Role of hypoxia and Toll-like receptor ligands in Matrix Metalloproteinase-7 regulation in primary human macrophages

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By

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To Ben

And my family,

Human and not

Abstract

The role of hypoxia and Toll-like receptor ligands in matrix metalloproteinase-7 regulation in primary human macrophages

Lorenza Francescut

Many solid tumours and other pathological sites such as infected wounds are characterised by hypoxic regions (defined by an O_2 tension of <1%), which are often heavily infiltrated by macrophages. Macrophages respond to hypoxia by up-regulating a number of genes likely to promote tumour growth and spread, including MMP-7. MMP-7 has been shown to be up-regulated in various tumours and it also has important roles in protection against microbial infections including release of the pro-inflammatory cytokine TNF and activation of pro-defensins.

The aim of this project was therefore to determine the mechanisms of hypoxic up-regulation of matrix metalloproteinase-7 (MMP-7) in primary human macrophages. An important aspect of this project was the analysis of the MMP-7 promoter in an attempt to identify the DNA elements required for hypoxic up-regulation, using wild-type and mutated luciferase reporter constructs transfected into primary human macrophages. A -296 bp construct was shown to be up-regulated 3-fold in primary human macrophages exposed to 5 days of hypoxia. The luciferase expression from the constructs containing mutations in the Ets and AP-1 transcription factor binding sites was not detectable, suggesting that these sites were essential for basal MMP-7 gene expression.

In this project, it was shown that MMP-7 mRNA was indeed up-regulated in severe hypoxia (0.2% O_2 for 18 hrs) in primary human macrophages. However, further experiments produced the surprising finding that hypoxia alone was not able to up-regulate MMP-7 mRNA; rather, the gene was induced by co-stimulation with hypoxia and TLR ligands such as LPS. The use of Polymyxin B, which neutralises LPS, blocked MMP-7 hypoxic up-regulation. Therefore, my data indicate that the observed and previously published "hypoxic" up-regulation of MMP-7 mRNA is actually most likely to be due to the synergistic interaction of hypoxia with LPS or other TLR ligands. MMP-7 has previously been shown to be induced by TLR ligands, but my finding of synergy between these and hypoxia in up-regulation of MMP-7 mRNA and protein is novel, and challenges current opinion on MMP-7 regulation by hypoxia.

Since the PI3K/Akt pathways is involved in TLR signaling and has been reported to be involved in hypoxic up-regulation of MMP-7, this pathway was investigated using two inhibitors, LY294002 and wortmannin. LY294002, and to a lesser extent wortmannin, inhibited LPS-induced MMP-7 up-regulation, linking MMP-7 LPS-regulation with the PI3K pathway. Another TLR signaling pathway, NF-κB, was investigated as a possible MMP-7 regulating pathway. NF-κB seems to be involved in MMP-7 up-regulation. Therefore, both PI3K and NF-κB pathways can be essential in MMP-7 up-regulation.

These findings regarding the regulation MMP-7 expression will expand knowledge of its important role, especially in innate immunity in the context of hypoxia and infection.

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Abbreviations

Acronym	Full name
β2m	Beta-2-microglobulin
BSA	Bovine Serum Albumin
AMV	Avian Myeloblastosis Virus
CAPE	Caffeic Acid Phenylethyl Ester
cDNA	Complementary DNA
CSF	Colony Stimulating Factor
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
dNTPs	Deoxynucleotide triphosphate
ECM	Extracellular Matrix
EMSA	Electromobility Shift Assay
EPO	Erythropoietin
EtOH	Ethanol
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
x g	Times Gravity
GLUT-1	Glucose Transporter-1
H	Hypoxia
HCC	Hepatocellular Carcinoma
HIF-1	Hypoxia Inducible Factor-1
hMDM	Human Monocyte-Derived Macrophages
HRE	Hypoxia-responsive element
LA	Low Attachment
LAR I	Luciferase Assay Reagent I
LAR II	Luciferase Assay Reagent II
LB	Luria-Bertani
LEF	Lymphoid-enhancer factor

LPS	Lipopolysaccharide
LUC	Luciferase
MAPK	Mitogen Activated Protein Kinase
M-CSF	Macrophage-Colony Stimulating Factor
MHC-II	Major Hystocompatibility Complex Class II
Min	Minute/minutes
ml/mg	Millilitre/milligram
MMP-7	Matrix Metalloproteinase 7
MMPs	Matrix Metalloproteinases
N	Normoxia
NF-kB	Nuclear Factor Kappa B
ng	Nanogram
NO	Nitric oxide
NSCLC	Non-small cell lung cancer
nt	Non-treated
O_2	Oxygen
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PGK	Phosphoglycerate Kinase
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3 Kinase
PMA	Phorbol 12-myristate 13-acetate
PMB	Polymyxin B
PBS	Phosphate Buffered Saline
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
P/S	Penicillin/Streptomycin
ROS	Reactive Oxygen Species
rpm	Rotation Per Minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Trancriptase
SAE	Salmonella abortus equi
Sec/s	Second/seconds

siRNA	Short interference RNA
TAMs	Tumour-Associated Macrophages
Tcf	Transcription Factor T-Cell Factor
TF	Transcription Factor
TIMPs	Tissue Inhibitor of Metalloproteinases
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-α
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
µl/µg	Microliter/microgram

Chapter 1 Introduction

Preface

It is widely recognized that many solid tumours and other pathological sites are characterised by extensive hypoxic regions (where hypoxia is defined as an O_2 tension of less than 1%); these regions are normally heavily infiltrated by macrophages, which can respond to hypoxia by up-regulating a number of genes that are likely to promote tumour growth and spread. The expression of one such gene, MMP-7, is explored in detail in this thesis. MMP-7 is often up-regulated in solid hypoxic tumors and correlates with poor prognosis and cancer progression, therefore representing a promising therapeutic target in the treatment of tumours. MMP-7 also was demonstrated to be up-regulated by hypoxia in primary human macrophages. The work presented here adds to the understanding of the mechanisms up- regulating MMP-7, and thus contributes to the knowledge of the mechanisms supporting metastasis, and potentially a basis for therapeutic approaches to cancer.

In the following introduction, I will firstly present an overview of the immune system with particular focus on macrophages, which was the main cell type employed in this project. Macrophages are able to express MMP-7 amongst an array of matrix metalloproteinases, and can infiltrate into hypoxic areas such as solid tumours. I will then introduce the concept of hypoxia and its involvement in cancer. Finally, I will link MMP-7 with all the elements aforementioned.

1.1 Immune system

The immune system is the body's coordinated reaction to non-self agents including pathogens and consists of two arms: the innate and the adaptive immune system which comprise both humoral and cellular responses. The cellular innate immune system is the non-specific first line of defence against foreign antigens and comprises white blood cells or leukocytes, which are a varied group including monocytes, macrophages and dendritic cells (mononuclear leukocytes), neutrophils, basophils and eosinophils (polymorphonuclear leukocytes) and mast cells (Campbell, 1976; Medzhitov, 2007). In contrast, the acquired or adaptive immune system is highly specific and composed mainly of T lymphocytes and B lymphocytes. All of these cells originate from a single progenitor, the hematopoietic stem cell in the bone marrow (Fogg et al., 2006). Epithelial surfaces (skin, lung and gut lining) are also part of the immune system, they constitute a physical barrier against exogenous agents and are thus primarily involved in immune protection. The mucosal epithelial layer in the lung and the gut also produces pro-defensins, which are small cationic peptides with a conserved cysteine motif that exert anti-microbial activity against both Gram-positive and -negative bacteria (Baragi et al., 1994).

1.1.1 Macrophages

Amongst leukocytes, the "mononuclear phagocyte system" is a subgroup which includes bone marrow promonocytes, circulating blood monocytes and tissue macrophages (van Furth et al., 1972). Circulating monocytes represent 10% of the mononuclear cells in peripheral blood (Gordon and Taylor, 2005), and originate from a precursor in the bone marrow (myeloid cell) that differentiates into pro-monocytes (as well as polymorphonuclear leukocytes, red cells and platelets), which then enter the circulation (Gordon and Taylor, 2005). Peripheral monocytes are an early component of

the infiltrate at inflammatory sites and are recruited to sites of infection along a gradient of a variety of chemoattractants, such as colony stimulating factor-1 (CSF-1), the anaphylatoxins C3a and C5a, produced by the activated complement system, and bacterial products (Webb et al., 1996). Peripheral monocytes make endothelial cell contact, roll, adhere and migrate into peripheral tissues where they differentiate into macrophages. Resident tissue mononuclear phagocytic cells have an average diameter of 10-20 µm, and play pivotal roles in both innate and adaptive immunity (Woessner, 1991; Murdoch et al., 2004). Macrophages can survive in tissues for 30-90 days where they undergo a continuous slow turnover and rarely divide (Tetley, 2002). According to the tissue in which they reside, macrophages acquire different morphology and are given different names: in the liver, macrophages are known as Kuppfer cells; in the brain, microglia; in the lungs, alveolar macrophages; in the lymph and blood sinuses, reticular cells; in the connective tissue, histiocytes (van Furth et al., 1972).

1.1.1.1 Roles of macrophages

The primary role of macrophages is the phagocytosis of microorganisms, necrotic and apoptotic cell debris via non-specific recognition. Following initial recognition, particles are ingested and enveloped in phagosomes, which fuse with lysosomes to form phagolysosomes (Stuart and Ezekowitz, 2005). The contents of the lysosome are released and the ingested particles are digested by enzymes such as lysozyme, protease, nucleases and glycosylases (Kwiatkowska and Sobota, 1999). Macrophages can act as antigen-presenting cells (APC): they capture and process antigens, and present the digested peptides on major histocompatibility complex class II molecules (MHC-II) which are in turn recognised by lymphocytes. When T cells receive an appropriate co-stimulatory signal (such as IL-2, Kasahara et al., 1983), they produce IFN-γ which in turn activates macrophages (positive feedback, Ma et al., 2003). Upon response to

inflammatory stimuli, macrophages can release cytokines; tumour necrosis factor alpha (TNF-α) and interleukins (IL-6, IL-10, IL-12 and IL-17) which are important mediators of host defence (Lin and Karin, 2007); colony-stimulating factors which regulate the growth and division of granulocyte and monocyte precursors (Shi et al., 2006); complement components (Rediske and Pickering, 1985), proteases (Finlay et al., 1997), reactive oxygen species (ROS, Gelderman et al., 2007), IL-1 and prostaglandins (Bachwich et al., 1986), growth factors and enzymes such as lysozyme (which acts against Gram-negative organisms such as *E. coli*, Ma et al., 2003). Macrophages are involved in further recruitment of monocytes and neutrophils via secretion of monocyte chemoattractant protein-1 (MCP-1, Graves and Valente, 1991; Cushing and Fogelman, 1992). Acute injury evokes an adaptive inflammatory response which leads to wound healing, extracellular matrix production, cell proliferation and vascularisation. Macrophages participate integrally in tissue remodeling via secretion of enzymes such as matrix metalloproteinases (Welgus et al., 1990). This role is pertinent to my project and thus will be described in detail in section 1.3.

1.1.1.2 Macrophages surface molecules

Macrophages are characterised by the expression of cluster of differentiation markers which enable them to recognise different ligands (Gordon, 2002). These markers include CD68 (lysosomal-associated transmembrane glycoprotein, Holness and Simmons, 1993), CD14 (a surface glycoprotein, part of the receptor complex required for LPS sensing, see section 1.2.2) and CD16 amongst others. Differential expression of CD14 and CD16 allows distinction of monocytes/macrophages into two classes: $CD14^+$ $CD16^-$ (classic inflammatory monocytes) and $CD14^+$ $CD16^+$ (mature tissue resident macrophages, Ziegler-Heitbrock, 2007 and Gordon and Taylor, 2005). Macrophage surface receptors include TLRs (for a more extensive explanation, see section 1.2)

which recognise pathogen-associated molecular patterns (PAMP) carried by pathogens and not by the host (Takeuchi et al., 1999). Other macrophage receptors regulate many diverse functions including differentiation, phagocytosis, adhesion and migration (Pearson, 1996). These include: interferon-gamma (IFN-γ) receptor which binds to IFN-γ, an inflammatory cytokine (Ohashi et al., 2000); receptors for the complement component C3, and for the Fc portion of antibodies - FcγR family - (particulate materials coated with antibody and C3 bind avidly to macrophage surfaces, Griffin and Mullinax, 1985). They also express mannose receptor, which binds to residues on microbes to trigger the phagocytic response and is involved in the clearance of cell debris (Kwiatkowska and Sobota, 1999), and scavenger receptor-A, which binds to pathogens in order to mount cellular and humoral response (Krieger, 2001). Classically activated macrophages (M1), activated by PAMPs and IFN-γ, exhibit a pro-inflammatory and microbicidial phenotype, whilst alternatively activated macrophages (M2), which are differentiated under the influence of IL-13 and IL-4, exhibit an increased endocytic and tissue repair activity. Macrophage functions are down-regulated by IL-10, an anti-inflammatory cytokine (Gordon and Taylor, 2005, Figure 1-1).

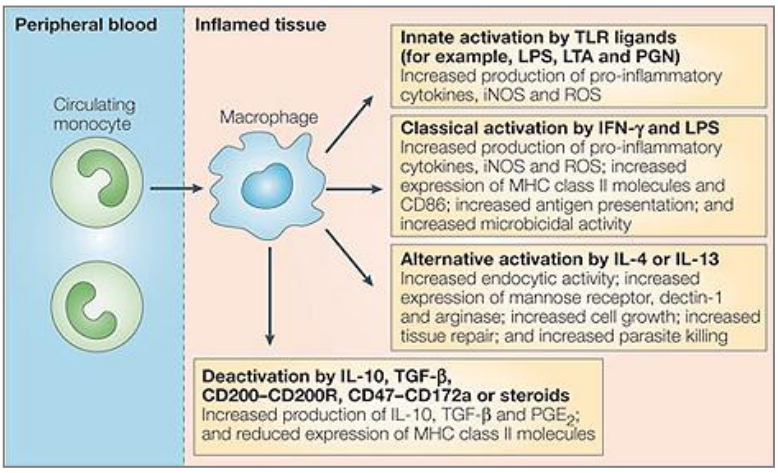


Figure 1-1: Tissue macrophage activation. **In inflamed tissues, the peripheral-blood monocytes can acquire distinct phenotypes. When stimulated with IFN-γ, macrophages show high microbicidal activity and production of reactive oxygen species (ROS), whilst when stimulated with interleukins IL-4, IL-10 or transforming growth factor-β (TGF-β), macrophages are able to promote tissue repair and suppress inflammation. CD200R, CD200 receptor; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PGE2, prostaglandin E2; PGN, peptidoglycan; TLR, Toll-like receptor; TNF, tumour-necrosis factor (Gordon and Taylor, 2005).**

1.2 Toll-like receptors (TLR)

As mentioned in the previous section, macrophages express many different TLRs. Toll-like receptors are a family of transmembrane receptors with an extracellular leucine-rich repeat domain and an intracellular domain homologous to the mammalian IL-1 receptor (Guha and Mackman, 2001). TLRs recognise PAMPs expressed by foreign organisms and PAMP engagement activates different signaling pathways which ultimately activate inflammatory pathways to regulate innate and adaptive immune responses, and are thus highly expressed on monocyte/macrophages, dendritic cells, mast cells and B lymphocytes (Zarember and Godowski, 2002). Homologous to the *Drosophila* transmembrane receptor protein Toll (Rock et al., 1998), to date thirteen mammalian TLRs are known (Atkinson, 2008). They are located both on the plasma membrane, where they recognise PAMPs accessible on the surface, and in endosomal organelles, where they mainly recognise microbial nucleic acids (Table 1-1). Some TLRs are monospecific (eg, TLR3 and TLR5), while others have broad specificity (eg, TLR2).

TLR	Location	Ligand(s)
TLR 1	Transmembrane	Lipoproteins
TLR 2	Transmembrane	Peptidoglycan from Gram-positive bacteria; MALP-2; zymosan from fungi
TLR 3	Endogenous	Viral/synthetic double stranded RNA
TLR 4	Transmembrane	LPS from Gram-negative bacteria
TLR 5	Transmembrane	Bacterial flagellin
TLR 6	Transmembrane	Lipopeptides
TLR 7	Endogenous	Single stranded RNA, Imiquimod
TLR 8	Endogenous	Single stranded RNA
TLR 9	Endogenous	Unmethylated CpG-rich bacterial DNA
TLR 10	Not known	Not identified
TLR 11	Not known	Profilin, uropathogenic bacteria
TLR 12	Not known	Not known
TLR 13	Not known	Not known

Table 1-1: Mammalian TLR ligands. **TLR1 and TLR6 require heterodimerization with TLR2 (Atkinson, 2008).**

All TLRs (except TLR3) share a conserved intracellular domain (TIR, Toll-like/interleukin-1 receptor) that activates a signal transduction cascade via recruitment of downstream adaptor protein myeloid differentiation factor-88 (MyD88). After stimulation, MyD88 is recruited to the TIR where it supports the association of IRAKs (IL-1R-associated kinases) and TRAF-6 (TNF-receptor associated factor 6). For more in depth explanation of this pathway, see section 1.2.2. The MyD88-independent pathway for TLR3 involves TRIF (TIR domain-containing adapter inducing IFN-β) rather than TIR. Engagement of TLRs by microbial components leads to a cascade of signals that ends in:

- Phosphorylation of IκB and consequent activation of NF-κB (for more detailed information on the NF-κB signaling pathway, see section 1.2.2), which translocates to the nucleus and mediates gene transcription (Carmody and Chen, 2007). All TLR-induced responses converge on the master regulator NF-κB so

the question is how responses to different PAMPs can translate into transcription of different genes. It is hypothesized that post-translational phosphorylation and acetylation of NF-κB or interaction with other factors could modulate the different responses (Carmody and Chen, 2007).

- Activation of mitogen-activated protein kinase (MAPK) pathways and c-Jun N-terminal kinase (JNK) pathway leading to up-regulation of AP-1 (Guha and Mackman, 2001);
- Activation of IRF3 transcription factor (via MyD88-independent pathway);
- Activation of phosphoinositide 3-kinase (PI3K) pathway, NO production and IFN-γ (TLR2 and TLR4). The Akt/PI3K pathway is composed by enzymes which regulate cell survival, growth and proliferation. Deregulation of the PI3K/Akt pathway is linked to cancer progression and tumorigenesis (Liu et al., 2009). Refer to section 1.2.1 for PI3K/Akt pathway description;
- Chemokine secretion (interleukin-8 aand -6, IL-8, IL-6 and MCP-1 amongst others, Schaefer et al., 2004).

MyD88 deficiency causes life-threatening bacterial infections (for example with *S. pneumoniae, S. aureus* and *P. aeruginosa*, von Bernuth et al., 2008). Therefore, MyD88-dependent TLRs play an essential role being involved in host defence.

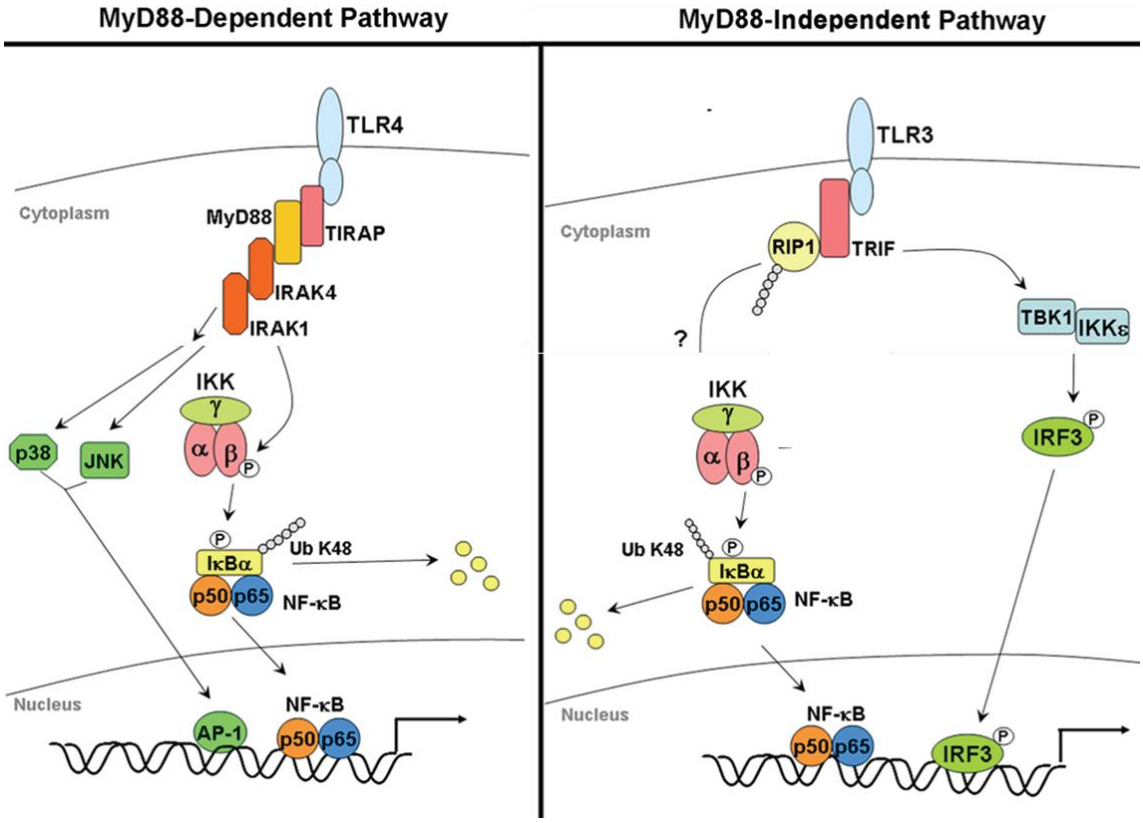


Figure 1-2: TLR signalling MyD88-dependent and independent pathways. **The two pathways both end in the activation of NF-κB. In the MyD88-dependent pathway, MyD88 directly binds to the TIR domain of the TLR or as in the case of TLR4, through the bridging adaptor TIRAP. A complex is then formed which includes IRAK1, IRAK4 and TRAF6 which eventually leads to phosphorylation and activation of IKK β and I κ B α which is degraded. Also JNK and p38 are activated, which lead to the activation of the AP-1 transcription factor. NF-κB is activated by MyD88-independent pathway, which requires the adaptor TRIF, which binds directly to TLR3. The exact mechanism of NF-κB activation is unclear. In addition, TRIF activates downstream via phosphorylation the IRF3 transcription, which regulates the expression of interferons (modified from Carmody and Chen, 2007).**

Heat shock proteins 60 (Hsp60) and 70 (Hsp70) were believed to be endogenous TLR4 agonists that could be produced by dying or damaged cells (Ohashi et al., 2000). However it was recently shown that a recombinant Hsp60 was expressed in *E. coli*, hence traces of LPS were present, which were actually responsible for mediating the interaction with TLR4 (Griffiths et al., 2000; Tsan and Gao, 2004; Osterloh and Breloer, 2008). In order to try and eliminate LPS contamination from any putative ligand of TLR4, various methods have been employed: use of Polymyxin B (see section 1.2.2) or other LPS inhibitors for LPS removal, sample boiling (LPS is heat-resistant, therefore

the leftover effect after protein degradation, was due to LPS contamination), treatment with proteinase K (LPS is proteinase K-insensitive), although all these techniques have limitations making it very difficult to identify new TLR ligands (Tsan and Gao, 2004).

TLRs (-1, -2, -3, -4, -5, -6, -9) are expressed on murine (Huang et al., 2005) and human tumour cells (Huang et al., 2008). Drugs that target TLRs could therefore be a future avenue for anti-cancer therapies (Yu and Chen, 2008).

1.2.1 Phosphoinositide 3-kinase (PI3K)/Akt pathway

As discussed in section 1.2, PI3K is a regulator involved in TLR signaling (Hazeki et al., 2007). PI3K is part of the highly conserved PI3K/Akt pathway. In response to different growth factors and cytokine stimulation, the lipid kinase PI3K, made up of a p110 catalytic subunit and a p85 regulatory one, is recruited to the membrane where it interacts with G-coupled protein receptors and phosphorylates phosphoinositides to generate phosphatidyl inositol-3,4,5-triphosphate (PIP_3). PIP_3 activates downstream signaling pathways, including the pivotal kinase Akt, which is recruited by PIP_3 and phosphorylated on Thr^{308} and Ser^{473} at the membrane by phosphoinositide-dependent kinase (PDK). Akt is then responsible for the phosphorylation of other proteins, including glycogen synthase kinase 3β (GSK-3β), which regulates a wide range of cellular processes (Jones et al., 2001). Amongst other functions, Akt activates downstream the NF-κB pathway resulting in NF-κB translocation into the nucleus (for NF-κB pathway description, see section 1.4.1.2). Phosphorylation of GSK-3β leads to its deactivation and translocation of β-catenin in the nucleus, where it binds to transcription factors bound to the DNA (Tcf/LEF and β-catenin are described in section 1.4.1.5) and modulates transcription of target genes (Doble and Woodgett, 2003),

including c-myc (He et al., 1998), cyclin D1 (Shtutman et al., 1999) and MMP-7 (Crawford et al., 1999).

Disruption of the PI3K/Akt pathway at different stages, such as inappropriate activation of PI3K, Akt over-expression or cytoplasmic or nuclear accumulation of β-catenin has been observed in many human cancers (Mann et al., 1999; Paez and Sellers, 2003). The importance of PI3K/Akt pathway in the regulation of MMP-7 is experimentally explored in section 5.8.

1.2.2 Lipopolysaccharide (LPS)

One of the most common PAMPs recognised by TLRs is LPS, the major component of the outer wall of Gram-negative bacteria which acts to protect and maintain the integrity of the cell by creating an impermeable barrier to macromolecules (Liu et al., 2002). Moreover, LPS is responsible for inducing a variety of effects in the host, including stimulation of pro-inflammatory cytokine production (TNF-α) that can often result in septic shock, thus the denomination "endotoxin". LPS is a glycolipid which is comprised of three regions: an outer long polysaccharide called O-antigen, a middle core (containing oligosaccharides) and an inner lipid A region anchored to the bacterial outer membrane (containing β-hydroxyfatty acids, Raetz and Whitfield, 2002, Figure 1-3). Lipid A has a highly conserved structure between Gram negative species and is therefore a major component recognised by the immune system.

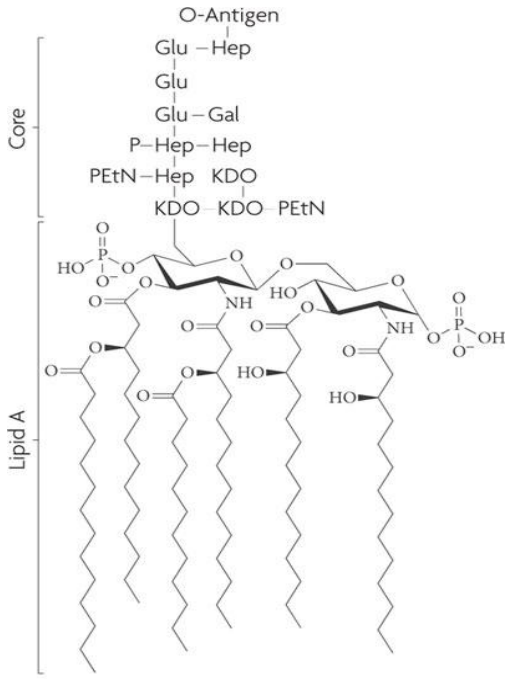


Figure 1-3: The structure of *E. coli* LPS. **The structure of LPS among different species is very diverse and bacteria can further modify the typical LPS structure in response to environmental signals. EtN, ethanolamine; Gal, D-galactose; Glu, D-glucose; Hep, L-glycero-D-manno-heptose; KDO, 3-deoxy-D-manno-oct-2-ulosonic acid; P phosphate (Frede et al., 2006).**

Macrophages are activated by LPS and respond to concentrations as low as 1 pg/mL (Fenton and Golenbock, 1998). LPS is one of the most studied macrophage-activating factors and induces a complex array of responses (including the expression of both TNF-α, and IL-10, Wanidworanun and Strober, 1993), stimulating multiple transcription factors through one or more TLRs (Monick et al., 2001). LPS signals mainly through TLR4, which was the first Toll homologue identified in humans (Medzhitov et al., 1997). Both free LPS and whole Gram-negative bacteria can be phagocytosed by macrophages via a CD14- and LBP- dependent pathway (Grunwald et al., 1996). Circulating LPS binds to serum LPS-binding protein (LBP) and this complex interacts with CD14, the cellular receptor for LPS-LBP, and MD-2, a soluble receptor for LPS. CD14 lacks a transmembrane and intracellular domain and thus needs to physically associate with TLR4, in order to signal intracellularly. The interaction of CD14 and MD-2 with TLR4 activates an extensive range of signaling pathways in

human monocytes/macrophages, from the PI3K and MyD88 pathway to the NF-κB pathway and MAPK cascade (p38, ERK1/2, JNK pathways), which stimulates the synthesis of TNF-α, IL-6, IL-1β, iNOS and COX-2 (Mendes Sdos et al., 2009). MyD88 interacts with the serine/threonine kinase IRAK which associates with another adaptor protein, TRAF6. TRAF6 signaling leads to phosphorylation and activation of IKK which, in turn, phosphorylates the NF-κB inhibitor IκB inducing its degradation and release of NF-κB. Other intracellular signaling pathways affected by LPS are ERK1 and 2, JNK (with AP-1 activation) and p38 (Feng et al., 1999). The main transcription factor activated by LPS associated with inflammatory response is NF-κB, which is responsible for controlling the transcription of many genes (Dos Santos et al., 2007). Transcription factor binding to LPS-responsive elements in the promoter region of various genes are NF-κB/Rel (p65/p50) and AP-1 (c-Fos/c-Jun) proteins, which are activated by phosphorylation (Guha and Mackman, 2001, Figure 1-4).

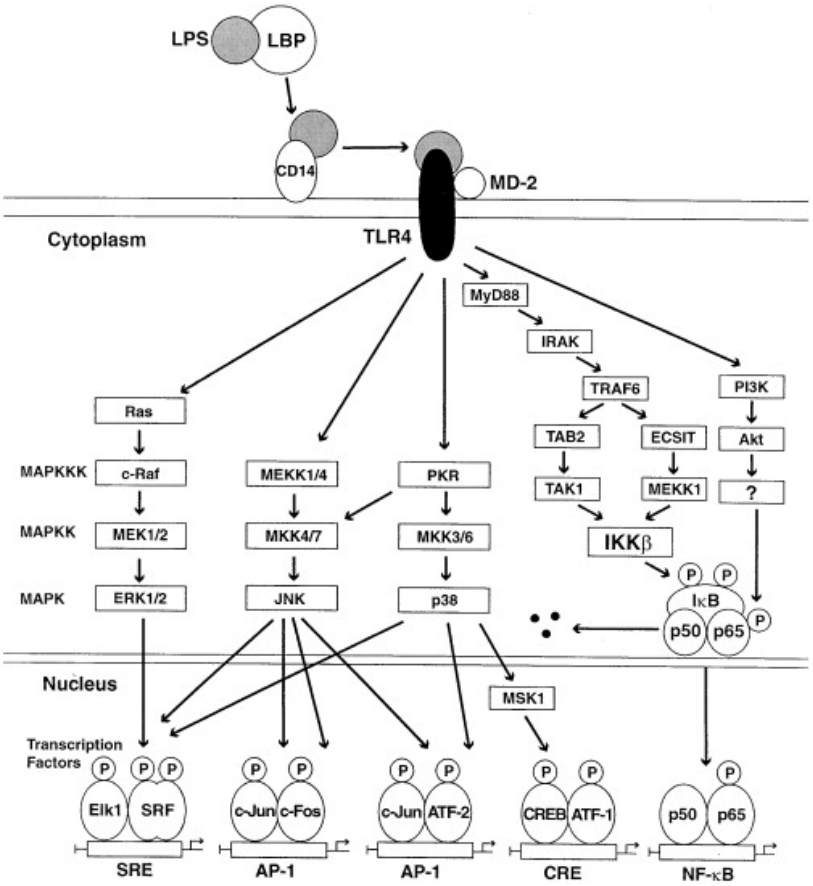


Figure 1-4: LPS signalling pathway in monocytes. **LPS binds to the serum protein LBP and is transferred to the CD14 at the cell surface where it interacts with TLR4 and the accessory protein MD-2. LPS stimulates the activation of various pathways, including the ERK, JNK, and p38 pathways which phosphorylate and activate various transcription factors, including c-Jun, c-Fos and CREB. In addition, LPS activates NFkB via the MyD88 pathway. The PI3K/Akt pathway phosphorylates and activates p65 via an unknown kinase (Guha and Mackman, 2001).**

1.3 Matrix Metalloproteinases (MMPs)

The extracellular matrix (ECM) serves as a barrier between tissue compartments. Composed of collagen, fibronectin, elastin, proteoglycans and other glycoproteins, the ECM supports cells and contributes to different cellular functions, from cell migration to development (Berrier and Yamada, 2007). ECM remodeling is necessary for cell growth, wound repair and migration and it must be finely tuned (Ii et al., 2006).

Matrix metalloproteinases (MMPs) are a family of more than 25 genetically related Zn^{2+}- and Ca^{2+}-dependent endoproteases found in humans, which regulate the turnover of specific extracellular matrix components.

Secreted-type MMPs: These MMPs are secreted either as inactive pro-enzymes (zymogens) - which are proteolytically activated extracellularly by cleavage of the N-terminal pro-peptide (cysteine-zinc domain), or intracellularly activated by furin.

Membrane-bound MMPs: These MMPs contain a transmembrane domain and have a furin cleavage sequence at the end of the pro-peptide (Shiomi et al., 2010).

MMPs are produced by different cell types such as epithelial cells, fibroblasts, inflammatory cells and monocytes (Bar-Or et al., 2003). MMPs share conserved motifs: a catalytic domain activated by zinc binding, a region targeting for secretion and a region that maintains the protein in an inactive form (Woessner, 1991). As stated above, MMP members have been classified into secreted and membrane-anchored types (see Table 1-2) and further divided according to substrate-specificity (which can overlap, Shiomi et al., 2010):

MMPs	Type	Substrate
Secreted/soluble-type		
Collagenases	MMP-1	Collagens I, II, III
	MMP-8	Aggrecan, collagen I, fibrinogen, gelatin
	MMP-13	Collagen I, II, III, fibrinogen, gelatin
Gelatinases	MMP-2	Collagens IV, V, elastin, fibronectin
	MMP-9	Collagen IV, V, elastin, gelatin I
Stromelysins	MMP-3	Collagen IV, procollagen I, gelatin, laminin
	MMP-10	Casein, gelatin
	MMP-11, -18	Not known
Matrilysins	MMP-7	Casein, collagen IV, elastin, fibronectin, laminin, gelatin I
	MMP-26	Collagen IV, fibronectin, fibrinogen, gelatin I
Furin-activated MMPs	MMP-28	Casein
Others	MMP-12, -19, -20, -21, -27	fibrinogen, factor XII, casein, collagen IV, fibrin, fibronectin, gelatin, laminin,
Membrane-bound-type		
Type I transmembrane	MMP-14	Aggrecan, collagen I, II, III, casein, fibronectin,

(MT)		fibrinogen, gelatin
	MMP-15	Aggrecan, fibronectin, laminin
	MMP-16	Pro-MMP-212
	MMP-17	Fibrin, fibrinogen, gelatin
	MMP-24	Gelatin
	MMP-25	Collagen IV, fibrin, fibronectin, gelatin
Type II transmembrane (MT)	MMP-23	Not known

Table 1-2: MMPs classification. **Adapted from Rydlova et al., 2008.**

MMPs are not generally expressed in healthy tissues, but their levels increase dramatically during the repair and remodelling processes in pathologies (Parks and Shapiro, 2001), a list of which can be found in

Table 1-3.

Physiological processes	Pathological processes
Embryonic development	Cancer invasion
Angiogenesis	Rheumatoid arthritis
Tissue remodelling	Atherosclerosis
Endometrium cycling	Liver cirrhosis
Bone remodelling	Aortic aneurysm
Wound healing	Osteoarthritis

Table 1-3: Roles of MMPs in normal and diseased processes **(adapted from Rydlova et al., 2008).**

At least eight MMP genes are clustered on chromosome 11 (Yin et al., 2010). MMP expression and activity is regulated by hormones and growth factors (Hulboy et al., 1997), chemical agents (eg, phorbol myristate acetate, PMA) and cytokines (Roomi et al., 2009), physical stress (Ergul 03) and oncogenes such as Src (Kuo et al., 2006) and c-jun and c-fos (McDonnell et al., 1990).

It has been reported that the AP-1 binding site interacting with PEA3 elements is essential for basal activity of many MMP promoters (Yin et al., 2010). MMP expression is transcriptionally regulated by different transcription factors such as AP-1, PEA3, NF-

κB and β-catenin/Tcf (Yan and Boyd, 2007). MMP-deficient mice to date (excluding the knockout mice for MMP-14, which die by three weeks of age, Holmbeck et al., 1999) do not show any major phenotype, therefore implying that these proteases are not involved in vital functions in development, or at least that there is redundancy of function (for an extensive review, see Parks and Shapiro, 2001).

1.3.1 MMPs and their inhibitors

MMP activity is regulated at different levels by gene transcription, pro-enzyme activation and inhibition by the physiological tissue inhibitor of metalloproteinases, TIMPs. TIMPs are a family of four secreted proteins which bind non-covalently to MMPs in 1:1 complexes, these complexes are inactive and therefore MMPs are unable to bind to their substrate (Woessner and Taplin, 1988, Nelson et al., 2000, Lewis and Pollard, 2006, Rydlova et al., 2008). TIMP-1, -2 and -4 are highly expressed in monocytes compared with T or B cells (Bar-Or et al., 2003), whilst TIMP-3 is associated with the ECM (Langton et al., 1998). It has been previously shown that a discrepancy in MMP/TIMP expression is correlated with carcinogenesis (Overall and Lopez-Otin, 2002). ECM remodeling requires tight regulation of MMPs: MMPs can be associated with tumour invasion, therefore synthetic TIMPs have been developed as anti-cancer therapies, which were reported to have a beneficial effect in less advanced gastric cancer patients (Coussens et al., 2002) – see Section 1.5.3 for further discussion on the role of MMPs in cancer.

MMPs can also be inhibited by general protease inhibitors like α_2-macroglobulin, present in plasma (Overall and Lopez-Otin, 2002) and the synthetic inhibitor batimastat (Coussens et al., 2002).

1.3.2 MMPs and TLR ligands

LPS, acting through TLR4, is the most potent inducer of MMP expression (Welgus et al., 1990, Busiek et al., 1995). The production of MMP-1 (interstitial collagenase) and MMP-9 (gelatinase B) by human monocytes/ macrophages after stimulation with LPS occurs in a PGE_2-cAMP and ERK1/2-dependent fashion; the p38 (for MMP-1 only) pathway is also essential (Lai et al., 2003). LPS stimulates cells to produce MMP-2 in lung tumour nodules (Harmey et al., 2002) and collagenases in U937 cells (Saarialho-Kere et al., 1993). Bacterial peptidoglycan and LPS can induce expression of MMP-1, -3 and -13 in synovial fibroblasts (Kim et al., 2006a). MMP-9 is induced in THP-1 and RAW264.7 cell lines (both macrophage-like cell lines derived from a tumour) by LPS, Pam_3Cys, CpG DNA and poly (I:C); and in primary peritoneal macrophages by LPS. This has been demonstrated by using decursin, a natural anti-inflammatory agent, which suppresses LPS-dependent MMP-9 induction in macrophages. Decursin has been shown to perform a variety of activities some of which are anti-cancerous and results in the reduction of tumour volume and prolongation of survival time. Therefore, the regulation of MMP-9 could be an additional reason for decursin anti-tumoural activity (Kim et al., 2006b). In human monocytes, LPS induces the mRNAs of MMP-1, MMP-9, MMP-10 and MMP-14 (Reel et al., 2011); LPS up-regulates collagenase, stromelysin (Welgus et al., 1990) and matrilysin in human macrophages (Busiek et al., 1995) and in human bone-marrow derived monocytes (Busiek et al., 1992).

1.4 Hypoxia

A common denominator of pathologies such as atherosclerosis and cancer is the presence of hypoxic areas (Vaupel et al., 1989, Grunwald et al., 1996). Normal air contains about 20.9% O_2 (158 mmHg) at sea level and hypoxia is defined as reduced

oxygen tension relative to normal oxygen level. Physiologically, hypoxia is often observed in healthy organs such as the spleen, thymus, brain and tissues like retina, whose oxygen tension spans from 24 to 70 mmHg – 2.5%-9% O_2 (Lewis et al., 1999, Trayhurn et al., 2008, Santilli et al., 2010). Tissues are normally characterised by a lower O_2 pressure (pO_2 25-70 mmHg) than in arterial blood (pO_2 100-150 mmHg) depending on the distance from the nearest capillary (Sell and Eckel, 2008). Cell cultures are usually exposed to 21% O_2 instead of the more physiological level of 4% O_2 (Semenza, 2004). Pathologically, hypoxia is a hallmark of ischæmic wounds (Lee et al., 2009), atherosclerotic lesions (Osada-Oka et al., 2008) and solid tumours (Brown and Wilson, 2004). Acute infection is also associated with hypoxia due to the oxygen consumption of pathogens (Zhao et al., 2009). Inflammatory sites are also characterised by decreased oxygen levels due to oxygen consumption from cells surrounding the inflammation site, either recruited or residential, and because of tissue and blood vessel damage which often accompanies infection (Kuhlicke et al., 2007, Kim et al., 2010). Reduction of oxygen levels and glucose in tissues with poor vascularisation contributes to cell adaptation by causing them to switch to anaerobic metabolism (Bosco et al., 2008). Approximately 1% of the genome is transcriptionally responsive to hypoxia, although the response in different cell types is very heterogenous (Denko et al., 2003).

Hypoxia induces transcription of a range of genes whose expression is fundamental to survival (Kuhlicke et al., 2007, Kim et al., 2010), such as Akt (Figueroa et al., 2002). MMP expression can be dysregulated in hypoxic macrophages: MMP-25 is diminished whilst MMP-1, -16, -19 (Bosco et al., 2006) and MMP-7 (Burke et al., 2003) are increased.

1.4.1 Hypoxia-responsive transcription factors

Hypoxia has a profound impact on cell behaviour and activates many different transcription factors, which are covered in turn below.

1.4.1.1 Hypoxia inducible factors (HIF)

HIFs are transcription factors, whose members include HIF-1 and HIF-2, which are considered to be the most important hypoxia-regulated transcription factors. HIF-1 is expressed ubiquitously and controls the transcription of over 70 genes (Semenza, 2004, Weinmann et al., 2005). HIF-1 is a heterodimeric protein composed of two subunits, the 120-kDa HIF-1α (the component responsive to oxygen, expressed in the cytoplasm) and the 91-94 kDa HIF-1β (constitutively expressed in the nucleus). The N-terminus of each subunit regulates heterodimerisation and DNA binding, whilst the C-terminus interacts with co-activators (CBP and p300, Semenza, 2004). HIF-1α is detectable at very low levels under normoxic conditions, being rapidly degraded in the presence of oxygen: its half-life in normoxic conditions is less than 5 min (Wang et al., 1995). Under normoxia, the HIF-1α subunit is hydroxylated on two different conserved residues: towards the N-terminus, proline residues 402 and 564 are hydroxylated by prolyl hydroxylase domain-containing proteins (PHDs), a family of Fe^{2+}-dependent enzymes requiring oxygen and iron as substrates; and on the C-terminus, the asparagine Asn^{803} is hydroxylated by Factor Inhibiting HIF (FIH), another Fe^{2+}-dependent enzyme requiring oxygen and iron (Mahon et al., 2001). The hydroxylation enables the binding by the tumour-suppressor protein von Hippel-Lindau (pVHL), resulting in the HIF-1α subunit being tagged with ubiquitin, leading to degradation by the ubiquitin-proteasome pathway. Also, the hydroxylation inhibits the binding of HIF-1α with p300 and CBP co-activators (Semenza, 2004).

In hypoxia (<5% O_2), prolyl and asparaginyl hydroxylation is blocked because PHDs require oxygen as a substrate, therefore hydroxylation and subsequent proteolytic degradation are reduced and the cytoplasmic level of HIF-1α protein is post-transcriptionally stabilised (Kim et al., 2010). The α subunit can then translocate into the nucleus where it binds to the constitutively expressed β subunit and this transcriptional complex binds to core hypoxia-responsive element (HRE) DNA motifs of 5'-($^{G}/_{A}$)CGTG-3' in the promoter of target genes, activating them with the help of recruited transcriptional co-activators p300/CBP (Fedele et al., 2002, Blouin et al., 2004). Structurally and functionally, HIF-2 (also known as EPAS-1) is similar to HIF-1: HIF-2α subunit is hydroxylated on another asparagine residue (Asn^{851}) and can heterodimerise with HIF-1β. HIF-2 is not expressed in all cell types (Semenza, 2004).

Genes up-regulated by HIF-1 are involved in anaerobic glycolysis (such as phosphoglycerate kinase, PGK , and glucose transporter-1, GLUT-1), increase of blood supply by angiogenesis (eg, vascular endothelial growth factor, VEGF) (Semenza et al., 1994), production of red blood cells via erythropoiesis (erythropoietin, EPO) (Semenza, 1994) and vasodilatation (eg, inducible nitric oxide synthase iNOS) (Todd et al., 1991, Schofield and Ratcliffe, 2004), which are all beneficial for tissue survival in ischæmic conditions (Cummins and Taylor, 2005).

Early studies reported that HIF-1α mRNA is not increased during hypoxia, but the up-regulation is due to protein stability (Blouin et al., 2004). However, a recent study has showed increases in HIF-1α mRNA levels after long term severe hypoxia (5 days, 0.2% O_2) in primary human macrophages and there are reports of this in other cell types also (Staples et al., 2011). HIF-1 can also be induced by non-hypoxic pro-inflammatory stimuli, such as LPS, ROS and cytokines (Dery et al., 2005, Frede et al., 2006). LPS induces HIF-1α mRNA transcription (Blouin et al., 2004, Dery et al., 2005) and protein

in macrophages (Blouin et al., 2004). TLR4 is regulated by HIF-1 by binding of HIF-1 to the TLR4 promoter under hypoxic conditions; in fact, hypoxic stress can increase the response to bacteria by up-regulating TLR4, thus enhancing macrophage activation (Kim et al., 2010).

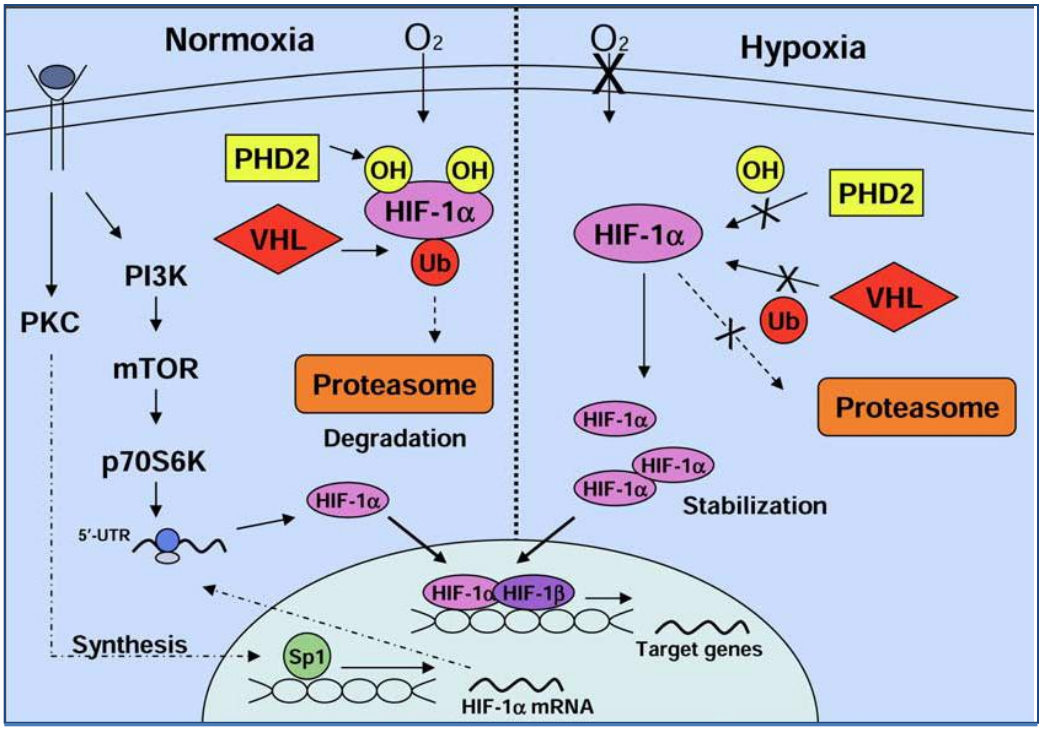


Figure 1-5: HIF-1 is regulated by normoxia, hypoxia and non-hypoxic stimuli. **In normoxic conditions, HIF-1 is hydroxylated on proline by PHD2, ubiquitinated by pVHL and degraded by the proteasome. In hypoxic conditions, PHD2 is blocked and HIF-1α can translocate in the nucleus where, with HIF-1β, it binds to the promoter of many genes essential to the adaptive response to hypoxia. Non-hypoxic stimuli amplify the transcription of HIF-1 gene by PKC. The PI3K pathway can increase HIF-1 mRNA translation. Figure from (Dery et al., 2005).**

HIF-1α and HIF-2α are expressed *in vitro* in human macrophages subjected to severe hypoxia (Staples et al., 2011), with high amounts of HIF-1α protein in respect to HIF-2α (Burke et al., 2002) and in human tumours, at both the protein and mRNA level (Talks et al., 2000). Cobalt chloride (a transition metal) and desferrioxamine (an iron chelator) mimic hypoxia by causing stabilisation and accumulation of HIF-1α in the cytosol: cobalt chloride acts by inhibiting PHDs, FIH and the binding of VHL via competition with free Fe^{2+} ions, whilst desferrioxamine inhibits PHDs and FIH via removing intracellular ions and substituting the Fe^{2+} active site (Asikainen and White, 2007).

Around 35% of genes which are inducible by hypoxia lack the HRE sequence in the promoter region, which implies other mechanisms of induction independent from, or at least not directly controlled by, HIFs (Bosco et al., 2008). A core HRE sequence (CGTG) is necessary but not sufficient for target gene activation by HIFs, meaning that the binding of HIF to the promoter does not necessarily cause efficient activation, since other transcription factors collaborate to produce hypoxic inducibility (Wenger, 2002). Also high cell density, which mimicks the solid tumour environment, can up-regulate a number of hypoxia-inducible genes in an HIF-1-independent fashion (Semenza, 2004).

1.4.1.2 NF-kB

Another transcription factor involved in the response to hypoxia is NF-κB (nuclear factor kappa-B), a family of five related members (p50, p52, p65, cRel and RelB) which regulate cell growth and immune/inflammatory responses (Cummins and Taylor, 2005). NF-κB is constitutively present in cells in the form of homodimers or heterodimers (the most abundant heterodimer is composed of the p50 DNA-binding subunit and the p65 trans-activating subunit) which is kept inactive in the cytoplasm by the inhibitor of κB (IκB) (Cummins and Taylor, 2005). When proinflammatory stimuli (bacterial products, UV light, etc) activate the NF-κB pathway, IκB is targeted for phosphorylation, which leads to its degradation via the proteasomal pathway, allowing NF-κB to translocate into the nucleus and bind to DNA κB binding sites (5'-GGGRNWYYCC-3', where R is a purine A/G, N is any base, W is an A/T and Y is a pyrimidine T/C) and activates expression of around 100 target genes (Abate and Schroder, 1998).

Hypoxia activates NF-κB probably by phosphorylation and subsequent degradation of IκB (Koong et al., 1994) and triggers transcription of target genes including cyclo-oxygenase-2 (COX-2), tumour necrosis factor-α (TNF-α), EPO (Figueroa et al., 2002),

interleukin-6 (IL-6) (Cummins and Taylor, 2005) and CCL20 (CC-chemokine MIP-3α, a chemoattractant for immune cells) in human macrophages (Battaglia et al., 2008). NF-κB activation by hypoxia is required to activate genes responding to stress of low oxygen tension (Koong et al., 1994). Inhibition of NF-κB causes blockage of HIF-1 protein expression (Figueroa et al., 2002). HIF-1α mRNA and protein expression can be modulated by NF-κB, via NF-κB binding sites in the HIF-1α promoter (van Uden et al., 2008).

1.4.1.3 Ets family transcription factors

Ets (E26 transformation-specific sequence) is a family of transcription factors defined by a conserved core purine-rich DNA binding site, the ets domain, 5'- ($^{C}/_{A}$)GGA($^{A}/_{T}$) - 3' with flanking sequences defining binding specificity (Marecki and Fenton, 2000). Ets factors can act both as repressors and activators of gene expression. There are around 30 mammalian ets family members (such as PU.1, PEA3, Ets-1, Ets-2 and Elk-1) which regulate different processes, from cellular proliferation and differentiation to apoptosis. The transcriptional activity of ets is modulated by other transcription factors (AP-1 and CBP/p300) which bind with weak affinity (Li et al., 2000). Ets-1 and ets-2 overexpression in malignant cancer correlates with negative prognosis (Lamm et al., 2005). Hypoxia induces ets-1 expression through HIF-1 in a human bladder cancer cell line (Oikawa et al., 2001), but in another study TX-402, a repressor of HIF-1α expression, does not affect the expression of genes such as ets-1 in hepatocellular carcinoma cells, demonstrating how ets-1 expression is HIF-1-independent (Miyoshi et al., 2006).

Ets also regulates tumour invasiveness via up-regulation of MMPs and angiogenesis via regulation of production of VEGF by endothelial cells (Dittmer, 2003).

PEA3 group factors are involved in transcriptional regulation of MMPs and play a role in tumourigenesis via regulation of genes involved in metastasis (de Launoit et al., 2006). In high density human and mouse cell populations, which mimics growing tumours, ets-1 (but not PEA3) is up-regulated and regulates several hypoxia-inducible genes (Salnikow et al., 2008). Using chromatin immunoprecipitation, it was found that LPS stimulates the binding of ets-1 to the TNF-α promoter in macrophages (Tsai et al., 2000).

Ets-1 or PEA3 can activate MMP promoters in cooperation with AP-1 (Oikawa and Yamada, 2003).

1.4.1.4 AP-1

AP-1 (activating protein-1) is a dimeric transcription factor complex composed by c (cellular)-*jun* and c-*fos*-related families of proteins, involved in apoptosis, cell proliferation and differentiation, tumour invasion and angiogenesis. Formed when c-*fos* and c-*jun* proteins heterodimerise, AP-1 binds to the specific DNA sequence 5'-TGA($^{C}/_{G}$)TCA-3' to induce transcription of target genes (Bosco et al., 2008). The basal level of c-*fos* is generally low in the majority of cell types and an extracellular signal is required for its up-regulation. c-*jun* and c-*fos* can induce oncogenic transformation (Vogt, 2001). Hypoxia can induce AP-1-dependent transcription of VEGF and endothelial NOS (Cummins and Taylor, 2005) and AP-1 can cooperate with HIF-1 and NF-κB to activate many other genes. The mechanism by which AP-1 is up-regulated by hypoxia is not fully elucidated, but there are various hypotheses: via enhancing cytoplasmatic Ca^{2+} influx since AP-1 transcription factors are Ca^{2+} sensitive (Fantozzi et al., 2003); and via the Jun N-terminal kinase (JNK) pathway (Minet et al., 2001).

1.4.1.5 β-catenin

Genes of the Wnt family (secreted glycoproteins) have an essential role in proliferation and migration. The deregulation of the Wnt pathway is associated with 70-80% of colorectal and 20-30% of hepatocellular tumours (Kolligs et al., 2002).

In the absence of Wnt ligand, free cytoplasmic β-catenin (a component of Wnt family which is highly conserved across species) is phosphorylated by glycogen synthase kinase-3β (GSK-3β)/Axin and degraded via the ubiquitin and proteasome pathway; when the canonical Wnt pathway is activated via Frizzled receptor, β-catenin's phosphorylation is inhibited via inhibition of GSK-3β and it accumulates in the cytoplasm and translocates into the nucleus (Deguchi et al., 2009) where it activates DNA-bound transcription factor T-cell factor/lymphoid-enhancer factor (TCF/LEF) leading to increased transcription of target genes (including cyclin D1, c-Myc and MMP-7). The β-catenin interacts with Tcf/LEF providing a transcription activation domain for Tcf/LEF to bind to a conserved DNA sequence (5'-(A/T)(A/T)CAA(A/T)G-3') through interaction with transcriptional coactivator p300/CBP (Gustavson et al., 2004).

Wnt signaling has been implicated in tumour progression: many cancer cells show increase in β-catenin accumulation in the nucleus (Le Floch et al., 2005). In epithelial cells, β-catenin interacts with the cytoplasmic domain of E-cadherin in adherens junctions mediating the connection between cadherins and actin (Aberle et al., 1996). Activation of the Wnt pathway, leading to phosphorylation of β-catenin, causes dissociation from E-cadherin and increased invasion and metastasis. Chronic moderate hypoxia has been shown to activate Akt (see section 1.2.1 for discussion of Akt/PI3K pathway) and β-catenin pathway via inactivation of GSK-3β in primary human macrophages (Deguchi et al., 2009). Severe hypoxia activates the Akt/PI3K pathway for

survival and this pathway deregulation can lead to diseases, from cancer to inflammatory disorders. HIF-1α is able to negatively regulate β-catenin during hypoxia by dissociating the β-catenin-Tcf/LEF complex, whilst HIF-2α facilitates gene transcription by assembling with β-catenin-Tcf/LEF and determining the growth of tumour cells during hypoxia (Choi et al., 2010).

1.5 Cancer

Cancer is a heterogeneous disease and its induction is a multistep process where different alterations can dictate malignant growth; the majority of cancers are linked to environmental factors (such as tobacco smoke and asbestos) and obesity. Other causes include infectious agents, which may be responsible for 15-20% of cancers worldwide (Balkwill and Mantovani, 2001) and somatic mutations (Grivennikov et al., 2010).

The process starts with cells acquiring mutations that allow uncontrollable growth and resistance to apoptosis. This is followed by invasion of adjacent tissues and metastatic spread, characterised by the detachment of tumour cells from the primary tumour, spread into the blood and lymphatic circulation and attachment to other tissues of the body to become secondary tumours (Hanahan and Weinberg, 2000). Dissemination of malignant tumour cells (metastasis) is the major cause of terminal illness in cancer patients. At this late stage, epithelial tumour cells differentiate into mesenchymal cells which can invade and migrate through the ECM, a critical event for metastatic transformation (Vincent-Salomon and Thiery, 2003). Degradation of ECM is due to proteolytic enzymes (MMPs, see section 1.3 and 1.5.3 for cancer and MMPs). Reduced cell-to-cell adhesiveness supports tumour cell invasion of neighbouring tissues (Borchers et al., 1997). The formation of new blood vessels, angiogenesis, is necessary

for growth once the tumour is larger than 1-2 mm^3, as will be discussed in section 1.5.1, and also for metastasis (Folkman, 1971).

1.5.1 Cancer, hypoxia and macrophages

Exposure to hypoxia induces an array of genes (the hypoxic transcriptome) in primary murine (Fang et al., 2009) and human macrophages (Burke et al., 2003) involved in regulation of angiogenesis (which requires cleavage of ECM components, proliferation and migration of endothelial cells, as well as synthesis of new matrix components) and inflammation (Bosco et al., 2006). In monocytes, hypoxia has been shown to up-regulate the amount of MMP-1, -16, and -19 (Bosco et al., 2006), whilst hypoxic macrophages can up-regulate MMP-7 (Burke et al., 2003) and MMP-12 (White et al., 2004). These findings indicate that macrophages in hypoxic conditions can modulate ECM stability.

Human monocyte-derived macrophages have been shown to survive chronic or acute hypoxia (Deguchi et al., 2009; Burke et al, 2003).

The presence of hypoxia is a common feature of solid malignant human tumours, with a mean pO_2 of < 25 mmHg in breast carcinoma tissue compared to a mean pO_2 of 65 mmHg in normal breast tissues (Vaupel et al., 1989). Solid tumours reaching a threshold size of 1-2 mm^3 can become oxygen- and nutrient-deprived, having outgrown the pre-existing local blood supply, and therefore contain hypoxic regions (pO_2 < 25 mm Hg or 2% O_2, Folkman, 1971). These poorly oxygenated regions are a good environment for angiogenesis and have been shown to be resistant to radio and chemotherapy (Brown and Wilson, 2004; Brizel et al., 1999; Durand, 1994). In fact, chemotherapeutic agents mostly act on proliferative cells, therefore hypoxic areas, which are mainly non-proliferative, show more resistance to these drugs (reviewed by

Murdoch et al., 2004). In tumours, new vessels develop in a disorganised fashion and are inadequate to provide oxygen and glucose to the growing cellular mass (Vaupel et al., 1989). These hypoxic areas of malignancies are heavily infiltrated by macrophages (Murdoch and Lewis, 2005; Lewis and Pollard, 2006) which are so-called tumour-associated macrophages (TAMs). During cancer, macrophages from circulating monocytes are recruited into the hypoxic tumour microenvironment by chemokines (MCP-1, RANTES), cytokines (CSF-1 and VEGF) and cell debris from lysis of tumour cells going through necrosis (Murdoch et al., 2004) and mature into macrophages (Balkwill and Mantovani, 2001). TAMs respond to the new microenvironment by acquiring a hypoxic "phenotype" and modifying their gene transcription (Bosco et al., 2008) by up-regulating a number of oxygen-sensitive genes, most of which are regulated by HIF-1, the main transcription factor induced by hypoxia, and HIF-2 (see 1.4.1.1 for details). Moreover, hypoxic macrophages can up-regulate genes that can inactivate some chemotherapeutic agents (eg, doxorubicin and MMP-7, see section 1.6.5). Macrophages are then likely to promote the growth and the spread of malignant tumours via a diverse array of hypoxia-driven mechanisms (Murdoch and Lewis, 2005), being involved in angiogenesis, invasion and metastasis (Grivennikov et al 2010.).

During hypoxia, total protein translation is reduced, a part from a group of proteins crucial for survival which keeps being translated (Koh et al., 2010).

TAMs can act as a double-edged sword: they can destroy neoplastic cells presenting tumour-associated antigens on the surface which stimulate lymphocytes, up-regulating the pro-inflammatory mediator TNF-α and producing ROS and NO to enhance tumour cells cytotoxicity (Murdoch et al., 2004); conversely, macrophages, as previously described, can also promote tumour growth by stimulating angiogenesis (secretion of VEGF) and metastasis (partly via secretion of MMPs, reviewed more extensively in

section 1.5.3) (Bosco et al., 2006, Condeelis and Pollard, 2006), inhibiting lymphocyte activity via up-regulation of IL-10 and therefore correlate negatively with prognosis (Elgert et al., 1998). It has been previously studied how, in fact, co-cultivation of a breast cancer cell line and macrophages leads to increased invasiveness of cancerous cells (Hagemann et al., 2004).

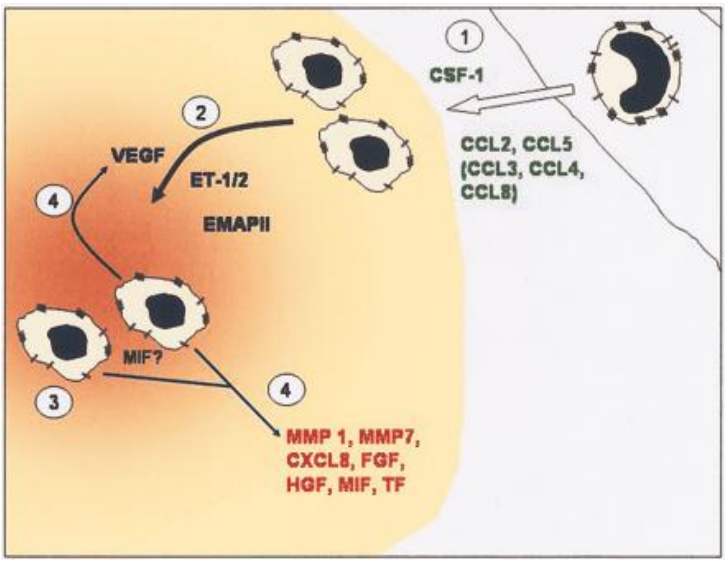


Figure 1-6: Monocyte recruitment in tumours. **Chemokines of the CC family and CSF-1 produced by tumour cells recruit monocytes (1) into the tumour (in orange) where they differentiate into TAMs (2). TAMs are attracted by chemoattractants such as VEGF to the hypoxic region (in brown, 3) where they are retained and express VEGF which amplifies the loop and up-regulates genes involved in angiogenesis and metastasis (4) (Murdoch et al., 2004).**

Immune cells are able to remove malignant cells before they become fully grown tumours; combination radiation treatment and immunotherapy is a new frontier in cancer treatment, since radiation is known to trigger anti-tumour immunity (Hodge et al., 2008). How this happens is subject to debate: one hypothesis is that suppressor T cells are more radiosensitive than effector T cells; it has also being suggested that radiated cells release a substance which acts as an endogenous TLR4 ligand; and also that radiated-injured gut releases endotoxin that, again, stimulates TLR (Oblak and Jerala, 2011). APCs such as macrophages can present tumour-associated antigens which can stimulate T-cells to raise anti-tumour immunity (Campton et al., 2000).

In the hypoxic microenvironment typical of solid tumours, the transcription factor HIF-1 promotes tumour growth, cell survival and resistance to chemotherapeutic drugs (Cummins and Taylor, 2005). This property makes macrophages good candidates for targeted hypoxia-regulated gene therapy to human tumours (Griffiths et al., 2000). This idea is based on the concept that macrophages would be isolated from patients, differentiatied *ex vivo*, transfected with a therapeutic gene construct designed to be up-regulated by hypoxia and re-introduced into the patients where they would migrate to hypoxic areas and express HIF-1 and HIF-2 which can activate therapeutic-genes HRE-driven expression. In a previous study, macrophages infected with a hypoxia-regulated adenovirus carrying human cytochrome P450, a pro-drug-activating enzyme, were co-cultured with breast tumour cell spheroids in the presence of cyclophosphamide (CPA, the pro-drug). CPA is used as an anticancer pro-drug, which is normally activated in the liver. Macrophages migrate into the spheroids and express P450 enzyme in the hypoxic centre which converts the pro-drug in its cytotoxic metabolite which causes surrounding tumour cell death (Griffiths et al., 2000). This tactic is so-called gene-dependent enzyme pro-drug therapy (GDEPT).

1.5.2 Cancer, Inflammation and Infection

Originally, it was Virchow in the 19th century who observed the presence of leukocytes in tumours. It is now widely recognized that cancer is associated with inflammatory (Balkwill and Mantovani, 2001) and infectious processes, both viral (Pagano et al., 2004) and bacterial, such as *Helicobacter pylori* (Pagano et al., 2004). For example, inflammatory bowel disease can increase the risk of colorectal cancer and chronic airway inflammation can lead to lung carcinogenesis (Karin, 2006). Obesity, for example, could induce chronic inflammation that could increase liver and pancreatic

cancer risk (Grivennikov et al., 2010). Amongst others, chronic infections with Hepatitis B and C viruses can lead to hepatocellular carcinoma whilst *H. pylori* infection can cause gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Pagano et al., 2004); human papillomavirus is associated with cancer of the anus and cervix; in human immunodeficiency virus (HIV) patients there is increased occurrence of primary central nervous system lymphoma, Kaposi's sarcoma and cervical cancer (Ambinder, 1995). LPS from Gram-negative bacteria introduced during open surgical procedure on a mouse model is associated with metastatic growth via increase of serum VEGF (and angiogenesis) and higher tumour cell proliferation (Pidgeon et al., 1999).

H. pylori is a Gram-negative, microaerophilic, spiral-shaped bacterium. It is a human pathogen, which colonises the gastric mucosa and it is responsible for acid secretion which can lead to stomach ulcers and chronic gastritis (Joo et al., 2010). Stomach infections cause gastric mucosa inflammation which can last a lifetime and can lead to stomach carcinoma (Megraud and Lehours, 2004). *H. pylori* infects around 70% of the population of developing countries and 20-30% of industrialized countries (WHO report 2011) and it has been classified as a Class I carcinogen since 1994 by World Health Organisation (Pandey et al., 2010). ROS produced by *H. pylori* can lead to stabilisation of HIF-1α, and this can lead to gastric cancer development (Griffiths et al., 2005).

In contrast, a mixed killed microbial preparation (e.g., the so-called "Coley's toxin") can be used to stimulate acute inflammation and treat some cancers such as bladder cancer (Rakoff-Nahoum and Medzhitov, 2009). Administration of attenuated IL-2-expressing Salmonella strains, which preferentially home in to the tumour environment, can lead to tumour inhibition in mice inoculated with B16 melanoma cells (Al-Ramadi et al., 2008b).

1.5.3 Cancer and Matrix Metalloproteinases

There is extensive evidence on the role of MMPs in promoting tumour growth, invasion and metastasis, since these proteases are over-expressed in many tumours (Rydlova et al., 2008). Invasive tumour cells require the degradation of ECM components and therefore experience enhanced activity of proteolytic enzymes (Berrier and Yamada, 2007). In order to gain a metastatic phenotype, multiple steps need to be taken by a primary tumour: tumour cell attachment, angiogenesis, disruption of basement membrane, intravasation into the blood stream, extravasation at distant sites and growth at the new site (Nelson et al., 2000). Elevated expression of MMPs is associated with increased metastatic potential in many tumour cells (Coussens et al., 2002). MMPs are involved in a microenvironment which helps the angiogenesis process, where degradation of ECM is an essential step for the growth of new blood vessels (Ii et al., 2006). Because of the involvement of MMPs in cancer progression, the inhibition of MMP transcription can be useful for controlling early stage tumours. In clinical trials, the first molecule to be tested was batimastat, which blocks MMP-1, -2, -3 and -9. Marimastat came as a second-generation inhibitor followed by synthetic TIMPs, but no therapeutic efficacy was observed, possibly because those inhibitors were given to patients with advanced rather than early stage cancer (Coussens et al., 2002, Coussens and Werb, 2002). NF-κB is a transcription factor that can also be targeted to inhibit MMP transcription in cancer, such as multiple myeloma (Overall and Lopez-Otin, 2002).

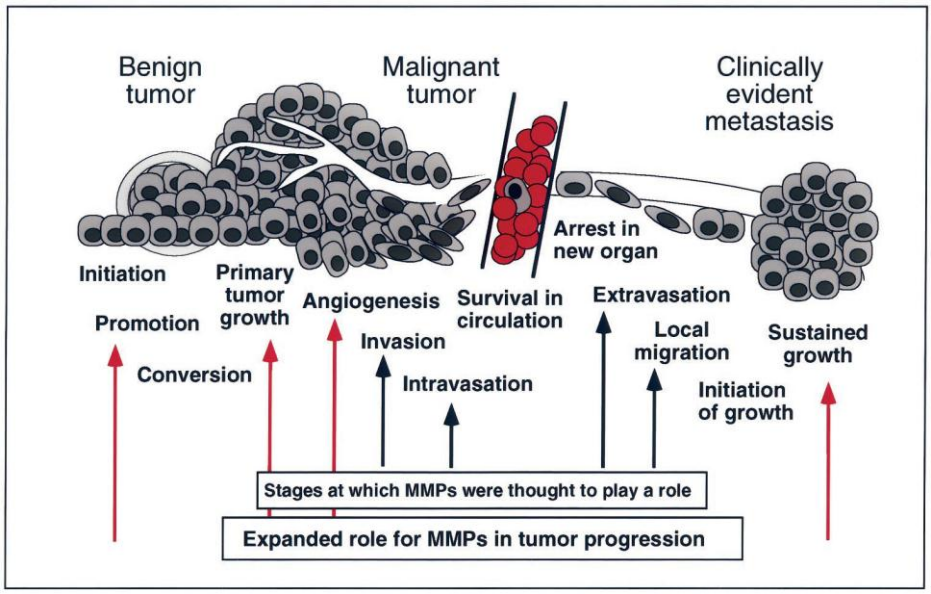


Figure 1-7: MMPs in tumour progression. **MMPs contribute to different steps required for metastasis, from cells invasion, intra/extravasation and migration (Nelson et al., 2000).**

MMPs are mainly expressed by macrophages and tumour cells, however it is not clear which proportion of the two populations of cells is involved in their up-regulation during cancer. Examples of cancers often expressing MMPs are breast, colorectal, lung, prostate, pancreatic and ovarian, however there is evidence that MMPs are actually expressed more by stromal cells than by tumour cells (Nelson et al., 2000), with the exception of MMP-7, which is expressed in many tumours in their epithelia, such as human prostate carcinoma (Knox et al., 1996) and gastric cancer (McDonnell et al., 1991). Co-incubation of human breast cancer cell lines and macrophages leads to increase in the supernatant of MMP-2, -3, -7 and -9 and an increase in the *in vitro* invasiveness of the tumour cells (Hagemann et al., 2004), indicating that tumour cells and macrophages may work together to up-regulate MMPs which facilitate tumour invasion. MMP expression and the stage of tumour are generally correlated (Nelson et al., 2000).

1.6 Matrix metalloproteinase-7 (MMP-7)

1.6.1 MMP-7 structure

Matrix metalloproteinase-7 (MMP-7), also known as *matrilysin* or PUMP-1 (putative metalloproteinase-1), is a secreted protease expressed mainly by epithelial cells (both lumenally and basally, (Wilson and Matrisian, 1996), blood monocytes, in a very variable way in mature monocyte-derived macrophages (Busiek et al., 1992, Busiek et al., 1995), B and T-lymphocytes (Bar-Or et al., 2003) and many tumour cells (Adachi et al., 1999) and reviewed in Wilson and Matrisian, 1996. Production of MMP-7 in healthy cell types is restricted to human endometrium (Rodgers et al., 1993), developmental brain (Szklarczyk et al., 2008), renal mesangial cells (Marti et al., 1992) and gastric epithelium (Honda et al., 1996). MMP-7 mRNA is found in Paneth cells (epithelial cells of the small intestine) where MMP-7 protein is released apically (Wilson et al., 1999). MMP-7 is found to be constitutively expressed in regions with heavy bacterial load, such as the airway lumen and lungs of both healthy (weak expression) (Wadsworth et al., 2010) and cystic fibrosis patients (strong expression) (Dunsmore et al., 1998).

Discovered originally in rat uterus (Woessner and Taplin, 1988), MMP-7 is the smallest member of the MMP family: in its zymogen latent form (pro-MMP-7), the molecular weight is 28 kDa whilst 19 kDa is the molecular weight of the active form (Busiek et al., 1992), which is generated by other proteinases (such as MMP-3 and MMP-10), by trypsin and by auto-activation (Wilson and Matrisian, 1996, Lopez-Boado et al., 2000). MMP-7 is generated from pro-MMP-7 by removal of the N-terminal containing the cysteine domain (approximately 9 kDa).

MMP-7 activated protein (267 amino acids, codified by 6 exons on the human mRNA, NCBI accession number NM_002423.3) consists of five β-sheets, three α-helices and two domains: a pro-domain and a catalytic domain (Figure 1-8), but it lacks the COOH-terminal encoded by other MMPs (Wilson and Matrisian, 1996). The COOH-terminus usually determines MMPs substrate specificity, and the natural lack of it in MMP-7 could explain the broad variety of substrates. As the other MMPs, MMP-7 requires Zn^{2+} (for the catalytic and structural domains) and Ca^{2+} (for the structural domain) for its activity. Amongst the MMPs, MMP-7 is the only other one with MMP-26 (de Coignac et al., 2000, Zhao et al., 2009) and MMP-23 (Velasco et al., 1999) that spontaneously lacks the haemopexin domain in the C-terminal. The haemopexin domain of proMMP-2 and -9 binds the MMP inhibitors TIMP-2 and -1 respectively, possibly suggesting that MMP-7 inhibition by TIMP-1 is reduced in diseased areas (Dufour et al, 2008)).

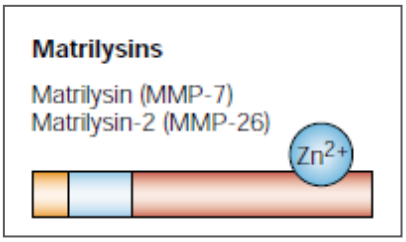


Figure 1-8: **MMP-7 protein structure** - **adapted from Overall and Lopez-Otin, 2002. MMP-7 contains the minimal domains necessary for secretion (the orange portion on the left of the MMP-7 gene), latency (propeptide, the blue region) and catalytic activity (the red region, which binds Zn^{2+}). MMP-26, so-called matrilysin-2, is associated with human cancers of epithelial origin.**

MMP-7 mRNA expression in human monocyte/macrophages is increased after 3 days in culture *in vitro*, peaking at 5-7 days and decreasing at 9 days *in vitro* (Filippov et al., 2003). MMP-7 mRNA is induced rapidly and dramatically by adherence in monocytes and also by differentiation to macrophages for 7 days (Reel et al., 2011). Classically activated macrophages (mainly by LPS) express increased TNF-α and MMP-7 (Busiek et al., 1992, Song et al., 2000).

MMP-7 expression is correlated to the extent of cell-cell contact, which is a determinant of tumours invasive behaviour: in human squamous cell carcinoma cell line, cell-cell contact is mediated by E-cadherin and, as the cells reach confluence, MMP-7 expression is increased (Borchers et al., 1997).

Factor	Effect	Cell type	Ref
TNF-α	Induction	Human mesangial cells	(Marti et al., 1992)
IL-1β		Human mesangial cells	(Marti et al., 1992)
Flagellin		Lung epithelial cells	(Lopez-Boado et al., 2000)
LPS		Macrophages	(Busiek et al., 1992)
H. pylorii		Gastric epithelium	(Wroblewski et al., 2003)
zymosan		MDMs	(Busiek et al., 1995)
Hypoxia		MDMs	(Burke et al., 2003)
IL-4	Inhibition	MDMs	(Busiek et al., 1995)
IFN-γ		MDMs	(Busiek et al., 1995)
IL-10		MDMs	(Busiek et al., 1995)
Dexhametasone		MDMs	(Busiek et al., 1995)
Retinoic acid		MDMs	(Busiek et al., 1995)
Green tea catechins		N/A	(Oneda et al., 2003)
Indomethacin		MDMs	(Busiek et al., 1995)
Glucocorticoids		MDMs	(Busiek et al., 1995)

Table 1-4: factors influencing MMP-7 expression.

1.6.2 MMP-7 promoter structure and gene regulation

MMP-7 is encoded by a single gene within a cluster of human MMP genes on chromosome 11, including MMP-20, -27, -8, -10, -1, -3, -12 and -13. The coding region spans 10 kb and contains six exons.

Gene regulation is essential to modulate gene expression. Gene promoters contain core elements (eg, the TATA box and downstream promoter elements) which are necessary for correctly positioning the RNA Polymerase II for mRNA transcription. MMP-7 is highly regulated at the transcription level and its promoter contains a number of transcription factor (TF) binding sites which regulate transcription (Figure 1-9), including: three binding sites for ets transcription factors located from -55 to -52, -144

to -141 and -168 to -165 (PEA3 group, which consisted of Ets transcription factors ER81, PEA3 and ERM); two functional consensus Tcf binding sites, one in an inverted orientation between -194 and -188 and another between -109 and -103; an AP-1 site located between -67 and -61. All the above mentioned sites are enclosed in the early proximal promoter, -300 bp upstream of the transcription start site (Crawford et al., 2001). The human MMP-7 promoter contains three TIE elements (transforming growth factor β inhibitory element, Figure 1-11) which downregulate the expression of MMPs (Wilson and Matrisian, 1996). Three potential STAT3 binding sites were also found and STAT3 has been implicated in MMP-7 expression in a breast cancer cell line (Yuan et al., 2008). In a study investigating cooperative effects of TF overexpression, maximum activation of the MMP-7 promoter was obtained by combining TCF4/β-catenin/Ets-1/c-Jun and c-Fos vectors (Crawford et al., 2001).

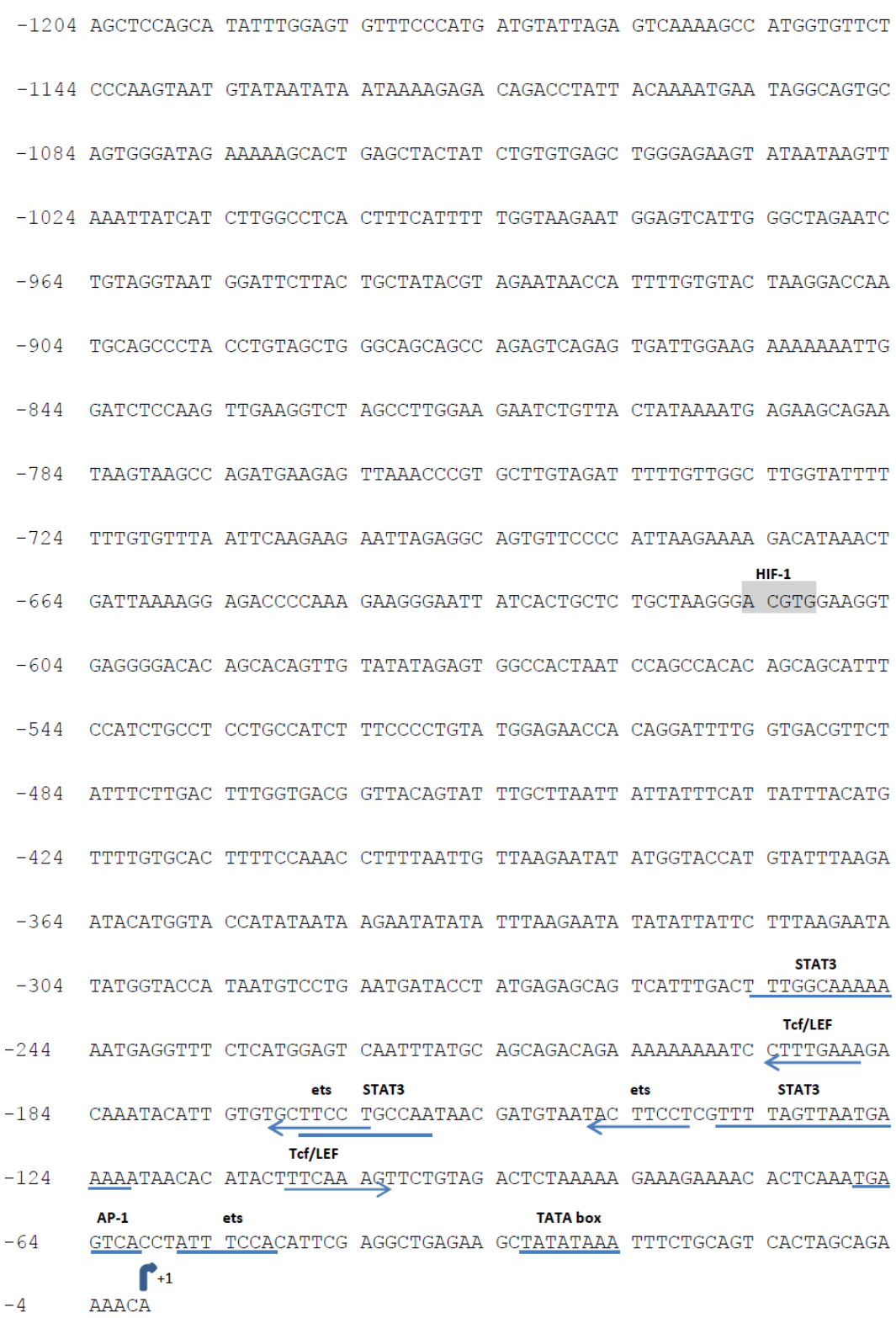


Figure 1-9: human MMP-7 promoter. +1 is the transcription start site, TATA box, ets, HIF, STAT3, AP-1 and Tcf/LEF binding sites are indicated (Gaire et al., 1994). The human MMP-7 promoter sequence accession numer in GenBank is L22525.1.

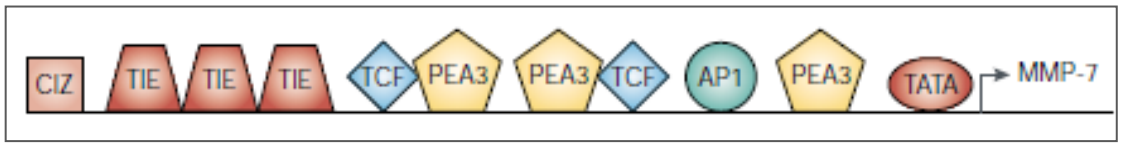


Figure 1-10: Human MMP-7 promoter showing TFs and transcription start site indicated by the arrow (Overall and Lopez-Otin, 2002). CIZ=CAS-interacting zinc-finger protein, TIE= transforming growth-factor-β inhibitory element, TATA= TATA-box

Two single nucleotide polymorphisms (SNP) in the MMP-7 human promoter are known: -181 A/G and -153 C/T. The -181 A/G promoter polymorphism (Figure 1-11) is known to affect MMP-7 gene transcription and is associated with increase risk of gastric cancer (Malik et al., 2011) and of esophageal squamous cell carcinoma, gastric cardiac adenocarcinoma and non-small cell lung carcinoma (Zhang et al., 2005). Both of these polymorphisms influence the binding of nuclear proteins and the combination of the two rare alleles can increase promoter activity (Jormsjo et al., 2001).

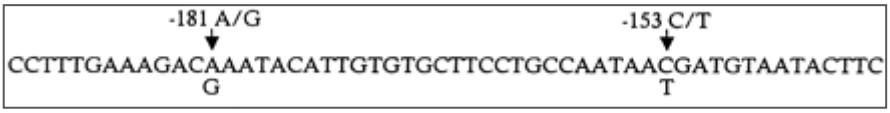


Figure 1-11: The two single nucleotide polymorphisms of MMP-7 human promoter (Jormsjo et al., 2001).

MMP-7 expression is thought to be regulated by ets transcription factors (Crawford et al., 2001) and by AP-1, since inactivation of AP-1 binding site in the promoter leads to down-regulation of MMP-7 gene and protein by Wnt (Le Floch et al., 2005).

Ets can up-regulate MMP-7 expression in human hepatocellular carcinoma (HCC) and hepatoma-derived cell lines, where MMP-7 and ets-1 are co-expressed (Ozaki et al., 2000). Ets is up-regulated in tumours, where it regulates the expression of angiogenic and extracellular matrix remodeling factors promoting an invasive phenotype (Lincoln and Bove, 2005) and in several cancer cell lines by hypoxia (Miyoshi et al., 2006). When PEA3 expression is down-regulated, MMP-7 expression is decreased and gastric

carcinoma cells are shown to be less invasive (de Launoit et al., 2006). PEA3 is the specific ets group member that activates the target matrilysin gene in gastric cancer. PEA3 co-operates with AP1 and regulates β-catenin in mouse colon tumour cells to up-regulate the MMP-7 promoter, thus contributing to tumorigenesis (Crawford et al., 2001). MMP-7 mRNA stimulation by epidermal growth factor and the tumor promoter TPA (12-O-tetradecanoyl-phorbol-13-acetate) is dependent on AP-1 and PEA3 elements (Gaire et al., 1994).

MMP-7 is a transcriptional target of the β-catenin/Tcf/LEF-1 complex (Brabletz et al., 1999). β-catenin is a known proto-oncoprotein: by itself, β-catenin is not sufficient for the induction of MMP-7 expression, but requires co-expression of PEA3, c-Jun and LEF-1 in HEK293 cell line (Crawford et al., 2001). β-catenin interacts with p300 and CREB-binding protein (CBP), its transcriptional coactivators (Hecht et al., 2000). p300 is able to transactivate MMP-7 transcription in synergy with PEA3 and β-catenin-LEF-1 (Crawford 2001). β-catenin and MMP-7 can be used as markers for human pancreatic cancer (Li et al., 2005) and Wnt/β-catenin/Tcf pathway deregulation is involved in many gastrointestinal cancers. The Wnt pathway – which leads to translocation of β-catenin in the nucleus – up-regulates MMP-7 mRNA and protein in HEK293 cells (Le Floch et al., 2005).

1.6.3 MMP-7 functions

MMP-7 performs many different functions. First and foremost, MMP-7 is involved in epithelial-matrix remodeling, degrading many substrates such as casein, gelatin of types I, III, IV and V, elastin, proteoglycans, fibronectin (Powell et al., 1993), laminin and versican (Gaire et al., 1994). ADAM28, a proteinase member of the disintegrin and metalloproteinase family (ADAM), is also a MMP-7 substrate. Constituted by a

membrane and a secreted form, the precursor form of ADAM28 is activated by MMP-7 via removal of the pro-peptide. Its activated secreted form digests the insulin-like growth factor binding proteins, which can favour cancer cell growth and survival (Mochizuki et al., 2004).

As a protease, MMP-7 cleaves and activates other proteases, the structurally similar gelatinases MMP-2 and MMP-9 (Wilson and Matrisian, 1996; Imai et al., 1995), which are also up-regulated in tumours (Yu et al., 1995; Lindenmeyer et al., 1997).

MMP-7 releases TNF-α, a pro-inflammatory cytokine, by cleavage from the membrane of macrophages producing soluble TNF-α which, among many other effects, increases apoptosis (Gearing et al., 1995). Other than soluble TNF-α, MMP-7 sheds the membrane-bound Fas ligand (FasL) generating soluble FasL, affecting the apoptotic pathway (Powell et al., 1999; Vargo-Gogola et al., 2002). Fas is a ubiquitously expressed receptor and FasL is expressed on immune cells. IL-13, present in inflamed airways in asthma patients, can up-regulate MMP-7 mRNA and its activity in airway epithelial cells inducing the release of soluble FasL (Wadsworth et al., 2010). In MMP-7-deficient mice, apoptosis is in fact reduced (Powell et al., 1999).

Many of the MMP-7 functions are related to tumour progression and metastasis. There are reports of MMP-7 inhibiting doxorubicin-induced apoptosis in cancer cells (Mitsiades et al., 2001), although there are contradictory data in the literature (Fingleton et al., 2001). Doxorubicin kills tumour cells via up-regulation of FasL on tumour cell surface and activation of cell death pathway, therefore the ability of MMP-7 to reduce the expression of FasL from cell surface, causes promotion of tumour survival. MMP-7 inactivation (eg, by BB-1101 inhibitor, Leib et al., 2001) can enhance the effectiveness of cancer chemotherapy.

MMP-7 can shed E-Cadherin at the cell surface generating soluble E-Cadherin (Figure 1-12) which can promote migration and invasion of tumour cells (Noe et al., 2001; Ii et al., 2006). The cleavage of β4-integrin (an ECM protein receptor involved in cell adhesion and migration) by MMP-7 *in vitro* and, therefore, its absence, has been associated with different invasive carcinomas (von Bredow et al., 1997).

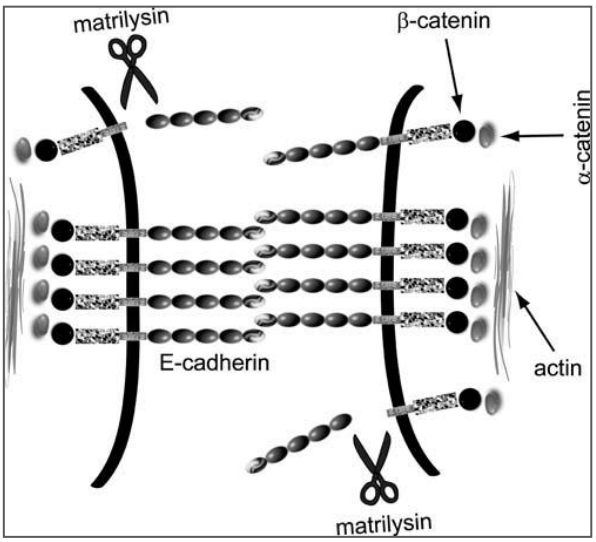


Figure 1-12: MMP-7 sheds E-cadherin adherence junctions, disrupting the E-cadherin/catenin complex. **The extracellular domain of E-cadherin interacts with other E-cadherin proteins on adjacent cells. The cytoplasmic domain of E-cadherin interacts directly with β/α-catenin in a protein complex that is linked to the actin cytoskeleton (Ii et al., 2006).**

MMP-7 can affect the angiogenesis process, which is defined as the process of growth of new blood vessels: MMP-7 can be both pro-angiogenic, via ECM degradation, and anti-angiogenic via the production of polypeptides (angiostatin and endostatin) with this function (Patterson and Sang, 1997; Ii et al., 2006). VEGF is essential for angiogenesis and it has been demonstrated that MMP-7 can cleave ECM-bound VEGF which becomes soluble and is less effective in stimulating vessel growth (Lee et al., 2005).

1.6.4 MMP-7 and hypoxia

Using cDNA array hybridisation, real time RT-PCR and promoter reporter constructs, human MMP-7 has been discovered for the first time to be up-regulated by 16 hrs of hypoxia in primary human macrophages and in a mouse macrophage cell lines (RAW264.7) at the mRNA level (circa 9-fold induction for cDNA array and 6-fold induction for real time RT-PCR) and at the protein level in the cytoplasm of human macrophages in breast carcinoma (Burke *et al*, 2003). MMP-7 hypoxic inducibility has been confirmed also by other groups who found that both MMP-7 mRNA (via RT-PCR) and protein (via Western blot) are up-regulated by hypoxia (1% O_2) in human pancreatic cancer cell lines (Ide et al., 2006) and HepG2 and Hep3B cell lines (Miyoshi et al., 2006). Since MMP-7 is up-regulated in both macrophages and tumour cells, it is then possible to hypothesise a synergy between neoplastic cells and TAMs of which the significance is still unclear; however it is likely that in the early stages of a tumour, before it begins to overexpress MMP-7, macrophage-expressed MMP-7 may be important for tumour progression. Most genes up-regulated by hypoxia are controlled by HIF-1, but MMP-7 appears to be an exception. A weak consensus HIF-binding HRE exists in the MMP-7 promoter (around -600 bp, Figure 1-119), but it is unable to mediate transcriptional up-regulation of a luciferase reporter under hypoxia in macrophage cell lines, even when trimerised (Burke et al., 2003). Recent data indicates that HIF-1 is not in itself sufficient for, or required for, up-regulation of MMP-7, in contrast to the known HIF-1-dependent hypoxia inducible gene PGK, in two HCC cell lines (Miyoshi et al., 2006). Also, LPS could increase HIF-1 (see section 1.4.1.1) and therefore modulate hypoxic gene activation, identifying a novel pathway by which LPS can activate genes in macrophages (Blouin et al., 2004). However, the mechanisms for hypoxic induction of MMP-7 remain mostly unknown.

Alternatively, other elements in the MMP-7 promoter could be responsible for the observed hypoxic induction. A number of HIF-independent mechanisms of transcriptional up-regulation by hypoxia are known, involving transcription factors such as NF-κB**,** which has been discovered as the pathway involved in tranilast-dependent suppression of MMP-7 in response to LPS-stimulation, where tranilast is an agent which inhibits the formation of keloid scarring (Shimizu et al., 2006). Chronic moderate hypoxia has been shown to activate Akt and β-catenin pathway which, in turn, can up-regulate MMP-7 expression in primary human macrophages (Deguchi et al., 2009). In addition to MMP-7, other MMPs are up-regulated by hypoxia at the protein and/or mRNA level, including MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13. For MMP-2, the AP-1 complex mediates hypoxic up-regulation. Interestingly, a search for transcription factor binding sites within the human MMP-7 promoter region shows an AP-1 site in an almost identical position as MMP-2. Thus it is possible that AP-1 could also be involved in MMP-7 up-regulation by hypoxia, since it is a common feature of MMP promoters and it mediates basal promoter activity (Benbow and Brinckerhoff, 1997).

1.6.5 Roles of MMP-7 in cancer

MMP-7 is often up-regulated in solid hypoxic tumors and correlates with poor prognosis and cancer progression (Gustavson et al., 2004), therefore representing a promising therapeutic target in the treatment of tumours (Wielockx et al., 2004; Ii et al., 2006). MMP-7 is expressed in the malignant epithelia of the majority of human colorectal adenocarcinomas, in over 90% of intestinal adenomas (Crawford et al., 2001), in human prostate, gastric and breast adenocarcinomas (Gaire et al., 1994; Honda et al., 1996), in pancreatic cancer (Jones et al., 2004) and in different types of brain

tumours, even though MMP-7 distribution was quite variable (Rome et al., 2007). Moreover, approximately 50% of benign adenomas show low level of MMP-7 expression (Newell et al., 1994). MMP-7 affects tumour size and metastasis in pancreatic ductal adenocarcinoma (Fukuda et al., 2011) and it is present at the invasive front of gastric carcinomas affecting patient's survival (Liu et al., 2002). MMP-7 expression has been found in Ewing's sarcoma, a rare cancer of the bone or the soft tissue, by immunoblotting and immunohistochemistry analysis (Mitsiades et al., 2001). MMP-7 mRNA is overexpressed in liver metastases (Zeng et al., 2002).

In 41% of non-small cell lung cancer (NSCLC), MMP-7 is overexpressed and artesunate, an anti-malaria drug, has been shown to inhibit MMP-7 by suppressing invasion and metastasis (Rasheed et al., 2010). In fact, it also has been shown that inhibition of MMP-7 via fibulin-5, a protein of the ECM, through the ERK pathway can inhibit the tumour invasion process in NSCLC (Yue et al., 2009).

MMP-7 is highly expressed in the early stages of human colorectal tumours and an MMP-7-deficient mouse showed a reduced intestinal tumorigenesis (Wilson et al., 1997). It was also shown that MMP-7 – and other intestinal adenomas markers – was not up-regulated in MyD88-deficient mice crossed with APC^{min+} mice (prone to forming spontaneous intestinal tumours), which suggests that MyD88 regulates expression of genes involved in tumourigenesis, including MMP-7 (al-Ramadi et al., 2008a).

Emodin, an anti-bacterial and anti-tumoural compound, has been shown to inhibit the levels of MMP-7 in SH-SY5Y cells (human neuroblastoma cancer cells) as well as reducing cancer cells invasiveness via suppression of MMP-9 through AP-1 and NF-κB pathway inhibition (Lu et al., 2009). Co-incubation of human breast cancer cell lines and macrophages leads to increase in the mRNAs of several MMPs (MMP-2, -3, -7 and

-9) and an increases in the *in vitro* invasiveness of the tumour cells (Hagemann et al., 2004), indicating that tumour cells and macrophages may work together to up-regulate MMPs which facilitate tumour invasion.

In pre-clinical studies, an antisense oligonucleotide specific for MMP-7 inhibited metastasis of gastric and colon cancers in mice studies, however there were concerns about its stability, efficacy, and off-target effects which could overwhelm its potential as a therapeutic drugs (Miyazaki et al., 1999; Yonemura et al., 2001).

1.6.6 Further roles of MMP-7 in diseases

MMP-7 can play an important role in cardiovascular diseases: it is highly expressed in human atherosclerotic plaques, possibly with the role of weakening the fibrous cap and influencing its rupture (Halpert et al., 1996). In fact, rosuvastatin, a statin used in reducing cardiovascular mortality, reduces MMP-7 (Furman et al., 2004). A role for MMP-7 in neurodegenerative disease has also been acknowledged. Normally, the mature central nervous system contains low levels of MMPs which can become up-regulated in diseases such as multiple sclerosis (MS) and stroke (Bar-Or et al., 2003). Various inflammatory diseases of the central nervous system (such as MS) show enhanced expression of MMP-7 which is induced in infiltrating macrophages in demyelinating MS lesions (Cossins et al., 1997).

MMP-7 cleaves a subunit of the NMDA receptor, a glutamate receptor which is involved in synaptic functions like learning and memory (Szklarczyk et al., 2008).

1.6.7 MMP-7 induction by bacteria

MMP-7 is induced by a variety of bacteria in different epithelial tissues. MMP-7 expression is up-regulated by bacterial exposure (*E. coli, S. typhimurium* and *P. aeruginosa*) in epithelial cells (Lopez-Boado et al., 2000; Lopez-Boado et al., 2001). Flagellin has been identified as an inducer of MMP-7 and the constitutive secretion of MMP-7 in the airway lumen suggests its involvement in innate mucosal immunity (Lopez-Boado et al., 2001). Exposure of HT29 colon cells to *E. coli* increases MMP-7 mRNA after 2 hrs which remains stable at 24 hrs after infection; the hypothesis was that some soluble factor from bacteria (LPS) could be involved in MMP-7 regulation (Lopez-Boado et al., 2000). MMP-7 mRNA levels are markedly stimulated by exposure of monocyte- and bone marrow-derived macrophages to LPS, but with considerable variability (Busiek et al., 1992; Busiek et al., 1995). Polymyxin B, an antibiotic which binds to LPS, added to the human monocytic cell line U937 blocks the induction of MMP-7 by LPS (Lopez-Boado et al., 2000). In addition, MMP-7 was also independently reported to be induced by LPS (100 ng/mL) in the same cell line (Maldonado et al., 2004). MMP-7 is also up-regulated by opsonized zymosan (Busiek et al., 1995). MMP-7 is induced by *H. pylori* in gastric cells *in vitro* via NF-κB and Erk1/2 pathways (Wroblewski et al., 2003) and *in vivo* in gastric biopsy samples at the mRNA and protein level (Bebb et al., 2003). Induction of MMP-7 by *H. pylori* requires contact between viable bacterium and epithelial cells (Crawford et al., 2003) and is partially dependent on gastrin (Yin et al., 2010). Immunocytochemistry for MMP-7 shows epithelial staining after co-culture of gastric epithelial cells with *H. pylori* (Yin et al., 2010). The MMP-7 -181 A>G promoter variant has been associated to the development of gastric ulcers in *H. pylori*-infected patients (Hellmig et al., 2006). A recent study

shows up-regulation of MMP-7 both at the mRNA and protein level in gastric cancer after catecholamine stimulation; this regulation was AP-1-dependent (Shi et al., 2010; Yin et al., 2010). Up-regulation of MMP-7 expression can therefore represent a part of a larger response to bacterial infection.

1.6.8 MMP-7 and innate immunity

MMP-7 has also important functions in the immune system especially in lung and intestine, where it proteolytically activates anti-bacterial peptides (pro-defensins) in response to microbial products such as lipopolysaccharide, LPS (Wilson et al., 1999; Burke, 2004). MMP-7-deficient mice are more susceptible to bacterial infection *in vivo* and *in vitro* because pro-defensins are not processed in their active forms (Wilson et al., 1999); the same MMP-7-deficient mouse has severe defects in wound-healing (Dunsmore et al., 1998).

1.7 Previous work that led to the current project

Some preliminary experiments from our group on the possible regulation of MMP-7 promoter in hypoxia, showed that an HIF-1α over-expression construct had no effect on the up-regulation of a -2.3 kb MMP-7 promoter reporter construct which is regulated by hypoxia (in CHO13.5 KA HIF-knockout cells, Figure 1-13) or on both the -2.3 kb and the shorter -296 bp MMP-7 constructs (in HCT-116 cells, Figure 1-14). Therefore, these results suggest that MMP-7 -2.3 kb promoter reporter construct seems to be up-regulated by hypoxia, but in a HIF-1α-independent fashion. However, these data do not exclude the possibility that HIF-1 might be involved in MMP-7 induction in macrophages or tumour cells.

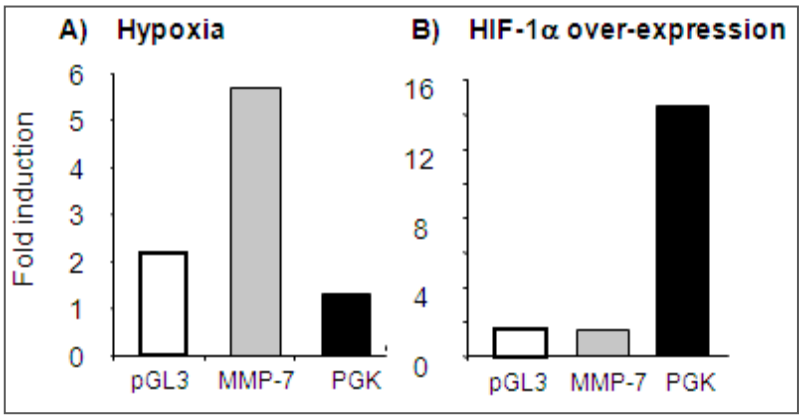


Figure 1-13: Luciferase expression in response to A) 16 hrs hypoxia (0.5% O_2) and B) HIF-1α overexpression of pGL3-Basic (negative control), MMP-7 -2.3 kb promoter reporter, and PGK (phosphoglycerate kinase, a positive control) constructs transfected into CHO 13.5 KA HIF- knockout cells. Fold inductions are calculated relative to expression in normoxia (20.9% O_2) (A) or to expression levels obtained by co-transfection with an empty control plasmid not expressing HIF-1α (B). A representative example of 3 independent experiments is shown (*unpublished results from Dr B. Burke, personal communication*).

An ets-1 over-expression construct, however, did up-regulate the -296 bp promoter reporter construct in HCT-116 cells (Figure 1-14), suggesting the possibility of involvement of the ets-1 transcription factor in MMP-7 hypoxic up-regulation, since ets-1 is known to be up-regulated by hypoxia (Miyoshi et al., 2006).

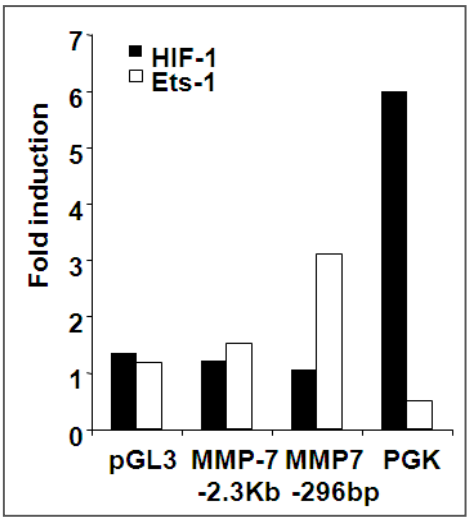


Figure 1-14: Effect of over expression of transcription factors HIF-1 and ets-1 on pGL3-Basic (negative control), MMP-7 -2.3 kb and -296 bp and PGK (positive control) luciferase reporter constructs in HCT-116 colon tumour cell line under normoxic condition (20.9% O_2). Cells were incubated with over expression and reporter plasmids for 24 hrs, lysed and assayed for luciferase. Fold induction relative to cell co-transfected with each reporter construct and an empty plasmid control (*unpublished results from Dr B. Burke, personal communication*).

It also has been demonstrated that the up-regulation of MMP-7 mRNA is not due to an effect of hypoxia on mRNA stability, since mRNA stability was unaffected by exposure of hMDM to hypoxia (*unpublished results from Dr B. Burke*, Figure 1-15).

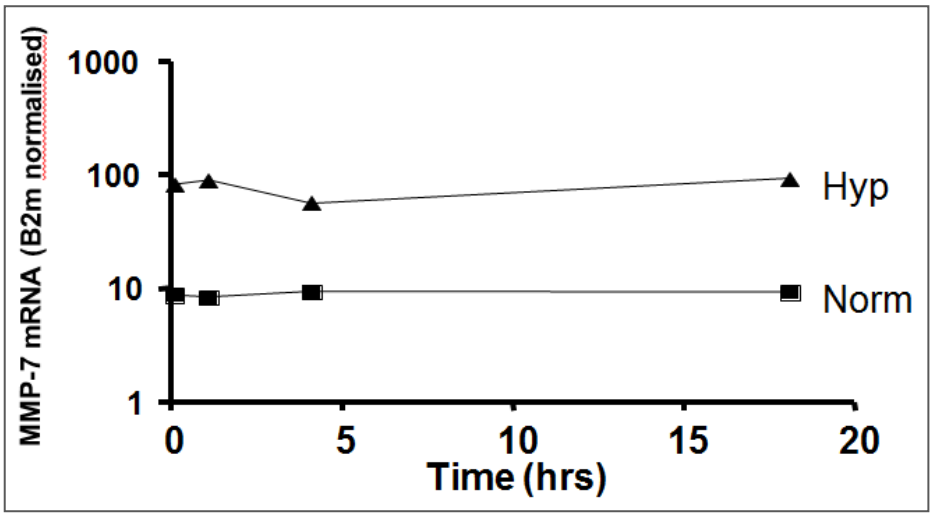


Figure 1-15: MMP-7 mRNA stability in primary human blood monocyte-derived macrophages. **Adherence-purified macrophages 4 days *in vitro* (2 x 10^6 per well in 6-well plates) were incubated at normal oxygen level (20.9% O_2; Norm) or in hypoxia (0.2% O_2; Hyp) for 18 hrs. Time zero wells were harvested followed by addition of 10 µg/ml actinomycin D to the remaining Norm and Hyp wells. Cells were returned then to the appropriate oxygen tensions. Wells were lysed after 0,1,4,18 hrs. Real Time RT-PCR was carried out for MMP-7 mRNA and data was normalised using the β-2 microglobulin housekeeping gene.**

Aims of this project

As previously outlined, several studies have now highlighted the up-regulation of MMP-7 during hypoxia. Hypoxia is a common feature in many diseased tissues where macrophage can accumulate and activate the transcription of many genes, including MMP-7. The above gives the rationale behind my interest in the mechanism regulating MMP-7 hypoxic induction.

The main aims of this project therefore were:

1. Investigation of the effect of hypoxia on MMP-7 mRNA and protein expression in human monocyte-derived macrophages subjected to hypoxia (0.2% O_2).
2. Determination of the mechanism responsible for MMP-7 promoter regulation using wild-type and mutated promoter reporter constructs, band shift assays, transcription factor over-expression and silencing.
3. Analysis of the pathways involved in MMP-7 hypoxic regulation via the use of specific inhibitor of those pathways.

Chapter 2 Materials and Methods

2.1 General consumables

Unless otherwise stated, consumables were purchased from Axygen Biosciences, VWR International, Beckton Dickinson and Costar (Corning).

2.2 Provided solutions recipes

The following solutions were prepared and autoclaved by the media kitchen in the Infection, Immunity and Inflammation Department of the University of Leicester.

1x PBS

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4

LB (LURIA BERTANI)-MEDIUM

10 g/L NaCl
5 g/L Yeast extract
10 g/L Tryptone

LB-AGAR

10 g/L NaCl
5 g/L Yeast extract
10 g/L Tryptone
15 g/L Agar

2.3 Luciferase constructs

Reporter assays are used to test for transcription activity of a promoter: a reporter gene which encodes a protein (in this project, luciferase) is attached to an upstream promoter of interest.

A number of luciferase reporter constructs were received as a kind gift from Dr H.C. Crawford (Pharmacological Sciences - State University of New York at Stony Brook, USA). These constructs consist of different lengths of the MMP-7 human promoter cloned into the pGL3-Basic firefly luciferase reporter plasmid (Promega, E1751) which lacks a eukaryotic promoter (Figure 2-1).

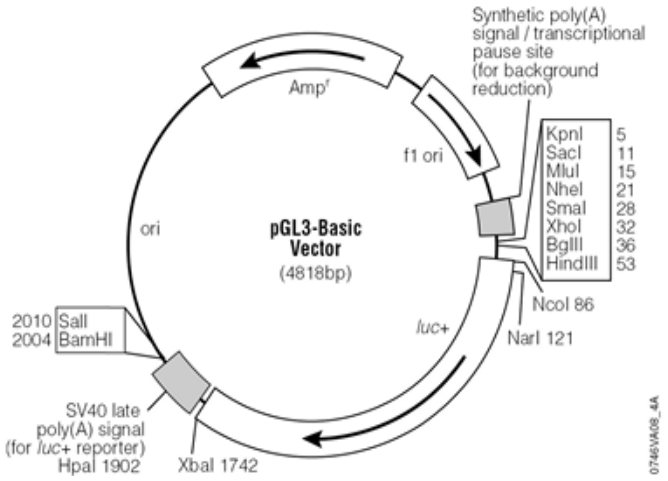


Figure 2-1: pGL3-Basic promoterless vector (www.promega.com).

The -2.3 kb and -296 bp MMP-7 human promoter constructs (Crawford *et al*, 2001) were extensively used in my work. The longer construct (-2.3 kb) was generated by cutting a 4.2 kb genomic clone of the MMP-7 promoter (Crawford et al., 2001).

Dr. Crawford also provided us with eight more luciferase constructs (Crawford et al., 2001) containing mutated versions of the MMP-7 -296 bp promoter sequence in which transcription factor sites were altered or ablated in order to study the effect of those transcription factors on gene transcription (Figure 2-2). Sense oligonucleotides used by

(Crawford et al., 2001) for mutagenesis were as follows, with mutated positions underlined:

-163 Ets: 5'-GTGTGCTTCTGCCAATAACGATG-3';

-139 Ets: 5'-GTAATACTTCTTCGTTTTAGTTAATG-3';

-51 Ets: 5'-CCTATTTCTACATTCGAGGC-3';

-188 Tcf: 5'-GACAGAAAAAAAAATCATTGGCGATACAAATACATTGTGTG-3';

-105 Tcf: 5'-TAACACATAATCGCCAACTTCTGTAGACTC-3';

mAP-1, 5'-CAAACGAGTGACCTATTTCCAC-3'.

Two further constructs contain double mutated transcription factor binding sites (-163 Ets /-139 Ets and -188/-105 Tcf).

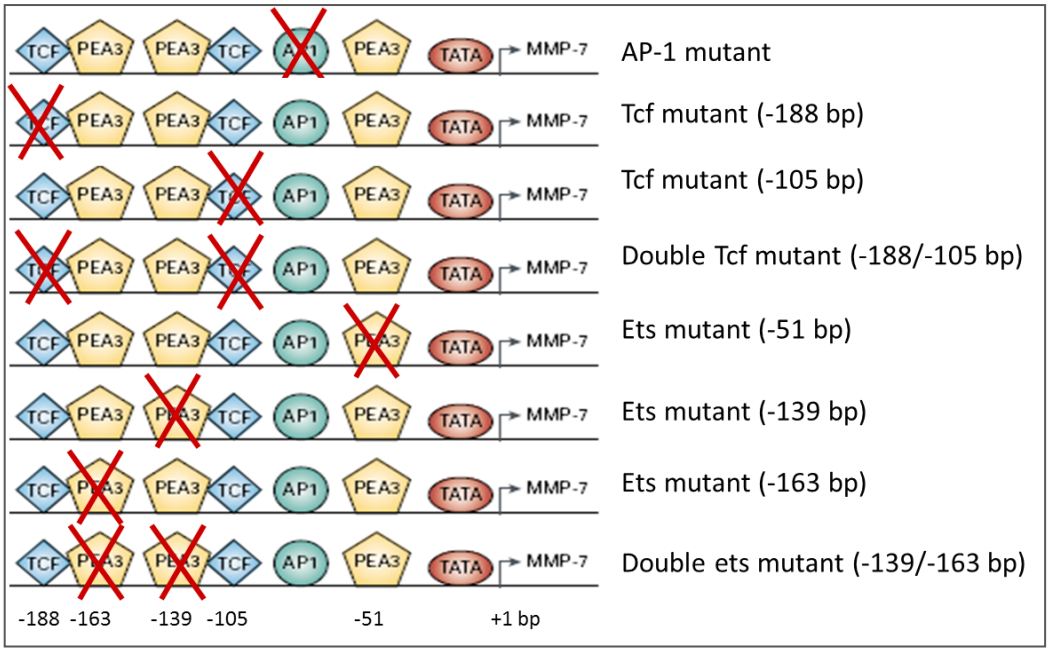


Figure 2-2: The different MMP-7 promoter reporter mutants constructs.

Also, an MMP-7 promoter luciferase construct extending to -965 bp, derived by a *Sma*I-*Sna*BI restriction fragment deletion from the -2.3 kb MMP-7 luciferase promoter, was kindly provided by Dr B. Burke.

As an internal plasmid reporter control for normalization of luciferase data, the *Renilla* pRL-TK vector (Promega, E2241, Figure 2-3), was co-transfected with other MMP-7 promoter luciferase reporter plasmids. *Renilla* luciferase, derived from the marine organism *Renilla reniformis* (sea pansy) is a 36 kDa enzyme which does not require post-translational modification for activity, and therefore, like firefly luciferase, functions as a genetic reporter immediately following translation. The pRL-TK vector contains the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide constitutive low-to-moderate levels of *Renilla* luciferase expression.

The PGK construct, used as a hypoxia-inducible positive control, is a luciferase reporter construct which promoter is a trimer of the HRE from the murine PGK-1 gene (Ameri et al., 2002).

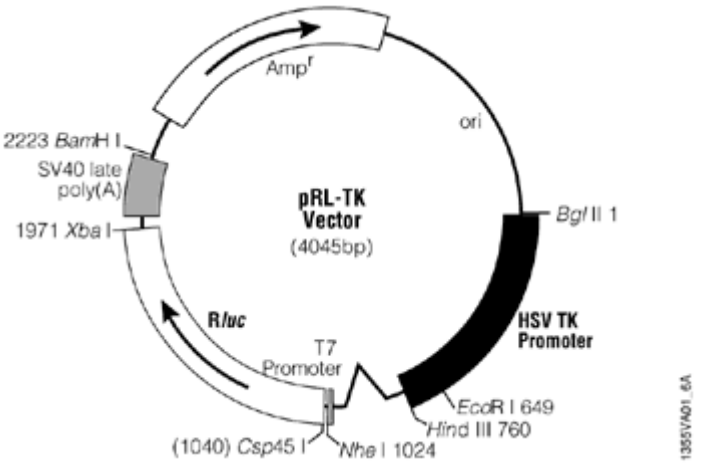


Figure 2-3: Renilla pRL-TK vector (www.promega.com).

2.3.1 Transcription factor over-expression

An ets-1 over-expression construct pcDNAneo-hEts-1 (Ozaki et al., 2000) was provided by Dr M. Oucida, Department of Molecular Genetics, Graduate School of Medicine and Dentistry, Okayama University, JAPAN.

2.4 Peripheral Blood Mononuclear Cell Isolation and Monocytes Culture

Blood samples for the preparation of mononuclear cells were obtained from healthy volunteer donors after written consent was obtained from them. University of Leicester Ethics committee approval was obtained for this study. All blood samples were kept anonymously and it was not possible to link the outcome of the results to individuals. Blood was removed by trained phlebotomists via a sterile syringe (Fisher, SZR-205-160T) containing Heparin solution (Leo Laboratories Ltd. UK, provided by Glenfield Hospital, Leicester, UK) sufficient to give a final concentration of 10 U/mL, in order to prevent blood coagulation.

PBMCs were isolated from whole blood samples by density gradient centrifugation on Ficoll-Paque™ PLUS (GE Healthcare 17-1440-03). After dilution of the blood 1:1 in HBSS (Hank's Balanced Salt Solution, SIGMA H-6648) to maintain the pH and osmotic balance, anti-coagulated whole blood was layered carefully over Ficoll-Paque™ PLUS and centrifuged at 400 x g for 30 min at room temperature, with minimum acceleration and no brake. After the centrifugation step, the following layers were observed: the bottom of the tube contained erythrocytes and granulocytes, PBMCs were located at the Ficoll-Paque/plasma interface, and the plasma/serum layer was on top. The mononuclear layer at the interface containing monocytes and lymphocytes was carefully extracted, transferred to a clean tube and washed twice (400 x g for 5 min) with HBSS (to remove platelets and plasma) and once with fresh medium. The medium used for preparation and culture of human mononuclear cells was Iscove's Modified Dulbecco's Medium (SIGMA, I-3390), with 2.5% human AB serum (Biosera, S4190, batch number 017K0443, 1 EU/mL endotoxin contamination), 1% L-glutamine (SIGMA, G-7513), at a final concentration of 2 mM, and 1% Penicillin/Streptomycin solution (SIGMA, P-0781), at a final concentration of 50,000 U Penicillin and 50 mg

streptomycin. The medium used for the majority of this project was filtered through a Gambro U-2000 ultrafiltration column to remove contaminating LPS (Benkhart et al., 2000). LPS quantification using the Endosafe PTS instrument (Charles River Laboratories, PTS-100) of unfiltered and filtered Iscove's medium gave readings of ~2.6 EU (Endotoxin Units)/mL (~267 pg/mL) for the unfiltered and <0.1 EU/mL (<10 pg/mL) for the filtered, confirming the effectiveness of the filtration procedure.

Cell count was performed using a hemocytometer, after diluting the cell suspension 1:10 and loading in it around 10 µL. The chamber is 0.1 mm deep. 25 central squares of the grid pattern bounded by triple lines (1 mm x 1 mm) were counted and the cell concentration (cells per mL) calculated as follows:

$$\text{Cells per mL} = \text{cells counted} \times 10^4 \times 10 \text{ (dilution factor)}$$

Where 10^4 is the number of mm^3 in 1 mL.

PBMCs were plated in either 6-well low attachment (LA) plates (Appleton Woods, CC227) to obtain the whole PBMC population or in 6-well normal attachment tissue culture-treated plates (Nunc, VWR International, 734-0991) to generate adherence-purified human monocyte-derived macrophages (hMDM), depending on the experiments performed. The seeding density was adjusted to 2×10^6 cells per well (each well contains 2 mL of medium) in normal attachment plates and 4×10^6 cells per well in LA plates. After 2 hrs incubation (Kendro Laboratory Products, Hera Cell 150 incubator; conditions set at 5% CO_2 and 37°C), the medium in the normal attachment plate was changed and replaced with fresh medium in order to remove non-adherent cells, mainly lymphocytes (Burke et al., 2003) and retain the monocytes which are adherent to plastic (Reel et al., 2011). With this method, CD68 marker used to identify macrophages found >95% purity in the adherent population (Burke et al., 2003). In the LA plates the medium was not replaced as the whole PBMC population was in

suspension. The adherent monocytes in the normal plates and the PBMC in the LA plates were then cultured for 5 or 7 days at 37°C, which was previously reported as an appropriate time for the generation of macrophages from monocytes (Fedele et al., 2002). Macrophages derived from monocytes by culturing them *in vitro* in normal attachment plates and represent 7-11% of the starting PBMCs (Arbeit et al., 1982).

2.5 Cell lines

The cell lines used in this project were:

Name	Cell line type	Adherence status
AGS-1	gastric cancer cells	adherent
HCT-116	human colon tumour intestinal epithelial cells	adherent
HEK293	human embryonic kidney cells	adherent
HepG2	human hepatocellular carcinoma cells	adherent
MM6	MonoMac6, human monocytic cells	adherent
RAW 264.7	mouse leukaemic monocyte macrophage cells	adherent
THP-1	human acute monocytic leukemia cells	non adherent
U937	human leukemic monocyte lymphoma cells	non adherent

U937, THP-1, RAW 264.7, HepG2 and HEK293 were grown in filtered RPMI 1640 medium (SIGMA, R-0883) supplemented with 10% Fetal Bovine Serum, FBS (Seromed, S0115), 1% Penicillin/Streptomycin (P/S) Solution, 1% L-glutamine and 1x NEAA (non-essential amino acids, provided as 100x, Autogen Bioclear, K-0293). AGS were grown in F-12 Ham medium (SIGMA, N-6658) supplemented with 10% FBS, 1% P/S Solution, 1% L-glutamine. HCT-116 cells were grown in McCoy's 5a medium (SIGMA, M8403) supplemented with 10% FBS, 1% P/S Solution and 1% L-glutamine. For cell plating numbers, refer to legend in the text figures.

2.6 Hypoxia conditions

Hypoxic cultures were incubated in a hypoxia incubator (Galaxy ® CO_2 incubator, RS Biotech Ltd; 0.2% O_2, 5% CO_2, 94.8% N_2, 37°C) for 18 hrs (or different times as indicated). The level of oxygen was independently verified using a Mini O2 oxygen analyser (Analox Sensor Technology) positioned inside the hypoxic incubator.

2.7 H. pylori

H. pylori are Gram negative, motile, helix-shaped and microaerophilic (requiring less O_2 concentration than in the atmosphere for growth) bacteria. Mostly residing in the stomach, they have been demonstrated to induce stomach cancer (Jankowski, 1991).

Strain NCTC (National Collection of Type Cultures) 11637 (generously donated by Dr Jeroen Stoof, School of molecular medical science, University of Nottingham) were streaked on Columbia Agar plates and grown in a VAIN incubator (variable atmosphere incubator, at 10% CO_2. 7% O_2, 84% N_2, 41°C).

COLUMBIA BLOOD AGAR

Columbia blood agar base, autoclaved	Oxoid (CM0331)
7% defibrinated horse blood*	Oxoid (SR0050C)
10 μg/mL vancomycin	kindly provided by Dr R. Haigh, Dept of Genetics, University of Leicester

***Defribinated horse blood was lysed for 2 hrs at 37°C in a 2.3% saponin solution (from a 10% freshly prepared saponin solution, SIGMA, S7900).**

H. pylori colonies were washed from Columbia agar plates and frozen down at -80°C in Brain Heart Infusion Broth (Oxoid, CM1135, supplemented with 0.1% yeast extract, Oxoid, CM0019, 10 μg/mL vancomycin and 10% FBS) and 25% glycerol (SIGMA G-7757). *H. pylori* can grow in liquid medium without agitation and its growth is quite slow, over 3 to 4 days. Viable counts were determined as Colony Forming Unit

(CFU)/mL: *H. pylori* grown in medium were serially diluted 10-fold in filtered PBS (for recipe see section 2.2) and spotted drop-wise on Columbia blood agar plates until visible enough to count them. DNA extraction, 16S RNA PCR and sequencing were performed at the Department of Genetics (thanks to Dr Richard Haigh) to confirm the identity of the bacteria.

For experimental use, AGS cells were plated at 2 x 10^5 cells per well in 6-well plates (see Section 2.5) and live *H. pylori* added in medium without antibiotics at 1 and 0.1 MOI (multiplicity of infection) 4 hrs after plating cells. Following 18 hrs normoxic or hypoxic conditions, infected and non-infected AGS cells were prepared for RNA isolation (see section 2.15).

2.8 LPS stimulation of macrophages

Lipopolysaccharide (LPS, serotype R515, Enzo Life Science, ALX-581-007-L002) used for cell stimulation was purified from *E. coli*. Some work has also been performed using SAE LPS (*Salmonella abortus equii*, Alexis, 581-009-L002) at different concentrations. After 5 or 7 days incubation under normal oxygen tensions according to the protocol, 2 × 10^6 adherent hMDMs were treated with different LPS concentrations and incubated under normoxia or hypoxia for a further 18 hrs prior to RNA isolation (see section 2.15).

2.9 Cell treatments with inhibitors

LY294002 (New England Biolabs, 9901) and wortmannin (EMD Biosciences, 681675) are inhibitors of PI3-Kinase (PI3K, Powell et al., 1999). After 5 days incubation under normal oxygen tensions, adherent hMDMs prepared as stated in section 2.4 were treated with LY290042 at a final concentrations of 10 μM and/or wortmannin at a final concentration of 200 nM. Both LY294002 and wortmannin were pre-incubated with the

cells at 37°C for 1 hr before LPS treatment. All wells received 2 μL of DMSO/inhibitor solution and the same volume of DMSO (SIGMA, D-5879) only was added to cells as a carrier control. The wells were subjected to normoxia or hypoxia for further 18 hrs before RNA isolation (see section 2.15).

CAPE (Caffeic acid phenylethyl ester, Enzo Life Science, ALX-270-244-M010), a potent and specific inhibitor of NF-κB activation (Natarajan et al., 1996), was pre-incubated with the cells at 37°C for 1 hr before LPS treatment. All wells received 2 μL of DMSO/inhibitor solution and the same volume of DMSO (SIGMA, D-5879) only was added to cells as a carrier control. The wells were subjected to normoxia or hypoxia for further 18 hrs before RNA isolation (see section 2.15). Polymixin B Sulfate Salt (PMB, SIGMA, P-1004) is an inhibitor of LPS and it was pre-incubated for 45 min with LPS in a dilution 1:1000 in respect of the LPS concentration before addition to the cells.

2.10 Detection of cell viability by flow cytometry

Macrophage viability in normoxia and hypoxia was assessed by propidium iodide (PI, SIGMA P4170) exclusion. PI is a non-permeant dye that can enter dead cells because of their loss of normal membrane function; by DNA intercalation, PI produces a "red" fluorescence (550-670 nm). In a flow cytometer, cells are passed single-file through a laser beam by continuous laminar flow of a fine stream of the cell suspension. Each cell scatters some laser light and also emits fluorescent light from the fluorochrome, excited by the laser. The cytometer measures several parameters simultaneously for each cell, including: forward scatter intensity (proportional to cell size), 90 degree or right angle side scatter intensity (proportional to the quantity of granular structures within the cell) and fluorescence intensities at several wavelengths (for determination of antigens on or

in the cell and the number of cells expressing the antigen, if fluorochrome-labelled antibodies are used) which is also known as mean fluorescence intensity.

FACSCalibur was used to determine the percentage of PI-stained cells.

After 5 days in culture, PBMCs were incubated for 18 hrs or 5 days in normoxia or hypoxia, then harvested from low attachment (LA) wells and centrifuged (Eppendorf, Centrifuge 5417R) at 400 x g for 5 min. The pellet was then resuspended in 2 mL cold PBS and divided into two aliquots, one of which was left untreated and one treated with PI, added to the cells at a concentration of 250 ng/mL and incubated at room temperature for 5 min. Background readings were obtained from unstained cells. Controls for PI efficiency were obtained from cells treated with 1 mL Triton X-100 0.1% (SIGMA, T-8532) in PBS, which is a detergent used for cell permeabilisation and 1 mL 0.1% saponin (SIGMA, S-7900) in PBS, a natural surfactant, which is used to cause permeabilisation by solubilising cell membrane cholesterol. Acquisition of data was followed by analysis using CellQuest software (Becton Dickinson). The instrument settings are shown in Table 2-1.

Parameter	Voltage	AMPGain	Mode
FCS	E-1	6.53	Lin
SSC	250	1.0	Log
FL2	366	1.0	Log

Table 2-1: Instrument settings for the flow cytometric acquisition of human PMBCs. FCS: Forward Scatter, SSC: Side Scatter, FL2: Filter 2 (propidium iodide fluorescence is usually assigned to the FL2 channel).

2.11 Molecular Cloning of the human MMP-7 promoter

Two new MMP-7 promoter constructs were generated:

MMP-7 343 bp in pGL3-Basic construct: the MMP-7 343 bp consists of the -296 bp construct and +47 bp downstream of the transcription start site into pGL3-Basic

luciferase vector (Figure 2-1) was created by PCR cloning. Using PubMed (human MMP-7 promoter sequence, Accession number L22525.1), primers were designed for this cloning.

Forward primer (*Nhe*I site, NEB, R0131):

5'-AATTGCTAGCGGTACCATAATGTCCTGAATGA–3'

Reverse primer (*Hind*III site, NEB, R0104):

5'-AATTAAGCTTCCGTCCAGAGACAATTGTTCT-3'

The insert was cut with *Nhe*I and *Hind*III and inserted then in pGL3-Basic cut with the same restriction digestion enzymes (see below for detailed explanation).

MMP-7 -296bp in pGL4.10[*luc*2]: the MMP-7 -296 bp in pGL4.10[*luc*2] construct was generated via a "cut and paste" cloning technique. The insert was removed from the previous MMP-7 -296 bp pGL3 construct with *Kpn*I/*Hind*III and inserted in pGL4.10[*luc2*] (Figure 2-4) using the same sites.

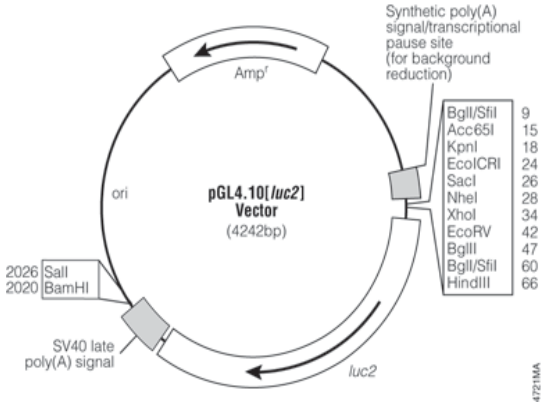


Figure 2-4: pGL4.10[luc2] (www.promega.com).

2.11.1 PCR cloning

The correspondent MMP-7 promoter sequence is amplified from genomic DNA by polymerase chain reaction (PCR), which occurs in three stages; denaturation of the

template DNA, primer annealing and primer extension. In a PCR, the DNA polymerase is able to synthesize a new strand of DNA complementary to the template strand by using two primers, short pieces (usually around 20 base pairs) of synthetic DNA. Amplification of the target sequence on the template is achieved by repeated cycles which elongate the primers according to the target sequence.

The enzyme used for PCR cloning was *Pfu* DNA polymerase (Fermentas, EP0571), a highly thermostable DNA polymerase which catalyzes 5'→3' DNA polymerization and exhibits 3'→5' exonuclease activity for correction of errors in nucleotide insertion.

Reagent	Final concentration	Volume
Pfu buffer (10x)	1x	2.5 µL
$MgSO_4$ (25 mM)	2 mM	2 µL
dNTPs (10 mM)	0.2 mM	0.5 µL
Genomic DNA		1 µL
Primer 5'(10 µM)	1 µM	2.5 µL
Primer 3'(10 µM)	1 µM	2.5 µL
Pfu DNA polymerase (2.5 U/µL)	1.25 U	0.5 µL
Sterile dH_2O		x µL
Final volume		25 µL

Table 2-2: PCR reaction mix for the generation of MMP-7 fragments.

Step	Temperature and duration	cycle
Initial denaturation	95°C, hold 30 secs	1
Denaturation	95°C, hold 30 secs	35
Annealing	56°C, hold 30 secs	
Elongation	72°C, hold 1 min	
Final extension	72°C, hold 15 min	1
Final hold	4°C	∞

Table 2-3: PCR conditions for generation of MMP-7 promoter fragment.

PCRs were performed in either Techne TC-3000 or Progene 231-103 thermocyclers.

2.11.2 Agarose gel electrophoresis

Electrophoresis is used to separate DNA fragments by applying an electric field to a gel. DNA is negatively charged and migrates towards the positive pole of an electric field. The rate of migration of DNA in a gel depends on the concentration of the agarose, the applied voltage, and conformation and length of the DNA molecules. Generally, longer DNA fragments migrate more slowly through the gel.

The sizes of PCR amplified fragments of the MMP-7 promoter were confirmed by agarose gel electrophoresis. To prepare these gels, a solution of a suitable percentage of agarose (SIGMA, A-5093) in 1x Tris-acetate EDTA, pH 8.0 (TAE, see recipe at the end of this section) was heated in a microwave oven until the agarose was dissolved.

The solution was left to cool and ethidium bromide (EtBr, SIGMA, 46065) was added to give a final concentration of 0.25 µg/mL; the solution was poured into a gel tray with a comb in it and allowed to set. EtBr is a fluorescent dye which intercalates between double stranded DNA strands and allows visualisation by exposure to UV light. Once the agarose solution was solidified and complete wells were formed, the comb was removed and the gel was placed in an electrophoresis tank, which was filled with enough 1x TAE buffer to cover the gel. DNA samples were mixed with 6x gel loading buffer (see recipe below) and water to give a final concentration of 1x, and loaded into the wells. A voltage of 100 V was applied for about 2 hrs. The gel run was terminated when the bromophenol blue had migrated about 3/4 of the length of the gel. The gel was then examined under UV light and photographed and, in the analysis, the size of a fragment was estimated by comparison with a DNA molecular weight marker containing fragment of known length.

50X TAE

Reagent	Company (cat number)
2 M Tris-acetate	SIGMA (T-1503)
50 mM EDTA	SIGMA (E-7889)

6X GEL LOADING BUFFER

Reagent	Company (cat number)
0.25% (w/v) bromophenol blue	Merck (111746)
30% (v/v) glycerol	SIGMA (G-7757)
0.25% (w/v) xylene cyanol FF	SIGMA (X-4126)

2.11.3 Restriction digestion

Endonucleases can be used for cleaving double-stranded DNA at specific positions called restriction sites. The cleavage site is located within a specific recognition sequence (or, for certain enzymes such as *Mnl*I and *Fok*I, the cleavage site is found at a definite distance from their recognition sites) and, depending on the enzyme, DNA fragments with blunt ends or cohesive (sticky) ends are generated.

After the PCR reaction, the 343 bp MMP-7 promoter construct insert and the pGL3-Basic vector (Figure 2-1) were both digested with *Nhe*I and *Hind*III for 1 hr 30 min at 37°C in buffer 2 (NEB, R0131). The purpose of the digestion was to create in both insert and vector the same ends for insertion of the insert into the vector.

*Nhe*I recognition site

*Hind*III recognition site

Reagent	Final concentration	Vector	Insert
10x Buffer 2	1x	2 µL	2 µL
100x BSA	1x	2 µL	2 µL
*Nhe*I (20 U/µL)	10 U	0.5 µL	0.5 µL
*Hind*III (20 U/µL)	10 U	0.5 µL	0.5 µL
DNA	0.5-2 µg	x µL	x µL
Sterile dH_2O		x µL	x µL
Final volume	20 µL		

Table 2-4: restriction digestion reaction for the MMP-7 343 bp construct and pGL3-Basic plasmid.

The MMP-7 -296 bp insert in pGL3 construct was removed using a *Kpn*I/*Hind*III digestion and inserted into the pGL4.10[*luc2*] vector (Figure 2-4) which had been cut using the same sites.

*Kpn*I recognition site

Reagent	Final concentration	Vector	Insert
10x Buffer Multi Core	1x	2 µL	2 µL
*Kpn*I (20 U/µL)	10 U	0.5 µL	0.5 µL
*Hind*III (20 U/µL)	10 U	0.5 µL	0.5 µL
DNA	1 µg	x µL	x µL
Sterile dH_2O		x µL	x µL
Final volume	20 µL		

Table 2-5: restriction digestion reaction for the MMP-7 -296 bp construct and pGL4.10[*luc*2] plasmid.

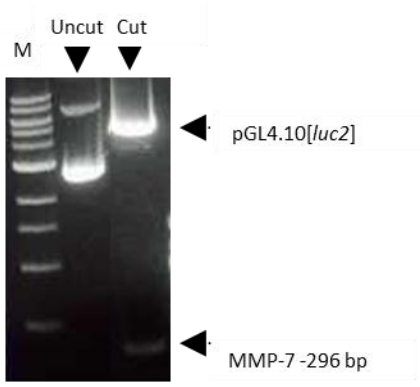


Figure 2-5: cloning of MMP-7 -296 bp in pGL4.10[*luc*2] vector. 1% agarose gel electrophoresis of restriction digested plasmid of -296 bp of MMP-7 promoter and pGL4.10[*luc*2] construct. The clone was digested with *Kpn*I and *Hind*III and run with an undigested -296 bp MMP-7 in pGL4.10[*luc*2] construct. M=marker (1 Kb DNA ladder, NEB, N3232). Uncut=undigested -296 bp MMP-7 in pGL4.10[*luc*2] construct. Cut=digested -296 bp MMP-7 in pGL4.10[*luc*2] construct.

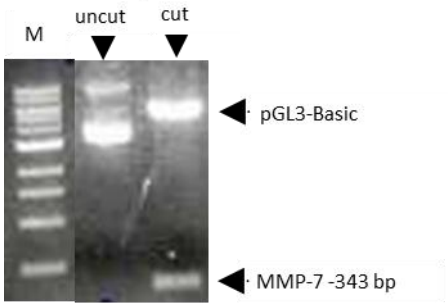


Figure 2-6: cloning of MMP-7 343 bp in pGL3-Basic vector. 1% agarose gel electrophoresis of restriction digested plasmid of 343 bp of MMP-7 promoter and pGL3-Basic construct. The clone was digested with *Nhe*I and *Hind*III and run with an undigested 343 bp MMP-7 in pGL3 construct. M=marker (1 Kb DNA ladder). Uncut=undigested 343 bp MMP-7 in pGL3 construct. Cut=digested 343 bp MMP-7 in pGL3 construct.

2.11.4 QIAquick DNA Agarose Gel Extraction

The digested pGL3-Basic, pGL4.10[*luc*2] and fragments of MMP-7 promoter sequences were extracted and purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen, 28704) according to the manufacturer's instructions.

Three volumes of QG Buffer were added to one volume of extracted gel slice (e.g. 300 µL of QG Buffer to 100 mg of extracted gel) in a tube and then incubated at 50°C for 10 min. When the gel slice was dissolved, one volume of isopropanol was added to one volume of extracted gel slice (e.g. 100 µL of isopropanol to 100 mg of extracted gel) to increase the yield of DNA fragments. The resulting mixture was transferred to a

QIAquick spin column and centrifuged at 13,000 x g for 1 min to allow DNA binding to the column. 750 µL of PE Buffer containing ethanol was then added to the column and spun down at 13,000 x g for 1 min to wash the DNA. Then, the column was placed over a new tube and 30 µL of EB Buffer was added to the centre of column for DNA elution, left for 1 min and then centrifuged at 13,000 x g for 1 min. The purified DNA fragments were analysed by agarose gel electrophoresis.

2.11.5 Ligation of MMP-7 DNA into plasmid vector

DNA ligation is the process of joining together two DNA molecule ends, most commonly to ligate an insert DNA molecule into a plasmid vector, ready for bacterial transformation. It creates a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. This reaction is usually catalyzed by a DNA ligase enzyme such as T4 DNA ligase.

After running the digested vector and insert on an agarose gel (see section 2.11.4), the DNA concentration was estimated by eye by comparison of that particular band with the known DNA ladder band which it resembles the most. This step is necessary to determine vector and insert concentration for ligation purposes. Vector (V) and insert MMP-7 promoter (I) were ligated at V:I ratio of 1:3 according to the formula:

$$\frac{50 \text{ ng V x kb size of I}}{\text{kb size of V}} \text{ x 3 = ng I}$$

To prepare the ligation mixture the vector, MMP-7 DNA fragments and sterile dH_2O were incubated in the Thermocycler (PCR machine) at 42°C for 2 min and then allowed to cool down to room temperature. The rest of the reagents, T4 ligase (Invitrogen, Y90001), ligase buffer and rATP (required for ligase reaction) were then added and the reaction was incubated at 4°C for 16 hrs (over-night) to complete ligation and followed by storage at -20°C.

Reagent	Final concentration	Volume
Digested vector	50 ng	x µL
Digested insert	x ng	x µL
T4 DNA Ligase (4 U/µl)	4 U	1 µL
10x T4 DNA Ligase buffer	1x	2 µL
rATP (10 mM)	1 mM	2 µL
Sterile dH_2O		x µL
Final volume		20 µL

Table 2-6: **Reaction mixture for ligation of the digested MMP-7 promoter sequences into the vector.**

2.11.6 Bacterial transformation

Bacterial transformation is a technique used to introduce a DNA such a plasmid into bacteria, enabling amplification of the plasmid in order to produce large quantities of it. Library Efficiency® DH5α™ competent cells (Invitrogen, 18263-012) and XL-2 Blue Supercompetent cells (Stratagene, 200150) were used for transformation following the respective manufacturer's protocol.

Library Efficiency® DH5α™ competent cells were thawed on ice, mixed and aliquot 100 µl in pre-chilled tubes. Ligated DNA was diluted 1:5 with TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) and 1 µL of the diluted ligation product (1–10 ng) was added to the competent cells. As a transformation efficiency control, 5 µl (50 pg) of the pUC19 DNA (included in the kit) were added to a different aliquot of competent cells, swirled and incubated on ice for 30 min. Tubes were heat-pulsed in a 42°C pre-warmed water bath for 45s (the duration of the heat pulse is critical for maximum efficiency) and incubated on ice for 2 min. To follow, 900 µl of room temperature S.O.C. medium (included in the kit) was added in each tube and they were incubated at 37°C for 1 hr shaking at 225–250 rpm. After this, for the pUC19 control the reaction was diluted 1:10 with S.O.C. medium and 100 µL were spread on a previously prepared LB agar plate

(LB agar was prepared by the media kitchen III Dept of University of Leicester, see section 2.2 for recipe) containing 100 μg/mL of antibiotic ampicillin (SIGMA, A-9393) for colony selection. For the experimental DNA, 100-200 μL of the transformation mixture were plated on LB agar plates with ampicillin. Plates were incubated at 37°C over-night. For the pUC19 control, around 50 colonies were expected ($\geq 1\times10^8$ cfu/μg pUC19 DNA). For the experimental DNA, the number of colonies will vary according to this formula:

$$\text{CFU/}\mu\text{g} = \frac{\text{CFU on control plate}}{\text{pg pUC19 DNA}} \times \frac{1 \times 10^6 \text{ pg}}{\mu\text{g}} \times \frac{\text{volume of transformants}}{\text{volume plated}} \times \text{dilution factor}$$

XL-2 Blue Supercompetent cells were thawed on ice, mixed and aliquot 100 μl in pre-chilled tubes. 2 μl β-mercaptoethanol (provided with the kit) were added to each aliquot of cells, the contents were mixed gently and incubated on ice for 10 min, swirling gently every 2 min. 0.1–50 ng of the experimental DNA and 10 pg of the pUC18 control DNA (to check transformation efficiency, included in the kit) were added to different aliquot of cells, swirled and incubated on ice for 30 min. Tubes were heat-pulsed in a 42°C water bath for 30s (the duration of the heat pulse is critical for maximum efficiency) and incubated on ice for 2 min. To follow, 900 μl of pre-heated at 42°C NZY medium (provided with the kit) was added in each tube and they were incubated at 37°C for 1 h shaking at 225–250 rpm. After this, for the pUC18 control 5 μL of the reaction was spread on a LB agar plate containing 100 μg/mL of antibiotic ampicillin for colony selection. For the experimental DNA, ≤200 μL of the transformation mixture were plated on LB agar plates with ampicillin. Plates were incubated at 37°C overnight. For the pUC18 control, around 250 colonies were expected ($\geq 5 \times 10^9$ cfu/μg pUC18 DNA). For the experimental DNA, the number of colonies would vary.

2.11.7 Plasmid isolation: Mini-prep

Isolation of the cloned construct was performed in the first instance using QIAprep Spin MiniPrep Kit (Qiagen, 27104) and following manufacturer's instructions. This mini-prep is based on bacterial cell lysis, DNA adsorption and elution.

Single colonies from a plate were picked with a loop and inoculated overnight in a 3 mL LB medium (LB medium was prepared by the media kitchen III Dept of University of Leicester, see section 2.2 for recipe) with 100 μg/mL of antibiotic ampicillin, shaking at 300 rpm at 37 ^{o}C. Bacterial cells were harvested by centrifugation of 1.5 mL culture in a microcentrifuge tube at 6,800 x g for 3 min at room temperature and the pellet was resuspended in 250 μL Buffer P1, where RNaseA was previously added. 250 μL of Buffer 2 (NaOH/SDS) was then added for bacterial lysis under alkaline conditions and the suspension mixed by tube inversion 4-6 times, followed by 5 min incubation. SDS solubilises lipids and protein content of cell membrane, leading to cell lysis and release of the plasmid and chromosomal DNA which is denatured by alkaline conditions. To follow, 350 μL of buffer N3, which neutralizes the pH, were added and the suspension mixed immediately by inverting the tube 4-6 times and centrifugate for 10 min at 18,000 x g. Buffer N3 also provides high salt concentration which causes protein and chromosomal DNA denaturation, cell debris and SDS precipitation; the plasmid stays in the clear supernatant which is applied to the QIAprep spin column inserted in a clean microcentrifuge tube, centrifuged for 30-60s at 18,000 x g and the flow-through is discarded. The spin column contains a silica membrane for plasmid DNA adsorption. The QIAprep spin column is then washed by addition of 750 μL Buffer PE (wash buffer), centrifuged as above, the flow-through is discarded and the column is centrifuged an additional minute to remove residual wash buffer. The spin column is moved to a clean microcentrifuge tube and 50 μL of Buffer EB (10 mM Tris-HCl, pH

8.5) are added, let stand for 1 minute and centrifuge at 18,000 x g for 1 min for DNA elution. Following miniprep, the DNA Absorbance and concentration is determined (see section 2.11.9) and the construct is checked via restriction digestion (see section 0) (200-500 ng DNA are normally enough). If the restriction digestion is satisfactory, the positive mini-prep are sent to PNACL (Protein and Nucleic Acid Chemistry Laboratory, based in the Hodgkin Building, University of Leicester) for sequencing with RVPrimer 3 and 4 (for both the pGL3-Basic and pGL4.10[*luc*2] constructs). Once the sequencing confirms the right construct, this is followed by maxi-prep (see following section). Mini-prep DNA is stored at -20°C for future use and the leftover from the bacteria culture is kept at -80°C following addition of glycerol 50% in PBS (for the positive sequences).

2.11.8 Plasmid isolation: Maxi-prep

Plasmid DNA was prepared using the Qiagen EndoFree^{TM} Plasmid Maxi Kit (Qiagen, 12362) according to manufacturer's instructions.

A 3 mL culture of LB medium containing 100 µg/ml ampicillin was inoculated with a single bacteria colony picked from a selective plate and incubated ~8 hours at 37^{o}C shaking at 300 rpm. The culture was diluted 1:1000 in 100 mL of selective LB medium containing 100 µg/mL ampicillin and grown for 12-16 hrs (over-night) at 37^{o}C shaking at 300 rpm. Over-night grown bacterial cultures were pelleted at 6,000 x g for 30 min at 4°C. The bacteria pellet was resuspended in 10 mL of Buffer P1 (which contains RNase A) followed by lysis by addition of 10 mL of Buffer P2. The buffer and bacteria were mixed vigorously by inverting the tube five times and then incubated 5 min at room temperature. During the incubation time, the QIAfilter cartridge was prepared by screwing the cap onto the outlet nozzle of it and placing into a convenient tube. 10 mL

of chilled neutralisation Buffer P3 were added to the lysate which was mixed vigorously by inverting the tube 5 times followed by transferring it into the QIAfilter cartridge and incubation at room temperature for 10 min. After incubation, the QIAfilter cartridge was placed over a centrifuge tube, the cap was removed and the lysate was filtered into the tube by inserting the plunger - insoluble complexes containing chromosomal DNA, salt, detergent and proteins are removed by passing the lysate through a filter. Plasmid DNA is bound to the column under low salt and pH conditions and RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash. The filtered lysate was mixed gently with 2.5 mL of buffer ER - a specific removal buffer which prevents the binding of LPS molecules to the column in the subsequent step allowing purification of DNA containing <0.1 endotoxin U/µg of DNA - and incubated on ice for 30 min. A QIAGEN-tip column was equilibrated by applying 10 mL of QBT buffer and allowed to empty by gravity flow. Then, the filtered lysate was transferred into the QIAGEN-tip column and allowed to enter the resin by gravity flow. The QIAGEN-tip column was washed twice with 30 mL Buffer QC and the DNA was eluted with 15 mL Buffer QN into a centrifuge tube - plasmid-DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. The DNA was precipitated by addition of 10.5 mL isopropanol and centrifugation at 15,000 x g for 30 min at 4^{o}C. The supernatant was carefully decanted and the DNA pellet was washed with 5 mL of 70% ethanol - this final wash step removes residual salt - and centrifuged at 10,000 x g for 10 min at 4^{o}C. The DNA-pellet was air dried for 10-15 min, resuspended in 300-450 µl endotoxin-free Buffer TE and stored at -20^{0}C. Finally, the DNA concentration was measured at 260 nm (see section 2.11.9).

2.11.9 DNA absorbance and concentration measurements

DNA absorbs UV light at a wavelength of 260 nm (A_{260}). DNA absorbance was measured using a spectrophotometer with a quartz cuvette in the early phases of the project and a Nanodrop 1000 (Nanodrop Products) in the later phases. DNA purity was checked by the $A_{260/280}$ ratio for determination of protein contamination, since proteins absorb maximally at 280 nm. Pure preparations of DNA have A_{260}/A_{280} values of ~1.8 whereas contamination with protein results in lower values.

The DNA concentration was determined by spectrophotometer at A_{260} and calculated using the following formula, which relates the amount of light absorbed to the concentration of the absorbing molecule:

$$\text{Concentration } (\mu g/mL) = \varepsilon \, d \, A_{260}$$

ε=extinction coefficient (ε=50 for DNA, since when A_{260}=1, ε=50 µg/mL)

d=dilution factor

Again, the DNA construct can be further checked with restriction digestion and/or sequencing with appropriate primers.

2.12 Eukaryotic cell transfection

All transfection experiments were performed in non-filtered media.

2.12.1 Transfection of primary human macrophages

Primary human macrophages were transiently transfected with jetPEI® transfection reagent (Polyplus, 101-40).

jetPEI® is a linear polyethylenimine (PEI) which condenses the DNA to be transfected into positively charged particles, allowing its interaction with anionic residues on the cell surface (eg, proteoglycans and phospholipids); the DNA-jetPEI® complex is then

internalized via endocytosis. In the endosomes, PEI works as a proton sponge by accepting protons leading to increase of endosomal pH which prevents activation of endosomal enzymes resulting in protection of DNA from degradation. This mechanism eventually leads to endosome swelling and rupture, releasing the jetPEI®/DNA complexes into the cytoplasm, thereby allowing nuclear transport for subsequent transcription. The ionic charge of the jetPEI®/DNA complex is essential for efficient transfection. This charge is identified by a number called N/P ratio, where N represents the number of nitrogen residues on the jetPEI® reagent and P the phosphate residues on the DNA; to have a positively charged complex, an N/P ratio > 3 is required. The following formula, recommended by the manufacturer of jetPEI®, was used in order to calculate the N/P ratio which takes into account the volume of jetPEI® reagent used for any given amount of DNA:

$$N/P = \frac{7.5 \times \mu L \text{ jetPEI}^{®}}{3 \times \mu g \text{ DNA}}$$

where 7.5 is the concentration (mM) of nitrogen residues in jetPEI® and 3 represents the nmoles of phosphate per µg of DNA.

Following a number of optimization experiments, the optimal conditions for transfection of the promoter reporter constructs into macrophages were as follows:

- hMDM were prepared as described in section 2.4 and incubated 5 or 7 days *in vitro* before transfection, to allow the monocytes to differentiate in mature macrophages by adherence. On the 5th or 7th day as specified, macrophages were co-transfected with 1.5 µg of MMP-7 reporter DNA or PGK or CMV-GFP per well and *Renilla* pRL-TK (see Section 2.3) plasmid DNA (used as an internal control in order to normalize transfection results) at the specified concentration. Briefly, for each transfection, 1.5 µg of DNA and 4.8 µL of jetPEI® (following

optimization, the N/P ratio used in all experiments was 8) were separately diluted in 100 µL of 150 mM NaCl (Polyplus, 702-50). The jetPEI® dilution was vortexed for 10s followed by its addition to the DNA. The solution was vortexed for 15s, briefly spun down and incubated for 15-30 min at room temperature before complexes were added drop-wise to the well; the plate was swirled to ensure homogenous mixing of the complexes. Following 1 hr or more (as specified) of incubation in normoxia after transfection ("rest" time, to allow the macrophages to recover from the transfection procedure), macrophages were either kept in normoxia or moved to the hypoxia incubator for 18 hrs or 5 days, as specified. After that, cells were lysed and dual luciferase assay was performed as described in Section 2.13.1.

- PBMC were prepared and plated in LA plates, 4 x 10^6 cells per well, as described in section 2.4 and incubated 5 or 7 days *in vitro* as specified before transfection. The wells were prepared in order to include two transfections in the same well (this would minimize variability when normalizing later on). Since PBMC preparations plated in LA plates include both monocytes and lymphocytes in suspension, on the day of the transfection cells were resuspended, pooled together in a tube and kept at 37°C until ready for transfection. Briefly, for each transfection (using two wells per transfection) 3 µg of DNA and 9.6 µL of jetPEI® were separately diluted in 200 µL of 150 mM NaCl. The jetPEI® dilution was vortexed for 10s followed by its addition to the DNA dilution. The solution was vortexed for 15s, briefly spun down and incubated for 15-30 min at room temperature before the 400 µL of complex was added drop-wise to the 4 mL of complete Iscove's medium containing 4 × 10^6 PBMC which had been previously resuspended as described above. Transfected

cells were split equally in two and plated in 2 mL at a density of 2×10^6 per well in 6-well normal adherence plates and returned to normoxia for 1 hr (or more, as specified) to allow the macrophages to recover from the transfection procedure; to follow, macrophages were either kept in normoxia or moved to the hypoxia incubator for 18 hrs or 5 days. After the experimental time, cells were lysed, single luciferase assay was performed as described in Section 2.13.2 and protein concentration was measured for normalization purposes.

2.12.2 Transfection of cell lines

Different cell lines were transfected with different transfection reagents according to optimized methods developed in the laboratory by previous members.

U937 and RAW264.7 cell lines were transfected with Effectene (Qiagen, 301425), a lipid reagent used with an enhancer (which permits DNA condensation) which coats the condensed DNA with cationic lipid for efficient eukaryotic cell uptake (Figure 2-7).

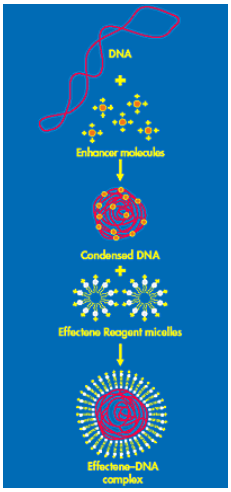


Figure 2-7: Mode of action of Effectene transfection reagent (www.qiagen.com).

Effectene transfection requires two steps:

1. DNA is combined with the Enhancer which allows DNA condensation (2-5 min at room temperature)
2. Effectene is added to the condensed DNA (in a ratio of 10:1) to form complexes (5-10 min at room temperature).

Component	Volume
Buffer EC	149 µL
MMP-7 constructs	1 µg
Renilla construct	10 ng
Enhancer	8 µL
Effectene reagent	10 µL

The complexes were mixed with growth medium and added to the cells.

RAW264.7 cells were plated at 4-6 x 10^5 cells per well in 6-well plates after the transfection complex was added. MMP-7 -2.3 Kb or -296 bp (both at 1 µg) and *Renilla* pRL-TK 10 ng were co-transfected. When the pcDNAneo-hEts-1 plasmid was tested, it was co-transfected at concentrations of 150 ng to 1 µg.

U937 cells were plated at 4-6 x 10^5 cells per well in 6-well plates 24 hrs before transfection, and 50 ng/mL PMA (Phorbol 12-myristate 13-acetate) was added before transfection, since it has been demonstrated that U937 cells up-regulate MMP-7 expression after PMA-treatment (Jormsjo et al., 2001).

THP-1, HCT-116 and HepG2 cells were transfected with FuGENE 6 (Roche, 1 815 091). THP-1 and HCT-116 cells were plated at 2 x 10^5 and 4 x 10^5 (respectively) cells per well in 6-well plates 24 hrs before transfection and co-transfected with *Renilla* pRL-TK, MMP-7 -2.3 Kb or -296 bp in a ratio 1:3 with the transfection reagent; FuGENE 6 and medium were mixed and incubated 5 min at room temperature (Table 2-7). The

DNA (1 µg) and *Renilla* pRL-TK (50 ng) were then added and the complex incubated for 15 min at room termperature. The day after transfection, dual luciferase assay was performed (see section 2.13.1).

HepG2 cells were plated at 2 x 10^5 cells per well in 6-wells plate 24 hrs before transfection and transfected with MMP-7 -296 bp (1 µg) in a ratio 2:3 with the transfection reagent; 4 hrs after transfection, the cells containing the same construct were pooled and split into two new wells for protein assay (used for normalisation). One sample of each construct was then incubated in hypoxia and one in normoxia for 18 hrs before single luciferase assay was performed (see section 2.13.2).

Component	Volume
DNA	1 µg
Renilla	50 ng
FuGENE 6 reagent	3 µL
Serum-free medium	x µL
Final volume	100 µL

Component	Volume
DNA	2 µg
FuGENE 6 reagent	3 µL
Serum-free medium	x µL
Final volume	100 µL

Table 2-7: HCT-116 and THP-1 (left) and HepG2 (right) mixture component for MMP-7 transfection with FuGENE 6.

2.12.3 RNA interference

RNA interference (RNAi) is a method of modulating and silencing gene expression. Short interfering RNAs (siRNA) are small molecules that bind to mRNA and mediate its degradation. The RNAi pathway is initiated by the enzyme Dicer, which cleaves long double-stranded RNA (dsRNA) into short fragments of ~20 nucleotides (siRNAs). Each siRNA is unwound into two single-stranded RNAs, the passenger strand which is degraded and the guide strand which is incorporated into the RNA-induced silencing complex (RISC). The guide strand pairs with a complementary sequence of mRNA and

induces cleavage by the RISC complex. Most often, siRNA are used instead of dsRNA since dsRNA initiated an innate immune response (interferon pathway) in the organism.

jetPEI® is also an efficient reagent for oligonucleotide delivery and is recommended for transfection of siRNA. The protocol for the *in vitro* DNA oligonucleotide transfection was followed and the amount of jetPEI® was calculated according to this equation:

$$\frac{\text{oligo base number x pmol x } 8\text{x}10^{-3}}{\text{jetPEI concentration (mM)}} = \mu\text{L jetPEI}$$

0.25-0.5 μM or 250-500 pmol of ets-1 and HIF sense and antisense oligonucleotides were used (Table 2-8). jetPEI® co-transfections with ets-1 or HIF oligonucleotides, MMP-7 -296 bp or -2.3 Kb 1 μg and *Renilla* pRL-Tk 100 ng were performed. After 4 hrs, the medium with the complexes was removed and exchanged for fresh medium which was kept for further 20 hrs before RNA isolation.

Gene	Oligonucleotide sequence
Ets-1 (Kessler et al., 2006)	Sense:5'-CCGCCTTCATGGTGCCAGGAGTG-3' Antisense: 5'-CACTCCTGGCACCATGAAGGCGG-3'
HIF-1 (Caniggia et al., 2000)	Sense: 5'-ATGGAGGGCGCCGGC-3' Antisense: 5'-GCCGGCGCCCTCCAT-3'

Table 2-8: Oligonucleotides used for ets-1 and HIF-1 mRNA silencing.

2.13 Luciferase assay

During the course of the project, both single and dual luciferase assays were employed.

2.13.1 Dual-Luciferase® Reporter Assay System (Promega, E1960)

In a luciferase dual-reporter assay, cells are co-transfected with a firefly luciferase gene under the control of a promoter of the gene of interest (the "experimental" construct) and a *Renilla* luciferase gene under the control of a constitutively expressed promoter (the "control" reporter, see section 2.3). This assay is used to determine the transfection

and experimental efficiency at the same time. One problem can be that control plasmids can be suppressed or "squelched" by other plasmids and therefore affect the interpretation of results (Farr and Roman, 1992).

Cells were lysed with 250 µl/well Passive Lysis Buffer 1x (supplied as a 5x concentrate, diluted before use with distilled water) included in the Dual-Luciferase Reporter assay System after one wash in sterile PBS in order to remove dead cells and residual growth medium for adherent cells; for suspension cells (like U937), the content of a well was transferred to a tube, centrifuged at 400 x g for 5 min and resuspended in 250 µl 1x Passive Lysis Buffer. The plate or tube was then incubated for 15 min at room temperature and frozen for 15 min at -80°C to complete cell lysis. The firefly luciferase reporter is measured firstly by adding 100 µL of Luciferase Assay Reagent II (LAR II) to 20 µL of sample in a luminometer tube (Falcon, 352008) in order to generate a luminescent signal which is then measured by a luminometer. After this, firefly luminescence is quenched by addition of 100 µL of Stop & Glo® reagent (prepared by adding 1 volume of 50x Stop & Glo® Substrate to 49 volumes of Stop & Glo® Buffer) and *Renilla* luciferase signal is then measured in the same tube. All measurements were carried out at room temperature (temperature affects luciferase activity). The luminometer used was a SIRIUS single-tube luminometer (Berthold Detection System), with a 30s pre-read delay and a 10s measurement period. Background luminescence from empty tubes or tubes with LAR II only or non transfected control cell, as appropriate, were subtracted from all measurements. Background luminescence can result from autofluorescence and static electricity discharges from the plastic tube samples: the key step taken to avoid this was not using gloves during the procedure.

Samples were kept frozen for future reference at -20°C or -80°C.

2.13.2 Luciferase Assay System (Promega, E1501)

This system is used to measure the firefly luciferase in cells transfected with the "experimental" reporter construct only. Subsequent analyses involve protein concentration assay (see section 2.14) for normalisation based on cell number. Cells are lysed with 250 µL/well Cell Culture Lysis Reagent 1x supplied as a 5x concentrate (diluted before use with distilled water) included in the Luciferase Reporter Assay System. For suspension cells (like PBMC), the content of a well is transferred to a tube, centrifuged at 400 x g for 5 min and resuspended in 250 µl 1x Passive Lysis Buffer. The tubes are then incubated for 15 min at room temperature and frozen for 15 min at -80°C to allow complete cell lysis. The firefly luciferase reporter expression is measured by adding 100 µL of Luciferase Assay Reagent I (LAR I) to 20 µL of sample in a luminometer tube in order to generate a luminescent signal. Measurements are analysed after subtraction of the correspondent background value (blank tube or tube with LAR I only or non transfected control).

Samples were kept frozen for future reference in the -20°C or -80°C.

2.14 Protein concentration assay

Protein concentration was assessed with Pierce® 660 nm Protein Assay Reagent (Thermo Scientific, 22660). This method is a colorimetric assay: the binding of the dye (reddish-brown colour) with certain amino acids in the protein (basic residues such as histidine, arginine and lysine) causes a shift in the absorbance from 450 nm to 660 nm (green colour) which is detectable via spectrophotometer. Bovine serum albumin (BSA, SIGMA, A-3294) was used to prepare a set of standards: the standard curve was prepared in the range of 50-500 µg/mL with 5 concentrations included in this range; this standard curve corrects for variation in experiment conditions and is used for estimation

of protein concentration in relation with absorbance. Protein sample and protein standard are processed in the same fashion.

For the assay, 10 µL of blank, standard or sample were added in a 96-well plate. 150 µL of the Protein Assay Reagent was added to each well; the plate was then rocked on a plate shaker for 1 min and incubated at room temperature for 5 min, whilst eliminating any bubbles in the well which can produce false readings in the spectrophotometer. The blank wells were used to zero the plate reader and the absorbance was read at 660 nm with the Microplate Reader (model 680, Bio-Rad) using the Microplate Manager 5.2.1® software (Bio-Rad). The absorbance readings obtained from the standard curve were used to build a graph of absorbance as a function of protein concentration (Figure 2-8). The concentration of unknown samples is obtained by building a linear regression curve using the BSA standard curve, with the known BSA concentration on the *x* axis and the absorbance on the *y* axis; the unknown sample concentration is interpolated from the linear regression of the standard curve.

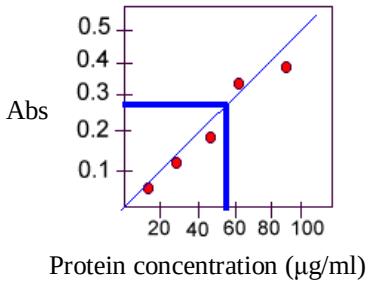


Figure 2-8: Graph of absorbance in relation to protein concentration.

2.15 Total RNA Isolation

Obtaining good quality RNA is very important to every subsequent step in molecular biology experiments. To isolate intact RNA, this method includes disruption of cells, denaturation of RNA/protein complexes, inactivation of endogenous ribonucleases and purification from contaminating DNA and proteins.

After the treatment in normoxia or hypoxia, cells were lysed directly on the culture plate using 500 µL per well in a 6-well-plate of cold TRI Reagent® (SIGMA, T-9424) and transferred in a pre-chilled tube, which minimises the release of endogenous RNAses. TRI Reagent® is a guanidine thiocyanate and phenol-based reagent which allows cell disruption and recovery of RNA, DNA and proteins. Lysis was then made complete by vigorous pipetting of the solution. After adding 100 µL chloroform (SIGMA, C-2432) to form a biphasic mixture, vortexing for 15s and incubating at room temperature for 5 min, centrifugation was performed at 12,000 x g for 15 min at 4°C after which the mixture separates in 3 phases: a top colorless aqueous phase containing RNA, an interphase containing DNA and a bottom red organic phase containing proteins. The aqueous phase was transferred in a clean tube followed by addition of isopropanol (Fisher Scientific, P/7500/15), which precipitates the RNA, and centrifugation at 12,000 x g for 10 min at 4°C; the RNA pellet was then washed with 1 mL of ethanol 70% (provided by University of Leicester Chemistry Department) in distilled water, vortexed for 15s and centrifuged at 12,000 x g for 5 min at 4°C; the pellet was then left to dry in the tissue culture hood, resuspended in 20 µL of RNase-free deionized water (treated with 0.1% DEPC [Diethyl pyrocarbonate, SIGMA, D-5758] incubated overnight, then autoclaved to destroy the DEPC), incubated for 10 min at RT to allow dissolution of the RNA pellet and stored at -20°C for later use. The red organic phase and the interphase (containing protein and DNA respectively) were kept at 4°C for eventual later use. Pure RNA solutions should have an $A_{260/280nm}$ ratio ≥ 1.7 with RNA absorbing at A_{260nm} and protein absorbing at A_{280nm}.

2.16 Reverse transcription Real Time -PCR:

2.16.1 Reverse transcription (RT) reaction

Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT, Promega, M515A) was used to synthesise cDNA using RNA as a template. This step was non-specific since all the RNA present was copied into cDNA, because random hexamer primers (Eurofins MWG) were used. The main reaction (RT mastermix) includes dNTPs 10 mM (composed of dATP, Promega U120D, dCTP, Promega U122D, dGTP, Promega U121D and dTTP, Promega, U123D), RNasin® ribonuclease inhibitor (Promega, N251B), random hexanucleotides as primers, AMV RT enzyme and appropriate buffer (Table 2-9).

Reagent and stock	Final concentration	Volume
Total RNA	-	9 µL
dNTP mix (10mM)	1 mM	2 µL
Hexanucleotides (0.2 µg/µL)	0.2 µg	1µL
RNAsin® Plus (40 U/µL)	20 U	0.5 µL
AMV RT Buffer, 5x	1x	4 µL
AMV RT enxyme (10 U/µL)	8 U	0.8 µL
DEPC dH_2O	-	2.4 µL
Final volume		20 µL

Table 2-9: RT reaction mix.

DEPC water was used in place of RNA as a negative control.

Before adding all the reagents, RNA samples were denatured at 70°C for 5 min; samples were then allowed to cool to 42°C, the program was paused and the tubes kept on ice for addition of the RT reagents. The tube reactions were then incubated at 42°C for 1 hr (annealing and extension) and a final incubation at 90°C for 4 min was used to deactivate the enzyme. The reactions were performed in either Techne TC-3000 or Progene 231-103 termocyclers. cDNA samples were then diluted 1:4 with sterile deionized water before use in Real Time PCR.

2.16.2 Real Time PCR

Due to its high sensitivity, real time PCR is one of the most used techniques to measure gene expression. In a double stranded DNA (dsDNA) dye-based real time PCR, the fluorescence emitted during the cycles is an indication of the production of dsDNA: the signal increases proportionally to the amount of product.

Reagent	Final concentration	Volume
SYBR® green JumpStart™ *Taq* ReadyMix™, 2x	1x	10 µL
cDNA		3 µL
Primer 5'(5 µM)	0.5 µM	2 µL
Primer 3'(5 µM)	0.5 µM	2 µL
Sterile dH_2O		3 µL
Final volume		20 µL

Table 2-10: qPCR reaction components.

SYBR® green JumpStart™ *Taq* ReadyMix™ (SIGMA, S-1816) contains SYBR Green I dye, with an excitation of 494 nm and emission of 521 nm, which binds to double-stranded DNA; hot start JumpStart *Taq* DNA polymerase; JumpStart *Taq* antibody which inactivates the DNA polymerase at room temperature; 7 mM $MgCl_2$ and deoxynucleotides. Reactions were performed in LightCycler capillaries (Roche, 04 929 292 001) in a LightCycler Real Time-PCR machine (Version 3, Roche).

Gene	Size	Sequence
β_2m	116 bp	F:5'-GGCTATCCAGCGTACTCCAAAG-3' R: 5'-CAACTTCAATGTCGGATGGATG-3'
MMP-7	393 bp (spanning ~4 kb intron)	F:5'-ATGGACTTCCAAAGTGGTCACCTACAG-3' R: 5'- GGATACATCACTGCATTAGGATCAGAG-3'
	78 bp (Yin et al., 2010)	F: 5'-GATGGTAGCAGTCTAGGGATTAACTTC-3' R:5'-GGAATGTCCCATACCCAAAGAA-3'
Ets-1	233 bp	F: 5'-TGGAGTCAACCCAGCCTATC-3' R: 5'- TCTGCAAGGTGTCTGTCTGG-3'

HIF-1	237 bp	F: 5'-AATGCCACCACTACCACTGC-3' R: 5'- TAACACGTTAGGGCTTCTTGGA-3'
GLUT-1	209 bp	F: 5'-CAACTGGACCTCAAATTTCATTGTGGG-3' R: 5'-CGGGTGTCTTATCACTTTGGCTGG-3'

Table 2-11: List of primers used for Real Time RT-PCR. All these primers were disegned to span an intron and to cross exon-exon boundaries.

In order to compare the expression of the gene of interest, the values need to be normalised to an internal reference gene (housekeeping gene). β-2-microglobulin (β_2m), the β chain of MHC class I molecule, is a widely expressed cell-surface protein and was used as the reference gene. This reference gene has been previously used by several groups (Semenza et al., 1994; Piehler et al., 2010; Staples et al., 2011). Standard curves, with three serial 1:5 dilutions of an initial pool of all the cDNA samples to be used, were made fresh for every PCR reaction. The standard curve is necessary for relative quantification and allows convertion of the C_T (crossing threshold) number into relative cDNA concentration.

Initial denaturation	94°C, hold 30 secs
Cycling (45-60reps)	Step 1 @ 94°C, hold 10 sec
	Step 2 @ 60°C, hold 10 secs
	Step 3 @ 72°C, hold 25 secs
Melt	Step 1 @ 96°C, hold 2 secs
	Step 2 @ 65, hold 10 secs
	Step 3, continous ascending gradient of 1°C intervals to 96°C
Cool down	40°C, hold 30 secs

Table 2-12: Real time PRC cycle used for MMP-7, GLUT-1, HIF-1 and β2m primers.

The specificity of the real time RT-PCR product was checked via a melting curve obtained at the end of the cycles. At the melting temperature, the dsDNA strand of each specific product separate and the fluorescence from SYBR Green decreases dramatically. Pure samples should have all one peak, which corresponds to one PCR product (see Figure 2-9A).

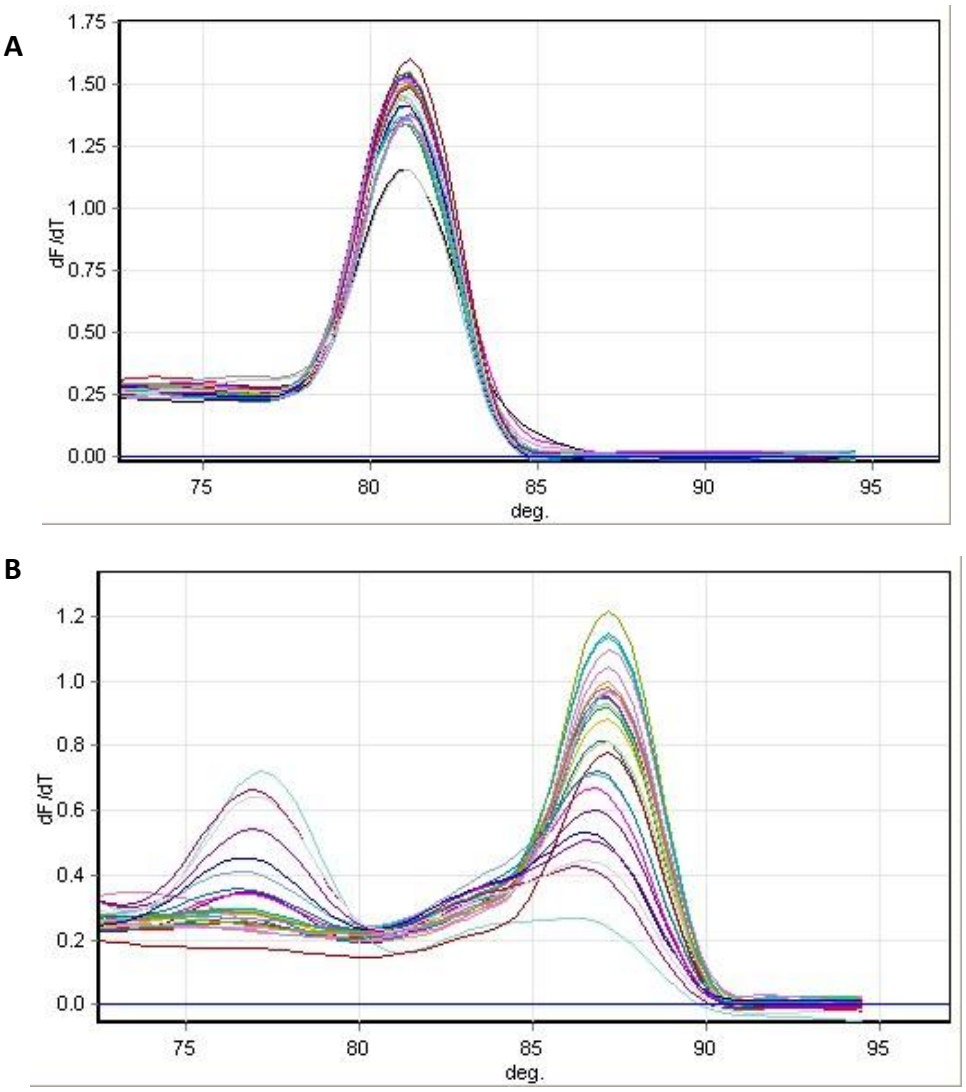


Figure 2-9: Example of one peak melting curve (A) and two peaks melting curve (B). **B shows contamination with another PCR product or primer-dimer product, shorter than the expected one (78°C against 87°C).**

Initial denaturation	94°C, hold 30 secs
Cycling (55 reps)	Step 1 @ 94°C, hold 10 sec
	Step 2 @ 65°C, hold 10 secs
	Step 3 @ 72°C, hold 30 secs
	Step 4 @ 82°C, hold 1 sec
Melt	Step 1 @ 96°C, hold 2 secs
	Step 2 @ 65, hold 10 secs
	Step 3, continous ascending gradient of 1°C to 96°C
Cool down	40°C, hold 30 secs

Table 2-13: Real time PCR cycle used for ets-1 primers. **After optimization, the real time PCR performed had addition of $MgCl_2$ 2 mM (SIGMA, M8787).**

RT negative (the water-only negative control from the RT step) and PCR negative (water instead of the cDNA sample) samples must also be added to confirm the PCR specificity and to rule out contamination.

Analysis of the data from real time RT-PCR falls in two categories: absolute quantification, which is based on the calibration to a standard of known concentration, and relative quantification, based on comparision to a housekeeping or reference gene.

In the analysis, the number of cycles is plotted against the fluorescence. C_T is defined as the first cycle at which a detectable increase in fluorescence emitted is seen due to the presence of a PCR product.

2.17 ELISA (enzyme-linked immunosorbent assay)

Total MMP-7 levels in cell culture supernatants were measured in triplicate using the commercially available Quantikine® MMP-7 ELISA kit (R&D Systems, DMP700) according to the manufacturer's instructions. The kit recognizes both active and inactive forms of MMP-7. The sensitivity of this kit was 0.005-0.094 ng/mL. This assay, containing recombinant human MMP-7 and an anti-MMP-7 monoclonal antibody, is a sandwich enzyme immunoassay, where a 96-well plate is pre-coated with the monoclonal antibody against MMP-7. When samples or standards were added to the well, MMP-7 was bound to the immobilised antibody. After washes, an enzyme-linked polyclonal antibody specific to MMP-7 and its substrate solution were added, and colour develops in proportion to the amount of MMP-7 bound. When the colour development is stopped, the intensity is measured at 450 nm with a background correction of 550 nm (Microplate Reader, model 680, Bio-Rad).

Human MDMs were plated at a density of 9 x 10^6 per well, grown for 5 days as described previously, treated with LPS *E. coli* at the appropriate concentrations and incubated in normoxia or hypoxia for 18 hrs. After incubation, the supernatant was collected and centrifuged at 18,000 x g for 5 min in order to remove any particulate, and used for subsequent ELISA. The supernatant was stored at -20°C until enough samples were collected for the immunoassay.

All the ELISA reagents were brought at room temperature before use; the wash buffer was diluted from 25x to 1x with dH_2O and the MMP-7 protein standard was reconstituted with 1 mL of dH_2O to produce a stock solution of 100 ng/mL. Before making dilutions of the standard, the MMP-7 protein standard was incubated for 15 min with gentle agitation. Serial dilutions from 10 ng/ml to 0.156 ng/ml (max of 7) were produced with Calibrator Diluent RD6-28 and the samples were diluted 2-fold in Calibrator Diluent RD6-28. Just before their use, Colour Reagent A and B were mixed in equal volumes.

Twelve removable 8-well strips were placed in a holder frame. After removing only the number of wells needed for one experiment, 100 µL of assay diluent RD1-52 were added to each well and 50 µL of standard, control, or sample were added, allowing MMP-7 to bind to the immobilized antibody. The 96-well plate was covered with adhesive strip and was incubated for 2 hrs at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. After incubation, each well was washed with 400 µL wash buffer for three times. The complete removal of liquid at each step was essential to good performance. After the last wash, any remaining wash buffer was removed by aspirating and inverting the plate against clean paper towels. Next, 200 µL of MMP-7 conjugate (an enzyme-linked polyclonal antibody specific for MMP-7) were added to each well, the plate was covered with a new adhesive strip and incubated for 2

more hrs at room temperature on the shaker. The aspiration/wash steps were repeated as before. 200 µL of substrate solution were added to each well and the plate incubated - protected from light - for 30 min at room temperature**.** 50 µL of Stop Solution were added to each well so the color in the wells should change from blue to yellow in proportion to the amount of MMP-7 present. Within 30 min, the optical density of each well was determined using a microplate reader at 450 nm with background correction at 540 nm or 570 nm, which corrected for optical imperfections in the plate.

For the calculations, the zero standard was subtracted by each value and the optical density of the standards was plotted versus the concentration, interpolating the points to get the best fit. To determine MMP-7 concentration, the optical density was used in relation with the curve to read the concentration (see Figure 2-10).

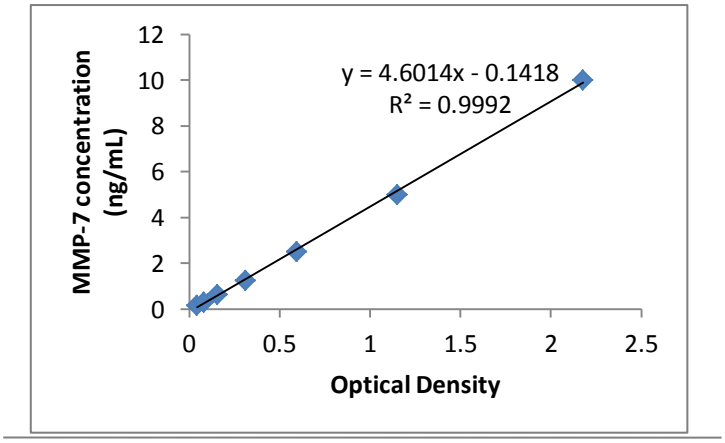


Figure 2-10: Example of a standard curve obtained for the ELISA assay. **The curve is described by the equation Y=mX+b, where Y is the MMP-7 concentration, m is the slope, X is the optical density and b is the y intercept.**

2.18 Nuclear extracts

hMDM and THP-1 cells were plated at a density of 10 x 10^6 and 8 x 10^6 respectively in 6-well plates (hMDM) or flasks (THP-1). For hMDM, after 5 days *in vitro*, treatments with LPS *E. coli* at various concentrations in normoxia and hypoxia for 18 hrs were performed. For THP-1 cells, treatments with 100 ng/ml LPS *E. coli* and hypoxia for 18

hrs were performed the same day of plating. After washing cells twice with ice-cold PBS, cells were scraped off with 1 mL ice-cold PBS and transferred to a cold new tube where they were pelleted at 1,700 x g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 4 volumes of ice-cold buffer A and allowed to swell for 5 min on ice. Nonidet NP40 (Igepal, SIGMA I-3021) was added to a final concentration of 0.6%, vortexed for 10 sec and centrifuged for 30s at 18,000 x g at 4°C. The supernatant was transferred to another tube and stored at -80°C as "cytoplasmic extract" (5 µL were kept for protein assay). Pellet was resuspended in 1 volume of ice-cold buffer B and glycerol was added to a final concentration of 20%. Tubes were rotated at 4°C for 15 min and centrifuged for 5 min at 18,000 x g at 4°C. The supernatant was transferred to another tube to be kept in -80°C as "nuclear extract" and 2 µL were kept for protein assay. Both the supernatants and the cytoplasmic extract were measured with Nanodrop ($A_{280\ nm}$) for protein concentration.

NUCLEAR EXTRACT BUFFER A (final concentrations shown):

Reagent	Company (cat number)
10 mM Hepes pH 7.5	SIGMA (H-4034)
0.1 mM EDTA pH 8.0	SIGMA (E-7889)
0.1 mM EGTA pH 8.0	SIGMA (E-8145)
10 mM KCl	Fisher (P/4280/60)

Added just before use:

Reagent	Company (cat number)
1 mM DTT	SIGMA (D-9163)
0.5 mM PMSF	SIGMA (P-7626)
100 mM sodium metavanadate	SIGMA (590088)

NUCLEAR EXTRACT BUFFER B (final concentrations shown):

Reagent	Company (cat number)
20 mM Hepes pH 7.5	See above
1 mM EDTA pH 8.0	See above
1 mM EGTA pH 8.0	See above
0.4 mM NaCl	Fisher (S/3160/63)

Added just before use:

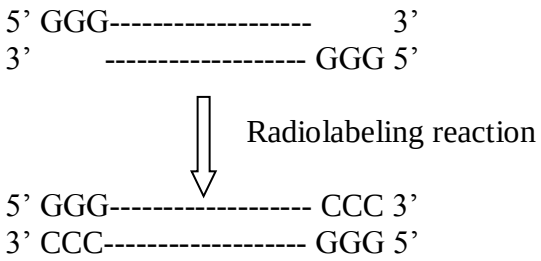
1 mM DTT	See above
1 mM PMSF	See above
1 mM sodium metavanadate	See above
10 µg / ml Leupeptin	SIGMA (L-2023)
10 µg/ml Pepstatin	SIGMA (P-4265)
10 µg/ml Aprotinin	SIGMA (A-1153)

2.19 EMSA (Electrophoretic Mobility Shift Assay)

EMSA is an electrophoretic technique which allows characterization of protein-DNA interactions. A radiolabelled double stranded oligonucleotide (oligo) probe is run with a protein extract to determine the binding of proteins to the oligo: the protein:DNA complexes migrate more slowly than free DNA fragments on a non-denaturing polyacrylamide gel due to a higher combined mass than the DNA oligo alone. After the addition of an antibody specific for the protein of interest, the band can be supershifted due to increased mass/size of the DNA-protein-antibody complex.

Complementary oligonucleotides (with wild-type or mutated binding sites) were synthesised (MWG-Eurofins) such that when annealed they woud result in a double-stranded oligo with over-hanging 5' ends (Table 2-14). EMSA oligonucleotides are generally 20-50 bp long. The oligonucleotides were resuspended at a concentration of 100 µM and annealed in a final volume of 25 µL (final concentration of each oligonucleotide was therefore 50 µM) at 95°C for 5 min in a heat block followed by

slow cooling to room temperature. Radiolabelling was carried out by 5'-end filling using DNA polymerase I, Large (Klenow) fragment (Invitrogen, cat number 18012-021) in a total volume of 50 µL. The Klenow fragment can fill in 5' overhangs (5'→3' DNA polymerase activity) and exhibits 3'→5' exonuclease activity. [α^{32}P]dCTP was provided in 5 µL aliquots which equal 1.85 MBq by the Department of Genetics (University of Leicester).



RADIOLABELLING REACTION:

3 µL of 1:25 diluted annealed oligos (0.12 µM)
5 µL of 10x Klenow buffer
1 µL of 10 mM dNTPs (mix of dTTP, dGTP and dATP)
2 µL fresh [α^{32}P]dCTP (1-0.74MBq)
38 µL dWater
1 µL diluted Klenow polymerase 1 U/µL (add last to minimise risk of exonuclease activity)

The reactions were incubated at 30°C for 30 min.

Unincorporated [α^{32}P]dCTP was removed from radiolabelled oligonucleotides ("hot") using the QIAquick nucleotide removal kit (Qiagen, 28304) according to the manufacturer's protocol, and eluted in 50µL of EB buffer. The purity of the probe was assessed by running 1 µl of probe in a TLC (Thin Layer Chromatography, Merck, 1.05735) plate using TLC separation buffer.

TLC SEPARATION BUFFER

Reagent	Company (catalogue)
1.2 M HCl	Fisher (H/1100/PB17)
0.8 M ammonium acetate	SIGMA (A-1542)

TLC shows how many components are present in a mixture: a small amount of the mixture is spotted near the bottom of the plate and the plate is placed in a shallow pool of solvent which slowly rises up by capillary action. The different components have a different solubility so some of them will run further up than others. TLC plates were air dried, exposed to film, and visualised by autoradiography after overnight exposure (Figure 2-11).

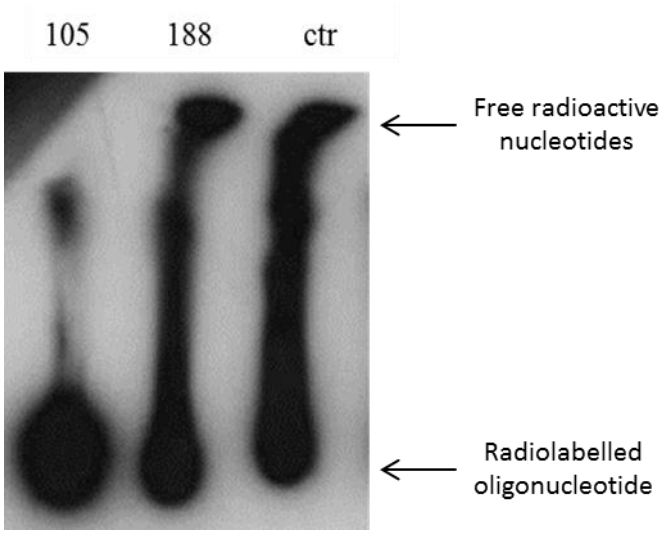


Figure 2-11: An example of TLC radiography for some radiolabelled oligonucleotides.

The competitor DNA was an un-labelled ("cold") oligonucleotide (in this project, an oligo with mutated base pairs for the specific transcription factor) which was used to quench the binding of non-specific proteins to the "hot" oligonucleotide. Competitor DNA was used in excess of 100-200 folds over the radiolabelled oligonucleotide.

BANDSHIFT REACTION

1x BSRB buffer
Nuclear extract (10 µg protein)
Competitor DNA
In dH_2O to a total of 20 µL (allowing for the probe).

Incubate for 10 min at room temperature then 5 µL radioactive oligo were added. For supershift experiments, 1-2 µg of β-catenin polyclonal antibody (SIGMA, C-2206) was added where indicated and incubated 10-15 min at room temperature.

5X BSRB BUFFER:

1 M Tris pH 8.0
1 M NaCl
0.5 M EDTA pH 8.0
20% glycerol
2.5 mM DTT

Separation was performed on a 1-2 hrs pre-run 5% polyacrylamide gel (Acrylamide/bis-Acrylamide 40% solution, SIGMA, A-9926) run in 0.5x TBE buffer.

5X TBE:

Reagent	Company (cat number)
0.45 M boric acid 0.45 M	Merck (1.00165)
0.45 M Tris	See above
0.01M EDTA pH 8.0	See above

5% POLYACRYLAMIDE GEL:

Reagent	Company (cat number)
0.5X TBE buffer	N/A
Acrylamide/bis-Acrylamide 40% solution	SIGMA (A-9926)
0.08% TEMED	SIGMA (T-9281)
0.08% of 10% APS	SIGMA A-3678

The samples were loaded without loading dye (bromophenol blue), which interferes with bandshifts and the gel was run at 300V for about 90 mins.

FIXATIVE:

Reagent	Company (catalogue)
20% methanol	Fisher (M/4000/17)
10% glacial acetic acid	SIGMA (A-9926)

The gel was fixed, still attached to the plate, in the fixative buffer for 15-20 min at room temperature without agitating. The gel was dried under vacuum (BioRad Gel dryer, model 583), at 80°C for 30 min, exposed to film at -70°C for 12-72 h and developed.

Any radioactive spill was immediately cleaned with 5% Decon 90 (Appleton Woods, GC231). All work involving the use of radioactive probe was done in an appropriately appointed room where plexiglass was used as a shield and a Geiger counter was employed for β-particle detection.

EMSA primers:

AP-1 binding site	
Wt sense:	5' – GGG TAG GTG ACT CAT TTG AGT GT – 3'
Wt anti:	5'- GGG ACA CTC AAA TGA GTC ACC TA – 3'
Mut sense:	5' – GGG TAG GTG AAT TAT TTG AGT GT – 3'
Mut anti:	5'- GGG ACA CTC AAA TAA TTC ACC TA – 3'
-139 binding site for ets	
Wt sense:	5' – GGG AAA ACG AGG AAG TAT TAC ATC GT– 3'
Wt anti:	5'- GGG ACG ATG TAA TAC TTC CTC GTT TT– 3'
Mut sense:	5' – GGG AAA ACG AGC AAG TAT TAC ATC GT – 3'
Mut anti:	5'- GGG ACG ATG TAA TAC TTG CTC GTT TT – 3'
-163 binding site for ets	
Wt sense:	5' – GGG TAT TGG CAG GAA GCA CAC AA– 3'
Wt anti:	5'- GGG TTG TGT GCT TCC TGC CAA TA– 3'
Mut sense:	5' – GGG TAT TGG CAG CAA GCA CAC AA – 3'
Mut anti:	5'- GGG TTG TGT GCT TGC TGC CAA TA – 3'

-188 binding site for β-catenin	
Wt sense:	5' – GGG AAA AAA TCC TTT GAA AGA CAA ATA CAT – 3'
Wt anti:	5'- GGG ATG TAT TTG TCT TTC AAA GGA TTT TTT – 3'
Mut sense:	5' – GGG AAA AAA TCC TCA GAA AGA CAA ATA CAT – 3'
Mut anti:	5'- GGG ATG TAT TTG TCT TTC TGA GGA TTT TTT – 3'
-105 binding site for β-catenin	
Wt sense:	5' – GGG ACA CAT ACT TTC AAA GTT CTG TAG ACT – 3
Wt anti:	5'- GGG AGT CTA CAG AAC TTT GAA AGT ATG TGT – 3'
Mut sense:	5'- GGG ACA CAT ACT GGC TAA ATT CTG TAG ACT – 3'
Mut anti:	5'- GGG AGT CTA CAG AAT TTA GCC AGT ATG TGT – 3'
-1.5 kb NF-kB site	
Wt sense:	5' –GGG AAA TCA AAA GGC ATT TCC TCA AGT – 3'
Wt anti:	5'- GGG ACT TGA GGA AAT GCC TTT TGA TTT – 3'
Mut sense:	5'- GGG AAA TCA AAA GG*C* ATG TAC TCA AGT – 3'
Mut anti:	5'- GGG ACT TGA GTA CAT GCC TTT TGA TTT – 3'
-2.5 kb NF-kB site	
Wt sense:	5'- GGG CCC TTG GAA AAG GGC ATT CCA GGA AG – 3'
Wt anti:	5'- GGG CTT CCT GGA ATG CCC TTT TCC AAG GG – 3'
Mut sense:	5'- GGG CCC TTG GAA AAG GGC ATG CCA GGA AG – 3'
Mut anti:	5'- GGG CTT CCT GGC ATG CCC TTT TCC AAG GG – 3'

Table 2-14: EMSA oligonucleotides used. Red bases are the mutated bases inserted for ablation of transcription factor binding site. Wt is the wild-type primer, whilst "mut" is the primer carrying mutation for a trsncription factor binding site.

2.20 Software for transcription factor binding site analysis

Genomatix *MatInspector* software:

http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html

was used for transcription factor binding sites analysis.

2.21 Casein zymography for detection of MMP-7 activity in culture medium

Zymography is an electrophoretic technique used to detect enzymatic activity under non-reducing conditions. The basis of this technique is a polyacrylamide gel co-polymerised with casein, a substrate for MMP-7 (Woessner and Taplin, 1988). Enzymes

that are able to digest those substrates will show up as clear band against a dark blue background after Coomassie blue (SIGMA, B8647) staining, which has high affinity for casein (Fernandez-Resa et al., 1995).

RESOLVING GEL, 10%, (10 ML)

Substance	Company (cat number)	Final concentration	Volume
Casein 3 mg/mL	SIGMA (C8654)	1 mg/mL	3.4 mL
3 M Tris-HCl pH 8.3	See above	375 mM	1.25 mL
10% SDS	SIGMA (L-5750)	0.1%	100 µL
30% Acrylamide/Bisacrylamide 37.5:1	SIGMA (A-3699)	10%	3.4 mL
10% APS	See above	0.1%	100 µL
TEMED	See above	0.3%	30 µL
dH_2O	N/A	-	1.72 mL

STACKING GEL, 4%, (4 ML)

Substance	Final concentration	Volume
1.5 M Tris-HCl pH 6.8	127 mM	340 uL
10% SDS	0.1%	100 µL
30% Acrylamide/Bisacrylamide 37.5:1	4%	533 µL
10% APS	0.1%	50 µL
TEMED	0.3%	10 µL
dH_2O	-	3 mL

Adherent 7 days old hMDM at 8 x 10^6 cell per well, were switched to serum-free medium after two washes with HBSS. Where appropriate, LPS *E. coli* was added and the plates put in normoxia or hypoxia for 18 hrs. The conditioned medium (supernatant) was collected, spun down at 18,000 x g for 5 min at room temperature and concentrated around 10 fold with Amicon Ultra 10K concentrator (Millipore, UFC501024): briefly, 500 µL of conditioned medium was loaded into the concentrator which was spun down

at 14,000 x g for 5-15 min until around 50 µL were left. After, the filter was reversed in another clean tube, spun down at 1000 x g for 2 min and the concentrate collected for gel loading.

Casein gels were pre-run at 40 mA (~240 V) at room temperature in 1x running buffer (see recipe below) until the loading buffer dye loaded into a well ran out of the gel. The pre-run allows the excess casein to migrate out. After pre-running, the samples were loaded in 1x loading buffer (see recipe below) and the gel run at 20-40 mA on ice for 1 hr. Plasmin (SIGMA, P-8644) was used as a positive control, since it has been demonstrated that plasmin can cleave casein (Liu et al., 2009). As a marker, unstained protein molecular weight marker was used, boiled before loading (ThermoScientific, 26610, former Fermentas, SM0431).

Linearised proteins (due to SDS denaturation) do not show enzymatic activity and require renaturation to restore enzymatic activity. Renaturation stage is required to fold enzymes back to their original conformation in order to digest the substrate.

Gels were washed in wash buffer for 60 min (changing the buffer twice) in order to remove SDS and developed over-night at 37°C. Following this, gels were fixed for 1 hr at room temperature, washed with wash/de-staining buffer for more than 30 min up to over-night, Coomassie-stained over-night and de-stained. All buffer solutions are found below. Gels were soaked for 15 min in equilibration buffer; one sheet of cellophane drying film was moistened in the same buffer and laid onto the gel drying frame (see Figure 2-12). The gel was rolled onto the film, bubbles and wrinkles were removed before another previously moistened gel drying sheet was laid on top. The frames were clamped together and the gel left to dry overnight in an upright position. The day after, the clamps were removed, the excess film was cut and the gel was ready for storage.

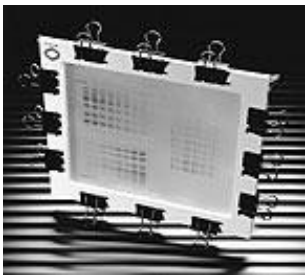


Figure 2-12: Example of a gel drying structure
http://www.fishersci.com/ecomm/servlet/fsproductdetail?catalogId=29104&productId=767619&langId=-1&storeId=10652&distype=2&isChemical=false&fromSearch=0

ZYMOGRAPHY RUNNING BUFFER, 10X

Reagent	Company (cat number)	Final concentration	Volume
Tris	See above	25 mM	340 uL
Glycine	SIGMA (G-8898)	250 mM	100 µL
SDS	See above	0.1%	50 µL
dH_2O	N/A	-	3 mL

LOADING BUFFER, 2X

Reagent	Final concentration
SDS	10%
glycerol	10%
Bromophenol Blue	0.004%
Tris-HCl pH 6.8	125 mM

ZYMOGRAPHY WASH BUFFER FOR SDS REMOVAL

Reagent	Final concentration
Triton X-100	2.50%

ZYMOGRAPHY DEVELOPING BUFFER

Reagent	Company (cat number)	Final concentration
Tris pH 7.5	See above	50 mM
NaCl	See above	100 mM
$CaCl_2$	Merck (172570)	10 mM
Brij	SIGMA (P-6153)	0.05%

ZYMOGRAPHY FIXING BUFFER

Reagent	Final concentration
Ethanol*	50%
Acetic acid	10%

***Provided by Chemistry Dept, University of Leicester**

COOMASSIE-STAINING SOLUTION

Reagent	Final concentration
Coomassie Blue	0.5%
Acetic Acid	10%
Methanol	20%

ZYMOGRAPHY WASH/DE-STAINING SOLUTION

Reagent	Final concentration
Acetic Acid	10%
Methanol	50%

ZYMOGRAPHY EQUILIBRATION BUFFER

Reagent	Final concentration
Glycerol	3%
Acetic Acid	10%
Methanol	40%

2.22 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Unless otherwise stated in figure legends, results are presented as means ± SEM; comparisons were made using a Student's paired *t*-test, and were considered significant (*) at a p value of <0.05 and highly significant (**) at p value of <0.01.

Chapter 3 Hypoxic regulation of MMP-7 gene in primary human macrophages and cell lines

3.1 Introduction

Hypoxia, as described in detail in section 1.4, is a feature of many pathological conditions, including atherosclerotic plaques (Crawford and Blankenhorn, 1996), solid tumours (Vaupel et al., 1989), wounds, arthritic joints (Ng et al., 2010) and sites of infection and inflammation (Wenger et al., 2005). The average O_2 tension in normal human tissues is between 20-70 mmHg but in ischaemic sites is <20 mmHg (Vaupel et al., 2003). However some healthy tissues such as spleen (Caldwell et al., 2001) can also contain regions of hypoxia.

A common denominator of the healthy tissues and the above mentioned pathological conditions is the presence of monocyte/macrophages (Lewis et al., 1999). Macrophages accumulate in hypoxic sites and respond to hypoxic conditions via up-regulating genes required for survival and adaptation to lower O_2 tension (Burke et al., 2003, Murdoch and Lewis, 2005).

Matrix metalloproteinase-7 (MMP-7) is a secreted protease mainly expressed by epithelial cells (Wilson and Matrisian, 1996), blood monocytes (Busiek et al., 1992), B and T-lymphocytes (Bar-Or et al., 2003) and many tumour cells (Adachi et al., 1999); reviewed in (Wilson and Matrisian, 1996). MMP-7 mRNA expression in human monocyte/macrophages is increased after 3 days in culture *in vitro*, peaking at 5-7 days and decreasing at 9 days *in vitro* (Filippov et al., 2003). MMP-7 mRNA is induced

rapidly and dramatically by adherence in monocytes and also by differentiation to macrophages for 7 days (Reel et al., 2011).

It has been previously reported that under hypoxic conditions, MMP-7 mRNA is up-regulated, 9-fold by cDNA array and 5.6-fold by real time RT-PCR analysis in primary human macrophages exposed to short term severe hypoxia, 0.2% O_2 for 16 hrs (Burke et al., 2003). In a more recent study, MMP-7 mRNA up-regulation between 50 to 100 fold was detected in primary human macrophages subjected to chronic moderate hypoxia, 2% O_2 for ten days (Deguchi et al., 2009). MMP-7 mRNA is also up-regulated in HepG2 and Hep3B cell lines exposed to 1% O_2 from 4 to 24 hrs (Miyoshi et al., 2006).

In this project, the effect of short term acute hypoxia (18 hrs at 0.2% O_2) on MMP-7 mRNA up-regulation was investigated, in both cell lines and in adherence-purified primary human macrophages from different donors.

3.2 MMP-7 mRNA expression in cell lines

Initially, cell lines were tested for MMP-7 mRNA expression and hypoxic inducibility, to enable the use of a reproducible and convenient model such as an immortalised cell line for this study. Cell lines have the important advantage of usually being much easier to transfect than primary cells, especially macrophages, which would be important for my later studies using MMP-7 promoter reporter constructs. When using real time RT-PCR (see section 2.16), MMP-7 mRNA values were normalised by the housekeeping gene β-2-microglobulin (β2m) mRNA values (MMP-7/β2m) and the fold induction was defined as the hypoxic value divided by the normoxic one. β2m mRNA was found not to be induced by hypoxia (*data not shown*). MMP-7 PCR product was checked for the correct size by agarose gel. There was no detectable MMP-7 mRNA in THP-1, MonoMac-6, HeLa and HEK293 cell lines (*data not shown*). In contrast, U937 (*data*

not shown), HCT-116 and AGS cell lines showed high MMP-7 mRNA expression (Figure 3-1).

After 18 hrs of hypoxia a difference in the level of MMP-7 transcript was detected, but not statistically significant, showing that the MMP-7 gene is not hypoxia-inducible in these cell lines.

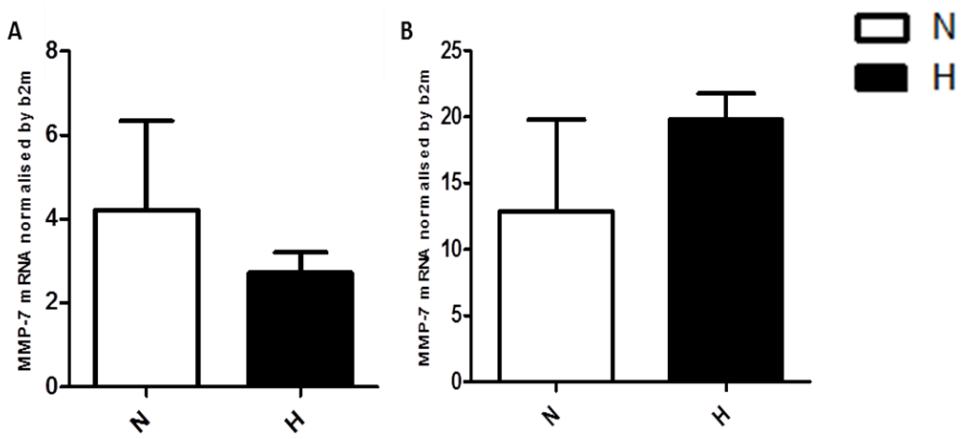


Figure 3-1: MMP-7 mRNA levels in hypoxia (H) in (A) HCT-116 and (B) AGS cells as determined by real time RT-PCR (n=3). **The y axis is an arbitrary value obtained normalising MMP-7 calculated concentration by the housekeeping gene (β2m) calculated concentration. N=normoxia.**

3.3 Regulation of MMP-7 by *Helicobacter pylori*

As mentioned in section 1.6.7, MMP-7 is up-regulated by bacterial infection. AGS cells, as shown in Figure 3-1, express MMP-7 both in normoxia and hypoxia, but no significant hypoxic induction is observed. It is known that gastric cells (*in vitro* and *in vivo*) respond to *H. pylori* infection by up-regulating MMP-7 via NF-κB and AP-1 pathways (Wroblewski et al., 2003). The choice of AGS cells and *H. pylori* in this project is due to their biological relevance: AGS cells are derived from a gastric tumour and MMP-7 is known to be up-regulated in gastric cancer (Honda et al., 1996), moreover *H. pylori* is known to cause ulcers and gastric cancer (Uemura et al., 2001, Crawford et al., 2003, Ogden et al., 2010). In AGS cells, *H. pylori*-dependent MMP-7 up-regulation correlates with enhanced migration and invasion, determined by

Transwell migration and invasion assay (Wroblewski et al., 2003). Therefore, we sought to investigate the expression of MMP-7 in AGS cells incubated with *H. pylori* in both normoxic and hypoxic conditions.

In AGS cells, levels of MMP-7 mRNA were detected and *H. pylori* infection led to a significant increase in MMP-7 mRNA induction in normoxia (around 3 fold for AGS infected with an MOI of 0.1, Figure 3-2), an evidence in line with a study where MMP-7 gene was seen to be up-regulated by the pathogenic *H. pylori* at MOI 200 (Gry et al., 2009, Yin et al., 2010). Hypoxia did not cause any further up-regulation, even though the standard error of the mean is very large, hindering what might be a trend in hypoxic induction of MMP-7. Given the absence of hypoxia inducibility in these conditions, AGS were not further investigated for MMP-7/hypoxia studies.

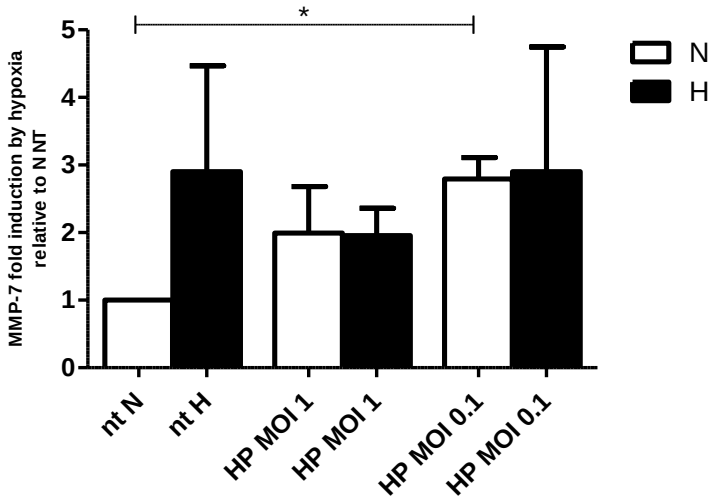


Figure 3-2: Effect of *H. pylori* in MMP-7 mRNA levels in AGS cells. ***H. pylori*** **(HP) was added at multiplicity of infection (MOI) 1 and 0.1 and incubated in normoxia (N) or hypoxia (H) for 18 hrs (n=3). MMP-7 mRNA was quantified via real time RT-PCR and normalised against β2m mRNA levels. The graph represents difference in fold induction in different conditions compared to non treated (nt) normoxic sample. *=p<0.05.**

3.4 Human primary macrophages as a model

For the investigation of MMP-7 mRNA expression, we employed primary human macrophages, which have been shown to express and induce MMP-7 mRNA in hypoxia (Burke et al., 2003). At the same time, the use of primary human macrophages allows a

better insight into inflammatory events *in vivo*, since their state of differentiation, unlike cell lines, allows a more physiological interpretation of the role of MMP-7.

3.4.1 Hypoxic viability

There are contradictory data about macrophages viability in hypoxia: after 10 days moderate hypoxia (2% O_2), primary human macrophages were reported not to survive (Deguchi et al., 2009); apoptosis was induced in a murine macrophage cell line subjected to hypoxia (1% O_2 for 24-48 hrs, Fong et al., 2007) whilst a subpopulation of a murine macrophage cell line was reported to be resistant to hypoxia-induced apoptosis (2% O_2 for 24-48 hrs, Yun et al., 1997). 1% O_2 for 48 hrs promotes the survival of murine bone-marrow derived macrophages, and also human monocytes and macrophages were shown to increase their survival under 1% O_2 for 24 hrs (Roiniotis et al., 2009).

Therefore, for this project, the viability of macrophages was investigated for periods of hypoxia shorter than 10 days, namely 18 hrs and 5 days. Cell viability was assessed by staining with propidium iodide (PI) followed by flow cytometry. PI is a fluorescent dye that intercalates into DNA in non-viable cells, due to its capacity to penetrate only cells with a compromised membrane. By using flow cytometry, macrophages can be distinguished by size and granularity (Figure 3-3 and 3-4, dot plots in panel on the left) and the dead cells (PI-positive) can be detected by the high FL-2 fluorescence signal (Figure 3-3 and 3-4, histograms in panel on the right). The region marked "M1" was set to include fluorescent cells stained by PI, and therefore it identifies dead cells. The histograms presented in Figure 3-3 E and 3-4 E show the percentage of live cells in both normoxia and hypoxia. As no difference in macrophage viability was observed, we

concluded that hypoxic incubation for 18 hrs or 5 days did not cause macrophages death.

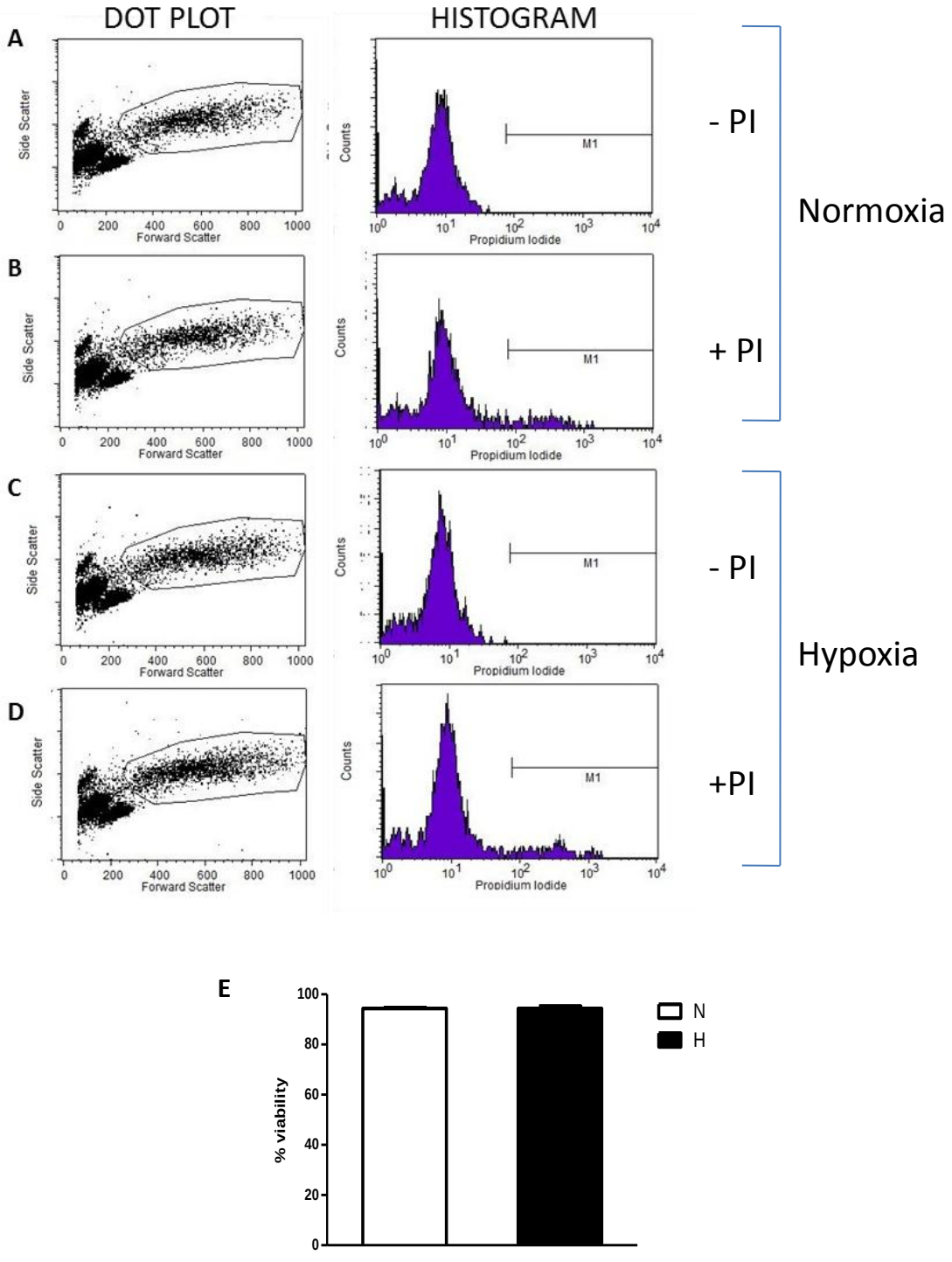


Figure 3-3: Macrophages viability after 18 hrs hypoxia (H, 0.2% O_2). **A. Normoxia (N) control (PI-negative); B. Normoxia PI-positive; C. Hypoxia control (PI-negative); D. Hypoxia PI-positive; the gated area/M1 represents the macrophage population. E. histogram representing the percentage of viable cells in normoxia and hypoxia (n=3).**

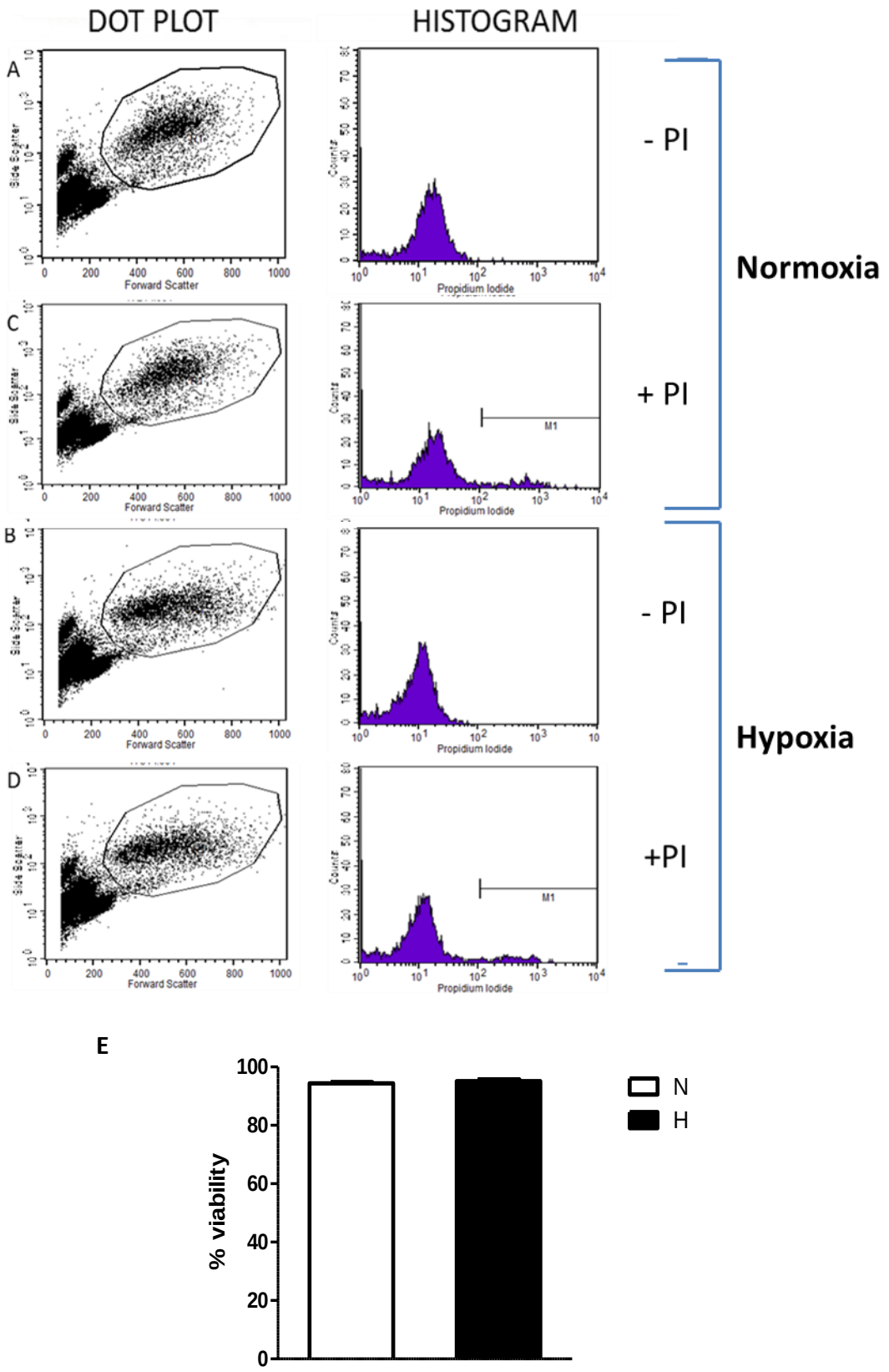


Figure 3-4: Macrophage viability after 5 days hypoxia (H, 0.2% O_2). A. Normoxia (N) PI-negative; B. Normoxia PI-positive; C. Hypoxia PI-negative; D. Hypoxia PI-positive; E. histogram representing the percentage of viable cells in normoxia and hypoxia (n=3).

3.5 MMP-7 mRNA expression in hMDM under hypoxia

After confirming that hypoxia did not cause primary macrophage cell death (see section 3.4.1), transcriptional up-regulation of MMP-7 was tested in human monocyte-derived macrophages (hMDM) under hypoxic conditions: this induction was previously demonstrated (Burke et al., 2003), but needed confirmation in our specific model. Hypoxia caused a statistically significant 2.6-fold induction in MMP-7 mRNA ($p = 0.027$, Figure 3-5).

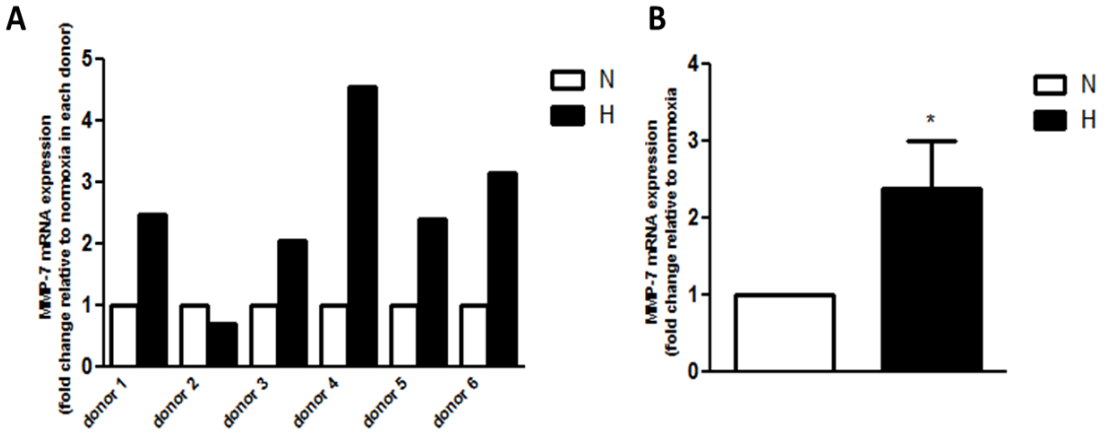


Figure 3-5: MMP-7 mRNA levels after exposure of 5 days-old hMDM to hypoxia (H, 0.2% O_2) for 18 hrs. **(A) fold increase in hypoxia compared to normoxia (N) in 6 different donors; (B) mean fold increase in all donors. MMP-7 mRNA was up-regulated 2.6-fold (n=6). *=p<0.05.**

3.6 Discussion

In this chapter, the effect of 18 hrs of hypoxia in MMP-7 mRNA induction was investigated in different cell lines and in primary human macrophages generated by adherence from peripheral blood mononuclear cells (PBMC) from different donors. By real time RT-PCR, some of the cell lines tested (THP-1, MM-6, HeLa and HEK293 cells) were shown not to express MMP-7, whilst HCT-116, U937 and AGS cells showed a marked MMP-7 expression (Figure 3-1), but MMP-7 does not appear to be hypoxia-inducibile. To further investigate MMP-7 expression, based on a paper from

Wroblewski et al., 2003 which showed that MMP-7 expression is up-regulated in the gastric epithelium by *H. pylori*, we studied the effect of the bacterium *H. pylori* on MMP-7 mRNA expression in the AGS gastric cancer cell line, chosen for their expression of MMP-7 mRNA (Figure 3-1) and for their physiological importance as a cancer cell line. AGS cells derived from a gastric tumour and MMP-7 is known to be up-regulated in gastric cancer (Honda et al., 1996). Moreover, *H. pylori* is known to cause ulcers and gastric cancer. We found a trend of up-regulation of MMP-7 following infection for 18 hrs with *H. pylori*, especially at the lowest MOI, but this up-regulation did not correlate with any further effect from hypoxia (Figure 3-2). An MOI of 10 was also tried, but incubation with *H. pylori* for 18 hrs resulted in AGS cell death. It is possible therefore that the highest MOI used in the experiment (MOI=1) could damage AGS cells blocking any induction of MMP-7, whilst an MOI of 0.1 could be optimal for both AGS cell viability and MMP-7 expression. Clinical studies (Yeh et al., 2010) correlate the increase of MMP-7 due to *H. pylori* as a marker for poor survival, however a very recent study (Ogden et al., 2010), unexpectedly, demonstrated that *H. pylori*-induced inflammation is increased in an infected MMP-7-knockout mice. Therefore, the role of MMP-7 in *H. pylori* infection is still controversial.

We next used adherence-purified peripheral mononuclear blood cells from healthy volunteers. As shown in Figure 3-3 and Figure 3-4, hypoxic macrophages retain levels of viability comparable to the normoxic macrophages after both acute severe hypoxia (18 hrs at 0.2% O_2) and chronic severe hypoxia (5 days at 0.2% O_2). Macrophages viability under severe hypoxia could be considered an evolutionary adaptation for macrophage survival, advantageous because of their constant tendency to accumulate in hypoxic sites such as wounds and solid tumours. In Figure 3-5, real-time RT-PCR on hMDM showed that MMP-7 mRNA is induced approximately 2.6-fold (p value =

0.027) after 18 hrs hypoxia (0.2% O_2). As shown in Figure 3-5, variability was noticed in MMP-7 mRNA expression between different donors (ranging from no induction at all in donor 2 to 4.5-fold in donor 4). Five possibilities for this variability were hypothesised: 1) donor-to-donor variation, possibly because of the well known single nucleotide polymorphisms (SNP) in the first 200 bp of the promoter - see section 1.6.2 (Jormsjo et al., 2001); 2) random experimental error; 3) fluctuation in the percentage of hypoxia in different donors experiments; 4) the presence of variable amount of endotoxin (LPS) in the medium in different experiments and 5) differences in transcription factors in macrophages from different individuals due to genetic or environmental factors (such as ongoing illness, for example). The -181 A/G promoter polymorphism (Figure 1-10) is known to affect MMP-7 gene transcription and the combination of the two rare alleles (the second being -153 C/T) can influence the binding of nuclear proteins and can increase promoter activity (Jormsjo et al., 2001). The presence of one of those polymorphisms or indeed both of them and the difference in transcription factors for different donors are factors that could not be controlled. The other factors could actually be indistinguishable, but every care was taken to avoid any experimental error and to independently monitor the oxygen percentage in the hypoxic chamber.

As mentioned in section 2.4, the medium used for macrophage culture was filtered in order to avoid LPS contamination, which will be discussed thoroughly in Chapter 5. It is possible that different bottles/batches of media/sera used for different experiments could carry variable amount of LPS, which could vouch for the variability in MMP-7 induction.

In Deguchi et al., 2009, MMP-7 mRNA up-regulation in primary human macrophages exposed to chronic (10 days) moderate hypoxia (2% O_2) is almost 53-fold. This huge

difference in up-regulation of MMP-7 between our study (2.6-fold) and Deguchi's could be possibly explained by the length of the hypoxic treatment (18 hrs in our study, 10 days in Deguchi's) and the difference in the oxygen percentage (0.2% O_2 in our study, 2% O_2 in Deguchi's). It has been reported that chronic moderate hypoxia (which occurs physiologically in the arterial wall (Crawford and Blankenhorn, 1991) is able to activate the Akt and β-catenin pathway (Deguchi et al., 2009), which can further amplify the inflammatory response, causing a massive increase in MMP-7 regulation. In contrast, a shorter severe bout of hypoxia could activate different pathways, such as NF-κB (shown to be activated by 1.8% O_2 for 2 hrs in alveolar macrophages, Leeper-Woodford 99) and AP-1 (maximal activation at 5 hrs in HeLa cells, Rupec and Baeuerle, 1995) which could regulate MMP-7 differently or which could result in less MMP-7 up-regulation from the PI3K pathway mentioned above. The sensitivity of AP-1 and NF-κB transcription factors to mild hypoxia is much less than other hypoxia-regulating transcription factors like HIF (Denko et al., 2003) or β-catenin (Deguchi et al., 2009, Liu et al., 2010).

Secondly, it has been observed that chronic hypoxia (5 days) during differentiation from monocytes to macrophages induces much higher pro-angiogenic cytokine VEGF mRNA levels in adherence-purified macrophages (27-fold) compared to acute (24 hrs) hypoxia (Staples et al., 2011), which could be part of the explanation for differences observed in MMP-7 mRNA up-regulation.

Chapter 4 Use of promoter reporter constructs to investigate MMP-7 hypoxic inducibility and the role of different transcription factors

4.1 Introduction

In eukaryotes, gene expression is tightly regulated and any step of it can be modulated: from chromatin condensation to RNA transcription to post-transcriptional regulation such as messenger RNA (mRNA) stabilisation. Of these, the most common control point is transcriptional initiation. Transcriptional regulation of a gene takes place at the promoter region, which generally is located directly upstream of the start of transcription site and is the region RNA polymerase binds to. A promoter contains specific DNA sequences for binding transcription factors which control the flow of gene transcription (Wray, 2003). Regulation of gene expression is coordinated by interaction of different transcription factors (Phillips, 2008). By itself, a basal promoter initiates transcription at a low rate (basal transcription) and needs additional proteins (transcription factors) to be activated accurately (when required) and at high levels (Roeder, 1996). The composition of transcription factor binding sites near a gene determines its expression profile. Identification of the transcription start gives clues as to the location of the promoter of a gene but the exact length of a promoter can often only be defined experimentally. As discussed in section 1.4.1, many transcription factors are regulated by hypoxia.

Most of the MMP promoters share a TATA box at position -30 bp (relative to the transcription binding site) and an AP-1 site at around -70 bp, with a PEA3 element adjacent to the AP-1 site which cooperates with it in regulating MMP transcription (Benbow and Brinckerhoff, 1997).

Amongst many other biologically active molecules, macrophages can secrete MMP-7, which has been shown to be up-regulated in hypoxia (Burke et al., 2003), however the mechanism responsible for this up-regulation is currently not fully characterised.

The human MMP-7 promixal promoter contains a number of different transcription factor binding sites (Figure 1-11 and Table 4-1).

Transcription factor	**General consensus sequence**	**Specific consensus sequence**	**Location**
PEA3 (ets family member)	5'-GGA[$^{A}/_{T}$]-3'	5'-GGAA-3' (inverted orientation)	-55 / -52
			-144 / -141
			-168 / -165
AP-1	5'-TGA[$^{G}/_{C}$]TCA-3'	5'-TGAGTCA-3'	-67 / -61
Tcf/LEF-1	5'-[$^{A}/_{T}$] [$^{A}/_{T}$] CAAAG-3'	5'-CTTTGAA-3' (inverted orientation)	-194 / -188
		5'-TTCAAAG-3'	-109 / -103

Table 4-1: Transcription factor binding sites for human MMP-7 proximal promoter.

MMP-7 is a known transcriptional target of β-catenin (Le Floch et al., 2005); it is co-localised with β-catenin and PEA3 in mouse intestinal tumours (Crawford et al., 2001) and it is closely associated with ets-1 expression in human hepatocellular carcinoma cells (Ozaki et al., 2000; Miyoshi et al., 2006). PEA3 and AP-1 synergise with β-catenin (binding to the Tcf/LEF-1 binding site) to *trans*-activate the MMP-7 promoter in intestinal tumours (Crawford et al., 2001). MMP-7 promoter expression in HEK293 cell line was *trans*-activated up to 250-fold by co-transfection with different vectors encoding PEA3/c-Jun (AP-1 factor)/LEF-1/βcatenin when compared to the non co-transfected MMP-7 construct only (Crawford et al., 2001). Deletion analysis of the MMP-7 promoter showed that MMP-7 promoter activity seems to be regulated by ets-1

(Ozaki et al., 2000), which is up-regulated at the mRNA level by hypoxia (1% O_2) in hepatocellular carcinoma cells in a HIF-1-independent way (Miyoshi et al., 2006).

MMP-7 does not appear to be regulated at the mRNA level through increased mRNA stability (unpublished data, Dr B. Burke, Figure 1-15). A previous study using reporter gene constructs containing a trimer of the putative hypoxia-responsive elements present in the MMP-7 promoter in RAW 264.7 murine macrophage cells showed that this putative HRE was not active. This could suggest that MMP-7 is regulated in a HIF-independent fashion, even though it is still possible that other elements are required for that particular HRE to function, which are not present in the MMP-7 construct, or that a functional HRE could be present in the distal promoter or even downstream of the gene (Burke et al., 2003).

In the present study, promoter reporter constructs were used to investigate the regions of MMP-7 promoter responsible for hypoxic transcriptional regulation. In order to define the precise mechanism of hypoxic up-regulation, -2.3 kb and -296 bp (the latter consists of both wild-type and mutated constructs) MMP-7 promoter constructs cloned into a luciferase expression vector were used (Crawford et al., 2001) and transfected into different cells lines or primary human monocyte-derived macrophages and exposed to hypoxia (0.2% O_2). In addition, two further MMP-7 constructs (-296 bp in pGL4.10[*luc2*] and -343 bp in pGL3-Basic) were cloned in order to test if hypoxia inducibility in these new constructs was enhanced. Since primary macrophages cultured in ordinary attachment plates as described in Section 2.4 have been shown not to be a good model in these transfection experiments (due to hypoxic induction of the control reporter, see Section 4.8), another method of culturing primary human macrophages was employed, as described in Section 2.4. The total population of peripheral blood mononuclear cells (PBMCs), including both macrophages and lymphocytes, was

cultured in low attachment (LA) plates. The aim of this was to allow the cells to be transfected in an LA plate, where they do not adhere, allowing them to then be divided equally into two ordinary attachment plates, thus reducing well-to-well variations in transfection efficiency. Before the luciferase assay, PBMCs were transferred to normal attachment plates and lymphocytes were washed off, obtaining a pure macrophages population. The precise method will be described in Section 4.9.

4.2 Transcription factor analysis

Using Genomatix MatInspector software, potential transcription factor binding sites in the -296 bp MMP-7 promoter were identified. In addition to the already known transcription factor binding sites (described above, Table 4-1), two more hypoxia-related transcription factor sites were found, for cEBP and NFAT (Figure 4-1). cEBP (Ccaat/Enhancer Binding protein) was shown to be regulated by hypoxia (Min et al., 2011) and NFAT (Nuclear Factor of Activated T cells) forms complexes with AP-1 in DNA binding (Macian et al., 2001).

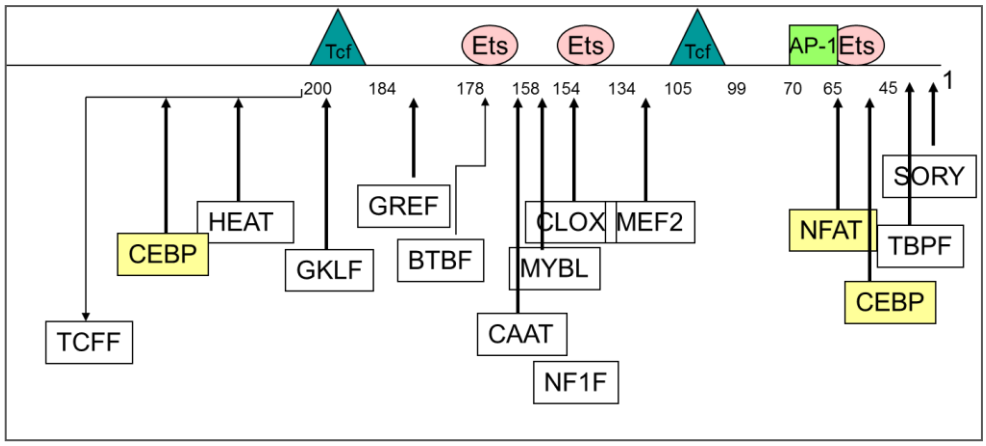


Figure 4-1: Genomatix promoter analysis for MMP-7 human promoter. **Novel potential binding sites involved in hypoxia are highlighted in yellow.**

4.3 MMP-7 promoter constructs transfected into cell lines

Table 4-2 lists all the MMP-7 promoter constructs used in this project.

MMP-7 promoter	Plasmid
-2.3 kb	pGL3-Basic
-965 bp	pGL3-Basic
-343 bp	pGL3-Basic
-296 bp	pGL4.10[*luc*2]
-296 bp	pGL3-Basic

Table 4-2: List of all the MMP-7 promoter luciferase constructs used in this project **(see section 2.11 for cloned constructs and section 2.3 for provided constructs).**

The -2.3 kb MMP-7 reporter construct was transfected into different immortalised cell lines in order to assess its hypoxia inducibility. Cell lines have the advantage of being permanently established and of proliferating indefinitely. The cell lines which were chosen for this project were either monocytic cells (U937, THP-1 and RAW 264.7, the first two human and the third murine) or human hepatocellular carcinoma cells (HepG2, which were shown to express MMP-7 in a hypoxia-inducible fashion, Ozaki et al., 2000; Miyoshi et al., 2006). The longer construct was chosen as a way to replicate results obtained in RAW 264.7 cells (Burke et al., 2003). None of the monocytic cell lines showed expression or hypoxia-inducibility of the -2.3 kb luciferase construct, with RLU/s (relative light units/second) values which were at the same levels of the promoterless control plasmid, pGL3-Basic, or indistinguishable from background values, in contrast to the positive control PGK hypoxia-inducible construct (Table 4-3).

Cell line	Construct	Condition	Firefly (RLU/s)
RAW 246.7	MMP-7 -2.3 Kb	normoxia	58
	MMP-7 -2.3 Kb	hypoxia	45
	PGK	normoxia	78
	PGK	hypoxia	929

U937	MMP-7 -2.3 Kb	normoxia	60
	MMP-7 -2.3 Kb	hypoxia	49
	PGK	normoxia	83
	PGK	hypoxia	868
	pGL3-Basic	normoxia	57
	pGL3-Basic	hypoxia	58
THP-1	MMP-7 -2.3 Kb	normoxia	82
	MMP-7 -2.3 Kb	hypoxia	58
	PGK	normoxia	158,822
	PGK	hypoxia	1,277,135
	pGL3-Basic	normoxia	60
	pGL3-Basic	hypoxia	51

Table 4-3: Firefly luciferase values (RLU/s) obtained with MMP-7 -2.3 kb promoter luciferase construct transfection in different cell lines. **MMP-7, PGK or pGL3-Basic constructs (1 µg) were transfected in the cell lines specified. The blank reading (LARII reagent only, in an empty tube with no sample) 40 RLU/s. PGK-1 was used as positive control for checking hypoxia inducibility.**

Therefore, these cells lines were deemed unsuitable for MMP-7 -2.3 kb transfection. It is possible that the failure to express the -2.3 kb construct could have been due to difficulty in transfecting a large plasmid. One solution could have been a control transfection with an equal size plasmid (2.3 kb plus the pGL3-Basic plasmid size).

Next, preliminary experiments were carried out using the shorter MMP-7 promoter construct (-296 bp) in U937 (Table 4-4) and HepG2 (Figure 4-2). The U937 cells were treated with PMA (a potent inducer of AP-1 activity), since it was previously found that undifferentiated U937 cells produced only small amounts of MMP-7 mRNA, however MMP-7 was strongly induced in cells differentiated with PMA for 24 hrs (Jormsjo et al., 2001). The MMP-7 -296 bp construct showed consistently higher basal luciferase values than the MMP-7 -2.3 kb construct, but no significant induction was seen after subjecting the cells to hypoxic conditions (Table 4-4).

Construct	Condition	RLU/s
MMP-7 -296 bp	normoxia	507
MMP-7 -296 bp	hypoxia	763
MMP-7 -2.3 Kb	normoxia	67
MMP-7 -2.3 Kb	hypoxia	58

Table 4-4: Firefly luciferase values (RLU/s) obtained with MMP-7 -2.3 kb and MMP-7 -296 bp transfection in U937 cells pre-treated with PMA. **MMP-7 -2.3 kb and -296 bp (1 µg) were transfected in U937 cells with Effectene. The blank reading (LARII reagent only) was an average of 65 RLU/s.**

Studies were also performed in HepG2 cells since it was shown that MMP-7 mRNA expression was induced by hypoxia in this cell line (Ozaki et al., 2000; Miyoshi et al., 2006). Research by our group has demonstrated that the MMP-7 -296 bp construct was induced 1.7-fold in hypoxia (Figure 4-2, data from Samantha Reading, an undergraduate project student in Dr Burke's group, working under my supervision).

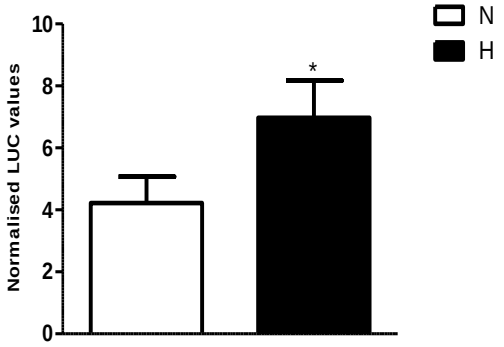


Figure 4-2: MMP-7 -296 bp luciferase construct hypoxic induction in HepG2 cells. **Cells were transfected with MMP-7 -296 bp construct (2 µg) and firefly luciferase values (LUC) were normalised by protein concentration (n=4). N=normoxia, H=hypoxia. Data from Samantha Reading.**

Based on the significant and reproducibile hypoxic inducibility of the MMP-7 -296 bp construct in this cell line, this model was employed to test the -296 bp mutant promoter reporter constructs (see section 2.3 and Figure 2-2). The mutant constructs contain one or more mutated bases located in a transcription factor binding site, affecting the binding of the appropriate transcription factor. All the different constructs were tested in HepG2 cells in order to investigate if these constructs were showing a different response

to hypoxia than the wild-type construct. If the observed hypoxic induction was inhibited in any of the mutated constructs in respect to the wild-type, then that particular transcription factor binding site could be considered as essential to the hypoxic regulation of MMP-7. As seen in Figure 4-3, the mutated binding sites for the ets transcription factor in position -163 bp ("163ets") and the "188Tcf" (mutated binding site for Tcf/LEF-1 in position -188 bp) appear to be important for both MMP-7 expression and hypoxic regulation. However, the -51 and -139 bp binding sites for ets do not appear to be required for MMP-7 expression or hypoxic regulation. Interestingly, the mutated binding sites for ets in position -51 and -139 bp actually increase MMP-7 expression, which also is in accordance with the results from Crawford et al., 2001, suggesting a possible inhibitory role of the ets transcription factor in those positions. Nothing definite could be concluded for the AP-1 mutated construct with regards to hypoxic inducibility, since the level of MMP-7 expression was below the level of detection in the majority of the experiments involving that construct. However, it can be concluded that the AP-1 site also seems to be essential for MMP-7 basal expression.

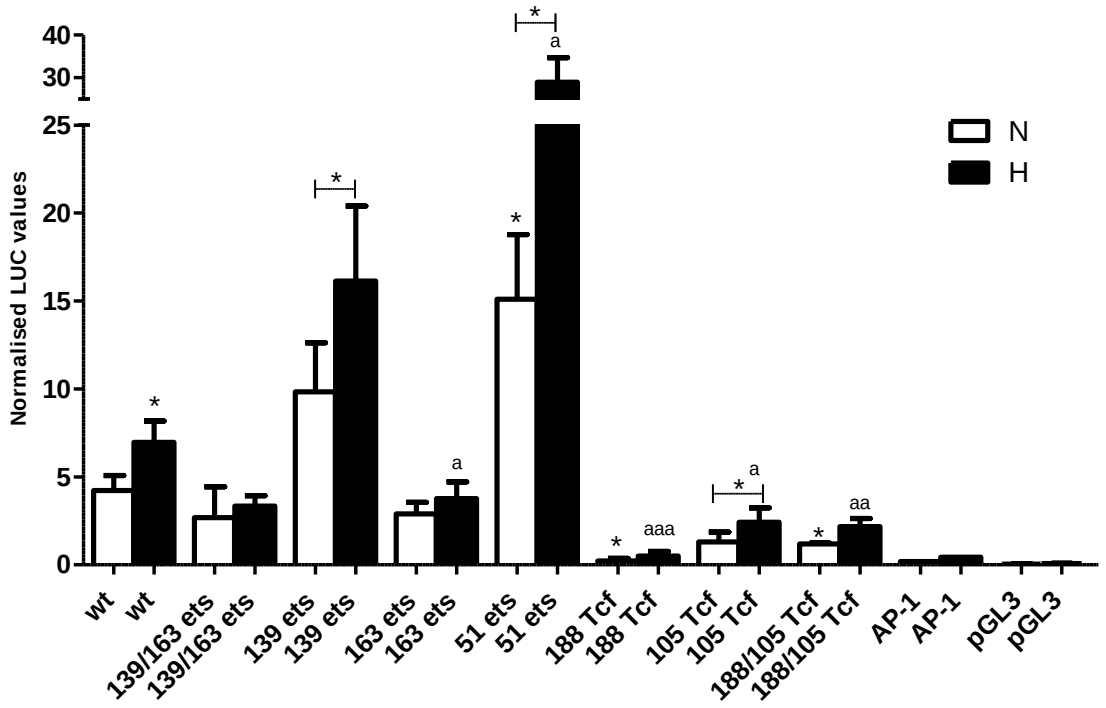


Figure 4-3: MMP-7 -296 bp wild-type and mutant luciferase promoter reporter constructs analysed for hypoxic inducibility in HepG2 cells. **Cells were transfected with the MMP-7 -296 bp construct (2 µg). Results were normalised by protein concentration (n=3). *, a=p<0.05, aa=p<0.01, aaa=p<0.001. * compares the normoxia (N) wild-type (wt) or the correspondent normoxic sample, as indicated, whilst "a" compares mutated constructs hypoxic (H) samples versus wild-type hypoxic sample. Data from Samantha Reading.**

4.4 Transfection of reporter constructs in primary human macrophages

The same methods as for tumour cell lines were applied to primary human macrophages, in an attempt to pinpoint the mechanism for MMP-7 hypoxic induction and find a connection between regulation of MMP-7 in both tumour cells and macrophages.

The effect of 18 hrs acute hypoxia (0.2% O_2) was investigated on luciferase expression driven by the MMP-7 promoter in primary adherence-purified hMDMs prepared from different healthy donors. Using JetPEI® as the transfection reagent, primary human macrophages cultured for 7 days *in vitro* were co-transfected with MMP-7 human promoter constructs and *Renilla* luciferase pRL-Tk construct. For the analysis, the experimental reporter was normalised to the constitutive reporter to control for

differences in transfection efficiencies. For a list of all the MMP-7 promoter reporter constructs used in this project, refer to Table 4-2.

Following initial optimisation of JetPEI® transfection using the positive control PGK (Figure 4-4), an N/P ratio of 8 was used for subsequent transfections (see section 2.12.1 for a thorough explanation of N/P ratio).

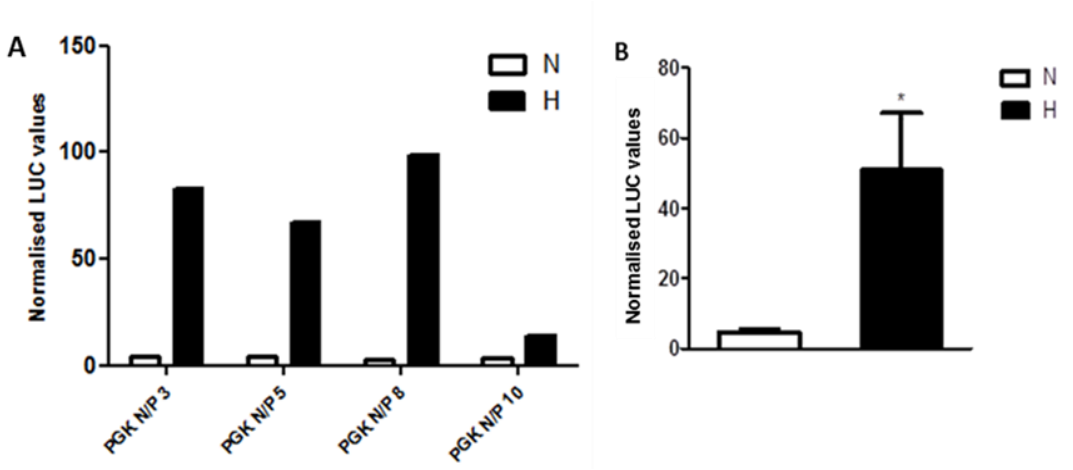


Figure 4-4: optimisation of N/P ratio in hMDMs transfected with PGK promoter reporter construct. **Co-transfection of hMDMs 7 days *in vitro* with (A) PGK plasmid (1.5 µg) and *Renilla* pRL-Tk (100 ng) subjected to hypoxia for 18 hrs. Graph (B) shows PGK transfection with N/P=8 and its up-regulation by hypoxia (n=7). Firefly luciferase results were normalised by *Renilla* luciferase values. * = p<0.05. N=normoxia, H=hypoxia.**

The efficiency of transfection in hMDMs was tested via transfection with 1.5 µg of DNA of a Green Fluorescent Protein (GFP)-expressing construct (CMV-GFP). hMDMs were washed twice in HBSS before looking at the cells under a fluorescence microscope (Olympus CXK41). The transfection efficiency was 38.5% (*data not shown*).

As was the case with the cell lines, the MMP-7 -2.3 kb construct expression was barely detectable in hMDMs; often the luciferase values were below background, therefore no solid conclusions could be made (Table 4-5).

Construct	Condition	RLU/s
MMP-7 -2.3 kb	Normoxia	49
	Hypoxia	54
	Normoxia	31
	Hypoxia	47
MMP-7 -296 bp in pGL3-Basic	Normoxia	915
	Hypoxia	3,095
	Normoxia	1,445
	Hypoxia	2,632
PGK	Normoxia	525
	Hypoxia	873
	Normoxia	1,274
	Hypoxia	27,308
Non transfected	Normoxia	42
	Hypoxia	39
	Normoxia	26
	Hypoxia	27

Table 4-5: Examples of hMDM 7 days *in vitro* transfected with 1.5 µg DNA constructs as specified. The blank reading (LARII reagent only) was between 30 and 40 RLU/s.

The -296 bp MMP-7 construct gave relatively high expression (Table 4-5), and therefore this was the construct chosen for subsequent experiments. The MMP-7 -296 bp construct transfected into human monocyte-derived macrophages was found not to be induced in hypoxia (Figure 4-5). It could be possible that, when the *Renilla* construct was co-transfected with this construct, MMP-7 expression was lowered, probably because of the "squelching" effect (a strong promoter like Tk for *Renilla* could soak up general transcription factors competing with the investigated promoter for gene transcription). Consistent with this notion, firefly Luc values decreased as increasing amounts of Renilla plasmid were co-transfected (Figure 4-5).

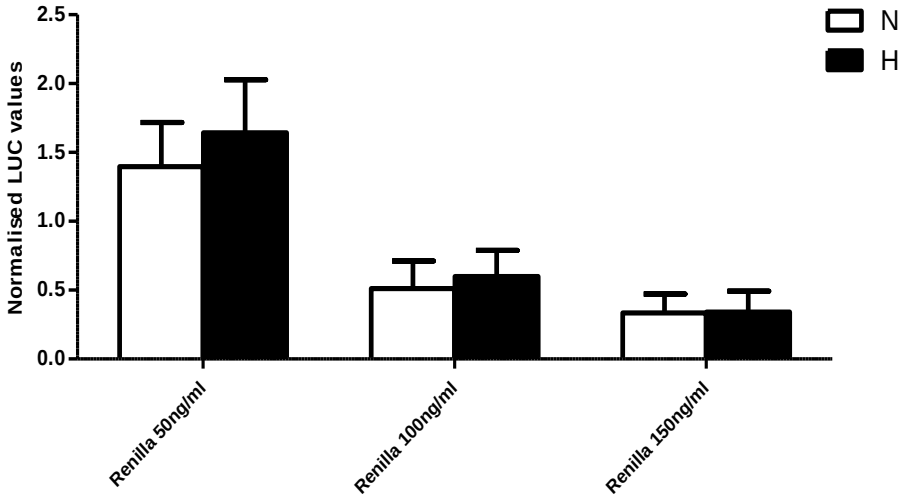


Figure 4-5: MMP-7 -296 bp promoter reporter construct induction by hypoxia (H). **hMDM 7 days *in vitro* were co-transfected with the -296 bp MMP-7 firefly luciferase constructs (1.5 µg) and *Renilla* luciferase construct 50 ng/mL (n=9); *Renilla* luciferase construct 100 ng/mL (n=4); *Renilla* luciferase construct 150 ng/mL (n=4). N=normoxia.**

An additional longer construct (-965 bp, see section 2.3) was transfected using the same method. Although the luciferase values from the -965 bp promoter were detectable, MMP-7 expression was not significantly induced by hypoxia (Figure 4-6).

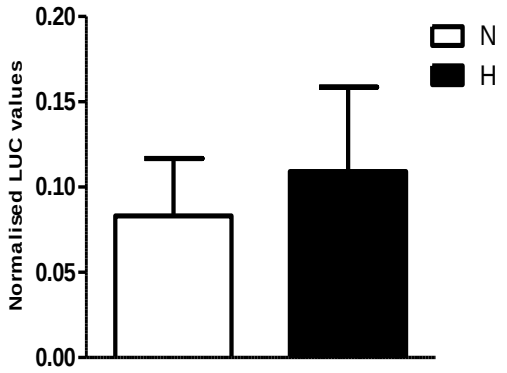


Figure 4-6: MMP-7 -965 bp promoter reporter construct expression. **hMDM 7 days *in vitro* were co-transfected with 1.5 µg MMP-7 -965 bp and *Renilla* luciferase construct 50 ng/mL (n=3). N=normoxia, H=hypoxia.**

4.5 Cloning of the MMP-7 downstream promoter element

Since none of the constructs used so far (-2.3 kb, -296 bp and -965 bp) showed expression or hypoxia inducibility of luciferase, the cloning of a new MMP-7 construct which included the region immediately downstream of the transcription start site was

attempted, since it was considered that it might be beneficial for increasing the expression and the fold induction of the MMP-7 construct in hypoxia (section 2.11). Promoters can be transactivated by interaction with a downstream promoter element (DPE), such as a downstream HRE sequence in the EPO gene which interacts with the upstream promoter for EPO full hypoxia inducibility (Sanchez-Elsner et al., 2004). An MMP-7 promoter construct of 343 bp, which included the previous -296 bp sequence and +47 bp downstream of the transcription start site, was cloned into pGL3-Basic, in order to make it consistent with the previous constructs.

When transfected into primary human macrophages, the level of firefly luciferase expression was very low, indicating a lack of basal expression of this new construct (Table 4-6).

Construct	Condition	RLU/s
MMP-7 -343 bp	Normoxia	38
	Hypoxia	34
	Normoxia	31
	Hypoxia	32

Table 4-6: Example of hMDM 7 days *in vitro* transfected with 1.5 µg MMP-7 343 bp construct. The blank reading (LARII reagent only) was between 30 and 40 RLU/s.

This could perhaps be explained by considering a possible negative interaction between different transcription elements. For example, the promoter that directs the transcription of genes of capside proteins in a parvovirus (P38) can be repressed *in vitro* in HeLa cells by a downstream promoter element (Krauskopf and Aloni, 1994).

4.6 Cloning of the MMP-7 -296 bp promoter fragment into pGL4.10[*luc*2] to reduce spurious basal expression

In an attempt to reduce background or spurious luciferase expression which could hinder the construct inducibility, the same -296 bp portion of the MMP-7 promoter was then cloned into another vector, pGL4.10[*luc*2] (Figure 2-4), as described in section

2.10. pGL4.10[*luc*2] is a next generation luciferase reporter plasmid, which has the advantage of having less transcription factor binding sites in its backbone (therefore promoting less spurious transcription of the luciferase gene).

The cloning was successful and preliminary experiments showed a high basal luciferase expression, but no hypoxia inducibility was seen (Table 4-7).

Construct	RLU/s	Condition
MMP-7 -296 bp in pGL4	103,482	Normoxia
	75,779	Hypoxia
MMP-7 -296 bp in pGL3	1,445	Normoxia
	2,632	Hypoxia

Table 4-7: Examples of hMDM transfected with 1.5 µg MMP-7 constructs. **hMDM 7 days *in vitro* were co-transfected with MMP-7 -296 bp in pGL4.10[*luc*2] (1.5 µg) and *Renilla* pRL-Tk 50 (ng) and incubated in hypoxia for 18 hrs. Blank value readings were between 30 and 40 RLU/s.**

Because all the MMP-7 constructs used so far did not show expression or hypoxia inducibility, the project proceeded with the study of regulation of MMP-7 via transcription factors as outlined below.

4.7 Analysis of the role of the transcription factors ets-1 and HIF

Because ets-1 is a potential transcription factor involved in the regulation of hypoxia-induced MMP-7 and is up-regulated by hypoxia (Miyoshi et al., 2006), I sought to investigate its involvement.

4.7.1 Ets-1 and HIF silencing

To investigate the importance of ets-1 for MMP-7 regulation, siRNA was used to knock-down the ets-1 mRNA expression, inducing gene silencing. Ets-1 siRNA oligonucleotides at a concentration of 0.5 µM were transfected in hMDM using jetPEI (see section 2.12.3). Before proceeding with the study of MMP-7 expression, the ets-1 mRNA concentration was measured, to make sure the siRNA was effectively down-

regulating ets-1 gene expression. This was found not to be the case, and the ets-1 siRNA actually seems to up-regulate ets-1 mRNA expression (*data not shown*). This problem was compounded by the very low expression of ets-1 in macrophages, which was barely detectable with real time RT-PCR.

In an attempt to investigate if HIF-1 was an important transcription factor for hypoxic up-regulation of MMP-7, HIF-1α siRNA was attempted. As seen in Figure 4-7, this attempt at silencing did not show a down-regulation of HIF-1 α mRNA, therefore a further investigation on MMP-7 hypoxic regulation was not pursued.

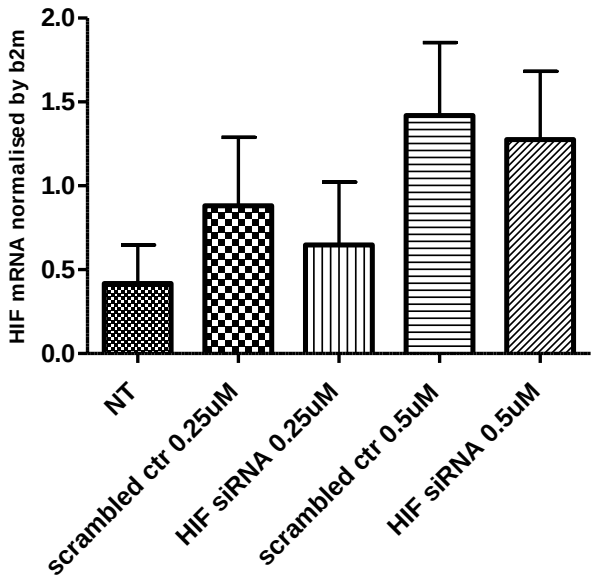


Figure 4-7: HIF-1α silencing via siRNA. **hMDM 7 days *in vitro* were transfected with HIF-1α siRNA or scrambled oligonucleotide (scrambled ctr) at two different concentrations as indicated. HIF-1 mRNA was measured via real time RT-PCR and normalised by β2m (3<n<6).**

It was also investigated if ets-1 and HIF both siRNA and scrambled oligonucleotides were producing non-specific effects via analysis of β2m housekeeping gene expression: from Figure 4-8, β2m expression seems to be down-regulated by the oligonucleotides in respect of the non-treated sample, which could explain the observed up-regulation of both ets-1 and HIF mRNA when the value was normalised by β2m. Therefore, further

work with RNA interference was not pursued. It has to be kept in mind that future optimisation could be tried in order to improve siRNA experiments.

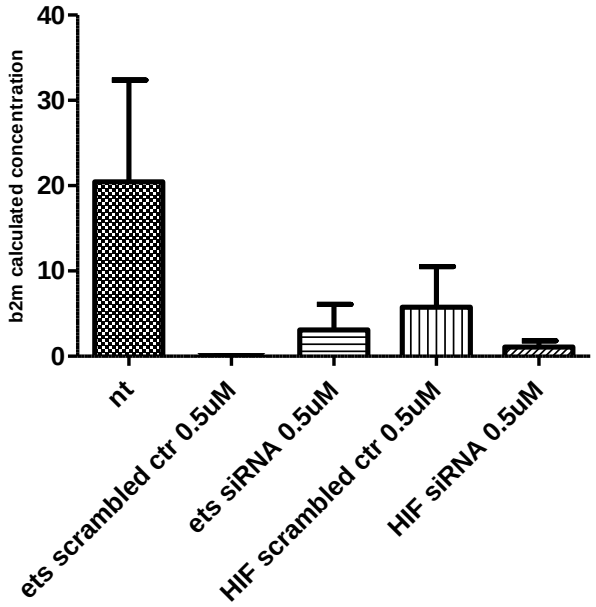


Figure 4-8: Short interfering RNA oligonucleotides non-specific silencing. **hMDM after 7 days *in vitro* were transfected with HIF and ets-1 siRNA or scrambled control (ctr) oligonucleotides. β2m mRNA was investigated via real time RT-PCR (2<n<8).**

4.7.2 Use of an Ets-1 inhibitor

As another method to down-regulate ets-1 expression, an inhibitor of ets-1 and ets-2, nimesulide (SIGMA, N-1016), was used. Used at a final concentration of 600 μM for 48 hrs (the ets transcription factor members are down-regulated in a relatively short time), nimesulide showed both ets-1 and ets-2 protein down-regulation in squamous cell carcinoma cell line (Lamm et al., 2005). A pilot experiment did not show down-regulation of MMP-7 mRNA by nimesulide, rather it seemed that DMSO (in which nimesulide was dissolved) was down-regulating MMP-7 (*data not shown*). The final DMSO concentration in the cell culture medium, which caused this effect, was 0.5%.

4.7.3 Overexpression of ets-1

Since the down-regulation of ets-1 could not be achieved by siRNA or the inhibitor nimesulide, the role of ets-1 was also investigated in the RAW 264.7 cell line via over-

expression of the protein from the plasmid pcDNAneo-hEts-1. RAW 264.7 cells were chosen as a starting point to investigate the optimal ets-1 concentration and were co-transfected with MMP-7 -2.3 kb or -296 bp (both at 1 µg), pcDNAneo-hEts-1 construct at increasing concentrations (from 150 ng to 1 µg) and Renilla pRL-Tk 10 ng/mL. Transfections of this cell line (see section 2.12.2), showed maximal induction of both MMP-7 constructs in normoxia when 300 ng of the ets-1 over-expression construct was transfected (4 fold for the -2.3 kb construct and 8 fold for the -296 bp one, Figure 4-9).

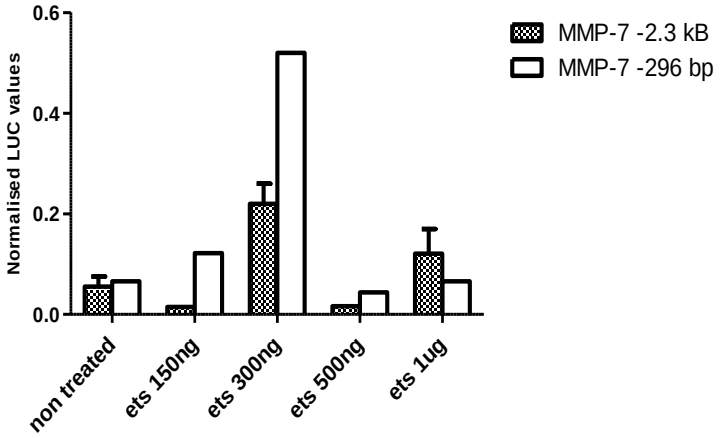


Figure 4-9: Ets-1 over-expression in RAW 264.7 cells. **Cells were co-transfected with MMP-7 -2.3 kb (n=2) or -296 bp (n=1) constructs (1 µg) and Renilla (10 ng/mL) in normoxia (20.9% O_2). pcDNAneo-hEts-1 construct was over-expressed in different concentration as indicated. Firefly luciferase values were normalised by *Renilla* values.**

Primary human macrophages similarly showed an induction of MMP-7 -296 bp (Figure 4-10), -2.3 kb and -965 bp constructs (*data not shown*) by ets-1, but no increase in MMP-7 regulation was seen in hypoxia, therefore it was decided to not pursue this work further. No clear role for the ets-1 transcription factor in hypoxic up-regulation of MMP-7 could be demonstrated, possibly because MMP-7 up-regulation in hypoxia is dependent on the interaction of many different transcription factors (Crawford et al., 2001).

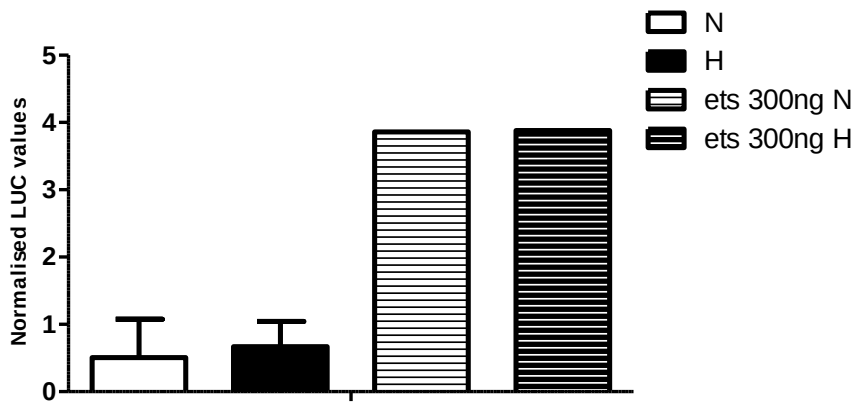


Figure 4-10: Ets-1 overexpression in hMDM. **hMDM cultured for 7 days *in vitro* were co-transfected with MMP-7 -296 bp construct (1.5 µg), pcDNAneo-hEts-1 (300 ng) and Renilla pRL-Tk (50 ng) and moved to hypoxia (H) for 18 hrs (n=2).**

4.8 *Renilla* pRL-Tk hypoxic induction

The *Renilla* control reporter allows the evaluation of experimental variation – ie, transfection efficiency and cell viability. A number of factors could affect the level of control of the reporter expression, such as culture conditions or the type of promoter used or the activity of the gene of interest (Vesuna et al., 2005). As all the transfection experiments so far were performed with the *Renilla* control reporter, we sought to analyse the expression of the pRL-Tk *Renilla* luciferase reporter plasmid under both normoxic and hypoxic conditions. It was revealed that the *Renilla* luciferase reporter construct itself was significantly induced by hypoxia (Figure 4-11, p value=0.0093). This *Renilla* construct, therefore, was revealed not to be a useful internal control for the experiments since its hypoxia inducibility would influence the investigated hypoxic inducibility of the co-transfected MMP-7 reporter construct. Therefore, the *Renilla* luciferase reporter construct was not used in subsequent experiments and was substituted with another internal control (see section 4.9).

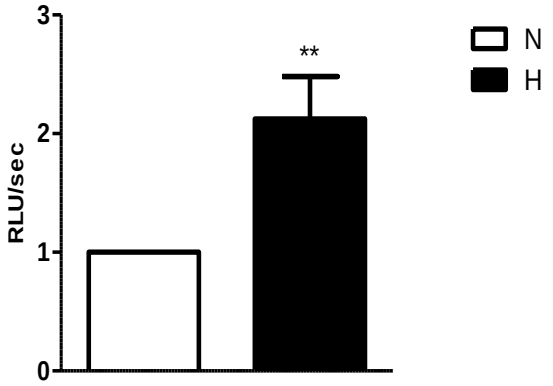


Figure 4-11: *Renilla* luciferase pRL-Tk plasmid induction by hypoxia (H). ***Renilla*** **luciferase plasmid pRL-Tk was transfected in hMDM 7 days *in vitro* at a concentration of 50 ng/ml (n=12). **= p<0.01. N=normoxia.**

4.9 Protein assay normalisation method for MMP-7 transfections

In an attempt to get around the *Renilla* luciferase pRL-Tk construct normalisation problem (since it proved not to be a good control because of its hypoxic inducibility, Figure 4-11), protein concentration was used as an alternative method to normalise the firefly luciferase values of the MMP-7 -296 bp construct. Although this method was not able to correct for transfection efficiency, it proved to be a valuable method of normalisation for determination of the protein amount in each well and therefore correction for the cell number per well.

In order to perform this normalization, an alternative method to plate macrophages has been developed. As mentioned in section 2.4, during macrophage preparation peripheral blood mononuclear cells (PBMCs) extracted from healthy donors were plated in low attachment wells in a 6-well plate, 4 x 10^6 cell per well, obtaining a suspension of both monocytes and lymphocytes. After 7 days in normoxia, PBMCs from one well were resuspended and transfected with the appropriate construct (see section 2.12.1) and subsequently split in two different normal attachment wells, of which one was kept in normoxia and the other one was moved to hypoxia for the appropriate amount of time.

The aim of this was to allow the cells to be transfected in an LA plate, where they do not adhere, allowing them to then be afterwards divided equally into two ordinary attachment plates, thus reducing well-to-well variations in transfection efficiency. After 18 hrs of normoxia or hypoxia, attached cells were washed before performing the luciferase assay, in order to obtain a pure macrophage population and remove the non-adherent lymphocytes. However, no hypoxic inducibility was observed using protein assay normalisation in these conditions (Figure 4-12).

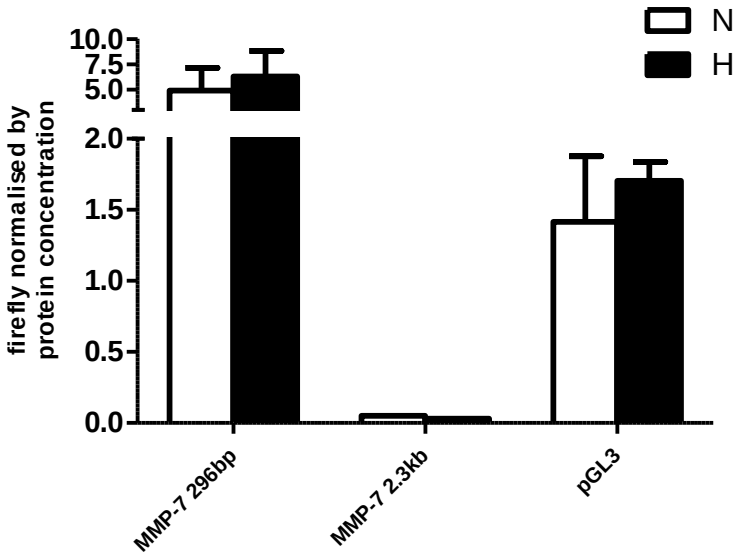


Figure 4-12: MMP-7 promoter luciferase constructs induction by hypoxia (H) in primary macrophages. PBMC-derived macrophages after 7 days *in vitro* were transfected with MMP-7 -296 bp, MMP-7 -2.3 kb and pGL3-Basic contruct (negative control) (1.5 µg per well) and incubated in hypoxia for 18 hrs. Firefly luciferase values were normalised by protein concentration (n=3). N=normoxia.

As further optimisation, PBMC transfection was coupled with the extension of the hypoxia length, from 18 hrs to 5 days, which, in preliminary studies, gave the best results in terms of MMP-7 -296 bp expression (Figure 4-13), suggesting that longer periods of cultivation in hypoxia might up-regulate MMP-7 basal levels.

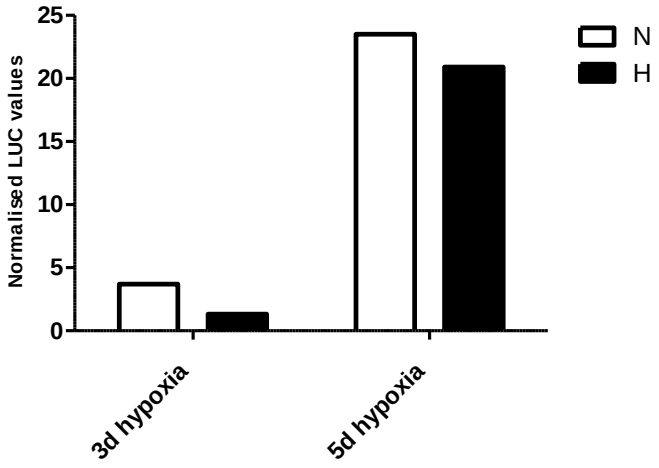


Figure 4-13: Optimisation of hypoxia length for MMP-7 -296 bp construct in PBMC-derived macrophages. **PBMC 7 days *in vitro* were transfected with MMP-7 -296 bp (1.5 µg per well) and incubated in hypoxia (H) for 3 and 5 days. Firefly luciferase values were normalised by protein concentration (N=normoxia).**

Additional optimisation experiments involved varying the "resting" time (the period macrophages were allowed to recover after the transfection procedure before moving them to hypoxia). 24 or 48 hrs "rest" period appeared to up-regulate significantly MMP-7 induction in hypoxia versus normoxia (Figure 4-14). Thus, the optimum conditions to obtain a significant fold induction of MMP-7 -296 bp construct in PMBC-derived macrophages 7 days *in vitro* were transfection with 1.5 µg DNA and exposure to 5 days hypoxia following a 24 or 48 hrs "resting" period (p value = 0.028 and 0.042, respectively, Figure 4-14).

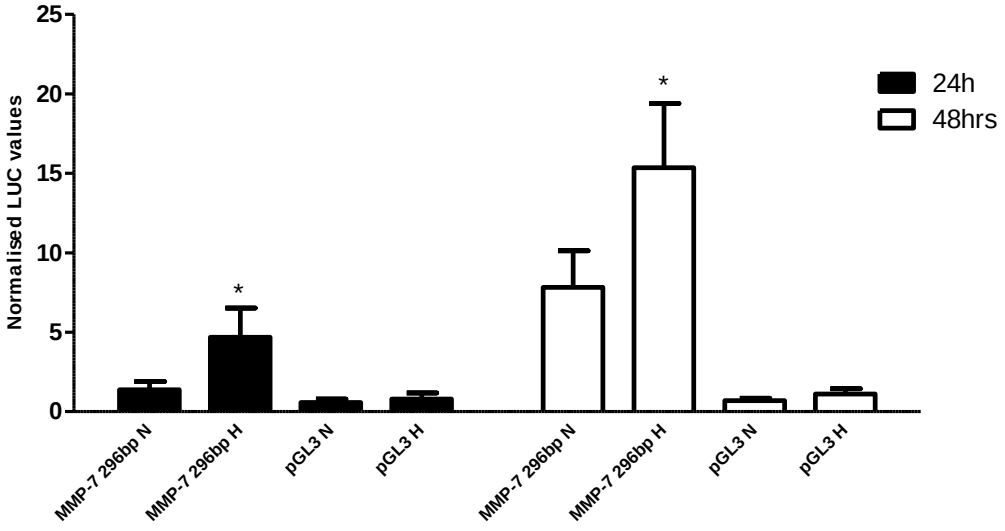


Figure 4-14: MMP-7 -296 bp construct induction by hypoxia with different "resting" time after transfection. **PBMC 7 days *in vitro* transfected with MMP-7 -296 bp (n=18) and pGL3-Basic (n=10) and allowed to rest for 24 and 48 hrs followed by 5 days in hypoxia (H). Firefly luciferase values were normalised by protein concentration. * = p < 0.05. * compares the hypoxic sample towards the correspondent normoxic (N) sample.**

As described in section 4.6, the cloned MMP-7 -296 bp in pGL4.10[*luc*2] construct has been shown to express higher levels of luciferase activity compared to the MMP-7 -296 bp pGL3-Basic construct. Despite showing a trend in up-regulation of the MMP-7 -296 bp pGL4.10[*luc*2] construct, 5 days hypoxia did not show any statistically significant fold induction of that construct (Figure 4-15), therefore indicating that the MMP-7 -296 bp pGL4.10[*luc*2] construct had enhanced basal luciferase expression but not hypoxic induction. The MMP-7 -296 bp in pGL4.10[luc2] construct did show a trend towards increased induction in hypoxia, therefore a few more repetitions could possibly have shown significance.

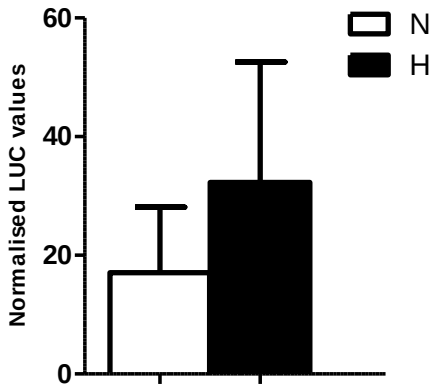


Figure 4-15: Transfection with MMP-7 -296 bp construct in pGL4.10[*luc*2] plasmid. **PBMC 7 days *in vitro* transfected with MMP-7 -296 bp in pGL4.10[*luc*2] (1.5 µg per well), moved into hypoxia (H) after 48 h and kept in hypoxia for 5 days (n=3). N=normoxia. Firefly luciferase values were normalised by protein concentration.**

4.10 Investigation of MMP-7 -296 bp mutant constructs

Using the transfection condition as described above (PMBCs after 7 days *in vitro* transfected with 1.5 µg DNA and exposed to 5 days hypoxia following a 48 hrs "resting" period), a number of the mutant constructs were investigated.

As shown in Figure 4-16, the MMP-7 -296 bp wild-type construct was significantly induced by hypoxia (3-fold, p value = 0.0349). All the mutated constructs showed very low basal expression, comparable or lower than the pGL3-Basic-transfected cells, suggesting that those transcription factors are required for basal MMP-7 gene transcription. Raw values of the transfection of the wild-type and mutated constructs are reported in Table 4-8.

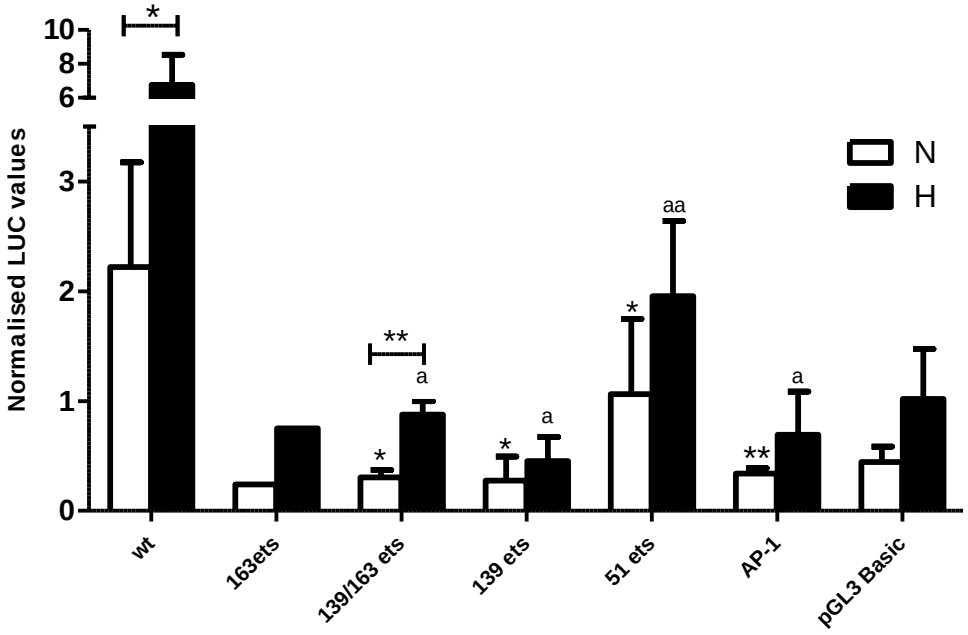


Figure 4-16: Wild-type and mutated MMP-7 -296 bp constructs inducibility by hypoxia (H). PBMC-derived macrophages 7 days *in vitro* transfected with MMP-7 -296 bp wild-type, 139ets, 163ets, 139/163ets, 51ets, AP-1 and pGL3-Basic (negative control). After transfection, the cells were subjected to a 48 hrs "resting" period and subsequently incubated for 5 days in hypoxia. Firefly luciferase values were normalised by protein concentration values (n=4). *, a=p<0.05, **, aa=p<0.01. * compares the sample with the wild-type (wt) normoxic (N) sample, "a" compares the sample towards hypoxia wild-type sample.

Construct	Reading	Normoxia				Hypoxia			
Wild-type	Background	44	52	33	36	37	52	40	36
	Firefly LUC	446	526	105	198	764	1242	766	738
-51ets	Background	42	54	34	34	39	59	29	30
	Firefly LUC	132	89	38	56	134	74	49	339
-139ets	Background	41	42	41	42	45	40	28	30
	Firefly LUC	40	68	42	52	56	40	43	97
-163ets	Background	48	44	32	36	52	43	44	41
	Firefly LUC	39	46	39	38	45	42	46	35
-139/-163 ets	Background	36	42	42	41	47	40	36	39
	Firefly LUC	156	80	54	95	251	207	88	92
AP-1	Background	49	49	30	48	40	42	37	35
	Firefly LUC	150	170	64	124	372	107	73	84
pGL3	Background	50	44	32	34	51	40	41	32
	Firefly LUC	90	184	164	155	81	99	166	165

Table 4-8: Firefly luciferase reading for MMP-7 -296 bp wild-type and mutants as indicated. The RLU/s background values (grey rows) correspond to the reading of the LARI reagent only and in subsequent analysis that value was subtracted from all Firefly luciferase (Firefly LUC) readings.

Since it has been shown in the literature that hypoxia (0.02% O_2) can cause down-regulation of protein synthesis via phosphorylation of translation initiation factor eIF2, which leads to inhibition of new protein synthesis (Koumenis et al., 2002), I sought to

ascertain if in fact the protein amount measured was affected by hypoxia. As shown in Figure 4-17, 5 days hypoxic conditions caused a statistically significant decrease in protein concentration in primary human macrophages.

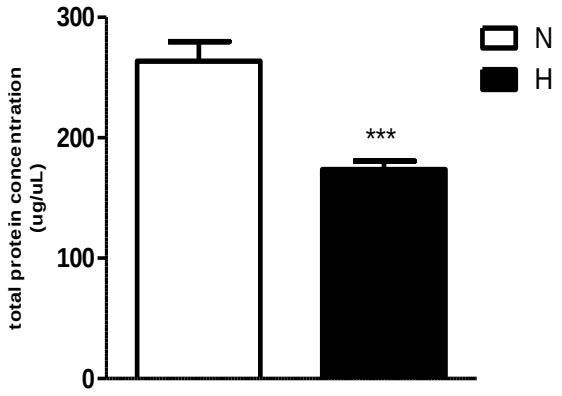


Figure 4-17: Protein concentration assay. **PBMC 7 days *in vitro* transfected with different constructs. After transfection, the cells had a 48 h "resting" period and were subsequently incubated for 5 days in hypoxia (H). ***p<0.0001 compares the normoxic (N) protein concentration with the hypoxic sample (n=25).**

Figure 4-16 was re-analysed taking this new finding into consideration using the Firefly luciferase raw data – ie, without normalisation (Figure 4-18): this resulted in an expected lowering of the wild-type MMP-7 -296 bp construct hypoxic induction (from 3-fold to 2-fold), however the final readout did not change in terms of mutated construct expression. Therefore this study did not produce a definitive answer on the role of any specific transcription factor.

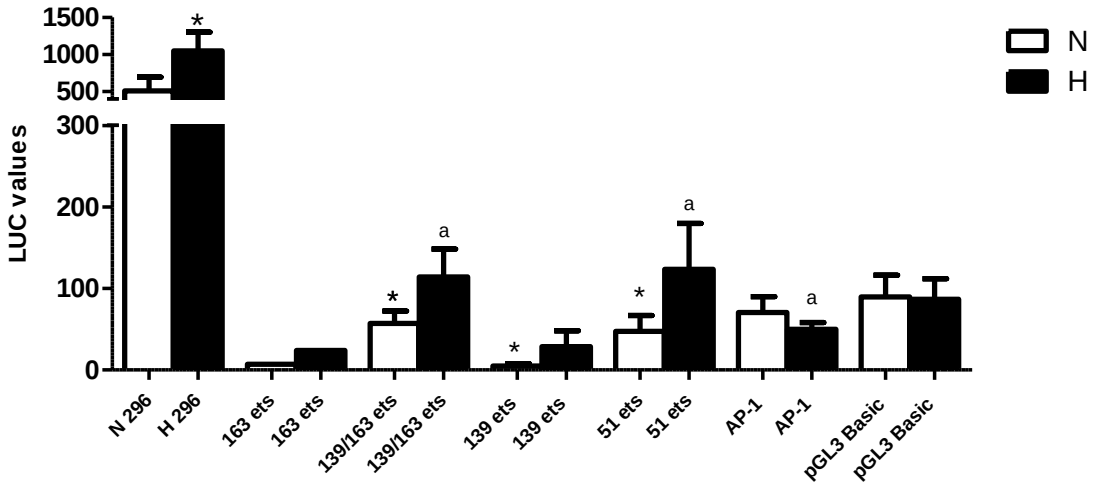


Figure 4-18: Re-graphing of wild-type and mutated MMP-7 -296 bp constructs transfection in PBMC-derived macrophages. **PBMC after 7 days *in vitro* transfected with MMP-7 -296 bp wild-type (wt), 139ets, 163ets, 139/163ets, 51ets, AP-1 and pGL3 as a control. After transfection, the cells had a 48 h "resting" period and were subsequently incubated for 5 days in hypoxia (H). On the y axis, raw firefly luciferase values (n=4). *, a=p<0.05. * is comparing the sample with the wild-type normoxic (N) sample, "a" compares the sample towards hypoxia wild-type sample.**

4.11 Discussion

In this chapter, the effect of 18 hrs and 5 days hypoxia on MMP-7 expression using transfection of different length promoter luciferase constructs was investigated in adherence-purified MDM from donor PBMCs. Two different methods to culture adherence-purified macrophages were used: hMDMs refers to the plating of the peripheral blood mononuclear cells (PBMCs) on a normal attachment well and quick removal of lymphocytes in suspension, which results in a pure monocyte population; PBMC-derived macrophages where instead initially plated in low attachment plates, therefore the monocytes are in suspension with the lymphocytes, then transfected and moved to a normal attachment plate were, subsequently, lymphocytes in suspension were removed, to obtain again a pure adherent monocyte population. The PBMC-derived macrophages method aims to allow transfection in LA plate, where they do not adhere, followed by equal division into two ordinary attachment plates, thus reducing well-to-well variations in transfection efficiency.

The aim of this chapter was firstly to identify the shortest construct induced by hypoxia. Initial transfections using the -2.3 kb MMP-7 promoter in pGL3-Basic construct showed undetectable levels of firefly luciferase in the investigated cell lines (Table 4-3), in hMDM (Table 4-5) and in PBMC-derived macrophages (Figure 4-12). The -296 bp MMP-7 construct expression was then investigated in cell lines (Figure 4-2 and Table 4-3), hMDM (Table 4-5) and PBMC-derived macrophages (Figure 4-12) and showed consistent detectable levels of luciferase activity, and therefore this construct was selected for futher analysis. DNA sequence elements controlling transcription by RNA polymerase II are generally divided into proximal and remote elements. Proximal elements reside near the start point of transcription, usually within 100 bp, whilst remote elements, including enhancers and upstream activating sequences, are positioned at greater distances, usually from a few hundred to a few thousand base pairs away. Both types of elements are involved in raising transcription to levels higher than that set by the basal transcription machinery, which generally includes TATA sequences. Most mammalian promoters require both proximal and remote elements for appropriate transcription. Silencers, in contrast, are regions of the promoter which repress gene expression when the appropriate transcription factor binds to them (Ogbourne and Antalis, 1998). My experiments showed that the MMP-7 -296 bp promoter construct produces higher luciferase expression than the -2.3 kb construct, which could be due to the presence of silencer regions in the longer construct, which could then inhibit MMP-7 gene expression. The low MMP-7 343 bp readings for luciferase activity (Table 4-6) could possibly be explained by the presence of a negative regulatory element, either "genuine" or produced artifactually due to the precise transcription factor binding sites included or partly included in the cloned downstream portion. In the extra +47 bp, I found a site for Tcf/LEF-1 binding site by Genomatix MatInspector analysis; it is

known that the murine MMP-7 promoter contains a single Tcf site located downstream the transcription start site (+8 bp), which represses the activation of MMP-7 promoter (Gustavson et al., 2004). Therefore, it could be possible that this downstream promoter found in the +47 bp in the human MMP-7 promoter can repress MMP-7 promoter activity as well.

Initially, transfections were performed in hMDMs using a *Renilla* luciferase pRL-TK plasmid as an internal control. The MMP-7 -296 bp (Figure 4-5) and the MMP-7 -965 bp (Figure 4-6) constructs showed no hypoxic inducibility. Because all the MMP-7 constructs used so far did not show expression or hypoxia inducibility, the project proceeded with the study of regulation of MMP-7 via transcription factors.

Gene expression in eukaryotic cells is regulated by alteration of gene transcription as well as other methods (e.g. speed of RNA processing, mRNA stability, efficiency of mRNA translation, Phillips, 2008). Different ets transcription factors may have different effects on target gene expression by cooperating with or opposing target gene expression (Le Floch et al., 2005). In order to investigate the role of ets in MMP-7 regulation, various techniques have been employed: from RNA silencing to ets inhibitors to ets over-expression. Test for the down-regulation of either ets (*data not shown*) or HIF-1 (Figure 4-7) failed to show functionality of the silencing assay, therefore a conclusive result for MMP-7 regulation by ets or HIF-1 cannot be stated. It is known however that stable siRNA transfection in primary human macrophages is difficult to achieve (Asplund et al., 2010).

It has been shown in the literature that ets-1 in macrophages, without the presence of a stimulus such as TNF-α, shows a low expression (Goetze et al., 2001), therefore the low detectability of ets-1 could also be due to the little ets-1 expression.

MMP-7 constructs (both -2.3 kb and -296 bp) were up-regulated by ets-1 over-expression construct in RAW 264.7 and hMDM cells (Figure 4-9 and Figure 4-10). However, a role for ets-1 in MMP-7 regulation is already known (Miyoshi et al., 2006) and a further hypoxia contribution was not found, therefore the ets-1 study was not pursued further.

Interestingly, it was found that DMSO at 0.5% could down-regulate MMP-7 expression (section 4.7.2); DMSO has also being shown to down-regulate NF-κB in murine macrophages (Kelly et al., 1994; Munshi et al., 2004). The possibility of MMP-7 expression being affected by NF-κB pathway will be explored in section 5.9.

Before proceeding to further optimisation, the *Renilla* pRL-Tk construct was checked for hypoxic inducibility and it was shown to be induced by hypoxia (Figure 4-11). Alternative normalisation techniques have been suggested such as co-transfection with GFP (Vesuna et al., 2005) or normalisation to total protein content (Goldman and Shalev, 2006). Therefore, subsequent transfections were carried out in PBMC-derived macrophages using protein concentration as a method of normalization since its ease and availability. Although this method was not able to determine the transfection efficiency, it could still prove to be a valuable method of normalisation for determination of the protein amount in each well and therefore correction for the cell number per well. Combined optimisation of hypoxia length (5 days) and resting time after transfection (24 or 48 hrs) led to a statistically significant MMP-7 hypoxic induction (2- and 3.4-fold respectively, Figure 4-14).

Next, the mutant promoter construct luciferase expression was analysed in order to determine their hypoxia inducibility, as a lack of induction would have revealed that transcription factor as essential for hypoxia induction. Using the 5 days hypoxia length coupled with 48 hrs “resting” time after transfection protocol, selected mutants were

transfected in PBMCs (Figure 4-16). All the mutants constructs employed in these experiments showed a decreased level of luciferase expression, comparable to or below the pGL3-Basic-transfected negative control cells, suggesting that those transcription factors are required for basal MMP-7 gene transcription and promoter functionality. These results are in contrast to data from HepG2 cells (Figure 4-3), where only the mutation in the -163 bp binding site for ets transcription factor and mutation in the AP-1 binding site showed decrease in MMP-7 expression; however, it has to be kept in mind that these data are collected from two different cell types in two different hypoxic condition (18 hrs in HepG2 and 5 days for primary macrophages), therefore a different cell type could be responsive to different transcription factor in the regulation of MMP-7. The same conditions (18 hrs) of HepG2 cells were not tested in primary human macrophages since the wild-type construct was seen not to be hypoxia-inducible after that period (Figure 4-5). As mentioned in section 4.10, the "163ets" mutated promoter had almost undetectable luciferase values (only one experiment produced detectable luciferase values, represented in Figure 4-16), which probably indicates that this transcription factor binding site is essential for MMP-7 basal expression. It seems therefore that all the ets sites and AP-1 site are essential for MMP-7 expression and possibly (which could not be confirmed from this set of experiments) hypoxic regulation.

CEBP, a transcription factor for which transcription binding sites have been found by Genomatix analysis in the proximal promoter of human MMP-7, has been shown to be elevated in carcinoma and is increased by hypoxia; CEBP also regulates HIF-1α (Min et al., 2011). NFAT, the other novel transcription factor which I identified in the proximal MMP-7 promoter, has also been shown to be activated by chronic hypoxia (de Frutos et

al., 2007). It would be therefore of interest in the future to study the possible involvement of these two hypoxia-related transcription factors in MMP-7 regulation.

Chapter 5 Investigation of synergy between TLR ligands and hypoxia in regulation of MMP-7 transcription and studies of possible mechanisms of up-regulation

5.1 Introduction

A secreted protease expressed mainly by epithelial cells, blood monocytes, mature monocyte-derived macrophages, B and T-lymphocytes and many tumour cells, MMP-7 mRNA levels are markedly stimulated by exposure to LPS of monocyte-, bone marrow-derived macrophages and the human monocytic cell line U937, but with great variability (Busiek et al., 1992; Busiek et al., 1995; Fang et al., 2009). Polymyxin B (PMB) is an antibiotic derived from the bacterium *Bacillus polymyxa* and it is used to treat a variety of infections, such as meningitis, respiratory, urinary and gastrointestinal infections caused by Gram-negative bacteria (Falagas et al., 2006, Zavascki et al., 2007). PMB is a cationic protein that binds to the bacterium outer membrane (specifically to the lipid A component of the LPS layer, which is negatively charged) and alters it making it more permeable to water, which results in bacterial death. Moreover, PMB can bind to and neutralise free endotoxins, inhibiting their activity (Tsan and Gao, 2004). Addition of PMB to PBMC cultures stimulated with LPS causes a reduction of both TNF-α and IL-10 (Cardoso et al., 2007). Polymyxin B, interacting with and neutralising LPS, blocks the LPS-induction of MMP-7 in the human monocytic cell line U937 (Lopez-Boado et al., 2000).

Non-adherent monocytes express low levels of MMP-7 but monocyte adherence to plastic (which is a pre-requisite for their differentiation into macrophages) can increase

MMP-7 mRNA expression from undetectable to transiently detectable after just 2 hrs, in a COX-independent fashion (Table 5-1); immediately *ex vivo* PBMCs in fact do not express MMP-7 (Bar-Or et al., 2003). In the same study, stimulation of monocytes with 100 ng/mL LPS was reported not to up-regulate MMP-7 gene, although the monocytes were analyzed 8 hrs after preparation from blood, thus it might not have given the monocytes enough time to differentiate into macrophages (Bar-Or et al., 2003). In constrast, LPS stimulation (25 µM) of adherent monocytes was shown to up-regulate MMP-7 mRNA (Table 5-1) in a p38-dependent way (Reel et al., 2011). Previous studies showed that monocyte adhesion, LPS-treatment and differentiation into macrophages increased the mRNA levels of MMP-7 to a greater degree than the increase in TIMP expression, implying a shift in the protease/anti-protease balance which tends to promote matrix degradation (Reel et al., 2011).

	Effect of adhesion	**LPS induction**	**Effect of differentiation**
MMP-7	~100 fold	132.59 fold	126.99 fold

Table 5-1: Increase in MMP-7 mRNA levels by different stimuli. Monocytes were prepared from buffy coats and left to adhere for 2 hrs after preparation (adhesion effect). *E. coli* LPS 026:B6 100 ng/mL was added for 18 hrs during the adhesion stage in the presence of the COX inhibitor indomethacin (LPS induction effect). CD16- monocytes were purified and left to adhere and mature into macrophages for 7 days in the presence of M-CSF (effect of differentiation). Fold changes relative to the fresh unadhered and undifferentiatied monocytes (control). Adapted from Reel et al., 2011.

The human MMP-7 gene and protein were found to be up-regulated by 16 hrs of hypoxia in primary human macrophages and in a mouse macrophage cell lines (RAW264.7, Burke *et al*, 2003). However, recent unpublished results from our group have shown that hypoxia as a single treatment was not sufficient for MMP-7 gene induction. Studies on the involvement of LPS in transcriptional regulation of MMP-7 were carried out by an MSc project student in Dr Burke's lab, Valentinos Kounnis, as part of his project, whom I supervised. In these studies, LPS treatments (1 ng/mL and

10 ng/mL) were seen to enhance the basal expression of MMP-7 mRNA and, when in synergy with hypoxia, caused a significant increase in MMP-7 mRNA level compared to non-treated hypoxic cells (Figure 5-1).

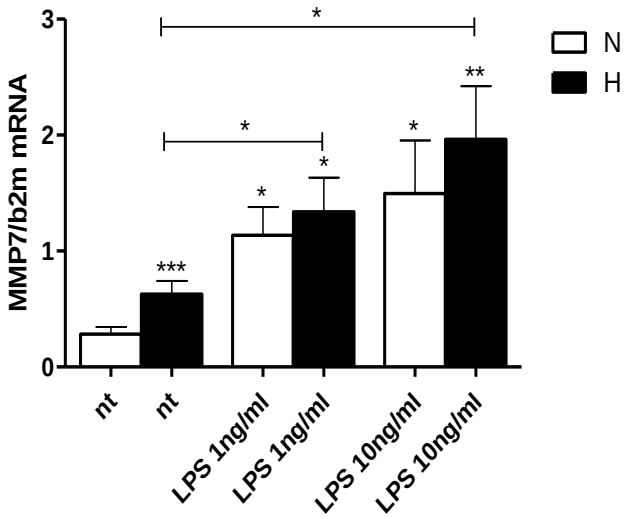


Figure 5-1: Synergistic up-regulation of MMP-7 mRNA by LPS and hypoxia (H). **SAE LPS was added at the specified concentration to hMDM 5 days *in vitro* and incubated in hypoxia for 18 hrs either not treated (nt, n=18) or with LPS 1 ng/mL (n=5) and LPS 10 ng/mL (n=6). * is compared with the nt normoxic (N) sample and the other * follow the specific lines. Data from Valentinos Kounnis and Dr Bernard Burke.**

As described in the Introduction, section 1.2, TLRs are ubiquitous receptors for PAMP recognition. LPS and Flagellin (TLR ligands for TLR4 and TLR5, respectively) have been demonstrated in the literature to induce MMP-7 (Busiek et al., 1992; Lopez-Boado et al., 2001), and thus the next question to answer was if LPS and Flagellin were the only compounds able to up-regulate MMP-7 or if a panel of TLR ligands could have the same effect. Figure 5-2 shows that up-regulation of MMP-7 mRNA in primary human MDMs occurs when MDMs are treated with a range of TLR ligands, with the exception of Imiquimod. Also, hypoxia up-regulation seen in the non-treated sample is preserved amongst all the TLR ligands, in the majority of cases inducing a bigger up-regulation than the hypoxia-treated sample only.

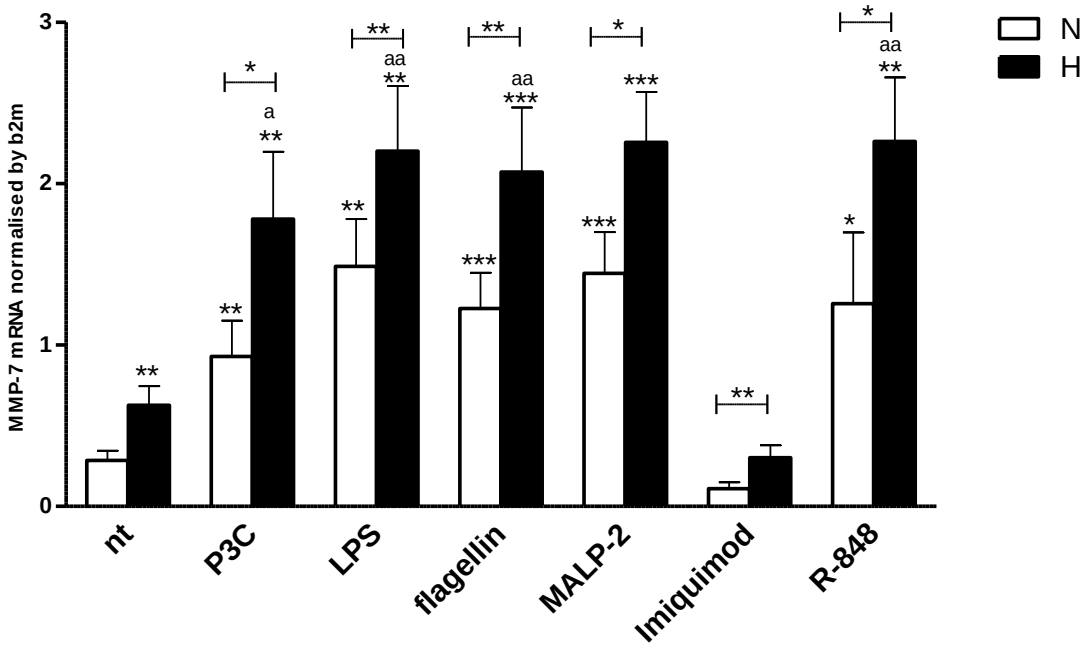


Figure 5-2: MMP-7 is up-regulated in macrophages in response to stimulation of TLRs 1-2-4-5-6 and -8. ***, a=p<0.05, **, aa=p<0.01, ***=p<0.001. a=towards non-treated (nt) in hypoxia (H), *=towards nt in normoxia (N). Bars represent means ± SEM. TLR ligands were used at the following concentrations: P3C at 10 ng/ml, *E.Coli* LPS at 40 ng/mL, flagellin at 100 ng/mL, MALP-2 at 20 ng/mL, Imiquimod at 2.5 µg/mL and R-848 at 2.5 µg/mL (5<n<8). This figure was produced by Valentinos Kounnis.**

Taking these results into consideration, the next part of my project focuses on the interaction between hypoxia and other TLR agonists in up-regulation of MMP-7.

5.2 TLR involvement in MMP-7 up-regulation

MMP-7 expression in HCT-116 and HEK293 cells was investigated. HCT-116 cells do not express some of the TLR receptors, namely TLR2, TLR4 and TLR7, whilst expressing TLR5 and TLR9. HEK293 cells do not express TLR2 and TLR4 (Zhao et al., 2007). The aim of this study was to check for MMP-7 hypoxia inducibility in cell lines lacking expression of some TLRs. HEK293 cells were shown not to express MMP-7 gene (*data not shown*), whilst HCT-116 cell express MMP-7 which is neither responsive to LPS, according to the lack of TLR4 in this cells line, nor hypoxia, which could be explained as well by the lack of LPS response (*data not shown*). The presence of flagellin, whose receptor is present in the HCT-116 cells, produced a trend of

increased MMP-7 expression, though not significant. This study could validate the hypothesis of the necessity of an LPS response in order to obtain a hypoxic up-regulation of MMP-7.

5.3 LPS involvement in endogenous MMP-7 regulation

Results obtained from a PhD student in our laboratory (Abdulkareem AlHerz) from his whole-genome Affymetrix U133 cDNA microarray work also showed that LPS seems to have a bigger effect than hypoxia alone on MMP-7 induction (Figure 5-3).

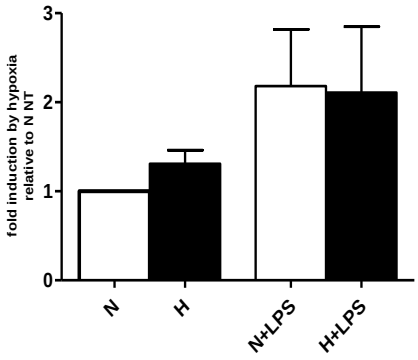


Figure 5-3: cDNA microarray analysis data for MMP-7. Data kindly provided by Abdulkareem AlHerz (n=2). Human MDMs were plated in flasks at a concentration of $1.5x10^6$ cells/mL and after 5 days, SAE (*Salmonella Abortus Equi*) LPS was added at a concentration of 100 ng/mL followed by incubation in either normoxia (N) or hypoxia (H) for a further 18 hrs. RNA was isolated using RNeasy QIAGEN Kit and samples from the four conditions (Normoxia, hypoxia, normoxia+LPS and hypoxia+LPS) were sent for microarray cDNA analysis at the University of Nottingham.

The effect of increasing doses of LPS and hypoxia on MMP-7 mRNA expression was then investigated. As seen in Figure 5-4, MMP-7 mRNA is progressively up-regulated by LPS in normoxia and the effect is dose-dependent up to 40 ng/mL. Also, the cells exposed to the two stimuli together, hypoxia and LPS, show higher MMP-7 mRNA expression than cells exposed to hypoxia only, again up to 40 ng/mL. These results suggest a synergy between LPS and hypoxia in up-regulating MMP-7 expression. Of interest is the observed reaching of a "plateau" in MMP-7 up-regulation at LPS

concentration of 40 ng/mL (ie, any further up-regulation of MMP-7 expression was not observed at 400 ng/mL). LPS seems to cause saturation of the MMP-7 response, which is supported by the fact that above a certain LPS concentration (40 ng/ml), additional LPS does not induce further MMP-7 expression. MMP-7 was not significantly up-regulated by the hypoxic treatment by itself (Figure 5-4).

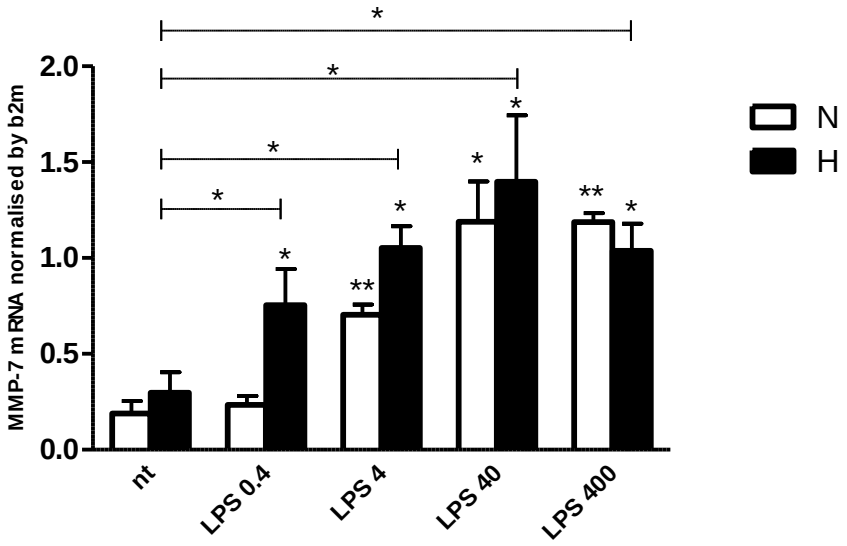


Figure 5-4: Dose-response of MMP-7 mRNA to increasing concentrations of *E. coli* LPS (ng/mL) in normoxia (N) or hypoxia (H) for 18 hrs. **hMDMs were cultured for 5 days prior to experiments (n=3). *=p<0.05 compared to non treated (nt) normoxic sample except where indicated by horizontal lines.**

5.4 LPS specificity in MMP-7 up-regulation

Polymyxin B (PMB), as explained in section 5.1, is an antibiotic used in Gram-negative infections. My data shows that PMB inhibits MMP-7 hypoxic fold induction in both non-LPS-treated samples and its LPS-dependent induction (Figure 5-5); therefore, the hypoxic MMP-7 induction in the untreated sample may be solely due to LPS contamination still present in filtered medium, since this induction disappears in the PMB-treated hypoxic sample.

To investigate the specificity of MMP-7 responses to LPS, we investigated MALP-2 (a TLR2 ligand which is able to up-regulate MMP-7 expression, Figure 5-2), to determine

if PMB blocks other TLR-ligand-induced MMP-7 up-regulation. PMB is not capable of downregulating MALP-2-dependent MMP-7 induction in a statistically significant way (Figure 5-5), and the hypoxic induction of MMP-7 is still preserved. Therefore, MMP-7 hypoxic up-regulation seems to be regulated by LPS. As stated previously, it is evident that the hypoxic up-regulation of MMP-7 is due to remaining LPS contamination in synergy with hypoxic conditions, which contradicts previous published papers (Burke et al., 2003) showing MMP-7 being up-regulated by hypoxia only.

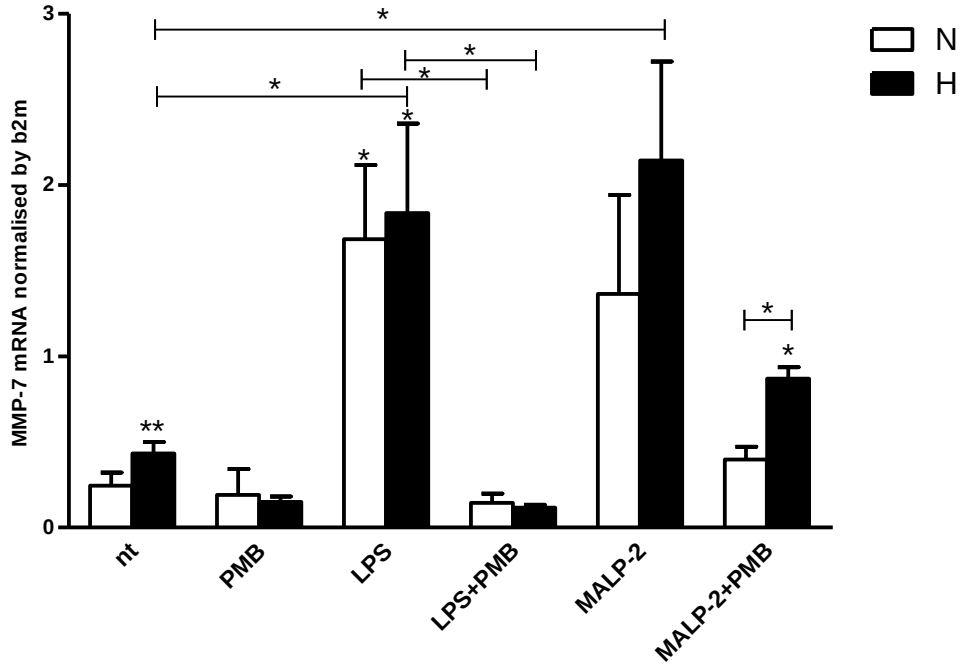


Figure 5-5: Effect of polymyxin B on MMP-7 induction in normoxia (N) and hypoxia (H). **MDMs 5 days *in vitro* were treated with PMB (40 µg/ml), LPS (40 ng/ml) and MALP2 (20 ng/ml). LPS+PMB and MALP2+PMB complexes were pre-incubated 45 min before addition to the wells. Real time RT-PCR. n=3. *=p<0.05 (towards non treated, nt, sample in normoxia or between specific samples indicated by the horizontal line).**

5.5 Regulation of the MMP-7 promoter by LPS

After investigating MMP-7 mRNA up-regulation by LPS, the MMP-7 promoter response to LPS was re-analysed from previous experiments, in order to define the region involved in LPS regulation.

Human MDMs cultured *in vitro* for 7 days were transfected with three different MMP-7 promoter constructs (-2.3 kb, -296 bp and -965 bp) and treated with 100 ng/mL LPS. The plates were then incubated in normoxia or hypoxia for further 18 hrs. As stated in Section 4.4, the MMP-7 -2.3 kb expression was not detectable, the -965 bp expression was very low, whilst MMP-7 -296 bp promoter reporter construct luciferase values were detectable and the expression was slightly increased both in the presence of hypoxia and LPS (Figure 5-6). The induction was not statistically significant, but it appears that the segment in the MMP-7 promoter responsive to LPS can be located in the first -296 bp upstream of the transcription start site.

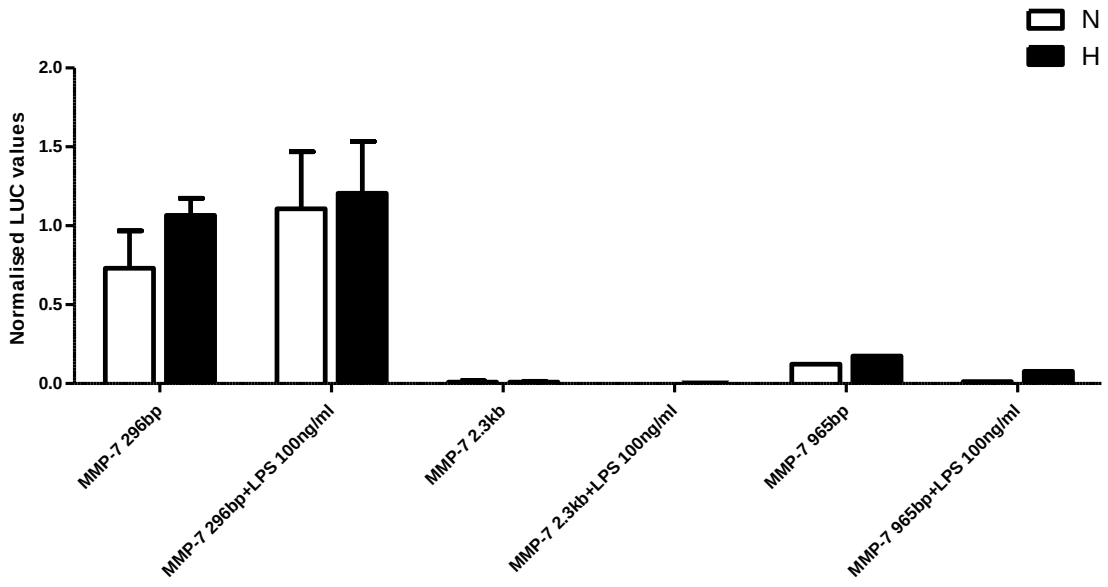


Figure 5-6: MMP-7 promoter constructs expression in LPS-treated human MDM. **hMDMs 7 days *in vitro* were transfected with MMP-7 -296 bp, -2.3 kb, -965 bp wild type construct (1.5 µg) and *Renilla* pRL-Tk (50 ng). SAE LPS (100 ng/mL) added were described. Firefly luciferase values were normalised by *Renilla* luciferase values (n=3 for the -296 bp construct, n=1 for -2.3 kb and -965 bp). N=normoxia, H=hypoxia.**

5.6 LPS involvement in MMP-7 protein induction

Measurement of protein level is as necessary as mRNA level for a more complete understanding of how the cell works. Therefore, the next step was to investigate if MMP-7 protein was regulated in the same way as the MMP-7 mRNA, assuming that

there should be a correlation between mRNA and protein level (Kleijn and Proud, 2002). The ELISA assay was performed as described in Section 2.17. In our assay (Figure 5-7) the average concentration of MMP-7 in the supernatant was 160 ng/mL.

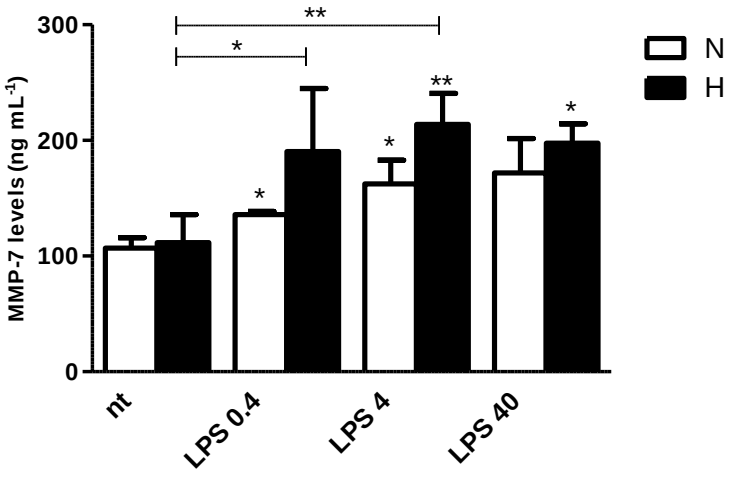


Figure 5-7: Effect of LPS and hypoxia (H) on MMP-7 protein induction. **ELISA was carried out on hMDMs 9 x 10^6 cells per well cultured for 5 days *in vitro*. *E. coli* LPS was then added at the concentration specified (ng/µL) and the plates incubated for 18 hrs in hypoxia (n=3). The supernatant was then collected, centrifuged and the ELISA assay was performed. *=p<0.05, **=p<0.01 compared to non treated (nt) normoxic (N) sample except where indicated by horizontal lines.**

The samples were diluted 1:10, 1:20 or 1:50 and the concentrations were determined through interpolation of the values from a standard curve. For control purposes, cell-free medium with or without AB serum were tested and gave similar readings to the blank (*data not shown*). MMP-7 protein (Figure 5-7) shows an increase with the increased LPS concentration and an even higher up-regulation when both hypoxia and LPS (at concentrations of 0.4 and 4 ng/mL) were present rather than hypoxia alone.

5.7 Hypoxia and LPS involvement in MMP-7 enzymatic activity

Casein and gelatin zymography are commonly used for assaying MMP activity. Zymography is based on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) in non-denaturing conditions where casein or gelatin co-polymerise with acrylamide (Fernandez-Resa et al., 1995).

In Figure 5-8, samples in normoxia and hypoxia+LPS analysed via casein zymography showed a clear band with an approximate size of 28,000 Da which possibly corresponds to the latent form of MMP-7, 28 kDa (Lopez-Boado et al., 2000). A clear pro-MMP-7 band is present in normoxia and a slightly brighter band in hypoxia treated with LPS (Iscove's medium without the addition of human serum). When the AB serum was present in the medium in sample treated with hypoxia and LPS, a much brighter band of the same molecular weight was seen (Figure 5-8).

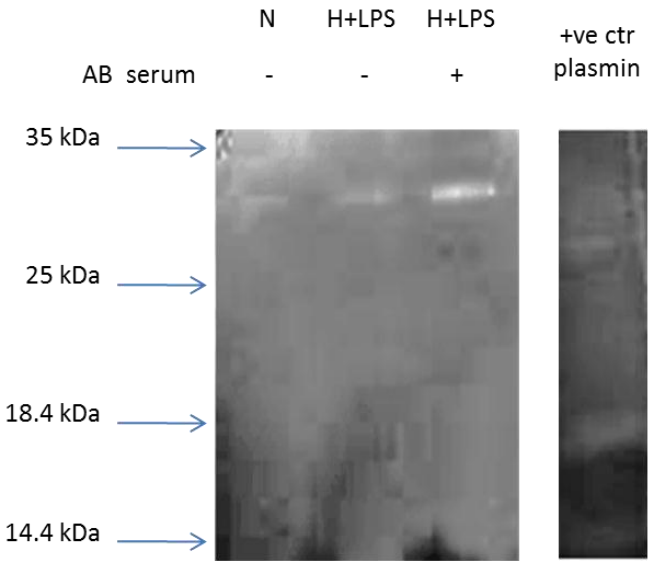


Figure 5-8: MMP-7 enzymatic activity in the presence (+) or absence (-) of AB serum. **Normoxic (N) and hypoxic (H)+LPS samples, AB serum-negative: 7-days-old hMDM were plated 8x10^6 per well. On the 4th day, the medium was changed to non-serum medium, SAE LPS was added and the samples incubated for further 18 hrs in N or H. H+LPS sample AB serum-positive: 7-days-old hMDM were plated 8x10^6 per well. On the 4th day, SAE LPS was added and the samples incubated for further 18 hrs. The supernatants were collected, spun down and concentrated as described in section 2.21. Normoxic and hypoxic+LPS samples were analysed by casein zymography. Plasmin was used as a positive control (+ve ctr).**

A possible explanation for a brighter band present in the lane with the AB serum could be the interaction of the cells with the serum, which could increase MMP-7 expression. In order to investigate this, medium only (with or without AB serum) was analysed.

An MMP-7 band was present when the medium was supplemented with serum (Figure 5-9), but not in the non-serum lane. This suggests the presence of MMP-7 in the human

serum added to the medium, which is known since MMP-7 is a secreted protease. The band present with both hypoxia and LPS conditions could therefore be explained by the presence of the serum in the medium.

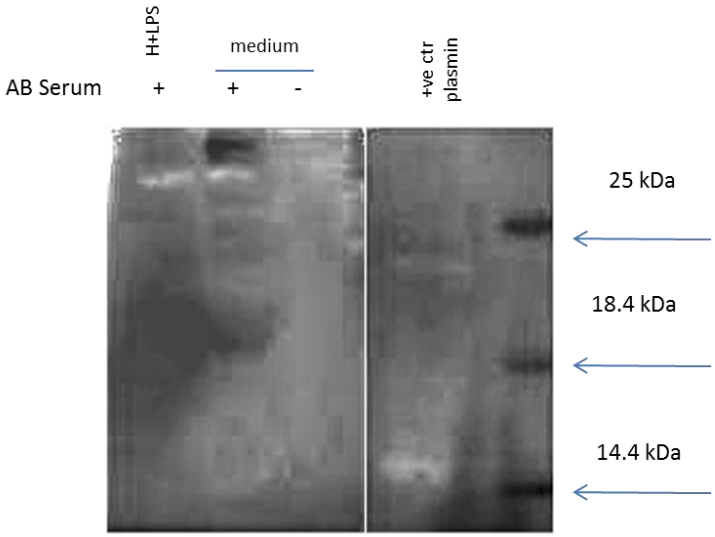


Figure 5-9: MMP-7 enzymatic activity in the presence (+) or absence (-) of AB serum. H+LPS sample AB serum-positive: 7-days-old hMDM were plated 8x10^6 per well. On the 4th day, SAE LPS was added and the samples incubated for further 18 hrs. The supernatants were collected, spun down and concentrated as described in section 2.21. Lanes indicated by "medium" indicated the absence of cells, with or without the addition of serum. Plasmin was used as a positive control (+ve ctr).

Therefore, the use of medium without addition of serum was employed for the next set of experiments. In the absence of serum, no difference was found in pro-MMP-7 levels in a complete set of conditions (Figure 5-10). An apparent band appears in the normoxic sample around 25 kDa but on a closer inspection, it is a defect present on the dried gel.

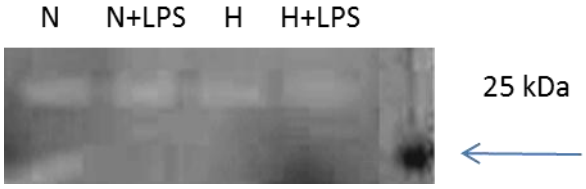


Figure 5-10: MMP-7 enzymatic activity for samples in different conditions (normoxia, hypoxia, normoxia+LPS and hypoxia+LPS) without serum. 7-days-old PBMC were split, plated 6x10^6 per well, SAE LPS (100 ng/mL) was added and the samples incubated in normoxia (N) or hypoxia (H) for further 18 hrs. Supernatants were analysed via casein zymopgraphy.

I can conclude that, in the samples analysed above, it appears that MMP-7 was present as a pro-enzyme, therefore a non-active form, because of its size (28 kDa). Therefore, increased levels of MMP-7 mRNA and protein observed previously (Figure 5-4 and Figure 5-7) do not necessarily correspond to augmented enzymatic activity. No difference in pro-MMP-7 levels in normoxic or hypoxic condition with and without LPS addition was observed. Further studies were not carried out using this method.

5.8 Use of PI3K inhibitors to investigate mechanism of up-regulation of MMP-7 by hypoxia and LPS

Hypoxic up-regulation of MMP-7 was reported to be driven by the PI3K pathway (Deguchi et al., 2009), therefore this pathway was investigated as a possible mechanism of up-regulation of MMP-7 by both hypoxia and LPS. LPS activates the PI3K pathway which results in β-catenin accumulation in the nucleus (Monick et al., 2001). LY294002 and wortmannin, known inhibitors of the the PI3K pathway, have been used to examine the role of PI3K in MMP-7 regulation. Wortmannin, an antifungal compound, binds irreversibly to PI3K, whilst LY294002 is a competitive inhibitor (Liu et al., 2009). In the literature, LY294002 plus LPS was shown to reduce β-catenin accumulation in the nucleus which is enhanced in the presence of LPS only (Monick et al., 2001).

Preliminary studies were performed to determine the optimal concentration of LY294002 (*data not shown*). From this study, the chosen concentration of LY294002 was 10 µM, based on the reduction of MMP-7 expression and hypoxic fold induction. Hypoxic fold induction decreases with the increase of LY294002 concentrations (around 60% hypoxic up-regulation reduction by LY294002 10 µM). After optimisation experiments, the use of LY294002 at the concentration of 10 µM, preincubated for 1 h before the addition of *E. coli* LPS (40 ng/mL) was determined.

To investigate if LY294002 could inhibit LPS-dependent MMP-7 up-regulation, MDMs were pre-incubated with this inhibitor and treated with LPS both in normoxia and hypoxia for 18 hrs. As seen in Figure 5-11, LPS treatment of hMDM leads to a significant MMP-7 up-regulation in both normoxia and hypoxia, however cells pre-treated with LY294002 and subjected to LPS, showed a statistically significant inhibition of MMP-7 mRNA LPS-dependent up-regulation. DMSO (at a final concentration of 0.1%) did not markedly affect MMP-7 expression. This suggests a role for the PI3K pathway in the induction of MMP-7 mRNA by LPS. LY294002 alone is able to down-regulate MMP-7 expression, even if not significantly (Figure 5-11), and possibly this effect is due to the blockage of PI3K pathway, which is a known pathway of LPS stimulation (1.2.1). The effect of LY294002 on MMP-7 down-regulation could possibly be due to the non specific effects of the inhibitor, in fact LY294002 has also been shown to inhibit lipid kinases, such as casein kinase 2, and genes involved in metabolism such as GAPDH (Gharbi et al., 2007) in addition to the PI3 kinase.

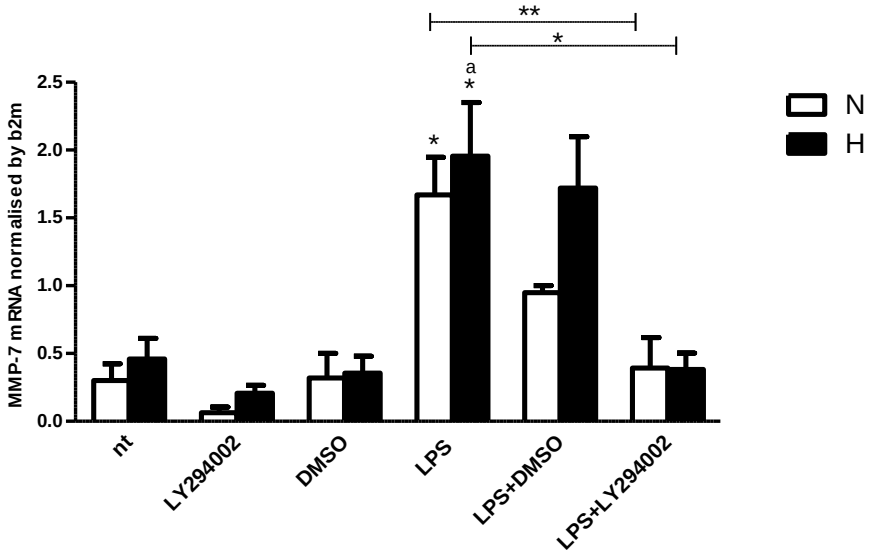


Figure 5-11: MMP-7 mRNA expression in the presence of the PI3K inhibitor LY294002. **hMDM 5 days *in vitro* were treated with *E.coli* LPS 40 ng/ml, LY294002 (LY) 10 µM (pre-incubated for 1 hr) and DMSO 2 µL and subjected to normoxia (N) or hypoxia (H) for 18 hrs (n=4). MMP-7 mRNA was quantified by real time RT-PCR and normalised against β2m mRNA levels. *=p<0.05 (compared to non treated (nt) normoxic sample except where indicated by horizontal lines), a=p<0.05 (compared to non treated hypoxic sample).**

The concentration of wortmannin used, based on a literature search, was 200 nM as it was previously employed in studies on human macrophages and murine peritoneal macrophages (Tripathi and Sodhi, 2007). Wortmannin was pre-incubated with the cells for 1 hr before addition of LPS. Wortmannin appears to inhibit MMP-7 hypoxic induction, but although it did inhibit LPS-dependent MMP-7 induction, especially in hypoxia, the inhibition was not significant (Figure 5-12). It would be informative to further optimize this experiment by using different concentration of wortmannin to see if the effect of inhibition of LPS-dependent MMP-7 induction could be dose-dependent.

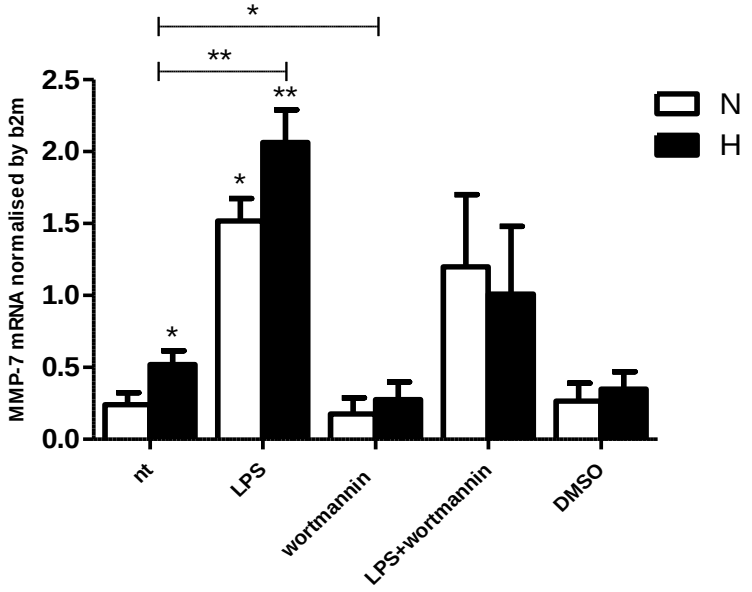


Figure 5-12: MMP-7 mRNA expression in human MDM treated with wortmannin. **hMDM 5 days *in vitro* were treated with *E. coli* LPS 40 ng/ml, wortmannin 200 nM (pre-incubated 1 hr) and DMSO (the medium in which wortmannin is dissolved) and subjected to normoxia (N) or hypoxia (H, 0.2% O_2) for 18 hrs. MMP-7 mRNA was quantified by real time RT-PCR and normalised against β2m mRNA levels (n=3). *=p<0.05 (compared to non treated, nt, normoxic sample except where indicated by horizontal lines compared to nt N or between specific samples indicated by the line connecting the column).**

5.9 NF-κB inhibitor

To investigate the possible role of NF-κB in LPS/hypoxia induction of MMP-7 mRNA, CAPE (a specific inhibitor of NF-κB) was used. In previous studies (Borthakur et al., 2008), CAPE was used at concentrations of 10-25-50 μM. CAPE seemed to down-

regulate MMP-7 expression, suggesting that NF-κB may have a role in MMP-7 mRNA expression (Figure 5-13). Although there is no statistical significance, a noticeable decrease in MMP-7 inducibility under LPS and hypoxic conditions with the higher concentration of CAPE is observed (Figure 5-13). The NF-κB pathway could therefore be involved in hypoxic and LPS regulation. Further experiments with alternative NF-κB pathway inhibitors will be necessary to confirm this hypothesis.

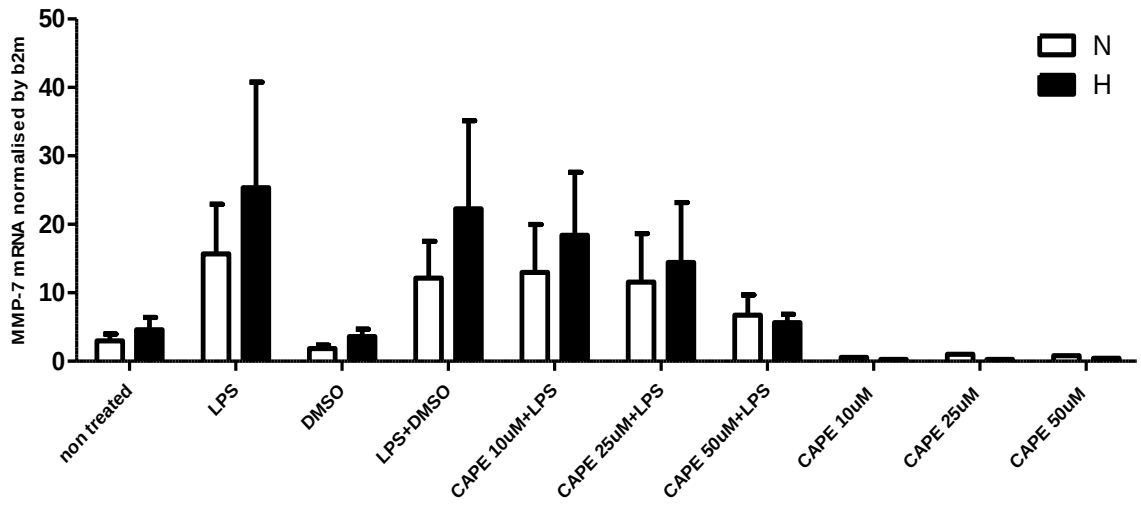


Figure 5-13: CAPE inhibition of MMP-7 up-regulation by LPS. **hMDM 5 days *in vitro* treated with LPS 40 ng/mL, CAPE at different concentration and DMSO as specified (n=4). N=normoxia, H=hypoxia.**

5.10 EMSA

Electromobility shift assays (EMSAs) were used in an attempt to determine which transcription factors (TFs) were binding to the MMP-7 promoter in hypoxia. The objective was to determine whether a particular TF was involved in hypoxic regulation. If that particular transcription factor was involved in MMP-7 regulation, a band should appear when the nuclear lysate interacts with the particular radiolabelled oligonucleotides based on the sequence of regions of the MMP-7 promoter. In the

presence of the antibody for that particular transcription factor, the band should be "supershifted", due to the increase in size of the "DNA-protein-antibody complex".

From my EMSA gels, there was no band showing interaction of AP-1 and HIF-1 with their binding sites on the MMP-7 promoter, therefore suggesting that those binding sites are not functional in primary human macrophages (*data not shown*). This is in contrast with Yuan et al., 2008 for the AP-1 site but is in accord with many papers stating the independence of MMP-7 from HIF-1 (Miyoshi et al., 2006). The radiolabelled oligonucleotide for the ets binding site in position -139 bp and -163 bp gave variable results according to different donors (*data not shown*). A band appearing in the ets EMSA gels meant that ets as a transcription factor was involved in the regulation of MMP-7, and those binding site were functional, but the extent of involvement of ets in hypoxic and LPS conditions could not be interpreted clearly.

5.10.1 EMSA for β-catenin binding site

β-catenin transcription factor binding site (Tcf/LEF-1) was investigated next, since β-catenin has been shown to transactivate MMP-7 (Crawford et al., 1999). Two Tcf/LEF-1/β-catenin binding sites (Figure 1-11) have been found on the MMP-7 promoter, in positions -105 bp and -188 bp. Therefore, I designed two oligonucleotides (see section 2.19) based on the MMP-7 promoter sequences spanning these two TF positions.

As seen in Figure 5-14, no difference in band intensity is noticed in THP-1 cell line nuclear extract in normoxic and hypoxic+LPS conditions for both Tcf/β-catenin probes. For the hMDM nuclear extracts, a faint band appearing in hypoxia+LPS conditions was seen for bot the -105 bp and the -188 bp Tcf/LEF-1/β-catenin probe, suggesting that the

β-catenin transcription factor binding to both the Tcf/LEF-1 -105 and -188 bp sites is involved in MMP-7 up-regulation in hypoxia+LPS conditions.

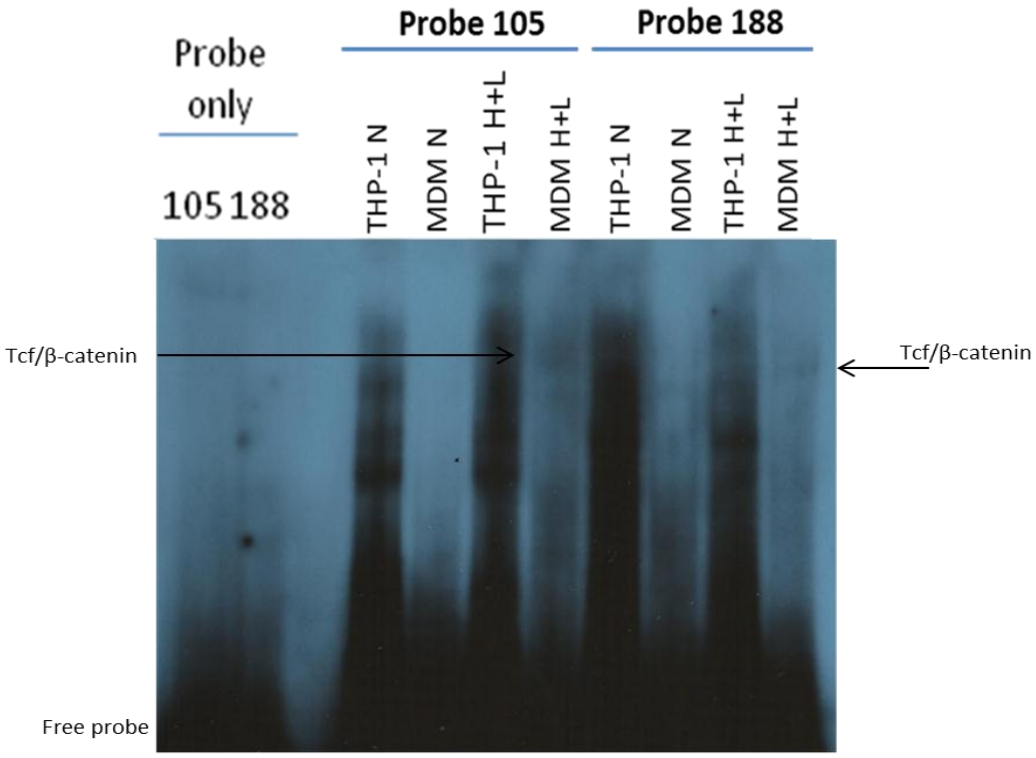


Figure 5-14: β-catenin involvement in MMP-7 regulation. **Gel shift assay for THP-1 and hMDM (MDM) nuclear extracts in normoxia (N) and hypoxia (H)+LPS (L). LPS added for THP-1 was 100 ng/mL, whilst for hMDM was 40 ng/mL. Probe 105 represents the -105 bp site for Tcf/β-catenin, whilst Probe 188 represents the -188 bp site. The "probe only" lanes represent lanes where only the radioactive nucleotide is present and they are used as a control for non-specific bands. Representative of an n=2.**

From Figure 5-15, it looks like both positions (-105 and -188 bp) for Tcf/LEF-1/β-catenin transcription factor could be relevant for MMP-7 hypoxic and LPS inducibility. There was a band appearing when the conditions were changed to hypoxia and LPS, and when β-catenin antibody was added, the same band present in hypoxia+LPS disappeared, ie the band was supershifted (β-catenin antibody is a polyclonal antibody which would bind to many epitopes on the surface of the protein, making the complex too heavy to even enter into the gel to be detected). The addition of β-catenin antibody to the normoxic samples still showed a band (even stronger in probe 188), which could

be a problem for the identification of that particular band as the complex Tcf/LEF-1/β-catenin (Figure 5-15).

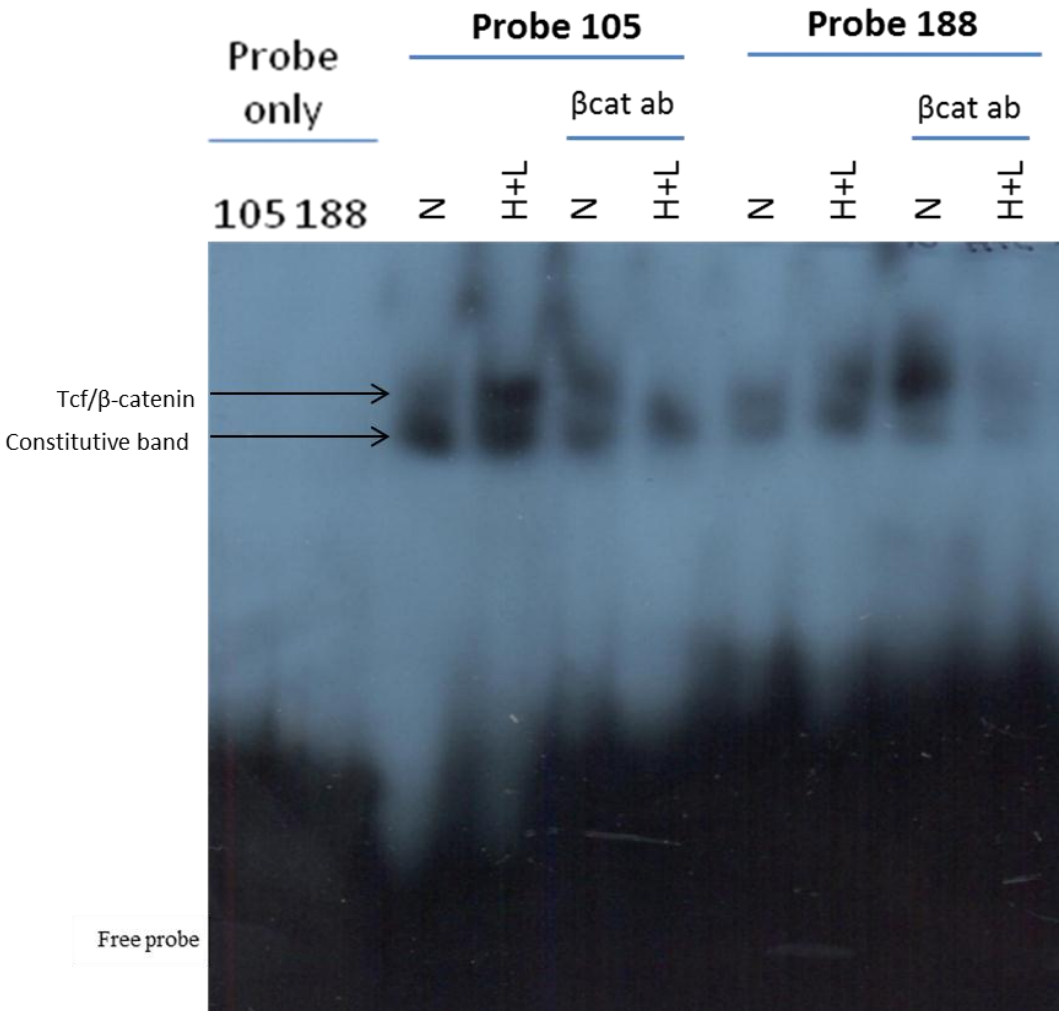


Figure 5-15: β-catenin and MMP-7 interaction in different conditions. **hMDM were treated in normoxia (N) and hypoxia (H) + LPS 40 ng/mL (H+L). The nuclear extracts were run on an EMSA with two probes, -105 and -188, both for Tcf/β-catenin transcription factors. EMSA representative of an n=2.**

5.10.2 NF-kB sites

With the help of Genomatix software, the MMP-7 promoter was found to have two putative NF-κB sites in position -1.5 kb (aaaggcatTTCCt) and -3.6 kb (aaagggcaTTCCa), where the underlined bases are highly conserved according to the Genomatix software (see Section 2.20) and the capital letter bases are the core sequence

for NF-κB binding. The NF-κB consensus sequence is GGGRDTYYCC, where R is a purine A/G (adenine/guanine), D is C/A/T (cytosine/adenine/thymine) and Y is a pyrimidine (C/T). The MatInspector software (from Genomatix) is not able to predict functional binding sites, only potential. As observed from the MMP-7 sequences, the NF-κB binding sites do not exactly correspond to the accepted consensus sequence, which might suggest that the binding sites could not be functional in the MMP-7 promoter. The preliminary EMSA did not show a clear band (*data not shown*), so no conclusion can be drawn so far for the NF-κB binding sites.

5.11 Discussion

My experiments have demonstrated that MMP-7 is regulated both at the mRNA (Figure 5-4) and protein level (Figure 5-7) by TLR ligands. Studies of hypoxic up-regulation of MMP-7 mRNA have shown that not only MMP-7 was up-regulated slightly by hypoxia (and with very variable readouts, Figure 3-5, Figure 5-2 and Figure 5-5), but the main up-regulation was obtained when macrophages were treated with TLR ligand (Figure 5-2 and Figure 5-4). In some instances the TLR treatment and the hypoxia treatment cooperate to increase MMP-7 regulation even further than each treatment alone (Figure 5-2 and Figure 5-4). Amongst the TLR ligands investigated, Imiquimod was the only one that did not cause up-regulation of MMP-7 expression (Figure 5-2). Interestingly, Imiquimod (imidazoquinoline 5%) is used in clinical practice as a topical cream to treat skin melanoma metastasis which interferes with neovascularisation and down-regulates MMP-9 (Hesling et al., 2004). An interesting question to answer would be if the mechanism of imidazoquinoline of inhibiting metastasis and invasion could be also due to the down-regulation of MMP-7 gene.

Notably, MMP-7 mRNA is progressively up-regulated by LPS in normoxia and the effect is dose-dependent up to 40 ng/mL (Figure 5-4). Also, the cells exposed to the two stimuli together, hypoxia and LPS, showed higher MMP-7 mRNA expression than cells exposed to hypoxia only, again up to 40 ng/mL (Figure 5-4). These results suggest a synergy between LPS and hypoxia in up-regulating MMP-7 expression. Of interest is the observed reaching of a "plateau" in MMP-7 up-regulation at LPS concentration of 40 ng/mL (ie, any further up-regulation of MMP-7 expression was not observed at 400 ng/mL). LPS seems to cause saturation of the MMP-7 response, which is supported by the fact that above a certain LPS concentration (40 ng/ml), additional LPS does not induce further MMP-7 expression. This is observed also in human dendritic cells, where LPS causes a plateau of different cytokines amounts (such as TNF, IL-2 and IL-6) at concentrations of 10 ng/mL (Verhasselt et al., 1997). MMP-7 was not significantly up-regulated by the hypoxic treatment itself (Figure 5-4).

Messenger RNA levels are informative in predicting protein expression, however protein does not necessarily match mRNA expression: protein translation often needs a stimulus to be activated, such as high glucose and high insulin in kidney (Kasinath et al., 2008) or activation of T cells (Kleijn and Proud, 2002). In addition, complex post-transcriptional mechanisms are utilized by the cell to turn mRNA into protein and proteins can have different half-lives which determine very different amount of protein at different times (Greenbaum et al., 2003). However, in my experiments, the levels of MMP-7 protein were strikingly similar to the MMP-7 mRNA levels: increasing concentration of LPS cause increased amounts of both MMP-7 mRNA and protein, and the hypoxia treatment when LPS was present at 4 ng/mL caused an even bigger increase than hypoxia alone (Figure 5-7). Notably, lack of hypoxic induction of the MMP-7

protein in the untreated sample was seen, possibly because regulation of the MMP-7 protein *in vivo* requires an additional signal not present in this *in vitro* study.

The up-regulation of mRNA and protein did not necessarily correlate with enzymatic activity since MMP-7, as seen from casein zymography, seemed to be secreted as a latent precursor (Figure 5-10). MMP-7 is normally secreted as a precursor (pro-MMP-7) which is subsequently activated (Gaire et al., 1994). The observed production of pro-MMP-7 at high levels was demonstrated to be due to the presence of the human serum added to the medium (Figure 5-9) rather than the MMP-7 produced by the cells, which is expressed at lower levels (Figure 5-10). The lack of observed differences of pro-MMP-7 in different conditions (normoxia or hypoxia, with or without LPS) is in contrast with the increased production of MMP-7 protein in hypoxia+LPS conditions from ELISA analysis (which detects both the precursor and the active form of MMP-7 enzyme, Figure 5-7). This suggests that the inducible MMP-7 in the ELISA assay was the active form of MMP-7. It is possible that the level of active MMP-7 may be below the detection limit of our casein zymography system. In fact, in a previous study, the same issue arose with the detection of the active MMP-7 form in an immunoblotting assay (Sarkissian et al., 2008).

In this part of the project, the importance of LPS in MMP-7 regulation was investigated. LPS is known to activate NF-κB, but also the PI3K/Akt pathway is suggested to have a role in LPS-induced gene expression (see section 1.2.1 and 1.2.2). LPS activates the PI3K/Akt pathway through phosphorylation of Akt on Thr^{308} and Ser^{473}, which inactivates GSK-3β and ends in β-catenin translocation in the nucleus, which transactivates many target genes, of which MMP-7 is one (Brabletz et al., 1999). In my study, LPS-induction of MMP-7 is inhibited by LY294002 but not by wortmannin (Figure 5-11 and Figure 5-12). LY294002 and wortmnannin are both PI3K inhibitors,

acting in different ways: LY294002 competitively inhibits an ATP binding site on the p85 subunit of PI3K (Vlahos et al., 1994), whilst wortmannin targets irreversibly the p110 subunit of PI3K (Powis et al., 1994). In a 2009 study, microarray analysis of LPS-stimulated RAW 246.7 cells reported MMP-9 as being up-regulated by LPS via the NF-κB pathway, but not via the PI3K pathway: MMP-9 regulation was inhibited by LY294002 but not by wortmannin (Mendes Sdos et al., 2009). In this array, MMP-7 was not identified (Mendes Sdos et al., 2009), possibly because RAW 264.7 cells do not show normal expression of MMP-7. In contrast, α-tomatine (a compound from immature green tomatoes) was shown to inhibit NF-κB nuclear translocation, inactivating the PI3K/Akt pathway and decreasing MMP-7 activity, gene expression and protein showing a marked reduction in human NSCLC cells invasion and migration (Shieh et al., 2011). This latest study connects the PI3K and the NF-κB pathways (Figure 5-16).

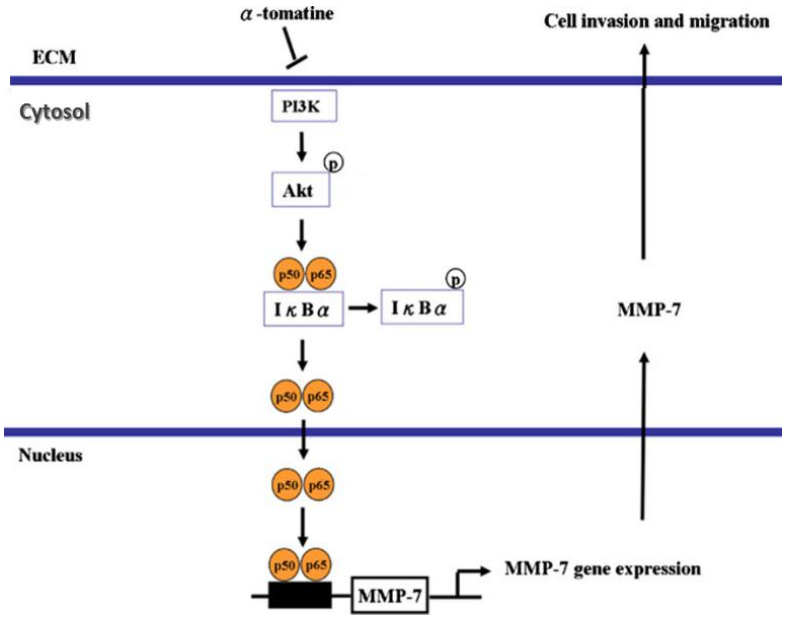


Figure 5-16: Proposed mechanism of α-tomatine role in regulation of MMP-7 in cell invasion and metastasis. Adapted from Shieh et al., 2011.

Moreover, LY294002 inhibits the DNA-binding activity of NF-κB (Kim et al., 2005) whilst wortmannin has been shown to enhance IκB degradation and p65 activation, therefore increasing NF-κB activity (Zhao et al., 2008). Therefore, the enhancement of NF-κB caused by wortmannin could be the cause for the lack of inhibition of LPS-induced MMP-7 (Figure 5-12).

Although the LY294002 and wortmannin results for MMP-7 regulation appear to be contradictory, in the literature there are many examples of the same mechanism. LPS-induced nitric oxide synthase (iNOS), in fact, is suppressed with LY294002 treatment, but not with wortmannin in RAW 264.7 cells and in glomerular mesangial cells (Kim et al., 2005, Tsai et al., 2012). It has been suggested that LY294002 inhibition of iNOS partly occurs via inhibition of NF-κB pathway (Kim et al., 2005). A very recent study has shown that LPS-induction of IFN-β is inhibited by LY294002, but not wortmannin, in an IRF3-dependent and PI3K-independent manner (Zhao et al., 2012).

Therefore definitive data on a role for the PI3K pathway in LPS-dependent MMP-7 regulation is still not clear. It is possible that wortmannin concentration could require optimisation, but the support from the literature above mentioned suggests that MMP-7 could be regulated in the same way NO is. It is suggested that LY294002 and wortmannin act through different pathways: wortmannin inhibits Akt phosphorylation, but the same effect is not observed with LY294002 (Tsai et al., 2012). The effect of LY294002 could be due to the interference of the inhibitor with other signaling pathways (off-target effects, Gharbi et al., 2007). Therefore, the proposed mechanism of PI3K pathway in hypoxic up-regulation of MMP-7 (Deguchi et al., 2009) using the LY294002 PI3K inhibitor, could be not accurate.

A study of an NF-κB inhibitor, CAPE, showed a down-regulation of MMP-7 expression and hypoxic+LPS induction (Figure 5-13). This suggests an involvement of NF-κB in MMP-7 regulation. In fact, NF-κB has been already found to up-regulate an MMP-7 luciferase construct in AGS cells (Wroblewski et al., 2003).

In order to define the transcription factors involved in MMP-7 regulation during hypoxic and LPS treatments, gel shift assay (EMSA) were employed. The EMSA data suggest that both the Tcf/LEF-1/β-catenin binding sites in position -105 and -188 bp could be involved in MMP-7 hypoxic and LPS up-regulation. These two binding sites for β-catenin have been previously shown to be functional in the human MMP-7 promoter (Crawford et al., 2001) and Tcf was found to be the transcription factor involved in hypoxic up-regulation of MMP-7 according to Deguchi et al., 2009.

Chapter 6 Discussion

The focus of this project was the investigation of the mechanism of up-regulation of MMP-7 by hypoxia in human primary macrophages. The use of human primary MDMs rather than cell lines can allow better understanding of *in vivo* inflammatory events.

MMP-7 is a secreted protease mainly expressed by epithelial cells (Wilson and Matrisian, 1996), blood monocytes (Busiek et al., 1992), B and T-lymphocytes (Bar-Or et al., 2003) and many tumour cells (Adachi et al., 1999; Wilson and Matrisian, 1996). Busiek was the first to confirm the expression of MMP-7 protein and mRNA in primary human macrophages (Busiek et al., 1992). MMP-7 mRNA expression in human MDM was seen to be increased after 3 days in culture *in vitro*, peaking at 5-7 days and decreasing at 9 days *in vitro* (Filippov et al., 2003). MMP-7 mRNA was induced rapidly and dramatically by adherence in monocytes, LPS stimulation and by differentiation to macrophages, steps which are likely to be involved in early inflammation (Reel et al., 2011).

This study followed on from previously published papers, which demonstrated that MMP-7 was up-regulated by hypoxia in primary human macrophages, macrophage cell lines (Burke *et al*, 2003) and human cancer cell lines (Miyoshi *et al*, 2006; Ide *et al*, 2006). Moreover, MMP-7 mRNA and protein have been demonstrated to be up-regulated in pancreatic cancer cells under 1% O_2 for 24 hrs (Ide et al., 2006).

MMP-7 is known to be over expressed in the majority of carcinomas (Honda et al., 1996; Crawford et al., 2001; Gustavson et al., 2004), and it correlates with poor prognosis, being involved in tumour progression and metastasis (Rudolph-Owen and Matrisian, 1998; Ii et al., 2006; Lu et al., 2011). MMP-7 inhibition could, therefore,

potentially be used as a therapy to block disease progression and metastasis (Coussens et al., 2002). Macrophages accumulate in poorly vascularised and hypoxic sites including solid tumours, wounds and sites of infection and inflammation where they can be exposed to low levels of oxygen for long periods (Lewis et al., 1999; Vaupel et al., 2001). The link between macrophages, hypoxia and MMP-7 expression in diseases, was the rationale behind my investigation of the mechanisms involving the hypoxic regulation of MMP-7.

In human monocyte-derived macrophages from 6 different donors, my data showed that MMP-7 mRNA was up-regulated 2.6-fold in acute (18 hrs) severe hypoxia (0.2% O_2) (Figure 3-5). In comparison with a 2009 study (Deguchi et al., 2009), where MMP-7 mRNA was up-regulated up to 53-fold in hypoxic conditions, the hypoxic up-regulation observed in my study is very low. This huge difference in up-regulation of MMP-7 between our study and Deguchi's could be explained by the length of the hypoxic treatment (18 hrs in our study, 10 days in Deguchi's). As mentioned above, MMP-7 expression changes in response to adherence and monocyte to macrophage differentiation (Reel et al., 2011), so it is likely that part of the difference noticed could be due to these factors. Secondly, it has been observed that chronic hypoxia (5 days) during differentiation from monocytes to macrophages induces much higher pro-angiogenic cytokine VEGF mRNA levels in adherence-purified macrophages (27-fold) compared to acute (24 hrs) hypoxia (Staples et al., 2011), which could be part of the explanation for differences observed in MMP-7 mRNA up-regulation. It has been reported that chronic moderate hypoxia (which occurs physiologically in the arterial wall, Crawford and Blankenhorn, 1991), activates the Akt and β-catenin pathway (Deguchi et al., 2009), which could possibly further amplify the inflammatory response, causing increased MMP-7 RNA levels. In contrast, a shorter severe bout of hypoxia

could activate different pathways, such as NF-κB (shown to be activated by 1.8% O_2 for 2 hrs in alveolar macrophages, Leeper-Woodford and Detmer, 1999) and AP-1 (maximal activation at 5 hrs in HeLa cells, Rupec and Baeuerle, 1995) which could regulate MMP-7 differently from the PI3K pathway mentioned above.

The up-regulation of MMP-7 by hypoxia has been demonstrated not to be due to increased mRNA stability in hypoxia, unlike increases in mRNA levels of genes such as VEGF in hypoxia, which are due in part to increased mRNA stability (Levy et al., 1998). In actinomycin D experiments using hMDM, the half-life of the MMP-7 mRNA was not affected by hypoxia (*unpublished data by B. Burke, personal communication,* Figure 1-15). Thus the mRNA increases observed in hypoxia must be due to transcriptional up-regulation.

Therefore, to investigate the mechanism of MMP-7 up-regulation by hypoxia, different MMP-7 promoter constructs were used in order to determine firstly the hypoxia inducibility of the wild-type MMP-7 constructs in primary human macrophages or tumour cell lines and subsequently, using the mutant versions, the transcription factor(s) (TFs) implicated in this up-regulation. The aim was to obtain the shortest possible promoter construct which showed still hypoxia inducibility and eventually narrow down the TFs involved by deletion and/or mutagenesis of the promoter sequences in the reporter vectors. Because the MMP-7 -2.3 kb and the cloned MMP-7 343 bp (see section 2.11) promoter constructs were shown not to express detectable levels of luciferase both in cell lines (Table 4-3) and hMDM (Table 4-5 and Figure 4-12), the MMP-7 -296 bp luciferase reporter construct was used, since it showed high luciferase values both in cell lines (Table 4-4) and hMDM (Table 4-5, Figure 4-5 and Figure 4-12).

The great variability and therefore the non-reproducibility of the results obtained from the transfection of human primary macrophages with this -296 bp construct made these experiments very challenging. Transfections in hMDMs were performed initially utilising the pRL-Tk *Renilla* luciferase reporter plasmid as an internal control for normalisation of the firefly luciferase values from the MMP-7 reporter construct. However, even after optimisation to determine the best transfection conditions in hMDM (Figure 4-4), the MMP-7 -296 bp promoter reporter construct was shown not to be induced by hypoxia (Figure 4-5). On closer analysis, the pRL-Tk *Renilla* luciferase construct was found to be up-regulated by hypoxia as well (Figure 4-11). This finding has been confirmed by a recent study, which has shown that hypoxic conditions (5% O_2) can cause a significant increase in the level of constitutive luciferase reporter activity, including the pRL-Tk construct (Doran et al., 2011). Therefore, the use of this or similar *Renilla* luciferase constructs as a normalisation method in hypoxia experiments can lead to erroneous conclusions.

To try and overcome the above difficulties, which are intrinsic to this kind of experiment, a new way of transfecting and culturing macrophages was developed, as described in section 4.9: this method involved the use of a suspension of PBMC on low-attachment 6-wells plate. PBMC in suspension consisted of monocytes (~10%) and lymphocytes (~90%). The PBMC in each well were sufficient to generate two normal attachment wells. The transfection of MMP-7 promoter luciferase constructs was performed on the cell suspension and, after transfection, the cells were re-plated at equal numbers in two normal attachmment wells, one that was subjected to normoxia and one to hypoxia. In this way, the transfection efficiency was comparable in the two wells, without the need of an internal transfection efficiency control. Moreover, protein concentration in each sample was assayed to normalise for cell number. This method

presented some disadvantages as well, since this normalisation did not give any information on relative levels of gene expression/protein translation, only on the amount of total protein present in different samples.

Using this method, no hypoxic induction of the MMP-7 -296 bp construct (Figure 4-12) was observed in PBMC under 18 hrs of hypoxia.

As a next attempt to optimise this experiment, PBMC transfection was coupled with the extension of the hypoxia length, from 18 hrs to 5 days; under these conditions, a significant induction of the MMP-7 -296 bp construct was observed (3.4-fold, Figure 4-14). As previously discussed, MMP-7 mRNA was demonstrated to be highly up-regulated by chronic hypoxia in primary human macrophages (Deguchi et al., 2009) and a recent study has shown that chronic hypoxia in macrophages induces higher VEGF mRNA levels compared to acute hypoxia (Staples et al., 2011). It therefore seems likely that chronic hypoxia can similarly produce increased induction of the MMP-7 -296 bp promoter luciferase reporter construct.

Following the successful transfection method mentioned above, which resulted in MMP-7 promoter -296 bp being induced in hypoxia, analysis of hMDM transfected with several different mutated MMP-7 -296 bp constructs (Figure 2-2) was performed. The mutated constructs analysed showed statistically lower luciferase expression compared to the wild-type MMP-7 -296 bp construct, comparable to or below the pGL3-Basic-transfected negative control cells, which suggests that these sites are important for the functionality of the promoter construct in primary human macrophages. The markedly decreased luciferase readings from the mutant construct could hinder the detection of possible hypoxia inducibility, because the readings were very close to background (Table 4-8), therefore nothing concrete could be determined about hypoxia inducibility of these mutated constructs.

This is in contrast with data from HepG2 cells (Figure 4-3), where only the mutation in the -163 bp binding site for the ets transcription factor and in the AP-1 binding site decreased luciferase expression. These differences are most likely due to the use of different cell types (HepG2 and hMDM), which could be responsive to different transcription factors in hypoxic regulation of MMP-7.

In hMDMs, the "163ets" mutated promoter had almost undetectable luciferase values (only one experiment produced luciferase values above the background reading, represented in Figure 4-16), which probably indicates that this transcription factor binding site is essential for MMP-7 basal expression both in HepG2 cells and hMDM. MMP-7 promoter reporter constructs have been shown to be responsive to over-expression of PEA3, c-Jun and Tcf/β-catenin in HEK293 cells (Crawford et al., 2001). It thus seems that in hMDM all the ets sites and the AP-1 site are essential for basal MMP-7 expression; therefore a conclusive answer on the role of these transcription factor binding sites in hypoxic induction could not be reached.

An important finding, which is agreeable with previous literature (Koumenis et al., 2002), was that protein concentration during 5 days of hypoxia was significantly down-regulated (Figure 4-17). Therefore, the protein concentration assay employed in the PBMC transfections was discovered not to be appropriate for hypoxic conditions.

In conclusion, the transfection experiments proved to be very challenging, from the transfection in primary human macrophages, to the use of internal controls, to the low expression of the mutated MMP-7 promoter constructs. These difficulties have granted a switch of the focus to the endogenous MMP-7, bypassing therefore the transfection problems.

The finding in the literature of the LPS importance in MMP-7 regulation (Busiek et al., 1992) and several pieces of data from this lab (Figure 5-1, Figure 5-2 and Figure 5-3), triggered a partial change of the project, which focussed on the interaction between hypoxia and other non-hypoxic stimuli such as TLR agonists in up-regulation of MMP-7. Interestingly, when Polymyxin B (an antibiotic which binds to LPS and inactivates its effects) was added to the cells, the hypoxic inducibilty of MMP-7 which was observed in the non-treated sample was completely abolished (Figure 5-5). Moreover, PMB effect was reversed on MMP-7 regulation (ie, the hypoxic inducibility was re-established) by addition of another TLR ligand such as MALP2, which is not affected by PMB (Figure 5-5), suggesting that the so-called and previously reported "hypoxic" regulation (Burke et al., 2003; Deguchi et al., 2009) could be actually due to TLR ligand and hypoxia combination.

LPS is a potent activator of macrophages: concentrations in the order of 10 pg/mL are sufficient for TNF protein release in human PBMC (Tsan and Gao, 2004), so this level of LPS (still present even after filtration in the medium used for this study) in combination with another factor, hypoxia in this case, could certainly be responsible for regulation of a range of genes, including MMP-7.

In accordance with the finding that MMP-7 was not up-regulated by hypoxia alone, MMP-7 did not appear as an up-regulated gene in a cDNA array study of hypoxic primary human macrophages (White et al., 2004). Also, another cDNA array study of primary human macrophages exposed to 16 hrs of hypoxia (1% O_2) did not show up-regulation MMP-7 (Bosco et al., 2006). Moreover, unpublished cDNA array data from this lab have also shown the lack of hypoxic inducibility of MMP-7 when filtered medium was used (Figure 5-3). Differentiated macrophages primed with LPS increased their TNF-α, iNOS and VEGF response to hypoxia reviewed in (Lewis et al., 1999), and

similarly MMP-7 expression also seems to be augmented in hypoxic macrophages primed with LPS. Hypoxic conditions can modify the transcriptional response in macrophages stimulated with LPS (Mi et al., 2008); transcriptional activation of inducible nitric oxide synthase (iNOS) mRNA and protein levels was highly stimulated in rat (Agorreta et al., 2003) and mouse (Mi et al., 2008) macrophages when hypoxia and LPS were present together. HIF-1α transcription and protein level were also induced by LPS (Blouin et al., 2004, Mi et al., 2008); therefore in these studies they hypothesized a cross-talk amongst hypoxic and non-hypoxic signaling pathways in the regulation of genes expressed by macrophages. Thus, MMP-7 could be regulated via both hypoxic and pro-inflammatory stimuli. In fact, MMP-7 gene and protein expression under LPS plus hypoxia is dose-dependent and is up-regulated already in macrophages treated with LPS 0.4 ng/mL (Figure 5-4 and Figure 5-7). These data provide strong evidence of a synergy between inflammatory signals and hypoxia in mediating regulation of MMP-7. In a recent study, COX-2 has been demonstrated to be synergistically regulated by HIF-1 and NF-κB (Bruning et al., 2011). Like the iNOS promoter (Mi et al., 2008), one possibility is that the synergistic up-regulation of MMP-7 by hypoxia and LPS is determined by both the putative HRE sequence (located in the MMP-7 promoter around -600 bp) and the upstream putative NF-κB sites (see section 5.10.2), albeit many studies have reported the independence of MMP-7 up-regulation in hypoxia by HIF-1 (Burke et al., 2003; Miyoshi et al., 2006). Moreover, from the White et al, 2004 study, it appears that over-expression of an HIF-1α and HIF-2α adenoviral construct during hypoxic conditions (0.1% O_2) can amplify the expression of some hypoxia-induced genes, but from their microarray data MMP-7 did not appear, even when HIF-1α and HIF-2α were over-expressed. It must be borne in mind that these studies did not combine hypoxia and LPS treatment; it is possible that in the presence of

co-stimulation by LPS, HIF-1 may play a role in MMP-7 up-regulation, given the numerous studies which have now delineated synergy between these two stimuli in regulation of other promoters (Mi et al., 2008) and A. AlHerz (PhD thesis from Dr B Burke's laboratory).

In primary human macrophages, hypoxia in the presence of an inflammatory signal (LPS) causes a pronounced inflammatory and angiogenic response (Carmi et al., 2009). By itself, hypoxia modestly stimulated macrophage-induced inflammatory and angiogenic responses, but both were increased by LPS, ie HIF-1α is highly translocated to the nucleus after LPS and hypoxic stimulation (Carmi et al., 2009). Hypoxia enhances the expression of TLR4 in macrophages, rendering the cells more responsive to LPS, therefore enhancing susceptibility to infection (Kim et al., 2010). The fact that macrophage responses to hypoxia *in vitro* are enhanced by a macrophage stimulant, such as LPS, suggests an important role for these co-stimuli in coordinating macrophage activity in disease. As tissue hypoxia usually follows infection, the presence of LPS may potentiate macrophage responses that are appropriate to dealing with sepsis and enhancing wound healing or prime macrophages to a subsequent hypoxic challenge.

It was observed that the MMP-7 -296 bp construct was slightly inducible by hypoxia and by LPS in normoxia, but no synergy was observed in the induction by both LPS and hypoxia (Figure 5-6), unlike the endogenous MMP-7 gene, which shows synergy, (Figure 5-4). One hypothesis is that in the gene, in which there are several NF-κB sites and also other binding sites lacking in the short -296 bp construct, the presence of LPS (which activates mainly NF-κB) causes the activation of these sites which, in turn, creates cooperative protein-protein interactions with other transcription factors. In the literature it is well known that LPS up-regulates HIF-1α mRNA (Blouin *et al*, 2004) and, in turn, HIF-1 up-regulates ets (Oikawa *et al* 2001); the MMP-7 -296 bp promoter

contains at least three binding sites for ets which can then be activated either by hypoxia and/or by LPS through the mechanism cited above. In the MMP-7 -296 bp construct, there are no NF-kB binding sites, which could account for the lack of synergy between LPS and hypoxia. Still, in the construct, LPS can increase MMP-7 induction via the ets sites. LPS is able to induce the pro-inflammatory COX-2 in monocyte/macrophages via the ets family transcription factor ESE-1 via cooperation with NF-κB and NFAT (Grall et al., 2005), and the regulation of MMP-7 by PEA3 could theoretically follow the same pathway since NFAT has been found in the MMP-7 -296 bp promoter as a novel transcription factor that could be involved in MMP-7 hypoxic regulation (Figure 4-1).

In my experiments, the up-regulation of MMP-7 protein levels analysed by ELISA were strikingly similar to the MMP-7 mRNA levels: increasing concentration of LPS cause increased amounts of MMP-7 mRNA and protein, and the hypoxia treatment when LPS was present at 4 ng/mL caused an even bigger increase than hypoxia alone (Figure 5-4 and Figure 5-7). MMP-7 protein was not induced by hypoxia alone (Figure 5-7), as the MMP-7 mRNA (Figure 5-4), but it was induced when LPS was present in the medium, again in a dose-dependent fashion. MMP-7 mRNA was up-regulated by hypoxia up to 5.5-fold when macrophages were treated with 4 ng/mL LPS (Figure 5-4), whilst the protein was up-regulated only up to 2-fold (Figure 5-7). In fact, previous studies comparing RNA and protein profiles have shown that there is a certain degree of correlation between mRNA and protein levels in human cell lines (Gry et al., 2009).

The up-regulation of mRNA and protein did not necessarily correlate with enzymatic activity since MMP-7, as seen from casein zymography, seemed to be secreted as a latent precursor (Figure 5-10). MMP-7 is normally secreted as pro-MMP-7 which is subsequently activated (Gaire et al., 1994). The observed production of pro-MMP-7 at high levels was demonstrated to be due to the presence of the human serum added to the

medium (Figure 5-9) rather than the MMP-7 produced by the cells (Figure 5-10). The lack of observed differences of pro-MMP-7 in different conditions (normoxia or hypoxia, with or without LPS) is in contrast with the increased production of MMP-7 protein in hypoxia+LPS conditions from ELISA analysis (which detects both the precursor and the active form of MMP-7 enzyme, Figure 5-7). This suggests that the inducible MMP-7 in the ELISA assay was the active form of MMP-7. It is possible that the level of active MMP-7 may be below the detection limit of our casein zymography system.

LPS (like other pro-inflammatory microbial stimuli) activates the main NF-κB pathway (Pomerantz et al., 1990), however an LPS signaling pathway involving activation of PI3K followed by Akt phosphorylation that results in inactivation of GSK-3β has also being described (Guha and Mackman, 2001; Monick et al., 2001). This pathway effects in β-catenin accumulation and transportation in the nucleus where it activates various transcriptional targets (MMP-7 is a known β-catenin transcriptional target, Crawford et al., 1999). The same pathway was highlighted as the possible mechanism for hypoxic up-regulation of MMP-7 by Deguchi et al., 2009, who reported that moderate chronic hypoxia (2% O_2 for 10 days) in primary human macrophages activates Akt and β-catenin pathway and expression of downstream genes like MMP-7. Although the Deguchi data is not without flaws, taken together with my data, it suggests a possible common pathway between LPS (and its downstream effector NF-κB) and hypoxia in regulation of the MMP-7 gene.

Moreover, it was reported that in macrophages stimulated with Gram-positive or -negative bacteria, HIF-1α activates NF-κB and in turn NF-κB controls HIF-1α transcription (Rius et al., 2008).

To investigate the possibility of MMP-7 being regulated via the PI3K and NF-κB pathways, the PI3K inhibitors LY294002 and wortmannin (both of which disrupt the ATP-binding pocket of PI3K, Paez and Sellers, 2003) were used, whilst CAPE inhibitor (which prevents the translocation of the p65 subunit of NF-κB in the nucleus, Natarajan et al., 1996) was employed for investigation of the NF-κB pathway. NF-κB plays an important role in the macrophage response to LPS, but also the PI3K pathway partially contributes to it (Dos Santos et al., 2007).

From the PI3K investigation, LY294002 inhibits LPS-dependent over-expression of MMP-7, but wortmannin did not have such a marked effect (Figure 5-11 and Figure 5-12). A similar result has previously been found for nitric oxide (NO) production: LY294002 causes inhibition of LPS-dependent up-regulation of NO, whilst wortmannin causes a slight increase of NO production, even though the results were not statistically analyzed (Kim et al., 2005). The effect of LY294002 could be due to the interference of the inhibitor with other signaling pathways (off-target effects, Gharbi et al., 2007); in a 2009 study, microarray analysis of LPS-stimulated RAW 246.7 cells identified MMP-9 as up-regulated by LPS via the NF-κB pathway but not via the PI3K pathway; this regulation was inhibited by LY294002 but not by wortmannin. Also, LY294002 inhibits the DNA binding activity of NF-κB (Kim et al., 2005), which is of great importance given my findings of LY294002 down-regulation of LPS-induced MMP-7 (Figure 5-11), which could be really due to the NF-κB contribution. A connection between PI3K and NF-κB pathways was found, suggesting that these pathways are intricately connected and cannot be separated via the use of inhibitors (Shieh et al., 2011). The use of CAPE as a NF-κB inhibitor shows a decrease in LPS-induced MMP-7 up-regulation with the highest CAPE concentration (Figure 5-13), however the presence of the CAPE inhibitor only causes a down-regulation of MMP-7 expression, which could be the

rationale behind the observed reduction of hypoxic regulation. Therefore, taking all these results into consideration, the role of NF-κB and PI3K pathways is still controversial.

Gel shift assay (EMSA), used in order to define the transcription factors involved in MMP-7 regulation during hypoxic and LPS treatments, suggested that both the Tcf/LEF-1/β-catenin binding sites in position -105 and -188 bp could be involved in MMP-7 hypoxic and LPS up-regulation (Figure 5-14 and Figure 5-15). These two binding sites for β-catenin have been previously shown to be functional in the human MMP-7 promoter (Crawford et al., 2001) and Tcf was found to be the transcription factor involved in hypoxic up-regulation of MMP-7 according to Deguchi et al., 2009.

My results, taken together, suggest that the up-regulation of MMP-7 mRNA and protein in primary human macrophages is caused by the presence of both LPS and hypoxia acting synergistically. This is the most significant finding of my thesis, and while perhaps somewhat controversial, should be publishable and will hopefully be of interest to other workers in this area. My data show that TLR activation is essential for hypoxic up-regulation of MMP-7 and that in fact hypoxia is not capable of significantly up-regulating MMP-7 expression in the absence of a second stimulus such as TLR activation. Since most of the TLR agonists used have a synergistic effect with hypoxia, the effect was not limited to LPS. MMP-7 gene expression, hypoxic and LPS up-regulation may be regulated, at least in part, by AP-1 sites and ets binding sites (based on the mutated -296 bp promoter reporter constructs analyses and the ets overexpression experiments). It is also likely that the Tcf/β-catenin sites present at -105 and -188 bp are involved in MMP-7 hypoxic and LPS regulation (based on gel shift assays). Several preliminary experiments I carried out suggest involvement of both the NF-kB (via

CAPE inhibition) and PI3K (via LY294002 and wortmannin inhibition) pathways in the TLR/hypoxia synergistic co-up-regulation of MMP-7 and this area will be of great interest for further work. Finally, a cross-talk between hypoxia and a pro-inflammatory stimulus such as LPS could be envisaged as the mechanism on the basis of MMP-7 up-regulation: hypoxia and LPS, acting both through the PI3K and NF-kB pathway and via up-regulation of AP-1 and Ets transcription factors, can coordinate the regulation of MMP-7 mRNA (Figure 6-1).

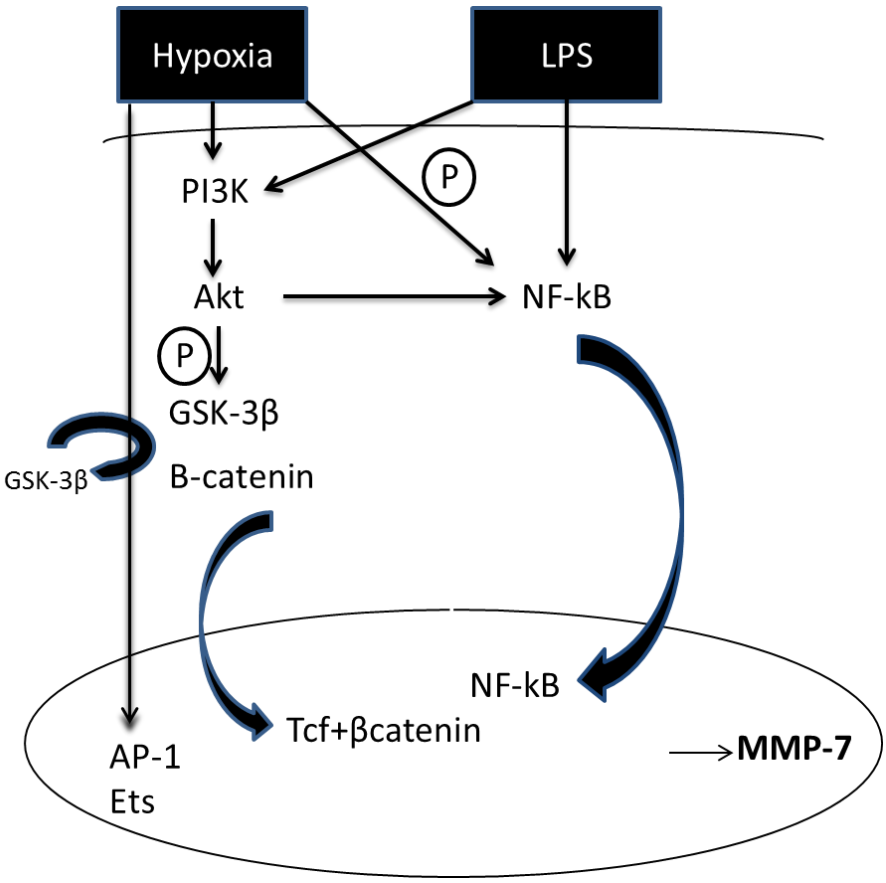


Figure 6-1: hypothesis of the mechanism of action of hypoxia and LPS in the activation of MMP-7 mRNA.

A better understanding of how MMP-7 is regulated in hypoxia in human macrophages would be helpful for development of future therapies for different disease which involve areas of hypoxia (such as atherosclerosis and tumours) and therefore up-regulation of MMP-7.

Future work

A number of further experiments would be required to confirm if indeed there is a unique pathway of up-regulation of MMP-7 during hypoxia or rather its regulation is dependent on a multitude of transcription factors acting through different pathways. EMSA work on NF-κB sites possibly would be a good starting point, since NF-κB is seen as central in MMP-7 regulation.

Deletion of the MMP-7 -296 bp construct would be recommended to pinpoint the shortest sequence involved in LPS response and hypoxia inducibility; eventually, for the shortest sequence obtained which still shows hypoxia inducibility, band shift analysis will need to be performed to study which protein(s) binds to the sequence in normoxia or hypoxia.

An interesting experiment would be the addition of ROS scavengers. From a previous study, it has been shown that addition of ROS-scavengers completely abrogated the synergistic induction of HIF-1 transcriptional activity by both LPS and hypoxia (Mi et al., 2008).

Further studies to investigate the role of HIF-1 in the presence of co-stimulation by LPS in MMP-7 up-regulation, given the numerous studies which have now delineated synergy between these two stimuli in regulation of other promoters (Mi et al., 2008) and A. AlHerz PhD thesis), would also be recommended.

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Appendix

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