Novel stromal cell signalling systems in oesophageal cancer

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Disclaimer

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

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List of Abbreviations

Abbreviation	Meanings
AC	Adenocarcinoma
ADAM	A desintegrin and metalloprotease
ATM	Adjacent tissue myofibroblast
ATP	Adenosine-5'-triphosphate
BCH	Basal cell hyperplasia
BSA	Bovine serum albumin
C-9	Chemrein-9
CAF	Cancer-associated fibroblasts
CAM	Cancer-associated myofibroblasts
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CCL-2	Chemokine (C-C motif) ligand 2
CCRL2	Chemokine (CC motif) receptor-like 2
Ch	Chemerin
ChemR	Chemerin receptor
CM	Conditioned media
CMLR1	Chemokine like-receptor 1
COX	Cyclooxygenase
CuSO4	Copper sulphate
CXCR	Chemokine receptor
DAG	Diacylglycerol
DE	Differential gene expression
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
EMT	Epithelial mesenchymal transition
ER	Endoplasmic reticulum
ESCC	Oesophageal squamous cell carcinoma

FACS	Fluorescence-activated cell sorting
FDR	False Discovery Rate
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FM	Full medium
GABA	Gamma-aminobutiryc acid
GAG	Glycosaminoglycans
GAP	GTPase-activating proteins
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal tract
GIF	Glycosylation-inhibiting factor
GIP	Gastric inhibitory polypeptide
GIST	Gastrointestinal stromal tumour
GLP	Glucagon-like peptide
GnRH	Gonadotropin releasing hormone receptor
GORD	Gastro-oesophageal reflux
GPCR	G-protein-coupled receptors
GPR1	G-protein-coupled receptor 1
GRK	G protein-coupled receptor kinases
GRO	Growth-related oncogenes
GTP	Guanosine-triphosphate
H.felis	Helicobacter felis
H.pylori	Helicobacter pylori
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
Hh	Hedgehog
HIF	Hypoxia induced factor
HPLC	High-performance liquid chromatography
HREs	Hormone response elements
HRP	Horseradish peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
ICC	Immunocytochemistry
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein

IGF-IR	Insulin-like growth factor-receptor type 1
IHC	Immunohistochemistry
IHH	Indian hedgehog homolog
IL	Interleukin
IP_3	Inositol 1,4,5-trisphosphate
iTRAQ	Isobaric tags for relative and absolute quantitation
KGF	Keratinocyte growth factor
M	Molar
MALDI	Matrix-assisted laser desorption/ionisation-
TOF	Time of flight
MS	Mass spectrometry
MAPK	Mitogen-activate protein kinase
MAQC	Microarray Suite 5.0 QC Metrics
MAS5	Microarray Analysis Suite 5
MCP	Monocyte chemoattractant protein
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloprotease
MS	Mass spectrometry
MSC	Mesenchymal stem/stromal cell
MSCBM	Mesenchymal Stem Cell Basal Medium
MT1-MMP	Membrane type-Matrix metalloprotease
MTT	(3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide)
nAChR	Nicotinic acetylcholine receptors NK Natural killer
NRs	Nuclear receptors
NSCLC	Non-small cell lung cancer
NTM	Normal tissue myofibroblast
OPN	Osteopontin
PA	Pernicious anaemia
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor
PET	Perforated polyethylene terephthalate

PFA	Paraformalydehyde
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PIP$_2$	Phosphatidylinositol 4,5-bisphosphate
PK	Protein kinase
PTCH	Patched
PYY	Peptide YY
rh	Recombinant human
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RTK	Receptor tyrosine kinases
SCTR	Secretin receptor
SDF	Serum derived factor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SF	Serum free
SHH	Sonic hedgehog
SILAC	Stable Isotope Labelling by Amino-acid in Culture
siRNA	Small interference RNA
-SMA	-smooth muscle actin
TE	Tris/EDTA buffer
TGF	Transforming growth factor
TGFig-h3	Transforming growth factor- inducible protein
TIMP	Tissue inhibitors of matrix metalloproteases
TNF	Tumour necrosis factor
TOFT	Tissue Organisation Field Theory
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
WB	Western blot

Abstract

Myofibroblasts are recognised to play an important role in wound healing and the maintenance of tissue integrity. In addition, they are increasingly recognised to provide a supporting microenvironment for cancer cells. They secrete a variety of chemokines, cytokines, growth factors and proteases that collectively regulate cell proliferation, migration and invasion. Specific chemokines are known to recruit mesenchymal stromal cells (MSCs) to both tumours and normal tissue which may then give rise to myofibroblasts. Proteomic studies by Holmberg and Varro (unpublished observations) identified chemerin as an upregulated chemokine in conditioned media (CM) from oesophageal cancer associated myofibroblasts (CAMs) compared to adjacent tissue myofibroblasts (ATMs). Chemerin is potent chemo-attractant for immune and inflammatory cells. The objectives of this thesis were (a) to functionally characterise the oesophageal myofibroblasts,(b) validate the findings of previous proteomic studies and (c) determine the role of chemerin in MSC migration.

Oesophageal CAMs from both squamous and adenocarcinoma tumours were shown to be more proliferative than their paired ATMs, or normal tissue myofibroblasts (NTMs). In addition, CAM conditioned media increased the proliferation and migration of two oesophageal cancer cell lines (OE21 and OE33) and stimulated MSC migration compared to ATM CM. The data suggest oesophageal CAMs promote an aggressive tumour microenvironment.

Western blotting and ELISA confirmed increased chemerin secretion by squamous carcinoma CAMs. Chemerin and conditioned media from squamous carcinoma CAMs, stimulated MSC and OE21 cell migration; Chemerin neutralizing antibody reversed these effects and siRNA knockdown of chemerin in CAMs, or of its cognate receptor ChemR23 in MSCs, decreased migratory responses. Studies

using pharmacological inhibitors or Western blot of cellular proteins indicated that chemerin stimulated MSCs via PKC, and p42/44, p38 MAP and JNK kinases.

Macrophage Inhibitory Factor (MIF) was identified as a putative chemerin target in MSCs and validated by ELISA and Western blot of MSC media and cell extracts. MIF inhibited MSC migration in response to low or moderate concentrations of chemerin, indicating that it might restrain MSC migration in normal tissues but not in cancers where chemerin is elevated.

Finally, confirmation of chemerin-chemR23 interactions was obtained using a chemR23 antagonist, CCX832. Chemerin induced MSC and OE21 cell migration was inhibited by CCX832. Moreover, transendothelial migration of MSCs in response to chemerin or CAM conditioned media was reversed by CCX832. Transendothelial migration was also shown to depend on chemerin-stimulated MMP-2 secretion. These findings indicate a molecular mechanism by which MSCs are recruited to tumours.

Taken as a whole, this work indicates that myofibroblasts derived from oesophageal cancers differ from those in adjacent or normal tissue. The finding of increased chemerin in these cells is novel and may be relevant to MSC recruitment. Since it is possible to inhibit the effects of chemerin on MSCs using CCX832, there is the potential for a novel therapeutic approach to prevent cancer progression.

Chapter -1

Introduction

1.1   Overview

In multicellular organisms there are evolutionary advantages conferred by the differentiation of cells into different types with specialised functions. Coordinated cell-cell interactions then become vital in maintaining the organisation and function of tissues containing multiple cells types, both in development and in tissue repair. In the gastrointestinal tract, for example, interactions between epithelial cells and stromal cells control cell proliferation, differentiation and apoptosis. These interactions are disrupted in a variety of diseases including inflammatory conditions and, most strikingly, in cancer. Since inflammation is recognised as a driver of cancer progression (Balkwill and Mantovani 2001) there has been a growing appreciation of the role that cellular microenvironments play in carcinogenesis. In particular work by several groups over the last decades has led to the idea of a specific niche that determines the fate of mutated cancer cells (Bissell, Radisky et al. 2002) and is one of the hallmarks of cancer (Hanahan and Weinberg 2011) as well as being a potential target for therapy (Hainaut and Plymoth 2013).

Amongst the various cells contributing to the tumour niche, myofibroblasts have been highlighted by many studies. These cells normally play an important role in the maintenance of tissue architecture and wound healing (Roland 1976; Tomasek, Gabbiani et al. 2002; Radisky, Kenny et al. 2007; Klingberg, Hinz et al. 2013). They are a rich source of growth factors, cytokines, chemokines, extracellular matrix (ECM) proteins, proteases and their inhibitors (Powell, Mifflin et al. 1999a). Disrupted autocrine and paracrine signalling by myofibroblasts influences cancer cell proliferation, metastasis, infiltration of immune cells and recruitment of mesenchymal stromal cells (MSCs) (Powell, Mifflin et al. 1999a; Direkze, Hodivala-Dilke et al. 2004). The latter are of interest because they are

thought to be myofibroblast progenitors and as well as potentially exerting other effects that determine tumour progression (Klopp, Gupta et al. 2011).

In spite of recent progress, the functional role of myofibroblasts in many cancers, including those of the oesophagus, is not well understood. In this thesis, the overall aim was to investigate novel signalling pathways that are altered in cancer-derived myofibroblasts and their influence on other cell types including cancer cells and the recruitment of MSCs.

1.2 The Organisation of the Gastrointestinal Tract

1.2.1 The basic pattern of organisation

The wall of the gastrointestinal tract is divided into layers: i) there is an outer muscle layer and ii) within this a mucosal layer consisting of epithelial cells that continuously line the luminal surface and an underlying stromal layer, the lamina propria consisting of blood vessels, ECM, lymphatics, inflammatory and immune cells, fibroblasts and myofibroblasts (Wu, Jackson et al. 1999). One of the ECM components, basement membrane, provides the anchorage for epithelial cells. Between these two layers, there is a submucosal layer of connective tissue (Streuli, Bailey et al. 1991). Nerve plexuses are found between the circular and longitudinal muscle (myenteric plexus) and in the submucosa (submucosal plexus). There are marked differences in mucosal organisation in different regions of the gut; the present studies focus on the upper gastrointestinal tract.

1.2.2   Oesophagus

The mucosal organisation of the oesophagus is relatively simple compared with many other regions of the gut. In particular, the lining consists of squamous type stratified epithelial cells that lack absorptive and secretory functions. The oesophageal submucosa includes seromucous glands. This pattern of organisation is changed dramatically in preneoplastic conditions e.g. Barrett's oesophagus.

1.2.3   Stomach

In the stomach, the epithelium is folded into gastric glands that are of two main types: in the body of stomach there are corpus or acid-secreting gastric glands while distally there are pyloric antral glands that do not release acid but do contain G-cells releasing the hormone gastrin. Mucus-secreting surface epithelial cells that have a protective role are found in both cases, as are pepsinogen-secreting chief cells. Acid is released by parietal cells in corpus glands in response to histamine secreted by enterochromaffin-like (ECL) cells. The latter are stimulated by gastrin released from antral G-cells.   Other endocrine cells include D-cells producing somatostatin in both regions of the stomach, and X-cells producing ghrelin in the corpus.

The maintenance of glandular organisation is achieved by cellular proliferation and differentiation, and the migration of differentiating cells to their final destination. In the corpus, proliferating cells occur in the isthmus region that also includes pluripotent gastric stem cells (Figure 1.1). The precursor cells exhibit bi-directional movement from this region towards the surface or towards the base of the glands (Barker, Huch et al. 2010). Cells moving up to the pit region

differentiate into mucus-secreting cells, whereas cells migrating in the opposite direction differentiate into pepsinogen cells, parietal cells, or endocrine cells (Yang, Tsuyama et al. 1997; Karam 1999). Epithelial cell populations survive for varying periods of time: mucus cells are reproduced every 2-6 days compared with >3 weeks for parietal and chief cells (Karam 1993; Kirton, Wang et al. 2002). There is a tight network of myofibroblasts around the gastric glands that are thought to determine organisation and function of the epithelial cells.
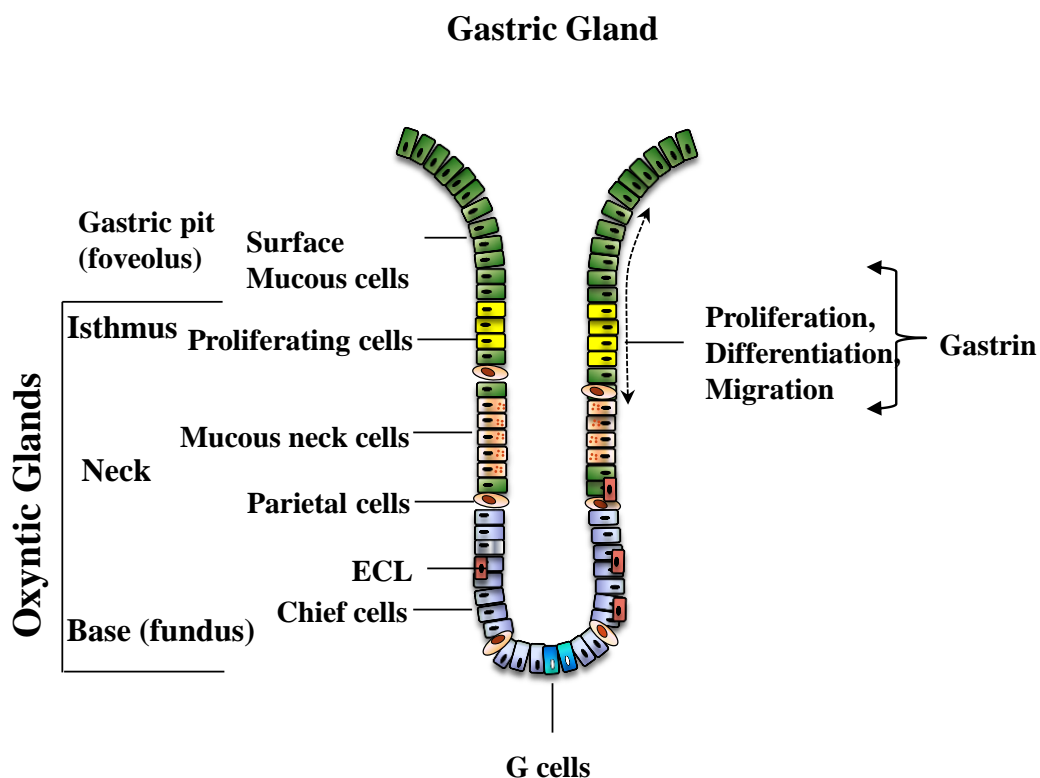
Gastric Gland

Figure.1.1   Gastric gland cell organisations.
Diagrammatic representation of different cell types: proliferating cells, mucous neck cells, parietal cells, ECL cells and chief cells. The bi- directional migration of cells leaving the cell-cycle occurs from isthmus region to gastric pits and to the fundus region generating the differentiated cell types.

1.3   Tissue Remodelling and Repair

The events that characterise wound healing in the gut include (a) restitution and haemostasis after tissue damage, (b) inflammation, (c) neo-vascularisation and

re-epithelisation and (d) resolution or tissue remodelling (Shaw and Martin 2009). In some tissues e.g. liver and pancreas there are stellate cells which resemble myofibroblasts but in general myofibroblasts are sparse in healthy tissue (Omary, Lugea et al. 2007). In the gastrointestinal tract, however, myofibroblasts are normally relatively abundant and their numbers increase still further in wound healing.

1.3.1   Restitution and Haemostasis: Blood Platelets and Fibrin

Restitution is an epithelial migratory response to superficial damage in the gut by which a protective layer of epithelial cells forms over exposed basement membrane. This is characterised by initial epithelial cell migration about 30 minutes after tissue damage and does not involve proliferation (Lacy and Ito 1984). The initial step in healing upon more substantial damage is formation of a blood clot and platelet aggregation. In conjunction with blood clotting factors, anaphylatoxin, C3a and C5a in the matrix (Ghebrehiwet, Silverberg et al. 1981) aggregate platelets on fibrin (Clark 2001). In damaged tissue, fibrin forms a provisional ECM (Clark, Lanigan et al. 1982; Clark 2001) that embeds platelets which are an early source of growth factors and chemokines e.g. platelet derived growth factor (PDGF) and monocyte chemotactic protein-1 (MCP-1) required to further drive coordinated wound healing (Gawaz, Neumann et al. 1998; Kim, Gittes et al. 1998).

1.3.2   Inflammation: Infiltration of inflammatory cells

Inflammatory cells/leucocytes including macrophages, neutrophils, natural killer cells and eosinophils circulate in largely undifferentiated forms that are recruited to damaged tissue. The release of anaphylatoxins and histamine from mast cells

act as vasodilators (Fernandez, Henson et al. 1978). This facilitates successful infiltration of inflammatory cells in response to growth factors such as PDGF and MCP-1 (Deuel, Senior et al. 1982; Gross, Leavell et al. 1997; Gawaz, Neumann et al. 1998). The first to migrate into wounds are neutrophils which release matrix degrading enzymes such as collagenase (matrix metalloproteinase-8, MMP-8) (Nwomeh, Liang et al. 1999; Dovi, Szpaderska et al. 2004) and shield against further infection as well as removing dead cells via phagocytosis.

Monocytes infiltrate the tissue microenvironment and differentiate into macrophages in response to TGF-β (Turley, Falk et al. 1996). Resident macrophages act as anti-inflammatory agents by phagocytosing microbes and secreting IL-10 (Monteleone, Platt et al. 2008). The modification of ECM by macrophage-secreted proteins including MMPs and plasmin also provokes the activation and release of ECM-stored angiogenic factors e.g. vascular endothelial growth factor (VEGF), PDGF and TGF- β (Cordon-Cardo, Vlodavsky et al. 1990).

1.3.3   Proliferation: Vascularisation and Re-epithelialisation

1.3.3.1 Vascularisation

Neoangiogenesis is required to meet the oxygen requirements of healing tissues. Oxygen stress induces the transcription factor, hypoxia inducible factor (HIF) in endothelial cells which enhances the expression of VEGF (Gerber, Condorelli et al. 1997). Fibroblast growth factor (FGF) (Gospodarowicz, Bialecki et al. 1978) and endothelial cell growth factors (ECGF) (Maciag, Cerundolo et al. 1979) also increase the proliferation of endothelial cells. In addition, other growth factors e.g. FGF-2, insulin growth factor (IGF)-I and IGF-II also support neoangiogenesis (Chang, Garcia-Cardena et al. 2004; Bjorndahl, Cao et al. 2005). Increased

endothelial cell proliferation is followed by initiation of collagen deposition (Prockop, Kivirikko et al. 1979).

1.3.3.2 Re-epithelialisation

Re-epithelisation is the process of epithelial cell proliferation and migration in damaged tissue. In gastric inflammation, macrophage or platelet derived TGF-β stimulates interleukin-1β (IL-1β), interferon-γ (IFN- γ) and EGF expression which promote epithelial cell proliferation (Dignass and Podolsky 1993). The increased expression of integrin (Gailit, Welch et al. 1994) and IGF-I, EGF and FGF facilitates cell adhesion and pro-migratory roles in re-epithelialisation (Giancotti and Ruoslahti 1999; Eslami, Gallant-Behm et al. 2009).

1.3.4   Matrix Deposition and Tissue Remodelling

The concluding event in wound healing involves scar maturation and crosslinking of collagen fibrils. Simultaneously, there is removal of blood clots, inhibition of platelet driven signalling events and remodelling of tissue architecture.

1.3.4.1 Fibroblasts

In the final stages, mechanical force is required for closure of wounds and matrix deposition. The vital cell types in this process are derived from fibroblast lineages. Fibroblasts are activated in response to TGF-β to become "activated fibroblasts" also called myofibroblasts. The expression of α-smooth muscle actin (α-SMA) and other actin filaments in myofibroblasts provides the capacity for contractile tension for wound closure (Hinz 2007). These cells are also an important source of ECM proteins e.g. collagens. In addition, they secrete proteases such as MMPs to maintain a balance in ECM deposition and degradation (Hinz 2007).

1.3.4.2 ECM Proteins

The adhesion of infiltrated cells occurs via integrin receptors and interactions with fibronectin, collagen, vitronectin or other ECM molecules (Schwartz, Schaller et al. 1995). Adhesion, migration and proliferation are mediated by increased expression of integrin and fibronectin in tissue repair (George, Georges-Labouesse et al. 1993; Wijelath, Murray et al. 2002). In tissue remodelling all types of collagen contribute to tissue strength and elasticity. The intestinal submucosa demands elasticity and is predominantly constituted of Type III and V collagen (Graham, Diegelmann et al. 1988). However, fibrous collagens, type I and type IV, are the primary structural unit in ECM and basement membrane, respectively, of all tissues (Timpl, Wiedemann et al. 1981; Prockop and Kivirikko 1995). Other vital players in the ECM are glycosaminoglycans (GAG) e.g. hyaluronan (Chen and Abatangelo 1999; Trabucchi, Pallotta et al. 2002). In addition, other GAG molecules e.g. heparin sulphate regulates cell proliferation and angiogenesis (Andres, DeFalcis et al. 1992; Iozzo 1998). Proteolytic cleavage of sydencan, laminin and elastin releases bioactive fragments known as matrikines (Maquart, Bellon et al. 2005). They are equally important in proliferation, adhesion and signalling in completion of wound repair (Adair-Kirk and Senior 2008).

1.3.5   Failure in Tissue Remodelling and Repair

Failure of the normal wound healing processes to restore tissue architecture may lead to the development of conditions such as fibrosis and chronic inflammation.

1.3.5.1 Fibrosis

Fibrosis is the result of excessive deposition of ECM proteins often with loss of some resident cell types, but increased numbers of myofibroblasts and related

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cells. Early in wound healing, fibrin and other scar matrix proteins provide a favourable scaffold for adhesion, migration and proliferation of cells. However, subsequent inefficient removal of fibrin, and further matrix protein deposition, leads to fibrosis (Olman, Mackman et al. 1995). The role of plasminogen activator inhibitor-1 (PAI-1), for example, is well studied in this case. Thus PAI-1 is an inhibitor of tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Plasmin abundance is regulated by uPA which determines the degradation of fibrin and ECM proteins; these enzymes also play a role in activating MMPs (Madlener, Parks et al. 1998). The mouse bleomycin induced-lung fibrosis model illustrates some of the relevant issues: in bleomycin-treated mice there is upregulation of TGF-β and procollagen I and III (Gurujeyalakshmi and Giri 1995); TGF-β enhances PAI-1 expression (Kutz, Hordines et al. 2001; Vayalil, Olman et al. 2005) and PAI-1 in turn contributes to pulmonary fibrosis by inhibiting proteases that degrade ECM proteins (Olman, Mackman et al. 1995). Similar evidence is available for other systems including liver and kidney fibrosis, but less is understood of the stomach. Even so, however, there is evidence of increased PAI-1 in epithelial cells in conditions associated with atrophy e.g *H.pylori* infection (Kenny, Duval et al. 2008). Interestingly, in some clinical situations fibrosis might develop from cancer cell-secreted growth factors. For example, the fibrosis of the peritoneum in scirrhous gastric cancer (Yashiro, Chung et al. 1996) is characterised by extensive growth of fibroblasts possibly due to TGF-β_1 secretion from scirrhous gastric cancer cells (Mahara, Kato et al. 1994).

1.3.5.2 Chronic Inflammation

One characteristic of chronic inflammation is the continuous reiteration of inflammation steps in wound healing. An excess of cytokines and MMP's due to continuous infiltration of inflammatory cells causes increased degradation of ECM (Wysocki, Staiano-Coico et al. 1993; Widgerow 2011). Proteases also cleave laminin and elastin triggering further inflammatory responses (Adair-Kirk and Senior 2008; He, Turino et al. 2010). These phenomena cause repetition of repair events (Adair-Kirk and Senior 2008; He, Turino et al. 2010) resulting in deformed tissue architecture, a loose bed of ECM proteins, damaged basement membrane and infiltrated macrophages and fibroblasts or resident activated myofibroblasts. Chronic inflammation leads to a state in which wounds never heal and predisposes to cancer (Figure 1.2).

1.4   Chronic inflammation in the gastrointestinal tract

The gastrointestinal tract is normally exposed to many different potentially damaging agents including acid, ingested toxins, resident and ingested microbiota. In the upper gastrointestinal tract, failure of the normal protective mechanisms is a feature of several common conditions including pathology associated with gastric infection with *H.pylori,* and reflux of gastroduodenal contents into the oesophagus. In both cases chronic inflammation is associated with increased risk of cancer.

A. Normal Epithelial Tissue   B. Inflammatory Tissue   C. Cancer Tissue

	A	B	C
Epithelial cells	Organised	Partially disorganised	Disorganised
Basement membrane	Intact	Partially broken	Absent
Extracellular Matrix	Active	Reactive	Hyper reactive
Fibroblasts	+	++	++
Macrophages	+	++	+++
Neutrophils	+	++	+++
Myofibroblasts	+	++	+++
Blood vessels	+	++	+++
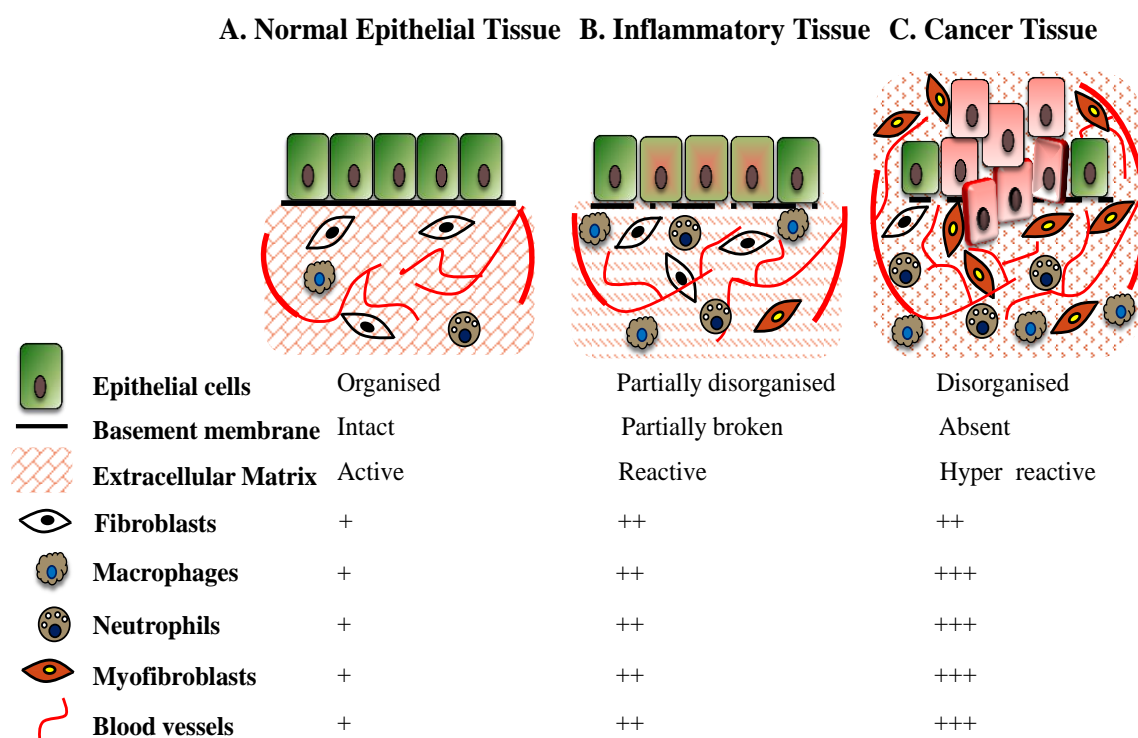
Figure 1.2   Changes in tissue intergrity and microenvironment components. Schematic representation of changes in epithelial cell organisation and cellular microenvironments **A.** in normal tissue, **B.** inflammed and **C.** in cancer. The loss of basement memebrane and increased stromal cell population, particulary myofibroblasts, contributes to the development of cancer on a background on chronic inflammation.

1.4.1   Chronic Inflammation and Gastric cancer

H.pylori infection is associated with chronic gastritis (Fox and Wang 2007). The cascade of events initiated by *H. pylori* in the stomach triggers immune responses involving secretion of cytokines and chemokines (Bodger and Crabtree 1998). Subsequently infiltration of neutrophils and monocytes enhances the secretion of proinflammatory cytokines like TNF-α and chemokines from epithelial cells (Bodger and Crabtree 1998). In some patients this leads to chronic inflammation that is associated with a sequence of pre-neoplastic changes including atrophy, metaplasia and dysplasia (Correa, Piazuelo et al. 2010). In other patients there is

an increased risk of duodenal ulcer (McColl 2012). It seems that antrum-predominant infection leads to increased acid secretion by parietal cells which are associated with duodenal ulcer. In contrast, infection in the antrum and the corpus is associated with development of the pre-neoplastic progression leading to gastric cancer (Fox and Wang 2007). There is also some evidence of increased fibrosis in atrophy (Truong, Farhood et al. 1992; Kang, Hughes et al. 2001).

1.4.2   Barrett's oesophagus

Barrett's oesophagus results from persistent gastro-oesophageal reflux (GORD) by which the oesophagus is continuously exposed to gastric acid and bile salts (Souza, Krishnan et al. 2008). The resulting chronic inflammation can lead in some patients to a transition from squamous to columnar epithelia characterised by the presence of increased goblet cells i.e. intestinal metaplasia, and known as Barrett's oesophagus (Naef, Savary et al. 1975; Nicholson, Graham et al. 2012). The condition is important because it may lead to oesophageal adenocarcinoma (AC). Interestingly, infection of the gastric corpus with *H. pylori* may decrease acid secretion and mitigate the effects of gastric acid reflux thereby decreasing the risk for developing Barrett's oesophagus (Chow, Blaser et al. 1998).

1.4.3   Oesophagitis and Oesophageal Squamous Cell Carcinoma

Other states of oesophageal inflammation are recognised including excess eosinophil infiltration. Eosinophil oesophagitis is defined as an increase in eosinophils (>20 /high power field) (Rothenberg, Mishra et al. 2001) in the mid and upper oesophagus (Noffsinger 2009). The condition may progress to involve basal cell hyperplasia (BCH), with >15% epithelial thickness and extended lamina

propria papillae, leading to squamous dysplasia and squamous carcinoma of the oesophagus (Wang, Abnet et al. 2005).

1.5 Cancer

Modern theories of oncogenesis are based on observations that go back well over a century. The "Tissue Organisation Field Theory" (TOFT) of cancer development (Soto and Sonnenschein 2011) originated with Franz Boll in 1876 who considered cancer to be a self-sustaining entity with enhanced cellular proliferation (Garrison 1926). The concept of "false embryo" was put forward by Thiersch, Cohnheim in 1876, i.e. displaced embryonic rest, by which a portion of undifferentiated embryonic cells in adult tissue initiate cancer; this was later also called the theory of "displaced embryonic cell in space and time" (Garrison 1926). Modern ideas are based on the observation of genetic and somatic mutations in epithelial cells determining the cancer phenotype (Knudson 2001).

Over the last decade or so, however, it has become clear that genetic changes in cancer cells do not, alone, account for cancer progression. Instead there are important tumour-promoting changes in the microenvironment. The term niche is sometimes used to define the combination of stromal cells such as myofibroblasts and ECM proteins that together interact with cancer cells to provide the conditions for tumour growth (Hanahan and Weinberg 2000; Coussens and Werb 2002; Hanahan and Weinberg 2011).

1.5.1 Cancer Niche

The idea of a cancer-promoting microenvironment is an extension of an old question which is "why do certain organs support cancer metastasis?" i.e. provide an appropriate microenvironment that nurtures cancer cells. The question was

posed by Stephen Paget in 1889, who suggested the "seed (cancer) and soil (microenvironment)" theory of cancer progression. Later, the idea of a cancer-specialised niche emerged that sustains mutated epithelial cell capacities for tumorigenicity (Weaver, Fischer et al. 1996; Bissell 2007).

The cancer niche depends on stromal cells that include resident and inwardly migrating non-malignant cells. The non-malignant cells can be from local or healthy neighbouring tissue, e.g. myofibroblasts, or can migrate in from distant source e.g. MSCs and inflammatory cells (Wels, Kaplan et al. 2008). A reactive stroma encourages tumour growth through multiple mechanisms. For instance, extensive neo-vascularisation results from enhanced endothelial cell proliferation. In such cases upregulation of MT1-MMP and VEGF in stromal cells have been identified (Sounni, Janssen et al. 2003). In addition, vascularisation facilitates metastasis, i.e. cancer cell migration and invasion of other tissues. Abnormal basement membrane facilitates the motility of cancer cells in solid tumours of the gut (Ozzello 1959; Li, Chen et al. 2001). This includes basement membrane degradation and imbalances between collagen deposition, hyaluoran GAGs, laminin, vitronectin and fibronectin (Noble, McKee et al. 1996; Kalluri 2003).

However, in addition to interactions with ECM proteins, various stromal cells in the niche determine cancer cell proliferation, survival and metastatic properties (Weaver, Petersen et al. 1997). Stromal cells progressively increase in cancer either by inward migration or transdifferentiation from other cells as well as by proliferation. For example, in response to signalling molecules such as TGF-β, fibroblasts and infiltrated monocytes differentiate to myofibroblasts and macrophages, respectively (Roberts and Sporn 1993). In addition, recruitment of bone marrow derived MSCs to tumour sites is increasingly recognised (Kidd,

Spaeth et al. 2009). These pluripotent cells infiltrate in response to multiple signalling molecules including chemokines, VEGF and IL-10 (Serafini, Borrello et al. 2006); they may differentiate into various stromal cells including myofibroblasts (Spaeth, Dembinski et al. 2009; Quante, Tu et al. 2011). In addition they have their own signalling systems that influence the microenvironment and cancer cell function (Whiteside 2008).

1.6   Myofibroblasts

Myofibroblasts are activated fibroblasts; they are dynamic, spindle-like cells sharing the functional characteristics of both fibrocytes and smooth muscle cells (Hinz, Phan et al. 2007). They are rare in most tissues, but in the gastrointestinal tract they are relatively abundant where they are localized in a subepithelial compartment (in the lamina propria mucosae). Myofibroblasts express alpha-smooth muscle actin (α-SMA) and vimentin, which are specific histopathological markers of these cells. A variety of different origins of these cells has been discussed (Figure. 1.3), including (a) rapid stimulation of local fibroblasts to differentiate into myofibroblasts (Serini, Bochaton-Piallat et al. 1998), (b) epithelial cell transdifferentiation into myofibroblasts via epithelial mesenchymal transition (EMT) (Radisky, Kenny et al. 2007), (c) local or bone-marrow-derived mesenchymal stem cells transformation into myofibroblasts (Spaeth, Dembinski et al. 2009; Quante, Tu et al. 2011).

The dynamic secretory profile of myofibroblasts includes growth factors, MMPs, and tissue inhibitor of metalloproteinase (TIMPs) that regulate tissue remodelling and influence cancer progression (Figure 1.3). In case of *H. pylori* gastritis, gastric epithelial cells secrete matrix metalloproteinase-7 which cleaves insulin-like growth factor binding protein-5 (IGFBP-5) secreted by myofibroblasts leading

to release of IGF-II which stimulates both epithelial and myofibroblast proliferation (McCaig, Duval et al. 2006). In breast cancer, stromal derived factor-1 (SDF-1), HIF-1 and VEGF are highly expressed by CAMs and promote cancer cell proliferation and angiogenesis (Orimo, Tomioka et al. 2001; Orimo, Gupta et al. 2005). The profound effect of CAMs can be shown in xenografts where they increase tumour growth when co-injected with cancer cells compared to cancer cell alone (Gleave, Hsieh et al. 1991; Quante, Tu et al. 2011; Holmberg, Quante et al. 2012).

1.6.1   Myofibroblasts Associated With Cancers

Fibroblasts residing in the cancer niche are known as peritumoural fibroblasts. Their activated forms express α-SMA, vimentin, P4H and are negative for cytokeratin; they are designated "cancer associated myofibroblasts" (CAMs) (De Wever and Mareel 2003). Differences in gene expression (Boussioutas, Li et al. 2003; Farmer, Bonnefoi et al. 2009) and changes at the epigenetic level have been detected in CAMs from gastric cancer compared with myofibroblasts derived from normal tissues (Jiang, Gonda et al. 2008). The differential expression of genes is not only involved in classifying myofibroblast associated with tumour tissue (CAMs/CAFs) or non-tumour/normal tissue (NTMs) but also, relates to various cellular functions such as cancer cell proliferation, migration and invasion (Bhowmick, Neilson et al. 2004; Singer, Gschwantler-Kaulich et al. 2008; Grugan, Miller et al. 2010). It has been demonstrated that CAMs exhibit increased migration and/ or proliferation compared to myofibroblasts from non-cancerous stroma (Schor, Schor et al. 1988; Holmberg, Quante et al. 2012).

Tumour myofibroblasts have been shown to have decreased sensitivity to chemotherapy at least partly due to CpG DNA hypermethylation and reduced

expression of caspase and STAT-1 resulting in escape from apoptosis of cancer cells (Muerkoster, Werbing et al. 2008).
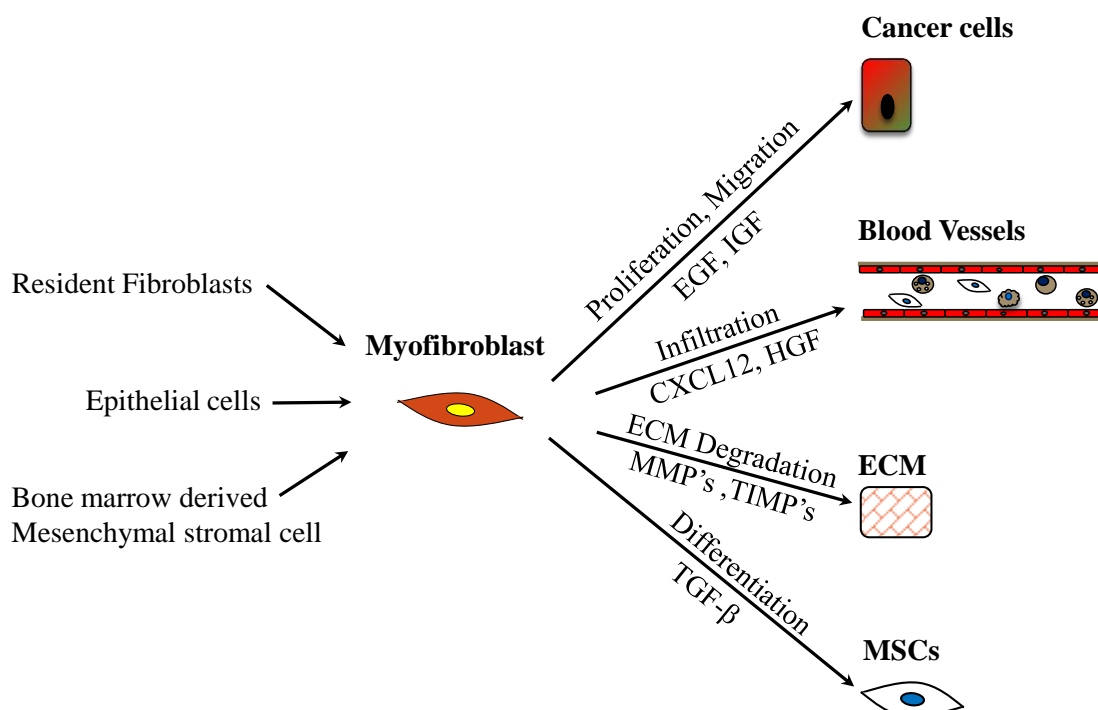
Figure 1.3 Myofibroblasts: single cell with multiple, origins and functional roles. Schematic representation of origin of myofibroblasts from multiple progenitors (fibroblasts, mesenchymal stromal cells orepithelial cells). Myofibroblasts secrete various growth factors e.g. EGF, IGF, HGF; chemokines e.g. CXCL12, TGF-β; preoteases, MMPs; and protease inhibitors, TIMPs that regulate cell proliferation, migration, and infiltration of immune cells and stromal cells, e.g. MSCs, cell differentiation, as well as ECM remodelling.

1.6.2 Myofibroblast Secretomes in Cancer

Differential secretome analysis has suggested upregulated stromal-derived factor-1α and Rantes in CAFs associated with colon cancer compared to bone marrow derived MSCs (De Boeck, Hendrix et al. 2013). Moreover secretome analysis of CAMs in gastric cancer compared to ATMs showed decreased transforming growth factor-β-induced gene-h3 (TGFβig-h3) in advanced disease. A role for this protein in suppression of cancer cell proliferation and migration was shown

indicating that myofibroblasts may also release tumour suppressors (Holmberg, Quante et al. 2012).

Myofibroblast are a prime source of extracellular MMP's that have potential as prognostic markers in cancer (Vihinen and Kahari 2002) as well as promoting cancer progression (Holmberg, Ghesquiere et al. 2013). Also, secretome analysis has provided a potential marker, collagen type XII, for differentiating myofibroblast in colon cancer (Karagiannis, Petraki et al. 2012). These studies highlight the value of protein profiling of myofibroblasts in understanding cancer growth and treatment.

However, there are still large gaps in knowledge and, for example, myofibroblasts in oesophageal cancers have been largely ignored to date. Unpublished studies in our group have shown differentially upregulated secretory proteins in CAMs vs ATMs from oesophageal squamous cell carcinoma (ESCC) and adenocarcinoma (AC) (Holmberg & Varro, unpublished data). The data have provided a potential candidate, namely the chemokine chemerin, as differentially upregulated in CAMs vs ATMs and this provides the starting point for the present experimental work.

1.7   Mesenchymal Stromal Cells

Bone marrow-derived MSCs have shown to populate the gastrointestinal tract and in bone transplant patients there is evidence of differentiation into epithelial cells (Ferrand, Noel et al. 2011). In Barrett's adenocarcinoma, X/Y fluorescence in situ hybridization (FISH), indicates MSC homing and differentiation to stromal and epithelial cells (Sarosi, Brown et al. 2008; Hutchinson, Stenstrom et al. 2011). Moreover, in mice with *H. felis*-induced gastric cancer, 20% of CAMs have been shown to originate from bone marrow-derived MSCs (Quante, Tu et al. 2011). In

other studies, sublethally irradiated severe combined immunodeficient (SCID) mouse (H-2d) tumours were shown to harbour bone marrow derived myofibroblasts: 12.7 % on day 14 and 39.8 % on day 28 (Ishii, Sangai et al. 2003). The infiltrated bone marrow derived MSCs differentiate into myofibroblasts in response to osteopontin (OPN) or TGF-β secreted by cancer cells or stroma (Popova, Bozyk et al. 2010; Mi, Bhattacharya et al. 2011). Moreover, TGF-β_1-stimulated MSCs shared 16 proteins with CAFs in colon cancer, including stromal-derived factor-1α and Rantes (De Boeck, Hendrix et al. 2013), suggesting that in the presence of TGF-β_1 MSCs might potentially give rise to CAFs. In other studies, IGF-1 transactivation of CXCR4 increased MSC migration in response to growth factor SDF-1 (Li, Yu et al. 2007). Recruited MSCs secrete CCL5 which interacts with its cognate receptor CCLR5 to increase breast cancer cell motility (Karnoub, Dash et al. 2007). While, on the other hand, MSCs have also been ascribed anti-tumorigenic properties (Kidd, Spaeth et al. 2008). In Kaposi's sarcoma, recruited MSCs inhibited tumour growth perhaps via moderation of Akt signalling (Khakoo, Pati et al. 2006).

Collectively the data suggest that the homing of bone marrow derived MSCs to cancer involves multiple ligands eg EGF, HGF, MCP-1, and ligand-receptor interactions, SDF-1α/CXCR4 and CXCL5/CXCR2 (Karnoub, Dash et al. 2007; Yang, Huang et al. 2008; Feng and Chen 2009; Quante, Tu et al. 2011). Nevertheless many aspects of the relationships between MSCs and tumour stroma remain uncertain, including mechanisms of homing in oesophageal cancer.

1.8   Mechanisms of inter-cellular signalling

Cell-cell signalling is mediated by various substances including hormones, growth factors, cytokines and chemokines. In each case, a ligand activates receptors on target cells. Where ligands are released in close proximity to target cells the mechanisms are collectively known as paracrine signalling. Where they are carried in the blood stream to their targets the mechanisms are known as hormonal signalling; in some cases, ligands may even act on their cell of origin, known as autocrine signalling. Within a tissue, there are frequently interactions that are two-way, for example epithelial-stromal cell interactions where both cell types can be the source of signalling ligands and can respond to them by paracrine mechanisms.

1.8.1   Humoral transmission

The idea of chemical messengers mediating cell-cell interactions originated with the discovery of the first hormone, secretin, in 1902 by Bayliss and Starling (Bayliss and Starling 1902). Many gut hormones were subsequently discovered and shown to be involved in regulating gut secretion, motility and growth (Dockray 2006). It was only much later that the cell surface receptors at which these hormones act were discovered. The well characterized gut hormones include gastrin, cholecystokinin (CCK), secretin, gastric inhibitory polypeptide (GIP), peptide YY (PYY), glucagon-like peptide (GLP)-1, and somatostatin (SST); their respective receptors are CCK-2 (gastrin/CCK-B), CCK-1, human secretin receptor (SCTR), GIP-R , Y-2 receptor, GLP-1 and the SSTR1-5 receptors (Konturek, Konturek et al. 2004; Murphy and Bloom 2006).

1.8.2 Growth factors

Growth factors (GFs) are polypeptides which are secreted and act in relatively close proximity to their cells of origin. Like polypeptide hormones they bind specific membrane receptors. However, while hormones typically act via GPCRs (Walsh 1993), growth factors typically act at receptor tyrosine kinases (Lemmon and Schlessinger 2010). There are multiple classes of growth factors including the families of EGF, IGF, VEGF, TGF-β, HGF, FGF, PDGF, Wnt, Hedgehog, Notch; each of these act at their cognate receptors and most play a role in gastrointestinal mucosal regeneration (Jones, Tomikawa et al. 1999). Cancer cells (Sporn and Roberts 1985), stromal cells e.g. myofibroblasts, bone marrow-derived MSCs and epithelial cells are all potential sources of growth factors (Sporn and Roberts 1985; Orimo, Tomioka et al. 2001; Chen, Tredget et al. 2008)

EGF family. The EGF family consists of EGF, heparin-binding EGF (HB-EGF), TGFα, amphiregulin, epiregulin, βcellulin, and neuregulin-1 to -4. The expression of TGFα, and HB-EGF in gastric parietal cells (Murayama, Miyagawa et al. 1995) may induce mitogenic signals within the epithelium regulating tissue integrity (Wang, Dangler et al. 2000). Members of the family may also influence stromal cells to enhance healing for example of gastroduodenal ulcers (Schultz, Rotatori et al. 1991). The exocrine secretion of EGF from salivary gland may stimulate proliferation of gastrointestinal epithelial cells and plays a role in the healing of gastric ulcers (Olsen, Poulsen et al. 1984; Konturek, Dembinski et al. 1988).

IGF family. As its name suggests, members of the IGF family of growth factors are related in structure to insulin; however, the two members of this family, IGF-I and IGF-II, each consist of a single peptide chain which is homologous to the

single chain of pro-insulin prior to its cleavage to generate the A and B chains of mature insulin (Rinderknecht and Humbel 1978). Both IGF-I and –II act at the IGF-1 receptor to stimulate cell proliferation and migration; there is also an IGF-II receptor which is not functional. In the extracellular space, IGFs are sequestered by binding to IGF-binding proteins (IGFBPs1-6). In the upper gastrointestinal tract, IGF-II has been shown to be produced by myofibroblasts and to act on both epithelial cells and myofibroblasts to stimulate proliferation and migration (McCaig, Duval et al. 2006). It has been shown that MMP-7 released by epithelial cells cleaves IGFBP-5 released by myofibroblasts to liberate the active growth factor (Hemers, Duval et al. 2005).

TGFβ/activins. The TGF-β family comprises five members, TGF-β_{1-5} of which TGF-β_{1-3} are expressed in mammals. The release of TGF-β is attributed to cleavage by plasmin of latent TGF-β (Sato and Rifkin 1989); TGF-β_1, TGF-β_2 and TGF-β_3 are mainly expressed by mesenchymal, epithelial and connective tissue cells respectively (Massague 1990). TGF-β acts via two receptors, TGF-β receptors type I and II. The binding of ligand to a type II receptor dimer, recruits a type I receptor dimer forming a hetero-tetrameric complex in which both receptors are required for the transduction of signal (Wrana, Attisano et al. 1992). Receptor type-III does not transduce signals; however, binding of type I and II is catalysed by type III receptors (Wrana, Attisano et al. 1994). In gastric cancer, cell invasiveness and migration may be mediated by TGF-β_1 (Fu, Hu et al. 2009). As already noted, TGF-β stimulates conversion of fibroblasts to myofibroblasts and consistent with this increased TGF-β in scirrhous gastric carcinoma cells increases the number of myofibroblasts associated with the tumour (Fuyuhiro, Yashiro et al. 2011).

FGF Family. There are 23 mammalian forms of FGF, including FGF-1 (acidic FGF), FGF-2 (basic FGF) and FGF-7/keratinocyte growth factor (KGF) which interact with FGFRs to influence cellular growth and differentiation. FGF-2/bFGF is secreted by fibroblasts and immobilised by binding to heparin and heparan sulphate in the ECM. The binding of FGF-2 to ECM proteins may confer protection from proteases and regulate activity and binding affinity to the receptor, FGFR2 (Guimond, Maccarana et al. 1993). FGF-7 is expressed in mesenchymal cells (Finch, Rubin et al. 1989) and studies have shown it signals via FGFR2 expressed on epithelial cells (Mason, Fuller-Pace et al. 1994). There is increased expression of FGF-7 in gastric ulcer (Hull, Brough et al. 1998) which in turn stimulates epithelial cell proliferation and migration (Cordon-Cardo, Vlodavsky et al. 1990).

PDGF Family. This is a family of dimeric glycoproteins with isoforms e.g. PDGFA, PDGFB acting through two different receptors, PDGFRA, PDGFRB. In gastric ulcer PDGF has been shown to be a potent mitogen for fibroblasts (Piazuelo, Lanas et al. 1998). Other studies have shown that gastric cancer cells (MKN-1) secrete PDGF which interacts with PDGFRA to increase the proliferation of gastric fibroblasts (Wada, Sakamoto et al. 1998). The isoform PDGF-B regulates the expression of other growth factors, e.g. VEGF and HGF by stromal cells; it increases the proliferation of epithelial cells and myofibroblasts in epithelial cancers (Lederle, Stark et al. 2006).

HGF Family. Inactive HGF produced by stromal cells is cleaved by serine proteases to release the active form that acts on the protoncogene c-Met receptor. CAF-derived HGF has been shown to act at c-Met expressed on ESCC

to increase cell invasion (Grugan, Miller et al. 2010). Other studies have shown increased c-Met in TE-1 and TE-4 cells (ESCC lines) that depends on the level of Ets-1 transcription factor (Saeki, Oda et al. 2002). Further, these cells show increased motility in response to HGF.

VEGF Family. As the name suggests, VEGF is a growth factor for endothelial cells and is vital in angiogenesis. The family consist of the isoforms, VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGF-A regulates cell proliferation and migration via VEGFR-1 and VEGFR-2. The expression of VEGF depends upon enhanced HIP-1 expression in an oxygen-stressed environment and has been observed in ESCC (Kimura, Kitadai et al. 2004). Further, VEGFs are extensively studied in gastrointestinal tract injury (Milani and Calabro 2001) and they have been shown to have relevance in carcinogenesis (Hyodo, Doi et al. 1998).

Wnt Family. These are a family of cysteine rich glycoproteins comprised of 19 members in humans. There are distinct intracellular signalling pathways for different Wnt proteins. For example Wnt3a, binds to cognate receptors of the Frizzled (Fz) family and a low-density lipoprotein receptor-related (LRP) protein to activate the so-called canonical pathway mediated by β-catenin; while Wnt5a activates the non-canonical pathway (Cong, Schweizer et al. 2004). Collectively, Wnt molecules act as growth factors for stem cells and maintain the stemness of epithelial cells (Willert, Brown et al. 2003). Increased accumulation of β-catenin as a result of canonical pathway activation is seen in a majority of upper gastrointestinal cancers (Woo, Kim et al. 2001). Moreover, Wnt2 derived from tumour fibroblasts increases oesophageal cancer cell proliferation and migration via increased cyclin D and c-Myc expression and via Wnt/β catenin signalling (Fu,

Zhang et al. 2011). In contrast, both gastric epithelial and myofibroblasts-derived Wnt-5a mediates a β-catenin-independent pathway to enhance the gastric cancer cell migration (Kurayoshi, Oue et al. 2006; Wang 2013).

Hedgehog Family. There are three hedgehog (Hh) homologues, Desert hedgehog homolog (DHH), Indian hedgehog homolog (IHH), and sonic hedgehog (SHH) of which SHH is widely studied in mammals. SHH undergoes processing to yield the active form by a mechanism involving translocation to the endoplasmic reticulum and autocatalytic processing to produce an active N-terminal domain. The activation of Patched (PTCH), a cell-surface transmembrane protein, by Hh increases the level of a 7-transmembrane spanning receptor, Smoothened (SMO) which in turn is coupled to activation of GLI transcription factors (Brink, Peppelenbosch et al. 2006). In gastric glands SHH is expressed in neck cells (van den Brink, Hardwick et al. 2001) while IHH is expressed by pit epithelial cells (Fukaya, Isohata et al. 2006). Sonic hedgehog plays a vital role in cell differentiation and is lost in atrophic gastritis (Zavros 2008). The loss of SHH in parietal cells increases the expression of E-cadherin, nuclear localisation of β-catenin, the transcription factor Gli1, and activation of Wnt pathways. In addition, the expression of IHH was shown to regulate the events occurring in the absence of SHH in gastric epithelium. Thus Hh and related molecules might play an important role in maintaining gastric epithelial architecture (Xiao, Ogle et al. 2010). Overexpression of sonic hedgehog (SHH) and abnormal hedgehog signalling has been shown to increase proliferation of both gastric and oesophageal cancer cells (Berman, Karhadkar et al. 2003).

Notch Family. Notch proteins are of 4 types, Notch1-Notch4, and possess transmembrane domains. The activation of notch receptors occurs mostly via the transmembrane ligands, delta and jagged, thus cell-cell contact is essential. Increased notch-1 activity and increased jagged expression are associated with Barrett's adenocarcinoma (Mendelson, Song et al. 2011) and inhibition of notch-1 activation increases the colony formation in oesophageal cancer cells (Subramaniam, Ponnurangam et al. 2012).

1.8.3 Cytokines

Cytokines are small cell signalling molecules found in both extracellular and membrane-bound forms. Many cytokines are upregulated in cancer. They are named on the basis of their ability to stimulate growth, differentiation and activation of immune cells and can be grouped into immunoregulatory cytokines e.g. IL-2 and pro-inflammatory cytokines, e.g. TNF-α.

Immunoregulatory cytokines. Immunoregulatory cytokines regulate various aspects of inflammation, and modulate the biological impact of pro-inflammatory cytokines. The expression of some cytokine receptors such as IL1R, IL-4R in resting immune cells is constitutive providing for readily responsive cells. In addition, activating stimuli may increase expression and activation of cytokine receptors e.g. IL 2R, (Ozaki, Kikly et al. 2000; Dumoutier, Lejeune et al. 2003). The interaction of activated cytokine receptors and upregulated cytokines regulates the migration and proliferation not just of the immune cells, but also of stromal and epithelial cells (Trinchieri and Scott 1995). Gastric cancer cells show increase expression of IL 2Rβ and in response to IL-2 exhibit enhanced cell proliferation (Lin, Yasumura et al. 1995). Some cytokines also regulate immune

responses via induced secretion of other cytokines. Thus increased IL-10 in oesophageal cancer regulates cell proliferation (Gholamin, Moaven et al. 2009) and secretion of IL-8 and IL-12 (Buelens, Verhasselt et al. 1997).

Proinflammatory Cytokines. These cytokine are generally not expressed until an external stress stimulus triggers their expression. Infection with *H. pylori* provides an example, and is associated for instance with increased the secretion of IL-1β (Jung, Kim et al. 1997). As the name suggests, the main function of this class of cytokine is to enhance inflammation. In the case of gastric cells, co-culture of cancer cells (MKN45) with *H. pylori* caused increased IL-8 which was attributed to activation of NF-kB and regulated proinflammatory cytokine transcription (Aihara, Tsuchimoto et al. 1997). Moreover, increased IL-1β in response to *H.pylori* may regulate gastric acid secretion (Beales and Calam 1998). Increased abundance of proinflammatory cytokines can be a driver for cancer development. For instance, increased IL-1 expression is associated with increased risk of upper gastrointestinal cancer (El-Omar, Carrington et al. 2001).

Macrophage migratory inhibition factor (MIF): Macrophage migration inhibitory factor (MIF) is also known as glycosylation-inhibiting factor (GIF), L-dopachrome isomerase, or phenyl pyruvate tautomerase. It was discovered in activated T-lymphocytes as an inhibitor of the migration of peritoneal cells (David 1966). Later, it was shown that macrophages possess a receptor for MIF (Remold and David 1971) and associated with this was inhibition of macrophage migration (Rocklin 1976). Now it is well known that MIF interacts through CD74 and its activation regulates recruitment of CD44 (Bernhagen, Calandra et al. 1993) The recruitment of CD44 then activates non-receptor tyrosine kinases leading

ultimately to extracellular signal-regulated kinase phosphorylation (Shi, Leng et al. 2006). It plays a vital role in proinflammatory signalling in the control of local and systemic immune responses. In *H. pylori* induced gastritis and gastric cancer there is increased MIF which enhances gastric epithelial proliferation (Xia, Lam et al. 2005; He, Yang et al. 2006). In addition, MIF indirectly promotes angiogenesis in ESCC by stimulating tumour cells to produce angiogenic factors, such as IL-8 and VEGF (Ren, Law et al. 2005). Moreover, it was recently shown that MIF transduces inhibitory signals via CD74 regulating MSC migration (Barrilleaux, Phinney et al. 2009). Amongst specific downstream targets of MIF is activation of MAPK pathways, e.g. ERK1/2 which depends on activation of protein kinase A (PKA) and protein kinase C (PKC) (Mitchell 2004); there is also down-regulation of the expression of the tumour-suppressor protein p53 (Hudson, Shoaibi et al. 1999).

1.8.4   Chemokines

Chemokines are defined as chemotactic cytokines vital for infiltration of immune cells and MSCs. They are also involved in cancer metastasis. The expression and regulation of chemokines and their receptors is widely modulated in chronic inflammation. The inflammatory chemokines recruit neutrophils, inflammatory cells, and stimulate neoangiogenesis. In general, chemokines bind to soluble and cell membrane-bound GAGs. The interaction with GAG has varying consequences; thus binding to soluble GAG impairs chemokine activity whereas immobilised membrane GAGs increases their local availability (Kuschert, Coulin et al. 1999). Chemokine receptors undergo oligomerization or dimerization and interact with chemokine-GAGs complexes to stimulate cancer cell migration and angiogenesis (Folkman and Shing 1992). Broadly, the chemokines expressed in

cancer belong to inflammatory group. Inflammatory chemokines are induced or upregulated by pro-inflammatory stimuli e.g. TNF-α. This group of chemokines, e.g. CXCL10, CCL5 attract immune and inflammatory cell to the site of damage. The other group, homeostatic chemokines, includes members regulating the normal recruitment of neutrophils e.g. CXCL12 and CCL14. The inflammatory and homeostatic groups consist of various classes of chemokines which can be characterised on the basis of their structure, namely the spacing of their first two cysteine residues i.e. CXC, CC, C and CX_3C.

CXC Family. The α-chemokines, i.e. CXC, consist of 17 members that can be further classified depending on the presence or absence of a glutamine-leucine-arginine (ELR) motif ie ELR+ or ELR- chemokines. The interaction of ELR+ or ELR- CXCs with their cognate G-protein-coupled seven trans-membrane (GPCR) receptors (CXCR) is essential for paracrine or autocrine signalling. In addition, growth-related oncogenes (GROs) (GROα, GROβ, and GROγ) of the ELR+ CXC group are potent promoters of cell proliferation and angiogenesis via interactions with CXC chemokine receptor 1 or 2 (CXCR1 or CXCR2). Autocrine signalling via GROβ/CXCR2 interaction increases ESCC proliferation by stimulating ERK1/2 (Wang, Hendricks et al. 2006). In breast cancer, CAM-derived CXCL12/SDF-1 interacts with CXCR4 on cancer cell enhancing their invasion (Orimo, Gupta et al. 2005) and stimulating angiogenesis (Luker and Luker 2006).

CC Family. The β-chemokines, ie CC, consist of 28 members that act via CCRs which are mostly of the GPCR type (Murphy 2002). Two different studies in gastric cancer showed increased CC family members, CCL7 and CCL21 (Hwang, Lee et al. 2012) and their receptor, CCR7 (Mashino, Sadanaga et al. 2002),

associated with cancer cell invasion and metastasis. CCL5 derived from CD4+ T

blockade significantly impaired gastric cancer progression and has been shown to

induce apoptosis of CD8+ T cells (Sugasawa, Ichikura et al. 2008). Oesophageal

cancer cell derived MCP-1 stimulates both infiltration of immune cells and

secretion of angiogenic factors such as VEGF suggesting role in angiogenesis.

Further, there is a significant correlation of MCP-1 expression by cancer cells and

CCR2 via infiltrating stromal cell macrophages (Koide, Nishio et al. 2004) thereby

suggesting cancer and stromal interactions that are important in tumour growth.

C Family: A small family of chemokines with two members named, XCL1

(lymphotactin-α) and XCL2 (lymphotactin-β). These ligands are expressed by

immune cells and act as chemo-attractants for T cells via binding to XCR1 e.g. G-

protein receptor 5 (GPR5)(Kroczek and Henn 2012) as well as modulating cancer

growth (Cairns, Gordon et al. 2001).

CX$_3$C Family: This subgroup of chemokines consists of single member called

fractalkine (or CX$_3$CL1). It exists in soluble form and in an immobilized form on

cell membranes (Bazan, Bacon et al. 1997). Fractalkine supports adhesion and

migration of stromal cells, e.g. monocytes and endothelial cells via the G$_\alpha$i

coupled receptor CX$_3$CR1(White and Greaves 2009)

1.8.5   Adipocytokines

As the name suggested these are cytokine-like molecules secreted from

adipocytes, also known as adipokines. The well-known members include

chemerin, PAI-1, retinol binding protein 4 (RBP4), visfatin, and leptin. The most

abundant and well-studied adipokine is leptin which may also be produced in

gastric chief cells and D1 cells in stomach (Bado, Levasseur et al. 1998). Similarly, other adipokines such as visfatin and chemerin may be derived from non-adipocytes including macrophages, neutrophils and fibroblasts (Moschen, Kaser et al. 2007; Albanesi, Scarponi et al. 2009). Generally, the adipokines are involved in energy metabolism, obesity and insulin resistance (MacDougald and Burant 2007; Rabe, Lehrke et al. 2008). However, recent evidence has identified a link to cancer (MacDougald and Burant 2007). Leptin exerts a mitogenic effect in oesophageal cancer cells (OE33) via PI3K/Akt and p38 MAPK (Beales and Ogunwobi 2007; Kendall, Macdonald et al. 2008). In contrast, adiponectin inhibited ERK1/2 and Bcl-2 expression, and increased Bax abundance, in OE19 cells thereby exerting anti-proliferative and pro-apoptotic effects (Konturek, Burnat et al. 2008).

1.9   Intracellular Signal Transduction Pathways

Multiple different types of ligand-activated receptors have been defined that can be conveniently classified as (1) receptor tyrosine kinases (RTK) e.g. EGFR, (2) ligand gated ion channels, e.g. nicotinic cholinergic receptor, (3) the G-protein coupled receptors, GPCRs, e.g. CXCR4 and chemR23 and (4) nuclear receptors e.g. receptors for oestrogen, corticosteroids, thyroxin.

Receptor tyrosine kinases. Members of the RTK family have a single transmembrane domain and typically act in dimers. They can be grouped into 20 different classes including for example EGF receptors, IGF and PDGF receptor families respectively. The EGFR family illustrates some of the general issues and is linked to a well-defined intracellular signalling cascade. The members of the family are ErbB 1 to ErbB4 (or HER1-4). They are widely expressed in epithelial,

mesenchymal, and neuronal-derived cells where they influence cell proliferation and differentiation. The extracellular binding of ligand causes RTK receptor dimerization (Lemmon and Schlessinger 2010). Auto-phosphorylation of specific intracellular tyrosine residues creates binding sites for Src homology 2 (SH2) domain- and phosphotyrosine binding (PTB) domain-containing proteins (Pawson 1995). The recruitment of Src causes downstream activation of a signal transduction pathway (Pawson 1995) in which isoforms of Raf (Raf1, A-Raf or B-Raf) interact with Ras to activate MEK1/2 followed ERK1/2 (Roberts and Der 2007) (Figure 1.4). In addition conformational changes in Ras transduce signals by PI3 kinase and Ral guanine nucleotide exchange factors (RalGEFs) (Hamad, Elconin et al. 2002; Yin, Pollock et al. 2007) (Figure 1.4). The loss of ErbB signalling results in failure of organ development and embryonic death (Miettinen, Berger et al. 1995). Mutations in Ras family members are common being found up to 40% of upper gastrointestinal cancers (Arber, Shapira et al. 2000; Lord, O'Grady et al. 2000). There may also be mutations in erbB receptors which, since they are upstream of Ras, can lead to constitutive activation of Ras/Raf/MEK in cancer (Roberts and Der 2007). Constitutive activation of Ras can also be result of germline mutation either in Ras itself or Raf isoforms. Mutations found in human cancers include Ras residues G12 (glycine to valine mutation at residue 12), Q61 and infrequently at G13. In the case of Raf, V600E (valine substitution by glutamate at position 600) occurs in high proportion of cancers (Garnett and Marais 2004; Tan, Liu et al. 2008). Signalling through the MAPK cascade is transduced by GTP loading of Ras leading to the activation of Raf kinase;, constitutive activation of Ras is due to inhibition of GTPase activity that is longer be stimulated by GAP (Mitin, Rossman et al. 2005).

G-Protein coupled receptors. The GPCRs are a family of approximately 800 receptors characterised by 7-transmembrane domains and exhibiting sequence homology and functional similarity; they can be grouped into 6 different classes (A-F). The binding of ligand to a GPCR causes a conformational change and regulates G-protein/guanine nucleotide-binding protein signals via the exchange of GDP to GTP on the alpha (α) subunit of a heterotrimeric G-protein complex; the other components of the complex are beta (β) and gamma (γ) subunits (Hurowitz, Melnyk et al. 2000; Krumins and Gilman 2006). G_α exists in different forms each possessing unique function: $G_{\alpha s}$ is linked to activation of adenylate cyclase and increased production of cyclic adenosine monophosphate (cAMP) while $G_{\alpha i}$ inhibits the production of cAMP from adenosine-5'-triphosphate (ATP); $G_{\alpha q/11}$ stimulates membrane-bound phospholipase C beta, and $G_{\alpha 12/13}$ are involved in Rho family GTPase signalling and control cell cytoskeleton remodelling and cell migration (Etienne-Manneville and Hall 2002; Vega and Ridley 2008). The major pathways resulting from GPCR activation are therefore stimulation of cAMP and phosphatidylinositol (PI) (Figure 1.4) (Gilman 1987). The increase of cAMP acts as secondary messenger to induce downstream events via kinases such as PKA. In the case of phospholipase C (PLC) (Boyer, Waldo et al. 1992), there is digestion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-trisphosphate (IP_3) which increases intracellular Ca^{2+}, and diacylglycerol (DAG) which activates protein kinase C (Takai, Kishimoto et al. 1979).
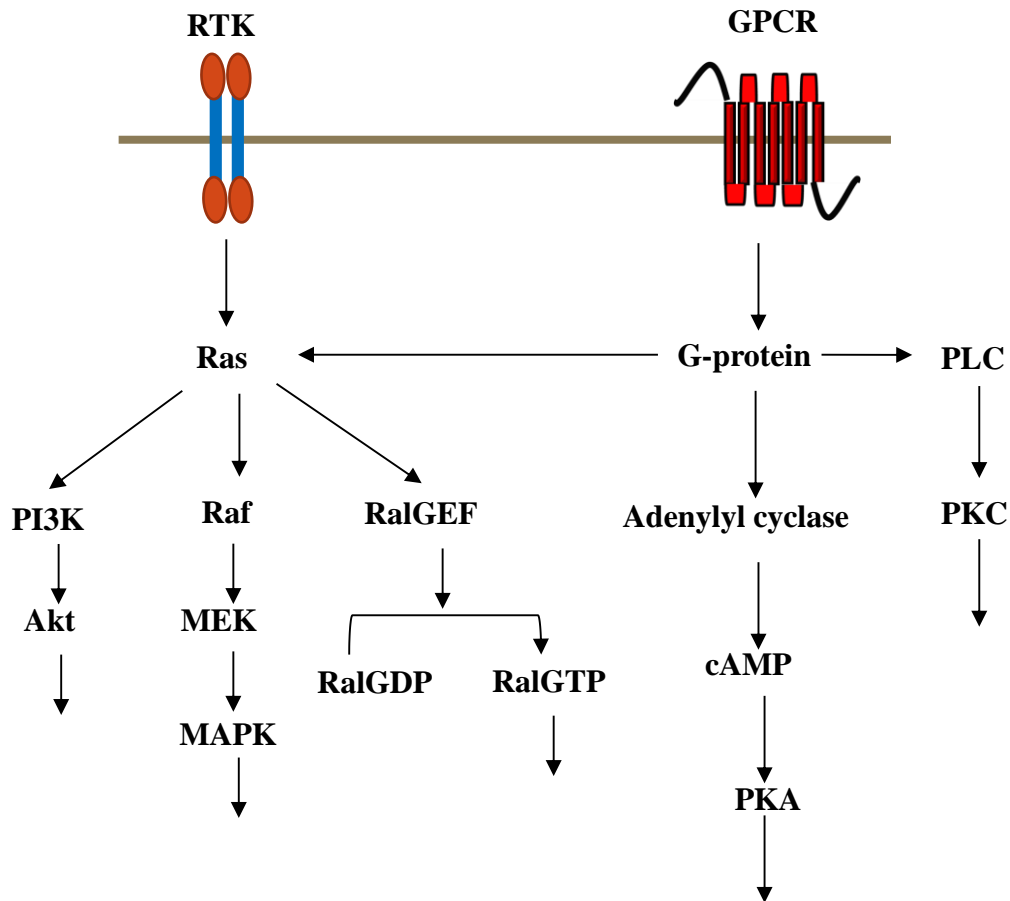
Figure 1.4 Receptor Tyrosine Kinases (RTK) and G-protein Coupled Receptor (GPCR) signal transduction pathways.
Ligand binding results in activation by phosphorylation of tyrosine residues and recruitment of docking proteins. Ras proteins then become activated, resulting in the activation of Ras kinase, which initiates the phosphorylation cascade of MEK and MAPK. The activation of phosphatidylinositol 3 kinase (PI3K) and Ral guanine nucleotide exchange factors (RalGEFs) also mediate MAPK signalling. GPCR activation leads to G-protein dependent activation of phospholipase C (PLC) or cyclic adenosine monophosphate (cAMP). The Ras-Raf-MEK-MAPK/ERK pathway can also be activated by GPCR agonists.

Regulators of G protein signalling (RGS), guanine nucleotide exchange factors (GEF), switch on GPCR activity, while β-arrestin, G protein-coupled receptor kinases (GRK) and GTPase-activating proteins (GAP) all switch off GPCR signalling (Dohlman 2009). The GRKs have kinase activity and phosphorylate the intracellular domain of GPCRs at serine and threonine residues (Ribas, Penela et al. 2007). The phosphorylated residues serve as binding sites for β-arrestin and

disrupt conformation thereby inhibiting the reactivation of the signal transduction pathways (Gurevich and Gurevich 2006). GTPase-activating proteins (GAP) involved in hydrolysis of GTP regain the status of inactive G-protein-GDP complex causing inactivation. The regulation of RGSs is also important, and for instance GPCR activation releases PIP_3, which itself binds to RGS4 GAP and causes temporary inactivation (Ishii, Fujita et al. 2005). Some GPCR regulatory mechanisms also involve PKC and PKA which may activate GRKs stop GPCR signalling (Lefkowitz 1998). The desensitised GPCR may be degraded via clathrin-associated receptor translocation to lysosomes (Hanyaloglu and von Zastrow 2008), but there is also recycling of internalised GPCRs to the cell surface.

GPCR signalling is misdirected in various ways in cancer (Marinissen and Gutkind 2001). For instance, signalling via CXCL12/CXCR4 activates protein kinase B (PKB) associated with increased proliferation and decreased apoptosis in gastric cancer (Yasumoto, Koizumi et al. 2006).

The ligand gated ion channel receptors. These are transmembrane receptors that regulate permeability to various ions such as Ca^{2+}, Na^+, K^+, or Cl^- upon binding of appropriate ligands. The receptor class is named according to its ligand: for example, nicotinic acetylcholine receptors (nAChR), ionotropic glutamate receptors and ATP-gated channels (P2XR). The binding of ligands induces conformational changes in the receptors that allow the movement of ions. Receptor structure varies and for example nAChR is a complex pentamer structure, (ααβγδ), of which (α9)5 is expressed in epithelial and neuronal cells and shown to be overexpressed in cancer cells (Lee, Huang et al. 2010).

Nuclear receptors. The nuclear receptors (NRs) are typically localised in the cytosol in their inactive forms but on stimulation they move to the nucleus where they bind to DNA and change gene expression. (Mangelsdorf, Thummel et al. 1995). The binding of lipophilic ligand to type I NRs activates them causing translocation from cytosol to nucleus and binding at specific hormone response elements (HREs) on DNA (Gronemeyer, Gustafsson et al. 2004). While type II NRs occur in the nucleus in the inactive state associated with the relevant HRE. The activation of type II NRs involves co-regulators (Aranda and Pascual 2001). The inactive state is maintained by a small protein called a co-repressor. Co-repressors directly associate with repressors and indirectly bind to DNA to limit gene transcription. In the presence of ligand the co-repressor is replaced by a co-activator, a protein molecule recruited to DNA along with a transcriptional complex required for gene expression.

Cross talk. Networking amongst different transduction pathways occurs widely in health and disease. Examples include the intracellular activation of signalling pathways by stimulation of neighbouring receptors collectively known as transactivation. Thus GPCR-mediated transactivation of tyrosine kinase receptors has been extensively studied. Transactivation includes ligand-dependent and independent activation of RTK pathways; for instance, prostaglandin E_2 and endothelin act at their GPCRs to transactivate EGFR secondary to cleavage of HB-EGF or TNF-α with subsequent increases in phosphorylation of ERK2 and cell proliferation (Prenzel, Zwick et al. 1999; Pai, Soreghan et al. 2002; Ogunwobi and Beales 2008). Other relevant mechanisms include ligand-independent transactivation, for example GPCR activation of intracellular molecules such as Src and followed by autophosphorylation of EGFR (Hsieh, Sun et al. 2008).

1.10   Chemerin and ChemR23

Chemerin is 18kDa chemokine/adipokine expressed in adipocytes and immune cells e.g. macrophages (Zabel, Zuniga et al. 2006). It is a potent chemoattractant that recruits macrophages, natural killer (NK) and dendritic cells via interacting with a GPCR, chemokine like-receptor 1 (CMLR1) also called chemerin receptor 23 (chemR23) (Zabel, Zuniga et al. 2006; Yoshimura and Oppenheim 2008). Chemerin was originally identified in 3-D co-culture experiments of keratinocytes and fibroblasts from psoriatic skin using a subtraction hybridization approach (Nagpal, Patel et al. 1997).   The ligand was then confirmed to interact with chemR23 and two other putative receptors, G-protein-coupled receptor 1 (GPR1) and chemokine (CC motif) receptor-like 2 (CCRL2) (Nagpal, Patel et al. 1997; Bondue, Wittamer et al. 2011). Chemerin was subsequently isolated from haemofiltrates using HPLC followed by peptide analysis by MALDI-TOF-MS (Meder, Wendland et al. 2003; Wittamer, Franssen et al. 2003). The C-terminal sequence of full length chemerin (163 residues) is proteolytically cleaved to produce the active forms. Cleavage may be mediated by different proteases (Figure. 1.5). Elastase cleaves chemerin 163 between amino acids at positions 5 and 6 from the C-terminus to give active chemerin-157 (Zabel, Allen et al. 2005). Similarly, processing at the C-terminus by cathepsin G produces active chemerin 156 (Zabel, Allen et al. 2005); however cleavage by chymase of chemerin 157 and chemerin 156 leads to the inactive chemerin 154 (Guillabert, Wittamer et al. 2008; Du and Leung 2009).

Various studies have determined chemerin or its transcripts in serum, media or cell extracts using ELISA, Western blot, RT-PCR or gene microarray (Parlee, McNeil et al. 2012). In parallel, chemR23 expression has been studied in various

stromal cells, endothelial, macrophage, NK cells, dendritic cells using gene microarray, fluorescence-activated cell sorting (FACS), immunocytochemistry (ICC) and immunohistochemistry (IHC) (Albanesi, Scarponi et al. 2009; Kaur, Adya et al. 2010; Muruganandan, Parlee et al. 2011).
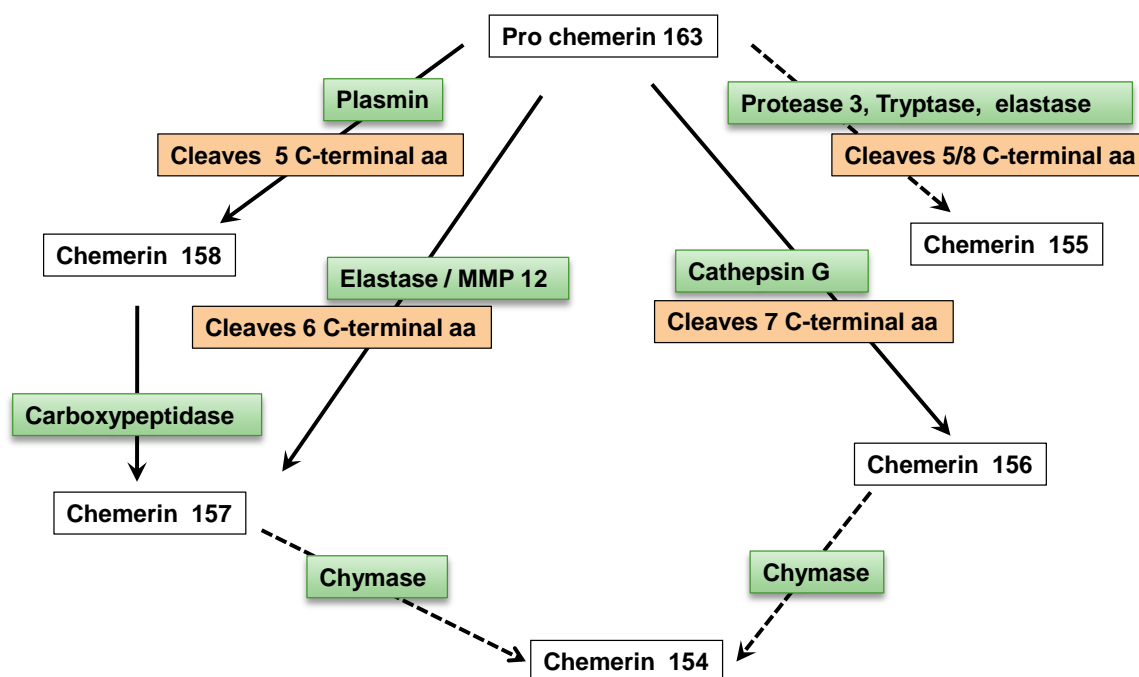
Figure 1.5   Isoforms of chemerin.
Schematic of proteolytic cleavage in the C- terminal region of full length prochemerin 163 and subsequent chemerin derivatives. Solid arrows represent active chemerin, while black dotted arrows represent inactive isoforms of chemerin.

Ligand-receptor interactions have been studied using calcium influx measurement in adipocytes and macrophages (Bondue, Wittamer et al. 2011) and decreased accumulation of cAMP using cell based FLIPR assays (Meder, Wendland et al. 2003; Wittamer, Franssen et al. 2003). Chemerin binding to chemR23 also activates downstream signalling pathways involving p44-42, p38, JNK-II, Akt, PI3K, and NFκβ (Berg, Sveinbjornsson et al. 2010; Hart and Greaves 2010;

Kaneko, Miyabe et al. 2011) (Figure 1.5). Chemerin binding to GPR1 and CCRL-2 does not activate intracellular signalling pathways but may increase the local availability of chemerin to chemR23 expressing cells (Barnea, Strapps et al. 2008; Zabel, Nakae et al. 2008).

Chemerin

ChemR23

PI3K/Akt and MAPKs	ERK1/2 , Ca^{+2} and NF-κB	G2/M cyclins (cyclin A2/B2)	PI3K, Akt, and p38
Endothelial cell survival	Adipocyte and Skeletal muscle cell metabolism	BMSC clonal expansion	Macrophage recruitment and adhesion to ECM
Angiogenesis	Adipogeneis and insulin resistance	Adipogeneis and Differentiation	Inflammation
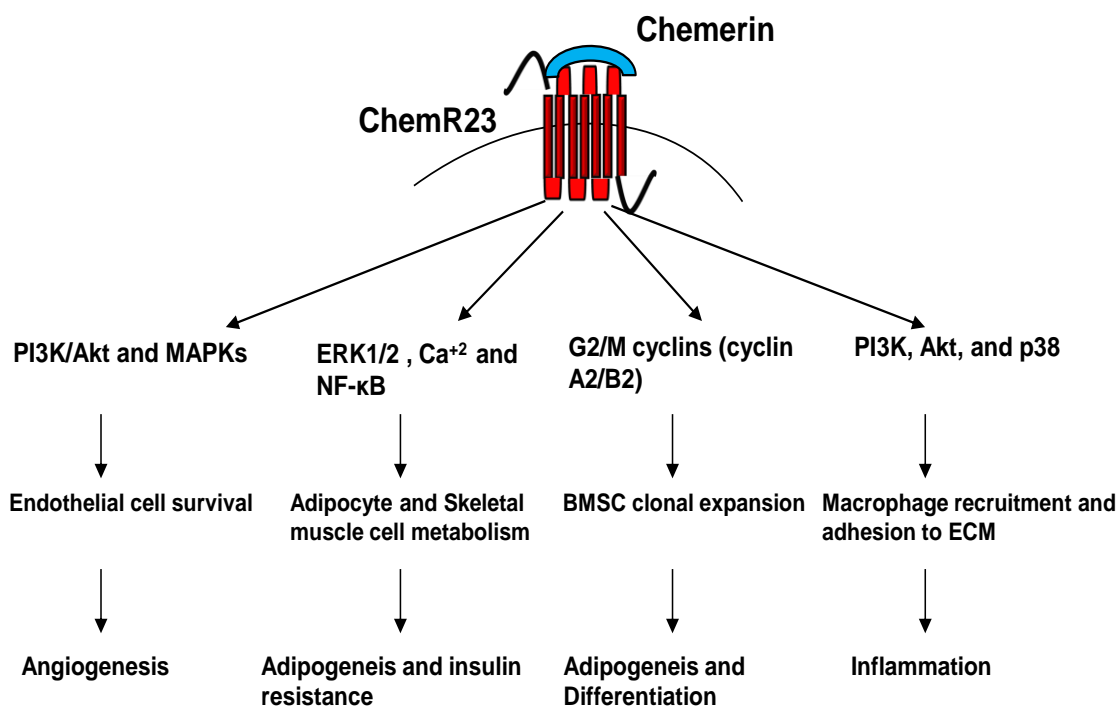
Figure 1.6   Chemerin mediated cellular functions.
Shcematic representation of action of chemerin in activating signal pathways in varied cell types and subsequent cellular funcitons.

Chemerin has been implicated in autocrine and paracrine signalling in maturation and differentiation of adipocytes (Roh, Song et al. 2007). The inflammation mediated by chemerin is associated with infiltration of macrophages and dendritic cells in inflamed tissues. Chemerin also increases lipolysis and insulin signalling in adipocytes (Goralski, McCarthy et al. 2007) and acts on endothelial cell to increase angiogenesis (Kaur, Adya et al. 2010).

The role of chemerin in arthiritis, liver fibrosis and obesity has attracted attention. Chemerin regulation of IL-6 and TNF-α, and vice versa, and also chemerin-stimulated adiponectin, has indicated a role as anti-inflammatory cytokine (Cash, Hart et al. 2008). In contrast, it has been suggested that the ability of chemerin to stimulate TGFβ secretion (Cash, Hart et al. 2008) might be critical in liver fibrosis and chronic inflammation. In obesity, increased chemerin expression in mature adipocytes mediates insulin-dependent glucose uptake and cell metabolism (Takahashi, Takahashi et al. 2008) and chemerin concentrations in blood correlate with body mass index and plasma triglyceride levels (Zabel, Allen et al. 2005). Relatively little is known about possible roles of chemerin in cancer. However, previous findings in our group (Holmberg & Varro, unpublished observations) showed chemerin is increased in oesophageal CAMs from SC compared to ATMs. This raises the possibility of a role in oesophageal cancer.

1.11   Aims :

This investigation focuses on the properties of myofibroblasts in oesophageal cancer and in particular on the mechanisms by which they communicate with cancer cells and with bone marrow derived MSCs. The specific aims were:

1. To characterise myofibroblasts derived from ESCC and EAC (CAMs) and from adjacent tissue (ATMs) and to compare their properties with myofibroblasts from normal donor patients (NTMs).

2. To investigate the role of chemerin in chemotactic responses of MSCs and oesophageal cancer cells and to define the signal transduction pathways.

3. To determine the role of MIF in local modulation of the responses to chemerin.

4. To establish mechanisms of transendothelial migration of MSCs and to validate the use of a chemR23 antagonist

Chapter -2

Materials and Methods

2.1   Materials

Oesophageal cancer cell lines (OE21, OE33 and OE19) were obtained from American type culture collection, ATCC, VA, US. Human mesenchymal stromal cells (MSC; MSC7F3914) and Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza (Cambridge, UK). Cell culture materials namely 0.25 % w/v trypsin-EDTA, Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI)-1640, L-glutamine, non-essential amino acids, antibiotic-antimycotic solution, penicillin-streptomycin solution and phosphate buffered saline (PBS) were obtained from Sigma (Dorset, UK); Recovery™ cell freezing medium came from Invitrogen (Paisley, UK). Fetal bovine serum (FBS) was purchased from Lonza. Cell culture reagents for MSCs and HUVECs i.e MSC Trypsin (0.25% w/v)-EDTA, Mesenchymal Stem Cell Growth Medium bulletkit, (MSCGM, includes basal medium and MSC growth supplement, MSCGS) and Endothelial Growth media (EGM) were purchased from Lonza Transfection kits, Amaxa™ Fibroblasts Nucleofector™ kit and Amaxa™ Human MSC Nucleofector® kit were also purchased from Lonza. Competent *E. coli* cells for plasmid amplification were purchased from Invitrogen. PKH67 membrane labelling reagent was purchased from Sigma. Chemerin silencing RNAs were obtained from Sigma and a chemerin overexpression plasmid from Origene, Rockville, USA. Macrophage migration inhibiting factor (MIF) siRNA was obtained from Invitrogen. Plasmid DNA extraction mini Kit and RNeasy kit were obtained from Qiagen (Manchester, UK). Protease Inhibitor cocktail set III, EDTA-free, phosphatase Inhibitor cocktail set III, EDTA free were obtained from Calbiochem (Darmstadt, Germany) and RIPA buffer was purchased from Cell Signalling (Hitchin, UK). Western blot reagents were obtained from Bio-Rad Laboratories (Hercules, CA, US) namely Immun-Star™ WesternC™ Chemiluminescence Kit,

Lowry DC protein assay, ChemiDoc XRS system and the nitrocellulose membrane came from Amersham Pharmacia (Biotech, Little Chalfont, UK). PageRuler[TM] prestained protein ladder was purchased from Fermentas (York, UK). Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for immunocytochemistry were purchased from Jackson Immunoresearch (PA, USA) and Sigma, respectively. Vectashield with DAPI for nuclear staining was obtained from Vector laboratories (Peterborough, UK). The Click-iT EdU Alexa Fluor 488 Imaging kits were purchased from Invitrogen. Clonogenic reagent, (1, 9-dimethyl-methylene blue) was obtained from Sigma. Chemerin ELISA kits were obtained from Adipo Bioscience (Santa Clara, CA, USA) and MIF ELISA kits from R&D Systems Europe Ltd (Abingdon, UK). Boyden chamber control cell culture inserts and BD BioCoat™ Matrigel™ Invasion Chamber for migration and invasion assays were purchased from SLS (Nottingham, UK). The migration cell fixing and staining kit (DiffQuick) was from Dade Behring Inc (Newark, DE). Drugs including Ro320432, SB202190, SP600125, Uo126, ISO-1 were purchased from Calbiochem and LY294002 was obtained from Cell Signaling. Chemerin-9 was purchased from Genscript (Piscataway, NJ, USA). Chemerin was purchased from R&D Systems Europe Ltd (Abingdon, UK) and IGF-II from Calbiochem (Darmstadt, Germany). ChemoCentryx (Mountain View, CA, USA) provided the ChemR23 antagonist CCX-832 and a control compound (CCX-826). GeneChip©Human Genome U133 plus 2.0 arrays was obtained from Affymetrix (Wycombe, UK).

2.2   Human primary myofibroblasts

Human primary myofibroblasts were obtained from resected oesophagael cancers and adjacent non-cancerous tissue during surgery for removal of tumours

at First Department of Surgery, University of Szeged, Szeged, Hungary. Myofibroblasts were prepared in the Department of Medicine, University of Szeged, Hungary as previously described (McCaig, Duval et al. 2006) and transported to University of Liverpool in liquid nitrogen and cryopreserved until use. Myofibroblasts were derived from four oesophageal squamous cancers and three Barrett's adenocarcinoma, recovered from the oesophagus and oesophagus/cardiac junction. From each patient, CAMs and ATMs were cultured. Also, 6 myofibroblast lines were obtained from the oesophagus of normal patients who were transplant donors (NTMs: 3 males, 3 females, mean average age 48.7 ± 5.8 yrs and 49.3 ± 2.7 yrs respectively). The details of tumour staging (pathology tumour size, lymph nodes, metastasis) and cancer classification are shown in Table 2.1.

2.3 Tissue culture

2.3.1 Human myofibroblasts

Myofibroblasts were maintained in T-75 flasks in DMEM supplemented with 10% v/v FBS, 1% v/v penicillin-streptomycin, 2% v/v antibiotic-antimycotic and 1% v/v non-essential amino acids. This is referred to as "full medium" (FM) unless otherwise stated. Cells were grown at $37^{o}C$ in a 5% v/v CO_2 atmosphere and the medium was changed every 48 h.

For passaging, cells at 80% confluence or above were washed twice with PBS followed by incubation for 5-8 min in 2ml 0.25 % w/v trypsin-EDTA. Trypsinised single cell suspensions were then added to 8ml of FM and $8x10^5$ to 10^6 cells were re-plated in T-75 flasks. Aliquots of 10µl of cell suspensions were used for cell counting using a haemocytometer. The cells used in experiments were obtained from passages 5 to 15.

A

Patient No.	Age	Gender	Survival (months)	Tumour Staging	Tumour Location	Classification	Adjacent Tissue
1	72	M	18	pT3N1M0	oesophagus	Barrett adenocarcinoma	Barrett's
2	64	M	19	pT2N3M0	cardia	Barrett adenocarcinoma	Barrett's
3	70	F	>25	pT3N**1**M1	cardia	Barrett adenocarcinoma	Barrett's
4	56	M	34	pT3N1M0	oesophagus	squamous carcinoma	CIM, PAM (cardia)
5	52	M	31	pT3N1M0	oesophagus	squamous carcinoma	normal
6	49	M	35	pT2N1M0	oesophagus	squamous carcinoma	GERD
7	69	M	14	pT3N1M0	oesophagus	squamous carcinoma	PAM (cardia)

B

Patient No.	Age	Gender	Location
8	44	F	oesophagus
9	45	M	oesophagus
10	52	F	oesophagus
11	60	M	oesophagus
12	52	F	oesophagus
13	41	M	oesophagus

Table 2.1 Characteristics of patients used to provide oesophageal myofibroblasts. A) Age, gender, post-operative survival (>, alive at time of submission), tumour staging, tumour localisation, tumour classification, and pathology assessment of tissue taken adjacent to the tumour for patients used to provide oesophageal CAMs and ATMs. Tumour staging is defined in terms of pathology of tumour (pT) where 0 defines no sign of tumour and 3 maximum size and/or extensions. Similarly, the involvement of local and metastasis to proximal lymph nodes is stated as N0 - N3 and metastasis severity by M0, no metastasis and M, distant metastasis. The gastro-intestinal junction tissue possessing the intestinal metaplasia and metastasis of pancreatic acinar cells are called cardiac intestinal metaplasia (CIM) and pancreatic acinar metaplasia (PAM) respectively in adjacent tissue, last column. B) Myofibroblasts isolated from 6 normal transplant donors tissue, NTMs (3 were males, 3 females mean average age 48.66 ± 5.78 yrs and 49.3 ± 2.66 yrs respectively).

2.3.2 Human mesenchymal stromal cells (MSC)

Mesenchymal stromal cells were maintained in an undifferentiated state in T-75 flasks in MSCGM containing 440ml basal medium and MSC growth supplements. Cells were maintained at 37^oC in 5% v/v CO_2 atmosphere. Every 3-4 days, media was aspirated and replaced with 20ml fresh media. Cultures were split and passaged at 80% confluence or over. Cells were detached with 2ml of trypsin (0.25% w/v)-EDTA for 2-3 min, and $8x10^5$ to 10^6 cells were then added to 8ml FM and re-plated in T-75 flasks. The cells used in experiments were obtained from passages 5 to 12 and maintained their undifferentiated phenotype.

2.3.3 Human Umbilical Vein Endothelial Cells (HUVEC)

Human umbilical vein endothelial cells were maintained in T-25 flasks in EGM medium. Cells were maintained at 37^oC in 5% v/v CO_2 atmosphere. Every 2nd day, media was aspirated and replaced with 10ml fresh media. At 80% confluence cells were trypsinised with 1ml of trypsin (0.25% w/v)-EDTA for 2-3 min, and then suspended in 4ml FM and re-plated in T-25 flasks at a density of 1000-2000 cells/ml. The cells used in experiments were obtained from passages 5 to 9.

2.3.4 Human oesophageal cancer cell lines

Oesophageal squamous cancer (OE21), adenocarcinoma (OE33), and Barrett's oesophagus (OE19) cell lines were cultured in RPMI-1640 supplemented with 10% v/v FBS, 1% v/v penicillin-streptomycin, 2% v/v L-glutamine. Cells were maintained at 37^oC in a 5 % v/v CO_2 atmosphere, and media were changed every 48-72 h. Confluent cells at 80% were washed twice with PBS, trypsinised using

0.25% w/v trypsin-EDTA and 8×10^5 to 10^6 cells added 8ml FM and re-plated in T-75 flasks.

2.3.5   Cryopreservation of cell lines

Cells were trypsinised at 80% confluence, centrifuged at 800 x g for 7 min at $4^{o}C$ and supernatants discarded. Pellets were resuspended in 1 ml recovery cell freezing medium and cell suspensions stored in 1.5 ml cryovials. These vials were placed in a plastic holder in a bath containing propane-1, 2,-diol and then transferred to a -80°C freezer overnight and then to liquid nitrogen for long-term storage.

2.3.6   Recovering frozen cell lines

Cells were removed from liquid nitrogen, thawed by hand or in a water bath at $37^{o}C$ and added to a T-75 culture flask with 19 ml FM and maintained $37^{o}C$ in a 5 % v/v CO_2 atmosphere

2.4   Preparation of condition media

Myofibroblasts (1.5×10^6 cells) were plated in T-75 falcon flasks and maintained at $37^{o}C$ in a 5% v/v CO_2 atmosphere for 24 h in FM. Cultures were then washed 3 times with sterile PBS and incubated in 15ml serum free (SF) media (FBS excluded from FM) for 24 h. Conditioned media were collected in 20 ml Universal tubes and centrifuged for 7 min 800 x g, $4^{o}C$. Aliquots of 5 ml were stored at -$80^{o}C$ until further use.

2.5   Immunocytochemistry

Cells were cultured in T-75 flasks and trypsinised to yield single cell suspensions that were seeded at 10,000 cells per well on cover slips in 24 well plates. Cells were incubated for 24 h and fixed using 4% w/v paraformaldehyde for 30 min at room temperature (RT). Cells were then washed twice with PBS before permeabilisation with 500µl of fresh 0.2% Triton X-100 in PBS (PBS-T) for 30 min at RT. Cells were washed twice with PBS and incubated with 500µl of 5% w/v BSA for 30 min at RT. To prevent non-specific binding of secondary antibody, cells were incubated with 500 µl 10% v/v donkey serum for 1 h at RT. Cells were then washed with PBS twice and incubated with primary antibody diluted in PBS, overnight at 4°C in a humid atmosphere. Next day, the cells were washed with 500µl 0.14M NaCl for 10 min, 0.5M NaCl for 10 min and 0.14M NaCl for 10 min. Secondary antibodies were prepared in HEPES. Slides were incubated with the appropriate secondary antibody for 1 h at RT in the dark. Cover slips were then washed in PBS for 10 min each and transferred to glass slides. Vectashield containing DAPI was added to cover slips that were mounted, sealed with nail varnish and kept overnight in the dark at 4°C. Staining was observed using 40 x magnification using a fluorescence Zeiss Axioplan-2 microscope and Axiovision software V 4.8.0. Images (MicrOptik, Deursen, Netherlands) were captured using a JVC-3 charged coupled device camera with KS300 software. Emissions of fluorescence (FITC) were captured with the XF22 filter (excitation 493 nm, emission 520 nm) and Texas Red was detected using the XF33 filter (excitation 596 nm, emission 620 nm, Omega Optical, Essex, UK). Details of primary and secondary antibodies provided in Table 2.2.

Antibody	Species	Dilution	Supplier
Anti-α smooth muscle actin	Mouse	1:100	Fitzgerald, USA
Anti-vimentin	Guinea pig	1:100	Fitzgerald, USA
Anti-desmin	Rabbit	1:100	Fitzgerald, USA
Anti-chemR23	Rabbit	1:100	Millipore, UK
Anti-GPCR GPR1	Rabbit	1:250	Abcam, UK
Anti-CD105	Mouse	1:50	Bayport, USA
FITC-conjugated donkey-anti-mouse	Donkey	1:400	Jackson, USA
Texas-Red-conjugated donkey-anti-guinea pig	Donkey	1:400	Jackson, USA
FITC-conjugated donkey-anti-rabbit	Donkey	1:400	Jackson, USA

Table 2.2 Antibodies and their working dilution used in immunocytochemistry.

2.6   Bioassays

2.6.1   Cell migration assays

2.6.1.1 Boyden chamber-chemotactic migration assays

Migration assays were applied to myofibroblasts, cancer cells and MSC following the manufacturer's instructions. Cells were seeded in 500µl SF media on perforated polyethylene terepthalate (PET) membranes with 8.0 µm pores; the inserts were placed in wells containing 750µl of appropriate media. Cells were obtained by trypsinising 70% confluent cultures and resuspension in 5 ml of medium with 0.1% FBS. A 10 µL aliquot of the cell suspension was counted in a haemocytometer. Stimulants and inhibitors, conditioned media, cell types, seeding density and incubation times are described in the relevant sections.

After incubation, migrated cells on the underside of the insert were stained with DiffQuick according to manufacturer's instructions. Cells on the upper side of the membrane were removed by scrubbing with a cotton bud and those on the lower side of the membrane were fixed, stained and counted under 10 x magnifications using a Zeiss 25 Axiovert Microscope (Carl Zeiss Microscopy, NY, USA). Cells were counted in five fields on a single membrane and means were calculated.

2.6.1.2 In *vitro* scratch wound healing assays

Cells were plated at 80% confluence in 6-well plates and incubated in 2ml FM until monolayers of cells were achieved. A single scratch was created across the monolayer in each well using a p2 pipette tip perpendicular to the surface and through the centre of the well. Experiments were performed in duplicates or

triplicates. Cells migrating across the wound were determined in one or two fields per well using time-lapse microscopy with Hamamatsu Orca ER Camera and Kinetic Imaging AQM-2001 software (Hamamatsu, Town NJ, USA).

2.6.1.3 Transendothelial migration assays

Mesenchymal stromal cells were labelled with 1μM PKH67 per 10^7 cells following the manufacturer's protocol. Transendothelial migration assays were applied to PKH67 labelled MSCs using BD BioCoat™ Matrigel™ Invasion Chambers according to manufacturer's instructions. Endothelial cells, HUVEC were trypsinised and seeded at 10^6 cells per insert and topped up with 500 μl of EGM. The HUVEC cells were incubated for 48 hr at 37^oC in a 5 % v/v CO_2 atmosphere to allow the formation of a monolayer. Subsequently the procedure used to study MSC migration was performed as described in section 2.6.1.1. Mesenchymal stromal cells were counted with green fluorescence for PKH67 for five fields on a single membrane and means were calculated.

2.6.2 Cell proliferation assays

2.6.2.1 EdU labelling

Proliferating cells were labelled with 5-ethynyl-2´-deoxyuridine (EdU) and detected using the Click-iT™ EdU Alexa Fluor 488 Imaging kit according to the manufacturer's instructions. In 24 well plates, 10,000 cells/well were cultured on coverslips in 1ml FM for 24 h. Cells were washed twice with PBS and serum starved for 24 h to synchronize the cells at G0/G1 of the cell cycle. Fresh SF media (1ml) was added with 10μM EdU and stimuli or inhibitors for 24 h. Cultured cells were fixed in 4% PFA in PBS for 30 min at RT, then washed three times with

PBS followed by PBT (0.5% Triton X-100 in PBS) for 20 min at room temperature. Cells were incubated for 30 min with 3% 500µl of BSA. The Click-iT™ reaction cocktail was prepared freshly just before use by mixing 430 µL of reaction Click-iT buffer, 20µL of 100 mM copper sulphate (CuSO4), 1.2µL of Alexa Fluor dye, and 50µL of reaction buffer additive solution in a final volume of 500 µL (sufficient for one well). Cells were washed three times with 3% 500µl of BSA and incubated with Click-iT for 30 min in the dark. Cover slips were then washed three times in PBS for 10 min each and transferred to a glass slide. Vectashield containing DAPI was added to the cover slips which was then mounted, and sealed with nail varnish, and kept overnight in the dark at 4^oC until counting at 10x magnification using a Zeiss Axioplan-2 microscope. Alexa Fluor 488 light emission was captured with the XF22 filter (excitation 495 nm, emission 519 nm) and Axiovision software V4.8. Images were captured using a JVC-3 charged coupled device camera with KS300 software.

2.7   Gene expression arrays

Myofibroblasts and MSCs were cultured as described in section 2.3.1 and 2.3.2 respectively and RNA extracted by Dr. Islay Steele using the RNeasy kit. Similarly, the RNA was extracted from cultured oesophageal cancer cells, (OE21, OE19 and OE33) according to manufacturer's instruction. Samples were analysed using GeneChip©Human Genome U133 Plus 2.0 arrays at the Liverpool Genome Facility by Dr. Lucille Rainbow, according to the manufacturer's instruction. The GeneChip©Human Genome U133 Plus 2.0 array measures the expression of 47,000 transcripts. GeneChip® Scanner 3000 enabled high-resolution scanning of the arrays. To provide stringent microarray assessments confirming reproducibility and avoiding the experimental artifacts, Microarray Suite 5.0 QC Metrics (MAQC) was used as a quality control. Further, the data were extracted using the software GeneSpring GX V.10. Data were normalised with a MAS5 algorithm and filtered using "flags present (P)", "marginal (M)", and "absent (A)". Statistical analysis was performed using student paired t-test, unpaired t-test and ANOVA within the GeneSpring software. For large datasets of differential gene expression (DE), the percentage of false positives among defined significant results, ie False Discovery Rate (FDR), was used. The expression of individual genes was reported relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.8   Western Blots

2.8.1   Preparation of whole cell lysates

Cells were cultured in 10 cm Petri dishes at a seeding density of 10^6 cells and incubated for 24 h in FM. Cells were then washed three times with PBS and fresh 10 ml SF media containing stimuli or inhibitors was added and incubated for 24 h. Media was then removed and 10ml sterile PBS added to the cells on ice. Cells were washed twice with 10ml PBS and recovered by 100µl RIPA buffer (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 Protease Inhibitor cocktail set III, EDTA-free and 1% v/v Phosphatase Inhibitor cocktail set III, EDTA free. Cell suspensions were pipetted into clean labelled tubes, sonicated for 5 min and incubated on ice for 30 min. The extracts were then centrifuged at 12000 x g at 4^oC for 30 min and supernatants stored at -80°C.

2.8.2   Protein quantification

To ensure equal loading in Western blots, protein concentrations were quantified using modified Lowry DC protein assays. Bovine serum albumin standard (2 µg/ml-0.125 µg/ml) was serially diluted in PBS and 5 µL was added in triplicate. Similarly, triplicates of 5 µL of sample diluted in RIPA lysis buffer (1:10) were added followed by the addition of 25 µL reagent A and 200 µL of reagent B. The samples were incubated for 30 min and absorbance was measured at 450 nm.

2.8.3   SDS-poly-acrylamide gel electrophoresis

Proteins were resolved by discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE). A separating gel of 10% or 15% w/v SDS-poly-acrylamide was

layered with H_2O-saturated iso-butyl alcohol. The chamber and gels were assembled as described in the manufacturer's protocol. Each gel was loaded with 5 μL of PageRuler[TM] Plus prestained protein ladder to provide molecular weight standards and to monitor electro-blotting. Cell extracts were denatured by pre-heating for 5 min at $100^{o}C$ with 5x loading buffer. Gels were run at a constant current of 100V for 1 h and were blotted once the bromophenol blue reached the lower end of the gel.

2.8.3.1 Electrophoretic transfer

Proteins were electotransferred onto nitrocellulose at a constant voltage of 100 V for 1 h at RT. Nirtocellulose membranes were then rinsed in Tris-buffered saline containing 0.1% Tween (TBS-T) three times for 10 min. Membranes were blocked for at least 1 h at RT with blocking buffer (TBS-T containing 5% W/V Marvel milk powder). Primary antibody diluted in 5 ml blocking buffer was applied to membranes overnight at $4^{o}C$. On the next day, membranes were washed four times in TBS-T and horseradish peroxidase (HRP)-conjugated secondary antibodies were applied for at least 1 h. Membranes were exposed using the Immun-Star™ WesternC™ Chemiluminescence Kit.

2.8.3.2 Densitometry evaluation of band intensity

Protein bands were quantified from captured blot images using a ChemiDoc XRS system (Bio-Rad, Hemel Hempstead, UK). The intensity of individual bands was quantified with the BioRad software version 2.3.1. The samples were normalised to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) unless otherwise stated.

Antibody	Species	Dilution	Source
GAPDH	Mouse	1:1000	Meridian Life Science, USA
Chemerin	Goat	1:250	R&D Systems, UK
MIF	Goat	1:200	R&D Systems, UK
Phospho- p44/42 MAPK	Rabbit	1:2000	CellSignal, USA
Phospho-p38 MAPK	Rabbit	1:1000	CellSignal, USA
Phospho- SPAK/JNK-II	Rabbit	1:1000	CellSignal, USA
Total p44/42 MAPK	Rabbit	1:2000	CellSignal, USA
Total p38 MAPK	Rabbit	1:2000	CellSignal, USA
Total SAPK/JNK-II	Rabbit	1:2000	CellSignal, USA
Anti-Mouse	Goat	1:20000	Sigma, UK
Anti-Goat	Rabbit	1:10000	Sigma, UK
Anti-Rabbit	Goat	1:10000	Sigma, UK
Anti-Rabbit	Goat	1:2000	Sigma, UK

Table 2.3 Primary antibodies and secondary antibodies and their dilutions used for Western blot analysis.

2.9   Sandwich - enzyme linked immunosorbent assay (ELISA)

Media samples were collected as mentioned in section 2.4 and were stored at -80°C until use. Chemerin and MIF concentrations in media were measured using commercially available Chemerin ELISA Kits and Human MIF Immunoassays respectively.

2.10   Amplification of plasmid DNA

Chemically competent *E. coli* were used to amplify plasmids expressing DNAs of interest. A 50 µL aliquot of competent *E. coli* cells was thawed on ice, mixed with 100 ng of plasmid DNA and then incubated for 30 min on ice, followed by 30 sec heat shock at 37°C and 2 min incubation on ice to induce uptake of DNA. The cells were allowed to recover by the addition of 250 µL of Luria broth (LB) medium in a shaking incubator at 37°C for 1 h. Two aliquots of the suspension (20 µL and 100 µL) were then plated onto pre-warmed (37°C) LB agar plates containing 100 µg/ml ampicillin. The plates were observed the next day after overnight incubation at 37°C for the antibiotic-resistant colonies. A single colony isolated from a fresh LB plate was used to inoculate 4 ml of LB medium containing 100 µg/ml ampicillin, the cells were then incubated at 37°C in a shaking incubator at 225 rpm for 4-8 h. Bacterial cultures were collected by centrifugation (6000 rpm for 5 min) and processed for plasmid DNA extraction using Qiagen Plasmid Mini Kit. DNA concentrations were measured by absorbance at A260, and the ratio of A260/A280 was used as an indicator of purity. When long-term storage of clones was needed, glycerol was added to an

aliquot of culture at a final concentration of 15% v/v, and the stocks were stored in required aliquots at -20°C. A small aliquot was kept at 4°C for usage till 6 months

2.11   Transient transfection of myofibroblasts and MSCs

Cells were cultured as described in section 2.3 and maintained at low passage number (<9) and 85% confluence before transient transfection. Amaxa™ Fibroblasts Nucleofector™ kits and Amaxa™ Human MSC Nucleofector® kits were used according to manufacturer's instructions. Each transfection employed 2 µg of DNA (5 x 10^5 cells) and 100 µL of complete nucleofector solution along with media. The required number of cells was centrifuged at 200 x g for 10 min and the supernatant was discarded. The pellet was resuspended at RT Human MSC Nucleofector Solution to a final concentration of 5 x10^5 cells/100µL. Transfection was achieved by using the program U-23 (for high transfection efficiency) by adding 500 µL of the pre-warmed culture full medium to the cuvette and using the plastic pipette (provided in kits) and transfer sample to the T-75 with 20 ml of freshly prepared FM.

2.12   Statistics

The final results were calculated as mean ± standard error of means (SEM). Student t-test and ANOVA were performed on the data as appropriate with significance at p≤0.05, unless otherwise stated. Experiments with standards included the assay efficacy, reliability, and calculation of unknown samples which were based on coefficient of regression, R^2 and line equation, y= mx + c (where m= slope of line and c is the intercept at y-axis)

Chapter 3

Morphological and functional characterisation of oesophageal myofibroblasts

3.1   Introduction

Myofibroblasts are often described as activated fibroblasts and are observed in the stroma of many, probably all, solid cancers. The isolation of myofibroblasts from tumours and their characterisation by morphology and function has been widely applied to studies of breast cancer, prostate cancer and gastric cancer (Ronnov-Jessen, Petersen et al. 1995; Tuxhorn, Ayala et al. 2002; McCaig, Duval et al. 2006; Holmberg, Quante et al. 2012). In oesophageal cancer, some studies have showed that fibroblasts influence tumour growth and development (Noma, Smalley et al. 2008; Grugan, Miller et al. 2010). However, in general little is known of how myofibroblasts obtained from oesophageal cancer (CAMs) differ from those obtained from neighbouring tissue (ATMs) or normal tissue (NTMs).

Myofibroblasts share some markers, e.g. αSMA, vimentin with other stromal cells notably smooth muscle cells (Sappino, Schurch et al. 1990; Meran and Steadman 2011). Some studies have therefore used a panel of positive (αSMA, fibroblast activated protein, fibroblasts secreted protein), and negative (pan cytokeratin, desmin) markers to distinguish myofibroblasts from epithelial cells and other stromal cells (Spaeth, Dembinski et al. 2009; Kidd, Spaeth et al. 2012). However, αSMA has been used in virtually every study characterising myofibroblasts and increased αSMA abundance is considered to be one of the strongest signatures of myofibroblasts.

Recent studies of gastric myofibroblasts have shown clear differences between CAMs and ATMs with respect to proliferation and migration (Holmberg, Quante et al. 2012). To our knowledge, so far there has been no comparable study of CAMs and ATMs in oesophageal SC or AC. However, Zhang *et al.*, 2009 compared

oesophageal fibroblasts from cancer and non-cancer tissue and confirmed increased proliferation using MTT assays. In the present study we used two different proliferation assays (EdU and clonogenic) and two different migrations assays (Boyden chamber and wound healing) to characterise oesophageal SC and AC CAMs and ATMs, and NTMs, and studied the responses of cancer cell lines (SC, OE 21 and AC, OE19 and OE33) to CM from these cells.

3.1.1 Objectives

Specific objectives were:

1. To characterise CAMs and ATMs from SC and AC, and to compare with myofibroblasts from normal transplant donors (NTMs).

2. To determine the effect of conditioned media from oesophageal CAMs, ATMs and NTMs on cancer cell proliferation and migration.

3.2   Material and Methods

3.2.1   Cell culture

Oesophageal SC and AC myofibroblasts (both CAMs and ATMs), and oesophageal NTMs, were cultured as described in sections 2.3, and 2.3.1. Oesophageal cancer cell lines (OE19, OE21, and OE33 cells) were cultured as described in section 2.3.3.

3.2.2   Conditioned media

Conditioned media were prepared from myofibroblasts as described in section 2.4.

3.2.3   Immunocytochemistry

Myofibroblasts were seeded at a density of 10,000 cells/well in 24 well plates on cover slips (see section 2.3.1) and probed for each marker. Approximately 500-800 cells were counted for each marker and images were taken as described in section 2.5.

3.2.4   EdU labelling

To determine differences in proliferation between CAMs, ATMs and NTMs, cells were seeded at a density of 10,000 cells/well in 24 well plates on cover slips. The effect of myofibroblast CM was determined on OE19, OE21 and OE33 cells using EdU labelling as described in section 2.7.1. Myofibroblasts labelled with EdU were counted in 5-10 fields and cancer cells in 5 fields (100-200 cells/well). Images were taken as described in section 2.7.1. Data are presented as mean proliferating cells in 5 fields for cancer cells and 10 fields for myofibroblasts per

well; experiments were performed in triplicate and data expressed as mean ± S.E.M; significance was determined by ANOVA, $p < 0.05$.

3.2.5   Boyden chamber chemotactic migration assays

Chemotactic migration assays were performed using Boyden chambers as described in section 2.6.1.1. Inserts were seeded with 25,000 OE21 or OE33 cells in 500µl 0.1% FBS in appropriate media. The bottom well contained 750µl CM from CAMs, ATMs or NTMs. The incubation time was 24 h. Cell staining and counting was performed as described in section 2.6.1.1. Data are presented as mean cells migrating in 5 fields/well; experiments were performed in triplicate and data expressed as mean ± S.E.M; significance was determined by student t-test or ANOVA as appropriate, $p < 0.05$.

3.2.6   Scratch wound assays

Wound healing assays were performed in 6 well plates. Cells were allowed to form monolayers and subsequent steps were performed as described in section 2.6.1.2. OE21 and OE33 cells were incubated for 8 h and 10 h respectively with 2 ml myofibroblast CM after creating wound edges. Data analysis and presentation were performed as described in section 2.6.1.2. Thus, the distance between wound edges was measured at 5 equally spaced positions in each field and presented as mean percentage of wound healing/well; experiments were performed in triplicate and data expressed as means ± S.E.M; significance was determined by student t-test or ANOVA as appropriate, $p < 0.05$.

3.3   Results

3.3.1   Oesophageal myofibroblasts express α-SMA and vimentin

The primary oesophageal tumours employed in this study exhibited increased numbers of myofibroblasts typically with disordered architecture and morphology compared to adjacent tissue or normal tissue. They also stained with α-SMA and vimentin and they were negative for the smooth muscle and pericyte marker desmin (Appendix, Figure 1; Data obtained by Dr Tiszlavicz, Szeged, Hungary). Initially, cultured myofibroblasts were examined for the expression of two characteristic markers, namely α-SMA and vimentin. Expression of α-SMA and vimentin was found in both SC and AC CAMs and ATMs, and in NTMs (Figure 3.1 A, B). Distinct intermediate filaments were visible with vimentin staining while with α-SMA there was a network of immunopositive structures that were clustered within the plasma membrane (Figure 3.1 A). The majority (99%) of myofibroblasts were positive for α-SMA and vimentin, with no structural differences between CAMs, ATMs or NTMs. All oesophageal CAMs, ATMs, and NTMs were negative for desmin markers for pericytes.

3.3.2   CAMs show increased proliferation compared to ATMs

Myofibroblast characterization was then extended to include a search for functional differences between SC and AC CAMs, ATMs and NTMs. The incorporation of EdU was used to measure the proportion of CAM, ATM and NTM cultures that were entering S-phase of the cell cycle (Figure 3.2 A). In individual pairs of SC (n=4) and AC (n=3) cells, CAMs showed greater proliferation than their ATM counterparts (Figure 3.2 B & C). Taken together, the mean data reveal

significantly greater numbers of CAMs in S-phase compared to ATMs or NTMs (Figure 3.2 D & E).

3.3.3 Optimisation of functional assays for oesophageal cancer cell lines, OE21, OE33 and OE19

In order to study the effect of CM on oesophageal cancer cells it was first necessary to optimize assays of proliferation and migration for these cells. EdU incorporation assays showed full media significantly increased labeling of OE21, OE19 and OE33 cells by approximately 30% compared to SF conditions (Figure 3.3 A & B). In wound healing assays, there was significantly increased migration of OE21 and OE33 cells in response to FM compared to SF conditions (Figure 3.3 C). OE21 and OE33 cells both migrated as a layer of cells (Figure 3.3 D). There was poor adhesion of OE19 cells after wounding and serum starvation and so these cells were not included in wound healing assays.
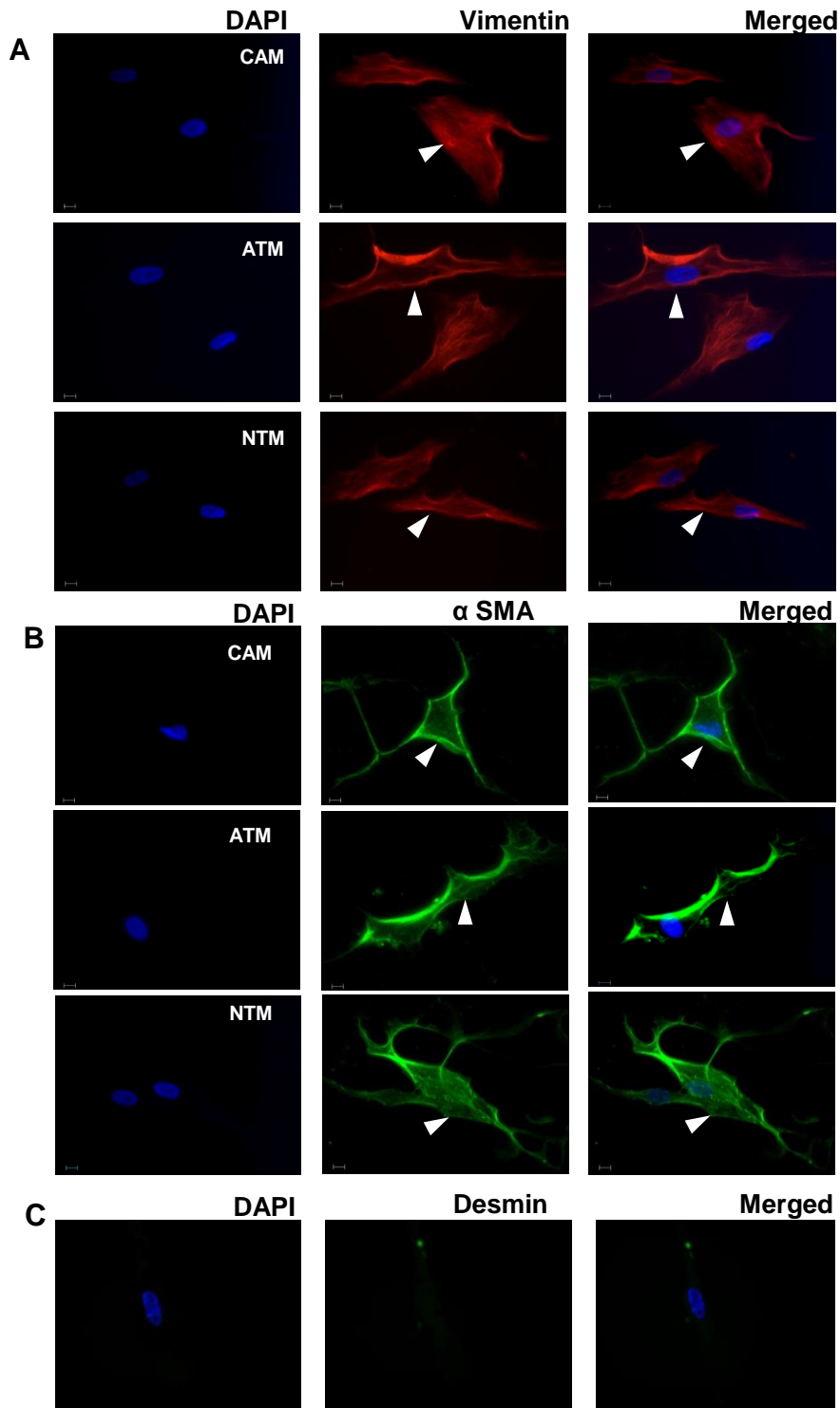
Figure 3.1 Characterisation of cultured oesophageal myofibroblasts. A) Positive vimentin (red) staining of cultured oesophageal myofibroblasts (arrow) from squamous cancer and normal oesophageal tissue (nuclear staining with DAPI, blue); top, CAMs; middle, ATMs; lower, NTMs. B) Positive α-SMA (green) staining in cultured oesophageal myofibroblasts (arrow) from squamous cancer and normal oesophageal tissue (nuclear staining with DAPI, blue); top, CAMs; middle, ATMs; lower, NTMs. C) Negative desmin staining in cultures oesophageal CAMs. Scale bar: 20 µm.
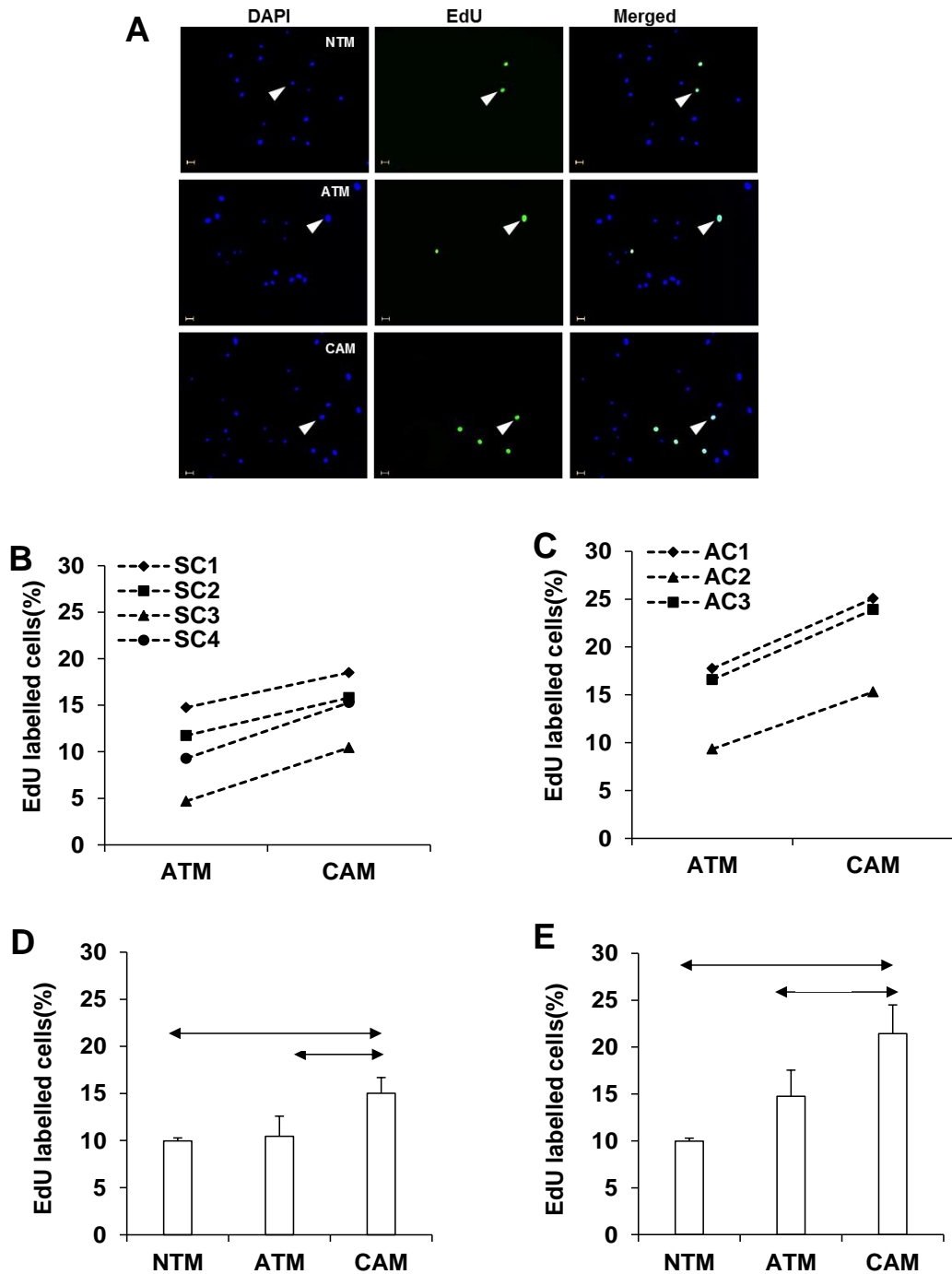
Figure 3.2 Increased proliferation of cancer associated myofibroblasts. A) Representative data from EdU proliferation assays. Staining of CAM, ATM, and NTM nuclei with DAPI (blue, first column, arrow), EdU (green; middle, arrow) and merged (third column, arrow). B) Individual pair-wise comparison of EdU labelling in CAMs versus ATMs in 4 pairs of SC cells. C) Individual pair-wise comparison of EdU labelling in CAMs versus paired ATMs in 3 pairs of AC cells. D) Group mean of data in panel B; CAMs (n=4), ATMs (n=4) and NTMs (n=6). E) Group mean of data in panel C; CAMs (n=3), ATMs (n=3) and NTMs (n=6). Horizontal arrows mean $p<0.05$ by ANOVA; Bars, SEM; Scale bar= 20μM.
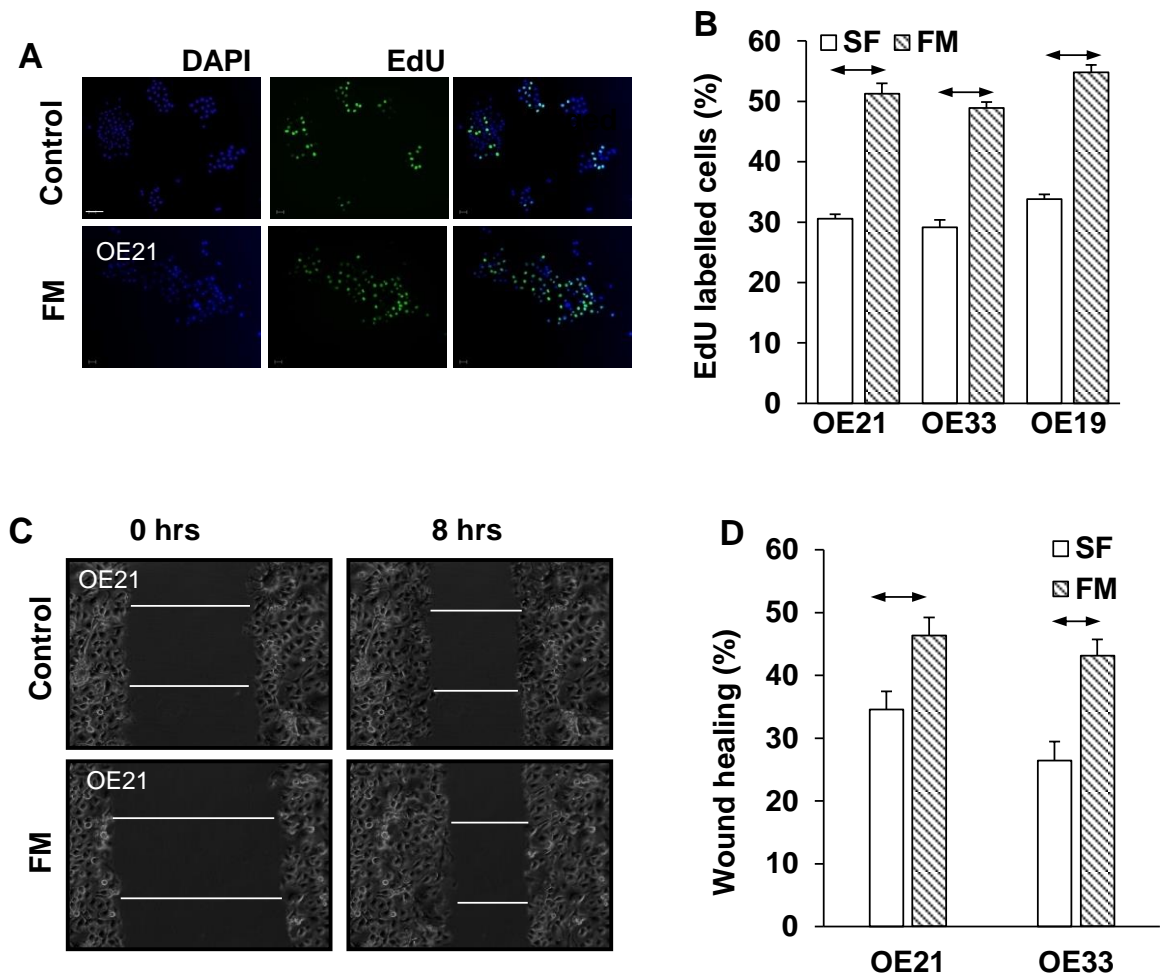
Figure 3.3 Optimisation of EdU incorporation, and wound healing assays for OE19, OE21 and OE33 cells. A) Representative EdU incorporation assays. Staining of OE21 nuclei with DAPI (blue, first column), EdU (green; middle) and merged images (third column) of cells treated with serum free media (SF, control) and full media (FM). B) Mean EdU labeling of OE19, OE 21 and OE33 cells (10,000 cells/well, 2cm^2) 24 h in SF or FM. C) Wound healing assays of OE21 and OE33 cells, scored at 8 h and 10 h when the closure of the wounds was approximately 50%. D) Mean wound healing of OE 21 and OE33 cells 8 h in SF or FM. Horizontal arrows mean $p<0.05$ by ANOVA; Bars, SEM; Scale bar- 20µM.

3.3.4   Enhanced proliferation of OE21 and OE33 cells in response to CM from CAMs

EdU incorporation was then used to measure the proliferation of OE21 and OE33 cells in response to myofibroblast CM. In individual pair comparisons, CM from all 4 SC CAMs (SC1- SC4) stimulated OE21 cell proliferation and CM from all 3 AC CAMs (AC1- AC3) stimulated OE33 cell proliferation compared to the relevant ATM CM (Figure 3.4 A & B). The group mean increase in OE21 cell proliferation in response to CAM CM was significant for SC (n=4) compared to either ATMs or NTMs (n=6) (Figure 3.4 C). However, CAM CM stimulated OE33 cell proliferation was significantly greater compared to NTM CM (n=6) but not ATM CM (Figure 3.4 D). In addition, the increase in OE21 and OE33 cell proliferation was significant in response to CM from SC ATM and AC ATM respectively, compared to control (Figure 3.4 C & D).
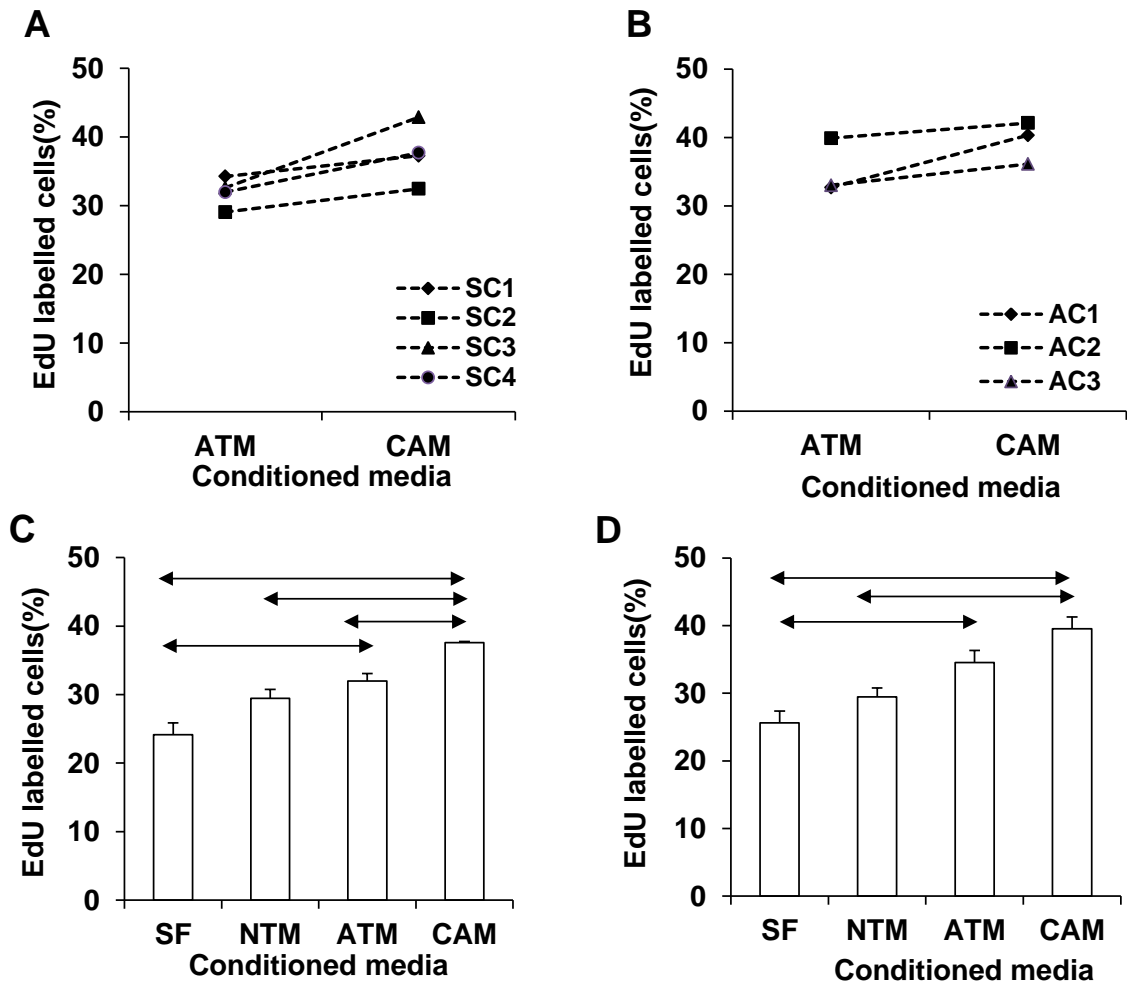
Figure 3.4 Increased proliferation of OE21 and OE33 cells treated with CM from CAMs. A) EdU labelling of OE21 cells treated with CM from SC CAMs compared to the corresponding ATM CM. B) EdU labelling of OE33 cells treated with CM from AC CAMs compared to the corresponding ATM CM. C) Group mean data for EdU labelling of OE21 cells treated with CM from SC CAMs (n=4) compared to ATM CM (n=4) and NTM CM (n=6). D) Group mean data for EdU labeling of OE33 cells treated with CM from AC CAMs (n=3) compared to ATM CM (n=3). Horizontal arrows mean $p<0.05$ by ANOVA; Bars, SEM.

3.3.5 Wound healing assays show CAM CM induces greater OE21 and OE33 cell migration

In individual pairwise comparisons, CM from SC CAMs increased OE21 cell migration compared to CM from corresponding ATMs (Figure 3.5 A). Similar results were obtained for OE33 cells in response to CM from AC CAMs compared with ATMs (n=3) (Figure 3.5 B). The group mean data showed significant increases in OE21 cell migration in response to CM from SC CAMs compared to the paired ATMs, or NTMs (Figure 3.5 C). Increase OE33 cell migration was also seen in response to CM from AC CAMs compared to the corresponding ATM CM. In addition, a significant increase in OE33 cell wound healing was seen in response to CM from AC ATM compared to control (Figure 3.5 D).

3.3.6 CAM CM induces OE21 and OE33 chemotaxis

Finally, studies of cell migration were extended to include a different type of assay namely chemotaxis in Boyden chambers. In individual paired comparisons, OE21 cells treated with CM (in the lower chamber) from SC CAMs showed enhanced migration compared to CM from the corresponding ATMs (Figure 3.6 C). Similar results were obtained for OE33 cells in response to CM from AC CAMs compared with ATMs (Figure 3.6 D). The group mean data showed statistically significant increases in OE21 and OE33 cell migration in response to CM from CAMs compared to ATMs or NTMs in both, SC and AC group (Figure 3.6 E & F). In addition, a significant increase in OE21 and OE33 cell migration was seen in response to CM from SC ATMs and AC ATMs, respectively, compared to control (Figure 3.6 E & F).
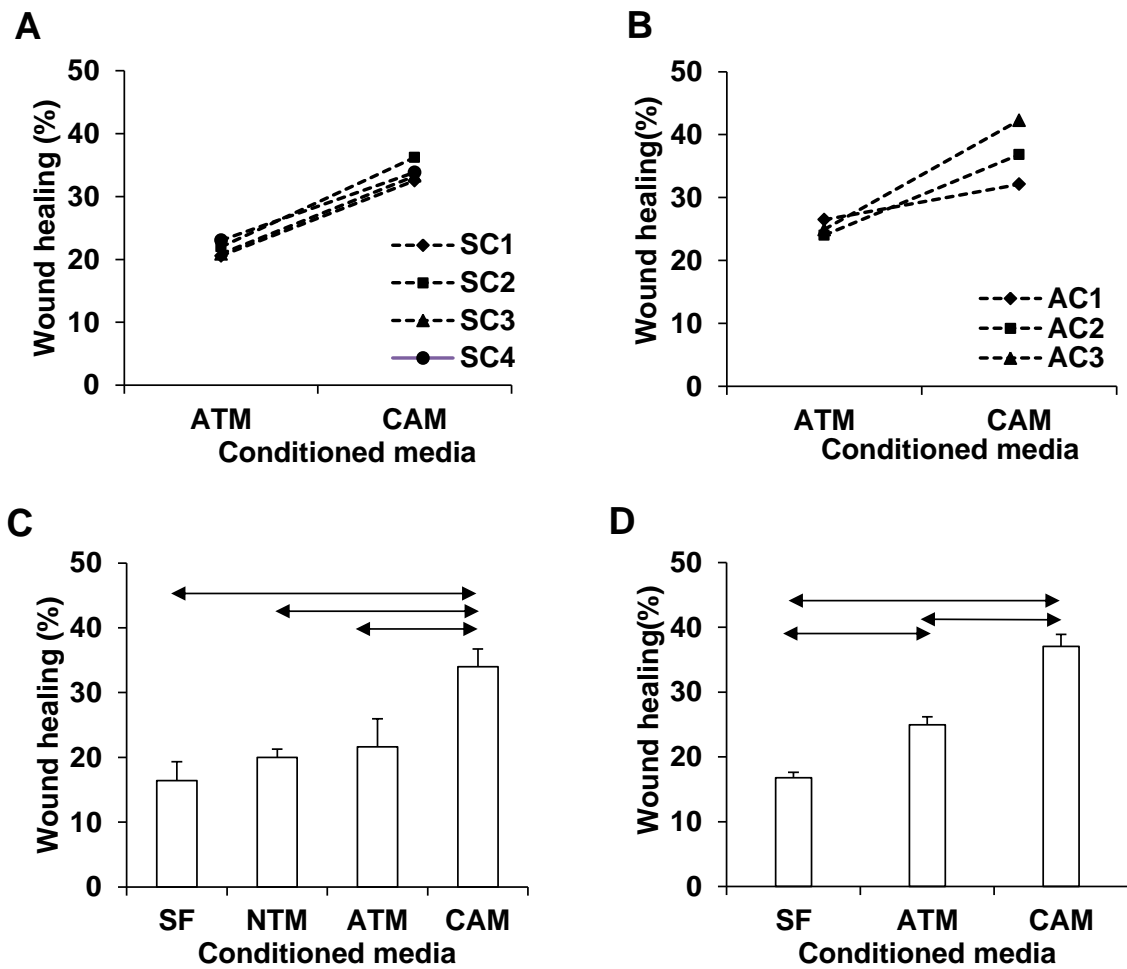
Figure 3.5 Increased migration in wound healing assays of OE21 and OE33 cells treated with CM from CAMs. A) Wound healing responses of OE21 cells treated with CM from SC CAMs versus corresponding ATMs. B) Wound healing responses of OE33 cells treated with CM from AC CAMs versus corresponding ATM CM. C) Group mean wound healing data for OE21 cells treated with CM from SC CAMs (n=4), ATMs (n=4) and normal tissue myofibroblasts, NTMs (n=4). D) Group mean wound healing data for OE33 cells treated with CM from AC CAMs (n=3) and ATMs (n=3). Horizontal arrows mean p<0.05 by ANOVA; Bars, SEM.

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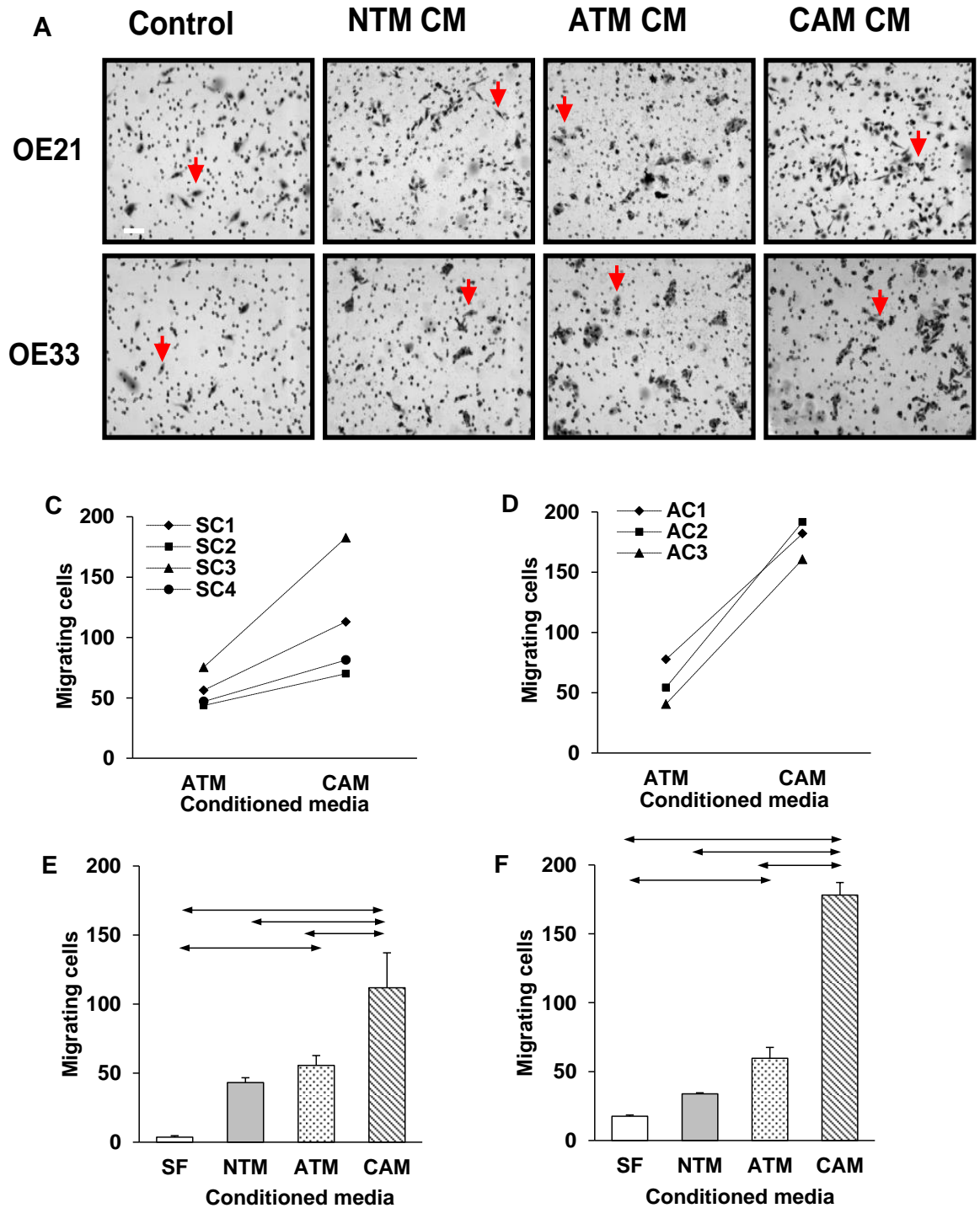
Figure 3.6 Enhanced chemotaxis of OE21 and OE33 cells in response to CM from CAMs. A) Representative Boyden chamber migration assays of OE21 cells treated with CM from NTMs, ATMs, and CAMs (red arrow). B) Representative Boyden chamber migration assays of OE33 cells treated with CM from NTMs, ATMs, and CAMs (red arrow). C) Boyden chamber migration assays of OE21 cells treated with CM from SC CAMs and corresponding ATMs. D) Group mean data of OE21 cell migration in response to CM from SC CAMs (n=4), ATMs (n=4) and NTMs (n=4). E) Boyden chamber migration assays of OE33 cells in response to CM from AC CAMs and corresponding ATMs. F) Group mean data of OE33 migration responses to CM from AC CAMs (n=3), ATMs (n=3) and NTMs (n=2). Horizontal arrows mean $p < 0.05$ by ANOVA; Bars, SEM.

3.4   Discussion

The main finding of these studies is that conditioned media from myofibroblasts increases the proliferation and migration of oesophageal squamous cancer i.e. OE21 cells and Barrett's adenocarcinoma i.e. OE33, cells. The myofibroblasts used for these studies were characterised by positive staining for α-SMA and vimentin expression. In the case of both types of oesophageal cancer, the results indicate clear functional differences in proliferation *in vitro* between CAMs compared with ATMs or NTMs. Importantly, the functional differences also include an increased capacity for CM from SC and AC CAMs to stimulate the proliferation and migration of OE21 and OE33 cells compared to CM from ATMs or NTMs. Expression of α-SMA and vimentin are widely used as markers for myofibroblasts. In particular increased expression of α-SMA occurs when fibroblasts are transformed to myofibroblasts and is a marker for this transformation (Hinz, Celetta et al. 2001). The present data are therefore consistent with observations that myofibroblasts or CAFs from various other cancers such as lung, breast and pancreatic cancer also express α-SMA and vimentin (Serini and Gabbiani 1999; Micke and Ostman 2004; Surowiak, Suchocki et al. 2006; Brentnall, Lai et al. 2012). However, expression of α-SMA also occurs in smooth muscle cells, and possible contamination of pericytes and epithelial cells might conceivably occur in myofibroblast cultures. This was tackled by showing positive co-staining for vimentin and negative staining for desmin and pan-cytokeratin thereby confirming that the myofibroblast cultures differ from smooth muscle cells pericytes or epithelial cells.

Functional studies showed clear differences between CAMs versus ATMs or NTMs. In particular, SC and AC CAMs proliferate at greater rates than ATMs or

NTMs as indicated by EdU incorporation (which measures cells in S-phase). It would have been interesting to measure the apoptosis in myofibroblasts although previous studies have suggested no difference in cell death between gastric CAMs, ATMs and NTMs (Holmberg, Quante et al. 2012). Moreover, other work in the group has found no difference in apoptosis using cleaved caspase assays in these myofibroblasts (Holmberg, Quante et al. 2012). The present data are nevertheless consistent with previous studies using cell growth assays such as MTT (3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide) which show increased biomass of tumour fibroblasts, and flow cytometry to measure cells in the S and G_2-M phases of the cell cycle which has confirmed increased tumour fibroblast proliferation compared with non-tumorous fibroblasts derived from oesophageal SC (Zhang, Fu et al. 2009).

In view of the potential importance of myofibroblasts in regulating the tumour microenvironment and promoting cancer cell growth, the effect of myofibroblast CM was then studied on several different oesophageal cancer cell lines. For these studies CM from SC and AC myofibroblasts were applied to an SC cell line (OE21) or to AC cell lines (OE33 and OE19), respectively. Myofibroblast CM stimulated proliferation and migration of the cancer cell lines, and CAM CM was more potent than ATM CM. Proliferation was measured as EdU incorporation into DNA of dividing cells. Unlike BrdU incorporation assays, EdU incorporation assays are rapid to perform and do not require DNA denaturation; in principle though, the two assay both measure cells in S-phase. In previous studies, oesophageal cancer cell proliferation has been measured by MTT and XTT (sodium3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate incorporation (Liu, Jamieson et al. 2005; O'Donovan, O'Sullivan et al. 2011). These assays measure tetrazolium salt

reduction in metabolically more active cells by mitochondrial dehydrogenase. Although convenient, they do not therefore measure cell proliferation directly. Moreover, the tetrazolium salt in the presence of PMS (phenozine methosulfate) is toxic and can result in unreliable assays. It is conceivable that increased proliferation could be accompanied by increased apoptosis in which case EdU incorporation might not indicate an increase in cell numbers. In response to CM from SC and AC CAMs, OE21 and OE33 cells exhibited both increased EdU incorporation and greater clonal expansion compared with ATM CM. Previously, other models have been used to study stromal-cancer cell interactions; for example co-culture studies suggest that direct contact of breast cancer cells, UACC-81 and MCF7 with fibroblasts is essential to induce cancer cells proliferation or migration (Maeda, Alexander et al. 2004; Samoszuk, Tan et al. 2005). Even so, studies using CM from myofibroblasts in CFU and Boyden chamber migration assays have shown enhanced cancer cell proliferation and migration, respectively, in human breast cancer and in gastric cancer (Gioni, Karampinas et al. 2008; Mi, Bhattacharya et al. 2011; Holmberg, Quante et al. 2012). Hence, the data from multiple models supports the idea of functional roles for myofibroblasts driving cancer cell proliferation.

The present study examined migration of cancer cells using two different assays: wound healing and transwell Boyden chamber assay. In wound healing assays the ability of cells to migrate in an open field is measured; this type of assay is suitable for cells that migrate as a sheet ie without loss of cell-cell contact. Boyden chamber assays also determine migration but in this case in response to a gradient of growth factor; they therefore measure chemotaxis. Moreover these assays are suitable for cells that migrate individually ie exhibit EMT. In response to CM from oesophageal SC and AC CAMs, both OE21 and OE33 cells showed

enhanced migration compared with CM from ATMs or NTMs. The migration of cancer cells in response to myofibroblast CM has previously been determined in several other studies, for example Holmberg et al., showed increased AGS cell migration in response to CM from gastric cancer CAMs and ATMs(Holmberg, Quante et al. 2012).

In conclusion oesophageal myofibroblasts in culture express α-SMA and vimentin markers, which are important markers of their differentiation. However, there are important functional differences between CAMs, ATMs and NTMs: CAMs proliferate more than ATMs and NTMs. In addition CM from CAMs consistently induces more proliferation and migration of OE21 and OE33 cells compared to CM from paired ATMs, or from NTMs. One obvious mechanism to explain these observations would be the increased abundance of paracrine factors in CAM CM. In subsequent chapters, therefore, this possibility will be explored in order to define myofibroblast secreted factor(s) involved in stimulating oesophageal cancer cell proliferation or migration.

3.5   Conclusion

1. CAMs, ATMs, and NTMs exhibit a myofibroblast phenotype.

2. CAMs are more proliferative than ATMs in both SC and AC.

3. Conditioned media from SC and AC CAMs increased proliferation and migration of OE21 and OE33 cell, respectively, more than CM from ATMs, or NTMs.

Chapter-4

Chemerin and Mesenchymal Stromal Cell migration

4.1   Introduction

Chemerin is a comparatively new protein that has emerged as a modulator of cancer growth. Its potential to recruit stromal cells, e.g. macrophages and dendritic cells has been studied in obesity (Landgraf, Friebe et al. 2012), insulin resistance (Takahashi, Takahashi et al. 2008) and inflammatory diseases (Landgraf, Friebe et al. 2012). In addition, recent findings have shown increased chemerin concentrations in ascites fluid resulting from ovarian and liver cancer (Wittamer, Franssen et al. 2003) although no functional relevance was attached to the observation. Other studies have shown increased chemerin in association with a good prognosis for non-small cell lung cancer (NSCLC) (Shen Zhao 2011), and suppression of tumorigenesis in melanoma (Pachynski, Zabel et al. 2012). Nevertheless, little attention has been given to chemerin interactions with its cognate receptors, chemR23 and GPR1, or to the activation of signalling cascades, in cancer. To our knowledge, so far nothing has been published relating chemerin to oesophageal cancer.

Proteomic studies using iTRAQ analysis to compare the secretome of SC CAMs and ATMs have been performed by Dr C Holmberg (Holmberg 2009) The results revealed chemerin to be an upregulated chemokine in the media of CAMs relative to paired ATMs in all 4 SC patients examined (see Appendix 2). In the present study these observations were validated and the effect of chemerin on MSC, OE21 and OE33 cell migration was investigated, together with the role of the two putative chemerin receptors: chemR23 and GPR1.

4.1.1 Objectives

The specific objectives were:

1. To validate the expression and secretion of chemerin by myofibroblasts.

2. To investigate the effect of chemerin on MSC, OE21 and OE33 cell proliferation and migration.

3. To study ligand–receptor interactions and dissect the transduction pathways mediating chemerin-stimulated MSC migration.

4. To identify targets of chemerin expressed by MSCs.

4.2   Material and Methods

4.2.1   Cell culture and condition media preparation

Oesophageal SC and AC myofibroblasts (CAMs and ATMs), and oesophageal NTMs, were cultured as described in section 2.3.1. Conditioned media were prepared as described in section 2.4. Oesophageal cancer cell lines (OE19, OE21 and OE33 cells) and MSCs were cultured as described in sections 2.3.2 and 2.3.3 respectively.

4.2.2   Immunocytochemistry

Expression of chemR23 and GPR1 in MSC was confirmed using ICC as described in section 2.5. Approximately 500-800 cells were counted in each case and images were taken as described in section 2.5.

4.2.3   EdU labelling

Proliferation was determined by EdU labelling as described in section 2.7.1. Cells were incubated in 1 ml SF media containing human recombinant chemerin at 100ng/ml for 24 h. Labelled cells were counted in 5-10 fields (approximately 100-200 cells/well) in triplicate. Images were taken as described in section 2.3. Data are represented as mean % EdU-labelled cells ± S.E.M, and significance determined by student t-test, or ANOVA as appropriate and considered significant at $p < 0.05$.

4.2.4   Boyden chamber- chemotactic migration assays

Chemotactic migration assays were performed as described in section 2.6.1. Inserts were seeded with 10,000 cells in 500µl SF and the bottom well contained

either 750µl CM or SF media containing chemerin (4 - 100ng/ml) or other treatment, as appropriate. The effect of CM from myofibroblasts overexpressing chemerin or in which chemerin had been knocked-down was studied on MSC and OE21 cell migration as described in section 2.6.1.1. The effect of chemerin receptor (chemR23 and GPR1) knock down in MSCs was studied in response to CM or chemerin. Inhibitors of MAPK (SP600125, 50µM; UO126, 10µM; SB202190, 3µM), PKC (Ro320432, 2µM) and PI3K (LY294002, 50µM), were used. Cell staining and counting were performed as described in section 2.6.1.2. Data are represented as mean of cells migrating in 5 fields/well in triplicate ± S.E.M and significance was determined by student t-test or ANOVA as appropriate and considered significant at $p < 0.05$.

4.2.5 Scratch wound assays

The effect of chemerin (20ng/ml, 100ng/ml) on OE21, OE33 and MSC migration in scratch wound assays was studied in 6 well plates as described in 2.6.1.2.

4.2.6 Western blot analysis

Western blot analysis was used to validate expression of chemerin in myofibroblasts after overexpression and knockdown and to determine activation of MAPK pathways after chemerin and PMA stimulation, as described in section 2.9.

4.2.7 Gene Expression analysis

Transcript profiles of myofibroblasts, MSCs and OE21 cells were analysed using GeneChip©Human Genome U133 Plus 2.0 arrays as described in section 2.8.

4.2.8 Sandwich - enzyme linked immunosorbent assay (ELISA)

Chemerin concentrations in media from myofibroblasts, MSCs and oesophageal cancer cell lines were determined by ELISA as described in section 2.10.

4.2.9   Transient transfection of myofibroblasts and MSCs

Squamous cancer CAMs and ATMs were transfected with a chemerin overexpression plasmid (3µg) or with 3 different silencing RNAs (siRNA) (3µM) as described in section 2.11 and 2.12.

4.3   Results

4.3.1   Gene arrays indicate reciprocal expression of chemerin and chemR23 transcripts in CAMs and MSCs

In initial studies, the relative abundance of chemerin and chemR23 transcripts was examined using existing microarray datasets from oesophageal CAMs, ATMs, MSCs and cancer cell lines. The abundance of chemerin transcripts relative to GAPDH was highest in CAMs and ATMs (both SC and AC), followed by MSCs, OE19, OE21 and OE33 cells. A comparison between CAMs and ATMs from SC or AC showed no difference in chemerin transcript abundance. Similarly the abundance of chemerin transcripts, relative to GAPDH, in NTMs resembled that in CAMs and ATMs and was higher than in MSCs and cancer cells (Figure 4.1 A).

The abundance of chemR23 transcripts relative to GAPDH was significantly higher in MSCs than in myofibroblasts or cancer cells (Figure 4.1 A). There was no difference in chemR23 transcript abundance in the different types of myofibroblast. A microarray analysis was also performed for the other putative chemerin receptor, GPR1. The abundance of GPR1 transcripts relative to GAPDH was higher in MSCs than in CAMs, ATMs (either SC or AC), NTMs or cancer cells. There was no difference of GPR1 transcripts between CAMs and ATMs in AC, but in SC there was higher abundance in ATMs than CAMs.
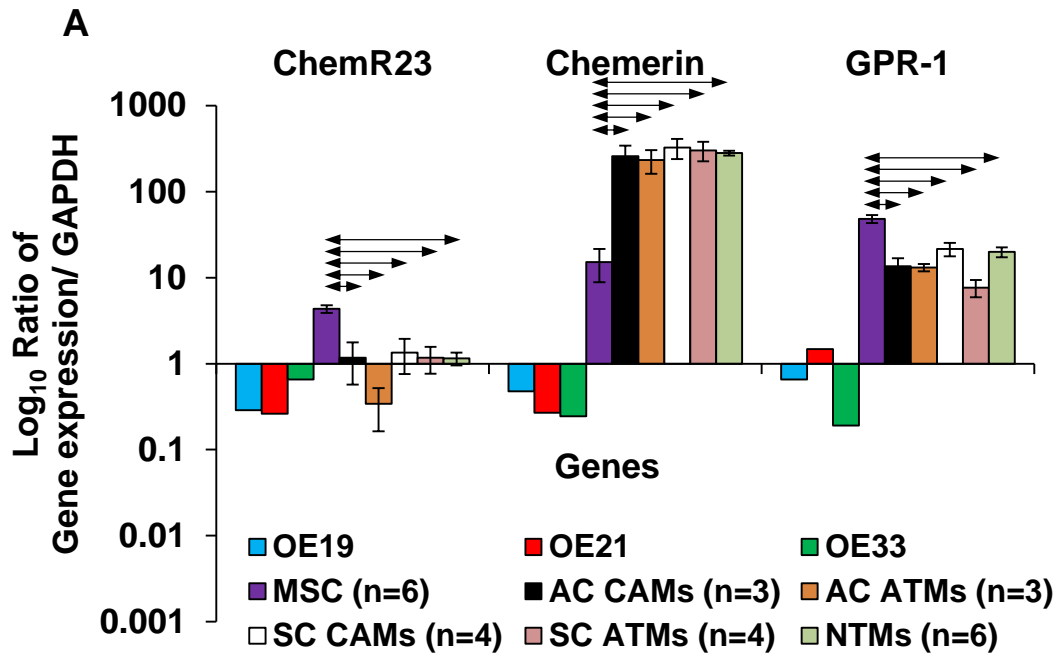
Figure 4.1 Chemerin expression is higher in myofibroblasts while that of its putative receptors, chemR23 and GPR1, is higher in MSCs. Transcript abundance of chemerin, chemR23 and GPR1 relative to GAPDH in OE19, OE21, OE33, MSCs, and different groups of myofibroblasts (CAMs, ATMs, NTMs). Horizontal arrows mean $p < 0.05$ by ANOVA; vertical bars, SEM.

4.3.2   Increased chemerin expression in CAMs

Given the microarray data, it was then considered particularly important to validate the observation by proteomic analysis of increased chemerin in CAMs using Western blot and ELISA. Western blot analysis of media and cell extracts from SC CAMs and ATMs showed greater expression of chemerin in CAMs than in ATMs (Figure 4.2 A and B). In contrast, cell extracts from MSC and OE21 cells showed either no, or barely, detectable expression of chemerin (Figure 4.2 C). The group mean data for densitometry analysis Western blots of SC cell extracts and media confirmed significantly increased chemerin expression in CAMs compared to ATMs (Figure 4.2 D and E).

To further validate the findings, individual pairwise comparisons were then made of chemerin expression measured by ELISA of media from SC and AC CAMs compared to ATMs (Fig 4.3 A & C). The range of chemerin concentrations in CAM media was 200 to 3250 pg/ml compared with 90 pg/ml to 1450 pg/ml in ATMs. The group mean chemerin concentration in CAM media was significantly higher than in their paired ATMs for both SC and AC (Figure 4.3 B & D).

4.3.3   Chemerin receptor, chemR23 and GPR1, expression in MSC

Since the gene array data suggested chemR23 and GPR1 were relatively well expressed in MSCs compared to myofibroblasts and cancer cells the expression of these was then validated by immunocytochemistry. Specific antibodies for each receptor showed strong chemR23 and GPR1 expression in MSCs (Figure 4.2 F). Approximately 90-95% of MSCs were positive for chemR23 and GPR1 suggesting most cells expressed both receptors. Myofibroblasts and cancer cells showed weak chemR23 and GPR1 expression.
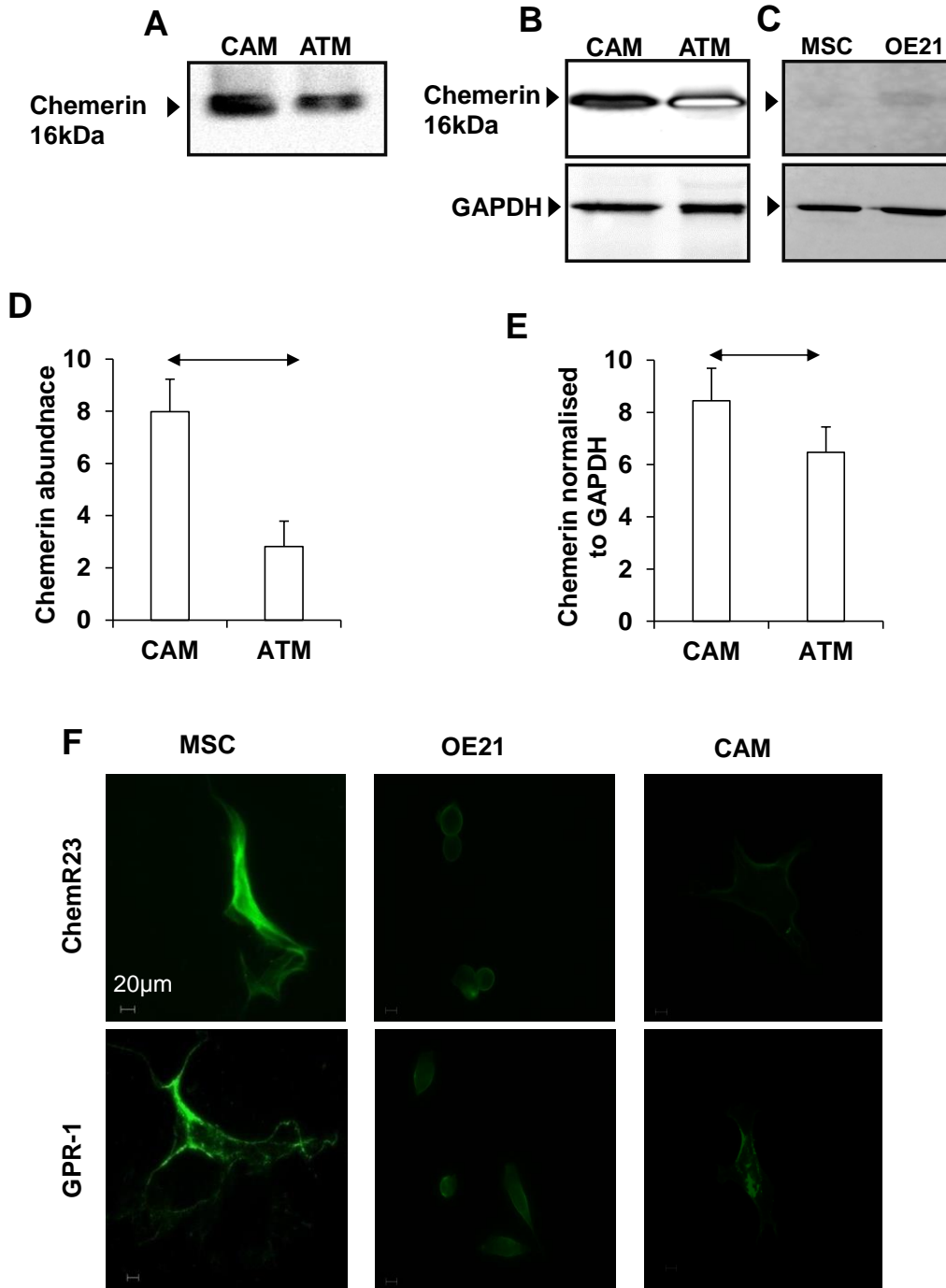
Figure 4.2 Increased chemerin expression in CAMs and ChemR23 receptor expression in MSCs. A) Western blots analysis of chemerin in media from SC CAMs and ATMs, and B) cell extracts. C) Western blots of chemerin in MSC and OE21 cell extracts. D) Quantitative analysis of chemerin abundance in SC CAM and ATM media by densitometry (n=4), and E) cell extracts (n=4). F) Immunohistochemical staining of ChemR23, and GPR1 in MSCs. Horizontal arrows p<0.05 by t-test; vertical bars, SEM; scale bar, 20µM; magnification, 40x.
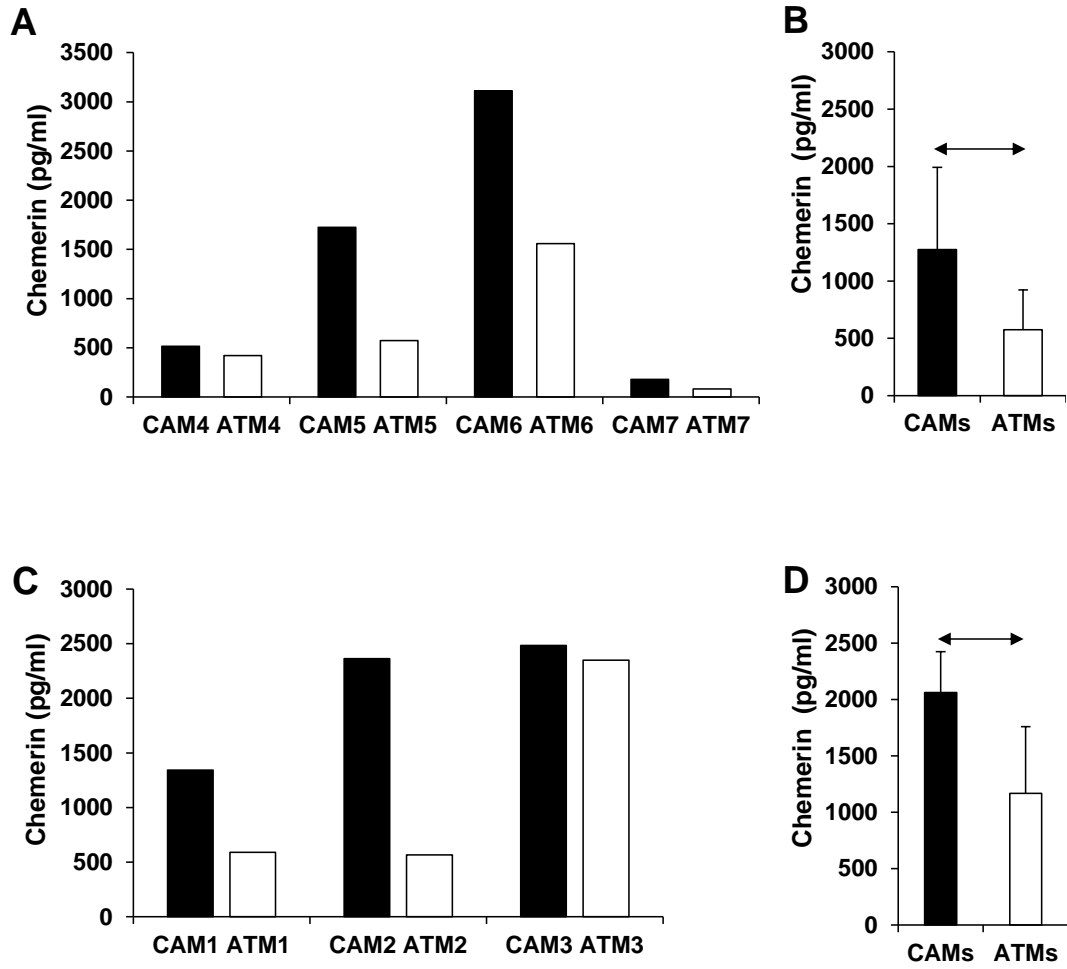
Figure 4.3 ELISA indicates increased chemerin in media of CAMs compared to ATMs. A) Chemerin concentrations (pg/ml) in individual pairs of CAMs (filled bars) and ATMs (open bars) from SC. B) Mean chemerin concentration from SC CAMs and ATMs (n=4). C,D) Similar data for AC (n=3). Horizontal arrows, $p < 0.05$, paired t- test; vertical bars, SEM.

4.3.4   Chemerin increased EdU incorporation

Next, the role of chemerin was investigated using human recombinant chemerin in cell proliferation assays, by EdU. EdU incorporation assays showed increased proliferation in MSC and OE21 cells in response to 100ng/m chemerin (Figure 4.4 B & C).

4.3.5   Chemerin in CM from CAMs stimulated MSC proliferation

The role of chemerin released from CAMs was then studied by examining the effect of CM from SC myofibroblasts, and from NTMs, on MSC proliferation. Thus CM from CAM6 significantly increased MSC proliferation compared to CM from the corresponding ATM or from NTMs (Figure 4.4 D). The stimulatory effect of CAM CM on MSC proliferation was inhibited using chemerin neutralising antibody (Figure 4.4 E).

4.3.6   Recombinant chemerin enhanced MSC, OE21, OE33 and myofibroblast chemotactic cell migration

To study the effect of chemerin in chemotactic cell migration, Boyden chamber assays were performed. Chemerin increased MSC, OE33 and OE21 cell migration in these assays (Figure 4.5 A, B, C, D, & F). It also increased CAM and ATM migration significantly compared to SF media (Figure 4.5 G & H).
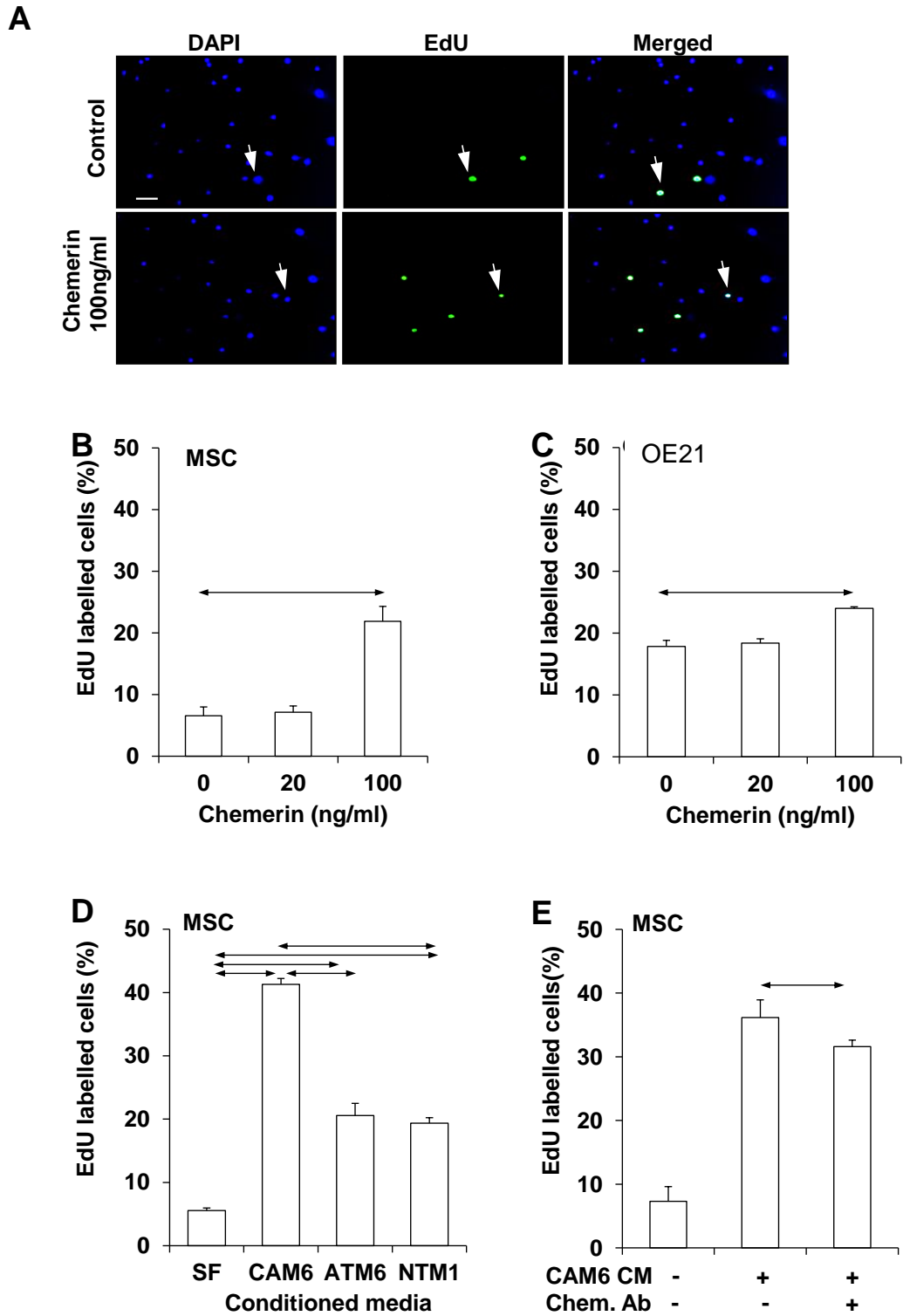
Figure 4.4 Increased proliferation of MSCs in response to chemerin. A) Representative image of EdU staining (arrow) of MSCs in response to chemerin, 100 ng/ml. B) Dose dependent increase in EdU labelling of MSCs in response to chemerin. C) Similar data for EdU labelling of OE21 cells. D) Increased EdU labelling of MSCs in response to CM from CAMs, ATMs and NTMs. E) Chemerin immune-neutralisation (Chem Ab) significantly decreased EdU labelling of MSCs treated with CM from CAM6. Horizontal arrows, $p<0.05$, ANOVA; vertical bars, SEM.
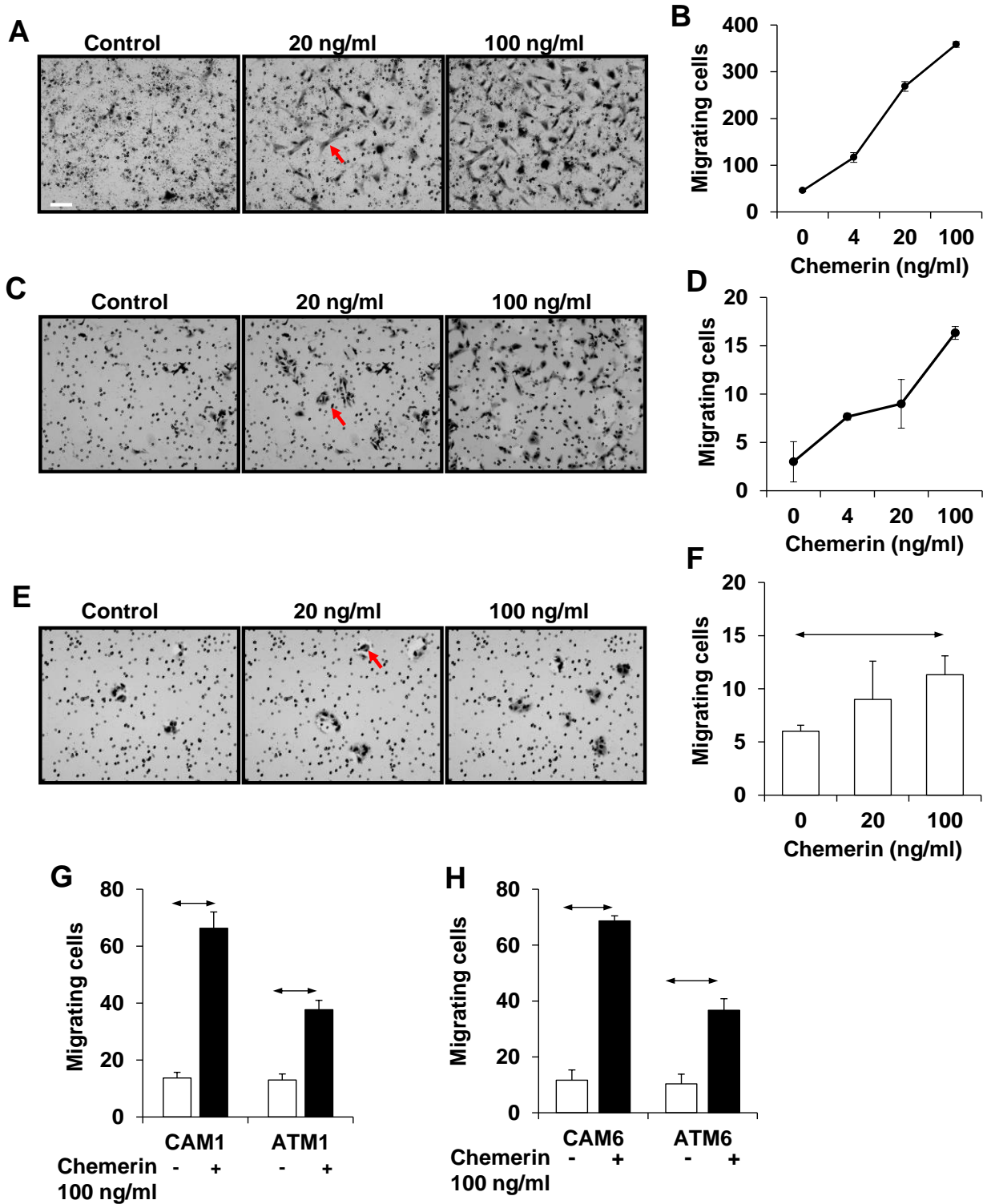
Figure 4.5 Chemerin stimulation of cell migration in chemotaxis assays. A) Representative image of migrating MSCs (red arrow). B) Dose-dependent MSC migration in response to chemerin. C) Representative image of OE21 migrating cells (red arrow). D) Dose-dependent OE21 migration in response to chemerin. E) Representative image of OE33 migrating cells (red arrow). F) OE33 cells migrated in response to dose dependent stimulation by chemerin. G) Chemerin (100 ng/ml) stimulated CAM1 and ATM1 and H) CAM6 and ATM6 migration (n=3). Horizontal arrows means $p < 0.05$, t- test; vertical bars, SEM. Scale bar, 20 μm

4.3.7 Recombinant chemerin increased MSC, OE33 and OE21 cell migration in wound healing assays

As a final step in characterising the biological properties of chemerin, its effects in a different cell migration assay, namely scratch wound healing, was studied. In these assays MSCs, OE33 and OE21 cells exhibited significantly more migration in response to chemerin at doses of 20 and 100 ng/ml compared to SF controls (Figure 4.6 A-F).

4.3.8 Conditioned media from CAMs enhances MSC and OE21 cell migration

To study whether chemerin produced by myofibroblast increases MSC migration studies were performed using CM and a chemerin neutralising antibody. In Boyden chamber assays, CM from SC CAMs and ATMs increased MSC migration. Individual pairwise comparisons showed MSCs migrated more in response to CM from CAMs than the corresponding ATMs (Figure 4.7 A). The group mean data also showed CAM CM significantly increased MSC migration compared to either ATM or NTM CM (Figure 4.7 B). The effect of chemerin (100 ng/ml) on MSC and OE21 cell migration was inhibited by a neutralising antibody (Figure 4.7 C & D). Moreover, the specificity of chemerin neutralising antibody was showed by no effect on IGF-II stimulated migration (100 ng/ml) (Figure 4.7 C). Importantly, MSC and OE21 cell migration in response to CM from SC CAMs was significantly inhibited by chemerin neutralising antibody (Figure 4.8 E & F).
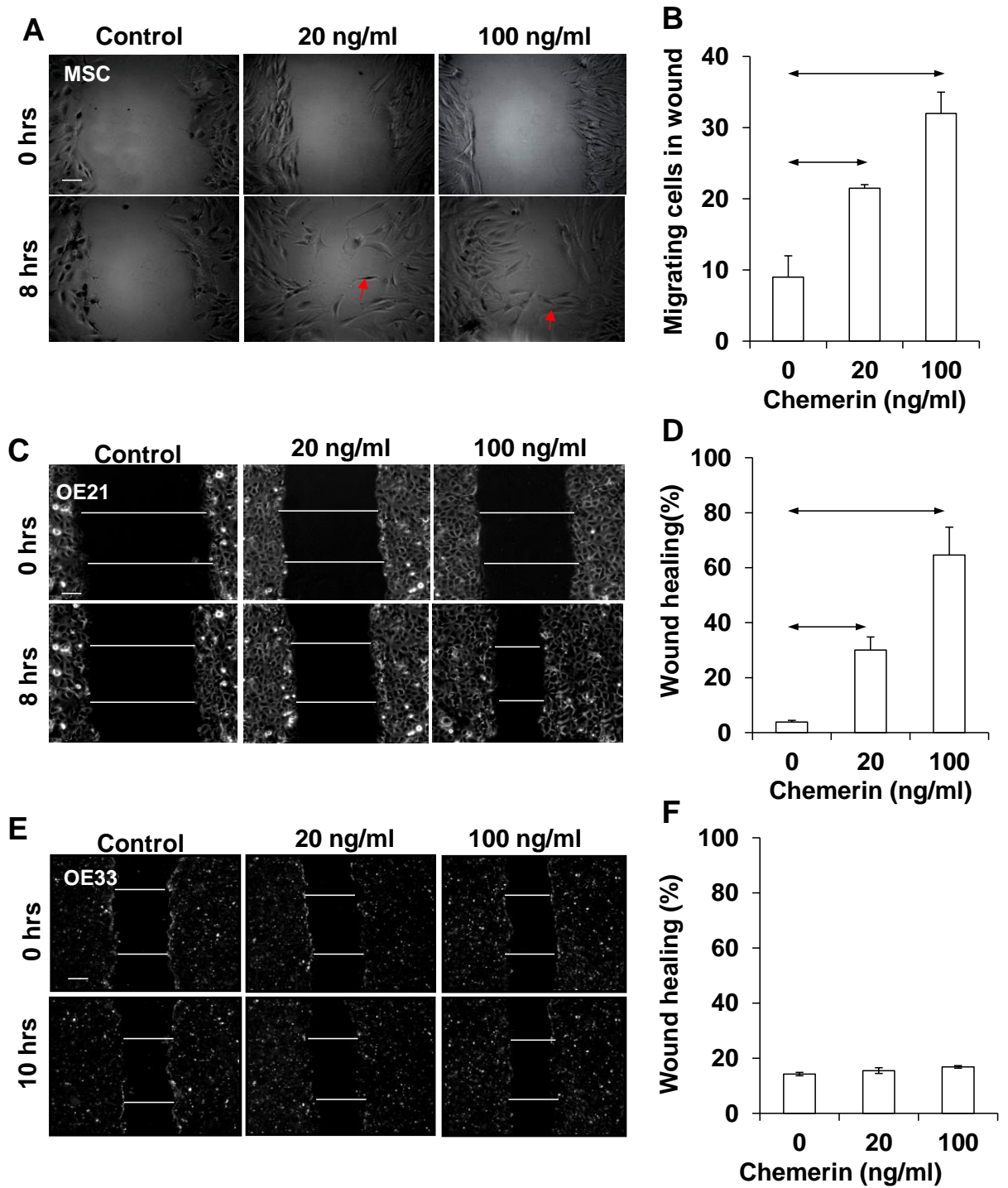
Figure 4.6 In wound healing assays, chemerin stimulated cell migration in a dose dependent manner. A) Representative images of MSCs migrating individually in scratch wound assays (red arrow) in response to chemerin stimulation. B) Dose dependent chemerin stimulation of MSC migration. C) Representative image of OE21 cells migrating as a sheet. D) Dose dependent chemerin stimulation of OE21 cell migration. E) Representative image of OE33 cells in wound healing assay in the presence of chemerin. F) Chemerin has little or no effect on OE33 cell migration. n=3. Horizontal arrows, $p<0.05$, t-test; vertical bars, SEM. Scale bar, 20µm.
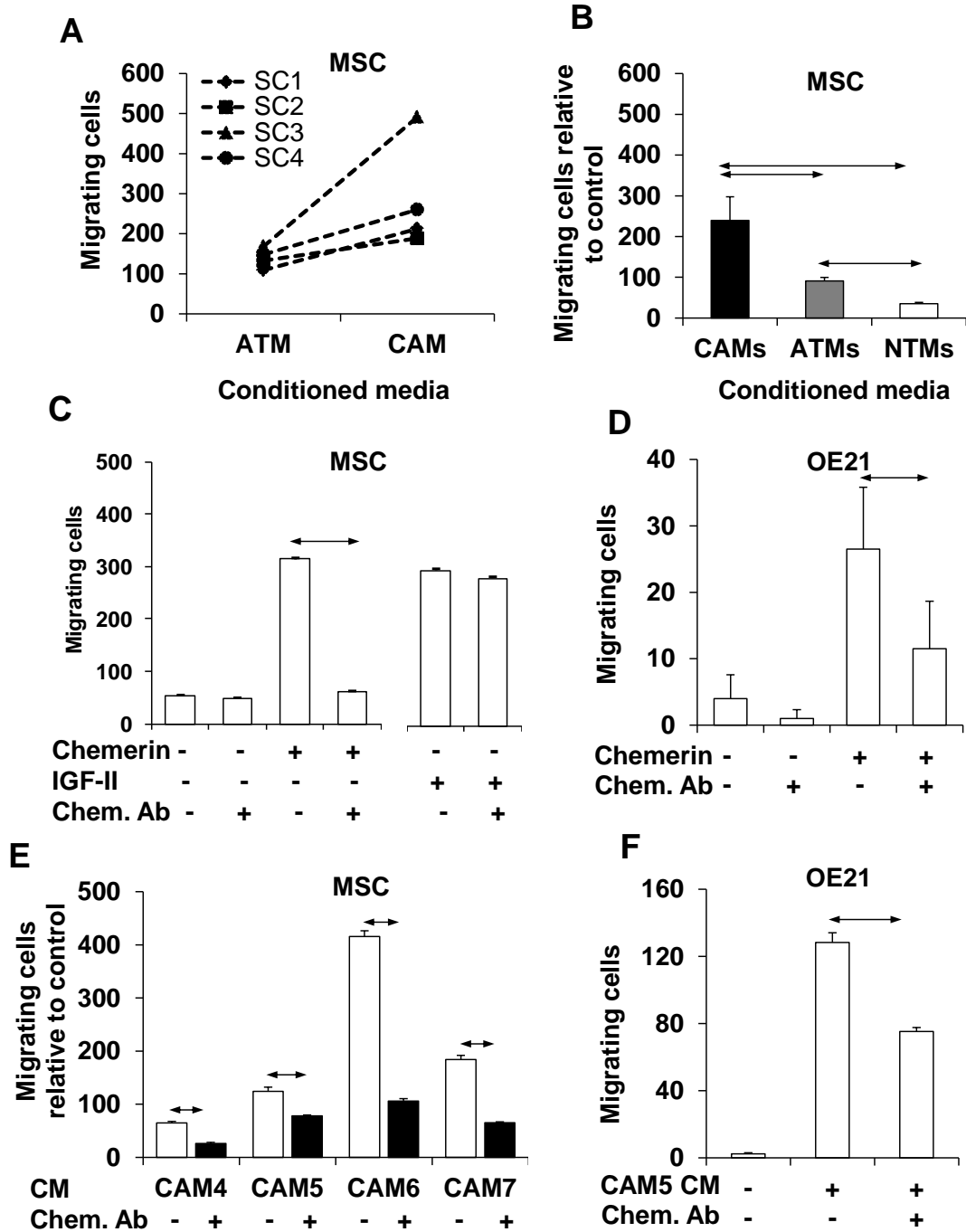
Figure 4.7 In Boyden chamber migration assays, SC CAM CM increased MSC and OE21 cell migration. A) Comparison of MSC migration in response to CM from paired SC CAMs versus ATMs. B) Group mean data of MSC migration in response to CM from SC CAMs (n=4), ATMs (n=4) and NTMs (n=4). C) Chemerin neutralising antibody inhibited MSC migration in response to chemerin (100 ng/ml) but not IGF-II (100 ng/ml). D) Chemerin stimulation of OE21 cell migration is decreased by chemerin immuno-neutralisation (Chem Ab). E) Stimulation of MSC migration by CAM4, CAM5, CAM6 and CAM7 CM was inhibited by chemerin neutralising antibody (n=3). F) OE21 migration in response to CAM5 CM treatment was partially inhibited by chemerin immuno-neutralisation. Horizontal arrows, p<0.05, ANOVA; vertical bars, SEM.

4.3.9 Chemerin neutralisation inhibits CM stimulated OE21 wound healing

In wound healing assays, chemerin (100ng/ml) and CM from CAM4 significantly increased OE21 cell migration compared to SF media (Figure 4.8 A & B). The stimulatory effect was significantly inhibited by chemerin neutralising antibody (Figure 4.8 A & B).

4.3.10 Knock-down of chemerin inhibits and overexpression increases cell migration

In addition to the neutralising antibody approach, knock-down and overexpression of chemerin were used to confirm a role in cell migration. Chemerin knock-down in CAMs using siRNA was confirmed by Western blots (Figure 4.9 A). MSCs showed significantly reduced migration in response to CM from CAMs treated with silencing RNA compared to control CAM CM (Figure 4.9 B). In the case of OE21 cell migration, there was a significant reduction by CAM4 and CAM7 CM after chemerin knock-down (Figure 4.9 D).

Overexpression of chemerin after transfection with an expression vector of CAM4 and ATM7 cells was confirmed by Western blot (Figure 4.9 A). The CM from myofibroblasts overexpressing chemerin significantly increased in MSC and OE21 cell migration relative to control (Figure 4.9 C & E).
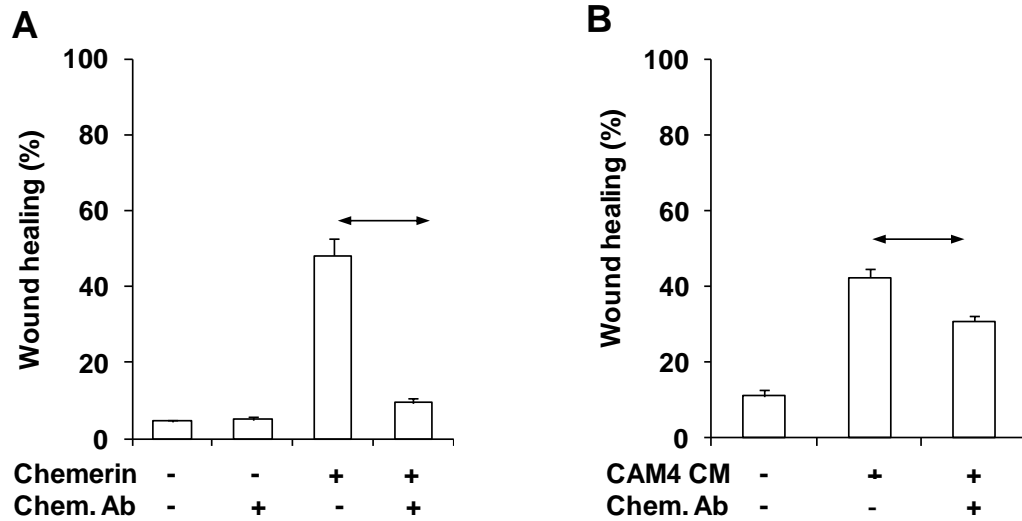
Figure 4.8 In wound healing assays, chemerin immuno-neutralisation decreased OE21 migration. A) Effect of immuno-neutralisation on OE21 migration stimulated by chemerin (100ng/ml). B) OE21 migration in response to CM from CAM4 was inhibited by chemerin neutralising antibody (n=3). Horizontal arrows, $p<0.05$; ANOVA; vertical bars, SEM.
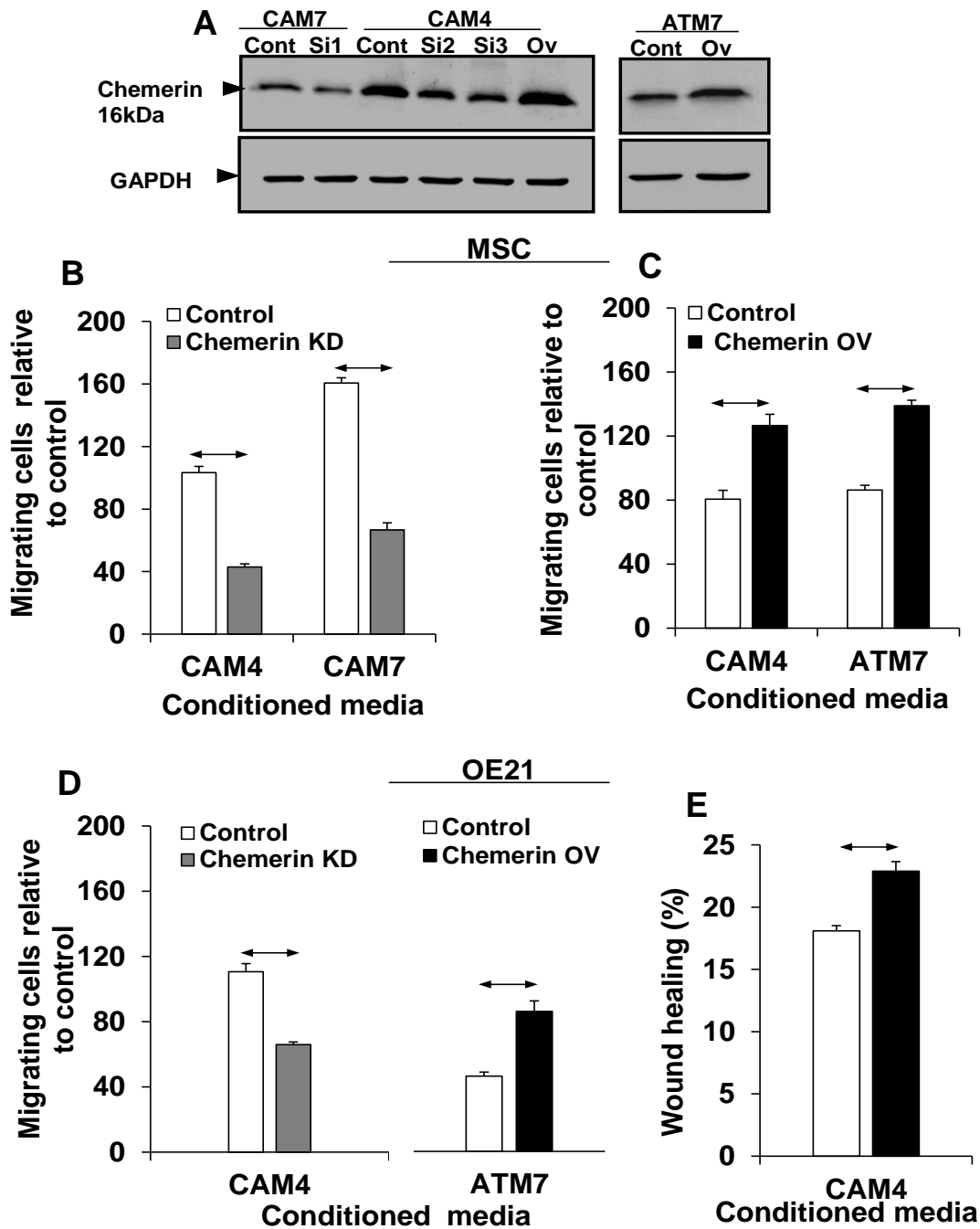
Figure 4.94 Chemerin knock-down decreased, and overexpression increased, MSC migration in Boyden chamber migration assays. A) Western blots showing chemerin knock-down in siRNA treated (Si1) CAM7 and (Si2 and Si3) CAM4 cells, and overexpression after chemerin plasmid transfection in CAM4 and ATM7 cells. B) MSC migration is decreased in response to CM from CAM4 and CAM7 transfected with chemerin silencing RNA, Si1 and Si3 respectively. C) Increased MSC migration in response to CM from CAM4 and ATM7 overexpressing chemerin. D) OE21 cell migration is decreased in response to CM from CAM4 siRNA transfected cells, and increased by CM from chemerin overexpressing ATM7 cells. E) In wound healing assays, CM from CAM4 cells overexpressing chemerin increased OE21 cell migration. Horizontal arrows, p<0.05, t- test; vertical bars, SEM.

4.3.11 ChemR23 knock-down in MSCs reduce the migratory response to CM and chemerin

The data above indicate a role for chemerin in mediating the effect of myofibroblast CM on migration of several cell types including MSCs. The relevant receptors in MSCs were then examined by separate knock-down of chemR23 and GPR1. The knock-down of receptors by validated siRNAs was confirmed by immunocytochemistry (Figure 4.10 A & B). Scoring of chemR23 and GPR1 after knock-down indicated a reduction in immunoreactive cells by 80.7% and 75.9%, respectively, relative to control MSCs (Figure 4.10 C & D). In both cases, a negative control, vimentin, showed no change after treatment with siRNA for chemR23 and GPR1 confirming the specificity of the receptor suppression (Figure 4.10 C & D).

In response to CAM CM, MSCs treated with chemR23 siRNA showed significant decreases in migration compared to control cells (Figure 4.11 A, Left panel). Similar results were obtained in response to chemerin (Figure 4.11 B, Left panel). However, GPR1 knock-down in MSCs had only modest effect on migration in response to CM from CAM6 but no effect to chemerin (Figure 4.11 A & B, Top and bottom right panel respectively).

4.3.12 Chemerin activation of chemR23 stimulates MAPK pathways

Having established a functional role for chemR23 in mediating chemerin effects on MSCs, the next step was to determine the signal transduction pathways involved in cell migration. In response to chemerin, MSC cell extracts showed increased phosphorylation of p42-44, p38 and JNK-II kinases in Western blots

using phosphor-specific antibodies and in all cases this was decreased by chemR23 knock-down (Figure 4.12).

4.3.13 Chemerin stimulated MSC migration via activating MAPK pathways

The role of signalling pathways activated by chemerin was then studied using specific pharmacological inhibitors targeting p42-44, p38, JNK-II, and PKC pathways. Chemerin-induced MSC migration was significantly inhibited by SP600125 (JNK-II inhibitor), UO126 (p42/44 inhibitor), SB202190 (p38 inhibitor), Ro320432 (PKC inhibitor) but not LY294002 (PI3K inhibitor) (Figure 4.13 A). The PKC inhibitor showed the highest inhibition of chemerin-stimulated MSC migration. Combinations of inhibitors i.e. two or three together virtually abolished chemerin stimulated MSC migration (Figure 4.13 B).
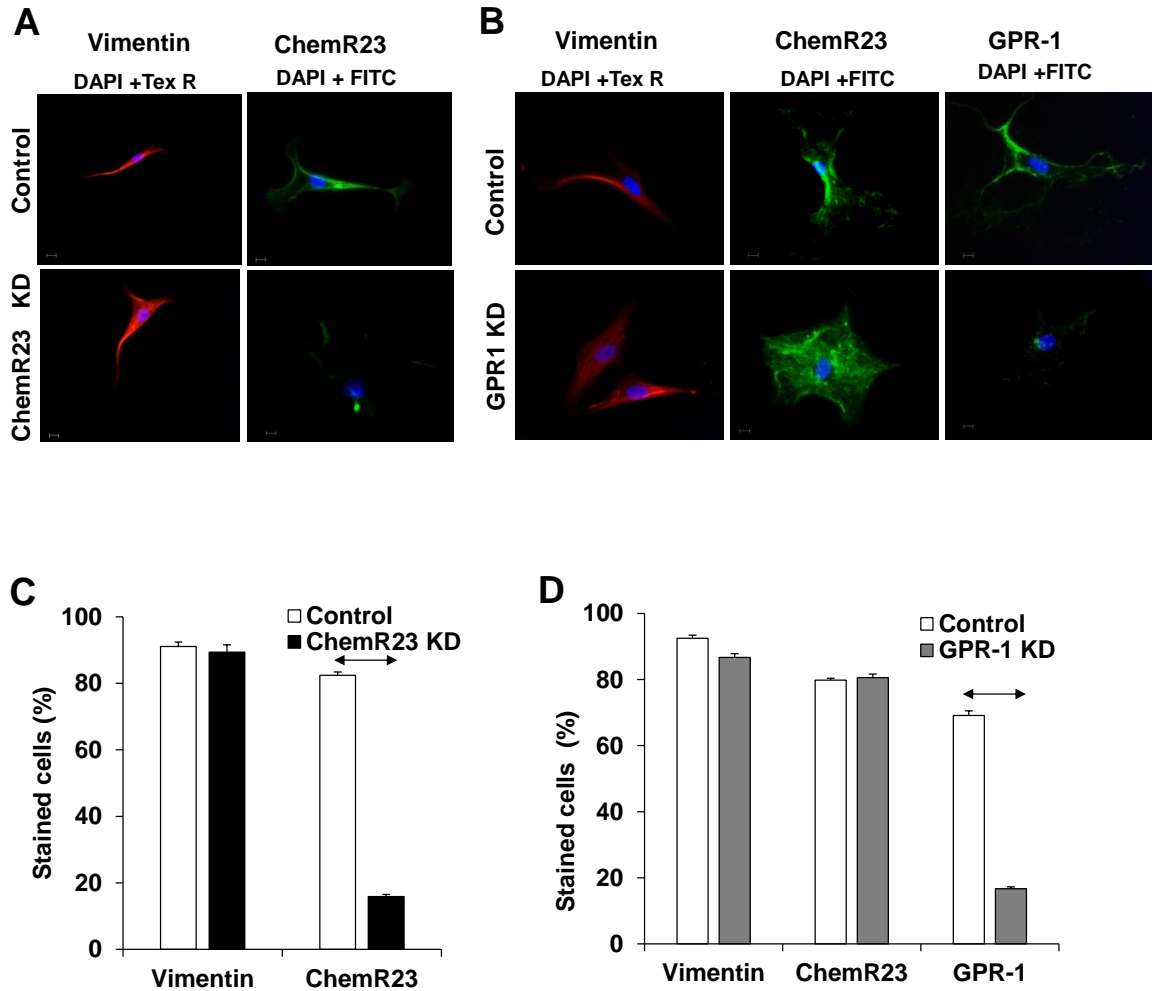
Figure 4.5 Immunocytochemical validation of receptor expression after chemR23 and GPR1 knock-down. A) Representative images from MSCs stained for vimentin (positive control) and chemR23. B) Representative image from MSCs stained for vimentin (positive control) chemR23 and GPR1.   C) Quantitative data show decreased chemR23 expression after its knock-down and D) quantitative data showing GPR1 expression after its knock-down. Horizontal arrows, $p < 0.05$, t-test; vertical bars, SEM. Scale bar, 20μm.
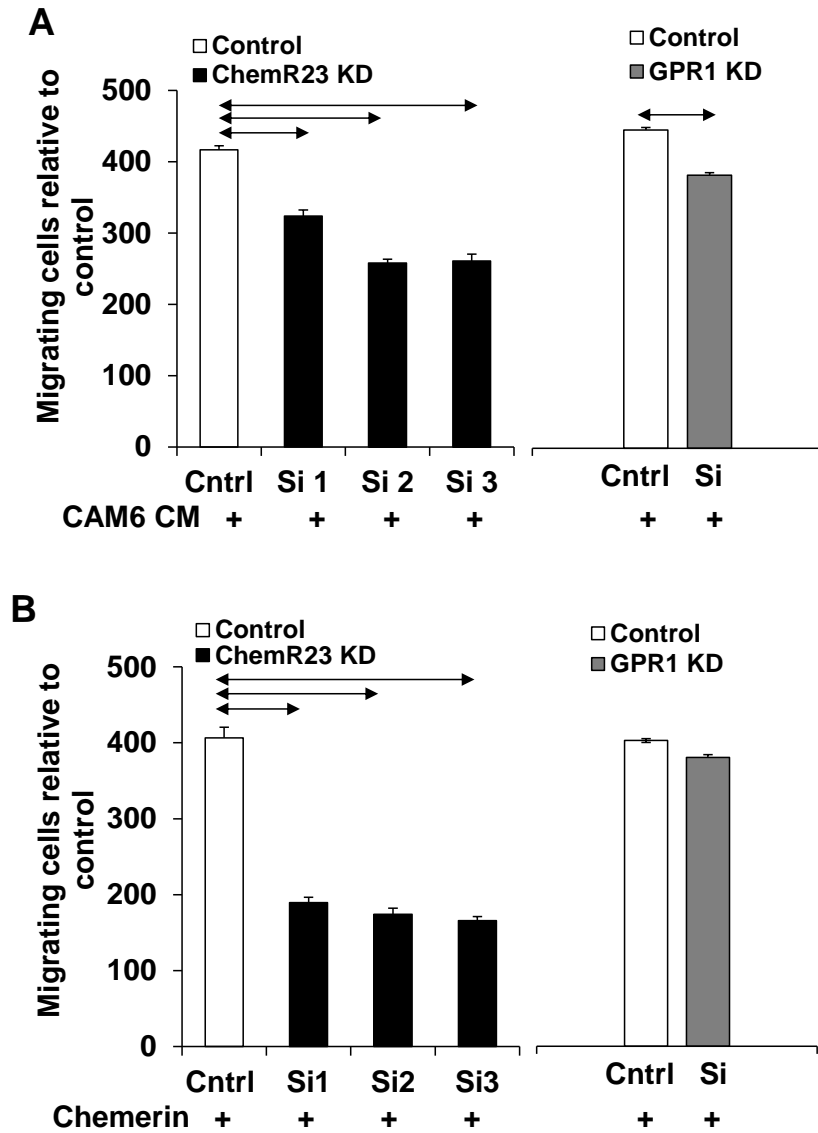
Figure 4.61 In Boyden chamber migration assays chemR23 knock-down decreased MSC migration in response to chemerin (100ng/ml) and CM from CAMs. A) Effect of 3 different chemR23 silencing RNAs (Si) (left) and a GPR1 validated siRNA (right) on MSC migration in response to CAM6 CM. B) MSC migration after chemR23 and GPR1 knock-down in response to chemerin. Horizontal arrows, p<0.05, t- test; vertical bars, SEM.
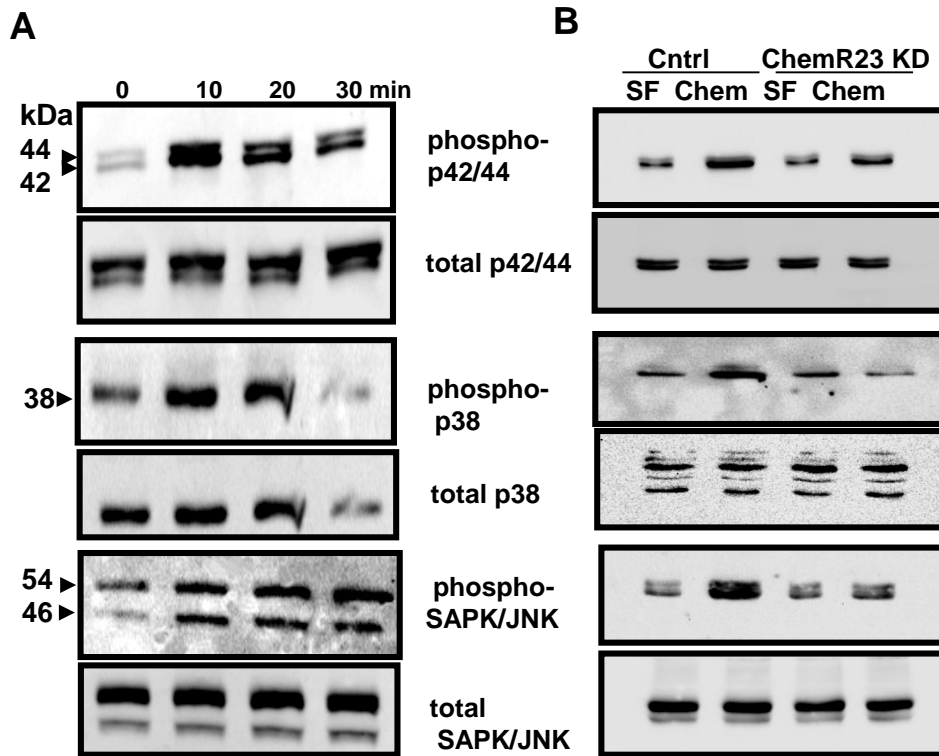
Figure 4.12 Chemerin stimulated p42/44, p38, and JNK-II in MSCs and the stimulation is reduced after chemR23 knock-down. A) Representative Western blot analysis of phospho- and total p42/44 kinase, p38 kinase, and JNK-II in MSCs treated with chemerin (100 ng/ml) for 0, 10, 20, 30 mins. B) Western blot of phospho and total p42/44 kinase, p38 kinase, and JNK-II expression in response to chemerin (Ch) (100 ng/ml) in control MSC cells and after chemR23 knock-down.
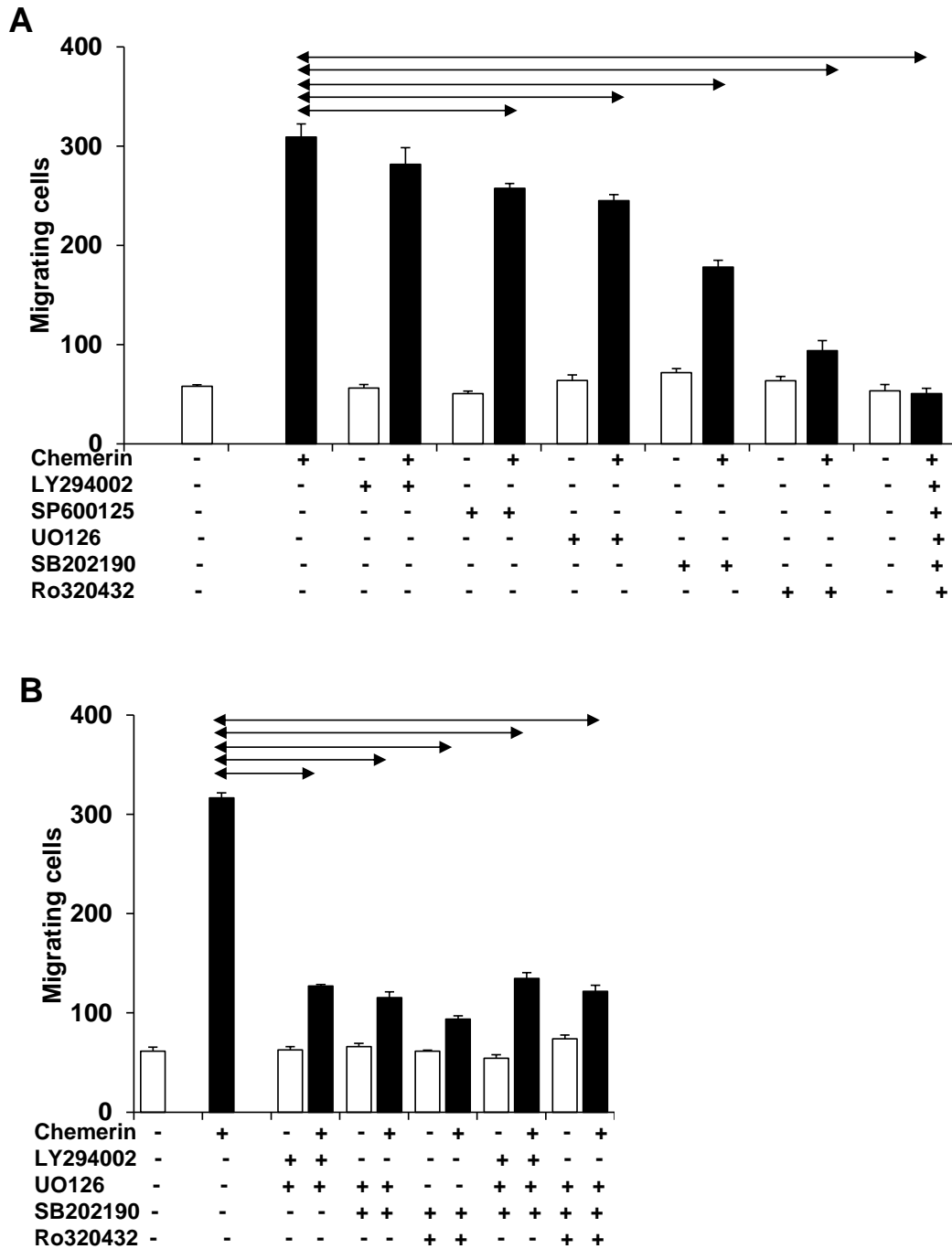
Figure 4.73 Chemerin stimulated MSC migration is mediated by several protein kinases. A) In Boyden chamber assays, chemerin stimulated MSC migration is inhibited by SP600125 (JNK-II inhibitor, 50µM), UO126 (p42/44 inhibitor, 10µM), SB202190 (p38 inhibitor, 3µM), Ro320432 (PKC inhibitor, 2µM) but not LY294002 (PIK3 inhibitor, 50µM) (n=3). B) Chemerin stimulated MSC migration is significantly inhibited by combinations of two of UO126, SB202190, and Ro320432, and by combination of three of SP600125, UO126, SB202190 and Ro320432 (n=3). Horizontal arrows, p<0.05, Anova; vertical bars, SEM.

4.3.14 PKC acts upstream of MAPK activation

The observation that the strongest inhibition of chemerin-stimulated MSC migration was by the PKC inhibitor suggested the existence of signalling cascades downstream of PKC. This hypothesis was then investigated by using p42/44, p38, JNK-II inhibitors against PMA-stimulated MSC migration. MSCs showed a significant increase in migration in response to the PKC activator, PMA (Figure 4.14 A). The MAPK inhibitors SP600125, UO126 and SB202190 significantly inhibited PMA-stimulated migration (Figure 4.14 A). The combination of all three inhibitors virtually abolished PMA stimulated MSC migration compared to control (Figure 4.14 A). In addition, Western blot analysis of cell extracts from PMA-stimulated MSCs showed increased phospho - p42/44, p38 and pJNK-II (Figure 4.14 B & C respectively) that was reduced by the PKC inhibitor Ro320432 (Figure 4.14 C).
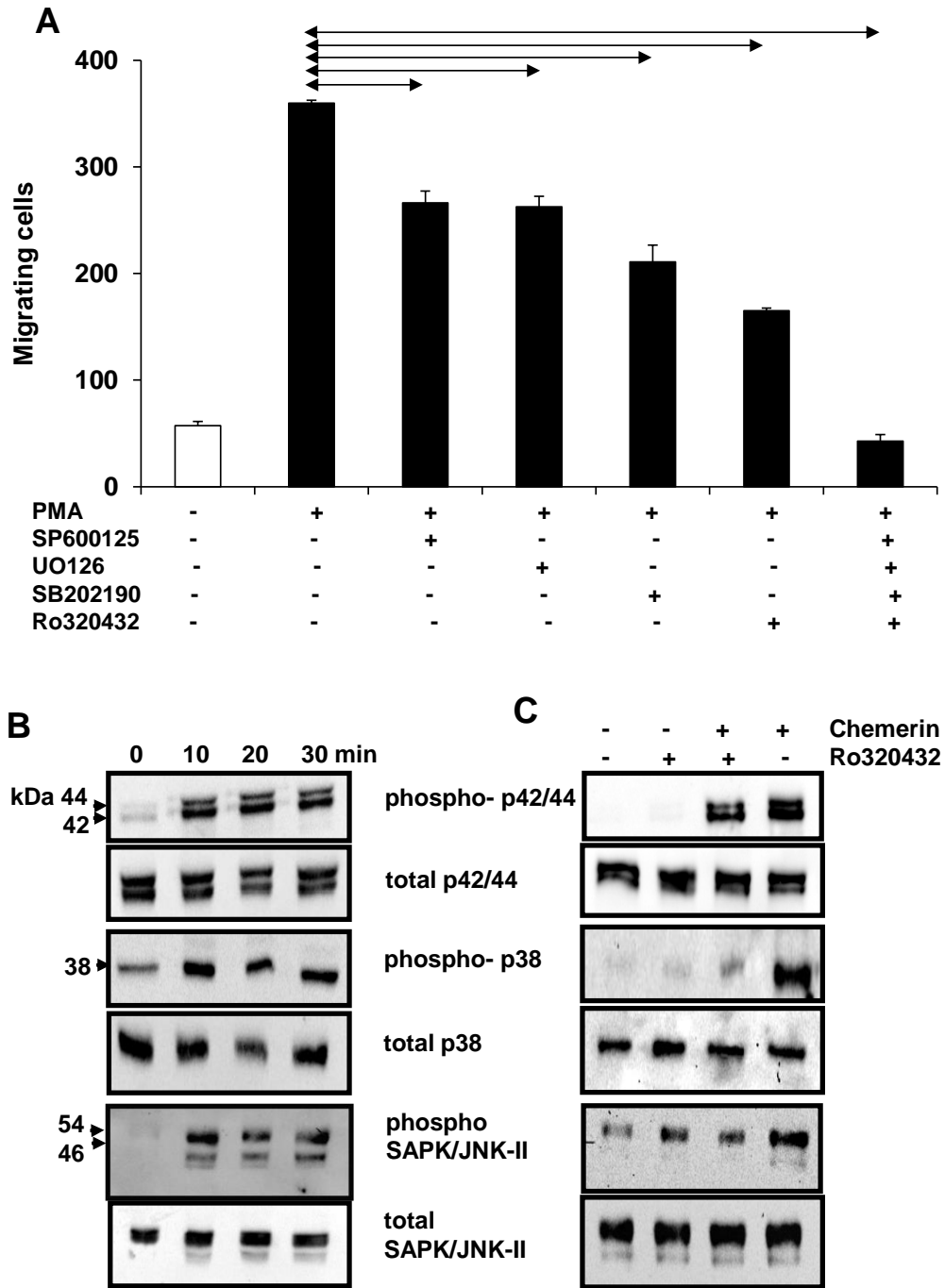
Figure 4.14. PMA stimulated MSC migration is mediated by activation of p42/44 kinase, p38 kinase, JNK-II, and PKC. A) PMA (100nM) stimulated MSC migration in Boyden chamber assay and was inhibited by SP600125 (JNK-II inhibitor, 50µM), UO126 (p42/44 inhibitor, 10µM), and SB202190 (p38 inhibitor, 3µM). B) Representative Western blot analysis of p42/44, p38 and JNK-II kinases from MSC cell extracts treated with PMA. C) Western blots analysis of p42/44, p38 and JNK-II kinases from MSC cell extracts treated with Ro320432 (Ro, PKC inhibitor) and chemerin (100 ng/ml). Horizontal arrows, $p < 0.05$, ANOVA; vertical bars, SEM.

4.4   Discussion

The main finding of this chapter is that oesophageal SC CAMs are a source of increased chemerin expression compared with ATMs. Bone marrow derived MSCs strongly express the putative chemerin receptors, chemR23 and GPR1, and have a strong migratory response to chemerin. Knockdown and overexpression studies have confirmed that chemerin in CAM CM acts at chemR23 to induce MSC migration. The latter is mostly mediated by activation of PKC which in turn stimulates the p42/44, p38 and JNK-II MAPK pathways.

In this study, the initial discovery of chemerin expression by myofibroblasts was made by iTRAQ proteomic analysis comparing CM from SC CAMs and ATMs. Chemerin was the only chemokine identified as upregulated in all 4 CAMs from SC patients. Interestingly, microarray studies did not show a difference in chemerin transcript abundance in CAMs *versus* ATMs emphasising the importance of validation of the proteomic data. Thus a combination of methods (ELISA and Western blot) was used to validate the increased chemerin abundance both in media and cell extracts of CAMs from oesophageal SC. These confirmed increased chemerin in CAM media. Presumably the different conclusions from microarray and protein studies indicate regulation of chemerin expression at a translational or post-translational level.

Upregulation of chemerin in fibroblasts related to pathology (skin psoriasis compared to paired normal fibroblasts) has previously been reported using RT-PCR (Albanesi, Scarponi et al. 2009). In addition, other studies have shown increased abundance of chemerin in plasma, media and cell extracts using ELISA and Western blot (Parlee, Ernst et al. 2010; Parlee, McNeil et al. 2012). However, the existence of various chemerin isoforms (Du and Leung 2009) limits the

interpretation of assays using a single antibody in ELISA or Western blot. Chemerin-9 (which has high bioactivity) was undetectable by the ELISA used here, emphasising the need for further studies to precisely define the active forms of chemerin in CAM media; these in turn will depend on the use of multiple antibodies of different specificities. Nevertheless the combination of the two methods, taken together with the iTRAQ data, provides strong evidence of increased expression in CAMs. Consequently, for the first time, it has been possible to identify myofibroblasts as a source of elevated chemerin, at least in oesophageal cancer.

The microarray data analysis showed that MSCs have greater chemerin receptor expression, both chemR23 and GPR1, compared to myofibroblasts followed by oesophageal cancer cells, OE21, OE19 and OE33 cells. Immunohistochemistry confirmed the expression of chemR23 and GPR1 in MSCs and showed weak staining for both receptors in CAMs and OE21 cells. Techniques such as RT-PCR and FACS have previously been applied to other cells eg adipocytes and macrophages, to establish chemR23 and GPR1 expression (Huang, Zhang et al. 2010; Bondue, De Henau et al. 2012). In the present study, the finding of increased ligand (chemerin) in myofibroblasts and of receptor expression (chemR23) in MSCs provided a basis for further detailed functional studies, namely with regard to cell migration.

Recent work has shown that MSCs are an integral part of the tumour microenvironment and are key factors for tumour growth and development (Feng and Chen 2009; Kidd, Spaeth et al. 2009). Undifferentiated MSCs can contribute to various stromal cell populations including potentially CAMs, as well as epithelial cells in oesophageal AC (Worthley, Ruszkiewicz et al. 2009; Hutchinson,

Stenstrom et al. 2011; Lecomte, Masset et al. 2012). It therefore becomes important to understand the mechanisms involved in recruitment of MSCs to the tumour microenvironment. In this context the chemotactic property of chemerin demonstrated in this study suggests a potential novel mechanism.

Recombinant chemerin increased MSC migration and neutralising antibody to chemerin decreased MSC migration stimulated by CM from CAMs and ATMs indicating a role for the native protein. Chemerin is known to interact with chemR23 to induce trafficking of inflammatory cells and dendritic cells (Ernst and Sinal 2010; Bondue, Wittamer et al. 2011). In good agreement with this, siRNA knock-down of chemR23 decreased chemerin stimulated MSC migration. Albanesi *et al.,* have used anti chemR23 antibody to inhibit chemerin stimulated *in vitro* transmigration of pDC thereby confirming the chemerin interaction with chemR23 in chemotaxis (Albanesi, Scarponi et al. 2009). However, the transient knockdown by siRNA was not 100% efficient and other reporter based *in vitro* assays, such as Tango assays, ought to be considered as an alternative approach in the future (Barnea, Strapps et al. 2008). The latter assay depends upon the exogenous ligand activation of receptor followed by protein-protein interactions subsequently activating the binding of protease to specific sites of cleavage in the fusion of a membrane receptor and transcriptional activator. The release of transcriptional activator amplifies the reporter gene and produces appropriate signals for quantification (Barnea, Strapps et al. 2008). This method might provide a further rigorous approach to define receptor specificity, and its activation by chemerin in our cell systems.

Other studies showed GPR1 has equal binding affinity to chemerin, however no signal transduction ability was observed suggesting of no functional significance (Ernst and Sinal 2010; Bondue, Wittamer et al. 2011). We investigated the functional significance of GPR1, and in agreement with previous studies GPR1 knock-down had no effect on chemerin stimulated MSC migration.

The action of chemerin on MSCs was shown to be mediated by activation of p44/42, p38, JNK-II and PKC signal transduction pathways. Furthermore, chemerin-mediated MSC migration was significantly reversed by a PKC inhibitor suggesting PKC as upstream of MAPK signalling pathways. Nevertheless many of the inhibitors used produced only partial inhibition of migratory responses. There are two components to interpreting these data First there is partial PKC-dependent downstream activation of MAPKs signals (Figure 4.15). Second, chemerin chemotaxis might independently activate p44/42, p38 and JNK-II. It is known that chemerin activates Akt, and PI3K to facilitate macrophage adhesion to ECM (Hart and Greaves 2010). However, in our studies the PI3K pathway had no role to play in chemerin stimulated MSC migration.
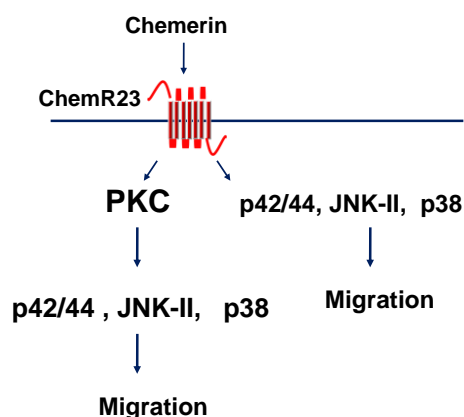
Figure 4.15. The PKC activation partially activates downstream p44/42, p38, JNK-II pathways. In addition, individual activation of p42/44, p38 and JNK-II stimulates cell migration.

Although not studied in as much detail, we have also investigated and confirmed the effect of chemerin on cancer cells. In response to chemerin and CM from SC CAMs, OE21 cells showed increased proliferation and migration whereas OE33 cells did not respond to chemerin. These observations suggest cancer-restricted tumour-stromal interaction mediated by chemerin.

The results presented here raise the first indication of a role for chemerin in oesophageal cancer. Firstly, myofibroblasts in oesophageal SC are a major source of chemerin. Secondly, chemerin secreted from CAMs interacts with the putative receptor chemR23, expressed by MSC and cancer cells and stimulates their *in vitro* migration. Thirdly, the chemerin-chemR23 interaction regulates cell migration via activation of signalling pathways, notably PKC that is upstream of p44-42, p38, and JNK-II. Berg *et al.,* and others have shown chemerin influences the secretion of TNF-α, interleukins and MMPs that further regulate the recruitment and migration of cells across stromal compartment (De Becker, Van Hummelen et al. 2007; Berg, Sveinbjornsson et al. 2010; Lecomte, Masset et al. 2012). It is possible that there are comparable extracellular mediators in the present system and, if so, their identification will provide valuable insight in understanding molecular mechanism involving the tumour microenvironment and recruitment of cells to it.

4.5   Conclusions

1. Oesophageal SC CAMs secrete chemerin and MSCs express the putative chemerin receptors, chemR23 and GPR1.

2. Chemerin in CM stimulates the migration of MSCs via interaction with chemR23.

3. Chemerin-stimulated chemotaxis is the result of activation of PKC followed by partial downstream activation of p-44/42, p38, and JNK-II MAPKs but not PI3K.

4. Chemerin in CM also induces OE21 cell migration.

Chapter-5

Identification of MIF as a Chemerin Target in MSCs and its Functional Significance

5.1   Introductions

The findings in chapter-4 confirmed an increase of chemerin in myofibroblasts associated with oesophageal SC and showed its significance in enhancing MSC migration *in vitro*. In general, chemokines activate multiple paracrine signalling pathways regulating functions such as migration and invasion (Wels, Kaplan et al. 2008). For example, Zhiyong Mi *et al.,* showed increased expression of CCL5 in BM-derived MSCs along with their recruitment to a metastatic niche in response to distant osteopontin originating from breast cancer cells (MDA-MB231) (Mi, Bhattacharya et al. 2011). In the present study, it was considered reasonable to suppose that chemerin might also activate paracrine signalling mechanisms in MSCs that were functionally significant.

Proteomic studies in the group (Holmberg, Varro, unpublished observations) using SILAC-labelled MSCs identified increase MIF abundance in response to chemerin stimulation. It has been reported that MIF acts as a negative regulator of MSC migration at sites of tissue damage (Ozaki, Nishimura et al. 2007; Barrilleaux, Fischer-Valuck et al. 2010). It was therefore hypothesised that MIF might regulate MSC migration in response to chemerin. To our knowledge there have been no previous studies of the effect of chemerin on MIF release. Hence, the rationale for the studies in this chapter was to investigate and determine the role of MIF in MSC migration.

5.1.1 Objectives

The specific objectives of this chapter were:

1. To study the effect of chemerin on MIF secretion from MSC and OE21 cells.

2. To determine the functional significance of MIF on chemerin stimulated MSC migration.

5.2   Material and Methods

5.2.1   Cell culture

Myofibroblasts, MSCs, and oesophageal cancer cell lines were cultured as described in sections 2.3.

5.2.2   Boyden chamber migration assays

The effect of MIF on migration was investigated using Boyden chamber assays as described in sections 2.6.1.1 and 2.6.1.2. The bottom well contained 750µl serum-free media, with or without chemerin (4-100ng/ml), MIF (200ng/ml), IGF-II (100ng/ml) or ISO-1 (50µM).

5.2.3   Transient transfection of myofibroblasts and MSCs

MSCs were transfected with validated MIF siRNA (100µM) as described in sections 2.11 and 2.12 following the manufacturer's instruction.

5.2.4   Western blot analysis

The expression of MIF in normal and transiently transfected myofibroblasts, MSCs and OE21 cells extracts and media was investigated by Western blot as described in section 2.9.

5.2.5   Sandwich - enzyme linked immunosorbent assay (ELISA)

The concentration of MIF in media from myofibroblasts, MSCs, OE21 and OE33 cells was determined by ELISA as described in section 2.10.

5.3   Results

5.3.1   Chemerin stimulated MSC expresses MIF

Because proteomic studies using SILAC had shown increased MIF abundance in MSC media after 24 hr stimulation with chemerin, validation studies using ELISA and Western blot were undertaken to confirm this observation.

The analysis of MIF by ELISA showed relatively low concentrations in media from unstimulated MSCs and increased abundance in response to chemerin and IGF-II (Figure 5.1 A). However, in OE21 cell media there were relatively high concentrations basally and no effect with IGF-II or chemerin (Figure 5.1 B). Similarly, the basal concentrations of MIF in control MSC media were relatively low in Western blots, but in response to stimulation by chemerin or IGF-II there was increased MIF; there was also increased MIF in MSC cell extracts after stimulation (Figure 5.1 C top panels). In contrast, chemerin and IGF-II had very modest effects on MIF detected by Western blot in OE21 cell extracts and media (Figure 5.1 C bottom panels). ELISA also showed the mean MIF concentration in ATM (ATM4-ATM7) media was significantly higher than in the corresponding CAM media (CAM4-CAM7) (Figure 5.1 E).
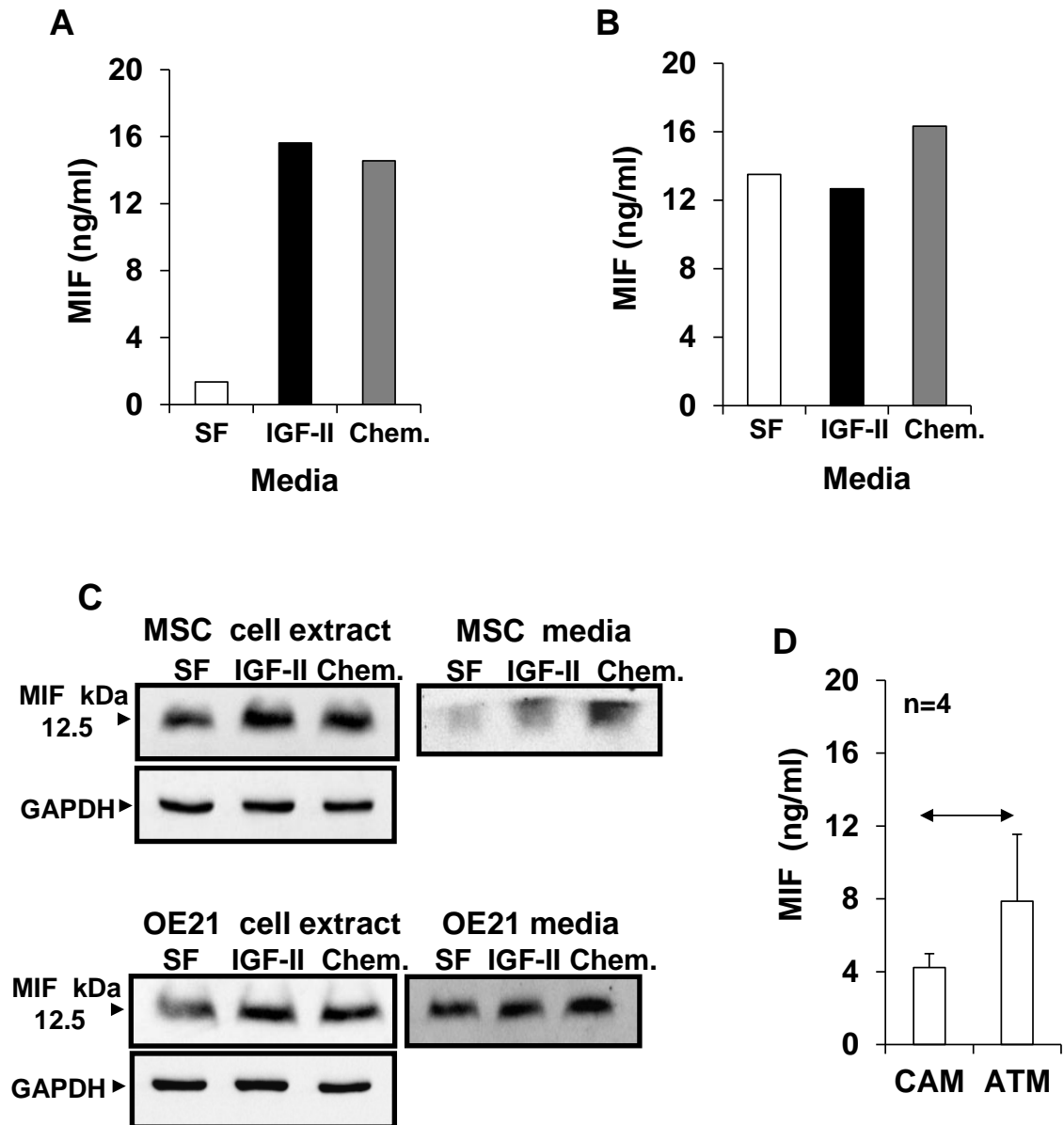
Figure 5.1 Increased MIF secretion in MSCs in response to IGF-II (100 ng/ml) and chemerin (100 ng/ml). A) ELISA showed MIF secretion in MSC (n=2) but not B) OE21 media in response to IGF-II or chemerin (n=2). C) Representative Western blots showing secretion of MIF by MSCs (top panel) but not by OE21 cells (bottom panel) treated with chemerin or IGF-II for 15 min. D) Myofibroblasts from squamous cancer (CAMs) secreted less MIF compared to ATMs (n=4). Horizontal arrows, p<0.05, t-test; vertical bars, SEM.

5.3.2 MIF inhibits chemerin and IGF-II stimulated MSC migration

The role of MIF was then investigated with respect to cell migration in response to chemerin and IGF-II using Boyden chambers. Chemerin and IGF-II stimulated MSC migration was significantly inhibited by MIF (200ng/ml) (Figure 5.2 A). The inhibitory effect of MIF on chemerin-stimulated MSC migration was significantly reversed by ISO-1, a MIF antagonist, at 50μM (Figure 5.2 C). A role for endogenous MIF in modulating MSC migration was then indicated by the observation that ISO-1 dose-dependently increased migration in response to chemerin (Figure 5.2 B).

5.3.3 MIF inhibits chemerin and IGF-II stimulated MSC migration

Finally, knock-down studies were performed to further validate the role of MIF. Thus knock-down of MIF expression in MSCs using siRNA was confirmed by Western blot (Figure 5.3 A). In cells transfected with MIF siRNA there was a significant increase in migration in response to 4ng/ml chemerin compared to control (Figure 5.3 B); however, at 20ng/ml chemerin, which produced a stronger stimulation of MSC migration, there was no significant effect of MIF knockdown (Figure 5.3 B)

Medium from chemerin-stimulated MSCs pre-treated with siRNA to knock-down chemR23 expression was then studied by ELISA to establish the role of chemerin-chemR23 interactions in MIF secretion. As expected, ELISA showed a significant decrease in MIF secretion in chemerin stimulated MSCs pre-treated with chemR23 siRNA compared to control MSCs (Figure 5.3 C). Interestingly, however, while MSC migration in response to chemerin (4 ng/ml) after chemR23

knockdown was, as expected, decreased (p<0.05) compared to control cells, ISO-1 strongly increased migration further supporting the idea that MIF plays a part in modulating responses to modest chemerin stimulation (Figure 5.3 D).
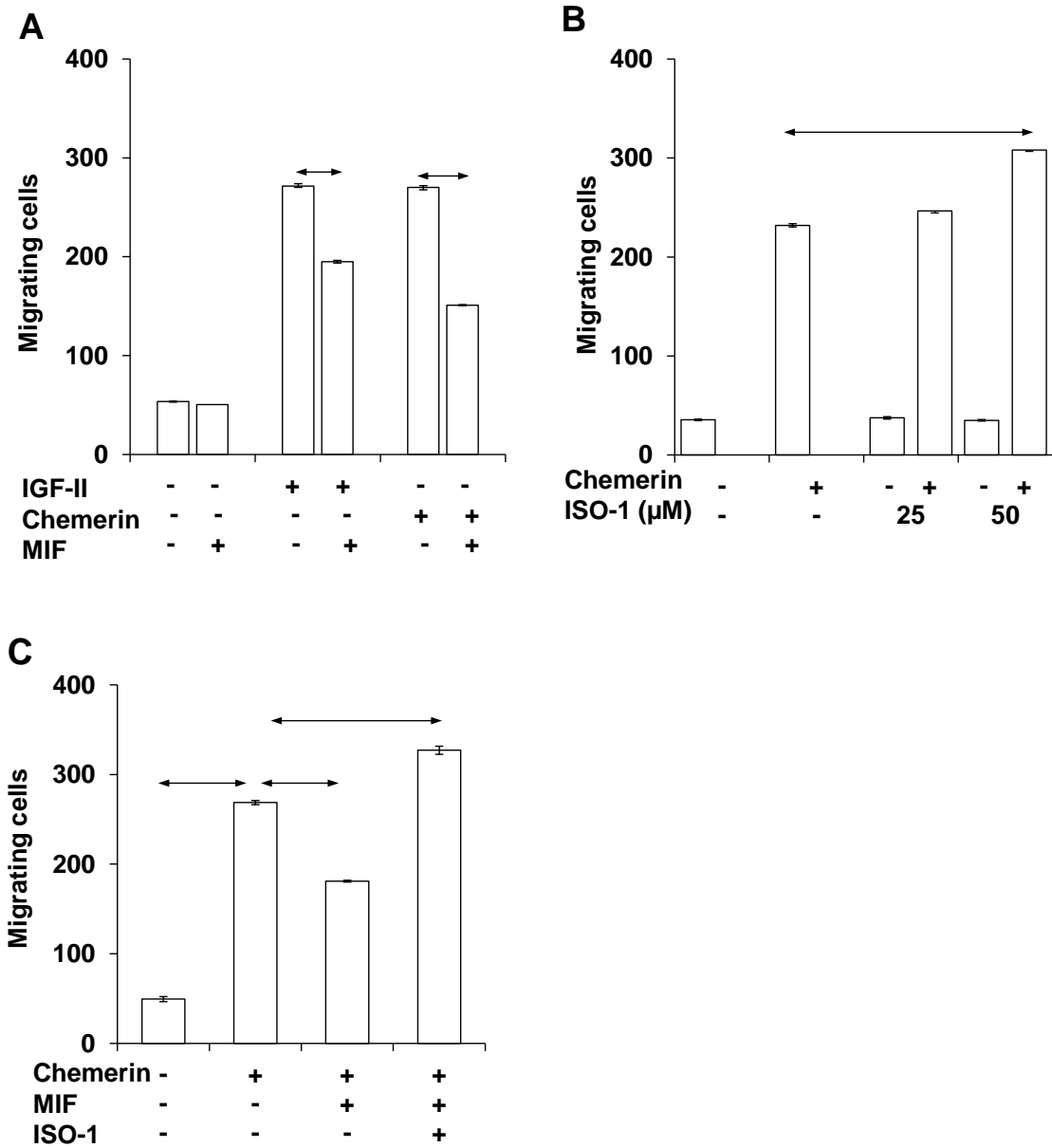
Figure 5.2 MSC migration in response to ISO-1 (50μM) treatment. A) MSC migration in response to chemerin or IGF-II (100 ng/ml) was inhibited by MIF (200 ng/ml). B) ISO-1 increased chemerin-stimulated MSC migration in a dose-dependent manner and C) ISO-I reversed the effect of MIF on chemerin-stimulated migration. Horizontal arrows, $p < 0.05$, ANOVA; vertical bars, SEM.
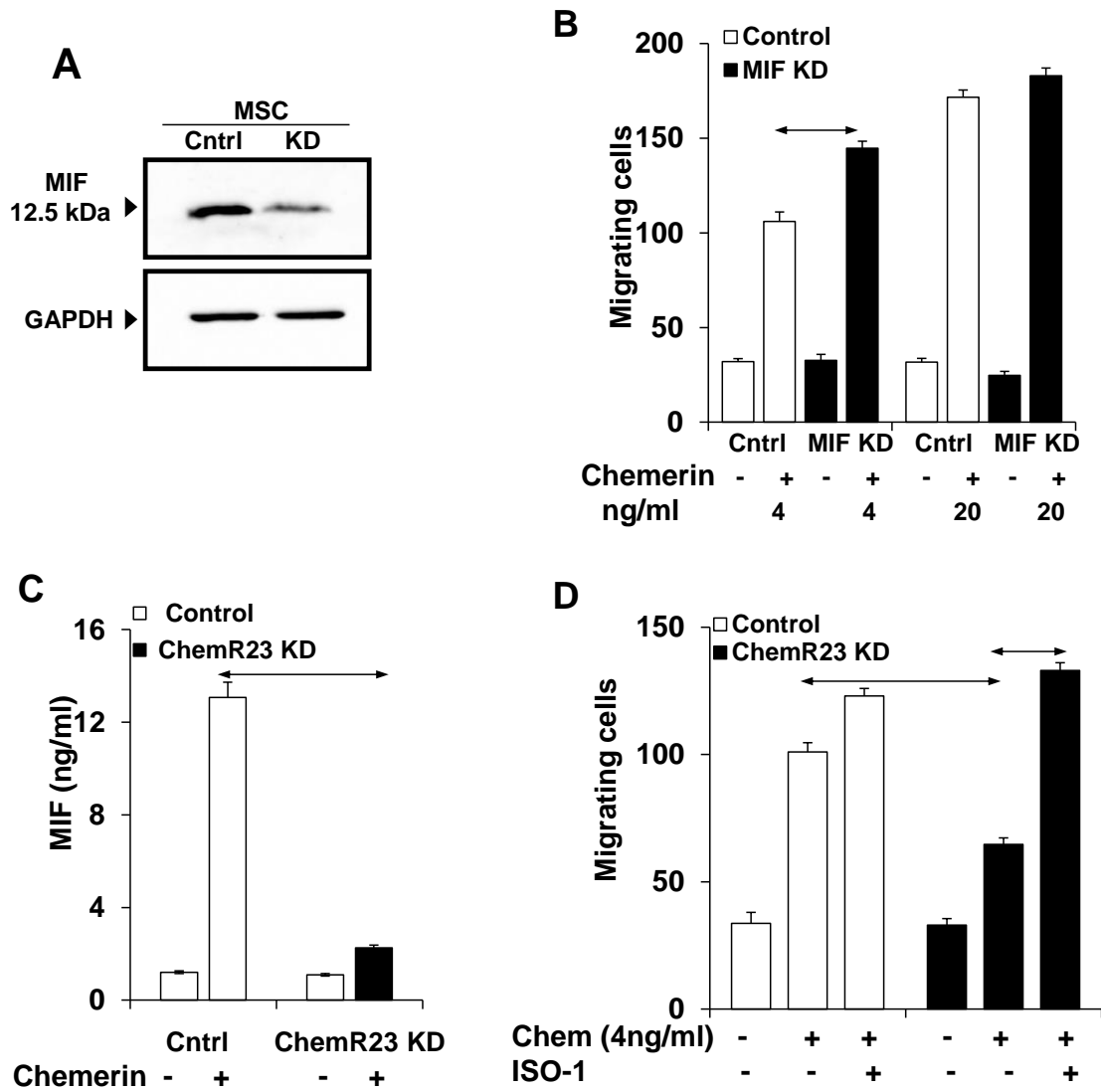
Figure 5.3 In Boyden chamber assays, MSC migration in response to chemerin was significantly increased by MIF knock-down. A) Western blots showing MIF knock-down in MSC cell extracts after siRNA treatment. B) MSC migration in response to 4 ng/ml, but not 20 ng/ml, chemerin was increased by MIF knock-down. C) ChemR23 knock-down decreased MIF release in response to chemerin. D) MSC migration in response chemerin (4 ng/ml) was decreased after chemR23 knock-down in MSCs but was increased by ISO-1 (50µM). Horizontal arrows, $p < 0.05$, ANOVA; vertical bars, SEM.

5.4   Discussion

Initial proteomic studies using SILAC identified increased MIF in chemerin-stimulated MSC media. The main point of the work in this chapter was to validate this observation and explore its biological significance. Thus, studies using ELISA and Western blot confirmed an increase in concentration of MIF in media and cell extracts of MSCs after chemerin stimulation. Chemerin enhanced OE21 cell migration (chapter 3) but, in contrast to MSCs, it did not stimulate MIF secretion in these cells, although it should be noted that basal secretion of MIF is already relatively high in OE21 cells. Using siRNA treatments and the antagonist ISO-1 evidence was obtained to indicate that MIF significantly inhibited MSC migration in response to chemerin.

Interestingly, the present findings indicated that MIF restrained the MSC migratory response to relatively low concentrations of chemerin (4ng/ml) but not higher concentrations (20ng/ml and 100ng/ml). Presumably, maximal secretion of MIF occurs at concentrations of chemerin lower than those that are maximal for migration so that relatively strong chemerin stimulation overwhelms MIF-inhibitory effects. This finding may be relevant in understanding MSC recruitment in cancer. Thus the high concentrations of chemerin in CAM CM promote MSC migration while in CM from ATMs and NTMs, where chemerin concentration is lower, MIF is able to act more effectively as an auto-inhibitor thereby restraining MSC recruitment to normal or preneoplastic tissues (Figure 5.4).

The proposed role of MIF in the present system would appear to be novel. However, the existence of similar phenomena is known for other growth factors in cancer biology. Well known example of biphasic roles is TGF-β which at

physiological concentrations acts as a negative regulator of proliferation of epithelial cells (Blobe, Schiemann et al. 2000).
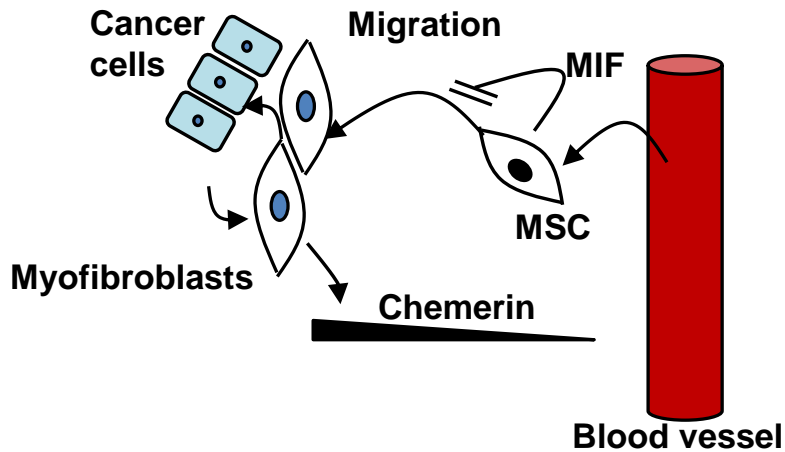
Figure 5.4 MSC migration regulated by chemerin.
Chemerin stimulated MSC migration and MIF secretion. The migration of MSCs was inhibited by autocrine release of MIF only at relatively modest chemerin concentrations.

However, in cancer the neoplastic cells become insensitive to this inhibitory effect of TGF-β and simultaneously increased TGF-β expression promotes tumour growth via stimulating cancer cell invasion and metastasis (Maehara, Kakeji et al. 1999).

The present functional studies were made using both an antagonist for MIF, ISO-1 (Dessein, Stechly et al. 2010) and siRNA, knock-down approaches. A hydrophobic pocket in MIF constitutes a catalytic domain which is important for its tautomerase action on substrates such as asp-hydroxyphenylpyruvic acid. The

binding of ISO-1 at the catalytic site abolishes MIF tautomerase activity and suppresses the production of cytokines, e.g. TNFα (Al-Abed, Dabideen et al. 2005). Previously, it was shown that MIF inhibits migration of MSCs and that this was rescued by ISO-1 *in vitro (*Barrilleaux, Phinney et al. 2009*),* which is in broad agreement with the present findings*.* In addition, though, knock-down studies here using validated MIF siRNA also confirmed the inhibitory effect of MIF on MSC migration. Intriguingly, ISO-1 rescued chemerin-stimulated migration of MSCs pre-treated with chemR23 siRNA. This could suggest chemerin regulation of MIF through a mechanism independent of chemR23, for example via GPR-1 or CCLR2; or alternatively, it may reflect the fact that MIF is an effective inhibitor of MSC migration at low level chemerin stimulation (in this case achieved by receptor knock-down). Further studies to clarify this interpretation would be useful.

The functional significance of MIF in oesophageal cancer is not well understood. However clinical studies showed increased MIF expression in cancer tissues compared to non-cancerous tissues in oesophageal SC by tissue staining and RT-PCR for mRNA expression. Moreover, ELISA has shown elevated levels of MIF in a SC cell line, Eca-109 (Xia, Zhang et al. 2005), while MIF has been shown to regulate angiogenic factors in oesophageal cancer cells, HKESC-1, HKESC-2, such as VEGF and IL-8 suggesting positive regulation of tumour growth (Ren, Law et al. 2005). The current findings using ELISA and Western blot suggest that MIF abundance in media and cell extracts from OE21 cells is not increased by chemerin. A possible explanation could be that expression levels of chemerin receptor, chemR23, that are sufficient to trigger the biological function are inadequate for effective regulation of MIF in OE21 cells. More plausible, however, is that MIF release is already close to maximal and chemerin stimulation

has little or no additional effect. In the future it would be useful to study other validated SC cell lines for MIF secretion such as HKESC-1, HKESC-2 (Ren, Law et al. 2005) and Eca-109 (Xia, Zhang et al. 2005) to determine whether the observations on OE21 cells are representative.

The current findings suggest MSC responses to chemerin are modulated by autocrine effects of MIF and that the relative concentrations of chemerin and MIF determine the migratory response. It will now be necessary to establish the *in vivo* significance of these findings in order to better understand the role of chemerin in carcinogenesis. Nevertheless the data do raise the interesting possibility of an auto regulatory role for MIF production by MSCs that restricts recruitment when chemerin concentrations are relatively low (e.g. from normal and preneoplastic stromal cells) and which is overcome by high chemerin concentrations.

5.5 Conclusions

1. Chemerin effect on MSC is release of chemokine, MIF.

2. Chemerin stimulated MSC migration was inhibited by MIF.

3. The regulatory effect of MIF in CM might depend on the relative abundance of chemerin to MIF in media.

Chapter-6

Characterisation of chemerin-stimulated transendothelial migration using a ChemR23 antagonist

6.1   Introduction

The potential role of chemerin in MSC recruitment to oesophageal cancers was raised in chapter 4. Further, MIF expression by MSCs was identified in chapter 5 as an effect of chemerin which was proposed to supress MSC recruitment to sites of moderate or low chemerin production. However, there remains the question of whether MSC recruitment by chemerin also includes stimulation of transendothelial migration (given that MSCs are presumably recruited from the circulation). While this study was in progress a chemR23 antagonist became available providing additional opportunities to study the role of chemerin in MSC migration and in transendothelial migration in particular. First, however, it was considered necessary to establish that the antagonist (CCX832) inhibited chemerin effects in the assays that had already been employed in this study.

The extravasation of MSCs through endothelial monolayers is regarded as an integral part of recruitment to tumours. This process is thought to be regulated by several factors including MMP-2 and MMP-9 (Vande Broek, Asosingh et al. 2004; De Becker, Van Hummelen et al. 2007; Ries, Egea et al. 2007). Previous work in our group using SILAC-based proteomics (Holmberg & Varro, unpublished observations) identified increased MMP-2 secretion in IGF-II stimulated MSCs. IGF-II is a relatively well-established chemotactic growth factor secreted by CAMs (Holmberg, Quante et al. 2012), and was shown in chapter 4 to strongly stimulate MSC migration. It was therefore hypothesised that chemerin stimulated MSC transendothelial migration at least in part by a mechanism involving MMP-2.

The present studies made use of the chemR23 antagonist, CCX832, generated by a collaborator, Chemocentryx, who generously provided a sample (ChemoCentryx 2011). To the best of our knowledge there are, so far, no published studies of the effect of chemR23 antagonists in oesophageal SC. Hence, CCX832 was used firstly in validation studies employing assays used earlier and then secondly to directly study chemerin/ChemR23 interactions in MSC transendothelial migration.

6.1.1   Objectives

The specific objectives were:

1. To characterise the action of the chemR23 antagonist CCX832 in inhibiting chemerin-stimulated MSC and OE21 cell migration *in vitro*.

2. To use CCX832 to study the effect of chemerin and CM in stimulating transendothelial migration by MSCs using a Matrigel-coated invasion chamber.

3. To determine the role of MMP-2 in transendothelial migration of MSCs in response to chemerin.

6.2   Material and Methods

6.2.1   Cell culture

MSCs and OE21 cells were cultured as described in sections 2.3.2 and 2.3.3 respectively.

6.2.2   Boyden chamber migration assays

The effect of CCX832 (0.1nM - 1µM), and a control compound CCX826 (1 µM), on MSC and OE21 cell migration was investigated using Boyden chamber as described in section 2.6.1.1. The bottom well contained 750µl serum-free media, CM from CAM4, chemerin or chemerin-9 (6.25nM of chemerin-9 equivalent to 100ng/ml chemerin) with or without CCX832 or CCX826.

6.2.3   Scratch wound healing assays

Wound healing assays were performed with OE21 cells to determine the effect of CCX832 (1 µM) on chemerin-9 stimulated migration (6.25nM) as described in sections 2.6.1.2 and 2.6.1.3.

6.2.4   Labelling of MSCs

MSCs were labelled using the fluorescent membrane dye, PKH67, and validated for functional studies. The labelling has been described in section 2.13.1.3.

6.2.5   Transendothelial migration assays

The effect of CCX832 (1µM) and CCX826 (1 µM) on transendothelial migration of labelled MSCs across a monolayer of HUVECs overlaid on Matrigel coated membrane was investigated using BD BioCoat™ Matrigel™ invasion chambers as described in section 2.6.1.3. Furthermore, chemerin-9 (6.25nM) and CAM4

CM-stimulated transendothelial migration of labelled MSCs was investigated using a chemerin neutralising antibody. The effect of human recombinant MMP-2 in these assays was studied using specific inhibitor for MMP-2 (MMP-2 inhibitor I) (Holmberg, Ghesquiere et al. 2013).

6.2.6   MMP activity assay

Enzyme activity assays for MMP-2 were performed using the selective substrate MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$ and CM from chemerin-stimulated MSCs as described in section 2.18.

6.3   Results

6.3.1 The ChemR23 antagonist, CCX832, inhibits chemerin-stimulated MSC and OE21 cell migration

Initially, the action of CCX832 against both full length chemerin and its C-terminal active fragment chemerin-9 was evaluated in both Boyden chamber and scratch wound migration assays. In Boyden chamber assays, chemerin-9 (6.25nM) exhibited similar efficacy to the recombinant full length chemerin (100ng/ml) for stimulation of MSC migration (Figure 6.1 A). MSC migration in response to chemerin-9 was inhibited in a dose-dependent manner by CCX832 (Figure 6.1 B & C). At a concentration of 1µM (which was the highest concentration of CCX832 used) the control compound CCX826 had no effect (Figure 6.1 D). Furthermore, CCX832 and AF2325 (chemerin neutralising antibody) also inhibited significantly CAM CM-induced MSC migration (Figure 6.1 E). Chemerin-9 stimulation of OE21 cell migration in Boyden chamber assays was also reversed in a dose-dependent manner by CCX832 (Figure 6.2 A & B). Moreover, CCX832 (1µM) and chemerin neutralising antibody, AF2325, inhibited CAM CM-stimulated OE21 cell migration (Figure 6.2. C). In wound healing assays, CCX832 (1µM) inhibited also OE21 cell migration in response to C-9 while the control compound, CCX826, had no effect (Figure 6.2 D)
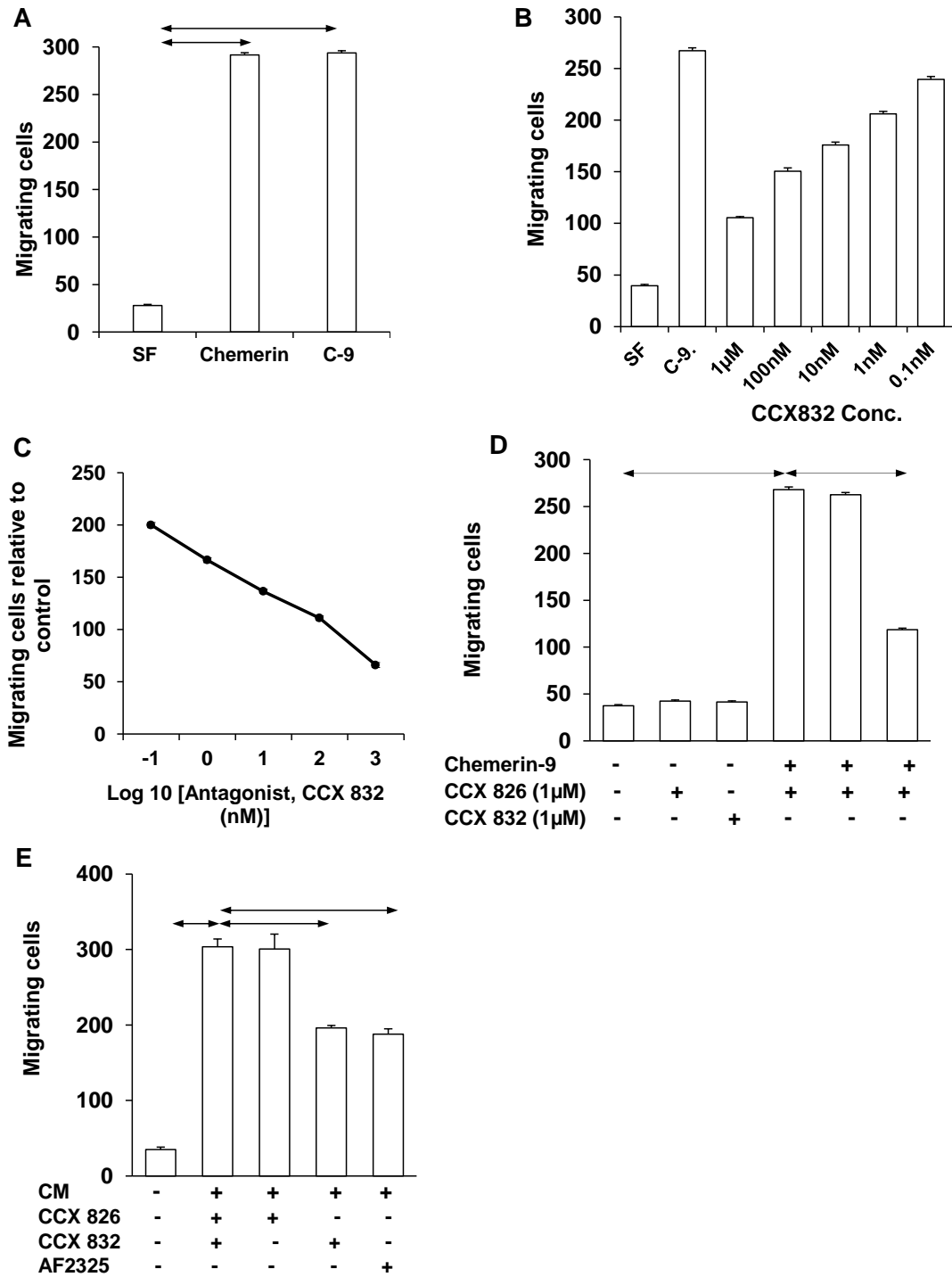
Figure 6.1 In Boyden chamber migration assays, the chemR23 antagonist CCX832 significantly inhibited MSC migration. A) Chemerin-9 (C-9) and mature chemerin-157 (chemerin) similarly stimulated MSC migration. B) Dose-dependent inhibition of MSC migration in response to chemerin-9 by CCX832 (0, 0.1nM, 1nM, 10nM, 100nM, and 1µM) (n=3). C) Data from panel B with basal subtracted. D) CCX832 (1µM) significantly inhibited chemerin-9 stimulation of MSC migration while the control, compound CCX826 (1 µM) did not (n=3). E) CCX832 (1µM) and chemerin neutralising antibody, AF2325, inhibited MSC migration in response to CAM CM while the control compound, CCX826 (1 µM), did not. Horizontal arrows, $p < 0.05$, t- test; vertical bars, SEM.
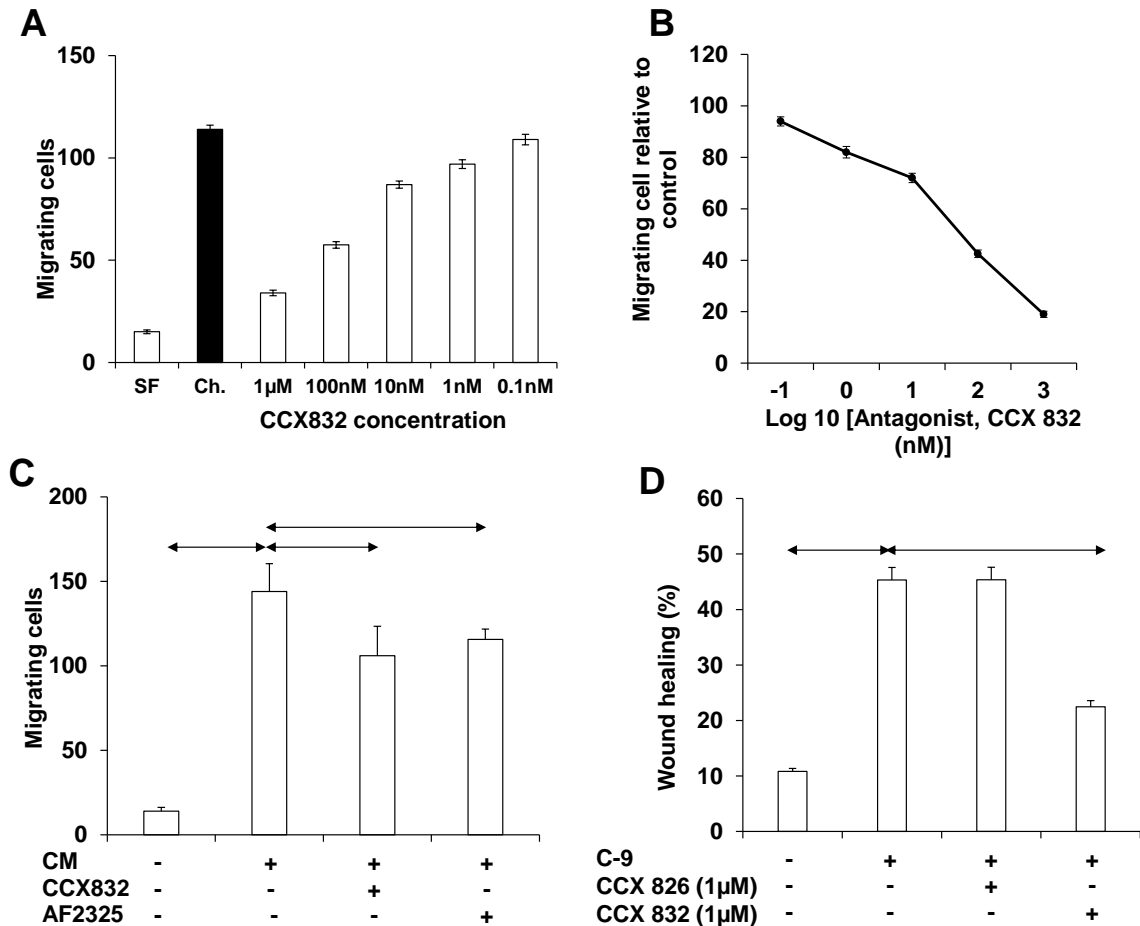
Figure 6.2 The chemR23 antagonist, CCX832, significantly inhibited OE21 cell migration in Boyden chamber and scratch wound assays. A) Dose-dependent inhibition of OE21 migration in response to CCX832 (0, 0.1nM, 1nM, 10nM, 100nM, and 1μM). B) The data from panel A with basal subtracted. C) CCX832 (1μM) and chemerin neutralising antibody, AF2325, inhibited OE21 migration in response to CM. D) In wound healing assay, CCX832 (1μM) inhibited OE21 migration while the control compound CCX826 (1 μM) did not. Horizontal arrows, p<0.05, t- test; vertical bars, SEM.

6.3.2 Chemerin stimulates transendothelial migration of MSCs via ChemR23

MSCs labelled with the membrane-inserting dye, PKH67 (Figure 6.3 A) were then used to investigate the ability of MSCs to migrate through a HUVEC monolayer (transendothelial migration). First, we confirmed using Boyden chamber assays that migration in response to chemerin-9 was similar for labelled and unlabelled cells (Figure 6.3 B). Chemerin-9 and CM from CAM4 stimulated transendothelial migration of MSCs and in both cases the response was significantly inhibited by both CCX832 (1µM) and chemerin neutralising antibody, AF2325 (Figure 6.3 C, D & E) while the control compound, CCX826, had no effect (Figure 6.3 D & E).

6.3.3 Chemerin stimulated transendothelial migration of MSCs requires MMP-2

Previous data indicated expression of MMP-2 by MSCs and increased abundance in the media after growth factor stimulation (IGF-II). We considered the possibility that chemerin might stimulate MMP-2 release that was required for MSC transendothelial migration. Initially, then, enzyme activity was shown to be significantly increased in the medium in response to chemerin and IGF-II stimulation of MSCs (Figure 6.4 A). Human recombinant MMP-2 was then shown to stimulate MSC transendothelial migration and the response was inhibited in a dose-dependent manner by a selective MMP-2 inhibitor (MMP-2 inhibitor I) (Holmberg, Ghesquiere et al. 2013). Since the MMP-2 inhibitor at 60.18 µM was found to have no further inhibitory effect compared to 20.16 µM in human MMP-2 stimulated MSC migration. (Figure 6.4 B) we used this concentration in further studies. Chemerin and IGF-II stimulated MSC transendothelial migration was significantly inhibited by the MMP-2 inhibitor at this concentration (Figure 6.4 C).
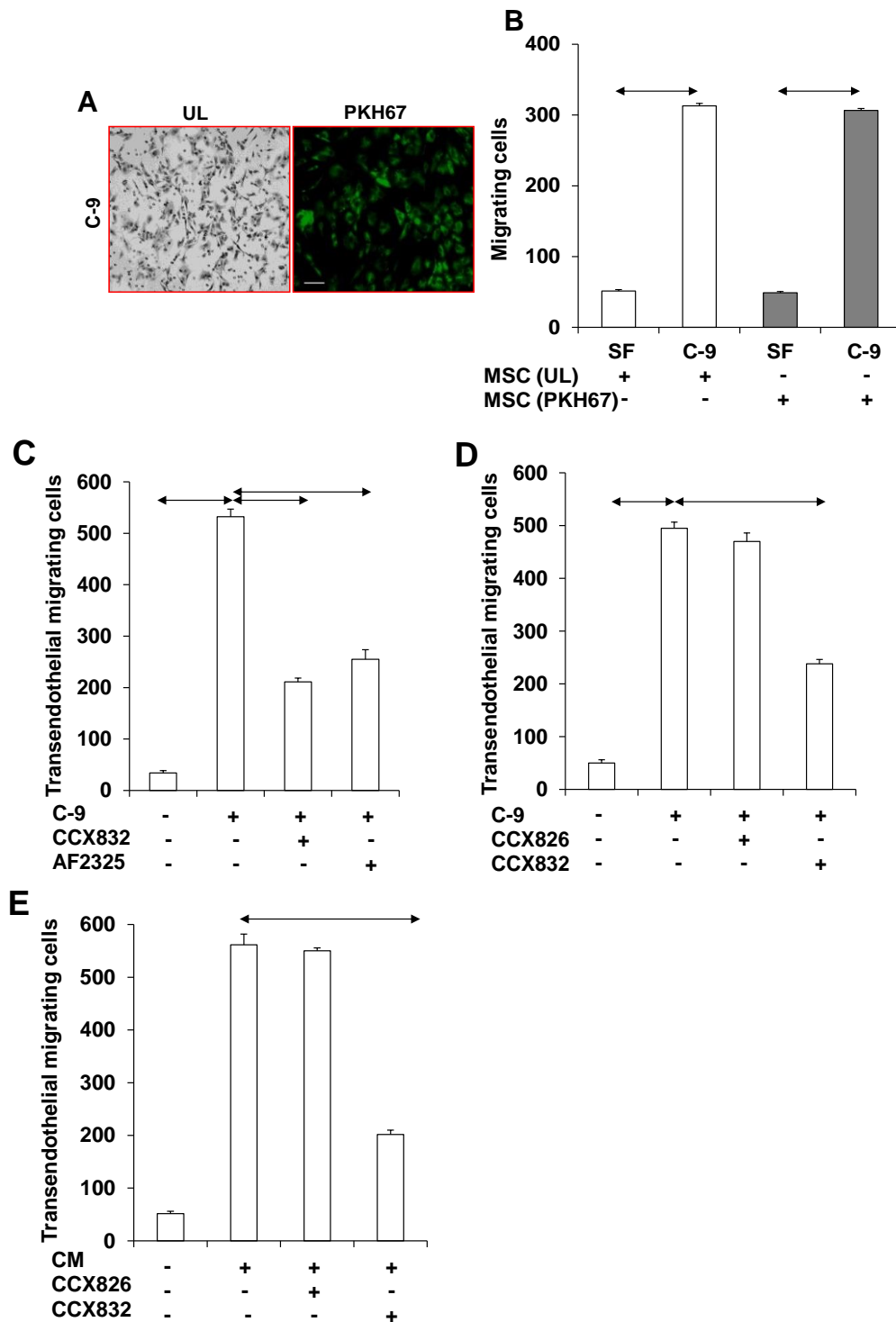
Figure 6.3 The chemR23 antagonist, CCX832, significantly inhibited MSC transendothelial migration. A) Representative fields from transendothelial MSC migration experiments showing migrating unlabelled (left panel) and labelled MSCs (right panel). B) Membrane labelling did not effected MSC migration in response to chemerin-9 (C-9) compared to unlabelled MSCs. C) CCX832 and chemerin neutralising antibody, AF2325, inhibited MSC transendothelial migration stimulated by chemerin-9 D) The inactive compound CCX826 (1 µM) did not affect chemerin-9 stimulated MSC transendothelial migration. E) Conditioned media (CM) from CAMs stimulated MSC transendothelial migration and this was significantly inhibited by CCX832 (1µM) but not CCX826 (1 µM). Horizontal arrows, p<0.05, t- test; vertical bars, SEM.
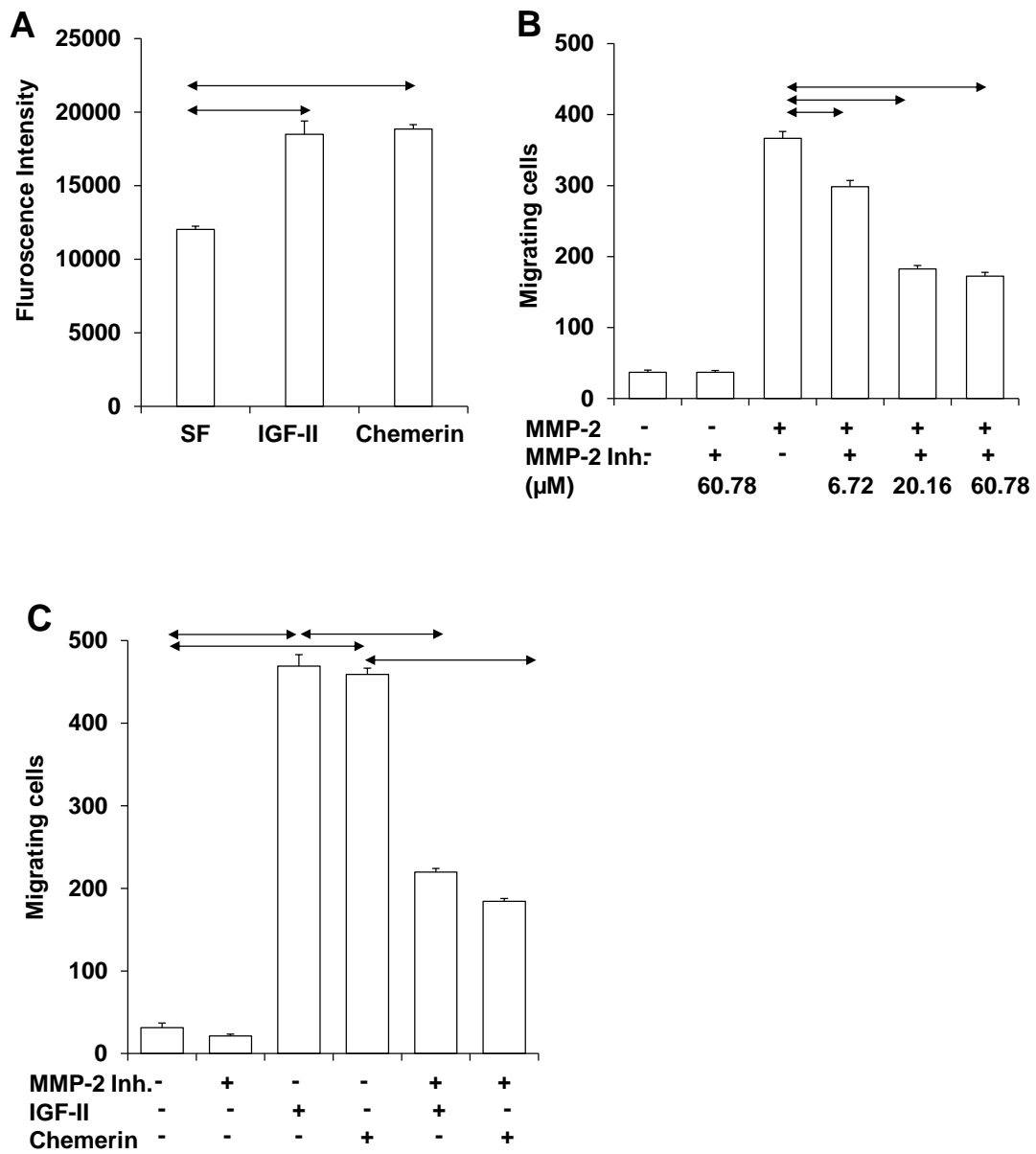
Figure 6.4 MMP-2 is released by chemerin and stimulates transendothelial migration of MSCs. A) IGF-II and chemerin significantly increased MMP-2 enzyme activity in MSC media detected by the selective substrate MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2. B) MMP-2 stimulates transendothelial migration and there is dose-dependent inhibition by an MMP-2 selective inhibitor (MMP-2 inhibitor I). C) An MMP-2 inhibitor significantly inhibited IGF-II and chemerin stimulated MSC transendothelial migration. Horizontal arrows, $p < 0.05$, t- test; vertical bars, SEM.

6.4   Discussion

In chapters 4 and 5 chemerin-regulated MSC migration was characterised. Since circulating MSCs presumably gain access to tissues after crossing the endothelium, an assay was then established to study transendothelial migration of these cells *in vitro*. Both chemerin and CM were shown to stimulate transendothelial migration of MSCs. Transendothelial migration involves MMP-2, and in addition chemerin was shown to stimulate MMP-2 secretion suggesting a molecular mechanism for MSC extravasation. Characterisation of a chemR23 antagonist, CCX832, confirmed the role of chemerin/chemR23 interactions in CM and chemerin-stimulated MSC and OE21 migration, and this compound was then used to establish a role for these interactions in MSC transendothelial migration. The data presented here are the first of their kind to study chemR23 function using CCX832 in oesophageal cells and in MSCs. The validation of CCX832 in these systems provides a basis for future *in vivo* studies.

Transendothelial migration of MSCs requires protease activity for passage of the cells across the endothelial barrier. The activity of MMP-2 in MSC media was measured using a selective fluorogenic substrate, MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$, (Holmberg, Ghesquiere et al. 2013) and shown to be increased in response to chemerin. Others have previously demonstrated increased MMP-2, MT1-MMP and MMP-9 levels in MSCs in response to inflammatory cytokines, TNF-α and IL-1β (Ries, Egea et al. 2007). Moreover, these proteases, along with protease inhibitor, TIMP-3 regulate the chemotaxis of BM-derived MSCs to the matrix environment (De Becker, Van Hummelen et al. 2007; Ries, Egea et al. 2007). Our findings are in agreement with previous studies showing MMP-2 induce MSC transendothelial migration (De Becker, Van Hummelen et al. 2007; Ries, Egea et al. 2007). Chemerin also increases MMP-2 and MMP-9 in

endothelial cells (Kaur, Adya et al. 2010). In the future it will be necessary to expand the present studies including, for example, using gelatin zymography to determine the ability of chemerin to activate other gelatinases (eg MMP-9) in MSCs. There is also a need for Western blots to probe active and inactive MMP-2, and other MMPs, in both cell extracts and media in response to chemerin.

In chapters 4 and 5, chemerin-stimulated cell migration was studied using knockdown and overexpression approaches. Although the findings were robust and reproducible, these approaches do not lend themselves to future studies *in vivo*. However, the use of a chemR23 antagonist, CCX832, suggests a novel method to target chemerin-chemR23 interactions involving MSC function *in vivo*. Previously, CCX832 has been used in studies of psoriasis (ChemoCentryx 2011) but so far no studies of the compound in cancer have been published. Nevertheless the use of other antagonists targeting GPCRs in cancer is well established; for example, a recognised target in prostate cancer is the gonadotropin releasing hormone receptor (GnRH) which regulates testosterone and dihydrotestosterone and can be blocked by antagonists resulting in impaired cancer cell proliferation (Cook and Sheridan 2000; Pommerville and de Boer 2010). Other examples include studies associated with increased chemokine CCL5 expression in breast cancers and targeting the receptor, CCR5, by the antagonist maraviroc which attenuates invasion of breast cancer cell *in vitro* and *in vivo* (Velasco-Velazquez, Jiao et al. 2012*)*. In these cases, the targets were cancer cells themselves, rather than the tumour microenvironment.

Our results have shown for first time that blocking chemR23 decreases transendothelial migration of MSCs *in vitro* and clearly it will now become interesting and important to devise experiments to test the effects *in vivo*.

The present studies have shown chemerin-stimulated OE21 cell migration was also inhibited by CCX832. These findings therefore suggest a potential paracrine signalling pathway in oesophageal cancer cells involving myofibroblasts that might be exploited therapeutically. Other studies in oesophageal cancer cells that involve targeting of GPCRs include the findings of EGF/EGFR transactivation of β-adrenoceptors (Liu, Wu et al. 2008) and in particular the demonstration that EGF stimulated oesophageal SC cell (HKESC-1) proliferation was inhibited by Atenolol and ICI 118,551 which are antagonists of β-adrenoceptor (Liu, Wu et al. 2008). Although not extensively studied, increased OE21 cell proliferation was observed in response to chemerin (data not shown). Thus it would now be interesting to study the blocking effect of CCX832 on OE21 cell proliferation in response to chemerin.

These present findings confirm the role of chemR23 in MSC and OE21 cell migration and they extend these findings by demonstrating a role in transendothelial transit. It now becomes important to ascertain whether chemerin-stimulated cancer cell migration, or recruitment of MSCs to the oesophageal cancer niche, is important *in vivo*. Xenograft studies in mice using various strategies to track MSCs (Kang, Shin et al. 2012) and by targeting ligand-GPCR axis or biomolecules modulation may help here (De Becker, Van Hummelen et al. 2007; Placencio, Li et al. 2010). Moreover, the *in vitro* validation of CCX832 provides a platform for future xenograft experiments to study chemR23 more generally in tumour growth. These studies are now underway. Potentially the data from such studies will indicate whether this is an appropriate candidate for cancer drug development.

6.5   Conclusions

1. Chem23 antagonist, CCX832, inhibited chemerin-stimulated MSC and OE21 cell migration.

2. Chemerin-stimulated transendothelial migration of MSCs was inhibited by CCX832.

3. Chemerin-stimulated secretion of active MMP-2 from MSCs was required for MSC transendothelial migration.

Chapter 7

Final Discussion

7.1   Overview

The main findings of this thesis are that (a) oesophageal CAMs are functionally distinguishable from their paired ATMs in both SC and AC, (b) conditioned media from oesophageal CAMs increases cancer cell migration and proliferation compared to CM from ATMs, (c) there is increased chemerin abundance in SC CAMs compared to their respective ATMs and (d) chemerin stimulates migration of chemR23- expressing MSC and OE21 cells. The migration of MSCs is mediated through chemerin-chemR23 dependent activation of PKC, p44/42, p38 and JNK-II pathways. In addition, (e) chemerin increases MIF secretion by MSCs which tends to reduce the migratory response at low chemerin concentrations and (f) migration of MSCs across a monolayer of endothelial cells was also stimulated by chemerin and required induction of MMP-2. The effect of chemerin MSC transendothelial migration was reversed by a chemR23 antagonist, CCX832, which provides a basis for future *in vivo* studies.

7.1.1   Myofibroblasts and the tumour microenvironment

7.1.1.1 CAMs are functionally distinct from ATMs

Morphology and expression of markers such as α-SMA and vimentin confirmed the CAMs and ATMs used in the present studies as myofibroblasts. These are phenotypically different from other stromal cells and are considered to be subset of fibroblasts (Gabbiani, Ryan et al. 1971). Previously, heterogeneity of myofibroblasts has been associated with a role in tissue organisation (Powell et al., 1999). The present study on oesophageal myofibroblasts, and previous studies of gastric, breast and oesophageal myofibroblasts have shown CAMs to

be more aggressive than their corresponding ATMs, or myofibroblasts from normal tissue (Holmberg, Quante et al. 2012) (Noma, Smalley et al. 2008; Peng, Zhao et al. 2013).. The differences are evident both in vitro, for example with respect to proliferation and migration, and in vivo when xenografts composed of co-injected cancers cells and myofibroblast/CAMs are studied (Quante, Tu et al. 2011).

What mechanisms account for the differences between CAMs, ATMs and NTMs? Previously, hypomethylation was identified in gastric CAMs which might account for differences in gene expression (Jiang, Gonda et al. 2008) and in protein secretion (Balabanova 2012). In addition, the profiling of miRNA in gastric myofibroblasts showed differential regulation in CAMs which might change protein expression (Wang, 2013). At the phenotypic level, the activation of proteolytic systems (Bernstein, Twining et al. 2007; Holmberg, Ghesquiere et al. 2013), differences in NHE1 activity (Czepan, Rakonczay et al. 2012) and in IGF-II secretion (Grotendorst, Rahmanie et al. 2004) could all contribute to increased myofibroblast proliferation and migration.

Because myofibroblasts critically influence the microenvironment it seems possible that they are rate limiting factors in determining disease progression via their rich pool of extracellular secreted proteins involved in autocrine and paracrine signalling (Powell, Mifflin et al. 1999; Kalluri and Zeisberg 2006; Hinz, Phan et al. 2007). In addition, the recent recognition of polyclonal tumour origins was hypothesised to be due to paracrine signalling from secreted factors such as Wnt-1 from myofibroblasts (Thliverisa A. T. et al. 2013). Collectively, these signalling mechanisms control the growth and invasiveness of cancer cells, infiltration of MSCs, and differentiation of various cells including myofibroblasts

themselves (Hinz, Phan et al. 2007; De Wever, Demetter et al. 2008; Quante, Tu et al. 2011).

7.1.1.2 Differential secretory profile of CAMs vs ATMs: chemerin is a novel example

Proteomic analyses of CM from oesophageal myofibroblasts (Holmberg & Varro, unpublished data) have revealed an impressive array of secreted proteins. The list includes families of proteases, chemokines and inflammatory cytokines that could play crucial roles in cancer progression. Other studies have shown increased SDF-1 in CAMs that might encourage angiogenesis and tumour growth as well as MSC recruitment (Orimo, Gupta et al. 2005; Quante, Tu et al. 2011; Jung, Kim et al. 2013). Similarly, compared to NTMs increased Wnt-2 was reported in oesophageal CAMs and shown to regulate the invasiveness of cancer cells (Fu, Zhang et al. 2011). The present validation of a novel target in the oesophageal CAM secretome, chemerin, should therefore be seen as a further (previously unsuspected) example of differential expression of signalling molecules. Interestingly, there was no marked difference in chemerin transcripts observed in microarray datasets suggesting the existence of post transcriptional mechanisms regulating expression, possibly due to differential miRNA expression (Wang 2013). There is extensive evidence of post-translational processing of chemerin that determines biological activity. The precise processing mechanisms in myofibroblasts have not yet been addressed and this question should now be resolved. However, prochemerin is known to be processed by various enzymes, such as elastase, tyrptase, and plasmin at least some of these may also be produced by myofibroblasts indicating that the machinery for post-translational processing is present in these cells (Du and Leung 2009). Interestingly, chemerin

was not detected in adenocarcinoma myofibroblasts despite the presence of gene transcripts. This might be attributed to its complete enzymatic degradation in AC and studies of these are now required.

7.1.2 Biological outcomes of chemokine-ChemR23 interactions

7.1.2.1 Chemokine-ChemR23

Recruitment of inflammatory cells and MSCs is an important step in inflammation-driven cancers and is orchestrated to a large extent by chemokines. Chemokines such as SDF-1/CXCL12 (Levesque, Hendy et al. 2003) and MCP-1 to MCP-4 (Uguccioni, D'Apuzzo et al. 1995) are recognised as potent chemoattractants. Studies have also demonstrated chemokine (C-C motif) ligand 2 (CCL-2)/MCP-1 (Lin, Chuang et al. 2012) and SDF-1/CXCL12 derived from CAMs (Orimo, Gupta et al. 2005) could interact with epithelial and other stromal cells in microenvironment, macrophages and endothelial cells. The interaction is crucial for triggering cell proliferation, angiogenesis and downstream activation of other chemokines, cytokines and proteases (Balkwill 2004).

In this study, chemerin was confirmed to be approximately equally efficacious as IGF-II as a chemoattractant for MSCs and cancer cells. Nevertheless the function of chemokines is not restricted to chemotaxis. Indeed, chemerin has been shown to be responsible for cell differentiation and proliferation as well (Muruganandan, Roman et al. 2010; Yang, Li et al. 2012). For example, chemerin induces adipocyte differentiation, clonal expansion, and basal proliferation of bone marrow MSCs (Muruganandan, Roman et al. 2010; Muruganandan, Parlee et al. 2011). Stimulation of proliferation was not studied in detail, but chemerin shown in pilot

experiments to increase cancer cell proliferation. In addition, differentiation of MSCs into adipocytes was confirmed in response to chemerin (data not shown). In addition, in response to CAM CM and chemerin, the specific marker study of osteogenic (Alizarin Red staining), chondrogenic (Alcian Staining) and adipogenic (Oil Red O) differentiation of MSC might suggest chemerin inducing differentiation capability. However, the relevance of this findings remains uncertain given that in oesophageal cancer adipocytes in the stromal environment are unlikely to be functionally important, in contrast to some other cancers notably breast cancer (Dirat, Bochet et al. 2011).

The GPCRs, chemR23 and GPR1, have previously been shown to interact with chemerin in various cell types (Barnea, Strapps et al. 2008; Huang, Zhang et al. 2010). Other studies showed chemerin also binds efficiently to CCLR2 (Zabel, Nakae et al. 2008; Otero, Vecchi et al. 2010). In this study, chemR23 and GPR1 expression was confirmed at the transcript level, and also by immunohistochemistry, in MSCs and oesophageal cancer cells. However, the functionally significant chemerin interaction was shown to be with chemR23. Although interactions with the two receptors might have varying biological significance the current findings indicate that chemerin stimulation of MSC migration requires only chemR23. Other studies also confirmed the chemerin/chemR23 axis is primary to transduce signals and to execute the cell functions (Huang, Zhang et al. 2010). Despite equal binding affinity to GPR1 and CCLR2, it may be that these only amplify the preceding effects on chemotaxis for example by increasing local availability of chemokine to cells expressing primary receptors (Zabel, Nakae et al. 2008; Otero, Vecchi et al. 2010).

7.1.2.2 Chemerin signalling in migration

The various downstream pathways involved in chemokine signalling include activation of PKC, PI3K/Akt, MAPK, Src, and Jak/STAT and activation of Rac and Rho GTPase (Raftopoulou and Hall 2004; O'Hayre, Salanga et al. 2008). Chemerin triggers p44/42 and Akt in chrodocytes (Berg, Sveinbjornsson et al. 2010), and p44/42, p38 and Akt in fibroblast-like synoviocytes (Kaneko, Miyabe et al. 2011); it also increases calcium influx and inhibits cAMP in endothelial cells (Hart and Greaves 2010) all linked to cell motility. Here, chemerin induced MSC cell migration via increased phosphorylation of p44/42, p38 and JNK-II, while PI3k/Akt was not crucial for chemotaxis notwithstanding the finding that PI3K and Akt are considered key signalling mediators in adhesion of macrophages (Hart and Greaves 2010). Chemerin increases calcium influx and triggeres MAPKs pathways via Gi family members regulating dendritic cell chemotaxis (Wittamer, Franssen et al. 2003). It would be worth examining the role of calcium influx in MSCs in the future. The activation of p44/42, p38 and JNK-II was presumably part of a cascade following PKC activation as occurs in various other cell functions including migration (O'Hayre, Salanga et al. 2008). Pharmacological inhibitors showed partial PKC-dependent downstream MPAKs activation, but there also seems to be PKC independent responses that sustain activation. This raises the possibility of cross talk following transactivation of RTK downstream of GPCR activation which should be further studied. In this context, and based on earlier studies of chemokine/GPCR interaction, it would be useful to determine whether there is activation of proteases such as ADAM's that cleave and release active EGF ligands to trigger intracellular pathways via EGFR (Gschwind, Zwick et al. 2001).

7.1.2.3 Chemerin, cytokines and proteases

Chemerin is known to enhance chondrocyte chemotaxis via increased secretion of TNF-α, interleukin-6 and interleukin 8 (Berg, Sveinbjornsson et al. 2010). Other studies have confirmed TNF-α and IL-6 strongly stimulate MSC migration (Fu, Han et al. 2009; Anton, Banerjee et al. 2012). Chemokines such as osteopontin have also been shown to induce MSC-derived CCL5 to enhance migration and increased cancer cell invasion (Mi, Bhattacharya et al. 2011). In the present study, chemerin was shown to target MIF and MMP-2 in MSCs, which has not been previously demonstrated (Figure 7.1).

The inflammatory cytokine, MIF, exerts a dual role both intracellular and extracellular which are considered to be anti- and pro-tumorigenic, respectively (Verjans, Noetzel et al. 2009). This is reminiscent of the dual function of TGF-β which inhibits cell migration in normal tissue, but in cancer it acts as a tumour enhancer (Derynck, Akhurst et al. 2001). The present *in vitro* findings indicated that MIF inhibition of MSC migration was exhibited only at moderate chemerin concentrations. This suggests the strength of the stimulus provided by chemerin decides the degree to which MSCs are recruited*.*

It is well-known that for cells to cross the endothelial monolayer (either in recruitment or in metastatic migration) there is a requirement for MMP activity (Przybylo and Radisky 2007). Chemerin was shown to induce MMP-2 secretion in MSCs, which extends studies showing stimulation of MMP-1, MMP-3, MMP-8 and MMP-13 (Berg, Sveinbjornsson et al. 2010) and MMP-9 (Kaur, Adya et al. 2010) in other cells.

Previously MMP-2 and also MMP-14 were shown to be crucial for MSC migration *in vitro* (De Becker, Van Hummelen et al. 2007; Lu, Li et al. 2010). MMP-2 targets laminin and collagen which are constituents of BM and ECM (Giannelli, Falk-Marzillier et al. 1997). Moreover, the co-localisation of MMP-2 with integrin has been shown at the leading edge of the migrating cells (Ogier, Bernard et al. 2006). A similar mechanism is considered to control the MSC migration across endothelial monolayers and suggests a mechanistic target for *in vivo* MSC homing in microenvironment. The existence of other proteases in this process cannot be ruled out. For example, MMP-9 and MMP-14 shown to increase cell migration via interacting with integrin (Egeblad and Werb 2002; Sabeh, Ota et al. 2004).
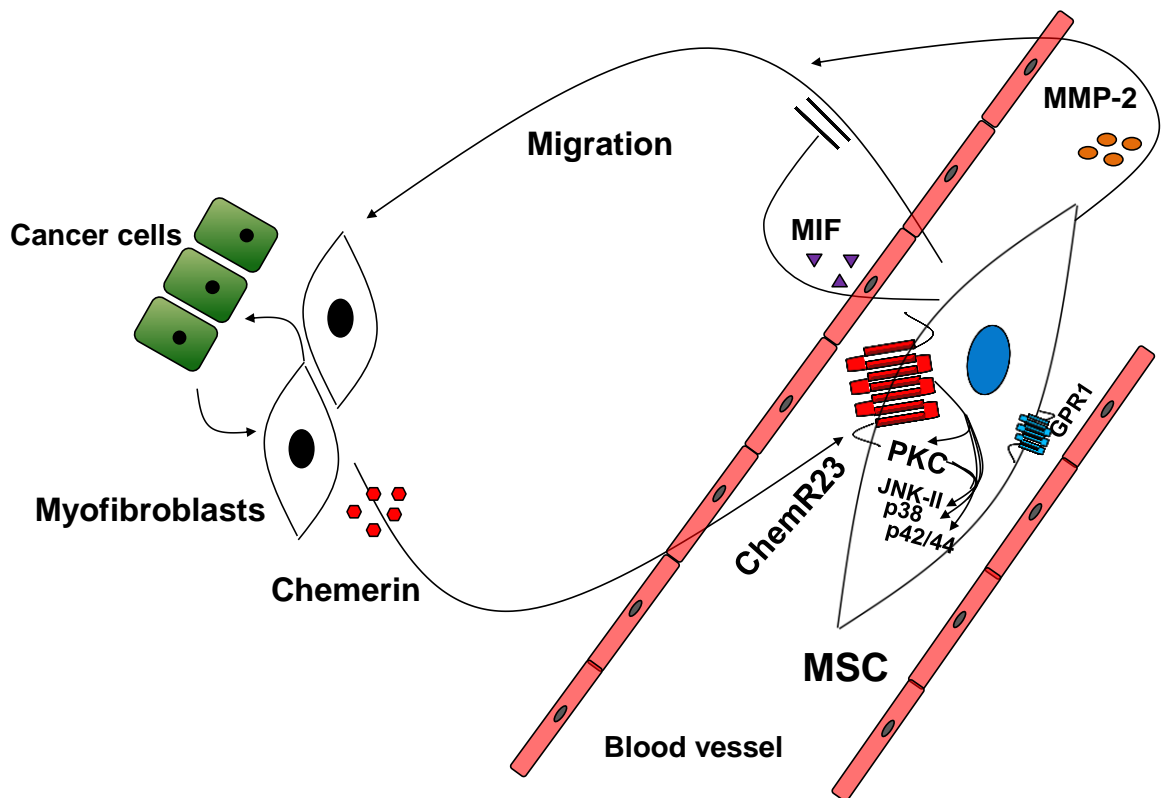
Figure 7.1 Novel stromal cell signalling for MSC migration.
Schematic showing cancer associated myofibroblasts secretion of chemerin and stimulation of MSC migration via chemR23. The chemerin/chemR23 interaction activates PKC and MAPKs pathways. Chemerin targets in MSCs include MIF and MMP-2 which enhance transendothelial migration.

7.1.2.4 Targeting chemerin-ChemR23 interactions

The identification of chemokine-receptor interactions opens up the prospect of therapeutic intervention. For example the antagonist maraviroc for CCR5 decreases tumour growth in response to CCL5 (Velasco-Velazquez, Jiao et al. 2012). In this study, the chemR23 antagonist, CCX832, was shown to significantly inhibit chemerin stimulated MSC and cancer cell migration. Very recently, the supplier of CCX832, ChemoCentryx, have reported that this compound antagonises the effect of chemerin in stimulating human monocyte migration (Watts, Dorrance et al. 2013). The binding affinity of CCX832 was significantly higher for chemR23 than GPR1 and CCLR2, (Watts, Dorrance et al. 2013) thereby confirming its specificity. Together these two studies provide a platform for future *in vivo* work. In particular it now becomes possible to address the specific question of whether chemerin acts as a chemoattractant for MSCs *in vivo*, as well as more generally to determine whether chemerin influences tumour progression.

7.2 Methodology

The strong positive correlation of increased CAMs and tumour growth has been established (Orimo and Weinberg 2006). The use of primary cells in our research is considered to provide advantages over immortalised myofibroblasts and fibroblast cell lines (Pan, Kumar et al. 2009). There are limitations, however; using primary cells for research tends to be associated with small sample sizes and limited life span (passages) of the cells. There is also a concern that even primary myofibroblasts may have adapted to culture conditions. Nevertheless several studies have shown that co-injection of CAMs and cancer cells in mice stimulates xenograft growth supporting the idea that CAMs do indeed promote cancer

progression and that this property is retained when the cells are expanded in vitro (Hwang, Moore et al. 2008; Holmberg, Quante et al. 2012).

The identification of chemerin abundance was by the robust proteomic technique of iTRAQ (Holmberg & Varro, unpublished observations). Further, Western blot and ELISA was used to validate chemerin abundance in cultured myofibroblast cell extracts and media. Other approaches, such as IHC for primary tumour tissues would provide data on protein expression in tissues. The exploitation for selective cell type from clinical samples for gene expression could provide better chances for prognostic and diagnostic markers. For studies of the expression of specific genes in a defined cell type in a clinical samples, it would be possible to use laser capture microdissection (Gillespie, Ahram et al. 2001). This allows targeting of cells with defined properties and that after microdissected can be processed for either gene microarray or RT-PCR (Bonner, Emmert-Buck et al. 1997).

The present study made extensive use of different *in vitro* assays for proliferation and migration of cells in response to CM. The proliferation was studied by labelling cells in S phase (EdU incorporation). Migration was studied both by Boyden chamber and by wound healing assays. The cell functions of proliferation and migration reflect integrative responses of host cells (Teoh and Anderson 1997). However, it would be useful to extend this to the study of cells in ECM. Thus an *in vitro* 3D culture system might be an approach to investigate cell behaviour in response to environment mimicking the possible tissue physiology/pathology (Noma, Smalley et al. 2008; Kimlin, Casagrande et al. 2013).

There is ample scope for further studies of *in vivo* tumour growth using imaging methods that might extend the current *in vitro* findings. Mice transgenic for GFP expressing bone marrow can be used to provide evidence of homing of BM-MSCs (Quante, Tu et al. 2011; Lecomte, Masset et al. 2012). Simultaneously, the tumour can be grown and accessed for increased cancer cell population by co-injection of transfected cancer cells for red fluorescence e.g. (renilla luciferase-monomeric red fluorescence reported gene) (Wang, Cao et al. 2009) and myofibroblasts. There are also future prospects for the use of knockout mice for chemerin and chemR23. In response to chemerin the recruitment of NK cells, and dendritic cell have been studied in chemR23$^{-/-}$ mice for inflammation and tumour model (Luangsay, Wittamer et al. 2009; Pachynski, Zabel et al. 2012). The mouse chemR23 expressing dendritic and macrophages were shown to respond to human and mouse chemerin equally confirming the conserved chemerin and chemR23 cell signalling system homologous to human (Luangsay, Wittamer et al. 2009). Double transgenic mice for chemR23$^{-/-}$ and GFP-BM-MSCs would provide a strong tool to study the recruitment of MSC in response to tumours harbouring primary myofibroblasts.

7.3 Future prospects

In this thesis the ligand-receptor interaction identified *in vitro* provides a notion of homing of MSCs, with extended scope of specific receptor and ligand which might be a potential therapeutic target. Orthodox conventional chemotherapy using single cytotoxic agents were ineffective in oesophageal tumours, thereby, later combinational therapy was carried out. Such as a combination of Cisplatin/5-FU, and Epirubicin/Cisplatin/5-FU are recently being used in clinical trial stage II and III respectively in oesophageal squamous cell carcinoma (Ross, Nicolson et al.

2002; Nishimura, Hiraoka et al. 2012). The advancement in molecular oncogenesis provided better therapeutics opportunities by targeted therapy chemotherapy. Such example includes targeting receptors, e.g. EGFR, with monoloclonal antibodies and Gefitinib, however was not impressively effective against oesophageal SC in clinical trial II (Janmaat, Gallegos-Ruiz et al. 2006; Ilson 2008). Others examples of molecular targets are VEGF, HGF, and COX-2 in oesophageal SC with inhibitors in clinical trial phase I/II (Tabernero, Macarulla et al. 2005; Tew, Kelsen et al. 2005; Ilson 2008; Hong, Wo et al. 2013). The small molecule drugs and targeted therapy proved to be efficient and at least circumvent the conventional problem over using cytotoxic drugs. Hence, the novel target in oesophageal squamous cancer, chemerin-chemR23 axis with available small molecule drug, CCX832 might holds a great promise in oesophageal cancer treatment. However, in this research the significance of chemerin-chemR23 interaction was established using small sample size. Heterogeneity in patient population and their individual response to treatment is always a challenge to benefit from specific treatments. Therefore, large panel of pre-clinical and clinical studies are further required to introduce CCX832, as potent drug in the cancer treatment.

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Appendices

Appendix-1

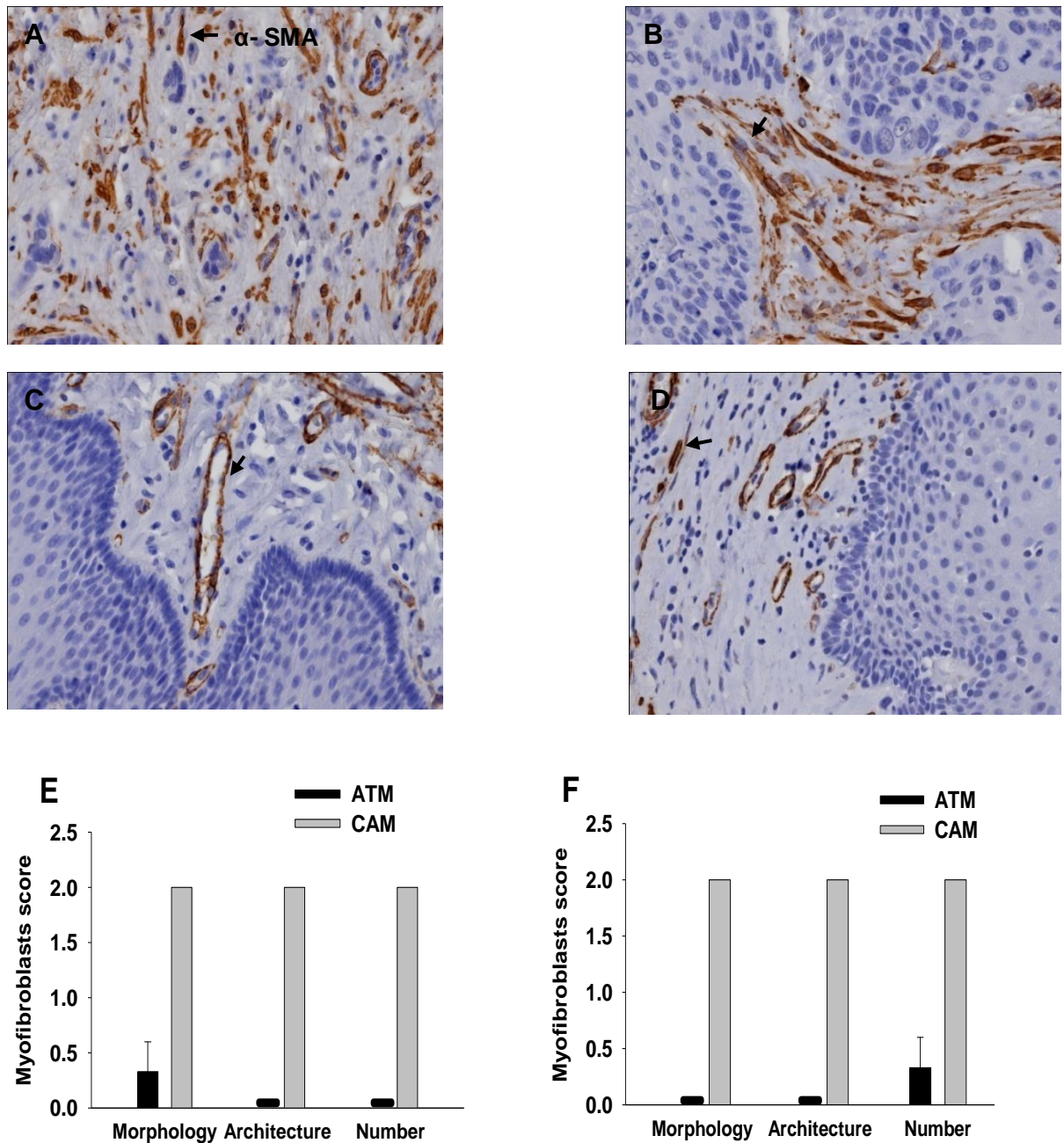# Myofibroblasts characterisation in oesophageal cancer tissues

Figure 1. α-SMA staining identified myofibroblasts in increased numbers, and with disordered morphology in oesophageal adenocarcinoma cancer and squamous cancer. Myofibroblasts in a oesophageal A) adenocarcinoma and B) squamous cancer. Myofibroblast morphology in adjacent tissue (ATM) from the same patients C) adenocarcinoma and D) squamous cancer. Arrows indicate α-SMA positive myofibroblasts (brown). E and F) Quantification of myofibroblast morphology, architecture and number in cancer, and adjacent tissue. The scoring system for

myofibroblast morphology was: 0, normal; 1, mildly distorted; 2, severely distorted. Myofibroblast architecture was scored: 0, restricted to periglandular or subepithelial localisation; 1, both in periglandular/subepithelial regions and elsewhere in the interstitium; 2, severe architectural damage with meshwork-like appearance. Myofibroblast number was scored: 0, as NTMs; 1, mild to moderately increased; 2 substantially increased. For the histopathological assessment, myofibroblasts were defined as stellate/spindle-shaped cells with consistent α-SMA and vimentin coexpresssion. Smooth muscle fibers were excluded based on their characteristic morphology. * p<0.05, n=7.

Appendix-2

Isobaric tagging for relative and absolute quantitation (iTRAQ)

Table 1.

Uniprot ID	Name	Relative change CAM vs ATM			
		Patient #1	Patient #2	Patient #3	Patient #4
Q99969	Chemerin (Retinoic acid receptor responder protein 2)	1.66	4.63	1.83	4.7

A. MRRLLIPLAL WLGAVGVGVA ELTEAQRRGL QVALEEFHKH PPVQWAFQE SVESAVDTPF PAGIFVRLEF KLQQTSCRKR DWKKPECKVR PNGRKRKCLA CIKLGSEDKV LGRLVHCPIE TQVLREAEEH QETQCLRVQR AGEDPHSFYF PGQFAFSKAL PRS

Red **–** identified in 4 patients
Green **–** identified in 1 patient

Table 1. iTARQ identified chemerin as upregulated in media in all 4 CAMs compared to ATMs. A) Tryptic peptides in chemerin identified by iTRAQ in 4 (red) and 1 (green) patients.

Isobaric tagging for relative and absolute quantitation (iTRAQ)

Myofibroblast media was concentrated to 500 µl and 80µg of protein was precipitated in cold acetone, reduced with 2µl 50mM tris-(2-carboxyethyl) phosphine (TCEP) at 60^oC for one hour and alkylated with 1µl 200mM methyl methanethiosulfonate (MMTS) for 10 min at room temperature. Samples were then digested using 8 µg sequencing-grade modified trypsin overnight at 37^oC. The iTRAQ tag reagents were prepared by adding 70 µl ethanol to the samples and incubated for 1 h at ambient temperature. Samples were then mixed and dried by vacuum centrifugation to less than 30 µl. Protein samples labelled by iTRAQ were diluted to 2 ml with 25% v/v ACN and pH adjusted to less than 3 with H_3PO_4. The sample was then loaded onto a PolyLC PolySULFOETHYL A (4.6 x 200mm i.d.)

cation exchange (CEX) column using an Agilent 1100 HPLC system. Fractions (27) were collected and dried by vacuum centrifugation. Samples were then resuspended in 5% v/v ACN, 0.05% w/v TFA and loaded onto a PepMap C_{18} (75μm x 180 mm i.d.) reverse phase column with C_{18} Trap (300 μm x 5 mm i.d.) using an LC Packings Ultimate nano-LC system run in-line with an Applied Biosystems QStar Pulsar mass spectrometer via a nano-electrospray source head and 10μm inner diameter PicoTip (New Objective, Massachusetts, USA). Spectra from both MS and MS/MS were acquired using an information-dependant acquisition (IDA) in positive ion mode. Protein identification and quantification were performed using the ProteinPilot^{TM} v3.0.1 software (Applied Biosystems; MDS-Sciex). The Paragon algorithm was selected as the default search program, with the digestion agent set as trypsin and cysteine modification as methyl methanethiosulfonate. Only proteins with an identification confidence of at least 95% based on the assignment of at least two tryptic peptides, and a local FDR calculated using the PSPEP algorithm of <1%, were reported. Differentially abundant proteins were defined as having a fold change of 1.5 or more, and an associated p-value of <0.05.