Studies on regulation of versican gene expression by hypoxia in primary human macrophages

Thesis submitted for the degree of Doctor of Philosophy

by

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July 2011
Acknowledgments

Firstly I wish to thank my supervisor Dr. Bernard Burke who provided the opportunity to do my PhD and has given great supervision, advice and wisdom over the years. His ability to see a bright side, broad knowledge, helpful suggestions, guidance and being available when needed played a major part in the course of my PhD.

My heartfelt thanks go to my parents and my sister for their constant love, supporting a large part of my education and moral encouragement throughout. Their support and faith gave me the initial encouragement and confidence to undertake a PhD. I also gratefully acknowledge my father, Mohammad Sotoodehnejad for providing the funding for this research.

I would like to thank Dr. Rojer James and Dr. Cordula Stover, members of my PhD committee meeting, for looking over this research and being available when needed. Thanks for critically discussing my data and your suggestions for my thesis.

I would also like to thank all the members of the Department of Infection, Immunity and Inflammation, who have given up their time and blood and for generosity in sharing equipments and chemicals especially the members of renal group (lab 117 and 118). Many thanks also to Lory, Abdulkareem and particularly Helen Pearson for great experimental advice. I couldn’t have done it without all of you! I extend equally important thanks to Elvina Chrysanthou for her friendship and especially producing valuable data during her MSc project under my supervision.

Last but not least I wish to express my gratitude to my wife, Shayesteh Mashayekhi for all of her love and great encouragement. Her faith in me and the daily conversation kept me going through the final stages. Thanks for letting me go.
Abstract

Author: Fattah Sotoodehnejadnematalahi

Title: Studies on regulation of versican gene expression by hypoxia in primary human macrophages

Hypoxia is a hallmark of many pathological tissues. Macrophages accumulate in hypoxic sites and up-regulate a number of hypoxia-inducible genes. The extracellular matrix glycoprotein versican has recently been identified as one such gene, but the mechanisms responsible for hypoxic induction are not well characterised. Here, hypoxic up-regulation of versican was investigated in primary human monocyte-derived macrophages. Flow cytometry of isolated peripheral blood mononuclear cells demonstrated a three-fold increase in versican protein in macrophages after 5 days incubation in hypoxia. Subset analysis showed that macrophages, and not lymphocytes, are the main peripheral blood mononuclear cells which express, and show hypoxic up-regulation of, versican protein and mRNA. This study showed that versican mRNA is up-regulated 34-fold after exposure of primary human macrophages to hypoxia for 18hrs. Further investigation showed that versican mRNA decay rates are not affected by hypoxia, indicating that hypoxic induction of versican mRNA is mediated by increased promoter activity rather than increased mRNA stability. Extensive deletion and transfection analysis of proximal versican promoter luciferase reporter constructs identified two regions which are required for high level activity of the promoter in hypoxic primary human macrophages. A recent publication suggested that hypoxic induction of versican mRNA in macrophages is mediated by the hypoxia inducible transcription factor HIF-1α. Here, bacterial lipopolysaccharide and the hypoxia mimetic agents desferrioxamine and cobalt chloride, three stimuli which are known to induce HIF-1α, were used to investigate the role of HIF-1 in the up-regulation of versican mRNA. Neither LPS nor cobalt chloride caused up-regulation of versican mRNA, although control HIF-1 regulated genes were up-regulated, suggesting that high-level transcription of the versican promoter in hypoxia occurs via a HIF-1 independent mechanism. Lastly, two specific inhibitors of PI3-kinase, LY294002 and Wortmannin, were shown to down-regulate hypoxic induction of versican mRNA, suggesting a possible role for PI3-kinase.
Abbreviations

α- Alpha
ALDA- Aldolase A
Amp- Ampicillin
AP-1- Activator Protein-1
APCs- Antigen Presenting Cells
APS- Ammonium Persulphate
AREs- Adenylate Uridylate Rich Elements
ARNT- Aryl hydrocarbon Receptor Nuclear Translocator
ASMCs- Arterial Smooth Muscle Cells
ATF- Activating Transcription Factor
ATP- Adenosine Triphosphate
β- Beta
β-2M- β-2 microglobulin
bFGF- basic Fibroblast Growth Factor
bp- base pair
BSA- Bovine Serum Albumin
°C- Celsius
CBP- CREB Binding Protein
CBP/P300- CREB Binding Protein p300
CD- Cluster of Differentiation
cDNA- Complementary Deoxyribonucleic acid
ChiP- Chromatin Immunoprecipitation
CNS- Central Nervous System
CO2- Carbon dioxide
COX-2- Cyclooxygenase- 2
CREB- CAMP-Response Element Binding protein
CS- Chondroitin Sulphate
CSF- Colony Stimulating Factor
CSPGs- Chondroitin Sulphate Proteoglycans
Abbreviations

DEPC- Diethylpyrocarbonate
DFO- Desferrioxamine
dH2O- Deionised Water
DMSO- Dimethyl Sulfoxide
DNA- Deoxyribonucleic acid
dNTPs- Deoxynucleotide Triphosphates
D-PBS- Degassed Phosphate Buffered Saline
DTT- Dithiothreitol
E.coli- Escherichia coli
ECM- Extracellular Matrix
EDTA- Ethylenediaminetetraacetic acid
eEF2- eukaryotic Elongation Factor-2
EGF- Epidermal Growth Factor
ELISA- Enzyme-Linked Immunosorbent Assay
EMSA- Electrophoretic Mobility Shift Assay
EPO- Erythropoietin
ER- Endoplasmic Reticulum
ERK- Extracellular Regulated Kinase
ET-1- Endothelin-1
EtBr- Ethidium Bromide
FACS- Fluorescence-Activated Cell Sorting
FCS- Feta Calf Serum
FGF2- Fibroblast Growth Factor 2
FIH- Factor Inhibiting HIF
FITC- Fluorescein Isothiocyanate
FSC- Forward Scatter
γ- Gamma
g- Gramme or gravitational acceleration
G3PDH- Glyceraldehyde 3-Phosphate Dehydrogenase
GAG- Glycosaminoglycans
GC- Golgi complex
GLUT-1- Glucose transporter 1
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</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>HCl</td>
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<td>HIF-1</td>
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<tr>
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<td>inducible Nitric Oxide Synthase</td>
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<td>Kb</td>
<td>Kilo-base</td>
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<td>KCL</td>
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<td>kDa</td>
<td>Kilo-Dalton</td>
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<td>LB-Luria Bertani Medium</td>
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<td>LDHA-</td>
<td>Lactate Dehydrogenase A</td>
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<td>LDL</td>
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<tr>
<td>LPS</td>
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<tr>
<td>MACS</td>
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<td>mRNA</td>
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<td>N₂-</td>
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Abbreviations

NaCl- Sodium Chloride
NF-κB- Nuclear Factor-Kappa Binding
NO- Nitric Oxide
O₂- Oxygen
ODD- Oxygen Dependent Degradation domain
PAI-1- Plasminogen Activator Inhibitor-1
PAMP- Pathogen Associated Molecular Patterns
PBMC- Peripheral Blood Mononuclear Cells
PBS- Phosphate Buffered Saline
PCR- Polymerase Chain Reaction
PDGF- Platelet Derived Growth Factor
PEI- Polyethyleneimine
PGK- Phosphoglycerate kinase
PHD- Prolyl Hydroxylase Enzymes
PI3K- Phosphoinositide 3-kinase
PKB- Protein Kinase B
PKC- Protein Kinase C
PRPs- Pattern Recognition Receptors
RLU- Relative Light Units
RNA- Ribonucleic Acid
RNAi- Ribonucleic Acid interference
RNase- Ribonuclease
rpm- Revolutions Per Minute
RT- Room Temperature or Reverse transcriptase
RT-PCR- Real Time Polymerase Chain Reaction
SDS- Sodium Dodecyl Sulphate
SDS-PAGE- Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
siRNA- small interfering Ribonucleic Acid
SLC- Secondary Lymphoid tissue Chemokine
SNP- Single Nucleotide Polymorphisms
SP-1- Specific Protein- 1
SR- Scavenger Receptors
Abbreviations

SSC- Side Scatter
TAD- Transactivation Domains
TAMs- Tumour Associated Macrophages
TBE- Tris Borate EDTA buffer
TBS- Tris Buffered Saline
TCF/LEF- Transcription Factor Lymphoid Enhancer binding Factor
Tess- Transcription Element Search System
TGF- Transforming Growth Factor
Th-T helper
TLR- Toll Like Receptor
TNF- Tumour Necrosis Factor
ULA- Ultra Low Attachment
UTR- Untranslated Region
UV- Ultraviolet light
VEGF- Vascular Endothelial Growth Factor
VHL- Vo Hippel Lindau protein
V-Volts
XRE- Xenobiotic Responsive Element
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Chapter 1: Introduction

1.1 Macrophages

1.1.1 Origin of macrophages

Leukocytes are a diverse group of cells which regulate the body’s immune response against infectious disease and foreign materials (Crowell et al., 1977; Stock et al., 2000). They circulate in the blood and/or lymphatic system so they can be recruited to areas where tissue damage or an infection has developed (van Furth and Cohn, 1968). Leukocytes consist of different subsets including lymphocytes, neutrophils, eosinophils, basophils, mast cells and monocytes (which differentiate into macrophages). These subsets are distinguished by functional and physical characteristics but all are derived from cells in the bone marrow known as hematopoietic stem cells (Fig 1.1) (Leder et al., 1967; Fogg et al., 2006). The large phagocytic mononuclear leukocytes represent a population of bone marrow-derived (myeloid) cells which are known as monocytes (Leder et al., 1967; Beekhuizen et al., 1993). Monocytes constitute 5-10% of leukocytes in the peripheral blood, where they circulate for several days before populating tissues as macrophages, in the steady state or during inflammation (Issekutz et al., 1981; Gordon and Taylor, 2005). At sites of injury or microbial invasion, monocytes express chemokine receptors such as CCR2 and chemoattractants such as MCP-1 (Monocyte Chemoattractant Protein 1, also called CCL2, the ligand for CCR2) which elicits increased recruitment of monocytes to peripheral sites where they differentiate into macrophages and contribute to host defence, tissue remodelling and repair (Kuziel et al., 1997; Mutsaers et al., 1997; Lu et al., 1998; Smith et al., 2005).
The appearance of macrophages differs according to the tissues in which they reside, where they are classified and named differently. For instance, macrophages are known as Kupffer cells in the liver, red pulp macrophages in the spleen, microglia in the brain, reticular cells in the sinuses of spleen and lymph nodes, Langerhans cells in the skin and alveolar macrophages in the lungs (Dijkstra et al., 1985; Gordon and Taylor, 2005). Macrophages are “professional” phagocytic cells which act as an early line of defence in the immune system by recognising and engulfing pathogens such as bacteria and viruses (Serbina et al., 2008).

![Diagram illustrating hematopoietic stem cell derived from the bone marrow which gives rise to all types of blood cells.](http://stemcells.nih.gov/StaticResources/info/scireport/images/figure51.jpg)
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1.1.2 Role of macrophages in the body

Phagocytosis is the uptake of particles and it is an important function of macrophages, which are key players in the immune response and remove microorganisms and damaged or apoptotic cells of the host (Dentan et al., 1996; Kwiatkowska et al., 1999). An initial event, which is required for phagocytosis, is adhesion of particles by macrophages (Cougoule et al., 2004). It has been shown that adhesion structures (podosomes) and integrins which mediate cell adhesion to the extracellular matrix are involved in adhesion of macrophages to particles (Berton and Lowell, 1999; Calle et al., 2006). Phagocytosis is believed to be involved in macrophage activation and results in the release of cytokines such as IL-1 (Interleukin-1), IL-6 and TNF (Tumour Necrosis Factor) which promote inflammation (Nathan, 1987; Uchimura et al., 1997; Patel et al., 2003; Tripathi and Sodhi, 2008). After phagocytosis, membrane remodelling of macrophages leads to the complete enveloping of the particles in endosomes known as phagosomes. Phagosomes are membrane-bound organelles released into the cytoplasm where lysosomes fuse with them and release their contents into the phagosomes to kill, digest and degrade the contents (Fig 1.2) (Jutras et al., 2005; Stuart and Ezekowitz, 2005).
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Figure 1.2: Schematic diagram of macrophage structure and mechanisms of phagocytosis. Phagocytosis begins with the macrophage flowing around the pathogen and engulfing it. Pathogens are enclosed in a phagosome (phagocytic vesicle). The next step is the fusion of lysosomes with the phagosome. The result is called a phagolysosome. Lysosomes are derived from the Golgi apparatus and their contents are focused on destroying microorganisms.


Macrophage activation is closely related to defence against microbial infection, causing inflammation and other reactions which act against foreign body or particle invasion (van Rooijen et al., 1997; Smythies et al., 2005). Macrophages can act as antigen presenting cells (APC), which participate in the initial capture and processing of antigens (innate immunity) and then in the activation of specific T and B lymphocyte mechanisms (adaptive immunity). The activated T lymphocytes in turn cooperate with macrophages and enhance destruction of intra- and extra-cellular pathogens through phagocytosis (Grey et al., 1982; Unanue, 1984; Gordon and Taylor, 2005).
The first step in the macrophage response to foreign organisms is to recognize them via pattern recognition receptors (PRRs) which are activated and affected immediately by conserved molecular patterns (Pathogen Associated Molecular Patterns, or PAMPs) carried by microbes, and are able to recognize many different classes of macromolecules (Gordon, 2002; Janeway and Medzhitov, 2002).

Macrophages express PRRs such as toll like receptors (TLRs) which are transmembrane proteins implicated in differential recognition of microbial and foreign molecular targets (Akira et al., 2001). For example, TLR4 is known to be involved in the recognition of lipopolysaccharide, a major cell wall component of Gram-negative bacteria (Triantafilou, M and Triantafilou, K, 2002). Recognition of LPS also requires other molecules such as CD14, a co-receptor expressed by monocytes and macrophages, in addition to TLR4. Activation of the CD14-TLR4 complex in monocyte/macrophages results in the induction of the adaptive immune response and triggers expression of multiple genes involved in immune responses (Akira et al., 2003; Miyake et al., 2004; Takeda and Akira, 2005).

Many other PRRs are expressed by macrophages, including scavenger receptors (SR) and mannose receptors (MR) (Krieger et al., 2001; Taylor et al., 2005). Scavenger receptors are primarily involved in the recognition, binding, phagocytosis and clearance of cellular components, such as modified low-density lipoprotein (LDL) and apoptotic cells (Peiser et al., 2002), whereas the mannose receptor is involved in innate and adaptive immune responses (East and Isacke, 2002). The mannose receptor is an endocytic and phagocytic receptor which binds to carbohydrate structures on the
surface of many pathogens, including bacteria, fungi, parasites and viruses, and leads to phagocytosis of these particles by macrophages (Llorca et al., 2008). It has been clearly demonstrated that activation of PRRs such as TLRs, SRs and MRs lead to increased phagocytosis of microorganisms by macrophages (Pearson, 1996; Fraser et al., 1998; Underhill et al., 1999).

1.1.3 The inflammatory macrophages

Inflammation is a response of a tissue to injury which could be a simple wound or a complex autoimmune inflammation such as rheumatoid arthritis (Schmid-Schonbein, 2006). It has been shown that macrophages are major players in the inflammatory response and secrete pro-inflammatory and antimicrobial mediators (Gordon, 1998; Ma et al., 2003). For example it has long been known that macrophages activated in vitro by interferon-γ (IFN-γ) followed by a microbial trigger can increase production of pro-inflammatory cytokines such as tumour necrosis factor and interleukins including IL-1 and IL-6 (Gordon, 2003). Also innate activation of macrophages by ligation of TLRs such as TLR-4 with LPS is associated with microbicidal activity and production of other pro-inflammatory cytokines such as IFN-α and IFN-β (Gao et al., 1998; Fujihara et al., 2003). Evidence to date suggests that macrophages-derived cytokines such as transforming growth factor-β (TGF-β), basic fibroblast growth factor and platelet-derived growth factor are important in tissue repair and remodelling (Nathan et al., 1987; Ricardo et al., 2008). In addition, it has also been shown that deactivation of macrophages, which is induced by presence of cytokines such as IL-10 or TGF-β, is associated with increased production of IL-4, an anti-inflammatory cytokine (Goerdt and Orfanos, 1999; Gordon, 2003).
Several studies have suggested that macrophages can be classified into two major groups, M1 and M2 (Edwards et al., 2006; Mantovani et al., 2006; Gordon and Martinez, 2010). M1 macrophages are activated by IFN-γ, TNF or pathogen-associated molecular patterns such as LPS and can effectively destroy invading pathogens, tumour cells and foreign materials (Gordon, 2003; Mantovani et al., 2004). They act as antigen presenting cells and release pro-inflammatory cytokines such as TNF, IL-6, IL-1 and IL-12 and participate as inducer and effector cells in T helper 1 (Th1) responses (Gordon, 2003; Mantovani et al., 2004; Van Ginderachter et al., 2006). Accumulating evidence suggests that M2 macrophages, which result from culture in IL-4, IL-13, IL-10 or TGF-β, can release anti-inflammatory cytokines, growth factors and mediators which are involved in wound repair and tissue remodelling, and contribute as inducers in T helper 2 (Th2) responses (Gordon, 2003; Mantovani et al., 2004; Benoit et al., 2008; Laskin, 2009).

Overall there are many stimuli which can push macrophages toward the activation phenotype. The hypoxia which often occurs in tumours and sites of infection can therefore activate macrophage expression of a broad range of genes including pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Ghezzi et al., 1991; Scannell et al., 1993; Albina et al., 1995).
1.2 Hypoxia

Molecular oxygen is essential for aerobic metabolism to maintain intracellular bioenergetics and to serve as an electron acceptor in many reactions (Freeman, 2000). Ambient air is 21% O\(_2\) (150 mm Hg) at sea level; however, most mammalian tissues have O\(_2\) levels of 24 to 66 mm Hg (2%–9% O\(_2\)) (Vaupel et al., 1989). Hypoxia is a term which describes low oxygen concentrations (Semenza, 2010), which can affect and regulate many physiological and pathophysiological processes, including embryonic development (Simon and Keith, 2008) and wound healing (Lee et al., 2009). In biological systems, hypoxia usually occurs in pathological tissues including tumours, ischemic tissues, chronic obstructive pulmonary disease, atherosclerotic plaques (Bjornheden et al., 1999; Brahimi-Horn and Pouyssegur, 2006), and arthritic joints (Stevens et al., 1991). It is known that a major obstacle to cell survival is reduction in oxygen availability, which is often confronted by cancer cells (Sutherland, 1998; Vaupel et al., 2004). In general, rapid growth and abnormal angiogenesis at the site of the tumour leads to insufficient blood supply and consequent depletion of oxygen. This eventually results in the formation of necrotic and hypoxic regions in the inner parts of the tumour (Griffiths et al., 2000; Vaupel et al., 2004). Work by Vaupel and Meyer showed that O\(_2\) concentrations within cancers are reduced compared to surrounding normal tissue, with severe hypoxia correlating with invasion, metastasis and patient death (Vaupel and Mayer, 2007). The oxygen concentration in these pathological tissues ranged from 0 to 15mmHg (Hockel and Vaupel, 2001). Hypoxia is also found in healthy tissues such as the spleen (oxygen levels as low as 0.5% or 3 mmHg) (Caldwell et al., 2001) and it is also a condition encountered in embryogenesis, in which hypoxic
signalling is considered necessary for normal development (Brahimi-Horn and Pouyssegur, 2006).

A role for a hypoxic microenvironment in the pathogenesis and progression of human cancer was first proposed by Gray et al, when intratumoral hypoxia was correlated with reduced efficiency of radiation therapy (Gray et al., 1953; Brizel et al., 1999; Overgaard, 2007). Hypoxia has also been shown to be linked to increased mutation rates (Yuan and Glazer, 1998), tumour invasion (Pennacchietti et al., 2003) and metastasis (Subarsky and Hill, 2003).

Genomic tools, including DNA microarrays, have enabled study of the global gene expression of many different cells and tissues under hypoxic stress (Burke et al., 2003; Greijer et al., 2005; Weinmann et al., 2005) and more than 100 genes have been shown to be up-regulated by hypoxia. For example, hypoxia induces erythropoietin (EPO) (Wang and Semenza, 1993a), and angiogenic cytokines such as vascular endothelial growth factor (VEGF) (Liu et al., 1995) and basic fibroblast growth factor (bFGF), which are required for the adaption of the whole organism to general hypoxia by enhancement of blood oxygen-carrying capacity and oxygen delivery (Egger et al., 2007). Also hypoxic up-regulation of glucose transporter-1 (GLUT-1), which facilitates the transport of glucose across the plasma membranes of mammalian cells, has been detected in a variety of malignant tissues (Airley et al., 2001; Macheda et al., 2005).

It is well known that a variety of signalling pathways are activated by hypoxia (Haddad, 2004; Benizri et al., 2008). Among these, the activation of the transcription factor hypoxia-inducible factor 1 (HIF-1) is a key element responsible for embryogenesis and
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up-regulation of numerous hypoxia inducible genes (Kallio et al., 1998; Sutter et al., 2000). HIF-1-mediated gene expression allows an organism to respond to hypoxia by increasing oxygen delivery or adapting to decreased oxygen availability (Wenger, 2002). Such targets of HIF-1 play critical roles in glycolysis, oxygen homeostasis, tissue remodelling, fat metabolism, angiogenesis, erythropoiesis, and proliferation (Semenza, 2001b; Hirota, 2002).

1.2.1 Macrophages in hypoxia

It has been known for some time that macrophages are recruited and retained in poorly vascularised, hypoxic and necrotic sites including in breast (Leek et al., 1999; Bingle et al., 2002) and ovarian carcinomas (Negus et al., 1997), wounds (Crowther et al., 2001), atherosclerotic plaques (Husain et al., 1999) and arthritic joints (Kinne et al., 2000). In addition, it has been reported that chemoattractants such as colony stimulating factor 1 (CSF-1), MCP-1, VEGF and endothelin 1 recruit peripheral monocytes to tumour regions which are characterised by extremely low levels of oxygen and trigger differentiation into tumour associated macrophages (TAMs) (Murdoch et al., 2004). Several studies have shown that these TAMs release a variety of enzymes and cytokines which promote tumour invasion, angiogenesis and metastasis, such as epidermal growth factor (EGF) and VEGF (Lewis et al., 2000; Goswami et al., 2005; Bingle et al., 2006; Lewis and Pollard, 2006; Mantovani et al., 2006).

A study by Burke et al., (2003) showed that certain genes are up-regulated by macrophages under hypoxic conditions. They used cDNA array hybridization to
determine the effect of hypoxia on mRNA of 1185 genes in primary human monocyte-derived macrophages (HMDM). This study showed hypoxia induced mRNA up-regulation of the enzyme matrix metalloproteinase-7 (MMP-7), neuromedin B receptor and DNA-binding protein inhibitor (Id2) as well as known hypoxia inducible genes such as VEGF and GLUT-1 (Burke et al., 2003). Other cDNA array work by White et al., (2004) also revealed more than 30 mRNA pro-angiogenic genes which were up-regulated by hypoxia in primary macrophages. The best characterized of these, apart from VEGF, were fibroblast growth factor 2 (FGF2), IL-8, macrophage migration inhibitory factor (MIF) and cyclooxygenase-2 (COX-2) (White et al., 2004). In addition, it has been demonstrated that hypoxic macrophages up-regulate a number of transcription factors, such as HIF-1, which in turn up-regulate a broad array of genes including VEGF and GLUT-1 whose products promote tumour growth and angiogenesis (Burke et al., 2002 & 2003; Leek et al., 2002; Elbarghati et al., 2008).

1.2.2 Hypoxia-responsive transcription factors

Hypoxia activates a diverse array of transcription factors such as activator protein-1 (AP-1) (Bandyopadhyay et al., 1995; Rupec and Baeuerle, 1995; Fantozzi et al., 2003), cAMP-response element binding protein (CREB) (Beitner-Johnson and Millhorn, 1998; Leonard et al., 2008), specific protein 1 (SP-1) (Xu et al., 2000; Lee et al., 2004; Sanchez-Elsner et al., 2004) and most importantly HIF-1 (Semenza et al., 1994), which in turn activates a broad array of mitogenic, proinvasive, proangiogenic, and prometastatic genes (Semenza, 1999 & 2000). Since the discovery of HIF-1 by the Semenza lab in the early 1990s, it has been recognised as being of central importance
and has been described as the “master regulator” of the transcriptional response to hypoxia (Semenza, 1999).

### 1.2.3 Hypoxia-Inducible Factors (HIFs)

There are two main types of HIF, HIF-1 and HIF-2 (Wang and Semenza, 1995; Ema et al., 1997) which are the predominant transcription factors mediating the effects of hypoxia on gene expression (Semenza, 2001a; Bertout et al., 2008). HIF-1 is the most ubiquitously expressed and best characterised of the family and is recognised as a master regulator of hypoxic signalling whose activation has been shown to regulate expression of over 70 genes at the transcriptional level (Semenza, 2004).

Both HIF-1 and 2 are heterodimeric molecules consisting of α and β subunits which belong to a family of basic helix-loop-helix proteins (Dery et al., 2005). The HIF-β subunit, also known ARNT (Aryl hydrocarbon Receptor Nuclear Translocator) is found in the nucleus in both normoxia and hypoxia (Wang and Semenza, 1995), whereas the α subunit is also constitutively produced but is subject to rapid degradation in the presence of oxygen (half-life less than five minutes), only becoming stable in the absence of oxygen (i.e. hypoxia) (Semenza, 2004). The HIF-1α subunit contains an oxygen-dependent degradation domain (ODD) and two transactivation domains (TAD) which are required for the transcriptional activation activity of HIF-1, being capable of binding to two transcriptional co-activators, CREB binding protein (CBP) and p300 (Kallio et al., 1998; Ema et al., 1999; Jiang et al., 1997; Pugh et al., 1997 & 2003). In normoxia, specific proline residues at positions 402 and/or 564 in the ODD of the HIF-α subunit are hydroxylated by prolyl hydroxylase enzymes (PHD) (Semenza, 2000a;
1. Introduction

Jaakkola et al., 2001). PHD is a family composed of prolyl 4-hydroxylases (PHD1-4) which require iron (Fe (II)), 2-oxoglutarate, O₂ and ascorbate as substrates; their activity is reduced in hypoxia due to the limitation of O₂ (Epstein et al., 2001; Ivan et al., 2001). Hydroxylation of HIF-1α acts as a signal for recognition by the tumour suppressor VHL (von Hippel–Lindau protein), leading to ubiquitination and proteasomal degradation (Fig 1.3) (Maxwell et al., 1999; Kamura et al., 2000; Tanimoto et al., 2000).

In addition to prolyl hydroxylation by PHDs, another oxygen-dependent modification occurs in the transactivation domains of HIF-α subunit. It is dependent on the presence of an asparagine hydroxylase enzyme known as factor inhibiting HIF (FIH) (Mahon et al., 2001; Cockman et al., 2009). In this oxygen-dependent regulatory mechanism, FIH blocks the interaction between HIF-α with p300 and CBP by hydroxylating an asparagine residue at position 803, thus inhibiting the activity of the HIF-1α transactivation domain (Fig 1.3) (Hewitson et al., 2002; McNeill et al., 2002).

In hypoxia, PHD activity decreases which enables rapid accumulation of HIF-α in the nucleus where it dimerises with the HIF-β subunit and binds to hypoxia response elements (HREs) in the promoters of various genes (Sutter et al., 2000; Jewell et al., 2001). The decrease in oxygen availability also impairs FIH which results in a decrease in HIF-α subunit asparagine hydroxylation, allowing increased recruitment of transcriptional co-activators (p300/CBP) which eventually leads to the enhanced transcriptional activation of HIF target genes (Fig 1.3) (Wenger et al., 2005; Lando et al., 2002; Mahon et al., 2001) which are implicated in many different aspects of oxygen
delivery and metabolism including vasodilatation (nitric oxide synthases), iron metabolism (transferrin) (Ratcliffe et al., 1998), glucose transporters (GLUT-1), angiogenesis (VEGF), enhanced blood oxygenation (erythropoietin) (Gleadle et al., 1997) and glycolysis (phosphoglycerate kinase) (Semenza et al., 1994).

**Figure 1.3:** Regulation of HIF-1 in normoxia and hypoxia. Further details are given in the text.
1.2.4 Hypoxia Responsive Elements (HREs)

Previous studies showed that HIF-1 binds to hypoxia responsive elements, a consensus sequence in the promoter of about 200 HIF target genes (among which around 100 genes have been confirmed) and initiates transcription by recruiting transcriptional co-activators such as p300/CBP (Semenza, 2000; Wenger et al., 2005).

The minimal cis-regulatory element (CGTG) required for hypoxic induction of gene transcription was first identified by Semenza who also determined that this core HRE consensus sequence is required but not sufficient for effective gene activation in response to hypoxia (Semenza et al., 1994 & 1996). Analysis of 107 HIF-1 responsive genes has shown that neighbouring nucleotides occur with non-random frequency, especially in the 5′ flanking bases, demonstrating that a fully functional HRE requires neighbouring DNA binding sites for additional transcription factors or co-activators, which may act to amplify the hypoxia response (Wenger et al., 2005).

1.2.5 Role of HIF-1 in macrophages

As previously mentioned, macrophages are associated with a number of inflammatory sites such as atherosclerotic plaques (Bjornheden et al., 1999), myocardial infarcts (Jurgensen et al., 2004), rheumatoid arthritis (Hollander et al. 2001), healing wounds (Stevens et al., 1991), sites of bacterial infection, and malignant tumours (Blouin et al., 2004; Murdoch et al., 2005; Pouyssegur et al., 2006) in which hypoxia is present. In hypoxia, macrophages rely heavily on HIFs for energy generation and activity, and express HIF-1α protein abundantly and increase transcriptional activation of HIF target genes (Burke et al., 2002 & 2003). Unusually, however, macrophages are also
significantly dependent on HIF-1 regulated genes for energy generation in normoxia (Cramer et al. 2003).

Some early studies using a rat alveolar macrophage-derived cell line and the human monocytic cell line THP-1 reported that short term hypoxia did not increase HIF-1α mRNA, suggesting instead that HIF-1α is regulated by hypoxia by decreased protein stability (Blouin et al., 2004; Frede et al., 2006). However, a very recent study by us (Staples et al., 2011; see Appendix) showed increases in HIF-1α mRNA levels after long term hypoxia (5 days) in human primary macrophages and reported that this up-regulation is mediated by increased transcription rather than increased mRNA stability. Similar increases in HIF-1 mRNA in hypoxia have been reported by other groups in non-macrophage cell types (discussed in Staples et al., 2011) but the subject is still somewhat controversial.

An increased level of HIF-1α protein in activated macrophages was first demonstrated by Hollander et al., (2001) in inflamed joints of patients suffering from rheumatoid arthritis and later by Talks et al., (2000) in tumour sections and Burke et al., (2002) in isolated hypoxic human primary macrophages in vitro. Also other studies showed increased levels of HIF-1α in the inflammatory cells of healing wounds and it was suggested that this could be due to a release of inflammatory cytokines such as TNF-α which can strongly increase HIF-1α protein levels in cells after injury, leading to increased expression of HIF-1 responsive genes such as VEGF which regulate the process of tissue repair (Albina et al., 2001). Other groups have also investigated whether HIF-1 activity is induced during differentiation from monocytes to
It was shown by Oda et al., (2006) that both HIF-1α and HIF-1β protein levels markedly increase during the differentiation of monocytes to macrophages in the monocytic cell line THP-1 and in monocytes from human peripheral blood. They suggested that activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)-signalling pathways are responsible for this increase in HIF-1 gene expression (Oda et al., 2006).

### 1.2.6 Non-hypoxic up-regulation of HIF-1

Despite the name, numerous studies have now shown that HIF-1α can be induced by a variety of stimuli in addition to hypoxia. The key studies in this area are reviewed below.

#### 1.2.6.1 Lipopolysaccharide (LPS)

LPS is a component of the cell wall of gram-negative bacteria (Hajjar et al., 2002). It binds to the CD14 and TLR4 cell surface receptors of monocyte/macrophages (Hoshino et al., 1999; Moynagh, 2003) leading to the activation of a number of genes often associated with hypoxia, many of which are believed to be up-regulated independently of HIF-1 (Pocock et al., 2003; Covert et al., 2005; Karin and Greten, 2005; Karin, 2006). Several studies have shown that LPS treated macrophages up-regulate genes such as VEGF, GLUT-1 and iNOS which are known to be regulated by HIF-1 (Blouin et al., 2004; Frede et al., 2006). In contrast to hypoxia, which is generally considered not to up-regulate HIF-1α mRNA, LPS has been shown to stimulate HIF-1α expression at the transcriptional level under normoxia in alveolar-derived rat macrophages and human primary macrophages through a NF-κB site in the promoter of the HIF-1α gene (Blouin...
et al., 2004; Frede et al., 2006). Blouin et al., (2004) showed that LPS increases HIF-1α protein expression in a time and dose-dependent manner which in turn modulates hypoxic gene activation. Frede et al., (2006) reported induced HIF-1α mRNA and protein expression in differentiated THP-1 cells treated with LPS under normoxia. This study, using RNAi against MAPK and also a specific inhibitor of this pathway, showed down-regulation of the LPS-induced HIF-1α mRNA and protein in THP-1 cells suggesting a role for the MAPK pathway in LPS-dependent HIF-1α induction (Frede et al., 2006).

1.2.6.2 Phosphoinositide (PI) 3-kinase signalling
PI3-Kinase activities have been found in all eukaryotic cell types and are linked to a diverse set of key cellular functions, including cell growth, proliferation, motility, differentiation and survival (Rameh and Cantley, 1999; Fry, 2001; Katso et al., 2001). PI3-kinase phosphorylates the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (Zvelebil et al., 1996). PI3-kinase has been the focus of intense study as increasing evidence suggests a key role for the PI3-kinase pathway in many human diseases including allergy, inflammation, heart disease and cancer (Stein, 2001; Liu et al., 2009). An interesting mechanism was proposed by which the activation of PI3-kinase could increase the rate of HIF-1α translation in normoxia (Page et al., 2002). It has been previously reported that activation of PI3-kinase by growth factors and hormones leads to the recruitment and activation of a downstream effector of PI3-kinase, known as the mammalian target of rapamycin (mTOR) (Treins et al., 2002; Alam et al., 2004). mTOR activation results in increased phosphorylation and inactivation of 4E-binding protein 1 (4E-BP), the eukaryotic translation initiation factor,
and activation of p70-S6 kinase 1 which leads to increased protein synthesis (Zhong et al., 2000; Treins et al., 2002). Inactivation of 4E-BP and activation of p70S6K has been shown to increase translation of HIF-1α mRNA through the 5′ untranslated region (5′UTR) (Richard et al., 2000; Page et al., 2002). This is believed to be the main effector mechanism responsible for HIF-1α induction through the PI3-kinase dependent pathway, resulting in increased VEGF expression in vascular smooth muscle cells and human tumour cell lines (Jiang et al., 2001; Laughner et al., 2001).

1.2.6.3 Cobalt (CoCl₂) stabilisation of HIF-1α
It has been demonstrated that CoCl₂ induces hypoxia-regulated genes by stabilising HIF-1α in normoxia (Maxwell et al., 1999). As outlined in section 1.2.3, hydroxylation of the proline residues, which reside in the oxygen-dependent degradation domain of HIF-1α, by prolyl hydroxylase is one of the key mechanisms that mediate the binding of VHL with HIF-1α which eventually leads to proteasomal degradation of HIF-1α (Ivan et al., 2001; Jaakkola et al., 2001). A study by Epstein et al., (2001) suggested that iron is a critical factor for the activity of PHD as these enzymes have an iron-binding centre. In addition, this study suggested that CoCl₂ may act as a competitor for iron, inactivating PHD by binding and engaging an iron-binding site in the proline hydroxylase. Due to this enzymatic inhibition, HIF-α is not targeted for proteasomal degradation (Epstein et al., 2001). Beside the inactivation of PHD by CoCl₂, an investigation by Yuan et al., (2003) also suggested another mechanism in which HIF-α could be stabilized by cobalt. In this mechanism, cobalt stabilizes HIF-1α protein by direct binding to the ODD in HIF-1α, thereby preventing the interaction between HIF-
1α and VHL protein and subsequently inhibiting proteasomal degradation which results in HIF-1α stabilisation (Yuan et al., 2003).

1.2.6.4 Desferrioxamine (DFO) stabilisation of HIF-1α
Since the introduction of DFO in the 1960s, it has been widely used as a chelating agent to bind free iron in the bloodstream and removing excess iron from the body (Breuer et al., 2001). Several studies have demonstrated that normoxic cells treated with DFO induced HIF-1 target genes such as EPO (Wang and Semenza, 1993b), VEGF (Agani and Semenza, 1998) and GLUT-1 (Dongiovanni et al., 2008) by inducing the accumulation of HIF-1 protein. An early study by Maxwell et al., (1999) demonstrated that DFO disrupts pVHL–HIF-α complex formation which is required for ubiquitination and proteasomal degradation of HIF-1α in normoxia. It has been demonstrated that DFO inhibits hydroxylation of HIF-1α by chelating the iron required for activity of the PHD enzyme (Yuan et al., 2003). Therefore due to inhibition of HIF-α hydroxylation, the pVHL–HIF-α complex formation is inhibited causing HIF-α stabilisation which results in induction of HIF-1 target genes (Mole et al., 2001; Pham et al., 2002; Woo et al., 2006).
1.3 Extracellular matrix

The extracellular matrix (ECM) provides structural support for organs and tissues (Badylak, 2002), cell layers in the form of basement membranes (Daley et al., 2008) and individual cells as substrates for migration (Werb, 1997). ECM is composed of collagens and elastic fibres which are embedded in a viscoelastic gel that comprises proteoglycans (e.g. versican and hyaluronan), glycoproteins and water (Berrier and Yamada, 2007; Campbell et al., 2010). ECM forms a complex, three-dimensional network among the cells of different tissues in an organ-specific manner (Adams and Watt, 1993) and plays vital roles in the differentiation, proliferation and survival of cells (Boudreau and Bissell, 1998; Streuli, 1999; van Horssen et al., 2007).

Proteoglycans are the main components of ECM, and are characterised by a protein portion (core protein) and one or more unbranched, long and negatively charged polysaccharide chains called glycosaminoglycans (GAG) which are covalently attached to the core protein (Hardingham and Fosang, 1992; Iozzo and Murdoch, 1996). Depending upon the nature of the GAG chains, proteoglycans can be categorised as heparan sulphate proteoglycans, chondroitin sulphate proteoglycans (CSPGs) and dermatan sulphate proteoglycans, or keratan sulphate proteoglycans (Iozzo, 1998; Kresse and Schonherr, 2001). Of these, the CSPGs such as versican are the most abundant type of proteoglycan in the ECM of mammalian tissues (Zimmermann and Ruoslahti, 1989; Carulli et al., 2005).
1.3.1 Versican structure

Versican is a large chondroitin sulphate proteoglycan which is a major component of the ECM (Naso et al., 1994). Versican is transcribed from a single gene which is localized on chromosome 5q 12–14 in the human genome and extends over 90 kb (Iozzo et al., 1992) which is divided into 15 exons which range in size from 76 to 5262 bp (Naso et al., 1994). The alternative mRNA-splicing of these exons gives rise to four different versican isoforms which are distinguished by different core-middle regions (Dours-Zimmermann and Zimmermann, 1994). Versican is comprised of three domains. The amino terminal G1 domain interacts with a glycosaminoglycan (GAG) called hyaluronan present in the extracellular matrix (Zimmermann and Ruoslahti, 1989). The carboxyl terminal domain of versican is called the G3 domain and it contains a C-type lectin binding domain, two epidermal growth factor repeats and a complement regulatory region. The versican core protein contains the GAG attachment region and the chondroitin sulphate chains extend from this region of the protein (Fig 1.4) (Zimmermann and Ruoslahti, 1989; Zako et al., 1995; Yang et al., 2000).
Figure 1.4: Schematic model of versican structure. Versican contains globular domains at the amino terminus (G1) and carboxyl terminus (G3). The G1 domain is composed of an immunoglobulin-like motif, followed by two proteoglycan tandem repeats which bind hyaluronan (HABR; hyaluronan binding region). The G3 domain contains two epidermal growth factor-like repeats, a carbohydrate recognition domain (a lectin-binding domain) and complement binding protein (ELC). The core-middle region of versican contains GAG attachment regions that are encoded by exons 7 and 8 which give rise to four different versican isoforms. GAG chondroitin sulphate chains extend from the core protein (Wu et al., 2005)

1.3.2 Versican isoforms

The central area of versican is encoded by two exons that specify chondroitin sulphate attachment regions (Zimmermann and Ruoslahti, 1989). RNA splicing of these two exons generates four isoforms of versican named V0, V1, V2 and V3 core protein molecular weight of about 370 kDa, 263 kDa, 180 kDa, and 74 kDa, respectively (Dours-Zimmermann and Zimmermann, 1994). V0, the largest versican isoform, is encoded by exons 7 and 8 and contains the GAG-α and β domains. The V1 isoform contains GAG-β attachment domain which is encoded by exon 8 (lacking exon 7) whereas the V2 isoform contains a GAG-α domain which is encoded by exon 7 (lacking exon 8) (Lemire et al., 1999). V3 does not include either exon 7 or 8 and consequently
has no GAG attachment sites (Fig 1.5) (Zako et al., 1995; Lemire et al., 1999). An interesting study on human adult tissues showed that all four versican isoforms are transcribed in more than 50% of tissues including the brain and skin, although, intriguingly, only the V1 isoform is expressed in liver and spleen (Cattaruzza et al., 2002). Another study suggested that V1 versican enhances cell proliferation and protects mouse fibroblast cell lines from apoptosis whereas the V2 isoform exhibits opposite activities by inhibiting cell proliferation (Sheng et al., 2005). In addition, it has been reported that V0 and V1 are the predominantly expressed isoforms in tumours suggesting that these isoforms are mainly involved in tumour development (Touab et al., 2002; Arslan et al., 2007). In contrast, over-expression of V3 (the smallest versican isoform, which consists of only the G1 and G3 domains) in melanoma cancer cells markedly reduced cell growth, suggesting a role for V3 versican as an inhibitor of tumour growth (Serra et al., 2005; Hernandez et al., 2010).

**Figure 1.5:** Cartoon of versican isoforms generated by alternative splicing of the mRNA transcript. Different colours show specific domains in the gene. G1 and G3 are shown at the amino and carboxyl termini respectively. Purple = hyaluronan binding region (HABR); yellow = α GAG exon; red = β GAG exon and green = epidermal growth factor repeats (E), a lectin binding domain (L) and a complement regulatory region (C). The glycosaminoglycan chondroitin sulphate chains are shown as (/).
1.3.3 Versican promoter

The transcription start site for the versican gene was first identified by Naso et al. (1994). This study reported that the versican promoter contains a typical TATA box located approximately 16 base pairs upstream of the transcription start site (Fig 1.6). Transient transfections of a reporter construct driven by an 876-bp (-632/+240) fragment of the versican promoter showed significant expression in HeLa cells and IMR-90 embryonic lung fibroblasts. Furthermore, deletion constructs of the 876-bp confirmed that the human versican 5′-flanking sequence contains promoter, enhancer and repressor elements which are able to drive the expression of the versican reporter gene in different cells (Naso et al., 1994). In addition, sequence analysis has revealed potential binding sites for several transcription factors in the 876-bp versican promoter region including CREB (Naso et al., 1994), T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) (Rahmani et al., 2005), AP-1 and SP1 (Domenzain et al., 2009) (Fig 1.6).
1. Introduction

Figure 1.6: Sequence analysis of versican gene promoter and exon 1. Naso et al., (1994) revealed a typical TATA box located approximately 16 bp upstream of the transcription start site and binding sites for a number of transcription regulatory factors including CREB at position -36bp, SP-1 at position -370bp, XRE at position -509bp and AP-2 at positions -426bp, -355bp, -218bp and -190bp respectively. Rahmani et al., (2004) showed two binding sites for TCF/LEF at positions ~546bp and -492bp respectively. Also further investigation by Domenzain et al., (2009) revealed one binding site for SP-1 at position -166bp and one binding site for AP-1 at position -36bp. Positions of all transcription factors are relative to the transcription start site. The sequence of exon 1 is shaded. CREB; cAMP response element-binding, AP-1 & 2; (activator protein 1 & 2), XRE; xenobiotic responsive element, TCF/LEF; T-cell factor/lymphoid enhancer factor.
1.3.4 Versican function

Versican is a main component of the ECM where its hygroscopic properties create a loose and hydrated matrix which is necessary to support key events in development (Lemire et al., 2002; Hinek et al., 2004). Increased expression of versican is observed in sites of tissue injury (Landolt et al., 1995) and in cancers including breast (Kischel et al., 2010), ovarian (Voutilainen et al., 2003), gastrointestinal tract (Theocharis, 2002), prostate (Sakko et al., 2003), brain (Schwartz and Domowicz, 2004), cervical (Kodama et al., 2007) and melanoma (Domenzain et al., 2003). Several reports have also highlighted the role of versican in wound healing (Cattaruzza et al., 2002), angiogenesis, tumour growth (Zheng et al., 2004) and in vascular diseases, especially atherosclerosis (Talusan et al., 2005; Kenagy et al., 2006). It has been demonstrated that versican binds low-density lipoprotein (LDL) particles and it is believed that accumulation of versican in blood vessels promotes extracellular lipoprotein retention, suggesting roles in lipid accumulation, inflammation, and thrombosis (Olin et al., 1999; Wight and Merrilees, 2004). Due to versican’s structural composition and its widespread expression in the body it is able to regulate cell adhesion and survival, cell proliferation, cell migration and ECM assembly (Ricciardelli et al., 2009) that the key studies in these areas are reviewed below.
1.3.4.1 Cell adhesion

Early studies reported that most chondroitin sulphate proteoglycans may be considered as anti-adhesion molecules for the regulation of cell adhesion to the substratum, which is essential for various cell and tissue functions (Yamagata et al., 1989 & 1993). Different studies presented evidence suggesting that this inhibitory activity could be due to the G1 domain of versican (Yamagata et al., 1989; Sugiura et al., 1993). They showed that selective exclusion of versican from podosomes of cultured human osteosarcoma cells suppresses the malignant cell-adhesive phenotype, suggesting that versican can act as an anti-adhesive molecule (Yamagata et al., 1994). However there is evidence that the carboxy-terminal domain of versican interacts with the β1 integrin of brain tumour cells leading to the activation of focal adhesion kinase (FAK), promoting cell adhesion and protecting the cell from apoptosis (Wu et al, 2002 & 2004).

Interaction of versican with selectins and chemokines has been studied. It has been shown that versican binds to L-selectin, adhesion molecules on the surface of activated endothelial cells, through its chondroitin sulphate chains (Kawashima et al., 2002). In addition, versican has been shown to bind secondary lymphoid tissue chemokine (SLC) through chondroitin sulphate chains and this binding tends to down-regulate chemokine function for recruitment of lymphocytes (Hirose et al., 2001). Taken together the data suggest that versican, which is induced in inflammatory conditions such as arthritis (Wight and Merrilees, 2004), asthma and lung disease (Johnson, 2001; Merrilees et al., 2004), may regulate inflammation by regulating interaction with selectins and chemokines (Hirose et al., 2001).
1.3.4.2 Cell Proliferation

Abundant expression of versican in fast growing tissues and cells suggested a key role for versican in cell proliferation (Bode-Lesniewska et al., 1996; Touab et al., 2002). For example, high expression of versican is detected in the loose connective tissue of various organs including the central and peripheral nervous system (Bode-Lesniewska et al., 1996), blood vessels (Zheng et al., 2006), dermis and in the proliferative zone of the epidermis (Zimmermann et al., 1994).

Versican is also involved in the proliferation of smooth muscle cells (SMC) (Schonherr et al., 1997; Cardoso et al., 2010). Several studies have reported proteins such as platelet-derived growth factor (PDGF) and transforming growth factor-β 1 (TFG-β1) increase versican synthesis in arterial smooth muscle cells (ASMCs) (Schonherr et al., 1991 & 1997). It was demonstrated that increases in versican and the associated protein hyaluronan in response to PDGF and TFG-β1 cause increases in the pericellular matrix of the cells and expansion of the ECM that is required for the proliferation and migration of these cells (Evanko et al., 1999). In addition, it was shown that proliferation of ASMCs treated with PDGF is blocked by inhibition of the formation of versican-hyaluronan complex which serves as an important mechanism for controlling cell shape and cell division (Evanko et al., 2001).

Other studies have suggested a role for versican in cell proliferation through its two epidermal growth factor sequences in the G3 domain of the molecule (Zhang et al., 1998; Wu et al., 2001). These studies showed that expression of the G3 domain of versican promotes proliferation in NIH3T3 fibroblasts cells whereas this effect can be inhibited by removing EGF motifs in the versican G3 domain (Zhang et al., 1998). These
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Results suggested that the EGF-like motifs in the versican G3 domain may promote cell proliferation through direct or indirect interaction with the EGF receptor (EGFR) (Zhang et al., 1999).

1.3.4.3 Cell migration

Controversial studies have demonstrated that versican is widely expressed at both mRNA and protein level in neural crest pathways and influences neural cell migration (Perissinotto et al., 2000) whereas a number of other studies have shown that versican prevents migration of these cells (Landolt et al., 1995; Perris et al., 1996). These contradictory findings are believed to be due to different versican isoforms which differ in the core-middle region (Dutt et al., 2006). Some studies have investigated the role of versican in the nervous system and axonal outgrowth (Fidler et al., 1999; Jones et al., 2003; Tang et al., 2003). These studies showed that chondroitin sulphate (CS) chains of versican isotype V2 are involved in inhibiting axonal outgrowth and migration of the mature nervous system. The role of versican in axonal migration was investigated by Asher et al., (2002) who showed up-regulation of versican following central nervous system (CNS) injury and suggested these changes in versican regulation are associated with the failure of nerves to regenerate.

As mentioned earlier, the G3 domain of versican can interact with integrin β1 which is able to form clusters with EGF receptors (Yamada and Even-Ram, 2002). Growing evidence indicates that interaction of integrins with EGF receptors induces downstream signal to extracellular regulated kinase (ERK) which is crucial in regulating a range of cell activities, such as migration (Adelsman et al., 1999; Cabodi et al., 2004;
Wu et al., 2004). Also, the role of versican in the migration of embryonic cells in the development of the heart has been studied (Henderson and Copp, 1998; Kern et al., 2006). It has been reported that versican mRNA and protein is strongly expressed during the development of mouse heart suggesting a key role for versican in cardiac development (Henderson and Copp, 1998).

Furthermore other studies have reported that the G3 domain of versican directly interacts with fibronectin, another extracellular matrix glycoprotein (Yamagata et al., 1986) which showed that formation of a complex of versican G3 domain and fibronectin with VEGF can enhance endothelial cell migration which this process was reversed by removal of the complex (Zheng et al., 2004). This study and other investigation by Wijelath et al., (2002) indicated that expression of versican G3 enhanced brain tumour growth, suggesting the role of versican G3 fragment on promoting angiogenesis and tumour growth that it suggests targeting versican G3 fragments may help to develop a new approach for anticancer and antiangiogenic therapies.

1.3.4.4 Extracellular matrix assembly
Versican interacts with different ECM molecules and has been reported to have an important role during ECM assembly (Wu et al., 2005). Possibly the best known is a specific interaction between the G1 domain of versican and hyaluronan (LeBaron et al., 1992). Hyaluronan is a large polysaccharide in the ECM and is able to create a lattice structure which may regulate cell adhesion and migration (Lee and Spicer,
1. Introduction

Versican binds hyaluronan and this binding requires the double tandem repeat present in the G1 domain of versican (Mukaratirwa et al., 2004; Suwiwat et al., 2004).

1.3.5 Versican regulation

1.3.5.1 Signal transduction pathways

The signalling pathways which modulate versican synthesis are not fully understood although studies on the intracellular pathways have reported that PDGF-stimulated versican expression in arterial smooth muscle cells (SMC) is regulated by endogenous tyrosine kinase activity of the PDGF receptor which up-regulates versican synthesis at both mRNA and protein levels in vascular smooth muscle cells (Schonherr et al., 1997; Syrokou et al., 1999). In addition, another study has suggested the role of protein kinase C (PKC) and ERK in the PDGF stimulated versican gene expression in non-human ASMC (Cardoso et al., 2010).

An interesting study by Rahmani et al., (2005) demonstrated the role of the PI3-K/ PKB (Protein kinase B) pathway in versican expression in SMC. They suggested that phosphorylation and inactivation of glycogen synthase kinase-3β (GSK-3β), a downstream effector of PI3K/PKB (Harwood, 2001), leads to activation and nuclear accumulation of β-catenin which binds to the TCF/LEF transcription factors in the versican promoter and then increases versican transcription in SMC (Fig 1.7) (Rahmani et al., 2005). Further investigation by this group using a specific inhibitor of the PI3-Kinase pathway, LY294002, which inhibited the activation of downstream PKB, showed significant reduction of versican promoter reporter activity and also versican mRNA expression in SMC. A similar mechanism has been observed for other genes such as
MMP-7 (Deguchi et al., 2009) and VEGF (Zhang et al., 2001), which have also been shown to be targeted by PI3-K/PKB and up-regulated by formation of a β-catenin TCF/LEF complex.

**Figure 1.7:** Schematic model of versican promoter regulation via PI3/PKB signalling and β-catenin TCF transcription factor complex suggested by Rahmani et al., 2005.Activation of PI3K signalling by growth factors leads to phosphorylation and inactivation of GSK-3β which results in β-catenin cytoplasmic accumulation and subsequent translocation to the nucleus. Nuclear accumulation of β-catenin leads to complex formation with TCF/LEF transcription factors and transactivation of TCF/LEF target genes. Specific inhibitors of the PI3K signalling pathway such as LY294002 and wortmannin lead to activation of GSK-3β and subsequently to β-catenin degradation.
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1.3.5.2 Transcription factors
Analysis of the versican promoter by Rahmani et al., (2005) revealed two putative binding sites for TCF/LEF transcription factors at positions -546 and -492 bp relative to the transcription start site (Fig 1.6). They showed that site-directed mutagenesis of the TCF sites in the versican promoter markedly diminished reporter luciferase activity in SMC. Furthermore, electrophoretic mobility shift assay (EMSA) and supershift assays revealed that the β-catenin / TCF transcription factor complex is essential for versican expression in SMC (Rahmani et al., 2005).

A recent study by Domenzain et al., (2009) identified several other transcriptional regulatory elements including AP1, SP1 and AP2 on a 620-bp (-618/+2 relative to the transcriptional start site) in a proximal versican promoter reporter construct (Domenzain et al., 2009). This study demonstrated that mutagenesis of the AP-1 site at position -36bp completely abolished versican promoter activity in human melanoma cell lines. Also further investigation by EMSA confirmed the importance of the AP-1 binding site for versican promoter transcription in these cell lines. In addition, versican promoter activity in a TCF/LEF mutated construct was reduced by half, suggesting that versican expression is also up-regulated via the β-catenin/TCF pathway in human melanoma cell lines (Domenzain et al., 2009).

Yoon et al., (2002) investigated the role of the transcription factor p53 in versican gene expression in a broad range of human carcinoma cell lines. p53 is a transcription factor involved in important cellular processes such as cell cycle checkpoint regulation, DNA damage and apoptosis (Harris, 1996; Bossi and Sacchi, 2007). Oligonucleotide-array gene expression analysis of human carcinoma cell lines by Yoon et al., (2002)
demonstrated high expression of the versican gene in wild type p53 (p53 +/+ ) cells but lower expression in p53 -/- cells, suggesting that versican is a direct target of p53. Further investigation using wild type p53 over-expression in p53-null cells transfected with versican promoter reporter constructs showed 200-fold increases in luciferase activity in comparison with the control plasmid. EMSA and super-shift assays confirmed the interaction of p53 protein and the versican p53 binding site (Yoon et al., 2002).
1.4 Aims of the project

As previously outlined, several studies have highlighted the role of versican in angiogenesis and tumour growth (Zheng et al., 2004) where macrophages and hypoxia are present. In addition, another study has shown macrophage activation by versican over-expression through pattern recognition receptor engagement leading to the release of pro-inflammatory cytokines (Kim et al., 2009). Therefore, taking into account the above findings linking disease outcomes with macrophages, hypoxia, and versican expression, I aimed in this study to:

- Identify the principle mononuclear cells which show hypoxic up-regulation of versican mRNA by analysing adherence-purified HMDM, CD14+ purified monocyte/macrophages and lymphocytes.

- Investigate the effect of hypoxia on versican protein expression in PBMC after either 24 hours or 5 days hypoxia.

- Study whether the observed up-regulation of versican mRNA by hypoxia is due to increased transcription or increased versican mRNA stability.

- Elucidate in detail the mechanisms responsible for the up-regulation of versican mRNA by hypoxia in primary HMDM using promoter reporter constructs and transcription factor over-expression.
1. **Introduction**

In addition, unpublished data by Staples and Burke et al., (Bernard Burke, personal communication) using Real-Time RT-PCR showed that versican is up-regulated over 600-fold at the mRNA level in 5 day old hypoxic human peripheral blood mononuclear cells (PBMC) (Fig 1.8). This preliminarily investigation set me out to establish how the versican expression is regulated by hypoxia in human primary macrophages.

![Figure 1.8: Effect of hypoxia (0.2% O₂) on versican mRNA expression in peripheral blood mononuclear cells (PBMC).](image)

PBMCs were quantified by Real-Time RT-PCR to analysis the effect of 24 hours and 5 days hypoxia on versican mRNA expression (See section 3.3 for RNA isolation and Real-Time RT-PCR experiment). 2 x10^6 Cells per well were exposed to normoxia for 5 days (5 d N) or hypoxia for 5 days (5 d H) or 4 days in normoxia followed by 1 day in hypoxia (4 d N, 1 d H; See section 3.2.1). Data were normalised to β2M mRNA levels. Data from 8 independent experiments using different donors are expressed as means ± SEM. The normoxic value in each experiment was assigned an arbitrary value of 1. Data were further analysed for significant induction using two-tailed, paired t-tests. ** p < 0.01, * = p <0.05. Staples, Burke et al., unpublished data.
### Chapter 2: Materials

#### 2.1 Reagents

##### 2.1.1 Cell culture

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<td>Ferric citrate</td>
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<td>Heparin (5000 U/ml)</td>
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<td>Iscove’s Modified Dulbecco’s medium</td>
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<td>LY294002 (PI3 kinase inhibitor)</td>
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<td>Penicillin-Streptomycin 100 mM</td>
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<td>Salmonella minnesota lipopolysaccharide (SMI LPS)</td>
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### 2. Materials

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<td>VLE RPMI 1640 medium (Very Low Endotoxin)</td>
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<td>Wortmannin</td>
<td>681675</td>
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**Table 2.1:** List of reagents used in the cell culture

#### 2.1.2 Reverse transcription and Real-Time PCR

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**Table 2.2:** List of reagents used in RNA isolation, reverse transcription and RT-PCR
### 2.1.3 Molecular cloning

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<td>New England Biolabs, Hitchin, UK</td>
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<td>Bioline, London, UK</td>
</tr>
<tr>
<td>Ethidium Bromide (EtBr)</td>
<td>46065</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>EP0571</td>
<td>Fermentas, York, UK</td>
</tr>
<tr>
<td>REDAccuTaq® LA DNA Polymerase</td>
<td>D-1313</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>SfiI Restriction Enzyme</td>
<td>R0123S</td>
<td>New England Biolabs, Hitchin, UK</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>600011</td>
<td>Stratagene, Stockport, UK</td>
</tr>
<tr>
<td>Xylene cyanol</td>
<td>X4126</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

**Table 2.3:** List of reagents used in general molecular cloning
## 2.1.4 Nuclear extraction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>A-1153</td>
<td>Sigma Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (Na₂HPO₄)</td>
<td>1.06580.5000</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>D-9163</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Ethylenediaminetetra-acetic acid (EDTA) 0.5 M, pH 8.0</td>
<td>E-7889</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Ethylene glycol tetra-acetic acid (EGTA)</td>
<td>E-8145</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Glycerol</td>
<td>G-7757</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>L-2023</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)</td>
<td>H-4034</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Nonidet NP40 (Igepal)</td>
<td>I-3021</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>P-4265</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>P-7626</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>P/4280/60</td>
<td>Fisher, Loughborough, UK</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (KH₂PO₄)</td>
<td>A323473</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>S/3160/63</td>
<td>Fisher, Loughborough, UK</td>
</tr>
<tr>
<td>Sodium vanadate</td>
<td>590088</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

**Table 2.4:** List of reagents used for nuclear protein extraction
2. Materials

2.1.5 Western blotting

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bisacrylamide</td>
<td>161-0158</td>
<td>Bio-Rad Laboratories, Hertfordshire, UK</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>A-3678</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>8122</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>ECL advance™ western blotting detection kit</td>
<td>RPN2135</td>
<td>GE healthcare, Buckinghamshire, UK</td>
</tr>
<tr>
<td>Glycine</td>
<td>G-8898</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Methanol</td>
<td>M/4000/17</td>
<td>Fisher, Loughborough, UK</td>
</tr>
<tr>
<td>Pre-Stained High Molecular Weight Protein Standard</td>
<td>LC5699</td>
<td>Invitrogen Life Technology, Paisley, UK</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulphate (SDS)</td>
<td>L-5750</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
<td>T-9281</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Trizma™ (Tris base)</td>
<td>T-1503</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Tween-20</td>
<td>P-5927</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

_Table 2.5:_ List of reagents used in western blotting

2.1.6 Flow cytometry

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brefeldin A</td>
<td>B-6542</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Paraformaldehyde (formalin)</td>
<td>F-8775</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Saponin</td>
<td>S-7900</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

_Table 2.6:_ List of reagents used in flow cytometry (for antibodies used see table 2.14)
### 2.1.7 Reagent Kits

<table>
<thead>
<tr>
<th>Reagent Kit</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dual luciferase® Reporter Assay System*</td>
<td>E1910</td>
<td>Promega Corporation Southampton, UK</td>
</tr>
<tr>
<td>EndoFree™ Plasmid Maxi Kit**</td>
<td>12362</td>
<td>QIAGEN, West Sussex, UK</td>
</tr>
<tr>
<td>JetPEI™ DNA transfection reagent</td>
<td>101-40N</td>
<td>Polyplus-transfection Inc, New York, USA</td>
</tr>
<tr>
<td>Library Efficiency® DH5α™ competent cells***</td>
<td>18263-012</td>
<td>Invitrogen Life Technology, Paisley, UK</td>
</tr>
<tr>
<td>Luciferase assay system</td>
<td>E1501</td>
<td>Promega Corporation Southampton, UK</td>
</tr>
<tr>
<td>Pierce 660 nm Protein Assay</td>
<td>22660</td>
<td>Thermo Scientific, Rockford, USA</td>
</tr>
<tr>
<td>QIAquick Gel Extraction kit</td>
<td>28704</td>
<td>QIAGEN, West Sussex, UK</td>
</tr>
</tbody>
</table>

**Table 2.7:** List of reagent kits used in the project

* Dual luciferase® Reporter Assay System kit contains 5x Lysis buffer, Luciferase Assay Reagent II and Stop & Glo reagent

** EndoFree™ Plasmid Maxi Kit contains TE buffer (10mM Tris-HCl [pH7.5] and 1mM EDTA)

*** Library Efficiency® DH5α™ competent cells Kits contains control pUC19 DNA and S.O.C medium

**** Luciferase assay system kit contains 5x Lysis buffer and Luciferase Assay Reagent
## 2.2 Consumables

<table>
<thead>
<tr>
<th>Material</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml Centrifuge tube</td>
<td>311-02-051</td>
<td>Axygen Bioscience, California, USA</td>
</tr>
<tr>
<td>1.5 ml Centrifuge tube</td>
<td>311-08-051</td>
<td>Axygen Bioscience, California, USA</td>
</tr>
<tr>
<td>15 ml Centrifuge tube</td>
<td>430791</td>
<td>Corning Incorporated, Tampaulipas, Mexico</td>
</tr>
<tr>
<td>50 ml Centrifuge tube</td>
<td>21008-178</td>
<td>VWR International, West Chester, USA</td>
</tr>
<tr>
<td>5 ml Falcon tube (polystyrene)</td>
<td>352008</td>
<td>Becton Dickinson, New Jersey, USA</td>
</tr>
<tr>
<td>14 ml Falcon tube</td>
<td>352059</td>
<td>Becton Dickinson, New Jersey, USA</td>
</tr>
<tr>
<td>Hybond-P PVDF membrane</td>
<td>RPN303F</td>
<td>GE healthcare, Buckinghamshire, UK</td>
</tr>
<tr>
<td>LightCycler ® Capillaries (20 µl)</td>
<td>04929292001</td>
<td>Roche Diagnostics, Burgess Hill, UK</td>
</tr>
<tr>
<td>MACS MS column</td>
<td>130-042-201</td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td>Multidish 6-Well Nunclon plates (adherent plates)</td>
<td>S3127XC</td>
<td>NUNC A/S, Roskilde, Denmark</td>
</tr>
<tr>
<td>20 ml Syringe</td>
<td>SZR-205-110L</td>
<td>Fisher, Loughborough, UK</td>
</tr>
<tr>
<td>50 ml Syringe</td>
<td>SZR-205-160T</td>
<td>Fisher, Loughborough, UK</td>
</tr>
<tr>
<td>75 cm² Tissue culture flasks</td>
<td>658170</td>
<td>Greiner Bio-One, Stonehouse, UK</td>
</tr>
<tr>
<td>75 cm² Tissue culture flasks filter cap</td>
<td>658175</td>
<td>Greiner Bio-One, Stonehouse, UK</td>
</tr>
<tr>
<td>VarioMACS separator</td>
<td>130-090</td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td>6-Well Costar ultra low attachment plates</td>
<td>CC227</td>
<td>Appleton Woods, Birmingham, UK</td>
</tr>
<tr>
<td>96-well cell culture cluster</td>
<td>3596</td>
<td>Corning Incorporated, New York, USA</td>
</tr>
</tbody>
</table>

**Table 2.8:** List of materials used in the project
2.3 Media

2.3.1 Media for cell culture

<table>
<thead>
<tr>
<th>Medium</th>
<th>Reagents</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMPLETE Iscove’s Dulbecco’s Modified Medium</td>
<td>Iscove’s Dulbecco’s Modified Medium 2 mM L-glutamine 200 U/ml Penicillin 200 µg/ml Streptomycin 2.5 % Human AB serum</td>
<td>500 ml Iscove’s Dulbecco’s Modified Medium 5 ml 200 mM L-glutamine 10 ml 10000 U/ml Penicillin / 10000 µg/ml Streptomycin 12.5 ml Human AB serum store at 4°C</td>
</tr>
<tr>
<td>COMPLETE RPMI 1640 medium</td>
<td>VLE RPMI1640 medium 2 mM L-glutamine 200 U/ml Penicillin 200 µg/ml Streptomycin 10x Non-essential amino acids 10 % FBS</td>
<td>500 ml VLE RPMI1640 medium 5 ml 200 mM L-glutamine 10 ml 10000 U/ml Penicillin / 10000 µg/ml Streptomycin 5 ml 100x NEAA the medium was passed through a Gambro U-2000 ultrafiltration column to eliminate potential contaminants such as endotoxin and other bacterial components then add 50 ml FBS and store at 4°C</td>
</tr>
</tbody>
</table>

*Table 2.9:* Media used for cell culture
### 2.3.2 Media for bacteria

<table>
<thead>
<tr>
<th>Medium</th>
<th>Reagent</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-medium with Ampicillin*</td>
<td>10 g/l NaCl</td>
<td>10 g Luria broth powder 400 ml dH$_2$O autoclave and let cool to 55°C</td>
</tr>
<tr>
<td></td>
<td>5 g/l Yeast extract</td>
<td>add 400 µl 100 mg/ml Ampicillin</td>
</tr>
<tr>
<td></td>
<td>10 g/l Tryptone</td>
<td>store at 4°C</td>
</tr>
<tr>
<td></td>
<td>100 µg/ml Ampicillin</td>
<td></td>
</tr>
<tr>
<td>LB-Agar medium with Ampicillin*</td>
<td>10 g/l NaCl</td>
<td>10 g Luria broth 6 g Agar 400 ml dH$_2$O autoclave and let cool to 55°C</td>
</tr>
<tr>
<td></td>
<td>5 g/l Yeast extract</td>
<td>add 400 µl 100 mg/ml Ampicillin</td>
</tr>
<tr>
<td></td>
<td>10 g/l Tryptone</td>
<td>pour into plates store at 4°C</td>
</tr>
<tr>
<td></td>
<td>15 g/l Agar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µg/ml Ampicillin</td>
<td></td>
</tr>
</tbody>
</table>

*LB-medium and LB-agar were prepared by the medium kitchen, Department of Infection, Immunity and Inflammation, University of Leicester*
2.4 Primers

2.4.1 Primers for Real-Time PCR

<table>
<thead>
<tr>
<th></th>
<th>5’ Forward</th>
<th>3’ reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2M</td>
<td>5’-GGCTATCCAGCGTACTCCAAAG-3’</td>
<td>5’-CAACTTCAATGTCGGATGGATG-3’</td>
</tr>
<tr>
<td>Versican</td>
<td>5’-ACAAGCATCCTGTCTCAG-3’</td>
<td>5’-TGAAACCATCTTTGCACTGG-3’</td>
</tr>
<tr>
<td>VEGF</td>
<td>5’-CAGCGCAGCTACTGCAATCGAGAGA-3’</td>
<td>5’-GCTTGTCACATCTGCAAGTACGTTCGTTTA-3’</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>5’-CAACTGGACCTCAAATTTCATTGGGA-3’</td>
<td>5’-CGGGGTGTTTATCATTGGCTGG-3’</td>
</tr>
</tbody>
</table>

Table 2.11: List of primers used for Real Time-PCR (β2M: Beta-2 Microglobulin, VEGF: Vascular Endothelial Growth Factor, GLUT-1: Glucose Transporter -1)

Note: Versican primer sequences were derived from NCBI (National Centre for Biotechnology Information) nucleotide bank accession no. NM_004385. Forward primer is from nucleotides 9879 - 9897; reverse primer is from nucleotides 10254 – 10273. β2M, VEGF and GLUT-1 primer sequences were obtained from published work (Burke et al., 2003; Gao et al., 2005). All primers (Eurofins MWG Operon, Ebersberg, Germany) were diluted with autoclaved dH2O to make 100 μM laboratory stocks which were then further diluted 1:20 with autoclaved dH2O to produce 5 μM working stocks.
2.4.2 Primers for PCR cloning

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3.956kb versican 5’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>-646bp versican 5’</td>
<td>5’-aattGGCCTAACTGGCCAGGTCAGTGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>-296bp versican 5’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>-56bp versican 5’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>-26bp versican 5’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>+54bp versican 3’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>+104bp versican 3’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>+154bp versican 3’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>+184bp versican 3’</td>
<td>5’-aattGGCCTAAGGCTCGCTGCTCGAGGCCAGGCGCTC-3’</td>
</tr>
<tr>
<td>Random 5’</td>
<td>5’-TGGCCTAGCTAGCTAGCTAGCTAGATGGCGCTCGG-3’</td>
</tr>
<tr>
<td>Random 3’</td>
<td>5’-AGGCGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCGCTCGG-3’</td>
</tr>
</tbody>
</table>

Table 2.12: List of primers used for PCR cloning

**Note:** Versican promoter sequences were generated by PCR from human genomic DNA (See section 3.3) with the appropriate sets of primers designed using Primer3 web-based software (available at: [http://frodo.wi.mit.edu/primer3/input.htm](http://frodo.wi.mit.edu/primer3/input.htm)) based on the published versican promoter sequence (Naso et al., 1994, accession number U15963). The shaded sequence is the SfiI restriction site. The sequence “aatt” is a random sequence added to both ends of the clones in order to facilitate restriction endonuclease activity.
### 2.5 Recipes

All solutions and buffers were prepared using nanopure deionised water (dH$_2$O).

Where stated, buffers and solutions were autoclaved (20 minutes, 121°C).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>X % Agarose gel</td>
<td>X % (w/v) Agarose</td>
<td>X g Agarose</td>
</tr>
<tr>
<td></td>
<td>1x TBE</td>
<td>100 ml 1x TBE</td>
</tr>
<tr>
<td></td>
<td>0.25 µg/ml EtBr</td>
<td>2.5 µl 10 mg/ml EtBr</td>
</tr>
<tr>
<td></td>
<td>bring to the boil in a microwave oven, let cool and pour into the</td>
<td>gel tray</td>
</tr>
<tr>
<td></td>
<td>gel tray</td>
<td></td>
</tr>
<tr>
<td>Ampicillin stock solution</td>
<td>100 mg/ml Ampicillin</td>
<td>1 g Ampicillin</td>
</tr>
<tr>
<td></td>
<td>add to 10 ml autoclaved dH$_2$O, filter sterilise and store aliquots at -20°C</td>
<td></td>
</tr>
<tr>
<td>2 mg/ml Aprotinin</td>
<td>2 mg/ml Aprotinin</td>
<td>5 mg in 2.5 ml dH$_2$O</td>
</tr>
<tr>
<td></td>
<td>Store in -20°C</td>
<td></td>
</tr>
<tr>
<td>10% APS</td>
<td>10% (w/v) Ammonium persulphate</td>
<td>10 g APS in 100 ml dH$_2$O</td>
</tr>
<tr>
<td>1 mg/ml BSA</td>
<td>1 mg/ml BSA</td>
<td>10 mg BSA</td>
</tr>
<tr>
<td></td>
<td>1x luciferase lysis buffer</td>
<td>10 ml 1x luciferase lysis buffer</td>
</tr>
<tr>
<td>50 µg/ml BSA</td>
<td>50 µg/ml BSA</td>
<td>100 µl 1mg/ml BSA</td>
</tr>
<tr>
<td></td>
<td>1x luciferase lysis buffer</td>
<td>1.9 ml 1x luciferase lysis buffer</td>
</tr>
<tr>
<td>100 µg/ml BSA</td>
<td>100 µg/ml BSA</td>
<td>100 µl 1mg/ml BSA</td>
</tr>
<tr>
<td></td>
<td>1x luciferase lysis buffer</td>
<td>900 µl 1x luciferase lysis buffer</td>
</tr>
<tr>
<td>250 µg/ml BSA</td>
<td>250 µg/ml BSA</td>
<td>100 µl 1mg/ml BSA</td>
</tr>
<tr>
<td></td>
<td>1x luciferase lysis buffer</td>
<td>300 µl 1x luciferase lysis buffer</td>
</tr>
<tr>
<td>500 µg/ml BSA</td>
<td>500 µg/ml BSA</td>
<td>100 µl 1mg/ml BSA</td>
</tr>
<tr>
<td></td>
<td>1x luciferase lysis buffer</td>
<td>100 µl 1x luciferase lysis buffer</td>
</tr>
<tr>
<td>750 µg/ml BSA</td>
<td>750 µg/ml BSA</td>
<td>100 µl 1mg/ml BSA</td>
</tr>
<tr>
<td></td>
<td>1x luciferase lysis buffer</td>
<td>30 µl 1x luciferase lysis buffer</td>
</tr>
<tr>
<td>Degassed PBE buffer (D-PBE)</td>
<td>1x PBS 1% FBS 2 mM EDTA</td>
<td>50 ml 1x filtered PBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 µl FBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 µl 0.5 M EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>place under vacuum for 45 min for degassing</td>
</tr>
</tbody>
</table>
### Materials

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC-dH2O</td>
<td>0.1 % Diethyl pyrocarbonate 1 litre dH2O add 1 ml DEPC and incubate at RT overnight, then autoclave</td>
</tr>
<tr>
<td>75% DEPC-Ethanol</td>
<td>75% (v/v) Ethanol 37.5 ml 100% Ethanol 12.5 ml 0.1% DEPC-dH2O store at -20°C</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>10 mM dATP 10 mM dTTP 10 mM dCTP 10 mM dGTP 100 µl 100 mM dATP 100 µl 100 mM dTTP 100 µl 100 mM dCTP 600 µl DEPC-dH2O store aliquots at -20°C</td>
</tr>
<tr>
<td>1 M DTT</td>
<td>1 M Dithiothreitol 0.54 g DTT in 3.5 ml dH2O filter sterilise and store at -20°C</td>
</tr>
<tr>
<td>DNA 6x gel loading buffer (10 ml)</td>
<td>0.25% Bromophenol blue 0.25% Xylene cyanol 30% Glycerol 7.0 ml dH2O 250 mg Bromophenol blue 250 mg Xylene cyanol 3.0 ml Glycerol store aliquot at 4°C</td>
</tr>
<tr>
<td>10 mg/ml EtBr</td>
<td>10 mg/ml EtBr g EtBr adjust to 10 ml with dH2O and store in the dark at RT</td>
</tr>
<tr>
<td>0.1M EGTA pH 8.0</td>
<td>380 g/l EGTA 1.9 g Adjust PH to 8.0 adjust to 50 ml with dH2O and autoclave</td>
</tr>
<tr>
<td>1% Formalin</td>
<td>1x PBS/2% FBS 1% Formalin 100 µl formalin 9.9 ml 1x PBS/2% FBS</td>
</tr>
<tr>
<td>1M Hepes pH7.5</td>
<td>238.3 g/l Hepes 11.915 g adjust pH to 7.5 adjust to 50 ml with dH2O and autoclave</td>
</tr>
<tr>
<td>1M KCl</td>
<td>74.56 g/l KCl 3.72 g in 50 ml dH2O and autoclave</td>
</tr>
<tr>
<td>2 mg/ml Leupeptin</td>
<td>2 mg/ml Leupeptin 5 mg in 2.5 ml dH2O Store in -20°C</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>58.44 g/l NaCl 2.92 g in 50 ml dH2O and autoclave</td>
</tr>
<tr>
<td>Nuclear extract buffer A (50 ml)</td>
<td>10 mM Hepes pH7.5 0.1 mM EDTA pH 8.0 0.1 mM EGTA pH 8.0 10 mM KCl 48.94 ml dH2O 500 µl 1 M Hepes pH7.5 10 µl 0.5 M EDTA pH 8.0 50 µl 0.1 M EGTA pH 8.0 500 µl KCl</td>
</tr>
</tbody>
</table>
2. Materials

<table>
<thead>
<tr>
<th>Nuclear extract buffer B (50 ml)</th>
<th>20 mM Hepes pH 7.5 1 mM EDTA pH 8.0 1 mM EGTA pH 8.0 0.4 M NaCl</th>
<th>28.4 ml dH2O 1 ml 1 M Hepes pH7.5 100 µl 0.5 M EDTA PH 8.0 500 µl 0.1 M EGTA 20 ml 1 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x PBS (Phosphate buffered saline)*</td>
<td>137 mM NaCl 2.7 mM KCl 10 mM Na2HPO4 2 mM KH2PO4</td>
<td>40 g NaCl 1 g KCl 7.2 g Na2HPO4 1.2 g KH2PO4 adjust to 5 litre with dH2O autoclave passed through a Gambro U-2000 ultrafiltration column to eliminate potential contaminants such as endotoxin and other bacterial components store at 4°C</td>
</tr>
<tr>
<td>1x PBS/2% FBS</td>
<td>1x PBS 2% FBS</td>
<td>98 ml 1x PBS 2 ml FBS</td>
</tr>
<tr>
<td>1 mg/ml Pepstatin A</td>
<td>1 mg/ml Pepstatin A</td>
<td>5 mg in 5 ml dH2O Store in -20°C</td>
</tr>
<tr>
<td>0.2 M PMSF</td>
<td>174.19 g/l PMSF</td>
<td>0.35 g in 10 ml 100% Ethanol Store in 4°C</td>
</tr>
<tr>
<td>4% polyacrylamide gel (stacking gel)</td>
<td>0.13 M Tris/HCl 6.8 0.25% SDS 4% Acrylamide/bisacrylamide 0.125% APS 0.25% TEMED</td>
<td>3 ml dH2O 340 µl ml 1.5 M Tris/HCl 6.8 100 µl 10% SDS 500 µl 30% Acrylamide/bisacrylamide 50 µl 10% APS 10 µl TEMED After addition of APS and TEMED the gel was poured immediately and left at least 30 min at RT to polymerise</td>
</tr>
<tr>
<td>8% Polyacrylamide gel (resolving gel)</td>
<td>0.4 M Tris/HCl 8.3 0.1% SDS 8% Acrylamide/bisacrylamide 0.1% APS 0.3% TEMED</td>
<td>5.8 ml dH2O 1.27 ml 3 M Tris/HCl 8.3 100 µl 10% SDS 2.7 ml 30% Acrylamide/bisacrylamide 100 µl 10% APS 30 µl TEMED After addition of APS and TEMED the gel was poured immediately and left at least 30 min at RT to polymerise</td>
</tr>
</tbody>
</table>
2. Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% Saponin</td>
<td></td>
<td>0.1% Saponin in 100 ml 1x PBS/2% FBS</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10% (w/v) sodium dodecyl sulfate</td>
<td>10 g SDS in 100 ml dH₂O</td>
</tr>
<tr>
<td>0.1 M Sodium vanadate</td>
<td>121.93 g/l Sodium vanadate</td>
<td>0.12 g in 10 ml dH₂O, Store in 4°C</td>
</tr>
<tr>
<td>Solution A (fresh prepared nuclear buffer A) (500 µl)</td>
<td>Nuclear extract buffer A 1 mM DTT 0.5 mM PMSF 1 mM sodium vanadate</td>
<td>493.25 µl Nuclear extract buffer A 0.5 µl 1 M DTT 1.25 µl 0.2 M PMSF 5 µl 0.1 M Sodium vanadate</td>
</tr>
<tr>
<td>Solution B (fresh prepared nuclear buffer A) (500 µl)</td>
<td>Nuclear extract buffer B 1 mM DTT 1 mM PMSF 10 µg/ml Leupeptin 10 µg/ml Pepstatin 10 µg/ml Aprotinin 1 mM sodium vanadate</td>
<td>482 µl Nuclear extract buffer B 0.5 µl 1 M DTT 2.5 µl 0.2 M PMSF 2.5 µl 2 mg/ml Leupeptin 5 µl 1 mg/ml Pepstatin 2.5 µl 2 mg/ml Aprotinin 5 µl 0.1 M Sodium vanadate</td>
</tr>
<tr>
<td>10x TBE (Tris-borate-EDTA)</td>
<td>0.9 M Tris base 0.9 M Boric acid 2 mM EDTA PH 8.0</td>
<td>108 g Tris base 55 g Boric acid 40 ml 0.5 M EDTA Adjust to 1 litre with dH₂O autoclave</td>
</tr>
<tr>
<td>1x TBE with EtBr</td>
<td>90 mM Tris-borate 0.5 mM EDTA PH 8.0 0.25 µg/ml EtBr</td>
<td>100 ml 10x TBE 25 µl 10 mg/ml EtBr Adjust to 1 litre with dH₂O</td>
</tr>
<tr>
<td>10x TBS (Tris buffered saline)</td>
<td>200 mM Tris base 1.368 M NaCl</td>
<td>24.2 g Tris base 80 g NaCl Adjust Ph to 7.6 Adjust to 1 litre with dH₂O and autoclave</td>
</tr>
<tr>
<td>1x TBS/Tween-20</td>
<td>1x TBS 0.1% Tween-20</td>
<td>100 ml 10x TBS 1 ml Tween-20 900 ml dH₂O</td>
</tr>
<tr>
<td>1.5 M Tris/HCl</td>
<td>121.14 g/l Tris base</td>
<td>90.855 g Tris base adjust to pH 6.8 with HCl adjust to 500 ml with dH₂O and autoclave</td>
</tr>
<tr>
<td>3 M Tris/HCl</td>
<td>121.14 g/l Tris base</td>
<td>181.71 g Tris base adjust to pH 8.3 with HCl adjust to 500 ml with dH₂O and autoclave</td>
</tr>
<tr>
<td>Western blot blocking buffer</td>
<td>5% Skimmed milk powder</td>
<td>5 g Skimmed milk powder</td>
</tr>
<tr>
<td>1x TBS</td>
<td></td>
<td>100 ml 1x TBS/Tween-20</td>
</tr>
</tbody>
</table>
2. Materials

| Western blot gel loading buffer 5x | 250 mM Tris/HCl 6.8  
|                                  | 10% SDS  
|                                  | 0.5% Bromophenol blue  
|                                  | 50% glycerol  
|                                  | 500 mM DTT  
|                                  | 3.4 ml dH₂O  
|                                  | 1.6 ml 1.5 M Tris/HCl 6.8  
|                                  | 1 g SDS  
|                                  | 0.05 g Bromophenol blue  
|                                  | 5 ml glycerol  
|                                  | 25 µl of 1 M DTT added to  
|                                  | 25 µl 5x gel loading buffer before use |
| Western blot running Buffer 10x | 250 mM Tris  
|                                  | 2 M Glycine  
|                                  | 10% SDS  
|                                  | 30.29 g Tris  
|                                  | 144.13 g Glycine  
|                                  | 10 g SDS  
|                                  | adjust to 1 litre with dH₂O  
|                                  | and autoclave  
|                                  | Dilute 1:10 with dH₂O to use |
| Western blot transfer buffer     | 25 mM Tris  
|                                  | 200 mM Glycine  
|                                  | 20% Methanol  
|                                  | 3.03 g Tris  
|                                  | 14.41 g Glycine  
|                                  | 200 ml Methanol  
|                                  | 800 ml dH₂O |

**Table 2.13:** List of recipes for all buffers and solutions

*1x PBS was prepared by the media kitchen of the Department of Infection, Immunity and Inflammation, University of Leicester*
2.6 Antibodies

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human CD14 microbeads</td>
<td>120-000-305</td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td>Monoclonal anti-human versican antibody</td>
<td>MAB3054</td>
<td>R&amp;D Systems, Abingdon, UK</td>
</tr>
<tr>
<td>Rabbit anti-Actin polyclonal antibody</td>
<td>A-2066</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Rabbit anti-β-Catenin polyclonal antibody</td>
<td>C-2206</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Polyclonal anti-Rabbit Immunoglobulins/HRP</td>
<td>P0448</td>
<td>Dako, Cambridgeshire, UK</td>
</tr>
<tr>
<td>Anti-rat IgG conjugated to FITC</td>
<td>F-1763</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Rat IgG₁ isotype control</td>
<td>MAB005</td>
<td>R&amp;D Systems, Abingdon, UK</td>
</tr>
</tbody>
</table>

Table 2.14: List of antibodies used for immunoblotting and flow cytometry experiments

2.7 Reporter plasmids

2.7.1 pGL4.10 [luc2] Luciferase reporter vector (#E6651, Promega, Southampton, UK)

The pGL4 luciferase reporter vectors are reporter vectors optimized for expression in mammalian cells (Fig 2.1). Numerous configurations of pGL4 vectors are available, including those with the synthetic firefly luc2 (*Photinus pyralis*) and Renilla hRluc (*Renilla reniformis*) luciferase genes, which have been optimized for more efficient expression in mammalian cells. Furthermore, both the reporter genes and the vector backbone such as Ampicillin (Amp<sup>+</sup>) gene and mammalian selectable marker genes have been engineered to reduce the number of consensus transcription factor binding sites.
sites, reducing background and the risk of anomalous transcription. The multiple cloning region of the pGL4 vectors has been synthetically constructed and is based on the multiple cloning region of the pGL3 vectors (Figure 2.1 and 2.2). However, differences between the two multiple cloning regions exist. The pGL4 vector multiple cloning region includes the following restriction sites: *BglII, SfiI, Acc65I, KpnI, EcoICRI, SacI, Nhel, Xhol, EcoRV, BgIII, and HindIII*. The *BglII, SfiI* and *EcoRV* restriction sites have been added to the pGL4 vector multiple cloning region and are not found in the pGL3 vector multiple cloning region. This increases the number of choices in choosing restriction sites during cloning. The *MluI* and *SmaI* restriction sites found in the pGL3 multiple cloning region have not been included in the pGL4 multiple cloning region. The two *BglII/SfiI* restriction sites in pGL4 facilitate the moving the DNA of interest such as response elements, enhancers and promoters between vectors. Additionally, transfers between pGL4 vectors using either the *BglII* or *SfiI* restriction enzymes retain the desired orientation of the DNA of interest due to the unique DNA recognition properties of *BglII* and *SfiI*. Also pGL4 vectors contain a SV40 late poly (A) signal which increases the level of luciferase expression due to polyadenylation signals (Fig 2.1). In addition to the features listed, all pGL4 vectors contain a synthetic poly(A) signal/transcriptional pause site which is located upstream of the multiple cloning region. This site is present to reduce the effects of spurious transcription on luciferase reporter gene expression (Fig 2.1). In this project the *SfiI* restriction site of pGL4 luciferase reporter vector was chosen for versican promoter cloning (See section 3.3).
2. Materials

Figure 2.1: Generic pGL4.10 [luc2] vector map

Figure 2.2: The multiple cloning region of the pGL4 vectors
2.7.2 pRL-TK vector (#E2241, Promega, Southampton, UK)

The pRL-TK vector serves as an internal control reporter construct which can be used in combination with any experimental reporter vector (e.g. pGL4) to co-transfect mammalian cells. The pRL-TK vector contains a cDNA encoding Renilla luciferase, originally cloned from the marine organism *Renilla reniformis* (sea pansy) which can be used to normalize the firefly luciferase activity from the targeting constructs. In this project, HMDM were transfected with versican promoter pGL4.10 [luc2] reporter vector and co-transfected with Renilla plasmid (pRL-TK) to normalise the transfection results.
### 2.8 Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Model</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balance</td>
<td>BD202</td>
<td>Mettler Toledo, Greifensee, Switzerland</td>
</tr>
<tr>
<td>Centrifuges</td>
<td>5717R Sorvall Legend</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thermo Scientific, Germany</td>
</tr>
<tr>
<td>Micro plate reader (ELISA reader)</td>
<td>Model 680</td>
<td>Bio-Rad Laboratories, Hertfordshire, UK</td>
</tr>
<tr>
<td>Incubators</td>
<td>Hera cell Galaxy R</td>
<td>Heraeus, Hanau, Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td>New Brunswick Scientific / RS Biotech</td>
</tr>
<tr>
<td>LightCycler® Systems for Real-Time PCR</td>
<td>Version 3</td>
<td>Roche, Burgess Hill, UK</td>
</tr>
<tr>
<td>Luminometer</td>
<td>Sirius</td>
<td>Berthold detection system, Pforzheim, Germany</td>
</tr>
<tr>
<td>Microscopes</td>
<td>H600 Wilovert S</td>
<td>Hund, Wetzlar, Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hund, Wetzlar, Germany</td>
</tr>
<tr>
<td>NanoDrop spectrophotometer</td>
<td>1000</td>
<td>NanoDrop products, Wilmington, USA</td>
</tr>
<tr>
<td>Oxygen analyser</td>
<td>Mini O₂</td>
<td>Analox sensor technology, North Yorkshire, UK</td>
</tr>
<tr>
<td>pH-meter</td>
<td>MP225</td>
<td>Mettler Toledo, Greifensee, Germany</td>
</tr>
<tr>
<td>Shaker</td>
<td>Innova44 Celloshaker</td>
<td>New Brunswick Scientific, Hertfordshire, UK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glotec-Fischer, Reiskirchen, Germany</td>
</tr>
<tr>
<td>Thermocycler (PCR machine)</td>
<td>TC-3000 Progene 231-103</td>
<td>Techne, Cambridge, UK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Techne, Cambridge, UK</td>
</tr>
<tr>
<td>UV-visible Spectrophotometer</td>
<td>Cary 50 Bio</td>
<td>Varian, Santa Clara, USA</td>
</tr>
<tr>
<td>Western blot apparatus</td>
<td>Xcell II Minicell</td>
<td>Novex San Diego, USA</td>
</tr>
</tbody>
</table>

**Table 2.15**: List of equipment
3. Methods

Chapter 3: Methods

3.1 Cell culture

3.1.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Blood samples from healthy donors were collected into a standard laboratory syringe which contained sufficient heparin to give a final concentration of 10 U/ml, to prevent blood clotting. The blood sample was diluted 1:1 with Hank’s Balanced Salt Solution and then 30 ml of diluted blood was gently layered onto 15 ml of Ficoll-Paque™ Plus in a 50 ml centrifuge tube, followed by 30 minutes centrifugation (minimum acceleration and brake off) at 400 g at room temperature (RT). Following centrifugation a thick concentrated PBMC layer, containing monocytes and lymphocytes, was visible at the interface between the original blood sample and the Ficoll-Paque Plus. The monocyte/lymphocyte layer was carefully transferred to a new 50 ml centrifuge tube and washed twice with Hank’s Balanced Salt Solution and spun down at 400 g for 10 minutes at RT. Finally the PBMC were washed once with Iscove’s Modified Dulbecco’s medium and spun down at 400 g for 10 minutes at RT. Isolated cells were re-suspended in 10ml of Iscove’s Dulbecco’s medium containing 2.5% human AB serum, 1% L-glutamine, and 1% penicillin and streptomycin (See tables 2.1, 2.8 and 2.9 for the reagents and materials) followed by cell counting using an improved Neubauer haemocytometer with a chamber depth of 0.1 mm (Staples et al., 2007) (See section 3.1.2).
3. Methods

Note: Iscove’s Dulbecco’s medium containing 2.5% human AB serum, 1% L-glutamine, and 1% penicillin and streptomycin is described as complete Iscove’s medium in this project. Hank’s Balanced Salt Solution and complete Iscove’s medium were used at RT.

3.1.2 Determination of cell concentration
An aliquot of the cells was diluted 1:10 in hank’s balanced salt solution (HBSS). 10 µl of the diluted cells was transferred to an improved Neubauer haemocytometer with a chamber depth of 0.1 mm. 25 central squares of the grid pattern bounded by triple lines (1mm x 1mm) were counted and the cell concentration (cells/ml) calculated as follows:

<table>
<thead>
<tr>
<th>Cells/ml = Cells counted x 10⁴ x dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>• 10⁴ = Conversion factor of volume of counting chamber into ml</td>
</tr>
<tr>
<td>• Dilution factor=10</td>
</tr>
</tbody>
</table>
3. Methods

3.1.3. Generation of adherence-purified human monocyte-derived macrophages (HMDM)

The PBMC concentration was adjusted to 1 x 10^6 cell/ml in complete Iscove’s medium. 6-well adherent Nunclon plates were seeded with 2ml of medium per well giving 2x10^6 cells per well and incubated at 37°C, 5% CO_2 for 2 hours. After 2 hours, the medium containing the non-adherent cells (mainly lymphocytes) was removed and replaced with 2ml of fresh complete Iscove’s medium. Adherent monocytes were incubated at 37°C with 5% CO_2 and 20.9% O_2 for 5 days to allow differentiation into macrophages (Burke et al., 2003; Staples et al., 2011). Previous analysis of maturation markers (CD68) and morphology of monocyte-derived macrophages (MDMs) over a period of 3-5 days incubation concluded that this length was appropriate for the generation of macrophages (Staples et al., 2007) (See table 2.1 and 2.8 for the reagents and materials).

3.1.4 Magnetic cell sorting system (MACS)

The Miltenyi Biotec MACS technology separates different cell populations based on the expression of specific surface antigens on these cells using microbeads. MACS microbeads are super-paramagnetic particles that are coupled to monoclonal antibodies. CD14^+ purified monocytes were prepared according to the manufacturer’s instructions, as described below.

PBMC pellet was re-suspended in D-PBE buffer (See table 2.13), using 80 µl per 10^7 cells. Microbeads conjugated to monoclonal anti-human CD14 antibody (See table 2.14) were added (20 µl per 10^7 cells) followed by 15 minutes incubation at 4°C. After
incubation, cells were washed by adding 1.5 ml of D-PBE buffer per $10^7$ cells and spun down for 5 minutes at 300g. The cell pellet was re-suspended in 500 µl of D-PBE buffer and then loaded into a MACS MS column, located in the magnetic field of the VarioMACS separator (See table 2.8). The MS column was washed twice with 500 µl of D-PBE buffer, which was allowed to run through into a discard pot. Cells pass through the column slowly so that CD14 positive cells bound to the magnetic beads attach to the column. The column was then washed twice with D-PBE to ensure that there were no CD14 negative cells trapped within the column. To retrieve the CD14 positive cells (monocytes) from the column, the MS column was removed from the VarioMACS unit and placed over a 15 ml sterile centrifuge tube. The CD14 expressing monocyte/microbeads complex was eluted by adding 1 ml of D-PBE to the column and forcefully applying a plunger. The collected CD14$^+$ purified monocytes and CD14$^-$ cells (mainly lymphocytes) were washed once with 1 ml of complete Iscove’s medium and spun down at 300 g for 5 minutes at RT. Cells then were re-suspended in 1 ml of complete Iscove’s medium and counted (See section 3.1.2). The CD14$^+$ purified monocytes and CD14$^-$ cells were seeded in 2ml complete Iscove’s medium at a density of $1\times10^6$ cells per well in 6-well adherent Nunclon plates and incubated at $37^\circ$C with 5% CO$_2$ and 20.9% O$_2$ for 5 days.

3.1.5 Culture of U937

U937 cells are a non-adherent human monocytic cell line derived from a histiocytic lymphoma. U937 cells were cultured in 75cm$^2$ tissue culture flasks using filtered RPMI 1640 medium containing 10% FCS, 1% L-glutamine, 1% penicillin and streptomycin and 1% non-essential amino-acid (See table 2.1 and 2.9). Cells were split once every 3 days
Methods

to keep the cells healthy; an aliquot of cells was diluted 1:1 with 1% filtered Trypan blue and then counted as described in section 3.1.2. Trypan blue stains the cytoplasm of apoptotic and dead cells blue but it is unable to penetrate the cell membrane of living cells. After determining the cell concentration, cells were diluted to $2.0 \times 10^5$ cells/ml and 10 ml transferred to a 75cm$^2$ flask. The flask was labelled with the date split and the cell concentration and returned to incubation at 37°C with 5% CO$_2$ and 20.9% O$_2$ (See table 2.1 and 2.8 for the reagents and materials).

**Note:** RPMI 1640 medium containing 10% FCS, 1% L-glutamine, 1% non-essential amino-acids and 1% penicillin/streptomycin has been described as complete RPMI1640 medium in this project. Complete RPMI1640 medium was used at RT.

3.2 Cell treatment

3.2.1 Normoxic & Hypoxic Incubation

Depending on the experiment, cells were either cultured under normal oxygen concentrations (normoxia) in a humidified atmosphere of 95% air (20.9% O$_2$), 5% CO$_2$, or under hypoxia in a humidified multi-gas oxygen control Galaxy R incubator at 0.2% O$_2$, 5% CO$_2$, 94.8% N$_2$ (See table 2.15). Oxygen levels indicated on the incubator display screens were verified using a separate oxygen analyser (Analox). Cells were incubated under normoxia or hypoxia for 18 hours, 24 hours or 5 days depending on the experiment.
3. Methods

3.2.2 Lipopolysaccharide (LPS) stimulation of adherence-purified HMDM
The LPS used for cell stimulation was commercially isolated and purified from *Salmonella abortus equii* (SAE) or *Salmonella minnesota* (SMI) (See table 2.1), and were diluted with RPMI 1640 medium to make 10 ng/µl working stock solutions. After 5 days incubation under normal oxygen tensions, $2 \times 10^6$ adherent HMDM were treated with either SAE or SMI LPS at final a concentration of 100 ng/ml and incubated under normoxia or hypoxia for a further 18 hours (See section 3.2.1) prior to RNA isolation (See section 3.4).

3.2.3 Cobalt chloride (CoCl$_2$) stimulation of adherence-purified HMDM
CoCl$_2$ is one the common used agent which stabilises HIF-1 (Maxwell et al. 1999). The manufacturer’s CoCl$_2$ (See table 2.1) was dissolved by dH$_2$O to prepare laboratory stock solution with a concentration of 30 mM. CoCl$_2$ was added to $2 \times 10^6$ adherent HMDM at final concentration of 300 µM, for 18 hours in normoxia, prior to RNA isolation (See section 3.4).

3.2.4 Desferrioxamine (DFO) stimulation of adherence-purified HMDM
DFO is an iron chelating agent which primarily uses for HIF-1 stabilisation (Maxwell et al., 1999). The manufacturer’s DFO (See table 2.1) was dissolved by dH$_2$O to prepare laboratory stock solution with a concentration of 20 mM. DFO was added to $2 \times 10^6$ adherent HMDM at final concentration of 200 µM, for 18 hours in normoxia, prior to RNA isolation (See section 3.4).
3.2.5 Ferric citrate stimulation of adherence-purified HMDM

The manufacturer’s ferric citrate (See table 2.1) was dissolved by dH₂O to prepare laboratory stock solution with a concentration of 20 mM. For assessment of iron chelating activity of the DFO and its effect on versican gene expression, cells were treated simultaneously with 200 µM DFO and ferric citrate at different concentrations (e.g. 100 µM, 200 µM, 400 µM) to saturate the agent and reverse its activity.

3.2.6 Actinomycin D treatment of adherence-purified HMDM

Actinomycin D is an antineoplastic antibiotic that inhibits transcription by binding DNA at the transcription initiation complex and preventing elongation by RNA polymerase (Sobell, 1985). The manufacturer’s actinomycin D (See table 2.1) was dissolved by sterile DMSO to generate a laboratory stock with a concentration of 1 mg/ml. For mRNA half-life analysis, actinomycin D at a final concentration of 10 µg/ml was added to 2 × 10⁶ adherent HMDM after incubation for 5 days in normoxia. Cells were incubated under either normoxia or hypoxia (See section 3.2.1) for a further 2 hours, 8 hours, 12 hours, 24 hours and 36 hours prior to RNA isolation (See section 3.4).

3.2.7 PI3-Kinase inhibitor treatment of adherence-purified HMDM

LY294002 and wortmannin are shown to act in vivo as highly selective inhibitors of PI3-Kinase. The manufacturer’s LY294002 and wortmannin (See table 2.1) were dissolved by sterile DMSO to generate laboratory stock solutions with a concentration of 10 mM and 0.2 mM respectively. After 5 days incubation under normal oxygen tensions, 2 × 10⁶ adherent HMDM were treated with LY290042 at final concentrations of 2 µM and 5 µM for 18 hours in normoxia and hypoxia (See section 3.2.1), prior to RNA isolation.
(See section 3.4). Wortmannin at a final concentration of 200 nM was added to $2 \times 10^6$ adherent HMDM after incubation for 5 days in normoxia. Dilutions were prepared such that all wells received 2 µl of DMSO/inhibitor solution. The same volume of DMSO (2 µl) was added to cells as a carrier control. Cells then were incubated under normoxia or hypoxia (See section 3.2.1) for a further 18 hours prior to RNA isolation (See section 3.4).

3.3 General molecular cloning

3.3.1 Amplification of the versican promoter by polymerase chain reaction

Polymerase chain reaction (PCR) is a powerful method developed by Kary Mullis in the 1980s and occurs in three stages; denaturation of the template DNA, primer annealing, and primer extension. PCR is based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand by using two primers which are short pieces (usually around 20 base pairs) of synthetic DNA. Amplification of the targeting sequence on the template is achieved by cycles of reaction that elongate the primers according to the targeting sequence by DNA polymerase. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify.

Forward and reverse primers which were used to amplify regions of the versican promoter for PCR cloning are shown in table 2.12. Below is the reaction mix and PCR set up to amplify different regions of the versican promoter using a thermocycler (See table 2.3 and 2.13 for reagents and materials).
3. Methods

**Note:** in order to generate a 29-bp of random negative control, a reaction mix including 1 µl of 10x buffer with MgSO₄ (1x), 1 µl of forward (2.5 ng/µl) and 1 µl of reverse primers (2.5 ng/µl) were made up to 10 µl with Sterile dH₂O. The reaction then was placed in a thermocycler (PCR machine) for 1 minute at 95°C to anneal the primers. All PCR reaction mixes were prepared on ice.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>REDAccuTaq® LA 10x Buffer</td>
<td>5 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
<td>2.5 µl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Human genomic DNA (50pg/µl)</td>
<td>1 µl</td>
<td>50 pg</td>
</tr>
<tr>
<td>Forward primer (20mM)</td>
<td>1 µl</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Reverse primer (20mM)</td>
<td>1 µl</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>37 µl</td>
<td>-</td>
</tr>
<tr>
<td>REDAccuTaq® LA DNA Polymerase</td>
<td>2.5 µl</td>
<td>0.05 U/µl</td>
</tr>
<tr>
<td>Final Volume</td>
<td>50 µl</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3.1:** PCR reaction mix for 4.140-Kb (-3.956Kb/+184bp), 830-bp (-646bp/+184bp), 480-bp (-296bp/+184bp) and 240-bp (-56bp/+184bp) of versican promoter construct
3. Methods

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Pfu buffer with MgSO₄</td>
<td>5 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>dNTP (10 mM)</td>
<td>1 µl</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Genomic DNA (50 pg/µl)</td>
<td>1 µl</td>
<td>50 pg</td>
</tr>
<tr>
<td>Forward primer (20 mM)</td>
<td>1 µl</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Reverse primer (20 mM)</td>
<td>1 µl</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>50.5 µl</td>
<td>-</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>0.5 µl</td>
<td>0.025 U/µl</td>
</tr>
<tr>
<td>Final Volume</td>
<td>50 µl</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3.2:** PCR reaction mix for 210-bp (-56bp/+154bp), 160-bp (-56bp/+104bp) and 110-bp (-56bp/54bp), 210-bp (-26bp/+184bp), 180-bp (-26bp/+154bp), 130-bp (-26bp/+104bp), 80-bp (-26bp/+54bp) constructs of versican promoter

<table>
<thead>
<tr>
<th>Segment</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>96°C</td>
<td>30 sec</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>15 sec</td>
<td>40</td>
</tr>
<tr>
<td>Annealing</td>
<td>68°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>68°C</td>
<td>15 min</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>68°C</td>
<td>20 min</td>
<td>1</td>
</tr>
<tr>
<td>Final Extension</td>
<td>4°C</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3.3:** PCR set-up for amplification of 4.140-Kb (-3.956Kb/+184bp), 830-bp (-626bp/+184bp), 480-bp (-298bp/+184bp) and 240-bp (-56bp/+184bp) of versican promoter

**Note:** Annealing temperature and Extension time used for PCR amplification of 4.140-Kb was 66°C and 5 min respectively.
Table 3.4: PCR set-up for amplification of 210-bp (-56bp/+154bp), 160-bp (-56bp/+104bp) and 110-bp (-56bp/+54bp) 210-bp (-26bp/+184bp), 180-bp (-26bp/+154bp), 130-bp (-26bp/+104bp), 80-bp (-26bp/+54bp) constructs of versican promoter

3.3.2 Agarose gel electrophoresis

Electrophoresis is used to separate DNA fragments by applying an electric field to a gel. DNA is negatively charged, independent of the pH of the solution, 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 pore size of the gel, the applied voltage, and conformation and length of the DNA molecules. In general bigger fragments migrate slower through the gel.

The sizes of PCR amplified fragments of the versican promoter were confirmed by running on an agarose gel and compared with a DNA marker (See table 2.3 and 2.13 for all the reagents and buffers). For agarose gel electrophoresis, a solution of agarose in 100ml of 1x TBE containing 0.25 µg/ml ethidium bromide (See table 2.13 and 3.5)
was heated in a microwave oven until the agarose was dissolved. The solution was left to cool for a few minutes and then poured into a gel tray with a 1.0 mm comb in it and let the gel set. Ethidium bromide (EtBr) is a fluorescent dye which intercalates between stacked bases of double stranded DNA and allows visualisation of nucleic acids 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 the electrophoresis tank which was filled with sufficient 1x TBE buffer (containing EtBr) to cover the gel (See table 2.13). The DNA samples of amplified fragments of versican promoter were diluted with 1:6 with 6x gel loading buffer and slowly loaded into the complete wells. The size of a fragment is estimated by comparison with a DNA molecular weight marker which contains fragment of known length. 250 ng of DNA molecular weight marker was loaded at the same time as the samples into a different well. A voltage of 100 V was applied for 2 hours. The gel run was terminated when the bromophenol blue migrated about 3/4 of the length of the gel. The gel was then examined under UV light and photographed.
### Table 3.5: Electrophoretic separation of PCR amplified fragments of the versican promoter

<table>
<thead>
<tr>
<th>Amplified fragment of versican promoter</th>
<th>Agarose concentration (%w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.140-Kb (-3.956Kb/+184bp)</td>
<td>0.6</td>
</tr>
<tr>
<td>830-bp (-646bp/+184bp)</td>
<td>1.5</td>
</tr>
<tr>
<td>480-bp (-296bp/+184bp)</td>
<td>1.5</td>
</tr>
<tr>
<td>240-bp (-56bp/+184bp)</td>
<td>1.5</td>
</tr>
<tr>
<td>210-bp (-56bp/+154bp)</td>
<td>1.5</td>
</tr>
<tr>
<td>160-bp (-56bp/+104bp)</td>
<td>2.0</td>
</tr>
<tr>
<td>110-bp (-56bp/+54bp)</td>
<td>2.0</td>
</tr>
<tr>
<td>210-bp (-26bp/+184bp)</td>
<td>1.5</td>
</tr>
<tr>
<td>180-bp (-26bp/+154bp)</td>
<td>2.0</td>
</tr>
<tr>
<td>130-bp (-26bp/+104bp)</td>
<td>2.0</td>
</tr>
<tr>
<td>80-bp (-26bp/+54bp)</td>
<td>2.0</td>
</tr>
</tbody>
</table>
3.3.3 Restriction digestion

Restriction endonucleases can be used for cleaving double-stranded DNA molecules at specific positions which are called restriction sites. The cleavage site is located within the recognition sequence and depending on the enzyme, DNA fragments with blunt ends or cohesive (sticky) ends are generated (See table 2.3).

In this project, for versican promoter cloning, the SfiI restriction site was chosen, which is cleaved by the SfiI restriction enzyme isolated from an E.coli strain that carries the SfiI gene from Streptomyces fimbriatus. The SfiI endonuclease cleaves DNA at the sequence 5’-GGCCNNNN↓NGGCC-3’ (where N is any base and ↓ is the point of cleavage) and creates sticky ends. The SfiI restriction enzyme was used for the digestion of PCR amplified fragments of the versican promoter which carry SfiI restriction sites and also the pGL4.10 luciferase reporter plasmid (See section 2.7.1). Restriction digestion was carried according to the manufacturer’s instructions (See table 3.6 and 3.7). The reaction was incubated at 50°C for 2 hours to allow digestion. After 2 hours, digested DNAs were separated on agarose gels as described in section 3.3.2.

**Note:** Concentrations of the amplified versican promoter PCR products were determined by comparing to known concentrations of DNA molecular weight marker separated on agarose gels (See section 3.3.2).
3. **Methods**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SfiI Restriction Enzyme (20 U/µl)</td>
<td>1 µl</td>
<td>1 U/µl</td>
</tr>
<tr>
<td>DNA</td>
<td>11 µl</td>
<td>1-5 µg/20 µl</td>
</tr>
<tr>
<td>10x SfiI Buffer</td>
<td>2.0 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>10x BSA</td>
<td>2.0 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>4.0 µl</td>
<td>-</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td>20 µl</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3.6:** *SfiI* digestion reaction mixture for PCR amplified fragments of versican promoter

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SfiI Restriction Enzyme (20 U/µl)</td>
<td>1 µl</td>
<td>0.8 U/µl</td>
</tr>
<tr>
<td>pGL4.10 vector (1 µg/µl)</td>
<td>3 µl</td>
<td>12 ng/25 µl</td>
</tr>
<tr>
<td>10x SfiI Buffer</td>
<td>2.5 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>10x BSA</td>
<td>2.5 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>16 µl</td>
<td>-</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td>25 µl</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3.7:** *SfiI* digestion reaction mixture for pGL4.10 luciferase reporter plasmid
3.3.4 QIAquick DNA Agarose Gel Extraction

The digested pGL4.10 vector and PCR amplified fragments of versican promoter sequences were extracted and purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s instructions (See table 2.7).

First, 3 volume of QG Buffer was added to 1 volume of extracted gel slice (e.g. 300 µl of QG Buffer to 100 mg of extracted gel) in a 1.5 ml sterile centrifuge tube and then incubated at 50°C for 10 minutes. When the gel slice was dissolved 1 volume of isopropanol was added to 1 volume of extracted gel slice (e.g. 100 µl of isopropanol to 100 mg of extracted gel) to increase the yield of DNA fragments. Then the resulting mixture was transferred to a QIAquick spin column and centrifuged at 13000 g for 1 minute to bind DNA to the column. 750 µl of PE Buffer containing ethanol was then added to the column and spun down at 13000 g for 1 minute to wash the DNA. Then, the column was placed over a new sterile 1.5 ml centrifuge tube and finally, to elute DNA, 30 µl of EB Buffer was added to the centre of column and after 1 min in RT it was centrifuged at 13000 g for 1 minute. The purified DNA fragments were analysed by agarose gel electrophoresis (See section 3.3.2).

3.3.5 Ligation of 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 involves creating 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 which is the most commonly used DNA
ligase to ligate sticky or blunt ends. Typically, insert and plasmid vector DNA are individually cut to yield complementary ends, then both are added to a ligation reaction to be circularised by DNA ligase (See table 2.3 and 2.8 for the reagents and materials).

2.5-Kb of versican promoter cloned into pGL3-basic luciferase reporter plasmid was kindly provided by Dr Burke. In this project the digested pGL4.10 plasmid vector and digested fragments of versican promoter sequence were ligated at X:Y ratio according to the manufacturer’s instructions (See table 3.9, Fig 2.1 and 2.2 for the cloning region of the pGL4.10 vector) where X is the amount of vector plasmid and Y the amount of insert DNA (See table 3.8). To prepare the ligation mixture, first PGL4.10 plasmid vector, versican DNA fragments and sterile dH₂O were transferred into a 0.5 ml sterile centrifuge tube and incubated in the Thermocycler (PCR machine) at 42°C for 2 minutes and then let it to cool down in RT. The rest of the reagents were then added and incubated at 4°C for 16 hours to complete ligation and followed by storage at -20°C (See table 3.9).

Note: Concentrations of the digested fragment of versican promoter products and digested pGL4.10 plasmid vector were estimated by comparing to known concentrations of DNA molecular weight marker separated on agarose gels (See section 3.3.2).
3. Methods

**Table 3.8:** Different ratios of vector DNA (pGL4.10 vector): insert DNA (digested versican promoter product). pGL4.10 vector was used at 25 ng/µl, 50 ng/µl and 14 ng/µl.

<table>
<thead>
<tr>
<th>Digested fragment of versican promoter</th>
<th>pGL4 vector X</th>
<th>Insert DNA Y</th>
<th>pGL4 vector (ng)</th>
<th>Insert DNA (ng)</th>
<th>pGL4 vector (µl)</th>
<th>Insert DNA (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.140-kb (-3.956kb/+184bp) (30ng/µl)</td>
<td>1</td>
<td>2</td>
<td>100</td>
<td>200</td>
<td>4</td>
<td>6.6</td>
</tr>
<tr>
<td>830-bp (-646bp/+184bp) (3ng/µl)</td>
<td>1</td>
<td>3</td>
<td>50</td>
<td>30</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>480-bp (-296bp/+184bp) (18ng/µl)</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>2</td>
<td>5.5</td>
</tr>
<tr>
<td>240-bp (-56bp/+184bp) (5ng/µl)</td>
<td>1</td>
<td>20</td>
<td>50</td>
<td>50</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>210-bp (-56bp/+154bp) (3ng/µl)</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>25</td>
<td>3.5</td>
<td>8.3</td>
</tr>
<tr>
<td>160-bp (-56bp/+104bp) (8ng/µl)</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>12.5</td>
<td>3.5</td>
<td>1.5</td>
</tr>
<tr>
<td>110-bp (-56bp/+54bp) (4ng/µl)</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>12.5</td>
<td>3.5</td>
<td>3.1</td>
</tr>
<tr>
<td>210-bp (-26bp/+184bp) (8ng/µl)</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>25</td>
<td>3.5</td>
<td>3.1</td>
</tr>
<tr>
<td>180-bp (-26bp/+154bp) (8ng/µl)</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>20</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>130-bp (-26bp/+104bp) (4ng/µl)</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>12.5</td>
<td>3.5</td>
<td>3.1</td>
</tr>
<tr>
<td>80-bp (-26bp/+54bp) (2ng/µl)</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td>3.5</td>
<td>5</td>
</tr>
</tbody>
</table>
### Table 3.9: Reaction mixture for ligation of the digested versican promoter sequences into the pGL4.10 vector

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested pGL4.10</td>
<td>See table above</td>
<td>2.5-5ng</td>
</tr>
<tr>
<td>Digested versican promoter</td>
<td>See table above</td>
<td>0.5-10ng</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>Variable</td>
<td>-</td>
</tr>
</tbody>
</table>

**Note:** The pGL4.10 digested plasmid and 29-bp random sequence were ligated at 1:10 ratio where 1 is the amount of vector plasmid and 10 the amount of insert DNA. The ligation mixture included 2 µl of T4 DNA ligase (0.4 U/ µl, Stratagene), 2 µl of 10x Buffer (1x), 2µl of ATP (1mM), 4 µl of pGL4.10 vector plasmid, 2 µl of 29-bp random sequence (0.5 ng/µl) and were made up to 20 µl with sterile dH₂O. The water, plasmid and versican promoter DNAs were first incubated at 42°C for 2 minutes and were let to cool down. The rest of the reagents were then added and left at 4°C for 16 hours followed by storage at -20°C.
3.3.6 Transformation of plasmid-DNA into DH5-α competent cells

Bacterial transformation is a technique to introduce a foreign plasmid into bacteria and to use those bacteria to amplify the plasmid in order to make large quantities of it. This is based on the natural function of a plasmid.

For this project, Library Efficiency DH5-α Competent Cells were used as these cells are suitable for cloning experiments in which limiting amount of plasmid DNA will be used (See table 2.7 and 2.8 for the reagents and materials). For the transformation, first the ligation product (pGL4.10 plasmid-versican promoter sequence) was diluted 1:5 with TE buffer (10mM Tris-HCl [pH7.5] and 1mM EDTA). A 200 µl of aliquot of DH5-α competent cells was thawed on ice and 100 µl of cells were transferred into 14 ml Falcon tubes. 1 µL (1-10 ng) of the diluted ligation product and 5 µl (50 pg) of the control pUC19 DNA were added into each tube and gently shaken to mix. The bacteria were incubated on ice for 30 minutes, then heat-shocked at 42°C in a water bath for 45 seconds followed by 2 minutes incubation on ice. Then, 900 µl of room temperature S.O.C medium was added to each tube, and the competent cells were incubated in 37°C with shaking at 225 rpm for 1 hour. 100 µl, 300 µl, and 500 µl of competent cells were plated out onto LB-agar plates containing 100 µg/ml ampicillin for selection (See table 2.10) and the plates incubated overnight at 37°C.
Transformation efficiency of competent cells was determined by transforming 50 pg of the control pUC19 DNA into competent cells, with 100 µl of cells being plated out;

\[
\text{Transformation efficiency (CFU/µg)} = \frac{\text{NO. of colonies} \times 10^6}{\text{Used pUC19 DNA in pg}}
\]

- Factor 10: 100 µl transformed bacteria streaked onto LB-agar
- Factor \(10^6\): conversion factor of pg to µg

### 3.3.7 Purification of plasmid-DNA

Plasmid DNA was prepared using the Qiagen EndoFree™ Plasmid Maxi Kit according to the manufacturer’s instructions. The plasmid purification protocol is based on a modified alkaline lysis procedure. Insoluble complexes containing chromosomal DNA, salt, detergent and proteins are removed by passing the lysate through a filter. The filtered lysate is incubated on ice with a specific removal buffer which prevents the binding of LPS molecules to an anion-exchange column in the subsequent step allowing purification of DNA containing less than 0.1 endotoxin units per µg of DNA. Plasmid DNA is bound to an anion-exchange column under low salt and pH conditions and RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid-DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. A final wash step with 70% ethanol
removes residual salt. The purified DNA is briefly air-dried and dissolved in a small volume of TE buffer (See table 2.7 and 2.8 for the reagents and materials).

The procedure is as follows. A starter culture of 3 ml LB medium containing 100 µg/ml ampicillin (See table 2.10) was inoculated with a single bacteria colony picked from a selective plate and incubated -8 hours at 37°C shaking at 224 rpm. The starter culture was diluted 1:1000 into 100 ml of selective LB medium containing 100 µg/ml ampicillin and grown 12-16 hours at 37°C shaking at 224 rpm. 50 ml of over-night grown bacterial culture were centrifuged at 6000 g for 30 minutes at 4°C. The bacteria pellet were lysed by re-suspending in 10 ml of buffer P1 followed by adding 10 ml of buffer P2. The buffer and bacteria were mixed gently by inverting the tube 5 times and then incubated 5 minutes at RT. 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 buffer P3 was added to the lysate and was mixed gently by inverting the tube 5 times followed by transferring lysate into the QIAfilter cartridge and incubated in RT for 10 minutes. After incubation, the QIAfilter cartridge was placed over a sterile 50 ml centrifuge tube and the cap was removed and then the lysate was filtered into the tube by applying a plunger. The filtered lysate was mixed gently with 2.5 ml of buffer ER and incubated on ice for 30 minutes. 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 sterile 50 ml centrifuge tube. Addition of 10.5 ml isopropanol and centrifugation at 15000 g for 30
3. Methods

hours at 4°C precipitated the DNA. The supernatant was decanted and the DNA pellet was washed with 5 ml of RT endotoxin-free 70% ethanol and centrifuged at 10000 g for 10 minutes at 4°C. The DNA-pellet was air dried for 10-15 minutes and re-suspended in 300-450 µl endotoxin-free buffer TE and stored at -20°C.

A small volume of purified plasmid DNA was used to determine DNA concentration (See section 3.3.8). DNA sequencing to confirm the sequence of cloned fragments was done by the Protein Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester.

3.3.8 Quantification of nucleic acids

Absorption spectroscopy is a non-destructive method of measuring the concentration of deoxyribonucleic acid (DNA). Nucleic acids absorb light maximally at a wavelength of 260 nm, and this physical property can be used as a basis to determine the concentration of the nucleic acids in a given solution. DNA samples are sometimes contaminated with trace of protein which has a maximum absorption at 280 nm. So the purity of the nucleic acids can be estimated by calculating the ratio of readings taking at 260 nm and 280 nm (OD_{260}/OD_{280}). Pure preparations of DNA have OD_{260}/OD_{280} values of ~1.8 whereas contamination with protein results in lower values.

The measurement of purified DNA (pGL4.10 plasmid-versican promoter) was carried out in a special quartz cuvette since glass or plastic cuvettes show a high self-absorption at 260 nm. In general, to save the samples, they were diluted 1:100 with TE
buffer. Then the DNA concentration was determined by spectrophotometer at OD$_{260}$ and calculated using the following formula:

\[
\text{Concentration of the nucleic acid (µg/ml) = OD}_{260} \times \varepsilon \times d
\]

- $\varepsilon$: extinction coefficient (50 µg/ml for dsDNA)
- $d$: dilution factor

### 3.4 Quantification of mRNA

#### 3.4.1 Isolation of total RNA

Total RNA was isolated from either normoxic or hypoxic adherent HMDM (See section 3.1.3) and CD14$^+$ purified monocytes and CD14$^-$ (lymphocyte) (See section 3.1.4) using TRI reagent lysis and isopropanol precipitation according to the manufacturer’s instructions as described below (Staples et al., 2000 & 2007 & 2011) (See table 2.2 and 2.8 for the reagents and materials).

Cells were lysed in 500 µl TRI reagent. The lysate, containing RNA, DNA and protein was transferred into a new autoclaved 1.5 ml centrifuge tube and allowed to stand for 5 minutes at RT to permit complete dissociation of nucleoprotein complexes. At this stage the lysate was either stored at -80°C or used for mRNA isolation. 100 µl of chloroform was added to the lysate and vortexed vigorously for 15 seconds to form a white emulsion which was allowed to stand for 10 minutes at RT. The resulting mixture
was centrifuged at 12000 g for 15 minutes at 4°C. This separates the mixture into 3 phases; a colourless upper aqueous phase containing RNA, an interphase containing DNA and a red organic phase containing protein. The top aqueous layer was transferred into a new autoclaved 1.5 ml centrifuge tube and 500 µl of isopropanol was added for RNA precipitation. The sample was incubated at RT for 10 minutes and then centrifuged at 12000 g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% Ethanol and spun down at 12000 g for 5 minutes at 4°C. The supernatant was discarded and the pellet air-dried for 10 minutes at RT under a laboratory fume hood. The RNA pellet was re-suspended in 20 µl of DEPC-dH₂O and either immediately reverse transcribed (See section 3.4.2) or stored at -80°C.

3.4.2 Reverse transcription

In an enzymatic reaction, isolated total RNA is reverse transcribed into cDNA. For this reaction, 9 µl of total RNA (See section 3.4.1) was transferred into an autoclaved 0.5 ml centrifuge tube and incubated in a thermocycler (PCR machine) for 5 minutes at 70°C to denature RNA secondary structure. The sample was cooled to 42°C and then 11 µl of reaction mix (See table 3.10) was added and the reaction was incubated at 42°C for 1 hour to allow reverse transcription of RNA into cDNA. The reaction was terminated by heating to 90°C for 4 minutes to denature the reverse transcriptase enzyme. The cDNA was stored at -20°C or used immediately for Real-Time- PCR (See table 2.2, 2.8 and 2.13 for the reagents and materials).
Table 3.10: Reaction mix for reverse transcription. Reagents and reaction mix were kept on ice.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanucleotides (0.2 µg/µl)</td>
<td>1 µl</td>
<td>10 ng/µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>2 µl</td>
<td>1 mM</td>
</tr>
<tr>
<td>DEPC-dH₂O</td>
<td>2.4 µl</td>
<td>-</td>
</tr>
<tr>
<td>5x AMV Buffer</td>
<td>4.0 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>AMV Reverse Transcriptase (10 U/µl)</td>
<td>0.8 µl</td>
<td>0.4 U/µl</td>
</tr>
<tr>
<td>RNasin® Ribonuclease Inhibitor (40 U/µl)</td>
<td>0.8 µl</td>
<td>1.6 U/µl</td>
</tr>
<tr>
<td>RNA</td>
<td>9 µl</td>
<td>-</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
<td>-</td>
</tr>
</tbody>
</table>

3.4.3 Real-Time PCR

Semi-quantitative PCR was performed using a Roche LightCycler, an integrated thermal cycler and fluorimeter that amplifies the target nucleic acid and monitors the development of the amplification products via measurement of fluorescence. Amplification of target DNA is carried out in the presence of the fluorescent dye SYBR Green which binds to the minor groove of the DNA double helix. The fluorescent intensity is directly proportional to the amount of the amplified product. Subsequent to an amplification run, a melting curve analysis of the PCR product is performed to allow discrimination between specific products and non-specific products. The melting cycle comprises three steps; rapid heating to 94°C to denature all DNA, cooling the
reaction below the annealing temperature of the DNA fragments, and finally slowly heating to monitor the double-stranded DNA as it melts at a characteristic temperature.

For each PCR assay, four 1:5 serially diluted standard samples from a pool of the cDNAs were prepared to be tested. These four standard PCRs were plotted by LightCycler software to calculate the relative concentration of each tested cDNA. Then 3 µl of samples (standard samples and tested cDNA) were transferred into a Roche LightCycler capillary and 17 µl of PCR master mix (See table 3.11) was added to each reaction capillary. The PCR master mix contains SYBR Green JumpStart with Taq DNA polymerase which is inactive at RT, due to the presence of heat-labile blocking groups on some of the amino acid residues of the enzyme and subsequently minimizes non-specific amplification products as no elongation takes place during the period when primers can bind non-specifically. The polymerase is activated by removing the blocking groups at high temperatures in the initial denaturation step of the Real-Time PCR. One extra capillary was prepared using 3 µl of autoclaved dH₂O with 17 µl of PCR master mixture as a negative control to confirm that the PCR master mix is not contaminated with DNA. Then all the capillaries were capped and spun down at 400 g for 10 seconds at 4°C. LightCycler capillaries were transferred to a Roche PCR LightCycler real-time PCR instrument for amplification (See table 2.2, 2.8, 2.11 and 2.15 for the materials, reagents and equipment). The reaction mix and set up was prepared according to the following tables;
3. Methods

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x SYBR® Green JumpStart™ Taq ReadyMix</td>
<td>10 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>5’ Primer (5 µM)</td>
<td>2 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>3’ Primer (5 µM)</td>
<td>2 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>3 µl</td>
<td>-</td>
</tr>
<tr>
<td>Total volume</td>
<td>17 µl</td>
<td>-</td>
</tr>
<tr>
<td>DNA template</td>
<td>3 µl</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3.11:** Reaction master mix for Real-Time PCR. Primers have been listed in table 2.4.1

<table>
<thead>
<tr>
<th>Segment</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>96°C</td>
<td>10 sec</td>
<td>45</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>25 sec</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.12:** PCR cycle used for amplification and detection mRNA of β2-M, Versican, VEGF and GLUT-1 genes
3.4.4 Quantification of mRNA

First Real-Time PCR was performed using primers for the housekeeping gene β-2 microglobulin (β-2M) which is present in all nucleated cells (Gussow et al., 1987; Lee et al., 2007). Different studies have shown that β-2M expression does not alter under low oxygen conditions (Jogi et al., 2002; Foldager et al., 2009; Watson et al., 2009) and previously has been used as an internal control in 0.2% O$_2$ (Staples et al., 2011). Then the amount of cDNA of interest was determined in a second Real-Time PCR using versican primers. In each run, four internal standards were used consisting of serial 1:5 dilutions of a cDNA pool containing cDNA from each sample (See section 3.4.3). These four standard samples were plotted by LightCycler software to calculate the relative concentration of each cDNA of interest. Then each sample was normalised by dividing the calculated cDNA value of gene of interest by the calculated amount of β-2M of the same sample. The following formulas were used to calculate the relative level and hypoxic induction of the gene of interest (versican in this example):

\[
\frac{\text{Versican calculated value for normoxic cDNA}}{\text{β-2M calculated value for normoxic cDNA}} = \frac{\text{Relative versican abundance in normoxia}}{\text{Relative versican abundance in hypoxia}}
\]

\[
\frac{\text{Versican calculated value for hypoxia cDNA}}{\text{β-2M calculated value for hypoxia cDNA}} = \text{Relative versican abundance in hypoxia}
\]

\[
\frac{\text{Relative versican abundance in hypoxia}}{\text{Relative versican abundance in normoxia}} = \text{Versican hypoxic fold induction}
\]
3. Methods

3.5 Transfection

3.5.1 JetPEI™ DNA transfection reagent

PBMCs were transfected using JetPEI™ transfection reagent to assess the hypoxic inducibility of versican promoter constructs. JetPEI™ is a powerful and useful delivery vehicle and ensures effective and reproducible DNA and oligonucleotide transfection into mammalian cells with low toxicity. JetPEI™ is a linear polyethylenimine (PEI), synthesized and purified by PolyPlus LTD which condenses DNA into positively charged particles. The particles then interact with anionic cell surface residues such as proteoglycans and phospholipids and are taken into the cell via endocytosis. Once inside the lysosomes, PEI works as a proton sponge, resulting in an influx of counterions by accepting protons leading to endosomal pH increasing. Raising endosomal pH could alter protein folding and inactivate endosomal enzymes results in protection of DNA from degradation. This mechanism also leads to endosome swelling and rupture, permitting the release of the JetPEI/DNA complexes into the cytoplasm, thereby allowing nuclear transport for subsequent transcription.

The overall charge of the JetPEI™/DNA complexes is therefore crucial for efficient transfection. It is determined by the DNA to reagent ratio. This ratio, which represents the ionic balance within the complexes, is defined as the N/P ratio which refers to the number of nitrogen residues (N) in the JetPEI™ per phosphate (P) of DNA. To obtain positively charged complexes, an N/P ratio of >3 is required. The following formula, recommended by PolyPlus, was used in order to calculate the N/P ratio which is taking into account the volume of JetPEI™ reagent used for a given amount of DNA;
3. Methods

\[
N/P \text{ ratio} = \frac{7.5 \times \mu{l} \text{ of } \text{jetPEI}^{TM}}{3 \times \mu{g} \text{ of DNA}}
\]

- Factor 7.5: concentration of nitrogen residues in jetPEI™
- Factor 3: 1 µg of oligonucleotide contains 3 nmoles of anionic phosphate

In preliminary investigation to optimise the transfection procedure, HMDM generated from PBMC were also transfected by JetPEI™ (See table 2.7) according to the manufacturer’s instruction. 2 × 10⁶ PBMC cultured in 6-well adherent plate with complete Iscove’s Dulbecco’s medium and after 5 days incubation under normal oxygen condition (See section 3.1.3), HMDM were transfected with DNA-plasmid and aid of JetPEI DNA transfection reagent is described below. In order to normalise the transfection results, HMDM were co-transfected with Renilla plasmid (pRL-TK) which contains Renilla luciferase and serves as an internal control for normalization (See section 2.7.2). Cells were then incubated under normoxia or hypoxia for 24 hours or 5 days (See section 3.2.1) prior to luciferase assay (See section 3.5.3).

In this project, my preliminary experiments established that the best PBMC transfection results were obtained using an N/P ratio of 8 (1 µg of DNA, 3.2 µl of JetPEI™). PBMC were isolated (See section 3.1.1) and seeded in 4ml complete Iscove’s medium at a density of 4×10⁶ per well in 6-Well Costar ultra low attachment plates and incubated at 37°C with 5% CO₂ and 20.9% O₂ for 5 days to allow differentiation of
monocytes to macrophages. On the day of transfection, cells were de-adhered by repetitive pipetting using a 1 ml Gilson and transferred into a sterile 50 ml centrifuge tube. Cells were returned to 37°C prior to transfection. For each transfection (using two wells per transfection) 2 µg of DNA was diluted with 200 µl of 150mM NaCl into a sterile 0.5 ml centrifuge tube. In a new sterile 0.5 ml centrifuge tube, 6.4 µl of JetPEI™ reagent was diluted with 200 µl of 150 mM NaCl. Both tubes were gently mixed and spun down briefly. The 200 µl of diluted JetPEI™ solution were then added all at once to the 200 µl of diluted DNA solution and immediately mixed by vortexing and spun down briefly to bring the drops to the bottom of the tube. The JetPEI™ / DNA mixture was incubated for 30 minutes at RT and then 400 µl of mixture was added to 4 ml of complete Iscove’s medium containing 4×10⁶ PBMC which had been previously de-adhered as described above. Transfected cells were plated in 2 ml at a density of 2×10⁶ per well in 6-Well Nunclon plates (adherent plates) and returned to 37°C with 5% CO² and 20.9% O₂ for 1 hour. Cells were then incubated under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 24 hours or 5 days (See section 3.2.1) prior to luciferase assay (See section 3.5.3).

3.5.2 Reporter gene assay
In order to study the activity of a specific promoter, this promoter is cloned into a vector containing a reporter gene (e.g. luciferase). Transfection of the vector into cells results in expression of luciferase using the host cell’s transcription factors as well as its transcription and translation machinery.
Luciferase catalyses the formation of light from ATP and luciferin (Reaction is shown below). The emitted light intensity is linearly related to the amount of luciferase expressed and is measured using a luminometer (See table 2.15). The measurements are expressed in relative light units (RLU) per second.

\[
\text{ATP} + \text{luciferin} + \text{O}_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PP}_i + \text{CO}_2 + \text{light}\]

**3.5.3 Luciferase assay system**

PBMC were transfected with versican promoter regions which were cloned into the pGL4.10 luciferase reporter vector (See section 3.3 and 3.5.1) and cultured under normal oxygen concentration or hypoxia (See section 3.2.1) prior to luciferase assay. The assay was performed according to the manufacturer’s instructions (See table 2.7 and 2.8 for the reagents and materials).

Medium was first removed from the wells and the cells were washed twice with 1 ml of 1x PBS. To each well 250 µl of 1x Lysis buffer were added. The cell lysates were incubated at -80°C for 10 minutes followed by thawing at RT for 30 minutes. 100 µl of luciferase assay reagent (LAR) was added to a 5 ml polystyrene Falcon tube and then was measured as background in the luminometer (See table 2.15). The luminometer measured the emitted light units for 30 seconds and converted the collected light units into RLU/s. Then 20 µl of the cell lysate were added to the tube containing 100 µl
LAR and mixed gently and measured in the luminometer. All transfection experiments were performed triplicate. Additionally, the protein concentration of each lysate was determined using a Pierce 660 nm Protein Assay to normalize luciferase values (See section 3.5.4).

In addition, a dual luciferase assay system (See table 2.7) was used according to the manufacturer’s instructions to measure the luciferase signals expressed by HMDM which were transfected with DNA-plasmid (e.g. versican promoter pGL4.10 plasmid) and co-transfected with pRL-TK (See section 2.7.2). Medium was first removed from the wells and to each well 250 µl of 1x Lysis buffer were added. The cell lysates were incubated at -80°C for 10 minutes followed by thawing at RT for 30 minutes. The luminometer measured the emitted light units for 30 seconds and converted the collected light units into RLU/s. To measure the firefly luciferase expressed by pGL4.10 plasmid, 20 µl of the RT cell lysate were added to the Falcon tube containing 100 µl luciferase assay reagent II (LAR II) and mixed gently and measured in the luminometer. Then by adding 100 µl of Stop & Glo reagent to the same tube, the firefly luciferase activity is quenched and the Renilla luciferase expressed by pRL-TK, will be activated and measured in the luminometer. For each experiment, 100 µl of LAR II and then 100 µl of Stop & Glo reagent were sequentially added to the 5 ml polystyrene Falcon tube and the RLU quantified, and the values used as firefly and Renilla background values.
3.5.4 Measuring protein concentration

The Thermo Scientific Pierce 660 nm protein assay is a quick, ready-to-use colorimetric method for total protein quantification (See table 2.7). The assay is reproducible, rapid and more linear than coomassie-based Bradford assays and compatible with higher concentrations of most detergents, reducing agents and other commonly used reagents. The Pierce 660nm protein assay is based on the binding of a proprietary dye-metal complex to protein in acidic conditions that causes a shift in the dye's absorption maximum, which is measured at 660nm. The dye-metal complex is reddish-brown and changes to green upon protein binding. The colour change is produced by deprotonation of the dye at low pH facilitated by interactions with positively charged amino acid groups in proteins. Therefore, the dye interacts mainly with basic residues in proteins, such as histidine, arginine and lysine and to a lesser extent tyrosine, tryptophan and phenylalanine.

Protein concentrations are estimated by reference to absorbance obtained for a series of standard protein dilutions assayed alongside the unknown samples. In this project, bovine serum albumin (BSA) was used as standard protein and the assay was performed according to the manufacturer’s instructions (See table 2.7). First a serial dilution of BSA in 1x luciferase Lysis buffer was prepared as follow: 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml and 750 µg/ml (See table 2.13). For the assay, 10 µl of standards, luciferase cell lysate and 1x Lysis buffer as blank were pipetted into a 96-well plate. 150 µl of protein assay reagent was then added to each well and the plate was covered and mixed on a plate shaker at 200 rpm for 1 minute following 5 minutes incubation at RT. After incubation the protein concentration was measured in an ELISA
reader at 660 nm (See table 2.15). To calculate the protein expression, a standard curve was prepared by plotting the average blank-corrected 660 nm measurement for each BSA standard versus its concentration in µg/ml. Then the standard curve was used to determine the protein concentration of each luciferase cell lysate sample.

### 3.6 Nuclear Extraction

The method originally described by Schreiber et al., (1989) was used for nuclear protein isolation, with slight modifications as described below.

The non-adherent monocytic cell line U937 was cultured in 75cm² tissue culture flasks using complete RPMI1640 medium (See section 3.1.5). Cells were counted using Trypan blue (See section 3.1.2) and 5.0 x 10⁵ cells/ml were transferred into 10 ml of fresh complete RPMI1640 medium in the 75cm² flask which was incubated either under normoxia or hypoxia for 24 hours prior to nuclear extraction (See section 3.2.1). In order to inhibit proteolysis, two freshly cocktail of proteases inhibitors; solution 1 from nuclear extract buffer A and solution 2 from nuclear extract buffer B were prepared fresh before starting and kept on ice prior to use (See table 2.13). U937 cells were removed from normoxia and hypoxia incubators and transferred into a sterile 15 ml centrifuge tube followed by 5 minutes centrifugation at 400 g. The supernatant was decanted and cell pellet was washed twice with 10 ml of ice-cold 1x PBS and centrifuged at 400 g for 5 minutes. Then, cell pellet was re-suspended in 1 ml of ice-cold 1x PBS and transferred into a 1.5 ml sterile centrifuge tube followed by 5 minutes centrifugation at 4°C for 400 g. The supernatant was removed and the cell pellet was re-suspended in 4 packed cell volumes of ice-cold solution 1. The pellet was incubated...
on ice for 5 minutes during which the cells swell due to the hypotonic buffer. After ice incubation, Nonidet NP40 (Igepal) was added to a final concentration of 0.6% and the mixture was then vortexed for 10 seconds and centrifuged at 12000 g for 30 seconds at 4°C. The supernatant was removed (cytoplasmic extract) and transferred to a new sterile 1.5 ml centrifuge tube and kept in -80°C. The pellet was washed with 1 ml of 1x PBS and spun down at 12000 g for 30 seconds at 4°C. This stage was applied to make sure there would not be any trace of cytoplasmic proteins in the nuclear extract proteins. Then the supernatant was removed and the pellet was re-suspended in 1 packed volume of ice-cold solution 2 followed by addition of glycerol to a final concentration of 20%. The mixture was incubated at 4°C for 15 minutes on a rotator. After incubation, the mixture was centrifuged at 12000 g for 5 minutes at 4°C and supernatant (nuclear extract) was transferred to a new sterile 1.5 ml centrifuge tube and kept in -80°C and used in western blot analysis (See section 3.7). 2 µl of cytoplasm or nuclear extract proteins were applied to a Nanodrop cuvette-free spectrophotometer to determine the protein concentration of each sample. 2 µl of solution 2 were used as a blank to zero the Nanodrop (See table 2.15).

**Note:** please see table 2.4, 2.8 and 2.13 for the reagents, buffers and materials which were used for nuclear protein extraction
3.7 Western blotting

Western blotting is a technique used to identify and locate proteins based on their ability to bind to specific antibodies. Western blot analysis can detect a protein of interest from a mixture of a great number of proteins. In western blotting, prior to protein immobilization on the PVDF or nitrocellulose membrane, sample proteins are separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) which can give information about the size of the protein with comparison to a size marker or ladder, and also give information on protein expression with comparison to a control such as untreated sample or another cell type or tissue.

The Xcell II Minicell electrophoresis and blotting system was used according to the manufacturer’s instruction (See table 2.15). For electrophoresis, nuclear protein extract samples (90 µg) (See section 3.6) were diluted 1:6 with 6x loading buffer to a maximum of 18 µl (Kept on ice at all times) following 5 minutes incubation at 95ºC in the thermocycler and then loaded to the gel. Samples were then size fractionated on SDS-PAGE gel containing 8% (resolving gel) and 4% polyacrylamide (stacking gel) in 1x western blot running buffer for 60 minutes at 40 mA. Pre-stained high molecular weight protein ladder was also used to assess product size. Following electrophoresis, proteins were electro-botted onto Hybond-P PVDF membrane in western blot transfer buffer for 90 minutes at 20 V.

**Note:** please see table 2.5 and 2.13 for the reagents and which were used for western blotting.
3. Methods

3.7.1 Immunodetection of β-catenin
Membranes were washed three times in 1x TBS/Tween 20 buffer for 5 minutes at RT and then were blocked overnight at 4°C in western blot blocking buffer. Next day, the membranes were washed twice for 10 minutes in 1x TBS/Tween 20 buffer followed by incubation for 1 hour at RT in a 1:2000 dilution of rabbit polyclonal anti-β-Catenin antibody in western blot blocking buffer. The membranes then were washed twice for 20 minutes and twice for 10 minutes in 1x TBS/Tween 20 buffer. Then the membranes were probed with secondary antibody for 1 hour at RT in a 1:4000 dilution of polyclonal anti-rabbit immunoglobulins/HRP in western blot blocking buffer. Again, the membranes were washed twice for 20 minutes and twice for 10 minutes in 1x TBS/Tween 20 buffer and the antibody-labelled proteins were detected using ECL Advance western blotting detection reagents (Amersham) according to the manufacturer’s instructions and visualised on film (See table 2.5).

Note: please see table 2.13 and 2.14 for the buffers and antibodies which used in Immunodetection of hypoxic accumulation of β-catenin

3.7.2 Re-probing the membranes
Membranes were re-probed for β-actin protein as an internal control for western blotting analysis. For re-probing, the membranes were washed three times for 10 minutes in 1x TBS/Tween 20 buffer following incubation overnight at 4°C in western blot blocking buffer. Rabbit polyclonal anti-actin antibody was used at a 1:2000 dilution in western blot blocking buffer for 1 hour at RT. The membranes were probed with secondary antibody for 1 hour at RT in a 1:10000 dilution of polyclonal anti-rabbit
immunoglobulins/HRP in western blot blocking buffer. All the immunodetection procedure was performed as described in section 3.7.1.

**Note:** please see table 2.13 and 2.14 for the buffers and antibodies which used in Immunodetection of β-actin protein.

### 3.8 Flow cytometric analysis of versican protein expression

Flow cytometry is commonly used to identify and quantify cell surface or intracellular markers expressed by cells in mixed cell populations, following staining of cells using fluorescent monoclonal antibodies specific for the target markers. The cells may be alive or fixed at the time of measurement. 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 typically measures several parameters simultaneously for each cell, including: Forward scatter intensity (approximately proportional to cell size), 90 degree or right angle side scatter intensity (approximately proportional to the quantity of granular structures within the cell) and florescence intensities at several wavelengths (allowing the determination of the density of antigen on or in the cell and the number of cells expressing the antigen) which is also known as mean florescence intensity.

In order to detect the expression of versican protein, PBMC were treated with brefeldin A (See table 2.6 and 2.13). Brefeldin A is an inhibitor of intracellular protein transport (Hunziker et al., 1992). Incubation of cells in culture with brefeldin A leads to
blockade of protein transport to the golgi complex (GC) and accumulation of proteins in the endoplasmic reticulum (ER), leading to inhibition of protein secretion. Thus the protein of interest accumulates within the cell (Sciaky et al., 1997; Nebenfuhr et al., 2002).

PBMCs were isolated (See section 3.1.1) and seeded in 2ml complete Iscove’s medium at a cell density of 2×10^6 per well in 6-Well Costar ultra low attachment plates and incubated at 37°C with 5% CO₂ and 20.9% O₂ for 5 days to allow differentiation of monocytes to macrophages (See section 3.1.3). After 5 days, PBMCs were incubated under either normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 4 days (See section 3.2.1). Cells were then treated with 2 μg/ml brefeldin A and incubated under the same oxygen tension for a further 24 hours prior to quantification of versican protein by fluorescence-activated cell sorting (FACS). First cells were harvested and transferred into the 5 ml polystyrene Falcon tube followed by 5 minutes centrifugation at 200 g. Cell pellets were then washed with 1 ml of 1x PBS and centrifuged at 200 g for 5 minutes. Supernatant was removed and cells were fixed using 1% paraformaldehyde (formalin) in 1x PBS/2%FCS followed 20 minutes incubation in RT and then centrifuged at 200g for 5 minutes. Supernatant was discarded and to permit intracellular staining cells were permeabilised with 0.1% saponin in 1x PBS/2%FCS and incubated for 15 minutes at RT. Saponin a detergent-like molecule, has been used to cause permeabilisation by solubilising cell membrane cholesterol, thereby, allowing cytometric analysis of nuclear and other intracellular antigens (Jacob et al., 1991; Krutzik and Nolan, 2003). Cell suspensions were then centrifuged at 200 g for 5 minutes and cell pellets were washed twice with 1 ml of PBS/2%FCS followed by 5
3. Methods

minutes centrifugation at 200 g. Cell pellets were re-suspended in 1 ml of PBS/2%FCS and then equally divided into two 5 ml polystyrene Falcon tubes. Cells were then incubated on ice for 45 minutes in a 1:50 dilution of monoclonal anti-human versican antibody or rat IgG1 isotype control with 0.1% saponin in 1x PBS/2%FCS buffer. Cell suspensions were centrifuged at 200 g for 5 minutes and then washed with 1 ml of PBS/2%FCS followed 5 minutes centrifugation at 200 g. Cell pellets were re-suspended in 500 µl of 0.1% saponin in 1x PBS/2%FCS buffer and incubated with 1:40 diluted anti-rat IgG conjugated to FITC on ice for 45 minutes. Cell suspensions were centrifuged at 200 g for 5 minutes and pellets were washed with 1 ml of PBS/2%FCS followed by 5 minutes centrifugation at 200 g. Cell pellets were then re-suspended in 500 µl of 1x PBS and stored at 4°C in the dark, prior to cell acquisition on a FACSCalibur flow cytometer followed by analysis using CellQuest software (Becton Dickinson). The instrument settings were obtained from published work (Staples et al., 2011) and shown in table 3.13.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Voltage</th>
<th>AMPGain</th>
<th>Mode</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>E-1</td>
<td>6.53</td>
<td>Lin</td>
<td>52</td>
</tr>
<tr>
<td>SSC</td>
<td>250</td>
<td>1.0</td>
<td>Log</td>
<td>0</td>
</tr>
<tr>
<td>FL1</td>
<td>490</td>
<td>1.0</td>
<td>Log</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 3.13:** Instrument settings for the flow cytometric acquisition of human PMBCs. FCS: Forward Scatter, SSC: Side Scatter, FL1: Filter 1.

**Note:** please see table 2.6, 2.13 and 2.14 for all the reagents, buffers and antibodies used in flow cytometry experiments.
3.9 Database analysis

The versican proximal promoter sequence was searched using MatInspector (Genomatix, http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html) and transcription element search system (TESS) software (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) for putative transcription factor binding sites.

3.10 Statistics

Statistical analysis was done using the statistical software GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA). Student’s one tailed and two-tailed, paired t-test was selected for the analysis after inputting the data. P-value was calculated for significance level. The results were considered statistically significant if ***, p \leq 0.001; **, p \leq 0.01; and *, p \leq 0.05.
Chapter 4: Results

Hypoxic regulation of versican transcription in primary human macrophages

4.1 Introduction

Hypoxia, which refers to low oxygen tension in biological systems, is a feature of many pathological tissues such as solid tumours (Hockel et al., 1996; Vaupel et al., 2001), wounds (Lee et al., 2009), atherosclerotic plaques (Simanonok et al., 1996), arthritic joints (Mapp et al., 1995) and sites of infection and inflammation (Wenger et al., 2005). The median oxygen tension in normal tissues is usually between 20 and 70 mmHg but in ischaemic pathological sites can reach zero mmHg (Vaupel et al., 2001). However, hypoxia is also found in healthy tissues such as spleen (oxygen level as low as 0.5%) (Caldwell et al., 2001). Cells of the monocyte/macrophage lineage are involved in all of the above pathologies (Lewis et al., 1999; Griffiths et al., 2000; Lewis et al., 2000). It has been known for some time that macrophages accumulate in poorly vascularised, hypoxic sites and respond rapidly to the hypoxia present by altering their expression of a wide array of genes (Murdoch et al., 2005; Burke et al., 2003).

Unpublished work by Staples and B. Burke et al., (personal communication) found that the extracellular matrix (ECM) proteoglycan versican is one such hypoxia inducible gene which can be up-regulated up to 700-fold at the mRNA level in human peripheral blood mononuclear cells (PBMC) after 5 days incubation under 0.2% O₂ (Fig 1.8). Versican is a member of the large aggregating chondroitin sulphate proteoglycans, and is distributed in a wide variety of tissues during development and injury (Yee et al.,
Recent studies by Asplund et al., (2010) showed the hypoxic inducibility of the versican gene in human monocyte-derived macrophages (HMDM) after 24 hours under 0.5% O$_2$.

Here, the effect of short term (18 hours) hypoxia (0.2% O$_2$) on versican mRNA expression was first investigated in adherence-purified HMDM in different donors. Then in order to investigate whether macrophages are the principle cell type showing hypoxic up-regulation of versican in our PBMC cultures, Real Time RT-PCR was used to compare the level of versican mRNA expression in CD14$^+$ monocyte-derived macrophages with lymphocytes (CD14$^-$). In addition, the decay of versican mRNA was investigated in normoxic and hypoxic adherence-purified HMDM.

### 4.2 Short term hypoxia induces versican mRNA in adherence-purified HMDM

The effect of 18 hours (short term) hypoxia (0.2% O$_2$) was investigated on versican mRNA expression in primary HMDM, purified by adherence from PBMC from 15 different healthy donors. As described in section 3.1.1, PBMCs were cultured in 2ml complete Iscove’s medium at a cell density of 2×10$^6$ per well in 6-well adherent Nunclon plates and incubated at 37°C with 5% CO$_2$ and 20.9% O$_2$ for 5 days to allow differentiation of monocytes into macrophages (See section 3.1.3). Lymphocytes are non-adherent so this procedure typically yields a cell population of >95% monocyte-macrophages (Burke et al., 2003). After 5 days incubation, cells were either cultured under normal oxygen concentrations (20.9% O$_2$) or hypoxia (0.2% O$_2$) for a further 18
4. Results

hours prior to RNA isolation followed by determination of β-2M (a housekeeping gene) and versican mRNA levels by Real-Time RT-PCR (See section 3.4).

As previously described, first Real-Time PCR was performed on cDNA samples using primers for the housekeeping gene β-2M. Studies in our lab (Staples et al., 2011) have shown that β-2M has a constant level of expression under normoxia and hypoxia and has also been used as an internal control in a variety of studies (Jogi et al., 2002; Watson et al., 2009). As figure 4.1 shows, to obtain relative concentration, four five-fold serially-diluted standards (STD1-4; 1, 0.2, 0.04 and 0.008) were prepared and were run in parallel with the samples and negative control; PCR H2O (PCR –ve control) and the standard curve were plotted by the Lightcycler (Roche) software to calculate the relative concentration of β-2M cDNA for each sample (See section 3.4.3). The specificity and purity of the quantified samples was confirmed by examining the melting curve. As figure 4.2 indicates, all the PCR products (standards and samples) showed a similar melting temperature except the negative control (PCR –ve) which by comparison with previous experiments in which the product size had been confirmed on agarose gels, confirms that the correct PCR product was amplified and that no contamination was observed in the reaction. Then the amount of cDNA of the gene of interest, versican, was determined in second Real-Time PCR using versican primers, on the same standards, samples and negative control (Fig 4.3 and 4.4). As previously described in section 3.4.4, to normalise the amount of versican cDNA for each sample (normoxia or hypoxia), the calculated cDNA value of versican was divided by the calculated amount of β-2M cDNA in the same sample. To calculate the hypoxic fold induction of versican in adherence-purified HMDM of a particular donor, normalised
abundance of versican in hypoxia was divided by normalised abundance of versican in normoxia.

Figure 4.1: LightCycler PCR amplification of β-2M cDNA in normoxic (N, 20.9% O₂) and hypoxic (H, 0.2% O₂) adherence-purified HMDM. The figure shows four standards (STD1-4) produced by serial 1:5 dilution (1, 0.2, 0.04 and 0.008) which were prepared and run in parallel with the samples (HMDM N and HMDM H) and negative control, H₂O (PCR -ve). The standard curve was plotted by the software to calculate the relative concentration of β-2M cDNA for each sample.
Figure 4.2: LightCycler melting curve of amplified β-2M cDNA in the samples described in Fig 4.1. The figure shows similar melting temperatures in the four standards (STD1-4) and samples (HMDM N and HMDM H) but not in the negative control; H₂O (PCR –ve) which, by comparison with previous experiments in which the product size had been confirmed on agarose gels, confirms that the correct PCR product was amplified. The small non-specific product peak in the negative control could be due to primer dimer formation and confirms no contamination in the reaction.
Figure 4.3: LightCycler PCR amplification of versican cDNA in normoxic (N, 20.9% O$_2$) and hypoxic (H, 0.2% O$_2$) adherence-purified HMDM. The figure shows four standards (STD1-4) with a serial dilution of 1:5 (1, 0.2, 0.04 and 0.008) which were prepared and run in parallel with the samples (HMDM N and HMDM H) and negative control, H$_2$O (PCR -ve). The standard curve was plotted by the software to calculate the relative concentration of versican DNA for each sample.
Figure 4.4: LightCycler melting curve of amplified versican cDNA in samples detailed in Fig 4.3. The figure shows similar melting temperatures in the four standards (STD1-4) and samples (HMDM N and HMDM H) but not in the negative control; H$_2$O (PCR -ve) which, by comparison with previous experiments in which the product size had been confirmed on agarose gels, confirms that the correct PCR product was amplified and that no contamination was observed.
This method was applied to 15 different donors to confirm the hypoxic up-regulation of versican mRNA. As figure 4.5 shows, I observed substantial induction of versican mRNA (average 34-fold) in all 15 different donors after 18 hours hypoxia despite high variability in hypoxic fold induction among these donors, ranging from 11 to 125 fold. These data clearly confirmed the hypoxic up-regulation of versican mRNA in adherence-purified HMDM.

**Figure 4.5**: Effect of short term (18 hours) hypoxia on versican mRNA expression in adherence-purified HMDM. Real-Time RT-PCR analysis was used to determine the effect of 18 hrs hypoxia on versican mRNA expression in 15 different donors. $2 \times 10^6$ cells per well were exposed to normoxia (20.9% O$_2$) or hypoxia (0.2% O$_2$) for 18 hours. All data were normalised to β-2M mRNA levels in the same samples determined by separate PCR. Fold induction was calculated by dividing the normalized versican mRNA level in hypoxia by the normalised versican mRNA level in normoxia.
4.3 Comparison of versican mRNA hypoxic up-regulation in macrophages and lymphocytes

Real-Time RT-PCR was used to compare the induction of versican mRNA expression in macrophages and lymphocytes, to determine the relative importance of these cell types in preliminarily experiments using PBMC cultures (Fig 1.8), which contain a mixture of monocyte-macrophages and lymphocytes, with the majority, typically 90-95%, being lymphocytes (Burke et al., 2003). As described in section 3.1.4, PBMCs were first prepared and then CD14+ monocytes were separated from CD14- cells (mainly lymphocytes) using Miltenyi MACS magnetic beads linked to an antibody specific for the monocyte-specific surface antigen CD14. Cells were then seeded in 2ml complete Iscove’s medium at a cell density of 1×10⁶ per well in 6-well adherent Nunclon plates and incubated at 37°C with 5% CO₂ and 20.9% O₂ for 5 days to allow differentiation of CD14+ purified monocytes into macrophages (See section 3.1.3). After 5 days incubation, cells were either cultured under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for a further 18 hours prior to RNA isolation followed by determination of versican mRNA level by Real-Time RT-PCR (See section 3.4).

As figure 4.6 indicates, 18 hours of hypoxia significantly induced versican mRNA expression in adherence-purified HMDM (34-fold) and CD14+ magnetic bead purified monocyte-derived macrophages (25-fold) but not in lymphocytes (CD14- cells). These data clearly suggest that macrophages, and not lymphocytes, are the main type of peripheral blood mononuclear cell responsible for hypoxic up-regulation of versican.
Figure 4.6: Effect of short term (18 hours) hypoxia on versican mRNA expression in human primary macrophages and Lymphocytes. Versican mRNA levels were quantified by Real-Time RT-PCR in HMDM (n=15), CD14\(^+\) magnetic bead purified monocyte-macrophages and CD14\(^-\) cells (lymphocytes) (n=3). 2 x 10\(^6\) cells per well were exposed to normoxia (20.9% O\(_2\)) or hypoxia (0.2% O\(_2\)) for 18 hours. All data were normalised to β2-M mRNA levels determined by separate PCR. Data are from independent experiments using different donors and are expressed as means ± SEM. Data were further analysed for significant induction using paired t-tests. *** = p <0.001, * = p <0.05 when compared to the normoxic samples.
4.4 Hypoxic up-regulation of versican mRNA occurs at the transcriptional level

To determine whether the observed up-regulation of versican mRNA by hypoxia is due to increased transcription or increased versican mRNA stability, the decay of versican mRNA was investigated in normoxia and hypoxia in adherence-purified HMDM. In order to block further transcription, cells were treated with actinomycin D, and the amounts of versican mRNA at a series of time points were determined by RT-PCR. For these experiments, adherence-purified HMDM were isolated and cultured as normal (See sections 3.1.1 and 3.1.3) and after 5 days maturation, were incubated under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for a further 18 hours prior to actinomycin D treatment. Cells were then treated with actinomycin D at a final concentration of 10 µg/ml and returned to normoxia or hypoxia for a further 2 hours, 8 hours, 12 hours, 24 hours and 36 hours respectively prior to RNA isolation followed by determination of versican mRNA level by Real-Time RT-PCR.

As Figure 4.7 shows, very similar levels of versican mRNA degradation were observed in normoxia and hypoxia at all time points up to 36 hours. Since these data demonstrate that versican mRNA stability is not increased by hypoxia, it appears that the increase observed in versican mRNA in hypoxic adherence-purified HMDM is due to transcriptional up-regulation rather than increased mRNA half-life by hypoxia.
Figure 4.7: Effect of hypoxia on versican mRNA decay in adherence-purified HMDM. Adherence-purified HMDM were quantified by Real-Time RT-PCR to assess the decay of versican mRNA in normoxia and hypoxia. 2 x 10^6 cells per well were first treated with actinomycin D at a final concentration of 10 µg/ml and then were exposed to normoxia (20.9% O_2) or hypoxia (0.2% O_2) for a further 2h, 8h, 12h, 24h and 36h prior to RNA isolation. Data were normalised to β2-M mRNA levels, and are shown as a percentage of the initial time zero (Actinomycin D addition time) mRNA level in normoxia or hypoxia. Data from 5 independent experiments using different donors are expressed as means ± SEM.

As a control to validate the RNA samples generated by this actinomycin D mRNA stability approach, Real-Time RT-PCR using GLUT-1 primers was performed on the same samples to determine the decay of GLUT-1 mRNA, a gene known to be transcriptionally induced by hypoxia (Burke et al., 2003). As figure 4.8 shows that, as for versican, similar levels of GLUT-1 mRNA degradation were observed in normoxia and hypoxia at all time points examined. This data confirms, as expected, that hypoxia-induced increases in GLUT-1 mRNA are mediated by transcriptional up-regulation rather than increased mRNA stability in hypoxia.
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![Graph showing GLUT-1 mRNA decay](image)

**Figure 4.8**: Effect of hypoxia on GLUT-1 mRNA decay in adherence-purified HMDM. Adherence-purified HMDM were quantified by Real-Time RT-PCR to assess the decay of GLUT-1 mRNA in normoxia and hypoxia. Experimental details as explained for figure 4.7. Data from 5 independent experiments using different donors are expressed as means ± SEM.

4.5 Summary

In this chapter, the effect of 18 hours period of hypoxia on versican mRNA expression was investigated in adherence-purified HMDM generated from PBMC in 15 different donors. As figure 4.5 showed, Real-Time RT-PCR on adherence-purified HMDM in 15 different donors indicated substantial induction of versican mRNA after 18 hours hypoxia. There was high variability in hypoxic fold induction among these donors (average induction 34-fold, range 11-125 fold). Further study was carried out to investigate which cell type was principally responsible for versican expression, since adherence-purified HMDM cultures are typically no more than 95% macrophages (Burke et al., 2003). As figure 4.6 showed, 18 hours of hypoxia significantly induced versican mRNA expression in adherence-purified HMDM (34-fold) and CD14+...
monocyte-derived macrophages (25-fold) but not in lymphocytes (CD14+), indicating that macrophages as the principle cell type showing hypoxic up-regulation of versican mRNA. In addition, analysis of the decay of the mRNA of versican (Fig 4.7) and GLUT-1 (Fig 4.8) showed similar kinetics of mRNA degradation in normoxic and hypoxic adherence-purified HMDM at all time points which clearly confirmed that hypoxic up-regulation of versican and GLUT-1 mRNA is not due to changes in mRNA stability and must occur at the transcriptional level. The data presented in this chapter, showing that the strong up-regulation of versican mRNA by hypoxia occurs at the transcriptional level, indicate that analysis of the versican promoter must be carried out to elucidate the mechanism of up-regulation. This work is described in chapter 6.
Chapter 5: Results

Hypoxic regulation of versican protein synthesis in human primary macrophages

5.1 Introduction

Some previous studies have investigated the level of versican protein expression in human (Zimmermann et al., 1994; Kischel et al., 2010) and mouse tissues (Russell et al., 2003). Du Cros et al., (1995) also showed versican protein expression in skin and blood vessels of adult human tissue and during hair follicle development in mouse. Further study by Domenzain et al., on media from subconfluent cultures by immunoblotting, demonstrated that invasive human melanoma SK-m1-131 cells express versican protein isoforms V0 and V1 (Domenzain et al., 2003 & 2009). A recent study observed that versican is present in the CD68-positive macrophage-rich areas of human carotid lesions (Asplund et al., 2010). However, to date there has been only one study addressing the question of whether versican protein is induced by hypoxia. Asplund et al., using Immunoblotting, did not show hypoxic induction of versican protein level in normoxia and hypoxia in HMDM. They incubated HMDM for 24 hours under normoxia (21% O₂) or hypoxia (0.5% O₂) and then cell lysates were separated by SDS–PAGE on 5% gels and immunoblotted for versican protein. However further investigation, using radioactive quantification of versican-bound GAG chains using ³⁵S-sulphate and D-[⁶⁻³H]-glucosamine hydrochloride showed 3 and 7-fold increases in GAG chains respectively, in cells exposed to hypoxia for 24 hours compared to cells incubated in normoxia (Asplund et al., 2010).
In this project, further study was carried out to investigate in more detail the effect of hypoxia on versican protein synthesis in human primary macrophages using intracellular flow cytometry. Based on previous studies on quantification of versican protein in conditioned medium (Serra et al., 2005; Hasegawa et al., 2007) and using intracellular flow cytometry (Wang et al., 2008), PBMCs were treated with brefeldin A, an intracellular protein transport inhibitor which causes blockade of protein transport to the Golgi complex and accumulation of proteins in the endoplasmic reticulum, leading to inhibition of protein secretion (Hunziker et al., 1992; Sciaky et al., 1997; Nebenfuhr et al., 2002). Here, versican protein expression was analysed in PBMC after 24 hours or 5 days incubation under normoxia (20.9% O\textsubscript{2}) or hypoxia (0.2% O\textsubscript{2}) using intracellular staining followed by flow cytometry. In addition, the level of versican protein expression and hypoxic induction in monocytes/macrophages was compared to those in lymphocytes.

5.2 Long term hypoxia induces versican protein expression in human primary macrophages

The effect of 5 days (long term) hypoxia (0.2% O\textsubscript{2}) was investigated on versican protein expression in PBMC from 5 different healthy donors using intracellular flow cytometry. In order to optimize the protocols being used, pilot experiments were performed to investigate whether brefeldin A is necessary for the detection of intracellular protein as described previously. As described in section 3.1.1, PBMCs were cultured in 2ml complete Iscove’s medium at a cell density of 2×10\textsuperscript{6} per well in 6-Well Costar ultra low attachment plates and incubated under normoxia (20.9% O\textsubscript{2}) for 5 days. After 5 days incubation, PBMCs were incubated under either normoxia or hypoxia (0.2% O\textsubscript{2}) for a
further 5 days prior to intracellular staining. As described in section 3.8, in order to quantify intracellular protein level, a cell permeabilisation protocol was used prior to addition of a 1:50 dilution of rat monoclonal anti-human versican antibody or rat IgG1 isotype control followed by incubation with a 1:40 dilution of anti-rat IgG conjugated to FITC. Samples were then acquired using a FACSCalibur flow cytometer, followed by analysis with CellQuest software (See section 3.8).

As figure 5.1 shows, brefeldin A-untreated normoxic (A) and hypoxic (C) PBMCs were plotted onto a dot-scatter plot which exhibited lymphocytes with low forward scatter (FSC), side scatter (SSC) and monocytes/macrophages with higher FSC and SSC (Smyth et al., 2005; Alexis et al., 2006; Lay et al., 2009). When a rough population of monocyte/macrophages (R1) was gated onto a histogram-plot, despite having some background with isotype control (green line) which could be due to non-specific binding, no difference of versican fluorescent intensity (n=3) were observed in hypoxia in comparison with normoxia (blue line). Increasing the size of the monocyte/macrophage region on the dot-scatter plot did not show any increase in hypoxia versican fluorescent intensity (data not shown).
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Figure 5.1: Effect of long term (5 days) hypoxia on versican protein expression in brefeldin A-untreated PBMC-derived macrophages. Untreated PBMCs were subjected to flow cytometry to analyse the effect of 5 days hypoxia on versican protein expression. 2 x 10⁶ cells per well were cultured under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 5 days prior to flow cytometry analysis. Normoxic and hypoxic PBMCs were plotted onto a dot-scatter plot (A and C). The monocyte/macrophage region (R1) with high forward (FSC) and side scatter (SSC) was gated onto a histogram-plot and then examined for normoxic and hypoxic versican fluorescent intensity (B and D) (blue line) against isotype control (green line). A representative example of 3 independent experiments is shown.
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Identical studies, except for the addition of brefeldin A, were performed on PBMC treated with brefeldin A to investigate the effect of 5 days (long term) hypoxia on versican protein expression. Human PBMCs were isolated and cultured as described in section 3.1.1. After 5 days incubation, PBMCs were incubated under either normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 4 days prior to brefeldin A treatment. Cells were then treated with 2 μg/ml brefeldin A and incubated under the same oxygen tension for a further 24 hours prior to quantification of protein by intracellular flow cytometry as described in section 3.8.

As figure 5.2 (plots A and C) demonstrates, when a rough population of monocytes/macrophages (R1) was gated onto a histogram-plot, despite having some isotype background which could be due to non-specific binding (green line), a remarkable increase of versican fluorescent intensity was observed (n=5) in hypoxia (D) (blue line) in comparison with normoxia (B) (blue line) against isotype control (green line). As figure 5.2, graph E shows, flow cytometric analysis of versican protein expression on PBMC derived macrophages from 5 different donors indicated significant induction of versican protein (3-fold) after 5 days in hypoxia. In order to eliminate any possible background due to non-specific binding, the versican fluorescent intensity was subtracted from isotype fluorescent intensity in normoxia and hypoxia in each separate experiment. As well as demonstrating clear hypoxic induction, these experiments demonstrated the need for brefeldin A in order to allow accumulation of versican protein within the cells to allow detection and quantification by flow cytometry.
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Figure 5.2: Effect of long term (5 days) hypoxia on versican protein expression in brefeldin A treated PBMC-derived macrophages. Treated PBMCs were subjected to flow cytometry to analyse the effect of 5 days hypoxia on versican protein expression. 2 x 10^6 cells per well were cultured under normoxia (20.9% O_{2}) or hypoxia (0.2% O_{2}) for 4 days followed brefeldin A treatment and incubated under the same oxygen tension for a further 24 hours. Normoxic and hypoxic PBMCs were plotted onto a dot-scatter plot (A and C). The monocyte/macrophage region (R1) with high forward (FSC) and side scatter (SSC) was gated onto a histogram-plot and then examined for normoxic and hypoxic versican fluorescent intensity (B and D) (blue line) against isotype control (green line). A representative example of 5 independent experiments is shown. (E) Versican protein fold induction in region R1 cells from 5 independent experiments using different donors is expressed as means ±SEM. Isotype control levels were subtracted from the data. The normoxic value in each experiment was assigned an arbitrary value of 1. Data is from 5 independent experiments and were further analysed using paired two-tailed t-tests. ** p < 0.01
5.3 Short term hypoxia did not induce versican protein expression in human primary macrophages

The effect of 24 hours (short term) hypoxia (0.2% O$_2$) was also investigated on versican protein expression in PBMC treated with brefeldin A from 3 different healthy donors using intracellular flow cytometry. PBMCs were cultured in 2ml complete Iscove’s medium at a cell density of 2×10$^6$ per well in 6-Well Costar ultra low attachment plates and incubated under normal oxygen condition for 5 days (See section 3.1.3). After 5 days, PBMCs were treated with 2 μg/ml brefeldin A and incubated under either normoxia (20.9% O$_2$) or hypoxia (0.2% O$_2$) for 24 hours prior to quantification of protein by intracellular flow cytometry as described in section 3.8.

As figure 5.3 shows, normoxic (A) and hypoxic (C) PBMCs were first plotted onto a dot-scatter plot. When the same region of monocytes/macrophages (R1) as previously showed in Fig 5.1 and 5.2 (plot A and C) was selected and gated onto the histogram-plot, despite having some background with isotype control (green line) which could be due to non-specific binding, no increase of versican fluorescence intensity (n=3) was observed in hypoxia (D) in comparison with normoxia (B) (blue line) against isotype control (green line). Increasing the monocyte/macrophage region size on the dot scatter plot did not result in any increase in versican fluorescent intensity in hypoxia (data not shown). This finding is concordant with the observations of Asplund et al., (2010) who failed to show hypoxic up-regulation of versican protein in HMDM after 24 hours.
Figure 5.3: Effect of short term (24 hours) hypoxia on versican protein expression in PBMC derived macrophages treated with brefeldin A. PBMCs were subjected to flow cytometry followed to analyse the effect of 24 hours hypoxia on versican protein expression. 2 x 10^6 cells per well were treated with brefeldin A and then exposed to normoxia (20.9% O_2) or hypoxia (0.2% O_2) for 24 hours. Experimental details as explained for figure 5.2. A representative example of 3 independent experiments is shown.
5.4 Hypoxia-induced increases of versican protein expression are confined to monocyte/macrophage cells

To investigate whether the hypoxic up-regulation of versican protein in monocytes/macrophages demonstrated by intracellular flow cytometry (Fig 5.2, plot E) is affected by monocyte maturation/differentiation, as indicated by side scatter (cell granularity) and forward scatter (cell size) (Alexis et al., 2006; Lay et al., 2009), different sub-populations of 5 days old normoxic and hypoxic monocyte/macrophages were analysed for versican fluorescent intensity.

When looking at macrophages with different forward signals, the cells with high forward and side scatter (large, more mature macrophages) showed a higher versican specific mean fluorescent intensity compared to cells with a low forward and side scatter (small, less mature monocyte/macrophages) (Fig 5.4, plot C and F). To further analyse this, the monocyte/macrophage populations on the dot-scatter plots were divided into 3 separate regions (R3-R1) on the basis of increasing FSC, as shown in figure 5.4, plots A and D. The proportion of cells in each of these regions differed in normoxia and hypoxia (Fig 5.4, plots B and E), likely reflecting the effect of prolonged hypoxia on the increase in cell size (Liu et al., 2006) which is associated with macrophage maturation (Alexis et al., 2006; Lay et al., 2009). In both normoxia and hypoxia, increasing cell size (forward scatter) significantly correlated with increasing versican mean fluorescent intensity (Fig 5.4, plots C and F) but not with isotype control (data not shown). Comparison of R1 mature macrophages from normoxia and hypoxia (Fig 5.4 Plots C and F) with R2 and R3 regions, demonstrated a statistically significant overall increase in versican protein expression. Similar hypoxia-induced increases in
versican mean fluorescent intensity were also observed in the monocyte/macrophages in regions R2 and R3. Interestingly, very low levels of versican fluorescent intensity, and no hypoxic induction, were detected in the population assumed to contain the majority of lymphocytes (R4; Fig 5.4 plots C and F) however, histogram-plot analysing of cells in R4 showed few strongly positive cells (data not shown). As expected, this matches well with the low versican mRNA level, and lack of hypoxic induction, which was found in CD14^- cells (lymphocytes) (Fig 4.6). Taken together, this data suggests that hypoxic up-regulation of versican protein is confined to monocyte/macrophage lineage cells and is not detectable in lymphocytes.
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Figure 5.4: Intracellular flow cytometry analysis of hypoxic regulation of versican protein in human PBMC. PBMCs were subjected to flow cytometry to analyse the effect of 5 days hypoxia on versican protein expression. $2 \times 10^6$ cells per well were cultured under normoxia (20.9% $O_2$) or hypoxia (0.2% $O_2$) for 4 days followed by brefeldin A treatment and incubated under the same oxygen tension for a further 24 hours. Normoxic and hypoxic PBMCs were plotted onto a dot-scatter plot (A and D). Monocyte/macrophages are subdivided into 3 regions R3-R1 in respect of increasing cell size (forward scatter, FSC). Lymphocytes are included in region 4 (A and D). Dot scatter plots were used to calculate the percentage of the total monocyte/macrophage population present in regions R1, R2, and R3 in normoxia (B) or hypoxia (E). Monocyte/macrophage regions R3-R1 and lymphocytes region (R4) were gated onto the histogram-plot and then examined for normoxic and hypoxic versican fluorescent intensity (C and F) against isotype control. Isotype control levels were subtracted from the data. Data are from 5 independent experiments and were further analysed using paired two-tailed t-tests, * = p <0.05
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### 5.5 Summary

In this chapter, the level of versican protein expression was quantified in normoxic or hypoxic PBMC after 24 hours and 5 days incubation using intracellular staining followed by flow cytometry. Preliminary investigations showed the role of brefeldin A in blocking the protein secretion into the culture medium when the results from untreated PBMC (Fig 5.1) were compared to PBMC treated with brefeldin A (Fig 5.2). Flow cytometry analysis of PBMCs treated with brefeldin A followed 24 hours incubation under normoxia or hypoxia did not show any increase in versican fluorescent intensity in hypoxia in comparison with normoxia (Fig 5.3 plots B and D) whereas further investigation demonstrated significant induction of versican protein expression (3-fold) in monocyte/macrophages after 5 days hypoxia (n=5) (Fig 5.2, graph E). Also further investigation on PBMCs after 5 days hypoxia demonstrated that increasing monocyte/macrophage cell size (forward scatter) on the dot scatter plot is associated with increasing versican fluorescent intensity in normoxia and hypoxia (Fig 5.4, plot A, D, C and F). In addition, as figures 5.4, plots C and F show, no hypoxic induction of versican protein was detected in region which contains the lymphocyte population (R4) although a very low level of versican mean fluorescent intensity was observed in both normoxia and hypoxia. The data suggested that versican protein expression is largely confined to monocyte/macrophage and is not detectable in lymphocytes.
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Versican promoter analysis

6.1 Introduction

The versican promoter which was characterised by Naso et al., (1994), contains a typical TATA box located approximately 16 base pairs upstream of the transcription start site and several putative regulatory sites for a number of transcription factors such as AP-2 and SP-1 (Fig 1.6). Furthermore, a reporter construct driven by an 876-bp (-632/+240 relative to the transcriptional start site) piece of the versican promoter showed significant expression in HeLa cells and IMR-90 embryonic lung fibroblasts. Deletion constructs revealed enhancer and repressor elements on the 876-bp of versican promoter which were responsible for the basal expression in different cells (Naso et al., 1994). In addition, oligonucleotide arrays on human carcinoma cell lines such as MCF7, suggested a role for the Pax3 and P53 transcription factors in versican regulation and expression (Mayanil et al., 2001; Yoon et al., 2002). In addition, two putative binding sites for the TCF/LEF which are localized at position -546-bp and -492-bp on the versican promoter, are revealed by Rahmani et al., (2005) (Fig 1.6). Site-directed mutagenesis of the TCF sites in the versican promoter markedly reduced reporter luciferase activity in smooth muscle cells (SMC) and further investigation confirmed the role of the β-catenin / TCF transcription factor complex in expression of versican in SMC (Rahmani et al., 2005). In addition, studies in human melanoma cells identified several transcriptional regulatory elements in a 620-bp (-618/+2) versican promoter construct, including SP-1, AP-2 and AP-1 binding sites (Domenzain et al., 2009). Further investigation using mutagenesis of the AP-1 site at position -36
suggested that AP-1 is the main regulatory factor which directs versican production during melanoma progression (Domenzain et al., 2009).

In this project, promoter reporter constructs were used to determine the parts of the versican promoter responsible for the hypoxic up-regulation of versican in human primary macrophages. A 2.5-Kb piece of DNA containing the versican promoter was initially cloned into pGL3-basic in Dr Burke’s lab prior to this project, and during this project I produced a series of versican promoter constructs including 4.140-Kb (-3.956kb/+184bp), 830-bp (-646bp/+184bp), 480-bp (-296bp/+184bp) and 240-bp (-56bp/+184bp) which were cloned into the more modern, improved pGL4.10 luciferase reporter vector. These constructs were then transfected into HMDM and tested for inducibility by hypoxia. In addition, to determine which parts of the proximal promoter are required for increased expression in hypoxia, a series of versican promoter deletion constructs were made and transfected into HMDM. Also sequence analysis was carried out using Genomatix MatInspector and TESS software to search for putative binding sites for hypoxia-inducible transcription factors.
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6.2 PCR amplification of different regions of versican promoter

To determine the DNA sequences which are required for hypoxic expression of versican in HMDM, the different segments of versican promoter were amplified from human genomic DNA first by PCR and then were cloned into the pGL4.10 luciferase reporter plasmid (See section 3.3 for general molecular cloning and table 2.12 for primers).

A 4.140-Kb (-3.956kb/+184bp) region of the versican promoter was PCR-amplified using -3.956kb versican-5’ (forward) and +184bp versican-3’ (reverse) primers and then run on a 0.6% agarose gel against a 1-Kb DNA molecular weight ladder to confirm the correct size of DNA fragment is amplified (Fig 6.1).

![Image of agarose gel electrophoresis]

**Figure 6.1:** 0.6% agarose gel electrophoresis of PCR amplification of the 4.140-Kb (-3.956kb/+184bp) versican promoter sequence from human genomic DNA. Lane 1 = 1-Kb DNA ladder, lane 2 = 4.140-Kb
In addition, shorter sections of the versican promoter including 830-bp (-646bp/+184bp), 480-bp (-296bp/+184bp) and 240-bp (-56bp/+184bp) were PCR-amplified and run on a 1.5% agarose gel. The sizes of the amplified segments of the versican promoter were confirmed against a 100-bp DNA molecular weight ladder (Fig 6.2).

**Figure 6.2:** 1.5% agarose gel electrophoresis of PCR amplification of the 240-bp (-56bp/+184bp), 480-bp (-296bp/+184bp) and 830-bp (-646bp/+184bp) versican promoter sequences from human genomic DNA. Lane 1 = 100-bp DNA ladder, Lane 2 = 240-bp, Lane 3 = 480-bp, Lane 4 = 830-bp.
In order to define the DNA sequence of the versican promoter responsible for hypoxic expression in HMDM, a deletion series of the 240-bp (-56bp/+184bp) of versican proximal promoter was generated, including, 210-bp (-56bp/+154bp), 160-bp (-56bp/+104bp), 110-bp (-56bp/+54bp), 210-bp (-26bp/+184bp), 180-bp (-26bp/+154bp), 130-bp (-26bp/+104bp), 80-bp (-26bp/+54bp). As figure 6.3 shows, two amplified 210-bp (-56bp/+154bp and -26bp/+184bp), 180-bp, 160-bp, 130-bp, 110-bp and 80-bp of versican promoter sequences were successfully PCR-amplified and run on a 2.0% agarose gel and compared to a DNA molecular weight ladder (HyperLadder™ V, Bioline) to confirm that the correct fragments of the versican promoter have been generated (Fig 6.3).

Figure 6.3: 2% agarose gel electrophoresis of PCR amplification of the 110-bp (-56bp/+54bp), 160-bp (-56bp/+104bp), 210-bp (-56bp/+154bp), 80-bp (-26bp/+54bp), 130-bp (-26bp/+104bp), 180bp (-26bp/+154bp) and 210-bp (-26bp/+184bp) versican promoter sequences from human genomic DNA. Lane 1 = HyperLadder V, Lane 2 = 110-bp, Lane 3 = 160-bp, Lane 4 = 210-bp, Lane 5 = 80-bp, Lane 6 = 130-bp, Lane 7 = 180-bp and Lane 8 = 210-bp
In addition, a 29-bp random nucleotide sequence (CTAGCTAGCTAGCTAGCTAGCTAGCTAGC) was cloned into the pGL4.10 luciferase reporter plasmid for use as a negative control in transfection experiments. The annealed 29-bp random sequence was loaded into a 2.0% agarose gel and the size was confirmed in comparison with a DNA molecular weight ladder (HyperLadder™ V, Bioline) (Figure 6.4).

![Figure 6.4: 2% agarose gel electrophoresis of the annealed 29-bp random nucleotide sequence (CTAGCTAGCTAGCTAGCTAGCTAGCTAGC). Lane 1 = HyperLadder V, lane 2 = 29-bp](image-url)
6.3 Cloning and screening of different fragments of versican promoter

The successfully amplified different regions of the versican promoter, and the pGL4.10 [luc2] vector, were digested by the SfiI restriction enzyme isolated from an E. coli strain that carries the SfiI gene from Streptomyces fimbriatus (See section 2.7.1 and 3.3.3). The digested versican promoter DNA sequences and pGL4.10 vector were run on agarose gels and extracted with the QIAquick Gel Extraction Kit, and the concentration estimated by comparison with known standards in ethidium bromide-stained agarose gels. Purified DNA fragments were cloned into the pGL4.10 vector and DNA sequencing was performed to confirm the sequences of the different fragments cloned (See section 3.3 for general molecular cloning).

In order to confirm that a positive 4.140-Kb construct had been generated, all three putative constructs obtained were digested using SfiI and run on a 0.6% agarose gel beside an undigested 4.140-Kb construct to determine if the correct size insert has been cloned (Fig 6.5). As figure 6.5 indicates, all 3 digested 4.140-Kb versican promoter pGL4.10 constructs showed the correct insert size at 4-Kb in comparison with DNA ladder. Due to the fact that the insert and vector are similar in size, an incomplete separation of 4.140-Kb versican promoter and pGL4.10 vector was observed (Fig 6.5).
In addition, to confirm that the 830-bp, 480-bp and 240-bp fragments of the versican promoter had been successfully cloned into the pGL4.10 plasmid, three clones of each construct were digested using SfiI and run on 1.5% agarose gels beside undigested constructs. As figure 6.6 indicates, of three putative clones of the 480-bp versican pGL4.10 construct, only one was digested by SfiI and showed the correct size insert. Also all 3 selected clones of the 240-bp (Fig 6.7) and 830-bp (Fig 6.8) versican pGL4.10 constructs showed the correct insert size when they were digested by SfiI and compared to undigested constructs and DNA ladder.
6. Results

Figure 6.6: 1.5% agarose gel electrophoresis of restriction digested plasmid preps of 480-bp of versican promoter pGL4.10 constructs. Three putative purified 480-bp versican pGL4.10 clones were digested with SfiI and run with an undigested 480-bp construct. Lane 1 = 1-Kb DNA ladder, Lane 2 = digested 480-bp versican pGL4.10 construct (No insert), Lane 3 = undigested 480-bp versican pGL4.10 construct, Lane 4 = digested 480-bp versican pGL4.10 construct, Lane 5 = undigested 480-bp versican pGL4.10 construct, Lane 6 = digested 480-bp versican pGL4.10 construct (No correct insert), Lane 7 = undigested 480-bp versican pGL4.10 construct, Lane 8 = 100-bp DNA ladder

Figure 6.7: 1.5% agarose gel electrophoresis of restriction digested plasmid preps of 240-bp versican promoter pGL4.10 constructs. Three putative purified 240-bp versican clones were digested with SfiI and run with an undigested 240-bp construct. Lane 1 = 100-bp DNA ladder, Lane 2 = digested 240-bp versican pGL4.10 construct, Lane 3 = undigested 240-bp versican pGL4.10 construct, Lane 4 = digested 240-bp versican pGL4.10 construct, Lane 5 = undigested 240-bp versican pGL4.10 construct, Lane 6 = digested 240-bp versican pGL4.10 construct, Lane 7 = undigested 240-bp versican pGL4.10 construct, Lane 8 = 1-kb DNA ladder
6. Results

Figure 6.8: 1.5% agarose gel of restriction digested plasmid preps of 830-bp versican promoter pGL4.10 constructs. Three putative purified 830-bp versican clones were digested with SfiI and run with an undigested 830-bp construct. Lane 1 = 1-Kb DNA ladder, Lane 2 = undigested 830-bp versican pGL4.10 construct, Lane 3 = digested 830-bp versican pGL4.10 construct, Lane 4 = undigested 830-bp versican pGL4.10 construct, Lane 5 = digested 830-bp versican pGL4.10 construct, Lane 6 = undigested 830-bp versican pGL4.10 construct, Lane 7 = digested 830-bp versican pGL4.10 construct

Due to detection of normoxic and hypoxic luciferase activity in the 240-bp of versican proximal promoter construct, further work was carried out to generate a deletion series of the 240-bp, by inserting shorter sequences of versican promoter into the pGL4.10 vector. Three putative clones of the 110-bp versican pGL4.10 construct (Fig 6.9) were analysed by SfiI restriction digestion and run on a 2.0% agarose gel beside an undigested 110-bp construct to confirm successful cloning. As figure 6.9 shows, only two digested purified DNA-plasmid constructs showed a band of the correct size. Also three and two selected clones of the 160-bp and 210-bp (-56bp/+154bp) constructs showed one and two positive clones respectively on 2.0% agarose gels (Fig 6.9). All digested versican promoter pGL4.10 constructs were examined against the same
undigested constructs alongside 1-Kb and 100-bp DNA ladder to confirm the correct size of insert.

**Figure 6.9:** 2% agarose gel electrophoresis of restriction digested plasmid preps of 110-bp, 160-bp and 210-bp (-56bp/+154bp) versican promoter pGL4.10 constructs. Three putative purified 110-bp and 160-bp clones and two purified 210-bp clones digested with SfiI and run with an undigested relative construct. Lane 1 = 1-Kb DNA ladder, Lane 2 = undigested 110-bp versican pGL4.10 construct, Lane 3 = digested 110-bp versican pGL4.10 construct (No insert), Lane 4 = undigested 110-bp versican pGL4.10 construct, Lane 5 = digested 110-bp versican pGL4.10 construct, Lane 6 = undigested 110-bp versican pGL4.10 construct, Lane 7 = digested 110-bp versican pGL4.10 construct, Lane 8 = 100-bp DNA ladder, Lane 9 = undigested 160-bp versican pGL4.10 construct, Lane 10 = digested 160-bp versican pGL4.10 construct (No insert), Lane 11 = undigested 160-bp versican pGL4.10 construct, Lane 12 = digested 160-bp versican pGL4.10 construct (No insert), Lane 13 = undigested 160-bp versican pGL4.10 construct, Lane 14 = digested 160-bp versican pGL4.10 construct, Lane 15 = 100-bp DNA ladder, Lane 16 = undigested 210-bp versican pGL4.10 construct, Lane 17 = digested 210-bp versican pGL4.10 construct, Lane 18 = undigested 210-bp versican pGL4.10 construct, Lane 19 = digested 210-bp versican pGL4.10 construct, Lane 20 = 100-bp DNA ladder
Also 4 selected clones of the 80-bp versican promoter pGL4.10 construct were analysed by SfiI restriction digestion against undigested 80-bp versican promoter construct on a 1.5% agarose gel. As figure 6.10 shows, three out of four digested 80-bp versican promoter pGL4.10 construct were positive and showed the correct insert size.

Analysis of four putative clones of the 130-bp, 180-bp constructs (Fig 6.11) and three putative clones of the 210-bp (-26bp/+184bp) of versican promoter pGL4.10 construct (Fig 6.12) showed three, two and three positive clones, respectively, in comparison with undigested constructs and DNA ladders.

**Figure 6.10:** 2% agarose gel electrophoresis of restriction digested plasmid preps of 80-bp versican pGL4.10 construct. Four putative purified 80-bp clones digested with SfiI and run with an undigested 80-bp construct. Lane 1 = 1-Kb DNA ladder, Lane 2 = undigested 80-bp versican pGL4.10 construct, Lane 3 = digested 80-bp versican pGL4.10 construct (No insert), Lane 4 = undigested 80-bp versican pGL4.10 construct, Lane 5 = digested 80-bp versican pGL4.10 construct, Lane 6 = undigested 80-bp versican pGL4.10 construct, Lane 7 = digested 80-bp versican pGL4.10 construct, Lane 8 = undigested 80-bp versican pGL4.10 construct, Lane 9 = digested 80-bp versican pGL4.10 construct, Lane 10 = 100-bp DNA ladder.
Figure 6.11: 2% agarose gel electrophoresis of restriction digested plasmid preps of 130-bp and 180-bp versican pGL4.10 construct. Four putative purified 130-bp and 180-bp versican pGL4.10 clones digested with SfiI and run with an undigested relative construct. Lane 1 = 100-bp DNA ladder, Lane 2 = undigested 130-bp versican pGL4.10 construct, Lane 3 = digested 130-bp versican pGL4.10 construct (No insert), Lane 4 = undigested 130-bp versican pGL4.10 construct, Lane 5 = digested 130-bp versican pGL4.10 construct, Lane 6 = undigested 130-bp versican pGL4.10 construct, Lane 7 = digested 130-bp versican pGL4.10 construct, Lane 8 = undigested 130-bp versican pGL4.10 construct, Lane 9 = digested 130-bp versican pGL4.10 construct, Lane 10 = 100-bp DNA ladder, Lane 11 = undigested 180-bp versican pGL4.10 construct, Lane 12 = digested 180-bp versican pGL4.10 construct (No insert), Lane 13 = undigested 180-bp versican pGL4.10 construct, Lane 14 = digested 180-bp versican pGL4.10 construct, Lane 15 = undigested 180-bp versican pGL4.10 construct, Lane 16 = digested 210-bp versican pGL4.10 construct (No insert), Lane 17 = undigested 180-bp versican pGL4.10 construct, Lane 19 = digested 180-bp versican pGL4.10 construct, Lane 19 = 100-bp DNA ladder
Figure 6.12: 2% agarose gel electrophoresis of restriction digested plasmid preps of 210-bp (-26bp/+184bp) versican pGL4.10 construct. Three putative purified 210-bp versican pGL4.10 clones digested with SfiI and run with an undigested 210-bp construct. Lane 1 = 100-bp DNA ladder, Lane 2 = undigested 210-bp versican pGL4.10 construct, Lane 3 = digested 210-bp versican pGL4.10 construct, Lane 4 = undigested 210-bp versican pGL4.10 construct, Lane 5 = digested 210-bp versican pGL4.10 construct, Lane 6 = undigested 210-bp versican pGL4.10 construct, Lane 7 = digested 210-bp versican pGL4.10 construct.
In order to generate a random pGL4.10 construct, 3 putative clones were digested using *SfiI* and loaded onto a 2.0% agarose gel. Digested random pGL4.10 constructs were compared to undigested construct in addition to 100-bp DNA ladder. Weak bands indicate that three positive clones had been obtained (Fig. 6.13).

**Figure 6.13:** 2% agarose gel electrophoresis of restriction digested plasmid preps of random pGL4.10 construct. Three putative purified 29-bp random pGL4.10 clones digested with *SfiI* and run with an undigested random construct. Lane 1 = HyperLadder V, Lane 2 = undigested 29-bp random pGL4.10 construct, Lane 3 = digested 29-bp random pGL4.10 construct, Lane 4 = undigested 29-bp random pGL4.10 construct, Lane 5 = digested 29-bp random pGL4.10 construct, Lane 6 = undigested 29-bp random pGL4.10 construct, Lane 7 = digested 29-bp random pGL4.10 construct.
6.4 Transfection optimisation using JetPEI™ in HMDM

As pilot experiments, several transfections using a 2.5-Kb versican promoter pGL3-basic luciferase reporter construct kindly provided by Dr Burke were carried out on HMDM. Cells were transfected with this firefly luciferase expressing reporter plasmid and co-transfected with the pRL-TK plasmid (Promega) which contains a Renilla luciferase reporter gene and serves as an internal control for normalization of transfections (See section 3.5 for transfection).

Five-day old HMDM (prepared as described in section 3.1.1) were cultured at a cell density of $2 \times 10^6$ per well in, a volume of 2ml in 6-well adherent Nunclon plates. Cells were transfected with 2 µg, 1.5 µg or 1 µg of 2.5-Kb versican promoter pGL3-basic construct and co-transfected with either 20 ng, 15 ng or 25 ng of pRL-TK plasmid, giving firefly luciferase to Renilla luciferase construct ratios of 1:100, 1:100, and 1:40, respectively, using JetPEI DNA transfection reagent (PolyPlus) (See section 3.5.1). Cells were then incubated under normoxia (20.9% O$_2$) or hypoxia (0.2% O$_2$) for 24 hours prior to luciferase assay (See section 3.5.3).

HMDM transfected with 2 µg versican promoter-plasmid/20 ng pRL-TK using 6.4 µl JetPEI reagent (using one well per transfection), expressed firefly luciferase reporter activity after 24 incubation under normoxia and hypoxia in three independent experiments with different donors (Table 6.1). Although the level of firefly luciferase expression was higher in hypoxia (overall 1.9-fold), the level of internal control Renilla luciferase expression in all three experiments was too low (in many cases below the background level obtained using only LAR II and Stop & Glo reagent, in the absence of
cell lysate (See section 3.5.3)) to be reliable. In further optimisation experiments, HMDM transfected with 1.5 µg of versican promoter-plasmid/15 ng pRL-TK using 4.8 µl JetPEI reagent (using one well per transfection) expressed versican luciferase reporter construct using three different donors in independent experiments, although no luciferase increase was observed in hypoxic HMDM. In addition, the level of Renilla luciferase expression was also close to the background level (Table 6.1). To optimise the level of Renilla luciferase expression, further experiments in HMDM from three different donors, using 1 µg DNA promoter-plasmid/25 ng pRL-TK with 3.2 µl JetPEI reagent (using one well per transfection), showed very high luciferase expression in normoxia and hypoxia, and Renilla luciferase levels well above background. As table 6.1 shows, although the level of firefly luciferase using 1 µg of versican promoter-plasmid/25 ng pRL-TK was higher in hypoxia (average 2-fold), the level of Renilla luciferase was also increased by hypoxia in all three independent experiments. Overall the pRL-TK plasmid either failed to show signals that could be distinguished from background or was affected by hypoxia, so it was not useful for normalization. In an attempt to circumvent the Renilla normalization problem, the Thermo Scientific Pierce 660 nm protein assay was used to normalize in some transfections, which is not a perfect solution but at least allowed correction for the number of cells in each well.
### Table 6.1: Transfection optimization in HMDM using different amounts of 2.5-Kb versican promoter pGL3-basic construct and JetPEI DNA transfection reagent.

HMDM were incubated under normoxia for 5 days and co-transfected with different amounts of 2.5-Kb versican promoter pGL3-basic construct and the internal control pRL-TK Renilla luciferase plasmid using different amounts of JetPEI DNA transfection reagent. Transfected cells were incubated under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 24 hours prior to luciferase assay. Renilla luciferase values were compared to the background RLU obtained using only LAR II and Stop & Glo reagent, in the absence of cell lysate. Data are from 3 independent experiments using different donors and experiments were performed in triplicate.

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<th>JetPEI DNA transfection reagent (µl)</th>
<th>Firefly Luciferase (RLU/s 20.9% O₂ 24 hrs)</th>
<th>Renilla Luciferase (RLU/s 0.2% O₂ 24 hrs)</th>
<th>Background (RLU/s)</th>
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Data are from 3 independent experiments using different donors and experiments were performed in triplicate.
6. Results

Taken as a whole, the initial experiments on the 2.5-Kb versican promoter pGL3-basic luciferase reporter construct confirmed the basal promoter activity of this construct and showed hypoxia-induced increases (2-fold). These findings led me to further dissect the versican promoter in order to try to determine the DNA sequences which are required for the expression and hypoxic regulation of versican in HMDM, by making a series of versican reporter constructs including 4.140-Kb (-3.956Kb/+184bp), 830-bp (-646bp/+184bp), 480-bp (-296bp/+184bp) and 240-bp (-56bp/+184bp) which were generated and cloned into the pGL4.10 reporter vector via the SfiI restriction site.

6.5 Effect of 24 hours hypoxia on versican promoter reporter constructs expression in HMDM

Based on the data from the initial optimisation experiments described in section 6.4, five-day old HMDM which were cultured at a cell density of 2x10^6 per well, were transfected with 1 µg of versican reporter constructs and co-transfected with 25 ng pRL-TK using 3.2 µl of JetPEI reagent (using one well per transfection). Cells were then incubated under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 24 hours prior to luciferase assay (See section 3.5).

In these experiments, the Renilla luciferase expression by the internal control plasmid pRL-TK was unfortunately not high enough above background to be useful. Therefore the “raw” (non-normalised) firefly luciferase values were plotted (Fig 6.14). Negative control promoterless plasmids, pGL3-basic and pGL4.10 (Promega), did not show luciferase expression that could be distinguished from background (data not shown).
As figure 6.14 shows, 24 hours hypoxia did not induce luciferase activity in HMDM transfected with the 4.140-Kb pGL4.10 or 480-bp pGL4.10 constructs, whereas cells transfected with 2.5-Kb pGL3-basic or 830-bp pGL4.10 constructs showed hypoxia-increased luciferase expression in addition to the 240-bp pGL4.10, the shortest versican promoter reporter construct. As expected, hypoxia significantly induced the level of luciferase expression in HMDM transfected with PGK, a hypoxia-inducible positive control luciferase reporter construct (Ameri et al., 2002). Although little hypoxia inducibility was observed after 24 hours with any construct, this data indicated that all the versican promoter constructs were functional.

![Figure 6.14: Effect of 24 hours hypoxia on versican promoter reporter construct activity in HMDM.](image-url)

**Figure 6.14: Effect of 24 hours hypoxia on versican promoter reporter construct activity in HMDM.** HMDM incubated under normal oxygen condition for 5 days, and transfected with 1 µg of versican reporter construct DNA and 25 ng of pRL-TK Renilla normalization plasmid using 3.2 µl of JetPEI reagent according to the manufacturer’s protocol. Cells were incubated under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for a further 24 hours prior to luciferase assay. Luciferase raw value data from minimum 3 independent experiments using different donors are expressed as means ± SEM. Data were further analysed for significant induction using paired two-tailed t-tests.* = p <0.05 when compared to the normoxic PGK.
6.6 Effect of 5 days hypoxia on versican promoter reporter constructs
expression in HMDM

Preliminary investigations by Staples and Burke et al., (unpublished data) had shown that versican mRNA is up-regulated much more highly by long term hypoxia (5 days) than by short term hypoxia in PBMC (Fig 1.8). In addition, my early investigation on versican protein, showed hypoxic induction of versican protein level in macrophages after 5 days (Fig 5.2, graph E). Therefore, the effect of 5 days hypoxia was investigated on HMDM transfected with versican promoter reporter constructs in addition to the PGK positive control plasmid (Fig. 6.15). Negative controls (pGL3-basic and pGL4.10 [luc2]) were also used and showed negligible expression similar to background even after 5 days (data not shown). Five-day old HMDM which were cultured at a cell density of 2x10^6 per well, were transfected with 1 µg of versican reporter constructs and co-transfected with 25 ng pRL-TK using 3.2 µl of JetPEI reagent (using one well per transfection) (See section 3.5). Cells were then incubated under normoxia (20.9% O_2) or hypoxia (0.2% O_2) for 5 days prior to luciferase assay.

As expected 5 days hypoxia significantly induced PGK luciferase expression in HMDM (Fig 6.15). In addition, all versican promoter reporter constructs including 4.140-Kb pGL4.10, 2.5-Kb pGL3-basic, 830-bp pGL4.10 and 480-bp pGL4.10 showed hypoxia-induced luciferase activity after 5 days. As figure 6.15 indicates, also 5 days hypoxia markedly induced 240-bp pGL4.10 (2.7-fold), the shortest versican reporter construct which determined me for further investigation on the versican promoter in order to elucidate the DNA sequences which are required for the hypoxic activation of versican in HMDM. Although hypoxia inducibility was observed after 5 days with any construct (Fig 6.15), overall these data confirmed that the all versican promoter luciferase
constructs are functional however the data could not be normalised by internal control due to very low level of Renilla luciferase expression from the co-transfected pRL-TK plasmid.

**Figure 6.15**: Effect of 5 days hypoxia on versican promoter reporter construct activity in HMDM. HMDM were generated from $2 \times 10^6$ per well PBMC in 6-well adherent Nunclon plates, incubated under normal oxygen condition for 5 days, and transfected with 1 µg of versican reporter construct DNA and 25ng of pRL-TK Renilla normalization plasmid using 3.2 µl of JetPEI reagent according to the manufacturer’s protocol. Cells were incubated under normoxia (20.9% $O_2$) or hypoxia (0.2% $O_2$) for a further 5 days prior to luciferase assay. Luciferase raw value data from minimum 3 independent experiments using different donors are expressed as means ± SEM. Data were further analysed for significant induction using paired one-tailed t-tests.* = p <0.05 when compared to the normoxic PGK.
To address the problem of poor Renilla luciferase expression levels, for further transfection experiments, luciferase values were normalized to the protein concentrations obtained using the Thermo Scientific Pierce 660 nm protein assay. Also, in order to minimise variability from cell plating, pipetting inconsistencies, and toxicity during transfection, PBMCs were first transfected and then split equally into adherent plates which were incubated under normoxia or hypoxia for 5 days prior to luciferase assay (See section 3.5). In addition, to solve the problem of the negative controls expressing too little luciferase to be detected, a 29-bp random nucleotide sequence cloned into pGL4.10 to use as a negative control in further transfection experiments (See section 3.3), with the intention of producing detectable signals. In addition, sequence analysing using Genomatix MatInspector and TESS software did not show any putative transcription factor binding sites within the 29-bp random nucleotide sequence.

6.7 Expression of versican promoter reporter constructs in hypoxia
As described above, high levels of luciferase activity were detected after 5 days with all the versican promoter constructs analysed, including the shortest, the 240-bp (-56bp/+184bp) construct which was markedly induced by hypoxia (2.7-fold) (Fig 6.15). Therefore, to further elucidate the DNA sequences responsible for hypoxic activity of the versican promoter in HMDM a deletion series was made to dissect the putative transcription factor binding sites (Fig 6.17) within the -56bp/+184bp construct.

Shorter derivatives including 210-bp (-56bp/+154bp), 210-bp (-26bp/+184bp), 180-bp (-26bp/+154bp), 160-bp (-56bp/+104bp), 130-bp (-26bp/+104bp), 110-bp (-
56bp/+54bp) and 80-bp (-26bp/+54bp), were generated by PCR from human genomic DNA with the appropriate sets of primers and cloned into the pGL4.10 [luc2] using standard techniques. In order to transfect PBMC, cells were isolated (See section 3.1.1) and seeded in 4ml complete Iscove’s medium at a cell density of 4×10^6 per well in 6-Well Costar ultra low attachment plates and incubated at 37°C with 5% CO₂ and 20.9% O₂ for 5 days to allow differentiation of monocytes to macrophages. After 5 days incubation, 4×10^6 PBMCs were transfected with 2 µg of versican promoter reporter construct DNA followed by 6.4 µl of JetPEI reagent (using two well per transfection) and were plated in 2 ml at a cell density of 2×10^6 per well in 6-Well Nunclon adherent plates and returned to 37°C with 5% CO₂ and 20.9% O₂ for 1 hour followed by incubation under either normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 5 days (See section 3.5.1). After 5 days, medium was removed and cells were washed twice with 1x PBS, lysed, and luciferase activities were quantified using luciferase assay reagents according to the manufacturer’s protocol (See section 3.5.3). In addition, protein concentrations were measured using the Thermo Scientific Pierce 660 nm protein assay and luciferase values were normalized to the obtained protein concentrations (See section 3.5.4).

As figure 6.16 indicates, versican promoter reporter construct -56bp/+184bp (240-bp) in addition to those carrying deletions at the 3’ end of the 240-bp starting sequence, containing the sequences -56bp/+154bp (210-bp) and -56bp/+104bp (160-bp), showed high levels of luciferase expression in both normoxia and hypoxia after 5 days. However, luciferase activity was markedly lower in the -56bp/+54bp (110-bp) construct which additionally showed significant hypoxia-induced luciferase expression.
in comparison with the random construct. Overall, these data indicate that the region +54 to +104 is important for high level expression in both normoxia and hypoxia in HMDM, although hypoxic induction was still observed with the -56bp/+54bp construct, suggesting that the region +54 to +104 is not the only region involved in hypoxic inducibility (Fig 6.16).

Next, a series of 5’ deletion constructs of the 240-bp (-56bp/+184bp) versican promoter was also generated containing the sequences -26bp/+184bp (210-bp), -26bp/+154bp (180-bp), -26bp/+104bp (130-bp) and -26bp/+54bp (80-bp) and transfected into HMDM followed by 5 days incubation under normoxia and hypoxia. As figure 6.16 shows, although hypoxia activity was significantly lower in the shortest versican reporter construct -26bp/+54bp in comparison with -56bp/+54bp, transfection analysis data demonstrated that luciferase expression is markedly lower in the constructs beginning at -26 compared to that beginning at -56, suggesting that the sequence from -56 to -26 encompasses a region required for high level activity of these versican promoter reporter constructs in HMDM. In addition, as figures 6.16 shows, although normoxic and hypoxic expression of luciferase was observed in the 29-bp random negative control construct, the luciferase activity was significantly lower than the versican promoter constructs.
Figure 6.16: Effect of 5 days normoxia and hypoxia on 240-bp (-56bp/+184bp) and deleted versican promoter reporter construct in HMDM. PBMCs were transfected with deleted versican promoter or 29-bp negative control random pGL4.10 constructs to assess the level of luciferase activity in normoxic and hypoxic HMDM. 4×10^6 PBMCs were transfected with 2 µg of versican reporter or 29-bp random constructs and plated in 2 ml at a cell density of 2×10^6 per well in 6-Well Nunclon adherent plates. Cells were then incubated under normoxia (20.9% O_2) or hypoxia (0.2% O_2) for 5 days prior to luciferase assay. Data were normalised to the obtained protein concentrations. Data from minimum 6 independent experiments each done in triplicate using cells from different donors are expressed as means ± SEM. Data were further analysed for significant induction using paired two-tailed t-tests. * = p <0.05 when compared to the random construct in hypoxia.
In addition as figure 6.17 indicates, analysis of the 240-bp versican promoter sequence using Genomatix MatInspector and TESS software revealed binding sites for a number of transcription factors which are known to be hypoxia-inducible: CREB (Leonard et al., 2008) and AP-1 at -34, AP-1 at -4 (Uenoyama et al., 2006), SP1 at +25 (Xu et al., 2000; Szalad et al., 2009), E2F at +80 (O’Connor and Lu, 2000; Cummins and Taylor, 2005) and a putative binding site at +60 for the master regulator of hypoxia-inducible gene expression, the transcription factor HIF-1 (Wenger et al., 2005).

**Figure 6.17:** Putative transcription factor binding sites within the 240bp (-56+184) versican promoter sequence. Genomatix MatInspector and TESS software revealed putative transcription binding sites for hypoxia inducible factor (HIF), cAMP responsive element binding (CREB), activator Protein 1 (AP1), SP1, nuclear factor 1 (NF-1) and E2F within the 240bp (-56+184) versican promoter sequence. The TATA box and transcription start site were identified by Naso et al., (1994).
6. Results

Overall the data first showed that region +54 to +104, which contains a putative HIF-1 binding site (Fig 6.17), is crucial for high level versican promoter activity in hypoxia in HMDM (Fig 6.16). In addition, constructs which lack the -56 to -26 region showed reduced hypoxia activity suggesting that transcription factors which bind in this region are important for versican promoter activity in HMDM. Thus, the data show that both the -56 to -26 and +54 to +104 regions are required together in the same construct for high expression from the versican promoter in hypoxic HMDM (Fig 6.16).

6.8 Summary

In this chapter, in order to determine the DNA sequences of the versican promoter which are responsible for hypoxic regulation, a 2.5-Kb versican promoter pGL3-basic construct (provided by Dr B. Burke) and a series of versican promoter pGL4.10 constructs including 4.140-Kb, 830-bp, 480-bp and 240-bp (made during this project) were transfected into HMDM. Initial transfections using the 2.5-Kb versican promoter pGL3-basic construct showed detectable levels of firefly luciferase in HMDM which were transfected with 2 µg or 1.5 µg of versican promoter construct and co-transfected with 15ng or 20ng of pRL-TK plasmid DNA respectively (Table 6.14). However, the level of Renilla luciferase expressed from the normalisation control pRL-TK plasmid was so close to the background as to make the data of no use for normalization. Further experiments using 1 µg of versican promoter pGL3-basic construct and 25 ng of pRL-TK plasmid showed increased versican luciferase expression in hypoxia (2-fold) in comparison with normoxia with a level of Renilla luciferase expression which was above background however it showed hypoxia induced increases (Table 6.14). Based on these preliminary investigations on the 2.5-
Kb versican promoter pGL3-basic luciferase reporter construct, further study analysing the raw value of luciferase expression by a series of versican promoter constructs showed basal luciferase activity in the 4.140-Kb, 830-bp, 480-bp and 240-bp versican promoter reporter constructs and confirmed that they are all functional after 24 hours or 5 days exposure to normoxia and hypoxia (Fig 6.14 and 6.15). But these data could not be normalised against an internal control due to the problematic hypoxic induction of luciferase expression by the pRL-TK plasmid. Therefore in further transfection experiments, luciferase values were normalized to the obtained protein concentrations using a protein assay. In addition, as figure 6.14 and 6.15 showed, hypoxia significantly induced PGK luciferase expression, a hypoxia-inducible positive control in HMDM whereas the level of luciferase expressed by negative controls reporter plasmids, pGL3-basic and pGL4.10 [luc2] was undetectable (data not shown). In order to have a negative control which produces detectable luciferase activity, a 29-bp random construct was transfected into HMDM.

Based on initial transfection experiments using a 240-bp construct (-56bp/+184bp), the shortest versican promoter luciferase reporter construct which showed hypoxia-induced increases (2.7-fold) after 5 days (Fig 6.15), HMDM were transfected with a deletion series of this 240-bp construct, to analyse which parts are required for high level activity of the versican promoter in hypoxia. As figure 6.16 showed, hypoxic promoter activity markedly dropped in the -56 to +54 construct compared to constructs which contain the region +54+104, indicating that the sequence from +54 to +104 is crucial for high level versican promoter activity in hypoxic HMDM. Analysis of this promoter sequence (Fig 6.17) identified several putative binding sites for
hypoxia inducible transcription factors, the most important one being HIF-1 at +60bp, the master regulator of hypoxia-inducible gene expression (Wenger et al., 2005; Semenza, 2009). The data also showed that promoter activity is significantly reduced in constructs which lack -56 to -26, suggesting that this region also contains regulatory factors which are important for versican promoter activity in hypoxic HMDM (Fig 6.16). Taken together, the data suggested that both the -56 to -26 and +54 to +104 regions are required together in the same construct for high expression from the versican promoter constructs.
Chapter 7: Results

Role of HIF-1 in regulation of versican gene expression in human monocyte-derived-macrophages

7.1 Introduction
A recent study by Asplund et al., (2010) using siRNA in THP-1 cells suggested that the hypoxic inducibility of the versican gene in human monocyte-derived macrophages (HMDM) is controlled by hypoxia inducible factor-1 (HIF-1). HIF-1, a principle mediator of angiogenesis and glycolysis (Semenza, 2003), is the main hypoxia-inducible transcription factor, and regulates numerous genes in response to hypoxic stress (Semenza, 2004). HIF-1 is a heterodimeric molecule which is composed of α and β subunits. HIF-1β is constitutively expressed and found in the nucleus whereas HIF-1α is rapidly degraded under normal oxygen levels by the ubiquitin-proteasome pathway which is directed by the tumour suppressor VHL (von Hippel–Lindau protein) (Adams et al., 2009; Semenza, 2009). In the absence of oxygen (i.e. in hypoxia) HIF-1α protein degradation is reduced in many cell types including macrophages (Talks et al., 2000; Burke et al., 2002). HIF-1α then accumulates and translocates into the nucleus where it binds to the HIF-1β subunit and transactivates certain genes via binding to the hypoxia responsive elements (HRE) in their promoters (Semenza, 2009). In addition, accumulating evidence has demonstrated that HIF-1 also responds to non-hypoxic stimuli such as lipopolysaccharide (LPS), cobalt chloride (CoCl$_2$) and the iron chelator desferrioxamine (DFO) resulting in the transcription of hypoxia-inducible genes under normal oxygen concentrations (Dery et al., 2005; Frede et al., 2006). CoCl$_2$ and DFO are chemical reagents that are commonly used to induce HIF-1α protein in normoxia (Guo
et al., 2006). Maxwell et al., (1999) and Yuan et al., (2003) demonstrated that cobalt chloride and DFO are capable of inducing HIF by stabilizing HIF-1α using mechanisms other than hypoxia.

In this project study, I investigated whether versican promoter reporter constructs containing potential HIF binding sites are induced by HIF-1α over-expression. Furthermore, the role of HIF-1 in versican mRNA expression was studied in normoxic HMDM by inducing HIF-1 using LPS, CoCl₂ and DFO treatment. In addition as previously shown the role of PI3-kinase in normoxic up-regulation of versican in SMC (Rahmani et al., 2005), here also the possible role of this pathway was investigated in hypoxic up-regulation of versican mRNA in HMDM.

7.2 HIF-1α over-expression does not affect the expression of versican promoter reporter constructs in normoxic HMDM

Versican promoter analysis to locate the HRE consensus sequences proposed by Wenger et al., (2005), identified one putative HIF binding site in the sense (position: +60, 5’-GGCGTGCG-3’) (Fig 6.17) and two in the antisense strand of the promoter (position: -507, 5’-AACGCGT-3’ and -373, 5’-TGGCGGG-3’). To investigate whether these putative HIF binding sites are induced by HIF-1, HIF-1α was over-expressed in normoxic HMDM which were co-transfected with the 2.5-Kb or 240-bp versican promoter luciferase reporter constructs. In order to transfect PBMC, cells were isolated (See section 3.1.1) and seeded in 4ml complete Iscove’s medium at a cell density of 4×10⁶ per well in 6-Well Costar ultra low attachment plates as described previously. After 5 days incubation, 2×10⁶ PBMCs were transfected with 1 µg of DNA of
versican luciferase reporter construct (2.5-Kb or 240-bp), Phosphoglycerate kinase-1 (PGK), empty pGL4.10 [luc2] or pGL3-basic plasmids and co-transfected with 300 ng of HIF-1α over-expressing plasmid (pCDNA3.1/HIF-1α; a kind gift from Professor Chris Pugh, University of Oxford) or the negative control plasmid (pCDNA 3.1 CAT) followed by 3.2 µl of JetPEI reagent (one well per transfection) according to the manufacturer’s protocol (See section 3.5). After 24 hours incubation in normoxia (20.9% O₂), medium was removed and HMDM were washed twice with 1x PBS, lysed, and luciferase activities were quantified. Luciferase values were normalized to the obtained protein concentrations (See sections 3.5.3 and 3.5.4).

As figure 7.1 indicates, HIF-1α over-expression in normoxic HMDM did not affect the expression of the 2.5-Kb (-2.316Kb/+184bp) versican promoter luciferase construct, which contains potential HIF-1 binding sites in the sense (position: +60) and antisense (position: -507 and -373) strands. In contrast, the control PGK-1 reporter construct, which is known to be regulated by HIF-1 (Carmeliet et al., 1998; Ryan et al., 1998; Ameri et al., 2002), showed significant induction in comparison with the versican reporter construct, demonstrating that the HIF-1α over-expression plasmid was functional (Fig 7.1). Since the 2.5-Kb versican promoter is cloned into the pGL3-basic reporter vector, pGL3-basic empty plasmid was used as a negative control and as expected it was not induced by HIF-1α over-expression (Fig 7.1).
7. Results

**Figure 7.1:** Effect of over-expression of HIF-1α on the 2.5-Kb (-2.316Kb+184bp) versican promoter construct in normoxic HMDM. HIF-1α was over-expressed in normoxic HMDM which were transfected with the 2.5-Kb versican promoter luciferase reporter construct to investigate whether the construct is responsive to HIF-1. 2×10⁶ PBMCs were transfected with 1 µg of 2.5-Kb versican promoter luciferase pGL3-basic construct, PGK (positive control) or empty pGL3-basic plasmid (negative control). Cells were then co-transfected with 300ng of HIF-1α over-expressing plasmid (pCDNA3.1/HIF-1α) or the negative control plasmid (pCDNA 3.1 CAT) with aid of 3.2 µl of JetPEI reagent according to the manufacturer’s protocol and were plated in 2 ml per well at a cell density of 1×10⁶ /ml in 6-Well Nunclon adherent plates. Cells were then incubated under normoxia (20.9% O₂) for 24 hours prior to luciferase assay. Data were normalised to the obtained protein concentrations. Triplicate data from 3 independent experiments using different donors are expressed as means ± SEM. The pGL3-basic value in each experiment was assigned an arbitrary value of 1. Data were further analysed for significant induction using paired two-tailed t-tests. **= p < 0.01
Similarly, as figure 6.17 showed, although the putative HIF-1 binding site within the +54 to +104 region (position: +60) is present in the 240-bp versican promoter construct, induction was not observed in normoxic HMDM co-transfected with the HIF-1α over-expressing plasmid (Fig 7.2). The pGL4.10 empty plasmid was used as a negative control because the 240-bp construct is cloned into it. In contrast, the positive control PGK-1 reporter construct, which contains HIF-1 binding sites, was significantly induced by HIF-1α over-expression (Fig 7.2). Overall this data suggest that either these HIF binding sites on the sense and antisense strand of the versican promoter are not functional HIF-1 binding sites, or hypoxic activation of versican promoter is not controlled by HIF-1.

Figure 7.2: Effect of over-expression of HIF-1α on the 240-bp (-56bp+184bp) versican promoter construct in HMDM. HIF-1α was over-expressed in normoxic HMDM which were transfected with the 240-bp the versican promoter luciferase reporter construct to investigate whether the construct is responsive to HIF-1. Experimental details as for figure 7.1. Triplicate data from 3 independent experiments using different donors are expressed as means ± SEM. The pGL4.10 value in each experiment was assigned an arbitrary value of 1. Data were further analysed for significant induction using paired two-tailed t-tests. * = p <0.05
7.3 LPS slightly induce versican mRNA expression in normoxic HMDM

Previous studies have shown that LPS induces HIF-1α mRNA and protein, as well as expression of a range of HIF-1 inducible genes including vascular endothelial growth factor (VEGF), in macrophages (Blouin et al., 2004; Oh et al., 2008). So to investigate whether LPS might induce versican mRNA, adherence-purified HMDM were treated with LPS in normoxia followed by analysis of versican mRNA levels by Real Time RT-PCR. As previously described, PBMC were cultured at a cell density of 2x10^6 per well in 6-well adherent plates and incubated for 5 days under normal oxygen condition. After 5 days incubation, adherence-purified HMDM were treated at final concentrations of 100 ng/ml with LPS from either Salmonella abortus equii (SAE) or Salmonella minnesota (MINN), two different gram negative bacteria species which both activate macrophages through TLR4 ligation (Gomes et al., 2010) (See section 1.1.2). Cells were then incubated under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for a further 18 hrs prior to RNA isolation (See section 3.4).

Treatment of HMDM with either LPS from two different bacterial species or hypoxia caused similar induction of the mRNA for the known HIF-1 up-regulated gene, VEGF, which has been shown to be induced by LPS via a HIF-1 dependent mechanism (Ramanathan et al., 2007) (Fig 7.3). However, in the same RNA samples, LPS only slightly induced versican mRNA (MINN: 2.4-fold, SAE: 1.8-fold), in contrast to hypoxia (15.6-fold) (Figure 7.4). This data suggests that hypoxic induction of versican mRNA is likely to be a HIF-1 independent mechanism.
7. Results

**Figure 7.3:** Effect of LPS on VEGF mRNA expression in comparison with hypoxia. Adherence-purified HMDM were analysed by Real Time RT-PCR to determine the effect of two different preparations of LPS (SAE: Salmonella abortus equii LPS; MINN: Salmonella Minnesota LPS) on VEGF mRNA expression in comparison with hypoxia. 2 x10^6 cells per well were treated with MINN LPS and SAE LPS at 100 ng/ml and then exposed to normoxia (20.9% O_2) or hypoxia alone (0.2% O_2) for a further 18 hours prior to RNA isolation. Data from 3 independent experiments using different donors were normalised to β-2M mRNA levels and are expressed as means ± SEM. The normoxic value in each experiment was assigned an arbitrary value of 1. Data were further analysed using paired two-tailed t-tests. * = p <0.05 compared to normoxia.

**Figure 7.4:** Effect of LPS on versican mRNA expression in comparison with hypoxia. Adherence-purified HMDM were analysed by Real Time RT-PCR to determine the effect of two different preparations of LPS (SAE: Salmonella abortus equii LPS; MINN: Salmonella Minnesota LPS) on versican mRNA expression in comparison with hypoxia. Experimental details as explained for figure 7.3. Data from 3 independent experiments and are expressed as means ± SEM. The normoxic value in each experiment was assigned an arbitrary value of 1. Data were further analysed using paired two-tailed t-tests. * = p <0.05
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7.4 Desferrioxamine induces versican mRNA in HMDM but not cobalt chloride

To further investigate whether HIF-1 is involved in the hypoxic induction of versican gene expression, adherence-purified HMDM were treated with the hypoxia mimetic agents DFO and CoCl₂ which stabilise the hypoxia-inducible α subunit of HIF-1, increasing HIF-1 protein levels in normoxia, leading to induce the expression of HIF-1 dependent hypoxia-inducible genes (Wang and Semenza, 1993b; Minchenko et al., 2002 & 2004; Mojsilovic-Petrovic et al., 2007). As previously described, PBMC were cultured at a cell density of 2x10⁶ per well in 6-well adherent plates and incubated for 5 days under normal oxygen conditions. After 5 days incubation, adherence-purified HMDM were treated with either DFO or CoCl₂ at final concentrations of 200 µM and 300 µM respectively and incubated under normoxia (20.9% O₂) or hypoxia (0.2% O₂) for a further 18 hours (See section 3.2) prior to RNA isolation followed by determination of versican, VEGF and GLUT-1 mRNA levels by real time RT-PCR.

Hypoxic inducibility of versican, VEGF and GLUT-1 was first confirmed. As expected, 18 hours hypoxia significantly induced versican (15.5-fold), VEGF (11.7-fold) and GLUT-1 mRNA (15.5-fold) (Fig 7.5). As figure 7.6 indicates, treatment of normoxic HMDM with DFO also significantly induced versican mRNA expression in normoxia (10-fold), as well as inducing the two known HIF-1 inducible control mRNAs, coding for VEGF (11-fold) and GLUT-1 (29.5-fold), in the same RNA samples. In contrast, as figure 7.7 shows, CoCl₂ markedly induced VEGF (10.6-fold) and GLUT-1 mRNAs (6.3-fold) but not versican mRNA expression, in the same RNA samples. DFO and CoCl₂ have been previously shown to induce VEGF and GLUT-1 via the induction of HIF-1 protein
(Hayashi et al., 2004; Mace et al., 2007; Lin et al., 2008). Therefore these data suggest that versican is regulated differently to VEGF and GLUT-1, via mechanisms which can be activated by hypoxia and DFO but not by CoCl₂. Since cobalt chloride is known to induce HIF-1 protein (Maxwell et al., 1999), HIF-1 is clearly not sufficient in itself to induce the versican promoter, suggesting that a HIF-1 independent mechanism up-regulates versican hypoxic induction in HMDM.

**Figure 7.5:** Effect of 18 hours hypoxia on versican, VEGF and GLUT-1 mRNA expression in adherence-purified HMDM. HMDM were analysed by Real Time RT-PCR to determine the effect of 18 hours 0.2% O₂ on versican mRNA expression in comparison with VEGF and GLUT-1. 2 x 10⁶ cells per well were exposed to normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 18 hours prior to RNA isolation. Data from minimum 4 independent experiments using different donors were normalised to β-2M mRNA levels and are expressed as means ± SEM. The normoxic value in each experiment was assigned an arbitrary value of 1. Data were further analysed using paired two-tailed t-tests. ** p < 0.01, * = p <0.05 compared to the normoxia samples.
Figure 7.6: Effect of desferrioxamine on versican, VEGF and GLUT-1 mRNA expression in adherence-purified HMDM. HMDM were analysed by Real Time RT-PCR to determine the effect of DFO on versican mRNA expression in comparison with VEGF and GLUT-1. Experimental details as explained for figure 7.5. Data were further analysed using paired two-tailed t-tests. ** p < 0.01,* = p < 0.05 compared to the normoxia samples.

Figure 7.7: Effect of cobalt chloride on versican, VEGF and GLUT-1 mRNA expression in adherence-purified HMDM. HMDM were analysed by Real Time RT-PCR to determine the effect CoCl$_2$ on versican mRNA expression in comparison with VEGF and GLUT-1. Experimental details as explained for figure 7.5. Data were further analysed using paired two-tailed t-tests.* = p <0.05 compared to the normoxia samples.
7.5 Desferrioxamine induces versican mRNA due to iron chelating

It is well known that desferrioxamine (DFO) is an iron chelating agent which binds and removes free iron from the bloodstream (Piga et al., 2003). In addition, studies have suggested that DFO stabilises HIF-α in normoxia by acting as an iron chelator that depletes the iron required for HIF-α hydroxylation (Maxwell et al., 1999; Yuan et al., 2003). Therefore following the finding of induction of versican mRNA by DFO in normoxic adherence-purified HMDM, further investigation was carried out to confirm that induction could be blocked by addition of iron. As previously described, PBMC were cultured at a cell density of 2x10^6 per well in 6-well adherent plates and incubated for 5 days under normal oxygen condition. After 5 days incubation, adherence-purified HMDM were treated simultaneously with DFO at a final concentration of 200 µM and ferric citrate at final concentrations of 100 µM, 200 µM and 400 µM (See section 3.2.5) to saturate DFO and nullify the effects of its iron-chelating activity. Cells were then incubated under normoxia (20.9% O₂) for a further 18 hours prior to RNA isolation followed by determination of versican mRNA levels by Real Time RT-PCR.

As expected, DFO treatment alone significantly induced versican mRNA (Fig 7.8) whereas versican gene expression was gradually and significantly reduced as the concentration of the iron was increased and finally reached a similar level of versican mRNA expression in untreated normoxic cells. This data shows that the induction of the versican gene by DFO was completely blocked by the addition of iron, confirming that the iron-chelating activity of DFO, rather than any other activity or contaminant, was responsible for versican induction.
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**Figure 7.8:** Effect of iron on versican mRNA induction by desferrioxamine in HMDM. Adherence-purified HMDM were analysed by Real Time RT-PCR to determine the effect of added iron on versican mRNA induction by desferrioxamine (DFO). 2 x 10⁶ cells per well were treated simultaneously with DFO at final concentration of 200 µM and ferric citrate at final concentration of 100 µM, 200 µM and 400 µM and then were incubated in normoxia (20.9% O₂) for a further 18 hours prior to RNA isolation. Data from 3 independent experiments using different donors were normalised to β-2M mRNA levels and are expressed as means ± SEM. The normoxic value in each experiment was assigned an arbitrary value of 1. Data were further analysed using paired two-tailed t-tests. *** = p <0.001 ** p < 0.01, * = p <0.05
7.6 PI3-kinase inhibitors reduce hypoxic induction of versican mRNA in adherence-purified HMDM

It has been previously reported that versican can be up-regulated in normoxic smooth muscle cells (SMC) by the PI3-kinase pathway, via accumulation of β-catenin in the nucleus and binding of a β-catenin / TCF complex to the versican promoter (Rahmani et al., 2005). In addition, several studies have shown that hypoxia activates the PI3-Kinase signaling pathway leads to induce increases of β-catenin mRNA and protein (Alvarez-Tejado et al., 2001; Liu et al., 2010) which enhances expression of various genes such as MMP7 in human primary macrophages (Deguchi et al., 2009) and IL-8 (Xu et al., 2004). To investigate the possible role of this pathway in hypoxic up-regulation of versican, HMDM were treated with two distinct specific inhibitors of PI3-kinase, LY290042 and wortmannin, to analyse their effects on hypoxia-induced increases in versican mRNA in HMDM. As previously described, PBMC were cultured at a cell density of 2x10^6 per well in 6-well adherent plates and incubated for 5 days under normal oxygen conditions. Five day matured adherence-purified HMDM were treated with LY290042 (2 µM and 5 µM) or wortmannin (200 nM) and then incubated under either normoxia (20.9% O_2) or hypoxia (0.2% O_2) for a further 18 hours (See section 3.2) prior to RNA isolation followed by determination of versican and GLUT-1 mRNA levels by Real Time RT-PCR.

As figure 7.9 indicates, reduced induction of versican mRNA by hypoxia was markedly observed in HMDM treated with two specific inhibitors of PI3-Kinase, LY294002 (2 µM: 3-fold and 5 µM: 4.5-fold reduction) and wortmannin (2.6-fold reduction) in comparison with DMSO treated cells although this data was not statistically significant.
due to high variability in hypoxic fold induction among these donors as it was earlier shown in this project (Fig 4.5). In addition, since previous studies showed that PI3-Kinase activation by hypoxia increased HIF-1α synthesis in different cell lines (Mazure et al., 1997; Chen et al., 2001; Xu et al., 2004; Lee et al., 2006), in this project, HMDM treated with PI3-Kinase inhibitors were investigated for a known HIF-1 inducible mRNA, coding for GLUT-1. As figure 7.9 shows, no reduction of hypoxic induction was observed in GLUT-1 mRNA following treatment with PI3-Kinase inhibitors, suggesting that the previous findings, showing the possible activation of PI3-K/HIF-1 pathway by hypoxia, could be confined to specific cell types. Overall this data suggests a possible role for the PI3-Kinase pathway in hypoxic up-regulation of versican mRNA in HMDM and shows that versican is affected differently by PI3-Kinase inhibitors compared to a classical HIF-1 regulated gene, GLUT-1.

Figure 7.9: Effect of PI3-kinase inhibitors on versican gene expression in adherence-purified HMDM. Versican and GLUT-1 mRNA levels were quantified by Real Time RT-PCR to determine the effect of LY294002 and wortmannin, two specific inhibitors of PI3 kinase on expression of these two genes. 2 x 10⁶ cells per well were treated with LY290042 at final concentration of 2µM and 5µM and wortmannin at a final concentration of 200nM. Inhibitors were dissolved in DMSO which the same volume (2 µl) was added to the cells as a carrier control. Cells were then exposed to normoxia (20.9% O₂) or hypoxia (0.2% O₂) for a further 18 hours prior to RNA isolation. Data from 3 independent experiments using different donors were normalised to β-2M mRNA levels and are expressed as means ± SEM. NT; Not Treated
As previously discussed, in the absence of PI3-Kinase activity, β-Catenin, a cytoplasmic phosphoprotein, is phosphorylated by GSK-3, which is targeted for degradation by the ubiquitin-proteasome system (Polakis, 2000). Several studies suggested a correlation between β-catenin accumulation in the nucleus and hypoxic activation of PI3-kinase through GSK-3β degradation (Demir et al., 2009; Du et al., 2010; Mazumdar et al., 2010). More in-depth studies showed β-Catenin can interact with transcription factors such as TCF/LEF (Clevers and van de Wetering, 1997; Cui et al., 2010) and control the transcriptional activation of various genes such as versican in smooth muscle cells (Rahmani et al., 2005). In the present study, in a preliminary investigation, I investigated the possible hypoxic up-regulation of nuclear β-catenin protein level in U937 cells, a human monocytic cell line since they are able to grow rapidly and reach a high level of density in comparison with HMDM which take 5 days to be generated from PBMC. For this experiment, U937 cells were cultured at a cell density of 5x10^6 per flask and incubated under either normoxia (20.9% O2) or hypoxia (0.2% O2) for 24 hours prior to nuclear protein extraction followed by immunoblotting for β-catenin and actin (See sections 3.6 and 3.7).

As figure 7.10 shows, hypoxia markedly up-regulated the nuclear β-catenin protein in U937 cells in comparison with normoxia, suggesting the possible involvement of this signaling pathway in hypoxic regulation of nuclear β-catenin in U937 cells. This data is in accordance with previous studies which showed hypoxia-induced increases in nuclear β-catenin in several cell lines and indicated a correlation between β-catenin accumulation and activation of PI3-kinase by hypoxia (Deguchi et al., 2009; Liu et al., 2010; Mazumdar et al., 2010).
**7. Results**

**Figure 7.10:** Effect of hypoxia on β-catenin protein expression in U937 cell nuclear extracts. Nuclear extracts were immunoblotted to assess the presence of β-catenin in normoxia and hypoxia. 5 x 10^5 cells per ml in 10 ml complete RPMI1640 were exposed to normoxia (20.9% O₂) or hypoxia (0.2% O₂) for 24 hours prior to nuclear protein extraction. 100 µg of nuclear protein was then immunoblotted for β-catenin and actin using 1:2000 and 1:4000 dilutions of primary antibody, respectively, followed by a 1:10000 dilution of secondary antibody. A blot representative of 2 independent experiments is shown.

**7.7 Summary**

In this chapter, study was first investigated whether the versican promoter was induced by HIF-1α over-expression as a previous study suggested that the hypoxia-induced increases in versican mRNA are controlled by HIF-1 (Asplund et al., 2010). As figures 7.1 and 7.2 showed, HIF-1α over-expression did not affect the expression of the 2.5-Kb or 240-bp versican promoter constructs, which both contain HIF binding sites. However, the positive control PGK-1 reporter construct, which contains HIF-1 binding sites, was strongly induced by HIF-1α over-expression in all experiments, proving that the HIF-1α over-expression construct was functional and effective at the ratios of reporter to over-expression plasmid DNA. In addition, as figure 7.4 showed, LPS treatment of HMDM slightly induced versican mRNA in contrast with the mRNA of the HIF-1 regulated gene VEGF which has been shown to be up-regulated by LPS via HIF-1.
(Ramanathan et al., 2007). To further investigate the possibility of up-regulation of versican mRNA expression by HIF-1, HMDM were treated with DFO and CoCl\(_2\), two hypoxia mimetic agents. As figures 7.6 and 7.7 showed, only DFO, and not CoCl\(_2\), significantly induced versican mRNA, whereas the HIF-1 regulated genes VEGF and GLUT-1 were up-regulated by both DFO and CoCl\(_2\), in the same RNA samples. These findings suggested that hypoxic induction of versican mRNA is regulated differently to VEGF and GLUT-1, via mechanisms which can be activated by hypoxia and DFO but not by CoCl\(_2\). In addition, as figure 7.8 indicated, the level of versican mRNA induction was significantly dropped when the iron concentration was increased, strongly suggesting that the iron chelating activity of DFO induced versican mRNA in HMDM, rather than any other activity, or chemical or microbial contaminant. Further investigation, using two inhibitors of the PI3-kinase pathway, LY294002 and wortmannin, markedly reduced induction of versican mRNA by hypoxia (Fig 7.9), suggesting a role for PI3-kinase in hypoxic up-regulation of versican mRNA in adherence-purified HMDM. Finally, accumulation of β-catenin protein was shown in the nucleus of hypoxic U937 cells (Fig 7.10) that it is in line with previous studies which showed hypoxia induced nucleus accumulation of β-catenin in different cell lines (Liu et al., 2010; Mazumdar et al., 2010).
Chapter 8: Discussion

Versican is a large extracellular matrix proteoglycan which is expressed from the early stages of development (Westergren-Thorsson et al., 1998) through to adult tissues (Perides et al., 1992; Zimmermann et al., 1994). Versican expression is also elevated in pathological sites, including in healing wounds (Landolt et al., 1995; Theocharis et al., 2000) and in a variety of tumours such as breast (Kischel et al., 2010) brain (Schwartz and Domowicz, 2004) and melanoma (Domenzain et al., 2003). On the other hand, 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). In addition, several studies have demonstrated that versican expression is related to tumour progression in many malignant tumours (Ricciardelli et al., 1998; Touab et al., 2002; Voutilainen et al., 2003) where hypoxia is present. Furthermore macrophages also accumulate in atherosclerotic plaques in which both hypoxia and high versican expression have been reported (Husain et al., 1999; Talusan et al., 2005; Kenagy et al., 2006). Moreover unpublished data by Staples and Burke et al., (B. Burke, personal communication) showed that hypoxia produced very high versican mRNA fold inductions in macrophages (data not shown). Taking into account the above findings linking disease outcomes with macrophages, hypoxia, and versican expression, in this study I set out to establish how versican expression is regulated by hypoxia in macrophages, principally by a thorough deletion analysis of the versican promoter, which has not previously been carried out.
A recent study by Asplund et al., (2010) demonstrated that versican mRNA and protein are induced by exposure to hypoxia (0.5% O₂) for 24 hours in primary HMDM. The previously mentioned unpublished data by Staples and Burke et al., (B. Burke, personal communication) showed that extended periods of severe hypoxia (0.2% O₂, 5 days) in HMDM (data not shown) and PBMC (Fig 1.8) produce far higher versican mRNA fold inductions (140 and 700-fold respectively) than those which were obtained with overnight hypoxia (37 and 20-fold respectively) or which Asplund et al., (2010) obtained in HMDM with 24 hours hypoxia (40-fold). This finding is similar to those in our recent publication which showed that 5 days of hypoxia induced much higher mRNA levels of the pro-angiogenic cytokine VEGF and the glucose transporter GLUT-1 in PBMC compared to 24 hours hypoxia (Staples et al., 2010). No published work to date has investigated the effect of long term hypoxia on versican gene expression in primary HMDM, and compared it with short-term hypoxia. This was one of the key aims of my studies.

Initially, I investigated the effect of 18 hours hypoxia (0.2% O₂) on versican mRNA expression in HMDM from 15 different healthy donors. Substantial hypoxic induction of versican mRNA was observed in all 15 different donors after 18 hours exposure to 0.2% O₂ (Figure 4.5). Despite high variability in hypoxic fold induction among these donors (average 34-fold, range 11-125-fold), these data clearly confirmed the hypoxic up-regulation of versican mRNA in adherence-purified HMDM. In addition, since similar hypoxic inductions were observed when some of the same donors were used in further experiments, the high degree of variability in hypoxic fold induction of versican mRNA could possibly be due to single nucleotide polymorphisms (SNP) in the versican
promoter, although no studies to date have reported these. Overall this finding is similar to Asplund’s observation which showed an average of 40-fold hypoxic induction in HMDM from 8 different donors after 24 hours exposure to 0.5% O₂, although the method of blood preparation and generation of adherence purified HMDM were slightly different to this project (Asplund et al., 2010). Furthermore my experiments on versican protein expression, using intracellular flow cytometry analysis, did not show hypoxia-induced increases of versican protein after 24 hours (Fig 5.3) whereas significant induction of versican protein (3-fold) was observed after exposure to hypoxia for 5 days in PBMC-derived macrophages (Fig 5.2). These findings therefore are in line with the versican mRNA findings which suggest far higher production of versican after long term hypoxia (Fig 1.8).

Versican has been shown to form a complex with fibronectin and VEGF leading to enhanced endothelial cell adhesion, proliferation, and migration, and thus enhanced angiogenesis (Zhang et al., 1998; Zheng et al., 2004). In addition, another study has reported that versican activates macrophages through TLR2 engagement leading to the release of pro-inflammatory cytokines which promote metastatic growth (Kim et al., 2009). Versican has been shown by other studies to play important roles in cancer invasion and metastasis (Zheng et al., 2004; Ricciardelli et al., 2009). Therefore this finding of unexpectedly high up-regulation of versican in primary human macrophages exposed to long term hypoxia is biologically relevant, because macrophages entering hypoxic tissues remain there for extended periods (Lewis and Murdoch, 2005). Taken together, these findings suggest that macrophages may be a more important source of versican in hypoxic sites than previously realised.
In this project, I carried out experiments on PBMC (a mixed population of monocytes and lymphocytes) to test my hypothesis that the hypoxic up-regulation of versican mRNA is limited to macrophages. In order to compare the level of hypoxic induction of versican mRNA in macrophages and lymphocytes, monocytes (CD14⁺) were separated from lymphocytes (CD14⁻ cells) using MACS magnetic beads linked to an antibody raised to the surface antigen CD14. As figure 4.6 indicated, versican mRNA levels in CD14⁺ purified monocyte-derived macrophages were compared to adherence-purified HMDM and to lymphocytes. These data demonstrated that 18 hours of hypoxia significantly induced versican mRNA expression in adherence-purified HMDM (34-fold) and CD14⁺ monocyte-derived macrophages (25-fold) but not in lymphocytes (CD14⁻), indicating that macrophages are the principle mononuclear cells showing hypoxic up-regulation of versican mRNA.

This finding was also confirmed when I analysed versican protein expression in PBMC after both 24 hours and 5 days incubation in normoxia or hypoxia using intracellular staining followed by flow cytometry. As figures 5.4, graph C and F showed, very low versican mean fluorescent intensities were observed in lymphocytes (in the R4 region) in both normoxia and hypoxia. These findings suggest that among PBMC, versican protein expression is confined to monocyte/macrophage lineage cells and is not detectable in lymphocytes, which matches well with the low versican mRNA level, and lack of hypoxic induction, which I found in CD14⁻ cells (lymphocytes) (Fig 4.6).

It was also of interest to examine whether macrophage maturation affected versican protein expression. As figure 5.4 demonstrated, increasing monocyte/macrophage cell
size (forward scatter) on the dot-scatter plot was associated with increased versican mean fluorescent intensity in both normoxia and hypoxia; the versican mean fluorescent intensity was higher in the R1 region (large mature macrophages), in both normoxic and hypoxic macrophages, in comparison with R2 and R3 (smaller, less granular, less mature monocytes/macrophages). Since increased side and forward scatter correlate with macrophage maturation (Alexis et al., 2006; Lay et al., 2009), these novel data indicate that versican protein expression increases with monocyte to macrophage differentiation / maturation.

The recent study by Asplund et al., (2010) using immunoblotting, failed to observe induction of versican protein in HMDM after 24 hours hypoxia (0.5% O₂) in comparison with normoxia. However, Asplund et al., reported 3 to 7-fold hypoxia increases in versican bound GAG chains which were detected by [⁴⁵S]-sulphate and D-[6-³H]-glucosamine hydrochloride using indirect radioactive evaluation of the levels of versican in normoxic and hypoxic HMDM. The reasons for the conflicting data from Asplund are not clear, but one possibility is a simple failure of their immunoblotting procedure, as versican is known to be quite difficult to detect using this technique because its large, variable size and variable post-translational modifications. A second possibility is donor-to-donor variability; as can be seen from figure 4.5, hypoxic induction, at least at the mRNA level, varies markedly.

With these conflicting data from Asplund et al., in mind, a key aim of my work was to clarify the facts regarding hypoxic up-regulation of versican protein in macrophages. When I analysed my flow cytometry data, the monocyte/macrophage population,
after 24 hours in either normoxia or hypoxia, showed constitutive versican protein expression, but no hypoxia-induced increases (Fig 5.3, plots B and D). However, analysis of versican protein expression in PBMC-derived macrophages after 5 days of hypoxia indicated significant hypoxic induction of versican protein (3-fold) (Fig 5.2, plot B, D and E).

My data allow an interesting observation which was not mentioned by Asplund et al., (2010): despite the high level of versican mRNA induction by hypoxia (average 140-fold) in HMDM, the level of hypoxia-induced increases of versican protein remained very low (average 3-fold in my data, 3-7 fold in Asplund et al.,). This may be explained by previous findings showing that protein translation is reduced under hypoxia (Heerlein et al., 2005; Liu et al., 2006). Indeed, several studies have reported that hypoxia activates 4E-binding protein-1 (4E-BP1), a repressor of mRNA translation following hypoxic inactivation of eukaryotic elongation factor-2 (eEF2) which is an essential factor for protein synthesis (Tinton and Buc-Calderon, 1999; Arsham et al., 2003; Connolly et al., 2006). On the other hand, several other studies have investigated the correlation between the mRNA expression levels and protein abundance in many cell types suggesting significant correlations in mRNA changes and protein expression (Chen et al., 2002; Guo et al., 2008; Gry et al., 2009; Maier et al., 2009). In addition, a ground-breaking study by Pascal et al., (2008) using gene arrays and immunohistochemistry on different cell lines, demonstrated a poor relationship between mRNA and protein level for many genes. Therefore I conclude it is possible that versican could be one such gene, for which either the mRNA level does not
correspond closely with the protein expression level, or translation of versican is affected by hypoxia.

To summarise my versican mRNA and protein data, which I have discussed above, they provide good evidence in support of previous data on the biological role of versican, which suggested versican accumulation in atherosclerotic plaques may be mediated by macrophages (Seidelmann et al., 2008) and demonstrated co-localization of versican in poorly vascularised and hypoxic macrophage-rich areas such as carotid lesions (Asplund et al., 2010). As previously described, accumulation of macrophages has been reported in hypoxic and necrotic sites in breast cancers (Leek et al., 1999) and ovarian carcinomas (Negus et al., 1997), wounds (Crowther et al., 2001), atherosclerotic plaques (Hansson et al., 1988; Takahashi et al., 2002; Collot-Teixeira et al., 2007) and arthritic joints (Hansch et al. 1996; Hollander et al., 2001). Several reports have shown that versican is highly expressed in the above pathologies and highlighted the role of versican in wound healing (Theocharis et al., 2000 & 2002) and in vascular disease, especially atherosclerosis which is thought in part to be mediated by binding of versican to low density lipoproteins via its large number of GAG chains (Camejo et al., 2002; Khalil et al., 2004; Seidelmann et al., 2008). My results showing up-regulation of versican mRNA and protein by macrophages exposed to prolonged hypoxia provide further evidence for a likely important role for macrophages in versican accumulation in the blood vessel walls, which is believed to promote extracellular lipoprotein retention and uptake leading to foam cell formation, the key step in the atherosclerotic process (Wight and Merrilees, 2004).
Regarding the mechanism of versican mRNA induction by hypoxia, in this project I also investigated the decay of versican mRNA in normoxic and hypoxic adherence-purified HMDM to determine whether the observed up-regulation of versican mRNA by hypoxia is due to increased transcription or increased mRNA stability. Interesting studies by Chen and Shyu, (1995) and subsequently several other groups have identified specific RNA-binding proteins which can bind to adenylate-uridylate rich elements (AREs) such as AUUUUA sequences, present in 3’ untranslated regions (UTR) of many mRNAs, including growth factors and cytokines, and increase stability of the mRNA by protecting them from degradation (Chen and Shyu, 1995; Bakheet et al., 2001; Barreau et al., 2005). HuR is one such RNA-binding protein, which in addition has been found to be induced by hypoxia, and binds to the AU-rich elements of the VEGF 3’ UTR, playing a critical role in the hypoxic stabilization of VEGF mRNA (Levy et al., 1998; Goldberg-Cohen et al., 2002; Leandersson et al., 2006).

My analysis of the versican 3’UTR identified several putative AU-rich elements containing the sequence AUUUUA (Fig 8.1) which are predicted by computer analysis to be potentially bound by the HuR protein, which could lead to mRNA stabilization. Therefore I decided to investigate whether the observed up-regulation of versican mRNA by hypoxia is due to increased mRNA stability. Analysis of versican mRNA decay indicated very similar rates of versican mRNA decay in normoxic and hypoxic adherence-purified HMDM at different time points up to 36 hours (Fig 4.7). This finding shows that hypoxic induction of versican mRNA does not occur via changes in mRNA stability in hypoxia. This finding provided the justification for further analysis of
the versican promoter as an essential step on the way to elucidate the mechanism of hypoxic up-regulation of versican.

**Figure 8.1:** The versican 3′-untranslated region (UTR) from base pairs 2188-2428 is shown. The underlined letters indicate putative HuR binding sites.

In this project, in order to define the DNA sequences of the versican promoter and possible regulatory elements which are responsible for the hypoxic regulation of versican, a 2.5-Kb (-2.316Kb/+184bp) versican promoter pGL3-basic construct and a series of versican promoter pGL4.10 constructs including 4.140-Kb (-3.956Kb/+184bp), 830-bp (-646bp/+184bp), 480-bp (-296bp/+184bp) and 240-bp (-56bp/+184bp) were made and transfected into HMDM. These sequences were chosen for analysis because it has previously been shown that an 876-bp (-632/+240) piece of the versican DNA sequence constitutes an active promoter. This region contains a typical TATA box and has putative binding sites for a number of transcription factors including CREB, AP-1, AP-2, SP1 and TCF/LEF (Naso et al., 1994; Rahmani et al. 2005; Domenzain et al., 2009) some of which are known to be hypoxia inducible (Xu et al., 2000; Uenoyama et al., 2006; Leonard et al., 2008). Since previous preliminary investigations showed that versican mRNA is up-regulated much more highly by long term hypoxia (5 days) than
by short term (24 hours) in PBMC and HMDM (Staples and Burke et al., unpublished), in addition to my finding which demonstrated hypoxia-induced increases of versican protein after 5 days, I decided to investigate the effect of 24 hours as well as 5 days hypoxia on HMDM transfected with the versican promoter reporter constructs.

My initial experiments to optimise the transfection in HMDM which were co-transfected with the pRL-TK Renilla luciferase plasmid to serve as an internal control for normalization showed hypoxia-induced increases of Renilla luciferase expression which was very problematic. In accordance with my findings, a very recent study by Doran et al., (2011) examined the effect of hypoxia on luciferase reporter constructs and showed that hypoxia enhanced Renilla luciferase expressed by the pRL-TK plasmid, which demonstrates that caution must be used when interpreting transfection data obtained under hypoxic conditions. Taking this problem into account, I initially had to use the “raw” (non-normalised) firefly luciferase values for analysis of transfection experiments.

In further experiments I normalised the firefly luciferase values from the versican promoter reporter constructs to the obtained protein concentrations. My preliminary investigation analysing the non-normalised luciferase expression by the 2.5-Kb versican pGL3-basic construct, which was made by Dr Burke before I started this project, showed hypoxia-induced increases (2-fold) and confirmed the promoter activity of this construct in HMDM after 24 hours hypoxia (Table 6.1). Because of this relatively low fold induction, compared to the fold inductions of the endogenous versican mRNA obtained in RT-PCR experiments (Fig 4.5), I made a longer reporter
construct, since I hypothesized that increasing the length of 2.5-Kb versican promoter from the 5’ end may include transcription factor binding sites which are required for hypoxic up-regulation. As figure 6.14 showed, however this 4.140-Kb versican pGL4.10 construct was expressed in HMDM but no hypoxic induction was observed. This suggested that no extra hypoxic regulatory elements had been included in this longer construct in comparison with 2.5-Kb (Fig 6.14). Given the complete lack of induction, it is even possible that negative regulatory elements had been included in this longer construct. I therefore decided to make a series of constructs shorter than 2.5-Kb to examine the possibility that negative regulatory elements may be present in the promoter. Shorter constructs also have the advantage of containing fewer potential transcription factor binding sites to analyse. My further experiments using 830-bp, 480-bp and 240-bp pGL4.10 constructs as well as the 2.5-Kb pGL3-basic construct indicated that all the versican promoter constructs were functional under normoxia and hypoxia and some showed inducibility after short term hypoxia (Fig 6.14). I then studied the effect of longer hypoxia on HMDM transfected with 4.140-Kb pGL4.10, 2.5-Kb pGL3-basic, 830-bp pGL4.10 and 480-bp pGL4.10 constructs, which showed hypoxic induction of non-normalised luciferase activity in all the versican promoter reporter constructs after 5 days (Fig 6.15).

My data demonstrated that 5 days hypoxia markedly induced the shortest promoter reporter construct (240-bp pGL4.10; 2.7-fold), suggesting the possible existence of hypoxia regulatory elements in the 240-bp promoter fragment (Fig 6.15). Therefore I made a deletion series of the 240-bp (-56bp/+184bp) versican proximal promoter
luciferase reporter construct aiming to elucidate the DNA sequences responsible for hypoxic activity of the versican promoter in HMDM.

Another problem during the transfection experiments was that the level of luciferase expressed by the negative control reporter plasmids, pGL3-basic and pGL4.10, was very low and often undetectable. To solve this problem, and to act as a more comparable control for the very short versican promoter deletion constructs I made, a 29-bp random nucleotide sequence was cloned into pGL4.10 and used as a negative control. Unexpectedly, I observed hypoxia-induced changes in luciferase expression from this random construct, although the actual luciferase expression values were significantly lower than luciferase expression from the versican promoter constructs (Fig 6.16). However, these data indicated that great caution must be used when interpreting transfection data from short promoter reporter constructs obtained under hypoxic conditions.

As figure 6.16 showed, high levels of luciferase expression were observed in normoxic and hypoxic HMDM transfected with all versican promoter reporter constructs which contain the region from +54 to +104 whereas luciferase activity was markedly lower in constructs which do not contain this region, suggesting that +54 to +104 is important for high level expression of versican promoter constructs in normoxia and hypoxia. Further experiments on shorter versican promoter constructs showed that luciferase activity is noticeably lower in constructs beginning at -26 in comparison with -56, suggested that the region from -56 to -26 is also required for high level activity of versican promoter reporter constructs in normoxia and hypoxia (Fig 6.16).
Further analysis of the 240-bp (-56bp/+184bp) promoter construct, using Genomatix MatInspector and TESS software, revealed several putative binding sites for hypoxia inducible transcription factors, including CREB (Leonard et al., 2008), AP-1 (Uenoyama et al. 2006), SP-1 (Xu et al., 2000; Szalad et al., 2009), E2F (O’Connor and Lu, 2000) and most importantly one site for HIF-1, the “master regulator” of hypoxic gene expression, at position +60 (Wenger et al., 2005) (Fig 6.17). This putative HIF-1 site was not identified in the recent initial report of versican hypoxia responsiveness (Asplund et al., 2010). As mentioned above, my data demonstrated reduced hypoxic activity in the -56 to +54 construct compared to constructs which contain the region +54 to +104, which includes the putative HIF binding site at +60bp (Fig 6.17). Furthermore, my data showed that promoter activity is markedly reduced in constructs which lack -56 to -26, regardless of whether they contain the putative +60 HIF site or not, suggesting that regions which do not contain a HIF-1 binding site are still important for versican promoter activity. Overall these data showed that both the -56 to -26 and +54 to +104 regions are required together in the same construct for high expression from 240-bp versican promoter (Fig 6.16).

Taking the above data into account, I propose two possible alternative mechanisms which could mediate high level transcription of the versican promoter in hypoxia. Firstly, I speculate that two or more independent hypoxia inducible transcription factors such as CREB, AP-1, E2F or HIF-1, could bind in these two regions and induce increases in versican promoter activity. Several studies have confirmed the hypoxia inducibility of CREB (Beitner-Johnson and Millhorn, 1998; Leonard et al., 2008), AP-1 (Uenoyama et al., 2006; Sermeus et al., 2008) and E2F (O’Connor and Lu, 2000) as well
as HIF-1, the main hypoxia-inducible transcription factor in many different cell lines (Semenza et al., 1994; Wenger et al., 2005). My second hypothesis proposes that transcription factors binding in these two regions may interact to facilitate the transcription of the versican promoter in hypoxia. Such regulatory elements include AP-1 (position: -34 and -4) which can interact with NF-1 (position: +94) (Shi et al., 1999), CREB (position: -34) with HIF-1 (position: +60) (Kvietikova et al., 1995) or AP-1 with HIF-1 (Yamashita et al., 2001; Salnikow et al., 2002). Further studies are required to investigate these alternative hypotheses.

In further experiments I decided to test whether the putative HIF binding site at position +60, within the +54 to +104 region of the versican promoter, is induced by HIF-1. As Figure 7.2 showed, constitutive HIF-1α over-expression did not affect the expression of the -56bp/+184bp (240-bp) versican promoter luciferase reporter construct in normoxic HMDM, in contrast to the control PGK-1 reporter construct which is known to be regulated by HIF-1 (Carmeliet et al., 1998; Ryan et al. 1998, Ameri et al. 2002). In addition, my further analysis of the versican promoter sequence identified two other candidate HIF binding sites at positions -507 and -373 which were identical to the consensus HRE sequences proposed by Wenger et al., (2005). As figure 7.1 showed, HIF-1α over-expression in normoxic HMDM did not affect the expression of the 2.5-Kb versican promoter luciferase reporter construct, which contains all three potential HF-1 binding sites (position -507, -373 and +60). Therefore, taking these data into account, I hypothesise that:
(I) These three potential hypoxia responsive elements which have not previously been identified, are non-functional HIF-1 binding sites; several studies have reported the existence of apparently non-functional HIF-1 binding sites in the proximal promoter of different genes which are known to be induced by hypoxia. For example, a study by Kietzmann et al., (1999) demonstrated that the promoter of the hypoxia-inducible plasminogen activator inhibitor-1 (PAI-1) gene contains two potential HIF binding sites, but further investigation using HIF-1 over expression and gel shift assays confirmed the role of only one of these potential HIF binding sites in the hypoxic induction of PAI-1 and HIF-1 binding (Kietzmann et al., 1999). In addition, a study by Garayoa et al., (2000) showed hypoxic induction of adrenomedullin (AM) via a HIF-1 dependent pathway. This study also identified several HIF binding sites on the sense and antisense strands of the AM promoter and showed that luciferase promoter constructs containing HIF binding sites close to the TATA box are not inducible in response to DFO treatment, which is known to induce HIF-1 protein in normoxia, suggesting that these potential sites are non-functional (Garayoa et al., 2000).

(II) These three HIF binding sites are not sufficient for efficient induction of the versican promoter; a study by Yamashita et al., (2001) despite showing the critical role of HIF-1 in hypoxic induction of endothelin-1 (ET1), suggested that the ET-1 HIF-1 binding site alone on the promoter sequences is not sufficient to mediate transcriptional responses to hypoxia and requires the additional DNA-binding elements such as AP-1 and GATA-2 (GATA-binding transcription factor-2) in the vicinity of the HIF-1 binding site (Yamashita et al., 2001). Therefore further investigation is required to determine whether the three potential HIF-1 binding sites in the versican
promoter are functional binding sites and whether they need to interact with other factors to direct hypoxia inducible transcription of the promoter.

In addition, to investigate the possible role of HIF-1 in inducing versican promoter reporter constructs, I also studied the role of HIF-1 in the hypoxic up-regulation of endogenous versican mRNA. Previous studies have shown the up-regulation of HIF-1α mRNA and protein by LPS in monocyte/macrophage lineage cells and suggested the activation of hypoxia-regulated genes by LPS through a HIF-1 dependent pathway in normoxic cells (Blouin et al., 2004; Oh et al., 2008). Therefore I decided to investigate whether this mechanism also is involved in the up-regulation of versican mRNA. I treated normoxic adherence-purified HMDM with LPS, an inducer of HIF-1 (Blouin et al., 2004), and as figure 7.4 showed, LPS from two different bacterial species slightly induced versican mRNA, in contrast with significant induction by hypoxia, whereas adherence-purified HMDM treated with LPS or hypoxia indicated significant induction of the mRNA level of the known HIF-1 regulated gene VEGF (Fig 7.3). VEGF has been shown to be up-regulated by LPS via a HIF-1-dependent mechanism (Ramanathan et al., 2007). Therefore I conclude that hypoxic induction of versican mRNA is regulated differently to VEGF and that hypoxic induction of versican mRNA is regulated through a mechanism which is not activated by LPS stimulation under normoxia. Thus these data cast doubt on the role of HIF-1 in hypoxic up-regulation of versican in human primary macrophages, as proposed by Asplund et al., 2010. Therefore to assess this, I used DFO and CoCl₂ in further experiments to investigate the possibility of up-regulation of versican mRNA expression by HIF-1 in HMDM.
Several studies have reported that DFO and CoCl₂, two hypoxia mimetic agents, stabilise HIF-1α protein, resulting in increased HIF-1 protein in normoxia, and inducing the expression of HIF-1 dependent hypoxia-inducible genes such as VEGF and GLUT-1 (Wang and Semenza, 1993b; Minchenko et al., 2002; Yuan et al., 2003; Mojsilovic-Petrovic et al., 2007). Therefore I treated HMDM with DFO and CoCl₂ to determine whether versican mRNA expression is induced by these two agents as well as by hypoxia. I first confirmed the hypoxic inducibility of versican mRNA (15.5-fold) as well as VEGF (11.7-fold) and GLUT-1 (15.5-fold) (Fig 7.5). As figures 7.6 and 7.7 showed, I observed that only DFO, but not CoCl₂, significantly induced versican mRNA (10-fold), whereas the known HIF-1 regulated genes VEGF (DFO: 11-fold, CoCl₂: 10.6-fold) and GLUT-1 (DFO: 29.5-fold, CoCl₂: 6.3-fold) were up-regulated by both. Induction of versican mRNA by DFO but not by CoCl₂ which is known to up-regulate HIF-1α strongly, suggests that versican is regulated differently to VEGF and GLUT-1, possibly via a HIF-1 independent mechanism which can be activated by hypoxia and DFO but not by CoCl₂.

An interesting study by Vengellur et al., (2005) using microarray analysis, compared the transcriptional responses to three treatments: hypoxia, DFO and CoCl₂. Comparison between these treatments showed that some mRNAs can be up-regulated by hypoxia and DFO but not by CoCl₂, such as human monocarboxylate transporter 2 (MCT2), glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and homeodomain protein (Prox 1) (Vengellur et al., 2005). This finding is in line with my speculation of a HIF-1 independent mechanism which can be activated by hypoxia and DFO but not by CoCl₂. For example, a regulatory factor such as Prox1, a transcription factor essential for the development of numerous tissues and which has previously been shown to be
induced by hypoxia (Ota et al., 2007), and as described above has been shown by Vengellur et al., (2005) to be up-regulated by hypoxia and DFO but not by CoCl₂, could be involved in the hypoxic up-regulation of versican in human MDM.

Moreover, the iron chelating activity of DFO is well known (Maxwell et al., 1999; Yuan et al., 2003). A study by Woo et al., (2006) in a mouse macrophage cell line reported that DFO increases cyclooxygenase-2 (COX-2) mRNA and protein, an important inducible enzyme in inflammation, and showed that COX-2 protein expression was reduced by addition of iron, suggesting that the iron chelating function of DFO induced increased COX-2 expression (Woo et al., 2006).

Therefore I decided to investigate whether versican mRNA induction by DFO could be blocked by addition of iron in normoxic adherence-purified HMDM, to confirm that the iron chelating activity, and not some other activity or contaminant, was responsible for the ability of DFO to induce versican mRNA. As figure 7.8 showed, DFO alone significantly induced versican mRNA and that versican mRNA expression was gradually and significantly reduced as the concentration of iron was increased and finally reached a similar level as in untreated normoxic adherence purified HMDM. This finding is presumably due to the fact that as the concentration of iron increases DFO molecules become saturated with iron, and DFO’s activity is reduced and finally eliminated when each molecule becomes bound to iron. This data confirms that the iron chelating activity of DFO was responsible for versican induction in human MDM, rather than any other activity.
Given the lack of inducibility of versican by LPS and CoCl₂, two known inducers of HIF-1 protein, DFO presumably acts on the versican promoter by stimulation of a different pathway. Such a candidate pathway involves p38 mitogen-activated protein kinases (MAPK) which has been recently shown to be activated by DFO due to iron chelating in the human macrophage cell line THP-1 (Fan et al., 2010). Thus I hypothesise that the MAPK pathway, which has been additionally shown to be activated by hypoxia (Blaschke et al., 2002; Xu et al., 2004), may play a role in modulation of versican mRNA up-regulation in hypoxic human MDM. Further investigation is required to study the possible role of MAPK in hypoxic regulation of versican.

Taken together, the lack of induction of versican mRNA in response to the HIF-1 inducers LPS and CoCl₂ suggests that versican induction by hypoxia is mediated via a HIF-1 independent mechanism, in contrast to what has recently been published by Asplund et al., (2010). There are many examples of genes being up-regulated by hypoxia independently of HIF-1; for example previous studies by Dong et al., (2001) and Ameri et al., (2004) reported that inhibitor of apoptosis protein-2 (IAP-2) and activating transcription factor-4 (ATF-4), respectively, respond to severe hypoxia independently of the HIF-1 signalling pathway. In addition other studies using microarray analysis of murine HIF-1α null (HIF-1α−/−) fibroblasts or MCF-7 cells treated with HIF-1α siRNA showed hypoxic up-regulation of several genes in a HIF-1 independent manner (Greijer et al., 2005; Elvidge et al., 2006). However, I conclude that my current data do not completely rule out a possible role for HIF-1 in hypoxic up-regulation of versican. Further experiments to directly target HIF-1α, such as siRNA, should be carried out to elucidate whether hypoxia-induced increases of versican is via
a HIF-1 independent mechanism. HIF-1α siRNA data was shown by Asplund et al., 2010, but the data was weak as it lacked appropriate controls. Using the THP-1 macrophage cell line, they showed only a very minor reduction in versican mRNA level in hypoxia (approximately 20%) compared to approximately 40% reduction for the known HIF-1 regulated gene GLUT-1. It is known that macrophages rely heavily on HIFs for energy production and a range of other cellular activities even in normoxia (Cramer et al., 2003), and therefore many mRNAs are likely to be down-regulated non-specifically by such an approach (Bosco et al., 2006; Fang et al., 2009). Therefore the specificity of the modest reductions in hypoxic versican mRNA levels observed in the Asplund et al., siRNA experiments cannot be confirmed in the absence of suitable controls, (which were not carried out) such as evidence of a HIF-1 independent gene which is unaffected by siRNA treatment. Therefore further investigation on HMDM using siRNA to target HIF-1α and appropriate controls, which is currently taking place in the lab, needs to be carried out to completely rule out HIF-1 involvement in the hypoxic up-regulation of versican.

My data showing the possible lack of an important role for HIF-1 in hypoxic induction of versican encouraged me to further investigate alternative mechanisms which could be involved in the hypoxic regulation of versican in human MDM. A study by Rahmani et al., (2005) has demonstrated that the PI3-K / PKB pathway plays a critical role in versican mRNA expression in smooth muscle cells (SMC). This study suggested that phosphorylation (inactivation) of GSK-3β, a downstream effector of PI3-Kinase, allowed β-catenin-TCF complex formation in the nucleus and increased versican transcription in normoxia (Rahmani et al., 2005). In addition, several studies have
reported activation of the PI3-K / PKB pathway by hypoxia and shown that this mechanism is involved in the induction of target genes such as MMP-7 (Alvarez-Tejado et al., 2001; Xu et al., 2004; Deguchi et al., 2009; Liu et al., 2010). Therefore I decided to investigate the possible role of PI3-Kinase in hypoxic up-regulation of versican mRNA. As figure 7.9 showed, the PI3-Kinase inhibitor LY290042 markedly reduced the hypoxic induction of versican mRNA. Furthermore, a similar effect was observed when wortmannin, a different inhibitor of the PI3-Kinase pathway, was used. Based on this preliminary finding, I speculate that hypoxic induction of versican mRNA may be dependent, at least in part, on the PI3-Kinase pathway in HMDM.

In addition, as previously outlined, different studies have demonstrated hypoxia-induced increases of β-catenin via the PI3-Kinase pathway (Demir et al., 2009; Du et al., 2010; Mazumdar et al., 2010) which in turn results in the nuclear accumulation of β-catenin, which can bind to the TCF transcription factor on the promoter and increase expression of target genes in different cells, including human macrophages and smooth muscle cells (Polakis, 2000; Rahmani et al., 2006; Deguchi et al., 2009; Cui et al., 2010). In preliminary immunoblotting experiments to study whether nuclear β-catenin accumulation could be detected under hypoxia in my hands, I observed an increase in β-catenin in the nuclei of hypoxic human monocytic U937 cells (Fig 7.10). These findings are in line with my hypothesis that hypoxic activation of PI3-Kinase may lead to the nuclear accumulation of β-catenin, a downstream effector of the PI3-Kinase pathway and consequently cause β-catenin-TCF complex formation on the versican promoter, resulting in hypoxia-induced versican transcription in HMDM. Other studies have shown increased HIF-1 via hypoxic activation of the PI3-K / PKB
pathway in different cell lines (Chen et al., 2001; Xu et al., 2004). However, in my experiments, no decrease in the hypoxic induction of GLUT-1 mRNA, a well known HIF-1 regulated gene, was observed in HMDM treated with LY294002 or wortmannin (Fig 7.9). This finding firstly shows that possible activation of HIF-1 by the PI3-Kinase pathway in hypoxia may be confined to specific cell types, as has been previously shown by Mazure et al., (1997) and Lee et al., (2006), and secondly shows that versican is regulated differently to GLUT-1, possibly via a mechanism such as β-catenin-TCF complex formation which can be activated by PI3-Kinase pathway rather than HIF-1 in hypoxic HMDM.
Conclusion and future work

In the present study, I provided evidence that primary human macrophages are the principle peripheral blood mononuclear cell showing expression and hypoxic up-regulation of versican mRNA and protein. In addition, my data demonstrate that increases observed in versican mRNA in hypoxic adherence-purified HMDM are due to transcriptional up-regulation rather than increased mRNA stability in hypoxia. This finding led me to analyse the versican promoter by making, and transfecting primary macrophages with, a series of versican promoter deletion constructs. The data from these experiments indicated two regions which are required for high expression from the promoter in hypoxic primary human macrophages. Further experiments suggested that high-level transcription of the versican promoter in hypoxia appears to occur via a HIF-1 independent mechanism which can be activated by hypoxia and DFO not by CoCl₂. I also carried out inhibitor experiments which lead me to propose a possible role for the PI3-Kinase pathway in the hypoxic up-regulation of versican.

However, a number of further experiments are required to confirm and extend the findings presented in this thesis. As I discussed earlier, studies of versican induction in HIF-1α knockout cells or in HMDM transfected with HIF-1α siRNA are required to finally confirm whether HIF-1 is or is not responsible for the induction of versican in hypoxia. Likewise, further experiments are required to confirm whether MAPK or PI3-Kinase pathway activation by hypoxia and subsequent β-catenin-TCF complex formation are essential for versican transcription in HMDM.
In addition, site-directed mutagenesis on the regions identified in the present study using versican promoter deletion reporter constructs could be used to indicate the specific transcription factor binding-sites which are essential for high level expression from the promoter in hypoxic primary human macrophages. Electrophoretic mobility shift and supershift assays could reveal specific binding of such transcription factors to oligonucleotides corresponding to their potential binding sites in the versican promoter, and Chromatin Immunoprecipitation (ChIP) could be used to confirm the ability of these factors to bind to the promoter under hypoxic conditions in living cells.

A better understanding of how versican is regulated by hypoxia in human macrophages will hopefully be helpful for the development of future therapies for a range of different disease such as vascular disorders, including atherosclerosis, where versican accumulation plays a key role (Wight and Merrilees, 2004). As macrophages have been shown to accumulate in the areas of low oxygen tension where versican is up-regulated, the knowledge of how the versican promoter is induced by hypoxia by elucidation of the hypoxia responsive elements could be an additional advantage for future tumour gene therapy whereby a therapeutic gene could be engineered to be regulated by the hypoxia responsive promoter. Macrophages transfected with this construct could be used in the delivery of the therapeutic gene to radiotherapy and chemotherapy resistant hypoxic tumour sites where the gene would be locally induced. In addition, hypoxic up-regulation of versican by human macrophages which are recruited and retained in hypoxic and necrotic sites could be a potential prognostic factor in patients with malignants tumours.
References


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Appendix

Publication