UNIVERSITY OF LIVERPOOL

Matrix metalloproteinase-7 in Barrett's oesophagus and oesophageal adenocarcinoma: expression, metabolism and functional significance

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Disclaimer

This thesis is result of my own work. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification. The research was performed in the Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool. All other parties involved in the research presented here, and the nature of their contribution, are listed in the ‘Acknowledgements’ section of this thesis.

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List of Abbreviations

Abbreviation	Meanings
ADAMs	Adisintegrin and metaproteinases
AGS	Gastric adenocarcinoma cell line
AGS-GR	Gastric adenocarcinoma cell lines stably transfected with CCK2 receptor
AJCC	American Joint committee of cancer
Akt	Serine-threonine kinase
Arg	Arginine
ATM	Adjacent tissue myofibroblasts
AVCs	Angiogenic vascular cells
BFA	Brefeldin A
BMDCs	Bone marrow derived cells
BO	Barrett's oesophagus
CAFs	Cancer-associated fibroblastic cells
CAMs	Cancer associated myofibroblasts
CAP	Cancer-associated proteins
CFP	C-terminal flanking peptide
CM	Conditioned media
CNS	Central nervous system
COX	Cyclooxygenase
CSCs	Cancer stem cells
CV	Coefficient of variation
DAG	Diacylglycerol
DMEM	Dulbecco's Eagle Modified medium
DNA	Deoxyribonucleic acid
ECL	Enterochromaffin-like
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptors

ELISA	Enzyme-linked immunoassay
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FAP	Fibroblast activation protein
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FM	Full media
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCAMs	Gastric adenocarcinoma associated myofibroblasts
GORD	Gastro-oesophageal reflux disease
GPCRs	G protein–coupled receptors
H.pylori	*Helicobacter pylori*
HDACs	Histone deacetylases
HGD	High grade dysplasia
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IICs	Infiltrating immune cells
IL	Interleukin
IM	Intestinal metaplasia
IND	Indefinite for dysplasia
JAK2	Janus kinase 2
LGD	Low grade dysplasia
LGR5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
LOH	Loss of heterozygosity
Lys	Lysine
M	Molar
MAPK	Mitogen-activate protein kinase
MEK	mitogen-activated protein kinase or extracellular signal-regulated kinase kinase

MMPs	Matrixmetallproteinase
miRNA	MicroRNA
mRNA	Messenger RNA
MT-MMPs	Membrane-type matrix metalloproteinases
mTOR	Mammalian target of rapamycin
MUC	Mucin glycoprotein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTM	Normal tissue myofibroblasts
OAC	Oesophageal adenocarcinoma
OE19	Oesophageal adenocarcinoma of gastric cardia/oesophageal gastric junction cell lines
OE21	Oesophageal squamous carcinoma cell lines
OE33	Oesophageal adenocarcinoma cell lines
OE33-GR	Oesophageal adenocarcinoma cell lines stably transfected with wild type CCK2 receptor
OEC	Oesophageal cancer
PAI-1	Plasminogen activator inhibitor-1
PAM	Peptidyl alpha-amidating monooxygenase
PCNA	Proliferating cell nuclear antigen
PDAF	Platelet derived angiogenesis factor
PDGFR	Platelet derived growth factor receptor
PDK1	Phosphoinositide-dependent protein kinase 1
PEA3	Polyoma enhancer activator protein
PGs	Proteoglycans
PI3Kinase	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PK	Protein kinase
PMA	Phorbol 12-myristate 13-acetate
pmol	Picomole
PPIs	Proton pump inhibitors
PTEN	Phosphatase and tensin homolog
RAF	Proto-oncogene

RAS	Ras superfamily of proteins
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
SD	Standard deviation
SEM	Standard error of the mean
Ser	Serine
SF	Serum free
SILAC	Stable Isotope Labelling by Amino-acid in Culture
SOX2	Sex determining region Y (SRY)-box 2
STAT3	Signal transducer and activator of transcription 3
TGF-α	Transforming growth factor-α
TGF-β1	Transforming growth factor-β1
TGN	Trans Golgi network
TIMPs	Tissue inhibitors of metalloproteinases
TLR-4	Toll-like receptor-4
TMB	3,3’,5,5’-tetramethylbenzidine
TMEs	Tumour microenvironments
TNF-α	Tumour necrosis factor-α
TSG	Tumour suppressor genes
Tyr	Tyrosine
UPA	Urokinase plasminogen activator
UTRs	Untranslated regions
VEGF	Vascular endothelial growth factor
VPS34	Vesicle-mediated vacuolar protein sorting 34
α-SMA	α-smooth muscle actin

Abstract

Oesophageal adenocarcinoma (OAC) is an entity of increasing clinical importance due to an unexplained rise in incidence in recent decades in most Western countries. The prognosis is dismal. The most important risk factor is Barrett's oesophagus (BO) and so identifying those patients with BO at risk of progression to OAC becomes important.

A growing body of evidence implicates MMP-7 in tumorigenesis in a number of different organs, including roles in cellular transformation, cell survival, tumour growth and angiogenesis. MMP-7 expression is typically associated with epithelial cells but the regulatory mechanisms remain uncertain. The objectives of this thesis were (a) to evaluate the pattern of expression of MMP-7 in OAC and BO, (b) to investigate the mechanisms regulating its expression in an OAC cell line, and its functional significance, (c) to assess the action of gastrin on MMP-7 secretion by oesophageal cancer cell lines and (d) to characterise interactions between OE33 cells and myofibroblasts cells in modulating MMP-7 secretion and metabolism.

Expression of MMP-7 in all OAC cases was localized to carcinoma cells with relatively low expression in normal squamous oesophageal epithelium and progressively increased expression in BO, low grade dysplasia, high grade dysplasia and OAC with highest expression at the invasive front. In the stroma, myofibroblasts, identified as spindle-shaped cells, exhibited strong MMP-7 expression in the invasive part of the tumour. The data suggest that MMP-7 could be a novel predictive marker for progression of BO to OAC, as well as a potential target driving malignant transformation in BO.

Western blotting and ELISA revealed OE33 but not OE21 or OE19 cells secreted abundant proMMP-7 that was insensitive to phorbol 12-myristate 13-acetate (PMA) and both classical (amidated) and non-classical forms of gastrin. Factors other than hypergastrinaemia are therefore likely to be responsible for the increased MMP-7 that occurs in BO and OAC. MMP-7 secretion was inhibited by brefeldin A in OE33-GR cells. Additionally, blocking PI3kinase signalling decreased the expression and secretion of MMP-7 in OE33 cells, while inhibition of protein kinase C or MAP kinase activation had no effect. Cultured cancer-associated myofibroblasts expressed MMP-7, but there was no evidence of secretion into the media. However, MMP-7 was cleaved in the presence of myofibroblasts. Finally, CAMs exhibited increased migration in response to conditioned medium from OE33 cells and the response was reversed by MMP-7 neutralising antibody.

This work indicates that MMP-7 should now be formally evaluated as a marker for progression of BO to OAC. Increased expression in OAC may be attributable to enhanced PI3K signalling and may be functionally important in driving invasion and metastasis

CHAPTER 1

INTRODUCTION

1.1 Cancer: A historic perspective and overview

Cancer has been recognized since antiquity and there have been many different views of its origins. For instance, in the 17th century, it was thought that tumours grew from lymph constantly thrown out by the blood (lymph theory). Muller in 1838 then demonstrated that cancer is made up of cells not lymph, giving rise to the blastema theory. His student, Virchow (1821–1902) noted that all cells, including cancer cells, were derived from other cells. Virchow proposed that chronic irritation was the cause of cancer (chronic irritation theory)**.** Later, in the 1920s, cancer was thought to be caused by trauma (trauma theory) (Kardinal and Yarbro, 1979, Gallucci, 1985).

By the middle of the 20th century, the complex problems of chemistry and biology underlying the origins of cancer started to become clearer. Reviewing its first century of publication, *The New England Journal of Medicine* recorded that at the start of the period medical practitioners could observe tumours, weigh them, and measure them but had few tools to examine the workings of the cancer cell (DeVita and Rosenberg, 2012). Today, it is clear that cancer is caused by mutations and over 100 carcinogens that are mutagenic have been identified. As a consequence, something of the mechanism by which cancers are created has become clearer which in turn has provided insight into ways to prevent it.

The history of oesophageal cancer dates back to ancient Egyptian times, around 3000BC (Eslick, 2009). In recent years, progress in the diagnosis and treatment of oesophageal cancer has been steady rather than impressive. Despite improvements in the detection of premalignant changes and advanced preventative strategies, the overall incidence of oesophageal carcinomas has risen.

A clear association has been established between the development of oesophageal cancer and gastroesophageal reflux disease, smoking, and heavy alcohol use (Layke and Lopez, 2006).

Oesophageal cancer (OEC) is the eighth most common cause of cancer death worldwide. OEC has two main histopathological subtypes; squamous cell carcinoma (SCC) and adenocarcinoma (OAC). SCC arises from squamous epithelium that undergoes inflammatory, hyperplastic and dysplastic changes whereas OAC arises through metaplastic intestinal type changes (Barrett's oesophagus, BO) that replace the squamous epithelium. SCC is the predominant histological subtype. OAC is mostly a disease of developed society (Melhado et al., 2010). However, the growing number of newly diagnosed OAC cases is recognised as a problem. Many studies over several decades (Cairns, 1975, Lao-Sirieix et al., 2008, Reid et al., 2011) support the hypothesis that Barrett's specialized intestinal metaplasia represents an adaptation to the harsh intraoesophageal environment of chronic gastroesophageal reflux disease and predisposes to oesophageal adenocarcinoma (Naef et al., 1975, Barrett, 1950). The ultimate public health importance of BO therefore lies in its association with OAC (Falk, 2000).

OAC became the most common histological type of OEC in Western countries in the late 1990s. Data from genomic, transcriptomic, and proteomic studies have revealed the complexity of changes that develop during neoplastic evolution to OAC, including genome-wide chromosomal instability, disruption of regulatory pathways, and dynamic clonal evolution (Reid et al., 2010, Murugaesu et al., 2015, The Cancer Genome Atlas Research, 2017, Hayakawa et al., 2016). Current approaches for controlling OAC incidence and mortality are largely based on the endoscopic investigation of symptomatic gastroesophageal reflux disease and histology-guided

surveillance (Fornari and Wagner, 2012). To date, no prevention or early detection strategy has yet been conclusively established to reduce OAC. There is, therefore, an urgent need to recognise more precisely and with higher resolution (biological, and clinical measures) the risk of OAC and the factors determining the malignant transformation of BO.

1.2 Hallmarks of cancer

Tumorigenesis is a multistep process reflect molecular alterations and genetic instability. During the multistep development of malignant tumours, cancer cells acquire six biological capabilities (known as the hallmarks of cancer) that together constitute an organizing principle accounting for the complexities of neoplastic disease (Hanahan and Weinberg, 2000). The essential alterations in cell physiology that collectively direct malignant self-sustaining proliferative signalling are: evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis. Recently Weinberg and Hanahan proposed additional hallmarks: abnormal metabolic pathways, evading the immune system, genome instability, inflammation and signalling interactions in the tumour microenvironments (TMEs) (Hanahan and Weinberg, 2011). One new dimension of cancer complexity is, therefore, recognition of the reciprocal interactions between the neoplastic cells and the supporting stromal cells (heterotypic signalling) which are reflected by histopathological progression in cancer cells and can be extended to include the final stage of multistep tumour progression i.e. distant dissemination. The premetastatic niches may be induced by intrinsic factors in the new tissue site (Talmadge and Fidler, 2010), circulating factors released by the primary tumour

(Peinado et al., 2011), tumour-promoting inflammatory cells (DeNardo et al., 2010, Qian and Pollard, 2010, Mader, 2007) and the extracellular matrix (ECM).

1.3 Extracellular matrix: a dynamic niche in cancer

The ECM is a fundamental component of the microenvironment. There are two main types of ECM that differ with regard to their location and composition: the interstitial connective tissue matrix which surrounds cells and provides structural scaffolding for tissues, and the basement membrane which is a specialized form of ECM that separates the epithelium from the surrounding stroma (Bonnans et al., 2014a, Hynes and Naba, 2012). The ECM is composed of two main classes of macromolecules: proteoglycans (PGs) and fibrous proteins (Jarvelainen et al., 2009). PGs fill the majority of the extracellular interstitial space forming a hydrated gel; the main fibrous ECM proteins are collagens, elastins, fibronectins, and laminins. Most ECM proteins are large and complex with multiple distinct domains. PGs can modulate cell adhesion, migration and proliferation (Schaefer and Schaefer, 2010). However, basement membrane modular PGs (perlecan, agrin and collagen type XVIII) have a dual functions as pro- and anti-angiogenic factors (Iozzo et al., 2009). The physical properties of the ECM can be defined in terms of its porosity, insolubility, rigidity, structural arrangement and topography (orientation), as well as other physical features that together determine its role in scaffolding to support tissue structure and integrity (Frantz et al., 2010).

The interactions between ECM and cells are changeable and allow cells to continually remodel the ECM in their immediate microenvironment (Hynes, 2009). Feedback regulatory mechanisms between cells and their ECM also allow cells and

tissues to quickly accommodate to their environment (Samuel et al., 2011). The reciprocal interactions between the ECM and its resident cellular constitutes are essential in ECM remodelling, and it follows that the ECM contributes to the assembly of individual cells into tissues, transmits signals through ECM receptors to influence cell characteristics and activities (Yue, 2014). For instance, myofibroblasts are embedded in ECM and exhibit both fibrous junctions with other cells and specialized junctional complexes with the ECM. Large mature focal adhesions also allow strong attachments, contract and remodel the ECM, and provide a means of transducing mechanical force in the tissue (Darby et al., 2014).

In addition, components of the ECM constantly interact with epithelial cells by acting as ligands for cell receptors such as integrins, thereby transmitting signals that regulate adhesion, proliferation, apoptosis, migration, and survival or differentiation (Bonnans et al., 2014b). However, the importance of the ECM goes beyond providing physical support for tissue integrity and elasticity, since impairment of ECM composition leads to several pathological conditions.

1.4 Protein secretion pathways

Secretory proteins are required for cell growth, cell differentiation, proliferation, blood coagulation, and immune defense (Jackson et al., 1992, Hughes, 1999). Secretory proteins are also crucial in cancer angiogenesis, invasion, and metastasis (Pavlou and Diamandis, 2010, Ralhan et al., 2011, Jimenez et al., 2010). Pro-hormones often undergo wide-ranging cellular processing before secretion. These post-translation processing events occur in eukaryotic cells of the constitutive or regulated secretory pathway are responsible for protein secretion (Kelly, 1985). For

example it was indicated that N-terminal fragments of progastrin are secreted along the constitutive pathway, whereas endoproteolytically processed C-terminal fragments are secreted through the regulated or constitutive-like pathways (Bundgaard and Rehfeld, 2008)

1.4.1 *Constitutive secretory pathway*

The classical secretion mechanism depends on an N-terminal leader sequence that directs newly synthesized proteins on cytoplasmic ribosomes to the ER; they then pass from the last cisterna of the Golgi to secretory vesicles. The N-terminal signal peptide sequence is cleaved from the protein when the polypeptide chain is continuing to grow on the ribosome (Walter et al., 1984, Mellman and Warren, 2000). Secretion is constitutive if proteins are secreted as fast as they are synthesized.

1.4.2 *The regulated secretory pathway*

In regulated secretion, newly synthesized proteins destined for secretion are stored at high concentration in secretory vesicles until the cell receives an appropriate stimulus. Condensation takes place in the Golgi apparatus and exocytosis is regulated at the cell surface. It appears that those cells that regulate their secretion also condense their secretory products, whereas those that secrete constitutively do not (Kelly, 1985).

1.4.3 *Non- classical secretory pathway*

The non-classical secretory pathway (unconventional), i.e. proteins without N-terminal signal peptide, is also known as leaderless secretion. These proteins are secreted through direct translocation of proteins across the plasma membrane likely through membrane transporters (non-vesicular transport), or through organelle carriers (vesicular transport) by using intracellular vesicles such as blebbing,

lysosomal secretion, and release via exosomes derived from multivesicular bodies. These proteins are therefore secreted by ER-to-Golgi independent secretion pathways.

1.5 Gastrin

The pyloric antral hormone gastrin was discovered by John Sydney Edkins (Edkins, 1905) and isolated by Gregory and Tracy in Liverpool in the 1960s (Gregory et al., 1960). Gastrin is one of the best studied hormones of the digestive system. Besides assisting in the stimulation of gastric acid secretion via the CCK2 receptor (CCK2R), it also facilitates the proliferation of the gastric epithelial cells, tissue remodelling and angiogenesis (Burkitt et al., 2009, Dockray et al., 2005a, Dockray et al., 2012). At the cellular level, secretion of gastrin from G-cells is via Ca^{2+}-dependent release from secretory vesicles of the regulated pathway (Campos et al., 1990). It is well established that gastrin release occurs in response to both gastric luminal amino acids, neuronal stimulation (Walsh, 1994), and that the extracellular calcium-sensing receptor plays some role (Buchan et al., 2001).

1.5.1 *Synthesis and processing*

Human gastrin is the product of single gene located on chromosome 17q. Its transcription is regulated by somatostatin and by ligands of epidermal growth factor (EGF) (Merchant et al., 1991). Following transcription, gastrin mRNA is translated to pre-progastrin at the ER; preprogastrin is a 101 amino acid peptide including a signal peptide at its N-terminus and is rapidly converted to progastrin (Varro and Dockray, 1993).

In G-cells, progastrin is translocated through the Golgi complex where it may be modified by Tyr sulphation and Ser-phosphorylation(Varro et al., 1994). It is then sequestered in vesicles of the regulatory secretory pathway.

The cleavage of progastrin at two pairs of Arg residues occurs relatively rapidly, within 10 min, after exiting from the *trans*-Golgi network (TGN) to secretory vesicles, further cleavage at a pair of Lys residues occurs more slowly (within 60 min). These cleavages are thought to be mediated by the prohormone convertases PC1/3 and PC2 (Macro et al., 1997). As shown in Figure 1.1, endopeptidase cleavage is then followed by carboxypeptidase trimming of C-terminal Arg or Lys residues to create the extreme C-terminal flanking peptide (CFP) together with G34-Gly or G17-Gly, as well as G34-CFP or G17-CFP (Dockray et al., 2001). Collectively, progastrin and the COOH-terminal Gly-gastrins are known as the non-classical gastrins (Dockray et al., 2005b). Further conversion to matured amidated gastrin-17 (G17) or G34 then occurs following the action of peptidyl alpha-amidating monooxygenase (PAM) in secretory vesicles of the regulated secretory pathway (Dockray et al., 2001). The peptides G34 and G17 are known as the classical gastrins, they possess the defining biological property of the hormone: the stimulation of acid secretion.

1.5.2 *Classical gastrins*

In humans, amidated gastrins occur mainly as G17 and G34 both of which may be sulphated on their solitary tyrosine residue. G34 and G17 were originally characterized and their biological properties defined by Gregory and Tracy (Gregory et al., 1964, Gregory and Tracy, 1964, Gregory and Tracy, 1972).

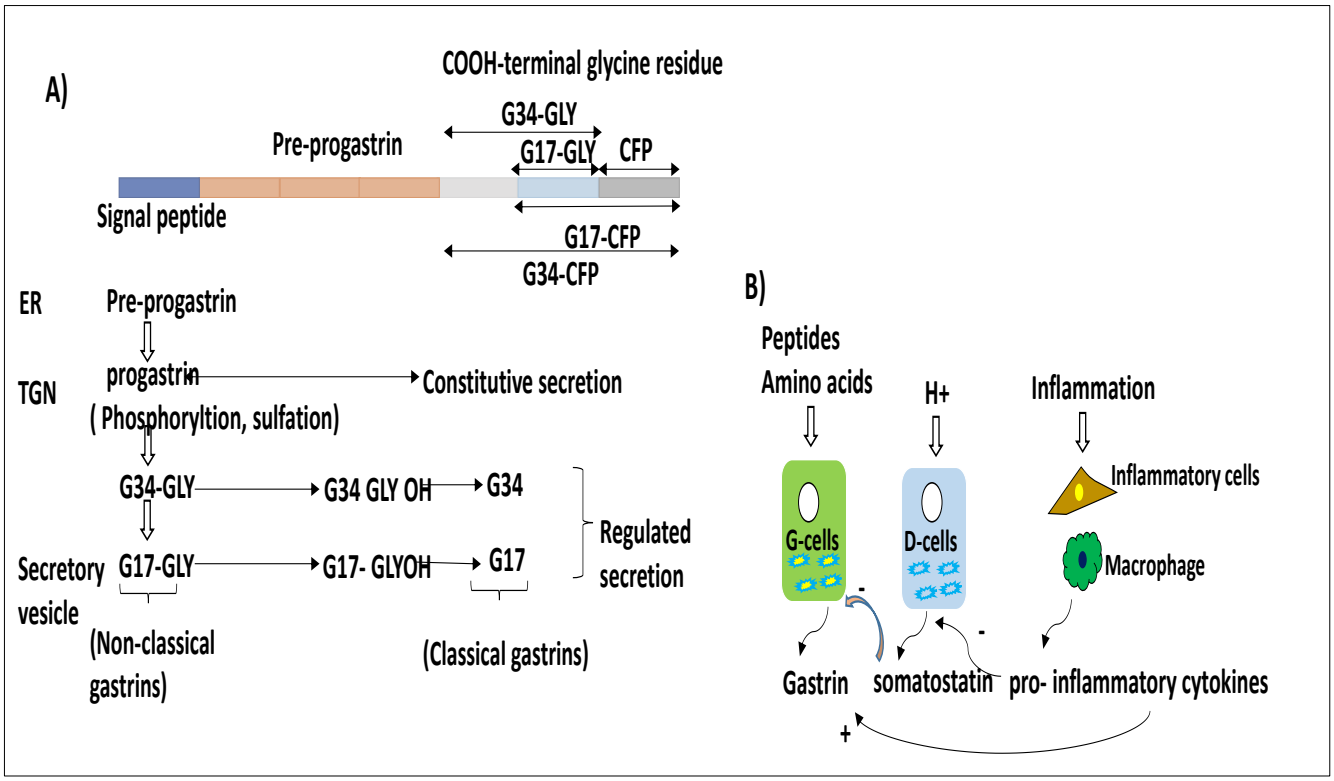

Figure 1.1: Conversion of pre progastrin to mature amidated gastrin A) Schematic representation of the biosynthesis of gastrin showed that the earliest cleavage products of pre progastrin B) Schematic representation of cellular relationships in the pyloric antral epithelium. The release of gastrin from G-cells is stimulated by peptides and amino acids, gastrin release inhibited by somatostatin secreted by D cells. In the case of inflammation (e.g. *Helicobacter pylori*), pro-inflammatory cytokines may inhibit D-cells and stimulate G-cells, so enhance gastrin release indirectly and directly.

Variable cleavage of the Lys74-Lys75 bond means that the relative proportions of G34 and G17 varies between different tissues: G17 predominates in the antral mucosa where about 95% is this form (Dockray, 1999) while G34 is more prevalent in the duodenum (Walsh, 1994). In addition to stimulation of acid secretion, amidated gastrins stimulate gastric epithelial proliferation and differentiation (Dockray et al., 2001). Gastrin may also contribute to the progression to gastric cancer particularly in association with the carcinogenic bacterium *Helicobacter pylori* (Dimaline and Varro, 2007, Wang et al., 2000).

1.5.3 *Non-classical gastrins*

The conversion of progastrin to smaller peptides is governed by prohormone phosphorylation and secretory vesicle pH. The CFP together with G34-Gly or G17-Gly, as well as G34-CFP or G17-CFP, together with intact progastrin, may all be found in cells lacking the regulated secretory pathway (Dimaline and Varro, 2014, Dockray et al., 2001). It has been suggested that progastrin, as well as intermediate processing products such as the glycine-extended gastrins, have their own distinctive biological activities, especially in the colon (Dockray et al., 2001, Nemeth et al., 1993). Progastrin and Gly-gastrins stimulate colonic epithelial proliferation and may influence gastric epithelial differentiation and function at least in part by the release of paracrine factors that include histamine, epidermal growth factor (EGF)-receptor ligands (Dockray et al., 2001). Additionally, they may also be expressed in cell lines and cancers that are unable to process progastrin to the classical amidated peptides such as in oesophagus (Yuan et al., 2008, Dockray et al., 2001). Plasma levels of progastrin but not amidated gastrin or glycine-extended gastrin are elevated in patients with colorectal carcinoma (Siddheshwar et al., 2001).

In some cancer cells, progastrin may be secreted by the constitutive pathway direct from the TGN to the cell surface (Dockray, 1999). Also, it was reported that overexpression of progastrin induced the metastatic potential of embryonic epithelial cells (Sarkar et al., 2012). The proliferative action of the Gly-gastrins has been reported in both transformed and non-transformed cells (Watson et al., 1996).

1.5.4 *Gastrin receptors*

Amidated gastrins act at the cholecystokinin-2 (CCK2R; also known as gastrin/CCK-b) receptor which is normally expressed on gastric parietal and enterochromaffin-like (ECL) cells, some smooth muscle cells and is highly expressed in central nervous system (CNS) neurons. The CCK2R is a member of the 7-transmembrane domain G-protein-coupled receptor superfamily. It acts via $G\alpha_{q/11}$ to increase intracellular calcium concentrations and protein kinase C activity (Dufresne et al., 2006, Wank, 1995, Prinz et al., 1993, Kinoshita et al., 1998, Sachs et al., 1997). This receptor has high affinity for both gastrin and CCK (Berna and Jensen, 2007, Ashurst et al., 2008); however, gastrin is the main ligand for receptors on parietal and ECL cells because its plasma concentrations are roughly 10 times higher than those of CCK (Miller and Gao, 2008, Kopin et al., 1992). However, CCK is the main ligand for CNS receptors because there is abundant CCK, but little or no gastrin, in the brain (Pisegna et al., 1992). Other gastrin products such as progastrin and the Gly-gastrins (Seva et al., 1994, Beales and Ogunwobi, 2009) do not have the COOH terminal amide of G17 and while they may stimulate cell proliferation it is not via CCK2R (Dockray et al., 2012, Varro and Ardill, 2003); both are reported to have their own characteristic pattern of biological activities that are mediated by non-CCK1/non-CCK2 receptors (Ahmed et al., 2004, Singh et al., 2003).

1.5.5 *Gastrins and MMP upregulation*

Gastrin regulates the expression of a number of molecules that exert important effects on extracellular matrix remodeling (Dimaline and Varro, 2014). Initial array studies of the gastric cancer cell line, AGS-GR (i.e. expressing CCK2R), identified plasminogen activator inhibitor (PAI)-2, MMP-7 and -9 as previously unsuspected gastrin-regulated genes (Varro et al., 2002, Wroblewski et al., 2002). Moreover, stimulation of gastric MMP-7 is associated with elevated plasma gastrin (Varro et al., 2007b). Hypergastrinaemic mice showed enhanced expression of MMP-9 and -13 (Takaishi et al., 2009). Gastrin increases MMP-9 expression which in turn stimulates gastric epithelial cell invasion (Wroblewski et al., 2002). In addition, in gastric epithelial cells, MMP-1 expression is a target of gastrin (Kumar et al., 2015). G-Gly renders colon cancer cells more invasive by increasing MMP-1 and MMP-3 expressions via the putative G-Gly receptor (Baba et al., 2004).

1.5.6 *Gastrin and oesophageal cancer*

The role of PPIs in the management of gastro-oesophageal reflux disease (GORD) erosive oesophagitis has been well established. However, PPIs at therapeutic doses cause physiological secondary hypergastrinaemia in Barrett's patients (Wang et al., 2010a). There is increased oesophageal carcinomas in animals receiving long-term PPIs (Dall'Olmo et al., 2014) and CCK-2R mRNA has been shown in the inflamed oesophageal squamous mucosa, Barrett's mucosa (2-fold higher than those of the normal squamous mucosa), and OAC (Haigh, 2003). Additionally, human oesophageal adenocarcinoma cell lines (SEG-1, BIC, and SKGT-4) have been reported to express CCK2R (Moore et al., 2004). Another study revealed that BO cells have a high CCK2R expression and that gastrin

stimulated proliferation, anti-apoptosis and loss of cell to cell adhesion in these cells (Ferrand and Wang, 2006). Quite recently CCK2R has been localized to a LGR5+ stem cell population in BO (Lee et al., 2016).

Non-classical gastrins, for example Gly-gastrin, stimulate proliferation in oesophageal cancer cell lines (OE19 and OE33) via COX-2 expression (Ogunwobi and Beales, 2008). Moreover, Beales et al concluded that G-Gly inhibits apoptosis in BO and OAC via mechanisms distinct from those activated by G-17 and involving JAK2 and STAT3 activation (Beales and Ogunwobi, 2009). Together this evidence supports the idea that gastrin may have an essential role in oesophageal tumourigenesis.

1.6 Intercellular signalling pathways

Intracellular signalling pathways are basic cellular mechanisms controlling cell growth, proliferation, metabolism, apoptosis, carcinogenesis and many other processes (Duronio and Xiong, 2013, Buchen, 2011b). Signal transduction pathways are molecular events by which extracellular signals acting at membrane receptors (in the case of hydrophilic messenger molecules) to influence cell function and allow cellular responses to be coordinated (Papin et al., 2005, Buchen, 2011a). Signalling pathways frequently allow the convergence of multiple extracellular messengers on common mechanisms of action. They mediate, therefore, the action of extracellular stimuli such as growth factors, cytokines, and neurotransmitters that bind cell surface receptors and give rise to a cascade of biochemical events which may involve nuclear responses involving activation of transcriptional machinery or other responses including secretion and cell movement. Autoimmune diseases and cancer are caused by errors in signalling interactions and cellular information processing. The cancer phenotype is acquired by activation or inhibiting of key signalling pathways as a result of genetic or epigenetic mechanisms. Signalling pathways in cancer cells are commonly unregulated and resistant to feedback inhibition resulting in maintained activation of signalling pathways (Keld and Ang, 2011).

1.6.1 *PI3Kinase pathway*

Phosphatidylinositol-3 kinases (PI3Kinases) comprise a lipid kinase family activated by a large number of cell surface receptors, especially those related to tyrosine kinase and Ras superfamily of proteins (RAS). The initial catalyst activity of PI3kinase is the phosphorylation of the 3-OH position which produces a second

messenger that plays essential roles in various critical signalling pathways (Parikshit et al., 2014), including those correlated with cell survival and proliferation.

Based on structure and substrate specificity, PI3kinase can be classified into three sub-classes (I, II, and III) (Faes and Dormond, 2015). Class I are activated by growth factor receptor tyrosine kinases (RTKs). In the inner side of plasma membrane phosphatidylinositol-4, 5-bisphosphate (PIP_2) is phosphorylated by class I enzymes to produce phosphatidylinositol-3, 4, 5-trisphosphate (PIP_3). According to signalling receptors, class I is further divided into two groups; class IA are heterodimers of a p110 catalytic subunit with three isoforms (α, β, and δ), and a regulatory subunit (with three isoforms: p85α, p85β, and p55γ). Class IB consists of a p101-regulatory subunit and a p110γ catalytic subunit and activated by G protein–coupled receptors (GPCRs). Class II are activated by tyrosine kinase-coupled receptor and remain poorly characterized (Engelman, 2007). Class III have a single catalytic subunit called VPS34 (vesicle-mediated vacuolar protein sorting 34) and are involved in autophagy (Herman and Emr, 1990). Also VPS34 plays role in intracellular vesicle transport and protein sorting (Schu et al., 1993). Dysregulation of PI3Kinase and its downstream effectors is implicated in many cancer types. The p110α catalytic subunit is the only PI3K gene established with common mutations in cancer (Ligresti et al., 2009). The serine-threonine kinase Akt (also known as protein kinase B, PKB) is the most substantial downstream effector of PIP_3. Akt is phosphorylated at Thr308 by 3-phosphoinositide-dependent protein kinase 1 (PDK1) leading to partial Akt activation; full enzymatic activity of Akt occurs after phosphorylation at Ser473 by mTORC2. Akt has three tightly related isoforms (Akt1, Akt2, and Akt3) (Hers et al.,

2011). Akt activity is inhibited by phosphatase and tensin homolog (PTEN), however, PTEN is frequently lost or mutated in cancer (Salmena et al., 2008).

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase involved in cell growth and proliferation (Hay and Sonenberg, 2004). It is activated through the phosphorylation and inactivation of the repressor of mRNA translation. mTOR has two separate protein kinase complexes mTORC1 and mTORC2. mTOR dysfunction has been correlated with human tumours (Porta et al., 2014). It has been demonstrated that protein serine/threonine kinase-3′-phosphoinositide-dependent kinase 1 (PDK1) acts in cancer cells harbouring PI3Kinase mutations through MAPK (mitogen-activated protein kinase) or PKCα (protein kinase C alpha) rather than Akt.

1.6.2 *Mitogen-activated protein kinases (MAPKs)*

The mitogen-activated protein kinases (MAPKs) are a family of proteins that are key regulators for various cellular activities, and mediate a variety of extracellular stimuli including growth factors, cytokines, hormones, and several cellular stressors such as (oxidative stress and endoplasmic reticulum stress). They act on pathways that include GTPase signal transducer proteins. The RAS and the RAFa families of related proteins. The main groups of MAPKs are the extracellular signal-regulated protein kinases (ERK1/2 or p44/42), the p38 MAP kinases (isoforms α, β, γ, δ), and the c-Jun NH2-terminal kinases (JNK1/2/3) (Chen et al., 2001). MAPK cascades uniformly conduct a wide set of cellular processes such as cell proliferation, differentiation, metabolism, motility, survival and apoptosis.

Abnormal functioning of these cascades plays a part in the development and progression of cancer, as well as in determining responses to cancer therapy.

Mutations of the components of the MAPK cascade are common and may even contribute to the majority of cancers (>1/2 of cancer) (Montagut and Settleman, 2009, Schubbert et al., 2007). For instance, ERK MAPK is active in 60% of OAC. Also it was reported that there is an apparent association between the presence of BRAF and RAS mutations in cancer suggesting that RAFs are regulated by binding RAS. The possibility that in KRAS and BRAF mutant cancer cells differential signalling mechanisms that involve MEK, supported the mutual exclusivity of these two mutations (Oikonomou et al., 2014). For example, it was indicated that mutations of KRAS/NRAS/BRAF predict the response to cetuximab therapy in metastatic colorectal cancer patients (Hsu et al., 2016). The development of kinase inhibitors directed at the RAF and MEK kinases has attracted considerable attention; some inhibitors have already given encouraging results in initial clinical studies (Montagut and Settleman, 2009).

1.6.3 *Protein kinase C (PKC) signalling*

PKC refers to a large family of protein kinases involved in various cellular functions, including regulating cell proliferation and apoptosis, controlling gene transcription and translation. They regulate the activity of proteins by phosphorylating hydroxyl groups of their threonine and serine residues. PKC is composed of a catalytic domain and a regulatory region. Typical PKCs (PKCα, βI, βII and γ) need both calcium and diacylglycerol (DAG) for their activation. Structural conformational changes in PKC allow it to phosphorylate its substrates. Finally, atypical PKCs do not need either DAG or Ca^{2+} for their activation, although they do rely on diverse lipid metabolites and second messengers.

PKC acts as the main receptor for the tumour promoting phorbol esters. Phorbol esters have a potent capability to activate two of the three classes of PKC isoenzymes, so they provide useful pharmacological tools for directly increasing PKC activity (Wu-Zhang and Newton, 2013). Many biological conditions are governed by PKC and so perhaps not surprisingly its dysregulation is implicated in a variety of diseases including cancer (Griner and Kazanietz, 2007). Targeting PKC for cancer therapy has been the subject of recent work (Mochly-Rosen et al., 2012, Kang, 2014).

1.6.4 *Signalling pathways in oesophageal adenocarcinoma (OAC)*

It is generally accepted that the progression of BO to OAC is a multistep process characterized by genomic instability which aids accumulation of lesions that target proto-oncogenes, tumour suppressor genes (TSG), mismatch repair genes, and mitotic checkpoint genes thereby facilitating tumour progression (Rabinovitch et al., 1989, Lengauer et al., 1998). It has been suggested that EGFR activation through ligands such as EGF and TGFα activates an autocrine signalling mechanism that may be an early event in the BO metaplasia-dysplasia-OAC sequence (Jankowski et al., 1992a, Jankowski et al., 1991). The EGFR has been recognized as significantly upregulated in the progression of BO to OAC (Hector et al., 2010). Downstream of this there are Ras /ERK and PI3K /Akt activation which also appear to be important in the development of BO in response to acid and bile salts resulting in enhanced proliferation, inhibition of apoptosis, and upregulation of mucin glycoprotein (MUC-1, -4) and COX-2 (Ogunwobi et al., 2006, Beales et al., 2007, Mariette et al., 2008, Song et al., 2007b, Sagatys et al., 2007), all of which are involved in Barrett's carcinogenesis. The active members of the MAPK pathway were established to be

upregulated in about 40% of OAC (Paterson et al., 2013). MAPK signalling is an important driver of PEA3-mediated transactivation of MMP-1 in OAC cell lines (Keld et al., 2010a). Phospho-Akt (active Akt signalling) is increased along the progression from the normal oesophagus to BO, dysplasia, and OAC (Beales et al., 2007, Sagatys et al., 2007). Pharmacological inhibition of PI3 kinase which is a potential upstream activator of Akt has been shown to decrease proliferation and induce apoptosis in cultured oesophageal cancer cell lines (Vona-Davis et al., 2005). All the above-mentioned data revealed the complexity of the factors involved in OAC development.

1.7 An overview of inflammation

Elimination of the initial cause of cell injury (foreign invaders), removal of necrotic cells and tissue, and initiation of the repair process are crucial to tissue responses to injury and are achieved via complex mechanisms collectively known as inflammation. In response to tissue injury, a complex network of chemical signals initiates and maintains host responses focused on healing the damaged tissue. The initial inflammatory response is defined by five cardinal signs; heat (calor), redness (rubor), swelling (tumor), pain (dollar), and loss of function (laesa). It is initiated and accelerated by platelet secreted proteins such as transforming growth factor-β1 (TGF- β1), platelet-derived growth factor (PDGF), and platelet derived angiogenesis factor (PDAF) which recruit leukocytes including neutrophils, macrophages/monocytes, eosinophils and natural killer cells to sites of injury. Also, tissue mast cells play a significant role by releasing histamine and anaphylatoxins that act as vasodilators (Fernandez et al., 1978).

Neutrophils initiate wound healing by acting as a source of early response pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1α and IL-1β (Hubner et al., 1996, Feiken et al., 1995). These cytokines mediate leukocyte adherence to the vascular endothelium, thereby targeting and restricting leukocytes to areas of repair. They initiate repair by inducing expression of MMPs (Dovi et al., 2004), and phagocytosis. As the number of neutrophils subsequently declines, monocytes/macrophages differentiate into mature macrophages or immature dendritic cells (Osusky et al., 1997). Active macrophages modulate tissue repair by releasing TGF-β1, PDGF, b FGF, TGF-α, insulin-like growth factor (IGF)-I and -II, TNF-α and lL-1. Moreover, macrophages are implicated in remodeling the ECM by stimulating the production of proteolytic enzymes (for example, MMPs and urokinase plasminogen activator (uPA), clearing apoptotic and necrotic cells, and modulating angiogenesis through local production of thrombospondin-1 (DiPietro, 1995).

Angiogenesis (neovascularisation) represents an essential part of wound healing and is a tightly regulated processes. Some factors enhance proliferation of endothelial cells and support neo-angiogenesis, such as endothelial cell growth factors (Scrofani et al., 2000), FGF-2, IGF-I and IGF-II (Chang et al., 2004). uncontrolled angiogenesis has been correlated with several conditions including arthritis and cancer development (Folkman, 1990).

1.7.1 *Cancer and inflammation*

In 1863 Virchow hypothesized that cancer developed at sites of chronic inflammation (Balkwill and Mantovani, 2001). Meta-analysis studies have since demonstrated that

greater than 15% of malignancies worldwide can be attributed to infections (Kuper et al., 2000, Blaser et al., 1995, Shacter and Weitzman, 2002). For instance, long-standing *H.pylori* infection leads to gastric cancer (Watanabe et al., 1998), and human papillomavirus plays a central role in cervical cancer worldwide (Bosch et al., 1995). As the fundamental feature of chronic inflammation is persistence for long duration (weeks, months and years) the longer the inflammation persists, the higher the risk of associated carcinogenesis. Repeated tissue damage involves persistent generation of reactive oxygen and nitrogen species produced by leukocytes and other recruited cells (phagocytic) at the site of inflammation that in turn are associated with DNA damage in proliferating cells resulting in the acquisition of permanent genetic changes including deletions, point mutations and rearrangements (Maeda and Akaike, 1998). The association between inflammation and cancer has been captured in the well-known expression (Dvorak, 1986) “tumours are wounds that never heal”. Later (Dvorak, 2015) suggested the tumour co-opted the healing response of wounds to generate its stroma required for cancer cell survival and growth.

In addition to infection, there are other conditions that are correlated with chronic irritation and subsequent inflammation leading to cancer (Balkwill and Mantovani, 2001, Coussens and Werb, 2002). Thus, colon carcinogenesis arises more often in individuals with inflammatory bowel diseases (chronic ulcerative colitis and Crohn’s disease), and oesophageal adenocarcinoma is usually preceded by years of inflammation caused by gastro-oesophageal reflux.

1.8 Gastroesophageal reflux disease (GORD)

The initiating event in GORD is increased exposure of the oesophageal squamous epithelium to gastric contents (acid, pepsin, trypsin, and bile acids) (Dodds et al., 1982), which leads to mucosal injury and a nonspecific inflammatory infiltrate (neutrophils, eosinophils, and macrophages) surrounding the damaged epithelium. The associated inflammatory processes contribute to the well-known complications of GORD namely abnormalities in oesophageal defense mechanisms, abnormal motility, fibrosis and carcinogenesis (Rieder et al., 2010). In GORD inflammation, oxidative stress with subsequent reactive oxygen species (ROS) production (Lee et al., 2001) may activate a number of cancer-associated signalling pathways such as PI3Kinase/Akt, ERK1/2, and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Song et al., 2007b). Exaggerated regeneration of the oesophageal epithelium and increased cellular turnover (Dvorak et al., 2007), work together to produce neoplastic progression. The relationship between longstanding and sever GORD and the development of Barrett's oesophagus is now well accepted (Ronkainen et al., 2005, Conio et al., 2002, Souza et al., 2008, Dresner et al., 2003). Proton pump inhibitors (PPIs) are the first drug of choice to treat GORD. Oesophageal acid exposure is reduced by PPIs which decreases inflammation and proliferation. However, PPIs treatment also increases circulating gastrin concentrations which may cause proliferation, COX-2 upregulation, and possibly promote the development of Barrett's metaplasia and its progression to dysplasia or cancer (Dall'Olmo et al., 2014, Miyashita et al., 2013, Wang et al., 2010b).

1.9 Barrett's oesophagus (BO)

BO was described in 1950 by Norman Barrett (Barrett, 1950) and defined as replacement of normal oesophageal squamous epithelium in the distal portion by columnar epithelium. It was reported to be associated with GORD (Allison and Johnstone, 1953), and was later identified as a precursor lesion for development of oesophageal adenocarcinoma (Naef et al., 1975). The prevalence of BO is significantly higher in Caucasian males living in Western countries of higher socioeconomic status; it is lower among Asians and blacks; prevalence increases with age (Ford et al., 2005). An epidemiologic survey indicated the median age for developing BO is 40 years even though the mean age at diagnosis is 63 years (Cameron, 1997). The progression of BO to OAC is generally accepted to be a multistep processes (Spechler and Goyal, 1996). Barrett's specialised intestinal metaplasia is frequently regarded as an adaptation to the intra-oesophageal environment generated by chronic gastroesophageal reflux; the lesion acquires several functions not present in the normal oesophageal squamous epithelium (Orlando, 2006, Lao-Sirieix et al., 2008). Products of the stem cells that maintain the squamous epithelium undergo altered differentiation (trans- differentiation) due to changes in chemical environment giving rise to intestinal-type metaplasia (Guillem, 2005, Jankowski et al., 1999). Normally, the endoderm develops into a stratified squamous epithelium supported by a confluent basal layer of p63 expressing progenitor cells. However, loss of p63 results in the failure of proper squamous epithelial expansion, and instead leads to the persistence of mucus-producing ciliated columnar epithelial cells in distal oesophagus (Xian et al., 2012).

In addition to metaplastic columnar epithelium with intestinal-type goblet cells (IM), there are two other histological subtypes of Barrett's metaplasia: the fundic (oxyntic) subtype exhibits parietal and chief cells, and the cardiac (junctional) subtype exhibits mucus-secreting glands. Several factors are thought to be responsible for the heterogeneity of metaplasia including the degree of local stem cell enrichment, multiple independent clones, and altered gene expression profiles (Leedham et al., 2008, Kaz et al., 2015). The importance of this subgrouping of the histological subtypes of BO lies in their potential to progress to cancer.

To diagnose BO according to most recent British Society of Gastroenterology guidelines (Gregson et al., 2016) the intestinal columnar epithelium should be visible endoscopically and confirmed histologically in the distal oesophagus (Fitzgerald et al., 2014). In the American Gastroenterological Association guidelines, IM is a requirement for the diagnosis of BO (Spechler et al., 2011), since BO with IM is biologically more unstable than other metaplastic subtypes and is more likely to progress to dysplasia and neoplasia (Bhat et al., 2011). Even so, the role of IM in the carcinogenic process remains a subject of discussion. Thus IM with goblet cells and cardiac metaplasia have higher risk of malignancy than fundic type (Spechler and Goyal, 1996, Voltaggio et al., 2011).The risk for development of OAC is therefore not solely restricted to IM and better designed and powered studies are required to assess properly the true risk of progression in each subtype (Riddell and Odze, 2009).

1.9.1 *Dysplasia in Barrett's oesophagus*

Dysplasia is defined as neoplastic epithelium that remains confined within the basement membrane of the epithelial surface. The Vienna classification system is used for dysplasia grading, i.e. no dysplasia, low grade dysplasia (LGD) and high grade dysplasia (HGD) (Schlemper et al., 2000). Correlation between pathologists for the diagnosis of LGD is still poor (Curvers et al., 2010). About 75% of LGD patients regress with no indication of dysplasia at subsequent biopsies; however, 60 -90% of HGD patients progress to OAC (Chang and Katzka, 2004, Kaye et al., 2009, Montgomery et al., 2001) . To improve the risk stratification in BO patients, repeated endoscopic biopsies and histological assessment of dysplasia are used (Reid et al., 2010). At the molecular level, alteration in microsatellite instability, expression of matrix metalloproteinases (MMPs), epidermal growth factor receptor (EGFR) and transforming growth factor α (TGFα), are all thought to push the Barrett's epithelium to cancer as shown in (Figure 1.2). These and related changes therefore offer potential areas of intervention to prevent and manage oesophageal adenocarcinoma (Wijnhoven et al., 2001).

1.10 Oesophageal adenocarcinoma (OAC)

Cancer of the oesophagus is the fourth most common cause of cancer death in men in the UK (Graham et al., 2016) and has two main histological subtypes: squamous cell carcinoma (SCC) with squamous cell differentiation and adenocarcinoma (OAC) with glandular differentiation. Both are typically located in the distal oesophagus. The majority of OAC cases occur in Western countries where the incidence is greatly influenced by sex, stage, and race/ethnicity (Nordenstedt and El-Serag, 2011). OAC is characterised by high mortality which is improved by early diagnosis, advances in

surgical care and new treatment modalities. As a result of long-term use of PPIs, hypergastrinaemia may be present and has been suggested as another possible risk factor for OAC (Lee et al., 2017, Wang et al., 2010b, Haigh et al., 2003a). Additionally, obesity has been identified as strong risk factor for OAC through inflammatory and metabolic changes (Lagergren, 2011).The increasing prevalence of obesity in Western populations could, therefore, partly explain the increasing incidence of OAC. In this context it is relevant that studies have demonstrated a correlation between serum leptin and insulin in blood, as well as metabolic syndrome components, with increased risk of BO (Kendall et al., 2008, Chandar et al., 2015).

Heterogeneity of OAC, sometimes referred to as complex pathogenesis, is accompanied by multiple changes at the molecular level (Hayakawa et al., 2016). OAC is diagnosed by endoscopic biopsy (Stahl et al., 2013) and the treatment is based on cancer staging. Accurate tumour staging and histological grading is therefore crucial to improve cancer prognosis. As displayed in table 1.1 the most recent and commonly used American Joint Committee of Cancer (AJCC) staging system (Rice et al., 2010) is based on depth of tumour invasion, regional lymph node involvement, and the presence or absence of distant metastasis. Grading of OAC is traditionally based on cytological and histological features similar to cell of origin. OAC is characterized by high frequency of heterogeneous genomic alterations with complex chromosome instability and mutations, and disruption of intracellular regulatory pathways.

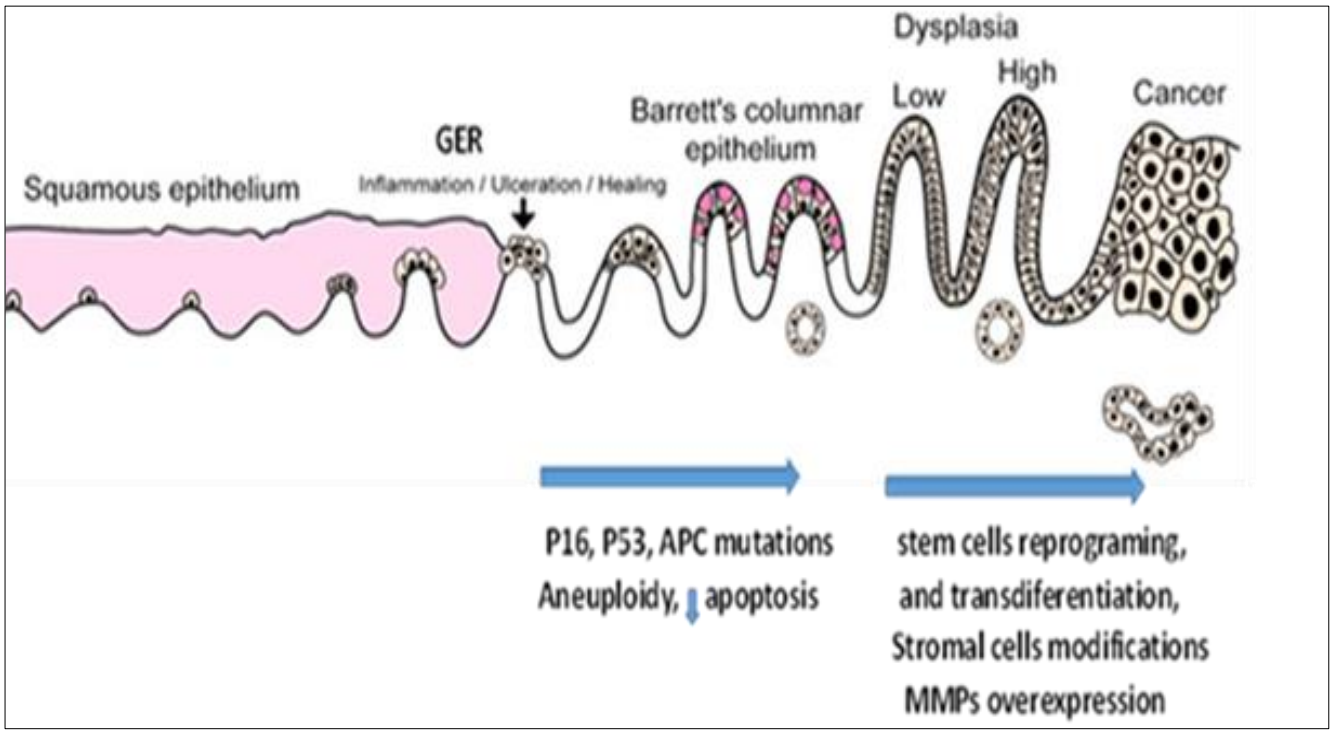

Figure 1.2: BO Progression to OAC. Schematic representation of changes in BO in progression to OAC. Reprogramming and/or trans-differentiation of stem cells situated in the basal layer of the normal squamous epithelium, as well as modifications of stromal cells characterized by mesenchymal-to-epithelial transition are part of the progression.

Tumour		
T_{is}		High-grade dysplasia
T1		Invasion into the lamina propria, muscularis mucosae, or submucosa
T2		Invasion into muscularis propria
T3		Invasion into adventitia
T4		Invasion of adjacent structures
	T4a	Invades resectable adjacent structures (pleura, pericardium, diaphragm)
	T4b	Invades unresectable adjacent structures (aorta, vertebral body, trachea)
L.N status		
N0		No regional lymph node metastases
N1		1 to 2 positive regional lymph nodes
N2		3 to 6 positive regional lymph nodes
N3		7 or more positive regional lymph nodes
M status		
M0		No distant metastases
M1		Distant metastases
Histologic grade		
G1		Well differentiated
G2		Moderately differentiated
G3		Poorly differentiated
G4		Undifferentiated

Table1.1 TNM staging system. As displayed in the table, is based exclusively on the anatomical extent of the disease tumour depth (T), number of lymph nodes involved (N), and presence or absence of metastasis (M).

1.11 Biomarkers in BO and OAC

Exploring the molecular alterations that direct the progression of BO to OAC might help to identify clinically relevant biomarkers of progression (Clemons et al., 2013). The criteria required for selecting biomarkers suitable for clinical use are not well defined. However, the Early Detection Research Network (EDRN) has identified five stages for defining biomarkers for risk of progression (Pepe et al., 2001); there has also been work on prognostic biomarkers (McShane et al., 2005). Most putative biomarkers that have been studied have not progressed into clinical use either due to problems developing precise assays or because the biomarker lacks sufficient sensitivity and specificity in validation studies (Srivastava et al., 2001). In spite of much work, there remain no biomarkers suitable for replacement of the current golden standard: dysplasia. Important factors determining whether molecular signatures are accepted for clinical application include the type of methods e.g. immunohistochemistry, data reproducibility and validation in independent samples (Altman et al., 2009). Identification of biomarkers tested in biological samples (blood or endoscopic biopsies) may help to improve clinical treatment at different stages of the disease. For instance, unrecognized cases of BO could be diagnosed in the population by screening biomarkers, whereas predictive biomarkers could be used in addition to, or replacing, the current surveillance program for the detection of dysplasia which has inter and intra-observer error, and one of the most difficult tasks for the pathologist (Reid et al., 1988).

Numerous studies in BO (Reid et al., 2001, Rabinovitch et al., 2001) have correlated aneuploidy and specific DNA abnormalities with the progression; however, these features are not easy to apply to the clinical use. Other studies have demonstrated that the abnormal surface staining of proliferation markers

(mini chromosome maintenance protein (Mm) 2, 5 and Ki67) in dysplastic Barrett's mucosa could be used to provide a novel method for detecting patients at risk for developing OAC. However, large prospective studies are still needed before the application of these methods in routine clinical practice (Sirieix et al., 2003, Hong et al., 1995, Going et al., 2002). Cell cycle markers such as cyclin A, D, and p53 have been reported to be useful biomarkers of malignant progression (Trudgill et al., 2003, Bani-Hani et al., 2000a). More recently, Jin et al have indicated a methylation biomarker panel containing eight genes might accurately determine the risk of progression in BO in a retrospective, multicentre validation study (Jin et al., 2009). However, the utility of hypermethylation as a biomarker is problematic, because the techniques are technically demanding and time consuming for routine application (Laird, 2010). Interestingly, NF-κB over-expression was reported to be specific to BO and OAC, but not reflux oesophagitis, which suggests that it may be a marker of metaplasia-dysplasia-adenocarcinoma progression rather than simple inflammation (O'Riordan et al., 2005). NF-κB was also associated with shortened disease-free and overall survival in patients with OAC (Izzo et al., 2007). It is, therefore, challenging to predict the progression of BO to OAC by using biomarker approaches (Gregson et al., 2016). There is some indication that MMP-7 and -9 have been reported as elevated in non-dysplastic BO, with even higher levels found in dysplastic Barrett mucosa and OAC (Salmela et al., 2001a, Herszenyi et al., 2007b, Zhang et al., 2010). Recently, the absence of SOX2 expression has been reported as a highly specific marker to predict the neoplastic progression in BO (van Olphen et al., 2015).

1.12 Tumour microenvironment (TME)

The TME has attracted increasing attention in recent years (Spill et al., 2016) although the precise functions of different TME constituents are still not fully appreciated. In particular the TME has been recognized as a key contributor to cancer progression (Chen et al., 2015, Quail and Joyce, 2013) and drug resistance (Trédan et al., 2007, Tsai et al., 2014). The TME is made up of various (stromal) cell populations, signalling factors and structural molecules that interact with tumour cells and support all stages of tumourigenesis (Hanahan and Weinberg, 2011, Whiteside, 2008) indicating that cancer is a result of a cooperation of multiple uncontrolled cell types (Figure 1.3).

Stromal cells increase in cancer by inward migration of normal cells, trans-differentiation of fibroblasts to myofibroblasts in response to TGF-β, or by recruitment of bone marrow-derived mesenchymal stem cells. The latter migrate in response to multiple signalling molecules and may differentiate to many different stromal cell types (Quante et al., 2011). Cancer stromal cells can be broadly divided into three classes; Angiogenic vascular cells (AVCs), infiltrating immune cells (IICs) and cancer-associated fibroblastic cells (CAFs) (Hanahan and Coussens, 2012). The relative importance of each of these stromal cell types depends on tumour type and organ. A reactive microenvironment supports tumour growth and invasion through various mechanisms including upregulation of growth factors (e.g. VEGF) and extracellular proteases (e.g. MMPs) in stromal cells and endothelial cell leading to extensive neoangiogenesis and facilitation of cancer cell invasion and metastasis (Sounni et al., 2003).

It is well recognised that stromal cell types that are normally associated with responses to injury, such as fibroblasts, have a prominent role in the initiation,

progression and eventual spread of tumours. However, there is also substantial evidence that a specific subset of fibroblasts, called cancer-associated fibroblasts (CAFs) or cancer associated myofibroblasts (CAMs), are common to all stages of cancer (Kalluri and Zeisberg, 2006).

1.12.1 *Cancer associated myofibroblasts (CAMs)*

Myofibroblasts are activated fibroblasts that acquire contractile elements. They are large spindle-shaped cells and are probably a sub-set of CAFs. Myofibroblasts tend to assemble around and encircle carcinoma cells forming a desmoplastic reactive stroma which correlates with poor prognosis (Liu et al., 2012). A number of mechanisms have been implicated in the conversion of fibroblasts to CAMs including changes in miRNAs (Mitra et al., 2012), activation by TGFβ, and DNA methylation (Jiang et al., 2008). Bone marrow derived cells (BMDCs) may also be a source of CAMs including in patients with OAC (Hutchinson et al., 2011). The activated phenotype of CAMs can be defined by expression of α-smooth muscle actin (α-SMA) and fibroblast activation protein-α (FAP), both of which are considered to be specific biomarkers (Orimo and Weinberg, 2007). Other markers that have been reported include vimentin, desmin, platelet derived growth factor receptor-α and β (PDGFR α and β), and fibroblast specific protein-1 (FSP-1) (Strutz et al., 1995, Togo et al., 2013). Cytokeratin, and CD31 are considered as negative markers (Xing et al., 2010). CAMs secrete growth factors and alter the ECM to create a tumour niche and enhance tumour cell migration and metastasis (Lin et al., 2016).

Recent studies have suggested that CAMs undergo specific genetic and epigenetic changes compared with normal tissue myofibroblasts (NTM), and adjacent tissue

myofibroblasts (ATM). These changes determine the functional properties of each subset; for example, CAMs in gastric cancer showed increased rates of migration and proliferation compared with ATMs or NTMs (Holmberg et al., 2012). Moreover, CAMs may have a neuroendocrine-like phenotype characterized by Ca^{2+}-dependent regulated secretion that is lost with cancer progression (Balabanova et al., 2014). Moreover, conditioned medium from CAMs strongly enhances cancer cell proliferation, migration and invasion compared with ATMs, and NTMs (Kumar et al., 2016) (Kumar et al., 2014) (De Wever et al., 2014). Interestingly, Wang et al have demonstrated that there are different miRNA profiles in normal and gastric cancer myofibroblasts, which may promote an aggressive phenotype in gastric cancer cells that is of significance in Wnt signalling (Wang et al., 2016)

In the case of oesophageal cancer (Okawa et al., 2007), CAMs provide an environment for carcinogenesis, proliferation, angiogenesis and invasion via a number of secreted factors (Zhang et al., 2009, Rahman et al., 2010, Grugan et al., 2010) (Hayden et al., 2012) (Underwood et al., 2015b). Verbeek et al reported that CAM-derived Toll-like receptor-4 (TLR-4) (an innate immune system activator) was involved in the progression from BO to OAC (Verbeek et al., 2014). Additionally, Underwood et al found that CAMs were able to accelerate OAC growth in mice: the tumour volume in mice injected with OAC cells plus CAMs reached $500mm^3$ earlier than OAC cells alone (Underwood et al., 2015a). In terms of OAC cell invasiveness, Underwood et al demonstrated that two OAC cell lines FLO-1 and OE33 showed a more than a two-fold higher invasion in transwell invasion assays when exposed in conditioned medium from CAMs. This supported their belief that OAC invasiveness was fueled by periostin secreted by CAMs.

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1.12.2 *Epithelial-mesenchymal transition (EMT)*

The concept of EMT has gained considerable attention in the cancer research community not least because it accounts for the altered phenotype of epithelial cells in the metastasis of solid tumours. Basically, the term defines a process in which cells lose epithelial and gain a mesenchymal phenotype; this is accompanied by a loss of cell-cell cohesiveness, leading to enhanced migratory capacity.

Up- or down-regulation of multiple genes (Hur et al., 2013) as well as protein expression play a central role in EMT, and their possible correlation with tumor invasion and metastatic spread (Polyak and Weinberg, 2009) (Figure 1.3). Also, there is also great interest in EMT as a pharmaceutical target. In the 1990s, growing evidence indicated that EMT was associated with cancer progression which correlated with EMT-related signal pathways (Thiery, 2002, Moustakas and Heldin, 2007). The molecular basis of EMT is thought to be under the control of many regulatory pathways, although TGF-β has a predominant role and acts to regulate expression of so-called EMT master genes via the Smad family transcription factors. Other downstream signalling pathways activated mediating TGF-β signaling in EMT include Rho-like GTPases, PI3Kinase and MAPK (Lamouille et al., 2014). The hall mark of EMT is the cadherin switch (the balance change between members of cadherin family): E-cadherin is down-regulated and N-cadherin is up-regulated (Hazan et al., 2004). After loss of cohesiveness due to degradation of cell-cell junction complexes, mesenchymal-like tumour cells are able to invade through the basement membrane into underlying tissue by the secretion of lytic enzymes such as matrix metalloproteinases (Bourboulia and Stetler-Stevenson, 2010). TGF-β signaling by stromal myofibroblasts can induce secretion of hepatocyte growth factor (HGF) which promotes cancer cell proliferation and invasion (Lewis et al., 2004).

The clinical significance of EMT is clearest in its link to tumor cell invasion and metastasis. Thus EMT is required for epithelial tumour cell invasion into the vasculature as a prerequisite for metastatic seeding, together with invadopodia formation and increased MMP activity (Yoo et al., 2011, Karihtala et al., 2013). For example, mesenchymal-like tumour cells secrete MMPs such as MMP-1, MMP-7, and MMP-9 that enable them to invade the basement membrane and ECM (Ridley, 2011, Bourboulia and Stetler-Stevenson, 2010).

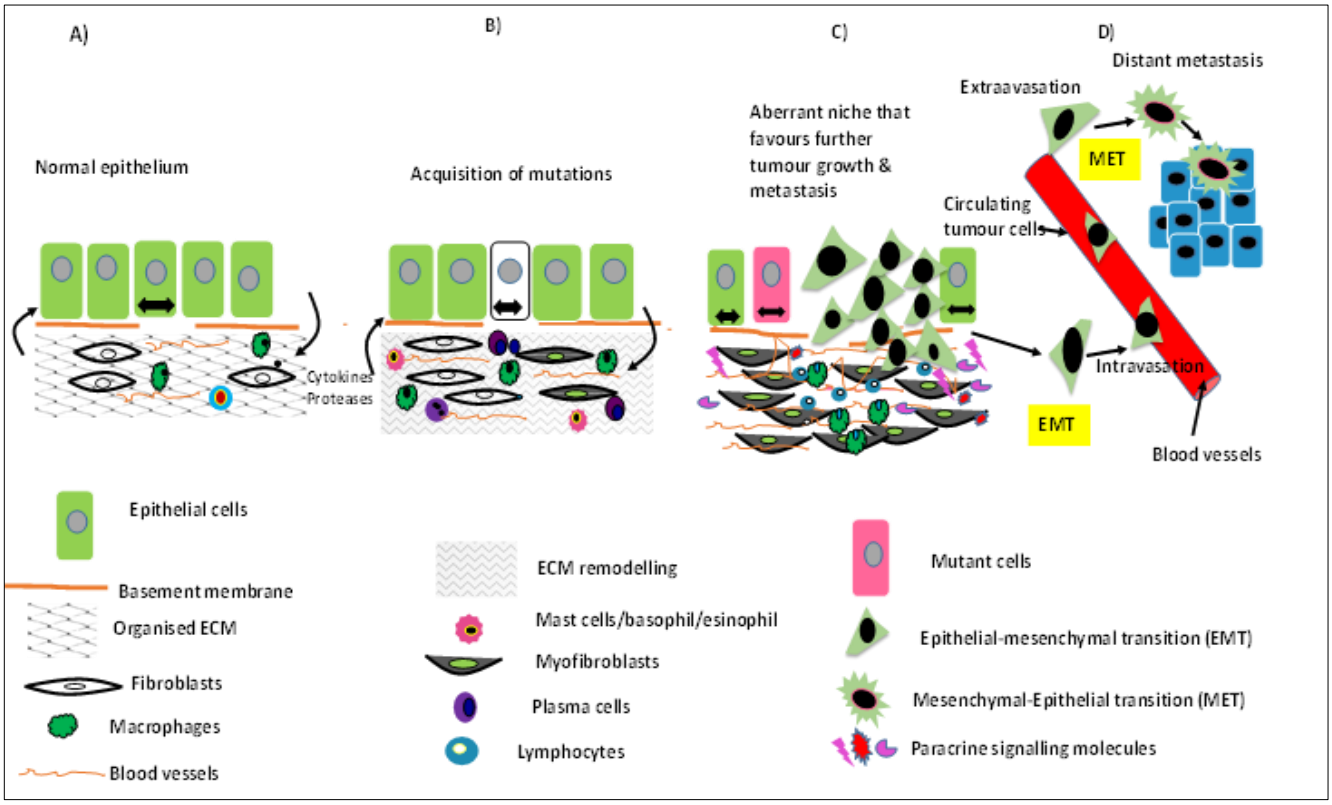

Figure 1.3: Cancer progression. Schematic representation of changes in epithelial cell organization and cellular microenvironments during tumorigenesis. A) Normal epithelial cells and ECM organization. B) In chronic inflammation, active fibroblasts, chronic inflammatory cells recruitment (these cells comprise mononuclear phagocyte system), with the increase of cytokines, MMP, and chemokines, and epithelial-stroma; cells signalling. C) Cancer initiation, increase the number of myofibroblasts (CAM), increased proliferation decreased apoptosis, and epithelial mesenchymal transition (EMT), and mesenchymal – epithelial transition. D) Cancer cells migration through blood, and lymphatic vessels and invade distant sites (Metastasis).

1.13 Matrix metalloproteinases (MMPs)

MMPs (matrixins) are a family of highly conserved zinc-binding endopeptidases. The first member of the family was discovered by Gross and Lapiere in 1962 and named collagenase (Gross and Lapiere, 1962). Since then extensive research has led to the discovery a many structurally related proteinases - 23 in human and 24 in mice (Page-McCaw et al., 2007b). Recent clinical data indicates a relationship between MMPs and disease that is not simple although high expression is often connected with poor prognosis (Egeblad and Werb, 2002). The proteolytic activity of MMPs can be inhibited by tissue inhibitors of metalloproteinases (TIMPs). MMPs are either secreted or exist as transmembrane pro-enzymes that require activation to exert their proteolytic activity; their active site contains a zinc ion and requires a second metal cofactor such as calcium, and enzyme activity is optimal in the physiological pH range (Nagase and Woessner, 1999). All cell types express distinct patterns of MMPs. While the substrate specificity of different MMPs varies. MMPs are now known to accomplish a wide range of distinct functions in addition to they are all involved in ECM turnover in many physiological processes with implications for the control of processes such as cell migration (Chen and Parks, 2009), immunity (Khokha et al., 2013) and angiogenesis (Pepper, 2001), bone development, and uterine and mammary involution. MMPs are expressed at basal levels in the normal tissue. When tissue remodeling is required as in wound healing, MMPs can be rapidly expressed and activated. Also MMPs expression is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell–cell and cell–matrix interaction (Nagase et al., 2006).

The MMPs are generally classified according to their substrate specificity. There are four sub-classes: the collagenases (MMP-1, -8 and -13), the gelatinases (MMP-2

and -9), the stromelysins (MMP-3, -10 and -11) and a heterogeneous group containing matrilysin (MMP-7), metallo-elastase (MMP-12), enamel metalloproteinase or enamelysin (MMP-20), endometase (MMP-26) and epilysin (MMP-28) (Klein and Bischoff, 2011). Nevertheless, with the discovery of new MMP family members, it has become attractive to categorize MMPs according to their domain structure. Thus, MMPs can be divided into four sub-classes with respect to the following domains: the minimal domain which is comprised of pre- and pro-sequences connected to the catalytic domain by a hinge region, a haemopexin like domain (absent in MMP-7), a fibronectin domain and, in some cases, a trans-membrane domain (Figure 1.4). The pre-domain directs the protein to ER for secretion and is rapidly removed during and just after translation. The pro domain contains a highly conserved sequence of eight amino acids PRCGVPDV that includes a cysteine residue those complexes with a zinc molecule, thereby ensuring that the N-terminal region is folded around the latent enzyme; the pro-domain therefore maintains the protein in an inactive state and must be removed by cleavage in order to activate the enzyme. Following cleavage, which generally occurs after secretion, a conformational change dissociates the pro-domain cysteine from the zinc atom at the catalytic site and replaces it with water (cysteine switch) - this step results in MMP activation (Van Wart and Birkedal-Hansen, 1990). Proteolytic removal of the pro-domain is mediated by other endopeptidases including other MMPs and furin (a paired basic amino acid cleaving enzyme) (Pei and Weiss, 1995).

The catalytic domain contains the conserved sequence HEXGHXXGXXHS which constitutes the active site; two calcium ions are present in the catalytic domain – these are not required for enzymatic activity but may be essential in inhibitor binding

(Yuan et al., 1994). MMP-7 (Matrilysin) and MMP-26 (matrilysin-2) are composed of the three minimal domains. In other MMPs the haemopexin terminal domain has been implicated in determining the distinct substrate specificities of the MMPs.

Membrane-type matrix metalloproteinases (MT-MMPs) contain a transmembrane domain near their carboxyl termini which localizes the enzyme to the plasma membrane e.g. MT1-, MT2-, MT3- and MT5-MMP; some MT-MMPs e.g. MT4- and MT6-MMP are localized at the cell surface through glycosylphosphatidylinositol anchor (Zucker et al., 2003). The 3D structures of different domains of a number of MMPs have been identified, even so it is still considered necessary to increase the number of available full-length structure for MMPs in order to better understand the substrate-specificity of the MMPs and therefore to improve inhibition strategies (Visse and Nagase, 2003).

The function of MMPs includes cleavage and rearrangement of ECM, as well as cleavage of the precursor forms of other MMPs, proteinase inhibitors e.g. α-1-antitrypsin and urokinase-type plasminogen activator (uPA) (Chambers and Matrisian, 1997); MMPs are also involved in activation of growth factors such as tumour necrosis factor alpha (TNF-α), and growth factor receptors e.g. interleukin-6 (Gearing et al., 1994). Recently, MMPs have been found to act in a non-proteolytic manner via the haemopexin domain (Kessenbrock et al., 2010). Substrate availability and accessibility determine the degree to which MMP activity is used (Page-McCaw et al., 2007a).

The ADAMs family (A disintegrin and metalloproteinase) differ from MMPs in that they are anchored to the cell membrane. Moreover, ADAMs function as transmembrane proteases or shedases (Bonnans et al., 2014a).

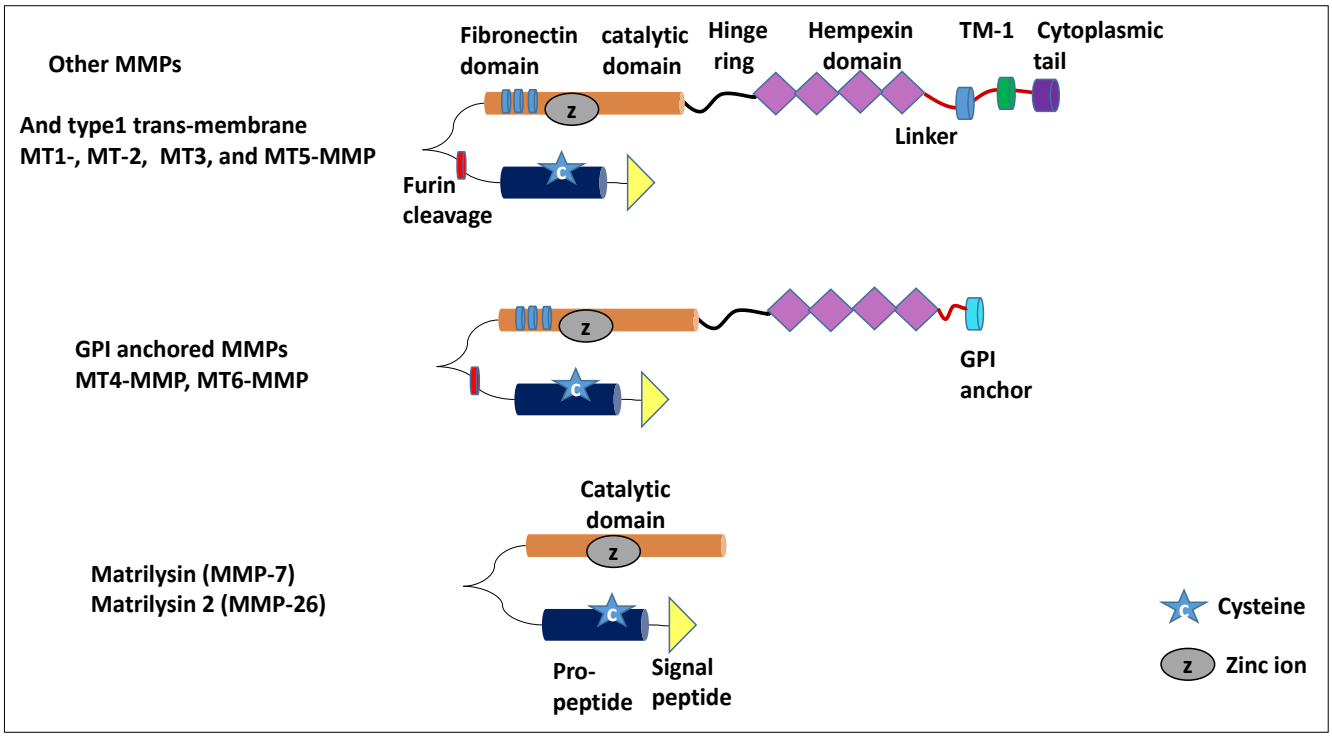

Figure 1.4 Domain Structure of MMPs: As shown in the figure the typical MMP structure composed of distinct domains; predomain, propeptide, catalytic domain, and hemopexin domain. MMP-7 (matrilysin-1), and MMP-26 (matrilysin-2), both have no hinge region and hemopexin domain.

1.14 Regulation of MMP gene expression

MMPs are frequently either co-expressed or co-repressed in response to stimuli such as inflammatory cytokines, growth factors, glucocorticoids or retinoids, (Yan and Boyd, 2007, Westermarck and Kahari, 1999, Guo et al., 1997).

1.14.1 *Transcriptional regulation*

MMP promoters harbour several *cis*-elements or key transcription binding sites, e.g. a TATA box at approximately 30 base pairs (bp) relative to the transcription start site and activator proteins (AP) -1 and -2 sites in the proximal promoter at approximately 70 bp in most MMP genes (Pendas et al., 1997). The proteins encoded by the Fos and Jun family of oncogenes form heterodimeric complexes that bind AP-1 sites (Abate et al., 1991), thereby providing an interesting connection between transcription factors related to malignant transformation and transcriptional activation of MMP genes (Folgueras et al., 2004). However, some Fos and Jun family members may act as transcriptional repressors, for example Junβ inhibit Jun-induced MMP-1 gene expression and is associated with less aggressive and more differentiated tumours (Curran and Murray, 2000). Most of the promotor inducible genes contain a polyoma enhancing activator-3 (PEA3) site which binds the Ets family of oncoproteins. In many MMPs the PEA3 site is located adjacent to the AP-1 site, and together they are recognised as an oncogene responsive unit that may act cooperatively to promote MMP production by cancer cells (Yan et al., 2008). Modulation of these two sites is therefore a potential therapeutic approach to down-regulating expression of MMPs in cancer.

In addition to AP-1- and PEA3-binding sites, a Tcf-4 site (for β-catenin/LEF binding) occurs in the MMP-7, -14, -12, and -26 promoters. The Tcf-4 site mediates MMP expression via Wnt signalling. For example, MMP-7 expression increases in response to Wnt-2 expression (Le Floch et al., 2005, Gustavson et al., 2004).

1.14.2 *Post-transcriptional regulation of MMPs*

A series of post-transcriptional events has also been shown to regulate MMP expression. MMP transcripts harbour specific sequences in their 5′- or 3′-untranslated regions (UTRs) which are potential targets for different UTR-binding proteins with the capability to stabilize or destabilize these mRNAs (Fanjul-Fernández et al., 2010). Other mechanism may also control MMP expression such as mRNA stability, and regulation of translational efficiency. For example, in murine prostate carcinoma cells an increase in the binding of mRNA to polysomes results in significantly increased MMP-9 expression (Jiang and Muschel, 2002). Also microRNAs (miRNAs) have been reported to participate in MMP regulation either as direct targets (Xia et al., 2009) or indirectly through miRNAs involved in various signalling pathways responsible for MMP activation. For instance, in myocardial infraction miR-21 in response to the high phospho-Akt level caused by the knockdown of PTEN mRNA lead to upregulation of MMP2 expression (Roy et al., 2009).

1.14.3 *Epigenetic regulation of MMPs*

Epigenetic mechanisms, such as DNA methylation (Chicoine et al., 2002) or histone acetylation (Kouzarides, 2007), may also influence expression of MMP genes. There is an inverse correlation between promoter methylation of MMP-9 and MMP-2 expression levels, adding evidence that methylation of the promoter region is functionally important for MMPs gene expression (Chicoine et al., 2002, Shukeir et

al., 2006). An additional level of epigenetic control of MMPs gene expression derives from post-translational modifications of histones through acetylation processes. Histone acetyl transferases (HAT) are the enzymes responsible for producing chromatin in a relaxed state that allows the entry of transcription factors and other transcriptional machinery to promoter regions (Kouzarides, 2007). Thus, the induction of MMP-1 and MMP-13 by IL-1α and oncostatin M is almost completely abolished by two independent histone deacetylase (HDAC) inhibitors (Young et al., 2005).

1.14.4 Proenzyme activation

All MMPs either secreted or attached to the membrane are produced as inactive zymogens. Activation of pro-MMPs therefore represents a further step in regulating MMP activity. Agents that activate MMPs in vitro include reactive oxygen radicals, low pH and high temperature (Nagase, 1997). In vivo, it has been suggested that plasmin is an endogenous activator for MMPs (He et al., 1989). Activation of MMPs acts in cascades for example MMP-7, MMP-3, MMP-10 and the MT1-MMP all activate other MMPs by proteolytic cleavage. The activity of MMPs may be also controlled by a series of endogenous inhibitors; for example in plasma and tissue fluids, α2-macroglobulin blocks MMP activity, whereas other inhibitors such as TIMPs (tissue inhibitors of metalloproteinases) are more specific. In order to achieve regulated functions representing MMPs, a balance has to be preserved between MMPs/TIMPs, therefore, any interruption in this balance can finally lead to a variety of abnormal conditions (Visse and Nagase, 2003, Kessenbrock et al., 2010, Hua et al., 2011).

1.15 MMP roles in cancer

MMPs are considered as to be critical in four hallmarks of cancer (migration, angiogenesis , invasion and metastasis) because they degrade various cell adhesion molecules, thereby modulating cell–cell and cell–ECM interactions (Gialeli et al., 2011) (Hotary et al., 2003). MMPs may exert either apoptotic or anti-apoptotic activities in cancer cells. Thus by activating indirectly the serine／threonine kinase Akt／protein kinase B through the signaling cascades downstream of EGFR MMPs may contribute to the anti-apoptotic effect (Ch et al., 2009). Conversely, MMPs may initiate apoptosis in malignant cells via the cleavage of ligands or receptors that transduce proapoptotic signals e.g. MMP-7 in doxorubicin-treated cancer cells surface cleaves Fas ligand (Mitsiades et al., 2001). Moreover, MMP activities are linked to a diversity of escape mechanisms by which cancer cells bypass host immune response (Coussens and Werb, 2002) (Kataoka et al., 1999). During carcinogenesis MMPs promote vascular alteration and neovascularization at sites of tumour metastasis (Folgueras et al., 2004). Nevertheless, and antagonistic to these proangiogenic roles of MMPs, the recent explanation of mechanisms by which these enzymes negatively regulate angiogenesis has provided to increase the functional complexity of this proteolytic system in cancer. Some of MMPs are able cleave the precursors of angiostatin and endostatin (endogenous inhibitors of angiogenesis) and generate the active forms (Ferreras et al., 2000). Taken together, these discoveries represent the diversity of MMPs capacities related with cancer and highlight the significance of MMPs protective activities in tumour progression, an angle that had been to a great extent disregarded in this field. Hence, it is basic to recognize the physiological role of every individual MMP and its particular support in

the numerous stages of tumour development to better create powerful therapeutic interventions.

1.15.1 *Matrilysin (MMP-7)*

MMP-7 was initially described as PUMP-1 (putative uterine metalloprotease-1) in 1988 (Muller et al., 1988). MMP-7 is the smallest member of the MMP family; its inactive form is just 28 kDa and the active form is 18kDa. MMP-7 plays a crucial role in the degradation of ECM proteins such as collagens I, III, IV, V, glycoproteins, laminin, vitronectin, aggrecan and entactin, and proteoglycans (Overall, 2002). It catalyzes the ectodomain shedding of various cell surface molecules such as Fas ligand (Wang et al., 2006), syndecan-1 (Li et al., 2002), E-cadherin (McGuire et al., 2003).

MMP-7 was initially characterized by Woessner et al. It digests components of the extracellular matrix, cleaves the α 2 (I) chain of gelatin, and digests the B chain of insulin at Ala-Leu, and Thyr-Leu (Woessner and Taplin, 1988). It was reported that MMP-7 was presented in some hepatocytes and endothelial cells in the 6th gestational week, but was restricted to hematopoietic cells after that (Quondamatteo et al., 1999). Active MMP-7 is recruited to the plasma membrane of epithelial cells inducing membrane-associated growth factor processing for epithelial repair and proliferation. In human endometrium, the expression of MMP-7 mRNA increases at menstruation and remains high during the proliferative phase; it promotes endometrium regeneration after menstrual breakdown (Gaide Chevronnay et al., 2012). Huang et al. reported that the proteolytic activity of MMP-7 plays a major role in tissue remodeling in biliary atresia-associated liver fibrosis (Huang et al., 2005). The evidence from mice, including knockouts, suggests that it also regulates the activity of defensins in the intestinal mucosa.

MMP-7 could have a prominent diagnostic and/or prognostic role in tumours and merits in-depth investigation (McGuire et al., 2003). MMP-7 has been implicated as a participant in the progression, invasion and migration of many tumour types such as gastric cancer (Wroblewski et al., 2003, McDonnell et al., 1991, Yamashita et al., 1998), oesophageal cancer (Salmela et al., 2001a, Ohashi et al., 2000, Yamashita et al., 2000), colorectal cancer (Maurel et al., 2007), bladder cancer (Szarvas et al., 2010), renal carcinoma (Li et al., 2016), prostate cancer (Grindel et al., 2014), pancreatic cancer (Yamamoto et al., 2001) and breast cancer (Bucan et al., 2012). An increase in MMP-7 mRNA level was noticed to correspond with increased colon cancer dedifferentiation and metastasis (Mori et al., 1995). Also, MMP-7 can regulate IGF bioavailability in the surrounding tumour stroma by acting on binding proteins for insulin-like growth factors (IGFBPs). Cleavage of the latter liberates IGF, so assisting cancer cell growth and survival (Hemers et al., 2005, McCaig et al., 2006).

1.15.2 Summary of previous work about MMP-7 and MMP-1 in in BO-OAC progression

Studies that have provided information on MMP-7 and MMP-1 expression in OAC between 1998 and 2015 were identified from electronic databases through screening the title and keywords. The following data were extracted from these studies and are summarized in tables 1.2 and 1.3: surname of first author, year of publication, sample type, antibody used, detection method for protein expression and main conclusion.

Study	Cancer	Sample	Method	Antibody	Conclusion	Year
Yamashita et.al	SCC/OAC	Oesophageal carcinoma surgical tissue specimens, SCC(n=48), OAC(n=2)	Northern Blot Hybridization Western Blot IHC	mouse monoclonal primary antibody	MMP-7 might be a novel prognostic marker for patients with oesophageal carcinoma	2000
Salmela et .al	OAC	Formalin-fixed, paraffin-embedded specimens Of (OAC n=16, and IM n=5)	In sit hybridizations IHC	S labelled RNA probe	MMP-7 is expressed by malignant cells in Barrett's carcinoma and is up-regulated early in oncogenesis	2001
Tanioka et al	SCC/OAC	Oesophageal carcinoma surgical tissue specimens, SCC(n=44), OAC(n=1)	IHC	Primary mouse monoclonal anti-human MMP-7/ anti-human MMP-9	Combined MMP-7 and MMP-9 expression may be a good marker for the malignancy level of oesophageal cancer	2003
CHEN Yan et. al	OEC	OEC (n= 45)	Reverse transcription-PCR(RT-PCR)		The expressions of MMP-1 and MMP-7 play an important role in carcinogenesis of oesophageal cancer	2012
Shuai Miao et. al	MMP-7 expression in OEC	Databases	a meta-analysis	-	Overexpression of MMP-7 may be a suitable diagnostic biomarker for variation in EC clinicopathological features	2015
Agnieszka Juchniewicz et. al	MMP-10, MMP-7, TIMP-1 and TIMP-2 mRNA expression in esophageal cancer	Oesophageal carcinoma surgical tissue specimens, SCC(n=29) OAC(n=32)	Reverse transcription-PCR(RT-PCR) and correlated the results with the patient clinicopathologic features		Increased mRNA expression of MMP-7, MMP-10 and TIMP-1 correlated with clinicopathologic features. We suggest that these genes may play a role during progression of the disease	2017

Table 1.2 Summary of previous work on MMP-7 in BO-OAC progression. The studies listed were published between 2000 and 2017. Papers were chosen from electronic databases through screening the title and keywords. The following related data were extracted from elective studies: surname of first author, year of publication, sample type, antibody used, detection method for protein expression, and conclusion.

Study	Cancer	Sample	Method	Antibody	Conclusion	Year
Murray et al	OEC	formalin-fixed, wax-embedded sections of oesophageal cancers	IHC		MMP-1 is associated with poor prognosis in oesophageal cancer	1998b
Yamashita et.al	SCC/OAC	SCC n=49 OAC n=1	RT-PCR Northern blot hybridization IHC Western blot	mouse monoclonal primary antibody to MMP-1	MMP-1 could be a novel prognostic factor independent in oesophageal carcinoma	2001
Grimm et. al 2010	BO, OAC	OAC with BO n = 41, OAC without n = 19, SCC n = 10, IM n =18, and the cell line OE-33.	IHC		MMP-1 plays a role as preinvasive factor OAC with BO	2010
Keld et. al	OAC	OE33 cells OAC with BO n= 28 healthy controls n=55	-	-	ERK-PEA3-MMP-1 axis is upregulated in oesophageal adenocarcinoma cells and is a potentially important driver of the metastatic progression of oesophageal adenocarcinomas	2010

Table 1.3 Summary of previous work on MMP-1 in BO-OAC progression. The studies listed were published between 1998 and 2010. Papers were chosen from electronic databases through screening the title and keywords. The following related data were extracted: surname of first author, year of publication, sample type, antibody used, detection method for protein expression, and conclusion

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1.16 Aims:

This thesis was based on the general hypothesis that expression of MMP-7 is a marker of, and participant in, the progression to oesophageal adenocarcinoma. The specific aims were:

1. To evaluate by immunohistochemistry the expression of MMP-7 in OAC and BO, and to determine the relationship with different stages of progression.
2. To develop a novel ELISA for detection of MMP-7 release from cell lines.
3. To establish MMP-7 expression, mechanisms of secretion and metabolism in an OAC cell line.
4. To define the role of downstream signalling pathways in MMP-7 regulation in OE33 cells.

CHAPTER-2

MATERIALS AND METHODS

2.1 Materials

Oesophageal cancer cell lines OE21 cells also known as JROECL21 were established in 1993 from a squamous carcinoma with moderate differentiation of the mid oesophagus of 74 year- old male patient. OE33 cells also known as JROECL33 were established from the adenocarcinoma with poor differentiation of the lower oesophagus (Barrett's metaplasia) of a 73 year old female patient.OE33-GR derived from the OE33 cell line stably transfected with wild type CCK2 receptor (Haigh et al, Gastroenterology). OE19 cells also known as JROECL19 was established in 1993 from an adenocarcinoma with moderate differentiation of gastric cardia/oesophageal gastric junction of a 72 year old male patient. OE cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC), via the Culture Collection of Public Health England. AGS cell line derived from an adenocarcinoma of the stomach of a 54 year-old Caucasian female with no prior anti-cancer treatment was obtained from American Type Culture Collection (ATCC Manassas, VA, USA). AGS-GR cells stably transfected with full length cDNA encoding the human gastrin-CCKB receptor was described previously (Varro et al., 2007c).

Cell culture materials, namely Nunc flask T75, was obtained from Fisher Scientific (Loughborough, UK), Dulbecco's Eagle Modified medium (DMEM), Roswell Park Memorial Institute medium (RPMI)-1640, penicillin and streptomycin, antibiotics-antimycotic, non-essential amino acids, and trypsin-EDTA Solution (0.25%) were obtained from Sigma (Poole, UK). Recovery cell freezing medium came from Invitrogen (Paisley, UK), foetal bovine serum (FBS), and Ham's F-12 medium was purchased from Gibson-Life technologies (Paisley Glasgow, UK). Phosphate buffered saline (PBS) was from Invitrogen. Protease Inhibitor cocktail set III (EDTA-free), phosphatase Inhibitor cocktail set III (EDTA free) were obtained from

Calbiochem (Darmstadt, Germany) and RIPA buffer was purchased from Cell Signalling (Hitchin, UK). Human recombinant pro-MMP-7 and active recombinant MMP-7 antibodies were purchased from Millipore (Billerica, MA, USA). G17-gly, G17-CFP from obtained from University of Liverpool, progastrin was a gift from Tim Wang (New York, Columbia), G17 and G34 were obtained from Sigma.

Western blot reagents, namely Immun-Star™ WesternC™ Chemiluminescence Kit, Lowry DC protein assay, and ChemiDoc XRS system were obtained from Bio-Rad Laboratories (Hemel Hempstead, UK). NuPAGE Novex Bis-Tris Mini protein Gels, Gel Novex Mini Cell, NuPAGE MOPS SDS Running Buffer (20x), NuPAGE LDS Sample buffer (4X), NuPAGE Reducing agent (10x), NUPAGE antioxidant, NuPAGE Transfer buffer (20x), and Magic Mark™ XP Western Protein Standard were obtained from Invitrogen. Amersham Protran Premium 0.45 nitrocellulose membrane came from Fisher Scientific. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was obtained from Bio design (Saco, Maine, USA). Primary and secondary antibodies and their dilutions used for Western blot analysis are shown in table 2.1.

For immunocytochemistry Vectashield with DAPI was obtained from Vector laboratories (Peterborough, UK). 4-(2-hydroxyethyl)-1-piperazineethanesuulfonic acid (HEPES), Triton-X-100 and NaCl were obtained from Sigma. Bovine serum albumin (BSA) was purchased from Jackson Immunoresearch (Suffolk, UK). Paraformaldehyde (PFA) was from Agar scientific (Stansted, UK).

For migration assays, Boyden chamber control cell culture inserts were purchased from SLS (Nottingham, UK), and the migration cell fixing and staining kit (DiffQuick) was from Dade Behring Inc (Glasgow, DE, USA). Thermo scientific microscope slides were from Fisher scientific.

Drugs, including PKC inhibitor Ro32043, and U0126, were purchased from Calbiochem. PI3Kinase inhibitors, such as TG100713, rapamycin, and MK-2206 were obtained from Selleckchem (Newmarket, UK); LY294002 was obtained from Cell Signalling and wortmannin, and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem. Brefeldin A (BFA) was obtained from Cambia (Dry Drayton, UK).

In the indirect ELISA we used the following materials; the coating buffer plasma sample diluent (PSD), OBT1998G, from Bio-Rad, 96 well MaxiSorp (Nunc) plate from Thermo-scientific, the washing buffer sodium barbitone (veronal) with Tween-20 from Sigma. The rabbit polyclonal antibody (Ab), L522 (G), specific to pp95 (YSLFPNSPKWTSKVVC) of MMP-7 was generated by Cambridge Research Biochemicals (CRB, Billingham, Cleveland, UK), the secondary antibody, biotin conjugated anti-rabbit goat IgG, was purchased from Sigma. High sensitivity Streptavidin-HRP was obtained from Thermo-Fisher scientific, and the substrate solution, 3, 3´, 5, 5´-tetramethylbenidine (TMB) was purchased from Sigma.

2.2 Human primary myofibroblasts

Human primary myofibroblasts were derived from resected oesophageal cancer (CAM) and adjacent macroscopically normal tissues (ATM) as described previously (Kumar et al., 2014). Normal myofibroblasts (NTM) were generated from deceased transplant donors with normal oesophageal morphology.

2.3 Cell culture

2.3.1 *Human oesophageal cancer cell lines*

OE21, OE33, OE33-GR and OE19 cells were cultured in DMEM supplemented with 10% v/v FBS, 1% v/v penicillin-streptomycin, 2% v/v antibiotic/antimycotic. This is referred to as "full medium" (FM) unless otherwise stated. Cells were routinely checked for viability and media were changed every 48 hours. Cells were passaged at 1:2, 1:4 ration at 80-100% confluency with 0.25 % w/v trypsin-EDTA.

2.3.2 *Human myofibroblasts*

Myofibroblasts were maintained in T-75 flasks in DMEM supplemented with 10% v/v FBS, 1% v/v penicillin-streptomycin, 2% v/v antibiotic-antimycotic and 1% v/v non-essential amino acids. Cells were grown at 37°C in a 5% v/v CO2 atmosphere and the medium replaced routinely every 48 hours and cells were passaged at 1:2, 1:3 at 100% confluency, with 0.25% w/v trypsin -EDTA.

2.3.3 *Gastric adenocarcinoma cell lines*

AGS and AGS-GR cells were cultured in HAMS-F12 medium supplemented with 10% foetal bovine serum (FBS), and 1% w/v penicillin/ streptomycin.

2.4 Preparation of conditioned media

Cells (3 million) were plated and allowed to attach overnight. The next day serum free (SF) medium (8ml) was added to the cells with or without the appropriate treatments up to 24 hours. After the incubation period, media was centrifuged at 800g 4°C for 7 minutes to remove the cell debris and an aliquot was kept for ELISA at -80°C. The rest of the medium was concentrated using Strata Clean TM Resin (Agilent technologies). Briefly, 10µl of resin was added per 1ml of media. Sample

with resin was vortexed for 1 minute and centrifuged at 2000rpm for 1 minute at 4°C followed by two washes with 500 µl ammonium bicarbonate. Pellet was resuspended with 100µl ammonium bicarbonate, aliquoted, and stored at -80°C until use. Media processed with Strata Clean TM Resin was used for Western blots.

2.5 Cell extraction

Cells (3 million) were cultured in in T-75 flasks, and incubated for 24h in 20ml FM. Cells were then washed three times with sterile 1XPBS and fresh 8ml serum free media (SF) containing stimuli or inhibitors was added and incubated overnight (16hr). On the next day, media was removed and 10ml non-sterile 1X PBS added to the cells which were placed on ice. Cells were then washed twice with 10ml ice-cold 1XPBS, scraped and lysed with 100µl ice cold RIPA buffer containing 1% v/v Protease Inhibitor cocktail set III and 1% v/v Phosphatase Inhibitor cocktail set III. Cell suspensions were collected, sonicated for 5 min and incubated on ice for 30 min. The extracts were then centrifuged at 12000 x g at 4°C for 3 min and supernatants were aliquoted and kept at -80°C until further use.

Protein concentrations in cell extracts were quantified using modified Lowry DC protein assays. Bovine serum albumin standard (10mg/ml BSA) was serially diluted in RIPA lysis buffer, and 5µl was added in triplicate (in 96 well plates). Similarly, triplicates of 5µl of sample diluted in RIPA lysis buffer (1:2.5 and 1:5) were added followed by the addition of 25µl reagent A/S and 200 µl of reagent B to each well and incubated for 30 min at room temperature. The plate was read in Genios Plus microplate reader (Tecan, Reading, UK) at 750 nm.

2.6 Western blots

2.6.1 *Electrophoresis*

NuPAGE Novex Bis-Tris Gels (12% or 4-12%) were used into Novex Mini Cell Gel Electrophoresis System (Invitrogen). MOPS buffer at 1X final concentration was used as running buffer. Samples (20-60µg) were denatured by pre-heating for 4 min at 100°C with 4x loading buffer. NuPAGE Reducing Agent (10X) and NuPAGE antioxidant (500µl) were added to the running buffer of the upper buffer chamber to prevent sample reoxidation and maintain the proteins in a reduced state. Each gel was loaded with 5µl of PageRulerTM plus prestained protein ladder (Magic Marker) to provide molecular weight standards. Gels were run at a constant current of 200 voltage for 50 minutes.

2.6.2 *Electrophoretic transfer*

After electrophoresis, proteins were electro transferred (wet transfer) onto nitrocellulose membranes at constant voltage of 30V for 1 hr at room temperature (RT). The transfer contained NuPAGE transfer buffer (50ml), 20% methanol (200ml), NUPAGE antioxidant (1ml) and 749ml deionized water. After transfer step was finished, nitrocellulose membranes were rinsed in 1XTris-buffered saline (TBS) containing 0.1% Tween-20 (TBST washing buffer) three times for 10 min each. Membranes were blocked for 1hr at RT with blocking buffer (TBST containing 5% W/V Marvel milk powder).

2.6.3 *Incubation with primary, and secondary antibodies*

Membranes were incubated with primary antibodies (Ab) diluted in blocking buffer overnight at 4°C (cold room, shaker). On the next day, membranes were washed three times in TBST and incubated with secondary antibodies for a 1 hr on a shaker at room temperature.

2.6.4 *Development of blot*

For development of blots, membranes were exposed to Immun-Star™ WesternC™ Chemiluminescence Solution, and developed with a ChemiDoc XRS system.

2.6.5 *Densitometry evaluation of band intensity*

Protein bands were quantified from captured blot images using the Bio Rad software version 2.3.1. Blots were normalised to GAPDH unless otherwise stated.

Antibody	Species	Dilution	Source
MMP-7(L522, L523and L524)	Rabbit	1:1000	Cambridge Research Biochemicals
Human MMP-7 Antibody	Mouse	1:200	R&D System
MMP-1(BAF 901)	Goat	1:500	R&D System
MMP-3 (BAF 513)	Goat	1:250	R&D System
Phospho- Akt	Rabbit	1:1000	Cell Signalling
total-Akt	Rabbit	1:1000	Cell Signalling
GAPDH	Mouse	1:1000	Bio-Design
Anti-Mouse	Goat	1:10000	Sigma
Anti-Rabbit	Goat	1:10000	Sigma
Anti- Rabbit	Goat	1:20000	Cell Signalling

Table 2.1 Primary and secondary antibodies used in Western blotting

2.7 Indirect ELISA

2.7.1 *Antigen coating*

We used indirect ELISA to detect MMP-7 and its degraded products in cell media following a series of optimisation experiments. Media samples were collected as described in section 2.4, and were stored at -80°C until use. Briefly, media were serially diluted in different concentration of plasma sample diluent (PSD) coated on the 96 well MaxiSorp (Nunc) microtitre plate in duplicates for 2hr on a shaker (500+/-50rpm). After removal of the coating solution plates were washed 4 times with 400µl 0.02M sodium barbitone (Veronal) buffer pH 8.4 containing 0.05% Tween-20 (Veronal-T).

2.7.2 *Blocking*

The remaining protein-binding sites in the coated wells were blocked by adding 300 µl blocking buffer (1% BSA in Veronal buffer) per well and incubated for 2 hours at room temperature. Plates were washed 4 times with Veronal-T prior of addition of primary antibody.

2.7.3 *Incubation with primary and secondary antibody*

100µl of primary polyclonal rabbit L522 (G) (1:5000) antibody were added to each well (diluted in 0.1% BSA in Veronal-T) and incubated on shaker overnight at 4°C (500 ± 50 rpm). Plates were then washed 4 times with Veronal-T; 100µl of conjugated secondary, goat anti-rabbit IgG, Biotin conjugated (1:20,000) antibody, diluted in 0.1%BSA in Veronal-T was added to the wells and incubated for 2 hours at room temperature on a shaker (500 ± 50 rpm). After incubation plates were washed 4 times with Veronal-T prior to the detection step.

2.7.4 *Detection*

Detection was performed using high sensitivity streptavidin-HRP in 1% BSA in Veronal (1:20,000), by adding 100 µl of diluted High sensitivity Streptavidin-HRP to each well, incubated for 30 minutes at RT, followed by washing with Veronal-T and addition of 100 µl of TMB (3, 3', 5, 5'-tetramethyl benzidine) substrate solution per well. Wells were then incubated for 15-30min, to allow sufficient colour to develop. The TMB substrate reacts with immobilized horseradish peroxidase (HRP) conjugated secondary antibody to produce a blue solution. Stop Solution was used to terminate the enzyme substrate reaction, after attaining the desired intensity of colour by adding 100 µl of stopping solution (0.5M H_2SO_4) just before reading the plates at 450nm using the X fluro4 program, and Genios Plus microplate reader. The indirect ELISA protocol assay optimization and validation will be described in relevant result chapter.

2.8 Patients

Surgical specimens from 14 patients that had undergone surgical resection for oesophageal adenocarcinomas (OAC) with associated Barrett's oesophagus (BO) were used in this study. The specimens formed part of an archival collection for the period 2007‑2014 in the Department of Pathology at the University of Szeged, Hungary. In addition, endoscopic biopsies (n=17) from patients diagnosed with different type of metaplasia (intestinal, cardia, and fundic), and different degrees of dysplasia, low grade (n=17) and high-grade (n=12) intraepithelial neoplasia within the Barrett's mucosa were assessed. The study was approved by the Ethics Committee of the University of Szeged. Clinicopathological parameters were obtained from the patients' admission case sheets and pathology reports (mean age, 70±3 years; range, 49–83 years). Sections from all available tumour blocks of all

cases underwent intensive histopathological assessment, prior histopathology reports were issued.

2.9 Immunohistochemistry (IHC)

To localize MMP-7 in oesophageal adenocarcinoma (OAC) tissue specimens and biopsies, an immunohistochemical analysis was performed following the immunohistochemistry protocol published earlier (Kumar et al., 2016). In brief, sections of 5µm thickness were taken, deparaffinised in xylene, rehydrated in ethanol and then in water. Immunohistochemistry was performed using three steps indirect streptavidin methods using a monoclonal mouse anti‑human matrix metalloproteinase‑7 (MMP‑7) antibody (Millipore). Staining was reviewed by an expert gastrointestinal pathologist to ensure specificity of the staining. Adjacent sections were restained with MMP-1 antibody, and used as positive control.

2.9.1 *Quantification of Immunohistochemistry*

MMP-7 and MMP-1 were quantified in OAC, different types of metaplasia, low and high grade dysplasia. Scoring was done by two independent pathologists counting the percentage of stained cells (epithelial, and stromal cells) and staining intensity on a four point scale (0 - 3) negative (0), weak (1), moderate (2), and strong (3). The percentage of stained cells at each intensity was recorded. The results were expressed as percentages of total cells stained.

2.10 Immunocytochemistry

Briefly, cells were seeded at 15,000 cells per well on cover slips in 4 well chamber slide until they were 60% confluent. Cells were fixed in 4% (v/v) paraformaldehyde (PFA) for 30 min at room temperature (RT), then permeabilised with 0.2% Triton X100 for 30 minutes at RT. Cells were washed twice with PBS, blocked with 500µl of 5% w/v BSA for 30 min followed by blocking with 350ul of 10% donkey serum per well and incubated for 30min at RT. Cells were then washed with PBS twice prior of addition of 250ul MMP-7 antibody (1:200) per well and incubated overnight at 4°C in a humidified chamber. Next day, the cells were washed with 500µl 0.14M NaCl for 10 min followed by 0.5M NaCl for 10 min and then 0.14M NaCl for 10 min. Finally cells were incubated with FITC- conjugated donkey anti-mouse Ab (1:400) for 1 hr at RT in dark. Cover slips then were transferred to glass slides containing Vectashield with DAPI. Slides were viewed at 40 x magnification using a fluorescence Zeiss Axioplan-2 microscope and Axiovision software V 4.8.0 (Zeiss vision, Welwyn Garden city, UK). Images were captured using a JVC-3 charged coupled device camera with KS300 software. Emissions of fluorescence (FITC) were captured with the XF22 filter (excitation 493nm, emission 520nm).

2.11 Cell migration assays

Cells were obtained by trypsinzing 80% confluent cultures and resuspension in medium containing 0.1% FBS. 25,000 cells were added to 24 well plates in 500µl serum-free medium, on perforated polyethylene terephthalate (PET) insert with 8.0µm pores in Boyden chambers inserts. The inserts were placed in wells containing 750µl of SF media with or without the neutralising MMP-7 antibody. Cells migrating through the membrane after 16 hr at 37^{o}C in a 5 % v/v CO2 atmosphere

were detected on the lower surface. Briefly, media from each insert was removed and non-migrated cells from inner membrane surface of insert were removed using wet cotton buds. Migrated cells were then fixed and stained using DiffQuick. The membranes were then excised and mounted on a drop of immersion oil on a slide with a coverslip to avoid air bubbles. Cells in 5 fields per well were counted, under 10x magnifications using a Zeiss 25 Axiovert Microscope (Zeiss Vision), and the mean of 3 wells per experiment was taken.

2.12 Statistics

Results were expressed as mean ± standard error of the mean (SEM), unless otherwise stated. One way analysis of variance ANOVA (SysTest Software, Inc., Hounslow, UK.) were performed on the data as appropriate to determine statistical significance. Data were considered significant at $p < 0.05$. Graphs were produced using Sigma plot 13.0 software (Systat software UK Ltd, London, UK).

CHAPTER 3

MMP-7 AS A POTENTIAL IMMUNOHISTOCHEMICAL BIOMARKER OF THE PROGRESSION OF BARRETT'S OESOPHAGUS TO OESOPHAGEAL ADENOCARCINOMA

3.1 Introduction

None of the currently used clinical and endoscopic criteria have sufficient predictive power to identify progression of BO in a useful manner. There has, therefore, been considerable interest in the identification of molecular biomarkers for progression (Prasad et al., 2010). In this study it is suggested that up-regulation of MMP-7 expression could be an early event in the development of BO.

Multiple factors including alterations in MMPs seem to be associated with the creation and maintenance of a microenvironment that facilitates angiogenesis, and the growth of tumours at both primary and metastatic sites (Nelson et al., 2000, Auvinen et al., 2002). MMP-7 and -9 have been found to be increased in non-dysplastic BO, with even higher levels found in dysplastic Barrett's mucosa and OAC (Salmela et al., 2001b, Herszenyi et al., 2007a). Moreover, it has been reported that expression of MMP-7 plays an essential part in tumour invasion and may be strongly implicated in oesophageal tumourigenesis (Saeki et al., 2002, Tanioka et al., 2003).

Malignant progression within BO is regarded to follow a sequence of well-characterized histopathological changes from intestinal metaplasia (IM), thorough low-grade and high-grade intraepithelial dysplasia towards invasive OAC (Spechler, 2002). There is always the concern that IM may be missed due to sampling bias. For this reason, the British Society of Gastroenterology guidelines do not require IM for BO diagnosis but suggest that its presence or absence should be taken into consideration for patient management in terms of frequency of follow-up endoscopies (Gregson et al., 2016).

The present study has characterised MMP-7 expression using immunohistochemical analysis in the development and progression of the BO-dysplasia-adenocarcinoma

sequence in endoscopic biopsies, including different types of metaplasia (cardiac, intestinal, and fundic), LGD and HGD, as well as in Barrett's adenocarcinoma in surgical tissue specimens. The contribution of stromal cells (myofibroblasts) in BO progression to OAC remains poorly defined. This study also assessed the pattern of MMP-7 expression in myofibroblasts compared with cancer cells during Barrett's disease progression.

3.1.1 Objectives

1. To evaluate by immunohistochemistry the expression of MMP-7 (and for comparison MMP-1) in Barrett's adenocarcinoma and stromal cells.
2. To correlate expression with different stages of Barrett’s progression.
3. To evaluate the potential of MMP-7 as a pre-invasive factor in BO and to compare expression levels with adjacent OAC

3.2 Material and Methods

3.2.1 *Tissue samples and biopsies*

Patients enrolled in this study are described in section 2.8. The mean patient age for surgical tissue resection cases was 73 ± 3 (male, M; n = 9) and 59 ± 2yrs (female, F; n = 5) patients. The mean age for patients providing endoscopic biopsies was: intestinal metaplasia, 65 ± 3 (M; n = 12) and 66 ± 6 (F; n = 5); cardiac metaplasia, 47 ± 6 (M; n = 12) and 68 ± 4 (F; n = 5); fundic metaplasia, 57 ± 3 (M; n = 7) and 59 ± 3 (F; n =10); LGD, 64 ± 6 (M, n = 14), and 70 ± 3 (F; n = 3); HGD, 66 ± 2 (M; n = 11) and 70 (F, n =1).

3.2.2 *Immunohistochemistry*

Sections were stained using mouse monoclonal anti-human MMP-7 and goat anti-human MMP-1 antigen affinity purified polyclonal antibody (as described in section 2.9). Slides were scanned by light microscopy and representative fields selected for analysis.

3.2.3 *Quantification of Immunohistochemistry*

Scoring of immunohistochemical staining is described in section 2.9.1. The stromal and epithelial compartments were scored separately. Sections were separately evaluated by three independent blinded investigators.

3.2.4 *Histopathological analysis, tumour staging and definition of Barrett's mucosa*

Immunohistochemically stained sections of OAC were analysed with a special focus on tumour infiltrated areas and stromal areas. Tumour staging was performed according to the 6th edition of the TNM staging system by the UICC/AJCC (Sobin et al., 2011). Grading was performed according to WHO criteria (Hamilton and Aaltonen, 2000). All patient characteristics were documented in a database (EXCEL, Microsoft) in the Department of Pathology at the University of Szeged, Hungary. Other clinic-pathological variables for fourteen clinical cases of OAC are summarised in Table 3.1

Parameters	Values (%)
Age	
Mean	70±3
Range	49 -83 years
Gender	
Male	68.75%
Female	31.25%
Depth of invasion	
pT1	2/14 (14.29%)
pT2	3/14 (21.43%)
pT3	9/14 (64.29%)
Lymph nodes, distant metastasis	
pN0	6/14 (42.86%)
pN1	6/14 (42.86%)
pN2	1/14 (7.14%)
pN3	1/14 (7.14%)
M0	13/14 (92.86%)
M1	1/14 (7.14%)

Table 3.1: The clinicoplathological parameters of the OAC. Patients (n=14) studied with respect to age, gender, depth of invasion, lymph node involvement, and systemic metastasis.

3.3 Results

3.3.1 *Simple regression analysis of score validation*

Immunohistochemically stained slides were initially scored by three independent pathologists for intensity of staining on a range of 0 to 3 point scale. The correlation coefficient R_2 is shown in (Fig 3.1 A, and B**).** Linear regression showed excellent inter-observer agreement.

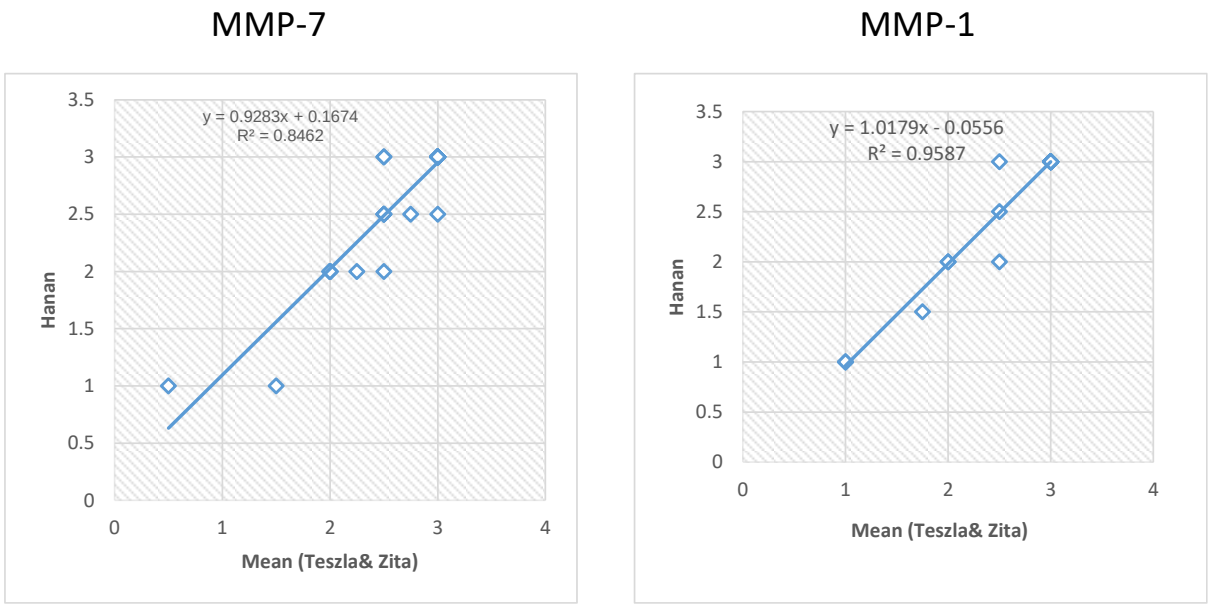

Figure 3.1: Score validation. A) MMP-7 visual scores were highly consistent; there was an excellent concordance between pathologists. B) The agreement was excellent for MMP-1 scoring between observers.

3.3.2 *High MMP-7 expression was associated with invasive grade in OAC patients*

MMP-7 was expressed with varying staining intensity. As shown in table 3.1 the majority of OAC cases involved in this study were stage pT3 (64.3%), which means the cancer was growing into the outer layer of the oesophagus; we found that MMP-7 exhibited strong staining intensity (score 3) in OAC patients with invasive grade T3 that was higher than in patients with invasive grade T1–2.

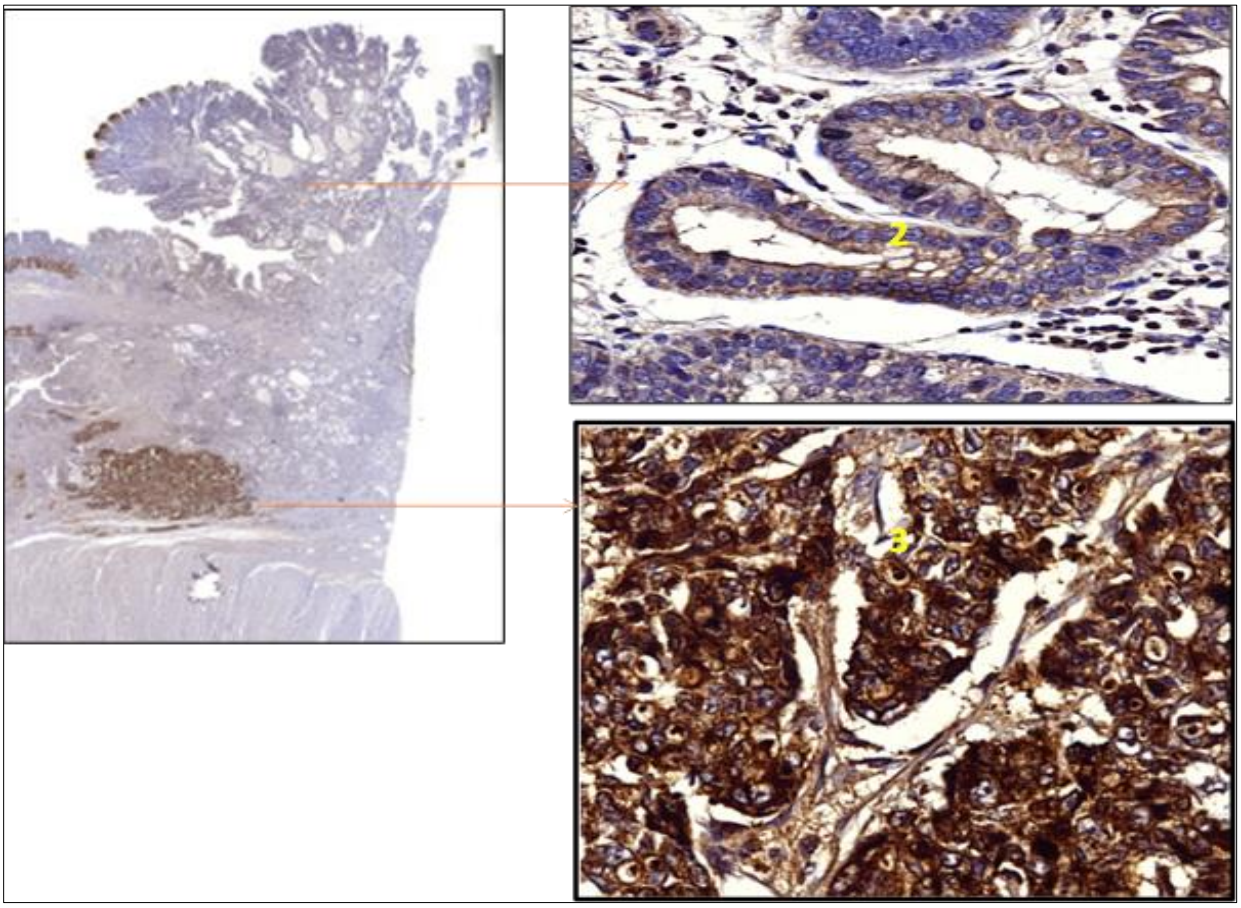

Figure 3.2: The relationship between MMP-7 expression and depth of tumour invasion in OAC patients. MMP-7 exhibited strong staining intensity with more aggressive tumour characteristics such as deeper invasion (PT3).

3.3.3 *Expression of MMP-7 is up-regulated in the progression from BO to OAC*

In order to determine the pattern of MMP-7 expression across the Barrett's metaplasia, dysplasia and carcinoma sequence, immunohistochemical staining was then performed and both cancer cells and stromal cells (myofibroblasts) scored. The data indicated that MMP-7 was expressed throughout the tumour, and most abundant expression was detected at the invasive part of tumour. As shown in (Figure 3.3 A upper panel) MMP-7 expression correlated well with histological progression in BO: thus MMP-7 exhibited a progressive increase in expression as indicated by intensity of staining in the histological progression from metaplasia to indefinite/LGD to HGD, and to OAC. In particular (Figure 3.3 A lower panel) the intensity of staining was weak in normal oesophagus (<50% of normal epithelial cells were positive) but there was a progressive increase in intensity of staining in epithelial cells in adjacent premalignant lesions, metaplastic epithelium (BO), dysplastic epithelium, non-invasive, and invasive carcinoma cells. Surprisingly, in the stroma putative myofibroblasts identified as spindle-shaped cells that surround tumour cells showed MMP-7 expression. In these cells, the intensity of staining was greatest in cells close to the invasive portion of the tumour, compared with adjacent tissue (Figure 3.3 B).

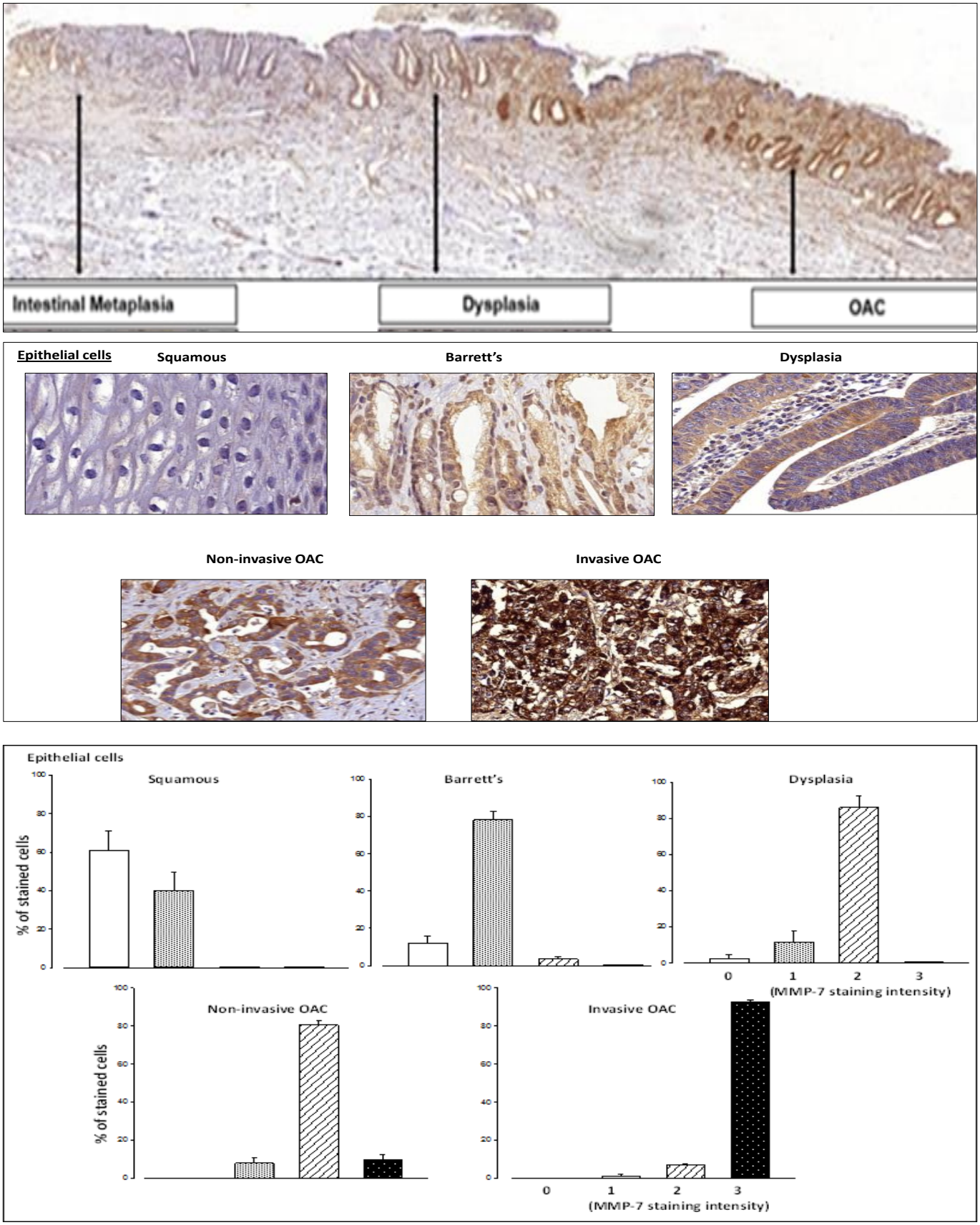

Figure 3.3A: MMP-7 is highly expressed in OAC, up-regulated early in oncogenesis. Upper panel photomicrograph illustrating the expression pattern of MMP-7 during Barrett's disease progression to OAC. Lower panel in epithelial cells, progressive MMP-7 expression from squamous epithelium to metaplasia, dysplasia, non-invasive OAC and the invasive front of OAC.

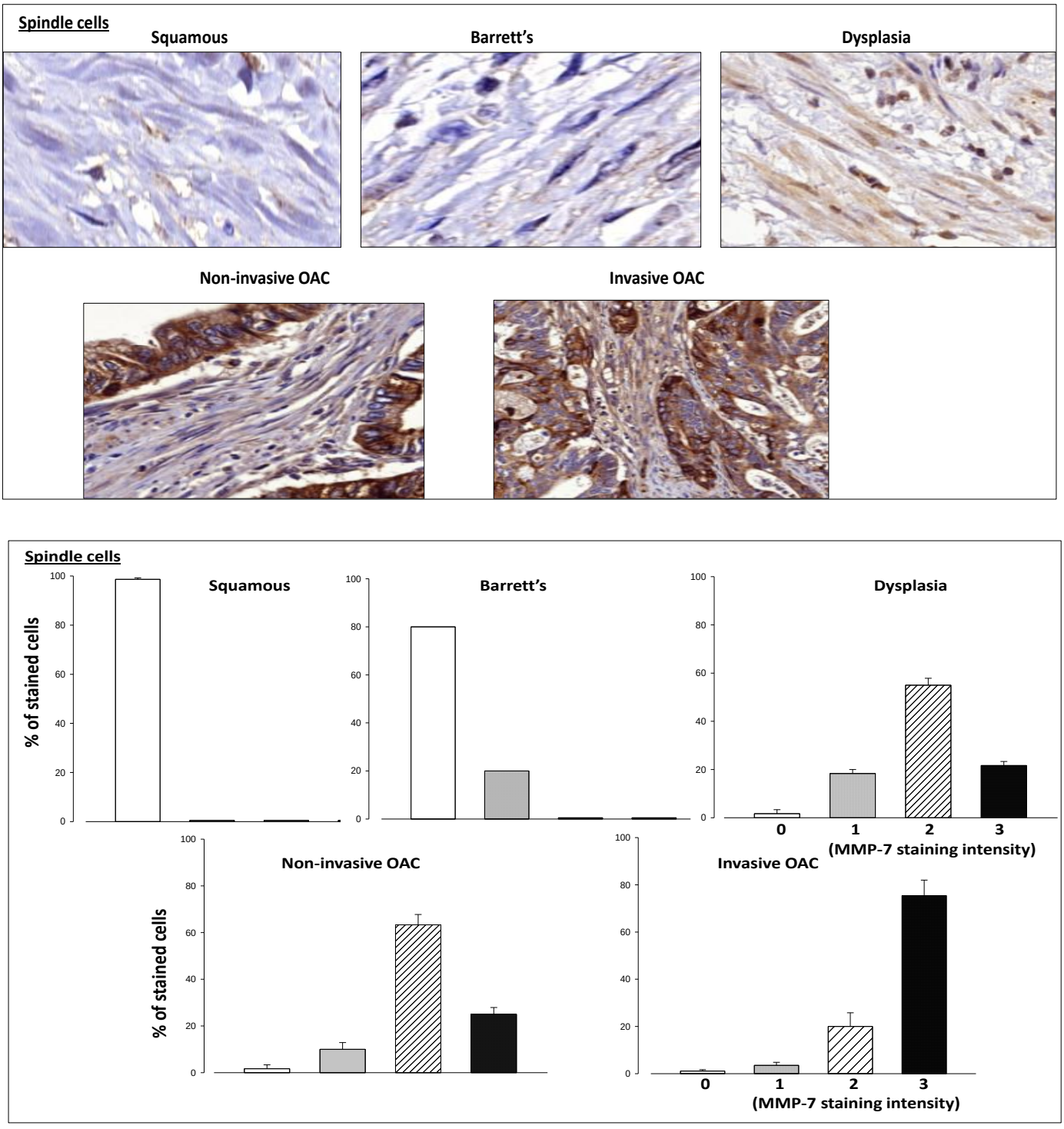

Figure 3.3 B: Unexpected high MMP-7 expression by stromal cells in OAC. In spindle cells, there is also a progressive increase in expression in spindle-shaped cells in the stroma, corresponding to myofibroblasts.

3.3.4 *MMP-7 is expressed abundantly in HGD*

In order to establish the expression of MMP-7 during BO progression to adenocarcinoma, the pattern of immunostaining in endoscopic biopsies from different types of metaplasia (cardiac, intestinal, and fundic), as well as LGD and HGD was examined. Fundic metaplasia and HGD cases exhibited a strong staining pattern. The extent of staining of the intestinal metaplasia and low grade cases was intermediate compared to the cardiac type metaplasia (Figure 3.4). Since the amount of tissue in endoscopic biopsies of the precursor lesions was tiny, the pattern of expression in myofibroblasts was analysed in a different way. Thus, the Immunohistochemical localization of MMP-7 in myofibroblasts in LGD, HGD and non-dysplastic BO, was limited to those cases in which detectable MMP-7 was found. As shown in Table 3.2, MMP-7 immunoreactivity in myofibroblasts was seen in most cases of intestinal metaplasia (10/17) and HGD (9/12) as compared with fundic, cardiac type of metaplasia, and LGD where positive myofibroblasts were scarce.

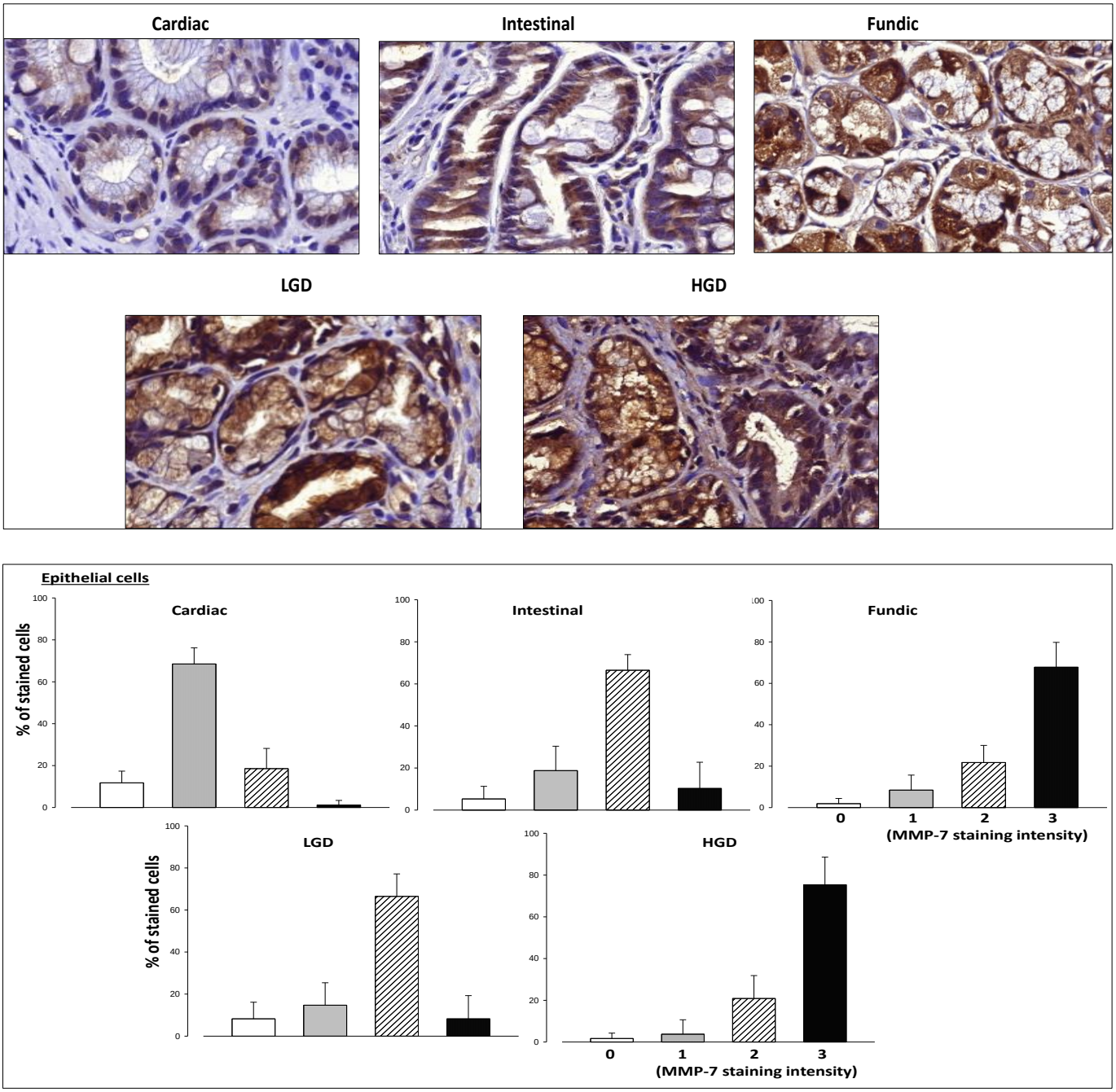

Figure 3.4: Different expression pattern of MMP-7 in different types of metaplasia, LGD, and HGD. Upper panels representative photomicrographs of MMP-7 expression in cardiac, intestinal, and fundic metaplasia; low grade dysplasia (LGD), and high grade dysplasia (HGD). Lower panels, representative graphs covering the all scoring range showed that MMP-7 exhibiting different staining intensity (0-3) during the multistep sequence of Barrett's disease progression.

Epithelium	Myofibroblasts not detected	Myofibroblasts negative	Myofibroblasts positive
A) Metaplasia			
Cardiac	5/17(29.4%)	8/17(47%)	4/17 (23.5%)
Intestinal	1/17 (5.9%)	6/17(35.3%)	10/17 (59%)
Fundic	1/17(5.9%)	9/17(53%)	7/17(41.2%)
B) Dysplasia			
LGD	9/17(53%)	5/17(29.4%)	3/17(17.6%)
HGD	1/12(8.3&)	2/12(16.6%)	9/12 (75%)

Table 3.2: Immunohistochemical analysis of myofibroblasts in biopsies material. Myofibroblasts exhibited abundant expression of MMP-7 in intestinal metaplasia and in HGD.

3.3.5 *MMP-1 expression in BO and OAC*

To provide a comparison for the quantitative analysis of MMP-7 expression in OAC, we examined MMP-1 expression in 11 out of 14 cases that were scored for MMP-7. MMP-1 immunostaining was detected in all samples of OAC examined. Oesophageal carcinoma cells showed a varying degree of immunostaining for MMP-1. MMP-1 expression was seen in metaplasia, dysplasia within Barrett's mucosa, as well as cancer cells suggesting that it is upregulated early in oncogenesis. The mean value of percentage of positively stained carcinoma cells at each staining intensity on 4 point scale of 0-3 as shown in representative graphs covering the full scoring range of OAC cases (Figure 3.5 A, epithelial cells). The expression levels of the MMP-1 in non- invasive OAC (80%) did not differ significantly from invasive part of the tumour, but showed decrease compared to the invasive front (65%), suggesting that MMP-1 may plays a role as pre-invasive factor in OAC. Myofibroblasts exhibited different staining patterns in all OAC cases, the most abundant expression (>60%) was at the invasive edge (Figure 3.5 B, spindle cells).

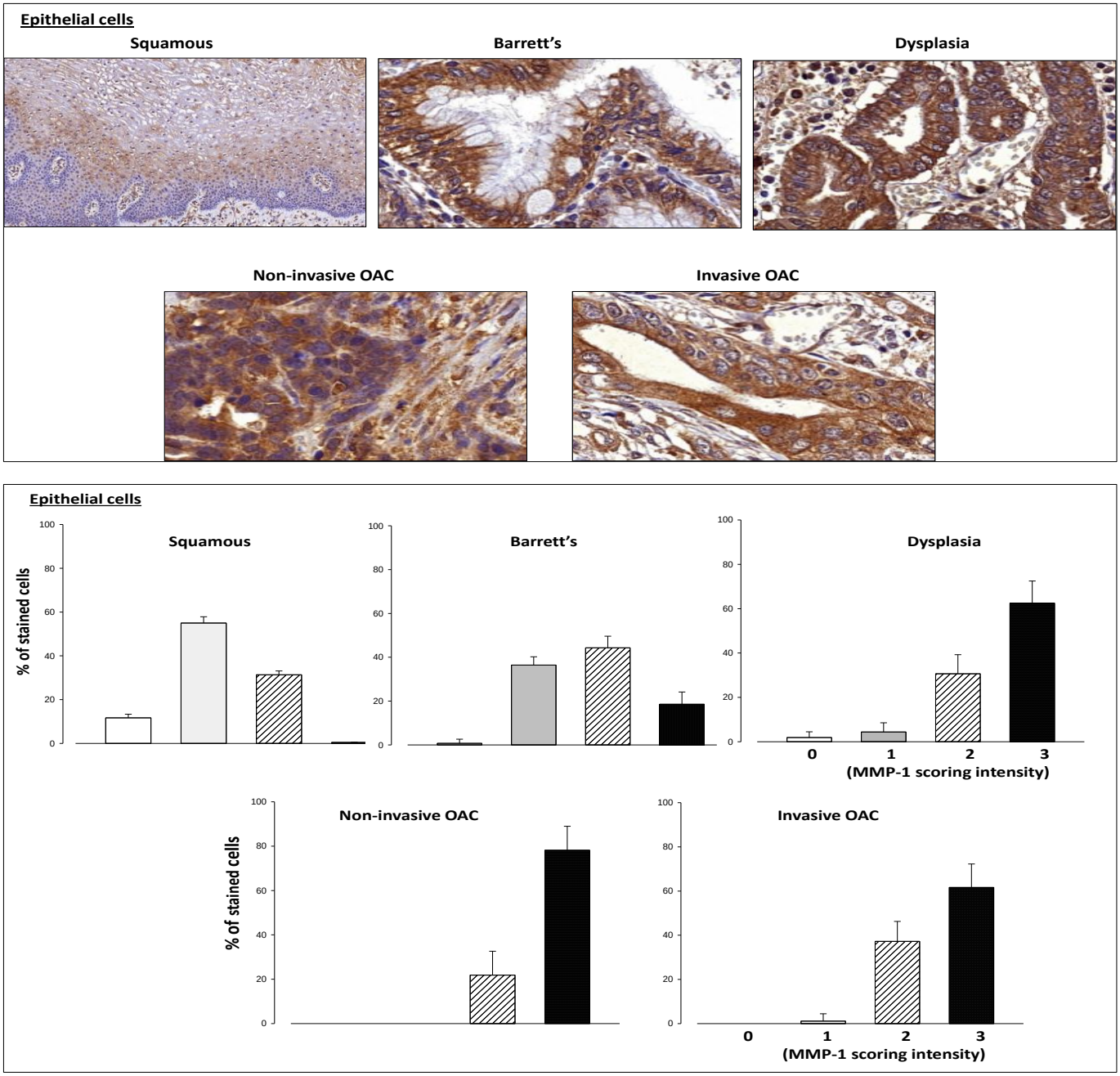

Figure 3.5 A: Immunohistochemical localization of MMP-1 in Barrett's adenocarcinoma.
In epithelial cells, MMP-1 expression (as positive control) immunostaining in dysplasia with concomitant non-invasive OAC, and invasive OAC show the most intensive expression of MMP-1.

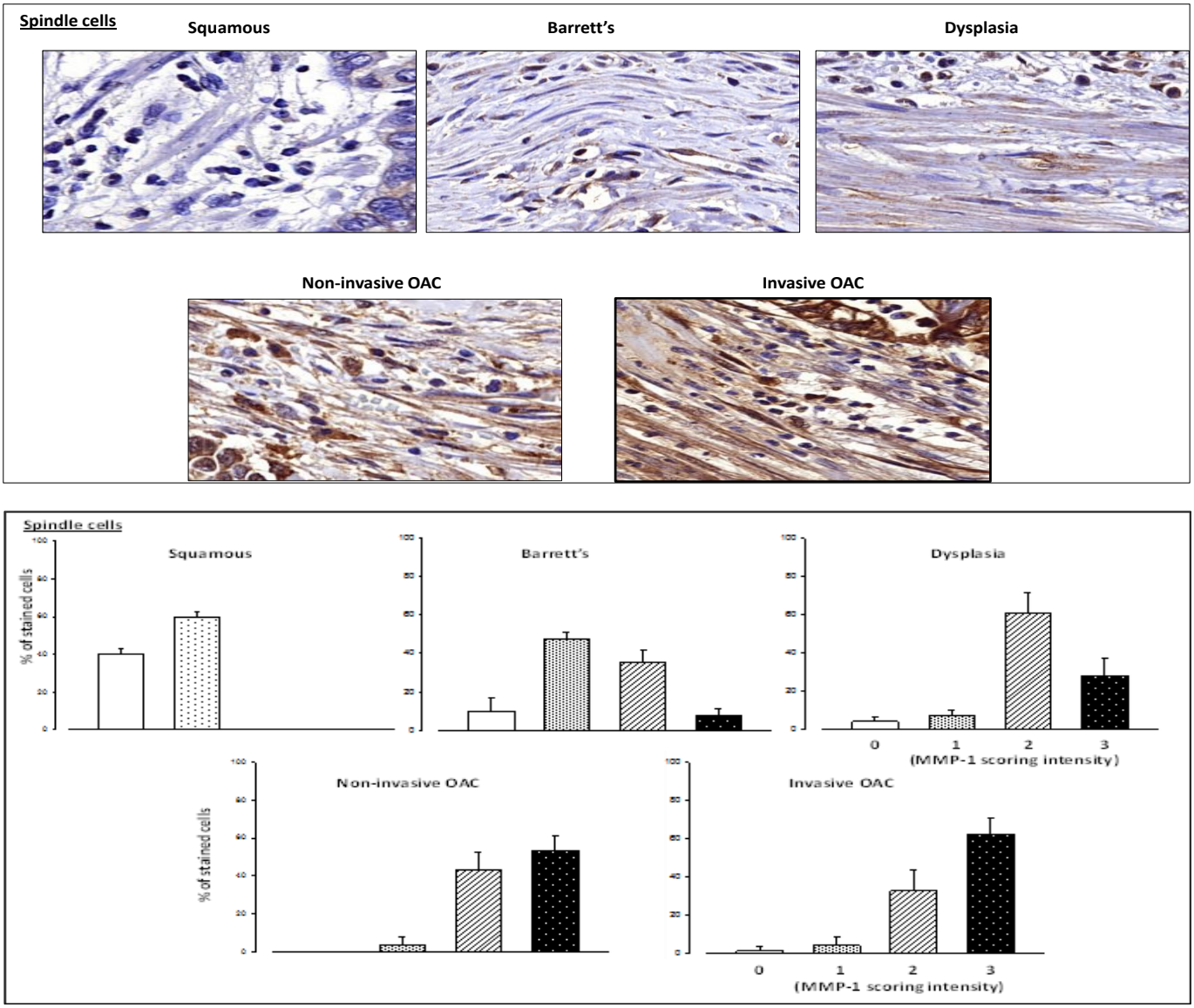

Figure 3.5 B: MMP-1 expression by spindle cells in OAC. Myofibroblasts, representative images, and graphs of MMP-1 expression by myofibroblasts exhibiting different pattern of staining intensity in OAC.

3.3.6 *MMP-1 is expressed early during the Barrett's malignancy sequence*

In order to identify MMP-1 as a marker in combination with MMP-7 to predict progression of dysplasia to OAC, and to understand the involvement of MMP-1 in Barrett's progression, we compared the abundance of MMP-1 between different types of metaplasia, LGD, and HGD. Immunohistochemical analysis was performed on biopsy samples. Biopsies revealed the extent of staining of MMP-1 in intestinal metaplasia and LGD cases was intermediate as compared to the cases of fundic type metaplasia, and HGD exhibited a strong staining pattern (Figure 3.6). The myofibroblasts were positively stained with MMP-1 mostly in the cases of intestinal metaplasia (64.7%), and HGD (83.33%) as shown in (Table3.3).

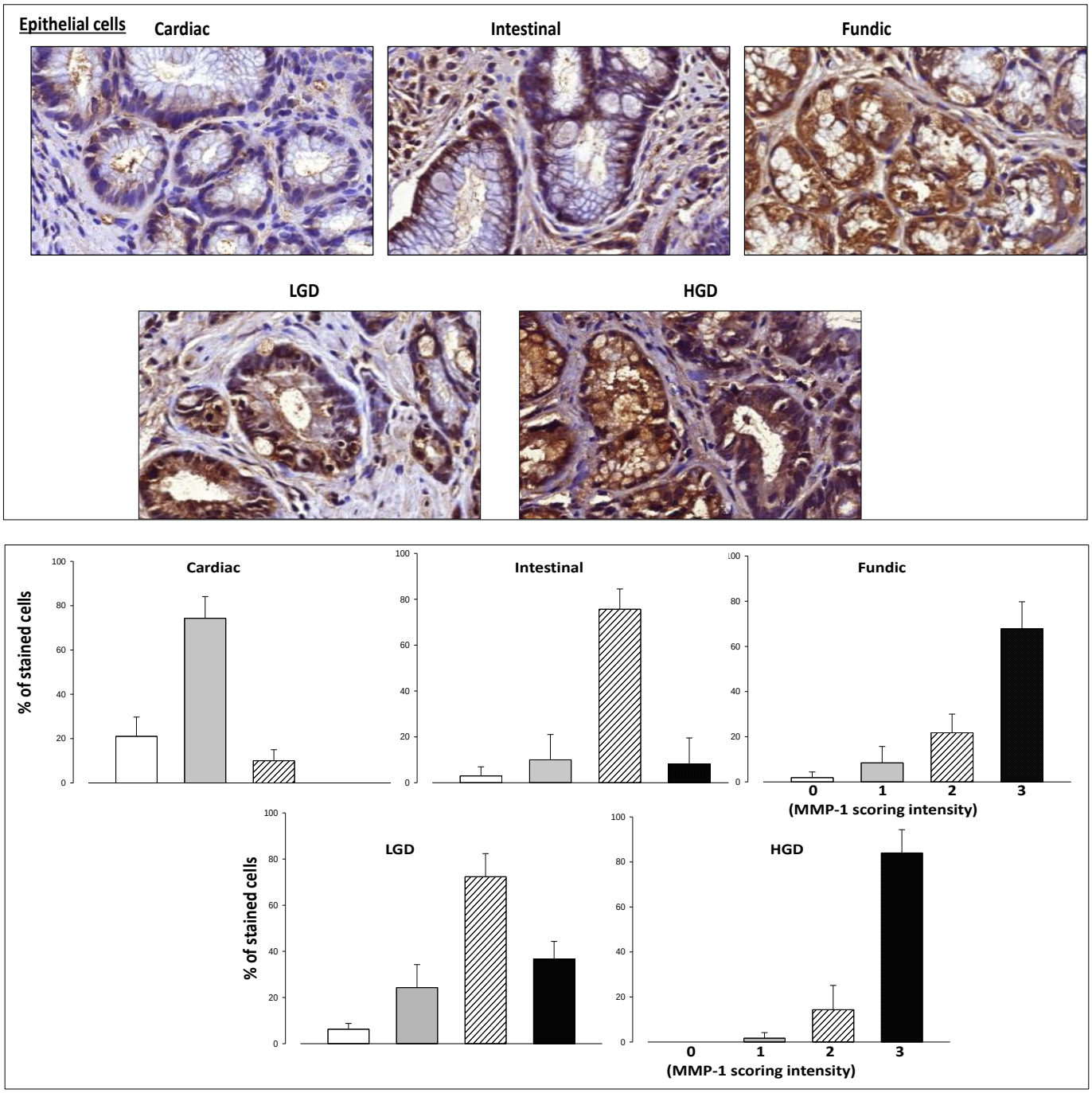

Figure 3.6: MMP-1 expression is an early event in the carcinogenesis of Barrett's-associated adenocarcinomas. Epithelial cells, representative images of MMP-1 expression in early BO. MMP-1 expression is strongly associated with fundic type metaplasia and high-grade dysplasia within Barrett's mucosa. The extent of staining was patchy in cases of intestinal, cardiac metaplasia, and LGD exhibited weak to moderate staining pattern.

Epithelium	Myofibroblasts (Not detected)	Myofibroblasts (No stain)	Myofibroblasts With stain)
A) Metaplasia			
Cardiac	2/17(11.7%)	9/17(53%)	6/17(35.3%)
Intestinal	1/17(5.9%)	5/17(29.4%)	11/17(64.7%)
Fundic	3/17(17.6%)	6/17(35.3%)	8/17(47%)
B)Dysplasia			
LGD	4/17(23.5%)	4/17(23.5%)	9/17(53%)
HGD	0/12(0%)	2/12(16.7%)	10/12(83.3%)

Table 3.3: Immunohistochemical analysis of Myofibroblasts in biopsies material. The table displayed Myofibroblasts exhibited abundant expression of MMP-1 in intestinal metaplasia 64.7%, and 83.3% in HGD

3.4 Discussion

It is generally accepted that the development of OAC in BO is a gradual process in which the disruption of biological processes at the cellular level accumulates in a cascade from non-dysplastic BO, through LGD and HGD, and finally OAC (Buttar and Wang, 2004, Haggitt, 1994, Hamilton and Smith, 1987).

One of the fundamental goals of translational research in BO is to distinguish the small number of individuals who progress to OAC from the majority who do not. Currently, periodic endoscopic biopsies with histological assessment of dysplasia are used to assess the risk of progression to OAC in patients with BO (Reid et al., 2010). The main finding of this study is that MMP-7 expression increases in the progression to OAC, with low expression in normal squamous oesophageal epithelium, and progressively increased expression in BO, LGD, HGD; MMP-7 is particularly abundant in the invasive part of the tumour. Additionally, MMP-7 is not expressed by normal oesophageal spindle shape cells, however, expression is gradually increased in spindle shape cells in the progression to adenocarcinoma. This work also suggests that MMP-7 and MMP-1 might together be useful markers for evaluating progression of BO to adenocarcinoma, and may lead to a new paradigm for early diagnosis, improved prognostication and individually tailored therapeutic options.

Biopsy samples derive from endoscopically recognized abnormalities, and in spite of inter-observer variability, histologic assessment of dysplasia remains the primary approach for diagnosis and surveillance (Voltaggio et al., 2011). However, patients with BO often develop cancer without prior biopsy detection of each stage of progression. It is recommended, therefore, that in all patients with BO targeted biopsies should be taken from visible lesions suspicious for dysplastic changes of the mucosa, as well as four ‘random’ quadratic biopsies at 2 cm intervals over the

entire extent of the Barrett's segment – the so-called Seattle protocol (Gregson et al., 2016). In this study, we investigated MMP-7 expression in both surgically resected material and endoscopic biopsies examining MMP-7 expression throughout BO tumorigenesis (BO, LGD, and HDG biopsies) to allow for a more definitive comparison of the MMP-7 expression pattern. Since MMP-7 was found to be highly expressed in HGD relative to BO, and LGD it may be particularly useful in providing a novel basis for the diagnosis of HGD.

Histological subgroupings of BO are associated with different capabilities to develop malignancy. The metaplastic columnar epithelium with intestinal type goblet cells and the junctional (cardia) type have a more significant risk of malignant transformation (Voltaggio et al., 2011). The three main histological subtypes (Intestinal, fundic, and cardiac metaplasia) of BO, LGD and HGD were included to provide insights into the expression pattern of MMP-7 through the natural history of BO. There has been a considerable interest in defining a subgroup of high risk patients. Our data revealed alterations in MMP-7 expression pattern as an early event in BO, and more abundantly expressed at the invasion front of OAC, so MMP-7 could be has potential role in the surveillance of patients with BO at high risk of developing OAC although the assessment of epithelial cells alone may be problematical given that MMP-7 was also expressed in fundic metaplasia which has low potential for progression.

It was reported (Underwood et al., 2015a) that the most OAC (93%) contained CAFs with a myofibroblastic (α-SMA-positive) phenotype, which correlated significantly with poor survival, and this may explain the aggressive, highly infiltrative nature of the disease. CAFs isolated from OAC have a functional myofibroblastic phenotype, and promote tumour cell invasion *in vitro* and growth *in vivo*, signalling to OAC cells via secretion of the ECM protein, periostin, and perhaps chemerin (Kumar et al., 2014).

However, very little is known about the role of the CAMs in Barrett's disease progression to adenocarcinoma. Another study (Yamashita et al., 2000) of the localization of MMP-7 in a series of 48 oesophageal carcinoma specimens (43/48 squamous oesophageal carcinoma, 2/48 oesophageal adenocarcinoma, 2/48 basal cell carcinoma, and 1/48 carcinosarcoma) showed that carcinoma cells facing the stroma also showed an intense staining especially at the invasive front, despite almost no staining in the stromal components. However, our finding that MMP-7 expression was abundant in stromal cells at the invasive part of the tumour appears to be novel and may provide a useful indicator of invasive activity in OAC.

In order to provide a point of comparison for the MMP-7 data we also examined expression of MMP-1 in the same surgical specimens and biopsy material. Previous work has found MMP-1, -3, -7 and -10. along with TIMP-1, were increased along the sequence from BO to OAC (Salmela et al., 2001b). MMP-9 exhibits a similar trend (Herszenyi et al., 2007a) suggesting that alterations in these MMPs may be early events in oesophageal carcinogenesis and indeed it has been suggested that MMP-1 plays a role as pre-invasive factor in BO associated OAC, consistent with malignant proliferation following the clonal expansion (Grimm et al., 2010a). The present study indicates that MMP-7, as well as MMP-1, should be included in the functionally relevant MMPs is implicated in the progression of BO to OAC.

Oesophageal adenocarcinoma is frequently precipitated by the loss of p53 (Gregson et al., 2016), and p53 can be used to predict progression from BO to OAC. Patients with dysplasia are now advised to be examined using p53 immunohistochemistry to improve diagnostic accuracy (Fitzgerald et al., 2014), since abnormal p53 immunohistochemistry is associated with three to eight times more rapid progression of this disease (Sikkema et al., 2009). The value of p53 as a biomarker of malignant

progression in BO has been confirmed in other studies, but the sensitivity of this marker alone in these studies was too low to predict cancer risk (Bani-Hani et al., 2000a, Murray et al., 2006). Increased expression of cyclin D has also been implicated in the predisposition to transform from metaplastic epithelium to cancer (Trudgill et al., 2003, Bani-Hani et al., 2000b).

Other markers as proliferating cell nuclear antigen (PCNA), which is a cofactor of DNA synthase and an indicator of cell cycle progression at the G1/S transition phase in the cell cycle has been suggested in several studies to be increased in HGD/OAC (Gillen et al., 1994, Jankowski et al., 1992b, Krishnadath et al., 1997). This was however not confirmed in another study (Scholzen and Gerdes, 2000), in which PCNA was found to be of limited value in differentiating between BO, LGD and 'indefinite for dysplasia' (IND) in BO. A disadvantage of PCNA staining is that it is affected by the fixation method of the tissue, with consequently staining of quiescent cells (G0 phase) during antigen retrieval. Therefore, PCNA is probably not a reliable marker that can be used for the prediction of patients at risk of neoplastic progression in BO. It seems likely that panels of biomarkers are more helpful in predicting cancer risk in BO compared to a single biomarker. For instance, it was reported that the combined use of the biomarkers 17p13 LOH (p53), 9p21 LOH (p16), and DNA ploidy improved the detection of the subgroup of BO patients with an increased risk of progression to OAC, compared to using only a single biomarker (Maley et al., 2004, Reid et al., 2001).

Our finding suggests that it may be possible to use both stromal and epithelial derived MMP-7, and MMP-1 as biomarkers of progression from Barrett's oesophagus to dysplasia and cancer. It is however now necessary to validate this result using larger panel of pre-clinical and clinical patients. Heterogeneity in patient

populations and sampling bias are always a challenge in histopathology. Other approaches have been suggested (McManus et al., 2004) including blood or urine biomarkers. Thus recent studies have shown that MMP-7 could be detected in the serum of patients, with ovarian cancer (Palmer et al., 2008) and colorectal cancer (Klupp et al., 2016). Proteomic methods combined with serology have also led to the identification of serum pro-MMP-7 as a marker of renal cell carcinoma and represent a powerful tool in searching for candidate biomarker proteins (Sarkissian et al., 2008). Other suggestions (Kerkhof et al., 2007) for future studies aiming at risk stratification in BO are that these should be performed in a multicentre setting in order to investigate large cohorts of BO patients. Further technological developments that allow panels of biomarkers to be determined in a (semi-) automated setup, may be expected to eliminate observer bias (Fiore et al., 2012).

3.5 Conclusions

1. MMP-7, and MMP-1, expression increases in the progression to OAC. The patterns of MMP-7 and MMP-1 expression in non-dysplastic BO, HGD to invasive carcinoma could be helpful in predicting cancer risk in BO patients.

2. High expression levels of MMP-7 may be related to aggressive invasive grade in OAC patients.

3. Unexpectedly, MMP-7 is expressed by myofibroblasts in a progressive manner especially at the invasive front of the OAC.

CHAPTER 4

VALIDATION OF A NOVEL INDIRECT ELISA FOR MMP-7

4.1 Introduction

The validation of analytical methods is a systematic process of establishing that the method is acceptable for its intended purpose. Validation is therefore defined as the evaluation process that determines fitness for a specific use (Chandran and Singh, 2007b). In the present study, validation of an indirect ELISA for MMP-7 was undertaken with respect to its application to the assay of samples of cell media. The validation included determination of assay specificity, recovery, inter-assay precision, intra-assay precision, linear dilution and sensitivity. In this study we were committed to delivering a highly-validated assay capable of yielding accurate results.

An indirect ELISA format was chosen because it is characterized by high sensitivity, flexibility and cost-saving. A key step in indirect ELISAs is the immobilization of the antigen of interest, which is then detected by a primary antibody and then indirectly using labeled secondary antibody (Figure 4.1). This chapter describes the validation of an assay to be used in order to better understand the secretion mechanism of MMP-7, and to detect MMP-7 and its degraded products in cell media samples in OE33 cells.

4.1.1 Objectives

*The specific objectives were***:**

1. To have available an optimised ELISA which would facilitate the quantitative assay of large numbers of media samples.

2. To validate a specific and sensitive indirect ELISA for detecting MMP-7, and its cleavage products in cell media.

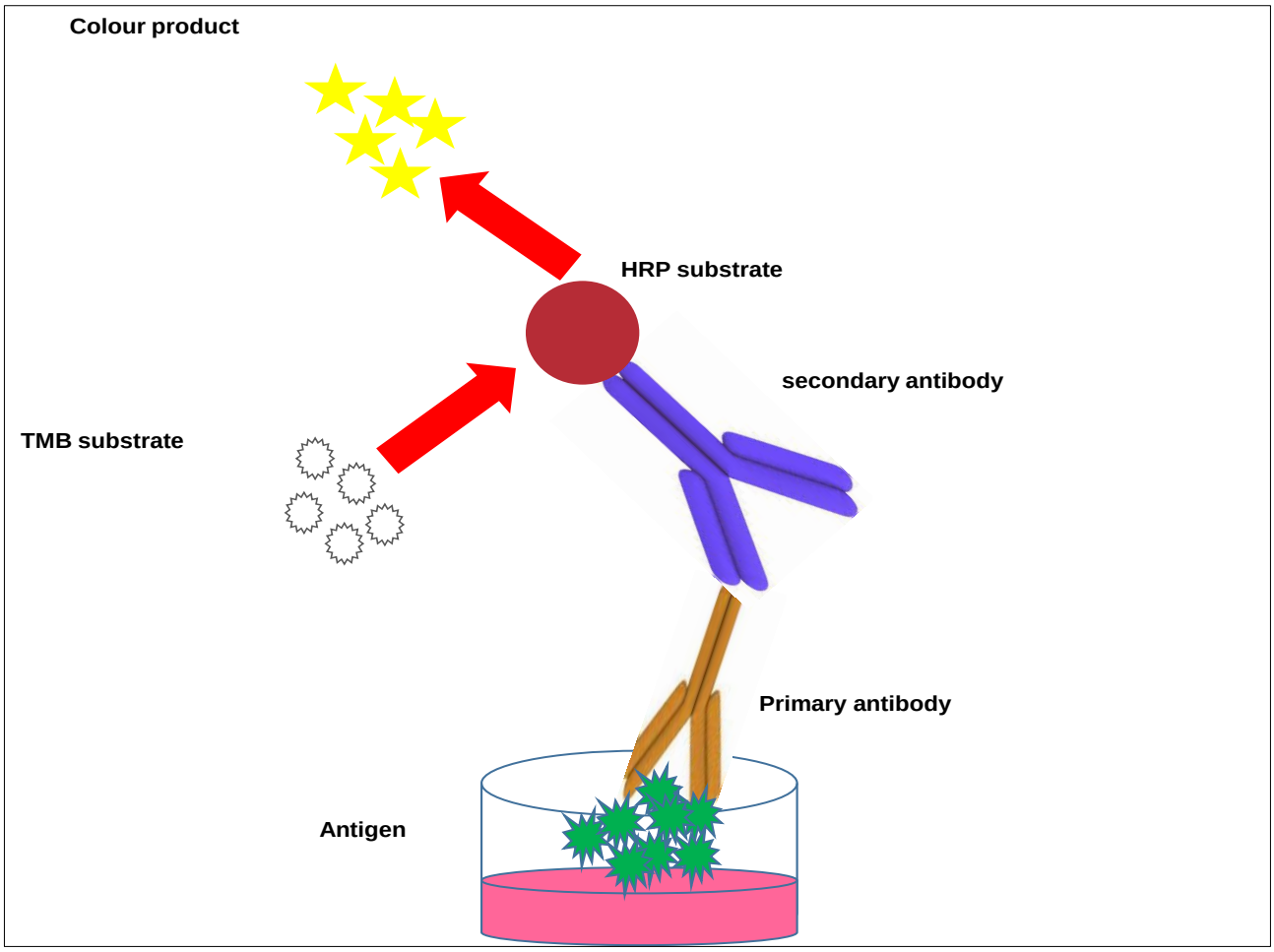

Figure 4.1: Schematic model of indirect ELISAs. There are five steps: (1) coating of microplate wells with antigen, (2) blocking and incubation with appropriate primary antibody, (3) attachment of the secondary biotinylated antibody to primary antibody, (4) detection of biotin by streptavidin-HRP, and finally (5) enzymatic activity monitored by addition of 3,3 ,5,5 tetramethylbenzidine (TMB) chromogenic substrate

4.2 Material and Methods

4.2.1 *Coating the plate with antigen*

The coating of 96-well ELISA plates with antigen was described in section 2.7.1. This was achieved through passive adsorption of the protein to the plastic of the microplate.

4.2.2 *Blocking the plates*

The remaining protein-binding sites in the coated wells were blocked by 300 µl of 1% BSA blocking buffer as described in section 2.7.2.

4.2.3 *Incubation with primary and secondary antibody*

Primary polyclonal rabbit L522 (G) (1:5000) antibody was added to each well followed by secondary antibodies as described in section 2.7.3.

4.2.4 *Detection (TMB application)*

TMB (3, 3', 5, 5'-tetramethylbenzidine) was then added to each well followed by 1M sulphuric acid (1 M H_2SO_4)to stop the enzymatic reaction. Optical density was read at 450 nm as described in section 2.7.4.

4.2.5 *Analysis of data (calibration curve)*

A calibration curve was constructed using rh-MMP-7 standard over the range 0.39-100 pmol/ml.

4.3 Results

4.3.1 *Optimal dilution of primary antibody*

The optimal dilution of primary antibody was determined by examining different concentrations of antibody L522 (1:5000, 1:10,000, 1:20,000, and 1:50,000) as well as standard curves using rh-MMP-7. A dilution of 1:5000 of L522 gave rh-MMP-7 standard curves with good sensitivity and dynamic range (Figure 4.2).

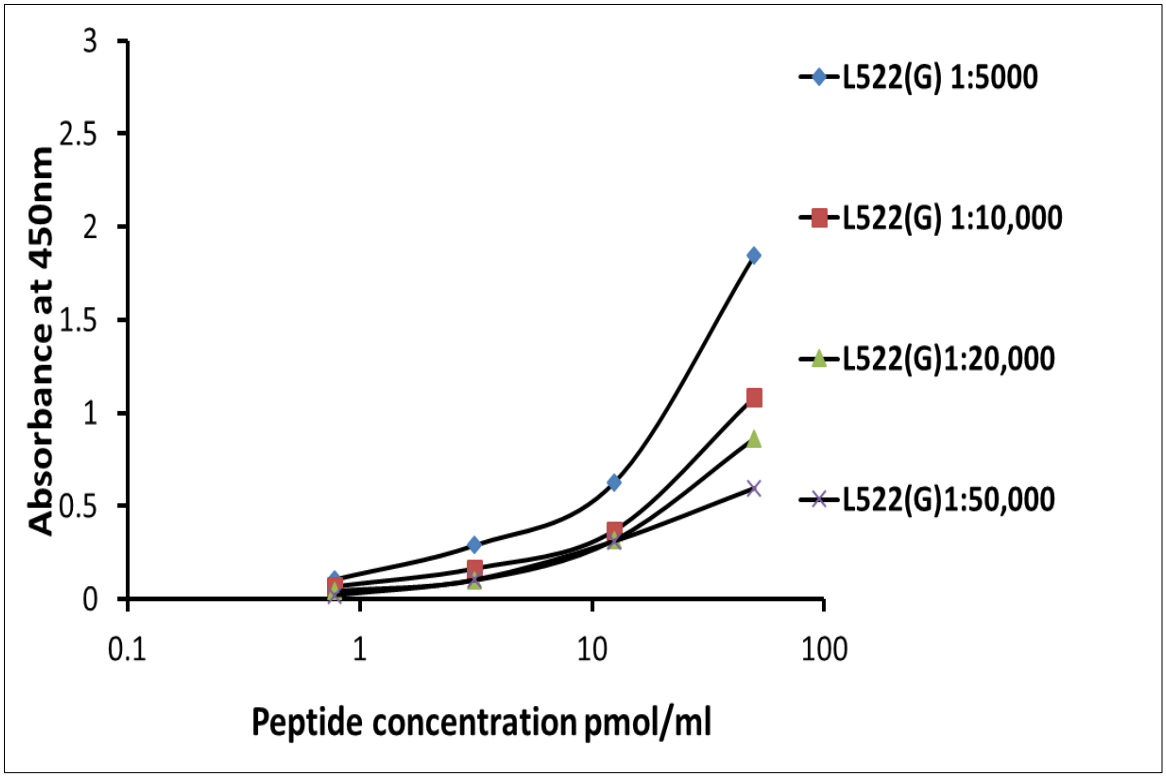

Figure 4.2: ELISA Validation: Optimizing the concentration of detecting antibody. The data revealed a strong signal and low background with 1:5000 of L522.

4.3.2 *Parallel dilution curves of rh-MMP-7 with OE33 cell media and cell extracts*

To ensure that the estimated concentration of MMP-7 in an unknown sample was independent of dilution, a sample was then tested at various dilutions. Linearity of dilution for sample and standard was therefore examined for both representative cell medium and also cell extracts (Figure 4.3). OE33 cell media at different concentrations clearly exhibited parallel dilution with standard, at least at dilutions greater than 1:10 thereby establishing that the assay is valid for OE33 cell media diluted in this range. Similarly cell extracts exhibited parallel dilution with standard.

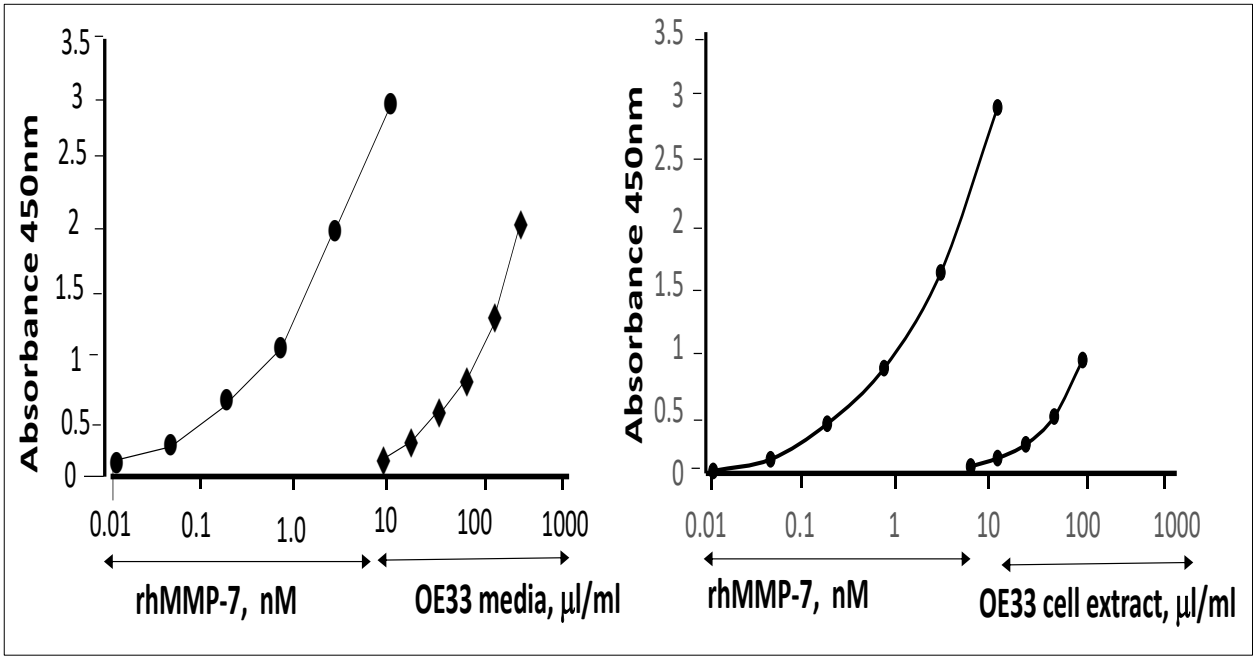

Figure 4.3: ELISA Validation: parallel dilution of sample and standard. Dilution curves of rh-MMP-7, OE33 cell medium and OE33 cell extract. Samples were serially diluted in PSD

4.3.3 *L522 reacts with 97-108 sequence of pro-MMP-7*

To study specificity of the assay, the reactivity of various peptides was tested by comparison of signal *vs* peptide concentration. Synthetic peptides were used to profile the substrate specificity of MMP-7 in the assay e.g. preproMMP-7(95-110, 97-108, 102-113, 114-125, and 134-145), pro-MMP-7 and MMP-7. The ELISA reacted well with pro-MMP-7, MMP-7 and a peptide fragment corresponding to the 97-108 sequence of preproMMP-7, although the latter exhibited slightly lower affinity than the former (Figure 4.4 top panel). The fragments 114-125 and 102 -113 did not react up to 100 pmol/ml (Figure 4.4 lower panel).The data indicate that L522 binds the sequence of pro-MMP-7 in the region of 97-101 and tolerates extensions to the C- and N-terminus of this sequence; any peptide containing the region is therefore expected to react in the assay.

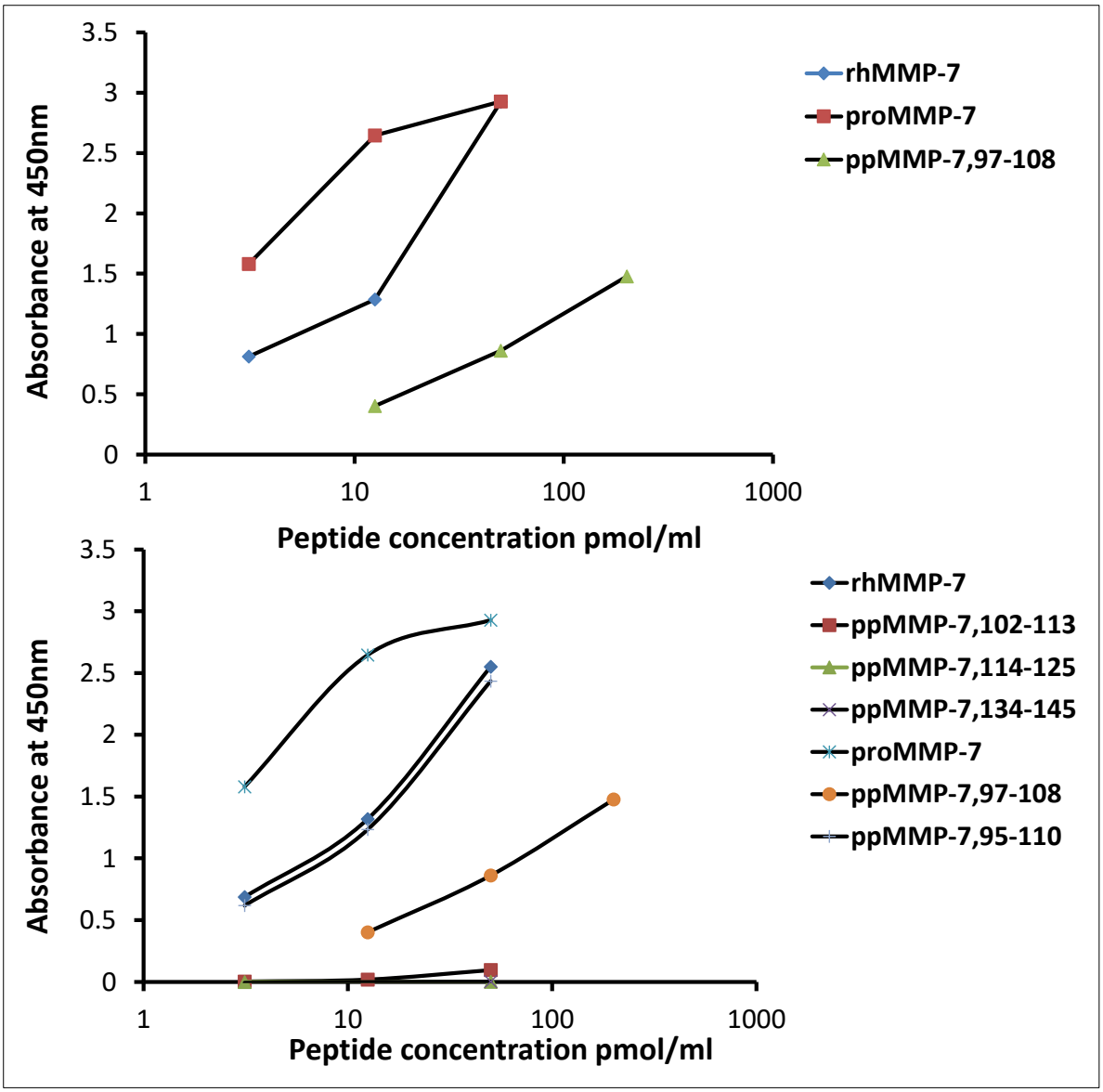

Figure 4.4: ELISA Validation: Specificity - L522 reacts with 97-108 sequence of pro-MMP-7. The ELISA data are typically graphed as signal *vs* log peptide concentration in pmol/ml. A) The average of three specificity assay of three peptides; pro-MMP-7 and rh-MMP-7 show higher affinity with L522 and ppMMP-7 97-108 reacts but with lower affinity. B) Other peptides fragments are studied, namely fragments 114-125,102-113 do not react with L522

4.3.4 *Recovery of spiked MMP-7*

Known concentrations of MMP-7(0.5-100pmol/ml) were spiked into OE33 media samples and assayed against rh-MMP-7 as standard. The data revealed that the recovery of spiked MMP-7 in OE33 cell media was approximately 100%; comparison between the standard curve and the spiked samples showed that the MMP-7 concentration stays within the linear section of the standard curve (Figure 4.5).

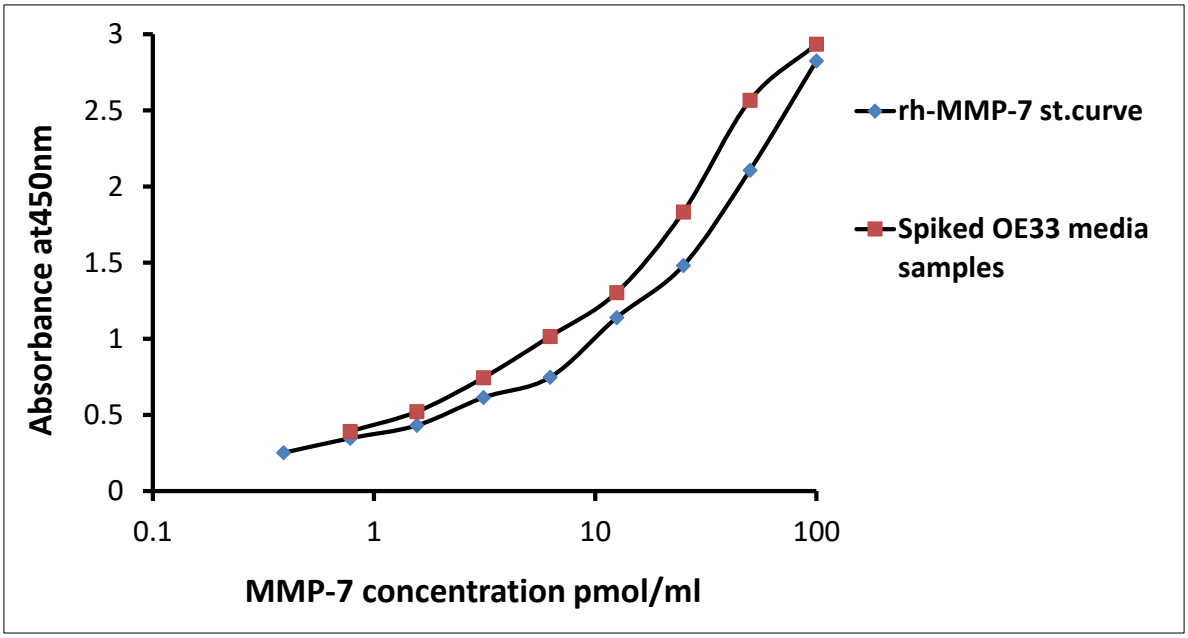

Figure 4.5: ELISA Validation: recovery. Known concentrations of MMP-7 (0.5-100pmol/ml) were spiked into OE33 media sample. The data revealed that both spiked sample and standard curves were parallel and recovery approximated to 100%.

4.3.5 *Intra-assay variation (precision)*

Assay precision was evaluated by determining the variability of results within a data set obtained within a single assay. It was expressed as the intra-assay coefficient of variation (intra-assay CV) and reflects deviation within the same assay. The % CV is calculated by dividing the standard deviation (SD) of a set of measurements by the set mean, and multiplying by 100. The CV was 7.3% (n = 10 samples).

4.3.6 *Inter-assay variation (reproducibility)*

Assay reproducibility was evaluated by determining the variability between assays. Inter-assay variation was determined from assays of three dilutions of an OE33 media sample in 6 independent assays. The results showed a CV of 9.6 %.

4.3.7 *Sensitivity*

Sensitivity is defined as the lowest level of analyte that can be distinguished from background. This is often referred to as analytical sensitivity or limit of detection. Here, we measured replicates of a blank sample, calculated the mean result and from this the standard deviation. The minimum detectable concentration of MMP-7 was then determined as three standard deviations above the blank and corresponded to approximately 10 pmol/l which equates to 1 fmol in the assay.

4.4 Discussion

A validated ELISA for the assay of MMP-7 was developed with excellent specificity and linearity allowing detection of nanomolar concentrations of MMP-7 in OE33 cell media samples. Validation is a mandatory step for all analytical laboratories and is required by health authorities and regulatory bodies such as the FDA (Chandran and Singh, 2007a).

Since our ELISA assay was an in-house method, robustness (the ability of a method to remain unaffected by small variations in method parameters) was firstly investigated as part of method development and the obtained results reflected in the assay protocol (optimization). The reason for this is that validation is linked to an assay protocol and changes in the latter might demand a new validation to be performed. The validation parameters for a bioanalytical method were then performed according to international guidelines and included selectivity/specificity, precision, recovery and linearity (Chandran and Singh, 2007a, Vassault et al., 2010). Stability of the analyte is considered in a separate chapter (see chapter 5). Obviously, validation of an assay cannot eliminate all the problems likely to arise during implementation of the methodology, but it does ensure that major problems are prospectively seen and a mechanism to control the variability developed. Hence, the indirect ELISA assay with step-by-step validation is included in the present work. The validated ELISA was developed with the intended use for measurement of MMP-7 in cancer cells media in tissue culture.

Most previous relevant publications either used ELISA commercial kits or incompletely/partially validated ELISAs. It is striking that while validation of a commercial assay ought to include all parameters except for robustness, and should

be covered by the manufacturer during method development the relevant information can be difficult to obtain, and may not exist (Andreasson et al., 2015).

A number of researchers have used the same commercial kit; none of these reports described the detection method explicitly, and full validation has not been reported for estimation of serum MMP-7 concentrations. One study reported the intra-assay variability for duplicate measurement was 4.3 % while the inter-assay variability was 7.7 % (Niedworok et al., 2016). MMP-7 concentrations were determined by using commercial ELISA kits without any validation parameter mentioned (R & D Systems) (Tzouvelekis et al., 2016, Pukrop et al., 2006). In spite of the requirements for regulatory submission many studies do not include the essential data on specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness to ensure that the analytical methodology in question gives timely, accurate, reproducible and reliable data which are adequate for the intended purpose of use (Preparations, 1990, Preparations, 2002, Pasteelnick, 1993, González and Herrador, 2007). In the light of this, our study, for the first time, has provided a fully validated and optimised MMP-7 ELISA appropriate for assay of MMP-7 in OE33 cells media. Moreover, it is easy to perform and presents lower costs than commercial kits.

The specificity of an antibody is dependent, in part, on the immunogen used: the latter can be a synthetic peptide or a purified protein. Synthetic peptides provide the advantage of knowing the amino acid sequence to which the antibody is directed (Ramos-Vara, 2005). It was reported (Ohuchi et al., 1996) that a pair of mouse monoclonal antibodies for determination of human MMP-7 concentration were able to specifically recognize pro-MMP-7 in colon carcinoma cells. In contrast, our MMP-7 antibody (L522) is able to react with a well-defined peptide fragment of the MMP-7

sequence, with the highest affinity, and specific to prepro 97-108 sequence of pro-MMP-7 which is appropriate for detection of pro-, active-, and cleaved MMP-7 from OE33 cells.

Antibody specificity is further complicated by the choice of monoclonal versus polyclonal antibodies. Polyclonal antibodies represent a pool of antibodies against the immunogen and may therefore detect a range of different epitopes, whereas a monoclonal antibody by definition detects a single epitope. Having said this, at high dilutions, many polyclonal antibodies appear to be specific for a single epitope: L522 may be an example of this. Most companies did not provide any in-depth descriptions of antibody validation procedures. In fact, it is now clear that the responsibility for proof of specificity is with the purchaser, not the vendor (Bordeaux et al., 2010) although this responsibility is frequently not met.

Together, these results demonstrate that our novel ELISA assay is a robust, sensitive and high throughput assay for a large number of samples successful in detecting MMP-7 in OE33 cell media in tissue culture, with defined specificity for detection of the N-terminal region of MMP-7. The use of a more accurate and quantitative MMP-7 measurement may improve the accuracy and sensitivity of the testing methodology.

4.5 Conclusion

1. A specific and sensitive indirect ELISA for pro-MMP-7, active MMP-7, and its cleavage products has been validated.
2. The assay makes use of antibodies that react at a sequence in the N-terminus of MMP-7 and is informative in ELISA for OE33 cell medium.

CHAPTER 5

VALIDATION OF OE33 AND OTHER CELL LINES FOR MMP-7 EXPRESSION AND SECRETION

5.1 Introduction

To gain insight into the cell biology of MMP-7 in OAC, we examined and validated its expression and secretion in various oesophageal cancer cell lines using Western blotting, indirect ELISA and immunocytochemistry.

Increased gastric expression of MMP-7 occurs in hypergastrinaemia, and may regulate myofibroblasts function via cleavage of IGFBP-5, the release of IGF and stimulation of MAPKinase and PI3Kinase pathways contributing to stromal deposition (Hemers et al., 2005, McCaig et al., 2006, Varro et al., 2007c). In addition, it is known that MMP-1 expression is also a target of gastrin and is implicated in mucosal remodelling (Kumar et al., 2015). Less is known about the factors that might regulate MMP-7 expression and secretion in oesophageal cancer and in view of the evidence for an involvement of gastrin in BO (Haigh, 2003, Wang et al., 2010b) we examined the hypothesis that gastrin stimulates expression of MMP-7 in OE33 and OE33-GR cells. Furthermore, as MMP-7 is secreted as pro-MMP-7, we asked if pro-MMP-7 is stable in OE33 cell media. Finally, as MMP-7 influences cells of the surrounding microenvironment, we asked if there are epithelial-stromal interactions in which MMP-7 is implicated.

5.1.1 *Objectives*

1. To determine MMP-7 expression and secretion in oesophageal cancer cell lines.

2. To assess the action of gastrin on MMP-7 secretion by oesophageal cancer cell lines.

3. Analyse the metabolism of MMP-7 by oesophageal cancer cells and conversion of pro-MMP-7 to MMP-7.

4. Study the expression and secretion of MMP-7 in oesophageal myofibroblasts.

5. Characterise interactions between OE33 cells and myofibroblasts in modulating MMP-7 secretion and metabolism.

5.2 Materials and Methods

5.2.1 *Cell culture, and Conditioned media*

Oesophageal cancer cell lines (OE19, OE21, OE33 cells, and OE33-GR cells) were cultured as described in section 2.3.1. Oesophageal myofibroblasts (NTMs, ATMs, and CAMs) were cultured as described in sections 2.3.2. AGS-GR cells were maintained as previously described in section 2.3.3. Conditioned media were prepared from, OE33 cells, and myofibroblasts as described in section 2.4.

5.2.2 *Western blot analysis*

Western blot analysis was used to determine the expression of MMP-7 and to characterise the possible role of myofibroblasts in modulating MMP-7 metabolism. Routinely the MMP-7 rabbit polyclonal antibody L522 was used, but in some experiments two other MMP-7 antibodies (L523, L524) were compared. Some experiments also employed antibodies to MMP-1 (BAF 901), MMP-3 (BAF 513) and GAPDH as described in section 2.6.

5.2.3 *Indirect ELISA*

The concentration of MMP-7 in media from OE cells and myofibroblasts was determined by ELISA using antibody L522 that detects pro-MMP-7, MMP-7 and its metabolic cleavage products as described in section 2.7.

5.2.4 *Immunocytochemistry*

Expression of pro-MMP-7 in CAMs was confirmed using immunocytochemistry as described in section 2.10. Images were taken as described in section 2.10.

5.2.5 *Cell migration*

The effect of MMP-7 on CAMs migration was investigated using Boyden chamber assays as described in section 2.11. Inserts were seeded with 25,000 cells in 500µl SF and the bottom well contained either 750µl OE33 CM or OE33 CM media containing MMP-7 neutralising antibody (mouse monoclonal anti-human MMP-7, 1:200). The effect of CM from OE33 cells overexpressing MMP-7 was studied on CAMs cell migration after 24 hours. Cell staining and counting were performed as described in section 2.11. Data are represented as mean of cells migrating in 5 fields/well in triplicate ± S.E.M and significance was determined by ANOVA and considered significant at $p<0.05$.

5.3 Results

5.3.1 *OE33 but not OE19 cells express and secrete abundant of MMP-7*

In order to establish the relative expression and secretion of MMP-7 in OAC cell lines we compared OE33, OE21 and OE19 cells using Western blot and indirect ELISA. Western blot analysis of media and cell extracts from OE19, OE21 and OE33 cells using antibody L522 revealed that the most abundant expression was detected in OE33 cells and media. The major band in OE33 cell extracts and media was at 28 kDa corresponding to pro-MMP-7; there was a minor band corresponding to MMP-7 at 18kDa. Western blot analysis also clearly showed that the secretion of pro-MMP-7 or MMP-7 was insensitive to PMA in OE33 cells (Figure 5.1A, B). In contrast, cell extracts from OE19 cells showed either no, or barely, detectable expression of pro-MMP-7 or MMP-7 and there was no evidence of OE19 cell secretion of pro-MMP-7 or MMP-7. To further validate the findings, individual pairwise comparisons were then made of MMP-7 secretion measured by ELISA of media from OE33 and OE19 cells. The data confirmed Western blot results: the mean MMP-7 concentrations in OE33 cell media was 8 pmol/ml, there was a slight decrease with PMA, and MMP-7 was undetectable in OE19 cell media (Figure 5.1C). Interestingly, there was no detectable pro-MMP-7 or MMP-7 in OE21 cell media, although OE21 cell extracts exhibited low expression of pro-MMP-7 with evidence of increased expression in response to PMA stimulation (Figure 5.1D, E).

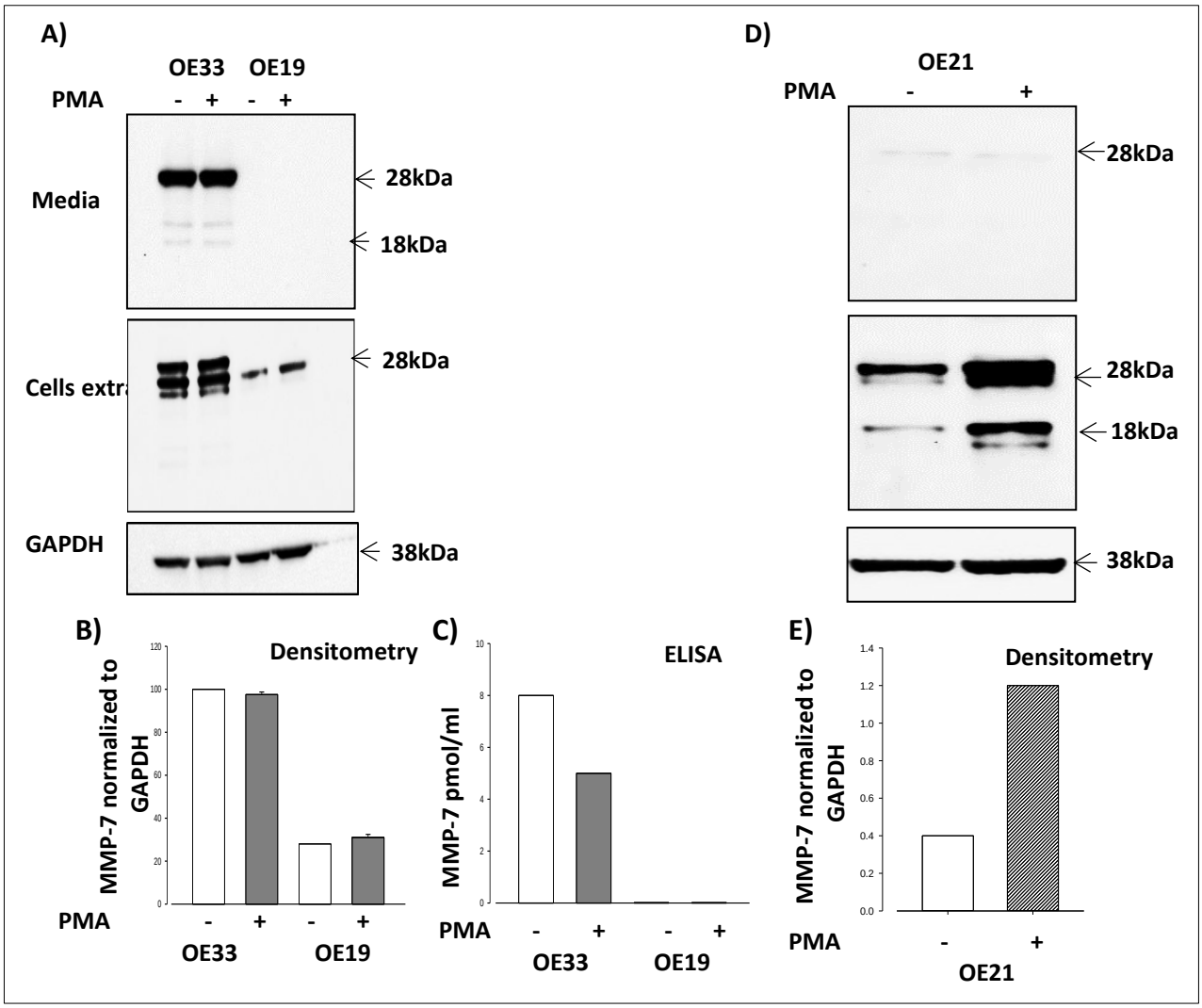

Figure 5.1: OE33 but not OE19 or OE21 cells secrete pro-MMP-7. A) Representative Western blots revealed that OE33 cells secrete high basal pro-MMP-7 compared with OE19 cells; there is no effect of PMA (100nM) on pro-MMP-7 secretion in OE33 cells, but with evidence of PMA effect on MMP-7 expression in OE19 cells line. B) Quantitative analysis by densitometry (n=4) of pro-MMP-7 abundance in OE33 and OE19 cells extract after normalizing with GAPDH (lower panels); note pro-MMP-7at 28kDa and MMP-7 at18kDa. Vertical bars, ± SEM. C) ELISA indicates increased MMP-7 in OE33 cells media compared to OE19 cell media where MMP-7 was below the limit of detection. D, E) OE21 cells exhibited low expression of pro-MMP-7 with clear stimulation by PMA but no evidence of secretion.

5.3.2 *Antibodies L522, 523, 524 react with pro-MMP-7 and MMP-7 in Western blot*.

To identify the optimum antibody for future work, three rabbit polyclonal antibodies that had recently been generated in the laboratory were compared using Western blotting. As part of the same experiment, to examine thermo-stability of pro-MMP-7 or MMP-7 in OE33 cell media, we collected media at different times (0h, 2h, 4h) after incubation at different temperatures (4°C, 37°C), and probed blots with the three antibodies (L522, L523, L524). Western blot data showed that all three antibodies revealed a band corresponding to pro-MMP-7 at 28 kDa; cleavage products estimated to be between 18-16 kDa corresponding to MMP-7 were clearly evident in the blots probed with L522 and L523, but were barely detectable with L524. These results also show that at 4°C, and 37°C, and at 0h, 2h, and 4h, pro-MMP-7 is stable with a low level of cleaved products detected (Figure 5.2).

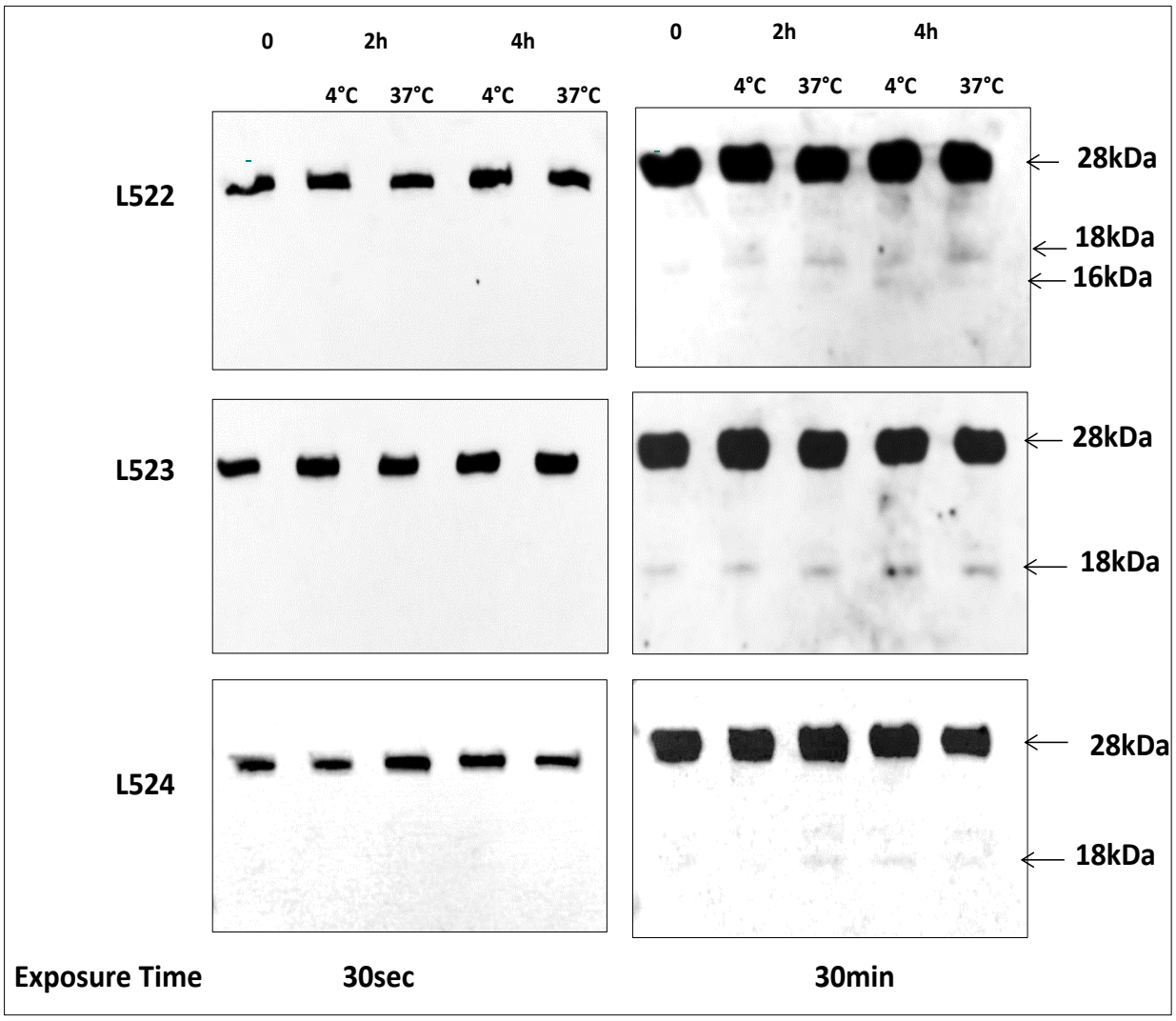

Figure 5.2: L522 is the antibody of choice for detection of MMP-7 related proteins in OE33 cells using Western blot. OE33 cell media was collected at 0h, 2h, or 4h after incubation at 4°C or 37°C. Samples were resolved on 15% gels and blots probed with 1:1000 L522, L523 or L524. At short exposures (left, 30 sec) in all cases there was a band corresponding to pro-MMP-7 at 28kDa. With prolonged exposure (right, 30 min) there are minor bands corresponding to MMP-7 (18 kDa) and a possible degradation product (16kDa); these are most evident with L522 and L523, and barely detectable with L524. Pro-MMP-7 relatively stable at both indicated time and temperature.

5.3.3 *pro-MMP-7 is relatively stable after secretion in media*

In view of the difficulty of detecting MMP-7 related proteins in OE19 and OE21 cells we then asked whether these cells degraded pro-MMP-7. Thus, we carried out a stability experiment using recombinant human pro-MMP-7 incubated with OE19 and OE21 cells for 2 h. Western blot did not reveal a significant change in active MMP-7 or of cleaved products. Therefore, pro-MMP-7 appears to be relatively stable in the media of these cells and failure to detect wild type MMP-7 in the medium is unlikely to be due to rapid degradation (Figure 5.3).

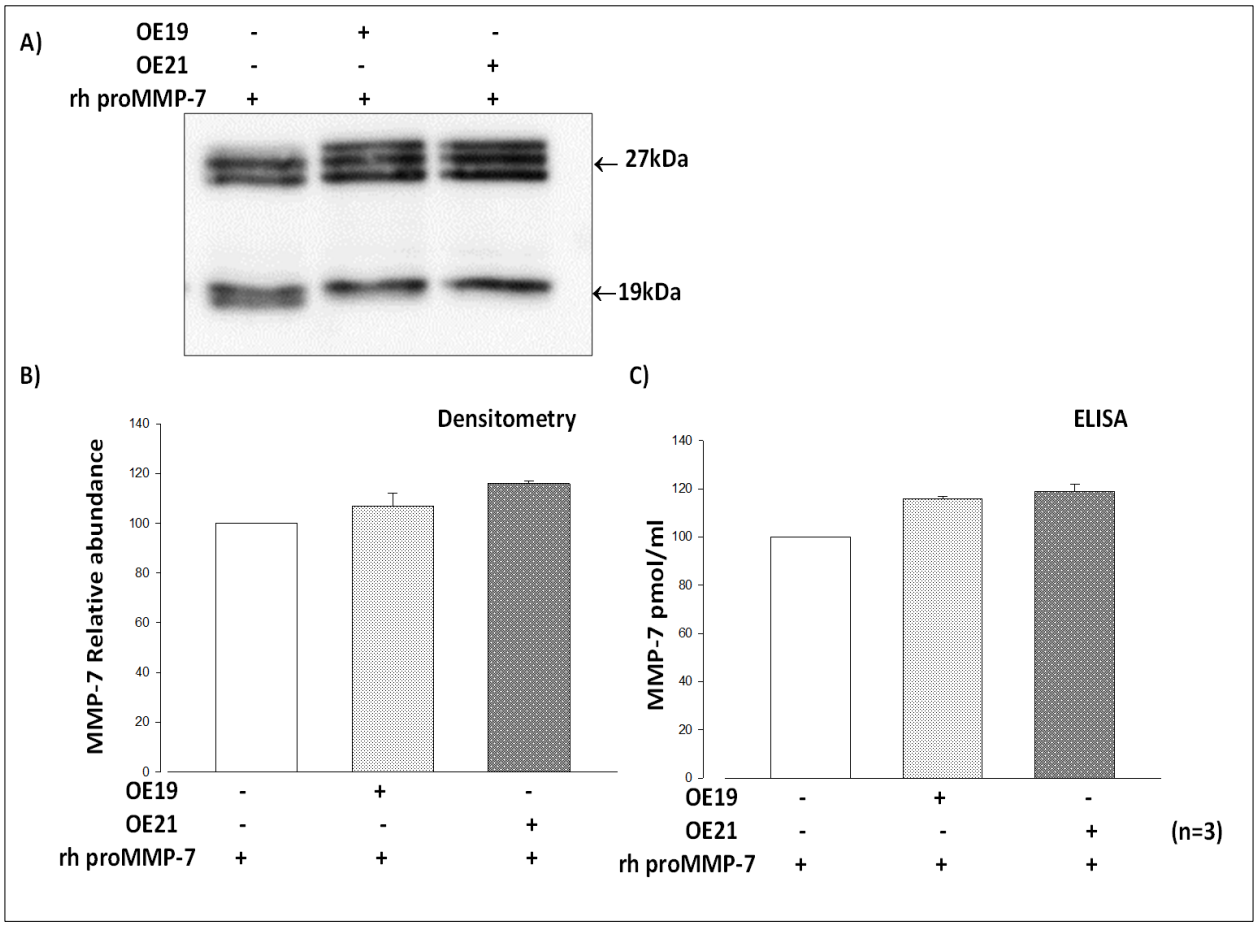

Figure 5.3: pro-MMP-7 stability in media. A) Incubation of 36pmol/ml of rh-pro-MMP-7 with OE19 and OE21 cells for 2 h: representative Western using L522 revealed pro-and active MMP-7, but no cleaved active products up to 30 min exposure. B) Densitometry analysis of 27 kDa band (pro-MMP-7) relative to control sample C) ELISA data demonstrated that there is no significant change in MMP-7 in media, or in presence of cells. The mean of three experiments is shown.

5.3.4 *MMP-7 abundance in OE33 cell media increases with incubation time*

Validation studies using ELISA were undertaken to confirm the observation MMP-7 accumulates in OE33 cell media with incubation time. When OE33 cell media was analysed by ELISA at 4h, 6h, and 20 h there was a progressive increase in MMP-7 (Figure 5.4).

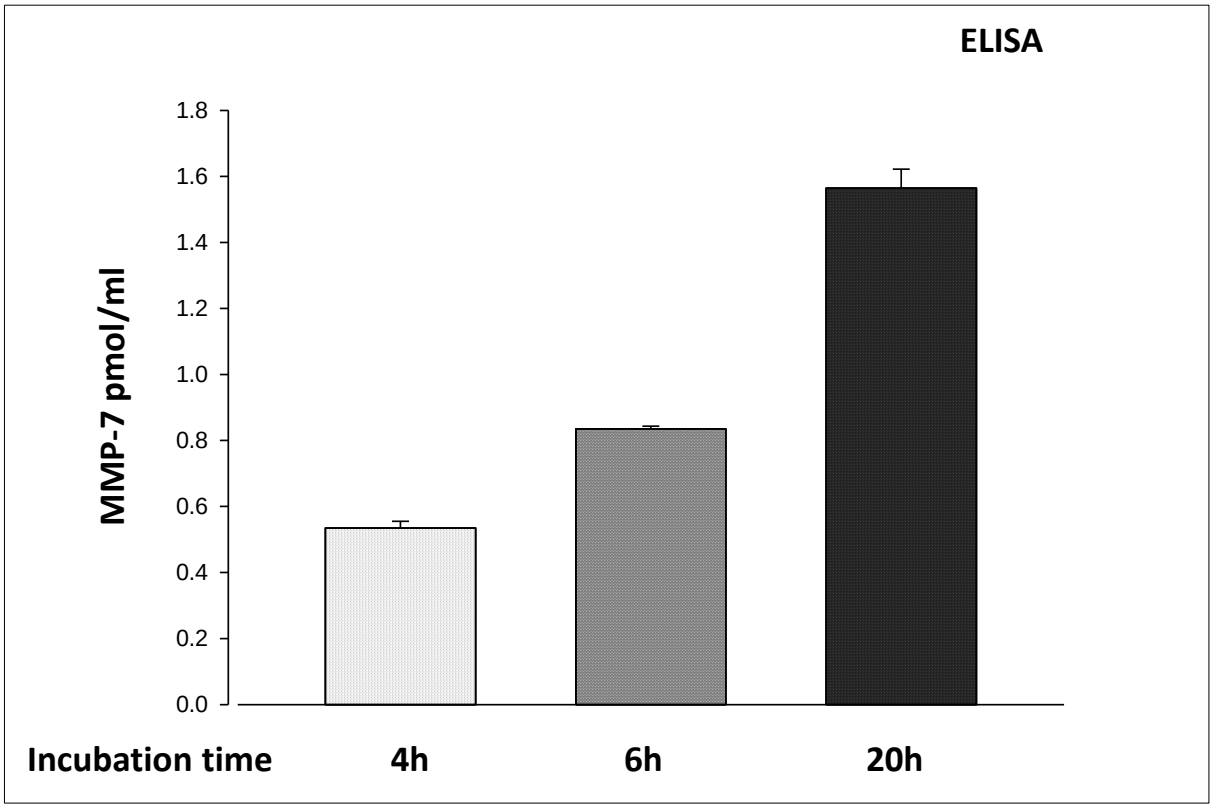

Figure 5.4: Increased MMP-7 secretion with incubation time. Indirect ELISA revealed that OE33 cell media showed a progressive increase in MMP-7 activity with time (n=3).

5.3.5 *Gastrin has no significant effect on pro-MMP-7 in OE33-GR cell media*

We next tested the specific hypothesis that gastrin stimulates MMP-7 expression. Thus medium was examined from OE33 and OE33-GR cells (expressing CCK-2 receptors) treated with G17 (10nM) or PMA (100nM). In unstimulated samples, L522 revealed pro-MMP-7 at 28 kDa, and a minor band at approximately 18 kDa. Neither G17 nor PMA influenced relative abundance of the two bands in media from either cell type. To further explore the possible effects of gastrin on the expression of other MMPs, with the same samples we probed the blots with antibodies to MMP-1 and MMP-3. The results indicated that while PMA stimulated the abundance of pro-MMP-1, and to a lesser extent pro-MMP-3, in the media of both OE33 and OE33-GR cells, gastrin had no effect in either case (Figure 5.5).

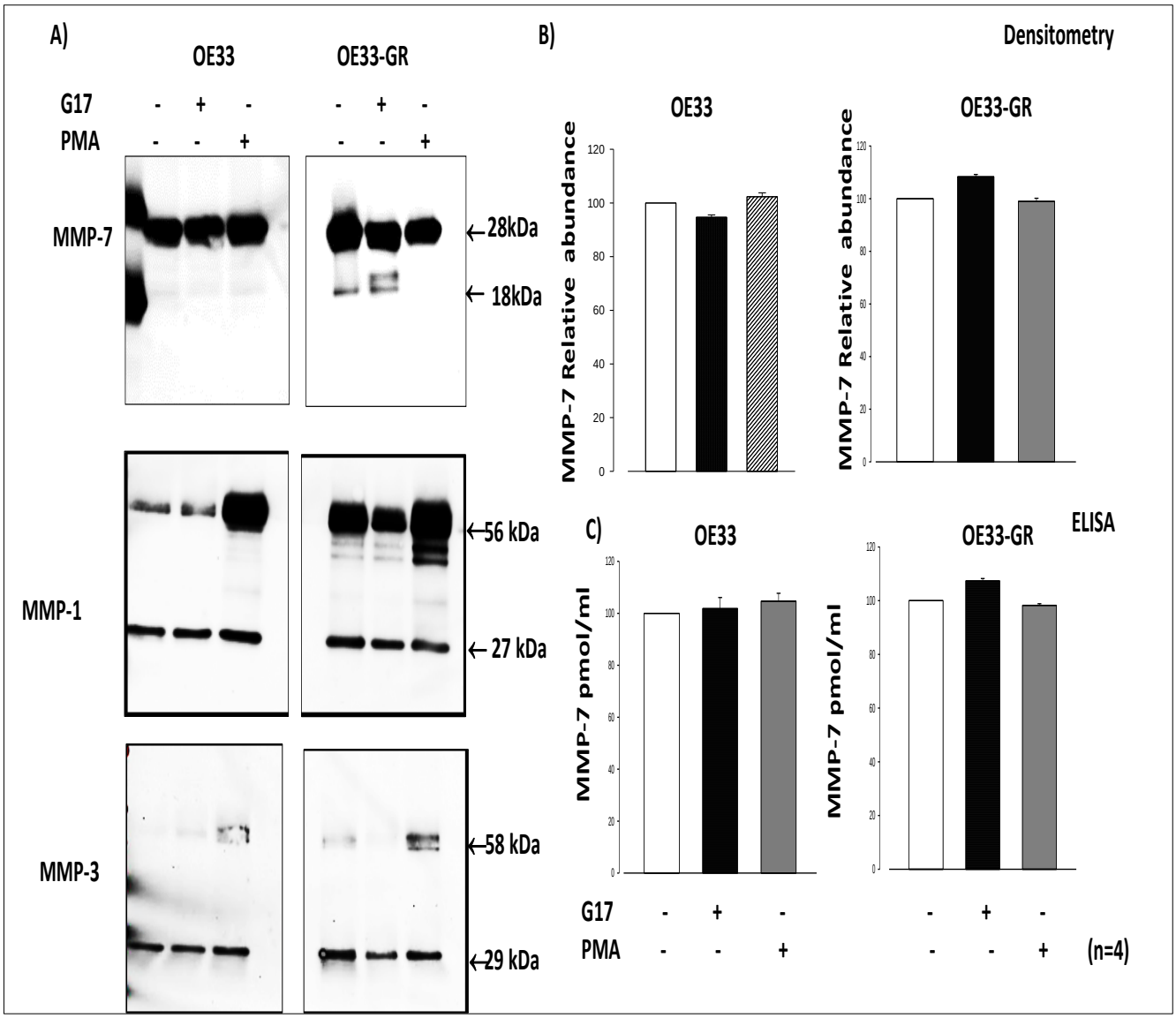

Figure 5.5: pro-MMP-7, pro-MMP-1 and pro-MMP-3 in OE33 and OE33-GR cell media treated with gastrin and PMA. A) Western blot analysis of pro-MMP-7, pro-MMP-1, and pro-MMP-3 in media from OE33 and OE33-GR cells. B) Quantitative analysis of MMP-7 abundance in OE33 and OE33-GR cell media by densitometry (n=4). C) ELISA results confirmed that MMP-7 secretion was not stimulated by either G17 or PMA.

5.3.6 *Non-classical forms of gastrin do not influence pro-MMP-7 in OE33 cell media*

We then compared the effect of G17 and non-classical gastrins namely progastrin, Gly-extended gastrin-17 (G17-Gly) and C-terminal flanking peptide extended gastrin (G17-CFP) in OE33 cells. Western blot analysis revealed that there was a major band corresponding to pro-MMP-7 at 28kDa in both media and cell extracts, but there was no association between pro-MMP-7 abundance and G17 at 0.1, 1, and 10nM concentration. Moreover, it was confirmed by ELISA that in response to concentrations of G17 in the range of 0.1 nM to 10 nM, there was no change in MMP-7 release in OE33 cells (Figure 5.6 A). We then analysed the impact of non-classical gastrins on pro-MMP-7 secretion. In different experiments, cells were treated with G17-Gly, progastrin, and G17-CFP at 1 and 10nM concentration. Western blot of OE33 cell extracts and media showed a major band corresponding to pro-MMP-7 at 28 kDa; there was no correlation between pro-MMP-7 abundance and non-classical gastrins (Gly-extended gastrins, G17-CFP and progastrin) (Figure 5.6B, C).

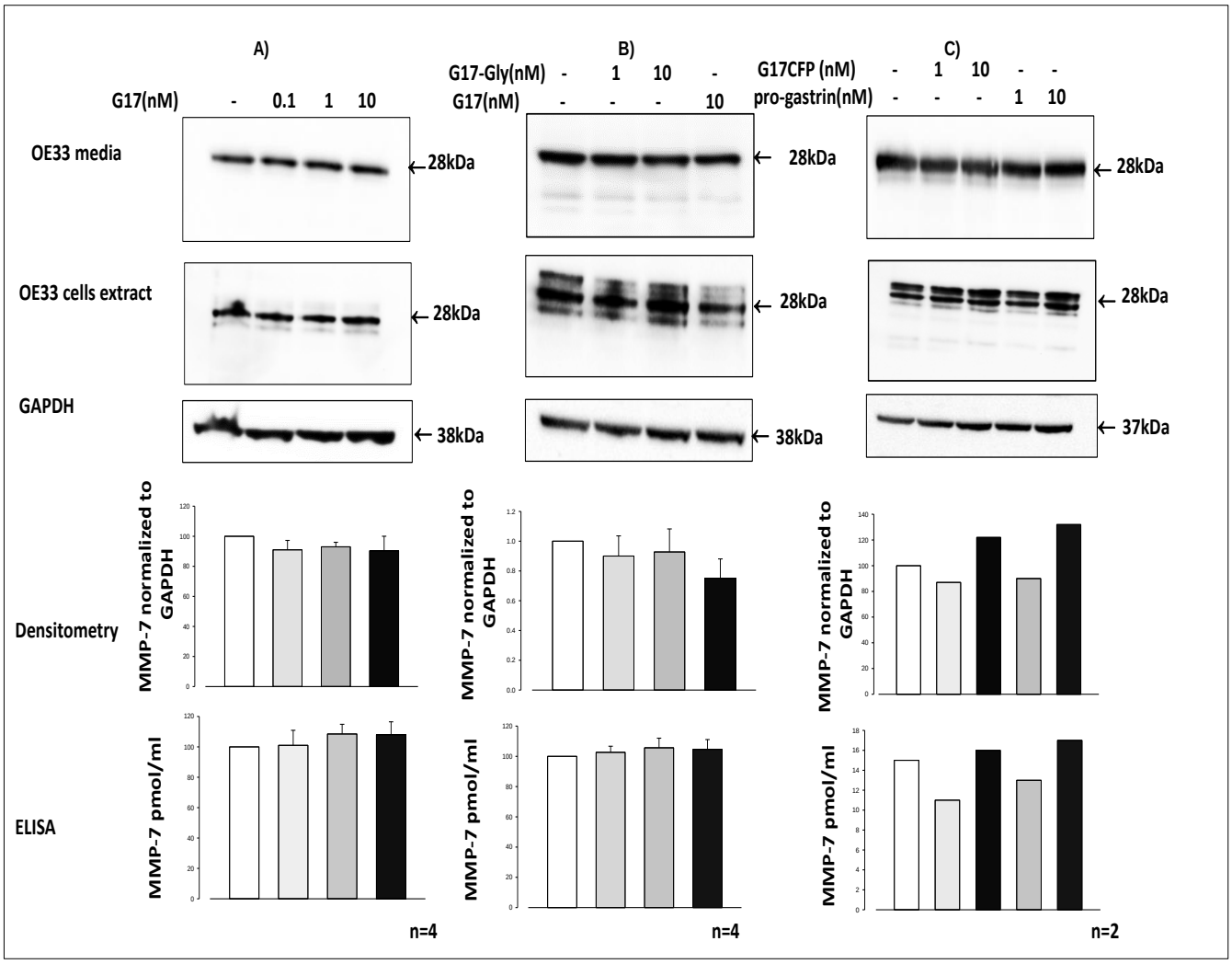

Figure 5.6: Expression of pro-MMP-7 in OE33 cells is insensitive to various forms of gastrin. A) Amidated gastrin (G17) (0.1, 1, and 10 nM) did not influence pro-MMP-7 abundance in OE33-GR cells or media, while there was no difference in cellular GAPDH. B) Gly-extended G17 (G17-Gly, 1 and 10 nM) had no significant effect on pro-MMP-7 abundance in media or cells of OE33 cells; there was no change in GAPDH abundance in cell extracts. C) G17 extended to include the C-terminal flanking peptide of progastrin (G17-CFP, 1 and 10 nM) and intact progastrin had no effect on pro-MMP-7 abundance in OE33 media or cells. No change in GAPDH confirmed equal loading. ELISA data in all cases were in parallel with Western results (bottom panels).

5.3.7 *Increased expression of pro-MMP-1 and pro-MMP-3, but not pro-MMP-7, in AGS-GR cells in response to G17, and PMA*

To establish whether gastrin is capable of stimulating secretion of MMPs, a similar approach to that used for OE33-GR cells was then applied to AGS-GR cells (i.e. used as positive control). AGS-GR cells treated with G17 (10nM) and PMA (100nM) were probed with MMP-7, MMP-1 and MMP-3 antibodies. Western analysis of media showed greater release of pro-MMP-1 and pro-MMP-3 at 58 kDa, and 56 kDa respectively in response to G17 and PMA. However, AGS-GR cells media showed either no, or barely, detectable secretion of pro-MMP-7. The densitometry analysis of Western blots of OE33-GR and AGS-GR cells media confirmed significantly increased pro-MMP-1 secretion in response to G17 and PMA in AGS-GR cells compared to OE33-GR cells which did not respond (Figure 5.7).

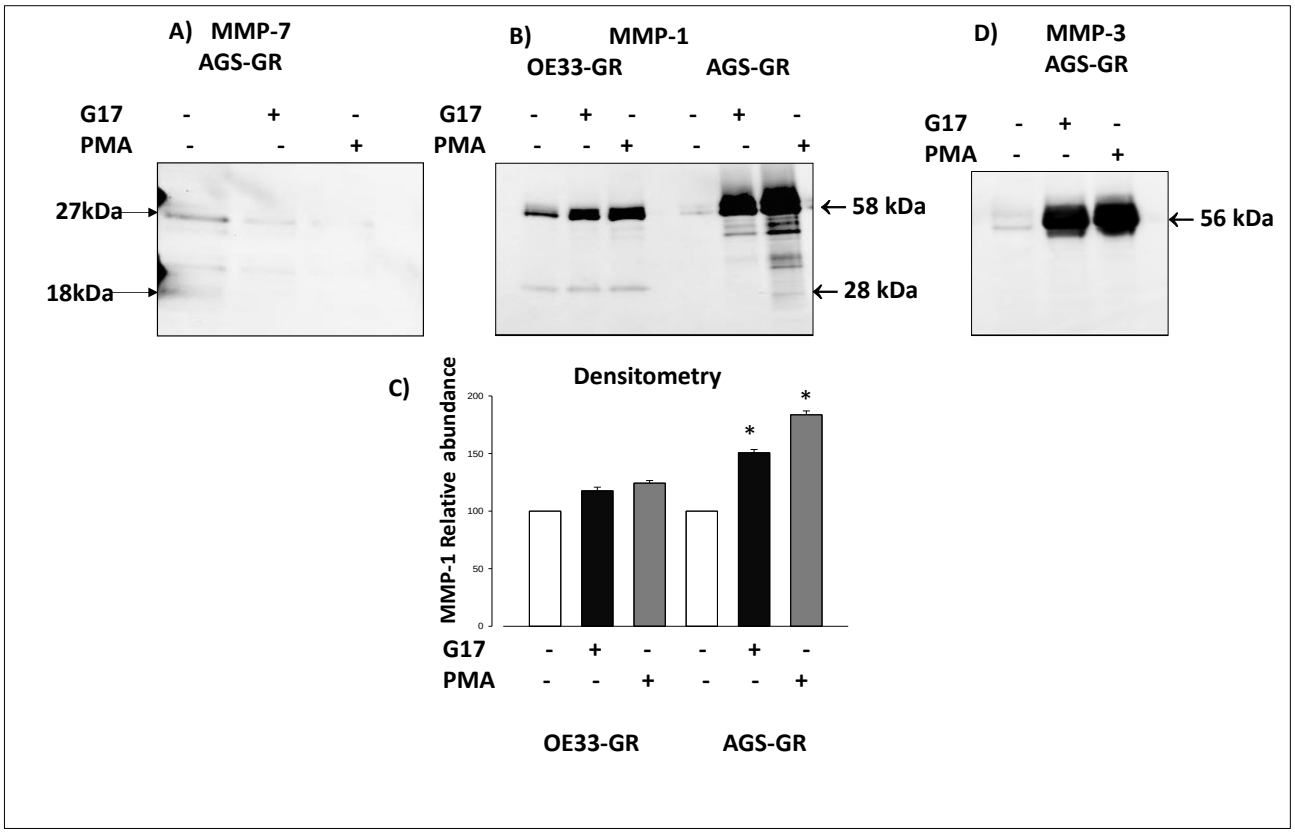

Figure 5.7: Gastrin and PMA enhance pro-MMP-1 and pro- MMP-3 but not pro-MMP-7 secretion in AGS-GR media. A) Western blot data revealed AGS-GR cells media produce barely detectable band of pro-MMP-7 at 27 kDa. B) Representative Western blot analysis of pro-MMP-1 in OE33-GR, and AGS-GR cells treated with G17 (10 nM), and PMA (100nM). C) Quantification of Western blots showed an 2–fold significant increase in pro-MMP-1 in AGS-GR cells media treated with G17 and PMA (n=3) compared with MMP-1 release in OE33-GR cells not responded to G17, and PMA $p<0.05$, ANOVA; vertical bars, ±SEM. D) Western data showed that pro-MMP-3 secretion was sensitive to G17 and PMA stimulation of AGS-GR cells.

5.3.8 *BFA inhibits MMP-7 secretion in OE33-GR cells media*

We hypothesized that brefeldin A (BFA) would inhibit MMP-7 transport from the endoplasmic reticulum to the Golgi apparatus thereby preventing formation of transport vesicles and inhibiting basal secretion, and we asked whether it might therefore be possible to show a small stimulatory effect of gastrin by stimulating exocytosis of preformed vesicles in the absence of high basal secretion. OE33-GR cells were therefore treated with BFA, G17 and the combination. The Western blot analysis clearly showed that BFA promptly inhibited basal secretion of pro-MMP-7 as expected, and this was accompanied by increased intracellular pro-MMP-7 attributable to accumulation of pro-MMP-7 arrested in the endoplasmic reticulum. There was no evoked secretion of pro-MMP-7 in response to G17 (Figure 5.8A). ELISA data confirmed the Western blot results that BFA inhibited pro-MMP-7 secretion in OE33-GR cells media (Figure 5.8C).

As shown in (Figure 5.8B) pro-MMP-1 (as a reference), demonstrated that BFA increased intracellular content of pro-MMP-1 in G17/BFA treated samples, and inhibited pro-MMP-1 basal secretion in OE33-GR cells media, gastrin had no effect.

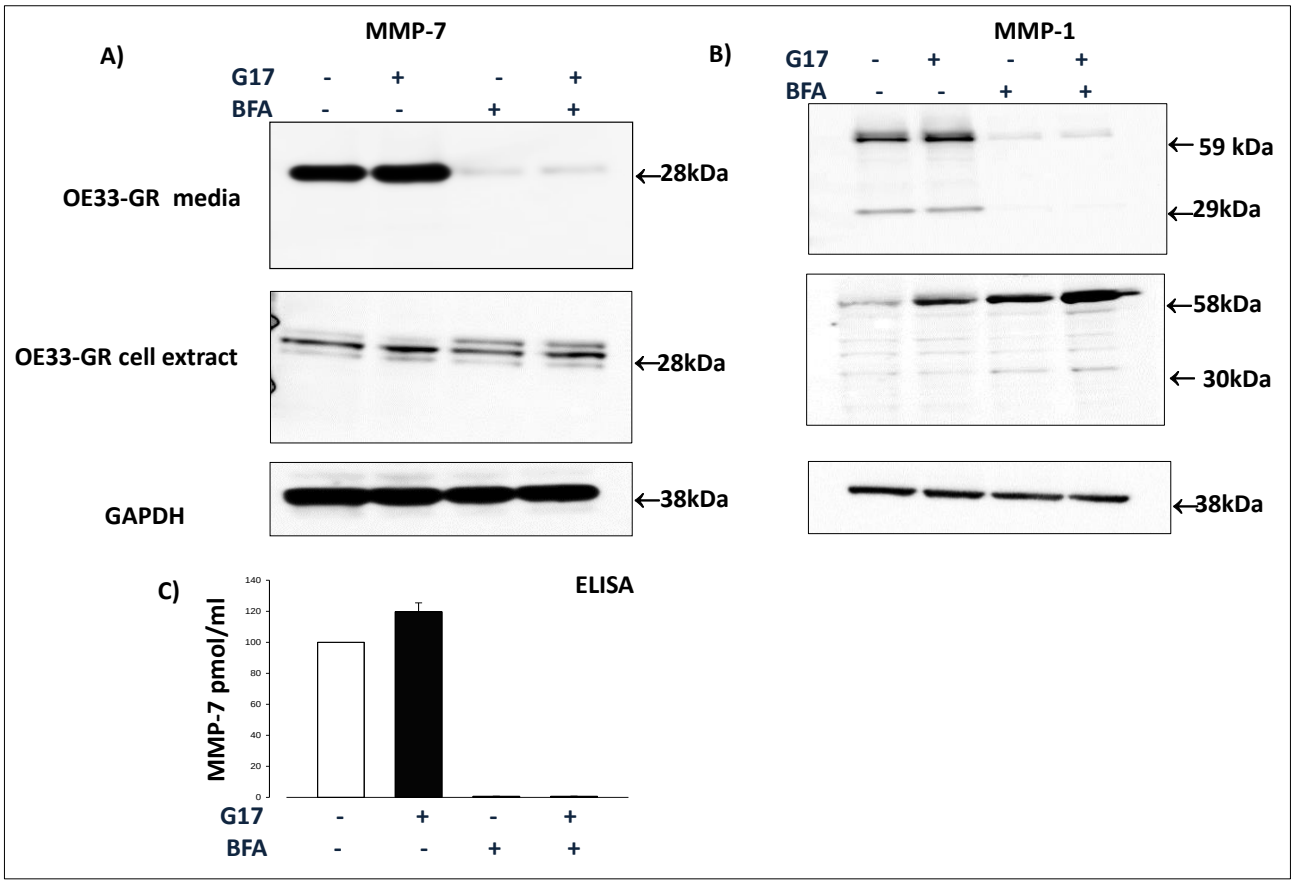

Figure 5.8: Effects of BFA treatment on pro-MMP-7 and pro-MMP-1 secretion in OE33-GR cells. A) Representative Western blots showing that BFA (10 µg/ml) inhibited pro-MMP-7 secretion in media of OE33-GR cells in response to G17 (10nM) treatment for 16 h. B) Similarly Western blot data demonstrated inhibition of pro-MMP-1 in OE33-GR cell media in BFA treated samples, with increase the intracellular contents in response to G17/BFA. C) ELISA data confirmed Western results that BFA (10 µg/ml) inhibits pro-MMP-7 release in media of OE33-GR cells by arresting it is secretion.

5.3.9 *Activation of pro-MMP-7 by OE33-GR cells, and BFA, and temperature-sensitive secretory responses*

We performed stop-flow experiments by using the ability of BFA to arrest secretion by blocking transport through the early secretory pathway, to determine the capacity of OE33-GR cells for pro-MMP-7 activation. Since there is a temperature sensitive step that is distal to the site of action of BFA (Varro et al., 1996), OE33-GR cells were incubated at 37°C and media were collected at 0h, 2h, 4h and 6h. The Western blot data clearly revealed that there was a little further conversion of pro-MMP-7 over the period of 6 h. The precursor form of MMP-7 is therefore relatively stable in OE33-GR cell media up to 6h incubation (Figure 5.9A). Other blots were probed for pro-MMP-1 and demonstrated that there is conversion of pro-MMP-1 to smaller bands corresponding to active MMP-1 at 28 kDa (Figure 5.9B). Moreover, a relatively abundant form of pro-MMP-1 was found in media and cells after 4h and 6 h of G17/BFA treatment. Collectively, BFA inhibited secretion of pro-MMP-7 but in delayed addition experiments had no effect on the activity of previously secreted pro-MMP-7, again confirming that in the bulk phase of the cell media there is little or no conversion of pro-MMP-7 to active MMP-7.

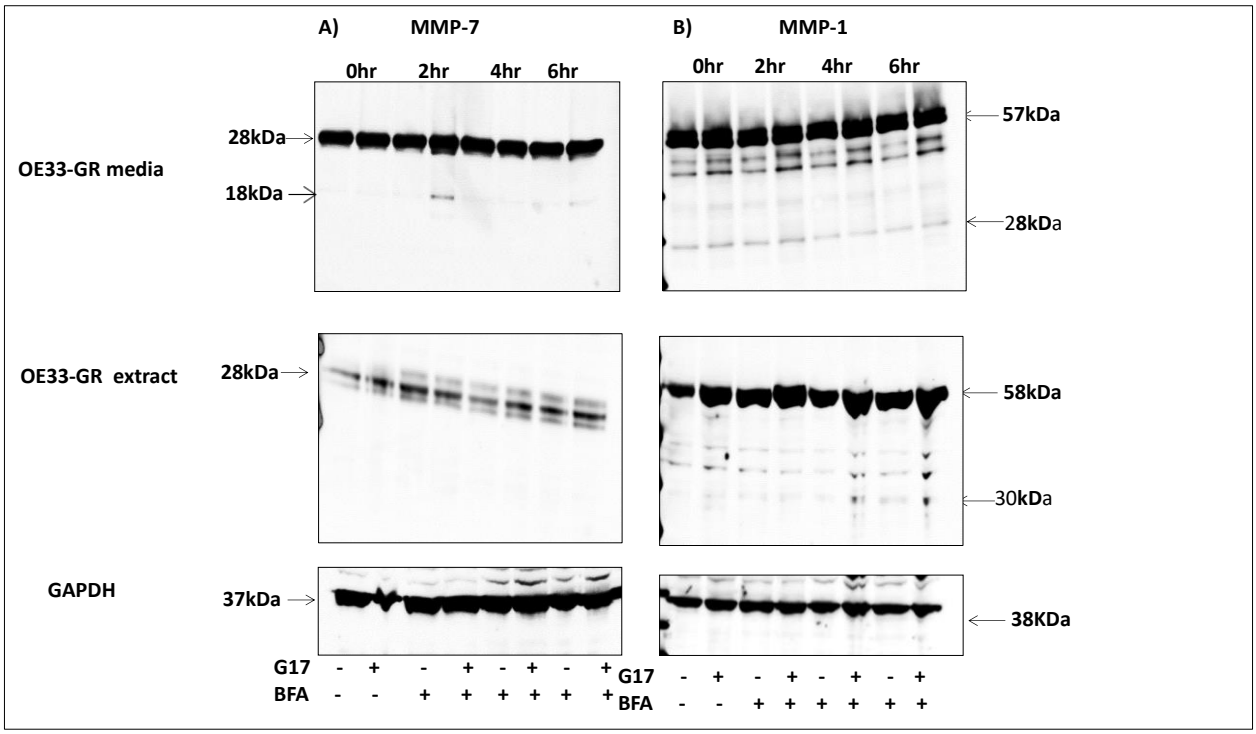

Figure 5.9: Capacity of OE33-GR cells for pro-MMP-7 activation. A) Western blot analysis of pro-MMP-7 in OE33-GR cells showed that when BFA is added to cells already treated with G17 for 16 h the pattern of bands in media and cells after a further 6 h (i.e., at 22 h) is similar to that at 16 h, suggesting a low capacity of OE33-GR cells for pro-MMP-7 activation or degradation. B) Western blots of MMP-1 in OE33-GR cells showed a major band corresponding to pro-MMP-1 at 57kDa and a relatively minor band corresponding to the active form at 28kDa, and 30kDa in media, and cells respectively. Thus OE33-GR cells have some capacity to activate pro-MMP-1.

5.3.10 *Pro-MMP-7 is stable in presence of NTMs and CAMs, but MMP-7 is partially cleaved in the presence of myofibroblasts*

To determine the influence of myofibroblasts on pro-MMP-7 and in modulating MMP-7 secretion and metabolism, OE33-GR cells were incubated with conditioned medium from myofibroblasts (NTMs, CAMs); in other experiments myofibroblasts (NTMs, CAMs) were co-cultured with OE33-GR cells and the effect of myofibroblasts on pro-MMP-7 degradation was determined by Western blot and ELISA. The results with conditioned medium (Figure 5.10A) from myofibroblasts were similar to those obtained in co-culture experiments (Figure 5.10B). In particular, there was no activation or degradation of pro-MMP-7 in the presence of either NTMs or CAMs.

To analyse possible MMP-7 cleavage in the presence of myofibroblasts, and to assess whether cleavage of MMP-7 is specific to a cancerous or pre-neoplastic microenvironment, 2 µg/ml recombinant human MMP-7 was incubated with different myofibroblasts (NTMs, ATMs, oesophageal and gastric CAMs) for 2hr. Western blots showed that in the presence of myofibroblasts MMP-7 is cleaved to bands with molecular weights of approximately 14kDa and 11kDa, but the fragments were less than 10% of total over 2 h (Figure 5.10C, D). Moreover, ELISA data confirmed Western results, indicating the limited role of myofibroblasts in MMP-7 metabolisms (Figure 5.10E).

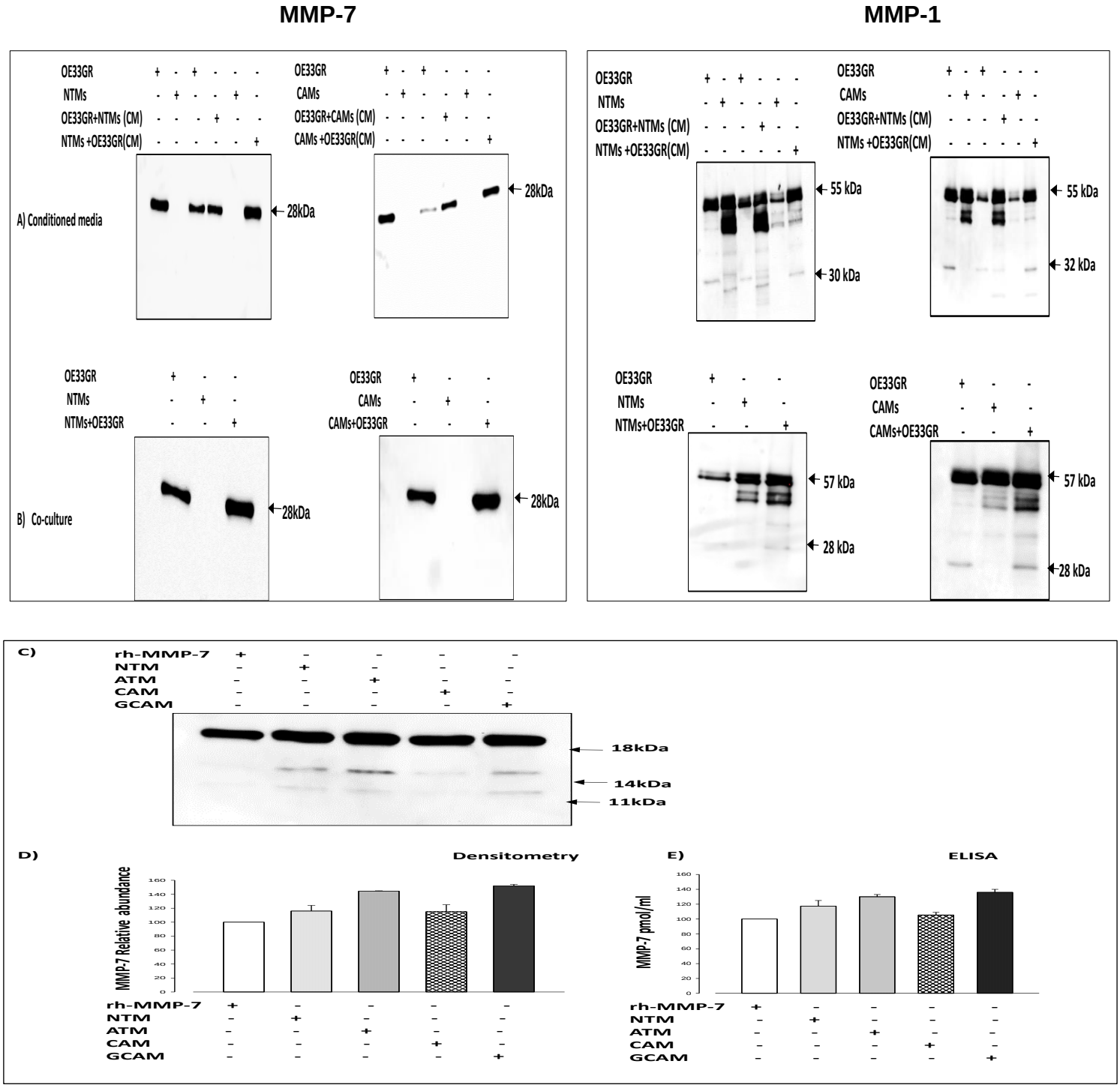

Figure 5.10: There are no cleaved pro-MMP-7 products after incubation with CAMs and NTMs, but MMP-7 is partially cleaved in the presence of myofibroblasts. Upper panel: A) Western blots analysis of MMP-7, and MMP-1 in media from of OE33-GR cells treated with CM from NTM compared to the corresponding CAMs CM. B) Representative Western images of MMP-7, and MMP-1 in OE33GR cells co-cultured with NTM, and OE33GR cells co-cultured CAMs. Lower panel: A) Incubation of rh-mmp-7 (2 µg/ml) with myofibroblasts for 2 hrs, and the blot was probed with L522. Representative Western image revealed that high level of full length at 18kDa and cleaved rh-MMP-7 at14kDa, and 11 kDa, after exposure to myofibroblasts (NTM, ATM, CAM, and G CAM). B) Graph of total MMP-7 concentration after cell treatment with rh-MMP-7(n=4). C) ELISA data confirmed Western results.

5.3.11 *pro-MMP-7 is unexpectedly expressed by CAMs, but is not secreted*

On the basis of our data described in Chapter 3 it was considered necessary to examine the expression of MMP-7 in myofibroblasts. ELISA and Western blot analysis of cell extracts from CAMs showed pro-MMP-7 expression in unstimulated CAMs (SF) but neither PMA nor IGFII induced changes in expression (Figure 5.11A, D). There was undetectable pro-MMP-7 in media. Disruption of Golgi trafficking and function using BFA showed that pro-MMP-7 accumulated in treated cells and remained virtually undetectable in media (Figure 5.11B, E). Expression of pro-MMP-7 in myofibroblasts was further examined in three different oesophageal adenocarcinoma-derived CAMs (CAM1, CAM2, and CAM3). As shown in Figure 5.11 C cell extracts express pro-MMP-7 at 28 kDa, in contrast media showed undetectable levels of pro-MMP-7; ELISA data confirmed Western blot results (Figure 5.11 F). The expression of pro-MMP-7 in CAMs was then validated by immunocytochemistry by using the same antibody that we used in IHC in Chapter 3. The immunocytochemical studies revealed punctuate staining of MMP-7 in CAMs, compatible with localization to secretory vesicles (Figure 5.11G).

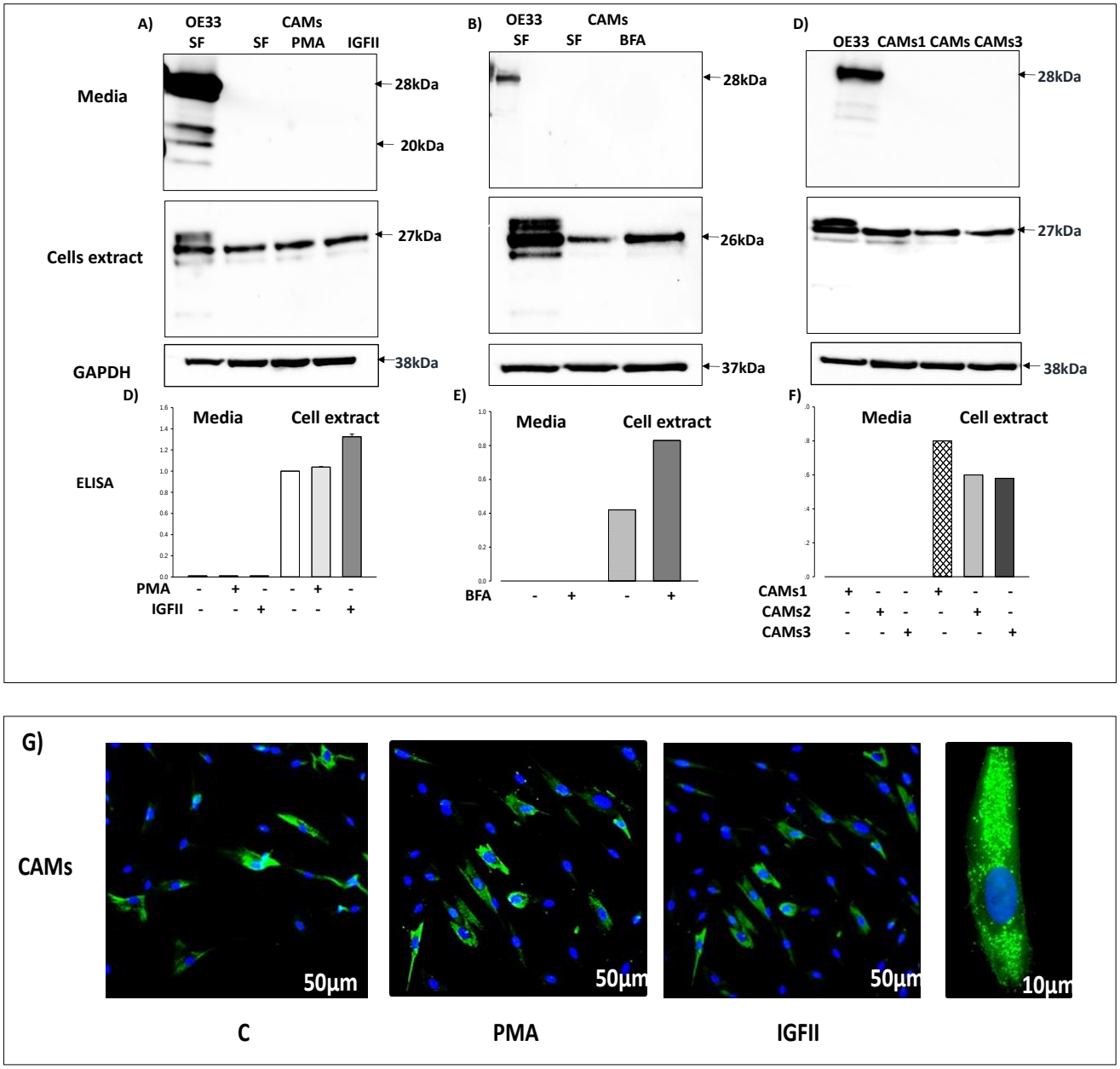

Figure 5.11: Expression of MMP-7 in myofibroblasts. A) Representative Western blot showed that unstimulated CAMs cell extract (c) but not media expressed a band corresponding to proMMP-7 at 28 kDa, there was no effect of either PMA or IGF-II on proMMP-7 release in CAMs or OE33 cells run as a positive control. B) In cell extracts of myofibroblasts treated with BFA was an increase in intracellular pro-MMP-7. C) In Western blots cell extracts of three different CAMs lines (CAMs 1, 2 and 3) there was a band corresponding to proMMP-7 at 28 kDa. OE33 cells run as a positive control in the three Western blot. D) ELISA data for media samples indicated that CAMs fail to secrete MMP-7. However, cell extracts of CAMs in all cases exhibited MMP-7 that was not influenced by PMA and only marginally by IGF-II. E) ELISA showed intracellular in MMP-7 release in response to BFA in CAMs cell extract samples but not CAMs cell media. F) ELISA data confirmed Western results three CAMs express but not secrete MMP-7. G) Immunocytochemistry of MMP-7 in myofibroblasts revealed expression in a high proportion of cells (left), and at a higher power (right) localisation is confirmed to vesicular organelles.

5.3.12 *Conditioned media from OE33 cells enhances CAM migration*

As a final step in characterising the biological relationship between MMP-7 and CAMs, migration studies were performed using OE33 cell CM and MMP-7 neutralising antibody. In Boyden chamber assays, CM from OE33 cells increased CAM migration relative to control (SF medium). The group mean data also showed OE33 cell CM significantly increased CAM migration. Importantly, CAM cell migration in response to CM from OE33 cells was significantly inhibited by MMP-7 neutralising antibody; moreover, the stimulatory effect of OE33 cell CM on CAM migration was significantly inhibited by MMP-7 neutralising antibody (Figure 5.12).

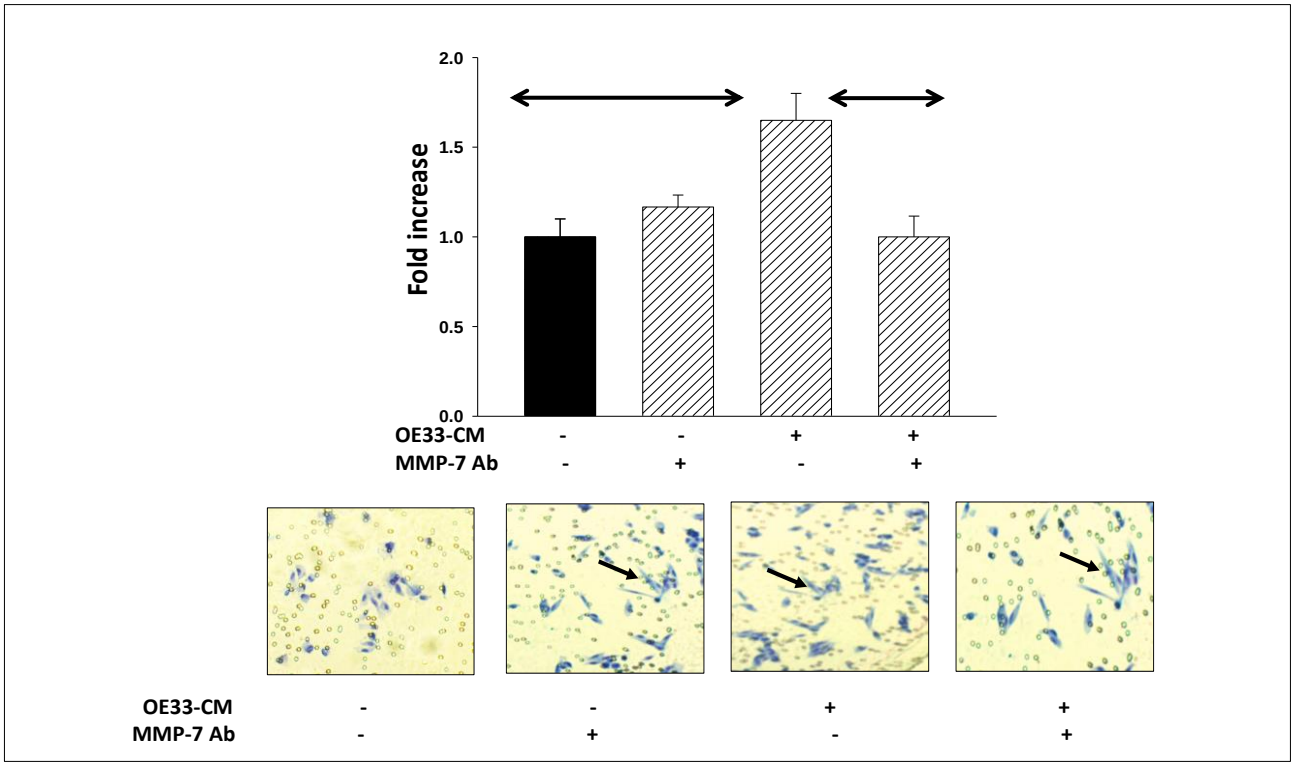

Figure 5.12: OE33 CM significantly increased migration of oesophageal cancer associated myofibroblasts. A) Representative Boyden chamber migration assays of CAM cells treated with CM from OE33 cells. Group mean data of CAMs cell migration in response to OE33 cells CM, vertical bars, and SEM. Horizontal arrows mean $p<0.05$ by ANOVA; Bars, SEM. B) Representative image of migrating CAMs treated with CM from OE33 cells.

5.4 Discussion

Western blot and indirect ELISA applied to an oesophageal adenocarcinoma cell line (OE33 cells) showed high pro-MMP-7 release. In contrast pro-MMP-7 abundance in OE19 and OE21 cells media was below the limit of detection. Cultured myofibroblasts expressed pro-MMP-7 as indicated by immunocytochemistry and Western blot of cell extracts, but we were unable to detect pro-MMP-7 or MMP-7 in myofibroblasts medium. This appears unlikely to be due to degradation of pro-MMP-7 in media. The present data showed gastrins and PMA have no effect on pro-MMP-7 secretion by OE33-GR cells; however, there was activation of pro-MMP-1 by G17 in OE33-GR cells media. There is some degradation of MMP-7 to fragments of 11 and 16 kDa in the presence of NTMs or CAMs in OE33-GR cells.

Abnormal ECM remodelling in cancer is primarily mediated by proteinases such as MMPs which collectively are capable of degrading the entire ECM. Normally, the regulation of MMPs is tightly controlled by the cells which secrete them, and most MMP family members share common cis-elements in their promoter sequences, which allow a tight control of cell-specific expression(Yan and Boyd, 2007). In this study, we investigated MMP-7 expression in oesophageal cancer cell lines in an attempt to provide baseline data for an exploration of the mechanisms regulating its expression. There have been no previous detailed studies of MMP-7 in these cells. However, other MMPs have been studied in OE cells; for example, Keld et. al examined the MMP-1 expression in OE33 and OE21 cells and established the role of ERK-PEA3-MMP-1 axis in MMP-1 regulation. In the same study MMP-7 expression in the same cells was examined for comparison purpose only, they found MMP-7 was only expressed to high levels in OE33 cells, but not in OE21, and Het1A, (a cell line derived from normal oesophageal epithelial tissue) (Keld et al., 2010a). Another

study (Grimm et al., 2010b) examined MMP-1 expression in OE33 cells in order to explore the role of MMP-1 as a preinvasive factor in BO-associated OAC. MMP-7 expression was also examined in human oesophageal squamous carcinoma carcinoma cell line (KYSE170) stably transfected with activin beta A: the authors conclude that activin A enhances MMP-7 activity via the transcription factor AP-1 in an oesophageal squamous cell carcinoma cell line (Yoshinaga et al., 2008). In related studies directed at elucidation of the molecular mechanisms regulating MMP-7 in gastric cancer cell lines (HGC-27 and MGC-803) a role for catecholamine stimulation through an adrenergic signaling pathway was demonstrated using conventional and real-time PCR, and Western blotting (Shi et al., 2010). The results presented here indicate that OE33 cells are a major source of pro-MMP-7, and suggest that MMP-7 in OE33 CM is functionally relevant in that it promotes CAM migration.

Multiple active products are produced from preprogastrin including the well-characterized amidated gastrins acting at the CCK2R (gastrin-CCK_B) receptor and other peptides that may act as growth factors in cancer (Dockray, 2004). Haigh et al showed that CCK2R expression was increased in Barrett's metaplastic tissue compared to normal oesophageal tissue (Haigh et al., 2003b). Recent work has further shown that in a L2-IL-1β mouse model, Barrett's like oesophagus arises from the gastric cardia and is a model for progression of BO to OAC in which CCK2R is expressed by a progenitor cell population (LGR5+ stem cell population). hypergastrinaemia in this model is associated with increased proliferation giving rise to metaplasia and dysplasia (Lee et al., 2016). Additionally, in gastric epithelial cells gastrin stimulates both MMP-1 and MMP-7 expression (Kumar et al., 2015), with potential significance for recruiting and maintaining myofibroblasts populations

(Varro et al., 2007b). In this study, it was therefore hypothesised that gastrin acting on Barrett's oesophageal cells triggers increased MMP-7 expression. The main findings of our study were that the classical or amidated gastrins (G17) had no effect on MMP-7 expression in OE33-GR cells; neither did stimulation of protein kinase C (which is a downstream mediator of CCK2R by PMA. To provide a positive control for studies of the biological action of gastrin we examined AGS-GR cells expressing the CCK-2R because AGS-GR cells are a relatively well studied model of gastrin-stimulated gene expression. The data suggest that while gastrin increases pro-MMP-1 release in the media of these cells via stimulation of CCK-2R, it does not trigger MMP-7 secretion. We also found no evidence that MMP-7 expression and secretion in OE33 cells varied with non-classical gastrins. Thus mechanisms other than hypergastrinaemia are likely to be responsible for the increased MMP-7 that occurs in BO, and OAC.

It is well established that MMPs are generally produced as inactive precursors that are activated by cleavage after secretion (Nagase et al., 2006). Our data clearly revealed stability of pro-MMP-7 after secretion up to 2h. Moreover, recombinant pro-MMP-7 is stable at 4ºC and 37ºC. It is nevertheless known that CAMs and NTMs have very different secretomes, and in the tumour microenvironment, there may be activation of myofibroblasts MMPs by epithelial or cancer-derived proteases (Holmberg et al., 2013a). Moreover, other work in the group has found selective MMP activation occurs in the CAM secretomes even when these cells are cultured in the absence of cancer cells (Holmberg et al., 2013b). There is already evidence that the proliferative responses of myofibroblasts to MMP-7 appear to depend on its proteolytic activity, since it was found that heat-inactivated MMP-7 has no effect (Hemers et al., 2005, Varro et al., 2007a). However, the precise mechanisms

responsible for MMP-7 proteolytic cleavages/ stability are presently unclear and should now be investigated.

In view of the potential importance of myofibroblasts in regulating the tumour microenvironment and promoting cancer cell growth, and given the finding in chapter 3 that MMP-7 might be expressed in myofibroblasts, the biology of MMP-7 in myofibroblasts was studied in three different OAC CAM lines. Our data identified expression in cell extracts yet no proof of MMP-7 release into the medium. It is conceivable that MMP-7 expression in myofibroblasts could be accompanied by rapid degradation to peptide fragments that were undetectable by antibody L522. However, L522 detects total MMP-7 (pro-, active and cleaved N-terminal fragments) suggesting that degradation is unlikely. Additionally, the results presented here showed a limited role for myofibroblasts in the metabolism of recombinant MMP-7.

The functional significance of MMP-7 released by OE33 cells was established by showing OE33 CM increased CAM migration and neutralising antibody to MMP-7 decreased CM-stimulated CAM migration. This is in broad agreement with the earlier observation that MMP-7 stimulated the proliferation and migration of human colonic myofibroblasts (Hemers et al., 2005). Hence, the data from multiple models supports the idea of functional roles for MMP-7 in driving cell migration.

5.5 Conclusions

1. OE33, but not OE19, cells secrete pro-MMP-7. Moreover, pro-MMP-7 accumulated in media with time. OE21 cells express but do not secrete pro-MMP-7

2. Novel polyclonal antibodies reacting at the N-terminus of MMP-7 are informative in Western blots and ELISA.

3. Gastrins (G17-CFP, progastrin, G-Gly and G17) have no effect on MMP-7 expression and secretion by OE33 and OE33-GR cells. Moreover, MMP-7 secretion in OE33-GR cells is insensitive to PMA (PKC stimulation).

4. Pro-MMP-7 is relatively stable in media. Stromal cells such as myofibroblasts play limited roles in the metabolism of MMP-7.

5. Secreted MMP-7 may modify the tumour microenvironment by stimulating stromal cell migration.

CHAPTER 6

PI3KINASE/AKT SIGNALLING PATHWAY CONTRIBUTES TO MMP-7 UP-REGULATION IN OE33 CELLS

6.1 Introduction

Signalling pathways in cancer cells may be unregulated and resistant to feedback inhibition, usually as a consequence of sustained activation of their components (Keld and Ang, 2011). Two key signalling pathways in oesophageal adenocarcinoma are the MAP kinase and PI3Kinase pathways. For instance, the MAPK pathway is active in 60% of oesophageal adenocarcinomas (Keld et al., 2010b). Moreover, the genes encoding the catalytic subunit of PI3Kinase and Akt are amplified in a wide range of tumour types, affecting 30–50% of malignancies such as digestive tract, breast, endometrial, lung and thyroid (Vivanco and Sawyers, 2002, Workman et al., 2006, Clarke and Workman, 2012).

Components of the PI3Kinase pathway are known to be up-regulated in oesophageal adenocarcinoma, and in particular the expression of phosphorylated Akt is increased in oesophageal adenocarcinoma tissue compared to normal epithelial and Barrett's tissue (Beales and Ogunwobi, 2007). Pharmacological inhibition of PI3Kinase has been shown to reduce proliferation and induce apoptosis in a cultured oesophageal cancer cell line (OE33 cells) (Beales et al., 2007).

We therefore hypothesised that high basal secretion of MMP-7 in OE33 cells might be due to activation of signalling pathways, and this chapter presents evidence to support the hypothesis.

6.1.1 Objectives

1. To determine the mechanisms regulating MMP-7 expression in an OAC cell line.
2. To study the signalling pathways in Barrett's adenocarcinoma cells, and to dissect the transduction pathway directly influencing the high basal secretion of MMP-7 in OE33 cell.
3. To assess the effect of transient inhibition of Akt phosphorylation and to correlate it with MMP-7 secretion in OE33 cells

6.2 Material and Methods

6.2.*1* *Cell culture, and conditioned media*

Oesophageal adenocarcinoma cells were cultured as described in section 2.3.1. Media were prepared from OE33 cells as described in section 2.4.

6.2.2 Preparation of whole cell lysate and protein quantification

Cell extracts were prepared in RIPA buffer containing protease and phosphatase inhibitors, the cellular protein concentration was quantified according to the protocol described in section 2.5.

6.2.*3* *Drugs and antibodies*

In order to explore which signalling pathways were involved in MMP-7 secretion in OE33 cells, cultured OE33 cells were washed with serum free medium (3 times) and treated with an inhibitor of the transport of newly synthesized proteins into the Golgi apparatus namely brefeldin A (BFA, 10µg/ml), or inhibitors of the activation of p42/44 MAPKinase (UO126, 10µM), PKC (Ro320432, 2µM) and PI3Kinase. With respect to the latter several inhibitors with different specificity were examined namely LY294002 (highly selective inhibitor of PI3Kinase, 50µM), TG100713 (pan-PI3Kinase inhibitor against PI3Kγ, PI3Kδ, PI3Kα and PI3Kβ, 2µM), rapamycin (extremely selective mTOR inhibitor, 100nM), wortmannin (potent and specific PI3Kinase inhibitor, 50nM), and MK 2206 2HCL (highly selective inhibitor of Akt1/2/3, 100nM).

6.2.4 *Western blot analysis*

Samples were separated by SDS-gel electrophoresis and subject to Western blot analysis as described in section 2.6. For antibodies and concentrations see Table 2.6.1.

6.2.5 *Indirect ELISA*

Media samples were collected as described in section 2.4. The concentration of MMP-7 in media from OE33 cells was determined by indirect ELISA using MMP-7 Ab (L522) as described in section 2.7.

6.3 Results

6.3.1 *BFA and the PI3Kinase inhibitor, LY 294002, inhibit MMP-1 and MMP-7 secretion by OE33 cells*

The possible role of different signalling pathways in MMP-7 secretion was studied using specific pharmacological inhibitors targeting PI3Kinase, PKC and activation of p42/44 MAP kinase. Incubation (6 h) of OE33 cells with inhibitors clearly indicated that secretion of MMP-7 (Figure 6.1 A, B), and MMP-1 as a reference (Figure 6.1 D, E), was inhibited by brefeldin A (BFA). Moreover, evidence that PI3Kinase activation contributes to MMP-7 and MMP-1 secretion was provided by the observation that these were inhibited by LY294002. However, inhibitors of PKC (Ro320432) and MAP kinase activation (U0126) had no significant effect on MMP-7 expression. To confirm the Western results, indirect ELISA was used (Figure 6.1 C): ELISA data confirmed that MMP-7 secretion in OE33 cells media was inhibited by LY294002 but there was no obvious contribution of other signalling pathways in MMP-7 secretion.

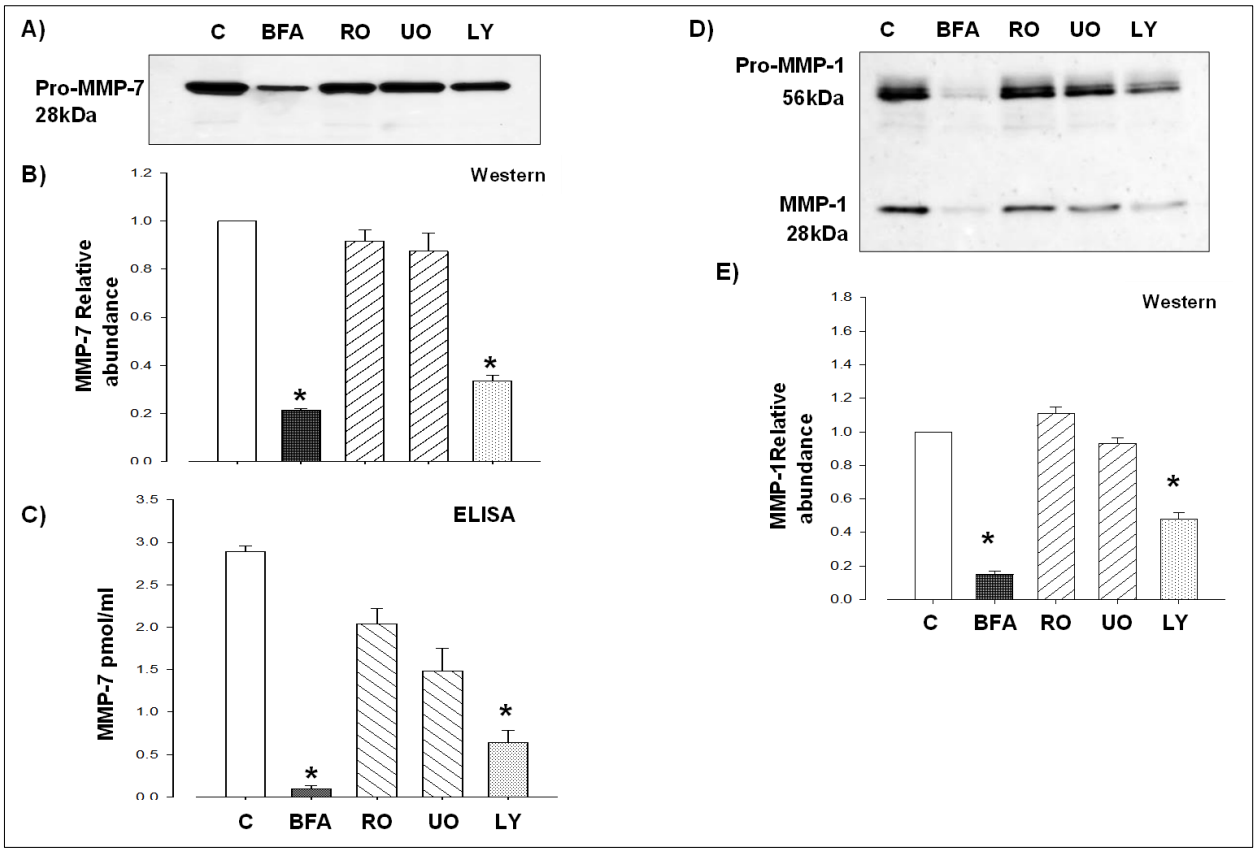

Figure 6.1: BFA and LY294002 inhibit MMP-7 and MMP-1 secretion by OE33 cells.
A) OE33 cells were treated with different inhibitors and probed with MMP-7 antibody; B) densitometry quantification of Western data revealed that BFA and a PI3Kinase inhibitor (LY294002, LY) inhibit MMP-7 secretion by OE33 cells. However, inhibitors of the p42/44 inhibitor, UO126 (10 mM), and the PKC inhibitor Ro320432 (2 µM) had no significant effect on MMP-7 release. C) ELISA data confirmed the Western result that MMP-7 was inhibited by BFA $p<0.01$) compatible with release by the constitutive pathway. The PI3Kinase inhibitor (LY294002) also inhibited MMP-7 secretion suggesting a role of the PI3Kinase pathway in MMP-7 secretion in OE33cells. D) MMP-1 antibody used as a reference, revealled BFA and LY294002 inhibit MMP-1 secretion in OE33 cells; E) densitometry. * $p<0.05$, ANOVA, (n=4 independent experiments).

6.3.2 *pan-PI3Kinase inhibitor (TG100713) inhibits MMP-7 secretion in OE33 cells*

The observation that MMP-7 secretion in OE33 cells was inhibited by the PI3Kinase inhibitor (LY294002) suggested the possible role of a signalling cascade involving the PI3Kinase pathway. This observation was then further evaluated using other inhibitors of the PI3Kinase signalling pathway, namely TG100713 a pan-PI3Kinase inhibitor against PI3Kγ, PI3Kδ, PI3Kα and PI3Kβ, and wortmannin a potent and irreversible inhibitor of PI3Kinase, but with little selectivity within the PI3Kinase family; in addition, LY294002 was used as a reference in these experiments. Western data (Figure 6.2, A, B) showed that MMP-7 secretion in OE33 cells was inhibited by TG100713, but only weakly by wortmannin. ELISA confirmed the result (Figure 6.2 C). In addition, Western blot analysis of cell extracts from OE33 cells showed Akt phosphorylation was significantly inhibited by both LY294002 and wortmannin; however, TG100713 had no inhibitory effect on Akt-phosphorylation after 6 hours incubation (Figure 6.2 D, E).

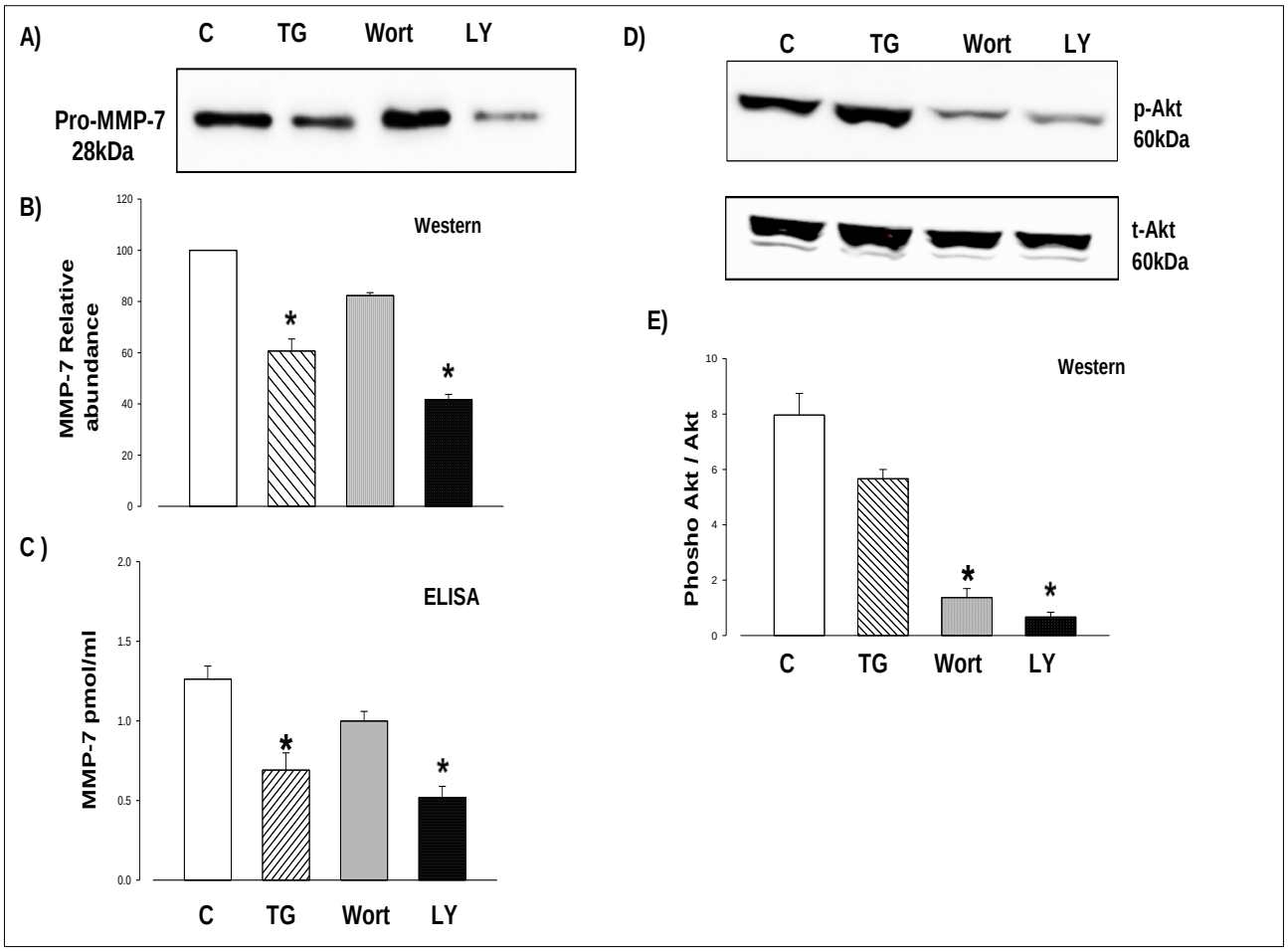

Figure 6.2: TG100713 and LY294002, but not wortmannin, inhibit MMP-7 secretion by OE33 cells. A) Representative Western blot analysis of MMP-7 shows LY294002 (50μM) and TG10073 (2μM) inhibit MMP-7 in media but wortmannin (Wort, 50nM) is less active. B) Densitometry. C) ELISA data confirmed Western results. D) Phospho-Akt was significantly inhibited in OE33 cell extracts (40μg of protein per lane) by LY294002 and Wortmannin, but not TG10073 after 6 hour incubation (n = 3). E) Densitometry. * $P < 0.05$ vs untreated control ANOVA.

6.3.3 *Activation of Akt mediates PI3Kinase pathway stimulated MMP-7 secretion*

To further characterise the role of downstream effectors of PI3K signalling pathway, we examined the effects of Akt and mTOR inhibition on MMP-7 secretion in OE33 cells using specific pharmacological inhibitors: MK 2206 2HCL (highly selective inhibitor of Akt1/2/3) and rapamycin (extremely selective mTOR inhibitor) both at 100nM; LY294002 was used as reference. After six hours incubation, Western blot (Figure 6.3, A, B) and ELISA (Figure 6.3, C) clearly revealed that MMP-7 secretion by OE33 cells was significantly inhibited by MK 2206 2HCL and LY294002 but not rapamycin. Corresponding results were obtained in OE33 cell extracts (Figure 6.3 D, E) with respect to inhibition of Akt phosphorylation. The results suggest that Akt but not mTOR activation is essential for MMP-7 upregulation in OE33 cells.

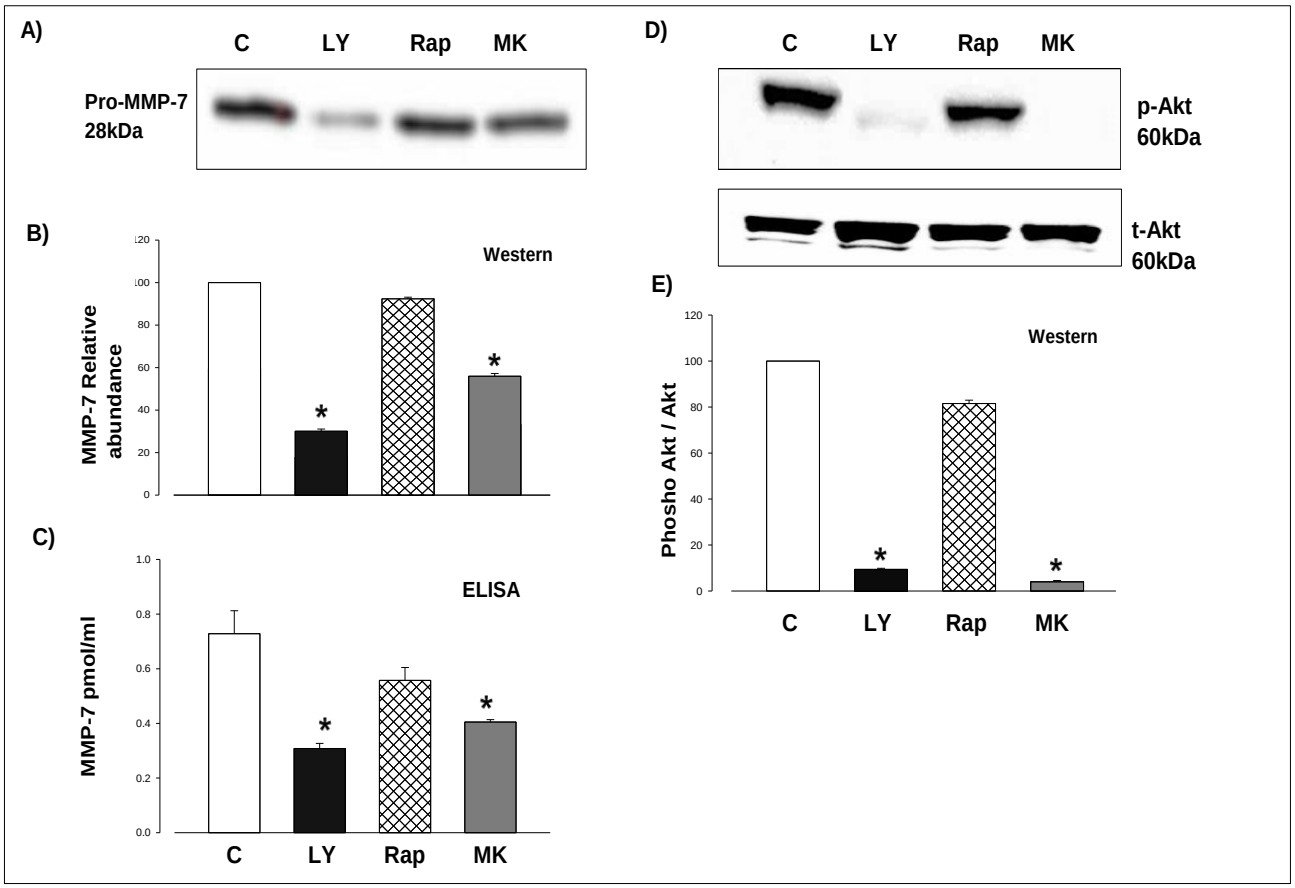

Figure 6.3: A selective inhibitor of Akt1/2/3 inhibits MMP-7 secretion in OE33 cell media. A) Representative Western blot, B) densitometry and C) ELISA of OE33 media showed MMP-7 secretion is significantly inhibited by LY294002 (LY, 50μM), and MK 2206 2HCL (MK, 100nM) but not rapamycin (Rap, 100nM)(n=3 in each case). D) Akt phosphorylation in OE33 cell extracts is significantly inhibited by MK 2206 2HCL and LY294002, but not Rapamycin. E) Densitometry. ***** $p<0.05$, ANOVA; vertical bars, SEM.

6.3.4 *TG100713 transiently inhibits Akt phosphorylation in OE33 cells*

TG100713 was associated with only very modest inhibition of Akt activity at the end of 6 hours (Figure 6.2). Then we asked if TG100713 inhibited Akt phosphorylation in a time dependent manner. To this end, studies were made after 20, 40, 80, and 160 minute's treatment with TG100713 (2μM). Interestingly, Western data revealed rapid transient inhibition of Akt phosphorylation ($p<0.05$) at 20, 40, and 80 minutes (Figure 6.4 A, B), thereafter phosphorylation of Akt gradually back toward the basal levels.

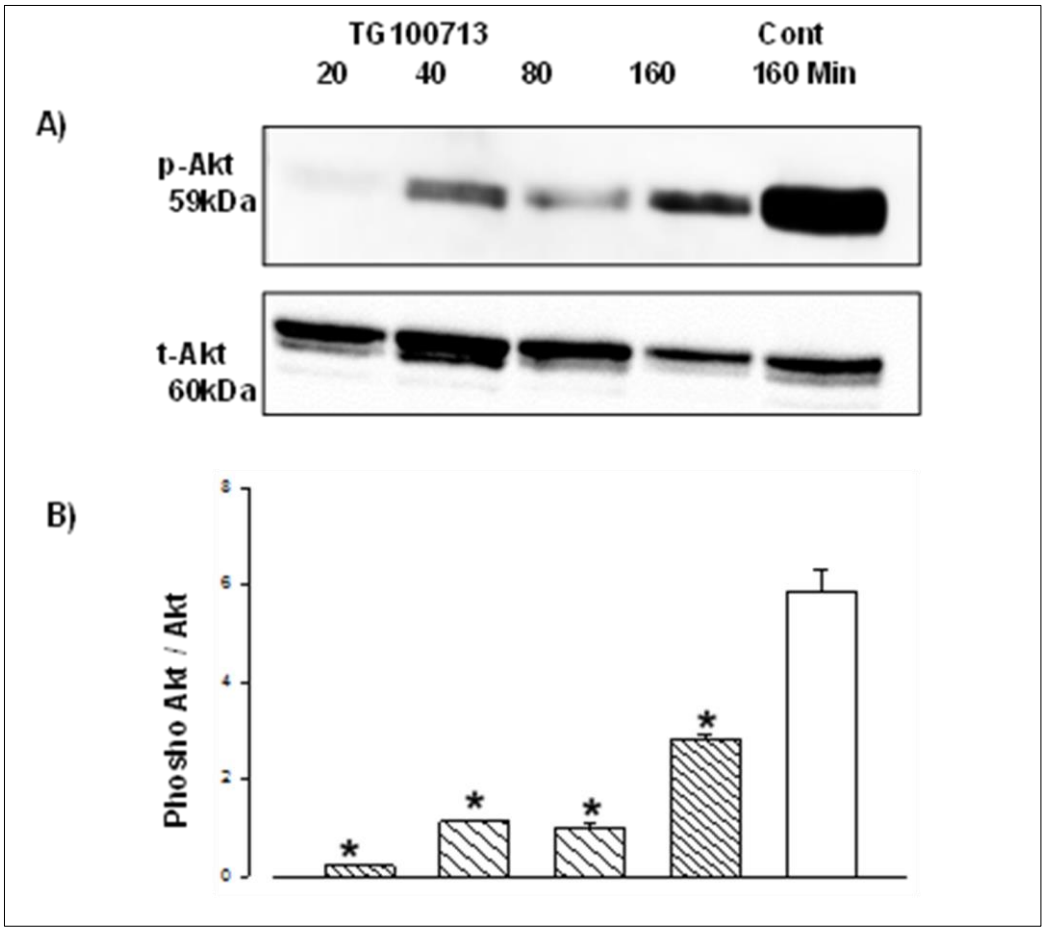

Figure 6.4: OE33 cells exhibited transient inhibition of Akt phosphorylation. A) Representative Western blot revealed transient inhibition of Akt phosphorylation in OE 33cells treated with TG100713 (2µM) at 20, 40, 80, and 160 minutes (n=3) using Phospho-Akt antibody (1:1000). B) Densitometry.* indicates that the difference between the time point and the one previous is significant ($p<0.05$, ANOVA).

6.3.5 Rapamycin does not inhibit Akt-phosphorylation

Given the previous finding with TG100713, the time course of Akt phosphorylation was then examined using other PI3Kinase inhibitors (LY294002, Wortmannin and MK 2206 2HCL) as well as rapamycin. Western data (Figure 6.5, A, B) demonstrated that rapamycin even transiently did not inhibit Akt phosphorylation, while other PI3Kinase inhibitors virtually abolished Akt phosphorylation compared to control as shown previously without any obvious effect for short time exposure on their inhibitory effect.

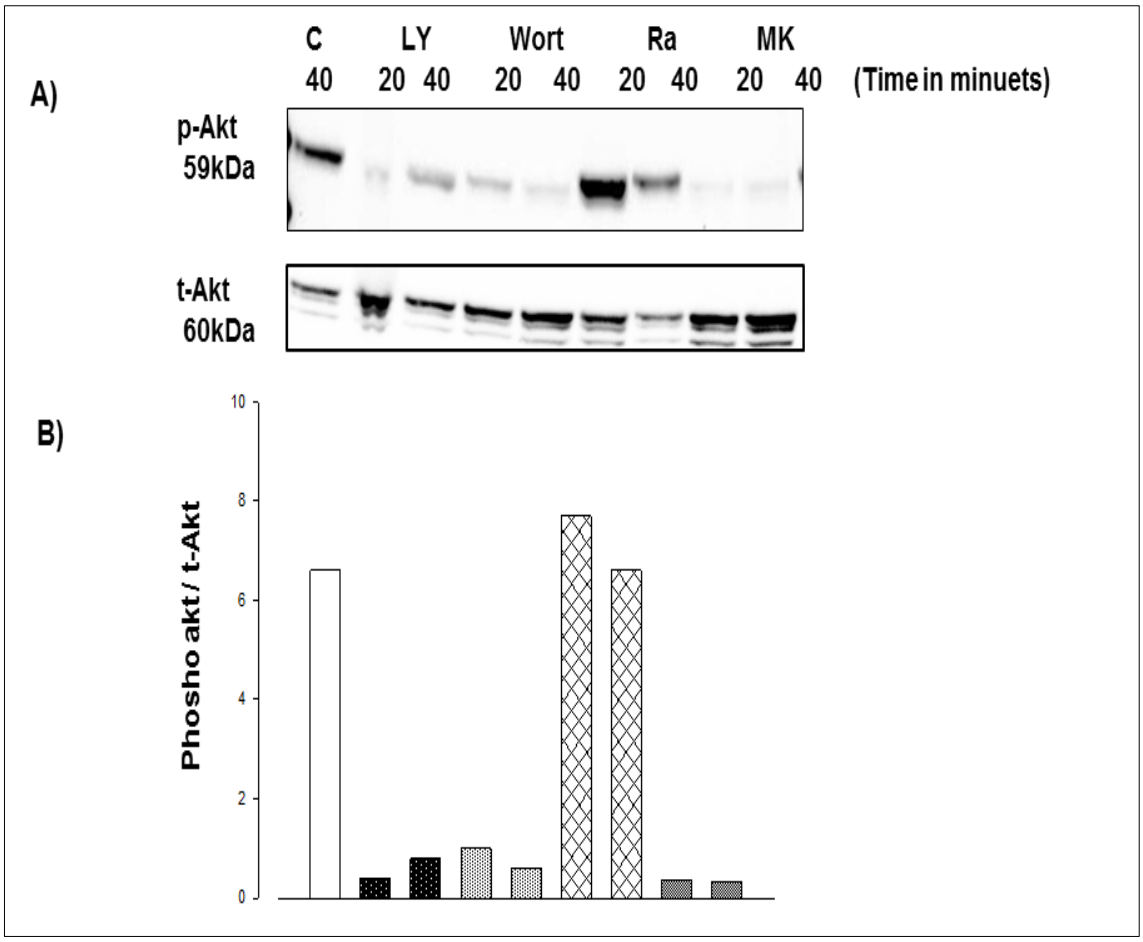

Figure 6.5: LY294002, wortmannin and MK 2206 2HCL, but not Rapamycin, rapidly inhibited Akt phosphorylation in OE33 cells. OE33 cells were treated with various inhibitors for 20 and 40 minutes and examined by Western blot. A) Representative Western blot, and B) densitometry quantification showed that Akt phosphorylation was inhibited by LY294002 (50μM), wortmannin (50nM), MK 2206 2HCL (100nM), but not rapamycin (100nM) (n=1).

6.4 Discussion

This study has shown that the PI3Kinase signalling pathway but not the MAP kinase or PKC pathways is associated, at least partly, with upregulation of MMP-7 and MMP-1 in OE33 cells. In addition, Western blot analysis confirmed by ELISA revealed the PI3Kinase signalling pathway regulated MMP-7 secretion via it is downstream effector Akt. In contrast, when similar experiments were done to determine the contribution of other downstream pathways in MMP-7 secretion, the data showed that an mTOR inhibitor (rapamycin) did not exhibit any effect on either Akt phosphorylation or MMP-7 secretion (Figure 6.3, 6.5). In addition, the data demonstrated that a pan-PI3Kinase inhibitor (TG100713) has an inhibitory effect in a time dependant manner (Figure 6.4).

Improving survival in oesophageal adenocarcinoma by targeting key signalling pathways is a fast growing field. However, this strategy has been restricted by drug resistance seen in therapies that target signal transducer proteins (Keld and Ang, 2011). The clinical use of PI3Kinase/Akt pathway inhibitors will be optimized if it is possible to identify biomarkers that predict response to therapy thereby allowing targeted treatment (LoPiccolo et al., 2008). The present results suggest that MMP-7 has potential as one such biomarker.

Currently over 30 small molecule PI3Kinase inhibitors are being evaluated in clinical trials either as monotherapy or combination therapy. The first authorization of a PI3Kδ inhibitor was idelalisib for multiple B-cell malignancies (as monotherapy for patients with relapsed follicular B-cell non-Hodgkin lymphoma and small lymphocytic lymphoma) and in combination with rituximab for those with relapsed chronic lymphocytic leukemia (Furman et al., 2014, Yang et al., 2015, Gopal et al., 2014).

Molecular targeted inhibitors effectively regulate overexpressed molecule in tumour cells. For example, it has been demonstrated that miRNA-34 inhibits EGFR signalling via downstream PI3K signalling cascades to regulate MMP7 expression in gastric carcinoma. Thus, miRNA-34a, EGFR, and MMP7 appear to be promising therapeutic targets for preventing the metastasis of gastric carcinoma(Liu et al., 2014).

Previous work has found PI3Kinase activates phosphorylation of Akt on Thr308 and Ser473 which inactivates GSK-3β resulting in β-catenin translocation to the nucleus and transactivation of many target genes including, potentially, MMP-7 (Brabletz et al., 1999a). It was reported (Brabletz et al., 1999b) that the MMP-7 gene contains two TCF binding sites, and is activated by β-catenin/TCF and mutations in the APC gene that occur in colon cancer. Moreover, In common with other MMP genes, the regulatory elements of the MMP-7 gene possess TGF-β inhibitory elements (Gaire et al., 1994). Further work could test the idea that both constitutive activation of β-catenin/TCF and inactivating mutations in the TGF-β pathway (functional cooperation of both pathways) are necessary for efficient activation of MMP-7 transcription. There is now an urgent need to clarify the clinical utilization of PI3K inhibitors with different isoform selectivity perhaps in rational combinatorial use with other antitumour agents, so as to maximally impact cancer, and provide patients with the greatest chance of valuable benefit (Yap et al., 2015). In each case, strong predictive biomarkers are needed that are tailored to the specific circumstances of each tumour.

For some years it has been clear that broad-spectrum MMP inhibitors (MMPIs) do not offer much promise for treating patients with advanced cancer perhaps due to the functional complexity of the MMP family (Folgueras et al., 2004). In order to block

the undesired activities of MMPs in cancer, it is first necessary to understand the precise mechanisms that regulate MMP expression and activity in cancer. In this study, we explored one of mechanisms responsible for overproduction of MMP-7 in OAC (PI3Kinase via Akt); this concept could be helpful to precisely identify the area targeted in OAC therapy, especially in light of the above mentioned other studies findings demonstrating the difficulties that prevent success of clinical trials with MMPIs.

The finding that MMP-7 secretion in OE33 cells was inhibited by LY294002 but not wortmannin was considered interesting. This may be explained by the fact that LY294002 competitively inhibits an ATP binding site on the p85 subunit of PI3Kinase, and also has been shown to block PI3Kinase-dependent Akt phosphorylation (Vlahos et al., 1994), whilst wortmannin targets irreversibly the p110 subunit of PI3Kinase (Powis et al., 1994). For this reason isoform-specific inhibitors are more difficult to develop because of the highly conserved nature of the ATP-binding pocket. Moreover, the lack of selectivity of these compounds, together with the instability of wortmannin and the insolubility of LY294002, means that neither has very promising pharmaceutical potential. However, in a recent systematic screening (Miller et al., 2009) of both compounds for activity against a broad range of kinases, LY294002 was reported to inhibit casein kinase 2 with a sensitivity comparable to that of p110α/p85α. This observation is important, because casein kinase 2 is involved in many cell regulatory processes. Recent studies (Chan et al., 2002, Cuevas et al., 2001) have demonstrated that tyrosine phosphorylation of p85 may also alter PI3Kinase activity. Moreover, recent studies (Kang et al., 2002, Song et al., 2007a) have shown p85 functions which are independent of the p110 catalytic subunit, including regulation of gene transcription. Another study (Mendes Sdos et

al., 2009) showed that MMP-9 regulation was inhibited by LY294002 but not by wortmannin.

It has been well documented that mTOR functions downstream of the PI3Kinase/Akt pathway and is phosphorylated (or activated) in response to stimuli that activate the PI3K/Akt pathway, and Akt has been placed upstream of mTOR in many cell types (Bjornsti and Houghton, 2004, Hay and Sonenberg, 2004). Rapamycin was initially considered as a promising candidate for blocking mTOR phosphorylation in several cancer types; however, cancer patients with high expression Akt may have little response to mTORC1 inhibitors (O'Reilly et al., 2006). In this study, we hypothesised that rapamycin would alter the phosphorylation levels of Akt (p-Akt). However, the data demonstrated that rapamycin does not have any inhibitory effect on MMP-7 secretion as shown in (figure 6.3). In agreement with our finding, it has previously been reported (Breuleux et al., 2009, Dos et al., 2004), using a large panel of cell lines representing different tumour histological subtypes that increased Akt S473 phosphorylation after mTORC1 inhibition is rictor- (novel protein rictor, rapamycin-insensitive companion of mTOR)-dependent and does not predict tumour cell response to PI3Kinase mTOR inhibition. Other studies (Hay and Sonenberg, 2004) have shown that the inhibition of mTOR by rapamycin triggers the activation of two survival signalling pathways; increased the phosphorylation of both Akt and eukaryotic translation initiation factor (eIF4E) that may contribute to drug resistance.

Further experiments will now be required to confirm if indeed there is a unique pathway of up-regulation of MMP-7 in oesophageal adenocarcinoma or rather its regulation is dependent on a multitude of transcription factors acting through different signalling pathways. Study of downstream substrates of Akt in more detail in OE33 cells, and work on NF-κB sites possibly would be a good starting point, since NF-κB is seen as central in MMP-7 regulation in other systems.

6.5 Conclusions

1. High constitutive expression of MMP-7 in an OAC cell line is partly attributable to activation of the PI3Kinase/Akt signalling pathway.
    a) TG100713, LY294002, and MK2206 inhibit MMP-7 secretion in OE33 cells; however, rapamycin and wortmannin have no inhibitory effect.
    b) LY294002, MK2206, TG100713 and wortmannin but not rapamycin inhibit phospho-Akt in OE33 cells.

CHAPTER 7

GENERAL DISCUSSION

7.1 Main findings

The main findings of this thesis are that MMP-7 is up-regulated in epithelial cells in the progression from BO to OAC including at the invasive front in OAC. Unexpectedly, MMP-7 is also expressed by myofibroblasts particularly at the invasive front in OAC. Although MMP-7 is expressed in cultured oesophageal myofibroblasts it was undetectable in media. A novel ELISA that measures MMP-7 and related peptides in cell media was validated for this purpose. OE33 but not OE21 or OE19 cells secrete high pro-MMP-7. High constitutive expression of MMP-7 in OE33 cells is partly attributable to activation of PI3kinase via Akt. However, MMP-7 expression and secretion were insensitive to gastrins and PMA. MMP-7 secreted by OE33 cells may modify the tumour microenvironment by stimulating CAM migration since the effect of OE33 cell conditioned medium on CAMs migration was reversed by a neutralizing antibody. The results suggest MMP-7 is a strong candidate for future studies directed at identification of biomarkers in the BO-OAC progression and potential novel therapeutic targets.

7.2 Methodology

The present study relied on the use of cell lines which are derived from a distinct population of cells that, it can be argued, may not reflect wild type tumour cells. However, cancer cell lines are frequently used as in vitro tumour models. Moreover, their use has advanced the understanding of cancer biology tremendously over the past decades. The possibility that cell lines acquire or lose key characteristics through phenotypic drift needs to be kept in mind (Welch et al., 1984). Early passage cells were utilized in the present study in an attempt to ensure that the characteristics of the cells still closely resembled those of the tissue of origin.

The identification of MMP-7 was based on immunohistochemistry, Western blot and ELISA. These approaches critically depend on antibodies. Alternative methods, for example focussing directly on DNA, mRNA or protein analysis, including -omics technologies are now needed for a comprehensive analysis of the molecular anatomy of both tissue sections and cell lines (Gillespie et al., 2001). For example, laser microdissection technology allows accurate separation of tumour stromal and normal cells within a single biopsy specimen and can be coupled with advanced analysis methods (Datta et al., 2015, Espina et al., 2006, Mustafa et al., 2008). The use of genetically modified mice can also be useful in replicating the progression of human disease, and have advantages of a functioning immune components, although manipulation of stromal compartment in this model is more difficult (Richmond and Su, 2008).

The present study made use of migration assays employing Boyden chambers. It would be useful to extend this to the study of cells in ECM to provide a 3D organotypic culture (Smalley et al., 2008, Kimlin et al., 2013) .

7.3 The pros and cons of IHC and the semi-quantitative scoring systems

IHC allows direct visualization of biomarker expression in histologically relevant areas and can be readily related to classification and grading the disease. IHC is typically performed on tissue sections processed by standard methods, can be applied to large numbers of specimens and is easy to implement in clinical practice. There are, however, some variables which remain problematic. These can be classified into three groups: first, pre-analytical variables of IHC include the steps in tissue processing, starting from receiving tissue samples (prolonged ischemia,

delayed fixation), type and length of fixation, and tissue handling, proper specimens orientation, and careful noting of surgical margins, slicing into sections (naming, and adequate). Unfortunately, pre-analytical variables cannot be controlled closely (Shi et al., 2007, Kaczorowski and Kaczorowska, 1992, Fritz et al., 1989). Second, there are analytical variables such as slide thickness, selection of detection system, and of antibodies, and antigen retrieval procedures (Yamashita, 2007). In this study, we have chosen monoclonal MMP-7 antibody in IHC due to the following points; since fixed antigens in surgical tissue samples processed by IHC have a slightly different conformational structure than those that have been removed from the cell and processed by SDS-PAGE (Shi et al., 2011). Also Monoclonal antibodies are usually preferable due to their specificity and batch-to-batch consistency. However, monoclonal antibodies (mAbs) may be too specific for IHC.

Third, post-analytical variables include data reporting and interpretation. Routinely, pathologists have visually scored IHC data; the most broadly used system is semi-quantitative scoring used to convert subjective perception of IHC-marker expression into quantitative data. However, visual scoring is fraught with problems due to subjectivity in interpretation. To overcome these limitations, alternative approaches using automated IHC measurements (IHC profiler) which are precise and produce continuous data are being developed. Additionally, automated IHC profilers considerably improves both intra- and inter-observer agreement (Varghese et al., 2014). Also, the digital methods for IHC quantifications are ideal for large samples size (Rizzardi et al., 2016).

7.4 Stromal MMP-7

Currently, it is broadly accepted that the interaction between tumour cells and surrounding TME is critical in understanding the mechanisms of cancer development, progression and metastasis. The TME also provides new routes for cancer detection and treatment. The novelty in the present study is the finding that MMP-7 is expressed in stromal spindle-shaped cells, and secreted MMP-7 from OE33 cells may modify the tumour microenvironment by stimulating stromal cell migration. These findings suggest the substantial involvement of TME stroma in OAC biology. It would be useful to extend this now to the study of stroma associated protein in more detail especially through proteomic analysis. For instance, using a combination of tandem mass spectrometry (MS/MS) with activity-based probes to determine the occurrence and quantification of proteins in addition to their functional activity is promising (Cardoza et al., 2012). Another approach involves stable isotope labelling of amino acids in cell culture (SILAC), which enables proteins that are newly produced by cells to be differentiated from culture media supplemented with serum, for the accurate quantitation of relative protein abundances (Ong et al., 2003). Newly imaging probes based on MMP-specific activities can also be utilized as an alternative approach that may help to overcome a number of technical difficulties. For instance, cell-penetrating peptides, which are activated by proteolysis and carry a fluorescent cargo that accumulates in cells that are in the proximity of active MMPs (Jiang et al., 2004), have been used favorably to visualize MMP-2 and -9 activity in cell culture systems and in mouse xenograft models and show active MMPs predominantly at the interface between tumor and stroma (Olson et al., 2009, Holmberg et al., 2013a). Another powerful tool is MRI reporter gene imaging that monitors gene expression patterns *in vivo* (Vandsburger et al., 2013)

7.5 Western blot and qPCR will give a more complete picture

Protein abundances reflect a dynamic balance of a series of connected processes, including transcription, processing and degradation of mRNAs and the translation, localization, modification and programmed destruction of proteins themselves. In this study, Western blot was used to compare the relative MMP-7 abundance from different cells and with different treatments. In order to progress, further study of MMP-7 regulatory mechanisms requires qPCR to determine whether mRNA abundance is increased, decreased, or unchanged. Even so, it should be noted that the concordance between mRNA and protein expression varies from poor to moderate (Pearson correlation ranged from 0 to 0.63) (Pascal et al., 2008, Xiao et al., 2012). Definitely, this will help to narrow down our research interest in the future and aid to build a more complete picture.

7.6 OE33 cells: necessary but not sufficient

In this study data were generated from OE33 cells; the dependence on a single cell line could be considered as a weakness. Nevertheless, since we were interested in examining the OAC in the presence of BO, OE33 cells are highly relevant since they exhibit a phenotype closely resembling that of the original OAC tumour (Boonstra et al., 2010). Moreover, OE33 cells line is the only commercially available adenocarcinoma cell line of the lower oesophagus (Barrett's metaplasia). Also, OE33 cells express and secrete a high level of MMP-7 compared to with another commonly used OAC cell line (Flo-1 cells) that does not express or secrete MMP-7 (Grimm et al., 2010b). A short tandem repeat (STR) comparison of OE33 with the

tumour of origin taken from formalin fixed paraffin embedded tissue has established the authenticity of current OE33 cell stocks (Rockett et al., 1997).

Although no genome-wide analysis of mutational status has been performed in OE33 cells, these cells have been used for karyotyping and cell surface antigen phenotyping. Nevertheless, some groups have applied an alternative approach to establishing in vitro models of OAC (Clemons et al., 2014). It is not yet clear if the latter are typical of the mutational profile of cells governing the malignancy in vivo. Interestingly, a multiple genome-wide platform has been used to create MFD-1 cells (Garcia et al., 2016). These show high expression of epithelial and glandular markers and a fingerprint of open chromatin. In addition, MFD-1 cells were tumorigenic in SCID mice and were proliferative and invasive in 3D cultures. Other models that outline the complexity of OAC in vivo, such as primary xenografts or spheroids, will be required to further address these issues. In this context it is worth stressing that research in OAC has been restricted due a paucity of high fidelity pre-clinical models and additional efforts should be conducted to bridge this gap.

7.7 MMPs as new targets for cancer therapy

A variety of strategies based on targeting endogenous regulation of MMPs are in development. In an attempt to block transcription of MMP genes, for example, inhibition of MAPK or ERK pathways, or NF-κB, may be feasible (Aggarwal et al., 2003, Pan and Hung, 2002, Ala-aho and Kahari, 2005). Another strategy is to reduce the conversion of pro-MMP into activated form, so blocking proteolytic activity. Besides natural TIMPs, MMPIs (matrix metalloproteinase inhibitors) have been synthesized and inserted at particular checkpoints within the MMP activation

cascade. Thus batimastat (BB-94) was the first synthetic inhibitor, and acts on MMP-1, MMP- 2, MMP-3, MMP-7 and MMP-9 (Hidalgo and Eckhardt, 2001).

However, there are limitations associated with broad range MMPIs that may also block antitumor activities of MMPs (Khalid and Javaid, 2016). The present study suggests that MMP-7 inhibitors could provide a new approach in treating OAC. In addition, there may be advantages to using imaging technique with MMPIs employing fluorescent or other labelled particles that could visualise events within the tumour milieu (Furumoto et al., 2003, Wagner et al., 2009).

7.8 Future prospects

This work identifies the need for further experiments to determine whether there is a unique pathway of upregulation of MMP-7 during Barrett's disease progression to OAC. Further studies of in vivo tumour model by using imaging methods that might also extend the current in vitro findings. Hence, a focussed approach using MMP-7 as a therapeutic target for in the BO - OAC progression is of potential promise. Also, MMP-7 could provide a useful marker for disease progression, risk stratification, or monitoring of treatment response.

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