The expression and regulation of matrilysin (MMP-7) in human colon cancer and leukaemia cell lines

A dissertation submitted for the degree of Ph.D

by

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Declaration

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.

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"Remembering you……how you used to be
Slow drowned, you were angels
So much more than everything
Hold for the last time then slip away quietly
Open my eyes, but I never see anything"

Robert Smith, 1989.

Abstract

Matrilysin (MMP-7, EC 3.4.24.23) is the smallest member of the matrix metalloproteinase (MMP) family and has been shown to be overexpressed in various tumours including breast and colon cancers. Matrilysin has also been shown to play an important role in several aspects of tumour biology including growth, progression, invasion and metastasis. With respect to colon cancer, matrilysin is unique in that it is the only MMP expressed exclusively by the malignant epithelia of colonic adenocarcinomas. These facts combine to make matrilysin a promising therapeutic target. However, in order to develop drugs which specifically inhibit matrilysin it is important to understand how matrilysin gene expression is controlled, something which to date remains poorly understood.

We have examined a panel of human colon tumour cell lines and have shown that matrilysin expression can be upregulated by a number of cytokines including EGF, IL-6 and bFGF. Analysis of the matrilysin promoter revealed the presence of a number of potential transcription factor binding sites including three ETS sites. We have shown that EGF treatment increased matrilysin gene expression by activation of PEA3 transcription factors using artificial promoter, western blot and EMSA analysis. 'Supershift' EMSA analysis showed that other PEA3 subfamily members such as ERM and ER81 may also be involved which is in agreement with other studies. In addition, we have found that EGF increased cellular levels of β-catenin through destabilisation of the E-cadherin/catenin complex which resulted in increased binding to the Tcf site within the matrilysin promoter.

We also examined the expression and regulation of matrilysin in the K562 and HL-60 myeloid leukaemia cell lines. Results showed that only the K562 cell line expressed matrilysin and *in vitro* invasion assays showed that the K562 cells were up to 4 times more invasive than the HL-60 cell line. Matrilysin antibody blocking experiments showed a significant decrease in invasion in the K562 cell line suggesting a role for matrilysin in leukaemia invasion. The MMP and TIMP profiles of these cell lines were also examined.

Our data suggests that EGF plays an important role in the regulation of matrilysin gene expression via a number of new mechanisms. Furthermore, we have shown that matrilysin plays an important role in leukaemia cell line invasion. These findings have identified possible new drug targets that will inhibit matrilysin expression which in turn should lead to decreased tumourigenesis and invasion and metastasis.

Abbreviations

β-gal	β-galactosidase
γP^{32}-ATP	Gamma phosphorous 32 labelled ATP
Abs	Absorbance
ACF	Aberrant crypt foci
ADAM	A disintegrin and metalloproteinase
ADAMTS	ADAM thrombospondin
aFGF	acidic fibroblast growth factor
ALL	Acute lymphoblastic leukaemia
AML	Acute myelocytic leukaemia
AOM	Azoxymethane
AP	Activator protein
APC	Adenomatous polyposis coli
APL	Acute premyelocytic leukaemia
AR	Amphiregulin
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BAD	Bcl associated death promoter
BCA	Bicinchoninic acid assay
BCR	Breakpoint cluster region
bFGF	basic FGF
BHK	Baby hamster kidney fibroblasts
BM-MNC	Bone marrow mononuclear cells
BP-1/6C3/APA	BP-1/6C3/aminopeptidase A
BSA	Bovine serum albumin
BTB/POZ-ZF	Broad complex Tramtack Bric a brac, Pox virus and zinc finger
BTC	betacellulin
C/EBP	CAATT/Enhancer binding protein
CAM	Cell adhesion molecule
CAT	Chloramphenicol acetyl transferase

CD10/NEP	CD10 neutral endopeptidase
CD13/APN	CD13 amino peptidase N
cDNA	Complementary DNA
CFC	Colony forming cells
CFU	Colony forming units
CML	Chronic myeloid leukaemia
CNTF	Ciliary neurotrophic factor
COMP	Cartilage oligomeric protein
CSF	Colony stimulating factor
CT-1	Cardiotrophin-1
CTP	Cytosine triphosphate
DAB	Diaminobenzidine
DCC	Deleted in colon cancer
DEPC	Diethylpyrocarbonate
dIdC	Deoxyinositol/deoxycytosine
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	Dithiothreitol
Dvl	Dishevelled
EBS	ETS binding site
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
EGF-r	EGF receptor
ELF	ETS like factor
EMSA	Electrophoretic mobility shift assay
EPA	Erythroid potentiating activity
ERK	Extracellular response kinase
Ets	E-twentysix

FAP	Familial adenomatous polyposis
FasL	Fas ligand
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FGF-r	FGF-receptor
GABP-α	GA binding protein-α
gp	Glycoprotein
GRB	Growth factor receptor bound protein
GSK	Glycogen sythase kinase
GTP	Guanosine triphosphate
HANs	Hyperplastic alveolar nodules
HB-EGF	Heparin bound EGF
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HNPCC	Hereditary nonpolyposis colon cancer
HRE	Hormone response elements
HRGs	Heregulins
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin like growth factor
IGF-BP	IGF-binding protein
IL	Interleukin
IL-6r	IL-6 receptor
IPTG	Isopropyl β-D-thiogalctopyranoside
JAK	Janus Kinase
JNK	Jun kinase
λ	Lambda
LB	Luria-Bertrani broth
LEF	Leukaemia enhancing factor
LIF	Leukaemia inhibitory factor
MAPK	Mitogen activated protein kinase

MDS	Myelodyplastic syndromes
Min	Multiple intestinal neoplasia
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
MOPS	3-[-N-Morpholino] propanesulfonic acid
mRNA	Messenger RNA
MT-MMP	Membrane type MMP
MW	Molecular weight
NF-κB	Nuclear factor-kappa B
NF-IL-6	Nuclear factor for IL-6
NHL	Non Hodgkins lymphoma
OD	Optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
OSM	Oncostatin M
$p120^{ctn}$	protein 120 catenin
p21	Protein 21
p53	Protein 53
PA	Plasminogen activator
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEA	Polyoma enhancing activator
PGE2	Prostaglandin 2
PI-3-K	Phosphoinositol-3-Kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C

PLZF	promyelocytic leukaemia zinc finger
PMSF	Phenylmethylsulfonyl fluoride
pol	polymerase
PTB	phosphotyrosine binding
PUMP	Putative metalloproteinase
RAR-α	Retinoic acid receptor-α
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPTPs	receptor protein tyrosine phosphatases
RT	Room temperature (25°C)
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription-PCR
RXR	Retinoid X receptor
SCID	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
SH	Src homology
SMAD	Sma and MAD related protiens
SMDF	Sensory and motor neuron derived factor
SOS	Son of sevenless
SP-1	Specificity protein-1
STAT	Signal transducers and activators of transcription
SV40	Simian virus 40
TACE	Tumour necrosis factor-α converting enzyme
TAF	TATA binding protein associated factor
TBE	Tris borate EDTA
TBP	TATA binding protein
TBST	Tris buffered saline plus Tween
TCF	T cell factor
TE	Tris EDTA
TEMED	N N N' N'-Tetramethylethylenediamine
TF	Transcription factor

TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TIE	TGF-β inhibitory element
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumour necrosis factor-α
TPA	12-O-tetradecanoyl-phorbal-13-acetate
tPA	Tissue-type plasminogen activator
TRE	TPA responsive element
Triton-X-100	t-Octylphenoxypolyethoxyethanol
TTP	Thymidine triphosphate
uPA	Urokinase-type plasmiogen activator
VCAM	Vascular cell adhesion molecule
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Units

°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometer
bp	base pairs
Ci/mmol	Curies/millimole
cm	centimetre
cpm	counts per minute
g	grams
hrs	hours
kbp	kilobase pairs
kDa	kiloDaltons
Kg	kilogram
L	litre
M	molar
mA	milliamps
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
ng	nanogram
nm	nanometre
s	seconds
V	Volts
v/v	volume per volume
w/v	weight per volume

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Talks

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Biochemical Society, Irish area section meeting. "EGF regulates matrilysin gene expression through the activation of PEA3 transcription factors". Belfast, UK, September, 2000.

Irish Association for Cancer Research annual meeting. "Cytokine regulation of matrilysin gene transcription". Galway Ireland, April, 2000.

Poster presentations (2000-2001 only)

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Chapter 1

Introduction to the Matrix Metalloproteinases

1. Introduction

1.1 The extracellular matrix

The extracellular matrix (ECM) is a complex and dynamic meshwork that is assembled outside cells from the specialised glycoproteins and proteoglycans secreted by them. As well as providing structural support in the form of bone, cartilage and tendon the ECM also plays an important role in many biological processes such as cell adhesion and migration, tissue morphogenesis, as well as cell proliferation and differentiation. The ECM can generally be divided into two categories: the basement membrane and the interstitial connective tissue (Boudreau and Jones, 1999).

The basement membranes are extracellular structures that usually separate cells from underlying connective tissue. They are found for example between endothelial and epithelial cells and underlying the connective tissue around muscle and adipose tissue. The major components of the basement membranes are type IV and V collagens, laminin, entactin and several glycoproteins which interact noncovalently to form a dense network. In the adult the basement membrane usually forms a barrier to most cell types and thus serves to compartmentalise tissues and organs. However, in some cases the basement membrane can be selectively penetrated, for example, during white blood cell diapedesis into surrounding tissue. The basement membrane in the kidney also serves as a biological filter whereby molecules are allowed to pass through on the basis of a certain molecular weight (Lukashev and Werb, 1998, Kawai *et al.*, 1999, De Arcangelis and Georges-Labouesse, 2000).

Included among the interstitial connective tissues are heterogenous regions such as the dermis and stroma, as well as more specialised tissues such as bone and cartilage. These tissues contain several cell types in addition to those responsible for producing the bulk of the ECM, i.e. fibroblasts in connective tissue and osteoblasts in bone. These additional cell types include macrophages, lymphocytes, granulocytes, melanocytes, fat, endothelial, muscle and nerve cells. The major protein of the connective tissue matrix is

collagen. Collagens type I and III are found in most connective tissues while types II and IX collagens are present in cartilage. Collagens type VI, VII, VIII, IX, X and XII have also been described in specific tissues. In addition to the collagens, interstitial connective tissue also contains fibronectin, elastin, chondroitin sulphate proteoglycans, heparin sulphate proteoglycans, tenascin and hyaluronic acid. Laminin and entactin are also found in interstitial connective tissues and are not exclusively basement membrane components.

The ECM plays an important role in cell signaling during proliferation, migration and differentiation and this is primarily achieved through the interaction of the cell with the ECM via the use of cell adhesion molecules (CAMs). There are four main members of the CAM family which are the cadherins, integrins, selectins and immunoglobulin like adhesion molecules. The interaction between the cells and the ECM is essential for several biological processes such as cell proliferation whereby cell adhesion to the ECM modulates the expression and functional state of several cell cycle regulators including cyclin dependent kinases, cyclins and cyclin dependent inhibitors (Lukashev and Werb, 1998). Several cytokines are also incorporated into the ECM such as, basic fibroblast (bFGF) and epidermal growth factor (EGF), which when released also provide the cell with valuable external stimuli which inform the cell of the surrounding environment (Aharoni *et al.*, 1997, Kaiura *et al.*, 2000).

From the description above it is clear that both the 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 biological processes. When considering the possible ways in which defects in the ECM may be generated it is important to remember that the quality and quantity of the matrix depends not only on the structural components such as collagen, laminin and proteoglycan, but also on the regulated expression of ECM degrading proteinases and their inhibitors. These proteinases and their inhibitors have, for the most part, been studied in the context of their possible role in tumour invasion and metastasis (McCawley and Matrisian, 2000). 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 (MMPs) e.g. matrilysin (MMP-7).

The MMPs are believed to be the primary contributors to ECM degradation as a result of several key characteristics: they are secreted into the extracellular space and function under normal physiological conditions, they are highly regulated and frequently induced in areas of active matrix remodeling and the members of this family are the only enzymes capable of degrading fibrillar collagens. The condition of the ECM 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 MMPs are a family of highly conserved zinc dependent endopeptidases which collectively are capable of degrading the components of the basement membrane and interstitial ECM. They are a continually growing family of enzymes which currently consist of at least 23 well defined members. The MMPs can be defined by the following characteristics: i) they share common amino acid sequences; ii) their proteolytic activity can be inhibited by tissue inhibitors of metalloproteinases (TIMPs) ; iii) they are either secreted or exist as transmembrane pro-enzymes that require activation to exert their matrix degrading activity; iv) the active site contains a zinc ion and requires a second metal cofactor such as calcium and v) enzyme activity is optimal in the physiological pH range (Nagase and Woessner, 1999). The properties of the human MMPs which have been cloned to date are summarised in table 1.1.

Table 1.1 Properties of the human matrix metalloproteinase family.

MMP Domain	Enzyme Name	MW kDa (latent)	MW kDa (active)	Substrates	Activation of other MMPs
MINIMAL					
MMP-7	Matrilysin, Pump-1.	28	19	Proteoglycans, laminin, fibronectin, gelatins, collagen IV, elastin, entactin, tenascin.	MMP-1, MMP-2, MMP-9
HEMOPEXIN					
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 I, II, III, IV	Unknown
MMP-12	Metalloelastase	53	45/22	Elastin, fibronectin, collagen IV	MMP-2, MMP-12
MMP-3	Stromelysin-1	57	45	Proteoglycans, laminin, fibronectin, collagen III,IV, V, X, 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					
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					
MMP-14	MT1-MMP	63	-	CD44	MMP-2
MMP-15	MT2-MMP	72	-	Unknown	Unknown
MMP-16	MT3-MMP	64	-	Unknown	MMP-2
MMP-17	MT4-MMP	70	-	Unknown	Unknown
MMP-24	MT5-MMP	60	-	Proteoglycan ECM components.	Unknown
MMP-25	MT6-MMP	62	-	Unknown	MMP-2
MISCELLANEOUS					
MMP-19	RASI-1	-	-	Gelatins, Aggrecan, cartilage.	Unknown
MMP-20	Enamelysin	54	-	Amelogenin, Aggrecan, cartilage.	Unknown
MMP-21	Recently cloned MMP	-	-	Unknown	Unknown
MMP-22	Recently cloned MMP	-	-	Unknown	Unknown
MMP-23	Recently cloned MMP	28	-	Unknown	Unknown
MMP-26	Endometase/matrilysin-2	28	18	Collagen type IV, Fibronectin, Fibrin, Gelatin.	MMP-9
MMP-27	Epilysin	59	-	Casein	Unknown

1.2.1 The classification and structure of MMPs

Previously, the MMPs were divided into subclasses with respect to their substrate specificity but this classification system became redundant as the family grew and new substrates were identified for individual members. MMPs are now classified according to their protein domain structure and on this basis the MMP family can be loosely divided into classes which contain the following domains; i) the minimal domain which is comprised of a pre, pro and catalytic region, ii) the hemopexin domain, iii) the fibronectin domain and iv) the trans-membrane domain. (See figure 1.1).

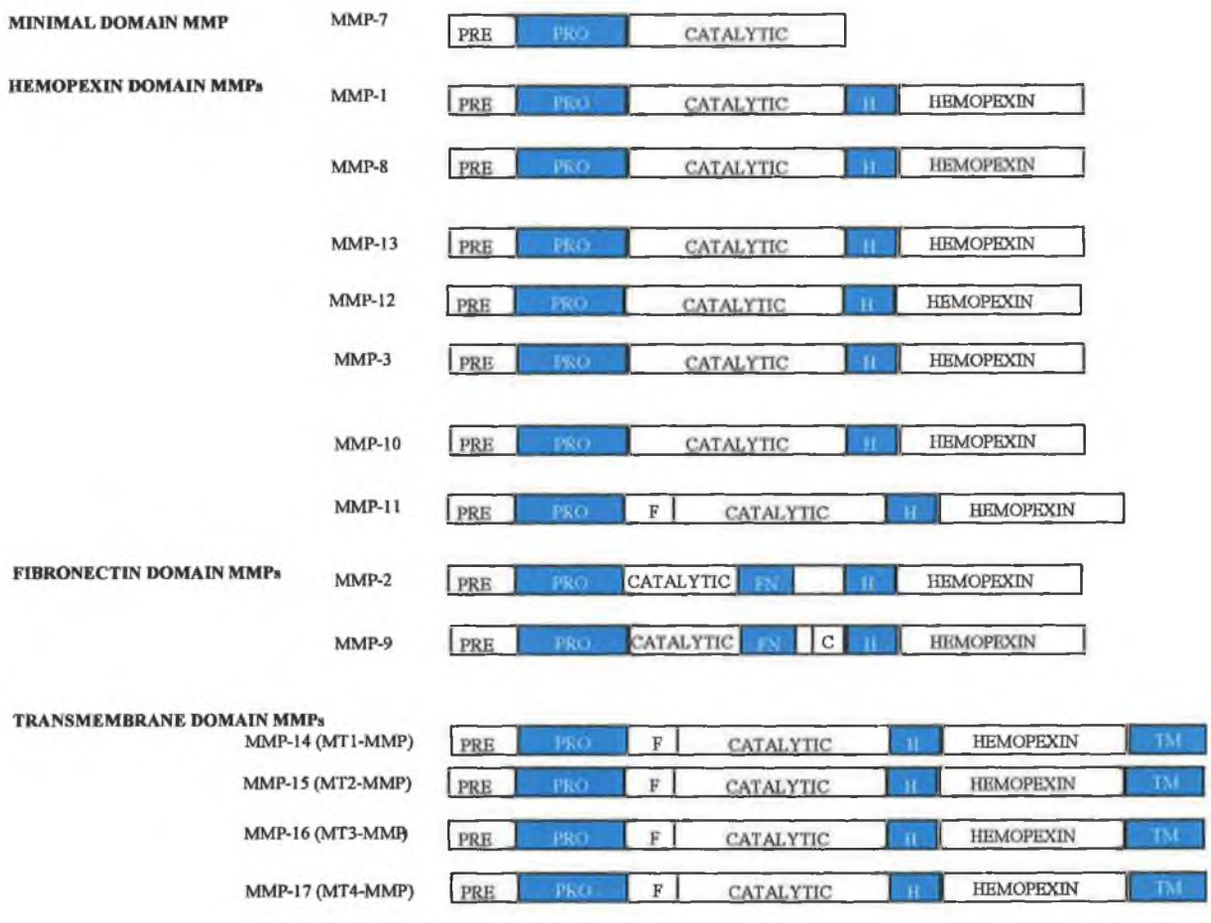

Figure 1.1 Domain structure of various MMP family members. [H = Hinge domain ; F = furin consensus site ; FN = Fibronectin-like domain ; C = collagen-like domain ; TM = transmembrane-like domain].

1.2.1.1 The minimal domain of MMPs

The minimal domain is comprised of the pre, pro and catalytic domain. The pre domain can be found at the amino terminus of all MMPs and is involved in directing the protein for cellular export. The pre domain is rapidly removed prior to secretion (Wilhelm et al., 1987).

The pro domain, which can be up to 100 amino acids long, contains a highly conserved sequence of eight amino acids, PRCGVPDV (Nagase *et al.*, 1990). The pro-domain must be removed through cleavage in order to activate the enzyme. This region is involved in maintaining the enzyme in a latent state since mutations in this area results in an enzyme which no longer requires proteolytic activation. *In vitro*, this activation can be achieved by a variety of agents including oraganomercurials, oxidants, sulphydryl alkylating agents and, in some cases, proteolytic cleavage by trypsin or plasmin (Nagase *et al.* 1990). This spectrum of activators suggests that a conformational change is required for activation and that a thiol bond is involved. Activation of MMPs involves a process whereby the N-terminal part of the molecule is folded around the latent enzyme so that the cysteine residue in the conserved PR**C**GVPDV region complexes with a zinc molecule in the active site. The activation results when a conformation change dissociates the cysteine from the zinc atom and replaces it with water. This has been referred to by Van Wart and colleagues as the 'cysteine switch' mechanism (See figure 1.2). The activated MMP is then capable of auto proteolysis. *In vitro*, other enzymes such as cathepsin G and neutrophil elastase have shown to activate stromelysin-1 and gelatinase A (VanWart *et al.*, 1990).

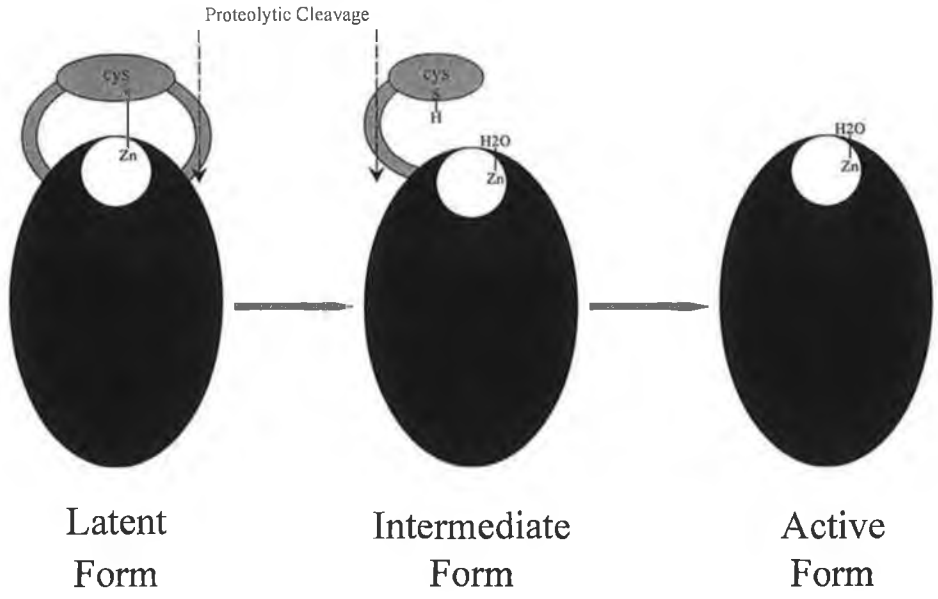

Figure 1.2 Cysteine switch mechanism for MMP activation. The pro domain of the latent molecule is folded around so that the cysteine residue can form a complex with the zinc molecule in the active site. An initial cleavage then causes a conformation change in the molecule that disrupts the cysteine-zinc 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).

The catalytic site is another element of the minimal domain and contains the conserved sequence **H**EXG**H**XXGXX**H**S. The active site holds a zinc ion by coordinate bonding to three histidines. Studies have also shown that some MMPs contain a second zinc binding site which plays an important role in stabilising tertiary structure (Yuan *et al.*, 1994). Two calcium ions are also present in the catalytic domain but these however, are not essential for enzymatic activity but may be important in inhibitor binding (Yuan, *et al.*, 1994). Matrilysin (MMP-7) and MMP-26, also known as matrilysin-2, are the smallest members of the MMP family. They are comprised of just these three essential minimal domains which may imply that MMP-7 and MMP-26 were the first MMP-family members to evolve.

The type IV collagenases, MMP-2 and MMP-9, have three repeats of fibronectin-type domain inserted into the catalytic domain which have been shown to be required for gelatinase/collagenase activity (Steffensen *et al.*, 1995). 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 remains unclear.

1.2.1.2 The MMP hemopexin terminal domain

The fourth domain, the C-terminal, of MMPs is involved in the recognition of macromolecular substrates and has been implicated in determining the distinct substrate specificities of the MMPs (Borkakoti, 1998). Matrilysin, until the recent cloning of MMP-26 (endometase/matrilysin-2), was the only family member which did 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. Although matrilysin does not contain this domain it still retains an ability to degrade a wide variety of substrates. The hemopexin domain is linked to the catalytic domain via a short but variable hinge region which is proline rich and is thought to be involved in substrate interaction (Nagase and Woessner, 1999). The presence of the hemopexin domain in MMP-1, MMP-8 and MMP-13 allows them to cleave fibrillar type I collagen (Hirose *et al.*, 1993).

1.2.1.3 The MMP transmembrane domain

The most recently described members of the MMP family contain a transmembrane domain near their carboxyl termini which localises the enzymes to the plasma membrane and thus, they are referred to as membrane type matrix metalloproteinases (MT-MMPs) (Polette and Birembaut, 1998). While these MMPs have a common domain structure with pre-, pro- catalytic and hemopexin-like domains, they have also three unique insertions. First there is an insertion of 11 amino acids between the pro peptide and catalytic domains. This sequence is also present in MMP-11. The sequence is recognised by furin, a pro-protein convertase, present in the Golgi apparatus which is able to activate recombinant MT1-MMP (Sato *et al.*, 1996). Furin can cleave MT1-MMP which subsequently results in MMP-2 activation although other reports have shown that MT1-MMP activity is not a pre-requisite for MMP-2 activation as plasmin has also been shown to activate MMP-2 extracellularly. The MT-MMPs have a second

insertion of 8 amino acids within the catalytic enzyme domain whose function remains undefined. The third insertion at the C-terminus contains a hydrophobic amino acid sequence which acts as a transmembrane domain. The MT-MMPs cannot apparently be activated by agents such as organomecurials (Pei and Weiss, 1995) but require proteolytic activation by molecules such as furin. It has also been shown that MT1-MMP and MMP-11 have a unique similarity in that neither enzyme can efficiently degrade ECM proteins. This observation suggests the possibility that there may be other functions associated with MMPs other than the degradation of ECM proteins.

1.2.2 Substrate Specificities of the MMPs

The degradation of the ECM is a multi-step process in which members of the MMP family are inextricably linked. Each MMP has a unique yet slightly overlapping substrate specificity. Prior to the classification of MMPs with respect to their protein domain characteristics, the MMPs were classified according to their substrate specificity. Broadly speaking the MMPs were divided into three subclasses: i) the type I collagenases; ii) the type IV collagenases and iii) the stromelysins (see table 1.1 for additional information on molecular weights and substrates). However, with the discovery of so many new MMP family members it has become more efficient to classify the substrate specificities of the MMPs according to their domain structure.

1.2.2.1 Substrate specificities of the minimal domain sub-class of MMPs

Matrilysin (MMP-7) has thus far been the only MMP family member to be classified under this heading although further studies involving MMP-26/matrilysin-2 should also place it in the minimal domain category. Matrilysin is the smallest member of the MMP family with the inactive form having a molecular weight of 28 kDa and the active form having a molecular weight of 19 kDa. Interestingly, matrilysin has the broadest substrate activity of all the MMPs with an ability to degrade collagens, proteoglycans and glycoproteins (Fingleton *et al.*, 1999). Matrilysin has been shown to play an important role in many normal biological process but has also been shown to play an active part in the progression and invasion of many tumour types including colon,

breast and prostate cancers (McDonnell *et al.*, 1991, Newell *et al.*, 1994 and Sundareshan *et al.*, 1999). The role of matrilysin in these processes will be discussed in more detail in chapter 3.

1.2.2.2 Substrates specificities of the hemopexin domain sub-class of MMPs.

The hemopexin domain sub-class is comprised of several members which are characterised by the presence of four hemopexin-like repeats in their C-terminal region. Hemopexin domain sub-class members include MMP-1, MMP-8, MMP-13, MMP-12, MMP-3, MMP-10 and MMP-11. MMP-1, MMP-8 and MMP-13 which are capable of degrading fibrillar collagens that include type I, II, III, VII, X, gelatin and proteoglycans. Interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8) have been well characterised and both these enzymes operate by cleaving the alpha chains of types I, II and III collagen at a single site. This results in an 'unwinding' of the helical structure which subsequently exposes the collagen to other proteolytic agents. The gene encoding interstitial collagenase was isolated from a human lambda (λ) phage cDNA library (Collier *et al.*, 1988a). Neutrophil collagenase was identified and sequenced from a cDNA library derived from peripheral leukocytes of a patient with chronic granulocytic leukaemia (Hasty *et al.* 1990) and its expression has been found to be restricted to cells of a neutrophil and chondrocyte lineage. MMP-13 also known as collagenase-3 was cloned from a cDNA library derived from breast tissue (Freije *et al.*, 1994). Increased expression of MMP-13 in breast carcinoma and the absence of detectable levels in normal tissues has been shown and therefore a possible link to tumourigenesis has been proposed. Analysis of MMP-13 substrate specificity has shown that collagen type II is preferentially hydrolysed by MMP-13 (Knauper *et al.* 1996).

The stromelysin subclass, containing stromelysin-1, -2, -3 (MMPs –3, -10 –11 respectively) and metalloelastase (MMP-12) are capable of cleaving many ECM components (with the exception of MMP-11) including proteoglycans, fibronectin, collagens and gelatins but have no proteolytic activity for native collagen type I, with the exception of stromelysin-3 which is a weak proteinase in comparison to other members of this subclass but has been shown to cleave laminin and fibronectin (Murphy *et al.* 1993).

Stromelysin-1 was identified in 1985 as a secreted MMP (Chin *et al.*, 1985). The molecule was originally cloned by virtue of its inducibility by oncogenes and growth factors and was referred to as transin (Matrisian *et al.*, 1985). Transin was later shown to be the rat homolog of human stromelysin-1 (Muller *et al.*, 1988). Stromelysin-2 (MMP-10) has been identified in both rat and human tissues and has a similar amino acid sequence (75-80% homology) and substrate specificity to stromelysin-1 (Nicholson *et al.*, 1989). However, the two genes are regulated differently by growth factors due to differences in transcription factor binding sites within each promoter. Stromelysin-3 (MMP-11) was initially isolated from stromal cells surrounding breast carcinomas and its expression has been associated with the invasion and metastasis of breast cancer (Bassett *et al.*, 1990). The stromelysins have been shown to be over expressed in several cancer types including colon, breast and prostrate cancer (McDonnell *et al.*, 1991, Pacheco *et al.*, 1998 and Sundareshan *et al.*, 1999).

The final member of the stromelysin subclass is metalloelastase (MMP-12). Metalloelastase is a macrophage specific member of the MMP family and was initially identified in the lung as a 22 kDa protein with elastinolytic activity (Feinberg *et al.*, 2000). MMP-12 degrades a number of proteins that not only includes elastin but also collagen type IV, fibronectin, vitronectin and laminin. Human and mouse MMP-12 have been shown to undergo auto-proteolytic processing and recombinant MMP-12 has also been shown to play an important role in the MMP activation cascade and is capable of activating MMP-2 and MMP-3. MMP-12 expression has also been shown to play an important role in arteriosclerosis, arthritis and emphysema (Shapiro, 1998 and Johnson *et al.*, 1998).

1.2.2.3 Substrate specificities of the fibronectin domain sub-class

The fibronectin domain sub-class currently contains two members, MMP-2 and MMP-9 or gelatinase A and gelatinase B respectively. MMP-2 and MMP-9 degrade denatured collagens (gelatins) and are specific for the degradation of type IV basement membrane collagen. MMP-2 was cloned by Collier *et al.* (1988b) from a human lambda (λ) phage cDNA library. It is secreted as a 72 kDa proenzyme and when activated cleaves

the following substrates in order of preference: gelatin, collagens type IV, V, VII and fibronectin. The expression of MMP-2 is widespread and is frequently elevated in malignant tumours. MMP-9 was traditionally thought of as a macrophage specific gelatinase but its expression has been described in malignant cells, neutrophils cytotrophoblasts and keratinocytes (Collier *et al.*, 1988 and Wilhelm *et al.*, 1989). MMP-9 was cloned from SV-40 transformed human lung fibroblasts (Willhelm *et al.*, 1989). It is secreted as a 92 kDa proenzyme and when activated degrades gelatins, collagens type IV and V and elastin.

1.2.3 The membrane type matrix metalloproteinases (MT-MMPs)

Using domain contents to classify the MMPs has resulted in the grouping together of various sub-families such as the MT-MMPs. The membrane type matrix metalloproteinases are comprised of 6 family members. The first member of the MT-MMP family (MT1-MMP) was isolated by Sato *et al.* (1994) who cloned a cDNA encoding a MMP of 63 kDa with a transmembrane domain. Expression of the gene product of this MT-MMP induced specific activation of MMP-2 on the cell surface *in vitro* and enhanced cellular invasion of reconstituted basement membrane. Following this discovery of MT1-MMP (MMP-14), five other MT-MMPs have been identified; MT2-MMP (MMP-15) which has a molecular weight of 72 kDa (Takino *et al.*, 1995), MT3-MMP (MMP-16) which has a molecular weight 64 kDa (Matsumoto *et al.*, 1997) and MT4-MMP (MMP-17) which has a molecular weight of 70 kDa (Puente *et al.*, 1996), MT5-MMP which has a molecular weight of ~ 60 kDa (Wang *et al.*, 1999) and MT6-MMP (MMP-25) which has a molecular weight of ~62 kDa (Kojima *et al.*, 2000. MT1-MMP has also been reported to act as a receptor for pro-MMP-2 (Will *et al.*, 1996). MT1-MMP has also been shown to be involved in the activation of pro collagenase-3 (MMP-13). The substrate characteristics of the MT-MMP group remain unclear.

1.2.4 Recently cloned MMPs

Several new and novel members of the MMP family have recently been discovered including MMP-18, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP-

26 and MMP-27. MMP-18 is a novel collagenase isolated from metamorphosing Xenopus *laevis* tadpoles and has essentially identical enzymatic activity to human interstitial collagense (Stolow *et al.*, 1996).

MMP-19 and MMP-20 exhibit previously described MMP characteristics but lack a series of structural features distinctive of the diverse MMP subclasses and have been proposed to be the first members of a new MMP subfamily (Pendas *et al.*, 1997 and Stracke *et al.*, 2000). MMP-19 may play a role in arthritis since serum anti-MMP-19 autoantibodies are frequently detected in rheumatoid arthritis patients (Sedlacek *et al.*, 1998). MMP-19 is mainly expressed in the lung, placenta, pancreas, ovary, spleen, and intestine suggesting that it may have specialised role in these tissues. MMP-20, also known as enamelysin was cloned from odontoblastic cells which are involved in matrix remodeling during enamel maturation, and the enzyme was shown to cleave the major component of teeth ECM, amelogenin (Llano *et al.*, 1997). MMP-20 expression is restricted to the enamel organ (Stracke *et al.*, 2000). MMP-19 has also been recently shown to cleave aggrecan, a proteoglycan of the cartilage ECM, and cartilage oligomeric matrix protein (COMP) which again may implicate MMP-19 in arthritic disease.

The gene for MMP-21 was identified together with the gene for MMP-22 in the Cdc2L1-2 locus at the end of the short arm of human chromosome 1, 1p36.3, which is deleted frequently in a number of tumours. The catalytic domains of MMP-21 and MMP-22 are related most closely to MMP-11 (stromelysin-3). These proteins do not contain highly conserved cysteine residues in the proenzyme domain and have been shown to be involved in the autocatalytic activation of other metalloproteinases. Multiple MMP-21 and MMP-22 mRNAs, some of which are derived by alternative splicing, are expressed in a tissue-specific manner (Gururajan *et al.*, 1998). MMP-23 was originally cloned from an ovarian cDNA library (Velasco *et al.*, 1999). This protein exhibits sequence similarity with MMPs, but displays a different domain structure in that MMP-23 lacks a recognizable signal sequence and has a short prodomain, although it contains a single cysteine residue that can be part of the cysteine-switch mechanism operating for maintaining enzyme latency. The C-terminal domain is considerably shortened and shows no sequence similarity to hemopexin, whereas all human MMPs, with the exception of

matrilysin and MMP-26, contain four hemopexin-like repeats. Furthermore, MMP-23 is devoid of structural features distinctive of the diverse MMP subclasses, including the specific residues located close to the zinc-binding site in collagenases, the transmembrane domain of membrane-type MMPs, or the fibronectin-like domain of gelatinases (Velasco *et al.*, 1999). MMP-23 is predominantly expressed in ovary, testis, and prostate, suggesting that this new MMP may play a specialized role in reproductive processes. The protein has a molecular weight of approximately 28 kDa.

MMP-26, also known as endometase and matrilysin-2, was isolated from human endometrial tumours and human placenta (Park *et al.*, 2000 and Uria and Lopez-Otin, 2000). The enzyme is similar to matrilysin in that it contains only three functional domains but has a low sequence homology to matrilysin and is specifically expressed in the human uterus. MMP-26 has a molecular weight of 28 kDa and is capable of cleaving a diverse spectrum of substrates including collagen type IV, fibronectin and gelatin and has also been shown to be expressed in malignant tumours. MMP-27, also known as epilysin, was cloned from kidney and testis cDNA libraries. MMP-27 has a molecular weight of 59 kDa and has been shown to degrade casein. The protein contains a signal sequence, a prodomain, a zinc-binding catalytic domain, and a hemopexin-like domain. In addition, epilysin has a furin activation sequence but has no transmembrane sequence. MMP-27 has a broad substrate specificity and is expressed in a number of tissues including testis, lungs, heart colon and brain (Lohi *et al.*, 2001).

1.2.5 Additional MMP substrates

In addition to ECM components, MMPs can cleave precursor forms of other MMPs, urokinase-type plasminogen activator (uPA) and proteinase inhibitor α-1-antitrypsin (see Chambers and Matrisian, 1997 for review). MMPs have also been implicated in the activation of growth factors, e.g. tumour necrosis factor alpha (TNF-α) and growth factor receptors such as interleukin-6 receptor (IL-6r), (Gearing *et al.*, 1994 and Chambers and Matrisian, 1997). A recent report also describes the ability of matrilysin and MMP-9 to inhibit angiogenesis via the hydrolysis of plasminogen to produce angiostatin fragments (Patterson and Sang, 1997). Novel functions attributed to

the MMPs such as those outlined above have helped to redefine the matrix metalloproteinases as important factors in not only ECM remodeling but in numerous biological processes including cell proliferation, migration and angiogenesis.

1.3 The ADAMs family

The ADAMs family (a disintegrin and metalloproteinase) are transmembrane proteins that contain a disintegrin and metalloproteinase domain and therefore, potentially have both cell adhesion and proteinase activities. The ADAMs are a rapidly growing family and thus far, 29 members have been characterized, although the function of many of these members has yet to be determined. An ADAM is a multi-domain protein ~ 750 amino acids long and includes : the pro-domain, the metalloproteinase, disintegrin, cysteine rich, EGF-like transmembrane domains and the cytoplasmic tail domain (see Primakoff and Myles, 2000 for review). Among the 29 known ADAMSs, 17 have been shown to have a metalloproteinase active site with the correct amino acid sequence (deduced from the cDNA) and therefore are thought to be functional proteinases. The other 12 ADAMs have one or more residues in the active site which are incompatible to those with metalloproteinase activity and are therefore thought to lack metalloproteinase activity. It is possible however, that although they may be poor proteinases they may still retain the ability to dock with substrates and present them to more suitable proteinases. Many members of the ADAMs family, 14 in all, are expressed in a wide variety of tissues while 15 are found exclusively in the testis which suggests that these ADAMs play an important role in the processes of spermatogenesis and fertilisation. Several of the ADAMs have been shown to act as sheddases and perhaps the best studied of these is Tumour Necrosis Factor (TNF)-α converting enzyme (TACE) or ADAM-17. TNF-α, which is involved in the inflammatory response is synthesised as a 26 kDa membrane anchored protein which is cleaved by TACE to give the active 17 kDa ligand. The generation of a TACE knockout mouse causes embryonic lethality as a result of the inability to shed TNF-α and transforming growth factor-α (TGF-α) (Black and White, 1998). These mice were also unable to shed the TNF receptor, L-selectin (an adhesion molecule) or amyloid protein precursor (APP) which suggests that TACE is a sheddase with multiple substrates (Peschon *et al.*, 1998 and Buxbaum *et al.*, 1998). Recently

another ADAMs family member, Kuzbanian (ADAM-10) has been shown to release Delta which is a Notch ligand (Qi *et al.*, 1999). Notch is a surface receptor that regulates cell fate determination in various aspects of development, such as neurogenesis. New data has also shown that Kuzbanian can mediate the transactivation of the EGF receptor by G-protein coupled receptors and via the cleavage of heparin bound EGF (HB-EGF) (Yubing Yan, personal communication).

Recently it has been made clear that ADAM proteins with a thrombospondin repeat motif (ADAMTS) represent an important and separate class of proteinases. ADAMTS which have been well charaterised include gon-1 (involved in gonad formation in *C. elegans*), ADAMTS-4 (aggrecanase-1) and ADAMTS-11 (aggrecanase-2) (see Tang and Hong, 1999 for review). The fundamental difference between ADAMs and ADAMTS proteins stem from their different locations in tissues. ADAMs are transmembrane proteinases, the known substrates of which are other transmembrane proteins. ADAMTS proteins are soluble extracellular matrix proteins that consist of a pro-domain, and metalloproteinase and disintegrin domains, but lack the ADAMs' cysteine rich, EGF-like transmembrane and cytoplasmic domains. The thrombospondin motifs present on the C-terminal side of the disintegrin domain of ADAMTS may function along with the disintegrin domain to maintain these proteins in an appropriate location in the ECM architecture.

1.4 Regulation of the MMPs

The constitutive level of expression of MMP genes is normally low, the enzymes being induced under various physiological circumstances when ECM remodeling is required, for example during embryogenesis, wound repair and bone remodeling. MMP expression is tightly regulated which indicates the biological importance of these enzymes. In addition, individual members of the MMP family are separately regulated and their expression is highly tissue specific. The regulation of MMPs is complex and occurs at a number of different levels including; i) the transcriptional level; ii) at the protein level via processing of the latent precursor to an active enzyme; and iii) by

modulating proteinase activity via the tissue inhibitor of metalloproteinases (TIMPs), (see section 1.5).

1.4.1 Transcriptional regulation of MMP genes

Several studies have shown that MMPs are regulated both positively and negatively at the transcriptional level by a variety of growth factors, cytokines, oncogenes, phorbol esters and adhesion molecules (Fingleton and McDonnell, 1993, Birkedal-Hansen *et al.*, 1993 and Crawford and Matrisian, 1996). In order for genes to be transcribed a number of elements of the cells transcriptional machinery need to be in place on the promoter of the gene. Within the promoter there are short sequences of DNA known as transcription factor binding elements to which transcription factors can bind (see figure 1.3). Although there is a clear demarkation in the patterns of MMP expression, the MMP gene promoters show remarkable similarities with respect to transcription factor binding sites (see figure 1.3). For example the majority of the promoters of the human MMP genes with the notable exception of gelatinase A (MMP-2) and stromelysin-3 (MMP-11) contain an activating protein-1 (AP-1) element which binds the Fos/Jun AP-1 protein complex (Crawford and Matrisian, 1996). Another notable element which is found throughout the MMP promoters is the polyoma enhancing activator-3 (PEA3) element.

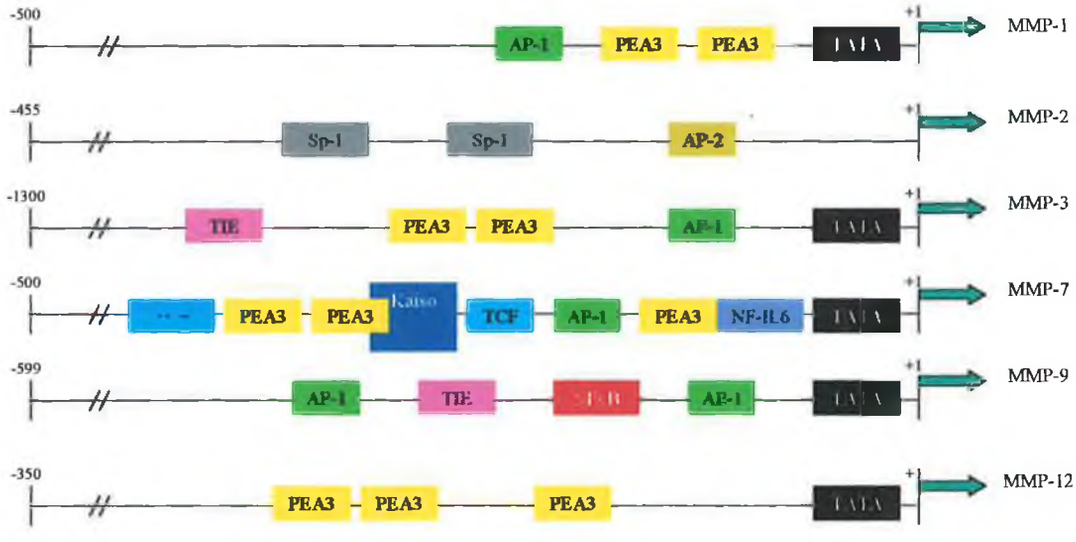

Figure 1.3 Transcription factor binding elements within various MMP promoters. Note that the figure is not drawn to scale.

1.4.2 Transcription factor binding elements within the MMP promoters

A single AP-1 element is normally present at approximately –70 base pairs (bp) upstream in the promoter region of each inducible MMP gene. The AP-1 site has been the subject of much recent research and it is now known to be essential for the basal transcription of MMP-1 (Curran and Murray, 2000). Molecules that inhibit the expression of inducible MMP genes also appear to act via the AP-1 site. The AP-1 site is necessary for the transcriptional response to a variety of signals, for example, interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), interferon-β (INF-β) and 12-O-tetradecanoylphorbol 13-acetate (TPA) (Curran and Murray, 2000). Other agents that modulate the expression of MMPs, such as glucocorticoids, retinoids and transforming growth factor-β (TGF-β) also require the AP-1 site to exert their function. Many studies have looked at the effects of these molecules on MMP-expression in isolation which is not typically the case *in vivo* as several agents may be required to initiate the transcription of MMP genes. In many cases it is possible that the effects may not be direct and the initiation of MMP transcription may be a downstream response. Other *cis*-acting elements are also necessary for both basal transcription and *trans*-activation by phorbol esters, cytokines and growth factors (Vincenti *et al.*, 1996, Benbow and Brinckerhoff, 1997). AP-1 transcription factors are protein complexes, composed of Jun and Fos subunits. These subunits form heterodimeric leucine zipper proteins that bind to a consensus DNA sequence, typically **TGAGTCA**, which is the AP-1 element within the MMP promoters. In normal cells, Jun and Fos proteins are transiently expressed following a mitogenic stimulus to the cell. The cellular concentration of these subunits is normally controlled by the stability of their mRNA (i.e. post transcriptional regulation), as well as by the rate of gene transcription. Binding of the Jun/Fos complexes to the AP-1 site is associated with transcriptional activation of the MMP genes, but some members of the Fos and Jun families act as transcriptional repressors. Fos/Jun complexes that are weak activators of AP-1 interact with this site, thereby blocking interaction with more potent activators. For example, Jun B has been shown to inhibit Jun induced MMP-1 gene expression (Westermarck *et al*, 1997). Expression of Jun B is associated with differentiated cells, which may contribute to the less aggressive biological behaviour observed in more differentiated tumours

(Curran and Murray 2000). A second activating protein complex (AP-2) has also been described and has been shown to play a significant role in the regulation of MMP-2 whereby it was found to be necessary for constitutive expression of MMP-2 (Qin *et al.*, 1999). The AP-2 transcription factor family is comprised of three members, AP-2α, AP-2β and AP-2 γ which bind to a site distinct to that of AP-1 (**GCCN$_3$GGC** for AP-2 versus **TGAGTCA** for AP-1) (Hilger-Eversheim *et al.*, 2000).

Most of the promoters of MMP inducible genes also contain a polyoma enhancing activator-3 (PEA3) site which binds members of the ETS (E-twenty six) family of oncoproteins. The combination of AP-1 and PEA3 binding sites has been referred to as an 'oncogene responsive unit' and it appears to be a recurring motif, found in the promoter regions of other genes (Gutman and Wasylyk, 1991). The AP-1 and PEA3 sites display functional cooperation and there is evidence of synergism between these sites (Crawford and Matrisian, 1996). Thus, inhibition or modulation of these two sites may represent a therapeutic approach to downregulate synthesis of MMPs.

Several compounds are also capable of inhibiting the synthesis of MMPs including retinoids, thyroid hormones, glucocorticoids, progesterone and androgens. All these agents bind to members of the nuclear receptor subfamily which control gene transcription via a number of different mechanisms. Firstly, nuclear receptors act on the promoter regions of MMP genes. To date, few hormone response elements (HRE) have been found within the MMP promoters and they appear to inhibit transcription by forming complexes with the DNA through interactions with AP-1 proteins. Glucocorticoids and retinoids also inhibit AP-1 activation by complexing with the site or by sequestering Fos and Jun proteins (Schroen and Brinckerhoff, 1996). Studies have also shown that TGF-β inhibits the EGF induction of stromelysin gene expression through an upstream element referred to as the TGF-β inhibitory element (TIE). Interestingly, Fos protein is present in the complex that recognises the TIE element (Kerr *et al.*, 1988). Thus it appears that proto-oncogene induction by growth factors can be involved in both positive and negative regulation of MMP gene expression.

1.4.3 Post Transcriptional Regulation of MMP Activity

Although sophisticated transcriptional control mechanisms play an important role in the modulation of MMP synthesis, post-transcriptional control mechanisms are central to the control of MMP activity as all the soluble MMPs are secreted as inactive zymogens requiring activation by cleavage of the N-terminal pro-domain. It has been suggested that the endogenous activator of MMPs is plasmin, produced from the plasminogen precursor by the action of plasminogen activators, (He *et al.*, 1989). Plasmin can convert both proMMP-1 and proMMP-3 to their active forms. MMP-7, MMP-3, MMP-10 and the MT1-MMP can also activate other MMPs by proteolytic cleavage. Pro-MMP-2 has been shown to be a substrate for MT1-MMP (Sato *et al.*, 1996). Interestingly, MT1-MMP also binds to TIMP-2 and it has been proposed that the MT1-MMP/TIMP complex acts as a receptor for pro-MMP-2. Once pro-MMP-2 has bound, other MT1-MMPs in proximity to the complex clip the pro domain resulting in active pro-MMP-2. Plasmin has also been implicated in the activation of pro-MMP-2 when it is bound to the MT1-MMP complex. (Mazzieri *et al.*, 1997). These proteolytic events are tightly regulated in a cascade style system and this system serves as a controlled but powerful mechanism to coordinate complete degradation of the multiple components of the ECM (see figure 1.4).

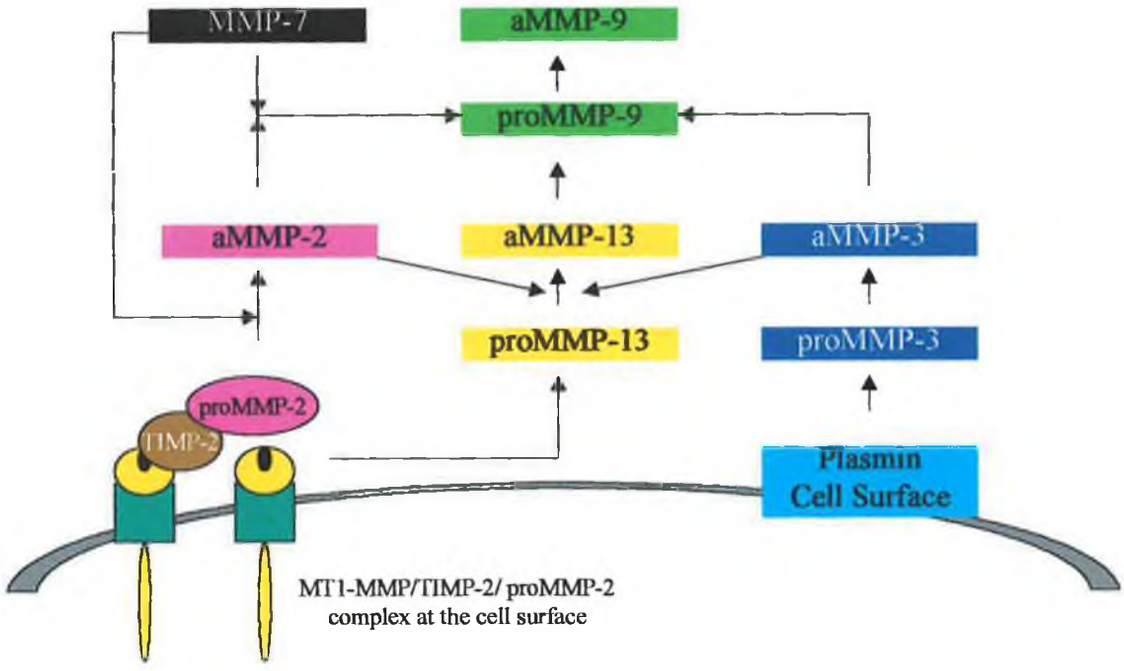

Figure 1.4 Matrix metalloproteinase activation cascades. Cell surface associated plasmin, generated by the activity of receptor bound urokinase-like plasminogen activator on cell bound plaminogen, is a key initiator of MMP activation, notably MMP-3, MMP-2 and MMP-9. Active surface MT1-MMP acts as a second focus of activation, cleaving proMMP-2 which subsequently acts in other cleavage reactions. MT1-MMP may also activate MMP-13 directly. A more likely event, is activation of MMP-13 by MMP-2. Both MMP-2, MMP-3 and MMP-13 can activate MMP-9. MMP-13 may also be activated by MMP-3.

1.5 Tissue Inhibitors of Metalloproteinases (TIMPs).

Serum proteinases, such as α-2-macroglobulin and other non specific proteinase inhibitors such as α_1 proteinase inhibitor are ubiquitously present in extracellular spaces and play an important role in controlling the overall proteolytic activity in tissues (Kennedy, 1998). In addition, the proteolytic activity of MMPs is specifically inhibited by the tissue inhibitor of metalloproteinases (TIMPs) family of proteins (Brew *et al.*, 2000). The TIMP family is comprised of at least 4 distinct members which are produced by a variety of cell types including fibroblasts, keratinocytes, endothelial cells and

osteoblasts. TIMP-1, TIMP-2 and TIMP-4 are secreted in soluble form while TIMP-3 is bound to the ECM (Carmichael *et al.*, 1986, Stetler-Stevenson *et al.*, 1989, Leco *et al.*, 1994 and Greene *et al.*, 1996). TIMPs mediate MMP inhibition by binding non-covalently to active MMPs in a 1:1 stoichiometric manner. TIMPs show a 30-40% homology at the amino acid sequence level and share several structural features; they possess 12 cysteine residues which are necessary for the formation of 6 loops; the N terminal domain is necessary for MMP inhibition and all TIMPs contain the consensus sequence VIRAK in this domain; the TIMPs also contain a 29 amino acid leader sequence which when cleaved gives the mature protein (DeClerck *et al.*, 1993 and Gomez *et al.*, 1997); the C terminal domain has enzyme binding sites which by interaction with the MMPs increases the rate of enzyme inhibition (Wojtowicz-Praga *et al.*, 1997).

1.5.1 TIMP-1

TIMP-1 is an extensively glycosylated protein with a molecular weight of 28 kDa. Human TIMP-1 is secreted by a variety of cell types and has a wide tissue distribution (Carmichael *et al.*, 1986). TIMP-1 has been shown to inhibit multiple MMPs with the exception of MT1-MMP but has been found to be most effective against MMP-9. In addition, TIMP-1 binds to the C-terminal hemopexin domain of proMMP-9 and slows its activation (Gomez *et al.*, 1997). The gene encoding TIMP-1 has been cloned and sequenced (Mahtani and Willard, 1988). The secreted protein has 184 amino acids and six intramolecular disulphide bonds. In the early mouse embryo, tissue localisation data showed that high levels of TIMP-1 mRNA transcripts were found at the sites of active matrix remodeling such as developing bone, where its expression overlapped significantly with that of members of the TGF-β family (Flenniken and Williams, 1990). TIMP-1 has also been shown in several studies to have many diverse biological functions including roles in gonadal steroidogenesis, ovulation, tissue remodeling during tumour progression, growth factor activity, including erythroid potentiating activity (EPA) and inhibition of angiogenesis *in vitro* (Gomez *et al.*, 1997). Recently it has been found to suppress apoptosis in B-cells (Guedez *et al.*, 1998) and accumulate in the nucleus of fibroblast cells during the S phase of the cell cycle (Zhao *et al.*, 1998). Expression of TIMP-1 is regulated at the level of transcription by various growth factors, cytokines and hormones.

Some studies have shown it to be coordinately regulated with MMPs while others have shown it to be reciprocally regulated. Phorbol esters and IL-1 stimulate both MMP and TIMP-1 expression (Overall, 1994) whereas MMP expression is decreased and TIMP-1 expression increased by TGF-β and retinoic acid (Gomez *et al.*, 1997). TIMP-1 expression is also stimulated by IL-6, EGF, oncostatin M and glucocorticoids (Kahari and Saarialho-Kere, 1999). TNF-α has also been shown to stimulate TIMP-1 at low concentrations while inhibiting expression at high levels (Iato *et al.*, 1990). The TIMP-1 promoter contains several transcription factor binding sites including AP-1, AP-2, Sp1 and PEA3 but does not contain a TATA box. The AP-1 site at –92 bp upstream of the initiation site is essential for basal activity (Clark *et al.*, 1997).

1.5.2 TIMP-2

TIMP-2 is a 21 kDa unglycosylated protein which was originally co-purified with MMP-2 from the supernatants of human melanoma cells. It consists of 192 amino acids residues with a 41% homology to TIMP-1 at the amino acid level (Stetler-Stevenson *et al.*, 1989). TIMP-2 forms a complex with proMMP-2 and has a complex role in the regulation of MMP-2 (Golberg *et al.*, 1989) (see figure 1.4). At low concentrations TIMP-2 promotes a ternary complex formation with proMMP-2 and MT1-MMP. Once the complex has formed a second TIMP-2 free MT1-MMP proteinase activates MMP-2 (Strongin *et al.*, 1995). When extracellular levels of TIMP-2 are high the activation of MMP-2 is prevented due to the inhibition of MT1-MMP activity by excess TIMP-2. Although a key element in MMP regulation, TIMP-2 has also been shown to play important roles in other biological processes, for example, in the protection of melanoma cells from apoptosis (Baker *et al.*, 1998, and Valente *et al.*, 1998). TIMP-2 tends to be constitutively expressed by many cell types in culture, although in cases where TIMP-2 expression can be modulated, the response is typically the opposite to that of TIMP-1 (Stetler-Stevenson *et al.*, 1990 and Overall, 1994). The TIMP-2 promoter has a number of potential transcription factor binding sites including several Sp-1 sites and an AP-1 site. However, in contrast to the TIMP-1 promoter, it is unresponsive to TPA and various cytokines (DeClerck *et al.*, 1994).

1.5.3 TIMP-3

Unlike other members of the TIMP family, TIMP-3 has poor solubility and is specifically localised to the ECM. The secreted mature protein contains 188 amino acids and shares 30% homology with TIMP-2. The unglycosylated protein has a relative molecular weight of 21 kDa, although a higher molecular weight form due to glycosylation has been reported (Anand-Apte *et al.*, 1996 and Baker *et al.*, 1998). The protein contains an N-linked glycosylation site suggesting that the natural protein may exist in a glycosylated form, although the functional consequences of glycosylation remain unclear (Anand-Apte *et al.*, 1996). TIMP-3 was first isolated as a transiently expressed protein in the ECM of transforming chick fibroblast cultures (Blenis and Hawkes, 1984) and was subsequently identified as a member of the TIMP family (Pavloff *et al.*, 1992). TIMP-3 inhibits a wide range of MMPs including MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13 and has also been shown to inhibit the activity of TNF-α converting enzyme (TACE). TIMP-3 is expressed in a number of tissues including cartilage, muscle and kidney (Leco *et al.*, 1994). The TIMP-3 promoter lacks a TATA box and contains several AP-1 sites (Kim *et al.*, 1997). TIMP-3 expression has been shown to be stimulated by a number of agents including EGF, TGF-β, platelet derived growth factor (PDGF), phorbol esters, dexamethasone, and IL-1β. TIMP-3, unlike TIMP-1 and TIMP-2, does not appear to have any growth potentiating activity but it has been reported to be involved in suppressing cell growth and inducing apoptosis (Ahonen *et al.*, 1998 and Baker *et al.*, 1998).

1.5.4 TIMP-4

TIMP-4 contains 195 amino acids and the protein has a molecular weight of 23 kDa (Greene *et al.*, 1996, Liu *et al.*, 1997). TIMP-4 shares a 37% sequence homology with TIMP-1 and a 51% homology to TIMP-2 and TIMP-3. In comparison to other TIMP family members TIMP-4 expression is restricted and is normally localised in the adult human heart and at lower levels in kidney, colon, placenta and kidney (Greene *et al.*, 1996). TIMP-4 has been shown to inhibit the activities of MMP-1, MMP-3, MMP-7,

MMP-9 and in particular has been shown to be a potent inhibitor of MMP-2 (Liu *et al.*, 1997).

The TIMP family of metalloproteinase inhibitors represent an important mechanism in the regulation of MMP activity which is essential in the maintenance of ECM homeostasis. Recent reports have shown that TIMPs, aside from MMP inhibition, have several other important biological functions the mechanisms of which have yet to be elucidated.

1.6 MMPs in cancer invasion and metastasis

MMPs have been implicated in a number of pathological conditions including rheumatoid arthritis (Cawston *et al.*, 1999) and atherosclerosis (Thompson and Parks, 1996) but it is their role in tumour invasion and metastasis which has been extensively studied (Parsons *et al.*, 1997).

1.6.1 Metastasis

Metastasis is the spread of cancer from a primary tumour to distant sites of the body and is the most lethal aspect of cancer (Chambers and Matrisian, 1997). In order to spread to these distant sites, invading tumour cells must overcome the barrier of the surrounding ECM. As MMPs are involved in the proteolytic cascade which is responsible for ECM degradation, many studies have investigated the links between tumour invasion and metastasis and MMP expression and in general it has been concluded that MMPs are inextricably linked to this process. Metastasis is regarded as a multi-step process separated into sequential steps which are as follows; the escape of cells from the primary tumour, intravasation (entry of cells into the lymphatic or blood circulation), survival and transport in the circulation, arrest in different organs, extravasation (escape of cells from the circulation) and growth of cells to form secondary tumours in the new organ environment. Angiogenesis, the formation of new blood vessels, is essential for the secondary tumour to grow beyond minimal size and the cell must escape immune destruction during each of the metastatic steps (see figure 1.5).

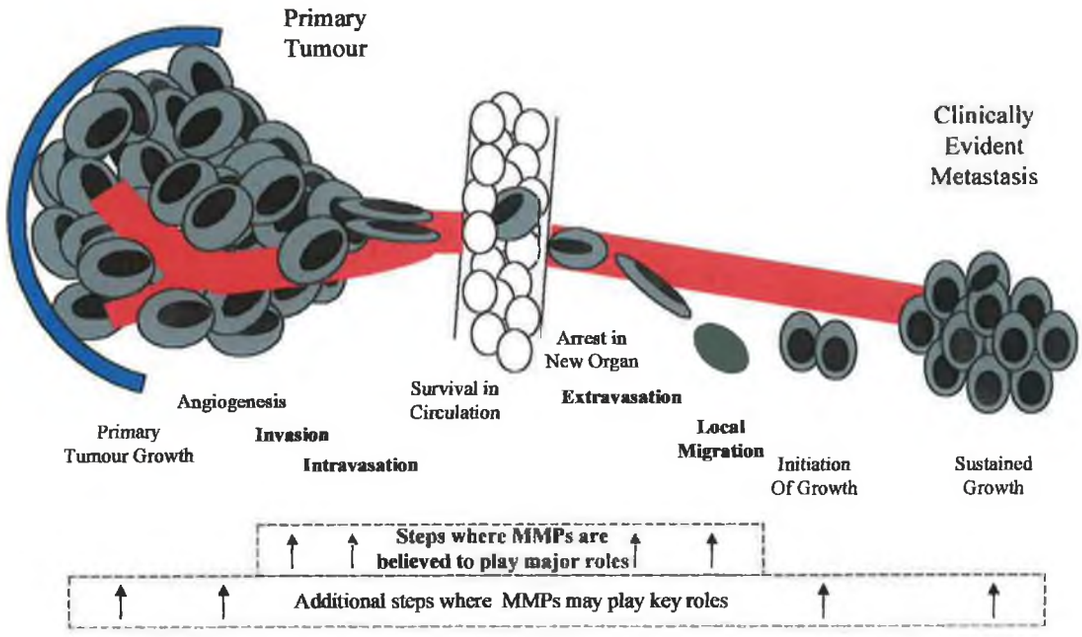

Figure 1.5 The role of MMPs in tumourigenesis and cancer metastasis. MMPs were traditionally thought of as being involved in tumour invasion and metastasis due to their ECM degrading capabilities (bold legend) but recent evidence has suggested that MMPs are implicated in several stages of primary and secondary tumourigenesis (adapted from Chambers and Matrisian, 1996).

MMPs have been associated with the malignant phenotype for many years (Chambers and Matrisian, 1997) and early work concentrated on collagen type IV destruction (Liotta *et al.*, 1980). Collagen type IV is a major component of the basement membrane and it has been demonstrated that MMP-2 and MMP-9 play a major part in its degradation. Subsequent studies demonstrated the association of MMP family members with tumour progression. Several generalisations can be made, i) the number of MMP family members that can be detected tends to increase with tumour progression, ii) the relative levels of any MMP family member tend to increase with increasing tumour stage and iii) the MMPs can be produced by tumour cells or by the surrounding host tissue. These generalisations are evident in several tumour types including, colon, breast and pancreatic cancer.

Numerous studies have investigated the expression of MMPs in various cancers with many showing that the level of MMP expression can be used as a prognostic marker for how aggressive a particular tumour is. For example, MMP-1 has been shown to be associated with poor prognosis in colorectal cancer whereby tumour sections were examined for the presence of MMP-1 (Murray *et al.*, 1996). Results showed that the presence of MMP-1 could be prognostic of tumour aggressiveness and that the prognostic value was independent of Duke's stage (the classification system used to define colon tumour aggressiveness). Matrilysin (MMP-7) has been shown to involved in the invasion and metastasis of several tumours, in particular, those of colon and breast origin and reports have shown that increased levels of matrilysin correlate well with increased invasion and metastasis. Matrilysin has also been recently shown to play an important role in early breast and colon tumourigenesis. The studies which identified matrilysin as being an important factor in colon and breast tumour progression will be discussed in more detail in **chapter 3**. In experiments examining patient biopsies, MMP-2 and MMP-9 levels were assessed and it was found that high levels of these enzymes were prognostic for a poor overall survival of patients (Sier *et al.*, 1996, Ogura *et al.*, 1997 and Baker *et al.* 2000). MMP-2 has been found to play an important role in breast cancer and has been detected very early in breast carcinoma but not in normal, resting breast tissue (Poulsom *et al.*, 1993). Once again MMP-2 expression has been found to increase with increasing tumour aggressiveness (Polette *et al.*, 1994). The protein has been localized in tumour cells (Daidone *et al.*, 1991, Höyhtyä *et al.*, 1990) and also stromal cells (Okada *et al.*, 1995) in breast carcinoma by immunohistochemistry. An association between MMP-9 positive cells and either invasive carcinoma cells or lymph node metastasis has also been found in breast carcinoma, although expression of the enzyme is not strictly confined to the neoplastic cell. Stromelysin-3 has also been shown to be expressed in breast carcinomas (Basset *et al.*, 1990) and it was found that the stromelysin-3 mRNA expression was restricted to the stromal cells immediately surrounding the islands of the malignant tumour epithelial cells. Many other MMPs have been detected in these tissues but it is beyond the scope of this study to examine them in detail.

Recent evidence suggests that MMPs play a much broader role in metastasis than previously believed, and that the action of MMPs at steps both before and after the

breakdown of the apparent physical barriers to metastasis may in fact be of greater importance (see figure 1.5). MMPs and their inhibitors appear to be important regulators in the growth of tumours, both at the primary site and at metastases. How MMPs mediate this growth regulation is not yet fully understood, but a number of mechanisms are possible. Firstly, MMPs appear to contribute to the initiation of growth, at both primary and secondary sites. One can speculate that this may involve regulation of the growth environment by, for example, regulating access to growth factors, such as EGF (Ravanti and Kahari, 2000), from the extracellular matrix surrounding the growing tumour, either directly or via a proteolytic cascade. Insulin like growth factor binding proteins (IGFBPs) are cleaved by a number of serine proteinases that are initially activated by MMPs such as MMP-2 and MMP-9 (Clemmons, 1998). Similarly, MMPs and their inhibitors appear to regulate the sustained growth of tumours. Beyond the maintenance of an appropriate growth environment, the role of MMPs in angiogenesis is likely to be important at this stage. Angiogenesis is required for growth of both primary and metastatic tumours and MMPs play a contributory role in regulation of angiogenesis.

1.6.2 The anti-tumour effect of TIMPs

There are several mechanisms that support the anti-tumour effect of TIMPs. Firstly, TIMPs have anti-angiogenic activity either by a direct effect on endothelial cell proliferation or by their ability to downregulate the activities of MMPs required for endothelial cell migration and invasion (Blavier *et al.*, 1999). As a second mechanism it has been recently recognised that MMPs can degrade proteins other than structural MMP proteins such as IGFBPs. Some of these proteins form complexes with growth factors and control their bioavailability. By preventing the degradation of IGFBP by MMPs, TIMPs can therefore play an important role in preventing the bioavailability of this potent growth factor. A third mechanism that supports the anti-tumour effect of TIMPs is based on the increasingly recognisable fact that the ECM can control essential cellular functions such as growth, differentiation and apoptosis. Thus by maintaining the integrity of the ECM via MMP inhibition, TIMPs can maintain an indirect control over malignant cell proliferation. TIMPs on the other hand have also been shown to have a tumour promoting effect. For example, in colon cancer, higher levels of TIMP-1 have been observed in

association with more invasive stages and in non-Hodgkin's lymphoma, TIMP-1 expression is a positive index of malignant expression. In bladder and breast cancers, elevated levels of TIMP-2 in tumour tissues predict an unfavorable rather than a favorable prognosis. The mechanisms supporting the paradoxically positive effect of TIMPs in tumour progression are not fully understood but are the focus of intensive investigations. It may be possible that the tumour cells increase the amount of TIMPs in response to increased levels of MMPs in order to halt ECM degradation and tumour progression. The role of TIMP-2 as a docking molecule for MMP-2 activation has also been well documented and therefore increased levels of MMP-2 may also result in increased levels of TIMP-2. TIMP-1 has also been shown to bind to the EPA receptor thus stimulating the growth of erythroid cells (Murate *et al.*, 1993).

Details of the mechanisms by which MMPs and their inhibitors contribute to creating an environment that favours the initiation and continued growth of primary and metastatic tumours remain to be elucidated, but are of key importance in cancer therapy. An understanding of the molecular role of MMPs at each of the sequential steps required to produce clinically evident metastases will be important in the design and appropriate use of novel therapeutics designed to combat metastasis.

1.7 Physiological roles of MMPs

The expression of MMPs by a variety of cell and tissue types has been extensively studied. Although the majority of the MMPs were first isolated and cloned from tumour-associated tissues, their roles in normal tissue formation and remodeling has been less frequently studied. Initial reports point to complex and highly individualised patterns of expression for the various family members. 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. The best defined correlations of MMP expression with normal functions are found during trophoblast invasion (Cross *et al.*, 1994) development (Matrisian and Hogan 1990), endometrial remodeling (Salamonsen, 1994) ovulation (Russell and Findlay, 1995) and angiogenesis. (Fisher *et al.*, 1994).

1.7.1 MMPs and reproduction

Development of the human foetus depends primarily on the embryo rapidly gaining access to the maternal circulation and the embryo achieves this by using specialised cells referred to as trophoblasts. In contrast to tumour cell invasion, trophoblast invasion is precisely regulated (Cross *et al.*, 1994). A number of investigators have shown that trophoblast cells employ a number of MMPs during their invasion of the uterus and in particular, MMP-2 and MMP-9 have been shown to play an important role in this respect (Librach *et al.*, 1991 and Fisher *et al.*, 1994).

Perhaps the most dramatic expression of MMPs in normal tissue is observed in the human cycling endometrium. The uterine endometrium is regularly shed throughout the human reproductive life which implies active remodeling. During the menstrual phase several MMPs including, MMP-1, MMP-3, MMP-10, MMP-11, MMP-2 and MMP-9 are all expressed (Rodgers *et al.*, 1994) which implicates the importance of MMPs in the breakdown and release of endometrial tissue during menstruation.

1.7.2 The role of MMPs in angiogenesis

MMPs, as stated earlier, have been shown to play an important role in angiogenesis during tumour development but are also involved in angiogenesis in normal conditions such as embryonic development, ovulation, bone formation, inflammation and wound repair. In order to form a new capillary in response to an angiogenic stimulus the ECM must first be degraded in order to make space for the advancing endothelial cells. The penetration of local ECM barriers by endothelial cells requires the strict control and regulation of a variety of proteinases including the MMPs. Angiogenic stimuli include fibroblast growth factor (FGF), angiotropin, angiogenin and EGF (Fisher *et al.*, 1994). Cultured endothelial cells have been shown to secrete a number of MMPs such as, MMP-1, MMP-2, MMP-3 and MMP-9 in response to different stimuli (Moscatelli and Rifkin, 1988, Mignatti *et al.*, 1989 and Fisher *et al.*, 1994). Other evidence also suggests that MMPs may negatively regulate angiogenesis. Recently matrilysin and MMP-9 have been

shown to cleave plasminogen thus giving rise to angiostatin which is a potent inhibitor of angiogenesis (Patterson and Sang, 1997). This would therefore imply that MMPs can play a direct role in the regulation of angiogenesis. Perhaps this may also explain why MMP inhibitors such as Agouron have been found to perform worse than placebo in recent phase III clinical trials (see section 1.8) whereby the inhibition of the MMPs may assist in the vascularisation of the tumour.

1.7.3 MMPs and apoptosis

The nature of the ECM can influence the apoptotic programme in mammalian cells, thus leading to the association of MMPs with apoptosis. It is hypothesised that the basement membrane is a survival factor for epithelial cells and that loss of contact with the basement membrane results in initiation of the apoptotic cascade (see Wiesen and Werb, 2000 for review). Several investigators have shown this to be the case. For example in the breast, a basement membrane rich in laminin is required for normal mammary development and when mammary epithelial cells are placed in culture without essential basement membrane components, the cells undergo apoptosis. MMPs may also be involved in processing factors which induce apoptosis such as Fas which when it interacts with its ligands induces apoptosis in T lymphocyte cells. 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 which induce apoptosis. Recently, Powell *et al* (1999) have found that Fas ligand is a substrate for matrilysin and plays an important role in the apoptotic cascade in breast cancer cell lines.

1.8 MMPs as therapeutic targets

As the role of MMPs in tumour development and progression became apparent, many potential inhibitors of these enzymes (matrix metalloproteinase inhibitors, (MMPIs)) were assessed for anticancer properties. TIMPs, in general, were unsuitable for therapeutic use due to the proteins short half-life and large size. Therefore a number of synthetic inhibitors have been designed.

1.8.1 Batimistat/Marimistat MMP inhibition

Batimistat (British Biotech Inc., Oxford, UK), a potent broad spectrum inhibitor of MMPs, is a synthetic hydroxamate derivative, whose structure imitates the MMP binding site of collagen. Batimistat functions by chelating the zinc ion present at the active site of each MMP enzyme. The drug proved successful in animal trials but its major drawback was its extreme insolubility. Recent *in vitro* studies have shown that Batimistat inhibits angiogenesis and this may be the basis of its anti tumour effects (Curran and Murray, 2000).

Marimistat is a second generation MMP inhibitor and phase I and II trials have shown the inhibitor, which is orally administered, to be effective against colon, ovarian, prostate, lung and pancreatic cancer. It can also be safely used in association with other chemotherapeutic agents. Potential drawbacks for both Batimistat and Marimistat are the dose limiting side effects which include inflammation of the joints and tendons which are reversible on discontinuation of the treatment (Tierney *et al.*, 1999). The drugs are also broad spectrum inhibitors which may have other undesirable side-effects as the exact functions of MMPs have not yet been fully elucidated. Thus the development of specific MMP-inhibitors may prevent cancer spread while having minimal interference in other normal biological processes involving MMPs. Recently phase three clinical trials involving marimastat were halted as it was found to be performing worse than the placebo. Thus further chemical modifications of these drugs are required in order to improve specificity and reduce cytotxicity.

1.8.2 Other Bryostatin MMP inhibitiors

The bryostatins are a group of naturally occurring macrocyclic lactones which inhibit MMP activity in a different manner to that of Batimistat and Marimistat. Although the exact mode of action remains unclear, it is thought that bryostatin downregulates the PKC pathway. Several genes including MMP-1, MMP-3, MMP-9 and MMP-11 are downstream targets of the PKC pathway and therefore bryostatin may prevent the transcriptional activation of these genes. Recently a number of companies including

Bayer and Agouron began testing byrostatin compounds for their effectiveness as tumouristatic agents. BAY 12-9566 (Bayer) was shown in pre clinical trials to be effective as an MMP inhibitor but unfortunately it was withdrawn from all clinical trials in 1999 when it was found to be less effective than the placebo. However, another bryostatin compound, AG3340/prinomostat developed by Agouron has proved to be more successful. Studies have shown prinomostat to inhibit the proliferation and invasion of glioma cells *in vitro* (Price *et al.*, 1999). Prinomostat is a selective MMP inhibitor and inhibits the activity of MMP-2, MMP-9, MMP-13 and MT1-MMP (Shalinsky *et al.*, 1999). In early 2001, Agouron halted phase III clinical trials which were examining the effects of prinomostat on prostate and lung cancers. Phase II trials involving patients with earlier stages of the disease, however, are set to continue. Redevelopment of these drugs by Bayer and Agouron using new chemistry techniques such as 'combinatorial' chemistry may lead to the discovery of new potent MMP inhibitors which have minimal side effects.

1.8.3 Indirect inhibition of MMPs by tyrosine kinase directed inhibitors

A number of other drugs are available which may inhibit MMP activity indirectly such as tyrosine kinase receptor (TK-r) inhibitors. Initially these drugs inhibited tyrosine kinase receptors in a non-specific manner which resulted in unwanted side effects. Chemical alteration of initial lead compounds has resulted in several new drugs being developed which specifically target various receptors. A recent example of this is the Her2/Neu anti-body based drug, Herceptin released by Genentech. Her2/neu is a tyrosine kinase receptor which was been found to be amplified in a majority of breast cancers and this drug has been shown in clinical trials to prevent tumour growth and to have no deleterious side effects (Cornez and Picart, 2000). Tyrosine kinase receptors, once bound to a ligand, mediate their response via a number of pathways including the mitogen activated kinase pathway (MAPK) and the extracellular response kinase (ERK) pathway. The endpoint of these interlinked pathways are transcription factors which carry out the desired effect by binding to the promoter of target genes. One of the transcription factors targeted by these pathways is the AP-1 complex proteins Jun and Fos. As shown earlier many of the inducible MMP genes have AP-1 transcription factor binding elements within

their promoter and therefore inhibition of the tyrosine kinase receptors may not only lead to the cessation of cell growth but also to the indirect inhibition of MMPs.

1.8.4 Novel approaches to MMP Inhibition

Another rapidly expanding area of cancer therapeutic research is the design of specific transcription factor inhibitors in order to prevent the transcription of target genes. This can be achieved in a number of ways including conventional drug design procedures and also by the development of triple helix oligonucleotide inhibitors. The triple helix oligonucleotide inhibitors act by binding to transcription factor DNA sequences within the target genes promoter thus preventing the binding of the transcription factor protein.

The construction of antisense nucleotides has also been shown to be a successful method in the inhibition of MMPs. Antisense oligonucleotides to matrilysin have been tested on two human colon cancer cell lines, CaR-1 and WiDR. The antisense oligonucleotide inhibited both the secretion of matrilysin and also inhibited *in vitro* metastasis (Miyazaki *et al.*, 1999).

Ribozyme technology has also been investigated as a possible MMP inhibitor and recently an MMP-9 ribozyme has been evaluated in an experimental model. The ribozyme works on the basis of cleaving MMP-9 mRNA transcripts thus preventing the translation of MMP-9 mRNA into protein (Peng *et al.*, 1997).

1.8.5 Drug delivery mechanisms

Although many new drugs have been shown to be potent inhibitors of MMP synthesis and enzymatic activity *in vitro*, their effects *in vivo* require more in depth analysis. One of the main barriers of successful *in vivo* treatment is the method of drug delivery. MMPs, as have been shown, are not only involved in matrix remodeling but also have a host of other functions in normal biological processes and thus it is essential that drugs which inhibit their synthesis or activity be delivered to the areas in which the tumours are developing. Most novel drug-delivery systems have stemmed from work on

new polymers, lipid vesicles, cyclodextrins, pro-drugs, and viral vectors. For example, some polymer systems can be rendered "smart", i.e. sensitive to a tissue environment. Thus, a drug-polymer complex can be designed to undergo a conformational change or enzymatic breakdown that results in release of the active drug only under certain conditions. The liposome is another construct that serves as an envelope for active drug particles and can, like polymers, deliver high concentrations of drugs to infected or neoplastic tissue. Once these technologies have become more refined, they can perhaps be tailor made for the delivery of MMP inhibitors to tumour sites within the body thus reducing the risk of undesirable side effects.

1.9 Summary

The MMP family continues to expand with the recent cloning of new MT-MMP family members, MMP-26 (matrilysin-2/endometase) and MMP-27 (epilysin). Although the enzymatic properties of the MMPs are becoming clearer the complex regulation of this large and multifunctional group of endopeptidases *in vivo* is poorly understood. An understanding of how the MMPs are regulated at the transcriptional and protein level is essential for predicting what happens during tumourigenesis. To date, individual MMPs have been shown to be of prognostic significance in several tumour types. Precise information on which MMPs are critical in invasion and knowledge of their regulatory mechanisms may help in the development of more effective MMP inhibitors. In the case of matrilysin, several reports have shown that this MMP is involved in the tumourigenesis and progression of many cancer types, in particular, in breast and colon cancers and it has also been shown to be involved in the early stages of tumour development. Therefore, matrilysin presents itself as an excellent therapeutic target and inhibitors directed against its transcription or proteolytic activity may serve as potent anti-tumourigenic/anti-metastatic agents.

1.10 Thesis Overview

The research presented in this thesis examines the regulation of matrilysin (MMP-7) in colon cancer and leukaemia and has been divided into three main chapters.

- Chapter 3:

  A panel of human colon cell lines was used to examine the effects of several cytokines on matrilysin expression at the transcriptional and protein levels. Matrilysin expression was analysed via reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis. Human matrilysin promoter (2.3 kbp and 335 bp) luciferase reporter constructs were also employed. The presence of silencers and enhancers within the murine matrilysin gene was also examined.

- Chapter 4:

  A thorough investigation into the mechanisms of EGF mediated matrilysin upregulation was carried out. The effects of EGF treatment on PEA3 transcription factors was analysed using artificial promoter constructs, immunoblot analysis and elctrophoretic mobility assays (EMSA). The effect of EGF on E-cadherin and β-catenin on PEA3 transcription factor and matrilysin levels was also examined.

- Chapter 5:

  The expression and regulation of matrilysin in leukaemia was investigated and the potential role of matrilysin in leukaemia invasion and metastasis was also addressed. The levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3 were investigated with respect to their importance in leukaemia invasion.

In order to provide clarity this thesis has been divided into seven chapters. Chapter 1 serves as a general introduction to the MMP family. There is a common materials and methods section (Chapter 2) and bibliography (Chapter 8). Chapters 3, 4 and 5 each have their own introduction, results and discussion sections while chapter 6 provides an overall summary of the thesis.

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-Aldrich Chemical Company; Dublin, Ireland, BDH Chemicals Ltd., Poole, Dorset, England and Riedal De Haen AG, Seelze, Hannover, Germany.

Cell culture medium and transfection reagents (Lipofectamine, Cellfectin and Superfect) were obtained from Sigma-Aldrich, Dublin, Ireland, Gibco BRL, Paisley, Scotland and Qiagen, Crawley, West Sussex, England.

Foetal calf serum was supplied by Sigma-Aldrich, Dublin, Ireland.

Disposable plastics for animal cell and microbiological culture, and 96-well plates were obtained from Sarstedt, Sinnottstown Lane, Drinagh, Co. Wexford, Ireland.

Human matrilysin promoter constructs, artificial promoter constructs and the matrilysin anti-body were kindly donated by Prof. Lynn Matrisian, Vanderbilt University, Nashville, TN, USA.

HCA7 human adenocarcinoma colon cell line was a gift from Dr. Susan Kirkland, University of London, England.

BHK cells transfected with the human MMP-9 cDNA (BHK 92) were obtained from Prof. Dylan Edwards, University of East Anglia, Norwich, England.

All other cell lines used in this study were obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury, Wiltshire, England.

TIMP primers were obtained from Hugh McGlynn Ph.D., University of Ulster at Coleraine, Northern Ireland.

PBS tablets were purchased from Oxoid Ltd., Basingstoke, Hampshire, England.

Taq polymerase and other components of RT-PCR reactions were purchased from Sigma Aldrich, Dublin, Ireland and Promega Corp., Southampton, Hampshire, England.

Anti-MMP-9, MMP-2 and TIMP antibodies were purchased from Chemicon, Temecula, California, USA and Calbiochem, Beston, Nottingham, England.

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

L-glutamine, trypsin, penicillin/streptomycin were purchased from Sigma-Aldrich, Dublin, Ireland.

The BCA reagent for protein determination and Supersignal West ECL detection kit were obtained from Pierce Chemicals, Rockford, Illinois, USA.

Cytokines were obtained from, Promega, Southampton, England, Sigma Aldrich, Dublin, Ireland and Roche-Boehringer Mannheim, Lewes, East Sussex, England.

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, England.

Specific primers for PCR were made to order by MWG-Biotech, Ebersberg, Germany.

Qiagen kit for preparation of DNA purchased from Qiagen, Crawley, West Sussex, England.

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

SW480 (ATCC Cat# CCL-228), SW620 (ATCC Cat# CCL-227), HCT116 (ATCC Cat# CCL-247), LoVo (ATCC Cat# CCL-229), WiDR (ATCC Cat# CCL-218), HT1080 (ATCC Cat# CCL-121), HCA7 and BHK92 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) and 1 unit/ml penicillin and 1μg/ml streptomycin (see table 2.1 for colon cell line characteristics). All cells were cultured in 25cm^2 or 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 aspirated and the flask rinsed with 3 ml of phosphate buffered saline (PBS) to remove any residual FBS which contains a trypsin-inhibitor (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 had detached from the surface. The cell suspension was removed to 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 x 10^5 - 1 x 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 a Heraeus cell culture incubator.

Cell line	Ras	myc	p53	fos	ros	src	Comments
SW480	+	+	+	+	-	-	Established from a primary adenocarcinoma in the colon. Male patient, 50 years, classified as Dukes' B
SW620	+	+	+	+	-	-	The cell line was derived from the metastasis (lymph node) of the same tumour from which SW480 was derived. Male patient, 51 years, classified as Dukes' C
WiDr	+	+	+	+	-	-	Cell line is derived from the HT-29. Female patient, 78 years old
LoVo	+	+	+	+	-	-	LoVo was initiated in 1971 from the fragment of a metastatic tumour nodule in the left supra clavicular region of a 56 year old Caucasian male patient with a histologically proven diagnosis of adenocarcinoma of the colon
HCT116	+	+	+	+	+	+	Colorectal carcinoma isolated from a male patient

Table 2.1 Colon cancer cell lines used in the study. Positive expression of oncogene products in the cell is denoted by the + sign while expression of oncogene products not detected in the cell lines are denoted by a – sign.

2.2.1.2 Culture of cells in suspension

K562 (ATCC Cat# CCL-243) and HL-60 (ATCC Cat # CCL-240) leukaemia cell lines were maintained in DMEM supplemented with 10% (v/v) FBS, 1 mM HEPES, 2 mM L-glutamine, 1 unit/ml penicillin and 1μg/ml streptomycin (see table 2.2). Cultures were maintained at densities of 2.5 x 10^5 cells/ml, in 75 cm^2 flasks. Cultures were passaged by

diluting the cells with media. The cells were incubated in a humid 5% CO_2 atmosphere at 37°C.

Characteristics	K562	HL-60
Tissue	Chronic Myeloid Leukaemia (CML) : Bone marrow	Acute Promyelocytic Leukaemia (AML) : Peripheral blood : Promyeloblast
Morphology	Lymphoblast	Myeloblastic
Tumourigenic	Yes, in nude mice	Yes, form colonies in semi solid media and produce subcutaneous myeloid tumours in mice
Oncogene	N/A	Myc +
Comments	The K562 cell line was established from the plueral effusion of a of a 53 year old caucasian female with chronic myelogenous leukaemia in terminal blast crises. The cell population has been characterised as highly undifferentiated and of a granulocytic series. K562 blasts are multipotential, haematopoietic cells that spontaneously differentiate	HL-60 is a promyelocytic cell line from peripheral blood leukocytes. HL-60 cells spontaneously differentiate and differentiation can be induced by a number of compounds including TPA, DMSO, actinomycin D and retinoic acid. The cells exhibit phagocytic activity and responsiveness to chemotactic stimuli

Table 2.2 Characterisation of the K562 and HL-60 human leukaemia cell lines

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. The number of cells was calculated as follows (Average number of viable cells) x 1.2 (dilution factor) x $1x10^4$ = viable cells/ml.

2.2.1.4 Recovery and storage of cells

Long term storage of cells was achieved by storing the cells in liquid nitrogen and maintaining them in a cryofreezer (supplied and serviced by Cooper Cryoservice Ltd, Dublin, Ireland). Cells to be stored were centrifuged and the resulting cell pellet resuspended at a concentration of 1 x 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 and TPA treatment of cells in culture

Cells were routinely cultured for at least 2 passages prior to cytokine and/or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment. For these experiments, adherent 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 $75cm^2$ flask. Suspension cells were washed 3 times in PBS and seeded at $1x10^6$ cells/ml in 8 ml of serum free media in a $75cm^2$ flask. Both adherent and suspension cells were left in serum free media overnight at 37°C. 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): insulin like growth factor I and II (IGF-I and IGF-II) (100 ng/ml),

epidermal growth factor (EGF) (50 ng/ml), interleukin 6 (IL-6) (100 units/ml), basic fibroblast growth factor (bFGF) (50ng/ml) and TPA (100 ng/ml). The cells were then returned to the incubator for 8 hours. 2, 4, 6, 12 and 24 hour time points were also examined and 8 hours was found to be the optimal time period for RNA. The cells were harvested for total ribonucleic acid (RNA), or for 24-72 hr after which the conditioned medium was collected and MMP/TIMP expression analysed.

2.2.2 RNA isolation

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 diethylpyrocarbonate (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. The procedures must be carried out quickly and on ice to help prevent degradation of RNA by endogenous RNases.

2.2.2.1 RNA extraction from cultured cells

Total RNA was isolated from equal numbers of cells using RNA ISOLATOR (Genosys/Sigma Aldrich, Dublin Ireland), which is a modified version of the single step method by acid guanidinium thiocyanate-phenol-chloroform extraction. The cultured cells were lysed directly in 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, shaking for 15 s and incubating 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 a colour-less upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins partition to the interphase and

organic phase respectively. 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.

2.2.2.2 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) (0.08 M Tris-HCl pH 8.0: 0.04 M boric acid; 1 mM EDTA). 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.

2.2.3 Reverse transcription polymerase chain reaction (RT-PCR)

The PCR reaction 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 messenger RNA (mRNA). Total RNA is primed with oligo dT's and then converted to complementary DNA (cDNA) using reverse transcriptase. cDNAs of interest are then amplified in the PCR by inclusion of the appropriate primers. Details of the primers used are shown in table 2.3. The PCR used was semi-

quantitative as a constitutively expressed gene, β-actin, was also amplified from the same RNA sample along with the MMP or TIMP target. This acted as an internal control and, by calculating the ratio of MMP to β-actin, relative amounts of the targets could be determined and compared to different reactions.

2.2.3.1 Reverse transcription

1μg of total RNA (prepared as outlined in section 2.2.2.1) 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 1 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.

2.2.3.2 Polymerase Chain Reaction

A 45 μl PCR mix was prepared by adding 5 μl of 10X reaction buffer (supplied with the Taq polymerase enzyme), 1 μl each of dATP, dCTP, dGTP, and dTTP, each at a concentration of 10mM, 1μl of the forward and reverse primers required in the reaction, 0.5 μl Taq DNA polymerase and sterile water to bring the volume to 45 μl into a reaction tube. To this reaction mixture was added 5 μl of cDNA prepared as in section 2.2.3.1 and the mixture overlaid with 50 μl 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 s, annealing temperature as in Table 2.3 for 90 s 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 2.2.3.3 below.

Target	Primer sequence	Product size	Temp (°C)	Reference
MMP-2	5' TGA CAT CAA GGG CAT TTC AGG AGC3' 3' GTC CGC CAA ATG AAC GGT CCT TG 5'	180bp	53	Shimonovitz *et al.*, 1994
MMP-7	5' TGT ATC CAA CCT ATG GAA ATG 3' 3'CAT TTA TTG ACA TCT ACG CGC 5'	341bp	47	Witty *et al.*, 1994
MMP-9	5 'GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC 3' 3' GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT 5'	640bp	54	Onisto *et al.*, 1993
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 5'	551bp	53	Onisto *et al.*, 1993
TIMP-2	5'TGC AAT GCA GAT GTA GTG ATC AGG G 3' 3'TGC TTA TGG GTC CTC GAT GTC GAG A 5'	552bp	60	Stetler-Stevenson *et al.*, 1990
TIMP-3	5'CTG TGC AAC TTC GTG GAG 3' 3' TCG GTA CCA GCT GCA GTA 5'	250bp	55	Apte *et al.*, 1994
β- actin	5' TCA GGA GGA GCA ATG ATC TTG A 3' 3' GAA ATC GTG CGT GAC ATT AAG GAG AAG CT 5'	383bp	47-55	Nakajima-Iijima *et al.*, 1985

Table 2.3 Details of the primers used for RT-PCR experiments. The primers were chosen so that they amplified sequences specific to the particular gene and did not cross react with any other MMPs or TIMPs. The primers were chosen to span an intron within the gene which meant that any amplification of genomic DNA rather than cDNA would result in larger sized products than those expected.

2.2.3.3 Agarose gel electrophoresis

An 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). The percentage gel used depended on the sizes of the DNA being visualised with a lower percentage gels (0.6 - 1.2%, w/v) being used for visualising large sized DNA fragments (e.g. over 2Kb) and higher percentage gels (2-3%, w/v) reserved for small DNA fragments (e.g. 200-500bp). Once cooled to hand-hot, 1 µl of 10mg/ml ethidium bromide solution was added to the gel which was then 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 sample was mixed with 3 µl of loading buffer and the samples loaded into the wells of the gel. The gel was run at 100V in 1X TBE. Electrophoresis was completed when the blue loading dye had run to within 0.5cm of the bottom of the gel. The gel was visualised by placing on a UV transilluminator. The images of the gels were saved on disc using a Pharmacia Gel Documentation System.

2.2.4 Bicinchoninic acid (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 were supplied in the commercially available assay kit (Pierce Chemicals): **A**, an alkaline bicarbonate solution and B, a copper sulphate solution. Working solution was 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 microtitre plate reader (Rosys Anthos 2010). Protein concentrations were determined from a bovine serum albumin (BSA) standard curve in the 0 - 1 mg/ml range.

2.2.5 Protein electrophoresis

2.2.5.1 SDS Polyacrylamide Gel Electrophoresis (PAGE)

SDS- PAGE 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). For western blot analysis a similar sample buffer was used with the exception that β-mercaptoethanol at a final concentration of 1M was used. 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 milli amps (mA) per gel using an Atto vertical mini-electrophoresis system until the blue dye front reached the bottom of the gel.

2.2.5.2 Staining with Coomassie Brilliant Blue

Gels were stained for 2 hr in 0.5% (w/v) Coomassie Brilliant Blue in acetic acid : isopropanol : H_2O (1 : 3 : 6, v/v/v), and destained overnight in the same solvent system.

2.2.6 Western Blotting

Samples were boiled for 10 mins prior to electrophoresis in sample buffer (0.5M Tris, pH 6.8, 0.4% SDS, 30% glycerol, 10 % SDS 0.12 mg/ml bromophenol blue and 1M β-mercaptoethanol). Following electrophoresis as outlined in section 2.2.5.1, the gel was soaked for 30 min in cold transfer buffer (0.025 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 for 1 hr at 100 V. After transfer, the blot was blocked for 1 hr in blocking solution [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 diluted, according to manufacturers instructions in blocking solution. The following day, blots were washed three times for 10 min each with TBST and then incubated for 3 hr gently shaking at room temperature (RT) with a suitable biotinylated secondary antibody diluted according to the manufacturers recommendations in TBST. After incubation the blots were washed again three times in TBST for 10 min and a suitable streptavidin horse radish peroxidase (HRP) labelled tertiary antibody, diluted to the manufacturers instructions, was added and the blots which were then incubated for 1.5 hr at RT. Bands were identified using enhanced chemiluminescence (ECL) which involved covering the blots in a luminol containing substrate. Light emitted from bands present on the blots was detected using photographic film (Kodak BioMAX). In some cases (MMP-2 and MMP-9) the bands were detected colourimetrically. This procedure typically employed a secondary antibody linked to HRP. The substrate for HRP used in this procedure, diaminobenzidine (DAB), was prepared by dissolving one 5 mg tablet in 15 ml TBS, filtering and adding fresh hydrogen peroxide (H_2O_2) to a final concentration of 0.024 % (v/v). This was poured directly onto the blots and the colour precipitate was allowed to develop.

2.2.7 DNA preparation methods

2.2.7.1 Preparation of competent cells

Using a sterile loop, *Escherischia coli* DH5α were scraped from a frozen stock and inoculated into 5 ml of sterile 1X medium (1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; 10mM $MgCl_2$; 10mM $MgSO_4$). This culture was grown overnight with shaking (200 rpm) at 37°C. The following day, 1 ml of the DH5α culture was inoculated into 100 ml of fresh, sterile 1X medium and the culture grown at 37°C at 200 rpm until the O.D. at 550nm had reached 0.6 (approximately 2-3 hr). The culture was then centrifuged at 2,500 rpm for 12 min at 4°C. The pellet was resuspended in 33 ml of frozen storage buffer (FSB), pH 6.4 (10mM CH_3COOK, 100mM KCl, 45mM $MnCl_2.4H_2O$, 10mM $CaCl_2.2H_2O$, 3 mM $HACoCl_3$, 10% (v/v) glycerol). The resuspended pellet was left on ice for 10 min and then centrifuged at 2500 rpm for 10 min at 4°C. The pellet was resuspended in 8 ml of cold FSB. DMSO was then added dropwise until it reached a final concentration of 3.5% (v/v). The mixture was left for 10 min at 4°C after which the same quantity of DMSO was again added. The cells were quickly aliquoted into 200 µl amounts in pre-chilled eppendorfs and flash-frozen in liquid nitrogen prior to storage at -80°C.

2.2.7.2 Transformation of competent cells

10 ng of the required DNA was placed in a sterile microfuge tube. To this was added 100µl of the competent cells which had been briefly thawed between the fingers. The tube was swirled gently and placed on ice for 30 min. The cells were then heat-shocked by placing the tube in a waterbath at 42°C for 90 s after which they were returned to ice for 2 min. 1 ml of sterile Luria-Bertrani (LB) medium, pH 7.5 (1% (w/v) tryptone; 0.5 % (w/v) yeast extract; 1% (w/v) NaCl) was added to the tube and the cells incubated at 37°C for 1 hr with gentle agitation (200 rpm). The cells were centrifuged at 6,000 rpm for 1 min in a Hereaus Microfuge and the supernatant removed. The pellet was gently resuspended in 0.2 ml LB medium and then plated out on LB agar plus ampicillin (LB medium containing 1.5% (w/v) agar and 35 µg/ml ampicillin) plates which were incubated overnight at 37°C in an inverted position. Two

plates were used for each transformation, 90% of the transformation (i.e. 180 µl) was spread on one, while 10% (i.e. 20 µl) was spread on the second, to ensure single colonies were obtained. Two controls were included in every transformation experiment : (1) A mock transformation reaction that received no DNA, and (2) A transformation reaction that received a known amount of a standard preparation of plasmid DNA.

2.2.7.3 Minipreparation of plasmid DNA

Single colonies of bacteria from the transformed cells grown on the LB ampicillin plates were selected and each placed into sterile universal containers containing 5ml LB broth supplemented with 35µg/ml ampicillin. These minicultures were grown overnight at 37°C and 150 rpm. The following day, 1.5 ml of each of the cultures were transferred to sterile microfuge tubes and centrifuged at 10,000 rpm for 1 min after which the supernatants were aspirated. The pellets were resuspended in 100 µl of solution 1 (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10mM EDTA, pH 8.0) and vortexed. 200µl of solution 2 (0.2 M NaOH; 1% (w/v) SDS) was added to each tube and the tubes mixed by gentle inversion after which 150µl cold solution 3 (3M potassium acetate, pH 4.8) was added and the tubes were placed on ice for 5 min. The tubes were then centrifuged for 5 min at 10,000 rpm after which the supernatants were carefully transferred to fresh tubes and the pellets discarded. 450 µl of phenol : chloroform : 2 M Trizma (1 : 1 : 1, v/v/v) was then added to each tube and the mixtures vortexed and then centrifuged at 12,000 rpm for 10 min. The resultant aqueous phase was carefully removed to a fresh tube. 500 µl of 100% ethanol was added and the mixtures let sit at RT for 10 min prior to being centrifuged at 10,000 rpm for 10 min. The supernatants were removed and the pellets washed twice with 70% (v/v) ethanol and then dried at 37°C before being resuspended in 25µl TE, pH 8 and stored at 4°C until ready to digest.

2.2.7.4 Restriction digests

DNA was digested with restriction endonucleases for identification purposes or for linearization of plasmid. For minipreparation DNA, the digest mixture contained 5µl DNA, 2.5 µl of 10X reaction buffer (supplied with each enzyme, by the manufacturer), 16.5 µl of a 25µg/ml solution of RNase A and 1µl (15-20 units, approximately) of the appropriate restriction enzyme (details of plasmids and restriction enzyme used will be outlined in chapter 3). For maxipreparation DNA, 1µg of DNA was prepared in a reaction solution containing 10X reaction buffer and 1µl restriction enzyme. The digests were incubated at the appropriate temperature for the enzyme (usually 37°C) in a water bath for 3 hr.

2.2.7.5 Maxipreparation of plasmid DNA (Qiagen-tip 500 method)

A small aliqout of transformed bacteria glycerol stock containing the desired plasmid was inoculated into a starter culture of 5 ml LB medium containing 35µg/ml ampicillin, grown for 8 hr with shaking at 37°C. The starter culture was diluted 1/500 into 500 ml LB medium containing 35µg/ml ampicillin and grown at 37°C overnight at 250 rpm. Next day the bacterial cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C. The pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris-HCl, pH 8.0; 10mM EDTA; 100µg/ml RNase A) and 10 ml of Buffer P2 (200 mM NaOH; 1% SDS) added and mixed thoroughly by inverting the tube. After incubation at RT for 5 min 10 ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5) was added, mixed by inversion and incubated on ice for 20 min. The samples then became quite viscous with a large amount of white protein precipitate. In order to remove as much of the precipitate as possible the samples were centrifuged twice (the sample was centrifuged at 13,000 rpm for 30 min at 4°C and the supernatant re-centrifuged at 13,000 rpm for 15 min at 4°C). Once the protein precipitate was removed the supernatant was applied to an equilibrated Qiagen tip-500 [add 10 ml Buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100) and allow the column to empty by gravity flow], and allowed to enter the resin by gravity flow. The Qiagen tip was then washed with 2 x 30 ml Buffer QC (1 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). The DNA was eluted with

15 ml of Buffer QF (1.25M NaCl; 50 mM Tris-HCl, pH 8.5; 15% isopropanol) and precipitated by adding 0.7 volumes of RT isopropanol. The sample was centrifuged at 11,000 rpm for 30 min at 4°C. The DNA pellet was washed with 5 ml of 70% ethanol at RT and centrifuged at 11,000 rpm for 10 min. The pellet was allowed to air dry and dissolved in 500µl sterile TE.

2.2.7.6 Spectrophotometric analysis of nucleic acids

DNA or RNA concentration was determined by measuring the absorbance at 260 nm, the wavelength at which nucleic acids absorb light maximally ($\lambda_{max.}$). A 50µg/ml preparation of pure DNA has an absorbance of 1 unit at 260nm while a 40 µg/ml of pure RNA also has an absorbance reading of 1 at this wavelength. In order to calculate the concentration of DNA in samples the following calculation was used, ABS_{260nm} x 50 x 100 (dilution factor, 5µl sample in 495 µl of H_2O) = µg/ml of DNA. To determine the concentration of RNA a similar formula was used, ABS_{260nm} x 40 x 100 (dilution factor, 5µl sample in 495 µl of H_2O) = µg/ml of RNA. The purity of an RNA or DNA preparation was determined by reading absorbances at 260 nm and at 280nm, the λ_{max} for proteins and obtaining the ratio of these absorbances. Pure DNA with no protein contamination should have an ABS_{260}/ABS_{280} ratio of 1.8 while for pure RNA, the ratio is 2.0. Lower ratios indicate the presence of protein while higher ratios often indicate residues of organic reagents.

2.2.8 Transient transfection of human colon cell lines

2.2.8.1 Superfect (Qiagen) mediated transient transfections

The day prior to transfection the cells were seeded at $1x10^5$ cells/ml in 1 ml of growth medium in a 24 well plate and incubated overnight in a humidified incubator at 37°C with 5% CO_2. 1 µg of total DNA, including 10 ng of normalising plasmid DNA (pCH110 and pRenilla), was diluted in 150 µl of cell culture media containing no serum, proteins or

antibiotics. The solution was mixed for 30 s and briefly spun in order to collect the contents to the bottom of the tube. 10 µl of Superfect was added to the tube and the contents vortexed for 10 s. The samples were then incubated at room temperature for 30 min in order to allow DNA-liposome complex formation. While complex formation was taking place the media covering the cells was removed and the cells were washed once in 0.5 ml of PBS. After 30 min 1 ml of normal growth containing media was added to the reaction tube containing the transfection complexes and the contents were mixed by pipetting up and down twice. The contents of the tube were then added into three wells of a 24 well plate (~ 333 µl each). The cells were then incubated with the complexes for approximately 6 hr. Afterwards the media containing the complexes was removed and the cells were then fed with 500 µl of fresh serum containing media and allowed to recover overnight. The following day the cells were serum starved for at least 12 hr prior to cytokine treatment. Subsequently the cells were treated with cytokines of interest (concentrations outlined in section 2.2.1.5) for 2-8 hr (depending on plasmid used) before being lysed for luciferase analysis (see section 2.2.8.3/4/5).

2.2.8.2 *In situ* assay of β-galactosidase

During the transfection optimisation procedure, the plasmid pCH110, encoding for the β-galactosidase enzyme, was used, as its presence could be easily detected and subsequently used as an assessment of transfection efficiency. The enzyme activity of β-galactosidase can be detected *in situ*, on the plate, using the synthetic substrate 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). 48 hr following transfection, cells were washed twice with PBS and then fixed with 5 ml fix solution (0.2% (v/v) glutaraldehyde; 0.02M phosphate buffer, pH 7.3; 0.004M EGTA; 0.002 M $MgCl_2$) for 10 min at RT. This step was repeated once. The cells were then washed twice for 10 min each with 5 ml rinse solution (0.02 M phosphate buffer, pH 7.3; 0.002M $MgCl_2$; 0.1mg/ml sodium deoxycholate; 0.2 µl/ml NP-40) before 1 ml of stain solution (1 mg/ml X-gal; 1.65 mg/ml potassium ferricyanide ($K_3Fe(CN)_6$) ; 1.65 mg/ml potassium ferro-cyanide K_4 Fe $(CN)_6$ in rinse solution) was added to each well and the plates returned to the incubator overnight. The following day, the cells were examined under the microscope. Cells that stained blue produced β-galactosidase and were therefore successfully transfected. Untransfected cells remained clear.

2.2.8.3 Cell Lysis

A commercial lysis buffer (Roche-Boehringer-Mannheim) used in β-galactosidase and luciferase assays was supplied as a 5x concentrate and prior to use this was prepared by diluting 1 part lysis buffer with 4 parts distilled water. Media was aspirated from the cells which were then washed once in PBS. Lysis buffer was then added to each well in the plate (100 µl for 24 well plate). The plates were then placed on a rocking platform for at least 15 min to allow for complete lysis of the cells.

2.2.8.4 Luciferase assays

After lysis cellular debris were spun out in a microtitre plate centrifuge and the resultant lysate was checked for desired enzymatic activity. The promoter constructs used contained a luciferase (*Photinus pyralis*) reporter gene and the level of luciferase within the extracted cell lysate was determined by analysing light emission on the addition of luciferin, ATP and O_2 (Dual-Luciferase™ Reporter Assay System, Promega). The supplied luciferase assay reagent was resuspended in 10 ml of luciferase assay buffer and 50 µl was pre-dispensed into a luminometer tube or luminometer assay plate. The luminometer (Labsystems Luminoskan RS) was programmed to perform a 2 second pre-measurement delay followed by a 10 second measurement period for each reporter assay. 20 µl of the cell lysate was transferred into the luminometer tube/well containing the luciferase assay reagent. The contents were mixed by pipetting prior to the initiation of the measurement period. After the measurement period had elapsed the reading on the luminometer was noted. A number of methods are available which allow for the normalisation of the transfection efficiency between each transfection. One of the simplest means of recording transfection efficiency was through the co-transfection of a second plasmid which encodes a different luciferase gene, in the case of the Dual-Luciferase™ assay this second plasmid contained the Sea pansy (*Renilla*) luciferase gene. The system works through the quenching of the first reaction using the Stop & Glo™ reagent and the initiation of the second reaction with a different substrate, for *Renilla* this was

coelenterazine and O_2. After the initial reading the background reading was measured through the addition of the Stop & Glo™ reagent (supplied in Promega Kit as a 50x concentrate which was diluted to 1x with luciferase assay buffer 1x). 50 µl of the Stop & Glo™ reagent was added to the vial and the background measurement was performed. Once the reading was noted the next set of reactions were carried out.

2.2.8.5 β-galactosidase assay

Initial experiments did not use the Dual Luciferase assay and therefore in order to normalise luciferase values the pCH110 plasmid which encodes β-galactosidase was used. For each sample to be assayed mix 3 µl of 100x Mg solution (0.1M $MgCl_2$ and 4.5M β-mercaptoethanol), 66 µl of 1x ONPG (*o*-nitrophenyl-β-D-galctopyranoside) (4 mg/ml ONPG in 0.1M sodium phosphate, pH 7.5 which is made by mixing 41 ml of 0.2M Na_2HPO_4. $2H_2O$, 9 ml of 0.2M $NaH_2PO_4.2H_2O$ and 50 ml H_2O), 30 µl of cell extract and 201 µl of 0.1M sodium phosphate. The reactions were incubated for 30 min at 37°C until a faint yellow colour had developed. The reactions were stopped by adding 500 µl of Na_2CO_3 to each tube. The optical density of each of the samples was then read at 420nm. β-galactosidase enzyme (Boehringer Mannheim) was used as a positive control for the reaction and also as a standard in order to determine the amount of enzymatic activity.

2.2.9 Cloning of PCR products

This protocol is based on the commercial PCR-Script™ Amp cloning kit which was supplied by Stratagene. The pPCR-Script plasmid (see appendix 1) allows the blunt ligation of PCR products into its multiple cloning site. In order to identify potential clones the plasmid also allows for blue white screening where white colonies indicate clones that are positive for insert. This is due to the disruption of the lac Z gene which encodes β-galactosidase.

2.2.9.1 Purification of PCR products

50 µl PCR reactions were carried out according to conditions described in section 2.2.3.2 with the exception that Pfu, a high fidelity polymerase was used instead of Taq. 50 µl of a DNA binding solution supplied by Stratagene was then added to the PCR reaction tube. The contents of the PCR tube were then transferred to a purification column. The column was spun in a microfuge for 30 s at maximum speed (13,000 rpm). During this stage the DNA is bound to the fibre matrix within the column while all other contaminants pass through. The DNA was then washed twice with an ethanol containing buffer (Stratagene) by adding 750 µl of wash buffer to the fibre matrix and spinning for 30 s at maximum speed. After the second wash, 50 µl of elution buffer was added to the column which was then incubated for 5 min at RT. Afterwards the column was placed in a microcentrifuge and spun at maximum speed for 30 s. The eluted DNA was stored at 4°C until required.

2.2.9.2 Ligation of the PCR products into pPCR-Script Amp

The concentration of DNA after PCR can be found by using the method outlined in section 2.2.7.6. The PCR cloning procedure requires a high ration of insert to vector for ligation. For sample DNA a range of 40: 1 to 100 : 1 is recommended. The following equation was used to optimize conditions for the insert.

$$X \text{ ng of PCR product} = \frac{(\text{number of bp of PCR product})\,(10 \text{ ng of pPCR Script cloning vector})}{2961 \text{ bp of pPCR Script cloning vector}}$$

where X is the quantity of PCR product (in nanograms) required for a 1:1 insert to vector molar ratio. The following table provides examples of optimal insert to vector molar ratios calculated using the above equation.

Size of PCR product (bp)	Quantity of PCR product required (ng) 40x-100x
250	33-85
500	67-169
750	101-253
1000	135-338
1500	202-506
2000	270-675
3000	405-1013

To prepare the ligation reaction the following components were added *in order* to a 0.5ml microcentrifuge tube

1 µl of pPCR-Script Amp cloning vector (10 ng/µl)
1 µl of PCR-Script 10x reaction buffer
0.5 µl of 10 mM ATP
2-4 µl of the blunt ended PCR product (40x-100x range)
1 µl of Srf I restriction enzyme (5 U/µl)
1 µl of T4 DNA ligase (4 U/µl)
Distilled water to a final volume of 10 µl

The reaction was mixed gently and left for 1 hr at RT. The ligation reaction was then heated to 65°C for 10 min. The mixture was kept at 4°C until transformation. The transformation procedure is similar to that outlined in section 2.2.7.2 with the exception that the LB Amp plates were spread 100 µl of a 2% X-gal and 100 µl of 10mM IPTG 30 min prior to spreading of the bacterial colonies, thus allowing for blue white screening. When the pPCR-Script plasmid ligates with an insert it's ability to process X-gal is disrupted as it no longer can encode for a functioning β-galatosidase enzyme. Therefore positive clones can easily be distinguished from negative clones which grow as blue colonies as they still retain their ability to process X-gal.

Positive clones (white colonies) were picked and grown over night at 37°C in LB Amp broth (50 ng/ml). The following day colonies were mini-prepped according to section 2.2.7.3. The plasmid DNA was then assessed for insert content by restriction digest which is outlined in section 2.2.7.4. Restriction enzymes used to cut out the insert were Xho I and Sac I. Once positive clones were identified they were grown overnight in 500 ml of LB Amp broth (50 ng/ml) and maxi-prepped according to section 2.2.7.5.

2.2.10 Ligation of matrilysin introns into pGL_2-Promoter

pPCR-Script clones, positive for matrilysin intron inserts were identified and maxi-prepped. In order to examine the introns for the presence of silencers/enhancers the introns were cut from the pPCR-Script plasmid and ligated into $pPGL_2$-Promoter vector (Promega, see appendix 1) which contains a luciferase reporter gene controlled by an SV-40 promoter. Insertion of the matrilysin introns before the SV-40 promoter will determine if silencers/enhancers exist within the intron.

Introns in the pPCR-Script plasmid were cut out using Sac I and Xho I restriction enzymes (see section 2.2.7.4 for restriciton digest protocol). The pGL_2-Promoter was also linearised using Sac I and Xho I which cut the plasmid in its multiple cloning site. Thus introns cut with Sac I and Xho I could be cloned directly into the pGL_2-Promoter plasmid. Once the restriction digests were complete the contents were run out on a low melting agarose gel (0.7%) in order to separate the pPCR-Script plasmid from the intron inserts and also to isolate linearised pGL_2-Promoter plasmid. The fragments of interest were cut from the gel, taking care to remove as much of the agarose as possible and inserted in to microcentrifuge tubes. The ligation of the DNA fragments was carried out using the following protocol which allows for the ligation of DNA fragments in gel slices.

The gel slices were melted in a 70°C water bath for at least 10 mins. This temperature was hot enough to melt the agarose without denaturing the DNA. An insert to vector ratio of approximately 10 : 1 was used for the ligation reaction. Because the concentration of DNA within the gel slice was unknown the ratio had to be estimated from the initial amount of DNA

used in the restriction digest. Both the intron and pGL_2-Promoter DNA were added to a single micro centrifuge tube with the final volume being 9 µl. The combined DNA fragments were kept at 37°C in order to keep the agarose from solidifying. To each tube containing the DNA mixture 11 µl of an ice cold mixture containing 2x buffer including ATP (final conc. will be 1x) and 2 µl of T4 DNA ligase (4 U/µl). The tubes are mixed immediately by flicking and are then incubated overnight at 15°C on a shaking platform. Even though the mixture solidifies the DNA fragments can still be ligated. After the ligation reaction is complete the mixtures are melted for 5 to 10 min using a 73°C waterbath. Competent bacteria were transformed according to section 2.2.7.2 with the exception that 5 µl of the ligated products were added to 200 µl of competent bacteria. Cells were grown on LB Amp (50 ng/ml) plates over night at 37°C. Individual colonies were picked and mini prepped (see section 2.2.7.3). Restriction digests of isolated plasmid DNA was carried out according to section 2.2.7.4. Clones that contained the correct inserts were then maxi-prepped. In order to determine if silencers and enhancers were present in the introns the plasmids were transiently transfected into various cell lines and luciferase activity was subsequently analysed (see section 2.2.8.4).

2.2.11. Electrophoretic mobility shift assay (EMSA)

2.2.11.1 Nuclear Cell Extracts

Buffers used in this protocol were kept at 4°C and prior to use the following was added (final concentration of each in brackets): DTT (0.5mM), PMSF (0.2mM), Leupeptin (1µg/µl), Aprotinin (1µg/µl), and Pepstatin (0.25 µM). Cells were grown to approximately 70% confluency in 100mm cell culture dishes under normal conditions (DMEM S_5 at 37°C). Cells were then serum starved overnight. The following day the cells were washed with PBS and treated with various cytokines (see 2.2.1.5 for dilutions) for 8 hr at 37°C in serum free media. After incubation the supernatant was removed and the cells were washed three times in PBS solution. 1 ml of PBS was added to each plate and using a cell scraper the cells were collected. The cells were spun out (pulse spin in microfuge ~ 13,000 rpm for 30 s), the supernatant was removed and the cells were resuspended in hypotonic buffer (10mM HEPES, pH 7.9 at 4°C

1.5mM $MgCl_2$, 10mM KCl, 50mM NaF, and 1mM $NaVO_4$). The cells were pulsed briefly in a microfuge and the resultant pellet was resuspended in two packed cell volumes (approx. 100-200 µl) of hypotonic buffer. The cells were then incubated on ice for 10 min. In order to promote cell lysis 1 µl of 5% NP-40 was added to the cells and the suspension was mixed by pipetting 3-4 times with a pasteur pipette. The cells were then analysed for lysis by taking 1µl of the cell suspension and adding trypan blue exclusion reagent. When approximately 60% of the cells were blue in colour, thus indicating that the cell walls had been disrupted, the nuclei were immediately pelletted and washed once in hypotonic buffer. The supernatant was removed and the cells were gently resuspended in a ½ packed nuclear volume (approx. 50-100 µl) of low salt buffer (20mM HEPES, pH 7.9 @ 4°C, 1.5mM $MgCl_2$, 0.02M KCl, 0.2mM EDTA and 25% glycerol). A ½ packed nuclear volume (approx. 50-100 µl) of high salt buffer (20mM HEPES, pH 7.9 at 4°C, 1.5mM $MgCl_2$, 1.2M KCl, 0.2mM EDTA and 25% glycerol) was added drop by drop with careful mixing after each drop. The suspensions were then placed on a rocker/rotator for 30 min at 4°C. One volume of dialysis buffer (20mM HEPES, pH 7.9 at 4°C, 100mM KCl, 0.2mM EDTA and 20% glycerol) was then added to the suspension which was then pulsed in order to remove debris. The supernatant was then placed in visking tubing and dialysed overnight at 4°C in dialysis buffer using an agitated vessel. Once dialysed the contents of the dialysis tubing were removed into separate tubes, flash frozen in liquid nitrogen and stored at -80°C. One tube from each sample was retained and a BCA protein assay (See section 2.2.4) was performed in order to determine the concentration of protein. Samples were also run on SDS-PAGE gels (see section 2.2.5.1) in order to confirm that the isolated nuclear proteins were intact.

2.2.11.2 Preparation of radioactively labelled probes

Oligonucleotide primers for PEA3 (optimal, proximal, upstream and downstream) and TCF were generated (MWG Biotech, UK) and N labelled using γP^{32}-ATP (see table2.4). Radioactive probes were made of each using the following protocol: 50 ng of the oligonucleotide of interest was aliquoted into sterile 0.5ml tube. To this was added 1µl of γ-P^{32} labelled dATP (3000 Ci/mmol, NEN Life science products, Zaventen, Belgium), 1µl 10x

buffer (supplied with enzyme), 1μl of T_4 polynucleotide kinase (NEB, Hitchin, Hertfordshire UK) in a final volume of 10 μl. The reaction was then carried out in a 37°C water bath for 1 hr. In order to separate unincorporated radioactive dATP from the reaction the contents of the tubes were inserted into post reaction purification columns (N' oligo post reaction purification columns, Sephadex G-10 size, Clontech, Basingstoke, Hampshire, UK). Prior to addition of the reaction contents the columns were prepared according to manufacturers instructions. Briefly this involved inverting the contents of the tube and spinning at 3000 rpm for 2 min. The reaction contents were then added to the top of the purification column. The tubes were spun at 3000 rpm for 2 min and labelled probes were collected at the bottom of receptacle tubes while unincorporated γP^{32}-ATP remained in the column. Scintillation counts were then performed on the labelled probes which were subsequently diluted to 50,000 counts per min (cpm) per μl. For the gel shift assay the reactions were prepared in the following manner. 10 μl of 2x dialysis buffer, X μl H_2O*, 1 μl poly dIdC (2 mg/ml stock solution, Amersham Int.), 5 μg of Nuclear extract protein and 1 μl of γP^{32} labelled oligo (50,000 cpm/μl). Asterisk denotes that water was added to take the final volume to 20 μl. The reaction was incubated in a 37°C water bath for at least 30 min.

Probe	Oligonucleotide Sequence
-168 PEA3	5'-GTGTGCTTCCTGCCAATAACGATGT-3'
-144 PEA3	5'-GTAATACTTCCTCGTTTTAGTTAATG-3'
-55 PEA3	5'-CCTATTTCCACATTCGAGGC-3'
-194 TCF	5'-GCAAAATCCTTTGAAAGACAAATCCCTCTCCTT-3'
-109 TCF	5'-CACATACTTTCAAAGTTCTGTAGACTCCCTCTCCTT-3'

Table 2.4 Oligonucleotide sequence of matrilysin PEA3 and TCF probes

2.2.11.3 EMSA gel preparation

Resolving gels used in the assay were 4% acrylamide/bis-acrylamide and made up to a final volume of 50 ml.

6.7 ml	30%(w/v) acrylamide/bis-acrylamide (37.5:1 ratio)
1 ml	Glycerol
41.75 ml	0.5x TBE Buffer
500 µl	10% APS
50 µl	TEMED

Once the gels had set they were placed in a vertical gel box and pre-warmed by running at 200V for 30 min at RT. Once the nuclear extract reactions were complete the samples were loaded (no loading buffer was added to the samples with the exception of the free probe sample which was added as a guidance aid for loading) and were run at 200V at RT. Once the dye front had migrated to approximately ¾ distance the gels were stopped. The gels were placed on filter paper, then transferred to a vacuum dryer and dried at a medium heat for 1 hr. The gels were then placed in a photo-intensifying cassette, covered with radiosensitive photography film (Kodak X-OMAT AR) and then placed at -80°C for up to 2 weeks. Films were developed using an Amersham-Pharmacia film developer.

2.2.11.4 'Supershift' Electrophoretic Mobility Shift Assays

In order to identify nuclear proteins binding to sequences of interest, antibodies generated against a particular protein were added to the nuclear extracts. The antibody-protein complexes were allowed to form by incubating at 4°C for 30 min. The other reactants (see section 2.2.11.3) were then added to the tubes and incubated at 37°C for 30 min prior to running on the gel. 'Supershift' bands migrate more slowly through the gel due to a higher molecular weight complex formed by the antibody and protein thus resulting in a higher band when compared to the control.

2.2.12 *In vitro* invasion assays

Biocoat Matrigel invasion chambers provided a system that allowed assessment of cells invasive activity *in vitro*. The cell culture inserts contained an 8μm pore size membrane that was coated with a layer of matrigel basement membrane matrix. The layer of matrigel 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. In contrast, invasive cells were able to migrate through the Matrigel coated membrane. The 6-well plate invasion chambers were removed from 4°C storage and allowed to come 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 wells of the plate. The plates were then incubated for 48 hr at 37°C in 5% CO_2 incubator. After incubation, the non-invading cells are 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 solution and rinsed in tap water several times. The membrane was then counterstained for 1 min using Eosin stain. The cells and membrane were then dehydrated by incubating 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 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 involving 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.

2.2.13 Zymography

Zymography was used to localise enzyme activity by molecular weight. The gel was prepared by incorporating the protein substrate of interest (gelatin) 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 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 reached 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).

2.2.14 Reverse Zymography

Reverse zymography was used to detect the presence of 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 2.2.13 except that the water component of the gel is reduced by 1 ml to allow for the addition of 0.5 ml of MMP-rich conditioned medium from both the BHK92 cells and the HT-1080 cells. Gels were loaded and ran as above, TIMP standard, containing TIMP-1, TIMP-2 and TIMP-3 (Chemicon Ltd.), 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.

2.2.15 Densitometry and statistics.

Densitometry of protein and RT-PCR gels was performed using a Pharmacia Amersham Densitometer with Imagemaster software. Two tailed T tests were performed using Microsoft Excel™.

Chapter 3

The effect of cytokines on matrilysin mRNA and protein expression in human colon cancer cell lines

3. Introduction

Matrilysin is one of the smallest members of the matrix metalloproteinase family and is capable of degrading many components of the basement membrane and interstitial ECM. Matrilysin has been implicated in many disease processes, such as, cancer invasion and metastasis, and rheumatoid arthritis (Adachi *et al.*, 1999 and Cawston, 1998). Recently, several reports have shown that matrilysin is involved, not just in ECM breakdown but in a wide range of normal biological processes including proliferation, wound healing, angiogenesis and the activation of other MMP members involved in the proteolytic cascade resulting in total ECM degradation (Sang *et al.*, 2000, Nagashima *et al.*, 1997, Lu *et al.*, 1999 and, Barille *et al.*, 1999)

3.1 Structure and function

Matrilysin is similar to the stromelysins in its substrate specificity and to interstitial collagenase in the crystal structure of its catalytic domain but the enzyme is unique in that it lacks the carboxyl terminal domains present in other MMPs (Wilson and Matrisian, 1996). Matrilysin has three domains consisting of a pre, pro and catalytic domain which are essential for cellular processing of the protein and enzymatic activity. Matrilysin is secreted as an inactive protein with a molecular weight of 28 kDa which is subsequently proteolytically cleaved to produce the active 19 kDa enzyme. The cDNA encoding human matrilysin was first isolated by Muller *et al.* (1988) from a mixed tumour library in an effort to clone stromelysin related genes involved in tumour invasion and metastasis. The cDNA, at 1078 bp, was shown to be derived from a previously undescribed gene by comparison to stromelysin and collagenase sequence to which it was 49% and 44% homologous, respectively. The gene was initially termed pump-1 to indicate that it encoded a putative metalloproteinase, with similarity to other MMPs but lacking sequence encoding the caboxyl-terminal hemopexin-like domain. Subsequent purification of the matrilysin protein and analysis of the enzymes substrate specificity revealed that it was capable of degrading a wide spectrum of high molecular weight proteins including fibronectin, gelatins, collagen, laminin, entactin, and elastin (Quantin *et al.*, 1989)

3.2 Matrilysin in disease processes

The MMPs are primarily involved in ECM remodelling and their activity is tightly regulated at the transcriptional and protein level. When the regulation of MMPs becomes aberrant in diseases such as cancer, then there is excessive ECM destruction which may ultimately lead to tumour invasion and metastasis to a secondary site. Matrilysin, which plays an important role in ECM degradation, has been reported to be overexpressed in a number of tumour types, in particular, breast, colon and prostate cancers (Rudolph-Owen *et al.*, 1998a, Wilson *et al.*, 1997 and Udayakumar *et al.*, 2001). Moreover, recent studies have shown that matrilysin is also involved in other aspects of tumourigenesis including, growth, migration and angiogenesis (Chambers and Matrisian 1997, Wilson *et al.*, 1997 and Patterson and Sang, 1997).

3.2.1 The genetics of human colon cancer.

Approximately 22,000 new cases of cancer arise in Ireland each year. Non-melanoma skin cancer is the commonest diagnosis but these are easily detected, treated and rarely cause death. Of the more serious cancers, colon cancer, is the most common accounting for approximately 9% or just under 2000 of total new cases. Males are at a slightly higher risk of developing the disease. The 5 year survival rates for colon cancer patients are less that 55% and colon cancer attributes to 13.5% of the total of cancer related deaths (National Cancer Registry of Ireland, 1997). Colon cancer can be sporadic or inherited. Inherited forms of colon cancer include familial adenomatous polyposis (FAP) and hereditary nonpolyposis colon cancer (HNPCC) which account for up to 20% of total colon cancer cases. The mean age for patients with sporadic colon cancer tends to be over 60 while FAP and HNPCC patients tend to present with colon cancer much earlier with the ages being approximately 20-30 and 40 years old respectively. In 1990, Fearon and Vogelstein put forward a hypothesis for colon cancer progression whereby a number of genetic mutations or 'hits' were required for the progression of colon cancer (see figure 3.1), (Fearon and Vogelstein, 1990).

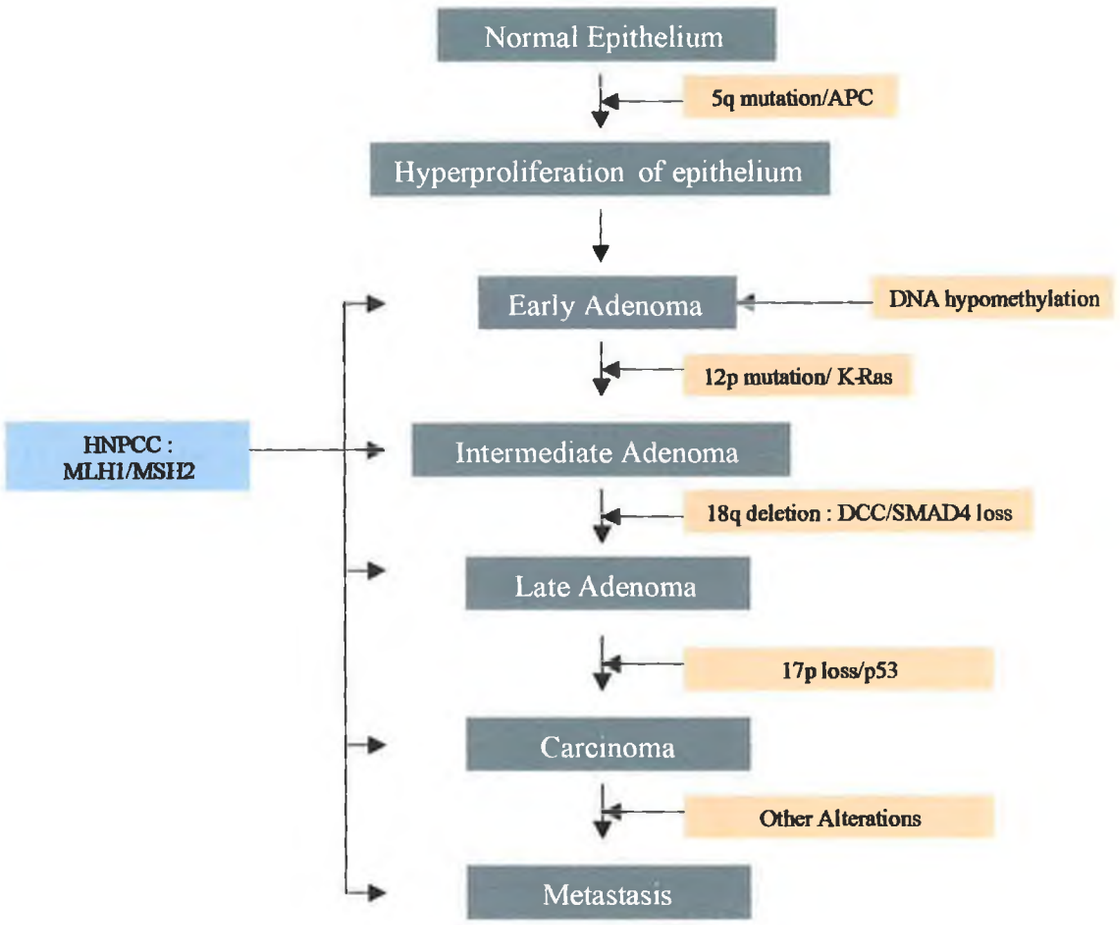

Figure 3.1 Flow diagram illustrating that colon tumourigenesis is a 'multi-step process'. One of the most common mutations in colon cancer is the APC gene. The mismatch repair system which is disrupted in patients suffering from HNPCC contributes to several stages of the tumourigenic process. DNA hypomethylation also leads to the 'switching on' of potential oncogenes. The accumulation of these genetic mutations, however, is more important than the order in which they occur.

3.2.1.1 The APC mutation

The APC (adenomatous polyposis coli) gene mutation is involved in an inherited form of colon cancer referred to as familial adenomatous polyposis (FAP). FAP is highly penetrant and patients usually develop hundreds to thousands of adenomatous polyps during their second and third decades of life. The risk of developing subsequent carcinomas and invasive carcinomas in these patients is greatly enhanced (Sieber *et al.*, 2000). Approximately 80% of sporadic colon cancer cases also contain *APC* mutations

which implies that the APC protein plays an important role in the development of both sporadic and familial colon cancer. A major function of the APC protein involves targetting of a cytoskeletal protein known as β-catenin for degradation. Mutated APC proteins do not have the ability to assist in the degradation of β-catenin, therefore excess β-catenin accumulates within the cytoplasm. The excess β-catenin is efficiently translocated to the nucleus where it effects the transcription of several target genes including cyclin D, c-myc (He *et al.*, 1998) and, as will be discussed later, the MMP matrilysin (see figure 3.2). APC also plays an important role in cytoskeletal organization as it has been shown to bind to the microtubule cytoskeleton via its basic domain. In normal epithelial cells this association occurs at actively migrating regions of the cell membrane. For example if cell migration is induced by wounding a cell monolayer or treating with a human hepatocyte growth/scatter factor APC clusters intensify at the edges of migrating cell membranes (Smith *et al.*, 1994 and Nathke *et al.*, 1996). APC has also been shown to indirectly modulate the organisation of the actin filament network and consequently affects cell morphology polarity and migration. This modulation occurs via the binding of Asef to APC which in turn enhances the interaction of Asef with Rac, a molecule which is involved in the regulation of the actin cytoskeleton (Kawasaki *et al.*, 2000).

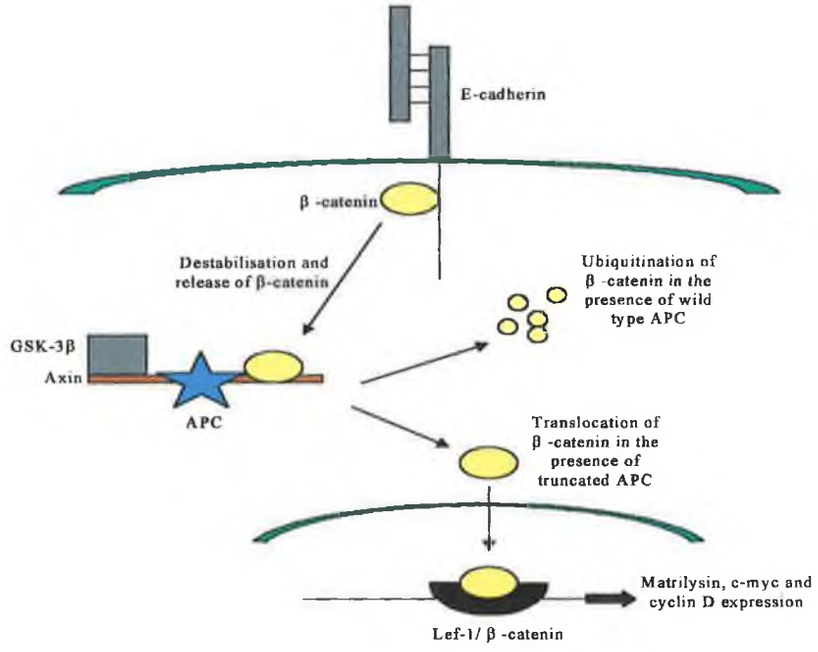

Figure 3.2 The role of APC in the regulation of β-catenin

3.2.1.2 The ras mutation

Another important early somatic alteration identified in colon tumours is the ras gene mutation. When transfected into suitable recipient host cells it was found that mutated ras genes conferred neoplastic properties. The importance of the ras gene mutation in early colorectal tumourigenesis was further bolstered by experiments examining a number of patient samples. It was found that 50% of colorectal carcinomas and a similar percentage of adenomas greater than 1cm in size had a ras gene mutation. In contrast such mutations have been identified in fewer than 10% of adenomas less than 1cm in size regardless of whether the tumours arose sporadically or in patients with an inherited predisposition for their formation (Bos *et al.*, 1987 and Vogelstein *et al.*, 1989). The ras gene product is a small membrane bound GTPase protein which plays an important role in the transduction of cell signals. It is involved in several signalling cascades which include the mitogen activated protein kinase (MAPK) pathway and phosphoinositol (PI) pathway which will be dealt with in more detail later. In its inactive state the Ras protein is GDP bound. Stimulation of receptor tyrosine kinases such as the EGF receptor leads to the activation of Ras, hence the activation of the subsequent signalling cascades associated with Ras. This is achieved via the tyrosine kinase activity of the receptor which subsequently activates SOS (son of sevenless, a GTP exchange molecule) via an intermediate docking protein known as GRB. SOS facilitates the activation of Ras via the exchange of GDP for GTP. Activated Ras is then involved in the activation of various signalling cascades. Once the required effects have been achieved by the cell, Ras reverts back to its inactive state via NF1-GTPase activating protein (see figure 3.3).

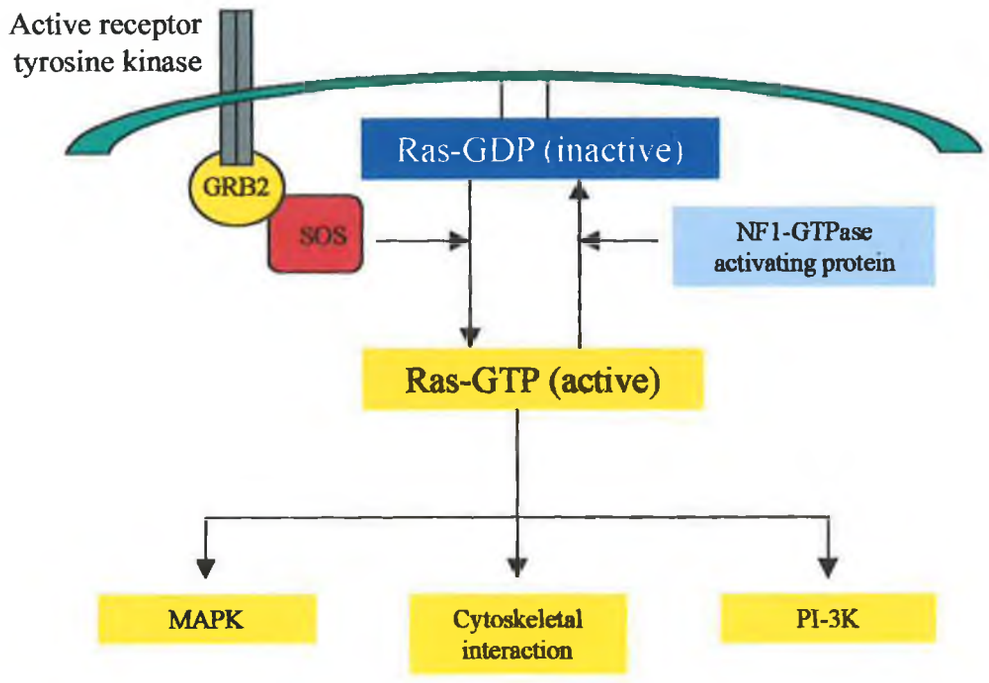

Fig 3.3 RAS activation. The ras protein is bound to the cytoplasmic cell membrane via a 15 carbon isoprenyl (farneysl group). Activation of the inactive GDP bound Ras molecule involves the GTP exchange molecule SOS. Ras mediates its effects via several pathways including the mitogen activated protein kinase (MAPK) and the phosphoinositol-3 kinase (PI-3-K) pathways. Ras is also an important modulator of the cell cytoskeleton. Once Ras has mediated its effects it reverts back to its inactive state via the activity of NF1-GTPase activity.

Ras gene mutation is a common event in human colon cancer and mutation typically results in the permanent activation of the Ras protein. Therefore the Ras protein constantly stimulates the cells with mitogenic signals which subsequently leads to enhanced tumourigenesis.

3.2.1.3 The 18q deletion (DCC/SMAD4/SMAD2)

One of the most common regions of allelic loss in carcinomas is chromosome 18q which is absent in more than 70 % of carcinomas and 50% of late adenomas (Vogelstein *et al.*, 1989 and Delattre *et al.*, 1989). Initially it was thought that the 18q deletion resulted in the loss of a single gene, DCC (deleted in colon cancer). However recent studies have shown that other candidate tumour suppressor genes such as SMAD4 and SMAD2 are also present in this chromosome region. The function of the DCC gene

protein product was initially thought to be involved in cell adhesion but it was later identified as a receptor (semaphorin) for extracellular chemotactic proteins (netrins) which are involved in cell migration. It is now thought, however, that the SMAD deletion is more important in colon tumourigenesis as SMAD4 knockout mice were shown to develop colon cancers whereas DCC knockout mice remained cancer free. The SMAD proteins are involved in TGF-β (transforming growth factor β) signalling. TGF-β is a multifunctional cytokine and has a wide variety of functions including the inhibition of cell proliferation and induction of ECM proteins. TGF-β mediates its effects via a Type I and Type II receptor which in turn utilise the SMAD molecules. Loss of the SMAD molecules would therefore result in the cell ignoring TGF-β mediated cell proliferation or inhibition. SMAD4 and SMAD2 function is commonly lost in colon cancer. The net result of the loss of SMAD4 and SMAD2 is a loss of differentiation, increased proliferation and altered cell adhesion (Tafara *et al.*, 2000).

3.2.1.4 The 17p loss (p53)

The loss of a large portion of chromosome 17p through chromosomal loss or mitotic combination has been detected in 75% of colorectal carcinomas but this loss is not apparent in early adenomas. Moreover, in several patients this loss was found to correlate with the progression of adenoma to carcinoma (Fearon *et al.*, 1987). Other cancers such as lung, breast and bladder have also shown a loss in this chromosomal region (Yokota *et al.*, 1987, MacKay *et al.*, 1987 and Hartmann *et al.*, 2000). The region lost on chromosome 17p has been identified and contains the p53 gene, which is essential in maintaining and regulating normality during the cell cycle. p53 is known as the 'guardian of the genome' and plays an important role in coordinately blocking cell proliferation, stimulating DNA repair and promoting apoptosis. p53 can mediate its effects directly by acting as a transcription factor and binding to specific sites within the promoter of target genes such as BAX, p21 and insulin like growth factor binding protein 3 (IGFB3). p53 can also indirectly inhibit the expression of proto-oncogenes such as jun and fos through its interaction with the TATA binding protein (TBP). By inhibiting the interaction of TBP with the TATA site within the promoter of these genes p53 can prevent gene transcription. Thus a point mutation and/or loss of the p53 gene is commonly associated with carcinomas from several tissues including colon tumours. p53 activity can also be

regulated by MDM2 (murine double minute clone 2) which directly controls the rate of p53 degradation.

Studies examining many colon cancer samples have shown that the genetic mutations outlined above take place during certain stages of colon tumourigenesis leading to the hypothesis that colorectal carcinoma development is a step wise procedure, however accumulation of these mutations is more important than the order in which they occur.

3.2.1.5 Hereditary non-polyposis colon cancer (HNPCC)

Other forms of inherited colon cancer include Lynch syndrome or Hereditary Non Polyposis Colorectal Cancer (HNPCC). HNPCC is a common cancer predisposition syndrome with a familial component that has been recognised for over a century (Bradley and Evers, 1997). Historically HNPCC, which is inherited in an autosomal dominant fashion, has been difficult to define but three minimum criteria, known as the Amsterdam criteria, must be met for a diagnosis of HNPCC: i) at least three relatives, one of which is first degree, should have histologically verified colorectal cancer, persons with FAP should be excluded, ii) at least two successive generations should be affected and iii) one of the three relatives must have a cancer that was diagnosed prior to the age of 50 (Vasen *et al.*, 1991). HNPCC is thought to account for approximately 1%-5% of all colorectal carcinomas but estimates of up to 15% have been reported (Cunningham and Dunlop, 1994 and Bellacosa *et al.*, 1997). Strict adherence to the criteria set out above should rectify these figures. By studying family histories via RFLP (restriction length fragment polymorphisms), scientists discovered the location of the genes involved in HNPCC on chromosome 2p16-15 and 3p and the role of the genes located at these positions and their role in the development of colorectal cancer are now being elucidated. The current theory for function of the mutated genes in HNPCC implicates a defective DNA mismatch repair system producing microsatellite instability in HNPCC tumours. The mismatch repair system is responsible for correcting deletions or expansions that occur as a result of physical damage to the DNA, misincorporation of nucleotides during DNA replication, or mismatched nucleotides that occur during genetic recombination (Fishel *et al.*, 1993). Thus the major function of the mismatch repair system is to correct the errors that escape proofreading (Fishel *et al.*, 1993 and Branch *et al.*, 1995). Reports have shown that

HNPCC tumours have at least a 100 fold increase rate in their mutation rate when compared to sporadic tumours (Parsons *et al.*, 1993) which may go some way to explaining why HNPCC affected individuals are diagnosed with colorectal cancer at such an early age (Parsons *et al.*, 1993). The mismatch repair system was first identified in *S. Cerevisiae* and *E. Coli* and there are a number of genes involved, *h*MSH2 and GTBP which are involved in mismatch binding while *h*MLH1, *h*PMS1 and *h*PMS2 are involved in complex formation. In the majority of HNPCC patients *h*MSH2 and *h*MLH1 mismatch repair enzymes have been found to be defective (Bradley and Evers for review, 1997).

3.2.2 The role of matrilysin in colon cancer

When the proto-oncogenes and tumour suppressor genes involved in hereditary and sporadic colorectal cancer development are mutated/deleted the regulation of many other genes within the cell are also affected. For example mutations in the ras gene leads to a protein product which constantly stimulates the cells to proliferate via signalling cascades such as the MAPK pathway. This in turn leads to an increase in transcription factors such as jun and fos which bind to the AP-1 sites within the promoters of target genes. MMP promoters contain several transcription factor binding sites such as AP-1 and therefore mutations in proteins such as ras may directly lead to an increase of MMPs including matrilysin. Matrilysin has been shown to be overexpressed in several colon tumour studies and the regulation of the matrilysin gene at the transcriptional level by oncogenes/tumour suppressor genes will be dealt with in detail later. Matrilysin has also been shown to correlate well with colon tumour aggressiveness and its value as a prognostic marker has been well documented (Ichikawa *et al.*, 1998 and Adachi *et al.*, 1999), however, matrilysin has recently been shown to play an important role in early colorectal tumourigenesis.

Initial studies focussed on the expression of matrilysin in gastrointestinal cancers and using various techniques such as immunohistochemistry and northern blot analysis it was established that matrilysin was produced by tumour epithelial cells, unlike most MMPs which are secreted by stromal cells surrounding the tumour, and that this expression was upregulated in tumour tissue in comparison to that of normal adjacent tissue (McDonnell *et al.*, 1991 and Newell *et al.*, 1994). Overall matrilysin mRNA was

detected in 80% of gastrointestinal tumours via northern blot analysis and in 90% of colorectal tumours examined by *in situ* hybridisation and northern blot analysis. More interestingly, studies examining matrilysin levels in a panel of gastrointestinal samples, found that matrilysin expression was often upregulated in lesions which were histologically classified as pre-invasive, i.e. adenomas and carcinomas *in situ*, which suggested that matrilysin could play a role in gastrointestinal cancer other than that of ECM degradation (Newell *et al.*, 1994).

Experimental evidence which supported the role of matrilysin in intestinal tumourigenesis was obtained via the genetically matched human colon adenocarcinoma derived cell lines SW480 and SW620 (Witty *et al.*, 1994). The SW620 cell line, derived from a lymph node metastasis, expressed matrilysin while the SW480 primary tumour cell line, derived from the same patient expressed no matrilysin. Both cell lines were analysed for their expression of other MMPs and with the exception of SW480 producing low amounts of MMP-9, neither cell line was found to express other MMPs. Transfection of the SW480 cell line with either a wild type or an activated form of matrilysin did not result in an increase in the *in vitro* invasive ability using invasion assays. Nor, did it result in liver metastasis following orthotopic injection into nude mice, however, an increase in tumour load was observed. It was found that when the SW480 parental cell line was injected into the cecum of nude mice that no tumours were detected while in the mice injected with the matrilysin transfected SW480 cell line, 50 % had detectable tumours. The complementary experiment also proved that matrilysin was involved in early intestinal tumourigenesis. This involved the transfection of the SW620 cell line with a full length anti-sense matrilysin cDNA. Once again no detectable difference between the parental and the transfected cell lines was observed with respect to *in vitro* invasive ability however, a significant reduction in tumour incidence was observed when both cell lines were injected into nude mice. In this case the incidence of primary tumours was halved from 80% to 40% which suggested that matrilysin, although important in tumour invasion and metastasis, plays a significant role in early tumourigenesis (Witty *et al.*, 1994).

Animal models have also been employed to demonstrate the relationship between matrilysin and early stage intestinal tumourigenesis. Colorectal tumour progression proceeds in a well defined series of steps from precursor lesions known as aberrant crypt

foci (ACF) to adenoma, to carcinoma, to invasive carcinoma (Muto and Bussey, 1975 and Smith *et al.*, 1994). The genetic lesions associated with this process, as outlined above, involve early mutations of the APC tumour suppressor gene and ras oncogene. The matrilysin promoter, as will be discussed later in chapter 4, contains an AP-1 transcription factor binding site and it has been shown previously that ras induces AP-1 transcription factor binding proteins which are essential for the basal transcription of matrilysin. The APC gene encodes a 300 kDa protein and as discussed earlier is involved in familial adenomatous polyposis (FAP). In FAP and the majority of sporadic colon cancers the APC gene is mutated. The mutation results in a truncated form of the protein which in turn is associated with the formation of multiple polyps in the colon, (Powell *et al.*, 1992 and Miyaki *et al.*, 1994). A mouse model of FAP known as Min (multiple intestinal neoplasia) was generated by treating pedigree mice with the mutagen ethylnitrosourea. Min mice carry an autosomal dominant germline mutation in the *Apc* gene which results in the development of intestinal polyps (Moser *et al.*, 1990). This and other similar models have proved to be invaluable tools in analysing the early development of colon tumours and the role that matrilysin plays in their development.

MMP expression was examined in a number of adenomas excised from Min mice. The majority of the tumours examined via immunohistochemistry were positive for matrilysin expression (22/25 = 88%) and this expression was confined to the tumour epithelial cells which was consistent with observations made in human studies (Wilson *et al.*, 1997). Similarly, other MMP family members, such as MMP-9 and MMP-3, were detected in the stromal cells surrounding the tumour which was again consistent with human studies. No matrilysin was detected in normal adjacent tissue which indicated that matrilysin was associated with benign tumour formation in the Min mice. In order to prove that matrilysin was involved in early tumourigenesis a set of experiments involving the crossing of Min mice with matrilysin null mice (*mmp7 -/-)* were performed. The matrilysin null mice were created by homologous recombination and displayed no overt phenotype. The matrilysin null mice were then back crossed into a C57/BL6 background and mated with Min mice creating an animal model whereby Min mice in which the gene for matrilysin was ablated, could be compared to wild type Min mice. The results showed that the average number of tumours in the $Min^{+/+}/mmp7^{-/-}$ decreased by approximately

58% when compared to the average number identified in the 'wild type'. A significant reduction in tumour size was also observed (Wilson *et al.*, 1997).

To further elucidate matrilysin's role in early tumourigenesis, the AOM (azoxymethane) animal model was employed as it provides a particularly useful system that mimics the adenoma-carcinoma sequence observed in humans. AOM treatment induces colon cancer that progresses in a step-wise fashion (Vivona *et al.*, 1993). The first lesions associated with carcinogen treatment are known as ACF and are regarded as pre-neoplastic lesions (McLellan and Bird, 1988 and Vivona *et al.*, 1993). Similar lesions are also detected in human colonic mucosa (Pretlow *et al.*, 1991). Both the matrilysin null mice (*mmp7* -/-) and wild type mice were treated with AOM and after six weeks the number of foci per animal were examined. A significant difference was observed whereby the number of foci formed in the matrilysin null mice was approximately 50% less than those which formed in the wild type mice mice. These results implicated that matrilysin expression was important in the initial stages of tumour development (Fingleton *et al.*, 1999).

Together these *in vitro* and *in vivo* studies demonstrate that matrilysin plays an important but as yet undefined role in early colon tumour development.

3.2.3 The role of matrilysin in other cancers

Matrilysin is also involved in the progression of other tumours such as breast, prostate, lung, esophageal, myeloma and head and neck squamous carcinomas (Chambers and Matrisian, 1996, Barille *et al*, 1999, Ocharoenrat *et al.*, 1999, Yamashita *et al.*, 2000 and Udayakumar *et al.*, 2001). Matrilysin is also involved in other diseases such as rheumatoid arthritis and osteoarthritis as overexpression of matrilysin leads to excessive cartilage destruction (Cawston, 1998).

3.2.3.1 Matrilysin's role in breast cancer tumourigenesis

After skin cancer, breast cancer is the most common cancer among women with approximately 1,800 new cases being diagnosed in Ireland each year and is responsible

for approximately 18% of cancer related deaths (National Cancer Registry Ireland, 1997). The 5 year survival rates are less than 55%. Breast cancer is the leading cause of mortality due to cancer in non-smoking women in the US (Parker *et al.*, 1997). Lethality is usually the result of local invasion and metastasis of neoplastic cells from the primary tumour into the underlying stroma, entry into the circulation and growth of the cancer cells at distant sites in the body (Liotta, 1986). By the time a patient presents with breast cancer, detectable metastases may be present. Breast cancer can arise sporadically due to a series of genetic mutations but approximately 10% of breast cancers occur as a result of highly penetrative germline mutations in cancer predisposition genes (Claus *et al.*, 1991). These genes include *BRCA1, BRCA2* and *BRCA3*. The *BRCA1* and *BRCA2* protein products have been shown to play an important role in DNA damage repair through their interaction with Rad51 (Gowen *et al.*, 1998, Abbott *et al.*, 1999). Rad51 has been shown to play a central role in mediating homologous recombination events and can promote strand exchange alone *in vitro* (Bhattacharyya *et al.*, 2000). It has also been suggested from studies examining *BRCA1* knockout mice that *BRCA1* and p53 may operate via a common functional pathway (Hakem *et al.*, 1997 and Ludwig *et al.*, 1997).

neu/ErbB-2 is a relative of the epidermal growth factor receptor and the gene protein product (Her2/Neu) is known to mediate cell mitogenesis via signalling pathways upon dimerisation. Hence overexpression of this mutated gene leads to increased mitogenic signaling in affected cells. Several studies have observed that a high degree of *neu/ErbB-2* amplification is associated with poor clinical outcome (Slamon *et al.*, 1987 and Varley *et al.*, 1987). MMPs have been described as being involved in proliferation and migration (Sang *et al.*, 2000 and Nagashima *et al.*, 2000) and therefore mutations in genes which regulate cell growth such as *BRCA1, BRCA2* and *neu/ErbB-2* may have a direct or indirect effect on MMPs and their deletion or amplification may play a role in the regulation of many MMPs including matrilysin.

The role of MMPs in breast cancer has traditionally focussed on their expression with respect to invasion and metastasis and several groups have reported that MMP expression increases with advancing tumour stage (Rudolph-Owen and Matrisian, 1998a). In particular, MMP-2 and matrilysin mRNA transcripts have been shown to be expressed at higher levels in malignant than in normal breast tissues (Pacheco *et al.*, 1998). Of all

the MMPs involved in mammary cancer, matrilysin is important in that it is the only MMP almost exclusively expressed in the epithelial component of the tumour, compared to the predominantly stromal expression of other MMPs (Rudolph-Owen *et al* 1998a). Matrilysin mRNA has been detected in the neoplastic epithelial tumour cells of 70%-91% of breast adenocarcinomas and also in benign breast fibroadenomas which indicates that matrilysin by logical inference is involved in not only tumour invasion and metastasis but also in early mammary tumourigenesis (Wolf *et al.*, 1993 and Rudoph-Owen *et al.*, 1998b). In a recent report matrilysin has been shown to influence early stage mammary tumourigenesis (Rudolph-Owen *et al.*, 1998b). These studies used transgenic mice that expressed human matrilysin under control of the mouse mammary tumour virus (MMTV) promoter. Overexpression of matrilysin in these animals did not produce any observable morphological changes during mammary gland development. However closer inspection showed that 50% of the transgenic mice contained abnormal structures in the mammary gland in comparison to control mice. These areas of hyperplasia strongly resemble structures previously termed HANs (hyperplastic alveolar nodules), which are considered to be premalignant precursors that are prone to develop into mammary carcinomas (Cardiff, 1984). HANs have been reported as being susceptible to carcinogens and exposure to agents such as chemical carcinogens or radiation increases the tumour incidence of the hyperplastic cells and usually decreases the tumour latency period (Cardiff, 1984). In order to assess if the HANs type structures observed in mice overexpressing matrilysin could be induced to form tumours, the mice were crossed with mice which overexpressed Her2/Neu. The result revealed a striking acceleration in the onset of mammary tumour formation by 13 weeks. It was suggested that the role of matrilysin may be involved in the production of soluble ligands that bind to the Her2/Neu receptor hence creating a loop whereby increased matrilysin expression led to increased Her2/Neu signalling which in turn leads to cell proliferation and increased matrilysin production. Matrilysin has therefore been shown to play an important role in mammary tumour formation in conjunction with other mutations.

3.2.3.2 The role of matrilysin in prostate cancer

Matrilysin has also been shown to play an important role in the invasion and metastasis of prostate cancer (Udayakumar *et al.*, 2001), a disease which afflicts males

only and the average age of diagnosis is approximately 65 (National cancer registry of Ireland, 1997). Recent evidence has shown that factors such as FGF, EGF or their receptors are commonly upregulated in prostate cancers and that this upregulation is associated with increased matrilysin expression (Udayakumar *et al.*, 2001 and Sundrashen *et al.*, 1999). Interestingly, a recent report by Powell *et al* (1999) has shown that matrilysin plays a role in the Fas ligand/Fas receptor (FasL/Fas) mediated apoptotic system in the normal involuting rodent prostate. Studies showed that matrilysin can process recombinant and cell associated FasL to soluble FasL (sFasL) which was subsequently capable of inducing apoptosis in a target epithelial cell population. Mice deficient in matrilysin also showed a 67% reduction in apoptotic index in the involuting prostate compared with wild type which once again implicates matrilysin in the FasL mediated apoptotic process (Powell *et al.*, 1999).

3.2.4 Therapeutic implications

Matrilysin has been shown to play an important role in several tumour types and evidence suggests that it is involved in the early tumour development of colon and breast tumours besides playing its more traditional role in tumour invasion and metastasis. Although we cannot yet define the mechanisms by which matrilysin expression aids tumour growth it is clear that its expression confers an important advantage with respect to tumour formation. It may therefore be of benefit to treat tumours at early stages using MMP inhibitors directed against matrilysin in order to prevent tumour growth, invasion and metastasis. As we shall see matrilysin also plays roles in other processes and with improvements in drug delivery mechanisms diseases such as breast and colon cancer could be treated without deleterious side effects.

3.3 Matrilysin in normal biological processes.

Many studies have investigated the role of matrilysin with respect to cancer tumourigenesis and invasion and metastasis but growing evidence suggests that matrilysin, has important roles in various normal biological processes which may have implications for tumour biology.

3.3.1 Matrilysin and angiogenesis

Angiogenesis involves the formation of new blood vessels in response to angiogenic stimuli secreted by cells (Risau, 1997). The process involves the destruction of the ECM by vascular endothelial cells in order to create room for the developing blood vessels. MMPs including matrilysin have been shown to play an integral part in the regulation of this process. Recent studies have shown that matrilysin is involved in the hydrolysis of plasminogen resulting in the production of angiostatin fragments (Patterson and Sang, 1997). Angiostatin is one of the most potent inhibitors of angiogenesis, however, the physiological relevance of the generation of angiostatin by matrilysin remains to be elucidated. This would appear to be contradictory to the role of matrilysin in tumour formation and growth as angiogenesis is a key element in tumour growth and progression. Models in which matrilysin is not expressed contain angiostatin fragments suggesting that other proteases are involved in the process (Patterson and Sang, 1997). Matrilysin may have a low catalytic activity for cleavage thus preventing other more suitable proteases producing angiostatin fragments thereby allowing the process of angiogenesis to proceed at a quicker rate.

3.3.2 Matrilysin and apoptosis

Matrilysin has been shown to be involved in epithelial cell apoptosis via the generation of soluble Fas ligand. The Fas ligand/Fas receptor (FasL/Fas) system was first described as an important mediator of immune cell apoptosis whereby the binding of FasL to Fas resulted in the initiation of the apoptotic cascade (Schulze-Osthoff *et al.*, 1998). Matrilysin has been shown to be involved in the cleavage of many of the high molecular weight proteins within the ECM and has also been reported to cleave a variety of secreted and cell surface proteins. These cell surface proteins include the membrane bound precursor to TNF-α, a member of the same protein family as FasL. Cleavage of the TNF-α precursor by matrilysin and other proteases can result in the release of active ligand from the cell surface (Gearing *et al.*, 1994). Further experiments showed that FasL is a substrate for matrilysin with cleavage resulting in soluble Fas ligand. The soluble Fas ligand was then shown to induce apoptosis in human epithelial cells (Powell *et al.*, 1999). This new biological role of matrilysin could be contrary with respect to its role in

tumourigenesis but it is quite possible that matrilysin may be involved in the generation of FasL resistant cells or that the cells may receive other mutations which render them resistant to FasL induced apoptosis (Personal communication from Barbara Fingleton).

3.3.3 Matrilysin's role in innate host defence.

Recently matrilysin has been shown to play an important role in innate host defence. A study by Wilson *et al.* (1999) reported that matrilysin was capable of activating molecules known as defensins which are antibiotic peptides secreted by epithelial Paneth cells located in the intestinal lumen. The defensins are a family of cationic peptides that kill bacteria by membrane disruption. Granulocytes and several epithelial tissues, including the Paneth cells of the small intestine of most mammals produce α-defensins as prepropeptides. These antibacterial agents are released from secretory granules in response to bacteria or cholingeric agents. The pro region of the peptides keeps them in an inactive state. Evidence has shown that the expression of defensins parallels that of matrilysin in the Paneth cells and that there is an accumulation of pro-peptide defensins in matrilysin null mice (Wilson *et al.*, 1999). Further studies revealed that matrilysin was capable of directly processing the active form of α-defensin (Wilson *et al.*, 1999). The study also showed that in comparison to the wild type mice ($MAT^{+/+}$), the matrilysin null mice ($MAT^{-/-}$) died much more quickly upon exposure to a virulent strain of *Salmonella typhimurium* with the LD_{50} for $MAT^{+/+}$ being 1.14×10^5 cells and $MAT^{-/-}$ being 1.41×10^4 cells. Other MMPs expressed in the mouse small intestine (MMP-10 and MMP-13) were also examined but their presence was not detected in the Paneth cells which again implicated matrilysin's involvement in the processing of defensins (Wilson *et al.*, 1999). These results clearly show that MMPs such as matrilysin may play an important role in the processing of a wide variety of proteins and not just those associated with the ECM.

3.3.4 Matrilysin in other biological processes

Matrilysin is also involved in several important normal biological processes including wound healing, menstruation and herniated disc resorption (Arumugam *et al.*, 1999, Koks *et al.*, 2000 and Haro *et al.*, 2000). Response to tissue injury begins with the

deposition of a fibrin rich clot of the provisional matrix. The provisional matrix consists of plasma borne matrix molecules that serve as scaffolding for the ensuing migration of cells. During wound repair, multiple cell types must migrate through the clot matrix scaffolding, and the migration of these cells through this matrix is dependent on the expression of MMPs such as matrilysin (Arumugam *et al.*, 1999). Matrilysin has been shown to be involved in the menstrual cycle and its expression has been found to be at its highest during the extensive remodeling of the endometrium (Koks *et al.*, 2000). In a recent report matrilysin has also been implicated in the release of soluble TNF-α which in turn plays an important role in herniated disc resorption (Haro *et al.*, 2000).

3.4 Cytokine function and mode of action

In order for cells to carry out their proper biological functions they need to be able to 'sense' their external environment and this is typically achieved by the expression of various receptors on the cell surface, for example, cell adhesion molecules (CAMs) and hormone and growth factor receptors (Parmiani *et al.*, 2000). When ligands bind to these receptors internal signaling pathways are activated which in turn activate the transcription of target genes. Cytokines are one of the most common signalling molecules used in biology. Cytokines are proteins or glycoproteins that mediate potent biological effects on many cell types. Cytokines mediate their effects through various signal cascades and have been found to play critical roles in almost all aspects of cellular functions including development, migration, haematopoiesis, inflammatory response and the development and maintainence of the immune system. Cytokines are also potent mediators of cell growth and many of the genes which express either cytokines or the receptors for them are considered to be proto-oncogenes and mutations in these genes can have catastrophic effects on cellular function including uncontrolled cell proliferation thus increasing the affected cells potential to become tumourigenic. Based on their mode of action cytokines can be divided into three groups, i) autocrine, in that they act on the cells which have secreted them, ii) paracrine, in that they are secreted by a cell but cause a cellular reaction in a neighboring cell, and iii) endocrine, in that they are normally secreted by specialised glands, transported in the blood and carry out their effects on distant target cells (see figure 3.4).

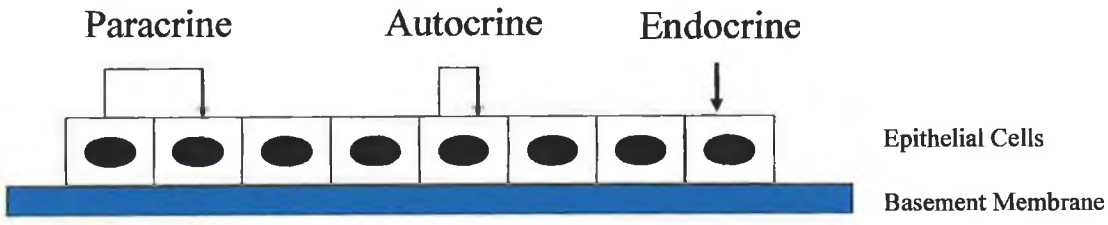

Figure 3.4 Mode of action of cytokines on epithelial cells. Cytokines may also act in a juxtacrine manner whereby they can signal a neighboring cell by using membrane bound signalling peptides and receptors.

Cells use cytokines and other factors to 'sense' their external environment and for example when a cell receives a stimulus to proliferate a number of events occur such as entry into the cell cycle and DNA replication. In order for the cell to divide it must loosen the bonds of the ECM which anchor it and this is achieved through the regulation of cell adhesion molecules and the expression of proteases such as MMPs (Beksac *et al.*, 2000 and Madri and Graesser, 2000). If MMPs are therefore expressed in response to external stimuli from cytokines it is possible that aberrant cytokine expression could result in the overexpression of MMPs resulting both in increased cell proliferation and increased ECM degradation.

This chapter focuses on the expression and regulation of matrilysin by several cytokines in a panel of colon cell lines. We have chosen the colon system as much work to date has shown that matrilysin expression is critical in colon tumour formation, proliferation, invasion and metastasis and is unique in that it is the only MMP expressed exclusively by the tumour epithelial cells. We have examined a panel of cytokines for their effect on matrilysin expression in these cell lines and hope to establish that cytokine regulation is an important feature in matrilysin expression during colorectal tumourigenesis. The cytokines examined here include IGF-I, IGF-II, EGF, bFGF, IL-6 and a known upregulator of MMP expression 12-O-tetradecanoyl-phorbol-13-acetate (TPA).

3.4.1 EGF and the EGF receptor (EGF-r)

EGF is part of a large family of cytokines which can be characterised by the fact that they contain at least one extracellular EGF structural unit (a conserved 6-cysteine

motif that forms three disulphide bonds). Family members include EGF itself, transforming growth factor-α (TGF-α), Heparin binding-epidermal growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), sensory and motor neuron derived factor (SMDF) and the heregulins (HRGs). EGF is secreted as a 9 kDa protein which is proteolytically cleaved to yield an active 6 kDa form. It is a multifunctional cytokine most commonly associated with stimulating the growth of epithelial cells and mediates this effect by binding to the EGF-r. The EGF-r is a common receptor for EGF, TGF-α, AR and HB-EGF and the genes encoding for each of these molecules are important in growth and development. The EGF-r has tyrosine kinase properties which are used in the transduction of external signals and is a member of the *src* group of oncogenes. Although the EGF-r is expressed by a wide variety of adult cell types *in vivo* and cell lines in culture, EGF was so named because of its first recognised activity in an *in vivo* assay for epidermal maturation. Tissues that continue to divide in order to renew themselves generally have more EGF-r present or are more responsive to EGF than other tissues. These tissues would include the epithelial lining of the gut and epidermal layer of the skin (Wilson and Gibson, 2000).

The EGF-r gene encodes a 170 kDa transmembrane receptor with an intracellular domain similar to that of the src gene product (see figure 3.5). The EGF-r is characterised by its ability to autophosphorylate and upon autophosphorylation dimerisation with another nearby EGF-r occurs. The tyrosine kinase receptor can then carry out its function via the phosphorylation of signaling molecules docked to its intracellular domain. The EGF receptor as has been stated is a trans-membrane receptor with intrinsic tyrosine kinase activity and there have been numerous reports on the expression of this receptor and its ligands, EGF and TGF-α, in colon tumours. In an immunohistochemical study, synchronous expression of EGF and EGF-r in highly invasive colon carcinomas suggested that an EGF autocrine loop may play a role in the regulation of these tumours (Karamerias *et al.*, 1993). Such autocrine loops have been described in several colon carcinoma cell lines where cell growth was inhibited by using antisense TGF-α and blocking antibodies to the EGF receptor (Karnes *et al.*, 1992). It has also been reported that the expression of EGF-r by colon carcinoma cells directly correlates with their metastatic potential (Radinsky *et al.*, 1995). EGF promotes the migration and invasion of a number of cell

types and has been linked to alterations in cell-ECM interactions. In fibroblasts c-src synergistically increases the oncogenic activity of the EGF receptor suggesting that these two kinases may be involved in the progression of malignant phenotype. (Brunton *et al.*, 1997).

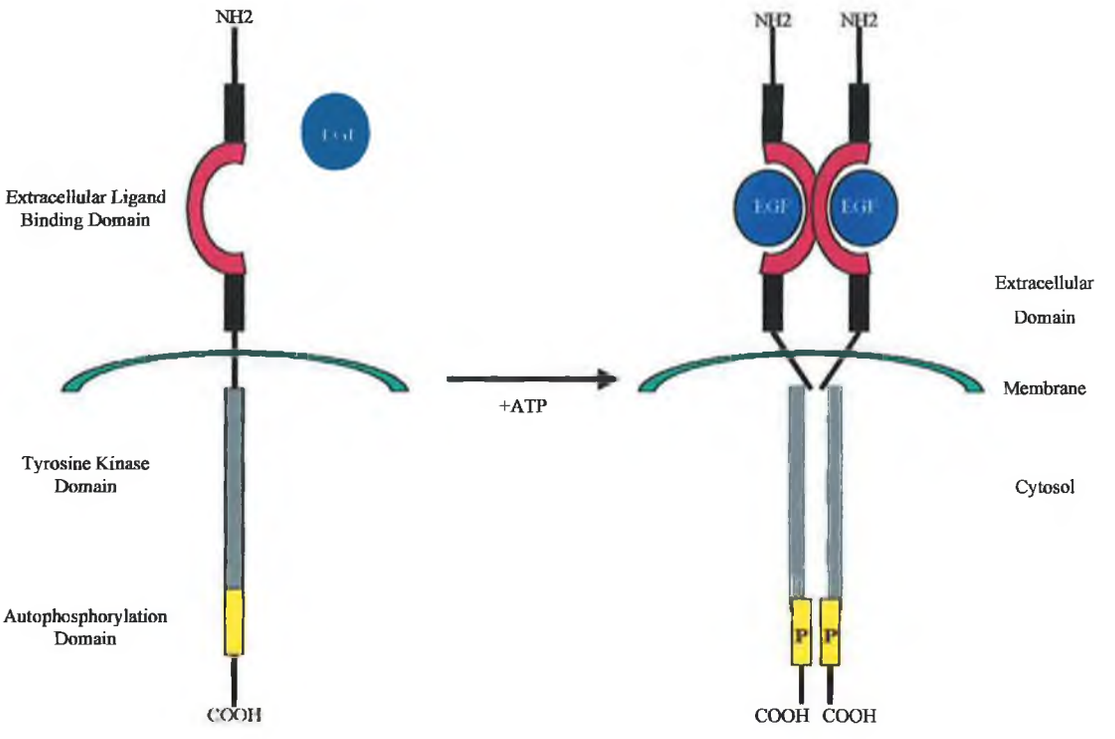

Figure 3.5 The epidermal growth factor receptor. EGF binds to the receptor causing a conformational change which allows for receptor dimerisation and autophosphorylation of the tyrosine kinase domain

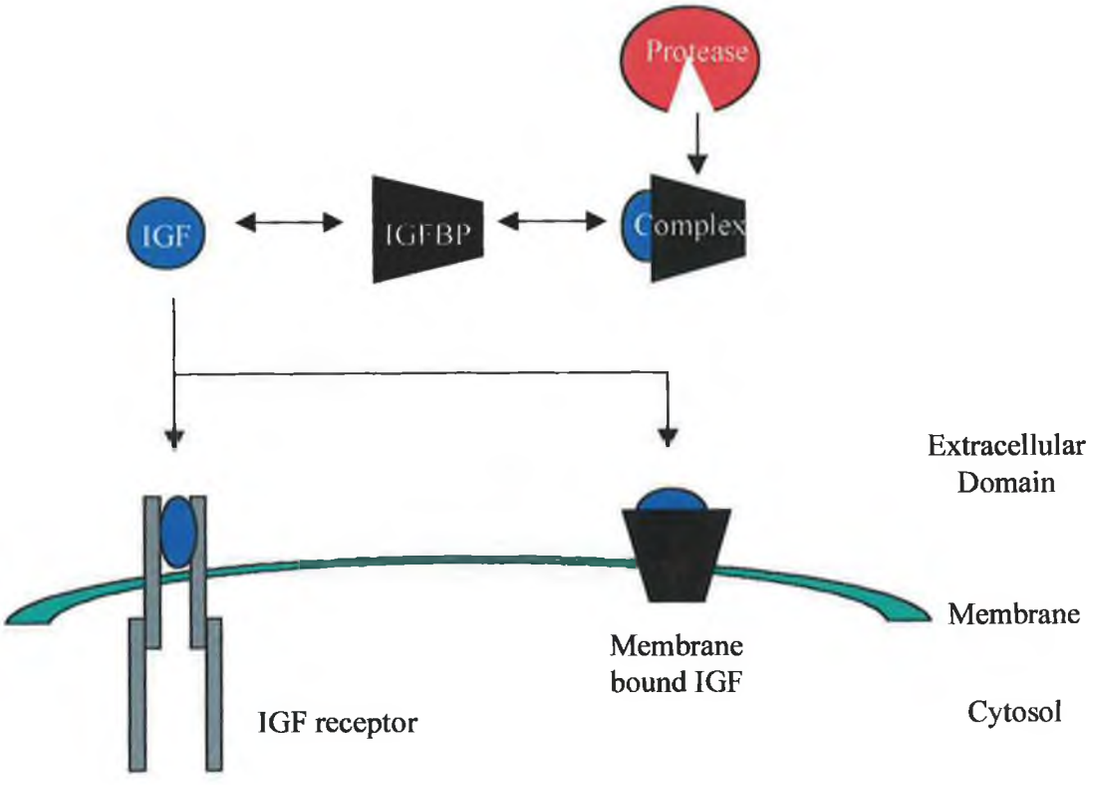

Figure 3.6 The IGF system. Several proteases including MMPs have been implicated in the production of biologically active IGF

3.4.2 IGF-I and IGF-II

Insulin like growth factors (IGF) are so called because of their ability to bind to the insulin receptor (also known as the IGF-I receptor). The IGF family is comprised of IGF-I and IGF-II, both of which have an active molecular weight of 7.5 kDa. IGFs are multifunctional polypeptides that are capable of regulating growth, differentiation and survival in several cell and tissue types. The IGF system includes ligands (IGF-I and IGF-II), receptors (IGF-IR and IGF-IIR), IGF binding proteins (IGFBP-1-7) and IGFBP proteases (see figure 3.6).

The IGF system regulates pre- and postnatal growth, the establishment and maintenance of differentiated cell function via paracrine, autocrine and endocrine signalling. IGFs are potent mitogenic and anti-apoptotic molecules involved in the regulation of cell proliferation in renewing epithelial cell populations of organs including

breast, prostate, colon and lung. Unlike many other regulatory peptides, IGFs have characteristics of both classic 'endocrine' hormones and also characteristics of tissue growth factors (Jones and Clemmons, 1995 and Rajaram *et al.*, 1997). Circulating levels of IGFs are subject to complex physiological regulation and the vast majority of IGFs are bound to high affinity binding proteins which need to be proteolytically cleaved in order to produce the active IGF cytokine. It has been shown that >90% of circulating IGF is bound to a high molecular weight complex comprised of IGFBP-3 and a separate protein known as the acid labile sub-unit. However, IGF bioactivity in tissues is not merely a function of circulating levels, as local expression of genes encoding IGFs, IGFBPs, and proteases that digest IGFBPs are also important (Ramajaran *et al.*, 1997). The classic source of IGF-I is the liver, where the peptide is produced in response to growth hormone. It is considered a primary growth regulator in pre and post-natal life. In contrast to IGF-I, the physiological function of IGF-II is mainly involved in stimulating undifferentiated cells and plays a key role in foetus development (Pollak, 2000). The IGF-I receptor is structurally very similar to insulin and is comprised of 2α subunits and 2β subunits which are linked by disulphide bonds (see figure 3.7). The receptor is comprised of an extracellular, transmembrane and intracellular domain. When a ligand binds, the IGF-I receptor undergoes rapid auto-phosphorylation and the intracellular domain once phosphorylated is a potent tyrosine kinase and mediates its effects through the phosphorylation of secondary signaling molecules. The IGF-II receptor is similar to the receptor for mannose-6-phosphate, is structurally very different to that of IGF-I, and is capable of binding both IGF-I and IGF-II. As IGFs are potent mitogenic agents it is quite possible that aberrant gene expression may result in an increased rate of epithelial cell division which in conjunction with other oncogene expression may induce malignant transformation.

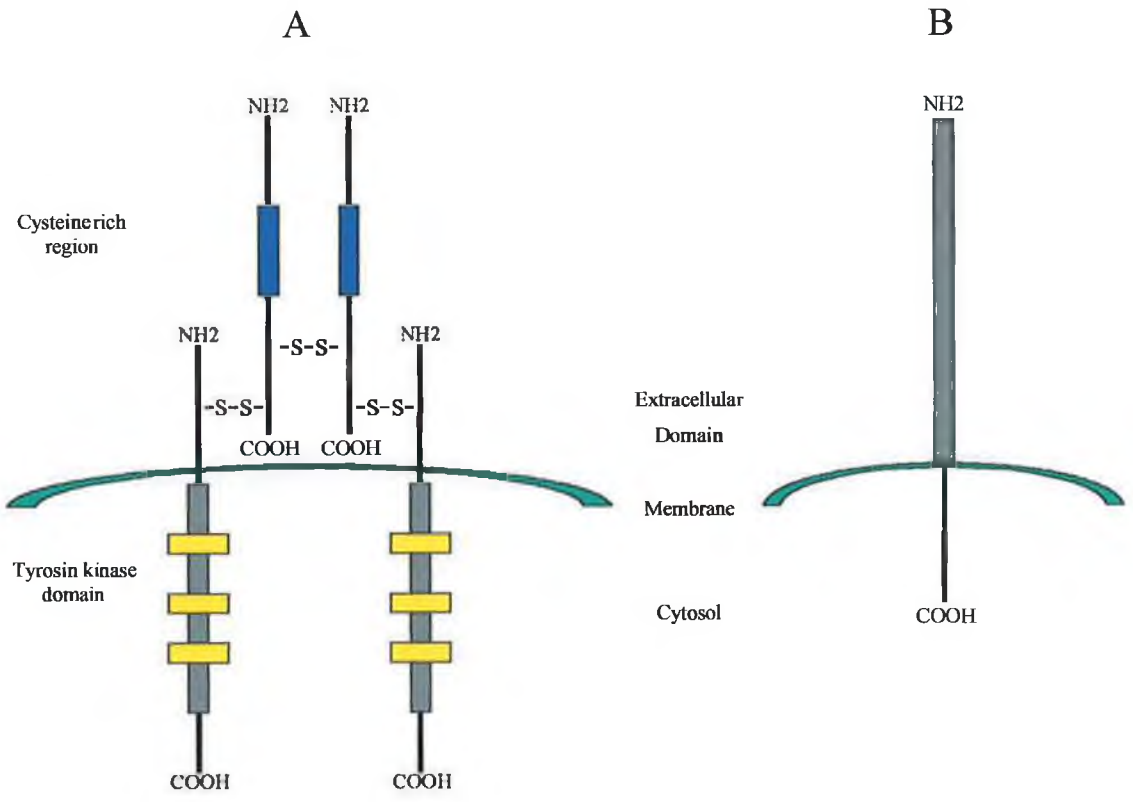

Figure 3.7 A The IGF-I receptor is comprised of 2α and 2β subunits. Once the IGF ligand has bound the intracellular tyrosine kinase domain is activated. **B** The IGF-II receptor which is similar in structure to the mannose-6- phosphate receptor

3.4.3 Interleukin-6

Interleukin-6 (IL-6) is a member of the family of cytokines collectively termed "the interleukin-6 type cytokines". IL-6 is variably glycosylated and the 22-27 kDa secreted glycoprotein serves as a prototype for a family of molecules that includes leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and IL-11. Although all these molecules possess a similar helical structure, their association is due to their functional redundancy and receptor interactions (Hibi *et al.*, 1996). IL-6 achieves its effects through the ligand-specific IL-6 receptor (IL-6R). Unlike most other cytokine receptors, the IL-6R is active in both membrane bound and soluble forms. Among its many functions, IL-6 plays an active role in immunology, bone metabolism, reproduction, arthritis, neoplasia, and aging. IL-6 expression is regulated by a variety of factors, including steroidal hormones, at both the transcriptional and post-transcriptional levels (Keller *et al.*, 1996).

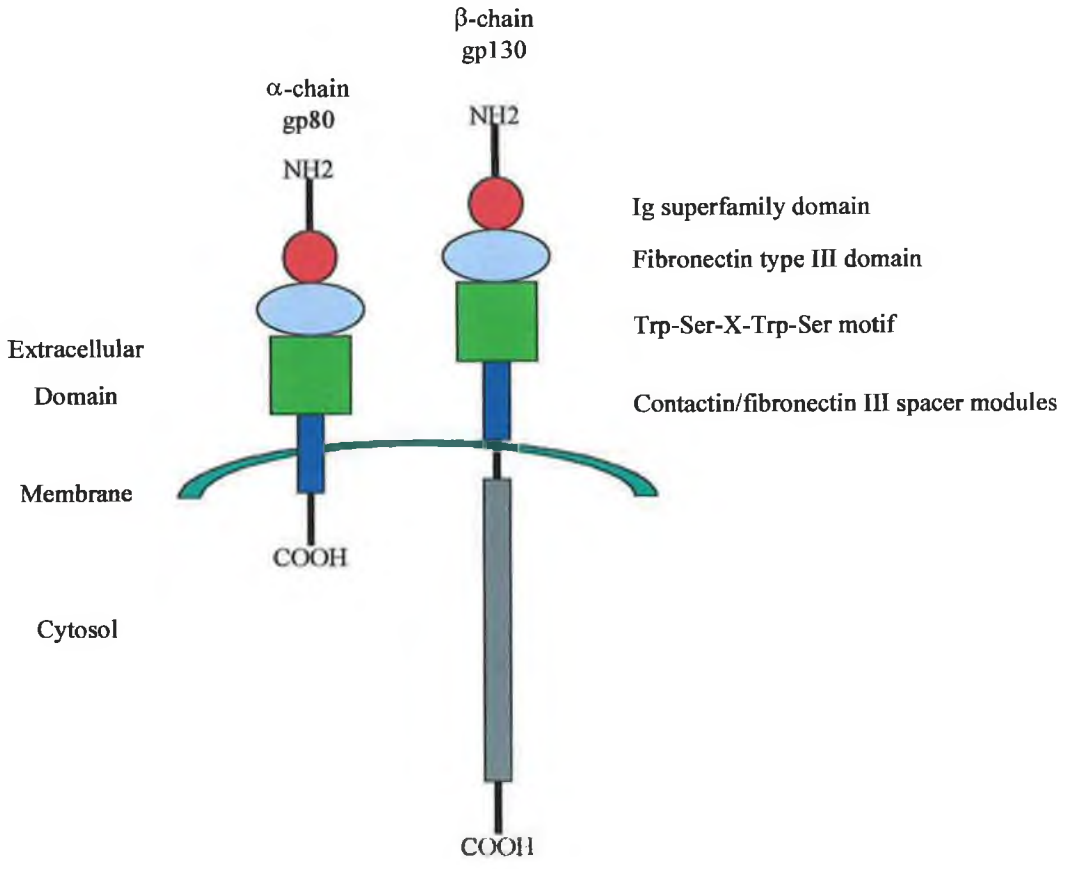

Figure 3.8 The IL-6 receptor is comprised of 2 chains, the α/gp80 chain and the β/gp130 chain.

The human IL-6 receptor, also known as gp80 and IL-6R, is an 80 kDa protein consisting of 467 amino acids (Keller *et al.*, 1996). The receptor is comprised of an extracellular region, a transmembrane region and an intracellular region. The IL-6R is not capable of mediating signal transduction directly and must initially bind to gp-80 which gives rise to a low affinity receptor complex. Homodimerisation of gp-130 is subsequently required for IL-6 signal transduction (Murakami *et al.*, 1993) (see figure 3.8). Although it was originally considered that one unit of IL-6 and the IL-6R bound to a gp-130 homodimer, the stoichiometry and number of this reaction appears to involve a hexameric complex consisting of two molecules each of IL-6, IL-6R gp-80, and gp-130 (Hammacher *et al.*, 1994). This complex forms a high affinity binding site for IL-6, as opposed to the low affinity binding observed with IL-6 and IL-6R gp-80 in the absence of gp-130.

The IL-6 receptor is expressed in a variety of cells and the number of receptors normally range between 100-2000 sites per cell. However, in myeloma cell lines up to 29,000 sites per cell have been observed (Snyers *et al.*, 1989). The receptor has also been shown to be upregulated in several cancers including bladder and prostate cancer (Meyers *et al.*, 1991 and Siegsmund *et al.*, 1994). IL-6 is a pleiotropic cytokine and is involved in initiating several cellular processes including proliferation and differentiation. IL-6 is also involved in the inflammatory response cascade and is often found at high levels in the stroma surrounding advancing tumours. Many of the promoters of MMP genes contain transcription factor binding elements to which transcription factors triggered by IL-6 such as NF-IL6 (nuclear factor interleukin-6), can bind and these will be discussed later. Therefore, a possible link with cancer proliferation and overexpression of MMPs, including matrilysin, due to increased expression of IL-6R or IL-6 by the tumour cell may exist.

3.4.4 Basic fibroblast growth factor (bFGF)

Fibroblast growth factors are a family of secreted peptide ligands containing at least 15 members. Signalling by the FGFs and their receptors, which are a family of 4 transmembrane protein tyrosine kinases in mammals, is critical for diverse aspects of embryonic, foetal and post natal development of a variety of tissues (see figure 3.9) (Matsui *et al.*, 1999). Acidic and basic fibroblast growth factors (aFGF and bFGF) were among the first members of the FGF family to be isolated. bFGF plays a role in autocrine and paracrine regulation of cell proliferation, migration, angiogenesis and vascular development (Olson *et al.*, 2000 and Sezer *et al.*, 2001). bFGF is secreted as an 18 kDa protein and mediates it's effects through binding to the FGF receptor-2 (FGFR-2). Various forms of bFGF also exist in that the gene which encodes for the bFGF protein has several transcription initiation start sites. bFGF has also been shown to be essential in the formation of monolayers of cells and has been found to be present as an insoluble proteoglycan incorporated into the ECM which indicates its role as a local regulator of cell migration, proliferation and regeneration during wound healing (Aktas and Kayton, 2001).

Angiogenesis is an essential requirement for tumour progression and because bFGF is involved in the angiogenic process its overexpression by developing tumours results in blood capilliary formation which in turn feeds the tumour with oxygen and other essential requirements. bFGF has been shown in various studies to play an important role in colon cancer invasion whereby the invasion of colon cancer cells using an *in vitro* invasion assay was promoted by the addition of exogenous bFGF (Galzie *et al.*, 1997). Reverse experiments which focussed on the inhibition of endogenous expression using an antisense strategy resulted in colon cancer invasion inhibition (Netzer *et al.*, 1999). As MMPs are involved in colon cancer invasion and metastasis and angiogenesis it is possible that bFGF may have a role in the direct or indirect regulation of MMPs including matrilysin.

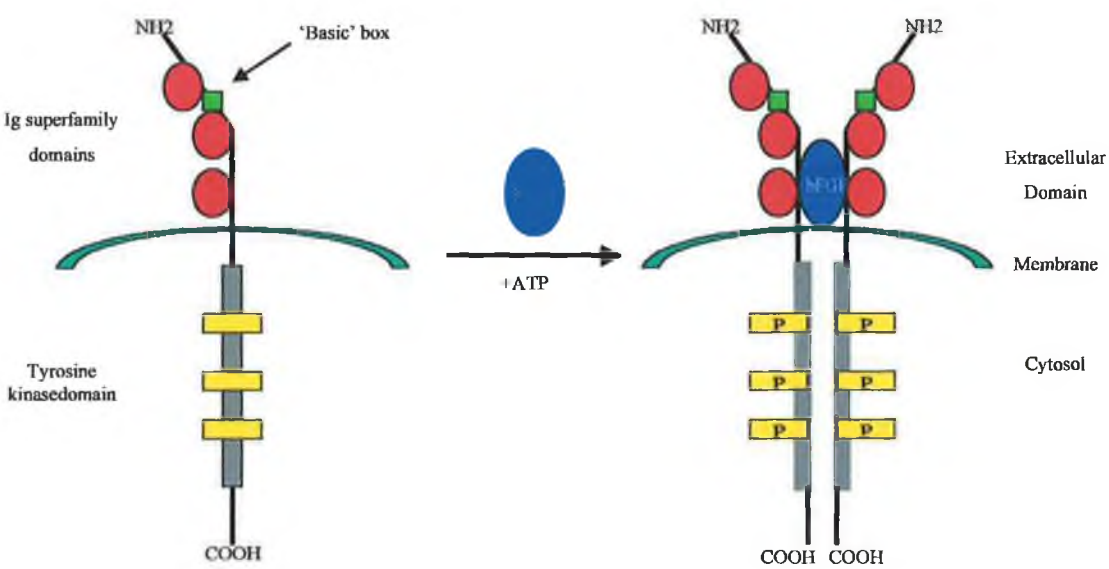

Figure 3.9 A representative diagram of the FGF receptor family. The FGF receptor is a potent tyrosine kinase and similar to other receptor tyrosine kinases it is activated after ligand binding.

3.5 Cytokine signalling networks

In order for cytokines to mediate their effects in a particular cell they must first bind to their receptor. The receptor mediates its action through the phosphorylation of molecules which interact with the tyrosine kinase portion of the receptor. These molecules subsequently phosphorylate other molecules and so on until the required response is carried out. The main targets of the signalling pathways are transcription

factors which bind to the promoter region of target genes. There are many important pathways involved in cell signalling and many of these 'cross talk' in that the pathways overlap each other or use the same signalling molecules. Signalling pathways can also overlap to enhance or silence a particular signal. The regulation of these pathways is also important and aberrant expression of mutated receptors which continually 'fire' signals at the nucleus wreak havoc on the cell and normally result in excessive proliferation which when accompanied by other mutations will result in cancer development.

There are many signalling pathways used to effect cellular processes, for example, the mitogen activated protein kinase (MAPK) pathway the phosphotidyl inositol-3 pathway (PI-3) and the janus kinase/ signal transducer and activator of transcription (JAK/STAT) pathway. Several of the receptors outlined above also engage these pathways in order to mediate their desired effect. One of the first molecules engaged by many of the receptor tyrosine kinases (RTKs) is the Ras protein.

The MAPK pathways can effect a wide variety of processes but they are primarily involved in stimulating cell proliferation. Common transcription factors stimulated by the MAPK effector molecules include those of the AP-1 and Ets family. As discussed earlier many of the MMP gene promoters including that of matrilysin contain DNA binding regions for these transcription factors and therefore stimulation of the MAPK pathway may lead to increased proliferation as well as increased MMP gene transcription.

3.5.1 Ras and the MAPK pathways

Ras proteins activate multiple signalling pathways (see figure 3.10). Ras binds to at least three types of effector protein : i) kinases of the raf family including MAPK and ERK, ii) phosphoinositide-3 kinases (PI-3) and iii) RalGDS (guanine nucleotide disassociation stimulator) proteins (McCormick, 1999). The molecular mechanisms that lead to the activation of Ras effectors are complicated and unclear and the downstream consequences of activation are more complicated still. Both the Raf and PI-3 pathways have been shown to be involved in proliferation, migration and angiogenesis via their signalling networks and their complex interaction or 'cross talk' with other networks. The

RalGDS is also involved in gene transcription and its mechanisms of signalling are now being elucidated.

Several receptor tyrosine kinase (RTKs) including the EGFr and the FGFr use Ras in order to signal to the cell nucleus. Tyrosine phosphorylation of specific residues on the internal domain or the receptor creates binding sites for several molecules including Src- homology 2 (SH2) and phosphotyrosine binding (PTB) domain containing proteins. Some of these proteins are enzymes that are tyrosine phosphorylated and thereby activated, such as Src, phospholipase Cγ (PLCγ) and PI-3 kinase, whereas Shc, Grb2, Grb7 and Nck are adaptor molecules that link RTKs to downstream signalling pathways (Hackel *et al.*, 1999).

Growth factor receptor bound protein 2 (Grb2) is a small adaptor protein that is essential in the Ras signalling pathway (Lowenstein *et al.*, 1992). It is comprised of one SH2 domain of approximately 100 amino acids surrounded by two SH3 domains, each containing about 60 amino acids. After receptor dimerisation and tyrosine trans-phosphorylation Grb2 docks with the internal domain of the receptor and upon phosphorylation Grb2 then recruits SOS proteins. SOS accelerates the change of GDP to GTP in Ras, which leads to Ras activation. Once loaded with GTP, Ras is then capable of interacting with effectors such as Raf which ultimately leads to the activation of the mitogen activated protein kinase (MAPK) pathway.

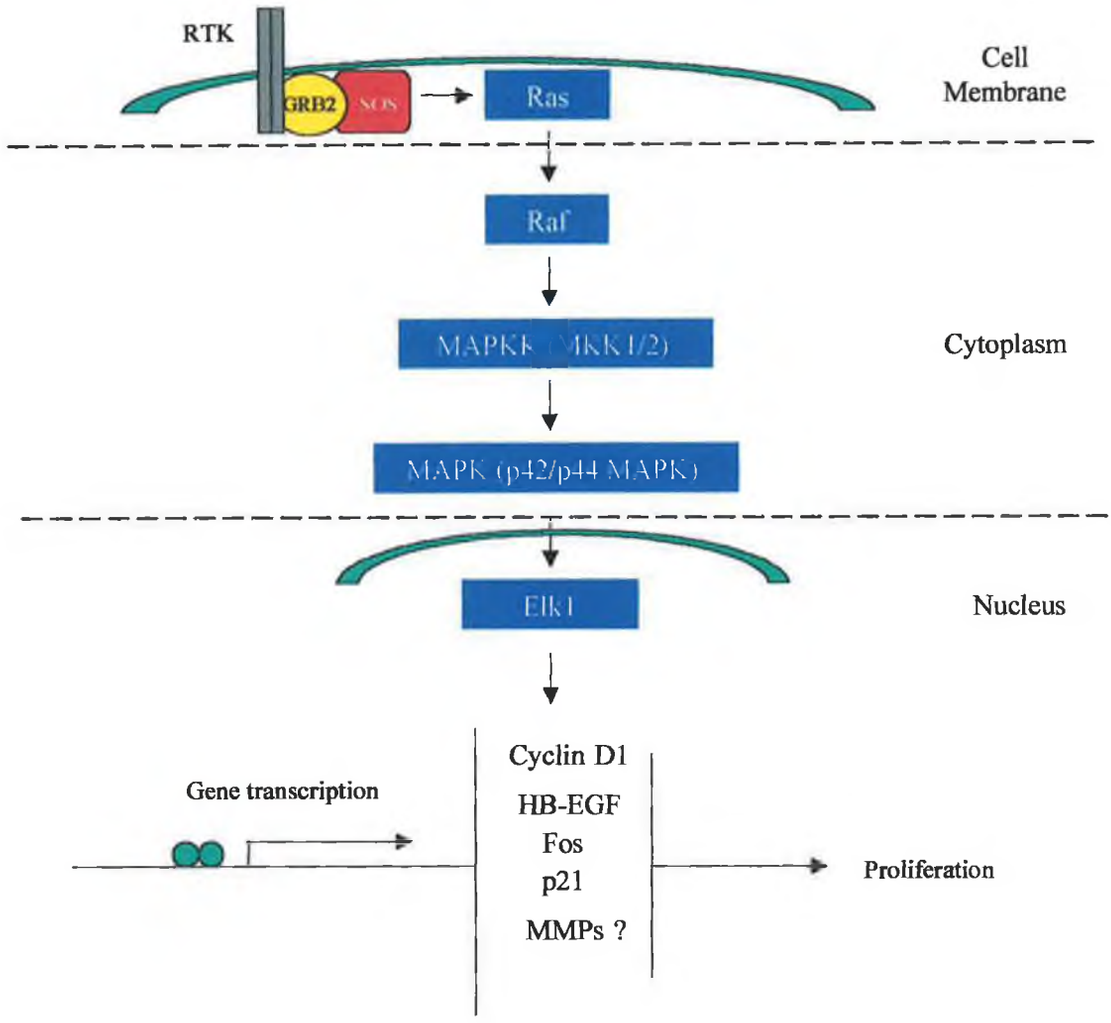

Figure 3.10 Once activated, membrane bound Ras proteins activate a number of signalling pathways including the mitogen activated protein kinase pathway. An increase in Fos and other AP-1 transcription factor family members may also lead to an increase in MMP activity.

The chain of events leading to MAPK activation take place within the cell cytoplasm. Having been recruited to the cell membrane and activated by Ras, Raf proceeds to phosphorlyate, hence activate, serine residues on the mitogen activated protein kinases 1 and 2 (MKK1, MKK2). The discovery of the first mammalian MAPK pathway was based on the identification of p42 and p44 MAPKs (Ray and Sturgill, 1987). p42/p44 MAPKs, once activated, have been shown to be translocated to the nucleus upon growth factor stimulation (Lenormand *et al.*, 1993). This nuclear translocation allows the MAPKs to contact and phosphorylate key substrates. *In vitro* studies show that p42/44 MAPKs have the ability to phosphorylate a number of substrates including EGF-r,

phospholipase A2, protein kinase p90RSK, Myc and Elk1, however, *in vivo* the p42/p44 MAPKs have been best characterised by their interaction with the nuclear transcription factor Elk1. After phosphorylation by MAPK, Elk1 is responsible for the induction of *c-fos* gene expression. The p42/p44 MAPK pathway can, therefore, integrate the signals coming from various growth factor receptors and by translocation to the nucleus and activation of transcription factors, relay this information to modulate gene expression accordingly.

3.5.2 Ras and the PI-3 kinase pathway

Oncogenic Ras suppresses apoptosis through the PI-3 kinase pathway, partly through the activation of protein kinase B (PKB) (see figure 3.11). The precise role of PKB in suppressing apoptosis is complex: PKB phosphorlyates BAD, a member of the Bcl-2 family that promotes cell death, and caspase 9, a protease that degrades cellular substrates during the process of cell death. However, PKB also regulates transcription through the phosphorylation of Forkhead proteins, which might themselves regulate other effectors of apoptosis, suggesting a much more complex and indirect route to cell survival (McCormick, 1997). Ras activated PI-3 kinase also plays a role in promoting cell growth through the inhibition of glycogen synthase kinase 3 (GSK3) by PKB. Activated GSK3 is capable of degrading, via ubiquitination, the cyclin D1 proteins which drive the cell cycle from the G1 to the S phase. Inhibition of GSK3 therefore promotes cell division through the accumulation of cyclin D1 (Garbay *et al.*, 2000).

The PI-3 kinase pathway may also stimulate the production of MMPs indirectly via the increase of transcription factors associated with cell proliferation. As discussed earlier matrilysin has been shown to cleave FasL into its soluble form and is involved in FasL/Fas mediated apoptosis. Since the PI-3 kinase pathway can increase levels of Fas L it may also be appropriate that the pathway lead to an increase in molecules which are capable of converting FasL to its soluble form such as matrilysin.

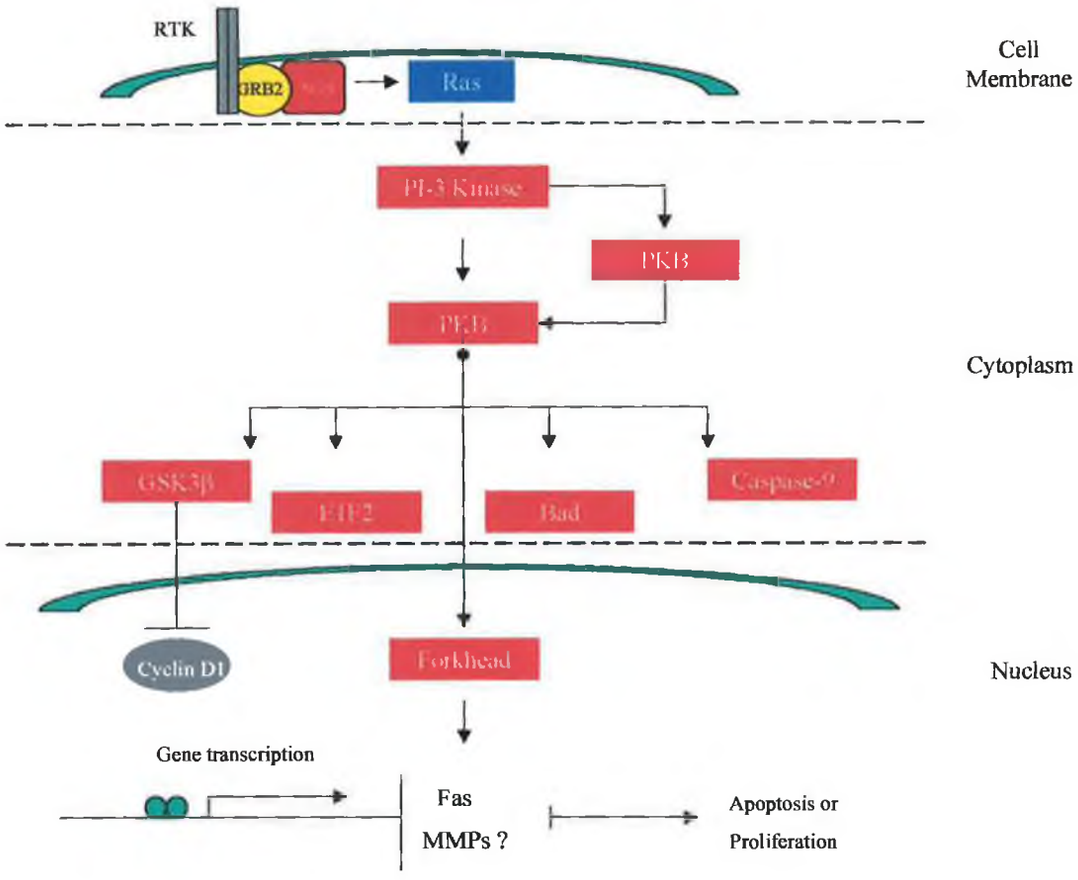

Figure 3.11 Ras is also capable of activating the PI-3 kinase pathway which has numerous effects.

3.5.3 The JAK/STAT pathway

The STATs (signal transducers of and activators of transcription) constitute a multiple member family of signal transduction proteins that are activated in the cytoplasm by the binding of extracellular polypeptides to the transmembrane receptors which then regulate the transcription of immediate response genes (Horvath and Darnell, 1997), (see figure 3.12). The Janus kinase-signal transducer and activator of transcription factor (JAK-STAT) signalling pathways were originally identified in the study of interferon signalling and are now believed to be the important pathways in signal transduction for many cytokines including IL-6. These pathways have been implicated in a variety of cellular functions in the haematopoietic, immunologic, neuronal and hepatic systems (Chen *et al.*, 1999). It is well established that binding of IL-6 to the IL-6 receptor-α chain induces homodimerisation of the signal transducing β chain, gp130, which is followed by

the activation of the receptor associated tyrosine kinases JAK1, JAK2 and Tyk2. A process assisted by ROS (reactive oxygen species). This receptor tyrosine kinase complex interacts with and activates the Src homology 2 (SH2) containing cytoplasmic STAT3 transcription factor, which then translocates to the nucleus to activate the transcription of many target genes including JunB, CCAAT enhancer binding protein (C/EBP) and $p21^{WAF1/Cip1}$ (Chen *et al*, 1999). Although it is clear that JAKs serve to phosphorylate STATs in receptors that lack intrinsic tyrosine kinase domains, it has also been shown that RTKs have the ability to activate STATs without recruiting JAKs (Horvath and Darnell, 1997).

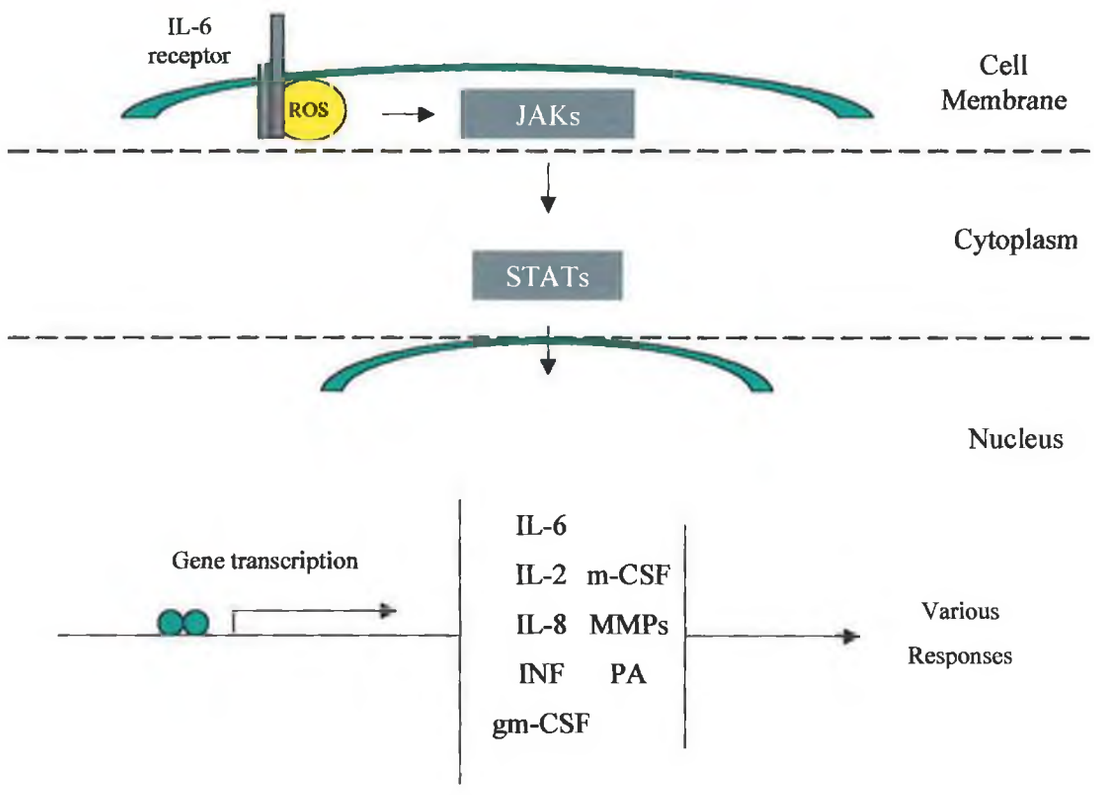

Figure 3.12 Activation of the JAK/STAT pathway leads to the activation of several transcription factors and the production of various gene products including IL-6 and MMPs

3.6 Summary

Matrilysin is the smallest MMP family member, has one of the largest substrate specificities and has been shown to play a role in many normal and disease processes. Much of the research into matrilysin thus far, has been associated with its role in cancer invasion and metastasis, in particular, its involvement in the spread of colon and breast tumours. More recently, matrilysin has been shown to play an important role in several aspects of colon and breast tumour progression with respect to initial tumour formation, growth and angiogenesis. Matrilysin is also unique in that it is the only MMP member expressed exclusively by the epithelial cells of advancing colon tumours. These characteristics of matrilysin have combined to make it an exciting therapeutic target. While much is known about the matrilysin protein and its substrates, relatively little is known about how matrilysin gene expression is regulated. Cells in order to function properly need to 'sense' their external environment and this is achieved through signalling molecules such as cytokines and signalling pathways which carry these signals to the nucleus. Overexpression of cytokines or their receptors in combination with other mutations leads to the uncontrolled proliferation of cancer cells. As matrilysin is involved in the growth and invasion and metastasis of colon cancer it is quite possible that aberrant cytokine signalling may subsequently lead to matrilysin overexpression in tumours however the mechanisms of this interaction between cytokine stimulation and matrilysin expression has yet to be elucidated. In order to determine if a link exists we have taken a panel of colon cell lines and investigated whether matrilysin expression at the transcriptional level and protein level can be affected by cytokines which employ several different signalling pathways in mediating their effects.

3.7 Results

A panel of colon cancer cell lines (HCT116, SW480, SW620, LoVo, WiDr and HCA7) were investigated with respect to their expression of matrilysin and if this expression could be regulated via cytokine treatment using IGF-I, IGF-II, EGF, IL-6 and bFGF. Properties of the cell lines used and a summary of the oncogenes expressed in each are illustrated in table 2.1 in section 2.2.1. The HCA7, LoVo and SW480 cell lines all represent early to late adenocarcinomas while HCT116, SW620 and WiDR represent colon carcinomas at later stages of development. The SW480 and SW620 cell line have both been isolated from the same patient and are a genetically matched pair. SW480 was established from a primary adenocarcinoma in the colon which was classified as Duke's B while the SW620 cell line was derived from a lymph node metastasis of the same tumour from which SW480 was derived. Interestingly, SW480 has been shown not to express matrilysin while SW620 has previously been shown to express matrilysin at the basal level (Witty *et al.*, 1994).

3.7.1 RNA isolation from colon cancer cell lines

Prior to reverse transcription polymerase chain reaction analysis the total RNA isolated from each cell line was examined for degradation by running an aliqout of each isolated RNA sample (see figure 3.13). The presence of 28s, 16s and 6s RNA ribosomal sub-units is typical of undegraded RNA.

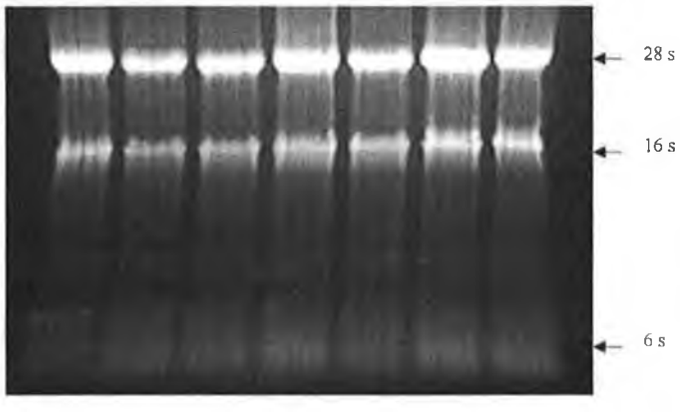

Fig 3.13 Total RNA was isolated and examined for the presence of 28s, 16s and 6s ribosomal sub-units. The samples shown here were isolated from the LoVo cell line (left to right : Control, IGF-I, IGF-II, EGF, IL-6, bFGF, TPA) and similar results were obtained for other cell lines.

Isolated RNA was quantified by measuring the absorbance of the samples at 260nm (1 OD unit equals 40 μg RNA). The quality of RNA was also determined by measuring the absorbance of the samples at 280nm. The absorbance $_{260/280}$ ratio is typically between 1.6 and 1.8 for total RNA free of DNA, protein and organic chemical contaminants.

3.7.2 RT-PCR/Western analysis of matrilysin expression in colon cancer cell lines

Once the total RNA had been isolated and purified, 1μg of sample was used in the RT-PCR procedure outlined in section 2.2.3. The RT-PCR procedure was semi-quantitative and β-actin, a constitutively expressed cytoskeletal structural protein, was used as an internal control. Due to the similarity in product size the β-actin PCR reaction was performed in a separate tube to that of the matrilysin PCR reaction. The levels of β-actin served to normalise the amount of matrilysin mRNA expression observed in control and cytokine treated cell lines. Figure 3.14 shows β-actin levels for control and treated samples for the LoVo cell lines. The figure illustrates that cytokine treatment of the LoVo cell line has no effect on β-actin levels. This result was consistent in all cell lines investigated and therefore the levels of β-actin in the LoVo cell line are illustrated as an example while the β-actin data for other cell lines are not shown.

Cytokines used in the study included IGF-I, IGF-II, EGF, bFGF. TPA was also used as a positive control as it has been previously shown to upregulate MMPs (Mackay *et al*, 1992). Figure 3.15 demonstrates RT-PCR analysis of matrilysin mRNA in the LoVo cell line in normal and cytokine treated samples. The lower panel in the figure shows the entire gel and illustrates that non-specific binding of the matrilysin primers did not occur and the expected product size of 341 bp was obtained. This was also observed in the other cell lines investigated. In order to clearly see increases/decreases in matrilysin mRNA levels, the section which contains the matrilysin PCR products has been enlarged. Subsequent RT-PCR and western blot analysis have also been presented in this enlarged manner.

Figure 3.14 RT-PCR analysis of β-actin mRNA expression in the LoVo cell line. The expected PCR product size for β-actin was 383 bp. MM; molecular marker, IGF-I; Insulin like growth factor I, IGF-II; Insulin like growth factor II, EGF; epidermal growth factor, IL-6; interleukin 6, bFGF; basic fibroblast growth factor, TPA; tetradecanoyl, phorbol acetate, E+T; cells treated with EGF and TPA, I+T; cells treated with IL-6 and TPA, B+T; cells treated with bFGF and TPA, E+B+I; cells treated with EGF, bFGF and IL-6, ALL: cells treated with EGF, IL-6, bFGF and TPA.

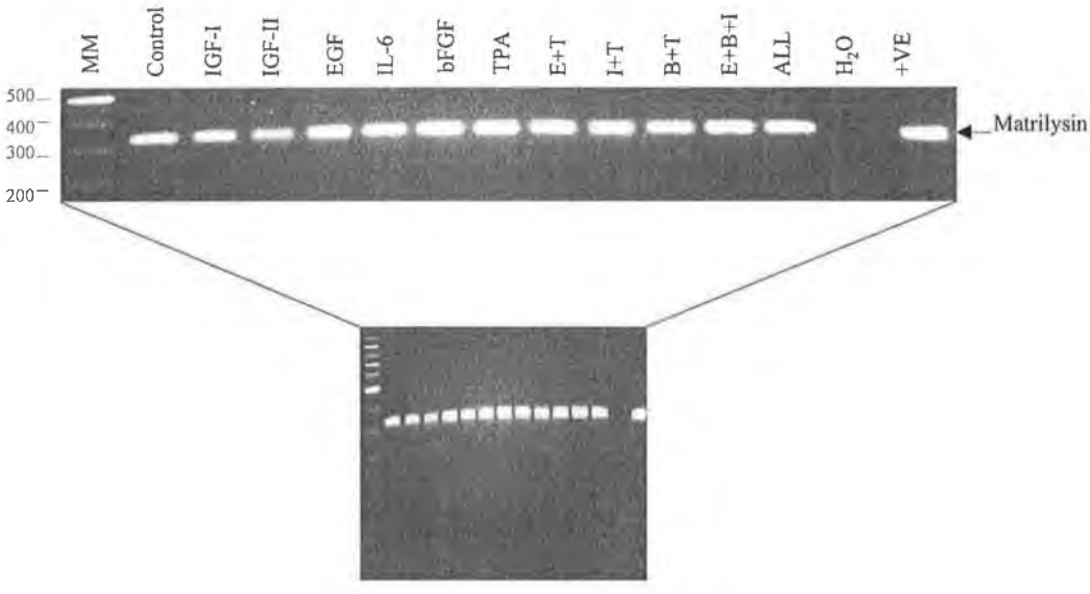

Fig 3.15 RT-PCR analysis of matrilysin expression in the LoVo cell line. Matrilysin PCR product size was 341 bp. Concentration of all cytokines and TPA were 50ng/ml. MM; molecular marker, IGF-I; Insulin like growth factor I, IGF-II; Insulin like growth factor II, EGF; epidermal growth factor, IL-6; interleukin 6, bFGF; basic fibroblast growth factor, TPA; tetradecanoyl, phorbol acetate, E+T; cells treated with EGF and TPA, I+T; cells treated with IL-6 and TPA, B+T; cells treated with bFGF and TPA, E+B+I; cells treated with EGF, bFGF and IL-6, ALL: cells treated with EGF, IL-6, bFGF and TPA, H_2O: sterile water used as a negative control in PCR, +ve; positive PCR control using matrilysin cDNA. Illustrated molecular marker units are in base pairs. The lower gel indicates that no non-specific binding occurred, an observation which was consistent in all cell lines examined.

The effects of cytokine treatments on matrilysin mRNA expression on each of the cell lines investigated are displayed in figures 3.17 to 3.21. In order to confirm that the cytokine effects on matrilysin expression at the mRNA level were resulting in higher levels of matrilysin protein, western blot analysis was also performed on the normal and cytokine treated cell lines (for methodology see section 2.2.6). Supernatants were measured for total protein content and equal amounts of total protein were loaded onto SDS-PAGE gels, ensuring that the protein levels detected are as a direct result of cytokine treatment. These results for each individual cell line are also shown in figure 3.17 to 3.21.

Matrilysin mRNA and protein was detected in all cell lines with the exception of SW480 (see figure 3.16 and data not shown).

Figure 3.16. Matrilysin was not detected at the mRNA level in the **SW480** using RT-PCR. Treatment with various cytokines and cytokine combinations failed to initiate the transcription of matrilysin. Western blot analysis also failed to detect the presence of matrilysin protein in SW480 samples (data not shown).

Experiments on both mRNA and protein expression were performed in triplicate. Densitometry was performed on the gels and blots and the average results with standard deviation from the mean (Microsoft Excel™) are detailed in figure 3.17-3.21.

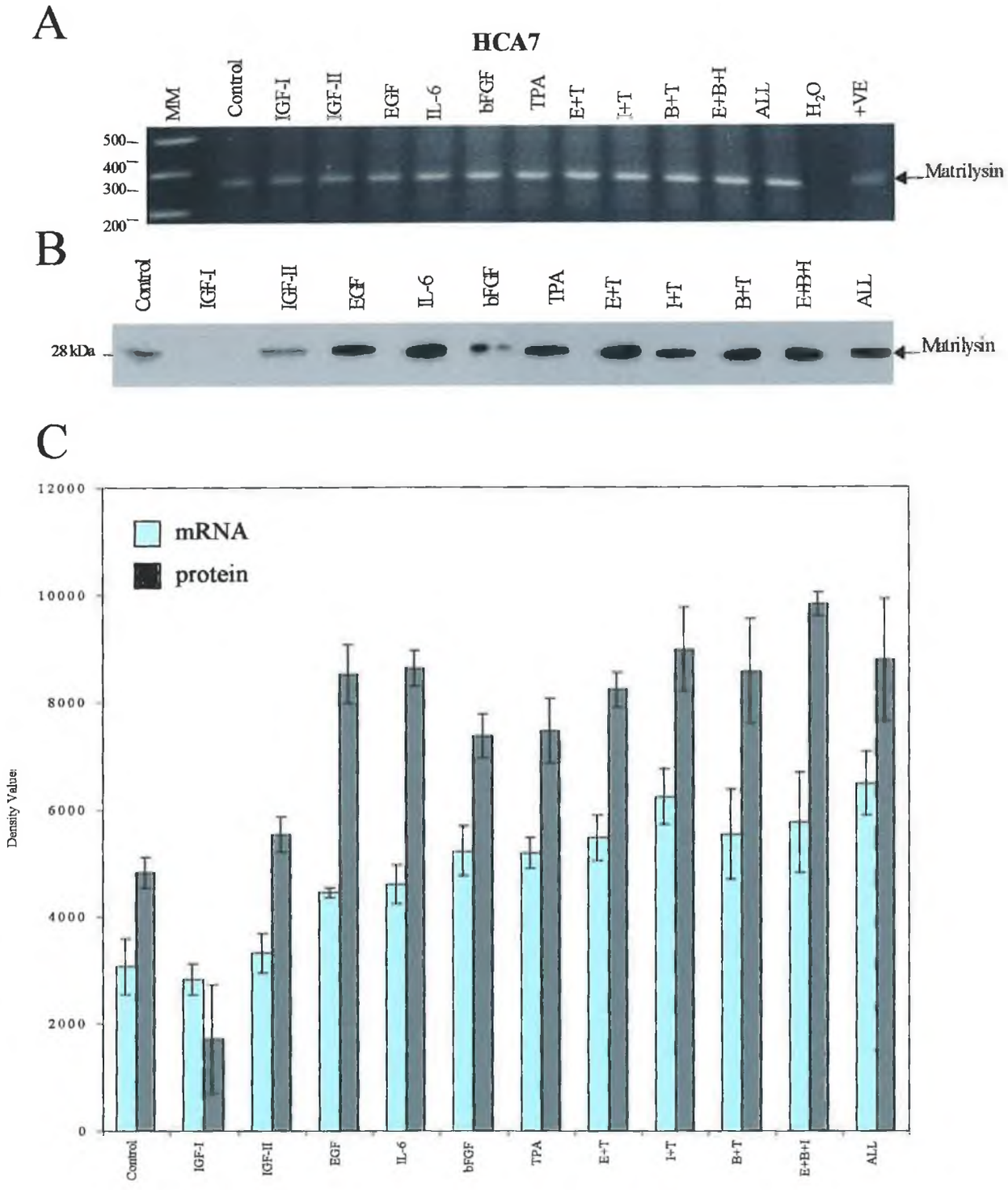

Figure 3.17 Analysis of matrilysin expression in the HCA7 cell line using **A**: RT-PCR analysis and **B**: western blot analysis. Figure legends have been previously described in figure 3.14. **C** Densitometric analysis of matrilysin mRNA and protein expression. Experiments were performed in triplicate and error bars represent standard deviation from the mean.

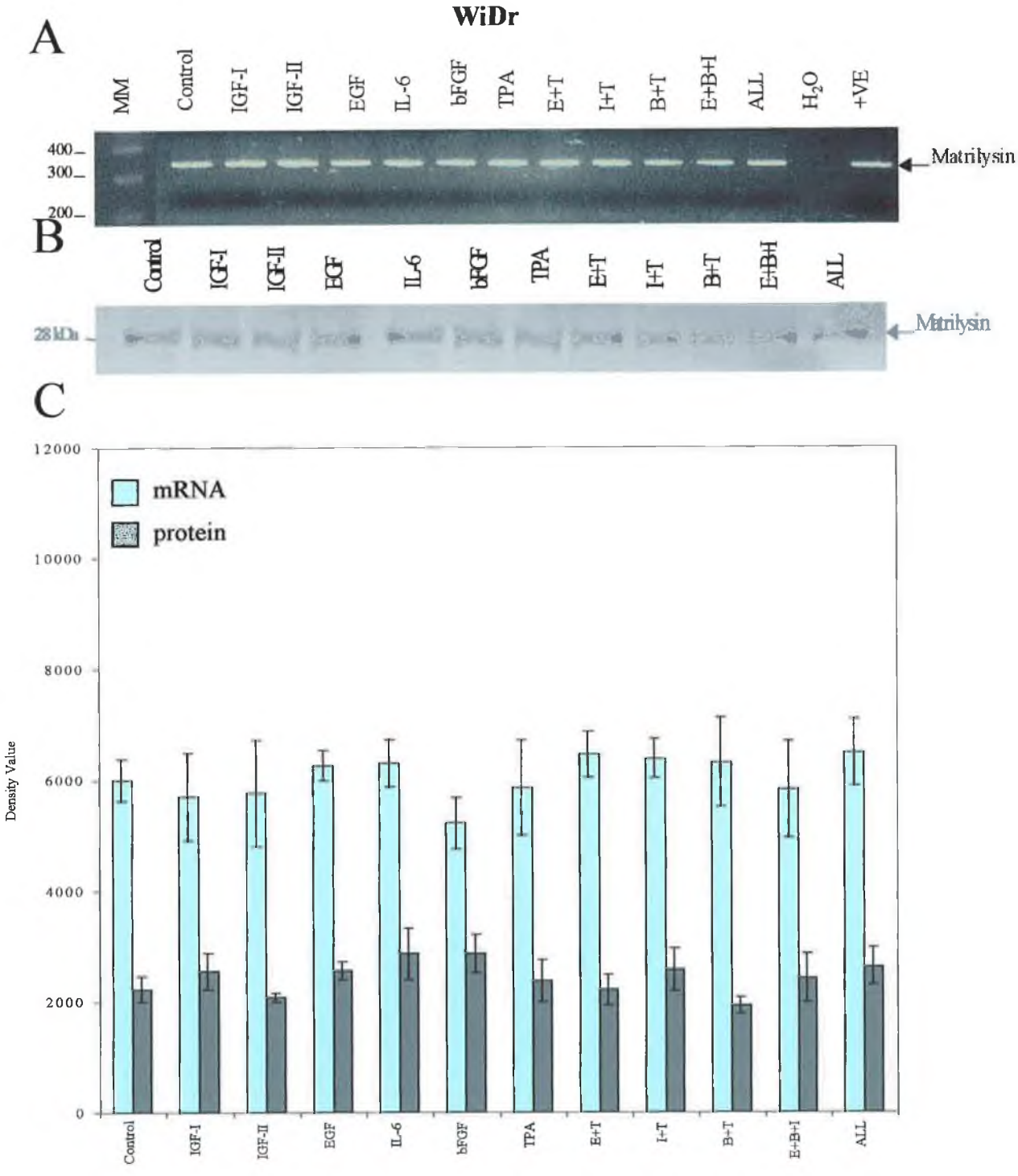

Figure 3.18 Analysis of matrilysin expression in the WiDr cell line using **A**: RT-PCR analysis and **B**: western blot analysis. Figure legends have been previously described in figure 3.14. **C** Densitometric analysis of matrilysin mRNA and protein expression. Experiments were performed in triplicate and error bars represent standard deviation from the mean.

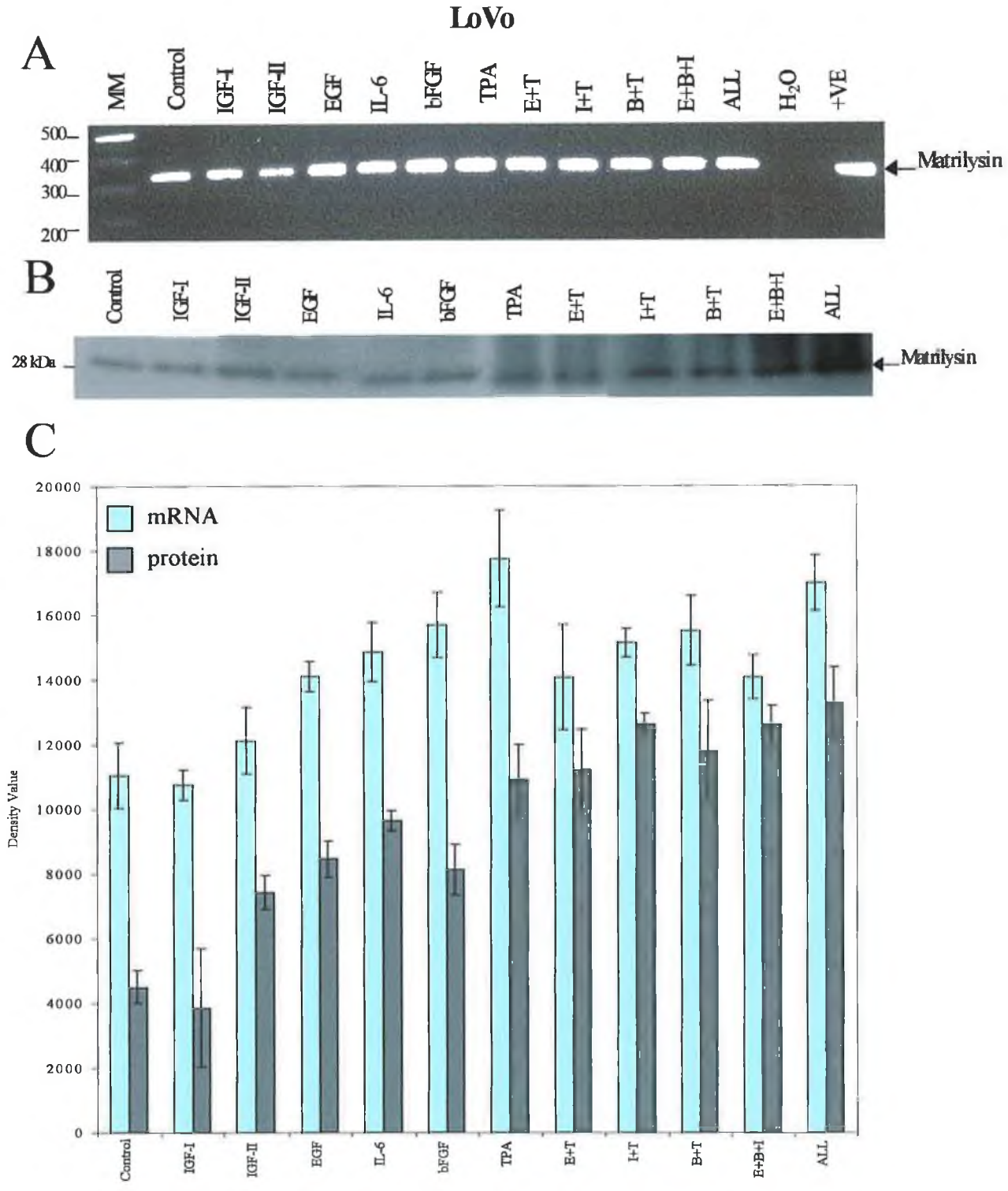

Figure 3.19 Analysis of matrilysin expression in the LoVo cell line using **A**: RT-PCR analysis and **B**: western blot analysis. Figure legends have been previously described in figure 3.14. **C** Densitometric analysis of matrilysin mRNA and protein expression. Experiments were performed in triplicate and error bars represent standard deviation from the mean.

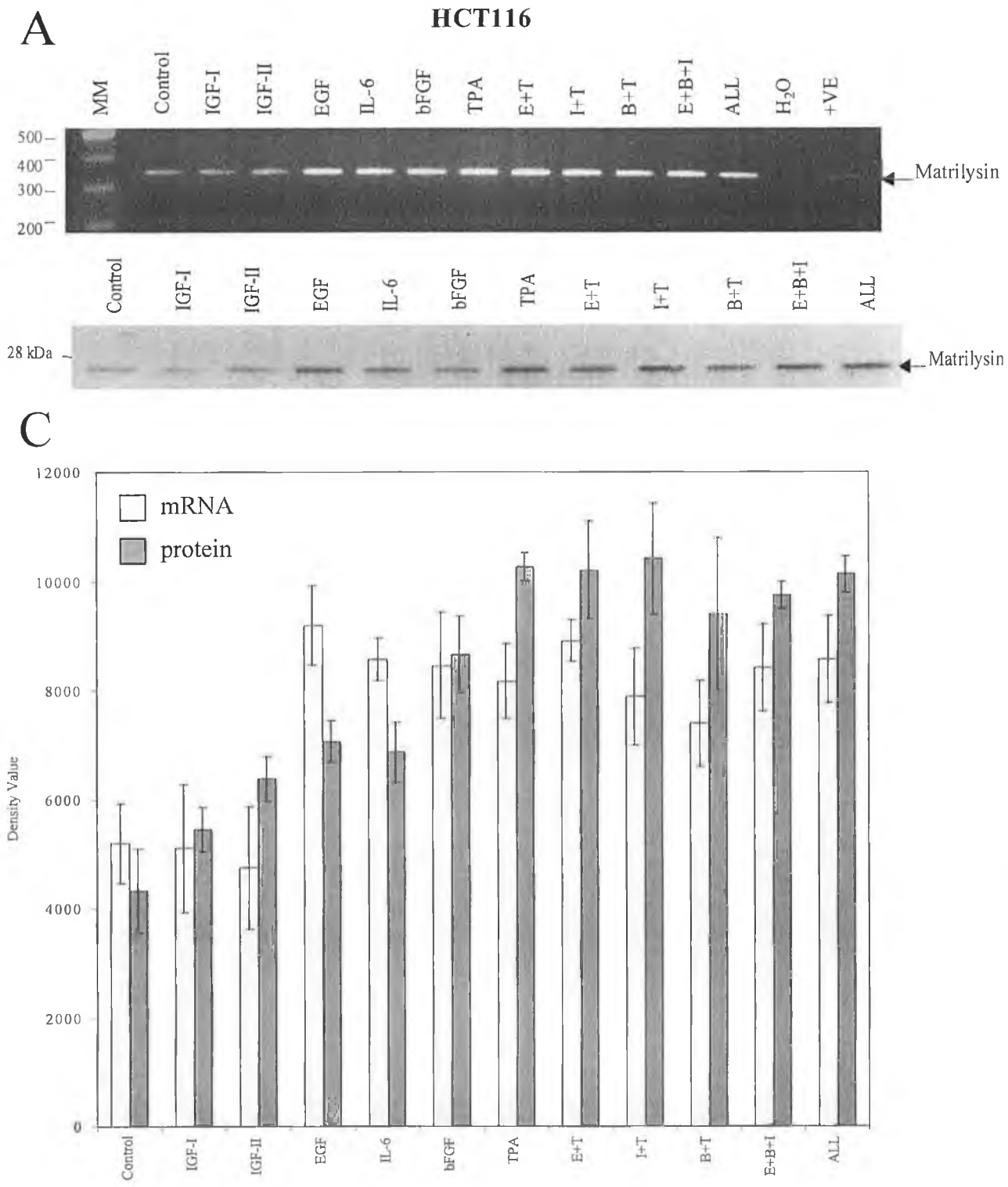

Figure 3.20 Analysis of matrilysin expression in the HCT116 cell line using **A**: RT-PCR analysis and **B**: western blot analysis. Figure legends have been previously described in figure 3.14. **C** Densitometric analysis of matrilysin mRNA and protein expression. Experiments were performed in triplicate and error bars represent standard deviation from the mean.

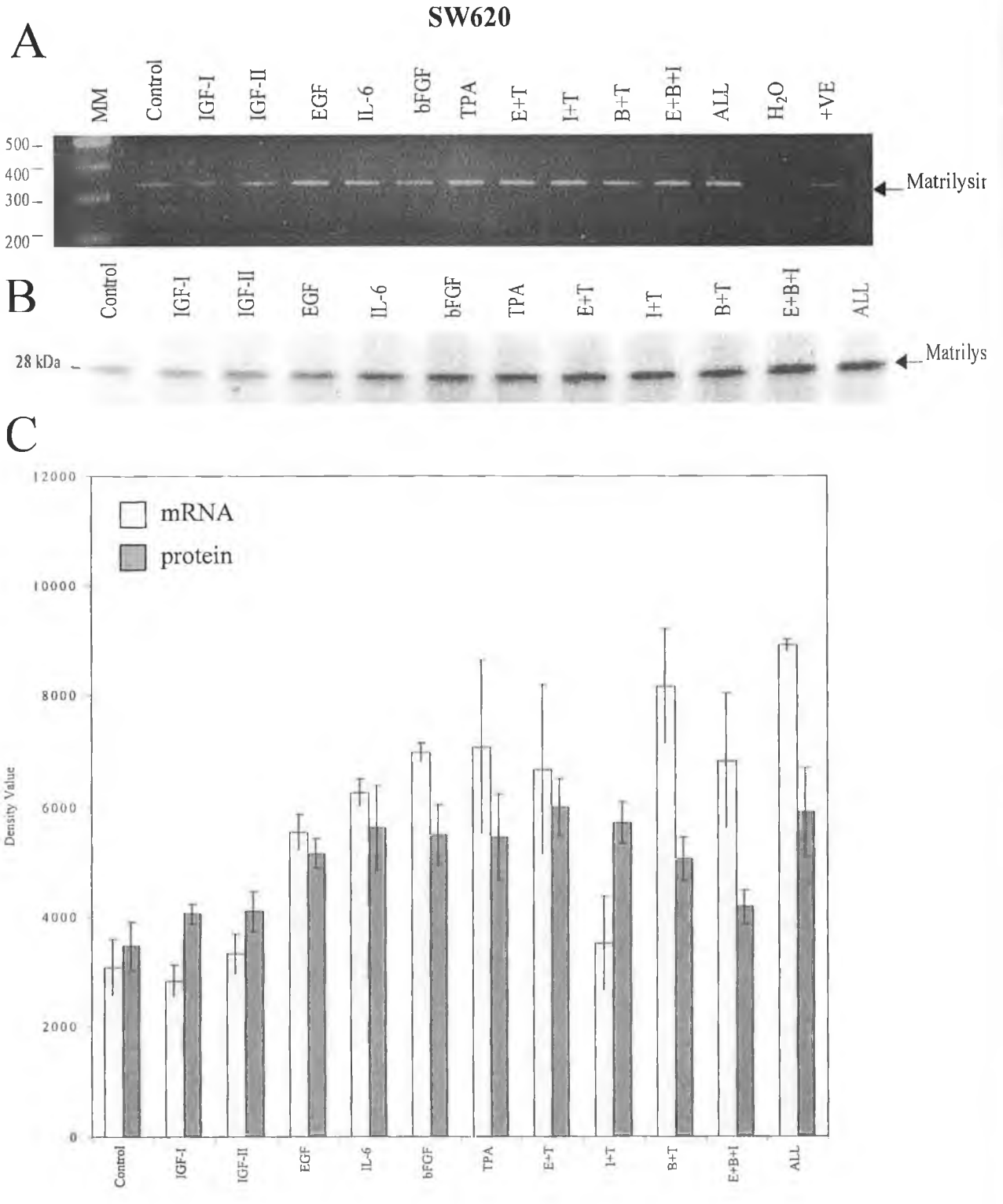

Figure 3.21 Analysis of matrilysin expression in the SW620 cell line using **A**: RT-PCR analysis and **B**: western blot analysis. Figure legends have been previously described in figure 3.14. **C** Densitometric analysis of matrilysin mRNA and protein expression. Experiments were performed in triplicate and error bars represent standard error from the mean.

Time and dosage experiments found that the optimal time point for endogenous matrilysin mRNA expression subsequent to cytokine treatment was 8 hours. Time points of 2, 4, 6 12 and 24 were also examined (data not shown). Cytokine concentrations of 10 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/ml were examined and 50 ng/ml of each cytokine was found to be the maximum concentration for induction (data not shown). For protein analysis, cell lines were incubated with 50 ng/ml of the appropriate cytokine for 48-72 hours.

Expression of matrilysin in the HCA7 cell line was observed at both the mRNA and protein levels (see figure 3.17). An increase in matrilysin mRNA expression was generally observed when the cells were treated with EGF, IL-6, bFGF or TPA. This increase was also observed at the protein level using western blot analysis. The effect of bFGF on matrilysin protein expression in the HCA7 cell line appears to be similar to that of the control level but subsequent studies showed that bFGF was capable of stimulating matrilysin protein expression in this cell line in a similar manner to that of EGF and IL-6 as shown by densitometric analysis. Interestingly, IGF-I appears to have little or no effect on matrilysin expression and seems to inhibit the production of matrilysin protein. However repeat experiments do not consistently show a significant inhibition in matrilysin protein expression in the HCA7 cell line upon treatment with IGF-I suggesting that IGF-I has little or no effect on the regulation of matrilysin expression. IGF-II also appears to have little or no effect on matrilysin regulation in the HCA7 cell line. *In vivo* there may be many signals from different signalling molecules converging on the matrilysin promoter in order to initiate its transcription. Therefore matrilysin expression may not necessarily be initiated/enhanced by a single cytokine but in response to a number of cytokines working in concert to promote matrilysin gene expression. We therefore investigated if treatment of the HCA7 cell line and other cell lines with a combination of cytokines would further enhance matrilysin gene transcription. Combination of cytokines (outlined in figure 3.17) showed an increase in matrilysin expression in the HCA7 cell line, in particular at the mRNA level, but these increases were not much greater than those observed when the cells were treated with individual cytokines such as EGF. Cells were also treated with IGF-I and IGF-II in combination with TPA but the observed effects were similar to individual TPA treatment which again suggested that neither IGF-I or IGF-II were involved in the regulation of matrilysin in the HCA7 cell line (data not shown).

The WiDr cell line was also found to express matrilysin at both the mRNA and protein level. However in contrast to HCA7, cytokine treatment appeared to have little or no effect on the expression of matrilysin (see figure 3.18) at either the mRNA or protein level.

The LoVo cell line expressed matrilysin at the mRNA and protein level and this expression could be modulated by a number of cytokines including EGF, IL-6 and bFGF. TPA stimulates the expression of matrilysin mRNA and protein expression to a greater extent than EGF, IL-6 and bFGF. Combination of cytokines with TPA or with each other do not appear to stimulate matrilysin mRNA expression any more than individual treatments but the effect on matrilysin protein appears to be more pronounced. IGF-I and IGF-II do not appear to have any effect on matrilysin mRNA or protein expression (see figure 3.19).

The HCT116 cell line expressed matrilysin at the mRNA and protein level and this expression could be modulated by EGF, IL-6 and bFGF. Treatment of the HCT116 cell line also increased matrilysin mRNA and protein. Combination treatments appear to have little effect on the level of matrilysin mRNA expression in comparison to individual cytokine treatment but the effect was more pronounced at the protein level in a similar manner to that observed in the LoVo cell line. This may suggest that the mRNA half life was increased by combination treatment of the cells with various cytokines (see figure 3.20).

The SW620 cell line exhibited a similar matrilysin expression pattern in comparison to the HCA7, LoVo and HCT116 cell line in that the normal levels of matrilysin expression can be enhanced by EGF, IL-6 and bFGF. IL-6 and bFGF in particular appeared to strongly enhance matrilysin at the protein level in the SW620 cell line. TPA treatment was again found to enhance matrilysin expression. Combination treatments slightly enhanced matrilysin mRNA and protein expression (see figure 3.21).

3.7.3 Analysis of matrilysin gene expression using a promoter-luciferase construct

In order to elucidate the mechanisms through which cytokines such as EGF, IL-6 and bFGF were stimulating matrilysin gene expression, two matrilysin promoter constructs were obtained. 2.3 kbp (pHMATPro-2.3-Luc) and 335 bp (pHMATPro-335-Luc) of the human matrilysin promoter were inserted into a luciferase reporter gene vector (see table 3.1). Thus increases in matrilysin promoter activity, as a response to cytokine treatment, could be determined by an increase in luciferase protein product. The regions of DNA within the matrilysin promoter responsible for transcription factor binding will be the subject of chapter 4. The promoter-reporter transient transfection technique is extremely sensitive and unlike RT-PCR is a quantitative method. The methodology involves transfection, cytokine treatment and luciferase detection which are outlined in the methods and materials section 2.2.8. On average transfection efficiencies of 10-20% were obtained (see figure 3.22)

Name	Size	Diagnostic Cut (Expected fragments)	Description
PHMATPro-2.3-Luc	7.9 kb	Eco RI (5.6 kb and 2.3 kb)	A 2.3 kb Mfe I fragment of the human matrilysin promoter was cloned into the EcoR1 site of pBluescript (Stratagene). The fragment was then cut using Hind III and BamHI and cloned into the Hind III and Bgl II sites in the pGL_2-Basic vector (Promega).
PHMATPro-335-Luc	5.9 kb	Eco RI (5.6 kb and 335 bp)	This plasmid was constructed in a similar manner to that of pHMATPro-2.3-Luc with the exception that only 335 bp of the human matrilysin promoter was inserted into the pGL_2-Basic vector
PRL-SV40	3.7 kb	BamHI and Pst I (1.4 kb and 2.3 kb)	Contains the SV40 early enhancer/promoter region which gives strong constitutive expression of Renilla luciferase.
PCH110	7.1 kb	Pvu II (3.5 kb, 2.6 kb, 700 bp and 360 bp)	Contains a functional Lac Z gene, the product of which is β-galactosidase. This plasmid can be transfected into cells and transfection efficiency can be determined by assaying for blue cells using an X-gal stain

Table 3.1 Characterisation of plasmids used in transfection studies (see appendix 1 for plasmid maps)

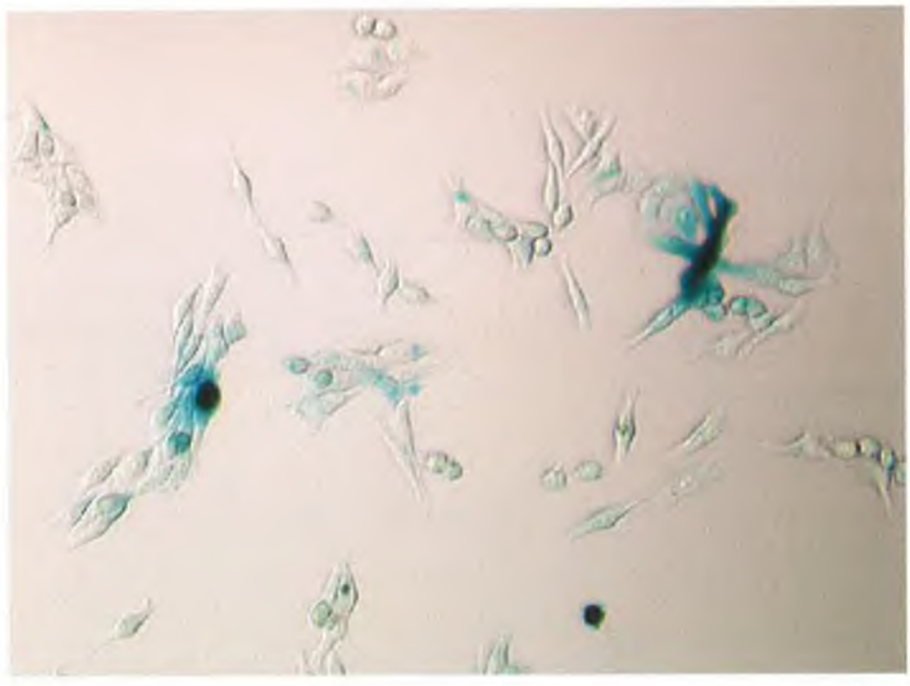

Figure 3.22 Transfection conditions for each cell line was optimised using β-galactosidase activity. Cells which were transfected attained the ability to process X-gal which results in a blue colour. **SW620** transfected cells can be clearly observed against non-transfected cells.

In order to normalise transfection efficiencies between each experiment an internal control plasmid (pRL-SV40 at a concentration of 10 ng) was also transfected with the matrilysin promoter construct. This ensured that increases in matrilysin promoter activity observed after cytokine treatment were in fact due to the cytokine and not due to variations in transfection efficiencies between each sample. The internal control in these experiments was *Renilla luciferase* and was a supplied component of the Promega Dual Luciferase™ kit. Stimulation of matrilysin promoter activity by several cytokines was observed in all cell lines using the promoter-reporter construct (see figure 3.23 to 3.28). This increase was even more pronounced when combination treatments were used, e.g. EGF with IL-6 and bFGF which may imply that a number of elements were required to bind to the matrilysin promoter before transcription could occur. Similar results were observed in the experiments using the 335 bp matrilysin promoter reporter construct which suggested that transcription factor binding sites important in effecting the transcription of matrilysin were located within the first 335 bp.

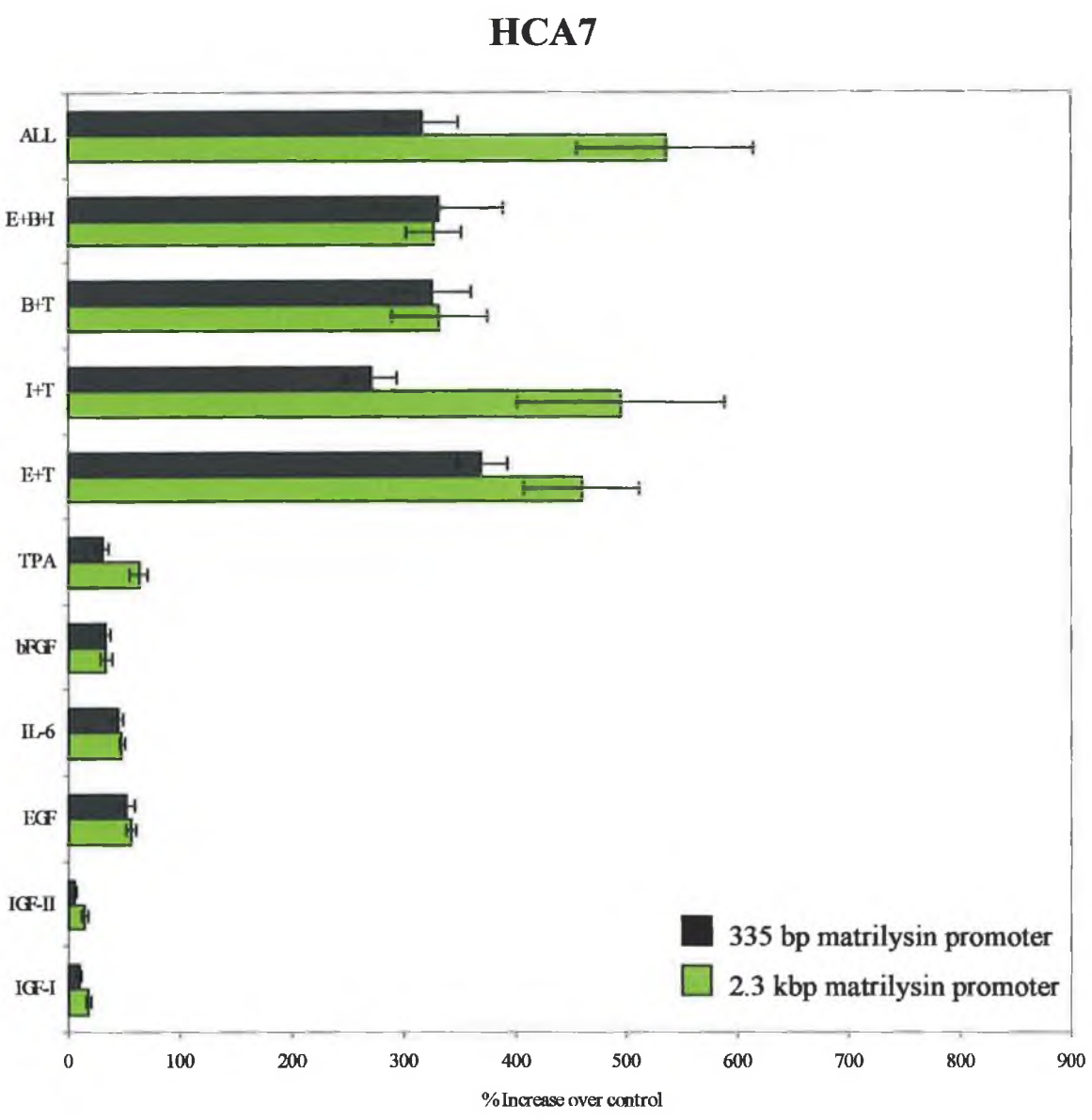

Figure 3.23 2.3 kbp and 335 bp promoter-luciferase analysis in the **HCA7** cell line. Treatment with EGF, IL-6, bFGF and combination treatments upregulate matrilysin expression. Legend definitions outlined in figure 3.14.

WiDr

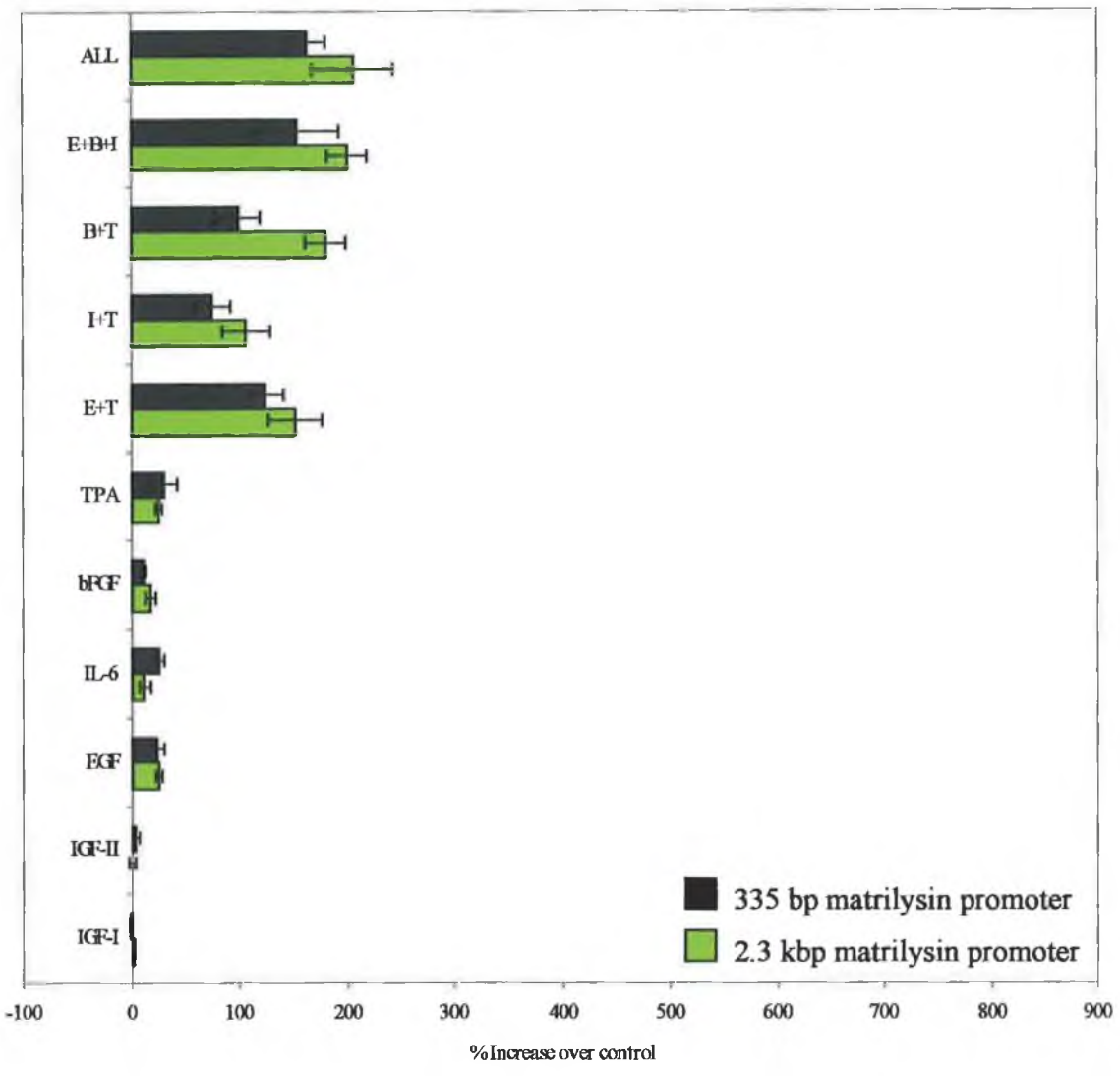

Figure 3.24 2.3 kbp and 335 bp promoter-luciferase analysis in the **WiDr** cell line. Treatment with EGF, IL-6, bFGF and combination treatments upregulate matrilysin expression. Legend definitions outlined in figure 3.14.

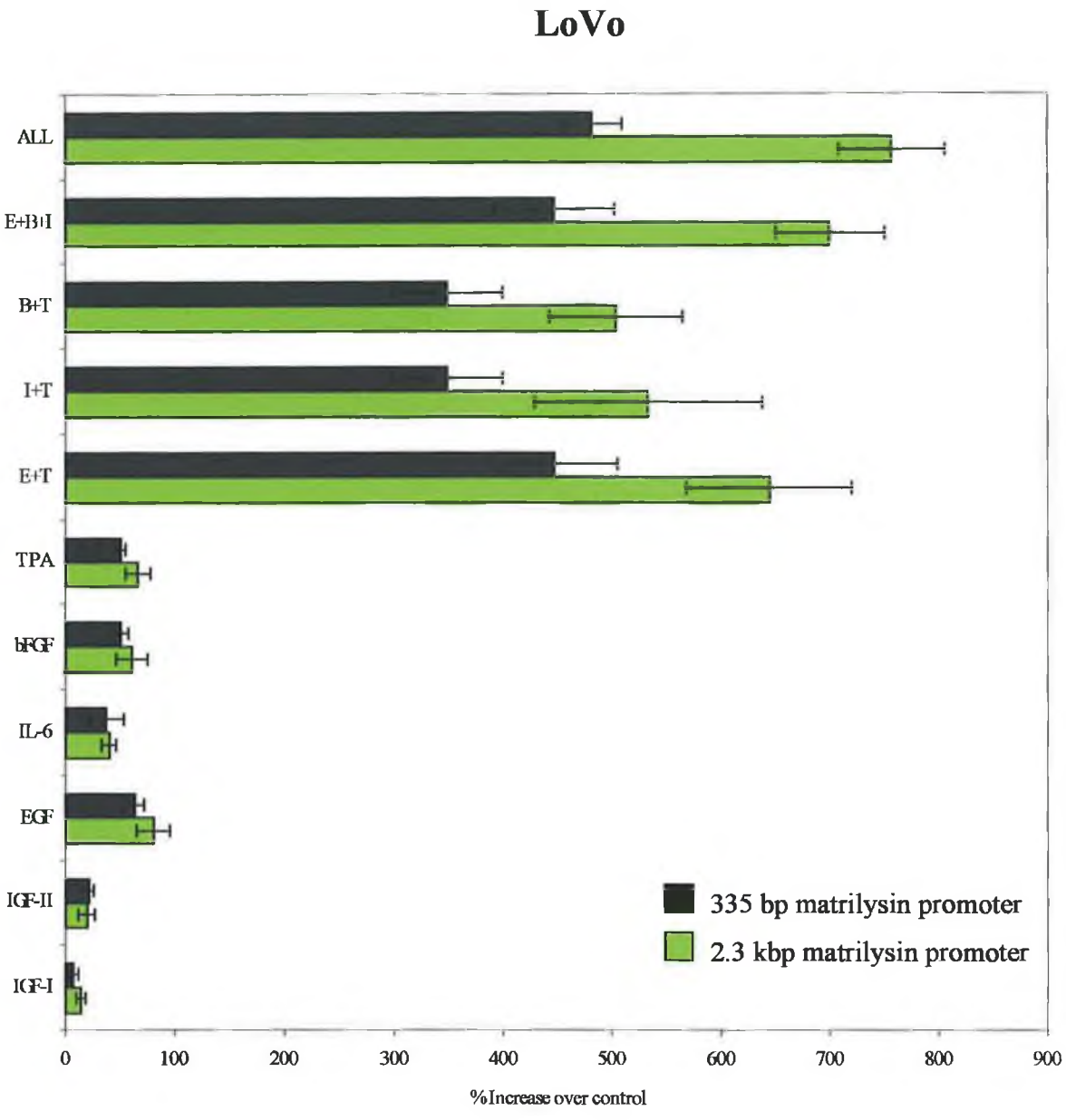

Figure 3.25 2.3 kbp and 335 bp promoter-luciferase analysis in the **LoVo** cell line. Treatment with EGF, IL-6, bFGF and combination treatments upregulate matrilysin expression. Legend definitions outlined in figure 3.14.

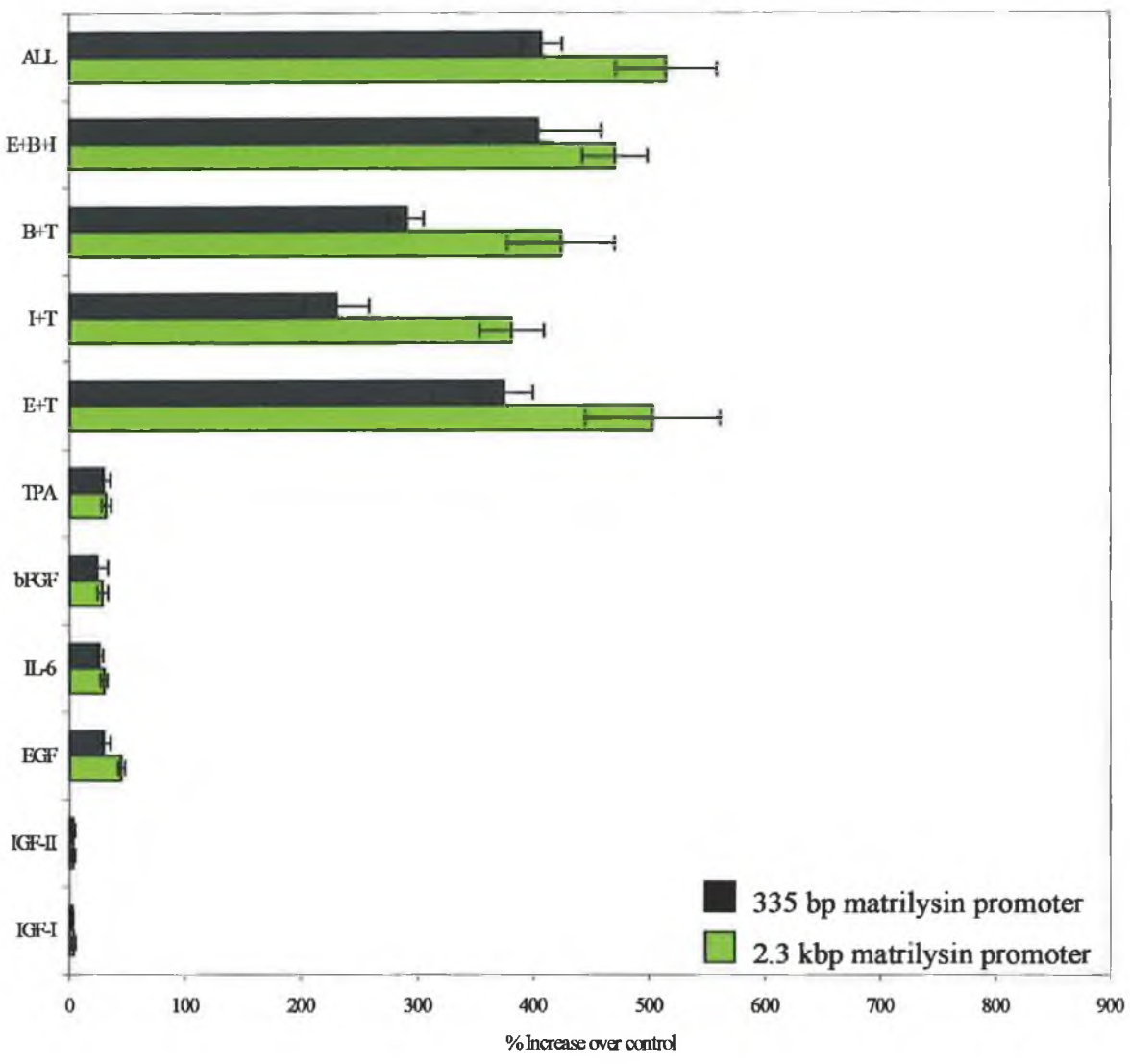

Figure 3.26 2.3 kbp and 335 bp promoter-luciferase analysis in the **HCT116** cell line. Treatment with EGF, IL-6, bFGF and combination treatments upregulate matrilysin expression. Legend definitions outlined in figure 3.14.

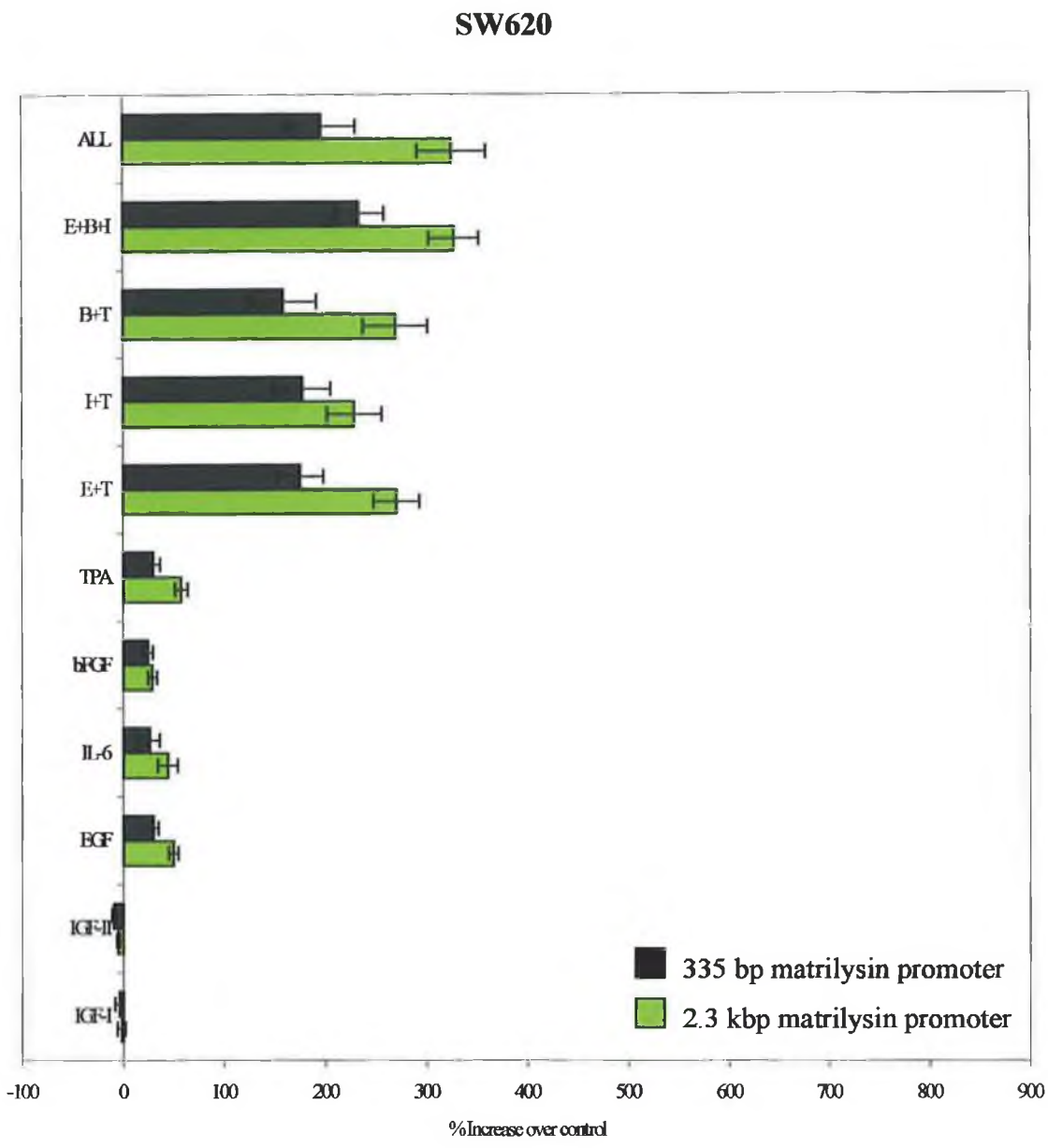

Figure 3.27 2.3 kbp and 335 bp promoter-luciferase analysis in the **SW620** cell line. Treatment with EGF, IL-6, bFGF and combination treatments upregulate matrilysin expression. Legend definitions outlined in figure 3.14.

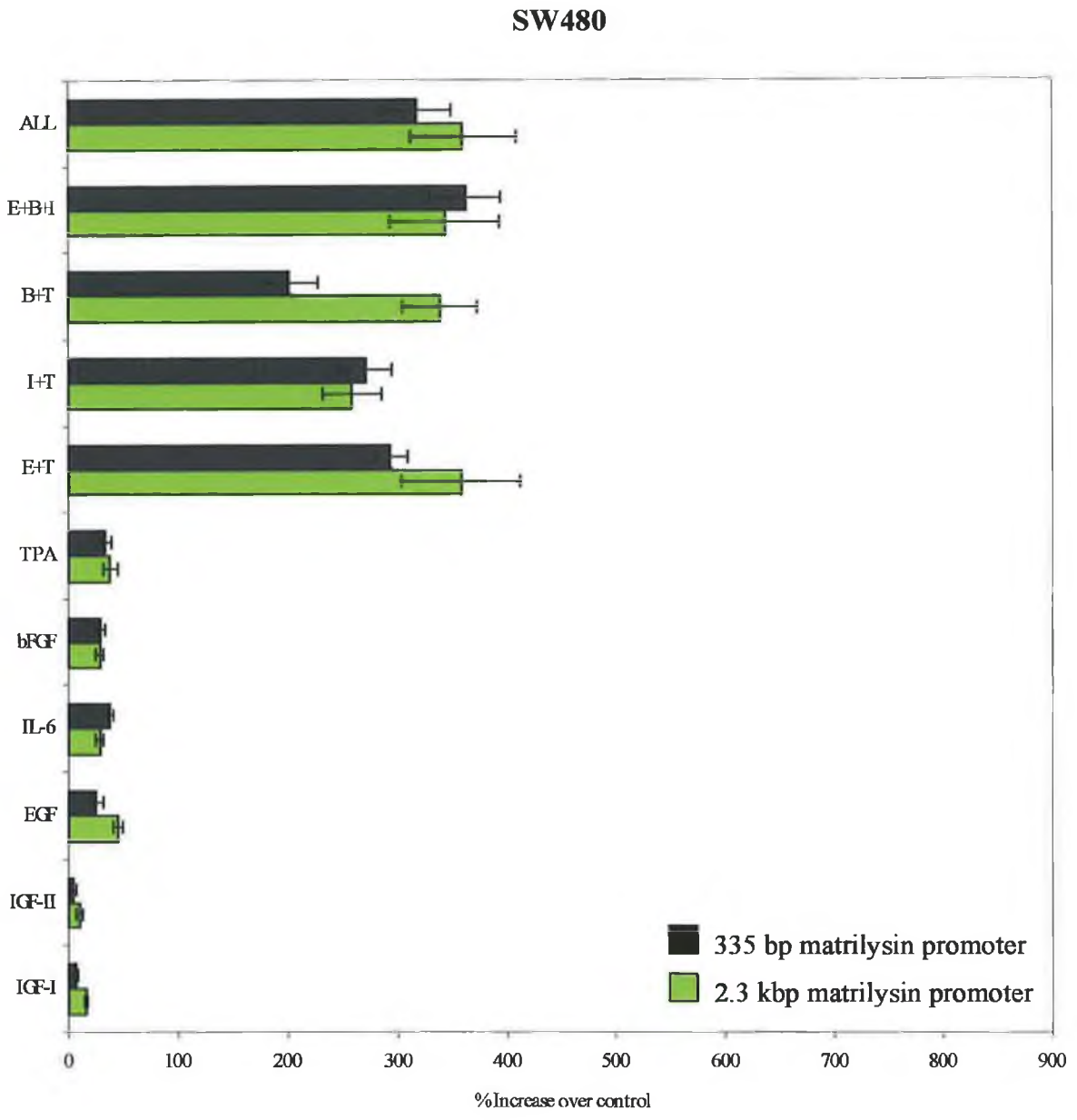

Figure 3.28 2.3 kbp and 335 bp promoter-luciferase analysis in the **SW480** cell line. Treatment with EGF, IL-6, bFGF and combination treatments upregulate matrilysin expression. Legend definitions outlined in figure 3.14.

For matrilysin promoter-reporter studies time points of 2, 4, 6, 8, 12 and 24 hours were examined and similarly to RT-PCR, 8 hours was found to be the optimal response time. In all the cell lines investigated there was a consistent increase in luciferase activity with both the 2.3 kb and 335 bp matrilysin promoter constructs when the cells were treated with EGF, IL-6, bFGF and TPA. Figure 3.23 demonstrates the effects of cytokine treatment on the 2.3 kb and 335 bp matrilysin promoter in the HCA7 cell line. Cytokines such as EGF, IL-6 and bFGF and the phorbol ester TPA all gave small but significant increases in promoter reporter activity. In general this was found to be the typical response in all the cell lines examined (see figure 3.24 to 28). IGF-I and IGF-II also showed stimulation of the matrilysin promoter in several of the cell lines treated, in particular for HCA7 and LoVo (see figure 3.23 and 3.25 respectively) while other cell lines such as WiDr and SW620 showed decreases or very slight increases in matrilysin promoter activity in comparison to control (see figure 3.24 and 3.28 respectively). The increases in matrilysin promoter activity that were observed after IGF treatment were low in comparison to those observed after treatment of the cells with EGF, IL-6, bFGF and TPA. These results confirmed the RT-PCR and western results whereby IGF-I and IGF-II treatment appeared to have little or no effect on matrilysin gene activity whereas EGF, bFGF and IL-6 gave a small but consistent increase in the majority of cell lines examined.

Perhaps the most interesting observation in these experiments was the fact that treatment of the cells with a combination of cytokines resulted in a consistently large increase in matrilysin promoter activity in comparison to the control. For example treatment of the majority of cell lines with EGF , bFGF and IL-6 resulted in an approximately 400-700% increase in matrilysin promoter activity in comparison to control levels (see figure 3.23 to 3.28). This suggested that the matrilysin promoter may require the effect of a number of signalling cascades before gene transcription can commence. Interestingly, similar effects with combination treatments were not observed via RT-PCR and western blot analysis. This may be due to the fact that the promoter-luciferase reporter assay system was much more sensitive than RT-PCR and western analysis or that the endogenous matrilysin gene activity within the cell lines investigated was already being stimulated and that combination treatments were only slightly able to augment endogenous matrilysin activity.

Surprisingly the SW480 cell line also showed similar increases in matrilysin promoter activity in response to cytokine treatment when compared to the other cell lines investigated (see figure 3.28). This result was in complete contrast to those obtained for SW480 using RT-PCR and western blot analysis whereby endogenous matrilysin was not detected nor could it be stimulated by treatment of individual or combination treatment of cytokines. We therefore hypothesised that although the SW480 cell line contained the signalling cascades and transcription factors required to initiate the transcription of matrilysin, the matrilysin gene within the SW480 cell line was unresponsive to the effect of these transcription factors, a process which may be explained by promoter hypermethylation.

In the cell lines investigated with the matrilysin promoter reporter constructs, LoVo (figure 3.25) appeared to be the most responsive while WiDr (figure 3.24) appeared to be least responsive to cytokine treatment. Many of these cell lines contain activated signalling cascades which may result in the cell nucleus being saturated with various transcription factors that can promote matrilysin transcription. It may also be possible that cytokine treatment only augments the levels of these transcription factors and therefore the initial level of transcription factors within the cell may dictate how responsive that cell line may be to cytokine treatment. For example the WiDR cell line may have reached a saturation point of transcription factors that are responsible for the stimulation of the matrilysin promoter and would therefore be unresponsive to further cytokine treatment.

In the majority of luciferase assays the 2.3 kb matrilysin promoter reporter construct typically gave higher values than those of the 335 construct which may be an artifact of RNA polymerase stabilisation as the 335 matrilysin promoter construct also gave significant increases after cytokine treatment in comparison to control. These data would suggest that the transcription factors responsible for the transcriptional regulation of the matrilysin gene bind to the promoter within the first 335 base pairs. However, the effect of enhancers or silencers which can be many thousands of base pairs away cannot be ignored.

3.7.4 Silencers and enhancers within the murine matrilysin gene

Silencers and enhancers are elements which repress or enhance gene transcription. These can be located up to several kilobase pairs upstream or downstream of the target gene or may also be located in the introns within the gene itself (Kamakaka, 1997). Enhancers are cis acting elements and stimulate gene expression by binding to transcription factors bound to the promoter of the gene in question and causing a conformational change which 'enhances' gene transcription. Repressor proteins bind to silencer elements and slow or 'silence' transcription of a particular gene. Even though these elements may be thousands of base pairs away silencers and enhancers can still mediate their effects due to the helical looping of DNA which brings them into contact with target genes.

The murine matrilysin gene is 15.5 kbp in size and contains 6 exons and 5 introns. In order to assess these introns for enhancing/silencing properties, the introns within the gene were inserted into a promoter plasmid (pGL_2Promoter, Promega), transfected into cells and analysed for luciferase activity. The promoter plasmid contains the SV40 gene upstream of the luciferase gene. DNA fragments containing putative promoter enhancer silencer elements can be inserted upstream/downstream of the promoter-luciferase transcriptional unit (see appendix 1 for plasmid map). Initial cloning of the whole murine matrilysin gene into the promoter vector was unsuccessful due to the size of the insert. Thus a different strategy involving PCR cloning of the introns was employed, see section 2.2.9 for details.

PCR primers, forward and reverse, were developed for each of the introns within the murine matrilysin gene and the expected basepair sizes were as follows; Intron 1, 3 kb; Intron 2, 250 bp; Intron 3; 650bp; Intron 4, 1.6 kbp and Intron 5, 1.7kbp. Following PCR and isolation of the products (see figure 3.29 A and B), the DNA encoding each intron was subsequently ligated into pBlueScript™ (Stratagene, see appendix 1 for plasmid map), see materials and methods section 2.2.9. Positive clones were miniprepped and the introns then ligated into pGL_2Promoter. The resultant plasmids were then transfected into several cell lines and examined for their effect on the pGL_2Promoter's luciferase activity (figure 3.30).

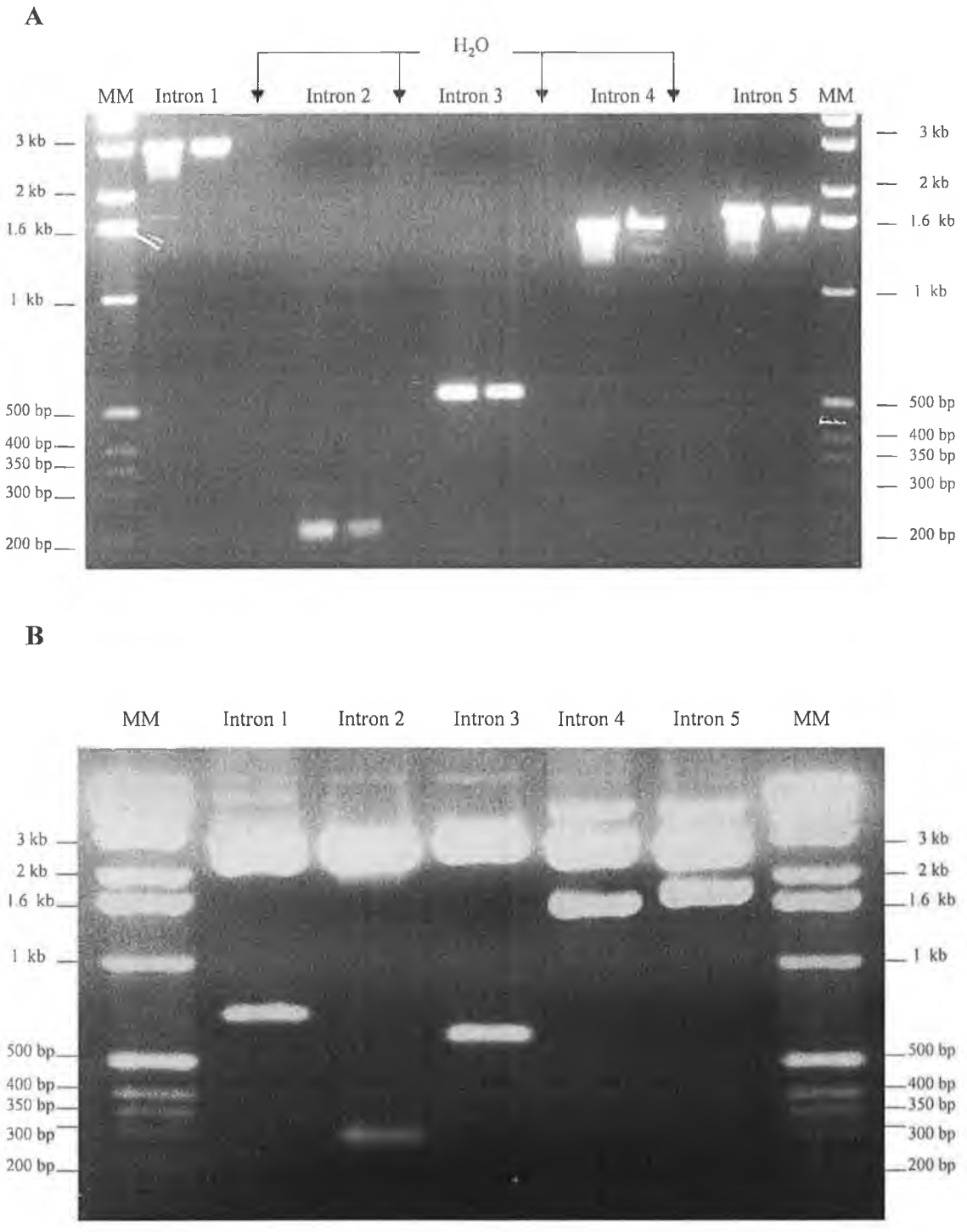

Fig 3.29 A PCR products of the five introns within the matrilysin gene. The expected base pair sizes were 3, 0.25, 0.65, 1.6 and 1.7 kbp for introns 1-5 respectively. **B** PCR products illustrated in 3.17 A were ligated into pBlueScript™. Restriction of the pBS plasmids with *Xho I* and *SacI* result in the separation of the insert from the pBS vector. The resultant stagger ended cDNA fragments were then ligated into pGL_2Promoter.

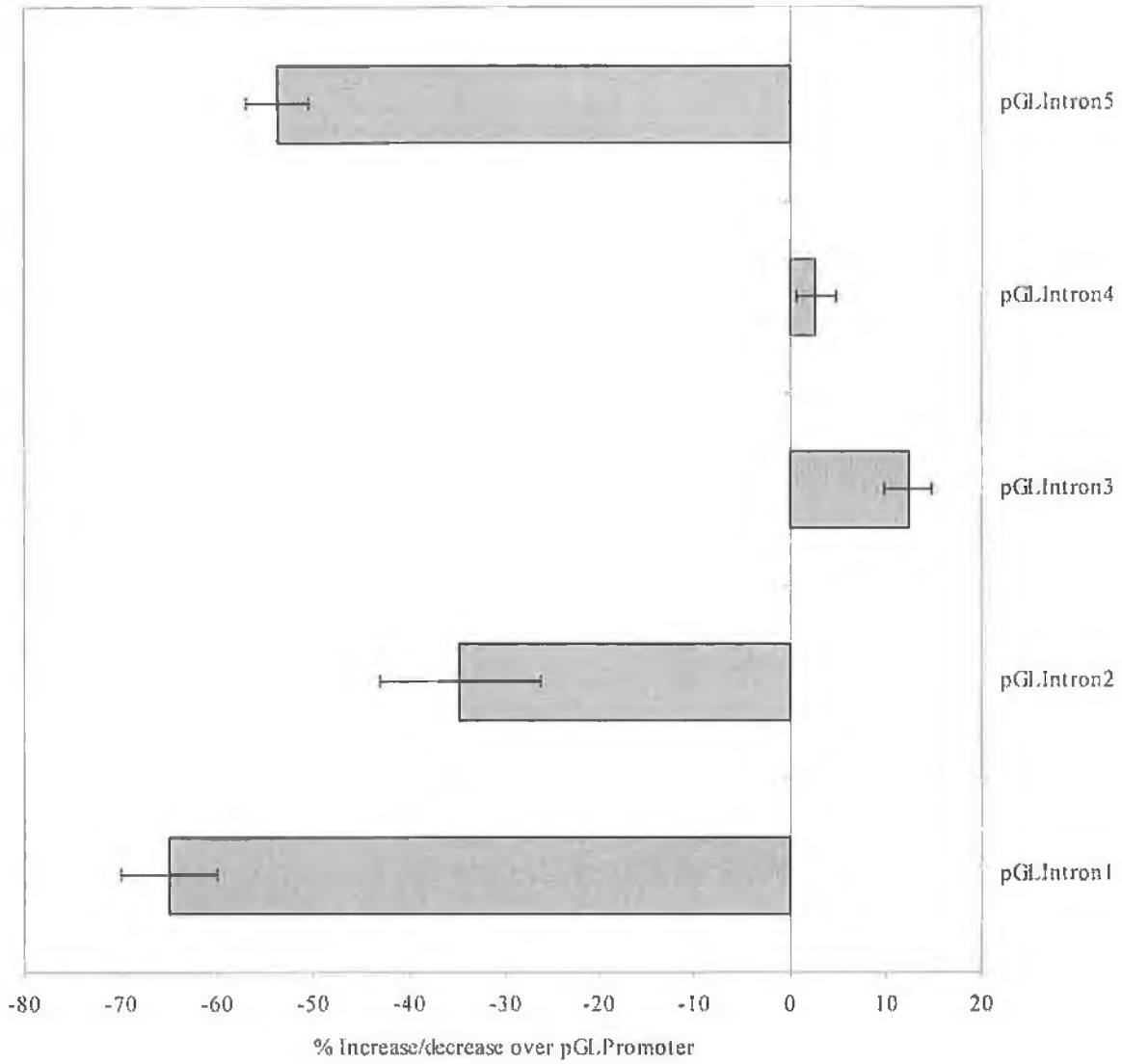

Figure 3.30 Transfection of the promoter intron constructs into the HCT116 cell line. A number of the constructs show a decrease in promoter luciferase activity suggesting the possible location of silencer elements within the introns.

3.8 Discussion

Previous reports have shown that MMP expression can be modulated by a variety of agents including cytokines. Matrilysin expression has been shown to be upregulated by EGF in colon, prostate, head and neck and cervical cancer (Gaire *et al.*, 1994, Witty *et al.*, 1994, Sundareshan *et al.*, 1999, O-Charoenrat *et al.*, 1999 and Ueda *et al.*, 1998) while bFGF and IL-6 have been shown to be involved in matrilysin expression in the prostate and mononuclear phagocytes (Klein *et al.*, 1999, Udayakumar *et al.*, 2001 and Busiek *et al.*, 1995). However, the mechanisms of how these agents mediate their effects, in particular with respect to matrilysin expression, are poorly understood. We have investigated the regulation and expression of matrilysin in colon cancer as several studies have implicated matrilysin to be clinically relevant in colon tumour invasion and metastasis and more recently in early stages of colon tumour growth and development. An understanding of how matrilysin is regulated at a gene level is therefore important in determining i) how its expression in early tumours is controlled and ii) the link between tumour growth and matrilysin expression. Understanding these questions will play a key role in the development of therapies which will be effective in inhibiting matrilysin's role in colon tumourigenesis.

In the panel of colon cell lines investigated in this chapter EGF, IL-6 and bFGF were found to upregulate matrilysin expression in HCT116, SW620, HCA7 and LoVo while in general WiDr matrilysin levels remained unaffected when treated with cytokines. The SW480 cell line did not express matrilysin at a basal level and could not be stimulated to do so by cytokine treatment.

Semi-quantitative RT-PCR analysis showed that in the 4 cell lines stimulated by EGF, IL-6 and bFGF, matrilysin mRNA expression increased by up to two fold on average. RT-PCR is a semi-quantitative method and gives an approximate indication of the levels of matrilysin mRNA in samples. In order to determine a more accurate portrayal of the levels of mRNA after cytokine treatments an internal positive control that is normally constitutively expressed was used. β-actin is a cytoskeletal protein expressed in the majority of cell types and was therefore used as a control in the RT-PCR experiments.

Fig 3.14 shows an example of β-actin expression in the LoVo cells following treatment with the various cytokines. In general levels of β-actin did not fluctuate in response to cytokine treatment in any of the cell lines. Slight inaccuracies in the addition of mRNA may result in a higher or lower yield of PCR products but using densitometric analysis on β-actin mRNA levels, these inaccuracies can be rectified and matrilysin levels subsequently calculated, thus yielding the accurate level of matrilysin mRNA expression. Although the RT-PCR used in these experiments is semi-quantitative, figures 3.17-3.21 show consistently that in many cell lines there is a distinct increase in matrilysin mRNA levels. RT-PCR can be made quantitative, but a means to negate the tube to tube variability which is inherent in the procedure must be found. The use of an internal standard is normally used to make RT-PCR more accurate. Data presented in figures 3.17-3.21 do not show the β-actin products as the matrilysin and β-actin reactions were performed in separate tubes. However earlier experiments did incorporate β-actin into the matrilysin PCR reaction and these results were consistent with those displayed in figures 3.17-3.21 with respect to cytokine stimulation of matrilysin at the mRNA level. Northern blot analysis is traditionally used in analysing mRNA levels but the procedure involves radioactive probes and therefore can be quite hazardous. Competitive RT-PCR and Northern blot analysis were not used to analyse matrilysin levels as promoter-reporter constructs were used to ensure that the cytokines chosen were having a direct effect on matrilysin gene expression and that the increase in mRNA levels observed via RT-PCR were not as a result of secondary signalling or stabilisation of the mRNA.

Although matrilysin mRNA expression was increased in response to EGF, IL-6 and bFGF, this may not necessarily result in an increase in protein expression. Therefore the effects of the cytokines on matrilysin protein levels was also examined. Figure 3.17-21 shows the detection of matrilysin protein via western blot analysis. The antibody used was a monoclonal antibody which detects the latent and intermediate forms of the matrilysin protein (Shattuck-Brandt *et al.*, 1999). Matrilysin protein was detected in all the cell lines with the exception of SW480 which is consistent with the mRNA analysis in that no matrilysin expression was observed via RT-PCR. Levels of matrilysin in the remaining cell lines could be increased via treatment with EGF, bFGF and IL-6 in varying degrees. WiDr however showed only slight increases which is also consistent with RT-

PCR analysis in that only small increases were observed. Figure 3.17 (HCA7) and figure 3.20 (HCT116) show that in general the effects on matrilysin mRNA expression by EGF, IL-6 and bFGF resulted in increased levels of matrilysin protein expression. This suggests that EGF, IL-6 and bFGF stimulate matrilysin mRNA expression which is subsequently translated into increases at the protein level and therefore the overexpression of these cytokines or their receptors in cancer cells could lead to an increase in matrilysin expression which, as we have discussed, may subsequently play a role in colon cancer tumourigenesis and colon cancer invasion and metastasis.

The use of matrilysin promoter reporter constructs also verified that the increases in matrilysin expression observed via RT-PCR analysis were as a result of direct stimulation at a transcriptional level by EGF, IL-6 and bFGF. Figures 3.23 to 28 illustrate that transient transfection of both the 2.3 kbp and 335 bp matrilysin promoter constructs and treatment with EGF, IL-6 and bFGF resulted in an increase in luciferase levels in all cell lines including the SW480 and WiDr cell lines. The increase in luciferase production for EGF was up to 50% greater than that of the control cells in many of the cell lines. Interestingly, some combination treatments with various cytokines showed an increase of 700% in the production of luciferase. For example, in Figure 3.23, treatment of the HCA7 cell line with a combination of EGF, IL-6 and bFGF resulted in a 500% increase in luciferase production in comparison to control values. These results indicated that the transcription factors stimulated by these cytokines converge on the matrilysin promoter and synergistically cooperate to enhance matrilysin transcription as an additive effect would have only given an increase of approximately 150%. These increases, observed in combination treatments, suggest that a number of transcription factors need to be present on the matrilysin promoter in order to enhance transcription of the matrilysin gene. It is important when examining the effects of a single signalling agent *in vitro* to realise that these agents do not operate in isolation. Many of the signalling pathways used by receptors engage molecules used by other receptors and therefore there is a great deal of 'cross talk' between signalling molecules within the signalling cascades. The 'cross talk' between receptor signalling pathways can serve to inhibit gene expression or enhance the signal and increase the rate of expression of target genes. It is therefore plausible that combination treatment with factors involved in proliferation, migration, wound response and inflammation will result in the 'cross talk' between various receptors and their

signalling molecules. This in turn would result in an increase of transcription factors geared towards the expression of matrilysin and other target genes. The transcription factors stimulated by these receptors converge on the matrilysin promoter and through contact with each other and via conformational change of the matrilysin promoter enhance the expression of matrilysin.

TPA, a known regulator of several MMPs, was shown in the majority of the experiments illustrated in figures 3.17 to 3.21 to increase matrilysin expression at the mRNA and protein level. TPA has been shown to act as a diacyl glycerol analogue in the PKC pathway as its effects can be blocked by the addition of staurosporine (Mackay *et al.*, 1992). The treatment of cells with TPA also resulted in an increase of the AP-1 binding site molecules Jun and Fos. Recent evidence has also shown that TPA is involved in the activation of the TNF-α converting enzyme (TACE) which converts TNF-α and TGF-α into their biologically active form (Jolly-Tornetta and Wolf, 2000). TNF-α mediates its effects through the PKC pathway while TGF-α exerts its effects through the EGF receptor. Therefore TPA uses a number of pathways which result in the increase of transcription factors such as jun and fos which in turn can bind to the AP-1 site located in the promoter of many of the MMPs including matrilysin (Fukasawa *et al.*, 2000). TPA was therefore used in experiments as a positive control. In matrilysin promoter reporter experiments illustrated in figures 3.23-3.28, TPA treatment resulted in an increase of approximately 40% luciferase production when compared to control in the majority of cell lines. Combination of TPA with EGF, IL-6 or bFFG enhanced matrilysin promoter activity by up to 500% over control. This suggested that although TPA in isolation can increase matrilysin via transcription factors such as jun and fos, the promoter also requires the presence of other transcription factors stimulated by EGF, IL-6 and bFGF to enhance its activity. The resultant increase in promoter activity when cells are treated with a combination of TPA and EGF, TPA and IL-6, TPA and bFGF or a combination of EGF, IL-6 and bFGF suggests a number of factors are required to stimulate matrilysin activity and that these factors act in a synergistic manner.

The large increases observed in promoter-reporter experiments after combination treatments were not observed to the same extent at the mRNA and protein level as evidenced in figures 3.17 to 3.21. Luciferase reporter experiments are extremely sensitive

and therefore differences in activity are much more pronounced. This is primarily due to the long half life of the luciferase protein and the sensitivity of the assay. It is also possible that matrilysin gene transcription is rate limiting as the gene can only be translated at a certain rate. The control levels of matrilysin were quite high in many of the cell lines investigated with the exception of SW480 and so the differences in matrilysin activity at the mRNA and protein levels were not as apparent. WiDr also showed that luciferase levels can be stimulated after cytokine treatment although these increases were not observed via RT-PCR and western blot analysis. The luciferase technique is quite sensitive and therefore increases in mRNA and protein level may be more difficult to observe via RT-PCR and western blot analysis in the WiDr cell line.

Using RT-PCR and western blot analysis, SW480 was found not to express matrilysin and could not be stimulated to do so when treated with cytokines in isolation or in combination. However matrilysin promoter reporter studies showed that the transcriptional machinery required for matrilysin transcription is present in the cells as the control experiments produced high levels of luciferase activity and these levels could be enhanced even further when the transiently transfected cells were treated with cytokines in isolation and in combination. Witty *et al.*, (1994) have shown that the SW480 cell line can be stably transfected with the matrilysin cDNA under control of a SV-40 promoter. The transfected SW480 cells as a result become much more invasive as was evidenced when the cells were injected into nude mice. It is therefore possible that the matrilysin gene may be hypermethylated which would suggest that the gene is 'silenced' and therefore not expressed in SW480. Increased methylation is associated with cancer with the genes being 'silenced' normally being tumour suppressor genes. It is therefore possible that other genes such as matrilysin may be the target of random hypermethylation in a GC area which results in a restriction of expression in the affected gene (Kamakaka *et al.*, 1997).

Cytokine treatments were initially performed on the cell lines transfected with the 2.3kbp human matrilysin promoter reporter construct. However, most of the potential transcription factor binding sites are located within the first 335 bp. Therefore in order to determine the sites within the promoter that were responsible for the enhanced activity after cytokine treatment a 335 bp promoter-reporter plasmid was constructed. The

plasmid was transfected into the same cell lines and the activity of the promoter was analysed after the various cytokine treatments. Although the luciferase levels were lower than those observed with the larger 2.3 kbp matrilysin promoter the percentage increases over control were comparatively similar. The lower values may have been due to RNA polymerase instability due to the length of the promoter but it is also possible that additional transcription factor binding sites or enhancers may exist further upstream. The fact that the increases over control were similar to those obtained with the 2.3 kbp promoter suggested that the key transcription factor binding DNA sites required for matrilysin gene transcription are present in the first 335 bp.

Interestingly, IGF-I and IGF-II, irrespective of dose, proved not to stimulate matrilysin expression at the mRNA and protein level or to stimulate matrilysin promoter activity. Previous studies have shown that certain MMPs are capable of activating IGF-I and IGF-II by cleaving bound inhibiting proteins, the IGFBPs (Lahm *et al*, 1994, Collett-Solberg and Cohen, 1996 and Fowlkes *et al.*, 1995). These studies suggest that a link between IGF activation and stimulation of MMP expression may exist. Although IGFs may be involved in the stimulation of other MMPs, experimental evidence in this chapter indicates that IGF does not play a direct role in the stimulation of matrilysin and therefore no autocrine loop between matrilysin stimulation and IGF activation exists.

Doses of IGF-I and IGF-II of up to 200ng/ml were examined but these failed to increase matrilysin expression (data not shown). Only slight increases were observed in matrilysin promoter studies but these increases were inconsistent, unlike other cytokines examined such as EGF, bFGF and IL-6. Matrilysin promoter experiments in which cells were treated with a combination of IGF-I and IGF-II failed to increase promoter activity. Addition of TPA with IGF-I and IGF-II increased expression by approximately 40% in a number of cell lines but this increase was due to the activity of TPA on the matrilysin promoter as TPA treatment on its own yielded a 40 % increase in promoter activity. In many cancer cell lines the production of cytokines and their receptors is elevated or the receptor may be continually firing irrespective of whether a cytokine is bound or not. Many of the cell lines examined in this study have high basal levels of matrilysin and this is not surprising since the majority of the cell lines studied express activated Ras which switches on pathways involved in cell proliferation, therefore increasing transcription

factors associated with cell proliferation, such as the AP-1 binding complex of Jun and Fos. This increase in transcription factors such as AP-1 in turn increase the expression of many of the MMPs including matrilysin. Slight increases in promoter activity after cytokine treatment may therefore be hard to identify in these colon cancer cell line model systems. In addition to Ras activation, the cell lines as stated previously may also have upregulated or activated mutated receptors. Our studies therefore suggest that while matrilysin may play an extracellular role in the activation of IGFs (either directly or through the activation of proteolytic cascades) the active IGFs in turn do not play a direct role in upregulating matrilysin gene expression.

The presence of enhancer and silencer elements within the introns of the murine matrilysin gene was also investigated. Enhancers and silencers can be many thousands of base pairs away from their target gene but the helical and looping nature of DNA ensures that these elements come in contact with the promoter region in the gene of interest. Enhancers and silencers can be upstream and downstream of the transcription initiation site but can also be located in the intron of the gene of interest. Having cloned the murine matrilysin introns into a promoter-luciferase reporter plasmid the effects of the introns on the promoter were examined in various cell lines. Figure 3.30 gives a representative example of the effects of the introns on the promoter-luciferase construct in the HCT116 cell line. Introns 1, 2 and 5 gave a significant reduction in promoter activity when compared to the control while introns 3 and 4 gave only slight increases. This may suggest that 'silencing' or repressing elements may be present in introns 1, 2 and 5 of the murine matrilysin gene. The presence of enhancer elements outside the gene are also possible and this will involve cloning of several kilobases at either side of the gene and their subsequent examination.

3.9 Conclusions

This chapter focussed primarily on the expression and regulation of matrilysin by IGF-I, IGF-II, EGF, IL-6 and bFGF in a number of colon cancer cell lines. All cytokines, with the exception of the IGF members, were found to increase matrilysin mRNA and protein levels in the cell lines tested with the exception of the SW480 cell line. These observations were confirmed via matrilysin promoter-reporter experiments which indicated that EGF, IL-6 and bFGF are capable of enhancing matrilysin gene transcription. Increased promoter activity was observed in all cell lines including SW480. Combination treatments of cytokines with or without TPA increased both the 335 bp and 2.3kb promoter activity considerably suggesting that the transcription factors stimulated by these agents act in a synergistic manner in the activation of the matrilysin promoter although these effects were not observed via RT-PCR and western blot analysis. Matrilysin promoter experiments coupled with RT-PCR analysis suggested that EGF, IL-6 and bFGF have a direct effect on the expression and regulation of matrilysin. The increases observed using the 2.3 kb promoter experiments were also observed with the 335 bp promoter experiments and this suggested that the transcription factor elements causing the increases were binding to DNA sequences within the first 335 base pairs of the transcription initiation site of the matrilysin promoter.

IGF-I and IGF-II were shown not to affect matrilysin expression thus indicating that although matrilysin may be involved in the processing of IGFs into biologically active molecules, IGFs are not involved in the direct stimulation of matrilysin gene expression. The presence of silencer and enhancer elements within the murine matrilysin gene was also examined. Potential silencer elements may exist within introns 1, 2 and 5. The existence of silencer and enhancer elements outside the matrilysin gene may also be possible but this will require further studies.

Although previous reports have indicated that EGF, IL-6 and bFGF have a stimulatory effect on MMPs, including matrilysin, this is one of the first studies examining the effect of these cytokines on a number of colon cell lines. However the exact mechanisms by which these cytokines and their receptors mediate their effects are poorly understood and will be examined in more detail in chapter 4.

Chapter 4

The mechanisms controlling matrilysin gene transcription : EGF upregulates matrilysin expression through activation of PEA3 transcription factors

4.1 Analysis of the human matrilysin promoter

Experiments carried out in chapter 3 showed that matrilysin gene transcription could be influenced by a number of cytokines including EGF, bFGF and IL-6. EGF treatment of colon cell lines, in particular, consistently resulted in an increase in matrilysin promoter activity. Other reports have shown that EGF can upregulate matrilysin transcription in various cancer cell lines including those of a prostate, colon and head and neck squamous carcinoma origin (Sundareshan *et al.*, 1999, Gaire *et al.*, 1994 and O-charoenrat, 1999). However, no reports have adequately addressed the mechanisms by which EGF, or other cytokines for that matter, can upregulate the transcription of matrilysin. As discussed earlier cytokines, such as EGF, mediate their effects by binding to their specific receptors. The receptor in turn activates a number of signalling molecules which then activates other molecules involved in a signalling cascade. This cascade converges on the nucleus resulting in the activation/enhanced expression of transcription factors. These transcription factors subsequently bind to the promoter of target genes and, in combination with other transcriptional elements, prepare the gene for transcription.

Gene transcription is the fundamental cellular process that regulates growth, development, cell maintenance and many responses to external stimuli. The transcription machinery is exceptionally complicated, consisting of three multisubunit RNA polymerases, complex polymerase associated transcription factors, co-activators and hundreds (currently defined) to thousands (the projected number) of upstream transcription factors that bind to DNA and interact with the promoter bound transcription complex. The transcription factor proteins bind to sequence specific elements within the promoters of target genes. The sequence of the human matrilysin promoter has been previously reported (Gaire *et al.*, 1994) and insertion of the matrilysin promoter into a transcription factor search engine (http://transfac.gbf.de/TRANSFAC/) reveals a number of key potential transcription factor DNA binding sequences within the first 500 base pairs (see figure 4.1). The potential transcription factor binding sites within the matrilysin promoter include TATA, AP-1, C/EBP, PEA3, Tcf/LEF and Kaiso binding elements.

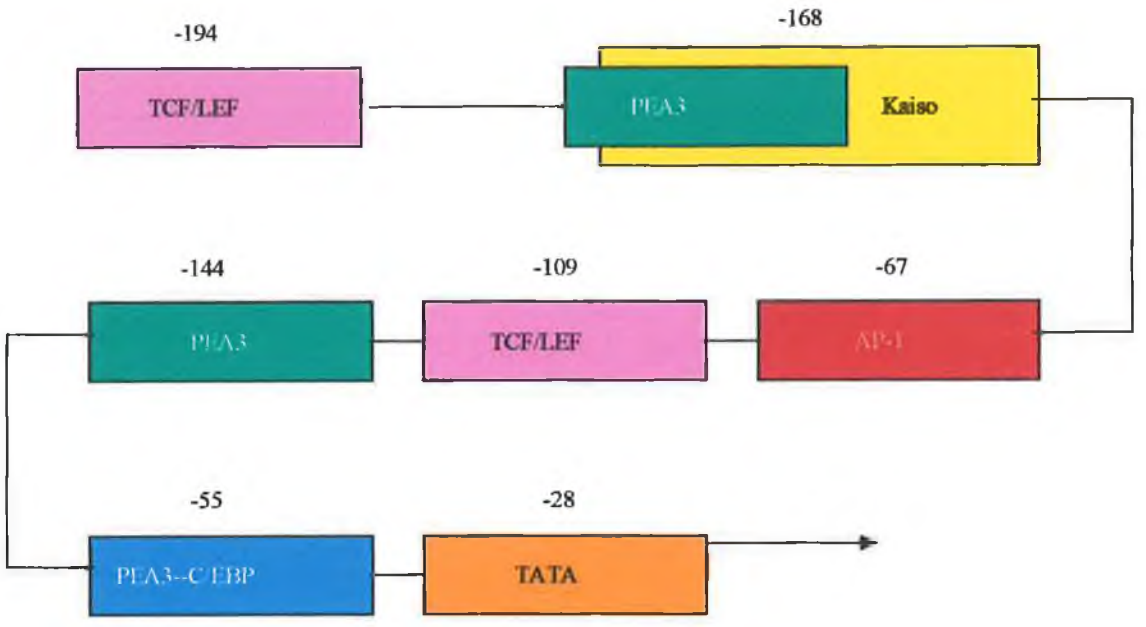

Figure 4.1 The structure of the human matrilysin promoter. The sequence of the human matrilysin promoter has been previously reported (GenBank accession number L22525). Indicated are the positions of a number of potential transcription factor binding sites within the promoter relative to the transcription start site which is located at +1. These sites were determined via the transcription factor search engine (Transfac). The Tcf site at –194 is inverted. Diagram is not drawn to scale.

4.1.1 The TATA binding protein (TBP)

The matrilysin promoter is similar to other eukaryotic promoters in that it is composed of a myriad of binding sites for gene specific regulatory transcription factors as well as a core that is composed of a TATA box. The general transcription machinery assembles over the core promoter and initiates transcription at the start site. The presence of the TATA binding protein (TBP) at the TATA box appears to be a pivotal intermediary step in transcriptional activation and deactivation. With the aid of a functional bound transcriptional activator, TBP is recruited to the TATA box along with a number of other regulatory proteins. This activator/TBP complex subsequently or simultaneously recruits the RNA polymerase (pol) II holoenzyme allowing it to effectively compete out the binding of TBP inhibitors and thus allowing gene transcription to commence (see figure

4.2). Once the transcription factor activator signal is removed, rendering the activator non-functional, TBP inhibitors may be at a competitive advantage over the pol II holoenzyme thus inhibiting gene expression (see Pugh, 2000 for review).

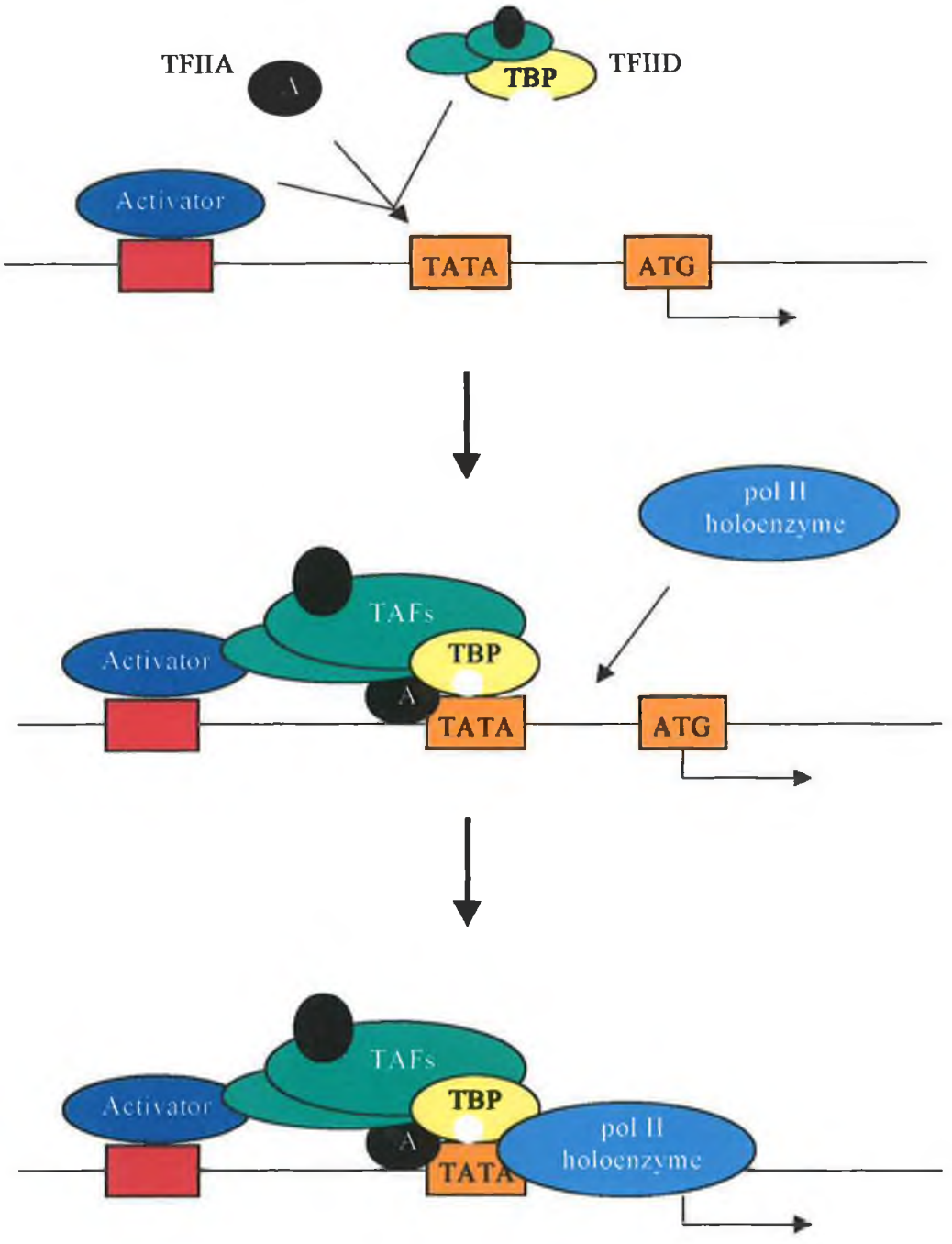

Figure 4.2 Simplified diagram of two of the major steps in transcription complex assembly. Activators bound to their cognate sites on promoter DNA recruit TFIIA and TBP/TBP associated factors (TAFs) complex TFIID to the core promoter. This complex then enables the pol II holoenzyme to bind and initiate transcription.

4.1.2 AP-1 transcription factor binding proteins.

The AP-1 DNA binding sequence, also known as the TRE site (TPA response element), has been shown to bind a number of AP-1 protein complexes. These are primarily Jun, JunB, Fos, FosB, Fra1, Fra2 and JunD. Each of these proteins can bind to form homo or heterodimers and are structurally grouped in the basic leucine zipper class of transcription factors (Borden and Heller, 1997). The resultant AP-1 complexes can bind to AP-1 DNA elements, and the content of the complex, i.e. Jun/Fos or JunB/Fos can dictate the speed at which gene transcription can take place. Thus within the cell the levels of each of the factors which combine and bind to the AP-1 DNA sequence are important in determining whether gene transcription can commence as some complexes are weaker activators than others.

The AP-1 DNA binding site has been shown to play a pivotal role in the regulation of MMP gene expression by growth factors, cytokines and oncogenes (Lafyatis *et al.*, 1990, McDonnell *et al.*, 1990 and Hu *et al.*, 1994). Many of the MMPs contain an AP-1 binding site within the first 100 bp of the transcription start site and this is also true for matrilysin (see figure 1.3). The general AP-1 sequence, found in many of the MMP and TIMP promoters is **TGAGTCA**. Human matrilysin has been shown to be induced by a number of factors such as EGF and TPA (Gaire *et al.* 1994). In these experiments matrilysin-chloroamphenicol acetyl transferase (CAT) promoter constructs were transfected into HeLa cells and treated with EGF which resulted in increased CAT activity over control. Gaire *et al.*, (1994) developed promoter-reporter constructs of various sizes which showed that the PEA3 and AP-1 sites were essential for increased promoter activity following EGF treatment. Several reports have shown that AP-1 is important in growth factor/TPA induction of many of the MMP genes but the AP-1 site alone is insufficient for full activity (Mattei *et al.*, 1990, Lafyatis *et al.*, 1990 and Matrisian *et al.*, 1991). Increases in AP-1 transcription factors in the cell nucleus would therefore contribute to increased expression of matrilysin. In normal cells the increase in AP-1 transcription factors can be as a result of stimulation by external stimuli such as EGF but in cancer cells mutations in oncogenes such as ras can lead to constitutively high levels of AP-1 transcription factor binding proteins. Various reports have shown that AP-1 activity can be induced by neoplastic transformation and expression of transforming oncogenes such

as activated H-ras in the colon and breast (Smith *et al.*, 2000 and Sassone-Corsi *et al.*, 1989). Yamamoto *et al.*, (1995) have also shown that matrilysin can be induced by activated Ki-ras via AP-1 in SW1417 colon cancer cells. This was achieved by the stable transfection of Ki-ras into the SW1417 cell line and its subsequent activation which resulted in increased levels of AP-1 factors (Yamamoto *et al.*, 1995). Thus, the increase in AP-1 levels resulted in increased levels of matrilysin in the SW1417 cell line. As previously discussed matrilysin is involved in early colorectal tumourigenesis and a ras mutation is one of the early genetic 'hits' in Fearon and Vogelstein's colorectal cancer progression model which may therefore suggest that mutations in ras lead to increased matrilysin expression which is subsequently involved in tumour growth and progression. The members of the AP-1 transcription factor family are also capable of forming dimers with ATF (activating transcription factor) family members, comprised of ATF1, ATF2, ATF3, ATF4, ATF6, CREB, E4TF3 and E2A.E which are also involved in cell growth and differentiation (Vinson *et al.*, 1993).

4.1.3 Potential activation matrilysin via the C/EBP element

As previously discussed in chapter 3, the IL-6 receptor is thought to mediate its signal via the JAK-STAT signalling pathways. IL-6 binding to the IL-6 receptor α-chain induces homodimerisation with the signal transducing β-chain, gp-130, which is followed by the activation of the receptor tyrosine kinases JAK-1, JAK-2 and Tyk-2. This receptor kinase complex then interacts with and activates the SRC homology 2 (SH2)-containing cytoplasmic STAT3 transcription factor which then translocates to the nucleus and aids the transcription of many target genes including, c-jun, c-myc and CCAAT enhancer binding proteins or C/EBPs. The C/EBPs encompass a family of transcription factors with structural as well as functional homologies. Similarities between C/EBP family members suggest an evolutionary history of genetic duplications with subsequent pressure to diversify. The resulting family of proteins vary in trans-activating ability and tissue specificity. Since the cloning of the family's original member, C/EBPα, five other C/EBPs have been identified that interact with each other and with transcription factors in other protein families to regulate mRNA transcription. The other five family members have been designated C/EBPβ, C/EBPγ, C/EBPδ C/EBPε and C/EBPζ (see Lekstrom-

Himes and Xanthopoulos, 1998 for review). C/EBPs in general, like many other transcription factors, are modular proteins consisting of an activation domain, a DNA binding basic region and a leucine rich dimerisation domain. The dimerisation domain or 'leucine zipper' is a heptad of leucine repeats that intercalate with repeats of the dimer partner, forming a coil of α-helices in parallel orientation. Electrostatic interactions between amino acids along the dimerisation interface determine the specificity of dimer formation among family members as well as with transcription factors of the NF-κB and Jun/Fos families (Vinson *et al.*, 1993). The presence of a potential C/EBP element (CAATT) adjoining the –55 PEA3 site on the matrilysin promoter may indicate that matrilysin can be directly activated by factors such as IL-6 via C/EBP stimulation. Previous results in Chapter 3 have shown that matrilysin gene activity was enhanced after the addition of IL-6 and therefore it is possible that IL-6 mediates its effects on matrilysin gene activity via the upregulation of transcription factors such as C/EBP.

4.1.4 The Ets family of transcription factors

There are three potential PEA3 transcription factor binding elements within the matrilysin gene suggesting that members of the ETS family of transcription factors are involved in matrilysin gene transcription. The *v-ets* oncogene, the first member of the *ets* gene family to be described, was discovered in the E26 ('E-twenty-six') acutely transforming retrovirus of chicken from which it derives it name (LePrince *et* al., 1983 and Watson *et* al., 1990). The main family members include Ets-1, Ets-2, GABP-α, Erg, Fli-1, D-Elg, E74A, Elf-1, Sap-1, Elk-1, PU.1 and PEA3 which were cloned from a variety of tissues (see table 4.1). In most family members, the conserved DNA binding domain, the ETS domain, is located at the carboxyl terminus of the protein with the exception of Elk-1, SAP-1, and Elf-1, where it is found at the amino acid terminus (Rao and Reddy, 1992 and Thompson *et al.*, 1992). The ETS domain, which is approximately 85 amino acids, has no structural homology to other DNA binding motifs such as the zinc finger, homeodomain, leucine zipper or helix turn motifs but has limited homology to the DNA binding domain of the c-myb oncogene product.

Protein	Molecular Weight	Amino Acid homology to ETS domain of Ets-1	Expression/Features
Ets-1	39-52	100%	Elevated expression in the thymus and endothelial cells; phosphorylated; alternatively spliced; positively autoregulates transcription
Ets-2	58/62	90%	Expression induced following macrophage differentiation and T-cell activation; alternatively spliced; phosphorylated
Erg	41/52	70%	Alternatively spliced; 98% homologous to Fli-1
Fli-1	51	68%	98% homologous to Erg
Elk-1	60	76%	Ets like factor –1; Ets domain located in amino terminus of the protein; forms ternary complex with SRF; Shows three regions of homology with Sap-1
Sap-1	52/58	75%	SRF accessory protein-1; which like Elk-1 forms a ternary complex with SRF; contains three regions of homology with Elk-1; including the Ets domain which is located at the amino terminus of the protein; the two isoforms, Sap-1a and Sap-1b, differ in their caboxy-termini
PEA3	68	68%	Expressed in brain, epididymis and in fibroblast and epithelial cells, down regulated in embryonic cell lines in response to retinoic acid induced differentiation; subfamily members include **ERM** and **ER-81**
TCF1-α	55	55%	Very limited homology to ETS domain exists within the HMG box of this factor; expression is generally restricted to the thymus and is induced following T-cell activation; regulates activity of the TCRα enhancer

Table 4.1 Details of selected members of the ETS family of transcription factors.

The sequence conservation of the proteins and chromosome locations of the genes provide clues to gene duplication and divergence events which gave rise to this gene family. For example the *ets-1* and *fli-1* genes both map to the same chromosome region, 11q23, while *ets-2* and *erg* both map to 21q22. This suggests that duplication of an ancestral *ets*-related gene may have occurred, giving rise to two sister genes, which subsequently diverged over time.

The expression of *ets* genes is controlled at several levels; the initiation of transcription, alternative splicing, post translational modification and protein stability. The expression of the *ets* gene family also varies between tissues. Regulation of transcriptional initiation is best understood for the human ***ets-2*** gene. Like all other *ets* promoters described and many other oncogene promoters, the *ets-2* promoter lacks the classical TATA and CCAAT box sequences found in many RNA polymerase II transcribed genes. A putative Ets-binding site (EBS) has been identified downstream of the promoter which may indicate that the *ets-2* gene is negatively autoregulated. Adjacent to this EBS there is a putative activator protein 1 site (AP-1) and factors bound at these sites may cooperate in trans activation. The promoter of the ***ets-1*** gene contains putative AP-1, EBS, AP-2 and Sp1 binding sites. Expression of exogenous AP-2 and Ets-1 increases the level of transcription from the *ets-1* promoter indicating an activating role for AP-2 in *ets-1* expression and positive auto regulation for *ets-1* transcription. The human *ets-1* mRNA can be alternatively spliced and gives rise to two forms of *ets-1* mRNA, p68 $^{c\text{-}ets\text{-}1}$ and p54 $^{c\text{-}ets\text{-}1}$. Phosphorylation of Ets-1 and Ets-2 results in the loss of non-specific binding activity and phosphorylation by a number of signalling molecules, including those of the PKC pathway, are thought to be involved (McLeod *et al.*, 1992).

Ets proteins bind to the polyoma enhancing activator 3 (PEA3) element and this DNA sequence can vary (core consensus sequence is typically **GGAA**) and gives rise to varying degrees of binding affinity depending on the nucleotide content. The p68 $^{c\text{-}ets\text{-}1}$ protein binds to the PEA3 element of the polyoma enhancer virus promoter. The DNA sequence AGCAGGAAGT is specifically recognised by p68 $^{c\text{-}ets\text{-}1}$ in the polyoma enhancer and binds p68 $^{c\text{-}ets\text{-}1}$ with moderate affinity as determined by electrophoretic mobility shift assays (EMSAs). Mutational analysis of the PEA3 element has identified several higher affinity EBS such as AGC**C**GGAAGT, in which the second A has been replaced by a C.

The identification of PEA3 elements in several genes including MMPs such as stromelysin-1 and matrilysin, suggests that these genes are regulated by Ets related proteins. Ets-1 and Ets-2 have been shown to activate human stromelysin and therefore may be implicated in the activation of other MMPs (Jayaraman *et al.*, 1999). Many of the Ets proteins which bind to PEA3 elements trans-activate gene expression through

cooperation with AP-1 bound protein complexes and this cooperation is usually in the form of interactions between the proteins bound to the promoter. This general combination of nuclear oncoprotein Ets and AP-1 binding sites has been dubbed the 'oncogene response unit' and is found in the promoters and enhancers of many other genes (see Gutman and Wasylyk, 1991 for review). These interactions result in conformational changes which activate expression of the target gene.

The role of *ets* gene family members as oncogenes has been tested in classical transformation experiments in which overexpression of c-*ets-1* and c-*ets-2* oncogenes in NIH-3T3 experiments mouse fibroblasts generated transformed foci in low serum conditions (Seth and Papas, 1990). These transformed cells grew in soft agar and formed tumours in nude mice. The transforming capacity of the c-*ets-1* proto-oncogene may be associated with its ability to auto-regulate its own expression. Evidence has also suggested that Raf-1 kinase is directly/indirectly involved in activating Ets driven promoters following Ras transformation of fibroblasts (Bruder *et al.*, 1992). Another interesting aspect of Ets proteins in growth control has been the observation that Ets-1 expression correlates with the proliferation of endothelial cells in normal blood vessel development during normal processes such as wound healing but also with the vascularisation of tumours during malignant progression. This evidence combined with the ability of Ets proteins to regulate MMPs such as matrilysin suggests that these transcription factors play an important role in tumourigenesis and in tumour progression (Wernert *et al.*, 1992 and Wasylyk *et al.*, 1991).

There are three inverted putative PEA3 elements within the matrilysin promoter located at, -168 (**GCTTCCTG**), -144 (**ACTTCCTC**) and –55 (**TTCCACAT**). However, it is not clear which members of the Ets family bind to the promoter and mediate its activation or what mechanisms promote the increases in Ets transcription factors which bind to the matrilysin promoter. It is perhaps likely that a number of Ets proteins are capable of binding to these elements but, in a similar manner to the AP-1 protein complexes, the Ets members which bind to PEA3 sites in the promoter may have different affinities and therefore may be weak or strong activators of matrilysin gene expression. However, it is clear that the Ets proteins that bind to MMP promoters such as collagenase and stromelysin, act in co-operation with AP-1 proteins which in turn activate

gene expression (White *et al.*, 1997). By considering only two *cis* elements which are common to most of the MMP promoters including matrilysin, the AP-1 and PEA3 sites, it is possible to speculate upon the conditions for the differential transcription of MMP genes. For instance, matrilysin expression may be promoted by the presence of Fos and JunD in epithelial cells but repressed by Ets-1 in stromal fibroblasts. Collagenase-3 expression may be dependent on either Ets-1 or Ets-2 in collaboration with an activating AP-1 complex. If we consider only two members of the *ets* family and assume full occupation of all AP-1 and PEA3 sites in the MMP promoters, there are at least 36 possible Fos/Jun/Ets combinations, each of which could have a different effect on MMP transcription. On the other hand, if we consider all of the Ets family members presently known and all possible Fos/Jun combinations acting on the stromelysin-1 promoter and that each PEA3 and AP-1 site acts independently, there are over 4,000 possible combinations. Despite the potential combinatorial complexity of Ets and AP-1 factors, for this complexity to have any meaning there must be mechanisms whereby various combinations of factors distinguish one MMP promoter from another. Differences between the number and spacing of the oncogene responsive units have been discussed and it is possible that these differences are sufficient for differential regulation of the MMP genes. However, such a mechanism of regulation remains highly idealised (Crawford and Matrisian, 1996).

4.1.5 The regulation of Ets transcription factors by various signalling pathways

In general the ultimate target of activated receptor tyrosine kinases and their associated signalling cascades are transcription factors within the cell nucleus. Several Ets transcription factors have been shown to be the target of the MAPK pathway, a pathway which is employed by the EGF receptor. Experiments carried out in the previous chapter showed that EGF upregulated matrilysin gene expression and so the next logical questions would be: Does EGF upregulate matrilysin gene expression through the activation of the EGF-r and its associated pathways which results in increased activation of Ets factors and, do these Ets factors bind to the PEA3 elements within the matrilysin promoter and enable its transcription?

Several studies have shown the involvement and relevance of PEA3 transcription factors in breast cancers and how these transcription factors are effected by Her2/*neu*. Her2/*neu* proto-oncogene (also known as c-*erb*B-2) encodes a 185 kDa class I receptor tyrosine kinase that is structurally related to the EGF receptor. The EGF receptor family includes EGF-r (c-*erb*B-1), c-*erb*B-2, c-*erb*B-3 and c-*erb*B-4. Overexpression of the human Her2/*neu* is implicated in the genesis of a number of carcinomas affecting several different organs including the breast, ovary, stomach, colon, kidney, bladder and salivary gland (Hynes and Stern, 1994). In breast cancer, Her2/*neu* is associated with poor prognosis for the patient (Slamon *et al.*, 1987). Breast tumours expressing Her2/Neu protein have an increased propensity to metastasize and respond poorly to hormonal and chemotherapeutic agents. The Her2/Neu protein is synthesized at elevated levels in breast tumours as a consequence of both Her2/*neu* gene amplification and transcriptional upregulation of those genes that are amplified; transcriptional activation accounts for a 6-8 fold increased abundance of Her2/*neu* mRNA molecules per gene copy number (Liu *et al.*, 1992 and Iglehart *et al.*, 1990). The enhanced rate of transcription of Her2/*neu* in breast tumour cells has been ascribed to the increased activity of transcription activators including AP-2 and any one of several Ets proteins that regulate transcription initiation from the Her2/*neu* promoter (Hollywood and Hurst, 1993 and Scott *et al.*, 1994). Under normal physiological conditions the Her2/Neu protein is activated by ligand binding which effects its homodimerisation or heterodimerisation with other EGF-r family members, and subsequent auto-phosphorylation at multiple specific tyrosine residues in the intracellular cytoplasmic portion of the protein. Effectors of the Her2/Neu receptor include GRB-2, GRB-7, SHC, phospholipase C-γ, PI-3-kinase, Ras, Src and protein tyrosine phosphatase (Pawson, 1995). One of the pathways used by the Her2/Neu receptor is the Ras/ERK pathway which, as discussed earlier involves several docking and membrane bound molecules which serve to activate signalling pathways such as ERK and JNK cascades (Hynes and Stern, 1994). These activated MAP kinases directly phosphorylate and elevate the activity of a subset of nuclear transcription factors including members of the fos, jun and ets families resulting in changes in gene expression.

Previous murine studies have shown that PEA3 transcription factors are expressed at a low level in normal mammary epithelium (Xin *et al.*, 1992). However, this

expression of PEA3 is substantially elevated in mammary tumours and lung metastases that develop in transgenic mice genetically modified to express normal HER2/Neu in their mammary epithelial cells suggesting a potential role for PEA3 in Her2/Neu induced mammary tumourigenesis (Trimble *et al.*, 1993). Her2/Neu receptors are overexpressed in 30% of breast cancers and 94% of these cases also show increased levels of PEA3 which suggests that PEA3 may also play a role in human mammary tumourigenesis (Scott *et al.*, 1994). To account for the increased abundance of PEA3 transcripts in Her2/*neu*-induced mammary tumours, it was suggested by O'Hagan and Hassell (1999) that the Her2/Neu tyrosine kinase enhances transcription of the PEA3 gene by stimulating the activity of transcription factors that bind to the PEA3 promoter. Recent data also suggests that the PEA3 protein itself can regulate the expression of the PEA3 gene by binding to sites in the PEA3 promoter. These findings have suggested the possibility that elevated levels of PEA3 protein may upregulate transcription of the PEA3 gene thereby leading to increased amounts of its transcript and protein product (O'Hagan and Hassell, 1999).

As we have discussed, PEA3/Ets transcription factors can be stimulated by EGF and EGF like receptors such as Her2/Neu. The PEA3 group of transcription factors belongs to the ETS family and is comprised of PEA3, ERM and ER-81 which are more than 95% identical within their respective DNA-binding domains, the ETS domain, and which demonstrate 50% amino acid similarity overall (Brown and McKnight, 1992 and Monte *et al.*, 1994) (see table 4.1). This group of transcription factors possess functional domains responsible for DNA-binding, DNA-binding inhibition and transactivation. New data suggests that the Ets transcription factors are also targets for signalling cascades such as the Ras dependent ones, and thus may contribute first to the nuclear response to stimulation and second, to Ras-induced cell transformation (O'Hagan and Hassell, 1999). The expression of PEA3 group members in numerous developing murine organs, and especially, in epithelial-mesenchymal interaction events, suggests that the PEA3 transcription factors play an important role in murine organogenesis. Moreover, the expression of PEA3 transcription factors in certain breast cancer cells suggests that the factors themselves or the proto-oncogenes encoding them are involved in the appearance, progression and invasion of malignant cells (de Launoit *et al.*, 1997).

Various reports have shown that Ets transcription factor activity can be modulated by the EGF-r and the MAPK pathway. Recently, Ets-1 and Ets-2 have been shown to activate the promoters of MMP-9 and urokinase genes in response to EGF (Watabe *et al.*, 1998). In these experiments, the Her2/Neu overexpressing cell line SK-BR-3 was stimulated with EGF which resulted in increased levels of Ets-1 and Ets-2 protein and in a more invasive phenotype. This increase in Ets transcription factors correlated with the induction of MMP-9 and uPA. Ets have also been shown to be involved in the regulation of EGF molecules such as heparin bound EGF (HB-EGF) (McCarthy *et al.*, 1997). HB-EGF gene transcription is rapidly activated in NIH-3T3 cells transformed by oncogenic Ras and Raf. Co-transfection of a 1.7 kbp HB-EGF promoter reporter construct with activated Raf was found to enhance HB-EGF promoter activity. This activity was dependent on a PEA3/AP-1 element located –974 and –988 bp upstream of the transcription initiation site. Co-transfection of Ets-2 and p44 MAPK enhanced this stimulation even further which suggested that increased levels of Ets-2, activated by p44 MAPK resulted in increased levels of HB-EGF.

A large proportion of the MMP family members have been designated as Ets target genes and this is mainly due to the fact that many of the MMP promoters contain PEA3 binding elements which suggests that increased levels of PEA3 may play a role in the upregulation of MMPs. In agreement with this, overexpression of PEA3 in the non-metastatic MCF-7 breast cancer cell line leads to an increase in its invasive and metastatic properties in cell culture which implies that there is an increase in MMP activity. These findings raise the prospect that elevated levels of PEA3 in Her2/Neu positive breast tumours may account for their increased metastatic potential (Kaya *et al.*, 1996).

4.1.6 Recent advances in our understanding of matrilysin gene expression

As discussed in the previous chapter the expression of matrilysin has been shown to play an important role in early colon cancer development. The mechanisms of how matrilysin is 'switched on' still remain unclear but several advancements have been made in the past 5 years which clarify to some extent the reasons why matrilysin plays an active role in cancer progression.

4.1.6.1 Matrilysin is a target of β-catenin/Tcf transcription factors

As mentioned earlier, matrilysin gene expression is dependent on the binding of the AP-1 transcription factor complex to the promoter, a state which can be induced by Ras signalling. Studies with colon cell lines have shown however that AP-1 binding is not sufficient for matrilysin expression. For example, the SW480 cell line carries a Ki-ras mutation which results in activated ras gene expression but nevertheless the cell line has been shown not to secrete matrilysin (see Fingleton *et al.* 1999 for review). The observation that a high proportion of Min tumours (mouse model of FAP), in which both of the alleles of the tumour suppressor gene *APC* are known to be lost or mutated, expressed matrilysin led to the speculation that the APC protein may be involved in matrilysin gene expression (Fingleton *et al.*, 1999). The 300 kDa APC protein has been shown to regulate cellular levels of the β-catenin protein (Rubinfeld *et al.*, 1997 and Polakis, 1997). β-catenin is a component of the cadherin complex at cell:cell junctions, however, it has also been found to interact with the family of T-cell factor (Tcf) DNA binding proteins and therefore can act as a transcriptional regulator.

The regulation of β-catenin is complex and poorly understood. A number of mechanisms are involved in controlling the levels of the cytoplasmic pool of β-catenin. In epithelial cells, β-catenin is normally found associated with the cytoplasmic tail domain of the homotypic cell adhesion molecule E-cadherin (see figure 4.3). E-cadherin is crucial to the intercellular adherens junctions which are involved in the organisation and structure of epithelial cells and the supression of tumour invasion (Kemler, 1993). Loss of E-cadherin expression by tumour cells has been shown to be associated with increased invasiveness (Hao *et al.*, 1997). E-cadherin is associated with the actin cytoskeleton via cytoplasmic proteins including α-catenin, β-catenin and γ-catenin, also known as plakoglobin, which together form the cadherin/catenin complex. α-catenin, has sequence similarities to vinculin and is responsible for linking E-cadherin to the actin filament network (Nagafuchi and Takeichi, 1988). Cellular levels of β-catenin are regulated by APC which binds excess β-catenin and directs phosphorylation by the serine/threonine kinase GSK-3β. The levels of active GSK-3β in the cytoplasm are determined by the Wnt signalling

pathway (see figure 4.3). Wnt binds to its receptor, frizzled which in turn activates dishevelled (Dvl). Activated Dvl prevents GSK-3β phosphorylation of β-catenin which inhibits β-catenin being 'tagged' for degradation by ubiquitin. It was poorly understood how Dvl, GSK-3β and APC combine to phosphorylate β-catenin directly but recently it has been shown that axin plays an important role in the preparation of β-catenin for ubiquitination (Kikuchi, 1999). Axin contains binding sites for APC, β-catenin, GSK-3β, PP2A and Dvl. In this complex, GSK-3β efficiently phosphorylates β-catenin, APC, and axin itself. The resultant serine phosphorylated β-catenin can then be degraded by the ubiquitin proteasome system. However in the presence of Dvl, the phosphorylation of β-catenin does not occur as GSK-3β is inactive and therefore β-catenin translocates to the nucleus and carries out its various functions including the activation of matrilysin gene transcription. Therefore truncating mutations in APC which prevent it binding to β-catenin, or mutations in β-catenin which prevent its phosphorylation by GSK-3β, result in an accumulation of β-catenin in the cytoplasm. This excess β-catenin can then translocate to the nucleus where it interacts with Tcf transcription factors and activates the transcription of specific genes containing Tcf sites such as c-myc and cyclin D and matrilysin (He *et al.*, 1998, Brabletz *et al.*, 1999 and Crawford *et al.*, 1999).

Analysis of both familial and sporadic colon cancers has revealed that in most tumours either the *Apc* (FAP and sporadic) or the *β-catenin* (sporadic) gene itself are mutated, resulting, in either case in an accumulation of stabilised β-catenin protein in the cell. This is then free to interact with members of the Tcf transcription factor family which could in turn activate genes involved in the tumour process. A correlation between β-catenin accumulation, which can be detected via immunohistochemistry, and matrilysin expression was observed in Min tumours. In cells with obvious matrilysin expression, β-catenin was always clearly detectable. This observation coupled with an examination of the matrilysin promoter which revealed the existence of Tcf binding sites in both the human and murine matrilysin promoters allowed Crawford *et al.* to show that matrilysin was a target of the APC/β-catenin/Tcf pathway (Crawford *et al.*, 1999).

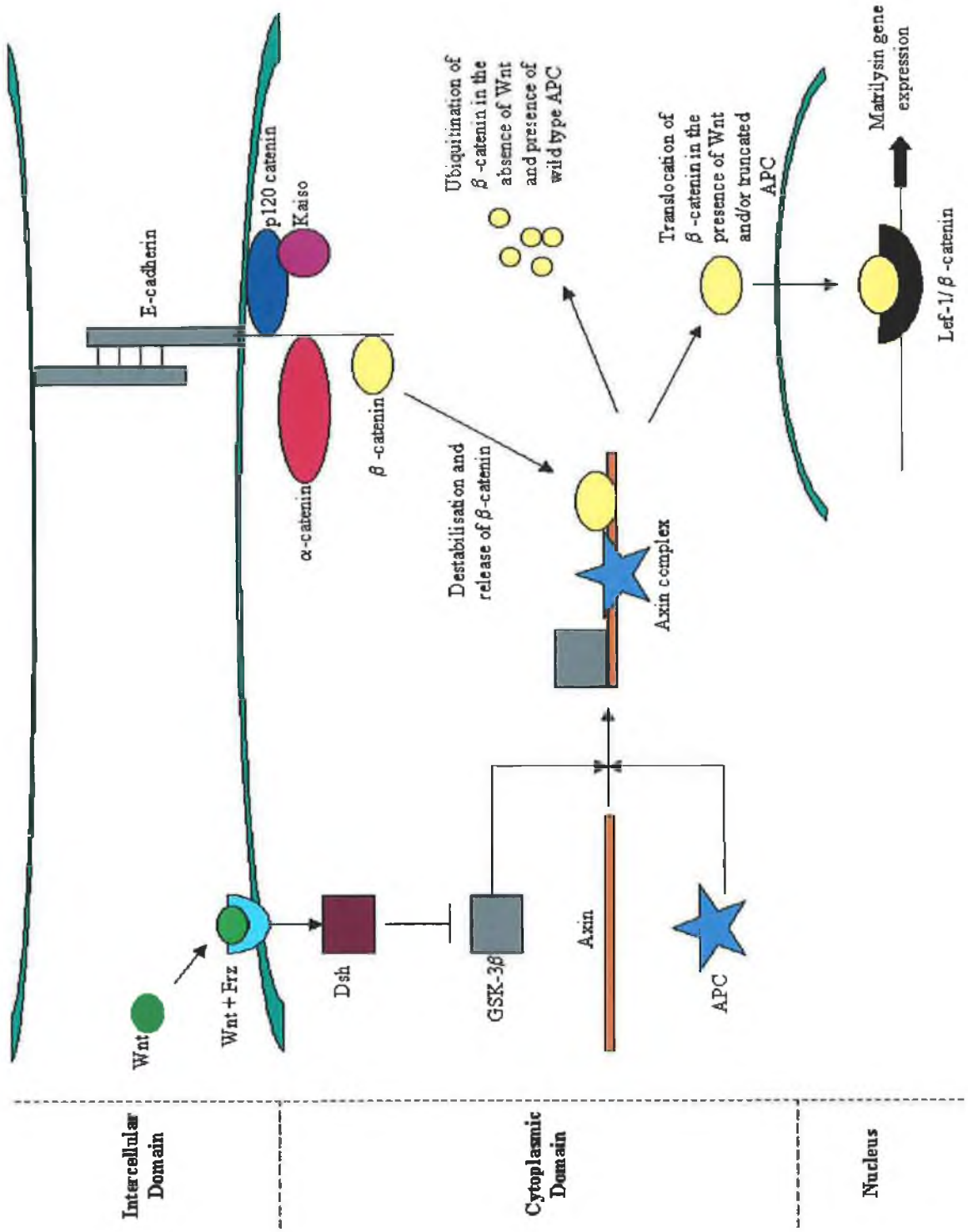

Figure 4.3 Recent developments in the regulation of matrilysin transcription. When β-catenin is released from the cadherin/catenin complex it is degraded via APC, axin and GSK-3β. If undegraded then β-catenin translocates to the nucleus and interacts with Tcf bound Lef-1 which results in derepression of the matrilysin promoter. In early colorectal tumourigenesis, the *APC* gene is often mutated and therefore the accumulation of β-catenin may lead to increased matrilysin gene expression.

Using matrilysin promoter luciferase constructs it has been shown that matrilysin can be upregulated up to 7-16 fold in response to β-catenin co-transfection (Crawford *et al.*, 1999). The human matrilysin promoter contains two putative Tcf sites at –194 (**CTTTGAA** in the reverse orientation) and at –109 (**TTCAAAG**). The upregulation observed in the matrilysin promoter after β-catenin transfection is dependent on the Tcf binding site located at –109 as mutation of this site partially abrogates the induction by β-catenin. Additionally, in cell lines in which a high level of β-catenin/Tcf complex exists (as determined by EMSA analysis), cotransfection of the matrilysin promoter with the cytoplasmic E-cadherin domain which binds β-catenin thus preventing the interaction of β-catenin with Tcf, significantly downregulated matrilysin promoter activity. Again this effect was demonstrated to be Tcf site dependent as the promoter with a mutated Tcf site did not show the same response. Crawford *et al.* (1999) also found that basal activity from the promoter construct containing the mutated Tcf binding site was higher than the wild type promoter construct in those cell lines with low amounts of β-catenin/Tcf complexes. This suggested that binding of the Tcf factor alone to the matrilysin promoter represses transcription from the promoter. This was confirmed by co-transfection of the promoter with the Tcf family member LEF-1 which resulted in a down regulation of matrilysin promoter activity. Tcf factors are known to induce DNA bending and this may be the mechanism of repression (Love *et al.*, 1995, Giese *et al.*, 1992). Binding of β-catenin to Tcf relieves its repressing bending activity, essentially freeing the DNA to be transcribed (Behrens *et al.*, 1996). This would therefore suggest that β-catenin is permissive for matrilysin gene transcription. Crawford *et al.* (1999) have also found areas in AOM tumours where β-catenin was present but not accompanied by matrilysin. This indicates that although β-catenin is essential for matrilysin transcription in a similar manner to AP-1 it is not sufficient on its own to stimulate matrilysin transcription. Matrilysin therefore appears to be a target for the APC/β-catenin pathway which is so often activated in colon tumours. As *APC* mutations are associated with the earliest stages of tumourigenesis, being a target of this regulatory pathway may help explain the early appearance of matrilysin as well as its strong association with intestinal adenomas in particular.

4.1.6.2 Potential role for p120^{ctn}/Kaiso in matrilysin gene activation

p120^{ctn} is an armadillo repeat protein which is related to β-catenin and γ-catenin. All members of this family interact with the cytoplasmic tail of E-cadherin which is a principal component of the adherens junctions. β-catenin and γ-catenin bind to the COOH domain of E-cadherin in a mutually exclusive manner (Daniel and Reynolds, 1995). p120^{ctn} binds to the proximal membrane region of E-cadherin (see figure 4.3) (Thoreson *et al.*, 2000). Unlike β-catenin, p120^{ctn} does not interact with α-catenin, APC or Lef-1, suggesting that it has unique binding partners and plays a distinct role in the cadherin/catenin complex (Lampugnani *et al.*, 1997). Using p120^{ctn} as bait a yeast two hybrid system recognised a novel transcription factor named Kaiso (Daniel and Reynolds, 1999). Kaiso's deduced amino acid sequence revealed an amino terminal BTB/POZ-ZF (Broad complex Tramtrack Bric-a-brac, Pox virus and zinc finger) protein/protein interaction domain and three carboxy-terminal zinc fingers of the C_2H_2 DNA binding type (Albagli *et al.*, 1995). Monoclonal antibodies specific for Kaiso, co-precipitated with a variety of p120 antibodies but not with antibodies to α-catenin, β-catenin, E-cadherin or APC suggesting that Kaiso interacts with different and as yet undefined binding partners (Daniel and Reynolds, 1999). Like other POZ-ZF proteins, Kaiso was found to localise to the nucleus and was associated with specific nuclear dots. Kaiso homodimerises via its POZ domain and translocates to the nucleus. Other proteins grouped in the same family, for example, PLZF (promyelocytic leukaemia zinc finger), form macromolecular complexes with nuclear transcriptional machinery which aid the transcription process (Chang *et al.*, 1996). It is therefore possible that Kaiso may be involved in mediating p120^{ctn} signalling. The presence of a putative Kaiso site overlapping a PEA3 site in the matrilysin promoter suggests that Kaiso, once released from the E-cadherin/p120^{ctn} complex may translocate to the nucleus and effect the transcription of several genes including matrilysin in a similar manner to that of β-catenin. It is also possible that Kaiso may be involved in matrilysin regulation at the various stages of colorectal tumour progression and excess Kaiso in the free cytoplasmic pool of proteins may translocate to the nucleus and facilitate the transcription of matrilysin.

4.1.6.3 Potential role for EGF-r in the release of β-catenin from E-cadherin

Several studies have examined the levels of E-cadherin and the cadherin/catenin complex in human colorectal tumours (Van Acken *et al.*, 1993 and Dorudi *et al.*, 1993). It has been shown that decreased expression of E-cadherin and its associated cytoplasmic proteins results in increased dysplasia. Therefore low levels of expression of the cadherin/catenin complex in cancer cells would result in decreased cell-cell interaction and allow the cells to invade more readily. Previous reports have shown that matrilysin activity can be modulated by E-cadherin. In squamous cell carcinomas of the head and neck the expression of matrilysin was found to directly correlate to the level of cell-cell interaction and E-cadherin played an important role in this observation. Therefore increased E-cadherin levels in the cell resulted in decreased matrilysin gene expression which makes sense in that, the levels of 'free pool' β-catenin are reduced due to increased interaction with the cytoplasmic tail of E-cadherin (Borchers *et al.*, 1997). In a normal cell, with wild type APC and β-catenin, the levels of cytoplasmic 'free pool' β-catenin can be controlled by the release of β-catenin from the E-cadherin/catenin complex and the activity of GSK-3β which is controlled through Wnt signalling. Therefore, if the Wnt pathway is activated and there is a release of β-catenin from the adherens junctions, there is an increase of free pool β-catenin in the cell cytoplasm which translocates to the nucleus and effects the transcription of several genes including matrilysin. The question that remains, however, with respect to the E-cadherin release of β-catenin is, under what circumstances in the normal cell does this process occur and what 'triggers' the release of β-catenin from the cadherin/catenin complex? Is it possible that the release of β-catenin by E-cadherin could be mediated by receptor tyrosine kinases such as the EGF-r? Previous studies have shown that overexpression of E-cadherin in human colon cell lines results in a decrease in proliferation as a result of an increase in the level of cyclin dependent kinase inhibitor $p27^{kip1}$, a molecule whose levels are decreased during EGF-r stimulated proliferation (St. Croix *et al.*, 1998). Subsequent experiments showed that inhibition of E-cadherin via antibody addition, and the addition of TGF-α to the media resulted in a 14-fold increase in growth rate. The mechanism of interaction between the EGF-r and E-cadherin was proposed to be via the increase in endogenous phosphatase activity by E-cadherin (through the addition of sodium vanadate which prevents

phosphatase activity). It has also been shown that several receptor protein tyrosine phosphatases (RPTPs) are activated across the cell membrane and have been shown to colocalise with the cadherin/catenin complex. Tight adhesion mediated by E-cadherin, may therefore serve to strengthen interactions with adjacent RPTPs, ensuring high continuous phosphatase activity which could result in the dephosphorylation of the EGF-r (St. Croix *et al.*, 1998). Interestingly, it has been shown via immunoprecipitation studies that β-catenin is linked to several receptor tyrosine kinase receptors including EGF-r and Her2/Neu (St. Croix *et al.*, 1998). It has also been shown in some studies that the EGF-r can interact with β-catenin through phosphorylation which in turn leads to the destabilisation of the cadherin/catenin complex and results in the release of β-catenin as phosphorylated β-catenin is not found at the cadherin/catenin complex. Earlier studies conflicted with these observations and have shown that β-catenin phosphorylation is not required for complex destabilisation (St Croix *et al.* 1998). However, other more recent reports have confirmed that phosphorylation of β-catenin does indeed cause cadherin/catenin complex destabilisation. *In vitro* experiments showed that tyrosine phosphorylation of β-catenin by $pp60^{c\text{-}src}$ resulted in decreased binding to the E-cadherin cytoplasmic domain (Roura *et al.*, 1999). Other studies have also shown that in src and ras transformed cells, tyrosine phosphorylation of adherens junction components is related to the impairment of cell-cell adhesion, indicating that increases in phosphorylation result in destabilisation of the cadherin/catenin complex (Lampugnani *et al.*, 1997).

These studies suggest that the phosphorylation of the cadherin/catenin complex leads to its destabilisation and subsequent detachment from the cadherin/complexes of other cells, hence, the 'rounding up' of the cell. This detachment is necessary in normal cell processes for migration and proliferation. It may be possible, therefore, that when a cell receives a mitogenic signal from its external environment, for example EGF/EGF-r interaction, the cadherin/catenin junctions destabilise through either direct or indirect action of the tyrosine kinase activity of the EGF-r cytoplasmic domain. A number of *in vitro* studies have demonstrated that EGF is a potent morphogen affecting cell shape and motility. Indeed, a link between the EGF-r and the cytoskeleton has been recognised for several years: activation of the EGF-r by EGF initiates a number of cellular changes including cell rounding, membrane ruffling, cytoskeletal reorganisation and redistribution

of the EGF-r (Kadowaki *et al.*, 1986). Furthermore, EGF-r has been demonstrated to bind to the actin cytoskeleton (den Hartigh *et al.*, 1992) and has been shown to co-localise with the cadherin/catenin complex at the lateral membrane of epithelial cells (Kirkpatrick *et al.*, 1995). Another report has shown that the EGF-r interacts with β-catenin and γ-catenin and catalyses their phosphorylation which, as mentioned earlier, leads to the destabilisation of the cadherin/catenin complex (Solic and Davies, 1997). Overall EGF has been shown to decrease cell-cell contact by affecting components of the adherens and desmosomal junctions and to increase cell-substratum interactions by enhancing integrin expression. As EGF and the EGF-r have been shown to play a role in the destabilisation of the E-cadherin/catenin complex it is plausible that EGF can regulate matrilysin through the release of β-catenin which translocates to the nucleus and interacts with the LEF bound Tcf site within the matrilysin gene promoter.

4.1.7 Summary

Analysis of the matrilysin promoter reveals a number of potential transcription factor binding sites. Previous experiments have shown that binding of AP-1 protein complexes to the AP-1 site within the matrilysin promoter were essential for gene transcription. Recently it has been demonstrated that β-catenin is involved in matrilysin gene transcription via its interaction with the LEF bound Tcf sites within the matrilysin promoter. In chapter 3, we observed a consistent increase in matrilysin gene activity following treatment with EGF. EGF and the EGF related receptor Her2/Neu have been shown to be involved in the upregulation of PEA3 transcription factors in breast cancer. In addition the EGF receptor has also been shown to be involved in modulating the cadherin/catenin complex in various colon cancer cell lines. In chapter 4 the mechanisms by which EGF regulates matrilysin gene transcription in number of colon cell lines (SW480, HCA7 and HCT116) were investigated.

4.2 Results

4.2.1 EGF increases PEA3 artificial promoter activity

The experiments detailed in chapter 4 used three cell lines; SW480 cells which did not express matrilysin at the mRNA or protein level, HCT116 cells which expressed intermediate levels of matrilysin at both the mRNA and protein level and HCA7 cells which expressed high levels of matrilysin at the mRNA and protein level.

Artificial promoters containing multiple sequence motifs for AP-1, ETS and PEA3 (see table 4.2) were constructed and transfected into the SW480, HCT116 and HCA7 cell lines. For example, the transcription factor binding site for PEA3 contains an AGCAGGAAGT core DNA consensus sequence which PEA3 transcription factors recognise via their ETS domain. In an artificial promoter this sequence is repeated a number of times and is attached to a luciferase reporter-gene. Therefore increases in PEA3 protein activity within the nucleus would result in increased expression of a PEA3 artificial promoter. As EGF has been shown to mediate its effect through the MAPK pathway we also analysed an AP-1 and ETS artificial promoters as AP-1 and Ets transcription factors can also be stimulated via the MAPK pathway. The results show that following the addition of EGF, the activity of the PEA3 promoter increased by up to 70% indicating that EGF may indeed be stimulating an increase in PEA3 transcription factors (see figure 4.4). Increases in AP-1 promoter activity were also observed but this activity was lower in comparison to that of the PEA3 promoter. The ETS artificial promoter showed only a slight increase in activity after EGF treatment. These data suggested that PEA3 activity was being stimulated in the colon cancer cell lines via EGF treatment. After transfection with the PEA3 artificial promoter the SW480 and HCT116 cell lines were found to be most responsive to EGF treatment. The increase in PEA3 artificial promoter activity observed in the HCA7 cell line was much lower in comparison to the SW480 and HCT116 cell lines which may indicate that PEA3 levels are already quite high in the HCA7 cell line (see figure 4.6).

Artificial promoter plasmid	Sequence	No. of repeats
PEA3	AGCAGGAAGT	3
ETS	GCCGGAAGT	5
AP-1	TGAGTCA	5

Table 4.2 Details of the PEA3, ETS and AP-1 artificial promoters

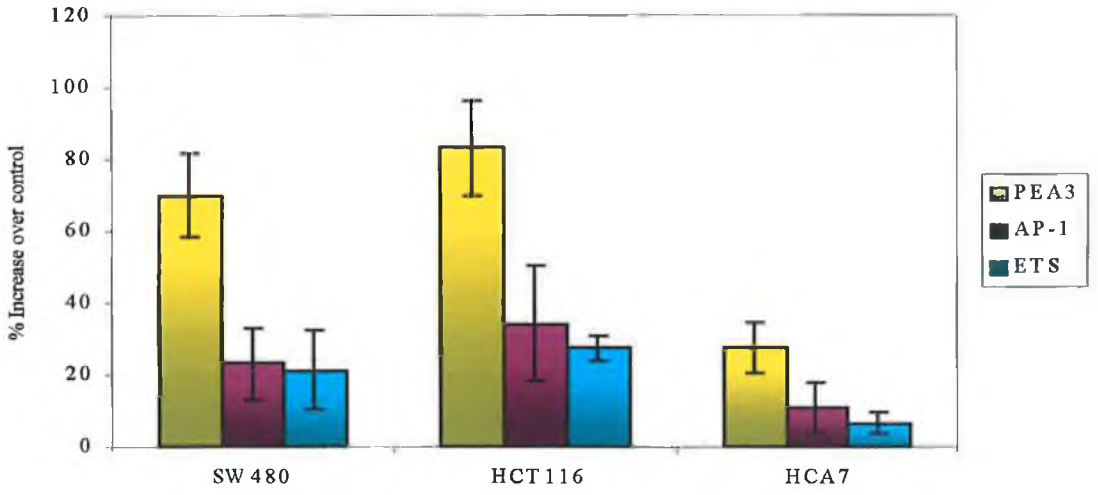

Figure 4.4 PEA3, AP-1 and ETS artificial promoters were transfected individually into various cell lines which were subsequently treated with EGF (50 ng/ml) for 6 hours. In many of the cell lines increases of up to 80% were observed in PEA3 activity.

4.2.2 EGF increases PEA3 protein and PEA3 activity

Having shown that PEA3 transcription factors were being stimulated by EGF using artificial promoter reporter experiments, we investigated whether nuclear levels of PEA3 protein were upregulated in order to determine if EGF was stimulating PEA3 protein expression. Nuclear extracts from cells were prepared in order to examine the levels of PEA3 protein within the nucleus after EGF treatment (see methods and materials section 2.2.11.1). The phosphorylated species of the PEA3 transcription factors were also examined and this was achieved through the addition of sodium vanadate which prevents phosphatase action and protects against the dephosphorylation of substrates. Once the nuclear protein was extracted it was quickly aliquoted into small volumes, flash frozen and maintained at –80°C. The concentration of protein in the samples was subsequently

assayed (see section 2.2.4) and prior to experimentation was analysed for signs of degradation by running the samples on an SDS-PAGE gel (see section 2.2.5.1). In figure 4.5 equal volumes of protein were loaded for the control and EGF samples of each cell line.

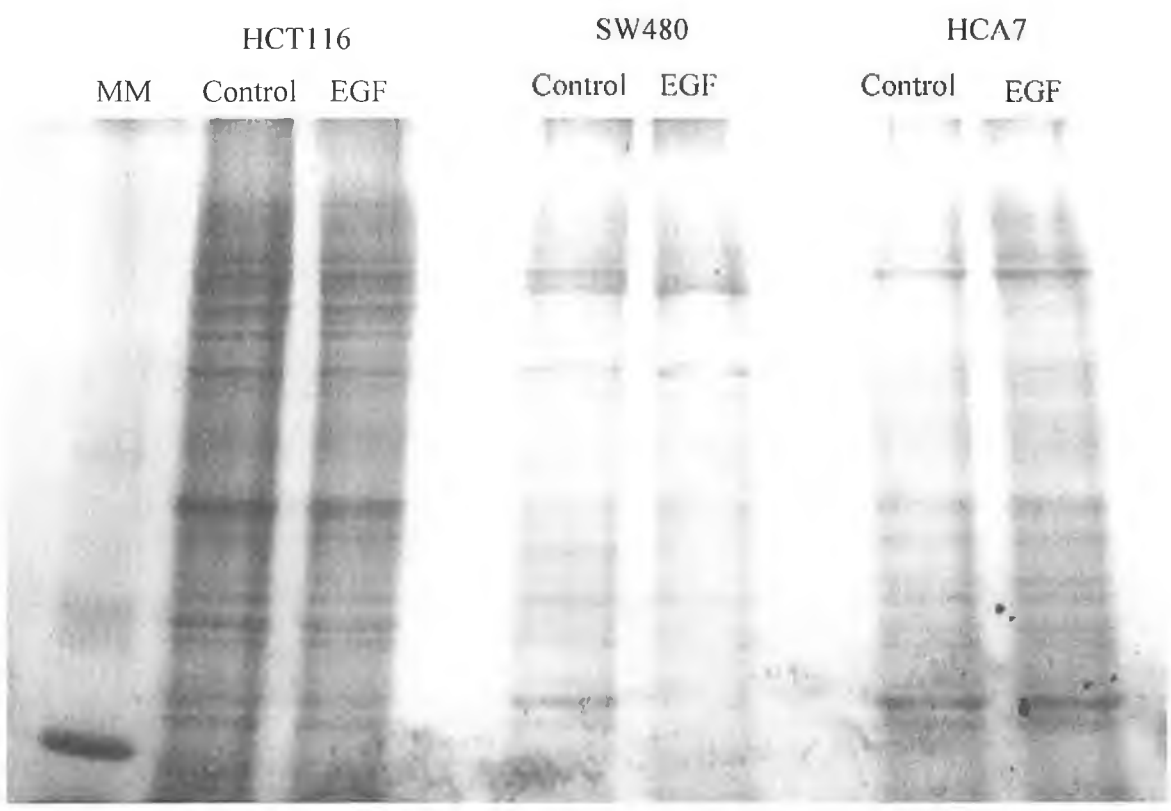

Figure 4.5 Nuclear extract samples (after 6 hours of EGF (50 ng/ml) treatment) from each cell line were analysed for protein degradation. Equal amounts of protein for each cell line were run on SDS-PAGE gels. The gels were stained with Coomassie brilliant blue and then destained.

Results, via western blotting, show that in the cell lines examined the level of PEA3 protein was increased after treatment with EGF, suggesting that EGF enhanced the expression of the PEA3 transcription factor (see figure 4.6). The phosphorylated species of the PEA3 protein migrates to a position slightly higher than that of the unphosphorylated PEA3 protein. Western blot analysis with a PEA3 antibody which recognises both species showed that EGF treatment also increased the amount of phosphorylated PEA3 within the nucleus.

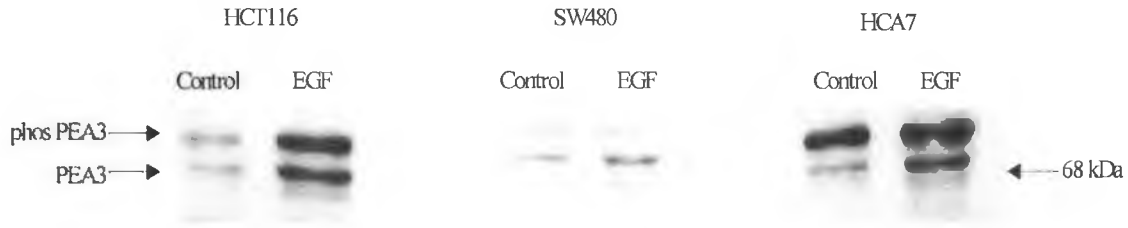

Figure 4.6 Western blot analysis of PEA3 protein levels in a variety of colon cell lines following EGF treatment (50 ng/ml) for 6 hours. The antibody used also recognises the phosphorylated form of PEA3. 50 μg of total protein was loaded for each sample.

4.2.3 Increased levels of PEA3 via EGF treatment result in increased binding to optimal PEA3 probes

Initial experiments showed that EGF treatment resulted in an increase in PEA3 protein expression and in the PEA3 phosphorylated form. However, this may not necessarily mean increased binding to the PEA3 sites within the matrilysin promoter. In order to ascertain first of all if the increase in PEA3 protein would result in increased binding to PEA3 DNA binding sites we used an optimal PEA3 probe. PEA3 DNA binding sites differ slightly in terms of nucleotide content but primarily are comprised of a GCAGGAAGTG core. The PEA3 probe used in these experiments was designed for optimal binding of PEA3. The effect of EGF on the binding of PEA3 protein to the optimal probes was analysed via electrophoretic mobility shift assay (see section 2.2.11). Briefly the technique involved mixing nuclear protein extract samples with radioactively labelled oligonucleotide probes and retard their migration through the gel. If PEA3 transcription factors were present then they would bind to the probes. The samples were run on an acrylamide gel which separates the transcription factor bound probe from the free probe. Results showed clearly that in all the cell lines assayed using this technique EGF stimulated PEA3 transcription factors which in turn led to increased binding to the optimal PEA3 oligonucleotide probe (see figure 4.7 and 4.8).

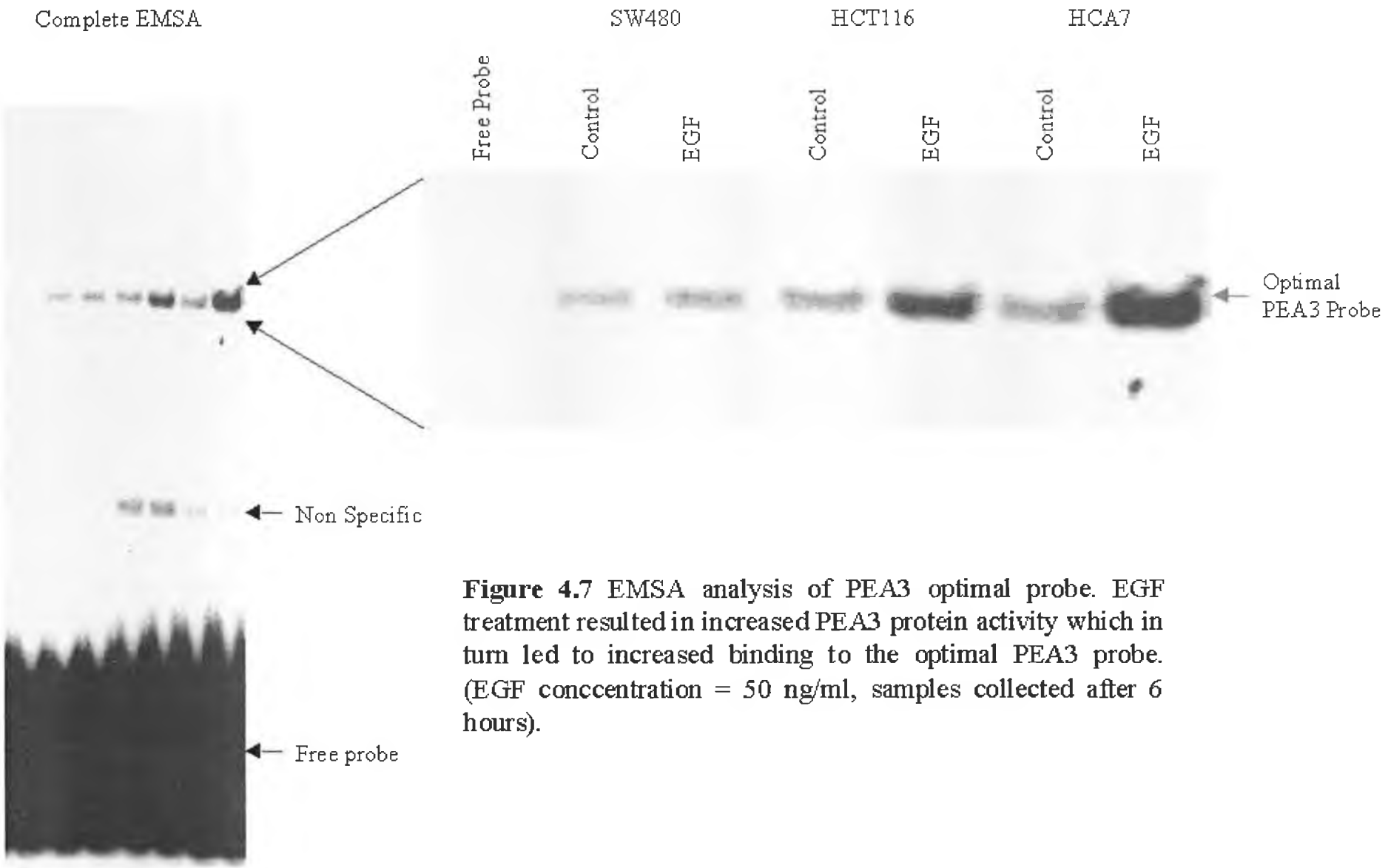

Figure 4.7 EMSA analysis of PEA3 optimal probe. EGF treatment resulted in increased PEA3 protein activity which in turn led to increased binding to the optimal PEA3 probe. (EGF conccentration = 50 ng/ml, samples collected after 6 hours).

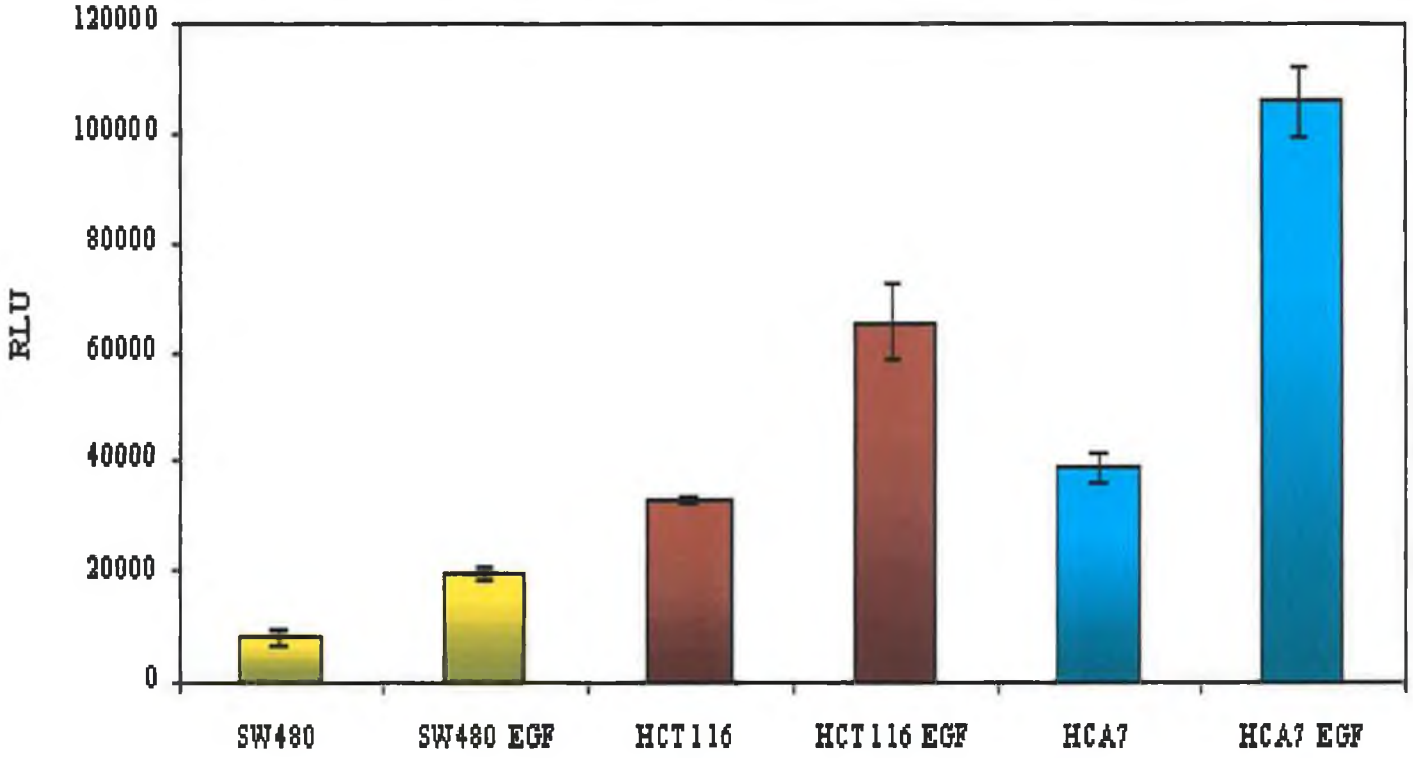

Figure 4.8 EMSA analysis of PEA3 optimal oligonucleotide probe. Graphical anaylsis shows that treatment of various cell lines results in a significant two fold increase in PEA3 transcription factor binding to the optimal PEA3 oligonucleotide probe.

4.2.4 EGF stimulated PEA3 protein binds to matrilysin promoter PEA3 sites

We examined if the PEA3 transcription factors would bind to the potential PEA3 oligonucleotide sites within the first 335 bp of the matrilysin promoter. Oligonucleotides for each of these PEA3 sites, located at –55, -144 and –168 bp down stream from the initiation site were radioactively labelled (see section 2.2.11 for probe sequence). The PEA3 sites contain a core GGAA sequence which is recognised by the ETS family of transcription factors. The flanking DNA however differs slightly at each of these sites and can dictate how well different transcription factors can bind. Results showed that in HCT116 nuclear extracts, PEA3 transcription factors bound to all of the PEA3 sites within the matrilysin promoter and that stimulation of PEA3 transcription factors via EGF treatment resulted in increased binding to these sites (see figures 4.9 and 4.10). Similar results were also found for other cell lines (SW480 and HCA7, data not shown). It was noted that PEA3 transcription factors bound with similar intensities to each of the sites suggesting that all three are important in the activation of matrilysin gene expression. Interestingly, the PEA3 sites at –168 and –55 overlap with potential Kaiso and C/EBP sites respectively and it is possible that these transcription factors may co-operate or compete with PEA3 transcription factors in binding to the matrilysin promoter and therefore offer an alternative means of regulating matrilysin gene transcription.

4.2.5 'Supershift' EMSA confirmation of PEA3 transcription factors

In order to confirm that PEA3 transcription factors were binding to the PEA3 oligonucleotide probes, the samples were mixed with a PEA3 antibody (see section 2.2.11). The PEA3 antibody complexes with the PEA3 transcription factor and the radiolabelled oligonucleotide probe which results in a 'supershift' as the complex has a much higher molecular weight than the probe and transcription factor combined (see figure 4.11). Results, using the optimal PEA3 probe show that the bands only partially 'supershift' which suggests that other factors belonging to the PEA3 subfamily such as ERM and ER-81 may also be involved in the activation of matrilysin gene expression. Previous reports have shown that other ETS family members such as Ets-1 and Ets-2 bind to the matrilysin promoter but are very weak activators even in the presence of c-jun (Crawford *et al.*, 2001).

Full EMSA

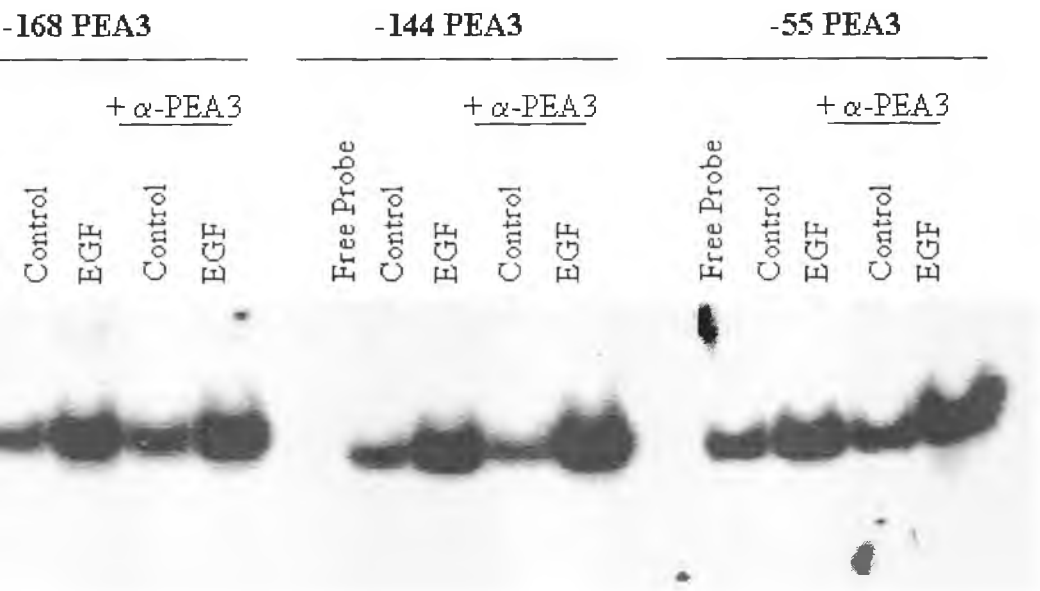

Figure 4.9 EMSA analysis of the PEA3 sites within the matrilysin promoter. EGF treatment (50 ng/ml) of the HCT116 cells results in increased PEA3 protein activity which in turn leads to increased binding to each of the PEA3 sites within the promoter. This may suggest that all three elements are important in the activation of matrilysin gene expression.

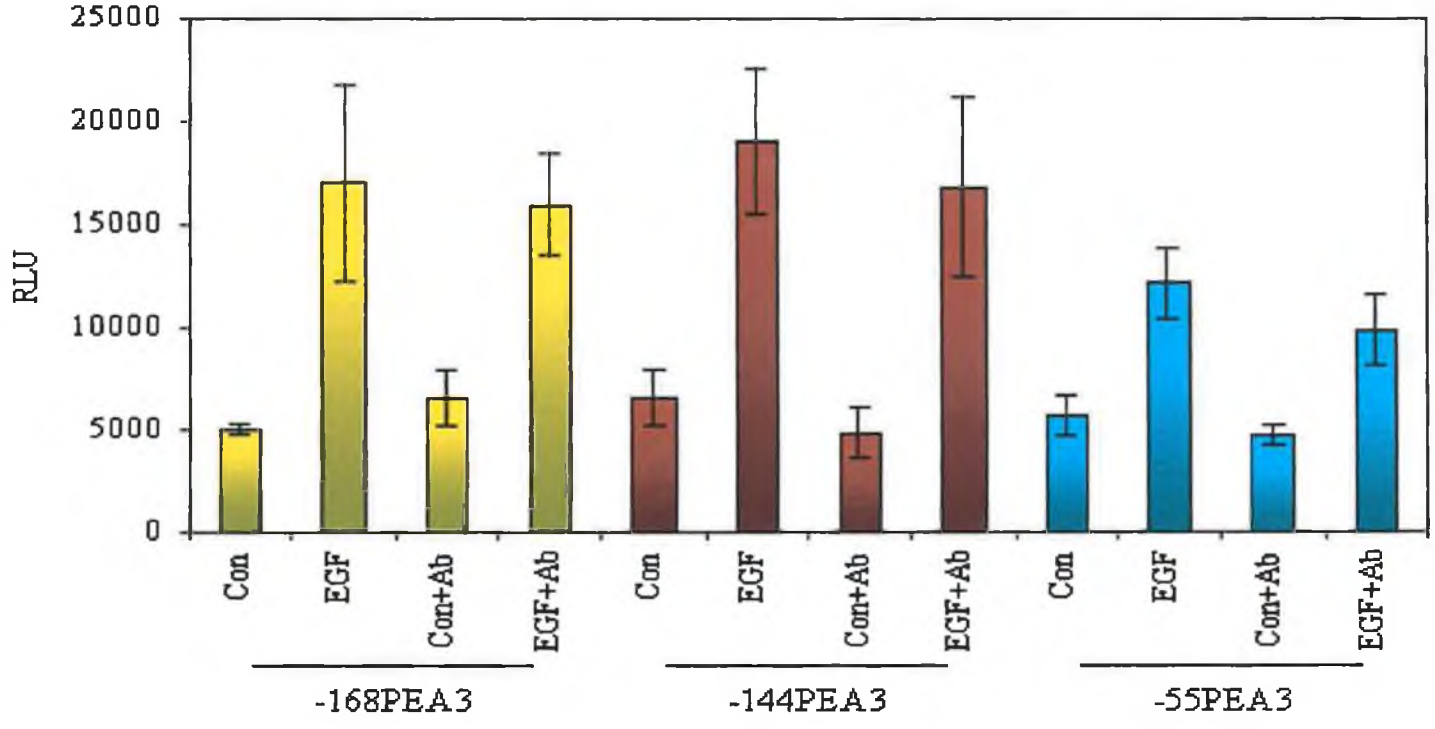

Figure 4.10 EMSA analysis of PEA3 binding sites within the matrilysin promoter. Graphical analysis shows that treatment of the HCT116 cell line with EGF (50 ng/ml) resulted in significant increases in the binding of PEA3 transcription factors to each of the PEA3 sites within the matrilysin promoter. Cont; Control samples, Con+Ab; Control sample plusPEA3 antibody, EGF; Epidermal growth factor, EGF+Ab; Epidermal growth factor plus PEA3 antibody

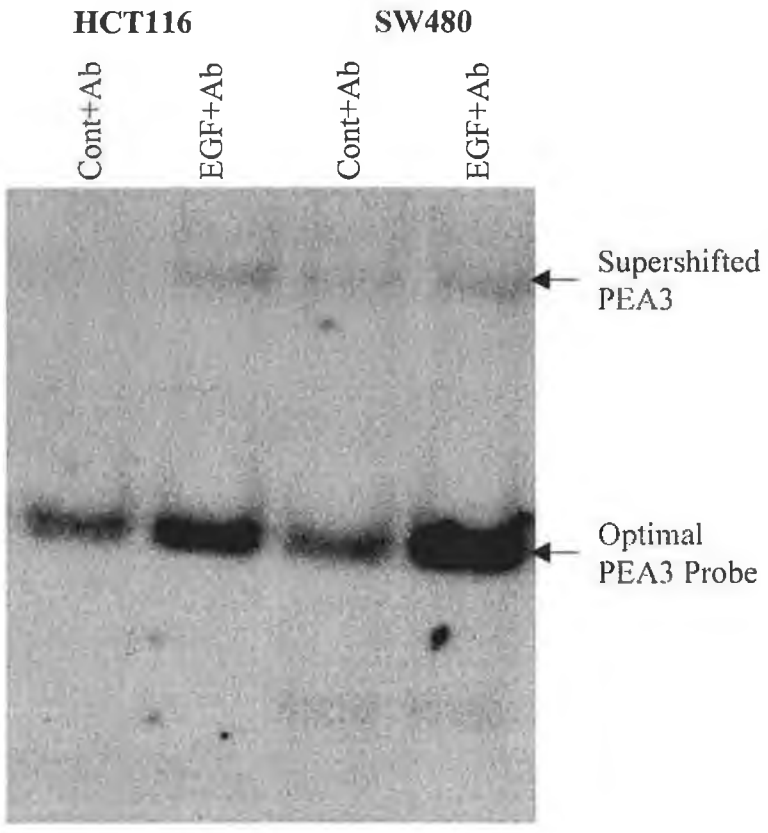

Figure 4.11 'Supershift' EMSA analysis of PEA3 optimal oligonucleotide probe shows partial 'supershifting' of the PEA3-probe complex on addition of the α-PEA3 antibody. This suggested that other members of the PEA3 subfamily, ERM and ER-81 may also be involved in the regulation of matrilysin gene expression. Con+Ab; Control sample plus PEA3 antibody, EGF; Epidermal growth factor, EGF+Ab; Epidermal growth factor plus PEA3 antibody.

4.2.6 Analysis of mutations in the PEA3 sites of the 335 bp matrilysin promoter-reporter construct.

The matrilysin promoter contains three PEA3 sites and in order to determine how essential each of these sites were for matrilysin gene expression, matrilysin promoter reporter constructs with a mutation in either the –168, -144 or the –55 sites, achieved by the PCR-splicing by overlap extension method (Horton *et al.*, 1989), were analysed (see table 4.3). Results showed that mutations in each of the sites resulted in a decrease in matrilysin gene expression in comparison to the wild type control (see figure 4.12). Treatment with EGF increased this activity but not to the level observed in wild type experiments. Each of the mutations resulted in a similar decrease in promoter activity which suggested that all three elements were equally important for matrilysin gene activity.

PEA3 Site	Wild Type	Mutations
-168 PEA3	5'-GTGTGCTTCCTGCCAATAACGATGT-3'	5'-GTGTGCTTCTTGCCAATAACGATGT-3'
-144 PEA3	5'-GTAATACTTCCTCGTTTTAGTTAATG-3'	5'-GTAATACTTCTTCGTTTTAGTTAATG-3'
-55 PEA3	5'-CCTATTTCCACATTCGAGGC-3'	5'-CCTATTTCTACATTCGAGGC-3'

Table 4.3 Mutated PEA3 positions within the matrilysin promoter (Crawford *et al.*, 2001). The mutated positions are underlined.

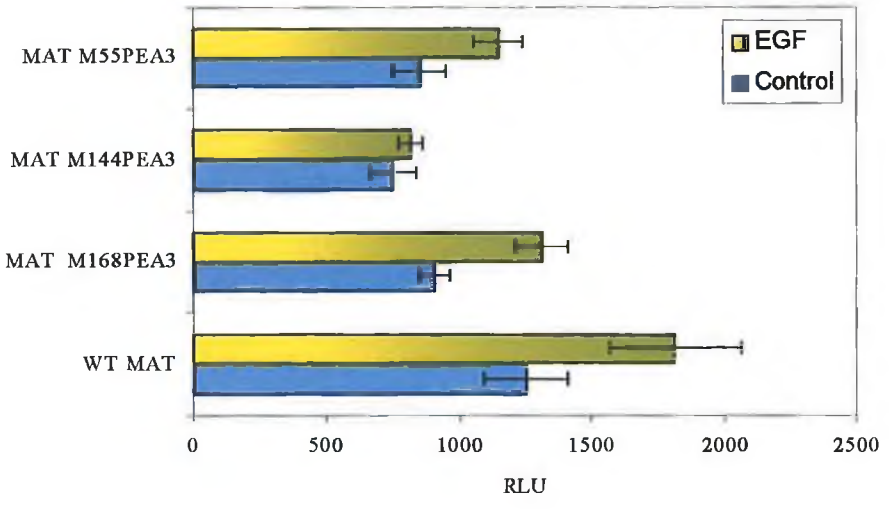

Figure 4.12 Matrilysin promoter constructs with mutations in each of the PEA3 sites were transfected into the **HCT116** cell line. Cells were treated with EGF and analysed after **8** hours. MAT M168PEA3 indicates that the 335 matrilysin promoter construct has a mutation in the –168 PEA3 site. MAT M144PEA3 indicates that the 335 matrilysin promoter has a mutation in the –144 PEA3 site while MAT M55PEA3 indicates that the 335 matrilysin promoter has a mutation in the –55 PEA3 site. WT MAT indicates the wild type 335 promoter. EGF concentration = 50 ng/ml

4.2.7 Does the EGF-r activity have a direct effect on the cadherin/catenin complex at the adherens junction?

Several reports have indicated that the EGF-r once activated plays an important role in the destabilisation of the cadherin/catenin complex (Lampugnani *et al.*, 1997, Roura *et al.*, 1999 and Solic and Davies, 1997). Other studies have also shown that the interaction between β-catenin and Tcf site bound Lef-1 is essential for matrilysin gene activation however it must cooperate with AP-1 and PEA3 for gene transcription to

commence (Crawford *et al.*, 1999). It has already been observed that only the ETS sub family members of PEA3 bind to the three PEA3 sites within the matrilysin promoter (Crawford *et al.*, 2001). Other studies have shown that PEA3 transcription factors can be directly stimulated by EGF, most likely via the MAPK pathway (O'Hagan and Hassell. 1999), but there is also another possibility which is that EGF may be able to stimulate matrilysin expression through its interaction with the cadherin/catenin complex. We have therefore investigated if the interaction between the EGF-r and the catenin/cadherin complex could promote the expression of PEA3 transcription factors by using the PEA3 artificial promoter construct.

These experiments involved the transient transfection of the PEA3 artificial promoter reporter with a number of plasmids that encoded for full length E-cadherin (**E-cad**), E-cadherin with a deletion in the region where β-catenin binds (**E-cadcytoΔ**) and a truncated stable form of β-catenin which does not interact with APC and therefore avoids ubiquitination (**b-cat**). The artificial PEA3 promoter was used as no PEA3 promoter-reporter plasmid was available. Results, illustrated in figure 4.13, showed that, EGF increased PEA3 artificial promoter activity (PEA3 vs. PEA3+EGF). This mechanism most likely involved stimulation of PEA3 transcription factors through the MAPK pathway (O'Hagan and Hassell, 1999). However, following co-transfection of the PEA3 artificial promoter with the full E-cadherin plasmid construct we observed a reduction in normal PEA3 promoter activity. We also observed that E-cadherin can inhibit the stimulation of the PEA3 promoter by EGF. Perhaps increases in E-cadherin levels within the cell also increased the receptor protein tyrosine phosphatases (RPTPs) associated with E-cadherin which in turn reduce the activity of phosphorylation cascades within the cell. Treatment with EGF appears to stimulate PEA3 promoter activity in the presence of E-cadherin which suggests that EGF can reverse the effects of E-cadherin through stimulation of the phosphorylation cascades. Co-transfection of the PEA3 promoter with E-cadcytoΔ, which has a deletion in the region that binds β-catenin, showed a decrease in PEA3 promoter activity but this decrease is less than that observed with experiments using the full length E-cadherin. Treatment with EGF again results in an increase in PEA3 promoter activity in the presence of E-cadcytoΔ. These effects may indicate that β-catenin plays a role in the regulation/activation of PEA3 transcription factors. We

therefore performed experiments whereby a stable β-catenin protein (b-cat) with a deletion which prevented interaction with the APC protein, hence degradation, was cotransfected with the PEA3 promoter and E-cadherin or E-cad cytoΔ. If β-catenin was playing a role in the regulation/activation of PEA3 transcription factors we would have expected a large increase in PEA3 promoter activity in the E-cad cytoΔ/β-catenin experiments as β-catenin, would translocate to the nucleus and effect the transcription of PEA3. No increase in PEA3 artificial promoter activity was observed after co-transfection with the E-cadherin and β-catenin constructs which suggested that EGF mediated release of β-catenin does not result in an increase of PEA3 transcription factors.

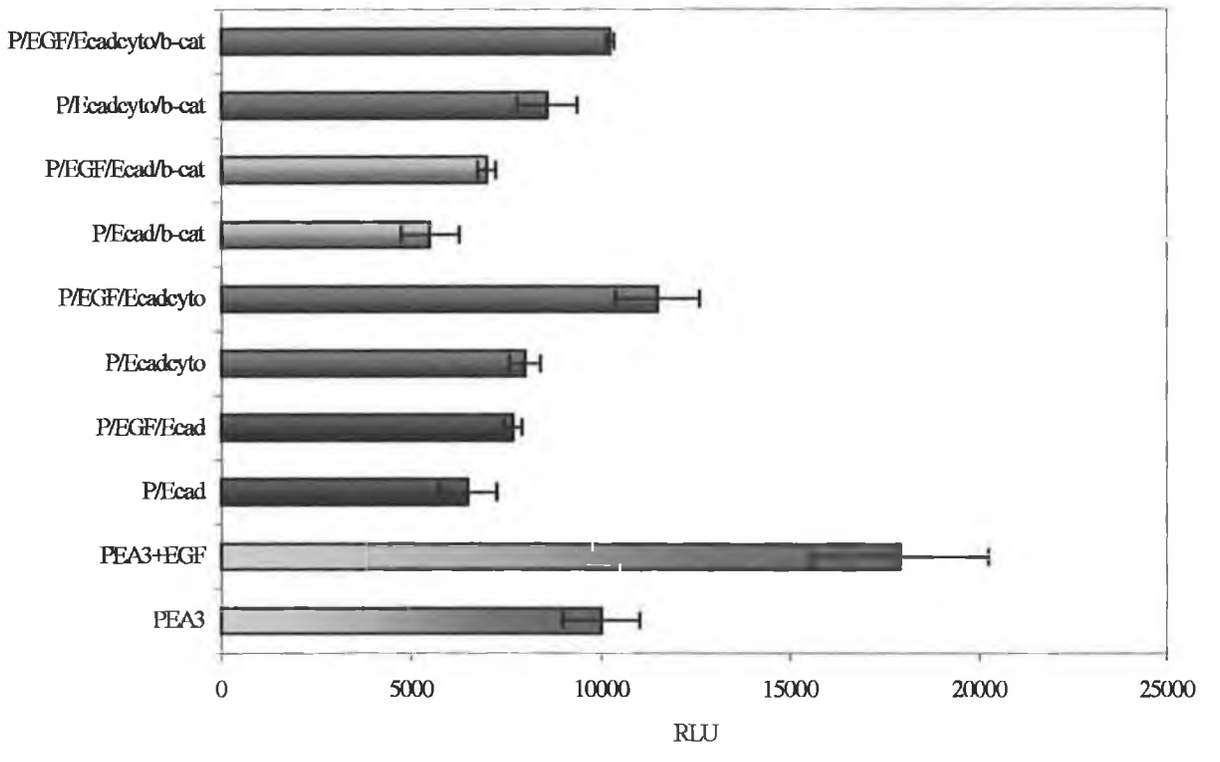

Figure 4.13 HCT116 cells transfected with PEA3 artificial promoter. A number of co-transfections were also performed and the effects on PEA3 promoter activity were observed. E-cad refers to the full length E-cadherin protein construct while E-cadcyto refers to the E-cadherin protein with a deletion in the region which binds β-catenin. b-cat refers to the β-catenin construct which encoded for a truncated β-catenin protein which could not interact with the APC protein. EGF concentration = 50 ng/ml

4.2.8 EMSA analysis of β catenin/Tcf interaction after EGF treatment.

Having shown that EGF-r interaction with the cadherin/catenin complex did not lead to increased stimulation of the PEA3 artificial promoter we analysed via EMSA the effect of EGF treatment on the interaction between β-catenin and the Tcf sites within the matrilysin promoter. Within the matrilysin promoter there are two potential Tcf sites in the region proximal to the transcription initiation start site and also further down stream (proximal cis Tcf site at –109 TTCAAAGT while inverted downstream Tcf site at –194 CTTTGAA) (Crawford *et al.*, 2001). As discussed earlier β-catenin mediates its effect through its interaction with Lef-1 which is bound to the Tcf site and causes derepression of the matrilysin promoter thus allowing gene transcription to commence. Previous studies have shown that the proximal Tcf site within the promoter is essential for the activation of matrilysin gene expression. Results show that in the SW480 and HCT116 cell line, in particular, that the level of interaction between β-catenin increases after treatment with EGF (see figure 4.14). Both of these cell lines have low levels of cytoplasmic pool β-catenin and therefore we suggest that EGF treatment activates the EGF receptor which in turn has been shown to destabilise the cadherin/catenin complex through its interaction with β-catenin (Crawford *et al.*, 2001). The resultant increase in free pool β-catenin subsequently leads to increased interaction with the Tcf site. The results also show that this effect was most obvious with the proximal Tcf site and not with the Tcf site located downstream of the transcription initiation start site.

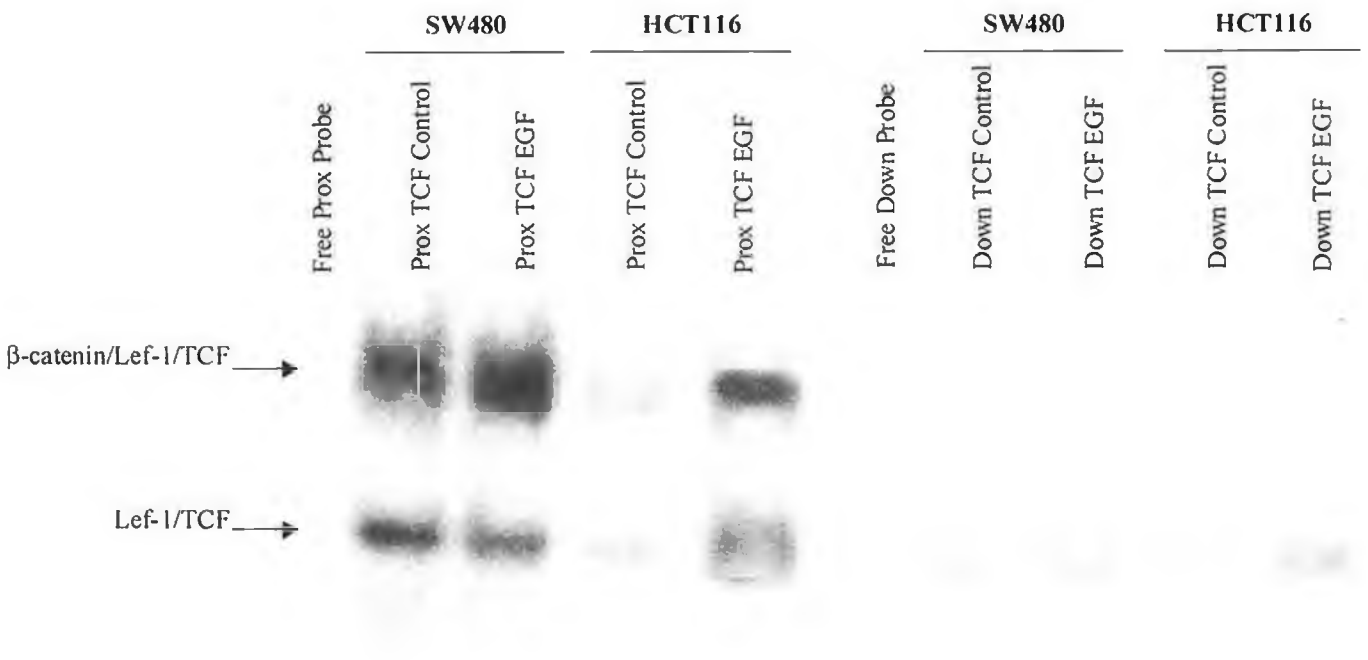

Figure 4.14 EMSA analysis of β-catenin interaction with the proximal (Prox) and downstream (down) Tcf sites located within the matrilysin promoter. The lower band indicates LEF-1 bound to the Tcf probe while the higher band represents the β-catenin/LEF-1/Tcf probe complex. Results indicate that the proximal Tcf element is important in matrilysin gene transcription activation (Howard C. Crawford, personal communication).

4.3 Discussion

Although matrilysin has been studied for many years and has been implicated in both normal and disease processes the regulation of this enzyme and what factors combine to switch on its expression still remain somewhat of a mystery. Recently, however, some exciting findings have brought us closer to understanding the conditions which trigger the expression of this important MMP family member. Initial studies showed that the matrilysin gene contained a TRE (TPA response element, now known as an AP-1 site) and several ETS sites within the promoter region. These studies also showed that removal of these sites reduced the promoter activity and suggested that the transcription factors bound to the ETS and AP-1 elements acted synergistically in the activation of the matrilysin promoter (Gaire *et al.*, 1994).

In the past two years a key finding by Crawford *et al.* (1999) has been the identification of the role which β-catenin plays in the activation of matrilysin gene expression through its interaction with the proximal Tcf site in the matrilysin promoter. APC is responsible for the 'tagging' of β-catenin for degradation by ubiquitin but when APC is mutated the degradation of β-catenin in the cytoplasmic pool does not take place. The β-catenin protein subsequently translocates to the nucleus and is involved in the transcription of several target genes including matrilysin. The fact that APC is mutated in many cases of early human colorectal cancer may also explain why matrilysin is associated with the early stages of colon tumourigenesis. This would indicate that matrilysin expression, although initiated as a result of the expression of mutated APC protein, may not be directly involved in early tumour growth. However, experiments by Wilson *et al.* (1997) have shown that elimination of the matrilysin gene results in decreased number and tumour size when matrilysin null mice are crossed with APC Min mice. Therefore, it is obvious that matrilysin expression, although switched on perhaps after cells receive an APC mutation, is subsequently involved in the tumour growth. Studies have also shown that while β-catenin derepression of the matrilysin gene is an essential requirement for transcription to take place the presence of β-catenin alone does not promote expression of the gene indicating that other factors are also required (Crawford *et al.*, 1999).

We have shown earlier in chapter 3 that EGF stimulated matrilysin gene expression via RT-PCR, western blot and promoter-reporter analysis in a variety of cell lines. Other factors such as IL-6 and bFGF were also shown to stimulate matrilysin gene activity, even more so when the cells were treated with combination 'cocktails' of these cytokines. However, in chapter 4 we were primarily interested in the mechanisms of how EGF was increasing matrilysin gene transcription and in determining the identity of the transcription factors which caused this increase.

EGF has been shown to stimulate a number of pathways which ultimately lead to the activation of several transcription factors including AP-1 and ETS family members (Lenormand *et al.*, 1993). We therefore initially tested a number of artificial promoters that contained either AP-1, ETS or PEA3 repeat sequence motifs to which AP-1, ETS or PEA3 transcription factors could bind. Treatment with EGF showed that the PEA3 artificial promoter gave up to a 100% increase over control in the cell lines examined (SW480, HCT116 and HCA7) suggesting that PEA3 transcription factors were being stimulated by EGF treatment. AP-1 and ETS artificial promoter responses were also increased but not to the same extent as the PEA3 promoter. This increase was expected since EGF can stimulate both AP-1 and ETS transcription factors via the MAPK pathway. From this data it was possible that PEA3 or any of the ETS family members could be responsible for the activation of the matrilysin promoter. As mentioned earlier, interesting work by O'Hagan and Hassell showed that in breast cancer, increases in the EGF receptor related Her2/*neu* gene product were associated with an increase in PEA3 transcription factors (O'Hagan and Hassell, 1999). PEA3 is a member of the ETS family of transcription factors and analysis of the matrilysin promoter reveals three potential PEA3 binding sites within the promoter. We therefore hypothesized that a possible method of stimulating matrilysin gene activity by EGF may be via an increase in ETS transcription factors such as PEA3. We subsequently analysed if EGF was capable of stimulating PEA3 transcription factors and if this increase was associated with increased binding to the PEA3 sites within the matrilysin promoter.

Analysis via western blotting showed that EGF increased PEA3 transcription factor levels after 6 hours and that the level of the phosphorylated species of PEA3 also increased. The increase in the level of phosphorylated species by EGF can be accounted for through EGF's activation of the MAPK and associated phosphorylation pathways. EGF has been shown to mediate its signalling cascade via Ras which in turn has been shown to upregulate a number of cascades including MAPK (O'Hackel *et al.*, 1999). The terminal signalling molecules in this pathway may therefore be responsible for the phosphorylation, hence activation, of the nuclear PEA3 transcription factors. The increase in levels of PEA3 protein may also be due to the fact that the PEA3 gene, in a similar fashion to other Ets family members is capable of (Chen *et al.*, 1995 and Seth and Papas, 1990). Therefore increases in phosphorylation of the PEA3 protein may result in the increased expression of the PEA3 gene which in turn could lead to increased nuclear levels of active PEA3 protein. To examine if the increase in PEA3 activity resulted in increased binding to the PEA3 sites within the matrilysin promoter we initially used an optimal PEA3 probe which showed that in all the cell lines tested, EGF treatment resulted in increased binding to the matrilysin promoter. The same was also found when the individual PEA3 sites within the matrilysin promoter were examined. It should be noted that there is some non-specific binding to the PEA3 probes. In order to clearly confirm that PEA3 was binding to the probes, various controls should be employed such as the use of recombinant PEA3 as a positive control and the use of mutated PEA3 probes as a negative control. Interestingly, PEA3 binding activity in the control samples was quite high and this may be due to the fact that several of the cell lines examined have activated Ras or other oncogenic mutations, such as p53, leading to an overall increase in growth promoting transcription factors, hence gene transcription. This may also explain why matrilysin levels in all the cell lines tested, with the exception of SW480, were so high.

Confirmation that PEA3 transcription factors were responsible for the increased binding to the PEA3 sites within the matrilysin promoter was achieved by 'supershift' EMSA. However only partial shifting of the band was observed indicating that subfamily members of the PEA3 family, ER-81 and ERM may also be involved. ER-81 and ERM have also been shown to be upregulated in breast cancers where Her2/Neu is activated (O'Hagan and Hassell, 1999).

Our findings suggest that PEA3 transcription factors play an important role in effecting the transcription of matrilysin and that the levels within the cell can be augmented by EGF. A recent report by Crawford *et al* (2001) is also in agreement with our studies in respect to the importance of the PEA3 family of transcription factors and their essential role in the regulation of matrilysin gene transcription. Crawford *et al.* (1999) had previously shown that the matrilysin gene was a target of the β-catenin/Tcf transcription factor complex and although this interaction was necessary for matrilysin gene activation, it was not enough on its own to activate matrilysin transcription (Crawford *et al.*, 2001). This inferred that co-activation of matrilysin gene transcription with other transcription factor proteins bound to the matrilysin promoter was necessary. Crawford *et al.* (2001) used a human embryonic kidney 293 (HEK293) cell line and co-transfected the HEK293 cells with the matrilysin promoter and a number of Ets family members including, Ets-1, Ets-2, PU.1, Fli-1, ER-81, ERM, PEA3 and the AP-1 binding protein Jun. Analysis of the results showed that all the Ets family members or Jun when transfected in isolation with the matrilysin promoter showed only minimal increases in promoter activity. However when the cells were transfected with Jun/PEA3 or Jun/ER-81 or Jun/ERM an induction in matrilysin promoter activity of over 50 fold was observed thus suggesting that the matrilysin promoter preferentially binds members of the PEA3 subfamily of transcription factors (Crawford *et al.*, 2001). These data suggested that a number of elements are required for activation of the matrilysin gene. This is in agreement with earlier studies in chapter 3 which showed that treatment of cells with EGF and TPA resulted in a significant increase in matrilysin promoter-reporter activity over control. From our findings it is therefore possible that all three members of the PEA3 family, which can be stimulated by EGF, interact with the PEA3 probes and this is why a full supershift with the α-PEA3 antibody was not observed. Reports have also shown that the majority of colon cell lines used in this study (HCT116, SW480, HCA7 and SW620) express large amounts of PEA3, ER-81 and ERM (Crawford *et al.*, 2001).

Further studies by Crawford *et al.* (2001) have shown that all matrilysin expressing tumours of the Min mouse (APC -/-) expressed all members of the PEA3 sub-family as well as all colon cell lines examined. This was also the first report of PEA3 subfamily members in intestinal tumours. Their frequent expression in Min mouse

tumour cells and human colon tumour cell lines suggests that members of the PEA3 subfamily are targets of a common early alteration in a tumour associated signalling pathway. Ets factors have been described as targets of Ras signalling (Wasylyk *et al.*, 1998). However, Min mouse adenomas do not have activated Ras (Shoemaker *et al.*, 1997). Thus if Ras signalling is involved in PEA3 regulation in intestinal tumours it is just as likely to be as a result of extracellular signals mediated by Ras such as EGF-r signalling. EGF-r signalling as discussed earlier has been shown to be of relevance in human colon tumour progression and has very recently been implicated in Min mice tumour formation (Coffey *et al.*, 1992 and Torrance *et al.*, 2000). This therefore is in agreement with our data which shows that EGF upregulates matrilysin gene expression through the activation of PEA3 transcription factors.

From gel shift assays examining the binding of PEA3 transcription factors it was observed that each of the PEA3 sites within the matrilysin promoter was capable of binding PEA3 and that each site did so with more or less the same affinity. Experiments where each of the PEA3 sites within the matrilysin promoter were mutated also showed that 'knocking out' one site led to only partial reduction in promoter activity in comparison to the wild type promoter. Treatment of the mutated promoters with EGF also increased their activity. These experiments suggest that all of the PEA3 sites within the matrilysin promoter play an important part in the regulation of matrilysin. Interestingly the PEA3 site at –144 overlaps with a potential Kaiso site, while the PEA3 site located at –55 bp downstream of the transcription initiation site overlaps with a potential C/EBP site. It may be possible that the interactions between PEA3 and Kaiso or C/EBP or their competition with each other may provide another means of matrilysin regulation.

Having determined that EGF has a direct effect on matrilysin expression through the activation of the PEA3 family of transcription factors we subsequently analysed if EGF could modulate the expression/activity of PEA3 transcription factors via its interaction with the E-cadherin/catenin complex. We also investigated if EGF mediated the release of β-catenin which would result in increased interaction with the Tcf sites within the matrilysin promoter. Results with the PEA3 artificial promoter show that cotransfection with the full length E-cadherin protein resulted in a decrease in PEA3 activity. The presence of excess E-cadherin in the cells may have resulted in an increase

of phosphatase activity as E-cadherin associates with a number of protein tyrosine phosphatases in order to form tight adherens junctions (St. Croix *et al.*, 1998). Thus an increase in the level of cellular E-cadherin may lead to a suppression of the phosphorylation cascades by these phosphatases which results in a decrease in the activity of the PEA3 promoter. Treatment of the cells with EGF showed only a slight increase in PEA3 promoter activity in the presence of E-cadherin. If EGF was involved in effecting PEA3 promoter activity via the destabilisation of the cadherin/catenin complex and the release of β catenin then a significant increase in PEA3 promoter activity would have been expected as the 'free pool' β-catenin 'mopped up' by the excess E-cadherin would have been released due to phosphorylation via EGF treatment. However, this was found not to be the case. In order to confirm that EGF did not affect PEA3 promoter activity via cadherin/catenin destabilisation we transfected an E-cadherin construct which had a deletion in the region where β-catenin binds into the cells. Once again if β-catenin was involved in the activity of PEA3 transcription factors then it would be expected that no decrease in PEA3 activity should have been observed as the mutated E-cadherin protein would be unable to 'mop up' β-catenin located in the cytoplasmic pool and this was found to be the case. In both the wild type and mutated E-cadherin transfections, treatment with EGF resulted in slight increases in PEA3 artificial promoter activity which may have been due to the increase in phosphorylation activity mediated by the activated EGF-r. Transfection of cells with the PEA3 artificial promoter, E-cadherin or mutated E-cadherin and β-catenin again showed no increase in the activity of PEA3 suggesting that EGF interaction with the E-cadherin catenin complex does not lead to an increase in PEA3 promoter activity.

Several studies have documented the effects of β-catenin on matrilysin gene expression and many have shown that mutations in APC lead to an increase in β-catenin levels which in turn aids in the transcription of matrilysin (Brabletz *et al.*, 1999 and Crawford *et al.*, 1999). In normal cells β-catenin is sequestered by E-cadherin in the formation of the adherens junctions which serves to form tight monolayers of cells. However, in order for matrilysin to be expressed by a normal cell line this would involve the release of β-catenin from the E-cadherin/catenin complex. Several earlier reports have also shown that increases or decreases in E-cadherin activity lead to repression or

expression respectively of several MMPs and proteases (Frixen *et al.*, 1993, Llorens *et al.*, 1998 and Miyaki *et al.*, 1995). As mentioned earlier EGF is thought to be involved in the destabilisation of the cadherin/catenin complex through its interaction with β-catenin (Kinch *et al.*, 1997, Lampugnani *et al.*, 1997, Roura *et al.*, 1999, Solic and Davies, 1997). The matrilysin promoter contains two potential Tcf sites with which β-catenin can interact.

EMSA analysis in chapter 4 examining the relationship between EGF and the E-cadherin/catenin complex showed that the proximal Tcf site located –109 bp downstream from the transcription initiation start site had increased binding of β-catenin protein. The Tcf site further downstream at –194, which is inverted, showed little or no binding of β-catenin. These data suggested that another possible mechanism of EGF upregulation of matrilysin gene expression was via its interaction with the E-cadherin/catenin complex and the subsequent release of β-catenin which in turn leads to enhanced matrilysin expression in the absence or mutation of the APC protein.

EGF has been shown for several years to be involved in many aspects of cell morphology. EGF is a potent mitogen and in order for the cell to replicate it must 'loosen' its bonds to other cells and this is most likely achieved via the destabilisation of the cadherin complex. One of the effects of this would be the upregulation of MMPs such as matrilysin which would subsequently be involved in the degradation of the ECM, thus aiding the 'rounding up process' of the cell. EGF has been shown to involved in the progression of many cancers including colon cancers (De Luca *et al.*, 1997 and Maleckapanas *et al.*, 1997). It may therefore be plausible that overexpression of EGF or EGF-r by the tumour cell could be involved in the increased proliferation and the upregulated expression of matrilysin which is also involved in increased tumour proliferation and increased invasiveness. Evidence also exists which links matrilysin directly to E-cadherin. Recently it has been shown that matrilysin is capable of cleaving the ectodomain of E-cadherin from the surface of MCF-7 breast cells. This soluble E-cadherin ectodomain can promote tumour invasion through its interaction with uncleaved E-cadherin thus preventing the cells forming tight junctions. The cells as a result are loosely bound to each other and subsequently can invade more readily (Noe *et al.*, 2000).

Overexpression of EGF or the EGF-r would therefore lead to the destabilisation of the cadherin/catenin complex. This destabilisation results in the increase of cytoplasmic β-catenin which translocates to the nucleus and effects the expression of matrilysin which in turn, once secreted, can prevent the formation of tight adherens junctions due to its 'sheddase' effect on E-cadherin. The inability of E-cadherin to form a stable cadherin catenin complex may subsequently lead to a reduction in receptor tyrosine phosphatase activity and stop the inhibition of the phosphorylation cascades and promote cell growth and tumourigenicity.

Thus far we have observed that EGF stimulated matrilysin gene transcription via the PEA3 family of transcription factors and also through the destabilisation of the cadherin/catenin complex. If EGF is capable of stimulating PEA3, β-catenin and as other studies have shown AP-1 transcription factors then one would expect to see a higher increase over control levels when cells were treated with EGF. Studies have shown that the interaction between each of these elements is essential for matrilysin gene transcription and that each of these factors when present act in a synergistic manner whereby high increases in matrilysin promoter activity are observed (Crawford *et al.*, 2001). Cell lines used in this study were colon cancer cell lines with various oncogene mutations including Ras and p53. EGF mediates its effects via several pathways and in many of these pathways, Ras is the effector molecule. Therefore one would expect to see a high level of activated transcription factors including those of the ETS and AP-1 family. This may partially explain why such high levels of endogenous matrilysin were observed in the cell lines used with the exception of the SW480 cell line. Some of the cell lines investigated also contain β-catenin mutations such as HCT116 which again would increase matrilysin as excess levels of β-catenin are not processed for degradation due to poor interaction with APC. Treatment of these cell lines with EGF may therefore only augment the level of matrilysin transcription which would explain why only slight increases in matrilysin gene expression were observed in the colon cancer cell lines.

4.3.1 Hypothesis of EGF regulation of matrilysin gene expression

Recent evidence has shown that in order for the matrilysin gene to be transcribed a number of factors/complexes such as PEA3, AP-1 and β-catenin/Tcf must be bound. We have shown here that EGF may regulate the transcription of matrilysin by stimulating the activity of PEA3 and also through its interaction with β-catenin via the destabilisation of the E-cadherin/catenin complex at the adherens junctions. As discussed earlier PEA3 is upregulated in breast cancer patients due to the overexpression of the EGF-r related receptor Her2/Neu and Crawford *et al.* (2001) have also shown that PEA3/ERM/ER-81, and not other Ets family members are required for matrilysin gene transcription. We therefore hypothesise that the increases observed in matrilysin gene activity in chapter 3 may be as a result of EGF stimulating the activity of the PEA3 gene and the activity of the PEA3 transcription factor through phosphorylation pathways associated with EGF-r signalling including MAPK. EGF may also cause increases in other transcription factors in the cell nucleus through its interaction with E-cadherin bound β-catenin (see figure 4.15).

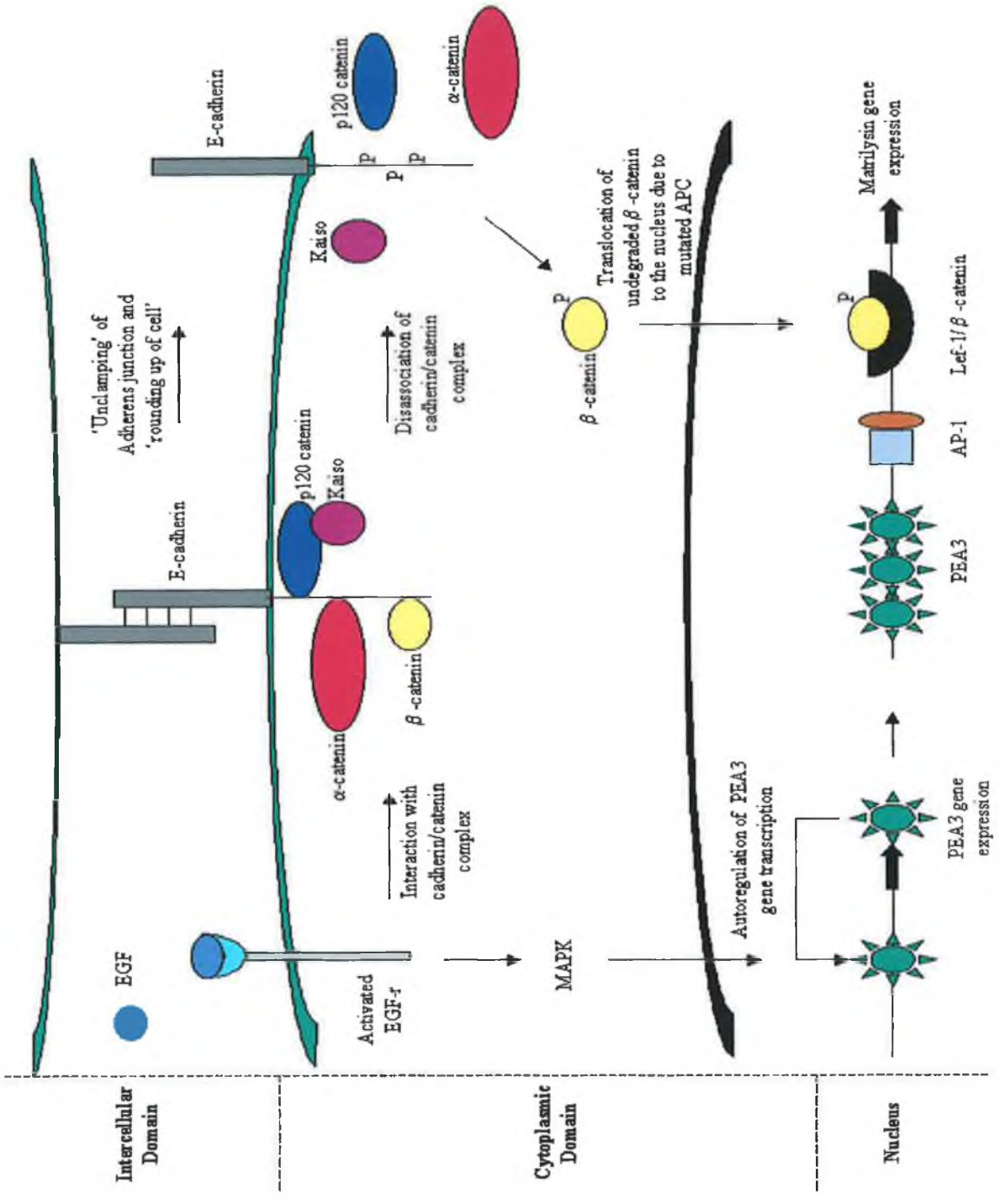

Figure 4.15 Hypothesis for the mechanism of EGF induction of matrilysin gene transcription. EGF may upregulate matrilysin gene transcription via increasing levels of PEA3 transcription factors which in turn bind to the PEA3 sites within the matrilysin promoter. The activated EGF receptor may also play a role in the destabilisation of the cadherin/catenin complex which results in the release of β-catenin into the cytoplasm. β-catenin which is not targetted for degradation translocates to the nucleus where it interacts with several target genes including matrilysin.

4.4 Conclusions

Matrilysin has been shown to be expressed in many cancers and is involved in several aspects of tumour biology including tumour growth, progression and angiogenesis. Combining these characteristics of matrilysin with the fact that it is unique amongst other MMPs whereby it is expressed only by the tumour epithelial cells makes matrilysin an interesting therapeutic target. In order to develop drugs which specifically inhibit matrilysin expression it is important to understand how it is 'switched' on during the tumourigenic process.

We have shown that matrilysin gene expression can be upregulated by EGF through its activation of the PEA3 transcription factor family. This conclusion is in agreement with other studies which have shown that PEA3 transcription factor family members preferentially bind to the matrilysin promoter and effect its transcription (Crawford *et al.*, 2001). We have also shown that each of the PEA3 sites within the matrilysin promoter are essential for activation of matrilysin gene transcription. Recent studies have shown that *APC* mutations, common in many colon cancer patients, lead to an increase in cytoplasmic β-catenin which tranlocates to the nucleus and effects the transcription of several target genes including matrilysin. We have shown that EGF interaction with the cadherin/catenin complex results in the release of β-catenin which does not affect the activity of the PEA3 transcription factors but does bind and interact with the proximal Tcf site located in the matrilysin promoter. This therefore represents another mechanism via which EGF can mediate its effect on the matrilysin gene.

Our data suggests that EGF plays an important role in the regulation of matrilysin gene expression via a number of different mechanisms. Aberrant expression of EGF or the EGF-r by tumour cells may therefore result in enhanced matrilysin expression which has been shown to be involved in early stages of colon/breast tumourigenesis and in the invasion and metastasis of several cancers.

Chapter 5

The expression and regulation of matrilysin in leukaemia cell lines and its involvement in leukaemia invasion and metastasis.

5.1 The haematopoietic process

The process of haematopoiesis can be described as the proliferation and differentiation of progenitor stem cells into mature blood cells and these events are orchestrated by the complex interactions between the haematopoietic stem cells and the surrounding bone marrow microenvironment (Sawyers *et al.*, 1991). The different types of blood cells and their immediate precursors can be recognised in the bone marrow by their distinctive appearances. In the marrow, the blood cells are intermingled with each other as well as with fat cells and stromal cells that produce a delicate supporting meshwork of collagen fibres and other extracellular matrix components. In addition, the whole tissue is richly supplied with thin walled blood vessels (also known as blood sinuses) into which the new blood cells are discharged. Within the bone marrow exist stem cells which are the precursors to all blood cells. These cells give rise to colony forming units (CFU) daughter cells of either the myeloid or lymphoid variety which in turn give rise to highly specialised sets of sub cells. Therefore the stem cell within the bone marrow matrix is known as a 'pluripotent' stem cell, in that it gives rise to all blood cell types. Pluripotent stem cells are found in low numbers in bone marrow and not many of them are actively cycling. The normal behaviour of stem cells has been inferred by transplantation experiments that use retroviral vector to create specific integration events and clonal markers. The independent viral integration sites are used to define clonal progeny of specific stem cells. The overall impression from such studies is that the growth pattern of the stem cells can best be described by the sequential entry of limited numbers of stem cells into cycle and these then dominate multiple lineages in the peripheral blood and lymphoid organs. Presumably, if the progeny of a limited number of stem cells can dominate the peripheral blood for extended periods of time there must be an appreciable expansion of that stem cell clone, as well as expansion of the committed progenitors for multiple lineages.

Once a cell has differentiated into an erythrocyte or granulocyte or some other type of blood cell there is no turning back, i.e. the state of differentiation is not reversible. Therefore at some stage in their development, some of the progeny of the pluripotent stem

cells must become irreversibly committed to a particular line of differentiation. It is clear from microscopic analysis that this commitment occurs long before the final division in which the mature differentiated cell is formed. One can also recognise specialised precursor cells that are still proliferating but already show signs of having begun differentiation. It thus appears that commitment to a particular line of differentiation is followed by a series of cell divisions that amplify the cells of a given specialised type.

The haematopoietic system can, therefore, be viewed as a hierarchy of cells. Pluripotent stem cells give rise to committed progenitor stem cells which are irreversibly determined as ancestors of only one or a few blood cell types. The committed progenitors divide rapidly but only a limited number of times. At the end of this series of amplification divisions, they develop into terminally differentiated cells, which usually divide no further and die after several days or weeks (see figure 5.1).

Although the proliferation rate of developing bone marrow cells is impressive there are a number of checks and balances which limit the overall cellularity of and response to stresses such as irradiation or bleeding. There are many examples in the literature on specific negative regulators of stem cell and other lineage committed cell types. Some of these effects are mediated by soluble factors such as transforming growth factor-β (TGF-β). Several reports have described the inhibition of stem cells by large granular lymphocytes that require cell-cell contact for function (Barlozzari *et al.*, 1987). In all these cases it is worthwhile considering how the normal stem cell and various leukaemias escape this down-regulation. An understanding of these controls could directly relate to the excessive-growth phenotype of leukaemias and lymphomas. Our understanding of the molecular mechanisms regulating the formation, growth and differentiation of haematopoietic stem cells is also important in identifying why blood cells become leukaemic and considerable advances in this respect have been made recently. Particular progress has been made in defining the cytokines, chemokines and extracellular matrix components which retain and maintain primitive haematopoietic cell populations in bone marrow. Furthermore signal transduction pathways that are critical in haematopoiesis and are activated by cytokines have also been identified and further characterised.

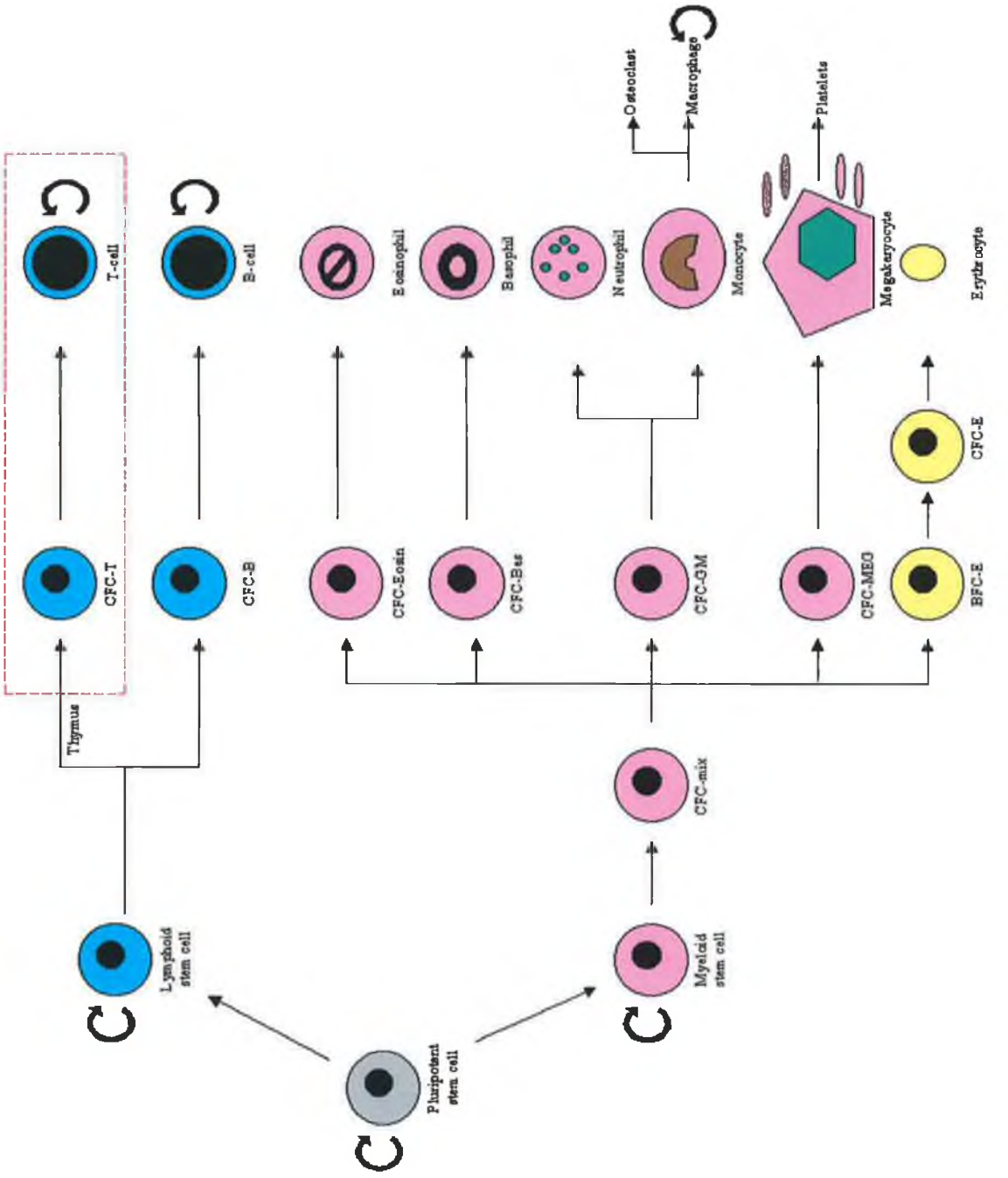

Figure 5.1 The haematopoietic process. The pluripotent stem cell normally divides infrequently to give rise to more pluripotent stem cells or committed progenitor cells, labelled CFC for colony forming cells. The progenitors are stimulated to proliferate by specific growth factors but progressively lose their ability to divide and develop into terminally differentiated blood cells which live for only a few days or weeks. In mammals all of the blood cells are made in the bone marrow with the exception of the T-cells which mature in the thymus (outlined in red).

5.2 Leukaemia

As discussed earlier, during the haematopoietic process, developing blood cells undergo rapid division and differentiation. Mutations in the machinery controlling these processes often leads to serious haematological disorders such as leukaemia. Leukaemic cells have escaped the constraints governing the growth of normal myeloid cells in that they do not require growth factors to survive and proliferate.

5.2.1 Chronic myeloid leukaemia

There are several types of leukaemia of which chronic myeloid leukaemia (CML) is perhaps the most extensively studied. CML cells have characteristic cytogenetic (Philadelphia chromosome) and molecular (the *bcr-abl* fusion gene) markers. The chromosomal abnormality involves the translocation of the c-*abl* tyrosine kinase proto-oncogene to a 6 kb breakpoint cluster region (*bcr*) composed of four small introns and exons, which are part of the larger gene called *bcr*. This translocation results in the transcription of a fused protein product, p120. Experimental analysis and patient studies have shown that the *bcr* sequence activates the tyrosine kinase activity of c-Abl and converts Abl to a transforming protein for haematopoietic cells (Sawyers *et al.*, 1991).

CML is a stem cell disease as the Philadelphia chromosome is found in all haematopoietic lineages in patients with this malignancy, but not in skin fibroblasts or bone marrow stromal cells. The clinical phenotype of the disease is expansion of the myeloid compartment during the initial phase but with retention of full differentiation. This initial phase termed chronic is followed by eventual progression to acute leukaemia which is characterised by circulating undifferentiated lymphoid or myeloid blast cells (see figure 5.2). Nearly all patients with chronic phase CML express a 210 kDa BCR-ABL protein whereas patients with Ph-positive acute lymphoblastic leukaemia express either a 210 kDa or a 190 kDa BCR-ABL protein (Sawyers, 1999). Recently, a larger 230 kDa BCR-ABL fusion protein was found in a sub group of patients with CML who presented with a lower white blood cell count than is usual for the disease and in whom progression

to blast crisis was slow (Pane *et al.*, 1996). The fact that fusion proteins of different sizes can be correlated with different outcomes has led to laboratory studies of the biologic activity of the proteins. The results indicate that the 190 kDa BCR-ABL protein has greater activity as a tyrosine kinase and is a more potent oncogene than the 210 kDa protein, suggesting that the magnitude of the tyrosine kinase activity affects the expression of the disease (Voncken *et al.*, 1995).

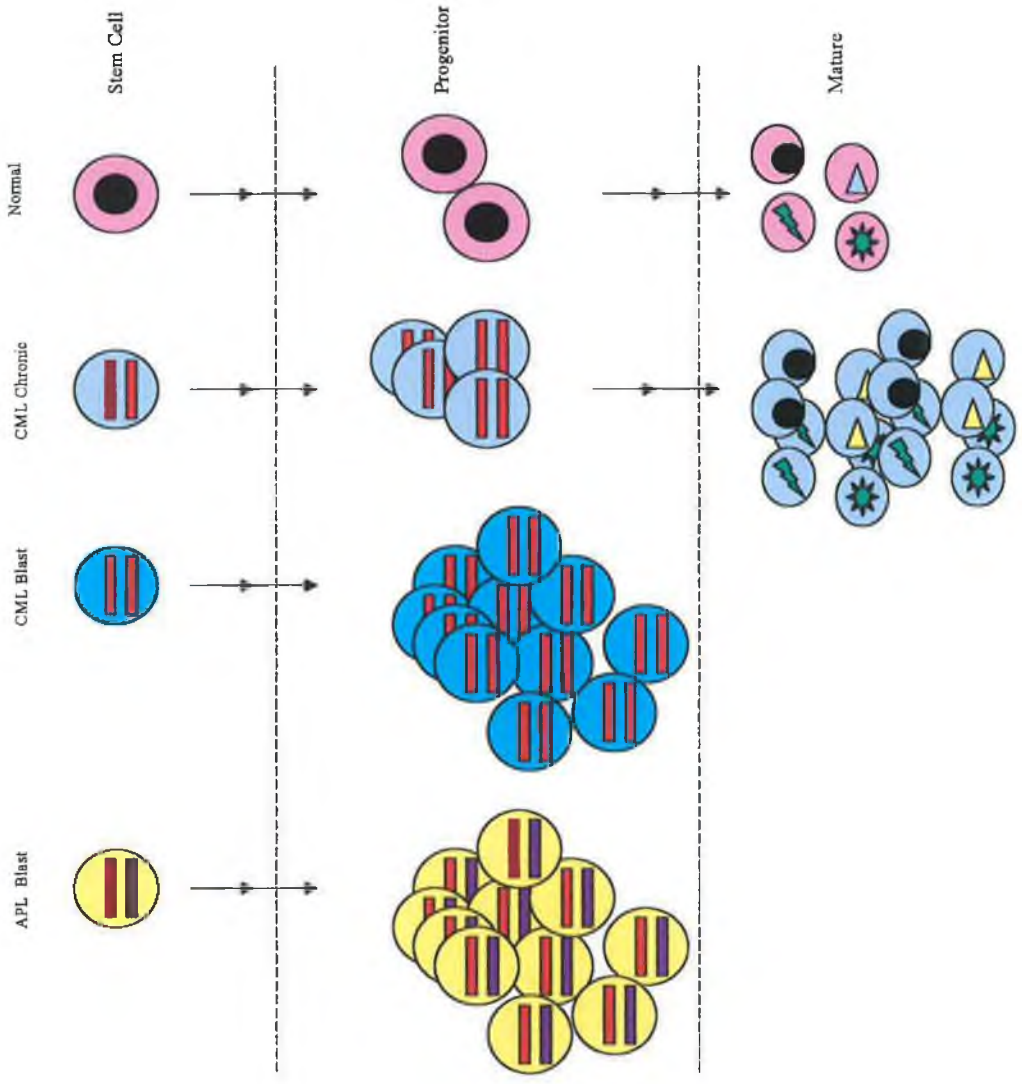

Figure 5.2 The progression of chronic myelogenous and acute myelogenous leukaemia. Mature blood cells develop from pluripotent stem cells though progenitor cells. In CML the *bcr-abl* fusion occurs in the stem cell and is present in all haematopoietic lineages. In the chronic phase of CML the number of myeloid progenitors and mature myeloid cells is increased, but differentiation occurs normally. During blast crisis CML, differentiation is arrested at the progenitor stage and the phenotype resembles that of acute leukaemia diseases such as APL (acute premyelocytic leukemia).

Studies of the *bcr-abl* gene fusion have established that it is a potent oncogene which induces leukaemias in animals. Transgenic expression of the 190 kDa BCR-ABL protein in mice causes acute leukaemia at birth suggesting that it confers a potent oncogenic signal in haematopoietic cells (Heisterkamp *et al.*, 1990). Another animal model examining the effects of the expression of the *bcr-abl* genes involved the infection of the gene into the haematopoietic stem cells of normal mice using a retro virus carrier. In these animals a range of acute leukaemias develops that varies in strains with different genetic backgrounds (Kelliher *et al.*, 1990). These findings suggest that the pathogenesis of CML is a multistep process. Studies into the function of the BCR-ABL proteins have revealed that the protein can transform haematopoietic cells so that they can grow independent of cytokines (Gishizky and Witte, 1992). It has also been shown to protect the cells from programmed cell death in response to cytokine withdrawal or radiation (Nishii *et al.*, 1996). However, in primary CML cells the effects of the tyrosine kinase appear to be less dramatic, in particular with respect to apoptosis. BCR-ABL has also been shown to increase adhesion of the haematopoietic cells to the ECM by increasing integrin activity (Bazzoni *et al.*, 1996). One mechanism of this effect may involve the BCR-ABL substrate CRKL, which induces adhesion when it is phosphorylated, by allowing the assembly of focal adhesion complexes (Senechal *et al.*, 1998). Curiously, primary CML cells adhere poorly to the bone marrow stroma *in vitro*. These data appear to be in contrast with the data presented by cells engineered to express the BCR-ABL protein and will require further studies. Nevertheless, the adhesion defect offers another mechanism for leukaemogenesis, since CML cells may escape negative regulatory influences that stromal cells normally exert on haematopoietic cells through contact between stromal and stem cells.

Biochemical studies show that the BCR-ABL protein is a constitutively active tyrosine kinase confined to the cytoplasm whereas wild type ABL shuttles between the nucleus and the cytoplasm (Lewis *et al.*, 1996). As a consequence of increased tyrosine kinase activity, the BCR-ABL protein can phosphorylate several substrates thereby activating various signal cascades affecting the growth and differentiation of the cells. Substrates of the BCR-ABL protein include CRKL, p62Dok, paxillin, CBL and Rin which

subsequently utilise pathways such as RAS, JNK PI-3K and STAT (see figure 5.3). It is not clear how BCR-ABL protein activates these pathways but an emerging theme is that it operates via the same mechanisms as cytokine receptors that control the growth and proliferation of haematopoietic cells. Since the *BCR-ABL* gene is constitutively expressed, these cells escape constraints on normal growth and become leukaemic.

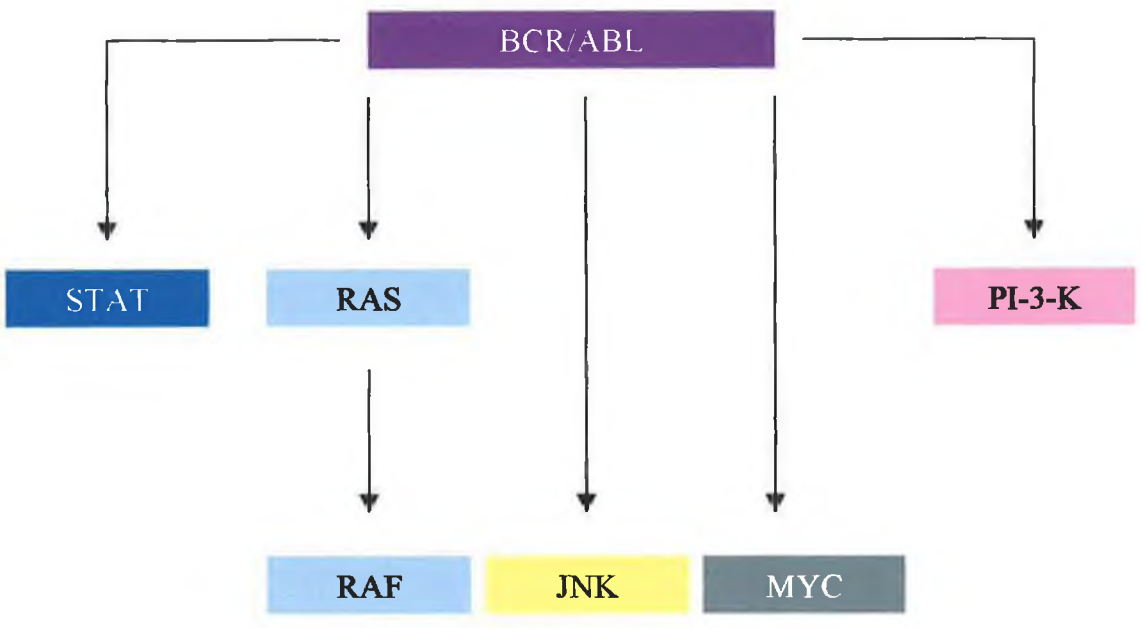

Figure 5.3 Signal transduction by the BCR/ABL protein. Constitutive activation of its tyrosine kinase domain causes the BCR/ABL protein to activate a number of cytoplasmic and nuclear signal transduction pathways that affect the growth and survival of haematopoietic cells.

5.2.2 Acute promyelocytic leukaemia

Other common forms of leukaemia include the acute myeloblastic leukaemias (AML). Of these, acute promyelocytic leukaemia (APL) accounts for approximately 10% of cases (Warrell *et al.*, 1993). APL was first recognised as a distinctive clinical entity in the 1950s and the disease typically presents with bleeding diathesis leading to a relatively high rate of early mortality, primarily from intracranial hemorrhage. The mechanisms underlying the haematorrhagic diathesis have been comprehensively studied and it has been shown that several processes are involved, including accelerated intravascular coagulation, hyperfibrinolysis, and thrombocytopenia (Tallman and Kwaan, 1992).

Individual differences in the severity of these processes may explain divergent findings among clinical studies. Malignant promyelocytes release pro-coagulant substances that activate the coagulation cascade, generate thrombin and deplete fibrinogen.

Early studies showed that APL was consistently associated with a non-random chromosomal abnormality characterised by balanced and reciprocal translocations between the long arms of chromosomes 15 and 17. In 1987, the gene encoding retinoic acid receptor alpha (RAR-α) was mapped to chromosome 17q21 (Mattei *et al.*, 1988). The location of the chromosomal breakpoint in APL and the observation that all-*trans*-retinoic acid was clinically effective, prompted studies on the potential role of the RAR-α gene in this translocation. Subsequently several groups cloned the breakpoint and showed that the RAR-α gene was rearranged in all the patients tested. Moreover, the breakpoints on chromosome 15 were found to cluster tightly in a region containing a previously unknown gene, named PML. The 15:17 translocation results in the fusion of genes for PML and RAR-α which results in the synthesis of two reciprocal fusion transcripts, PML/RAR-α or RAR-α/PML (Alcalay *et al.*, 1992). Thus in acute promyelocytic leukaemia (along with other certain acute myelocytic leukaemias and chronic myelocytic leukaemia) a translocation generates a disease specific fusion protein that is involved in carcinogenesis.

The PML/RAR-α fusion protein contains the amino section of PML (with its putative dimerisation and DNA binding domains) and the hormone binding domains of RAR-α (Goddard *et al.*, 1991). Although the RAR-α breakpoints on chromosome 17 always occur in intron 2 the breakpoints on chromosome 15 are clustered in two different introns and one exon of PML leading to various fusion proteins. In leukaemic promyelocytes PML/RAR-α seems to be considerably more abundant than the wild type retinoic acid receptor encoded by the intact chromosome 17 (Pandolfi *et al.*, 1992). These observations have suggested that the aberrant PML/RAR-α may exert a dominant effect over the normal RAR-α gene product. The HL-60 cell line, derived from a patient with acute myeloblastic leukaemia differentiates into granulocytes when treated with retinoic acid, an effect which is mediated in part by the retinoic acid receptor. If retinoic acid induces maturation in promyelocytes, the hybrid receptor may contribute to

leukaemogenesis by dominantly antagonising differentiation induced by retinoic acid. At the molecular level, several mechanisms could explain the dominant effect of the PML/RAR-α fusion protein over the wild type retinoic acid receptor and PML proteins. Unlike RAR-α, which requires retinoid X receptors (RXRs) for dimerisation and function, PML/RAR-α can form a homodimer and thereby effect efficient binding to retinoic acid response elements in DNA (see figure 5.4). PML/RAR-α also appears to dimerize with normal RXR and PML gene products, potentially leading to the sequestration of RXR. Finally PML/RAR-α can act dominantly to delocalise PML from its normal nuclear position, a process which can be reversed by all-*trans*-retinoic acid (Warrell *et al.*, 1993).

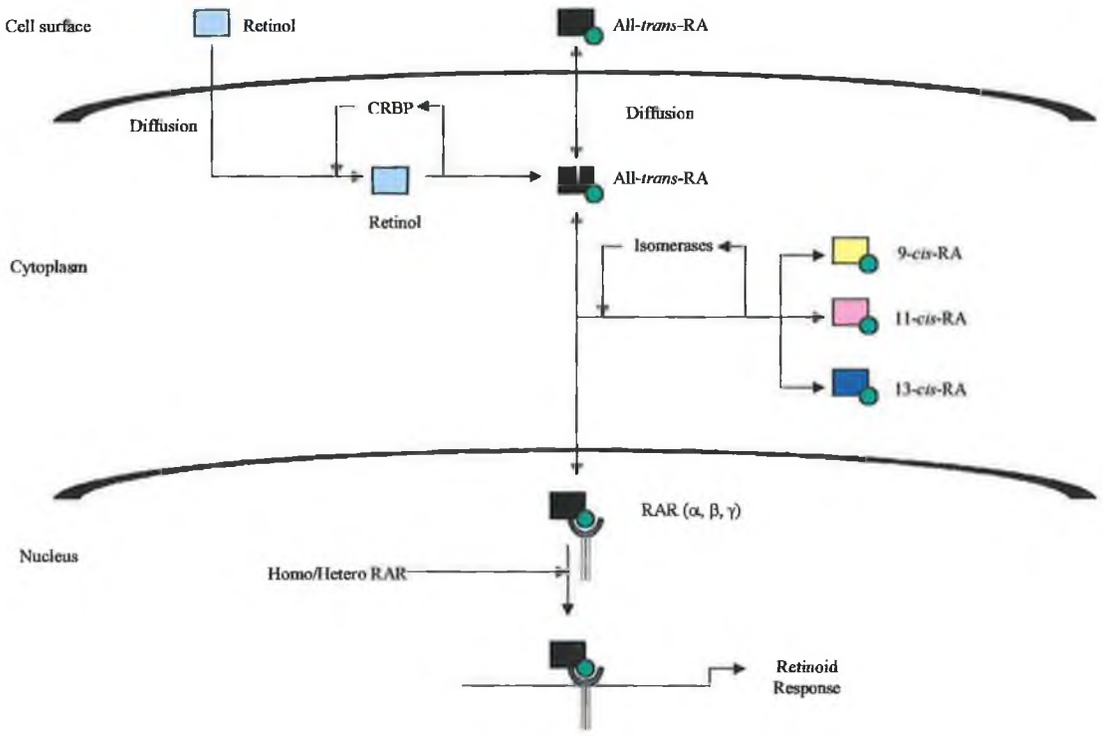

Figure 5.4 All-*trans*-retinoic acid enters the cell by simple diffusion or by conversion from retinol that has been absorbed from the gastro-intestinal tract. All-*trans*-RA (retinoic acid) can be metabolised immediately and located to the surface of the smooth endoplasmic reticulum (not shown). Alternatively, all-*trans*-RA or its isomers enter the cell nucleus and bind to RA receptors (RAR) or to retinoid X receptors (RXR). After dimerisation these RA activated receptors bind to specific DNA segments within the promoter region of target genes and facilitate their transcription. CRBP refers to cytochromic retinol binding protein.

5.3 MMPs and TIMPs in haematopoiesis

As previously discussed, the bone marrow microenvironment contains a heterogenous population of stromal cells which express a variety of ECM macromolecules and cytokines that serve to support developing blood cells and aid in their differentiation. The ECM macromolecules expressed by the stromal cells are mainly comprised of collagen types I-IV, fibronectin, vitronectin and laminin and are important not only in providing structural support but also in binding adhesion molecules and growth factors. Extensive studies have investigated the role of adhesion molecules and growth factors during haematopoietic cell development and in leukaemogenesis, however, the role of ECM degrading enzymes which are most probably produced by the haematopoietic and stromal cells has only recently begun to be explored.

The ECM of the haematopoietic microenvironment plays a pivotal role in the promotion of proliferation and differentiation of haematopoietic stem cells. The ECM within the bone marrow microenvironment is secreted by the stromal cells. In addition to providing a structural support for the cells the ECM provides discrete compartments in which different growth factors and cytokines are specifically localised and presented to the haematopoietic stem cells (Bussolino *et al.*, 1994). Specific domains in the ECM proteins have been identified as receptors for some growth factors and cytokines (Grimaud and Lortat-Jacob, 1994). Several studies have shown that the main mechanism of mature blood cell egress involves the down regulation of integrin receptors (Kerst *et al.*, 1993 and Levesque *et al.*, 1996). New evidence suggest that MMPs through their proteolytic action also aid in this process by virtue of their ECM degrading capacities. Several blood cell types have been found to secrete metalloproteinases, in particular MMP-2 and MMP-9. In cells of a lymphoid origin the expression of MMP-9 is uniform in comparison to MMP-2. MMP-9 expression is observed in reactive and malignant B-cells as well as unstimulated resting and activated T lymphocytes (Stetler-Stevenson *et al.*, 1997). Large granular lymphocytes isolated from human endometrium also expressed high levels of MMP-9 (Shi *et al.*, 1995). Activation of T-cells also resulted in the expression of active and latent MMP-9 as well as MMP-3 (Stetler-Stevenson *et al.*, 1997).

The activity of MMPs is effectively controlled by the tissue inhibitors of metalloproteinases whose characteristics have been outlined in chapter 1. TIMP-1 and TIMP-2 have been shown to be expressed in a variety of leukaemia cell lines including those of a CML and AML origin. TIMPs are multi-functional molecules and exert pleiotropic effects in that they not only regulate MMP protein activity but have also been shown to be involved in cell proliferation which suggests that they may exert some influence on cell growth modulation and cell signalling. Alternatively, this may be accomplished in a direct manner whereby TIMPs may positively or negatively regulate the actions of growth factors such as bFGF and TGF-β. The direct effects of TIMPs on cell growth modulation originated with the observation that TIMP-1 has erythroid potentiating activity (EPA). TIMP-1 is capable of stimulating the growth and differentiation of erythroid precursors, erythroleukaemic cell lines *in vitro* such as K562 as well as stimulating murine erythropoiesis *in vivo* (Hayakawa *et al.*, 1992 and Niskanen *et al.*, 1988). TIMP-2 has also been shown to promote the growth of erythroid precursors as well as acting as an autocrine growth factor for SV-40 transformed fibroblasts. A broad spectrum of mitogenic activity has been demonstrated for TIMP-2 in a number of cell lines including Raji and Burkitt's Lymphoma and HL-60 cells and the mitogenic activity of TIMP-2 was maintained when its inhibitory functions were destroyed through reductive alkylation (Edwards *et al.*, 1996)

5.3.1 Leucocyte associated metalloproteinases

A group of membrane associated enzymes that have been characterised as haematopoietic differentiation antigens have also been classified as zinc dependent endopeptidases. This group of enzymes which includes CD10/neutral endopeptidase 24.11 (CD10/NEP), CD13/amino peptidase N (CD13/APN) and BP-1/6C3/aminopeptidase A (BP-1/6C3/APA) have been well characterised and have been used as diagnostic markers for haematological malignancies (Shipp and Look, 1993). CD10 has been shown to be highly expressed in acute lymphoblastic lymphomas (ALL) and Burkitt's lymphomas, but is negative in more mature lymphocytes (Ritz *et al.*, 1981). CD13 is highly expressed by leukaemic blasts in acute myeloid leukaemias (Sobol *et al.*,

1987). Although little is known about their function in haematological cells these enzymes have been well characterised in non-haematological cells. All three enzymes have been found to be expressed in the epithelial brush border cells of the renal proximal tubules, small intestine and lung (Semenza, 1986). Various studies suggest that these enzymes are involved in the regulation of peptides which regulate cell proliferation. Bombesin like peptides are not only are potent mitogens, but also act in autocrine loops of small cell carcinomas of the lung. Hydrolysis of bombesin by CD10 inhibits cell growth, whereas inhibition of CD10 has been associated with tumour cell growth (Shipp *et al.*, 1991). Haematopoietic cells studies using mice suggest that CD10 regulates B-cell development by inactivating peptides that stimulate cell growth and differentiation. CD13 expression is restricted to cells committed to the granulocyte/monocyte lineage. Its enzymatic activity has been associated with the hydrolysis of chemotactic peptides which occurs in neutrophil mediated responses (Sakai *et al.*, 1987). The function of BP-1/6C3/APA has been studied in murine models and is believed to regulate the growth of early B cells activated by IL-7 induced proliferation.

5.3.2 MMPs/TIMPs and the inflammatory response

Metalloproteinases and their inhibitors are thought to play an important role in the ECM degradation that occurs as a consequence of the inflammatory response. Interactions between monocytes and T-cells induce the secretion of metalloproteinases at the site of inflammation (Goetzl *et al.*, 1996). Because of their potentially hazardous effects the MMPs are regulated at different steps during the inflammatory reaction. Although this control is mainly regulated by cytokines other factors such as prostaglandins can also induce metalloproteinases. For example, prostaglandin E 2 (PGE2) can induce MMP expression through a c-AMP dependent mechanism in monocytes and natural killer cells (Zeng *et al.*, 1996). Migration of monocytes and lymphocytes into extra lymphoid tissues requires penetration of the sub-endothelial basal lamina. After contact with the matrix proteins T-cells and monocytes are induced to produce MMPs. T-cells predominantly secrete MMP-2 and MMP-9 after stimulation with β_1 integrin, vascular cell adhesion molecule (VCAM) and by inflammatory mediators (Nikkari *et al.*, 1996). Expression of MMPs in T-cells is differentially regulated as studies

have shown that T-cells constitutively expressed MMP-9 whereas MMP-2 required induction (Leppert *et al.*, 1995). Macrophages have been shown to produce several types of MMPs depending on the antigens displayed on the T-cell surface. Cytokines from both Th1 and Th2 can either repress or stimulate the production of MMPs by macrophages (Campbell *et al.*, 1991). IL-1β and TNF-α are major pro-inflammatory cytokines produced by activated monocytes and they control interstitial collagenase, stromelysin-1 and MMP-9 in a post transcriptional manner. Furthermore these cytokines increase the expression of MMPs without affecting the levels of TIMP (Mauviel *et al.*, 1993 and Saren *et al.*, 1996). Unlike IL-1β or TNF-α, cytokines such as IL-4 and IFN-γ possess the capacity to inhibit MMP production and this also does not affect the expression of TIMPs. IL-10 is secreted by Th2 lymphocytes and is inhibitory to several macrophage functions. IL-10 and IL-6 have both been found to increase the expression of TIMP-1. IL-10 has also been shown to decrease the expression of MMP-9 (Lacraz *et al.*, 1995 and Roeb *et al.*, 1993). The ratio of MMPs:TIMPs changes throughout the inflammatory process in response to changes in the levels of cytokines. Therefore during the first hours of inflammation, pro-inflammatory cytokines are produced which enhance the basal levels of MMPs but not TIMPs. In the second phase of the response IL-6 is produced which enhances TIMP-1 expression. Following secretion of IFN-γ and IL-4, MMP expression is decreased without changing TIMP-1 levels. In the last phase the anti-inflammatory cytokine IL-10 is produced and maximum expression of TIMP-1 is reached whereas MMP expression returns to normal basal levels.

5.4 MMPs and TIMPs in leukaemia

The balance between MMPs and TIMPs in the bone marrow microenvironment during haematopoietic differentiation as well as leukaemic proliferation and dissemination/invasion has not been fully investigated. AML and CML in blast crisis are characterised by an excessive egress of leukaemic cells from bone marrow into peripheral blood followed by infiltration of organs, skin or mucous membranes. These movements which are normally restricted to functional mature leukocytes, include the necessity for the cells to cross matrix barriers and penetrate blood vessel walls, which is dependent on the catalytic modification of the ECM and basement membranes. As discussed in chapter

1 the expression of MMPs is an essential requirement for ECM and basement membrane degradation (Ries *et al.*, 1999).

Several reports have examined the expression of MMPs and TIMPs in various leukaemia patient samples and cell lines and have found in many cases that the expression of MMP-2 and MMP-9 often correlated with poor prognosis for the patient. Ries *et al.* (1999) studied the expression of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 in the bone marrow mononuclear cells (BM-MNCs) of patients with AML, CML, myelodysplastic syndromes (MDS) and healthy donors. Analysis of the samples found that all patients and donors expressed MMP-9 in the BM-MNC conditioned media. Interestingly MMP-2 was found to be expressed at high levels in patients suffering from primary (73%) and secondary (88%) AML while only 20% of the cases that were classified as AML in remission expressed MMP-2. In contrast MMP-2 expression was not detected in CML patients with the exception of those who had entered blast crisis. TIMP-1 expression was relatively low in all samples and interestingly, MMP-9 mRNA transcripts were found to be at a higher level in CML patients and healthy donors. TIMP-2 expression was also found in samples which expressed MMP-2 and the majority of MT1-MMP expression was found in primary AML samples. These data suggested that MMP-2 was a potential prognostic marker for the invasion of leukaemia cells. These observations for AML samples were in agreement with studies performed by Janowska-Wieczorek *et al.* (1999) who investigated AML patient samples and a number of leukaemic cell lines with respect to MMP-2, MMP-9, TIMP-1 and TIMP-2 expression. All were found to secrete MMP-9 and the majority of AML samples were found to secrete MMP-2. The AML patient samples were found to express TIMP-1 and TIMP-2 mRNA and protein. Normal steady state bone marrow immature progenitor cells were also examined and it was observed that neither MMP-2 or MMP-9 were expressed but more mature mononuclear cells from normal bone marrow expressed and secreted MMP-9. The upregulated expression of MMPs and TIMPs again links MMPs to the invasive phenotype of AML.

Although many studies have examined the expression of MMP-2 and MMP-9 in various leukaemias there are relatively few reports on how the MMPs are regulated in

leukaemias. However, one such study has investigated the relationship between IL-6 and the expression of MMP-2, MMP-9 and TIMP-1 in malignant Non-Hodgkin's Lymphomas (Kossakowska *et al.*, 1999). Non Hodgkin's lymphoma (NHL) represents a heterogenous group of tumours which vary in their biological aggressiveness and clinical course. High grade NHL have been shown to degrade ECM components *in vitro* and that MMPs, in particular MMP-2 and MMP-9, play an important role in this phenomenon. Elevated levels of MMP-2 and MMP-9 in high grade NHL correlate with a poor clinical outcome. Subsequent studies looked at the expression of cytokines and growth factors such as IL-1β, IL-10, IL-6, TNF-α, TGF-β and bFGF and determined whether these would affect the levels of MMP-2, MMP-9 and TIMP-1. Results showed that IL-6 correlated significantly with elevated mRNA expression of MMP-2, MMP-9 and TIMP-1. Use of *in vitro* invasion assays also showed that treatment of cells with IL-6 upregulated the invasive ability of a number of leukaemic cell lines which could be inhibited by recombinant TIMP-1 and α-MMP-9 and α-MMP-2 antibodies suggesting that the increase in *in vitro* invasive ability after IL-6 treatment was due to increased MMP-activity.

Reports have also shown that IL-6 is an important agent during the differentiation of various leukaemias. Various studies have shown that retinoic acid treatment of the acute premyelocytic cell line HL-60 induces differentiation of the cells towards the granulocytic lineage and has also been shown to induce the mRNA and protein expression of IL-6 and the IL-6 receptor subunit gp130. Subsequent analysis in a HL-60 cell line which was sensitive to RA differentiation showed that activation of the gp130 receptor by exogenous treatment with IL-6 potentiated the differentiating effects of retinoic acid (Xie *et al.*, 2000). The effect of IL-6 on the differentiation of CML cell lines such as K562 has also been observed. It has been extensively demonstrated that K562 cells can be induced to differentiate towards erythroid and megakaryocyte lineages by various differentiation inducers (Xie *et al.*, 1999). Treatment with phorbol myristate acetate induces the K562 cells to differentiate towards the megakaryocytic lineage and also induces the expression of IL-6 and the IL-6 receptor sub unit gp130. In a similar fashion to that observed in HL-60 cells, increased expression of IL-6 promoted the differentiation of K562 cells when treated with TPA.

5.5 Summary

All blood cells are derived from stem cells within the bone marrow. The bone marrow also provides 'scaffolding' for the developing blood cells in the form of extracellular matrix which is secreted by stromal cells. The ECM is important as it also docks various cytokines and presents them to the blood cells which in turn aids the differentiation process. Therefore remodelling of the ECM during haematopoiesis is an essential process which involves the expression of MMPs. Egress of mature blood cells from the bone marrow into the peripheral blood vessels also involves the expression of MMPs. However, leukaemia cells have also developed the ability to overexpress MMPs which aid in their invasion into the blood vessels. Studies thus far have focussed mainly on the expression of MMP-2 and MMP-9 and the MMP inhibitors TIMP-1 and TIMP-2 and many have determined that the expression of MMPs can be used as a diagnostic marker. Thus far no studies have examined the regulation or role of matrilysin in leukaemia invasion and metastasis. In chapter 5 we have examined the expression of matrilysin in two cell lines K562 and HL-60 and have also investigated whether matrilysin can be regulated by IL-6 or the phorbol ester, TPA.

5.6 Results

The K562 cell line is defined as a chronic myeloid leukaemia which was taken from a patient in blast crisis while the HL-60 cell line is an acute premyelocytic leukaemia. As discussed earlier the chronic myeloid leukaemias progress to blast crisis which phenotypically is similar to the acute form of leukaemia. The characteristics of both these cell lines are outlined in table 2.2.

5.6.1 *In vitro* invasive activity of leukaemic cell lines

Degradation of the ECM by leukaemia cells is an important event in the dissemination of cells and spread of disease (Kossakowska *et al.*, 1999 and Tapiovaara *et al.*, 1996). In this study we decided to examine the invasive activity of two leukaemia cell lines, K562 and HL-60 using an *in vitro* invasion assay. Briefly this assay involved plating the cells onto matrigel-coated filters and assaying the numbers of cells that invaded to the underside of the membrane. Our results showed that the K562 cell line was up to four times more invasive than the HL-60 cell line (2.33% +/- 0.21% for K562 vs 0.7% +/- 0.16% for HL-60) (Figure 5.5). Two tailed T-tests showed that the results obtained were significant (P value < 0.005).

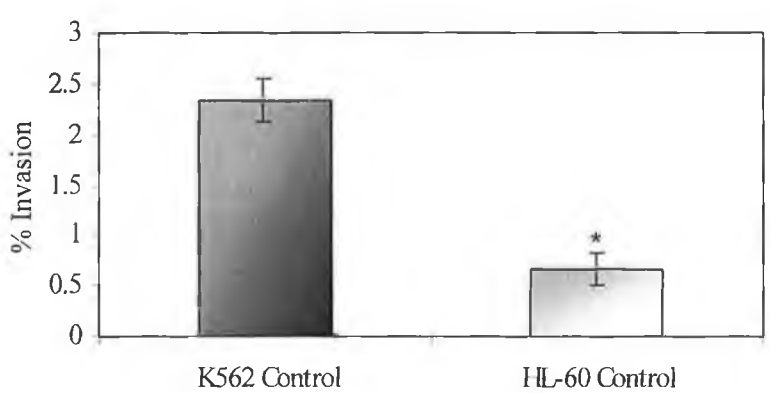

Figure 5.5 *In vitro* invasion analysis of the K562 and HL-60 cell lines using Biocoat™ matrigel invasion chambers demonstrated that K562 were up to 4 times more invasive than HL-60. Experiments were done in triplicate and repeated 3 times. The asterisk denotes that P<0.005.

5.6.2 Expression of matrilysin, MMP-2 and MMP-9 by the K562 and HL-60 cell lines.

Since the major enzymes implicated in ECM breakdown are the MMPs (McDonnell *et al.*, 1999) we examined the MMP expression profile of the K562 and HL-60 cell lines using RT-PCR, zymography and western blot analysis. Using RT-PCR the mRNA for MMP-2 and MMP-9 was detected in both cell lines while only the K562 cell line expressed matrilysin mRNA (Figure 5.6A). The mRNA from both cell lines was also amplified for the constitutively expressed gene β-actin (Figure 5.6A). We then looked at the MMP protein using zymography and western blot analysis of the conditioned media. Figure 5.6B shows that both K562 and HL-60 cell lines secreted MMP-2 and MMP-9 protein. The identity of these bands as MMP-2 and MMP-9 was confirmed by western blotting (data not shown). The levels of MMP-9 protein appeared similar in both cell lines while the K562 cells appeared to produce more latent MMP-2 than the HL-60 cells. However, the HL-60 cell line expressed low levels of active MMP-2 as seen by the presence of a faint lower molecular weight band. Expression of matrilysin protein was detected using western blotting as the primary antibody had a high affinity/specificity for matrilysin and was considered more sensitive than gelatin/casein zymography. Western blot analysis (Figure 5.6B) showed that only the K562 cell line produced matrilysin thus confirming the results obtained at the mRNA level.

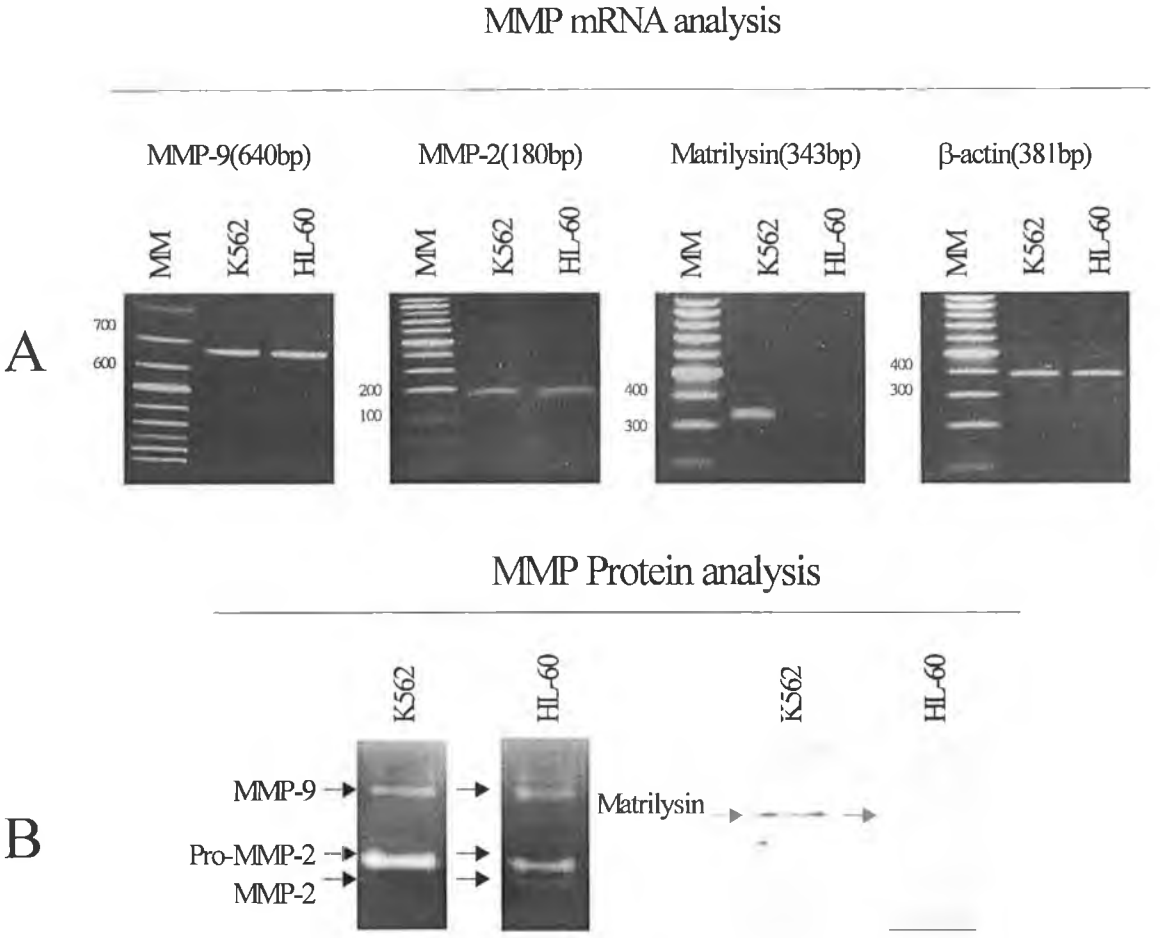

Figure 5.6 Analysis of MMP expression in the K562 and HL-60 cell lines at the mRNA (A) and protein level (B) suggested that matrilysin may be of importance in leukaemia cell invasion. Samples were normalised using β-actin which is a constitutively expressed gene.

5.6.3 Expression of TIMP-1, TIMP-2 and TIMP-3 by the HL-60 and K562 cell lines.

Although expression of MMPs has been shown to be critical for invasion of cells, the levels of their inhibitors, the TIMPs also play an important role. The K562 cell line constitutively expressed TIMP-1 mRNA while none was detected in the HL-60 cell line (Figure 5.7A). TIMP-2 mRNA was expressed in both cell lines with the HL-60 cells expressed approximately 2 fold (+/- 0.43) more than the K562 cells (Figure 5.7A). Reverse zymography showed that the K562 cells secreted large amounts of TIMP-1 protein while its presence in the HL-60 conditioned media was not detected (Figure 5.7B). In agreement with the RT-PCR analysis, TIMP-2 protein was observed in both cell lines, with approximately 2-fold higher levels in the HL-60 cells (Figure 5.7B). TIMP-3 was not detected at the mRNA or protein level in either cell line (data not shown).

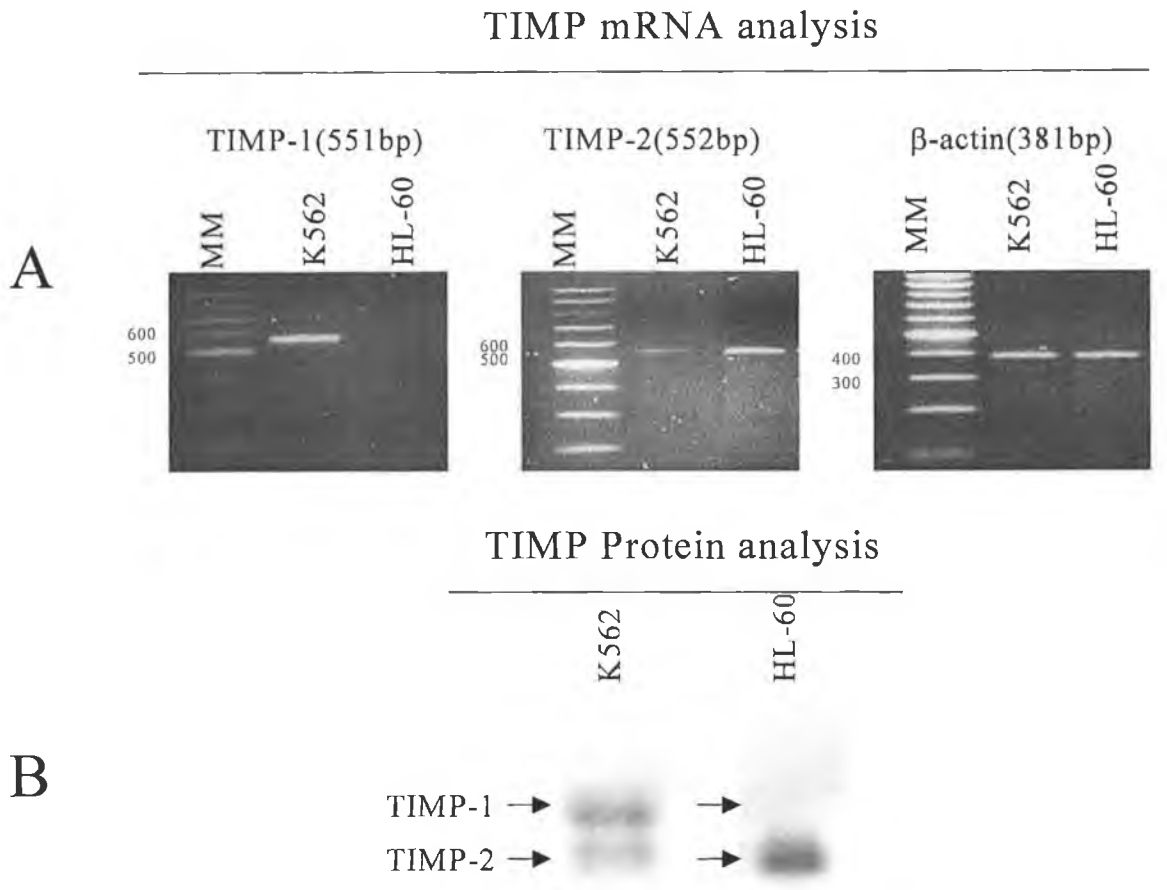

Figure 5.7 Analysis of TIMP expression in the K562 and HL-60 cell lines at the mRNA via RT-PCR (A) and protein level via reverse zymography (B). Samples were normalised using β-actin which is a constitutively expressed gene.

5.6.4 Effect of matrilysin blocking antibody on the in vitro invasiveness of the K562 cell line.

Following our initial observation that the K562 cell line was more invasive than the HL-60 cell line and subsequent MMP and TIMP analysis, the most striking difference observed between the two cell lines was in matrilysin expression. These results suggested that matrilysin expression in the K562 cell line may play a role in the increased invasiveness of this cell line. In order to address this question we used a matrilysin blocking antibody to investigate the invasive ability of the K562 cell line. Following the addition of 50μg/ml of matrilysin antibody we saw a significant 40% reduction in the invasiveness of the K562 cells (Figure 5.8). Cells treated with 50μg/ml anti-rat IgG showed no decrease in invasion in comparison to K562 control cells. Two tailed T-tests again showed that the results obtained were significant (P value < 0.005).

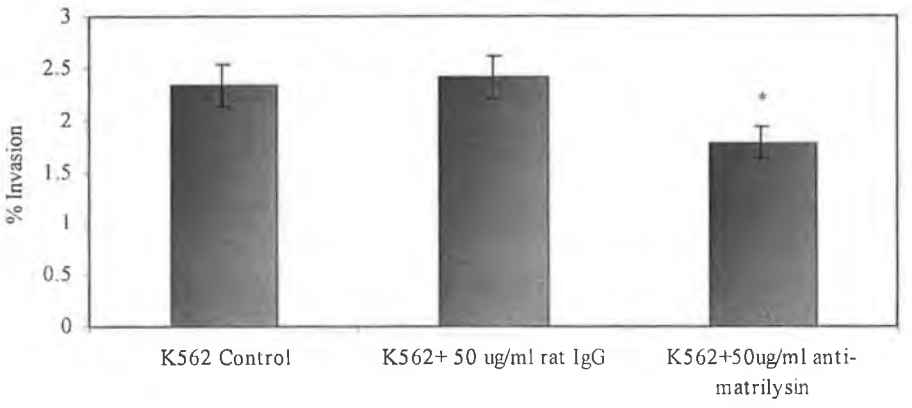

Figure 5.8 The effect of a matrilysin blocking antibody on the *in vitro* invasion activity of the K562 cell line shows that matrilysin is a relevant factor in the invasion process. Experiments were carried out in triplicate and repeated three times. The asterisk denotes that $P<0.005$.

5.6.5 Regulation of MMPs in the K562 and HL-60 cell lines

Earlier work carried out in chapter 3 involving the analysis of matrilysin promoter/reporter constructs in colon cell lines showed that matrilysin levels could be modulated by a range of cytokines and phorbol esters including IL-6 and TPA. Interestingly IL-6 has also been shown to have differentiating effects on the K562 and HL-60 cell lines. Previous studies have shown that upregulation of IL-6 in patient samples was indicative of poor prognosis for the patient and was nearly always accompanied by an increase in the expression of MMPs. We therefore examined if there was a relationship between IL-6 and matrilysin regulation in the K562 and HL-60 leukaemia cell lines. RT-PCR analysis of IL-6 and TPA treated RNA extracts showed an increase in matrilysin mRNA transcript production after cytokine treatment (Figure 5.9). However, cytokine treatment did not appear to induce matrilysin gene transcription in the HL-60 cells.

Similar observations were also noted at the protein level whereby production of MMP-9 in the K562 cell line can be upregulated through IL-6 and TPA expression (Figure 5.10 and 5.11) while HL-60 gives only a slight increase in MMP-9 protein

production following IL-6 and TPA treatment. For the K562 cell line, MMP-2 protein did not appear to be upregulated following IL-6 and TPA treatment while both active and latent forms of MMP-2 showed moderate upregulation. Western blot analysis (Figure 5.10) shows that matrilysin is expressed by the K562 cell line only and that it can be upregulated by IL-6 and TPA.

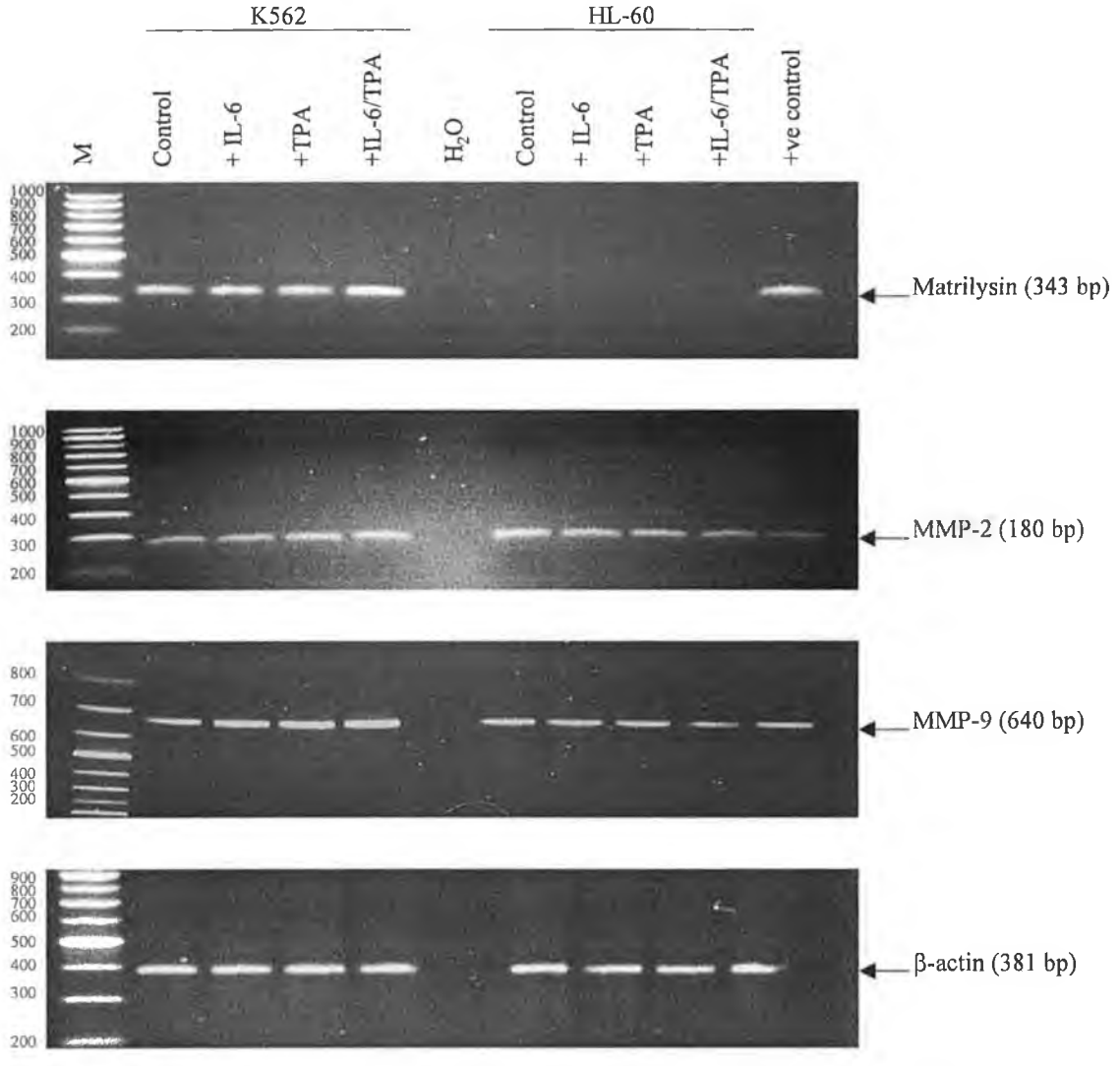

Figure 5.9 Regulation of matrilysin, MMP-2, and MMP-9 at the mRNA level following treatment with 100ng/ml IL-6 and/or 100ng/ml TPA. RT-PCR analysis negative control was H_2O and positive control was plasmid DNA containing the cDNA for the required gene.

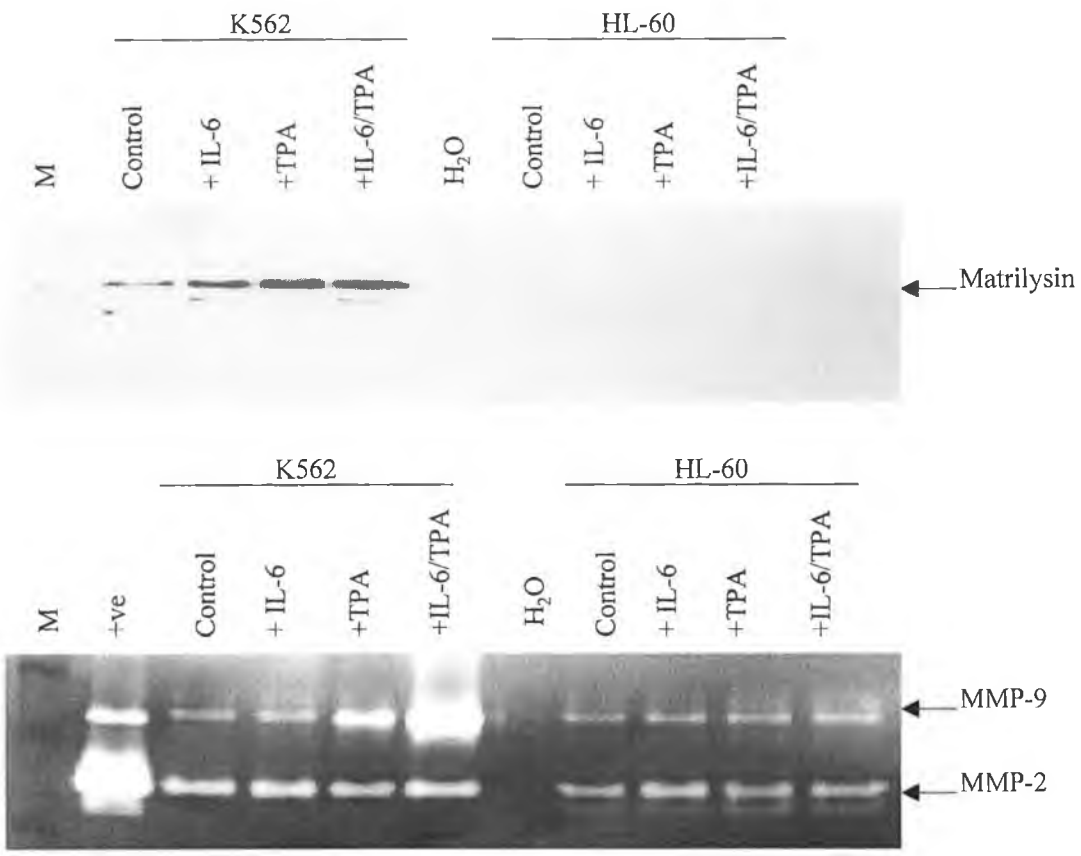

Figure 5.10 Regulation of matrilysin, MMP-2, and MMP-9 protein following treatment with 100ng/ml IL-6 and/or 100ng/ml TPA. Matrilysin protein was detected using western blot analysis while MMP-2 and MMP-9 protein were analysed using zymography.

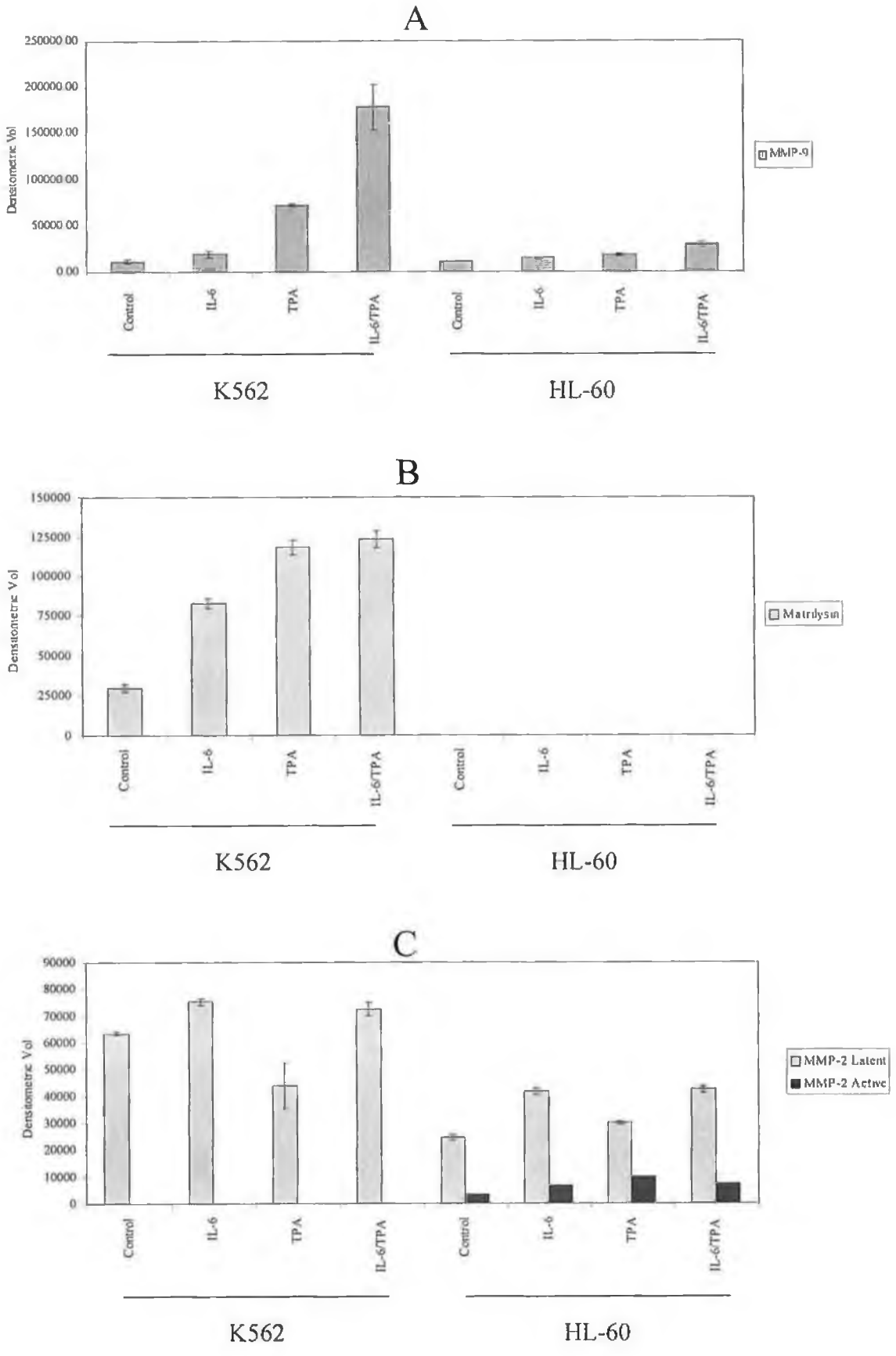

Figure 5.11 Graphical analysis of the expression of **A**; MMP-9 protein expression, **B**; matrilysin protein expression and **C**; MMP-2 expression.

5.6.6 Regulation of the TIMPs in the K562 and HL-60 cell lines

RT-PCR analysis on HL-60 and K562 RNA extracts demonstrated that only the K562 cell line expressed TIMP-1 while levels in the HL-60 cell line remained undetectable even upon treatment with IL-6 and TPA (figure 5.12). K562 cells expressed large constitutive amounts of TIMP-1 RNA which was only slightly increased through IL-6 and TPA treatment. RT-PCR analysis also revealed TIMP-2 expression in both cell lines with untreated HL-60 expressing approximately 3 fold (+/- 0.43) more than the K562 cell line (figure 5.12). IL-6 and TPA treatment also upregulated TIMP-2 expression in both cell lines. Reverse zymograms showed that K562 secreted large amounts of TIMP-1 protein while its presence in the HL-60 conditioned media was not detected. The effects of IL-6 and TPA treatment did not appear to have a great effect on TIMP-1 secretion in either cell line. TIMP-2 protein was observed in both cell lines and notably the differences in expression demonstrated at the mRNA level were not observed at the protein level (figure 5.13 and 5.14). This may indicate that TIMP-2 mRNA transcripts in the HL-60 cell line may have a longer half life which does not necessarily lead to an increase in protein levels. The presence of TIMP-1 and TIMP-2 protein in the conditioned media was also analysed via western blot and confirmed the findings of the reverse zymography experiments. TIMP-3 was not detected at the message or protein level in either cell line (data not shown).

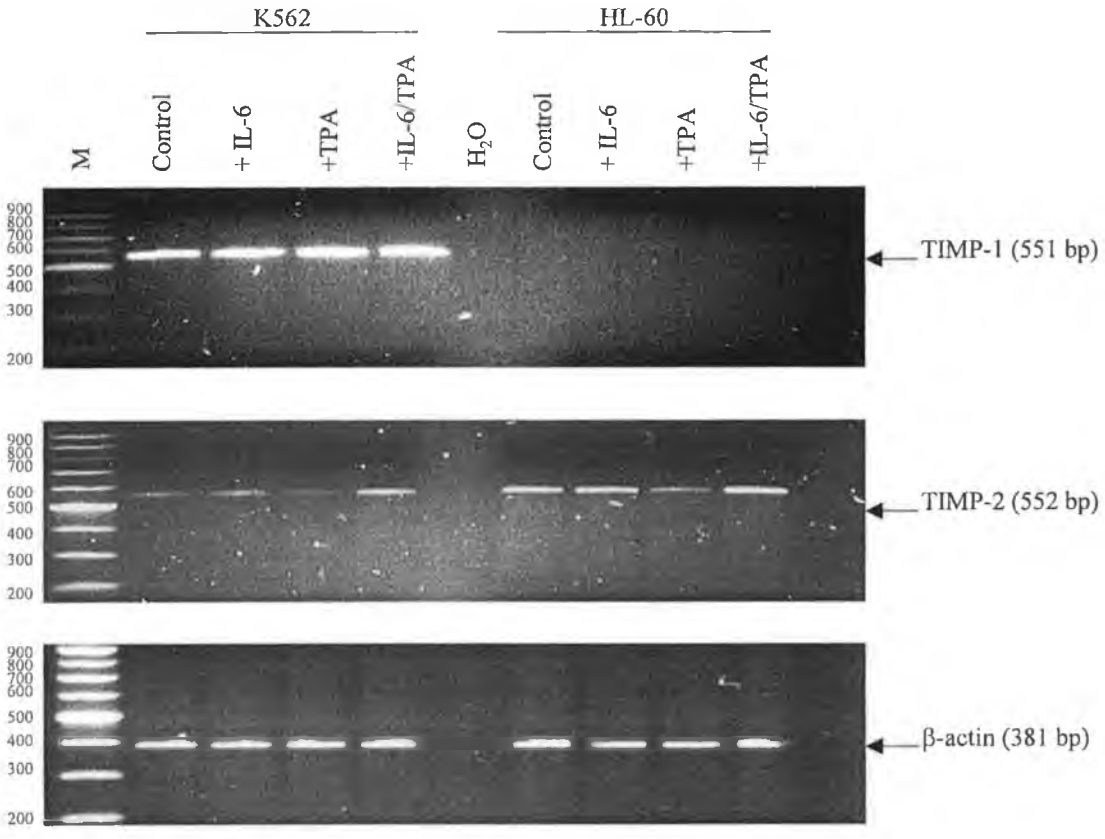

Figure 5.12 Regulation of TIMP-1 and TIMP-2 at the mRNA level following treatment with 100ng/ml IL-6 and/or 100ng/ml TPA. RT-PCR analysis negative control was H_2O.

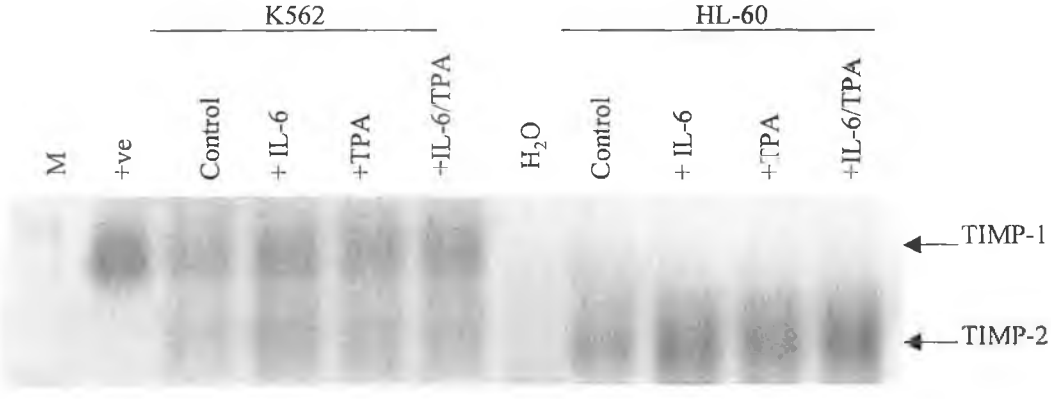

Figure 5.13 Regulation of TIMP-1 and TIMP-2 protein following treatment with 100ng/ml IL-6 and/or 100ng/ml TPA. TIMP-1 and TIMP-2 protein was detected via reverse zymography.

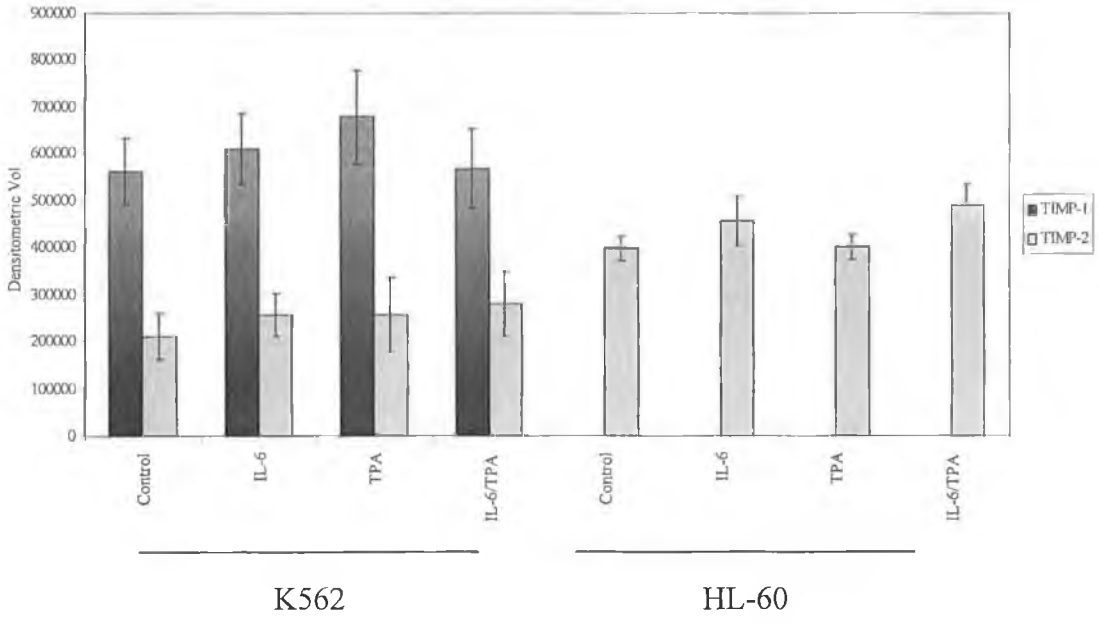

Figure 5.14 Graphical analysis of TIMP-1and TIMP-2 protein expression in the K562 and HL-60 cell lines

5.6.7 The effects of IL-6 on K562 and HL-60 *in vitro* invasion assays.

Having shown that IL-6 and TPA treatment were capable of upregulating matrilysin expression we analysed if this increase in matrilysin expression would result in the increased *in vitro* invasiveness of the K562 cell line. Results suggest that there is an increase in the invasion of K562 cells but this may be due to increased matrilysin or MMP-9 activity (see figure 5.15). Analysis also shows that the observed increases were not statistically significant therefore blocking experiments with matrilysin or MMP-9 antibodies would be unable to determine which MMP was responsible for the increase in invasion observed after IL-6 and TPA treatment.

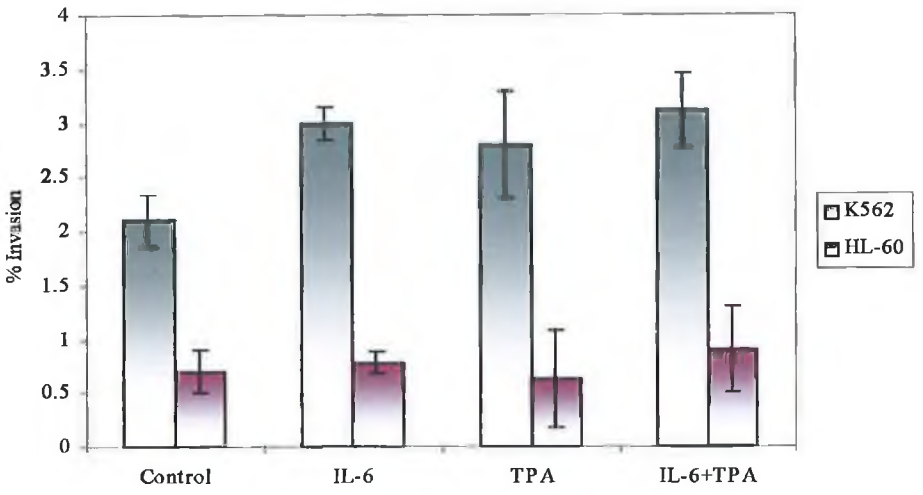

Figure 5.15 K562 and HL-60 cells were treated with IL-6 and/or TPA (each at 50 ng/ml) in order to determine if the subsequent increase in matrilysin and MMP activity would result in increased *in vitro* invasion activity.

5.7 Discussion

Previous studies investigating the role of MMPs in leukaemia analysed the expression of MMP-2, MMP-9 and their natural inhibitors TIMP-1 and TIMP-2 (Janowska-Wieczorek *et al.*, 1999, Ries *et al.*, 1999, Devy *et al.*, 1997, Stetler-Stevenson *et al.*, 1997 and Tapiovaara *et al.*, 1996). These studies suggested that leukaemias which have acquired the ability to express MMPs have more invasive phenotypes. In this chapter we examined two human leukaemia cell lines, K562 and HL-60 and found using an *in vitro* invasion assay that the K562 cells were four times more invasive than the HL-60 cells. In order to ascertain why the K562 cells were more invasive than the HL-60 cell line we analysed the expression of various MMPs and TIMPs in these cell lines.

Analysis of MMP-9 expression showed that both cell lines expressed similar levels of MMP-9 at both the mRNA and protein level. RT-PCR results were similar to those observed in other studies however some reports have shown that the HL-60 cell line expressed more MMP-9 than the K562 cell line (Janowska-Wieczorek *et al.*, 1999). RT-PCR analysis of the HL-60 and K562 cell line in other reports show similar levels of MMP-9 expression in these cell lines but examination of MMP-9 at the protein level

reveals that MMP-9 expression is much higher in the HL-60 samples suggesting post translational regulation (Janowska-Wieczorek *et al.*, 1999). Differences between the MMP-9 levels observed in our studies and in others may be due to differences in incubation times or different cell culture techniques. Only the latent form of the MMP-9 protein was observed in both the K562 and HL-60 cell lines. A band with a slightly higher molecular weight does appear in the HL-60 samples at approximately 96 kDa and this may either be a glycosylated form of MMP-9 or MMP-9 bound to another protein (Moses *et al.*, 1998). The band at 92 kDa matches the band observed in the BHK 92 positive control which secretes only the latent form of MMP-9. Previous reports have not identified active MMP-9 in either of these cell lines (Janowska-Wieczorek *et al.*, 1999 and Kossakowska *et al.*, 1999).

Similar results were observed for MMP-2 at both the mRNA and protein level with the exception that active MMP-2 was observed in the HL-60 samples. This suggests that there maybe an up-regulation of MMP-2 activators such as MT1-MMP in the HL-60 samples. The K562 cells were also found to express slightly more latent MMP-2.

Analysis of MMP-2 and MMP-9 expression alone would suggest that both of these cell lines should have similar invasion levels, however, the *in vitro* invasion assays showed this was not the case. Several investigators have shown that matrilysin expression correlates with the metastasis of colon and breast carcinomas and that over-expression of matrilysin in colon cells leads to increased invasion and tumourigenicity (Rudolph-Owen *et al.*, 1998b and Witty *et al.*, 1994). We analysed the expression and activity of matrilysin in both cell lines which hitherto had not been investigated in a leukaemia cell background. Matrilysin mRNA transcripts and protein were observed only in the K562 cell line. These data suggested that matrilysin expression may play a role in the increased invasiveness of the K562 cell line.

TIMPs are the natural inhibitors of MMPs and have been shown to inhibit invasion of metastatic cells (Alvarez *et al.*, 1990). TIMP-1 has been shown to bind preferentially to proMMP-9 while TIMP-2 binds to proMMP-2 but this is not a mutually exclusive situation as they have been shown to interact with other MMPs (Kahari *et al.*,

1999 and Gomez *et al.*, 1997). Recent studies have also shown that TIMPs are multifunctional molecules capable of not only preventing ECM degradation through MMP inhibition but also of stimulating growth proliferation through erythroid potentiating activator (EPA) receptors (Murate *et al.*, 1993). A recent report has shown that in MCF-7 breast cells, TIMP-1 binds and translocates to the nucleus although the exact function of this nuclear form of TIMP-1 remains unknown (Ritter *et al.*, 1999). In order to rule out the possibility that K562 increased invasiveness was due to little or no expression of TIMPs or that the reduced HL-60 cells invasion was due to high levels of TIMPs we investigated the expression of TIMP-1, TIMP-2 and TIMP-3.

Analysis of TIMP-1 showed that the K562 cell line expressed large quantities of this inhibitor at both the mRNA and protein level whereas TIMP-1 could not be detected in the HL-60 cell line. This would suggest that the K562 invasion should be retarded through TIMP-1 mediated MMP inhibition but, as has been shown by Von Bredow *et al.*, (1998) this inhibition may be circumvented by matrilysin's ability to activate TIMP-1.

Analysis of TIMP-2 showed that at the mRNA level the HL-60 cells produced approximately 2 fold more TIMP-2 mRNA than the K562 cells. This increase in TIMP-2 mRNA was also translated into a 2 fold increase in protein when samples were analysed via reverse zymography.

Analysis of the MMP profile of the K562 and HL-60 cell lines revealed that both cell lines expressed similar levels of MMP-2 and MMP-9 protein. However, only the K562 cell line was found to secrete matrilysin. This suggested that there may be a link between matrilysin expression by the K562 cell line and its increased invasiveness in comparison to the HL-60 cell line. In order to show that matrilysin plays a direct role in the invasion of the K562 cells we carried out *in vitro* invasion assays in the presence of a matrilysin blocking antibody. Results of these experiments showed that in the presence of the matrilysin blocking antibody there was a significant reduction in the invasion of the K562 cells. However, these experiments did not reduce the levels of K562 cell invasion to those observed with the HL-60 cell line i.e. 2.3% to 0.7% which suggested that additional factors may also be involved. Serine proteinases, cell adhesion molecules

(Murphy and Gavrilovic, 1999) and MMPs have all been implicated in the complex integrated events underlying cell migration and invasion.

Several reports have shown that a number of MMPs, in particular MMP-2 and MMP-9 localise to the cell surface of breast epithelial cells. MMP-2 has been shown to co-localise with αvβ3 integrins (Brooks *et al.*, 1996) at the cell surface and MMP-9 has also been found to associate with the CD44 antigen on the cell surface (Yu and Stamenkovic, 1999). CD44 is the receptor for hyaluran which is incorporated into the ECM by the stromal cells and binds to a location on CD44 distinct to that of MMP-9. This interaction between CD44 and MMP-9 has been implicated in increased tumour invasion. Expression of dominant negative soluble CD44 receptor disrupts this effect resulting in decreased invasiveness and in decreased vascularisation. Subsequent studies found that the increase in tumour vascularisation was due to MMP-9 activation of TGF-β and it was also shown that MMP-9 produces different TGF-β cleavage products (Yu and Stamenkovic, 2000). CD44 is present on a wide variety of blood cells including progenitor cells and therefore the interaction between CD44 and MMP-9 may represent a new and novel mechanism for blood cell/leukaemia egress/invasion. A recent study has also shown that α-MMP-9 antibodies significantly reduced the invasion of leukaemia cells (Kossakowska *et al.*, 1999). Studies have also shown that the invasive effect of the cells was dependent on the interaction of MMP-9 with CD44 as when the cells were transfected with MMP-9 secretion vector, tumour invasion was enhanced (Yu and Stamenkovic, 2000). We have observed that the expression of matrilysin by the K562 leukaemia cell line is directly involved with its increased invasion. The majority of the matrilysin produced by the K562 cell line was secreted into the culture medium but lysis of the cells also showed matrilysin was located inside the cells (data not shown). This may suggest that matrilysin also localises to the leukaemia cell surface by an as yet undetermined mechanism and could aid in the invasion/dissemination process. However, this will require further studies as the results suggest that the matrilysin detected in the cell lysates is protein which is waiting to be processed for secretion. Yu and Stamenkovic have also reported that matrilysin co-localises with CD44 on the surface of breast cancer cells (MMP GRC 2001, poster presentation). Matrilysin has also been well documented as a sheddase and is capable of processing many of the cell surface molecules including E-

cadherin and other integrin molecules (Noe *et al.*, 2000). To clarify the mechanism of how matrilysin enhances leukaemia invasion, either through secretion or localisation to the cell surface, will require more in depth studies.

In order to identify some of the regulatory mechanisms controlling matrilysin gene transcription we treated the cell lines with IL-6 and TPA. IL-6 is a multifunctional cytokine known to have a growth promoting/differentiating effect on heamatopoietic cells (Gao *et al*, 2000, Xie *et al.*, 2000, Xie *et al.*, 1999). A recent study has shown that IL-6 expression was generally associated with a poor prognosis in Non Hodgkin's Lymphoma patients (Kossakowska *et al,* 1999). This was not only as a result of IL-6 growth promoting activities but also due to its association with increased MMPs expression, suggesting the involvement of IL-6 in MMP regulation. However, the molecular mechanism whereby IL-6 up-regulates MMP expression remains unclear. Interestingly, the presence of a potential C/EBP (CCAAT/ Enhancer Binding Protein) element within the matrilysin promoter may indicate a possible mechanism (Wilson and Matrisian, 1996). Reports have shown that when IL-6 binds to its receptor it activates the JAK/STAT pathway and subsequently STAT3 has been shown to regulate levels of C/EBP transcription factor binding proteins (Chen *et al,* 1999). We feel that a possible mechanism may be the increased binding of the C/EBP transcription factor to the matrilysin promoter which in turn leads to increased matrilysin expression. We also examined the effects of IL-6 on MMP-2 and MMP-9 expression. TPA has been shown in several studies to modulate MMP expression and also to function as a potent differentiating agent (Mackay *et al,* 1992; Uchimaru *et al,* 1998). Combination treatments with IL-6 and TPA were also performed.

Treatment of the K562 cells with IL-6 and TPA treatment showed only slight increases in matrilysin expression at the mRNA level but the effect at the protein level was more pronounced (almost 2.5-fold over control for TPA/IL-6). Interestingly, we also observed an intermediary processed form of matrilysin (antibody does not recognise active form) in the IL-6 treated samples. This suggests that IL-6 may also regulate transcription of other proteases known to activate MMPs. Matrilysin was not detected at

either the RNA level or the protein level in the HL-60 cells and its transcription could not be induced through IL-6 and TPA treatment.

IL-6 and TPA increased MMP-9 mRNA and protein expression in the K562 cell line and when these factors were added in combination a marked increase in MMP-9 protein was observed by gelatin zymography. The HL-60 cells also responded to IL-6 and TPA treatment but not to the same extent as that observed in the K562 samples, which perhaps may be as a result of differences in IL-6 receptor expression.

The level of MMP-2 protein did not appear to increase in either cell line following treatment with IL-6. This is in contrast to Kossakowska *et al.* (1999) who have shown that IL-6 could upregulate MMP-2 expression in various types of leukaemia cell lines. The combination of IL-6 and TPA treatment did not affect MMP-2 expression to the same extent as MMP-9 expression suggesting that these genes have different regulatory mechanisms. Treatment of the K562 cell line with IL-6 and TPA appeared to have little or no effect on TIMP-1 mRNA levels. However at the protein level TIMP-1 appeared to be enhanced by IL-6 and TPA which may suggest stabilisation of TIMP-1 mRNA. The expression of TIMP-1 could not be induced by IL-6/TPA treatment of the HL-60 cell line. TPA treatment appeared to inhibit the expression of TIMP-2 at the mRNA level in both the K562 and HL-60 cell line but this effect was not observed at the protein level.

In order to determine if the increase in matrilysin production after IL-6/TPA treatment would result in increased invasiveness we performed *in vitro* invasion assays. An increase in the *in vitro* invasiveness in the K562 cell line was observed but this may have been possibly due to enhanced MMP-9 expression. Treatment with IL-6 and TPA appeared to have no significant effect on the HL-60 rate of invasion

5.8 Conclusion

In summary the experiments carried out in this chapter demonstrate the importance of matrilysin expression in leukaemia cell invasion. We have also shown that the expression of matrilysin can be regulated by IL-6 and the phorbol ester TPA. Therefore aberrant expression of IL-6 or the IL-6 receptor by leukaemia cell lines may result in the increased expression of matrilysin which in turn may lead to a more aggressive phenotype. Matrilysin may be of use as a potential prognostic marker for leukaemia aggressiveness. Therefore, in conclusion we feel that based on the results obtained in this study further investigations into matrilysin expression in additional leukaemia cell lines and primary samples are warranted.

Chapter 6

Final Summary and Future Directions

6.1 Final Summary

Matrilysin is the smallest member of the MMP family and has one of the broadest substrate specificities with an ability to degrade collagen type IV, gelatin, laminin, fibronectin, entactin and tenascin. The matrilysin enzyme is secreted from the cell in a latent 28 kDa form which is subsequently proteolytically cleaved to produce the active 19 kDa form.

The extracellular matrix can be viewed as a multifunctional dynamic meshwork of high molecular weight proteins which not only serves as an anchoring mechanism for various cell types but also in presenting the cells with an array of growth factors and other signalling molecules. During normal biological processes such as cell growth, wound healing and angiogenesis, the ECM must be remodelled to allow space for growing or advancing cells. This process involves the destruction of the ECM by a number of proteinases including the metalloproteinases which collectively are capable of degrading the entire ECM. The regulation of the MMPs must therefore be tightly controlled by the cells which secrete them as aberrant expression can lead to various disease processes. Increased MMP expression has been associated with cancer for many years and there are numerous reports linking the upregulation of MMPs with cancer invasion and metastasis (Chambers and Matrisian, 1997). In particular, matrilysin has been shown to be upregulated in several cancers, in particular those of a colon and breast origin and has been shown to correlate well with tumour aggressiveness (Wilson and Matrisian, 1996 and Rudolph-Owen *et al.*, 1998). Matrilysin is unique amongst other MMPs as it has been shown to be exclusively expressed by the epithelial cells of colon and breast tumours. Other MMPs which have been shown to be associated with cancer progression, such as MMP-2 and MMP-9, are normally found in the stroma surrounding the tumour cells and are secreted predominantly by stromal and macrophage cells.

Recent studies have also shown that matrilysin plays an important role in the early tumourigenesis of both breast and colon cancers (Wilson *et al.*, 1997 and Rudolph-Owen *et al.*, 1998). With respect to colon tumourigenesis a series of elegant experiments were performed in the Matrisian laboratory (Vanderbilt University, Nashville, TN.)

involving a mouse model of the human hereditary colon disease, familial adenomatous polyposis (FAP), in which there is a mutation in the *APC* gene. This mouse model, known as the $Min^{APC-/-}$ ($Min^{-/-}$) mouse for multiple intestinal neoplasia, was crossed with a matrilysin null mouse ($Mat^{-/-}$) resulting in an APC and matrilysin deficient mouse ($Min^{-/-}$ /$Mat^{-/-}$). The $Min^{-/-}$ mice develop numerous benign polyps in the intestine, in manner similar to that in FAP patients. Studies have shown that in the $Min^{-/-}$/$Mat^{-/-}$ mice there was a significant reduction in both size and number of the intestinal polyps which suggested that matrilysin played an important role in tumourigenesis (Wilson *et al.*, 1997). Azoxymethone (AOM) treatment of mice simulates early colorectal tumour development whereby lesions known as aberrant crypt foci, which are similar to the first lesions observed in human colon tumour development, are formed. Again there is a reduction in the number of lesions formed when matrilysin null mice were examined which again suggests that matrilysin is involved in the early stages of colon tumour development (see Fingleton *et al.*, 1999 for review). Matrilysin has also been shown to be involved in angiogenesis, apoptosis and the cleavage of a number of cell surface proteins, such as E-cadherin, which may have a potential role in tumourigenesis (Weisen and Werb, 2000, Powell *et al.*, 1999 and Noe *et al.*, 2001).

The association of matrilysin with enhanced tumour formation, growth and spread, have made it an exciting target for therapeutic drug development. Although many studies have investigated the expression of matrilysin in many systems, little is known about how matrilysin expression is regulated. In order to produce drugs which are effective in inhibiting matrilysin activity and have minimal side effects it is important to understand how this enzyme is regulated at the transcriptional and protein level.

Early studies investigating the regulation of matrilysin gene transcription observed that factors such as EGF and the phorbol ester, TPA, had positive regulatory effects on matrilysin gene transcription and suggested that the AP-1 and ETS transcription factor binding elements within the matrilysin promoter were important for this upregulation (Gaire *et al.*, 1994). Analysis of the matrilysin promoter using a transcription factor search engine revealed the presence of a number of potential transcription factor binding sites including an AP-1 site, three PEA3 sites, two of which

have the potential to act as either a Kaiso (-168 PEA3) or a C/EBP binding site (-55 PEA3) and two Tcf sites. Recent studies have shown that β-catenin, normally associated with E-cadherin at the adherens junctions acting as a cytoskeletal protein, can interact with the Tcf sites located within the matrilysin promoter and effect the transcription of matrilysin in conjunction with PEA3 and AP-1 bound factors (Crawford *et al.*, 1999). In a normal cell, excess β-catenin is 'mopped' up by APC which in association with axin and GSK-3β targets β-catenin for ubiquitination. However as mentioned earlier, APC is commonly mutated in many colon cancers including those of a sporadic nature and this mutation prevents β-catenin from being degraded and allows it to translocate to the nucleus and assist in the transcription of several target genes including matrilysin. Although β-catenin transactivation of the matrilysin promoter is essential for transcription to take place it is not enough on its own to initiate matrilysin transcription which means that other factors must also be in place (Crawford *et al.*, 2001).

In order to determine what these factors are we initially studied a panel of colon cell lines for their expression of matrilysin and found that with the exception of SW480 they all expressed matrilysin mRNA and protein. We then examined if growth factors such as EGF, IL-6, bFGF and IGF-I and IGF-II could upregulate matrilysin expression. Results showed that EGF, IL-6 and bFGF stimulated matrilysin transcription in many of the cell lines, in particular when the cytokines were treated in conjunction with each other or with the phorbol ester TPA which has been well documented as an upregulator of MMPs. Analysis using a matrilysin promoter reporter construct showed similar results and also showed that the transcription factor binding sites necessary for the stimulation of matrilysin gene transcription were located within the first 335 base pairs of the matrilysin promoter. Therefore, the transcription factors being stimulated by the cytokines were binding within the first 335 base pairs of the matrilysin promoter which in turn indicated that the transcription factors were of an ETS and/or AP-1 origin.

Crawford *et al.*, (2001) have shown that the ETS sub family of PEA3 transcription factors, including, PEA3, ER81 and ERM bind preferentially to the ETS sites within the matrilysin promoter while other ETS family members such as Ets-1, Ets-2, Fli-1 and PU.1 have little or no effect on matrilysin gene transcription. Interestingly, PEA3 protein has

been found to be expressed at higher levels in breast cancer than in normal tissue. This increase in PEA3 levels was almost always associated with an increase in the expression of the EGF receptor related Her2/Neu (O'Hagan and Hassell, 1998). In our preliminary studies we observed a consistent increase in matrilysin gene activity in all cell lines upon treatment with EGF with the exception of the SW480 cell line. We therefore suggested a potential model whereby EGF upregulated matrilysin by stimulating expression of the PEA3 sub family of transcription factors via the EGF receptor associated signalling cascades, for example RAS/MAPK. We initially examined the effect of EGF on AP-1, ETS and PEA3 artificial promoters which contained a number of transcription factor binding repeat motifs. Our results showed that EGF stimulated all three promoters but had a greater effect on the PEA3 promoter. The increases in ETS and AP-1 artificial promoter activity were expected as both these transcription factor families are targetted by the signalling pathways employed by the EGF receptor. Analysis of the nuclear proteins of treated cells also confirmed that there was not only an increase in PEA3 protein levels but also in PEA3 phosphorylation. These results suggested that PEA3 activity was increased via the phosphorylating signal cascades and the level of PEA3 protein was also increased suggesting a potential autoregulatory loop for PEA3. Our next step was to examine if the increase in PEA3 protein would lead to increased binding of an optimal PEA3 probe by using electrophoretic shift mobility assays (EMSA). Results indicated that this was indeed the case. Probes designed to mimic each of the PEA3 sites within the matrilysin promoter were then examined and it was found that the PEA3 bound to each of the probes with a similar intensity suggesting that all three sites were important for matrilysin gene transcription. This was also been confirmed via PEA3 site mutation analysis. Supershift assays revealed only partial shifting which implicated other members of the PEA3 sub family, ER-81 and ERM, in the regulation of the matrilysin gene. In addition, a recent paper has also shown that in many of the Min mice tumours, increased matrilysin levels were always accompanied with increased levels of the PEA3 family members (Crawford *et al.*, 2001).

Several reports have also shown that EGF interacts with β-catenin at the cell membrane and promotes the destabilisation of the E-cadherin/catenin complex. We therefore examined firstly if EGF was involved in the stimulation of PEA3 activity via β-

catenin. Analysis showed that β-catenin had little or no effect on PEA3 activity. Interestingly, EMSA analysis using Tcf probes with sequence similarity to those within the matrilysin promoter showed enhanced binding of β-catenin in EGF treated samples, in particular for the –109 Tcf site. This suggested that EGF through the destabilisation of the E-cadherin/catenin complex resulted in the release of β-catenin which, undegraded due to APC mutations, tranlocates to the nucleus and effects the transcription of matrilysin. As discussed earlier, matrilysin has been shown to be involved in early colorectal tumourigenesis and the mechanisms which 'switch on' the matrilysin gene in these early stages involves APC mutations. However, other aberrant regulatory mechanisms involved in matrilysin gene transcription such as, EGF receptor mutation or upregulation, may exist in cells where APC is not mutated. EGF or the EGF receptor has also been found to be upregulated in several cancers and through its stimulation and activation of PEA3 transcription factors and release of β-catenin from the E-cadherin/catenin complex may not only serve to drive cell proliferation but also the production of matrilysin. Matrilysin, in turn may therefore activate other growth promoting factors that enhance tumour growth and invasion. In addition, factors such as bFGF and IL-6 were also shown to stimulate matrilysin activity and we expect that bFGF may act in a similar manner to that of EGF, i.e. via the upregulation of PEA3 and AP-1 transcription factors which in turn bind to the sites within the matrilysin promoter and effect its transcription. We expect that IL-6 may mediate its effects on matrilysin by the activation of the JAK/STAT pathway which in turn activates C/EBP binding proteins. These proteins may then cooperate with PEA3 and bind to the PEA3/C/EBP site within the matrilysin promoter and synergistically act in the activation of the matrilysin gene.

Several reports have investigated the expression of MMPs in leukaemias and these have mainly focussed on MMP-2 and MMP-9 (Janowska-Wieczorek *et al*, 1999 and Ries *et al*., 1999). The studies showed that in general, MMP-2 and MMP-9 expression was elevated and was associated with leukaemia aggressiveness. Matrilysin has been implicated in the growth and invasion and metastasis of several tumours but to date no reports have been published on its expression by leukaemias. We therefore analysed the expression of matrilysin in the K562 and HL-60 leukaemia cell lines and found that only the K562 cell line expressed matrilysin. *In vitro* invasion assays showed that the K562

cell line was up to four times more invasive than the HL-60 cell line. Zymography analysis of both cell lines revealed that MMP-2 and MMP-9 levels were similar and reverse zymography showed that the K562 cells expressed more TIMP-1 than the HL-60 cells. These results suggested that matrilysin expression may lead to a more aggressive phenotype in the K562 cells and *in vitro* invasion experiments which incorporated a matrilysin blocking antibody proved this to be the case as there was a significant reduction in the K562 cell line invasion. Invasion was not completely inhibited which suggests that other MMPs such as MMP-2 and MMP-9 as well as cell adhesion molecules and other protease groups are involved in leukaemia invasion. We also examined the regulation of matrilysin in both cell lines and found that IL-6 could stimulate matrilysin expression. Other reports have also linked increased IL-6 expression with MMPs and a poor prognosis (Kossakowska *et al.*, 1999). Our data shows that matrilysin plays an important role in leukaemia invasion and may be of potential use as a prognostic marker.

In conclusion our data suggests that EGF plays an important role in the regulation of matrilysin gene expression via a number of new mechanisms. These findings suggest possible new drug targets, such as transcription factor inhibition and tyrosine kinase inhibition, which will inhibit matrilysin expression and in turn should lead to decreased tumourigenesis and invasion and metastasis. Furthermore, we have shown that matrilysin plays an important role in leukaemia cell invasion and may therefore be of use as a potential prognostic marker.

6.2 Future Directions

1) Data presented in chapter 3 consistently showed that EGF, IL-6 and bFGF stimulated matrilysin gene expression. However, the increase observed over the controls was typically only 0.5 to 1 fold. These increases were enhanced when cells were treated with a combination of cytokines, a result which was much more obvious with the matrilysin promoter-reporter construct. These studies were performed in colon cancer cell lines, the majority of which have mutations in signalling pathways such as Ras. This resulted in an increase of growth promoting transcription factors such as AP-1 and ETS. Therefore the treatment of these cell lines with various cytokines may only augment the level of transcription factors required for matrilysin gene expression thus resulting in the low level responses observed in chapter 3 via RT-PCR, western blot and promoter-reporter studies. In order to confirm that cytokines such as EGF, IL-6 and bFGF were having a direct effect on matrilysin gene transcription, we suggest that the matrilysin promoter should be studied in a cell line as close to 'normal' as possible. For example, Crawford *et al.* (2001) used a human embryonic kidney line (HEK293) which has low levels of the transcription factors thought to play an important role in matrilysin gene transcription such as PEA3, ETS and AP-1. Therefore treatment of this cell line with EGF, IL-6 and bFGF should greatly enhance the expression of these transcription factors and subsequently matrilysin.

2) Chapter 4 showed that PEA3 transcription factors may be responsible for the increased expression of matrilysin following EGF treatment. In order to confirm this, supershift assays with PEA3 antibodies were performed. Results showed that only 'partial' supershifts were obtained which suggested that the assay required further optimisation or that other members of the PEA3 family such as ER-81 and ERM were involved. Optimisation of the supershift protocol and the generation of specific antibodies for ER-81 and ERM should help to clarify which of these transcription factors were responsible for the increase in matrilysin gene transcription following EGF treatment. The use of negative controls such as competition experiments with 'cold' unlabelled PEA3 probes and the use of mutated probes would help to ensure that the binding of PEA3 proteins to PEA3 probes was specific. The relationship between EGF and the

release of β-catenin from the E-cadherin/catenin complex was also examined. Initial results showed that there was increased binding of β-catenin to TCF probes suggesting that EGF treatment may result in the release of β-catenin. Again these EMSA experiments should be repeated with the proper negative (mutated probes) and positive controls (recombinant PEA3 protein). Matrilysin has recently been shown to cleave E-cadherin at the cell surface which results in a more invasive phenotype (Noe *et al.*, 2001). We would therefore like to examine the relationship between matrilysin expression and E-cadherin, i.e. does overexpression of matrilysin or the addition of exogenous matrilysin result in the cleavage of E-cadherin at the cell surface which in turn results in a destabilisation of the E-cadherin/catenin complex. The subsequent release of β-catenin should result in increased matrilysin expression. These experiments would show another mechanism whereby matrilysin can contribute to the invasive phenotype of a cell.

3) Chapter 5 showed that the expression of matrilysin by K562 cells is associated with increased *in vitro* invasion. The involvement of matrilysin in leukaemia invasion could be confirmed by a number of experiments including the stable transfection of the HL-60 cell line with a matrilysin construct and analysing if there is a resultant increase in cellular invasion. The expression of matrilysin in other leukaemic cell lines and primary samples should also be examined in order to determine if there is a correlation between matrilysin expression and increased invasion. We would also like to further investigate the regulation of matrilysin in leukaemia cell lines by IL-6 as increased IL-6 expression in NHL samples has been associated with poor clinical outcome (Kossakowska *et al.*, 1996). Results in chapter 3 also indicate that matrilysin expression can be directly regulated by IL-6. Interestingly several reports have shown that MMPs associate with cell adhesion molecules at the cell surface, for example MMP-9 and CD-44 (Yu and Stamenkovic, 1999). We hypothesise that matrilysin may associate with cell adhesion molecules on the cell surface such as CD-44 and this may offer a potential mechanism of how matrilysin can assist in the invasion of leukaemic cells.

Chapter 7

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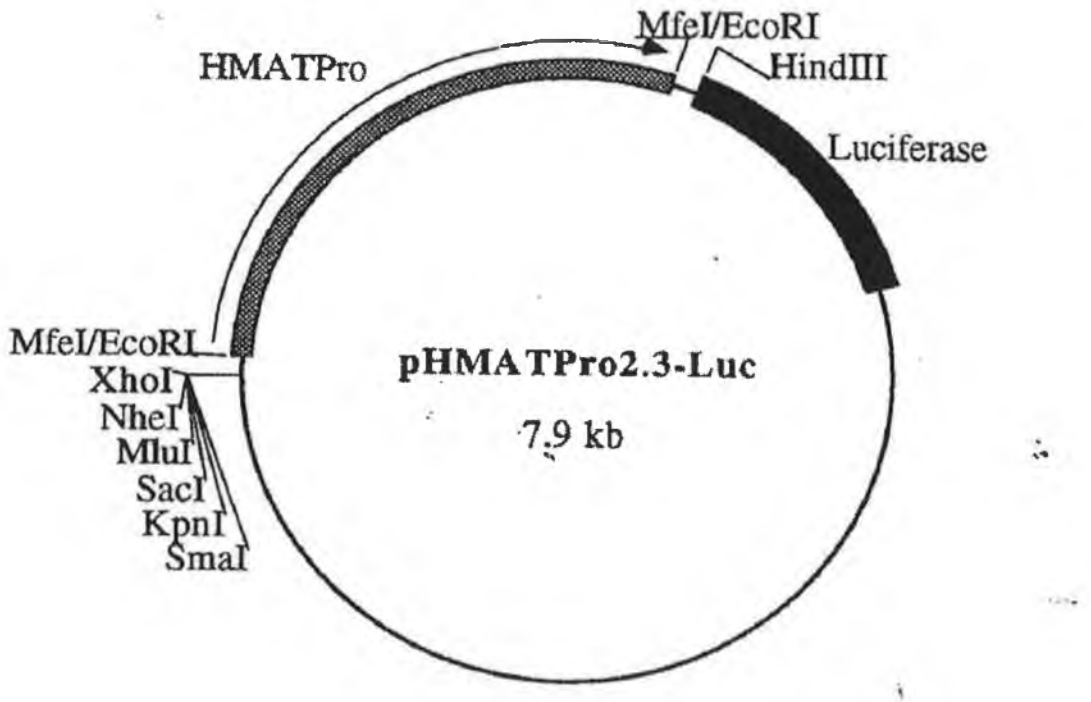
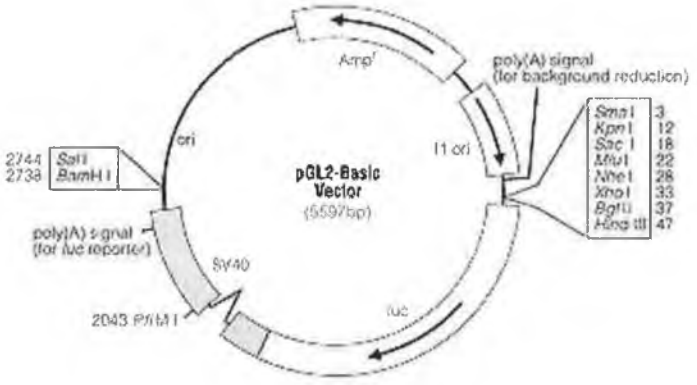
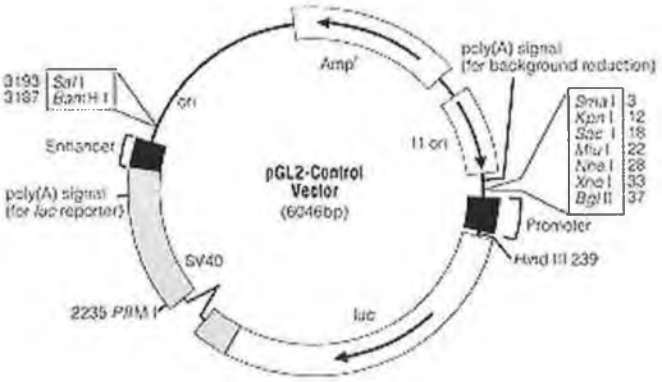
Appendix 1



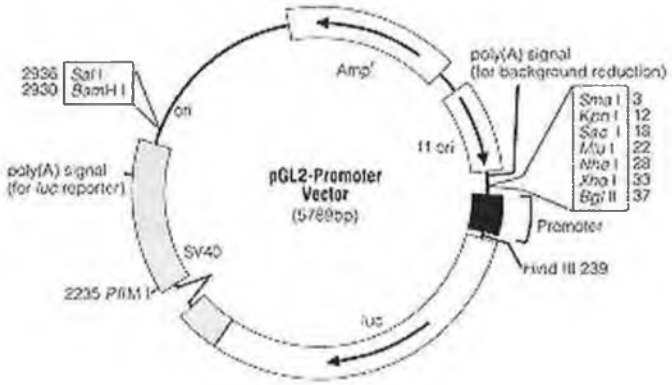
A) pHMATPro2.3-Luc plasmid containing 2.3kb of the human matrilysin promoter. The 335 bp matrilysin promoter construct is similar in structure. Both constructs were developed by Howard C. Crawford, Vanderbilt University, Nashville ,TN.



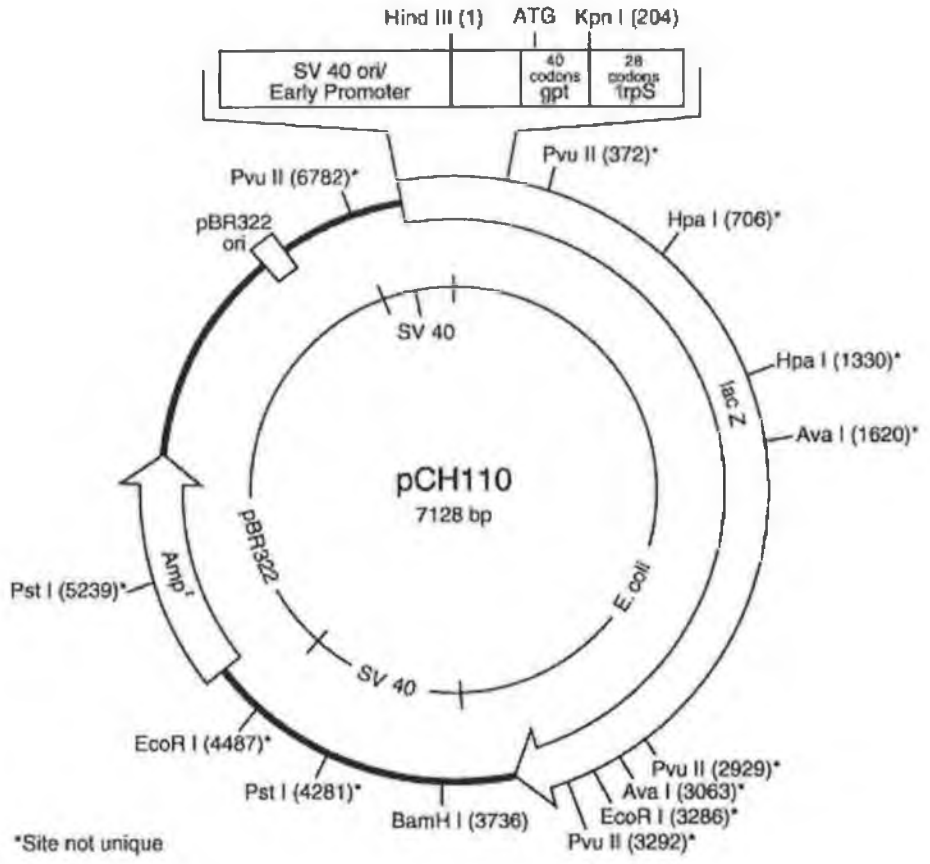
B) The 2.3 kb and 335 bp human matrilysin promoter fragments were cloned into the pGL_2-Basic vector (Promega).



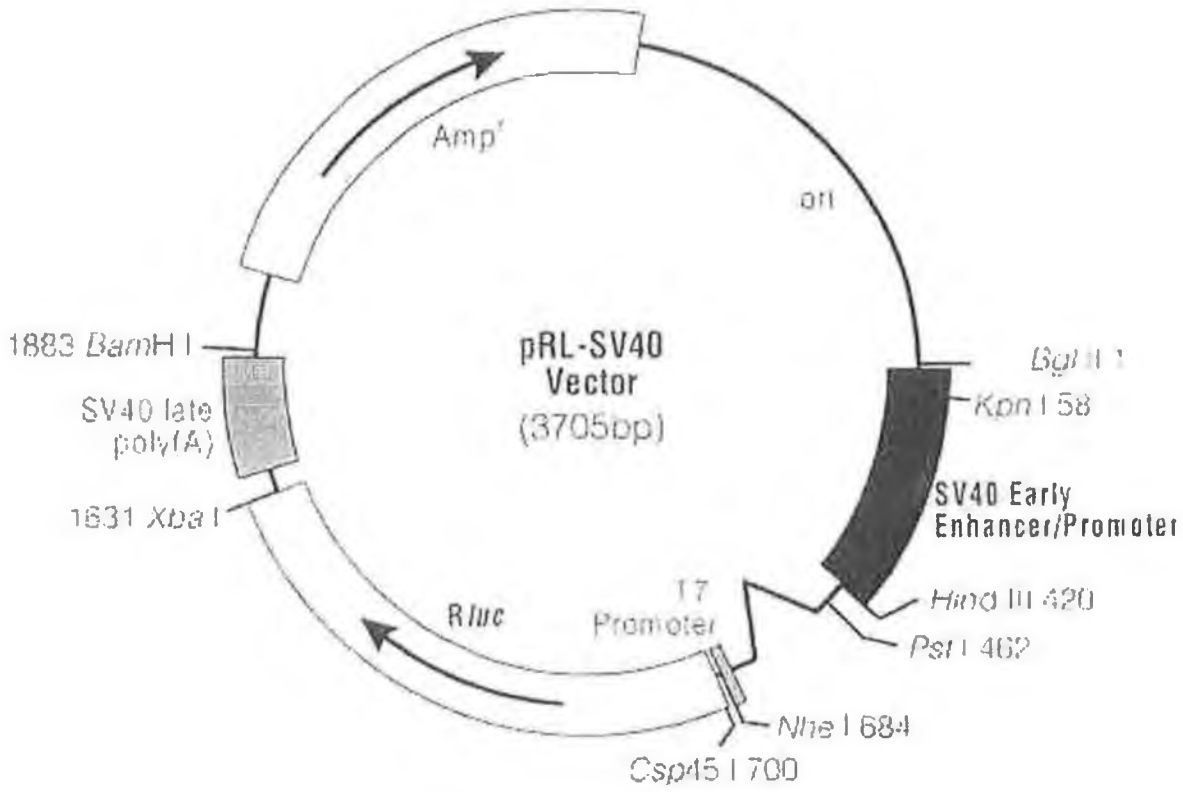
C) The pGL_2-Control plasmid (Promega) which was used as a positive control in transient transfection experiments.



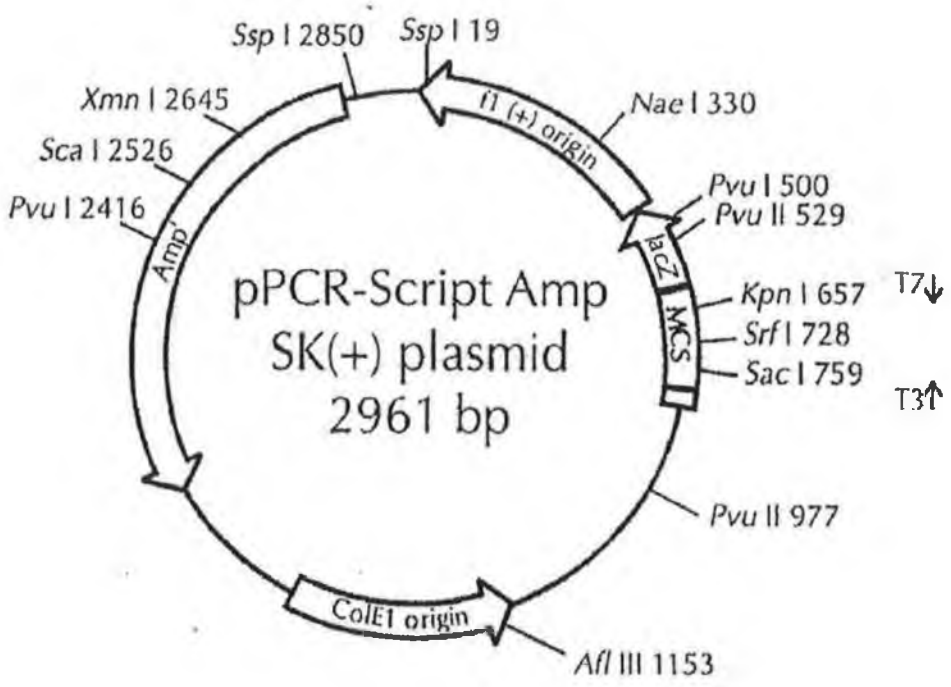
D) The pGL_2-Promoter (Promega) vector which was used to identify potential silencer/enhancer elements within the murine matrilysin gene.



E) The pCH110 (Pharmacia) plasmid used in transfection optimisation studies



F) The pRL-SV40 plasmid which was co-transfected with the matrilysin promoter reporter plasmids in order to normalise results.



G) The pPCR Script plasmid which was used for cloning PCR products

Matrilysin Promoter sequence

1 agctccagca tatttggagt gtttcccatg atgtattaga gtcaaaagcc atggtgttct
61 cccaagtaat gtataatata ataaaagaga cagacctatt acaaaatgaa taggcagtgc
121 agtgggatag aaaaagcact gagctactat ctgtgtgagc tgggagaagt ataataagtt
181 aaattatcat cttggcctca ctttcatttt tggtaagaat ggagtcattg ggctagaatc
241 tgtaggtaat ggattcttac tgctatacgt agaataacca ttttgtgtac taaggaccaa
301 tgcagcccta cctgtagctg ggcagcagcc agagtcagag tgattggaag aaaaaaattg
361 gatctccaag ttgaaggtct agccttggaa gaatctgtta ctataaaatg agaagcagaa
421 taagtaagcc agatgaagag ttaaacccgt gcttgtagat ttttgttggc ttggtatttt
481 tttgtgttta attcaagaag aattagaggc agtgttcccc attaagaaaa gacataaact
541 gattaaaagg agaccccaaa gaagggaatt atcactgctc tgctaaggga cgtggaaggt
601 gaggggacac agcacagttg tatatagagt ggccactaat ccagccacac agcagcattt
661 ccatctgcct cctgccatct ttcccctgta tggagaacca caggattttg gtgacgttct
721 atttcttgac tttggtgacg gttacagtat ttgcttaatt attatttcat tatttacatg
781 ttttgtgcac ttttccaaac cttttaattg ttaagaatat atggtaccat gtatttaaga
841 atacatggta ccatataata agaatatata tttaagaata tatattattc tttaagaata
901 tatggtacca taatgtcctg aatgatacct atgagagcag tcatttgact ttggcaaaaa
961 aatgaggttt ctcatggagt caatttatgc agcagacaga aaaaaaaatc ctttgaaaga
1021 caaatacatt gtgtgcttcc tgccaataac gatgtaatac ttcctcgttt tagttaatga
1081 aaaataacac atactttcaa agttctgtag actctaaaaa gaaagaaaac actcaaatga
1141 gtcacctatt tccacattcg aggctgagaa gctatataaa tttctgcagt cactagcaga
1201 aaaca

G) The sequence of the matrilysin promoter (Genbank accession number L22525)with the PEA3 sites at –168, -144 and –55 outlined.