia, the premise being that abnormal uteroplacental blood flow in pre-eclampsia might result from altered expression of MMPs by trophoblasts. A recent study showed that trophoblasts isolated from pre-eclamptic placentas (n=9) failed to modulate MMP-9 expression at the mRNA and protein levels compared to cells from normal placenta (n=8). Their invasive potential was also reduced (Lim *et al.*, 1997). Graham and McCrae (1996) carried out a similar study on cells isolated from normal (n=10) and pre-eclamptic (n=10) pregnancies and reported that cells from both cases secreted variable levels of MMP-9, but the cells from the pre-eclamptic placentas predominantly secreted MMP-9 in the inactive form. A report by Kolben *et al.* (1996) examined MMP-9, MMP-8 and TIMP-1 levels in placental extracts from control and pre-eclamptic/eclamptic placentas (n=21). In pre-eclampsia/eclampsia, they found significantly decreased MMP-9 concentrations in placental extracts. No significant difference in placental extract between pre-eclamptic and control groups was seen for MMP-8 or TIMP-1. These reports of decreased MMP-9 expression or expression of the zymogen form of the enzyme may be the manifestation of impaired compensatory transformation processes when there is circulatory placenta dysfunction due to pre-eclampsia.

The aim of this investigation was to localise expression of MMP protein in normal human placental biopsies using immunohistochemistry. In addition, we sought to examine potential changes in MMP expression in human placental tissue obtained from the pre-eclamptic disease state.

5.4 RESULTS

5.4.1 Placental Biopsies

Placental biopsies were obtained from the Pathology Department, Holles Street Maternity Hospital, Holles Street, Dublin. 9 normal and 10 pre-eclamptic samples were included in this study. All tissue sections had been examined and classified by Prof. Peter Kelehan. Status of placental biopsy specimens is given in Table 5.1.

Table 5.1 Placental Biopsy Specimens

Section No.	Status
12/3/95	Pre-eclamptic
12/6/95	Pre-eclamptic
12/34/95	Pre-eclamptic
12/78/95	Pre-eclamptic
12/127/95	Pre-eclamptic
12/237/95	Pre-eclamptic
12/267/95	Pre-eclamptic
12/420/95	Pre-eclamptic
12/455/95	Pre-eclamptic
12/489/95	Pre-eclamptic
12/54/95	Normal
12/109/95	Normal
12/112/95	Normal
12/142/95	Normal
12/186/95	Normal
12/193/95	Normal
12/345/95	Normal
12/357/95	Normal
12/422/95	Normal

5.4.2 Characterisation of antibodies by western blot analysis

The three antibodies used in this study; anti-MMP-2, -MMP-7 and -MMP-3 were first tested by western blot analysis (section 2.2.5) to ensure that each antibody specifically recognised its antigen. A sample of PMA-treated HT1080 conditioned medium spiked with recombinant MMP-7 or MMP-3 fusion protein was separated by SDS-PAGE, transferred to nitrocellulose and probed with each antibody. It was crucial to determine that the antibodies did not cross react and were suitable for immunohistochemistry. Figure 5.2 shows that all three antibodies specifically recognised only the antigen to which they were raised, with non-specific staining not detected.
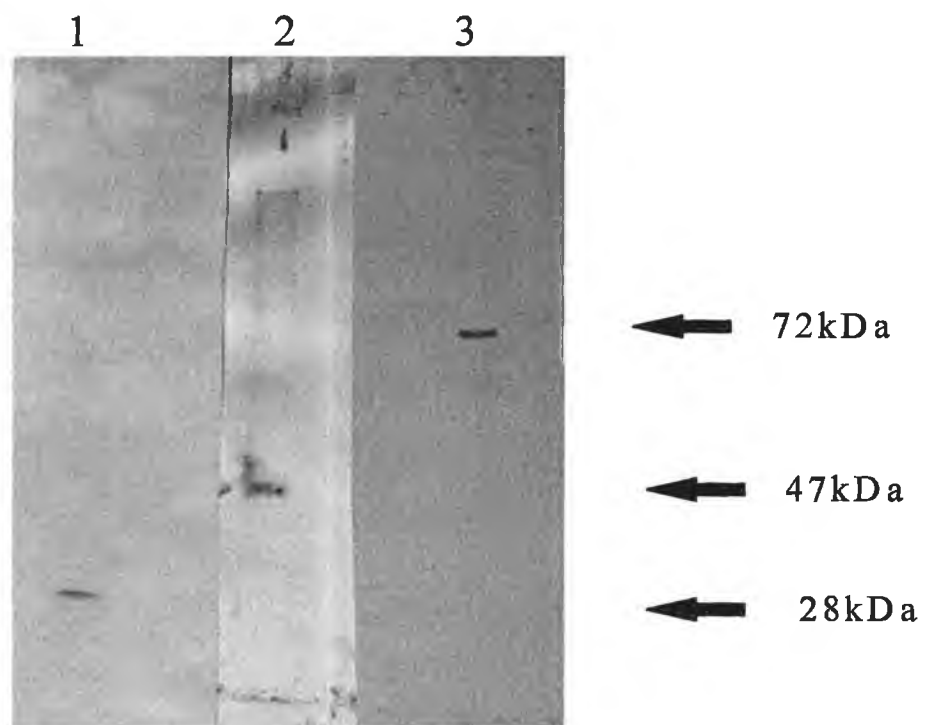

Figure 5.2 Western blots assessing the suitability of three antibodies for immunohistochemistry. Blot (1) probed with anti-MMP-7 antibody; (2) probed with anti-MMP-3 antibody; (3) probed with anti-MMP-2 antibody (GL-8).

5.4.3 Immunohistochemistry

5μm sections were cut from formalin-fixed, paraffin-embedded tissue blocks of placental biopsies. Prior to probing with the antibody, the sections were dewaxed in xylene and rehydrated by passage through a series of graded alcohols. The immunohistochemistry procedure was performed as outlined in section 2.2.10. Following development with DAB (positive reaction produced a brown colour product), the sections were counterstained with haematoxylin, mounted and viewed. For each specimen and antibody, control sections were incubated with pre-immune serum in place of the antibody. Little or no background staining was seen in all control sections. A section of each specimen was also stained with haematoxylin and eosin (H&E) to demonstrate histological features. Figures 5.3-5.10 show micrographs depicting examples of the staining patterns observed and showing the most prominent features of the placental biopsies. In general a similar staining pattern was seen for MMP-2, MMP-7 and MMP-3. This consisted of intense staining of syncytiotrophoblasts (Figures 5.4, 5.5, and 5.8) and light staining of stromal core areas of chorionic villi (Figure 5.6). Staining for MMP-2 was also seen localised around the periphery of fetal villous capillaries as shown in Figure 5.7. Specimens recovered from pregnancies complicated by pre-eclampsia showed a pattern of staining for MMP-2, MMP-7 and MMP-3 that did not differ from that noted for the placental samples recovered from uncomplicated pregnancy (Figures 5.9 and 5.10).

Four different antibodies to MMP-9 were used in the immunohistochemical procedure. However, none were found to be suitable for use on the formalin fixed paraffin embedded sections as no staining was achieved with any of the MMP-9 antibodies. Three antibodies were supplied by Oncogene Science and the fourth by Cell Tech. All four antibodies were tested at various concentrations for varying incubation periods and temperatures but without success. A modification of the immunohistochemical procedure which favours antigen retrieval (section 2.2.10.1) in formalin fixed paraffin embedded sections was made but this did not effect the outcome. Antibodies to TIMP-1 and TIMP-2 were also found to be unsuitable for immunohistochemistry of the formalin fixed sections. The immunohistochemical staining results are summarized in Tables 5.2 and 5.3.
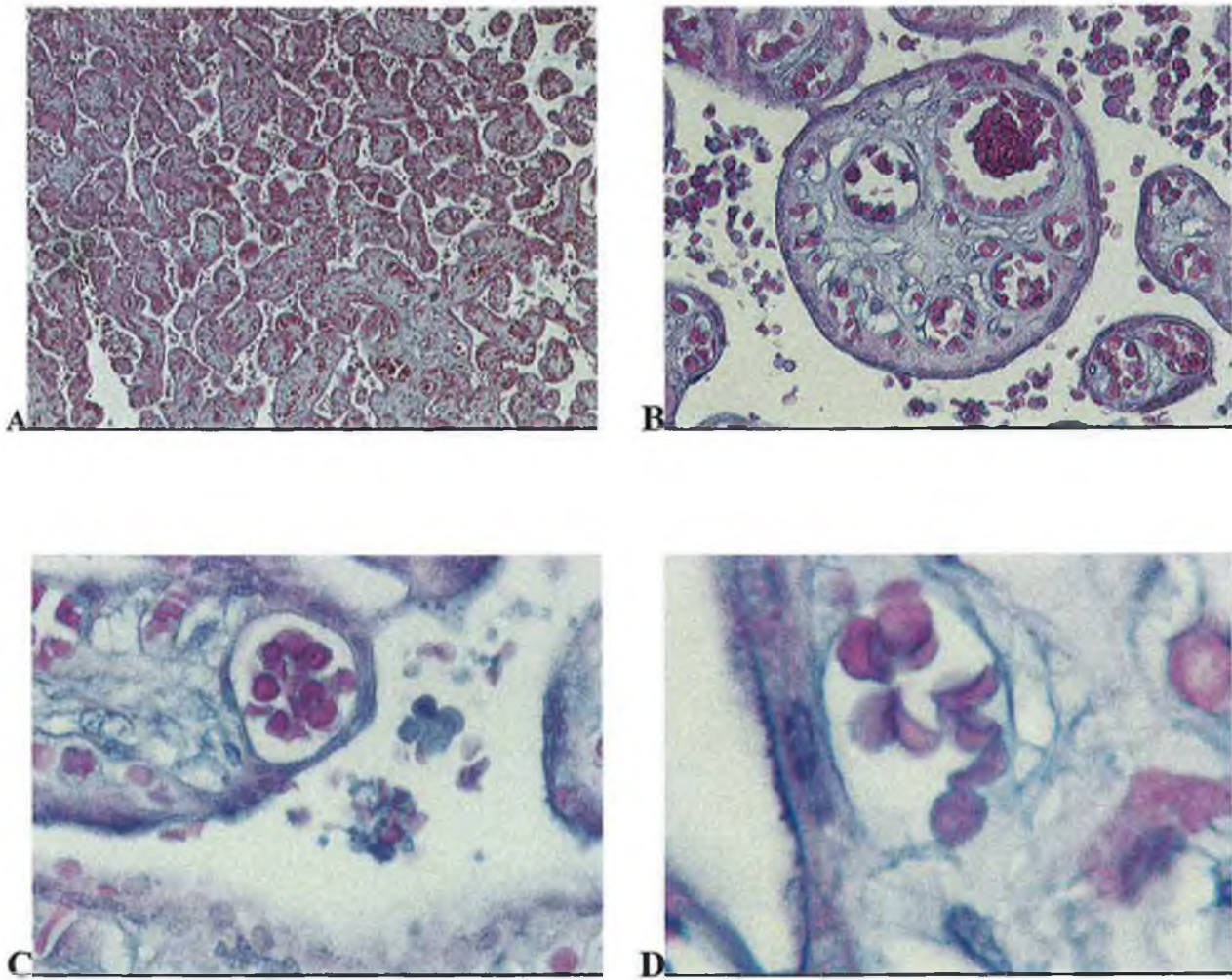
Figure 5.3 Micrographs illustrating features of the placenta from a full term fetus using H&E staining. **(A)** 40X magnification showing the large number of chorionic villi; **(B)** 200X magnification shows a highly vascularised floating villus surrounded by maternal blood; **(C)** 400X magnification focuses on a fetal capillary in a villus and the capillary's proximity to maternal blood cells; **(D)** 1000X magnification again focusing on a fetal capillary showing that there is only a single layer of syncytiotrophoblast separating the fetal capillary from maternal blood.
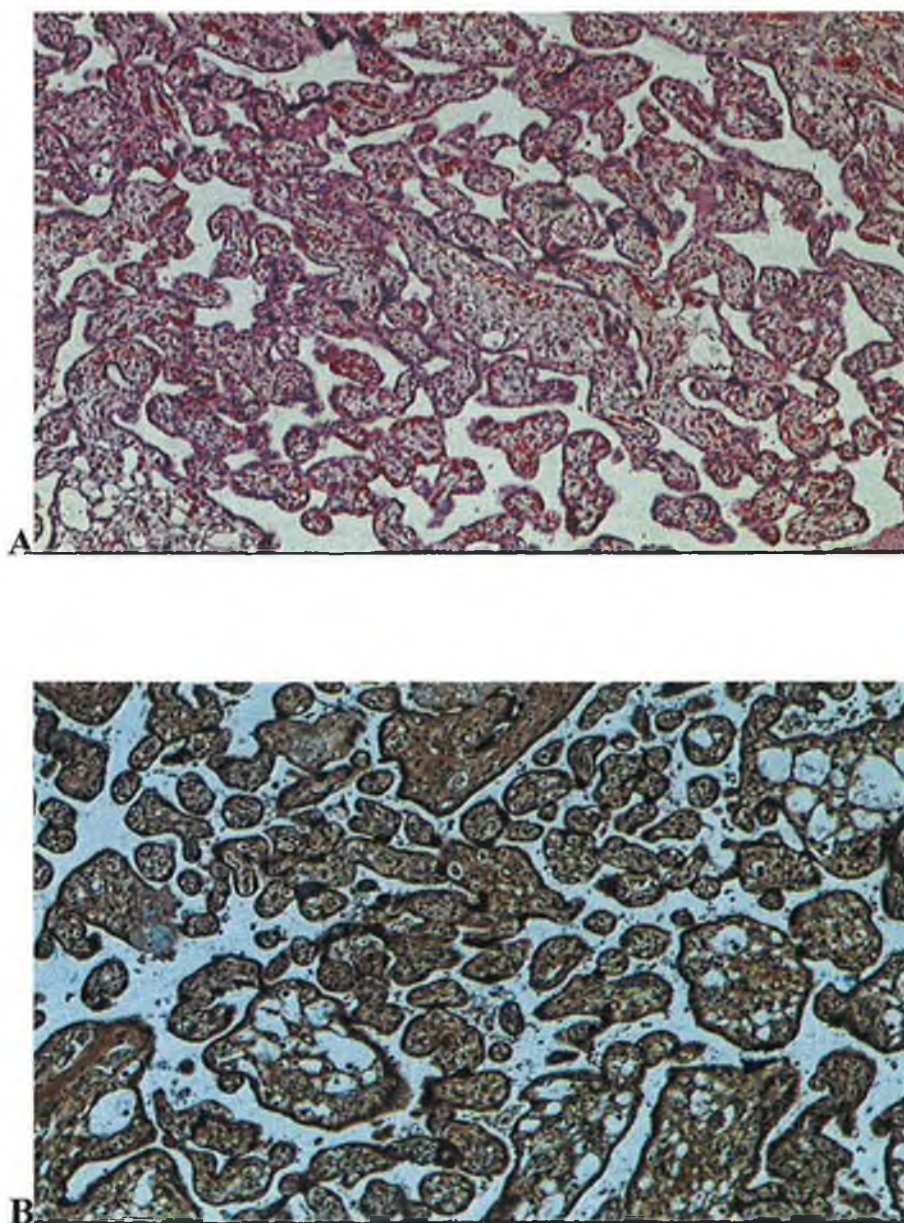
Figure 5.4 Localisation of MMP-2 staining in floating villi. **(A)** H&E staining of floating villi; **(B)** MMP-2 staining of syncytiotrophoblasts and stromal core of floating villi.[40X magnification].
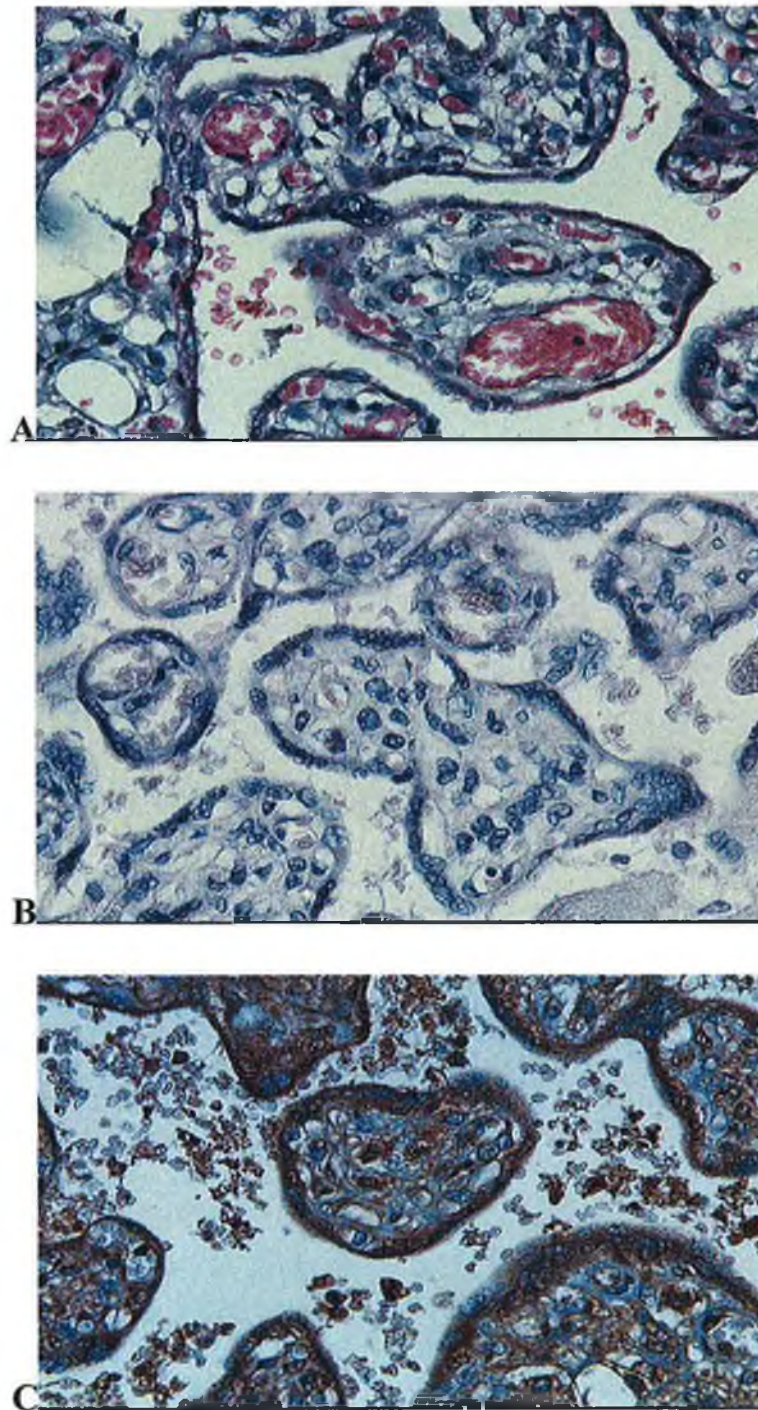
Figure 5.5 Localisation of MMP-2 in floating villi of placental biopsy. **(A)** H&E staining; **(B)** Pre-immune serum used; **(C)** Staining of MMP-2 in syncytiotrophoblasts of floating villi. Counterstained with haematoxylin; [200X magnification].
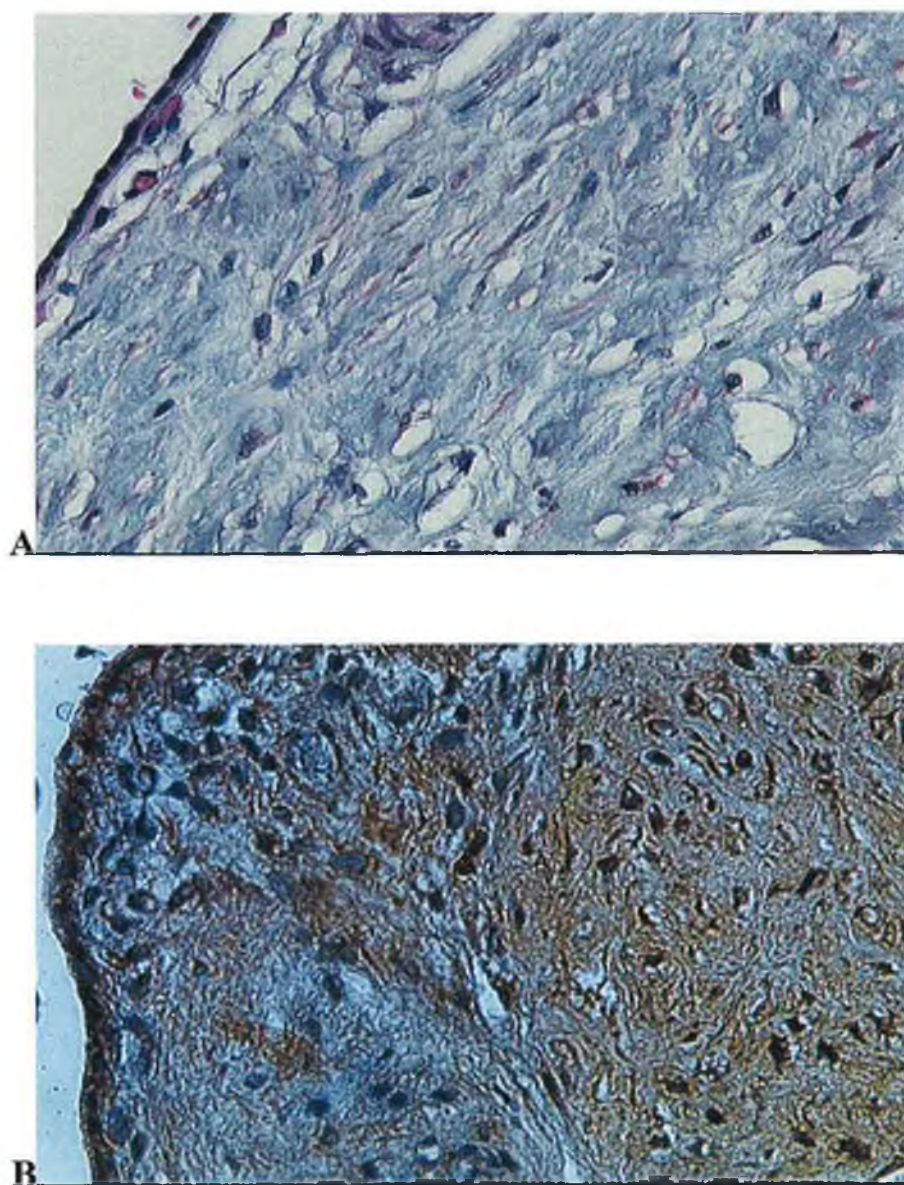
Figure 5.6 Localisation of MMP-2 in stromal core of villi. **(A)** H&E staining of stromal core region of villi; **(B)** MMP-2 staining of stromal core of villi, staining can also be seen at outer layer of syncytiotrophoblasts. [200X magnification]
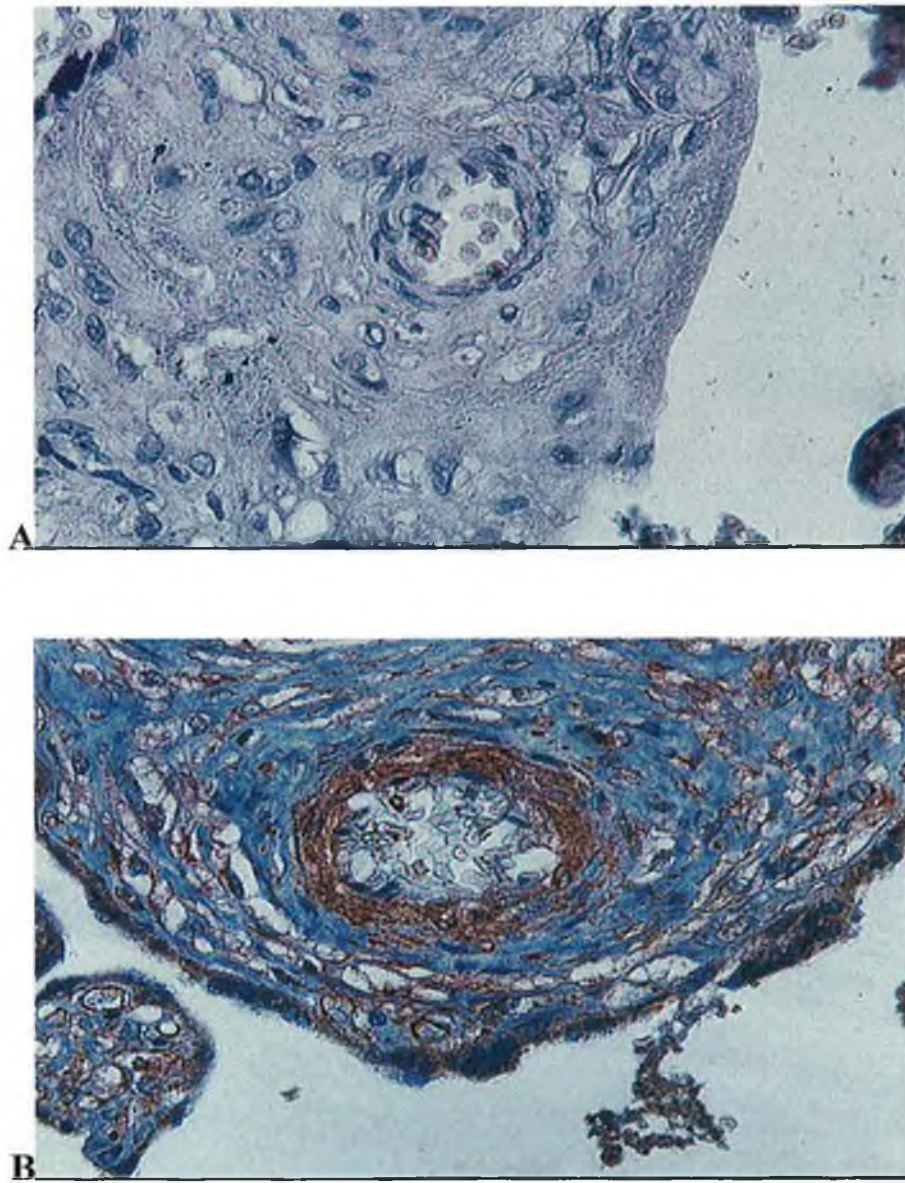
Figure 5.7 Localisation of MMP-2 in placental biopsy. **(A)** No staining when pre-immune serum used in place of antibody [200X]; **(B)** Staining for MMP-2 localised at the periphery of villus capillary [200X]
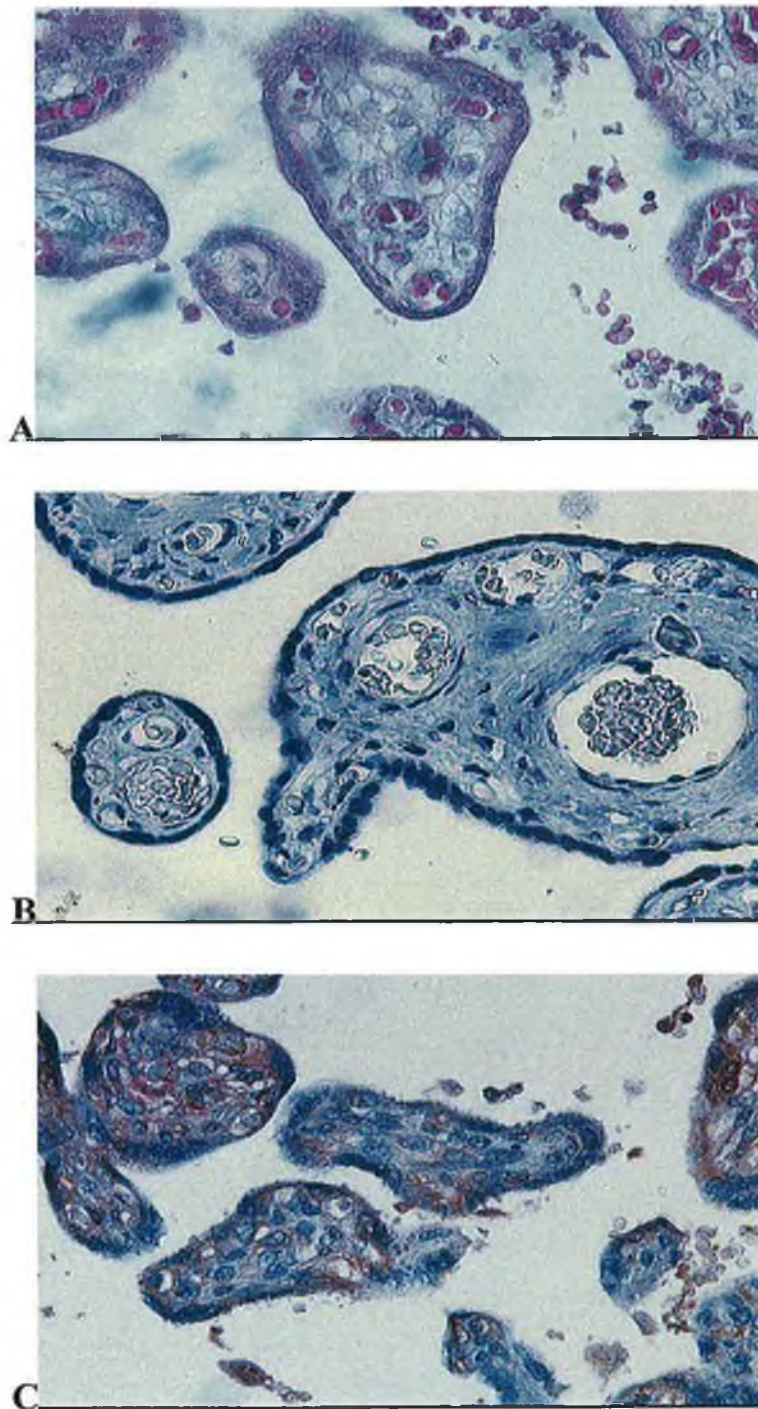
Figure 5.8 Localisation of MMP-3 in floating villi. **(A)** H&E staining of floating villi; **(B)** Pre-immune serum; **(C)** Staining of MMP-3 localised to syncytiotrophoblasts and some stromal staining in floating villi.[200X magnification].
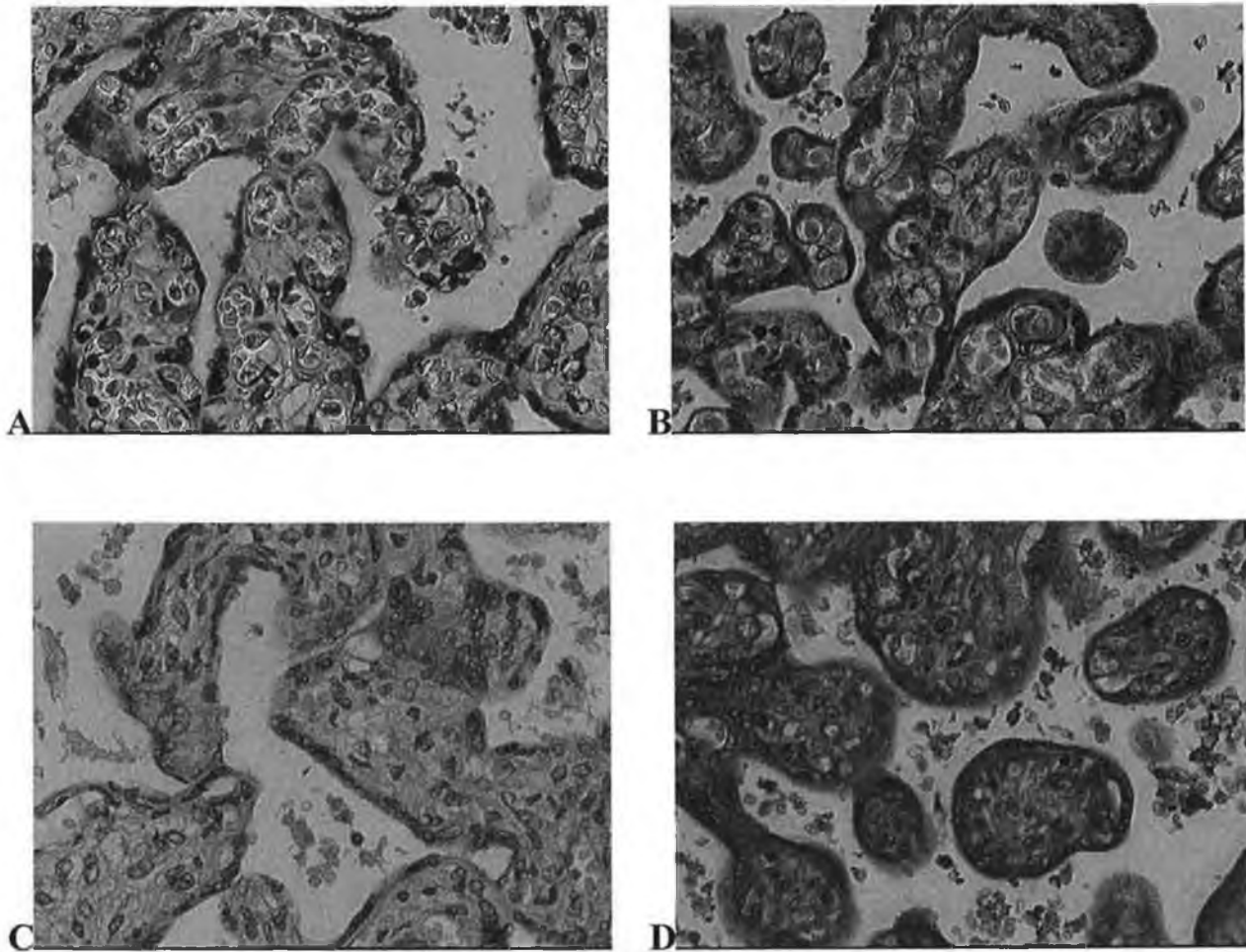
Figure 5.9 Comparison of MMP-2 expression in control and pre-eclamptic placentas. **(A) and (B)** Biopsy from normal placenta (section 12/127/95); (A) incubated with pre-immune serum; (B) MMP-2 staining of syncytiotrophoblasts in floating villi. **(C) and (D)** Biopsy from a pre-eclamptic placenta (section 12/375/95); (C) incubated with pre-immune serum; (D) MMP-2 staining of syncytiotrophoblasts in floating villi. [200X magnification]
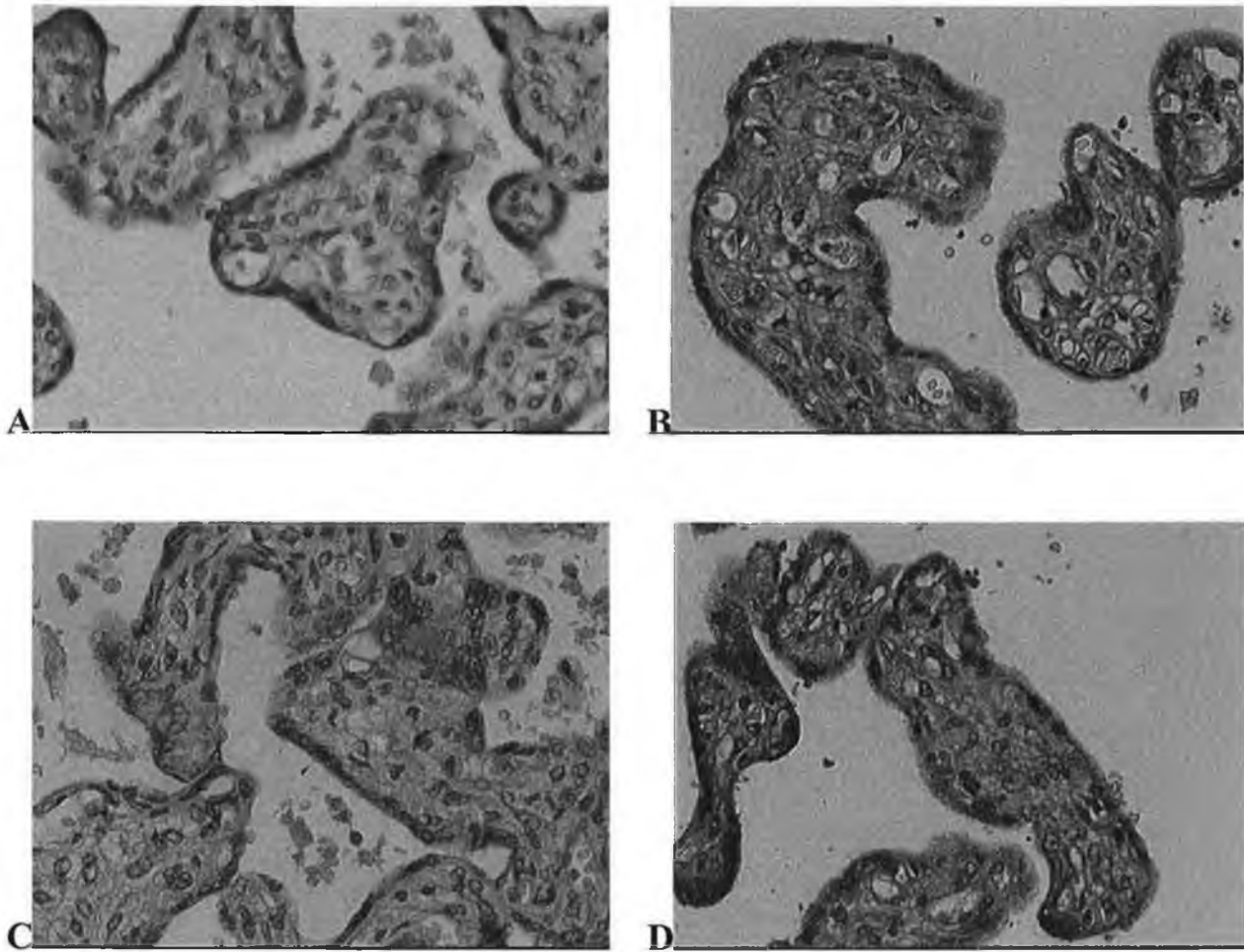
Figure 5.10 Comparison of MMP-7 expression in control and pre-eclamptic placentas. **(A) and (B)** Biopsy from normal placenta (section 12/112/95); (A) incubated with pre-immune serum; (B) MMP-2 staining of syncytiotrophoblasts in floating villi. **(C) and (D)** Biopsy from a pre-eclamptic placenta (section 12/34/95); (C) incubated with pre-immune serum; (D) MMP-2 staining of syncytiotrophoblasts in floating villi. [200X magnification]

Table 5.2 Summary of staining patterns observed for MMP-2, MMP-7 and MMP-3 protein expression in normal human placental biopsies.

MMP	General Staining Pattern Observed
MMP-2	Predominately strong staining of syncytiotrophoblasts in floating villi, weak staining of stromal core areas of villi, and localised staining around periphery of fetal blood vessels in villi.
MMP-7	Moderate staining of syncytiotrophoblasts in floating villi and weak staining of stromal core areas of villi.
MMP-3	Moderate staining of syncytiotrophoblasts in floating villi and weak staining of stromal core areas of villi.

Table 5.3 Staining intensity of MMP-2, MMP-7 and MMP-3 in normal uncomplicated and pre-eclamptic placental biopsies.

Specimen No.	Status	Intensity of MMP-2 staining		Intensity of MMP-7 staining		Intensity of MMP-3 staining	
		STB	villus core	STB	villus core	STB	villus core
12/3/95	Pre-eclamptic	++	+	++	−	++	+
12/6/95	Pre-eclamptic	+++	+	++	+	++	+
12/34/95	Pre-eclamptic	+++	+	++	+	+	+
12/78/95	Pre-eclamptic	+++	+	+	−	++	+
12/127/95	Pre-eclamptic	+++	+	++	+	++	+
12/237/95	Pre-eclamptic	++	+	+	−	+	+
12/267/95	Pre-eclamptic	+++	+	++	+	++	+
12/420/95	Pre-eclamptic	++	+	+	+	+	+
12/455/95	Pre-eclamptic	+++	-	++	-	++	−
12/489/95	Pre-eclamptic	++	+	+	+	+	+
12/54/95	Normal	++	+	+	+	+	+
12/109/95	Normal	+++	+	++	+	+	−
12/112/95	Normal	+++	+	++	−	++	+
12/142/95	Normal	+++	+	+	+	+	+
12/186/95	Normal	++	+	+	−	+++	+
12/193/95	Normal	+++	+	++	+	++	+
12/345/95	Normal	++	+	+	−	+	+
12/357/95	Normal	+++	+	+++	+	+	+
12/422/95	Normal	+++	+	++	+	++	+

The relative intensity and amount of staining of either syncytiotrophoblasts (STB) or villus core tissue in each section was assessed by eye and scored accordingly. '-' indicates no staining observed; '+' indicates low levels of staining; '++' indicates moderate staining; '+++' indicates a high level of intense staining.

5.5 DISCUSSION

In this investigation, we examined the localisation of MMP-2, MMP-7 and MMP-3 in normal human placenta. In addition, human tissue obtained from pregnancies complicated with pre-eclampsia was evaluated to access potential changes in MMP expression in human placenta obtained from this disease state. Our initial hypothesis was that the abnormal uteroplacental blood flow in pre-eclampsia (as a result of shallow invasiveness of the uterine arteries) might result from altered expression of MMPs by trophoblasts. Ideally placental biopsies would be obtained from young (i.e., first trimester) tissue, however it is impossible to predict whether pre-eclampsia will develop later in pregnancy. Therefore, in order to study trophoblast from pre-eclampsia-complicated pregnancies, we had to rely on material obtained from late (i.e., term or near-term) pregnancies.

A section from each specimen was stained with H&E to demonstrate histological features. H&E is the most commonly used technique in animal histology and routine pathology. The basic dye, haematoxylin, stains acidic structures a purplish blue. Nuclei, ribosomes and rough endoplasmic reticulum have a strong affinity for this dye owing to their high content of DNA and RNA respectively. In contrast, eosin is an acidic dye which stains basic structures red or pink. Most cytoplasmic proteins are basic and hence cytoplasm usually stains pink or pinkish red. In general, when the H&E staining technique is applied to animal cells, nuclei stain blue and cytoplasm stains pink or red.

The micrographs in Figure 5.3 illustrate the main structures of normal placenta from a full term fetus. At low magnification in micrograph (A), vast numbers of villi can be seen cut in various planes of the section and varying in diameter from main stem villi to very small terminal branch villi. Compared with early placenta, the villous pattern of term placenta is much more highly developed and the average villous diameter much less, reflecting the extensive branching growth of the villi as the placenta enlarges. Micrograph (B) demonstrates a villus at higher magnification. Compared to early placenta there is marked vascularity of the villous cores and greatly increased villous surface area exposed to the inter-villus spaces filled with maternal blood. Micrograph (C) focuses on a small branch villus and highlights the proximity of blood in fetal capillaries to maternal blood in the surrounding inter-villus space. The trophoblast is reduced to a thin layer of syncytiotrophoblast only and the capillaries tend to be located in the periphery of the core (micrograph (D)).

Since MMP-9 has been shown to play a critical role in trophoblast invasion it was the MMP of most interest to examine in the placental biopsies. Unfortunately, this was not possible with the antibodies for MMP-9 which are currently available. Four different commercial antibodies for MMP-9 were tested for immunohistochemistry but none were successful on the formalin fixed paraffin-embedded sections which were available to us. One of the antibodies, anti-MMP-9 Ab-3 supplied by Oncogene Science is marketed as being suitable for paraffin-embedded sections but this was found not to be the case in our hands and others (personal communication, Dr. Z. Zeng, Memorial Sloan-Kettering Cancer Center). This antibody has successfully been used on frozen sections however we unfortunately did not have access to frozen tissue specimens. Steps involved in producing paraffin sections are known to cause loss, diffusion, reduction, chemical alteration, or masking of tissue antigens, making immunohistochemistry difficult if not impossible (Herrington and McGee, 1992). Formalin fixation of tissue specimens which involves the formation of methylene bridges between amino groups, can also affect antigenic preservation. Formalin is believed to destroy some antigens completely, while many others undergo some reduction in number and others are masked by the fixation (Herrington and McGee, 1992). We also tested antibodies against TIMP-1 and TIMP-2 supplied by Oncogene Science, however neither were suitable for immunohistochemistry.

Antibodies to MMP-2, MMP-7 and MMP-3 were successfully used for immunohistochemistry. Each antibody specifically recognised its antigen as demonstrated by western blot analysis. MMP-2 was found to be expressed in syncytiotrophoblasts and villus core tissue of chorionic villi. The positive results for MMP-2 in syncytiotrophoblast, suggest that this cell type could be involved in active remodeling of the villi and modulation of matrix deposition. It has also been proposed that since syncytiotrophoblasts are in direct contact with maternal blood as it perfuses the placental intervillous spaces, expression of proteolytic activity by trophoblasts may contribute to the maintenance of blood fluidity at this site (Graham and McCrae, 1996). The positive staining of the villous fibroblasts for MMP-2 suggests that these cells play an important role in the process of production and remodeling of the villous stroma. In agreement with our findings for MMP-2, Polette *et al.* (1994) reported that MMP-2 and TIMP-2 mRNAs remained expressed at comparable levels in both first and third trimesters, supporting the idea that these molecules in addition to their role in trophoblast invasion, may mediate other processes such as extracellular matrix remodeling or vascular network establishment. Indeed, placental vascularization development persists during the late

gestation (Foidart *et al.*, 1992). Previous studies by Autio-Harmeinen *et al.* (1992) and Fernandez *et al.*, (1992) are also consistent with MMP-2 expression in term placentas.

MMP-7 protein was localised to syncytiotrophoblasts of chorionic villi. Weak staining for MMP-7 protein was also seen in stromal core areas of villi similar to MMP-2 staining. Only one previous study had examined MMP-7 protein localization in placental tissue (Vettraino *et al.*, 1996), and reported MMP-7 to be expressed in cytotrophoblasts and syncytiotrophoblasts during early pregnancy but only in cytotrophoblasts by the third trimester. MMP-3 showed a similar pattern of expression to MMP-2 and MMP-7, as staining was localised to syncytiotrophoblasts and villous core areas. Staining for MMP-3 was not as intense as that for MMP-2. Our results for MMP-3 are in agreement with other studies where MMP-3 was detected in syncytiotrophoblasts of last gestation placental biopsies (Vettraino *et al.*, 1996; Fernandez *et al.*, 1992).

Specimens recovered from pregnancies complicated by pre-eclampsia showed a pattern of staining for MMP-2, MMP-7 and MMP-3 that did not differ from that noted for the trophoblast samples recovered from uncomplicated pregnancy. There are no published reports investigating MMP-2 protein expression in placental biopsies from pre-eclamptic placentas. However, a study by Polette *et al.* (1994) examined expression of MMP-9 and MMP-2 by cells of early and term human placenta. They reported that first trimester trophoblast produced MMP-2 which was expressed less at term in these cells, and MMP-9 was no longer detected at term in these cells. For MMP-3, our results are in agreement with a study by Vettraino *et al.* (1996) who reported similar results for MMP-3 expression in normal and pre-eclamptic placentas. However Vettraino *et al.* (1996) reported that MMP-7 was over expressed and was produced by more cell types in placentas from pregnancies complicated by pre-eclampsia, leading the authors to speculate that the proteolytic activity of MMP-7 might contribute to the pathology of the condition. This study was based on only 4 pre-eclamptic specimens. Our finding for MMP-7 are in contrast to this but are based on the results from 10 pre-eclamptic samples which may be more representative of the disease state.

Thus it can be concluded from our study of MMP-2, MMP-7 and MMP-3 expression in term placental biopsies that no correlation can be made between the expression levels of these three MMPs and the pre-eclamptic disease state. It should be kept in mind, however, that the histological findings in the placental biopsy at term represent the end result of invasive events of early pregnancy. Therefore

a study of pre-eclamptic specimens from early gestation might have been more informative had it been possible to do so.

5.6 SUMMARY

Human pregnancy is associated with extensive growth and remodeling of the uterus and placenta. Restructuring of these tissues during specific stages of gestation has been shown to involve the degradative activity of various MMPs. In this investigation, we used immunohistochemistry to identify the cellular localisation of MMP-2, MMP-3 and MMP-7 in human tissues recovered from uncomplicated term pregnancies. Our results show prominent expression of all three MMPs in syncytiotrophoblasts and stromal core areas of chorionic villi at full term. We have shown that the cells composing the human placenta express the necessary MMPs to facilitate growth and tissue remodeling in the physiological progress of human gestation.

In normal human pregnancy, invasion of the uterus and its arterial system by trophoblasts extends through the entire decidua and the adjacent third of the myometrium. In the pregnancy related disorder pre-eclampsia, trophoblast invasion is limited to the superficial decidua, and few arterioles are breached. Since trophoblast invasion is abnormally shallow in pre-eclampsia, it is likely that some component of the invasion process is altered. The purpose of this study was to assess potential changes in MMP expression within human placenta in pre-eclampsia. Placental biopsies from normal pregnancies and those complicated by pre-eclampsia were stained with the anti-MMP antibodies. The results show that expression of MMP-2, MMP-3 and MMP-7 by trophoblasts in pre-eclamptic specimens was similar to MMP expression in normal uncomplicated pregnancy specimens. Therefore, no correlation can be made between the expression levels of these three MMPs and the pre-eclamptic disease state.

Chapter 6

Isolation and Characterisation of Antibody Fragments to MMP-9 using Phage Display Technology

6.1 Introduction to Antibodies

Antibodies are a modular weapon system of humans created by evolution to identify any foreign intruder. The genetic material for this huge 'library' of different antibodies is stored in the B-cell pool of human lymphatic tissue. With no advance knowledge of the molecular shapes of the foreign molecules (antigens) to be encountered in a lifetime, a 'catch-all' molecule or set of molecules has evolved. This antibody repertoire, in humans is generated by recombination events from a relatively small number of germline genes and is believed to contain antibodies recognizing, with moderate affinity, virtually any antigen. The rearrangement of the V gene segments creates a repertoire of virgin B cells, each displaying a single antibody species. Cells are selected by encountering and binding of antigen, and are triggered to differentiate to short-lived plasma cells that secrete antibody and to long-lived memory cells that persist in lymph nodes, spleen, and bone marrow. Many cycles of somatic mutation and selection of the antibody-producing cells in the presence of antigen then act to increase the affinity and abundance of a specific antibody.

The antibody molecule is made up of two identical heavy (H) and two identical light (L) chains held together by interchain disulfide bonds (Figure 6.1A). There are two major classes of L chains, called κ and λ. Both H and L chains can be divided into variable (V) and constant (C) regions. The antibody molecule consists of two Fab regions responsible for antigen binding and an Fc region responsible for effector functions that eliminate antigen. The Fab fragment consists of the entire light chain [composed of the variable (V_L) and constant (C_L) domain] and the first two domains of the heavy chain, the V_H and the C_H1 domains (Figure 6.1B). The smaller Fv fragment consists of V_H and V_L domains combined. The antigen binding site of an antibody is formed by two domains, the V_H and V_L chain variable domains which are on different polypeptide chains. These two variable domains can be expressed on the same polypepetide if they are joined artificially by a flexible linker to form single chain Fv fragments (scFv). Three hypervariable regions, the complementarity-determining regions (CDRs), located on both the heavy and light variable chains are crucial for antigen binding. The great diversity of the natural antibody repertoire is achieved by combinatorial association of many variants of these CDRs.

In 1988 Better and colleagues, demonstrated that single antibody-binding fragments (Fab and Fv) of an antibody molecule could be expressed in *E. coli*. The following year, several groups
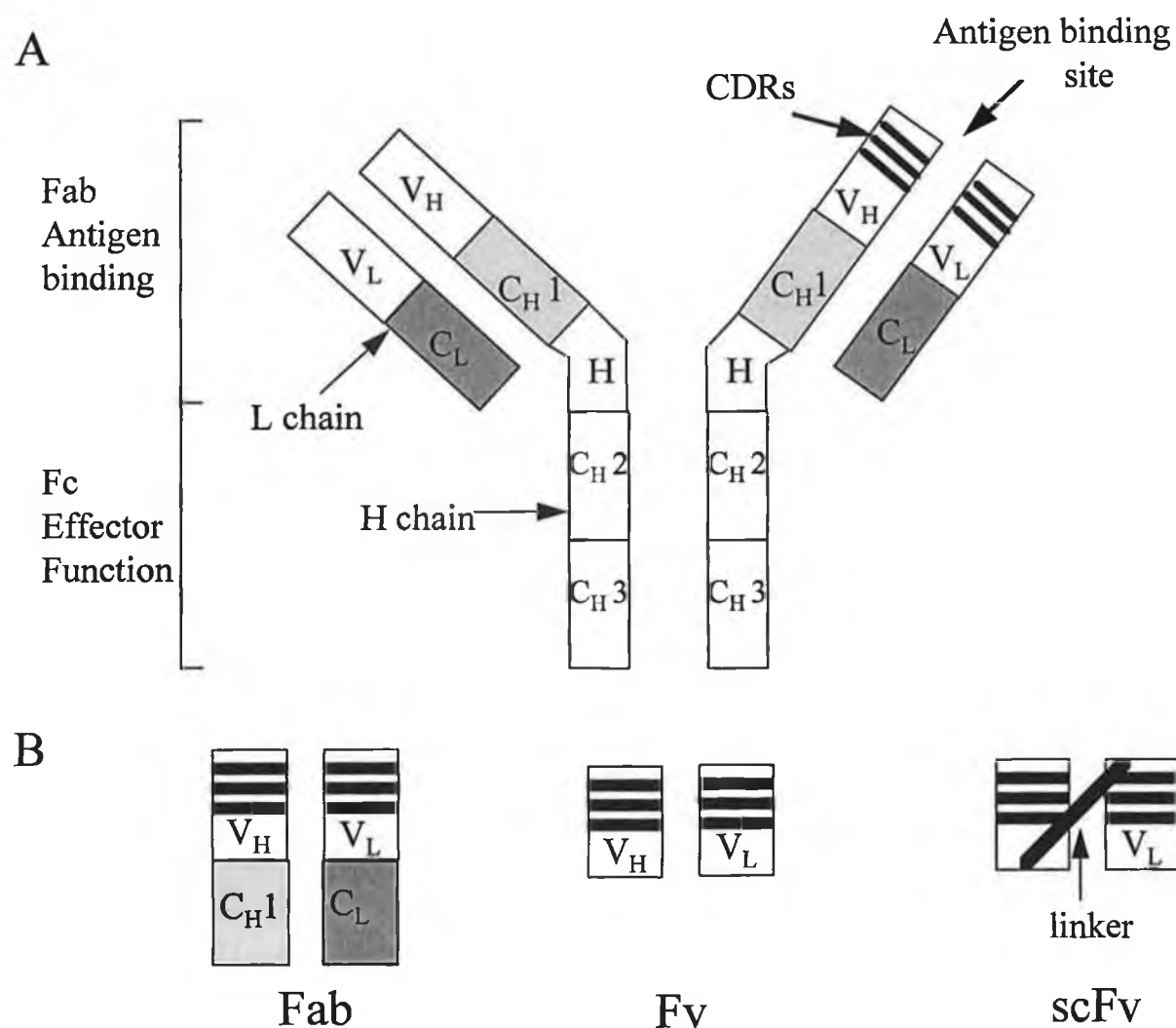
Figure 6.1 A schematic diagram of the antibody molecule and its fragments. (A) Structure of IgG antibody, showing arrangement of heavy and light chains. (B) Antibody fragments including Fab, Fv and scFv.

demonstrated that antibody genes could be amplified using PCR (reviewed by Orlandi *et al.*, 1989). These two discoveries paved the way for the generation of combinatorial antibody libraries. The combinatorial libraries from which the first antigen-selected antibodies were retrieved were expressed in phage λ (reviewed by Burton, 1991). Although the construction of libraries in phage λ was successful, the screening system was not optimal. This system, based on filter lift, was limited by two major factors. Firstly, screening was laborious and required a large amount of antigen.

Secondly, the properties of certain antigens caused them to stick to the filters in the absence of antibodies and thus resulted in false positive clones. These problems have been overcome with the advent of novel technology using filamentous bacteriophage which can display the antibody fragment on their surface and contain the genetic information for the antibody within the phage particle (Winter *et al.*, 1994). This technology gives access to rapid and efficient screening procedures and provides a direct link between the protein expressed and its genetic code.

6.2 Biology of the filamentous bacteriophage

A number of filamentous phage have been identified which are able to infect a variety of gram negative bacteria. They have a single-stranded, covalently closed DNA genome which is encased in a long cylinder of coat proteins. The best characterised of these phage are M13, fl, and fd, which infect *E. coli.* containing the F conjugative plasmid. The genomes of these three bacteriophage have been completely sequenced and are 98% homologous (Hill and Petersen, 1982), and are collectively referred to as Ff phage.

Infection of *E. coli* by Ff phage is initiated by the specific interaction of one end of the phage with the tip of the F pilus. This pilus is encoded by genes on the F conjugative plasmid. The F pilus is required for the conjugal transfer of the F plasmid DNA or chromosomal DNA containing the integrated plasmid DNA into recipient bacteria lacking the plasmid DNA. It consists of a protein tube which is assembled and disassembled by a polymerization and depolymerization process from pilin subunits in the bacterial inner membrane. In the case of phage infection, it is thought that the end of the phage attached to the pilus tip is brought to the bacterial membrane surface by depolymerisation of the F pilus. There, the major capsid proteins of the phage integrate into the membrane and the phage DNA is translocated into the cytoplasm. In the cytoplasm, bacterial enzymes synthesise the complementary strand and convert the infecting phage DNA into a supercoiled, double-stranded replicative form (RF) molecule. This molecule serves as a template for transcription and translation from which all of the phage proteins are synthesised. Some of the phage products, in concert with bacterial enzymes, direct the synthesis of single-stranded phage DNA which is converted to additional RF molecules. The production of phage proteins increases with the accumulation of these RF molecules. Capsid proteins and other phage proteins involved in

the assembly of the particle become integrated into the cell envelope. Proteins involved in DNA replication remain in the cytoplasm. When the phage specific single-stranded DNA binding protein, pV, reaches the proper concentration, it sequesters the newly synthesized phage single-stranded DNA into a complex. The DNA in this pV-DNA complex is not converted to RF but rather is assembled into new phage particles.

Assembly occurs at the bacterial envelope, at a site where the inner and outer membranes are in close contact. During the assembly process, the molecules of the pV single-stranded binding protein are displaced and the caspid proteins assemble around the DNA as it is extruded through the envelope. Assembly continues until the end of the DNA is reached and the phage is released into the media. The assembly process is tolerated quite well by the host, as the infected bacteria continue to grow and divide with a generation time approximately 50% longer than that of an uninfected bacteria.

The genome of the phage encodes 11 genes (gI- gXI), the protein products (pI-pXI) of which function either in DNA replication (pII, pX), the formation of capsid proteins (pIII, pVI, pVII, pVIII, p IX) or assembly proteins (pI, pIV, pV, pXI). The wild type Ff phage particle is approximately 6.5 nm in diameter and 930 nm in length. The DNA is encased in a somewhat flexible cylinder composed of approximately 2700 pVIII monomer units. At one end of the particle, there are about 5 molecules each of pVII and pIX. The other end contains approximately 5 molecules each of pIII and pVI (Figure 6.2).
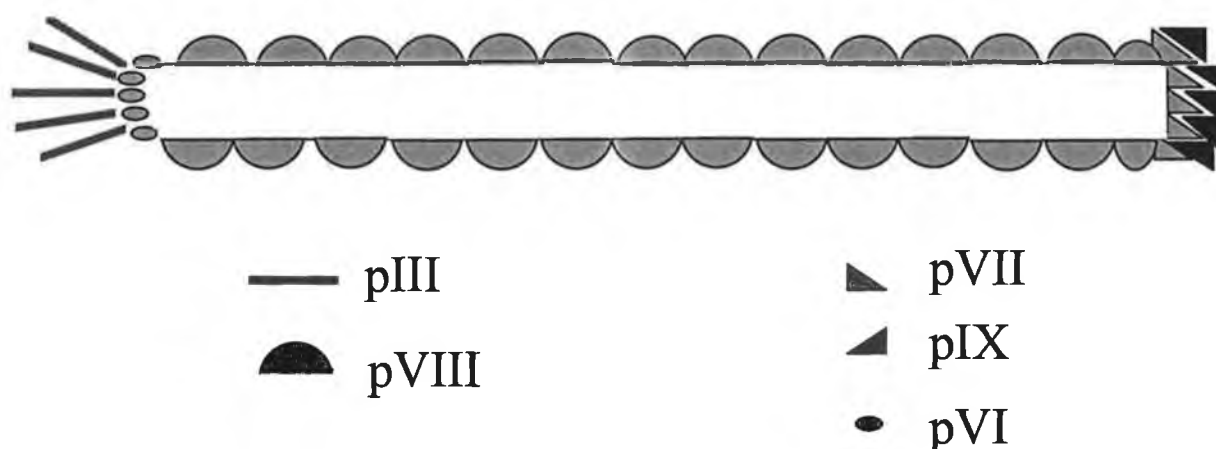

Figure 6.2 A schematic representation of the bacteriophage particle, showing the location of the capsid proteins.

As a consequence of their structure and life cycle, the phage have served as valuable tools for biological research. Since replication of DNA or assembly of the phage is not constrained by the size of the DNA, phage are excellent cloning vehicles. The result of an insertion of foreign DNA into a nonessential region merely results in a longer phage particle.

6.3 Principles and applications of phage display

The display of peptides and proteins on the surface of bacteriophage represents a powerful new methodology for carrying out molecular evolution in the laboratory. The ability to construct libraries of enormous molecular diversity and to select for molecules with predetermined properties has made this technology applicable to a wide range of problems. The origins of phage display date to the mid-1980s when George Smith first expressed a foreign segment of a protein on the surface of bacteriophage M13 virus particles. As a test case he fused a portion of the gene encoding the EcoRI endonuclease to the minor capsid protein gene gIII (Smith, 1985). Using a polyclonal antibody specific for the endonuclease, Smith demonstrated that phage containing the EcoRI-pIII fusion could be enriched more than 1000-fold from a mixture containing wild-type (nonbinding) phage with an immobilized polyclonal antibody. From these first experiments emerged two important concepts. First, using recombinant DNA technology, it should be possible to build large libraries (i.e., 10^8) wherein each phage displays a unique random peptide. Second, the methodology provides a direct link between phenotype and genotype. That is, every displayed molecule has an addressable tag via the DNA encoding that molecule. Within a few years of Smith's experiments the first phage-displayed random peptide libraries were assembled (Cwirla *et al.*, 1990; Scott and Smith, 1990), accompanied by reports that properly folded and functional proteins could also be displayed on the surface of M13 (McCafferty *et al.*, 1990).

Perhaps one of the most impressive aspects of phage display is the variety of uses for the technology, including mapping epitopes of monoclonal and polyclonal antibodies, generating immunogens, identifying peptide ligands, mapping substrate sites for proteases and kinases, directed evolution of proteins, cDNA expression screening, and of course isolation of high affinity antibody fragments. One of the more powerful applications of phage-display has been in the arena of antibody engineering. It has been possible to express both Fab and scFv antibody fragments on

the surface of M13 viral particle with no apparent loss of their affinity and specificity (McCafferty *et al.*, 1990; Barbas *et al.*, 1991). The coding regions of the V_L and V_H chains can be obtained from naive mice (Gram *et al.*, 1992), immunized mice (Clarkson *et al.*, 1991), or germline genes (Hoogenboom and Winter, 1992). Antibodies to diverse antigens have been successfully isolated using phage display technology including elongation factor EF-1α, immunoglobulin binding protein and rhombotin-2 oncogene protein (Winter *et al.*, 1994).

6.4 Phagmids

In the immune system, the B-cell provides a 'genetic display package', with antibody expressed (displayed) on the outside of the cell to encounter and bind to antigen and the genes encoding the antibody contained within. In recent years, vectors have been developed that allow the display of foreign peptides on the surface of the filamentous phage particles (Cesareni, 1992). By insertion of specific oligoneucleotides, or entire coding regions into genes encoding specific phage capsid proteins, chimeric proteins can be produced which are able to be assembled into phage particles (Winter *et al.*, 1994). This results in the display of the foreign protein on the surface of the phage particle.

By combining the best features of plasmids and the phage, new cloning vectors called phagmids have been constructed. They contain the replication origin and packaging signal of the filamentous phage together with the plasmid origin of replication and gene expression systems of the chosen plasmid. Phagmids can maintain themselves as plasmids directing expression of the desired proteins in the bacteria. Infection with a filamentous helper phage activates the phage origin of replication and the resulting phagmid single-stranded DNA is encapsulated into phage-like particles using helper phage proteins.

A variety of vectors have been used for the presentation of antibodies on the phage surface, examples include pHEN1 and pCOMB3. The display sites most commonly used are within genes III or VIII (Figure 6.3). Many short peptides and a variety of proteins have been displayed at the N-terminus of mature pIII. Phagmid vectors comprise the pIII fusion, as well as plasmid and phage origins of replication and antibiotic resistance markers. Helper phage e.g. VCS M13 provide the other functions for single-stranded replication and packaging. Helper phage are poorly packaged in

competition with phagmids due to a defective origin of replication (Vieira and Messing, 1987). VCS M13 also confers kanamycin resistance on their hosts. Because the phagmid pIII fusion competes with the helper phage pIII for incorporation into the phage coat, the majority of the phage particles display none or only one antibody molecule (Lowman *et al.*, 1991). Once the helper phage has made single-stranded phagmid DNA and packaged it in a bacteriophage coat, the phagmid is as infectious as a bacteriophage, however it cannot replicate to form infectious units without the aid of the helper phage.

In a number of vectors, there are drug resistance genes which have been introduced by bacterial transposons, in the case of pHEN1 there is an ampicillin resistance gene. Also several vectors have been engineered to express short peptide 'tags' which can be recognised by particular monoclonal antibodies. One such sequence is the c-myc epitope, EQKLISEEDLN, which is recognised by monoclonal antibody 9E10 (Evan *et al.*, 1985). In the pHEN1 vector, the c-myc epitope is upstream of the cloning site in gene III; this arrangement is useful in detecting chimeric pIII molecules on western blots and verifying that full length chimeric molecules exist on phage.

Suppressed stop codons (amber codons) have also been placed between displayed protein domains and pIII. When such recombinants are propagated in a suppressor carrying bacterial strain such as TG1, virus particles are produced incorporating the chimeric protein domain pIII into the capsids. In TG1 this amber codon is read as glutamic acid and a fusion between antibody and gene III is produced (Kay *et al.*, 1996). After screening phage populations for virus particles with certain properties (based on the particular protein domain displayed), individual isolates can be introduced into bacteria strains that lack suppressors (e.g. HB2151). In these hosts there is efficient translational termination at the stop codon in the chimeric gene III, consequently the protein domain is secreted by itself (Kay *et al.*, 1996). This has been a very effective means of generating soluble forms of the desired protein, for example antibodies as described by Hoogenboom *et al.* (1991). The vector pHEN1 can be used in this manner for the direct expression of the antibodies as soluble fragments, as an amber stop codon is encoded at the junction of the antibody gene and gene III.

In pHEN1 the production of the pIII fusion is under the control of the lacZ promotor which is inhibited by glucose and induced by isopropyl-β-D-thiogalactosidase (IPTG). Because the expression of gene III prevents infection with helper phage, the bacteria harboring the phagemid

vectors are grown in the presence of glucose. After infection with helper phage the bacteria are grown in the absence of glucose and presence of IPTG, consequently sufficient antibody-pIII fusion is produced for packaging into phage.
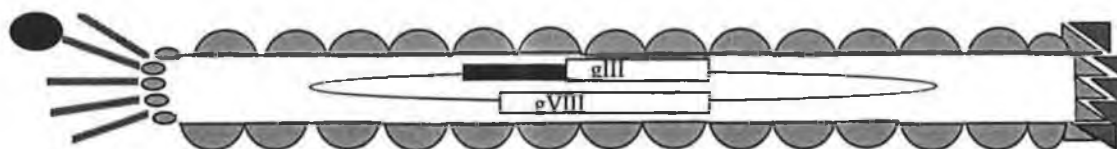
Fusion to gene VIII
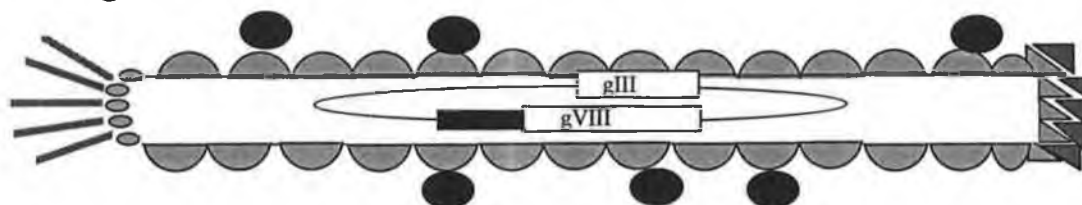
Fusion to gene III

Figure 6.3 Comparison of two different M13 phage-display vectors and viral proteins. The displayed proteins are fused to either proteins III or VIII on the surface of the phage, depending on where the foreign DNA has been inserted in the phage genome. [Black boxes and spheres correspond to the foreign genetic elements and their encoded peptides, respectively, other symbols as described in Figure 6.2].

6.5 Construction and Screening of Antibody Display Libraries

Libraries can be generated from the genetic material of both immunized or non-immunized animals or humans (Huse *et al.*, 1989; McCafferty *et al.*, 1990). Alternatively they may be constructed from naive (Marks *et al.*, 1991), synthetic or semi-synthetic genes (Barbas *et al.*, 1992; Lerner *et al.*, 1992). The source of mRNA may be B-lymphocytes from the spleens of hyperimmunised mice or from human B-lymphocytes obtained from peripheral blood or other lymphoid tissues such as tonsils or spleen if available. Once RNA has been extracted cDNA must be synthesized using reverse transcriptase. Reverse transcription uses a short RNA:DNA hybrid region as template and this can be generated on the RNA using random DNA hexamers or chain-specific primers which anneal with the various constant region. Having generated cDNA this is

used as a template for PCR. PCR amplification is carried out using primers complementary with the ends of the heavy- and light-chain variable regions. Alignment of known variable domain gene sequences demonstrates that there are regions of conservation within the variable domain, particularly at the 5' and 3' termini. This has lead to the determination of species-specific consensus sequences which have been used in the design of PCR primers. In this way whole repertories of antibody genes have been prepared by PCR. After PCR the various reactions from within a group can be mixed to give three pools (heavy, κ and λ chains). The separate heavy- and light-chain fragments are converted to a single-chain Fv gene by inserting a DNA sequence encoding a flexible linker peptide. The assembly of the VH, VL and linker fragments is carried out by PCR and is driven by homologies at the ends of the various fragments. The 3' end of the heavy chain is complementary to the 5' end of the linker and the 3' end of the linker DNA is complementary to the 5' end of the light chain (Kay *et al.*, 1996). This PCR step also introduces restriction enzyme sites at the termini for cloning. The products are cloned into an appropriate vector and electroporated into *E. coli* TG1 cells.

The scFv fragments expressed on the surface of the phage are screened against antigen-coated microtitre wells. This procedure is called 'panning' and is depicted in Figure 6.4. Following incubation with the phage preparation, unbound phage are removed by washing and discarded. Bound phage, those expressing a scFv which is specific for the antigen are eluted with acid. The eluted phage are amplified by transformation of *E.coli* and infection with helper phage. The panning procedure is repeated 3-5 times, each time enriching for those phage bearing antigen-binding scFvs. For each round the ratio of antigen-binding phage to non-binding phage is increased. For example, 1/5000 clones were positive from a library made from a human immunized with tetanus toxoid, however following one, two, and three rounds of panning the frequency increased to 1/4, 7/10 and 9/10, respectively (Kay *et al.*, 1996).

Following panning, individual colonies can be assayed directly for the ability to bind the antigen as a phage particle by immunoassay techniques such as ELISA. Phage that have bound an immobilised ligand via the expressed antibody (phage-antibody) are detected using an antiserum raised against bacteriophage fd. The principle component recognised by this antiserum is gene VIII protein of which there are approximately 2,800 copies, making up the bulk of the capsid, giving a convenient amplification system. Having detected positive phage clones, these can be transferred

into HB2151 which will give soluble expression of the antibody. Again these clones can be tested by ELISA, this time using an anti-c-myc antibody, which can recognise the c-myc tag on the scFv, to detect bound phage.
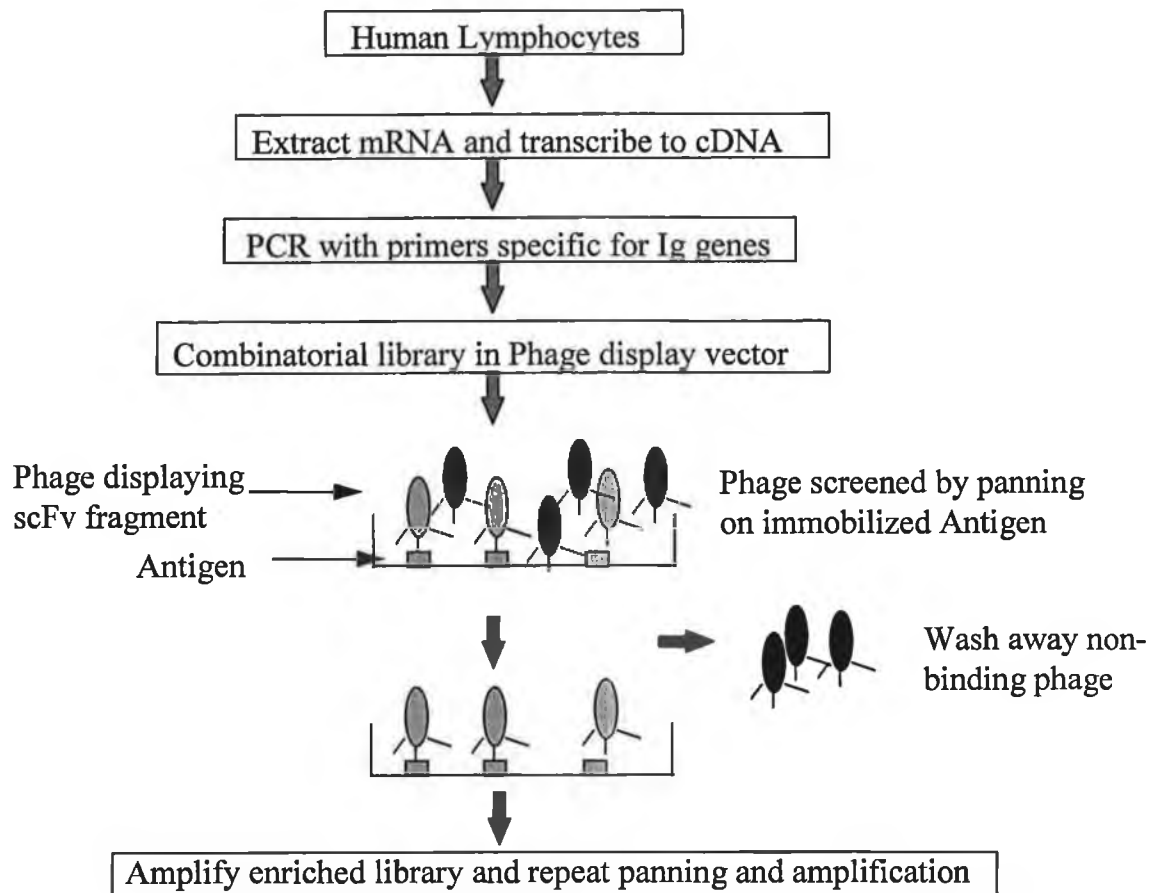
Figure 6.4 Outline of sequence of events in antibody phage library construction and panning procedure.

6.6 Nissim Library

The Nissim phage display antibody library was first developed in 1994 by Nissim *et al.* at MRC, Centre for Protein Engineering, Cambridge. This library was constructed from diverse repertories of rearranged human V_H gene segments from a bank of 50 cloned human V_H gene segments (Tomlinson *et al.*, 1992). The human synthetic library was constructed as in Hoogenboon and

Winter (1992) using PCR primers designed to introduce V_H-CDR3 of random sequence varying in length from 4 to 12 residues. The PCR products from each of the amplifications encoding the nine different CDR3 loop lengths were pooled, cut with NcoI and SalI, and cloned into NcoI-Xho I-cut pHEN1-Vλ_3 to provide nine phagmid libraries, each containing at least 10^7 clones. The *in vitro* V_H genes were paired with a single unmutated Vλ_3 light chain gene segment. Each library was rescued separately with helper phage VCS-M13, then pooled with each other, and with two earlier libraries (Hoogenboom and Winter, 1992), each of at least 10^7 different clones and encoding loops of five or eight residues to create a single pot library of $>10^8$ clones.

The Nissim library has been used to isolate immunochemical reagents to a range of antigens. From the same 'single pot' repertoire, phage were isolated with binding activities to each of 18 different antigens. These antigens included haptens, foreign and self- antigens, including secreted proteins (lysozyme and ovalbumin), cell surface proteins (T cell receptor), intracellular proteins from the cytoplasm (thyroglobulin), proteins from the lumen of the endoplasmic reticulum (BIP) and proteins from the nucleus (p53) (Nissim *et al.*, 1994). Both phage and scFv fragments secreted from infected bacteria were used as monoclonal and polyclonal reagents in western blots. Furthermore, the monoclonal reagents were used for epitope mapping and for immunocytochemical staining of cells (Nissim *et al.*, 1994).

6.7 Antibodies to MMP-9

MMP-9 has been implicated in many diseases involving ECM degradation (Brenner *et al.*, 1989; Powell *et al.*, 1996; Dean *et al.*, 1989). As described in chapters 1 and 2 of this thesis, MMP-9 is thought to have a critical role in mediating trophoblast invasion during placentation (Fisher *et al.* 1989; Librach *et al.*, 1991). In chapter 5 we sought to investigate the role of MMP-9 *in vivo* using immunohistochemistry of placental biopsies. However, currently available antibodies to MMP-9 were not suitable for performing immunohistochemistry on the formalin fixed paraffin-embedded sections. scFv fragments have been shown to perform better in penetrating tissue sections than whole IgG molecules because of their small size (Huston *et al.*, 1993).

In vivo the MMPs are secreted in a latent form and must be cleaved to yield an active degradative enzyme. Isolation of antibodies which specifically recognize the active form of this

enzyme has proved elusive by conventional methods, however this is essential as it is detection or localization of the active enzyme that is most clinically relevant. The display of repertoires of antibody fragments on the surface of filamentous bacteriophage offers a new way of making antibodies with predefined specificities and isolating very rare antibodies. Nissim *et al.* (1994) speculated that phage may recognize different epitopes, or at least those with a different bias, from monoclonal antibodies. For example, phage display technology isolated a novel antibody to p53 which recognized a sequence that no isolated monoclonal antibody had been capable of detecting, however, it remains to be seen whether this proves to be the case in general.

We set out to use the Nissim library to isolate novel scFv antibody fragments to MMP-9. It was hoped to characterise these fragments using BIAcore analysis. Real-time biospecific interaction analysis (BIA) is a general technique for observing the progress of biomolecular interactions as they occur. One of the interacting components is immobilized onto a sensor chip surface, and the change in the surface concentration resulting from binding of the other component is measured using surface plasmon resonance. We sought to isolate antibody fragments specifically against active MMP-9 (i.e. not detecting latent enzyme) which would be suitable for immunohistochemistry on paraffin-embedded sections.

6.8 RESULTS

6.8.1 Analysis of MMP-9 Protein

Our first consideration was the source of MMP-9 protein to be used for the panning procedure. Rather than go through lengthy purification protocols we chose to obtain the commercially available 'Gelatinase, 92 kDa' available from Boehringer Mannheim (Cat. No. 1758900). This MMP-9 protein was purified from human blood. To assess the purity of the MMP-9 protein, it was analysed by SDS-PAGE as described in section 2.2.4.1 and silver stained as in section 2.2.4.2.2. The protein appeared as a single homogenous band at 92 kDa (Figure 6.5). The purity of the protein was essential to ensure that antibody fragments were isolated specifically to MMP-9 and not to any contaminating antigens present in the preparation.
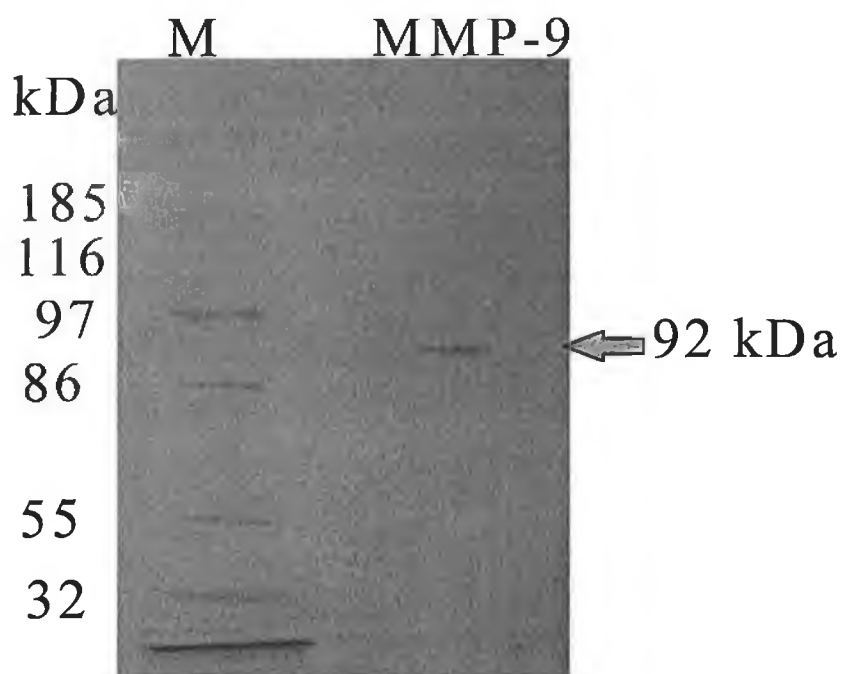

Figure 6.5 SDS-PAGE silver stained gel showing purity of MMP-9 protein. MMP-9 was loaded at a concentration of 1 ng.

6.8.2 Assessing the Activation State of the MMP-9 Protein

The MMP-9 protein supplied by Boehringer Mannheim was in the latent 92 kDa form. However, in order to isolate antibody fragments specific to the active form of the protein (88 kDa) some of the protein required activation. This was achieved by freeze-thawing which resulted in the loss of the pro domain of the protein leaving the 88 kDa active form (Figure 6.6). A sample of MMP-9 which consisted of a mixture of active and latent forms was used for panning. Freeze-thawing was chosen as the means of activation, rather than chemical or biological activation treatments (e.g. APMA or trypsin) which avoided the need to purify the protein from these agents which might have contaminated the subsequent panning procedure.

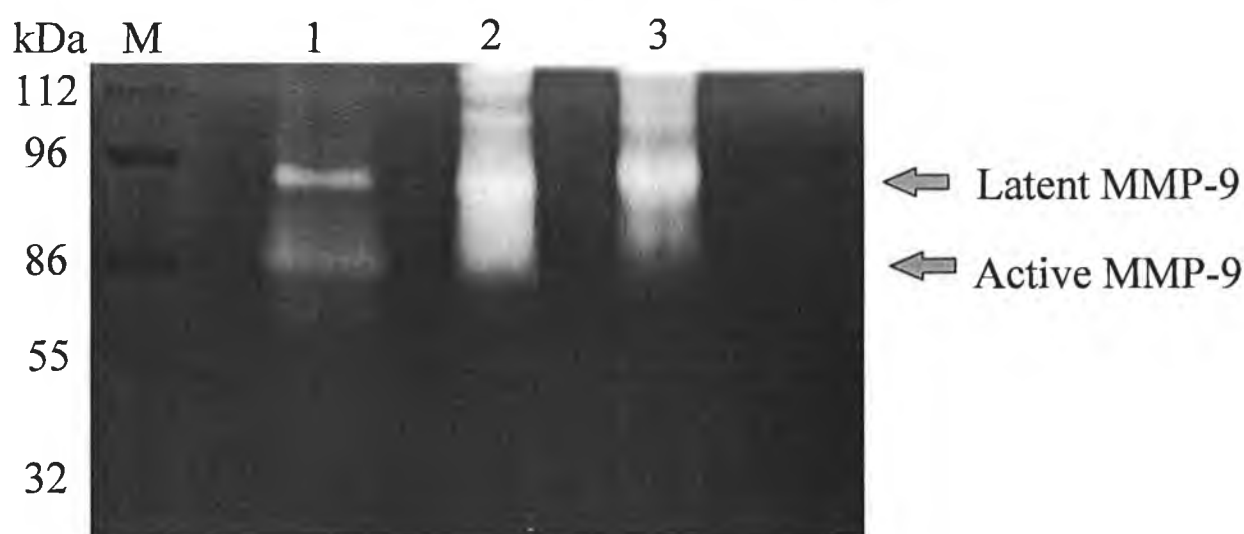**Figure 6.6** Zymogram showing active and latent forms of MMP-9 protein. Lanes (1) 1.5 ng MMP-9 protein after freeze-thawing, containing a mixture of active and latent MMP-9, (2) 15 ng MMP-9 protein after freeze-thawing, containing a mixture of active and latent MMP-9, (3) 15 ng MMP-9 protein prior to freeze-thawing, containing only latent MMP-9. As indicated the molecular weight of latent MMP-9 is 92 kDa and active MMP-9 is 88 kDa.

6.8.3 Assessment of Enrichment Following Panning

To monitor the success of the panning procedure (section 2.2.11.7) samples of polyclonal phage populations were taken at the end of each round of panning. These samples were tested by phage-antibody-ELISA (section 2.2.11.11) to determine if enrichment for phage specifically binding to MMP-9 after successive rounds of affinity selection had occured. Figure 6.7 shows a schematic representation of the phage-antibody-ELISA used. During each round of panning non-binding phage were washed away and binding phage were eluted, reinfected in *E. coli* and amplified prior to another round of panning. Therefore the concentration of binding phage should increase after each round. Figure 6.8 graphically represents this enrichment. After 4 rounds of panning sufficient enrichment had been achieved, as a 10 fold increase in binding activity was observed compared to the pre-selected library. Phage from the fourth round of panning were used for subsequent analysis.

Phage antibody ELISA

HRP-conjugated anti-rabbit antibody

anti-fd bacteriophage antibody (rabbit)

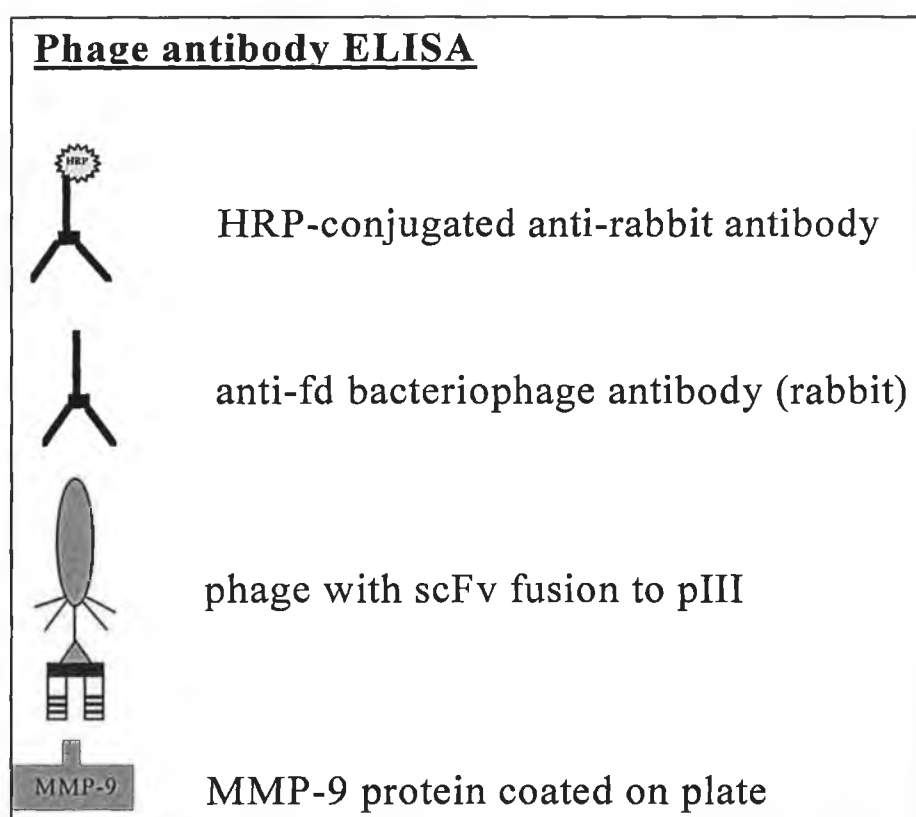phage with scFv fusion to pIII

MMP-9 protein coated on plate

Figure 6.7 Schematic representation of Phage-antibody-ELISA.
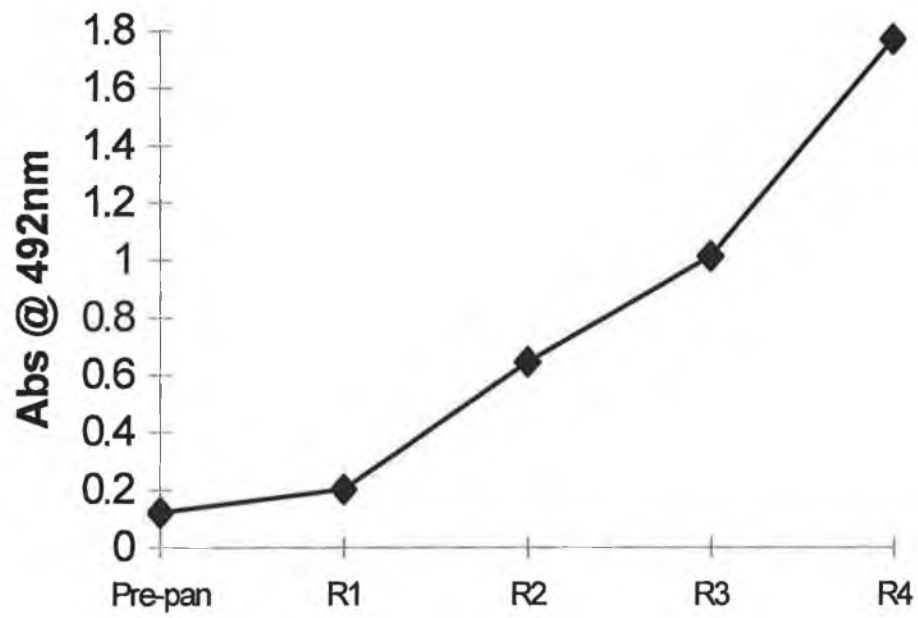
Figure 6.8 Graph showing enrichment results from polyclonal phage populations collected after each round of panning as determined by phage-antibody-ELISA.

6.8.4 Monoclonal Phage-Antibody-ELISA

Having determined that the panning procedure was successful by testing polyclonal phage samples, we then tested monoclonal phage from single colonies selected from round 4 by phage-antibody-ELISA (as in Figure 6.7) for binding activity to MMP-9 (section 2.2.11.12). 52% or 42 of the 82 clones tested were found to be positive. Positive clones were classified as those which gave approximately 10 fold higher absorbance readings over control wells in ELISA. The absorbance results of 20 representative positive clones are shown in Table 6.1.

Table 6.1 Phage-antibody-ELISA absorbance results for 20 clones.

Clone No.	Abs.@492nm
TG1-1	1.863
TG1-2	1.665
TG1-4	1.807
TG1-5	1.837
TG1-9	1.687
TG1-13	1.569
TG1-14	>2.000
TG1-15	1.264
TG1-17	1.761
TG1-20	>2.000
TG1-22	>2.000
TG1-23	1.892
TG1-24	1.522
TG1-25	1.606
TG1-28	1.655
TG1-32	1.993
TG1-36	1.999
TG1-37	1.095
TG1-40	1.645
TG1-41	1.833

6.8.5 Preparation of Anti c-myc Antibody

Having identified individual positive phage antibody clones, the next step was to produce soluble antibody fragments. The soluble scFvs cannot be detected by the anti-bacteriophage Fd antibody (used in the previous phage-antibody-ELISAs) as they are secreted as soluble proteins no longer fused to the phage pIII coat protein. These soluble fragments were detected using anti-c-myc antibodies which detected the c-myc tag incorporated into the scFv fragment (described in section 6.4). The anti-c-myc antibodies were produced by the hybridoma cell line 9E10 and secreted into the culture medium as described in section 2.2.1.2. The antibodies were isolated from the medium by ammonium sulphate precipitation as described in section 2.2.11.14. After precipitation the antibodies were tested by dot blot analysis. C-myc tagged proteins were immobilized onto nitrocellulose, incubated with anti-c-myc antibodies and detected using anti-mouse-HRP conjugated antibodies. Figure 6.9 shows that the anti-c-myc antibodies were present and functional.

Figure 6.9 Dot blot to detect anti-c-myc antibodies. Dot (1) 5 ul of c-myc-tagged protein sample immobilised onto nitrocellulose, incubated with anti-c-myc antibodies and, then detected with anti-mouse-HRP conjugated antibodies and detected with DAB. Dot (2) Negative control, BSA immobilised onto nitrocellulose instead of c-myc-tagged protein.

6.8.6 Monoclonal Soluble-scFv-Antibody-ELISA

The 42 phage clones which tested positive by phage-antibody-ELISA in section 6.8.4 were then infected into *E. coli* HB2151 and induced for soluble expression of scFv antibody protein as described in section 2.2.11.13. The nomenclature for each phagmid was retained when transferred from *E. coli* strain TG1 to strain HB2151, e.g. when the phagmid from clone TG1-4 was transferred to HB2151 it was labeled HB2151-4. These clones were then tested by soluble-scFv-antibody-ELISA for binding activity to MMP-9 (section 2.2.11.11). Figure 6.10 shows a schematic representation of the soluble scFv-antibody-ELISA. After transferring from TG1 to HB2151, 72% or 30 of the 42 clones tested produced soluble scFv fragments with binding affinity to MMP-9, as determined by soluble scFv-antibody-ELISA. However, positive clones only gave absorbances approximately 5 fold higher than control wells, considerably lower than phage-antibody-ELISA results. Table 6.2 shows the scFv-antibody-ELISA absorbance results for the 10 clones with greatest binding affinity for MMP-9. These 10 clones were analysed further in subsequent experiments.
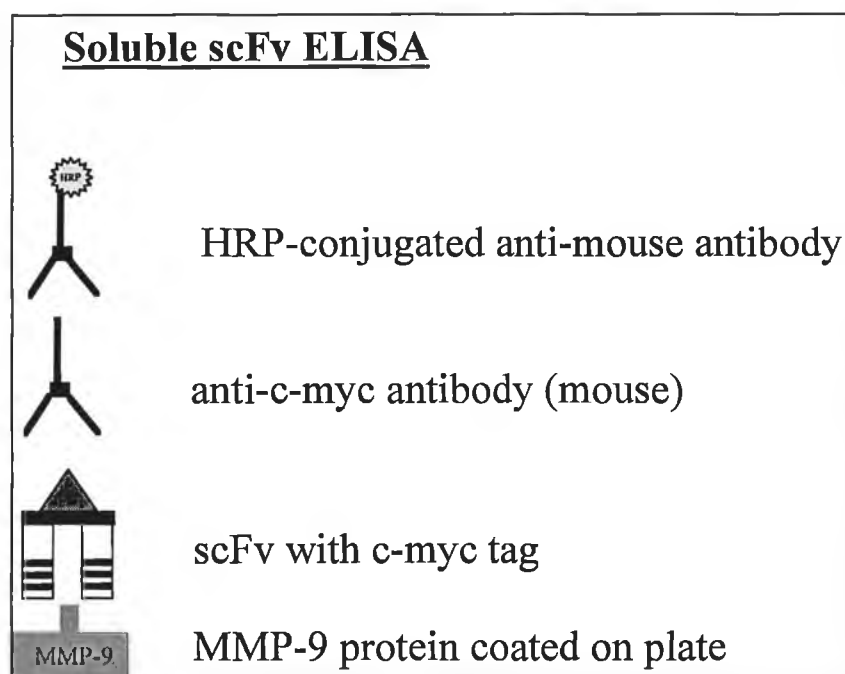
Figure 6.10 Schematic representation of scFv-antibody-ELISA.

Table 6.2 Soluble-antibody-ELISA absorbance results for top 10 clones.

Clone No.	Abs. @ 492nm
HB2151-4	1.333
HB2151-15	0.947
HB2151-18	0.821
HB2151-22	0.945
HB2151-25	1.231
HB2151-27	1.136
HB2151-28	1.050
HB2151-35	0.762
HB2151-39	0.946
HB2151-41	0.879

The specificity of the scFv fragments were tested by coating the wells with other proteins including BSA and the closely related metalloproteinase family member MMP-7. Clones HB2151-4 and-41 showed slight binding activity to MMP-7, however, for all other clones no non-specific binding was detected over background (data not shown). Clones HB2151-4 and-41 were not analysed further.

6.8.7 Optimisation of Soluble scFv Fragment Expression

As the levels of scFv in the culture supernatant were quite low we decided to look at periplasmic lysates as the source of scFv. A time course study of the production of scFv fragments after the addition of IPTG was carried out and the results are shown in Figure 6.11. The scFv fragment can be seen at 31 kDa which is the expected molecular weight of a scFv fragment. For expression of scFv in culture supernatant; 18 hours post induction gave optimum results, while for periplasmic scFv production 4 hours post induction was chosen as optimum. Periplasmic lysates were found to produce higher levels of scFv without the liquid handling problems associated with using culture supernatants and were used in subsequent experiments.
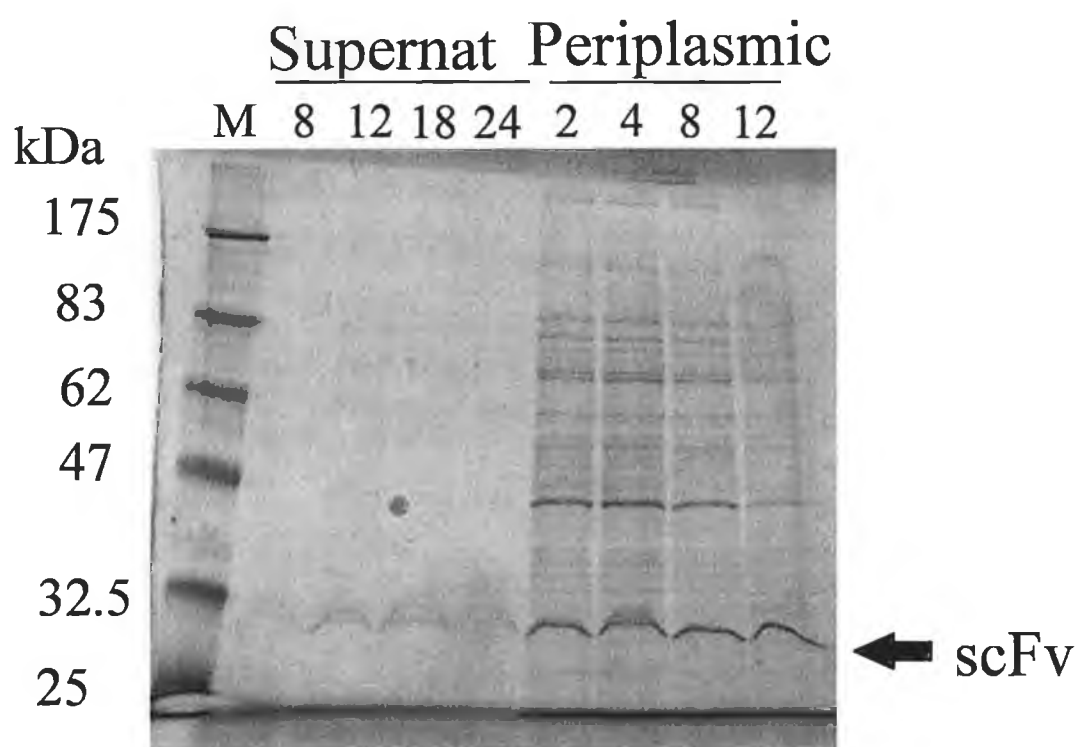

Figure 6.11 SDS-PAGE gel showing concentrations of scFv from clone HB2151-35 produced into the supernatant or periplasm at various time points (in hours) post induction.

6.8.9 Detection of scFv fragments by dot blot analysis

All of the clones were examined by dot blot analysis to ensure the c-myc tag was intact and could be detected by anti-c-myc antibodies followed with anti-mouse-HRP conjugated antibodies. Figure 6.12 shows that all the clones tested had a functional c-myc tag present. This was confirmed by western blot analysis, where the periplasmic lysates were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the anti-c-myc antibody (section 2.2.5). Figure 6.13 shows a single band at 31 kDa for the scFv fragments, confirming that scFv fragments were present in the lysates and the c-myc tag was present.
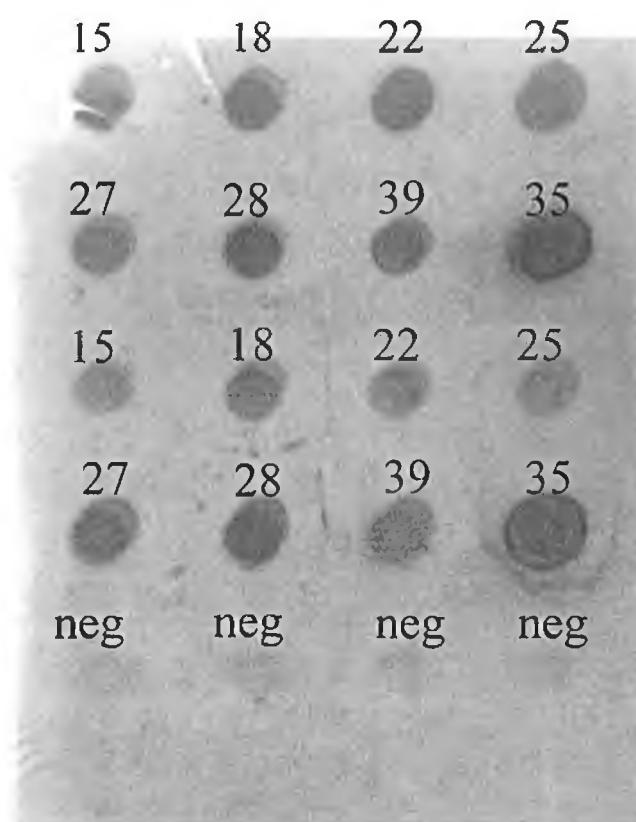
Figure 6.12 Dot blot analysis with 200μl culture supernatant from each clone (clones numbers HB2151-15, HB2151-18, HB2151-22, HB2151-25, HB2151-27, HB2151-28, HB2151-35, and HB2151-39) blotted onto nitrocellulose, probed with anti-c-myc followed by anti-mouse-HRP conjugated antibody. Clones were tested in duplicate and negative controls are also shown.
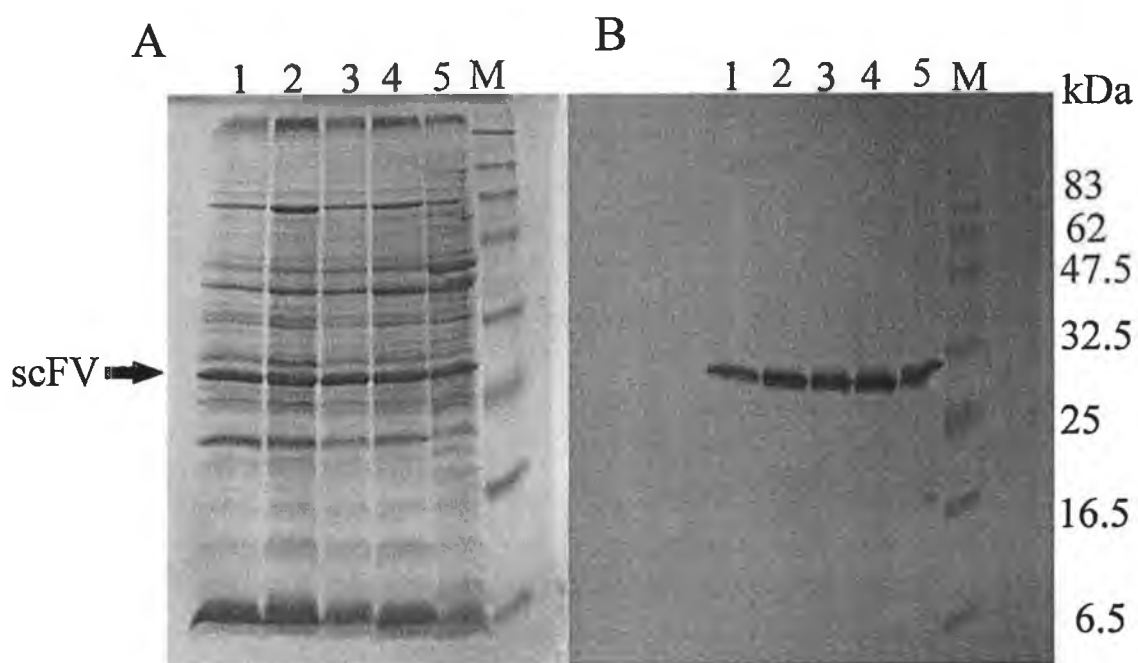
Figure 6.13 Analysis of periplasmic lysates by SDS-PAGE and western blot. (A) SDS-PAGE gel stained with coomassie blue showing proteins present in periplasmic lysates for the 5 clones. (B) Western blot of the same samples probed with anti-c-myc showing single band at 31 kDa for scFv fragment. Lanes contain periplasmic lysates from clones : (1) HB2151-22, (2) HB2151-27, (3) HB2151-28, (4) HB2151-35, (5) HB2151-39.

6.8.11 Titering of positive clones

The clone which gave the highest binding activity in section 6.8.6 (HB2151-35) was titered by scFv-antibody-ELISA. This involved serial dilution of the scFv in order to determine the lowest concentration of antibody fragment which could be used to generate a detectable difference in absorbance over background. As shown in Figure 6.14 clone HB2151-35 gave an antibody titre of approximately 1 in 100 dilution. This titre was too low to proceed with affinity ranking by BIAcore (Pharmacia Biosensor). A titre of approximately 1 in 1000 would be necessary for BIAcore analysis.
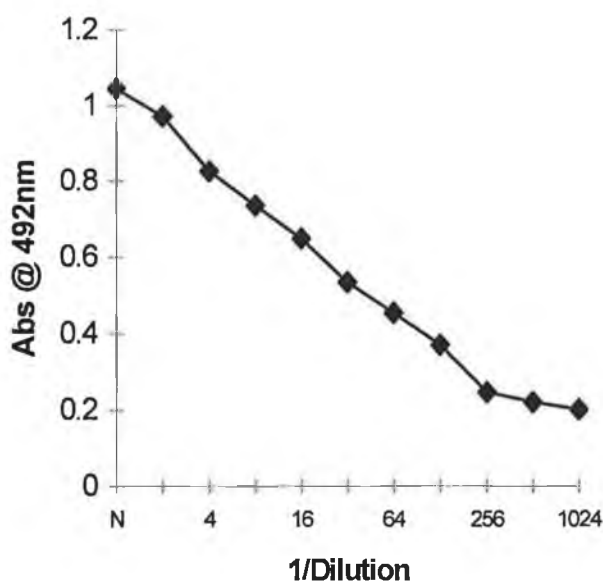

Figure 6.14 Graph of absorbance at 492nm vs. reciprocal dilution of scFv antibody from clone HB2151-35 showing antibody titre of approximately 1 in 100 dilution.

6.8.12 Use of the isolated scFv fragments as Immunological Reagents

Periplasmic lysates from all eight positive clones were tested by dot blot and western blot for binding activity to MMP-9. None of the clones tested were sensitive enough to detect the MMP-9 protein, even when high concentrations of MMP-9 protein were loaded (up to 15μg) (data not shown). Dot blots were carried out to ensure that the denaturing conditions of the PAGE were not affecting the

conformation of the MMP-9 protein, which might have prevented the scFvs recognising the protein. Figure 6.15 shows the comparison between a commercially available anti-MMP-9 antibody and the scFv from clone 35. No bands were detected by the scFv however the commercial antibody could detect 5 μg of MMP-9 protein. Chemiluminescence, a more sensitive method of western blot detection gave similar results. Westerns were also attempted using culture supernatants which had been concentrated 10 fold against PEG (to promote multimerization of scFv); using monoclonal phage-antibodies (because of the signal amplification afforded by the coat protein); and using polyclonal phage-antibodies however all of these were not successful (data not shown).

We also attempted immunohistochemistry using both soluble and phage-antibody scFvs. Placental biopsy sections, as well as BHK- H92 cells (which were known to express MMP-9 protein) were grown on slides and were tested with the scFvs, however there was no significant difference over background staining (data not shown).
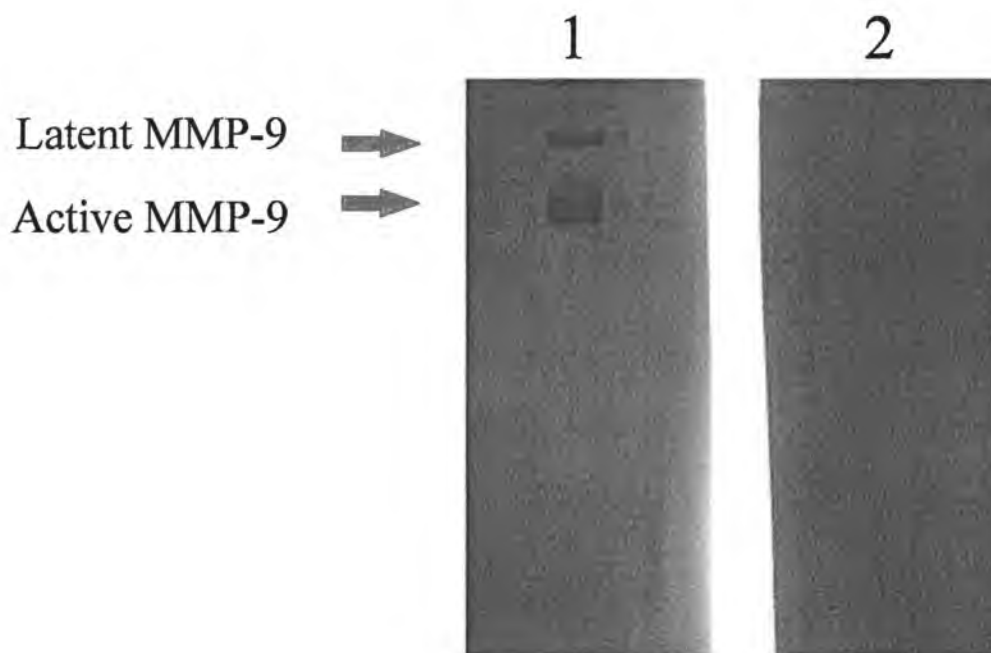

Figure 6.15 Western blots both loaded with 5μg MMP-9 protein. (1) Blot was probed with the commercial Ab-1 anti-MMP-9 antibody from Oncogene Science, detecting two bands for active and latent MMP-9. (2) Blot was probed with the scFv fragment produced by clone 35, no bands detected.

6.9 DISCUSSION

The fusion of peptides, or larger protein fragments, to the amino terminus of filamentous phage protein III, allowing display without disturbing the infective capacity of the phage, is one of the basic principles of phage display. This technology gives access to rapid and efficient screening procedures and provides a direct link between a protein presented at the surface of a phage and the corresponding genetic information packaged within the phage particle. Antibody phage display libraries may be generated from immunized or naive sources. Immunization enriches the mRNA for V_H and V_L genes encoding antigen binding activities (Winter and Milstein, 1991) but requires a different library for each antigen. By contrast, the use of larger and more diverse repertoires from V genes, rearranged *in vitro* (Hoogenboom and Winter, 1992) allows the isolation of antibodies of different binding specificities, without immunisation, from the same library as proposed by Milstein (1990). The Nissim library is one such 'single pot' library.

The wealth of knowledge currently available on antibodies has paved the way for the creation of antibodies entirely outside their natural host. To construct a synthetic antibody library, V genes are assembled by introducing a predetermined level of randomization of CDRs into germ line V-gene segments. The areas of synthetic diversity are chosen to correspond to areas of greatest natural sequence diversity of the primary antibody repertoire. From structural studies it has become apparent that five of the six CDR regions (all but the CDR3 of the heavy chain) have limited structural variation, and frequently follow a certain standard fold (Chothia and Lesk, 1987). The greatest structural diversity is found in the loop most central to the antigen combining site, the CDR3 of the heavy chain. This loop is diverse in composition and in length owing to the *in vivo* recombination procedure. The antibody CDR region may be partially randomized using PCR-based methods as in the Nissim library where 4 to 12 residues on the CDR3 were randomized and cloned for display as a scFv with a human λ light-chain. From this library many antibodies to haptens, and one against a protein antigen were isolated (Nissim *et al.*, 1994).

Developing antibodies in bacterial systems has practical advantages. For example, large amounts of phage-antibody or soluble antibody fragments sufficient for analysis can be produced after overnight growth from stocks. The stocks themselves are easier to establish and maintain than eukaryotic cell line stocks. Growth for production quantities of reagent are considerably simpler, faster and cheaper. Unlike traditional hybridoma techniques for generating antibodies, recombinant methods

immortalize the genes rather than the cells. However, even though *E. coli* allow folding of recombinant proteins and assembly of homo- or heterodimers (Winter and Milstein, 1991), there are biological limitations related to the host. It is well known that *E. coli* cannot glycosylate proteins or perform post-translational modifications like phosphorylations which often occur in eukaryotic systems. However, these limitations are common to all prokaryotic expression systems.

Our initial aim was to isolate antibodies which specifically recognised the active form of MMP-9 using phage display technology. This has proven elusive by conventional techniques of immunizing animals for polyclonal sera or by hybridoma technology. We hoped this might be possible using phage display technology as the affinity based selection and amplification inherent in this technology means that even rare phage ($< 1/10^7$) may be isolated over several rounds of panning (McCafferty *et al.*, 1990). In addition Nissim *et al.* (1994) had speculated that phage libraries could lead to the selection of a different spectrum of epitopes from natural immune systems. For example, mouse antibodies to human p53 bind to two main immunodominant regions of the protein, at the N-terminal region and at the C-terminal region. The epitope recognised by the anti-p53 scFv fragment lies outside these regions, which allowed the identification of a new epitope of p53 (Nissim *et al.* 1994).

The purity of the protein used in the panning procedure is critical since phage are chosen for subsequent amplification based on their ability to bind to the protein coated on the plate. Therefore, any contaminants present would dictate the affinity selection and scFv would also be isolated to them. The purity of the MMP-9 protein was analysed by silver staining and found to give a single homogenous band at 92 kDa indicating the absence of contaminating proteins and its suitability for use as an antigen for the affinity selection procedure. The MMP-9 protein was supplied in the latent form as the 92 kDa proenzyme. We used a mixture of active and latent forms of MMP-9 protein for affinity selection. This was achieved by freeze-thawing the protein which caused cleavage of the pro domain from the protein leaving the smaller active form (88 kDa). A mixture of active and latent protein was used for panning with the hope that scFv might be isolated to both forms of the enzyme. Activation was accomplished by freeze-thawing the protein, which did not require addition of compounds such as APMA or trypsin, which would have required removal from the protein prior to the panning procedure. The library was subjected to four rounds of panning against MMP-9 protein. The ratio of MMP-9 binding phage to non-binding phage was increased over each successive round of

panning. Three to five rounds of panning were recommended (Nissim *et al.*, 1994), and four rounds was found to be sufficient. All subsequent analysis was carried out on clones isolated from the fourth round of panning. 52% of phage-antibody monoclones tested were found to have binding activity to MMP-9. An important issue to keep in mind is that the displayed protein domain is susceptible to proteolysis. While M13 phage are resistant to many proteases (i.e. trypsin, chymotrypsin) (Schwind *et al.*, 1992), the displayed protein domains are not. Stocks of recombinant phage can lose their displayed element in hours to days when stored at 4°C in culture supernatant and this would result in false negative results. For short-term experiments monitoring binding properties of particular phage isolates, fresh (i.e. 6-18 hour) culture supernatants were used whenever possible. The pHEN1 vectors encoding these positive scFv were rescued and transferred into the non-suppressor strain HB2151. Due to the amber codon in pHEN1, HB2151 produce soluble scFv.

72% of these clones produced soluble scFv which had binding affinity to MMP-9, however the soluble scFv had considerably lower activity than the corresponding phage-antibody clones. In addition, not all clones which had tested positive as phage antibodies resulted in clones positive for soluble scFv antibody production. Nissim *et al.* (1994) also reported that for each of the antigens used in their study, they could isolate phage clones with binding activities, while they were sometimes unable to detect binding of the encoded scFv fragments using bacterial supernatants. This may reflect the amplified detection of the phage by virtue of the 2,800 subunits of the phage pVIII coat, if so a more sensitive assay should be used for the soluble fragments. It might also reflect the greater binding avidities afforded by multivalent display on phage and the moderate binding affinities of the monmeric scFv fragments from primary phage repertoires of 10^{7}-10^{8} clones (Hoogenboom and Winter, 1992; Griffiths *et al.*, 1993).

The production of soluble scFv was investigated and isolation of scFv from periplasmic lysates four hours after induction with IPTG was found to be optimum. While scFv could be found in the culture medium after longer induction periods there was the associated difficulty of concentrating down the large culture volumes. Periplasmic lysates on the other hand could be generated in small volumes avoiding the liquid handling difficulties. It has been reported that scFv fragments in supernatants could be more readily detected by concentrating the supernatant, or by multimerization of the fragments. Although scFv fragments can associate to form dimers in bacterial supernatants (Griffiths *et al.*, 1993), and the dimers have greater binding avidities to solid phase antigen,

concentration may also drive the formation of multimers. Presumably, multimerization is promoted by dissociation of V_H and V_L domains of the same chain, followed by pairing with another chain. Aggregation of moderate affinity scFv is probably important for their use as immunological reagents as it enhances the avidity of binding and slows the rate of dissociation during washing (Nissim *et al.*, 1994). We concentrated scFv fragments in bacterial supernatant by reverse dialysis against PEG to 1/10 their original volume. However, this did not lead to any significant improvement in binding affinity.

The scFv were found to be specific for MMP-9. Two clones showed slight cross reactivity when tested against MMP-7, which is a closely related family member and this may be due to sequence homology between the MMP-9 and MMP-7. For clone HB2151-35 titering of the scFv gave a very low titre of 1/100 indicating poor binding affinity of the scFv. This titre was too low to proceed with characterisation by BIAcore analysis, which would have provided real time kinetic information.

The scFv were tested for their suitability as immunological reagents. They were unable to detect high concentrations of MMP-9 protein by dot or western blot analysis. We initially tested the scFv by western blot analysis and in order to ensure that the denaturing conditions of the electrophoresis procedure were not affecting the scFv recognising the MMP-9 protein, dot blots were also carried out. These were however also unsuccessful. Western blots were attempted using periplasmic lysates, as they had a higher concentration of scFv. We also tried concentrated bacterial supernatant but again these were unsuccessful. Phage-antibodies were also tested but these did not detect protein by western or dot blot analysis. We then examined the possibility of using polyclonal phage-antibodies as polyclonal antisera can be more effective than monoclonal antibodies for some applications. The higher avidity of these sera gives increased sensitivity. Polyclonal populations of phage can be obtained after elution from an antigen-binding process. These populations may contain antibodies directed against a number of different epitopes, and are the equivalent of a polyclonal antibody serum with the notable difference that they can be grown *in vitro* repeatedly. Western blots using polyclonal phage antibodies were also unsuccessful.

The isolated scFv were used for immunocytochemistry on cells grown on slides. These cells were known to express MMP-9 protein and served as positive controls. The scFv showed no specific binding to MMP-9 protein over background staining. Consequently, these scFv could not be used for immunohistochemistry. It is possible that the isolated scFv only recognise MMP-9 protein bound to a

solid support such as plastic as this is the only format in which a response was seen using the scFvs. The affinity selection procedure was also carried out using MMP-9 coated onto plastic microtitre plates, thus selecting clones which recognise the protein in that format. Many proteins when adsorbed directly to solid phase by hydrophobic interaction, are altered in their three-dimensional structure (Butler, 1995), potentially creating different epitopes.

Although the scFv fragments against MMP-9 isolated from the library were of low affinity it is possible to improve the affinity and specificity of specific antigen-binding scFv fragments isolated from combinatorial libraries. The clones may be manipulated using various strategies (artificial affinity maturation). One strategy is 'chain shuffling' (Kang *et al.*, 1991). Chain shuffling involves combining a given chain from an isolated clone with complementary unselected chains. Alternative strategies for the refinement of clones involve mutagenesis and reselection. The enhancement of affinity *in vivo* can be contributed by a single point mutation, or several mutations (Berek and Milstein, 1987). Hypermutation of the genes corresponding to antibodies of low affinity should be easy to imitate *in vitro*. Point mutations could be introduced into the V genes by many techniques, for example, by using error-prone polymerases, PCR amplification through a large number of cycles, biased ratios of nucleotides triphosphates or 'spiked' oligonucleotide primers. Multiple mutations could be targeted throughout the body of the gene simultaneously, or to each of the hypervariable loops (Ward *et al.*, 1989). Ideally we would like to hypermutate specific segments of DNA within cells rather than in isolated DNA, and at the same time express the products on the cell surface, and select in a Darwinian fashion variants of steadily increasing affinity. Animals are superb at this job, and it will not be easy to compete with their efficiency. We may learn to imitate the animal strategy, but in the meantime for the production of high-affinity antibodies we will normally do better by stealing hypermutated V genes from immunized animals.

The binding affinities of the antibody fragments isolated from the Nissim library were disappointing, however, similar results have been seen by others working with the Nissim library (personal communication Dr. B. Manning and Tony Killard, BEST Center, DCU). An alternative phage display antibody library with a larger repertoire of antibody clones known as the Griffiths library has recently become available from the MRC. This library not only contains *in vitro* randomized CDR3 of heavy chains but also of the CDR3 in the λ light chain, leading to much larger repertoires (10^{10}) than had previously been possible. The synthetic repertoire of Fab fragments was

generated by recombination of heavy and light chain repertoires in bacteria using a process known as combinatorial infection. Soluble antibodies from this library have a histidine-myc tag which allows for purification using immobilised affinity chromatography. This library has also been used in our laboratory, but has so far yielded no positive results.

Although heralded as one of the greatest biological technologies of the 21st century, phage display has yet to live up to its promise. It has now been over a decade since phage antibody technology was introduced. However, the number of antibodies which have so far reached the market place has been minimal. Although unpublished, there is general recognition in the research community that there are serious difficulties, such as library instability, with the present generation of phage display vectors. The potential of the phage display system will only be fully realized when libraries with greater numbers of individual recombinants can be generated. The amplification step between rounds of panning with the phage display vector is associated with a number of problems even with pre-absorption steps, it is difficult to isolate a rare clone when its frequency is lower than the nonspecific background binding to the antigen affinity matrix. For the present at least, it seems that an antibody which specifically recognises active MMP-9 remains the holy grail of MMP antibodies.

Two emerging technologies could have the potential to 'compete' with phage display. Firstly, transgenic mice in which the human germ-line repertoire has replaced the animal's repertoire in part or totally (Jakobovits, 1995), however both screening and antigen limitations argue against it. Secondly, and of greater interest and potential are phage-free *in vitro* translation library systems in which expression on ribosomes is coupled to selection (Kawasaki, 1994). Such approaches are still only in their infancy and will require further developments before being readily applicable.

6.10 SUMMARY

We sought to isolate scFv fragments against MMP-9 using the Nissim library, a naive synthetic phage antibody library. Approximately 10^8 scFv antibody producing *E. coli* TG1 clones underwent 4 rounds of affinity selection (panning) to MMP-9 according to Nissim *et al.* (1994). Samples of polyclonal phage populations taken after each round of panning were tested by phage-antibody-ELISA and showed excellent enrichment of binding activity to MMP-9. To identify monoclonal phage antibodies the pHEN phage particles were rescued and 82 individual clones from round 4 were screened by phage-antibody-ELISA against MMP-9. 42 of the 82 clones (52%) were found to be positive. These positive phage clones were then infected into *E. coli* HB2151 and induced for soluble expression of scFv antibody protein. The production of antibody in the periplasm and supernatant was assessed. After transferring to HB2151 72% of clones produced soluble scFv fragments when tested by scFv-antibody-ELISA against MMP-9 protein. Culture supernatants were also tested by western blot analysis using an anti-c-myc antibody which detects the c-myc tag on the scFv fragment. Bands could be seen at 31 kDa for the scFv protein. Antibodies from clones with the greatest binding activity by ELISA were titered and found to be in the range of 1/100 dilution. Unfortunately, the scFv antibody titre was too low for further characterisation by BIAcore analysis. The binding affinity of the fragments was also too low for use as immunological reagents for western blots or immunohistochemistry.

Chapter 7

Final Summary

Final Summary

During early human pregnancy fetal trophoblasts rapidly invade the uterus. The extent and timing of this invasion is precisely regulated. The family of matrix degrading enzymes, called the matrix metalloproteinases (MMPs) have been implicated as major role players in this process. The research presented in this thesis was focused on investigating the expression, regulation and localisation of the MMPs involved in mediating trophoblast invasion. The aims of this research were to establish a model system using trophoblast continuous cell lines to examine the role of MMPs in trophoblast invasion. Using this model we sought to identify the factors which regulate expression of MMPs during the invasive process. We investigated the expression of MMPs *in vivo* and examined their role in pre-eclampsia. We also attempted to localise MMP-9 expression *in vivo* using an antibody fragment isolated using phage display technology.

Establishment of long term cultures of pure first-trimester human trophoblast cells with functional integrity provide reproducible *in vitro* systems for investigating the biological basis of invasive behaviour by cells. We have shown that long term replicating trophoblast cultures were free of contamination from other cell types, as judged by numerous cell-specific markers. Moreover, these long term monolayer cultures were proven to be functionally active since, they produced a significant amount of hCG and hPL, and were shown to be invasive using an *in vitro* invasion assay. These cell lines were unique as they were the first reported trophoblast cell lines to be established from CVS. We have successfully demonstrated that cell lines established from CVS do provide a useful *in vitro* system for studies of placental function. This opens up new avenues of research for investigators in countries where abortions are illegal or the use of abortus material is prohibited. We showed that two members of the MMP family, the type IV collagenases; MMP-2 and MMP-9 were expressed by the trophoblast cell lines. The choriocarcinoma cell line, BeWo produced large quantities of MMP-2 but little MMP-9. We also found that the BeWo cells were non-invasive under the culture conditions and assay used. The trophoblast cell lines were capable of invasion and it is likely that this is due to the expression of MMP-9. We proposed that in the model system described here MMP-9 is critical for invasion. We also reported the expression of TIMP-1 and TIMP-2 by the trophoblast cell lines but no TIMP expression by the BeWo cell line. Although it had previously been assumed that most metalloproteinase inhibitors that function in the

placental bed were probably of maternal origin, our results suggest that TIMP produced by the trophoblasts themselves may have a role in local regulation of ECM degradation.

Studies of the regulatory mechanisms controlling expression of MMP-9, are important for understanding the complex process of invasion which takes place during pregnancy. We examined the effects of three cytokines, IL-1β, EGF and TGF-β on trophoblast MMP production as possible regulators of the invasive process. Of those tested, only IL-1β had a significant effect, upregulating expression of MMP-9 at both the mRNA and protein level, and increasing the invasive ability of the cells. The trophoblasts were also grown on various ECM matrices, and laminin was found to upregulate MMP-9 mRNA expression. Other biological modifiers including progesterone, β-estradiol and hypoxic culture conditions were shown to have no effect on MMP-9 expression in the trophoblast cell lines. Expression of the MMP-2 gene could not be modulated by any of the factors investigated in this study, however due to the similarity of its promotor sequence to housekeeping genes this is not surprising. Considering that the type IV collagenases are developmentally regulated during placentation and also the differential response of these two proteinases to cytokine regulation, we speculated that cytokines and ECM components, in particular IL-1β and laminin are involved in controlling MMP-9 activity throughout placentation.

Human pregnancy is associated with extensive growth and remodeling of the uterus and placenta. Having shown using our tissue culture model system, that the degradative activity of MMPs are important in trophoblast invasion *in vitro*, we sought to extend our study to examine their role *in vivo*. Using immunohistochemistry the cellular localisation of MMP-2, MMP-7 and MMP-3 was identified in human tissues recovered from uncomplicated term pregnancies. Our results show prominent expression of all three MMPs in syncytiotrophoblasts and stromal core areas of chorionic villi at full term. These MMPs may be involved in active remodeling of the villi and/or modulation of matrix deposition. We have shown that the cells composing the human placenta express the necessary MMPs to facilitate growth and tissue remodeling in the physiological progress of human gestation. Interestingly, MMP-3 and MMP-7 were not expressed by the continuous trophoblast cell lines. However, the trophoblast cell lines originate from first trimester cytotrophoblasts which are not present at term. It may also be possible that the trophoblast cell lines lack some paracrine factor in culture which might stimulate the expression of MMP-7 and MMP-3 *in vivo*.

In normal human pregnancy, invasion of the uterus and its arterial system by trophoblasts extends throughout the entire decidua and the adjacent third of the myometrium. In the pregnancy related disorder pre-eclampsia, trophoblast invasion is limited to the superficial decidua, and few arterioles are breached. Since trophoblast invasion is abnormally shallow in pre-eclampsia, it is likely that some component of the invasion process is altered. Potential changes in MMP expression within human placenta in pre-eclampsia were assessed. Placental biopsies from normal pregnancies and those complicated by pre-eclampsia were stained with the anti-MMP antibodies. The results showed that expression of MMP-2, MMP-3 and MMP-7 by trophoblasts in pre-eclamptic specimens was similar to MMP expression in normal uncomplicated pregnancy specimens. Therefore, in placental biopsies at term, no correlation could be made between the expression levels of these three MMPs and the pre-eclamptic disease state. Unfortunately it was not possible to examine MMP-9 protein expression in this study.

Having failed to find a commercially available antibody to MMP-9 which could be successfully used for immunohistochemistry of formalin fixed paraffin embedded tissue specimens we sought to isolate scFv fragments against MMP-9 using phage display technology. The Nissim library, a naive synthetic phage antibody library was obtained from the MRC, Cambridge. The library of approximately 10^8 scFv antibody producing *E. coli* TG1 clones underwent 4 rounds of affinity selection to MMP-9. Excellent enrichment of binding activity to MMP-9 was seen after panning. Individual clones from round 4 were screened by phage-antibody-ELISA against MMP-9 and 52% were found to be positive. These positive phage clones were then infected into *E. coli* HB2151 and induced for soluble expression of scFv antibody protein. The production of antibody in the periplasm and supernatant was assessed. After transferring to HB2151 72% of clones produced soluble scFv fragments against MMP-9 protein. Culture supernatants were also tested by western blot analysis using an anti-c-myc antibody and bands were seen at 31 kDa for the scFv protein. Antibodies from clones with the greatest binding activity by ELISA were titered and found to be in the range of 1/100 dilution. Unfortunately, this scFv antibody titre was too low for further characterisation by BIAcore analysis. The binding affinity of the fragments was also too low for use as immunological reagents for western blots or immunohistochemistry. This was disappointing, however, similar results have been seen by other investigators working with the Nissim library. Although the scFv fragments against MMP-9 isolated from the library were of low

affinity it could be possible to improve the affinity and specificity of the scFvs using various artificial affinity maturation strategies such as chain shuffling.

Trophoblast invasion, due to its eloquently regulated nature both spatially and temporally presents a unique contrast to the unregulated invasion seen in metastatic cancer and provides an excellent model to study the regulation of MMP expression. Elucidating the mechanisms which control the MMPs in normal processes such as in trophoblast invasion may hold the key to unraveling their role in many pathological conditions. The next decade of research holds great promise for understanding the role of these enzymes in normal physiological functions in the body, and could lead the way for the use of MMP-related therapies in the treatment of ECM degradation-related diseases.

Chapter 8

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