

Investigation of the functions of Matrix Metalloproteinase-8 (MMP-8) in Mammary Carcinoma Cells

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Thesis submitted for the degree of Doctor of Philosophy

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April 2013

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Abstract

Breast cancer is the most common cancer in the UK today, with incidence rates rising steadily. Prognosis is improving but patients with metastatic disease only have a 15% chance of surviving 5 years beyond prognosis. Therefore, factors influencing the metastatic spread of breast cancer require better understanding.

The matrix metalloproteinases (MMPs) are a family of proteases thought to promote metastasis due to their matrix degradation capabilities. MMP-8 however has been discovered to be anti-tumourigenic in many cancers, and anti-metastatic in breast cancer. This occurs through unknown mechanisms, so this work sought to gain further insight into the functional effects of MMP-8. In the literature it has been suggested that MMP-8 is tumour protective through its role in regulating innate immune responses and preventing chronic inflammation. This occurs through activation cleavage of a pro-inflammatory chemokine, Interleukin-8 (IL-8).

Using an *in vitro* over-expression model it is shown in this thesis that MMP-8 reduced 2D random migration and scratch wound closure. It also increased cell adhesion and reduced colony formation and prevented primary tumour growth in mice. These data indicate an anti-tumourigenic role for MMP-8. Biochemically, MMP-8 induced expression of IL-8 in mammary carcinoma cells, and also the expression of a pro-inflammatory cytokine IL-6, dependent on its catalytic activity. This required NF κ B signalling and IL-6 also required Protease Activated receptor-2. This occurred following transient expression of MMP-8, and also in rare stably transfected clones that expressed MMP-8 long-term. However, wild-type MMP-8 was not tolerated by breast cancer cells and was epigenetically silenced, potentially as a mechanism to overcome growth inhibitory effects exerted by wild-type MMP-8. In these rare "long-term" MMP-8 expressing cells phenotypic alterations occurred, including elevated IL-6 and IL-8 expression independent of MMP-8, and a self-reinforcing loop between MMP-8, IL-6 and IL-8.

This pathway may contribute to the anti-tumourigenic and metastasis suppressive effects of MMP-8, or it may represent a cellular response to overcome the anti-tumour actions of the protease.

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Abbreviations

(v/v)	Volume per volume
(w/v)	Weight per volume
2D	2 Dimensional
3D	3 Dimensional
ADAM	A Disintegrin And Metalloprotease
ADAMTS	A Disintegrin And Metalloprotease with Thrombospondin Motifs
AP-1	Activator Protein 1 transcription factor
APMA	amino-phenylmercuricacetate
BM	Basement membrane
CBA	Cytometric Bead Array
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
DCIS	Ductal Carcinoma <i>In Situ</i>
DMBA	7,12-dimethylbenz(α)anthracene
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	Human Epidermal Growth Factor Receptor 2
hr	Human recombinant
HRP	Horseradish Peroxidase
Hsc70	Heat Shock Protein 70kDa

HSPC	Hematopoietic Stem Progenitor Cell
HUVEC	Human Umbilical Vascular Endothelial Cell
IgG	Immunoglobulin
IL-	Interleukin
IP	Immunoprecipitation
IR	Infra-Red
JNK	c-Jun N-terminal kinase
kb	Kilo base
kDa	kilo Dalton
LC	Liquid Chromatography
LIX	LPS-induced CXC chemokine
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MS	Mass Spectrometry
MT	Membrane-Type
NFκB	Nuclear Factor Kappa -light-chain-enhancer of activated B cells
NOD/SCID	Non-obese diabetic/ Severe Combined Immunodeficiency (mouse model)
nu/nu	Nude mouse
OS	Osteosarcoma
PAR	Protease Activated Receptor
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
pcDNA4	Plasmid vector
PCR	Polymerase Chain Reaction
PDCD4	Programmed Cell Death 4
PR	Progesterone Receptor
qRT-PCR	Quantitative reverse transcription PCR
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species

SCC	Squamous Cell Carcinoma
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
siRNA	Small interfering RNA
SNP	Single Nucleotide Polymorphism
STAT3	Signal transducer and activator of transcription 3
TBS	Tris Buffered Saline
TGF β	Transforming Growth Factor Beta
TIMP	Tissue Inhibitor of Metalloproteinases
TNF α	Tumour Necrosis Factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
VEGF	Vascular Endothelial Growth Factor

Acknowledgements

I would firstly like to thank Dylan for allowing me to carry out this very interesting, and at times very challenging and confusing project, and for encouraging me to persevere when times got tough. I would also like to thank Julie, who taught me everything I know in the lab and for her unwavering support and friendship through both the highs and the lows. I miss our coffee breaks greatly! I could not have carried out this work without funding from Big C- Norfolk and Waveney's own cancer charity, so am hugely appreciative of their funding, and also funding from Pamela Salter and the Norfolk Fundraisers. I must also thank our very own 'Big C', Christian Roghi, for providing us with entertainment in the lab along with contributing his immense knowledge to the project. Thanks also go out to Stephen, Helen, Graham and Grant for their contributions both in the lab, in meetings, and over a beer. I would also like to thank all lab members (and Ellis and Robinson lab members of course!), past and present, for making the lab a great, friendly environment to work in. Special thanks to Wout, Sally H and Carly for the PhD-moans, the drinks and the nights out.

I couldn't have done this without support and encouragement from my family and friends so thanks Mum and Dad for all the microwave dinners when I had been working late and Alice, Rose and Ruby for entertaining me with your beautiful singing and exceptional taste in music! I would like to thank my close group of friends: Anya, Becky, Carly, Hannah, Kate, Katherine, Roxanne and Sarah, for their encouragement and belief in me, especially Carly and Kate for all the dinners, gin and wine. For the same reasons I extend my thanks to Nanny and Granddad and to family friends such as the Brownes and the Bolts and also Book Group. Last but definitely not least I would like to thank Pete for your love and support whilst writing this thesis. Without you it probably wouldn't be written. Thanks everyone!

Chapter 1- Introduction

1.1 Breast Cancer

Breast cancer is largely a disease that affects Western Europe and the USA and is the most common cancer in the UK today, with women having a 1 in 8 lifetime risk of developing the disease, which is an increase from the 1 in 9 lifetime risk published until 2010 (Cancer Research UK). Incidence rates of breast cancer are increasing, with an increase of 6% over the last 10 years, presumably due to our aging population, environmental factors and life-style choices. However, survival rates in the UK have improved dramatically; with over three-quarters of women surviving at least 10 years post diagnosis and almost two-thirds of women surviving beyond 20 years of diagnosis. This is likely attributed to an increase in education through 'breast awareness' programmes and the introduction of wide-spread screening programmes, which have been estimated to save about 1300 lives a year. However, once a patient has aggressive, metastatic breast cancer which has spread to distant organs, survival is dramatically reduced to only 15% of patients surviving beyond 5 years from diagnosis (statistics from Cancer Research UK 22-03-13). Therefore, breast cancer progression, and especially factors influencing metastatic spread of the disease, need to be better understood in order to prevent cancers reaching this stage.

The normal breast is primarily made out of fatty tissue which contains lymph nodes, milk glands and milk ducts (figure 1.1). Cancer can arise from cell types of any of these structures. Carcinomas are the most common form of breast tumours and arise from epithelial cells such as those lining the milk duct and lobules (milk glands) while sarcomas arise from fatty tissue. Ductal carcinoma arising from the milk ducts is by far the most commonly seen type of breast cancer. The duct is lined by the basement membrane (BM), followed by myoepithelial cells and then the secretory luminal epithelial cells. The myoepithelial cells separate the luminal epithelial cells of the breast duct from

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the BM (see figure 1.9) surrounding the duct. Their main function in the breast is mediating interactions between the layers, which is necessary for maintaining normal breast function. They also have a structural role as they are contractile and are involved in ejaculation of milk from the ducts [6].

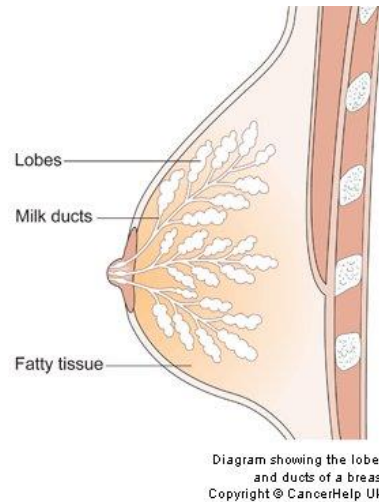


Figure 1.1: Normal breast structure. Cancer generally arises in the lobes and milk ducts (Cancer Research UK)

In the clinic breast cancer is assessed according to the TNM system (**T**umour size, **N**ode status, **M**etastasis). This system takes into account the size of the tumour (T), whether it has spread to the lymph nodes (N) and whether it has colonised distant organs (M), with the lungs and lymph glands being most commonly affected organs in breast cancer. Further classification comes from how fast the mass grows (stage), its differentiation status (grade) and its hormone receptor status. The first 3 stages of breast cancer are illustrated in figure 1.2, with stage 4 cancer involving metastatic spread to distant organs. Breast cancer subtypes are differentiated by the expression of the Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) (table 1.1). Luminal breast cancers are the most common, and easiest to treat due to their responsiveness to hormone therapies such as Tamoxifen or aromatase inhibitors, and HER2 positive cancers can be treated by Herceptin or other neoadjuvants. Basal type cancers on the other hand are non-responsive to such treatments so consequently patients with this sub-type have a worse prognosis.

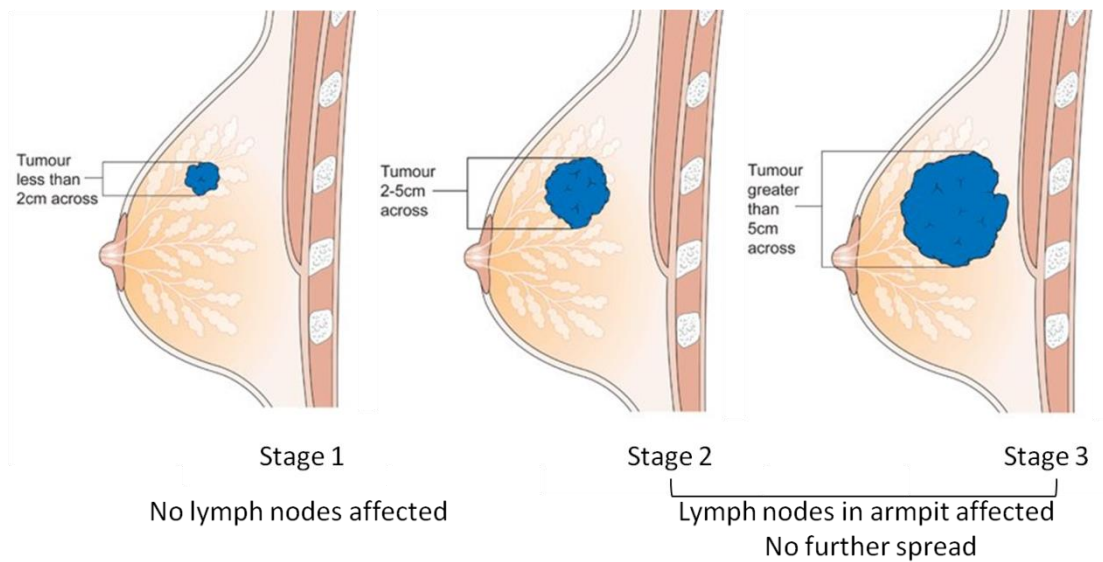


Figure 1.2: TNM grading system. Tumour is small with no lymph node involvement in stage 1 breast cancer, increasing through the 4 stages the tumour increases in size and spreads to the lymph nodes (stages 2 and 3) and to distant organs in stage 4. Adapted from Cancer Research UK (<http://www.cancerresearchuk.org/cancer-help/type/breast-cancer/treatment/tnm-breast-cancer-staging>).

Breast Cancer Subtype	ER	PR	HER2
Luminal A	+	+	-
Luminal B	+	+	+
HER2-enriched	-	-	+
Basal	-	-	-

Table 1.1: Breast cancer subtypes according to hormone receptor status. Generated from [2].

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Breast cancer occurs in a multi-step process from the original neoplasm to metastatic disease, (see figure 1.3), although it must be noted that not all cancers follow this strict progression. In the clinic, 75% of breast cancer cases diagnosed are at the Ductal Carcinoma In Situ (DCIS) stage which occurs when cells within the duct become hyperproliferative and take on an altered phenotype from the normal ductal cells due to genetic alterations. At this stage the tumour is contained and non-invasive so in most cases can be easily treated by surgery (either a lumpectomy or total mastectomy). If DCIS goes undetected the cancer cells can invade through the basement membrane and metastasise to distant organs such as the lymph nodes, lungs, liver, bone and brain [8]. This is the stage when prognosis is very poor, and treatment is generally just palliative.

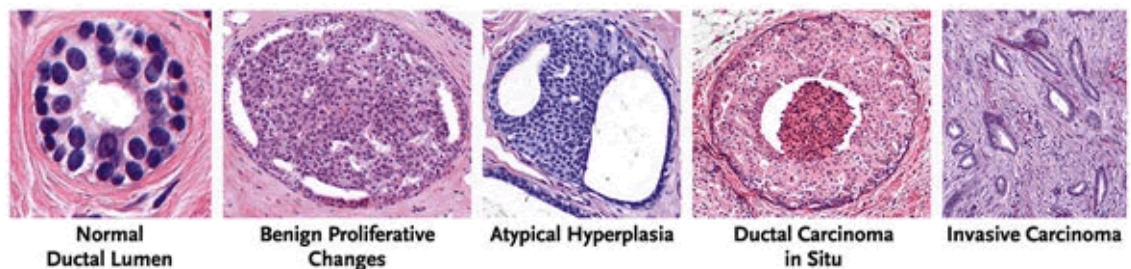


Figure 1.3: Breast cancer progression. Histological cross section of a milk duct showing a model of the progression from normal breast tissue to DCIS and then to invasive carcinoma. The cells of the duct acquire genetic changes which result in the cells undergoing uncontrolled division. These cells can then become 'atypical' as their shape changes. Women with such cells in their breast are at a high risk of developing DCIS [7].

1.2. Matrix Metalloproteinases (MMPs)

The matrix metalloproteinases form a 23 member subfamily of the metzincin superfamily of metalloproteinases. They are endopeptidases and are broadly classed according to their target substrate and domain structure: the collagenases (MMPs-1,-8,-13), cleaving collagen; the stromelysins (MMPs-3,-10) cleaving most extracellular proteins including collagens (not Type I), proteoglycans, laminin, fibronectin and elastin; the gelatinases (MMPs-2,-9) cleaving gelatin; the matrilysins (MMPs-7, -26) cleaving proteoglycans and fibronectin; and the membrane type (MT) MMPs cleaving range of substrates including collagens and Cadherins. All members are secreted and all are freely diffusible, with the exception of the MT-MMPs. The membrane-type MMPs (MMPs-14,-15,-16,-24, or MT-MMP -1,-2,-3,-5 respectively) have a transmembrane domain and an intracellular cytoplasmic tail, or a glycosyl phosphatidyl inositol (GPI) anchor (MMPs-17 and -25 and MT-4 and -6 MMPs). MMPs -23A and -23B are type II transmembrane proteases and have an IgG-like domain as opposed to a membrane spanning domain [9]. Structurally, all MMPs require a zinc ion at their catalytic site for activity, have a pro-peptide domain and a signal peptide but vary with respect to the other domains (figure 1.4). The majority of the MMPs are secreted as inactive zymogens (with the exception of MMP-11, -28 and the MT-MMPs), only being activated after removal of the propeptide domain through proteolytic cleavage. This releases the “cysteine switch” which is the inhibitory binding of the active-site zinc ion and a conserved cysteine residue that holds the MMP in an inactive conformation [1]. Activation is carried out by various proteases, such as the serine protease uPA and other MMPs (figure 1.5). They can also be activated artificially in the laboratory by amino-phenyl mercuric acetate (APMA) and trypsin.

Collectively MMPs can degrade all components of the extracellular matrix, which is primarily composed of collagens, but also elastins, laminins, glycoproteins and proteoglycans so it is imperative that expression is under tight regulatory control. MMP expression is low in most normal tissues, but can increase under certain physiological (embryogenesis, ovulation) and pathological conditions. Excess MMP expression is associated with disease

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states such as arthritis whereby increased MMP expression associated with the disease results in destruction of connective tissue and cartilage surrounding the joints [10]. Up-regulation of MMPs is however required in some pathological conditions. MMPs have a principal role in wound healing, with their expression levels being tightly regulated at different stages throughout the process. For example, MMP1 expression is increased during re-epithelialisation but switched off once the wound is repaired, whereas MMPs 7 and 9 are expressed at their highest levels during wound epithelial differentiation (reviewed in [11]), with some MMP knockout mice having wound healing defects (MMP3 [12], MMP7 [13], and MMP8 [14]).

MMP regulation is complex and occurs through a number of mechanisms on different levels. MMPs are regulated at the transcriptional level, with MMP promoters having binding sites for transcription factors associated with growth factor activity and inflammatory pathways such as NF κ B, AP-1, p53 and SP-1 [15]. Epigenetic regulation can also occur via histone acetylation and DNA methylation, with mRNA stability (possibly through the action of miRNAs) also being a factor [15]. In addition, some MMP promoters contain polymorphisms which can influence the level of the MMP expression [16]. Post-translational control is achieved by spatial and temporal control of pro-enzyme activation, and some MMPs are also inhibited by other proteins, such as plasma α -2 macroglobulin [17, 18]. The chief regulators of active MMPs are the endogenous tissue inhibitors of metalloproteinases (TIMPs). There are four human TIMPs (-1, -2, -3 and -4). TIMPs -1, -2 and -4 are secreted, and -2 and -3 are able to associate with the MT-MMPs at the cell membrane [18], where TIMP-3 is anchored [19]. Collectively, all the TIMPs can inhibit all the active MMPs, along with some members of related metalloproteases families such as the ADAMS and ADAMTSs (a disintegrin and metalloproteinase with a thrombospondin type 1 motif). The only exception is TIMP-1 which does not effectively inhibit the MT-MMPs. TIMP-2 is interesting as it has a dual role in both inhibition and activation of MMPs in that it is actually involved in the activation of proMMP2 by binding to it at the cell surface bringing it in close proximity to MT1-MMP, for activation [20].

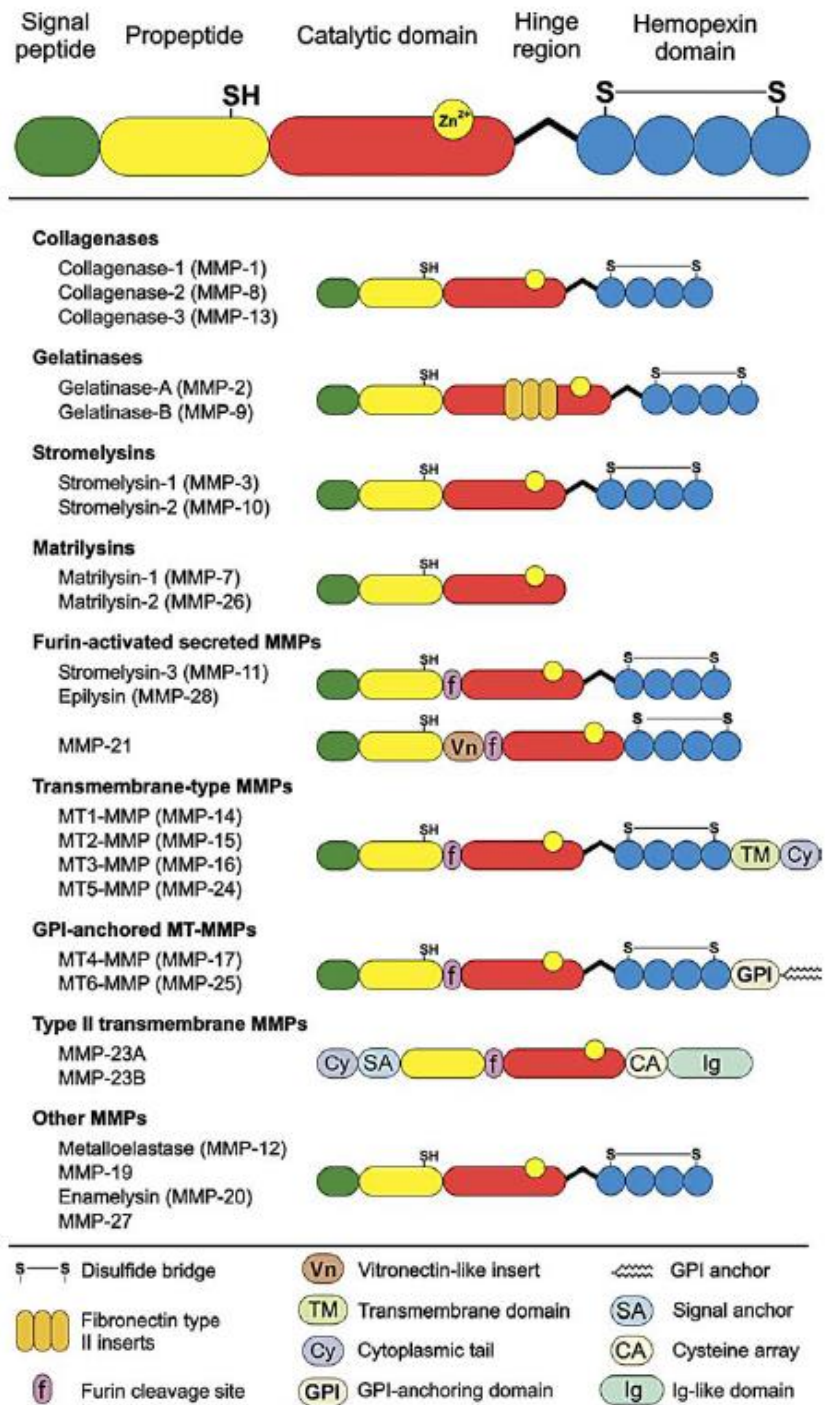


Figure 1.4: Domain structures and classification of the MMPs. MMPs are categorised according to their structure and function. Adapted from [1].

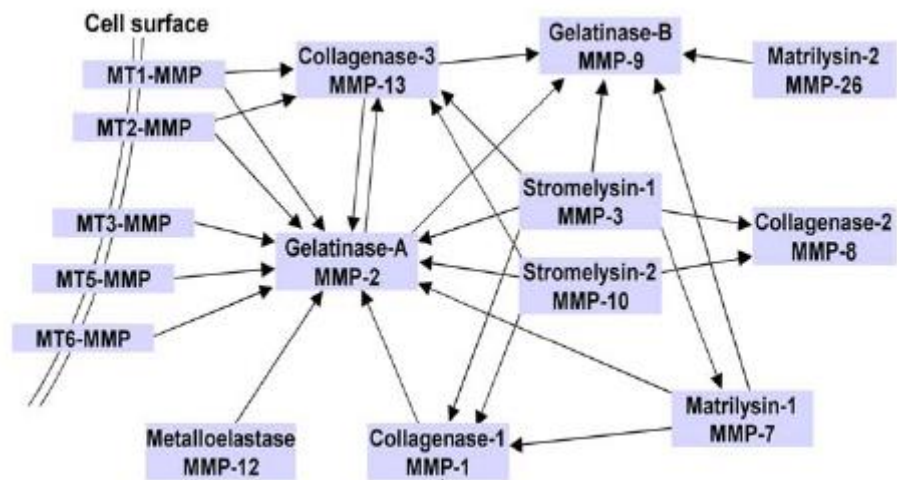


Figure 1.5: MMP cross-activation [1]. MMPs can activate each other through proteolytic cleavage to release the cysteine switch.

1.2.1 MMPs in Cancer

It was first postulated that MMPs were heavily involved in the promotion of metastasis, due to their collective ability to degrade the extracellular matrix surrounding the primary tumour and thus clearing a path for tumour cells to migrate through and disseminate to distant organs. However, more recently MMPs have been found to have roles in the entire process of tumourigenesis, from initial tumour promotion, to tumour survival and growth, angiogenesis, to the metastatic spread of the disease and the associated inflammation [4, 21] (figure 1.6). This is through manipulation of the tumour microenvironment in addition to matrix remodelling, as despite their complex involvement in cancer, MMPs are mainly produced by cells within the stroma and not the cancer cells themselves [22]. The only exceptions are MMP-7 which is predominantly expressed by cancer cells but can also be expressed by host inflammatory cells [22] and MT4-MMP, which in human breast cancers is seen in tumour epithelial cells as opposed to normal epithelial cells. Interestingly this is at the protein level only as mRNA levels between the two cell types is comparable [23]. The tumour stroma consists of a variety of cell types, such as fibroblasts, neutrophils and macrophages and endothelial cells, all of which produce MMPs. Many result

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in acceleration of tumour growth [22], and can induce the epithelial to mesenchymal transition (EMT) [24, 25], a key event that occurs within cancer cells as they progress to malignancy. As a consequence MMPs were the target of cancer therapeutic drug trials.

Clinical trials with synthetic broad spectrum MMP inhibitors were fraught with complications and were generally unsuccessful, resulting in the trials being abandoned [21]. Promising results were seen in animals treated with BB94, a broad spectrum MMP inhibitor, and was found to exert its most prominent effects in early stage cancer, reducing the angiogenic switch by 50% [11]. However, the human phase III trials were carried out on patients with advanced cancer. This was for both ethical considerations and at this time MMPs were only thought to influence the later stages of cancer, at which point the inhibitors appeared to be significantly less effective [21].

A highly probable additional reason for the failure of the clinical trials is that MMPs have subsequently been found to have differential roles in cancer progression. Some MMPs have been found to have protective effects against cancer (figure 1.7), making them anti-targets for MMP inhibitors, whereas some have both anti-and pro-tumourigenic effects [22, 26]. For example, expression of MMP3 has been found to be protective against squamous cell carcinoma [27] following chemical carcinogen treatment of *Mmp3* knockout and wild-type mice, but can also induce angiogenesis [28]. *Mmp9* knockout mice exhibit reduced incidence of tumours compared to wild-type mice, but the tumours are more aggressive [29] suggesting that it exerts different effects at late and early stages of tumourigenesis. For a comprehensive review of MMPs with anti-tumourigenic functions see Decock et al (2011) [3]. The first MMP discovered to predominantly exert protective effects in cancer, and thus making it an anti-target for cancer treatment, was MMP-8.

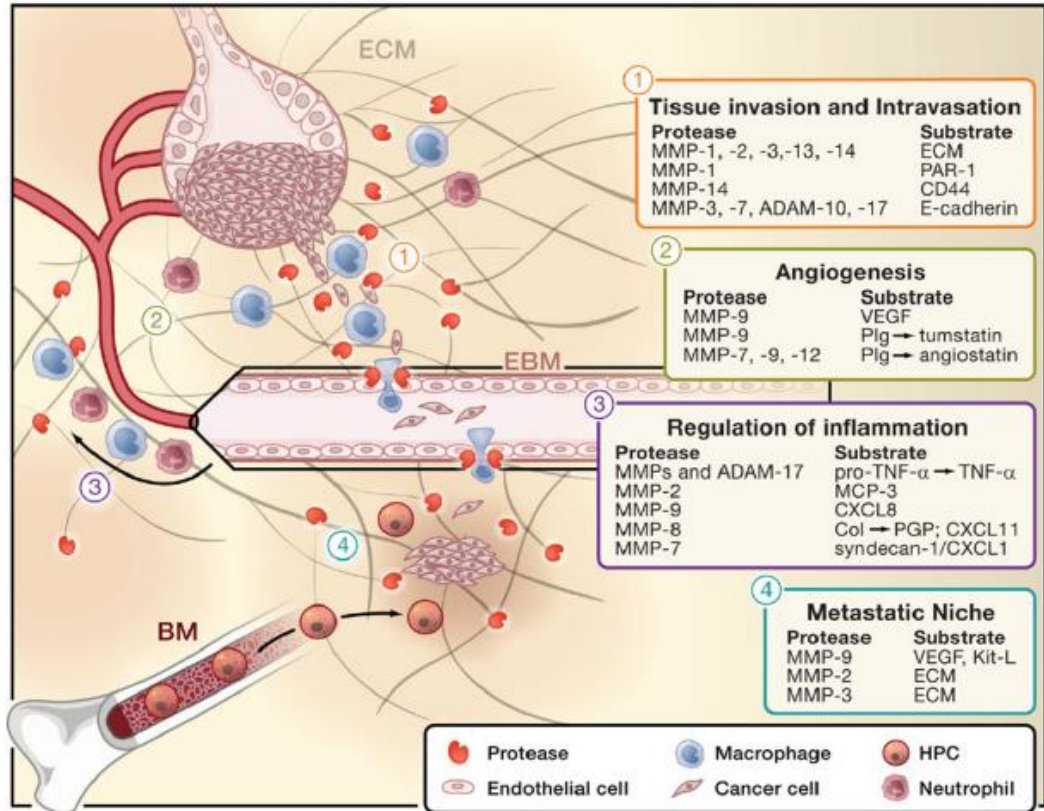


Figure 1.6: The multiple roles of MMPs in cancer progression. Recently MMPs have been discovered to be involved in all aspects of tumourigenesis from invasion, angiogenesis, regulation of inflammation, and the development of a metastatic niche [4].

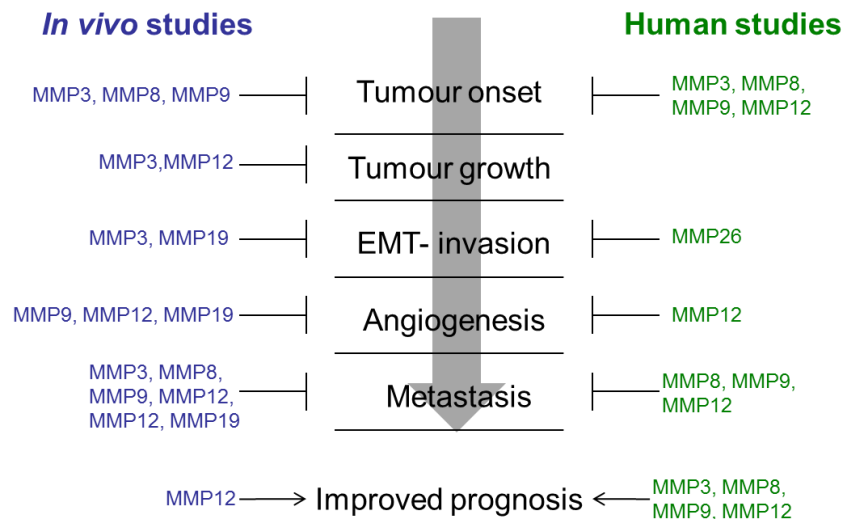


Figure 1.7: Anti-tumourigenic actions of some MMPs. The stage in tumour progression at which the MMP is acting to counteract tumour progression. Generated from data presented in a review on anti-tumourigenic MMPs by Decock *et al.*, (2011) [3].

1.3 MMP-8

MMP-8, also known as collagenase 2 or neutrophil collagenase, was first cloned in 1990 [30, 31]. Its main source is the neutrophils but has been shown to be expressed widely in different cell types such as epithelial and endothelial cells, fibroblasts, keratinocytes, smooth muscle cells and chondrocytes [32]. MMP-8 is secreted as an inactive zymogen. In the neutrophils, pro-MMP-8 is stored in the granules, due to its high level of glycosylation [30-32], and co-localises with lactoferrin [33] until required. It is activated extracellularly either by autocatalytic cleavage, chemical modification, reactive oxygen species, stromelysins 1 (MMP-3) and 2 (MMP-10) which release the cysteine switch (reviewed in [32]), and Cathepsin G and chromotrypsin [34]. A membrane-bound form of MMP-8 that is resistant to TIMP inhibition has also been reported [35, 36]. As for all MMPs, regulation of MMP-8 expression at both the protein and RNA level is under tight control, but has been shown to be upregulated by physiological factors such as hypoxia [37] and cytokines [38, 39]. Clinically, MMP-8 has mainly been implicated in a host of inflammatory conditions including atheroma [40], asthma [41], arthritis [42, 43] among many others (reviewed in [44]), and also in association with pathologies such as wound healing [14, 45-48], periodontitis [49, 50] and arterial disease [51]. Such research was primarily carried out using *Mmp8* knockout mouse models and *in vitro* model systems. The *Mmp8* knockout mouse provided the first evidence of MMP-8 having anti-tumourigenic properties, which has sparked further research into the anti-cancer functions of MMP-8.

1.3.1 MMP-8 in the normal breast

It has previously been discovered (Edwards Lab) that in the normal, non-cancerous breast, MMP8 is expressed in the myoepithelial cells ([52] and Simon Pilgrim, Edwards Lab, UEA, unpublished) (figure 1.8A), which are a cellular layer in the ducts and lobules of the breast that lay between the luminal epithelial cells and the basement membrane (figure 1.8B). The myoepithelial cells express a number of key markers (see table 1.2) including alpha-Smooth

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Muscle Actin (α -SMA) which is essential for their role in milk ejaculation. They also express integrins indicating that they are able to propagate and receive cellular signals and are involved in maintaining cell polarity. There is evidence that the myoepithelial cells are able to synthesise the ductal basement membrane [53], although this comes from rat rather than human models. Importantly, myoepithelial cells have a role in breast tumour suppression, giving out paracrine signals that result in the down-regulation of tumourigenic MMPs in surrounding cells. For example, when primary myoepithelial cells were grown with various invasive breast cancer cell lines in a co-culture invasion assay system there was up to a 2.5-fold decrease in invasion of the breast cancer cell lines. MMP expression analysis in the breast cell lines showed a reduction in the RNA levels of pro-tumourigenic MMP-2 and MT-1 MMPs. This reduction was not a result of TIMP up-regulation as there was no significant change in TIMP expression between cancer cells in mono-culture or co-culture with myoepithelial cells, suggesting paracrine regulation [54]. As further support for this paracrine signalling, another study has shown that conditioned media from cultured myoepithelial cells exerts anti-proliferative effects on epithelial breast cancer cell lines [55]. Interestingly, as breast cancer progresses past the DCIS stage the myoepithelial cells disappear or de-differentiate, through unknown processes [54]. It is only very occasionally that the myoepithelial cells themselves become malignant in the first instance, and these tumours may be more aggressive clinically [56]. There is evidence to suggest that although it is not the myoepithelial cells that initially become malignant they do exhibit genetic alterations in DCIS breast tissue compared to unaffected breast tissue [57] resulting in a loss of their ability to maintain the cellular organisation of the duct and thus facilitating tumour progression. It has been reported that changes in cytokine expression in myoepithelial cells during tumourigenesis alter proliferative and invasive capabilities of the cancerous luminal epithelial cells, enhancing the tumourigenic process [58].

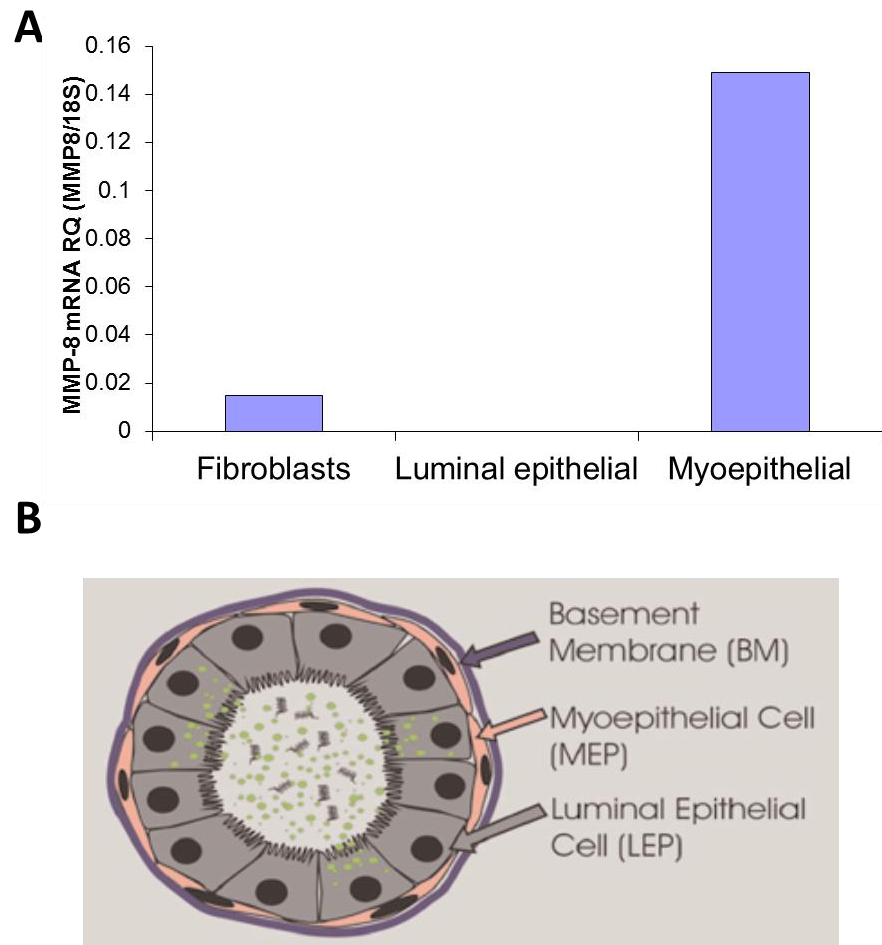


Figure 1.8: MMP8 is expressed in the myoepithelial cells of the normal breast. A) Gene expression analysis of MMP8 by Taqman RT-PCR in laser-dissected cell populations (Simon Pilgrim, Edwards Lab, UEA, unpublished). B) Cross section of duct in normal breast [6].

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Marker	Function	Reference
$\alpha 1\beta 1$ integrin	Cell-matrix interactions at focal contacts	[56]
$\alpha 2\beta 1$ integrin	Cell-matrix interactions at focal contacts	[56]
$\alpha 3\beta 1$ integrin	Cell-matrix interactions at focal contacts	[56]
$\alpha 6\beta 1$ integrin	Cell-matrix interactions at focal contacts	[56]
$\alpha 6\beta 4$ integrin	Cell-matrix interactions at hemi-desmosomes with $\beta 6$ integrin	[56]
CK5	Epithelial cytokeratin	[59]
CK14	Epithelial cytokeratin	[56]
CK17	Epithelial cytokeratin	[60]
Vimentin	Cytoskeletal intermediate filament	[56]
E-Cadherin	Component of adherens junction in epithelial cells	[56]
P-Cadherin	Placental Cadherin- component of adherens junction in myoepithelial cells	[61]
α -SMA	Contractile filament	[56]
Laminin	Basement membrane glycoproteins	[62]
Maspin	Serpin protease expressed in normal breast epithelial cells	[63]

Table 1.2: Key markers expressed by myoepithelial cells

1.3.2 Protective roles in Cancer

The earliest study showing the anti-tumourigenic effects of MMP-8 utilised *Mmp8* knock-out mice and found that *mmp8* deficiency in male nude mice caused more skin tumours when exposed to chemical carcinogens, DMBA and TPA, with a faster initiation rate compared to wild-type males [64]. This effect was reversed by injection of bone marrow from wild-type mice into the *Mmp8* knockouts, presumably because of the high levels of MMP-8 in the neutrophils. Interestingly, *Mmp8* reduced initial tumour progression, providing evidence that MMP-8 influences the early stages of tumour formation as well as later, metastatic stages, which is the stage at which research has shown most extensively where MMP8 has an impact [52, 65-67]. Montel et al. (2004) experimented on two breast cancer cell lines, M-4A4 and NM-2C5, from the same parental cell line (MDA-MB-435), that had different metastatic potentials in athymic mice [67]. The M-4A4 line is highly metastatic whereas the NM-2C5 line showed non-metastatic potential. Expression of MMP8 mRNA is 20 times greater in the NM-2C5, low metastatic line, than in the metastatic line (M-4A4). This is in contrast to MMPs 2 and 9 which were found to be expressed at the same levels in both cells lines [68], showing that MMP8 expression correlates with reduced metastatic capability of cells expressing it. Over-expression of MMP-8 in the highly metastatic M-4A4 line and ribozyme-mediated *MMP8* knockdown in the NM-2C5 line reversed the phenotypes of the two cell lines, again showing the capability of MMP8 for reducing metastasis [67]. Caution must be taken when interpreting these findings however as since this study it has been shown that that MDA-MB-435 cell line is actually a melanoma cell line rather than breast [69].

Further evidence for MMP-8 as a metastasis suppressor comes from Gutiérrez-Fernández *et al.*, (2008) [52]. Mice injected with B16F10 melanoma cells developed multiple lung metastasis, which were reduced in number when the B16F10 melanoma cells were transfected with murine *Mmp8* cDNA. An inactive mutant form of *Mmp8* did not have this effect, showing that proteolytic activity is required for MMP8 to exert its anti-metastatic properties [52]. MMP8 did not

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actually slow down the growth of melanoma cells but it did reduce the invasive ability of cells expressing it, and also increased cell adhesion to collagen-1 and laminin-1 [52], through presently unknown mechanisms. These studies contribute knowledge to the hypothesis that MMP8 is active at different stages of breast cancer progression, and can act through proteolytic processing of potentially, many different substrates, currently which are largely unknown.

In breast cancer, human studies involving the correlation of MMP8 expression with clinical diagnosis and prognosis show that cancer patients whose tumours express MMP8 are less likely to have lymph node metastasis [52]. Also, plasma MMP-8 levels have been found to be positively correlated with lymph node involvement, but negatively with distant metastasis [65]. It has been hypothesised that the more MMP-8 present in the plasma, the less there is contained within the tissue and vice versa, meaning that MMP-8 expression in the tumour may actually have a protective effect against lymph node metastasis. The DNA sequence of *MMP8* has also been shown to have an impact on prognosis. For example, Decock et al. (2007) found that four single nucleotide polymorphisms (SNPs) within the *MMP8* gene had a link to metastatic spread to the lymph nodes, especially striking in patients with the minor, *T* allele of the SNP rs11225395 showing reduced spread of breast cancer to the lymph nodes, along with increased survival and a reduction in relapse, as determined through 10 years of follow up [70]. This SNP, in the *MMP8* promoter, affected DNA binding, with the *T* allele resulting in higher promoter activity, so *MMP8* gene variation may exert some protective effects through changes in gene expression [70]. In a Korean study on gastric cancer however, this SNP was found to show no association with disease development and lymph node metastasis [71]. Further evidence for *MMP8* promoter SNPs affecting gene expression comes from studies into preterm premature rupture of membranes (PPROM) during pregnancy which showed reduced transcription factor binding to the minor allele of -799C/T SNP [72], with the *C* allele of the same SNP appearing to be protective against arterial disease [51].

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Further, MMP-8 has been investigated in squamous cell carcinomas [73, 74] whereby expression of MMP8 in the tumour cells was found to have protective effects [73]. Korpi et al (2008) found a positive correlation between MMP-8 protein expression in squamous cell carcinomas (SCC) cells and improved survival in female patients. On treatment with a chemical carcinogen, 4-Nitroquinoline-N-oxide (4NQO), female *Mmp8* knockout mice developed tumours whereas none of the wild-type female, or male, mice did [73]. The gender differences between the effects of MMP-8 are likely due to the influence of oestrogen in females, as data suggest that MMP-8 and oestrogen may impact on the expression of each other [64, 73, 75, 76]. MMP-8 has also been studied in melanoma, with Palavalli et al (2009), identifying 6 MMP8 mutants in melanoma cells from patients. Growth of human melanocyte Mel-STR cells stably transfected with wild-type MMP8 and 5 of the mutated versions identified was not affected, but wild-type MMP-8 inhibited colony formation in soft agar and reduced migration compared to the mutants. *In vivo*, injection of these same cells into NOD/SCID mice showed that wild-type MMP-8 protected against ulcerating lesions, and lung metastasis [77].

More recently, MMP-8 has been examined in osteosarcoma (OS) [78] and in colorectal cancer (CRC) [66]. Korpi et al (2011) looked at MMP-8 levels in OS biopsy, surgery samples and lung metastases. MMP-8 was detectable by immunohistological staining in the sections and biopsy samples but not in the lung metastases, whereas the other MMPs examined (-2, -13 and -26) were found in metastases as well as the primary specimens. Nothing can be concluded from this so far, as further research on a larger patient pool is required, but it may point to MMP-8 being involved in prevention of metastatic spread of OS. Also, single variable analysis did not uncover any correlation between MMP8 and survival, which may also be due to a limited sample size of only 25 patients [78]. Vayrynen et al (2011) carried out a larger scale study of 148 CRC patients and 83 healthy controls with 2-year patient follow-up, looking at MMP8 levels in the serum of patients with CRC and healthy controls. They found a greater than 3-fold increase in serum-MMP-8 (sMMP-8) levels in patients compared to controls, with highest levels in patients with stage IV,

metastatic, disease and an association between higher levels of sMMP-8 and depth of primary tumour invasion. Their short patient follow-up did find a correlation between sMMP-8 levels and recurrence, but they did hypothesise that because they did not see much sMMP-8 in patients with stage III disease, which is where patients would have lymph node spread, that MMP-8 may be protecting against lymph node metastasis in CRC [66], which is concurrent with research on MMP-8 in breast cancer [65].

In conjunction, all these studies point to MMP-8 being tumour protective, and specifically in preventing metastatic spread of the disease. However, the actions of individual proteins can differ wildly depending on their cellular environment, the type of cancer involved and the mechanism of study.

1.3.3 Conflicting roles of MMP-8 in cancer

As summarised above, the majority of studies into MMP-8 in cancer in the last 8 years provide very convincing evidence for it being tumour-protective. However, there have also been conflicting reports suggesting that MMP-8 accelerates tumour growth in other cancers. For example, in ovarian cancer, patient survival rates have been found to be higher in patients who express MMP-8 protein and mRNA at a low/non-existent level, with high levels of MMP8 expression being associated with a more aggressive cancer [38]. However, in this case MMP8 levels could not be used as an independent factor for prognosis. Higher MMP8 levels in ovarian cysts were also found to be associated with higher rates of malignancy [79]. In squamous cell carcinoma MMP-8 has been found to be expressed in cell lines derived from patient tumours, and also in islands of malignant cells *in vivo* as determined by histological staining and *in situ* hybridization. This could potentially indicate that MMP8 may have a role in the metastatic spread of the disease. However, MMP-8 was only expressed sporadically at low levels and no conclusions could be drawn with regards to patient outcome [74].

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The observed differences in the effects of MMP-8 may be attributed to the type and stage of tumour. Also, there is likely variation between the different *in vitro* models and whether RNA or protein levels of MMP8 were the focus of the study. Despite these conflicting reports, in breast cancer, the consensus is a reduction in metastatic disease and increased survival with MMP-8 expression. Therefore it is appropriate to harness the protective properties of MMP-8 to prevent metastatic spread of the disease in patients with breast cancer. As yet however the molecular mechanism and mode of action of MMP8 is largely unknown and this needs to be elucidated in order to determine potential therapeutic targets.

1.3.4 MMP-8- modulator of the immune response

The mechanism through which MMP8 exerts its anti-tumourigenic properties is largely unknown. There is however evidence that MMP8 is involved in modulating the acute immune response, and this alternative, but major role, may in part result in cancer protection.

Murine lipopolysaccharide (LPS)-induced CXC chemokine (LIX) has been found, by incubation of an array of chemokines with recombinant MMP-8, to be proteolytically cleaved by MMP-8. MMP-8 was found to be necessary for the initiation of the normal acute immune response to cell lesions and invading bacteria [80]. The human orthologues of LIX, CXCL8/IL-8 and CXCL5/ENA-78 have also been found to be cleaved by MMP-8. This cleavage, in both mice and humans is part of a 'feed-forward' mechanism that recruits neutrophils to the site of a wound or lesion. LIX, like human IL-8 is a potent neutrophil chemoattractant. Initiation of the innate immune response using the endotoxin LPS results in the release of LIX (from an unknown binding partner), resulting in migration of neutrophils to the affected site. Here, they de-granulate, releasing MMP-8 among other proteins. MMP-8 cleaves LIX, converting to its more hyperactive form which recruits and activates yet more neutrophils, resulting eventually in the resolution of the immune response, and a return to tissue homeostasis [80]. This does not occur efficiently in *Mmp8* null mice and they exhibit abnormal immune responses due to a heavily reduced number of

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neutrophils as LIX is not fully activated. This results in chronic inflammation, and this contributes to a microenvironment highly conducive to tumour development [81, 82].

Further evidence for a pro-inflammatory role of MMP-8 in immune responses comes from *Mmp8* knockout mice in an experimental model of periodontitis and TNF-induced lethal hepatitis. Hernández et al (2011) induced periodontitis in *Mmp8* knockout mice using the pathogen *Porphyromonas gingivalis* and observed that protein levels of LIX were significantly higher in the wild-type infected mice than the *Mmp8* knockout mice [49]. Van Lint et al (2005) showed a reduction in neutrophil influx to the liver and a reduction in apoptosis in *Mmp8* knockout mice with hepatitis [83]. Like Hernández et al (2011), they also found a reduction in LIX in the absence of MMP-8. Previous studies found an activation cleavage of LIX by MMP-8 [64, 80], but Hernández et al (2011) and Van Lint et al (2005) actually report a change in expression levels, possibly due to up-regulation at the RNA level or a release of LIX from ECM stores. Although the precise mechanism was not studied, it further complexity to the possible mechanistic actions of MMP-8.

Wound healing is delayed in *Mmp8* null mice compared to wild-type littermates, strongly indicating that MMP-8 is required for effective wound healing. The authors also report a delay of neutrophils migrating to the wound site, presumably as LIX is not cleaved resulting in a delayed immune response [14], or due to reduced collagen breakdown, obstructing the path of neutrophils through the ECM [84]. Interestingly, when the immune response was eventually initiated there was a greater accumulation of neutrophils which were more diffused around the wound site. Also, the immune response was sustained for up to 7 days post wounding [14, 64]. Normally the neutrophils would have undergone apoptosis at this point to reduce inflammation for healing to proceed. This delay in neutrophil apoptosis has been linked to reduced caspase 11 expression in *Mmp8* knockout mice in a model of autoimmune arthritis [42]. Vandembrouke *et al.*, (2012) also report the consequences of the pro-inflammatory functions of MMP-8 in a murine model of systemic inflammatory

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response syndrome (SIRS). However, in their study expression of MMP-8 was found to be detrimental to mouse survival. Using the same *Mmp8* knockout mice as [64], Vandenbrouke *et al.*, (2012) induced SIRS experimentally using LPS administration to induce TLR4 dependent inflammation. Using this model they showed that *Mmp8* knockout mice were protected from hypothermia and death, with prior exposure to an *Mmp8*-specific inhibitor having the same effects. The brain is an early target of the syndrome, and MMP8 was found to disrupt the brain-CSF-barrier (BCSFB), after LPS administration, through degradation of Type I collagen of the basement membrane underlying the choroid plexus. Cells comprising this epithelial layer secrete CSF and form the BCSFB. A local inflammatory response was observed in the CSF as observed by higher levels of pro-inflammatory cytokines IL-6, MIP-1 α , MIP-1 β , MIP-2 and MCP-1 in wild-type MMP-8 mice. Although this cytokine up-regulation was not mirrored in the choroid plexus cells, systemic inflammation as determined by examination of cytokine levels in blood sera, was present in the *Mmp8* wild-type mouse. This systemic inflammation resulted in organ damage and multiple organ failure in wild-type mice compared to *Mmp8* knockout mice. Interestingly, using this model they do not report any differences in neutrophil migration between wild-type and MMP8 knockout mice on LPS administration [85], a difference that was observed in other studies that used LPS to initiate inflammation [80], but this could be due to the difference in organs studied.

Predominantly in the literature, MMP-8 has been shown to have an effect on LIX (IL-8), but it has also been shown that MMP-8 can cleave both murine and human IL-10 (which in contrast to LIX and IL-8 is an anti-inflammatory cytokine), and that MMP-8 deficient mice show decreased levels of processed IL-10 [86]. Similarly, MMP-8 has been shown to solubilise IL-13 receptor α 2, which is involved in allergy-induced asthma [87] There is also a link with IL-1 β where addition of the cytokine to ovarian cancer cells increases MMP-8 protein secretion by 5.6 fold compared to controls [38], as does IL-6, which increases MMP-8 mRNA and protein levels in RPMI 8226 myeloma cells on exogenous addition for 24 hours [39]. Conversely, Transforming Growth Factor beta (TGF β), which is heavily involved in immune responses, has been shown to

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inhibit MMP-8 protein production in tumour and dermal fibroblasts from squamous cell carcinomas [74], and both MMP-8 mRNA and protein levels in human odontoblasts and dental pulp cells [88] (summarised in figure 1.9).

As aforementioned, MMP-8 is capable of cleaving CXC chemokines -8 and -5 [80] but recent evidence has shown that MMP-8 is also capable of cleaving chemokines of the CC family, which unlike CXC chemokines do not regulate neutrophils but act on monocytes, lymphocytes, basophils and eosinophils [89]. Through addition of recombinant MMP-8 to synthetic full length CCL chemokines Starr *et al.*, (2012) have shown that MMP-8 can cleave CCL15 (1-92) to either CCL15 (25-92) or CCL15(28-92), and CCL23(1-99) to CCL23(26-99) to generate products active for calcium mobilisation on transfection into murine pre-B B200-19 cells, and chemoattraction of human acute monocytic leukemia THP-1 cells. MMP-8 was also shown to cleave CCL16(1-97) to CCL16(8-85), although the generated cleavage fragment was inactive as a chemoattractant, as shown by looking at transwell migration of THP-1 monocytes. Increased glycosaminoglycan (GAG) binding was observed with the cleavage fragment however, which is a common requirement for the activity of chemokines, and CCL16 cleavage fragments were found to have modest chemoattractive activity at very high concentrations, when high levels of GAG were bound. It was also predicted from previous known cleavage sites and/or known cleavage sites generated by other MMPs that MMP-8 could be capable of cleaving CCL2-4, CCL-8, -13, and -17, although further investigation is required to confirm this [90].

MMP-8 has also been shown to have an anti-inflammatory role. Unexpectedly, intratracheal injection of LPS into *Mmp8* knockout mice resulted in a two-fold increase in neutrophil influx into the alveolar space compared to wild-type mice, thus MMP8 presence reduces the number of neutrophils within the lungs in this system, reducing acute inflammation [35, 91]. Similarly, in a mouse asthma model, on allergen stimulation *Mmp8* knockout mice showed a 10 fold increase in neutrophils, along with reduced neutrophil apoptosis [41], again indicating an anti-inflammatory role for MMP-8. Studies by Garcia-Prieto *et al.*,

(2010) and Quintero *et al.*, (2010) add further evidence to this. Garcia-Prieto *et al.*, (2010) link MMP-8 expression to increased activational cleavage of the anti-inflammatory cytokine IL-10, resulting in reduced neutrophil influx to the lungs [86]. Quintero *et al.*, (2012), like Herman *et al.*, (2001) [40] and Owen *et al.*, (2004) [35], show that *Mmp8* knockout mice exhibit increased neutrophil and macrophage accumulation after LPS and bleomycin-induced Acute Lung Injury (ALI) compared to wild-type mice. They also show that the presence of MMP-8 reduces alveolar capillary barrier injury, with the knockout mice showing a higher severity of inflammation and haemorrhage. These phenotypes were attributed to the inactivational cleavage of Macrophage Inflammatory Protein-1 α (MIP-1 α), a potent pro-inflammatory CC chemokine (alternatively known as CCL-3), that acts to recruit neutrophils to wound sites via binding to its receptor CCR-1 [36]. Further evidence has come from a more recent study by González-López (2012) that attributes anti-inflammatory properties in the lung to MMP8-mediated regulation of two alarmins, S100A8 and S100A9, in an endotoxemia mouse model after LPS stimulation. Both S100A8 and S100A9 are constitutively expressed in the neutrophils and are ligands for toll-like receptor 4 (TLR4) and Receptor for Advanced Glycation End-products (RAGE), but are increased in the lungs of *Mmp8* deficient mice in this model, resulting in an inflammatory response [91].

Collectively, these studies highlight the complexity of involvement of MMP-8 in the immune response in that it appears to be required for a normal immune response to prevent chronic inflammation [14, 64, 80], but in other cases may act to reduce acute inflammation in the first instance [35, 41]. The studies used different methods to stimulate an immune response, so this could be a possible explanation for the observed differences, but reactions could also be dependent on the tissue studied, the immune cells examined and the mouse model used.

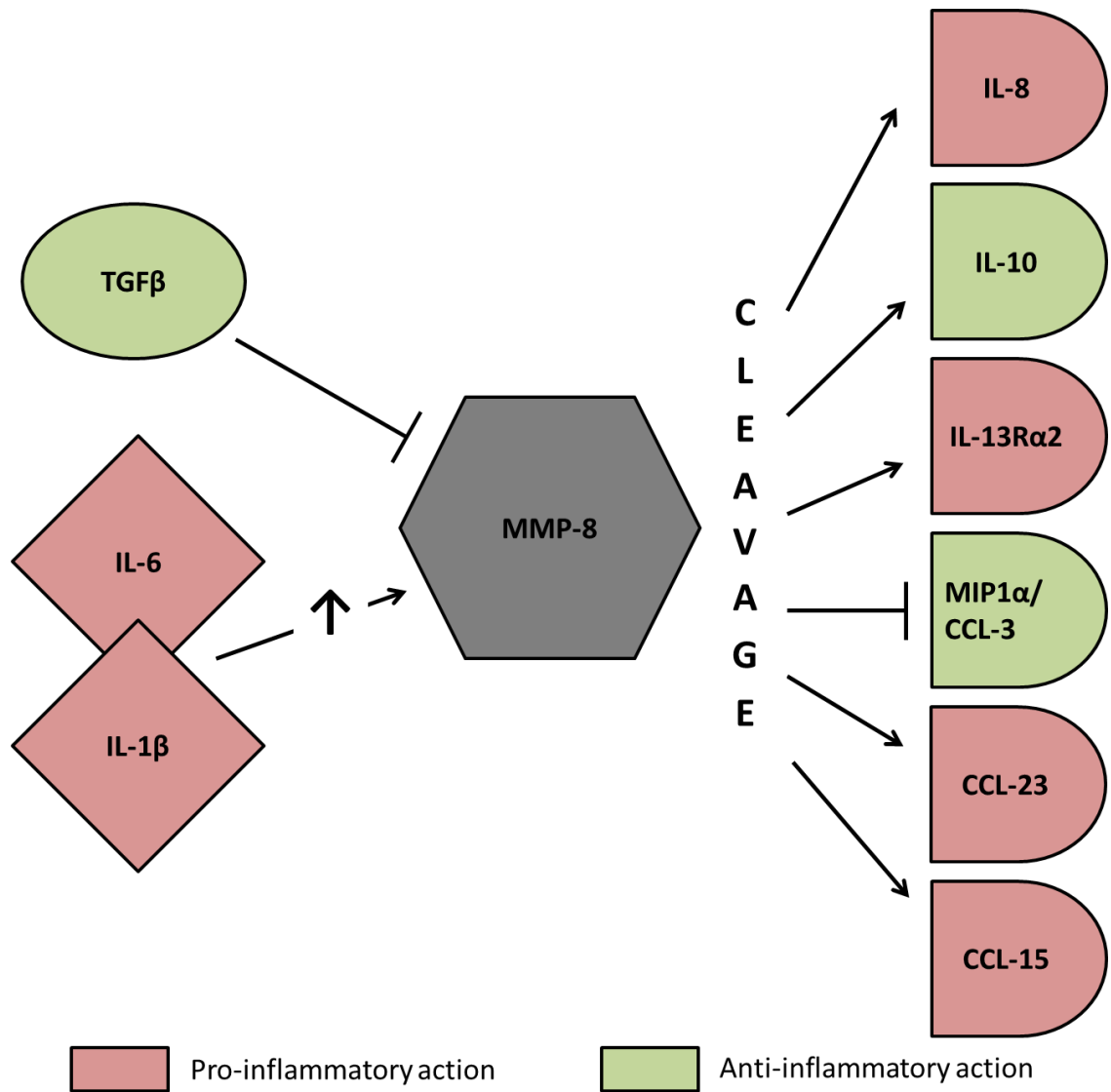


Figure 1.9: Summary of the inflammatory cytokine networks MMP8 is involved in. Key: directional arrow = activation, blunt ended arrow = inhibition.

1.4 Inflammation and Cancer

As briefly mentioned in section 1.2 (MMPs in tumour progression), the tumour microenvironment is instrumental to the initiation and development of cancer, with the tumour microenvironment hosting numerous inflammatory cells. During acute inflammation, in response to tissue wounding or an aberrant cell, the immune response is beneficial by removing and repairing the cause of the immune reaction, so plays an anti-tumourigenic role. However, a sustained, chronic inflammation, can drive cancer initiation and progression [92].

Neutrophils are the 'first response' immune cells to a wound site whereby they initiate the response. Guided by chemotactic factors (chemokines) macrophages emerge, secreting growth factors and cytokines to repair the wound. In a normal situation the inflammatory response would then be resolved; the inflammatory cells would undergo apoptosis and would be cleared by phagocytosis. If this does not occur effectively, or the cause of the immune response is not resolved, the inflammatory response is sustained chronically, resulting in tissue damage and an abundance of growth factors and mutagenizing reactive oxygen species (ROS) [81, 93].

Inflammation is driven by small signalling molecules, chemokines and cytokines, that are produced by inflammatory cells and also tumour cells. The balance of pro- and anti-inflammatory cytokines in the tumour microenvironment determine the outcome of tumour growth. Large amounts of pro-inflammatory cytokines result in chronic inflammation, and consequently in increased tumour growth, and angiogenesis. In contrast, tumours producing fewer pro-inflammatory cytokines than anti-inflammatory cytokines cannot use them to growth advantage [81].

Chronic inflammation not only accelerates tumourigenesis but can also initiate it. This is because the reactive oxygen and nitrogen species produced by inflammatory cells during an immune response can induce DNA mutagenesis. In

addition, an increasing number of cancers are associated with infections, for example cervical cancers caused by the human papilloma virus (HPV) [94, 95].

Parallels have been drawn between tumours and wound healing, in that both growth of new cells following wounding and tumourigenesis require cytokine signalling, up-regulation of growth factors, cell proliferation and survival. In the 1980's Dvorak described cancers as 'wounds that do not heal' [96]. A study by Chang *et al.*, (2005) showed that overall and metastasis-free survival of breast cancer patients is reduced if tumours express a "wound-response" gene signature [97], and more recently a study by Riss *et al.*, (2006) used microarray technologies to quantify the gene expression changes associated with renal regeneration and repair and renal cell carcinoma [98]. They showed that 77% of the genes expressed under both pathologies were regulated concordantly. Of the pathways investigated, the NFκB pathway significantly contained a high number of these concordant genes [98]. NFκB has been described as the key mediator between inflammation and tumourigenesis [92, 99]. This is due to its role in promotion of cell proliferation and inhibition of apoptosis [100], its central role as a transcription factor for pro-inflammatory cytokines (e.g. Interleukin 6 (IL-6) and its downstream effector STAT3) promoting cellular growth, and expression of tissue destructing MMPs such as MMPs 1,9 and 13 promoting invasion [99, 101]. NFκB is constitutively active in the majority of cancers, emphasising its pivotal role in both inflammation and tumourigenesis.

1.4.1 Inflammation in breast cancer

Inflammatory breast cancer is a rare type of breast cancer whereby cancer cells block lymphatic vessels, resulting in inflammation, but the cancer itself is not actually caused by inflammation [102]. The core inflammatory mediator, NFκB, however has been implicated in breast cancer progression through its role in the regulation of E-Cadherin, a marker of an epithelial, differentiated state of cancers and its role in the metastatic spread of the disease. Increased activity of some NFκB subunits have been found to be present at higher levels in breast cancer cells compared to adjacent non carcinoma cells [103], and is seen at

higher levels in Estrogen Receptor (ER) negative tumours and breast cancer cell lines than in ER positive lines [104, 105]. This indicates that NF κ B expression is involved in aggressiveness of cancer. Moreover, there is evidence that it has a role in the epithelial to mesenchymal transition (EMT) of breast cancer cells, thus propelling the cancer further in the direction of aggressive, metastatic disease. This has been shown in MCF10A breast cells through the repression of E-Cadherin, an epithelial marker and the induction of vimentin, a mesenchymal marker with the over-expression of the p65 NF κ B subunit [106] and also in Ras-transformed mammary epithelial cells, whereby blockade of NF κ B activity prevented EMT [107]. With regards to metastatic spread of breast cancer, NF κ B has been shown to be crucial in metastasis of breast cancer cells to the bone [108] so consequently, it is a prime target in breast cancer therapies. As aforementioned there are numerous down-stream targets of NF κ B that are involved in a whole host of cellular processes. A cytokine and a chemokine, IL-6 and IL-8 respectively, are such targets that have been discovered to be affected by over-expression of MMP-8 in the breast cancer cell model utilised in this thesis. The subsequent and concluding section of this chapter will investigate their roles in inflammation and cancer in general.

1.4.2 IL- 6 and IL-8- Introduction and Regulation

IL-6 and -8 are proteins involved in immune responses. IL-6 is a 21-28kDa cytokine [109], which has a pivotal role in the immune response but can also act on multiple cells in a range of organs and tissue systems. Due to these wide functions IL-6 is known as a 'pleiotropic' cytokine [110]. IL-6 has two signalling pathways, classical and trans. IL-6 actually signals through two receptors which aggregate to form the complete receptor, the first is an 80kDa protein, IL-6R α , and the second in a 130kDa protein, gp130. If both of the receptors are bound to the membrane of the target cell this is 'classical' signalling. However, soluble IL-6R α (sIL-6R α) can be generated from differential splicing at the mRNA level or from shedding of the bound receptor [111, 112]. IL-6 can bind sIL-6R α forming a heterodimeric complex which can bind to the membrane-bound gp130 on the target cell [113]. This 'trans' signalling occurs when the target cell does not have

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an endogenous IL-6R α . The IL-6R α is mainly expressed on cells of the immune system but also on neural and skeletal tissue [110], whereas the gp130 receptor is expressed by most cell types [114]. Signalling through the sIL-6R α has been implicated in many disease states such as arthritis, asthma, inflammatory bowel disease and cancers [112]. IL-6 downstream signalling is widespread and complex (summarised in figure 1.10). The main downstream signalling effector discussed in the literature is STAT3, which propagates pro-survival signals, and has been described as a 'multifaceted oncogene' through its ability to both promote cell survival and protect cells from immune surveillance [115]. IL-6 has been described as having both pro- and anti-inflammatory roles. It is anti-inflammatory in acute inflammation [116, 117] but pro-inflammatory in chronic inflammatory disease [118, 119]. Therefore, IL-6 is involved in the generation of an environment of chronic inflammation which is conducive to tumour initiation and progression.

IL-8, otherwise known as CXCL8, is a pro-inflammatory CXC-type 8kDa chemokine, with a predominant role as chemo-attractant for, and activator of neutrophils, thus playing a strong role in acute inflammation [120]. It signals through two cell-surface G-Protein Coupled Receptors, CXCR1 and CXCR2 resulting in mobilisation of calcium and downstream signalling [121, 122]. These receptors have different specificities; CXCR1 is only activated by IL-8 and GCP-2, whereas CXCR2 can be activated by multiple growth factors and cytokines [5]. Like IL-6, IL-8 downstream signalling is broad and complex, but can be summarised in figure 1.11. IL-8 does not solely regulate neutrophil chemotaxis but can regulate many other cellular pathways in a range of cell types.

As can be seen from figures 1.10 and 1.11 the majority of the down-stream signalling of both IL-6 and IL-8 is pro-inflammatory and pro-cell survival, and as a consequence the majority of studies on the roles of IL-6 and -8 in cancer points to both cytokines being pro-tumourigenic.

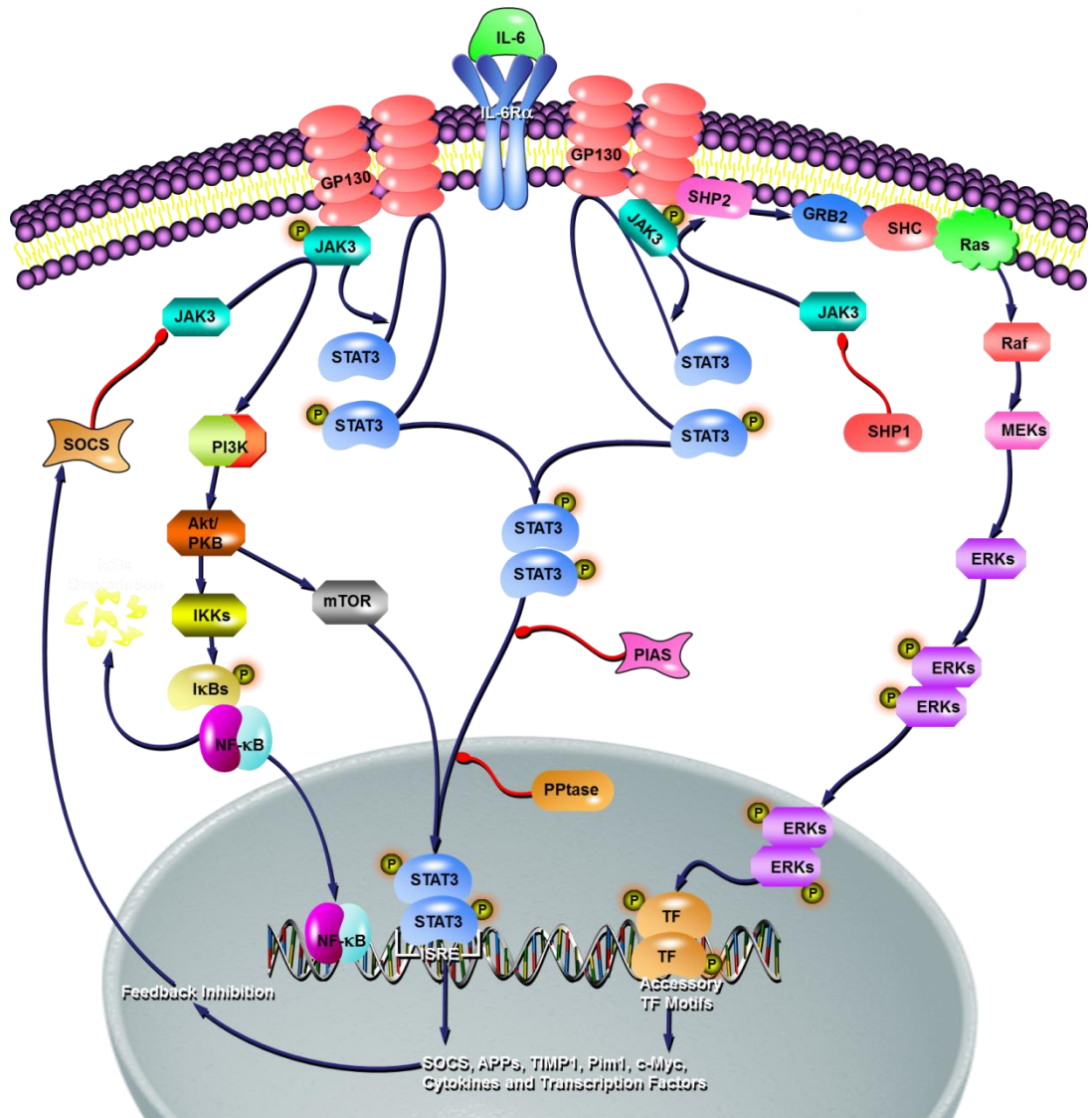


Figure 1.10: The IL-6 pathway in Cancer. On binding its receptor IL-6 initiates a cascade of kinase phosphorylation resulting in the activation of the transcription factor STAT3 which regulates genes involved in cell survival. Adapted from a publically available figure from Qiagen.

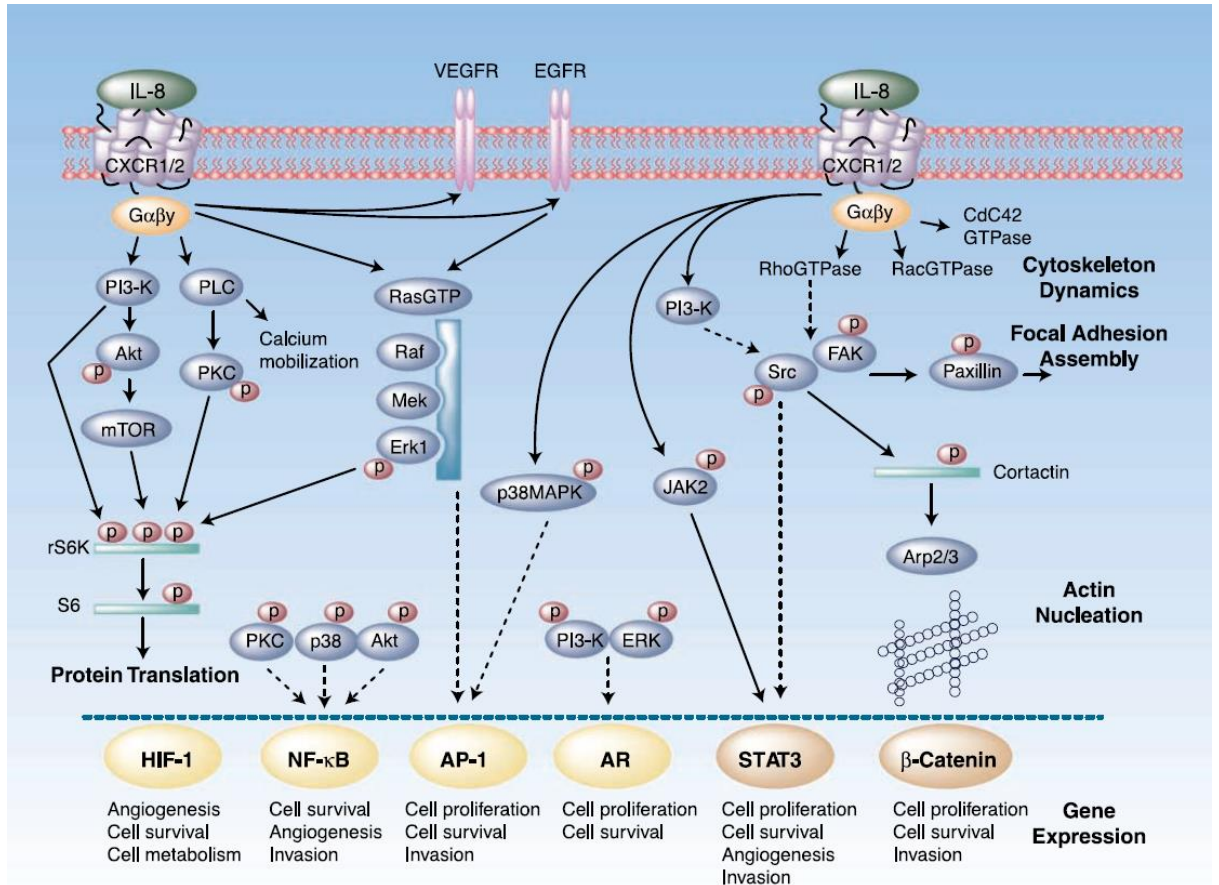


Figure 1.11: IL-8 downstream signalling in cancer. IL-8 initiates a kinase phosphorylation cascade resulting in activation of transcription factors involved in tumourigenesis [5].

1.4.3 IL-6 in Cancer

IL-6 has been implicated in a range of cancers and is predominantly described as a target for cancer treatment. *In vitro* studies have shown that exogenous addition of rhIL-6 to a range of human breast cancer cell lines induces the epithelial to mesenchymal transition which was characterised by a reduction in E-Cadherin expression (Sullivan 2009). In addition, MCF-7 cells over-expressing IL-6 exhibited an enhanced invasive capacity, induced vimentin expression and showed a lack of E-Cadherin expression showing IL-6 expression could reverse the phenotype of these cells. Similar results have been published in prostate cancer, with exogenous addition of IL-6 to a non-tumourigenic prostate cancer cell line, SV40T, resulting in morphological changes, loss of E-Cadherin expression and gain of vimentin expression [123]. In biliary tract cancer cell lines exogenous addition of rhIL-6 could induce EMT and cellular invasion [124]. Despite these pro-tumourigenic roles of IL-6 *in vitro*, in contrast Chiu *et al.*, (1996) showed that addition of rhIL-6 to some ER+ cells (but not MCF-7 cells) reduced proliferation. IL-6 produced by ER- cells such as MDA-MB-231 cells could exert this effect showing that IL-6 can act as a paracrine growth factor [125]. The high levels of IL-6 produced by ER- cells, which are predominantly cells which have come from a highly aggressive tumour however suggests that IL-6 may be involved in the high tumourigenicity of these cell lines.

IL-6 has also been implicated in ovarian cancer, for example, elevated IL-6 levels in ovarian cancer ascites was found to be an independent predictor of reduced progression free survival [126]. High IL-6 expression in ovarian tumours, with higher levels found in malignant cells compared to the tumour stroma, was found to be positively associated with shorter overall patient survival [127]. Studies have also described a dynamic cytokine network in ovarian cancer, in which IL-6 has been found to play a dominant role [127-129]. IL-6 has also been found to be a key driver in malignant progression of squamous cell carcinoma where it initiates a complex self-reinforcing cytokine-MMP network [130, 131]. It has also been found to be higher in the serum of lung cancer patients

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compared to healthy controls [132]. Additionally, tumour IL-6 mRNA levels have been found to be reduced in non-tumour cells compared to tumour cells, presumed to be due to promoter methylation in the non-tumour cells [133]. In nonsmall cell lung cancer IL-6 has been shown to be a predictor of poor survival in [134].

Within breast cancer tissue itself IL-6 protein and RNA has been detected (reviewed in [135]) but whether IL-6 can be used as a prognostic factor, or whether the IL-6 present in the tumours was actually produced by the tumour cells has led to great discussion and controversy [135]. However, serum IL-6 levels have been consistently shown to be higher in breast cancer patients [136], especially patients with higher grade tumours [137, 138], and in patients with re-current breast cancer [139, 140]. Serum IL-6 levels have also been linked to reduced relapse free survival and overall survival [138, 141] and so therefore has been described as a predictor of breast cancer survival.

To summarise, IL-6 has been implicated in a range of cancers, predominantly having a tumour promoting role. IL-8 plays slightly different roles in cancer, but is just as implicitly involved.

1.4.4 IL-8 in cancer

Like IL-6, IL-8 has been implicated in tumour growth and EMT but has also been heavily described as a promoter of angiogenesis via up-regulation of VEGF. Firstly the *in vitro* effects of IL-8 will be described, followed by results of human studies. IL-8 (and its receptors, CXCR1 and 2) has been found to be elevated in epithelial cells that have undergone EMT [142]. Endogenous expression of IL-8 in mammary carcinoma cell lines appears to correlate with metastatic potential, with high levels secreted by the metastatic MDA-MB-231 cells and low/non-existent levels secreted by the non-metastatic MCF-7 and T47D cell lines [143-145]. In addition, IL-8 induction by ROS increased the invasiveness of MDA-MB-231 cells [146] and IL-8 signalling blockade resulted in a reduction of the ability of MCF-7 and MDA-MB-231 breast cancer cells to invade through

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Matrigel *in vitro* [142]. Additionally, Lin *et al.*, (2004) examined the cytokine profiles of a range of ER+ and ER- breast cancer cell lines, with IL-8 being found to be positively correlated with vimentin status (a marker of EMT) and the cell's metastatic status. IL-8 expressing cells were more invasive through Matrigel which was reduced on exposure to IL-8 neutralising antibodies [144]. Knocking down IL-8 in MDA-MB-231 cells, which endogenously produce lots of IL-8, confirmed that *in vitro* invasion through Matrigel was reduced [147]. Both studies showed however that IL-8 had no effect on either cellular proliferation or cell cycle profile [144, 147]. Collectively these studies led to IL-8 being considered an important factor in breast cancer progression.

Early studies provide evidence for a role of IL-8 in the tumour microenvironment. Youngs *et al.*, (1997) showed that IL-8 induced migratory chemotaxis of MCF-7 cells through an 8µm-pored microchemotaxis chamber, even though the cells do not endogenously express it [148]. This chemoattractive activity of IL-8 on carcinoma cells, as well as its main role in neutrophil chemotaxis, suggests that IL-8 in the tumour microenvironments, may have a role in the metastatic spread of cancer. The most predominant role of IL-8 in the tumour microenvironment is in angiogenesis through its ability to activate the vascular endothelial growth factor receptor 2 (VEGFR-2) in endothelial cells, increasing the permeability of the endothelial barrier [149] and allowing for intravasation of tumour cells and neutrophils, and to induce angiogenesis through up-regulation of VEGF via CXCR2 [150] which can then co-localise with VEGFR-2 [149].

In vitro studies utilising Human Umbilical Vascular Endothelial Cells (HUVECS) as a model system for angiogenesis have shown that IL-8 can induce the migration of HUVECs through Matrigel, promote tube formation into vessel-like structures [144, 151, 152], and increase HUVEC proliferation [151, 152] while inhibiting apoptosis [152]. HUVECS express both IL-8 receptors, CXCR1 and 2, , suggesting that IL-8 propagates these actions through its traditional signalling pathways [152]. *In vivo*, subcutaneous injection of conditioned media (combined with Matrigel to form a plug) from IL-8 expressing cells induced

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blood vessel formation (validated by a CD31 stain which is specific for vascular cells) compared to media from low IL-8 expressing cells [144], and tumours resulting from subcutaneous injection of MDA-MB-231 cells displayed a higher microvasculature density than tumours arising from injection of MDA-MB-231 cells with suppressed IL-8 [147]. However, in this study, IL-8 suppression in MDA-MB-231 cells resulted in faster growth of tumours after subcutaneous injection, suggesting that IL-8 may have a tumour suppressive role in this model [147]. Additional support for the pro-angiogenic role of IL-8 comes from a nude mouse xenograft model in which Ras-induced IL-8 expressing HeLa cells form subcutaneous tumours. Tumours injected biweekly with an antibody to IL-8 exhibited a reduction in tumour growth rate which was due to an increase in necrosis attributed to a reduction in angiogenesis. Tumours that were not injected with the IL-8 neutralising antibody had significantly more vascularisation than those where IL-8 was inhibited [153].

There have not been many human studies looking into the prognostic significance of IL-8 in breast cancer. Generally IL-8 is associated with a more aggressive cancer subtype and therefore could potentially be used as a predictor of metastatic disease. A small scale study by Derin *et al.*, (2007) showed a correlation between serum levels of IL-8 and metastatic breast cancer but did not observe any differences in patients compared to healthy controls, nor any correlation with prognosis [154]. A slightly larger study by Benoy *et al.*, (2004) showed increased serum IL-8 levels in breast cancer patients compared to controls, with higher levels correlating with a more aggressive cancer and metastasis [155]. Higher IL-8 (and IL-6 and IL-10) serum concentrations have been found in patients with breast cancer compared to controls, with higher levels positively correlated to higher grade [137]. Therefore, although evidence is limited IL-8, like IL-6, appears to be associated with higher grade tumours, metastasis and a poorer prognosis.

Project Aims

This chapter has described in detail the anti-tumourigenic effects of MMP8 in cancer and has touched on its tumour protective role in human breast cancer. However, apart from the knowledge that MMP8 is required for cognate immune responses, little is known mechanistically about how it exerts these effects. Its influence on the immune system during acute immune responses suggests that MMP8 may be tumour-protective via its immuno-modulatory functions.

The aims of this project are to dissect the functions of MMP8 in mammary carcinoma cells through the generation of an MMP8 *in vitro* expression model. Using this model both the functional and molecular influence of MMP8 on the cells will be determined using a range of microscopic, biochemical and proteomic approaches.

Chapter 2- Materials and Methods

2.1 General Reagents, antibodies, plasmids and cell lines

2.1.1 General chemicals

Table 2.1 lists general chemicals used to make widely used buffers (see table 2.2). More specific reagents are described within the appropriate method.

	Chemical/Reagent	Source
Calcium Phosphate Transfection	Calcium Chloride dehydrate (CaCl ₂ ·2H ₂ O)	Fisher Scientific (C/1500/53)
	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Fisher Scientific (BPE310a)
General Buffers	Trisaminomethane (TRIS Base)	Fisher Scientific (BP152)
	Phosphate Buffered Saline (Dulbecco A) tablets	Oxoid (BR0014G)
	Glycerol	Fisher Scientific (G/0650/17)
	Sulphuric Acid (H ₂ SO ₄)	BDH Lab supplies
	Hydrochloric Acid (HCl)	Fisher Scientific (H/1200/PB17)
	Sodium Hydroxide (NaOH)	Fisher Scientific (S302)
	Triton X-100	Sigma (93443)
	Polysorbate 20 (TWEEN 20)	Sigma (P7949)
	Sodium Chloride	Fisher Scientific (S/3160/60)
Plasmid propagation	Agar	Formedium Ltd. (AGA02)
	Luria Broth (LB)	Merck (VMO40685)
	Tryptone	Fisher Scientific (BPE1421)

Table 2.1: Commonly used chemicals and reagents grouped according to their most common usage.

Chapter 2- Materials and Methods

	Chemical/Reagent	Source
Plasmid propagation	Yeast extract	Fisher Scientific (BP1422)
	Ampicillin	Formedium (AMP25)
	Agarose	Fisher Scientific (BP1356)
	Ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific (D/0700/53)
	Glacial Acetic Acid	Fisher Scientific (A/0400/BP17)
Western Blotting	Protogel- 30% (w/v) Acrylamide:0.8% (w/v) Bis-Acrylamide electrophoresis grade	National Diagnostics (EC-890)
	Tetramethylethylenediamine (TEMED)	Invitrogen (15524-010)
	Sodium Dodecyl Sulfate	Fisher Scientific (BP166)
	Ammonium Persulfate (APS)	Sigma (A3678)
	Bromophenol Blue	Sigma (B8026)
	Ponceau S	Electran (44083)
	Bovine Serum Albumin	Fisher Scientific (BPE9701)
	Skimmed Milk Powder	Oxoid (LP0031)
	SimplyBlue™ SafeStain	Invitrogen (LC6060)
	Methylene Blue	Sigma (M9140)

Table 2.1: Commonly used chemicals and reagents grouped according to their most common usage.

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Buffer	Recipe	Section used
LB Broth	5g yeast, 10g NaCl, 10g Tryptone in 1L dd H ₂ O and sterilised by autoclave	2.2.1
LB Agar	5g yeast, 10g NaCl, 10g Tryptone, 15g Agar in 1L ddH ₂ O and sterilised by autoclave	2.2.1
TAE (50x stock diluted in ddH ₂ O when required)	2M Tris-HCl and 1M glacial acetic acid with 100ml 0.5M EDTA made up to 1L with ddH ₂ O	2.2.1
Calcium Chloride	2M CaCl ₂ in ddH ₂ O and filter sterilised using a 0.2µm filter	2.3.2
2x HEPES Buffered Saline (HBS)	280mM NaCl, 1.5mM Na ₂ HPO ₄ , 50mM HEPES in ddH ₂ O and filter sterilised using a 0.2µm filter	2.3.2
Methylene Blue	2g Methylene Blue, 500ml ddH ₂ O and 500ml methanol	2.3.6 and 2.6.5
Destain	50% EtOH with 50% 0.1M HCl	2.6.5
PBS	1 PBS tablet (see table 2.1) in 100ml ddH ₂ O	multiple
TBS (10x stock diluted in ddH ₂ O when required)	0.2M Tris-HCl and 1.4M NaCl made up to 1L ddH ₂ O, pH 7.6	2.4.4
RIPA protein lysis buffer	50mM TRIS-HCl, 150mM NaCl, 1% TRITON-X, pH7.8	2.4.1
SDS Sample buffer for western blotting	125mM TRIS-HCl, 5% SDS, 15% Glycerol, Bromophenol Blue pH 6.8	2.4.3 and 2.3.4
Ponceau S	0.1% Ponceau in 0.5% Acetic Acid	2.4.4
Coating buffer for adhesion	0.1M Sodium Carbonate, pH 9.6	2.6.5
Acetate citrate buffer	0.3M sodium citrate, 145mM tri-sodium citrate, 28mM citric acid, 385ml propan-2-ol made up to 1 litre with ddH ₂ O and pH 6)	2.6.6.2
3,3'-Diaminobenzidine (DAB)	3M DAB in 70% perchloric acid	2.6.6.2

Table 2.2: Buffers

2.1.2. Antibodies

Table 2.3 lists the antibodies used throughout this project.

	Primary Antibody	Supplier	Concentration used (for Western Blot*)	Secondary Antibody
Cell line characterisation	MMP8	Abcam (ab81286)	1:2000	Anti-Rabbit
	V5 tag	Invitrogen (46-0705)	1:1000	Anti-Mouse
	Cytokeratin 18	Cell Signalling (#4548)	1:1000	Anti-Mouse
	β -Catenin	Cell Signalling (#9587)	1:1000	Anti-Rabbit
	Integrin β 1	NCA	1:1000	Anti-Mouse
	E-Cadherin	Cell Signalling (#4065)	1:1000	Anti-Rabbit
	Vimentin	Cell Signalling (#3932)	1:1000	Anti-Rabbit
	MMP14	NCA [156]	1:1000	Anti-sheep
	PAR2 (D61D5)	Cell Signalling (#6976)	1:1000	Anti-Rabbit
Cell Signalling	Total NF κ B p65 (F-8)	Santa Cruz (sc-8008)	1:1000	Anti-mouse
	Phospho NF κ B p65 (ser536)	Santa Cruz (sc-101752)	1:1000	Anti-Rabbit
	Total I κ β α	Santa Cruz (sc-371)	1:1000	Anti-Rabbit
	Phospho I κ β α (ser32)(14D4)	Cell Signalling (#2859)	1:1000	Anti-Rabbit
	Total IKK α	Cell Signalling (#2682)	1:1000	Anti-Rabbit
	Phospho IKK α (ser176)/IKK β (ser177) (C84E11)	Cell Signalling (#2078)	1:1000	Anti-Rabbit

Table 2.3: Antibodies used throughout this project primarily for western blotting grouped according to usage. NCA= not commercially available, * = with the exception of the functional blocking antibodies

	Primary Antibody	Supplier	Concentration used (for Western Blot*)	Secondary Antibody
	p38 MAPK	Cell Signalling (#9212)	1:1000	Anti-Rabbit
	Phospho p38 MAPK (Thr180/Tyr182) (28B10)	Cell Signalling (#9216)	1:1000	Anti-Rabbit
	p44/42 MAPK (Erk1/2)	Cell Signalling (#9102)	1:1000	Anti-Rabbit
	Phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signalling (#9101)	1:1000	Anti-Rabbit
Apoptosis and Cell Cycle	Caspase 3	Cell Signalling (#9662)	1:1000	Anti-Rabbit
	Caspase 7	Cell Signalling (#9492)	1:1000	Anti-Rabbit
	Caspase 9	Cell Signalling (#9502)	1:1000	Anti-Rabbit
	PARP	Cell Signalling (#9542)	1:1000	Anti-Rabbit
	PDCD4	Abcam (ab51495)	1:2000	Anti-Rabbit
	p53 (DO-1)	Santa Cruz (sc-126)	1:10 000	Anti-Mouse
	p21 Waf1/Cip1 (12D1)	Cell Signalling (#2947)	1:1000	Anti-Rabbit
Mass Spectrometry Validation	Annexin A1	Abcam (ab33061)	1.25µg/ml	Anti-Rabbit
	MRP8 (S100A8) [EPR3554]	Abcam (ab92331)	1:1000	Anti-Rabbit
Function-Blocking	IL-8	R&D Systems (MAB208)	100ng/ml	NA
	IL-6	R&D Systems (MAB2061)	100ng/ml	NA
Loading Controls	Heat Shock Protein 70 (hsc-70)	Santa Cruz (sc-7298)	1:1000	Anti-mouse
	GAPDH (14C10)	Cell Signalling (#3683)	1:1000	HRP-conjugated/anti-rabbit

Table 2.3: Antibodies used throughout this project primarily for western blotting grouped according to usage. NCA= not commercially available, * = with the exception of the functional blocking antibodies

		Supplier	Concentration used (for Western Blot*)	Secondary Antibody
Secondary Antibodies	Mouse HRP conjugated	DAKO	1:1000	NA
	Rabbit HRP conjugated	DAKO	1:1000	NA
	Sheep HRP conjugated	DAKO	1:1000	NA
	Donkey anti-Rabbit Infra-Red conjugated	Licor	1:10000	NA
	Donkey anti-Mouse Infra-Red conjugated	Licor	1:10000	NA

Table 2.3: Antibodies used throughout this project primarily for western blotting grouped according to usage. NCA= not commercially available, * = with the exception of the functional blocking antibodies

2.1.3. Plasmid constructs

Wild-type MMP8 has previously been cloned into the pcDNA4TM V5-His A plasmid (Invitrogen) using the *EcoRI* and *XhoI* restriction sites (figure 2.1) by Dr. Julie Decock. The vector backbone alone was used as a negative control for transfection, and E198A, a catalytically inactive version of MMP8 was generated using site-directed mutagenesis (section 2.1.3.1). Zeocin resistance was used for generating stable cell lines (see section 2.2.2) and ampicillin resistance was used for bacterial propagation of the plasmid (see section 2.2).

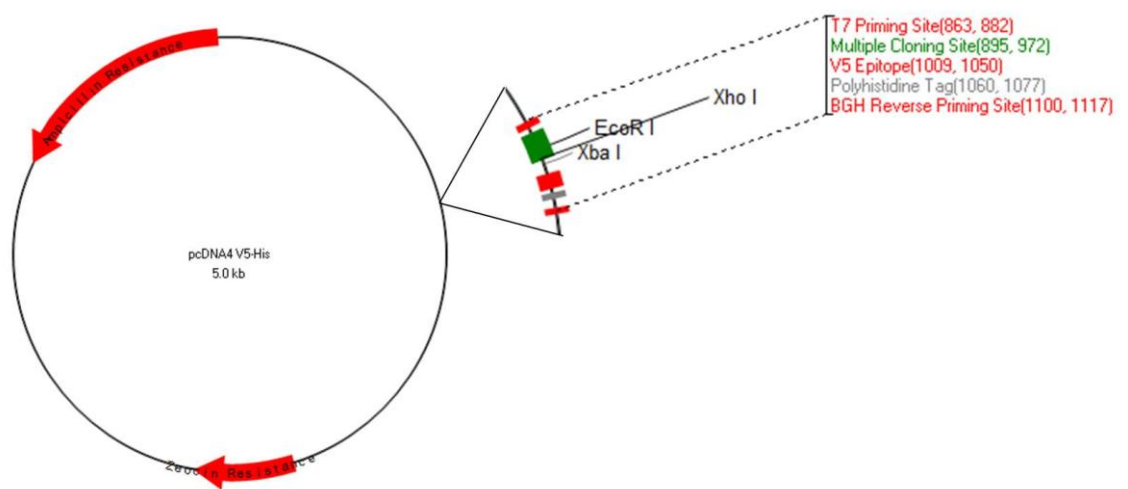


Figure 2.1. Plasmid map of pcDNA4 V5-His A. Ampicillin and Zeocin resistance sites are highlighted along-side the multiple cloning site containing *EcoRI* and *XhoI* restriction sights which were used to clone MMP-8 into the vector and the unique *XbaI* site which was used to linearise the plasmid. The T7 forward and BGH Reverse Priming sites were used for sequencing to confirm the presence of MMP-8 in the plasmid by sequencing.

2.1.3.1 Site-Directed Mutagenesis of MMP8

A mutagenesis PCR was used to generate a catalytically inactive MMP8

(5'- CTTGTTGCTGCTCATGAATTTGGC to
5'- CTTGTTGCTGCTCATGCATTTGGC).

This results in a switch from glutamic acid (E) to alanine (A) at position 198 (E198A). The reaction is carried out in duplicate and the mix consists of 5µl 10x reaction buffer , 45ng pcDNA4-WT MMP8 DNA, 125ng forward primer (5'- CTTGTTGCTGCTCATGCATTTGGCCATTCTTTGG), 125ng reverse primer (5'- CCAAAGAATGGCCAAATGCATGAGCAGCAACAAG), 1µl dNTPs and made up to 50µl with water. Lastly, 1µl Pfu Turbo polymerase (2.5U/µl) was added. All reagents were from Stratagene with the exception of primers from Sigma. The PCR cycles in a thermocycler were as follows: 30 seconds at 95°C, and 16 cycles of 95°C for 30 seconds and 68°C for 7 minutes (2 minutes per kilobase of plasmid). Following the PCR reaction the samples were incubated on ice for 2 minutes before the addition of 1µl DpnI (10U/µl)(Statagene) which causes the parental, methylated DNA to be digested. This is added to one of the reactions only, the other serves as a negative control. The reactions were centrifuged for one minute and then incubated at 37°C for hour for the parental DNA digestion to occur. Transformation of 4µl of the reaction was carried out (using standard transformation protocol section 2.2.1 and 2.2.2) and the entire transformation was plated out onto lysogeny broth (LB) agar plates containing ampicillin (100µg/ml) as a selection reagent. Following incubation at 37°C overnight colonies from the DpnI treated reaction were picked and put into 3ml LB broth (+ 100µg/ml ampicillin) and incubated at 37°C and shaken at 180rpm overnight. DNA was isolated from these cultures using the QIAprep Miniprep kit (QIAGEN), following manufactures instructions. The presence of the mutation was detected by sequencing at the BBSRC Genome Analysis Centre, Norwich Research Park.

2.1.4 Cell Culture

2.1.4.1 Cell Lines

MDA-MB-231 and MCF-7 breast cancer cell lines were cultured in Dulbecco's Modified Eagle Medium containing high glucose + GLUTAMAX (GIBCO, 21885). SKBR3 cells were cultured in McCoy's 5A (GIBCO, 16600-082), HMT-3522 S1 human breast epithelial cells [157] were cultured in Minimum Essential Media, MEM (GIBCO, 41090-028), and T47D cells were cultured in RPMI-1640 plus 4.5g/L D-glucose, 2.383g/L HEPES, L-Glutamine, 1.5g/L Sodium Bicarbonate, and 110mg/L Sodium Pyruvate (GIBCO, A10491). All culture medium was supplemented with 10% (v/v) Fetal Bovine Serum (HyClone, SV30160.03). Cells were passaged once a week when they reached 80- 90% confluency with the use of 0.25% (w/v) Trypsin and 1mM EDTA (GIBCO, 25200-072). All cells grown in 75cm² or 175cm² tissue culture flasks (NUNC) at 37°C and 5% (v/v) CO₂ in a 95% humidity incubator.

2.1.4.2 Routine Mycoplasma testing

Cells were tested weekly to ensure they were mycoplasma free. This was carried out through PCR reaction on 3-day culture supernatant. Culture supernatant was boiled for 5 minutes before spinning down for 1 minute at high speed. Each PCR reaction consisted of:

5.0µl 5x Phusion Buffer, 2.5µl 2mM dNTPs, 2.5µl 10µM Myco1 primer (GGGAGCAAACAGGATTAGATACCCT), 2.5µl 10µM Myco2 primer (TGCACCATCTGTCCTCTGTTACCCTC), 2.0µl boiled culture supernatant, 0.25µl Phusion high fidelity Taq Polymerase, 10.25µl Water (Finzymes). The samples were run for 30 seconds at 98°C, 40 cycles of: 10 seconds at 98°C, 20 seconds at 52°C, 30 seconds at 72°C finished with 2 minutes at 72°C. Products were resolved on a 2% agarose gel. If positive, a band will be seen at 270 base pairs.

2.2 DNA Propagation

2.2.1 Plasmid Transformation

To amplify the plasmid constructs pcDNA4, pcDNA4-WT MMP8 and pcDNA4-E198A MMP8, 1µl of DNA at 1µg/ml was added to 50µl of DH5α Competent *E.coli* (Invitrogen) and incubated on ice for 30 minutes. The *E.coli* was then heat-shocked for 5 minutes at 37°C and then cooled on ice for 2 minutes. To this, 250µl of room temperature LB broth (table 2.2) was added and the tubes incubated at 37°C, 180rpm for 1 hour. Either the entire transformation reaction, for the MMP8 constructs, or half, for the empty vector control was spread onto LB agar plates (see table 2.2) containing 100µg/ml ampicillin as a selection reagent for successful transformation and incubated at 37°C overnight.

2.2.2 Colony Expansion and DNA Extraction

A single colony was picked and put into 100ml LB containing 100µg/ml ampicillin and incubated at 37°C, 180rpm overnight. DNA was extracted using either QIAGEN Plasmid Midi Kit (cat #12143) or QIAGEN Plasmid Maxi Kit (cat #12963), following manufactures instructions, depending on the amount of DNA required. To check the sequence of the plasmid it was sent to the BBSRC Genome Analysis Centre, Norwich Research Park, and sequenced using primers specific to regions in the pcDNA4 plasmid either side of the cloning site

(T7-20mer, 5'-TAATACGACTCACTATAGGG-3' and BGH reverse, 5'- TAGAAGGCACAGTCGAGG-3'-)

2.3 Transfections

2.3.1 Transient Transfections

MCF-7, MDA-MB-231, SKBR3 and HMT-2552 S1 cells were plated 24-48 hours prior to transfection (400,000 cells/well of a 6-well tissue culture plate and 6×10^6 cells for a 10cm² tissue culture dish) and transfected at 80-90% confluency with pcDNA4, pcDNA4-WT MMP8 and pcDNA4- E198A MMP8 plasmid DNA. Two

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transfections mixes were initially made; the first containing 50µl serum-free DMEM (per well of a 6-well plate) or 250µl serum-free DMEM (per 10cm² dish) and 3µl (6-well plate) or 9µl (10cm² dish) of the transfection reagent LipoD293 (SigmaGen Laboratories). The second mix, the DNA mix, contains the same volume of serum-free DMEM as for the first mix with 1µg (6-well plate) or 3µg (10cm² dish) plasmid DNA. Each mix was first mixed thoroughly by vortex and then the contents of the tube containing the LipoD293 was transferred to the tube containing the DNA, which was then mixed by vortex. The mix was incubated at room temperature for 15 minutes. During incubation the media is removed and the cells were washed twice with PBS and media replaced: 900µl of serum-free DMEM per well of a 6-well plate and 4.5ml in a 10cm² dish. The transfection mix was added dropwise to the cells (100µl per well of a 6-well plate and 500µl per 10cm² dish) and cells were incubated for 6 hours at 37°C. The cells were washed twice with PBS and 1ml (6-well plate) or 5ml (10cm² dish) of serum-free media is then added. Conditioned media and cell lysate is harvested at time-points from 24 hours post transfection onwards.

2.3.2 Kill Curve

The pcDNA4 plasmid containing the MMP8 constructs contains a Zeocin resistance gene that was used when generating stable transfected MDA-MB-231 cells (section 2.3.3). Cells that had not transfected successfully would die after Zeocin treatment, leaving only the transfected cells. The amount of Zeocin used had to be optimised as too much would be toxic and too little would not kill the cells off fast enough.

To determine the optimum concentration of Zeocin MDA-MB-231 cells (100,000 cells per well of a 6 well plate) were treated with a range of Zeocin concentrations from 20-100µg/ml, with media containing Zeocin refreshed every 3 days over 2 weeks. The concentration of 40µg/ml was decided upon as most of the cells had died after 2 weeks of treatment, whereas cell death had occurred much earlier with higher concentrations.

2.3.3 Stable Transfection

MDA-MD-231 cells were engineered to constitutively over-express pcDNA4 wild type MMP8 plasmid DNA, pcDNA4 E198A mutant MMP8 plasmid DNA and a

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pcDNA4 vector control plasmid (empty vector). The calcium phosphate method of transfection was used. This method makes use of a co-precipitation reaction that occurs on the mixing of DNA together with Calcium Chloride and a HEPES-buffered solution, which, when introduced into the culture media adheres to the cells and is taken up by endocytosis or phagocytosis [158].

Cells (75,000 cells/well of a 6 well plate) were seeded 24 hours prior to transfection so that they were at 20-30% confluency. An hour before transfection, 1.5ml of fresh medium (supplemented with FBS) was put on the cells, and the reagents, 2 x HBS buffer and CaCl₂ (see table 2.2 for recipe) were brought to room temperature. In a sterile eppendorf tube a mix was made of 4µg plasmid DNA, 7.6µl 2M CaCl₂ and made up to 62µl total volume with sterilised distilled water. This is the mix produced for one well of a six well plate, but as six wells were transfected with each construct a mastermix is made. In a separate tube 62µl (per well to be transfected) of 2x HBS was added. The HBS was bubbled using an automatic pipette pump attached to a 1ml serological pipette, with a sterile 200µl pipette tip attached to the end whilst the CaCl₂-DNA mix was added dropwise into it. Once all the CaCl₂-DNA mix had been completely transferred the mix was mixed briefly by vortex and left to precipitate for 20 minutes at room temperature. Following incubation 124µl of the transfection mix was added to each well of the 6-well plate 1 drop/second whilst gently tilting the plate constantly. After 16 hours incubation at 37°C the transfection media was removed from the cells, they were washed twice in PBS to remove all traces of the transfection mix and 3ml of complete medium added. The selection reagent, Zeocin (Invitrogen, R250) was added at 40µg/ml (see section 2.3.2) to the media 48 hours after transfection. The selection media was replaced every 3 days throughout all experiments. When appropriate (after approximately 1 month) cells were grown up into larger culture vessels as pooled stably transfected cells. MMP8 expression was determined by western blotting and Taqman qRT-PCR.

Clonal isolates were generated from the pooled cell lines expressing MMP8 at the protein level by diluting out cells in a 96-well plate. Wells containing single cells were maintained under selection and taken up into larger vessels when appropriate.

2.3.4 Transfection Monitoring Experiment

MDA-MB-231 cells expressing wild-type MMP-8 and EA mutant MMP-8 six weeks post stable transfection were divided into two, with half of the cells maintained under Zeocin selection and the other half without. Every two weeks cells were plated (250,000 cells per well of a 6 well plate) and left to adhere for 24 hours, after which they were washed with PBS and 1ml serum-free media added. After 24 hours of culture in serum-free media the media was collected, centrifuged at 193xg to remove any cells, and tested for MMP-8 expression by western blot.

2.3.5 siRNA transfection

MDA-MB-231 cells over-expressing wild-type MMP8 were transfected at 60% confluency with 25nM of either IL-6 (siGENOME SMARTpool M-007993-02-0005, Dharmacon), IL-8 (siGENOME SMARTpool M-004756-00-0005, Dharmacon) or control siRNA (siGENOME Non-Targeting siRNA Pool #1 D-001206-13-05, Dharmacon) using DharmaFECT 1 Transfection Reagent (Dharmacon, T-2001-02). RNA was collected 48 hours later for analysis (for methodology for RNA collection see section 2.5.1-2.5.3). Cells transfected with MMP8 siRNA (siGENOME SMARTpool M-005969-00-0005, Dharmacon) or F2RL1 (PAR2) (siGENOME SMARTpool, M-005098-01-005, Dharmacon) were re-transfected 24 hours after the first transfection and serum free cell culture supernatant, RNA and cell lysate collected for analysis 48 hours after the second transfection.

2.3.6 Colony Formation Assay

MDA-MB-231 cells were transfected with pcDNA4 empty vector, wild-type MMP8 and EA mutant MMP8 as for stable transfection (section 2.3.2). To prevent cells becoming confluent before they started dying off from the addition of Zeocin they were split, with 1/3 of the transfected cells continuing in a fresh 6 well plate. To speed up the rate of death of untransfected cells Zeocin was added at 200 μ g/ml and was replaced every 3 days. One well of cells was not transfected and was used as a control for cell death. When the control cells had all died and when individual colonies were visible by eye (approximately 20 days post transfection) cells were

washed with PBS and stained with methylene blue for thirty minutes at room temperature to fix the cells and visualise the colonies. Excess methylene blue was washed off with water and the plate imaged (Syngene, G:Box) and the number of colonies quantified by eye and ImageJ software.

2.4 Protein Analysis

2.4.1 Protein Extraction

Serum-free conditioned media was removed from cells, spun down at 2152xg for 5 minutes to clear it of any cells, and the supernatant stored at -20°C. The cells were washed twice in ice cold PBS (table 2.2) to ensure total removal of media. Depending on the size of the vessel, cells are either scraped directly into ice cold RIPA lysis buffer (table 2.2) containing Protease Inhibitor (1 Complete Mini, EDTA-free tablet, Roche, per 10ml of lysis buffer) which inhibits a broad range of cysteine and serine proteases, but not metallo or aspartic proteases. Alternatively, cells were scraped into a larger volume of PBS which was centrifuged at 193xg for 5 minutes and the cell pellet resuspended in lysis buffer (plus protease inhibitor). This is then incubated on ice for 30 minutes to allow cell lysis. Following this cell debris is removed by centrifugation at high speed for 10 minutes and the pellet discarded. Lysate is stored at -20°C until required.

2.4.2 Protein Quantification- Bicinchoninic acid (BCA) Assay

Protein quantification was carried out using a BCA Protein Assay Kit (Thermo Scientific #23227) as this kit is compatible with the TRITON-X in the cell lysis buffer. It is a colorimetric assay consisting of two- reactions. The first reaction involves chelation of copper by protein in the sample, resulting in a blue colour. The second step is the reaction of the chelated copper with the BCA, forming a deep purple colour of which the absorbance when determined at 550nm is linear to the amount of protein present. Experimental samples are measure against a standard curve which is generated using bovine serum albumin (BSA) at known concentrations (0, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 mg/ml). Standards and experimental samples were put in a 96-well plate (10µl) and

200µl of working solution was added to each well. This solution consists of reagent B (containing 4% cupric sulphate) and reagent A (containing bicinchoninic acid) from the kit mixed in a 1:50 ratio. The plate was incubated at 37°C for 30 minutes before allowing it to cool at room temperature and reading the absorbance at 550nm on a plate reader (BMG labtech Fluostar). The absorbance value of the lysis buffer was deducted from the absorbance values of the standards and samples to correct for background signal. The concentration of protein in the sample was determined from the BSA standard using linear regression to obtain a straight line curve.

2.4.3 Conditioned Media Concentration- trichloroacetic acid (TCA) precipitation

To concentrate the proteins in conditioned media samples in order to analyse them by western blotting 10% (w/v) TCA was added to the media at a volume of the equivalent of 1/3rd of the media to be concentrated. The sample was then incubated on ice for 5 minutes during which time the highly acidic TCA denatures and precipitates the proteins in the sample. The sample was centrifuged at high speed for 5 minutes to pellet the precipitated protein. After discarding the supernatant the pellet was washed twice in 400µl of ice-cold acetone, centrifuging at 14550xg for 5 minutes. The pellet was air-dried for 5 minutes before being re-suspended in 50µl sample buffer (table 2.2). Unless otherwise stated the entire 50 µl sample was analysed by western blot.

2.4.4 Western Blotting

Denatured and reduced cell lysate (30ug of protein unless stated otherwise) and conditioned media samples (TCA precipitated) were boiled for 3 minutes with SDS sample buffer (table 2.) and unless otherwise stated run on 10% resolving gel (resolving gel: 4ml dH₂O, 3.33ml Acrylamide, 2.5ml 1.5M TRIS pH 8.8, 100µl SDS, 100µl APS, 5µl TEMED. Stacking top gel: 4ml dH₂O, 1ml acrylamide, 0.6M TRIS

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pH 6.8, 60µl SDS, 75µl APS, 7µl TEMED). The samples were run at 200 volts for 1 hour alongside a dual colour protein ladder (BioRad).

The gel was transferred onto Immobilon-FL transfer membrane (Millipore, IPFL00010) or nitrocellulose (GE Healthcare, RPN203D) using a semi-dry transfer module at 15 volts for 1 ½ hours. To ensure consistent transfer of protein had occurred membranes were incubated for 5 minutes in Ponceau S red stain (table 2.1). The membranes were then blocked in 5% (w/v) Bovine Serum Albumin (BSA) in PBS or Tris Buffered Saline (TBS) containing 0.1% (v/v) TWEEN-20 for at least one hour at room temperature. Primary antibodies (see table 2.3) were diluted in 5% BSA or skimmed milk prepared in either PBS or TBS containing 0.1% (v/v) TWEEN depending on the specific requirements for the individual antibody, and were left on the membrane overnight at 4⁰ C under constant agitation. The membrane was washed 3 times for 10 minutes each in PBS or TBS containing 0.1% (v/v) TWEEN under constant agitation before addition of the secondary antibody (see table 2.3). Unless otherwise stated the appropriate secondary antibody (see table 2.2 for secondary antibodies used) was used at 1:10,000 in 5% BSA made in PBS or TBS containing 0.1% (v/v) TWEEN for the Infra-Red conjugated antibodies and 1:1000 for the HRP-conjugated ones and incubated for 2 hours at room temperature. This was followed by another 3 x 10 minute washes in PBS or TBS containing 0.1% TWEEN, and then a final 10 minute wash in PBS, under constant agitation. If using Horse Radish Peroxidase (HRP) conjugated secondary antibodies blots were treated for 5 minutes with ECL (Thermo Scientific, 32106) and then imaged using the FujiFilm Intelligent Darkbox LAS-3000. If using Infra-Red antibodies blots were imaged using the Odyssey Infra-Red scanner (Licor).

2.4.5 Enzyme Linked Immunosorbent Assay (ELISA)

Human Interleukin-6, Interleukin-8 and Tumour Necrosis Factor (TNF)-α ReadySET-Go! ELISAs were purchased from eBioscience (catalogue numbers 88-7066-22, 88-7087-22 and 88-7346 respectively) and used to analyse serum free cell supernatant samples according to manufacturer's instructions. Media was diluted

1:10 where appropriate to ensure samples fell within the predetermined range of the standard curve.

2.4.6 Human Phospho-Kinase Array Kit

The Proteome Profiler™ Antibody array (R&D Systems, ARY003) utilises antibodies to an array of phospho-kinase proteins for detection pre-stamped on a nitrocellulose membrane. According to manufacturer's instructions lysate from MDA-MB-231 wild-type and EA mutant MMP8 over-expressing and vector control clonal isolates that had been serum starved for 24 hours was applied to the membranes. The membrane was then incubated with a secondary, HRP-conjugated antibody that bound to a different part of the protein. The resulting membranes were treated with ECL (Thermo Scientific, 32106) for 5 minutes and then chemiluminescence imaged using a FujiFilm Intelligent Darkroom imager. Pixel intensity was quantified using the 'gel analyser' tool in Image J (<http://rsweb.nih.gov/ij/>).

2.4.7 Cytometric Bead Array

Cells were transiently transfected with pcDNA4 vector control, Wild Type MMP8 and E198A Mutant MMP8 plasmid constructs (2.3.1) and transfection success confirmed by western blot of serum free cell culture media collected 48 hours post transfection (2.4.3 and 2.4.4). The commercially available Human Inflammatory Cytokine Kit was used (BD Bioscience, 551811) according to manufacturer's guidelines and using a BDFACSAria™II flow cytometer to determine the levels of IL-8, IL-1 β , IL-10, TNF, IL-6, IL-12p70 in the conditioned media from the transiently transfected cells.

2.4.8 Immunoprecipitation of MMP8 and Mass Spectrometry to determine MMP8 binding partners

2.4.8.1 Immunoprecipitation of MMP8 from conditioned cell culture media

MDA-MB-231 stably over-expressing pcDNA4 vector control, wild-type MMP8 and E198A mutant MMP8 DNA were grown to 80% confluency in T175 cell culture flasks before being washed with PBS and 15mls serum free media added overnight. Media was centrifuged to pellet any cells and 14.5mls of media was incubated overnight at 4°C under constant rotation with 150µl of anti-V5 Agarose beads (Sigma, A7345) that had been prewashed with RIPA lysis buffer (table 2.2). The bound beads were pelleted by centrifugation at 193xg for 5 minutes and washed 3 times using RIPA buffer. Protein was stripped from the beads by the addition of 60µl SDS sample buffer (table 2.2) containing 4% (w/v) DTT and boiled for 5 minutes. Finally the beads were pelleted by centrifugation and 50µl of the immunoprecipitation reaction, IP, (the equivalent to 12mls of media precipitated) was loaded onto a 4-20% precast Tris-Glycine gradient gel (NuSep, Peqlab, NB10-420) and run for 45 minutes at 200V. The gel was then stained using SimplyBlue™ SafeStain (Invitrogen, LC6060). Visible bands were then cut from the gel and sent for analysis at the Mass Spectrometry Services at the John Innes Centre, Norwich Research Park (see section 2.4.8.2). The remaining 10µl of the immunoprecipitation reaction was also run on a 4-20% precast Tris-Glycine gradient gel (NuSep, Peqlab, NB10-420) for 45 mins at 200V but the standard western blotting protocol (section 2.4.4) was followed and the blot probed with an anti-MMP8 antibody to confirm that the IP was successful.

2.4.8.2 Mass Spectrometry

With many thanks to Gerhard Saalbach, John Innes Centre, Norwich Research Park.

The excised immunoprecipitation gel bands (procedure described in section 2.4.8.1, above) were sent to the Mass Spectrometry Services at the John Innes Centre, Norwich Research Park where they were washed, reduced and

alkylated, and treated with trypsin according to standard procedures. Peptides were extracted with 5% formic acid/50% acetonitrile, dried down, and re-dissolved in 0.1% TFA. For LC-MS/MS analysis, a sample aliquot was applied via a nanoAcquity™ (Waters Ltd, Manchester, UK) UPLC™-system running at a flow rate of 250 nL min⁻¹ to an LTQ-Orbitrap™ mass spectrometer (Thermo Fisher, Waltham, MA). Peptides were trapped using a pre-column (Symmetry® C18, 5µm, 180 µm x 20 mm, Waters Ltd) which was then switched in-line to an analytical column (BEH C18, 1.7 µm, 75 µm x 250 mm, Waters Ltd) for separation. Peptides were eluted with a gradient of 3-37% acetonitrile in water/0.1% formic acid at a rate of 0.67% min⁻¹. The column was connected to a 10 µm SilicaTip™ nanospray emitter (New Objective, Woburn, MA, USA) attached to a nanospray interface (Proxeon, Odense, Denmark) for infusion into the mass spectrometer. The mass spectrometer was operated in positive ion mode at a capillary temperature of 200 °C. The source voltage and focusing voltages were tuned for the transmission of MRFA peptide (m/z 524) (Sigma-Aldrich, St. Louis, MO). Data dependent analysis was carried out in orbitrap-IT parallel mode using CID fragmentation on the 5 most abundant ions in each cycle. The orbitrap was run with a resolution of 30,000 over the MS range from m/z 350 to m/z 1800 and an MS target of 10⁶ and 1 s maximum scan time. Collision energy was 35, and an isolation width of 2 was used. Only mono-isotopic 2+ and 3+ charged precursors were selected for MS2. The MS2 was triggered by a minimal signal of 1000 with an AGC target of 3x10⁴ ions and 150 ms scan time using the chromatography function for peak apex detection. Dynamic exclusion was set to 1 count and 30 s exclusion with an exclusion mass window of ±20 ppm. MS scans were saved in profile mode while MSMS scans were saved in centroid mode.

Raw files were processed with MaxQuant version 1.3.0.3 (<http://maxquant.org>) [159] to generate re-calibrated peaklist-files which were used for a database search using an in-house Mascot® 2.3 Server (Matrix Science Limited, London, UK). Mascot-mgf files were generated from MaxQuant apl-files using a suitable perl script. Mascot searches were performed on the *Sprot_sptrembl20120711.fasta* database with taxonomy set to human and a

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common contaminants database using trypsin/P with 2 missed cleavages, 6 ppm precursor tolerance, 0.8 Da fragment tolerance, carbamidomethylation (C) as fixed, and oxidation (M) and acetylation (protein N-terminus) as variable modifications. Mascot search results were imported and evaluated in Scaffold 3 (proteomsoftware.com, Portland, OR, USA) resulting in a false discovery rate of 0% for both peptides and proteins.

2.5 Gene Expression Analysis

Depending on the amount of RNA required and whether conditioned media was also required from the same cells two different methods of RNA isolation were used. The predominant method used is described in sections 2.5.1 and 2.5.2 and was used when cells were cultured on 6 well plates or 10cm² dishes when conditioned media was required for other assays. If there was no requirement to harvest any conditioned media from the experiment it was carried out on a much smaller scale as described in section 2.5.3.

2.5.1 RNA Collection and Extraction

Cells were washed twice in PBS before being scraped into 500µl RNA-Bee RNA isolation reagent (ambio, CS-501B), transferred to a tube and stored at -80⁰ C until extraction. A chloroform extraction was performed in combination with the SV Total RNA Isolation System (Promega, Z3100). On ice, 100µl chloroform was added to the sample, mixed by inversion and returned to ice for 5 minutes. This was then centrifuged at high speed for 15 minutes at 4⁰ C. The upper clear layer was transferred to a fresh tube and the lower layer discarded. To the upper layer 200µl of 95% ethanol was added. The mixture was added to the spin column provided with the SV Total RNA Isolation System. RNA quantification were checked using a nano-drop (Thermo Scientific).

2.5.2 Reverse Transcription

RNA (1µg) was mixed with 2µl Random Primers(200 ng/ µl) and 1µl of dNTP mix (10mM), and the volume made up to 11 µl with ddH₂O. The mixture was heated at 70⁰ C for 10 minutes before being returned to ice. A was then made up for all samples consisting of 2µl 10 x MMLV buffer , 1µl Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase and 0.5µl RNase OUT Ribonuclease Inhibitor (all reagents from Invitrogen) per sample, with 9µl added per sample to give a 20µl reaction. Samples were incubated at 37°C for 1 hour, followed by deactivation of the MMLV by heating to 85°C for 10 minutes. This results in a total of 20µl cDNA at 50ng/µl.

2.5.3 Cells-to-cDNA

A maximum of 100,000 cells were lysed in 30µl cells-to-cDNA lysis buffer (Ambion, AM8723) and incubated at 75°C for 15 minutes. For each sample 1µl of DNase I (2U/µl, Ambion, AM2222) and 3µl 10x DNase buffer (AM8170G) was added and incubated at 37°C for 15 minutes followed by 75°C for 5 minutes. Of this mixture 8µl is transferred to a fresh tube along with 1µl dNTPs (10mM) and 2µl Random Primers (200ng/µl, both Invitrogen). Samples were incubated at 70°C for 5 minutes and then the remaining reverse transcription reagents were added: 4µl 5x Reverse Transcriptase buffer, 2µl 0.1M DTT, 1.5µl nuclease free water, 0.5µl MMLV reverse transcriptase and 1µl RNase OUT (all Invitrogen). This final mix was incubated at 37°C for 50 minutes followed by 75°C for 15 minutes to deactivate the MMLV Reverse Transcriptase. To this, 30µl of nuclease free water was added. When analysing the samples by Taqman qRT-PCR (section 2.5.4) 5µl of the resulting cDNA was used to look for a gene of interest and 5µl of a 1:10 dilution was used when looking at 18S expression levels as a normaliser gene.

2.5.4. Taqman Quantitative Real-Time PCR

Taqman Quantitative Real-Time PCR analysis was carried out to determine the level of expression of a gene of interest in RNA extracted from cells in culture. A total of 5ng cDNA (10µl of 0.5ng/µl dilution), or 5µl if using the cell-to-cDNA method (section 2.5.3) was loaded per well in combination with 8.33µl of mastermix (Primer Design), the appropriate primer-probe (see table 2.4) and nuclease free water to make the reaction up to 25µl. 18S ribosomal RNA was used as a normalising marker, and had previously been shown to be expressed at a consistent level in the type of samples analysed, into which 1ng is used due to it being highly abundant. When using a new primer-probe set samples were compared to a standard curve which assesses how well the probe is reacting to a variety of cDNA concentrations. Serial dilutions of a sample expected to show good levels of expression of the gene of interest were generated ranging from 20-0.625ng, and 1-0.03125ng for the 18S reference gene. The reaction was run on an ABI7500 machine (Applied Biosystems) using the following cycles: 2 mins at 50°C, 10 mins at 95°C followed

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by 40 cycles of 15 secs at 90°C and 1 min at 60°C. Data was analysed using the 2^{-ΔCt} method comparing Ct values of the gene of interest to 18 values.

Target		Sequence
Timp 1	Forward Primer: Reverse Primer: Probe:	5'- GACGGCCTTCTGCAATTCC -3' 5'- GTATAAGGTGGTCTGGTTGACTTCTG -3' 5'-FAM- ACCTCGTCATCAGGGCCAAGTTCGT -TAMRA-3'
Timp 2	Forward Primer: Reverse Primer: Probe:	5'- GAGCCTGAACCACAGGTACCA -3' 5'- AGGAGATGTAGCACGGGATCA -3' 5'-FAM- CTGCGAGTGCAAGATCACGCGC -TAMRA-3'
Timp 3	Forward Primer: Reverse Primer: Probe:	5'- CCAGGACGCCTTCTGCAA -3' 5'- CCCCTCCTTTACCAGCTTCTTC -3' 5'-FAM- CGACATCGTGATCCGGGCCA -TAMRA-3'
Timp 4	Forward Primer: Reverse Primer: Probe:	5'- CACCCTCAGCAGCACATCTG -3' 5'- GGCCGGAACCTTCTCACT -3' 5'-FAM- CACTCGGCACTTGTGATTCGGGC -TAMRA-3'
MMP-1	Forward Primer: Reverse Primer: Probe:	5'- AAGATGAAAGGTGGACCAACAATT -3' 5'- CCAAGAGAATGGCCGAGTTC -3' 5'- FAM-CAGAGAGTACAACCTTACATCGTGTTGCGGCTC-TAMRA -3'
MMP-2	Forward Primer: Reverse Primer: Probe:	5'- AACTACGATGACGACCGCAAGT -3' 5'- AGGTGTAAATGGGTGCCATCA -3' 5'- FAM-CTTCTGCCCTGACCAAGGGTACAGCC-TAMRA -3'
MMP-3	Forward Primer: Reverse Primer: Probe:	5'- TTCCGCCTGTCTCAAGATGATAT -3' 5'- AAAGGACAAAGCAGGATCACAGTT -3' 5'- FAM-TCAGTCCCTCTATGGACCTCCCCTGAC-TAMRA -3'
MMP-7	Forward Primer: Reverse Primer: Probe:	5'-CTTTGCGCGAGGAGCTCA -3' 5'-CAGGCGCAAAGGCATGA -3' 5'- FAM-CCATTTGATGGGCCAGGAAACACG-TAMRA -3'
MMP-8		Primer-probe ready-made assay from Primer Design
MMP-9	Forward Primer: Reverse Primer: Probe:	5'- AGGCGCTCATGTACCCTATGTAC -3' 5'- GCCGTGGCTCAGGTTCA -3' 5'- FAM-CATCCGGCACCTCTATGGTCCTCG -TAMRA -3'
MMP-10	Forward Primer: Reverse Primer: Probe:	5'- GGACCTGGGCTTTATGGAGATAT -3' 5'- CCCAGGGAGTGGCCAAGT -3' 5'- FAM- CATCAGGCACCAATTTATTCCTCGTTGCT-TAMRA -3'
MMP-11	Forward Primer: Reverse Primer: Probe:	5'-GGGTGCCCTCTGAGTCGA -3' 5'-TCACAGGGTCAAACCTCCAGTAGA -3' 5'- FAM-ATGCTGATGGCTATGCCTACTTCTGCG -TAMRA -3'
MMP-12	Forward Primer: Reverse Primer: Probe:	5'- CGCCTCTCTGCTGATGACATAC -3' 5'- GGTAGTGACAGCATCAAAACTCAAA -3' 5'- FAM-TCCCTGTATGGAGACCCAAAAGAGAACCA-TAMRA -3'
MMP-13	Forward Primer: Reverse Primer: Probe:	5'- AAATTATGGAGGAGATGCCATT -3' 5'- TCCTTGGAGTGGTCAAGACCTAA -3' 5'- FAM-CTACAACCTGTTTCTTGTGCTGCGCATGA-TAMRA -3'
MMP-14	Forward Primer: Reverse Primer: Probe:	5'-AAGGCAAATGTTCTGAAGGAA -3' 5'-GGCCTCGTATGTGGCATACTC -3' 5'- FAM-CAACATAATGAAATCACTTTCTGCATCCAGAATTACA -TAMRA -3'
MMP-15	Forward Primer: Reverse Primer: Probe:	5'- GCGCTTCAACGAGGAGACA -3' 5'- TCCAGTATTTGGTGCCCTTGT -3' 5'- FAM- CCTTCCTGAGCAATGACGCAGCCTAC -TAMRA -3'

Table 2.4: Taqman primer-probes. All primers and probes were purchased from Sigma with the exception of those noted.

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Target		Sequence
MMP-16	Forward Primer: Reverse Primer: Probe:	5'- ATGATTTACAGGGCATCCAGAAA -3' 5'- TGGAGGCCGAGGAGGTTT -3' 5'- FAM-CAAGATT CCTCCACCTACAAGACCTCTACCGAC-TAMRA -3'
MMP-17	Forward Primer: Reverse Primer: Probe:	5'- FAM- GCGGGTATCCTTCTCTACGT -3' 5'- CAGCGACCACAAGATCGTCTT -3' 5'- FAM- ATTGTCCTTGAACACCCAGTACCTGTCTCCTTTAA - TAMRA -3'
MMP-19	Forward Primer: Reverse Primer: Probe:	5'- GGCTACCGGCCCACTT -3' 5'- TGTCTCTTCTTCTTCTCATCCCTTA -3' 5'- FAM- ATCCAGGCTCTCTATGGCAAGAAGAGTCCA- TAMRA -3'
MMP-20	Forward Primer: Reverse Primer: Probe:	5'- CCTTTGACGCTGTGACAATGC -3' 5'- TCCTGTCCGCAAGTGAACCT -3' 5'- FAM-TCCTGTCTTCAAGGACCGGATTTTCTG - TAMRA -3'
MMP-21	Forward Primer: Reverse Primer: Probe:	5'-CCAGTGACACGGGCATCA -3' 5'- ATTATGGATCCCGTCTGTAGGT -3' 5'- FAM-CCTTCTCAAGGTGGCCGTCATGA-TAMRA -3'
MMP-23	Forward Primer: Reverse Primer: Probe:	5'- TCCACAAGAAAGGGAAAGTGTACTG -3' 5'- ACGGCGTTGGCGATGAT -3' 5'- FAM-TTCTCTACCCCGGCTACCTGGCC -3'
MMP-24	Forward Primer: Reverse Primer: Probe:	5'- CCAGTACATGGAGACGCACAA -3' 5'- TGCGGACGGGGAGTGT -3' 5'- FAM- CAGGGCATCCAGAAGATCTATGGACCC - TAMRA -3'
MMP-25	Forward Primer: Reverse Primer: Probe:	5'- GACGATGAGGAGACCTGGACTTT -3' 5'- CCTGGTAGAAGGGCCTCATAATG -3' 5'- FAM-CCGACCTGTTTGCCGTGGCTGTC -3'
MMP-26	Forward Primer: Reverse Primer: Probe:	5'- AATTCTGGAAATCCTGGAGTTGTC -3' 5'- CAAAGAATGCCCAATCTCATGA -3' 5'- FAM- CTGGTCAGCTTCAGACACTGGATATAATCTGTTCC- TAMRA -3'
MMP-27	Forward Primer: Reverse Primer: Probe:	5'- GTTTAGAAAGTGTGGAGCAAAGTCACT -3' 5'- ATAGCGAGGACACCGACCAT -3' 5'- FAM- CATCATGATTGCCTTAGGACTCGAGTCCA - TAMRA -3'
MMP-28	Forward Primer: Reverse Primer: Probe:	5'- TTTGAGACCTGGGACTCCTACAG -3' 5'- CCCAGAAATGGCTCCCTTA -3' 5'- FAM- ACTTCTCTTCGATGCCATCACTGTAGACAG - TAMRA -3'
Interleukin-6		Primer-probe ready-made assay from Applied Biosystems catalogue # Hs00985639_m1
Interleukin-8		Primer-probe ready-made assay from Applied Biosystems catalogue # Hs00174103_m1
PAR2 (F2RL1)	Forward Primer: Reverse Primer: Probe:	5'- CGTCCAGTGGAGCTCTGAGT -3' 5'- GCAGGAGAGAGAGGCTGCTA -3' Universal Probe#3 (Roche)

Table 2.4: Taqman primer-probes. All primers and probes were purchased from Sigma with the exception of those noted.

2.6 Functional Cell Assays

2.6.1 Proliferation

Cells (20,000 cells per well of a 24 well plate) were seeded in triplicate and maintained in media supplemented with 10% FBS. Accurate cell counts using a haemocytometer were taken after 24, 48, 72 and 96 hours.

2.6.2 2-D Time Lapse Microscopy

Cells (cells per well of a 24 well plate) were seeded in quadruplicate 24 hours prior to the experiment in media supplemented with 10% FBS. Cells were serum starved for 6 hours prior to imaging and throughout the course of the experiment. Using a Zeiss Axiovert with an inbuilt camera the same group of cells were imaged every 10 minutes over a 15 hour time period. Ten individual cells were tracked per well of the plate using Image J software utilising the ‘Manual Tracking’ tool (<http://rsweb.nih.gov/ij/plugins/track/track.html>) and the distance accumulated and velocity of the cells was determined using the ‘Chemotaxis’ application (http://ibidi.de/applications/ap_chemo.html). Three biological replicates were carried out.

2.6.3 Scratch Wound Assay

Cells (250,000 cells per well of a 12 well plate) were seeded in triplicate 24 hours prior to experiment in media supplemented with 10% FBS. By 24 hours post seeding the cells were 100% confluent. The media was removed from the cells and they were washed with serum-free media. The scratch was made through the centre of the well with a p10 pipette tip. The cells were then washed a further two times with serum-free DMEM to remove any detached cells before replacing it with 1ml fresh serum-free DMEM. Pictures were taken (x10 objective) immediately after the scratch wound was made and then 24 hours later. Cells were imaged at 9 hours and 24 hours post making the scratch wound. Wound closure was analysed using Axiovision software that enabled the scratch to be measured. Scratch wound closure was expressed as a percentage of the original scratch wound size. Three biological replicates were carried out.

2.6.4 Inverted Invasion Assay

Collagen type I gels were prepared by the mixing of Type I Collagen (Rat Tail Type I, First Link UK, 60-30-810) and 10X DMEM in a 1:10 ratio (10x DMEM: Type I Collagen) on ice. To polymerise the gels the pH was increased through the gradual addition of 10M NaOH until the gel maintains an orange/pink colour. This was put into the well of an 8.0µm Transwell (100µl per transwell), which was suspended in the well of a 24 well plate, and incubated at 37°C for 30 minutes until set. Once set the transwells were inverted and MDA-MB-231 clonal isolates (pcDNA4 empty vector, wild-type MMP8 and E198A Mutant MMP8) were seeded at a density of 50,000 cells in 100µl of complete media onto the underside of the transwell by reverse pipetting. Cells were allowed to adhere for 4 hours at 37°C before being reverted back so that the gel is on the upper facing side of the transwell membrane and the cells are on the lower side. The transwells were washed twice in pre-incubated serum-free media to remove any unattached cells and serum and left in 1ml pre-warmed serum free media. As a chemoattractant for the cells through the gel 100µl of complete media with 50ng/µl EGF was added on top of the gel in the transwell. The assay was then incubated for 48 hours at 37°C for invasion to occur before imaging. To allow for cell imaging live cells were stained with 4µM Calcein AM (Invitrogen) in 1ml of serum free media to cover the gel for an hour before imaging. Invasion was measured using a laser-scanning confocal microscope (Leica) that took images at 15µm sections throughout the gel starting at the bottom on the gel where the cells were initially seeded. Analysis was carried out using the 'Area Calculator' PlugIn (<http://rsb.info.nih.gov/ij/plugins/area.html>) in ImageJ to calculate the total area of cells in each 15µm section. Results are expressed as the percentage of cells (by area) that migrate past 30µm into the type I collagen plug over the total area of cells in the gel.

2.6.5 Adhesion Assay

Type I Collagen, Fibronectin and Laminin (100µl per well of a 96 well plate) were coated at 5µg /µl in coating buffer (table 2.2) and left to coat overnight at 4°C. Wells coated with coating buffer only were used as a control. Prior to seeding cells, the coating buffer was removed and the wells were washed twice with PBS. They were then blocked with 100µl of 1% BSA for 30 minutes at 37°C. Cells were trypsinised,

quenched with complete media, centrifuged at 1000rpm for 5 mins, resuspended in serum free media and counted. This centrifugation step is to remove as much serum as possible from the cells. The number of cells required were centrifuged and resuspended in serum free media with a final concentration of 6000 MDA-MB-231 cells per 100µl of serum free media. The wells were again washed twice with PBS. Cells were added (100µl serum free media containing 6000 cells per well) and allowed to adhere for 1 hour at 37°C. The plate was then inverted to remove media and the wells were washed gently twice with PBS. Adhered cells were fixed and stained with methylene blue (table 2.2) for 30 minutes at room temperature. Excess stain was removed by holding the plate under running water until it ran clear. To quantify adhesion the stain was eluted by the addition of 100µl destain (50% EtOH, 50% 0.1M HCl) for 10 minutes at room temperature and absorbance measured at 630nm on a plate reader (BMG labtech Fluostar). For analysis the values obtained for the coating buffer only control were subtracted from the values obtained for the matrix coatings to eliminate any background readings arising from the coating buffer.

2.6.6 MMP8 collagenase activity assay- Collagen Contraction Assay and Hydroxyproline Assay

2.6.6.1 Collagen Digestion Assay

Type I Collagen gels were made through the mixing of collagen type I (Rat Tail Type I, First Link UK, 60-30-810) and 10X DMEM in a 1:10 ratio (10x DMEM: Type I Collagen) on ice. To polymerise the gels the pH was increased through the gradual addition of 10M NaOH until the gel maintains an orange/pink colour. MDA-MB-231 clonal isolates were trypsinised, neutralised with complete media, centrifuged, resuspended in serum free media and counted. Cells (300,000 in 250µl serum free media) were mixed with 250µl collagen gel, and 500µl collagen gel-cell mix added to a well of a 24 well plate, resulting in a collagen gel concentration of 0.925mg/ml. The gel/cells were incubated at 37°C for 30 minutes for the gels to set, after which 500µl serum free media is added so as the gels do not dry out. Twenty four hours later the gels were released from the edges and bottom of the cell culture well using a small spatula. Collagenases within the gel digest the gel. A further 24

hours later the media from the gels was collected and analysed for collagen breakdown using a hydroxyproline assay (next section, 2.6.6.2).

2.6.6.2 Hydroxyproline Assay

Determining hydroxyproline release is an indirect method to measure collagen breakdown. Concentrated HCl was used to hydrolyse proteins and release hydroxyproline. The hydroxyproline is then oxidised to a pyrrole with Chloroamine T (7% Chloroamine T, BDH cat# 276704E) which can be detected at 560nm with the addition of 4-dimethylaminobenzaldehyde (DAB).

In triplicate, 200µl of media sample from the collagen digestion assay (above) was taken and 200µl concentrated HCl was added. This was boiled overnight at 105°C, resulting in a dark brown liquid if hydroxyproline is present. The HCl was then evaporated out of the sample by placing the samples in a speed vac heated to 60°C for 3 hours. Once dried, 200µl water was added to the samples and pipetted to re-dissolve the dried sample. Samples were loaded (40µl) onto a flat bottomed 96-well plate alongside a dilution standard from 0-30mg/ml of stock hydroxyproline. Samples and standards were oxidised for exactly 4 minutes through the addition of 25µl of Solution A (2ml Chloroamine T and 8ml acetate citrate buffer (table 2.2)) before the addition of 150µl Solution B (30ml propan-2-ol with 10ml DAB (table 2.2)). The plate was then sealed and incubated at 65°C for 35 minutes before cooling and reading the absorbance. Quantification was carried out through the extrapolation of the concentration of the experimental samples from the known concentrations of the standard curve.

2.6.7 Cell Cycle Analysis using Flow Cytometry

This method utilises Propidium Iodide (PI) staining to determine the stage of the cell cycle cells are in. PI is a nucleic acid stain, with staining intensity being proportional to the amount of DNA present within a cell.

Cells were grown to 70% confluency before all cells were collected (including any cells floating in the cell culture media), centrifuged at 193xg and washed with PBS and resuspended in 70% (v/v) EtOH to fix the cells. Cells were fixed overnight at -

Chapter 2- Materials and Methods

20°C before being pelleted, washed twice with PBS and resuspended with a PI, RNase (to remove and RNA which would bind the PI), TRITON X-100 solution. Per sample of approximately 200,000 cells the solution consisted of 200ul PBS, 8µl PI (1mg/ml stock- final concentration of 40µg/ml, Sigma, P 4170), 40 µl of RNase A stock (1mg/ml in PBS stock - final concentration 0.1mg/ml) (Sigma R6513)) and 0.1% TRITON X-100. Samples were incubated at 37°C for 30 minutes in the dark. They were then run at slow speed on the flow cytometer (Accuri C6), recording 10,000 events per sample. Doublets were gated for and a final analysis was carried out using FlowJo software that calculated the percentage of cells in each phase of the cell cycle.

2.6.8 MMP8-IL-6/IL-8 reciprocal activation

Recombinant human IL-6 (R&D Systems# 206-IL/CF) and IL-8 (R&D Systems #208-IL/CF) was added to serum-starved parental MDA-MB-231 cells at 0.3, 0.6 and 100ng/ul IL-6 and 0.5, 1.5 and 100ng/ul IL-8 for 24 hours. Cells were harvested for RNA extraction and mRNA levels of MMP8, IL-6 and IL-8 were determined by Taqman qRT-PCR (section 2.4). APMA activated recombinant human MMP8 (R&D Systems #908-MP) was added to cells up to 10ng/ml for 24 hours.

2.7 Molecular Pathway Inhibition

Small molecule inhibition was utilised to dissect the mechanism of action of MMP8 on the induction of IL-6 and IL-8. Cells were plated at $2.6 \times 10^4/\text{cm}^2$ in 6 well plates and allowed to settle for 24 hours. Inhibitors (Table 2.5) were added into serum-free DMEM. Cell supernatants were removed after 24 and 48 hours (inhibitor replenished at 24 hours), samples centrifuged to remove any cell debris and stored at -20°C for analysis by ELISA (section 2.4.5).

Target	Inhibitor	Concentration Used	Source
p38	SB203580	1µM	Caymen Chemicals Cambridge Bioscience (CAY13067)
	BIRB 0796	1µM	Caymen Chemicals Cambridge Bioscience (CAY10460)
MKK1	PD0325901	0.1µM	Caymen Chemicals Cambridge Bioscience (CAY13034)
	U0126	10µM	Caymen Chemicals Cambridge Bioscience (CAY70970)
PI3K	Wortmannin	1µM	Sigma (W1628-1MG)
	PI-103	0.5µM	Caymen Chemicals Cambridge Bioscience (10009209)
PKC	Bisindolylmaleimide I	360nm	Calbiochem (203290)
JNK	SP600125	20µM	Sigma (S5567)
NF-κB	BAY 11-7082	10µM	Calbiochem (196871)
TGFβR1	SB431542	10 µM	Caymen Chemicals Cambridge Bioscience (CAY13031)
MMPs	CT1746	10 µM	Gift from Gillian Murphy University of Cambridge
	GM6001	10 µM	UNK
MMP8	DL111	1µM	Gift from Vincent Dive CEA Saclay, Gif- sur-Yvette

Table 2.5. Pathway inhibitors

2.8 *In vivo*

MDA-MB-231 cells engineered to stably over-express wild-type and E198A mutant MMP8 and empty vector control transfected cells were injected subcutaneously into the left flank (1 million cells in 100µl PBS per mouse) of CD1 nu/nu mice. Six mice each were injected with wild-type MMP-8 and E198A mutant MMP-8 expressing cells and 8 mice were injected with cells transfected with the control empty vector. Primary tumour growth was measured by bi-weekly palpation using digital calipers and mice were sacrificed at day 54.

Chapter 3- Development and characterisation of mammary *in vitro* models for over-expressing MMP-8

MMP8 has been shown to be protective against mammary carcinoma in human studies, specifically against metastatic spread of the disease [65, 70], but through unknown mechanisms. This chapter concerns the development and characterisation of an *in vitro* model to investigate the functions of MMP-8 in mammary carcinoma cells. This chapter outlines the development of an expression model and characterisation of the functional effects of MMP-8 on carcinoma cell migration, invasion, adhesion and growth.

3.1 Generation of an MMP8 expression model

3.1.1 Determination of an appropriate *in vitro* system

MMP8 is produced by the myoepithelial cells in the normal breast, which are located between the epithelial cells and the basement membrane of the ductal lumen. The initial aim of this project was to elucidate the role of MMP8 in the normal breast using these cells and then transfer knowledge gained to cancerous breast cells. The 1089 immortalised myoepithelial cell line was going to be used (gift from Dr. Louise Jones, Queen Mary University of London), of which there are two forms, the original 1089 cell and one which was engineered to over-expresses $\beta 6$ integrin (denoted 1089 $\beta 6$), making the cell line more DCIS-like. Myoepithelial cells express a number of protein markers that enable them to be distinguished from the luminal epithelial cells. For example, myoepithelial cells express $\beta 4$ integrin, cytokeratin 14 (CK14), vimentin, tenascin, and α -smooth muscle actin, whereas luminal epithelial cells do not. Conversely, luminal epithelial cells express cytokeratin 18 (CK18), whereas myoepithelial cells do not. Both cell types however express E-Cadherin in the

normal, non-cancerous, breast [56]. The 1089 and 1089 β 6 cell lines proved to be very difficult to culture, in that their phenotypes drifted very rapidly. Cells that were originally expressing E-Cadherin and not CK18 (figure 3.1A), soon lost E-Cadherin expression and gained CK18 expression (figure 3.1B). Morphological differences also emerged in parallel to this alteration in expression markers. Cells in early culture exhibited a cobble-stone morphology and were round with large areas of cytoplasm. Once expression markers had altered cells became very elongated and visually more fibroblastic (figure 3.1C). This transition occurred within a week or two of culture making these cells highly impractical for long-term experiments as one could not be certain that phenotypic drifts were not occurring during experiments. In addition, the aim of this project was to over-express MMP8 permanently in a mammary cell line and then look at any changes in cellular characteristics. Firstly the generation of stable transfectants is time-consuming and cells would likely have drifted in phenotype during this time, and secondly, it would be impossible to examine the effect of MMP-8 on cellular markers such as markers of the epithelial-to-mesenchymal transition (EMT) which include E-Cadherin and CK18, as the phenotypic drift of cells would mask any MMP-8 dependent effects. As a result the non-metastatic, more epithelial-like, MCF-7 and the highly metastatic, more mesenchymal-like, MDA-MB-231 breast cancer cell lines were used, as these have been well characterised in the literature, have been shown to be representative of cancer cells from breast cancer patients [160, 161], and are less susceptible to phenotypic drifts. As can be seen from figure 3.2 the cells express key protein markers that distinguish their different subtypes. For example, vimentin, a mesenchymal marker is expressed by the MDA-MB-231 cells only, and E-Cadherin, an epithelial marker [162] is expressed in the MCF-7 cells only.

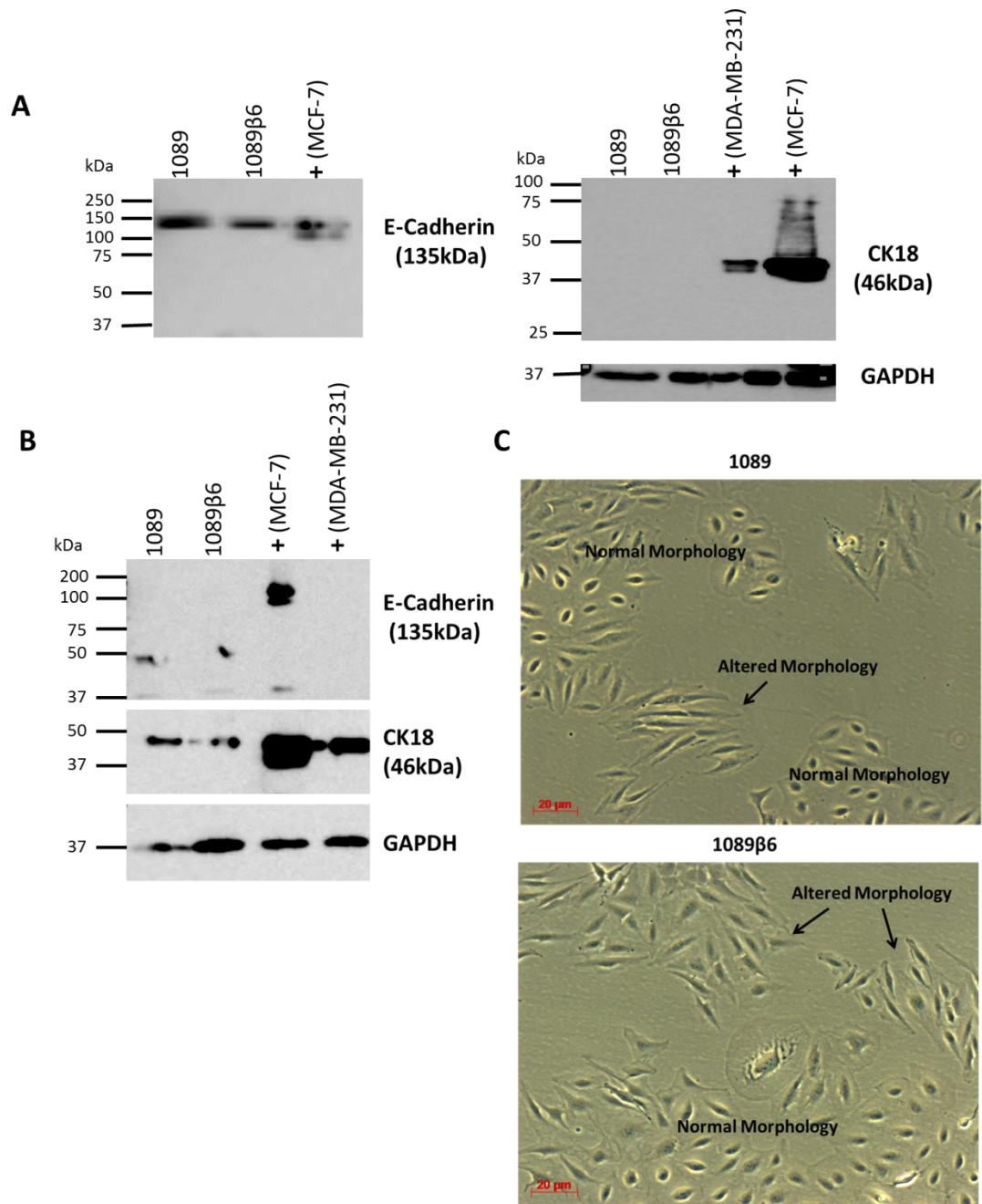


Figure 3.1: Characterisation of 1089 and 1089 β 6 myoepithelial cells. Western blot analysis of expression markers E-Cadherin (epithelial and myoepithelial) and CK18 (epithelial only) in (A) cells pre-phenotypic drift and (B) cells post-phenotypic drift. MCF-7 and MDA-MB-231 cell lines used as positive controls (+) for the markers and GAPDH as a loading control. C) Light microscopy of 1089 and 1089 β 6 cells in culture highlighting morphological changes.

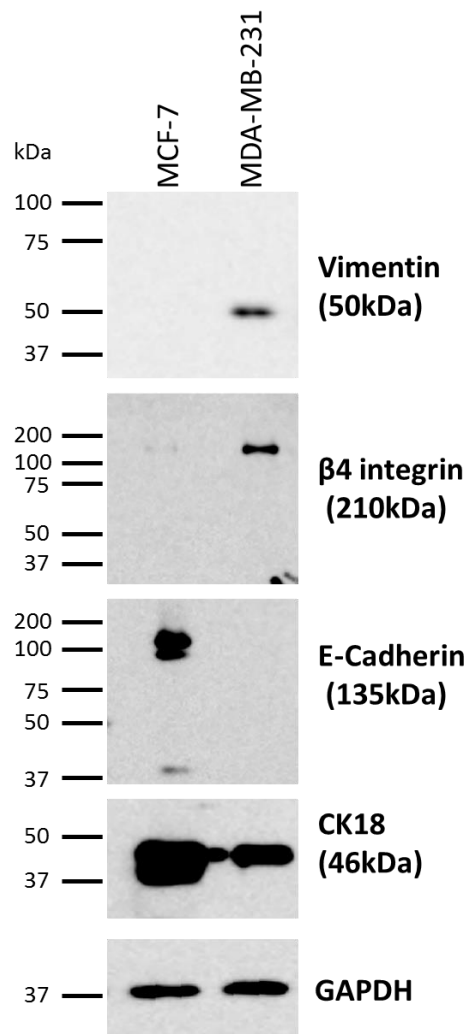


Figure 3.2: Characterisation of MCF-7 and MDA-MB-231 mammary carcinoma cells. Western blot analysis of markers of expression of epithelial cells (E-Cadherin, CK18) and mesenchymal cells (vimentin, β 4 integrin) using GAPDH as a loading control.

3.1.2 Generation and validation of MMP8 expression constructs

Wild-type MMP8 had previously been cloned into the pcDNA4TM V5-His plasmid in our lab prior to the start of this project. The V5 tag was used as at the time of construction there was no MMP-8 antibody for western blot commercially available. Using PCR a mutant version of MMP8 was generated within the same plasmid whereby a glutamic acid (E) residue at position 198 is substituted for an alanine (A) residue at the zinc binding site of the catalytic domain, rendering the protein catalytically inactive. This mutant is termed E198A in all subsequent experiments. The mutation was initially confirmed by sequencing (Appendix 1) and later by examination of the collagen-degrading ability of the cells that have been generated to over-express both wild-type MMP8 and the EA mutant MMP8 (figure 3.6). Prior to use in any experiments, plasmids were checked to be comparable to each other with regards to levels of linear, circular and supercoiled DNA. This was achieved by linearization and separation of the digest products on an agarose gel (figure 3.3).

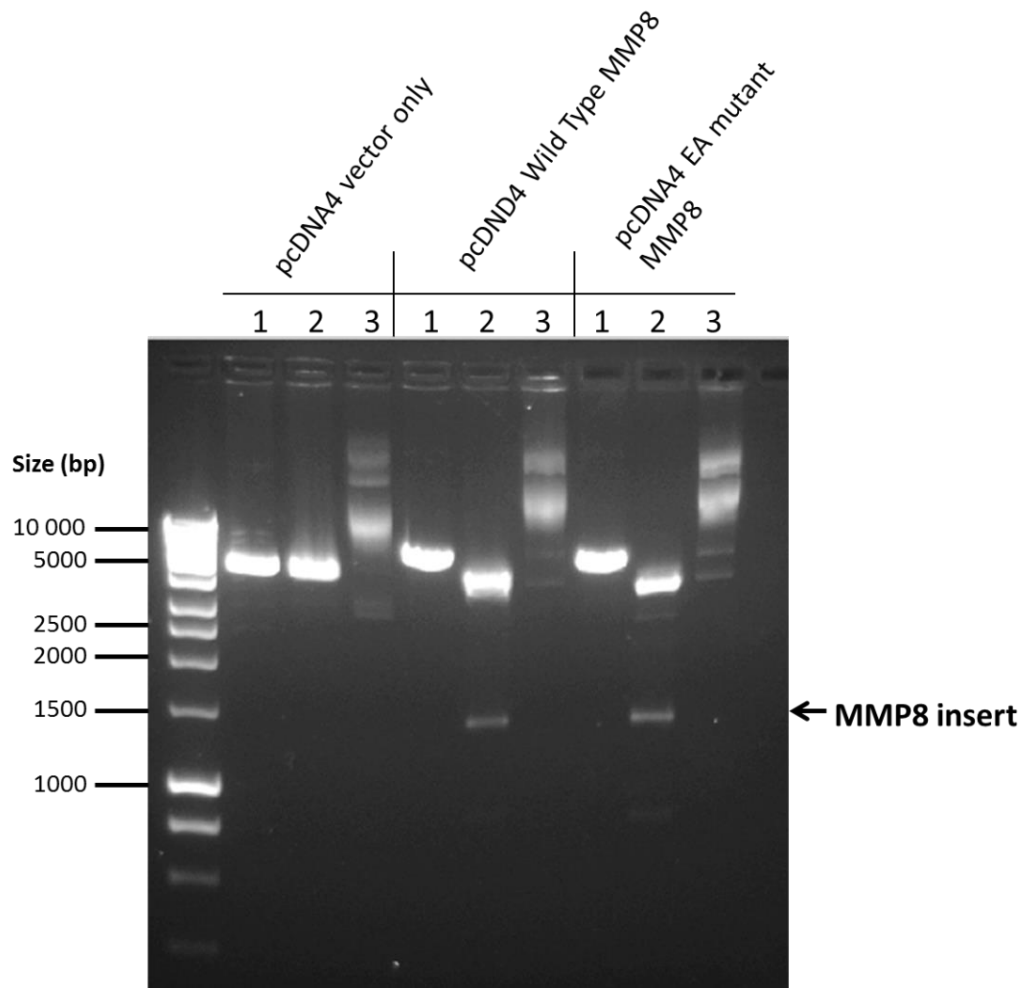


Figure 3.3: Restriction digest of pcDNA4™ V5-His and MMP8 constructs. 500ng pcDNA4 digested with: 1 – XbaI to linearise, 2- EcoRI and XhoI to cut out MMP8 (as seen at approximately 1500bp) and 3 - uncut , showing circularised and supercoiled DNA in equal amounts across the 3 plasmid constructs.

3.1.3 Transfection of MMP8 constructs into mammary carcinoma cells

To ensure that the plasmid constructs could be expressed in cells all constructs were transiently transfected into SK-BR-3, MCF-7 and MDA-MB-231 cells and MMP-8 protein detected by western blot. As can be seen in figure 3.4 a high level of expression was achieved, with both wild-type and E198A mutant MMP-8 observed in the cell lysate and secreted into the conditioned media at equivalent levels between the two constructs. MMP-8 is a secreted protease so high levels in the media was expected, plus this showed that the constructs were expressed effectively. Both pro (75kDa) and active MMP-8 (approximately 50kDa) was observed in the cell lysate. It is unclear whether secreted MMP-8 is active as when extending the exposure of the western blot to visualise and active band the pro-MMP-8 band became so over-exposed that it would have eclipsed any active-MMP-8 band.

Despite successful transient transfection of MMP8 into mammary carcinoma cells, generation of cells stably over-expressing MMP8 proved problematic. Three different protocols were followed in an attempt to make stable transfectants. Previously in the lab it was attempted using the Fugene 6 transfection reagent (Roche), which was unsuccessful as none of the resulting clones expressed MMP8 protein. Despite achieving a high level of expression of MMP8 on transient transfection into SK-BR-3, MCF-7 and MDA-MB-231 cells (figure 3.4) with LipoD293 transfection reagent (SigmaGen Laboratories), on stable transfection the reagent appeared to be toxic and once under selection cells died within a week.

Stable transfection was eventually achieved using the well-established calcium phosphate method of transfection. Both pooled stably transfected cells and isolated clones were generated for all three constructs in the MDA-MB-231 cell line (figure 3.5). Western blot analysis showed that MMP8 is only detected in the serum free conditioned media, so is secreted a very short time after production. Despite the use of a wide range of protein extraction buffers MMP-8

could not be observed in the cell lysate. The molecular weight of the protein detected was approximately 75kDa, which is in line with published data [32]. Two bands are seen with the MMP8 antibody, the larger, and more prevalent, likely corresponding to the pro-form, and a lower band likely corresponding to the active form. However, active MMP-8 is published to be approximately 50kDa, and the band observed here is slightly larger so caution must be taken when attributing identity. This lower band was not seen with the V5 tag antibody, as the tag may have been cleaved off. Despite the presence of a large pro-form band a collagenase assay consisting showed high collagenase activity and increased ability to contract a Type I Collagen gel with cells stably transfected with wild-type MMP8 but not in cells stably transfected with EA mutant MMP8 (figure 3.6). Confirmation that the anti-MMP-8 antibody is detecting MMP-8 comes from the use of the anti-V5 antibody which detects a protein of the same molecular weight as the anti-MMP-8 antibody. Additional validation of the antibody came from siRNA knockdown of MMP8 (chapter 5 section 5.1) where siRNA to MMP8 reduced this band. Although stable transfectants have successfully been generated there were problems associated with long-term expression of wild-type MMP-8 which will be addressed later in Section 5.1.

Loss of wild-type MMP-8 expression in the stable transfectants was observed in as little as two weeks of the cells being in culture. Protein expression of E198A mutant MMP-8 was not lost however, and was expressed at slightly higher levels than wild-type MMP-8 in the cells. This would suggest that the catalytic activity of MMP-8 is detrimental to the breast cancer cells, which can be presumed to be due to over-expressing an anti-tumourigenic protein in cancer cells. This loss of wild-type MMP-8 expression will be documented in Chapter 5. Despite this additional problem when generating the stably transfected cells, a subset of MMP-8 over-expressing clonal isolates (figure 3.5B) generated from the pooled cells shown in figure 3.5A maintained MMP-8 expression long-term.

Following the generation of these “long-term” MMP-8 over-expressing MDA-MD-231 clones it was determined whether MMP-8 expression had any

phenotypic effects on the cells. Functional cell behaviour, alterations in protein expression, changes in EMT and effects on the protease web were examined.

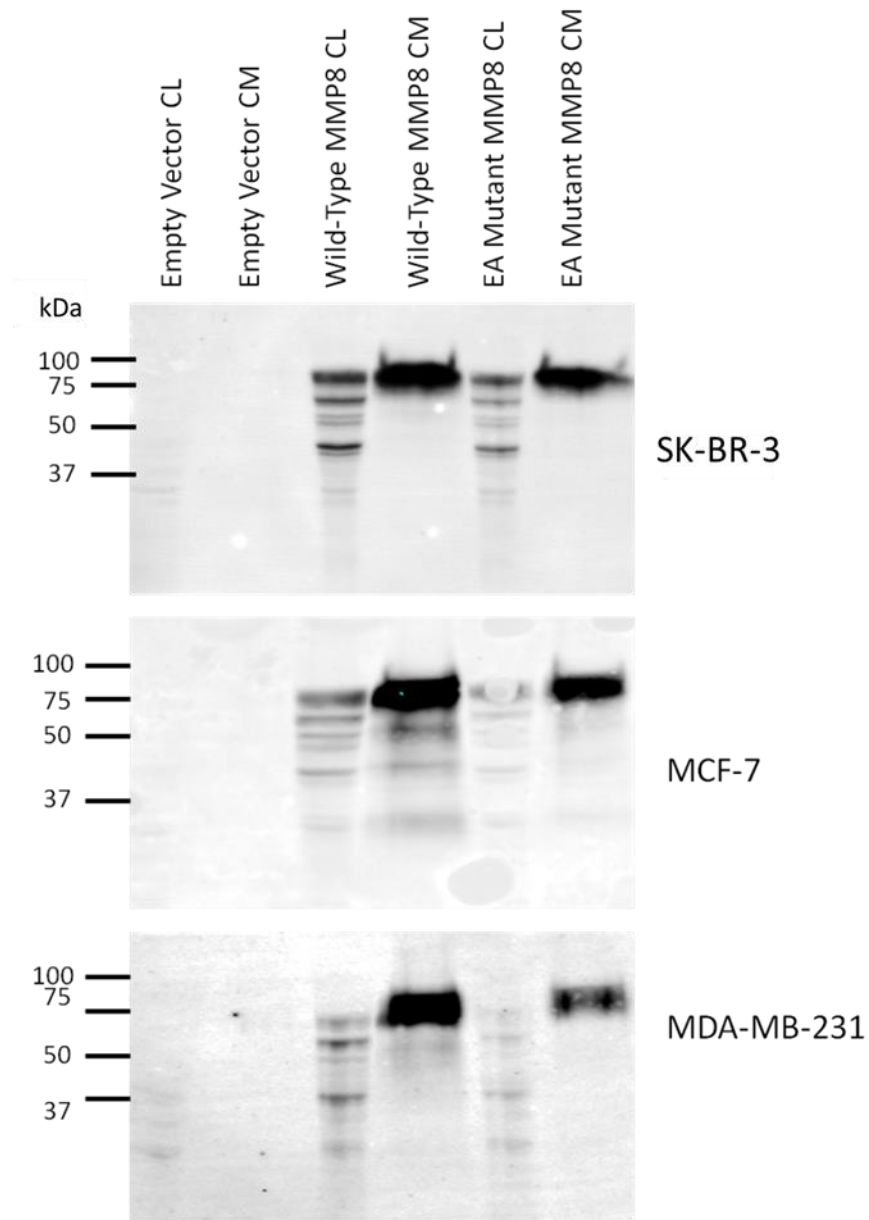


Figure 3.4: Transient transfection of wild-type and E198A mutant MMP-8 in breast cancer cell lines. Western blot analysis of MMP8 transient transfection of SK-BR-3, MCF-7 and MDA-MB-231 cell lines. An anti-MMP-8 antibody was used to detect MMP-8 in both the cell lysate (CL) and serum free conditioned media (CM) 48 hours post transfection.

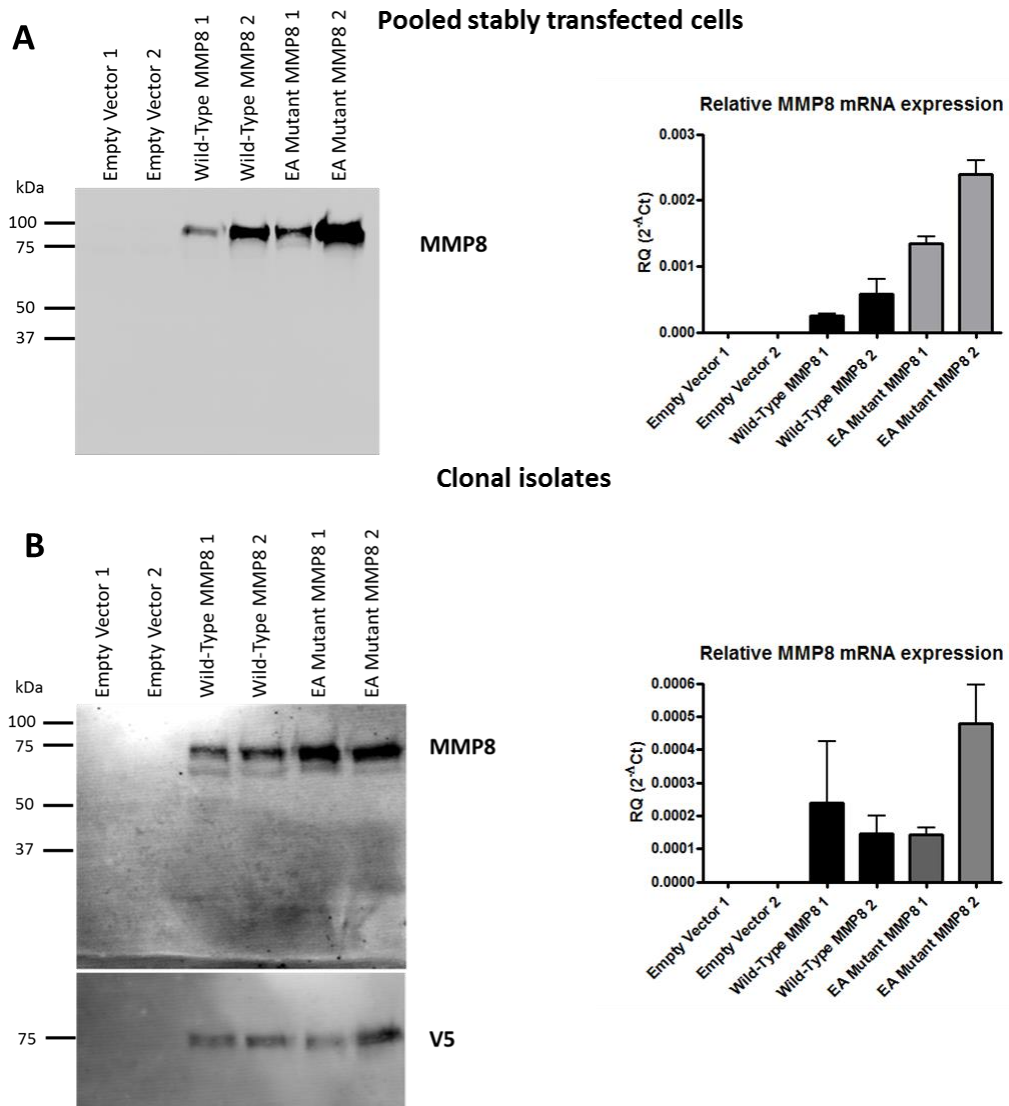


Figure 3.5: Stable expression of MMP-8 in MDA-MB-231 cells. Left panels: western blot analysis of MMP-8 in serum free conditioned media of cells over-expressing both wild-type and catalytically inactive (E198A) MMP8 using both anti-MMP-8 and anti-V5 tag antibodies. Right panels: RNA quantification by real-time TaqMan RT-PCR of MMP8 normalised to 18S rRNA. Clonal isolates (B) were generated from pools of stably transfected cells (A). Both 'empty vector' and EA Mutant MMP8 over-expressing clonal isolates were generated from their respective pool 1s.

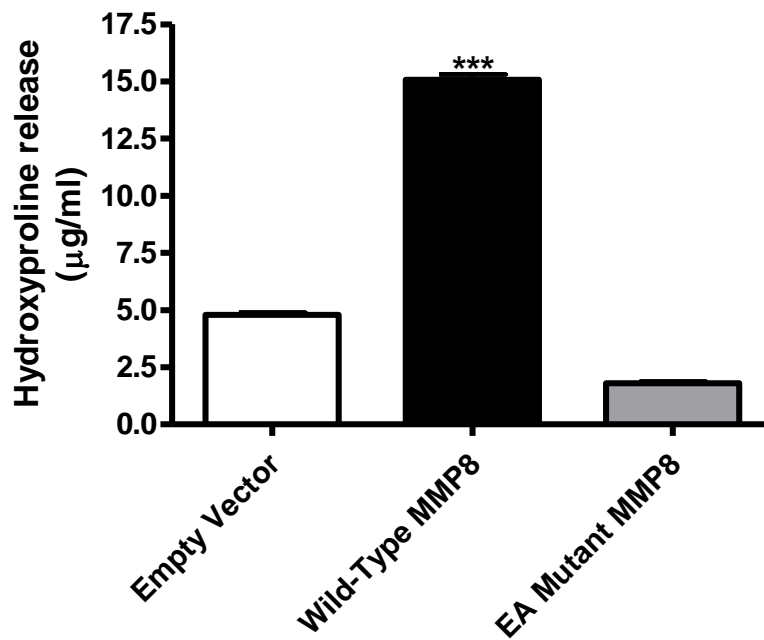


Figure 3.6: Collagenase activity as measured by hydroxyproline release in MMP-8 over-expressing MDA-MB-231 clonal isolates (set 1). Cells were seeded into type I collagen gels in serum free conditions. Gels were released from the edges of the plates and were digested for 24 hours. As an indirect measure of collagen breakdown hydroxyproline levels were measured in the serum free conditioned media. n=3, mean \pm SEM, * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$

3.2. Effects of MMP-8 on cell behaviour

Literature has shown that MMP-8 is a tumour suppressor, and has been shown to reduce invasion through Matrigel and to increase adhesion to type I collagen and laminin-1 when stably over expressed in B16F10 melanoma cells [52]. Therefore, the effects of MMP8 on invasion and adhesion on breast cancer cells were studied. This was also expanded to examine the effects of MMP8 on cell growth, cell cycle and 2D migration on breast cancer cells. One clone of control cells, wild-type MMP-8 over-expressing and E198A mutant MMP-8 over-expressing cells were studied in this section.

3.2.1. MMP-8 does not affect the growth or cell cycle profile of “long-term” MDA-MB-231 stably transfected cells

Cell growth was determined by cell counts every 24 hours for 72 hours. No significant differences in growth were observed between cells over-expressing wild-type and mutant MMP-8 and empty vector control cells (figure 3.7) This was further investigated by analysing the cell cycle profiles of the cells using propidium iodide staining of DNA followed by cell sorting by flow cytometry. All cell lines had the same proportion of cells in each phase of the cell cycle, and only very few cells (about 2%) were in the sub-G1 phase, which is used to give an indication of cells undergoing apoptosis (figure 3.8). Therefore it can be concluded that “long-term” MMP-8 over-expression (for a couple of months before the experiment) has no effect on either growth or apoptosis (when looking at sub G1 cells) of MDA-MB-231 cells.

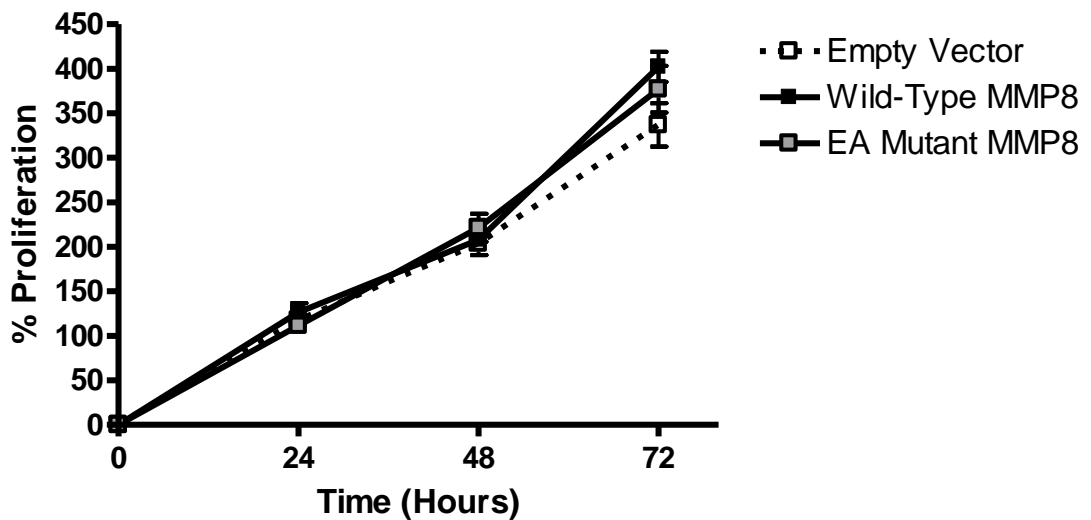


Figure 3.7: Growth of MDA-MB-231 stably expressing MMP8 “long-term”. Triplicate wells of MDA-MB-231 clonal isolates stably over-expressing pcDNA4 ‘empty vector, wild-type MMP8 and E198A mutant MMP8 were counted every 24 hours by haemocytometer and expressed as percentage proliferation from the number of cells initially seeded. n=5, mean \pm SEM.

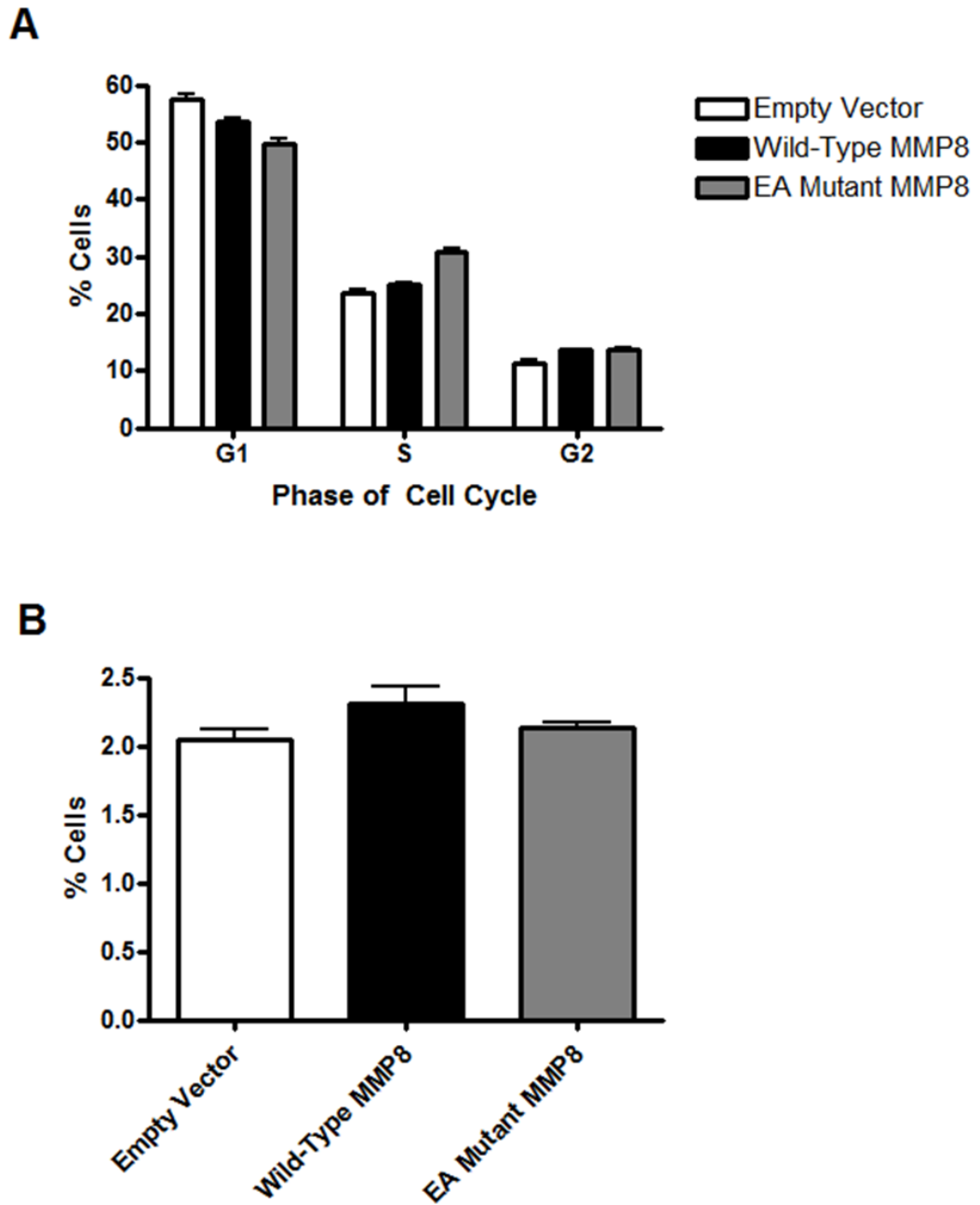


Figure 3.8: Flow cytometric analysis of cell cycle parameters of wild-type MMP8, E198A mutant MMP8 and empty vector control stably transfected MDA-MB-231 cells (clone 1). A) Percentage of cells in G1, S and G2 phase and B) percentage of cells in sub-G1 phase. n=3, mean \pm SEM.

3.2.3 Effects of “long-term” MMP-8 expression on 2D migration of MDA-MB-231 cells

The “long-term” MDA-MB-231 stable clones over-expressing either wild-type or EA mutant MMP-8 or an control ‘empty vector’ were seeded at a low density onto tissue culture plastic. Under serum-free conditions cells movement was captured by time-lapse video microscopy over 16 hours. Individual cell movements were tracked using Image J (‘manual tracking’ and ‘chemotaxis plugins’) and the total distance migrated and velocity of the cells was determined. Stable wild-type MMP-8 expression reduced both the overall distance migrated and the velocity of the MDA-MB-231 cell lines (figure 3.9A). MMP-8 did not only reduce cell migration compared to the control but also compared to the cells expressing the E198A mutant, indicating that this reduction in 2D, random cell migration, depends on the catalytic activity of MMP-8.

Scratch wound assays are similar to the 2D migration assay in that they are carried out in 2D on cell culture plastic, where the cells would have laid their own matrix on plating in the presence of serum. However, cells are seeded at a very high density and cell movement to fill a ‘scratch wound’ is monitored. Whereas the 2D migration assay looks at random cell migration this assay looks at collective migration. Almost total scratch wound closure (82% closure) was observed by 24 hours post scratch in the empty vector control MDA-MB-231 clonal isolates, compared to only 48% with cells over-expressing wild-type MMP-8 and 53% with cells expressing E198A mutant MMP8 (figures 3.9B-C). These data provide additional evidence for the anti-migratory actions of MMP-8; however reduction in migration occurred independently of the catalytic activity of MMP-8. These discrepancies may be due intrinsic features of the assay which will be discussed in section 3.4. Despite these differences it can be hypothesised that long-term expression of MMP-8 is capable of reducing migration of MDA-MB-231 cells.

To gain more insight into the disparities between the findings of these two migration assays we looked at 3D invasion and adhesion. Firstly this was because it is much more physiologically relevant to study cellular migration in a 3D matrix, and secondly, if the cells have different integrin-binding requirements it can be hypothesised that the cells may have different adherent properties to different cellular matrices.

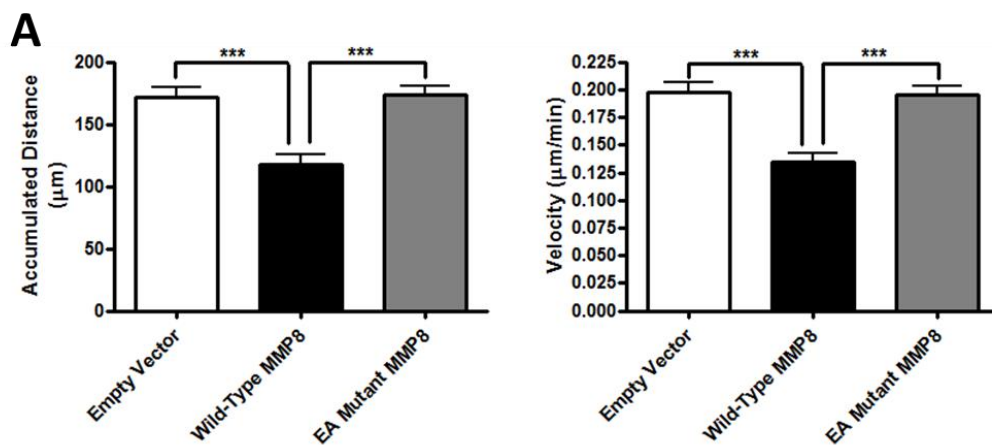


Figure 3.9: Effects of MMP-8 on MDA-MB-231 cell migration. A) Time-lapse light microscopy to determine random 2D migration of MDA-MB-231 clonal isolates (clone set 1) stably expressing empty vector control, wild-type MMP8 and E198A mutant MMP8. B and C) Scratch wound closure of the same cells over 24 hours. D) Western blot analysis of MMP-8 on serum free conditioned media showing expression of MMP8 throughout all experiments. n=3, >100 cells, mean \pm SEM, * = p < 0.05, ** = p < 0.01 *** = p < 0.001

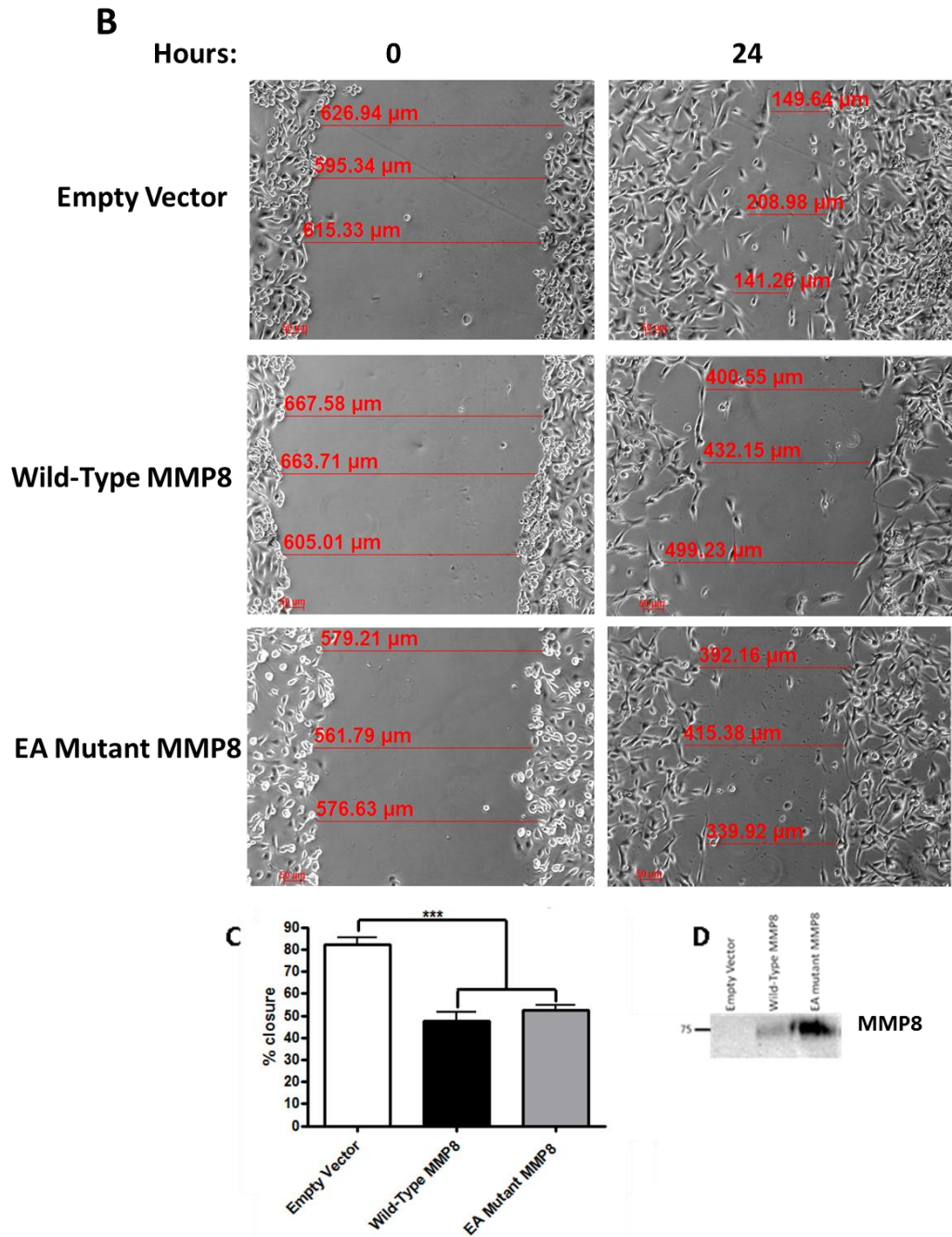


Figure 3.9: Effects of MMP-8 on MDA-MB-231 cell migration. A) Time-lapse light microscopy to determine random 2D migration of MDA-MB-231 clonal isolates (clone set 1) stably expressing empty vector control, wild-type MMP8 and E198A mutant MMP8. B and C) Scratch wound closure of the same cells over 24 hours. D) Western blot analysis of MMP-8 on serum free conditioned media showing expression of MMP8 throughout all experiments. n=3, mean \pm SEM, * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$

3.2.4 Effect of MMP8 on invasion and adhesion

Since MMP8 has an effect on 2D cell migration it was subsequently determined whether it has the same effect on 3D invasion. However, unlike the 2D migration assay which was carried out on plastic the 3D invasion was examined through a type I collagen matrix. This was because type I collagen is the main ECM substrate of MMP-8. To ensure that all cells were on a level plane at the start of the experiment, the inverted invasion assay, pioneered by Hennigan *et al.*, (1994) was used [163]. This requires cells to move upwards through a matrix, collagen in this case.. MDA-MB-231 clonal isolates (set 1) were seeded onto the underside of an 8.0µm transwell that had firstly had a collagen gel set within it. Media containing 10% FCS and 50ng/ml epidermal growth factor (EGF) was applied onto the top of the collagen gel as a chemoattractant and the cells invaded up through the collagen gel for 48 hours. Live cells were stained with CalceinAM and images were taken using a confocal microscope from the membrane through the gel at 15µm intervals. Prior to this any cells that had not invaded into the transwell and were just adhered to the underside were wiped off, although an image of the underside including adherent non-invading cells was taken first.

Invasion through type I collagen did not appear to be reproducible. Overall however there was a slight decrease in invasion of wild-type MMP-8 over-expressing cells, which is statistically significant with relation to the E198A mutant MMP8 over-expressing cells, but not the vector control cells (Figure 3.10 A, B). As we saw some differences in invasion, albeit with a lot of variability, total protein levels of MT1-MMP (MMP-14) were analysed by western blot (Figure 3.10E). MT1-MMP has long been thought of as the main collagenase responsible for cell invasion through cross-linked collagen [164, 165] so it was appropriate to determine whether MMP-8 over-expression in the MDA-MB-231 cells altered the level of MT1-MMP protein within the cells. No difference in pro- or active- MT1-MMP expression was seen between MDA-MB-231 clonal isolates expressing the control 'empty vector', and cells over-expressing wild-type MMP-8 or E198A mutant MMP-8.

Interestingly, despite inconsistencies between biological replicates with regards to cell invasion there were clearly less wild-type MMP-8 expressing cells at the bottom of the gel plug compared to empty vector cells and MDA-MB-231 cells expressing E198A mutant MMP8. Image J analysis of images taken at the very start of the experiment before non-invading cells are wiped off from the bottom of the transwell membrane shows that there are significantly more (3.5x) wild-type MMP-8 over-expressing cells adhering to the bottom of the transwell than with the other two cell lines (Figure 3.11). This is further supported by the slight decrease seen in total cells within the collagen gel (Figure 3.10C). Beta1 integrin is a major cell adhesion receptor (especially for fibronectin) [166] and a recent publication has suggested that MMP-8 can regulate β 1 integrin levels and also co-immunoprecipitate with it [167]. Therefore, to determine if β 1 integrin was involved in the increase in adhesion western blot analysis was carried out on both sets of clonal isolates of MMP-8 over-expressing cells and compared to control 'empty vector' and E198A mutant MMP-8 over-expressing clonal isolates. No differences were observed in β 1 protein expression with wild-type MMP-8 versus the other cell lines when quantifying levels relative to the GAPDH loading control (figure 3.11C). However, this does not discount any MMP-8-related changes in integrin activation.

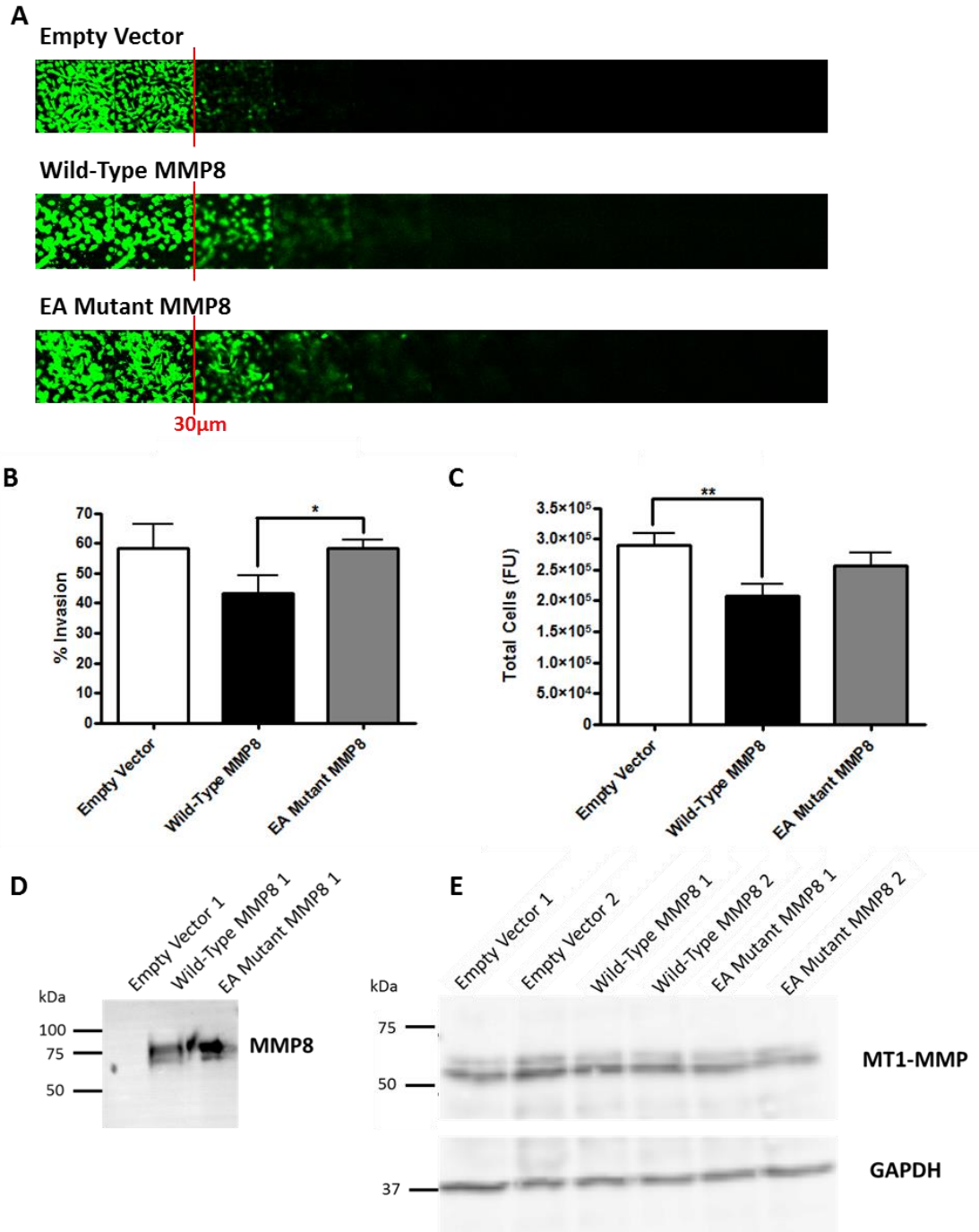


Figure 3.10: Effects of MMP8 on inverted invasion through type I collagen A) Montage generated by confocal microscopy of CalceinAM stained MDA-MB-231 clonal isolates (set 1) expressing pcDNA4 empty vector control, wild-type and E198A mutant MMP8 cells at 15µm sections. B) Percentage of cells that have invaded beyond 30µm sections relative to the total number of invading cells C) Total cells in the type I collagen plug (all quantified using Image J). D) Western blot to confirm MMP-8 expression throughout the experiments and (E) western blot analysis of MMP-14 (MT1-MMP) on total cell lysate of both sets of MDA-MB-231 stable clonal isolates. n=4, mean ± SEM, * = p < 0.05, ** = p < 0.01 *** = p < 0.001

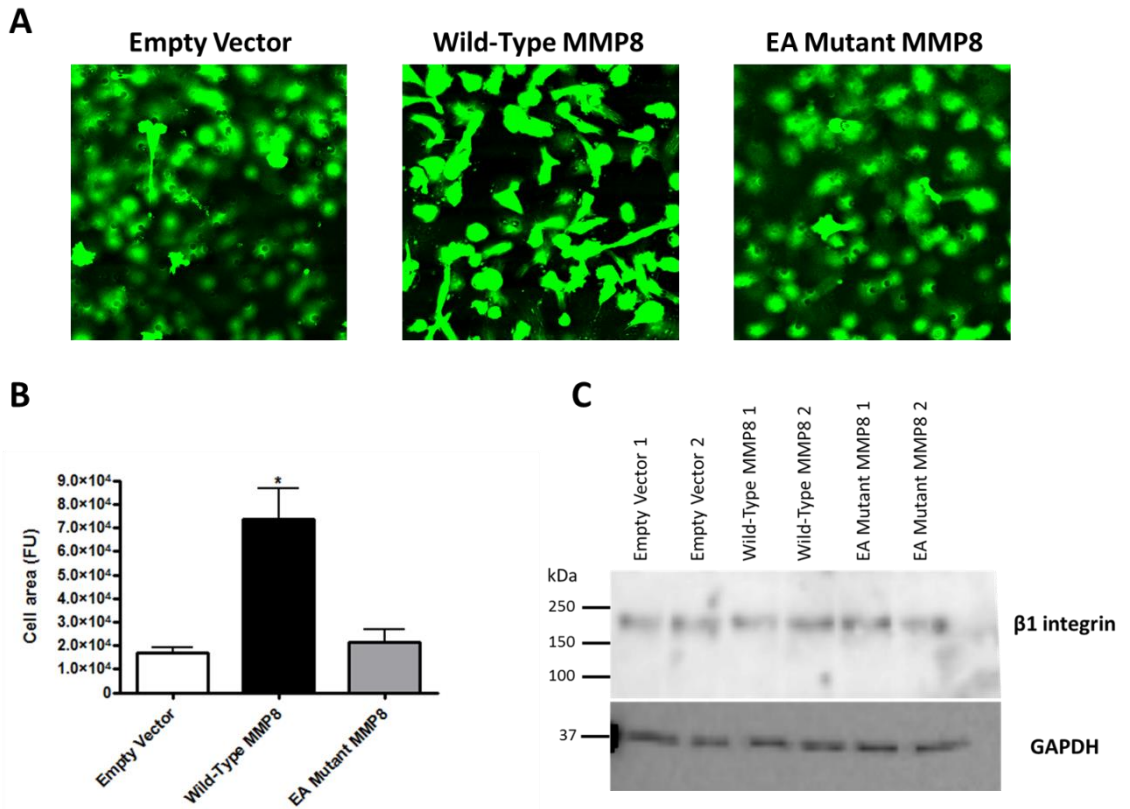


Figure 3.11: Cell adhesion to the under-side of the transwell used in the inverted invasion assay. A) Fluorescent confocal images of CalceinAM stained MDA-MB-231 cells adhered to the underside of the transwell. The cells in focus are on the underside, the out of focus cells are cells that have invaded into the gel. B) Quantification of total cell area of cells adhered to the transwell. n=4, mean ± SEM, * = p <0.05, ** = p<0.01 *** = p<0.001 C) Western blot analysis of β1 integrin in cell lysates of stably transfected MDA-MB-231 cells.

To further study the effect of wild-type MMP-8 on adhesion, a traditional adhesion assay [168] was carried out on clonal isolate sets 1 and 2. This assay showed that there were no consistent differences in adhesion to either type I collagen or fibronectin across all cell lines. However, the wild-type MMP8 expressing cells from clone set 2 showed a 2 fold increase of adhesion to type I collagen compared to the other cell lines (Figure 3.12A). This may be attributed to slight differences between the clones, as the cells were expressing MMP-8 equally (figure 3.12B). Cells also adhered more to collagen than to fibronectin. As the cells in the invasion assay were plated onto the underside of the transwells in serum, adhesion in absence of pre-laid matrices was also tested in

serum-containing conditions. No differences were found between cell lines under these conditions (data not shown).

Combined, these results suggest that MMP-8 may not have an effect on initial adhesion but adhesion contacts may be stronger, with cells adhering to the transwell membrane in preference to invading. Once cells have invaded however they invade to a similar extent. To fully confirm this however invasion assays would have to be carried out on the second set of clonal isolates as well as the first.

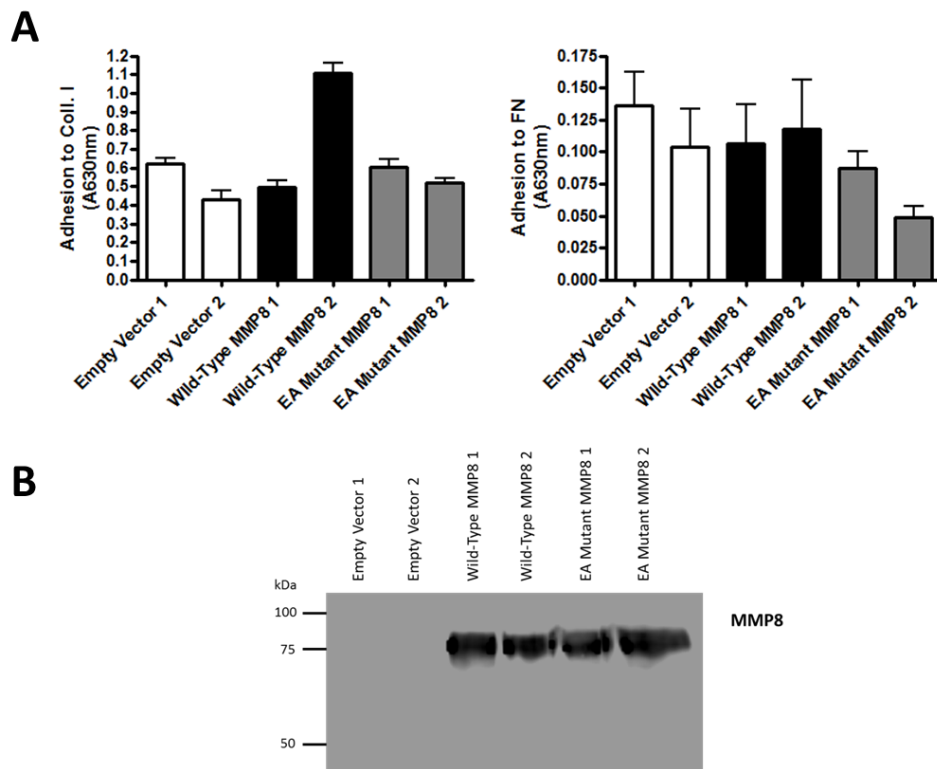


Figure 3.12: Effect of MMP-8 on adhesion of MDA-MB-231 cells. A) Adhesion of MDA-MB-231 clonal isolates stably transfected with pcDNA4 empty vector, wild-type and E198A mutant MMP8 to type I collagen and fibronectin (FN). n=5, mean \pm SEM. B) Western blot of MMP-8 in the serum free conditioned media of cells used in the adhesion assay.

3.2.5 “Long-term” MMP-8 expression has no effect on the epithelial to mesenchymal transition

The epithelial to mesenchymal transition (EMT) is a process through which cancer cells switch from an epithelial, differentiated state to a mesenchymal, de-differentiated state. It is in this state that cancer cells are more motile, and much more likely to metastasise. Some MMPs have been shown to have an effect on EMT through the activation of latent EMT-inducing growth factors (e.g. Epidermal Growth Factor) by proteolytic cleavage, coupled with evidence showing that cells that have undergone EMT produce more MMPs [169]. For example, MMP8 has been shown to be expressed during neural crest EMT in mouse embryogenesis [170].

MDA-MB-231 cells have already undergone EMT but we postulated that MMP-8 may have a role in reversing it; mesenchymal-to-epithelial- transition (MET), since MMP8 has been shown predominantly to have anti metastatic effects [52, 65, 77]. However, looking at E-Cadherin and Cytokeratin 18 (CK18) as markers of an epithelial state (figure 3.13A-B) and Vimentin and β -Catenin (figures 3.13C-D) as markers of a mesenchymal state no differences were seen with MMP8 over-expression. There is a slight increase in vimentin with both wild-type and E198A mutant MMP-8 expression compared to cells transfected with the empty vector control however, indicating that MMP8 may have some influence on EMT, and may actually be pushing it further forward. Therefore it can be concluded that over-expression of MMP8 in this cell line was not sufficient to induce MET, at least in relation to the markers examined here.

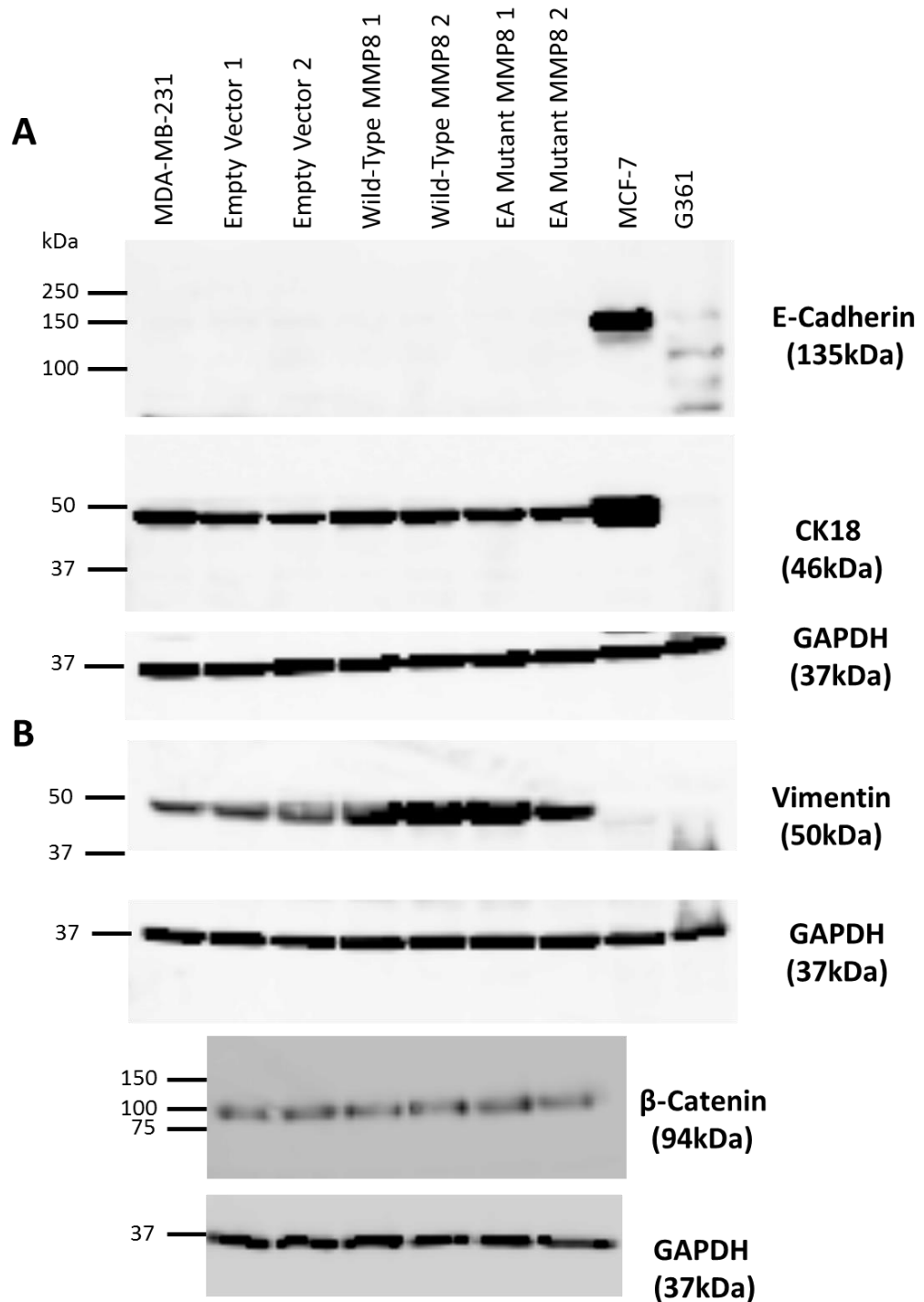


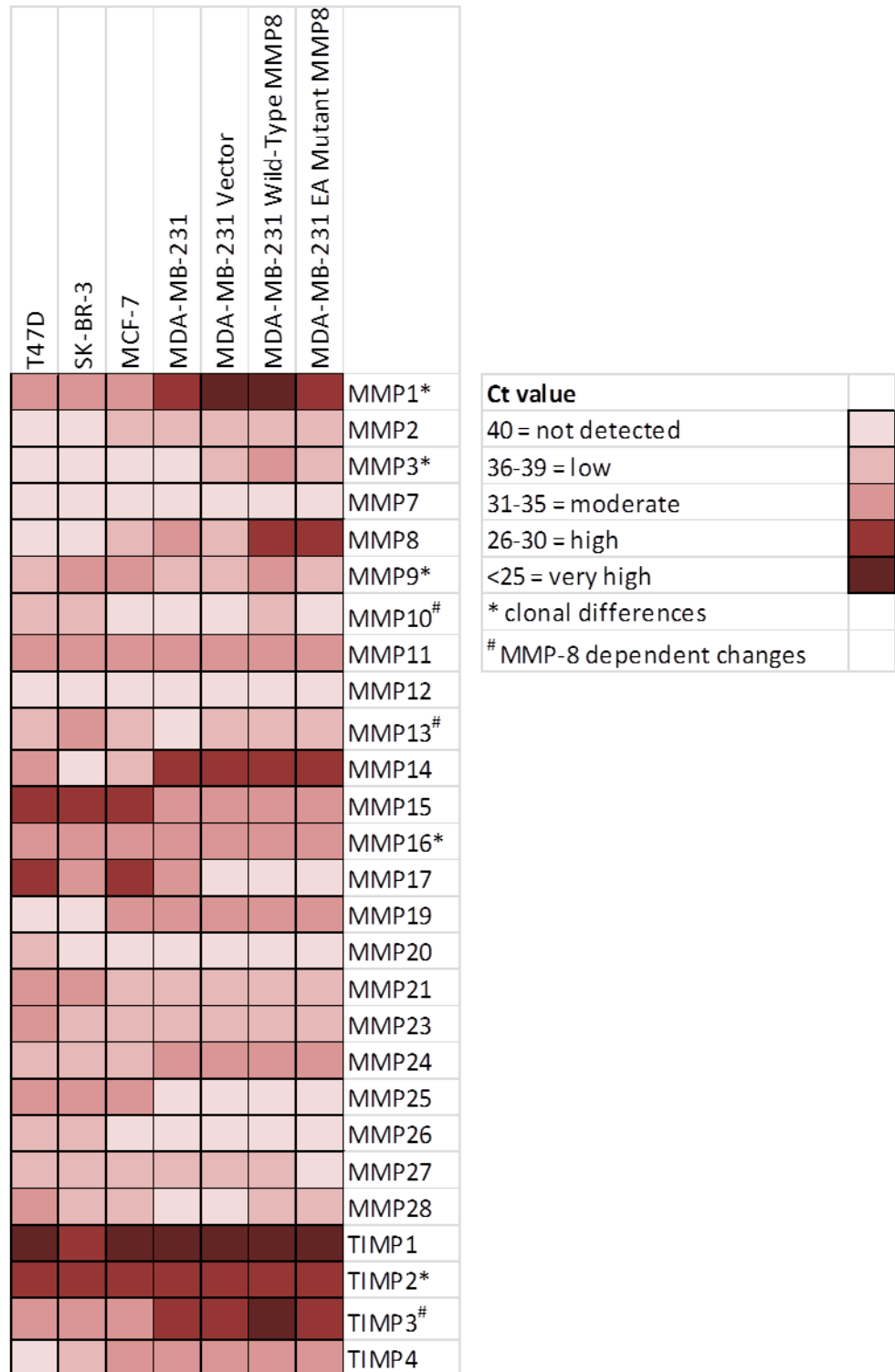
Figure 3.13: Characterisation of EMT markers in MMP-8 expressing MDA-MB-231 cells. A) Western blot analysis of epithelial markers (E-Cadherin and CK18) in the cell lysate of MDA-MB-231 clonal isolates (both sets) stably expressing wild-type and E198A mutant MMP8 and pcDNA4 empty vector control. B) Western blot analysis of mesenchymal markers (Vimentin and β -Catenin) in the same lysates. Parental MDA-MB-231 cells, MCF-7 cells and G361 melanoma cells were used as controls. Samples referenced against the GAPDH loading controls.

3.3 Degradome profiling of MMP-8 over-expressing cells

It has been widely accepted that MMPs are members of a complex “protease web”, with over-expression or deletion of an MMP resulting in the change in expression of other proteases. In addition, MMPs can have compensatory functions whereby a lack of phenotypic defect is observed when one MMP is deleted. To determine if this occurred in MDA-MB-231 breast cancer cells over-expressing MMP8, quantitative real-time PCR was carried out on all the MMPs and TIMPs. In addition, three other breast cancer cell lines were also profiled, SK-BR-3, T47D and MCF-7s, as well as parental MDA-MB-231 cells, to act as positive controls for some of the genes that are not expressed in the MDA-MB-231 cells, and to control for any influence of transfection alone. Figure 3.14A shows a heat map whereby expression levels are depicted broadly by cycle threshold (CT) values at which the genes are amplified above background in the PCR reaction. Few differences were found between clonal isolates independent of MMP8 expression which may be due to an indirect effect of transfection. Such differences may only show up on the heat map if differences cross the allocated CT boundaries but are indicated by an asterisk. MMP-8 dependent changes are marked by a hash and are shown in detail in figure 3.14B.

MMP-8 is classified as a collagenase along with MMP-1 and MMP-13. MMP-8 expression does not appear to have any effect on RNA expression of MMP-1 but wild-type MMP-8 causes a slight up-regulation of MMP-13 (Figure 3.14D). This is more marked in clone 1 of the wild-type MMP-8 over-expressing cells, which express MMP-8 to the greater extent (Figure 3.14B). MMP-8 also up-regulates MMP-10, a member of the stromelysin family (Figure 3.14C), with greater up-regulation in clone 1, which expresses higher levels of MMP-8. TIMP-3 is also up-regulated with wild-type MMP-8 over-expression (Figure 3.14E). Unlike MMP-10 and -13 however, TIMP-3 mRNA levels are higher in clone 2, which expressed less MMP-8.

A



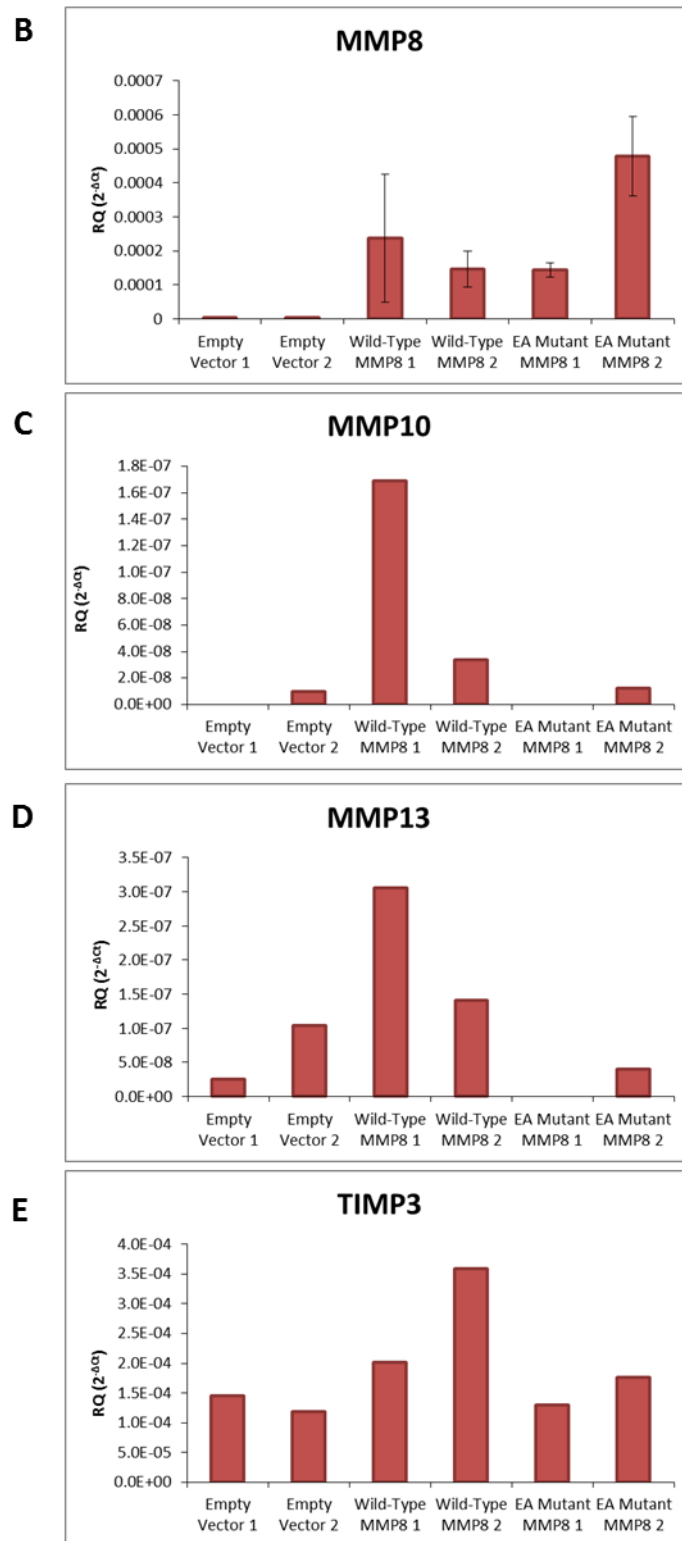


Figure 3.14: Taqman degradomic profiling of MMP-8 over-expressing MDA-MB-231 cells. A) Heat map showing the gene expression analysis of MMPs and TIMPs by Taqman PCR categorised according to their CT values. CT value is normalised to 18S rRNA and a mean generated from the two clonal isolates studied. B-E) Gene expression levels of B) MMP8, C) MMP10, D) MMP13 and E) TIMP3 in detail between the two sets of clonal isolates. n=2 except n=3 for MMP8.

Chapter 3- Development and characterisation of mammary *in vitro* models

To conclude this chapter, an *in vitro* expression model has been generated to examine the effects of wild-type and E198A mutant MMP8 in the pcDNA4 expression vector. “Long-term” stable transfection of MMP8 into MDA-MB-231 cells modulates cell behaviour through reducing 2D cell migration, increasing adhesion and slightly altering the protease web.

3.4 Conclusions and Discussion

The aim of this project was to determine the mechanisms of action of MMP8 in mammary carcinoma. To achieve this an *in vitro* model system has been generated. Whilst it is most physiologically relevant to examine the role of proteins within an *in vivo* system, when carrying out newly emerging research *in vitro* studies should be utilised until the research has reached a firm grounding. The most appropriate cells to use are non-commercially available breast myoepithelial cells, as in the normal, healthy breast these are the cells expressing MMP8. However these cells turned out to be unreliable, exhibiting significant phenotypic drifts in both their morphology and in expression of myoepithelial markers. As a result the commercially available aggressive breast cancer cell line MDA-MB-231 was used. This cell line was chosen over other widely used breast cancer cell lines such as MCF-7 as they have many characteristics, such as high motility and expression of mesenchymal markers that could potentially be reversed with over-expression of MMP8, a tumour/metastasis suppressing protease.

MDA-MB-231 cells were engineered to over-express MMP8 by stable transfection of a plasmid construct. Prior to transfection the plasmid constructs were validated by sequencing and also by a series of restriction digests to ensure restriction sites were as expected, and that MMP8 could be excised using the restriction sites used in the original cloning. As a control for the transfection a set of cells were transfected with a control 'empty vector', and to control for mechanisms of MMP8 action attributed to its catalytic activity cells were also transfected with a plasmid containing a catalytically inactive mutant MMP8, whereby a glutamic acid within the zinc binding site had been mutated to alanine (E198A). Firstly a polyclonal pool of stably transfected cells were generated which contained a heterogeneous mix of cells with various degrees of MMP-8 expression. This would result in variability between experiments so sets of clonal isolates were generated from these polyclonal pools resulting in a homogeneous cell system with reduced variability. Two pools of cells were generated to control for clonal differences, as the transfection strategy

employed in this project relies on random integration of the plasmid within the genome of the cells. This means that some observed effects could arise due to disruption of other genes by integration of the plasmid DNA. In the future a Flp-In™ system (such as supplied by Invitrogen) could be exploited, where stably transfected cells have to be made firstly to generate a Flp Recombination Target (FRT) site (sequencing or microarray analysis could firstly be used to ensure that this plasmid is not disrupting any genes), and then the DNA of interest has to be transfected. This removes the problem of random integration as the DNA of interest is only inserted into the genome by recombination at the FRT, resulting in isogenic clones. In most of the functional analysis experiments within this chapter only clone set 1 was utilised due to time constraints, although both sets were utilised when looking at markers of EMT and degradomic profiling of the protease web.

There is little known about the functional effects of MMP8 *in vitro* but there has been one study that looks at the effects of MMP8 on cell growth, adhesion and invasion, and another that examines the effects of MMP8 on cell growth. Gutiérrez-Fernandez *et al.*, (2008) generated MMP8 (murine MMP8) over-expressing B16F10 murine melanoma cells and found that MMP8 had no effect on cell growth (as measured by an MTS assay), but increased cell adhesion to type I collagen and laminin-1 and reduced invasion through Matrigel. Concurrent with this research project, Gutiérrez-Fernandez *et al.*, (2008) found that MMP8 did not have any effect on cell growth of MDA-MB-231 cells and neither did Palavalli *et al.*, (2009) who over-expressed MMP8 in Mel-STR melanoma cells [77]. Coupled to this, no difference in apoptosis was observed in this project. The effects of MMP8 on apoptosis have not been examined *in vitro* but it has been shown that neutrophils from MMP8 deficient mice accumulate at a wound site due to reduced apoptosis [14], attributed to reduced caspase 11 and caspase 3 cleavage [42]. In contrast to the research by Gutiérrez-Fernandez *et al.*, (2008) no effects were seen in this work on adhesion to type I collagen. This may be due to the different cell types used, with Gutiérrez-Fernandez *et al.*, (2008) using a mouse melanoma line while a human breast cancer line was used in this work. However, differences in adhesion were observed when combined

with the inverted invasion assay. Despite great variability in cell invasion between experiments wild-type MMP8 over-expressing cells consistently adhered to the underside of the transwell significantly more than the other cell lines, which corresponded to fewer cells invading into the type I collagen gel. In this assay cells were plated onto the bottom of the porous membrane of the transwell (polyethylene terephthalate (PET)) in serum-containing conditions onto a matrix provided by the serum proteins. This however could not be replicated in a traditional adhesion assay when the cells were plated directly onto tissue culture treated plastic in serum. An explanation could be that short-term adhesion of MDA-MB-231 cells is unaffected by MMP8, but once adhered, cells expressing wild-type MMP8 adhere more strongly. With regards to the invasion assay wild-type MMP8 expressing cells are adhering to the membrane in preference to invading into the collagen. In the future this could be tested by looking at the de-adhesion of the cells, as if the MMP8 expressing cells exhibit enhanced adhesive properties they would take longer to de-adhere. The actin cytoskeleton could also be examined by staining F-actin with phalloidin.

The inverted invasion assay used in this research was more complex than the invasion assay utilised by Gutiérrez-Fernandez *et al.*, (2008), which may explain the variability observed. Gutiérrez-Fernandez *et al.*, (2008) used a basic invasion assay whereby B16F10 cells (either over-expressing mMMP8 or a control plasmid) were seeded into transwell inserts with 8.0µm pores that had been pre-treated with Matrigel. Using 5% FCS as a chemoattractant they stained the cells that had reached the underside of the membrane after 8 hours and counted them by eye. Additionally, they coated their transwells in a layer of Matrigel, whereas in the inverted invasion assay the cells had to invade through a collagen plug which provides a more challenging environment for the cells to invade through. Matrigel is a protein mixture secreted from mouse sarcoma cells and consists of a mix of laminin, collagens and entactin, so therefore is said to mimic the extracellular matrix. However, when studying invasion *in vitro* it is important to ensure that the model used represents the *in vivo* situation and is appropriate to the molecule investigated. As a consequence type I collagen was used in preference to Matrigel. This was firstly because MMP-8 is a collagenase

that predominantly cleaves type I collagen, so thus is more physiologically relevant, and secondly, that it has been shown that invasion through Matrigel is not MMP dependent [165, 171]. For example MMP inhibition using the broad spectrum MMP inhibitor GM6001 blocked invasion of ovarian carcinoma cells through a collagen I coated transwell but only reduced invasion through a Matrigel-coated transwell by about 30% [172]. Studies into this effect have been carried out in our lab and have shown that tube-forming Human Umbilical Vein Endothelial Cells (HUVECs) require different proteases and integrins when in Matrigel than in type I collagen. These four major variables: the use of mouse cells, the possible effect of gravity, the use of a different matrix coating and the fact that a gel was used as opposed to a thin coating may explain the discrepancies observed in this work compared to Gutiérrez-Fernandez *et al.*, (2008). Additionally the variability observed with the inverted invasion assay may be explained by the multi-step complexity of the assay whereby variables thought insignificant may have had an influence on the outcome of the experiment.

In this chapter 2D cell migration was also examined, with MMP8 showing reduced random 2D migration on tissue culture treated plastic dependent on catalytic activity and also reduced scratch wound closure, although this occurred independently of the catalytic activity of MMP-8. There are differences between these assays in that random 2D migration relates to isolated cells and their cell-matrix interactions, whereas the scratch wound assay also relates to cell-cell interactions as well as cell-matrix interaction [173], suggesting that different adhesion and migration molecules may be required for the two different types of migration. Scratch wound assays can be criticised for differences in scratch wound closure being attributed to differences in cell proliferation rather than migration, however the growth rate between all cells was found to be equal. Whilst the effect of MMP-8 on *in vitro* cell migration in cancer cells has not been previously researched, the influence of MMP-8 on stem progenitor cell (SPC) migration *in vitro* has shown that MMP-8 is actually required for cell migration, with MMP8 knockout cells exhibiting reduced migration through both Matrigel and an endothelial monolayer, translating to

an *in vivo* situation whereby MMP-8 promotes the migration of SPCs into atherosclerotic plaques [174]. Interestingly within this model system incubation of embryonic Sca-1⁺ SPCs with MMP-8 reduced their adhesion to Matrigel, which is concurrent with their observation of MMP8 increasing migration, but the presence/absence of MMP-8 had no effect on cell growth [174], which is in line with this work. Drawing parallels to the work by Xiao *et al.*, (2013), Steinl *et al.*, (2012) examined the role of MMP-8 on the migration of haematopoietic stem progenitor cells (HSPCs) whereby MMP-8 was found to have an inhibitory role on their migration. This was hypothesised to occur through MMP-8 inactivational processing of CXCL12, a HSPC chemoattractant [175]. Therefore, the role of MMP-8 on cell migration *in vivo* is conflicting depending on the cell type studied and the model used, and due to a lack of *in vitro* research on this subject there are few data to which our results can be compared. To further confirm the data gained in this thesis and to dissect a potential mechanism for the reduction of migration observed, the experiments could be repeated on different matrices. Cell binding to, and migration on different matrices requires different integrins, for example integrins $\alpha 3\beta 1$, $\alpha\beta 1$ and $\alpha 6\beta 4$ bind laminin [176] whereas $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bind collagen [177]. Any changes in migration on these matrices may help elucidate the molecules and pathways involved.

Having looked at phenotypic changes as a result of MMP8 over-expression finally in this chapter it was sought to determine whether over-expression of MMP-8 altered the expression of the cellular markers of MDA-MB-231 cells and also the degradomic profile of the cells. MDA-MB-231 cells can be characterised by expression of mesenchymal markers as they have undergone EMT and also an array of MMPs. As aforementioned in this chapter MMPs can drive EMT indirectly through the liberation of matrix-bound EMT driving molecules, and also directly like MMP3 [169]. In addition, cells that have undergone the transition express more MMPs. We did not see any changes in total protein expression of EMT markers with MMP-8 over-expression, except a slight increase in vimentin in cells expressing both wild-type and E198A mutant MMP-8, an effect that is therefore independent of the catalytic activity of MMP-8. MDA-MB-231 cells have already gone through EMT so it was a surprise to see an

increase in the mesenchymal marker. In the literature, MMP8 has been shown to cause a small increase in E-Cadherin shedding via ADAM10 in stem progenitor cells [174], which occurs during loss of epithelial differentiation, but as the MDA-MB-231 cells used in this thesis do not express E-Cadherin we cannot confirm this in the breast cancer cells. We did however see some small changes in MMP expression with in the wild-type MMP8 over-expressing cells. We see a slight up-regulation of MMPs 10 and 13 with wild-type MMP8 over-expression. However this is very variable depending on the clone of MMP8 over-expressing cells examined, although expression levels do relate to the slightly differing levels of MMP8 expressed in the cells. To determine whether this is a true result or just due to clonal variation more clonal isolates need to be examined.

MMP-8 and MMP-13 have been shown to have some compensatory functions, for example on examination of the role of MMP-13 in wound healing there were no defects in wound healing in the *Mmp13* knockout mouse. Evidence suggests that MMP-8 performed a compensatory role, as expression of MMP-8 increased at the wound site from the first day of wounding [178]. This has also been paralleled in the bone of newborn mice, with *Mmp13* knockout mice exhibiting higher expression of MMP8 in RNA extracted from the distal femurs and proximal tibias of the newborn mice [179]. In this study however MMP-8 did not functionally compensate for the MMP-13 knockout phenotype. There have been reports of MMP-8 and MMP-9 having both compensatory functions and also direct interactions. A very recent study by Steinl *et al.*, (2012) reports that MMP-8 may be functionally compensating for MMP-9 in the mobilisation of hematopoietic stem progenitor cells as *Mmp9* knockout mice do not exhibit a defect in this, and levels of serum *Mmp8* rise as HSPC mobilisation occurs [175]. Conversely, Gutiérrez-Fernandez *et al.*, (2007) report that MMP-9 levels increased in the wounds of *Mmp8* deficient mice, although whether this is attributed to the accumulation of neutrophils at the wound site of *Mmp8* deficient mice or whether levels of *Mmp9* are increasing to compensate for the lack of *Mmp8* is unknown. In this situation however MMP-8 and MMP-9 existed in a stable complex with each other [14]. We did not see any consistent

difference in MMP9 expression at the RNA level, but clone 1 of the wild-type MMP8 over-expressing cells did exhibit slightly elevated MMP9 mRNA. When looking at MMP-9 protein by zymography the signal was very weak as MMP-9 is expressed at low levels in MDA-MB-231 cells. However, there was possibly more pro-MMP-9 in both clones of wild-type MMP8 over-expressing cells. This hints that MMP-8 and MMP-9 show some sort of interaction either at the expression level, protein interactions or both.

The most convincing change in degradome expression with wild-type MMP8 over-expression is seen in TIMP3 whereby expression in both sets of clones was elevated over levels expressed by the control 'empty vector' cells and the cells over-expressing E198A mutant MMP8. Despite being able to inhibit most MMPs, TIMP-3 is yet to be shown to inhibit MMP-8. However, perhaps TIMP-3 can actually inhibit MMP-8 and the increase in TIMP3 RNA in the wild-type MMP-8 expressing cells is part of a compensatory mechanism, or MMP-8 may have effects on other metalloproteinases such as ADAMs and ADAMTSs that were not investigated in this work which are regulated by TIMP3. To study this further the elevation of TIMP3 would firstly have to be confirmed at the protein level.

To conclude this chapter, this work has built on the limited knowledge of the cellular functions of MMP-8. Functional *in vitro* assays have previously only been carried out on mouse melanoma cells, so the work here has provided additional information on the wider functions of MMP-8. Whilst differences were drawn between these studies and the results in this chapter it has been shown that MMP-8 has no effect on the growth of MDA-MB-231 breast cancer cells, or the B16F10 melanoma cells used by Gutiérrez-Fernandez *et al.*, (2008) [52], has little or no effect on invasion through type I collagen but may have some longer-term effects on adhesion (dependent on its catalytic activity), reduces random 2D migration (dependent on its catalytic activity) and scratch wound closure (independent of its catalytic activity). MMP-8 did not have any effect on EMT, although this was expected as the MDA-MB-231 cells have already undergone this transition. It did however cause an increase in TIMP3 expression at the RNA level, potentially as a mechanism to reduce any adverse effects as a result of high expression of the protease. MMP8 does therefore have

some functional phenotypic effects on MDA-MB-231 breast carcinoma cells but without altering the fundamental molecular characteristics of the cells, with the exception of its role in the modulation of the two immune system mediators. This makes the system generated appropriate for further analysis into the functions of MMP8 in breast carcinoma cells. The following chapter involves further investigation of the molecular effects of MMP-8 with respect to its published roles in the innate immune response.

3.5 Summary

- Wild-type MMP8 and catalytically inactive E198A MMP8 expression constructs were generated and found to be functional by both transient and stable transfection into mammary carcinoma cells.
- MMP-8 is predominantly secreted from the cell.
- Stable transfection of MMP-8 was problematic but a set of MDA-MB-231 clonal isolates were generated that expressed wild-type MMP-8 “long-term”
 - Stable cells generated (clonal):
 - MDA-MB-231 pcDNA4 empty vector control x2
 - MDA-MB-231 pcDNA4 wild-type MMP8 x2
 - MDA-MB-231 pcDNA4 E198A mutant MMP8 x2
- Using the “long-term” MDA-MB-231 stably transfected cells behavioural characteristics were examined
 - MMP-8 did not affect cell growth or cell cycle profile
 - MMP-8 reduced random 2D migration dependent on catalytic activity
 - MMP-8 reduced scratch wound closure independent of catalytic activity
 - MMP-8 did not obviously affect EMT
 - MMP-8 potentially induced TIMP3 RNA levels dependent on catalytic activity

Chapter 4- Investigation of the regulation of Interleukins -6 and -8 by MMP-8

Having generated an *in vitro* system to discover the role of MMP-8 in mammary carcinoma we aimed to determine the actions of MMP-8 at the molecular level. The previous chapter showed that MMP8 had effects on migration and adhesion of breast carcinoma cells. This chapter examines a novel role for MMP-8 in the modulation the immune response through up-regulation of inflammatory mediators. Predominantly focussing on the “long-term” MMP-8 expressing MDA-MB-231 clonal isolates this chapter delves into this phenomenon aiming to discover the regulatory mechanisms and other factors involved. Some of this content has been published (Appendix 3).

4.1 MMP-8 has a role in modulation of immune responses

MMP-8 has been heavily implicated in diseases of the immune system [44] and *Mmp8* knockout mouse models have showed that MMP-8 cleaves both murine LIX, the orthologue of human interleukin 8 (IL-8), and IL-8 itself [64, 80]. Other studies have reported a decrease in LIX protein in the *Mmp8* knockout mouse [49], which is hypothesised as the result of a reduction in proteolytic release of LIX from stores in the extracellular matrix, which may primarily be carried out by MMP-8. Other evidence for the involvement of MMP-8 in immune system modulation comes from the study showing that MMP-8 can cleave the anti-inflammatory cytokine IL-10 in a mouse model of bleomycin-induced lung fibrosis, whereby in the *Mmp8* knockout mouse there was a decrease in IL-10 cleavage accompanied by a reduction in lung fibrosis [86]. In addition, MMP-8 expression has been found to be up-regulated by addition of IL-1 β to ovarian cancer cells in culture, with addition increasing MMP-8 protein levels by 5.6 fold compared to controls [38]. Therefore, it was appropriate to examine whether

MMP-8 expressed by breast cancer cells had any effect on individual inflammatory markers.

4.1.1 Cells transiently transfected with wild-type MMP8 induce IL-6 and IL-8 expression

Through looking at a panel of inflammatory markers (IL-8, IL-1 β , IL-10, TNF, IL-6, and IL-12p70) by FACS in conditioned media from MCF-7 cells transiently transfected with MMP8 for 48 hours it was discovered that, of the panel examined, MMP-8 had an effect on the levels of the cytokine IL-6 and the chemokine, IL-8. 'Cytokine' is a blanket term for these immune system modulators within which 'chemokines' reside. Chemokines are cytokines that have a role in inducing cellular chemotaxis. MMP-8, dependent on its catalytic activity, caused an increase in both IL-6 (20%) and IL-8 (65%) total protein secretion in MCF-7 cells (figure 4.1A). The other inflammatory molecules were not expressed by the cells so were not investigated further. To determine if this was due to liberation of the cytokines from cellular storage or an actual up-regulation of their expression, mRNA was examined by Taqman qRT-PCR. It was found that over-expression of wild-type MMP8 caused a 250% and 300% increase in mRNA levels of IL-6 and IL-8 respectively (figure 4.1B). Mutant MMP8 did not cause this response, and showed the same level of cytokine induction as the vector only negative control cells, meaning that the effect of MMP8 on cytokine expression is due to its catalytic activity. This effect can also be seen in SK-BR-3 cells (figure 4.1C) which are mammary gland adenocarcinoma cells isolated from a metastatic site of pleural effusion, but not in the non-tumourigenic HMT-3522 S1 cells (figure 4.2), suggesting that this phenomena is specific to cancer cells. None of the other inflammatory mediators studied in this assay were secreted from the MCF-7 cells at a measurable level, including IL-10, of which MMP-8 has been shown to cleave [86] and IL-1 β , which has been shown to control MMP8 expression [38] so were not examined further.

4.1.2 MMP8 up-regulates IL-6 and IL-8 in “long-term” MDA-MB-231 stable transfectants

Due to the differences observed by FACs and qRT-PCR in MCF-7 cells it was decided to look at IL-6 and 8 at both the protein and mRNA level in the MDA-MB-231 stable transfectants discussed in Chapter 3. Unlike the MCF-7 cells MDA-MB-231 metastatic breast cancer cells produce low levels of both IL-6 and 8 endogenously due to a lack of suppression by the estrogen receptor [180-183]. However, on stable transfection of wild-type MMP8 IL-6 protein levels were increased by 2-5x and mRNA level by 3-6x, and IL-8 protein levels by 2-10x and mRNA levels by 4-10x (figure 4.2). Two independent clonal isolates were examined, which confirms that the observed effect is due to MMP-8 rather than an effect of the transfection, but also explains the variation observed as IL-6 and 8 levels varied between clones. This effect is again due to the catalytic activity of MMP8, as the cells over-expressing the mutant show protein and mRNA levels comparable to the control. This effect was confirmed in the pooled stably transfected cells that the clonal isolates were derived from, at both the RNA and protein level (figure 4.4).

The observation of an induction of IL-6 and IL-8 with wild-type MMP8 over-expression is apparent in three different cells lines, after a short-term transfection time of 48 hours in the MCF-7 and SKBR3 cells, and also long-term stable transfection in MDA-MB-231 cells. This can be confirmed in two separate clonal isolates each for vector control cells, wild-type and EA mutant MMP-8 expressing cells. This trend is also apparent in the pooled stably transfected cells from which these cells were derived but there is more variation due to the heterogeneous nature of the cells.

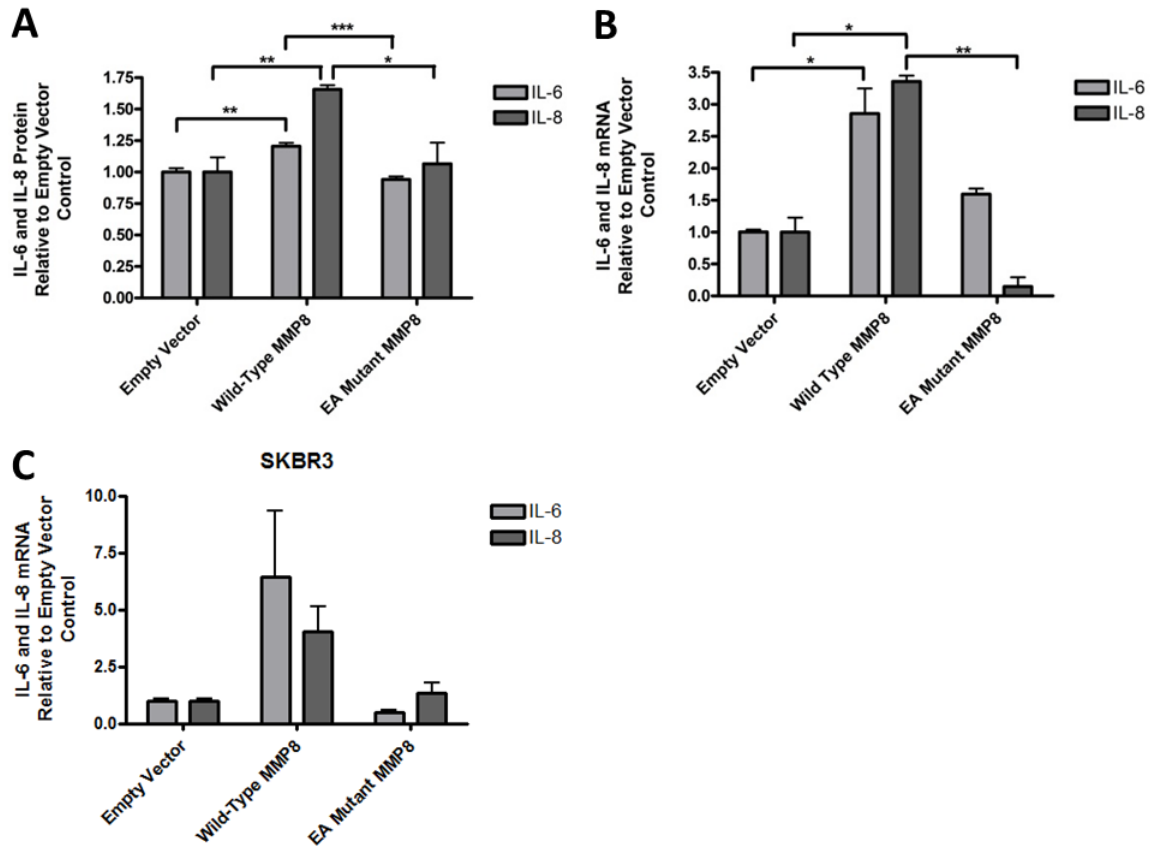


Figure 4.1: Transient transfection of MMP8 into MCF-7 and SKBR-3 cells. A) ELISA analysis of IL-6 and IL-8 protein levels in serum free conditioned media from MCF-7 cells transiently transfected with pcDNA4 empty vector, wild-type and E198A mutant MMP8 for 48 hours. B) Gene expression levels of IL-6 and IL-8 mRNA determined by Taqman in MCF-7 cells transiently transfected with pcDNA4 empty vector, wild-type and E198A mutant MMP8 for 48 hours. Values normalised to 18S and expressed relative to the EV control. C) Gene expression levels of IL-6 and IL-8 mRNA determined by Taqman in SK-BR-3 cells transiently transfected with pcDNA4 empty vector, wild-type and E198A mutant MMP8 for 48 hours. Values normalised to 18S and expressed relative to the EV control n=3, mean \pm SEM, * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$

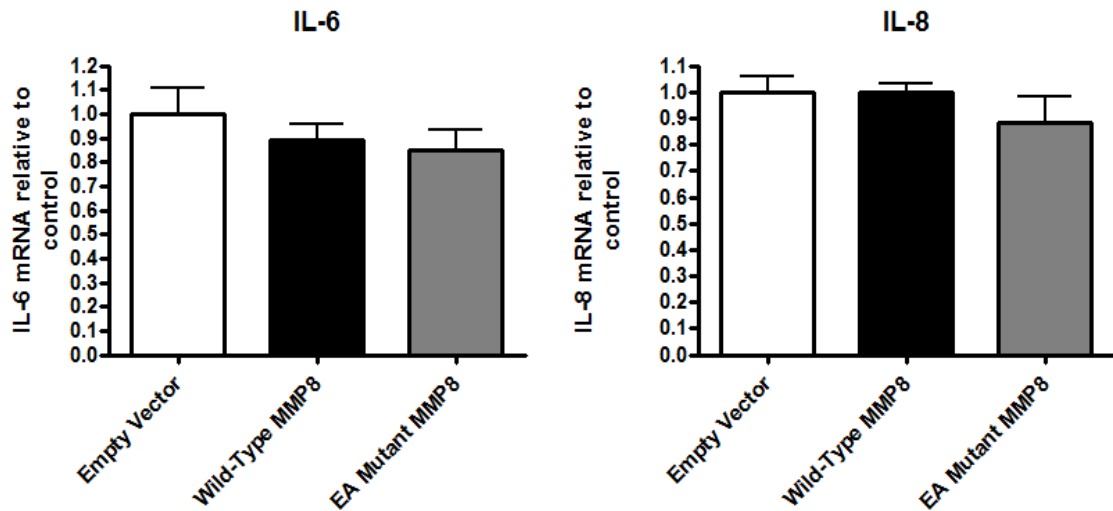
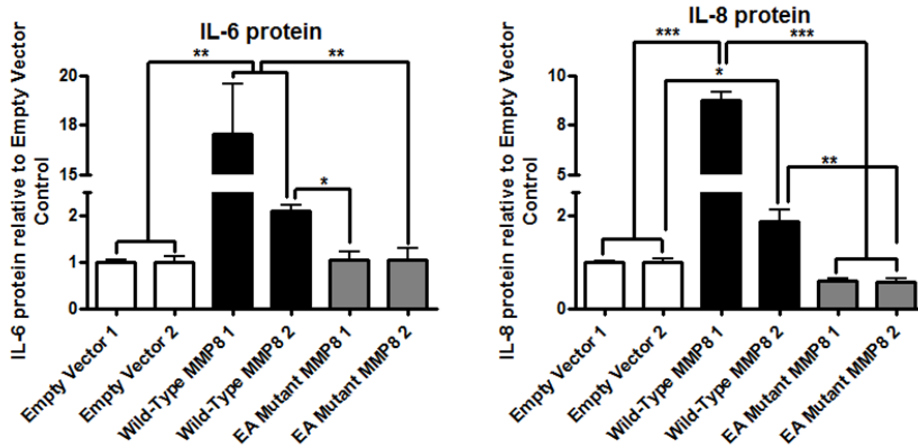


Figure 4.2: Transient transfection of MMP8 into HMT-3522 S1 cells. Gene expression levels of IL-6 and IL-8 mRNA determined by Taqman in HMT-3522 S1 cells transiently transfected with pcDNA4 empty vector, wild-type and E198A mutant MMP8 for 48 hours. n=3, mean \pm SEM

A



B

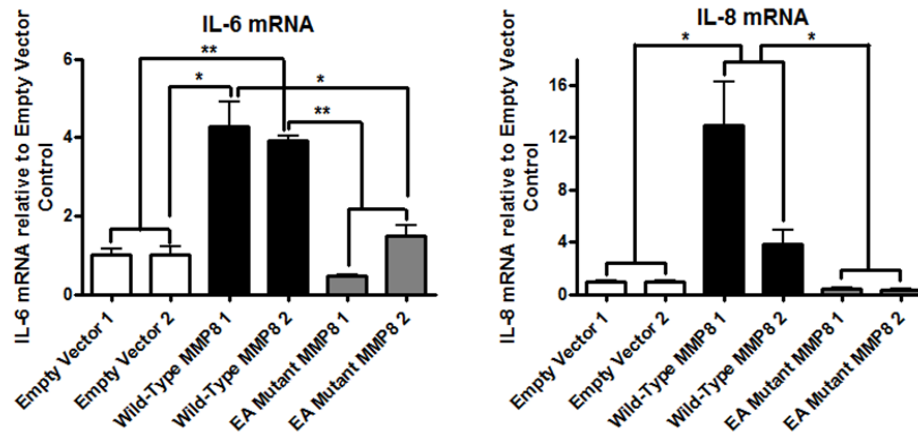


Figure 4.3: Effect of stable over-expression of wild-type MMP8 in MDA-MB-231 clonal isolates on IL-6 and IL-8 protein and RNA expression Two independent clones of MDA-MB-231 cells stably transfected with pcDNA4 empty vector, Wild-Type MMP-8 and E198A mutant MMP-8 were analysed. A) ELISA measurements of IL-6 and IL-8 protein levels in serum-free conditioned media collected 24hours after serum starvation. B) RNA quantification by real-time TaqMan RT-PCR of IL-6 and IL-8 mRNAs in these same cells. n=3, mean \pm SEM, Statistics: both wild-type MMP-8 clones 1 and 2 were different compared to all other cells by at least $p < 0.05$ or $p < 0.01$ unless where shown otherwise * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$.

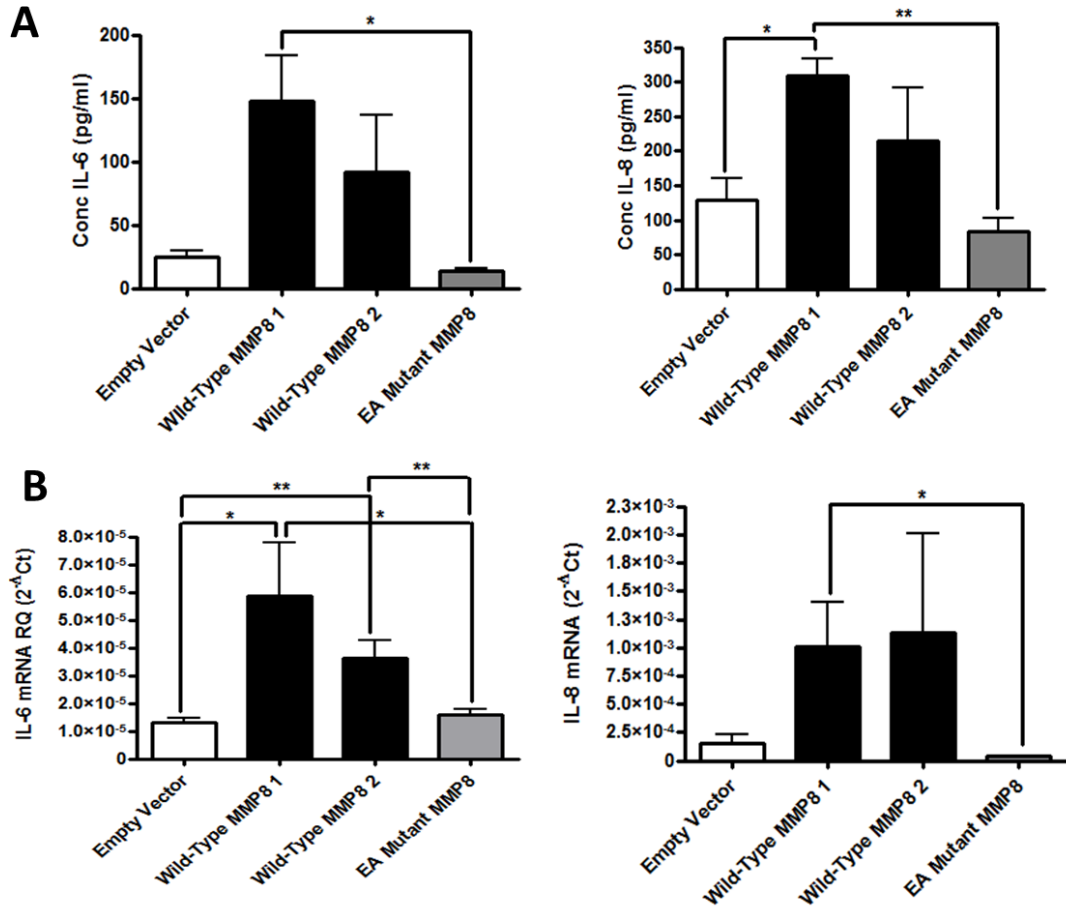


Figure 4.4: Effect of stable over-expression of wild-type MMP8 in MDA-MB-231 polyclonal pools on IL-6 and IL-8 protein and RNA expression. Two pools of wild-type MMP-8 expressing cells and one pool each of empty vector control and E198A mutant MMP-8 expressing cells were analysed. A) ELISA measurements of IL-6 and IL-8 protein levels in serum-free conditioned media collected 24 hours after serum starvation. B) RNA quantification by real-time TaqMan RT-PCR of IL-6 and IL-8 mRNAs in these same cells. n=3, mean ± SEM, * = p <0.05, ** = p<0.01 *** = p<0.001

4.1.3. MMP8, IL-6 and IL-8 exist as part of a self-reinforcing loop

Evidence widely suggests that cytokine levels within cells fluctuate dynamically in response to a wide variety of stimuli, and so will therefore exist in a range of varying feedback loops. To determine whether a feedback loop is operating in the MMP8 over-expressing MDA-MB-231 cells, cells over-expressing wild-type MMP8 were treated with siRNAs to IL-6 and IL-8 and levels of both cytokines measured by Taqman qRT-PCR. On siRNA knockdown of IL-6 no effect was observed on IL-8 RNA expression compared to the non-targeting control siRNA (figure 4.5B) indicating that IL-6 has no direct effect on IL-8 expression levels. However, on IL-8 siRNA knockdown IL-6 RNA levels were reduced by 75% compared to the non-targeting control (figure 4.5A). Finally, to determine if either of the cytokines had an effect on endogenous MMP8 expression human recombinant IL-6 and IL-8 were added to parental MDA-MB-231 cells for 24 hours, and the expression level of MMP8 determined by Taqman RT-PCR. Recombinant IL-6 was able to induce endogenous MMP8 expression by approximately 20%, but IL-8 had a very minor effect (figure 4.5C). This data suggests that there is an self-reinforcing loop occurring whereby MMP8 can induce expression of both IL-6 and -8, but also where IL-8 can reinforce IL-6 expression, and IL-6 expression can induce MMP8 expression (figure 4.5D), therefore resulting in a self-perpetuating loop. The remainder of this chapter concerns determination of the mechanisms involved in the MMP-8-driven induction of IL-6 and IL-8 expression.

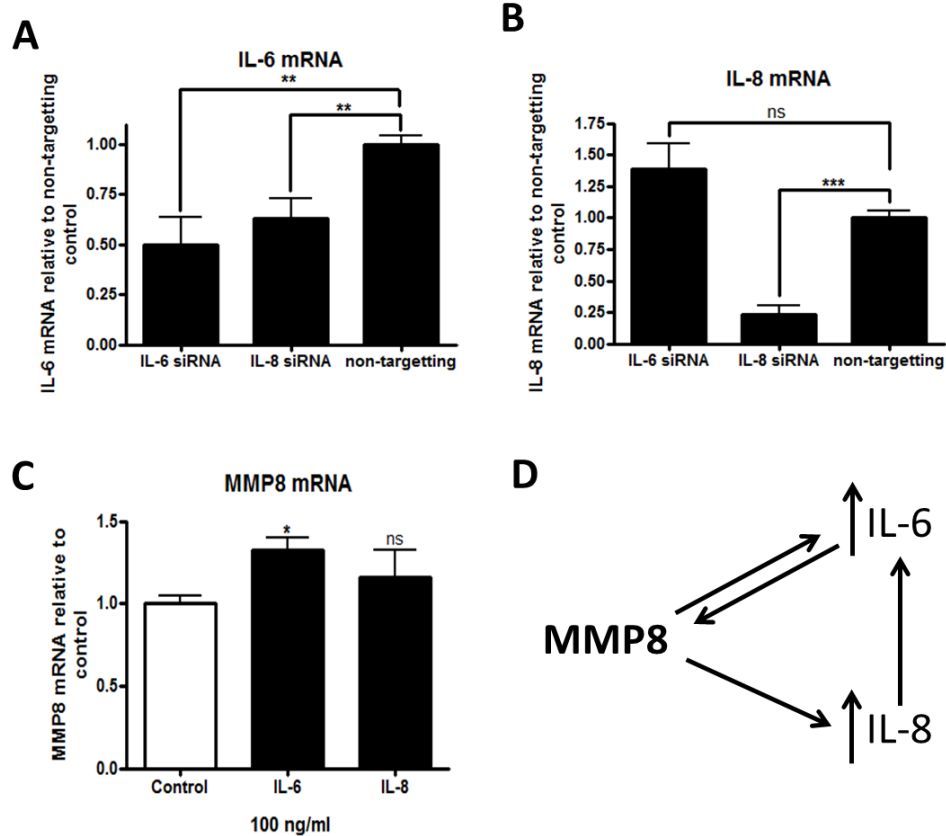


Figure 4.5: MMP8, IL-6 and IL-8 constitute a self-reinforcing loop. A) Gene expression analysis by Taqman of IL-6 levels after siRNA knockdown of both IL-6 and IL-8 for 48 hours. B) Gene expression analysis by Taqman of IL-8 mRNA levels after siRNA knockdown of both IL-6 and IL-8 for 48 hours. C) Gene expression analysis by Taqman of MMP8 mRNA after incubation with 100ng/ml rhIL-6 and rhIL-8 for 24 hours. D) The proposed self-reinforcing loop. n=3, mean \pm SEM, * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$

4.2 Molecular mechanisms of IL-6 and IL-8 up-regulation with wild-type MMP8 over-expression

Section 4.1 showed that wild-type MMP8 over-expression induces the expression of IL-6 and IL-8 in breast cancer cell lines, but not in normal breast cells. This effect is both dependent on the catalytic activity of MMP8 as the same effect is not seen in cells transfected with the E198A catalytically inactive mutant, and is dependent on malignant transformation. The aim of the rest of this chapter is to elucidate the mechanisms behind this up-regulation; firstly through a screen of molecular pathway inhibitors, then through an array that detects any changes in level of phosphorylated kinases, finishing with some mass spectrometry analysis of MMP8-binding partners to give further insight into the mechanisms involved. These experiments were carried out on clone set 1 of the MDA-MB-231 stable cell lines.

4.2.1 Effect of molecular pathway inhibitors on MMP-8 over-expressing cells

To determine the molecular mechanisms involved in the up-regulation of IL-6 and IL-8, various pathway inhibitors were used on the cells and IL-6 and IL-8 protein levels secreted into the cell culture media were measured by ELISA. These inhibitors were chosen on the basis of published literature into pathways known to affect cytokine production and key cell signalling constituents. The inhibitors used and their target pathways were; PD0325901 and U0126 (MAPK-Erk pathway), SB203580 (p38), Wortmannin and PI-103 (PI-3K), Bisindolylmaleimide 1 (PKC), SP600125 (JNK), BAY 11-7082 (NF- κ B) and SB431542 (TGF β R1). Concentrations used were in accordance to published guidelines [184] (see Chapter 2 table 2.5 for the concentrations used).

The only inhibitor that showed an effect on IL-6 and IL-8 expression selectively in the wild-type MMP-8 over-expressing cells only was BAY 11-7082. This is a Nuclear Factor Kappa Beta (NF κ B) inhibitor which acts by preventing phosphorylation of I κ B α , which in its un-phosphorylated form inhibits NF κ B by sequestering it in the cytosol [185, 186]. Inhibition of NF κ B signalling resulted

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in a 60-80% decrease in both IL-6 and -8 protein production in MDA-MB-231 cells over-expressing MMP-8 (figure 4.6A). The c-Jun-n-terminal kinase (JNK) inhibitor, SP600125, also had an effect in IL-6 and -8 protein levels but it did so across all three cell lines examined (data not shown). JNK kinases are involved in the activation of AP-1 [187] which is reported to be the second most influential transcription factor after NF κ B in IL-6 and -8 gene regulation [180]. IL-6 and -8 can also be differentially regulated, as seen in figure 4.6B after addition of SB431542, a TGF β inhibitor. This exerts its effects by blocking signaling through TGF β -Receptor 1 [188] and has no effect in IL-6 production but IL-8 production actually increases on inhibition across all three cell lines, showing that TGF β inhibits IL-8 production in MDA-MB-231 cells (figure 4.6B). This inhibitory effect has been reported in the literature in human bronchial epithelial cells [189]. Some inhibitors, such as PD0325901 and U0126 affected all cells dramatically and caused alteration in phenotype and induced cell death, probably due to the MAPK pathway having such a central role in signalling within these cells. Despite examination of components of the NF κ B pathway by western blot it was not possible to show how MMP8 affected its activity or vice versa, as no changes were observed in protein levels of either phosphorylated or total p65 NF κ B nor regulatory proteins, I κ K or I κ B α (figure 4.7). The phosphor I κ B α antibody did not work convincingly and showed many bands so lack of effect of MMP8 on the protein may be attributed to a poor antibody function. Additionally, the pathway is large and complex so it is wholly possible that MMP8 has an effect on other members not studied here.

Tumour necrosis factor alpha (TNF α) is a member of the TNF family of pro-inflammatory cytokines which has been shown to be an activator of NF κ B [190, 191]. It has also been shown to be solubilised by some MMPs [192, 193] to its active form. Furthermore, exogenous addition of TNF α to MDA-MB-231 cells can increase IL-6 production [194]. To determine whether the MMP-8 released from the cells is causing a release of pro-TNF α from the cell surface TNF α levels were determined in the cell media by ELISA. This would detect any TNF α that had been solubilised. Levels detected by ELISA were very low, and Taqman analysis showed that expression of TNF α is very low in MDA-MB-231 cells (CT values of

approximately 38). Therefore, it is not likely that MMP8 is acting on NF κ B via an increase in endogenous, active, TNF α , as the cells are not expressing it. To further support this, total TNF was analysed as part of the cytokine bead array (CBA) using FACS on conditioned media from MCF-7 cells transiently transfected with MMP8 and control constructs, and was undetected in this assay.

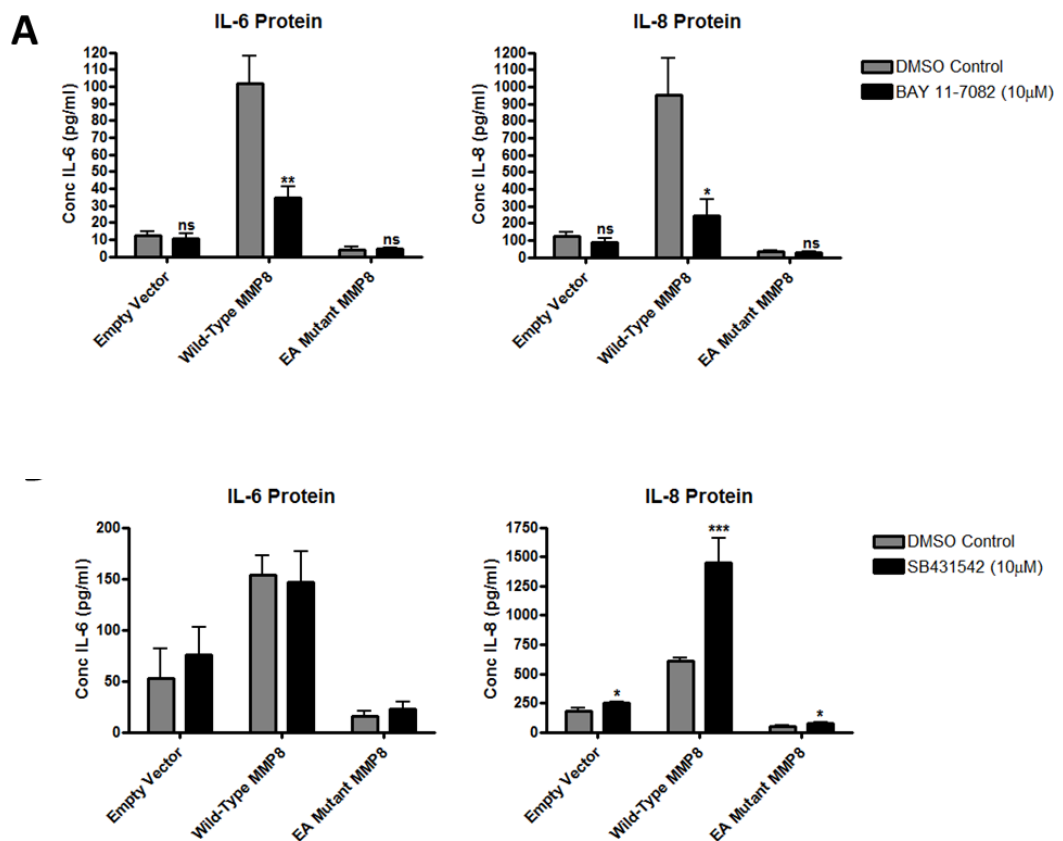


Figure 4.6: Molecular pathway inhibition in stably transfected MDA-MB-231 cells. A) IL-6 and IL-8 secreted protein levels were measured by ELISA after incubation of MDA-MB-231 clonal isolates stably expressing wild-type or E198A mutant MMP8 and empty vector control cells with (A) BAY 11-7082, an NF κ B inhibitor, of (B) SB431542, a TGF β RI inhibitor, for 48 hours. n=3, mean \pm SEM, * = p < 0.05, ** = p < 0.01 *** = p < 0.001

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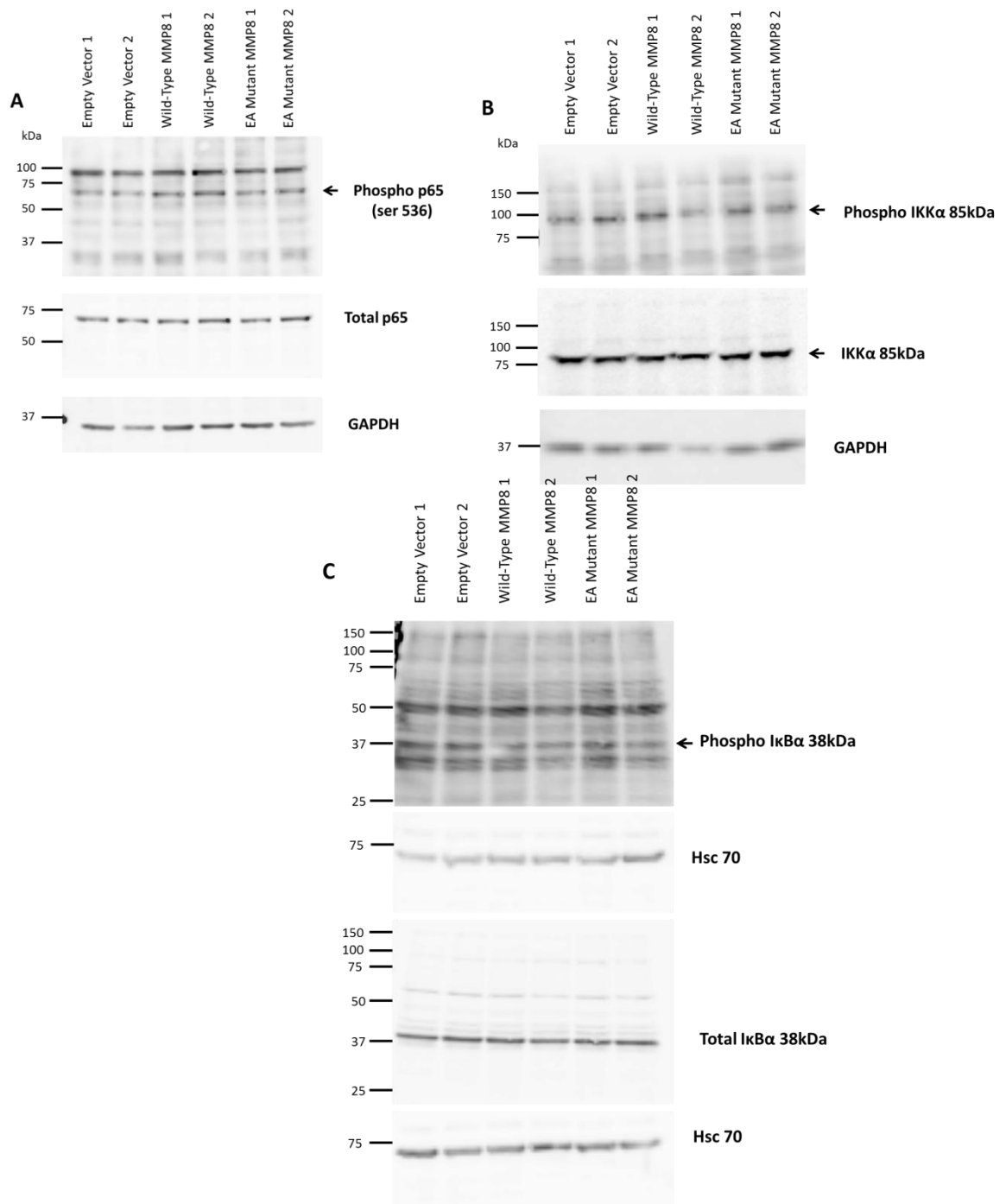


Figure 4.7: Investigation of the effects of MMP-8 on NFκB family members. Western blot analysis of (A) p65 (B), IKKα and (C) IκBα in MDA-MB-231 clonal isolates (both sets) stably expressing wild-type and E198A mutant MMP8 and an empty vector control.

4.2.2 Effects of MMP-8 on kinase phosphorylation

To dissect further the potential mechanisms through which MMP-8 is inducing IL-6 and -8 in the “long-term” stably transfected MDA-MB-231 cell lines, a commercially available array was used to determine kinase phosphorylation from cell protein samples. Proteins from MDA-MB-231 cells over-expressing both wild-type and E198A catalytically inactive MMP8, and an empty vector control (clone set 1) were applied to membranes pre-printed with antibodies to an array of phosphorylated kinases. After following the manufacturer’s instructions the membranes were imaged using HRP and the result was a membrane covered in dots of varying intensities (figure 4.8A). Pixel density was quantified using Image J and results can be seen in the heat map shown in figure 4.8B. The graph in the far right column provides a visual representation depicting the trend of the results, with those highlighted in yellow showing a potentially interesting trend discussed later (See appendix 2 for raw results).

Most interesting is the approximate 2 fold increase in phospho- p38 α MAPK in the cells over-expressing wild-type MMP-8 compared to cells expressing the vector control (EV) or EA mutant MMP8. This is accompanied by a reduction in phospho- ERK 1/2 (MAPK 42/44), MEK 1/2 and MSK 1/2. However, the changes in phospho p38 α or ERK 1/2 could not be confirmed by western blot. The MAPK pathway is involved in the activation of NF κ B, and thus IL-6 and IL-8 [195], via phosphorylation of p65 NF κ B by MSK1/2, a downstream effector of p38 [196]. Therefore, the increase in phosphorylation may indicate that p38 α is involved in the pathway downstream of MMP8 to up-regulate IL-6 and -8 expression. We actually saw a slight decrease in MSK1/2 signalling in the cells over-expressing MMP8 however, but as these signalling processes are very dynamic, and the samples used in the assay were only a ‘snapshot’ into the ever changing signalling pathways within the cells we cannot conclude that this pathway is not overactive in the wild-type MMP8 over-expressing cells. Despite this there is evidence for active p38 α signalling within the cells as levels of phosphorylated heat shock protein 27 (hsp27), a downstream target of p38 α via

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MAP kinase-activated protein kinase 2, were elevated in the cells over-expressing wild-type MMP8.

Other points to note include a slight increase in phosph-STAT3, the main downstream signal effector of IL-6, suggesting that IL-6 is signalling effectively within the cell. AMP-activated protein kinase alpha 1 (AMPK α 1) phosphorylation is slightly decreased with wild-type MMP8 expression. This protein senses changes in cellular energy, being activated by rising AMP levels and falling ATP levels [197]. It can also be activated by the tumour suppressor gene LKB1, by which this particular AMPK isoform is primarily regulated [198], thereby suggesting there may be some slight alterations in activity of this tumour suppressor in the cells over-expressing MMP8. This would need to be confirmed through other techniques however, especially considering the difference observed in AMPK α 1 in wild-type MMP8 over-expressing cells is only slight. Plus, p53 is a downstream target [199-201] and was not found to have any consistent changes in phosphorylation in this assay, although phosphorylation of p53 ser15, described as the target of AMPK [201] is reduced by a very small amount in the cells over-expressing wild-type MMP8. There was no effect on the AMPK α 2 isoform.

It can be concluded from these studies that up-regulation of IL-6 and IL-8 by wild-type MMP8 in MDA-MB-231 cells is dependent on NF κ B activity, which may be activated through the p38 MAPK pathway. Despite this, these pathways are intracellular and thus distant from MMP-8 which functions extracellularly so therefore, mass spectrometry analysis of MMP8 binding partners was carried out to uncover any of the missing mediating factors involved.

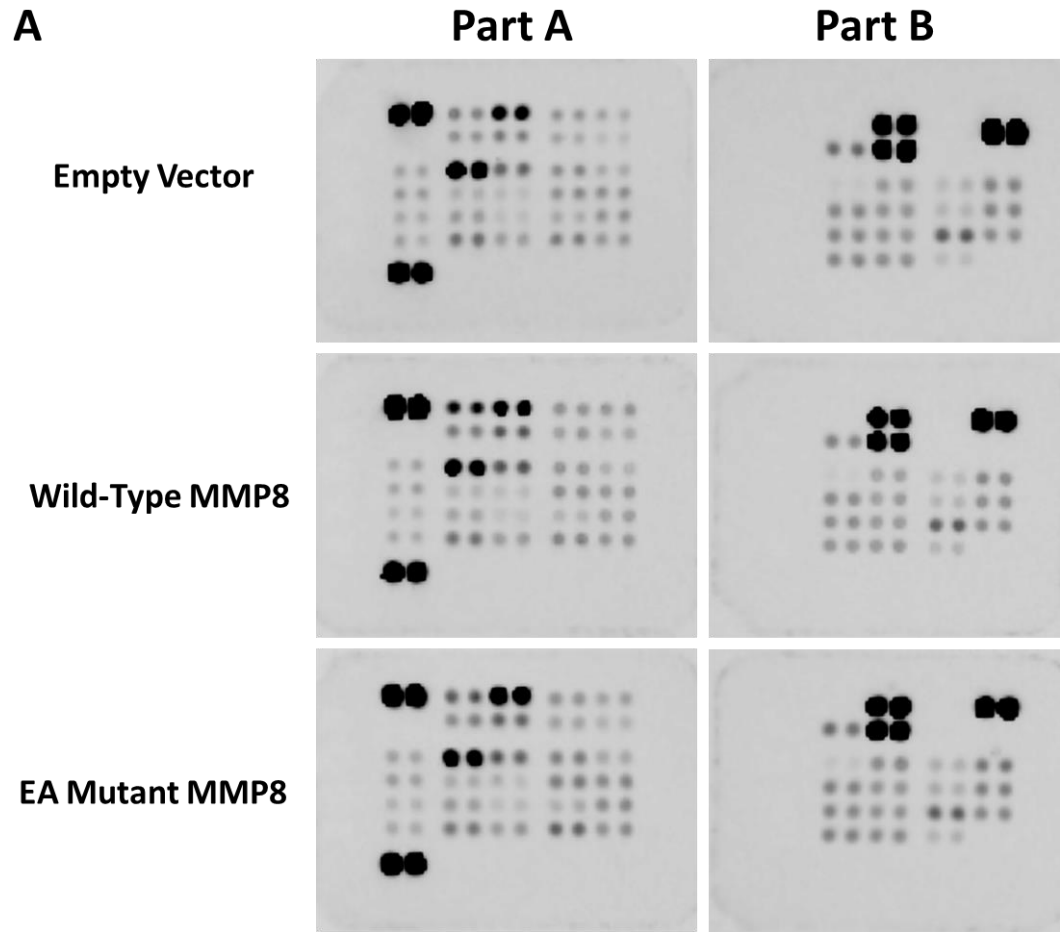


Figure 4.8: Effects of MMP-8 on kinase phosphorylation.

A) Phosphorylated kinase levels in MDA-MB-231 clonal isolates (set 1) stably transfected with wild-type and E198A mutant MMP8 and an empty vector control. Cell lysates were applied to membranes pre-printed with antibodies to an array of phosphorylated kinases. After following the manufacturer's instructions the membranes were imaged using HRP and the result was a membrane covered in dots of varying intensities. B) (next page) Phosphorylated kinase levels in MDA-MB-231 clonal isolates (set 1) stably transfected with wild-type and E198A mutant MMP8 and an empty vector control. Colours broadly relate to expression levels, expressed as pixel density on a sliding colour scale. Dark red colours relate to low values up to 1000, yellow relates to values around 2000 and dark green relates to the highest values of up to 8000. Right hand graphs give brief representations of the trends of the results, with those highlighted in yellow referred to in the text. Key: EV = 'empty vector' negative control cells, WT MMP8 = wild-type MMP8 over-expressing cells and EA MMP8 = E198A mutant MMP8 over-expressing cells.

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B

Kinase (phosphorylation site)	EV	WT MMP8	EA MMP8	
p38 α (T180/Y182)	1434.663	3529.5415	1717.37	
ERK 1/2 (T202/Y204, T185/Y187)	6223.941	4036.981	7972.5265	
JNK pan (T183/Y185, T221/Y223)	839.663	580.617	698.006	
GSK-3 α / β (S21/S9)	580.4455	510.7635	518.0315	
MEK 1/2 (S218/S222, S222/S226)	2945.103	2095.845	2990.1375	
MSK1/2 (S376/S360)	3887.31	3044.6375	4347.3445	
p53 (S392)	6941.32	7001.32	6738.456	
p53 (S46)	7286.284	6183.0165	5193.7235	
p53 (S15)	3866.088	3228.0775	3460.1025	
p27 (T189)	1562.906	1232.4555	940.5665	
p27 (T175)	1829.941	1402.749	1924.4915	
AMPK α 1 (T174)	1702.396	1180.249	1747.37	
AMPK α 2 (T172)	703.299	781.506	958.6275	
TOR (S2448)	429.2025	484.1775	522.9455	
CREB (S133)	7544.416	5543.4915	3653.3955	
HSP27 (S78/S82)	1284.396	2151.956	1607.749	
Akt (S473)	946.8345	768.067	1134.3095	
Akt (T308)	1449.199	1073.6025	1221.3095	
β -Catenin	313.228	335.1675	446.7635	
p70 S6 Kinase (T389)	90.364	100.657	392.849	
p70 S6 Kinase (T421/S424)	4605.052	4988.052	5349.2945	
p70 S6 Kinase (T229)	2377.81	3608.138	3164.32	
Paxillin (Y118)	5614.355	4038.163	2819.92	
Src (Y419)	3145.992	1746.663	1513.431	
Lyn (Y397)	991.213	776.0315	587.6525	
Lck (Y394)	960.749	713.274	641.6525	
PLC γ -1 (Y783)	6096.477	4188.9305	4617.784	
Fyn (Y420)	2682.345	2661.7595	1767.663	
Yes (Y426)	3811.077	4443.3445	3186.284	
Fgr (Y412)	973.9205	1209.42	1276.6025	
c-Jun (S63)	4838.87	4664.284	5744.5875	
Pyk2 (Y402)	2062.31	2966.406	2840.577	
Hck (Y411)	1531.456	1435.456	1413.724	
Chk-2 (T68)	5303.588	4189.87	3851.906	
FAK (Y397)	2190.931	1912.42	2380.4305	
RSK 1/2/3 (S380)	4404.588	3190.077	3361.138	
RSK 1/2 (S221/S227)	2076.163	2073.8955	2387.37	
eNOS (S1177)	861.213	1880.895	1201.456	
STAT1 (Y701)	5415.941	5204.113	5257.441	
STAT2 (Y689)	4912.26	3171.784	3065.4055	
STAT3 (S727/Y705)	1946.603	2193.542	1845.8955	
STAT4 (Y693)	4978.795	4570.87	4483.1735	
STAT5a (Y699)	4523.406	2602.6375	2896.502	
STAT5b (Y699)	4637.76	4305.8095	4567.9305	
STAT5a/b (Y699)	2610.527	1765.3595	2561.6375	
STAT6 (Y641)	4210.174	3033.8955	4924.2595	

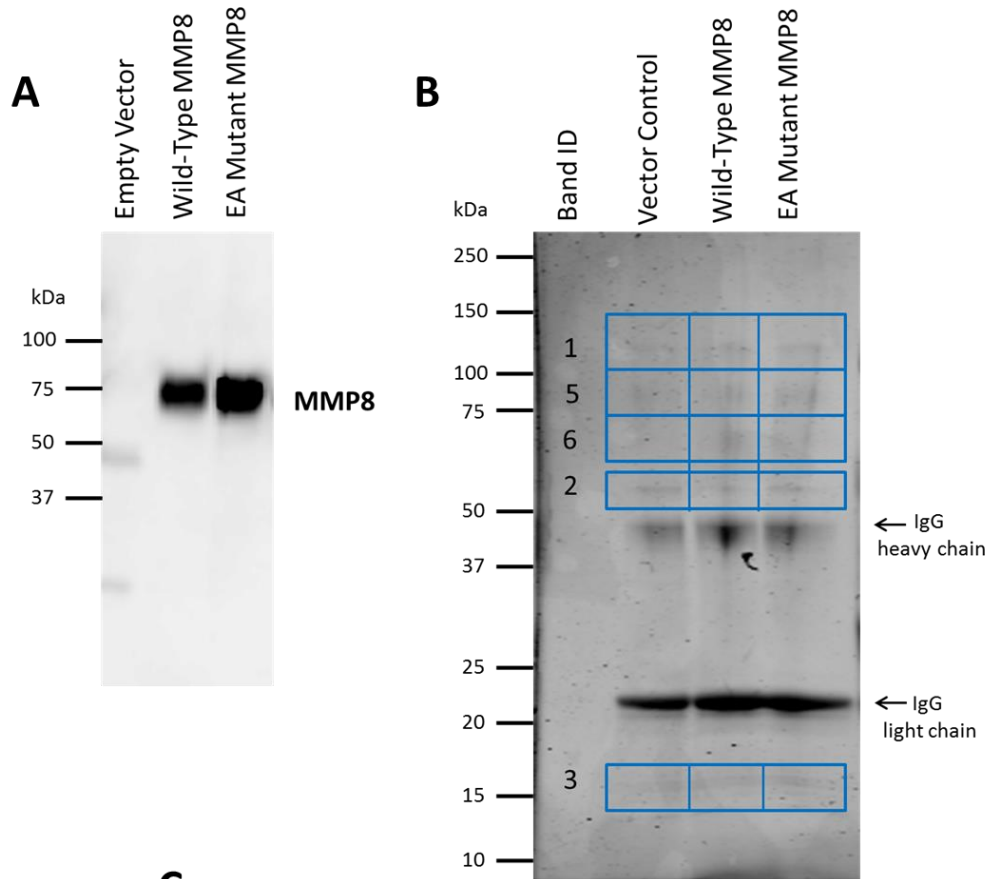
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4.2.3 Analysis of MMP8 binding proteins by mass spectrometry

To determine whether MMP8 binds to any of the mediating proteins involved in the up-regulation of IL-6 and IL-8, MMP-8 was immunoprecipitated out of serum free cell culture media conditioned by the engineered MDA-MB-231 cells expressing the control vector, wild-type and EA mutant MMP8. Serum free culture media was used as serum contains MMP-8 which would contaminate the results. The immunoprecipitation reaction was separated on a gradient gel, proteins stained and sections sent for analysis by mass spectrometry (figure 4.9B). Sections were chosen where visible bands were present. Figure 4.9A shows that both wild-type and E198A mutant MMP-8 were successfully immunoprecipitated out of the cell culture media by probing a blot that was prepared with a small portion of the immunoprecipitates. The results obtained were expressed as a probability of a protein being present. Hundreds of proteins gave a positive identification, but many of these were caused by the gel being contaminated by keratins for example, and these proteins were present in all samples. However, focus was centred on proteins that were either present in immunoprecipitates of both wild-type and E198A mutant MMP-8, present in the E198A mutant MMP-8 precipitate only or in the wild-type MMP-8 precipitate only. Binding to the E198A mutant MMP-8 only indicates these proteins are maybe MMP-8 substrates but are bound to the inactive enzyme as they have not been cleaved. In contrast, proteins that are bound to wild-type MMP-8 only may be cleavage products. There were some discrepancies with regards to the expected molecular weights of the proteins and the area of the gel from which the sample was extracted. However, the facility managers of the mass spectrometry services where the samples were analysed explained that some very large proteins do not run through the gel uniformly and could therefore appear at an unexpected weight and that the molecular weight marker is not 100% accurate. Also, some of the proteins may have undergone degradation so may be seen at multiple locations throughout the gel. Figures 4.9 C and D show the proteins identified that were found in the samples generated from the cells over-expressing both wild-type and EA mutant MMP8 or one of the other and thus indicating that MMP8 was involved. Figure 4.9C shows the proteins that

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are of interest with regards to the induction of IL-6 and -8 by wild-type MMP8 over-expressing MDA-MB-231 cells (S100-A8, Annexin A1 and Hornerin) and as follows is a brief introduction and description as to why they are potentially interesting.



C

Protein	MW	Empty Vector	Wild-Type MMP8	EA Mutant MMP8	Band
MMP-8	70kDa	0%	76%	100%	2
S100-A8	10kDa	0%	99%	98%	3
Annexin A1	31kDa	0%	99%	82%	5
		0%	79%	0%	6
Hornerin	245kDa	0%	100%	0%	2
		0%	85%	0%	3

Probability

- >95%
- 80-94%
- 50-79%
- 20-49%
- 0%

D

Protein	MW	Empty Vector	Wild-Type MMP8	EA Mutant MMP8	Band
Major vault protein	100kDa	0%	24%	100%	1
Calnexin	72kDa	0%	100%	73%	6
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	69kDa	0%	100%	81%	6
ATPase family AAA domain-containing protein 3A	58kDa	0%	100%	67%	6
Heat Shock 70 kDa Protein 6	71kDa	0%	100%	0%	6
threonyl-tRNA synthetase (TARS)	82kDa	0%	100%	0%	6

Protein	MW	Empty Vector	Wild-Type MMP8	EA Mutant MMP8	Band
DEAD box polypeptide 27	87kDa	0%	0%	99%	6
Glycine-tRNA ligase	83kDa	0%	100%	0%	6
Elongation factor 1-gamma	56kDa	0%	99%	0%	1
		0%	100%	0%	5
		0%	43%	0%	6
X-ray repair cross-complementing protein 5	83kDa	0%	100%	81%	6
Eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa (eIF3 B)	89kDa	0%	51%	76%	1
		0	100%	82%	5

Probability

>95%
80-94%
50-79%
20-49%
0%

Figure 4.9: Mass Spectrometry analysis of MMP-8 binding partners. A) Western blot analysis of MMP8 from the immunoprecipitation of MMP-8 from serum free conditioned media from MDA-MB-231 clonal isolates (set 1) expressing wild-type MMP8, E198A Mutant MMP8 and a control 'empty vector' B) Sections of the gel run from the immunoprecipitation analysed by mass spectrometry. Blue boxes indicate the sections analysed and the Row ID relates to the entire row, which was analysed in 3 separate sections, separated by genotype of the cells. C) Confirmation of protein presence by probability that the protein has been identified correctly of MMP-8 binding proteins considered of interest to this work. D) Confirmation of protein presence by probability that the protein has been identified correctly of other MMP-8 binding proteins that bind MMP8.

S100A8

There are two main reasons for investigating S100A8 further; its role in inflammation and its role in cancer.

S100A8, also known as S100 calcium binding protein A8, calgranulin A or MRP-8 is a pro-inflammatory calcium binding protein. It is mainly expressed by myeloid cells, but is found at very high levels in neutrophils, comprising 45% of the total cytosolic proteins [202] and is released during an inflammatory response. This has been demonstrated in both patients with inflammatory conditions such as periodontitis in heterodimeric form with S100A9 [203] and as a monomer [204], and *in vitro* [205, 206]. Both monomeric S100A8 and heterodimeric S100A8 (S100A8/S100A9) are chemoattractive to neutrophils *in vitro* [207] although S100A8 is sensitive to oxidation, with its chemoattractive abilities diminished under these conditions [208]. Extracellular S100A8 can also activate MAP kinase and NFκB signalling pathways, and thus release of pro-inflammatory cytokines, such as IL-8 [209] via binding to the receptor for advanced glycation end products (RAGE) [210-212]. Interestingly, this RAGE dependent activation of NFκB has been shown to be sustained long-term, resulting in a prolonged cellular change which can over-ride endogenous feedback controls [213]. Such downstream signalling effects have also been demonstrated through binding of S100A8 to the Toll-like receptor 4 (TLR4) [209].

S100A8 has been shown to be expressed in a variety of cancers such as gastric cancers [214], colorectal [215], pancreatic [216], prostate (the S100A8 ligand RAGE was also upregulated in this study) [217], bladder cancer [218-220] and oesophageal squamous cell carcinoma [221]. It is down-regulated in oesophageal squamous cell carcinoma but up-regulated in the others. Most importantly, and most relevant to this thesis, S100A8 has been found to be up-regulated in breast cancer [222-224]. Cancemi *et al.*, 2010 carried out a large-scale proteomic analysis of the S100 proteins in breast cancer tissues, comparing cancer tissue with non-cancerous adjacent tissue. S100A8 was found to be expressed only in the cancerous tissue, in 71% of the 100 patient samples

studied. Furthermore, S100A8 was present in the tumours at a significantly higher level (1.5x) in patients that had developed metastasis within 3 years after the initial study compared to patients that had remained disease free [222], suggesting that S100A8 expression is associated with more aggressive tumours. Interestingly, despite the wealth of literature suggesting that S100A8 and -A9 act in conjunction with each other, S100A9 was not identified in the breast tumour samples studied. In addition to this evidence, Mukhtar *et al.*, (2012) found positive associations between high levels of S100A8/S100A9 immunohistological staining in breast tumour samples and hormone receptor status (more S100A8/S100A9 in hormone receptor negative patients), higher grade tumours, with the majority of samples stained to a high level being grade 3 tumours, and age. The patients with higher levels of staining were younger than those with low level staining [223].

In vitro studies have given further evidence for pro-tumourigenic actions of S100A8. Moon *et al.*, (2008) carried out a genome microarray on H-Ras transformed MCF10A breast cells and found that compared to control cells; H-Ras transformed cells expressed S100A8 and A9. These cells were more invasive than control cells, but this effect was reduced dramatically on siRNA knockdown of both S100 proteins independently. Incidentally, S100A8 (and A9) can increase invasiveness irrespective of the presence of a Ras mutation, as over-expression of both S100 proteins (individually or in conjunction) in the MCF10A cells increased invasion significantly [225]. This effect was shown to be calcium dependent, as its presence is required for protein stability, and also on p38 MAPK and ERK signalling pathways [225]. S100A8 appears to act within a feedback loop with MAPK pathway members in that inhibition of p38 MAPK and ERK reduced S100A8 protein expression, and S100A8 siRNA reduced levels of phospho-p38 and phospho-ERK. Similar results have also been shown in LNCaP prostate cancer cells whereby cells treated with S100A8 and S100A9 proteins showed a dose-dependent increase in p38 MAPK and ERK phosphorylation. This was coupled to increased activation of NFκB, a well-known target of the MAPK pathway, and in increase in scratch wound closure [210]. Exogenous addition of S100A8 purified from neutrophils has also been shown to increase growth of

MCF-7 and MDA-MB-231 breast cancer cells at low concentrations, through binding to RAGE on the cell surface [212]. This effect was likely propagated through an increase in phosphotyrosylated p38 MAPK and ERK, as was evidenced within 30 minutes of S100A8 addition, and NFκB activation. Finally S100A7, S100A8 and S100A9 have been found to be repressed by the breast cancer tumour suppressor gene *BRCA1*, adding another piece of evidence for S100A8 acting as a tumour promoter [226]. Incidentally, a study by Rhee *et al.*, (2008), using an MCF10A breast cell model of tumourigenesis saw that S100A9 mRNA and protein expression decreased at the stages of the model representing benign proliferation and ductal carcinoma *in situ* (DCIS), but then increased in cells representing the invasive stages. Also at these early stages over-expression of S100A8 in cells representing the early stages of breast tumourigenesis actually inhibited cell growth [224]. This suggests that S100A8 is only involved in advanced cancers, but that forcing expression at early stages of tumourigenesis, at which stages it is not normally expressed may possibly be beneficial. This however is concluded from an *in vitro* model and, as human breast cancer studies show that tumour expression of S100A8 correlates with negative effects and poor prognosis care needs to be taken when interpreting such findings.

Here, S100A8 is pulled down with both wild-type and EA mutant MMP8 in band 3 which relates to a section from about 8-15kDa, so as S100A8 is about 10kDa it is detected at the correct molecular weight. Most importantly there has been a paper that proposed an anti-inflammatory role for MMP-8 showing that S100A8 (and S100A9) were increased in the lungs of *mmp8* deficient mice after LPS stimulation in an endotoxemia mouse model [91]. Another paper using a rat model to look at ischemia-reperfusion induced acute lung injury found that both *Mmp8* and *S100a8* were the 2 most up-regulated genes in a microarray screen (*Mmp9* and *S100a9* were also highly up-regulated). Tissue protein IL-6 levels were also highly up-regulated 60 mins after ischemia, although RNA levels were unchanged [227].

Consequently, due to the role of S100A8 in inflammation, breast cancer and its production from the same location as MMP8 it was deemed worthy of further investigation. Unfortunately the antibody available to validate this interaction by western blot did not work.

Annexin A1

Annexin A1 (also known as lipocortin 1) is a membrane binding protein that is regulated by calcium and has roles in trafficking of proteins in and out of the cell [228]. Some annexins however can be secreted, although through unknown mechanisms, with the majority of research highlighting a potential extracellular role for Annexin A1. For example, Christmas *et al.*, (1991) reported that despite Annexin A1 not having a 'traditional' signal sequence, it is secreted from epithelial cells within the prostate duct into the seminal fluid, whereas Annexin A4 which is expressed in the same cells is not secreted [229]. Annexin A1 is found at high levels in the cytoplasm of neutrophils [230-232], and one group have shown that it is stored in gelatinase granules in the cytoplasm of the resting neutrophil. On binding of the neutrophil to an endothelial barrier Annexin A1 is mobilised to the cell surface, whereby some is released. Therefore, the group postulate that annexin A1 is exocytosed following degranulation of the gelatinase granules [232]. Whether a similar mechanism occurs in prostate cells is unknown. Release of Annexin A1 from neutrophils *in vitro* has also been reported [233]. MMP-8 is stored within the specific granules of neutrophils [30-32] associated with lactoferrin [33], the majority of which also contain gelatinase [234] so it is plausible that MMP-8 and Annexin A1 are secreted together from the same granules on neutrophil activation. However there is a conundrum that the majority of the evidence points towards a pro-inflammatory role for MMP8, and Annexin A1 (and its active N-terminal Ac2-16 peptide), exerts anti-inflammatory roles through the prevention of neutrophil extravasation into the blood stream [235] [236, 237] [238] [239]. In contrast, there has been a report of a novel role for an Annexin A1 C-terminal cleavage product on activating ERK in endothelial cells and as a consequence enhancing neutrophil extravasation [240] but the majority of evidence points towards an anti-inflammatory function as in addition to inhibition of neutrophil

extravasation, Annexin A1 has been implicated in the promotion of neutrophil apoptosis [241] and phagocytosis of these neutrophils [242-244] and more recently has been implicated in the clearance of senescent neutrophils in the bone marrow by bone marrow-resident macrophages, thus preventing chronic inflammation.

Annexin A1 has been studied in many cancers such as lung whereby expression is seen at a much higher level in cancer tissue than normal lung tissue, with expression correlating with higher tumour grade, metastasis and reduced survival [245]. Similar correlations have also been found in gastrointestinal cancer [246, 247]. Conversely, loss of Annexin A1 expression has been reported in head and neck and prostate cancers [248, 249], whereby down-regulation of Annexin A1 was associated with poor patient outcome.

With regards to breast cancer conflicting reports have been published. Within the normal breast Annexin A1 has been found to be expressed in the myoepithelial cells [250-252] and also in the luminal epithelial cells [251, 252]. Some studies show an involvement of Annexin A1 in the early stages of breast cancer development [250] whereas it has also been found to be down-regulated in breast tumour samples compared to healthy tissue [251] [252].

In addition, a significant positive correlation has been shown between basal-like breast cancers and Annexin A1 expression, and a trend was observed between Annexin A1 protein expression and recurrence [252]. This can be further supported by the expression of Annexin A1 in breast cancer cell lines, with it being highly expressed in basal-like cell lines such as MDA-MB-231 and expressed at very low levels in luminal-line cell lines such as MCF7 and T47D [252, 253]. Khau *et al.*, (2011) even showed that Annexin A1 could be used to distinguish between basal and luminal breast cancer cell lines [254]. In contrast to MMP8, evidence points to Annexin A1 having a pro-metastatic role in breast cancer progression through its ability to constitutively activate NFκB. Constitutive NFκB expression is observed in solid tumours and also aggressive breast cancer cells lines and *in vitro* it has been shown that Annexin A1 is able to constitutively activate NFκB in MCF-7 and MDA-MB-231 breast cancer cells

through binding to the IKK complex, resulting in phosphorylation of I κ B α and activation of NF κ B [253] and driving the metastatic spread of the cancer cells [255]. The overall conclusion from studies into Annexin A1 in breast cancer suggests that its expression promotes metastasis.

Despite this contradictory evidence it is conclusive that Annexin A1 is expressed in myoepithelial cells in normal breast tissue, which is where MMP8 is expressed. This, plus Annexin A1 and MMP8 being expressed in the granules of neutrophil adds to the evidence of there being a link between the two proteins. In the mass spectrometry analysis Annexin A1 was found bound to wild-type MMP8, albeit in a segment of the gel of a higher molecular weight than expected for the protein. Despite validating a working antibody to confirm these data by western blot Annexin A1 could not be detected in an immunoprecipitation reaction, even when a large reaction volume was used. The mass spectrometry technique used was very sensitive and could detect very small amounts of protein so perhaps there was not enough protein bound to MMP8 to be detected by western blot.

Hornerin

Hornerin is another S100 protein, also known as S100A16 and S100A18 due to its calcium binding properties and homology to other S100 proteins, but studied to a lesser extent than other S100 proteins. Originally in 2001 it was identified in the mouse epidermis, and was found to be cleaved during epidermal differentiation [256], and most subsequent studies have focussed in the role of hornerin in the skin [257-260], under both normal and disease conditions. It is only in the last couple of years that hornerin has been examined in any other cells types or conditions, and the only condition studied has been breast cancer [261, 262]. Fleming *et al.*, (2010) carried out mass spectrometry on the extracellular matrix of whole breast tissue of African-American and Caucasian-American women in order to determine if the reason behind African-American women getting a higher rate of aggressive breast cancers was due to protein differences in the breast microenvironment between the two groups of women. A significant difference in hornerin expression between the two populations

was discovered, with expression being much higher in the ECM of breast tissue from Caucasian-American women [261] potentially indicating a tumour-suppressive role. This potential role can be heightened by evidence from their recent paper whereby hornerin was shown to be expressed more highly in less aggressive, early stage (TMN grade 1), lymph node negative tumours [262]. However, using the same MCF10A *in vitro* model of tumour progression employed by Rhee *et al.*, (2008) when examining the role of S100A8 [224], hornerin mRNA and protein expression increased with increasing invasiveness of the cells, potentially indicating a pro-tumourigenic role. Hornerin is expressed by primary breast fibroblasts and primary breast epithelial cells and also in breast cancer cell lines such as MCF10As, MDA-MB-231s, T47Ds and MCF7s, where it is seen to be heavily cleaved into many fragments ranging from 260-50kDa [262]. When Fleming *et al.*, 2012 examined hornerin expression using the MCF10A tumourigenesis model total hornerin mRNA increased, as did protein levels of the intact 260kDa protein. However, in these cells a 100kDa fragment is observed, but only in the non-invasive and DCIS-like cells. Perhaps this could indicate that this smaller fragment is exerting the anti-tumourigenic effects of hornerin, or alternatively this cleavage renders the 260kDa protein inactive, so the more cleavage that occurs, the less intact protein there is to exert pro-tumourigenic effects. However, as this is an *in vitro* model on one cell type so one cannot draw firm conclusions.

Interestingly in the mass spectrometry data generated from MDA-MB-231 cells hornerin was only detected immunoprecipitated to wild-type MMP8 and at approximate molecular weights of 50kDa and 15kDa. Products of up to 250kDa were not examined by the mass spec so intact protein would not have been detected. Fleming *et al.*, (2012) detect many hornerin cleavage products between 260kDa and 50kDa which we do not see. However, they examined cell lysates whereas our experiments were looking at secreted hornerin, which may account for the smaller products we observed. Hornerin is likely secreted from breast cancer cells in exosomes [262].

As hornerin has only been recently associated with breast cancer it would have been exciting to follow this up. Hornerin was only found bound to wild-type MMP8 and at two molecular weights possibly suggesting that it had been cleaved by MMP8. Unfortunately there was no available antibody to validate this result as the antibody used by Fleming *et al.*, (2012) [262] had been discontinued.

4.3 Conclusions and Discussion

Through looking at a panel of inflammatory mediators (IL-8, IL-1 β , IL-10, TNF, IL-6, and IL-12p70) secreted by MCF-7 cells transiently transfected with MMP8 and control constructs, secreted IL-6 and IL-8 levels were increased in cells transfected with wild-type MMP8. This effect did not occur in cells transfected with E198A mutant MMP8 so is dependent on catalytic activity. This effect was more evident in MDA-MB-231 cells engineered to stably over-express MMP8 and could be observed in both clonal isolates and the pooled stably transfected cells from which the clones were generated. Two groups have shown that MMP8 can proteolytically cleave, and thus activate LIX, the mouse orthologue of human IL-8, given its greater potency as a neutrophil chemoattractant [64, 80], which is a crucial event in the inflammatory cascade post infection [80]. Additionally, another group has shown that LIX protein levels are higher in the *Mmp8* wild-type compared to the *Mmp8* knockout mouse, which was postulated to be due to liberation of LIX from ECM stores by MMP8 [49]. *In vitro* MMP-8 has been shown to cleave human IL-8, resulting in the potent chemoattractive fragment [80]. This research has formed a solid functional link between MMP-8 and IL-8 *in vivo*, not only within the realms of resolving an inflammatory response, but also within cancer cells themselves.

Although it is unknown whether MMP-8 cleaves IL-8 in this work, the data show that MMP-8 can actually induce IL-8 expression. The increase in IL-8 in the culture media as a result of wild-type MMP-8 over-expression was not due to liberation of IL-8 that was already present, but due to an increase in expression level. This was evidenced from an increase in IL-8 mRNA within the cells over-

expressing wild-type MMP8. Interestingly MCF-7 cells do not produce any endogenous IL-8 as expression is suppressed by the estrogen receptor expressed in these cells [180, 181], so MMP-8 can actually induce cells to express IL-8, thus over-riding this suppression. This is plausible as studies have shown the IL-8 promoter is still functional [180, 181], but the NF κ B binding site is hypoacetylated [182]. Therefore, the data obtained in this thesis is credible due to the already established action of MMP-8 on IL-8, but it adds greater depth to the literature as MMP-8 can actually induce IL-8 expression too. The induction of IL-6 in cells expressing wild-type MMP-8 is new and was unexpected. So far there has been no literature to suggest that MMP8 has any effect on IL-6. Some data has been published to suggest that IL-6 has an effect on MMP expression (MMP1 [263], MMP8 [39] and 13 [39, 264], MMP2 and 9 [265], MMPs 2,3,9 and 13 [266], and MMP10 [267] but not vice versa. However, in the last year MMP-2 has been shown to regulate IL-6/STAT3 survival signalling in glioma indirectly, through formation of a complex with α 5 β 1 integrin [268].

To conclude so far, a new phenomenon has been discovered involving the ability of MMP8, through its catalytic activity, to induce the expression of IL-6 and IL-8 in breast carcinoma cells, but not in normal breast cells (at least in the one cell line examined). This phenomenon occurs as part of a self-reinforcing loop, with IL-8 having a role in promotion of IL-6 expression levels, and IL-6 being able to induce MMP8 expression. This is the second report of IL-6 having an effect on MMP8 expression, as previously Wahlgren *et al.*, (2001) showed that addition of recombinant IL-6 to RPMI 8226 myeloma cells increased MMP8 (and MMP13) protein and RNA levels by up to nine-fold [39]. Only a 20% increase in MMP8 RNA levels were observed on addition of the same concentration of rhIL-6 used by Wahlgren *et al.*, (2013) to naïve MDA-MB-231 cells, but this could be attributed to differences in cell line. The cytokine network is complex and dynamic [269], and a similar loop has been described by Lederle *et al.*, (2011) where IL-6 is involved in a cytokine network which include the up-regulation of MMP1 [130]. Unfortunately we do not know whether this signalling pathway is functionally active, as we do not know if IL-8 is proteolytically processed to its super-active form by MMP8 due to unavailability of appropriate techniques.

Also, despite the effect of IL-8 on IL-6 expression, IL-8 must not be signalling through its cognate receptors CXCR1 and CXCR2 as they are not expressed in these cells (as determined by Taqman qRT-PCR). There is evidence that IL-8 can activate these receptors [270] in endothelial cells, but this is not observed, probably due to lack of expression in the first place. We do know from the slight increase in phosphorylated STAT3 in cells over-expressing wild-type MMP8 that IL-6 is probably signalling (at least via STAT3) though.

A big disadvantage of using the *in vitro* model of stably transfected breast cancer cells in isolation is that there is only one cell type so it is not physiologically relevant when looking events on the tumour microenvironment. IL-8 is not signalling through its receptors as the cells do not express them. However, if these engineered cells were cultured in a multi-cellular environment, such as the organotypic breast cancer model pioneered by Holliday *et al.*, (2009) where luminal epithelial cells, myoepithelial cells and fibroblasts were grown together in a 3D collagen gel [271], much more could be discovered about the functionality of the up-regulated signalling pathways. This would be enhanced further by the addition of immune cells to the mix, although this would add massive complications to the assay due to the short life of the immune cells. Another option would be to generate MMP-8 expressing murine breast cells and inject them into mice. This would give the benefit of a multi-cellular environment, plus the influence of an immune system and would therefore give further insight into whether the same loop occurs in mice, both in the inflammatory system and within a cancer environment.

One of the published key regulators of IL-6 and -8 is NF κ B [180, 269], and this has been confirmed in this work. Inhibition of NF κ B with addition of an inhibitor reduced IL-6 and -8 protein levels in cells over-expressing wild-type MMP8 to near base-line levels, although not totally, suggesting that other factors are involved that would need inhibiting in conjunction with NF κ B to see a total reduction. We have no evidence of how/if MMP8 is affecting NF κ B, as no changes have been found in either phosphorylated or total levels of either p65 NF κ B or two regulatory molecules, IKK α or I κ B α . There are other aspects of the pathway to be looked at such as p50 NF κ B

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and IKK β and γ , along with changes in promoter binding of NF κ B which could be determined by carrying out an Electrophoretic Mobility Shift Assay (EMSA). As NF κ B is intracellular and MMP-8 is extracellular further investigation to determine mediators between them is required. Literature investigation of intracellular pathways up-stream of NF κ B showed that IL-8 can be induced via the Ras pathway resulting in increase in NF κ B transcriptional activity [153]. However, conclusions could not be made as inhibition of some members of this pathway, specifically MAPK inhibitors, were toxic to the cells so cell death masked any potential effects. Therefore, the array to look at levels of phosphorylated kinases within the cells was aimed to define the pathway responsible for IL-6 and IL-8 induction further. Interestingly phospho-p38 MAPK emerged as being up-regulated 3 fold in cells over-expressing wild-type MMP8 compared to cells expressing the control vector and also E198A mutant MMP8. Two inhibitors against p38 were used in the inhibitor screen but they adversely affected the cells so it was impossible to determine if there were any real effects on IL-6 and -8, so it is still possible that the p38 MAPK pathway is somehow activated by MMP8 which in turn activates NF κ B and IL-6 and IL-8 expression.

The mass spectrometry analysis of MMP8 binding partners aimed to identify further members that could be involved. Two proteins that have been shown to be involved in both breast cancer and inflammation and that draw some parallels to MMP8 were identified: S100A8 and Annexin A1. A third protein, Hornerin, was also identified and considered of interest due to its recently found role in breast cancer. S100A8 was definitely worthy of consideration as it has been shown that levels of S100A8 are increased in MMP8 deficient mice [91]. Perhaps S100A8 is bound to, and sequestered by MMP8, so higher levels are seen in the knockout mouse as a result of this not occurring. Additionally, both S100A8 and Annexin A1, like MMP8, are predominantly produced in the neutrophils [30, 31, 202, 230-232], and in the normal breast both Annexin A1 and MMP8 are expressed in the myoepithelial cells [250-252] and have published roles in inflammation. The S100A8/A9 heterodimer has been shown to induce expression of pro-inflammatory cytokines through activation of p38 MAPK and NF κ B [210, 272]. In contrast, Annexin A1 has been found to negatively regulate p38 [273] and NF κ B [274, 275], although reports exist to the contrary [253, 255]. This experiment could not be confirmed by western blot as a

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working antibody to S100A8 could not be found and the antibody to Hornerin was no longer available. A working Annexin A1 antibody was found when tested on total cell lysate, but Annexin A1 was not detected by western blot after MMP8 immunoprecipitation. This could mean that it was secreted at such low levels into the media that could only be detected by the sensitive mass spectrometer but not by an antibody for western blot.

Therefore, although some potential mediators of MMP8 involved in the up-regulation of IL-6 and -8 were identified they could not be validated so cannot be confirmed to be involved, although they are still worthy of follow-up. In addition, it would be wise to expand the search for MMP8 effectors especially as the up-regulation of IL-6 and IL-8 with MMP8 over-expression is dependent on its catalytic activity. Due to this it would be worthwhile in the future to carry out a more comprehensive mass spectrometry analysis of the total cell culture media from cells over-expressing both wild-type and the catalytically inactive E198A mutant MMP8 to determine protein cleavage products generated by wild-type MMP8. Intact proteins detected in the culture media of cells over-expressing E198A MMP8, the catalytically inactive form whereby the proteins should not be cleaved, should be used as a comparison. Techniques such as Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC) [276] and Isobaric Tag for Relative and Absolute Quantification (iTRAQ) [277] could be used to quantify changes in both protein levels and protein cleavage. These techniques utilise Liquid Chromatography-MS/MS, (LC-MS/MS) a tandem MS system whereby proteins separated by LC are passed through the MS system twice: quantified in terms of a ratio between the abundance of a particular protein between the conditions in the first run (MS1) and being further fragmented on the second run (MS2) and identified. With SILAC cells are labelled by growing them in the presence of either a heavy or a light isotope of an amino acid in the culture media, which is incorporated into its proteins as they are expressed. The protein source is then identified by the isotope [276]. With iTRAQ the proteins are chemically labelled directly with an "iTRAQ tag" consisting of an *N*-hydroxysuccinimide (NHS) group linked to an isobaric tag. These are distinguished by molecular weight. Eight tags are available with different molecular weights which allows for multi-plexing as

many as eight different conditions that can be compared in one reaction [277]. These methods could also be used to determine whether MMP8 is cleaving IL-8, as unless MMP8 binds to IL-8 it would not have been detected in the mass spectrometry screen carried out in this thesis.

To conclude this chapter it has been shown that the catalytic activity of MMP8 induces and up-regulates the expression of two inflammatory mediators, IL-6, a predominantly pro-inflammatory cytokine, and IL-8, a pro-inflammatory chemokine, in breast cancer cells, both on acute exposure (transient transfection) and on “long-term” exposure (stable transfection) This is most likely occurring through the p38 MAPK- NF κ B pathway although further validation is required to determine exactly how. In addition, three proteins have been identified that bind to MMP8 in the cell culture media and that have a role in inflammation. These may provide the missing link between MMP8 and the MAPK and NF κ B pathways but further analysis is required for validation. The next chapter of this thesis seeks to address the reasons underpinning the up-regulation of the inflammatory mediators.

4.4 Summary

- MMP-8 induced expression of IL-6 and IL-8 in malignantly transformed mammary carcinoma cells on acute exposure (transient transfection), dependent on its catalytic activity.
- MMP-8 also induced/up-regulated expression of IL-6 and IL-8 on stable transfection, in “long-term” expressing MDA-MB-231 cells, again dependent on catalytic activity.
- In the “long-term” wild-type MMP-8 expressing MDA-MB-231 clonal isolates MMP-8 initiated a self-reinforcing loop with IL-6 and IL-8
- IL-6 and IL-8 up-regulation by wild-type MMP-8 was dependent on NF κ B activity and possibly on p38 MAPK.
- In this system IL-8 was inhibited by TGF β signalling.
- MMP-8-binding proteins (S100A8, Annexin A1 and Hornerin) were discovered by mass spectrometry that may be involved in IL-6 and IL-8 up-regulation, but they require further investigation.

Chapter 5- Further insights into the cellular effects of MMP-8

In the previous chapter it was shown that MMP-8 induced expression of IL-6 and IL-8 at both the RNA and protein level. This occurred on an acute basis as shown by transient transfection of MMP-8 into multiple cell lines, and also in long-term MMP-8-expressing stably transfected MDA-MB-231 cells. This effect was dependent on the catalytic activity of MMP-8, as no such induction was seen in cells over-expressing EA mutant MMP8. In these “long-term” stably transfected cells MMP-8, IL-6 and IL-8 were found to be part of a self-reinforcing loop whereby IL-8 was found to be partly responsible for IL-6 induction, and IL-6 was found to be capable of inducing endogenous MMP8. However, as introduced in chapter 3, MMP-8-expressing stably transfected cell lines were difficult to generate, with wild-type MMP-8 expression being routinely switched off. These “long-term” MMP-8 stably expressing MDA-MB-231 cells used in this thesis and characterised in chapters 3 and 4 are therefore rare clones that emerged that could maintain MMP-8 expression for months. This chapter aims to determine why MMP-8 stable clones were so difficult to generate and why wild-type MMP-8 is switched off by the cells in the majority of stable transfectants. The behaviour of these “long-term” MMP-8 expressing cells *in vivo* will also be examined. Some of this content has been published (Appendix 3).

5.1 Loss of wild-type MMP-8 expression

As aforementioned in chapter 3, generation of wild-type MMP8 over-expressing MDA-MB-231 stably transfected cells was difficult. The majority of stable cell lines lost wild-type MMP8 expression within a few weeks of culture. The stable cell lines used in this thesis were those that maintained wild-type MMP8 expression for months enabling their use in multiple experiments without fear of MMP-8 expression loss mid-experiment. Figure 5.1A documents the loss of

wild-type MMP8 from pools of stably transfected MDA-MB-231 cells from 7 to 11 weeks post-transfection. Three different pools of wild-type MMP8 over-expressing pools were monitored alongside an empty vector control and a pool of cells over-expressing E198A mutant MMP8.

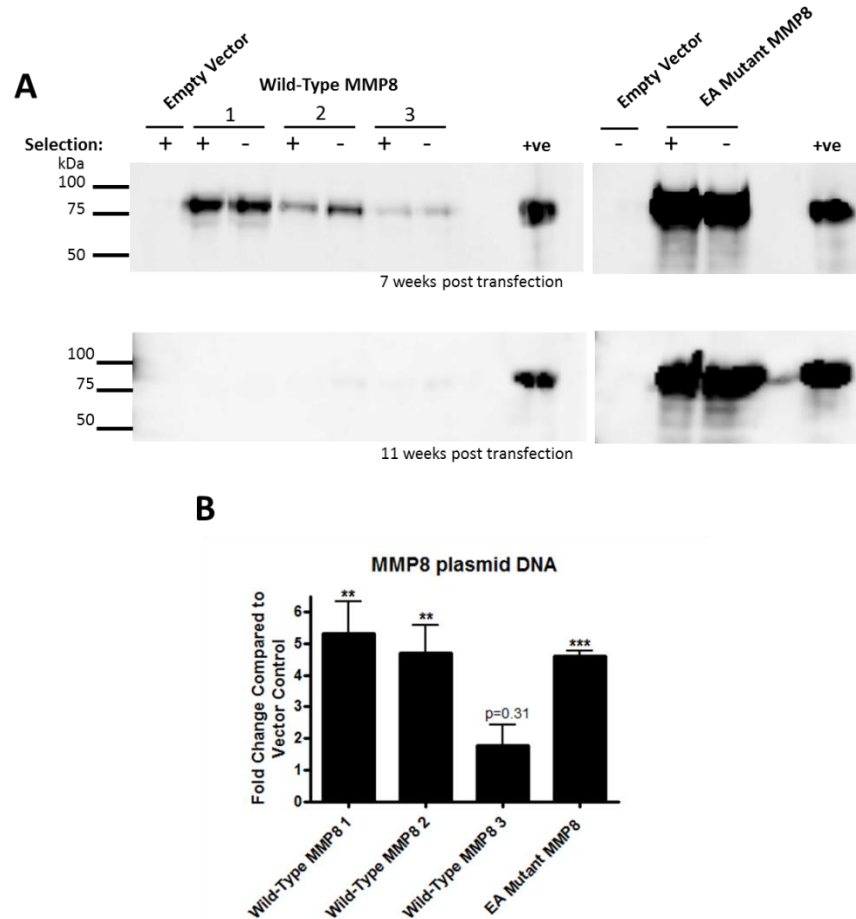


Figure 5.1: Unstable expression of wild-type MMP-8 under serial passage and sustained selection in culture. MDA-MB-231 cells were transfected with pcDNA4 wild-type MMP8, pcDNA4 E198A MMP8 and pcDNA4 vector control and maintained under zeocin selection for 6 weeks. At this point MMP-8 expressing pools of cells were divided and half maintained and passaged either under zeocin selection (+) and the other half without (-). MMP-8 expression was determined every other week by western blot. Three pools of cells expressing wild-type MMP-8 (divided into 2 at 6 weeks with half under selection and half without) and 1 pool each of cells expressing E198A Mutant MMP-8 and the ‘empty vector’ control (half of the pool under selection and half maintained without) were monitored. A) Western blot analysis of MMP-8 in 20x concentrated serum free conditioned media from pools of stably transfected cells at 7 weeks post transfection (top panel), and 11 weeks post transfection (bottom panel). B) Analysis of MMP-8 DNA by Taqman PCR on DNA isolated from the cells 11 weeks post transfection. * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$

Pools of cells expressing MMP-8 at 6 weeks post transfection were divided into two, with half being passaged and maintained under continuous zeocin selection and the other half maintained and passaged with no selection reagent. Loss of wild-type MMP-8 effect occurred independently of whether the cells were maintained under Zeocin selection or not, as pools expressing MMP-8 at 7 weeks post transfection lost wild-type MMP-8 expression at the same rate as their sister cells from the same original pool, irrespective of selection reagent. E198A mutant MMP-8 expression was consistently high across the 4-week monitoring period, and was expressed to a higher level than wild-type MMP-8 initially. DNA analysis showed that the exogenous MMP8 inserted into the genome was still present after 11 weeks (figure 5.1B), suggesting that the cells still contain the plasmid DNA. Based on the equivalent retention of plasmid DNA in cells that were expressing wild-type MMP-8 and cells still expressing E198A mutant MMP-8 it is likely that the cells may have epigenetically silenced MMP8. However, it is also possible that an expression control element of the plasmid may have been deleted. In the cells that had lost wild-type MMP-8 expression IL-6 and -8 were only expressed at baseline levels comparable to levels expressed by the empty vector control cells, with cells expressing slightly more MMP8 expressing slightly more IL-6 and IL-8 (data not shown). This proves a causal link between MMP8 expression and IL-6 and -8, as the cells that lose MMP8 expression also lose IL-6 and -8 expressions. Therefore, the rare clones used throughout this work that are able to express wild-type MMP-8 “long-term” have most likely undergone permanent phenotypic changes in order to maintain wild-type MMP-8 expression.

5.2 Detrimental effects of MMP-8 on MDA-MB-231 cells

Based on the progressive loss of wild-type MMP-8 expression over prolonged culture (figure 5.1), it is possible that the active MMP-8 enzyme may exert deleterious effects on some aspects of cell growth or survival that result in a negative selection pressure on wild-type MMP-8 expressing cells. As shown in chapter 3 wild-type MMP-8 had no effect on cell growth or cell cycle profile of

MDA-MB-231 cells expressing MMP-8 “long-term”. Therefore it was hypothesised that MMP-8 may affect cell behaviour either acutely (within days) or in a short (within a couple of weeks) time-frame.

5.2.1 MMP8 has no acute effects on apoptosis

Although it was shown in figure 3.8 (section 3.2.1), through looking at the Sub-G1 peak by flow cytometry, that MMP-8 has no effect on apoptosis in the stably transfected cells, it was deemed appropriate to determine whether MMP-8 affected apoptosis after only short exposure to the protease. Caspases 3, 7, 9 and poly (ADP-ribose) polymerase (PARP) were examined (7, 9 and PARP in MCF-7 cells) as a measure of apoptosis by western blot in MDA-MB-231 and MCF-7 cells transiently transfected with wild-type MMP8, EA Mutant MMP8 and an ‘empty vector’ control. IL-6 and -8 up-regulation was observed by 72 hours post transfection as seen in chapter 4 (figure 5.2A-B). A staurosporine time-course was conducted in MDA-MB-231 and MCF-7 cells to determine their sensitivity to apoptosis-inducing agents (figure 5.2C). These samples were consecutively used as positive controls for other western blots.

Some caspase cleavage was observed across all samples which was probably due to stress from the transfection and not MMP8 specifically (figure 5.4D for MDA-MB-231 cells and E for MCF-7 cells). In MDA-MB-231 cells caspase 3 and PARP cleavage products only observed in the positive control and not in transfected cells. The 116 and 89kDa bands appear rectangular but this is an artefact of the imaging device. Caspases 7 and 9 however were cleaved in all transfected cells in response to transfection. In addition, in MCF-7 cells two products of caspase 7 cleavage were observed. The 25kDa fragment was observed in the positive control and in all transfected samples and a lower band of approximately 17kDa was detected in all samples transfected for 48 and 72 hours. In addition, an 89kDa PARP cleavage product was observed in all transfected samples, peaking at 48 hours post transfection. There was always a strong PARP band across all samples in both cell lines (including positive and negative controls) at approximately 37kDa. This could not be identified in the

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literature and was not explained in the anti-body specification. It could therefore just be an artefact or a phosphorylated form. It was difficult to observe a caspase 9 cleavage product, even in the positive control. This showed that acutely, MMP8 is not having any observable adverse effects on these cells. Added confirmation comes from examination of p21, p53 and PDCD4, of which none were affected by MMP-8 expression (data not shown).

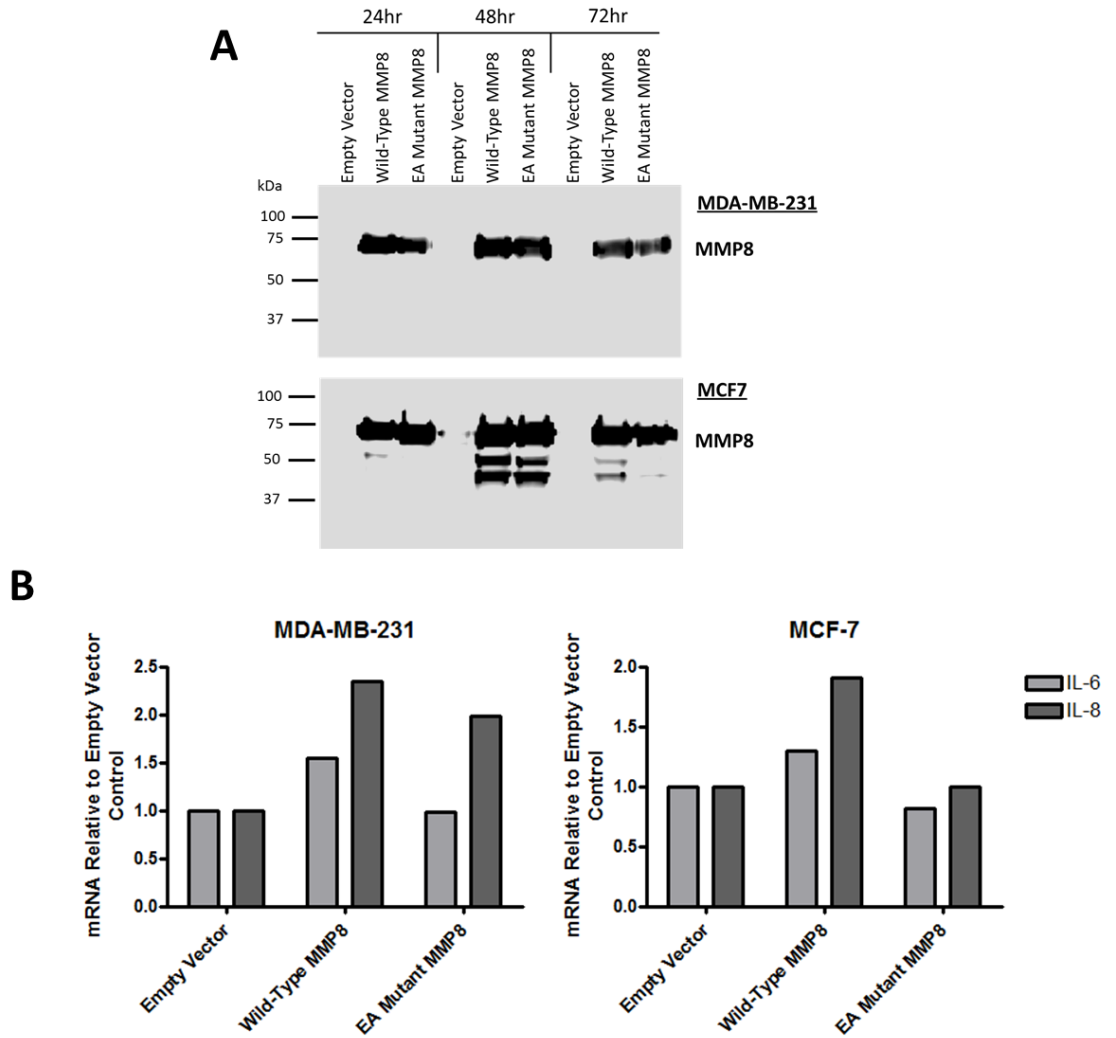


Figure 5.2A-B: Examination of the acute effects of MMP-8 on apoptosis- transient transfection of MMP8 into MDA-MB-231 and MCF-7 cells. MDA-MB-231 and MCF-7 cells were transiently transfected with pcDNA 4 constructs (wild-type and E198A mutant MMP8 and empty vector control) for 24, 48 and 72 hours. A) Western blot analysis of MMP-8 on 20x serum free conditioned media. B) Gene expression analysis of IL-6 and IL-8 by Taqman RT-PCR in samples collected 72 hours post transfection.

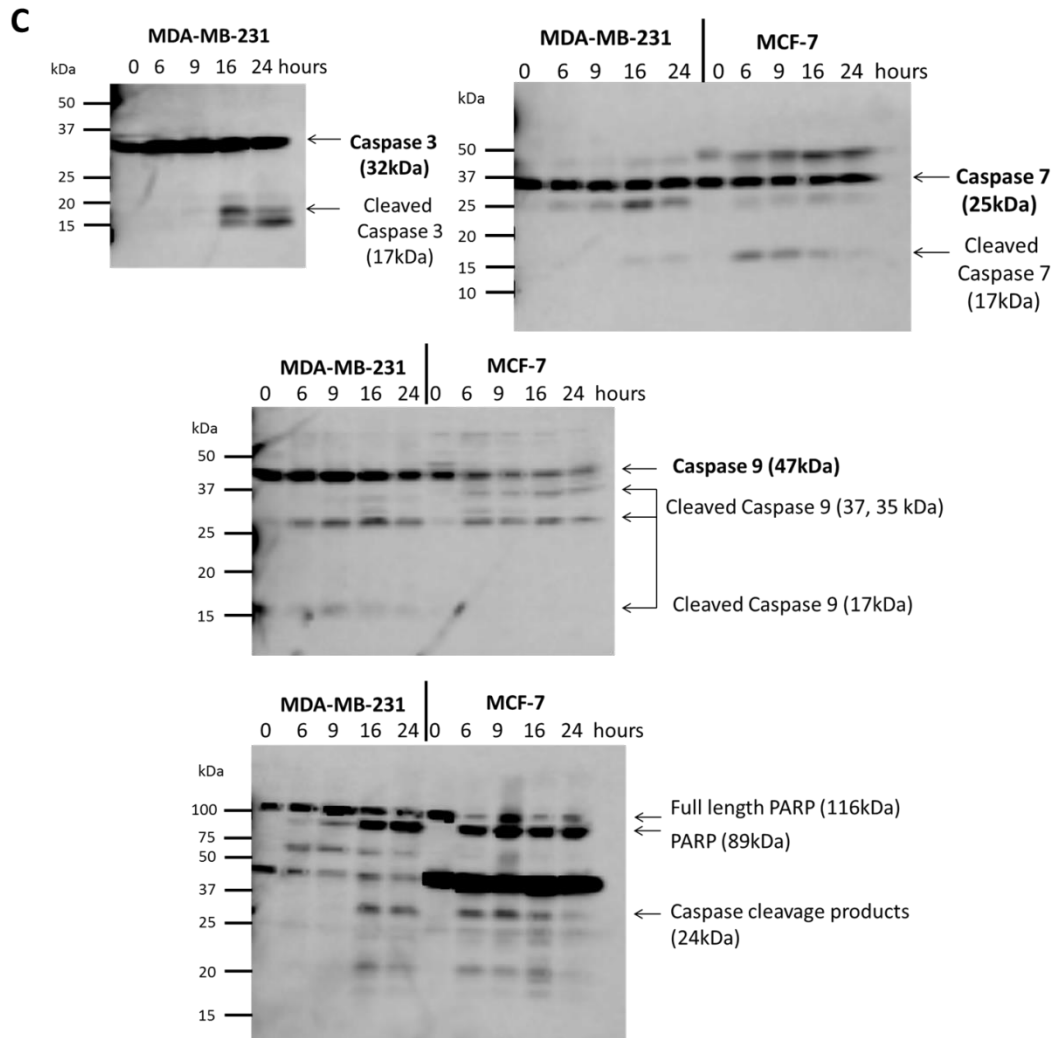


Figure 5.2C: Examination of the acute effects of MMP-8 on apoptosis- Staurosporine timecourse in naïve MDA-MB-231 and MCF-7 cells. MDA-MB-231 and MCF-7 cells were treated with 10 μ M Staurosporine for 6-24 hours to induce apoptosis. C) Western blot analysis of Caspase 3 (MDA-MB-231 cells only as not expressed by MCF-7 cells) Caspase 7, Caspase 9 and PARP.

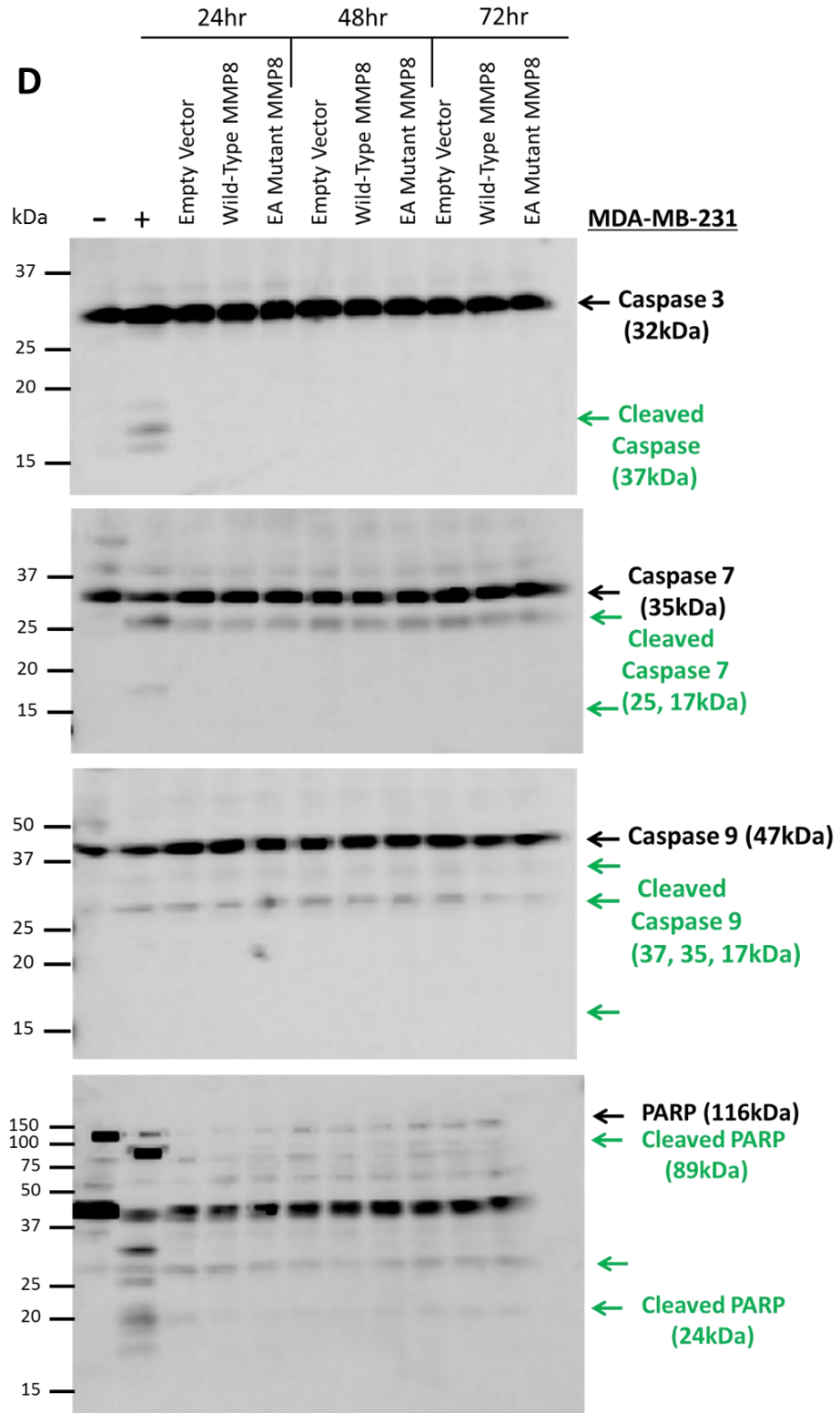


Figure 5.2D: Examination of the acute effects of MMP-8 on apoptosis- MDA-MB-231 cells. D) Western blot analysis of Caspases -3, 7, 9 and PARP in MDA-MB-231 cells transiently transfected with wild-type, E198A mutant MMP8 and empty vector control. Positive control was MDA-MB-231 cells treated with staurosporine for 16 hours.

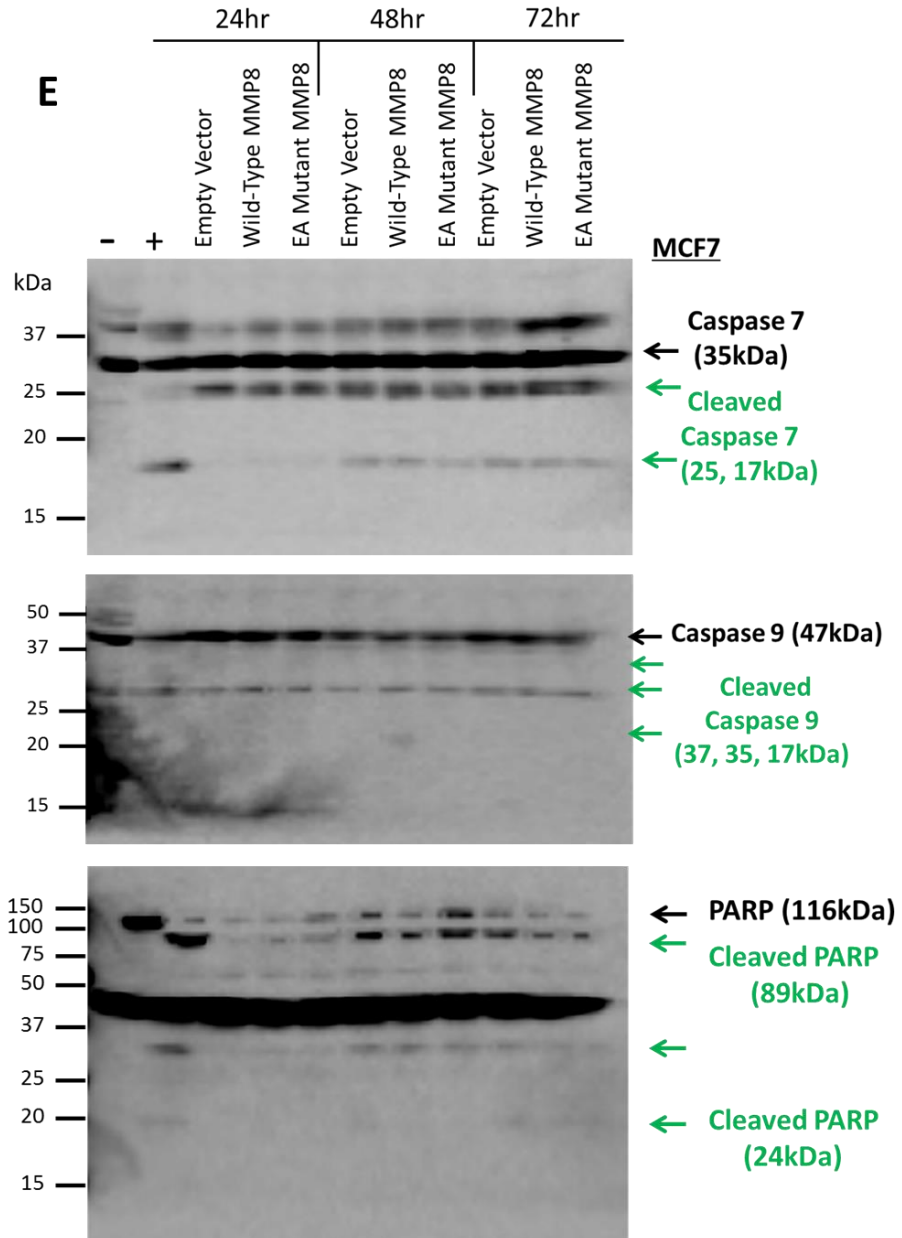


Figure 5.2E: Examination of the acute effects of MMP-8 on apoptosis- MCF-7 cells. D) Western blot analysis of Caspases- 7 ,9 and PARP in MCF-7 cells transiently transfected with wild-type, E198A mutant MMP8 and empty vector control. Positive control was MCF-7 cells treated with stataurosporine for 6 hours.

5.2.2 “Mid-term” exposure to wild-type MMP8 reduces colony formation

On an acute basis MMP-8 did not appear to affect apoptosis. Colony formation assays on the other hand examine effects over 2-3 weeks. There were 3 times fewer colonies in the plate transfected with wild-type MMP8 than those transfected with either the empty vector control or E198A mutant MMP8 (figure 5.3). There were no differences in size of the colonies. This showed that wild-type MMP8 has detrimental effects between 2 and 3 weeks post-transfection. The colonies that are able to grow may either be cells that had switched off wild-type MMP-8 or cells that had undergone alterations in order to cope with its presence.

To conclude, wild-type MMP8 is detrimental to cells after medium term exposure as exhibited by reduced colony formation, but not on an acute term as determined by apoptosis on western blot and neither in the “long-term” MMP-8 expressing stably transfected cells as shown in Chapter 3. However, as some caspase activity (as determined by small cleavage products) was observed due to transfection alone, any effects of MMP8 may have been masked. Transient transfection of MMP8 can induce IL-6 and -8 acutely due to the high levels of MMP8 being expressed, but it is only a limited number of cells that are able to overcome the detrimental effects of MMP8 and maintain wild-type MMP8 expression long term. Thus, the MDA-MB-231 clonal isolates used in this thesis seem to be rare cells that underwent phenotypic changes to cope with the presence of wild-type MMP8. This change may be permanent and may include the observed elevation of IL-6 and -8 expression levels. Such changes will be examined next.

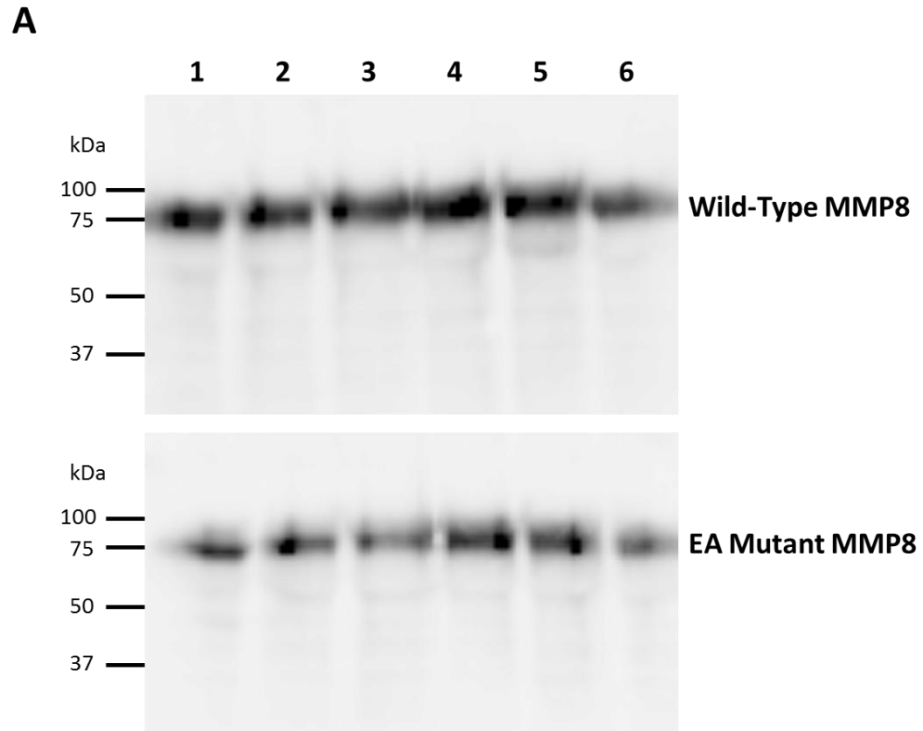
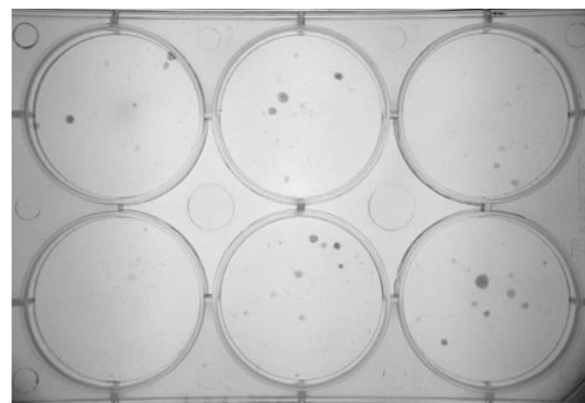
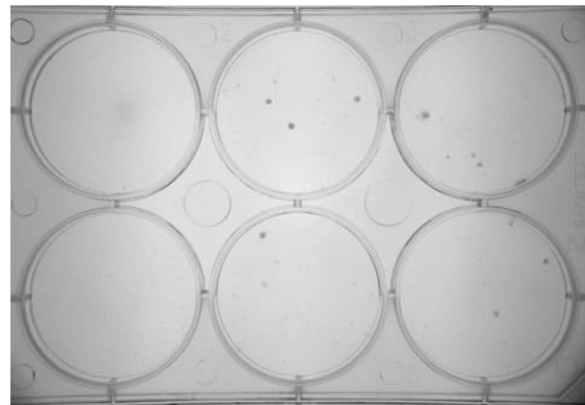
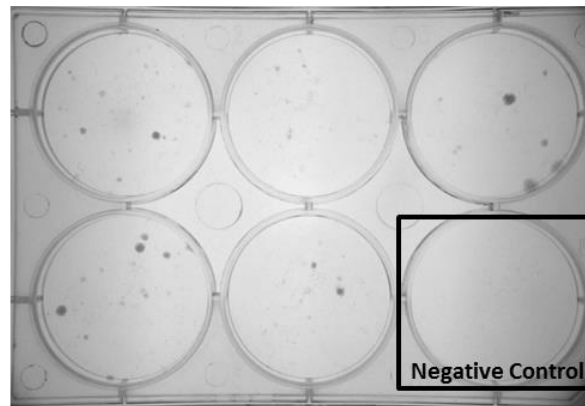
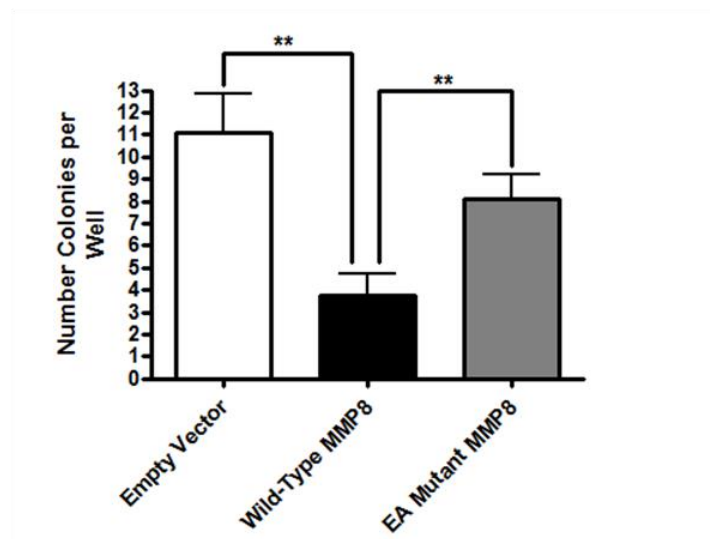


Figure 5.3: Effect of wild-type MMP-8 on colony formation of MDA-MB-231 cells. MDA-MB-231 cells stably transfected with wild-type and E198A mutant MMP8 and empty vector control were maintained under Zeocin selection for 20 days before staining of colonies with methylene blue. A) Western blot analysis of MMP-8 in 20x concentrated serum free conditioned media at the start of the experiment B) Methylene blue stained colonies and C) Colony counts. n=6 (except EV where n=5), mean \pm SEM, * = p <0.05, ** = p<0.01 *** = p<0.001

B



C



5.3 “Long-term” MMP-8 expressing cells have undergone permanent phenotypic changes

It was hypothesised that to enable expression of MMP-8 “long-term”, MDA-MB-231 clonal isolates had undergone permanent phenotypic changes to overcome the growth inhibitory effects of MMP-8 on colony formation (figure 5.3). Such changes may include up-regulation of IL-6 and IL-8. Having showed a causal link between wild-type MMP-8 expression and IL-6 and IL-8 regulation in section 5.1, this section will focus on the hypothesised changes that have occurred in these “long-term” MMP-8 expressing cells, including examination of whether elevated IL-6 and IL-8 levels may be a permanent feature of these cells, and whether MMP-8 is actually still required for their up-regulation. MDA-MB-231 cells are metastatic breast cancer cells, and MMP-8 has been shown to be an anti-metastatic protease [52, 65], so it is plausible that the cells will react to the presence of MMP-8. IL-6 and IL-8 have been widely shown to be pro-tumourigenic inflammatory modulators so up-regulation of these may compensate for the negative effects of MMP-8.

5.3.1 MMP-8 is dispensable in the up-regulation of IL-6 and IL-8 after long-term culture

It was sought to determine whether MMP-8 presence was still required in the self-reinforcing loop to maintain the enhanced IL-6 and -8 expression observed in MDA-MB-231 cells stably over-expressing wild-type MMP8 long-term (shown in figure 4.5), or whether this phenomenon was a permanent phenotypic change as a result of “long-term” wild-type MMP-8 expression. MMP inhibitors and siRNA were used to investigate this. The pan-MMP inhibitor CT1746 reduced IL-6 and IL-8 levels slightly whereas GM6001 had no effect (figure 5.4A-B), suggesting that metalloproteinases (MMPs, ADAMs or ADAMTs) may have some role to play depending on the inhibitor used. These two inhibitors are not specific and can inhibit most MMPs as well as some ADAMs and ADAMTs. They do show a stronger specificity towards MMP2 and 9 above others however. Next a specific MMP8 inhibitor DL111 was added and this also had no effect on IL-6 and -8 levels (figure 5.4C). These data suggest that MMP-8 is no longer required

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for maintained elevated IL-6 and IL-8 levels in “long-term” wild-type MMP-8 over-expressing MDA-MB-231 clones, although other metalloproteinases may be involved. As further confirmation of this lack of MMP-8 requirement, siRNA knockdown of MMP8 had no effect on IL-6 RNA levels whereas IL-8 expression increased 3 fold (figure 5.5).

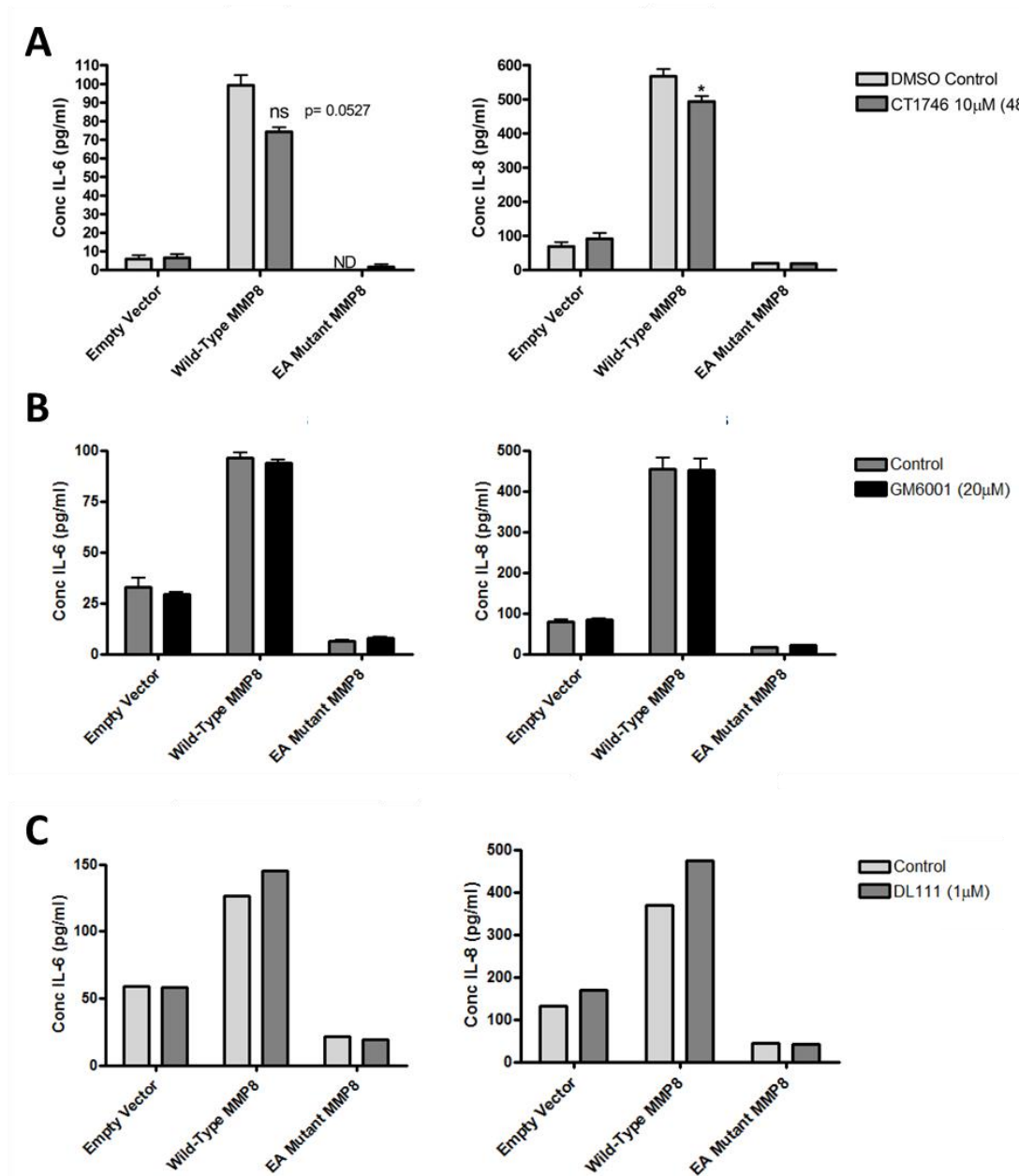


Figure 5.4. Effect of MMP inhibition on IL-6 and IL-8 levels. IL-6 and IL-8 protein secretion as measured by ELISA from “long-term” MMP-8 expressing stable MDA-MB-231 clonal isolates and cells expressing E198A mutant MMP-8 and empty vector control cells after incubation with broad spectrum MMP inhibitors CT1746 (A), GM6001 (B) and an MMP-8 specific inhibitor (C). n=3 (DL111 n=1), mean \pm SEM, ND= not detected, * = p < 0.05, ** = p < 0.01 *** = p < 0.001

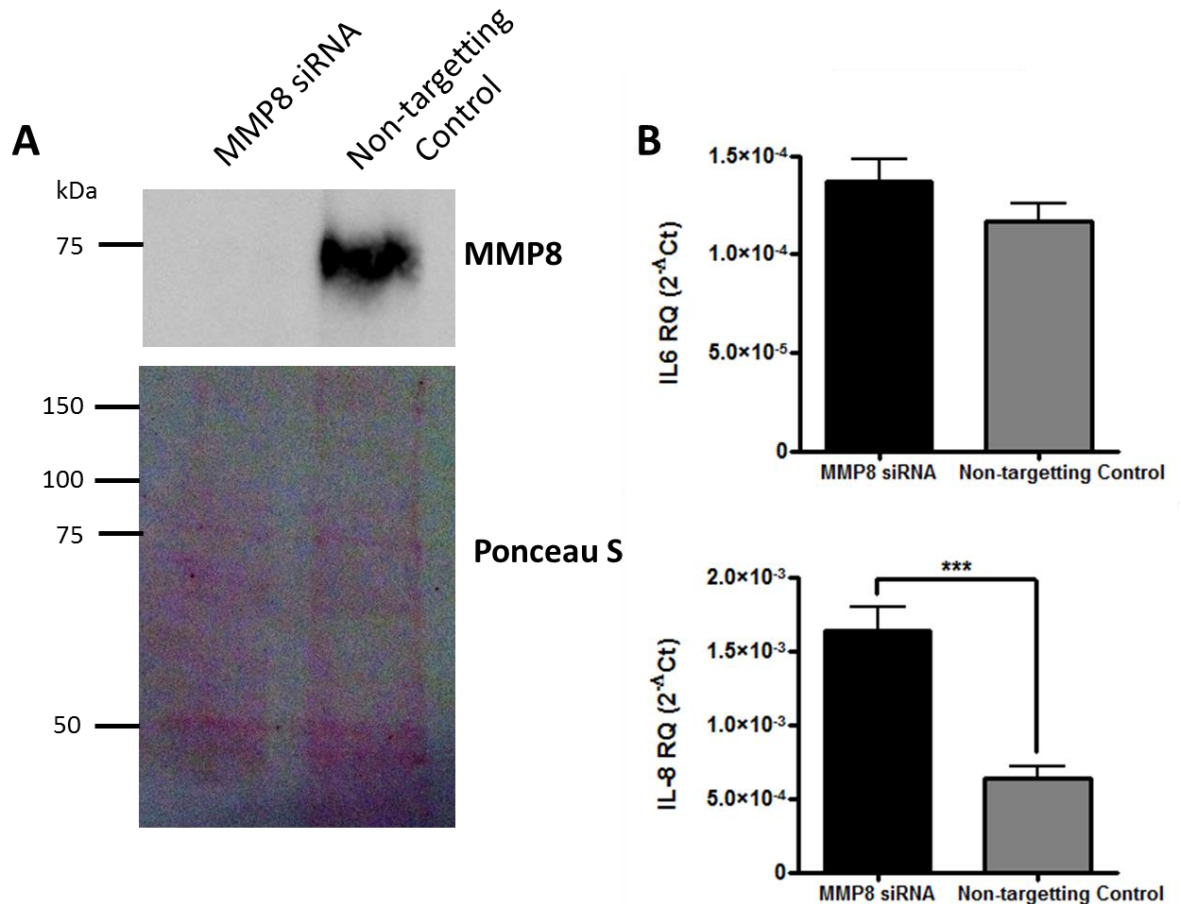


Figure 5.5: MMP8 siRNA in wild-type MMP-8 over-expressing MDA-MB-231 cells. A) Western blot analysis of MMP-8 in 20x concentrated serum free conditioned media using an anti-MMP-8 antibody after siRNA knockdown. The Ponceau S stain shows the presence of protein on both lanes of the western blot. B) Gene expression analysis of IL-6 and IL-8 by Taqman RT-PCR after siRNA knockdown of MMP8 fold. n=4, mean ± SEM, * = p < 0.05, ** = p < 0.01, *** = p < 0.001

Potentially, IL-8 expression increased with MMP8 knockdown in order to maintain the self-reinforcing loop described in figure 4.5. MMP8 was not easy to silence with siRNA, it required two consecutive siRNA transfections before MMP8 was seen knocked down at the protein level so as to be undetectable in the cell culture media (figure 5.5A). In addition, cells were washed frequently with serum free media during the knockdown to ensure that any residual MMP-

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8 secreted post-transfection was removed. Therefore it was concluded that despite extra efforts to knock MMP-8 down totally at the protein level and ensure that there was no MMP-8 still present in the culture media, IL-6 was not reduced and IL-8 increased. There is possibility though that molecules downstream of MMP-8 would have to be silenced in combination with MMP-8 to observe a decrease in expression of the cytokines.

This suggests that MMP-8 is no longer required in to induce IL-6 and IL-8 and that the cells have undergone a permanent phenotypic change whereby MMP-8 is no longer required. Another interesting discovery was that MMP8 *expression* is required for these adaptations to occur, as exogenous addition of 4-aminophenylmercuric acetate (APMA) activated human recombinant MMP8 to naïve MDA-MB-231 cells had no effect on IL-6 and IL-8 levels (figure 5.6).

It can therefore be concluded that cells expressing MMP-8 “long-term” have undergone permanent changes to over-come the detrimental effects of wild-type MMP-8. These effects are evident by 20 days post transfection and result in epigenetic silencing of wild-type MMP-8 in the majority of clones. Changes in the “long-term” wild-type MMP-8 expressing cells include the permanent elevation of IL-6 and IL-8 independent of the presence of MMP-8. A proposed model of IL-6 and IL-8 up-regulation in these cells follows.

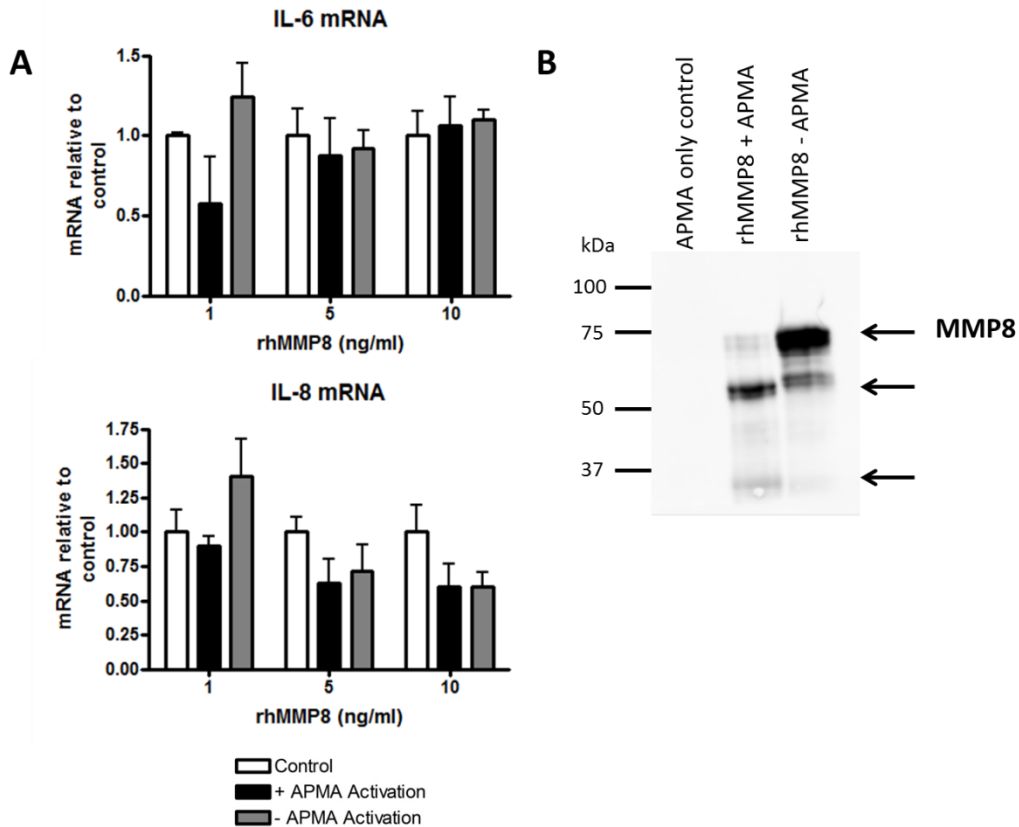


Figure 5.6: Addition of recombinant human MMP8 to naïve MDA-MB-231 cells A) Gene expression analysis by IL-6 and IL-8 by Taqman PCR after rhMMP8 addition, either inactive or activated with APMA, to naïve MDA-MB-231 cells. B) Western blot analysis of rhMMP8 either activated by APMA or incubated with the control buffer at 37°C for 2 hours. MMP8 pro-form = 75kDa, active forms = 50 and 37kDa. n=4, mean ± SEM

5.3.2 Additional regulatory mechanisms required for IL-6 and IL-8 up-regulation

Based on preliminary data and evidence from the literature, a model of IL-6 and IL-8 up-regulation involving Protease Activated Receptor 2 (PAR2), otherwise known as F2RL1, was proposed. The PAR family is a family of 4 (PARs 1-4) G-Protein Coupled Receptors (GPCRs) which can be activated, or inactivated depending on the site of cleavage, by proteases. This cleavage, historically carried out by serine proteases, results in a tethered ligand which can then bind back to the receptor irreversibly [278, 279]. PAR2 is the only PAR other than

PAR1 to be expressed in epithelial cells [279], although PAR1 was not found to be expressed in the MMP-8 expressing stably transfected MDA-MB-231 cells used in this thesis (J. Decock, unpublished data), and has been heavily linked to inflammation [280]. It is expressed in neutrophils [281] and plays a role in neutrophil migration [282] and IL-6 and IL-8 up-regulation and/or secretion from a range of cell types, including neutrophils [282], epithelial cells [283, 284] and endometrial stem cells (IL-6 only) [285]. It can signal through p38 MAPK and NF κ B [280, 285]. Lastly it has been shown that PARs can be cleaved and activated by MMPs. For example, MMP1 has been shown to activate PAR1 in melanoma [286], in the breast microenvironment [287] and during endothelial cell activation [288]. MMP-3, -8 and -9 could cleave a synthetic peptide containing conventional PAR1 cleavage sites [289]. Therefore, it is plausible that MMP8 may be influencing PAR2, either by changing expression levels or activating/inactivating it through cleavage.

Firstly total PAR2 levels were examined in the MDA-MB-231 clonal isolates (sets 1 and 2) at both mRNA and protein levels. Although only partially significant, an up-regulation of PAR2 mRNA was observed in both sets of wild-type MMP8 over-expressing clones (figure 5.7A). There was no difference in protein level, but this could be due to it being recycled rapidly, which would explain the increase in mRNA expression. There were many bands corresponding to PAR2 on the western blot, which reflect its phosphorylated forms [290]. MMP-8 probably does not act directly on PAR2 as siRNA knockdown of MMP8 had no effect on PAR2 levels (figure 5.7B), but was effecting its expression levels somehow.

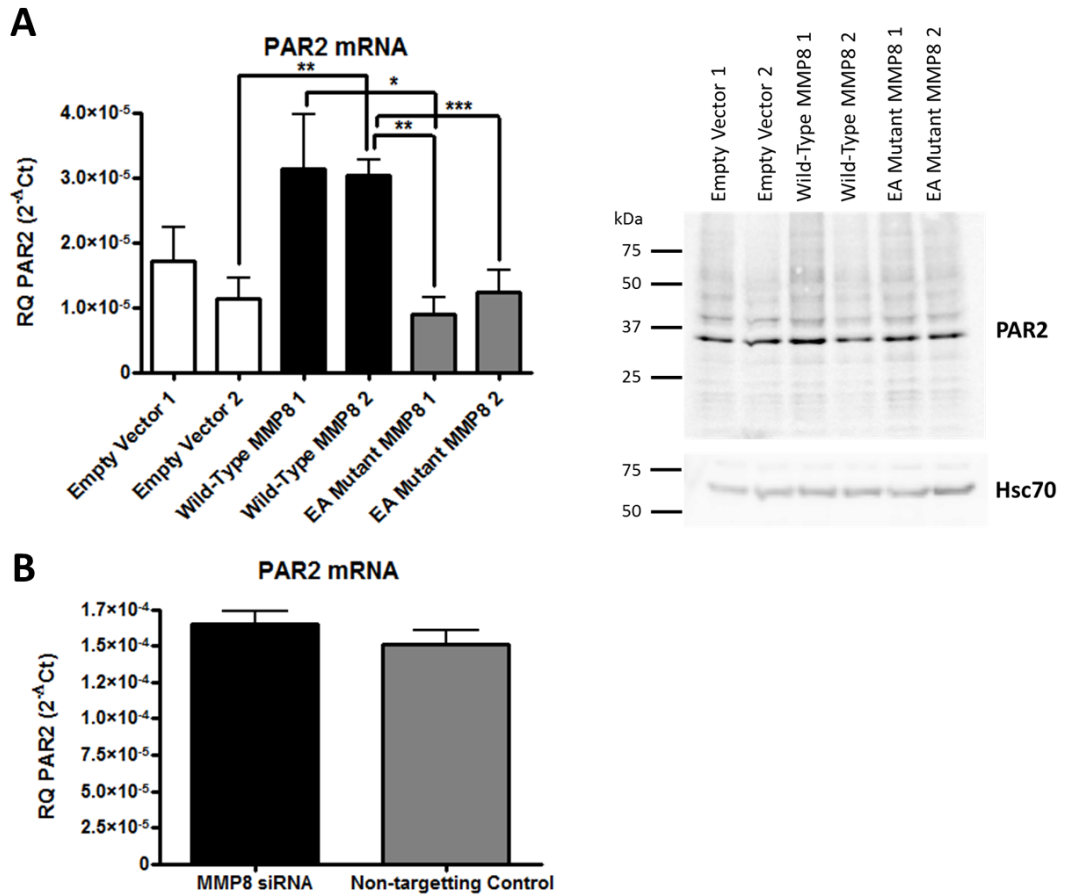


Figure 5.7: Effects of MMP-8 on PAR2. Gene expression levels of PAR2 by Taqman RT-PCR and western blot analysis of PAR2 in (A) “long-term” MMP-8 expressing MDA-MB-231 clonal isolates and (B) after MMP8 siRNA knockdown in clone 1 of wild-type MMP-8 expressing cells compared to the non-targetting control (gene expression only). n=3, mean ± SEM, * = p < 0.05, ** = p < 0.01, *** = p < 0.001

To test whether PAR2 acts on IL-6 and IL-8 in this system PAR2 siRNA was applied, both alone and in conjunction with MMP8 siRNA. PAR2 knockdown alone reduced IL-6 protein by 90% and mRNA expression levels by 60% (figure 5.8B). IL-8 levels actually increased compared to the non-targetting siRNA control (figure 5.8C); by 80% at mRNA level and 60% at the protein level. MMP8 knockdown in conjunction with PAR2 knockdown did not have any exaggerated effects on the cells on IL-6, but IL-8 mRNA levels were slightly reduced with combined PAR2 and MMP8 knockdown. However, IL-8 levels were still slightly higher than that of the non-targetting control and this effect was not mirrored at

the protein level. This gives further suggestion of the redundancy, long-term, of MMP-8 in this system but may indicate that MMP-8 and PAR2 may be acting through the same pathway.

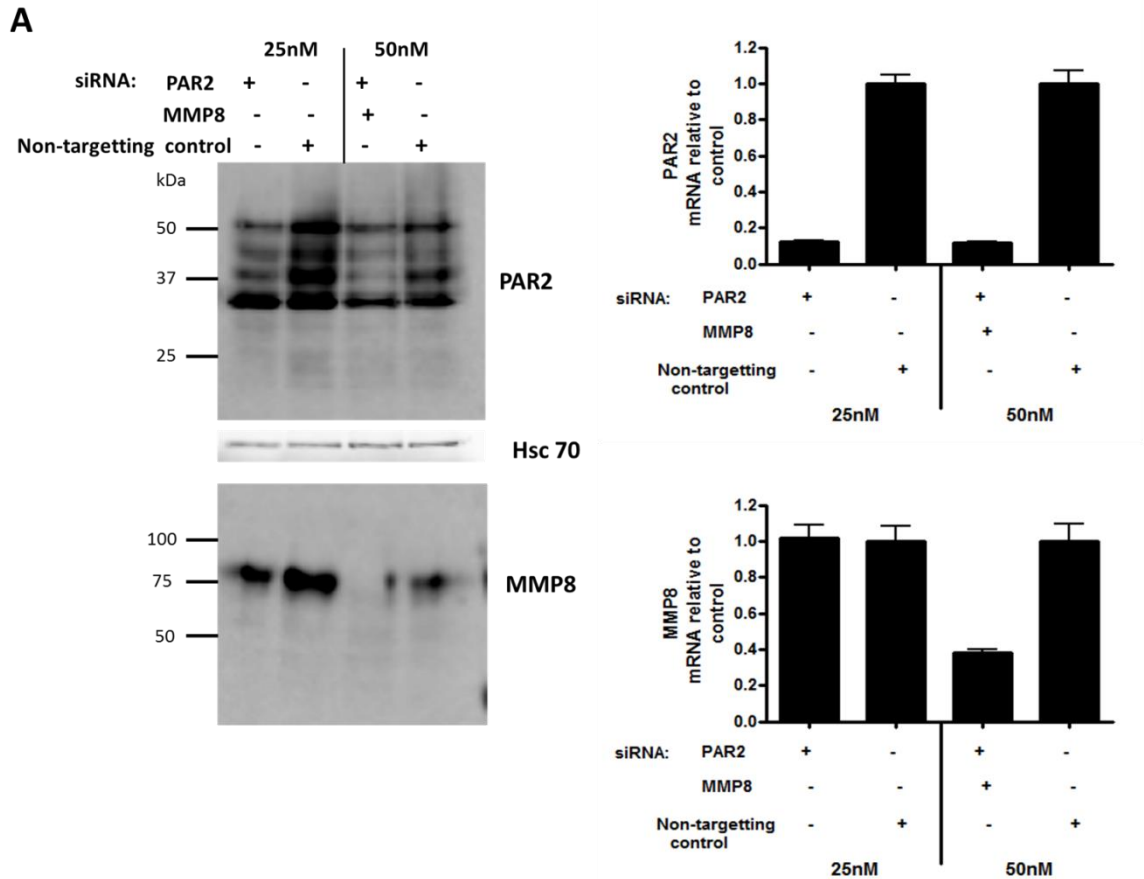


Figure 5.8A: Effect of PAR2 and MMP8 siRNA on IL-6 and IL-8. A) *Left panel:* Western blot analysis of PAR2 (in cell lysate) and MMP-8 (in 20x concentrated serum free conditioned media) in “long-term” wild-type MMP-8 expressing MDA-MB-231 clone set 1 after siRNA knockdown of PAR2 alone or in conjunction with MMP8 knockdown. *Right panel:* Gene expression analysis of PAR2 and MMP8 by Taqman RT-PCR in the same cells. Mean \pm SEM

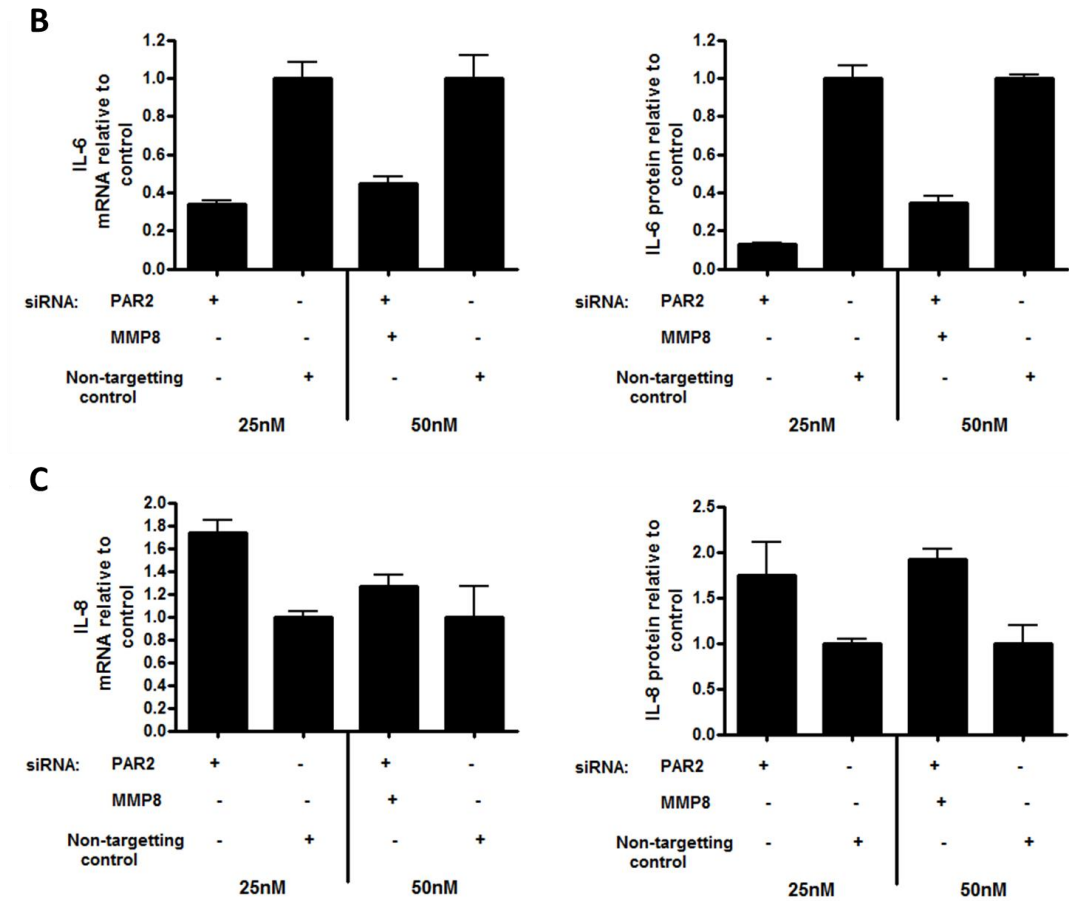


Figure 5.8 B-C Effect of PAR2 and MMP8 siRNA on IL-6 and IL-8.

B) *Left panel:* Gene expression analysis of IL-6 measured by taqman RT-PCR in “long-term” wild-type MMP-8 expressing MDA-MB-231 clone set 1 after siRNA knockdown of PAR2 alone or in conjunction with MMP8 knockdown alone. *Right panel:* IL-6 protein secretion measured by ELISA in the same cells. C) *Left panel:* Gene expression analysis of IL-8 measured by taqman RT-PCR in “long-term” wild-type MMP-8 expressing MDA-MB-231 clone set 1 after siRNA knockdown of PAR2 alone or in conjunction with MMP8 knockdown alone. *Right panel:* IL-8 protein secretion measured by ELISA in the same cells.

This has introduced a novel pathway through which MMP8 acts to induce IL-6 and IL-8 (summarised in figure 5.9) via its catalytic activity in the “long-term” MMP-8 expressing cells. MMP8 induced PAR2 expression, and then MMP8, or another unknown protease, activated the receptor, resulting in increased NFκB activity (possibly via p38) and an up-regulation of IL-6 and IL-8. IL-8 was then able to compensate for removal of MMP8 by siRNA knockdown by increasing expression levels, thus perpetuating a self-reinforcing loop that once established occurred independently of MMP8. These changes may have occurred to cope with the negative effects of wild-type MMP-8.

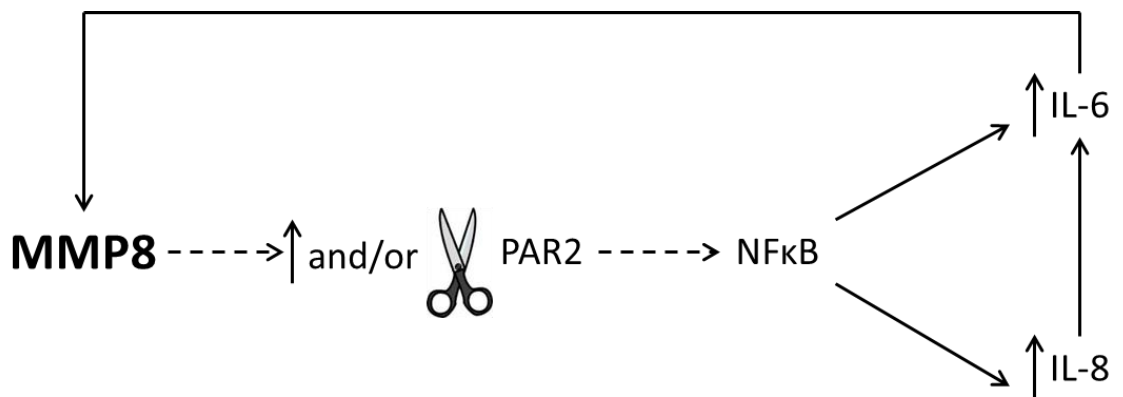


Figure 5.9: Summary of the proposed mechanistic actions of MMP8 leading to the up-regulation of IL-6 and IL-8. Wild-type MMP8 has been shown to increase PAR2 expression, which it likely indirect. Wild-type MMP8 may also cleave and activate PAR2 but this is unknown. PAR2 has been shown in the literature to activate NFκB as part of pro-inflammatory pathways, and in this thesis it has been shown that NFκB is the key factor responsible for the up-regulation of both IL-6 and IL-8 in MDA-MB-231 cells stably transfected with wild-type MMP8. It has already been shown in chapter 4 that IL-8 has a role in up-regulating IL-6, and IL-6 can act back on MMP8 itself to maintain this self-reinforcing loop. Solid arrows = pathways indicated from this thesis. Dashed arrows = pathways inferred from a combination of the literature and preliminary studies in this thesis.

To end this chapter, the question arose whether these cells would exhibit the tumour-repressive features observed by Gutiérrez-Fernández *et al.*, (2008)[52], or whether the elevated IL-6 and -8 levels would cause the “long-term” MMP-8 expressing MDA-MB-231 cells to be more aggressive *in vivo*. This experiment was not carried out earlier as immunocompromised mice would have to be used to avoid reaction to the human cells. However, if MMP-8 was dynamically influencing cytokine levels, as was initially thought, using such mice would render these experiments meaningless. This is likely not the situation however as subsequent research presented in this chapter has strongly suggested that cells expressing wild-type MMP-8 “long-term” have undergone permanent phenotypic changes where IL-6 and IL-8 levels are maintained independent of MMP-8 expression. Therefore, it was subsequently sought to determine how these ‘altered’ cells behaved *in vivo*.

5.4 Effects of MMP8 *in vivo*

As shown in chapter 4 “long-term” wild-type MMP-8 over-expressing cells exhibited reduced random 2D migration, dependent on its catalytic activity, and reduced scratch wound closure, independent of catalytic activity. This indicates that MMP-8 is exerting a tumour-suppressive role *in vitro*. To determine the effect of MMP-8 over-expression in these cells *in vivo*, set 1 of the wild-type MMP8, E198A mutant MMP8 and empty vector MDA-MB-231 “long-term” clonal isolates (figure 5.10A), were injected subcutaneously into CD1 nu/nu mice. These mice do not produce T cells but do have all other immune cells and hence will not react to the injection of human cells but should still be reactive to the higher levels of IL-6 and -8 produced by the wild-type MMP8 over-expressing cells. Presence of tumours was assessed by weekly palpation. Interestingly by day 54, 62% of mice injected with MDA-MB-231 cells expressing the empty vector control had a tumour (5/8), compared to 16% of mice injected with wild-type MMP-8 expressing cells (1/6), while none of the mice injected with E198A mutant MMP-8 expressing cells had a tumour (0/6) (figure 5.10B). Nothing can be concluded about tumour size due to the small sample size, but figure 5.10C shows the range of tumour size from mice injected with control ‘empty vector’

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transfected cells. This potentially shows that MMP-8, irrespective of catalytic activity, is tumour-protective in this model. It also suggests that this occurs irrespective of IL-6 and -8, and their pro-survival signals. It must be noted however, that the mice are partially immunocompromised which may have an effect on the response to the cytokines. Therefore, in line with other research, MMP8 is exerting anti-tumourigenic effects *in vivo*, which is likely independent of raised IL-6 and -8 levels. This aspect suggests that IL-6 and IL-8 may be a non-functional by-product of “long-term” MMP-8 expression that occur as a result of changes in other proteins and pathways that are yet to be determined and highlights that further investigation is required into the functional effects of MMP-8 in mammary carcinoma. Additionally it shows the complexities of the protease and how it can exert different effects *in vitro* and *in vivo*.

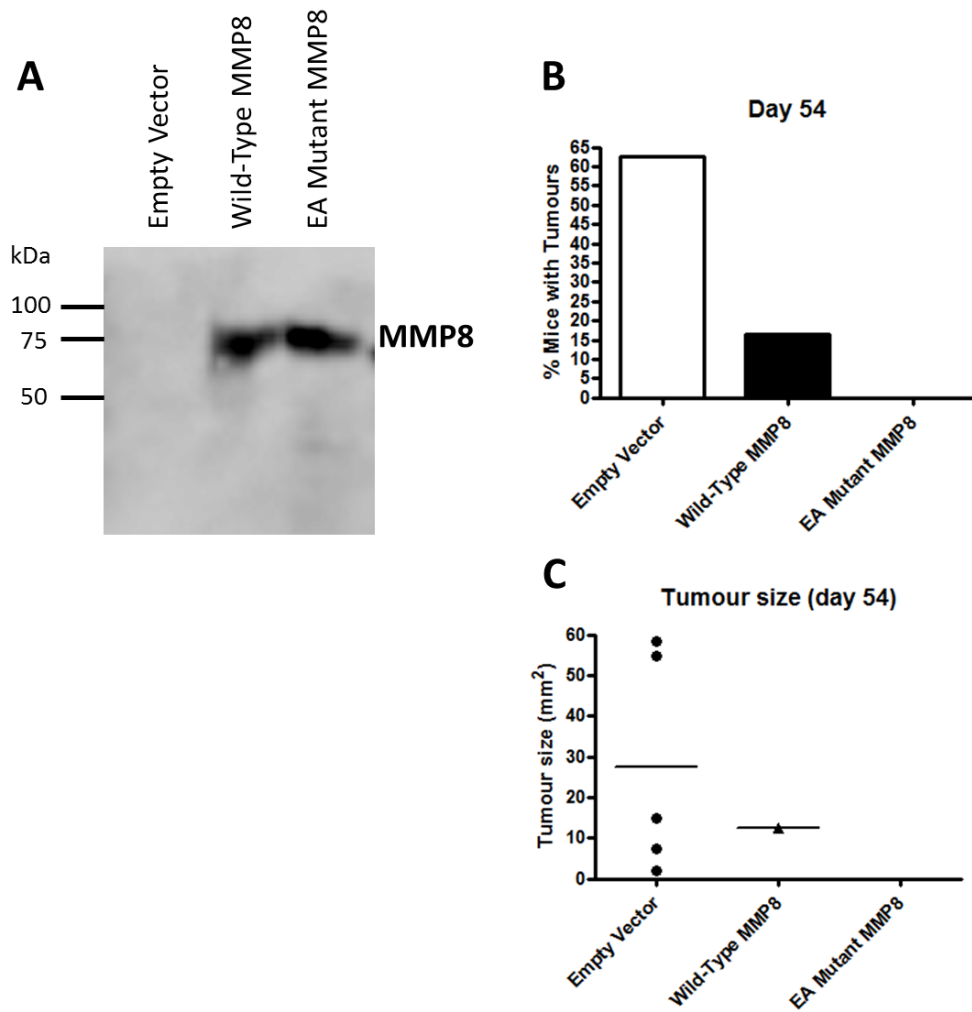


Figure 5.10: Effects of MMP- 8 *in vivo* on subcutaneous injection of “long-term” MMP-8 expressing MDA-MB-231 clonal isolates. “Long-term” MMP-8 expressing MDA-MB-231 clonal isolates (clone set 1) stably over-expressing wild-type MMP8, E198A mutant MMP8 and the ‘empty vector’ control were injected subcutaneously (1^6 cells per mouse in PBS into the left upper flank) into CD1 nu/nu mice. A) Western blot analysis of MMP-8 in 20x concentrated serum free conditioned media from the cells prior to subcutaneous injection. B) Tumour load (B) and tumour size (C) by day 54. n =6-8.

5.5 Conclusion and Discussion

This chapter aimed to determine the reason why MMP-8 stably expressing cells were so difficult to generate, and whether the observed induction and up-regulation of IL-6 and IL-8 with wild-type MMP-8 expression had any involvement. The most common outcome when generating the MDA-MB-231 stable transfectants used in chapters 3 and 4 of this work was that wild-type MMP-8 was lost from cells. This chapter documented wild-type MMP-8 loss in pools of stably transfected MDA-MB-231 cells and showed that expression was likely lost through epigenetic silencing. These cells expressed IL-6 and IL-8 at baseline levels showing a causal link between MMP-8 and IL-6 and IL-8 up-regulation. In a rare set of “long-term” wild-type MMP-8 expressing MDA-MB-231 clones it has been hypothesised that permanent phenotypic changes occur to allow continued expression of wild-type MMP-8, which may include IL-6 and IL-8 up-regulation. However, it has been shown that in these rare clones MMP-8, IL-6 and IL-8 expression has become decoupled and MMP-8 is no longer required for maintained elevated expression, emphasising the likelihood of permanent changes within the cells; Levels of IL-6 and IL-8 in the “long-term” wild-type MMP-8 expressing clones were only slightly reduced by a broad spectrum MMP inhibitor CT1746, but not by GM6001, nor by an MMP-8 specific inhibitor, DL-111. IL-6 levels were also unaffected by MMP8 siRNA and IL-8 levels actually increased. Exogenous addition of rhMMP-8 to cells had no effect on IL-6 and IL-8 levels. This firstly suggests that MMP8 expression is required to propagate pathways involved in regulation of the cytokines and secondly that the mechanism involving the induction of IL-6 and -8 may occur as a “private” mechanism that can not be affected by exogenous inhibitors or proteins. This is plausible as IL-6 has been reported previously to exert autocrine effects on cells as part of a “private” intracellular mechanism that could only be diminished by siRNA transfection, and not by the addition of exogenous inhibitors [291].

Further experiments aimed to determine what detrimental effects MMP-8 had on cells. Acute exposure to MMP-8 through transient transfection had no effect on apoptosis as shown through lack of MMP-8 dependent caspase cleavage on

western blot. Lack of acute effects cannot be discounted completely however as transfection alone induced apoptosis, which may have masked the effects of MMP-8. In the future more quantitative techniques could be used to examine apoptosis such as flow cytometry or a Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. As shown in chapter 3, wild-type MMP-8 in the “long-term” expressing cells had no effect on cell growth or cell cycle profile. However, wild-type MMP8 was found to have effects on mid-term exposure. Wild-type MMP8 reduced colony formation of MDA-MB-231 cells at 20 days post transfection, confirming that MMP-8 adversely affects the cells. The colony formation assay examines whether stable expression of a protein can cause either cell cycle arrest or cell death, and also the ability of individual cells to form colonies. Although the effects of MMP-8 on colony formation have not been examined in the literature, MMP-8 has been shown by Palavalli *et al.*, (2009) to reduce colony growth on soft agar [77]. This assay is different from the assay employed in this thesis as it examines the ability of anchorage independent growth. Anchorage independent growth is a marker of a stem cell phenotype [292], thus this study indicates that MMP-8 may be reducing the stem cell fraction. More experiments could in future be carried out to unravel the effects of medium-term exposure to wild-type MMP-8 including the soft agar focus formation assay. In addition, caspase cleavage, such as that examined on short-term transfection could be examined at 20 days post transfection. This therefore confirms that wild-type MMP8 adversely affects these cells, and this can first be documented 2 weeks post transfection, with total wild-type MMP8 expression shut down occurring approximately after 7 weeks post transfection. It is only the cells that have phenotypically altered in order to maintain wild-type MMP8 expression long-term over a period of months that exhibit up-regulation of IL-6 and -8 protein and RNA levels.

Further analysis of the mechanism behind IL-6 and IL-8 up-regulation in these rare “long-term” wild-type MMP-8 expressing cells resulted in the proposal of a model involving PAR2. PAR2 has been shown in the literature to be involved in cytokine up-regulation [280, 282-284], specifically IL-6 and -8, and can act through NF κ B [280]. NF κ B was shown in chapter 4 to be a key factor involved in

IL-6 and -8 up-regulation in this system. It is also expressed by the neutrophils, as is MMP8 *in vivo* [281]. PAR2 is likely to act downstream of MMP8 as it need to be proteolytically processed to become active. PAR2 mRNA was found to be up-regulated in wild-type MMP-8 expressing MDA-MB-231 clonal isolates. However, MMP8 siRNA had no effect on PAR2 mRNA levels, so the effects of MMP8 on PAR2 are likely to be indirect, and may even act through multiple factors which are yet to be determined. PAR2 was confirmed to be part of the MMP8, IL-6 and IL-8 self-reinforcing loop as siRNA knockdown of PAR2 reduced IL-6 mRNA and protein levels almost to baseline. Similarly to MMP8 siRNA however, PAR2 siRNA also increased IL-8 protein and mRNA levels. This indicates that this self-reinforcing loop, partly propagated by IL-8, is very difficult to impair, but also gives evidence that PAR2 and MMP-8 are functioning from the same side of the loop, and possibly in conjunction. There is also the possibility that IL-8 may be increasing to maintain the circuit but this could not be proven unless numerous other factors were examined. There are likely many unknown molecules involved in this circuit which may be uncovered using broad screens such as a microarray, or a broader cytokine array. Only a handful of inflammatory mediators were examined in the initial cytokine screen and many other cytokines and chemokines may also be altered as a result of wild-type MMP8 over-expression. Despite this unknown complexity, it is interesting to note that the TGF β small molecule inhibitor described in chapter 4 had the same effect on IL-8 expression as both the MMP8 and PAR2 siRNA, in that IL-8 levels increased when TGF β was inhibited. This suggests that TGF β may comprise part of this ever-expanding self-reinforcing loop involving proteases, receptors and cytokines which may in part serve to compensate for the deleterious effects of MMP-8 on breast cancer cells.

Initially it was considered fallacious to examine the behaviour of these “long-term” MMP-8 stable transfectants *in vivo*. This was because immunocompromised mice would have to be used to avoid a reaction to the human cells, and this would be meaningless when looking at the effect of raised levels of immune system modulators. However, after the discovery that the cells were not exhibiting dynamic changes in IL-6 and IL-8 levels and that elevated

levels appeared to be a permanent feature of the “long-term” expressing cells it was deemed acceptable to carry out an *in vivo* assay.

It was unknown whether these cells would exhibit the tumour-repressive features observed by Gutiérrez-Fernández *et al.*, (2008), whereby *Mmp8* over-expressing B16F10 cells reduced experimental lung metastasis [52], or by Palavalli *et al.*, (2009), whereby wild-type MMP8 expressing Mel-STR melanoma cells resulted in fewer metastatic lesions, or whether the elevated IL-6 and -8 levels would constitute a more aggressive cellular phenotype. IL-6 and -8 have been heavily implicated in the literature to promote tumourigenesis [123, 124, 130, 132, 138, 142, 143, 146, 147, 155, 293]. Immunodeficient CD1 nu/nu mice were used so that the presence of human tumour cells would be tolerated. They are only deficient in T cells so should respond to the higher levels of IL-6 and IL-8 produced by the wild-type MMP8 over-expressing cells; mice express the IL-6 receptor and the IL-8 receptors CXCR1 and CXCR2, which are functional against human IL-8 [294]. Additionally, human CXCR2 can even functionally replace the mouse version. Although mice do not express IL-8 and use LIX instead, human IL-8 will still activate their CXCR receptors [295], rendering this a valid model.

No tumour growth was observed in nu/nu mice injected with cells over-expressing either wild-type, or EA mutant MMP-8 compared to 63% of nu/nu mice injected with empty vector control cells. This shows that in this model system MMP-8 is anti-tumourigenic, independent of its catalytic activity, but also indicates that IL-6 and -8 are not pro-tumourigenic in this *in vivo* system. Both Gutiérrez-Fernández *et al.*, (2008) and Palavalli *et al.*, (2009) used the same model system of subcutaneous injection of MMP8 over-expressing cells [52, 77]. Palavalli *et al.*, (2009) used human melanoma cells over-expressing human MMP8 and injected them into NOD/SCID immunocompromised mice [77]. However, in contrast, Gutiérrez-Fernández *et al.*, (2008) used murine melanoma cells as opposed to human breast cancer cells and their cells were engineered to over-express murine *Mmp8* so they could be injected into mice with an intact immune system [52]. Gutiérrez-Fernández *et al.*, (2008) found no difference in primary tumour growth, whereas a stark difference was observed

in this thesis. Their main observation was a reduction in experimental lung metastasis which was dependent on the catalytic activity of MMP-8. However, this was determined from tail vein injection of transfected B16F10 cells, and not spontaneous lung metastasis resulting from subcutaneous injection. Palavalli *et al.*, (2009) saw a reduction in ulcerating lesions, which is a characteristic of melanoma metastatic potential, in mice injected with wild-type MMP8 over-expressing cells. This protective effect was dependent on the catalytic activity of MMP8 [77]. They did not use a transitional EA mutant but instead a panel of mutants observed in patient samples which they had demonstrated were catalytically inactive. They only found lung metastasis in mice injected with the cells expressing the range of catalytically inactive MMP8 mutants and so concluded that mutant MMP8 may have a dominant negative function. In this work no lung metastasis were found, even in the mice that had primary tumours. To summarise, MMP8, independently of its catalytic activity, reduces tumour formation on subcutaneous injection of MMP8 over-expressing MDA-MB-231 cells. This may also be independent of the raised IL-6 and -8 secretions by the wild-type MMP8 over-expressing cells. These conflicting reports are likely due to differences in the mouse model used, the type of cancer cells studied and the species of MMP8 examined. Further *in vivo* experiments could be carried out to further dissect the role of MMP-8 in mammary carcinoma. An *Mmp8* knockout mouse model has already been established in the laboratory which examines the rate of mammary tumour growth using the PyMT mouse model of mammary carcinogenesis (Dr. Julie Decock), with preliminary data suggesting that MMP8 reduces the rate of tumour growth and lung metastasis. Another option would be to generate MMP-8 over-expressing mouse mammary carcinoma cells (EMT6 mammary epithelial carcinoma cells isolated from the BALB/cCrgl mouse) which could then be injected sub-cutaneously into a mouse with an intact immune system. However, within the scope of this thesis the model used was most appropriate for the human tools we had available.

To summarise and conclude this chapter, expression of wild-type MMP-8 is detrimental to MDA-MB-231 cells on mid-term culture, dependent on its catalytic activity. In addition, wild-type MMP-8 expression is shut off in the

majority of stably transfected cell lines, whereas cells transfected with the E198A mutant MMP-8 maintain their expression long-term. In these cells IL-6 and IL-8 levels are at baseline. Rare clones of stably transfected MDA-MB-231 cells have been established that express wild-type MMP-8 “long-term” for months and within these cells permanent phenotypic changes have occurred allowing for the continued elevated IL-6 and IL-8 levels even in the absence of MMP-8. Within these rare cells MMP-8 initiated a self-reinforcing loop whereby IL-8 is partly responsible for the increase in IL-6 levels and IL-6 can induce MMP8 expression by a small amount. This loop is dependent on NF κ B signalling and also in this chapter has been partly been found to be dependent on PAR2. PAR2 mRNA levels are increased in clones over-expressing wild-type MMP8, but levels in clones expressing the vector control and E198A mutant MMP8 are equivalent. PAR2 is likely acting downstream of MMP-8, although indirectly, or in combination with another protein, as MMP8 siRNA did not have any effect on PAR2 mRNA levels over 48 hours of siRNA knockdown. However, PAR2 siRNA reduced IL-6 levels indicating its involvement in this self-reinforcing loop. IL-8 levels actually increased on PAR2 knockdown, as was the case in MMP8 siRNA knockdown and also TGF β inhibition, although TGF β inhibition had no impact on the expression of IL-6. This shows that IL-6 and -8 regulation can be uncoupled from each other and interdependently regulated as well as coordinately. Despite this knowledge there are many other factors yet to be discovered in this system, and the data gained from the behaviour of the stably transfected MDA-MB-231 cells *in vivo* add a further level of complexity as MMP-8, independent of its catalytic activity, and also independent of elevated IL-6 and IL-8 levels, reduce/prevented primary tumour growth.

This chapter has shown that wild-type MMP-8 is routinely switched off by MDA-MB-231 mammary carcinoma cells and prevents colony growth. Phenotypic changes may have occurred in cells that do express MMP-8 long-term as a coping mechanism. Changes, in addition to up-regulation of IL-6 and IL-8, have likely occurred in such cells, and these require consideration in the clinic.

5.6 Summary

- Wild-type MMP-8 is detrimental to mammary carcinoma cells and expression is epigenetically silenced, through presently unknown mechanisms.
- Rare wild-type MMP-8 expressing stable clones emerge that can express wild-type MMP-8 continuously for months
- Permanent phenotypic changes occurred within these “long-term” wild-type MMP-8 expressing cells to cope to allow continued MMP-8 expression, and this may include the permanent up-regulation of IL-6 and IL-8
- “Long-term” wild-type MMP-8 expressing cells are no longer exhibiting dynamic fluctuations in IL-6 and IL-8 and have altered permanently
- MMP-8, independent of its catalytic activity suppresses primary tumour growth after subcutaneous injection into CD1 nu/nu mice
 - IL-6 and IL-8 may not be functional *in vivo* as both wild-type and catalytically inactive E198A mutant MMP-8 expressing cells prevent primary tumour growth. This however cannot be confirmed as it is not known for certain whether the mice can react to the human IL-6 and -8, neither whether IL-6 and IL-8 were still being expressed in the injected cells.

Chapter 6- General Discussion and Future Directions

MMP-8 has a tumour protective role in human breast cancer and other cancers. However, apart from the knowledge that MMP8 is required for cognate immune responses, little is known mechanistically about how it exerts these effects. Its influence on the immune system during acute immune responses suggests that MMP8 may be tumour-protective via its immuno-modulatory functions. The aims of this work were to dissect the functions of MMP8 in mammary carcinoma cells through the generation of an MMP8 *in vitro* expression model.

To summarise this work in its entirety, wild-type and catalytically inactive E198A MMP8 expression constructs were generated. These were functional in both transient and stable transfection into mammary carcinoma cells, with MMP-8 predominantly secreted from cells into the culture media and wild-type having activity against type I collagen. To determine the long-term effects of MMP-8, MDA-MB-231 cells were transfected to stably express wild-type MMP8, catalytically inactive MMP8 to control for effects dependent on the catalytic activity of MMP-8, and an empty vector control to control for effects of transfection. Stable transfection of MMP-8 proved problematic but a set of MDA-MB-231 clonal isolates were generated that expressed wild-type MMP-8 “long-term”. Using these “long-term” MDA-MB-231 stable transfectants behavioural characteristics were examined and it was found that MMP-8 did not affect cell growth or cell cycle profile, but it reduced random 2D migration dependent on catalytic activity and MMP-8 reduced scratch wound closure independent of catalytic activity. MMP-8 also increased cellular adhesion in some experiments. Additionally, MMP-8 prevented primary tumour growth after subcutaneous injection of MDA-MB-231 stable cell lines into CD1 nu/nu immunocompromised mice. MMP-8 did not affect EMT (at least in the markers examined) and expression had minimal effects on the protease web, with only an increase in TIMP3 mRNA levels in cells expressing wild-type MMP-8.

Interestingly, MMP-8 induced expression of IL-6 and IL-8 in malignantly transformed mammary carcinoma cells on acute exposure (transient transfection) dependent on its catalytic activity. This same up-regulation also occurred in the “long-term” expressing MDA-MB-231 stable cells, again dependent on catalytic activity. Within these “long-term” wild-type MMP-8 expressing MDA-MB-231 clonal isolates MMP-8 initiated a self-reinforcing loop with IL-6 and IL-8, with IL-8 having a role in IL-6 promotion and IL-6 having a role in the promotion of MMP8 expression. Mechanistically this effect was dependent on NF κ B activity and also possibly on p38 MAPK signalling. However, IL-6 and IL-8 could be differentially regulated, as IL-8 was inhibited by TGF β signalling and also by PAR2, whereas IL-6 levels were unaffected by TGF β signalling and attenuated on PAR2 siRNA knockdown. Wild-type MMP-8 resulted in a modest increase in PAR2 mRNA expression levels although this is likely indirect as MMP-8 siRNA did not affect PAR2 levels. Potential additional players in this system were identified by mass spectrometry but require further investigation.

Along with some tumour suppressive phenotypic characteristics and MMP-8 driven IL-6 and IL-8 up-regulation, the fundamental take-home message of this work is that wild-type MMP-8 is detrimental to mammary carcinoma cell growth on medium term exposure (approximately 20 days exposure) and apparently as a direct consequence cells switch off the expression of wild-type MMP-8 transfected gene, likely through epigenetic silencing. In these cells that have lost wild-type MMP-8 expression, IL-6 and IL-8 levels returned to baseline, supporting a causal link between MMP-8 and IL-6 and -8. However, the majority of this work was carried out on a rare set of wild-type MMP-8 expressing stable clones that can express wild-type MMP-8 continuously for months. It was inferred that permanent phenotypic changes had occurred within these “long-term” wild-type MMP-8 expressing cells to allow continued MMP-8 expression, and this may include the permanent up-regulation of IL-6 and IL-8, independently of MMP-8, rather than a dynamic interaction between MMP-8 and the inflammatory molecules. It is also unclear whether IL-6 and IL-8

Chapter 6- General Discussion and Future Directions

from these cells are functional *in vivo* as both wild-type and catalytically inactive E198A mutant MMP-8 expressing cells prevent primary tumour growth after subcutaneous injection into CD1 nu/nu mice. This could not be certified however as although the mice should be able to react to higher IL-6 and IL-8 levels they were immunocompromised so the model was not entirely valid. Despite this, the tumour-protective roles of MMP8 were reinforced by this experiment and throw up some interesting ideas to develop this project further. As a whole this work has contributed to the limited knowledge of the role of MMP-8 in breast cancer cells and has also highlighted an additional role for MMP-8 in the immune system with respect to IL-6 and IL-8 up-regulation which has so far been unreported.

Due to the published pro-tumourigenic roles of both IL-6 and IL-8 [123-125, 130, 142-144] it at first appeared paradoxical that anti-tumourigenic MMP-8 would up-regulate these proteins. However, MMP-8 may exert different effects acutely and chronically, which may influence its effects on immune system mediating molecules, and thus tumourigenesis. It is already widely acknowledged that MMP-8 can cleave IL-8 *in vitro*, and that MMP-8 presence is required for cognate immune responses [80]. Absence of MMP-8 results in chronic inflammation [14, 64, 80, 83] which can promote tumour development [21, 296]. It has also been hypothesised that MMP-8 may influence IL-8 levels, as it was shown that *Mmp8* knockout mice in an experimental model of periodontitis had reduced IL-8 levels, although this was hypothesised to be a reduction in release of IL-8 from extracellular matrix stores in the absence of *Mmp8* [49]. This work has shown that MMP-8 may operate to induce IL-8 at the expression level, although it is also plausible that MMP-8 may also liberate IL-8 from ECM stores. There have been no previous reports suggesting that MMP-8 can influence IL-6 levels, so this aspect of this work is novel.

MMP-8 produced by neutrophils is thought to be vital for normal innate immune responses. However, it is plausible that MMP-8 from tumour cells themselves could initiate an immune response against the expressing cell in order to destroy themselves and reduce cancer growth and spread. Acute

expression of MMP-8 by tumour cells may drive the expression and release of IL-8, attracting neutrophils to the cancer cell and eventually resulting in destruction of the cancer cell. This hypothesis can also be applied to the mechanism behind which MMP-8 reduces metastasis, as any MMP-8 expressing micrometastasis will be party to their own destruction. This work has shown that induction of IL-8 expression in acute response to MMP-8 only occurs in malignantly transformed cells, making this a valid hypothesis. In addition, the IL-6 secreted by the cells, which in these “long-term” MMP-8 expressing cells is partly driven by IL-8 can be translated to an *in vivo* situation where IL-6 induced by MMP-8 and IL-8 may act to promote the immune response. Whether IL-8 can induce IL-6 in an immune situation is not studied in the literature. However, this may be plausible as there are many pathological and inflammatory conditions where they are up-regulated together [297-301]. Further support for MMP-8 initiating destruction of the host cell comes from a recent study showing that IL-6 and IL-8 are secreted by apoptosing cells and may function to recruit phagocytes to destroy and remove the cell [302].

As cancer progresses cells accrue mutations that enable immune system evasion [303]. If this occurs then IL-6 and IL-8 secreted from MMP-8 expressing cells will switch from having a role in cancer destruction, to a role in cancer promotion. IL-6 and IL-8 can induce Cancer-Stem Cell (CSC) -like changes in mammary carcinoma cells, such as self-renewal [304-306]. For example The IL-8 signalling pathway is up-regulated and active in breast CSC [307]. Inhibition of IL-8 signalling by CXCR1/2 small molecule has been shown to be effective at selectively targeting CSC in xenograft models [304] and at reducing mammosphere formation of primary breast cancer cells [307]. In addition, it has been shown that IL-8 levels in breast cancer patient metastatic fluid correlated with increased mammosphere formation of tumour cells from the same patient, which was propagated through CXCR1/2 [306]. IL-6 can transform breast cells to mammosphere forming CSCs in an epigenetic switch involving NFκB, that resulted in long-term, stable, transformed cells that maintained a CSC state even when the initial inducing signal was removed [305, 308].

Chapter 6- General Discussion and Future Directions

The “long-term” MMP-8 expressing MDA-MB-231 cells used throughout this work exhibit elevated IL-6 and IL-8 levels independent of MMP-8 presence, suggesting that the cells have undergone permanent genetic or epigenetic changes. These changes may be driving the cells towards a Cancer Stem Cell (CSC) phenotype. The definition of a CSC is “...a cell within a tumour that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour” [309]. A small proportion of cells in commercially available mammary carcinoma cell lines, including MDA-MB-231, exhibit the molecular signature of CSCs such as positive staining for ALDH [310], a commonly used marker to separate stem from non-stem breast cancer cells, and ability to grow in mammosphere culture [311]. The mammosphere assay, developed by Dontu [292], is a non-adherent *in vitro* culture where differentiated cells that require adherence for growth die, and only cells that have the capacity for self-renewal can survive and proliferate. These cells form spherical floating cultures called mammospheres [292]. This assay, plus the ability for cells to colonise *in vivo*, are the two ‘gold standard’ methods for detecting CSC activity.

MMP-8 appears to induce permanent alterations in the “long-term” expressing MDA-MB-231 stable transfectants. Due to the published of IL-6 and IL-8 in driving the self-renewal of breast CSCs, especially the evidence that IL-6 can drive permanent transformation, it would therefore be appropriate to determine whether MMP-8, via IL-6 and IL-8 up-regulation, is driving differentiated cells to be CSC. Alternatively, MMP-8 may be supporting the stem cell niche. Future work could include the mammosphere assay and looking at the ALDH levels in the cells, as if MMP-8 was driving cells to a CSC phenotype we would expect to see increased mammosphere formation and self-renewal of the MMP-8 expressing cells, and also a higher proportion of these cells would be ALDH positive.

Within these “long-term” MMP-8 expression cells it is still not known whether cells up-regulate IL-6 and IL-8 as a coping strategy to over-come the detrimental effects of wild-type MMP-8 on mid-term culture, or whether they are up-

regulated as a by-product. Acute exposure to MMP-8 by transient transfection did not appear to have any detrimental effects on the mammary carcinoma cells, but they still induced IL-6 and IL-8 expression. Perhaps on this time-frame MMP-8 is still dynamically affecting the cells, and this reflects the system observed *in vivo* in acute inflammation to a cancer cell. However, the “long-term” MMP-8 expressing cells have overcome the detrimental effects on cell growth and viability evidenced by reduced colony formation 20 days post transfection. Possibly IL-6 and IL-8 levels are initially maintained in response to MMP-8, but perhaps continued exposure may have caused both MMP-8 dependent and independent changes within the cell whereby their expression profiles have permanently altered. If IL-6 and IL-8 were up-regulated as a coping mechanism it would be expected that if IL-6 and IL-8 were removed from the cells the MMP-8 dependent negative effects would dominate resulting in reduced cell growth and even maybe cell death. Knock-down of IL-6 and IL-8 by siRNA had no effect on the cell cycle profile of the “long-term” MMP-8 expressing cells, neither was there an increase in cell population in the subG1 phase, which indicates apoptosing cells. This suggests that IL-6 and IL-8 are not sufficient to compensate for the deleterious effects of MMP-8, and that there may be additional growth factor/cytokine loops altered within these cells that have not yet been discovered, and that are acting in conjunction with IL-6 and IL-8 to maintain cell viability. Another suggestion is that if cells have been driven to a CSC-like phenotype anchorage dependent growth may not be affected, but the cells may exhibit reduced anchorage independent growth on siRNA knockdown of IL-6 and IL-8. Additionally, it may take longer for the negative effects of MMP-8 to prevail, and a short-term siRNA transfection may not be enough. In contrast, the suggestion by Cullen *et al.*, (2013) that IL-6 and IL-8 are secreted by cells undergoing apoptosis as a “Find Me” signal, fits with observations within this work that MMP-8 may be inducing apoptosis on medium-term exposure to the protease, and thus resulting in IL-6 and IL-8 secretion [302]. The majority of MMP-8 stable transfectants generated switched off MMP-8 expression gradually over-time, with a corresponding reduction in IL-6 and IL-8 levels, suggesting that it may take time for such effects to be

reversed. These are all strong possibilities and in the future require further investigation and clarification.

The hypothesis that long-term MMP-8 may be driving cells to a CSC-like phenotype would constitute a more aggressive cell. However, this work has also shown that the “long-term MMP-8 expressing cells have some tumour suppressive functions. On the other side of the table this work has shown that MMP-8 has anti-tumourigenic effects in the functional studies looking at migration, invasion and adhesion. Some of these effects were independent of proteolytic activity. In addition, MMP-8, independent of its catalytic activity, prevented primary tumour growth in immuno-compromised mice. The ‘gold-standard’ for assessing the stem cell capabilities of cells is the ability for cells to develop primary tumours after injection into immuno-compromised mice [312], and this work has therefore given some indication that MMP-8 may actually reduce the stem cell fraction. All of these assays suffer from the same flaw however as effects of MMP-8 are examined in isolated cell lines. Obviously the *in vivo* assay is much more physiologically relevant but the mice do not have a fully functioning immune system. They should react to human IL-6 and IL-8 but the complexities of the immune system may mean that the absence of T-Cells in these mice may have some unknown profound effects on their reactions to the cytokines. This experiment may also suggest that MMP-8 employs different molecular mechanisms *in vivo* than *in vitro*, with different molecules and pathways affected by MMP-8 expression. To confirm this further analysis of MMP-8 *in vivo* is required.

Although it has been observed consistently that wild-type MMP-8 induces IL-6 and IL-8 expression in malignantly transformed cells the precise mechanisms involved remain elusive. As summarised in figure 5.9, this induction is dependent on NF κ B signalling, and IL-6 is also dependent on PAR2. IL-6 and IL-8 are also differentially regulated as TGF β and PAR2 signalling inhibit IL-8. However, it is unlikely that the changes in IL-6 and IL-8 expression are caused by direct interactions between MMP-8, NF κ B and PAR2. S100A8, Annexin A1 and Hornerin were identified in chapter 4 as MMP-8 binding partners, so may fit

into the IL-6 and IL-8 regulatory mechanism proposed in this work. Like MMP-8 and PAR-2, S100A8 and Annexin A1 are produced by the neutrophils [202, 230-232]. There have been no published reports linking Annexin A1 to PAR2 but recently two studies have shown that S100A8 exerts anti-oxidant [313], anti-inflammatory [314] effects on neutrophils via PAR-2. This left the initial inflammatory response unaffected but dampened it down later [313], suggesting its requirement for cognate innate immune responses. This mechanism was uni-directional as PAR-2 was required for S100A8 functionality but S100A8 had no effect on PAR-2 activation [313]. This work hypothesised that MMP-8, which can bind S100A8, was acting up-stream of PAR-2. Lack of effect of MMP-8 siRNA knockdown on PAR2 mRNA levels suggested that MMP-8 was not acting on PAR-2 directly, but by other undetermined proteases. However, perhaps PAR-2 may be up-stream of MMP-8 in the signalling cascade and the up-regulation of PAR-2 in MMP-8 expressing cells may be the consequence of unknown factors, or even IL-6 and IL-8 as part of the self-reinforcing loop discussed in chapter 4. No evidence could be found in the literature to suggest that IL-6 and IL-8 can induce PAR2 though. In addition, no evidence could be found linking Hornerin to PAR2. Both PAR-2 and TGF β inhibited IL-8 expression in this work, and one study has shown that addition of hrTGF β to endometrotic stem cells increased PAR2 gene expression levels, resulting in an increase in IL-6 [285]. It is widely acknowledged in the literature that TGF β can induce IL-6 [315-317] but in this work a TGF β inhibitor had no effect on IL-6 protein levels. However, if TGF β is acting through PAR-2, residual PAR-2 protein present may be enough to sustain IL-6 protein levels. PAR-2 appears to have a slow turnover as in this work siRNA knockdown of PAR-2 reduced mRNA levels by 90% but protein levels were not reduced by nearly as much. To add further complexity, TGF β had been shown to down-regulate MMP-8 RNA and protein expression [88], although this was in dental pulp cells rather than carcinoma cells or neutrophils. Therefore, no further light has been shed on the mechanisms underpinning the up-regulation of IL-6 and IL-8 with wild-type MMP-8 expression, except that TGF β and S100A8 may also be involved. To gain further clarity into the signalling cascades initiated by MMP-8 it would be useful in the future to study the growth and other phenotypic

characteristics of these “long-term” MMP-8 expressing MDA-MB-231 cells in co-culture with other cell types such as stromal fibroblasts in an organoid model such as those used by Holliday *et al.*, (2009) and Nyström *et al.*, (2005) [271, 318].

To conclude, this work has contributed to the understanding of the molecular mechanisms of MMP-8 under-pinning its anti-tumourigenic roles. Despite many unanswered questions, unconfirmed conjecture and hypothesising, it is clear that MMP-8 is detrimental to carcinoma cells and has a more commanding role in the immune system than previously thought, to which its anti-tumourigenic functions may be attributed. Continuation of this work will include determination of the underlying function of MMP-8, such as whether MMP-8 drives cells towards a CSC phenotype, or whether MMP-8 actually reduces the stem cell population, and whether IL-6 and IL-8 up-regulation is a consequence of prolonged MMP-8 expression, is a cellular coping mechanism for MMP-8, or whether they serve any functionality at all. Additionally further *in vivo* work is required to define how MMP-8, irrespective of catalytic activity, reduces primary tumour growth. This work has answered some questions, but also set up a host more and revealed further stepping stones, to increase understanding of the elusive anti-tumourigenic protease, MMP-8.

6.1 Summary of all experiments

This table (table 6.1) shows a summary of all the functional cell experiments within this thesis.

Experiment	Section	Cells	Result	Dependent on MMP-8 Catalytic Activity
Collagen Digestion	3.1.3	MDA-MB-231 stably transfected cells	Wild-type MMP-8 over-expressing cells degrade collagen 3x more than control cells	Yes
Cell growth	3.2.1	MDA-MB-231 stably transfected cells	No difference	N/A
Cell cycle	3.2.1	MDA-MB-231 stably transfected cells	No difference	NA
2D Migration	3.2.3	MDA-MB-231 stably transfected cells	Wild-type MMP-8 reduced migration	Yes
Scratch wound assay	3.2.3	MDA-MB-231 stably transfected cells	Wild-type and E198A mutant MMP-8 reduced scratch wound closure	No
3D inverted invasion through type I collagen	3.2.4	MDA-MB-231 stably transfected cells	Wild-type MMP-8 slightly reduces invasion	Yes
Adhesion	3.2.4	MDA-MB-231 stably transfected cells	Wild-type MMP-8 increased adhesion depending on the assay	Yes
EMT	3.2.5	MDA-MB-231 stably transfected cells	No	NA
Changes in degradome profile	3.3	MDA-MB-231 stably transfected cells	TIMP3 RNA levels may be increased with wild-type MMP-8 expression	Yes

Table 6.1: A summary of the main experiments within this thesis

Chapter 6- General Discussion and Future Directions

Experiment	Section	Cells	Result	Dependent on MMP-8 Catalytic Activity
IL-6 and IL-8 up-regulation	4.1	Transient transfection of MMP8 into MCF-7, MDA-MB-231 and SK-BR-3 cells and MDA-MB-231 stably transfected cells	Wild-type MMP-8 increased IL-6 and IL-8 protein and RNA levels	Yes
PAR2 up-regulation	5.3.2	MDA-MB-231 stably transfected cells	Wild-type MMP-8 increased PAR2 mRNA	Yes
Mass Spectrometry analysis of MMP-8 binding partners	4.2.3	MDA-MB-231 stably transfected cells	Wild-type and E198A mutant MMP-8 bound S100A8 and Annexin A1, wild-type MMP-8 bound to Hornerin	NA
Loss of MMP-8 expression in stably transfected cells	5.1	Pools of stably transfected MDA-MB-231 cells	Wild-type MMP-8 expression was lost from cells	Yes
Acute effects of MMP8 on apoptosis	5.2.1	Transiently transfected MDA-MB-231 and MCF-7 cells	No difference	NA
Colony formation	5.2.2	MDA-MB-231 cells	Transfection of wild-type MMP8 reduced colony formation	Yes
Subcutaneous injection of cells into CD1 nu/nu immuno-compromised mice	5.4	MDA-MB-231 stably transfected cells	Both wild-type and E198A mutant MMP-8 prevented primary tumour growth	No

Table 6.1: A summary of the main experiments within this thesis

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Appendix 1- MMP8 sequence

Key: **Bold underlined** = start codon, **blue** = wild-type base, **red** = mutant base

Wild-type MMP8 sequence using T7 primer:

DNA sequence:

CCAGGCTGACCTGCCGTTTAACTTAAGCTTGGTACCGAGCTCGGATCCAC
TAGTCCAGTGTGGTGGGAATTCATGTTCCTCCCTGAAGACGCTTCCATTCT
GCTCTTACTCCATGTGCAGATTTCCAAGGCCTTTCCTGTATCTTCTAAAGA
GAAAAATACAAAAATTGTTCAAGACTACCTGGAAAAGTTCTACCAATTA
CCAAGCAACCAGTATCAGTCTACAAGGAAGAATGGCACTAATGTGATCG
TTGAAAAGCTTAAAGAAATGCAGCGATTTTTTTGGGTTGAATGTGACGGGG
AAGCCAAATGAGGAACTCTGGACATGATGAAAAAGCCTCGCTGTGGAG
TGCCTGACAGTGGTGGTTTTATGTTAACCCAGGAAACCCCAAGTGGGAA
CGCACTAACTTGACCTACAGGATTCGAAACTATACCCACAGCTGTCAGA
GGCTGAGGTAGAAAGAGCTATCAAGGATGCCTTTGAACTCTGGAGTGTT
GCATCACCTCTCATCTTACCAGGATCTCACAGGGAGAGGCAGATATCAA
CATTGCTTTTTACCAAAGAGATCACGGTGACAATTCTCCATTTGATGGAC
CCAATGGAATCCTTGCTCATGCCTTTCAGCCAGGCCAAGGTATTGGAGGA
GATGCACATTTTGATGCCGAAGAAACATGGACCAACACCTCCGCAAATT
ACAACCTGTTTCTTGTGCTGCTCATGAAATTTGGCCATTCTTTGGGGCTCG
CTCACTCCTCTGACCCTGGTGCCTTGATGTATCCCAACTATGCTTTCAGGG
AAACCAGCAACTACTCACTCCCTCAAGATGACATCGATGGCATTACAGCCA
TCTATGGACTTTCAAGCAACCCTATCCAACCTACTGGACCAAGCACACCC
AAACCCTGTGACCCAGTTTGACATTTGATGCTATCACCACACTCCGTGG
AGAAATACTTTTCTTTAAAGACAGGTA CTTCTGGAGAGGCATCCTCAGCT
ACAAGAGTCGAAATGAATTTTATTTCTCTATTCTGGCCATCCCTTCCAAC
GGTAAACAGGCTGCTTATGGAGATTTTGACGAGACCTCATTCTATTAAG
GCACATACTGGGCTCTGAGTGCTATGATATTCTGCAAGTTATCCCAGATA
TTTTCAAACATATGCCTCCCCAAGCAGCGTCAAGCATTGAACGCGACTGGT
TTCTACGAAGTAACTTACTCTGAATGACATTCTGGAATTGGATACAGAC
ATTTCCATGAAGCAGGATTATTCACAA

Translated protein sequence 5'-3':

ccaggctgacctgccgtttaacttaagcttggtagccgagctcggatccactagtccagtgtg
R L T C R L T - A W Y R A R I H - S S V
gtggaattcatgtctccctgaagacgcttccatttctgctcttactccatgtgcagatt
V E F M F S L K T L P F L L L L H V Q I
tccaaggccttctctgtatcttctaagagaaaaatacaaaaattgttcaggactacctg
S K A F P V S S K E K N T K I V Q D Y L
gaaaagttctaccaattaccaagcaaccagtatcagtctacaaggaagaatggcactaat
E K F Y Q L P S N Q Y Q S T R K N G T N
gtgatcgttgaaaagccttaagaaatgcagcgattttttgggttgatgtgacggggaag
V I V E K L K E M Q R F F G L N V T G K
ccaaatgaggaaaactctggacatgatgaaaaagcctcgctgtggagtgcctgacagtgg

Appendix 1- MMP8 sequence

P N E E T L D M M K K P R C G V P D S G
ggttttatgtaaccccaggaaccccaagtgggaacgcactaacttgacctacaggatt
G F M L T P G N P K W E R T N L T Y R I
cgaaactatacccccacagctgtcagaggctgaggtagaaagagctatcaaggatgccttt
R N Y T P Q L S E A E V E R A I K D A F
gaactctggagtggtgcatcacctctcatcttcaccaggatctcacagggagaggcagat
E L W S V A S P L I F T R I S Q G E A D
atcaacattgctttttaccaaagagatcacggtgacaattctccatttgatggaccaat
I N I A F Y Q R D H G D N S P F D G P N
ggaatccttgctcatgcctttcagccaggccaaggtattggaggagatgcacattttgat
G I L A H A F Q P G Q G I G G D A H F D
gccgaagaaacatggaccaacacctccgcaaattacaacttgtttcttggtgctgctcat
A E E T W T N T S A N Y N L F L V A A H
gaatttggccattctttggggctcgtcactcctctgaccctggccttgatgatccc
E F G H S L G L A H S S D P G A L M Y P
aactatgctttcagggaaaccagcaactactcactccctcaagatgacatcgatggcatt
N Y A F R E T S N Y S L P Q D D I D G I
cagccatctatggactttcaagcaaccctatccaacactactggaccaagcacacccaaac
Q P S M D F Q A T L S N L L D Q A H P N
cctgtgaccccagtttgacatttgatgctatcaccacactccgtggagaaatacttttct
P V T P V - H L M L S P H S V E K Y F S
ttaaagacaggtacttctggagaggcatcctcagctacaagagtcgaaatgaattttatt
L K T G T S G E A S S A T R V E M N F I
tctctattctggccatcccttccaactggtaaacaggctgcttattggagattttgacgag
S L F W P S L P T G K Q A A Y G D F D E
acctcattttctattaaggcacatactgggctctgagtgctatgatattctgcaagttatc
T S F L L R H I L G S E C Y D I L Q V I
ccagatattttcaaactatgcctcccccaagcagcgtcaagcattgaacgcgactggtttc
P D I F K L C L P K Q R Q A L N A T G F
tacgaagtaaacttactctgaatgacattctggaattggatacagacatttccatgaagc
Y E V N L L - M T F W N W I Q T F P - S
aggattattcacia
R I I H

Appendix 1- MMP8 sequence

E198A mutant MMP8 sequence using T7 primer:

DNA sequence:

TCGGCCTTAGCCTGCCGTTTACTTAAGCTTGGTACCGAGCTCGGATCCAC
TAGTCCAGTGTGGTGGAAATTCATGTTCTCCCTGAAGACGCTTCCATTTCT
GCTCTTACTCCATGTGCAGATTTCCAAGGCCTTTCCTGTATCTTCTAAAGA
GAAAAATACAAAAATTGTTTCAGGACTACCTGGAAAAGTTCTACCAATTA
CCAAGCAACCAGTATCAGTCTACAAGGAAGAATGGCACTAATGTGATCG
TTGAAAAGCTTAAAGAAATGCAGCGATTTTTTGGGTTGAATGTGACGGGG
AAGCCAAATGAGGAACTCTGGACATGATGAAAAGCCTCGCTGTGGAG
TGCCTGACAGTGGTGGTTTTATGTTAACCCACAGGAAACCCCAAGTGGGAA
CGCACTAACTTGACCTACAGGATTCGAAACTATACCCACAGCTGTCAGA
GGCTGAGGTAGAAAGAGCTATCAAGGATGCCTTTGAACTCTGGAGTGTT
GCATCACCTCTCATCTTACCAGGATCTCACAGGGAGAGGCAGATATAAA
CATTGCTTTTTACCAAAGAGATCACGGTGACAATTCTCCATTTGATGGAC
CCAATGGAATCCTTGCTCATGCCTTTCAGCCAGGCCAAGGTATTGGAGGA
GATGCACATTTTGTGATGCCGAAGAAACATGGACCAACACCTCCGCAAATT
ACAACCTGTTTCTTGTGCTGCTCATGCATTTGGCCATTCTTTGGGGCTCG
CTCACTCCTCTGACCCTGGTGCCTTGATGTATCCCAACTATGCTTTCAGGG
AAACCAGCAACTACTCACTCCCTCAAGATGACATCGATGGCATTTCAGGCC
ATCTATGGACTTTCAAGCAACCCTATCCAACCTACTGGACCAAGCACACC
CAAACCCTGTGACCCAGTTTGACATTTGATGCTATCACCACACTCCGTG
GAGAAATACTTTTCTTTAAAGACAGGTAATTCTGGAGAAGCATCCTCAGC
TACAAAGAGTCGAAATGAATTTTATTCTCTATTCTGGCCATCCCTTCCAA
CTGGTATACAGCTGCTATGAGATTTGACAGAGACCTCATTTTCTATTTAA
GCAACCAATACTGGGCTCTGAGTGCTATGAATATCTGCAAGTTATTCCCA
GAAATATCAACTATGCCTTCCCAGCACGGTTCAGCATTGACGCAGCTGTT
TTCTACGAGTAAACACTTCTTGTAAATGACATTTCGAATGAACAGACATCAT
GACAGTATCTCCAAGCATTATCAGGTTCCCATAGC

Translated protein sequence 5'-3':

tcggccttagcctgccgtttactttaagcttggtaaccgagctcggatccactagtccagtgtg
G L S L P F T - A W Y R A R I H - S S V
gtggaattcatgttctccctgaagacgcttccatTTctgctcttactccatgtgcagatt
V E F M F S L K T L P F L L L L H V Q I
tccaaggcctttcctgtatcttctaagagaaaaatacaaaaattgttcaggactacctg
S K A F P V S S K E K N T K I V Q D Y L
gaaaagttctaccaattaccaagcaaccagtatcagtctacaaggaagaatggcactaat
E K F Y Q L P S N Q Y Q S T R K N G T N
gtgatcgttgaaaagcttaagaaatgcagcgatTTTTTGGGTTgaatgtgacggggaag
V I V E K L K E M Q R F F G L N V T G K
ccaatgaggaaactctggacatgatgaaaaagcctcgctgtggagtgcctgacagtgg
P N E E T L D M M K K P R C G V P D S G
ggttttatgttaaccccaggaaacccaagtgggaacgcactaacttgacctacaggatt
G F M L T P G N P K W E R T N L T Y R I
cgaaactatacccccacagctgtcagaggctgaggtagaaagagctatcaaggatgccttt
R N Y T P Q L S E A E V E R A I K D A F

gaactctggagtgttgcatcacctctcatcttaccaggatctcacagggagagggcagat
E L W S V A S P L I F T R I S Q G E A D

Appendix 1- MMP8 sequence

ataaacattgctttttaccaaagagatcacggtgacaattctccatttgatggaccaat
I N I A F Y Q R D H G D N S P F D G P N
ggaatccttgctcatgcctttcagccaggccaaggattggaggagatgcacattttgat
G I L A H A F Q P G Q G I G G D A H F D
gccgaagaaacatggaccaacacctccgcaaattacaacttgtttcttgttgctgctcat
A E E T W T N T S A N Y N L F L V A A H
gcatttgccattctttggggctcgctcactcctctgaccctggggccttgatgtatccc
A F G H S L G L A H S S D P G A L M Y P
aactatgctttcagggaaaccagcaactactcactccctcaagatgacatcgatggcatt
N Y A F R E T S N Y S L P Q D D I D G I
caggccatctatggactttcaagcaaccctatccaacctactggaccaagcacacccaaa
Q A I Y G L S S N P I Q P T G P S T P K
cctgtgaccccagtttgacatttgatgctatcaccacactccgtggagaaatacttttc
P C D P S L T F D A I T T L R G E I L F
tttaaagacaggtacttctggagaagcatcctcagctacaaagagtcgaaatgaatttta
F K D R Y F W R S I L S Y K E S K - I L
tttctctattctggccatcccttccaactgggtatacagctgctatgagatttgacagaga
F L Y S G H P F Q L V Y S C Y E I - Q R
cctcatttcctatttaagcaaccaataactgggctctgagtgctatgaatatctgcaagtt
P H F L F K Q P I L G S E C Y E Y L Q V
attcccagaaatatcaactatgccttcccagcacgggttcagcattgacgcagctgttttc
I P R N I N Y A F P A R F S I D A A V F
tacgagtaaacacttcttgtaatgacattcgaatgaacagacatcatgacagtatctcca
Y E - T L L V M T F E - T D I M T V S P
agcattatcaggttcccatagc
S I I R F P -

Appendix 2- Phosphokinase array raw results

Raw data for the phosphokinase array described in chapter 4 section 4.2.2.

Figure A2.1 shows the membranes annotated with co-ordinates

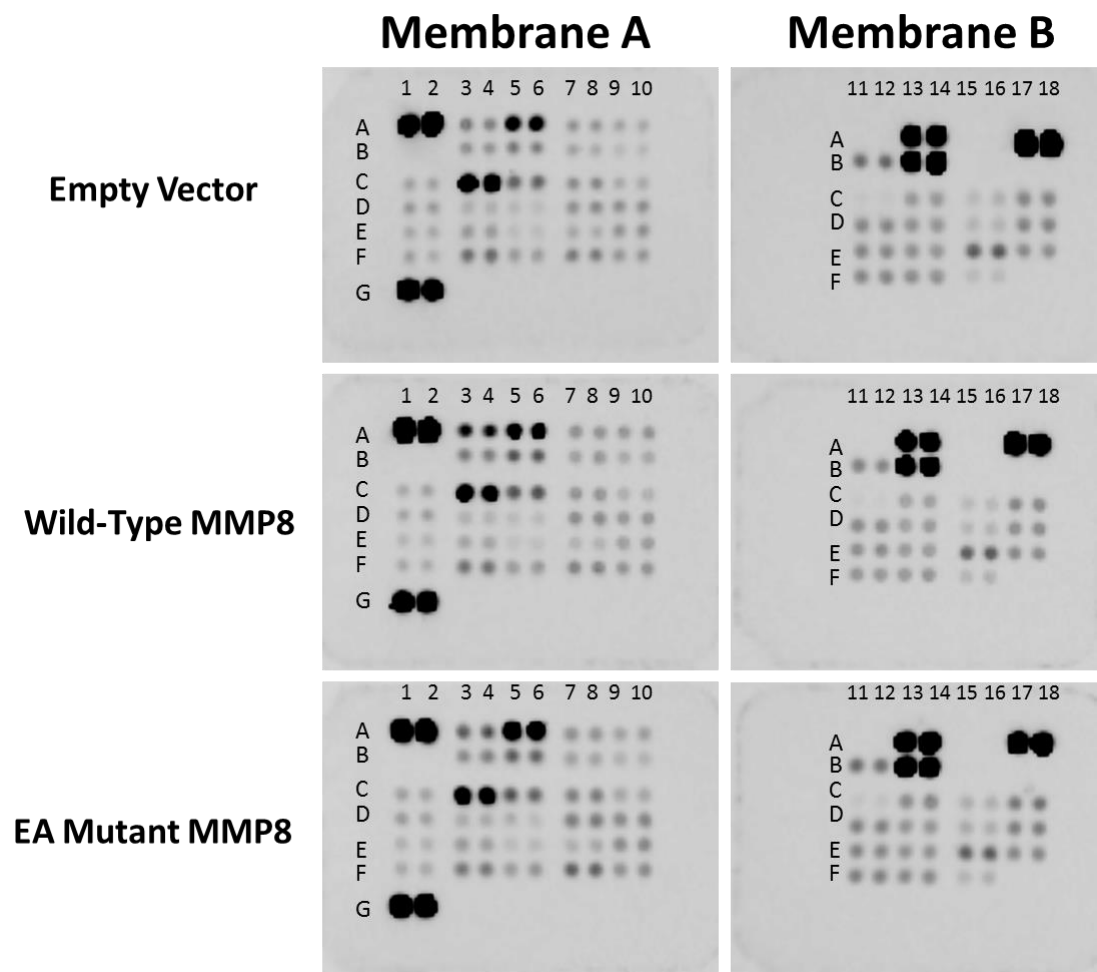


Figure A2.1: Effects of MMP-8 on kinase phosphorylation.

Phosphorylated kinase levels in MDA-MB-231 clonal isolates (set 1) stably transfected with wild-type and E198A mutant MMP8 and an empty vector control. Cell lysates were applied to membranes pre-printed with antibodies to an array of phosphorylated kinases. After following the manufacturer's instructions the membranes were imaged using HRP and the result was a membrane covered in dots of varying intensities.

Appendix 2- Phosphokinase array raw results

Table A2.1 show the pixel densities of the dots on the membranes in figure A2.1.

Membrane	Coordinate	Target	Pixel Density		
			Empty Vector	Wild-Type MMP8	EA Mutant MMP8
A	A1	Positive control			
A	A2	Positive control			
A	A3	p38 α (T180/Y182)	1491.749	3798.456	1559.042
A	A4	p38 α (T180/Y182)	1377.577	3260.627	1875.698
A	A5	ERK 1/2 (T202/Y204, T185/Y187)	6387.941	3215.92	8158.355
A	A6	ERK 1/2 (T202/Y204, T185/Y187)	6059.941	4858.042	7786.698
A	A7	JNK pan (T183/Y185, T221/Y223)	806.456	652.678	630.385
A	A8	JNK pan (T183/Y185, T221/Y223)	872.87	508.556	765.627
A	A9	GSK-3 α/β (S21/S9)	643.92	478.435	479.678
A	A10	GSK-3 α/β (S21/S9)	516.971	543.092	556.385
A	B3	MEK 1/2 (S218/S222, S222/S226)	2977.749	1965.749	2576.991
A	B4	MEK 1/2 (S218/S222, S222/S226)	2912.456	2225.941	3403.284
A	B5	MSK1/2 (S376/S360)	4149.163	3067.698	4659.698
A	B6	MSK1/2 (S376/S360)	3625.456	3021.577	4034.991
A	B7	AMPK α_1 (T174)	1875.042	1071.456	1734.991
A	B8	AMPK α_1 (T174)	1529.749	1289.042	1759.749
A	B9	Akt (S473)	927.627	791.335	1085.456
A	B10	Akt (S473)	966.042	744.799	1183.163
A	C1	TOR (S2448)	434.556	427.263	557.506
A	C2	TOR (S2448)	423.849	541.092	488.385
A	C3	CREB (S133)	8310.77	5511.406	2993.749
A	C4	CREB (S133)	6778.062	5575.577	4313.042
A	C5	HSP27 (S78/S82)	1250.042	2319.163	1714.335
A	C6	HSP27 (S78/S82)	1318.749	1984.749	1501.163
A	C7	AMPK α_2 (T172)	723.506	844.92	932.335
A	C8	AMPK α_2 (T172)	683.092	718.092	984.92
A	C9	β -Catenin	317.728	326.021	489.971
A	C10	β -Catenin	308.728	344.314	403.556
A	D1	Src (Y419)	3482.82	1539.335	1736.113
A	D2	Src (Y419)	2809.163	1953.991	1290.749
A	D3	Lyn (Y397)	920.213	836.092	535.092
A	D4	Lyn (Y397)	1062.213	715.971	640.213
A	D5	Lck (Y394)	971.749	685.506	699.627
A	D6	Lck (Y394)	949.749	741.042	583.678

Table A2.1. Effects ofMMP-8 on levels of kinase phosphorylation. Raw pixel density of the dots on the membranes in figure A2.1

Appendix 2- Phosphokinase array raw results

Membrane	Coordinate	Target	Pixel Density		
			Empty Vector	Wild-Type MMP8	EA Mutant MMP8
A	D7	STAT2 (Y689)	4344.406	3099.698	3107.82
A	D8	STAT2 (Y689)	5480.113	3243.87	3022.991
A	D9	STAT5a (Y699)	4851.82	2823.284	2250.87
A	D10	STAT5a (Y699)	4194.991	2381.991	3542.134
A	E1	Fyn (Y420)	2547.577	2529.406	1578.749
A	E2	Fyn (Y420)	2817.113	2794.113	1956.577
A	E3	Yes (Y426)	3785.87	4300.991	3269.577
A	E4	Yes (Y426)	3836.284	4585.698	3102.991
A	E5	Fgr (Y412)	1066.042	1433.92	1356.163
A	E6	Fgr (Y412)	881.799	984.92	1197.042
A	E7	STAT3 (S727/Y705)	1975.749	2072.749	1615.749
A	E8	STAT3 (S727/Y705)	1917.456	2314.335	2076.042
A	E9	STAT5b (Y699)	4401.406	3999.335	4555.577
A	E10	STAT5b (Y699)	4874.113	4612.284	4580.284
A	F1	Hck (Y411)	1821.163	1453.042	1469.406
A	F2	Hck (Y411)	1241.749	1417.87	1358.042
A	F3	Chk-2 (T68)	4725.113	4451.284	3952.406
A	F4	Chk-2 (T68)	5882.062	3928.456	3751.406
A	F5	FAK (Y397)	2293.698	1876.627	2226.284
A	F6	FAK (Y397)	2088.163	1948.213	2534.577
A	F7	STAT6 (Y641)	4032.82	3184.456	4992.406
A	F8	STAT6 (Y641)	4387.527	2883.335	4856.113
A	F9	STAT5a/b (Y699)	2704.527	1840.213	2364.577
A	F10	STAT5a/b (Y699)	2516.527	1690.506	2758.698
B	A13	p53 (S392)	6750.941	6424.234	6738.456
B	A14	p53 (S392)	7131.698	7578.406	4514.627
B	B11	Akt (T308)	1269.577	1081.163	1122.749
B	B12	Akt (T308)	1628.82	1066.042	1319.87
B	B13	p53 (S46)	7286.284	7113.406	5311.87
B	B14	p53 (S46)	2882.042	5252.627	5075.577
B	C11	p70 S6 Kinase (T389)	77.364	141.364	301.435
B	C12	p70 S6 Kinase (T389)	103.364	59.95	484.263

Table A2.1. Effects ofMMP-8 on levels of kinase phosphorylation. Raw pixel density of the dots on the membranes in figure A2.1

Appendix 2- Phosphokinase array raw results

Membrane	Coordinate	Target	Pixel Density		
			Empty Vector	Wild-Type MMP8	EA Mutant MMP8
B	C13	p53 (S15)	3496.527	3165.406	3596.456
B	C14	p53 (S15)	4235.648	3290.749	3323.749
B	C15	p27 (T189)	1286.991	1393.991	994.92
B	C16	p27 (T189)	1838.82	1070.92	886.213
B	C17	Paxillin (Y118)	5655.062	4586.991	2845.92
B	C18	Paxillin (Y118)	5573.648	3489.335	2793.92
B	D11	p70 S6 Kinase (T421/S424)	4459.406	5002.82	5678.355
B	D12	p70 S6 Kinase (T421/S424)	4750.698	4973.284	5020.234
B	D13	RSK 1/2/3 (S380)	4666.355	3111.284	3230.87
B	D14	RSK 1/2/3 (S380)	4142.82	3268.87	3491.406
B	D15	p27 (T175)	1690.648	1287.335	2014.406
B	D16	p27 (T175)	1969.234	1518.163	1834.577
B	D17	PLCy-1 (Y783)	6084.477	4200.698	4332.577
B	D18	PLCy-1 (Y783)	6108.477	4177.163	4902.991
B	E11	p70 S6 Kinase (T229)	2408.87	5002.82	3333.82
B	E12	p70 S6 Kinase (T229)	2346.749	2213.456	2994.82
B	E13	RSK 1/2 (S221/S227)	2200.87	2302.042	2399.577
B	E14	RSK 1/2 (S221/S227)	1951.456	1845.749	2375.163
B	E15	c-Jun (S63)	4681.284	1990.749	5889.941
B	E16	c-Jun (S63)	4996.456	4664.284	5599.234
B	E17	Pyk2 (Y402)	2013.456	4944.991	2754.456
B	E18	Pyk2 (Y402)	2111.163	2966.406	2926.698
B	F11	STAT1 (Y701)	5299.355	2447.577	5179.234
B	F12	STAT1 (Y701)	5532.527	5204.113	5335.648
B	F13	STAT4 (Y693)	5091.648	4543.87	4547.941
B	F14	STAT4 (Y693)	4865.941	4597.87	4418.406
B	F15	eNOS (S1177)	941.506	1863.87	1126.456
B	F16	eNOS (S1177)	780.92	1897.92	1276.456
B	F17	Negative	0	0	0
B	F18	Negative	0	0	0

Table A2.1. Effects ofMMP-8 on levels of kinase phosphorylation. Raw pixel density of the dots on the membranes in figure A2.1

Appendix 3-

Thirkettle *et al.*, Matrix metalloproteinase-8 (collagenase-2) activates expression of interleukins-6 and -8 in breast cancer cells. J Biol Chem. 2013

Matrix Metalloproteinase 8 (Collagenase 2) Induces the Expression of Interleukins 6 and 8 in Breast Cancer Cells*

Received for publication, February 26, 2013, and in revised form, April 30, 2013. Published, JBC Papers in Press, April 30, 2013, DOI 10.1074/jbc.M113.464230

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Background: Matrix metalloproteinase 8 is a metastasis-suppressing metalloprotease that regulates neutrophil chemotaxis in inflammation.

Results: MMP-8 expression by breast cancer cells is deleterious to long-term growth, but it induces IL-6 and IL-8, factors that conventionally promote malignancy.

Conclusion: MMP-8 influences signaling that regulates expression of proinflammatory factors.

Significance: These effects may contribute to how MMP-8 controls the onset and resolution of inflammation.

Matrix metalloproteinase 8 (MMP-8) is a tumor-suppressive protease that cleaves numerous substrates, including matrix proteins and chemokines. In particular, MMP-8 proteolytically activates IL-8 and, thereby, regulates neutrophil chemotaxis *in vivo*. We explored the effects of expression of either a WT or catalytically inactive (E198A) mutant version of MMP-8 in human breast cancer cell lines. Analysis of serum-free conditioned media from three breast cancer cell lines (MCF-7, SK-BR-3, and MDA-MB-231) expressing WT MMP-8 revealed elevated levels of IL-6 and IL-8. This increase was mirrored at the mRNA level and was dependent on MMP-8 catalytic activity. However, sustained expression of WT MMP-8 by breast cancer cells was non-permissive for long-term growth, as shown by reduced colony formation compared with cells expressing either control vector or E198A mutant MMP-8. In long-term culture of transfected MDA-MB-231 cells, expression of WT but not E198A mutant MMP-8 was lost, with IL-6 and IL-8 levels returning to base line. Rare clonal isolates of MDA-MB-231 cells expressing WT MMP-8 were generated, and these showed constitutively high levels of IL-6 and IL-8, although production of the interleukins was no longer dependent upon MMP-8 activity. These studies support a causal connection between MMP-8 activity and the IL-6/IL-8 network, with an acute response to MMP-8 involving induction of the proinflammatory mediators, which may in part serve to compensate for the deleterious effects of MMP-8 on breast cancer cell growth. This axis may be relevant to the recognized ability of MMP-8 to orchestrate the innate immune system in inflammation *in vivo*.

The matrix metalloproteinases (MMPs)³ are a family of 24 secreted proteases in humans that have fundamental roles in the catabolic turnover of extracellular matrix structures. Their activities are important in both normal and pathological tissue remodeling processes. However, the past decade has witnessed a growing appreciation of the fact that although these enzymes were originally identified by virtue of their ability to break down extracellular matrix components, they also control cell behavior via precise regulatory proteolytic cleavage of growth factors, receptors, adhesion molecules, and chemokines (1). Moreover, MMPs were originally considered to play an exclusively facilitatory role in tumor cell invasion and metastasis, but following the failure of synthetic metalloproteinase inhibitors in the clinic (2, 3), it has become recognized that MMPs and proteases of other classes can have powerful actions as suppressors of malignancy (4, 5). This is particularly the case for MMP-8, which has been reported to suppress tumor formation or metastasis, depending on the model system (6–10).

Originally identified as neutrophil collagenase, MMP-8 was the second member of the MMP family shown to cleave triple-helical collagen fibrils (11). It is found in specific granules in neutrophils but is also expressed by diverse cell types, including epithelial cells, fibroblasts, macrophages, and endothelial cells (12). Several studies have demonstrated tumor- or metastasis-suppressive activities of MMP-8. Firstly, male *Mmp8*-null animals show a dramatic increase in the incidence of carcinogen-induced skin papillomas (6). Secondly, in clonal cell lines derived from MDA-MB-435 cancer cells that display high (M-4A4) or low (NM-2C5) metastatic ability, overexpression of MMP-8 reduced the metastatic ability of M-4A4 cells, whereas ribozyme-mediated gene knockdown of MMP-8 enhanced metastasis of the NM-2C5 line (7, 13). A C/T single nucleotide polymorphism in the *MMP8* promoter that affects MMP-8 expression has been linked to survival of breast cancer patients with early-stage disease, with the high expression T allele equating to better survival (8). Higher *MMP8* RNA levels in

* This work was supported by a Big C PhD studentship (to S. T.); by the Breast Cancer Campaign, Cancer Research-UK; and by European Union FP6 (Cancerdegradome) and FP7 (SaveMe) projects.

⌘ Author's Choice—Final version full access.

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³ The abbreviations used are: MMP, matrix metalloproteinase; LIX, LPS-induced CXC; EV, empty vector; PAR, protease-activated receptor.

primary breast cancers are associated with reduced lymph node metastasis and with improved relapse-free and overall survival in node-negative patients (9). The metastasis-suppressive properties of MMP-8 were confirmed in this study and shown to be dependent on its MMP activity (9). Expression of MMP-8 has also been found to be protective in human squamous cell carcinoma of the tongue and in a carcinogen-induced mouse model (10).

In addition to its suppressive roles in cancer progression, MMP-8 is protective in bleomycin-induced lung fibrosis (14), ventilator-induced lung injury (15), periodontitis (16), and allergen-induced airway inflammation (17). In contrast, *Mmp8*-deficient mice show reduced development of experimental autoimmune encephalomyelitis, suggesting that MMP-8 promotes pathology in this model of multiple sclerosis (18). It is likely that underlying all of these pathologies is the influence of MMP-8 on innate immune responses via its actions on chemokines and other immunomodulators. Cleavage of murine LPS-induced CXC chemokine (LIX) at Ser⁴-Val⁵ and Lys⁷⁹-Arg⁸⁰ by MMP-8 activates the chemokine, leading to enhanced neutrophil chemotaxis (19). Although other MMPs can process LIX *in vitro*, MMP-8 appears to be essential *in vivo* for LPS-stimulated neutrophil chemokinesis. MMP-8 also executes comparable processing of interleukin 8 (CXCL8/IL-8) and CXCL5/ENA-88, the human orthologues of LIX (19). Thus, MMP-8 released from neutrophils and potentially other cellular sources at sites of inflammation activates LIX/IL-8, creating a "feed-forward" response that drives further neutrophil recruitment and potentially orchestrating subsequent events in the inflammatory process, including its resolution. In the absence of MMP-8, inflammation is persistent and non-resolving, which is the situation found in the delayed skin wound healing seen in *Mmp8*-null mice (20).

On the basis of the known tumor- or metastasis-suppressive effects of MMP-8 in several cancers and its links to inflammatory responses, we sought to explore in more detail its actions on cytokine and chemokine networks elaborated by breast cancer cells. We show here that expression of catalytically active MMP-8 leads to increased production of the proinflammatory mediators IL-6 and IL-8 in multiple breast cancer cell lines but not in normal mammary epithelial cells. In turn, IL-6 enhances endogenous *MMP8* expression in breast cancer cells, and IL-8 is responsible in part for the increased expression of IL-6. Our data support the idea that MMP-8 can create a self-reinforcing protease-immunomodulatory circuit that may underpin its function in diverse physiological repair and defense mechanisms. However, we also show that expression of catalytically active MMP-8 is not sustainable in breast cancer cells in long-term culture without additional stochastic events that allow the emergence of high-expressing clones. These results may, therefore, help to integrate the roles of MMP-8 in inflammation and as a negative regulator of tumor progression.

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmids—MDA-MB-231 and MCF-7 breast cancer cell lines (ATCC) were maintained in DMEM + GlutaMAXTM (Invitrogen) supplemented with 10% fetal bovine serum. The SK-BR-3 breast cancer and G361 melanoma cell

lines (ATCC) were maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum.

The full-length coding sequence of human MMP-8 was cloned into the eukaryotic expression vector pcDNA4TM V5-His A plasmid (Invitrogen) using the BamH I and EcoR I restriction sites. A catalytically inactive mutant (E198A) was generated using a mutagenesis PCR kit (Stratagene, La Jolla, CA) and the following primers: 5'-CTTGTTGCTGCTCATGCATTGGCCATTCTTTGG and 5'-CCAAAGAATGGCCAAATGCA-TGAGCAGCAACAAG. Empty vector pcDNA4TM V5-His A was used as a control.

Transient and Stable Transfection—To obtain transient overexpression of MMP-8, cells were plated 24 h prior to transfection at a density of 3.1×10^4 /cm² and transfected at 80–90% confluency with 1 μ g of plasmid DNA using LipoD293 (SigmaGen Laboratories, Rockville, MD). Conditioned medium and RNA were collected 48 h post-transfection.

Stable overexpression of MMP-8 in MDA-MB-231 cells was achieved using calcium phosphate transfection, and selection in DMEM + GlutaMAXTM supplemented with 10% fetal bovine serum and 40 μ g/ml zeocin (Invitrogen). Isolated clones were generated by serial dilution of a polyclonal pool of stably transfected cells, and expression of MMP-8 was assessed by Western blot analysis and real-time RT-PCR.

Preparation of Cell Lysates and Conditioned Media—Cell lysates were prepared in radioimmune precipitation assay lysis buffer supplemented with protease inhibitors (Roche). Conditioned media was collected from MDA-MB-231 stably transfected cells after 24 h serum starvation or 48 h after siRNA transfection. For Western blot analysis, the conditioned media were concentrated 20 \times and resuspended in reducing Laemmli sample buffer.

Cytometric Bead Array—Serum-free conditioned media from cells transiently transfected with pcDNA4 vector control, WT MMP-8, and E198A mutant MMP-8 plasmid constructs for 48 h were used in the commercially available human inflammatory cytokine kit (BD Biosciences, catalog no. 551811) according to the guidelines of the manufacturer, and a BDFACSAriaTM II flow cytometer was used to determine the levels of IL-8, IL-1 β , IL-10, TNF, IL-6, IL-12p70 in the conditioned media from the transiently transfected cells.

Western Blot Analysis—Cell lysates and 20 \times concentrated conditioned media samples were reduced and denatured in Laemmli sample buffer, and equal amounts of total protein were loaded on a 10% polyacrylamide gel. Protein samples were transferred onto polyvinylidene fluoride membrane (Millipore, Billerica, MA), blocked in PBS-0.1% Tween containing 5% bovine serum albumin, and incubated overnight at 4 $^{\circ}$ C with primary antibodies diluted in PBS-0.1% Tween containing 5% bovine serum albumin (MMP-8, Abcam, catalog no. ab81286 at 1:2000; V5, Invitrogen, catalog no. 460705, at 1:5000). Membranes were washed with PBS-0.1% Tween and incubated for 2 h with infrared-labeled secondary antibodies diluted in PBS-0.1% Tween with 5% bovine serum albumin (donkey anti-mouse IRDye 800CW and donkey anti-rabbit IRDye 800CW, Li-Cor Biosciences, Lincoln, NE). Finally, membranes were washed in PBS-0.1% Tween and imaged using an infrared scanner (Odyssey, Stamford, CT).

MMP-8 Induces Interleukin 6 and 8 Production

ELISA—Conditioned media collected after 24 h serum starvation were used to assess the total levels of IL-6, IL-8, and TNF α using specific ELISAs (eBiosciences, San Diego, CA) following the guidelines of the manufacturer.

Quantitative RT-PCR—Cell pellets were harvested in RNA-Bee RNA isolation reagent (Amsbio, Lake Forest, CA) followed by a combination method of chloroform extraction and RNA isolation using the SV Total RNA isolation system (Promega, Madison, WI) and stored at -80°C .

RNA quantity and quality were assessed by A_{260}/A_{280} and A_{230}/A_{280} absorbance ratios using the Nanodrop (Thermo Fisher Scientific, Waltham, MA). A total of 1 μg of RNA was reverse-transcribed to 50 ng/ μl cDNA, and real time RT-PCR was performed on an ABI 7700 PCR machine (Invitrogen) using the following cycles: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 90°C and 1 min at 60°C . IL-6 and IL-8 cDNA expression was quantified using human-specific 5'FAM-3'TAMRA Taqman gene expression primer/probe sets (Hs00985639_m1 and Hs00174103_m1, Invitrogen), and PAR-2 cDNA expression was quantified using the following primers: 5'CGTCCAGTGGAGCTCTGAGT-3' and 5'-GCAGGAGA-GAGAGGCTGCTA-3' and universal probe #3 (Roche). Expression levels were normalized to levels of 18 S rRNA (forward primer 5'-GCCGCTAGAGGTGAAATTCTTG-3', reverse primer 5'-CATTCTTGGCAAATGCTTTTCG-3', and probe ACCGGCGCAAGACGGA). For detection of human MMP8 expression, custom-made primers (Primer Design) were used.

Cell Stimulation and Inhibition Experiments—Recombinant human IL-6 (R&D Systems, Minneapolis, MN, catalog no. 206-IL/CF) and IL-8 (R&D Systems, catalog no. 208-IL/CF) were added to serum-starved (2 h) MDA-MB-231 cells at 0.3, 0.6, and 100 ng of IL-6 and 0.5, 1.5, and 100 ng of IL-8 for 24 h.

For inhibitor treatments, cells were plated at $2.6 \times 10^4/\text{cm}^2$ 24 h before addition of inhibitors in serum-free conditions. Inhibitors used were as follows: p38 inhibitors: 1 μM SB203580 and 1 μM BIRB (catalog nos. CAY13067 and CAY10460, Cayman Chemicals); MKK1 inhibitors: 0.1 μM PD0325901 and 10 μM U0126 (catalog nos. CAY13034 and CAY70970, Cayman Chemicals); PI3K inhibitors: 1 μM Wortmannin and 0.5 μM PI-103 (catalog no. W1628, Sigma, and catalog no. 10009209, Cayman Chemicals); PKC inhibitor: 360 nm bisindolylmaleimide (catalog no. 203290, Calbiochem); JNK inhibitor: 20 μM SP600125 (catalog no. S5567, Sigma); NF κB inhibitor: 10 μM BAY 11-7082 (catalog no. 196871, Calbiochem); TGF β inhibitor: SB431542 (catalog no. CAY13031, Cayman Chemicals); and MMP-8 inhibitor: 1 μM DL111 (a gift from Prof. Vincent Dive, CEA Saclay, Gif-sur-Yvette, France). Inhibitors were replenished after 24 h, and conditioned media were collected after a total incubation of 48 h.

siRNA Knockdowns—MDA-MB-231 cells stably overexpressing wild-type MMP-8 were transfected at 60% confluency with 25 nM of either IL-6 (siGENOME SMARTpool M-007993-02-0005, Dharmacon, Lafayette, CO), IL-8 (siGENOME SMARTpool M-004756-00-0005, Dharmacon), or control siRNA (siGENOME non-targeting siRNA pool #1 D-001206-13-05, Dharmacon) using DharmaFECT 1 transfection reagent (Dharmacon, catalog no. T-2001-02). RNA was collected 48 h

later for analysis. Cells transfected with MMP-8 siRNA (siGENOME SMARTpool M-005969-00-0005, Dharmacon) or F2RL1 (PAR-2) (siGENOME SMARTpool, M-005098-01-005, Dharmacon) were transfected a second time 24 h later. Serum-starved conditioned media and cell pellets for RNA and protein isolation were collected 48 h after the second transfection.

Colony Formation Assay—MDA-MB-231 cells were transfected using the calcium phosphate method for stable overexpression and selected using 200 $\mu\text{g}/\text{ml}$ zeocin. Approximately 20 days after transfection, individual colonies were visualized by staining with methylene blue for 30 min at room temperature.

Statistical Analysis—Unless stated otherwise, data were tested using two-tailed Student's *t* tests and are presented as mean \pm S.E.

RESULTS

MMP-8 Up-regulates of IL-6 and IL-8 Production by Breast Cancer Cells—To explore the effects of MMP-8 on inflammatory mediators generated by breast cancer cells, we generated a full-length wild-type human MMP-8 expression construct in pcDNA4 and a mutant in which the essential glutamic acid residue in the catalytic core motif HE¹⁹⁸FGH was altered to alanine (E198A mutant MMP-8). These constructs were transiently transfected into MCF-7 cells, and after 24 h, serum-free conditioned media were analyzed by a FACS cytokine bead array for levels of IL-6, IL-8, IL-10, IL-1 β , TNF α , and IL-12p70 released. Like IL-8, IL-10 is known to be a substrate of MMP-8 (14), whereas IL-1 β has been shown to up-regulate MMP-8 expression (21). Expression of IL-6 and -8 in MCF-7 cells is reported to be low because of inhibition by the estrogen receptor (22–25). We detected a 20% increase in IL-6 and a 65% increase in IL-8 protein levels in the conditioned media in WT-MMP-8-expressing cells compared with empty vector (EV) (Fig. 1A, upper panels). This increase was dependent on the catalytic activity of MMP-8 because cells transfected with the E198A mutant MMP-8 showed levels similar to the control cells. None of the other inflammatory mediators were detected in the MCF-7 cell-conditioned media at measurable levels. Wild-type and E198A mutant MMP-8 were expressed and released into the conditioned media at equivalent levels as determined by Western blotting (Fig. 1B). We anticipated that the increased detection of the inflammatory mediators might be attributable to release of matrix- or cell-associated IL-6 and IL-8 by MMP-8. However, the increases in IL-6 and IL-8 protein were mirrored at the mRNA level, with WT MMP-8 increasing both IL-6 and IL-8 mRNA levels 2- to 3-fold. Similar results were obtained with another breast cancer cell line (SK-BR-3) transiently transfected with WT MMP-8 and E198A MMP-8 (Fig. 1C), but this phenomenon was not seen on transient transfection into a non-cancerous breast epithelial cell line, HMT-3522 S1 (D).

The human breast cancer cell lines we examined (MDA-MB-231, MCF7, and SK-BR-3; Fig. 2A and data not shown) did not express MMP-8 at a level that was detectable by Western blot analysis, and only MDA-MB-231 cells expressed a very small amount of endogenous MMP8 that was detectable by TaqMan quantitative real-time RT-PCR (data not shown, see also Fig.

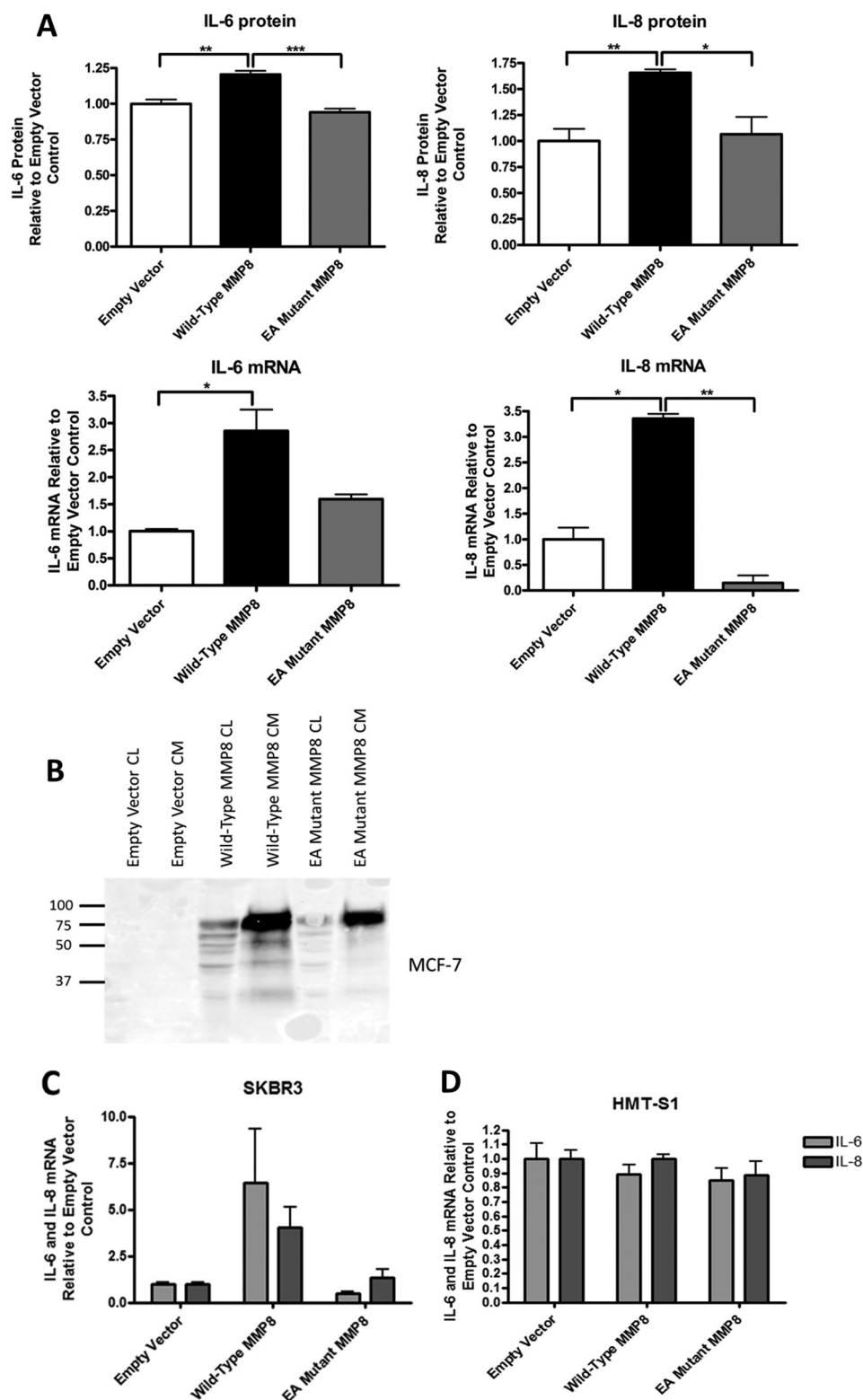


FIGURE 1. Transient expression of WT MMP-8 causes an up-regulation of IL-6 and IL-6 protein and mRNA expression that is dependent on the catalytic activity of MMP-8. *A*, MCF-7 cells were transiently transfected with pcDNA4 empty vector, wild-type MMP-8, and E198A mutant MMP-8 (EA). *Top panel*, FACS cytokine bead array measurements of IL-6 and IL-8 protein levels in serum-free conditioned media collected 24 h after transfection. *Lower panel*, RNA quantification by real-time TaqMan RT-PCR of IL-6 and IL-8 mRNAs in cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, Western blot analysis of WT MMP-8 and E198A MMP-8 expression in transfected MCF-7 cells. *C*, RNA quantification by real-time TaqMan RT-PCR of IL-6 and IL-8 mRNAs in SK-BR-3 cells transiently transfected with pcDNA4 empty vector, WT MMP-8, and E198A mutant MMP-8. *D*, RNA quantification by real-time TaqMan RT-PCR of IL-6 and IL-8 mRNAs in HMT-3552 S1 cells transiently transfected with pcDNA4 empty vector, WT MMP-8, and E198A mutant MMP-8. CL, cell lysate; CM, conditioned media.

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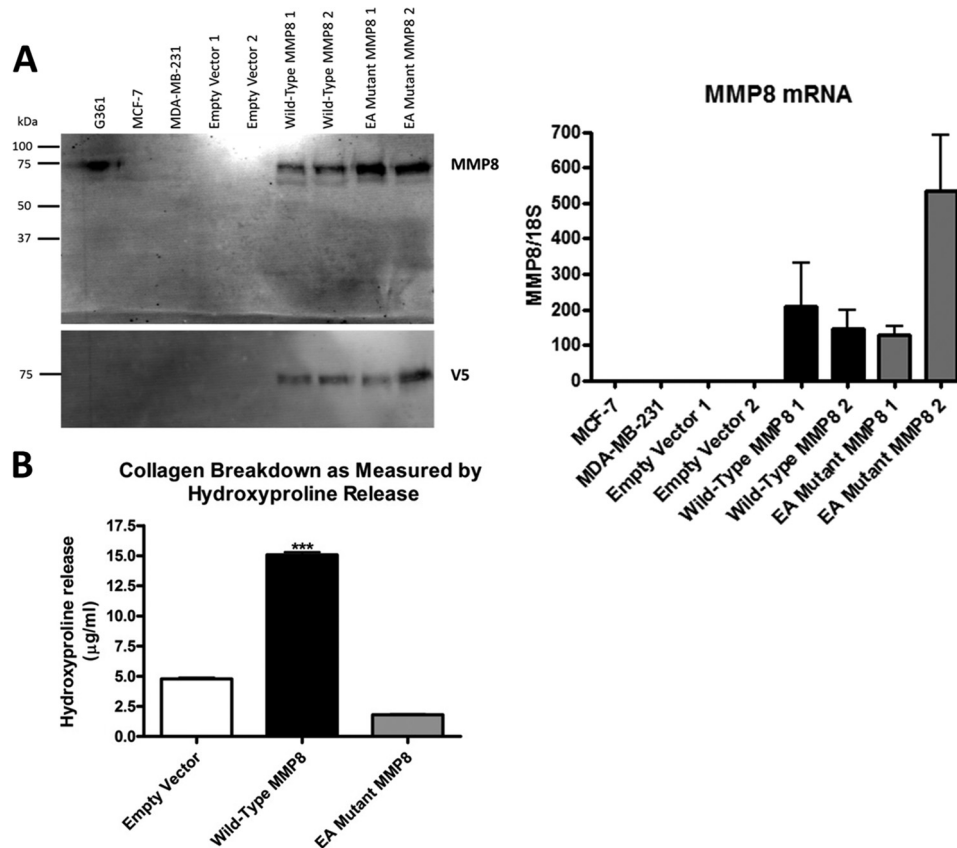


FIGURE 2. Generation of an *in vitro* model to examine the effects of MMP-8 expression in MDA-MB-231 breast carcinoma cells. *A*, expression of MMP-8 in serum-free conditioned media of MDA-MB-231 cells stably transfected with pcDNA4 empty vector, wild-type MMP-8, and E198A mutant MMP-8 as shown by Western blot analysis using both anti-MMP8 antibody (*top panel*) and anti-V5 antibody (*bottom panel*). Media from G361 melanoma cells were included as a positive control for endogenous MMP-8 and parental MCF-7 and MDA-MB-231 cell conditioned media as negative controls. All media were concentrated 20×. *Right panel*, RNA quantification by real-time TaqMan RT-PCR of MMP-8 mRNAs in the same cells. *B*, collagen-degrading activity of MDA-MB-231 cells stably transfected with pcDNA4 empty vector, WT MMP-8, and E198A mutant MMP-8 cultured within collagen gels and measured indirectly by hydroxyproline release. ***, $p < 0.001$.

5C). Using the highly metastatic MDA-MB-231 cells (estrogen receptor-/progesterone receptor-/HER2), we generated stably transfected clones carrying the WT MMP-8, E198A mutant, or EV control vector. Fig. 2A shows that comparable levels of the wild-type and catalytically inactive MMP-8 proteins were expressed and released into the conditioned media and that MMP8 transcript levels originating from the transfected genes were also similar. The levels of MMP-8 protein expression achieved in these transfected clonal lines were similar to that of endogenous MMP-8 in conditioned media from the G361 melanoma cell line, which was included as a control. The major MMP-8 band was 75 kDa, likely corresponding to pro-MMP-8, but a faster migrating band was detected by the MMP-8 antibody, although this could be a C-terminally processed form because it was undetectable by the antibody toward the V5 tag. However, functional collagenolytic activity of the wild-type MMP-8 was confirmed by demonstration of elevated hydroxyproline release compared with the E198A mutant-expressing cells and the empty vector control (Fig. 2B). Analysis of IL-6 and 8 protein production by ELISA of conditioned media from two independent clonal isolates of WT MMP-8 stably transfected MDA-MB-231 cells (Fig. 3, *upper panels*) showed a 2- to 12-fold increase in IL-6 and an 2- to 8.5-fold increase in IL-8 protein in comparison to EV control cells. Again, the E198A inactive

mutant MMP-8-expressing cells showed no increases compared with the EV control cells. Also, as with the transiently transfected MCF-7 cells, we found that in the MDA-MB-231 stably transfected cells, mRNA levels were increased 4-fold for IL-6 and 4- to 12-fold for IL-8 in stable WT MMP-8-expressing MDA-MB-231 clones (Fig. 3, *lower panels*), mirroring the increased levels of the soluble proteins.

We conclude that the effect of MMP-8 on the inflammatory mediators is primarily at the level of gene expression and that its catalytic activity is required because all cells overexpressing the E198A mutant show IL-6 and IL-8 protein and mRNA levels comparable with those of control-transfected cells. Furthermore, the induction of IL-6 and IL-8 was observed for all breast cancer cell lines examined (MCF-7, SK-BR-3, and MDA-MB-231) but not for HMT-3522 S1 normal mammary epithelial cells.

MMP-8 Expression Is Detrimental to Breast Cancer Cell Growth—When MDA-MB-231 cells were transfected with our suite of expression vectors and placed under antibiotic (zeocin) selection, after 20 days we observed a 50–60% reduction in colony formation by cells expressing WT MMP-8 compared with EV or E198A mutant MMP-8 (Fig. 4A). However, in shorter-term experiments following transient transfection of either WT or E198A mutant MMP-8 into MCF-7 or MDA-MB-231 cells

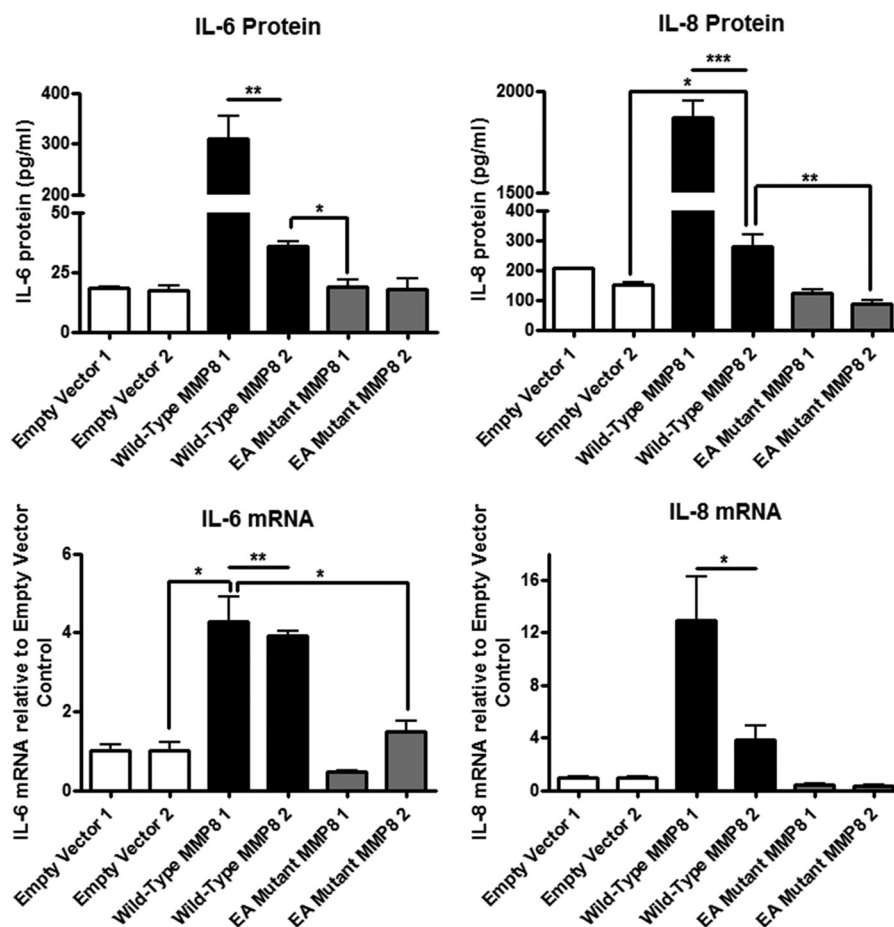


FIGURE 3. Long-term stable MMP-8 overexpression also causes an up-regulation of IL-6 and IL-8 protein and mRNA expression that is dependent on the catalytic activity of MMP-8. Two independent clones of MDA-MB-231 cells stably transfected with pcDNA4 empty vector, wild-type MMP-8, and E198A mutant MMP-8 were analyzed. *Top panel*, ELISA measurements of IL-6 and IL-8 protein levels in serum-free conditioned media collected 24 h after serum starvation. *Lower panel*, RNA quantification by real-time TaqMan RT-PCR of IL-6 and IL-8 mRNAs in the same cells. Both wild-type MMP-8 clones 1 and 2 were different compared with all other cells by at least $p < 0.05$ or $p < 0.01$ unless shown otherwise *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. EA, E198A mutant MMP-8.

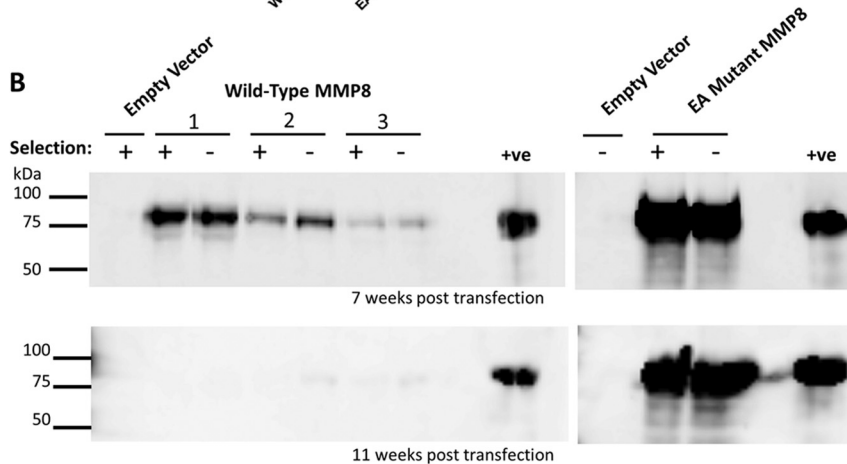
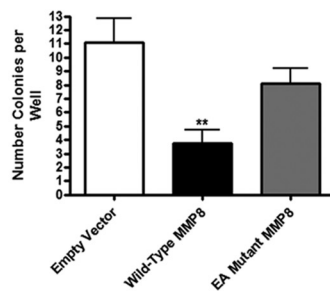
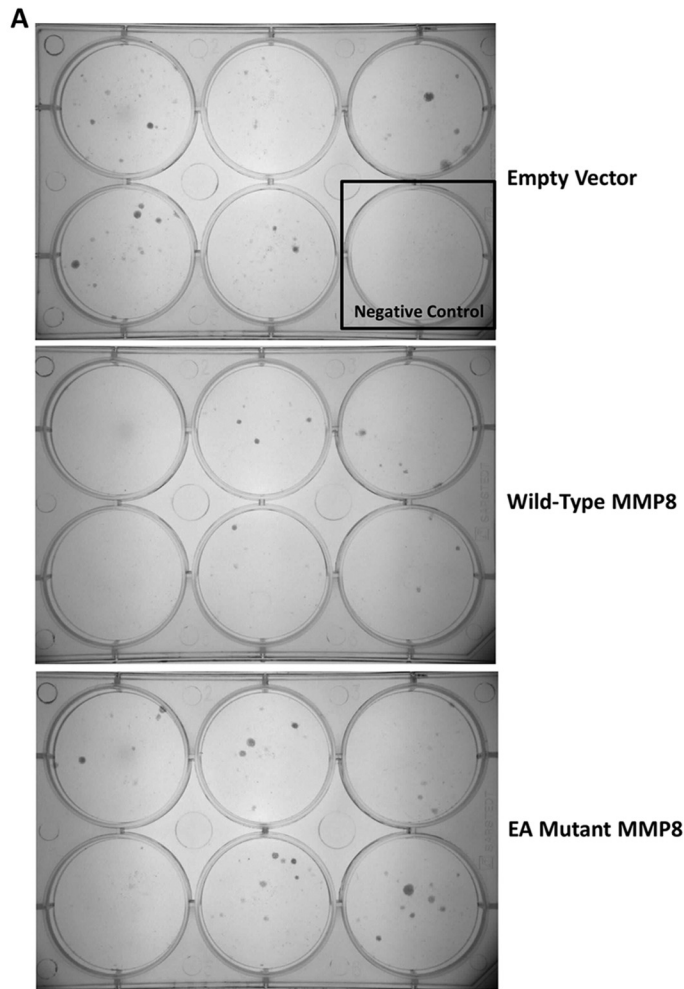
for up to 72 h, we saw no effects on cell growth or apoptosis (data not shown). We therefore monitored independent pools of transfected MDA-MB-231 cells carried forward for up to 3 months in culture with or without zeocin selection (Fig. 4B). Although expression was initially comparable early after transfection (data not shown), by 7 weeks, levels of WT MMP-8 were reduced compared with E198A mutant MMP-8 (Fig. 4B, upper panel). By 11 weeks in culture, WT MMP-8 expression was barely detectable in any of the cell pools regardless of zeocin selection, whereas expression of E198A mutant MMP-8 remained at a consistently high level, again regardless of selection (Fig. 4B, lower panel). PCR analysis of genomic DNA isolated from the cell populations from this experiment confirmed that the transfected *MMP8* expression vectors were present and at the same levels in both WT and E198 mutant MMP-8 transfected cells after 11 weeks (data not shown), suggesting that the WT *MMP8* expression cassette was silenced during continued passage of the cells. Moreover, IL-6 and IL-8 expression returned to base line in the cell pools that lost MMP-8 expression (data not shown).

These data, therefore, support a causal link between MMP-8 and up-regulation of the expression and secretion of IL-6 and IL-8. Furthermore, they argue that the occasional stably trans-

fected clones that maintain WT MMP-8 expression long-term, for longer than 3 months, such as those shown in Fig. 3, have likely undergone phenotypic alteration to compensate for the detrimental effects of MMP-8. This conclusion was supported by the observations that neither knockdown of MMP-8 in the “long-term” WT MMP-8-expressing cells or blockade of its activity using a selective small molecular weight inhibitor, KL-111, led to a reduction in IL-6 or IL-8 production (data not shown). These data emphasize that additional compensatory mechanisms that are independent of MMP-8 are operational to allow breast cancer cells to maintain elevated expression of the otherwise deleterious MMP.

MMP8, IL-6, and IL-8 Are Components of a Self-reinforcing Loop—Because IL-6 and IL-8 are powerful proinflammatory mediators that exert effects on the expression of many genes, we sought to determine whether their up-regulation might link them into a dynamic self-regulating network. To determine whether a feedback loop is operating in the long-term WT MMP-8-expressing MDA-MB-231 cells, the cells were treated with siRNAs to IL-6 and IL-8 and levels of both cytokines measured by TaqMan qRT-PCR. siRNA-mediated knockdown of IL-8 expression led to a concomitant reduction of IL-6 mRNA levels by ~40% compared with the non-targeting control (Fig.

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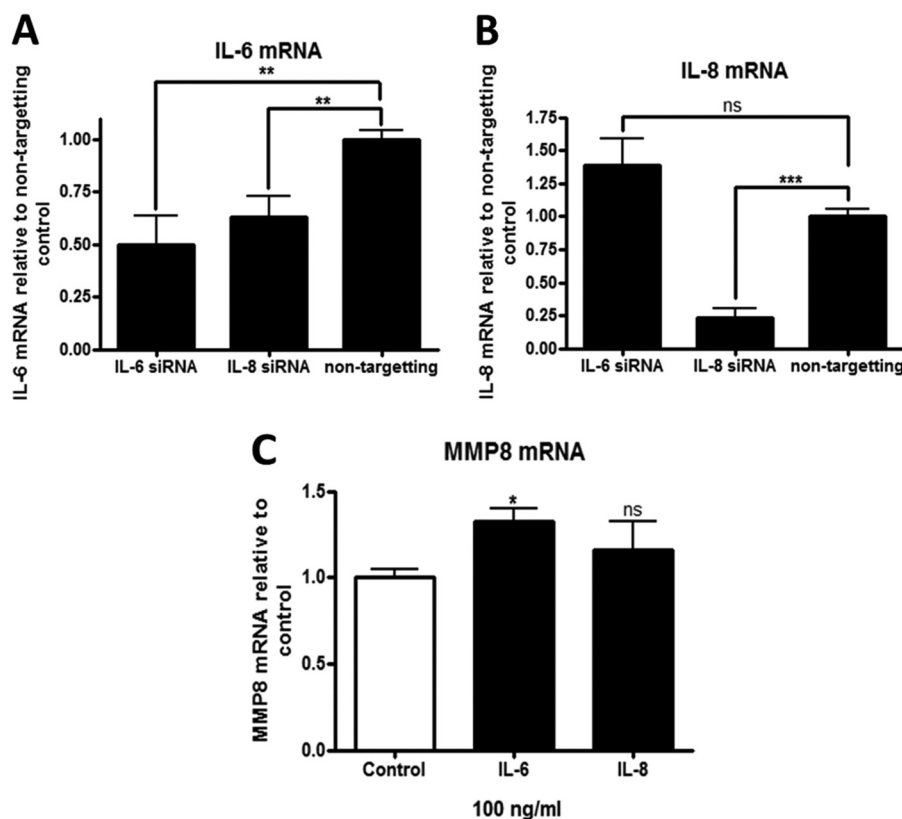


FIGURE 5. **MMP-8, IL-6, and IL-8 exist as part of an interconnected regulatory circuit.** A, RNA quantification by real-time TaqMan RT-PCR of IL-6 mRNA levels in WT MMP-8 overexpressing MDA-MB-231 cells after siRNA knockdown of IL-6 or IL-8 for 48 h. B, RNA quantification by real-time TaqMan RT-PCR of IL-8 mRNA levels in WT MMP-8 overexpressing MDA-MB-231 cells after siRNA knockdown of IL-6 or IL-8 for 48 h. C, RNA quantification by real-time TaqMan RT-PCR of endogenous MMP-8 in naïve, non-transfected MDA-MB-231 cells treated with recombinant human IL-6 and IL-8 for 24 h. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

5A). In contrast, knockdown of IL-6 had no effect on IL-8 mRNA expression compared with the non-targeting control siRNA (Fig. 5B). In separate experiments, human recombinant IL-6 and IL-8 were added to parental MDA-MB-231 cells for 24 h, and the expression level of *MMP8* determined by RT-PCR. Addition of IL-8 alone did not affect expression of *IL6* (data not shown) or *MMP8* mRNAs, but recombinant IL-6 induced endogenous *MMP8* expression by ~20% (Fig. 5C). Taken together, these data suggest that MMP-8, IL-6, and IL-8 may constitute a self-reinforcing loop. MMP-8 can induce expression of IL-6 and IL-8 in short-term exposure to the protease, and IL-8 reinforces IL-6 expression, whereas the induced IL-6 may augment *MMP8* expression. In the long-term WT MMP-8 clones, where IL-6 and IL-8 production is no longer dependent upon MMP-8, IL-8 may help to sustain elevated IL-6 expression.

IL-6 and IL-8 Expression in Long-term MMP-8-expressing Cells Is Dependent on NF- κ B Signaling and PAR2 Is Linked to IL-6 Expression—We explored the effects of pharmacological inhibition of major cell signaling pathways to determine the

relevance of the particular pathways to the increased expression of IL-6 and IL-8 in the long-term WT MMP-8-expressing MDA-MB-231 cells. The inhibitors used and their target pathways were as follows: PD0325901 and U0126 (MAPK-Erk pathway), SB203580 (p38), wortmannin and PI-103 (PI-3K), bisindolylmaleimide 1 (PKC), SP600125 (JNK), BAY 11-7082 (NF- κ B), and SB431542 (TGF β R1). Of the inhibitors examined, only the NF- κ B inhibitor BAY 11-7082 reduced secretion of both IL-6 and IL-8 by 60% compared with the vehicle control in the cells overexpressing wild-type MMP-8 (Fig. 6A). The TGF β R1 inhibitor SB431542 had no effect on MMP-8-induced IL-6 levels, but IL-8 levels were increased by 100% in the WT MMP-8 overexpressing cells, and the control EV and E198A mutant MMP-8-expressing cells also showed a slight induction, showing that TGF β signaling is inhibitory to IL-8 production in MDA-MB-231 cells (Fig. 6B).

We also considered the possible involvement of protease-activated receptor (PAR) signaling because MMP-1 is known to cleave and activate PAR-1 (26–28). However, PAR-1 was not expressed by the parental MDA-MB-231 cells or the WT

FIGURE 4. **Wild-type MMP-8 reduces colony formation of MDA-MB-231 cells, and expression is shut down by cells.** A, colonies visible by 20 days post-transfection stained with methylene blue. The control well consisted of cells that were untransfected and so died under zeocin selection as a control for cell death. EA, E198A mutant. B, Western blot analysis using an anti-MMP-8 antibody on 20 \times concentrated serum-free conditioned media, tracking the loss of expression of wild-type MMP-8 from stably transfected MDA-MB-231 cells. Three pools of wild-type MMP-8 stables (1–3), were cultured alongside a pool of cells transfected with the control vector, and E198A mutant MMP-8 were maintained with (+) or without (-) zeocin selection. *Top panel*, cells 7 weeks post-transfection. *Bottom panel*, cells 11 weeks post-transfection. The positive control used was conditioned media from HEK-293T cells transiently transfected with WT MMP-8. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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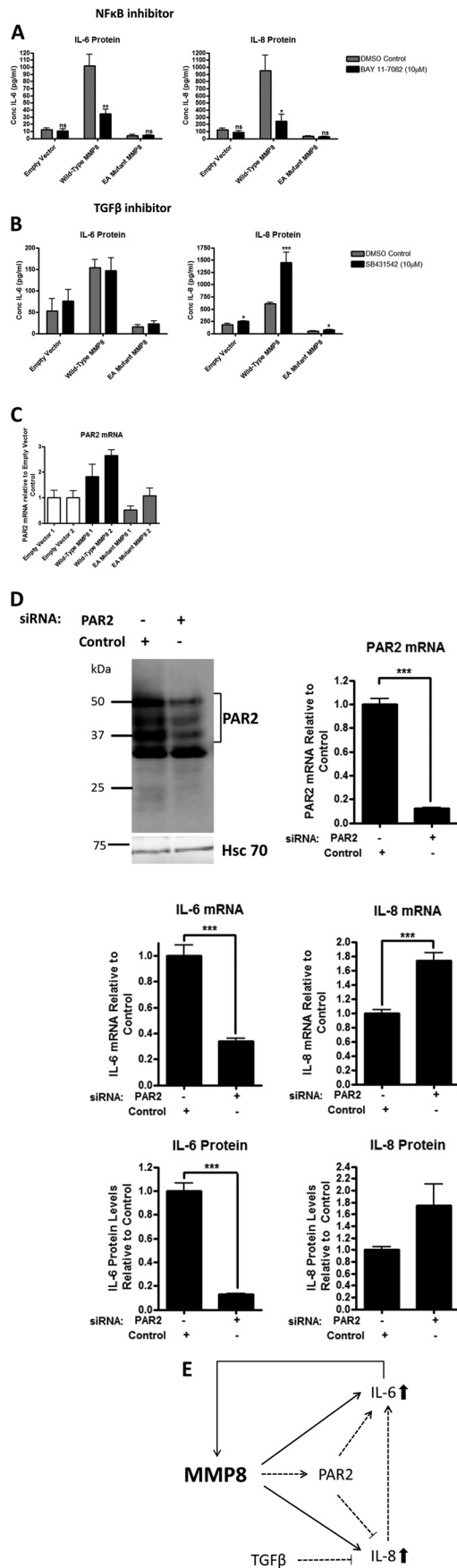


FIGURE 6. Both IL-6 and IL-8 up-regulation occurs via NF-κB, but IL-8 is also negatively regulated by TGFβ and PAR-2. A, ELISA measurements of

MMP-8-expressing clones (data not shown). The G protein-coupled receptor PAR-2 was considered because it is expressed by epithelial cells (29) and neutrophils (30), has a role in neutrophil migration (31), and has reported involvement in IL-6 and IL-8 up-regulation and secretion (31–33). We observed that PAR-2 expression was consistently up-regulated in the long-term WT MMP8-expressing MDA-MB-231 cells (Fig. 6C). Knockdown of PAR-2 by siRNA reduced IL-6 protein and mRNA expression by 90 and 60%, respectively, in the WT MMP-8 expressing cells but had the reverse effect on IL-8 expression, causing an approximate 80% increase in both RNA and protein levels (Fig. 6D). The schematic in Fig. 6E summarizes the relationships between MMP-8, IL-6, and IL-8 under acute and long-term exposure of breast cancer cells to catalytically active MMP-8 and to PAR-2 and TGF-β signaling.

DISCUSSION

Matrix metalloproteinase 8 was the first member of the MMP family that was revealed to be tumor- or metastasis-suppressive *in vivo* (6, 7, 13). Subsequently, MMP-8 expression was found to be a marker of a lower incidence of lymph node metastasis and, consequently, to confer a better prognosis in human breast carcinoma (9). It has also been found to be protective in cancer of the tongue (10). In human melanoma, function-inactivating mutations in *MMP8* have been found at high frequency, and wild-type but not mutant forms suppressed tumor formation in soft agar assays, supporting its role as a tumor suppressor (34). This study extends these findings using a series of human mammary carcinoma cell lines, where we demonstrate that transient expression of catalytically active WT MMP-8, but not an inactive E198A mutant form, induced the expression of IL-6 and IL-8. However, sustained expression of WT MMP-8 was deleterious to breast cancer cell growth in colony formation assays, and, upon extended culture, the exogenous *MMP8* expression cassette was silenced in most cell pools, accompanied by a coordinate reduction in IL-6 and IL-8 expression. In a few instances, stable WT MMP-8-expressing

IL-6 and IL-8 protein levels after incubation of MDA-MB-231 clonal isolates stably transfected with pcDNA4 empty vector, wild-type MMP-8, and E198A mutant (EA) MMP-8 after incubation with 10 μM BAY 11-7082, an NF-κB inhibitor, for 48 h. DMSO, dimethyl sulfoxide. B, ELISA measurements of IL-6 and IL-8 protein levels after incubation of MDA-MB-231 clonal isolates stably transfected with pcDNA4 empty vector, wild-type MMP-8, and E198A mutant MMP-8 after incubation with 10 μM SB431542, a TGFβR1 inhibitor, for 48 h. C, RNA quantification by real-time TaqMan RT-PCR of PAR-2 in MDA-MB-231 clonal isolates stably transfected with pcDNA4 empty vector, wild-type MMP-8, and E198A mutant MMP-8. D, top panel, Western blot analysis and RNA quantification by real-time TaqMan RT-PCR of PAR-2 to confirm the knockdown of PAR-2 after siRNA knockdown for 48 h in wild-type MMP-8 overexpressing MDA-MB-231 clonal isolate 1. Center panel, RNA quantification by real-time TaqMan RT-PCR of IL-6 and IL-8 in cells after PAR-2 siRNA knockdown. Bottom panel, ELISA measurement on serum-free conditioned media of IL-6 and IL-8 protein levels after PAR-2 siRNA knockdown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant. E, schematic showing the relationships between MMP-8, IL-6, and IL-8 in a self-reinforcing loop and pathways that may contribute to this system in breast cancer cells. On acute exposure to catalytically active WT MMP-8, breast cancer cells up-regulate the expression of IL-6 and IL-8 (black lines and arrows). IL-6 is also able to induce MMP-8 expression. Red lines and arrows show the pathways that are evident in breast cancer cells that adapt to long-term expression of WT MMP-8. Not shown on the diagram is that IL-6 and IL-8 expression is also dependent on NF-κB signaling, which may be activated by PAR-2 as well as other signaling pathways, including autocrine loops resulting from IL-6 and IL-8 induction.

clones of MDA-MB-231 cells were obtained, and these retained elevated IL-6 and IL-8 production, although we infer that additional genetic or epigenetic changes had likely occurred in these cells because their IL-6/IL-8 expression levels were no longer dependent upon MMP-8. Instead, increased IL-6 levels may in part be due to the action of IL-8 acting in an autocrine fashion and also to PAR-2, which was found to be up-regulated in the long-term WT MMP-8-expressing cells.

We detected increased levels of IL-6 and IL-8 proteins by ELISA in conditioned media of MCF-7, MDA-MB-231, and SK-BR-3 breast carcinoma cells transfected transiently with WT MMP-8, indicating that the response is likely a general one and not restricted to cancer cells that have a particular estrogen receptor status or amplified HER2. The effects on IL-6 and IL-8 were specific because we saw no increases in several other inflammatory mediators, including TNF α , IL1 β , IL-10, and IL-12p70. It is known that MMP-8 carries out N-terminal processing of IL-8 and its murine ortholog LIX, leading to activation of the chemokines and increasing their neutrophil chemoattractant activity *in vivo* (19). We considered that MMP-8 might thus have led to increased detection of IL-6 and IL-8 proteins in cell-conditioned media through proteolytic cleavage of precursor or cell-associated forms of these molecules. We cannot rule this out as a contributory factor, but because the increased protein levels were reflected in increased IL-6 and IL-8 mRNA abundance in WT MMP-8-transfected cells, we conclude that the principal effect of the protease is at the level of induction of gene transcription. A normal immortalized mammary epithelial cell line (HMT-3522 S1) showed no effect of WT or E198A mutant MMP-8 on the production of the interleukins, indicating that malignant transformation is a prerequisite for induction.

Interleukins 6 and 8 are well established as tumor-promoting, proinflammatory factors in numerous cancer scenarios (35, 36). Interleukin 6 acts via the membrane-bound or soluble IL-6 receptor, which binds the cytokine and then interacts with the gp130 signaling receptor, leading to activation of downstream pathways, including the JAK-1-STAT-3 pathway, MAP kinases, and NF- κ B (37). Activation of IL-6 secretion, for instance via HER2 amplification, creates an autocrine signaling loop involving JAK-1/STAT-3-mediated IL-6 expression (38). Also, Src-mediated transformation of mammary epithelial cells has been shown to involve IL-6 acting in a positive feedback loop via activation of NF- κ B (39). The IL-8 chemokine activates multiple pathways following its engagement with two G protein receptors (CXCR1 and CXCR2), leading to the promotion of the proliferation, survival, and migration of cancer cells (36). Recently, both IL-6 and IL-8 have also been shown to regulate breast cancer stem cell renewal (40, 41). Therefore, it appeared paradoxical to us that MMP-8, on the basis of its history as an antitumorogenic protease, would elicit the production of factors that have profoundly protumorogenic actions. Two points are critical for consideration, however, the first being the impact of MMP-8 on innate immunity and the role of the innate immune response in tumor progression and the second being the distinction between early, acute effects of catalytically active MMP-8 upon breast cancer cells and how cells cope with sustained production of the protease.

Neutrophils are the first wave of innate immune cells that are recruited to sites of acute inflammation. These triggered neutrophils release and autoactivate pro-MMP-8 from the specific granules, which then acts upon LIX (mouse) and IL-8 (human), enhancing further neutrophil chemotaxis in a feed-forward mechanism (19). The systemic absence of MMP-8 in *Mmp8*^{-/-} mice has profound effects on inflammation, the outcomes of which depend on the tissue site and the nature of the inflammation. In carcinogen-induced skin tumors or in skin wounds there is a delay in neutrophil infiltration at early times in *Mmp8*-null *versus* wild-type mice, but this eventually leads to a persistent accumulation at later time points, which exacerbates tumor development (6) and impairs wound repair (20). This is also evident in the more severe alveolar bone loss in *Mmp8*^{-/-} mice compared with the wild type in *Porphyromonas gingivalis*-induced periodontitis (16, 42) and in LPS-induced corneal inflammation (43). In all of these situations, MMP-8 is required to promote both the initial onset and the subsequent clearance of the inflammatory neutrophilic response. In some tissues, such as the lung and the liver, the absence of MMP-8 and the attendant initial reduction in neutrophil influx and reduced chemokine activation can be protective, as has been observed in models of ventilator-induced injury in the lung (15), bleomycin-induced lung fibrosis (14), and TNF α -induced lethal hepatitis (44). All of these situations are accompanied by alteration of the levels of numerous chemokines and cytokines, including reduced levels of LIX (15, 42, 44). Thus, the induction of IL-6 and IL-8 expression attributable to the catalytic activity of MMP-8 in breast cancer cells is consistent with a role for MMP-8 in the initial triggering of an acute inflammatory response. Within the microenvironments of emerging tumors, this activation may set in motion subsequent macrophage-dependent and adaptive immune system responses that act to resolve inflammation and suppress tumor progression (45).

One of the most important points to emerge from this study is that sustained, elevated expression of MMP-8 by breast cancer cells eventually impaired their growth. This did not occur as an acute response to WT MMP-8 because we saw no appreciable effects on cell growth or apoptosis over the 3–4 days during which cells were expressing the proteins following transient transfection (although subtle effects may be hard to detect in transient assays). Instead, the growth-suppressive effect of WT MMP-8 was apparent in longer-term colony formation assays. Palavalli and colleagues (34) also saw no effect on short-term growth of cultured Mel-STR melanocytes following expression of human WT MMP-8, but there was substantial inhibition of colony growth in soft agar. These effects on growth were not apparent in an earlier study that had transfected a mouse *Mmp8* expression vector into murine B16-F10 cells, so it is possible that the growth effects may be species-specific (9). In our experiments, serial passage of transfected breast MDA-MB-231 carcinoma cells expressing WT MMP-8 led, over time, to a progressive loss of expression of the protease. This was not observed for cells expressing the E198A catalytically dead mutant, indicating that it was a result of MMP-8 protease activity. The suppression occurred even when the cells were maintained under continuously enforced zeocin selection. We confirmed that the full *MMP8* expression cassette was retained in

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these cells, indicating that expression was likely lost through epigenetic silencing. A comprehensive analysis of the transcriptional status of the entire *MMP* family in relation to their epigenetic parameters (DNA methylation, histone modifications, and microRNAs) identified *MMP8* as displaying strict epigenetic silencing in multiple cancer cell lines, consistent with its antitumorigenic role (46). In our long-term MDA-MB-231 transfected cell cultures, loss of expression of WT MMP-8 coincided with a return to base-line levels for the expression of IL-6 and IL-8, which further supports a causal connection between MMP-8 and the elevated expression of the inflammatory mediators.

In the rare MDA-MB-231 clones that gave sustained elevated expression of WT MMP-8, although IL-6 and IL-8 levels were also maintained, the expression of the interleukins was no longer dependent on the presence of MMP-8 because neither siRNA-mediated knockdown of MMP-8 nor treatment with DL-111, an MMP-8-selective inhibitor, attenuated their expression. We infer from these data that IL-6 and IL-8 may act to overcome the growth-suppressive effects of WT MMP-8. However, these factors possibly act together with other growth-regulatory factors whose expression is sensitive to WT MMP-8 because their combined knockdown did not decrease cell growth in short-term assays (data not shown). We do not yet know the identities of the molecules that confer the continued ability of breast cancer cells to grow while expressing catalytically active MMP-8. It is clear from our knockdown experiments that IL-8 is, at least in part, responsible for the sustained expression of IL-6. Data from other groups support an autocrine feedback loop for IL-6 in breast cancer cells involving NF- κ B signaling linked to control of IL-6 expression through the microRNAs let-7 (39) and miR-200c (47). Consistent with this we see a dependence of both IL-6 and IL-8 production on NF- κ B signaling. We therefore speculate that IL-6 and IL-8 create a self-reinforcing loop that allows the MDA-MB-231 cells to continue growth independently of the inhibitory action of MMP-8.

It has been observed previously that the delayed wound healing seen in *Mmp8*^{-/-} mice correlated with reduced levels of active TGF- β 1 (20). Altered TGF β signaling could contribute to the WT MMP-8-mediated up-regulation of IL-8 expression we have observed because IL-8 levels were enhanced in MDA-MB-231 cells by pharmacological inhibition of TGF β signaling. Another potential contributory mechanism for the sustained growth of WT MMP-8-expressing MDA-MB-231 cells may be the increased expression of PAR-2 that we observed in these cells compared with cells expressing the E198A mutant MMP-8. Protease-activated receptor 2 is a widely expressed member of the PAR family of G-protein-coupled receptors that is known to be activated by several serine proteases, including trypsin and matriptase, leading to activation of NF- κ B signaling (48, 49). Knockdown of PAR-2 expression in the WT MMP-8-expressing cells led to a reduction of IL-6 production, but, in contrast, this led to a further augmentation of IL-8 expression. Although MMP-1 (collagenase 1) is known to cleave and activate PAR-1 (26–28), we have no evidence to suggest that MMP-8 is acting directly through PAR-2. Indeed, as discussed above, in the long-term WT MMP-8-expressing clones, IL-6/

IL-8 are decoupled from MMP-8 action. It is possible, however, that among the genetic/epigenetic changes during clonal evolution that have allowed MDA-MB-231 cells to tolerate WT MMP-8 may be the increased production of proteases that act through PAR-2. However, this likely contributes only to the IL-6 expression status because PAR-2 negatively regulates IL-8 expression (Fig. 6E).

In conclusion, therefore, our experiments reveal a novel action of catalytically active MMP-8 in breast cancer cells that results in increased production of key regulators of tumor growth and inflammation, IL-6, and IL-8. These effects occur upon acute expression of the enzyme and are not apparent in non-transformed mammary epithelial cells, suggesting that they may be linked with tumor progression. Although the identities of the MMP-8 substrates that are the direct mediators of its effects on growth and the expression of the inflammatory mediators remain to be firmly established, our data implicate the involvement of both TGF β - and PAR-2-mediated signaling. We suggest that the induction of proinflammatory mediators such as IL-6 and IL-8 is relevant to the mechanisms by which MMP-8 orchestrates the onset and resolution of inflammation *in vivo* in tumors and during tissue repair.

Acknowledgments—We thank Norfolk Fundraisers, Margaret Doggett, and the Suzie Wright Appeal.

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