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The neuroendocrine-like phenotype of gastric myofibroblasts and its significance in cancer

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ABSTRACT

Cell-cell communication, and specifically epithelial-mesenchymal signalling, is a key factor determining normal tissue development and organisation in hollow organs such as gastrointestinal tract. Mechanisms governing normal epithelial-mesenchymal communication have been studied for many years, but the changes occurring during tissue damage, infection and inflammation, and in cancer, remain unclear. Myofibroblasts are recognised as key mesenchymal cells involved in this communication in health and in disease. Myofibroblasts produce and secrete many proteins responsible for the assembly of extracellular matrix (ECM), tissue organisation and morphogenesis. Initial studies from this laboratory suggested that myofibroblasts might exhibit regulated secretion. The aim of this project was to determine the secretory mechanisms of myofibroblasts from the upper gut of normal and cancer tissues, and to address their significance in cancer.

Gastric myofibroblasts were shown to exhibit calcium-dependent secretion of the small ECM protein, Transforming growth factor-β inducible protein or TGFβig-h3, in response to acute stimulation (30 min) with insulin-like growth factor (IGF)s. Inhibitors of protein synthesis (actinomycin D and cycloheximide) and of protein transport from endoplasmic reticulum (ER) to Golgi (brefeldin A) inhibited basal, but not IGF-stimulated secretion, as seen from Western blot analyses of media. These observations support the idea that evoked secretion occurs from a pre-formed pool of vesicles.

Myofibroblasts from the upper gut showed differences in their secretory phenotype; specifically normal myofibroblasts from stomach exhibited regulated secretion, but their counterparts from oesophagus did not. Moreover, gastric cancer-associated myofibroblasts (CAMs) from patients with poor survival tend not to exhibit regulated secretion. These findings suggest a role for the tissue microenvironment in determining the secretory phenotype of myofibroblasts.

Secretome-wide analysis of myofibroblasts media collected after IGF stimulation revealed that about 85% of the secretome exhibits evoked release. The relevant proteins belonged to different classes including ECM proteins, ligands, binding proteins, carbohydrate-binding proteins, proteases and protease inhibitors. These data indicate that myofibroblasts may contribute to tissue organisation by rapid release of substances involved in re-modelling the tissue microenvironment.

The regulated secretory phenotype of myofibroblasts was associated with the expression of the chromogranin-like protein, secretogranin II. Knock-down of secretogranin II inhibited regulated secretion, whereas over-expression in myofibroblasts that lacked regulated released - induced it. Expression of secretogranin II by myofibroblasts coincided with the expression of dense core secretory vesicles that were similar to those found in neuroendocrine cells.

This work indicates that there is a neuroendocrine-like secretory phenotype in myofibroblasts, as illustrated by the expression of neuroendocrine cell protein secretogranin II and the presence of regulated secretion. However, not all normal myofibroblasts exhibit the regulated phenotype, and in gastric cancer the phenotype correlates with early stage of disease. These findings may be

exploitable both in the development of novel therapies and in understanding cancer progression.

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ABBREVIATIONS

3T3	Fibroblast cell line
α-SMA	α-smooth muscle actin
ADAM	A desintegrin and metalloprotease
ATM	Adjacent tissue myofibroblast
ATP	Adenosine triphosphate
BFA	Brefeldin A
BSA	Bovine serum albumin
CAM	Cancer-associated myofibroblasts
CCK	Cholecystokinin
CHO cells	Chinese hamster ovary cells
CNS	Central nervous system
COX	Cyclooxygenase
CRD	Carbohydrate-recognition domain
CS	Constitutive secretion
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECL	Enterochromaffin-like cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunoassay
EM	Electron microscopy

ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein isothiocyanate
H.felis	*Helicobacter felis*
H.pylori	*Helicobacter pylori*
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-IR	Insulin-like growth factor-receptor type 1
IL-1β	Interleukin-1β
GABA	Gamma-aminobutiryc acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal tract
GIST	Gastrointestinal stromal tumour
GTP	Guanosine-triphosphate
LB	Lysogeny broth
LC	Liquid chromatography
M	Molar
MAPK	Mitogen-activate protein kinase
MAS5	Microarray Analysis Suite 5

MMP	Matrix metalloprotease
Mo	Month
MS	Mass spectrometry
MSC	Mesenchymal stem cell
MSCBM	Mesenchymal Stem Cell Basal Medium
ND	Not done
NI	Not identified
NRSF	Neuron-restrictive silencer factor
NSF	N-ethylmaleimide-sensitive fusion protein
NTM	Normal tissue myofibroblast
PA	Pernicious anaemia
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PFA	Paraformalydehyde
PI3K	Phosphatidylinositol-3-kinase
REST	RE-1–silencing transcription factor
rh	recombinant human
RNA	Ribonucleic acid
RS	Regulated secretion
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SILAC	Stable Isotope Labelling by Amino-acid in Culture

siRNA	small interference RNA
SLRP	Small leucine-rich proteoglycan
SNAP	Soluble NSF attachment protein
SNAP-25	Synaptosome-associated protein of 25kDa
SNARE	SNAP receptor protein
SOC	Super Optimal Broth with Catabolite repression
SPARC	Secreted protein acidic and rich in cysteine
TE	Tris/EDTA buffer
TEM	Transmission electron microscopy
TIMP	Tissue inhibitors of matrix metalloproteases
TIRF	Total internal reflection fluorescence imaging
TGF	Transforming growth factor
TGFβig-h3	Transforming growth factor-β inducible protein
TGN	*trans*-Golgi network
tPA	Tissue-type plasminogen activator
t-SNARE	Target-membrane associated SNARE
UniProt	Universal Protein Resource database
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VAMP	Vesicle-associated membrane proteins
VGLUT	Vesicular glutamate transporter
v-SNARE	Vesicle-associated SNARE
WB	Western blot

CHAPTER 1

INTRODUCTION

1.1 Mechanisms of cell-cell communication

1.1.1 Overview

In multicellular organisms, communication between cells is crucial in maintaining normal health and development. The concept of an internal balance within complex organisms emerged from the idea of the "milieu de l'intérieur" introduced by the French physiologist Claude Bernard in 1854 (Gross, 1998). Initially, "milieu de l'intérieur" was considered to relate to the chemical composition of the blood, but later Bernard extended the principle to other areas e.g. temperature. The American physiologist Walter Bradford Cannon expanded on Bernard's view of "milieu de l'intérieur" and introduced the term *homeostasis* in his book *The Wisdom of the Body*, first published in 1932. To maintain homeostasis within the body it was anticipated that tightly controlled communication mechanisms existed at different levels of organisation within an organism including cell-cell communication.

1.1.2 Signal transduction

It is now well recognised that cell-cell communication occurs both by soluble secreted factors, by cell-matrix interactions and by interactions between membrane proteins. Water-soluble cell-cell signalling molecules generally act by binding to cell surface receptors to trigger changes in intracellular messenger molecules, which are followed by execution of the appropriate physiological responses; an early example of this process was elucidated by Rodbell who used the term signal transduction (Rodbell, 1980) to describe binding of the hormone

glucagon to a receptor on rat liver cell membranes which activated a G-protein that subsequently triggered a signalling cascade leading to changes in cell metabolism.

1.1.3 The first hormone

The first clear example for cell-cell communication was provided by the discovery of communication via soluble factors released into the blood stream and exerting their effects at a distance. In 1902, Bayliss and Starling discovered the first hormone, which they termed *secretin* (Bayliss and Starling, 1902). They showed that a substance was secreted by epithelial cells of the small intestine after stimulation with acid and was transported through the blood stream to the pancreas, where it stimulated the secretion of the pancreatic juice. The term *hormone* was then introduced in 1905 to describe the class of substances acting in this way (Starling, 1905). The process is now also termed endocrine regulation and a distinction is recognised between this and paracrine regulation, where secreted molecules influence local, or nearby, cells; similarly, in autocrine regulation secreted molecules act on the cell that released them in the first place.

1.1.4 Discovery of neurotransmitters

Prior to the discovery of hormones, the neuroanatomist Ramón y Cajal described the anatomy of junctions between neurons (Cajal., 1888), which afterwards were termed *synapses* by Sherrington to stress the functional importance of these structures in neuronal communication (Sherrington, 1897). Slightly later, the idea

of chemical transmission at synapses was developed independently by Loewi (Loewi, 1921) and by Dale who identified the first neurotransmitter, acetylcholine (Dale, 1914; Loewi, 1924).

1.1.5 Other types of signalling molecule

Although hormones (endocrine regulation) and neurotransmitters (neuronal regulation) were the first mediators of cell-cell communication to be studied in detail, it has been clear for several generations that there are many other examples including locally active substances such as cytokines, chemokines and growth factors.

Cytokine is a broad term for signalling molecules applied initially to proteins modulating immune cell function. However, it is now known that cytokines exert their effects on many different cell types. The unexpected discovery by Nowell in the 1960s that a plant lectin stimulated T-lymphocyte proliferation in culture triggered studies leading to identification of the first of the family of T-cell growth factors (Nowell, 1960), later identified as interleukin-2 (Morgan et al., 1976).

Chemokines are a large group of proteins that are released by cells to act as lures for other cells in acute and chronic inflammation, during infection and in cancer. The first chemokine to be described was interleukin-8, synthesised by various cell types including macrophages (Koch et al., 1992) and endothelial cells (Wolff et al., 1998) and acting as chemoattractant for memory T cells and monocytes (Schall et al., 1990).

Growth factors are substances that mediate cell growth and differentiation (Ray and Melmed, 1997). In the early 1960s, Levi-Montalcini and Cohen described the first growth factor, nerve growth factor (Levi-Montalcini, 1965). This was followed by the discovery of epidermal growth factor (EGF) by Cohen, while working in Levi-Montalcini's lab (Carpenter et al., 1978). Many other types of growth factor have since been characterised including, for example, platelet derived growth factor (PDGF), transforming growth factor (TGF)-α (which is chemically related to EGF) and TGF-β (which is chemically unrelated to EGF and TGF-α) and the insulin-like growth factors (IGFs).

1.1.6 The insulin-like growth factor system

1.1.6.1 IGFs

Insulin-like growth factors illustrate the relationship between hormones and growth factors. They were originally identified in 1957 by Daughaday (Salmon and Daughaday, 1957) and then chemically characterised as belonging to the same group as the islet hormone insulin, although unlike insulin they consist of a single aminoacid chain (Daughaday and Rotwein, 1989). In 1977, a family of IGF-binding proteins was described (Hintz and Liu, 1977). At the moment, the IGF system includes two IGFs (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR/mannose-6-phosphate receptor) and several IGF binding proteins (IGFBPs) (see below) (Ye et al., 2007).

The mature IGF polypeptides consist of A, B, C and D domains, A and B of which are homologous to insulin (Duan and Xu, 2005). Both IGFs are widely

expressed in fetal and prenatal stages in different body tissues, however hepatic IGF-I production is the major source of circulating IGF-I, regulated by growth hormone secretion (Duan and Xu, 2005). The IGFs regulate cell growth and development through endocrine, paracrine and autocrine mechanisms (Le Roith et al., 2001) via binding to IGF-IR, which is linked to activation of two major cellular signalling pathways: mitogen-activate protein kinase (MAPK) and phosphatidylinositol-3-kinase(PI3K)-Akt (Coolican et al., 1997).

1.1.6.2 IGFBPs

IGF-binding proteins regulate the bioavailability and effects of the IGFs. Depending on the stability of the bond when complexed with IGF, there are six IGFBPs with high affinity and ten with low affinity, which are also known as IGFBP-related proteins (Burger et al., 2005). The different IGFBPs are expressed in a tissue- and developmental stage-specific manner (Jurgeit et al., 2007). Although there is a high structural resemblance, each IGFBP has distinct features and specific roles. For example, IGFBP-3 and IGFBP-5 are found in the circulation in complex with IGFs (Guler et al., 1987; Twigg and Baxter, 1998), thereby mediating their systemic effects. Except for IGFBP-1 that is mainly expressed in liver, other IGFBPs are expressed by different cell types, including fibroblasts and myofibroblasts, in various organs where they are thought to exert their effects locally (Duan and Xu, 2005). Moreover, some IGFBPs inhibit (IGFBP-4 and -6), while the rest can either potentiate or inhibit IGF actions (Duan and Xu, 2005). In addition, IGFBPs may exhibit IGF-independent effects. The IGFBP-related proteins exhibit 20- to 100-fold lower affinity in comparison

with IGFBPs toward IGFs (Oh et al., 1996) and they too are believed to have IGF-independent effects (Hwa et al., 1998).

Two IGFBPs were identified in the current project: IGFBP-5 and IGFBP-7. The former was described about 20 years ago. In addition to its systemic IGF-related effects via the circulation, IGFBP-5 binds to IGF-IR on the cell surface and different extracellular matrix (ECM) proteoglycans that can lead to both inhibitory and stimulatory IGF effects depending on the cell type, method of administration or experimental conditions (Beattie et al., 2005; Yin et al., 2004). Besides IGF-related effects, IGFBP-5 exhibits some IGF-independent effects such as activation of the plasminogen system associated with tissue remodelling (Sorrell et al., 2006). Also, IGFBP-5 is reported to bind a specific cell surface IGFBP-5 receptor, followed by cellular uptake and activation of cellular pathways of growth and apoptosis (Andress, 1995; Andress, 1998). Moreover, IGFBP-5 is identified in the nucleus of different cell types, where it possibly acts as a signalling molecule (Schedlich et al., 2000). Multiple studies demonstrate that IGFBP-5 induces apoptosis (Hung et al., 2008), inhibits tumour growth and tumour vascularisation both *in vitro* and *in vivo* (Butt et al., 2003; Rho et al., 2008).

Insulin-like growth factor binding protein-7 (IGFBP-7) is the first member of the IGFBP-related proteins, and hence it is also called IGFBP-related protein-1 (Hwa et al., 1999). It acts as a tumour suppressor to inhibit tumour growth in a breast cancer cell line (Amemiya et al., 2011) and to induce apoptosis in prostate cancer cells (Mutaguchi et al., 2003).

1.1.6.3 The IGF system and the gut

The local role of the IGF-system is well illustrated by mechanisms in the gastrointestinal (GI) tract (Lund, 1999). A local or paracrine action of IGFs predominantly produced by mesenchymal cells (fibroblasts and myofibroblasts) determines epithelial cell turnover (Williams et al., 2002). The high turnover of gut epithelial cells i.e. the process of cell renewal, is determined in part by IGFs acting in a paracrine way on cell growth, proliferation and differentiation (Williams et al., 2002). In addition, IGF-I may also influence intestinal transport of sugar (Castilla-Cortazar et al., 1997) and normalise aberrations in ion transport (Peterson et al., 1996). Previous work from our laboratory identified IGF-II as an autocrine growth factor from colonic and gastric myofibroblasts (Hemers et al., 2005). Subsequent unpublished work from the laboratory showed that IGF-II increases intracellular calcium in gastric myofibroblasts (McCaig, Burdyga & Varro, unpublished observations), thereby raising questions as to whether there might not be other effects on cellular function besides mitogenic effects.

1.2 Biology of protein secretion

1.2.1 Secretion – classical and unconvential pathways

For many years after their discovery, hormones and neurotransmitters were considered to be part of quite different biological processes. It was recognised, however, that both were released by cells in response to stimulation and with the elucidation of cellular mechanisms of secretion it became clear that similar cellular events were involved. The so-called classical pathway of secretion

(exocytosis) of proteins from eukaryotic cells was largely elucidated by Palade and his colleagues working on enzyme secretion from pancreatic acinar cells (Caro and Palade, 1964; Jamieson and Palade, 1967; Palade, 1956) and later shown to apply to peptide hormones. In 1978, Tartakoff and Vassalli suggested that there are two types of secretory process: constitutive and regulated (Tartakoff et al., 1978). Constitutive secretion involves the release of newly synthesised protein within minutes and is unregulated, whereas regulated secretion involves storage of secretory product, often in large quantities, in secretory vesicles with dense cores that are released after stimulation through a mechanisms involving increased intracellular calcium (Douglas and Rubin, 1961; Douglas and Rubin, 1963) (Figure 1.1). Although some cases of calcium-independent secretion were reported, even in these systems calcium remained an obligatory element in the regulated secretion mediated by other secondary messengers (Hutton, 1986).

Additionally, neurons exhibit calcium dependent exocytosis from small clear synaptic vesicles docked near the presynaptic membrane and involved in rapid release of small, nonprotein, neurotransmitters such as acetylcholine, glutamate and gamma-aminobutiryc acid (GABA) (Kelly, 1993). Unlike dense core secretory vesicles, synaptic vesicles do not originate from Golgi, as they are recycled and refilled near the pre-synaptic membrane (Heuser and Reese, 1973).

There are some examples of cells specialised for regulated secretion, which are not of neuronal, endocrine or exocrine origin. For instance, juxtaglomerular cells

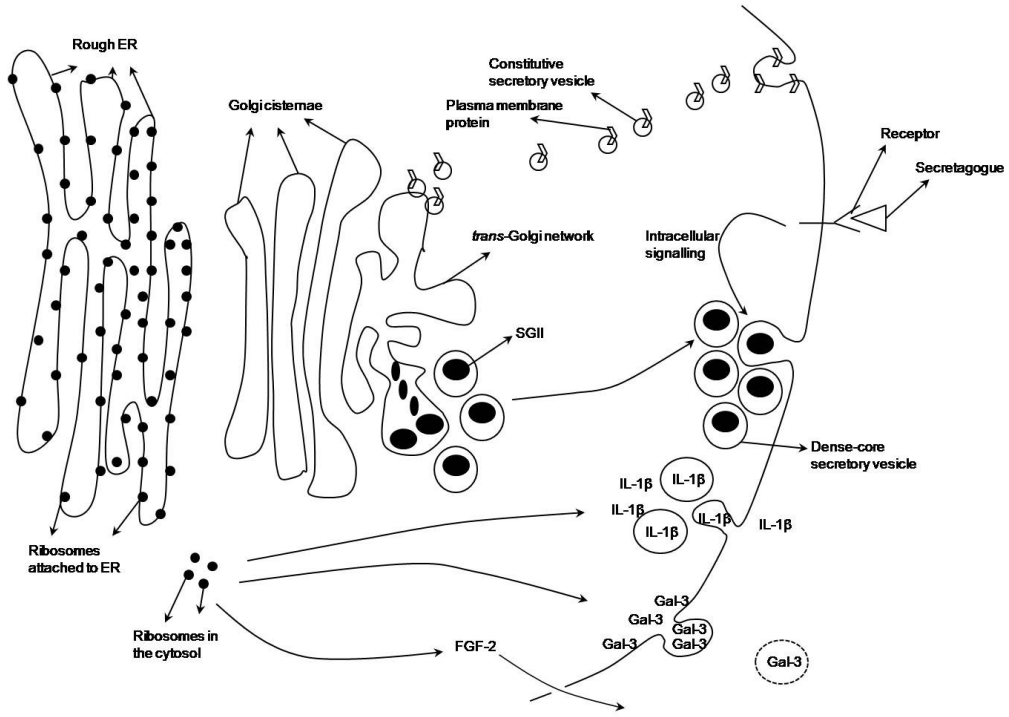


Figure 1.1 Secretion pathways. Cells release substances via two main secretion pathways: constitutive and regulated. Proteins are synthesised on ribosomes of the rough ER, transported through Golgi cisternae and sequestered within constitutive secretory vesicles labelled with membrane proteins for the constitutive pathway, or within dense core secretory vesicles containing core made of granins for the regulated pathway. Regulated secretion requires an extracellular stimulus or secretagogue and increased intracellular calcium. However, some proteins (eg FGF-2, IL-1β, galectin-3) leave the cell via unconventional secretion, i.e. not via the ER-Golgi pathway.

of the kidney secrete renin (Friis et al., 2000), endothelial cells secrete Weibel-Palade bodies (Birch et al., 1992), and non-neuronal cells of the CNS (astrocytes) may secrete neurotransmitters (Volterra and Meldolesi, 2005).

More recently, it was discovered that some proteins may leave cells through routes that do not involve the ER-Golgi pathway, termed unconventional secretion (Figure 1.1). The first proteins shown to exhibit unconventional secretion were the cytokine interleukin-1β (Rubartelli et al., 1990) and the carbohydrate-binding protein galectin-1(Cooper and Barondes, 1990). However,

over the last two decades the list has grown to include other proteins such as fibroblast growth factor-1 (Jackson et al., 1992), fibroblast growth factor-2 (Mignatti and Rifkin, 1991) and galectin-3 (Sato and Hughes, 1994). Also, there are a number of membrane-anchored precursors that are released on stimulation through mechanisms involving cleavage (or shedding) converting them into soluble forms for example members of the EGF family such as heparin-binding EGF-like growth factor (Goishi et al., 1995; Higashiyama et al., 1991).

1.2.2 Mechanism of secretion

The classical secretory pathway can be dissected into a number of functional stages (Burgess and Kelly, 1987). It starts with synthesis, i.e. addition of aminoacids, at ribosomes attached to the rough ER where the protein is passed through the membrane of the ER to the lumen; some post-translational modifications can occur at this stage including signal peptide cleavage, glycosylation and folding even before protein synthesis is complete. Secretory proteins entrapped within transport vesicles then leave the ER and are directed to the Golgi apparatus, where they fuse with Golgi membranes and their cargo passes into the Golgi lumen. The Golgi apparatus consists of three functionally distinct regions *cis-, medial-* and *trans-*, the latter also giving rise to the terminal compartment the *trans*-Golgi network (TGN). Within the Golgi apparatus proteins are modified by glycosylation and in the TGN there may be phosphorylation or sulphation, followed by sorting and packing within secretory vesicles. Sorting of proteins to the appropriate pathway depends on multiple factors, and those with relevance to this study are discussed later. Proteins

packaged into secretory vesicles pass through the cytosol to the plasma membrane for release via exocytosis. The process of membrane fusion involves multiple stages, including tethering, docking, fusion and release (Burgoyne and Morgan, 2003). Although constitutive and regulated secretory pathways share the basic cellular exocytotic machinery, there is specificity in the molecular mechanisms most notably there is an obligatory calcium-dependent mechanism involved in regulated exocytosis.

1.2.3 Cellular machinery for regulated secretion

1.2.3.1 Regulated secretory proteins

A number of proteins have been shown to be involved in the formation and maturation of the dense core secretory vesicles, most notably members of the chromogranin family. These are acidic, soluble secretory proteins present in cells exhibiting regulated secretion from dense core secretory vesicles of neuronal and endocrine lineages (Wiedenmann and Huttner, 1989). The first members of the group to be identified were the "classic" granins, namely chromogranin A which was initially isolated from chromaffin cells of the adrenal medulla (Banks and Helle, 1965; Helle, 1966), chromogranin B (or secretogranin I) which was isolated from a rat pheochromocytoma cell line (Lee and Huttner, 1983) and secretogranin II, previously known as chromogranin C which was initially described in anterior pituitary (Fischer-Colbrie et al., 1995; Rosa and Zanini, 1981). Six other acidic soluble proteins, which are less well known, have since been identified including secretogranins III (1B1075)(Ottiger et al., 1990), IV

(HISL-19 antigen)(Neuhold and Ullrich, 1993), V (neuroendocrine secretory protein 7B2)(Seidah et al., 1983), VI (NESP55)(Ischia et al., 1997), VII (the nerve growth factor inducible protein VGF)(Levi et al., 1985) and pro-SAAS (Fricker et al., 2000).

The *Chromogranin A* and *B*, and *secretogranin II* genes, contain cAMP-response elements within their proximal promoters, which play a crucial role in neuroendocrine-specific gene expression (Mahapatra et al., 2000; Mahata et al., 1999; Wu et al., 1995). Granin synthesis seems also to respond to agents like steroid hormones and neurotrophin growth factors (Taupenot et al., 2003).

The primary structure of granin molecules consist of a single polypeptide chain with an amino-terminal signal peptide sequence and a similar carboxy-terminal motif. Chromogranin A and B also share a highly-homologous structure in their amino-terminal region, namely a disulfide-bonded loop (Benedum et al., 1987). There are multiple sites for proteolytic processing (i.e. pairs of basic amino acid residues) suggesting that these molecules act as precursors for smaller secreted peptides. Thus cleavage of the granins by prohormone convertases-1 and -2 (Eskeland et al., 1996; Laslop et al., 1998), and plasmin (Jiang et al., 2001), leads to generation of biologically active products such as pancreastatin (Tatemoto et al., 1986) and vasostatin from chromogranin A (Aardal et al., 1993; Ratti et al., 2000) and secretoneurin from secretogranin II (Fischer-Colbrie et al., 1995), which can act as autocrine, paracrine or endocrine factors.

The granins bind calcium with low affinity, but high capacity and tend to aggregate at low pH and in the presence of calcium, a process believed to determine aggregation in the TGN and thought to be part of a sorting mechanism

for secretory vesicles of the regulated pathway, so-called "sorting by retention" (Gerdes et al., 1989; Reiffen and Gratzl, 1986; Yoo and Albanesi, 1991). Through condensation and aggregation at low pH and high calcium, the granins are believed to be an essential component of the dense core of secretory vesicles of the regulated secretory pathway by controlling vesicle biogenesis. Also, granins serve as chaperones in the sorting of other regulated secretory proteins into dense core secretory vesicles (Gerdes et al., 1989; Gorr et al., 1989; Huttner and Natori, 1995). Thus the granins have been suggested to serve as markers of dense core secretory granules in neurons and endocrine cells in both normal and neoplastic tissues (Huttner et al., 1991). Immunoreactivity for chromogranins A and B, and to some extent of secretogranin II and other granins, has been linked with different neuroendocrine tumours and their circulating levels may reflect the secretory activity of these tumours (Taupenot et al., 2003).

While chromogranin A and B have been quite intensively studied, secretogranin II is somewhat less well understood. It exhibits relatively weak homology with chromogranins A and B (Taupenot et al., 2003). Nevertheless, recent reports have shown that both N- and C-terminal regions of the polypeptide structure contain independent sorting signals that are sufficient to direct secretogranin II into the dense core granules of the regulated pathway (Courel et al., 2010).

1.2.3.2 Membrane fusion proteins

A central role in membrane fusion events is played by the membrane-bound SNAP receptor proteins (SNAREs) (Sollner et al., 1993b). SNARE proteins are

receptors for N-ethylmaleimide-sensitive fusion protein (NSF) and α-soluble NSF attachment protein (α-SNAP) (Burgoyne and Morgan, 2003). All SNARE proteins have a characteristic heptad repeat sequence, termed the SNARE motif (Kloepper et al., 2007). Of the many SNAREs in the human genome only a dozen or so are fusogenic (Sudhof and Rothman, 2009). Individual SNARE proteins are unfolded, but they spontaneously assemble into a stable four-helix bundle (Sutton et al., 1998) that forms between the two membranes and catalyzes fusion by forcing membranes together as they zipper up (Hanson et al., 1997). SNAREs can be separated in two groups: present on the vesicle (donor) membrane i.e. v-SNAREs (vesicle-associated membrane proteins such as VAMP and synaptobrevin), and present on the target (acceptor) membrane i.e. t-SNAREs (such as syntaxin and SNAP-25) (Gerst, 1999). According to the SNARE hypothesis, v-SNAREs and t-SNAREs form a stable complex that targets the vesicle to the correct destination at the plasma membrane (Rothman and Orci, 1992). Subsequent binding of the core SNARE complex to α-SNAP and adenosine triphosphate(ATP)ase NSF, which brings a significant energy input associated with ATP, disassembles the SNARE complex (Sollner et al., 1993b).

Synaptobrevin/VAMPs are small membrane proteins of about 120 amino acids, that exhibit extended or two short amphipathic α-helical segments, able to form coiled-coil structures (Gerst, 1999). Synaptobrevin/VAMPs are components of synaptic vesicle membranes (Baumert et al., 1989) and bind α-SNAP (Sollner et al., 1993a). They are substrates for clostridial neurotoxins, i.e. botulinum and tetanus toxins, which block synaptic transmission at the presynaptic membrane

(Montecucco and Schiavo, 1993). There are at least four known synaptobrevin/VAMP isoforms, associated with secretory granule and synaptic vesicle release in mammalian cells (Gerst, 1999). It has been discovered that the toxin-sensitive VAMP2, and the toxin-insensitive VAMP1A, are present in secretory granules, dense core vesicles and synaptic vesicles (Baumert et al., 1989). Other VAMP isoforms are thought to be related to the constitutive pathway (Gerst, 1999).

Syntaxins are t-SNARE proteins of around 300 amino acids with at least three putative coiled-coil forming regions (sites for protein binding) and a single transmembrane domain (Bennett et al., 1993). There are at least four known syntaxins (syntaxin 1A, B and possibly C, syntaxins 2 to 4) present in the plasma membrane and associated with exocytosis (Bennett et al., 1993). Syntaxins have roles in the earlier steps of the secretory pathway (Gerst, 1999), they can interact with calcium-sensing protein synaptotagmin (Shao et al., 1997) and the t-SNARE SNAP-23 associated with the constitutive secretion (Wang et al., 1997).

SNAP-25 is expressed by neurons (Oyler et al., 1989) and by many non-neuronal cells exhibiting regulated secretion (Kannan et al., 1996; Sadoul et al., 1995). Like other SNAREs, SNAP-25 has heptad repeats, is able to form coiled-coils and α-helices (Gerst, 1999) with synaptobrevin/VAMP and syntaxin (Chapman et al., 1994). SNAP-25 is linked to calcium-sensing via binding to synaptotagmin (Schiavo et al., 1997) and via interaction with the calcium channels (Wiser et al., 1996).

1.2.3.3 Calcium-sensing proteins

Many different proteins with calcium-binding properties have been described. For example, specific C_2 domains in the primary structure of protein kinase C, synaptotagmin, rabphilin 3A, calmodulin, calcyclin and annexin allow them to bind phospholipids in a calcium-dependent manner (Burgoyne and Morgan, 1998). One of the best characterised families of putative calcium sensors is the family of 13 proteins called synaptotagmins (Brose et al., 1992). Synaptotagmins also bind other molecules involved in regulated exocytosis including calcium-channels (Leveque et al., 1992), SNARE complex (Sollner et al., 1993a) and calmodulin (Fournier and Trifaro, 1988).

1.2.3.4 Other regulated secretory proteins

SNAREs have been identified as essential components for the fusion event, but they are far from being the only proteins involved. For example, Rab GTPases have been long known to play a role in regulated secretion by regulating the transport of vesicles through the actin cytoskeleton and by regulating the assembly of the tethering proteins between vesicle and target membrane (Zerial and McBride, 2001). Other important regulators belong to the SM (Sec1/Munc18) protein family, where Sec1 is a yeast protein and Munc18 stands for the Munc18 mammalian proteins (Burgoyne et al., 2009). SM proteins have been extensively studied in relation to membrane fusion events. They consist of around 600 amino acids that fold into an arch-shaped “clasp” structure (Misura et

al., 2000) and are capable of interacting with SNAREs and either inhibiting (Sudhof and Rothman, 2009) or promoting fusion (Yamaguchi et al., 2002).

Other regulators of SNARE assembly and disassembly may act to activate or inhibit fusion depending on the conditions. These include "grappling" proteins involved in membrane fusion, i.e. they keep the cell machinery in a "ready-to go" state so that a small stimulus can trigger the fusion event (Sudhof and Rothman, 2009). Synaptotagmin and complexins are the best described grappling proteins, which are responsible for the precise timing and place of release (Rizo and Rosenmund, 2008). SNAREs complexes are clamped by complexins in an activated state (Tang et al., 2006). Increases in intracellular calcium, followed by binding to synaptotagmin serve as a trigger to release the clamp and for the SNARE complex to proceed to fusion (Pang et al., 2006).

Other protein families described as SNARE function regulators include synaptophysin (for v-SNAREs), DOC2 (modulator of syntaxin-MUNC18 interaction), protein kinase C (a calcium-dependent tyrosine kinase); moreover, there is a large group of proteins implicated in regulated secretion including syncollin, Hrs-2, SV2, CAPS and VAP33 (Gerst, 1999).

1.3 The gastrointestinal tract and epithelial-mesenchymal communication

1.3.1 Epithelial-mesenchymal communication in health

Tissue organisation in hollow organs (e.g. gastrointestinal tract, airways, urogenital system) is maintained by interactions between epithelial cells and underlying mesenchymal (stromal) cells that are the source of growth factors

such as IGF and TGF-β (Powell et al., 1999a). Epithelial-mesenchymal cell communication is recognised to be crucial in normal development and differentiation, and in wound healing, inflammation and cancer (Castro and Arntzen, 1993; Perdue and McKay, 1994).

There is a common pattern of organisation along the length of the gastrointestinal tract or the gut, although each region has its own characteristic structure. Typically there is: mucosa, consisting of an epithelial layer facing the lumen; the underlying lamina propria; a thin layer of smooth muscle (muscularis propria); a dense irregular layer of connective tissue called submucosa that includes a nerve plexus (the submucosal plexus); two layers of smooth muscule (inner circular and outer longitudinal) with a nerve plexus (the myenteric plexus) between them; and adventitia or serosa consisting of several layers of connective tissue (Lund, 1999).

Each compartment of the gut wall consists of characteristic cell types, but in recent years myofibroblasts have emerged as a key component of sub-epithelial tissues that play a role in determining tissue organisation (Figure 1.2). These cells can be divided into two sub-groups depending on their exact localisation: subepithelial myofibroblasts are found in the lamina propria, and interstitial cells of Cajal are in the muscularis propria and in the submucosa (Powell et al., 1999b). There is a tight perycriptal sheath of myofibroblasts of the lamina propria in the intestine (Pascal et al., 1968), which forms a syncytium extending throughout lamina propria (Joyce et al., 1987). In the stomach, myofibroblasts do not form a tight sheath (Mutoh et al., 2005), but they are found scattered within the gastric mucosa (Wu et al., 1999), and represent one of the major cell types

producing factors influencing the organisation, development and differentiation within the mucosa.

1.3.2 Epithelial-mesenchymal communication in disease

1.3.2.1 *H. pylori* infection and other gastric inflammatory conditions

Changes in tissue architecture such as occurring in inflammation and wound healing are associated with increased proliferation and migration of cells in both epithelial and subepithelial compartments, and include changes in myofibroblast numbers and activity (Figure 1.2).

In the case of the upper gastrointestinal tract, the point is well illustrated by the changes that occur with *Helicobacter pylori* infection. Originally identified in 1982 by Barry Marshall and Robin Warren, *H.pylori* infection is associated with chronic gastritis and peptic ulcer (Marshall and Warren, 1984). It is now known that *H.pylori* infection is related to changes in the tissue microenvironment including changes in gene expression in gastric epithelial cells (Mills et al., 2001) and increased gastrin secretion (Levi et al., 1989). Increased gastrin secretion leads to increased acid secretion and activates local defence systems e.g. increasing epithelial cell migration and cell renewal, and growth factor secretion (Kirton et al., 2002; Noble et al., 2003).

Prolonged elevation of plasma gastrin is also associated with other conditions such as achlorhydria e.g. pernicious anaemia (Dockray et al., 2005; Jensen, 2002). Pernicious anaemia is a condition of chronic inflammation in the stomach,

which develops on the basis of atrophic gastritis and is associated with generation of auto-antibodies to gastric parietal cells, leading eventually to their

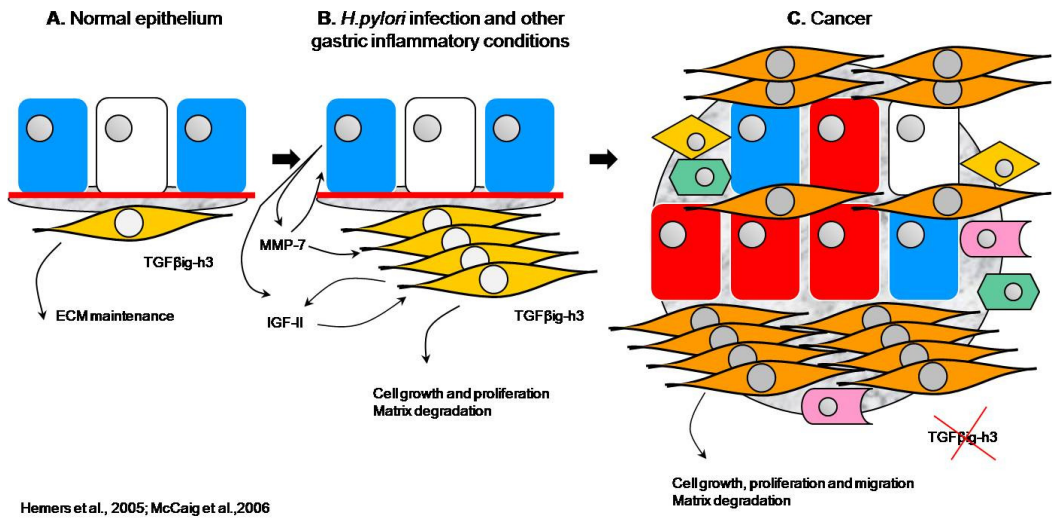


Figure 1.2 Epithelial-mesenchymal signalling in health and disease, and in cancer. Myofibroblast is a key cell type in epithelial-mesenchymal cross-talk, determining normal tissue organisation (**A**). In *H.pylori* infection and other gastric inflammatory conditions there is increased secretion of growth factors and matrix proteases, i.e. normal epithelial-mesenchymal signalling is impaired, leading to cell growth and proliferation and ECM degradation (**B**). Transition from normal conditions to cancer is associated with changes in epithelial-mesenchymal signalling, including changes in the genome, proteome and secretome of myofibroblasts e.g. decreased secretion of ECM protein TGFβig-h3 (**C**).

loss. Gastric acid, produced by parietal cells, inhibits G-cell function, so following atrophy there is increased G-cell numbers and increased plasma gastrin. Elevated plasma gastrin causes hyperplasia of the histamine-secreting enterochromaffin-like cells (ECL) in the gastric corpus (Bordi et al., 1995), and in some cases this may lead to dysplasia and formation of ECL carcinoid tumours (Dockray et al., 2005; Gibril et al., 2004; Peghini et al., 2002). Importantly, elevated gastrin also stimulates production of MMP-7, which in turn

stimulates proliferation of gastric myofibroblasts and epithelial cells via cleavage of IGFBP-5 and liberation of IGF-II (Varro et al., 2007) (Figure 1.2).

1.3.2.2 Gastric cancer and tumour microenvironment

Many reports associate chronic inflammation with *H.pylori* in the stomach with an increased risk of gastric cancer (Parsonnet et al., 1991). Gastric cancer is one of the most common malignancies of the gastrointestinal tract. Despite its declining incidence, 5-year overall survival is generally accepted as <15% for patients diagnosed in late stages (Gao et al., 2008; Siewert et al., 1998), which has been attributed to its late clinical onset and rapid progression (Jemal et al., 2009; Lochhead and El-Omar, 2008). A multistep model for progression to gastric cancer was proposed by Correa, starting with chronic gastritis, through atrophy, metaplasia and dysplasia leading to carcinoma (Correa, 1992).

Most gastric cancers (~95%) are adenocarcinomas of intestinal, diffuse or mixed type according to the Lauren histological classification (Lauren, 1965). The other 5% are rare cancers of the smooth muscle (leiomyosarcomas), lymph (lymphomas), neuroendocrine (carcinoids) or stromal tissue (gastrointestinal stromal tumours or GIST) (Rotterdam, 1989). Beside gastric cancer another malignancy of the upper gut is the oesophageal cancer. Oesophageal cancers predominantly are squamous cell carcinomas (in the upper or middle part of the oesophagus) and adenocarcinomas (in the lower part of oesophagus) (Daly et al., 2000).

Increasingly, a role for the microenvironment in promoting preneoplastic tissue remodelling has been recognised. The idea that tumour development requires an appropriately supportive tissue microenvironment, can be found in the "soil and seed hypothesis" first discussed by Paget in 1889 (Paget, 1889). The term "tumour microenvironment" was introduced in the late 1970s to stress the importance of the microenvironment (Cunha and Matrisian, 2002). The tumour microenvironment consists of the interconnected host cells of the cancer stroma including connective tissue cells, blood vessels and inflammatory cells, and the surrounding ECM, as well as cancer cells themselves (Figure 1.2). It is recognised that the tissue microenvironment can exert dual effects both protecting the host from cancer cell expansion and promoting cancer cell growth.

In some solid tumours the stromal compartment can account for more than 90% of the tumour mass (Ronnov-Jessen et al., 1996). Myofibroblasts have been recognised as a particularly abundant cell type in the tumour stroma, and more recently were identified as an active contributor in carcinogenesis (Bhowmick et al., 2004).

1.4 Myofibroblasts

1.4.1 The origin of myofibroblasts

Myofibroblasts are derived from fibroblasts which are non-vascular, non-epithelial and non-inflammatory cells that constitute the principal cellular component of connective tissue (Kalluri and Zeisberg, 2006). Various stimuli, including tissue injury and inflammation, and mediators such as TGFβ and

PDGF (Desmouliere et al., 2003), convert fibroblasts to myofibroblasts (sometimes called activated fibroblasts). Similar processes are thought to occur during cancerogenesis (De Wever and Mareel, 2002), and are associated with changes in gene expression including increased expression of α-smooth-muscle actin (Gabbiani et al., 1971). However, another hypothesis suggests that myofibroblasts share some common features with, and may arise from, smooth muscle cells. For example, intestinal myofibroblasts were described to originate histologically from the thin layer of smooth muscle of the muscularis mucosae (Powell, 2005). This was also the case for at least part of the cancer-associated myofibroblasts (CAMs) in the invasive gastric carcinoma (intestinal type) (Jiang et al., 2008).

Recent data has shown that bone marrow-derived mesenchymal stem cells (MSCs) may contribute to the development of inflammation-based gastric cancer (Houghton et al., 2004), and that these cells may also to be transformed into CAMs at the tumour site (Quante et al., 2011). Notably, 15-25% of CAMs in the tumour stroma of mouse gastric cancers exhibited markers of bone marrow-derived MSCs (Direkze et al., 2004).

Adult bone-marrow is a source of two different pluripotent cell populations: haematopoietic stem cells (Lewis and Trobaugh, 1964) and MSCs (Beresford, 1989; Caplan, 1991). MSCs under appropriate conditions are able to differentiate into different cell lineages, including fibroblast/myofibroblasts, osteoblasts (Haynesworth et al., 1992), chondrocytes (Johnstone et al., 1998) and adipocytes (Pittenger et al., 1999). By doing so, stem cells are efficient in maintaining tissue and organ homeostasis through regeneration (Urbanek et al., 2005). In addition,

however, during the multi-step lineage progression, MSCs respond to a spectrum of stimuli via synthesis and secretion of a number of growth factors and cytokines so that they also have both direct and indirect effects on other cells in their proximity (Haynesworth et al., 1996).

1.4.2 Role in health

Myofibroblasts are usually found in relatively low density. They regulate epithelial morphogenesis (Bhowmick et al., 2004) and maintain the structural integrity of the ECM by secreting many of its components including collagen type I, III and V and fibronectin (Rodemann and Muller, 1991; Tomasek et al., 2002). By secretion of type IV collagen and laminin they also contribute to the formation of basement membrane (Chang et al., 2002). They produce and secrete many growth factors including TGF-β (Border and Noble, 1994), PDGF (Bostrom et al., 1996; Leveen et al., 1994), HGF (Goke et al., 1998), IGF-I and IGF-II (Lund, 1999), growth factor binding proteins, proteases and protease inhibitors (Kalluri and Zeisberg, 2006). Myofibroblasts are found in increased numbers during the repair processes involved in the response to tissue injury, damage or infection (De Wever and Mareel, 2002) when they release increased amounts of ECM proteins and ECM-degrading proteases such as matrix metalloproteinases (MMP)-2, MMP-3 and MMP-9, implicated in increased ECM turnover (Rodemann and Muller, 1991). Additionally myofibroblasts participate in local immune response, via secretion of cytokines, such as IL-1, and chemokines (Rollins et al., 1989; Strieter et al., 1989).

Following wound healing, the number of myofibroblasts decreases and they regain their resting phenotype or undergo apoptosis (Tomasek et al., 2002). However, in some circumstances myofibroblasts remain activated leading to tissue fibrosis (Kalluri and Zeisberg, 2006) and their contraction accounts for the distorted architecture of scar tissue.

Besides their phenotypic and functional similarities, myofibroblasts from different tissues may exhibit degrees of specificity e.g. intestinal subepithelial myofibroblasts participate in electrolyte transport (Valentich, 1994) and interstitial cells of Cajal are implicated in the control of motility throughout the gut (Sanders, 1996).

1.4.3 Role in cancer

Recent work emphasises that CAMs differ from normal tissue myofibroblasts in number, cellular architecture, localisation, and in functional properties (De Wever and Mareel, 2002). Nevertheless, like normal myofibroblasts they participate in paracrine and autocrine control mechanisms involving secretion of signalling molecules such as growth factors and their binding proteins, proteases and protease inhibitors, and multiple ECM proteins, that collectively support cancer development (Kalluri and Zeisberg, 2006). In addition to secretion of soluble factors, myofibroblasts participate in the recruitment of other stromal cell types including macrophages, pericytes and endothelial cells (Rodemann and Muller, 1991), however in the later stages they support cancer progression simply by blocking immune and inflammatory cells from the tumour site

(Lieubeau et al., 1999). Also, myofibroblasts contribute to cancer progression by facilitating the invasiveness, i.e. metastasis, of otherwise non-invasive cancer cells (Dimanche-Boitrel et al., 1994). Further, myofibroblasts in the metastatic site support cancer cell growth similar to the myofibroblasts in the initial tumour site (Olaso et al., 1997).

A significant body of the work on myofibroblasts in cancer, has come from studies of breast and prostate cancer and by comparison much less is known of gastric cancer. Even so, however, recent work has shown that gastric myofibroblasts contribute to tumour development (Yuhiko Fuyuhiro and Hirakawaawaawa, 2010) and angiogenesis (Guo et al., 2008). Moreover, unpublished work from this laboratory has shown that gastric CAMs unlike normal gastric myofibroblasts can stimulate cancer cells proliferation, invasion and migration both *in vitro* and *in vivo* (Charlotte Woodcock, MPhil thesis); and a myofibroblasts-derived ECM protein TGFβig-h3 acts to inhibit these effects (Kumar & Varro, unpublished observations).

1.4.4 The myofibroblast secretome

Previous studies on colon and gastric myofibroblasts, showed that IGFBP-5 was a target of matrix metalloprotease-7 (MMP-7) and that following cleavage there was release of IGF-II (Hemers et al., 2005) which in turn stimulated proliferation and migration of both epithelial cells and gastric myofibroblasts (Hemers et al., 2005; McCaig et al., 2006). Other proteins identified as secreted by myofibroblasts in these studies included collagen α1 types I, III and VI, and

fibronectin. Subsequently, a number of differentially secreted proteins in CAMs compared to normal myofibroblasts were identified including e.g. TGFβig-h3, which was decreased in the media of CAMs from patients with advanced disease compared with myofibroblasts from early stage disease, or normal cells (Holmberg & Varro, unpublished observations). However, these studies leave unanswered the question of the mechanism of secretion by myofibroblasts and of the changes occurring in cancer. The following sections briefly introduce some of the main protein groups known to be released by myofibroblasts and so potentially accounting for the difference between CAMs and normal myofibroblasts.

1.4.4.1 Matrix proteases and their inhibitors

Myofibroblasts produce and secrete a range of matrix proteases and protease inhibitors including members of zinc superfamily of proteases (containing Zn^{2+} ion in the catalytic centre): matrix metalloproteases (MMPs) and desintegrin and metalloproteases (ADAMs) (Powell et al., 1999a). The MMPs are a family of 23 zinc-dependent endopeptidases, and they are separated into two major groups: soluble and membrane-anchored (Klein and Bischoff, 2011). In turn, soluble MMPs are characterised according to their substrate specificity as collagenases, gelatinases, stromelysins and a heterogenous group including for example MMP-7. Initially, MMPs were believed to modulate and regulate ECM turnover by directly degrading ECM proteins such as collagen, proteoglycans and fibronectin (Woessner, 1991). However, MMPs also degrade other components of the ECM including growth factors, cytokines, adhesion molecules and protease inhibitors

such as serine protease inhibitors (Nagase and Woessner, 1999). Also, MMPs liberate active forms of growth factors, cytokines and chemokines from their membrane-anchored pro-forms, a process termed shedding (Klein and Bischoff, 2011).

Most MMPs are produced by subepithelial cells such as myofibroblasts, but MMP-7 is an exception that is predominantly synthesised by epithelial cells, including those of the gastrointestinal tract (Lopez-Boado et al., 2000). Recent reports suggest that MMP-7 mediates responses to bacteria in different systems including in *H. pylori* infection in the stomach (Crawford et al., 2003; Wroblewski et al., 2003), is elevated in hypergastrinaemia (Varro et al., 2007); as noted above, liberates IGF-II by cleavage of IGFBP-5 and it has been suggested to play a role in mucosal remodelling (Hemers et al., 2005).

Tissue inhibitors of matrix metalloproteases (TIMPs) are the natural inhibitors of MMPs (Gomez et al., 1997). There are four known members of the family (TIMPs 1-4), numbered depending on the order of discovery, that exhibit similarities but with specific structure and functional roles (Greene et al., 1996). TIMPs were described to exhibit effects on cell growth and apoptosis independent from their protease inhibitor properties. Data on their role in cancer is controversial: some studies show that lower secretion rate of TIMPs is conducive for the development of distant metastases or tumour growth (Sakata et al., 2000; Vizoso et al., 2007), while others show that higher TIMP expression correlates with poor prognosis (Egeblad and Werb, 2002). It is believed that these effects may depend on the type and stage of disease, however the exact mechanisms are still unclear.

MMPs are secreted as inactive precursor molecules proteolytically activated by different matrix proteases and non-proteolytic agents (Nagase and Woessner, 1999). Two proteases that can activate pro-MMPs are urokinase type (uPA) and tissue type (tPA) plasminogen activators (Carmeliet et al., 1997; Yepes and Lawrence, 2004; Zhao et al., 2007). Both plasminogen activators convert plasminogen into its active form plasmin (Robbins et al., 1967) that in turn digests fibrin and other ECM proteins (Andreasen et al., 1997; Blasi et al., 1987; Robbins et al., 1967). The plasminogen activation system also includes three inhibitors: plasminogen activator inhibitor (PAI)-1, -2 and protease nexin, and the uPA receptor (uPAR).

Increased expression of the uPA system is associated with different tissues responses to injury and infection (Andreasen et al., 1997). For example, infection with *H.pylori* is associated with increased expression of uPA, uPA receptor, PAI-1 and PAI-2 in the gastric mucosa (Kenny et al., 2008; Varro et al., 2004). Moreover, increased expression of members of the uPA system was reported in gastric cancers with poor prognosis (Ito et al., 1996; Kaneko et al., 2003). Although little information is available about protease nexin, there is evidence for promoting the invasive phenotype of pancreatic and breast cancer cells via modifying ECM environment (Buchholz et al., 2003; Candia et al., 2006), and it is suggested to be a poor prognostic marker (Gao et al., 2008).

Different organisation of tPA molecule determines its high affinity for plasma fibrin (van Zonneveld et al., 1986a), and therefore, tPA is primarily studied as a thrombolytic agent (van Zonneveld et al., 1986b), although other functions are known.

1.4.4.2 Extracellular matrix proteins

Formation and remodelling of ECM is essential in tissue growth, wound healing and fibrosis, and in cancer. ECM consists of very many different components including a large group of protein-polysaccharide complexes called proteoglycans and protein fibrils (e.g. collagens, fibronectin, and laminin), both synthesised and secreted by specialised cells of the connective tissue like myofibroblasts.

Small leucine-rich proteoglycans (SLRPs) are a family of nine proteoglycans divided according to their gene and protein structure in three classes (Iozzo, 1998). Class I comprises of two proteins: decorin and biglycan, class II includes fibromodulin, lumican, keratocan, and PRELP, and class III consists of epiphycan and osteoglycin.

Decorin is the best known SLPR. Decorin has roles in the assembly of ECM via binding to collagen I, II and VI fibrils, and fibronectin *in vivo* (Iozzo, 1998), and in physiological process like inflammation and wound healing (Ferdous et al., 2007). Also, decorin is one of the major SLRPs of the human sclera and its expression is directly related with the maintenance of corneal transparency (Rada et al., 2000).

Reduced expression of decorin has been found in many epithelial tumours including colon, pancreas, breast (Iozzo and Cohen, 1993) and has been associated with carcinogenesis (Bi et al., 2008). Moreover, it has been shown that decorin inhibits tumour growth via different mechanisms: down-regulation of epidermal growth factor receptor (EGFR) (Iozzo et al., 1999), ErbB2 (Santra et

al., 2000) and hepatocyte growth factor (HGF or Met) receptor (Goldoni et al., 2009), as well as neutralising TGF-β signalling (Yamaguchi et al., 1990) and up-regulating cell cycle inhibitors p21 and p27 (Xaus et al., 2001). Decorin may therefore act as an extracellular tumour suppressor (Augoff et al., 2008). In addition, altered decorin expression in the stroma was reported in breast cancer (Kelemen et al., 2008). *In vivo* experiments linked altered decorin gene expression in the stroma with cancer-host cell signalling (Adany et al., 1990).

Lumican is another SLRP which, similarly to decorin, is a potent regulator of collagen fibrillogenesis (Rada et al., 1993) in dermal and muscle connective tissue (Chakravarti et al., 1998). Also, lumican in the human sclera and cornea is crucial for the maintenance of corneal transparency (Rada et al., 1993; Young et al., 2003). Vuillermoz et al. reported that lumican inhibits melanoma cell growth both *in vitro* and *in vivo* (Vuillermoz et al., 2004). It is has been reported that reduced expression of both decorin and lumican was associated with poor outcome in invasive breast cancers (Troup et al., 2003).

1.4.4.3 Small ECM proteins

Initial proteomic studies identified increased abundance of the small ECM protein TGFβ-inducible protein (TGFβig-h3, TGFBI, BIGH3, BIG-H3, RGD-CAP or kerato-epithelin) in the media of gastric myofibroblasts after treatment with MMP-7 (Duval & Varro, unpublished observations). TGFβig-h3 was first identified as a secreted protein in human adenocarcinoma cell lines after treatment with TGF-β (Skonier et al., 1992). TGFβig-h3 is believed to play a role

in cell adhesion, proliferation and migration through interaction with a number of ECM molecules like collagens, fibronectin, laminin, and also functioning as a ligand for several integrins (Kim et al., 2000). In the cornea, TGFβig-h3 co-aggregates with decorin in complexes with collagen VI (Reinboth et al., 2006). It is expressed in various cells, and mutations of the gene are associated with multiple forms of hereditary corneal distrophies (Zamilpa et al., 2009).

Down-regulation or loss of expression of TGFβig-h3 has been reported in primary tumours and cancer cell lines from lung and breast, hence it was described as a tumour suppressor and proposed as a diagnostic marker (Calaf et al., 2008; Zhang et al., 2009; Zhao Y., 2006). However, there are studies showing that TGFβig-h3 supports tumour growth and metastasis, suggesting that its pro- or anti-oncogenic effects may depend on tissue type and experimental conditions (Ma et al., 2008; Tang et al., 2007).

1.4.4.4 Carbohydrate-binding proteins

Galectins are a family of lectins binding β-galactoside through an evolutionary preserved carbohydrate-recognition domain (CRD) in their structure (Barondes et al., 1994). Based on the number and organisation of CRDs they are classified in three subtypes (Hirabayashi and Kasai, 1993). Galectin-3 has a unique structure amongst the vertebrate galectins, as it contains proline- and glycine-rich N-terminal domain (Houzelstein et al., 2004). Galectin-3 is known to lack an N-terminal signal peptide sequence directing a protein to ER-Golgi secretory pathway and thus it is released via an unconventional secretion pathway

(Hughes, 1999). Galectin-3 is ubiquitously expressed and can be localised to the nucleus and cytoplasm, or be secreted depending on various factors (Dumic et al., 2006). Intra- and extracellular localisation of galectin-3 is associated with specific biological functions in cell communication, development, in the immune response, in disease and in carcinogenesis.

1.5 Studies of secretome and SILAC

There is increasing evidence, including from this laboratory, that myofibroblasts undergo multiple changes in the transition to cancer and that these include changes in the secretome, i.e. the total of all secreted proteins (excluding membrane-bound proteins). In part these studies have been made possible through the recent technological advances in the proteomic techniques, notably the development of mass spectrometry, and the availability of well annotated gene and genome databases that made possible proteomic characterisation of complex protein samples.

One widely used mass-spectrometry based method that is applicable in secretome studies is stable isotope labelling by amino-acid in culture or SILAC (Alvarez-Llamas et al., 2007; Greco et al., 2010). In SILAC, cells are cultured in media supplemented with normal amino-acids (Light label), or with amino-acids labelled with stable isotopes (Heavy label). Thus light and heavy peptides in two or more samples can be identified and relatively quantified. Also, SILAC makes it possible (a) to exclude serum contaminants, which is not possible with some other proteomic methods; (b) to reduce sources of error introduced during sample

preparation prior to mass spectrometry; and (c) provides more accurate quantification of low signal-to-noise samples (Ong et al., 2002). Therefore, SILAC is routinely used in the assessment of protein expression as a function of time or stimulus (Ong and Mann, 2005). Moreover, SILAC can be used in the evaluation of mechanisms controlling protein secretion (Greco et al., 2010), for example, in myofibroblasts, where they are poorly understood.

1.6 Aims and objectives

The primary objective of this thesis was to determine the mechanisms by which myofibroblasts from normal tissue or cancers of upper gastrointestinal tract secrete proteins that might influence the cellular microenvironment. This study therefore investigated the secretion by primary myofibroblasts from normal tissue, cancers of stomach and oesophagus, and tissue adjacent to these cancers; for comparison studies were also made on human MSCs. The specific aims were:

1. To establish a model system for studying calcium-dependent secretion of myofibroblasts.

2. To assess the influence of the tissue microenvironment in determining the secretory phenotype of myofibroblasts.

3. To identify and study putative secreted proteins with potential as biomarkers.

4. To identify and characterise the molecular mechanisms determining regulated secretion by gastric myofibroblasts.

CHAPTER 2

MATERIALS AND METHODS

2.1 Primary cell cultures

2.1.1 Primary human myofibroblasts

Many of the primary human myofibroblasts used for these studies had been isolated from stomach or oesophagus obtained during surgery for cancer resection. In these cases, tissue had been taken from the tumour (CAM) and usually from an immediately adjacent, macroscopically normal, region within 10mm of the resection border (ATMs).

In addition, some myofibroblasts (NTM) were obtained from normal stomach or oesophagus of transplant donors, and some from the antrum of patients with pernicious anaemia undergoing antrectomy for treatment of ECL cell neuroendocrine tumours. A list of all the cell lines used is provided in Table 2.1. The cultures had been initially prepared by either Dr Peter Hegyi (First Department of Medicine, University of Szeged, Hungary) or by Dr Islay Steele within this research group. In all cases, the method used to prepare myofibroblasts was that described by (Mahida et al., 1997; Wu et al., 1999). Ethical Committee approval was provided by both the University of Szeged and the Royal Liverpool University Hospital; written consent was obtained. The cells had in each case been previously characterised in the laboratory by immunocytochemistry showing positive staining for α-SMA and vimentin, and negative for cytokeratin and desmin.

Patient No.	Origin	Type	Age	Gender	Survival (Mo)	Tumour location	Classification	Regional lymph nodes (N)	Adjacent Tissue
1	gastric	ATM CAM	72	M	>59	antrum, corpus, border	medullar	N0	intestinal metaplasia, atrophy
2	gastric	ATM CAM	82	M	3	antrum	intestinal	N2	intestinal metaplasia, chronic gastritis
3	gastric	ATM CAM	66	F	5	antrum, corpus, border	mixed	N4	chronic gastritis
4	gastric	ATM CAM	50	F	22	antrum, corpus, border	diffuse	N1	chronic gastritis
5	gastric	ATM CAM	76	M	25	antrum	intestinal	N0	intestinal metaplasia, chronic gastritis
6	gastric	ATM CAM	77	M	>34	antrum	intestinal	N0	intestinal metaplasia, chronic gastritis
7	gastric	ATM CAM	76	M	15	antrum	intestinal	N2	intestinal metaplasia, atrophy, chronic gastritis
8	gastric	ATM CAM	72	M	>34	corpus	mixed	N1	chronic gastritis
9	gastric	ATM CAM	84	F	>31	antrum, corpus, border	intestinal	N0	chronic gastritis
10	gastric	ATM CAM	59	F	17	antrum and corpus	diffuse	N2	chronic gastritis
11	gastric	ATM CAM	51	M	9	antrum, corpus, border	mixed	N3	chronic gastritis
12	gastric	ATM CAM	67	M	>31	antrum	intestinal	N1	chronic gastritis
13	gastric	CAM	39	F	>43	antrum, corpus, border	intestinal	N2	No adjacent tissue

14	gastric	CAM	54	M	>45	antrum, corpus, border	diffuse	N0	No adjacent tissue
15	gastric	ATM	61	M	n.a.	n.a.	n.a.	n.a.	Normal
16	gastric	ATM	65	M	n.a.	n.a.	n.a.	n.a.	intestinal metaplasia, chronic gastritis
17	gastric corpus gastric antrum oesophagus	NTM	44	F	n.a.	n.a.	n.a.	n.a.	n.a.
18	gastric corpus gastric antrum oesophagus	NTM	45	M	n.a.	n.a.	n.a.	n.a.	n.a.
19	gastric corpus gastric antrum oesophagus	NTM	52	F	n.a.	n.a.	n.a.	n.a.	n.a.
20	gastric corpus gastric antrum oesophagus	NTM	60	M	n.a.	n.a.	n.a.	n.a.	n.a.
21	gastric corpus gastric antrum oesophagus	NTM	52	F	n.a.	n.a.	n.a.	n.a.	n.a.
22	gastric corpus gastric antrum oesophagus	NTM	41	M	n.a.	n.a.	n.a.	n.a.	n.a.
27	oesophagus	ATM CAM	72	M	18	oesophagus	adenocarcinoma	n.a.	Barrett's oesophagus
28	cardia	ATM CAM	70	F	>36	cardia	adenocarcinoma	n.a.	chronic gastritis
29	cardia	ATM CAM	63	M	19	cardia	adenocarcinoma	n.a.	chronic gastritis, intestinal metaplasia
30	gastric antrum	PA	49	F	n.a	corpus	ECL cell carcinoids	n.a.	n.a
31	gastric antrum	PA	43	M	n.a	corpus	ECL cell carcinoids	n.a.	n.a

32	gastric antrum	PA	71	F	n.a	corpus	ECL cell carcinoids	n.a.	n.a
33	bone marrow	MSC	19	M	n.a.	n.a.	n.a.	n.a.	n.a.
34	bone marrow	MSC	43	M	n.a.	n.a.	n.a.	n.a.	n.a.
35	bone marrow	MSC	22	F	n.a.	n.a.	n.a.	n.a.	n.a.
36	bone marrow	MSC	36	M	n.a.	n.a.	n.a.	n.a.	n.a.
37	bone marrow	MSC	21	M	n.a.	n.a.	n.a.	n.a.	n.a.
38	bone marrow	MSC	19	M	n.a.	n.a.	n.a.	n.a.	n.a.

Table 2.1 Primary human myofibroblasts and MSC with corresponding patient data. Patient No, type of cells generated, origin of cells, age, gender, tumour location, tumour classification, post-operative survival and pathology assessment of tissue taken adjacent to the tumour are presented. A post-operative survival indicates survival at the time of thesis submission. (Key: PA-Pernicious anaemia, ECL cell-Enterochromaffin-like cell, MSC-Mesenchymal stem cell, NTM-Normal tissue myofibroblasts, CAM-Cancer, ATM-Adjacent tissue, N-Regional lymph nodes: N0-No regional lymph node metastasis, N1-Metastasis in 1 to 6 regional lymph nodes, N2-Metastasis in 7 to 15 regional lymph nodes, N3-Metastasis in more than 15 regional lymph nodes).

2.1.2 Human mesenchymal stem cells

Six human bone-marrow derived mesenchymal stromal (stem) cell (MSC) types were purchased from Lonza, USA and PromoCell, UK (Table 1). These cells had been routinely characterised by the producer as positive for surface antigens CD29, CD44, CD105 and CD166, and negative for CD14, CD34 and CD45, and by functional *in vitro* assays for differentiation into adipocytes, chondrocytes and osteocytes.

2.1.3 Tissue culture

Primary myofibroblasts were used at passages 4 to 15. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, Poole, Dorset, UK) supplemented with 10% v/v fetal bovine serum (FBS, BioWhittaker, Lonza, Belgium), 1% v/v antibiotic-antimycotic solution (Sigma), 1% v/v penicillin-streptomycin (Sigma) and 1% v/v non-essential amino acids (Sigma). Media was prepared fresh and changed every 48 – 72 h. Human MSCs were cultured in a special media containing Mesenchymal Stem Cell Basal Medium and SingleQuots® kit (MSCBM, Lonza, Belgium). The kit includes mesenchymal cell growth supplement, L-glutamine and gentamicin sulphate/amphotericin-B. Media was prepared and changed every 72 - 96 h. All cells were incubated at 37^0C in an atmosphere of 5.0% v/v CO_2. At about 90% confluence, cells were split into new flasks.

2.1.4 Release assay

Cells were washed twice with 10ml sterile phosphate buffered saline (PBS, Invitrogen, Paisley, Renfrew, UK) and harvested with 0.25% w/v trypsin (Sigma) or trypsin-EDTA in the case of MSCs. Trypsin was inactivated with media containing serum, and cells were counted with a haemocytometer (Weber Scientific International Ltd, Middlx, England). Two 10cm dishes were plated for each condition and were left overnight for cells to attach. Media was removed the following day and cells were washed 3 times with 10ml sterile PBS. Afterwards, cells were starved in 5ml serum-free media for 1 h, which was then replaced. Cells were stimulated, as appropriate, with 100ng/ml rhIGF-II (R&D Systems Inc., Oxfordshire, UK), 50ng/ml rhIGF-I (Calbiochem, Merck4Bioscheinces, Beeston, UK), 2µg/ml rhMMP-7 (Calbiochem) and 1µM ionomycin calcium salt (Sigma-Aldrich). Preincubations with 10µg/ml brefeldin A (Epicentre Biotechnologies, Cambio Ltd, Cambridge, UK), 10µg/ml cycloheximide (Sigma), 2µg/ml actinomycin D (Sigma), 3.2µM AG1024 (Calbiochem) and 5µM marimastat (Calbiochem) were performed in particular experiments. After stimulation for 30 min, media was collected and centrifuged (800g 4^0C, 7 min) to remove cell debri.

2.2 Protein chemistry

2.2.1 Protein extraction from media and cell lysates

Media was concentrated to a volume of about 100µl using Amicon Ultra-15 3kDa centrifugal filter devices (Millipore, Watford, UK). For proteomic studies

(subsequently performed by Dr Chris Holmberg), proteins in the media were extracted using StrataClean™ Resin (Agilent Technologies, USA) following the manufacturer's protocol. To recover cellular protein, cells were washed with 10ml sterile PBS on ice, scraped and cell protein was extracted using 100µl/dish RIPA cell lysis buffer (Upstate, USA) (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% w/v NP-40, 1% w/v sodium deoxycholate and 0.1% w/v sodium dodecyl sulfate), containing 1% v/v Phosphatase Inhibitor Cocktail set II, EDTA-Free (Calbiochem, USA) and 1% v/v Protease Inhibitor Cocktail Set III, EDTA-Free (Calbiochem, USA). Cell suspensions were sonicated (Sonicor, Sonicor Instrument Corporation, USA) for 5 min to break down membranes, incubated on ice for 30 min for the membranes to sediment and centrifuged (12 000g 4^0C, 3 min); supernatants were transferred into clean tubes and stored at -80^0C until further processed.

2.2.2 Protein assays

Total protein was determined using the D_c Protein Assay kit (Bio-Rad Lab Inc., Life Science Group, USA), containing alkaline copper tartrate (Reagent A), surfactant solution (Reagent S) and diluted Folin reagent (Reagent B) (Lowry et al., 1951). Standard solutions of bovine serum albumin (BSA, Jackson Immuno Research Lab., Suffolk, UK) at concentrations of 0 mg/ml, 0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml and 1 mg/ml were used to generate a standard curve. Cell protein samples were diluted 1:5 or 1:10 in RIPA buffer to ensure that absorbance at 750 nm would fall within the range of the standard curve. Media samples were assayed undiluted. Absorbance at 750nm was

measured with a SpectraCount spectrophotometer (Packard Bioscience Company, software: PlateReader v3.0) and the values were then used for the quantification of total protein using MSOffice Excel.

2.2.3 Western Blot analysis

Depending on the molecular weight of protein (kDa) different percentages of SDS polyacrylamide gel were run (Laemmli, 1970; Shapiro et al., 1967). PageRuler™ Plus Prestained Protein Ladder (Fermentas, York, UK) was used as a protein size marker. Depending on protein abundance, different quantities of total protein were loaded (10 µg to 60 µg). Protein extracts were mixed with Laemmli Buffer (4% w/v SDS, 2mM EDTA, 10% w/v sucrose, 125mM HEPES, 1% v/v 2-mercaptoethanol, 10% v/v glycerol, Bromophenol Blue) and heated for 4 min at 100^0C (except for the media treated with StrataClean™ Resin). Gel electrophoresis was conducted at 100 V in running buffer, prepared from a 5X stock buffer with a pH 8.3, containing 25mM tris base, 192mM glycine and 0.01% w/v SDS. Proteins were transferred onto nitrocellulose membranes (Hybond™-ECL Nitrocellulose membrane, GE Healthcare Limited, UK) (Towbin et al., 1979) for 1h at 100 V in transfer buffer, containing 0.2mM tris base, 150mM glycine and 20% v/v methanol. Membranes were washed 3 times for 10 min in 0.1% Tween 20 (Sigma) in tris buffered saline (TBS, Sigma) (450ml dH_2O, 50ml 10xTBS, 500µl Tween20). This procedure was followed by blocking for 1 h with 5% w/v non-fat dry milk (Marvel™, Premier Foods, UK), prepared as solution in 1xTBS/0.1%Tween 20. Membranes were then incubated

with the primary antibody solution in dilutions as described in Table 2 overnight at 4^0C on a shaker.

The following day, membranes were washed 3 times for 10 min in 1xTBS/0.1%Tween 20, followed by incubation for 1h with secondary antibody conjugated to horseradish peroxidase (HRP) (Table 2.2). Primary and secondary antibodies were prepared in 5% w/v non-fat dry milk dissolved in 1xTBS/0.1%Tween 20. Finally membranes were washed 3 times for 10 min and exposed for 5 min to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pierce Biotechnology, USA) or Immun-Star™ WesternC™ kit (Bio-Rad Lab.Inc.), both used as a working solution in volume ratio 1:1 from each stock reagent. For one membrane 2.5 ml from each stock solution was sufficient for the light sensitive reaction. Membranes were imaged with ChemiDoc XRS+ (Bio-Rad Lab.Inc.) or were exposed to High performance chemiluminescence film (Amersham Hyperfilm ECL, GE Healthcare Limited,

Antigen/antibody	Titre (Western Blot)	Species	Origin
Decorin	1:500	Goat	R&D – AF143
Galectin-3 (D-20)	1:200	Goat	Santa Cruz – SC19283
GAPDH	1:5000	Mouse	Meridian, Life Science, Inc. – H86504M
IGFBP-5	1:500	Goat	R&D Systems – AF875
IGFBP-7	1:500	Goat	R&D Systems – AF1334
Lumican (H-90)	1:500	Rabbit	Santa Cruz – SC33785
PAI-1	1:500	Rabbit	GeneTex – GX100550
Secretogranin II (H-300)	1:200	Rabbit	Santa Cruz – SC50290
Serpin E2 (nexin)	1:500	Goat	R&D Systems – AF2980
TIMP-1	1:1000	Rabbit	Chemicon – AB8112
TIMP-2	1:5000	Rabbit	Chemicon – AB8107
TGFβig-h3	1:1000	Goat	R&D – AF2935
Anti-goat IgG	1:10 000	Rabbit	Sigma A5420
Anti-mouse	1:10 000	Goat	Sigma A4416
Anti-rabbit	1:10 000	Goat	Santa Cruz SC-2004

Table 2.2 Primary and secondary HRP-conjugated antibodies used in Western blot.

UK). Films were developed with developer/replenisher and fixer/replenisher solutions (Sigma).

2.2.4 Densitometry

Densitometric analysis of the band intensities from Western blot analysis was performed using either ImageLab software v2.1 (Bio-Rad Lab, Inc.) for the film-free developing method or Multi-Analyst v1.1 build 34 (Bio-Rad Lab, Inc., Life Science Group, USA) for films.

2.2.5 Coefficient of variation

To determine the intra-assay variability in Western blots, a single sample was loaded 5 times and run on an SDS polyacrylamide gel followed by protein

transfer to nitrocellulose membrane and blotting for TGFβig-h3. Densitometric analysis of the bands was performed and the data were used to calculate the coefficient of variation, which was 6% (Figure 2). Based on this, in subsequent studies differences in relative abundance of protein between two samples run on the same gel above 1.2 were considered informative as this allowed a further margin of error for other possible sources of variation.

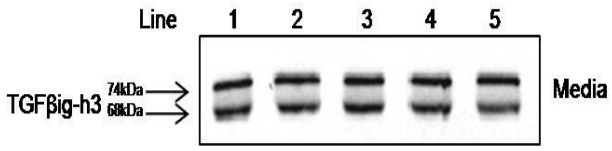


Figure 2 Coefficient of variation. Western blot analysis of TGFβig-h3 of a single myofibroblast media sample, loaded 5 times on one SDS-PAGE gel in equal quantity. Densitometric analysis of the bands intensity determined coefficient of variation of 6% (Mean band intensity ± stand error, $202.9x10^4 \pm 2.7x10^4$, n=5).

2.2.6 Variability of biological response

To determine biological variability across different experiments, identical stimulation experiments were conducted with IGF-II on separate days; media was processed via two different methods (see Section 2.2.1) and analysed by Western blot for TGFβig-h3 with subsequent densitometric analysis. Based on the band intensities the ratio of treated-to-control i.e. fold difference, was determined. The mean differences for IGF-II stimulation ± standard error were determined for two different myofibroblast cell lines using either filters or beads to concentrate the samples. For patient 14 (CAM), IGF-II stimulation of TGFβig-h3 was estimated to be 1.47±0.06 (n=3) using filters compared with 1.54±0.10

(n=9) using beads to concentrate the samples; for patient 21 (NTM), the corresponding values were 2.01±0.39 (n=6) and 2.05±0.29 (n=3) respectively.

2.2.7 Immunofluorescence

Cells were harvested, counted and 1 x 10^4 plated on 4-well chamber slides (BD Flacon, Belgium) or on cover slips placed in 24-well plates. The following day cells received experimental treatments (see Section 2.1.4) followed by washing twice with PBS and fixation with 500 µl 4% v/v paraformalydehyde (PFA, Agar Scientific Ltd.) (30 min). Fixed cells were washed twice in PBS and permeabilised (30 min) using filtered PBS containing 0.1% triton X-100. Permeabilised cells were washed in PBS and incubated with 5% w/v BSA (30 min) and then washed in PBS and incubated with 10% v/v donkey serum (Jackson ImmunoResearch Lab.) (30 min). Donkey serum was removed, cells were washed twice in PBS and primary antibody (diluted in PBS) was added overnight at 4^0C in a humidified atmosphere (Table 2.3).

Antigen/antibody	Titre (Immunofluorescence)	Species	Origin
Decorin	1:50	Goat	R&D – AF143
Galectin-1	1:400	Goat	Santa Cruz – SC19277
Galectin-3	1:400	Goat	Santa Cruz – SC19283
PAI-1	1:100	Goat	Santa Cruz – SC6644
Secretogranin II	1:1000	Rabbit	Thermo Sci. – PA1-10838
SPARC	1:200	Rabbit	Santa Cruz – SC25574
TGFβig-h3	1:50	Rabbit	Cell Signalling – Cat.2719
Fluorescein-conjugated anti-rabbit	1:400	Donkey	Jackson ImmunoResearch Lab. – 711-095-152
Texas Red-conjugated anti-goat	1:400	Donkey	Jackson ImmunoResearch Lab. – 705-075-147
Fluorescein-conjugated anti-goat	1:400	Donkey	Jackson ImmunoResearch Lab. – 705-095-147

Table 2.3 Primary and fluorescence dye-conjugated secondary antibodies used in Immunofluorescence.

Cells were washed the following day, with NaCl at 0.14M, 0.5M and 0.14M (5 min each) to minimise non-specific staining. The appropriate secondary antibody (prepared in 10mM HEPES pH7.5) was applied for 1 h in a dark, humidified, environment (Table 2.3). Secondary antibodies were removed and cells were washed 3 times in PBS (10min) and mounted with Vectashield containing 4',6-diamidino-2-phenylindole DAPI (Vector laboratories, Peterborough, UK). For confocal imaging Vectashield without DAPI was used. Slides were observed using a Zeiss Axioplan-2 microscope (Zeiss Vision, Welwyn Garden City, UK) and images were taken using an Axiocam HRm camera (Carl Zeiss) and processed by AxioVision 4.5 (Carl Zeiss). Non-specific binding was determined in the absence of primary antibody. Confocal microscopy was performed using Leica SP2 AOBS confocal microscope (Leica Microsystems, Milton Keynes, UK) and images were processed by Leica confocal software (Leica Microsystems) with the help of Miss Nantaporn Lertkowit.

2.3 Silencing and overexpression of secretogranin II

2.3.1Silencing RNA

Mission® Silencing RNAs (SASI_Hs01 00132008, SASI_Hs02 00335724, and SASI_Hs01 00132010) for secretogranin II were purchased from Sigma-Aldrich (Table 5). Upon arrival RNA was resuspended in 100 µl Tris/EDTA buffer (TE) to give 100 µM and was stored in aliquots in a freezer at -20^{0}C.

2.3.2 Plasmid DNA

An expression vector encoding human secretogranin II (10 µg, NM_003469.3) was purchased from OriGene Tehcnologies, Inc (Rockville, USA) and stored dried at -20^{0}C. After reconstitution with 100 µl 5mM Tris-HCl to give 0.1µg/ml, secretogranin II plasmid was stored at -20^{0}C.

2.3.3 Transformation of *E.coli* and extraction of plasmid

Competent cells α-Select™ Gold efficiency (Bioline Ltd., London, UK) were kept at -80^{0}C. Cells were thawed at room temperature and secretogranin II plasmid corresponding to 10 µg DNA was added to 50 µl competent cells. Cells were incubated on ice (20 min), heat shocked at 42^{0}C for 45 sec, incubated on ice for a further 2 min, followed by addition of 450 µl Super Optimal Broth with Catabolite repression (SOC) medium (20g tryptone, 5g yeast extract, 0.5g NaCl, 20ml 250mM KCl, 5ml sterile 2M $MgCl_2$ and 20ml sterile 1M glucose for

1000ml solution pH 7.0). Samples were shaken on a roto-shaker at 37^0C for 60-90min and the mixture plasmid/competent cells was plated onto ampicillin-resistant agar plates and incubated overnight at 37^0C.

For a Maxi-Prep inoculation, a single colony was picked the following day and grown in 1ml of freshly autoclaved Lysogeny broth (LB, 10g tryptone, 5g yeast extract, 10g NaCl for 1000ml solution of pH 7.0), containing ampicillin, for 8 h at 37^0C on a roto-shaker. The cultured colony was then added to 100ml ampicillin-containing LB and incubated overnight on a roto-shaker. Ampicillin 10mg/ml was prepared fresh, stored at 4^0C and filter-sterilised prior to use. The stock solution was used at 0.5ml per 100ml LB. Finally the QIAGEN protocol for Plasmid DNA purification Maxi Kit was used (see manufacturer's instructions). Concentration of the plasmid DNA was determined using NanoDrop 2000c device and NanoDrop2000 software. Secretogranin II plasmid concentration was 1139.8ng/ml and 260/280 ratio was 1.85. Reconstituted DNA plasmid was kept at 4^0C.

2.3.4 Transient transfection

Transfections were performed using Amaxa Human AoSMC Nucleofector kit (Lonza) or Amaxa Human Dermal Fibroblast Nucleofector kit (Lonza) on a Nucleofector® I device with the appropriate program for each kit following manufacturer's instructions. Cells were counted and $12x10^6$ cells were pulled down at 800g at 4^0C for 7 min. Media was decanted, the cell pellet gently dried and 800 µl of Nucleofector® solution was added, gently mixed and divided into

two fractions. Four transfections per fraction were done in volumes of 100 µl. Each nucleofection volume was transfected with 1µM siRNA or 3µg plasmid DNA. Control fraction was transfected either with a scrambled RNA or an empty vector pcDNA3 (a gift from Prof Rod Dimaline). After nucleofection 500 µl full media were added and samples were collected in clean sterilins, mixed and each fraction was equally plated into four 10cm dishes. Post-transfection cells were incubated for 96 h before release assays were performed and samples were processed as described earlier.

2.4 Gene expression analysis

Prior to this project, total RNA was extracted from primary myofibroblasts by Dr. Islay Steele, labelled and hybridised to a GeneChip©Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, USA) at the Liverpool Genome Facility Unit by Dr. Lucille Rainbow, following the manufacturer's instructions. The U133 Plus 2.0 array determines the relative expression of over 47,000 transcripts. A GeneChip® Scanner 3000 (Affymetrix) was used to image the arrays. Data analysis was performed using Gene Spring GX v.10 (Agilent Technologies, USA).

For the purpose of this project, the raw data were re-analysed. Gene expression profiles of myofibroblasts exhibiting regulated secretion of TGFβig-h3 were compared to those of myofibroblasts without regulated secretion. Firstly, data were normalised using the Affymetrix MAS5 normalization algorithm. Oligonucleotide probes marked as 100% present in all members of at least one of

the two groups were identified. Statistical analysis of the data was performed using unpaired t-test with no adjustments for multiple comparisons (i.e. No Correction false discovery rate). Further analysis was performed to identify the corresponding gene products using MetaCore™, a data-mining tool developed by GeneGo (www.genego.com); and to annotate the gene products by cellular component using Gene Ontology database (www.geneontology.org).

2.5 Proteomic analysis

Prior to this project, a list with putative secretory proteins identified in gastric myofibroblasts (Patient 14) and in MSCs (Patient 37) was generated by Dr Chris Holmberg using the procedures briefly described below.

2.5.1 SILAC labelling

Gastric myofibroblasts from patient 14 and MSCs from patient 37 were cultured in media supplemented with either $^{12}C_6$ lysine and $^{12}C_6$ arginine (Light label), or $^{13}C_6$ lysine and $^{13}C_6$ arginine (Heavy label) for at least 6 population doublings.

2.5.2 Sample processing and identification of proteins

Media protein was extracted using StrataClean™ Resin (Agilent Technologies, USA) following the manufacturer's protocol, and further processed for HPLC-MS/MS identification. SILAC data were searched and analysed using MaxQuant

1.1.1.36 (Cox and Mann, 2008) against the human Universal Protein Resource (UniProt) database v3.68.

2.6 Statistics

Statistical analysis was performed using Sigma Plot v11. Results were presented as mean ± standard error of the mean (SEM), where needed, and were considered statistically significant if $p<0.05$.

CHAPTER 3

CHARACTERISATION OF CALCIUM-DEPENDENT REGULATED SECRETION IN GASTRIC MYOFIBROBLAST

3.1 Introduction

Recent work in a number of different cell types has suggested that regulated exocytosis might be a property of a wider range of cells than previously supposed (Borgonovo et al., 2002; Coorssen et al., 1996; Ninomiya et al., 1996). For example, in addition to neurons, endocrine and exocrine cells that have been studied in detail for many decades, emerging evidence from this laboratory suggests that myofibroblasts might also exhibit regulated secretion.

Regulated exocytosis is calcium-dependent. Unpublished work in this laboratory has shown that rhIGF-II increased intracellular calcium concentration in gastric myofibroblasts and this required extracellular calcium (McCaig, Burdyga & Varro, unpublished observations). Subsequently it was shown that MMP-7 also increased intracellular calcium in these cells (McCaig, Burdyga & Varro, unpublished observations). Since increased intracellular calcium triggers secretory vesicle fusion with plasma membrane and hence exocytosis (Peters and Mayer, 1998), these observations raised the question of whether there might be regulated release of secretory proteins in gastric myofibroblasts. In other studies, a proteomic approach using stable isotope labeling with amino acids in cell culture (SILAC) identified increased abundance of the ECM protein TGFβ-inducible protein (TGFβig-h3) in the media of gastric myofibroblasts after acute treatment with rhMMP-7 (Duval & Varro, unpublished observations); the same studies also raised the possibility of stimulated release of decorin.

Based on these findings it was considered important to determine whether gastric myofibroblasts might exhibit regulated protein secretion, not least because this might be expected to be important in influencing the local tissue

microenvironment in health and disease. On the basis of previous studies it was therefore hypothesised that IGF- and MMP-7 stimulated protein secretion by gastric myofibroblasts and that TGFβig-h3 and decorin were putative markers of this process.

3.1.1 Aims and Objectives

The aim of this chapter was to characterise secretory mechanisms in gastric myofibroblasts and specifically:

1. To characterise the secretion of the ECM proteins decorin and TGFβig-h3 from gastric myofibroblasts.
2. To assess the effects of calcium-free media, ionomycin, brefeldin A, cycloheximide and actinomycin D on secretion.
3. To characterise the effects of MMP-7 in stimulating protein secretion.

3.2 Materials and Methods

3.2.1 Primary myofibroblasts

The work in this chapter utilised gastric myofibroblasts from patients 14 and 21. These cells were characterised in previous immunocytochemical studies as positive for α-SMA and vimentin, and negative for cytokeratin and desmin (See Chapter 2).

3.2.2 Immunocytochemistry

Myofibroblasts were stained for decorin and TGFβig-h3 using goat anti-decorin at 1:50 (R&D, AF143) and rabbit anti-TGFβig-h3 at 1:50 (Cell signalling, 2719). Secondary antibodies were FITC-conjugated donkey anti-goat and donkey anti-rabbit used in dilution 1:400 (Jackson ImmunoResearch Laboratories, 705-095-147 and 711-095-152).

3.2.3 Western blot analysis

Primary antibodies used for Western blotting were goat anti-decorin at 1:500 (R&D, AF143), goat anti-TGFβig-h3 at 1:1000 (R&D, AF2935) and mouse anti-GAPDH at 1:5000 (Meridian Life Science, H86504M). Secondary antibodies were rabbit anti-goat and goat anti-mouse used at 1:10 000 (Sigma, A5420 and A4416).

3.2.4 Confocal microscopy

Myofibroblasts from patient 21 were processed according to the immunofluorescence protocol in section 2.2.6 (Chapter 2) and mounted in Vectashield without DAPI prior to observation. Images were taken with the technical support of Dr Nantaporn Lertkowit.

3.3 Results

3.3.1 Gastric myofibroblasts produce and secrete ECM proteins decorin and TGFβig-h3

Intracellular localisation of decorin and TGFβig-h3

Immunocytochemical studies showed expression of TGFβig-h3 and decorin in myofibroblasts from patient 21 (Figure 3.3.1.1). Typically, there was perinuclear localisation and staining of a reticular-like network that could correspond to the endoplasmic reticulum (ER) and Golgi apparatus. There was distinct punctate staining of both proteins compatible with localisation to vesicles, and in some cases small numbers of vesicles were observed close to the cell membrane (Figure 3.3.1.1). Using confocal imaging, TGFβig-h3 and decorin were found co-expressed not only in regions correspond to the ER and Golgi complex, but also in secretory vesicles distributed through the cytosol and close to the plasma membrane (Figure 3.3.1.2).

Corpus and antrum NTMs have similar profiles of decorin secretion

To determine whether different populations of gastric myofibroblasts exhibited similar patterns of secretion, studies were initially made on cells from two anatomically different parts of stomach: the gastric corpus and pyloric antrum. Western blots revealed a band of approximately 90 kDa corresponding to glycosylated decorin in media in both cases. Moreover, media from both corpus

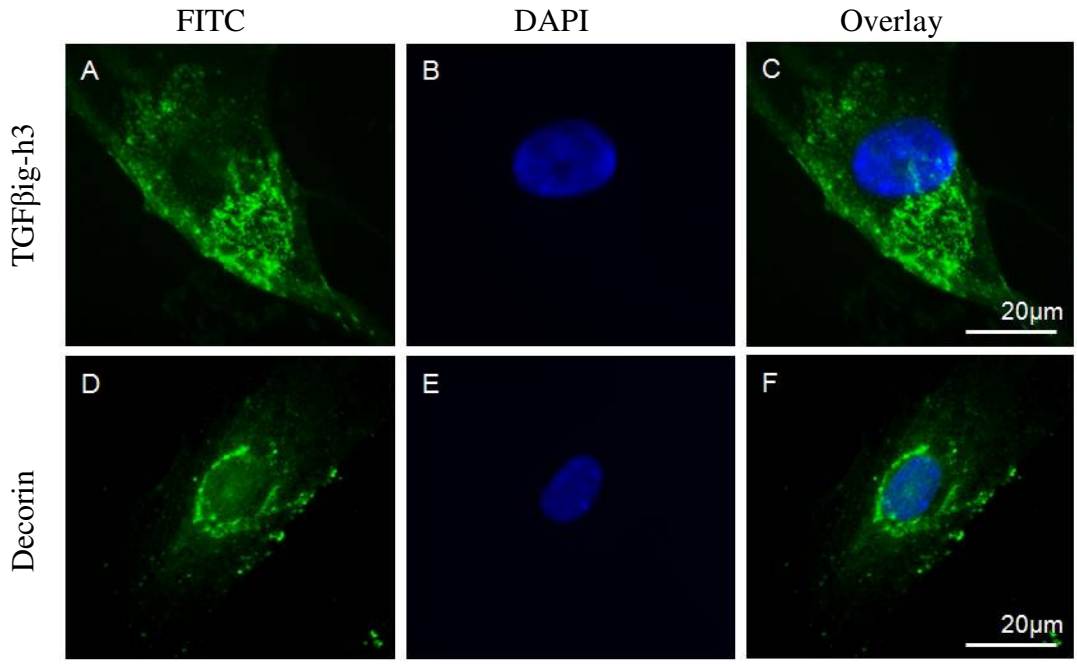


Figure 3.3.1.1 Gastric myofibroblasts express decorin and TGFβig-h3. Immunocytochemical localisation of TGFβig-h3 (A and C, green) shows punctate staining compatible with localisation in ER, Golgi or secretory vesicles. Immunocytochemical localisation of decorin (D and F, green) shows similar distribution. DAPI (B and E, blue) staining was used for the nuclei.

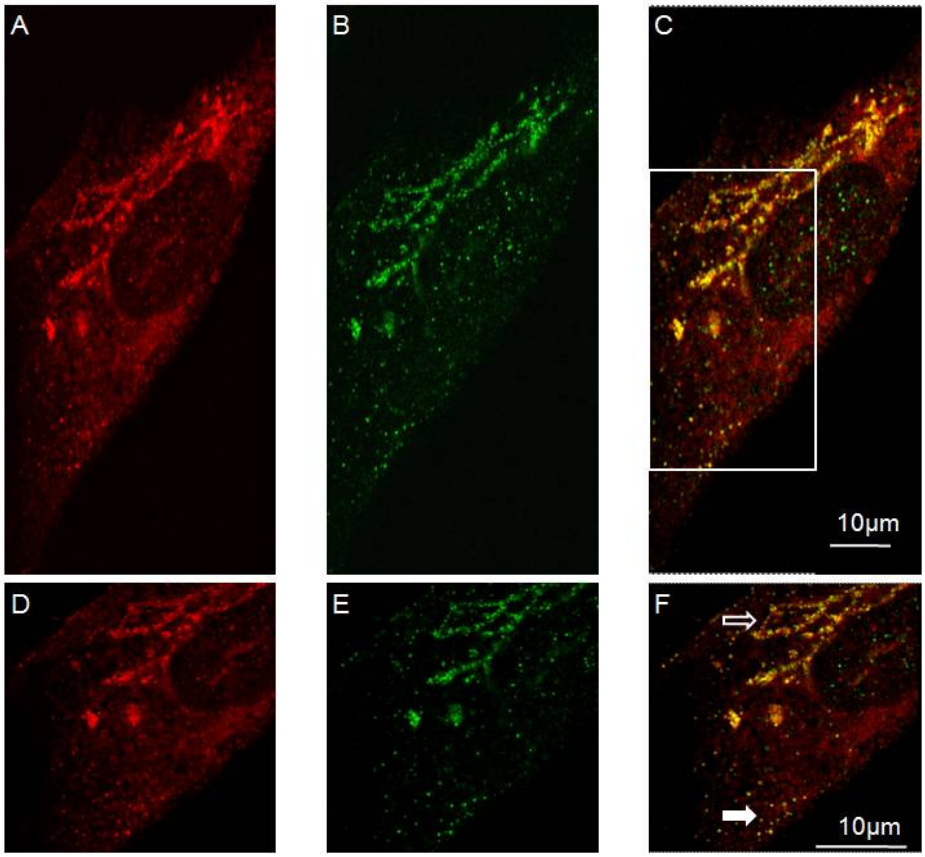


Figure 3.3.1.2 TGFβig-h3 and decorin localise to the same secretory vesicles in myofibroblasts with regulated secretion. Confocal microscopy of decorin (red) and TGFβig-h3 (green) in myofibroblasts with regulated secretion (see below). Myofibroblasts express decorin (**A, D**) and TGFβig-h3 (**B, E**) in regions corresponding to ER and Golgi complex (**open arrow**), and in the same secretory vesicles (**closed arrow**) (**C, F**).

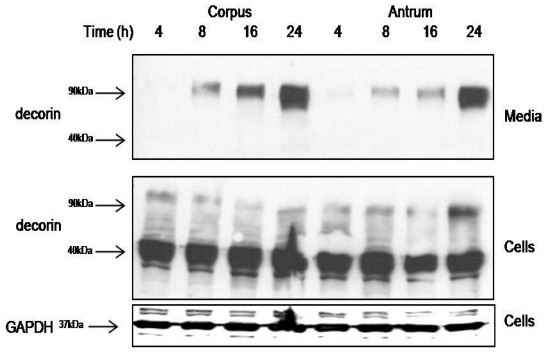


Figure 3.3.1.3 Similar time course of decorin release from corpus and antrum myofibroblasts. Western blots of decorin in myofibroblast media (top panel) and cell lysates (middle and bottom panels) from corpus and antrum myofibroblasts. Abundance of a band corresponding to glycosylated decorin (90kDa) increased progressively in the media of corpus and antrum myofibroblasts up to 24 h. Cellular abundance of the decorin core protein of approximately 40 kDa was unchanged. GAPDH, shown in the bottom panel, was used as a loading control for the cell extracts.

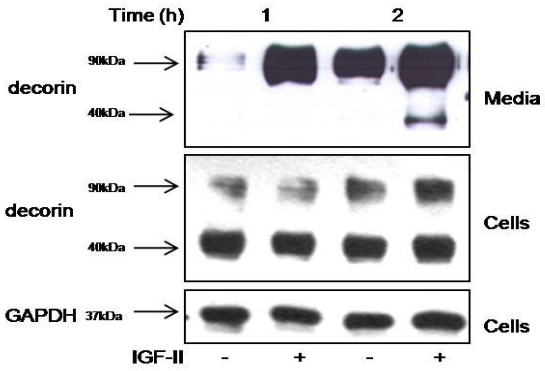


Figure 3.3.1.4 IGF-II stimulation increases secretion of decorin. Western blot analysis of decorin in myofibroblast media (top panel) and cell lysates (middle and bottom panels). IGF-II (100ng/ml) increased decorin abundance in the media after incubation for 1 and 2 h. Decorin cell abundance was not influenced by IGF-II (middle panel. GAPDH was used as a loading control for the cell extracts (bottom panel).

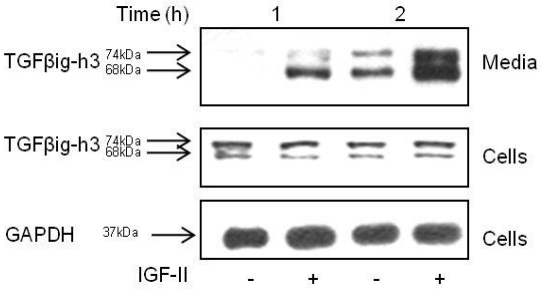


Figure 3.3.1.5 IGF-II stimulation increases secretion of TGFβig-h3. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panels). IGF-II (100ng/ml) increased TGFβig-h3 abundance in the media at 1 and 2 h. TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).

and antrum myofibroblasts exhibited a similar progressive increase in intensity of this band from 8 to 24 h of incubation (Figure 3.3.1.3 top panel); at an early time point, 4 h, decorin was undetectable. There was no difference in this respect between cells originating from the antrum or the corpus. In cell extracts, decorin was detected mainly as a band corresponding to the 40kDa core protein and this remained unchanged over 24 h (Figure 3.3.1.3 - middle and bottom panel). In view of the similarity in decorin secretion in antrum and corpus myofibroblasts, further studies were focused on corpus myofibroblasts.

IGF-II treatment increases secretion of decorin and TGFβig-h3

In view of previous findings in the laboratory (see above), the effect of IGF-II on secretion of decorin and of TGFβig-h3 was examined. Recombinant human IGF-II (rhIGF-II) was used in concentration of 100ng/ml, which was previously determined to increase proliferative and migratory responses of myofibroblasts (Hemers et al., 2005). Treatment of cells with IGF-II increased the abundance of decorin and TGFβig-h3 in media (Figure 3.3.1.4-3.3.1.5 top panels). Following pilot experiments to optimise conditions, secretion of decorin was detectable at 1 h and there was a clear increase in abundance with IGF-II treatment. Cellular decorin remained unchanged with IGF-II treatment (Figure 3.3.1.4 - middle and bottom panel). Similarly, there was little or no detectable TGFβig-h3 in the media of unstimulated cells after 1 h in these experiments, but there were clearly detectable bands of approximately 68 and 74kDa after 2 h (Figure 3.3.1.5). Strikingly, after 1 h in the presence of IGF-II there was evidence of increased TGFβig-h3 abundance in media, and there was a further increase after 2 h.

Cellular TGFβig-h3 was detected as bands of 74 and 68kDa respectively, corresponding to bands in control samples of media. There was no change in abundance or size of cellular TGFβig-h3 with IGF-II treatment but interestingly, in the media IGF-II treatment generated a band of 60-62kDa in addition to higher molecular weight material.

Actinomycin D does not change IGF-stimulated secretion of decorin and TGFβig-h3

To determine whether IGF-II might act by increasing decorin and TGFβig-h3 expression, the effect of actinomycin D (2µg/ml), which inhibits DNA transcription, was examined. There was no decrease in cellular decorin and only a slight reduction in cellular TGFβig-h3 over 2 h (Figure 3.3.1.6-3.3.1.7 middle and bottom panels). The abundance of decorin and TGFβig-h3 in the media was increased by IGF-II stimulation, regardless of whether actinomycin D was present or absent (Figure 3.3.1.6-3.3.1.7 top panel), suggesting that transcription was not required for secretory responses to IGF-II.

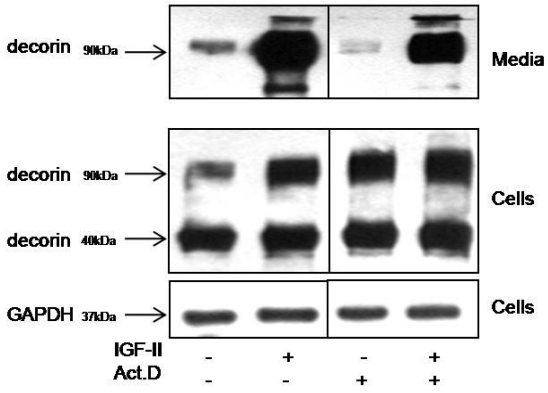


Figure 3.3.1.6 Actinomycin D does not influence decorin secretion in response to IGF-II. Western blot analysis of decorin in myofibroblast media collected after 2 h (top panel) and cell lysates (middle and bottom panels). IGF-II increased protein secretion and this increase was still present after actinomycin D treatment (top panel). There was no decrease in decorin cellular abundance after actinomycin D treatment (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).

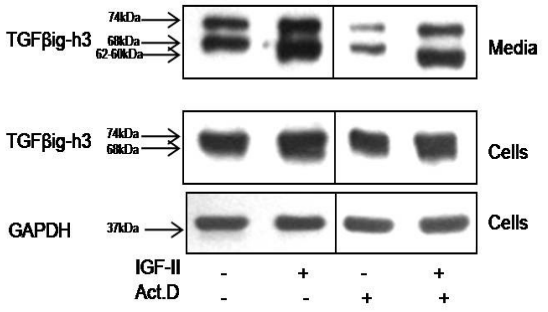


Figure 3.3.1.7 Actinomycin D does not influence TGFβig-h3 secretion in response to IGF-II. Western blot analysis of TGFβig-h3 in myofibroblast media collected after 2 h (top panel) and cell lysates (middle and bottom panels). IGF-II increased protein secretion and this increase was still present after actinomycin D treatment (top panel). TGFβig-h3 cell abundance was slightly decreased after actinomycin D treatment (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).

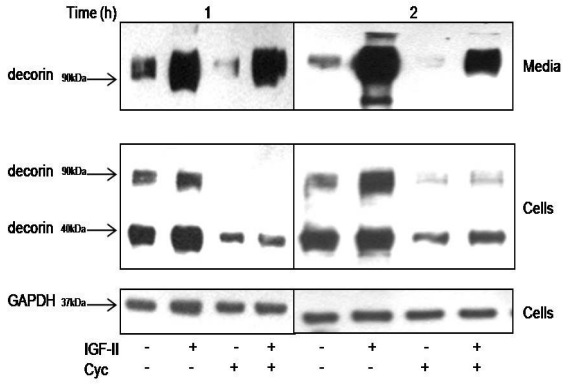


Figure 3.3.1.8 Decreased intracellular decorin after cycloheximide treatment. Western blot analysis of decorin in myofibroblast media collected after 1 and 2 h (top panel) and cell lysates (middle and bottom panels). IGF-II stimulation increased decorin in the media both with and without cycloheximide treatment (top panel). Cycloheximide decreased cellular glycosylated form of decorin more notably at 1 h. GAPDH was used as a loading control for the cell extracts (bottom panel).

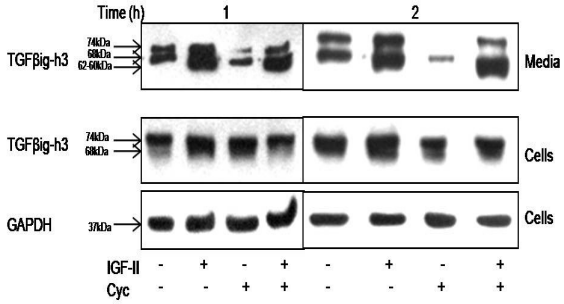


Figure 3.3.1.9 Decreased intracellular TGFβig-h3 after cycloheximide treatment. Western blot analysis of TGFβig-h3 in myofibroblast media collected after 1 and 2 h (top panel) and cell lysates (middle and bottom panels). IGF-II stimulation increased media TGFβig-h3 both with and without cycloheximide treatment (top panel). TGFβig-h3 cell abundance was decreased at 2 h (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).

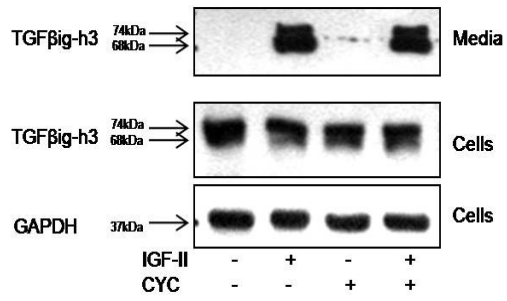


Figure 3.3.1.10 Cycloheximide does not inhibit TGFβig-h3 secretion in response to IGF-II. Western blot analysis of TGFβig-h3 in myofibroblast media collected after 30 min (top panel) and cell lysates (middle and bottom panels). Cycloheximide had no effect on the secretion in response to IGF-II (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).

Cycloheximide does not change IGF-stimulated secretion of decorin and TGFβig-h3

To further examine the role of protein synthesis in the secretory response to IGF-II, myofibroblasts were treated with cycloheximide (10µg/ml), a reversible inhibitor of protein translation, and then stimulated with IGF-II. Both, decorin and TGFβig-h3 were detected in the media after 1h but in unstimulated cells there was decreased abundance after 2 h (Figure 3.3.1.8-3.3.1.9 top panel); at both time points, there was increased abundance in media in the presence of IGF-II stimulation. Cycloheximide therefore inhibited basal secretion of decorin and TGFβig-h3, but it did not inhibit the response to IGF-II. In addition, cycloheximide decreased intracellular decorin, with a strong effect on the glycosylated form (Figure 3.3.1.8 middle and bottom panel). In contrast, TGFβig-h3 abundance in the cell was only slightly decreased at 2 h (Figure 3.3.1.9 middle and bottom panel).The data support the idea that IGF-II evokes secretion from a store of preformed vesicles, while basal secretion was maintained by newly synthesised material. In order to minimise the different contributions of regulated and constitutively secreted proteins in the media, the possibility of studying release over even shorter periods of time was explored. It proved difficult to detect decorin in studies of release over a period of 30 min, but TGFβig-h3 release was detectable in response to IGF-II stimulation (Figure 3.3.1.10 top panel) and this was resistant to treatment with cycloheximide (Figure 3.3.1.10 top panel). Cell protein abundance was unchanged (Figure 3.3.1.10 middle and bottom panels).

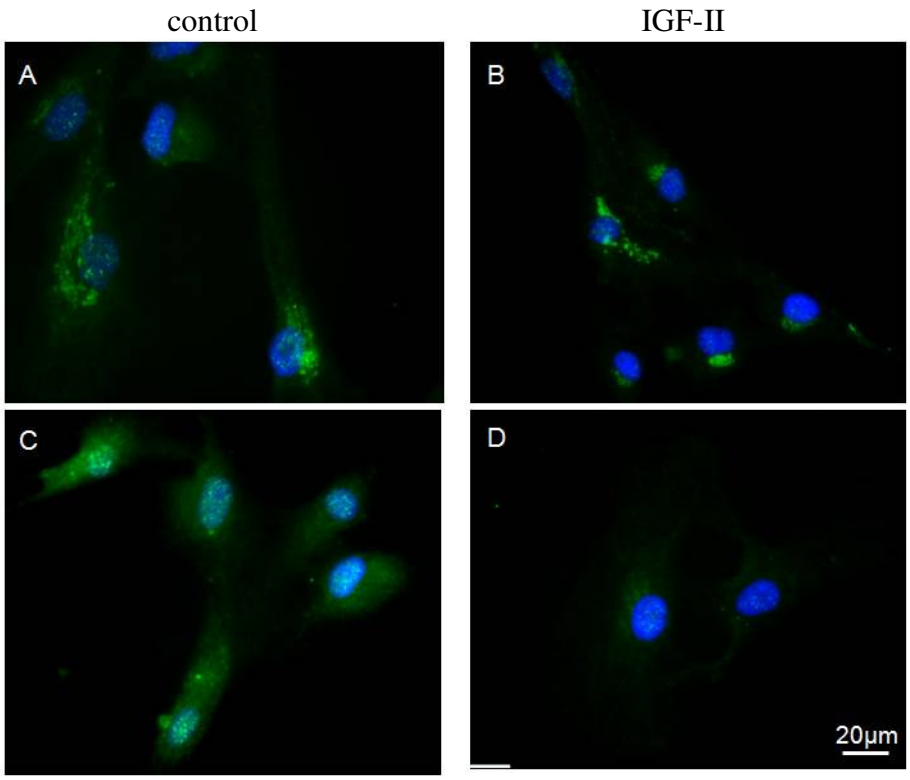


Figure 3.3.1.11 Cell depletion for TGFβig-h3. Immunocytochemical analysis of TGFβig-h3 (green) in NTM treated with cycloheximide (10µg/ml) and IGF-II (100ng/ml). Nuclei were stained with DAPI (blue). Untreated controls expressed TGFβig-h3 as punctated staining throughout the cytoplasm (**A**). IGF-II stimulation decreased cytoplasmic TGFβig-h3 (**B**). Cycloheximide decreased cell TGFβig-h3 abundance (**C**). Cells were entirely depleted of TGFβig-h3 (**D**). Images of all four treatments were taken on the same day, using identical software settings for each channel, including time of exposure and picture brightness and contrast.

Immunocytochemical studies of TGFβig-h3 after cycloheximide treatment

Immunocytochemical analysis for TGFβig-h3 in myofibroblasts pre-treated with cycloheximide and IGF-II showed that TGFβig-h3 was expressed in several cellular compartments (Figure 3.3.1.11). Nearly all (98%) control cells had staining compatible with localisation to ER, Golgi apparatus and secretory vesicles (Figure 3.3.1.11 A). Cells treated with IGF-II had decreased cytoplasmic TGFβig-h3 fluorescence (Figure 3.3.1.11 B). A majority (89%) of the cells preincubated with cycloheximide exhibited decreased TGFβig-h3 abundance (Figure 3.3.1.11 C). IGF-stimulation of cycloheximide-treated cells was associated with even weaker staining, consistent with decreased cellular TGFβig-h3 abundance (Figure 3.3.1.11 D).

3.3.2 Characterisation of short term (30 min) IGF-stimulated TGFβig-h3 secretion

Both IGF-II and IGF-I stimulate TGFβig-h3 secretion

In view of the data described above, it was considered that TGFβig-h3 offered advantages over decorin as a model protein for short term (30 min) secretion studies. To further explore the secretory response, a comparison was made of rhIGF-I and IGF-II. As noted previously, there was stimulation of TGFβig-h3 secretion in response to IGF-II at 30 min compared to the control (Figure 3.3.2.1 top panel); there was also comparable secretion of TGFβig-h3 at 30 min in response to 50 ng/ml rhIGF-I (Figure 3.3.2.1 top panel). There was no detectable

stimulation of secretion at 10 ng/ml rhIGF-I. Cellular TGFβig-h3 abundance was unchanged by both IGF-I and IGF-II (Figure 3.3.2.1 middle and bottom panels).

IGF-stimulated TGFβig-h3 secretion is decreased in calcium-free media

To define the role of extracellular calcium, experiments in the absence of extracellular calcium were then performed. IGF-stimulated TGFβig-h3 secretion was present in control media and decreased over 40% in calcium-free media (Figure 3.3.2.2 top panel). Cell protein abundance was unchanged by these treatments (Figure 3.3.2.2 middle and bottom panels).

Ionomycin stimulates TGFβig-h3 secretion

To further study the role of calcium in IGF-stimulated TGFβig-h3 secretion the calcium ionophore, ionomycin (1 μM), was employed. Myofibroblasts treated with ionomycin exhibited a small increase in TGFβig-h3 abundance in the media (Figure 3.3.2.3 top panel). The cell protein abundance was unchanged (Figure 3.3.2.3 middle and bottom panels).

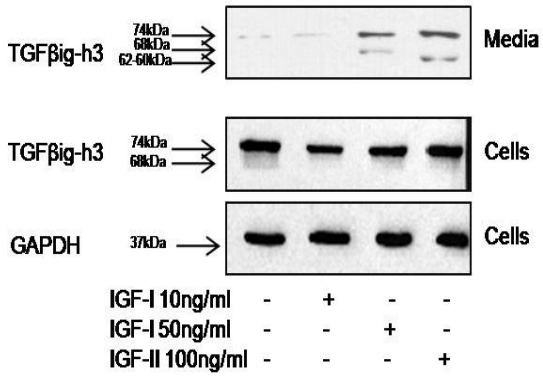
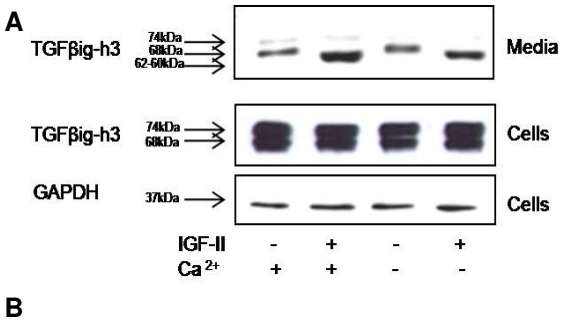


Figure 3.3.2.1 IGF-stimulated TGFβig-h3 secretion in NTM. Western blot analysis of TGFβig-h3 myofibroblast media (top panel) and cell lysates (middle and bottom panels). Both, IGF-II and IGF-I stimulated TGFβig-h3 secretion. TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).



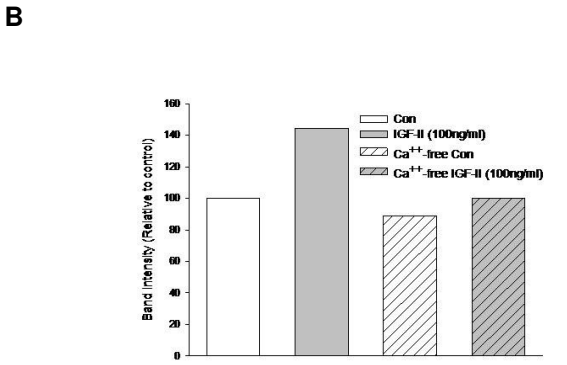


Figure 3.3.2.2 IGF-II-stimulated TGFβig-h3 secretion is inhibited in calcium-free media. A. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panel). IGF-II stimulated secretion was inhibited in calcium-free media (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel). **B.** Densitometric analysis of media Western blot showing over 40% decrease in IGF-stimulated secretion in the calcium-free media (bar chart). Representative results from n=2 separate experiments.

Brefeldin A does not inhibit IGF-stimulated TGFβig-h3 secretion

In order to further define the pool of secreted TGFβig-h3, studies were undertaken using brefeldin A (BFA) which inhibits ER-to-Golgi and intra-Golgi vesicular transport (Donaldson et al., 1992; Fujiwara et al., 1988) and thus can separate constitutive secretion from release of a preformed pool of storage vesicles. Pilot experiments indicated that pre-incubation with 10μg/ml BFA for 30 min was sufficient to reduce basal (constitutive) secretion of TGFβig-h3 (Figure 3.3.2.4 top panel). However, myofibroblasts pre-incubated with BFA and treated with IGF-II retained stimulated TGFβig-h3 secretion comparable to IGF-II treated control cells (Figure 3.3.2.4 top panel). Cell protein abundance remained unchanged (Figure 3.3.2.4 middle and bottom panels).

3.3.3 MMP-7 and short term stimulation of TGFβig-h3 secretion

MMP-7 stimulates TGFβig-h3 secretion through IGF-IR

In view of findings from previous proteomic studies (see above) suggesting that MMP-7 might evoke TGFβig-h3 release, a direct analysis of the action of MMP-7 was made. Thus the abundance of TGFβig-h3 in the media was shown to be increased after treatment with 0.2μg/ml rhMMP-7 (Figure 3.3.3.1 top panel). To determine if this effect was mediated by IGF-IR (which is the main receptor binding both IGF-I and IGF-II) the IGF-IR tyrosine kinase inhibitor AG1024 (3.2μM) was used. The secretion of TGFβig-h3 in response to MMP-7 was

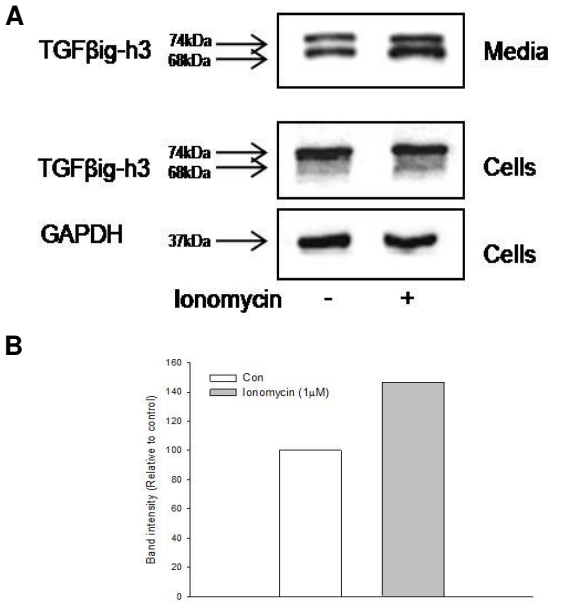


Figure 3.3.2.3 Ionomycin stimulates TGFβig-h3 release. A. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panel). Ionomycin slightly stimulated protein secretion in myofibroblasts (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel). **B.** Densitometric analysis of media Western blot showing ionomycin-stimulated TGFβig-h3 secretion (bar chart).

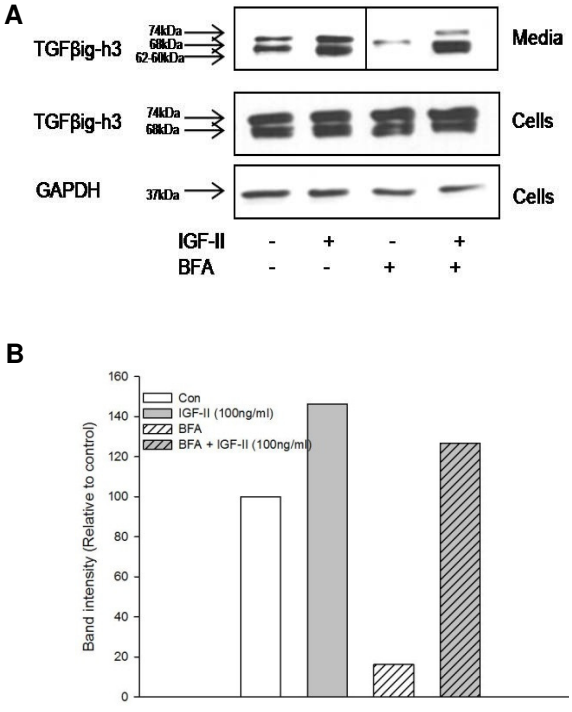


Figure 3.3.2.4 Brefeldin A does not inhibit IGF-stimulated TGFβig-h3 secretion. A. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panel). Brefeldin A inhibited basal secretion, but not the IGF-stimulated (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel). Representative results from n=2 separate experiments. **B.** Densitometric analysis of media Western blot showed lack of inhibition of IGF-stimulated TGFβig-h3 secretion by BFA (bar chart).

reduced approximately 40% by AG1024 (Figure 3.3.3.1 top panel). Cellular abundance of TGFβig-h3 remained unchanged (Figure 3.3.3.1 middle and bottom panels).

MMP-7-stimulated TGFβig-h3 secretion is decreased in calcium-free media

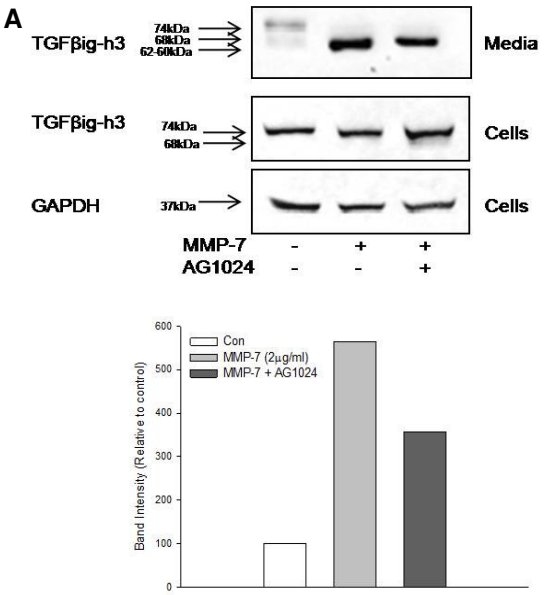
The effect of MMP-7 in stimulating TGFβig-h3 secretion was decreased in calcium-free media (Figure 3.3.3.2 top panel). Cellular abundance of TGFβig-h3 was unchanged (Figure 3.3.3.2 middle and bottom panels).

Marimastat inhibits MMP-7 stimulated TGFβig-h3 secretion

Lastly, the action of marimastat (5μM), a broad spectrum matrix metalloproteinase inhibitor, was examined. This fully inhibited MMP-7 evoked TGFβig-h3 secretion (Figure 3.3.3.3 top panel). Cell TGFβig-h3 abundance was unchanged (Figure 3.3.3.3 middle and bottom panels).

3.3.4 Other proteases and short term stimulation of TGFβig-h3 secretion

In addition, another proteolytic enzyme, urokinase-type plasminogen activator uPA, that has been shown previously by proteomic methods to increase TGFβig-h3 in the media (Duval & Varro, unpublished observations), also stimulated secretion of TGFβig-h3 from myofibroblasts (Fig 3.3.4). Cell TGFβig-h3 abundance was unchanged (Figure 3.3.4).



decreases MMP-7 effect on stimulated TGFβig-h3 secretion. A. Western blot analysis of TGFβig-h3 myofibroblast media (top panel) and cell lysates (middle and bottom panel). AG1024 decreased MMP-7 stimulated TGFβig-h3 secretion (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel). **B.** Densitometric analysis of media Western blot showed MMP-7-stimulated secretion with and without AG1024 (bar chart).

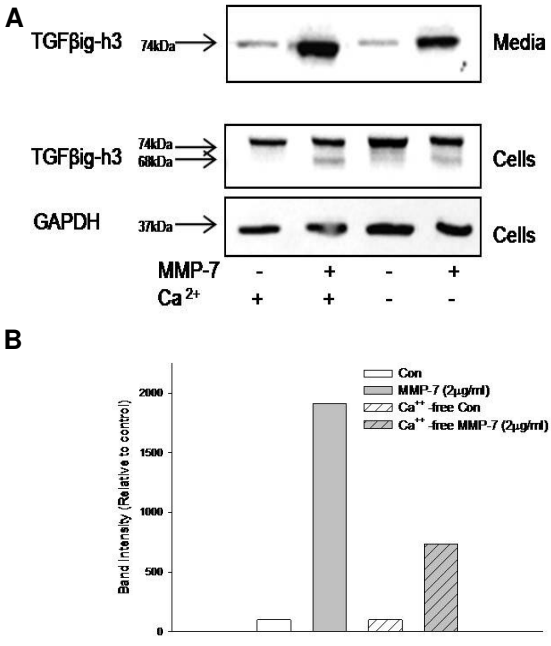


Figure 3.3.3.2 MMP-7-stimulated TGFβig-h3 secretion is decreased in calcium-free media. A. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panel). MMP-7 stimulated secretion was decreased in calcium-free media (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel). **B.** Densitometric analysis of media Western blot showed some inhibition of MMP-7-stimulated secretion in calcium-free media (bar chart).

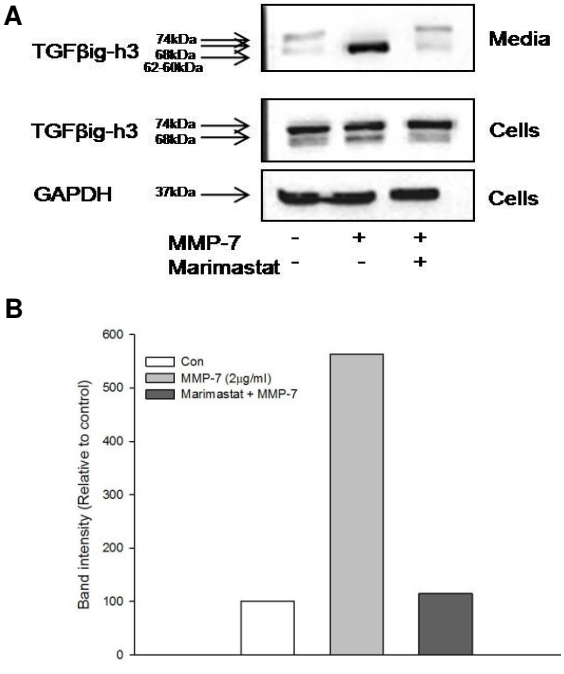


Figure 3.3.3.3 Marimastat inhibits MMP-7 stimulated TGFβig-h3 secretion. A. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panel). Marimastat fully inhibited MMP-7 evoked TGFβig-h3 secretion (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel). **B.** Densitometric analysis of media Western blot showed full inhibition of MMP-7 stimulated secretion by marimastat (bar chart).

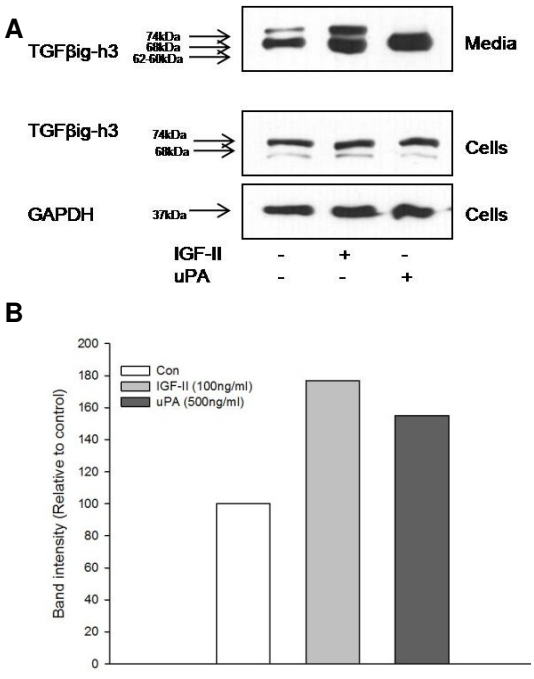


Figure 3.3.4 TGFβig-h3 secretion is stimulated by uPA. A. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panel). TGFβig-h3 secretion was stimulated by uPA (500ng/ml) (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel). **B.** Densitometric analysis of media Western blot showed uPA-stimulated secretion of TGFβig-h3 (bar chart).

3.4 Discussion

The present chapter provides evidence that normal gastric myofibroblasts exhibit stimulated secretion at least of the ECM proteins TGFβig-h3 and decorin. Regulated secretion is generally considered to be property of neurons, neuroendocrine, endocrine and exocrine cells although some cells with transitory secretory functions (eg sperm, egg) also have dense-core vesicles of the regulated secretory pathway. Even so, however, there is evidence that other cell types may exhibit evoked secretion too. For example, 3T3-L1 adipocytes secrete the adipokine apelin from a pre-formed pool after stimulation with eicosapentanenoic acid (Lorente-Cebrian et al., 2010); pancreatic stellate cells, a mesenchymal cell located in close proximity to the basolateral side of the acinar cells and playing role in maintenance of ECM, have CCK-stimulated acetycholine secretion (Phillips et al., 2010). The present work indicates that regulated release of ECM proteins occurs in response to IGF stimulation of gastric myofibroblasts.

Immunofluorescence staining revealed that decorin and TGFβig-h3 are co-expressed in compartments compatible with ER and Golgi and further studies to co-localise these proteins with ER, Golgi and *trans*-Golgi network (TGN) markers are now required. In this context, it is worth noting that in cornea there is perinuclear expression of TGFβig-h3 which was shown to co-localise with TGN38 staining, a marker for TGN (Kim et al., 2009). In some cases clusters of vesicles were observed in the cytoplasm and/or near the plasma membrane.

Decorin and TGFβig-h3 were co-expressed in these vesicles, which, however, could correspond to vesicles of either the constitutive or regulated secretory

pathways. However, in cells treated with IGF-II there was decreased cellular TGFβig-h3 immunofluorescence which was more pronounced after cycloheximide treatment to inhibit new synthesis, compatible with depletion of a pool of storage vesicles.

Initial studies showed that IGF-II increased secretion of decorin and TGFβig-h3, and since only the latter exhibited a relatively robust response at short time periods it was selected for further analysis. Two major bands of TGFβig-h3, 74 and 68kDa, were identified in media together with a 60-62kDa band in some IGF-treated samples. TGFβig-h3 from whole corneal extracts migrated on 2-D gels as multiple spots of the same molecular weight, but different isoelectric points (Korvatska et al., 2000) and this was ascribed to posttranslational modifications particularly phosphorylation at multiple sites (threonine, tyrosine and serine). Jurkunas et al. used anti-TGFβig-h3 antibody binding to the C-terminal of the protein and showed age-related differences in the TGFβig-h3 turnover in normal corneal endothelium (Jurkunas et al., 2009). They identified three species (57, 39 and 29kDa), whose production was increased in disease. Another study showed rapid proteolytic degradation of TGFβig-h3 in the media and formation of multiple size bands (69, 62 and 60kDa) which was required for protein's pro-apoptotic properties in human osteosarcoma cells (Zamilpa et al., 2009). This report corroborated earlier studies that an integrin-binding sequence in the C-terminus of the molecule is implicated in triggering of apoptosis (Kim et al., 2003). In the present study, bands corresponding to the intact protein (74kDa) and two minor bands (68 and 60-62kDa) were identified, but there was no relation between the ages of the patients. Interestingly, there were no changes in

the size of the bands for TGFβig-h3 (74 and 68kDa) in cell extracts suggesting that there was some, limited, proteolytic degradation after secretion (Adlerz et al., 2007).

Several different lines of evidence contribute to the view that IGF stimulated secretion of TGFβig-h3 is from preformed storage vesicles (Figure 3.4).

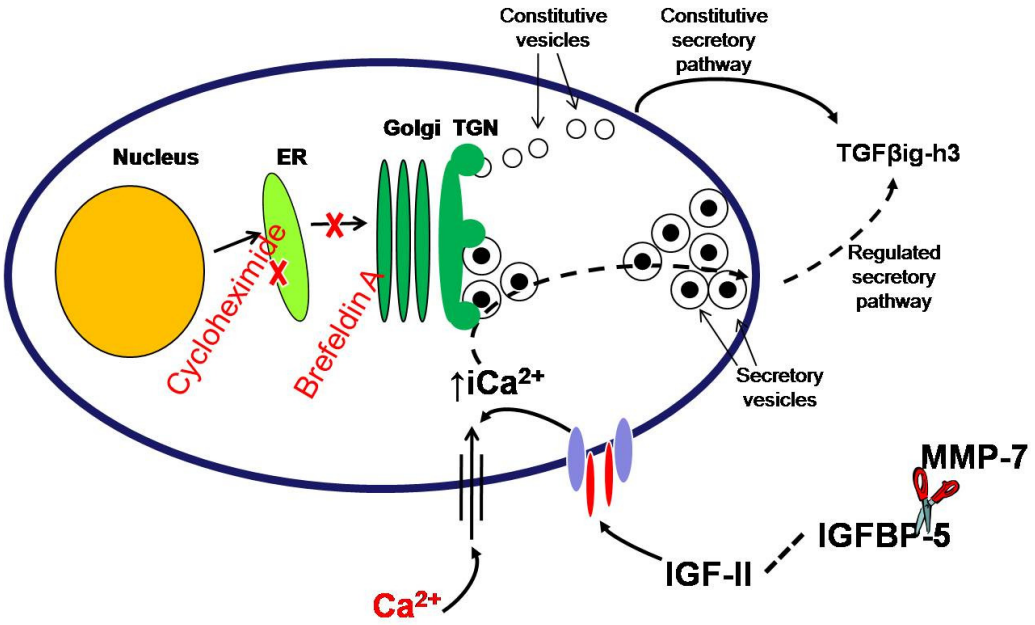


Figure 3.4 Schematic outline of the experimental approach for studies of myofibroblast regulated secretion. Regulated TGFβig-h3 secretion was studied in calcium-free media, with an inhibitor of protein transport (brefeldin A) and with inhibitors of protein synthesis (cycloheximide, actinomycin D), after stimulation with exogenous IGF-I and IGF-II (recombinant proteins) and endogenous IGFs (released after MMP-7 cleavage of IGFBP-5).

First, secretion was not dependent on protein synthesis; the transcriptional inhibitor actinomycin D did not alter either intracellular protein abundance or secretion, implying that rates of DNA transcription were relatively low in this system. However, the translational inhibitor cycloheximide inhibited basal

secretion, which is presumably constitutive, but had no effect on the secretion in response to IGF-II compatible with secretion from pre-existing storage vesicles. Second, an inhibitor of a GTP-exchange factor responsible for the recruitment of coat proteins for transport of vesicles from ER to Golgi namely brefeldin A (Jackson and Casanova, 2000) also inhibited basal (constitutive) TGFβig-h3 secretion, but it did not inhibit the IGF-stimulated secretion. Again, this is compatible with the release of a pre-formed protein pool. Third, IGF-stimulation exhibited a partial requirement for extracellular calcium. It is known that both IGF-I and IGF-II interact with IGF-IR, a receptor tyrosine kinase (Baker et al., 1993); the IGF-II receptor or type II IGF/mannose-6-phosphate receptor binds mainly IGF-II (Kar et al., 1997). Upon binding to IGF-IR the two IGFs activate downstream pathways including those related to calcium signalling leading to increased intracellular calcium (Kojima et al., 1988). In contrast, the IGF-II receptor has been suggested to have no functional activity but instead to be linked to receptor uptake and degradation (Jones and Clemmons, 1995) although a calcium influx was described in primed BALB/c3T3 fibroblast cells in which IGF-II receptor was found coupled to a calcium channel by a G protein (Nishimoto, 1993; Nishimoto et al., 1987). The present data suggest that both IGF-I and IGF-II evoke regulated secretion in gastric myofibroblasts and that while there was reduction of stimulated TGFβig-h3 secretion in the absence of extracellular calcium, it was not entirely inhibited. Conversely there was a relatively modest response to ionomycin.

Cell types differ in respect to the source of calcium ions for regulated secretion, i.e. calcium from internal stores, external calcium, or both. For example, for

regulated secretion adrenal chromaffin cells require calcium influx (Cheek and Thastrup, 1989; O'Sullivan et al., 1989), pancreatic exocrine cells - release of calcium from ER (Wasle and Edwardson, 2002), and pituitary corticotrophs – use both sources (Hille et al., 1999; Tse and Tse, 1999). In gastric myofibroblasts intracellular calcium was increased only in the presence of extracellular (personal communication, McCaig and Burdyga). Ionomycin triggers calcium influx in *Xenopus* oocytes by translocation from the smooth endoplasmic reticulum into the cytoplasm and by facilitating calcium entry (Cavarra et al., 2003). Similar dual mechanisms were demonstrated in other cell types like Ehrlich ascites tumour cells, murine peritoneal neutrophils, macrophages, and T-lymphocytes (Dedkova et al., 2000; Imboden and Weiss, 1987). Further studies are now required to define more precisely the mechanisms by which IGF controls intracellular calcium in myofibroblasts and to delineate the relevance for regulated secretion.

The finding that MMP-7 stimulated TGFβig-h3 secretion was compatible with the previous proteomic studies (Duval & Varro, unpublished observations). Inhibition of MMP-7 stimulated secretion with IGF-IR inhibitor, although modest, was an expected effect considering the described effect of MMP-7 on IGFBP-5 secretion (Hemers et al., 2005). Secretion of TGFβig-h3 stimulated by MMP-7, like IGF stimulated secretion, was inhibited in calcium-free media suggesting that both occur via common mechanisms involving IGF-IR. Since inhibition of MMP-7 stimulated secretion in calcium-free media was not complete, further studies to clarify the Ca-independent effect are required. The broad spectrum matrix-metalloprotease inhibitor marimastat fully inhibited

MMP-7 stimulated TGFβig-h3 secretion suggesting that the observed effects were due to MMP-7 enzymatic activity (Hemers et al., 2005).

3.5 Conclusions

1. Myofibroblasts from anatomically different stomach parts (corpus and antrum) have similar secretion of the ECM protein decorin.

2. TGFβig-h3 provides a useful model for studies of regulated secretion in response to IGF stimulation.

3. IGF-stimulated secretion of TGFβig-h3 is not dependent on DNA transcription, mRNA translation or ER to Golgi transport, but is partially dependent on extracellular calcium.

4. There is MMP-7 stimulated TGFβig-h3 secretion that appears to be mediated by IGF-IR.

CHAPTER 4

THE TISSUE MICROENVIRONMENT AS A DETERMINANT OF REGULATED SECRETION IN MYOFIBROBLASTS

4.1 Introduction

Myofibroblasts from different tissues show functional heterogeneity (Powell et al., 1999). Moreover, there is increasing evidence for differences between myofibroblasts from tumour stroma compared with normal tissue including, for example, evidence of epigenetic changes in tumour-derived myofibroblasts (Jiang et al., 2008). Previous work in this laboratory using proteomic approaches showed decreased TGFβig-h3 abundance in the media of CAMs from patients with poor survival compared with their corresponding ATMs (Holmberg & Varro, unpublished observations). In view of the evidence presented in the previous chapter, it was therefore considered important to determine whether regulated secretion was a uniform property of all myofibroblasts. A unique opportunity to study this issue in detail was provided by the availability of a panel of primary myofibroblasts derived from normal tissue (stomach and oesophagus), from inflammatory, non-cancer, tissue (pernicious anaemia), as well as from two different cancers (gastric and oesophageal) and their adjacent tissues. Additionally, another stromal cell type was available for comparison, namely bone-marrow derived MSCs.

4.1.1 Aims and Objectives

The aims of the work in this chapter were:

1. To study regulated secretion of TGFβig-h3 in myofibroblasts derived from normal stomach, gastric cancer and adjacent tissue.

2. To study regulated secretion of TGFβig-h3 in myofibroblasts derived from inflammatory gastric tissue (pernicious anaemia) and from normal oesophagus and oesophageal cancer.

3. To characterise regulated secretion of TGFβig-h3 in MSCs.

4.2 Materials and methods

4.2.1 Primary cell cultures

The work in this chapter utilised human myofibroblasts and MSCs as described in Chapter 2 (Table 2.1).

4.2.2 Release assay

Secretory responses to IGF-II were studied using a method similar to the one described in Chapter 3.

4.2.3 Immunocytochemistry

Gastric myofibroblasts and MSCs were stained for TGFβig-h3 using rabbit anti-TGFβig-h3 antibody at 1:50 (Cell signalling, 2719) and donkey anti-rabbit at 1:400 (Jackson ImmunoResearch Laboratories, 705-095-147 and 711-095-152). Deconvolution of the images was performed using the AxioVision 4.5 software (Carl Zeiss).

4.2.4 Transmission electron microscopy (TEM)

Myofibroblasts and mesenchymal stem cells were cultured in 10cm dishes at 80% confluency and processed for TEM by Simon Oliver (EM Unit, Physiology Department, University of Liverpool) using a resin pellet embedding protocol as published previously (Taylor et al., 2010). Multiple images were taken of 5-10

cells from three myofibroblast lines: one exhibiting (Patient 14) and two lacking regulated secretion (Patient 2 and 11), and one MSC line (Patient 37); the analysis of images for the presence of dense core secretory vesicles was conducted by an observer who was blind to the identity of the cells. Size of the secretory vesicles was measured using AnalySIS Pro 3.2 (Build 678) program.

4.2.5 Statistics

Results were presented as mean ± SEM, as appropriate. The Fisher exact statistical test was used to compare groups of patients segregated on the basis of survival after surgery with regard to the proportion of CAMs exhibiting regulated secretion in each group; the difference was considered significant if $p<0.05$.

4.3 Results

4.3.1 Gastric myofibroblasts and regulated secretion

Regulated secretion of TGFβig-h3 is a property of some gastric myofibroblasts

Initially, studies of regulated secretion of TGFβig-h3 were performed on myofibroblasts derived from normal stomach, gastric cancer and from tissue adjacent to tumours, using protocols based on the work described in the previous chapter. Stimulation of TGFβig-h3 secretion in response to IGF-II (100ng/ml) was defined as an increase of >20% compared with control; examples of cells either exhibiting stimulated secretion, or not, are presented in Figure 4.3.1.1 A and B. On this basis, regulated secretion was identified in 7 out of 8 NTMs (88%), in 10 out of 14 ATMs (71%) and in 6 out of 14 CAMs (43%) (Table 4.3.1, Figure 4.3.1.1C).

To further explore the properties of cells that did not exhibit regulated secretion of TGFβig-h3 in response to IGF-II, experiments were performed using the calcium ionophore ionomycin; there was no stimulation of TGFβig-h3 secretion in this experiment (Figure 4.3.1.2).

It was considered possible that if rates of constitutive secretion were high this might mask a relatively modest response to IGF-II. To test this possibility, myofibroblasts that appeared not to exhibit regulated secretion were studied using BFA to inhibit constitutive secretion. After BFA treatment there was no

Patient No.	Type of cells	Relative abundance IGF/control	Stimulation
1	ATM	1.57	Y
	CAM	0.95	N
2	ATM	1.31	Y
	CAM	0.85	N
3	ATM	4.00	Y
	CAM	1.17	N
4	ATM	1.40	Y
	CAM	1.71	Y
5	CAM	1.80	Y
6	ATM	1.22	Y
	CAM	1.33	Y
7	ATM	5.50	Y
	CAM	0.98	N
8	ATM	1.06	N
	CAM	0.94	N
9	ATM (proximal)	1.80	Y
	ATM (distal)	0.91	N
	CAM	5.00	Y
10	ATM	3.68	Y
	CAM	1.17	N
11	ATM	0.92	N
	CAM	0.88	N
12	ATM	0.91	N
	CAM	0.95	N
13	CAM	3.00	Y
14	CAM	1.98	Y
15	ATM	1.89	Y
16	ATM	1.33	Y
17	NTM Corpus	1.25	Y
18	NTM Corpus	1.27	Y
19	NTM Corpus	1.45	Y
20	NTM Antrum	1.17	N
21	NTM Corpus	3.00	Y
	NTM Antrum	2.33	Y
22	NTM Corpus	3.50	Y
	NTM Antrum	1.27	Y
30	PA Antrum	1.02	N
31	PA Antrum	3.25	Y
32	PA Antrum	1.39	Y

Table 4.3.1 Responses of gastric myofibroblasts to IGF-II stimulation. Densitometric analyses of band intensities from Western blots were used to calculate relative abundance between IGF-II treated and control samples. A relative abundance above 1.2 was considered as stimulation (see Materials & Methods section 2.2.4. for more information)

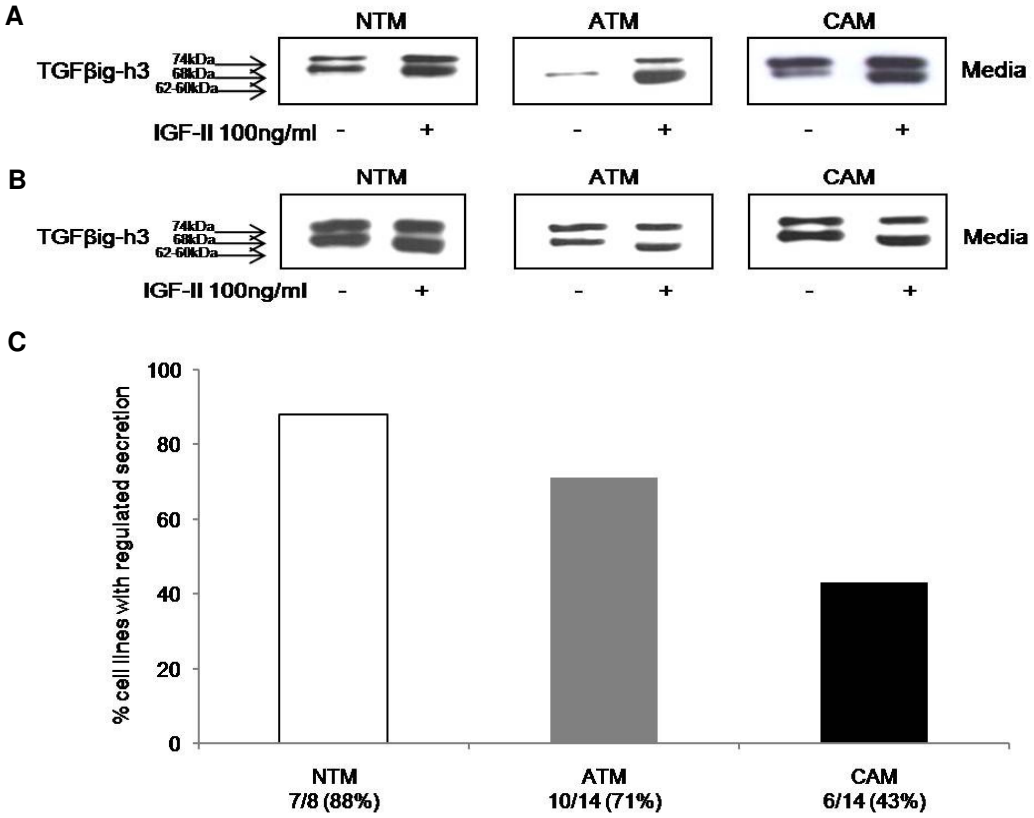


Figure 4.3.1.1 Comparison of different types of gastric myofibroblasts. Representative Western blots of TGFβig-h3 in media from NTMs, ATMs and CAMs with (**A**) and without (**B**) regulated secretion. Bar chart indicates the proportion of gastric myofibroblasts exhibiting regulated secretion of TGFβig-h3 from normal, adjacent and cancer tissues (**C**).

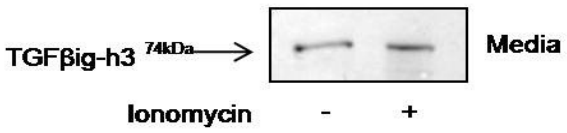


Figure 4.3.1.2 Lack of calcium sensitive TGFβig-h3 secretion in myofibroblasts without regulated secretion. Representative Western blot showing lack of ionomycin stimulated TGFβig-h3 release.

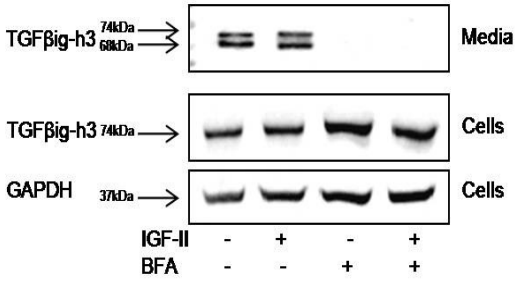


Figure 4.3.1.3 BFA inhibits secretion of TGFβig-h3 in the absence or presence of IGF-II in myofibroblasts that do not exhibit regulated secretion. Western blot analysis of TGFβig-h3 in myofibroblast media (top panel) and cell lysates (middle and bottom panel). BFA inhibited TGFβig-h3 secretion in both absence and presence of IGF-II in a myofibroblast line that failed to exhibit regulated exocytosis. TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).

detectable release of TGFβig-h3 in either control or IGF-treated samples (Figure 4.3.1.3). Cell protein abundance was unchanged (Figure 4.3.1.3).

Regulated secretion of TGFβig-h3 correlates with patient survival

Interestingly, six out of 9 CAMs that came from patients with good survival (>22 months) exhibited a secretory response to IGF-II (Figure 4.3.1.4). In contrast, in the group of patients with poor survival (<22 months) none of the 5 CAMs exhibited regulated secretion of TGFβig-h3 (Fisher exact, $p<0.05$) (Figure 4.3.1.4). There were no co-relations between regulated secretion of TGFβig-h3 and patient gender or the pathology in adjacent tissue (data not shown).

Regulated secretion of TGFβig-h3 in gastric myofibroblasts from pernicious anaemia

The presence of inflammatory and immune cells in solid tumours raised the possibility that differences in stromal cell function might be a reflection of an inflammatory/immune response. It was therefore considered interesting to see whether myofibroblasts from patients with pernicious anaemia in whom there is chronic atrophic gastritis would exhibit regulated secretion of TGFβig-h3. Regulated secretion of TGFβig-h3 was exhibited by two of three pernicious anaemia-derived myofibroblasts (67%) (Figure 4.3.1.5).

4.3.2 Oesophageal myofibroblasts and regulated secretion

To investigate further the role of tissue microenvironment in determining the capacity for regulated secretion, a panel of primary oesophageal myofibroblasts was studied, from normal tissue (n=6) and from adenocarcinoma (n=3) (Table 4.3.2). Surprisingly, only one out of 6 oesophageal NTMs exhibited regulated secretion of TGFβig-h3 (Figure 4.3.2.1A), whereas the remaining five NTMs had no signs of regulated secretion (Figure 4.3.2.1B). In contrast, all ATMs and CAMs from oesophageal adenocarcinoma exhibited regulated secretion of TGFβig-h3 (Figure 4.3.2.2).

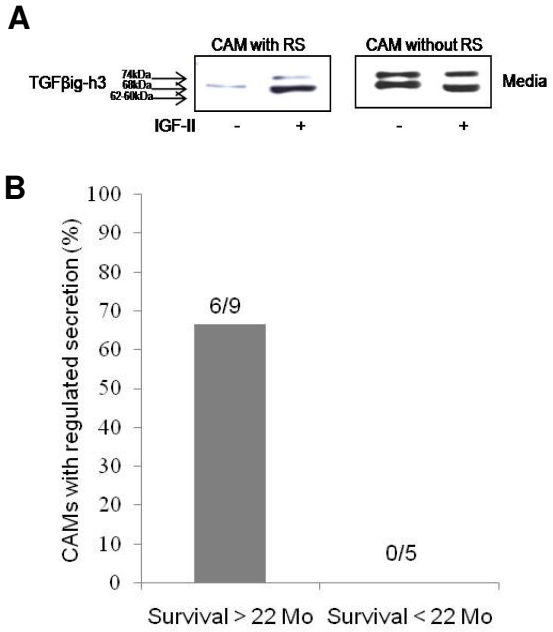


Figure 4.3.1.4 Demonstration of regulated TGFβig-h3 secretion in CAMs correlates with patient survival. **A.** Representative Western blots of TGFβig-h3 in myofibroblast media from CAMs from patients with good (>22Mo, left) and poor survival (<22Mo, right). **B.** CAMs from 6 out of 9 patients with good survival (>22 months) had regulated TGFβig-h3 secretion compared with 0 out of 5 patients with poor survival (<22 months) (Fisher exact, $p<0.05$).

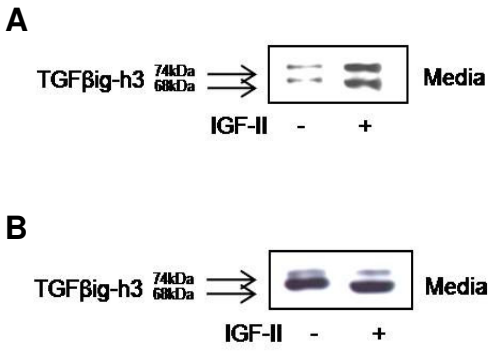


Figure 4.3.1.5 Secretion of TGFβig-h3 in pernicious anaemia-derived gastric myofibroblasts. **A.** Representative Western blot analysis of TGFβig-h3 in media from pernicious anaemia-derived antral myofibroblast. Examples of a myofibroblast with (**A**) and without (**B**) regulated secretion of TGFβig-h3.

Patient No.	Type of cells	Relative abundance IGF/control	Stimulation
17	NTM	0.79	N
18	NTM	0.93	N
19	NTM	0.63	N
20	NTM	1.91	Y
21	NTM	0.97	N
22	NTM	1.06	N
27	ATM	1.28	Y
	CAM	1.30	Y
28	ATM	3.39	Y
	CAM	1.25	Y
29	ATM	1.48	Y
	CAM	2.56	Y

Table 4.3.2 Responses of oesophageal myofibroblasts to IGF-II stimulation. Densitometric analysis of band intensities from Western blots was used to calculate relative abundance between IGF-II treated and control samples. Relative abundance above 1.2 was considered as stimulation.

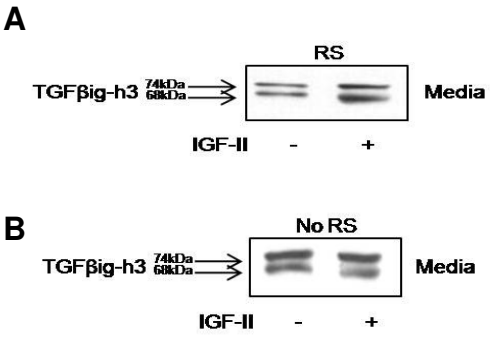


Figure 4.3.2.1 Secretion of TGFβig-h3 by normal oesophageal myofibroblasts. Representative Western blot analysis of TGFβig-h3 in media from oesophageal myofibroblast. Only 1 out of 6 (17%) of oesophageal normal myofibroblasts exhibited regulated secretion of TGFβig-h3 **(A),** and five did not **(B).**

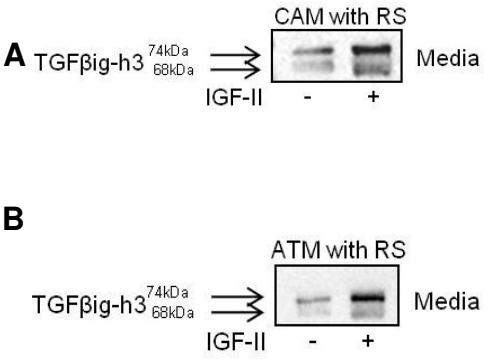


Figure 4.3.2.2 Secretion of TGFβig-h3 by myofibroblasts from oesophageal adenocarcinoma. Representative Western blot analysis of TGFβig-h3 in media from oesophageal cancer myofibroblasts**.** All CAMs (representative example in **A)** and all ATMs (representative example in **B)** exhibited regulated secretion of TGFβig-h3 after stimulation with IGF-II (mean increase 1.70±0.43, n=3 and 2.05±0.67, n=3 respectively).

4.3.3 MSCs and regulated secretion

Human MSCs exhibit regulated TGFβig-h3 secretion

To further characterise the capacity of mesenchymal cells to exhibit regulated secretion a set of six human MSC lines was examined. Using immunofluorescence, TGFβig-h3 was found to be localised to a perinuclear zone with some cytoplasmic, possibly vesicular, localisation (Figure 4.3.3.1). All six MSC lines exhibited increased secretion of TGFβig-h3 in response to stimulation with IGF-II (Table 4.3.3, Figure 4.3.3.2).

Since all lines of MSCs showed evidence of regulated secretion of TGFβig-h3, further studies were performed to characterise the response. In a pilot experiment studying a range of IGF-I concentrations (10ng/ml, 50ng/ml and 100ng/ml), there was stimulation of TGFβig-h3 secretion, as compared with the 100ng/ml IGF-II stimulation, and the secretory response to IGF-I was dose-dependent (Figure 4.3.3.3).

Regulated secretion of TGFβig-h3 by MSCs is decreased in calcium-free media

There was a decrease in IGF-stimulated TGFβig-h3 secretion in calcium-free media compared with normal media (Figure 4.3.3.4). Cell TGFβig-h3 abundance was unchanged (Figure 4.3.3.4). Additionally, the calcium ionophore, ionomycin, stimulated TGFβig-h3 secretion from MSCs comparable to that by both IGF-II and IGF-I (Figure 4.3.3.4). Cell abundance was unchanged (Figure 4.3.3.4).

Regulated secretion of TGFβig-h3 by MSCs is present after BFA treatment

Basal secretion of TGFβig-h3 in MSCs was almost entirely blocked by BFA (Figure 4.3.3.5). IGF-stimulated TGFβig-h3 secretion, however, was still present (Figure 4.3.3.5). Cell protein abundance was unchanged (Figure 4.3.3.5).

Regulated secretion of TGFβig-h3 by MSCs is present after cycloheximide treatment

Finally, cycloheximide was used to inhibit protein synthesis before stimulation with IGF-II. IGF-stimulated secretion of TGFβig-h3 was slightly lower, but still

Patient No.	Type of cells	Relative abundance IGF/control	Stimulation
33	MSC	2.75	Y
34	MSC	4.00	Y
35	MSC	16.00	Y
36	MSC	1.32	Y
37	MSC	3.33	Y
38	MSC	2.51	Y

Table 4.3.3 Responses of human MSCs to IGF-II stimulation. Densitometric analysis of band intensities from Western blots was used to calculate relative abundance between IGF-II treated and control samples. Relative abundance above 1.2 was considered as stimulation.

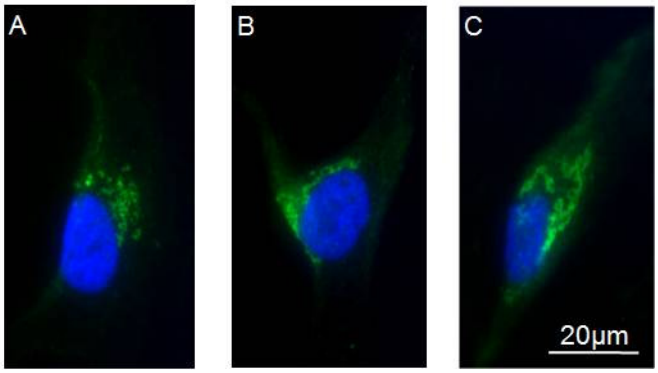


Figure 4.3.3.1 TGFβig-h3 expression in MSC. Immunocytochemical images of TGFβig-h3 (green) in MSCs showed perinuclear and some vesicular localisation. Nuclei were stained with DAPI (**A, B, C**).

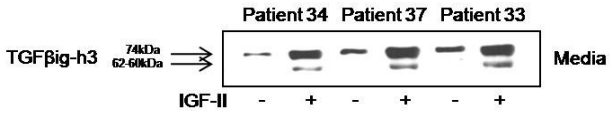


Figure 4.3.3.2 Regulated TGFβig-h3 secretion in MSCs. Representative Western blots of TGFβig-h3 in MSC media. All MSCs had increased TGFβig-h3 secretion after IGF-II treatment (mean fold increase 4.98±2.23, n=6).

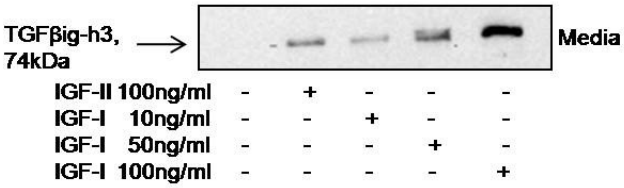


Figure 4.3.3.3 IGF-stimulated TGFβig-h3 secretion in MSC. Western blot analysis of TGFβig-h3 in MSC media. Both, IGF-II and IGF-I stimulated TGFβig-h3 secretion. There was dose-dependent stimulation of secretion by IGF-I.

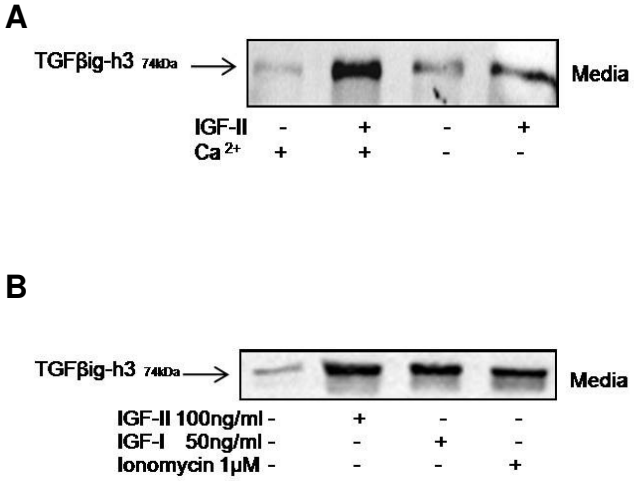


Figure 4.3.3.4 Regulated TGFβig-h3 secretion is inhibited in calcium-free media. *Western blot analysis of TGFβig-h3 in media from MSC. IGF-II stimulated secretion was inhibited in calcium-free media (**A**). Calcium ionophore, ionomycin stimulated TGFβig-h3 secretion comparable to IGF-I and IGF-II (**B**). *Data were obtained jointly with Mr Jin Rui Liang.

present after preincubation with cycloheximide (Figure 4.3.3.6). There was no observable change in cell protein abundance (Figure 4.3.3.6).

4.3.4 Dense-core secretory vesicles in the cytoplasm of myofibroblasts with regulated secretion and MSCs

Based on the findings described above, it was reasoned that in myofibroblasts exhibiting regulated exocytosis it might also be possible to identify dense-cored secretory vesicles using TEM, while it was not expected to find these structures in cells that did not exhibit regulated secretion. In myofibroblasts with regulated secretion (Patient 14, panel A) and in an MSC line (Patient 37, panel B) there were dense-cored secretory vesicles in the close vicinity of the Golgi complex (Figure 4.3.4). Typically the electron dense core occupied a relatively small proportion of the vesicle. Similar vesicles were occasionally found scattered throughout the cytosol. The diameter of the secretory vesicles was similar in both myofibroblasts and MSCs, respectively 197.4 ± 10.3 nm and 219.9 ± 11.2 nm (Table 4.3.4). Rough ER and Golgi stacks were observed routinely in all cells whether they exhibit regulated exocytosis or not. Dense cored secretory vesicles with diameter 202.5 ± 13.1nm were occasionally observed in a myofibroblast line that did not exhibit regulated secretion (Patient 11, Table 4.3.4). However, no secretory vesicles were observed either close to the Golgi, or in the cytosol of another myofibroblasts that did not exhibit regulated secretion (panel C, Figure 4.3.4; Patient 2, Table 4.3.4).

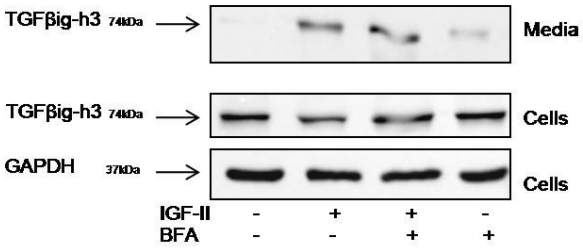


Figure 4.3.3.5 Regulated TGFβig-h3 secretion is maintained after brefeldin A treatment. *Western blot analysis of TGFβig-h3 in MSC media (top panel) and cell lysates (middle and bottom panel). Brefeldin A had no effect on the secretion in response to IGF-II (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).* Data were obtained jointly with Mr Jin Rui Liang.

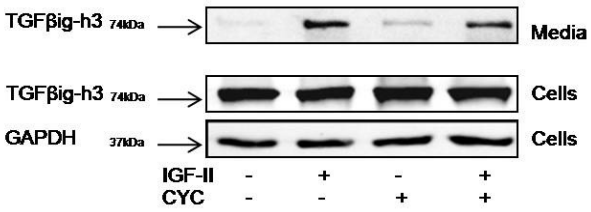


Figure 4.3.3.6 Regulated TGFβig-h3 secretion is maintained after cycloheximide treatment. *Western blot analysis of TGFβig-h3 in MSC media (top panel) and cell lysates (middle and bottom panel). Cycloheximide had no effect on the secretion in response to IGF-II (top panel). TGFβig-h3 cell abundance was unchanged (middle panel). GAPDH was used as a loading control for the cell extracts (bottom panel).* Data were obtained jointly with Mr Jin Rui Liang.

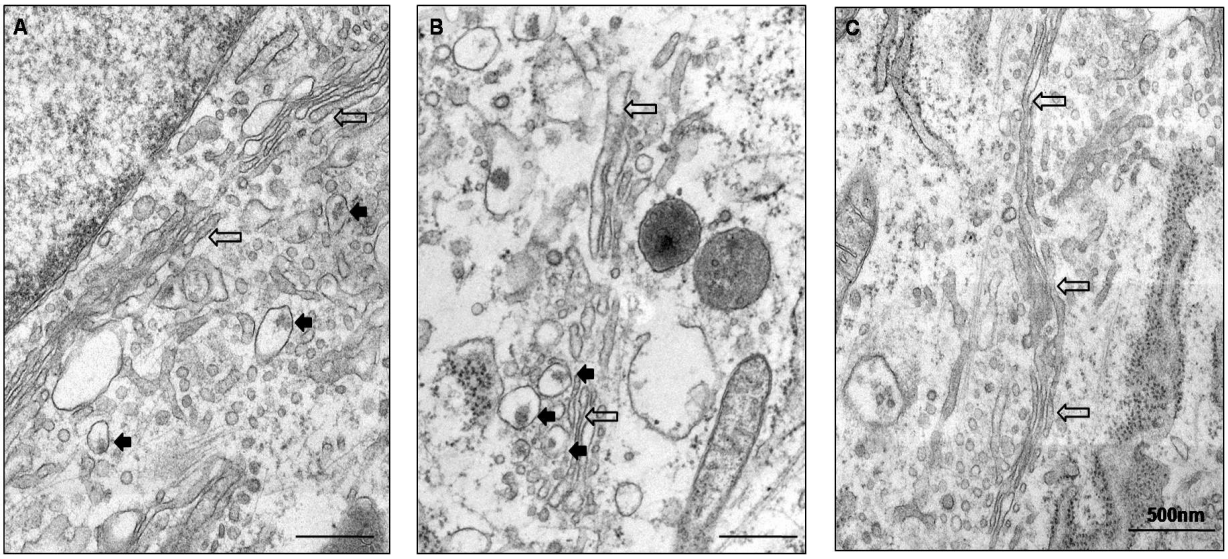


Figure 4.3.4 Transmission electron microscopy (TEM) identifies dense core secretory vesicles in myofibroblats exhibiting regulated secretion and in MSCs. Representative photomicrographs taken at magnification of 60,000 x showing dense core secretory vesicles (closed arrows) in myofibroblasts (**A**) and MSCs (**B**) exhibiting regulated secretion. Lack of dense core secretory vesicles in myofibroblasts without regulated secretion (**C**) while the Golgi apparatus is clearly visible (open arrows). Scale bar 500nm.

Patient No.	Cell type	No. of cells counted	No. of fields counted	No. of secretory vesicles counted	Mean diameter of secretory vesicles
Patient 2	CAM	9	14	0	-
Patient 11	ATM	15	31	3	202.5 ± 13.1nm
Patient 14	CAM	6	7	17	197.4 ± 10.3nm
Patient 37	MSC	5	8	37	219.9 ± 11.2nm

Table 4.3.4 Similar diameter of dense core secretory vesicles from myofibroblasts and from MSCs. The diameter of secretory vesicles was measured from photomicrographs taken at magnification of 60,000 x. For vesicles with a not round shape average diameter was calculated on the basis of diameter via long and short axis. For each cell type mean diameters were calculated ± SEM. Myofibroblasts with regulated secretion were taken from Patient 14 and without - from Patient 2 & 11.

4.4 Discussion

The work in this chapter describes differences between myofibroblast lines in the ability to respond to IGF with increased secretion of TGFβig-h3. The differences were apparent in myofibroblasts from distinct regions of gastrointestinal tract (stomach *vs* oesophagus), from the same region but from normal and cancerous tissue, as well as between different mesenchymal cell types (e.g. MSCs). In addition, electron microscopy images of myofibroblasts with regulated secretion showed dense core secretory vesicles in their cytosol whereas these were not identified in myofibroblasts that did not exhibit regulated secretion.

There is increasing evidence that myofibroblasts play an important role in tissue organization in health and in disease (Powell et al., 1999) includes roles for myofibroblasts from skin (Darby et al., 1990; Hebda et al., 1993), brain (Mucke and Eddleston, 1993), heart and pericardium (Weber et al., 1997), and small intestine and colon (Joyce et al., 1987). These studies showed local regulation exerted by myofibroblasts with respect to their respective tissue or organ of origin. For example, subepithelial intestinal myofibroblasts participate in the modulation of intestinal motility, in the regulation of intestinal water and electrolyte transport and in tissue responses to inflammation (Powell, 2005). The present data now suggest, conversely, that the local tissue microenvironment plays a role in determining the secretory phenotype of myofibroblasts. Thus, this study showed that almost all of the normal gastric myofibroblasts exhibited regulated secretion while normal oesophageal myofibroblasts did not, raising the possibility that differences in secretory pattern may be related to functional differences within the gastrointestinal tract. This is the first demonstration of

differences in secretory function in myofibroblasts from normal upper gastrointestinal tract and the functional significance, *in vivo*, will require further study.

Less than half of CAMs exhibited regulated secretion and, surprisingly, these cells were from patients with good survival. There is increasing evidence that changes occur in stromal cells alongside changes in tumour cells as cancers progress; for example, altered gene expression was detected in breast cancer stromal cells with tumour progression (Ma et al., 2009). Moreover, comparisons using microarray analysis between intestinal myofibroblasts from normal, adenomatous and adenocarcinomatous human colonic tissues found quite distinctive gene expression profiles (Powell, 2005). Furthermore, Jiang et al. showed epigenetic changes in myofibroblasts from gastric adenocarcinoma, which were not associated with genomic instability but with an altered gene expression instead (Jiang et al., 2008). Thus, the data raise the possibility that stromal myofibroblasts acquire changes during cancer development that modify their phenotype, including their secretory capacity. More generally, the observations with CAMs support the idea that the cellular microenvironment in cancer, as well as normal tissue, is a determinant of myofibroblast functional properties.

Based on these findings a test for regulated secretion in cancer stromal myofibroblasts could be used as a disease biomarker. Use of cancer stroma-derived markers to define cancer progression has been already discussed in the literature. For example, analysis of the fibrotic focus in the tumour was used as a useful histological prognostic marker for patients with breast cancer (Van den

Eynden et al., 2007). In the case of regulated secretion of TGFβig-h3, a possible biomarker assay might assess, for example, TGFβig-h3 abundance in the blood before and after local administration of IGF-II (Urbanek et al., 2005). Urbanek et al. injected a mixture of growth factors in the hearts of infarcted mice to trigger migration and growth, and proliferation of the cardiac stem cells and early committed cells to the damaged area, thus improving myocardial regeneration and increasing survival of the mice. This approach could be applied with some modifications in the staging of gastric tumours, and therefore in the assessment of therapeutic options. In addition, plasma proteomics in an experimental cancer model identified differentially abundant proteins derived from both cancer itself and cancer stroma (Hung et al., 2006). The latter study employed mass spectrometry to compare plasma from mice with moderate to high intestinal adenoma burden to healthy control mice of the same age. Despite the complex protein content of the plasma, the analysis identified differentially secreted proteins, including from the stromal cells, hence suggesting that the proteome of the cancer stromal cells could be used as a source of biomarkers in conjunction with the classic tumour markers; and to increase sensitivity and specificity of disease identification (Zhang et al., 2004).

Regulated secretion in myofibroblasts was associated with the presence of dense cored secretory vesicles in the cytosol. These vesicles were relatively large in size and exhibited an electron opaque core resembling the dense core secretory vesicles identified in endocrine, neuroendocrine and exocrine cells. Recently, astrocytes from brain were reported to exhibit ultrastructural features similar to the dense core vesicles of the neuronal cells (Prada et al., 2011). In contrast,

myofibroblasts without regulated secretion occasionally exhibited dense core secretory vesicles in their cytosol, if any at all. Additionally, the higher number of secretory vesicles per field in the MSCs was consistent with the higher rate of regulated secretion from these cells.

There is substantial work relating chronic inflammation with cancer development (Balkwill and Mantovani, 2001). For example, there is a direct relation between stomach infection with *H.pylori* and gastric cancer (Parsonnet et al., 1991), between inflammatory bowel disease and colorectal carcinoma (Coussens and Werb, 2002), between Hepatitis C infection and liver carcinoma (Tsukuma et al., 1993) and between chronic pancreatitis and pancreatic cancer (Lowenfels et al., 1993). Pernicious anaemia is a condition of chronic inflammation in the gastric antrum related to changed mucosal morphology and hypergastrinaemic environment. Two of three myofibroblast lines taken from pernicious anaemia stomachs exhibited regulated secretion of TGFβig-h3. Due to the small group size it would be speculative to draw any other conclusions besides that these myofibroblasts resembled the normal cells from the gastric antrum in terms of their capacity for regulated secretion.

Bone marrow-derived MSCs have some features of fibroblast morphology (Majumdar et al., 1998). MSCs cultured in special supplemented medium to remain undifferentiated express and secrete TGFβig-h3, as shown by immunofluorescence and Western blot. These findings are consistent with data from proteomic studies on the media of human embryonic stem cell (hESC)-derived MSCs (Sze et al., 2007) which identified 201 unique proteins in the secretome of hESC-MSCs, including TGFβig-h3 and other ECM proteins,

growth factors and their binding proteins, and matrix proteases and their inhibitors.

In the present study, MSCs were shown to exhibit regulated secretion of TGFβig-h3 that was triggered by IGFs; the response was partially dependent on extracellular calcium and occurred from a preformed store. Mesenchymal stem cells are reported to express IGF-IR (Ponte et al., 2007) and are able to respond to IGF-I treatment (Worster et al., 2001). Based on the relative changes in abundance of protein in the media, the secretory response of MSCs was stronger than that of myofibroblasts, suggesting that MSCs may have a larger pool of stored protein (see above). Growth factor-triggered regulated secretion by MSCs arriving at a peripheral destination, including for example in preneoplastic stomach, could be functionally significant. In this context it is interesting that Houghton *et al*. showed that bone-marrow derived MSCs contribute to the development of gastric tumours in mice with chronic *H.felis* infection (Houghton et al., 2004). Moreover, there is evidence that myofibroblasts develop from MSCs in the same model (Quante et al., 2011). These studies suggest an active role for bone marrow-derived MSCs in gastric cancer progression, and further suggest a functional link to myofibroblasts; the secretory phenotype of these cells might therefore help to define the identity of the cellular niches following growth factor stimulation.

In conclusion, the results described in this chapter show consistent differences in the secretion of myofibroblasts derived from different tissues, thus providing further evidence of their heterogeneity. The data are consistent with a role for the local tissue microenvironment in defining myofibroblast phenotype in normal

and in cancer tissue; however, the relevant factors remain to be determined. In the meantime, differences in the secretory phenotype between normal and cancer-associated gastric myofibroblasts might become useful as a diagnostic disease marker. Additionally, the presence of a regulated secretion in the bone-marrow derived MSCs might be important in the studies of their emerging tumour-promoting role.

4.5 Conclusions

1. Myofibroblasts from gastric tissue tend to exhibit regulated secretion if they are from normal tissue and from tumours from patients with good survival.
2. Myofibroblasts from normal oesophageal tissue tend not to exhibit regulated secretion of TGFβig-h3, but do so when from oesophageal adenocarcinoma.
3. MSCs exhibit regulated secretion of TGFβig-h3 that requires extracellular calcium and occurs from a preformed vesicle pool.
4. Regulated secretion of myofibroblasts is not a ubiquitous cell property as it may vary depending on different local tissue factors.

CHAPTER 5

IDENTIFICATION OF PROTEINS IN THE SECRETOMES OF GASTRIC MYOFIBROBLASTS AND MSCs THAT EXHIBIT REGULATED AND CONSTITUTIVE SECRETION

5.1 Introduction

In recent years, secretome studies have become increasingly important in understanding both mechanisms of intercellular communication and the way that cells define their extracellular niche. Based on the findings described in the previous chapter it was considered important to identify the range of proteins in the secretomes of gastric myofibroblasts and MSCs that exhibited stimulated release in response to IGF-II (termed here "the regulated secretome") and by exclusion those that were released via constitutive secretory pathways (and so could be used as controls in mechanistic studies).

There are multiple possible approaches to defining secretomes (Skalnikova et al., 2011). The work in this chapter is based on previous experience in the group that utilised a mass spectrometry-based proteomic method, namely Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC) combined with identification and relative quantification of proteins in IGF-treated compared to control samples using LTQ OrbitrapVelos mass spectrometer.

Although relatively new, the method has been successfully applied to secretome studies of several different primary cell types (Greco et al., 2010; Spellman et al., 2008). Amongst the various bioinformatic tools available for the analysis of mass spectrometry datasets, the MaxQuant software package has proven advantageous when applied to SILAC data (Cox and Mann, 2008) and was used to generate the information used here.

Thus, this chapter makes use of data from an analysis of the regulated secretome of myofibroblasts and MSCs performed by Dr Chris Holmberg; the validation of

these datasets is described with a particular focus on the identification of proteins that might act as markers of either the regulated or constitutive secretory pathways.

5.1.1 Aims and Objectives

The specific aim of the work in this chapter was:

To identify putative constitutive and regulated secretory proteins in the secretomes of gastric myofibroblasts and MSCs, thereby validating targets identified by proteomic methods.

5.2 Materials and methods

5.2.1 Identification of proteins for validation studies

A list of putative secreted proteins in the media of myofibroblasts (Patient 14) and MSCs (Patient 37) was generated by Dr Chris Holmberg and provided for this study (Appendix). Candidate proteins for release by the constitutive and regulated pathway were selected from this list and validated using Western blot.

5.2.2 Sample generation

Cells were plated at $1x10^6$ cells in 10cm dishes and left to attach overnight. On the following day cells were washed 3 times with PBS, serum-starved for 1 h and treated with 100ng/ml IGF-II for 30 min. Media was collected, centrifuged (800g 4^0C, 7 min) and processed with StrataClean™ Resin (10µl resin for ml sample) for extraction of proteins. The resin suspension was vortexed for 1 min and centrifuged (2000rpm 4^0C, 1 min) to separate waste media from the resin. After removal of waste, resin was washed twice with 500µl 50mM ammonium bicarbonate ($(NH_4)_2CO_3$), resuspended in 100µl $(NH_4)_2CO_3$, aliquoted and stored at -80^0C until further processed.

5.2.3 Western blot analysis

Media samples processed with StrataClean™ Resin were analysed by Western blot without the boiling step with Laemmli buffer. A full list of the primary antibodies used in this chapter is presented in Chapter 2 Table 2.2.

5.3 Results

The secretomes of gastric myofibroblasts and MSCs

Proteomic analysis initially identified approximately 250 proteins in the media of myofibroblasts and MSCs and from this a list of 143 putative secreted proteins had been generated (see Appendix). Using the Universal Protein Resource (UniProt) database the 143 proteins were manually identified as (a) soluble secretory proteins, (b) shed membrane proteins, i.e. plasma membrane proteins with a sequence exposed on the extracellular surface providing a target for shedding mechanisms, or (c) existing in both forms (membrane/secreted).

There were 106 putative secreted proteins in the media of gastric myofibroblasts, and co-incidentally there was exactly the same number of putative secreted proteins in the media of MSCs. More than half of the secretomes of the two cells were shared between them (~60%). Thus of the total of 143 proteins that had been identified, there were 69 present in the media of both cell types (Table 5.3.1). From the pool of common proteins, 63 belonged to the group of soluble secretory proteins and 51 out of these exhibited increased relative abundance in IGF vs control media of >1.2, i.e. were considered to be secreted via the regulated pathway. None of the common secreted proteins exhibited a relative abundance in IGF vs control media of <1.2 in both cell types; in other words, there were no common proteins that were candidates for secretion by the constitutive pathway. However, 12 proteins were identified as exhibiting a relative abundance in IGF vs control media of <1.2 in either myofibroblasts or MSCs and were therefore candidates for constitutive release in one or other of these cells.

Protein category	Number of proteins
Common secretome	69
Common soluble secretory proteins	63
Common soluble secretory proteins with relative abundance >1.2	51
Common soluble secretory proteins with relative abundance <1.2	0
Common soluble secretory proteins with relative abundance <1.2 in either myofibroblasts or MSCs	12

Table 5.3.1 The secretomes of gastric myofibroblasts and MSCs. From all identified proteins a list of 143 putative secreted proteins was generated (see Appendix I). There were 106 putative secreted proteins present in the media of myofibroblasts, and the same number of different proteins was present in the media of MSCs. The two secretomes shared 69 proteins, 63 of these were secreted soluble factors and 51 of the latter exhibited relative abundance of IGF to control >1.2. The remaining 12 exhibited secretion by a different pathway in the two cell types, leaving none shared proteins with relative abundance <1.2.

The secretome of gastric myofibroblasts

Based on the UniProt database, 92 of 106 proteins in the gastric myofibroblast secretome were identified as soluble secreted proteins, 12 as membrane-associated and 2 as membrane/secreted proteins (see above). From the 92 secreted proteins, 79 exhibited an increased relative abundance in IGF vs control media of >1.2, while 13 exhibited <1.2 (Appendix). As outlined in Chapter 2, a difference >1.2 was considered to be indicative of regulated secretion.

Individual proteins in the myofibroblast secretome were then assigned to different functional groups namely ECM proteins, binding proteins, ligands, proteases, protease inhibitors, receptors, enzymes, other and unknown, again using the UniProt database (Figure 5.3.1). The analysis was conducted separately for proteins potentially released by regulated and constitutive pathways. Although the number of protein candidates for secretion by the constitutive pathway was relatively low, there were examples in all the main functional classes.

Validation of the secretome data of gastric myofibroblasts

Validation of the proteomic data was performed on ten secreted proteins using Western blot. These proteins were selected on the basis of representation in different functional classes, with examples drawn from both putative regulated and constitutively secreted proteins.

Table 5.3.2 shows the relative abundance in the media and some cell extracts from samples treated with IGF-II compared to the control samples from both mass spectrometry and Western blot. Eight proteins exhibited an increased relative abundance after IGF treatment of >1.2 in both proteomic studies and Western blots namely decorin, IGF-binding protein (IGFBP)-5, IGF-binding protein (IGFBP)-7, lumican, nexin, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), TIMP-2 and TGFβig-h3 (Table 5.3.2, Figure 5.3.2). Two proteins, galectin-3 and plasminogen activator inhibitor-1 (PAI-1), exhibited relative abundance of <1.2 in response to IGF treatment determined by both mass spectrometry identification and Western blot, compatible with constitutive release (Figure 5.3.3).

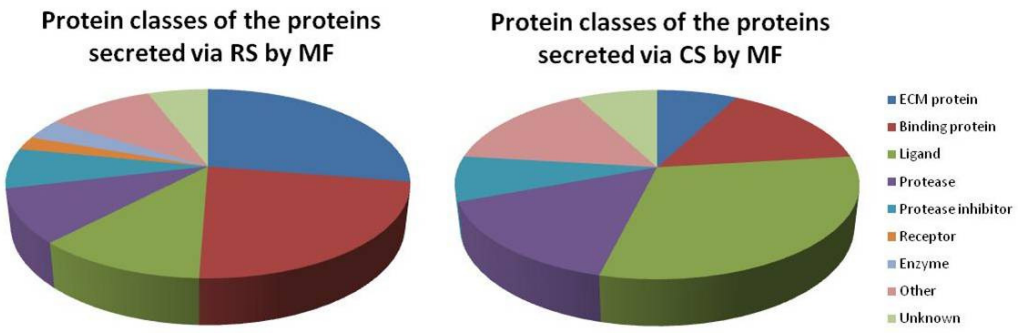


Figure 5.3.1 Separation by protein class of the secretome of gastric myofibroblasts. Nine functional categories were identified amongst proteins secreted via regulated secretion (RS) with the two predominant classes being ECM proteins and binding proteins. Proteins secreted via constitutive secretion (CS) belonged to seven categories. Allocation to different classes was based on UniProt protein database.

Protein name	Media		Cell extracts	
	SILAC-MS	Western Blot	SILAC-MS	Western Blot
Decorin	1.23	2.94	NI	0.98
Galectin-3	0.88	0.84	1.29	0.95
IGFBP-5	1.48	4.67	NI	1.01
IGFBP-7	1.72	1.50	NI	ND
Lumican	1.78	2.84	NI	ND
Nexin	2.47	1.59	1.69	ND
PAI-1	1.02	1.10	NI	ND
TIMP-1	1.54	1.36	1.15	ND
TIMP-2	1.80	2.35	NI	ND
TGFbigh-3	2.00	1.56	NI	1.09

Table 5.3.2 Relative abundance of selected proteins in the media and cell extract from myofibroblasts after IGF-II stimulation. Relative abundance of ten selected proteins from SILAC and Western blot identifications. Relative abundance for SILAC analysis was average of n=3 (n-number of replicates). NI-Not Identified, ND-Not Done.

Only three out of ten of the selected proteins were identified in cell extracts by mass spectrometry. Western blot analysis of one of these and of three other proteins in cell extracts indicated no change in relative abundance with IGF stimulation (Table 5.3.2).

The secretome of MSCs

Based on the UniProt database, 94 of 106 proteins in the secretome of MSCs were identified as soluble secreted proteins, 9 as membrane-associated and 3 as membrane/secreted proteins (see Appendix). From the 94 secreted proteins, 87 exhibited an increased relative abundance in IGF vs control media >1.2, while 7 exhibited <1.2 (Appendix).

Individual proteins in the MSCs secretome were then assigned to the same functional groups as above: ECM proteins, binding proteins, ligands, proteases, protease inhibitors, receptors, enzymes, other and unknown (Figure 5.3.4). The analysis was conducted separately for proteins potentially released by the regulated and constitutive pathways, and taking into account of the relatively low number of protein candidates for secretion by the constitutive pathway.

Validation of the secretome data of MSCs

Validation of the proteomic data was performed on seven secreted proteins using Western blot. These proteins were selected on the basis of representation in

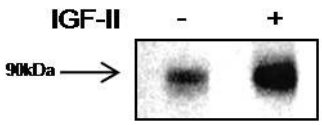
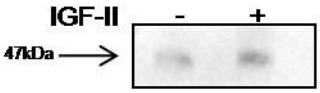
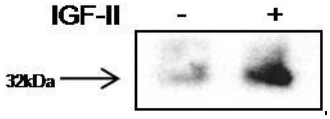
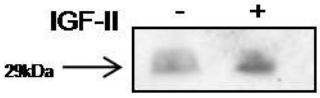
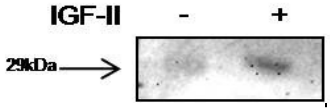
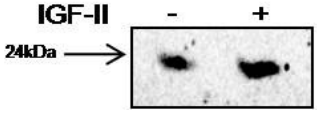
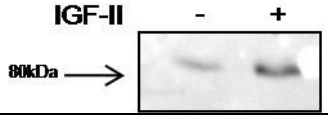
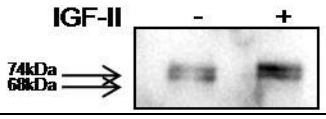
Protein name	Western Blot	Protein name	Western Blot
Decorin		Nexin	
IGFBP-5		TIMP-1	
IGFBP-7		TIMP-2	
Lumican		TGFβigh-3	

Figure 5.3.2 Representative Western blots of media from myofibroblasts after 30 minutes IGF-II stimulation. Eight proteins were chosen for validation after proteomic analysis suggested they were released via regulated secretion. Western blot identification confirmed the proteomic data.

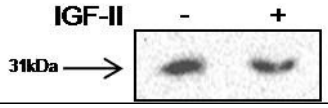
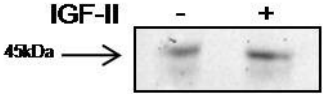
Protein name	Western Blot	Protein name	Western Blot
Galectin-3		PAI-1	

Figure 5.3.3 Representative Western blots of media from myofibroblasts after 30 minutes IGF-II stimulation. Two proteins were chosen for validation after proteomic analysis suggested they were secreted via constitutive secretion. Western blot identification confirmed the proteomic data.

different functional classes, with examples drawn from the putative regulated secreted proteins.

Table 5.3.3 shows the relative abundance in the media and some cell extracts from samples treated with IGF-II compared to the control samples from both mass spectrometry and Western blot. All seven proteins exhibited an increased relative abundance after IGF treatment of >1.2 in both proteomic studies and Western blots namely decorin, IGFBP-5, IGFBP-7, lumican, PAI-1, TIMP-2 and TGFβig-h3 (Table 5.3.3). Interestingly, PAI-1 exhibited relative abundance >1.2 in the media of MSCs, but <1.2 in the media of gastric myofibroblasts. An eighth protein, galectin-3, had not been identified by mass spectrometry in the media of MSCs, but in view of its identification as a candidate for constitutive release by myofibroblasts it was also studied by Western blot in MSC media. Interestingly, galectin-3 exhibited relative abundance in IGF-treated vs control media >1.2, suggesting that it is secreted in a regulated fashion by MSCs (Table 5.3.3, Figure 5.3.5).

Only one out of seven of the selected proteins was identified in the cell extracts by mass spectrometry. Western blot analysis of TGFβig-h3 in cell extracts indicated no change in relative abundance with IGF stimulation (Table 5.3.3).

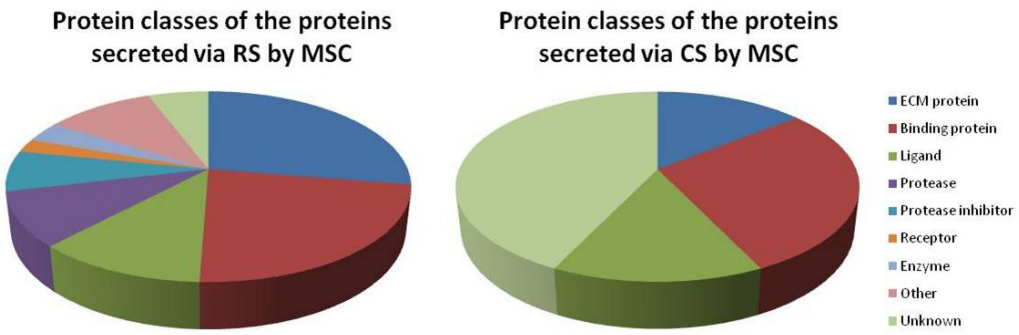


Figure 5.3.4 Separation by protein class of the secretome of MSCs. Nine functional categories were identified amongst proteins secreted via regulated secretion (RS) with the dominant being ECM proteins and binding proteins. Proteins secreted via constitutive secretion (CS belonged to four functional categories. Allocation to different classes was based on UniProt protein database.

Protein name	Media		Cell extracts	
	SILAC	Western Blot	SILAC	Western blot
Decorin	1.72	3.98	NI	ND
Galectin-3	NI	3.01	NI	ND
IGFBP-5	2.05	1.52	NI	ND
IGFBP-7	2.59	2.20	1.28	ND
Lumican	2.37	1.70	NI	ND
PAI-1	2.05	1.71	NI	ND
TIMP-2	2.28	2.16	NI	ND
TGFβig-h3	1.74	3.44	NI	1.03

Table 5.3.3 Relative abundance of selected proteins in the media and cell extract from MSCs after IGF-II stimulation. Relative abundance of seven specifically selected proteins from SILAC and Western blot identifications. Galectin-3 was identified in the media only by Western blot. Relative abundance for SILAC analysis was average of n=3 (n-number of replicates). Key: NI-Not Identified, ND-Not Done.

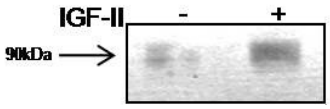
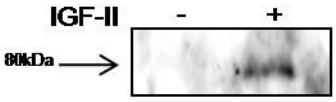
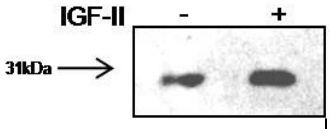
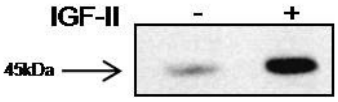
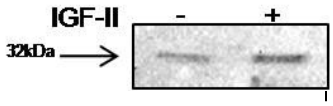
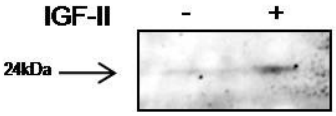
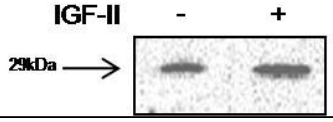
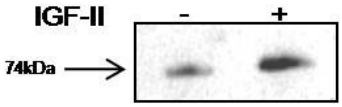
Protein name	Western Blot	Protein name	Western Blot
Decorin		Lumican	
Galectin-3		PAI-1	
IGFBP-5		TIMP-2	
IGFBP-7		TGFβig-h3	

Figure 5.3.5 Representative Western blots of media from MSCs after 30 minutes IGF-II stimulation. Eight proteins were chosen for validation after proteomic analysis suggested they were released via regulated secretion. Western blot identification confirmed the proteomic data.

5.4 Discussion

The present chapter describes for the first time data from a secretome-wide analysis of proteins exhibiting stimulated release by gastric myofibroblasts and MSCs after acute exposure to IGF-II. The study identifies candidates for release by both regulated and constitutive mechanisms. Interestingly, the former predominate. The data also reveal unexpected differences between myofibroblasts and MSCs in the capacity for regulated release of individual proteins.

Overlap in the secretomes of gastric myofibroblasts and MSCs (about 60% of each cell type) was not unexpected since these are both mesenchymal cell types. Moreover, there is evidence that myofibroblasts can develop from bone marrow-derived MSCs in murine gastric tumours with chronic *H.felis* infection (Quante et al., 2011). However, it was unexpected to see that 80% of the shared secretome was sensitive to IGF stimulation, and there were no examples of constitutively secreted proteins released by both cell types. It seems, then, that proteins released via the constitutive pathway in one cell type can be released via regulated secretion in a related cell type. It is of course, entirely feasible that further studies will indeed identify common constitutively released proteins. However, the finding that the same protein may exhibit IGF-stimulated release in one cell type but not the other, raises questions about the mechanisms determining release routes which will be taken up in the next chapter.

It is important to note that all proteins selected for validation exhibited relative abundance in the Western blot analysis consistent with the proteomic data, thereby showing robustness of the performed secretome analysis.

Separation by functional class for both myofibroblasts and MSCs demonstrated that secreted proteins represented a wide range of protein classes associated with the constitution and maintenance of the extracellular environment. These data are consistent with the large body of evidence indicating the role of myofibroblasts in secreting a range of different proteins involved in the formation of extracellular matrix, and in the repair and regeneration processes in inflammation and tissue injury (Powell et al., 1999). Similarly, utilising two different proteomic approaches, a recent study has identified a number of factors involved in tissue repair and regeneration in the media of human embryonic stem cells-derived MSCs (Sze et al., 2007). Additionally, our analysis showed for no particular preferences of protein functional class for a cell type or a release route.

Interestingly, although many similarities were identified between the myofibroblasts and MSCs, these were related to proteins exhibiting regulated release. In the first place it was unexpected to observe that shared secretome did not include any proteins potentially exhibiting constitutive release. There were, however, proteins with relative abundance <1.2, but not in the both cell types.

For example, PAI-1 was found not to exhibit increase in abundance in response to IGF stimulation of myofibroblasts, indicative for a non-regulated secretion, whereas there was increased abundance in response to IGF in MSCs compatible with release via regulated secretion. Multiple reports on human umbilical cord endothelial cells give evidence for a constitutive secretion of PAI-1 (Diamond et al., 1989; MacGregor and Booth, 1988; Stiko-Rahm et al., 1990). Conversely, a recent report found PAI-1 to be targeted to catecholamine storage vesicles of chromaffin cells and co-released with catecholamines in regulated fashion (Jiang

et al., 2011). These studies therefore support the idea that the same protein can be secreted via different release routes in different cell types.

Furthermore, in the validation studies galectin-3 exhibited relative abundance in response to IGF stimulation <1.2 in the media of myofibroblasts, but >1.2 in the media of MSCs. To understand this observation, however, it is important first to understand the release mechanism of galectin-3. As opposed to secreted proteins containing N-terminal signal sequence which targets them to ER (Kelly, 1985), galectins are known to lack N-terminal signal sequences, and thus they are example of an unconventional secretion (Muesch et al., 1990). A number of other proteins are known to exhibit unconventional secretion e.g. interleukin-1β (Rubartelli et al., 1990), fibroblast growth factor-2 (Mignatti and Rifkin, 1991; Schafer et al., 2004) and annexins (Christmas et al., 1991), however, the molecular mechanisms remain poorly understood. Galectin-3 has been found in culture supernatants entrapped within cytoplasmic vesicles, and hence it has been suggested that secretion occurs via membrane blebbing or ectocytosis (Mehul and Hughes, 1997; Stein and Luzio, 1991); it has also been found entrapped within exosomes secreted by dendritic cells (Thery et al., 2001). In both cases, galectin-3 was released inside vesicles and subsequently liberated after their lysis. Further, some reports show that galectin-1, another member of the lectin family, is secreted constitutively by Chinese hamster ovary (CHO)-K1 line (Cho and Cummings, 1995a; Cho and Cummings, 1995b). Conversely, there are cells where galectin-3 secretion is regulated to varying extents (Lindstedt et al., 1993; Sato and Hughes, 1994). Results from our study indicated that galectin-3 secretion was not regulated in gastric myofibroblasts, and thus raising the

possibility to use galectin-3 as a secretion control for these cells in further research.

In conclusion, the work in this chapter identified a number of proteins that are released in response to IGF and some that were not sensitive to IGF suggesting release by the constitutive pathway. Furthermore, this chapter presented similarities in the secretion profile of myofibroblasts and MSCs, along with some interesting differences.

5.5 Conclusions

1. A high proportion of proteins identified in the secretomes of both gastric myofibroblasts and MSCs exhibit increased abundance in response to IGF stimulation.
2. Individual proteins can exhibit IGF-stimulated secretion in one cell type, but not in another.
3. Protein markers for release by constitutive secretion were identified that can be used in future studies.

CHAPTER 6

INVESTIGATION OF THE MOLECULAR MECHANISMS DETERMINING REGULATED SECRETION BY MYOFIBROBLASTS

6.1 Introduction

Previous chapters reported that gastric myofibroblasts exhibited regulated secretion that tended to be absent in CAMs from patients with poor survival. However, the factors determining the presence or absence of regulated secretion in these cells are unknown.

Studies of secretory incompetent clones of neuroendocrine PC12 cells showed that the capacity for regulated secretion was associated with a specific gene and protein expression profile (Grundschober et al., 2002). Thus, in order to understand why some myofibroblasts exhibited regulated secretion while others did not, an obvious starting point was to study the gene expression profiles of these cell lines. The expression profile was therefore scanned for genes specifically associated with the regulated exocytotic machinery, including ones involved in biogenesis of the secretory granules, also known as regulated secretory proteins. The latter are known to be markers of both regulated secretion and disease progression in neuroendocrine tumours (Ischia et al., 2000; O'Connor and Deftos, 1986).

The results reported in this chapter utilised gene expression data from a previous microarray analysis performed within the group of all available lines of gastric myofibroblasts with the aim of identifying differences in expression profiles that would inform experimental studies of the underlying mechanisms. The experimental work in this chapter focuses on the characterisation of one specific protein, secretogranin II, which may play a role in determining the regulated secretory phenotype of gastric myofibroblasts.

6.1.1 Aims and Objectives

The specific aims of this chapter were:

1. To compare the transcriptomes of myofibroblasts exhibiting regulated secretion with those that do not, in order to identify differentially expressed genes.
2. To characterise secretogranin II and its role in the stimulated secretion of selected proteins.

6.2 Materials and methods

6.2.1 Experimental grouping

For the purpose of this study, myofibroblasts exhibiting regulated secretion (termed "responders") were compared with myofibroblasts without regulated secretion (termed "non-responders"). To increase accuracy of the analysis responders were defined on the basis of relative abundance of TGFβig-h3 in media in response to IGF of >1.5 compared with control, and non-responders as ≤1.17, thereby avoiding edge effects for cells that were on the margins (Table 6.2.1).

6.2.2 Data analysis

Using transcriptome data obtained by Dr Islay Steele (see section 2.4 Chapter 2) analysis of the data presented in this chapter was performed using Gene Spring GX v.10 (Agilent Technologies, USA). This included normalisation of gene expression data by the MAS5 algorithm, followed by checks for hybridisation and data quality, and identification of an "All Entities" list. Only transcripts flagged as "present" in all samples from one of the groups were used in the subsequent analysis. Given the experimental grouping, differentially expressed genes were identified using an unpaired t-test with no adjustment for multiple comparisons. A list of the genes regarded as significantly expressed ($p<0.05$) was refined by focussing on those with differential abundance of >2 in responders *vs* non-responders. Gene lists were uploaded into the Metacore™ database (Ekins et al., 2007) to generate corresponding protein lists. Differentially expressed

Responders			Non-Responders		
Patient ID	**Cell Type**	**Relative abundance IGF/Control**	**Patient ID**	**Cell Type**	**Relative abundance IGF/Control**
Patient 3	ATM	4.00	Patient 1	CAM	0.95
Patient 5	CAM	1.80	Patient 2	CAM	0.85
Patient 7	ATM	5.50	Patient 3	CAM	1.17
Patient 9	CAM	5.00	Patient 7	CAM	0.98
Patient 9	ATM	1.80	Patient 8	CAM	0.94
Patient 10	ATM	3.68	Patient 8	ATM	1.06
Patient 13	CAM	3.00	Patient 9	ATM	0.91
Patient 14	CAM	1.98	Patient 10	CAM	1.17
Patient 15	ATM	1.89	Patient 11	CAM	0.88
Patient 21	NTM	3.00	Patient 11	ATM	0.92
Patient 21	NTM	2.33	Patient 12	CAM	0.95
Patient 22	NTM	3.50	Patient 12	ATM	0.91
Patient 31	ATM	3.25	Patient 30	ATM	1.02

Table 6.2.1 Myofibroblasts used for gene expression analysis. Gastric myofibroblasts were divided into two groups (n=13 each3 according to the presence or absence of regulated secretion (responders *vs* non-responders), regardless the sample origin (NTM, ATM or CAM).

proteins were annotated by cellular localisation using the Gene Ontology database (www.geneontology.org).

6.2.3 Transfection

Myofibroblasts were transfected with siRNA or plasmid DNA using Nucleofection™ (Gresch et al., 2004). Protein secretion was studied 96 h post-transfection of myofibroblasts from Patient 14 (CAM, responder) and Patient 11 (ATM, non-responder).

6.2.4 Deconvolution analysis

Immunofluorescence images were analysed using an algorithm-based deconvolution method in the AxioVision 4.5 software, which reduced optical distortion and achieved sharper images.

6.2.5 Confocal microscopy

Myofibroblasts were prepared for confocal imaging and images were taken as described in section 3.2.4 (Chapter 3).

6.3 Results

6.3.1 Transcriptomic analysis of myofibroblasts with regulated secretion

To identify the gene expression profiles of responder and non-responder myofibroblasts, microarray data that had already been generated in the group were re-analysed. The designation of responder and non-responder cells was made regardless of tissue origin i.e. normal, adjacent or tumour tissue (Table 6.2.1).

The analysis identified a total of 18,861 expressed oligonucleotides flagged as "present". These oligonucleotides coded for 10,597 gene products, and 1,178 of them were significantly expressed by responders, i.e. passed the statistical test with $p<0.05$. Only 30 out of 1,178 differentially expressed genes were identified using a cut-off of 2-fold difference in abundance in the two datasets (Table 6.3.1). Of these differentially expressed genes 19 were up-regulated and the remaining 11 were down-regulated. Differentially expressed genes were identified from Metacore™ as coding for proteins belonging to nine different functional classes: metalloprotease (1), generic kinase (1), generic binding protein (11), generic enzyme (3), generic protein (3), transcription factor (3), generic receptor (3), generic protease (2), RNA (1) and receptor ligand (2). However, 8 out of 30 differentially expressed transcripts encoded soluble secreted proteins (in bold, Table 6.3.1). Two of the 8 transcripts (mannan-binding lectin serine protease 1 and stanniocalcin-1) were decreased in the responder dataset, while the remaining six were increased: disintegrin and metalloprotease domain-containing protein 12 (ADAM-12), glia-derived nexin,

#	Protein name	Protein class	Fold change	P-value
1	**Disintegrin and metalloproteinase domain-containing protein 12**	Metalloprotease	2.88	0.025
2	Adenylate kinase isoenzyme 4, mitochondrial	Generic kinase	2.28	0.003
3	Cadherin-2	Generic binding protein	2.16	0.017
4	Calponin-1	Generic binding protein	2.16	0.033
5	Cystathionine gamma-lyase	Generic enzyme	2.14	0.018
6	DENN domain-containing protein 2A	Generic protein	-2.06	0.025
7	ETS translocation variant 3	Transcription factor	2.41	0.002
8	Protein FAM65C	Generic protein	-2.09	0.027
9	FLJ35700 (gene symbol)	Generic protein	-3.16	0.034
10	Polypeptide N-acetylgalactosaminyltransferase 12	Generic enzyme	2.08	0.033
11	Transcription factor GATA-6	Transcription factor	2.15	0.047
12	Interleukin-17 receptor D	Generic receptor	2.35	0.043
13	Integrin beta-like protein 1	Generic receptor	2.38	0.033
14	**Mannan-binding lectin serine protease 1**	Generic protease	-2.64	0.015
15	MEG3 (gene symbol)	RNA	2.44	0.026
16	Neuron navigator 3	Generic binding protein	2.16	0.006
17	Protein naked cuticle homolog 2	Generic binding protein	-2.35	0.015
18	cAMP-specific 3',5'-cyclic phosphodiesterase 4B	Generic enzyme	-2.28	0.011
19	PDZ and LIM domain protein 3	Generic binding protein	2.83	0.013
20	**Trypsin-3**	Generic protease	2.20	0.010
21	Protein S100-A4	Generic binding protein	-2.12	0.012
22	**Secretogranin-2**	Receptor ligand	3.03	0.014
23	**Plasminogen activator inhibitor 1**	Receptor ligand	2.24	0.005
24	**Glia-derived nexin**	Generic binding protein	2.25	0.001
25	SH3-containing GRB2-like protein 3-interacting protein 1	Generic binding protein	-2.11	0.009
26	**Stanniocalcin-1**	Generic binding protein	-2.95	0.036
27	Transcription factor 21	Transcription factor	-2.09	0.012
28	Transmembrane 4 L6 family member 1	Generic receptor	-2.53	0.037
29	Tensin-1	Generic binding protein	2.02	0.016
30	**Versican core protein**	Generic binding protein	2.45	0.008

Table 6.3.1 Differentially expressed genes in responder compared with non-responder cells. Gene expression analysis indentified 30 differentially expressed genes defined as exhibiting a 2-fold difference in abundance in responders and non-responders (unpaired t-test, $p<0.05$, no adjustment for multiple comparisons); these encoded proteins in nine different functional classes. There were eight soluble secretory proteins (**in bold**).

integrin beta-like protein 1, PAI-1, trypsin-3, versican and secretogranin II. The latter exhibited the greatest difference between the two datasets (3.03).

6.3.2 Secretogranin II and its role in the regulated secretion of myofibroblasts

Myofibroblasts express secretogranin II

On the basis of the transcriptomic analysis, secretogranin II was selected for further study at the protein level using immunofluorescence and Western blot. Secretogranin II distribution in myofibroblasts exhibiting regulated secretion was predominantly punctuate consistent with vesicular localisation (Figure 6.3.2.1, **A-D**). Secretogranin II was present throughout the cell although in varying intensity in different cell lines. However, in non-responder myofibroblasts it was difficult to identify secretory vesicles (Figure 6.3.2.1, **E-H**). Consistently, Western blot analysis revealed that cellular secretogranin II abundance was higher in responder myofibroblasts than non-responders (unpaired t-test, $p<0.05$, $n=6$, n-number of cell lines in each group) (Figure 6.3.2.2). Additionally, responder myofibroblasts exhibited increased secretogranin II secretion after stimulation with IGF-II for 30 min (Figure 6.3.2.3) whereas non-responders did not (see below, Figure 6.3.2.9).

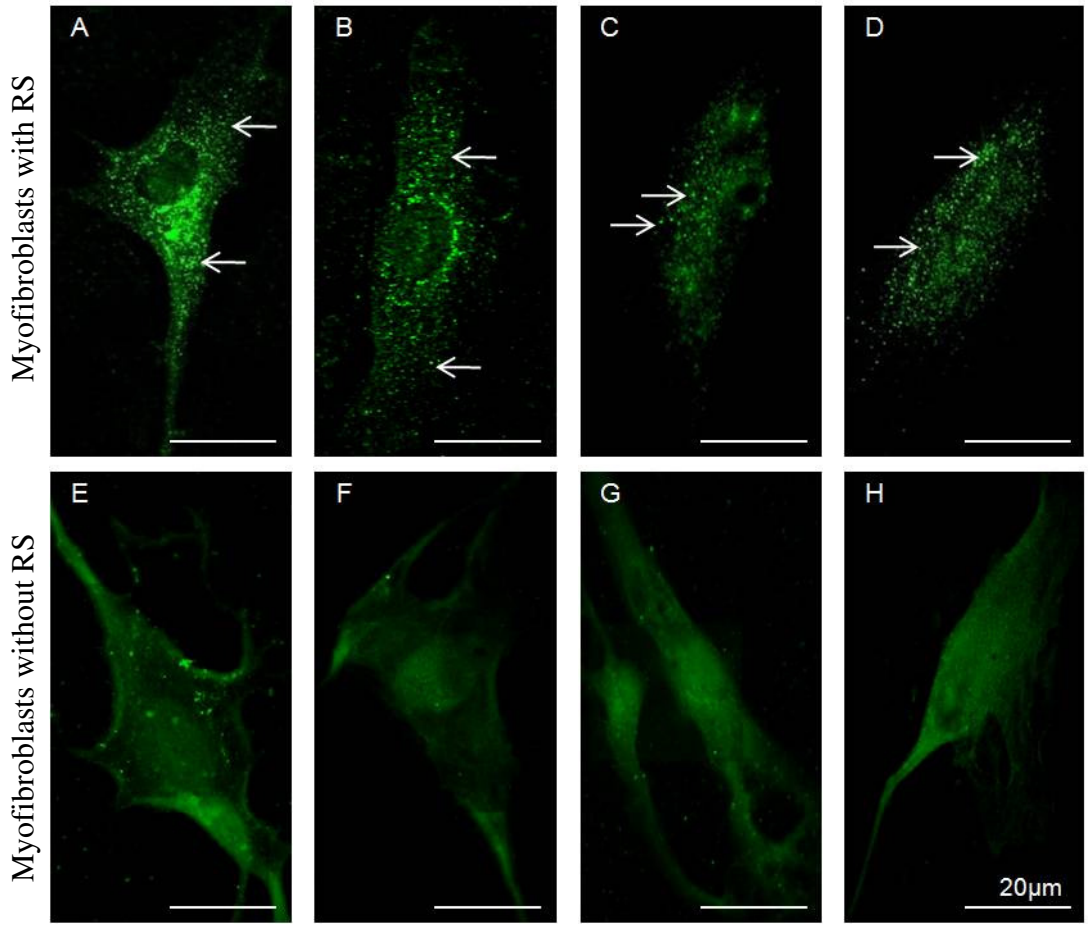


Figure 6.3.2.1 Responder myofibroblasts express secretogranin II. Immunocytochemical localisation with deconvolution analysis of secretogranin II (green) showed punctuate and vesicular staining (white arrows) throughout the cytosol in responder myofibroblasts (**A-D)** but not in non-responder myofibroblasts (**E-H).**

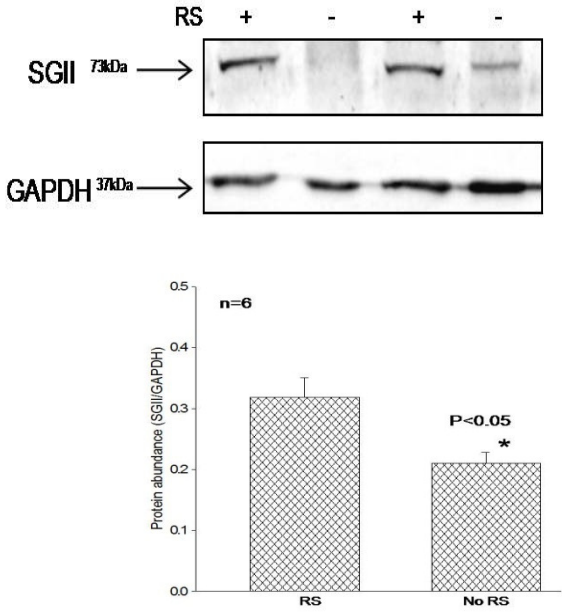


Figure 6.3.2.2 Increased expression of secretogranin II in responder myofibroblasts. A. Representative Western Blot analysis of cellular secretogranin II in two responder and two non-responder myofibroblasts (top panel). GAPDH was used as a loading control (bottom panel). **B.** Cumulative densitometric analysis (n=6 in each group; reference samples were loaded on every gel for normalization) of cellular secretogranin II showed significantly higher relative protein abundance in the responders (paired t-test, $p<0.05$).

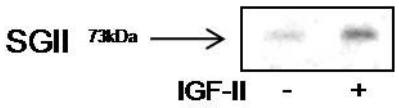


Figure 6.3.2.3 Stimulated secretion of secretogranin II in responder myofibroblasts. Representative Western Blot analysis of media from a responder myofibroblast showed increased abundance of secretogranin II after IGF-II treatment (100 ng/ml) for 30 min.

Consequences of inhibition of secretogranin II expression in responder myofibroblasts

To study the role of secretogranin II in regulated secretion, secretory responses to IGF-II were examined after secretogranin II gene was knocked down using siRNA. Using a myofibroblast cell line (Patient 14, CAM) that consistently exhibited robust secretory responses to IGF-II, and a short doubling time, the most effective of three siRNA molecules (siRNA3) in down-regulating secretogranin II gene expression was identified. With this construct there was

approximately 72% reduction in secretogranin II cellular protein reduction (Figure 6.3.2.4).

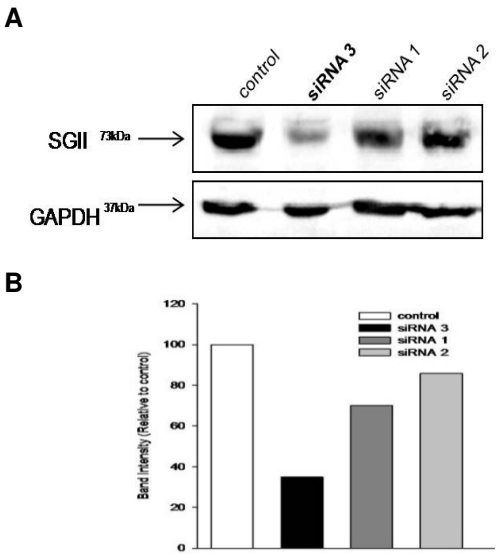


Figure 6.3.2.4 Decreased expression of secretogranin II in response to siRNA silencing. A. Western Blot analysis of secretogranin II after gene silencing with three different siRNAs (top panel). GAPDH was used as a loading control (bottom panel). **B.** Densitometric analysis of corresponding band intensities showed that secretogranin II expression was reduced by 72% with siRNA3, 26% with siRNA1 and 12% with siRNA2. (n=2)

Responses to IGF-II stimulation of myofibroblasts after secretogranin II knock down were then studied by monitoring release of three representative proteins. Secretogranin II itself, TGFβig-h3 and galectin-3 were selected for this purpose: TGFβig-h3 because it had previously been shown to exhibit regulated secretion, galectin-3 because it had previously been shown not to exhibit regulated secretion and secretogranin II to indicate the efficacy of knockdown. After secretogranin II silencing there was inhibition of the stimulated secretion of secretogranin II (Figure 6.3.2.5), and secretion of TGFβig-h3 was completely blocked (Figure 6.3.2.6). There was no change, however, in galectin-3 secretion (Figure 6.3.2.7).

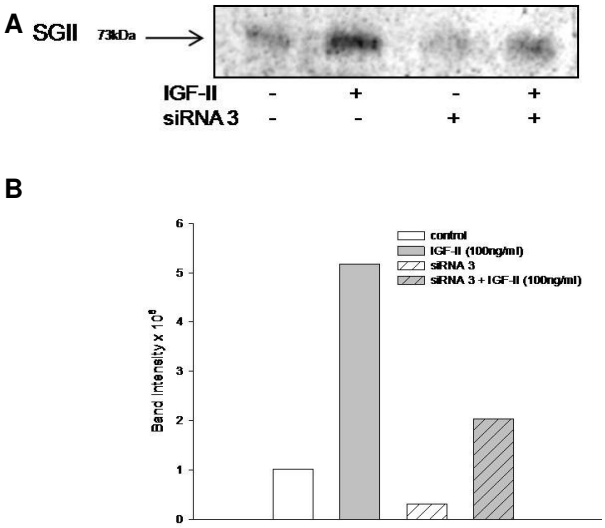


Figure 6.3.2.5 Decreased stimulated release of secretogranin II from myofibroblasts treated with siRNA3. A. Representative Western Blot analysis of secretogranin II in the media from responder myofibroblasts. There was a decrease in the secretory response to IGF-II after gene silencing. **B.** Densitometric analysis of the Western blot showed that IGF-stimulated secretion of secretogranin II was inhibited in siRNA3-treated cells. (n=2)

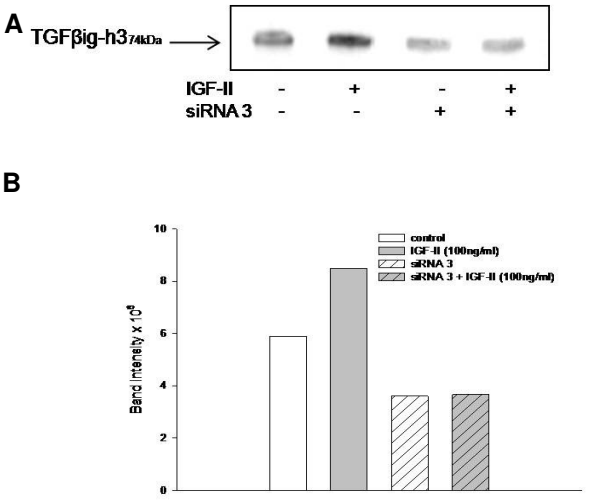


Figure 6.3.2.6 Inhibition of stimulated release of TGFβig-h3 from myofibroblasts treated with siRNA3. A. Representative Western Blot analysis of TGFβig-h3 in the media from responder myofibroblasts. There was inhibition of the secretion response to IGF-II after gene silencing. **B.** Densitometric analysis of the Western blot showed that IGF-stimulated secretion of TGFβig-h3 was inhibited in siRNA3-treated cells. (n=2)

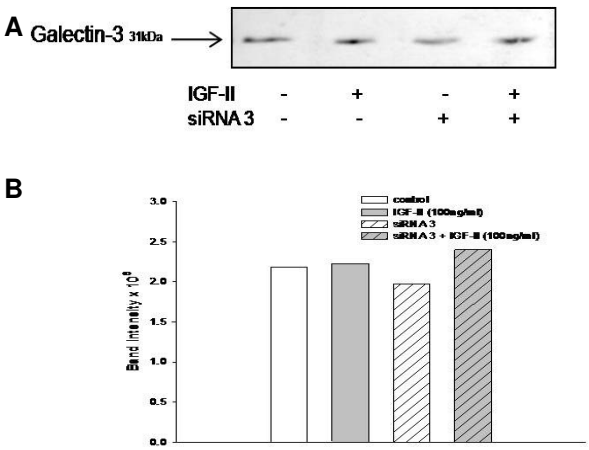


Figure 6.3.2.7 No change of galectin-3 release from myofibroblasts treated with siRNA3.A. Representative Western Blot analysis of galectin-3 in the media from responder myofibroblasts. There was no change in the secretion response to IGF-II after gene silencing. **B.** Densitometric analysis of the Western blot showed no change in the secretion of galectin-3. (n=2)

Consequences of overexpression of secretogranin II gene in non-responder myofibroblasts

To further explore the role of secretogranin II in the regulated secretion of gastric myofibroblasts, secretogranin II was then overexpressed in non-responder myofibroblasts. Many non-responder lines exhibited low levels of secretogranin II expression and for this experiment the line with the lowest protein expression according to Western blot analysis was chosen (Patient 11, ATM).

Using Western blot, cellular secretogranin II abundance after transfection with a secretogranin II expressing plasmid, was about 1.8 times greater than control untransfected cells (Figure 6.3.2.8). The secretory response to IGF-II after overexpressing secretogranin II was assessed using Western blot of secretogranin II itself, TGFβig-h3 and galectin-3 (see above). There was a substantial increase in secretogranin II stimulated secretion (Figure 6.3.2.9), and a modest increase in TGFβig-h3 stimulated secretion (Figure 6.3.2.10) in the transfected cells. However, there was no change in the galectin-3 secretion after secretogranin II overexpression (Figure 6.3.2.11).

6.3.3 Co-localisation studies of selected secreted proteins in myofibroblasts exhibiting regulated secretion

Finally, the sub-cellular localisation of proteins exhibiting regulated and non-regulated secretion was examined by confocal microscopy. Secretogranin II and galectin-3 were found to be co-expressed in the ER and Golgi region, while they were expressed in different secretory vesicles in the cytosol (Figure 6.3.3).

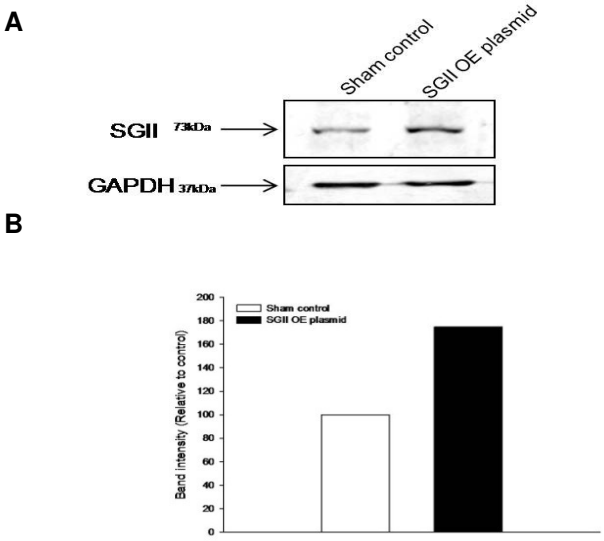


Figure 6.3.2.8 Increased cellular protein abundance after overexpression of secretogranin II gene. A. Western Blot analysis of cellular secretogranin II in control and transfected myofibroblasts (top panel). GAPDH was used as a loading control (bottom panel). **B.** Densitometric analysis of corresponding band intensities showed increased secretogranin II protein expression after secretogranin II overexpression (n=2).

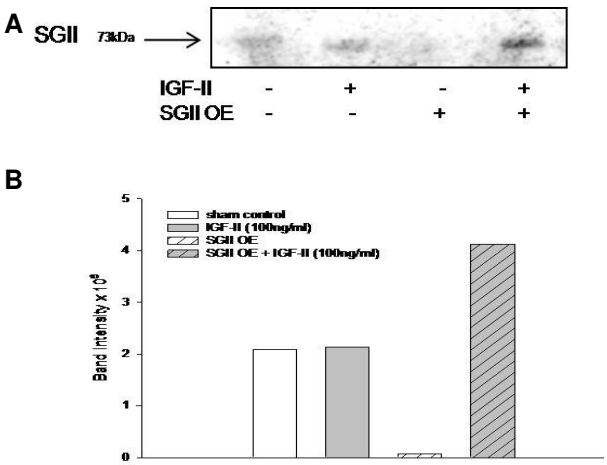


Figure 6.3.2.9 Stimulated release of secretogranin II in non-responder myofibroblasts after secretogranin II overexpression. A. Representative Western Blot analysis of secretogranin II in the media from myofibroblasts without RS. There was an increase in the secretion response to IGF-II after secretogranin II overexpression. **B.** Densitometric analysis of the Western blot showed the presence of IGF-II stimulated secretion after secretogranin II overexpression (n=2).

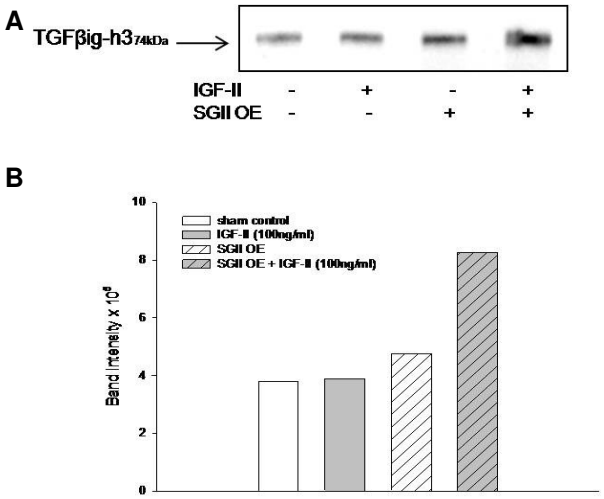


Figure 6.3.2.10 Stimulated release of TGFβig-h3 in non-responder myofibroblasts after secretogranin II overexpression. **A.** Representative Western Blot analysis of TGFβig-h3 in the media from myofibroblasts without RS. There was an increased secretion response to IGF-II after secretogranin II overexpression. **B.** Densitometric analysis of the Western blot showed the presence of IGF-II stimulated secretion of TGFβig-h3 after secretogranin II overexpression. (n=2)

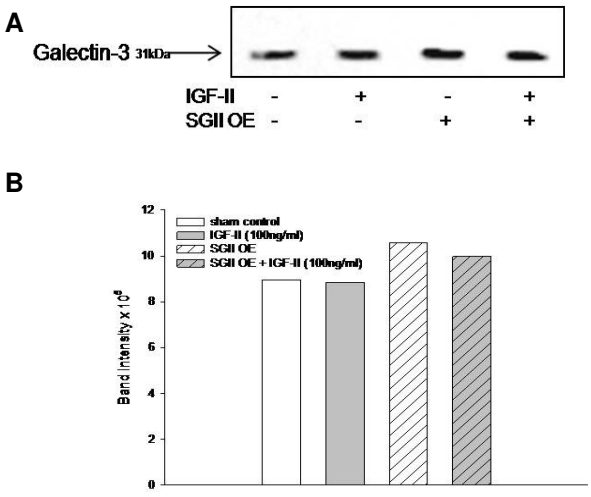


Figure 6.3.2.11 No change of galectin-3 release from non-responder myofibroblasts transfected with secretogranin II. **A.** Representative Western Blot analysis for galectin-3 in the media from myofibroblasts without RS. There was no change in the secretion response to IGF-II after secretogranin II overexpression. **B.** Densitometric analysis of the Western blot showed no change in the secretion of galectin-3 after IGF-II stimulation. (n=2)

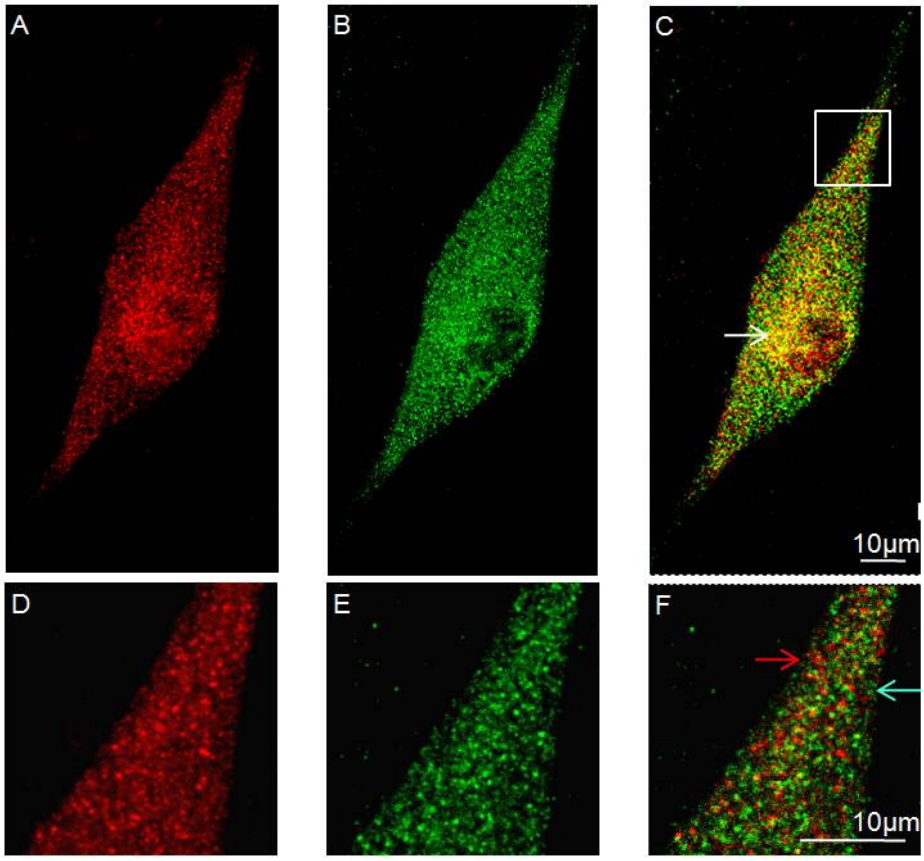


Figure 6.3.3 Secretogranin II and galectin-3 localise to different secretory vesicles in myofibroblasts with regulated secretion. Confocal microscopy of secretogranin II (green, **green arrow**) and galectin-3 (red, **red arrow**) in myofibroblasts with regulated secretion. Galectin-3 (**A, D**) and secretogranin II (**B, E**) co-localise in ER and Golgi (**white arrow**), but they belong to different types of secretory vesicle (**C, F**).

6.4 Discussion

The work in this chapter focuses on differences in the transcriptomes of responder and non-responder myofibroblasts and identifies the regulated secretory protein secretogranin II as a differentially expressed secretory protein. The results suggest that expression of secretogranin II plays a role in determining the regulated secretory phenotype and that there are distinct vesicular compartments for this protein and for galectin-3, which does not exhibit regulated exocytosis.

Based on the results presented in Chapter 4, it was not unexpected to identify differences in the gene expression profiles of responder and non-responder myofibroblasts. Given the functional difference between the two groups it was anticipated that differential genes might encode proteins related to the regulated exocytotic machinery including t-SNAREs and v-SNAREs, chromogranins and secretory peptides. Surprisingly, of 30 differentially expressed genes only one was directly associated with the regulated exocytotic phenotype – secretogranin II. Thus, secretogranin II is a member of the granin family (as called chromogranin C) and has been previously proposed as a marker for the neuroendocrine secretory phenotype (Ozawa and Takata, 1995; Rosa et al., 1985).

In PC12 cells there is a considerable body of research on the differences between secretory competent and secretory incompetent clones. The results indicate that regulated exocytosis is a complex program that includes regulation at multiple levels: transcriptional (Grundschober et al., 2002), post-transcriptional (Bruce et

al., 2006; Knoch et al., 2004), translational (Courel et al., 2010) and protein turnover or degradation (Kim and Loh, 2006; Kim et al., 2003).

Chromogranins (granins) are acidic soluble proteins that were initially identified to be co-secreted with catecholamines (Banks and Helle, 1965). It is now known that chromogranins are implicated in the biogenesis of secretory granules and in sorting them at the *trans*-Golgi network. Two mechanisms have been investigated: "sorting-for-entry" (based on specific primary structure of the granin molecules) and "sorting-by-retention" (based on protein aggregation under certain conditions). During the latter, in the lumen of the *trans*-Golgi high concentrations of calcium and low pH promote the aggregation of granins (chromogranin A, chromogranin B and secretogranin II) to generate the electron dense core of secretory vesicles (Chanat and Huttner, 1991). During aggregation granins may serve as chaperones for recruitment of other regulated secretory proteins (Gerdes et al., 1989; Gorr et al., 1989). "Sorting-for-entry" hypothesis is based on the amino acid sequence of the granin molecules. A N-terminal hydrophobic disulfide-bonded loop in the primary structure of chromogranin B was identified to serve as a sufficient sorting motif to direct this granin into the dense core vesicles (Chanat et al., 1993). An additional amphipathic α-helix was needed for chromogranin A to direct it to the regulated pathway (Taupenot et al., 2002). The primary structure of secretogranin II, however, lacks the N-terminal hydrophobic disulfide-bonded loop (Taupenot et al., 2003). It was reported that sorting information of secretogranin II to the regulated pathway was contained within both N- and C-terminals of the molecule, and involved specific α-helix-containing amino acid sequences (Courel et al., 2008).

Down-regulation of the chromogranin A and chromogranin B was associated with the loss of secretory granules in PC12 cells, whereas their overexpression, including in non-secretory cells, has triggered granule biogenesis (Huh et al., 2003; Kim et al., 2001). Mice with deletion of either the *chromogranin A* or *chromogranin B* genes nevertheless still exhibit regulated exocytosis, due to activation of compensatory mechanisms (Borges et al., 2010). In a secretory-deficient pheochromocytoma PC12 cells that are unable to store and release catecholamines via regulated secretion, expression of secretogranin II was sufficient to rescue the secretory phenotype (Courel et al., 2010). This was consistent with the data from the present study showing that expression of secretogranin II in responder myofibroblasts correlates with a distinct secretory response to IGF-II, which, however, was depressed after silencing of secretogranin II expression. Interestingly, the non-responder phenotype was associated with a low level of secretogranin II expression, which was not sufficient to generate a secretory response to IGF-II.

Initially, Kim *et al.* suggested that chromogranin A controls the stability of dense core granules by regulating degradation of granule proteins (Kim et al., 2003). Further they showed that overexpression of protease nexin-1 stabilised dense core granule proteins in the Golgi apparatus and thus promoted granulogenic properties and stimulated the regulated secretory phenotype (Kim and Loh, 2006). Based on these observations, they proposed a mechanism by which chromogranin A up-regulated protease nexin-1 expression, which further supported the granulogenic properties of chromogranin A and inhibited the proteolytic degradation of granule proteins in Golgi. It is important to note that

our analysis also identified protease nexin amongst the nine differentially expressed genes alongside secretogranin II (and in previous chapters regulated release of protease nexin was demonstrated), so that a similar stabilising effect might exist between protease nexin and secretogranin II in myofibroblasts.

Notwithstanding the evidence discussed above, there is nevertheless a view that chromogranin A and chromogranin B are simply cargo proteins, and thus their overexpression may not be sufficient to reinstall the regulated secretion (Day and Gorr, 2003; Malosio et al., 2004). Malosio *et al.* demonstrated that the newly formed organelles held features of lysosomes, but not secretory vesicles, thus raising the need to study the exact nature of the secretory vesicles of gastric myofibroblasts.

Based on the data from Chapter 5 galectin-3 was successfully employed as a control protein, i.e. protein secreted by non-regulated secretion. In this sense, it was not unexpected to see that secretogranin II and galectin-3 did not co-localise to the same vesicles.

Interestingly, the *secretogranin II* gene was expressed by all gastric myofibroblasts regardless of the capacity for regulated secretion, but it was considerably higher in responders, thereby implying transcriptional down-regulation in non-responder myofibroblasts. Recent studies identified transcriptional repressor RE-1–silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) as suppressing the regulated secretory phenotype in astrocytes (Bruce et al., 2006; Prada et al., 2011), but it was not identified as present in our microarray data, suggesting that other transcriptional

factors may regulate secretogranin II in myofibroblasts, and hence may influence the secretory phenotype.

The results of the current chapter indicate that a defect in secretrogranin II expression, occurring at transcriptional, translational and/or post-translational level influences the regulated secretory phenotype. Together with the evidence presented in the previous chapters, the data suggest that the non-responder phenotype might be a result of multiple defects in the regulated exocytotic machinery of these cells, and specifically due to defective secretogranin II expression as well as impaired intracellular calcium signalling. The present data suggest that secretogranin II expression in gastric myofibroblasts might provide a marker for the responder phenotype and its loss an indicator of cancer progression similar to that in some malignant neuroendocrine tumours (Schmid et al., 1994).

6.5 Conclusions

1. Differentially expressed genes in responder myofibroblasts included at least one encoding a protein involved in regulated exocytotic machinery - secretogranin II.

2. Loss of secretogranin II expression was associated with impaired regulated secretion of an independent protein, TGFβig-h3, and overexpression – with restored secretion.

3. Lack of regulated secretion capacity in myofibroblasts appears to be determined by defects in multiple levels.

CHAPTER 7

DISCUSSION

7.1 Overview

The main findings of this thesis are (a) that gastric myofibroblasts exhibit calcium-dependent secretion in response to stimulation by IGF; (b) the capacity for regulated secretion depends on the tissue microenvironment, since normal gastric myofibroblasts exhibited regulated secretion while those from the oesophagus did not; (c) some gastric CAMs, particularly from patients with poor survival, lack regulated secretion; (d) the secretory response to IGF includes the release of many different types of protein including ECM proteins, ligands, binding proteins, carbohydrate-binding proteins, proteases and protease inhibitors; (e) the capacity for regulated secretion of gastric myofibroblasts is associated with the expression of secretogranin II, which in turn may provide a useful biomarker for the regulated secretory phenotype of myofibroblasts.

7.1.1 Myofibroblasts exhibit regulated secretion

A growing body of literature indicates that in addition to neuronal, endocrine and exocrine cells a wide variety of other cell types exhibit regulated secretion; examples include 3T3-L1 adipocytes (Lorente-Cebrian et al., 2010) and Chinese hamster ovary cells (Chavez et al., 1996; Coorssen et al., 1996) in which secretion occurs from Golgi-derived vesicles, but in addition pancreatic stellate cells (which resemble myofibroblasts) (Phillips et al., 2010) and hippocampal astrocytes (Bezzi et al., 2004; Kreft et al., 2004; Zhang et al., 2004) exhibit regulated secretion by small, clear synaptic-like vesicles (Table 7.1.1). In all

cases, the cells respond to a range of stimuli with increase in intracellular calcium triggering release of small neurotransmitters or proteins.

The evidence is based on the use of a wide range of methods, including ELISA, subcellular fractionation, liquid chromatography with mass spectrometry and calcium signalling measurements (Table 7.1.1). Thus, secretion by 3T3-L1 adipocytes of the neuropeptide apelin was demonstrated before and after treatment with actinomycin D and cycloheximide using ELISA. Alternatively, Philips et al. used liquid chromatography with mass spectrometric analysis to study secreted acetylcholine (Phillips et al., 2010), and Chavez et al. used subcellular fractionation of different secretory vesicles to separate the vesicles of the regulated and constitutive pathways (Chavez et al., 1996). Electron microscopy studies of pancreatic stellate cells (Phillips et al., 2010) and immunogold labelling in hippocampal astrocytes (Bezzi et al., 2004) revealed secretory vesicles resembling synaptic-like vesicles. Some of the techniques used in these studies were employed in the present project, and the application of others (e.g. sub-cellular fractionation, EM immunogold labelling, pulse-chase labelling: see below) to the study of regulated secretion of myofibroblasts is now required.

Cell type	Marker of secretion	Type of vesicles	Stimulus	Methods	Reference
3T3-L1 adipocytes	Neuropeptide apelin	Golgi-derived	Eicosapentaenoic acid	ELISA, actinomycin D and cycloheximide	(Lorente-Cebrian et al., 2010)
CHO cells	$[^{35}S]SO_4$-labeled glycosaminoglycan chains	Golgi-derived	Ionophore A23187 and phorbol esters	N-ethylmaleiimide, subcellular fractionation, WB, indirect immunofluorescence	(Chavez et al., 1996)
CHO cells, 3T3 fibroblasts	Membrane capacitance	Golgi-derived	Different concentrations of calcium	Calcium signalling	(Coorssen et al., 1996)
Pancreatic stellate cells	Neurotransmitter acetylcholine	Clear synaptic-like vesicles	Cholecystokinin	LC-MS/MS, EM	(Phillips et al., 2010)
Hippocampal astrocytes	Gliotransmitter glutamate	Clear synaptic-like vesicles	Different stimuli	Total internal reflection fluorescence imaging (TIRF)c, immunogold labelling	(Bezzi et al., 2004)

Table 7.1.1 Methods used in studies of regulated secretion. Examples of non-specialised for regulated secretion cells and of approaches utilised to study their regulated secretion.

7.1.2 Characteristics of regulated secretion by gastric myofibroblasts

7.1.2.1 Role of the tissue microenvironment

Myofibroblasts from different tissues are phenotypically diverse. Initially, myofibroblasts were described as a sub-type of fibroblasts expressing smooth muscle-like features (Gabbiani et al., 1971), localised either close to epithelial cells as in the intestine (Pascal et al., 1968) or further away as in the stomach (Wu et al., 1999). The first studies of gastric myofibroblasts derived from the lamina propria established that these retained the phenotype seen *in vivo* with

respect to the expression of the cytoskeletal markers α-SMA and vimentin (but negative for desmin) and functional enzymes such as cyclooxygenase(COX)-1 and -2 (Wu et al., 1999). In addition, studies on cultured colonic myofibroblasts demonstrated expression of collagen type IV, laminin-β1 and γ1, and fibronectin resembling that *in vivo* (Mahida et al., 1997). The present project was based on cells obtained by the method used by Wu et al. (1999) coupled with immunocytochemical characterisation. These cells are thought to be identical to, or at least closely resemble, those studied by others (Mahida et al., 1997; Wu et al., 1999).

The heterogeneity of myofibroblasts has been associated with their role in tissue organisation (Powell et al., 1999). For example, myofibroblasts from small and large intestine form tight pericryptal sheath below the epithelial cells (Mutoh et al., 2005), gastric myofibroblasts are scattered throughout all levels of gastric mucosa (Wu et al., 1999), while there are no myofibroblasts in the normal heart tissue (Baum and Duffy, 2011). Moreover, stromal CAMs are shown to acquire genetic mutations including loss of heterozygosity (Hill et al., 2005; Patocs et al., 2007) and epigenetic changes like global DNA hypomethylation and focal gain of DNA methylation (Jiang et al., 2008). These observations suggest that the phenotype of gastric myofibroblasts, including secretory phenotype, might be influenced by factors from tissue microenvironment such as type and function of the organ, physiological and pathophysiological status. The present study is the first to identify differences in the secretory phenotype of myofibroblasts from the upper gut, as well as between cancer and normal tissue, but the factors determining this need to be further studied.

7.1.2.2 Expression of dense core secretory vesicles

The regulated secretory phenotype of myofibroblasts is associated with the expression of dense core secretory vesicles with diameter about 100nm and located close to the Golgi apparatus (Table 7.1.2). Two previous studies using TEM show abundant rough endoplasmic reticulum, stress fibres (longitudinal bundles of actin microfilaments), Golgi apparatus and mitochondria, but give no information on dense core secretory vesicles (Mahida et al., 1997; Valentich et al., 1997). Dense core vesicles are not mentioned by these authors, but interestingly some dense core vesicle-like structures can be seen in the cytosol of the colonic myofibroblasts in the photomicrographs, and it would be interesting to study the secretory phenotype of these cells (Mahida et al., 1997).

7.1.2.3 Regulated release of ECM proteins

Regulated secretion by myofibroblasts was not found to be associated with specific classes of proteins, but there were many examples of regulated secretion of proteins associated with ECM assembly, organisation and maintenance. This presumably reflects the role of myofibroblasts as a major cell type responsible for ECM remodelling after tissue damage and in cancer (Desmouliere et al., 2004). However, the high proportion of all identified secretory proteins released

Type of vesicle	Cell types	Origin	Morphology	Diameter	Content	Examples of markers (Reference)
Constitutive vesicles	All cells	Golgi-derived	Small and clear	~100nm	Plasma membrane proteins, proteoglycans	Glycosaminoglycan (Burgess and Kelly, 1984), plasma membrane proteins (Holcomb et al., 1988)
Synaptic vesicles	Neurons, neuroendocrine cells	Non-Golgi-derived	Small and clear	~40nm	Neuro-transmitters	Vesicular transporters (Sudhof, 2004), ATP-dependent proton pump (Stadler and Tsukita, 1984)
Zymogen granules	Exocrine cells e.g. pancreatic acinar cells	Golgi-derived	Varying electron density	≥1µm	Inactive forms of digestive enzymes, water, ions	Pancreatic membrane protein GP2 (Hoops et al., 1993)
Dense core secretory vesicles	Neuroendocrine cells, endocrine cells	Golgi-derived	Electron-dense core	~100nm	Neuropeptides, hormones	Granins (Huttner et al., 1991), prohormone convertases PC2 and PC1/3 (Steiner, 1998)
Myofibroblast secretory vesicles	Gastric myofibroblasts, bone marrow-derived MSCs	Golgi-derived	Electron-dense core	~200nm	ECM proteins	Secretogranin II

Table 7.1.2 Types of exocytotic vesicles in classical pathway of secretion. Table of main features of different types of secretory vesicles in the classical pathway of secretion, and comparison with the secretory vesicles of gastric myofibroblast.

via regulated pathway is a new observation indicating a higher level of complexity for the role of myofibroblasts in tissue organisation compared to that previously described by Powell et al. (Powell et al., 1999). For example, regulated secretion of gastric myofibroblasts at the sites of tissue injury, infection or cancer in response to autocrine and/or paracrine soluble factors would mean rapid release of substances re-shaping the microenvironment to support or suppress host defence systems.

Regulated secretion by myofibroblasts involves a rise in intracellular calcium. Previously, increased cytosolic calcium was observed in cultured rat lamina propria fibroblasts following stimulation with endothelins, substance P, angiotensin II, ATP, bradykinin and 5-hydroxytryptamine, and was associated with the contractile properties of these cells (Furuya et al., 1994). In the earlier literature distinction between fibroblasts and myofibroblasts was sometimes obscure. Therefore, it is important to mention that Furuya et al. used the term fibroblast, but actually referred to the myofibroblast, as per nowadays definition. In the present project, IGF-II stimulation was used since (a) it has previously been shown to increase intracellular calcium in gastric myofibroblasts (McCaig, Burdyga & Varro, unpublished observations), (b) has previously been shown to be produced by these cells, and (c) is known to stimulate myofibroblast proliferation and migration (Hemers et al., 2005). Taken together the two studies raise the question of whether, as seems likely, there are many other potential physiological secretagogues for gastric myofibroblasts; a systematic study of this question is now needed.

7.1.2.4 Expression of secretogranin II

Chromogranin A and B, and secretogranin II, are prominent members of the granin family that are present in large dense core secretory granules in many neuroendocrine and endocrine cell types (Wiedenmann and Huttner, 1989). Several functions have been attributed to the granins, including in the biogenesis of dense core granules, as they aggregate at low pH and millimolar calcium concentrations in TGN, and thereby contribute to the formation of the electron

dense core (Courel et al., 2010; Helle et al., 1985). Also, granins are shown to act as chaperones via binding other soluble molecules e.g. catecholamines and thus improving the packaging of the latter inside the dense core granules (Helle et al., 1985). Recent report, however, showed that chromogranin B unlike chromogranin A can bind the inositol 1,4,5-triphosphate receptor at the neutral pH of the ER lumen (Yoo and Lewis, 2000). Schmidt et al. further identify a C-terminal domain within the molecule of chromogranin B that is crucial for the induction of calcium release from ER stores and thus capable of modulating intracellular calcium signals from within the ER lumen (Schmidt et al., 2011). This thesis showed that the regulated phenotype of gastric myofibroblasts is associated (a) with expression of secretogranin II, that (b) knockdown of secretogranin II inhibited regulated secretion, while (c) over-expression induced the capacity for regulated secretion in cells that otherwise lacked this phenotype. Based on previous work with this family of proteins, it now seems possible that secretrogranin II might determine the regulated secretory phenotype either by contributing to the formation of dense cored secretory vesicles, or by influencing intracellular calcium concentrations, or both. Further experimental work in this area should address these issues. Regardless of the outcome, the present data raise the possibility that *in vivo* identification of secretogranin II could be used as a marker for the regulated secretion phenotype that might be used, for example, in assessing cancer progression.

7.2 Methodology

Primary cell cultures, such as used in this study, have been very widely employed for studies of secretory mechanisms. Even so, there are limitations in interpreting the physiological relevance of the findings. For example, in introducing them to culture, the cells are taken out of their normal physiological environment and in the process of adapting to a new environment they may undergo changes in gene expression that influence the results of subsequent experimental studies. To extrapolate these findings to physiological conditions an *in vivo* model system needs to be employed. One possibility would be to use targeted expression in transgenic animals of secretogranin II to cells that do not normally express it (e.g. oesophageal myofibroblasts), or alternatively to selectively delete secretogranin II. The effects on secretion in isolated myofibroblasts could subsequently be studied together with examination of *in vivo* phenotypes and with studies of *in vivo* secretion mechanisms (in the case of secretogranin II expressing animals). A conditional knockout of secretogranin II has been already described in the literature and used to study the biological role of the secretogranin II product secretoneurin (Leierer, 2008). Knockout mice for chromogranin A or B are described as viable, although they exhibit compensatory expression of the opposite granin (Borges et al., 2010). These strains retained the capacity for granule biogenesis and secretion, but with impaired accumulation of vesicular catecholamine content. Another report described upregulated expression of a few secretogranins, including secretogranin II, and chromogranin B in a chromogranin A null mice (Hendy et al., 2006). Although much is still unknown about the biology of secretogranin II,

these studies demonstrate a close relation between expressions of different granins.

Cells exhibit both constitutive and regulated secretion because secretory vesicles of the two pathways exhibit different fusion machinery proteins, as well as different mechanisms of biogenesis. Tooze et al. showed distinct classes of vesicles using pulse-chase labelling with [^{35}S]sulphate of marker molecules of each pathway (Tooze et al., 1991). Subsequently, some samples of supernatant were immunoprecipitated for quantification of radiolabelled proteins, while other samples were subjected to velocity and equilibrium gradient centrifugation to characterise the fractions of secretory granules according to their density and size. In another study, formation, docking and fusion of the constitutive vesicles and immature vesicles of the regulated pathway were studied also using sulphate labelling, but in permeabilised, semi-intact cells (Grimes and Kelly, 1992). Besides studies that segregate proteins released via the constitutive and regulated pathways, multiple studies have employed *in vitro* and *in vivo* imaging approaches to visualise secretory vesicles and their fusion kinetics. The imaging techniques used in this project included fluorescence microscopy, confocal microscopy and TEM. However, these are limited insofar as they provide only a single time point snap-shot of localisation and have not localised proteins to specific vesicle types. There is now a need to perform immunogold labelling of two or three proteins using different sizes of gold particle to establish the localisation to vesicular structures of these proteins. As an example of this approach, Bezzi et al. used double-labelling with immunogold to co-localise glutamate transporters VGLUT1 and VGLUT2 to vesicles containing the v-

SNARE cellubrevin, but not VAMP2 (Bezzi et al., 2004). They also employed a live cell imaging technique, total internal reflection fluorescence microscopy (TIRF), to determine whether VGLUT-positive vesicles underwent regulated exocytosis and to determine their kinetics in response to different stimuli. This was possible, because vesicles lying just below the plasma membrane (Tsuboi et al., 2000) were double-labelled with fluorescent markers that allowed observations of their movement before and after the fusion event (Steyer and Almers, 2001).

Finally, although there have been some direct studies of calcium signalling in gastric myofibroblasts more needs to be done to link this to regulated secretion. Both IGF-I and IGF-II are known to trigger calcium influx in primed competent cells and this is associated with their mitogenic effects (Kojima et al., 1988; Nishimoto et al., 1987); however a similar transduction pathway may well be activated when IGFs act as secretagogues for myofibroblasts. These two studies followed the same experimental pattern with the main difference being the type of IGF investigated. They determined cytoplasmic calcium concentration and measured calcium influx rate at rest and after stimulation using radioactive calcium ^{45}Ca, and further identified the relevant plasma membrane calcium channels. With regard to the relation of secretogranin II and calcium increase, it would be interesting to see if secretogranin II could modify calcium levels in the ER lumen similarly to chromogranin B (Schmidt et al., 2011). This group studied calcium peaks in different transfected cell lines as a function of chromogranin B expression using calcium chemical indicators (fura-2 or fluo-4). Calcium

signalling experiments like these would be informative and could be performed in the cells used in this project.

7.3 Future perspectives

This project identified that some gastric CAMs tend not to exhibit regulated secretion. Therefore, it is important to consider whether these gastric CAMs have never exhibited regulated secretion, like their oesophageal counterparts, or whether this capacity is lost, and even more what could be the significance in cancer. It is now known that CAMs differ from normal myofibroblasts (for more details see section 7.1.2.1). Since gastric CAMs without regulated secretion exhibited transcriptional downregulation of the *secretogranin II* gene it is tempting to speculate that this is a consequence of epigenetic changes. However, other mechanisms are possible. In the recent years, short RNA molecules or microRNAs are gaining increasing attention, as they act as post-transcriptional regulators leading to gene silencing (Lee et al., 1993), however, there is no evidence yet of microRNAs regulating expression of any granin.

Also, it would be useful to study the influence of the secretory phenotype of myofibroblasts on cancer cell function for example using a co-culture system, and furthermore to translate this knowledge into an *in vivo* model system such as a xenograft model. For example, only cancer-associated myofibroblasts co-injected with breast carcinoma cell line into immunodeficient mice were promoting tumour growth but not the normal myofibroblasts (Orimo et al., 2005).

Additionally, the link between patients survival and regulated secretion of gastric CAMs suggests the latter could be used as a marker in determining cancer progression. Secretogranin II immunofluorescence studies would be useful in this context, although their validation for clinical use would need to be established. Since secretogranin II may be cleaved to any of a variety of different peptide fragments it may well be that there is cleavage pattern which is specific to myofibroblasts, in which case specific antibodies for particular peptide products could be especially useful. Taking into consideration that generated protein products are biologically active (Anouar et al., 1998; Fischer-Colbrie et al., 1995; Yajima et al., 2004), it would be interesting to explore their effects on myofibroblasts or on cancer cells.

To conclude, regulated secretion by gastric myofibroblasts is a newly identified property of these cells, the molecular basis of which needs to be further explored. There is preliminary data that it may impact myofibroblast cell function in promoting tumour progression and that it may provide novel markers for cancer progression. The prognosis of gastric cancer remains poor in spite of research over many years. Focusing on the novel phenotypes of stromal cells in this tumour may yield new insights leading to benefits for patients with this condition.

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APPENDIX

Protein name	UniProt ID	Localisation	Myofibroblast (Patient 14) IGF-II/Cont	MSC (Patient 37) IGF-II/Cont
200 kDa protein;Laminin subunit beta-1;95 kDa protein	P07942;Q8TAS6	Secreted	1.79	NI
28 kDa heat- and acid-stable phosphoprotein;Putative uncharacterized protein PDAP1	Q13442;C9J8N2	Secreted	1.24	NI
45 kDa calcium-binding protein;stromal cell derived factor 4 isoform 1 precursor;Isoform 3 of 45 kDa calcium-binding protein;Stromal cell derived factor 4	Q9BRK5-1;Q9BRK5;Q9BRK5-4;B1AME6;Q9BRK5-3;B1AME7	Secreted	0.93	2.40
5'-nucleotidase;cDNA FLJ90527 fis, clone NT2RP4001467, highly similar to 5'-nucleotidase;5'-nucleotidase, ecto (CD73), isoform CRA_c;5'-nucleotidase, ecto	P21589;B2RBH2;B3KQK1;Q53Z63;Q6NZX3;B3KQI8;Q5JRQ2;Q96B60;Q5JRQ1	Membrane	1.28	NI
72 kDa type IV collagenase;matrix metalloproteinase 2 isoform b	P08253;B4DWH3;Q2EF79	Secreted	1.70	2.07
ADM	P35318	Secreted	2.96	NI
Aminopeptidase N	P15144;B4DP01;B4DP96;B4DPH5;B4DV63;Q59E93;Q71E46;Q8IVL7	Membrane	NI	1.73
Annexin A1;cDNA FLJ51887, highly similar to Annexin A1	P04083;B5BU38;Q05BR2;Q5TZZ9;B4DL19	Secreted	1.27	2.05
Annexin A2;Isoform 1 of Annexin A2;24 kDa protein;Putative annexin A2-like protein;cDNA FLJ59138, highly similar to Annexin A2	P07355-2;P07355;P07355-1;A6NMY6;B4DNH8	Secreted	1.33	1.81
Annexin A5;Putative uncharacterized protein ANXA5 (Fragment)	P08758;B4DNG6;Q6FHB3;A8MTE3	Secreted	1.70	1.72
annexin IV;Annexin A4	Q6LES2;P09525;B4DDF9;B4DDZ4;B4DE02;B4E1S2;Q59FK3;Q6MZI0;Q6P452	Secreted?	1.07	1.62
Apolipoprotein B-100	P04114;A8K479;C0JYY2;P78482;Q13789;Q13828;Q4ZG63;Q53QC8;Q59HB3;Q9UE51;Q9UE52;Q9UE53	Secreted	9.37	5.70
Basement membrane-specific heparan sulfate proteoglycan core protein;Basement membrane-specific heparan sulfate proteoglycan core protein variant	P98160;B6EU51;Q2VPA1;Q59EG0	Secreted	1.54	1.99
Biglycan;cDNA FLJ35635 fis, clone SPLEN2011805, highly similar to BONE/CARTILAGE PROTEOGLYCAN I;Putative uncharacterized protein BGN;cDNA FLJ59360, moderately similar to Biglycan;cDNA FLJ35704 fis, clone SPLEN2020183, highly similar to Biglycan;cDNA FLJ598	P21810;A8K7E0;B4DDQ2;Q53FI4;Q53HU6;Q8NAB7;A6NLG9;B4DNL4;B3KS75;B4DDN7;B4DQD6	Secreted	NI	1.53
Bone-derived growth factor	Q13876;O00391-	Secreted	NI	2.26

(Fragment);Isoform 1 of Sulfhydryl oxidase 1;Putative uncharacterized protein QSOX1;Isoform 2 of Sulfhydryl oxidase 1	1;O00391;A8K477;A8K4C2;A8MXT8;O00391-2			
Calpain-2 catalytic subunit;cDNA FLJ58224, highly similar to Calpain-2 catalytic subunit	P17655;B2RCM3;B4DN77;B4E1R7	Membrane	0.74	NI
CALUMENIN;Isoform 1 of Calumenin;Isoform 2 of Calumenin	B3KPG9;B3KQF5;O43852-1;O43852;Q6IAW5;O43852-2;B3KNG6;B3KQK3	Secreted	1.45	1.96
Cathepsin B;cDNA FLJ58073, moderately similar to Cathepsin B;cDNA FLJ40065 fis, clone TESOP2000400, highly similar to CATHEPSIN B	P07858;A8K2H4;B4DMY4;Q6LAF9;B4DL49;Q5HYG5;B3KUJ8;Q8TAC7	Secreted	1.26	2.47
Cathepsin D;Putative uncharacterized protein CTSD;Putative uncharacterized protein CTSD	P07339;C9JTL0;C9JIH9	Secreted	1.44	1.93
Cathepsin K	P43235;Q6FHN2;Q6FHS6	Secreted	1.96	NI
Cathepsin L1;Cathepsin L1	P07711;A5PLM9;B3KQK4;Q6LAF7;Q9HBQ7;Q5K630;Q5T8F0;Q8NG13	Secreted	1.92	NI
C-C motif chemokine 2	P13500	Secreted	1.82	NI
CD166 antigen;cDNA, FLJ79012, highly similar to CD166 antigen;Isoform 2 of CD166 antigen;Activated leukocyte cell adhesion molecule soluble isoform;cDNA FLJ52980, highly similar to CD166 antigen	Q13740-1;Q13740;B3KNN9;B4DTU0;Q13740-2;Q6PEY4;B4DX43	Membrane	1.20	NI
CD44 antigen;Isoform 5 of CD44 antigen;Isoform 7 of CD44 antigen;Isoform 3 of CD44 antigen;Isoform 4 of CD44 antigen;Isoform 6 of CD44 antigen;Isoform 17 of CD44 antigen;Isoform 8 of CD44 antigen;Isoform 16 of CD44 antigen;Isoform 10 of CD4	P16070-1;P16070;P16070-5;P16070-7;P16070-3;P16070-4;C1PHC2;Q9H5A7;Q9P2Z2;P16070-6;P16070-17;P16070-8;B4XHE2;C1PHE6;P16070-16;P16070-10;B4E2X0;Q9H5A6;P16070-11;P16070-13;P16070-14;A6NDN4;P16070-12;A8K309;Q9H5A4;B6EAT9;B4DN59	Membrane	0.95	1.89
CD59 glycoprotein	P13987;Q6FHM9	Membrane	NI	3.96
Cell proliferation-inducing protein 41;73 kDa protein	P27658;A1KY36	Secreted	NI	1.29
Chitinase-3-like protein 1	P36222	Secreted	NI	1.40
Chondroitin sulfate proteoglycan 4	Q6UVK1;A2VCL1	Membrane	1.32	NI
Clusterin;54 kDa protein;Isoform 1 of Clusterin;CLU	P10909-2;P10909;P10909-1;B3KSE6;B4DW11;Q6LDQ3;Q8IWL5;Q8IWM0	Secreted	31.52	1.43
Coatomer subunit alpha;Isoform 1 of Coatomer subunit alpha	P53621-2;P53621;P53621-1	Secreted?	NI	1.62
Coiled-coil domain-containing protein 80;Isoform 1 of Coiled-coil domain-containing protein 80;Putative uncharacterized protein CCDC80;cDNA	Q76M96-2;Q76M96;A3KC71;A8KA16;Q76M96-1;C9JDY9;Q76M96-3;B4E342	Secreted	1.54	1.60

FLJ54405, highly similar to Homo sapiens steroid sensitive gene 1 (URB), transcript variant 2, mRNA				
COL6A3 protein;alpha 3 type VI collagen isoform 4 precursor;132 kDa protein	B7ZW00;C9JNG9;B4E0A4;B7ZMJ7;Q59F25;Q6MZG6;Q8N4Z1	Secreted	NI	2.54
Collagen alpha-1(I) chain	P02452;Q14042;Q6LAN8;Q9UMA6	Secreted	1.27	2.15
Collagen alpha-1(V) chain	P20908;B2ZZ86;Q59EE7;Q96HC0;Q9UML4	Secreted	NI	1.81
Collagen alpha-1(VI) chain	P12109;B4DRR8;Q05BT9;Q8TBN2	Secreted	1.70	1.89
Collagen alpha-1(VII) chain;Isoform 2 of Collagen alpha-1(VII) chain	Q02388-1;Q02388;Q59F16;Q02388-2;A9Z119;B0LXL0	Secreted	1.62	NI
Collagen alpha-1(XII) chain;Isoform 4 of Collagen alpha-1(XII) chain	Q99715-1;Q99715;B9EJB8;Q99715-4	Secreted	1.73	1.81
Collagen alpha-1(XXII) chain;Isoform 2 of Collagen alpha-1(XXII) chain;Isoform 3 of Collagen alpha-1(XXII) chain	Q8NFW1-1;Q8NFW1;Q8NFW1-2;Q8NFW1-3;C9K0G4	Secreted	1.75	NI
Collagen alpha-2(I) chain	P08123;A2TIK1;Q75N18;Q7KZ71	Secreted	1.30	2.14
Collagen alpha-2(V) chain;110 kDa protein;COL5A2 protein;cDNA FLJ53096, highly similar to Collagen alpha-2(V) chain	P05997;B4DNJ0;Q5PR22;B4DTC0	Secreted	1.44	1.55
Collagen alpha-2(VI) chain	P12110-1;P12110;Q9BUM6	Secreted	1.77	1.93
Collagen alpha-2(VI) chain;Isoform 2C2A' of Collagen alpha-2(VI) chain	P12110-2;P12110;P12110-3	Secreted		1.64
Collagen alpha-3(VI) chain;COL6A3 protein;322 kDa protein;Isoform 2 of Collagen alpha-3(VI) chain;alpha 3 type VI collagen isoform 4 precursor;325 kDa protein;132 kDa protein	P12111-1;P12111;B7ZW00;C9JNG9;P12111-2;B4E0A4;B7ZMJ7;Q59F25;Q6MZG6;Q8N4Z1	Secreted	1.76	1.93
Complement C1r subcomponent;cDNA FLJ54318, highly similar to Complement C1r subcomponent	P00736;A8K5J8;B4DPQ0;Q53HT9;Q53HU9;B4E1B0	Secreted	1.50	2.10
Complement C1s subcomponent;Putative uncharacterized protein C1S	P09871;A8K2N0;B3KNX0;A6NG18	Secreted	1.66	2.37
Connective tissue growth factor;Isoform 2 of Connective tissue growth factor	P29279-1;P29279;B2RCP7;B3KRV6;B3KWK5;Q5M8T4;Q6FHL8;P29279-2;Q9UDL6	Secreted	0.79	1.41
Cystatin-C	P01034	Secreted	1.46	2.18
Decorin;Isoform B of Decorin;Isoform D of Decorin	P07585-1;P07585;Q6FH10;P07585-2;P07585-4	Secreted	1.23	1.72
Deleted in malignant brain tumors 1 protein;Isoform 7 of Deleted in malignant brain tumors 1 protein;Isoform 1 of Deleted in malignant brain tumors 1 protein;261 kDa	Q9UGM3-6;Q9UGM3;A6ND64;Q9UGM3-7;Q9UGM3-1;Q9UGM3-3;Q9UGM3-5;Q9UGM3-2;B6V682;B7Z8Y2;Q9UGM3-8;Q9UGM3-	Secreted	2.24	NI

protein;Isoform 3 of Deleted in malignant brain tumors 1 protein;Isoform 5 of	4;A6NFE0;A6NDZ5;A8MYK5			
Dermcidin isoform 2;Dermcidin	A5JHP3;P81605;Q53YJ2	Secreted	4.67	1.22
EGF-containing fibulin-like extracellular matrix protein 1;Isoform 3 of EGF-containing fibulin-like extracellular matrix protein 1;Isoform 4 of EGF-containing fibulin-like extracellular matrix protein 1;Isoform 2 of EGF-containing fibulin-like	Q12805-1;Q12805;A8KAJ3;B2R6M6;Q59G97;Q12805-3;Q12805-4;Q12805-2;Q53TA7;B4DW75;B4DWH0;B3KS53	Secreted	1.54	1.95
EGF-like repeat and discoidin I-like domain-containing protein 3;Isoform 2 of EGF-like repeat and discoidin I-like domain-containing protein 3	O43854-1;O43854;Q8N610;O43854-2;B7Z865	Secreted	NI	1.56
EMILIN-1	Q9Y6C2	Secreted	1.40	NI
Endosialin	Q9HCU0-1;Q9HCU0	Membrane	NI	1.65
Extracellular matrix protein 1;Isoform 1 of Extracellular matrix protein 1	Q16610-4;Q16610;Q16610-1;C8CHS3	Secreted	1.88	2.77
Fibrillin-1	P35555;B4E3I6;Q75N88;Q75N89;Q9NP01	Secreted	1.57	2.07
Fibroleukin	Q14314;A4D1B8;Q53GD2;Q8WWE4	Secreted	1.31	NI
fibronectin 1 isoform 2 preproprotein	Q14328	Secreted	1.04	NI
fibronectin 1 isoform 4 preproprotein;FN1 protein;263 kDa protein	P02751-4;P02751;A6YID3;Q5CZ99;Q9UQS6;P02751-10;B4DN21;B7ZLE5;B7ZLF0	Secreted	1.35	1.73
fibronectin type III domain containing 1;Isoform 2 of Fibronectin type III domain-containing protein 1;FNDC1 protein;179 kDa protein	Q4ZHG4-1;Q4ZHG4;B3KXM5;B7ZBR5;B9EK49;Q4ZHG4-2;A6H8X2;B7ZBR4	Secreted	NI	0.96
Fibronectin;Isoform 3 of Fibronectin;Isoform 15 of Fibronectin;Isoform 7 of Fibronectin;Isoform 5 of Fibronectin;Isoform 8 of Fibronectin;Isoform 14 of Fibronectin;Isoform 9 of Fibronectin;Isoform 11 of Fibronectin;Isoform 13 of Fibronectin;Is	P02751-1;P02751;A6YID6;P02751-3;A6YID2;O95617;Q68CX6;P02751-15;P02751-7;Q6MZM7;P02751-5;A6YID5;P02751-8;P02751-14;A6YID4;Q59G22;Q6N084;P02751-9;B4DU16;P02751-11;P02751-13;P02751-6;P02751-12	Secreted	NI	1.93
Fibulin 1;Isoform C of Fibulin-1	B0QY41;P23142-4;P23142;B0QY42;B4DUV1	Secreted	6.82	12.46
Fibulin-1;Isoform B of Fibulin-1;fibulin 1 isoform B precursor;Isoform A of Fibulin-1	P23142-1;P23142;Q8NBH6;P23142-3;B0QY43;P23142-2;B1AHM7	Secreted	NI	1.87
Fibulin-1;Isoform D of Fibulin-1;fibulin 1 isoform B precursor;Isoform A of Fibulin-1	P23142-3;P23142;P23142-1;Q8NBH6;B0QY43;P23142-2;B1AHM7	Secreted	1.75	NI
Follistatin;Isoform 3 of Follistatin;Isoform 2 of Follistatin	P19883-1;P19883;Q6FHE1;P19883-3;P19883-2;B5BU94	Secreted	1.11	1.62
Follistatin-related protein 1;cDNA FLJ50214, highly	Q12841;A8K523;B4DTT5;B4DTZ8;Q9BZQ0	Secreted	1.64	2.16

similar to Follistatin-related protein 1				
Galectin-1	P09382;B2R5E8;Q15954	Secreted	0.90	1.78
Galectin-3	P17931;Q59FR8;Q6FGL0;Q6IBA7;Q6NVH9;Q86TY5;Q8IXB9	Secreted	0.88	NI
Galectin-3-binding protein;cDNA FLJ53509, highly similar to Galectin-3-binding protein;cDNA FLJ54583, highly similar to Galectin-3-binding protein	Q08380;B4DVE1;B4DWA8;B4DI70;B3KP88;B4DDG4	Secreted	2.09	2.28
Gelsolin;gelsolin isoform c;Isoform 2 of Gelsolin;cDNA FLJ53327, highly similar to Gelsolin;cDNA FLJ35478 fis, clone SMINT2007796, highly similar to Gelsolin	P06396-1;P06396;B7Z6N2;B7Z9A0;Q5T0I2;B7Z373;B7Z5V1;Q5T0I1;Q69YR8;P06396-2;A2A418;B7Z4U6;B7Z992;B7Z2X4;Q5T0H8;B3KS49;Q5T0H9	Secreted	1.23	NI
Gremlin-1;Isoform 2 of Gremlin-1	O60565-1;O60565;A6XAA7;O60565-2	Secreted	1.53	NI
Growth arrest-specific protein 6;Isoform 2 of Growth arrest-specific protein 6;Isoform 3 of Growth arrest-specific protein 6;growth arrest-specific 6 isoform 2;growth arrest-specific 6 isoform 3	Q14393-1;Q14393;Q14393-2;Q14393-3;B4DZY7;B3KRQ7;B3KVL4;Q658L2	Secreted	1.57	NI
Hepatoma-derived growth factor;hepatoma-derived growth factor isoform b;Hepatoma-derived growth factor;hepatoma-derived growth factor isoform c	B7Z525;Q5SZ07;P51858;B2RDE8;A8K8G0;B3KU21;B7Z958;Q5SZ08	Secreted	NI	4.21
Hyaluronan and proteoglycan link protein 1	P10915	Secreted	NI	2.87
Immunoglobulin superfamily containing leucine-rich repeat protein;Putative uncharacterized protein ISLR	O14498;C9J667	Secreted	NI	1.53
insulin-like growth factor 2 isoform 2;Isoform 1 of Insulin-like growth factor II;Isoform 2 of Insulin-like growth factor II	A8K6C9;C9JAF2;P01344-1;P01344;B2MUX6;P01344-2	Secreted	98.75	116.24
insulin-like growth factor binding protein 3 isoform a precursor;Insulin-like growth factor-binding protein 3;cDNA FLJ45630 fis, clone CHONS2001287, moderately similar to INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 3;cDNA FLJ52568, highly similar to Insulin	P17936;A6XND0;A6XND1;B3KVF9;B4DN53;A6XNC9;B3KTQ0;B3KWK7;B4DP07;B3KRZ1;B3KTS0;B3KVY6;Q6ZSD0;C9JMX4	Secreted	NI	2.15
Insulin-like growth factor-binding protein 4	P22692;B4E351	Secreted	1.69	1.59
Insulin-like growth factor-binding protein 5	P24593	Secreted	1.48	2.05
Insulin-like growth factor-binding protein 6	P24592;Q9H2B5	Secreted	2.34	3.33
Insulin-like growth factor-binding protein 7	Q16270;B4E1N2;B7Z9W7	Secreted	1.72	2.59
Integrin beta-1	P05556-1;P05556	Membrane	1.35	NI

Integrin beta-like protein 1;cDNA FLJ59733, highly similar to Homo sapiens integrin, beta-like 1 (with EGF-like repeat domains) (ITGBL1), mRNA;Osteoblast specific cysteine-rich protein	O95965;B3KTP1;B4DN32;B4DQ02;O14549	Secreted		2.47
Lactadherin;milk fat globule-EGF factor 8 protein isoform b	Q08431;B3KTQ2;B4E396;Q7Z3D2;Q9BTL9	Membrane	NI	1.58
Laminin subunit gamma-1	P11047;Q6NVY8	Secreted	1.78	1.28
Latent-transforming growth factor beta-binding protein 1;173 kDa protein;Isoform Short of Latent-transforming growth factor beta-binding protein 1;latent transforming growth factor beta binding protein 1 isoform 4 precursor;Isoform 3 of Lat	Q14766-1;Q14766;Q14766-2;B7ZLY3;C9J9Y0;Q14766-3;B4DNR7;B4DRF7;B4DST8;B4DZD1;C9JCG4;C9JD84	Secreted	1.97	NI
Latent-transforming growth factor beta-binding protein 2	Q14767;Q59EE6;Q6AZ94	Secreted	1.86	0.77
Lumican	P51884;Q53FV4	Secreted	1.78	2.37
Lysyl oxidase homolog 2;cDNA FLJ53707, highly similar to Lysyl oxidase homolog 2	Q9Y4K0;B2R5Q0;Q53HV3;B4DDQ4	Secreted	NI	1.30
Macrophage migration inhibitory factor	P14174;A6MUU8	Secreted	1.48	2.57
matrix metalloproteinase 14 preproprotein;Matrix metalloproteinase-14	B2R6P3;Q9UHK5;P50281	Membrane	0.98	2.12
mesencephalic astrocyte-derived neurotrophic factor;Mesencephalic astrocyte-derived neurotrophic factor;Putative uncharacterized protein MANF	A8K878;P55145;C9J3P1	Secreted	1.15	NI
Metalloproteinase inhibitor 1;TIMP metallopeptidase inhibitor 1;TIMP metallopeptidase inhibitor 1	P01033;B3KQF4;Q58P21;Q6FGX5;Q5H9A7;Q5H9A8	Secreted	1.32	2.00
Metalloproteinase inhibitor 2;22 kDa protein;cDNA FLJ57920, highly similar to Metalloproteinase inhibitor 2	P16035;B4DFW2	Secreted	1.80	2.28
Metalloproteinase inhibitor 3;TIMP metallopeptidase inhibitor 3	P35625;B4DNZ4;Q6LEP5;B1AJV7	Secreted	NI	1.67
Mimecan;Osteoglycin	P20774;A8K0R3;B4DI63;Q7Z532;Q5TBF5	Secreted	1.67	NI
Mucin-16;1518 kDa protein;mucin 16	Q8WXI7;B5ME49	Secreted/membrane	NI	0.09
Mutant p53 binding protein 1 variant (Fragment);EGF-containing fibulin-like extracellular matrix protein 2	Q59GI8;O95967;A8K7R4;B2RCM5;B3KM31;Q6FH22;Q96TF5;Q9H3D5	Secreted	NI	1.73
Neuropilin-2;Isoform A17 of Neuropilin-2;Isoform A0 of Neuropilin-2;neuropilin 2 isoform 4 precursor;neuropilin 2 isoform 5 precursor;neuropilin 2 isoform 6 precursor	O60462-1;O60462;B7ZL68;Q7LBX7;Q7Z3T9;O60462-3;Q7LBX6;O60462-2;Q9H2D4;Q9H2E3;A8K4G9;Q9H2D5;Q9H2E4;Q9H2E2	Membrane	1.32	NI

Nidogen-1;Isoform 2 of Nidogen-1	P14543-1;P14543;P14543-2	Secreted	1.84	NI
Nidogen-2;NID2 protein;Putative uncharacterized protein DKFZp686D12108 (Fragment)	Q14112;A8K6I7;B4DPX8;B4DU19;Q8IV28;Q5CZI2	Secreted	1.74	NI
Nucleobindin-2;Isoform 2 of Nucleobindin-2	P80303-1;P80303;P80303-2	Membrane	1.33	1.82
Olfactomedin-like protein 3;Isoform 2 of Olfactomedin-like protein 3	Q9NRN5-1;Q9NRN5;Q9NRN5-2;B4DNG0	Secreted	1.37	1.30
Pentraxin-related protein PTX3	P26022	Secreted	1.49	1.54
Periostin, osteoblast specific factor;Isoform 4 of Periostin	B1ALD9;Q15063-4;Q15063;B7Z6G1;C0IMJ4	Secreted	NI	1.73
Periostin;Periostin, osteoblast specific factor;Isoform 2 of Periostin	Q15063-1;Q15063;C0IMJ1;Q15063-3;B1ALD8;B4E3A7;C0IMJ3;Q15063-2;B7Z4V4;C0IMJ2	Secreted	NI	0.16
Pigment epithelium-derived factor;25 kDa protein	P36955;Q2TU83	Secreted	NI	0.96
Plasminogen activator inhibitor 1;plasminogen activator inhibitor-1 isoform 2 precursor	P05121;B7Z1D9;B7Z4S0;B7Z4X6;B7ZAB0;C9J272	Secreted	1.02	2.05
plasminogen activator inhibitor type 1, member 2 isoform c precursor;45 kDa protein;Isoform 1 of Glia-derived nexin;Isoform 2 of Glia-derived nexin;Putative uncharacterized protein SERPINE2;Putative uncharacterized protein SERPINE2	B4DIF2;P07093-1;P07093;P07093-2;B4DMR3;C9JYC8;C9JN98	Secreted	1.74	1.96
Procollagen C-endopeptidase enhancer 1;cDNA FLJ55126, highly similar to Procollagen C-endopeptidase enhancer 1	Q15113;A4D2D2;B4DPJ4	Secreted	NI	2.59
Proenkephalin-A;25 kDa protein	P01210;Q3B7B1	Secreted	1.67	3.64
Protein CYR61;cDNA FLJ58182, highly similar to Protein CYR61;CYR61 protein	O00622;Q53FA4;Q6FI18;B4DI61;Q9UID7	Secreted	1.26	NI
Protein disulfide-isomerase;cDNA FLJ59430, highly similar to Protein disulfide-isomerase	P07237;B2RDQ2;B4DJS0;B4DNL5;B3KTQ9;B4DLN6;B4DUA5;Q96C96	Membrane	1.35	1.72
Protein S100-A13	Q99584	Secreted	1.07	3.05
Protein-lysine 6-oxidase	P28300;B0AZT2;B4DN04;B4DQF8;B7ZAJ4;D0PNI2;Q71V04;Q96PQ9	Secreted	1.58	1.72
Putative killer cell immunoglobulin-like receptor-like protein KIR3DX1;Isoform 1 of Putative killer cell immunoglobulin-like receptor-like protein KIR3DX1;Putative uncharacterized protein KIR3DX1;Putative uncharacterized protein KIR3DX1	Q9H7L2-2;Q9H7L2;Q9H7L2-1;C9JDH6;C9JJY8	Secreted	NI	1.45
Putative uncharacterized protein CD81;CD81 antigen	A6NMH8;P60033	Membrane	1.38	NI
Putative uncharacterized protein COL5A1	A6NNQ0;Q5JR39	Secreted	NI	0.79

Putative uncharacterized protein GPC1;Glypican-1;Putative uncharacterized protein GPC1;Glypican 1, isoform CRA_a	C9JGZ5;P35052;Q53EX3;C9J1L8;B3KTD1;Q59GI7	Membrane/secreted	1.70	1.49
Retinol-binding protein 4;Retinol binding protein 4, plasma, isoform CRA_b;Retinol binding protein 4, plasma	P02753;A6NCP9;Q5VY30	Secreted	NI	8.00
Semaphorin-7A;semaphorin 7A isoform 2 preproprotein;semaphorin 7A isoform 3	O75326;B4DDP7;B3KMH6	Secreted	1.39	2.13
Serine protease 23;cDNA FLJ51190, highly similar to Serine protease 23	O95084;B3KQQ9;B4E2J3	Secreted	1.20	1.21
Serine protease HTRA1;cDNA FLJ34625 fis, clone KIDNE2015244, highly similar to Serine protease HTRA1	Q92743;B3KRN4	Secreted	NI	2.03
small inducible cytokine subfamily E, member 1 isoform b precursor;Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1;cDNA FLJ52127, highly similar to Multisynthetase complex auxiliary component p43	B4E1S7;Q12904;B4DNK3	Secreted	1.66	NI
SPARC	P09486;B2RDL6;B4DRV4;Q6QE20	Secreted	2.17	1.17
Stanniocalcin-2	O76061;B3KNF2;Q6FHC9	Secreted	NI	1.89
Sulfhydryl oxidase 1;Putative uncharacterized protein QSOX1;Isoform 2 of Sulfhydryl oxidase 1	O00391-1;O00391;A8K477;A8K4C2;A8MXT8;O00391-2	Secreted	1.67	NI
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1;polydom;Isoform 3 of Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	Q4LDE5-1;Q4LDE5;Q4LDE5-3;B3KQM1	Secreted	1.61	NI
Target of Nesh-SH3 precursor (Tarsh) (Nesh-binding protein) (NeshBP) (ABI gene family member 3-binding protein). Isoform 2;Isoform 1 of Target of Nesh-SH3;Putative uncharacterized protein ABI3BP (Fragment);Protein;cDNA FLJ56632, moderately similar to Targe	Q7Z7G0-3;Q7Z7G0;Q7Z7G0-1;C9J0V1;B4DSV9;Q7Z7G0-4;Q7Z7G0-2	Secreted?	NI	1.69
Tenascin;Isoform 4 of Tenascin;Isoform 3 of Tenascin;Isoform 2 of Tenascin;Isoform 5 of Tenascin;TNC protein;Isoform 6 of Tenascin	P24821-1;P24821;Q4LE33;Q5T7S3;P24821-4;C9J848;P24821-3;C9J6D9;P24821-2;P24821-5;C9J575;A7MBN2;P24821-6;C9IYT7	Secreted	1.40	2.50
Testican-1	Q08629;O60400	Secreted	1.34	1.60
Thrombospondin-1	P07996;A0PJG0;B4E3J7;Q59E99;Q7KYY3	Secreted	1.66	1.49

Thrombospondin-2;Thrombospondin 2	P35442;Q6MZL6;Q5RI53	Secreted	1.44	1.55
Thrombospondin-4	P35443;B7Z832	Secreted	0.79	NI
Thy-1 membrane glycoprotein;Thy-1 cell surface antigen variant (Fragment)	P04216;B0YJA4;Q59GA0	Membrane	NI	0.89
Tissue factor pathway inhibitor 2	P48307;Q8NAK6;Q8NE89;Q96QP2	Secreted	1.64	NI
Tissue-type plasminogen activator;Isoform 3 of Tissue-type plasminogen activator;cDNA FLJ59440, highly similar to Tissue-type plasminogen activator;cDNA FLJ59355, highly similar to Tissue-type plasminogen activator;cDNA FLJ59358, highly simila	P00750-1;P00750;B4DRD3;B8ZX62;P00750-3;B2R8E8;Q5HYM8;B4DV92;B4DN26;Q6LBF5;P00750-4;B4DNJ1	Secreted	0.93	1.76
Transforming growth factor-beta-induced protein ig-h3;60 kDa protein;Transforming growth factor, beta-induced, 68kDa, isoform CRA_a	Q15582;Q53GU8;Q53XM1;B9ZVW9;C7FFS5	Secreted	2.00	1.74
Tubulointerstitial nephritis antigen-like;cDNA FLJ55020, highly similar to Tubulointerstitial nephritis antigen-like	Q9GZM7-1;Q9GZM7;B4DVG5;B4DPK6	Secreted	NI	0.26
Tumor necrosis factor receptor superfamily member 11B	O00300	Secreted	1.43	NI
UPF0027 protein C22orf28;cDNA FLJ58027	Q9Y3I0;B4DNA0	Membrane?	1.87	NI
Vasorin	Q6EMK4	Membrane/Secreted	1.34	2.83
Versican core protein;Isoform Vint of Versican core protein;Isoform V1 of Versican core protein;Isoform V2 of Versican core protein;Isoform V3 of Versican core protein	P13611-1;P13611;Q59FG9;P13611-5;P13611-2;B7Z638;Q6MZK8;P13611-3;B7Z3R6;P13611-4;Q86W61	Secreted	1.55	2.66

Table 1. List of putative secreted proteins in the media of gastric myofibroblasts and of MSCs. From approximately 250 proteins that were initially identified, a list of 143 predicted secreted proteins was generated. Of these, 106 proteins were predicted as secreted into the media of myofibroblasts. An identical number of proteins were predicted to be secreted into the media of MSCs. The information provided in this Table includes protein name, unique UniProt identification number(s), predicted localisation and relative abundance in IGF-treated *vs* control media for both cell types. NI-Not Identified.