Investigation into the role of HMGb1 in relation to myofibroblasts and cancer cells exposed to various conditions

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Abstract

A dynamic stroma that changes with alterations occurring in the epithelium is fundamental for the maintenance of epithelial tissue. Glucose starvation, anoxia and acidosis are characteristic features of the central core of most solid tumours. Myofibroblasts are stromal cells present in many such solid tumours including those of the colon, and they are known to be involved in all stages of tumour progression. HMGb1 is a nuclear protein that plays an important role in nucleosome stabilisation and gene transcription. Whilst HMGb1 is a nuclear protein, it has been reported to takes part in the immune system when passively released by necrotic cells or actively secreted by inflammatory cells such as dendritic cells in the extracellular milieu. The data presented in this thesis suggests that the microenvironmental condition of glucose starvation is responsible for the active release of HMGb1 from different types of cancer cell lines (HT29, MCF-7 and A549) under normoxic conditions. Recombinant HMGb1 (10ng/ml) was shown to trigger proliferation in myofibroblasts cells via activation of PI3K and MEK1/2. Conditioned medium collected from glucose deprived HT29 cells was shown to stimulate migration and invasion of colonic myofibroblasts, and these processes were significantly inhibited by immunoneutralising antibodies to HMGb1, RAGE and TLR-4, along with specific inhibitors of PI3K and MEK1/2. In addition, MMP-2 and MMP-9 two major degraders of basement membrane were investigated for their involvement in CCD18 myofibroblasts migration and invasion in matrigel membrane invasion assay setup. The data suggested that these proteases are not upregulated in this set up and thought not to play any major role in the migration and invasion of myofibroblast cells. There remains a possibility for other proteases being released from myofibroblasts and subsequent digestion of matrigel matrix in this set up. Together these data suggest that HMGb1 released from the cancer cells under glucose starvation is involved in stimulating the CCD18 myofibroblasts migration and invasion and that this was through activation of RAGE and TLR-4, resulting in activation of the MAPK and PI3K signalling pathways. Thus, this study suggests that HMGb1 may be released by cancer cells in areas of low glucose in solid tumours with the resulting activation of myofibroblasts. Therefore, HMGb1 may be considered as potential therapeutic target to inhibit solid tumour growth.

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List of Abbreviations

(α-SMA)	α-smooth muscle actin
(A549)	Human Lung cancer cells
(AP1)	Activator protein-1 transcription factor
(APS)	Ammonium persulphate
(ATP)	Adenosine triphosphate
(β-FGF)	Fibroblast growth factor beta
(CAFs)	Cancer associated fibroblasts
(CCD18)	Human colonic myofibroblasts
(CDC-42)	Cell division cycle-42
(CDK)	Cyclin dependent kinase
(CLE)	Cutaneous lupus erythematosus
(CTAD)	C-terminal transactivation domain
(CXCR4)	Chemokine CXC receptor 4
(DC)	Dendritic cells
(ECM)	Extracellular matrix
(ED-A)	Extra domain A
(EGF)	Epidermal growth factor
(EGF)	Epidermal growth factor
(EJ138)	Human bladder cancer cells
(EMMPRIN)	Extracellular matrix metalloproteinase inducer
(ERK1/2)	Extracellular- signal related kinases
(esRAGE)	Expressed secretory RAGE
(FAK)	Focal adhesion kinases

(FCS)	Foetal calf serum
(FRAP)	FKBP-rapamycin associated protein
(HER2)	Human epidermal growth factor receptor 2
(HGF)	Hepatocyte growth factor
(HIF)	Hypoxia-inducible factor
(HMG)	High mobility group proteins
(HSCs)	Hepatic stellate cells
(HT29)	Human colon adenocarcinoma cells
(ICAM-1)	Intracellular adhesion molecule 1
(IFN-γ)	Interferon gamma
(IGF)	Insulin like growth factors
(IGF2)	Insulin like growth factor 2
(IL)	Interleukin
(IRAK1)	Interleukin-1 receptor-associated kinase 1
(IRAK2)	Interleukin-1 receptor-associated kinase 2
(IRAK4)	Interleukin-1 receptor-associated kinase 4
(LPS)	Lipopolysaccharides
(MAPK)	Mitogen activated protein kinase
(MCF-7)	Human breast cancer cells
(Mdm2)	Mouse double minute 2
(MEK1/2)	Mitogen-activated protein kinase kinase 1/2
(MMP)	Matrix metalloproteinase
(MSCs)	Mesenchymal cells
(MTT)	(3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
(NCAM)	Neural cell adhesion molecule

(NF-κB)	Nuclear factor kappa B
(NRU)	Neutral red uptake
(NSCLC)	Non-small cell lung cancer
(NtRAGE)	N-truncated RAGE
(PBS)	Phosphate buffer saline
(pDCs)	Plasmacytocoid dendritic cells
(PDGF)	Platelet derived growth factor
(PHDs)	Prolyl-4 hydroxylases
(PI3K-AKT)	Phosphatidylinositide 3-kinases-protein kinase B
(PTEN)	Phosphatase and tensin homolog
(pVHL)	von Hippel-Lindau protein
(RAG1/2)	Recombination activating proteins 1/2
(RAGE)	Receptor for advanced glycation end products
(RIPA)	Radioimmunoprecipitation assay buffer
(ROS)	Reactive oxygen species
(SDF)	Stromal cell-derived factor
(SDS-PAGE)	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
(SEM)	Standard error of the mean
(SFU)	S-fluorouracil
(TBS)	Tris-Buffered Saline
(TEMED)	Tetramethylethylenediamine
(TGF-β)	Transforming growth factor-beta
(TGFBR1)	Type I transforming growth factor beta receptor
(TGFBR2)	Type II transforming growth factor beta receptor
(TIMPs)	Tissue inhibitors of metalloproteinases

(TIRAP)	Toll-interleukin 1 receptor domain containing adaptor protein
(TLR)	Toll like receptor
(TNF-α)	Tumour necrosis factor-α
(TRAF-6)	TNF receptor associated factor-6
(VCAM-1)	Vascular cell adhesion molecule 1
(VDA)	Vascular disrupting agents
(VEGF)	Vascular endothelial growth factor

Chapter 1

Introduction

1. Cancer

Cancer is a disease associated with morbidity and mortality and is characterised by uncontrolled proliferation of cells within the body. There were 14.1 million new cases and 8.2 million deaths recorded in 2012 worldwide. Out of 8.2 million, lung cancer, liver cancer and colorectal cancer contributed to 1.6 million, 745000 and 723000 deaths respectively worldwide in 2012 (Ferlay *et al.* 2015). In spite of many advances in cancer research, the mortality associated with cancer is still a major concern. In the last couple of decades, the understanding of cancer at the molecular level has lead to the identification of a number of new drug targets for the development of novel drug therapies. Some of these drugs such as cyclin dependent kinase (CDK) inhibitors and brentuximab vedotin (antibody drug conjugate) targeted at CD30 antigen have already entered for use in clinics (de Claro *et al.* 2012; Sánchez-Martínez *et al.* 2015). Unfortunately, most cancers are still associated with mortality in human population around the world (Hanahan and Weinberg 2000).

1.1 Carcinogenesis

Carcinogenesis in humans can be induced by any one or combination of certain chemical, biological or physical damage that causes genetic changes to occur in the cells. The process of carcinogenesis can be divided into three different stages; initiation, promotion, and progression.

The first stage of carcinogenesis is initiation, which can be a result of irreversible genetic alteration (Balmain and Brown 1988; Sugimura and Ushijima 2000; Califano *et al*. 2015)**.** The stages of initiation have been studied in a number of experimental models *in vivo.* The genetic changes that occur during the process of initiation could arise from one or more simple mutations such as transitions, transversions, or small deletions (Goodman *et al*. 1991). Thus, the first stage in the development of cancer (initiation) is a common phenomenon that can occur spontaneously in humans. However, based on the studies carried out on rat and mouse systems, it appears that most initiated cells usually do not go on to develop into cancer, but may remain quiescent in the organism for a lifetime. It is believed that adults carry many initiated cells that do not develop into cancer (Pitot and Dragan 1991; Bajaj *et al.* 2015).

The second stage of carcinogenesis is 'promotion' which does not involve changes in the structure of DNA, however it can change the expression of the genome. During the 'promotion' stage, a promoting agent (ligand) binds to a specific receptor which results in an altered expression of genes (Dragan and Pitot 1992). This change in expression of genes is regulated by the availability of the receptor and other promoting molecules in the cells. Therefore, it is likely that specific promoting agents would promote specific subsets of initiated cells (Pardal *et al.* 2003). Interestingly, it has been shown in many experimental models *in vivo* that the process of carcinogenesis does not always involve the stage of promotion. For example, if the dose of a promoting agent is substantially high, the stages of initiation and even promotion can be bypassed. However, in humans the stage of promotion during carcinogenesis is normally easily identified (Pitot *et al.* 1989).

The final stage of carcinogenesis is progression. This is characterised by multiple molecular changes within the genome and karyotypic instability. The normal cells undergoing cell division

regulate the structure of their genome and karyotype but cancer cells are unable to do so (Fearon and Vogelstein, 1990). The malignant cells have capability of repeatedly altering the structure of their genome. This genomic shuffling becomes the basis for increased growth and metastatic potential, the ability to escape immune surveillance and acquired drug resistance (Stark 1986). A number of molecular targets for the different stages of carcinogenesis have been studied. These include proto-oncogenes, cellular oncogenes and tumour suppressor genes. However, alterations in both alleles of the tumour suppressor genes are found only in the progression stage of carcinogenesis (Vanden *et al.* 1990).

1.1.1 Metastasis in cancer

In spite of many advances in the cancer chemotherapy and better clinical outcomes, metastatic spread remains a big hurdle in treatment of this disease (Hanahan and Weinberg 2000). Metastasis is the process of movement of tumour cells from one organ to another distant organ within the body. Metastatic spread is considered as a major cause of treatment failure in most cancers. For example, approximately 80% of patients who died of prostate cancer had clinical evidence of bone metastasis (Coleman 2006). Cancer metastasis involves complex interactions between tumour cells and other cells with the tumour microenvironment followed by movement of the tumour cells via the blood or lymphatic system, eventually seeding in distant organs (Fokas *et al.* 2007). The migration of tumour cells might be effected by a combination the mobility of the cells together with the attractions induced by cytokines or other molecules and/or availability of right nutrients at the migratory site (*seed and soil theory)*. Early *in vivo* work by Hart and Fidler (1980) demonstrated the seed and soil effect where melanoma cells were injected into the circulation of mice and tumour growth appeared in the lungs. Surprisingly, metastatic lesions did not develop at the site of where cells were injected. This suggested that

sites of metastatic spread are characterised by the microenvironment of the specific host tissue such as lungs where cancer cells are provided with all the nutrients necessary for their growth (Hart and Fidler 1980; Fokas, Engenhart-Cabillic *et al.* 2007).

The steps of pathogenesis of metastatic spread include invasion in the local host tissue, lymphatic penetration by malignant cells and finally detachment. It has been established that once malignant cells enter into the lymphatic system, they can also find their way to blood vessels (Steeg 2006). Therefore, metastatic spread can be classified into an orderly sequence; invasion, intravasation, circulation, extravasation and colonization. The lymphatic channels are thin walled; thus, provide a negligible resistance to the penetration by the tumour cells. This is one of the reasons why lymphatic system has been considered as common pathway for the tumour cells to enter into the circulation (Chambers *et al.* 2002). Once the tumour cells have made their way into the lymphatic system, they can detach themselves and be carried away or remain proliferative at the site of invasion (Fidler 2003). Some tumour cells are aggregated by cell interactions and form large cluster of cells. Such types cells have increased potential to form tumours after their arrest into the circulation (Tanaka *et al.* 1977). In addition, during the circulation phase, tumour cells can interact with other tumour cells, platelets, lymphocytes and other host cells. The metastatic tumours have always been a cause of concern for successful cancer chemotherapy because of high intolerance of the drugs administered targeting more than one tumour type or acquired drug resistance (Carmeliet and Jain 2000; Khozin *et al.* 2015).

1.1.2 Colon adenocarcinoma

Colon adenocarcinoma is listed amongst the top ten malignancies in many countries. This colon cancer accounts for approximately 55,000 deaths every year in USA (Sanson-Fisher *et al.* 2000). Half of the patients who undergo surgical removal of the cancer encounter complications linked with metastatic spread and are consequently not expected to survive (Kamangar *et al.* 2006). The management of this disease include adjuvant therapy with the synthetic drug, S-fluorouracil (SFU). However, a number of side effects have been seen in patients taking SFU (Shepherd 2003).

Colon cancer is a result of several genetic and epigenetic alterations, which drive the transformation of normal colonic epithelial cells to colon adenocarcinoma cells. This process is called colon carcinogenesis. During this process, the genetic and epigenetic changes have direct impact on molecular signature of cancer cells in which they occur. The understanding about the molecular genetics of colon cancer has revealed that colon carcinogenesis is multistep process characterized by genomic (Fearon and Vogelstein 1990)**.** The genetic or epigenetic alterations in colon cancers activate oncogenes (genes that encodes for proteins like growth factors, growth factor receptors, transducers of growth factor responses and transcription factors that induce growth factors induced gene transcription) or suppress tumour suppressor genes (genes that regulate DNA damage repair, cell cycle arrest, mitogenic signalling, cell differentiation, migration and programmed cell death) in various signalling pathways such as mitogen activated protein kinase cascade (RAF-RAS-MAPK), transforming growth factor-beta (TGF-β) and Phosphatidylinositide 3-kinases-protein kinase B (PI3K-AKT) pathways. There are three forms of genomic instability been reported in colon cancers: 1)

microsatellite instability 2) chromosomal instability (gain and losses of chromosomal regions) and 3) chromosomal translocations (Shih *et al.* 2001; Vurusaner *et al.* 2012; Lovén *et al.* 2013).

Previously, it was believed that adenomatous polyps were transformed into malignant tumours. However, now it has been suggested that hyperplastic polyps may transform into malignant tumours via adenoma to adenocarcinoma progression route (Jass 2004). It has been suggested that colorectal cancer evolves multiple molecular pathway with different morphological and clinical characteristics and two most common pathways are chromosomal instability and microsatellite instability. It has been shown that molecular and morphologically heterogenic hyperplastic polyps do exist and one with extensive DNA methylation are likely to have significant malignant potential (Jass 2007).

Reportedly, about 75% of colon cancers are resistant to the growth inhibitory effect of transforming growth factor (TGF-β) (Elliott and Blobe 2005). TGF-β is a tumour suppressor that mediates its effects via a heteromeric receptor complex. This heteromeric receptor complex consists of type I transforming growth factor beta receptor (TGFBR1) and type II transforming growth factor beta receptor (TGFBR2). Upon activation, these receptors phosphorylate down-stream signalling proteins such as Smad2, Smad3, PI3K and p38-MAPK (Markowitz and Roberts 1996). The downstream transcriptional targets of TGF-β are genes involved in proliferation, extracellular matrix (ECM) production and apoptosis. Therefore, considering the central role of TGF-β in colon cancer, it may be considered as a logical target for deregulation of colon cancer (Fynan and Reiss 1993).

1.1.3 Hypoxia

Hypoxia (low oxygen levels) plays an important role in tumour progression and has been considered as an established prognostic factor in solid tumours (Dhani *et al.* 2015). Most solid tumours are characterised by disorganised vasculature that is needed for oxygen and nutrient supply (Chung *et al.* 2010). Typically, hypoxia in tumours occurs at a distance of 100-200μM from the nearest vasculature (Figure 1.1) (Pugh and Ratcliffe 2003). Those cells that do not get enough oxygen and nutrients can eventually become necrotic or undergo apoptosis. However, this is not always the case in hypoxic tumours. The response to meet the stress of low oxygen tension in cells is facilitated by a transcription factor known as hypoxia-inducible factor (HIF). The transcription factor HIF-1 is composed of HIF-1α and HIF-1β subunits. Although, HIF-1β is constitutively expressed however the expression of HIF-1α is induced by hypoxic cells where oxygen concentration is less than 6% in their microenvironment (Semenza 2003). Under hypoxic conditions, HIF-1 gets activated after the stabilization of HIF-1α subunit, however under normoxic conditions, HIF-1α is rapidly degraded via ubiquitinated proteasomal degradation and is virtually undetectable (Pugh and Ratcliffe 2003).

A number of HIF-1 regulated genes have been shown to play critical roles in cellular response to hypoxia, glycolysis, erythropoiesis, angiogenesis and vascular remodelling. In tumour cells loss of p53 activity has been shown to increase HIF-1α expression and transcription of downstream target genes including vascular endothelial growth factor (VEGF). In addition, activation of certain signalling pathways such as PI3K and the serine/theronine kinases protein B (AKT) and FKBP-rapamycin associated protein (FRAP) has been shown to induce the expression of HIF-1α and VEGF mRNA under normoxic conditions (Zhong *et al.* 2000; Li *et al.*

2015). Phosphatase and tensin homolog (PTEN) protein is encoded by *PTEN* gene is a tumour suppressor. Loss of *PTEN* activity also leads to increased expression of HIF-1α (Courtnay *et al.* 2015). Although, molecular mechanism by which cells senses hypoxia followed by HIF-1α stabilisation is unclear but there are experimental evidences suggesting the requirement of superoxide generated in mitochondrion followed by hydrogen peroxide for the induction of HIF-1 activity (Michiels *et al.* 2002).

The regulatory mechanism behind HIF-1 stability, expression and associated pathophysiological consequences remains an active area of research. In normoxia, oxygen dependent prolyl-4 hydroxylases (PHDs) hydroxylate oxygen dependant degradation domain (ODD) of HIF-1α. This hydroxylated HIF-1α bonds with von Hippel-Lindau protein (pVHL) followed by 26S-proteasomal degradation (Ivan *et al.* 2001). The von Hippel-Lindau (VHL) protein has been shown to play critical role in the ubiquitination of HIF-1α. The protein binds to the stabilization domain of HIF-1α. It has been suggested that metal chelators such as cobalt chloride or desferrioxamine (iron chelator) facilitate HIF-1α stabilization by dissociated VHL from HIF-1α. Interestingly, hypoxia does not cause dissociation of VHL from HIF-1α, rather in hypoxia, HIF-1α gets stabilised because its iron containing PHDs require oxygen as co-factor. Whilst oxygen is the limiting substrate for hydroxylation, under many pathophysiological conditions, PHD activity is also modulated by limiting iron and ascorbate availability (Brahimi-Horn and Pouysségur 2007). Another regulatory mechanism involves transactivation domain that is present on the C-terminus of HIF-1α. This domain is known as C-terminal transactivation domain (CTAD). The hydroxylation of CTAD renders the HIF-1α to the p300 co-activator which prevents transactivation of HIF-1α. This results in stabilisation of HIF-1α (Hirsilä *et al.* 2003).

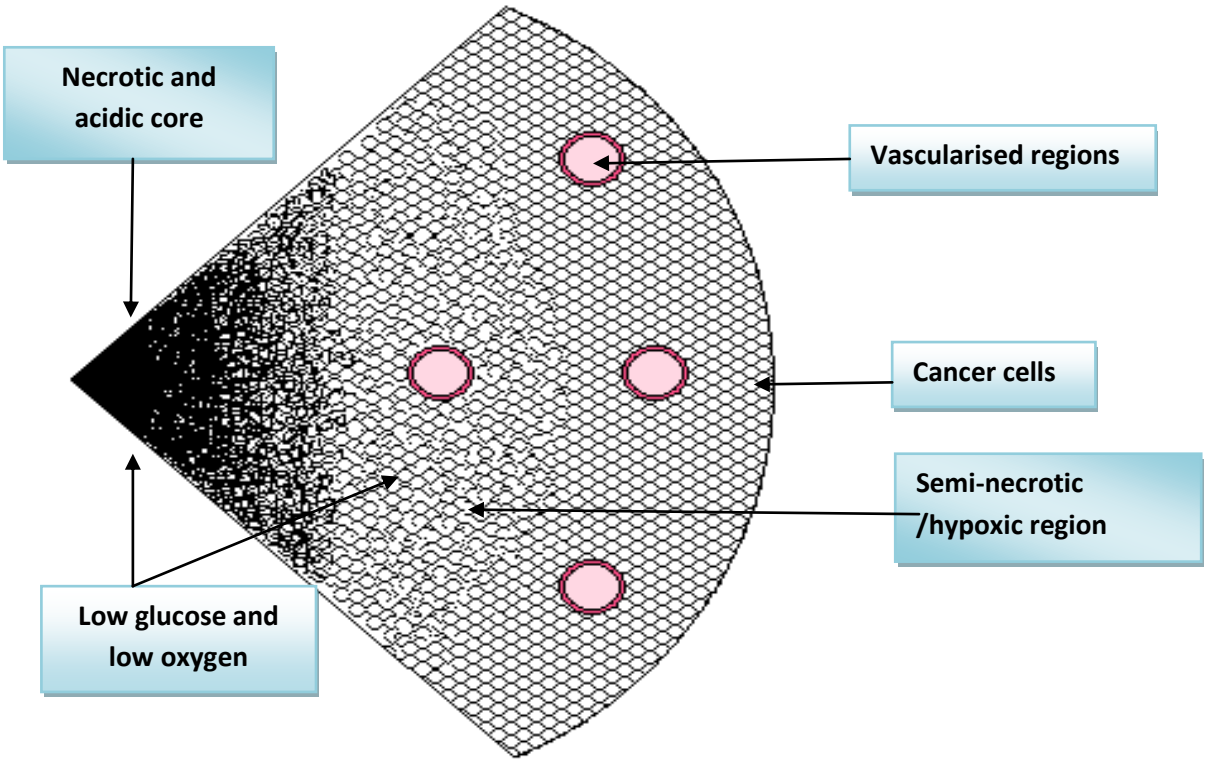


Figure 1.1: *Schematic representation of a typical solid tumour. There are well vascularised regions on and inside the periphery of tumour and seminecrotic areas towards the centre of the tumour. There is anoxic, acidic and glucose starved necrotic core in the centre of tumour* (Koh and Powis 2012).

Hypoxia can affect tumour growth in positive way by making tumour cells adapt to survive the local oxygen and nutrient deprived conditions. For example, hypoxia drives increased anaerobic glycolysis facilitated by increased levels of glycolyic enzymes, glucose transporters and neo-vascularisation (Pouysségur *et al.* 2006). Although, a clear explanation of pro-apoptotic and anti-apoptotic hypoxia is still lacking, however the interplay between p53 (which appears to be hypoxia inducible) and HIF-1 is can not be neglected (Carmeliet *et al.* 1998).

The tumour suppressor p53 gene undergoes mutational inactivation in many cases of solid tumour. Under hypoxic conditions, p53 undergoes post-translational modifications and gets stabilised. Upon stabilisation, p53 become active and promote cell cycle regulation and apoptosis (Oren *et al.* 2002). Under severe hypoxic or anoxic conditions, p53 interacts with HIF-1α directly or via mouse double minute 2 (Mdm2) -E3 ubiquitin-protein ligase pathway. In addition, in severe hypoxia or anoxia induced accumulation of p53 has been shown to inhibit HIF-1 transcriptional activity via Mdm2 targeted proteasomal degradation (Honda and Yasuda 1999). In principle, once p53 is activated, it either suppresses or destroys HIF-1α. Therefore, increased expression of p53 and thus destruction of HIF-1α might induce apoptosis (An *et al.* 1998).

It has been observed that cells surviving oxygen deficiency also usually survive apoptosis induced by chemotherapy (Schmaltz *et al.* 1998). This idea has been supported by the finding where vascular endothelial growth factor (VEGF) neutralizing antibodies blocked the anti-apoptotic effect of hypoxia on HepG2 cells (Baek *et al.* 2000). It has been shown that cancer cells secrete some factors that inhibit endothelial cell apoptosis by activating the extracellular-signal related kinases ERK1/2 pathway (Reinmuth *et al.* 2001). In addition, a number of growth factors such as VEGF, insulin like growth factor 2 (IGF2) and TGF-β can activate signal transmission that can lead to HIF-1α expression and cell survival (Tabatabai *et al.* 2006). This also includes hypoxia-induced platelet derived growth factor (PDGF) signalling and activation of the PI3K/Akt pathway. Therefore, HIF-1 can also stimulate autocrine signalling pathways crucial for cell survival under hypoxia (Zhang *et al.* 2003). It has been suggested that hypoxic tumours are more likely to acquire resistance against radiation and chemotherapy. In addition, these hypoxic tumour cells are more aggressive and have increased potential for invasion and metastasis than the normal tumour cells (Otrock *et al.* 2009).

1.2 The tumour microenvironment

The tumour microenvironment has been reported to play important roles in the progression of cancer (McAllister and Weinberg 2014). The tumour microenvironment is a complex system and consists of many cell types such as endothelial cells, pericytes, smooth-muscle cells, fibroblasts, myofibroblasts, neutrophils and other granulocytes, mast cells, macrophages and dendritic cells (Brennecke *et al.* 2015). These cells in addition to cancer cells might contribute to the acquisition of hallmark traits by creating 'tumour microenvironment' described by Hanahan and Weinberg previously. In addition, lymphatic vascular system plays a crucial supporting role in the microenvironment of metastatic tumours (Farber and Rubin 1991; Lee *et al.* 2015).

Whilst tumour associated fibroblasts and myofibroblasts are normal cells, they also support cancer in a positive way by releasing growth factors that are necessary for tumour growth (De Wever *et al.* 2008; Nagasaki *et al.* 2014). In addition, these cell types also constitute a substantial part of tumour stroma, which has an important role in the maintenance of tissue homeostasis (Figure 1.2) (Alcaraz and Roca-Cusachs 2015). The tumour stroma in the microenvironment provides structural support and facilitates the cross talk between the cells (Ohtani 1998).

Another key constituent of tumour microenvironment is the extracellular matrix (ECM), which is an important regulator of normal tissue behaviour. The ECM is typically composed of collagen, laminin, fibronectin and proteoglycans (Bosman and Stamenkovic 2003). The ECM separates the endothelium and underlies endothelial cells, pericytes, fibroblasts, myofibroblasts and other cell types (Figure 2). In normal tissue, ECM's role is to maintain homeostasis, which helps to

prevent the formation of a neoplasm (Kalluri 2003). However, in the tumour stroma, this is not achieved because of remodelling of the ECM. The ECM remodeling is mediated by a number of matrix degrading enzymes such as serine, cysteine, matrix metallproteases (MMPs) and endoglyosidases such as heparanase (Vlodavsky *et al.* 2002).

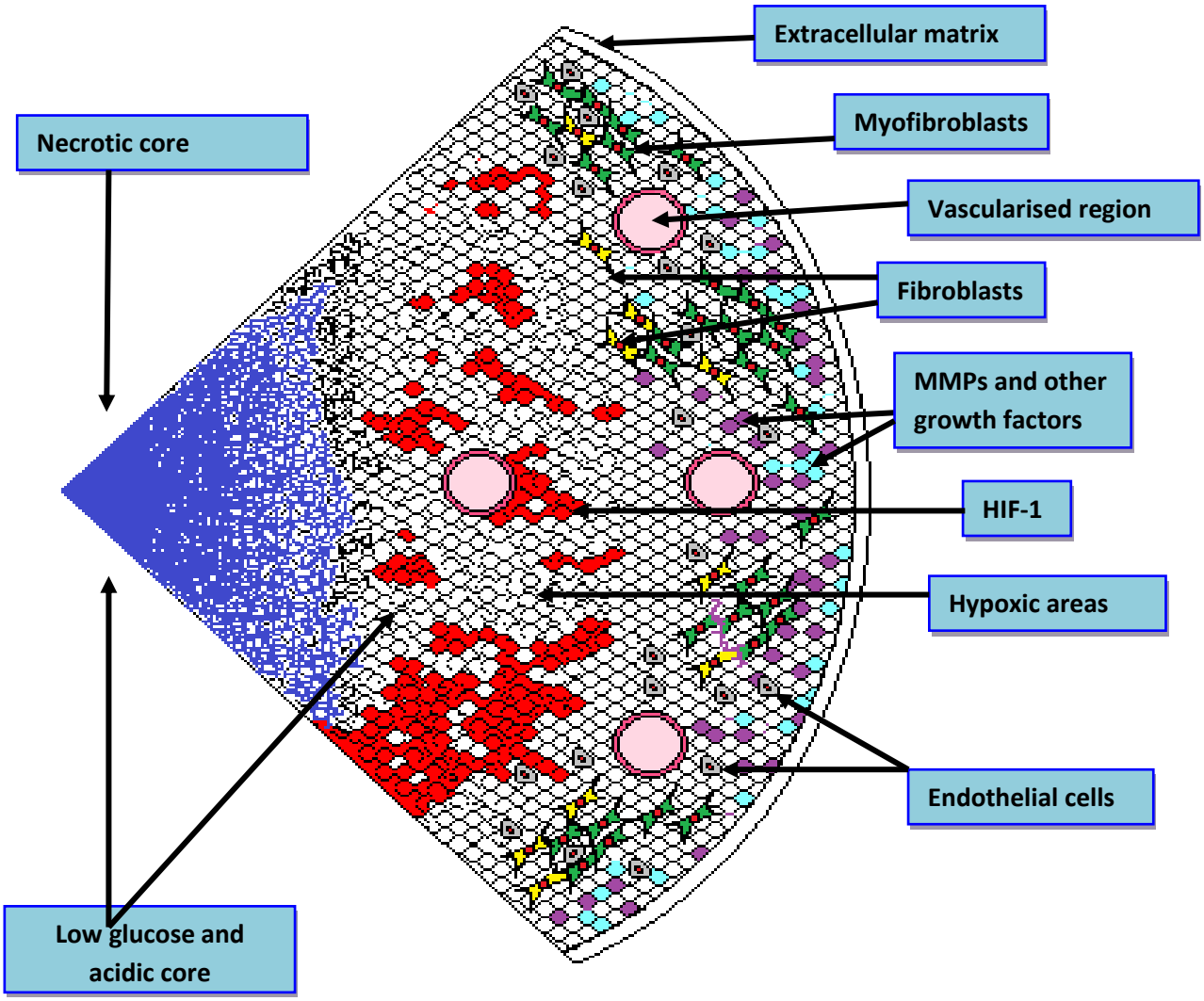


Figure 1.2: *The tumour stroma in microenvironment showing tumour cells (transparent), fibroblasts (in yellow), myofibroblasts (green) and endothelial cells (in grey). The necrotic areas are displayed in blue colour. Hypoxia, acidic core and lack of glucose are three hallmarks of tumour microenvironment displayed from centre to middle regions. The MMPs released from stromal cells such as fibroblasts and myofibroblasts degrade ECM (shown in purple and blue colours). Hypoxia induced stabilization of HIF-1 (red areas) contribute to promote tumour cells growth and angiogenesis by facilitating the release of growth factors such as VEGF.*

1.2.1 Extracellular matrix remodelling

The ECM is an important element of tumour microenvironment and an important regulator of normal tissue behaviour. One of the common aspects of many solid tumours is desmoplasia. This is caused by morphologically and functionally altered tumour stroma in the tumour microenvironment (Whatcott *et al*. 2015). This desmoplastic response is mediated by a number of growth factors and cytokines such as TGF-β and PDGF. It has been shown that both are responsible for the induction of signalling cascade that modulates the tumour stroma (Micke 2004). The altered expressions of α-smooth muscle actin (α-SMA), vimentin and desmin in fibroblasts are typical biomarkers for desmoplastic reaction in tumour microenvironment (Elenbaas and Weinberg 2001).

In the tumour microenvironment, the ECM is remodelled to support the neoplastic cells to proliferate and to allow them to enter into the circulation. The remodelling of ECM characterised by structural disruption or modification of ECM components which modulates the cell's ability to survive, proliferate and migrate (Figure 3). The ECM remodelling is mediated by cysteine proteases (cathepsin B) (Yan *et al.* 1998), aspartic proteases (cathepsin D), serine proteases (elastase and uPA) (Van den Steen *et al.* 2001) and MMPs (Matzner *et al.* 1985; Stamenkovic 2003).

It has been shown that most solid tumours exhibit increased expression of proteases that are correlated with tumour progression. However, during ECM remodelling, most of these proteases are produced by stromal cells of the host tissue. For example, in human breast cancer, stromal

fibroblasts have been reported to produce MMP-11 and urokinase plasminogen activator (uPA) (Pupa *et al.* 2002). In addition, MMP-1, 2 and 3 have been reported to be localised on the fibroblasts located in close proximity of invading cancer cells (Gomez *et al.* 1997). For example, extracellular matrix metalloproteinase inducer (EMMPRIN), a membrane bound glycoprotein is produced by cancer cells. The production of EMMPRIN stimulates stromal cells to synthesise MMPs in the microenvironment (Tang, *et al.* 2004). In addition, aspartic protease cathepsin B is found on plasma membrane of tumour cells and is correlated with invasion (Gangoda *et al.* 2015). A study has revealed that EMMPRIN stimulates MMP-1 production by fibroblasts followed by binding of MMP-1 to EMMPRIN on tumour cells. This process facilitates degradation of ECM by tumour cells (Biswas *et al.* 1995).

In tumour microenvironment, cancer cells stimulate stromal cells (fibroblast, myofibroblasts, endothelial cells, pericytes etc) to produce proteases which cleave matrix components (Figure 1.3). This cleavage or proteolysis of ECM modifies focal adhesion and cytoskeleton of ECM and triggers the release of focal adhesion kinases (FAK) (Fashena and Thomas 2000). In addition, the proteolysis of ECM components also exposes the binding sites such as integrin binding sites available on ECM. Furthermore, the laminin receptor (67LR), which is overexpressed by various tumour cells, interacts with integrins and modulates the interaction between tumour cells and laminin. This process has been shown to facilitate the metastasis of tumour cells (Martignone *et al.* 1993).

The ECM remodelling has a significant effect on tumour cell behaviour by stimulating various signalling pathways and molecules. A number of epidermal growth factor (EGF) family cytokines such as TGF-β, PDGF, fibroblast growth factor beta (b-FGF) and interferon gamma

(IFN-ɣ) bind to various components of ECM and remain inactive until they get signals from matrix proteases (Stamenkovic 2003; Park *et al.* 1993). Therefore, an increase in the protease activity on the ECM can lead to the activation and release of various growth factors that can stimulate tumour cells in the tumour microenvironment (Streuli 1999). The remodelling of the ECM also has a crucial role in angiogenesis. Once released from endothelial cells, the angiogenic factors like VEGF can be stored in the ECM. All these processes are initially guided by tumour cells but later on involve the microenvironment and the host cells to participate, thus disrupting intracellular signalling between tumour cells and the ECM within the tumour microenvironment (Park *et al.* 1993). The tumour cells remodel the matrix to override and modulate the homeostatic arrangement within the microenvironment. Moreover, the composition of tumour stroma is equally important for therapeutic response as the tumour phenotype is (Pupa *et al.* 2002).

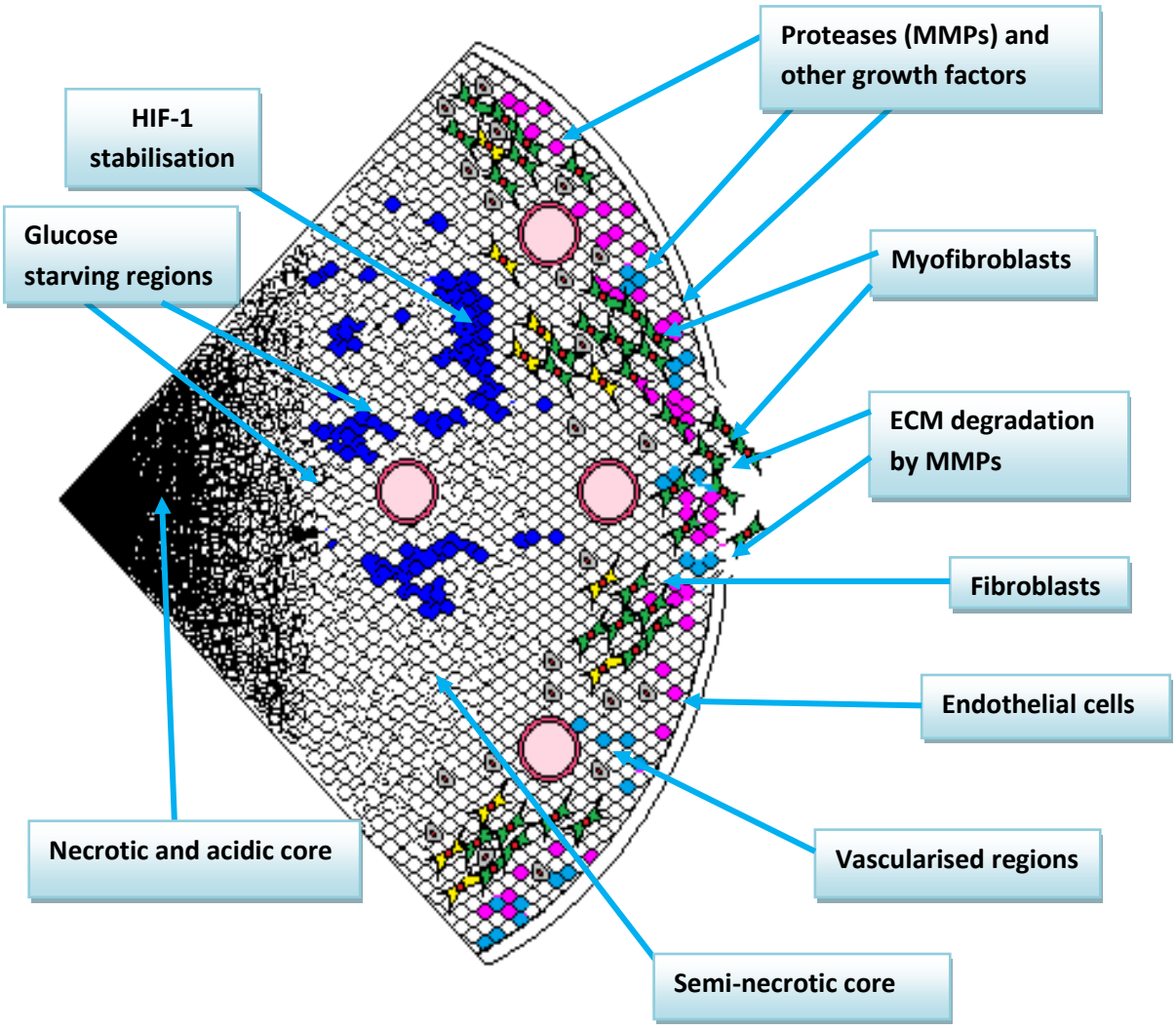


Figure 1.3: *Schematic representation of tumour stroma in microenvironment showing ECM degradation followed by escaping of cancer and stromal cells. Hypoxia, acidic core and lack of glucose are three hallmarks of tumour microenvironment. The MMPs produced from cancer and other stromal cells (fibroblasts and myofibroblasts) can degrade ECM. This degradation of ECM then can facilitate migration of cells.*

1.2.2 Cellular cross talk in the tumour microenvironment

The cross talk between the cells in tumour microenvironment is linked with the progression of the disease. For example, hepatic sinusoidal endothelial cells enhance the interaction between hepatocytes and kupffer cells to exchange nutrients that is needed for neo-vascularisation within tumours (Bhatia *et al.* 1999). It has been established that tumour cells promote VEGF expression in hypoxic conditions (Levy *et al.* 1996). Therefore, hypoxia induced VEGF expression and subsequent formation of neo-vascularisation promotes cell survival under harsh conditions within the microenvironment.

The expression of some angiogenic factors depends on organ specific cell-cell cross talk within the microenvironment. For example, interleukin-8 (IL-8) has been shown to induce proliferation of endothelial cells (Li *et al.* 2003). In contrast, the expression of IL-8 was inhibited in melanoma and hepatocyte co-cultures. Therefore, the expression of angiogenic factors in tumour cells is directly linked with individual organ specific microenvironments (Desbaillets *et al.* 1997).

The epithelial and mesenchymal cells are the drivers of differentiation and development. Furthermore, it has been shown that changes in the stroma can promote epithelial transformation (Park *et al.* 2000). For example, mesenchymal and epithelial cells regulate the ovarian cycle before and during the reproductive period. During the menopause, any loss in the cross talk between mesenchymal and epithelial cells is correlated with the promotion of ovarian cancer. Therefore, any interference in the cell-cell interaction during embryogenesis might be responsible for the onset of cancer (Aboseif *et al.* 1999).

The cellular cross talk is directly linked with motility, survival and invasion. For example, integrins present between the cells and the ECM are responsible for the adhesion of cells to the ECM. A disruption in the integrin mediated adhesion facilitates cellular translocation and apoptosis (Frisch *et al.* 1996). Similarly, the pro-survival and pro-invasive signals are needed for survival and invasion. For example, integrins activate a number of downstream molecules such as FAK and MAPK/ERK pathways. The phosphorylated FAK is necessary for the survival of cells combating anoikis (programmed cell death due to lack of cell-cell or cell-matrix interaction) (Frisch *et al.* 1996).

A number of other pathways such as PI3K, Ras, Rac, Rho and cell division cycle-42 (CDC-42) are involved in the regulation of cell motility within the tumour microenvironment. However, most of these pathways overlap in facilitating the process of motility, invasion and survival in cancer and stromal cells (Xue *et al.* 2000).

1.2.3 The interstitial fluid pressure in tumours

The central core of solid tumours is often necrotic due to a lack of nutrients and anoxia resulting from a poor blood supply. However, there is a viable rim on the periphery of this necrotic zone, below basement membrane of most solid tumours. Many chemotherapeutic drugs have been developed to target these cells and also the tumour vasculature (Shaked *et al.* 2006).

In spite of many advances in the target-oriented chemotherapy, most therapeutic agents are unable to kill the cells of the viable rim of tumours. The viable rim encapsulates the hypoxic and

necrotic core of solid tumour and thought to be one of the reasons why most drugs cannot penetrate and reach to the central core of tumours (Jain 1987; Minchinton and Tannock 2006). There could be a couple of explanations for the viable rim escaping effective treatment. One could be that the microenvironment within the tumour is dynamic and keep changing. This might result in an increase in the interstitial fluid pressure and cells from central core coming out and contribute in the formation of rim. Another explanation could be linked with the work done by Yuval Shaked *et al*. (2006) which suggested that vascular disrupting agents (VDA) may induce acute mobilisation of circulating endothelial cells in tumours. These cells may home to the viable rim in the tumours and may remain there after the therapy (Shaked *et al.* 2006)*.*

1.2.4 Hypoxia and glucose deprivation in solid tumours

The multistep development of most solid tumours involves six hallmarks of cancer reported by Hanahan and Weinberg previously. Recent conceptual advances have added two emerging hallmarks of cancer. These are reprogramming of energy metabolism and evading immune destruction. Unlike apoptosis or autophagy, necrosis in tumours might have direct impact on tumour growth and metastasis because necrotic cells might explode and release growth factors and other nutrients into the host tumour microenvironment. (Hanahan and Weinberg 2011). It is established that hypoxia is a common phenomenon that occurs in most solid tumours. Apparently, most hypoxic cells are resistant to radiotherapy and to a number of chemotherapeutic agents. It has been shown that there is a correlation between the oxygen levels and the response of tumours to the radiation therapy. In addition, the oxygen deprivation is also correlated to the metastatic potential of the tumour cells. Hypoxia has been considered

as one of three hallmarks of tumour microenvironment. The other two are glucose deprivation and acidosis (Tannock 1972; Schlappack *et al.* 1991; Hanahan and Weinberg 2011).

The microenvironment of solid tumour is not only characterised by hypoxia but also the formation of acidic environment within solid tumours (Raghunand *et al.* 2014). In solid tumours, lactic acid formation and hydrolysis of adenosine triphosphate (ATP) have been found to be acidic and a wider range of pH (5.85-7.68) has been observed in different regions of the same malignant tissue with median pH of 7.0. This pH is generally lower than the surrounding normal tissue where median pH is 7.5. The lower pH or acidosis is correlated with the decreased radiation sensitivity of solid tumours. In addition, it has been shown that the cytotoxic effect of doxorubicin (a DNA intercalating agent) and mitoxantrone (type II topoisomerase inhibitor) was reduced at low extracellular pH (Wike-Hooley *et al.* 1984; Mahoney *et al.* 2003).

It is clear that neoplastic tissues utilise a large quantity of glucose. For example, Walker carcinomas in rats were found to contain 0-5mg/ml of glucose in tumour interstitial fluid when compared to the normal subcutaneous interstitial fluid of rats with 90-100mg/ml of glucose (Woodward and Hudson 1954; Gullino *et al.* 1964). This might suggest that glucose passing through the tumour vasculature is rapidly utilised by the neoplastic cells (Wike-Hooley *et al.* 1984; Graff 2014). The effect of glucose deprivation and acidosis has been shown to increase metastasis in murine tumour cell lines *in vivo*. However, the underlying mechanism of glucose deprivation induced metastatic spread remains unknown (Schlappack, *et al.* 1991). Glucose deprivation and hypoxia have been shown to induce the accumulation of proteasomes in the nucleus of cancer cells (HT29 colon cancer cells). Proteasome is a major site for protein degradation, which plays a key role in the proteolysis to maintain homeostasis. It has been

shown that glucose deprivation and hypoxia can modulate the intracellular location of proteasomes. Inhibiting protein degradation by selective inhibitor of proteasome has been shown to restore the sensitivity to topoisomerase-IIα targeted drugs *in vitro* and *in vivo*. Therefore, an increased proteolysis in the nucleus of cells could be the survival strategy of cancer cells to survive under hypoxia and glucose deprivation (Ogiso *et al.* 1999).

1.3 High mobility group box 1 (HMGb1) protein

The high mobility group (HMG) proteins are chromosomal proteins, named according to their ability to move electrophoretically through polyacrylamide gels. There are three main known superfamilies; HMGa, HMGb and HMGn. The HMGb superfamily has a functional sequence motif, which is called DNA binding box (Bustin 2001). HMGb1 is a non-histone protein involved in stabilization of nucleosomes and the bending of DNA, which facilitates gene transcription (Lange *et al.* 2008). In addition, HMGb1 is responsible for modulating the activity of steroid hormone receptors by participating in the maintenance of nucleosome structure. As supporting evidence, HMGb1-deficient mice died shortly after birth, possibly because of inactivation of glucocorticoid receptor responsive genes (Wei *et al.* 2003). The HMGb1 is expressed in all cells of vertebrates and in yeast, plants and bacteria (Bustin *et al.* 1990). The cellular localisation of HMGb1 could be tissue specific with notably high levels found in lymphoid tissues and testis. In addition, an increased level of HMGb1 has been seen in cytoplasm of the cells of the liver and the brain. However, HMGb1 concentration is usually more in the nuclei of the cells than the cytoplasm (Mosevitsky *et al*. 1989).

Structurally, HMGb1 is composed of three main domains; the A box domain and B box domain are homologous DNA binding pockets and a negatively charged chain of 30 amino acids (Figure 1.4) (Chen *et al.* 2004; Ellerman *et al*. 2007). The A box domain plays an important role for its antagonistic and anti-inflammatory effect whereas the B box domain has a role similar to that of proinflammatory cytokines (Andersson, Erlandsson-Harris *et al*. 2002) (Figure 1.4). The amino acids 150-183 on the acidic tail are responsible for binding to the receptor for advanced glycation end products (RAGE) (Figure 1.4) (Ellerman *et al.* 2007). The HMGb1 binds to the minor grove of DNA without sequence specificity, and induces bends in the helical structure of the DNA. The formation of this complex facilitates interaction between DNA and other factors such as p53, nuclear factor kappa β (NF-kβ), recombination activating proteins 1/2 (RAG1/2) and some steroid hormone receptors (Bianchi 2004). The HMGb1 also interacts with other molecules including RNA, Lipopolysaccharides (LPS) or endotoxins and IL-1β. Thus HMGb1 may play a key role in facilitating the arrangement of many nucleoprotein complexes (Bianchi and Manfredi 2014; Keyel 2014).

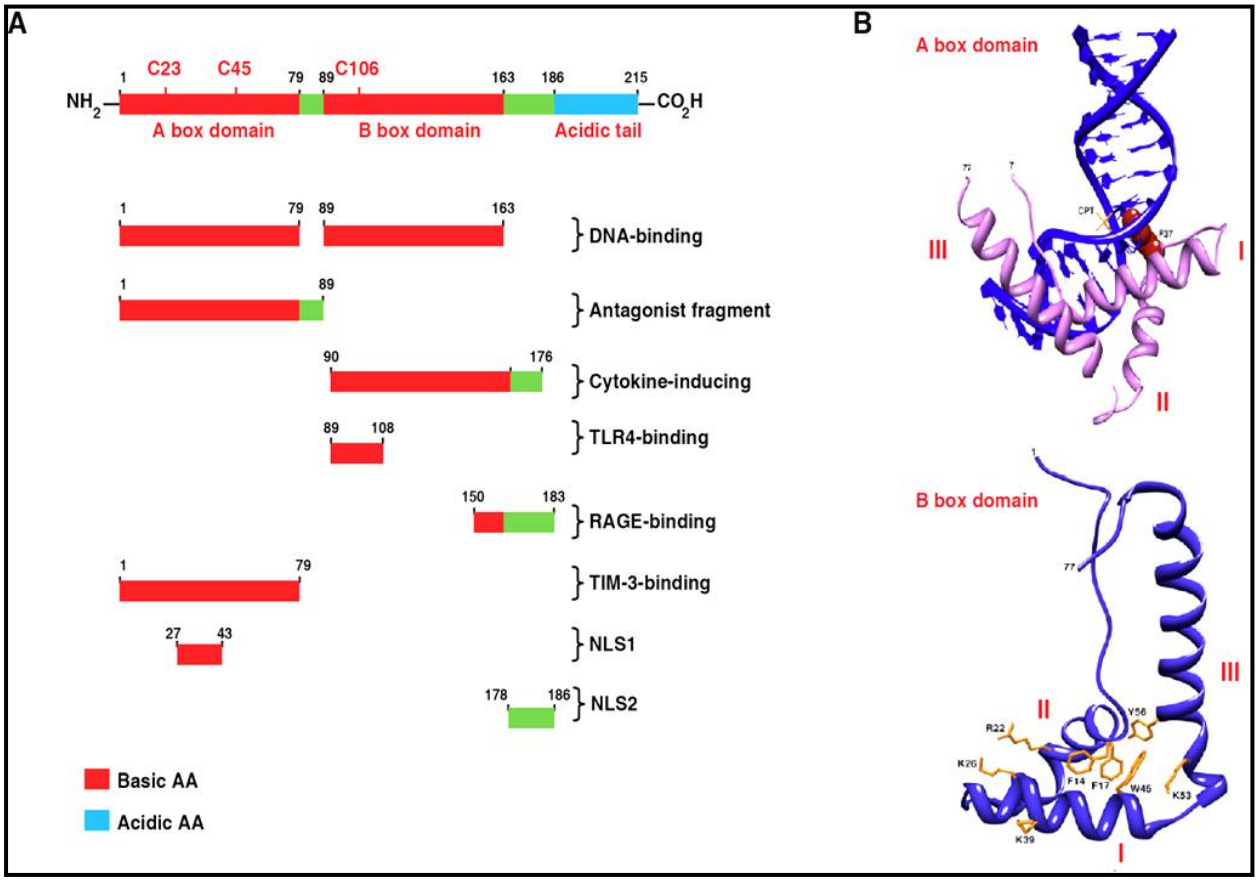


Figure 1.4. *Structure of HMGb1 protein. HMGb1 is a 215–amino acid (*AA*) protein of ~30 kDa. HMGb1 is composed of three domains: two positively charged domains (A box and B box) and a negatively char Acidic tail. A and B boxes are DNA-binding domains and B box is also responsible for cytokine activity by inducing macrophage secretion of proinflammatory cytokines. This cytokine activity is antagonized by recombinant A box. The protein structure involved in the binding of HMGb1 with RAGE is located between amino acid residues 150 and 183* (Ellerman *et al.* 2007).

1.3.1 Role of HMGb1 in disease

HMGb1 does not possess signal sequence and therefore, does not transverse the endoplasmic reticulum. However, it is released actively by various cells such as macrophages, pituicyte and erythroleukemia cells (Tang *et al.* 2007). In addition, HMGb1 is passively released from necrotic or damaged cells but not from cells undergoing apoptosis, these apoptotic cells retain HMGb1 within their nuclei (Zong *et al.* 2004). Therefore, apoptotic cells do not trigger inflammation even after the loss of the membrane (Bonaldi *et al.* 2003). Hence, HMGb1 can be considered as a critical stimulus of inflammation during cell death (Scaffidi *et al.* 2002). The HMGb1 has two lysine-rich nuclear localization sequences which direct its movement towards the nucleus of the cell (Bonaldi *et al.* 2003). Whilst it is a nuclear protein, HMGb1 is released from the cells under certain conditions to take part in the inflammatory process (Frank *et al.* 2015; Zhu *et al.* 2015).

During inflammation, extracellular HMGb1 activates infiltrating macrophages via the RAGE receptor. In addition, activated macrophages/monocytes are also responsible for the release of HMGb1 in the extracellular milieu (Figure 1.5) (Andersson *et al.* 2002; Huttunen and Rauvala 2004). There are three main steps have been suggested for HMGb1 secretion; 1) exit from the nucleus to the cytoplasm, 2) translocation from the cytoplasm into cytoplasmic organelles, and 3) exocytosis. Macrophages/monocytes upon activation by proinflammatory cytokines acetylate HMGb1 at their lysine-rich nuclear localization sequences. This leads to the translocation of HMGb1 into the cytoplasmic vesicles followed by extracellular release (Gardella *et al.* 2002), (Figure 1.5).

It has also been suggested that HMGb1 is involved in various diseases including autoimmune disorders, sepsis, chronic inflammatory disease and cancer (Wang *et al.* 2004; Sims *et al*. 2009). An increase in the concentration of HMGb1 has been observed in the plasma and epithelial lining fluids in patients with acute lung injury (Ueno *et al.* 2004)**.** In addition, HMGb1, TNF-α and IL-1 β have been suggested to be involved in the pathogenesis of cutaneous lupus erythematosus (CLE). In CLE, HMGb1 forms a pro-inflammatory loop between tumour necrosis factor-α (TNF-α) and IL-1β to sustain prolonged inflammation, suggesting an important role in this inflammatory autoimmune disorder (Barkauskaite *et al.* 2007). A study revealed that anti-HMGb1 antibody inhibited synovial inflammation by blocking HMGb1 in an experimental model of arthritis. However, this inhibition was independent of TNF-α pathway suggesting that TNF-α is not the only pathway necessary for extracellular release of HMGb1 (Pullerits *et al*. 2008).

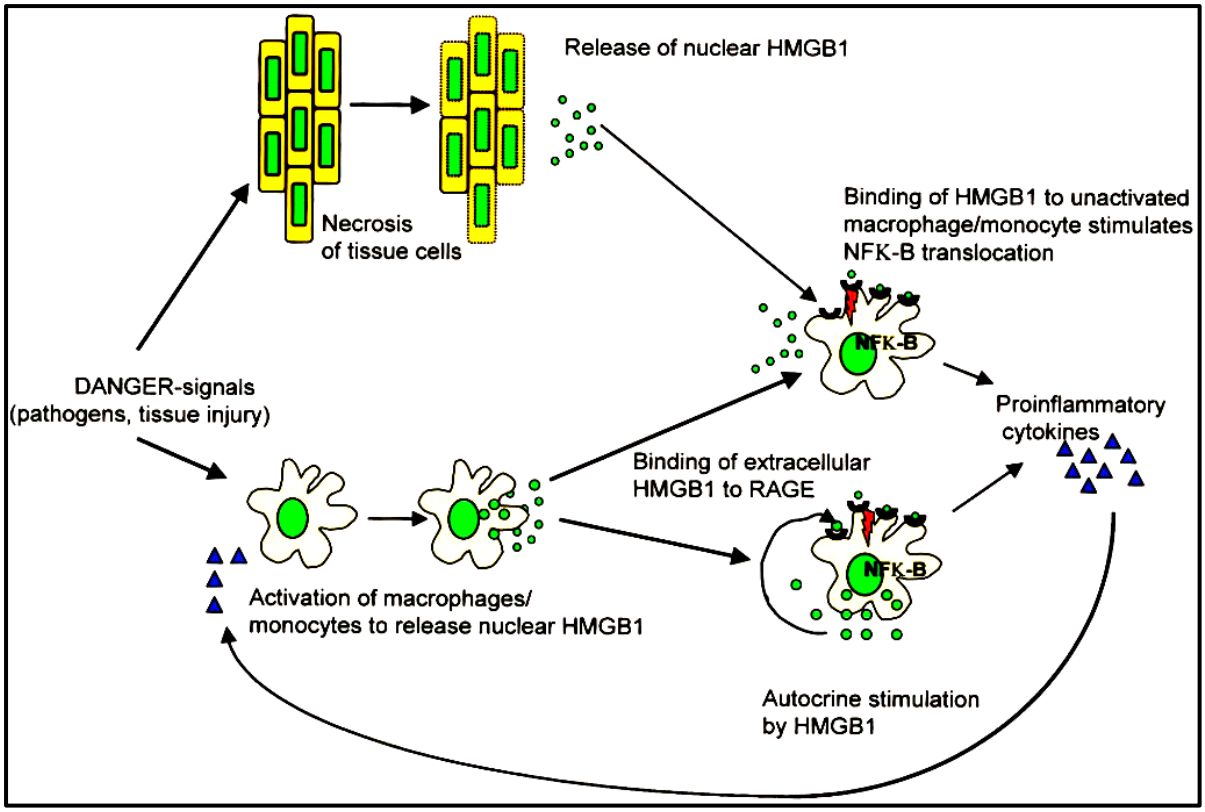


Figure 1.5*: Release of HMGb1 as a danger signal during inflammation. The figure shows a diagrammatic illustration of potential pathways for HMGb1 release leading to inflammatory responses. HMGb1 can be released extracellularly by passive secretion from any necrotic cell or by active secretion from activated macrophages/monocytes* (Andersson *et al.* 2002).

HMGb1 has been reported to be responsible for the impairment of intestinal barrier function in mice (Sappington *et al.* 2002). In addition, HMGb1 play an important role in increasing ileal mucosa permeability and bacterial translocation to lymph nodes (Sappington *et al.* 2002). Furthermore, elevated levels of HMGb1 have been observed in synovial fluids of experimental animal models of arthritis (Andersson and Erlandsson-Harris 2004). In addition, a clinical study has revealed that synovial fluid of rheumatoid arthritis patients had more elevated levels of HMGb1 than that of osteoarthritis patients (Taniguchi *et al.* 2003).

The hemorrhagic shock is characterised by activation of inflammatory cytokines. A clinical report revealed elevated levels of HMGb1 in the blood circulation of hemorrhagic shock patients. However, the elevated levels of HMGb1 went to normal as clinical conditions improved (Fan *et al.* 2007). In addition, the serum HMGb1 level goes significantly higher in sepsis patients compared to normal healthy volunteers. This observation was constant when compared to patients who died of sepsis versus the patients who had survived after recovering from sepsis (Yang *et al.* 2004).

Recently, the effect of HMGb1 on fibroblasts and keratinocytes has been explored. It has been shown that cytokine activity of HMGb1 stimulates keratinocyte scratch wound healing *in vitro* (Ranzato *et al.* 2009). In addition, HMGb1 was shown to induce proliferation and migration of keratinocytes via ERK1/2 pathway (Ranzato *et al*. 2010). To further support these findings, anti-RAGE antibody and selective MEK1/2 inhibitor (PD98059) were used to inhibit the HMGb1-RAGE-ERK1/2 pathway. This resulted in the inhibition of HMGb1 induced wound healing. Therefore, HMGb1 can be considered as a potential therapeutic target for the development of drugs for chronic inflammatory disease, chronic inflammatory autoimmune disorders and severe wounds (Ranzato *et al.* 2010). It has also been shown that HMGb1 stimulates vascularisation in chicken embryo chorioallantoic membrane *in vivo,* which suggests that HMGb1 plays an important role in angiogenesis (Mitola *et al.* 2006). In addition, HMGb1 triggers proliferation and migration of glioblastoma cells via ERK1/2 activation (Bassi *et al.* 2008). However, the proliferation of cells can not be considered as marker for the expression of HMGb1 *in vivo* (Ller *et al.,* 2004).

1.3.2 The role of HMGb1 in cancer

Hanahan and Weinberg in 2000 proposed a model that defined six hallmarks of cancer. These are 1) unlimited replicative potential, 2) ability to develop new blood vessels, 3) escape from surveillance, 4) insensitivity to inhibitors of growth, 5) self-sufficiency in growth signals and 6) invasion and metastasis (Hanahan and Weinberg 2000). Recently, seventh hallmark has been proposed which is inflammation. In addition, dysregulated cellular energies within tumour microenvironment and evading immune destruction are two additional emerging hallmarks that most or may be all tumours exhibit (Hanahan and Weinberg 2011). All these hallmarks of cancer are linked with the levels, localisation and alterations in HMGb1 (Mantovani 2008; Mantovani *et al.* 2008). Therefore, HMGb1 is now important to understand the molecular biology of cancer. Several solid tumours including melanoma, prostate cancer, breast cancer, pancreatic cancer and colon cancer exhibit markedly elevated levels of HMGb1 (Völp *et al*. 2006). These elevated levels of HMGb1 are associated with tumour formation, proliferation and metastasis and chemotherapeutic response. The presence of HMGb1 in the extracellular medium of cells is indicative of stress conditions (Lotze and Tracey 2005).

The HMGb1 has dual role in cancer. The first role is correlated with neovascularisation in solid tumours (Campana *et al.* 2008). A rapidly growing tumour causes reduction in the microvessel density followed by formation of necrotic areas within the tumour. The necrotic areas within tumour not only produce angiogenic factor such as VEGF but also attract macrophages. The macrophages have been reported to release HMGb1 in stress conditions such as necrosis. In addition, HMGb1 binds to its receptor RAGE and activates NF-$_{K}$B. Upon activation, NF-$_{K}$B upregulates the production of certain cytokines and angiogenic factors in endothelial cells (van

Beijnum *et al.* 2008). The direct inhibition of HMGb1 with immunoneutralising antibodies has been reported to inhibit angiogenesis *in vivo* and *in vitro* (van Beijnum *et al.* 2006). It has been suggested that HMGb1 might have a direct impact on migration of cells because of its ability to modulate the adhesive properties of the cells and ECM components (Ellerman *et al.* 2007). In addition, HMGb1 can enhance invasive and metastatic potential of tumour cells via the NF-κB pathway (Sasahira *et al*. 2008).

The other role of HMGb1 in cancer is related to the immune response against tumours. There is evidence that HMGb1 is released in response to specific chemotherapy or radiation therapy induced conditions, and this is thought to promote immune response against tumours (Campana *et al.* 2008). Extracellular HMGb1 has been shown to activate dendritic cells in murine models. Upon activation, these dendritic cells facilitate immune response against immunogenic apoptotic lymphoma cells (Ronchetti *et al.* 1999). In addition, HMGb1 has also been shown to trigger anti-neoplastic response from T cells (Campana *et al.* 2008). HMGb1 has been shown to induce antigrowth signals within tumours. These antigrowth signals usually act in two ways; a) cells may escape from the proliferation cycle and enter into the stage of quiescence (G0 phase) and b) permanent loss of proliferative potential by some alternations in their environment (Hanahan and Weinberg 2011). Contrary to this, it has been shown that MCF-7 cells overexpressing HMGb1 progress through to S2 phase when compared with HMGb1 knockdown MCF-7 cells (Yoon *et al*. 2004). However, other work has shown that HMGb1 overexpression has been shown to suppress the growth of MCF-7 xenografts in nude mice (Jiao 2007).

Recent work has suggested an important role of HMGb1 in the survival of myeloid derived suppressor cells followed by suppressing the immune response against tumours. These myeloid derived suppressor cells are found in abundance in most cancers and are representative of suppressed immune system (Parker *et al.* 2015). The extracellular HMGb1 due to its cytokine, chemokine and growth factor activity acts as protumour protein however, it does possess an ability to sustain genome stability during tumorigenesis. Thus, whilst HMGb1 may promote tumour cell survival during the early stages of chemotherapy however on the contrary intracellular HMGb1 led inhibition of autophagy might increase the effectiveness of anticancer treatment (Kang *et al.* 2013).

HMGb1 activates various signalling pathways such as MAPKs, protein kinase B (AKT) and PI3K. These pathways play important roles in proliferation and migration of normal cells and cancer cells. Supporting evidence includes activation of the PI3K pathway by HMGb1 in neutrophils and colon cancer cells (Kuniyasu *et al.* 2003). Furthermore, HMGb1 has been shown to induce toll like receptor-4 (TLR-4) mediated activation of MyD88-IRAK4-p38 and Myd88-IRAK4-AKT pathways. The activation of HMGb1-RAGE complex has been shown to activate NF-$_K$B, JNK kinases and ERK1/2 pathways (Degryse, *et al.* 2001). In addition to RAGE receptor, HMGb1 binding with TLR-2 and TLR-4 receptors also has been shown to activate the NF-$_K$B pathway. This suggests that NF-$_K$B is an important signalling pathway that requires activation by HMGb1 (van Beijnum *et al.* 2008).

1.3.3 HMGb1 and its receptors

The HMGb1 has been shown to modulate its cytokine like activity by interacting with multiple receptors including receptor for advanced glycation end products (RAGE) (Kokkola *et al.* 2005), toll like receptor 2 and 4 (TLR-2 and TLR-4) (Curtin *et al.* 2009; Kim *et al*. 2013), chemokine CXC receptor 4 (Schiraldi *et al.* 2012), and T cell immunoglobulin mucin 3 (TIM-3) (Baghdadi *et al.* 2013). However, RAGE is the most commonly researched receptor for HMGb1. This receptor belongs to immunoglobulin superfamily and is expressed in many cells including monocytes, macrophages, smooth muscle cells, dendritic cells (DC) and endothelial cells. The receptor for advanced glycation products is a receptor for multiple ligands that can therefore be activated by different ligands including HMGb1. However, the effects of this activation are dependent of the type of the cell this receptor is expressed upon. Activation of RAGE in monocytes/macrophages has been reported to trigger inflammatory response, neoplastic transformation and metastasis in neuroepithelial tumour cells (Taguchi *et al*. 2000). The fibroblasts have been shown to express RAGE and the activation of RAGE on fibroblasts was correlated with proliferation and migration of fibroblasts in tumour microenvironment (Liu *et al.* 2010; Rojas *et al.* 2010). It has been shown that RAGE overexpression is associated with chronic degenerative disease and cancer (Tanaka *et al.* 2000). In addition, activation of RAGE by HMGb1 is associated with increased expression of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) gene, which then inhibits apoptosis (Bierhaus *et al.* 2005)**.** The protein HMGb1 has been shown to interact with TLR-2 and TLR-4 and the activation of TLR-4 by HMGb1 was shown to inhibit migration of enterocytes and endothelial cells (Dai *et al.* 2010; Bauer *et al.* 2013).

1.3.4 Receptor for advanced glycation end products (RAGE)

The receptor RAGE known to play important roles in the many diseases such as diabetes, arthritis, Alzheimer's disease and cancer is localized on chromosome 6 in humans and mice (Wang *et al.* 2012).. The advanced glycation end products (AGEs) are common ligands for RAGE and involved in various diseases such as hyperglycaemia, renal failure and other inflammatory diseases. The N^{ε}-carboxymethylysin (CML) is one of the most common AGE ligands that can bind to RAGE (Wang *et al*. 2012). Apart from the AGEs, RAGE interacts with other ligands such as the S100 family of molecules (Schmidt *et al*. 2000). The S100 family of molecules have been reported to play important roles in inflammatory diseases. In addition, amyloid-β-peptide and β fibrils are other ligands of RAGE involved in the development of Alzheimer's disease. Interestingly, RAGE itself might not be overexpressed in most cancers but its ligands such as S100 family and HMGb1 are generally overexpressed in most type of cancers. However, amyloid-β-peptide and β fibrils have not been reported in cancers but they are overexpressed in neurological disorders (Hofmann *et al*. 1999).

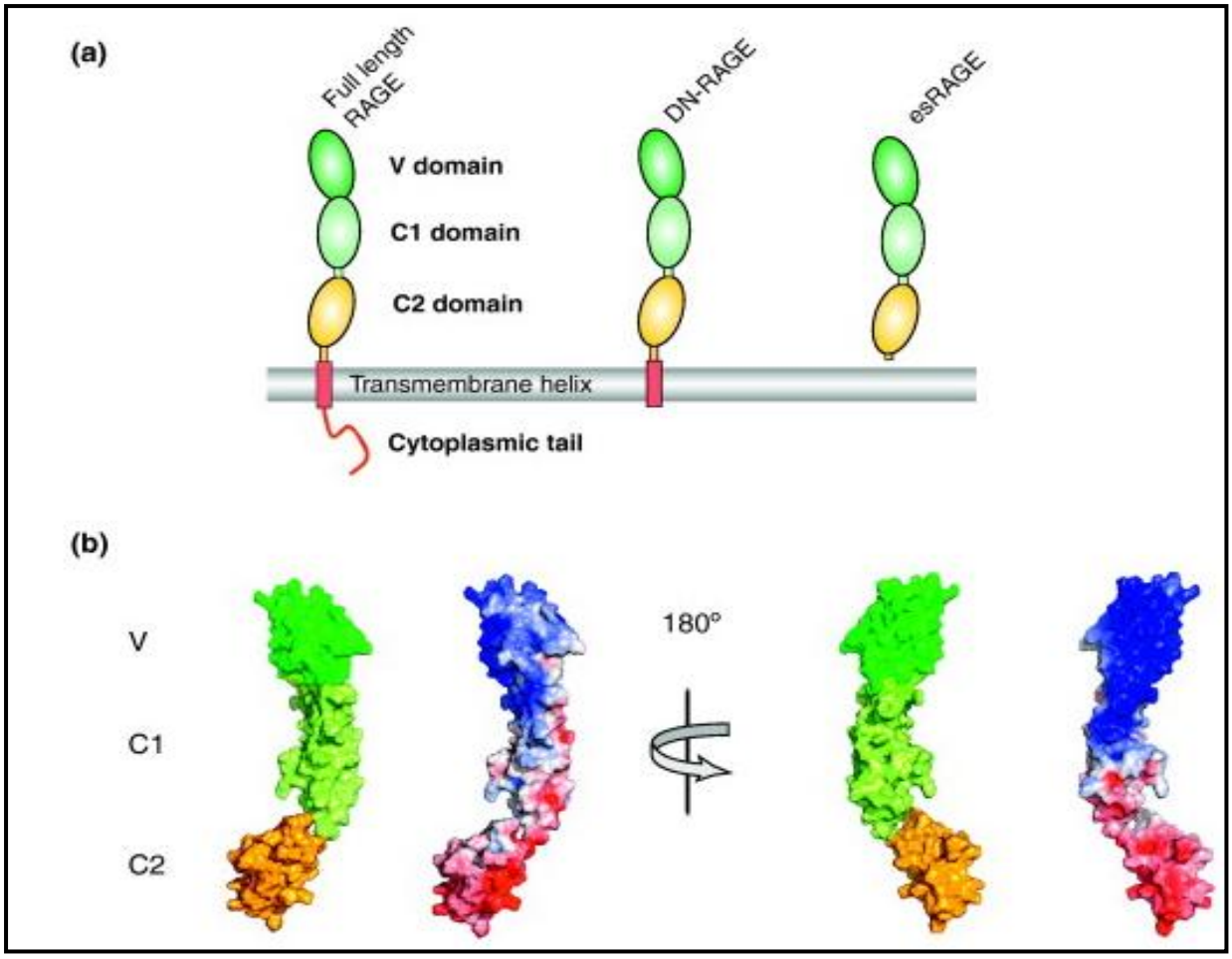


Figure 1.6: *Structure of RAGE: A) The structure of RAGE consists of one V and 2 C (C1 and C2) domains. There is also a transmembrane domain which joins cytoplasmic tail below the surface membrane of cells. B) Three dimensional structure showing V, C1 and C2 domains of RAGE* (Lin *et al.* 2009; G Fritz 2011)

The structure of RAGE consists of one V-type and two C-type domains, a short transmembrane domain and a cytoplasmic tail (Figure 1.6) (Bierhaus *et al.* 2005). There are three major RAGE isoforms have been reported; full-length RAGE, expressed secretory RAGE (esRAGE) and N-truncated RAGE (NtRAGE). However, a few other splice variants of RAGE have also been seen in pathological conditions. The mRNA of these splice variants lacks N terminal and C terminal (Yonekura *et al.* 2003). These splice variants have been seen on various cells including

endothelial cells and pericytes. However, the function of these isoforms is not fully understood (Schmidt *et al.* 1994)**.**

The esRAGE has similar immunoglobulin domains as that of a full length RAGE but lacks exons 10 and 11 that encode the transmembrane domain of full length RAGE. This is because esRAGE does express intron 9, which has a stop codon within the sequence. Therefore this stop codon does not allow expression of exons 10 and 11. The esRAGE has been reported to be secreted by cultured cells into the extracellular environment (Cheng *et al.* 2005). In the extracellular environment, esRAGE acts as decoy for many ligands which also bind to full length RAGE (Mahajan and Dhawan 2013). Before the discovery of esRAGE, a synthetic version of esRAGE was produced in baculovirus system, which was used as decoy for RAGE ligands. This system was used to inhibit the activation of RAGE. This synthetic version of esRAGE molecule has also been termed soluble RAGE (sRAGE) (Hofmann *et al.* 1999).

The NtRAGE has a stop codon which is located on intron 1 causing the loss of exons 1 and 2. Therefore, NtRAGE lacks the v-type immunoglobulin domain. However, it is expressed on plasma membrane. Han *et al*. detected a 42kDa protein which was similar to the full length RAGE on the plasma membrane (Han *et al.* 2011). However, considering the fact that NtRAGE lacks a signal peptide, the mechanism behind its expression on plasma membrane is unclear. In addition, its expression on plasma membrane does not interfere with AGE stimulated effects. However, its overexpression does correlate with inhibition in migration of endothelial cells. Therefore, it appears that NtRAGE might interact with other ligands and can interfere with various signalling pathways involving RAGE (Yonekura *et al.* 2003).

1.3.5 RAGE and Tumour Microenvironment

Many important cells of the microenvironment such as endothelial cells, pericytes, macrophages, fibroblasts and myofibroblasts are known to express RAGE (Logsdon, Fuentes *et al*. 2007). Therefore, RAGE ligands generated by cancer cells are likely to influence tumour microenvironment. Similarly, these cells in the tumour microenvironment can also produce RAGE ligands and these ligands can interact with RAGE on cancer cells (Rouhiainen *et al.* 2013). The most important role of RAGE that influences the tumour microenvironment is its ability to influence angiogenesis. It is established that angiogenesis play important roles in proliferation and migration of cancer cells, endothelial cells and pericytes (Papetti and Herman 2002). A number of RAGE ligands including HMGb1 have been shown to influence endothelial cells (Gupta and Massagué 2006). In addition, HMGb1 overexpression was correlated with angiogenesis in colon cancer and inhibiting HMGb1 abrogated the formation of new vessels (Schlueter *et al*. 2005). Furthermore, expression of RAGE was correlated with VEGF (an angiogenic stimulator) expression on endothelial cells. Also, a number of RAGE ligands have reported to induce other angiogenic factors such as IL-8. Therefore, it appears that RAGE and RAGE ligands are key players that participate in the tumour microenvironment (Folkman 1990).

Fibroblasts have been shown to express RAGE in tumour microenvironment. It is clear that the number of fibroblasts present is correlated with increased cancer progression (Rojas *et al.* 2010). In addition, they produce growth factors and ECM molecules which facilitate angiogenesis within the tumour microenvironment. RAGE has also been reported to influence fibroblasts by upregulating fibroblasts growth factor (FGF)**.** In addition, activation of RAGE on

fibroblasts was correlated with proliferation and chemotaxis of fibroblasts (Liu *et al*. 2010). However, the correlation of RAGE with cancer-associated myofibroblasts is unknown.

The macrophages are another major cell type stimulated by the activation of RAGE within the microenvironment. The macrophages exert their immunomodulatory effect by presenting tumour-associated antigens to T-cells and also by expressing immuno-stimulatory cytokines. The RAGE activity has been reported to stimulate macrophage function which was correlated with inflammation (Hofmann *et al*. 1999; Hasegawa *et al*. 2003).

1.3.6 RAGE in cancer

RAGE is expressed in many solid tumours including ovarian, lung, prostate, colonic, brain and melanomas. In addition, elevated levels of RAGE have been reported in many pathological conditions such as diabetes, arthritis and Alzheimer's disease (Hofmann *et al*. 2002; Fukami *et al*. 2015; Liu *et al*. 2015). These elevated levels are directly linked with the activation of NF-$_{K}$β which has been shown to play central role in activating downstream signalling in many tumours (Pikarsky *et al*. 2004). Therefore, this could be one of the reasons why most tumours have elevated levels of RAGE. However, there is conflicting evidence regarding the overexpression of RAGE in cancers. Some studies support the overexpression of RAGE in many cancers including colon, prostate and gastric (Sasahira *et al*. 2005; Sparvero *et al*. 2009). Contrary to this, no increase in the levels of RAGE in colon and pancreatic cancers has been reported by other researchers (Bartling *et al*. 2005). In addition, significantly low levels of RAGE have been observed in lung cancer. In particular, the levels of full length RAGE and esRAGE were

significantly low in non-small cell lung cancer (NSCLC). However, RAGE ligands have been reported to be increased in most tumours (Bartling *et al*. 2005).

There is conflicting evidence about the involvement of RAGE in lung cancer as reduced levels of RAGE were reported in NSCLC. In addition, esRAGE, which is an antagonist for the full length RAGE is downregulated in NSCLC. Furthermore, the overexpression of full-length RAGE was correlated with reduction in tumour size (NCI-H358 lung cancer cells) *in vivo* (Logsdon *et al*. 2007). However, several RAGE ligands such as HMGb1, S100A12 and S100P are overexpressed in the lung cancer (Diederichs *et al*. 2004). Furthermore, S100A4 overexpression was correlated with poor prognosis resulted from metastatic spread of pulmonary adenocarcinoma (Zou *et al*. 2004).

The role of RAGE in breast cancer is not fully understood. However, RAGE ligands appeared to play important roles in progression of the disease. For example, S100A4 has been shown to play crucial role in breast cancer growth. In addition, S100A4 negative patients' survival rate was much higher than those of S100A4 positive patients (Rudland *et al*. 2000). Also, elevated levels of HMGb1 were observed in human primary breast carcinoma. Therefore, there is much evidence that supports the involvement of RAGE ligands in breast cancer. However, it is nuclear that if these ligands mediate their effect via RAGE in breast cancer (Lum and Lee 2001).

The receptor for advanced glycation end products along with its ligands has been reported to be elevated in many stages of prostate cancer. For example, S100A8 and S100A9 have been reported to be overexpressed in human prostate cancer. The receptor (RAGE) has been shown

to be expressed by prostate cancer cells and its ligands S100A8 and S100A9 are apparently also secreted by prostate cancer cells (Gebhardt *et al*. 2006). In addition, S100s appeared to play key role in migration of prostate cancer cells by activating NF-$_{K}$β and MAPK pathway *in vitro.* Also, elevated levels of HMGb1 and RAGE have been reported in prostate cancer tissue when compared with normal prostate tissue (Ishiguro *et al*. 2005). Furthermore, HMGb1 and RAGE overexpression was observed in the PC3 prostate cancer cell line, which was related to androgen deprivation induced cancer cell invasion (Kuniyasu *et al*. 2003). Moreover, RAGE expression has been shown to be increased at various stages of colon cancer progression (Sasahira *et al*. 2005).

This receptor (RAGE) has been reported to play important role in a neoplastic model of IL-10 null mouse. The administration of sRAGE (decoy for RAGE ligands) significantly reduced the neoplasia related inflammation in IL-10 null mouse model (Berg *et al*. 1996). In addition, S100P and RAGE complex triggered proliferation and migration of SW480 colon cancer cells and inhibiting S100P/RAGE abrogated proliferation and migration in the SW480 cells. Furthermore, the expression of S100A4 was correlated with the invasive potential of the colon cancer cells as S100A4 is specifically overexpressed in invasive carcinoma. All these findings suggest that RAGE might play an important role in the interface between the inflammation and carcinogenesis (Yammani *et al*. 2006).

1.3.7 RAGE and HMGb1

RAGE is a multi-ligand receptor that binds to various molecules including HMGb1 and the S100 family of proteins. Structurally, RAGE has two N-glycosylation sites on its V domain. These N-glycans undergo carboxylation which enhances the ability of the receptor to bind to HMGb1 with subsequent signal transduction. The carbohydrate groups are then removed by PNGase F and therefore do not alter the conformation of protein complex. Upon confirmation, the protein complex (receptor-ligand complex) can initiate signal transduction necessary for cell growth (Wilton *et al*. 2006).

There is evidence that supports the involvement of RAGE and HMGb1 complex in the cancer. For example, blockade of HMGb1 and RAGE by immunoneutralising antibodies suppressed tumour growth in murine model of lung cancer (Schlueter *et al*. 2005). In addition, HMGb1 has been shown to induce proliferation and neovascularization in endothelial cells (Chavakis *et al*. 2007). Other research has shown that HMGb1 plays an important role in the activation of DCs. This activation is mediated via TLR-4 (another receptor for HMGb1). However, HMGb1 induced maturation and migration of DCs was shown to involve activation of RAGE via HMGb1 binding (Dumitriu *et al*. 2005).

Various cell surface receptors such as RAGE and TLRs play important roles in signal transduction. It was observed that nucleosomes containing HMGb1, derived from secondary necrotic cells, play an important role in inflammation by activating macrophages and DCs which also express RAGE and TLRs. The mechanism of action of this signal transduction was

explored using macrophages with defective RAGE and TLRs. Interestingly, the deletion of TLR-2 abrogated the activation of macrophages and DCs. This suggests when HMGb1 is associated nucleosome, the signal transduction will be strictly through TLR-2 (Urbonaviciute *et al*. 2008). Increased concentration of DNA containing complexes have been observed in autoimmune disorders such as Lupus. The HMGb1 interaction with DNA containing immune complexes has been extensively studied where these complexes bind to HMGb1 enter into the endosomal pathway of plasmacytocoid dendritic cells (pDCs) via CD32 mediated uptake where it encounters with TLR-9. Since, HMGb1 can directly bind to DNA, it is possible that when HMGb1 is bound to DNA it would signal through RAGE and the activation of TLR-9-MyD88 pathway (Tian *et al*. 2007).

The ligand binding of RAGE involves the activation of two major pathways which are CDC42/Rac and MAPK. It is evident that the activation of the MAPK pathway leads to the activation of NF-kβ and neurite outgrowth. The CDC42 pathway has been implicated in HMGb1 induced migration in cancer cells. The HMGb1 induced activation of RAGE lead to the activation of CDC42 and Rac1 and facilitates changes in the cytoskeleton. To support this, NF-kβ gene expression assay was carried out. It was found that expression of dominant negative RAGE eliminated the NF-kβ activation induced by HMGb1. Therefore, HMGb1 induced activation of RAGE was responsible for NF-kβ activation followed by neurite outgrowth (Huttunen *et al*. 1999). In addition, HMGb1 mediated activation of RAGE may amplify the expression of RAGE with subsequent activation of NF-kβ. The NF-kβ responsive genes play key role in inflammatory process (such as intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and few other cytokines e.g., TNFα, IL-1β and IL-8). Therefore, HMGb1 induced activation of RAGE can induce and sustain a pro-inflammatory phenotype (Park *et al*. 2003; Fiuza, Bustin *et al*. 2003). Apparently, RAGE was the first

receptor identified for HMGb1 binding but blocking RAGE did not abrogate the effect of HMGb1 completely in many experimental models. This suggested that an additional receptor for HMGb1 must exist. Subsequently, Toll like receptors were identified as HMGb1 binding receptors (Park *et al*. 2004).

1.3.8 Toll Like Receptors

The tolls like receptors (TLRs) are conserved proteins that help cells of the innate immune system to respond to endogenous danger molecules by activating intracellular signalling pathways. TLR signalling might require the receptors to dimerize or oligomerize for signal transduction to take place. Endothelial cells have been shown to express different TLRs on their membranes such as TLR-4, TLR-2 and TLR-9. These are multi-ligand receptors and when associated with HMGb1 and play important roles in activating immune response against inflammation via MyD88 pathway (Nogueira-Machado *et al*. 2011). However, these receptors can bind to endotoxin or LPS and gram positive and gram negative bacteria. For example, TLR-4 is the main receptor for endotoxin and also for gram-negative bacteria whereas TLR-2 is a receptor for gram-positive bacteria and also for fungi. Both play important roles in host response to fungal or bacterial infections (Yu *et al*. 2006).

1.3.9 TLRs and HMGb1

Various blood borne pathogens and cytokines usually target endothelial cells. Subsequently, endothelium expresses TLRs and gets activated. In particular, the activation of endothelium in response to microbial stimulation is mediated by TLR-2 (Iwasaki and Medzhitov 2004). In addition, it has also been shown that the activation of TLR-2 may lead to the activation and translocation of NF-kβ (Smith *et al*. 2003). The involvement of Rac1 and PI3K has been reported to activate TLR-2 in fimbrillin stimulated monocytes. The Rac1 and PI3K were recruited to the cytoplasmic domain of TLR followed by subsequent signal transduction (Harokopakis *et al*. 2006). The recruitment of Rac1 and PI3K resulted in CD18-dependent adhesion to ICAM-1. In addition, the dominant negative variant of PI3K and Rac1 inhibited the expression of NF-kβ reporter gene. This suggested an important role of PI3K and Rac1 in TLR-2 signalling (Arbibe *et al*. 2000).

Toll like receptor 2 has been reported as main receptor for HMGb1 in RAW264.7 macrophages. This was detected by using fluorescence resonance energy transfer (FRET) and immunoprecipitation assays. In addition, it was shown that the stimulation of HMGb1 increased TLR-2 mediated activation of NF-kβ. Furthermore, various dominant negative downstream regulators of TLR-2 such as MyD88, toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), Interleukin-1 receptor-associated kinase 1 (IRAK1), Interleukin-1 receptor-associated kinase 2 (IRAK2), Interleukin-1 receptor-associated kinase 4 (IRAK4), TNF receptor associated factor-6 (TRAF-6) and p38 inhibited HMGb1 induced activation of NF-kβ (Park *et al*. 2006). The interaction of HMGb1 was also substantiated by the finding where HMGb1 induced TNFα release was compromised in MyD88-/- and TLR-2-/- mice in comparison to wild type

mice. This suggests that HMGb1 interaction with TLR-2 can induce other pathways that may cross talk at several levels. However, this ultimately results in the activation of NF-kβ (Yu *et al*. 2006).

The ligand HMGb1 can signal through either TLR-2 or TLR-4. The signalling through TLR-4 has been shown to play important role for MyD88 and IRAK. To investigate the contribution of downstream proteins, RAW 264.7 macrophages were transduced with NF-kβ dependent luciferase reporter and dominant negative forms of MyD88, TIRAP, IRAK1, IRAK2 and IRAK4. It was found that dominant negative MyD88, TIRAP, IRAK1, IRAK2 and IRAK4 inhibited HMGb1 induced NF-kβ dependent luciferase reporter gene activity. In addition, it has been shown that PI3K can directly interact with TLR-4. Therefore, these data suggested key roles of these proteins in TLR-4 signalling cascade (van Beijnum *et al*. 2008; Park *et al*. 2006).

The signalling cascades that occur in TLR-2 and TLR-4 activation overlap at many points. However, the differentiation between two pathways might be visible at the level of proteins bound to the TIR domain of TLR. In addition, it has been shown that TLR-4 signalling could be MyD88 dependent or independent. However, in both cases, NF-kβ dependent gene expression will be seen followed by TLR activation. NF-kβ is a transcription factor belongs to five-member family of hetro or homodimers. These dimers remain in the cytoplasm in an inactive form by the inhibitor of kappa β kinase (Ikβ). The phosphorylation of Ikβ is required for activation of NF-kβ (Park *et al*. 2004).

The HMGb1 binding to TLR-4 is important in many pathological conditions including inflammation. This interaction triggers the activation of proinflammatory cascade. For example,

HMGb1 produced in tissues and serum during the inflammation triggers the release of TLR-4 dependent TNF-α (Tsung *et al*. 2005). In addition, HMGb1 has been shown to induce migration in vascular smooth muscle cells via TLR-4 dependent PI3K/Akt pathway (Inoue *et al*. 2007). The molecular understanding of HMGb1-TLR-4 interaction in inflammation may have significant implications for designing targeted therapeutics to suppress HMGb1 mediated tissue injury. Anti-HMGb1 antibody is one such example that has been highly effective in tissue injuries to animals with sepsis, ischemia and collagen induced arthritis (Yang *et al*. 2010). Though, TLR-4 has been implicated in migration and proliferation of neutrophil and vascular smooth muscle cells (Reaves *et al*. 2005; Rönnefarth *et al*. 2006; Pi *et al*. 2013). However, its role in cancer-associated myofibroblasts is not clear.

1.4 Myofibroblasts

Myofibroblasts are one type of mesenchymal cell that constitute a major part of stroma. Myofibroblasts are spindle shaped cells transiently found in early to mid-phase wound tissue and predominantly derived from mesenchymal stem cells (Quante *et al*. 2011). By secreting cytokines, growth factors and extracellular matrix proteins and proteases, they play important role in inflammation, repair and fibrosis (Marangoni *et al*. 2015). In addition, myofibroblasts play important roles in connective tissue remodelling by synthesising ECM components and providing cytoskeletal support from smooth muscle cells. It is well established that myofibroblasts contribute toward connective tissue remodelling by exerting contractile force, synthesise ECM component and undergo apoptosis in wound healing. However, in desmoplastic situations, myofibroblasts may persist and cause organ failure (Hinz *et al*. 2007).

The tumour associated myofibroblasts provide that mechanical environment (contractile force and ECM remodelling) which promotes tumour progression (Mareel *et al*. 2009).

The contractile force is necessary for generating tissue contraction. The myofibroblasts generate this contractile force by expressing alpha smooth muscle actin (α-SMA) encoded by the ACTA gene. This force is stronger than other forces generated by other isoforms of actin in myofibroblasts cells. The contractile force exerted by myofibroblasts followed by ECM synthesis and connective tissue remodelling is irreversible and thus can produce prolonged contractures (Follonier *et al*. 2010). It has been shown that myofibroblasts use a lockstep mechanism of contractile events that results in strong contractions mediated by RhoA/Rho-associated kinase *in vitro.* This study supported strong isomeric contraction generated in myofibroblast stressed collagen (Castella *et al*. 2010).

The origin of myofibroblasts remains controversial as it has been suggested that myofibroblasts originate from progenitor stem cells whereas some other studies suggest that they transdifferentiate from tissue fibroblasts (Gabbiani 1996; Powell *et al*. 1999), (Figure 1.7). Furthermore, pericytes and vascular smooth muscles share a close anatomical relationship, suggesting more than one route for transdifferentiation of myofibroblasts (Powell 2000).

The transdifferentiation of fibroblasts to myofibroblasts involves proto-myofibroblasts that express extra domain A (ED-A) variant of fibronectin at the cell surface (Figure 1.7). The proto-myofibroblasts are an intermediate between fibroblasts and myofibroblasts and have been shown to form actin containing stress fibres (Tomasek *et al*. 2002). These cells are capable of generating contractile forces necessary to close the wound during the healing process.

Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine that plays a central role in natural wound healing process. It has been shown that TGF-β1 increases the expression of ED-A fibronectin and modulates the differentiation of proto-myofibroblasts into myofibroblasts with *de-novo* expression of α smooth muscle actin (αSMA) (Figure 1.7) (Tomasek *et al*. 2002). Proto-myofibroblasts are different to myofibroblasts as myofibroblasts exhibit a higher organisation of extracellular fibronectin in fibrils, hence can generate greater contractile force than proto-myofibroblasts *in vivo* (Tomasek *et al.* 2002). Their positivity for α-SMA has been considered as a most widely used biomarker. However, it is a misconception that myofibroblasts must express α-SMA to be 'myofibroblasts'. The most important defining nature of myofibroblasts is the *de novo* development of stress fibre and contractile force (Follonier *et al*. 2010).

Certain other factors such as interleukin-1 (IL-1), tumour necrosis factor α (TNFα), PDGF and TGF-β play an important role at various stages of transdifferentiation of myofibroblasts (Kovacs and DiPietro 1994). TGF-β has been shown to be the most important cytokine for the transdifferentiation of myofibroblasts from fibroblasts. Myofibroblasts have been shown to have high levels of αSMA (Vaughan *et al*. 2000) (Figure 1.7). Although myofibroblasts share many of the features of fibroblasts and smooth muscle cells, smoothelin and caldesmon (components of smooth muscles) are absent in myofibroblasts (Van der Loop *et al*. 1996). In addition, several other modulators of the myofibroblast phenotype have been suggested such as endosialin in tumour associated fibroblasts, osteopontin in dermal fibroblasts and periostin (Gullberg and Reed 2011; (Lenga *et al*. 2008). Expression of these biomarkers has been reported as being unique to the myofibroblast phenotypes (Vi *et al*. 2009).

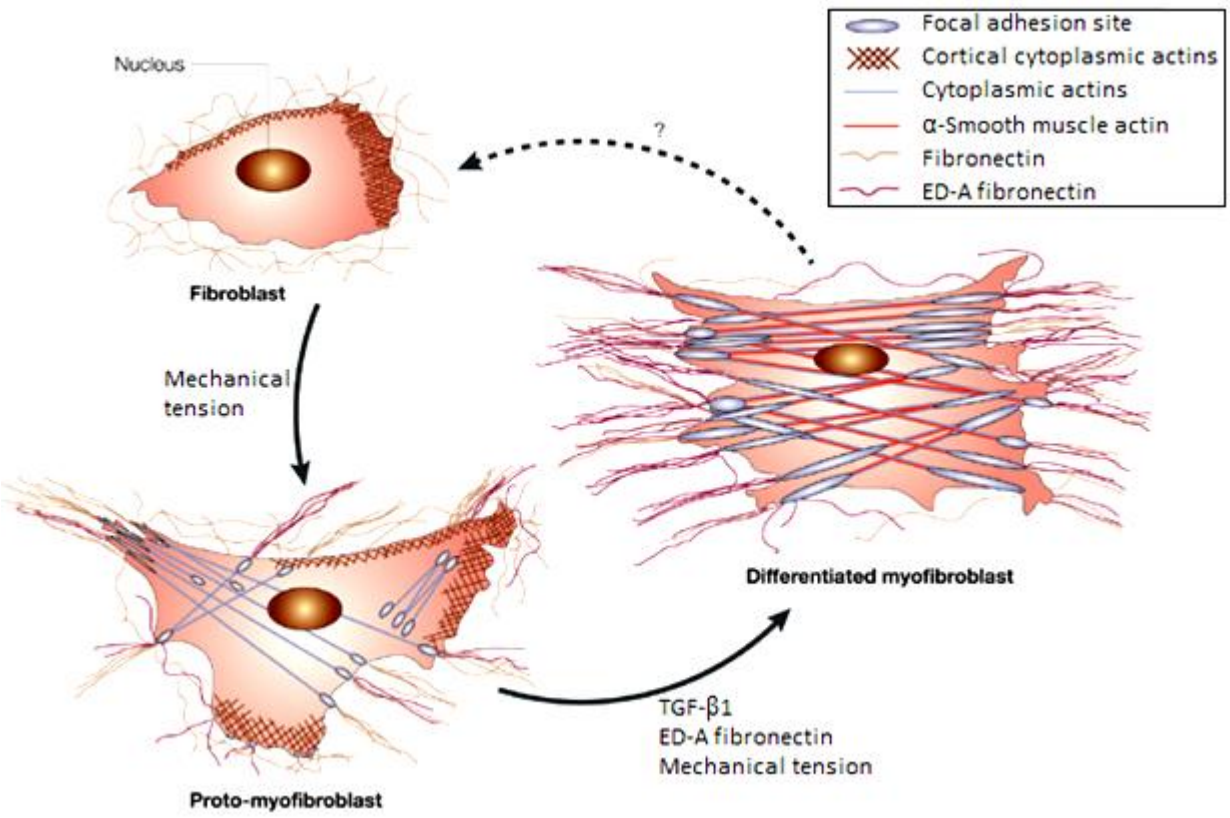


Figure 1.7: *TGF-β1 mediated transdifferentiation of myofibroblasts from fibroblasts. Proto-myofibroblasts share similar features of myofibroblasts but are weaker in generating the contractile force necessary for wound healing process. Myofibroblasts exhibit de novo expression of α SMA (Tomasek et al. 2002).*

Myofibroblasts play a significant role during the inflammatory response as they can produce both chemokines and cytokines (Hogaboam *et al.* 1998). The myofibroblasts induced secretion of cytokines and chemokines can augment or downregulate the inflammatory response (Tetsuka *et al*. 1994). In addition, myofibroblasts express key adhesion molecules such as vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM) and neural

cell adhesion molecule (NCAM). This suggests that the cells like lymphocytes, mast cells and neutrophils may get attached onto myofibroblasts to take part in the inflammatory response (Schroeder and Page 1972).

Myofibroblasts have been shown to play major roles in the pathogenesis of renal interstitial fibrosis (Yang and Liu 2001; Liu 2006). Although it is clear that myofibroblasts play important role in the pathogenesis linked to renal failure, the origin of renal myofibroblasts is poorly understood. There are several progenitor cells that have been suggested such as local fibroblasts, circulating fibrocytes and epithelial cells residing via epithelial to mesenchymal transition (EMT) (Zeisberg and Kalluri 2004). However, the presence of epithelial progenitor cells via EMT route remains controversial (Inoue *et al*. 2009).

The role of myofibroblasts in lung fibrosis has been established. The lung fibrosis may involve chronic pulmonary obstructive disease, pleural fibrosis and pulmonary hypertension. Indeed, many studies have shown that lung myofibroblasts are derived from bone marrow progenitor cells and lung epithelium cells. However, some studies contradicted the involvement of lung myofibroblasts in lung fibrosis. For example, there was no contribution from myofibroblasts in acute lung injury involving intra-tracheal instillation of bleomycin *in vivo* (Rock *et al*. 2011). The involvement of myofibroblasts in lungs may be linked with chronicity and irreversibility of the fibrotic process. The fate of myofibroblasts of any origin in lungs may determine the onset of normal healing process or end-stage fibrosis (Rock *et al*. 2011).

Myofibroblasts have been shown to escape immune surveillance and become apoptosis-resistant in many pathological conditions including liver fibrosis and cancer. The mechanism for

establishment of the apoptosis-resistant myofibroblast phenotype is not clear (Thannickal *et al*. 2004). However, various mechanisms have been suggested to play important roles in prolonged survival of myofibroblasts including their escape from immune surveillance. For example, CD34+ stellate precursor cells induce differentiation to myofibroblasts from lymphocytes and plasmocytes. The CD34+ stellate cells induce apoptosis in Fas-ligand positive lymphocytes. Therefore, apoptosis inducing Fas-ligand dependent signals would not be available to myofibroblasts. This escape from surveillance would consolidate myofibroblasts recruitment and enhance fibrosis. In addition, NF-$_{K}$β has also been shown to induce antiapoptotic effects in myofibroblasts in liver fibrosis (Wallach-Dayan *et al*. 2007).

It has also been shown that factors responsible for myofibroblast differentiation can also promote myofibroblast survival. For example, NADPH oxidase enzyme has been reported to promote TGF-β induced myofibroblast differentiation and the production of ECM components (Amara *et al*. 2010).

1.4.1 Myofibroblasts and cancer

The stroma is fundamental for the maintenance of epithelial tissue. The stroma also changes with alterations that occur in the epithelium. In cancer, these changes maintain an environment that supports proliferation and metastasis. It is established that desmoplasia, inflammation, and angiogenesis make a favourable environment for invasion and metastasis to occur (Mareel and Leroy 2003). In normal conditions, fibroblasts exhibit little ECM or cell matrix contacts. However, in the event of tissue injury or inflammation, fibroblasts transdifferentiate into myofibroblasts and migrate to damaged tissue and help in the formation of the ECM by producing collagen (Hinz *et*

al. 2007). As previously mentioned, myofibroblasts also secrete MMPs, major degraders of basement membrane, which also play an important role in the formation of new blood vessels (angiogenesis). Angiogenesis requires degradation of the basement membrane and the remodelling of the ECM (Rundhaug 2005).

It is evident that myofibroblasts are present in colon cancer, breast cancer, lung cancer, prostate cancer and pancreatic cancers (De Wever and Mareel 2003; Cho *et al*. 2012). However, their origin is controversial. Indeed, a number of origins of cancer-associated myofibroblasts have been suggested such as resident fibroblasts and bone marrow derived mesenchymal stem cells. In addition, endothelial cells, epithelial cancer cells, smooth muscle cells, adipocyte and stellate cells have also been suggested. This diverse possible origin of myofibroblasts may be related to the heterogeneous myofibroblast population that is observed within tumours (De Wever *et al*. 2008).

Myofibroblasts have been reported to refine the information about the response to cancer chemotherapy and prognostic outcome in patients with stromal tumours. For example, increased levels of α-SMA by myofibroblasts predict reoccurrence of disease in colorectal cancer (De Wever *et al*. 2008). The oncogenes derived paracrine signals trigger tumour growth and invasion. These signals may lead to myofibroblasts attraction, proliferation and invasion however identifying particular signals that influence myofibroblasts to infiltrate and proliferate on tumour site should be investigated (De Wever *et al*. 2004). The gene expression profile of tumour associated host cells including cancer associated fibroblasts and myofibroblasts resulted in prognostic signatures which were associated with poor survival in primary breast cancers. In the same study, the wound response signature obtained from 50 fibroblasts cultures

contained genes associated with tissue remodelling, migration and angiogenesis. In addition, breast cancer patients whose tumours expressed the wound response signature had a reduced survival rate than those patients whose tumours showed no expression of wound response signature. The protein types presented in wound response signature were expressed by myofibroblasts. This suggested an important role of myofibroblasts at prognostic front (Bissell and Hines 2011). In addition, myofibroblasts have shown to produce low density lipoprotein receptor (LOXL2) and α-SMA. The expression of PDGF-β, LOXL2, and CD10 in cancer associated with fibroblasts has been correlated with poor prognosis in breast, colon and pancreatic cancers (Paulsson, *et al*. 2009).

The interaction between two key cytokines (TGF-β and PDGF) and myofibroblasts appear to play an important role in cancer progression. It has been shown that PDGF is crucial for the development of proto-myofibroblasts at various stages of inflammation and during the wound healing process. However, PDGF does not induce the expression of αSMA in myofibroblasts (Tomasek *et al*. 2002).

Cancer cell derived TGF-β, an indirect pro-invasive factor has been shown to modulate myofibroblasts differentiation in stromal tumours (Mareel and Leroy 2003). In addition, TGF-β has been considered as tumour suppressor for its growth and cell cycle progression inhibitory properties in early stage tumours. However, late stage tumour cells not only become resistant to growth inhibition phenomenon induced by TGF-β but also start actively secreting TGF-β (Derynck *et al*. 2001). It has been observed that TGF-β has strong chemoattractant properties for fibroblasts. As a result, fibroblasts migrate towards TGF-β secreting cancer cells. This leads to transdifferentiation from fibroblasts into cancer associated myofibroblasts (Postlethwaite *et al*.

1987). The α-SMA negative fibroblasts do not stimulate invasion however after the TGF-β induced conversion into α-SMA positive myofibroblasts, they do (Watson *et al*. 2014). In addition, reactive oxygen species (ROS) induced oxidative stress has been shown to trigger myofibroblasts differentiation and secretion of pro-inflammatory cytokines such as IL-6 and IL-8 in primary tumours (Lane *et al*. 2011).

1.4.2 Role of myofibroblasts in metastasis

The tumour microenvironment plays an important role in the final outcome of cancer chemotherapy. The tumour stromal cells such as cancer associated fibroblasts (CAFs) have been shown to promote tumour proliferation and angiogenesis. Indeed, the inflammatory immune cells such dendritic cells and macrophages are highly recruited in carcinomas and play important roles in tumour growth and metastasis (Allinen *et al*. 2004) (Coussens and Werb 2002). Moreover, the likely role of myofibroblasts in tumour invasion front has been supported by a study which revealed that the myofibroblasts population gradually increases with the invasive stages of many cancers (Nakayama *et al*. 1998). In support of previous findings, it has been shown that CAFs play a significant role in the enhancement of metastatic spread by modulating α-SMA expression. For example, increased expression of α-SMA was seen in myofibroblasts present in human epidermal growth factor receptor 2 (HER2) positive breast cancers (Toullec *et al*. 2010). In addition, inactivation of the tumour suppressor, phosphatase and tensin homolog (P*ten*), resulted in increased tumour growth followed by ECM remodelling in stromal fibroblasts of tumours in mouse mammary glands (Trimboli *et al*. 2009).

There is evidence that support the involvement of stromal myofibroblasts in tumour development. For example, stromal myofibroblasts have been shown to stimulate growth in breast cancer cells *in vivo.* In addition, they have been shown to promote invasion of breast, pancreatic and squamous carcinoma cells *in vitro* (Casey *et al*. 2008; Hwang *et al*. 2008). The mesenchymal cells and their derivatives including myofibroblasts have been shown to promote breast cancer metastasis (Hu *et al*. 2008). The stromal myofibroblasts have been considered as predictors of human disease outcome and their abundance is correlated with poor survival in colorectal cancer (Tsujino *et al*. 2007).

The myofibroblasts population was significantly higher in breast adenocarcinomas and lung cancers that had metastasised to lymph nodes. For example, HER2 type invasive adenocarcinoma metastasising to lymph nodes have the highest proportion of myofibroblasts compared with basal or luminal (BLC or Lum-A) type cancers. This suggested that myofibroblasts population is correlated with tumour growth and metastasis (Toullec *et al*. 2010).

Myofibroblasts express genes that are responsible for the release of certain invasion associated factors, ECM proteins and protease. For example, the CAFs have been reported to express collagens, MMPs, cell adhesion molecules and cytoskeleton components. In addition, myofibroblasts have been shown to trigger deposition and proteolysis of ECM molecules which may facilitate migration of cancer cells (Gaggioli *et al*. 2007). Interestingly, the increased density of collagen present in myofibroblasts is directly correlated with tumour formation and this increased density of collagen is correlated with the invasive potential of lung cancers (Provenzano *et al*. 2008). These data further suggest an important of role of stromal myofibroblasts in cancer metastasis.

1.4.3 Interplay between myofibroblasts, the ECM and growth factors

Unlike normal stroma, tumour stroma exhibits an altered ECM and increased numbers of fibroblasts and myofibroblasts. In addition, the composition and amount of ECM generated may vary amongst tumours (Hinz and Gabbiani 2010). Myofibroblasts are known to play important roles in the formation and repair of the ECM. Recently, it has been accepted that the mechanical force generated by myofibroblasts is dependent upon the expression of αSMA. This mechanical force plays an important role in tissue remodelling, cytokine synthesis and ECM component production (Hinz and Gabbiani 2010). The ECM is made up of a mixture of matrix proteins including collagen and other glycoproteins and proteoglycans. Most of these proteins are produced by myofibroblasts and play important roles in parenchymal or epithelial cell differentiation (Fullár *et al*. 2015). This occurs by persistent accumulation of contractile cells (fibroblasts and myofibroblasts) and the formation of collagen in the ECM. This provides a scaffold for tissue formation and growth. This phenomenon is called fibrosis and usually resistant to treatments however, most of the time in cancer the ECM is remodelled (Hinz and Gabbiani 2010).

The mechanical resistance or contractile force generated during the formation of ECM with the action of TGF-β has been considered as primary stimulus for myofibroblasts differentiation. It has been shown that highly contractile cytoskeleton of myofibroblasts is stiffer than normal ECM stiffness threshold. It is known that various fibrotic organs and cancerous tissues exceed this threshold support the presence of myofibroblasts in pathological conditions (Hotary *et al*. 2000). The tissue stiffness has been reported as a consequence of myofibroblasts induced ECM remodelling. In addition, it has been shown that an increased liver stiffness lead to the activation

of fibroblastic cells such myofibroblasts and the accumulation of collagen *in vivo*. This suggested that early mechanical changes are sufficient to induce contraction cascade (Georges *et al*. 2007). In addition, myofibroblasts have been shown to activate TGF-β from stiffened ECM by integrin mediated contraction. Therefore, there could be a direct link between mechanical and chemical factors regulating myofibroblasts differentiation which can cause fibrosis (Wipff *et al*. 2007).

The degradation of the ECM is a physiologically regulated phenomenon and is important for tissue repair and remodelling. However, a dysregulated degradation of ECM can become a cause of many diseases including cancer (Nagase *et al*. 2006). The matrix metalloproteinase (MMP 1-3), major degraders of basement membranes and the matrix, are also secreted by myofibroblasts. However, these MMPs are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs) (Leivonen *et al*. 2013). Thus, a balance between MMPs and TIMPs is important for ECM remodelling in the tissue. These MMPs are classified on the basis of substrate they degrade; for example, MMP-1 digests collagen type I, II, and III, MMP-2 digests collagen type I, III and IV and MMP-3 degrades laminin, fibronectin and casein. However, many growth factors may bind to proteoglycans or collagens and thereby can neutralise the effect of MMPs temporarily (Benyon *et al*. 1996)**.**

The myofibroblasts also secrete growth factors, which play important roles in initiating cell mobility and proliferation through paracrine and autocrine mitogenic effect and also in the induction of cell differentiation and sometimes apoptosis. It has been shown that certain inflammatory cytokines, eicosanoids and growth factors released from damaged tissue may act more proximally on myofibroblast and influence myofibroblasts to secrete additional growth

factors *in vivo* (Powell *et al*. 1999). For example; hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) are secreted by myofibroblasts in response to IL-1 (Chedid *et al*. 1994). It has also been observed that growth factors such as epidermal growth factor (EGF) and TGF-α, insulin like growth factors (IGF-I and IGF-II), fibrosis growth factors (FGF-1, FGF-2, KGF), IL-6 and IL-11 are secreted by myofibroblasts and act as major paracrine growth factors for epithelial and parenchymal cells (Bamba *et al*. 2003) . In addition, CXC family of cytokines such as IL-6 and FGF family (FGF-1 and FGF-2) secreted by myofibroblasts are particularly important as they possess angiogenic properties necessary for wound healing (Powell *et al*. 1999).

1.4.4 Myofibroblasts differentiation in the tumour microenvironment

In the tumour microenvironment, cancer cells interact with local and distant host cells. This interaction may alter the whole system and trigger tumour initiation, invasion and metastasis. This is evident by desmoplastic reactions, response to inflammation by macrophages, lymphocytes and dendritic cells and neovascularisation in primary tumours (Mareel and Leroy 2003). In the tumour microenvironment, myofibroblasts induced secretion of pro-inflammatory cytokines may have an impact on the recruitment of inflammatory cells. These inflammatory cells may influence the invasive potential of tumour cells. For example, cancer associated fibroblasts isolated from multistep skin tumourigenesis expressed activated levels of IL-6 (Erez *et al*. 2010).

The tumour microenvironment undergoes continuous remodelling in primary tumours that initiates a metastatic niche and the recruitment of host cells. This suggests an important role for circulating secretory products in the microenvironment. The primary tumour signals to the bone marrow and triggers the release of mesenchymal cells (MSCs). The MSCs then enter into the circulation and are recruited to the tumour site by stromal cell-derived factor-1α (SDF-1α). At the tumour site, TGF-β triggers the MSCs to be converted into myofibroblasts (Quante *et al*. 2011).

Hypoxia, one of the hallmarks of the tumour microenvironment has also been shown to induce the transdifferentiation of fibroblasts to myofibroblasts. Hypoxia induced stabilisation of HIF-1α has been shown to play important role in this process. The inhibition of HIF-1α in junD deficient fibroblasts resulted in reduced expression of αSMA containing stress fibre. This suggested that HIF-1α is crucial for regulating contractile features of fibroblasts which would then transdifferentiate into myofibroblasts (Toullec *et al*. 2010).

JunD, a functional component of the activator protein-1 transcription factor (AP1), has key roles in controlling angiogenesis and damage from oxidative stress. The inactivation of JunD results in the accumulation of ROS in cells and tissues. In addition, junD inactivation facilitates tumour growth followed by extensive modification in the tumour stroma including differentiation of myofibroblasts from fibroblasts. In the tumour stroma, junD deletion caused fibroblasts to adopt myofibroblastic features such expression of α-SMA with high levels of adherens and focal junctions. This also included the enhanced expression of vinculin, tensin and focal adhesion kinase (FAK) contents (Gerald *et al*. 2004).

A large population of granulin-secretory cells are also found in tumours. The granulin accumulation in tumours converts resident fibroblasts to myofibroblasts and promotes tumour growth (Elkabets *et al*. 2011). The secretory cells in the tumour microenvironment recruit hematopoietic stem cells from the bone marrow, which can promote myofibroblast differentiation at distant sites. For example, TGF-β present in exosomes can convert fibroblasts into myofibroblasts in distant organs. The exosomes are secreted by most cell types including cancer cells (Pitt *et al*. 2014). Thus TGF-β is always in abundance in most cancers and can influence the stromal cells within the microenvironment. It has also been suggested that TGF-β might facilitate metastasis by producing fibronectin followed by trans-differentiation from proto-myofibroblasts to myofibroblasts (De Wever *et al*. 2004). During the process of ECM remodelling, cancer associated fibroblasts or myofibroblasts secrete various MMPs which are associated with ECM degradation. Some myofibroblasts cells can move along with metastatic cancer cells (Figure 1.3) to the distant sites including lungs. These migratory myofibroblasts have been shown to produce pro-survival signals in lung site (Duda *et al*. 2010). Therefore, transformed fibroblasts or cancer-associated myofibroblast may influence the tumour microenvironment and contribute towards malignant transformation in solid tumours. Although myofibroblastic contribution has been established in malignancies, the role of HMGb1 in myofibroblasts and the subsequent consequences have not been reported thus far. Therefore, it is logical to investigate the role of HMGb1 in myofibroblasts as this may be linked with invasion and metastasis of tumour cells.

1.5 Aims and objectives

This research is based on the hypothesis that HMGb1, a novel non-histone chromatin protein plays important roles in the stimulation of tumour associated myofibroblasts. Upon stimulation,

these myofibroblasts may proliferate in situ followed by their invasion and migration to distant sites. This HMGb1 led migration and invasion of tumour associated myofibroblasts might facilitate the migration of other neighbouring cells including cancer cells. Therefore, it is also likely that the levels of HMGb1 in tumour microenvironment are directly related to metastatic potential of those tumours.

The role of HMGb1 in the stimulation of tumour associated myofibroblasts has not been reported yet. Thus, the initial aims of this study will be to investigate if the recombinant HMGb1 can stimulate CCD18 myofibroblast cells to proliferate *in vitro*. In addition, the pathways involved in the proliferation will be investigated. It is known that HMGb1 is released from necrotic cancer cells however, if there is any connection of other microenvironmental stresses such as glucose starvation/hypoxia/lack of other nutrients such as glutamine with the release of HMGb1 is not clear. These stress conditions represent typical intra-tumoral stresses and very common in tumour microenvironment. Therefore, an investigation will be carried out to check if HMGb1 is released extracellularly from a panel of cell lines when exposed to different microenvironmental stress conditions. An investigation into the role of extracellular HMGb1 will be carried out to check if this extracellular HMGb1 released from different cancer cells under stress conditions can stimulate CCD18 myofibroblast cells to migrate and invade. In addition, the involvement of HMGb1 receptors such as RAGE and TLRs along with downstream signalling pathways such as PI3K and ERK1/2 will also be investigated. The involvement of receptors and/or downstream pathways will be validated by the inclusion of selective antagonists in each study to provide ‘proof of concept’ based outcomes. Based on the findings, a possible mechanism for tumour spread involving proliferation, migration and invasion of myofibroblast cells along with possible activation of some downstream pathways can be proposed.

Chapter 2

2. General Materials and Methods

2.1 Recombinant proteins, antibodies, antibiotics and cells

Human recombinant HMGb1, anti-RAGE, anti-TLR4, and anti-MMP-2 and anti-MMP-9 primary antibodies was obtained from R&D Systems. Anti-mouse, anti-goat and anti-rabbit secondary antibodies were also obtained from R&D Systems. The MEK1/2 inhibitor (U0126) and PI3K inhibitor (LY294002) were obtained from Cell Signaling Technology®. HT29 colon adenocarcinoma cells, EJ138 bladder cancer cells, A549 lung cancer cells, MCF-7 breast cancer cells and CCD18 myofibroblast cells were purchased from American Type Culture Collection (ATCC).

2.2 Cell culture

Myofibroblasts (CCD18) cells were cultured in T75 cm^2 flasks in MEM Eagle complete medium (Minimum Essential Medium Eagle) (Sigma Aldrich) supplemented with 10% foetal calf serum (FCS) (BioSera), 1% glutamine (GlutaMax-100x) (Gibco Ltd) and 1% antibiotic/antimycotic solution (100x) (Gibco Ltd) at 37°C, 5% CO_2 and 95% humidity. The cells were passaged when they had reached about 85-90% confluence. To passage the cells, the complete medium was aspirated off and cells were washed twice with phosphate buffered saline (PBS). The PBS was then aspirated off and cells were treated with 1ml of Trypsin-EDTA solution (0.025%(w/v))

(Invitrogen). The complete medium contains many substances including Mg^{2+} and Ca^{2+}, which are trypsin inhibitors. Hence, washing cells with PBS prior to the treatment with trypsin is important. After the treatment with trypsin, cells were incubated at 37°C until the cells had detached from the flask surface. The trypsin-EDTA solution was then neutralized by the addition of 5ml of complete medium and this cell suspension was used to seed more flasks.

Other cancer cell lines that were used such as HT29 colon adenocarcinoma cells, A549 lung cancer cells, MCF-7 breast cancer cells and EJ138 bladder cancer cells were maintained using RPMI1640 complete medium consisting of RPMI1640 (Sigma Aldrich) supplemented with 10% foetal calf serum (FCS) (BioSera), 1 % glutamine (GlutaMax-100x) (Gibco Ltd) and 1% antibiotic/antimycotic solution (100x) (Gibco Ltd) at 37°C, 5% CO_2 and 95% humidity.

2.2.1 Cryopreservation

Cells were routinely stored in liquid nitrogen for later use. After removing the medium from a T75 cm^2 flask (85-90% confluent), the cells were washed with PBS twice to remove the traces of complete medium before treating with 1ml of Trypsin-EDTA. The flask was then allowed to incubate at 37°C, 5% CO_2 and 95% humidity for a few minutes until the cells had detached from the surface of the flask. The trypsin was then neutralized by the addition of 5ml of complete medium. The cell suspension was then transferred to a universal tube and centrifuged at 1000rpm for 5 minutes at 4°C. The supernatant was then carefully discarded without disturbing the cell pellet. The cell pellet was then resuspended in 1ml of freeze mix (70% RPMI medium, 20% FCS and 10% dimethyl sulphoxide (DMSO). DMSO is a cryoprotectant and protects the

cells from rupture caused by the formation of ice crystals. The cryovial was then transferred to isopropyl alcohol containing 'Mister Frosty' which was slowly cooled to -80°C in a freezer before being transferred to liquid nitrogen for long term storage.

2.2.2 Treatment of CCD18 cells with recombinant HMGb1

To determine the effect of HMGb1 on proliferation of CCD18 myofibroblasts, cell suspensions of CCD18 cells ($6x10^4$ cells/ml) were used to seed 24-well plates (1ml/well). The plates were incubated at 37°C and 5% CO_2 for 24 hours to allow the cells to adhere and begin proliferating. After this period, the complete medium was aspirated from each well and the cells were then washed twice with PBS. Serum free MEM Eagle medium was added to each well (1ml/well) and plates were allowed to incubate for a further 24 hours at 37°C, 5% CO_2 and 95% humidity. The next day, cells were treated (1ml/well) for 96 hours with a range of concentrations of recombinant HMGb1 (R&D systems) (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100ng/ml) made up in serum free MEM Eagle (four wells per treatment per 24-well plate). In addition, four wells per plate were treated with serum free MEM Eagle medium as controls (serum free medium without HMGb1).

2.2.3 Treatment of CCD18 cells with recombinant HMGb1 and PI3K or MEK1/2 inhibitors (LY294002 or U0126)

In order to investigate the likely pathway/s that HMGb1 might utilise to signal proliferation, experiments were carried out using HMGb1 alone or in the presence of Phosphatidylinositide 3-kinases (PI3K) or mitogen-activated protein kinase kinase (MEK1/2) inhibitors. To achieve this, CCD18 myofibroblasts were seeded ($6x10^4$ cells/ml) into 24-well plates (1ml/well). The plates were incubated at 37°C and 5% CO_2 for 24 hours to allow the cells to adhere and begin proliferating. After this period, the complete medium was aspirated from each well and the cells were then washed twice with PBS. MEM Eagle (serum free) was added to each well (1ml/well) and plates were allowed to incubate for a further 24 hours at 37°C, 5% CO_2 and 95% humidity. The next day, the medium was removed and cells were treated (1ml/well) for 48 hours with HMGb1 (10ng/ml) alone, HMGb1 (10ng/ml) with LY294002 (5µM) and HMGb1 (10ng) with U0126 (50µM) (R&D systems) made up in serum free MEM Eagle medium (8 wells per treatment per 24-well plate with either U0126 or LY294002). In addition, eight wells were treated with serum free MEM Eagle only as controls (serum free medium without HMGb1, LY294002 or U0126). The 24-well plate treatment scheme was planned using 8 wells for controls (serum free medium), 8 wells for HMGb1 (10ng/ml in serum free medium) and 8 wells for combination treatment with HMGb1 (10ng/ml) and U0126 (50µM/ml) in serum free medium). Similar treatment scheme was implemented for HMGb1 and LY2904002 treatment which included 8 wells for controls (serum free medium), 8 wells for HMGb1 (10ng/ml in serum free medium) alone and 8 wells for combination treatment with HMGb1 (10ng/ml and LY2904002 (5µM/ml) in serum free medium).

2.2.4 Assessment of proliferation using the neutral red uptake (NRU) assay

The neutral red uptake assay has traditionally been viewed as an assay of toxicity however, it can also be used as an assay of proliferation. Neutral red (NR) is a weakly cationic dye which enters into the living cells by non-ionic diffusion through the cell membrane. Once internalised, the dye then accumulates in the lysosomes of living cells. The NR can then be extracted using 1% acetic acid and 50% ethanol in dH_2O. The absorption spectrum of the supernatant containing NR can be measured at 540nm (Borenfreund and Puerner 1985; Borenfreund and Puerner 1986).

A neutral red medium mix was made immediately prior to use by the addition of 380ul of neutral red solution (Sigma Aldrich, N2889) to 25ml of pre-warmed (37°C) MEM Eagle serum free medium to achieve a working concentration of 50ug/ml (w/v). The HMGb1 treatment medium was aspirated off from the cells. The neutral red medium mix was added (1ml/well) and the 24-well plates were returned to the incubator for 2 hours for the dye to be taken up by the cells. After 2 hours, the cells were washed with PBS (1ml/well) once and neutral red de-stain (50% ethanol, 49% dH_2O and 1% glacial acetic acid) was added (350µl/well) to lyse the cells and release the dye. Complete cell lysis and a homogenous colour was achieved by gentle shaking on a plate shaker. Aliquots of 100µl (x 3 per well) of resulting coloured solution were transferred to wells of 96-well plates. The absorbance of the accumulated neutral red was measured at 540nm using the Titertek Multiscan plate reader. The readings from treated cells were compared to those of non-treated control cells and these results expressed as percentages taking the control mean value as 100%.

2.2.5 Assessment of toxicity and proliferation using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

The MTT assay developed by Mossman (1983) can also be used to determine cytotoxicity or proliferation of cells in response to treatment with substances. In the ideal world, colorimetric assay for living cells should utilize a colourless substrate which can be converted into a coloured product by any living cell. The tetrazolium salt measures the activity of various dehydrogenase enzymes and it is cleaved in active mitochondria. MTT is cleaved by all living and metabolically active cells however it is not the case with dead cells. Therefore, the reaction will only occur in the living cells. The amount of formazan produced by active cells is more than the cells underwent senescence. Therefore, it can measure the activation even if the cells are not proliferating (Mosmann 1983).

The MTT solution was made prior to use by the addition of 2.5ml of MTT solution (5mg/ml) (Sigma Aldrich, M5655) to 25ml of pre-warmed (37°C) minimum essential medium eagle serum free medium (working concentration 0.5mg/ml (w/v)). The HMGb1, U0126 and LY294002 treatment medium was aspirated off from the cells. The MTT mix was added (1ml/well) and the 24-well plates were returned to the incubator for 2 hours for the dye to be taken up by the cells. After 2 hours, 2-propanol was added (350µl/well) to lyse the cells and release the dye. Complete cell lysis and a homogenous colour were achieved with help by gentle shaking on a plate shaker. Aliquots of 100µl (x 3 per well) of resulting coloured solution were transferred to the wells of 96-well plates. The absorbance of the accumulated MTT solution was measured at 540nm using the Titertek Multiscan plate reader. The readings from treated cells were

compared to those of non-treated control cells and these results expressed as percentages taking the control mean value as 100%.

2.3 Protein Assay

2.3.1 Sample preparation from CCD18, HT29, MCF-7, EJ138 and A549 cells

CCD18 myofibroblast cells, HT29 colon adenocarcinoma cells, A549 lung cancer cells, EJ138 bladder cancer cells and MCF-7 breast cancer cells were seeded into 17 x T25 cm^2 flasks at a density of $4.0x10^5$ per flask and allowed to grow overnight at 37°C, 5% CO^2 and 95% humidity. The following day, medium was removed and replaced with serum free medium (5ml/flask) and incubated for 24h. After 24h, the medium was aspirated off and replaced with fresh medium (RPMI 1640) with glucose (1.8 g/L) (9 flasks) and without glucose (8 flasks). The glucose free flasks were either incubated in normoxic or anoxic conditions (4 flasks for each condition). The flasks with glucose were also treated to the same conditions (4 flasks for each condition). The remaining flask was used as day 0 control (serum free medium with glucose). The medium was decanted into a labelled universal tube. This medium was immediately frozen at -80°C. For each of the following 4 days, 1 flask of cells from each condition (anoxia or normoxia with or without glucose) was removed and the medium again stored at -80°C. Between day 2 and 4, in some of the conditions, some cells appeared to be floating in the medium, these were presumed to be dead cells. These cells were removed from the medium by centrifugation (1,000rpm for 5 min) before the medium was frozen at -80°C. The medium was collected for investigation at 3 different occasions for each set of flasks.

In addition, HT29, A549, MCF-7 and EJ138 cells were treated with and without glutamine to assess the release of HMGb1 in low nutrient conditions. Two flasks of each cell type was treated with either glutamine (200mM/L) in serum free medium (RPMI 1640) or glutamine and serum free medium and incubated in normoxic conditions for 48h. The protocol for the collection of medium remained same as described above.

2.3.2 The extraction of proteins from the cells

The extraction of proteins from the flasks of cells (HT29 and CCD18) was carried out. To achieve this, a lysis buffer was prepared by adding phosphatase inhibitor cocktail set-II (10µl) and protease cocktail set-II (10µl) in RIPA buffer (980 µl) (Calbiochem reagents). To each flask, 100µl of lysis buffer was added and the cells were scraped using a cell scraper and transferred into a labelled eppendorf tube. The contents of the eppendorf tube were then homogenised for 30 seconds using a pellet pestle motor (Kontas). The eppendorf was then left on ice for 30min to achieve complete lysis. After 30 minutes, the tube was centrifuged for 3 minutes @10,000rpm at 4°C. The supernatant was carefully collected without disturbing the cell pallet in a labelled eppendorf tube before determining the protein concentration. This process was repeated for the cells from all flasks.

2.3.3 Determination of the sample protein concentration

To determine the protein concentration of the cell lysates and conditioned medium from each fl;ask, the BioRad DC protein assay was used. The BioRad protein assay is a colorimetric assay, which is modified from protein measurement technique using Folin phenol reagent described more than 60 years ago (Lowry, Rosebrough *et al*. 1951). This assay uses three different reagents namely reagent A (alkaline copper tartrate solution), reagent B (diluted Folin reagent) and reagent S (10% sodium dodecyl sulfate surfactant solution). This protein assay is based on the blue colour which is generated between the reaction of proteins with copper and the subsequent reduction of Folin by copper were amino acids, tyrosine and tryptophan are the reduced products. These reduced products upon loss of oxygen generate blue colour which is measured at 650-750nm. Bovine serum albumin (BSA) was prepared as a stock solution (1mg/ml) in dH_2O. This was then used to make up protein standards (0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75 and 1mg/ml).

To determine the protein concentration in the cell lysates, samples of each cell lysate (5µl) and each of the BSA standards (5µl) were added to wells of a 96-well plate (in triplicate). BioRad reagent 'AS' was prepared by adding reagent S (120µl) to reagent A (6ml). Reagent AS (25µl) was then added to each well of the 96-well plate. Then BioRad reagent B (200µl) was added to each well and the reagents were mixed well on orbital shaker for 10 minutes for the colour to develop. The absorbance was read on 690nm on a plate reader (Titertek multiscan) and background reading was measured for blanks using dH_2O. A BSA standard curve was prepared and the protein concentration of each sample was determined from this. The protein assay was carried out to ensure that all samples used for western blot were of approximately equal total

protein content. The same process was repeated to determine protein concentration in conditioned medium obtained from flasks of HT29 cells.

2.3.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the BioRad mini protean II electrophoresis system to separate the protein samples. Before pouring the gel, the gap (1.5mm) between the glass plates was filled with dH_2O to check for leakage. The dH_2O was removed and resolving gel was made up (Protogel (30%), 6ml; 4x resolving buffer, 5ml; dH_2O, 8.78ml; 10% (w/v) Ammonium persulphate (APS), 200µl; Tetramethylethylenediamine (TEMED), 20µl) and rapidly mixed without incorporating air bubbles quickly poured between the plates. The resolving gel buffer was then overlaid with 1ml of water saturated isobutanol and allowed to set. Isobutanol was used to prevent oxygen from getting into the gel which could oxidise it and inhibit polymerization. After the gel was set, this was removed and the top of the gel was washed with dH_2O and this was removed and the surface of the gel was carefully dried using filter paper. The stacking gel was again rapidly mixed without incorporating air (Protogel (30%), 1.3ml; 4 x stacking buffer, 2.5ml; bromophenol blue (0.05 mg/ml), 100µl; dH_2O, 6ml; 10% (w/v) APS, 50µl; TEMED, 10µl) was then poured on the top of the gel and a comb (10 well space) was inserted in the gel to make spaces for the protein sample wells. The gel was left for 30 minutes to set.

The samples were then diluted appropriately with Radioimmunoprecipitation assay (RIPA) buffer to make equal concentrations of total protein content (equalising to lowest protein

concentration of all the samples used on that occasion). The samples were prepared by mixing 50µl of sample with 10µl of 6x Laemmli loading buffer-reducing (1.5% SDS, 10% glycerol, 62.5Mm Tris-HCL (pH6.8), 0.0025% bromophenol blue, 2% β-mercaptoethanol). The samples were then denatured at 100˚C for 4 minutes. The gel was submerged in 1 x running buffer (25mM Tris, 192mM Glycine, and 0.1% (w/v) SDS, at pH8.3), and 45µl of each sample was loaded in each well in the stacking gel. The gel was run for 30 minutes at 26mA, or until the dye front had reached the bottom of the gel. Whilst the gel was running, 1 litre of transfer buffer was prepared by combining 100ml of 10x transfer buffer (Tris/Glycine Buffer, BioRad), 200ml methanol, and 700ml dH_2O. Once the gel had run, the gel was removed from the electrophoresis apparatus and soaked for about 15 minutes in transfer buffer.

2.3.5 Western blotting

A BioRad transfer tank was used to transfer the proteins from the gel to a nitrocellulose membrane (Amersham Hybond-ECL). Following electrophoresis, gels were allowed to soak in transfer buffer for 30 min to remove an excess salt. The nitrocellulose membranes and filter papers were cut in a size to cover the entire surface of the gel. The nitrocellulose membranes and filter papers were pre-soaked in transfer buffer for 20 min and a gel sandwich was prepared as illustrated in figure 2.1.

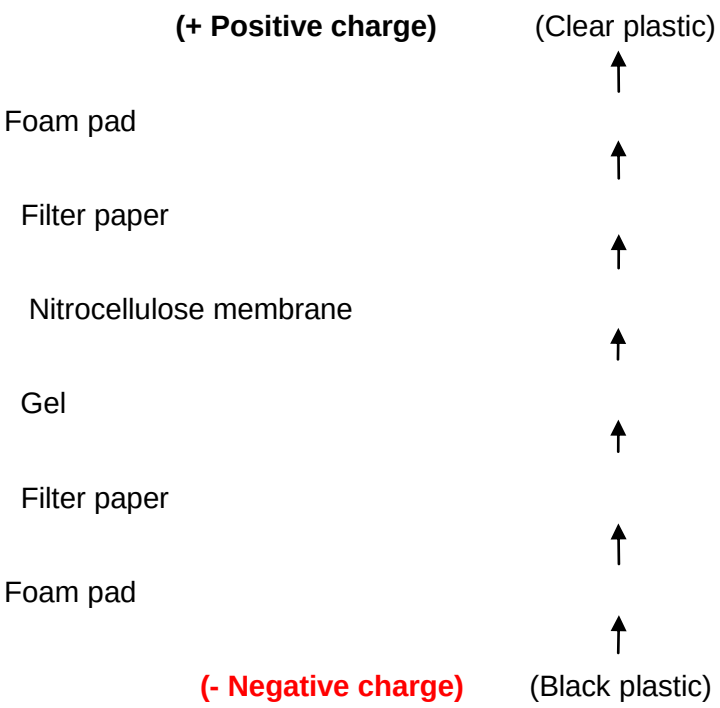


Figure 2.1 *Preparation of a gel/nitrocellulose transfer sandwich*

Note: *The arrows denote direction of protein migration with charge*

First a sponge was laid on black part of the gel holder, followed by filter paper, gel, nitrocellulose membrane, filter paper and finally another sponge, flooding with transfer buffer with each layer. A pasteur pipette was used as a rolling pin to remove any air bubbles from between the layers. A cooling block and the gel sandwich were fitted into the transfer tank and the tank filled with transfer buffer. The transfer of proteins was achieved running the gel at a constant 350 mA for 70 minutes.

One litre of Tris-Buffered Saline (TBS) (20mM Tris, 500mM sodium chloride, at pH 7.5) was made up. Nitrocellulose blots were washed (3 x 10 minutes) with TBS with 0.1% (v/v) Tween 20 (T) added (TBS-T). All non-specific protein binding sites were then blocked by incubating with

5% (w/v) non-fat milk (Marvel) in TBS-T for 1 hour at room temperature. Blocking buffer was poured off and the blot incubated with 10ng/ml mouse anti-human HMGb1 primary anybody (R&D Systems) or goat anti-human RAGE (R&D Systems) or TLR-4 antibody (R&D Systems) in 5% (w/v) Marvel (non-fat dry milk) in TBS-T at 4^{o}C overnight. The following day, the blot was washed with TBS-T (3 x 10 minutes). A secondary antibody (anti-mouse/goat/rabbit) conjugated to horseradish peroxidise (R&D systems) was diluted in 5% (w/v) Marvel in TBS-T (1 in 1000) and the blot was incubated in this for 1 hour at room temperature. The blot was then washed in TBS-T (3 x 10 minutes).

2.3.6 Blot development

Equal volumes each of ECL reagents (SuperSignal West Pico, Thermo Scientific) were mixed and added to the blot for 5 minutes on a shaking platform. Filter paper was used to remove excess reagent and the blot was placed on glass plate and wrapped in Clingfilm. The glass plate was placed into a cassette (Kodak) and taken to a dark room. Hyperfilm (Kodak) was loaded under the glass plate and the orientation of the blot was recorded. The cassette was then closed and the Hyperfilm exposed for the appropriate length of time. After this, the Hyperfilm was removed and developed and fixed (Kodak) according to the manufacturer's protocol.

2.3.7 Dot Blotting

To determine the working concentration of anti HMGb1, anti-RAGE and Anti-TLR-4 antibodies, a dot blot analysis was carried out prior to the western blot for each protein type. The nitrocellulose membrane was cut to an appropriate size enough for 8-10 sample dots. The samples were then spotted (2µl) onto the membrane. The nitrocellulose membrane was then allowed to air-dry for 2 hours. From this point on, nitrocellulose blots were treated and developed in the same way as western blots (see section 2.3.5).

2.3.8 Quantitative Analysis of Western Blots

The intensity of individual bands of western blots was determined by analysing scanned images of membranes using ImageJ software. The intensity is expressed as a percentage in terms of "normalised integrated density" of the control band for each blot. The "integrated density" is defined as the sum of the pixels in the image. The control was allocated an arbitrary value of 100%.

2.4 Migration assay

It is hypothesised that HMGb1 is released from cancer cells into the culture medium, particularly in conditions of glucose starvation. It is also possible that this release of HMGb1 might trigger migration of myofibroblasts. Therefore, a chemotaxis assay (migration assay) was set up. HT29 colon adenocarcinoma cell lines were seeded in 2 xT75cm^2 (2x10^6 cells per ml). Both flasks were incubated over night at 37°C, 95% humidity and 5% CO_2 to allow the cells to adhere. The following day, the complete medium was aspirated off and cells were washed twice with PBS. The medium on the cells was replaced with serum free medium with glucose in one flask and without glucose in the other flask. After 48 h, both lots of 'conditioned medium' (glucose containing and glucose free used medium) were collected into labelled universals and to be used to test their chemoattractant properties using the migration assay.

The CCD18 cell suspension was made in serum free medium (4.0x10^4 per ml). In a 24-well plate, glucose free and glucose containing conditioned medium were added (2 wells of each, 0.75ml per well). The CCD18 myofibroblast cells were then seeded (0.5ml per insert) in 4 chamber inserts (Biocoat®, 8µm pores) and 4 inserts were put into the 4 wells in the 24 well plate and ensured that insert bottom is touching the conditioned medium in the wells. The plate was incubated for 20h in the incubator at 37°C, 95% humidity and 5% CO_2.

Note: *Versene was used to detach myofibroblasts cells undergoing migration and invasion assays*

After the 20h incubation period, the 24-well plates were removed and cells which had migrated were stained for counting. Fixation and staining steps were carried out using ReaStain Quick-Diff kit. Each chamber insert was dried with cotton bud from inside and dipped in fixative solution for 2 minutes followed by red stain (Reastain Quick-Diff Red) and blue stain (Reastain Quick-Diff Blue) for 2 minutes each. Each chamber insert was then rinsed thoroughly and left upside down position on a filter paper to dry for 30 minutes. Once the chamber inserts are dried, membranes were cut out (using a scalpel and tweezers), and mounted on a microscope glass slide using a small drop of 'Microll' immersion oil followed a cover slip. The membranes were viewed using inverted fluorescence and phase contrast microscope (Olympus IMT2) with camera (QImaging-MP3.3-RTV-CLR-10) and images of random fields of vision were captured from each membrane (x20 magnification) using Image Pro Plus software. The cells that had migrated through the membrane were counted using ImageJ cell counter software and the average of the 5 fields of vision for each membrane calculated.

In an effort to determine which cytokines or pathways are involved in the migratory response, the assay was also carried out using known inhibitors of specific cytokines or pathways. These were added to the myofibroblast cell suspension used in the inserts or conditioned medium used in the wells. The ERK1/2 inhibitor U0126 and PI3K inhibitor LY294002 used to block the respective pathways (see table 2.7). These inhibitors have been shown to selectively inhibit these pathways (Satoh, Nakatsuka *et al*. 2000; Gottschalk, Doan *et al*. 2005).

2.5 Invasion assay

CCD18 myofibroblasts invasion assays were performed using 8µm pore matrigel matrix Biocoat® inserts according to the manufacturer's instruction (Becton Dickinson). The CCD18 cell suspension was made in serum free medium ($6.0x10^4$ per ml). The myofibroblasts cells were plated onto (0.5ml per insert) inserts in a 24-well plate supplied by the manufacturer. The inserts were put into the wells of 24-well plate. HT29 conditioned medium was used as chemoattractant and plated onto the lower chamber (0.5ml) and ensured that inserts were touching the conditioned medium (see section 2.4). The plate was incubated for 22h in the incubator at 37°C, 95% humidity and 5% CO_2. The myofibroblasts invasion, in response to the conditioned medium with and without various inhibitors and antibodies were determined by their inclusion in the lower chamber of the 24-well plate. Cells invaded through the matrigel matrix membrane were detected on the lower surface by using ReaStain Quick-Diff staining method and visualised using at x20 magnification under a microscope using the protocol described for migration assay above (same as section 2.4). In an effort to determine which cytokines or pathways are involved in myofibroblast invasion, the assay was also carried out using known inhibitors of specific cytokines or pathways added to the myofibroblast cell suspension used in the inserts or conditioned medium used in the wells. (see table 2.7)

2.6 Statistics

Results are presented as mean and +SEM. The results were compared using t-test where appropriate and were considered significant at $p<0.05$.

2.7 Table of inhibitors and antibodies used in migration and invasion assays

Inhibitor Name	**Manufacturer**	**Working Concentration**	**Assay Compartment Used**
Anti-HMGb1 antibody	R&D Systems	5µg/ml	Inserts and wells
Anti-RAGE antibody	R&D Systems	8µg/ml	Inserts
Anti-TLR-4 antibody	R&D Systems	2µg/ml	Inserts
U0126 (MEK1/2 inhibitor)	Cell Signalling	50µM/L	Inserts and wells
LY294002 (PI3K inhibitor)	Cell Signalling	5µM/L	Inserts and wells

Chapter 3

3. Investigating the role of HMGb1 in stimulating CCD18 myofibroblasts proliferation

3.1 Introduction

The high mobility group box 1 protein is a member of the HMGb superfamily. It is expressed in all vertebrate and non-vertebrate cells, yeast, plants and bacteria (Bustin *et al*. 1990). HMGb1 is a non-histone protein involved in stabilization of nucleosomes and the bending of DNA, which facilitates gene transcription (Lange *et al*. 2008). Whilst HMGb1 is a nuclear protein, it is released from the cells under certain conditions to take part in the inflammatory process. During inflammation, extracellular HMGb1 activates infiltrating macrophages, via the RAGE receptor. In addition, activated macrophages/monocytes are also responsible for the release of HMGb1 in the extracellular milieu (Andersson *et al*. 2002; Huttunen and Rauvala 2004).

It has also been suggested that HMGb1 is involved in various diseases including autoimmune disorders, sepsis, chronic inflammatory disease and cancer (Wang *et al*. 2004; Sims *et al*. 2009). An investigation into the influence of HMGb1 on fibroblasts and keratinocytes has been explored recently, where HMGb1 was shown to act as a cytokine and stimulate keratinocyte scratch wound healing *in vitro* (Clericuzio *et al.* 2013). The HMGb1 activates various signalling pathways such as MAPKs, protein kinase B (AKT) and PI3K. These pathways play important roles in proliferation and migration of cells involved in wound healing such as fibroblasts and keratinocytes (Taboubi *et al*. 2007; Song *et al*. 2012). For example, HMGb1 was shown to

induce proliferation and migration of keratinocytes via ERK1/2 pathway (Ranzato *et al*. 2009). To further support these findings, Ranzto *et al*. (2009) used anti-RAGE antibodies and selective MEK1/2 inhibitor (PD98059) to inhibit HMGb1-RAGE-ERK1/2 pathway. The inhibition of MEK1/2 by PD98059 abrogated HMGb1 induced wound healing. Both PI3K and MEK1/2 pathways have also been suggested to play important roles in proliferation of colonic myofibroblasts cells (Hemers *et al*. 2005). In addition, the pathways have also been implicated in proliferation and migration of cancer cells. Bassi *et al.* (2008) investigated the role of HMGb1 in glioblastoma cells *in vitro*. The results suggested that HMGb1 triggers proliferation and migration of glioblastoma cells via ERK1/2 activation.

Ranzato *et al*. (2010) suggested that HMGb1 could be considered as a potential therapeutic target for the development of drugs for chronic inflammatory disease, chronic inflammatory autoimmune disorders and severe wounds. Although HMGb1 has been shown to induce proliferation and migration in various cancer cells including glioblastoma and colon cancer cells, its role and involvement with myofibroblasts has not been explored thus far.

The myofibroblasts have been shown to play important roles in wound healing and inflammation (Lin *et al*. 2015). They share similar characteristic features as those of fibroblasts however get differentiated by *de novo* expression of α-SMA and stress fibres during the transition from quiescent fibroblasts to myofibroblasts. Other mechanisms may include epithelial to mesenchymal or endothelial to mesenchymal transition (Zeisberg *et al*. 2007). It has been established that myofibroblasts play important roles in the formation and repair of the ECM. For

example, myofibroblasts have been shown to be involved in connective tissue remodelling by synthesising ECM components and providing cytoskeletal support from smooth muscle cells (Klingberg *et al*. 2013). Unlike normal stroma, tumour stroma exhibits an altered extracellular matrix (ECM) and an increased expression of myofibroblasts. However, the composition and amount of ECM generated may vary amongst tumours (Mehner and Radisky 2013). Although myofibroblasts are important cells in the tumour ECM, their role in tumour proliferation remains unclear. In addition, little is known about how they interact with tumour cells and why they are found generally found on the tumour periphery.

Myofibroblasts play a significant role during inflammatory response as they can produce both chemokines and cytokines. This production of cytokines and chemokines by myofibroblasts can augment or down regulate the inflammatory response (Tetsuka *et al*. 1994). Many pathological conditions such as liver fibrosis and cancer are characterised by elevated levels of myofibroblasts which are able to escape immune surveillance and become apoptosis-resistant (Barcellos-de-Souza *et al*. 2013; Mallat and Lotersztajn 2013). Therefore, it is possible that CAFs may receive continuous proliferative signals within the tumour microenvironment and subsequently may contribute to tumour progression.

We hypothesise that HMGb1, which is a nuclear protein is released from cancer cells in extracellular milieu within microenvironment and trigger proliferation in myofibroblasts.

3.2 Aims and objectives

The aims of this study were to:

a) Determine if HMGb1 can induce proliferation in CCD18 myofibroblast cells.
b) Determine if the effect seen was time and/or dose dependent.
c) Investigate the intracellular signalling pathways involved in HMGb1 induced proliferation of myofibroblasts.

3.3 Materials and methods

3.3.1 CCD18 myofibroblasts cell culture

The myofibroblasts (CCD18) cells were cultured in T75 cm^2 flasks in complete medium (minimum essential medium eagle) (Sigma Aldrich, Dorset UK) supplemented with 10% foetal calf serum (FCS) (BioSera, East Sussex UK), 1% glutamine (GlutaMax-100x) (Gibco Ltd, Warrington UK) and 1% antibiotic/antimycotic solution (100x) (Gibco Ltd) at 37°C, 5% CO_2 and 95% humidity. The cells were passaged when they had reached 85-90% confluence. While growing CCD18 myofibroblast cells, it was observed that the growth slowed down after passage 12. Therefore, all assays were carried out between passage number 2 and 10.

3.3.2 Treatment of CCD18 cells with HMGb1 and PI3K and ERK1/2 inhibitors (U0126 and LY294002)

Cell suspensions of CCD18 myofibroblasts ($5x10^4$cells/ml) were used to seed into 24-well plates (1ml/well). The plates were incubated at 37°C and 5% CO_2, 95% humidity for 24 hours to allow the cells to adhere and begin proliferating. After this period, the complete medium was aspirated from each well and the cells were then washed twice with phosphate buffer saline (PBS). Minimum essential medium (MEM) eagle (serum free with no other supplement) was added to each well and plates were allowed to incubate for a further 24 hours at 37°C, 5% CO_2 and 95% humidity. The next day, medium was aspirated off and cells were treated (1ml/well) for 96 hours with a range of concentrations of recombinant HMGb1 (R&D systems) (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100ng/ml) made up in serum free minimum essential medium eagle (four wells per treatment). In addition, four wells were treated with serum free-minimum essential medium eagle only as controls (serum free medium without any supplement and HMGb1). For proof of concept inhibitor assay, cells were treated (1ml/well) for 48 hours with HMGb1 (10ng) alone and HMGb1 (10ng) with LY294002 (5µM) and HMGb1 (10ng) with U0126 (50µM) (R&D systems) made up in serum free - minimum essential medium eagle (8 wells per treatment). In addition, eight wells were treated with serum free-minimum essential medium eagle only as controls (serum free medium without HMGb1).

3.3.3 Assessment of proliferation using the NRU assay

The CCD18 myofibroblast cells have population doubling time of 4-5 days (Figure 3). Therefore, CCD18 cells were treated for 96h with HMGb1 to give sufficient time for the proliferation. The controls were washed with PBS three times and treated with serum free medium 24h prior to the treatment and the medium was changed to fresh serum free medium on the day of treatment to ensure unavailability of other nutrients to the cells. A neutral red medium mix was made immediately prior to use by the addition of 380ul of neutral red solution (Sigma Aldrich, N2889) to 25ml of pre-warmed (37°C) minimum essential medium eagle serum free medium (a working neutral red concentration of 50ug/ml (w/v)). The HMGb1 treatment medium was aspirated off from the cells. The neutral red medium mix was added (1ml/well) and the 24-well plates were returned to the incubator for 2 hours for the dye to be taken up by the cells. After 2 hours, the cells were washed with PBS (1ml/well) once and neutral red de-stain (50% ethanol, 49% dH_2O and 1% glacial acetic acid) was added (350µl/well) to lyse the cells and release the dye. Complete cell lysis and a homogenous colour were achieved with help by gentle shaking on a plate shaker. Aliquots of 100µl (x 3 per well) of resulting coloured solution were transferred to wells of 96-well plates. The absorbance of the accumulated neutral red was measured at 540nm using a Titertek Multiscan plate reader.

3.3.4 Assessment of toxicity and proliferation using the MTT assay

The MTT solution was made prior to use by the addition of 2.5ml of MTT solution (5mg/ml) (Sigma Aldrich, M5655) to 25ml of pre-warmed (37°C) minimum essential medium eagle serum

free medium (a working MTT concentration of 0.5mg/ml (w/v)). The HMGb1, U0126 and LY294002 treatment medium was aspirated off from the cells. The MTT mix was added (1ml/well) and the 24-well plates were returned to the incubator for 2 hours for the dye to be taken up by the cells. After 2 hours, 2-propanol was added (350µl/well) to lyse the cells and release the dye. Complete cell lysis and a homogenous colour were achieved with help by gentle shaking on a plate shaker. Aliquots of 100µl (x 3 per well) of resulting coloured solution were transferred to wells of 96-well plates. The absorbance of the accumulated MTT solution was measured at 540nm using a Titertek Multiscan plate reader.

3.4 Results

3.4.1 HMGb1 triggers proliferation in CCD18 myofibroblasts

The HMGb1 has been shown to play critical roles as cytokines and danger signalling molecules. HMGb1 has been shown to be released from necrotic cells but not apoptotic cells and play multiple roles in extracellular milieu. In addition, HMGb1 has been shown to induce proliferation certain other cancer cells such as glioblastoma and normal cells such as endothelial cells. However, the role of HMGb1 in myofibroblasts has not been explored. Thus, determining whether HMGb1 can trigger proliferation in CCD18 myofibroblasts would be of interest. Therefore, the proliferation assays (NRU and MTT) were carried out to test the proliferative effect of recombinant HMGb1 on CCD18 myofibroblast cells.

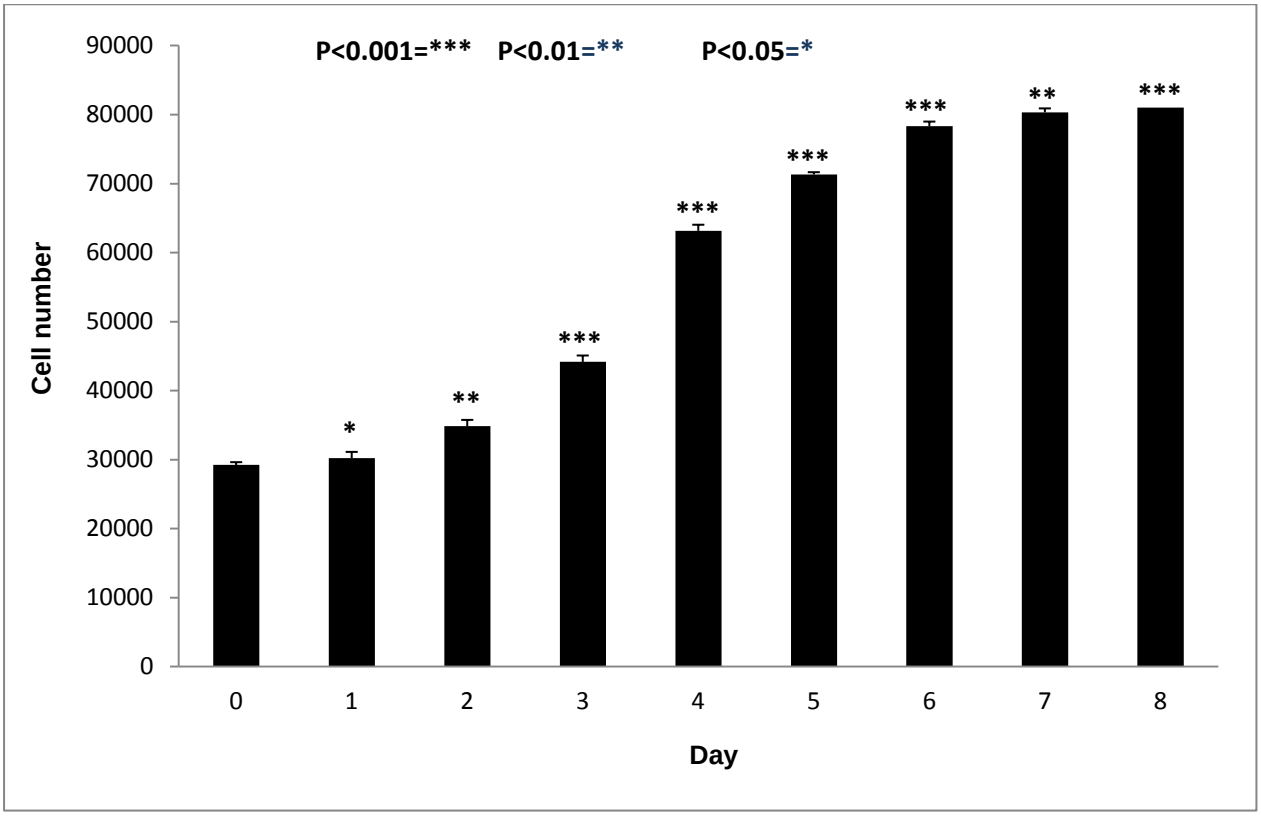


Figure 3.1: *CCD18 myofibroblasts growth curve showing growth over 8 days period. The cells were grown in MEM complete medium in 8x T25 cell culture flasks. The cells were trypsinised and manually counted on haemocytometer. The assay was carried out three times at three different occasions using different passage number of the cells each time.*

The log phase of the growth of CCD18 myofibroblast cells was determined. The CCD18 myofibroblast cells were seeded at a density of $3x10^4$ cells/ml in eight T25cm^2 flasks. Each flask was sacrificed every 24h from day 0-8, where day 0 being the day when cells were seeded. The cells were trypsinised and counted on a haemocytometer. The population doubling time of CCD18 myofibroblasts was around 3-5 days where log phase started when cells reached a density of approximately $4x10^4$ cells/ml (Figure 3.1).

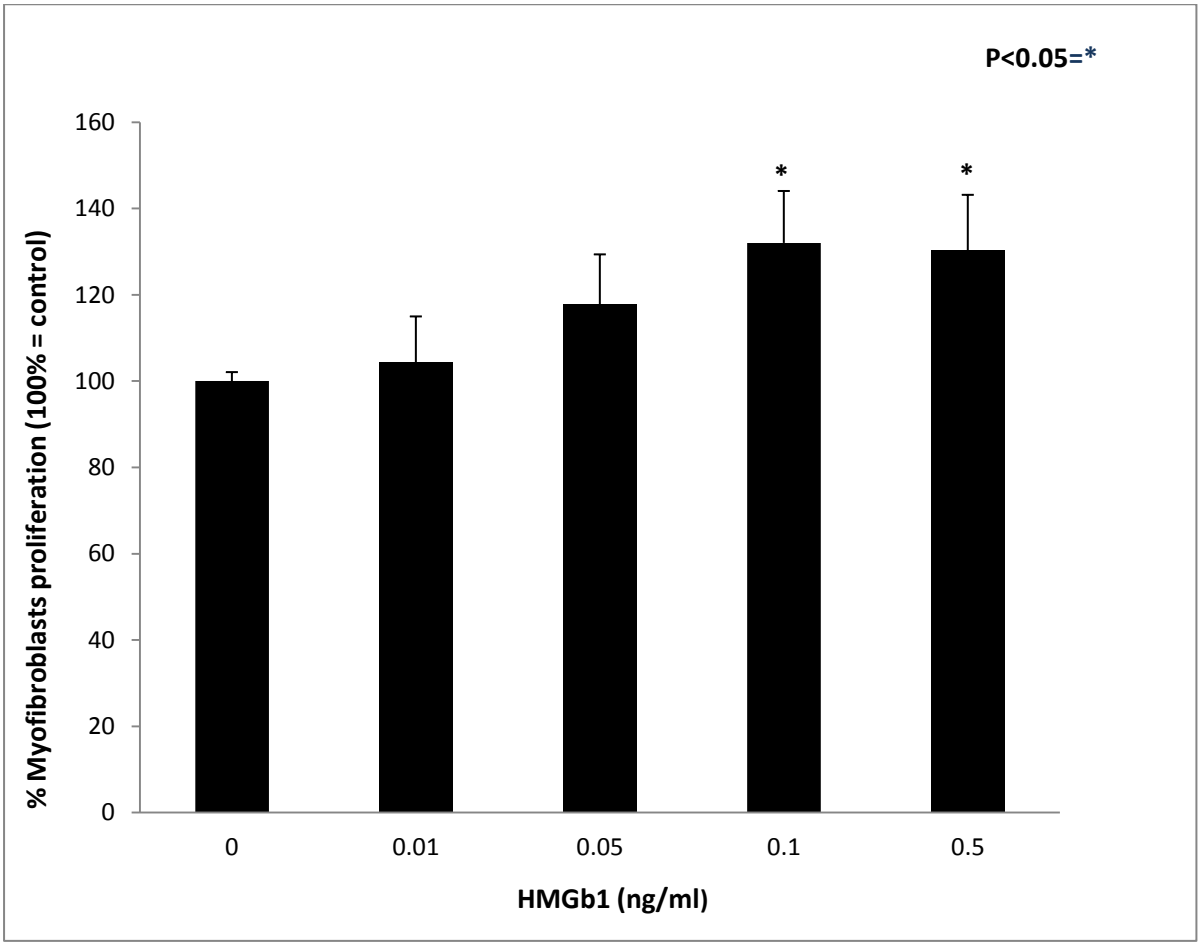


Figure 3.2: *HMGb1 induced proliferation in CCD18 myofibroblasts (NRU assay- HMGb1 using low concentration). Recombinant HMGb1 was used in a dose spectrum of 0ng/ml - 0.5ng/ml, where 0ng/ml served as control (serum free medium without HMGb1). The absorbance of accumulated NRU dye was measured at 540nm using the plate reader. HMGb1 appeared to trigger some proliferation at 0.01 and 0.05ng/ml but this was not statistically significant (P>0.05). The standard error of the mean (SEM) displayed as error bars. The student t-test was used to determine P values.* n=12 from 3 different occasions.

To determine the proliferative effect of HMGb1 on myofibroblasts cells, CCD18 myofibroblast cells were treated with HMGb1 with different concentrations for 96h before assessment using the NRU assay. It was observed that HMGb1 triggers proliferation in myofibroblasts in a dose-range of 0.1 to 50ng/ml.

The proliferation assays were carried out in two parts comprising of lower (0.01-0.5ng/ml) or higher doses (1-100ng/ml) of recombinant HMGb1. During the testing of lower doses, HMGb1 appeared to stimulate myofibroblasts cells in a dose ranging from 0.1 to 0.5ng/ml (Figure 3.2). This proliferation was statistically significant ($P<0.05$). Although, 0.05ng/ml of HMGb1also triggered proliferation in myofibroblasts cells however, this proliferation was lower than that of 0.1ng/ml HMGb1 and was not statistically significant either. In addition, no difference was observed between the proliferation induced by the treatment with 0.1ng/ml and 0.5ng/ml of HMGb1 (Figure 3.2). Thus, higher dose of HMGb1 were tested for further investigation.

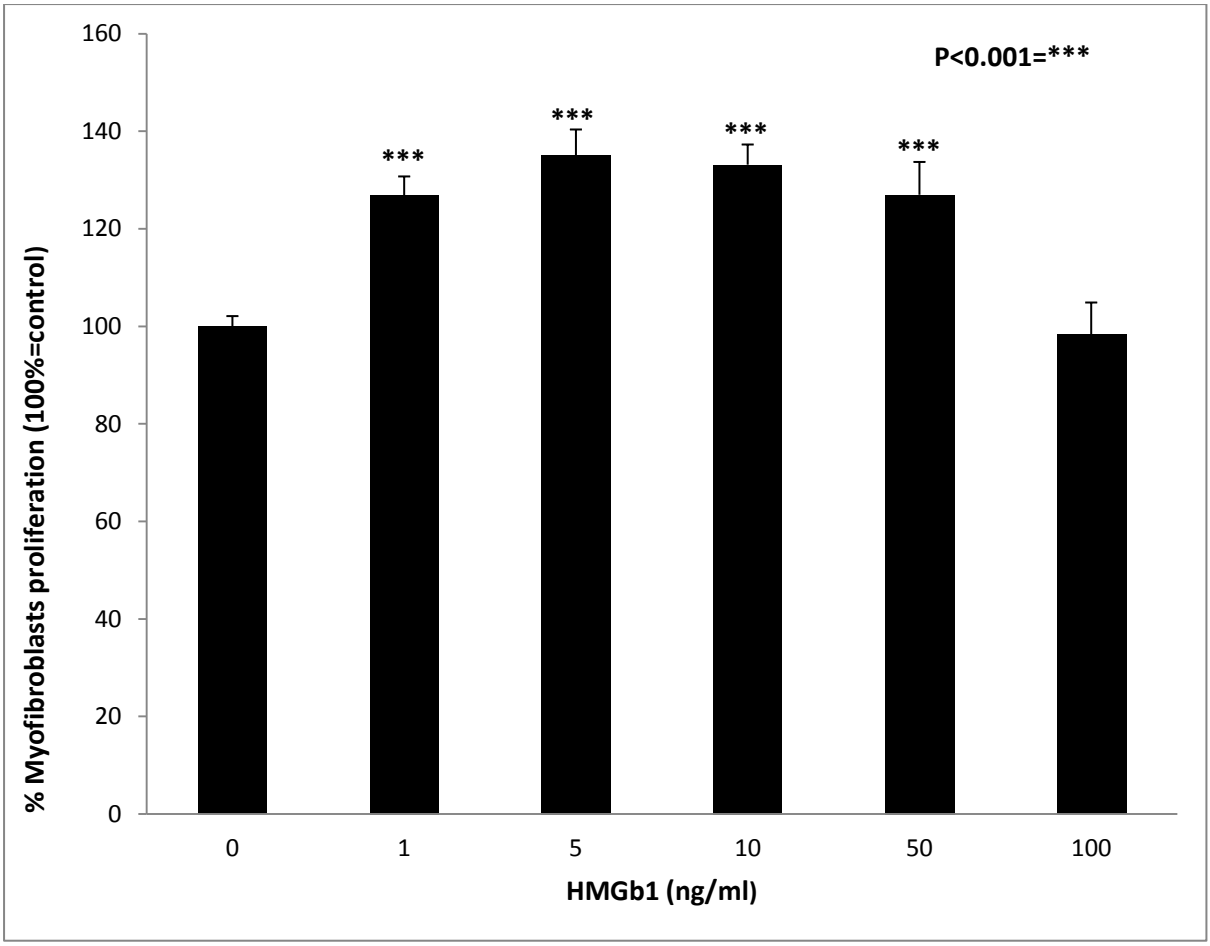


Figure 3.3: *HMGb1 induced proliferation in CCD18 myofibroblasts (NRU assay- HMGb1 using high concentration). Recombinant HMGb1 was used in dose spectrum of 0, 1, 5, 10, 50 and 100ng/ml where 0ng/ml served as control (serum free medium without HMGb1). The absorbance of accumulated NRU solution was measured at 540nm using the plate reader. The SEM displayed as error bars. The student t-test was used to determine P values.* n=29 from 8 occasions.

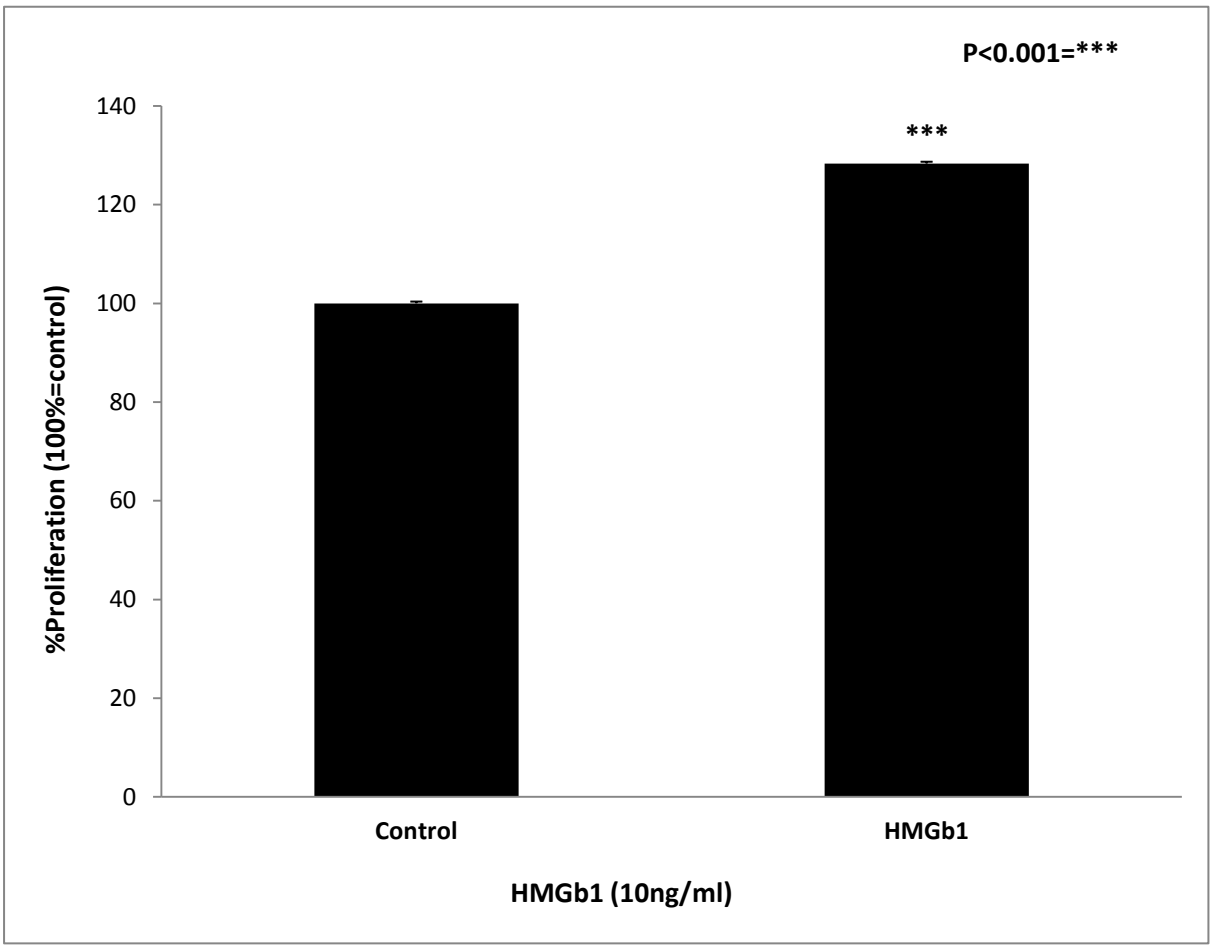


Figure 3.4: *HMGb1 induced proliferation in CCD18 myofibroblasts (MTT assay- HMGb1 single dose) at 48h. Recombinant HMGb1 was used at 10ng/ml and 0ng/ml served as control (serum free medium without HMGb1). The absorbance of accumulated MTT solution was measured at 540nm using the plate reader. The SEM displayed as error bars. The student t-test was used to determine P values.* n=30 from 8 occasions.

In the higher dose segment, the cells were treated with 1, 5, 10, 50 and 100ng/ml of HMGb1 (Figure 3.3). All four (1ng, 5ng, 10ng, 50ng) out of five doses significantly triggered the proliferation in CCD18 myofibroblast cells. Although, the results from all four (1ng, 5ng, 10ng

and 50ng/ml) doses of HMGb1 were statistically significant however 10ng/ml appeared to be the most effective dose. The reason for choosing 10ng/ml as most effective dose was the highest statistical significance ($P<0.001$) and lowest SEM value amongst all other doses which triggered proliferation. Approximately 30% increase in the proliferation of the myofibroblasts was observed when compared to the controls (serum free media) at 96h.

The 10ng/ml treatment with HMGb1 was also carried out on these cells using less exposure time (48h) and proliferation was assessed this time using the MTT assay (Figure 3.4). The reason for using MTT assay this time was to confirm that proliferative effect observed was not an anomaly found with the NRU assay. In addition, MTT being taken up by mitochondrion of living cells could measure different endpoints of proliferation. However, this resulted in a very similar proliferative response on the cells as seen after the 96h treatment (Figure 3.3).

3.4.2 HMGb1 triggers proliferation in CCD18 myofibroblasts via MEK1/2 pathway

These results show that recombinant HMGb1 can trigger proliferation in CCD18 myofibroblasts, but the mechanisms or pathways involved are not known. Therefore, an investigation into the involvement of key pathways was carried out. A number of pathways involving MEK1/2 have been implicated in cell proliferation. The MEK1/2 is a type of MAPK/ERK kinase and they have been implicated in a variety of proliferation pathways in cancer and normal cells (Liang *et al*. 2011; Li *et al*. 2015; Liu *et al*. 2015). Therefore, to determine if MEK1/2 is activated during HMGb1 induced proliferation, a selective inhibitor of MEK1/2 (U0126) was used. This was carried out using the MTT toxicity and proliferation assay.

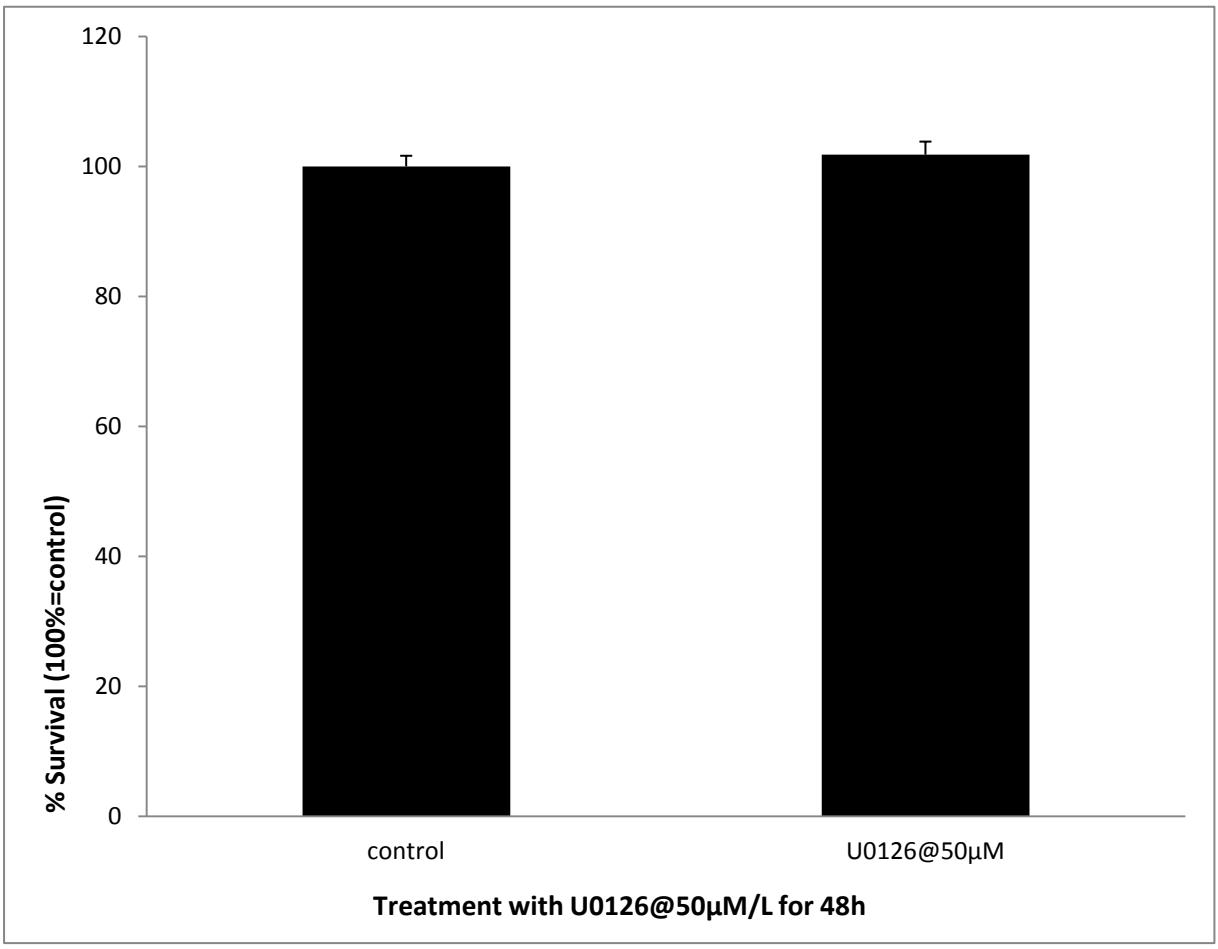


Figure 3.5: *Analysis of the toxicity caused by U0126 (MEK1/2 inhibitor) to CCD18 myofibroblast cells using MTT assay at 48h. The SEM displayed as error bars. The student t-test was used to determine P values.* n=12 from 3 occasions.

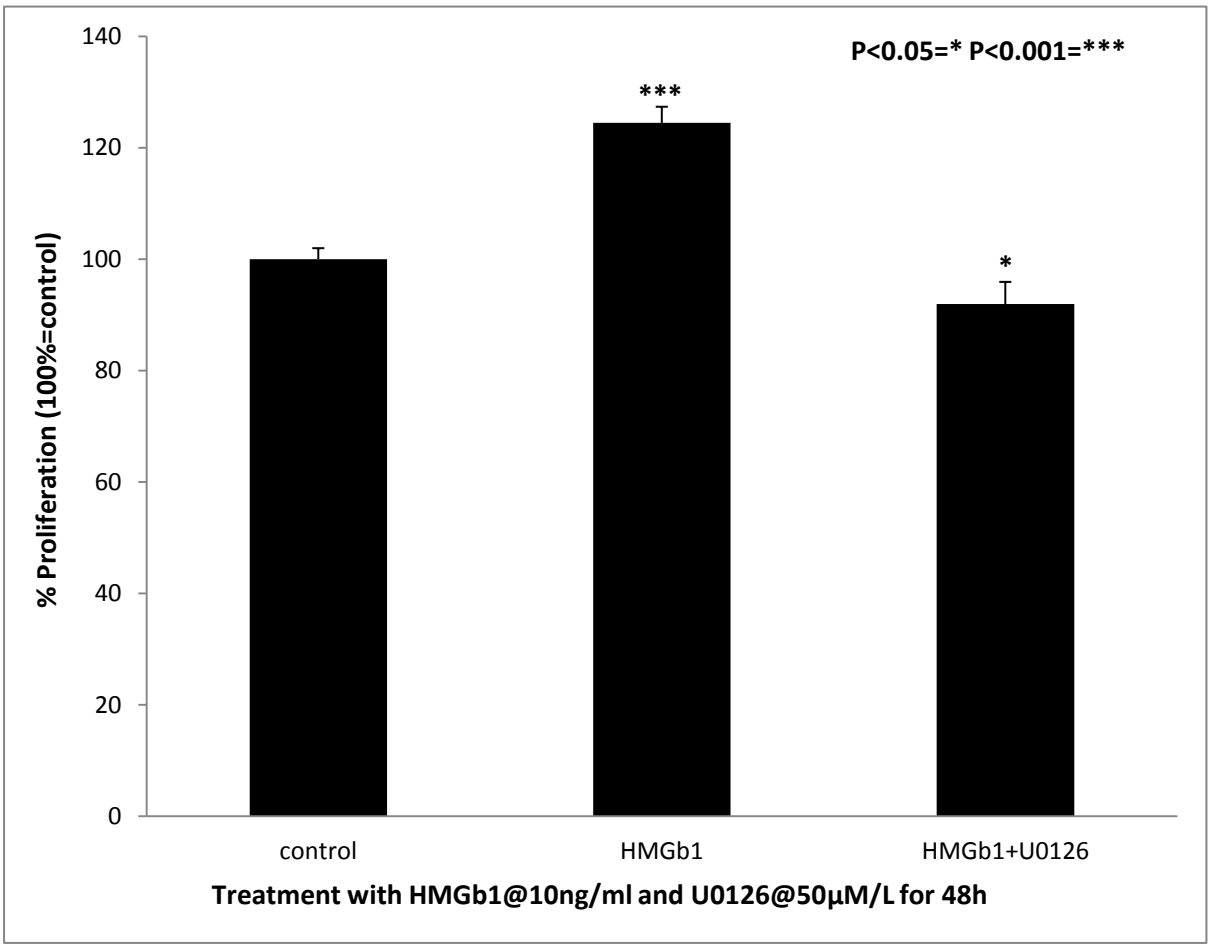


Figure 3.6: *The inhibitory effect of U0126 when combined with HMGb1 on CCD18 cells. The proliferation triggered by HMGb1 was 1/3 fold compared to the controls (serum free medium without HMGb1) in CCD18 myofibroblast cells. U0126 (50µM/L) completely abrogated the proliferative effect of HMGb1 in CCD18 cells at 48h when combined with HMGb1 (10ng/ml). The SEM displayed as error bars. The student t-test was used to determine P values.* n=12 from 3 occasions.

To determine a non-toxic dose of U0126, various concentration of U0126 were used to treat CCD18 myofibroblasts for 48h and toxicity was assessed using the MTT assay. The results suggested that U0126 (50µM/L) is not toxic to CCD18 myofibroblasts at 48h (Figure 3.5). This non-toxic dose (50µM/L) was used to inhibit the MEK1/2 pathway when combined a proliferative dose of HMGb1 (10ng/ml) in serum free medium. The CCD18 myofibroblast cells were treated with HMGb1 (10ng/ml) alone (as positive control), in combination with U0126 (50µM/L) or with serum free medium only (as negative control) for 48h before assessment using the MTT assay (Figure 3.6).

The proliferation triggered by HMGb1 (positive control) was approximately 30% compared to the negative control (serum free medium without HMGb1) in CCD18 myofibroblast cells at 48h. However, U0126 (50µM/L) completely abrogated the proliferative effect of HMGb1 in CCD18 cells at 48h when combined with HMGb1@10ng/ml. Therefore, results obtained from MTT assays suggested that HMGb1 induced proliferation in CCD18 myofibroblasts involves the MAPK/ERK pathway.

3.4.3 HMGb1 triggers proliferation in CCD18 myofibroblasts via the PI3K pathway

In addition to MEK1/2, role of intracellular signal transducer enzymes (PI3Ks) was explored using MTT toxicity and proliferation assays on CCD18 myofibroblasts. PI3Ks are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation,

motility, survival and intracellular trafficking, which in turn are involved in cancer (Foukas *et al*. 2010). They have been implicated in variety of proliferation pathways in cancer and normal cells (Ohashi *et al*. 2015; Song *et al*. 2015).

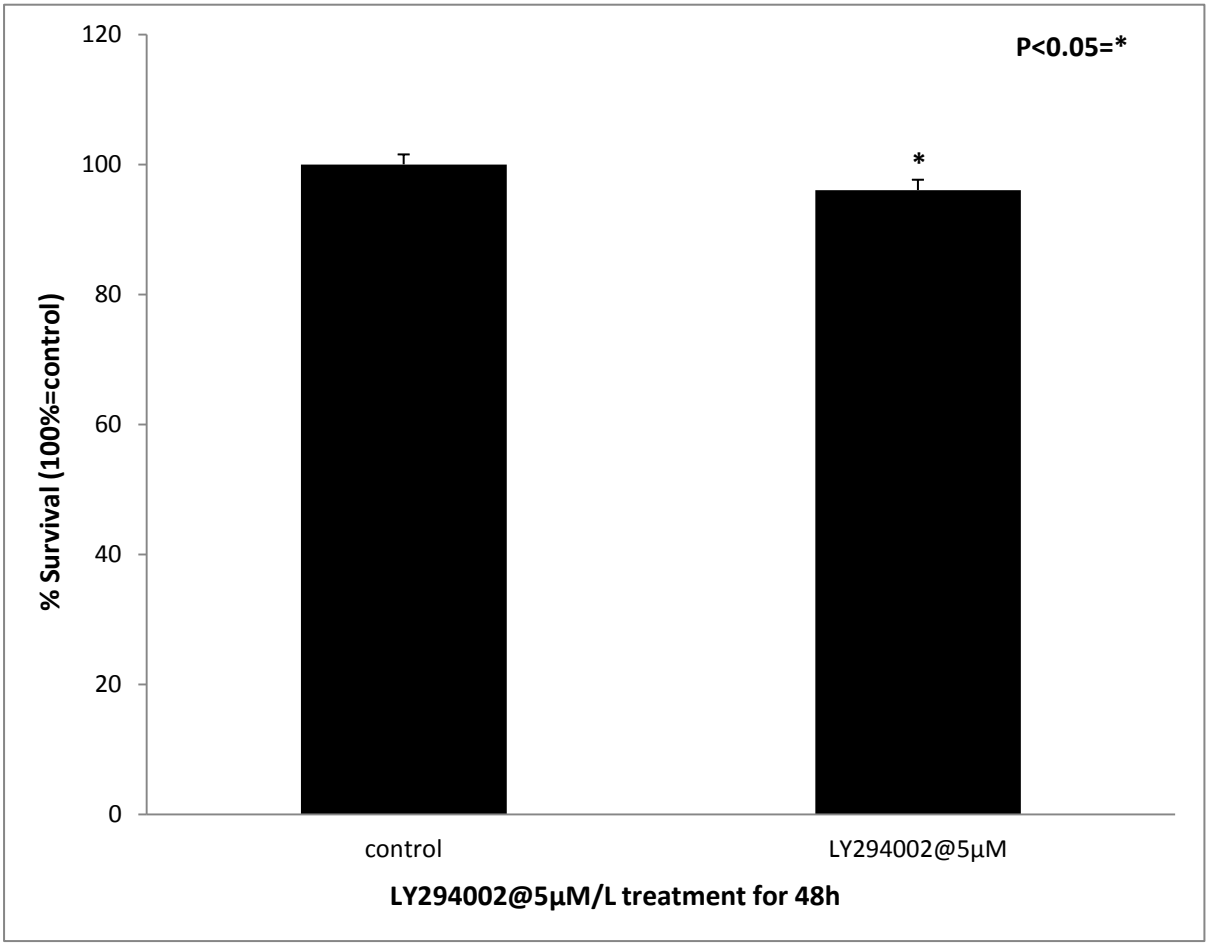


Figure 3.7: *Statistical analysis of the toxicity caused by LY294002 (PI3K inhibitor) to CCD18 myofibroblast cells using MTT assay at 48h. The SEM displayed as error bars. The treatment with LY294002 was slightly toxic at 5µM/Lat 48h. The student t-test was used to determine P values.* n=12 from 3 occasions.

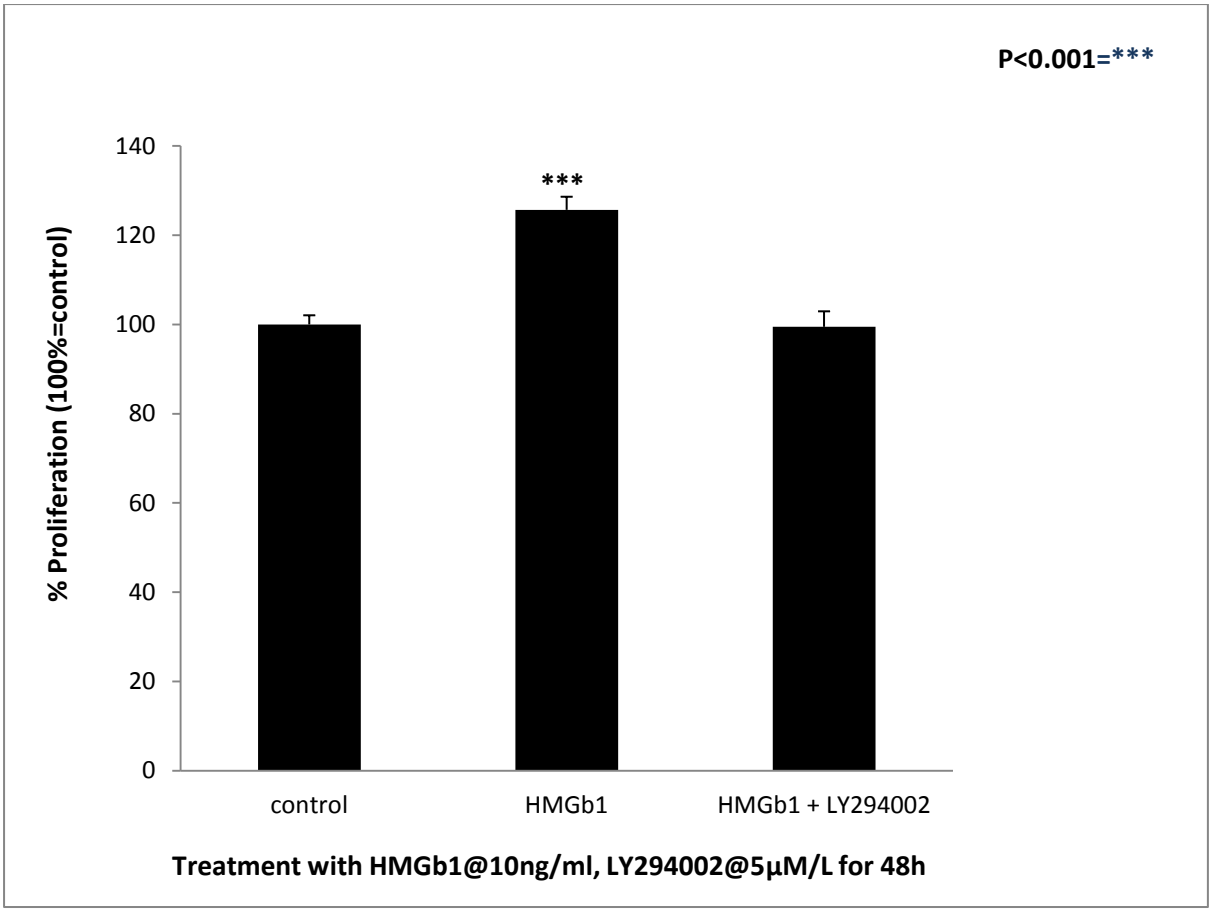


Figure 3.8: *Statistical analysis of inhibitory effect of LY294002 when combined with HMGb1 on CCD18 cells. The proliferation triggered by HMGb1 was 1/3 fold compared to the controls (serum free medium without HMGb1) in CCD18 myofibroblast cells. The student t-test was used to determine P values. This proliferation was statistically significant (P<0.001). The SEM displayed as error bars.*

Therefore, to investigate if PI3Ks are activated during HMGb1 induced proliferation, a selective inhibitor of PI3K (LY294002) was used. In addition, toxicity of LY294002 was determined using MTT toxicity assay on CCD18 cells. The results suggested that LY294002 (5µM/L) does not cause cell death in myofibroblasts at 48h (Figure 3.7). This non-toxic dose (5µM/L) was used to inhibit the PI3K pathway and combined with HMGb1 proliferative dose (10ng/ml) in serum free medium. The myofibroblasts cells were treated with HMGb1 (10ng/ml) alone and in combination

with LY294002 (5μM/L) for 48h and absorbance of accumulated MTT solution was measured at 540nm (Figure 3.8).

The results from MTT assays suggested that HMGb1 induced proliferation in CCD18 myofibroblasts involves PI3K pathway. The proliferation triggered by HMGb1 was 1/3 fold compared to the controls (serum free medium without HMGb1) in CCD18 myofibroblast cells. However, when combined with HMGb1@10ng/ml, LY294002 (5μM/L) completely abrogated the proliferative effect of HMGb1 in CCD18 cells at 48h (Figure 3.8).

3.5 Discussion

Myofibroblasts are present in colon cancer, breast cancer, lung cancer, prostate cancer and pancreatic cancer (Martin *et al*. 1996; Tuxhorn *et al*. 2002; Allinen *et al*. 2004; Bailey *et al*. 2008) and they are also useful in predicting the prognostic outcome in response to cancer chemotherapy (De Wever and Mareel 2003). However, the origin of cancer associated myofibroblasts is still controversial. Indeed, multiple origins of cancer associated myofibroblasts have been suggested such as transition from resident fibroblasts within tumour or from bone marrow derived mesenchymal stem cells. It has been proposed that the multiple origin of myofibroblasts may be related to the heterogeneous myofibroblasts population observed within tumours (De Wever *et al*. 2008).

Myofibroblasts have been shown to stimulate growth in breast cancer cells *in vivo.* In addition, they have also appeared to promote invasion of breast, pancreas and squamous carcinoma cells *in vitro* (Casey *et al*. 2008; Hwang *et al*. 2008). It has been shown that myofibroblasts

promote invasive breast carcinoma *in situ* (Hu *et al*. 2008). Furthermore cancer epithelial cells and their derivatives, including myofibroblasts have shown to promote breast cancer metastasis (Muehlberg *et al*. 2009). In addition, stromal myofibroblasts have been considered as predictors of human disease outcome and their abundance is correlated with poor survival in colorectal cancer (Tsujino *et al*. 2007).

Several solid tumours including melanoma, prostate cancer, breast cancer, pancreatic cancer and colon cancer exhibit markedly elevated levels of HMGb1 (Völp *et al*. 2006). These elevated levels of HMGb1 are associated with tumour formation, proliferation and metastasis and chemotherapeutic response (Sims *et al*. 2009). In addition, HMGb1 is involved in various diseases including autoimmune disorders, sepsis and chronic inflammatory disease (Wang *et al*. 2004; Sims *et al*. 2009). HMGb1 has been shown to trigger proliferation in many cells such as periodontal ligament fibroblasts, endothelial cells, keratinocytes and glioblastoma cells (Bassi *et al*. 2008; Ranzato *et al*. 2009; Chitanuwat *et al*. 2013; Hayakawa *et al*. 2015). Furthermore, HMGb1 has been shown to induce migration in endothelial cells and glioblastoma cells. HMGb1 activates various signalling pathways such as MAPK-AKT and PI3K (He *et al*. 2012; Kim *et al*. 2012). These pathways play an important role in the proliferation and migration of tumour cells. It is evident that HMGb1 activates the PI3K pathway in neutrophils and colon cancer cells (Kuniyasu *et al*. 2003).

The role of HMGb1 in myofibroblasts has not been explored thus far. Considering the proliferative effect of HMGb1 on other cell types such as dendritic cells, endothelial cells and glioblastoma cells, it was hypothesised that HMGb1 may trigger proliferation in myofibroblasts. Therefore, NRU proliferation assay was carried out using recombinant HMGb1 in a range of

doses (0-100ng/ml) for 96h. This dose and time range of HMGb1 has been shown to trigger proliferation in fibroblasts recently (Chitanuwat *et al*. 2013). The NRU assay revealed that HMGb1 treatment for 96h was able to trigger approximately a 30% increase in proliferation in CCD18 myofibroblasts over a range of doses over 96h (0.1ng/ml to up to a concentration of 50ng/ml) (Figures 3.2 and 3.3). However, 10ng/ml of HMGb1 appeared to trigger proliferation with the minimum variation in data (SEM). With 10ng/ml HMGb1, in keeping with the other doses within the effective range, an increase of approximately 30% in the cell number was seen when compared to the controls (Figures 3.2 and 3.3).

The NRU assay measures viability/proliferation of cells by the ability of the cells to take up the neutral red dye into the cellular lysosomes within the cells (Repetto *et al*. 2008). However, it is prudent to carry out a 2nd viability assay that measures a different endpoint to ensure confidence in findings. Unlike NRU assay, MTT assay measure viability/proliferation of cells by the ability of the cells to enzymatically reduce MTT solution to a blue crystalline formazan product. The previous NRU proliferation assays showed that HMGb1 could trigger proliferation in CCD18 myofibroblasts over 96h. However, this time the MTT assay was used to assess proliferation at 48h (HMGb1 10ng/ml). The similar dose (10ng/ml) and exposure time (48h) of HMGb1 has been shown to trigger proliferation in smooth muscle cells of atherosclerotic plaques previously (Porto *et al*. 2006).

The results from MTT assay show that HMGb1 (10ng/ml) triggers proliferation in CCD18 myofibroblast cells at 48h. This proliferation was observed as similar to that observed at 96h (approximately a 30% increase in proliferation) (Figure 3.4). In addition, U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor) have been shown to inhibit their respective pathways in

24-48 hour in various cell lines including fibroblasts (DeSilva *et al*. 1998; Wu *et al*. 2006). Thus, proliferation/inhibition assays were carried out using MTT at 48h to ensure sufficient time for measurable proliferation and inhibition to occur. The inhibitory effect on proliferation of cells including keratinocytes and lymphocytes using U0126 and LY294002 has previously been described at 48h using 10, 25 and 50µM/L of U0126 (Dolci *et al*. 2001; Yano *et al*. 2003) or 1, 5 and 10µM/L of LY294002 (Dolci *et al*. 2001; Du *et al*. 2001; Squires *et al*. 2003). Using this information, MTT assays were carried to find a non-toxic dose in CCD18 myofibroblasts of each of the inhibitors at 48h that might be an effective inhibitor. MTT assay revealed that U0126 at 50µM/L was non-toxic to CCD18 myofibroblasts (Figure 3.5). Similarly, a 5µM/L dose of LY294002 appeared to be non-toxic to CCD18 myofibroblasts (Figure 3.7). These non-toxic doses were selected to study whether MAPK/ERK (also known as MEK1/2) and PI3K pathways are involved in HMGb1 triggered proliferation in CCD18 myofibroblasts.

The CCD18 myofibroblast cells were treated with HMGb1 alone or in combination with U0126 or LY294002. The results obtained from MTT assays revealed that U0126 (50µM/L) completely abrogated the proliferative effect of HMGb1 on myofibroblasts cells (Figure 3.6). However, a slightly toxic effect was also seen using the combination of HMGb1 with U0126 (a reduction of about 10% in cell viability compared to control ($P<0.05$) however, this was not seen when CCD18 cells were treated with U0126 (50µM/L) alone. This suggested that U0126 (50µM/L) was not toxic to myofibroblasts cells. U0126 is described as a highly selective inhibitor of MEK1/2 however, its selectivity has been questioned recently by some research groups (Bain *et al*. 2007; Qin *et al*. 2010). Thus, the marginal toxic effect of combination treatment of HMGb1 and U0126 might be linked to the blockade of additional proliferative pathways involved in HMGb1 induced proliferation in myofibroblasts cells.

The proliferation triggered by HMGb1 in CCD18 myofibroblasts was also abrogated by LY294002, the selective inhibitor of PI3K (Figure 3.8). LY294002 was non-toxic to CCD18 myofibroblast cells when used alone at a concentration of 5µM/L (Figure 3.7). This dose of LY294002 successfully blocked the proliferative effect of HMGb1 at 10ng/ml on myofibroblast cells. The results obtained from MTT assay suggest that MEK1/2 or MAPK/ERK and PI3K pathways are involved in HMGb1 induced proliferation in CCD18 myofibroblast cells. These results are in line with previous observations establishing these pathways as most common signalling pathways involved in the proliferation and migration of various cells including colon cancer cells (Greenhough *et al*. 2007; Lai *et al*. 2010; Lee *et al*. 2010). Considering the contribution of myofibroblasts in the proliferation of tumours, these results provide a new insight of myofibroblasts biology in relation to the tumour microenvironment. The HMGb1 and its role in cancer has been explored to some extent, but it would be interesting to see how HMGb1 influenced normal cells such, as how myofibroblasts can contribute to the tumour spread after getting proliferative signals from HMGb1. In this study, an investigation into the role of HMGb1 in proliferation of myofibroblast cells was carried out and based on the results it is now clear that HMGb1 triggers proliferation in myofibroblast cells. In addition, this HMGb1 induced proliferation involves the activation of MEK1/2 and PI3K pathways.

Chapter 4

4. Investigation into the release of HMGb1 from cancer cells exposed to various microenvironmental stress conditions

4.1 Introduction

The HMGb1 is a non-histone protein involved in stabilization of nucleosomes and the bending of DNA and hence facilitating gene transcription (Lange *et al*. 2008). HMGb1 binds to the minor grove of DNA without sequence specificity, and thereby induces bends in the helical structure of the DNA. The formation of HMGb1-DNA complex facilitates interaction between DNA and other factors such as p53, NF-kβ, recombination activating proteins 1/2 (RAG1/2) and some hormone receptors (Bianchi 2004). Whilst HMGb1 is a nuclear protein, it is released from the cells under certain conditions to take part in the inflammatory process. During inflammation, extracellular HMGb1 activates infiltrating macrophages via the RAGE receptor. In addition, activated macrophages/monocytes are also responsible for the release of HMGb1 in the extracellular milieu (Andersson *et al*. 2002). Furthermore, HMGb1 is passively released by necrotic cells (Huttunen and Rauvala 2004; Andersson *et al*. 2002). The release of HMGb1 involves following steps; 1) exit from the nucleus to the cytoplasm, 2) translocation from the cytosol in to cytoplasmic organelles, and 3) exocytosis. Macrophages/monocytes upon activation by proinflammatory cytokines acetylate HMGb1 at lysine-rich nuclear localization sequences. This leads to the translocation of HMGb1 into the cytoplasmic vesicles followed by extracellular release (Gardella *et al*. 2002)

Several solid tumours including melanoma, prostate cancer, breast cancer, pancreatic cancer and colon cancer exhibit noticeably elevated levels of HMGb1 (Völp *et al*. 2006; Gnanasekar *et al*. 2009; Kostova *et al*. 2010). These elevated levels of HMGb1 are associated with tumour formation, proliferation and metastasis and chemotherapeutic response (Lotze and Tracey 2005). It has been suggested that HMGb1 might have a direct impact on migration of cells because of its ability to modulate the adhesive properties of the cells and ECM components (Ellerman *et al*. 2007).

Receptor for advanced glycation end products (RAGE) is the most commonly known receptor for HMGb1. The receptor belongs to immunoglobulin superfamily and is expressed in many cells including monocytes, macrophages, smooth muscle cells, dendritic cells and endothelial cells. RAGE is multi-ligand receptor that can be activated by several ligands including HMGb1. However, the effects of this activation are dependent of the type of the cell it is expressed upon. Activation of RAGE in monocytes/macrophages has been reported to trigger the inflammatory response and to trigger neoplastic transformation and metastasis in neuroepithelial tumour cells (Taguchi *et al*. 2000). It has been shown that RAGE overexpression is associated with chronic degenerative disease and cancer (Tanaka *et al*. 2000; Onyeagucha *et al*. 2013). The HMGb1-RAGE complex has recently been shown to enhance ATP production in tumours which then facilitates tumour proliferation and migration (Kang *et al*. 2013). RAGE is not the only receptor for HMGb1 and others include the toll-like receptors 2 and 4 (TLR-2 and TLR-4). Park *et al*. (2004) demonstrated that blockage or knockdown of these receptors resulted in decreased HMGb1 activation *in vitro* and *in vivo* (Park *et al*. 2004). In addition, HMGb1 has been shown to activate TLR-4 and p38MAPK pathways which play an important role in mediating acute lung injury (Yang *et al*. 2013). The effect of extracellular HMGb1 followed by its binding to RAGE and TLR-4 which also involves activation of NF-κB has been implicated in tumour cell growth and migration (Palumbo *et al*. 2007). However, the release of HMGb1 in the extracellular

environment and subsequent effects on myofibroblast cells in the tumour stroma remain unclear. In addition, the release of HMGb1 in response to low glucose conditions has not been explored.

4.2 Aims

The aims of this study were to:

a) Investigate whether HMGb1 is released from HT29 colon carcinoma cells in response to different stress conditions such as anoxia and/or the absence of nutrients such as glucose or glutamine.
b) Investigate if the release of HMGb1 is cell line specific or it is a common phenomenon that occurs in most of other cancer cell lines.
c) Investigate if CCD18 myofibroblast cells express RAGE and TLR-4 (receptors for HMGb1) and therefore might interact with HMGb1 released from tumour cells.

4.3 Material and methods

4.3.1 Cell culture

Myofibroblasts (CCD18) cells, HT29 colon adenocarcinoma cells, A549 lung cancer cells, EJ138 bladder cancer cells and MCF-7 breast cancer cells were cultured in T75 cm^2 flasks in complete medium (minimum essential medium eagle and RPMI1640) (Sigma Aldrich) supplemented with 10% foetal calf serum (FCS) (BioSera) and 1% antibiotic/antimycotic solution (100x) (Gibco Ltd) at 37°C, 5% CO_2 and 95% humidity as discussed in section 2.2.

4.3.2 Sample preparation

HT29 colon adenocarcinoma cells were seeded into 17 x T25 cm^2 flasks at a density of $4.0x10^5$ per flask and allowed to grow overnight at 37°C, 5% CO^2 and 95% humidity. The following day, medium was removed and replaced with serum free medium (5ml/flask) and incubated for 24h. After 24h, the medium was aspirated off and replaced with fresh medium (RPMI 1640) with glucose (9 flasks) and without glucose (8 flasks). The glucose free flasks were either incubated in normoxic or anoxic conditions (4 flasks for each condition). The flasks with glucose were also treated to the same conditions (4 flasks for each condition). The remaining flask was used as day 0 control (serum free medium with glucose). The medium from this flask was collected into a labelled universal tube and stored at -80^{o}C for later analysis. In addition, two flasks of each cell types (A549 lung cancer cells, MCF-7 breast cancer cells and EJ138 bladder cancer cells) were treated with serum free medium with and without glucose in normoxic conditions for 48h and medium were collected in labelled universal tubes and stored at -80^{o}C. In addition, HT29

cells were treated with and without glutamine incubated in normoxic conditions for 48h to assess the release of HMGb1 in low nutrient conditions. The protocol for the collection of medium remained same as discussed in section 2.3.1. The myofibroblast cells (CCD18) were treated with serum free medium for one day before incubating in normoxia with and without glucose for 24 and 48h. The medium from CCD18 cells were discarded and proteins were extracted from the cells to investigate if RAGE and TLR-4 are present on the myofibroblasts. This investigation was carried out using western blot analysis.

4.3.3 The extraction of cellular proteins

The extraction of proteins from the flasks of cells (CCD18) was carried out as discussed in 2.3.2.

4.3.4 Determination of cellular protein concentration

To determine the protein concentration of the cell lysates and conditioned medium, a BioRad DC protein assay was used (see section 2.3.3).

4.3.5 Dot Blot analysis for the detection of HMGb1, RAGE and TLR-4

A dot blot analysis was carried out to optimise the concentration of primary antibodies against HMGb1, RAGE and TLR-4 (See section 2.3.7).

4.3.6 SDS–PAGE and western blot analysis for the detection of HMGb1, RAGE and TLR-4

The protein extracts from CCD18 myofibroblasts and the culture medium from HT29, MCF-7, EJ138 and A549 were prepared as described in section 2.3.2 and subjected to western blot analysis (section 2.3.4). The samples were incubated with appropriate antibodies listed in section 2.7.

4.4 Results

4.4.1 The release of HMGb1 is triggered by glucose deprivation in HT29 colon adenocarcinoma cells

The extracellular HMGb1 has been shown to play important role in many diseases including cancer. A number of cells including inflammatory immune cells actively secrete HMGb1 as an immune response (Lu *et al*. 2014). However, necrotic cells have been reported to release HMGb1 passively in the extracellular environment (Scaffidi *et al*. 2002). In addition, HMGb1 has previously been shown to be released from cancer cells in hypoxic conditions (Kang *et al*. 2013). However, the release of HMGb1 under low glucose conditions has never been reported. It has been established that most solid tumours are acidic and hypoxic at placed within their tumour mass (Pellegrini *et al*. 2014). In addition, low glucose areas are characteristic feature of an aggressive tumour mass (Laderoute *et al*. 2006). Most solid tumours are composed of number cells with major part consisting of stromal cells such as myofibroblasts (Micke 2004). Thus, it was hypothesised that certain cancer cells may release HMGb1 into their

microenvironment and this HMGb1 might stimulate tumour grow by stimulating resident myofibroblasts. To this notion, a western blot analysis was developed to investigate if HT29 colon adenocarcinoma cells release HMGb1 into their culture medium under different stress conditions commonly observed in tumour microenvironment.

Our western blot analysis showed that HT29 cell release HMGb1 into their culture medium. This release of HMGb1 is triggered by glucose deprivation under normoxic conditions. This study compared the release of HMGb1 from HT29 cells under glucose deprivation or normal levels of glucose in anoxic and normoxic conditions. Surprisingly, the amount of HMGb1 released from the colon adenocarcinoma cell line under normoxic conditions without glucose was greater than the anoxic conditions with or without glucose. This release was triggered between 24 to 48h.

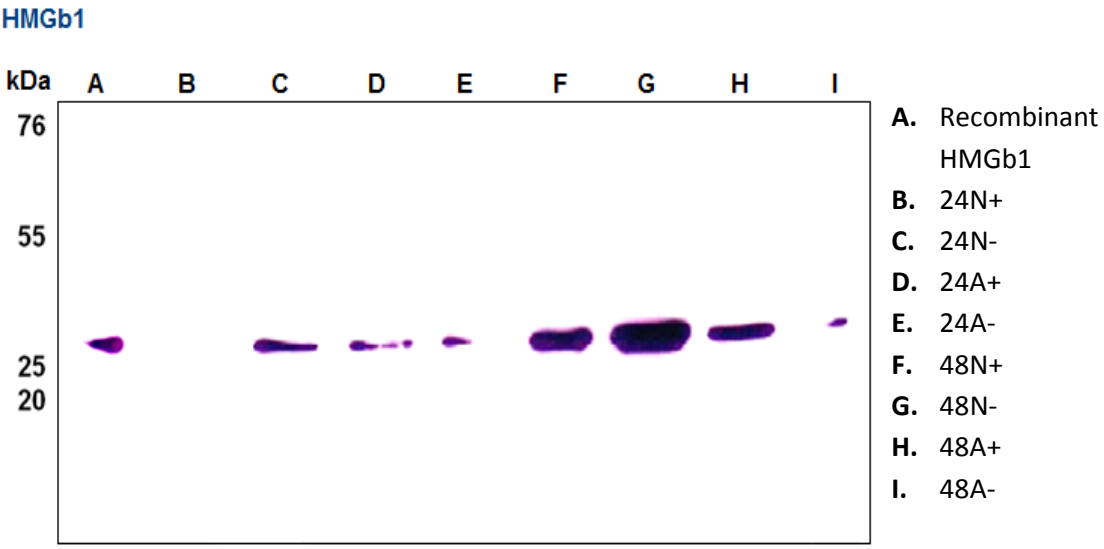


Figure 4.1: *Western blot analysis showing release of HMGb1 in the culture medium of HT29 cells. The samples were normalised to the lowest protein content in a sample. The recombinant HMGb1 was used at 50ng (total protein loaded). The assay was repeated more than 3 times.*

- ***A.*** *Recombinant HMGb1*
- ***B.*** *24N+ (Normoxia with glucose)*
- ***C.*** *24N- (Normoxia without glucose)*
- ***D.*** *24A+ (Anoxia with glucose)*
- ***E.*** *24A- (Anoxia without glucose)*
- ***F.*** *48N+ (Normoxia with glucose)*
- ***G.*** *48N- (Normoxia without glucose)*
- ***H.*** *48A+ (Anoxia with glucose)*
- ***I.*** *48A- (Anoxia without glucose)*

For western blot analysis, recombinant HMGb1 (50ng) was used as a positive control. Using western blot, recombinant HMGb1 and the HMGb1 in our samples was detected at 28kDa. The lack of glucose even in presence of oxygen had a major impact on the release of HMGb1. This was compared with the impact of glucose containing medium treatment in presence of oxygen.

There appeared to be a major quantitative difference amongst these two conditions with more HMGb1 being released when cells were treated with no glucose conditions (Figure 4.1). In addition, the presence of glucose appeared to trigger the release of HMGb1 but only after 24h (Figure 4.1). Interestingly, figure 4.1 also shows that harsher condition such as lack of oxygen (anoxia) did not show any significant impact on the release of HMGb1 when compared to oxygen rich conditions.

4.4.2 The release of HMGb1 in response to lack of glucose is a phenomenon in common with other cancer cell types

Western blot analysis from this study showed that glucose deprivation triggers the release of HMGb1 in HT29 cells (Figure 4.1). This release was greater than the release of HMGb1 in response to anoxia, a condition which is a known cause of HMGb1 release. To investigate whether this HMGb1 release in response to low glucose levels was specific to the colon adenocarcinoma cell line or whether it is a common phenomenon, other cancer cells were analysed. MCF-7 (breast adenocarcinoma), EJ138 (bladder carcinoma) and A549 (lung carcinoma) cells were treated with similar conditions and their culture medium at 48h were collected. A western blot was carried out to determine whether these cell lines followed the same pattern of HMGb1 release in response to glucose deprivation in HT29 cells.

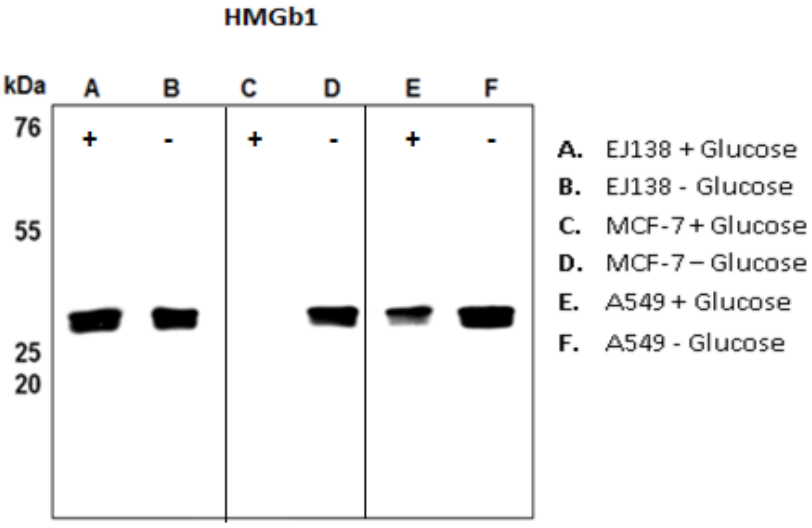


Figure 4.2: *Western blot analysis showing the release of HMGb1 from three different cancer cell lines in normoxia. The cells were treated with medium with glucose and without glucose for 48h. The protein concentration was normalised using BioRad protein assay in both conditions and compared.*

The western blot analysis suggested that glucose deprivation triggers the release of HMGb1 from some of these cell lines. The MCF-7 and A549 cells followed the same pattern with an increased amount of HMGb1 released in response to glucose starvation. The MCF-7 cells showed the greatest difference in the quantity of HMGb1 released. There was apparently no HMGb1 being released from the cell line in normoxia with normal glucose levels at 48h and yet a very strong band for HMGb1 was seen from the medium of the glucose starved cells in normoxia at the same time point. However, no major difference in HMGb1 release was observed between the normal and the glucose starved EJ138 cells in normoxia at 48h (Figure 4.2). Therefore, 3 out 4 cell lines investigated appeared to follow the same pattern of HMGb1 release in normoxic conditions without glucose.

4.4.3 Glutamine deprivation also stimulated the release of HMGb1

The results from western blot analysis suggested that HMGb1 was released from a selection of cancer cells including HT29, MCF-7, and A549 in glucose deprivation conditions under normoxia. However, whether HMGb1 also released in response to other nutrient deprived conditions is unclear. Therefore, the release of HMGb1 from HT29 cells was compared between normal and glutamine deprived conditions at 48h.

The western blot was set up in two sets; one was to compare the release of HMGb1 in response to glutamine deprived versus normal medium. The other one was to compare HMGb1 release in medium deprived of glutamine versus glucose. The results from the first set of western blot suggest that HMGb1 is released in glutamine deprived culture medium of HT29 cells (Figure 4.3 A).This release of HMGb1 was compared to glutamine deprived and glutamine containing medium. It was found that there was no major difference in the release of HMGb1 in glutamine deprived conditions compared to normal glutamine conditions. However, the second set of western blot comparing the release of HMGb1 between from HT29 cells in glucose and glutamine deprived medium revealed that only glucose deprivation triggers the release of HMGb1 (Figure 4.3 B).

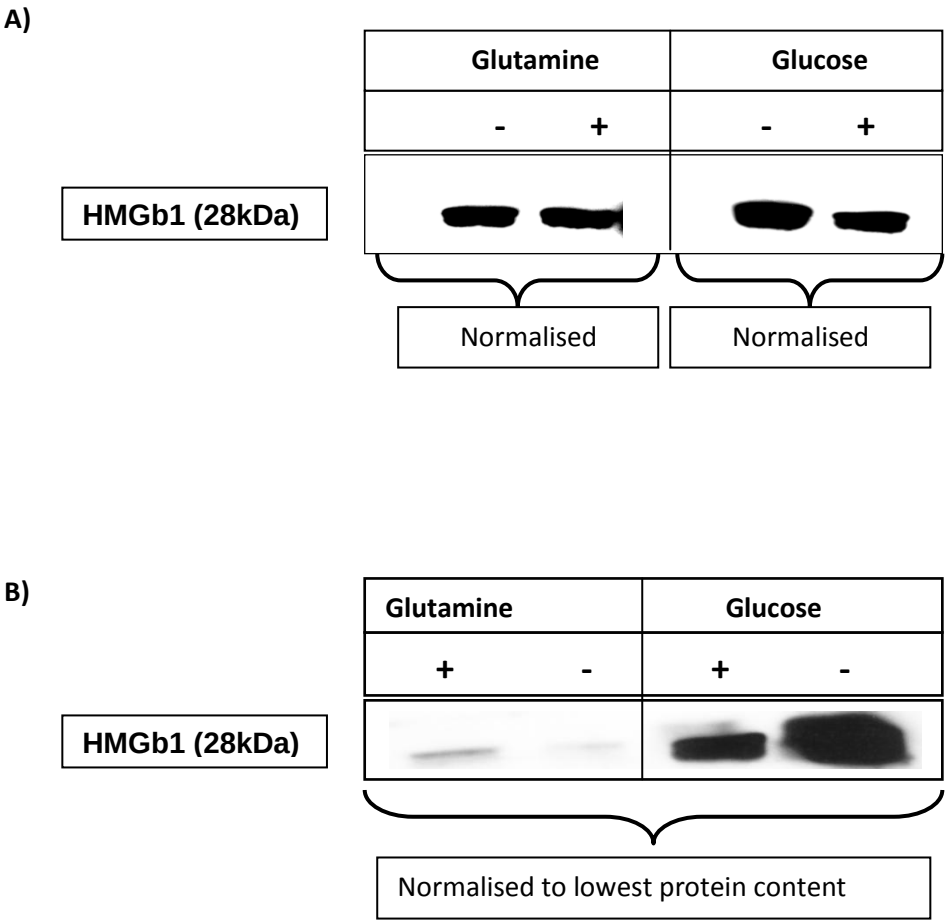


Figure 4.3: A) *Western blot showing release of HMGb1 in the culture medium from HT29 cells under glucose and glutamine deprived and normoxic conditions for 48h. The samples were equalised to the lowest protein content in each segment (glutamine and Glucose).* ***B)*** *Western blot analysis on samples equalised to lowest protein content in one sample (all 4 samples were equalised using Biorad protein assay)*

4.4.4 The CC18 myofibroblast cells express advanced glycation end product (RAGE)

Receptor for advanced glycation end products, a multiligand receptor and is also a receptor for HMGb1. The HMGb1 and RAGE complex has been implicated in many diseases including cancer (Nienhuis *et al*. 2009; Sims *et al*. 2009; Dzaman *et al*. 2015). The results from western blot analysis confirmed the presence of HMGb1 in the conditioned medium of cancer cells including HT29 cells. This study has shown that recombinant HMGb1 triggers proliferation in myofibroblasts cells. Therefore, there is a possibility that HMGb1 induced proliferation of myofibroblasts cells involves activation of RAGE which might be present on the membrane of CCD18 myofibroblast cells.

The CCD18 myofibroblast cells were treated with glucose free and normal glucose conditions in normoxia and anoxia for 24h and 48h and the cell lysates were collected for protein analysis. The protein concentration was equalised so that all samples contained the same concentration of total cellular protein before western blot analysis on all samples. Detection of GAPDH was used as loading control for the samples. The results from western blot analysis suggested that RAGE is present on myofibroblasts cells. The samples included with and without glucose in normoxic and with anoxic conditions. All conditions including 48h normoxia without glucose showed some levels of RAGE. There was no significant difference observed between any specific treatment but relatively higher levels of RAGE appeared to be correlated with high glucose levels (Figure 4.4). The ligand HMGb1 controlled upregulation or downregulation of RAGE would be difficult to investigate in this case because RAGE is a multiligand receptor and might be interacting with other ligands as well as HMGb1. Similarly, HMGb1 might be interacting with other receptors which may be present on myofibroblasts. Therefore, HMGb1

signalling in response to glucose deprivation might involve one or more other receptors which are present on myofibroblasts. Although, this study was focused on investigating similar conditions which triggered the release of HMGb1 from HT29 cells but there was always a possibility for HMGb1 binding to certain receptors which may not be upregulated in similar conditions as those with HT29 cells. Those receptors may include RAGE and TLR-4 amongst others. However, this experiment confirmed that CCD18 myofibroblast cells express RAGE in all treatment conditions (Figure 4.4).

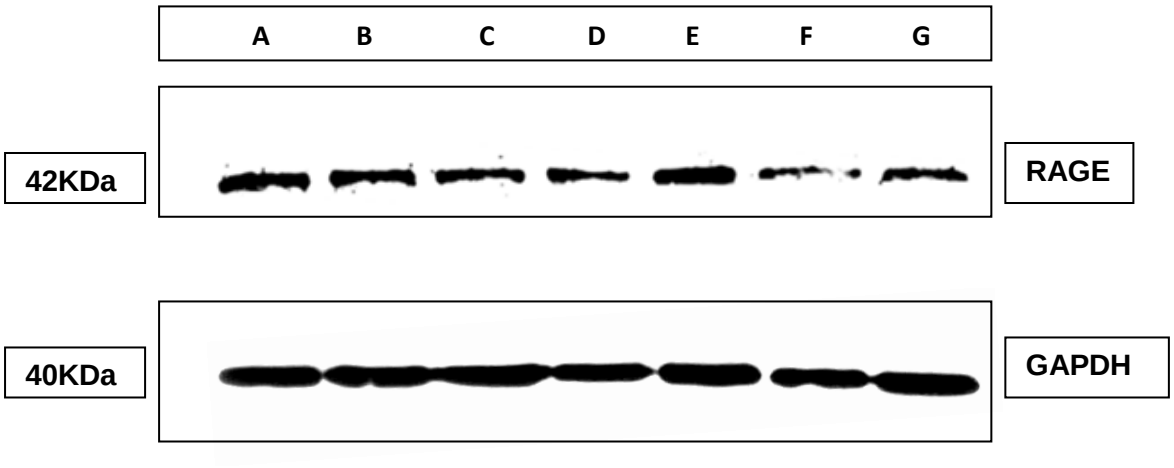


Figure 4.4: *Western blot confirming the presence of RAGE in the CCD18 cell lysates. The cells were treated with different conditions for 24 and 48h. The protein concentration was normalised using BioRad assay and GAPDH was used as loading control.*

- ***A.*** *24N+ (Normoxia with glucose)*
- ***B.*** *24N- (Normoxia without glucose)*
- ***C.*** *24A+ (Anoxia with glucose)*
- ***D.*** *24A- (Anoxia without glucose)*
- ***E.*** *48N+ (Normoxia with glucose)*
- ***F.*** *48N- (Normoxia without glucose)*
- ***G.*** *48A+ (Anoxia with glucose)*

4.4.5 The CCD18 myofibroblast cells express toll like receptor-4 (TLR-4)

It has been shown that the interaction between HMGb1 and TLR-4 triggers signalling pathways that may activate NF-Kb. The activation of NF-kB has been implicated in tumour cell survival and migration (Wu and Zhou 2010). In addition, TLR-4 mediated signalling has been shown to play important role in stimulating myofibroblasts (Pulskens *et al*. 2010). The western blot analysis has confirmed that myofibroblasts cells express RAGE. RAGE is not the only receptor for HMGb1 and TLR-4 has also been reported to interact with HMGb1. Therefore, a western blot was developed to investigate if CCD18 myofibroblast cells also express TLR-4.

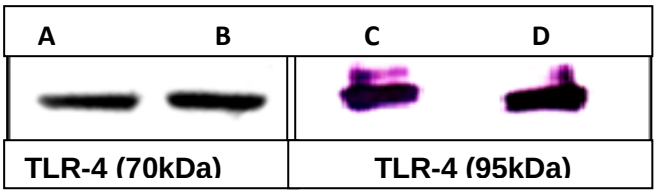


Figure 4.5: *Western blot confirming the presence of TLR-4 in CCD18 cell lysates. Lane A and lane B showing TLR-4 in cell lysates of CCD18 treated with glucose and without glucose in serum free medium for 24h followed by 22h treatment with glucose free and glucose containing serum free conditioned medium from HT29 cells. Lane C and Lane D showing TLR-4 under 48h treatment with glucose free and glucose containing serum free medium. Predicted molecular weight of TLR-4 is 95 kDa however soluble TLR-4 lacking a transmembrane domain has been detected at 70kDa previously (Hyakushima, Mitsuzawa et al. 2004)*

- **A.** 24N+ followed by treatment with conditioned media from HT29 cells for 22h
- **B.** 24N- followed by treatment with conditioned media from HT29 cells for 22h
- **C.** 48N+
- **D.** 48N-

The results from western blot confirmed that myofibroblasts cells express TLR-4. The cells were treated with glucose and without glucose for 24h in the absence of serum. The expression of TLR-4 was observed in both conditions (Figure 4.5).The cells were also treated with serum free medium with glucose for 24h followed by 22h treatment with medium taken from HT29 grown for 48h in the absence of glucose. As western blot analysis showed that TLR-4 is present in stress conditions such as lack of glucose and then it is possible that it might interact with HMGb1 to initiate signalling cascade in microenvironment. The TLR-4 was observed in at two different places at 95kDa with 48h treatment with glucose free and glucose rich conditions and 70kDa with fresh medium and HT29 conditioned medium treatment (24h+22h) (Figure 4.5). The predicted molecular weight of TLR-4 is 95kDa and therefore the 70kDa could be proteolytic degraded product that lacks transmembrane domain. The soluble TLR-4 which lacks transmembrane domain has been identified at 70kDa previously (Hyakushima *et al*. 2004).

4.5 Discussion

Tumour hypoxia facilitates the release of intracellular moieties including HMGb1 from tumours. It has also been shown that hypoxic regions of tumours attract macrophages that have strong pro-angiogenic properties which then facilitate the release of HMGb1. This facilitates more effective proliferation and metastatic spread in tumours (Ellerman *et al*. 2007). There is an increasing body of evidence that supports the hypothesis that neoplastic tissues utilise a large quantity of glucose. The effect of glucose deprivation and acidosis has been shown to increase metastasis in murine tumour cell lines *in vivo*. However, the underlying mechanism of glucose deprivation induced metastatic spread remains unknown (Schlappack *et al*. 1991; Xie and Huang 2003). Glioblastoma cells do not release HMGb1 in their culture medium. However,

necrotic glioma cells can release HMGb1 after its translocation from nucleus to cytosol (Bassi *et al*. 2008). The release of HMGb1 from immune cells as danger signal is well documented (Manfredi *et al*. 2009; Pisetsky 2011; Ganz *et al*. 2015). However, the release of HMGb1 from cancer cells under glucose starving conditions has, so far, not been reported.

A western blotting method was developed to detect the release of HMGb1 from HT29 cells. The western blot results showed that HMGb1 is released from HT29 cells when exposed to different stress conditions such as anoxia and/or the absence of glucose. Interestingly, absence of glucose appeared to stimulate the release of HMGb1 even in presence of oxygen at early time points (24 to 48h) in our system (Figure 4.1). This release of HGMb1 was compared to the release of HMGb1 in absence of oxygen. It was found that glucose deprivation is the main stimulus which facilitates the release of HMGb1 from HT29 cells.

The release of HGMb1 in anoxic conditions though appeared to be time dependent but it was slower than the normoxic conditions with glucose starvation. In addition, in an attempt to investigate the release of HMGb1 at higher time points (72 and 96h) it was found that number of cells were floating in the flasks. These cells were presumed to be dead cells. In that case, even if HMGb1 was released, being a nuclear protein, it was apparent that HMGb1 would have passively been released or spilled out from the dead cells into the culture medium. Therefore, carrying out an investigation using western blot would not represent the true data as actively and passively released HMGb1 can not be differentiated in terms of structure and molecular weight. Therefore, a western blot was not carried out on those samples.

The other aim was to investigate whether this release of HMGb1 is cell line specific or it's a common phenomenon that occurs in other cell lines derived from other types of tumour. The hypoxia induced HMGb1 release has been seen in a number of cells such as RAW264.7 macrophages, WM9 melanoma cells and hepatocytes (Wang *et al*. 2004; Tsung *et al*. 2007; Ito *et al*. 2007). The results from western blot analysis showed that the release of HMGb1 under glucose free normoxic conditions is not cell line specific and other cells such as MCF-7 and A549 followed the same pattern. However, EJ138 did not appear to release more HMGb1 in glucose free conditions. The levels of HMGb1 observed in the culture medium taken from EJ138 were not different in both glucose containing and glucose free conditions.

It has been documented that hypoxic or semi hypoxic regions inside the tumours are glucose deprived. However, glutamine is also an important nutrient for the cells to compete their cycle. The nutrient deprived conditions such as lack of glutamine in solid tumours have been shown to induce autophagy in the cells which in turn helps tumour cells to proliferate in the microenvironment (Ye *et al*. 2010). It has been shown that the glioblastoma cells that are destined to die (autophagy) release HMGb1 in their culture medium without the lysis of membrane or necrosis (Thorburn *et al*. 2008). Therefore, there is a possibility that HT29 cells were undergoing autophagy but it can not be confirmed because of poor understanding of the mechanism of autophagic cell death. However, the drivers of autophagic cell death such as lack of nutrients (glutamine) and oxygen were tested. Glutamine has been considered as major regulator of autophagy and lack of glutamine may promote cell death via apoptosis (Sakiyama *et al*. 2009). Therefore, it was also investigated whether this release of HMGb1 was triggered by absence of the nutrient glutamine or whether release was specific to the absence of the nutrient glucose. The results from our western blot showed that HMGb1 is released from both glutamine deprived and glutamine containing conditions and there was no major difference in release

observed between the two conditions. However, when glutamine deprived and glutamine containing conditions were compared with glucose deprived and glucose containing conditions, it was found that glucose deprived conditions serve as major stimulus which facilitates the release of HMGb1. These findings are novel and have not been published thus far.

Receptor for advanced glycation end products (RAGE) is the most commonly known receptor for HMGb1. RAGE is multi-ligand receptor, expressed in many cells including monocytes, macrophages and smooth muscle cells can be activated by several ligands including HMGb1. The activation of RAGE in monocytes/macrophages has been reported to trigger the inflammatory response and to trigger neoplastic transformation and metastasis in neuroepithelial tumour cells (Taguchi *et al*. 2000). A recent study has shown that HMGb1 binds to RAGE and not to the TLR-4 to promote resistance to Melphan by inducing beclin-1 dependent autophagy (Tang *et al*. 2010). The data from our proliferation assays demonstrated that HMGb1 triggers proliferation in myofibroblasts cells where blocking both MEK1/2 and PI3K pathways with selective inhibitors abrogated the proliferative effect of HMGb1 in CCD18 myofibroblast cells (Figure 3.6 and 3.8). Recently, it has been shown that HMGb1-RAGE complex increases the production of ATP, which is required by tumour cells to grow and migrate to the distant sites. This production of ATP was blocked by either inhibiting RAGE or HMGb1 by immunoneutralising antibodies which resulted in diminished proliferation and migration of tumour cells (Kang *et al*. 2013). The data from our western blot suggested that RAGE is expressed on myofibroblasts cells.

Although, the expression of RAGE was not correlated with the release of HMGb1 in different stress conditions from cancer cells but it was detected in all stress conditions such as glucose

deprivation and/or anoxia. Thus, there is a possibility for the activation of HMGb1-RAGE and subsequent activation of downstream signalling which facilitates proliferation in myofibroblasts cells such as activation of MAPK/ERK pathway in the HMGb1 induced proliferation in myofibroblasts shown in this study. Recently, it has been shown that RAGE expression is upregulated at various stages of transdifferentiation from hepatic stellate cells (HSCs) to myofibroblasts (Wang *et al*. 2013). However, here it is not confirmed yet whether HMGb1 induced proliferation of myofibroblasts involves activation of HMGb1-RAGE complex. The HMGb1-RAGE complex has been successfully detected by co-immunoprecipitation technique recently (Lai *et al*. 2013; Kang *et al*. 2014). Therefore, to further validate the interaction of RAGE and HMGb1 to trigger proliferation in myofibroblasts, co-immunoprecipitation should be carried out myofibroblasts cell lysates followed by affinity chromatography to elute RAGE or HMGb1 or both. The co-immunoprecipitation is a technique which detects the interaction of a protein with other proteins in a sample (Kaboord and Perr 2008).

RAGE is not the only receptor for HMGb1 and others include the toll-like receptors 2 and 4 (TLR-2 and TLR-4). Park *et al*. (2004) demonstrated that blockage or knockdown of these receptors resulted in decreased HMGb1 activation *in vitro* and *in vivo* (Park *et al*. 2004). The activation of HMGb1-TLR-4 complex has been shown to trigger migration in HSC cells via the activation of PI3K-AKT pathway (Wang *et al*. 2013). The results from our western blot suggested that TLR-4 is present on myofibroblasts cells treated with both conditions i,e. glucose containing and glucose deprived conditions for 48h. In addition, we also investigated whether TLR-4 is expressed on the CCD18 cells treated with serum fee medium for 24 hr then treated for 22h with used serum free medium taken from HT29 after 48h. These conditions mimic the migration assay conditions where inhibition TLR-4 significantly affected the migration response in myofibroblasts cells (explained in chapter 5).

The results from our western blot suggested that TLR-4 is present on the myofibroblasts cells that underwent treatment mimicking (treatment with HT29 culture medium) the migration assay treatment. However, the protein bands for TLR-4 were also observed at 70kDa which is presumably a proteolytic degraded product of TLR-4. This 70kDa band corresponding TLR-4 has previously been observed by others (Hyakushima *et al*. 2004). The role of HMGb1-TLR-4 complex has been implicated in predicting the outcome of cancer chemotherapy. The activation of this complex is correlated with relapse after the anthracycline based chemotherapy in breast cancer patients (Apetoh *et al*. 2007). The activation of HMGb-TLR-4 complex on dendritic cells was detected by co-immunoprecipitation technique (Apetoh *et al*. 2007). Therefore, it would be logical approach to apply co-immunoprecipitation to selectively detect the HMGb-TLR-4 complex in myofibroblast cell lysates. This would further confirm whether HMGb1 induced proliferation and migration of myofibroblasts cells involved the activation of HMGb1-TLR-4 complex.

Chapter 5

5. Role of HMGb1 in myofibroblasts migration and invasion

5.1 Introduction

Presence of stromal cells such as fibroblasts and myofibroblast in solid tumours plays an important role in predicting the metastatic behaviour of solid tumours. It has been established that stromal cells play important roles in carcinogenesis. The stromal myofibroblasts have been considered as predictors of human disease outcome and their abundance is correlated with poor survival in colorectal cancer (Tsujino *et al*. 2007). The likely role of myofibroblasts in tumour invasion has been explored in the past and it was found that the myofibroblasts population gradually increases with the invasive stages of many cancers (Nakayama *et al*. 1998). The expression of α-SMA, which is a biomarker for myofibroblasts, has been correlated with enhanced metastatic spread of tumours whose characterisation involves CAFs. For example, an increased expression of α-SMA has been seen in fibroblasts present in HER2 breast cancers (Toullec *et al*. 2010).

There is evidence that support the involvement of stromal myofibroblasts in tumour development. For example, myofibroblasts have been shown to stimulate proliferation in breast cancer cells *in vivo* (Khanna *et al*. 2015). In addition, they have also been shown to promote invasion of breast, pancreas and squamous carcinoma cells *in vitro* (Casey *et al*. 2008; Hu *et al*. 2008; Hwang *et al*. 2008). Furthermore, mesenchymal cells and their derivatives, including

myofibroblasts, have been shown to promote breast cancer metastasis. The myofibroblasts population was significantly high in breast adenocarcinoma and lung cancer that metastasised to lymph nodes compared to the non-metastatic tumours. This suggested that myofibroblasts contribute toward the spreading of tumours through metastasis (Toullec *et al*. 2010). Cancer cells have been shown to stimulate stromal cells such as myofibroblasts to produce proteases (Tuxhorn *et al*. 2002; Anborgh *et al*. 2010). These proteases cleave ECM components and remodel the ECM in the microenvironment (Hinz *et al*. 2012). A number of protease have been shown to degrade ECM in the tumour microenvironment however, gelatinase (MMP-2) and MMP-9 are two major proteases released from myofibroblasts and have been shown to degrade basement membrane (Cheng and Lovett 2003; Takahra *et al*. 2004).

The cytokine TGF-β is present in exosomes which are secreted by most cell types including cancer cells trigger the conversion of fibroblasts to myofibroblasts at distant organs. It has also been suggested that TGF-β might facilitate metastasis by stimulating cells to produce fibronectin. However, tumour cells may (also) move along with myofibroblasts to the distant sites, such as the lungs. These migrated myofibroblasts have been shown to produce pro-survival signals in the lung site (Duda *et al*. 2010). Therefore, transformed fibroblasts or cancer associated myofibroblasts may influence tumour microenvironment and may contribute towards malignant transformation in solid tumours. Therefore, it is logical to investigate the role of HMGb1 in myofibroblasts which may be linked with invasion and metastasis of tumour cells.

Necrotic areas are characteristic features of rapidly growing tumours. The necrotic areas within tumour not only produce angiogenic factors such as VEGF but also attract macrophages. In stress conditions such as necrosis, these macrophages release HMGb1 within the

microenvironment (van Beijnum *et al*. 2006). Several solid tumours including melanoma, prostate cancer, breast cancer, pancreatic cancer and colon cancer exhibit markedly elevated levels of HMGb1 (Völp *et al*. 2006). These elevated levels of HMGb1 are associated with tumour formation, proliferation and metastasis and chemotherapeutic response. In addition, presence of HMGb1 in the extracellular medium of cells is indicative of stress conditions (Lotze and Tracey 2005).

The direct inhibition of HMGb1 with immunoneutralising antibodies has been reported to inhibit angiogenesis *in vivo* and *in vitro* (Van Beijnum *et al*. 2006). The HMGb1 interacts with RAGE, TLRs (TLR-2 and TLR-4) and may be with other unknown receptors and activates various factors and kinases such as NF-κB and MAPK. It has been shown that HMGb1 can increase the metastatic potential of tumour cells by activating NF-κB pathway (Sasahira *et al*. 2008). In addition, HMGb1 can modulate the adhesive properties of cells and ECM components and thus can directly affect the migration of cells (Ellerman *et al*. 2007).

The HMGb1 activates various signalling pathways that involve the activation of MAPKs, AKT and PI3K. These protein kinases play an important role in the proliferation and migration of tumour cells. Supporting evidence includes HMGb1 activated PI3K pathway in neutrophils and colon cancer cells (Kuniyasu *et al*. 2003). In addition, HMGb1 has been shown to induce TLR-4 mediated activation of MyD88-IRAK4-p38 and Myd88-IRAK4-AKT pathways (Fan *et al*. 2007). These pathways have been implicated in tumour cell proliferation and survival. The HMGb1-RAGE complex has been shown to activate NF-$_{\kappa}$B, Jun N terminus kinase (JNK) and ERK1/2 pathways (Degryse *et al*. 2001). The activation of JNK is correlated with an increase in cell migration in different cell types including rat bladder tumour cells, keratinocytes and human

dermal fibroblasts. It has also been shown that MEK1 which is an upstream kinase in the JNK signalling pathway is essential for cell migration (Van Beijnum *et al*. 2008).

The data presented in chapter 3 suggested that HMGb1 triggers proliferation in myofibroblasts. This proliferation was inhibited by U0126 and LY294002 selective inhibitors of MEK1/2 and PI3K respectively. It has been established that HMGb1 is released in hypoxia, which is characteristic feature of most solid tumours (Tsung *et al*. 2007). Glucose deprivation is also a hallmark of tumour microenvironment. The central core of most solid tumours is characterised by lack of glucose and acidosis (Cuvier *et al*. 1997). Glucose deprivation in the solid tumour microenvironment and its consequences within and outside tumour microenvironment are largely unexplored. The data presented in chapter 4 indicates that HMGb1 is released from cancer cells in glucose deprived conditions and not from depravation of the nutrient glutamine. Previous studies have shown a correlation between myofibroblasts and metastasis in many solid tumours including breast cancer (Allinen *et al*. 2004; Muehlberg *et al*. 2009). Thus, there is a possibility that HMGb1 once released from cancer cells might influence the other stromal cells such as myofibroblasts to migrate and invade. In addition, MAPKs, which play a central role in migration of many cells including fibroblasts, might have a role in migration of myofibroblasts.

The HMGb1 and its interaction with RAGE and TLRs have important roles in intracellular signalling of various pathways including PI3K (Fan *et al*. 2007). Therefore, neutralising antibodies to these receptors will be used to confirm their role in migration and invasion of myofibroblasts

5.2 Aims and objectives

The aims of this study were to;

a) Investigate if glucose deprived conditioned medium of HT29 cells can induce migration and invasion in CCD18 myofibroblast cells.
b) Compare the impact of used medium, derived from HT29 cells deprived of glucose or rich in glucose with fresh medium rich in glucose on CCD18 myofibroblast cells migration and invasion.
c) Investigate whether recombinant HMGb1 can induce migration in myofibroblasts cells
d) Investigate whether HMGb1 present in the conditioned medium of HT29 cells is responsible for apparent migration and invasion in myofibroblasts.
e) Investigate whether inhibiting HMGb1 in the conditioned medium of HT29 cells by anti-HMGb1 neutralizing antibody can reduce migration and invasion in CCD18 cells.
f) Investigate whether Inhibition of RAGE by neutralizing antibodies can have negative impact of myofibroblasts migration and invasion.
g) Investigate whether inhibition of TLR-4 results in reduction in myofibroblastic migration and invasion.
h) Investigate whether MEK1/2 and PI3K pathways are involved in HMGb1 induced migration and invasion in myofibroblasts.
i) Investigate whether MMP-2 and MMP-9 are released from CCD18 myofibroblast cells following the treatment with HMGb1 containing conditioned medium collected from HT29 cells.

5.3 Material and Methods

5.3.1 Cell culture

The CCD18 myofibroblast cells were cultured in T75 cm^2 flasks in complete medium (minimum essential medium eagle) supplemented with foetal bovine serum (10% v/v). The cells were passaged when they had reached about 80-90% confluence. In order to carry out migration or invasion assays, the myofibroblast cells were first serum starved for 24h before they were used. The cells were dislodged from flasks using versene. Versene (1x) is less aggressive than trypsin and was used to dissociate the cells in the flask before plating them onto the 24-well plate. Versene is a chelating agent that binds to calcium and prevents joining of cadherins between cells thereby prevents clumping of cells in the medium. The cells suspension in versene was diluted with serum free medium to a concentration of $4.0x10^4$ cells per ml for use in migration assays or $6.0x10^4$ cells per ml for used in the invasion assays.

5.3.2 Migration assay

HT29 colon adenocarcinoma cell lines were seeded in 2x$T75cm^2$ ($2x10^6$ cells per ml). Both flasks were incubated over night at 37°C, 95% humidity and 5% CO_2 to allow the cells to adhere. The following day, the complete medium was aspirated off and cells were washed twice with PBS. The medium on the cells was replaced with serum free medium with glucose in one flask and without glucose in the other flask. After 48h, the conditioned medium from both flasks were collected into labelled universals and used as potential chemoattractants for the migration assay.

The CCD18 cell suspension was made in serum free medium ($4.0x10^4$ per ml) (see section 5.2.1 above). The CCD18 myofibroblast cells were then seeded (0.5ml per insert) in 2x2 chamber inserts (Biocoat®, 8µm pores) which were then transferred to 4 wells of the 24 well plate and ensured that insert bottom is touching the HT29 conditioned medium (0.75ml per well) in the wells. The plate was incubated for 20h in the incubator at 37°C, 95% humidity and 5% CO_2. The CCD18 cell suspension in the inserts and media at the bottom chamber were supplemented with antibodies and inhibitors to investigate the various pathways involved in myofibroblast migration (see chapter 2, table 2.7).

After 20h of incubation period, plates were removed and cells were stained (QuickDiff) for counting. The images of random fields of vision were captured from each membrane. The cells that had migrated through the membrane were counted using ImageJ cell counter software and the average of the 5 fields of vision for each membrane calculated.

5.3.3 Invasion assay

The invasion assays for CCD18 myofibroblasts were performed using 8µm pore matrigel matrix Biocoat® inserts according to the manufacturer's instruction (Becton Dickinson). The CCD18 cell suspension was made in serum free medium ($6.0x10^4$ per ml). The myofibroblasts cells were plated onto (0.5ml per insert) inserts in a 24-well plate supplied by the manufacturer. The inserts were put into the wells of 24-well plate. HT29 conditioned medium was used as chemoattractant and plated onto the lower chamber (0.5ml) and ensured that inserts were touching the conditioned medium (see section 2.4). The plate was incubated for 22h in the incubator at 37°C, 95% humidity and 5% CO_2. The myofibroblasts invasion, in response to the

conditioned medium with and without various inhibitors and antibodies were determined by their inclusion in the lower chamber of the 24-well plate. Cells invaded through the matrigel matrix membrane were detected on the lower surface by using ReaStain Quick-Diff staining method and visualised at x20 magnification under a microscope. The CCD18 cell suspension in the inserts and media at the bottom chamber were supplemented with antibodies and inhibitors to investigate the various pathways involved in myofibroblast invasion (see table 2.7)

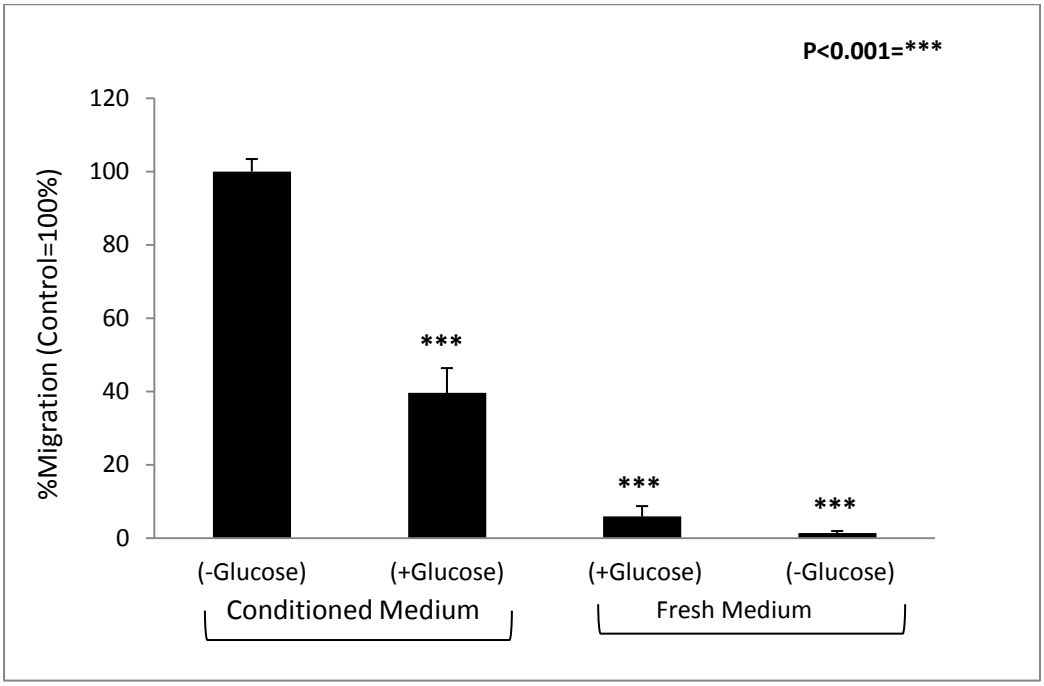
5.4 Results

5.4.1 Culture medium from HT29 cancer cells starved of glucose triggers migration in CCD18 myofibroblast cells

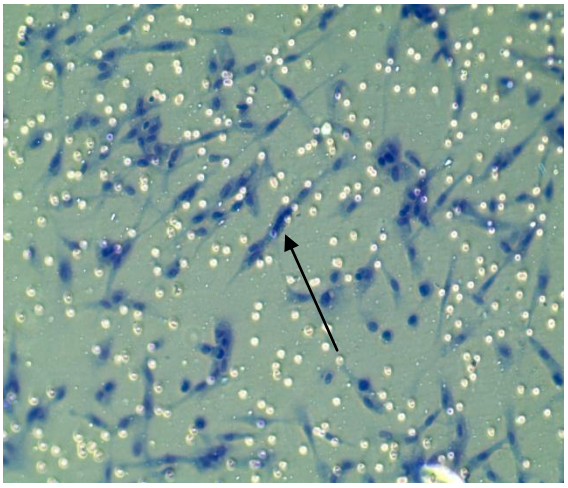
The glucose deprivation has been considered as hallmark in most solid tumours and has been reported to induce migration of cancer cells. The western blot results discussed in chapter 4 suggested that HMGb1 is released from cancer cells in glucose deprived conditions. However, the subsequent effect on tumour stromal cells such as myofibroblasts has not been explored. The myofibroblasts are stromal cells and constitute a major part of tumour stroma. In addition, they have been found in abundance at metastatic sites of solid tumours. Therefore, it is hypothesised that HMGb1 once released from cancer cells, reaches to the stroma to influence myofibroblasts to proliferate, migrate and invade along with neighbouring cancer cells by digesting the basement membrane. This might allow cancer cells to escape from their original location to the distant organs.

A migration assay was set up according to the instructions described in method section (section 5.2). HT29 colon cancer cells were grown in medium containing no glucose for 48h. This medium was then collected from the HT29 cells. This HT29 conditioned medium was used as chemoattractant as this was shown to contain the highest levels of HMGb1 when compared to medium that had been used on HT29 cells containing glucose or fresh unused medium with or without glucose (Chapter 4). This conditioned medium served as positive control (therefore counted as 100% migration) and was compared to myofibroblast migration in response to conditioned medium that contained glucose or the fresh serum free medium with and without glucose. The results obtained from this migration assay suggest that HT29 cells undergoing glucose deprivation release chemoattractants that trigger migration in myofibroblasts cells. In addition, conditioned medium with glucose does trigger migration in myofibroblasts cells; however, this migration was approximately 60% less than the conditioned medium without glucose at 100% (Figure 5.1 A). In addition, the effects of fresh serum free medium with and without glucose were examined. A negligible number of myofibroblasts cells migrated across the 8µm pore membrane when exposed to the fresh serum free medium with and without glucose (Figure 5.1 D and E). The data suggests that absence or presence of glucose in a fresh medium does not influence the myofibroblasts cells to migrate. All these results are statistically significant (Figure 5.1A).

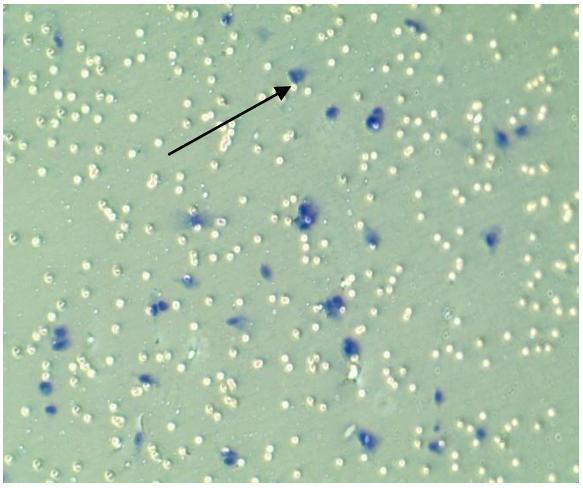
A)



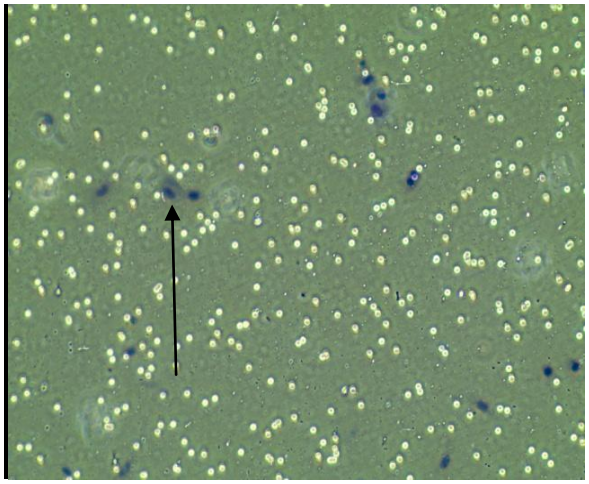
B) Control-Conditioned medium



C) Conditioned medium with glucose



D) Serum free fresh medium with glucose



E) Serum free medium without glucose

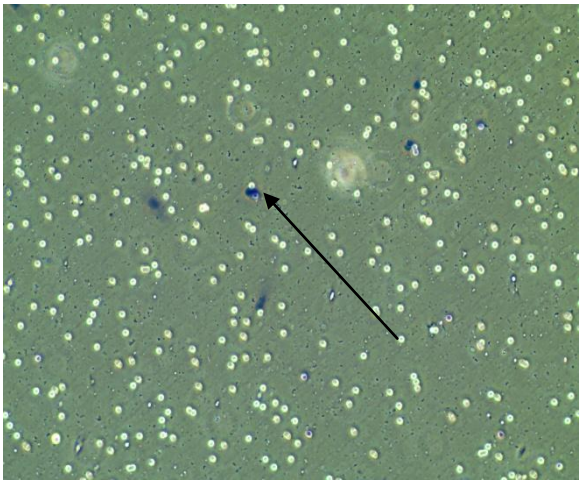


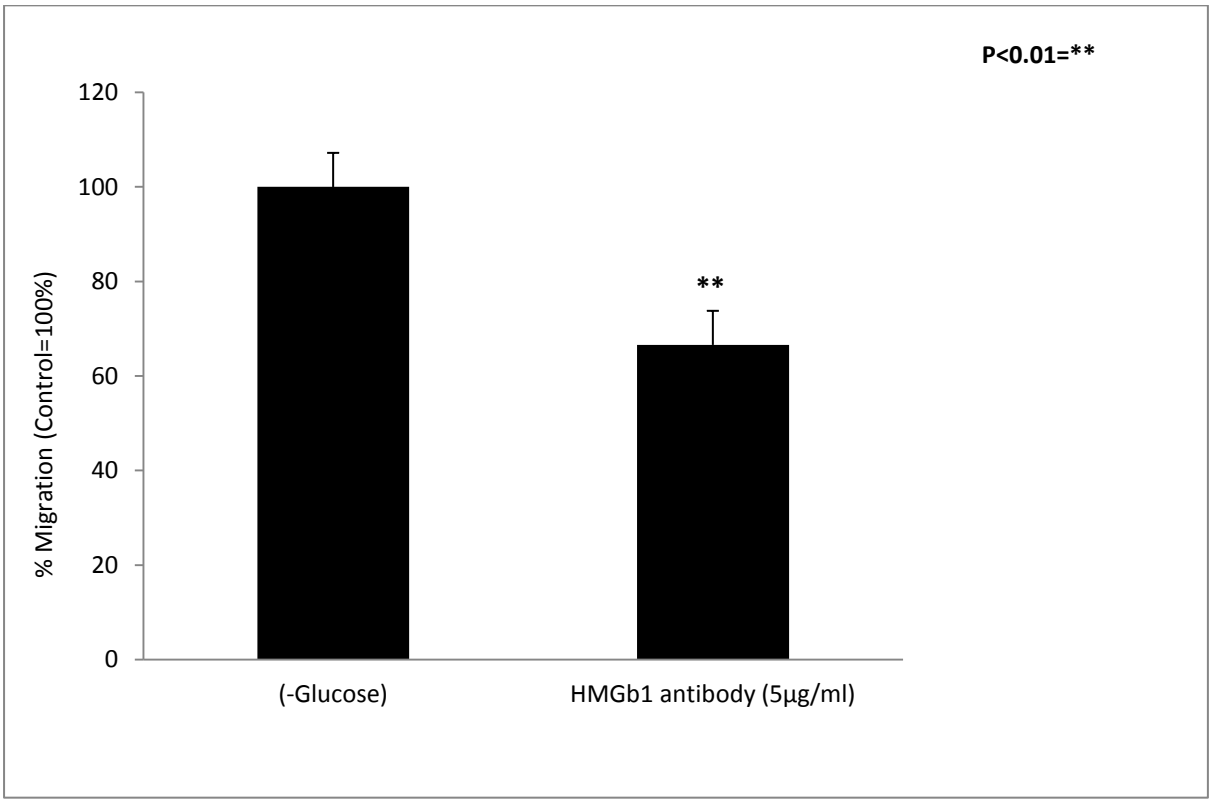
Figure 5.1: CCD18 myofibroblast cells migration assay in response to HT29 conditioned medium. A) *The migration assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control. The conditioned medium with glucose and fresh serum free medium with and without glucose were compared to the control (100%). The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars.*

Typical microscope images captured showing the underside of the membrane after completion of the migration assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Representative images from migration assays showing cells migrated in response to conditioned medium without glucose (Control=100%), C) cells migrated in response to the conditioned medium with glucose, D) cells migrated in response to fresh serum free medium with glucose and E) cells migrated in response to fresh glucose free and serum free medium. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken under x20 magnification.

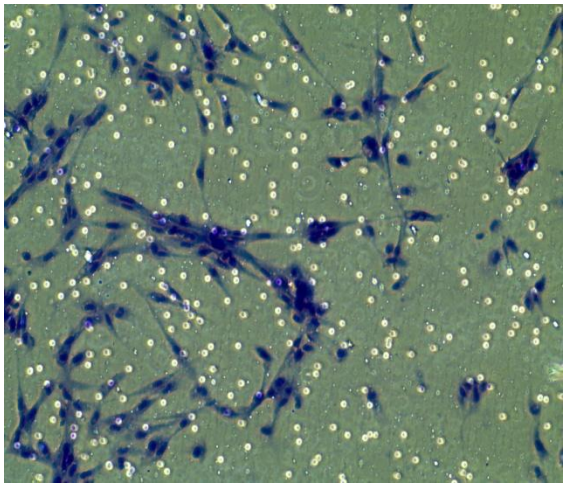
5.4.2 HMGb1 released from HT29 colon adenocarcinoma cells triggers migration in CCD18 myofibroblasts

The results discussed in chapter 3 suggested that recombinant HMGb1 triggers proliferation in CCD18 myofibroblast cells in a dose dependent manner. In addition, results from western blotting suggested that HMGb1 is released from HT29 and other cancer cells including MCF-7 and A549 cells under glucose deprivation normoxic conditions. To investigate whether HMGb1 present in the culture medium of HT29 cells can trigger migration in myofibroblasts, HT29 conditioned medium was used as chemoattractant. The glucose starving-conditioned medium worked as a strong chemoattractant in our migration assay. However, to validate HMGb1 mediated signalling in the migration of myofibroblasts cells, anti-HMGb1 antibody (5µg/ml) was added to the conditioned medium at the bottom chamber to neutralise the effect of HMGb1. If the HMGb1 was involved in the migration of myofibroblast cells, a decrease in the migration of cells was expected.

A)



B) Control



C) Anti HMGb1 antibody in the medium

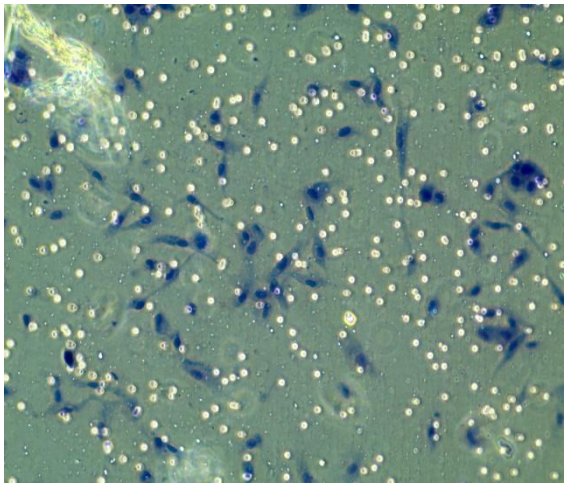


Figure 5.2: CCD18 myofibroblasts migration assay in response to HMGb1 present in the glucose free conditioned medium of HT29 cells. A) *The migration assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control. The conditioned medium with anti HMGb1 antibody (5µg/ml) was compared to the control (100%). The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert).Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars. Representative images captured showing the underside of the membrane after completion of the migration assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. B) Figure showing cells migrated through the membrane and C) cells migrated when anti-HMGb1 antibody was added to the medium. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken under x20 magnification.*

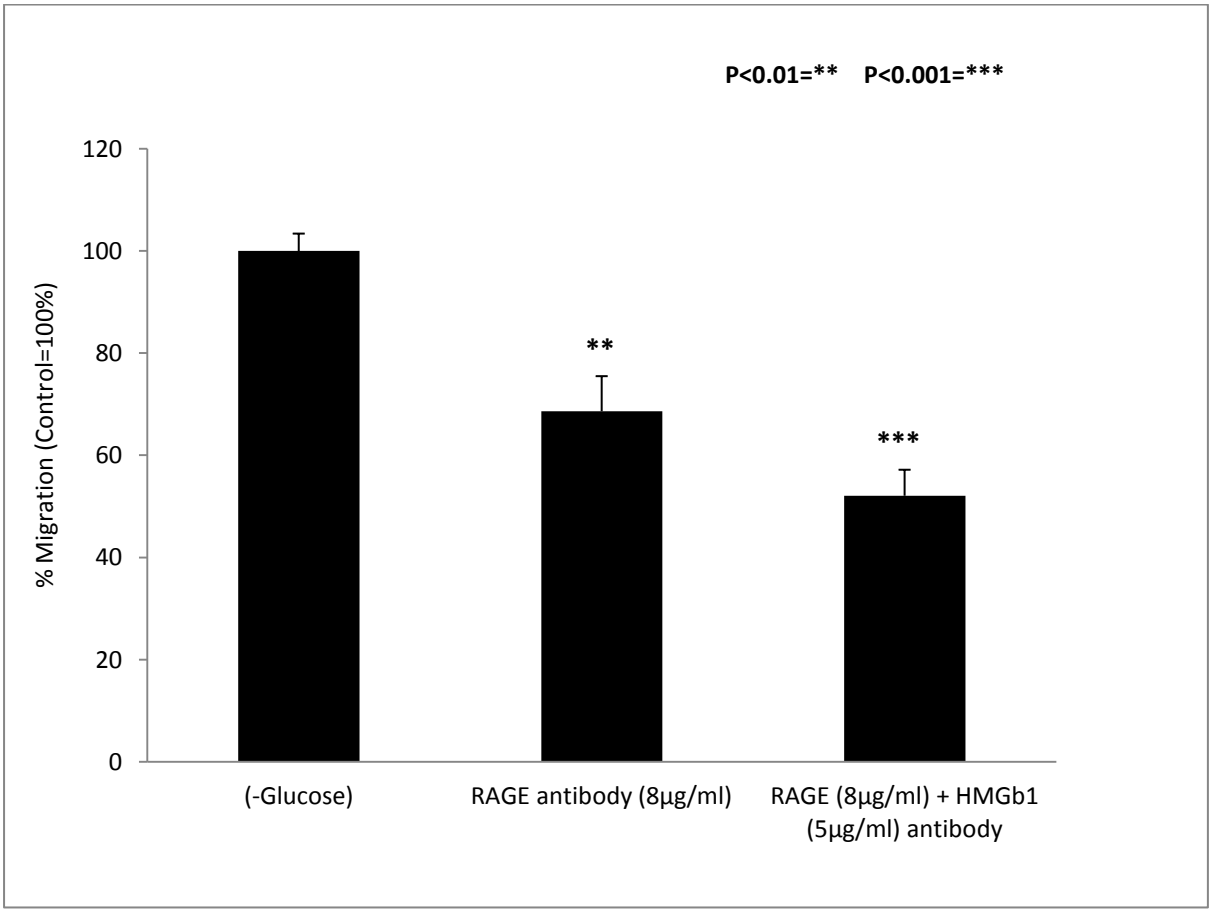
The results from the migration assay using the anti-HMGb1 antibody in conditioned medium suggested that HMGb1 play an important role in the migration of myofibroblast cells. This migratory effect was significantly inhibited by the addition of anti-HMGb1 antibody (5µg/ml) in the medium. This inhibition was 1/3 fold compared to the controls (Figure 5.2A). The results obtained from the migration assay are statistically significant (Figure 5.2 A).

5.4.3 HMGb1 triggers migration in myofibroblasts cells via RAGE signalling

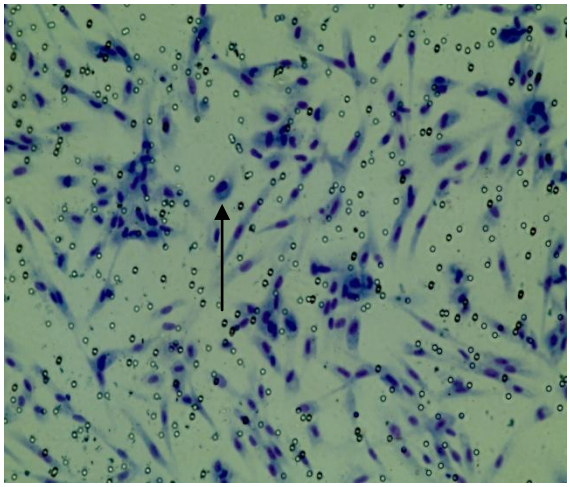
HMGb1 has been reported to interact with RAGE and this interaction has been implicated in variety of diseases including cancer (Stoetzer *et al*. 2013; Ingels *et al*. 2015). In addition, HMGb1 has been reported to trigger migration in mouse fibroblasts cells via RAGE receptor (Ranzato, *et al*. 2010). The presence of HMGb1 in conditioned medium (glucose deprived) from HT29 cells was confirmed by the western blotting (Chapter 4). In addition, the results from previous migration assay suggested HMGb1 mediated signalling play an important role in the migration of CCD18 myofibroblast cells.

One of the aims of this migration assay was to determine the involvement of RAGE in the HMGb1 induced migration of CCD18 myofibroblast cells. RAGE is the main receptor for HMGb1 however; HMGb1 does interact with other receptors including TLR-4. The expression of these receptors by CCD18 myofibroblast cells was confirmed by western blotting (Chapter 4). Therefore, it is likely that HMGb1 mediated migration of CCD18 myofibroblast cells involves activation of HMGb1-RAGE complex. This interaction may trigger downstream signalling cascade, which may facilitate the migration of myofibroblast cells.

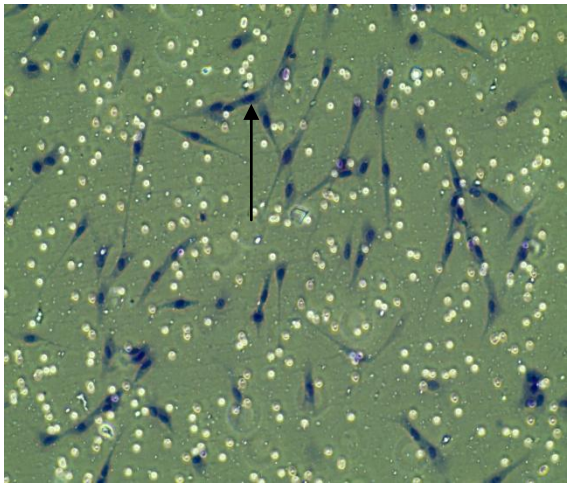
A)



B) Control



C) Anti RAGE antibody



D) Combination of anti RAGE and anti HMGb1 antibodies

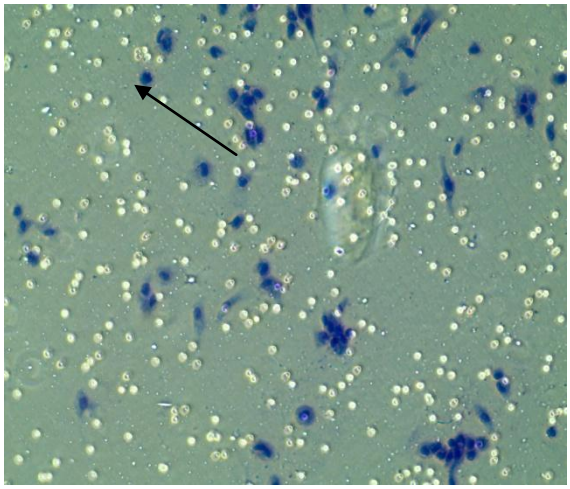


Figure 5.3: *Effect of inhibiting RAGE and HMGb1 by neutralising antibodies on CCD18 myofibroblast cells migration.*

A) *Migration assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control. The conditioned medium with immunoneutralising anti RAGE antibody was compared to the control (100%). A combination of anti RAGE and anti HMGb1 antibodies were also supplied to the conditioned medium and compared to the controls. The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars. Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars.*

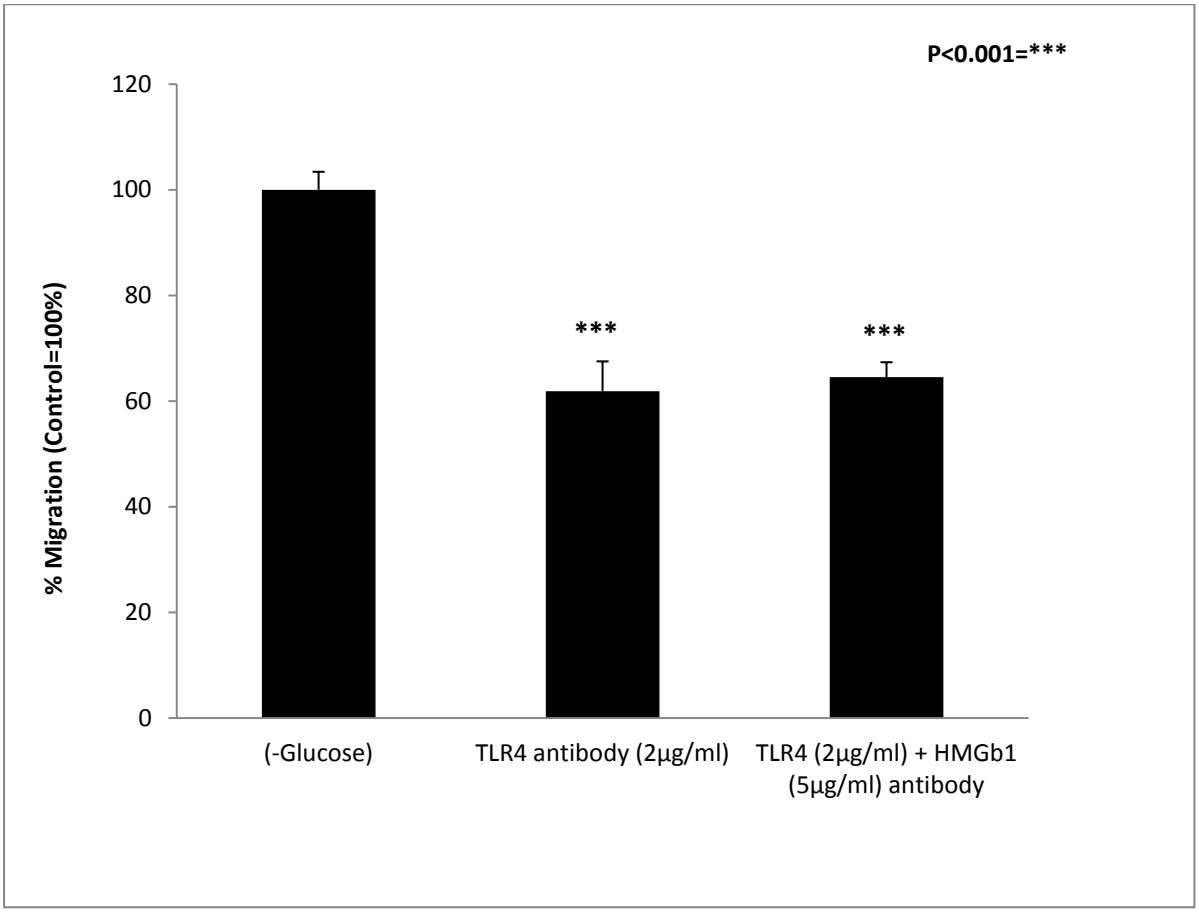
Representative images captured showing the underside of the membrane after completion of the migration assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Cells migrated in response to conditioned medium without glucose, C) cells migrated in response to immunoneutralising RAGE antibody in the conditioned medium without glucose and in the cell suspension in the inserts and D) cells migrated in response to the combination of immunoneutralising RAGE and HMGb1 antibodies in the medium and RAGE antibody in the cell suspension. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken under x20 magnification.

The effect of conditioned medium (control) on myofibroblast migration was compared to myofibroblast migration in response to two other treatments. In the first treatment, immunoneutralising anti-RAGE antibody (8µg/ml) was added to the conditioned medium and myofibroblast migration compared to that of the controls. In addition, another treatment, conditioned medium with a combination of anti-RAGE (8µg/ml) and anti-HMGb1 (5µg/ml) antibodies were used and the resulting myofibroblast migration was compared to the RAGE antibodies only treatments and controls. The results obtained from migration assay suggested that RAGE is involved in the migration of myofibroblasts cells as anti-RAGE antibodies significantly inhibited (approximately 38%) the migration of myofibroblasts. This inhibition was further increased by 10% when a combination of anti HMGb1 antibody and anti-RAGE antibodies were added to the chemoattractant (conditioned medium), confirming the involvement of HMGb1-RAGE complex in HT29 conditioned medium triggered migration of CCD18 myofibroblast cells. All results were statistically significant (Figure 5.3 A).

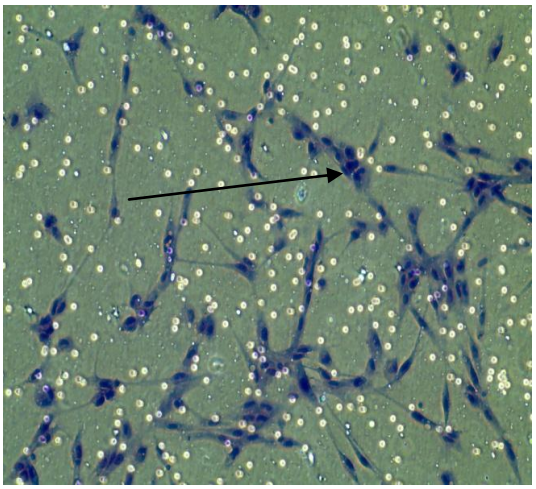
5.4.4 HMGb1 present in the conditioned medium triggers migration in CCD18 myofibroblast cells via TLR-4 signalling

The HMGb1 has been reported to interact with many receptors including RAGE and TLRs previously (TLR-2 and TLR-4) (Yu *et al*. 2006; Tian *et al*. 2007). The glucose free conditioned medium obtained from HT29 cells at 48h served as chemoattractant for myofibroblast migration as evident from this study. In addition, this study has also suggested the fact that conditioned medium obtained from HT29 contains significant amount of HMGb1 (Chapter 4). The results from migration assays suggested that both HMGb1 and RAGE are involved in the migration of CCD18 myofibroblast cells. In addition, inhibiting both (HMGb1 and RAGE) resulted in additive inhibition of migration in CCD18 myofibroblast cells. Therefore, it was important to investigate the involvement of another receptor TLR-4, which has been implicated in migration of other cell types previously (Fan and Malik 2003; Liu *et al*. 2014).

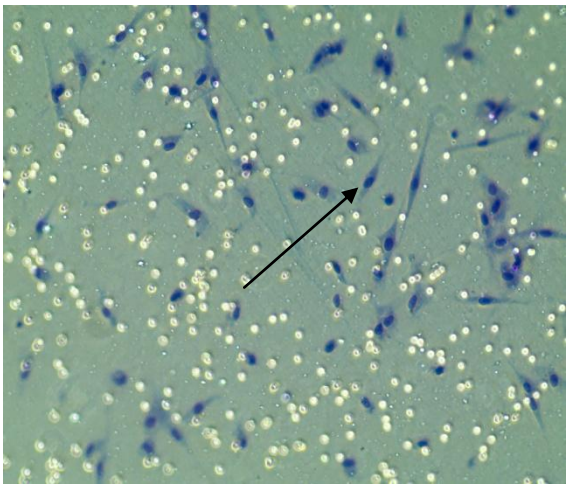
A)



B) Conditioned medium (control)



C) Anti-TLR-4 antibody



D) Combination of anti HMGb1 and anti TLR-4 antibodies

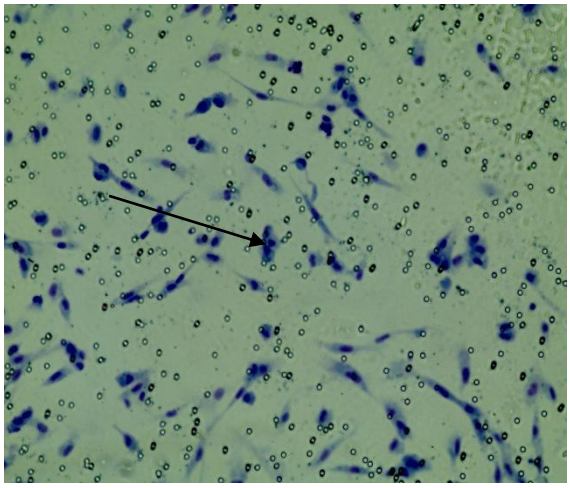


Figure 5.4*: Comparative analysis of CCD18 myofibroblast cells migration assay in response to HT29 conditioned medium and blocking TLR-4 or HMGb1/TLR-4 complex using immunoneutralising anti-HMGb1 or a combination of anti-HMGb1 and anti-TLR-4 antibodies.*

A) Migration assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control (control=100%). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars.

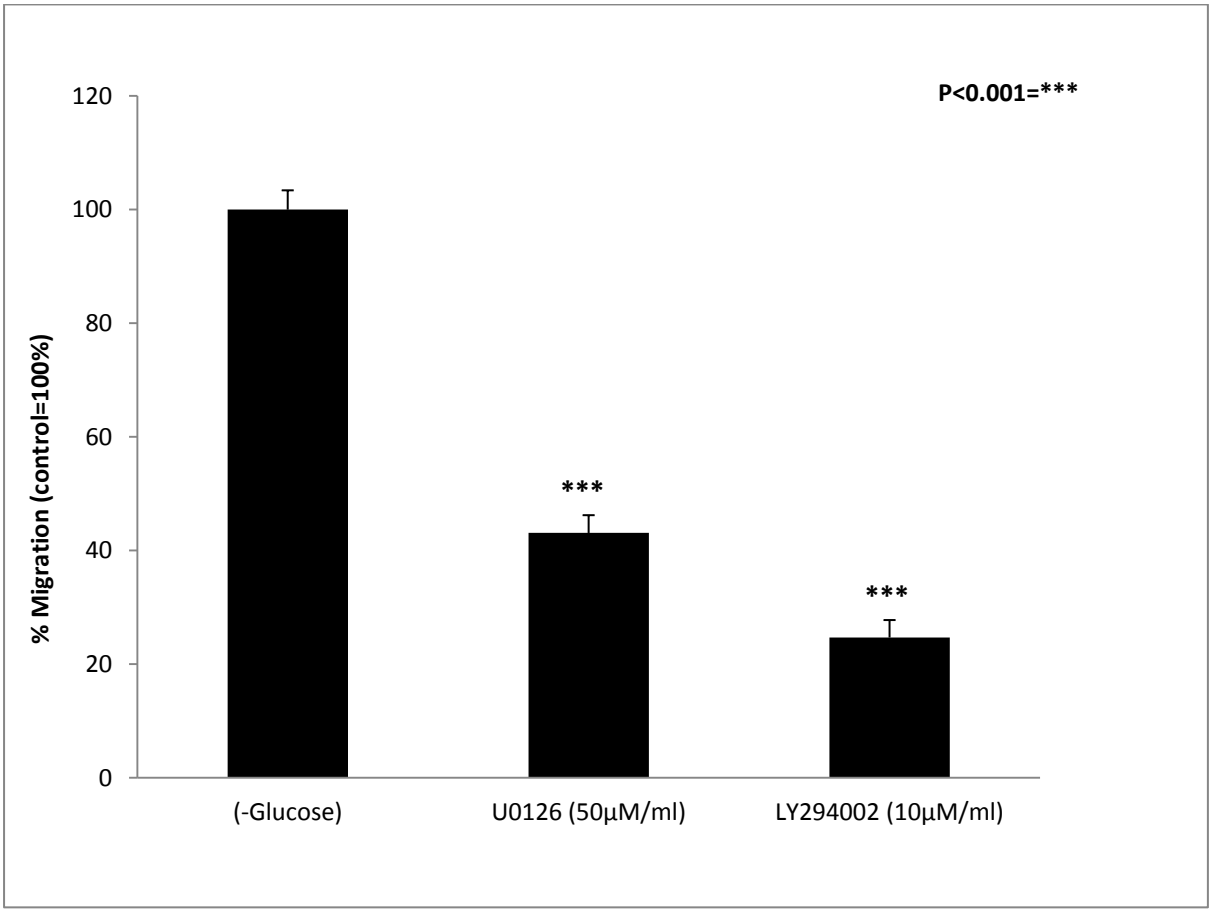
Representative images captured showing the underside of the membrane after completion of the migration assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Cells migrated in response to conditioned medium without glucose (Control=100%), C) cells migrated in response to immunoneutralising anti-TLR-4 antibody in the conditioned medium without glucose and in the cell suspension in the inserts and C) cells migrated in response to the combination of immunoneutralising anti-TLR-4 and anti-HMGb1 antibodies in the conditioned medium and in the cell suspension. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert). The photographs were taken under x20 magnification.

The conditioned medium (-glucose) served as a positive control and the number of myofibroblast cells migrating in response taken as 100% migration. Immunoneutralising antibodies against HMGb1 and TLR-4 were used to neutralise the effect of receptor and the ligand. In addition, both antibodies were also used in combination. The results obtained from this migration assay suggested that TLR-4 is involved in the migration of the myofibroblasts cells. However, results from combination treatment didn't show additive effect on the inhibition of migration of myofibroblasts. The results suggested that HMGb1 might bind to TLR-4 to induce migration in CCD18 myofibroblast cells. However, the inhibitory effects seen with both treatments (TLR-4 antibody alone and in combination with HMGb1 antibody) were statistically significant with approximately 40% reduction in migration of myofibroblasts cells (Figure 5.4 A).

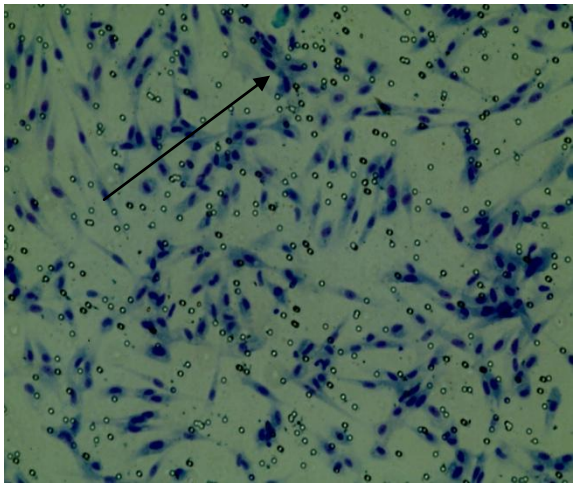
5.4.5 HMGb1 triggers migration in CCD18 myofibroblast cells via MEK1/2 and PI3K pathways

The results from previous assays suggested that HT29 cells release HMGb1 in their glucose deprived culture medium (chapter 4). This conditioned medium worked as a strong chemoattractant for myofibroblasts in our migration assays. The results from migration assays suggest that HMGb1 binds to its receptor RAGE and triggers migration in myofibroblasts cells. However, involvement of downstream signalling pathways is not clear. MEK1/2 and PI3K are two kinases that have been implicated in migration of many other cell types including glioblastoma cells, keratinocytes and rat fibroblasts (Jeong and Kim 2004; Mitchell *et al*. 2007; Bassi *et al*. 2008). Therefore, to investigate the involvement of these two kinases in the migration of CCD18 myofibroblast cells would be the next logical step.

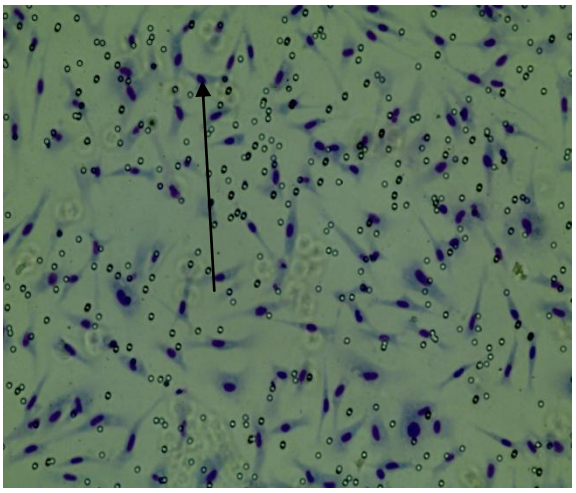
A)



B) Conditioned medium (control)



C) U0126 (MEK1/2 inhibitor)



D) LY294002 (PI3K inhibitor)

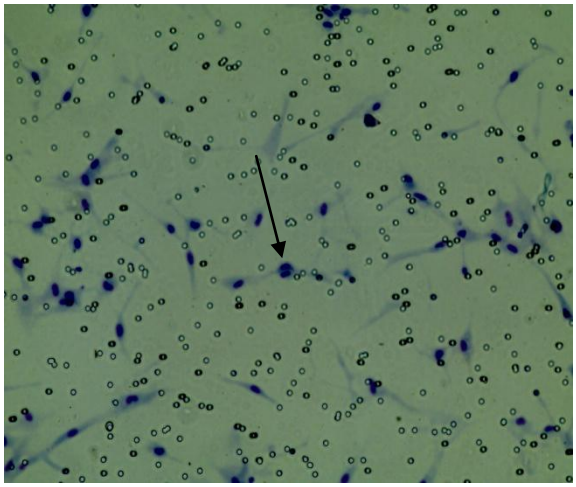


Figure 5.5: *Comparative analysis of CCD18 migration assay in response to the treatment with HT29 conditioned medium and MEK1/2 and PI3K inhibitors in the medium.*

A) Migration assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control (100%). The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars. The conditioned medium with MEK1/2 inhibitor (U0126) and PI3K inhibitor (LY294002) were compared to the control (100%). Representative images captured showing the underside of the membrane after completion of the migration assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Conditioned media without glucose showing cell migration (control=100%), C) migration of cells in response to U0126 MEK1/2 inhibitor and D) LY294002, PI3K inhibitor. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken under x20 magnification.

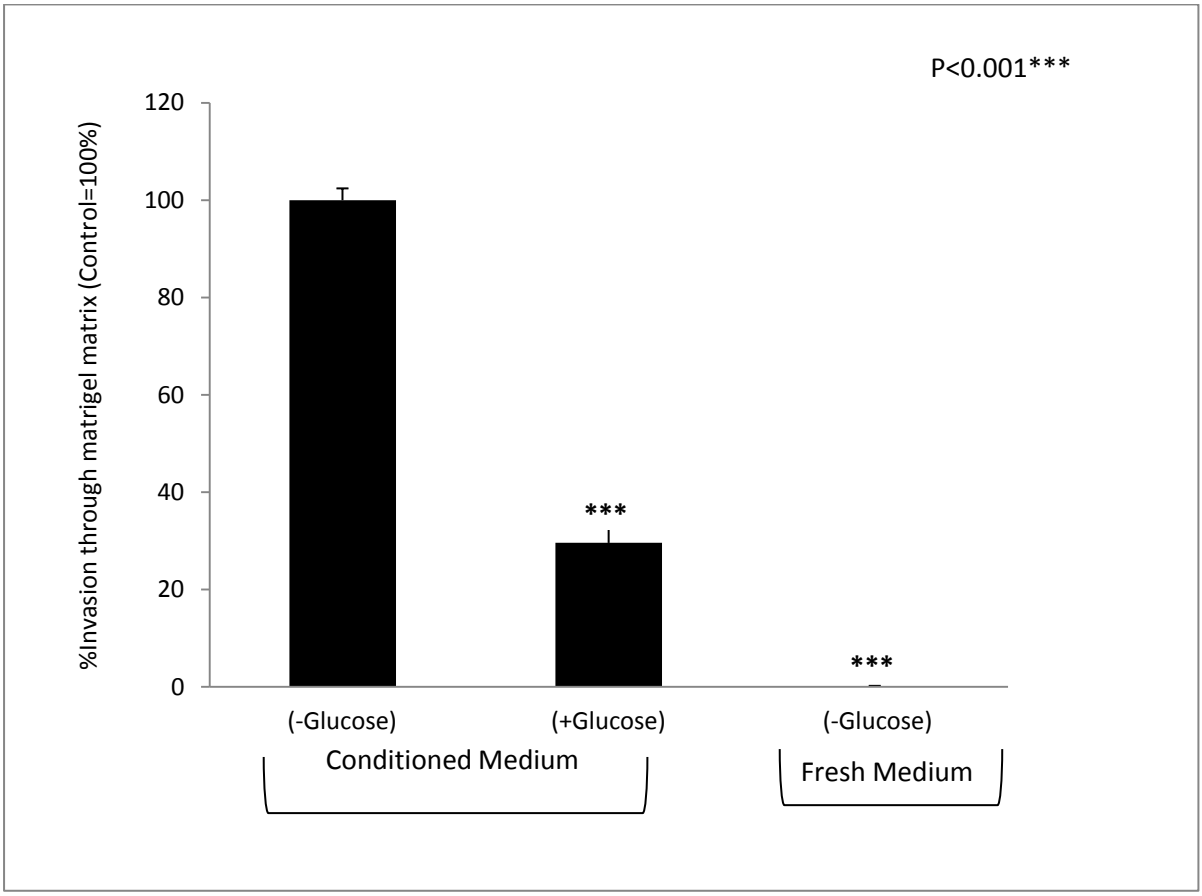
The involvement of MEK1/2 and PI3K was evaluated by comparing myofibroblast migration in conditioned medium without inhibitors (controls) with migration in the presence of inhibitors. The inhibitors U0126, a potent, selective and uncompetitive MEK 1/2 inhibitor was added to the conditioned medium used in the migration assay and LY294002, a selective inhibitor of PI3K was also added to the conditioned medium also used in the migration assay. The results from

the migration assay showed more than 55% reduction in the migration of CCD18 myofibroblast cells when treated with U0126 (50µM/ml) (Figure 5.5 A). However, LY294002 (10µM/ml) appeared to play major role with approximately 75% reduction in the migration of myofibroblasts cells (Figure 5.5 A). Therefore, results from these migration assays suggest that these pathways are activated during HMGb1 triggered migration in CCD18 myofibroblast cells.

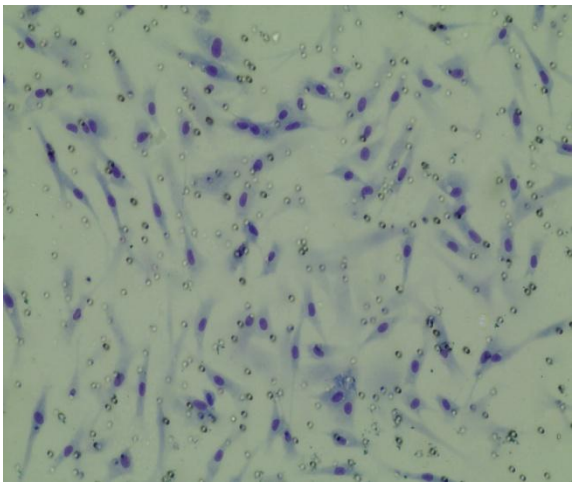
5.4.6 CCD18 myofibroblast cells invade through the matrigel matrix in response to conditioned medium

Myofibroblasts are stromal cells that are also found within tumours and their population also gradually increases in metastatic sites (Morotti *et al*. 2005). Previous migration assays carried out in this study has suggested that HT29 glucose starved conditioned medium containing HMGb1 triggers migration in CCD18 myofibroblast cells. Therefore, it was logical approach to determine whether this HT29 glucose starved conditioned medium can stimulate CCD18 myofibroblast cells to invade through the basement membrane. Thus, we developed Boyden chamber inserts invasion assays to determine whether CCD18 myofibroblast cells can degrade the basement membrane. If this was the case, then this may allow myofibroblasts to enter into the circulation to facilitate the metastasis of tumour cells. These invasion assays were based on a similar principle to the migration assay which involved placing a chemoattractant (conditioned medium without glucose) in the bottom chamber of the inserts. However, the insert membrane was different than the previously used migration kit. These inserts had matrigel matrix coating on top of the 8µm pores which is a well established basement membrane mimic available to test in vitro (Benton *et al*. 2011).

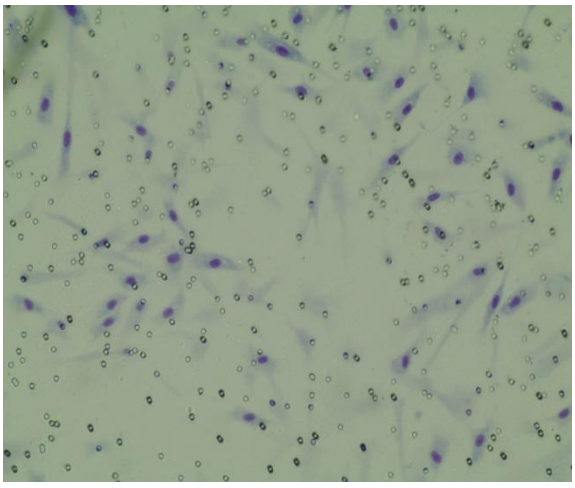
A)



B) Conditioned medium (Control)



C) Conditioned medium (with glucose)



D) Fresh serum free medium (without glucose)

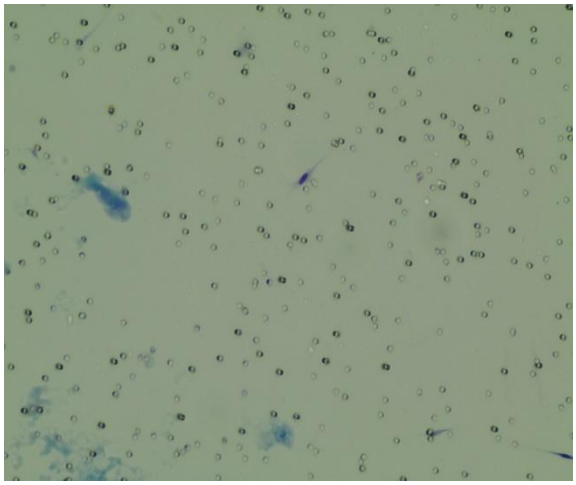


Figure 5.6: *CCD18 myofibroblasts invasion assay in response to the treatment with HT29 conditioned medium with and without glucose and fresh medium without glucose.*

A) *Invasion assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control. The conditioned medium with glucose and fresh serum free medium without glucose were compared to the control (100%). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars. The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert).*

Representative images captured showing the underside of the membrane after completion of the invasion assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Cells invaded in response to conditioned medium without glucose (Control=100%), C) cells invaded in response to the conditioned medium with glucose and D) cells invaded in response to the fresh serum free medium without glucose. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken under x20 magnification.

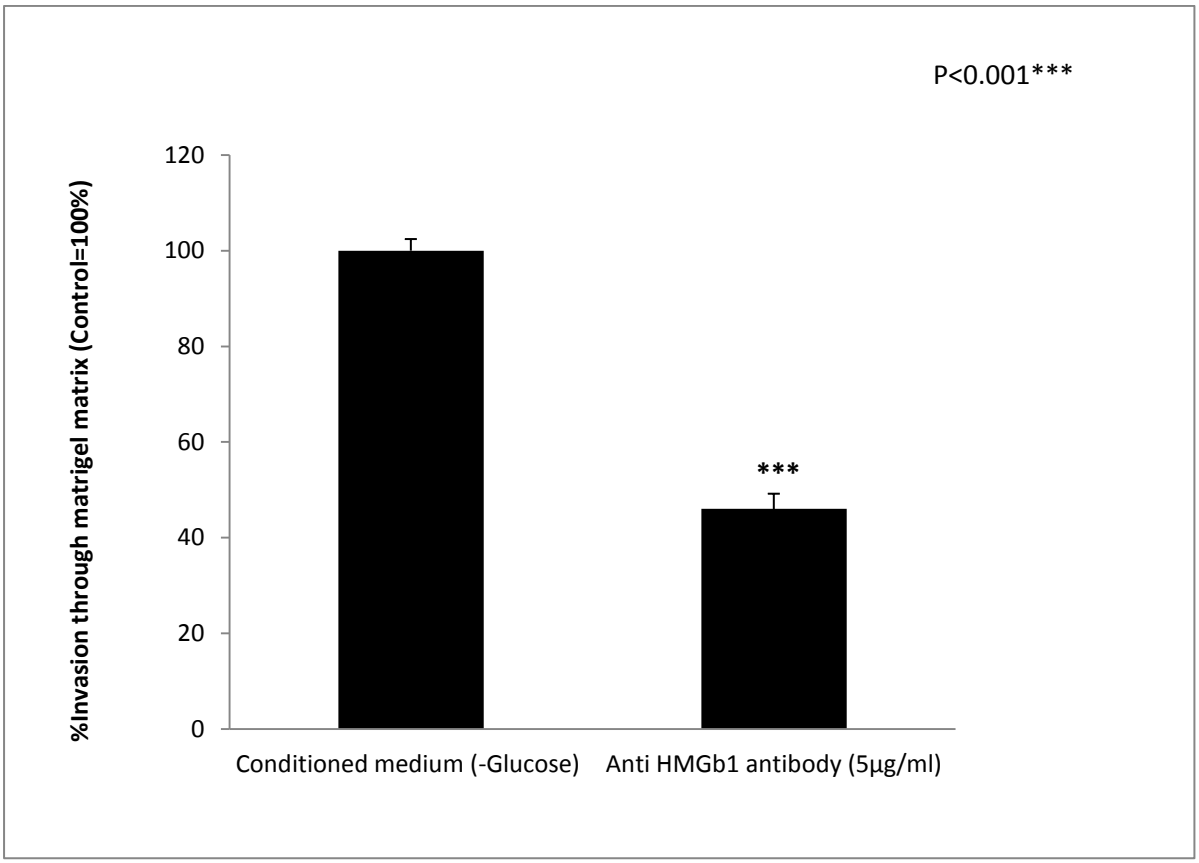
The conditioned medium (without glucose) from HT29 cells served as control (100%). The conditioned medium (with glucose) and fresh serum free medium (without glucose) were compared to the conditioned medium (without glucose). The results suggested that the conditioned medium (with glucose) although able to trigger invasion in myofibroblasts cells with approximately 30% invasion as compared to the conditioned medium (without glucose) at 100% (Figure 5.6 A). In addition, fresh serum medium (without glucose) was investigated against conditioned medium (without glucose). It was observed that cells did not invade through the matrigel membrane when exposed to glucose free fresh serum free medium (Figure 5.6 D). The results suggested that glucose deprivation on its own is not a trigger for migration. However, HT29 cells exposed to glucose deprivation appear to release of certain factors and/or chemokines which trigger myofibroblasts to invade. The results from this invasion assay support the results obtained from migration assays carried out using myofibroblasts cells.

5.4.7 HMGb1 in conditioned medium triggers invasion in CCD18 myofibroblast cells

Glucose deprivation triggers the release of HMGb1 from HT29 cells in normoxia. The chemoattractive properties of HMGb1 have been validated by migration assays carried out in our lab previously where anti-HMGb1 antibodies significantly reduced the migratory potential of myofibroblasts cells (Figure 5.2). The conditioned medium (without glucose) has shown positive results in our invasion assays and therefore investigating the involvement of HMGb1 in glucose free conditioned medium-induced invasion of myofibroblasts was important.

The immunoneutralising anti HMGb1 antibodies (5µg/ml) were added to the conditioned medium (without glucose) in the bottom chambers of the assay and the results compared to the control (conditioned medium without glucose). The results suggested that HMGb1 present in the conditioned medium stimulated CCD18 myofibroblast cells to invade through the matrigel matrix. A significant reduction in the number of cells that had invaded though the matrigel matrix was seen with the addition of the HMGb1 immunoneutralising polyclonal anti-HMGb1 antibodies into the conditioned medium (without glucose) at 22h. This reduction was more than 50% and statistically significant (Figure 5.7A).

A)



B) Conditioned medium (Control)

C) Anti HMGb1 antibody in the medium

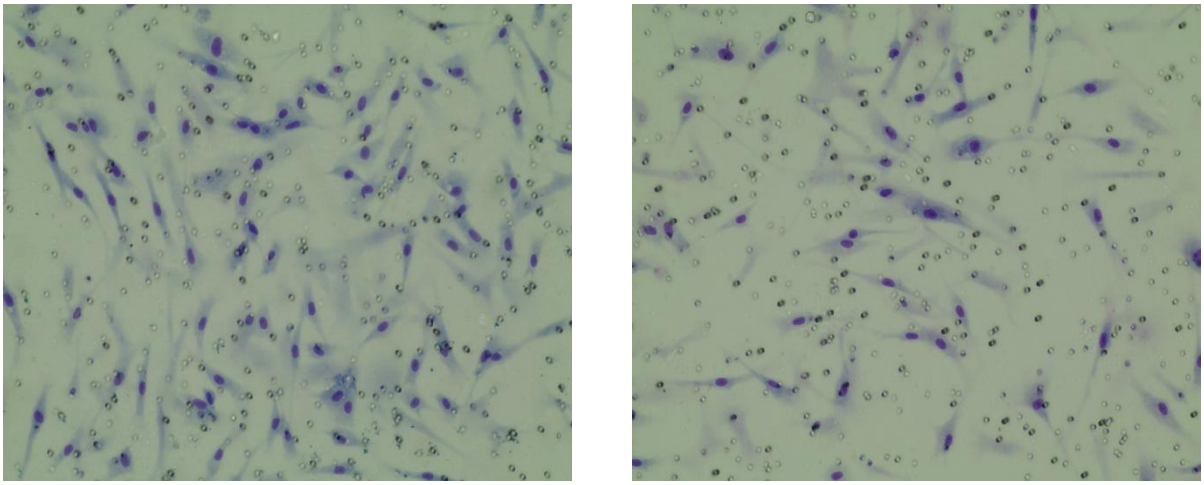


Figure 5.7: *CCD18 myofibroblasts invasion assay in response to HT29 conditioned medium and anti-HMGb1 antibodies in the medium.*

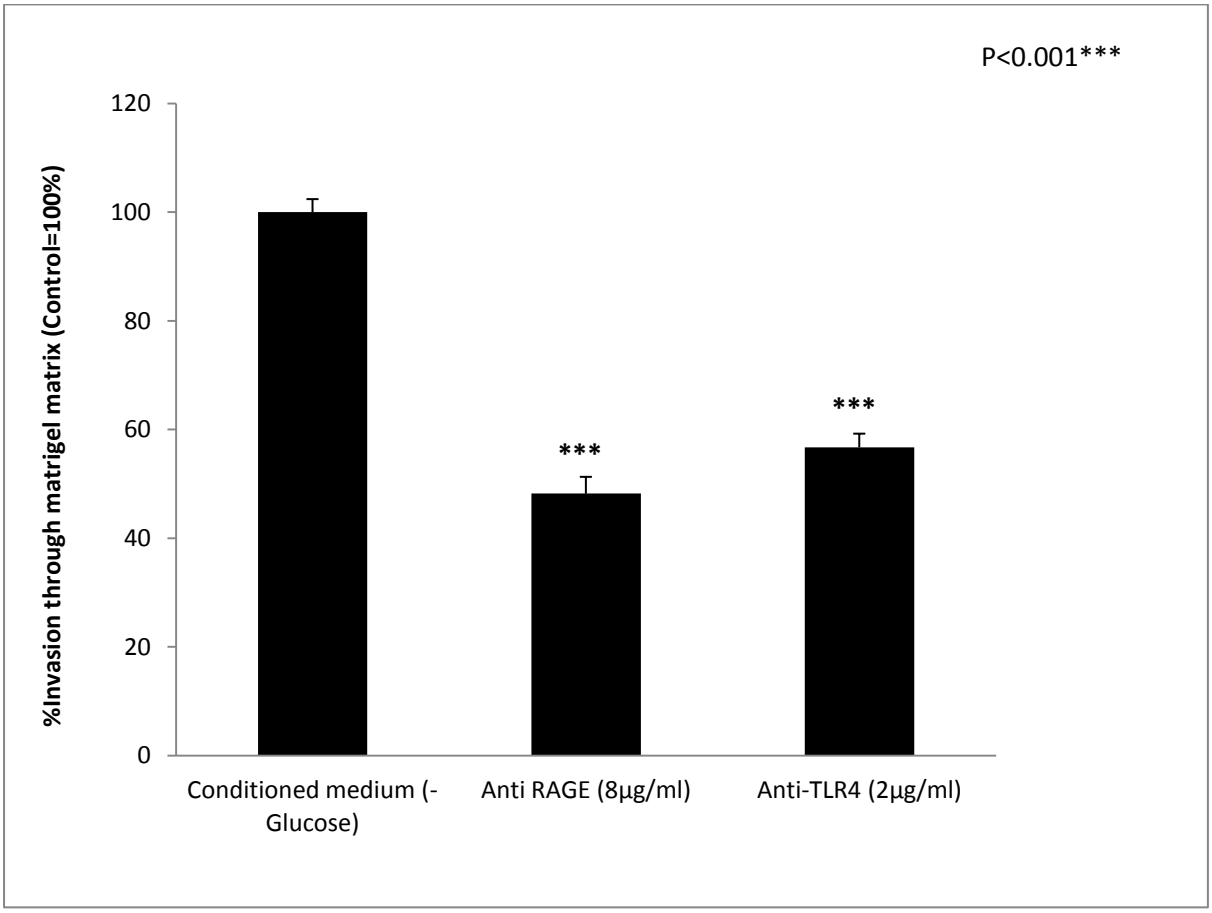
A) *Invasion assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control. The immunoneutralising HMGb1 antibody in the conditioned medium without glucose was compared to the control (100%). The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars. Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars.*

Representative microscopic images captured showing the underside of the membrane after completion of the invasion assay. Cells that had invaded though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Photographs showing number of cells invaded in response to the conditioned medium without glucose and C) HMGb1 antibody in the medium. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken at x20 magnification.

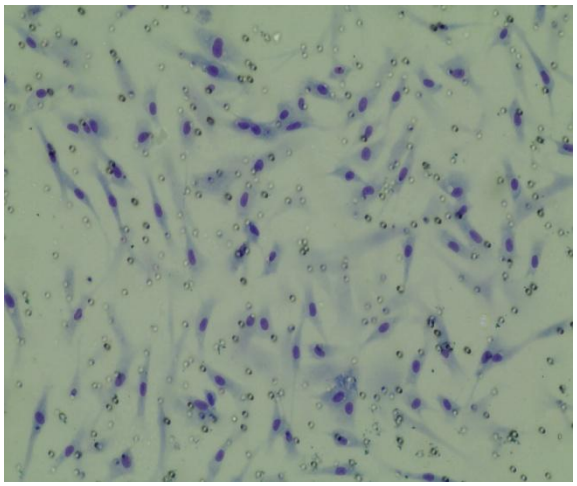
5.4.8 RAGE and TLR-4 facilitate CCD18 myofibroblast cells to invade through matrigel matrix

HMGb1 has been shown to interact with RAGE and TLR-4 and this interaction has been implicated in various malignancies including cancer (Wang *et al*. 2012; Zhang *et al*. 2012; Stoetzer *et al*. 2013; Agalave *et al*. 2014). Our western blot analysis has confirmed the presence of both RAGE and TLR-4 receptors expressed by CCD18 myofibroblast cells. In addition, previous migration assay carried out in our lab suggested that RAGE and TLR-4 are involved in the migration of CCD18 myofibroblast cells (Figure 5.3 and 5.4) therefore it is possible that these two receptors might also be involved invasion. Thus, investigating the involvement of RAGE and TLR-4 in the invasion of myofibroblasts cells would be a logical approach.

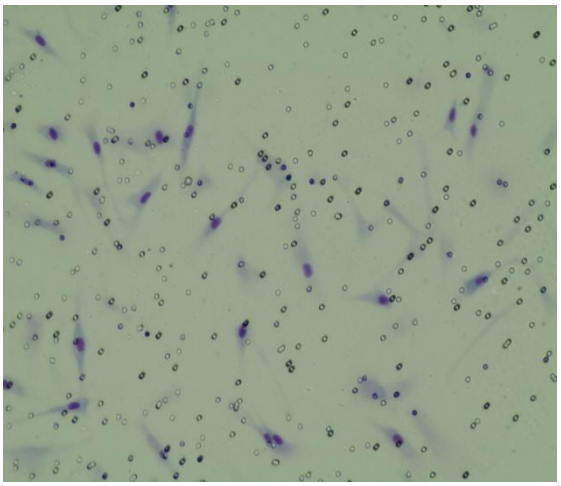
A)



B) Conditioned medium (Control)



C) Anti RAGE antibody in the medium



D) Anti TLR-4 antibody in the medium

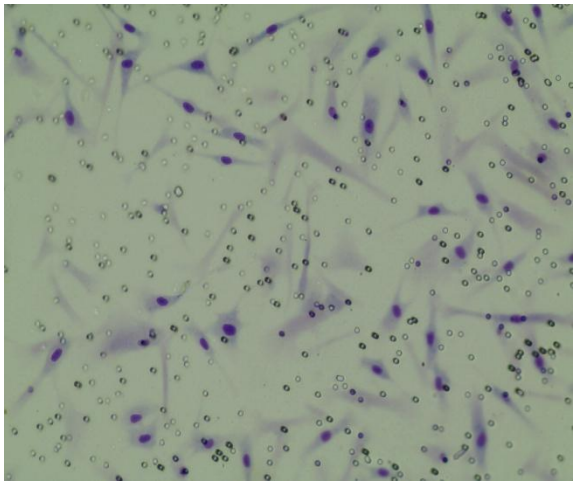


Figure 5.8: *CCD18 myofibroblasts invasion assay in response to the treatment with HT29 conditioned medium and anti-RAGE and anti-TLR-4 antibodies in the medium.*

A) *Invasion assay carried out using HT29 conditioned medium as chemoattractant. The HT29 conditioned medium (-glucose) served as control. The conditioned medium with glucose and RAGE and TLR-4 antibodies in the medium and inserts were compared to the control (100%). The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars. Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars.*

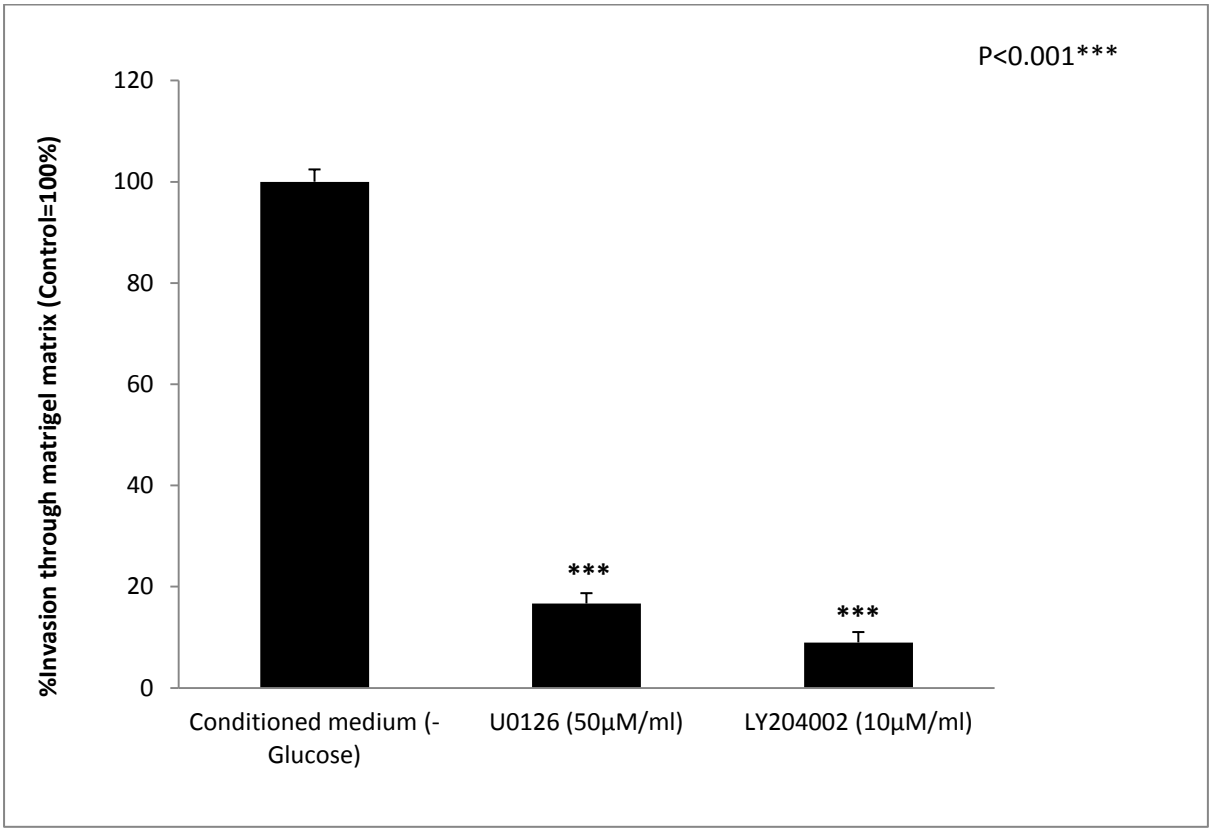
Representative images captured showing the underside of the membrane after completion of the invasion assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Cells invaded in response to the conditioned medium without glucose (Control=100%), C) immunoneutralising RAGE antibody in the conditioned medium without glucose and D) in response to immunoneutralising TLR-4 antibody in the conditioned medium without glucose. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken at x20 magnification.

The invasion assay carried out using immunoneutralising anti RAGE antibodies (8µg/ml) and anti TLR-4 antibodies (2µg/ml). The antibodies were added to conditioned medium in the wells and in CCD18 myofibroblast cell suspension in the inserts. The results after 22h of incubation suggested that RAGE plays an important role in the HMGb1 mediated invasion of myofibroblasts as immunoneutralising anti-RAGE antibodies significantly reduced the number cells invaded through the matrigel matrix. The addition of immunoneutralising anti-TLR-4 antibodies also resulted in significant reduction in number of cells invaded through the matrigel matrix. However, immunoneutralising RAGE appeared to be the most effective with marked reduction in invasion of more than 50% whereas immunoneutralising TLR-4 it was approximately 40% (Figure 5.8 A). These findings are in line with previous findings from the migration assays where inhibition of RAGE gave greater reduction in migration than inhibition of TLR-4 (Figure 5.3 and 5.4)

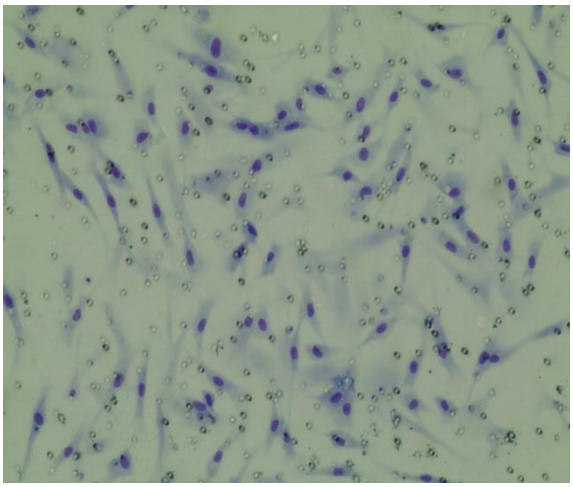
5.4.9 The invasion in CCD18 myofibroblast cells take place via activation of the MEK1/2 and PI3K signalling pathways

The activation of MEK1/2 and PI3K has been implicated in migration of many cells types including cancer cells (Yao *et al*. 2004; Ptak *et al*. 2014; Sobolik *et al*. 2014). Phosphorylation of these kinases activates many downstream signalling pathways including NF-kB. The migration assay carried out in our lab showed that MEK1/2 and PI3K are activated and involved in migration of CCD18 myofibroblast cells and inhibiting these pathways with selective inhibitors significantly reduced migration of myofibroblasts cells. Thus, it is possible that these pathways are involved HMGb1 induced invasion of CCD18 myofibroblast cells.

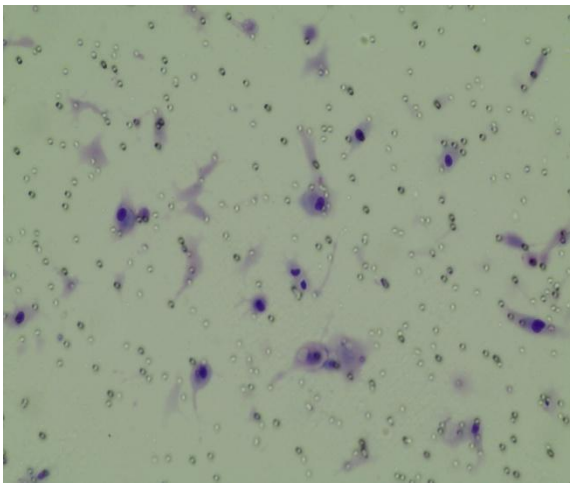
A)



A) Conditioned medium (control)



B) U0126 in medium (top and bottom)



C) LY294002 in medium (top and bottom)

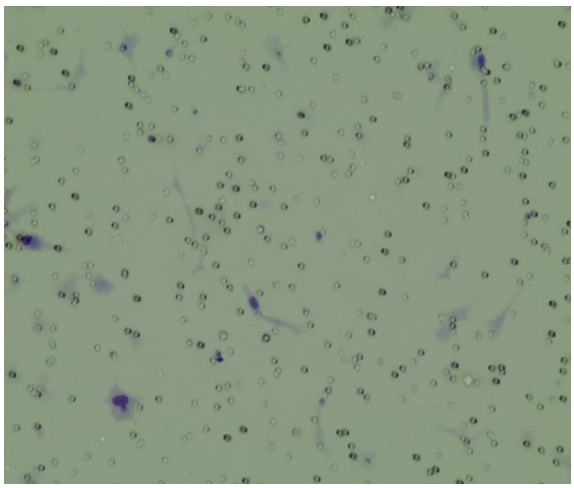


Figure 5.9: A) *Effect of MEK1/2 and PI3K inhibitors on CCD18 myofibroblasts invasion in response to HT29 conditioned medium as chemoattractant.*

The HT29 conditioned medium (-glucose) served as control. The MEK1/2 and PI3K inhibitors in the conditioned medium without glucose and in the inserts were compared to the control (100%). The assay was carried out three times with 2 inserts per condition per occasion (5 random fields of vision counted per insert). Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars. Student's t-test was used to determine statistical significance of the results. SEMs were displayed as error bars.

Representative images captured showing the underside of the membrane after completion of the invasion assay. Cells that had migrated though the 8µm pores of the membrane were stained blue using the ReaStain Quick-Diff staining method. The white dots are 8µm pores through which cells have not migrated. B) Cells invaded in response to the conditioned medium without glucose (Control=100%), C) MEK1/2 inhibitor (U0126) in the conditioned medium without glucose and D) in response to PI3K inhibitor (LY294002) in the conditioned medium without glucose. Cells that had migrated on these and other images were counted and the results were presented in A) of this figure. The photographs were taken under x20 magnification.

The invasion assay was carried out using selective inhibitors of MEK1/2 and PI3K. These inhibitors were added to medium in the bottom chambers and in the CCD18 cell suspension in the upper inserts. The data suggested that inhibiting MEK1/2 by its selective inhibitor U0126

(50µM/ml) significantly reduced the number of cells that invaded through the matrigel matrix. There was approximately an 80% reduction in the number of myofibroblasts invading through compared to the controls (100%). In addition, LY294002 (10µM/ml), a selective inhibitor of PI3K, resulted in a significant reduction (90%) in number of cells invading through the matrigel matrix (Figure 5.9A). The results suggested that both MEK1/2 and PI3K are involved in the invasion of myofibroblasts cells. However, based on the data from migration and invasion assays, it appears that PI3K-AKT pathways play an important role in CCD18 myofibroblast cells migration and invasion. Though, the involvement of MAPK-ERK pathway is also significant.

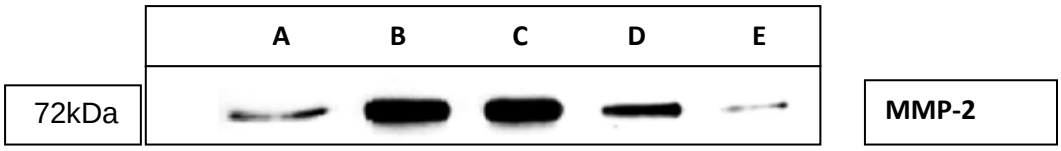
5.4.10 MMP-2 but not MMP-9 is produced and secreted by CCD18 Myofibroblast cells

The other aim was based on the novel findings from the invasion assays. The results from invasion assays suggested that CCD18 myofibroblasts are able invade through the matrigel matrix in response to glucose deprived conditioned medium taken from HT29 cells. Many MMPs including MMP-1,-2,-3,-7,-8, and MMP-9 have been shown to cleave the substrate during the invasion assay *in vitro* (Lutolf *et al*. 2003) and myofibroblasts have been shown to secrete MMP-2 and MMP-9 previously (Lewis *et al*. 2004; Turner *et al*. 2007). Thus we decided to investigate release of MMP-2 and MMP-9 from CCD18 cells.

Here, we investigate the release of MMP-2 and MMP-9 as these forms of MMPs have shown to degrade basement membrane previously (Lee *et al*. 2015; Shen *et al*. 2015). The CCD18 myofibroblast cells were treated with glucose free and glucose containing medium. In addition, the myofibroblasts cells were also treated with same conditions as those of migration assays

i.e., treatment with serum free medium for 24h followed by treatment with HT29 conditioned medium.

A)



B)

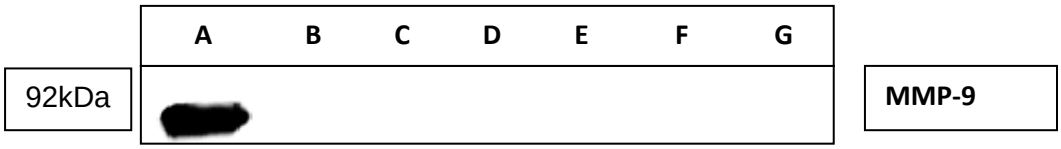


Figure 5.10: (A) *Western blot showing the release of MMP-2 from CCD18 cells. The CCD18 cells were also treated with HT29 conditioned medium (medium containing glucose and glucose free medium collected at 48h) for 22h.*

A= Recombinant MMP-2, B=CCD18 (+Glucose), C=CCD18 (-Glucose, D=CCD18 treatment with HT29 conditioned medium (+Glucose), E=CCD18 treatment with HT29 conditioned medium (–Glucose)

B) *Western blot analysis of the release of MMP-9 from HT29 cells and CCD18 cells. The cells were treated with presence and absence of glucose for 24h. The CCD18 cells were also treated with HT29 conditioned medium for 22h. The protein concentration was equalised in both conditions and compared.*

(+glucose = with glucose*) (-glucose = without glucose*). *A= Recombinant MMP-9, B=HT29 (+ Glucose), C=HT29 (–Glucose), D=CCD18 (+ Glucose), E=CCD18 (–Glucose), F=CCD18 treatment with HT29 conditioned medium (+ Glucose), G=CCD18 treatment with HT29 conditioned medium (–Glucose)*

The results from western blot suggest that CCD18 myofibroblast cells express and release MMP2 but not MMP-9. The protein bands for MMP-2 were detected at 74kDa (predicted molecular weight) resembling the molecular weight of recombinant MMP-2. A polyclonal primary antibody specific to MMP-2 was used to detect MMP-2. The recombinant MMP-2 served as positive control. The protein concentration of the medium from the treated CCD18 myofibroblast cells used in the western blot was equalised using BioRad protein assay. There was no difference observed in protein bands observed in treatment with glucose free fresh serum free medium and with glucose containing serum free medium at 24h. However, with conditions mimicking migration and invasion assay conditions, it appeared that MMP-2 is downregulated in response to glucose free conditioned medium taken from HT29 cells that had previously been cultured for 48h (Figure 5.10A).

Similar assay conditions were implemented to investigate the presence or upregulation or downregulation of MMP-9. The protein concentration of the medium from the CCD-18 cells was again equalised using the BioRad protein assay. A polyclonal primary anti-MMP-9 antibody was used to detect MMP-9 in the conditioned media samples. The results from western blot suggested that MMP-9 is not released from CCD18 cells in any of the conditions investigated i.e., first treatment being the treatment with fresh glucose free or glucose containing treatment for 24h and second treatment with glucose free condition treatment for 24h plus another 22h treatment with HT29 conditioned medium which was obtained at 48h (glucose free and glucose rich). Recombinant MMP-9 was detected at predicted molecular weight of 92kDa (Figure 6B). Therefore, both western blots were validated with positive controls being detected at correct molecular weight. These findings are indicative of possibilities for the involvement of other proteases in facilitating the HMGb1 induced migration and invasion of myofibroblasts.

5.5 Discussion

The tumour stromal cells such as fibroblasts and myofibroblasts play an important role in predicting the outcome of cancer chemotherapy (Yamashita *et al*. 2012). Cancer metastasis is a major hurdle in the treatment of cancer (Davidson *et al*. 1999; Spaderna *et al*. 2006; Li *et al*. 2011). It has been established that tumour stromal cells such as CAFs or myofibroblasts promote tumour proliferation and angiogenesis (Coussens and Werb 2002; Allinen *et al*. 2004). In addition, it has been shown that the myofibroblast population gradually increases with the invasive stages of many cancers (Nakayama *et al*. 1998). The degradation of ECM is one of the major events that occur during the migration of cells from one organ to another.

Myofibroblasts are known to play important roles in the formation and repair of the ECM (Vedrenne *et al*. 2012; Mia *et al*. 2014). The ECM is made up of various matrix proteins including collagen and other glycoproteins and proteoglycans (Robert 2015). Most of these proteins are produced by myofibroblasts and play important roles in epithelial cell migration and differentiation (Hinz and Gabbiani 2010). In addition, myofibroblasts have been shown to secrete MMPs (1-3). These MMPs are major degraders of basement membrane. However, the action of MMPs is inhibited by TIMPs during the ECM remodelling. Thus, a balance between MMPs and TIMPs is important for ECM remodelling in the tissue (Benyon *et al*. 1996)**.**

Most solid tumours are characterised by hypoxic and glucose deprived areas in their central core within the microenvironment (Laderoute *et al*. 2006; Jamieson *et al*. 2015). These

conditions have been shown to trigger the release various growth factors such as VEGF, which then can facilitate angiogenesis and metastasis. The results from our western blot analysis suggest that various cancer cell lines including HT29 (colon adenocarcinoma), MCF-7 (breast cancer), EJ138 (Bladder cancer) and A549 (Lung cancer) cells release HMGb1 under glucose deprivation in normoxia. HMGb1 is an inflammatory cytokine that is released as a danger signal during inflammation (Hreggvidsdottir *et al*. 2009; Lu *et al*. 2014). We have explored myofibroblast proliferation and found that HMGb1 appeared to drive proliferation in CCD18 myofibroblast cells. Other studies have shown that HMGb1 can trigger migration in many other cells including glioblastoma and neutrophils (Bassi *et al*. 2008; Berthelot, *et al*. 2011). However, its role in myofibroblasts migration and invasion had previously not been explored. Therefore, considering the cytokine-like activity of HMGb1, it was important to investigate its role in signalling migration and invasion of myofibroblasts cells.

The HMGb1 activates various signalling pathways such as MAPK-ERK and PI3K-AKT. These pathways play an important role in the proliferation and migration of tumour cells. Supporting evidence includes HMGb1 activated PI3K pathway in neutrophils and colon cancer cells (Kuniyasu *et al*. 2003). In addition, HMGb1 has been shown to induce TLR-4 mediated activation of MyD88-IRAK4-p38 and Myd88-IRAK4-AKT pathways. Also, HMGb1-RAGE complex has been shown to activate ERK1/2 pathways in rat smooth muscle cells (Degryse *et al*. 2001).

The presence of HMGb1 released into the culture medium from HT29 colon adenocarcinoma cells might contribute to advancing our knowledge of the cellular cross talk within tumours in

response to HMGb1 and subsequent impacts within the tumour microenvironment. One main question that has been answered by this work is that HMGb1 triggers proliferation in myofibroblasts and plays an important role in the activation of PI3K and MAPK-ERK pathways. In addition, presence of RAGE and TLR-4 on the membrane of myofibroblasts further indicates a possible interaction between HMGb1 and its receptors RAGE and TLR-4. Therefore, validating the involvement of these proteins by using immunoneutralising antibodies was a logical approach in our migration assays. The migration assays were carried using Boyden chamber inserts with 8µm pores membrane. This system has been used widely by many other researchers *in vitro* (Chen 2005).

The preliminary data from the CCD18 myofibroblast migration assays using the conditioned medium without glucose as a chemoattractant (conditioned by growing HT29 colon adenocarcinoma cells in it for 48h) showed significant migration of the myofibroblast cells during a 20h incubation period (Figure 5.1). Indeed, there was a reasonable chance for the existence of chemoattractants in the culture medium before exposure to the HT29 cells. However, the results from our western blots have confirmed the presence of HMGb1 in the culture medium of HT29 cells but not in the fresh medium (Chapter 4, Figure 4.1). A number of studies have shown the chemoattractant properties of HMGb1 in relation to a number of cells including mesoangioblasts and fibroblasts *in vitro* (Palumbo *et al*. 2007; Schiraldi *et al*. 2012).

Since our results suggested that HMGb1 present in the conditioned medium can trigger migration in myofibroblast cells, a selective immunoneutralising antibody to HMGb1 (5 µg/ml) was used to block HMGb1 in the conditioned medium to back up our findings. The antibody was allowed to interact with HMGb1 present in the conditioned medium for 20h. The results from this

migration assay revealed that HMGb1 present in the conditioned medium triggers migration in myofibroblasts cells. The addition of immunoneutralising antibody to HMGb1 (5µg/ml) to conditioned medium at the bottom chamber significantly reduced the number of myofibroblasts cells migrated through the membrane. This inhibition was approximately 40% when compared to the controls (100%) (Figure 5.2).

The interaction of HMGb1 with RAGE and TLR-4 has been established previously. For example, a recent study showed that HMGb1 is released from necrotic and severely stressed inflammatory cells and bind to TLR-4 and triggers migration in NIH/3T3 fibroblasts to the site of necrosis (Schiraldi *et al*. 2012). However, whilst this was shown in fibroblasts, whether the migration of myofibroblasts is facilitated by HMGb1 interaction with either RAGE or TLR-4 receptor interaction remained questionable. Therefore, it was logical to explore this by using immunoneutralising antibodies to RAGE and TLR-4 to specifically block these receptors.

In addition, HMGb1 antibodies were also used in combination with RAGE and TLR-4 antibodies to investigate synergistic effect. The RAGE and TLR-4 antibodies were added to both chambers in the migration assay (top chamber containing myofibroblast cell suspension and bottom chamber containing conditioned medium). The HMGb1 antibodies were added to medium at the bottom chambers. RAGE and TLR-4, both are multiligand receptors and known to activate various downstream signalling pathways (Palumbo *et al*. 2004). The blockade of RAGE by the use of immunoneutralising antibodies significantly reduced the number of myofibroblast cells that had migrated through the membrane (Figure 5.3). Interestingly, similar results were seen with the blockade of TLR-4 with immunoneutralising antibodies (Figure 5.4). This suggested that both receptors are present on the myofibroblasts cells and take part in signal transduction

necessary for the cells to migrate. In addition, an increased inhibition of migration was seen when both RAGE and HMGb1 were blocked by antibodies (Figure 5.3). This strengthens our evidence for the involvement of RAGE receptor interaction with HMGb1 and that HMGb1 primarily binds to RAGE to signal migration in CCD18 myofibroblast cells. However, blocking TLR-4 and HMGb1 together did not show any better inhibition than blocking TLR-4 alone. This suggests that TLR-4 might take part in signalling pathways in myofibroblasts cells but not primarily with HMGb1. Therefore it is possible that TLR-4 might interact with other proteins in the conditioned medium in addition to HMGb1.

The signalling pathways involved in the migration potential of the myofibroblast cells were also investigated. Our evidence suggests that CCD18 myofibroblast cells migrate in response to HMGb1 found at relatively high levels in the glucose-deprived medium conditioned by use on HT29 colon adenocarcinoma in the migration assay. Two key pathways MAPK-ERK and PI3K-AKT have been implicated widely in the study of migration of a variety of cells including endothelial, glioblastoma and colon cancer cells (Crean *et al*. 2002). Selective inhibitors of these pathways (U126 and LY294002) have been used by many researchers (Hayashi *et al*. 2008; Ghobrial 2009; Kobayashi *et al*. 2009) investigating the roles of MAPK-ERK or PI3K-AKT pathways. In this work, the inhibitors were added to the cell suspension and the medium to block the action of MEK1/2 and PI3K in CCD18 myofibroblast cells.

The results from the migration assays using these inhibitors revealed that both pathways are involved in HMGb1 triggered migration of CCD18 myofibroblast cells (Figure 5.5). However, inhibiting PI3K with LY294002 significantly blocked the migration by 80%, which suggested that migration of CCD18 myofibroblast cells involves PI3K and associated downstream signalling

which may include NF-kB. In addition MAPK-ERK pathway also plays an important role in the migration of CCD18 myofibroblast cells. The inhibition of MEK1/2 by U0126 significantly reduced the number of cells (55%) in our migration assay. Thus, according the results from these migration assays, it is likely that MEK1/2 is activated as a result of the interaction of HMGb1 and RAGE to trigger the migration of CCD18 myofibroblast cells.

Ability to invade plays a central role in metastasis. This occurs by the degradation of basement membrane followed by penetration of cells into the circulation or lymphatic system. The results from this work using western blotting analysis of CCD18 culture medium suggest that CCD18 myofibroblast cells produce and release MMP-2 (Figure 5.10 A). The MMP-2 has been implicated in the degradation of collagen type I, III and IV (Benyon *et al*. 1996).

The results from the invasion assays show that CCD18 myofibroblast cells invade through the matrigel matrix to reach to the bottom part of the membrane (Figure 5.6). This further suggests that CCD18 myofibroblast cells might release some MMPs which degrade the matrigel matrix that mimics the basement membrane in the invasion assay setup. However, treatment of myofibroblast with glucose free conditioned medium obtained from HT29 cells showed that there was a downregulation in the release of MMP-2 by CCD18 myofibroblast cells (Figure 5.10 A). However, there was a downregulation in MMP-2 levels in the culture medium of CCD18 myofibroblast cells when treated with conditioned medium with glucose (conditioned on HT29 colon adenocarcinoma cells) (Figure 5.10 A). In addition, MMP-9 another potential degrader of basement membrane was invested and our western blot results suggested that CCD18 cells do not produce or release MMP-9 when treated with either with or without glucose conditioned medium treatment (Figure 5.10 B). This suggested that the invasion which occurred in response

to the HT29 cell conditioned medium without glucose might upregulate the release of proteases other than MMP-2 or MMP-9, which might play important roles in degradation of basement membrane and facilitate invasion by myofibroblasts cells.

Interestingly, when HT29 cell conditioned medium with glucose was analysed for its chemoattractive properties, it was found that only 30% cells could invade through in comparison to the controls (100%) (HT29 conditioned medium without glucose) (Figure 5.6). This further raised a possibility for the release of additional proteases other than MMP-2 and MMP-9 that might have been released from the colon adenocarcinoma cell line during the glucose starvation. In addition, the results from invasion assays suggested that glucose deprivation on its own does not influence CCD18 cells to invade across the matrigel matrix because no cells invaded across the membrane when fresh medium without glucose was placed at the bottom chamber (Figure 5.6). Therefore, it was concluded that it's not the lack of glucose that is responsible for the invasive properties of CCD18 myofibroblast cells, rather it is the factors that are being released from the colon adenocarcinoma cells in response to glucose deprivation that give the myofibroblasts invasive properties.

Like migration, involvement of HMGb1 present in the conditioned medium of HT29 colon adenocarcinoma was analysed for invasion in myofibroblasts cells. It has been shown previously in chapter 4 that HMGb1 is present in the conditioned medium of HT29 cells especially in the glucose free conditioned medium. It was found that addition of anti-HMGb1 antibodies into the conditioned medium (without glucose) at the bottom chamber significantly reduced the number of cells invading through (Figure 5.7). In addition, RAGE and TLR-4 also appeared to be involved in initiating the signalling cascade that lead the degradation of the

matrigel matrix membrane and invasion of myofibroblast cells. Interestingly, the results from invasion assays were in line with those of migration assays when comparing the involvement of RAGE and TLR-4. The blockade of RAGE resulted in greater inhibition than TLR-4 (Figure 5.8). Though, a combination treatment with anti-HMGb1 antibodies was not investigated but would be logical approach to see if there is any synergistic effect.

The activation of MAPK-ERK and PI3K-AKT pathways have been implicated in the invasion of hepatocellular carcinoma and prostate cancer (Chen *et al*. 2005) The inhibitors of PI3K and MEK1/2 were used to selectively to inhibit both kinases in our invasion assay. The inhibition of both kinases resulted in marked reduction in invasion of CCD18 cells (Figure 5.9). However, PI3K inhibition by LY294002 was more effective than the U0126 like the migration assay. It has been shown that inhibition of PI3K and MAPKs results in the downregulation of MMP-2 and u-PA (urokinase-type plasminogen activator) which might suggest that why inhibiting PI3K and MEK1/2 result in the inhibition of invasion (Chen *et al*. 2005). However, in our invasion setup, glucose free HT29 cell conditioned medium triggered myofibroblast invasion and migration but our western blotting analysis revealed a downregulation of MMP-2 in these conditions. Therefore, it is likely that other MMPs or factors are involved and should be the subject of future work.

Chapter 6

6. Conclusions and future work

6.1 Major Findings

The HMGb1 is a nuclear protein has been shown to take part in the inflammatory process when released from immune cells (Scaffidi *et al*. 2002). In addition, it has recently been shown that the necrotic cells and the cells undergoing autophagy also release HMGb1 in the extracellular environment (Fiuza *et al*. 2003; Thorburn *et al*. 2009). Although, the release of HMGb1 from cancer cells has been shown previously (Ito *et al*. 2007) however, the conditions that promote HMGb1 release were unclear. The data presented in this thesis suggest that HMGb1 is released from HT29 colon adenocarcinoma cells grown under anoxic conditions and also from these cells grown under glucose deprivation particularly in presence of oxygen. In fact, the results from western blots show that glucose deprivation rather than anoxia is a major stimulus which triggers the release of HMGb1 from 3 out of 4 cancer cells tested (HT29 colon adenocarcimona), A549 lung cancer cells and MCF-7 breast cancer cells but not EJ138 bladder cancer cells) (Chapter 4, Figure 4.1 and 4.2). Therefore, it is possible that glucose deprivation induced HMGb1 release from cancer cells could be a common phenomenon in the tumour microenvironment of many tumour types.

In the tumour microenvironment, cancer cells and neighbouring tumour associated cells often undergo harsh ischemic conditions have alternate strategies to try to survive. For example, cancer cells are often exposed to an environment where there is not enough glucose and/or oxygen, especially in the core of tumour and so switch over to aerobic glycolysis (Warburg

effect). With anaerobic glycolysis, the tumour cells rapidly consume glucose and oxygen from the blood supply in order to get energy to survive (Vander Heiden *et al*. 2009). According to the data from this work, it may be likely that most cancer cells may release HMGb1 in within areas of the tumour microenvironment particularly where there is the shortage of glucose.

Glutamine deprivation has previously been shown to induce autophagy in cells (Sakiyama *et al*. 2009). The autophagy could result in translocation of HMGb1 from the nucleus followed by exocytosis in cancer and normal cells (Tang *et al*. 2010). The data presented here suggested that glucose deprivation should not be confused with glutamine deprivation that may be responsible for HMGb1 release from cancer cells via autophagy. The glutamine deprivation was also compared with glucose deprivation of the HT29 adenocarcinoma cell line and it was found that glucose alone serves as stimulating factor for HMGb1 release not glutamine. There was no evidence that glutamine deprivation resulted in additional HMGb1 being released into the medium when compared to normal glutamine and glucose conditions (control).

The HMGb1 has previously been shown to trigger proliferation in many cells including T lymphocytes, mesoangioblasts and smooth muscle cells (Palumbo *et al*. 2004; Porto *et al*. 2006; Sundberg *et al*. 2009). However, the role of HMGb1 interaction with myofibroblasts, a type of major mucosal but also tumour stromal cell is still unclear. The results from the proliferation assays suggest that recombinant HMGb1 triggers proliferation in myofibroblasts in a dose spectrum of 0.1 to 50ng/ml (Chapter 3, Figure 3.1B). Two major signalling pathways PI3K-AKT and MAPK were investigated for their role in HMGb1 induced proliferation in myofibroblasts. Both pathways have previously been reported to play active roles in HMGb1 induced proliferation and migration in glioblastoma and mesoangioblast cells (Feng *et al*. 2014). Thus, it was important to investigate the involvement of these pathways in HMGb1 induced

proliferation of myofibroblasts. The Inhibition of both pathways by selective inhibitors significantly reduced HMGb1 induced proliferation in myofibroblasts (Chapter 3, Figure 3.2 A and 3.2 B). This suggested that PI3K and MAPK both play important roles in the proliferation of myofibroblasts. Therefore, the data presented here in this thesis suggests that HMGb1 triggers proliferation in myofibroblasts via PI3K-AKT and MAPK-ERK pathways.

The results from western blot suggested that HMGb1 is present in the conditioned medium taken from HT29 colon adenocarcinoma cells after 48hrs. Therefore, HMGb1 containing conditioned media was chosen for use to investigate the effect of HMGb1 on myofibroblasts migration and invasion. After all, in the actual colon tumour environment, one would expect tumours to release factors (including HMGb1), that influence the behaviour of tumour-associated cells such as myofibroblasts. The HMGb1 released into the HT29 cell conditioned (glucose free) media triggered a chemotactic response in myofibroblasts in our transwell membrane migration assay setup (Chapter 5, Figure 5.2). The direct involvement of HMGb1 was demonstrated during migration assay by using immunoneutralising antibody against HMGb1. Neutralising the effect of HMGb1 by antibody significantly reduced migration response of myofibroblasts cells to the HT29 glucose free conditioned media (Chapter 5, Figure 5.2).

Once the involvement of HMGb1 in migration of myofibroblasts was validated, it was important to investigate which HMGb1 associated receptors that are being activated during the migration response in myofibroblasts. Thus, two well-known receptors, RAGE and TLR-4, were investigated for involvement in HMGb1 induced migration response in myofibroblasts. The presence of both receptors on myofibroblasts cell membrane was confirmed by western blotting (Chapter 4, Figure 4.4 and 4.5). The immunoneutralising antibodies against RAGE and TLR-4

were added to the myofibroblasts cells suspension in an attempt to inhibit the activation of both receptors present on the cell membrane of myofibroblasts. The blockade of RAGE or TLR-4 receptors individually by immunoneutralising antibodies significantly reduced migration in myofibroblasts cells in response to HT29 glucose free conditioned media (Chapter 5, Figure 5.3 and 5.4). In addition, combined inhibition of HMGb1 with RAGE showed some additive inhibition of migration of myofibroblasts cells (Chapter 5, Figure 5.3). However, no additive effect was seen with combined inhibition of HMGb1 and TLR-4 using immunoneutralising antibodies (Chapter 5, Figure 5.4). This further suggested the preferred route of HMGb1 induced migration of myofibroblasts may act via the activation of HMGb1-RAGE complex and subsequent downstream signalling leading to migration of myofibroblasts cells.

Indeed, PI3K and MAPK-ERK pathways are most widely studied pathways for migration in many cell types. The use of selective inhibitors of these pathways validated the involvement of these pathways in our migration assays. Blocking these pathways with selective inhibitors significantly reduced HMGb1 induced migration of myofibroblasts cells (Chapter 5, Figure 5.5). However, the inhibitor for PI3K-AKT pathways appeared to more greatly inhibit myofibroblasts migration when compared to the inhibitor for MEK1/2 pathways at non-toxic doses. Thus, based on this finding, it appeared that PI3K-AKT might be the main pathway involved in HMGb1 induced migration of myofibroblasts. However, whilst to a lesser extent, the inhibitor of MEK1/2 significantly reduced migration in myofibroblasts suggesting MEK1/2 is also an important signalling pathway in HMGb1 induced myofibroblasts migration.

Invasion assays were also carried out to investigate the invasive behaviour of myofibroblasts cells and the role of HMGb1 in promoting invasiveness of myofibroblasts. The results from

invasion assays suggested that HMGb1 present in the conditioned medium of HT29 cells triggers myofibroblasts cells to invade (Chapter 5, Figure 5.7). It was logical to investigate the role of the receptors that were reported to be involved in our migration assay. The resulting data suggested that HMGb1 mediated invasion of myofibroblasts cells again involved the activation of RAGE and TLR-4. The involvement of RAGE and TLR-4 was validated by immunoneutralising antibodies against RAGE and TLR-4 which significantly inhibited invasion of myofibroblasts cells through ECM mimicking matrigel membrane. In addition both pathways PI3K-AKT and MAPK-ERK which showed positive results in our migration assay were also investigated. Inhibiting both pathways by using selective inhibitors in the cell suspension significantly reduced HMGb1 induced invasion in myofibroblasts cells (Chapter 5, Figure 5.9). Thus, based on the findings, it appeared that HMGb1 mediated invasion take place via both the PI3K-AKT and MAPK-ERK pathways.

The results from the invasion assays suggest that myofibroblasts can invade through the basement membrane. To achieve that, basement membrane must be digested by proteases released from the cells that are responsible for ECM remodelling to facilitate the cells to move through the matrigel membrane. Hence, it was empirical to look for most prominent MMPs that might be released from myofibroblasts following the HMGb1 containing HT29 glucose free conditioned media treatment. Our western blot analysis suggested that MMP-2 is being released in the culture medium of myofibroblasts cells following the treatment with normal glucose containing conditioned medium from HT29 cells (Chapter 5, Figure 5.10). However, the levels of MMP-2 appeared to be reduced when myofibroblasts were treated with low glucose conditioned medium from HT29 cells (Chapter 5, Figure 5.10 A); a medium that we have previously shown to have strong chemo-attractive properties for myofibroblasts. In addition, MMP-9 another major degrader of basement membrane was also investigated. Our western

blot data suggested that MMP-9 is not being released from myofibroblasts following the treatment with glucose free or normal glucose conditioned medium of HT29 cells (Chapter 5, Figure 5.10 B). This further suggests that myofibroblasts are secreting MMPs other than MMP-2 and MMP-9 or other proteases that are actively taking part in the basement degradation and facilitating invasion of myofibroblasts cells.

On the basis of this work, we postulate that HMGb1 is released from the areas of low or no glucose rather than hypoxia in cancer cells. This areas typically found in the central core of most solid tumours which is also acidic (Schlappack *et al*. 1991; Rajendran *et al*. 2004). Once released from these areas, HMGb1 can reach to the myofibroblasts (may be due to interstitial fluid pressure) which are present in the stroma of most solid tumours. HMGb1 then can stimulate myofibroblasts to proliferate on site. The stromal myofibroblasts has been shown to release proteases to degrade basement membrane and have been related to poor prognosis in breast cancer (De Wever *et al*. 2008; Yamashita *et al*. 2012). Once the ECM has been digested, myofibroblasts along with other cells can make their way out of the tumour to the distant sites (Nielsen *et al.* 1996; Malik *et al.* 2015). The data presented in this thesis suggest that increasing number of myofibroblasts after getting signal from HMGb1 would likely to remodel basement membrane rapidly which would have then facilitate the migration of other cells too. In addition, levels of glucose in myofibroblastic tumours can become predictive tools for measuring the release of HMGb1 and its effects on the spreading of tumours. Increasing population of myofibroblasts on the tumour periphery will increase the tumour mass. It is also likely that larger tumour mass would have more glucose deprived areas and hence more HMGb1 being released. This HMGb1 then can further influence myofibroblasts and other cancer cells to grow, migrate and invade to the distant sites. Although, there is a clear need to identify other factors that may influence myofibroblasts and cancer cells migration and invasion

but the importance of HMGb1 and its crucial role in proliferation, migration and invasion of myofibroblasts can not be neglected.

6.2 Conceptual Advances

The tumour stroma in many solid tumour including colon cancer and breast cancer comprises a major part of the tumour mass (Peña *et al*. 2013). The myofibroblasts are predominant cell types in most carcinomas found on the periphery of most solid tumours (Desmoulière *et al*. 2004; Tripathi *et al*. 2012). The myofibroblasts have been shown to take part in tumour proliferation by secreting a number of growth factors including IGF-1, IGF-II and HGF (Hinz *et al*. 2007). These growth factors may play important roles in initiating certain pathways which are necessary for tumour cell survival and proliferation (Vanamala *et al*. 2010). However, contrary to this, the role of cancer cells in proliferation of myofibroblasts has not been explored, especially in the microenvironmental stress conditions.

The microenvironment of a tumour represents a number of conditions which may be unique to certain types of tumour such as hypoxic tumours. The hypoxic tumour may be characterised by a specific microenvironmental condition of glucose and oxygen deprivation in some parts within the tumour mass (Rajendran *et al*. 2004). In addition, the hypoxic tumours have been thought to be more aggressive in nature due to the adaptation of alternate survival strategy and are more prone to migrate and invade to the distant organs (Joseph *et al*. 2015). Although glucose deprivation has been considered as major hallmark of solid tumour microenvironment, yet this area remained unexplored with no major developments having been reported in the last decade

(Schlappack *et al*. 1991; Jang and Hill 1997). It has been established that hypoxic tumours are more likely to spread to distant sites because of the adaptation to adverse conditions and altered survival strategy (Jögi 2015) However, little is known about glucose deprivation related upregulation or downregulation of certain factors including ligands, receptors and proteases which may play important roles in aiding tumour survival, growth and tumour spread or metastasis in these conditions.

The data presented in this thesis shows that HMGb1, which is known to have role in bending of DNA in the nucleus of cells, is released from cancer cells that are deprived of glucose. The results of this work also yield evidence that HMGb1, a non-histone nuclear protein, is actively secreted by cancer cells that are not undergoing necrosis however may be under the state of reversible quiescence. This secreted HMGb1 may reach myofibroblasts present within the tumour stroma and attract myofibroblasts from elsewhere. Previous work has shown that necrotic cells are able to release HMGb1 in the extracellular environment (Vogel *et al*. 2015). Also, the cytoplasmic HMGb1 has been reported to regulate autophagy and may promote cancer cell survival (Zhang *et al*. 2015). The proliferative properties of HMGb1 has been explored recently and it has been shown that HMGb1 once released from damaged cells triggers proliferation in gingival fibroblasts (Chitanuwat *et al*. 2013). However, the stimulus for the release of HMGb1 from tumour cells and the interaction between HMGb1 and myofibroblasts has not previously been explored. The results from this work suggest that recombinant HMGb1 at 10ng/ml significantly stimulates proliferation, migration and invasion in myofibroblasts (Chapter 3, Figure 3.1, 3.2 and 3.3).

The tumour microenvironmental stress plays an important role in the outcome to chemotherapy. Within this microenvironment, it is likely that most solid tumours may have interstitial fluid pressure building up within which helps cell debris and some live cells to come out from internal core (Mori *et al*. 2015). It has been established that the central core of tumours is often hypoxic, acidic and may lack nutrients to feed the cells within (Sun *et al*. 2015). As a result, few cells may die but a few can become adaptive to the microenvironmental stress and continue to grow via altered route for survival (HIF pathway triggered by hypoxia and other physiological adaptations). Thus, one of the reasons for treatment failure while treating such tumours may be linked to interstitial fluid pressure led transfer of cells from the core to the periphery resulting in the expansion of tumour mass. Therefore, there is a strong possibility that interstitial fluid pressure inside the tumours may facilitate the transfer of HMGb1 towards the myofibroblasts from the glucose starving regions of solid tumour (Discussed in Chapter 1 – The interstitial fluid pressure). Once HMGb1 has reached to the myofibroblasts, it may trigger proliferation in myofibroblasts which then can result in further expansion of tumour mass.

Previously, it has been shown that the cells undergoing autophagy or necrosis release HMGb1 in the microenvironment (Dong *et al*. 2007; Ito *et al*. 2007; Thorburn *et al*. 2009; Beyer *et al*. 2012). Autophagy is induced by starvation, which may be caused by a lack of glucose, glutamine, pyruvate, oxygen and serum (Janji *et al*. 2013). However, it is not clear that which of these factors trigger the release of HMGb1 in microenvironment. The data presented in this thesis suggests that glucose deprivation is the trigger for the release of HMGb1 from HT29 cells in normoxic conditions. However, it is also released from anoxic cells. These cells (anoxic cells) represent typical oxidative stress conditions and therefore could undergo autophagy in order to fuel themselves. Surprisingly, the amount of HMGb1 released under glucose deprivation in normoxic conditions was much higher than any other conditions such as hypoxic or anoxic

conditions. In addition, cells exposed to low glucose but not anoxia were able to proliferate when treated with normal conditions i.e. with culture medium containing serum, glutamine and glucose after 48h. Therefore, this release of HMGb1 in glucose deprived normoxic conditions in our experimental design was an active release instead of passive released caused by necrosis.

The tumour metabolism is fundamental to cancer cell survival, growth and behaviour. It has been shown that tumour cells have enhanced demand for nutrients to provide energy to sustain their proliferative status (Brahimi-Horn and Pouysségur 2006). Increased glutamine intake is one of the key traits that has been reported in wide range of cancers (Márquez *et al*. 2015). In addition, glutamine deprivation has been considered as a potential inducer of autophagy and therefore may facilitate the release of HMGb1 out of the cells even though when cell membrane is intact (Thorburn *et al*. 2009; Zhang *et al*. 2015). Thus, the effect of glutamine deprivation was also investigated in this work. The data suggests that HMGb1 is released from glutamine-deprived conditions in normoxia (Chapter 4, Figure 4.3 A). However, when this release was compared to the release of HMGb1 in glucose deprivation conditions, a negligible amount of HMGb1 was detected in glutamine deprivation conditions (Chapter 4, Figure 4.3 B). This further suggests that the release of HMGb1 in glucose starving conditions may not be autophagic. The next question about this release could be if the cells were undergoing necrosis because HMGb1 has been reported to be released by necrotic cells previously (Scaffidi *et al*. 2002). In our experimental setup, the cancer cells investigated were not dead under glucose deprivation and were able to grow again when treated with fresh medium supplied with appropriate nutrients. Therefore, this release was suggested to be independent of necrosis and possibly involving a novel release mechanism. However, the underlying mechanism for this glucose dependent release of HMGb1 from cancer cells is not clear. Though, it is possible that low glucose may actively stimulate the release of HMGb1 demonstrates the likelihood that intracellular signalling

pathways maybe involved, which may facilitate the release of HMGb1 from the nucleus to the cytosol followed by translocation into the extracellular environment of the cells.

HMGb1 release has been linked with a number of cells including apoptotic jurkat cells (human T cell leukaemia) and U937 cells (human promonocytic) previously (Bell *et al*. 2006; Liu *et al*. 2011). However, release under glucose depravation has not been reported previously. It was important to check whether this release of HMGb1 was not cancer cell type specific, few other cancer cell lines (MCF-7 (breast cancer), A549 (lung cancer) and EJ138 (bladder cancer) were examined for their response to glucose depravation and compared to the response in the HT29 (colon adenocarcinoma) cell line. It was found that out of the four cell lines, three cell lines (HT29, MCF-7 and A549) followed the same pattern for HMGb1 release in response to low glucose. Although EJ138 cells did not appear to follow the same pattern, with increased HMGb1 release under glucose depravation, but showed an equal amount of HMGb1 was being released under normal and glucose deprived conditions (Chapter 4, Figure 4.2). With three out of the four cell lines investigated showing an increase in the HMGb1 release in response to low glucose, suggested that majority of cancer cells might release HMGb1 under glucose deprivation. Therefore, based on the results presented in this thesis, it is also likely that a large population of cancer cells will exhibit elevated levels of HMGb1 released in the tumour microenvironment. In addition, the stromal cells that are present on the periphery of tumour are potential targets for HMGb1. The data presented in this study suggest that HMGb1 can stimulate these stromal myofibroblasts, which might in turn facilitate tumour spread, invasion and metastasis.

The data from migration and invasion assays suggest that HMGb1 present in the glucose free culture medium of used on HT29 cells and collected at 48h triggers level of migration and invasion in myofibroblasts cells (Chapter 5, Figure 5.1 and 5.6). The presence of HMGb1 in the culture medium was supported by western blot data (Chapter 4, Figure 4.1). HMGb1 has previously been observed in culture medium of other cell types, macrophages and monocytes. However, this release was not due any microenvironmental stress conditions (Tang *et al*. 2007). The involvement of HMGb1 in migration and invasion of myofibroblasts was validated by immunoneutralising antibodies against HMGb1 (Chapter 5, Figure 5.2 and 5.7). The addition of immunoneutralising antibodies significantly reduced the migration of myofibroblasts cells, suggesting a key role of HMGb1 in stimulating myofibroblasts to migrate. Indeed, there is a potential chance that other factors are in the glucose and serum free culture medium taken from HT29 cells that could promote migration of myofibroblasts cells. However, the data from western blot confirmed that HMGb1 is present and at relatively high levels in the used glucose free medium than any other microenvironmental stress condition investigated. In addition, proliferation, migration and invasion are also relatively high in myofibroblasts exposed to this glucose free used medium. Furthermore, the fact that myofibroblast proliferation, migration and invasion were significantly reduced when HMGb1 immunoneutralising antibodies were used gives strong evidence that it is the HMGb1 that is interacting with the myofibroblasts.

The stimulation of myofibroblasts following the treatment with conditioned medium containing HMGb1 must involve activation of various receptors for HMGb1. Thus, known receptors (RAGE and TLR-4) for HMGb1 were investigated for their involvement. The western blot data indicates that myofibroblasts express both RAGE and TLR-4 (Chapter 4, Figure 4.4. and 4.5). The activation of TLR-4 has previously been shown to trigger migration in vascular smooth muscle cells (Yang *et al*. 2012). However, the activation of TLR-4 by HMGb1 has also been shown to

inhibit migration of enterocytes and endothelial cells (Bauer *et al*. 2013; Dai *et al*. 2010); Whereas, activation of RAGE by HMGb1 has been shown to modulate migratory properties in dendritic cells (DCs) and myoblasts (Dumitriu *et al*. 2007; Riuzzi, *et al*. 2006). Thus, it appears that the role of HMGb1 and its interaction with receptors might be cell-type specific. Here, in our migration assay setup, combined blockade of RAGE and HMGb1 resulted in additive inhibition in migration of CCD18 myofibroblasts (Chapter 5, Figure 5.4). This suggested that HMGb1 binds to RAGE which then may activate some downstream signalling pathways. However, combined blockade of TLR-4 and HMGb1 did not show any additive inhibitory effect on the migration of myofibroblasts (Chapter 5, Figure 5.5).

Though, it is possible that the effect of the antibodies used to block each of these pathways might have different efficiencies to each other or at completely different concentrations from each other. However, the concentration used for antibodies was enough to block more than 50% of the target protein according to the manufacturer data sheet (BioRad Laboratories UK). Therefore, the concentration of antibodies used was sufficient to block the target protein by at least 50%. The data suggests that HMGb1 binds to RAGE to trigger migration. However, it is also possible that some HMGb1 may also bind to TLR-4 and activate some downstream signalling. Another possibility behind signalling cascade following the interaction of HMGb1 with TLR-4 could be the involvement of TIRAP and Myd88 which are adaptor proteins for TLR-4. These downstream signalling molecules have been shown to play important roles in migration and invasion following the interaction with HMGb1 activated RAGE previously (Sakaguchi *et al*. 2011). Therefore, it is possible that both adaptor proteins may bind to HMGb1 activated RAGE and transduce signals to downstream molecules such as PI3K and MEK1/2.

The HMGb1 has been shown to interact with many receptors including RAGE and TLR-4 (Apetoh *et al*. 2007). The interaction of HMGb1 with TLR-4 has been implicated in proliferation of hepatic stellate cells (HSC) via PI3K-AKT pathway (Wang *et al*. 2013). Indeed, HMGb1 induced proliferation in myofibroblasts must involve receptor-ligand interaction. Therefore, future work should be to further investigate ligand-receptor interaction using the ligand-receptor binding assays (BiaCore) and co-immunoprecipitation followed by selective purification using affinity chromatography. This approach would further validate the activation and involvement of the HMGb1-RAGE complex. In addition, the potential involvement of other receptors such as CXCR4 (chemokine receptor 4) would be interesting because the role of CXCR4 in the recruitment of inflammatory cells has been explored recently where HMGb1 appeared to form a complex with CXCL12 (chemokine ligand 12) and triggered signalling via the activation of CXCR4 (Schiraldi *et al*. 2012). In addition, chemokine receptor CCR7 has been shown to play an important role in migration of DCs following the release of HMGb1 (Saïdi *et al*. 2008). Therefore, it is possible that both receptors (CCR7 and CXCR4) could play individual or combined roles in HMGb1 induced migration and invasion of myofibroblasts cells alongside of RAGE. Based on the data presented in this thesis, we propose a possible mechanism for tumour spread involving proliferation, migration and invasion of myofibroblasts (Figure 6.1)

Phosphorylation of ERK1/2 has been reported in HMGb1 induced migration of 3T3 fibroblasts and mesoangioblasts (Palumbo *et al*. 2007). The migration and invasion reported in this thesis also validated the involvement of PI3K and MAPK-ERK pathways. The role of proteases especially metalloproteases in degradation of basement membrane to facilitate migration was first established in 1983 (Kalebic *et al*. 1983). Therefore, Two major degraders (MMP-2 and MMP-9) of basement membrane were investigated and found not be involved in HMGb1 induced myofibroblasts migration and invasion. Recently, both proteases (MMP-2 and MMP-9)

have been shown to play important roles in promoting migration and invasion of prostate cancer cells (Ding *et al*. 2015). However, they do not play a significant role in HMGb1 induced migration and invasion of myofibroblasts in response to HMGb1 stimulation according to our western blot results (Chapter 5, Figure 5.10). Therefore, the next logical step for future work would be further investigation of all other potential proteases in the culture medium of myofibroblasts which have been treated with glucose free conditioned medium of HT29 cells collected at 48h.

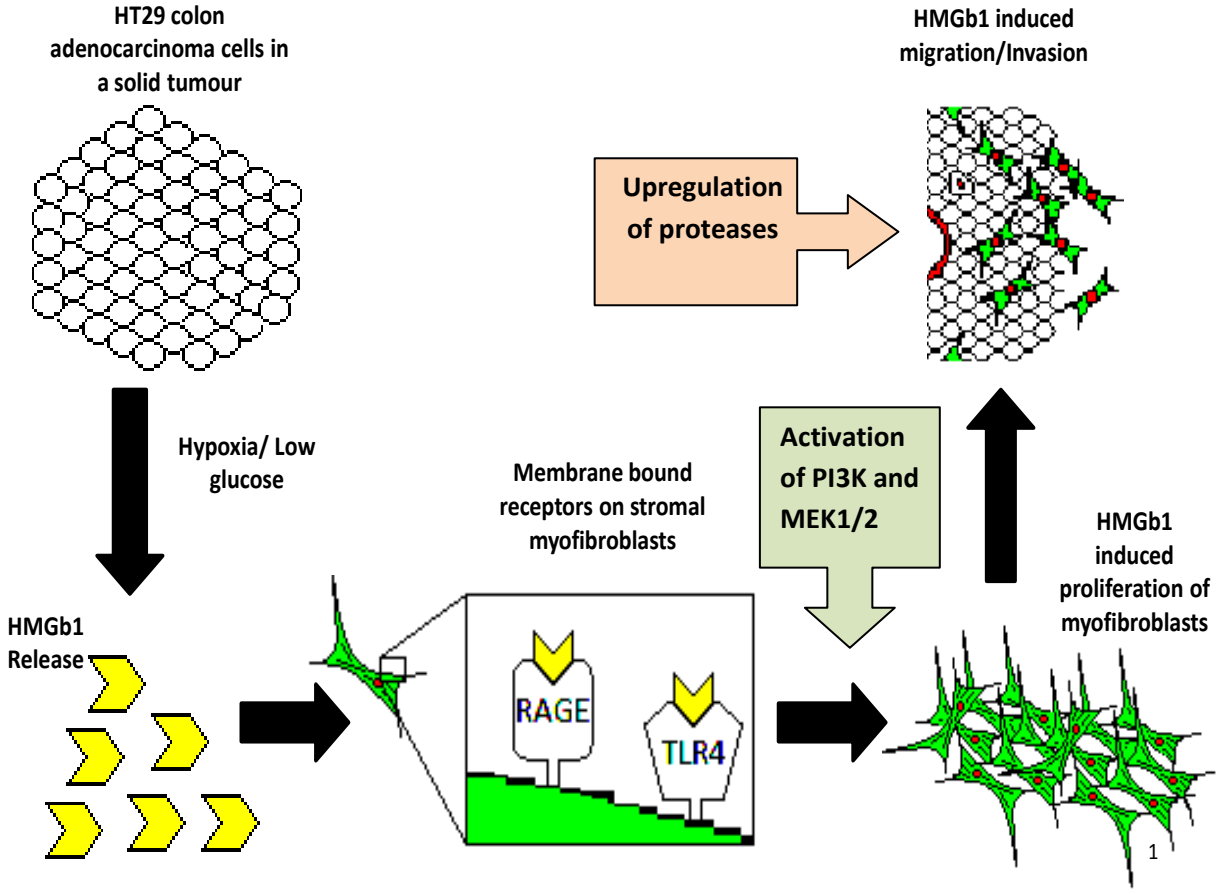


Figure 6.1: *Proposed mechanism of HMGb1 induced proliferation, migration and invasion in myofibroblasts*

The tumour cells starved of glucose in the tumour stroma release HMGb1 into the microenvironment. This HMGb1 then can interact with myofibroblasts (possibly by interstitial fluid pressure) and may bind to receptors RAGE, or RAGE and TLR-4. Once activated, these receptor complexes can activate downstream signalling pathways which may facilitate myofibroblasts proliferation, migration and invasion. Once myofibroblasts had made their way through the ECM, it is possible that some cancer cells can also spread out of the initial tumour site and possibly migrate towards distant organs.

Other researchers have reported these pathways (PI3K and MAPK) in both migration and proliferation in cells such as human melanoma cells, glioblastoma cells and renal cell carcinoma cells (Chen *et al*. 2015; Ji *et al*. 2015; Sun *et al*. 2015) However, involvement of these pathways in HMGb1 induced stimulation of myofibroblasts has not been reported. The data presented in this thesis suggests that HMGb1 induced proliferation in myofibroblasts involves activation of PI3K and MEK1/2 pathways. Although, PI3K appeared to be a preferential pathway in both settings (migration and invasion), however, it is possible that the effect of the antibodies or inhibitors used to block each of these pathways might have different efficiencies to each other or at completely different concentrations from each other. For example, the IC50 value for PI3K inhibitor could be less than the MAPK-ERK inhibitor. Therefore, it is difficult to estimate to what extent both pathways could have been inhibited using these antibodies. It was possible that an effective dose of these inhibitors might have been toxic to myofibroblasts. Therefore, we determined a non-toxic dose for both inhibitors that was able to inhibit the activation of these pathways.

Considering all evidence presented in this thesis, it is clear that both pathways and receptor RAGE and TLR-4 play important roles in HMGb1 induced proliferation, migration and invasion of myofibroblasts cells. Therefore, based on the findings presented in this thesis, the involvement of both pathways and receptors and a possible signalling flow path in HMGb1 induced proliferation, migration and invasion of myofibroblasts have been proposed using a schematic diagram (Figure 6.2).

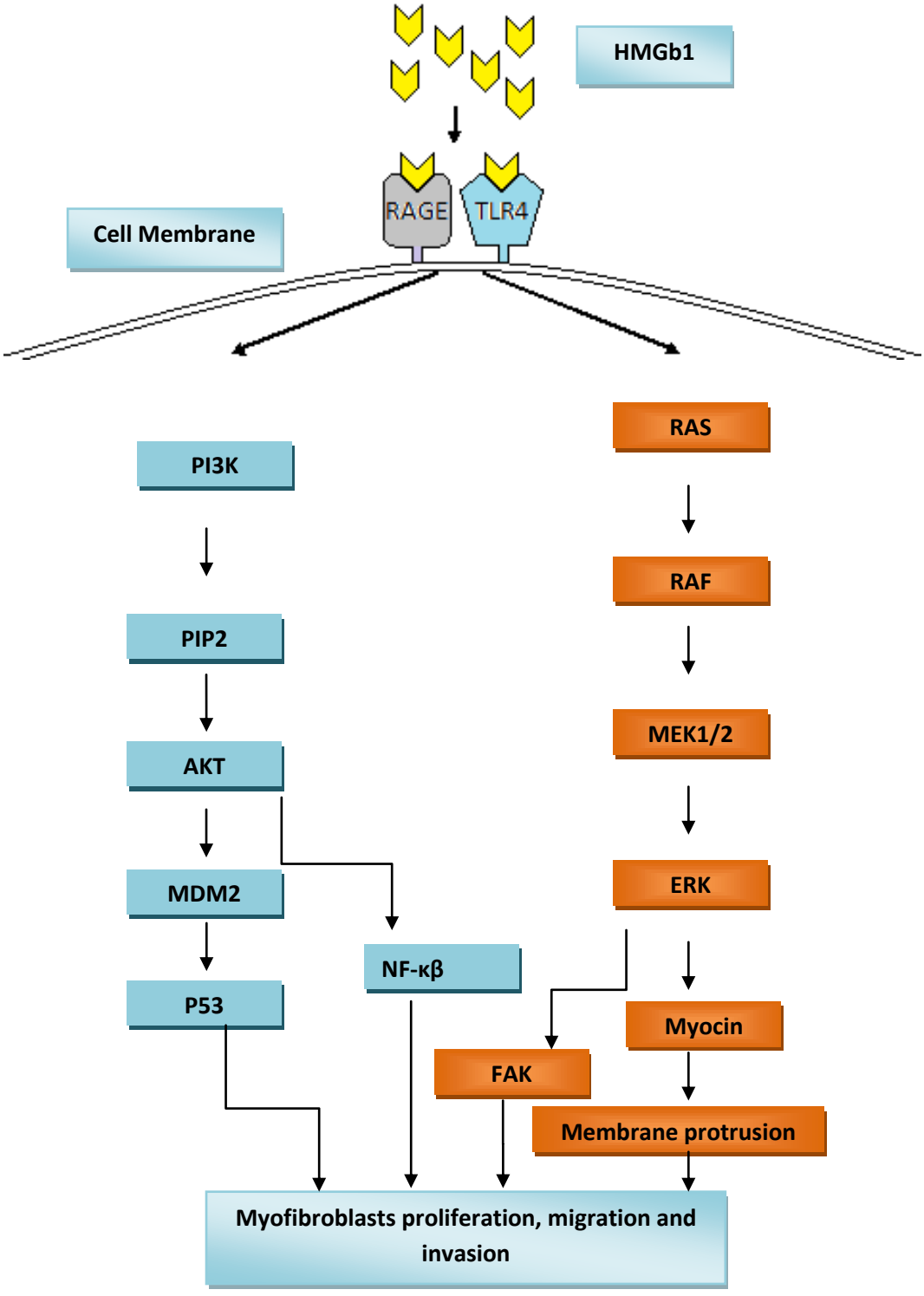


Figure 6.2: *Proposed activation of the pathways involved in proliferation, migration and invasion of myofibroblasts cells.*

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