STUDIES ON TRANSFORMING GROWTH FACTOR-β SIGNALLING AND THE REGULATION OF GENE EXPRESSION IN MACROPHAGES

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Abstract

Transforming growth factor-β (TGF-β) plays a crucial anti-atherogenic role. TGF-β classically signals through the Smad pathway but is known to activate other signalling pathways such as the mitogen-activated protein kinase (MAPK) cascades. Foam cell formation is inhibited by TGF-β through the regulation of expression of genes involved in macrophage cholesterol homeostasis. However, the molecular mechanisms underlying this regulation are yet to be fully elucidated. Studying such mechanisms may lead to identification of novel avenues for treatment of this disease and was therefore the main focus of these studies. TGF-β regulates the stability of atherosclerotic plaques through the regulation of expression of genes encoding proteins involved in the turnover of the extracellular matrix (ECM). The ADAMTS proteases cleave proteoglycans within the ECM and have recently been hypothesised to have roles in atherosclerosis. Cytokine regulation of these proteases and elucidation of the molecular mechanisms behind this regulation may enhance understanding of the roles these proteases play in atherosclerosis with a view to identifying novel avenues for treatment of this disease and was therefore an additional focus of these studies.

RT-qPCR and Western blot analysis demonstrated that TGF-β inhibited the expression of key genes involved in cholesterol uptake (LPL, SR-A, SR-B1 and CD36) and induced the expression of key genes involved in cholesterol efflux (ApoE, ABCA-1, ABCG-1) in the THP-1 cell line and identified an optimal time point for their regulation. Western blotting revealed that the Smad pathway was active in macrophages through the increased levels of phospho-Smad-2 and phospho-Smad-3 in response to TGF-β treatment. Small interfering RNA (siRNA) knockdown of Smad-2, Smad-3 and Smad-2 and -3 revealed crucial roles for the Smad pathway in the TGF-β-regulation of LPL, SR-A, SR-B1, CD36, ApoE, ABCA-1, and ABCG-1. Roles for the Smad pathway in the constitutive expression of SR-B1 and CD36 were also identified. Western blotting and non-radioactive kinase activity assays demonstrated that ERK, JNK and p38 kinase levels were induced by TGF-β in macrophages and this led to an increase in kinase activity. Individual knockdown of ERK 1/2, c-Jun and p38 kinase expression using siRNA revealed crucial roles for c-Jun and p38 kinase in the TGF-β-regulated expression of LPL, SR-A, SR-B1, CD36, ABCA-1, and ABCG-1 and a role for ERK 1/2 in the expression of CD36, ABCA-1 and ABCG-1. No discernable role for p38 kinase, c-Jun or ERK 1/2 in the expression of ApoE was identified.

Inhibition of ADAMTS-4 expression by TGF-β was observed using RT-qPCR and Western blot analysis. Knockdown of Smad and MAPK pathway components using siRNA revealed roles for the Smad pathway and for c-Jun and p38 kinase in this regulation. Transient transfection of a full length ADAMTS-4 promoter construct demonstrated that TGF-β down-regulated ADAMTS-4 promoter activity. Transfection of promoter deletion constructs located the minimal regulatory region for TGF-β action within the -506/-396 region of the ADAMTS-4 promoter and EMSA analysis demonstrated increased protein binding to this region. These studies have identified crucial roles for the Smad and MAPK pathways in the TGF-β regulation of expression of key genes involved in macrophage cholesterol homeostasis and have characterised the regulation of ADAMTS-4 by TGF-β.

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Abbreviations

Abbreviation	Full Term
ABCA-1/ABCG-1	ATP-binding cassette transporter A-1/ G-1
ACAT	Acyl-CoA: cholesterol acyltransferase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AP-1	Activator protein-1
ApoE	Apolipoprotein E
APS	Ammonium persulphate
ATP	Adenosine triphosphate
Bp	Base pairs
BSA	Bovine serum albumin
CBP	CREB (cAMP-responsive element binding protein) binding protein
cDNA	Complementary DNA
Da	Daltons
DMEM	Dubecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal regulated protein kinase
FCS	Foetal calf serum
g	Grams
g	Gravity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAT	Histone acetyltransferase
HDL	High-density lipoprotein
HI-FCS	Heat-inactivated FCS

HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
Hr	Hour
ICAM	Intercellular adhesion molecule
IFNγ	Interferon-γ
IL	Interleukin
JNK	c-Jun N-terminal kinase
Kb	Kilo bases
LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
Luc	Luciferase
LXR	Live X receptor
M/mM/nM	Molar/millimolar/nanomolar
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
Min	Minute
MMLV	Molony murine leukaemia virus
MMP	Matrix metalloproteinase
oxLDL	Oxidised low-density lipoprotein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen/strep	Penicillin/ Streptomycin
PMA	Phorbol 12-myristate 13-acetate
Pol	Polymerase
PPAR	Peroxisome proliferator activated receptor
Pro	Proline
RNA/mRNA	Ribonucleic acid/ messenger RNA
RNAi	RNA interference
RNase	Ribonuclease
Rpm	Revolutions per minute
RPMI	Rosswell park memorial institute

RT	Reverse transcription/ room temperature
RXR	Retinoid X receptor
SARA	Smad anchor for receptor activation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sec	Second
Ser	Serine
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SR-A/ SR-B1	Scavenger receptor A/B-1
SRC-1	Steroid receptor coactivator-1
TBE	Tris borate-EDTA
TE	Tris-EDTA
TEMED	N, N, N, N-tetra methyl ethylene diamine
TGF-β	Transforming growth factor-β
TGFβRI/ TGFβRII	TGF-β type 1 receptor/ type 2 receptor
Thr	Threonine
TIMP	Tissue inhibitors of metalloproteinases
TNFα	Tumour necrosis factor-α
Tyr	tyrosine
UT	untreated
UV	Ultra-violet
V	Volts
v/v	Volume/volume
VCAM	Vascular cell adhesion molecule
VLDL	Very low-density lipoprotein
VSMCs	Vascular smooth muscle cells
w/v	Weight/volume

CHAPTER 1

Introduction

1.1 Heart disease and atherosclerosis

Atherosclerosis is the primary cause of coronary heart disease. Coronary heart disease is the main cause of death in the UK and accounts for almost 94,000 deaths per year, making up almost half of all deaths from cardiovascular disease (Allender et al. 2010). The British Heart Foundation describes coronary heart disease as the narrowing of the coronary arteries that supply the heart with blood and oxygen. This process, known as atherosclerosis, is caused by a build up of fatty material (atheroma) within the walls of the arteries. Coronary heart disease often presents with angina or severe chest pains (British Heart Foundation 2006).

1.2 Atherosclerosis

Atherosclerosis was originally considered to be a lipid storage disease but it is now recognised as a form of chronic inflammation (Libby et al. 2010; Libby et al. 2002). Atherosclerosis begins as an immune response to vascular injury initiated by the accumulation of lipoproteins and other lipid aggregates in the intima of the arterial wall leading to the development of atherosclerotic lesions (Ross 1999). This widely accepted model for the disease is known as the 'response to injury' hypothesis and was first proposed by Russell Ross (Ross 1999).

Early lesions are known as fatty streaks and are made up of lipid-rich macrophage-derived foam cells (Lusis et al. 2004). Intermediate lesions are characterized by layers of foam cells, smooth muscle cells and T-lymphocytes (Ross 1999). Formation of more complex lesions, known as early atheroma, follows migration of smooth

muscle cells into the innermost layer of the artery, known as the intima (Hansson and Libby 2006; Singh and Ramji 2006). More advanced lesions are surrounded by a fibrous cap containing extracellular matrix proteins synthesized by smooth muscle cells present within the lesion. The lipid-rich core forms from the debris of foam cells that have undergone apoptosis (Lusis et al. 2004). Progression of atherosclerosis is summarised in Figure 1.1 and further described in Sections 1.2.2 to 1.6.

Although plaques can become large enough to block blood flow through the artery, the most clinically important complication of atherosclerosis is the formation of a blood clot (thrombus). This occurs on rupture or erosion of the plaque and it is this that can lead to myocardial infarction (Lusis et al. 2004). If atherosclerosis affects the arteries that supply the brain with blood and oxygen, it can cause an ischaemic stroke (Hansson and Libby 2006).

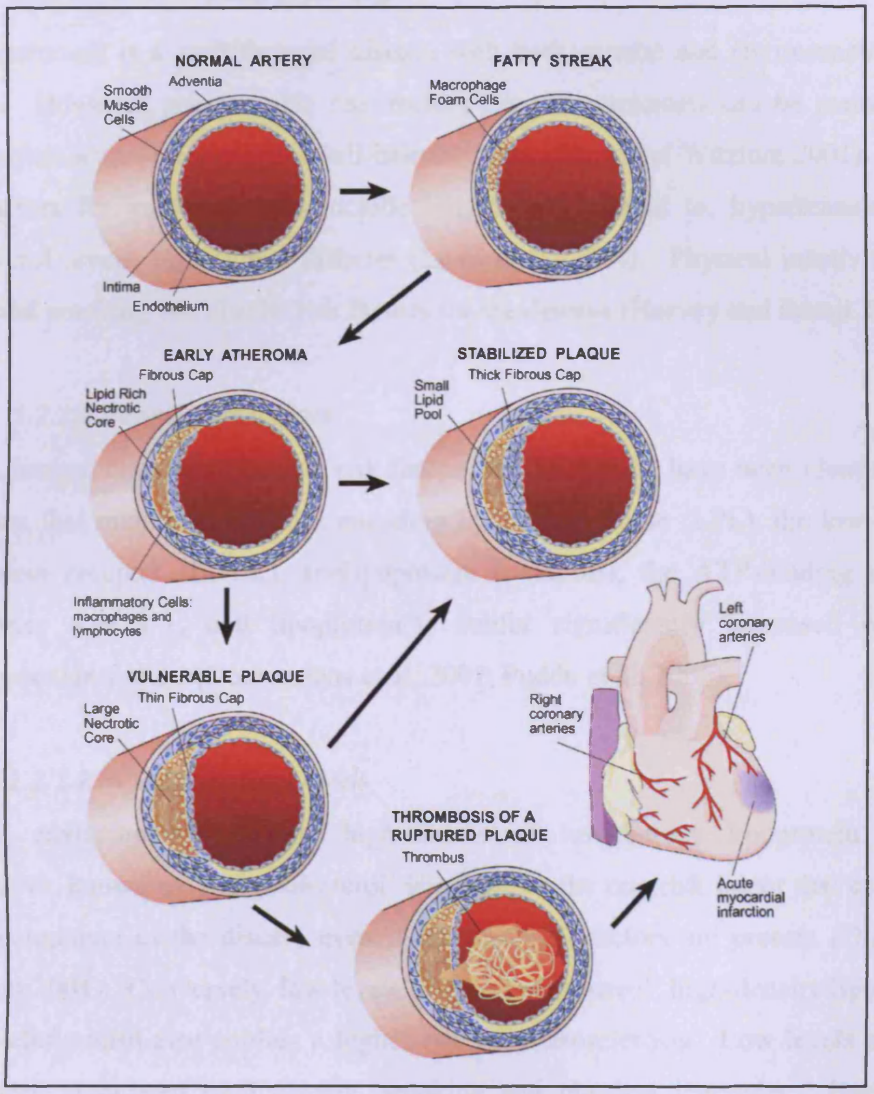

Figure 1.1 **Progression of atherosclerosis.** Regions of arterial damage attract monocytes and other inflammatory cells. Accumulation of cholesterol produces foam cells and leads to fatty streak formation. Migration of smooth muscle cells into the intima and production of a fibrous cap characterises development of more advanced lesions that become prone to rupture. For more details refer to text. Figure taken from Lusis et al. 2004.

1.2.1 Risk factors for atherosclerosis

Atherosclerosis is a multifactorial disease with both genetic and environmental risk factors. However, many single risk factors for atherosclerosis can be managed in conjunction with exercise and a well-balanced diet (Glass and Witztum 2001). Major risk factors for atherosclerosis include but are not limited to, hypertension, high cholesterol levels, obesity and diabetes (Lusis et al. 2004). Physical inactivity, age, stress and smoking can also be risk factors for the disease (Harvey and Ramji 2005).

1.2.1.1 Genetic risk factors

Only a limited number of genetic risk factors for the disease have been identified. It is known that mutations in genes encoding lipoprotein lipase (LPL), the low density lipoprotein receptor (LDLR), apolipoprotein E (ApoE), the ATP-binding cassette transporter ABCA-1, and lipoprotein(a) confer significantly increased risk for cardiovascular disease (Doevendans et al. 2001; Puddu et al. 2005).

1.2.1.2 High cholesterol levels

Of the environmental factors, high levels of low-density lipoprotein (LDL) cholesterol, known as 'bad cholesterol' is unique as the one risk factor that can drive the development of the disease even if no other risk factors are present (Glass and Witztum 2001). Conversely, low levels of 'good cholesterol' high-density lipoprotein (HDL) cholesterol also confers a higher risk of atherosclerosis. Low levels of HDL have been associated with obesity, smoking and physical inactivity. High LDL cholesterol levels cause damage to the endothelium, triggering an inflammatory response (Singh and Ramji 2006).

1.2.1.3 Obesity

Many of the risk factors for atherosclerosis and stroke can be related to lipoprotein metabolism. While the link between obesity and an increased risk of cardiovascular disease is clear, the mechanisms explaining this link remain poorly understood (Stocker and Keaney 2004). Obesity is known to predispose other risk factors for atherosclerosis, including diabetes. Studies suggest that high levels of fatty acids can stimulate the synthesis of very low-density lipoprotein (VLDL) by hepatocytes. This

in turn causes a reduction in HDL by exchange through the cholesterol ester transfer protein (Libby et al. 2002). Low plasma levels of HDL-cholesterol are also linked with smoking and lack of exercise (Doevendans et al. 2001).

1.2.1.4 Diabetes

The risk of atherosclerosis is 3-5 fold higher in diabetic patients (Stocker and Keaney 2004). Non-insulin dependent diabetes is caused by insufficient levels of insulin or defects in insulin signalling and results in hyperglycaemia. This can cause proteins to become modified leading to the production of advanced glycation end products. These modified proteins can then elicit the production of pro-inflammatory cytokines. In addition to this, the modified proteins can react with reactive oxygen species causing damage to the arterial wall and accelerating the development of atherosclerosis (Libby et al. 2002; Lusis et al. 2004).

1.2.1.5 Hypertension

Hypertension is a known risk factor for atherosclerosis and is defined as a diastolic blood pressure above 90mmHg or a systolic blood pressure above 140mmHg (Stocker and Keaney 2004). Genetic factors contributing to hypertension remain unclear, although some linkage studies have shown a role for the angiotensin II gene in Caucasian and African-Caribbean populations. The gene encoding the angiotensin converting enzyme has also been linked with coronary heart disease; however, this has no effects on blood pressure. The renin-angiotensin pathway may play a role in mediating the effects of high blood pressure on atherosclerosis (Lusis et al. 2004).

1.2.1.6 Infections

Both intravascular and chronic extravascular infections such as gingivitis and bronchitis may accelerate the development of atherosclerotic plaques through stimulation of inflammatory cytokines. Bacterial infections such as *Chlamydia pneumoniae* are also linked to atherosclerosis. The presence of bacteria and subsequent release of heat shock proteins and endotoxins such as lipopolysaccharide (LPS) stimulate the production of pro-inflammatory mediators (Libby et al. 2002).

1.2.2 Initiation of atherosclerosis

Damage to the endothelial wall from risk factors is thought to be mediated through oxidative stress, leading to changes in the permeability of the endothelium (Preiss and Sattar 2007). Under physiological conditions, pro-atherogenic factors in the vascular wall are balanced by the production of nitric oxide (NO) by endothelial cells (Preiss and Sattar 2007). NO has anti-atherogenic actions including vasodilation and inhibition of monocyte adhesion. However, in atherosclerosis decreased NO production, due to the damage to the vascular endothelium, may allow pro-inflammatory sites to develop (Preiss and Sattar 2007).

Fatty streaks, typically seen at the earliest stages of atherosclerosis, form through the recruitment of monocytes and other inflammatory cells to sites of damage in the arterial wall (Ross 1993). On activation by stimuli (risk factors and oxidative stress), endothelial cells produce adhesion molecules that recruit leukocytes, of which blood monocytes are the most numerous inflammatory cells (Libby et al. 2010). On adherence of monocytes to the endothelium, pro-inflammatory chemokines induce their uptake into the intima. Monocytes differentiate into macrophages which proliferate inside the intima and release growth factors and cytokines to enhance the inflammatory process and proteases to breakdown the extracellular matrix (ECM) (Libby et al. 2010). Macrophages also take up modified LDL such as oxidised LDL (oxLDL) which accumulates in atherosclerosis. This transforms them into lipid-laden foam cells, which are characteristic of early-stage atherosclerotic lesions (Glass and Witztum 2001).

1.2.3 Monocyte recruitment and migration

Recruitment of monocytes to lesions in the arterial wall occurs initially to remove cytotoxic molecules from the site of inflammation (Glass and Witztum 2001). This is regulated by cell adhesion molecules on the surface of endothelial cells (Lusis et al. 2004). Under normal conditions the endothelium does not bind white blood cells; however, in atherosclerosis endothelial cells express adhesion molecules on their surface that are able to bind leukocytes (Bobryshev 2006). Adhesion molecules are often expressed in areas within the artery prone to the development of atherosclerosis. Areas of the artery prone to the development of atherosclerosis are often branch points

where there is disturbed flow (Bobryshev 2006). Levels of adhesion molecules may be indicative of the severity of atherosclerosis and can be correlated with risk factors including smoking and hypertension (Bobryshev 2006; Libby et al. 2002).

In response to pro-inflammatory cytokines, endothelial cells not only express adhesion molecules but also increase the permeability of the endothelium (Hansson et al. 2002). The selective permeability of the endothelium can also be affected by fluid shear stress, disrupting its role in the regulation of inflammation (Lusis et al. 2004). Stress to the arterial wall may also induce proteoglycan production, increasing the inflammatory response through the binding of modified lipoproteins (Libby et al. 2002).

Adhesion of monocytes begins with a rolling interaction mediated by selectins including L-selectin expressed on monocytes and E-selectin expressed on the endothelial surface. P-selectin is mainly expressed on platelets but also on endothelial cells (Bobryshev 2006). The three selectins share a conserved glycoprotein structure and interact with highly fucosylated ligands but also with glycoprotein ligands. Both E- and P-selectins are activated at the transcriptional level by cytokines. Nuclear factor kappa B (NFκB) seems to be important for this induction (Preiss and Sattar 2007).

The rolling interaction is followed by a firm attachment to the endothelium, mediated by integrins (Li and Glass 2002). Integrins are transmembrane glycoproteins that exist in low-affinity conformations. Signals, possibly arising from changes in cellular activity, cause conformational changes to allow high affinity interactions with ligands. β1 and β2 integrins on monocytes interact with ligands of the immunoglobulin superfamily, namely the adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Preiss and Sattar 2007).

VCAM-1 is expressed by smooth muscle cells and endothelial cells and is present in fatty streaks (Preiss and Sattar 2007). In addition, the expression of VCAM-1 is up-regulated in response to oxLDL (Li and Glass 2002) while VCAM-1 deficient mice display reduced atherosclerosis (Ramji et al. 2006). Mice deficient in endothelial selectin and platelet selectins also develop less severe atherosclerosis. Endothelial

expression of VCAM-1 is followed by smooth muscle cell expression of this adhesion molecule (Hansson and Libby 2006). Inflammatory cells move across the endothelium by diapedesis after binding to adhesion molecules (Preiss and Sattar 2007).

1.2.3.1 Roles of chemokines in monocytes migration

Chemotactic factors (or chemokines) as well as adhesion molecules mediate the entry of monocytes into the intima and can be stimulated by oxLDL (Li and Glass 2002). Chemokines are low molecular weight proteins, typically 8-10kDa (Burke-Gaffney et al. 2002). There are approximately 50 human chemokines (or chemotactic cytokines). These can be divided into 3 families based on the spacing of conserved cysteine residues (Bobryshev 2006). Chemotactic factors include monocyte chemoattractant protein 1 (MCP-1), macrophage colony stimulating factor (M-CSF), migratory inflammatory protein 1 (MIP-1), transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α) (Bobryshev 2006). The largest group is the CC chemokines, of which MCP-1 is a member. Other members of this group include MIP-1α and MIP-1β. These chemokines are often found at sites of inflammation and attract mononuclear cells (Charo and Taubman 2004).

In response to chemoattractant signals, adherent leukocytes migrate into the intima. Chemokines such as MCP-1 may be involved in this process (Hansson et al. 2002). Chemokines are able to bind to proteoglycans in addition to binding to their cognate receptors (over 20 of which have been identified). The ability to bind to proteoglycans may facilitate the recruitment of monocytes by allowing the formation of chemokine gradients across the endothelium. Some chemokines contribute to angiogenesis and smooth muscle cell migration in atherosclerosis in addition to promoting monocyte recruitment (Burke-Gaffney et al. 2002).

MCP-1 is the most extensively studied member of the CC chemokines due to its role in atherosclerosis. MCP-1 and its receptor CCR2 are expressed in atherosclerotic plaques and also independently by endothelial and smooth muscle cells (Burke-Gaffney et al. 2002; Charo and Taubman 2004). MCP-1 is likely to play a key role in recruiting monocytes to atherosclerotic lesions and its expression may be induced

through interactions between monocytes and endothelial cells (Burke-Gaffney et al. 2002; Charo and Taubman 2004). Expression of MCP-1 has been shown to be induced by oxLDL while knockout of this gene in apoE-deficient or LDL receptor (LDLR)-deficient mice results in reduced development of atherosclerosis (Glass and Witztum 2001). MCP-1 is able to attract leukocytes through a CCR-2 receptor (Hansson et al. 2002) and absence of CCR2 can inhibit atherosclerosis by restricting the entry of monocytes into the intima (Hansson and Libby 2006). MCP-1 may also play a role in the development of thrombosis in more advanced plaques through the activation of tissue factor. Studies using bone marrow transplantation have shown that overexpression of MCP-1 in vessel wall macrophages results in increased foam cell formation while mice deficient in both LDLR and MCP-1 show reduced macrophage recruitment (Burke-Gaffney et al. 2002; Charo and Taubman 2004).

1.3 Foam cell formation-Cholesterol uptake

Once at the site of lesion formation, monocytes differentiate into macrophages (Libby et al. 2002). M-CSF is produced by both endothelial and smooth muscle cells and can mediate differentiation of these cells (Hansson and Libby 2006). Differentiation leads to the up-regulation of scavenger receptor expression on the surface of macrophages. Normally, scavenger receptors function to recognise pathogens and apoptotic cells for phagocytosis by macrophages. However, in atherosclerosis macrophages are able to take up cholesterol ester-rich lipoproteins through these receptors through receptor-mediated endocytosis (Rader and Pure 2005). This process is unregulated. Once internalised, lipoproteins are targeted to the lysosome where the cholesterol esters are hydrolysed to unesterified cholesterol to be delivered to the endoplasmic reticulum to be stored in lipid droplets following conversion back to cholesterol esters by acyl-CoA: cholesterol acyltransferase-1 (ACAT-1). Accumulation of these droplets is responsible for the soap bubble-like appearance of foam cells (Rader and Pure 2005). The physiological functions of key scavenger receptors and their pathophysiological roles in atherosclerosis are detailed in Section 1.3.3.

In addition to scavenger receptors, macrophages also express toll-like receptors (TLR) on their cell surface to mediate phagocytosis and host defence (Rader and Pure 2005).

These pattern-recognition receptors are expressed by macrophages in atherosclerotic plaques and may link innate immunity and atherosclerosis. TLRs can be activated by bacterial molecules such as LPS and also by modified lipoproteins such as minimally modified LDL (mmLDL). TLR signalling can promote pro-inflammatory gene expression through activation of NFκB and promote lipid accumulation through interaction with peroxisome proliferator-activated receptors (PPARs) and liver-X-receptors (LXRs) (Rader and Pure 2005). Macrophages therefore have critical roles in the initiation and progression of atherosclerosis. The importance of macrophages is demonstrated by their presence in lesions. Approximately 40% of cells present in atherosclerotic plaques express macrophage markers (Hansson and Libby 2006). The shoulder region of the plaque and the interface between the lipid rich core and the fibrous cap are particularly abundant in macrophages (Hansson and Libby 2006).

Quantity of circulating LDL is a well-known risk factor for cardiovascular disease (Glass and Witzum 2001). Mechanisms behind the entry of dietary cholesterol into the body, including absorption from the diet, are major causes of variation in cholesterol homeostasis. Under normal physiological conditions dietary cholesterol is packaged into chylomicrons and put into circulation where they accept apolipoproteins ApoC2 and ApoE from HDL to form mature chylomicrons (Daniels et al. 2009). This activates an enzyme known as lipoprotein lipase (LPL) to catalyse the hydrolysis of triglycerides to distribute fatty acids to tissues. As triglycerides and ApoC2 are lost from chylomicrons, ApoE is enriched; targeting the chylomicron remnants to the liver where lipids are hydrolysed into free fatty acids and free cholesterol for synthesis of very low-density lipoprotein (VLDL). VLDLs are processed in the same way as chylomicrons and mature into LDL (Daniels et al. 2009). Cholesterol-carrying lipoproteins are taken up through the LDL receptor into the liver/other cells or tissues (Daniels et al. 2009). Cholesterol synthesis also contributes to levels of cholesterol homeostasis. Cholesterol is synthesised in a series of steps that convert mevalonic acid into cholesterol using enzymes to catalyse each step. HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) is synthesised from acetyl-CoA and converted to mevalonic acid by HMG-CoA reductase as the first and rate-limiting step of cholesterol synthesis. Transcription of the HMG-CoA reductase gene is regulated by sterol-response element binding proteins (SREBPs) in response

to cholesterol levels in the cell providing a feedback mechanism for this pathway (Buhaescu and Izzedine 2007).

In atherosclerosis, the accumulation of cholesterol within macrophages and subsequent formation of foam cells can be enhanced by high levels of circulating LDL. LDL enters the intima by diffusion through endothelial cell junctions and it is retained through interactions between the apoB component of the lipoprotein and matrix proteoglycans (Lusis et al. 2004). Hydrolysis of lipoproteins by LPL contributes to the uptake of cholesterol by macrophages. The physiological function of LPL and its role in atherosclerosis is discussed in Section 1.3.2.

1.3.1 Oxidation of LDL

Modification of accumulated LDL through oxidation is a key step in its conversion to an atherogenic molecule (Ross 1993). Initial oxidation of LDL produces minimally modified LDL (mmLDL) that is still recognised by its native receptor. mmLDL stimulates the production and secretion of MCP-1 by smooth muscle cells and endothelial cells to promote monocyte recruitment. Immunolocalisation studies with antibodies to oxLDL demonstrate that oxLDL colocalises with macrophages in atherosclerotic lesions (Rader and Pure 2005; Stocker and Keaney 2004). Oxidation of LDL is promoted by oxygen free radicals from surrounding vascular cells and also by enzymes expressed by macrophages including myleoperoxidase, inducible nitric oxide synthase (iNOS), lipoxygenases and NADPH oxidases (Ohashi et al. 2004; Stocker and Keaney 2004). Most LDL filters through the endothelium by transcytosis but can be prevented by hydrodynamic resistance and thickness of the ECM explaining why areas of high shear stress are prone to atherosclerosis development (Newby 2000).

OxLDL is itself pro-inflammatory (Rader and Pure 2005). Oxidation of LDL generates cytotoxic by-products that can damage the vascular wall and oxLDL itself may also cause damage by initiating immune reactions as an autoantigen. Oxidative modifications to apoB can also elicit an immune response so contributing to atherogenesis (Stocker and Keaney 2004). Oxidation of LDL promotes foam cell formation by facilitating the recruitment of monocytes into the intima and enhancing

the rate of lipoprotein uptake, possibly through its own aggregation. The lipoprotein is also chemotactic for macrophages, preventing them leaving the intimal layer and further contributing to foam cell formation. It can also induce the expression of scavenger receptors and a number of pro-inflammatory genes in macrophages. The transcription factor peroxisome proliferator activated receptor-γ (PPAR-γ) may be involved in this regulation (Stocker and Keaney 2004). The importance of oxLDL in the initial events of atherosclerosis may explain why trials with antioxidants have been successful when administered at the outset of atherosclerosis in animal models but less successful in human trials where antioxidant treatment was given at later stages in the disease (Tedgui and Mallat 2006).

1.3.2 Lipoprotein lipase

The lipase family, comprising lipoprotein lipase, hepatic lipase and endothelial lipase are involved in the hydrolysis and regulation of lipid metabolism. LPL was the first of the lipases to be discovered and is consequently well studied. The gene for LPL resides on chromosome 8p22 and is 30kb in size, containing 10 exons or coding regions encoding a protein 473 amino acids in size (Hasham and Pillarisetti 2006; Merkel et al. 2002). LPL is synthesised mainly by the heart, adipose tissue and skeletal muscle and is secreted and attached to the endothelium by heparin sulphate proteoglycans (Hasham and Pillarisetti 2006; Merkel et al. 2002).

LPL hydrolyses VLDL and chylomicrons to produce free fatty acids and monoacylglycerols to be utilised by tissues (Hasham and Pillarisetti 2006). In addition to its catalytic action in lipid metabolism, LPL also has non-catalytic functions, in particular the enzyme can act as a molecular bridge between cell-surface receptors and other proteins to enhance lipoprotein uptake following hydrolysis. This was first discovered in 1975 when it was found that the enzyme remained associated with chylomicrons after their hydrolysis (Mead et al. 2002; Mead and Ramji 2002). Further studies confirmed that LPL was able to associate with a range of lipoproteins and receptors to enhance lipoprotein uptake and was also a ligand for the LDL receptor-related protein (LRP) (Mead et al. 2002; Mead and Ramji 2002).

1.3.2.1 LPL and atherosclerosis

The roles of LPL in lipid metabolism implicate the enzyme in atherosclerosis, where deregulation and retention of lipids and lipoproteins in the arterial wall can initiate inflammation and development of atherosclerotic plaques. High levels of LPL have also been observed in hypercholesterolemia and diabetes and mRNA and protein expression of the enzyme is also increased following damage to the arterial wall and upon monocyte-macrophage differentiation (Hasham and Pillarisetti 2006; Mead et al. 2002; Mead and Ramji 2002). Macrophages and smooth muscle cells are primary sources of LPL within atherosclerotic lesions (Mead and Ramji 2002; Stein and Stein 2003).

LPL was first hypothesised to be pro-atherogenic by Donald Zilversmit in 1973 who suggested that the action of the enzyme on circulating lipoproteins (chylomicrons and VLDL) would lead to high concentrations of cholesterol remnants that could contribute to development of atherosclerosis through their uptake into the arterial wall (Mead et al. 2002; Mead and Ramji 2002). This hypothesis proved correct. Accumulation of cholesterol esters and hydrolysis of VLDL to LDL undoubtedly contributes to atherosclerotic lesions as LDL undergoes oxidation by free radicals within the intima allowing uptake of both chylomicron remnants and oxLDL into macrophages through scavenger receptors, leading to enhanced foam cell formation (Mead et al. 2002; Mead and Ramji 2002).

Subsequently, Zilversmit showed that development of atherosclerotic lesions in rabbits fed a high cholesterol diet was associated with increased activity of LPL (Hasham and Pillarisetti 2006). Studies in mice confirmed that the association was due to raised levels of macrophage LPL. Mice overexpressing macrophage lipase showed accelerated atherosclerosis while mice deficient in lipases were shown to be resistant to the (high cholesterol diet-induced) disease (Hasham and Pillarisetti 2006). The hydrolysis of VLDL and chylomicrons by LPL produces smaller, cholesterol rich remnants that can be taken up by macrophages. This accumulation of cholesterol esters within macrophages is a key step in the formation of foam cells while hydrolysis of VLDL to LDL undoubtedly contributes to atherosclerotic lesions as they become oxidised by free radicals present in the intima of the artery. This modification increases the uptake of LDL by macrophage scavenger receptors to enhance foam cell development (Mead and Ramji 2002).

Interestingly, expression of macrophage LPL is associated with atherosclerosis development whilst plasma LPL has been demonstrated to exert anti-atherogenic actions suggesting that the enzyme has dual roles in atherosclerosis (Stein and Stein 2003). Significant reductions in lesion area and atherosclerosis incidence have been observed in transgenic mice and rabbits overexpressing human LPL and this is associated with lowered levels of plasma cholesterol and triglycerides (Stein and Stein 2003). Overexpression of LPL in wild-type mice also reduces atherosclerosis and can normalise the lipoprotein profiles of apoE$^{-/-}$ mice with atherogenic profiles. Cholesterol-lowering drugs such as statins and fibrates have also been shown to increase LPL expression in heart and adipose tissue (Mead and Ramji 2002).

In contrast to this, apoE$^{-/-}$ mice expressing human macrophage LPL showed significant increases in atherosclerotic lesion size and studies using mice deficient in macrophage LPL have demonstrated that atherogenic actions of LPL predominate during the early stages of the disease (Mead and Ramji 2002; Stein and Stein 2003). In addition to promoting foam cell formation through hydrolysis of VLDL and chylomicrons, LPL is also able to increase the production of smooth muscle cells to further disturb the endothelium and contribute to the development of the plaque. The capacity of LPL to act as a molecular bridge to aid binding of lipoproteins to cell surface receptors and heparan sulphate also promotes the migration of lipoproteins into the intima to promote foam cell formation (Hasham and Pillarisetti 2006; Stein and Stein 2003). The enzyme has been demonstrated to contribute to cholesterol ester uptake by vascular cells by acting as a cholesterol ester transferase and this may promote plaque formation by enhancing cholesterol uptake by vascular smooth muscle cells (VSMCs) (Stein and Stein 2003).

1.3.3 Macrophage scavenger receptors

Scavenger receptors were first identified by Brown and Goldstein in 1979; who described a binding site for acetylated LDL (AcLDL) on macrophages that mediates its uptake and degradation (de Winther et al. 2000). The general function of these receptors in clearing or 'scavenging' a broad range of ligands including apoptotic cells, anionic phospholipids, β-amyloid and pathogens such as *Staphylococcus aureas*

and *Mycoplasma pneumoniae* explains their naming as scavenger receptors (Bottalico et al. 1991; Greaves and Gordon 2009; Moore and Freeman 2006). Scavenger receptors are pattern recognition receptors which recognise pathogens through conserved pathogen associated molecular patterns. This allows the innate immune system to differentiate between 'self' and infectious 'non-self' (Kunjathoor et al. 2002; Moore and Freeman 2006). Scavenger receptors are found in organisms with both simple and complex immune systems. This conservation supports a critical role in the response to pathogens and in normal homeostasis (Febbraio and Silverstein 2007). Currently 8 classes of scavenger receptor have been identified as shown in Table 1.1 (Moore and Freeman 2006; Plüddemann and Neyen 2007).

Table 1.1 **Scavenger receptor classification**

Classification	Scavenger Receptors
Class A	SR-AI SR-AII SR-AIII MARCO (macrophage receptor with collagenous structure) SR with C-type lectin
Class B	CD36 SR-B1 SR-BII Lysosomal integral membrane protein II
Class C	Drosophila dSR-C (no human homologue identified)
Class D	CD68 Macrosialin (mouse homologue of CD68)
Class E	LOX-1 (lectin-like oxidised LDL receptor)
Class F	SREC-1 (Scavenger receptor expressed by endothelial cells) SREC-II
Class G	SR-PSOX (SR-phosphotidylserine and oxidised lipoproteins)
Class H	FEEL1 (fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain-containing scavenger receptor) FEEL2

In atherosclerosis, scavenger receptors promote atherogenesis by binding modified LDL that is not taken up by the classical LDL receptor (Greaves and Gordon 2009). Under normal physiological conditions LDL is taken up into cells through the LDLR in a process regulated by negative feedback. Uptake through scavenger receptors is unregulated. This results in foam cell formation leading to further development and progression of atherosclerosis. Of the group of scavenger receptors shown in Table 1.1, only a few have been found to be expressed in the arterial intima and have the ability to take up modified LDL. These include SR-A, CD36, CD68, lectin-like oxidised LDL receptor (LOX-1) and scavenger receptor for phosphatidylserine and oxidised lipoprotein (SR-PSOX). Of these, SR-A and CD36 have been demonstrated to be the principal players in atherogenesis (Moore and Freeman 2006).

Mice lacking both SR-A and CD36 demonstrate that these receptors are responsible for 75-90% of AcLDL and oxLDL uptake and degradation. Foam cell formation is also not evident in this knockout model. Further studies using knockouts crossed onto a hypercholesterolemic (ApoE deficient) background also show reduced formation of atherosclerotic lesions (de Villiers and Smart 1999; Kunjathoor et al. 2002; Moore and Freeman 2006; Plüddemann and Neyen 2007). It is thought that SR-A and CD36 are responsible for the uptake of different forms of modified LDL. In support of this, AcLDL and oxLDL have been shown to be trafficked to different intracellular compartments. AcLDL uptake is thought to be primarily driven by SR-A whilst oxLDL uptake is mediated through CD36. It is interesting to note that the contribution of SR-A to the uptake of oxLDL is determined by the extent of oxidation; the more oxidation, the greater the contribution of SR-A to its uptake (de Villiers and Smart 1999; Kunjathoor et al. 2002; Moore and Freeman 2006; Plüddemann and Neyen 2007).

In addition to the uptake of lipoproteins, scavenger receptors may contribute to the chronic inflammation of atherosclerosis through the promotion of pro-inflammatory signalling and macrophage activation and may have roles in macrophage retention and adhesion at sites of inflammation (Moore and Freeman 2006; Peiser and Gordon 2001). The role of scavenger receptors in the clearance of macrophages that have undergone apoptosis may also contribute to the development of atherosclerotic plaques (Moore and Freeman 2006; Peiser and Gordon 2001). The structure and

function of three principal scavenger receptors SR-A, SR-B1 and CD36 in atherosclerosis is outlined below.

1.3.3.1 SR-A

SR-A was the first scavenger receptor to be isolated and cloned in 1990. It has subsequently been shown to be present in human atherosclerotic plaques and is expressed in macrophages, vascular smooth muscle cells, endothelial cells and foam cells of lesions (Moore and Freeman 2006; Peiser and Gordon 2001). The gene for SR-A is located on chromosome 8 in humans and spans ~80kb. Three variants can be generated by alternative splicing of the same gene, SR-AI, II and III (de Winther et al. 2000). Analysis of the protein structure of SR-A has shown that the receptor consists of 6 domains. SR-A binds to LDL through its collagenous domain through recognition of the apoB protein component of the lipoprotein (Greaves and Gordon 2009; Moore and Freeman 2006; Peiser and Gordon 2001).

Studies using SR-A knockout mice in atherosclerosis susceptible backgrounds, (ApoE or LDLR deficient) showed reductions (60% and 20% respectively) in lesion size, suggesting a pro-atherosclerotic role for SR-A (Moore and Freeman 2006). A number of other studies using knockout mice models however have had varying results, with some studies showing increases in lesion area in SR-A knockout models. For example, SR-A deficient mice crossed onto an APOE3Leiden background (where mice carry a variant of the ApoE gene that results in sensitivity to diet-induced atherosclerosis and also hypercholesterolemia) showed increased lesion area following SR-A knockout. These conflicting sets of data are likely to be due to differences in the mouse models used. Interestingly, peritoneal macrophages from SR-A deficient mice have reduced levels of AcLDL and oxLDL degradation (80% and 30% respectively) but show no change in the *in vivo* clearance of modified LDL (de Winther et al. 2000; Moore and Freeman 2006; Peiser and Gordon 2001).

A number of roles for SR-A in macrophages have been identified. The clearance of apoptotic cells is an important function of macrophages in the inflammatory response and has been demonstrated to involve SR-A. A study using a monoclonal antibody against SR-A showed a 50% reduction in the uptake of thymocytes by macrophages in

the presence of the antibody. Macrophages from SR-A knockout mice also show a reduced capacity for apoptotic cell removal, however, *in vivo* studies with SR-A knockouts have not shown any change in apoptotic cell removal. This may be due to functional redundancy between scavenger receptors (de Winther et al. 2000; Moore and Freeman 2006; Peiser and Gordon 2001; Plüddemann and Neyen 2007). SR-A may also play a role in the retention and adhesion of macrophages at atherosclerotic lesions. In 1993, Fraser et al isolated an antibody that inhibited adhesion of RAW264.7 macrophages to tissue culture plastic. This antibody was subsequently identified as directed against SR-A and other studies demonstrated that it could also block adhesion of macrophages to various tissue sections (de Winther et al. 2000). SR-A may also play a role in adhesion of macrophages to the extracellular matrix of smooth muscle cells as biglycan and decorin have been identified as ligands for the receptor (Plüddemann and Neyen 2007). In addition, SR-A can modulate cytokine production as demonstrated by the high levels of pro-inflammatory cytokines in the myocardium of SR-A knockout mice as compared with wildtype mice, leading to impaired healing following myocardial infarction and lower survival rates (de Winther et al. 2000; Greaves and Gordon 2009). In summary, roles in the adherence of macrophages, binding and uptake of modified lipoproteins into macrophages and modification of macrophage activation suggest SR-A has a multi-functional role in atherosclerosis with some pro-atherogenic and some anti-atherogenic actions.

1.3.3.2 CD36

CD36 was first described as platelet glycoprotein IV in 1989 and subsequently identified as a receptor for oxLDL in 1993. The gene for CD36 encodes a protein of 471 amino acids, 88kDa in size, and is located on chromosome 7, spanning 28kb. CD36 is made up of a large extracellular domain (containing binding sites for fatty acids and oxLDL) and two transmembrane domains (Febbraio and Silverstein 2007). The receptor is expressed by a number of cell types including monocytes/macrophages, platelets, microvascular endothelial cells, cardiac and skeletal muscle and adipocytes (Febbraio and Silverstein 2007; Han et al. 1997; Moore and Freeman 2006; Nicholson et al. 2001). The receptor is able to bind to a broad range of ligands similar to SR-A but unlike SR-A it can interact with the native lipoproteins VLDL, LDL and HDL and also to fatty acids. Interestingly both SR-A

and CD36 are also able to bind the β-amyloid fibrils that characterise the plaques of Alzheimer's disease. β-amyloid fibrils have been detected in human atherosclerotic plaques and the increase in lipoprotein oxidation seen in atherosclerosis has also been observed in Alzheimer's disease (Plüddemann and Neyen 2007).

CD36 has been identified as the primary receptor for oxLDL. The role of macrophage CD36 in the uptake of oxLDL is pro-atherogenic and significantly contributes to foam cell formation (Febbraio and Silverstein 2007; Moore and Freeman 2006). Binding and degradation of oxLDL is increased 4-fold in CD36-transfected cells compared to vector-transfected controls and this binding occurs in a saturable manner (Nicholson et al. 2001). Antibodies against CD36 can inhibit binding of oxLDL by human monocyte-derived macrophages and macrophages from patients with a genetic polymorphism in the CD36 gene accumulate less cholesterol ester and bind ~40% less oxLDL than macrophages from normal control patients (Nicholson 2004; Nicholson et al. 2001). Unlike SR-A which binds to the apoprotein component of modified LDL, CD36 binds to the lipid moiety of the lipoprotein. This is evidenced by the observations that delipidated oxLDL will not bind to CD36 and binding of CD36 to oxLDL can be inhibited by anionic phospholipids. The binding of CD36 to oxLDL is mediated by oxidised phospholipids that associate with both parts of oxLDL (Nicholson 2004; Nicholson et al. 2001).

Knockout mice models have helped define the role of CD36 as a receptor for oxLDL. CD36 deficient mice show significantly less atherosclerosis than wildtype and show no foam cell formation in response to oxLDL. Mice deficient in macrophage CD36 also appear to be protected against lesion development (Febbraio and Silverstein 2007; Silverstein 2009). $CD36^{-/-}ApoE^{-/-}$ double knockout mice have 70% less lesions and also show reductions in lesion size. Re-introduction of CD36 into these mice results in lesion formation due to foam cell formation (Febbraio and Silverstein 2007; Nicholson 2004; Nicholson et al. 2001). In addition to this, $CD36^{-/-}ApoE^{-/-}$ mice and $CD36^{-/-}LDLR^{-/-}$ mice also present with significantly less macrophages in lesions. Treatment of ApoE knockout mice with a CD36 ligand (EP 80317) also showed decreased atherosclerosis. Macrophages isolated from these mice showed reduced uptake of modified LDL and up-regulation of expression of genes implicated in cholesterol efflux including ABCA1 and LXRα (Febbraio and Silverstein 2007).

Inactivation of CD36 in LDLR deficient mice fed a high cholesterol diet also show reductions in atherosclerotic lesion size. Interestingly, these mice also show higher levels of inflammation and oxidant stress and pro-inflammatory cytokine expression suggesting that in addition to hypercholesterolemia, a pro-inflammatory state also contributes to CD36-mediated atherogenesis (Kennedy et al. 2009).

Expression of CD36 can be up-regulated by oxLDL. This was first demonstrated by studies using the murine J774.2 macrophage cell line that showed that CD36 mRNA and protein expression could be up-regulated by lipoproteins, with the largest induction observed in response to oxLDL exposure. Treatment of cells with actinomycin D (an inhibitor of transcription) had no effects on mRNA decay suggesting that this effect was regulated at the transcriptional level (Nicholson 2004; Nicholson et al. 2001). The expression of CD36 in macrophages is regulated by oxLDL in a feed-forward loop where oxLDL increases CD36 expression which in turn stimulates the uptake of oxLDL resulting in foam cell formation (Nicholson et al. 2001). This loop is mediated through the transcription factor PPARγ. Studies have demonstrated that cells transfected with a PPARγ-response element attached to a reporter gene were able to increase reporter gene expression on exposure to oxLDL (Nicholson et al. 2001). On oxidation of LDL, oxidised fatty acids such as 9-hydroxyoctadecadienoic acid (HODE) and 12-HODE are formed. These are oxidised metabolites of linoleic acid and are ligands for PPARγ. PPARγ regulates the expression of a range of target genes involved in lipid metabolism and adipogenesis including CD36, by heterodimerising with the retinoid X receptor (RXR) (Nicholson et al. 2001; Silverstein 2009).

Interestingly studies using PPARγ agonists in $ApoE^{-/-}$ or $LDLR^{-/-}$ mice showed that despite increased CD36 expression, atherosclerosis development was inhibited (Nicholson 2004). Treatment of human macrophages with PPARγ agonists also does not result in increased foam cell formation. This may be due to the negative effects of PPARγ ligands/agonists on macrophage activation or through the effects of PPARγ ligands on cholesterol efflux (Nicholson 2004). Cholesterol uptake is countered by the induction in expression of genes involved in cholesterol efflux, such as the ATP-binding cassette transporter ABCA-1, through a liver X receptor (LXR) dependent pathway. PPARγ ligands are able to induce the expression of ABCA-1 by increasing

the expression of the oxysterol activated receptor LXRα, resulting in enhanced cholesterol efflux (Nicholson 2004).

The original identification of CD36 as a platelet receptor for thrombospondin-1 (TSP-1) still has relevance to cardiovascular biology. The interaction between CD36 and TSP-1 is likely to contribute to the role of the receptor in atherosclerosis and CD36 is abundantly expressed on platelets. The ability of oxLDL, but not LDL, to bind to platelets may play a role in atherosclerotic plaque thrombosis (Silverstein 2009). Exposure to oxLDL renders platelets more susceptible to platelet agonists such as collagen and thrombin and results in platelet activation and hence plaque development. Binding of oxLDL to platelets is CD36-dependent and can be blocked by an antibody to the receptor. Platelets from CD36-deficient mice also show no binding of oxLDL (Silverstein 2009). The interaction of CD36 with TSP-1 may also contribute to apoptotic cell phagocytosis, angiogenesis and the retention of macrophages at sites of vascular injury. Binding of TSP-1 by CD36 inhibits angiogenesis and causes endothelial cell apoptosis. Initial interaction of TSP-1 with CD36 is of low affinity but results in the release of phosphatases which de-phosphorylate CD36 to allow high affinity binding with TSP-1 (Draud and Lorenz 2001; Febbraio and Silverstein 2007; Plüddemann and Neyen 2007).

Absence of CD36 has been shown to have effects on fatty acid uptake by a number of tissues including the heart (Febbraio and Silverstein 2007). Under normal physiological conditions the heart uses fatty acids as a source of energy. During myocardial infarction however, the heart uses glucose as an energy source. Interestingly, this is also the case in the absence of CD36 (Febbraio and Silverstein 2007). CD36-deficient mice show higher glucose uptake and oxidation and reduced storage, and glucose has been shown to enhance CD36 expression in the J774 cell line. Insulin is also able to up-regulate CD36 suggesting a role for the receptor in insulin resistance/diabetes (Febbraio and Silverstein 2007). CD36-deficient mice do not show diabetes but are resistant to hepatic insulin while macrophages from diabetic $ApoE^{-/-}$ mice demonstrate increased uptake and accumulation of oxLDL cholesterol (Febbraio and Silverstein 2007).

1.3.3.3 SR-B1

SR-B1 (also known as CLA-1) was discovered in a study of scavenger receptor activity in CHO cells. The gene for SR-B1 is located on chromosome 12 in humans and encodes a 509 amino acid protein that contains two membrane spanning domains linked by a large extracellular region containing multiple sites for N-glycosylation. SR-B1 is expressed as an 82kDa protein with a similar structure to and sharing ~30% sequence homology with CD36. SR-B1 is expressed in the liver, steroidogenic tissues (such as the adrenal glands), tissue macrophages, monocyte-derived macrophages and a number of other cell types (de Villiers and Smart 1999; Gillotte-Taylor et al. 2001; Moore and Freeman 2006; Peiser and Gordon 2001).

In addition to being classed as a scavenger receptor SR-B1 is a HDL receptor (Gillotte-Taylor et al. 2001). HDL levels are inversely correlated with atherosclerosis risk and HDL is recognised to have a protective role in the disease through its roles in reverse cholesterol transport, endothelial cell signalling and protection against oxidative damage. Reverse cholesterol transport involves the efflux of unesterified ('free') cholesterol from tissues (such as macrophages) to the liver for excretion or recycling (see Section 1.4 for more detail). The major physiological role of SR-B1 is as a HDL receptor that mediates this binding and movement of cholesterol between HDL and cells. The exact mechanisms by which SR-B1 mediates uptake of lipids from lipoproteins and efflux of cholesterol from cells to lipoproteins are unclear (de Villiers and Smart 1999; Gillotte-Taylor et al. 2001; Moore and Freeman 2006). SR-B1 binds to HDL with high affinity through its protein component, apolipoproteins ApoE, ApoA-I and ApoC-I-ApoCIII of which ApoA-I is the most abundant. The amphipathic α-helices of ApoA-I, which are also present in the other HDL apolipoproteins, have been shown to be the recognition motif for SR-B1 binding (Moore and Freeman 2006; Plüddemann and Neyen 2007). Studies using purified SR-B1 have demonstrated that the receptor can mediate binding of HDL and lipid uptake essentially by itself with no requirement for other proteins (Trigatti et al. 2003). Deletion of SR-B1 in mice models results in increased levels of HDL and reduced cholesterol secretion by the liver leading to hypercholesterolemia, whilst overexpression of SR-B1 correlates with decreases in HDL levels (Moore and Freeman 2006).

SR-B1 mRNA has been found to be expressed in atherosclerotic plaques in both murine models and in humans. Expression is up-regulated following differentiation in macrophage cultures and is also expressed at significant levels in THP-1 macrophages (de Villiers and Smart 1999; Gillotte-Taylor et al. 2001; Moore and Freeman 2006; Zhang et al. 2003). SR-B1 is expressed by foam cells of atherosclerotic lesions and is likely to influence development of lesions through its roles in lipoprotein uptake and efflux. Macrophage SR-B1 appears to promote fatty streak formation but reduce the development of advanced plaques suggesting that SR-B1 may act in a pro- or anti-atherosclerotic way depending on context (Moore and Freeman 2006).

Studies in gene-targeted mice have demonstrated an anti-atherogenic role for both hepatic and macrophage SR-B1. This is thought to be mainly due to its role in uptake and secretion of HDL cholesterol and in reverse cholesterol transport (Trigatti et al. 2003). SR-B1 knockout mice develop significantly more atherosclerosis at the aortic sinus than wildtype mice and show impaired secretion of biliary cholesterol and accumulation of abnormally large HDL particles (Moore and Freeman 2006; Trigatti et al. 2004; Zhang et al. 2003). Other studies using SR-B1 knockout mice have observed a 2-fold increase in plasma cholesterol, reduced lipid storage in steroidogenic tissues and increased ratios of unesterified/esterified cholesterol in HDL in the absence of SR-B1. This is consistent with a role for the receptor in the removal of unesterified cholesterol from HDL (Trigatti et al. 2004).

Double knockout ($SR\text{-}B1^{-/-}$, $ApoE^{-/-}$) mice die prematurely due to development of hypercholesterolemia, accelerated coronary atherosclerosis and myocardial infarction. Deletion of SR-B1 in the $LDLR^{-/-}$ mouse model also results in a 6-fold increase in atherosclerotic lesion development. Hepatic overexpression of SR-B1 in the $LDLR^{-/-}$ mouse model suppresses atherosclerosis (Moore and Freeman 2006; Trigatti et al. 2004; Zhang et al. 2003). Taken together, these data suggests that hepatic SR-B1 has a protective (anti-atherogenic) role in atherosclerosis through its involvement in reverse cholesterol transport. Knockout of macrophage SR-B1 in an apoE knockout mouse model results in a 2-fold increase in lesion area compared to single apoE knockouts but show no changes in lipid or lipoprotein profiles (Zhang et al. 2003). This suggests that macrophage SR-B1 also plays a protective role in atherosclerosis.

A role for SR-B1 in atherosclerosis may be due to actions other than the role of the receptor in HDL cholesterol transport. Roles for SR-B1 in the clearance of LDL and ApoB-containing lipoproteins have been suggested by studies in transgenic mice (Trigatti et al. 2004). SR-B1 overexpression results in reduced LDL and VLDL cholesterol while attenuation of SR-B1 expression in $LDLR^{-/-}$ mice results in increased levels of LDL and ApoB (Trigatti et al. 2004). Cell culture studies have suggested a role for the receptor in preventing oxidative damage. Overexpression of SR-B1 increases uptake of α-tocopherol (a form of vitamin E) from HDL suggesting that the receptor may be important for uptake of this and protection against oxidative damage. A role in mediating HDL-dependent synthesis of nitric oxide in vascular endothelial cells has also been suggested as an anti-atherogenic action of SR-B1 (Trigatti et al. 2003; Zhang et al. 2003).

Alternative roles for SR-B1 may be of particular relevance in macrophages. Some of these actions may contribute to atherosclerosis development and progression. Much of the focus on SR-B1 research has been on its role as a HDL receptor, however SR-B1 deficiency does not lead to increases in ABCA-1 expression and AcLDL-loaded macrophages show no change in cholesterol efflux to HDL in the absence of SR-B1. This suggests that a role for macrophage SR-B1 in atherosclerosis does not involve cholesterol efflux (Trigatti et al. 2003; Zhang et al. 2003). Interestingly, SR-B1 deficiency can induce the expression of a number of genes involved in the adhesion and migration of monocytes in the arterial wall, suggesting there may be an enhanced inflammatory response in the absence of SR-B1 (Zhang et al. 2003).

SR-B1 is likely to have important roles as a true scavenger receptor, especially in macrophages. Similar to CD36, SR-B1 has a broad specificity for ligands including modified forms of LDL (acetylated and oxidised), native LDL, VLDL and HDL lipoproteins and anionic phospholipids (Gillotte-Taylor et al. 2001). Its similar structure to CD36, a principal player in cholesterol uptake in macrophages is suggestive of a similar role. SR-B1 has been demonstrated to be involved in the clearance of apoptotic cells and is able to bind oxLDL with high affinity (Gillotte-Taylor et al. 2001). Studies have shown that oxLDL associates with SR-B1 transfected Chinese hamster ovary (CHO) cells with a higher affinity than HDL. This binding could be competed out by oxidised LDL and to a lesser extent by oxidised

lipoproteins (Gillotte-Taylor et al. 2001). It has been suggested that SR-B1 has multiple binding sites and that both the oxidised lipid moieties and the protein moieties of oxLDL are important for its recognition by SR-B1. The oxLDL is internalised and degraded through an endocytic pathway and the ratio of binding to degradation was similar to that seen with SR-A (Gillotte-Taylor et al. 2001). Despite this finding, the exact contribution of SR-B1 to foam cell formation in macrophages remains unclear.

1.4 Foam cell formation-Cholesterol efflux

Foam cell formation in atherosclerosis can be prevented by the process of cholesterol efflux or RCT from macrophages. Reverse cholesterol transport is the process of moving cholesterol from macrophages and other cells to the liver or to other tissues (Daniels et al. 2009; Tall 2008). RCT can be grouped into three stages; firstly, cholesterol moves out of tissues, it is then transported in the plasma to the liver and it can then be excreted into the bile (Daniels et al. 2009; Tall 2008). The efficiency of RCT is determined by levels of HDL which stimulates the process (Ohashi et al. 2005).

HDL, like other lipoproteins is a spherical particle made up of a core of cholesterol and triglyceride surrounded by a monolayer of phospholipids punctuated by apolipoproteins; predominantly ApoA-I but also ApoC-I, ApoC-II, ApoC-III and ApoE (Daniels et al. 2009; Tabat and Rye 2009). Recycling of ApoA-I is important for HDL metabolism. Production of ApoA-I by the liver and its interaction with phospholipids and unesterified cholesterol forms discoidal HDL particles (Ohashi et al. 2005; Tall 2008). Discoidal HDL triggers cholesterol efflux from macrophages and other cells. Cholesterol from these cells is absorbed by the discoidal HDL and esterified by lecithin:cholesterol acyltransferase (LCAT) (Ohashi et al. 2005; Tall 2008). Accumulation of cholesterol esters inside HDL transforms them into larger, spherical HDL particles, normally observed in the plasma. This process requires the phospholipid transfer protein (PTP) (Tabat and Rye 2009). HDL enriched with triglyceride are processed by hepatic lipase while HDL enriched with cholesterol esters is processed by the cholesterol ester transport protein (CETP) which exchanges

cholesterol esters for triglycerides from other lipoproteins (VLDL and chylomicrons). Remnants are then taken up into the liver through the LDL receptor or through proteoglycans. Cholesterol in the liver can be utilised for a number of purposes or can be excreted into the bile via ABCG5 and ABCG8 transporters (Daniels et al. 2009; Ohashi et al. 2005; Tall 2008).

Key molecules involved in reverse cholesterol transport from macrophages include ABCA-1, ABCG-1 and ApoE. These proteins and their roles in atherosclerosis are discussed in detail in Sections 1.4.1 and 1.4.2 Whilst ABCA-1 promotes efflux of cholesterol to lipid-poor apoA-I and HDL formation in the liver, ABCG-1 promotes cholesterol efflux to HDL particles where it is esterified by LCAT without influencing overall levels of HDL (Tall 2008). Both ABCA-1 and ABCG-1 are targets of LXR, a transcription factor activated in response to accumulation of oxysterols in cells, suggesting a feedback mechanism for RCT. The exact mechanism of RCT and the relationships between key factors involved in RCT is currently unclear (Ohashi et al. 2005; Tall 2008).

In addition to its role in RCT, HDL has been demonstrated to have anti-inflammatory, anti-thrombotic and anti-apoptotic effects that are likely to contribute to its anti-atherogenic nature. A number of studies have demonstrated that HDL inhibits monocyte recruitment into the intima by inhibiting the pro-inflammatory cytokine-induced expression of adhesion molecules by endothelial cells and the expression of MCP-1 (Tabat and Rye 2009; Tall 2008). Oxidation of LDL and oxLDL-induced macrophage apoptosis is also inhibited by HDL. This action may be mediated through an inhibition of ROS production and restoration of vascular endothelial function by increasing endothelial NOS to increase NO availability, so inhibiting VSMC proliferation and monocyte adhesion to the endothelium (Tabat and Rye 2009; Tall 2008).

1.4.1 Apolipoprotein E

ApoE was first described in the 1970s by Shore and Shore. On its discovery it was found to be a component of VLDL, however, it has now been recognised as a component of a range of lipoproteins including chylomicrons and HDL (Greenow et

al. 2005; Horejsí and Ceska 2000). ApoE is a glycoprotein of 299 amino acids. It contains two domains linked by a protease sensitive loop and has recognition sites for the LDLR and the LRP. Binding sites in the N terminal region have been identified for heparin sulphate proteoglycans (HSPGs) and SR-B1 while the C terminal region of the protein is important for binding to lipids and has an α helical structure conserved throughout lipid binding apolipoproteins (Greenow et al. 2005; Mahley and Rall 2000).

The ApoE gene is located on chromosome 19 and encodes a 34kDa protein. Regulation of gene expression is complex with tissue specific regulation observed. The expression of the protein can be enhanced by factors including TGF-β, TNF-α and also by the differentiation of monocytes into macrophages. The cytokines IL-1 and IFN-γ both down-regulate ApoE expression (Greenow et al. 2005; Harvey and Ramji 2005; Kockx et al. 2008). Three common isoforms of apoE, derived from a single gene locus, exist and are known as apoE2, apoE3 and apoE4. The three allele products differ by a single amino acid substitution. The products of three alleles produce six phenotypes overall and the most common of these is the apoE3/3 phenotype. The isoforms show specificity in binding ability to both lipids and receptors. ApoE2 binds with only very low affinity to the LDL receptor while ApoE3 and ApoE4 bind with higher affinity. ApoE4 has high affinity for VLDL, a lipoprotein rich in triglyceride, while ApoE2 and ApoE3 preferentially bind to phospholipid rich HDL (Greenow et al. 2005; Horejsí and Ceska 2000; Mahley and Rall 2000). ApoE4 is linked with increased risk of cardiovascular disease (Kockx et al. 2008).

The main site of ApoE synthesis is the liver, however it is also synthesised by extrahepatic tissues including brain, lung, kidney and muscles (Greenow et al. 2005; Mahley and Rall 2000). In the vasculature apoE is secreted by smooth muscle cells and by macrophages. ApoE can undergo repeated cycles of secretion from and internalisation into macrophages, hepatocytes and a number of other cell types and this recycling is promoted by apoAI and HDL. Currently the importance of this recycling is unclear (Kockx et al. 2008).

ApoE derived from extra-hepatic sources makes up 20-40% of total plasma protein. The protein has roles in the hepatic uptake of lipoproteins and therefore also in cholesterol homeostasis, and is involved in the regulation of immune and inflammatory responses. The uptake and degradation of lipoproteins is regulated by ApoE through binding to the LDLR (Greenow et al. 2005; Mahley and Rall 2000). ApoE is a potent ligand for this receptor. The HSPG/LRP pathway functions in the liver to metabolise remnants of lipoproteins including chylomicrons. ApoE also stimulates RCT and the production of VLDL and triglycerides. Lipids are directed by delivery to either the liver (via VLDL) or the small intestine (via chylomicron remnants) (Greenow et al. 2005; Mahley and Rall 2000).

1.4.1.1 ApoE and atherosclerosis

Deficiency of apoE leads to the development of atherosclerosis and hypercholesterolemia and apoE-deficient mice are often used as a model for the disease. The protein is not expressed in normal vessel walls but is synthesised at high levels by macrophages present within atherosclerotic plaques. Reduced expression of the protein in atherosclerosis is associated with a pro-atherogenic lipoprotein profile whilst re-expression of apoE has been demonstrated to reduce the extent of atherosclerosis (Mahley and Rall 2000; Puddu et al. 2005).

The major anti-atherogenic property of apoE is the stimulation of cholesterol efflux from foam cells. It has been demonstrated that apoE can act as an acceptor of both cholesterol and phospholipids released from macrophages via ABCA-1 and is able to interact with ABCA-1, at least *in vitro* (Greenow et al. 2005).

ApoE can also exert anti-atherogenic actions that are independent of effects on cholesterol metabolism. For example, macrophage apoE can inhibit the expression of key proteins through binding to the apoE receptor 2 and causing the activation of nitric oxide synthase (NOS). Oxidation of LDL, proliferation of T-lymphocytes and smooth muscle cells, aggregation of platelets and production of the adhesion molecule VCAM-1 by endothelial cells are all inhibited by apoE and contribute to the anti-atherogenic nature of the protein (Greenow et al. 2005; Harvey and Ramji 2005).

1.4.2 ABCA-1 and ABCG-1

ABCA-1 and ABCG-1 are part of the ABC superfamily (Attie 2007). ABC transporters use ATP to facilitate the movement of lipids and metabolites across membranes and there are 49 human ABC transporters grouped into seven classes, A-G. Members of the ABC family are transmembranous transporters and have one of two structures. Whole transporters have a two domain structure that is joined by covalent bonds while half transporters are composed of single units that form homo- or hetero-dimers to become active (Oram and Heinecke 2005; Oram and Vaughan 2006).

1.4.2.1 ABCA-1

Of the ABC transporters, ABCA-1 is the best characterised and studied. The ABCA-1 gene has been mapped to chromosome 9q31 and encodes a whole transporter, 2216 amino acids in size (Cavelier et al. 2006). The protein is widely expressed with high mRNA levels in liver, small intestine and lung and has a broad substrate specificity (Oram and Heinecke 2005; Oram and Vaughan 2006). ABCA-1 is composed of 2 nucleotide binding folds exposed to the cytoplasm and 2 α helical transmembrane domains. Walker A and Walker B motifs present within the nucleotide binding folds are characteristic of ABC proteins (Attie 2007).

The primary role of ABCA-1 is the transport of cholesterol and phospholipids from cells to apolipoproteins in the bloodstream. This efflux of free cholesterol and phospholipids to lipid-poor apolipoproteins via ABCA-1 is the initial step in the formation of HDL (Wang and Tall 2003). In the intestine, cholesterol is moved out of epithelial cells into the bloodstream via ABCA-1 and this leads to over one third of HDL production (Attie 2007). ABCA-1 knockout mice present with HDL deficiency and reduced cholesterol efflux. Selective knockout of hepatic ABCA-1 also demonstrates a key role for the transporter in HDL production (Cavelier et al. 2006).

The central role of ABCA-1 in cholesterol efflux involves interaction with ApoA1, the protein component of HDL. It is thought that ApoA1 binds to ABCA-1 directly and the complex formed is essential for efflux of cholesterol. Cross linking studies have demonstrated that ABCA-1 associates with ApoA1 (Attie 2007; Fitzgerald et al.

2010; Wang and Tall 2003). However, it has also been suggested that the ApoA1 may not interact directly with ABCA-1 but that ApoA1 may dock at the plasma membrane following a local redistribution of lipids within the membrane, induced by the expression of ABCA-1. The formation of this complex stimulates lipid transfer from ABCA-1 to ApoA1 although how these two events are related remains to be clarified (Attie 2007; Fitzgerald et al. 2010; Wang and Tall 2003).

ABCA-1 targets specific regions of the membrane that are cholesterol-rich or sensitive to cholesterol accumulation, for secretion of lipids (Oram and Heinecke 2005). This may work through the targeting of excess cholesterol in transport vesicles to regions of the membrane where ABCA-1 is present or through the endocytosis of vesicles containing ABCA-1 and apolipoproteins to lipid rich deposits. It has been shown that ABCA-1 moves between endosomes and the plasma membrane and this may be involved in its regulation (Oram and Heinecke 2005).

The expression of the transporter is highly regulated and has a rapid turnover rate. This is likely to be due to the need to balance the beneficial cholesterol efflux and prevention of cytotoxicity effects, with the detrimental effects of altered membrane structure in the absence of cholesterol loading, seen on overexpression of ABCA-1 (Oram and Heinecke 2005; Wang and Tall 2003).

Levels of the transporter are increased in response to both ApoA1 and ApoE (Wang and Tall 2003). Studies in human THP-1 macrophages have demonstrated that endogenous synthesis of cholesterol up-regulates gene expression of ABCA-1 and other LXR-regulated genes involved in cholesterol efflux including ApoE and ABCG1 (Beyea et al. 2007). LXR is itself activated by cholesterol metabolites and is involved in 'sensing' cholesterol levels so activates ABCA-1 in cholesterol-loaded cells. Accumulation of modified lipoproteins and cholesterol in macrophages during atherosclerosis will therefore induce the expression of ABCA-1 in these cells (Oram and Vaughan 2006; Wang and Tall 2003).

OxLDL has also been demonstrated to up-regulate the expression of ABCA-1 at transcriptional and protein levels *in vitro* (Tang et al. 2004). Levels of ABCA-1 can be down-regulated by a decrease in LXR/RXR activation in response to cholesterol

efflux (Wang and Tall 2003). Interestingly, statins have also been shown to down-regulate the expression of ABCA-1 in human and mouse macrophage cell lines (Sone et al. 2004).

1.4.2.2 ABCG1

The human ABCG-1 gene has been mapped to chromosome 21q22.3. Unlike ABCA-1, ABCG-1 is a half-transporter. The protein is widely expressed but present at high levels in macrophage-rich tissues, macrophages, endothelial cells and lymphocytes (Ni et al. 2007). The mRNA expression of ABCG-1 is increased on transformation of macrophages into foam cells using acetylated or oxidised LDL and also on incubation of macrophages with cholesterol derivatives. Similar to ABCA-1, expression of ABCG-1 can be up-regulated by LXR, RXR, PPARγ and reduced by statins and cholesterol depletion (Ni et al. 2007).

The first evidence of ABCG1 activity was observed by Schmitz and colleagues in 2000 when they showed that the expression of ABCG1 was up-regulated by cholesterol in human macrophages. Studies using siRNA against ABCG-1 or overexpression of the protein have demonstrated that ABCG-1 stimulates cholesterol efflux to HDL but not to apoA-I (Fitzgerald et al. 2010; Ni et al. 2007). Further studies have indicated that ABCG-1 mediates cholesterol efflux to major HDL fractions to improve HDL efficiency. In addition to this, ABCG-1 also increases cholesterol levels in plasma membranes and this may control the availability of cholesterol to lipoprotein acceptors. These acceptors may in turn induce ABCG-1-mediated cholesterol efflux based on their phospholipid content (Ni et al. 2007).

1.4.2.3 ABCA-1, ABCG-1 and atherosclerosis

ABCA-1 is an anti-atherogenic protein. Deficiency of macrophage ABCA-1 in mice leads to accelerated atherosclerosis with excess accumulation of cholesterol independent of HDL levels, while overexpression reduces atherosclerosis (Cavelier et al. 2006; Oram and Vaughan 2006). ABCG-1 null mice exhibit increased accumulation of cholesterol and triglycerides in hepatocytes and macrophages when fed a high fat diet and overexpression of human ABCG-1 reduced accumulation in

these tissues. However, loss of ABCG-1 has not been linked with accelerated atherosclerosis (Cavelier et al. 2006; Fitzgerald et al. 2010; Ni et al. 2007).

The preventative role of ABCA-1 in atherosclerosis is also demonstrated by the association between ABCA-1 mutations and premature atherosclerosis observed in diseases including Tangier disease and Familial hypoalphalipoproteinemia (Attie 2007). For example, mutations in ABCA-1 in Tangier disease prevent cells moving cholesterol and phospholipids onto ApoAI and this transfer is the rate-limiting step of HDL biogenesis. Tangier patients have very low levels of circulating HDL but no changes in LDL cholesterol levels and are prone to foam cell formation and atherosclerosis due to accumulation of cholesterol esters in the liver, intestine and in macrophages (Attie 2007; Fitzgerald et al. 2010; Oram and Vaughan 2006; Wang and Tall 2003).

The function of ABCA-1 and ABCG-1 as removers of excess cholesterol from macrophages is responsible for their anti-atherogenic properties. In addition to negative feedback control of cholesterol synthesis, cholesterol levels can be regulated by a heterodimers of LXR and RXR transcription factors. Macrophages express high levels of ABCA-1 and ABCG-1 in response to the transcription factors, LXR, RXR and PPAR which can activate the ABC transporters (Attie 2007; Ni et al. 2007). The most likely ligand for this is 27-hydroxycholesterol which correlates with cellular cholesterol levels and may therefore provide a positive feedforward mechanism for ABCA-1-mediated cholesterol efflux (Beyea et al. 2007; Cavelier et al. 2006).

1.5 Development of lesions and plaque formation

Fatty streaks can be present in young individuals and rarely cause symptoms with most disappearing with time (Hansson and Libby 2006). Although fatty streaks are not always clinically significant, in the presence of risk factors for atherosclerosis such as hypertension or hyperlipidemia, they can develop into more advanced lesions. The migration of smooth muscle cells from the medial layer of the artery into the intima accompanies the progression of atherosclerotic lesions from fatty streaks to more complex plaques. Once inside, smooth muscle cells proliferate and take up

modified lipoproteins enhancing foam cell formation and further contributing to the necrotic core of the lesion. Smooth muscle cells also synthesise ECM proteins that form the fibrous cap often seen with in complex lesions (Glass and Witztum 2001; Li and Glass 2002).

1.5.1 The ECM

The ECM is composed of three different elements; elastins form elastic fibres to accommodate changes in blood flow; collagens provide tensile strength to the vascular wall; and proteoglycans interact with vascular cells and ECM components such as hyaluronan to form complexes that regulate permeability and structure (Raines 2000). Degradation and remodelling of the ECM by activated proteases is essential for processes such as growth and development but also has a role in a number of pathological processes. In atherosclerosis, degradation of the ECM that normally surrounds VSMCs results in the migration of VSMCs from the arterial media to the intima to form intermediate fibrofatty lesions composed of dense layers of VSMCs, macrophages and T-lymphocytes around a necrotic core. These lesions develop into plaques (or atheroma) which are characterised by the formation of a fibrous cap comprised of collagen, proteoglycans and fibrous elements (Libby et al. 2010).

1.5.2 Proteases and the atherosclerotic plaque

In atherosclerosis, macrophages (and monocytes) are known to secrete proteolytic enzymes at all stages of atherosclerosis to influence the development and/or stability of the plaque (Worley et al. 2003). Differentiation of monocytes to macrophages is accompanied by an increase in the expression of matrix-degrading proteinases (Whatling et al. 2004; Worley et al. 2003). In the later stages of atherosclerosis, high levels of proteases and correspondingly high levels of matrix degrading activity are seen in the vulnerable shoulder region of atherosclerotic plaques and macrophage-produced proteases have been demonstrated to induce the breakdown of collagen in the fibrous cap (Galis et al. 1995; Raines 2000).

Two important groups of ECM proteases are the matrix metalloproteinases (MMPs) and the ADAMTSs. MMPs are zinc-dependent endopeptidases that play a major role in ECM remodelling in a number of physiological and pathophysiological processes such as angiogenesis, wound healing, inflammation, arthritis and atherosclerosis. The MMP family is made up of 28 members divided into 4 classes based on their enzyme action. They are the interstitial collagenases, stromelysins, gelatinases and the membrane-type metalloproteinases. They are regulated by their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). ADAMTS proteases are secreted enzymes that are able to cleave proteoglycans such as aggrecan and versican. The structure and function of the ADAMTS proteases and their roles in atherosclerosis are described in detail in Chapter 6 of this thesis.

1.5.2.1 MMPs and atherosclerosis

Changes in the degradation of extracellular matrix by MMPs have been associated with a number of pathological conditions including rheumatoid arthritis and atherosclerosis (Galis and Khatri 2002; Galis et al. 1994; Malemud 2006; Wight 2005). This shift in the balance of synthesis and degradation is often driven by inflammation, injury and oxidative stress (Galis and Khatri 2002). Both MMPs and their endogenous inhibitors, the TIMPs are found in the heart and of the MMPs, MMP-2 is constitutively expressed in the heart and is found within human arteries (Chow et al. 2007). Matrix-degrading proteases are also constitutively expressed by foam cells of atherosclerotic plaques (Galis et al. 1995).

Investigation of normal and diseased human arteries showed that MMP-2 is constitutively expressed in both, however in normal arteries MMP-2 is associated with its endogenous inhibitor TIMP-2 and is inactive (Galis and Khatri 2002; Galis et al. 1994). MMP-1 and MMP-3 are also expressed by vascular smooth muscle cells and their expression can be induced by the pro-inflammatory cytokines TNF-α and IL-1 (Galis et al. 1994). MMP-9 expression can be induced by pro-inflammatory cytokines due to the presence of an activator protein-1 (AP-1) binding site and a nuclear factor-κB (NFκB) binding site in the promoter, and is associated with leukocytes and macrophages (Chow et al. 2007). Mice deficient in MMP-9 show

decreased intimal hyperplasia. This model supports a role for metalloproteinases in the promotion of VSMC migration and proliferation (Jönsson-Rylander et al. 2005).

Secretion of cytokines by activated macrophages results in the up-regulation of MMPs (Galis and Khatri 2002). Accumulation of lipids as seen in atherosclerosis can cause an increase in MMP expression while changes in oxidative stress associated with foam cell accumulation can also increase activation of latent MMPs in vascular cells (Galis and Khatri 2002). Increases in MMP production/activity have been associated with migration of smooth muscle cells and with the recruitment of inflammatory cells via interaction with adhesion molecules such as VCAM-1 (Galis and Khatri 2002).

1.5.3 T cells and plaque development

Inflammation and immune response continue to contribute to atherosclerosis development during plaque formation. T cells can be stimulated by the same adhesion molecules and chemokines as macrophages and can make up approximately 10-20% of advanced plaques (Hansson et al. 2002). CD4+ T cells are observed in human atherosclerosis and deletion of CD4+ and CD8+ T cells can reduce fatty streak formation in C57BL/6 mice. CD8+ T cells may contribute to disease progression by contributing to apoptosis within lesions however; CD4+ T cells are likely to play a larger role in development of the disease (Ohashi et al. 2004). Once activated, T cells can produce pro-inflammatory cytokines including the CD40 ligand, ligation of which promotes production of MMPs and the procoagulant tissue factor (TF) which initiates the coagulation cascade (Libby et al. 2010).

T cells present in atherosclerotic plaques are characterised according to the cytokines they secrete. Th1 cells are responsible for cell-mediated immunity, can activate macrophages and secrete pro-inflammatory cytokines including interferon-γ (IFN-γ). Th2 cells are involved in the production of antibodies by B cells and reduce macrophage activity through the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (Ohashi et al. 2004). Th1 type cytokines make up the highest proportion of cytokines in both mouse and human atherosclerotic plaques. These include IFN-γ, TNF-α and IL-12, -15 and -18. Studies have demonstrated a pathogenic role for Th1 cytokines in atherosclerosis. Hypercholesterolemic mice

deficient in any of these cytokines show reduced atherosclerosis (Hansson and Libby 2006; Tedgui and Mallat 2006). Th2 cytokines, such as IL-10 and TGF-β, inhibit the action of Th1 cytokines. The anti-inflammatory cytokines, IL-10 and TGF-β are atheroprotective. For example, mice overexpressing IL-10 under the control of the IL-2 promoter have reduced production of the pro-atherogenic cytokine, IFN-γ (Hansson and Libby 2006; Tedgui and Mallat 2006). Despite this, many studies suggest that balance of Th1/Th2 responses is not straightforward. IL-10 is not specific to Th2 cells and has been shown to inhibit the Th2 response while deficiency of the Th2 cytokine IL-4 has also been linked to reduced formation of atherosclerosis (Tedgui and Mallat 2006).

In addition to T helper cells, regulatory T (Treg) cells exist in two types. Natural Treg cells are characterised by expression of CD4, CD25 and the transcription factor FOXP3 and regulate immune responses to self-antigens to prevent autoimmunity (Taleb et al., 2010; Nilsson et al., 2009). Induced Treg cells are generated by the anti-inflammatory cytokines TGF-β or IL-10 during an immune response. Cells generated in response to TGF-β (Th3 cells) or IL-10 (Tr1 cells) mediate suppressor function through production of these two cytokines (Taleb et al., 2010). Both natural and induced Treg cells are therefore atheroprotective (Taleb et al., 2010; Nilsson et al., 2009). Roles for autoimmune responses in atherosclerosis were first suggested by the discovery of activated T cells and MHC class II antigens in atherosclerotic plaques (Nilsson et al., 2009). Deletion of T cells or their receptors is associated with enhanced progression of the disease and this is associated with depleted levels of Treg cells and enhanced inflammation (Nilsson et al., 2009). Low levels of Treg cells are observed in $ApoE^{-/-}$ mice and in human arterial lesions compared to normal arterial tissue (Nilsson et al., 2009). Co-culture of murine macrophages with Treg cells can reduce lipid accumulation (and therefore foam cell formation) through cell-cell contact and production of TGF-β and IL-10 associated with reduced expression of scavenger receptors CD36 and SR-A (Lin et al., 2010).

A recently identified T cell lineage is Th17 cells which have physiological roles in defence against bacterial and fungal infections (Taleb et al., 2010). Differentiation *in vitro* is mediated by TGF-β and IL-6. Roles for Th17 cells in autoimmune disease including rheumatoid arthritis have also been identified (Taleb et al., 2010). Their

role in atherosclerosis is currently unclear although a number of studies have suggested a pro-inflammatory role for IL-17 (Taleb et al., 2010). The cytokine can activate NFκB which is associated with the induction of pro-inflammatory factors including TNFα and IL-1. Cells expressing both IL-17 and IFNγ are present in atherosclerosis patients and enhance inflammation however; induction of IL-17 in the presence of TGF-β and IL-6 can have anti-inflammatory effects (Taleb et al., 2010).

1.6 Plaque rupture and Thrombosis

The composition and vulnerability of the atherosclerotic plaque determines development of thrombosis and subsequent acute coronary events. Plaques that are vulnerable to rupture have high numbers of inflammatory cells and a thin fibrous cap (Glass and Witztum 2001; Lusis et al. 2004). The fibrous cap is maintained through a balance of collagen and matrix production and degradation mediated by cytokines produced by inflammatory cells. In addition to thinning of the fibrous cap through breakdown of the ECM, other factors that may contribute to plaque stability include apoptosis which is promoted by the balance of cytokines and pro- and anti-apoptotic proteins within the arterial wall. Angiogenesis and calcification of lesions may also have effects on plaque stability (Glass and Witztum 2001; Lusis et al. 2004).

As the size of the atherosclerotic lesion increases, it initially expands outwards resulting in remodelling of the arterial wall. When the lesion becomes large enough, it extends inwards leading to narrowing of the artery and angina (Li and Glass 2002). Arterial thrombosis arising from disruption to the plaque can lead to myocardial infarction or stroke (Libby et al. 2002). This is the culmination of atherosclerosis.

1.7 Cytokines and atherosclerosis

In the context of disease or pathophysiology, cytokines are often grouped according to their pro-or anti-inflammatory actions. High levels of pro-inflammatory cytokines are observed in atherosclerotic lesions (Harvey and Ramji 2005). Pro-inflammatory cytokines include TNF-α, IFN-γ, IL-1 and IL-12 amongst others. Anti-inflammatory

cytokines include IL-10, IL-4 and TGF-β. Macrophages are the main source of cytokines in the atherosclerotic lesion alongside other vascular cells and platelets (Dinarello 2000; Tedgui and Mallat 2006). Cytokines mediate the development of the atherosclerotic lesion at all stages of the disease and have central roles in endothelial permeability, expression of adhesion molecules and scavenger receptors, migration of smooth muscle cells and induction of apoptosis (Tedgui and Mallat 2006).

1.7.1 Pro-inflammatory cytokines

Pro-inflammatory cytokines are expressed at high levels in the initial stages of atherosclerosis and can promote the expression of growth factors including vascular endothelial growth factor (VEGF), involved in plaque progression and stability, and platelet-derived growth factor (PDGF), involved in the proliferation and migration of smooth muscle cells (Tedgui and Mallat 2006). The most studied pro-inflammatory cytokine is IFN-γ which is a type II interferon. The main sites of IFN-γ synthesis are natural killer cells and T-lymphocytes (Th1 cells) but it is also synthesised by monocytes/macrophages. IFN-γ is important in the recruitment of immune cells to lesions in atherosclerosis. Expression of chemokines including MCP-1 and MIP-1 is induced by this cytokine (Harvey and Ramji 2005). Expression of adhesion molecules important for monocyte recruitment such as VCAM-1 is also increased by IFN-γ (Harvey and Ramji 2005). The cytokine can promote differentiation of monocytes into macrophages and can contribute to cholesterol accumulation by inhibiting the expression of key genes involved in cholesterol efflux (Harvey and Ramji 2005). Roles for IFN-γ in apoptosis of vascular smooth muscle cells and in plaque destabilisation have also been reported (Harvey and Ramji 2005).

1.7.2 Anti-inflammatory cytokines

Anti-inflammatory cytokines serve to limit inflammatory responses often through their ability to suppress the expression of pro-inflammatory cytokines. They also have effects on macrophage and T cell function. Major anti-inflammatory cytokines include IL-4, IL-10 and TGF-β (Dinarello 2000; Opal and DePalo 2000). TGF-β is the most studied anti-inflammatory cytokine. Knockout of TGF-β1 in mice results in uncontrolled inflammatory reactions, supporting its role as an anti-inflammatory

cytokine (Mallat et al. 2001). The cytokine has key roles in the regulation of cell proliferation and differentiation and can inhibit proliferation of B cells and T cells. Similar to IL-10, TGF-β can inhibit the production of pro-inflammatory cytokines TNF-α and IFN-γ by monocytes/ macrophages but these effects are less potent than those observed with IL-10 (Opal and DePalo 2000). TGF-β is discussed in detail in Section 1.9.

The balance between pro- and anti-inflammatory cytokines is important in the development and progression of atherosclerosis. For example, IFN-γ is known to inhibit the expression of apoE and ABCA-1, two proteins involved in reverse cholesterol transport whilst the anti-inflammatory cytokine TGF-β up-regulates their expression (Tedgui and Mallat 2006). Synthesis and degradation of ECM is important in the vulnerability of atherosclerotic plaques to rupture. This is likely to be mediated by the balance between pro- and anti-inflammatory cytokines. TGF-β and IL-10 inhibit the expression of MMPs which break down the ECM. TGF-β also induces synthesis of collagen to promote a stable plaque structure. In contrast, IFN- γ inhibits collagen synthesis while other pro-inflammatory cytokines, IL-1 and TNF-α induce the expression of MMPs (Dinarello 2000; Opal and DePalo 2000; Tedgui and Mallat 2006).

1.8 TGF-β

The TGF-β superfamily comprises 3 TGF-β isoforms, 4 activin β chains, the protein Nodal, 10 bone morphogenetic proteins and 11 growth and differentiation factors (Schmierer and Hill 2007). The three isoforms of TGF-β share between 64 and 82% homology in their amino acid sequence but are coded for by separate genes and have distinct patterns of expression. Whilst all three isoforms are expressed in the vascular wall, TGF-β1 is expressed at sites of inflammation and injury and is therefore most studied in relation to atherosclerosis (Ashcroft 1999). In the vasculature, the cytokine is expressed by endothelial cells, smooth muscle cells and macrophages and TGF-β mRNA is constitutively expressed by monocytes (Ashcroft 1999; Bobik 2006).

1.8.1 TGF-β synthesis

TGF-β is synthesised as a 24kDa dimeric precursor which is cleaved to become active. The cytokine is initially rendered inactive through association with the latency associated peptide (LAP), a 75kDa protein (Goumans et al. 2009). This complex is known as the small latent complex (SLC). The signal peptide is cleaved from the pre-pro-TGF-β on movement from the site of synthesis to the rough endoplasmic reticulum (RER) (ten Dijke and Arthur 2007). TGF-β has a cysteine knot motif in the mature protein formed by six conserved cysteine residues linked by three disulphide bonds, a structure conserved throughout the TGF-β superfamily (Schmierer and Hill 2007; Singh and Ramji 2006). Two monomers, 12-15kDa in size, of TGF-β dimerise through disulphide bridges and then undergo cleavage by furin-like proprotein convertases inside the cell. This cleavage produces the C-terminal mature TGF-β peptide and an N-terminal LAP, which remain attached through non-covalent bonds (Singh and Ramji 2006; ten Dijke and Arthur 2007).

The SLC can then covalently bond with a latent TGF-β binding protein (LTBP) to form the large latent complex (LLC) which can then be secreted. Four LTBPs have been identified and contain domains rich in cysteine residues and epidermal-growth factor-like repeats that allow interaction with components of the ECM (Singh and Ramji 2006; ten Dijke and Arthur 2007). The C-terminal of the LTBP interacts with microfibrils, possibly to stabilise TGF-β. Following secretion, the N-terminal of the LTBP interacts with components of the ECM, possibly to allow TGF-β to be targeted to specific sites and to regulate its activity. For TGF-β to become active and subsequently bind to TGF-β receptors to initiate signalling, the LLC must be released from interaction with microfibrils and ECM components. The exact mechanism that regulates this is unclear. The binding of Thrombospondin-1 to the LAP has been suggested to have a role in this by disrupting the bonds between TGF-β and the LAP. Binding of integrins to the LAP or the action of proteases on the LTBP may also have a role in this regulation (Singh and Ramji 2006; ten Dijke and Arthur 2007).

1.8.2 TGF-β receptors

TGF-β signals through its endogenous receptors also known as activin receptor-like kinases (ALKs). There are five type I receptors (TGFβRI) and seven type II receptors

(TGFβRII) in mammals (Goumans et al. 2009). TGF-β binds to a receptor complex made up of type I and type II receptors. The exact structure of the receptor complex is not known but is thought to be a heterotetramer consisting of two type I and two type II receptors. In the absence of TGF-β, the receptors exist as homodimers but studies using mutated receptors have suggested that at least two type I receptors are required in the signalling complex (ten Dijke and Hill 2004). The two receptor types are similar in structure and are the only known transmembranous, serine-threonine kinases. The receptors contain a small cysteine-rich extracellular domain, a single membrane spanning region and an intracellular kinase domain (ten Dijke and Arthur 2007). The kinase domain of the type II receptor is constitutively active and requires autophosphorylation of Ser213 and Ser409 for full kinase activity (Goumans et al. 2009). The receptor can also autophosphorylate specific tyrosine residues (259, 336 and 424) which may regulate its activity (Wrighton et al. 2009). Type I receptors act downstream of type II receptors and are phosphorylated by the type II receptors on specific serine and threonine residues within an intracellular region known as the GS domain as it is rich in glycine and serine residues (Bobik 2006; Goumans et al. 2009). This phosphorylation results in a conformational change which makes the L45 loop of the type I receptor accessible for binding of Smad proteins (Runyan et al. 2006). Two types of type I receptor exist- activin-like kinase-5 (ALK5) and ALK1 and these can often have opposing roles (see Section 1.9.3).

Proteins that interact with TGF-β receptors can positively or negatively regulate TGF-β signalling. The TGF-β receptor interacting protein (TRIP-1) binds to and becomes phosphorylated by the type II TGF-β receptor to inhibit TGF-β signalling (Wrighton et al. 2009). The TGF-β-related protein (TRAP-1) negatively regulates TGF-β receptor activation by binding to inactive type I receptors before being released upon receptor activation. Similar to this, the immunophilin FKBP12 binds to the type I receptor within the GS domain and is released on activation of type II TGF-β receptors. Disruption of FKBP12 and receptor interaction results in activation of receptors in the absence of ligand suggesting that the protein may safeguard against this (Runyan et al. 2006; Wrighton et al. 2009). In addition to receptor binding proteins, ligand binding proteins such as the type III TGF-β receptors endoglin and betaglycan can also regulate access to TGF-β receptors. These are abundant, transmembrane proteins that bind TGF-β with low affinity to facilitate signalling.

Endoglin only interacts with TGF-β when it is complexed with the TGF-β receptor but binds the BMPs directly. Betaglycan will interact with TGF-β before it is presented to the TGF-β receptor complex (Goumans et al. 2009; ten Dijke and Arthur 2007).

1.8.3 Endocytosis of TGF-β receptors

Like the majority of cell surface receptors, activated TGF-β receptors are internalized through clathrin-dependent endocytosis. Clathrin-coated vesicles contain a number of positive regulators of TGF-β signalling including the Smad anchor for receptor activation (SARA) (Schmierer and Hill 2007; ten Dijke and Hill 2004). Internalisation receptors, gathered around clathrin coated pits on the cytoplasmic side of the plasma membrane, recognise a di-leucine-like internalization signal (Ile218, Ile219, Leu220) found within the TGF-β receptors and conserved amongst most cell surface receptors (Chen 2009). The pits undergo invagination and pinch off from the membrane to move to the early endosomes where the clathrin coat is shed and vesicles fuse with the endosome to release the TGF-β receptors. Receptors are recycled back to the surface in the absence of the ligand (Chen 2009).

Following TGF-β signalling, TGF-β and its receptors can be internalized through clathrin-independent endocytosis. Cholesterol and sphingolipid enriched lipid rafts are found in the plasma membrane and associate with calveolin which is able to interact with type I TGF-β receptors to disrupt their interaction with Smads (Chen 2009). Endocytosis through lipid rafts attenuates TGF-β signalling as the lipid rafts contain Smad-7, Smurfs 1 and 2 and E3 ubiquitin ligases to promote receptor degradation as explained in more detail in Section 1.11.4 (Lönn et al. 2009). The factors that determine how TGF-β receptors are internalised remains unclear.

1.8.4 Roles of TGF-β

TGF-β has roles in numerous cellular processes including inflammation, fibrosis, wound healing and cell growth which are discussed briefly below.

1.8.4.1 The role of TGF-β in immune and inflammatory responses

The cytokine is recognised as a crucial negative regulator of the immune response and can control the threshold of activation for immune responses. The inhibitory role of TGF-β in the immune response was first recognised in the 1990s when mice deficient in the cytokine were found to display uncontrolled and widespread inflammation (Tedgui and Mallat 2006). Blockage of TGF-β signalling in T-lymphocytes using a dominant-negative form of the TGF-β type II receptor also results in inflammation spread across many organs (Li et al. 2006; Lutgens and Daemen 2001; Tedgui and Mallat 2006). Expression of MHC class II molecules by macrophages is down-regulated by the cytokine (Li et al. 2006). Inhibition of the co-stimulatory molecule CD40 by the cytokine is likely to impair the antigen presenting function of macrophages (Li et al. 2006). This would contribute to regulation of the immune response and may reduce secondary stimulation at sites of infection (Li et al. 2006). TGF-β can also inhibit the *in vitro* expression of LPS-induced inflammatory mediators including TNF-α, MMP-12 and the chemokines MIP-1α and MIP-2. Down-regulation of nitric oxide expression and inhibition of nitric oxide synthase by the cytokine has important implications for resolving inflammation and preventing immunopathology (Li et al. 2006).

TGF-β also plays important roles in other cell types including B lymphocytes, T cells and platelets. The cytokine affects the survival and inhibits proliferation of both T cells and B lymphocytes and also has regulatory roles in activated leukocytes (Singh and Ramji 2006; Grainger 2007). The cytokine inhibits the development of CD4 and CD8 T cells into mature T cells and can inhibit the proliferation of T cells via a number of pathways including inhibition of interleukin-2 or the c-myc transcription factor (Li et al. 2006; Singh and Ramji 2006). The cytokine also inhibits the differentiation of T cells to inhibit the acquisition of Th cell functions and also inhibits T cell activation of cytotoxic T cells and induces activation of regulatory T cells which function to suppress immune responses (Li et al. 2006). The extensive roles of TGF-β in immune and inflammatory mechanisms is beyond the scope of this thesis but is excellently reviewed by Li et al, 2006.

1.8.4.2 TGF-β and fibrosis

Overexpression of TGF-β under an organ specific promoter results in high levels of fibrosis in the target organ (Ghosh et al. 2005). TGF-β is the one of the most potent fibrogenic cytokines and excessive signalling via Smad-3 is thought to promote fibrogenesis (Ghosh et al. 2005). TGF-β promotes fibrosis through regulation of key components of the ECM. The cytokine can increase the synthesis of ECM proteins such as collagens, proteoglycans and fibronectin in a wide range of cell types (Ruiz-Ortega et al. 2007). The stimulation of TIMP expression and inhibition of collagenase production also contributes to the potent effects of TGF-β on matrix accumulation and fibrosis (Ruiz-Ortega et al. 2007). The pro-fibrotic effects of the cytokine have linked it with pulmonary fibrosis. This disease is characterised by impaired lung function and respiratory failure, caused by chronic scar formation and deposition of extracellular matrix (Ghosh et al. 2005).

1.8.4.3 Role of TGF-β in wound healing

The actions of the cytokine on endothelial cells are important for its role in wound healing. The cytokine inhibits migration and cell cycle progression of endothelial cells (Kim et al. 2005). It is also an inducer of apoptosis (Kim et al. 2005). At low concentrations the cytokine is associated with the invasion of endothelial cells. This may be critical in the migration of cells associated with wound healing. Higher concentrations of the cytokine cause an inhibitory effect on endothelial cells and may facilitate movement of vascular smooth muscle cells to sites of injury (Kim et al. 2005).

1.8.4.4 TGF-β and cellular growth and proliferation

TGF-β is a central mediator of growth inhibition in a range of cell types. Downstream targets of TGF-β signalling at the transcriptional level include the cyclin-dependent kinase inhibitor, $p21^{CIP1}$, which prevents progression through the cell cycle (Kim et al. 2005). The cytokine can induce anti-proliferative responses during the cell cycle through down-regulation of c-myc or cdk responses. Overexpression of c-Myc can cause cells to become resistant to growth suppression mediated by the cytokine (Kim et al. 2005; Massagué et al. 2000). The TGF-β signalling pathway is now recognized to have effects as a tumour suppressor pathway. Inactivation of type I or type II receptors underlies a wide range of pathologies including carcinogenesis (Kim et al.

2005). In human colon and gastric cancers mutation of TGF-β type II receptors results in cells becoming resistant to TGF-β-mediated growth inhibition (Kim et al. 2005; Massagué et al. 2000). However, these cancers often have high levels of TGF-β secretion which may contribute to pathogenesis through local immuno-suppression of immune responses or through induction of new blood vessel growth (angiogenesis). The cytokine potently induces angiogenesis by increasing expression of VEGF and up-regulation of MMP-2 and MMP-9 expression resulting in an increase in degradation of extracellular matrix to promote endothelial cell invasion and migration (Kim et al. 2005; Massagué et al. 2000).

1.9 TGF-β and atherosclerosis

1.9.1 Links between TGF-β and atherosclerosis

Evidence for the role of TGF-β in vascular biology was first proposed after studies in the balloon injured rat carotid artery, a model for neointima formation (Singh and Ramji 2006). Studies showed that levels of the cytokine were increased after the procedure and that overexpression or inhibition of the cytokine not only had effects on neointima formation but also on smooth muscle cell proliferation and extracellular matrix deposition (Singh and Ramji 2006). TGF-β is highly expressed in vascular cells of early stage fatty lesions and in more advanced atheroma and has been shown to have effects on all cell types present within lesions (Bobik 2006). Expression of the cytokine is higher in asymptomatic plaques (at both the mRNA and protein levels) and the cytokine accumulates in the shoulder regions of the plaque, in particular within macrophages and smooth muscle cells (Mallat et al. 2001; Singh and Ramji 2006).

Expression of TGF-β type I and type II receptors is higher in fatty streaks and decreases in more advanced lesions (Ramji et al. 2006; Singh and Ramji 2006). Expression profiles of TGF-β in lesions have revealed that lesional cells express type I TGF-β receptor at high levels whereas in the normal vessel wall, type II TGF-β receptors dominate (Grainger 2004; Lutgens and Daemon 2001). *In vitro*, type I

expressing VSMCs show increased production of extracellular matrix in response to the cytokine while typeII expressing VSMCs show no change in ECM production (Grainger 2004; Ghosh et al. 2005). It is therefore possible that excess levels of TGF-β maintain high levels of type II receptors allowing the vessel wall to retain normal differentiation and produce very little ECM. Changes in the expression of TGF-β can alter the ratio of type I and type II receptors. Inhibition of TGF-β in response to external factors switches the expression of receptors from type II to type I. This is accompanied by the increased proliferation, differentiation and migration of VSMCs into the intima, promoting lesion formation (Grainger 2004; Singh and Ramji 2006). Increases in endoglin and decorin accessory receptor have been associated with the disease but as these can act as positive or negative regulators of TGF-β signalling through either sequestering TGF-β into inactive pools or by facilitating formation of the active receptor complex it is difficult to interpret the meaning of these observations (Grainger 2007).

As detailed in Table 1.2, *in vivo* and *in vitro* studies have demonstrated the importance of TGF-β1 in regulation of vascular cell proliferation, plaque stability, differentiation and roles in immune and inflammatory mechanisms, with many studies pointing to a protective role for the cytokine in atherosclerosis (Kim et al. 2005). In the early 1990s it was proposed that TGF-β helped to maintain the normal structure of the arterial wall thereby protecting against the development of atherosclerosis. This was termed the protective cytokine hypothesis (Grainger 2004).

Studies in human and animal models support a primarily anti-atherogenic role for TGF-β. Serum levels of the cytokine correlate inversely with the development of atherosclerosis, with low serum levels seen in more advanced cases of the disease (Grainger 2004). Low levels of the cytokine are also linked to advanced atherosclerosis in angina patients (Kim et al. 2005). Regions of the aorta prone to development of lesions (sites with low shear stress) display low levels of TGF-β expression and levels of the cytokine are reduced in leukocytes following myocardial infarction (Grainger 2007; Redondo et al. 2007). High levels of active TGF-β1 have also been observed in patients with hypertension who have renal failure, end organ damage or obesity (Ghosh et al. 2005). The cytokine is thought to increase blood pressure by increasing peripheral resistance in the vascular wall through changes in

the levels of vaso-active mediators such as nitric oxide and endothelin-1 (a vasoconstrictor) (Ghosh et al. 2005; Lutgens and Daemon 2001). An arginine polymorphism in the TGF-β1 gene which causes increased production of the cytokine is associated with higher systolic blood pressure while other polymorphisms within the TGF-β1 gene have also been associated with cardiovascular disease (Ghosh et al. 2005; Kim et al. 2005). In contrast to these studies, the expression of TGF-β has also been demonstrated to be induced *in vitro* by shear stress and oxidised cholesterol and increased TGF-β expression is observed in clinical samples suggesting that the actions of TGF-β may be dependent on its bioavailability (Redondo et al. 2007). In support of this, human advanced atherosclerosis patients show increased levels of latent TGF-β and reduced levels of active TGF-β (Grainger 2007). Factors that may trigger suppression of TGF-β expression in the vessel wall remain poorly understood. One factor that is thought to contribute is the lipoprotein particle, Lp(a). Lp(a) is made up of two apolipoproteins, apo(a) and apoB, covalently bound to each other. Lp(a) can inhibit TGF-β activation *in vitro* and *in vivo*. Apo(a) has also been found to accumulate in regions where TGF-β activation is already suppressed forming a positive feedback loop at these sites (Grainger 2004).

TGF-$\beta^{+/-}$ mice fed a high cholesterol diet show higher levels of lipid retention and endothelial activation than controls and overexpression of TGF-β reduces atherosclerosis development (Grainger 2007). Targeted deletions of type I and II TGF-β receptors have demonstrated that loss of TGF-β signalling leads to abnormal vessel wall integrity and vascular structure; Embryos lacking these components die during gestation due to impaired vascular development (Goumans et al. 2009). Mice deficient in Smad-1, -2 or -4 present with pre-angiogenesis lethality and mice lacking Smad-3 die of impaired immunity (Goumans et al. 2009). Disruption of TGF-β signalling, using dominant negative forms of the receptor or neutralising antibodies to the cytokine, results in accelerated lesion formation, vascular inflammation and a tendency towards unstable plaques (Grainger 2007). Studies linking TGF-β with atherosclerosis are detailed in Table 1.2

Table 1.2 Studies on TGF-β and atherosclerosis

Aspect of atherosclerosis	Cell type or *in vivo* model	Key findings	Reference(s)
Atherosclerosis risk	Atherosclerosis patients	Serum levels of TGF-β are reduced in patients with advanced atherosclerosis.	Grainger et al. 1995
		Increased serum levels of TGF-β in atherosclerosis patients	Blann et al. 1996
Adhesion of monocytes to vascular endothelium	Porcine peripheral blood mononuclear cells	TGF-β decreases adhesiveness of the endothelium for monocytes.	Cai et al. 1991
	Human coronary artery endothelial cells	TGF-β expression is decreased in response to oxLDL and this reduces expression of adhesion molecules.	Chen et al. 2001
	THP-1 monocytes and human blood monocytes	TGF-β induced gene product (betaig-h3/TGFBIp integrin) is a chemoattractant for monocytes	Kim and Kim 2008
Monocyte migration	Differentiated U937 cells and human blood monocytes	TGF-β inhibits the expression of the CD11b leukocyte integrin and its activator Rap1 and their activation by chemokines reducing the migration of monocytes across a layer of endothelial cells.	Basoni et al. 2005
	Mouse RAW.264 macrophages	Short-term exposure to TGF-β increases macrophage migration whilst long-term exposure inhibits macrophage migration. This change is mediated through inhibition of RhoA expression and through increases in expression of chemokines such as MIP-1 and MCP-1.	Kim et al. 2006

Foam cell formation-Cholesterol uptake	Vascular cells and ApoE$^{-/-}$ mice	Cholesterol reduces the responsiveness of TGF-β in all vascular cells by increasing the accumulation of receptors in lipid rafts or caveolae and increasing degradation of TGF-β.	Chen et al. 2007
	Vascular cells and ApoE $^{-/-}$ mice	Cholesterol reduces binding of TGF-β to its receptors to reduce TGF-β responsiveness	Chen et al. 2008
Foam cell formation-Cholesterol efflux	Human plasma	TGF-β associates with and has higher levels of expression and bioactivity in high-density lipoproteins.	Tesseur et al. 2009
	J774 murine macrophages	TGF-β inhibits macrophage uptake of oxLDL to prevent accumulation of cholesterol esters and triglycerides. TGF-β also enhances cholesterol efflux through ABCA-1 and ABCG-1.	Argmann et al. 2001
Immune responses	ApoE$^{-/-}$ mice	Disrupted TGF-β signalling in T cells leads to accelerated atherosclerosis and increased activation of T cells.	Robertson et al. 2003
	LDLR$^{-/-}$ mice	Blockade of TGF-β signalling in T cells results in lesions that are smaller in size and have high levels of infiltrating T cells and MHC class II expression.	Gojova et al. 2003
	ApoE$^{-/-}$ mice	Elevated expression of TGF-β inhibits T cell responses and macrophage activation.	Zhou et al. 2009
Migration and proliferation of vascular smooth muscle cells	Rats with carotid balloon injury and vascular smooth muscle cells	Elevated expression of Smad-3 results in stimulation of VSMC proliferation by	Tsai et al. 2009

		TGF-β.	
	PAC-1 smooth muscle cells	TGF-β induces the expression of SMC differentiation marker genes and this requires activation of p38 kinase and the RhoA-PKN pathway.	Deaton et al. 2005
	Mouse VSMCs	TGF-β causes growth inhibition and inhibits proliferation of VSMCs and this requires activation of the p38 kinase pathway.	Seay et al. 2005
	Human arterial vascular smooth muscle cells	TGF-β stimulates the production of proteoglycans by VSMCs.	Chen et al. 1987
	Cultured rabbit aortic smooth muscle cells	TGF-β inhibits proliferation of SMCs during the G0/G1 stage of the cell cycle.	Morisaki et al. 1991

Plaque stability	$ApoE^{-/-}$ mice	Overexpression of TGF-β reduces expression of pro-inflammatory cytokines and MMPs and limits plaque growth to increase plaque stability.	Frutkin et al. 2009
	$ApoE^{-/-}$ mice	Inhibition of TGF-β results in accelerated atherosclerosis development and plaques with reduced collagen content and higher inflammatory content which are characteristically unstable.	Mallat et al. 2001
	Human carotid atherosclerotic plaques	TGF-β signalling is active in atherosclerotic plaques. Higher levels of endoglin and early growth factor-1 correlate with increased smooth muscle cell and collagen expression associated with a stable plaque phenotype.	Bot et al. 2009
	Human atherosclerotic plaques	Levels of TGF-β are higher in asymptomatic plaques and is associated with a increase in collagen content.	Cipollone et al. 2004
	$ApoE^{-/-}$ mice	Inhibition of TGF-β signalling using recombinant soluble TGF-β receptor II resulted in decreased size of plaques with decreased fibrosis and increased inflammatory and lipid content.	Lutgens et al. 2002
	Human coronary artery atherosclerotic plaques	Immunohistochemical staining shows that TGF-β is expressed at higher levels in stable plaques whilst expression of MMP-9 is higher in unstable plaques.	Jiang et al. 2004

	Human aortic vascular smooth muscle cells	PPARdelta regulates ECM turnover through increased expression of collagen and inhibition of VSMC apoptosis in response to oxLDL. This is mediated through TGF-β and Smad-3.	Kim et al. 2009

1.9.2 Actions of TGF-β on monocytes/macrophages in atherosclerosis

TGF-β has opposing stimulatory and inhibitory effects on monocytes and macrophages. A pro-inflammatory role for TGF-β in monocytes is supported by several studies. The cytokine is a potent chemoattractant for monocytes to facilitate their recruitment to the site of injury and inflammation (Ghosh et al. 2005; Li et al. 2006). The cytokine enhances the expression of integrins such as lymphocyte function-associated antigen-1 (LFA-1) and the fibronectin receptor to assist with monocyte attachment to the endothelial wall by interacting with ICAM-1 which mediates binding to fibronectin and collagen (Ashcroft 1999). Migration of monocytes is enhanced by the cytokine through the induction of MMP-2 and MMP-9 expression (Ashcroft 1999; Ghosh et al. 2005).

On monocyte-macrophage differentiation the expression of TGF-β is down-regulated and exhibits anti-inflammatory actions including the inhibition of pro-inflammatory genes including IL-1, IL-6, MCP-1 and MIP-1 and induction of anti-inflammatory cytokine IL-10 expression (Ashcroft 1999). The cytokine protects macrophages from apoptosis by stimulating ERK and attenuates macrophage activation by reducing iNOS expression through Smad3 (Werner et al. 2000). Macrophages produce inflammatory mediators and present antigens to T cells as part of the adaptive immune response. They also function to clear apoptotic cells and microbes by phagocytosis. It has been reported that phagocytosis of apototic cells can induce the secretion of TGF-β resulting in inhibition of expression of chemokines and pro-inflammatory cytokines (Li et al. 2006).

Many of the actions of TGF-β on macrophages inhibit foam cell formation (Mallat et al. 2001; Singh and Ramji 2006). Foam cell formation can be viewed as an imbalance between cholesterol uptake and efflux leading to cholesterol accumulation by macrophages (Sections 1.3 and 1.4). TGF-β reduces levels of cholesterol esters and triglycerides in macrophages by several mechanisms. Reduced uptake of lipoproteins is associated with decreased expression and activity of both LPL and the LDL receptor while uptake of oxidised lipoproteins can be reduced via inhibition of scavenger receptor expression (Mallat et al. 2001; Singh and Ramji 2006). In addition

to inhibiting the expression of genes implicated in cholesterol uptake, the cytokine induces the expression of genes involved in macrophage cholesterol efflux including apoE and ABCA-1 (Table 3.1). The actions of TGF-β on genes involved in cholesterol uptake and efflux and implications on foam cell formation are discussed in more detail in Chapter 3 of this thesis. This reduction in foam cell formation has been demonstrated in cultured macrophages and in $apoE^{-/-}$ mice where the presence of the cytokine prevents deposition of lipids within the intima (Grainger 2004).

1.9.3 Actions of TGF-β on VSMCs and endothelial cells in atherosclerosis

Formation of advanced atherosclerotic plaques is characterised by the migration and proliferation of smooth muscle cells and their subsequent production of extracellular matrix. TGF-β is a potent enhancer of both matrix deposition and collagen synthesis and inhibits the migration and proliferation of smooth muscle cells (Grainger 2004; Singh and Ramji 2006; Yokote et al. 2006). This contribution to fibrous cap formation and reduction of inflammation in the plaque promotes a stable plaque phenotype and the cytokine therefore has an important protective role in the development of atherosclerosis (Kim et al. 2005; Mallat et al. 2001).

In VSMCs TGF-β can attenuate activation by opposing the effects of pro-inflammatory cytokines and mitogenic growth factors as well as the expression of genes involved in vascular remodeling (Goumans et al. 2009). Activation of the expression of α2 type I collagen and inhibitor of metalloproteinase-1 is Smad-3 dependent (Bobik 2006). Signalling through Smad3 also prevents activation of SMCs by cytokines by binding to the CCAT/enhancer binding protein-β (Bobik 2006; Feinberg et al. 2004). Synthesis of VEGF is regulated through interactions which lead to p38MAPK activation (Bobik 2006). Differentiation of VSMCs is promoted by TGF-β by inducing a set of differentiation marker genes. At least one of these genes (the induction of SM22α actin) is known to be mediated through Smad-3 (Bobik 2006). The cytokine also inhibits growth of VSMCs through a Smad-3 and p38 kinase dependent mechanism (Feinberg et al. 2004; Goumans et al. 2009). Migration of VSMCs is also inhibited but through a Smad-independent pathway by up-regulation of cysteine-rich protein 2 expression through the recruitment of the transcription factor ATF-2 (Lin et al. 2008). SMCs within fibrous plaques also

express Smads which are not present in fatty lesions suggesting that TGF-β-mediated increase in collagen is only present in more advanced plaques. In $ApoE^{-/-}$ mice, a TGF-β neutralising antibody reduces collagen content by 50% and this is associated with reduced Smad-2 activation and increased lesion size, supporting a role for TGF-β in collagen production in lesions (Kalinina et al. 2004).

The cytokine regulates interaction between VSMCs and ECs. On interaction, production of TGF-β by ECs promotes differentiation of VSMCs while TGF-β produced by VSMCs stimulates VEGF to promote EC growth and differentiation (Bobik 2006; Goumans et al. 2009; ten Dijke and Arthur 2007; ten Dijke and Hill 2004). In endothelial cells, TGF-β can have contrasting actions depending on the receptor pathways used for signalling. For example, signalling through ALK5 inhibits migration and proliferation of ECs and also induces PAI-1 which contributes to the inhibition of EC migration. Signalling through ALK1 stimulates migration and proliferation of ECs through association with caveolin (a membrane protein involved in endocytosis). The two pathways can interact in ECs to regulate EC function. ALK5 is required for recruitment of ALK1 to the receptor complex whilst ALK1 is able to antagonise ALK5 signalling (Bobik 2006; Goumans et al. 2009; ten Dijke and Arthur 2007; ten Dijke and Hill 2004).

1.10 TGF-β-Smad signalling

1.10.1 The Smad signalling pathway

Smads are named after orthologs in *C. elegans* and *Drosophila*, known as Sma and Mad respectively. There are 8 Smad proteins, 42-60kDa in size (Heldin et al. 1997). Smads 1, 2, 3, 5 and 8 are receptor-regulated Smads (R-Smads) that interact with the TGF-β receptor complex. Smad-4 is a common mediator Smad (Co-Smad-4) that facilitates transport of Smads to the nucleus and Smads-6 and -7 are inhibitory Smads which negatively regulate TGF-β signalling. Smad proteins have a conserved structure consisting of two Mad-homology domains; an MH1 domain linked to an MH2 domain by a less conserved, proline-rich linker region (Bobik 2006; Ross and

Hill 2008; Schmierer and Hill 2007). The MH2 domain of Smad-2 has been analysed by crystallography and is made up of anti-parallel β-sheets capped at one end by three α-helices and at the other end by a loop/helix region consisting of three loops and an α helix (Heldin et al. 1997).

R-Smads are recruited to and phosphorylated by the TGF-β type I receptor. ALK4, 5 and 7 activate Smad-2 and -3 in response to TGF-β stimulation while ALK1, 2, 3 and 6 mediate activation of Smads -1, -5 and -8 in response to BMPs (Bobik 2006; Goumans et al. 2009). The type I receptor phosphorylates the R-Smad on two serine residues in the C-terminus. A positively charged pocket within the L3 loop of the MH2 domain of the R-Smad is essential for docking to the activated TGFβRI and is also required for binding to other Smad subunits (Ross and Hill 2008; ten Dijke and Arthur 2007; ten Dijke and Hill 2004). Binding of R-Smads to the type I receptor is facilitated by SARA. SARA contains a Smad binding domain that interacts with the β1 strand of the MH2 domain of Smad proteins to control the location of Smads following TGF-β stimulation. The C-terminal domain of SARA interacts with the TGF-β receptor to aid the receptor-Smad interaction (Runyan et al. 2006; ten Dijke and Hill 2004; Wrighton et al. 2009). The TRAP-1-like protein (TLP) is another positive regulator of TGF-β-Smad signalling. TLP is able to associate with active and inactive receptors (predominantly type II receptors) and in response to TGF-β is able to interact with Smad-4. Overexpression of the protein disrupts Smad complex formation (Runyan et al. 2006; Wrighton et al. 2009). TLP may differentially regulate Smad-2 and Smad-3 responses as overexpression of the protein blocks Smad-3 responses and potentiates Smad-2-mediated responses (Felici et al. 2003). Other receptor-interacting proteins that may be involved in the positive regulation of Smad signalling include the chaperone protein Hsp90 which stabilises TGF-β receptors and prevents them from becoming targeted for degradation (Wrighton et al. 2009). The Dab2 protein has been demonstrated to associate with Smad-2 and -3 in response to TGF-β and expression of this protein in a TGF-β-deficient cell line restores the activity of Smad-2 (Runyan et al. 2006).

R-Smads form complexes with co-Smad4 and other Smad subunits to translocate to the nucleus to regulate transcription of target genes. Smad complexes are formed through the binding of Smads to phosphorylated serines in the SXS motif in the C-

terminus of adjacent Smads. This also ensures that on complex formation, Smads are released from the type I receptor (Goumans et al. 2009; Ross and Hill 2008; ten Dijke and Hill 2004). The Smad signalling pathway is summarised in Figure 1.2 Recently, it has been demonstrated that some TGF-β responses can be mediated in a Smad-4 independent manner. Smad-4 deficient murine ES cells show no change in the TGF-β induced regulation of a subset of ECM genes and no change in the TGF-β regulated growth inhibition of fibroblasts, suggesting that signalling can be mediated through complexes lacking Smad-4 or may be Smad-independent (Sirard et al. 2000; ten Dijke and Hill 2004).

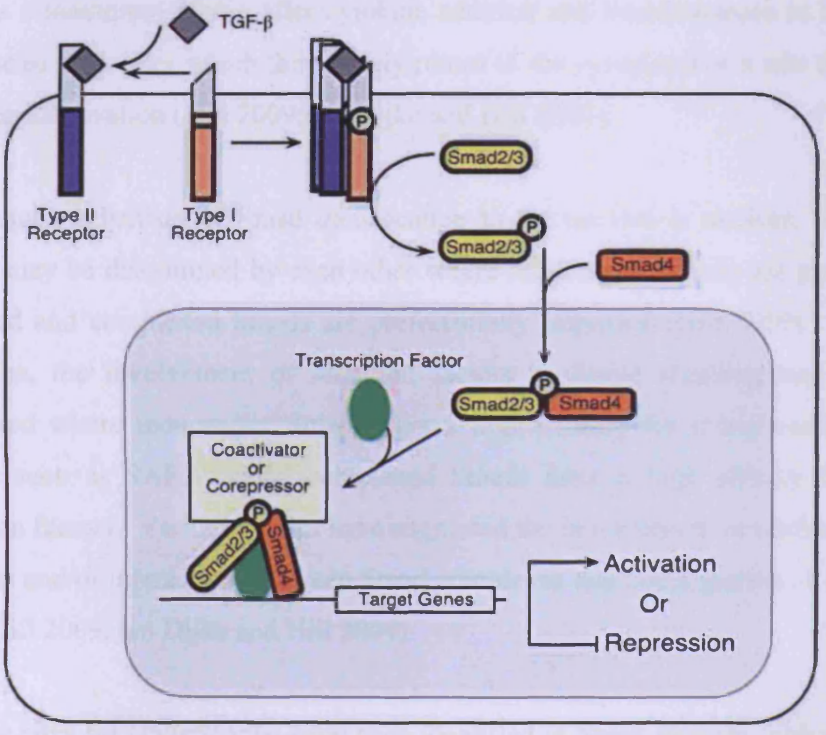

Figure 1.2 **The Smad signalling pathway.** On activation of the TGF-β receptor complex by TGF-β binding, the receptor-regulated Smads become phosphorylated by the type I receptor. Active Smads form complexes with Smad-4 to translocate to the nucleus where they can regulate gene transcription through binding to Smad binding elements within the promoter of target genes or through interaction with other transcription factors. For more details refer to text. Figure taken from Liu (2003).

TGF-β receptors. If the receptors are active Smads are re-phosphorylated and move back to the nucleus, if not low-level de-phosphorylation occurs and Smads move back to the cytoplasm. Fluorescence and GFP-fusion Smad proteins have demonstrated that in epithelial cells, TGF-β-stimulated nuclear accumulation of Smad-2, -3 and -4 reaches a maximum 45min after cytokine addition and Smads remain in the nucleus for at least 4-5h after which they slowly return to the cytoplasm at a rate that mirrors receptor inactivation (Hill 2009; ten Dijke and Hill 2004).

The exact mechanism of Smad translocation to the nucleus is unclear. Import and export may be determined by each other where monomeric Smads are preferentially exported and complexed Smads are preferentially imported (Hill 2009). Similar to this idea, the involvement of retention factors in Smad shuttling has also been suggested where monomeric Smads have a high affinity for cytoplasmic retention factors such as SARA whilst complexed Smads have a high affinity for nuclear retention factors. Various studies have suggested the involvement of nuclear transport proteins and/or interactions between Smad complexes and nucleoporins of the nuclear pore (Hill 2009; ten Dijke and Hill 2004).

Binding sites for nucleoporins have been identified in Smad proteins within the MH2 domain. Interestingly, these overlap with binding sites for SARA and for the Smad interacting motif (SIM) which suggests that interactions between Smads and cellular components may be exclusive and work in a competitive manner to move Smads to the nucleus (ten Dijke and Hill 2004). A lysine-rich NLS sequence (KKLKK) in the MH1 domain is conserved in all R-Smads and mutation of this sequence prevents nuclear accumulation of Smads in response to TGF-β. In Smad-3 the NLS mediates nuclear import by binding to importin-β1 and importin-α is not used. Smad-4 nuclear import uses both importin-α and -β1 as its NLS extends into the DNA-binding region

of the MH1 domain to allow interaction with importin-α. In Smad 2 the NLS motif is non-functional due to an insert located close to it which is likely to impair its binding to importin (Hill 2009).

Nuclear export of Smads relies on de-phosphorylation. So far, the protease PPM1A has been identified as being able to de-phosphorylate the SXS C-terminal motif of Smad-2 and -3 to facilitate their export to the cytoplasm. This interaction may involve phosphatase and tensin homolog (PTEN). Indeed Smad-2 and -3 phosphorylation is negatively correlated with the expression levels of both PPM1A and PTEN. Overexpression of PPM1A abolishes Smad-2 and -3 phosphorylation and shRNA-mediated knockdown of PPM1A enhances the duration of Smad phosphorylation and subsequent TGF-β-mediated responses (Itoh and ten Dijke 2007; Wrighton et al. 2009). It is likely that de-phosphorylation results in the dissociation of R-Smads from Smad-4 and that the two are exported by different mechanisms. Smad-4 contains a nuclear export signal (NES) within its linker region that is required for the binding of the nuclear exporter CRM1 (also known as exportin-1). Both of these components are required for nuclear export of Smad-4. Export of Smad-2 and -3 is independent of CRM1 but is likely to require other transport proteins such as exportin-4 which has been demonstrated to be required for export of Smad-3 (Hill 2009; ten Dijke and Hill 2004).

1.10.3 How Smads control gene transcription

Smad complexes control gene expression in a cell-type and ligand concentration-dependent manner. In Smad-3 and -4, direct contact to DNA is made through an 11-residue β-hairpin loop. It contacts the Smad binding element (sequence 5'-AGAC-3'). Smad -2 has an additional sequence N-terminal to this loop to prevent direct contact with DNA so binds DNA through Smad-4 (ten Dijke and Hill 2004; Wrighton and Feng 2008). Smads themselves have a low affinity for binding to DNA. Due to this, Smads recruit other factors such as co-activators and co-repressors that are able to bind specific sequences within target promoters to signal specificity and facilitate the binding of other transcription factors (Schmierer and Hill 2007).

A large number of Smad interacting proteins have been identified. The first Smad interacting transcription factor identified was the FastX1, a member of the *Xenopus* FoxH1 transcription factor family. FastX1 contains a Smad binding domain in its C-terminus and interacts with complexes of Smad-2 and -4 (ten Dijke and Hill 2004). The identification of other interacting factors has led to the identification of a common Smad interacting motif (SIM) which is rich in proline residues and shares similarity to the SBD of SARA and binds to the MH2 domain in the same way. One important difference between the two is that the SIM is shorter than the SBD and docks away from Smad interaction interfaces meaning that it binds equally well to monomeric and complexed Smads whereas the SBD of SARA binds with higher affinity to monomeric Smads (ten Dijke and Hill 2004)

Target genes are positively regulated through interaction with histone acetylases (HATs) such as CBP/p300. The addition of an acetyl coA to lysine residues in the Smad protein is TGF-β dependent. Interestingly, acetylation of Smad-2 is higher than Smad-3 suggesting that acetylation functions to improve Smad-DNA binding. Interaction between Smads and HATs is also thought to reduce nuclear export. CBP/p300 acetylates Smad-2 on lysines 19, 20 and 39 in the MH1 domain of Smad-2 and lysine 19 in the MH1 domain and lysine 378 in the MH2 domain of Smad-3 (Itoh and ten Dijke 2007; Ross and Hill 2008; Wrighton and Feng 2008). Another co-activator that has been demonstrated to interact with Smad proteins is P/CAF. This was discovered through its association with p300/CBP and like p300 has intrinsic HAT activity. The co-activator has been associated with the control of differentiation and growth inhibition. P/CAF interacts directly with Smad 3 on activation of the type I TGF-β receptor, in cultured mammalian cells. The co-activator has also been shown to enhance transcriptional responses mediated by the TGF-β/Smad3 pathway. p300/CBP further enhances these responses suggesting that the two co-activators may work cooperatively (Itoh and ten Dijke 2007).

Negative regulation of Smad target genes occurs through the recruitment of Smad co-repressors c-Ski and SnoN. In the absence of TGF-β, these bind to Smad binding elements in the promoters of target genes in a Smad-4 dependent manner and inhibit the basal expression of target genes through the recruitment of histone deacetylases (HDACs). HDACs remove acetyl groups from the lysine residues of histones to

increase their positive charge and promote high affinity binding with negatively charged DNA. TGF-β rapidly induces the degradation of c-Ski and SnoN to allow Smad complexes to interact with Smad binding elements. In addition to this, SnoN has been demonstrated to block the recruitment of co-activators to Smads and to block the formation of Smad complexes (Itoh and ten Dijke 2007; Ross and Hill 2008; Schmierer and Hill 2007).

1.10.4 Negative regulation of Smad signalling

Smad signalling is negatively regulated by Smad-6 and -7 also known as the inhibitory Smads (I-Smads). Smad-6 and -7 negatively regulate BMP and TGF-β signals respectively and of these, Smad-7 is the best studied. Smad-6 and -7 antagonise TGF-β-Smad signalling by competing with R-Smads for binding to the type I receptor and preventing their phosphorylation. The structure of Smad-6 and -7 is conserved within the MH2 domain, allowing them to bind to the TGF-β receptor, but show only limited similarity to other Smads in their MH1 domain (Schmierer and Hill 2007; ten Dijke and Hill 2004). The I-Smads are target gene for TGF-β so acts in a negative feedback loop to regulate TGF-β signalling. The I-Smads bind to TGF-β receptors to mediate their ubiquitination and subsequent targeting for degradation (Bobik 2006; Goumans et al. 2009). They can also carry phosphatase enzymes and disrupt interactions between Smads and receptors and also between R-Smads and Smad-4 to disrupt complex formation. (Lönn et al. 2009) Interaction of I-Smad and the type I receptor is stabilised by the serine-threonine kinase receptor associated protein (STRAP) (Runyan et al. 2006).

I-Smads recruit E3 ubiquitin ligases known as *Sm*ad *u*biquitination *r*egulatory *f*actor-1 and -2 (Smurf-1, and -2) within the nucleus. Smurf-1 binds Smads-1 and -5 whilst Smurf-2 has a broader specificity (Itoh and ten Dijke 2007; Ross and Hill 2008; Wrighton and Feng 2008). On binding to the TGF-β type I receptor, both the receptor and Smad-7 are ubiquitinated by the Smurf and targeted for degradation (Lönn et al. 2009). Ubiquitin is a 76 amino acid polypeptide that covalently attaches to lysine residues of a target protein. Ubiquitination is a three step process. Ubiquitin is covalently attached to lysine residues using E1 ubiquitin activating and E2 ubiquitin conjugating enzymes. The formation of ubiquitin chains linked from lysine residues

to the C-terminal glycine residue of ubiquitin is catalysed by E3 ubiquitin ligases. Polyubiquitination can take place as ubiquitin itself contains lysine residues to which more ubiquitin molecules can attach. This targets the protein to the 26S proteosome for degradation where the Smurf protein is also degraded (Lönn et al. 2009; Ross and Hill 2008; Wrighton et al. 2009).

Following TGF-β signalling, levels of Smurf-2 interaction with and ubiquitination of Smad-2 are enhanced (Lönn et al. 2009). Ubiquitination and degradation of Smad-2 requires Smurf-like proteins such as NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2)and WWP1/Tiul1 (WWP domain protein-1) which are activated by TGF-β and binds to Smad-2 (Itoh and ten Dijke 2007; Wrighton and Feng 2008). Although Smurfs are able to bind to Smad-3, it is not directly affected by Smurf interaction. Instead, Smad-7 induces the degradation of the co-repressor SnoN which interacts with the Smad-3-Smurf complex to disrupt it (Lönn et al. 2009; Wrighton and Feng 2008). Smad-4 can be ubiquitinated but requires R-Smads and TGF-β receptors to act as bridging proteins to mediate Smurf interaction (Ross and Hill 2008). The MH1 domain of Smad-4 can also become modified through the attachment of the small ubiquitin-related modifier (SUMO) to lysine residues which alters the transcriptional activity and stability of Smad-4 and has also been demonstrated to enhance its retention in the nucleus (Ross 1999; Wrighton and Feng 2008).

1.11 Smads and atherosclerosis

Relatively little is known about the roles of Smads in relation to atherosclerosis although as classical mediators of the TGF-β response they are likely to be central to many of the TGF-β-regulated effects observed in the vasculature during atherosclerosis. Smad signalling is reduced by shear stress through increased levels of I-Smad expression (Redondo et al. 2007). However, Smads are highly expressed in macrophages (and subsequent foam cells) of early lesions and smooth muscle cells of more advanced lesions (Kalinina et al. 2004). This expression profile supports the influence of TGF-β on macrophage foam cell formation and inflammatory responses. Smads are not expressed or are expressed at low levels in the smooth muscle cells of early lesions suggesting that enhanced collagen production characteristic in more

advanced, fibrous plaques, is impaired in early lesions (Kalinina et al. 2004). Smads have been implicated in a number of TGF-β-regulated responses in the vasculature including the induction of connective tissue growth factor (CTGF) expression, the production of ECM by VSMCs (Fu et al. 2003) and the inhibition of MMP-12 and iNOS, markers of macrophage activation (Werner et al. 2000). Smads have been demonstrated to be involved in the TGF-β-regulated induction of proteoglycan synthesis and subsequent binding to LDL in VSMCs (Dadlani et al. 2008). Knockout of Smad-3 in C57B1/6 mice suggests a role for Smad-3 in TGF-β-regulated inhibition of neointimal VSMC hyperplasia. Loss of Smad-3 demonstrated roles for the protein in the induction of α2 type I collagen and suppression of MMPs during the vascular response to injury (Kobayashi et al. 2005).

1.12 Non-Smad signalling

Although TGF-β classically signals through the Smad pathway it is able to activate other cellular signalling pathways to regulate target gene transcription. Of these the mitogen-activated protein kinases (MAPKs) are the best described and activation of these pathways by the cytokine has been demonstrated in a number of cell types.

1.12.1 Mitogen-activated protein kinase signalling pathways

Five distinct groups of mitogen-activated protein kinases (MAPKs) have been identified to date; extracellular-signal regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNK)/stress-activated protein kinases (SAPK), p38 kinase isoforms (α, β, γ, δ), ERK3 and ERK5. The best characterised of these are the vertebrate ERK1/2, JNK and p38 kinase pathways (Chang and Karin 2001; Johnson and Lapadat 2002). Similar to other protein kinases, the MAPKs consist of small N-terminal and large C-terminal domains connected by a linker region. Substrates bind at the surface while ATP is able to bind in a deep pocket at the interface between the two domains. Two phosphorylation sites are located in a surface loop known as the activation loop close to the activation site. In the unphosphorylated (inactive) state the substrate binding site is partially blocked but on phosphorylation (activation) of the MAPK this moves to help form the correct binding surface for the substrate (Cobb 1999).

MAPKs require phosphorylation on both a threonine and a tyrosine residue to become active. All MAPKs share a Thr-Xxx-Tyr phosphorylation motif, (where X is glutamic acid, proline, and glycine, for ERK, JNK and p38 respectively) located close to the ATP and substrate binding sites of the kinase (Bardwell 2006; Cobb 1999). MAPKs are phosphorylated by dual-specificity (serine/threonine-tyrosine) kinases known as MAPK kinases (MKKs) and this increases their activity by approximately 5000-fold (Cobb 1999). Phosphorylation of MAPKs enhances homodimerisation to produce symmetrical dimers of each of the MAPKs. Following activation MAPKs accumulate in the nucleus but the exact mechanism of nuclear import and export of MAPKs is unclear (Bardwell 2006; Cobb 1999).

Phosphorylation of MAPKs happens in a sequential manner where phosphorylation of the tyrosine residue is followed by threonine phosphorylation, with the MKK disassociating from the MAPK between each step (Harlaar and Brown, 1999). The specificity of the phosphorylation motif allows specific activation of MAPKs by particular MKKs. ERK is activated by MKK1, and MKK2, JNK is activated by MKK4 and MKK7 while p38 can be activated by MKK3, MKK4 or MKK6 (Bardwell 2006; Hommes et al. 2003; Johnson and Lapadat 2002).

In turn, the activity of the MKKs is controlled through phosphorylation by MAPK kinase kinases (MKKKs). MKKKs phosphorylate MKKs on activation by external stimuli. Little is known about the regulation of the MKKKs and they are numerous in number, adding both diversity and complexity to MAPK signalling (Hommes et al. 2003; Johnson and Lapadat 2002). The MAPK cascades are summarised in Figure 1.3. The MAPK cascades can be regulated by ligands of the receptor tyrosine kinases through binding of adaptor molecules (e.g. Grb2 and Shc) to phosphorylated receptor tyrosine kinases. Adaptor molecules interact with the guanine nucleotide exchange protein Sos through their SH3 domains and Sos enhances binding of GTP to Ras which is then in turn able to bind to Raf and other MKKKs to initiate the MAPK cascades (Cobb 1999; Wrighton et al. 2009). Adaptor molecules may also allow crosstalk between different signalling pathways.

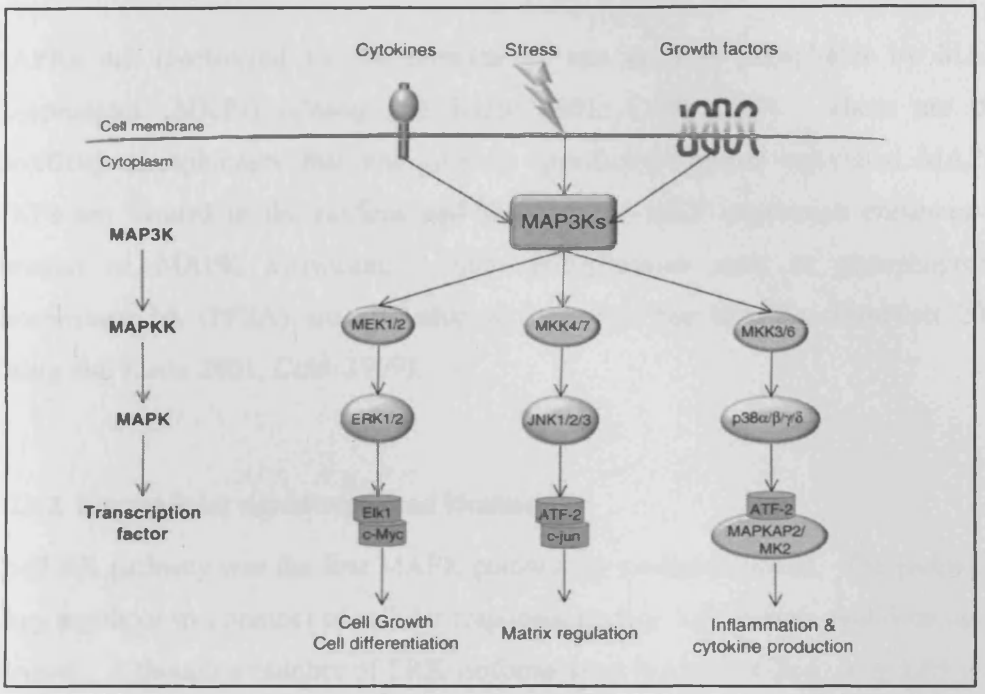

Figure 1.3 **The MAPK signalling pathways.** MAPKs are phosphorylated by MKKs which in turn are phosphorylated by MKKKs. Numerous MKKKs can be activated by a number of stimuli including cytokines, growth factors and cellular stress. For more details refer to text. Figure taken from Waldburger and Firestein (2009).

Activation of the MAPKs through this cascade allows the phosphorylation and activation of transcription factors and other kinases to regulate the expression of target genes (Hommes et al. 2003). MAPKs are serine/threonine proline-directed kinases and phosphorylate the serine or threonine residue within a S/T-P motif (also known as a phosphoacceptor site). However, this minimal target site is present within over 80% of proteins and so is by itself not enough to determine selectivity and specificity. Specificity may be enhanced by regions known as docking sites which are present on MAPK-interacting proteins and act to bind MAPKs and promote transactions such as MAPK-mediated substrate phosphorylation. These docking sites consist of a cluster of basic residues followed by a spacer region and several hydrophobic residues. In addition to this, scaffold proteins often play a key role in the specificity of MAPK signalling by binding to components of the MAPK pathways (Bardwell 2006; Chang and Karin 2001).

MAPKs are inactivated by the removal of one or both phosphates by MAPK phosphatases (MKPs) (Chang and Karin 2001; Cobb 1999). These are dual specificity phosphatases that also display specificity towards individual MAPKs. MKPs are located in the nucleus and blockage of MKP expression enhances the duration of MAPK activation. Other phosphatases such as phosphoprotein phosphatase 2A (PP2A) are also able to inactivate the MAPKs (Bardwell 2006; Chang and Karin 2001; Cobb 1999).

1.12.2 Extracellular signal-regulated kinases

The ERK pathway was the first MAPK pathway to be characterised. The pathway is a key regulator in a number of cellular responses such as cell growth, proliferation and survival. Although a number of ERK isoforms have been identified, only ERK1 and ERK2 are ubiquitously expressed (Hommes et al. 2003). ERK 1 and 2 are activated by a number of stimuli including cytokines, growth factors and ligands for G-protein coupled receptors. ERK 1 and 2 (or p44/42) are phosphorylated by either MEK1 or MEK2 and these are phosphorylated by the Raf proteins including cRaf1, B-Raf and A-Raf. Interestingly, the Raf proteins can be activated by the protooncogene Ras which is activated in many human tumours and induces the proliferation of tumour cells, so linking the ERK pathway with cancer (Johnson and Lapadat 2002). The duration of ERK activity can be modulated through removal of one or both phosphate groups from the MAPK. Transcription factors phosphorylated by ERK include but are not limited to Elk-1, c-Jun, c-Fos (AP-1) and ATF-2 (Hommes et al. 2003).

1.12.3 c-Jun N-terminal kinases

The JNKs were characterised as stress-activated protein kinases on the observation that they became activated in response to inhibition of protein synthesis. The JNK pathway is activated in response to cellular stress and cytokines and is primarily involved in the regulation of cell proliferation and apoptosis (Barr and Bogoyevitch 2001; Hommes et al. 2003). Three isoforms of JNK are formed by three genes JNK-1, JNK-2 and JNK-3. Disruption of the three JNK genes in mice results in defective stress-induced apoptosis and impaired immune responses. Each JNK is expressed as a

short form (46kDa) and a long form (55kDa) and of the three isoforms, JNK1 and JNK2 are ubiquitously expressed (Barr and Bogoyevitch 2001; Hommes et al. 2003). JNK can be activated by the upstream regulators MKK7 which is activated by cytokines such as TNF and IL-1 and MKK4 which is activated by stress. Regulation of JNK is complex and more than 13 MKKKs are able to activate the kinase (Hommes et al. 2003). The duration of strength of JNK activation is dependent on the balance between external stimuli and inactivation of the cascade. To date, four negative JNK regulatory factors have been identified; namely, MAPK phosphatase MKP7, heat shock protein 72, nitric oxide and evil oncoprotein. JNK phosphorylates two components of the AP-1 complex, c-Jun and ATF-2 (Hommes et al. 2003). The AP-1 transcription complex which regulates the expression of many cytokine genes and is activated in response to environmental stress and other factors that also activate JNKs (Johnson and Lapadat 2002). c-Jun is post-translationally activated by JNK through phosphorylation of Ser63 and Ser73 in the N-terminal domain. JNK also activates ATF-2 which heterodimerises with c-Jun to stimulate its expression (Barr and Bogoyevitch 2001).

1.12.4 p38 kinases

Like the ERK and JNK pathways, the p38 kinase pathway is associated with inflammation, cell growth, differentiation and death. The p38 kinases were first discovered during a screen for drugs inhibiting TNF-α-mediated inflammatory responses (Hommes et al. 2003; Johnson and Lapadat 2002). The p38 kinases are activated by a large number of inflammatory cytokines and growth factors in inflammatory cells (including TGF-β, TNF-α, IL-1, and IL-17) in addition to ligands for G-protein coupled receptors, hormones, and environmental stresses such as heat shock, pathogens, UV and oxygen radicals (Hommes et al. 2003; Johnson and Lapadat 2002). p38 was isolated as a 38kDa protein and four isoforms have been identified. p38α, β and δ are ubiquitously expressed while p38γ is predominantly expressed in skeletal muscle. In inflammatory cells such as macrophages, p38α is the major isoform activated. The kinase is activated by MKK6 and MKK3 although it is MKK6 that is able to activate all four isoforms whilst MKK3 preferentially activates α, γ and δ (Herlaar and Brown 1999; Ono and Han 2000). Downstream kinases such as MK2 or transcription factors such as ATF-2 are downstream targets of p38. The

p38 pathway promotes translation of proteins involved in the inflammatory response through AU-rich elements in the 3'-untranslated region of their mRNAs. These are often present in mRNAs of cytokines and inflammatory proteins. For example, the MAPK is able to regulate production of the TNF-α protein and inhibition of p38 in macrophage cell lines significantly reduces protein levels of TNF-α. Other proteins whose production is dependent on p38 include IL-1, MCP-1, VEGF and iNOS (Saklatvava 2004). p38 activation predominantly results in the production and activation of inflammatory mediators to initiate leukocyte recruitment (Herlaar and Brown 1999; Hommes et al. 2003).

1.12.5 MAPK and TGF-β-mediated responses

TGF-β activation of the ERK pathway was first suggested by studies in rat intestine and mink lung epithelial cells which demonstrated rapid activation of p21 (Ras) by the cytokine. ERK activation by TGF-β has subsequently been demonstrated in a number of cell types (Zhang 2009). One of the mechanisms by which TGF-β is able to induce the ERK pathway is through the activation of tyrosine kinase signalling pathways. Both the type I and type II receptors for TGF-β are phosphorylated on tyrosine residues following ligand stimulation. The type II receptor for TGF-β is autophosphorylated on three tyrosine residues (259, 336 and 424) and can also become phosphorylated at tyrosine 284 by Src which bridges TGF-β and MAPK activation by acting as a docking site for Grb2 and Shc recruitment. The type I receptor can recruit and phosphorylate ShcA to promote the formation of a Grb2/Sos complex which can activate Ras and Raf proteins which link receptor tyrosine kinases with sequential activation of ERK signalling components. Interestingly, activation of Grb2 has also been linked with the induction of TGF-β responses through p38 kinase (Lee et al. 2007; Wrighton et al. 2009; Zhang 2009).

TGF-β activation of the ERK pathway is required for TGF-β-induced epithelial to mesenchymal transistion which plays a physiological role in embryo development and pathophysiological roles in tumour metastasis (Moustakas and Heldin 2005; Zhang 2009).

TGF-β can activate the JNK and p38 kinase pathways through MKK3 or MKK6 in a number of cell lines and have been shown to be activated independently of the Smad pathway but can also act in conjunction with the Smad pathway. TGF-β is able to activate the JNK and p38 pathways through direct interaction between the type II receptor and the TGF-β-activated kinase 1 (TAK1). This interaction requires activation of TAK1 by TRAF6 which may also interact with the activated TGF-β receptor complex (Zhang 2009). TGF-β-activation of the JNK and p38 pathways is essential for TGF-β-induced apoptosis (Zhang 2009). The involvement of MAPKs in TGF-β-mediated apoptotic mechanisms is the most consistently observed role for MAPKs in TGF-β-mediated responses. The interaction between the type II receptor and a pro-apoptotic adaptor protein Daxx leads to activation of JNK and apoptosis in epithelial cells and hepatocytes (Moustakas and Heldin 2005).

1.13 MAPKs and atherosclerosis

MAPKs are expressed in the cardiovascular system and are activated by a number of stimuli including LDL, oxLDL, acute hypertension and mechanical stress (Metzler et al. 1999). ERK 1/2 has been shown to be activated and overexpressed in atherosclerotic lesions and phosphorylation and activation of ERK and JNK in arterial walls has been noted after balloon injury in animal models (Li and Xu 2000). Activation of MAPKs in response to shear stress has consistently been observed in a number of cell types and is often associated with cell proliferation and apoptosis. For example, ERK can be activated by shear stress in endothelial cells and cardiac myocytes and by mechanical stress in VSMCs. This activity is associated with AP-1-dependent transcription and with VSMC proliferation. Similarly activation of p38 in these cells is associated with proliferation and apoptosis (Cheng et al. 2008; Li and Xu 2000). MAPK activation may be mediated by Ras which is also activated in response to shear stress. Other factors that can activate MAPKs include angiotensin II and endothelin-1 in VSMCs (Siasos et al. 2007).

The exact roles of each of the MAPKs in atherosclerosis, is currently unclear. The pro- or anti-atherogenic nature of each MAPK is likely to be distinct and dependent on stimulus, cell-type and physiological context. Knockout studies in $ApoE^{-/-}$ mice

have demonstrated a pro-atherogenic role for JNK2 (Ricci et al. 2004). $JNK2^{-/-}$ (but not $JNK1^{-/-}$) mice showed less atherosclerosis than $ApoE^{-/-}$ counterparts and use of a pharmacological inhibitor against JNK resulted in reduced plaque formation in these mice. Macrophage-restricted deletion of JNK2 also resulted in a reduced uptake of lipoproteins, reduced foam cell formation and significantly less atherogenesis (Ricci et al. 2004).

Deficiency of macrophage p38 MAPK in $ApoE^{-/-}$ mice promotes apoptosis of macrophages and necrosis of advanced lesions. This increase in apoptosis was accompanied by thinning of the fibrous cap and reduced collagen content of lesions suggestive of enhanced lesion progression (Seimon et al. 2009). Pharmacological inhibition of p38 in cultured mouse macrophages also resulted in increased ER-stress induced apoptosis suggesting a role for p38 in apoptosis and lesion progression (Seimon et al. 2009). In contrast to this, models of inflammation have demonstrated a pro-inflammatory and possibly pro-atherogenic role for p38. A study in balloon-injured rat carotid arteries demonstrated that injury to the artery wall was followed by activation of p38 and that treatment with a p38-specific inhibitor produced a significant decrease in the numbers of proliferating cells implicating the kinase in neointimal formation (Ohashi et al. 2000). A role for the kinase in neointimal formation has also been suggested by a vascular injury model where inhibition of p38 expression in smooth muscle cells resulted in decreased neointimal formation (Proctor et al. 2008). Inhibition of p38 has been associated with reduced inflammation in models of skin injury and septic shock (Kim et al. 2008; Seimon et al. 2009). The kinase also plays a role in the transcriptional regulation of E-selectin and also of VCAM-1 in endothelial cells (Herlaar and Brown 1999). Production of TNF-α is regulated by p38 and JNK in macrophages and p38 also plays a role in TGF-β mediated chemotaxis of monocytes (Herlaar and Brown 1999).

In macrophages, roles for MAPKs in a number of processes have been defined. Apoptosis of macrophages in advanced atherosclerotic lesions has been demonstrated to require the p38 and JNK pathways. These pathways are activated by the accumulation of free cholesterol and engage the scavenger receptor, SR-A to promote apoptosis (Devries-Seimon et al. 2005). Inhibitors against p38 and ERK can inhibit oxLDL-induced macrophage proliferation suggesting roles for the kinases in this

process (Senokuchi et al. 2004). Macrophage proliferation in response to oxLDL is mediated by an increase in the production of GM-CSF, induced by oxLDL. Inhibitors against p38 and ERK can inhibit macrophage proliferation and indicate distinct roles for the two kinases in this process. ERK inhibitors can inhibit both production of GM-CSF and subsequent macrophage proliferation whilst inhibitors against p38 have no effect on GM-CSF mRNA or protein expression but maintain the inhibitory effect on macrophage proliferation, suggesting that p38 acts further down the pathway and may play a role in cell cycle progression which is negatively regulated by an oxLDL-ERK-GM-CSF cascade (Senokuchi et al. 2004).

Roles for the MAPKs in foam cell formation have been demonstrated by studies in mouse peritoneal macrophages where treatment of cells with oxLDL results in the rapid activation of ERK, p38 and JNK (Muslin 2008). Treatment of macrophages from $CD36^{-/-}$ mice with a JNK inhibitor blocks oxLDL-induced foam cell formation and ApoE null mice treated with a JNK inhibitor are also found to develop significantly less atherosclerosis than $ApoE^{-/-}$ untreated mice (Muslin 2008). In addition, JNK2 null mice crossed with $ApoE^{-/-}$ mice are resistant to atherosclerosis development and have reduced oxLDL and AcLDL-induced foam cell formation. Levels of phosphorylated SR-A are also reduced in macrophages from $JNK2^{-/-}$ mice suggesting that JNK may promote SR-A phosphorylation and therefore foam cell formation (Ricci et al. 2004). In J774 murine macrophages oxLDL-induced foam cell formation can be blocked with a p38 inhibitor (Muslin 2008). In addition, p38 has been demonstrated to be required for oxLDL-stimulated CD36 expression in macrophages (Muslin 2008).

1.14 Integration between Smads and other signalling pathways

A number of studies on TGF-β-regulated gene expression have evidenced crosstalk between Smad and MAPK pathways although the exact mechanism of crosstalk is often unclear. Interaction between signalling pathways can often be cell-type and gene specific. For instance, cells of mesangial origin tend to show a synergy between ERK and Smad pathways whilst epithelial cells tend to show that this interaction inhibits downstream events (Hayashida et al. 2003). Stimulation of collagen I and

fibronectin production by TGF-β in mesangial cells is mediated by the Smads but can be inhibited by blocking the expression of ERK (Hayashida et al. 2003). Interplay between signalling pathways has also been observed in relation to atherosclerosis. For example, in VSMCs, the ERK and Smad pathways have been implicated in the TGF-β-mediated induction of PAI-1 expression (Hong et al. 2006). PAI-1 promotes ECM accumulation and fibrosis therefore impacting on vascular remodelling, neointimal growth and VSMC migration and proliferation and is used as a biomarker for cardiovascular disease-associated mortality. The ERK and Smad pathways are required for PAI-1 induction by TGF-β. Activation of ERK by TGF-β is stimulated by oxLDL and this modulates the phosphorylation and nuclear accumulation of Smad-3 leading to an increase in PAI-1 expression (Hong et al. 2006; Samarakoon and Higgins 2008).

The linker region of Smads can be phosphorylated by MAPKs and cyclin-dependent kinases (CDKs) and may integrate signals from other pathways to mediate TGF-β responses (Schmierer and Hill 2007). Mitogens are able to initiate the ERK-mediated phosphorylation of both Smad-2 and Smad-3 at specific serine and threonine residues within the linker region (specifically, Ser245, 250, 255 and Thr220 of Smad-2 and Ser204, 208 and Thr179 of Smad-3) (Javelaud and Mauviel 2005). Phosphorylation of Smads by MAPKs can affect their ability to move to the nucleus or thereby affect transcriptional activity. For instance, ERK phosphorylation of Smad-1, -2 and -3 reduces the nuclear translocation of Smads following TGF-β stimulation (Javelaud and Mauviel 2005). TGF-β activated JNK can also phosphorylate Smad-3 to induce its nuclear translocation and activity. MSK1 kinase, a substrate of p38 kinase, can modulate the transcriptional activity of Smad-3 by promoting association with the p300 co-activator (Moustakas and Heldin 2005). Recently, phosphatases that dephosphorylate the linker region of Smads have been identified. SCP phosphatases dephosphorylate specific sites in the Smad-2 (Ser245, 250, 255 and Thr8) and Smad-3 (Ser204, 209, 213 and Thr8) linker region and have no effect on the SXS phosphorylation of the protein (Wrighton and Feng 2008; Wrighton et al. 2009).

The interaction of the MAPKs and Smads with transcription factor substrates adds another level of complexity to the interaction between signalling pathways. MAPK-associated transcription factors can modulate the activity of Smads and conversely

these transcription factors can be modulated by the Smad pathway. This regulation can be positive or negative and often occurs in a cell-type-, gene- and/or MAPK-specific manner (Javelaud and Mauviel 2005). The transcription factor AP-1 and Smads tend to cooperate to regulate AP-1-driven promoters while they antagonise each other on Smad-dependent transcription (Javelaud and Mauviel 2005). Smad-3 and c-Jun can interact at the transcriptional level through AP-1 sites. Interestingly, the c-Jun promoter itself contains both AP-1 and Smad3/4 binding sites and can be induced by TGF-β (Javelaud and Mauviel 2005). Repression of the Smad pathway by JNK activation in HepG2 cells is mediated by an interaction between Smad-3 and c-Jun (Dennler et al. 2000). Overexpression of constitutively active MKK4 and MEKK1 inhibited TGF-β-induced transcription and disrupts the c-Jun/Smad-3 interaction suggesting that this interaction impairs TGF-β-mediated transcription (Dennler et al. 2000). The inhibitory effect of c-Jun/Smad interaction is a result of the ability of c-Jun to stabilise the interaction of Smad-3 with co-repressors such as Ski and TGIF and block interactions with co-activators such as p300/CBP (Pessah et al. 2002; Pessah et al. 2001). Interaction between Smad and MAPK signalling pathways is discussed further in Chapter 5 of this thesis.

1.15 Target gene transcription

Transcription is the process of synthesising RNA that is complementary to the DNA sequence using RNA polymerase. A number of accessory proteins (TFIIA, B, D, E, F and H), known as general transcription factors, are required for RNA polymerase II-mediated transcription and make up the transcriptional machinery required for initiation of transcription (Thomas and Chiang 2006). Two models have been proposed for transcription initiation, the sequential assembly model and the holoenzyme model, reviewed by Thomas and Chiang 2006 and Malik and Roeder 2005 respectively. Transcription is regulated by trans-acting factors (or transcription factors) that are sequence specific and bind to cis-regulatory elements (CREs) within the promoter of the gene. Cis-regulatory elements are situated upstream of the transcription start site and interact with RNA polymerase and the associated transcriptional machinery to regulate transcription. They can act either as activators

or as repressors of gene expression dependent on other cofactors recruited to the gene promoter (Venters and Pugh 2009).

Transcription begins as a series of abortive initiations before the RNA polymerase enzyme reaches the +9 site where stable initiation begins. This transition is known as 'promoter clearance' (Buratowski 2009; Margaritis and Holstege 2003). As transcription elongation progresses levels of phosphorylated serine mediate interactions with histone methyltransferases which can then recruit complexes to modify chromatin structure and histone acetylation to allow access to the gene sequence for transcription to continue (Buratowski 2009; Venters and Pugh 2009). Transcription is terminated following the addition of a 5'CAP and 3' polyA tail (Schoenberg and Maquat 2009; Venters and Pugh 2009). The pre-mRNA is also spliced to remove introns from its sequence to form the mature mRNA (Bentley 2005; Buratowski 2009).

1.15.1 Histone acetylation and Chromatin remodelling

Genes are packaged and compacted within the nucleus in the form of chromatin. Chromosomal DNA is structurally similar to beads on a string where each bead is a nucleosome consisting of 147bp of DNA wrapped around an octomer of histones containing two copies of histones H2A, H2B, H3 and H4 (Cairns 2009; Venters and Pugh 2009). Nucleosomes are evenly spaced every 160-200bp throughout the genome. The packaging of chromatin can be modulated by ATP-dependent chromatin remodelling complexes such as SWI/SNF to regulate gene expression. This is achieved through competition between nucleosomes and transcription factors for access to cis-regulatory elements. While constitutively-expressed genes favour binding of transcription factors through a nucleosome-depleted region containing cis-regulatory elements, regulatory-transcribed genes are often covered using nucleosome positioning sequences that help position nucleosomes over key transcription factor binding sites. This then requires binding of a key transcription factor at an open site followed by remodelling of chromatin and modification to expose other sites (Cairns 2009; Venters and Pugh 2009). Chromatin remodelling is often preceded by histone acetylation by transcription factors with histone acetyltransferase (HAT) activity, known as co-activators. A large number of proteins with HAT activity that are linked

to transcription have been identified including p300/CBP (CREB (cAMP response element binding protein) binding protein), PCAF (p300/CBP associated factor) and SRC-1 (steroid receptor coactivator-1) (Gregory et al. 2001). Histone acetylation facilitates not only access for RNA polymerase but also binding of additional transcription factors to regulate transcription and gene expression. Acetylation occurs on specific lysine residues on the N-terminal tails of histone proteins (Gregory et al. 2001). The following section will focus on co-activators (transcription factors) involved in TGF-β signalling with a focus on the role of steroid receptor coactivator-1 (SRC-1).

1.16 Co-activators and TGF-β signalling

TGF-β-mediated changes in gene expression through the Smad and MAPK pathways are regulated by co-activators with HAT activity such as p300/CBP and SRC-1 or by co-repressors with HDAC activity such as NCoR and HDAC1 which bind to sites within the promoter of the target gene and to each other to regulate gene transcription (Spencer et al. 1997). Co-activators facilitate the assembly of a stable pre-initiation complex (PIC) of transcription machinery to enhance gene transcription while co-repressors bind to DNA to prevent assembly of the PIC and gene transcription. Recruitment of co-activators with intrinsic HAT activity target specific histones on the DNA resulting in the unpacking/unwinding of chromatin to facilitate access for other transcription factors and the basal transcriptional machinery while deacetylation of histones by co-repressors has the opposite effect (Spencer et al. 1997).

Co-activators and co-repressors, unlike general transcription factors are not part of the RNA polymerase II complex and interact with enhancer DNA-binding transcription factors. As the levels of co-activators within the cell is limiting, changes in their expression levels can have dramatic effects on gene expression and can serve to integrate signals from multiple pathways (Yuan and Xu 2007). Over 300 co-regulators of transcription have been identified to date (Lonard and O'Malley 2007). Co-activators and co-repressors do not work alone but in protein complexes made up of multiple components. These are dynamic in nature with some proteins stably bound and some less so (Lonard and O'Malley 2007). Reversible, post-transcriptional modifications to co-regulators (such as phosphorylation, acetylation, methylation and

ubiquitination) also contribute to this dynamic nature. These modifications can have a broad effect on gene expression through the interaction of co-regulators with a number of other transcription factors (Lonard and O'Malley 2007).

Interaction with co-activators or co-repressors with Smads is dependent on the MH2 domain of the proteins. The co-activator p300/CBP has been demonstrated to interact with the Smad proteins (Pouponnot et al. 1998). The co-activator is able to interact directly with the MH2 domain of Smad-2, -3 or -4 through its C-terminal domain to positively regulate transcription of target genes through its HAT activity and can enhance transcription of the TGF-β-responsive reporter plasmid 3TP-Lux (Dennler et al. 2005; Itoh et al. 2000; Nishihara et al. 1998). The transcriptional activity of both Smad-2 and -3 can also be enhanced by p300/CBP in response to TGF-β through acetylation of their MH2 domain (Inoue et al. 2007). Another co-activator demonstrated to interact with the Smad proteins is P/CAF, a co-activator associated with the control of differentiation, growth inhibition and apoptosis. Similar to p300/CBP, the co-activator is able to interact directly with Smad-3 to enhance Smad-dependent transcriptional responses following TGF-β stimulation in mammalian cells. Interestingly, the N-terminal of P/CAF is able to interact with p300/CBP suggesting that both co-activators may be required for some transcriptional responses (Itoh et al. 2000). Both p300/CBP and P/CAF co-activators are also able to regulate the expression of ERK in response to stimulation with retinoic acid (Chu et al. 2005).

1.16.1 Steroid receptor coactivator-1

SRC-1 is also thought to be involved in TGF-β signalling. SRC-1 (also known as nuclear receptor coactivator-1 (NCoA1)) is part of the p160 family of nuclear receptor co-activators and was the first of the p160 co-activators to be cloned. Other members of the p160 SRC family include SRC-2 (also known as NCoA2/transcriptional intermediary factor-2- TIF-2/glucocorticoid receptor-interacting protein- GRIP1) and SRC-3 (also known as NCoA3/amplified in breast cancer-1- AIB1/ retinoic acid interacting protein-3- RAC3/ thyroid hormone receptor-activated molecule-1- TRAM1/ p300/CBP-interacting protein- p/CIP). All SRCs are approximately 160kDa in size and expressed in a ubiquitous manner (Li and Shang 2007; Xu and Li 2003). Members of the family have been demonstrated to interact with nuclear receptors

(NR) including the oestrogen receptor, glucocorticoid receptor and the retinoid-X-receptor. In addition, they interact with several transcription factors including PPAR-γ, AP-1, NFκB and cAMP regulatory element-binding protein (CREB) (Leo and Chen 2000; Xu and Li 2003). SRC-1 knockout mice are prone to obesity, linking the co-activator to energy homeostasis, glycolysis and fatty acid synthesis (Lonard and O'Malley 2007; Xu et al. 2009). SRC-1 has also been linked with the vasoprotective effect of oestrogen. The co-activator is expressed in endothelial cells and VSMCs of normal arteries and in neointimal cells following vascular injury. Knockout mice have demonstrated a role in the inhibitory effects of oestrogen on neointimal growth and VSMC proliferation (Yuan and Xu 2007).

The SRC family members share a conserved structure. The N terminal domain is thought to be involved in intra-/inter-molecular interactions and the C terminal is involved in transcriptional activation. Between these domains, the receptor interacting domain contains conserved LXXLL (L-leucine, X-any amino acid) sequences known as NR boxes. These mediate interactions between the co-activator and nuclear receptors/other co-activators including p300/CBP which has been shown to interact with SRC-1 (Leo and Chen 2000; Li and Shang 2007; Lonard and O'Malley 2007; Xu and Li 2003). The co-activator has weak HAT activity mapped to its C-terminal domain and specific for histones H3 and H4 but is likely to require other co-activators for transcriptional activation through histone acetylation (Spencer et al. 1997).

Studies using the human PAI-1 promoter have demonstrated that like p300/CBP, the co-activator SRC-1 is able to enhance TGF-β/Smad transcriptional responses. In contrast to p300/CBP, SRC-1 does not bind directly to Smad-3 but instead is thought to interact with the p300/CBP co-activator to enhance association with Smad-3 and subsequent Smad-3 dependent transcriptional responses (Dennler et al. 2005). Using mammalian two-hybrid assays and transfection studies, Dennler and colleagues (2005) were able to show that interaction between p300/CBP and Smad-3 was enhanced by SRC-1 and inhibition of p300/CBP blocked the action of SRC-1 on Smad-3-dependent transcription (Dennler et al. 2005).

SRC-1 may also interact with MAPK pathways through phosphorylation (Rowan et al. 2000). Phosphorylation of SRC-1 is able to modulate its activity and generally enhances its transcriptional activity whilst methylation reduces transcriptional activity of the co-activator (Rowan et al. 2000). SRC-1 contains 7 serine/threonine phosphorylation sites. These contain consensus sites for proline-directed protein kinases (Rowan et al. 2000). The co-activator also contains consensus sites for MAPK (ERK 1/2) phosphorylation and the co-activator is able to potentiate AP-1 activity. cAMP/protein kinase A and NFκB pathways are also able to influence SRC-1 activity through phosphorylation (Li and Shang 2007).

1.17 Aims of study

Atherosclerosis is recognised as a progressive, inflammatory disorder and the underlying cause of cardiovascular disease, one of the leading causes of death in the Western world. Cytokines play crucial roles in the regulation of immune and inflammatory responses during atherosclerosis development, often mediated through the modulation of gene expression profiles associated with the disease. The study of the molecular mechanism regulating cytokine-mediated gene expression in atherosclerosis is crucial for the identification of novel targets for therapeutic intervention. TGF-β, an anti-inflammatory cytokine, acts as a key modulator of gene expression to prevent progression of atherosclerosis. TGF-β has been shown to modulate the expression of genes implicated in foam cell formation. However, the molecular mechanisms behind this regulation are yet to be elucidated. The classical Smad pathway plays a pivotal role in TGF-β signalling and in addition to this the cytokine can activate other cellular signalling pathways including the MAPK cascades. Studies into the TGF-β-regulation of these genes in macrophages, particularly through activation of TGF-β signalling pathways is therefore crucial to furthering understanding of the molecular basis of foam cell formation and atherosclerosis.

In the later stages of atherosclerosis, TGF-β modulates the expression of genes involved in turnover of the extracellular matrix to promote plaque stability. Recent identification of the ADAMTS family of proteases and their ability to cleave key

components of the vascular matrix has sparked interest in their role in atherosclerosis. Limited research currently exists on the functions of ADAMTS proteases, in particular ADAMTS-4 with relation to atherosclerosis. Studies on the cytokine-mediated regulation of ADAMTS-4 are therefore an important area for research.

The aims of the work presented in this thesis were therefore to:

- Investigate the regulation of expression of key genes implicated in cholesterol uptake and cholesterol efflux by TGF-β in macrophages (Chapter 3). This was to be achieved using RT-qPCR and Western blotting to analyse changes in mRNA and protein levels respectively.

- Investigate activation of key TGF-β signalling pathways in macrophages (Chapters 4 and 5). This was to be achieved using Western blotting and non-radioactive kinase activity assays to analyse protein levels of Smads, MAPKs and MAPK substrates respectively.

- Investigate the role of the classical Smad pathway in the TGF-β-regulation of key genes implicated in macrophage cholesterol homeostasis (Chapter 4). This was to be achieved using siRNA technology.
    - Following this, the aims were extended to investigate the role of three MAPK pathways (ERK, JNK and p38 kinase) using the same methodology (Chapter 5).

- Investigate the regulation of macrophage ADAMTS-4 by TGF-β (Chapter 6). This was to be achieved using RT-qPCR and Western blotting to analyse mRNA and protein levels respectively.
    - Following this, the aims were extended to investigate the action of TGF-β on ADAMTS-4 promoter activity using transient transfection and to characterise the minimal promoter binding region for the TGF-β response using transfection and EMSA analysis techniques.

CHAPTER 2

Materials and Methods

2.1 Materials

Table 2.1 lists the materials used during the course of this study, along with the suppliers from which they were purchased.

Table 2.1 **Materials and Suppliers**

Materials	Suppliers
Anti-ADAMTS-4	Affinity Bioreagents, Cambridge, UK
GAPDH negative control siRNA	Ambion, Cambridgeshire, UK
Acrylamide: Bisacrylamide (37.5:1) Acrylamide: Bisacrylamide (29:1)	Anachem, Luton, UK
INTERFERin™	Autogen Bioclear, Wiltshire, UK
Anti-ApoE	Biogenesis, Kiddlington, Oxford, UK
PCR grade magnesium chloride PCR grade NH_4 reaction buffer	Bioline, London, UK
Anti-mouse HRP-conjugate Anti-JNK Anti-phospho JNK Anti-phospho p38 kinase Anti-phospho p44/42 Anti-phospho Smad-2 Anti-phospho Smad-3 Anti-p44/42 Anti-p38kinase Anti-rabbit HRP-conjugate Anti-Smad-2/3	Cell Signaling Technology, New England Biolabs, Herts, UK
LB-medium and LB agar capsules	DIFCO Laboratories, Surrey, UK
Agarose	Eurogentec, Southampton, UK
Cell lines (Hep3B, THP-1, U937)	European Collection of Animal Cell Cultures, Salisbury, UK
EDTA Ethanol Hydrochloric acid Industrial methylated spirits Methanol Sodium chloride Sodium dodecyl sulphate Sodium hydroxide Tris-Base Other general chemicals	Fisher Scientific, Loughborough, UK

ECL Western blotting detection reagents Megaprime DNA labelling kit Nick columns Rainbow protein size markers Random Hexamers (PdN_6)	GE Healthcare, Buckinghamshire, UK
DMEM with GlutaMAX™ Foetal Calf Serum RPMI-1640 with GlutaMAX™	GIBCO® Invitrogen, Paisley, UK
1ml cryovials 96-well, 24-well and 12-well plates 50ml and 15ml Falcon polypropylene tubes Tissue culture flasks	Greiner, Gloucestershire, UK
Cell scrapers 0.2μM sterile filters 6-well plates	Helena Biosciences, Sunderland, UK
p38 kinase validated siRNA Smad-3 validated siRNA	Invitrogen, Paisley, UK
Penicillin/Streptomycin	Lonza Biologicals, Slough, UK
PVDF membrane Stericup® and Steritop™ 500ml filter	Millipore Ltd., Gloucestershire, UK
DNA molecular weight markers p44/42 MAPK kinase assay kit (non-radioactive) SAPK/JNK kinase assay kit (non-radioactive) Restriction endonucleases	New England Biolabs, Hertfordshire, UK
Anti- ABCA-1	Novus Biologicals, Suffolk, UK
Phosphate buffered saline tablets	Oxoid Ltd., Basingstoke, UK
[α-^{32}P]-dCTP	Perkin Elmer, Massachusetts, USA
Micro BCA protein assay kit	Pierce, Chester, UK
dNTPs Luciferase substrate M-MLV (5x) buffer M-MLV Reverse Transcriptase Passive lysis buffer (5x) pGL2 promoter vector RNasin® Shrimp alkaline phosphatase T4 DNA ligase	Promega, Southampton, UK
c-Jun validated siRNA ERK 1/2 validated siRNA QIAquick™ gel extraction kit QIAquick™ nucleotide removal kit Qiagen plasmid plus maxi kit QIAprep™ Spin mini kit RNeasy™ total RNA isolation mini kit Smad-2 validated siRNA SRC-1 (NCOA-1) genome-wide siRNA SuperFECT™	Qiagen, Crawley, UK
Anti-c-Jun Anti-LPL Anti-SR-A	Santa Cruz Biotechnology, California, USA
DNA oligonucleotides	Sigma Genosys, Cambridgeshire, UK
Ampicillin Aprotonin Benzamidine Bovine Serum Albumin Bromophenol blue Ethidium bromide Glycerol Kodak X-OMAT film	Sigma, Poole, UK

Leupeptin Molecular biology grade DMSO PMA PMSF Sodium fluoride Sodium orthovanadate Sodium pyrophosphate TBE (10x) TEMED Tissue culture grade DMSO Triton x-100 Trypsin/EDTA Tween 20 Zinc chloride 2-mercaptoethanol	
Human TGF-β	Totam (Tebu-Bio), Peterborough, Cambridgeshire, UK

2.2 Preparation of solutions and glassware

Solutions and glassware were autoclaved for 20-30 min at 121°C (975kPa) as necessary.

2.3 Cell culture techniques

2.3.1 Cell lines

2.3.1.1 THP-1

THP-1 is a human monocytic leukaemia cell line that can be differentiated with phorbol esters. Differentiated THP-1 cells display many of the properties and characteristics of human monocyte-derived macrophages and are therefore a useful model in which to study the properties of macrophages including cell signalling and gene expression (Auwerx, 1991).

2.3.1.2 U937

U937 is a human monocyte lymphoma cell line established by Sundström and Nilsson (1976) that has the characteristics of human monocytes. It is frequently used for promoter analysis in relation to monocyte/macrophage gene expression as it is easier to transfect with exogenous DNA. Similar to THP-1 cells, U937 is an immature cell

of the monocyte-macrophage lineage but can be differentiated using phorbol esters to induce a mature macrophage phenotype (Abrink et al. 1994; Tsuchiya et al. 1982).

2.3.1.3 Hep3B

Hep3B is a human hepatoma cell line established by Aden et al. (1979) that is often used for studies on cytokine-mediated gene expression during liver inflammation (acute-phase response). Cells are fully adherent hepatocytes derived from a human carcinoma. Hep3B cells show cytokine-mediated responses similar to that observed in inflammatory states (Coulouarn et al. 2005; Coulouarn et al. 2004; Hiron et al. 1992).

2.3.2 Maintenance of cell lines in culture

Both THP-1 and U937 cells were grown in RPMI-1640 with GlutaMAX™ (stabilized L-glutamine). Hep3B cells were grown in DMEM with GlutaMAX™. Medium was supplemented with 10% (v/v) heat-inactivated (at 56°C for 30min) foetal calf serum (HI-FCS) and penicillin (100U/ml) and streptomycin (100µg/ml) (complete medium). Both HI-FCS and penicillin/streptomycin were passed through a 0.2µm sterile filter prior to addition to the medium. Cells were maintained in a humidified incubator (37°C) with a 5% (v/v) CO_2 atmosphere. DMEM complete medium was replaced when required. Cells between passages 2 and 8 were used for experiments.

2.3.3 Sub-culturing of cells

2.3.3.1 THP-1 and U937 cells

Suspension cells were subcultured when they reached approximately 70% confluency (0.7×10^6 cells/ml). Confluent cells were split by diluting into fresh RPMI-1640 complete medium. Routinely cells were split in a ratio of 1:4.

2.3.3.2 Hep3B cells

Confluent (70%) Hep3B cells were washed once with DMEM. Cells were then trypsinised using 0.25% (v/v) trypsin/EDTA solution to cover the cell monolayer. Cells were incubated at 37°C, 5% (v/v) CO_2 until cells became visibly detached from the flask. DMEM complete medium was added and the cells were pelleted by

centrifugation at 110xg for 5min. The cell pellet was resuspended into an appropriate volume of fresh DMEM and cells were seeded at a ratio of 1:6.

2.3.4 Preserving and storing cells

Cell lines were preserved at -80°C or in liquid nitrogen. Only early passage cells (passages 1-3) were used for storage. Prior to freezing, cells were centrifuged at 110xg for 5min and resuspended in HI-FCS containing either 10% (v/v) glycerol (THP-1) or 10% (v/v) DMSO (U937 and Hep3B). Cells were aliquoted into 1ml cryovials and insulated with polystyrene. Cells were frozen at -80°C for short-term storage, or stored at -80°C overnight before being transferred to liquid nitrogen for longer-term storage.

2.3.5 Thawing frozen cells

Cells from liquid nitrogen were defrosted by placing in a water bath at 37°C. Cells were transferred to a polypropylene tube containing 10ml (v/v) HI-FCS and centrifuged at 110xg for 5min. After resuspension into the appropriate complete medium, cells were seeded into tissue culture flasks and incubated at 37°C with 5% (v/v) CO_2.

2.3.6 Counting cells

To count both U937 and THP-1 cells, a haemocytometer (Neubauer chamber) was used. After centrifugation (110xg for 5min), cells were re-suspended in an appropriate volume of medium containing 10% (v/v) HI-FCS. The haemocytometer was covered with a precision ground coverslip, then 7µl of cell suspension was used and the number of cells in the 5 x 5 grid was counted. The number of cells/ml was calculated by multiplying the number of cells in the counting area by 10^3. The appropriate volume of cells was diluted using fresh medium and seeded into tissue culture flasks or plates.

2.3.7 Treatment of cells with PMA and cytokines

THP-1 and U937 cells were differentiated with 0.16μM PMA for 24hr before cytokine treatment. Prior to cytokine treatment of Hep3B cells, the medium was aspirated and replaced. Cytokines were added directly to the culture medium and treated cells were incubated at 37°C with 5% (v/v) CO_2. Routinely TGF-β was added at a concentration of 30nM.

2.3.8 Human monocyte-derived macrophage cell culture

Ficoll-Hypaque purification was used to isolate human monocyte derived macrophages (HMDMs) from buffy coats. Blood was layered over Lymphoprep™ (Nycomed Pharmaceuticals) in Accuspin™ tubes (Sigma) and was centrifuged at 110xg for 30min to allow sedimentation of erythrocytes. Cells were transferred to a new centrifuge tube and an equal volume of PBS containing 0.4% (v/v) tri-sodium citrate was added before centrifugation (110xg, 5min). Pelleted cells were resuspended in 10ml 0.2% (v/v) saline solution, to lyse the red blood cells. Following centrifugation (110xg, 5min), platelets were removed from the mononuclear cell interface by washing several times with PBS containing 0.4% (v/v) tri-sodium citrate (with centrifugation (110xg, 5min) between washes). Monocytes were plated out in 12-well plates using RPMI-1640 supplemented with 5% (v/v) HI-FCS and penicillin (100U/ml) and streptomycin (100μg/ml). Cells were left to adhere for 7 days to allow for macrophage differentiation. Half of the volume of medium was replaced every 2 days and prior to use, cells were washed and the medium replaced to remove any non-adherent cells.

2.4 RNA/DNA related techniques

2.4.1 Isolation of Total RNA

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen). Briefly, the medium was aspirated from the cells and the cells washed once with PBS before being resuspended in 350-600μl buffer RLT (containing 10μl/ml β-mercaptoethanol), provided in the kit. This is a highly denaturing buffer which inactivates RNases to aid

in the collection of intact RNA. The lysate was homogenised either by passing the collected lysate through a QIA shredder column (Qiagen) or several times through a 0.9mm needle attached to a 1ml syringe. At this stage the lysate was stored at -80°C until a later date or immediately used for RNA extraction. The rest of the protocol was carried out according to the instructions provided by the manufacturer (Qiagen). Briefly, silica-based membrane spin columns were used to bind RNA. Addition of ethanol precipitated out any DNA to provide optimum binding conditions. Contaminants were washed away using the RW1 wash buffer (provided in the kit) before the RNA was eluted.

RNA concentration and purity was determined using a NanoDrop ND-1000 Spectrophotometer (Labtech International) according to the manufacturer's instructions. The integrity of the RNA was analysed by size-fractionating a small aliquot on a 1.5% (w/v) agarose gel (Section 2.4.4.1).

2.4.2 RT-PCR

Reverse transcription-polymerase chain reaction consists of two steps.

1. The synthesis of cDNA using reverse transcriptase.
2. PCR using gene-specific primers and optimised conditions to amplify a product of interest.

2.4.2.1 Reverse Transcription

RNA (typically 1µg or if the yield was low, 0.5µg) was mixed with 1µl random hexameric primers (200pmol, PdN6) and sterile water to a total volume of 13.5µl. Samples were incubated at 70°C for 5min and immediately cooled on ice. The following reagents were subsequently added to the reaction:

1µl dNTP mixture (10mM each of dATP, dCTP, dGTP, dTTP)
4µl 5 x M-MLV (Molony murine leukemia virus) reverse transcriptase buffer
0.5µl recombinant RNase inhibitor (RNasin®) (50U/µl)
1µl M-MLV reverse transcriptase (200U/µl)

The total mixture was incubated at 37°C for 1hr and the reaction terminated at 92°C for 2min. Synthesised cDNA was diluted by adding 80µl (or 30µl if 0.5µg RNA was used) RNase-free water and stored at -20°C.

2.4.2.2 Polymerase chain reaction

PCR conditions were specific to the primers used to amplify specific gene products. The primer sets used for PCR are shown in Table 2.2 with the product size in base pairs (bp) and the reference, if applicable, from which they were obtained. The reaction and amplification conditions are shown in Table 2.3 and Table 2.4 respectively. PCR conditions and cycle numbers were previously optimised in the laboratory so that the products were generated during the exponential phase of amplification, providing a direct correlation between the amount of product and the input cDNA template. PCR reactions were carried out using a Techne Thermal Cycler or a Peltier Thermal Cycler (PTC-200).

2.4.3 Real-time quantitative PCR

Real-time quantitative PCR (qRT-PCR) is an accurate method to analyse the amount of PCR products during the exponential stage of the amplification without reaching the plateau phase where the PCR reaction is not generating template at an exponential rate, one problem that can be encountered with endpoint quantification of RT-PCR reactions (Ginzinger 2002). qRT-PCR was carried out using the SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) and samples were prepared as 25µl reactions in duplicate using a 96-well plate format. The following reagents were added to 2.2µl cDNA:

27.5µl SYBR® Green JumpStart™ Taq ReadyMix™
10.5µl RNase-free H_2O
0.5µl forward primer
0.5µl reverse primer

Details of specific primers used are detailed in Table 2.5. SYBR Green is a dye that intercalates into dsDNA, similar to ethidium bromide used during agarose gel electrophoresis. The dye has undetectable fluorescence in its free form but fluoresces

on binding to dsDNA. SYBR Green can be used with any set of primers and although it is able to bind to any dsDNA target, correct amplification conditions can prevent the formation of primer dimers and ensure binding to a single product (Giulietti et al. 2001). Previously optimised amplification conditions are set out in Table 2.6 along with the reference, if applicable, from which the primer sequence was obtained. A melting curve was also constructed to verify correct, single product amplification.

For each transcript a standard curve was constructed using the purified PCR product generated for each specific set of primers. To create this, genes were blunt-end cloned into the pGEM-T vector (Promega) and correctly transformed colonies were identified by blue/white screening and sequenced. Plasmid DNA was prepared using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions (Section 2.4.8). Real-time PCR was carried out using a DNA Engine Opticon 2® real-time PCR detection system (MJ Research).

The comparative Ct method was used for analysis as described by Livak and Schmittgen (2001). The Ct method is a commonly used method for relative quantification of qPCR data. The Ct method relies on the number of cycles/rounds of amplification required for the fluorescence of a gene to surpass a pre-set threshold level (the Ct value) within the exponential phase of the amplification. This is directly proportional to the amount of starting template. The value of this for the gene of interest is compared relative to the equivalent Ct value for the control gene to normalise the data (Ginzinger 2002; Livak and Schmittgen 2001). The Ct method relies on two assumptions, firstly that the expression of the control gene does not vary under experimental conditions and secondly, that the PCR efficiencies for each gene are similar to each other. This can be assessed from the slope of the standard curve produced for each transcript (Ginzinger 2002). The transcript levels of 28SrRNA and RPL13A were used for normalisation in the studies presented in this thesis. These were used as normalising genes as they displayed stable expression in all samples. RPL13A is a ribosomal protein that has previously been shown to be a reliable standard in a number of studies analysing gene expression by qRT-qPCR including studies of chondrocyte and articular cartilage gene expression (Foldager et al. 2009; Pombo-Suarez et al. 2008) and gene expression during pancreatic diseases (Jesnowski et al. 2002) while 28S rRNA is an established housekeeping gene widely used for

analysis of gene expression, including in our own laboratory (Irvine et al. 2005; Singh and Ramji 2006).

Table 2.2 **Sequences of PCR primers used for the analysis of gene expression by RT-PCR**

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)	Reference
ABCA-1	GCTGCTGAAGCCAGGGCATGG	GTGGGGCAGTGGCCATACTCC	306	Kaplan et al. 2002
ADAMTS-4	AGGCACTGGGCTACTACTAT	GGGATAGTGACCACATTGTT	241	Arnaoutakis 2008
ApoE	TTCCTGGCAGGATGCCAGGC	GGTCAGTTGTTCCTCCAGTTC	270	Singh and Ramji 2006
β-2 microglobulin	TTTCTGGCCTGGAGGCTATC	CATGTCTCGATCCCACTTAACT	314	Ali 2007
GAPDH	CCCTTCATTGACCTCAACTA	AGTCTTCTGGGTGGCAGTGATGG	455	Sabatakos et al. 1998
LPL	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTTCTC	276	Irvine et al. 2005

Table 2.3 **Reaction conditions for RT-PCR**

Gene	Forward Primer (100µM)	Reverse Primer (100µM)	dNTPs (40mM)	$MgCl_2$ (50mM)	10x PCR buffer	10x Mg^{2+} PCR buffer	DMSO	Taq Polymerase (5µg/µl)	ddH_2O	cDNA
28S rRNA	0.50	0.50	1.00	2.00	5.00	-	5.00	0.25	25.75	10.00
ABCA-1	0.50	0.50	0.50	-	-	5.00	-	0.25	33.25	10.00
ADAMTS-4	0.50	0.50	0.50	0.50	5.00	-	-	0.50	32.50	10.00
ApoE	0.50	0.50	0.50	-	-	5.00	2.50	0.25	30.75	10.00
β-2 microglobulin	0.50	0.50	1.00	1.00	5.00	-	-	0.50	36.50	5.00
GAPDH	0.50	0.50	0.50	2.00	5.00	-	-	0.50	31.00	10.00
LPL	0.50	0.50	1.00	2.00	5.00	-	-	0.25	30.75	10.00

*All units given are µl and all PCR reactions were carried out in a total volume of 50µl.

Table 2.4 **Amplification conditions for RT-PCR**

Gene	Initial melting	Annealing	Extension	Melting	Final extension	Number of cycles
28S rRNA	94°C 2 min	62°C 30 sec	72°C 1 min	94°C 30 sec	72°C 10 min	11
ABCA-1	95°C 5 min	64°C 1 min	72°C 1 min	95°C 1 min	72°C 10 min	20
ADAMTS-4	96°C 5 min	60°C 1 min	72°C 1 min	93°C 30 sec	72°C 10 min	30
ApoE	96°C 5 min	62°C 1 min	72°C 2 min	93°C 30 sec	72°C 10 min	27
β-2 microglobulin	95°C 5 min	60°C 1 min	72°C 2 min	95°C 30 sec	72°C 8 min	19
GAPDH	95°C 5 min	60°C 1 min	72°C 2 min	93°C 30 sec	72°C 10 min	19
LPL	96°C 5 min	55°C 1 min	72°C 2 min	93°C 30 sec	72°C 10 min	24

Table 2.5 **Sequences of PCR primers used for the analysis of gene expression by qRT-PCR**

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
28S rRNA	TTAGACCGTCGTGAGACAGG	TTCAATAGATCGCAGCGAGG	Gibbs et al. 2002
ABCA-1	GCTGCTGAAGCCAGGGCATGG	GTGGGGCAGTGGCCATACTCC	Kaplan et al. 2002
ABCG-1	TGCAATCTTGTGCCATATTTGA	CCAGCCGACTGTTCTGATCA	Kaplan et al. 2002
ADAMTS-4	GGGATAGTGACCACATTGTT	AGGCACTGGGCTACTACTAT	-
ApoE	TTCCTGGCAGGATGCCAGGC	GGTCAGTTGTTCCTCCAGTTC	Singh and Ramji 2006
CD36	GAGAACTGTTATGGGGCTAT	TTCAACTGGAGAGGCAAAGG	Draude and Lorenz 2000
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTTGATGGGATTTC	-
LPL	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTTCTC	Irvine et al. 2005
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA	-
Smad-2	CCCATCAAATTCAGAGAGGTTC	TCACTTAGGCACTCAGCAAAAA	-
Smad-3	CTCCAAACCTATCCCCGAAT	GGCTCGCAGTAGGTAACTGG	-
SR-A	CCAGGGACATGGAATGCAA	CCAGTGGGACCTCGATCTCC	Draude and Lorenz 2000
SR-B1	ACGACACCGTGTCCTTCC	CGGGCTGTAGAACTCCAGCGA	Eguchi et al. 2006

Table 2.6 **Amplification conditions for qRT-PCR**

Gene	Initial melting	Annealing	Extension	Melting	Number of cycles
28S rRNA	94°C 2 min	62°C 30 sec	72°C 1 min	94°C 30 sec	40
ABCA-1	94°C 2 min	65°C 1 min	72°C 1 min	95°C 30 sec	40
ABCG-1	94°C 2 min	65°C 1 min	72°C 1 min	95°C 30 sec	40
ADAMTS-4	94°C 2 min	60°C 1 min	72°C 1 min	95°C 30 sec	40
ApoE	94°C 2 min	62°C 1 min	72°C 2 min	93°C 30 sec	40
CD36	94°C 2 min	58°C 1 min	72°C 1 min	95°C 30 sec	40
GAPDH	94°C 2 min	60°C 1 min	72°C 1 min	95°C 30 sec	40
LPL	94°C 2 min	55°C 1 min	72°C 2 min	93°C 30 sec	40
RPL13A	94°C 2 min	60°C 1 min	72°C 1 min	95°C 30 sec	40
Smad-2	94°C 2 min	63°C 1 min	72°C 1 min	95°C 30 sec	40
Smad-3	94°C 2 min	63°C 1 min	72°C 1 min	95°C 30 sec	40
SR-A	94°C 2 min	60°C 1 min	72°C 1 min	94°C 30 sec	40
SR-B1	94°C 2 min	60°C 30 sec	72°C 1 min 30 sec	95°C 40 sec	40

2.4.4 Agarose Gel Electrophoresis

For electrophoresis, 10x TBE [0.89M Tris-Borate (pH 8.3), 0.02M EDTA (Sigma)] was used to prepare a 1xTBE solution with UV-treated, double distilled water (ddH2O). Gels were made using 1-2% (w/v) agarose in 1xTBE with ethidium bromide (0.5µg/ml), a dye that fluoresces following intercalation into dsDNA, thereby allowing nucleic acids to be visualised. DNA/total RNA was visualised on the gel using a 5x loading dye (1x TBE, 50% (v/v) glycerol, 2.25% (w/v) bromophenol blue).

2.4.4.1 Resolving RNA on agarose gels

Routinely 1µg RNA was resolved on a 1.5% (w/v) agarose gel to examine its integrity. RNA samples were prepared with 5µl of sterile loading dye and RNase-free water (Qiagen) to a total volume of 20µl. Electrophoresis was performed in 1 x TBE at 50V for 10-15 min using a Fisherbrand horizontal gel unit.

2.4.4.2 Resolving DNA

To resolve DNA, agarose gels were prepared with between 1–2% (w/v) agarose depending on the size of the DNA to be fractionated. Size fractionation of PCR products was routinely carried out using 1.5% (w/v) agarose gels and fragment size compared to standard DNA molecular weight markers (Appendix I). DNA was prepared with 5µl of DNA loading dye and electrophoresis was carried out in 1x TBE buffer at 100V for 1hr using a Fisherbrand horizontal gel unit.

2.4.4.3 Analysis of RT-PCR amplification products

Nucleic acids were visualised under UV light using a Syngene Gel Documentation System. Signals for PCR products were quantified using the Syngene Gene Tools computer package (GRI).

2.4.5 Extracting DNA from agarose gels

DNA bands were extracted from ethidium bromide-stained agarose gels using the QIAquick™ gel extraction kit (Qiagen) according to the manufacturer's instructions.

Efficiency of purification was analysed by electrophoresis using a 1.5% (v/v) agarose gel.

2.4.6 Bacterial Strains and Vectors

All bacterial culture media (LB-Agar and LB-liquid media) was prepared according to manufacturer's instructions (DIFCO Laboratories). The strains and genotypes of *Escherichia coli* (*E. coli*) are shown in Table 2.7 below.

Table 2.7 Bacterial Strains and Genotypes

Bacterial Strain	Genotype	Reference
DH5α	*supE44 ΔlacU169 (ϕ80 lacZΔM15) hsd17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan 1983

2.4.7 Transformation of Competent cells

DH5-α competent cells (Invitrogen) were thawed on ice prior to transformation. Plasmid DNA (1-5µl) was added to 50µl of competent cells and kept on ice for 30min to allow the plasmid DNA to associate with the cell surface. The cells were heat-shocked at 42°C for 20sec to cause a temporary increase in membrane permeability and placed back on ice for a further 2min. Then, 950µl S.O.C. medium (Invitrogen) was added and the cells were incubated at 37°C with shaking (225rpm) for 1hr to allow the cells to express the newly-introduced gene. To select ampicillin-resistant bacterial colonies the cells were spread on LB-agar plates containing 100µg/ml ampicillin. Plates were incubated overnight at 37°C.

2.4.8 Small-scale preparation of plasmid DNA (Mini-prep)

LB-medium (5ml) containing ampicillin (100µg/ml) was inoculated with a single colony of transformed *E.coli* and incubated overnight at 37°C with shaking (200rpm). Preparation of plasmid DNA from the bacterial culture was carried out using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Briefly, alkaline lysis of cells was carried out, followed by the binding of plasmid

DNA to the silica membrane of the QIAprep column using a high-salt buffer. Endonucleases, salt and other contaminants were washed through and the DNA was eluted using a low-salt buffer.

2.4.9 Large scale preparation of plasmid DNA (Maxi-prep)

LB-medium (10ml) containing ampicillin (100μg/ml) was inoculated with a single colony of transformed *E.coli* and incubated for 8hr at 37°C with shaking (200rpm). This was then used to inoculate 500ml fresh LB-medium with ampicillin (100μg/ml) and this was incubated at 37°C with shaking (200rpm) overnight. Plasmid DNA was prepared using a QIAprep Spin Maxiprep kit according to the manufacturer's instructions (Qiagen). Briefly, transformed cells were resuspended in buffer P1 (a Tris-Cl buffer, provided in the kit). Alkaline lysis of the cells using NaOH and SDS (buffer P2, provided in the kit) was carried out before the plasmid DNA was bound to an anion-exchange column in the presence of a high-salt buffer. DNA was eluted using alcohol precipitation.

The concentration and purity of DNA was determined by measuring O.D.260 and O.D.280 using a U-1800 Hitachi spectrophotometer.

2.4.10 Restriction endonuclease digestion of recombinant plasmid DNA

Single and double restriction enzyme digests were carried out using 1μg/μl DNA in a 20μl reaction and were performed at 37°C for 3hr in the recommended buffer provided by the manufacturer (Promega) in the presence of acetylated BSA (10μg/μl). Typically 0.5μl of the appropriate restriction enzyme (10U/μl) was added to 1μl DNA. Restriction digests were analysed by agarose gel electrophoresis.

2.4.11 Cloning of Oligonucleotides

Oligonucleotides incorporating a 5'-XmaI or XhoI site were designed (Table 2.8) to be cloned directly into a restriction enzyme-digested pGL2 promoter vector (Appendix II). The vector was cleaved with the aforementioned restriction enzymes and subjected to agarose gel electrophoresis alongside 1kb DNA markers (Appendix

I). The product was excised from the gel and the DNA purified (Section 2.4.5). Oligonucleotides were prepared by incubating forward and reverse oligonucleotides together at 100°C for 10min. The mixture was then left to cool to room temperature to allow annealing to occur. The annealed oligonucleotides were then ligated into the cleaved pGL2 promoter vector. This was carried out by preparing a 10µl reaction mix containing 1µl 10x T4 DNA ligase buffer, 1µl T4 DNA ligase, 1µl pGL2 promoter vector and 1-7µl purified fragment. T4 DNA ligase forms phosphodiester bonds between the 3' hydroxyl group of one nucleotide and the 5' phosphate group of another to ligate the oligonucleotides to the pGL2 promoter vector. The reaction mix was incubated overnight at 4°C. Transformation of ligated products was carried out using *E.coli* DH5α competent cells (Section 2.4.7).

Table 2.8 **Oligonucleotide sequences used for insertion into pGL2 promoter vector and subsequent promoter analysis**

ADAMTS-4 promoter region	Annealed oligonucleotide inserts
Oligo 1 (-502 to -463)	5'-CCGG**ATGCCTCCTTACCTGTTCCCTACCTTCTTTTCTCAGGCA** -3' 3'- **TACGGAGGAATGGACAAGGGATGGAAGAAAAGAGTCCGT**AGCT -5
Oligo 2 (-464 to -439)	5'- CCGG**CAGCTCACTCAGTCCCCTCAGCCCTGG** -3' 3'- **GTCGAGTGAGTCAGGGGAGTCGGGACC**AGCT -5'
Oligo 3 (-443 to -423)	5'- CCGG**CCTGGAAACCAGCCACTAGG** -3' 3'- **GGACCTTTGGTCGG TGATCC**AGCT -5'
Oligo 4 (-423 to -390)	5'- CCGG**CCAAAGGGCAGCATGAGGGAGCCTTGAGAAAA** -3' 3'- **GGTTTCCCGTCGTACTCCCTCGGAACTCTTTT**AGCT -5'

The ADAMTS-4 promoter sequence is shown in bold text.

2.4.12 DNA sequencing

GL1 and GL2 primers that amplified any insert within the pGL2 multiple-cloning site were used for sequencing to detect correctly transformed colonies. Sequencing was carried out by the Molecular Biology Support Unit of Cardiff University.

2.5 DNA Transfections

2.5.1 SuperFect™ Transfection

Transfection work was carried out in U937 and Hep3B cell lines using conditions previously optimised in the laboratory. Cells were grown in medium containing 10% (v/v) HI-FCS. Confluent Hep3B cells were trypsanised and seeded as previously described (Section 2.3.3). Cells were plated in 12-well plates 24hr prior to transfection and incubated at 37°C with 5% (v/v) CO_2. Medium was replaced with fresh DMEM complete medium prior to transfection. Confluent U937 cells were harvested by centrifugation (110xg for 5 min). Cells were resuspended in medium supplemented with 3% (v/v) HI-FCS and plated out into 12-well plates at a density of 600,000 cells per well. Cells were incubated at 37°C with 5% (v/v) CO_2 for 4hr prior to transfection.

Transient transfection was carried out using SuperFect™ according to the manufacturer's instructions (Qiagen) with minor modifications. Briefly, the transfection mix was prepared in 50µl medium that contained no antibiotics and no HI-FCS. SuperFect™ Reagent and the plasmid DNA of interest (containing the promoter of the gene of interest linked to the firefly-luciferase reporter gene) was added in a 3:1 ratio. Plasmids maps and further information on each plasmid can be found in Appendix II. SuperFect™ has a dendrimer structure with positively charged amino groups that interact with the phosphate backbone of DNA to form a complex that can be taken up into the cell by endocytosis. The total mixture was allowed to incubate at room temperature for 10min before being added to complete medium (containing 10% (v/v) HI-FCS). The complete mix was added dropwise to cells. Cytokine treatment was carried out 30min following transfection (Hep3B cells). Cells were incubated at 37°C with 5% (v/v) CO_2 overnight before being harvested using 1 x Passive Lysis Buffer (Promega). In the case of U937 cells, PMA was added immediately following transfection and cytokines were added 2hr after transfection. Cells were left to differentiate overnight before being harvested using 1 x Passive Lysis Buffer (Promega).

2.5.2 Preparation of cell extracts for determination of reporter gene activity

To prepare cell extracts for analysis, medium was aspirated and replaced with PBS. The PBS was then aspirated and 200µl of 1 x Passive Lysis Buffer (Promega) was added directly to the cell monolayer and left to incubate for 15min at room temperature. Cells were then scraped into the buffer, transferred to a microcentrifuge tube and centrifuged at top speed for 5min. The supernatant was either stored at -20°C or used immediately for the measurement of luciferase activity.

2.5.3 Luciferase assay

Luciferase activity of samples was determined as described by the manufacturer (Promega). Firefly luciferase is an enzyme that catalyses the activation of luciferin by ATP to produce an anhydride intermediate which then reacts with oxygen to produce oxyluciferin, CO_2 and light (Reed et al. 2007). Luciferase Substrate (100µl) and sample were added to disposable plastic cuvettes in a 1:1 ratio. Luciferase activity was determined using a Luminometer (TD-20/20, Turner Designs, California, USA) set at 70% sensitivity level with a 2sec delay period and a 20sec integrate period. Measurements were taken in duplicate and luciferase counts were normalised to the protein concentration (µg/µl) of each sample (Section 2.7.4).

2.6 siRNA Transfections

2.6.1 siRNA transfection with Interferin™

siRNA transfections were carried out using validated siRNAs against target mRNAs (Table 2.9). Stock solutions were prepared from lyophilised siRNAs according to the manufacturer's instructions (Qiagen and Invitrogen). siRNA transfection was carried out in THP-1 cells prior to differentiation with 0.16µM PMA. Confluent THP-1 cells were harvested by centrifugation at 110xg for 5min. The cell pellet was resuspended in the appropriate volume of complete RPMI-1640 medium with no antibiotics. Cells were seeded into 6-well plates and incubated at 37°C with 5% (v/v) CO_2 for 4 hr prior to transfection. siRNA transfection was carried out according to the manufacturer's instructions (PolyPlus Transfection) with minor modifications.

The following procedure was carried out for the transfection of cells in one $2cm^2$ cell culture dish. Transfection mix was prepared in 100µl antibiotic-free and HI-FCS-free RPMI-1640 medium. Then, 7.5nM siRNA was added to this and the sample was briefly centrifuged in a microcentrifuge. Following addition of the transfection reagent Interferin™ (18µl), the mixture was again briefly micro-centrifuged and allowed to incubate at room temperature for 20min. The complete mixture (100µl) was then added dropwise to cells. Following transfection (24hr) cells were differentiated using 0.16µM PMA for 24hr. Cells were left untreated or stimulated with 30nM TGF-β for the requisite time period before being harvested for RNA extraction (Section 2.4.1) or protein extraction (Section 2.7.1).

Table 2.8 **siRNAs used during the course of this study**

Target mRNA transcript	NCBI accession number(s) of target	SI Reference number/Catalogue number*
GAPDH	NM_002046	*AM4604
Smad-2	NM_001003652 NM_001135937 NM_005901	SI02757496
Smad-3	NM_005902.3	*46-1718 / 46-1719
p38 MAPK	NM_0001315.1 NM_139012.1 NM_139013.1 NM_139014.1	*46-1471 / 46-1470
c-Jun	NM_002228	SI00300580
ERK 1/2	NM_002745 NM_138957	SI00300755
SRC-1 (NCOA-1)	NM_003743 NM_147223 NM_147233	SI00055342

2.7 Protein analysis

2.7.1 Preparation of protein extracts using Laemmli buffer

Cells were routinely grown in a $2cm^2$ well for protein analysis. The cells were washed with 1ml PBS. The PBS was then aspirated from the cells and 70µl freshly

prepared Laemmli buffer (Table 2.10) was added. Cells were scraped into the buffer and the collected extracts were micro-centrifuged at top speed for 5min. Lysates were stored at -80°C or used immediately for SDS-PAGE followed by western blotting (Section 2.7.7).

2.7.2 Preparation of Whole-cell protein extracts

Cells were washed and scraped into an appropriate volume of PBS before being transferred to a polypropylene (Falcon) tube and centrifuged at 110xg for 5min. Cells were resuspended in freshly prepared whole cell extracts buffer (100µl) (Table 2.10), transferred to microcentrifuge tubes and vortexed for 5sec. The extracts were left on ice at 4°C for 30min before being micro-centrifuged at 10,000rpm for 10min, also at 4°C. The supernatant containing the total cellular protein was removed and stored at -80°C. Protein concentration was determined using the Micro Protein Assay Reagent Kit (Pierce).

For preparation of phosphatase-free whole cell extracts, cells were washed twice with ice-cold PBS (1ml) containing 10mM NaF and 100µM sodium orthovanadate and micro-centrifuged (13,00rpm, 1min) between washes. The cell pellet was resuspended in whole cell extraction buffer (Table 2.10). Extracts were vortexed for 45sec and micro-centrifuged (10,000rpm, 10min at 4°C). The supernatant containing the total cellular protein was stored at -80°C and protein concentration was determined using the Micro Protein Assay Reagent Kit (Pierce) as described above.

Table 2.10 **Composition of stock solutions used for protein analysis by SDS-PAGE and Western Blotting**

Solution	Composition
Laemmli sample buffer	0.125M Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% (w/v) Glycerol, 10% (v/v) 2-mercaptoethanol
Phosphatase-free whole-cell extraction buffer	10mM Tris-HCl (pH 7.05), 50mM NaCl, 50mM NaF, 1% (v/v) Triton X-100, 30mM $Na_4P_2O_7$ (sodium pyrophosphate), 5µM $ZnCl_2$, 100µM Na_3VO_4 (sodium orthovanadate), 1mM DTT, 2.8µg/ml Aprotonin, 2.5µg/ml Leupeptin, 2.5µg/ml Pepstatin, 0.5mM Benzamidine, 0.5mM PMSF
Bromophenol blue	0.05% (w/v) bromophenol blue in ddH_2O
SDS PAGE gel loading buffer	50mM Tris-HCl (pH 6.8), 100mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol
SDS PAGE separating gel buffer	1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS
SDS PAGE stacking gel buffer	1M Tris-HCl (pH 6.8), 10% (w/v) SDS
SDS PAGE running buffer	25mM Tris, 250mM glycine, 0.1% (w/v) SDS
Western blot transfer buffer	25mM Tris, 192mM glycine, 20% (v/v) methanol
10x Tris buffered saline (TBS)	10mM Tris-HCl, 20mM NaCl, pH 7.4

2.7.3 Preparation of Nuclear Extracts

Nuclear extracts were prepared using the Active Motif Nuclear Extract Kit according to the manufacturer's instructions (Active Motif) with minor modifications. The following procedure was carried out for Hep3B cells grown in one 100mm^2 plate. Briefly, cells were washed once with 3ml ice-cold PBS/phosphatase inhibitors provided in the kit. This was aspirated and replaced with 5ml ice-cold PBS/phosphatase inhibitors. Cells were scraped into the solution and transferred to a polypropylene (Falcon) tube and pelleted by centrifugation (110xg, 5min at 4°C). The supernatant was discarded and the cell pellet was resuspended in 500µl 1x Hypotonic buffer (provided in the kit), transferred to a microcentrifuge tube and incubated on ice for 15min. Then, 25µl of detergent was added, and the mixture vortexed (10sec) and micro-centrifuged at 4°C (13,000rpm, 1min). The supernatant containing the cytoplasmic fraction was collected and stored at -80 °C. The cell pellet was resuspended in 50µl Complete Cell Lysis Buffer (provided in the kit), vortexed (10sec) and incubated on ice (30min, with rocking). After micro-centrifugation (13,000rpm for 10min at 4°C) the supernatant containing the nuclear fraction was collected and stored at -80°C. Protein concentration was determined using the Micro Protein Assay Reagent Kit (Pierce).

2.7.4 Determination of protein concentration

Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit as described by the manufacturer (Pierce). Samples were processed in 96 well plates and a standard curve was prepared for each assay using the appropriate buffer. Samples were routinely diluted using PBS at a ratio of 1 in 50 and 1 in 100. Reagents A, B and C, provided in the kit, were mixed according to the manufacturer's instructions (Pierce) and added to each well. The plate was covered and left to incubate at 37°C for 2.5hr or overnight at room temperature. A Model 680 Microplate Reader (Biorad) was used to measure the absorbance of each sample at a wavelength of 570nm.

2.7.5 SAPK/JNK Activity Assay

The SAPK/JNK assay was carried out using the SAPK/JNK non-radioactive kinase assay kit according to the manufacturer's instructions (Cell Signaling Technology) with minor modifications. Cell extracts were prepared using 1 x Cell Lysis Buffer (provided in the kit) supplemented with 1mM of the protease inhibitor PMSF. Then, 200µl of extract was added to 20µl of c-Jun fusion protein beads (a c-Jun fusion protein linked to agarose beads) and incubated at 4°C overnight, with gentle rocking to allow binding of SAPK/JNK to the c-Jun fusion protein. The sample was micro-centrifuged at 13,000rpm for 2min. The resultant pellet was washed three times with 500µl 1 x Cell Lysis Buffer (provided in the kit) and micro-centrifuged between washes (13,000rpm, 1min) to remove the agarose beads. The pellet was then washed three times with 1 x Kinase Buffer (provided in the kit) with micro-centrifugation (13,000rpm, 1min) between washes to remove any contaminants. The resulting pellet was resuspended in 50µl of 1 x Kinase Buffer (provided in the kit) supplemented with 200µM ATP and the mixture was incubated at 30°C for 30min to allow for the phosphorylation of c-Jun by SAPK/JNK. The reaction was terminated by addition of 25µl solubilising solution (SDS-PAGE gel loading buffer) (Table 2.10). The sample was boiled for 5min before being loaded onto a 10% (w/v) SDS-PAGE gel. SDS-PAGE and Western blotting were carried out as described in Sections 2.7.7 and 2.7.8 respectively. An antibody specific to this kit, phospho c-Jun (Ser63) was used for immuno-detection.

2.7.6 p44/42 MAPK Activity Assay

The p44/42 MAPK assay was carried out using the p44/42 MAPK non-radioactive kinase assay kit according to the manufacturer's instructions (Cell Signaling Technology) with minor modifications. Cells were prepared using 1 x cell lysis buffer (provided in the kit) supplemented with 1mM PMSF. Cell extracts (200µl) were added to 15µl Immobilised phospho p44/p42 (Thr202/Tyr204) MAPK mouse monoclonal antibody and incubated at 4°C overnight, with gentle rocking to allow the antibody to bind the p44/42 MAPK. Cells were pelleted and washed with 1 x cell lysis buffer followed by 1 x kinase buffer as described in Section 2.7.5. After the final wash, the pellet was resuspended in 50µl 1 x kinase buffer (provided in the kit) supplemented with 200µM ATP and 5µg Elk-1 fusion protein. The mixture was

incubated at 30°C for 30min to allow the active p44/42 MAPK to phosphorylate the Elk-1 substrate and then the reaction was terminated by addition of 25µl solubilising solution (SDS-PAGE gel loading buffer) (Table 2.10). The sample was boiled for 5min before being loaded onto a 10% (w/v) SDS-PAGE gel. SDS-PAGE and western blotting were carried out as described in Sections 2.7.7 and 2.7.8 respectively. An antibody specific to this kit, phospho Elk-1 (Ser383) was used for immuno-detection.

2.7.7 SDS polyacrylamide gel electrophoresis

SDS-PAGE was routinely carried out using gels composed of 10% or 12.5% (w/v) acrylamide. Stacking and separating gels were prepared from stock solutions (Table 2.11) with the composition of the separating gel determined by the size of the protein being fractionated. Electrophoresis was carried out using the Mini-PROTEAN II slab electrophoresis cell (Bio-Rad Laboratories). Glass plates were cleaned and prepared and the separating gel poured to within 3cm of the upper edge of the inner glass plate. Isopropanol was layered on top to remove any air bubbles and TEMED and APS were added to the gel mix to initiate polymerisation of the acrylamide/bisacrylamide gel. Once the gel had set, the isopropanol was washed off using ddH_20 and the upper surface of the plates dried with Whatman 3MM paper. The stacking gel was poured to the top of the plates and a well-forming comb added. Once the gel had set and polymerised, the comb was removed and any air bubbles in the wells removed using Whatman 3MM paper. The gel was then placed in the electrophoresis tank and the inner and outer compartments were filled with 1x running buffer, containing 0.1% (w/v) SDS.

Table 2.11 Composition of stacking and separating gels for SDS-PAGE

Component	5% (w/v) stacking gel	7.5% (w/v) separating gel	10% (w/v) separating gel	12.5% (w/v) separating gel
Upper buffer	1.25ml	-	-	-
Lower buffer	-	2.5ml	2.5ml	2.5ml
Acrylamide: Bisacrylamide (37.5:1)	0.625ml	1.875ml	2.5ml	3.125ml
10% (w/v) APS	50µl	100µl	100µl	100µl
TEMED	5µl	10µl	10µl	10µl
ddH_2O	3.07ml	5.625ml	5ml	4.26µl

Protein samples were prepared as described previously (Sections 2.7.1 and 2.7.2). Laemmli buffer and whole-cell extraction buffer preparation used the reducing agent 2-mercaptoethanol (Laemmli buffer-prepared extracts) or DTT (whole-cell extraction buffer-prepared extracts) to disrupt the disulphide bonds of the protein and cause denaturation. Both buffers also contained the detergent SDS which disrupts the non-covalent bonds of the protein. SDS deposits negative charges on the peptide backbone of the protein that are proportional to its molecular weight. The large negative charge causes electrostatic repulsion leading to the unfolding and denaturing of the protein into a rod-like shape that will run smoothly on the gel (Reed et al. 2007). To whole cell protein extracts, SDS-PAGE gel loading buffer (Table 2.10) was added and with the exception of ABCA-1 samples, the extracts were heated at 100°C for 5min. Laemmli buffer protein extracts (Section 2.7.1) were heated for 7min at 100°C following addition of bromophenol blue for visualisation of protein samples. Appropriate volumes of sample (routinely between 10 and 30µl of Laemmli buffer protein extract or 10-30µg whole cell protein extract) were added to wells and were size-fractionated alongside a full-range rainbow protein marker (GE Healthcare) (Appendix I). Electrophoresis was carried out at 200V for 45-50min. The gels were then used for western blotting.

2.7.8 Western Blotting

Following electrophoresis, the stacking gel was cut away and the separating gel equilibrated in transferring buffer (Table 2.10). PVDF membrane (0.45μm pore size, Millipore) was cut to the size of the gel and activated in methanol before also being placed in transferring buffer. The membrane was placed on top of the gel and both were sandwiched between Whatman 3MM paper and sponge pads which had also been soaked in transferring buffer and placed in a blotting cassette. Electrophoretic transfer of proteins was carried out using a Mini Trans-Blot cell (Bio-Rad Laboratories) containing the transfer buffer (Table 2.10). Electro-blotting was carried out at 150V for 75min at 4°C or at 15V for 12-18hr at 4°C.

2.7.9 Immuno-detection of proteins

Following blotting, the membrane was removed and placed in 5% (w/v) powdered milk for blocking, for 1hr with shaking. Proteins in the milk solution bind to spaces in the membrane to prevent non-specific binding of the antibody, ensuring that it can only bind to the target protein (Reed et al. 2007). The membrane was washed three times for 5-10min with 1 x TBS containing 0.1% (v/v) TWEEN-20 (Table 2.10). The membrane was then incubated with the primary antibody, diluted in 1x TBS with 0.1% (v/v) TWEEN-20 and 5% (w/v) skimmed milk powder or 5% (w/v) bovine serum albumin (BSA), for 1hr at room temperature or overnight at 4°C (Table 2.12). The membrane was washed as described above and incubated with the secondary antibody, diluted in 1x TBS containing 0.1% (v/v) TWEEN-20 and 5% (w/v) skimmed milk powder, for 1hr at room temperature (horse-radish peroxidise-conjugated, anti-rabbit IgG, anti-mouse IgG or anti-goat IgG diluted 1:2000 to 1:20,000). The membrane was washed as described above and then used for chemiluminescent detection (Section 2.7.10).

Table 2.12 **Antibodies used for the immuno-detection of proteins following Western blotting**

Primary Antibody	Dilution (in 1x TBS with 0.1% (v/v) TWEEN 20)	5% (w/v) non-fat milk or 5% (w/v) BSA	Protein size (kDa)
Anti- ABCA-1	1:1000	Milk	220
Anti- ADAMTS-4	1:1000	BSA	53 and 68
ApoE	1:1000	Milk	34
β-actin	1:10,000	Milk	42
c-Jun	1:1000	BSA	35 and 37
JNK	1:1000	BSA	46 and 54
LPL	1:500	Milk	56
p38 kinase	1:1000	BSA	43
p44/42 MAPK (ERK 1/2)	1:1000	BSA	42 and 44
Phospho c-Jun	1:1000	BSA	35 and 37
Phospho Elk-1	1:1000	BSA	62
Phospho p44/42 MAPK	1:1000	BSA	42 and 44
Phospho JNK	1:1000	BSA	46 and 54
Phospho p38 kinase	1:1000	BSA	43
Phospho Smad-2	1:1000	BSA	60
Phospho Smad-3	1:1000	BSA	60
Smad 2/3	1:1000	BSA	58
Smad 3	1:1000	BSA	58
SR-A	1:1000	Milk	75
SRC-1	1:1000	BSA	180

2.7.10 Detection of Chemiluminescence

Chemiluminescent detection was carried out according to the manufacturer's protocol (GE Healthcare). The chemiluminescent reagent is cleaved by the horse-radish peroxidise reporter gene present on the secondary antibody. This results in a luminescence that is proportional to the amount of protein present and that can be detected by photographic film. Membranes were placed in contact with Kodak X-ray film in a light proof cassette (Genetic Research Instrumentation, GRI) for varying periods of time. The film was developed using an automatic developer (Agfa-Gaevert).

2.8 Electrophoretic Mobility Shift Assay (EMSA)

2.8.1 Preparation of radiolabelled oligonucleotide probe

Forward and reverse oligonucleotide sequences were designed to leave 5' overhangs that contained at least one G residue following annealing to allow for the complementary binding of [α-^{32}P]-dCTP during radiolabelling (Table 2.13).

Table 2.13 Sequences of oligonucleotides used for EMSA

ADAMTS-4 promoter region	Annealed Oligonucleotide
Oligo 1 (-502 to -463)	5'- TTGATGCCTCCTTACCTGTTCCCTACCTTCTTTTCTCAGGCA -3' 3'- TACGGAGGAATGGACAAGGGATGGAAGAAAAGAGTCCGTCG -5'
Oligo 2 (-464 to -439)	5'- AGGCAGCTCACTCAGTCCCCTCAGCCCTGG -3' 3'- GTCGAGTGAGTCAGGGGAGTCGGGACCTTTG -5'
Oligo 3 (-443 to -423)	5'- GCCCTGGAAACCAGCCACTAGG -3' 3'- GGACCTTTGGTCGGTGATCCCGG -5'
Oligo 4 (-423 to -390)	5'- GGGCCAAAGGGCAGCATGAGGGAGCCTTGAGAAAA -3' 3'- GGTTTCCCGTCGTACTCCCTCGGAACTCTTTTCTCTTCGG -5'

2.8.1.1 Annealing of oligonucleotides

Forward and reverse oligonucleotides were incubated together (100°C, 10min) in the presence of a medium salt buffer (NEB buffer 3). The mixture was left to cool to room temperature and either used immediately for radiolabelling or stored at -20°C.

2.8.1.2 Radiolabelling of oligonucleotides

Oligonucleotides were radiolabelled using the reagents supplied in the MegaPrime™ Labelling Kit (Amersham). Annealed oligonucleotides (10µl) were incubated for 20min at 37°C with 1x labelling buffer, Klenow DNA polymerase (2µl) and [α-^{32}P]-dCTP (3µl) in a total volume of 50µl. Radiolabelled probe was separated from

unincorporated nucleotides using a Sephadex G50 nick column. The column was equilibrated using 1x TE buffer (10mM Tris-HCl (pH 7.5), 1mM EDTA). The reaction mixture (50µl) was added to the column and eluted using 1x TE buffer (350µl). The unlabelled probe was then discarded and the column further eluted using 400µl 1x TE buffer. The eluate (labelled probe) was collected and stored at -20°C.

2.8.2 DNA-protein binding reactions

Binding of the radiolabelled probe to the nuclear extracts was carried out in a 10µl reaction. Nuclear extract (2µg) was added to 2µl 5x binding buffer (Table 2.14), 2µl poly-(dI-dC) (1µg/µl) to prevent non-specific binding, and 3µl radio-labelled probe. The mixture was incubated at room temperature for 30min before being subjected to electrophoresis.

Table 2.14 **Composition of buffers used for EMSA**

Buffer	Composition
5x Binding buffer	1M Tris-HCl (pH 8), 2.5M KCl, 0.5M EDTA, 1M DTT, 62.5% (v/v) glycerol, 0.5% (v/v) Triton X-100

2.8.3 Competition binding studies

For competition binding studies a 250-fold molar excess of unlabelled competitor oligonucleotides was added to the protein binding reaction and incubated on ice for 20min prior to the addition of radiolabelled probe.

2.8.4 Electrophoresis of DNA: protein complexes

A nondenaturing, polyacrylamide gel (6%) was used to resolve DNA:protein complexes, the composition of which is outlined below.

7.5ml acrylamide:bisacrylamide (29:1)

2.5ml 10x TBE

40ml dH_2O

500µl APS (10%w/v)

50µl TEMED

Electrophoresis was carried out in 0.25% (v/v) TBE buffer at 150V for 3-4hr using vertical gel apparatus (Scotlab). Following electrophoresis the gel was transferred to Whatman 3MM paper and dried using a gel dryer (Model 583, Bio-Rad) for 1hr. The gel was exposed to Kodak XAR film (Sigma) in a light proof cassette at -80°C for varying time periods (12-72hr). Film was developed using an automatic developer (Agfa-Gevaert).

2.9 Densitometric analysis of data

The density of bands from agarose gel images, immunoblots and EMSA films was analysed using the GeneTools software (Syngene).

2.10 Statistical analysis of data

Data was analysed using a standard Student's T test with $P<0.05$ taken as statistically significant (Appendix III).

CHAPTER 3

TGF-β-regulated expression of key genes implicated in the control of macrophage cholesterol homeostasis

3.1 Introduction

3.1.1 Macrophage cholesterol homeostasis

The studies presented in this chapter focus on the regulation of expression of key genes implicated in macrophage cholesterol homeostasis by TGF-β. As detailed in Sections 1.3 and 1.4, the differentiation of macrophages into lipid-laden foam cells is a critical early event in atherosclerosis. Foam cell formation is caused by an imbalance between the uptake of modified LDL into cells and the efflux of cholesterol from these cells. Macrophages take up cholesterol through the scavenger receptors, SR-A, CD36 and SR-PSOX which normally serve to remove pathogens and cell debris but in atherosclerosis take up oxLDL. The enzyme lipoprotein lipase also contributes to the uptake of cholesterol by hydrolysing lipids into smaller cholesterol-rich remnants that can be easily taken up by macrophages. Cholesterol is removed from cells by reverse cholesterol transport; the movement of cholesterol from cells to HDL via ATP binding cassette transporters. This requires two apolipoproteins of HDL, ApoAI and ApoE that act as cholesterol acceptors and subsequently bind to lipoprotein receptors to enhance the degradation and clearance of cholesterol.

The expression of key genes involved in this balance between cholesterol uptake and efflux can be influenced by cytokines produced by macrophages. TGF-β negatively regulates foam cell formation and this is thought to be mediated through the inhibition of expression of genes implicated in cholesterol uptake and stimulation of expression of genes thought to be involved in cholesterol efflux (Ramji et al. 2006). For example, the cytokine inhibits cholesterol accumulation induced by VLDL remnants in the mouse macrophage J774.2 cell line and increases cholesterol efflux from macrophage-derived foam cells from ApoE-deficient mice (Argmann et al. 2001; Panousis et al. 2001). The studies presented in this chapter investigate the regulation of expression of a number of key genes implicated in the control of macrophage cholesterol homeostasis by the cytokine TGF-β, using the THP-1 macrophage cell line as a human model system. The genes selected for study have been employed in our laboratory as model genes which have well-defined and distinct roles in foam cell formation and atherosclerosis. The structure and function of these genes is detailed in Sections 1.3.1, 1.3.2, 1.4.1 and 1.4.2. The regulation of the expression of these genes by TGF-β has been reported but the majority of studies have been carried out using *in vivo* models or murine macrophage cell lines. The current knowledge regarding their regulation is outlined below.

3.1.2 Lipoprotein lipase (LPL)

A large number of studies have established that macrophage LPL is pro-atherogenic. Cholesterol-rich remnants produced by hydrolysis are readily taken up by macrophages and the ability of the enzyme to act as a molecular bridge promotes uptake of lipoproteins into the arterial intima as explained in more detail in Section 1.3.2 (Mead et al. 2002; Mead and Ramji 2002). Macrophages and smooth muscle cells are major sources of LPL in atherosclerotic plaques and the expression and activity of LPL is upregulated following monocyte-macrophage differentiation (Mead and Ramji 2002; Stein and Stein 2003).

Our laboratory has previously shown that TGF-β inhibits the mRNA and protein expression of LPL in mouse J774.2 macrophages and primary cultures of human monocyte-derived macrophages (HMDMs) and that this corresponds to a decrease in LPL activity (Irvine et al. 2005). LPL promoter-luciferase DNA constructs identified

the -31/+187 promoter region as containing the minimal TGF-β responsive elements and bioinformatic analysis of this region identified three Sp1/Sp3 binding sites (at positions +44, +62 and +65) that are crucial for inhibition of LPL gene transcription. Sp1 and Sp3 are widely expressed zinc finger transcriptional regulators that are involved in regulating constitutive gene transcription and cytokine-induced gene transcription. While Sp1 acts as a transcriptional activator, Sp3 contains a transcriptionally repressive domain allowing it to act as either a repressor or activator of transcription depending on cellular and promoter context (Black et al. 2001; Irvine et al. 2005). Mutation of these sites abolished the TGF-β response and DNA constructs containing multimers of the Sp1/Sp3 sites linked to the SV40 promoter of the pGL2 promoter vector showed that the sites were able to confer the TGF-β response to a heterologous promoter (Irvine et al. 2005). The absence of any change in the binding or expression of Sp1/Sp3 in macrophages suggested that TGF-β modulated LPL expression through the suppression of Sp1/Sp3 transactivation potential as opposed to having effects on Sp1/Sp3 ratio (Irvine et al. 2005; Ramji et al. 2006). Despite this work, the signalling pathways underlying the regulation of LPL gene transcription by TGF-β, along with how this cytokine modulates the transactivation potential of Sp1/Sp3 remains uncharacterised. Due to the critical role of this enzyme in cholesterol uptake and foam cell formation, further studies into the molecular mechanisms underlying its regulation will enhance our understanding of the molecular basis of atherosclerosis.

3.1.3 Scavenger receptor A (SR-A)

SR-A is expressed in both macrophages and foam cells of human atherosclerotic lesions where it plays a pro-atherogenic role through the uptake of acetylated and oxidised LDL (Moore and Freeman 2006; Peiser and Gordon 2001). Gene-targeted knockout of SR-A in $ApoE^{-/-}$ and $LDLR^{-/-}$ murine models results in significantly reduced atherosclerotic lesion size (de Winther et al. 2000; Moore and Freeman 2006; Peiser and Gordon 2001). SR-A expression and activity is upregulated following monocyte-macrophage differentiation and is often used as a marker for differentiation (Argmann et al. 2001). TGF-β inhibits the expression of SR-A in both THP-1 monocytes, macrophages and human monocyte-derived macrophages through a reduction of mRNA levels causing a decrease in cell-surface receptor number

(Bottalico et al. 1991; Draude and Lorenz 2000). In a functional context, this leads to reduced uptake of both acetylated and oxidised LDL (Argmann, 2001). Interestingly, pravastatin is able to down-regulate SR-A expression in a TGF-β-dependent manner in THP-1 macrophages (Baccante et al. 2004).

3.1.4 Scavenger receptor-B1 (SR-B1)

SR-B1 is expressed in cultured macrophages and immunohistochemical analysis has demonstrated that it is expressed in foam cells of human atherosclerotic lesions. Like SR-A, its expression is up-regulated following differentiation of monocytes into macrophages (de Villiers and Smart 1999; Gillotte-Taylor et al. 2001; Hirano et al. 1999; Moore and Freeman 2006; Zhang et al. 2003). The role of SR-B1 in atherosclerosis is complex with the receptor having two opposing functions. The primary and most well-studied function of SR-B1 as a receptor for HDL suggests an anti-atherogenic role. SR-B1 knockout mice present with hypercholesterolemia, accumulation of HDL and significantly more atherosclerosis than wild-type counterparts (Moore and Freeman 2006; Trigatti et al. 2004; Zhang et al. 2003). However, SR-B1 can also function as a classical scavenger receptor and this function may be more significant in macrophages. Indeed, knockout of macrophage SR-B1 in ApoE-deficient mice results in a 2-fold increase in atherosclerotic lesion area suggesting a pro-atherosclerotic role for the receptor (Zhang et al. 2003). Expression of SR-B1 at the mRNA and protein levels has been shown to be both induced and inhibited by oxLDL and it is likely that this effect is dependent on the differentiation state of macrophages with oxLDL decreasing SR-B1 expression in fully differentiated macrophages and increasing its expression in differentiating monocytes/macrophages (Han et al. 2001; Hirano et al. 1999). The levels of SR-B1 mRNA in macrophages are generally lower than that of CD36 (Zuckerman et al. 2001). TGF-β inhibits SR-B1 mRNA expression in mouse peritoneal macrophages and in macrophage-derived foam cells from $ApoE^{-/-}$ and $LDLR^{-/-}$ mice and this correlates with a decrease in HDL-macrophage interactions (Zuckerman et al. 2001).

3.1.5 CD36

CD36 acts in a pro-atherogenic manner as it stimulates the uptake of oxLDL and significantly contributes to foam cell formation (Febbraio and Silverstein 2007; Moore and Freeman 2006). CD36-deficient mice show significantly less atherosclerosis than wildtypes while $CD36^{-/-}ApoE^{-/-}$ double knockout mice have 70% less lesions and reduced lesion size compared to wildtype counterparts (Febbraio and Silverstein 2007; Nicholson 2004; Nicholson et al. 2001). The expression of CD36 is up-regulated following differentiation of monocytes into macrophages. The expression of CD36 is inhibited by both TGF- β and IL-10 (Febbraio and Silverstein 2007; Nicholson 2004). Down-regulation of CD36 expression by TGF-β has been shown in THP-1 macrophages, primary cultures of human macrophages and macrophages from $ApoE^{-/-}$ and $LDLR^{-/-}$ mice (Draude and Lorenz 2000; Han et al. 2000; Zuckerman et al. 2001).

Han et al. (2000) have demonstrated that TGF-β can inhibit the expression of CD36 at the mRNA and protein level in THP-1 macrophages. In addition to this, the cytokine inhibits CD36 expression induced in response to oxLDL and ligands of PPARγ. As detailed in Section 3.4, PPARγ is a nuclear receptor with important roles in inflammation and in the regulation of expression of genes involved in fatty acid metabolism and cholesterol homeostasis (Han et al. 1997; Han et al. 2000). The TGF-β-regulated inhibition of CD36 is accompanied by an increase in the phosphorylation of PPARγ and the p44/42 MAPK isoforms, and inhibitors of p44/42 MAPK reverse the TGF-β-mediated suppression of CD36. This suggested that TGF-β down-regulates CD36 expression via a mechanism involving cytokine activation of MAPK and subsequent inactivation of PPARγ through phosphorylation (Argmann et al. 2001; Han et al. 1997; Han et al. 2000; Nicholson et al. 2001). Interestingly, HDL inhibits the expression of CD36 by the same mechanism (Nicholson 2004). Down-regulation of CD36 expression by TGF-β is associated with a reduction in the uptake of oxLDL by macrophages, consistent with the role of CD36 as the primary receptor for oxLDL (Draude and Lorenz 2000).

3.1.6 Apolipoprotein E (ApoE)

ApoE stimulates cholesterol efflux from foam cells and can act as an acceptor for both cholesterol and phospholipids released from macrophages through ABCA-1 (Greenow et al. 2005; Mahley and Rall 2000). ApoE-deficient mice are widely used as a model for atherosclerosis as they are hypercholesterolemic and develop atherosclerosis even when placed on a low-fat diet. Reduced expression of the protein in atherosclerosis is associated with a pro-atherogenic lipoprotein profile (Greenow et al. 2005; Mahley and Rall 2000; Ramji et al. 2006).

ApoE is not expressed in normal vessel walls but is synthesised at high levels by macrophages present within atherosclerotic plaques. In monocytes and macrophages, ApoE expression is enhanced by differentiation and also by cholesterol loading of macrophage cultures. Pro-inflammatory cytokines IFNγ and IL-1 inhibit the expression of the protein (Greenow et al. 2005). TGF-β induces the mRNA expression of ApoE and its secretion from mouse peritoneal macrophages (Zuckerman et al. 1992). Our laboratory has demonstrated that TGF-β induces the expression of ApoE at the mRNA and protein levels in both THP-1 monocytes and macrophages (Ramji et al. 2006; Singh and Ramji 2006). Pharmacological inhibitors against JNK, p38 kinase and protein kinase CK2 attenuated the TGF-β-mediated induction of ApoE, identifying roles for these kinases in the ApoE response. The activity and/or expression of protein kinase CK2, p38 kinase, JNK and its downstream target c-Jun were induced by TGF-β in THP-1 monocytes and dominant-negative DNA constructs of these inhibited ApoE mRNA expression by this cytokine (Singh and Ramji 2006). EMSA analysis showed that the signals converge on the AP-1 binding site present within the ApoE promoter (Singh and Ramji 2006).

3.1.7 ATP-binding cassette transporters-A1 and –G1 (ABCA-1 and ABCG-1)

The primary role of ABCA-1 is the transport of cholesterol and phospholipids from cells to apolipoproteins in the bloodstream (Wang and Tall 2003). ABCA-1 is an anti-atherogenic protein. Deficiency of macrophage ABCA-1 in mice leads to increased atherosclerosis while overexpression reduces atherosclerosis (Oram and Vaughan 2006). The preventative role of ABCA-1 in atherosclerosis is also

demonstrated by the association between ABCA-1 mutations and premature atherosclerosis observed in Tangier disease (Attie 2007).

The function of ABCA-1 and ABCG-1 as removers of excess cholesterol from macrophages is responsible for their anti-atherogenic properties. Macrophages express high levels of ABCA-1 in response to the transcription factor, LXRα which is itself activated by cholesterol metabolites derived from excessive uptake of oxLDL by cells. LXRα upregulates ABCA-1 and ABCG-1 expression in various sources of cholesterol-loaded macrophages, including THP-1 macrophages, primary human macrophages and lipid-laden foam cells (Oram and Vaughan 2006; Wang and Tall 2003). Both ABCA-1 and ABCG1 expression is increased during monocyte-macrophage differentiation (Schmitz et al. 2001). TGF-β treatment of mouse peritoneal macrophages from $ApoE^{-/-}$ mice increases ABCA-1 expression and cholesterol efflux (Panousis et al. 2001). TGF-β enhances both ABCA-1 and ABCG-1 expression in THP-1 macrophages and this corresponds to an increased level of cholesterol efflux to apoA-I or HDL. The cytokine also increases levels of ABCA-1 and cholesterol efflux from macrophage-derived foam cells (Argmann et al. 2001).

3.2 Aims of experimental studies

The studies presented in this chapter have been carried out using the THP-1 cell line, a widely used model for macrophage function and gene expression during inflammatory disorders. THP-1 is a human monocytic leukaemia cell line first established by Tsuchiya et al. (1980) that can be differentiated into macrophages using phorbol esters. Differentiated THP-1 cells have similar characteristics to and behave in the same way as native monocyte-derived macrophages (Auwerx 1991). Conserved responses between THP-1 cells and primary cultures have been observed in numerous studies (Kohro et al. 2004). In addition to this, lipid accumulation can be observed in differentiated THP-1 macrophages cultured in medium containing acetylated LDL, making them a useful model for macrophage-derived foam cells (Kohro et al. 2004). These characteristics of THP-1 macrophages make them a suitable model for studies relating to atherosclerosis and foam cell formation (Kohro

et al. 2004). Table 3.1 details examples of studies on atherosclerosis that have been carried out using the THP-1 cell line and/or primary human monocyte-derived macrophages.

Table 3.1 **Studies utilising THP-1 cells and/or HMDMs to study various key aspects of atherosclerosis**

Aspect of atherosclerosis	Monocyte/ Macrophage source	Key findings	References
Monocyte adhesion to vascular endothelium	THP-1 monocytes	Adhesion of monocytes to endothelial cells is dependent on shear stress and is mediated by the platelet integrins, L-selectin and P-selectin.	Theilmeier et al. 1999
	THP-1 monocytes	MCSF induces the mRNA expression of MCP-1 and promotes the adhesion of monocytes to endothelial cells.	Shyy et al. 1993
Monocyte migration	THP-1 monocytes	Elevated LDL levels increase the expression of the MCP-1 receptor and enhance the chemotactic response to MCP-1, to help recruit monocytes to the endothelium.	Han et al. 1998
	THP-1 monocytes	PDGF-C and PDGF-D can stimulate the activity of MMP-1 and MMP-9 to enhance the migration of monocytes.	Wågsäter et al. 2009
	Human monocytes	Migration of monocytes is induced by VEGF and mediated through the VEGF receptor, Flt-1.	Barleon et al. 1996
Foam cell formation- Cholesterol uptake	THP-1 macrophages and HMDMs	IL-8 and MCP-1 expression are induced by cholesterol loading (AcLDL) of macrophages.	Wang et al. 1996
	THP-1 macrophages	IL-1 expression is induced by ligand binding to scavenger receptors.	Palkama 1991
	THP-1 macrophages	Adipocyte lipid binding protein expression is upregulated in foam cells, in response to oxLDL.	Fu et al. 2002
	THP-1 macrophages	IFN-γ induces foam cell formation.	Reiss et al. 2004
	THP-1 macrophages and HMDMs	Identification of LOX-1, an oxLDL receptor, expression in macrophages.	Yoshida et al. 1998

	THP-1 macrophages	Identification of CD36 as a receptor for oxLDL.	Endemann et al. 1993
	THP-1 macrophages	CD36 expression is stimulated by oxLDL and inhibited by IFN-γ.	Nakagawa et al. 1998
	HMDMs	SR-B1 is expressed in macrophages of atherosclerotic lesions and its mRNA and protein expression is increased by oxLDL and AcLDL.	Hirano et al. 1999
Foam cell formation-Cholesterol efflux	THP-1 macrophages and HMDMs	ABCA1 promotes the secretion of apoE from macrophages.	Von Eckardstein et al. 2001
	HMDMs	ApoAI promotes the recruitment of apoE and enhances cholesterol efflux from foam cells.	Bielicki et al. 1999
	THP-1 macrophages and HMDMs	Activators of PPARα and PPARγ induce the expression of ABCA1 and promote cholesterol efflux in macrophages.	Chinetti et al. 2001
Immune responses	THP-1 monocytes	Immune complexes of LDL bind to Fc receptors on monocytes to initiate atherogenic responses.	Ibeas et al. 2009; Kiener et al. 1995
	THP-1 monocytes and human monocytes	Inflammatory protein, phospholipase A2 type IIA, enhances the adhesion and migration of monocytes and the expression of cell surface markers and the T-cell proliferation capacity of these cells.	Ibeas et al. 2009
	THP-1 monocytes	Advanced ligation end products (formed following reactions between proteins and lipid intermediates) increase the expression of pro-inflammatory markers such as MCP-1, IL-6 and iNOS, and can also increase monocyte binding to endothelial and smooth muscle cells.	Shanmugam et al. 2008

	HMDMs	Immune complexes of LDL can activate macrophages.	Virella et al. 1995
	HMDMs	Toll-like receptor-4 (TL4), involved in innate immune and inflammatory responses, is expressed in macrophages and is upregulated by oxLDL.	Xu et al. 2001
Plaque stability	HMDMs	Increased density/activation of macrophages in atherosclerotic plaques can promote collagen breakdown through the enhanced secretion of MMPs.	Shah et al. 1995
	THP-1 monocytes	Interactions between monocytes and VSMCs increase the production of MMP-1 and this contributes to plaque instability.	Zhu et al. 2000

There are a number of advantages of using a cell line over HMDMs and *in vivo* models. In contrast to primary cells which are limited in availability and have a defined lifespan, cells lines can be cultured to grow indefinitely. It is also possible to extract larger amounts of RNA or protein from cell lines and to avoid the possible issue of donor-specific variations that arise with the use of primary cells. Use of a cell line can avoid complex interactions that occur *in vivo*. It also avoids the ethical issues that accompany the use of animal models or material from patients in scientific research.

The primary aims of the studies presented in this chapter were two-fold. The first aim was to verify the use of THP-1 macrophages as a human model system for studies on the regulation of macrophage gene expression. Although the cytokine regulation of a number of macrophage-expressed genes has been reported by our laboratory and others, the majority of studies have used murine macrophages or *in vivo* mice models of atherosclerosis as model systems (Table 3.2) (Harvey et al. 2007; Hughes et al. 2002; Singh and Ramji 2006). It was therefore important to establish the regulation of expression of key genes implicated in macrophage cholesterol homeostasis in the THP-1 macrophage cell line. This was the second aim of the studies presented in this chapter. The genes we investigated included LPL, SR-A, SR-B1 and CD36; genes implicated in cholesterol uptake by macrophages, and ApoE, ABCA-1 and ABCG-1; genes involved in cholesterol efflux by macrophages. The regulation of ApoE and LPL has previously been investigated by our laboratory using THP-1 monocytes and the mouse macrophage cell line J774.2 (Table 3.2) but their regulation has not been fully investigated in THP-1 macrophages (Irvine et al. 2005; Singh and Ramji 2006). Regulation of other key genes, including the class A and class B scavenger receptors, has been reported in the literature but not studied by our laboratory. It was our aim to firstly characterise the responses of these key genes to TGF-β stimulation and then to establish a time point of optimal induction or inhibition of expression by the cytokine in order to provide a basis for further studies into the mechanisms controlling the regulation of expression of these genes by TGF-β, as set out in Chapters 4 and 5. This would also confirm the use of THP-1 as a suitable cell line for these studies. The experimental strategy for this work is summarised in Figure 3.1.

Throughout the studies presented in this thesis, TGF-β was used at a concentration of 30ng/ml as our laboratory had previously determined this as the TGF-β concentration that optimally regulated ApoE and LPL expression in THP-1 macrophages (Irvine et al. 2005; Singh and Ramji 2006). Previous dose-response studies (using 0, 2, 5, 10, 20 and 30ng/ml TGF-β) carried out in our laboratory had demonstrated that mRNA and protein levels of ApoE were optimally upregulated following 30ng/ml TGF-β treatment and time course experiments (0, 1, 6, 12 and 24hr) demonstrated that this up-regulation was optimal after 24hr of TGF-β treatment (see Supplementary data-Singh and Ramji 2006).

Gene regulation was primarily studied at the mRNA level with key results also investigated at the protein level using western blotting. To confirm the use of THP-1 as a suitable model for human macrophages, the TGF-β regulation of ApoE expression was also studied in human monocyte-derived macrophages.

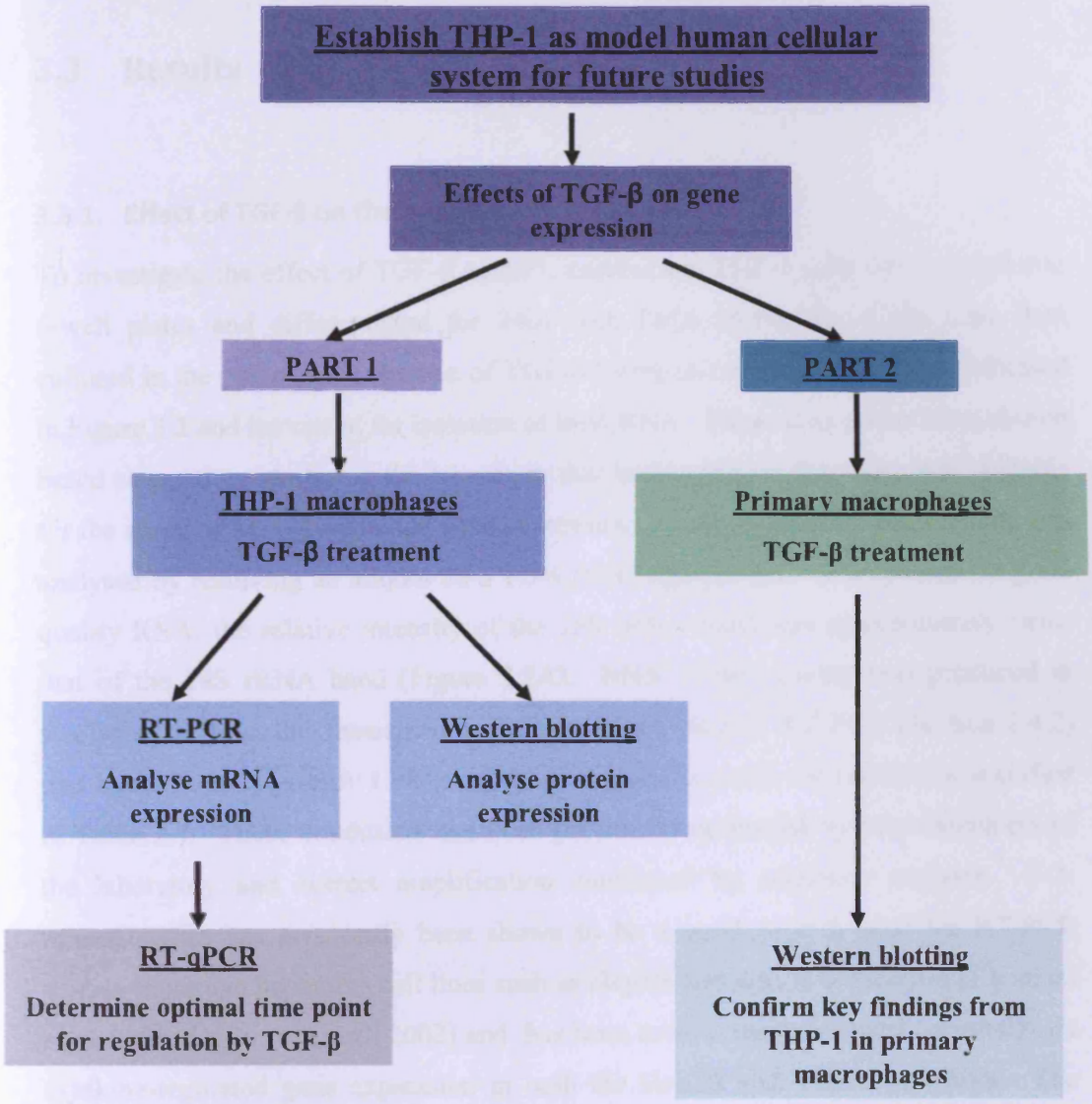

Figure 3.1 **Experimental Strategy**

Initially, studies on the regulation of gene expression by TGF-β used RT-PCR analysis. Although this is semi-quantitative in nature and cannot be used to gauge exact levels of mRNA expression, an indication of the changes in expression can be observed effectively by densitometric analysis of the data. Hence, as RT-qPCR analysis became available in the laboratory, the expression of later-studied genes was analysed using this method. Western blot analysis was used for analysis of protein expression where an antibody to the protein of interest was available in the laboratory. For ease of reading, results have been presented on a gene by gene basis.

3.3 Results

3.3.1. Effect of TGF-β on the mRNA expression of LPL

To investigate the effect of TGF-β on LPL expression, THP-1 cells were seeded into 6-well plates and differentiated for 24hr with PMA (0.16μM). Cells were then cultured in the presence or absence of TGF-β (30ng/ml) for 12hr or 24hr as indicated in Figure 3.2 and harvested for isolation of total RNA. These time points were chosen based on previous studies in the laboratory that had suggested that these were suitable for the study of TGF-β-regulated gene expression. Integrity of RNA preparations was analysed by resolving an aliquot on a 1.5% (w/v) agarose gel. As expected for good quality RNA, the relative intensity of the 28S rRNA band was approximately twice that of the 18S rRNA band (Figure 3.2A). RNA of this quality was produced in studies throughout this thesis and hence data is not shown. RT-PCR (Section 2.4.2) was carried out to amplify LPL and β-2 microglobulin using the conditions specified in Table 2.4. These conditions had been previously optimised by other members of the laboratory and correct amplification confirmed by sequence analysis. β-2-microglobulin has previously been shown to be a good control gene for RT-PCR studies in human hepatoma cell lines such as Hep3B and also in leukocytes (Cicinnati et al. 2008; Lupberger et al. 2002) and has been used in our laboratory for studies on cytokine-regulated gene expression in both the Hep3B and THP-1 cell lines. The PCR products were size-fractionated using agarose gel electrophoresis and correct amplification was verified by comparison to a standard DNA molecular weight marker (see Appendix I). Figure 3.2B shows the PCR products fractionated by agarose gel electrophoresis. The absence of any PCR product in the no reverse transcriptase (-RT) sample indicates that any product produced is due to correct amplification of the relative mRNA and not because of contamination of the samples with genomic DNA.

The relative intensity of LPL and β-2 microglobulin PCR products was determined using densitometric analysis (see Section 2.9). The Syngene GeneTools software assigns numerical values to each product that are related to its intensity. The intensity

of each LPL product was compared relative to the intensity of the equivalent β-2 microglobulin control (housekeeping) gene product. LPL expression was high in untreated cells and was dramatically inhibited by TGF-β at the 24hr and 48hr time points. The data is expressed as the fold change (mean± SD) with values from untreated samples arbitrarily assigned as 1 (Figure 3.2C). A standard statistical Student's t-test was carried out on the data. β-2 microglobulin remained unaffected by TGF-β throughout the time course verifying that TGF-β does not globally affect gene expression and that any changes in LPL expression are a result of TGF-β treatment and not to the state of the cells at the different time points.

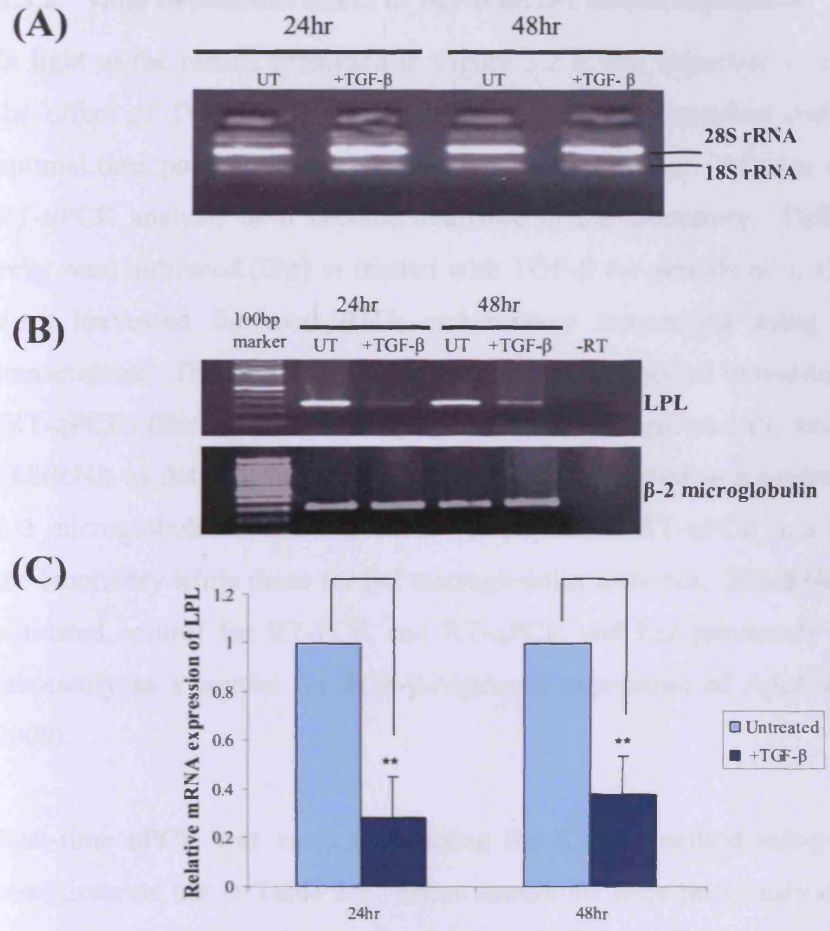

Figure 3.2 TGF-β down-regulates LPL mRNA expression in THP-1 macrophages. THP-1 cells were treated with TGF-β or left untreated (UT) for the time points indicated and then harvested for total RNA extraction. RNA integrity was analysed by agarose gel electrophoresis (Panel A). Total RNA was subjected to RT-PCR analysis and the PCR amplification products for LPL and β2-microglobulin were resolved by agarose gel electrophoresis (Panel B). The size of the product was determined by comparison with a standard DNA molecular weight marker (Appendix I). –RT denotes a reaction in which no reverse transcriptase was included in cDNA preparation (using 24hr, untreated RNA). Panel C shows the relative expression (mean ±SD) of LPL normalised to the expression of β-2 microglobulin (untreated samples for each time point assigned as 1) from three independent experiments (**P<0.01).

3.3.2 Time dependent effect of TGF-β on LPL mRNA expression

In light of the results presented in Figure 3.2 it was important to determine whether the effect of TGF-β on LPL expression was time-dependent and to determine an optimal time point for future studies on LPL expression. This was investigated using RT-qPCR analysis as it became available in the laboratory. Differentiated THP-1 cells were untreated (0hr) or treated with TGF-β for periods of 6, 12 and 24hr. Cells were harvested for total RNA and reverse transcribed using M-MLV reverse transcriptase. The resulting cDNA template was subjected to real-time qPCR analysis (RT-qPCR) (Section 2.4.3) using specific primers against LPL and the control gene 28SrRNA as detailed in Table 2.5. 28SrRNA was used as a control gene in place of β-2 microglobulin as the conditions for 28SrRNA RT-qPCR had been optimised in the laboratory while those for β-2 microglobulin were not. 28SrRNA is a widely used validated control for RT-PCR and RT-qPCR and has previously been used in our laboratory as a control for TGF-β-regulated expression of ApoE (Singh and Ramji 2006).

Real-time qPCR was carried out using the SYBR method using the amplification conditions set out in Table 2.6. These conditions were previously optimised by other members of the laboratory and correct amplification verified by sequence analysis. SYBR green is a DNA-binding dye that emits fluorescence on incorporation into dsDNA (Giulietti et al. 2001). A melting curve was constructed to ensure single-product amplification and the comparative Ct method was used for analysis as described by Livak and Schmittgen (2001). Data from qPCR methods can be analysed using absolute quantification (by comparison of transcript copy number with a standard curve) or using relative quantification (by comparison with a reference group such as an untreated control or the stable expression of a control gene, such as the housekeeping genes β-2-microglobulin and 28S rRNA) (Livak and Schmittgen 2001). The Ct method is a commonly used method for relative quantification of qPCR data. The Ct method relies on the number of cycles/rounds of amplification required for the fluorescence of a gene to surpass a pre-set threshold level (the Ct value). The value of this for the gene of interest is compared relative to the equivalent Ct value for the control gene to normalise the data (Ginzinger 2002; Livak and Schmittgen 2001).

The data presented in Figure 3.3 is displayed as the relative expression of LPL compared to the untreated (0hr) sample. LPL expression was high in untreated cells and significantly reduced by TGF-β, 6hr after treatment with the cytokine. Levels of LPL mRNA expression remained significantly inhibited by TGF-β over the 24hr time course with maximal inhibition at the 24hr time point. Levels of the control 28SrRNA were unaffected by the cytokine.

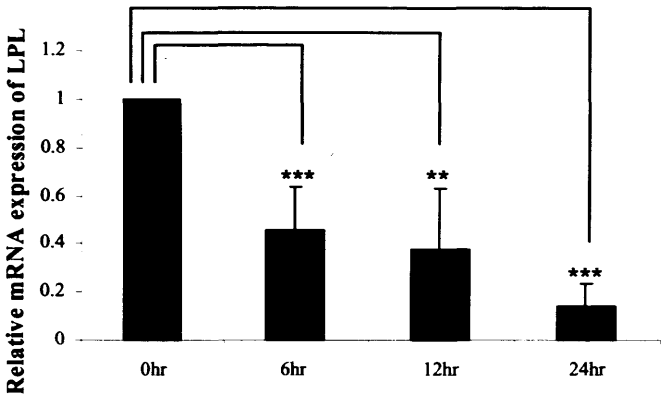

Figure 3.3 TGF-β inhibits LPL expression in a time-dependent manner in THP-1 macrophages. THP-1 cells were left untreated (0hr) or treated with TGF-β for the time points indicated before being harvested for total RNA extraction. Total RNA was reverse-transcribed and the resulting cDNA subjected to RT-qPCR analysis using primers specific to LPL and the control gene 28SrRNA. Data is presented as the relative expression of LPL (mean ±SD) normalised to 28S rRNA expression (0hr (untreated) sample assigned as 1) from four independent experiments (**P<0.01, ***P<0.001).

3.3.3 Effect of TGF-β on the protein expression of LPL

To confirm that the inhibition of LPL mRNA by TGF-β resulted in a corresponding change in the expression of the LPL protein, western blot analysis was carried out. THP-1 cells were seeded into 6-well plates and differentiated for 24hr with PMA.

Cells were then treated with TGF-β or left untreated for the requisite time. Total cellular protein was extracted using Laemmli buffer (Section 2.7.1), subjected to SDS-PAGE using a 10% (v/v) polyacrylamide gel and transferred to a PVDF membrane. The membrane was probed with an antibody against LPL and then re-probed with an antibody for a β-actin control to ensure equal loading of samples. The membrane was placed in contact with X-ray film and developed using chemiluminescent detection as described in Section 2.7.10. Figure 3.4A shows the images from western blotting. The relative density of LPL and β-actin protein was determined using densitometric analysis (Section 2.9). β-actin has been widely used as a control for protein expression and has been used as a control for TGF-β-regulated gene expression (Draude and Lorenz 2000; Irvine et al. 2005) and for cytokine-regulated protein expression (Foka et al. 2009; Harvey et al. 2007) by our laboratory and others. The data was normalised to the untreated control for each time point and expressed as the mean fold change relative to this. Figure 3.4B shows that TGF-β inhibited the expression of LPL protein at the 24hr and 48hr time points. This change is synonymous with the changes in mRNA expression shown in Figure 3.2.

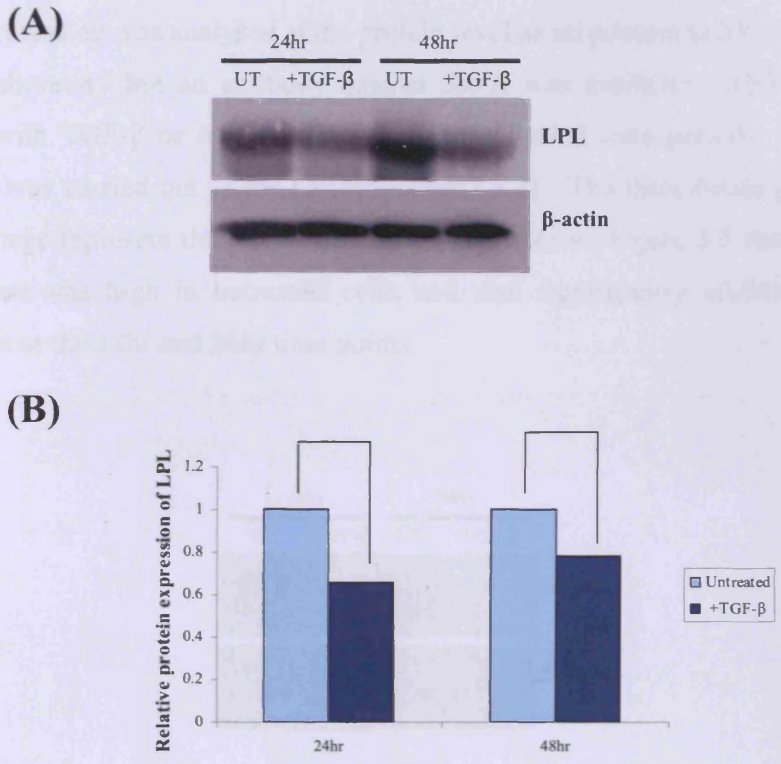

Figure 3.4 **TGF-β inhibits LPL protein expression in THP-1 macrophages.** THP-1 cells were left untreated (UT) or stimulated with TGF-β for 24 or 48hr. Whole cell protein extracts were subjected to SDS-PAGE and western blotting using antibodies against LPL and β-actin (Panel A). Protein size was determined by comparison against a standard protein molecular weight marker (Appendix I). β-actin was used as a loading control. Panel B shows the mean relative expression of LPL normalised to β-actin expression (untreated samples for each time point assigned as 1) from two independent experiments.

3.3.4 Effect of TGF-β on the protein expression of SR-A

SR-A expression was analysed at the protein level as no primers to SR-A were present in the laboratory but an antibody against SR-A was available. THP-1 cells were treated with TGF-β or left untreated for the indicated time periods. Western blot analysis was carried out as for LPL (Section 3.3.3). The three bands present on the SR-A image represent the three isoforms of the protein. Figure 3.5 shows that SR-A expression was high in untreated cells and was significantly inhibited by TGF-β treatment at the 12hr and 24hr time points.

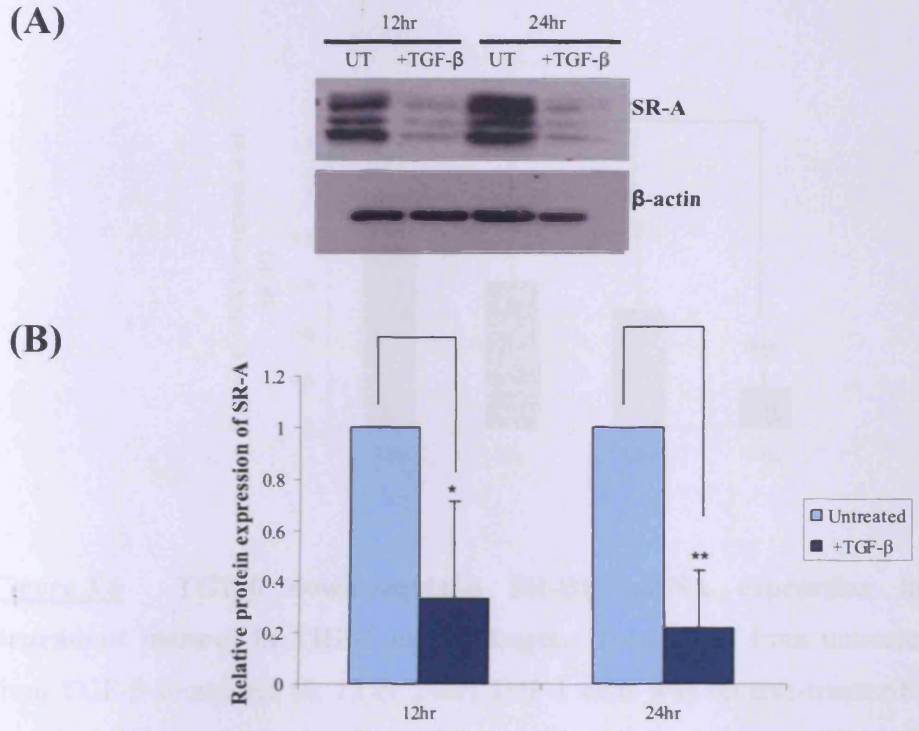

Figure 3.5 TGF-β inhibits SR-A protein expression in THP-1 macrophages. Whole cell-protein extracts from THP-1 macrophages that were untreated (UT) or treated with TGF-β for 12 or 24hr were subjected to SDS-PAGE and western blotting using antibodies against SR-A and β-actin (Panel A). Panel B shows the relative expression (mean± SD) of SR-A normalised to the expression of β-actin (untreated samples for each time point assigned as 1) from three independent experiments (*P<0.05, **P<0.01).

3.3.5 Effect of TGF-β on the mRNA expression of SR-B1

SR-B1 expression was analysed at the mRNA level as no antibodies to SR-B1 were available in the laboratory. A 24hr time course was prepared to study any time-dependent effects of TGF-β treatment on SR-B1 expression. THP-1 cells were left untreated (0hr) or treated with TGF-β for the requisite time period and then harvested for total RNA and RT-qPCR analysis. The cDNA template was subjected to RT-qPCR analysis as described in Section 3.3.2. SR-B1 expression was high in untreated cells and was significantly reduced by TGF-β, 6hr after treatment with the cytokine with maximal inhibition at the 24hr time point.

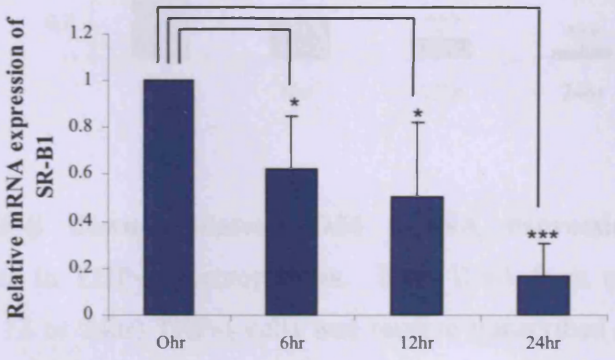

Figure 3.6 TGF-β down-regulates SR-B1 mRNA expression in a time dependent manner in THP-1 macrophages. Total RNA from untreated (0hr) or from TGF-β-stimulated (6, 12 or 24hr) THP-1 cells was reverse-transcribed and the resulting cDNA subjected to RT-qPCR analysis using primers specific to SR-B1 and the control gene 28S rRNA. Data is presented as the relative expression (mean± SD) of SR-B1 normalised to the expression of 28S rRNA (untreated (0hr) sample assigned as 1) from four independent experiments (*P<0.05, ***P<0.001).

3.3.6 Effect of TGF-β on the mRNA expression of CD36

CD36 expression was also analysed at the mRNA level as no antibodies to the protein were available in the laboratory. A time course was prepared to analyse any time-dependent effects of TGF-β treatment on CD36 expression. CD36 expression was high in untreated cells and was significantly attenuated by TGF-β, 6hr after treatment with the cytokine with maximal inhibition at the 24hr time point.

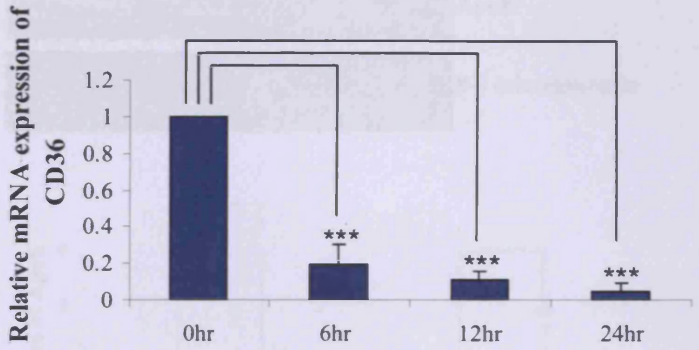

Figure 3.7 TGF-β down-regulates CD36 mRNA expression in a time-dependent manner in THP-1 macrophages. Total RNA from untreated (0hr) or TGF-β-treated (6, 12 or 24hr) THP-1 cells was reverse-transcribed and the resulting cDNA subjected to RT-qPCR analysis using primers specific to CD36 and the control gene 28SrRNA. Data is presented as the relative expression (mean± SD) of CD36 normalised to the expression of 28S rRNA (untreated (0hr) sample assigned as 1) from four independent experiments (***P<0.001).

3.3.7 Effect of TGF-β on the mRNA expression of ApoE

THP-1 cells were cultured in the presence or absence of TGF-β for the requisite time period and then harvested for total RNA extraction. Total RNA was quantified and then used for RT-PCR analysis. PCR was carried out to amplify ApoE and β-2 microglobulin using the conditions specified in Table 2.4 and the PCR products were size-fractionated using agarose gel electrophoresis. Figure 3.8A shows the PCR products fractionated by agarose gel electrophoresis. The results indicated that ApoE

mRNA expression was low in untreated THP-1 macrophages and was dramatically induced by TGF-β treatment with maximal induction at the 24hr time point (Figure 3.8B).

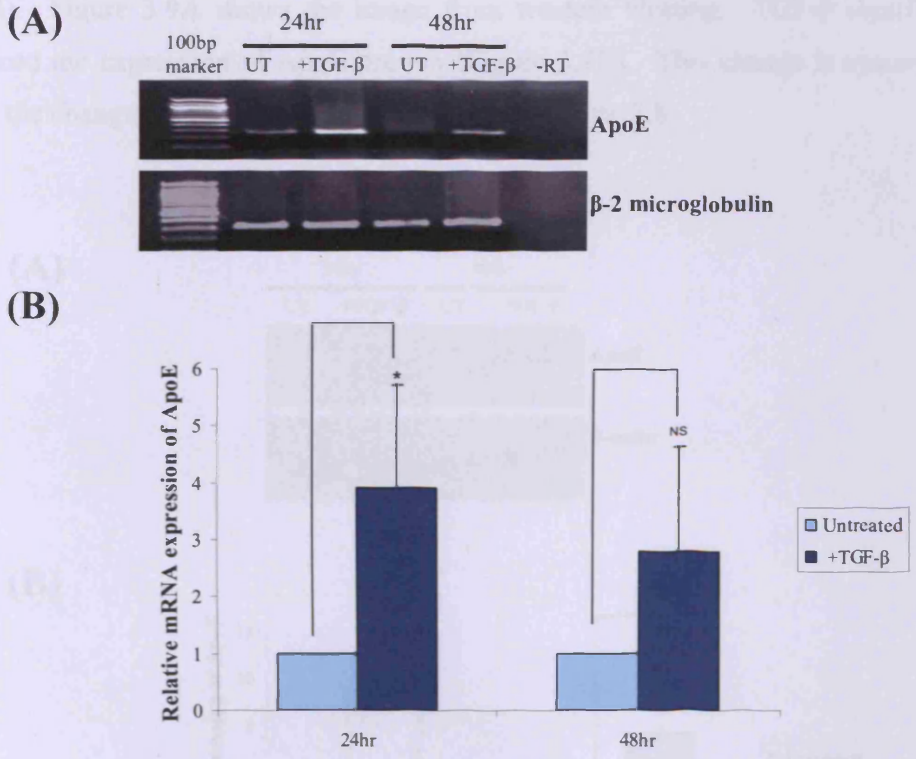

Figure 3.8 TGF-β up-regulates ApoE mRNA expression in THP-1 macrophages. Total RNA from untreated (UT) or TGF-β-treated (24 or 48hr) THP-1 cells was subjected to RT-PCR analysis. ApoE and β2-microglobulin amplification products were resolved by agarose gel electrophoresis (Panel A). The size of the product was determined by comparison with a standard DNA molecular weight marker (Appendix I). –RT denotes a reaction in which no reverse transcriptase was included in cDNA preparation (24hr, TGF-β-treated RNA). Panel B shows the relative expression (mean± SD) of ApoE normalised to the expression of β-2 microglobulin (untreated samples for each time point assigned as 1) from three independent experiments (*P<0.05, NS-not significant).

3.3.8 Effect of TGF-β on the protein expression of ApoE

To confirm that the induction of ApoE mRNA by TGF-β in THP-1 macrophages resulted in a corresponding change in the expression of ApoE protein, western blot analysis was carried out. THP-1 cells were treated with TGF-β or left untreated for the requisite time period. Western blot analysis was carried out as for LPL (Section 3.3.3). Figure 3.9A shows the image from western blotting. TGF-β significantly induced the expression of ApoE protein (Figure 3.9B). This change is synonymous with the changes in mRNA expression shown in Figure 3.8.

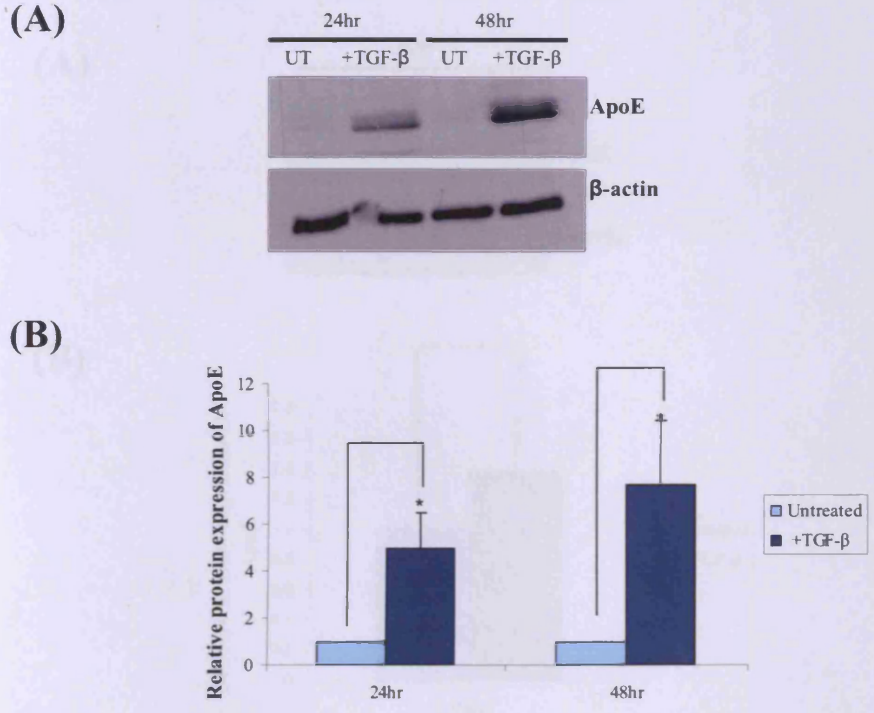

Figure 3.9 TGF-β up-regulates ApoE protein expression in THP-1 macrophages. Whole cell-protein extracts from untreated (UT) or TGF-β treated (24 or 48hr) THP-1 cells were subjected to SDS-PAGE and western blotting using antibodies against ApoE and β-actin (Panel A). Panel B shows the relative expression (mean± SD) of ApoE normalised to the expression of β-actin (untreated samples for each time point assigned as 1) from three independent experiments (*P<0.05).

3.3.9 Effect of TGF-β on ApoE protein expression in human monocyte-derived macrophages (HMDMs)

In order to confirm the use of THP-1 as a suitable model for HMDMs it was important to see if the responses observed in THP-1 macrophages were conserved in primary cultures of HMDMs. For this, the ApoE response was investigated in HMDMs. HMDMs were prepared in 12-well plates as described in Section 2.3.8. A single time point was chosen and total cellular protein extracts were subjected to western blotting as described in Section 3.3.3. Figure 3.10 shows that ApoE expression was low in untreated primary human macrophages and was significantly induced by TGF-β at the 24hr time point.

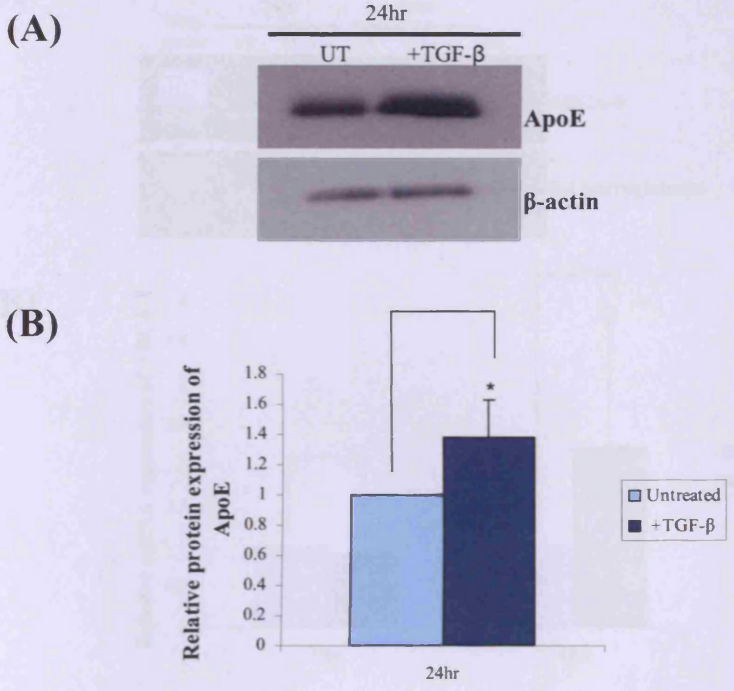

Figure 3.10 **TGF-β up-regulates ApoE expression in HMDMs.** HMDMs were treated with TGF-β or left untreated (UT) for 24hr. Whole cell-protein extracts were subjected to SDS-PAGE and western blotting using antibodies against ApoE and β-actin (Panel A). Panel B shows the relative expression (mean± SD) of ApoE normalised to β-actin expression (untreated sample assigned as 1) from four independent experiments (*P<0.05).

3.3.10 Effect of TGF-β on the mRNA expression of ABCA-1

THP-1 cells, cultured in the presence or absence of TGF-β, were harvested for total RNA extraction. Total RNA was quantified and then used for RT-PCR analysis of ABCA-1 and β-2 microglobulin. Figure 3.11(A) shows the PCR products fractionated by agarose gel electrophoresis. The results indicated that ABCA-1 mRNA expression was low in untreated THP-1 macrophages and was induced by TGF-β treatment but this induction was not statistically significant (Figure 3.11B). The effect of TGF-β on ABCA-1 expression was therefore investigated further using western blot and RT-qPCR analysis (Section 3.3.11 and Section 3.3.12).

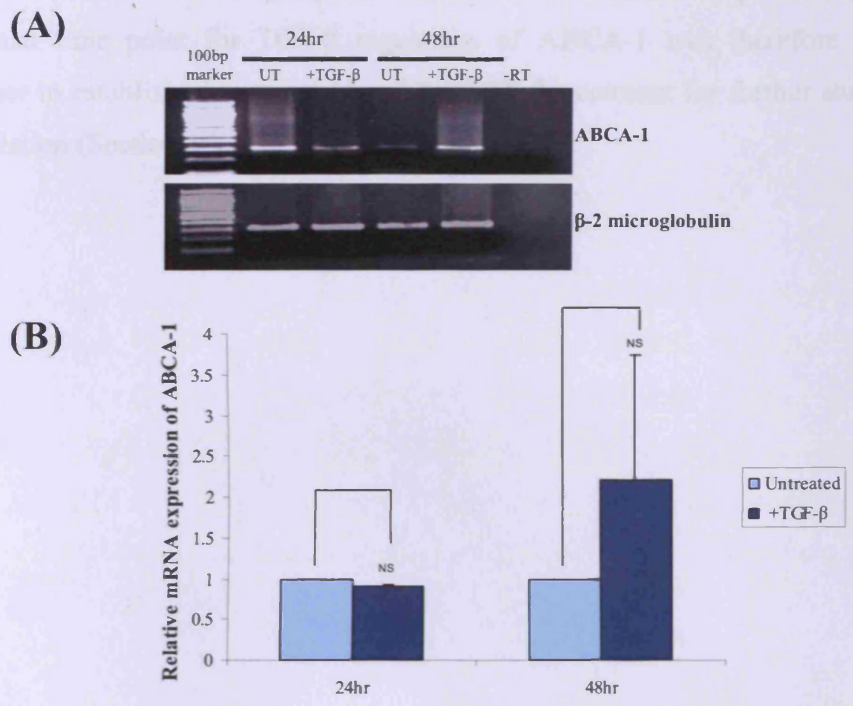

Figure 3.11 ABCA-1 mRNA expression is upregulated by TGF-β in THP-1 macrophages. Total RNA from untreated (UT) or TGF-β-treated (24 or 48hr) THP-1 cells was subjected to RT-PCR analysis and the PCR amplification products for ABCA-1 and β2-microglobulin were resolved by agarose gel electrophoresis (Panel A). Panel B shows the relative expression (mean± SD) of ABCA-1 normalised to β-2 microglobulin expression (untreated samples for each time point assigned as 1) from three independent experiments (NS-not significant).

3.3.11 Effect of TGF-β on the protein expression of ABCA-1

To confirm that the induction of ABCA-1 mRNA expression by TGF-β in THP-1 macrophages resulted in a corresponding change in the expression of the ABCA-1 protein, western blot analysis was carried out. THP-1 cells were treated with TGF-β or left untreated for the requisite time period. Total cellular protein was extracted using whole-cell extraction buffer (Section 2.7.2) and subjected to SDS-PAGE and western blot analysis using a 7.5% polyacrylamide gel. Figure 3.12A shows the image from western blotting. TGF-β induced the expression of ABCA-1 protein at the 24hr time point (Figure 3.12B). The change in expression is synonymous with the data presented in Figure 3.11 but the time point at which this occurs in a significant manner (24hr) is not in agreement with the data on mRNA expression (48hr). The optimal time point for TGF-β regulation of ABCA-1 was therefore investigated further to establish an optimal period for TGF-β treatment for further studies into its regulation (Section 3.3.12).

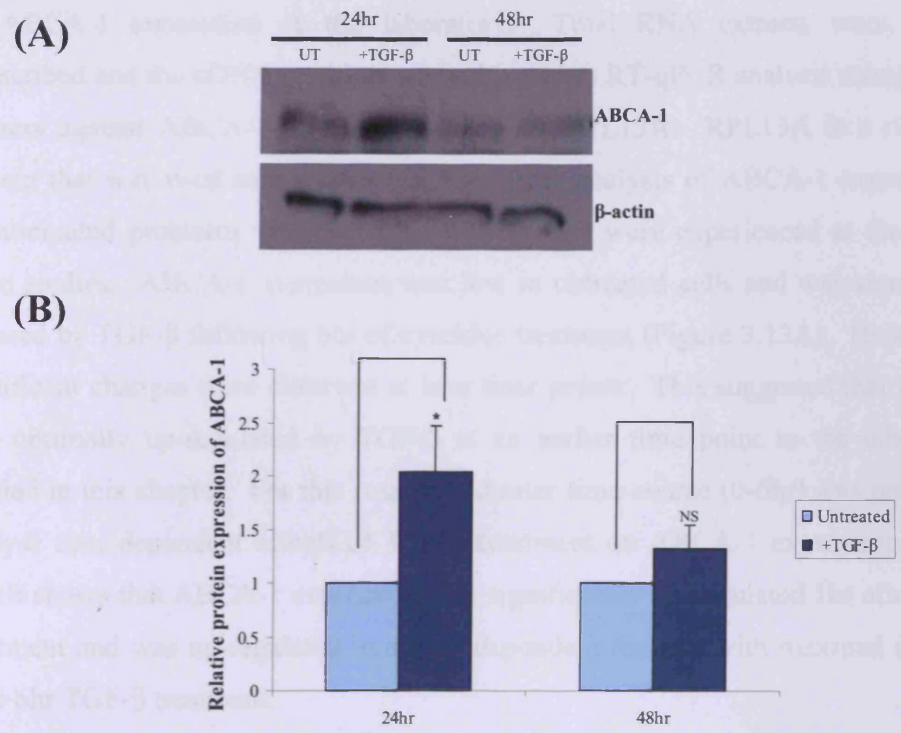

Figure 3.12 ABCA-1 protein expression is upregulated by TGF-β in THP-1 macrophages. Whole cell-protein extracts from untreated (UT) or TGF-β-treated (24 or 48hr) THP-1 cells were subjected to SDS-PAGE and western blotting using antibodies against ABCA-1 and β-actin (Panel A). Panel B shows the relative expression (mean± SD) of ABCA-1 normalised to β-actin expression (untreated samples for each time point assigned as 1) from three independent experiments (*P<0.05, NS-not significant).

3.3.12 Time-dependent effect of TGF-β on ABCA1 mRNA expression

In light of the results presented in Figures 3.11 and 3.12, a time course was prepared to analyse any time-dependent effects of TGF-β treatment on ABCA-1 expression. Due to difficulties of reproducibly obtaining good western blots with ABCA-1, because of the extremely large size of this protein (220kDa), it was decided that any further investigations into its regulation would be performed at the mRNA level. Initially a 24hr time course was prepared (Figure 3.12A) based on the regulation of other key genes by TGF-β as presented in this chapter and previous studies carried out

on ABCA-1 expression in the laboratory. Total RNA extracts were reverse-transcribed and the cDNA template was subjected to RT-qPCR analysis using specific primers against ABCA-1 and the control gene RPL13A. RPL13A is a ribosomal protein that was used as a control for RT-qPCR analysis of ABCA-1 expression as unanticipated problems with the 28S rRNA qPCR were experienced at the time of these studies. ABCA-1 expression was low in untreated cells and was significantly induced by TGF-β following 6hr of cytokine treatment (Figure 3.13A). However, no significant changes were observed at later time points. This suggested that ABCA-1 was optimally up-regulated by TGF-β at an earlier time point to the other genes studied in this chapter. For this reason, a shorter time course (0-6hr) was prepared to analyse time-dependent effects of TGF-β treatment on ABCA-1 expression. Figure 3.13B shows that ABCA-1 expression was significantly up-regulated 1hr after TGF-β treatment and was up-regulated in a time dependent manner with maximal induction after 6hr TGF-β treatment.

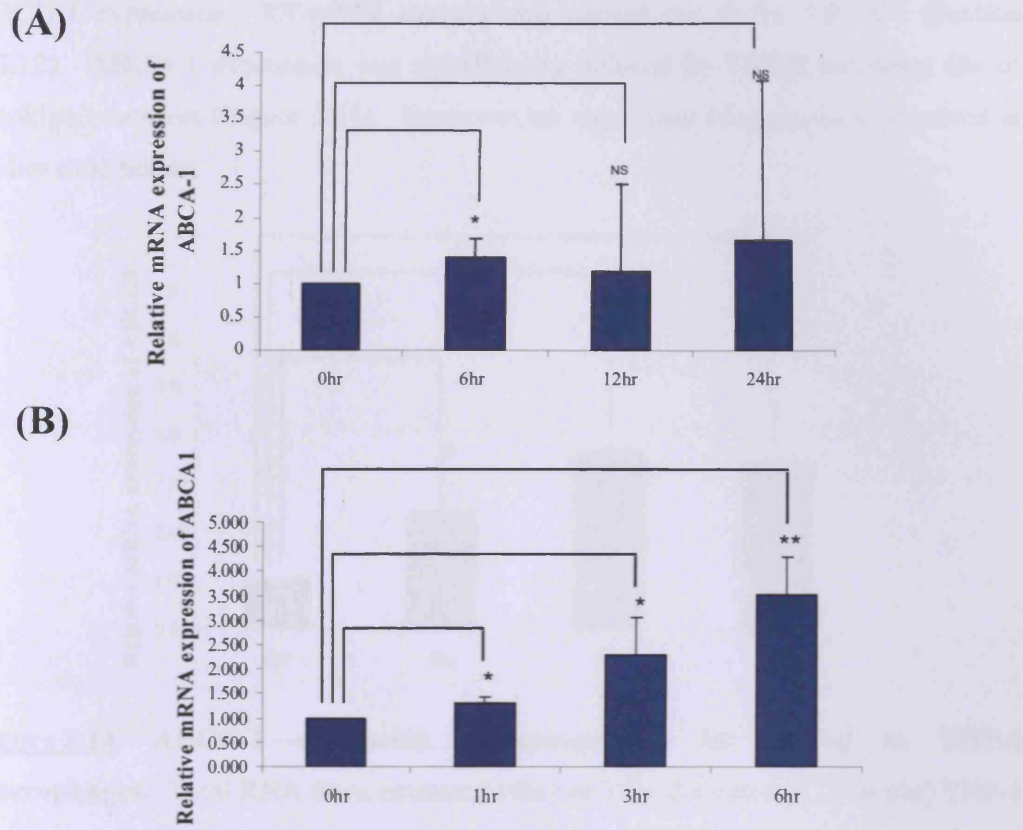

Figure 3.13 **Time-dependent changes in ABCA-1 expression by TGF-β.** Total RNA from untreated (0hr) or TGF-β-treated THP-1 cells was reverse-transcribed and the resulting cDNA subjected to RT-qPCR analysis using primers specific to ABCA-1 and the control gene RPL13A. The relative expression (mean± SD) of ABCA-1 was investigated over 2 time courses, a longer (24hr) time course (Panel A) and a shorter (6hr) time course (Panel B). Data is presented as the relative expression of ABCA-1 (mean± SD) from four independent experiments (*P<0.05, **P<0.01, NS-not significant).

3.3.13 Effect of TGF-β on the mRNA expression of ABCG-1

ABCG-1 expression was analysed at the mRNA level as no antibody against ABCG-1 was available in the laboratory. Based on our observations of TGF-β-regulated ABCA-1 expression and the similarities between the two proteins at the functional level, it was likely that ABCG-1 would be regulated by TGF-β in the same manner as

ABCA-1. We therefore decided to prepare a short time course (up to 6hr) to analyse ABCG-1 expression. RT-qPCR analysis was carried out as for ABCA-1 (Section 3.3.12). ABCG-1 expression was significantly induced by TGF-β following 6hr of cytokine treatment (Figure 3.14). However, no significant changes were observed at earlier time points.

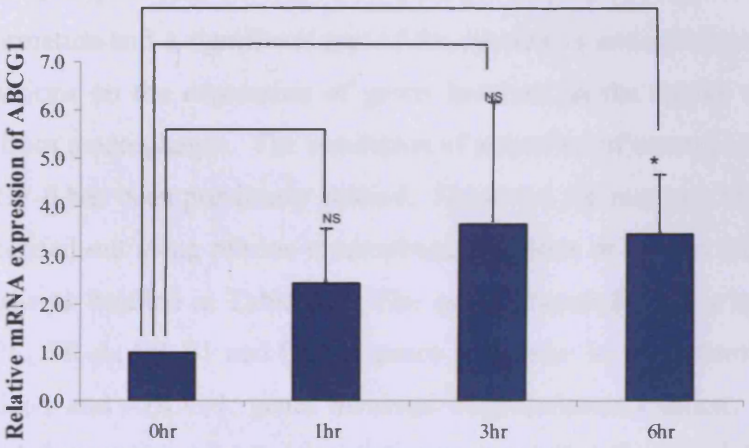

Figure 3.14 ABCG-1 expression is upregulated by TGF-β in THP-1 macrophages. Total RNA from untreated (0hr) or TGF-β treated (1, 3 or 6hr) THP-1 cells was reverse-transcribed and the resulting cDNA subjected to real-time quantitative PCR analysis using primers specific to ABCG-1 and the control gene RPL13A. Data is presented as the relative expression of ABCG-1 (mean± SD) from four independent experiments (*P<0.05, NS-not significant).

3.3 Discussion

The regulation of expression of key genes involved in macrophage cholesterol homeostasis has implications in foam cell formation. TGF-β is a negative regulator of foam cell formation and a significant part of the cytokine's anti-atherogenic effects is due to its actions on the expression of genes involved in the uptake and efflux of cholesterol from macrophages. The regulation of a number of macrophage-expressed genes by TGF-β has been previously studied. However, the majority of these studies have been carried out using murine macrophage cell lines or *in vivo* mice models of atherosclerosis as detailed in Table 3.2. The genes chosen for study in this chapter included LPL, SR-A, SR-B1 and CD36; genes with roles in cholesterol uptake; and ApoE, ABCA-1 and ABCG-1; genes involved with cholesterol efflux. These genes were chosen because their roles in atherosclerosis are well-defined and the expression of some of these in J774.2 macrophages and THP-1 monocytes had previously been studied in our laboratory (Table 3.2) (Hughes et al. 2002; Irvine et al. 2005; Singh and Ramji 2006). Although the action of TGF-β on several of these key genes expressed by macrophages has previously been described in the published literature, a detailed study of their regulation in THP-1 macrophages has not been carried out. To establish an optimal time point for TGF-β regulation and to provide a foundation for further studies into the mechanisms of TGF-β action on these genes (as presented in Chapters 4 and 5) it was important to determine how the cytokine regulates gene expression over time. The action of TGF-β on these key genes involved in cholesterol uptake and efflux was studied in THP-1 macrophages as a model system for human macrophages.

Table 3.2 **Studies on the TGF-β regulation of expression of genes implicated in macrophage cholesterol homeostasis**

Gene	Macrophage source	Regulation of expression by TGF-β	References
ABCA-1	Macrophage derived foam cells from ApoE$^{-/-}$ mice	mRNA and protein expression up-regulated	Panousis et al. 2001
	Murine J774A.1 macrophage cell line	mRNA expression up-regulated	Argmann et al. 2001
ABCG-1	Murine J774A.1 macrophage cell line	mRNA expression up-regulated	Argmann et al. 2001
ACAT-1	Primary human monocytes/ macrophages	mRNA and protein expression is up-regulated on monocyte-macrophage differentiation	Hori et al. 2004
ApoE	Cholesterol loaded and non-cholesterol loaded mouse peritoneal macrophages	mRNA expression and protein secretion up-regulated	Zuckerman et al. 1992
	THP-1 monocytes and THP-1 macrophages	mRNA and protein expression up-regulated	Singh and Ramji 2006
CD163	Primary human macrophages	mRNA and protein expression is down-regulated	Pioli et al. 2004
CD36	THP-1 monocytes and primary human monocytes	mRNA and protein expression down-regulated	Draude and Lorenz 2000
	Macrophages from non-atherosclerotic mice, ApoE$^{-/-}$ mice and LDLR$^{-/-}$ mice	mRNA expression down-regulated	Zuckerman et al. 2001
	THP-1 macrophages	mRNA and protein expression down-regulated	Han et al. 2000
LDL receptor	Murine J774A.1 macrophage cell line	mRNA expression and receptor activity down-regulated	Argmann et al. 2001
LOX-1 (lectin-like oxLDL receptor-1)	THP-1 monocytes and primary human monocytes	mRNA expression up-regulated	Draude and Lorenz 2000
	Murine peritoneal macrophages	mRNA expression upregulated	Minami et al. 2000

LPL	Murine J774A.1 macrophage cell line	mRNA expression and enzyme activity down-regulated	Argmann et al. 2001
	Murine J774.2 macrophages and primary human macrophages	mRNA and protein expression down-regulated	Irvine et al. 2005
SR-A	THP-1 macrophages	mRNA expression down-regulated	Bottalico et al. 1991
	THP-1 monocytes and primary human monocytes	mRNA expression down-regulated	Draude and Lorenz 2000
SR-B1	Macrophages from non-atherosclerotic mice, $ApoE^{-/-}$ mice and $LDLR^{-/-}$ mice	mRNA expression down-regulated	Zuckerman et al. 2001

As expected, the expression of LPL, SR-A, SR-B1 and CD36 was attenuated by TGF-β at both the mRNA and protein level. This is consistent with published studies on the regulation of expression of these genes in macrophages (Table 3.2). In all cases, maximal inhibition occurred following 24hr of TGF-β treatment and this time point was therefore used for further studies on these genes, as presented in Chapters 4 and 5. Also in agreement with the published literature (Table 3.2), TGF-β induced the expression of ApoE, ABCA-1 and ABCG-1 at both the mRNA and protein level. In addition, induction of ApoE by the cytokine was observed in primary human macrophage cultures thereby signifying that any changes in gene expression observed in THP-1 were indicative of changes that could be replicated in primary macrophages. This verified the use of the THP-1 cell line as a model system for the study of TGF-β regulation of gene expression in macrophages. Maximal and significant induction of ApoE mRNA expression occurred following 24hr of TGF-β treatment and maximal protein expression occurred after 48hr of TGF-β treatment. For consistency, it was decided that the 24hr time point would be used for further studies into ApoE regulation. In contrast to the other genes studied, ABCA-1 and ABCG-1 were not optimally up-regulated following 24hr TGF-β treatment. An extensive time course of ABCA-1 regulation by TGF-β was prepared to investigate the disparity between mRNA and protein induction. Maximal induction of ABCA-1 and ABCG-1 was observed after 6hr TGF-β treatment and this time point was used in further studies into the regulation of their expression by the cytokine.

Despite a number of studies, the mechanism of the regulation of expression of genes implicated in macrophage cholesterol homeostasis by TGF-β remains unclear. Studies by our laboratory have highlighted the importance of the JNK, p38 kinase and CK2 pathways in the ApoE response to TGF-β (Section 3.1.6) but the involvement of the classical and other TGF-β signalling pathways in gene regulation remains poorly understood. Only the mechanisms behind the regulation of LPL, ApoE and CD36 expression by TGF-β have previously been investigated. The current knowledge regarding the mechanisms of gene regulation by TGF-β in macrophages is summarised in Figure 3.15. The regulation of LPL expression by the cytokine occurs at the transcriptional level and is mediated through a change in transactivation potential of Sp1 and Sp3 by TGF-β (Irvine et al. 2005). The regulation of CD36 has also been described at the transcriptional level where the gene is a direct target for

PPARγ, which becomes inactivated by MAPK phosphorylation in response to TGF-β (Han et al. 2000). Interestingly, published literature suggests that PPARγ is able to regulate the expression of each of the genes studied in this chapter, either directly or indirectly. This may therefore be a potential link to their regulation by TGF-β.

PPARγ is part of a larger family of nuclear receptors, including PPAR-α, -β and –γ. Like other nuclear receptors the PPARs contain a ligand-binding domain and a DNA-binding domain. The primary role of PPARs is in metabolic regulation where they stimulate fatty acid breakdown. PPARγ is involved in the storage and release of fat from adipose tissue through the regulation of adipose gene expression (Fernandez 2008; Neve et al. 2000). PPARγ is expressed as two isoforms; PPARγ1 is expressed within monocytes and macrophages while PPARγ2 is restricted to adipose tissue (Kintscher et al. 2002). Both PPARα and PPARγ are expressed in vascular cells and have been associated with atherosclerosis. PPARγ can be detected in the macrophage-rich regions of atherosclerotic plaques and is overexpressed in foam cells (Duan et al. 2008; Fernandez 2008; Galetto et al. 2001; Worley et al. 2003). Ligands of PPAR have been associated with reduced progression of atherosclerotic lesions and reduced intimal thickness whilst activators of PPARγ can inhibit the proliferation of VSMCs by inhibiting the expression of MMP-9. Agonists of PPARγ can reduce the expression of pro-inflammatory cytokines TNFα and IL-6 and the chemokine MCP-1 in monocytes (Duan et al. 2008; Neve et al. 2000).

PPARγ activates target genes through heterodimerisation with RXRα, another nuclear receptor. Heterodimers can be activated by ligands of PPARγ and ligands of RXRα. Ligands for PPARγ include metabolites of arachidonic acid and fatty acid derivatives of oxLDL such as 16-deoxy-$\Delta_{-12,\ 14}$-prostaglandin J_2 (15-dPGJ$_2$). Thiazolidinediones (TZDs), used in the treatment of type 2 diabetes are also ligands for PPARγ (Neve et al. 2000). Heterodimerisation is followed by binding to a PPARγ response element (PPRE) in the promoter of target genes (Fernandez 2008; Worley et al. 2003). The consensus sequence for this response element is a repeat of AGGTCA. In addition to direct gene regulation, PPAR also negatively regulates inflammatory gene transcription by modulating the recruitment of cofactors or through interaction with other transcription factors such as AP-1, STAT and NFκB (Neve et al. 2000). This is mediated by a conformational change in the heterodimer complex following ligand

binding allowing DNA binding and release of corepressors. In macrophages, this allows the binding of PPARγ to NFκB and the recruitment of corepressors to negatively regulate NFκB target genes such as iNOS (Duan et al. 2008; Neve et al. 2000).

Similar to TGF-β, PPARγ has been suggested to play an anti-atherogenic role in foam cell formation (Akiyama et al. 2002). Macrophages deficient in PPARγ have reduced levels of oxLDL uptake and reduced cholesterol efflux (Akiyama et al. 2002). Bone marrow transfer of macrophages deficient in PPARγ into $LDLR^{-/-}$ mice produces larger atherosclerotic lesions than in wildtype counterparts and *in vivo* transfer of PPARγ into rat artery reduces neointima formation following balloon injury (Duan et al. 2008). Agonists of PPARγ reduce atherosclerosis in human and animal models, and in cultured human endothelial cells can inhibit the expression of chemokine genes and adhesion molecules such as VCAM-1 to reduce adherence of monocytes to endothelial cells (Duan et al. 2008; Fernandez 2008). Connections between TGF-β and PPARγ have been demonstrated in VSMCs where TGF-β transiently induces the expression of PPARγ before inhibiting its expression through activation of AP-1 and Smad-3. Interestingly, PPARγ is able to negatively-regulate the TGF-β pathway in these cells and stimulation of PPARγ disrupts Smad-3 activation to inhibit the TGF-β-induced expression of connective tissue growth factor. These observations suggest that crosstalk between TGF-β and PPARγ signalling does exist (Fu et al. 2003). The cytokine has also been shown to up-regulate PPARγ mRNA and protein expression in THP-1 monocytes (Kintscher et al. 2002).

As described in Section 3.1.5, CD36 is a direct target gene for PPARγ and induction of PPARγ results in the up-regulation of CD36 expression. Mice deficient in macrophage PPARγ have reduced levels of CD36 expression suggesting that the receptor is required for the basal expression of CD36. The reduced uptake of oxLDL observed in macrophage PPARγ deficient mice may partly be a result of the down-regulation of CD36 expression (Akiyama et al. 2002; Duan et al. 2008). Similar to the regulation of CD36, activators of PPARγ can induce the expression of SR-B1 in primary cultures of human macrophages and in the $ApoE^{-/-}$ mice. This indicates that PPARγ positively regulates class B scavenger receptor expression (Chinetti et al.

2000). TGF-β stimulation may therefore inactivate PPARγ through phosphorylation to achieve inhibition of CD36 and SR-B1 expression.

As an inhibitor of macrophage activation, PPARγ inhibits the expression of SR-A, through interaction with AP-1, STAT and NFκB transcription factors (Ricote et al. 1998). Transcriptional activation of SR-A by pro-inflammatory cytokines is also inhibited by PPARγ (Ricote et al. 1998). In addition to reduced scavenger receptor expression, mice deficient in macrophage PPARγ present with reduced expression of LPL suggesting that (as is the case for CD36) the receptor is required for basal expression of macrophage LPL. In support of this, a requirement for PPARγ in the basal expression of LPL has been demonstrated in adipose and liver tissue and a PPRE has been identified within the LPL promoter region (Akiyama et al. 2002; Schoonjans et al. 1996).

PPARγ has effects on both cholesterol uptake and efflux from macrophages. Macrophages deficient in PPARγ have reduced cholesterol efflux and reduced expression of ABCA-1, ABCG-1 and ApoE (Akiyama et al. 2002). The PPARγ ligand, ciglitazone and the RXRα ligand, 9-cis retinoic acid can up-regulate ApoE expression in THP-1 cells and a PPRE has been identified in the intergenic region of the ApoE and ApoCI promoters (Galetto et al. 2001). Ligands of PPARγ induce ABCA-1 and ABCG1 expression through the up-regulation of the oxysterol-regulated LXRα, leading to high levels of cholesterol efflux (Nicholson 2004). ABCA-1 and ABCG-1 are up-regulated through an LXRα-dependent pathway and contain LXR response elements within their promoters, whilst ApoE is also a direct target for the receptor (Akiyama et al. 2002; Nicholson 2004).

In conclusion, although there is a link between the regulation of expression of cholesterol uptake and efflux genes through PPARγ, the regulation of these genes by TGF-β has only been partly defined for CD36, LPL and ApoE. In addition to this, whilst there are some clues to the transcriptional regulation of these genes, very little is known about the molecular mechanisms behind their regulation by TGF-β. In particular, the involvement of the classical and other TGF-β signalling pathways in gene regulation remains poorly understood. Current knowledge regarding the TGF-β regulation of genes implicated in macrophage cholesterol homeostasis is summarised

in Figure 3.15. Specifically, the classical TGF-β/Smad signalling pathway has been comprehensively characterised but its role in the regulation of a large number of TGF-β target genes has not been described. This will be the focus of Chapter 4 of this thesis.

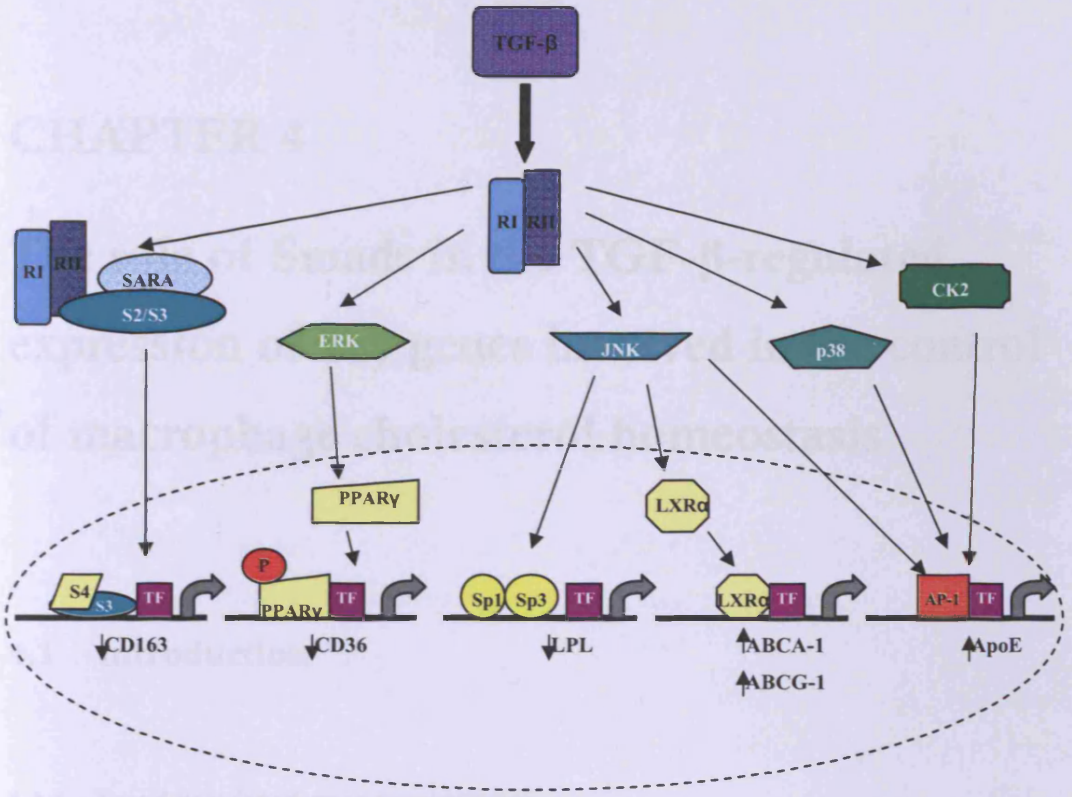

Figure 3.15 Current knowledge of TGF-β-regulated expression of genes implicated in macrophage cholesterol homeostasis and the involvement of TGF-β signalling pathways and transcriptional mediators in this regulation. CD163 is the only gene known to be directly regulated by the classical TGF-β-Smad signalling (Pioli et al. 2004). TGF-β-regulated expression of ApoE, LPL, CD36 and ABCA-1 is thought to involve components of the MAPK pathways but whether these act independently or in cooperation with the Smad pathway is currently unclear. Signalling pathways can act independently of each other or may interact with other signalling pathways to mediate the TGF-β response (Section 1.14). Nuclear receptors and transcription factors may also interact with other transcription factors or proteins to mediate the TGF-β response, such as PPARγ and LXRα in the case of ABCA-1. Abbreviations: AP-1-activator protein-1 (comprising the transcription factors c-Jun and c-Fos), CK2-casein kinase 2, ERK-extracellular signal-regulated kinase, JNK-c-Jun N-terminal kinase, LXR-liver-X-receptor, P-phosphate group, PPAR-peroxisome proliferator-activated receptor, SARA-Smad anchor for receptor activation, S2/S3/S4-Smad-2/-3/-4, TF-transcription factor.

CHAPTER 4

The role of Smads in the TGF-β-regulated expression of key genes involved in the control of macrophage cholesterol homeostasis

4.1 Introduction

4.1.1 Smad-regulated gene transcription

Although the TGF-β-Smad pathway is well characterised, less is known about how this pathway contributes to individual regulation of target genes. Genes known to be regulated through the Smad proteins are few in number. One of the most cited examples of a Smad-mediated TGF-β response is the induced expression of cyclin-dependent kinase inhibitors p15 and p21, in epithelial cells, which leads to growth arrest of these cells (Wrighton and Feng 2008). Sequence specific binding of Smads to promoters of target genes has been observed for plasminogen activator inhibitor-1 (PAI-1) where they bind directly to a Smad binding element in the promoter region (Mallat et al. 2001). Often the exact mechanism of regulation of these genes by Smads is rendered difficult by the interaction of Smad proteins with various other proteins and/or transcription factors to regulate gene transcription. An example of this is the activation of α1-collagen gene expression in response to TGF-β, which is mediated through the binding of Smad-2 and Sp1 to the promoter. Smad-2 binding to a TGF-β response element present in the promoter enhances the binding of Sp1 to activate the promoter (Sysa et al. 2009). TGF-β-mediated stimulation of type-I collagen expression in cultured human fibroblasts is mediated by an interaction

between activated Smads and p300/CBP, which accumulates at consensus Smad-binding elements (Ghosh et al. 2000).

Very little is currently known about the involvement of the Smads in atherosclerosis and the vasculature. Immunohistochemistry and RT-PCR have demonstrated that Smad-2, -3 and -4 are expressed in macrophages and foam cells of fatty lesions (Kalinina et al. 2004). Smad expression is up-regulated following monocyte-macrophage differentiation in both macrophages of lesions and in THP-1 cells. Such an expression profile suggests that the Smads may contribute to monocyte accumulation (Kalinina et al. 2004). Interestingly, the expression pattern of Smads in VSMCs is distinct from that in macrophages. Smad-2, -3 and -4 are not expressed in VSMCs of fatty lesions but are present in more advanced fibrous plaques, suggesting that they could contribute to the accumulation of collagen (Kalinina et al. 2004).

The identification of the Smad pathway as a mediator for the TGF-β-regulated expression of a number of genes implicated in lesion development and/or stability has connected the pathway with atherosclerosis. The Smad pathway has been shown to mediate the induction of cyclin-dependent kinase inhibitors expression in macrophages (Fu et al. 2003; Kalinina et al. 2004), which play roles in apoptosis and cell proliferation. The TGF-β-regulated induction of connective tissue growth factor (CTGF) involved in the production of the ECM by VSMCs is also mediated by the Smad pathway. In human aortic smooth muscle cells, the induction of CTGF promoter activity by ligands of PPARγ could be reversed by overexpression of Smad-3 and plasmids specifying for Smad-3/-4 could increase promoter activity in transfected cells (Fu et al. 2003).

In macrophages, the TGF-β-regulated activation of IL-23 expression is thought to involve Smad-3 (Al-Salleeh and Petro, 2008). The Smad pathway, in particular Smad-3, has also been demonstrated to mediate the inhibitory effects of TGF-β on macrophage activation. TGF-β inhibits the expression of MMP-12 and iNOS, both markers of macrophage activation. Transient transfection of murine RAW264.7 macrophages demonstrated that promoter activity of MMP-12 and iNOS could be inhibited by Smad-3 and that this was further enhanced by treatment with TGF-β (Werner et al. 2000).

In relation to foam cell formation, only the TGF-β-regulated expression of the CD163 gene is known to be directly regulated through the Smad pathway. CD163 is a scavenger receptor expressed exclusively by monocytes and macrophages. It is thought to mediate the endocytosis of haemoglobin complexes to attenuate inflammatory responses linked with haemolysis but can take up lipoproteins into cells in an atherogenic environment (Pioli et al. 2004). The expression of CD163 has been demonstrated to be reduced by TGF-β, at the transcriptional level (Pioli et al. 2004). The direct involvement of Smad-3 in this response was demonstrated by Pioli and colleagues in 2004. Transient transfection studies demonstrated that the inhibitory effect of TGF-β on CD163 promoter activity could be antagonised by co-transfection with a dominant-negative Smad-3 plasmid but not with dominant-negative Smad-2. This implicated Smad-3 as the primary mediator of this TGF-β response (Pioli et al. 2004).

4.1.2 RNA interference (RNAi)

RNAi describes the process by which double-stranded RNA (dsRNA) triggers the destruction of a target mRNA that shares the same sequence. This was initially discovered in transgenic plants where it was termed post-transcriptional gene silencing (Meister and Tuschl 2004). The RNAi pathway was discovered in eukaryotic cells in 1998 when it was shown that double-stranded RNA could induce gene silencing in *C.elegans* (de Fougerolles et al. 2007; Hajeri and Singh 2009; Hutvágner and Zamore 2002). This was followed by the finding in 2001 that 21-nucleotide siRNA duplexes could suppress gene expression in mammalian cell lines including human embryonic (293) and HeLa cells (Elbashir et al. 2001).

The RNAi pathway is an evolutionary conserved mechanism of cellular defence by which the expression of 'foreign genes' randomly integrated into the host genome (such as those from viruses) is regulated. Foreign genes often express dsRNA and the presence of this is used to initiate processes to post-transcriptionally inhibit the expression of these genes (Tuschl and Borkhardt 2002).

4.1.2.1 RNAi and small interfering RNA (siRNA)

As shown in Figure 4.1, long dsRNA is cleaved into smaller dsRNA fragments known as small-interfering RNAs (siRNA). This cleavage is carried out by the enzyme Dicer, a member of the RNase III family of endonucleases that is specific for dsRNA. Dicer contains both an RNA binding domain and a RNase domain. Dicer is located within the cytoplasm and interacts with a partner protein that also binds dsRNA known as TAR RNA-binding protein (TRBP). Dicer is the only member of the RNase III family of endonucleases that requires ATP for cleavage and this is due to the presence of an ATP-dependent RNA helicase domain within its amino terminus. Helicases use ATP to break the hydrogen bonds that join the two strands of DNA or, in this case, dsRNA. In dsRNA cleavage, the ATP is likely to be used for catalytic activity and may help to move Dicer along the dsRNA (Hajeri and Singh 2009; Hutvágner and Zamore 2002; Meister and Tuschl 2004; Rao et al. 2009).

The siRNAs produced following cleavage of dsRNA by Dicer are 21-25 nucleotides in length (length is species specific) and contain a phosphate group at their 5' end and a hydroxyl group at their 3' end (Hajeri and Singh 2009; Hutvágner and Zamore 2002). Duplexes of siRNA interact with a protein complex known as the RNA-induced silencing complex (RISC). RISC is a multi-enzyme complex that is made up of RISC and Argonaute 2 (AGO2/ EIF2C2-eukaryotic translation initiation factor C2) (de Fougerolles et al. 2007; Meister and Tuschl 2004). Fluoresence spectroscopy studies have indicated that RISC shuttles between the nucleus and the cytoplasm (Rao et al. 2009). siRNA fragments bind tightly to the AGO2 protein (Pratt and MacRae 2009). If the siRNA sequence has perfect sequence complementarity then AGO2 unwinds and cleaves the sense strand, leaving one guide strand (the antisense strand), complementary to the target mRNA, attached to RISC as shown in Figure 4.1. Binding of the RNA strand to the RISC protein activates the complex (now known as RISC*). The siRNA then targets active RISC to the target mRNA through complementary base pairing (Hajeri and Singh 2009; Hutvágner and Zamore 2002).

Radiolabelled siRNAs demonstrated that the target mRNA is cleaved at nucleotides close to the centre of the guide strand and further studies showed that the target mRNA is cleaved between nucleotides complementary to nucleotides 10 and 11 from the 5' end of the guide siRNA strand. This cleavage results in a disrupted reading frame for the encoded protein and promotes degradation of the mRNA by

exonucleases. Cleavage is independent of ATP but requires a catalytically active RNase, which has been aptly named Slicer. Slicer is thought to be the AGO2 protein, as it contains a catalytically active RNase domain (de Fougerolles et al. 2007; Meister and Tuschl 2004; Rao et al. 2009). Although the exact mechanism of mRNA cleavage is not clear, it has been suggested that a catalytic triad (aspartate, aspartate and aspartate or histidine) is used to coordinate catalytic metal ions and position a water molecule for nucleophilic attck of the phosphodiester backbone of the mRNA (Pratt and MacRae 2009). Although cleavage does not require ATP, multiple rounds of cleavage where products (including RISC) are released to go on to target new mRNAs, is enhanced by ATP (Meister and Tuschl 2004).

4.1.2.2 Short hairpin RNA (shRNA) and microRNA (miRNA)

Gene silencing can also be induced by short hairpin RNAs (shRNAs) or by micro RNAs (miRNA) which are derived from non-coding hairpin RNA structures. This is shown in Figure 4.1. shRNAs have a hairpin-like stem loop structure. miRNAs are encoded within introns or intergenic regions and are initially processed in the nucleus by an enzyme known as Drosha. Drosha is another member of the RNase III endonuclease family that like Dicer contains both an RNase and an RNA-binding domain. Drosha processes and shortens double-stranded shRNA and miRNA to form a precursor that can be exported from the nucleus using the nuclear export protein, exportin-5. The shRNA and miRNA undergo further processing by Dicer in the cytoplasm to form mature shRNA/miRNA (de Fougerolles et al. 2007; Hajeri and Singh 2009; Meister and Tuschl 2004; Rao et al. 2009). On loading of RISC with shRNA or miRNA, the sense strand is unwound to leave the guide strand (mature shRNA/miRNA) to target activated RISC to the mRNA target. In contrast to siRNA which is complementary to and results in the cleavage of the target mRNA sequence, mature shRNA/miRNA is not perfectly complementary to the target sequence and instead of cleaving the target mRNA, mediates repression of its translation as shown in Figure 4.1 (de Fougerolles et al. 2007; Pratt and MacRae 2009; Rao et al. 2009). Only 2-7 bases of the guide and target sequence must pair up to initiate translational repression of the target mRNA. Often, the miRNA recognises sites within the 3' untranslated region of the target mRNA to inhibit translation (de Fougerolles et al.

2007). The exact mechanism of translational repression by shRNA/miRNA is unclear but may be mediated through protein: protein interactions (Pratt and MacRae 2009).

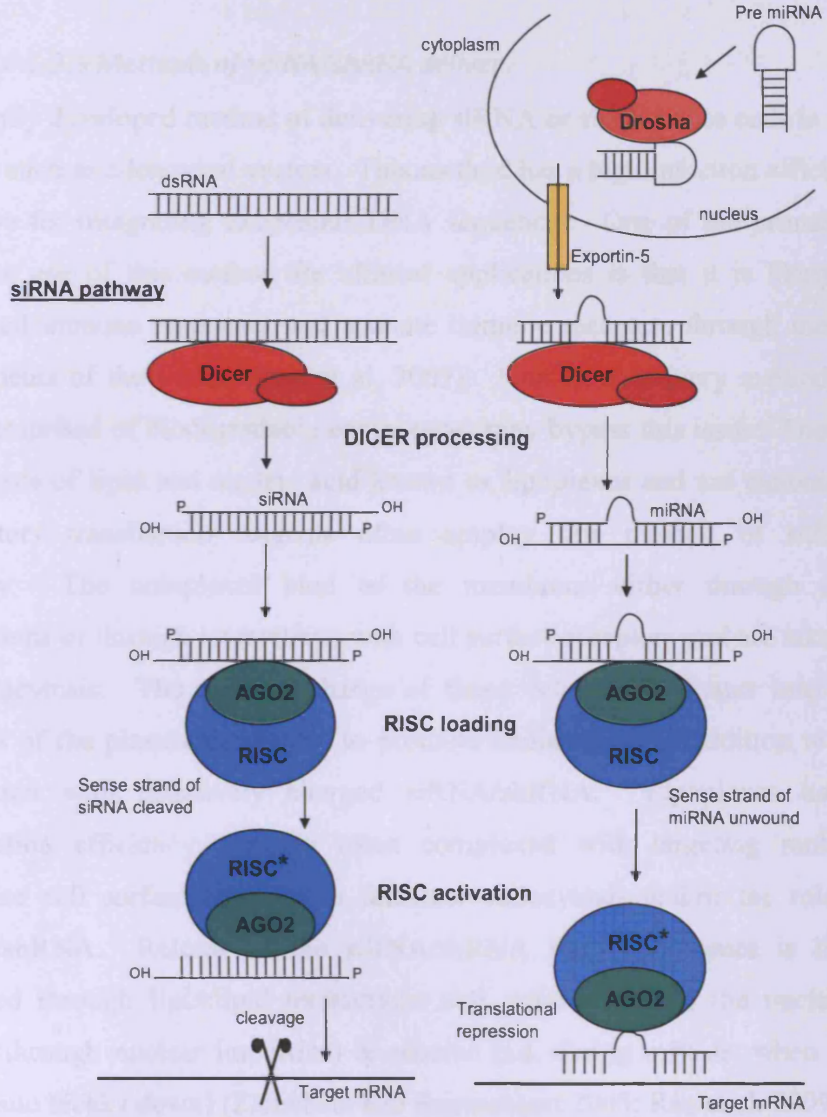

Figure 4.1 **The RNA interference pathway.** Gene silencing is induced by dsRNA. miRNA is processed by Drosha in the nucleus before undergoing processing in the cytoplasm by Dicer, where siRNA is also processed in an ATP-dependent process. Dicer produces siRNAs, 21-25 nucleotides in length which interact with the RISC complex. Duplexes are cleaved (siRNA) or unwound (miRNA) by the AGO2 protein to produce an activated RISC complex which is guided to the target mRNA through complementary base pairing. The target mRNA is cleaved (siRNA) or translationally repressed (miRNA) by an unknown mechanism. Abbreviations: dsRNA-double stranded RNA, miRNA-microRNA, RISC-RNA-induced silencing complex, siRNA-small interfering RNA (Adapted from de Fougerolles et al., 2007).

4.1.2.3 Methods of siRNA/shRNA delivery

A recently developed method of delivering siRNA or shRNA into cells is using viral-vectors such as adenoviral vectors. This method has a high infection efficiency and is effective for integrating exogenous DNA sequences. One of the primary concerns with the use of this method for clinical applications is that it is likely to induce unwanted immune responses and activate immune receptors through the adenoviral components of the vector (Rao et al. 2009). Non-viral delivery methods including ones comprised of biodegradable components may bypass this issue. These are often complexes of lipid and nucleic acid known as lipoplexes and are cationic in nature. Laboratory transfection reagents often employ this method of siRNA/shRNA delivery. The complexes bind to the membrane either through electrostatic interactions or through interactions with cell surface receptors and are taken into cells by endocytosis. The positive charge of these vehicles facilitates interaction with proteins of the plasma membrane to promote endocytosis, in addition to facilitating interaction with negatively charged siRNA/shRNA. Lipoplexes have a high transfection efficiency and are often complexed with targeting molecules that recognise cell surface markers to facilitate endocytosis and/or the release of the siRNA/shRNA. Release of the siRNA/shRNA from endosomes is likely to be mediated through lipid:lipid interactions and movement into the nucleus may be active (through nuclear importins) or passive (i.e. during mitosis, when the nuclear membrane breaks down) (Elouahabi and Ruysschaert 2005; Rao et al. 2009).

4.2 Aims of experimental studies

The primary aim of the studies presented in this chapter was to determine the role of the Smad pathway in the TGF-β-regulated expression of key genes implicated in the control of macrophage cholesterol homeostasis. The TGF-β-regulation of expression of these genes (LPL, SR-A, SR-B1, CD36, ApoE, ABCA-1 and ABCG1) has been characterised by previous studies, as presented in Chapter 3 of this thesis. Although the Smad signalling pathway is the classical mediator of TGF-β responses, its involvement in the TGF-β-mediated regulation of expression of these key genes has

not been investigated. As explained in more detail in Section 1.10, the Smad pathway is triggered following activation of the TGF-β receptor complex by TGF-β1. This results in the phosphorylation of R-Smad-2 or R-Smad-3 which form a complex with the common mediator Smad protein, Smad-4 to translocate to the nucleus to regulate transcription of target genes. The only macrophage expressed gene known to be regulated directly through the Smad pathway is the scavenger receptor CD163 as described in Section 4.1.1.

The expression of Smads is up-regulated following monocyte-macrophage differentiation in both HMDMs and in THP-1 cells and Smad activation has been observed in macrophages of fatty lesions. In order to carry out studies on the involvement of the Smad proteins in the TGF-β-regulated expression of key genes, it was necessary to characterise the activation of Smad proteins by TGF-β in THP-1 macrophages. This aim was carried out using western blot analysis of the activated (phosphorylated) forms of Smad-2 and Smad-3 to determine the kinetics and levels of Smad activation in this cell line. Following this, the involvement of Smad-2 and/or Smad-3 in the regulation of gene transcription was carried out using siRNA-mediated knockdown. Figure 4.2 outlines the experimental strategy used during these studies.

The use of stable RNAi against the Smad proteins has been used in a number of studies to determine the role of Smads in TGF-β-regulated responses. siRNA-mediated knockdown of Smads has identified Smad-3 as a mediator of the TGF-β-mediated activation of inhibitor of differentiation-1 (Id1) in epithelial cells (Liang et al. 2009) and also as a regulator of TGF-β-mediated apoptosis in the Hep3B cell line (Yu et al. 2008). siRNA-mediated knockdown of Smad-2, -3 or -4 demonstrated an involvement for all three Smads and therefore implicated the Smad pathway in the TGF-β stimulation of the angiotensin II type I receptor expression in human lung fibroblasts (Martin et al. 2007). A role for Smad-3 in the autoinduction of TGF-β1 in proximal tubular epithelial cells was also verified using siRNA against the Smad-3 gene (Zhang et al. 2006). Based on these studies and the commercial availability of validated siRNAs against Smad-2 and Smad-3, it was decided that siRNA-mediated knockdown of Smad expression would be used to determine the role of the pathway in gene expression. This method had previously been optimised in the laboratory and the technique had been used previously in the laboratory to investigate the

involvement of signalling pathways in cytokine-mediated gene expression. RNA interference has a number of advantages over alternative techniques for knockdown of gene expression or function such as the use of pharmacological inhibitors. The use of RNAi in low concentrations avoids possible problems with off-target effects and specificity issues that can arise with the use of inhibitors, in particular with higher concentrations of inhibitor. Cell cultures are also prone to issues with toxicity arising from the use of pharmacological inhibitors which can be avoided with the use of RNAi. The ability to use RNAi in cell lines also means that functional studies do not have to rely solely on experiments in knockout animal models which are often time consuming and difficult and conclusions from which cannot always be reconciled with studies in human cells or subjects. RNAi can allow functional studies to be carried out in human cells.

The role of each Smad was investigated first. Differential contributions of Smad-2 and Smad-3 had to be considered based on studies that have established that some TGF-β responses are mediated by Smad-3 as opposed to Smad-2 and vice versa (Brown et al. 2007). The use of siRNA against Smads has also identified subsets of genes that are regulated by Smad-2 and Smad-3 (Yu et al. 2008). In addition to this, the effects of siRNA-mediated knockdown of both Smads on gene expression was investigated as it has been suggested that competition between the two Smads for gene transcription may occur and also that functional redundancy between the Smad proteins may exist (Yu et al. 2008).

In order to confirm that any responses observed in THP-1 macrophages would be indicative of what would be observed in human macrophages, adenoviral infection of HMDMs with shRNA against Smad-2 shRNA was carried out in conjunction with Dr Daryn R. Michael. TGF-β-regulated expression of key genes was studied. Data from siRNA knockdown studies in THP-1 cells was analysed for effects on basal and TGF-β regulated expression. Mostly, the effects on mRNA expression were analysed using RT-qPCR and initially, where antibodies to the protein were available, effects on protein expression were investigated with further studies carried out at the mRNA level. For ease of reading studies are presented on a gene by gene basis.

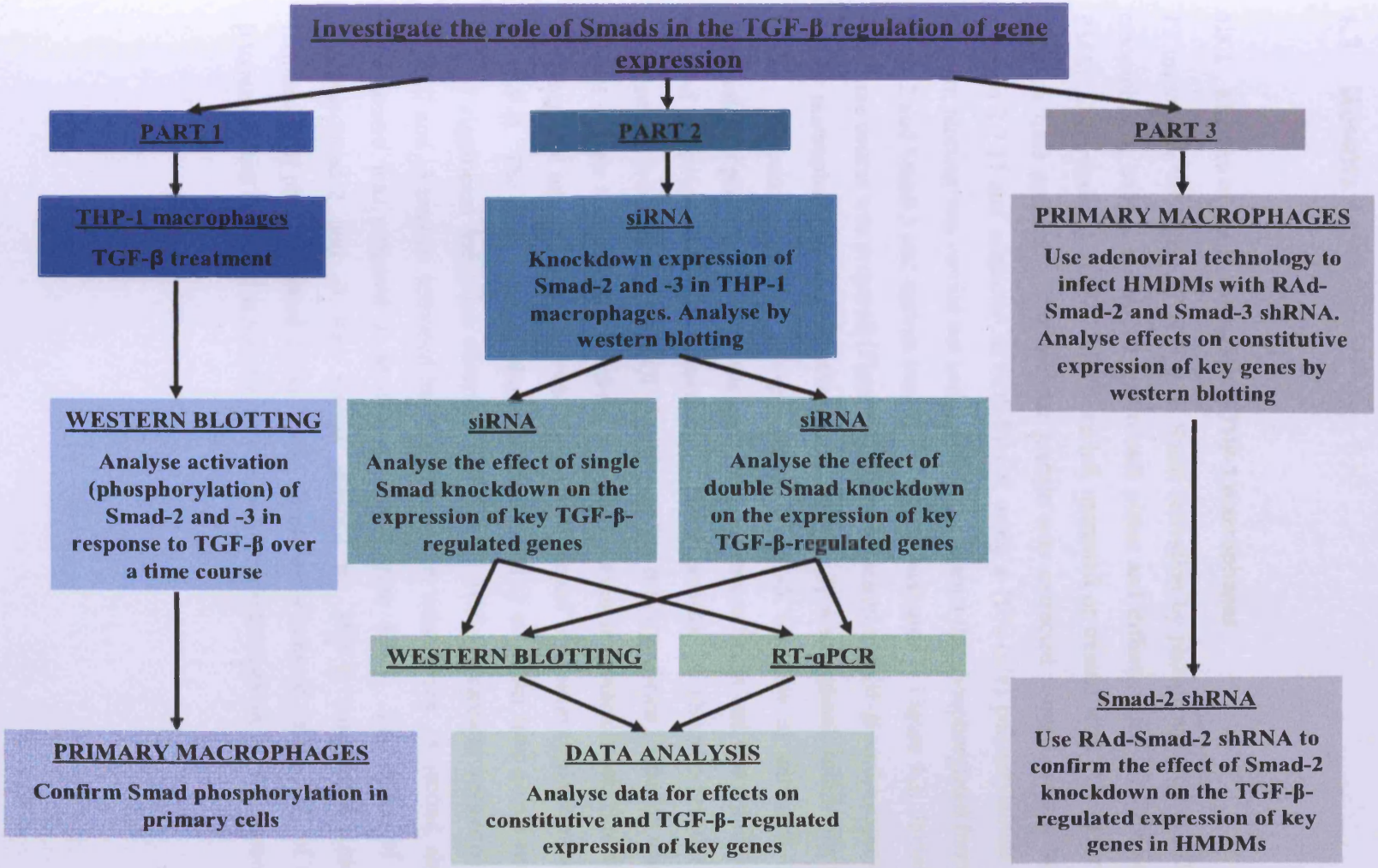

Figure 4.2 Experimental Strategy Diagram

4.3 Results

4.3.1 Kinetics of Smad activation in THP-1 macrophages

To investigate the effect of TGF-β on Smad activation by phosphorylation in THP-1 macrophages, cells were seeded into 6-well plates and differentiated for 24hr using PMA. Differentiated THP-1 cells were left untreated or treated with TGF-β for the requisite time periods. Total cellular protein was extracted using Laemmli buffer (Section 2.7.1) and subjected to SDS-PAGE using a 10% (v/v) polyacrylamide gel. Western blotting was carried out using antibodies against the phosphorylated forms of Smad-2 and Smad-3 and against total Smad-2+3 as indicated in Figure 4.3. Initially a 24hr time course was prepared (Figure 4.3A) as the action of TGF-β on the expression of key macrophage genes (as presented in Chapter 3) was optimal within this time frame. Expression of both phospho-Smad-2 and -3 was low in untreated cells. Expression of phospho-Smad-2 was high in TGF-β-treated cells and was significantly induced following 3hr TGF-β treatment. The expression of phospho-Smad-3 was significantly induced after 6hr TGF-β treatment but this significant induction was not evident at later time points. No changes were observed in levels of total Smad-2+3, verifying that any changes observed were due to Smad activation (phosphorylation) by TGF-β. The high levels of phospho-Smad-2 and -3 at earlier time points and the loss of significant induction observed with longer TGF-β treatment suggested that Smad-2 and -3 may be activated by TGF-β at earlier time points. A second, shorter time course was prepared to investigate this (Figure 4.3B). Expression of both phospho-Smad-2 and -3 was indeed induced by TGF-β with rapid kinetics. Expression of phospho-Smad-2 and -3 was significantly induced within 5min of TGF-β treatment and remained at significantly induced levels throughout the time course.

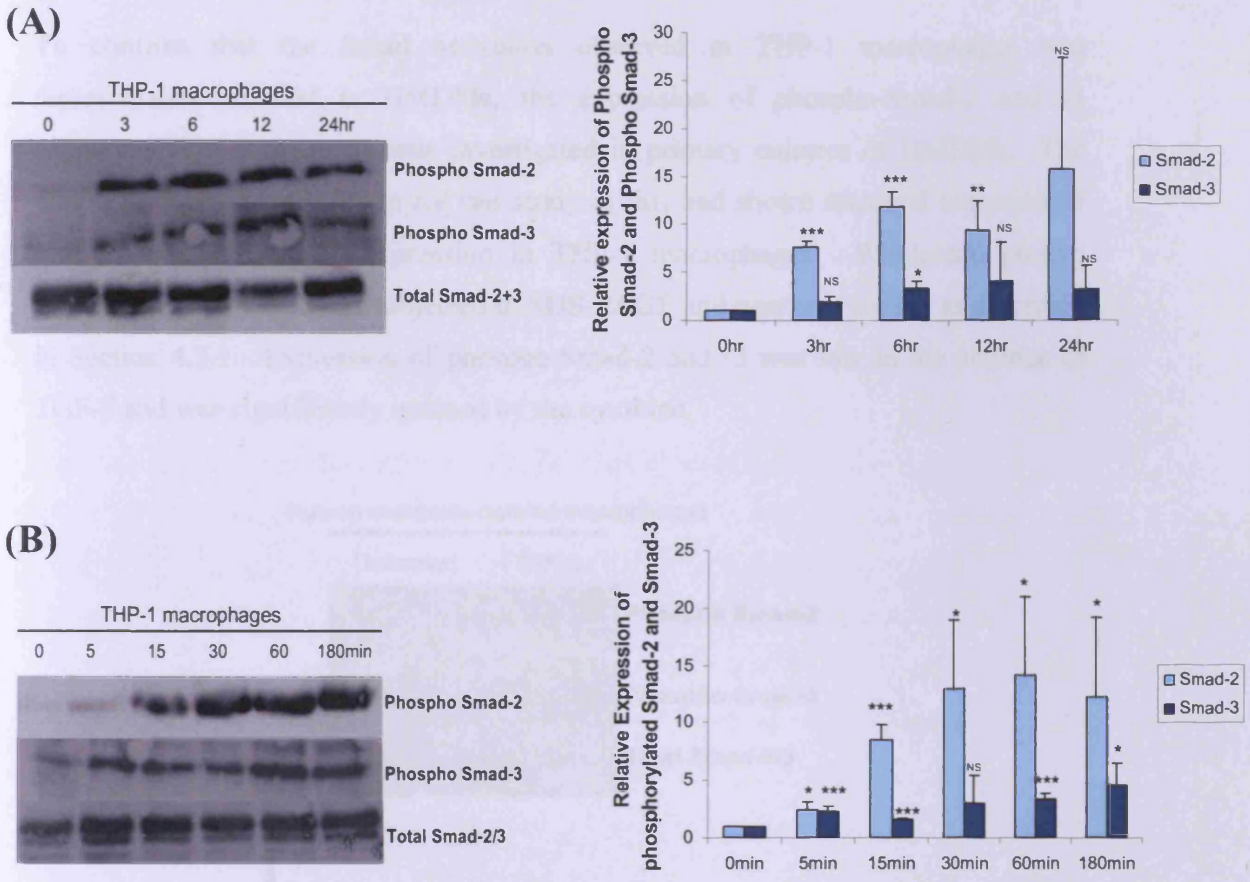

Figure 4.3 **TGF-β activates Smad-2 and Smad-3 with rapid kinetics in THP-1 macrophages.** THP-1 macrophages were left untreated (0hr) or treated with TGF-β for 3, 6, 12 or 24hr (Panel A) or for 5, 15, 30, 60 or 180min (Panel B), as indicated. Whole-cell protein extracts were subjected to SDS-PAGE and western blotting and levels of phospho-Smad-2 and -3 were compared to levels of total Smad-2+3. Protein size was determined by comparison against a standard molecular weight marker (Appendix I). The relative expression (mean± SD) of phospho-Smad-2 and -3 normalised to total Smad-2+3 expression (untreated samples (0hr) arbitrarily assigned as 1), from three independent experiments is shown (*P<0.05, **P<0.01, ***P<0.001, NS-not significant).

4.3.2 Smad activation in HMDMs

To confirm that the Smad activation observed in THP-1 macrophages was representative of that in HMDMs, the expression of phospho-Smad-2 and -3 following TGF-β treatment was investigated in primary cultures of HMDMs. The 60min time point was chosen for this study as this had shown maximal induction of phospho-Smad-2 and -3 expression in THP-1 macrophages. Whole-cell protein extracts were prepared and subjected to SDS-PAGE and western blotting as described in Section 4.3.1. Expression of phospho-Smad-2 and -3 was low in the absence of TGF-β and was significantly induced by the cytokine.

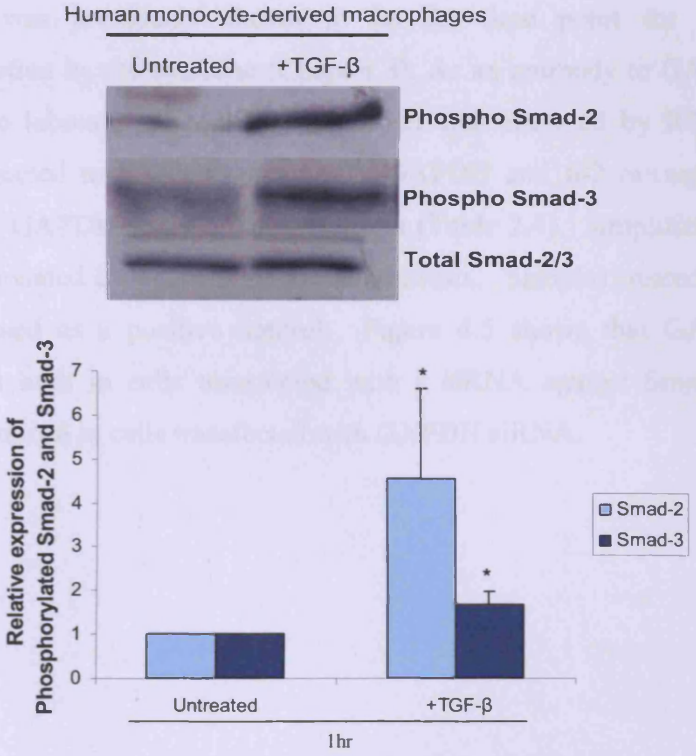

Figure 4.4 **TGF-β activates Smad-2 and Smad-3 in HMDMs.** HMDMs were left untreated or treated with TGF-β for 1hr. Whole-cell protein extracts were subjected to SDS-PAGE and western blotting using antibodies against phospho-Smad-2, -3 and total Smad-2+3. Relative expression (mean± SD) of phospho-Smad-2 or phospho-Smad-3, normalised to levels of total Smad-2+3 (untreated samples arbitrarily assigned as 1) from three independent experiments is shown (*P<0.05).

4.3.3 siRNA-mediated knockdown of control gene, GAPDH

Control siRNAs used for siRNA interference assays include scramble siRNA or siRNAs against housekeeping genes such as GAPDH. A number of studies have successfully employed GAPDH as a control for siRNA-mediated knockdown (Chinetti-Gbaguidi et al. 2005; Larigauderie et al. 2004) and based on this, and its previous successful use in the laboratory, GAPDH was chosen as a control for these studies. THP-1 monocytes were transfected with 7.5nM siRNA as described in Section 2.6.1. This concentration was chosen following an initial dose-response experiment based on previous work carried out in the laboratory. Cells were differentiated for 24hr using PMA and then left untreated or treated with TGF-β for 24hr as this was previously shown to be the time point for optimal gene inhibition/induction by the cytokine (Chapter 3). As an antibody to GAPDH was not available in the laboratory, GAPDH expression was analysed by RT-PCR. Total RNA was subjected to RT-PCR to amplify GAPDH and β-2 microglobulin using primers against GAPDH and β-2 microglobulin (Table 2.4). Amplification products were size-fractionated by agarose gel electrophoresis. Samples treated with Smad-2 siRNA were used as a positive control. Figure 4.5 shows that GAPDH mRNA expression was high in cells transfected with a siRNA against Smad-2 and were significantly reduced in cells transfected with GAPDH siRNA.

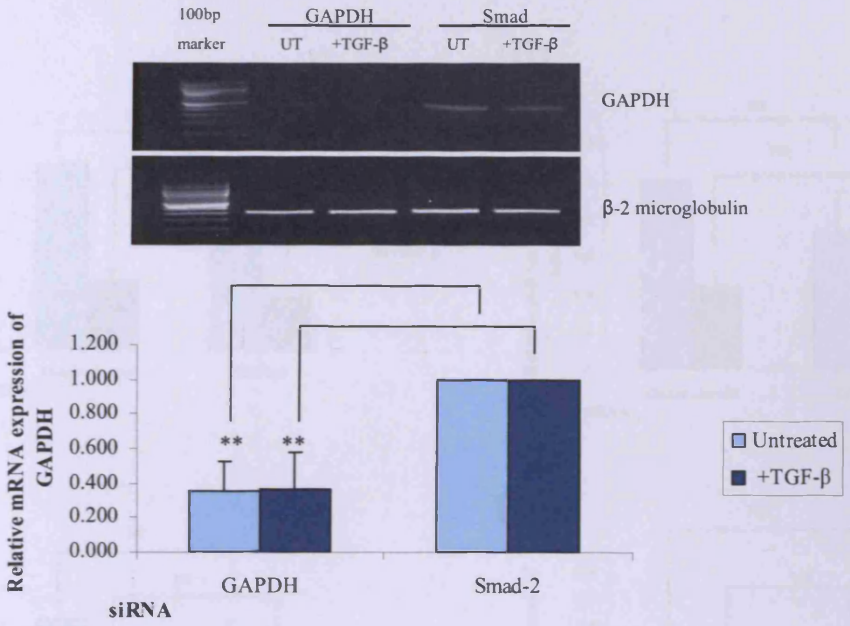

Figure 4.5 GAPDH siRNA-mediated knockdown in THP-1 macrophages. THP-1 cells were transfected with 7.5nM siRNA (Smad-2 or GAPDH) using INTERFERin™ (Section 2.6.1). Total RNA from untreated and TGF-β-treated cells (24hr) was subjected to RT-PCR using specific primers against GAPDH and β-2 microglobulin. Amplification products were resolved by agarose gel electrophoresis (Panel A). Product size was determined by comparison against a standard DNA molecular weight marker (Appendix I). Relative expression (mean± SD) of GAPDH from three independent experiments is shown, with values from Smad-2 transfected cells arbitrarily assigned as 1 (**P<0.01).

The suitability of GAPDH siRNA as a control for these studies was validated by RT-qPCR studies analysing the constitutive and TGF-β-regulated expression of the genes studied in this chapter in GAPDH-transfected cells compared to mock-transfected cells (Figure 4.6). Knockdown of GAPDH had no effect on the constitutive expression of any of the genes studied with the exception of LPL whose constitutive expression was reduced following transfection of GAPDH siRNA. No significant change in the TGF-β-regulated expression of any of the genes was observed and the TGF-β response was conserved following transfection making GAPDH a suitable control for siRNA studies investigating the expression of these genes in the THP-1 macrophage cell line.

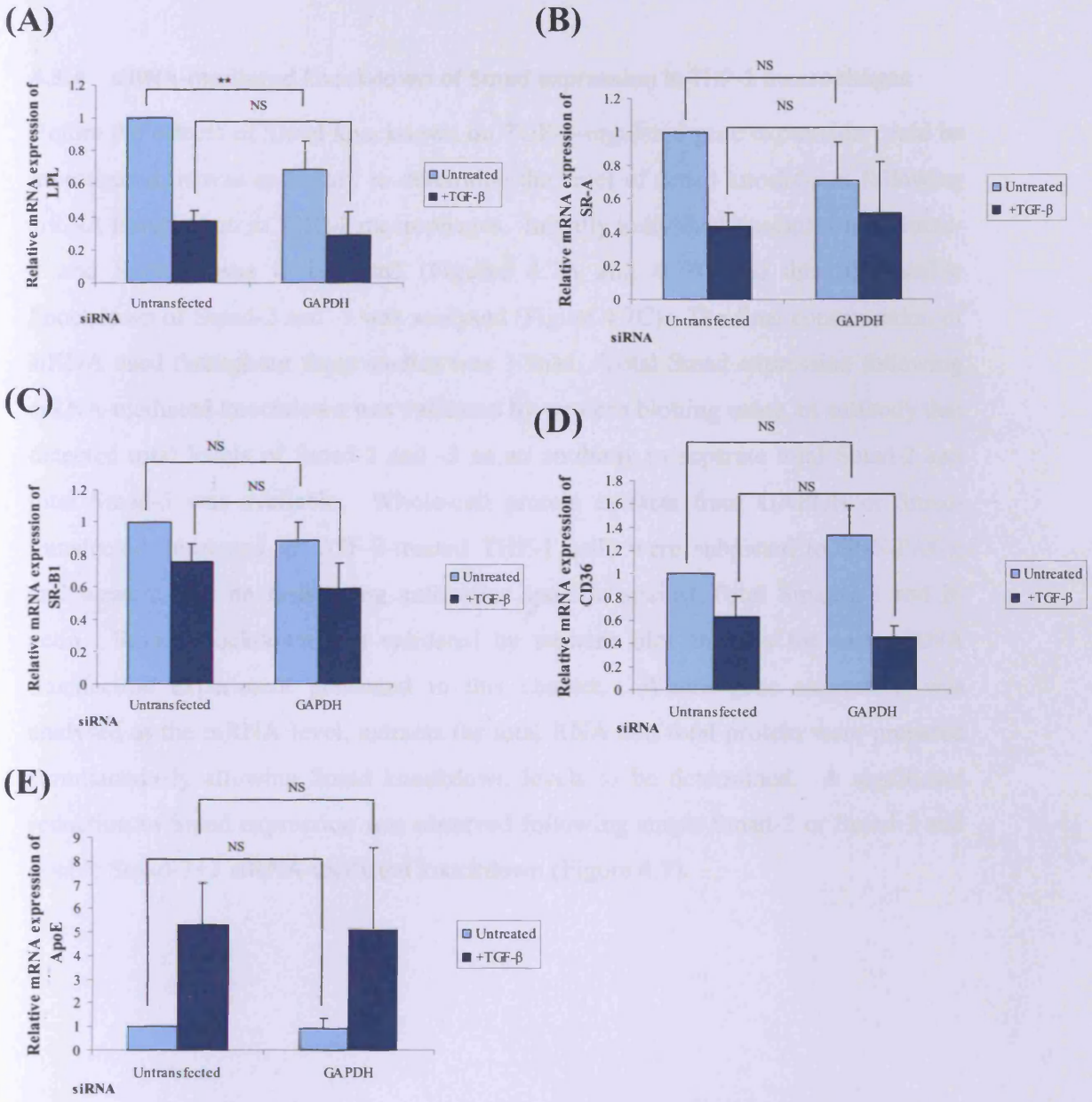

Figure 4.6 **GAPDH siRNA control has no effect on the TGF-β-regulated expression of key genes in THP-1 macrophages.** Total RNA from untransfected or GAPDH siRNA-transfected cells, left untreated or treated with TGF-β, were subjected to RT-qPCR using primers specific to LPL (Panel A), SR-A (Panel B), SR-B1 (Panel C), CD36 (Panel D), ApoE (Panel E) and RPL13A. Relative expression (mean± SD) of each gene, normalised to RPL13A with the values from untransfected and untreated cells arbitrarily assigned as 1, from three independent experiments is shown (**P<0.01, NS-not significant).

4.3.4 siRNA-mediated knockdown of Smad expression in THP-1 macrophages

Before the effects of Smad knockdown on TGF-β-regulated gene expression could be investigated, it was necessary to determine the level of Smad knockdown following siRNA transfection in THP-1 macrophages. Initially individual knockdown of Smad-2 and Smad-3 was investigated (Figures 4.7A and 4.7B) and then the double knockdown of Smad-2 and -3 was analysed (Figure 4.7C). The final concentration of siRNA used throughout these studies was 7.5nM. Total Smad expression following siRNA-mediated knockdown was validated by western blotting using an antibody that detected total levels of Smad-2 and -3 as no antibody to separate total Smad-2 and total Smad-3 was available. Whole-cell protein extracts from GAPDH or Smad-transfected, untreated or TGF-β-treated THP-1 cells were subjected to SDS-PAGE and western blot analysis using antibodies specific against Total Smad-2/3 and β-actin. Smad knockdown was validated by western blot analysis for each siRNA transfection experiment presented in this chapter. Where gene expression was analysed at the mRNA level, extracts for total RNA and total protein were prepared simultaneously allowing Smad knockdown levels to be determined. A significant reduction in Smad expression was observed following single Smad-2 or Smad-3 and double Smad-2+3 siRNA-mediated knockdown (Figure 4.7).

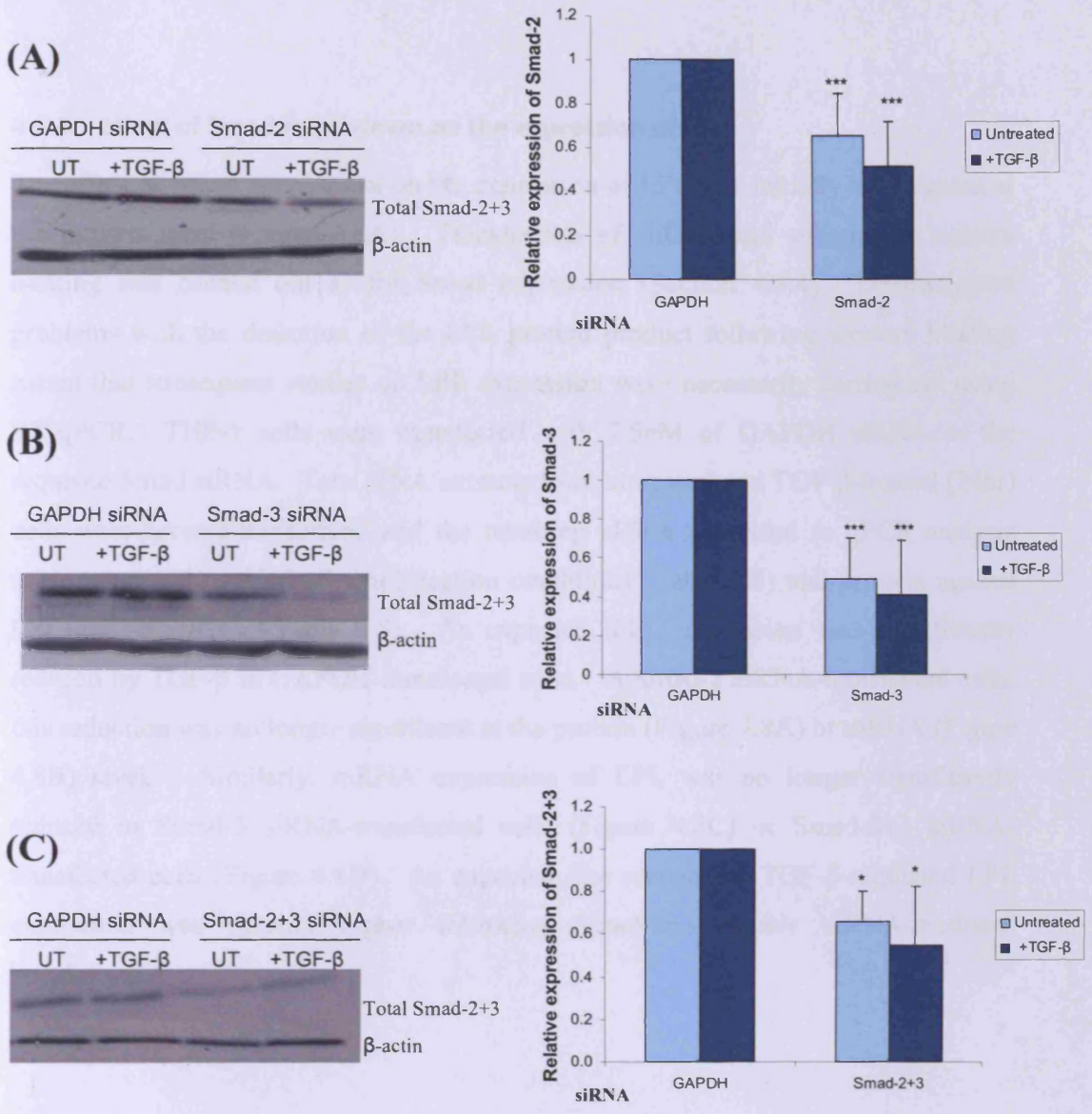

Figure 4.7 **Smad expression following siRNA-mediated knockdown in THP-1 macrophages.** Whole-cell protein extracts were prepared from untreated or TGF-β-treated THP-1 cells transfected with validated GAPDH siRNA or Smad-2 (Panel A), Smad-3 (Panel B) or Smad-2 and Smad-3 (Panel C) siRNA as described in Section 2.6.1. Extracts were subjected to SDS-PAGE and western blotting and levels of total Smad-2/3 were compared against levels of β-actin. Relative expression (mean± SD) of Smad-2/3 normalised to β-actin levels, with values from GAPDH-transfected cells arbitrarily assigned as 1, from four (Panel A) or five (Panel B) or three (Panel C) independent experiments is shown (*P<0.05, ***P<0.001).

4.3.5 Effect of Smad knockdown on the expression of LPL

The effect of Smad knockdown on the expression of LPL was initially investigated at the protein level (Figure 4.8A). Transfection of siRNA and subsequent western blotting was carried out as for Smad expression (Section 4.3.4). Unanticipated problems with the detection of the LPL protein product following western blotting meant that subsequent studies on LPL expression were necessarily carried out using RT-qPCR. THP-1 cells were transfected with 7.5nM of GAPDH siRNA or the requisite Smad siRNA. Total RNA extracts from untreated and TGF-β-treated (24hr) cells were reverse-transcribed and the resulting cDNA subjected to qPCR analysis using previously optimised amplification conditions (Table 2.6) and primers against LPL and 28SrRNA (Table 2.5). As expected, LPL expression was significantly reduced by TGF-β in GAPDH-transfected cells. In Smad-2 siRNA-transfected cells, this reduction was no longer significant at the protein (Figure 4.8A) or mRNA (Figure 4.8B) level. Similarly, mRNA expression of LPL was no longer significantly reduced in Smad-3 siRNA-transfected cells (Figure 4.8C) or Smad-2+3 siRNA-transfected cells (Figure 4.8D). As expected, the reversal of TGF-β-regulated LPL expression was notably higher following Smad-2+3 double siRNA-mediated knockdown.

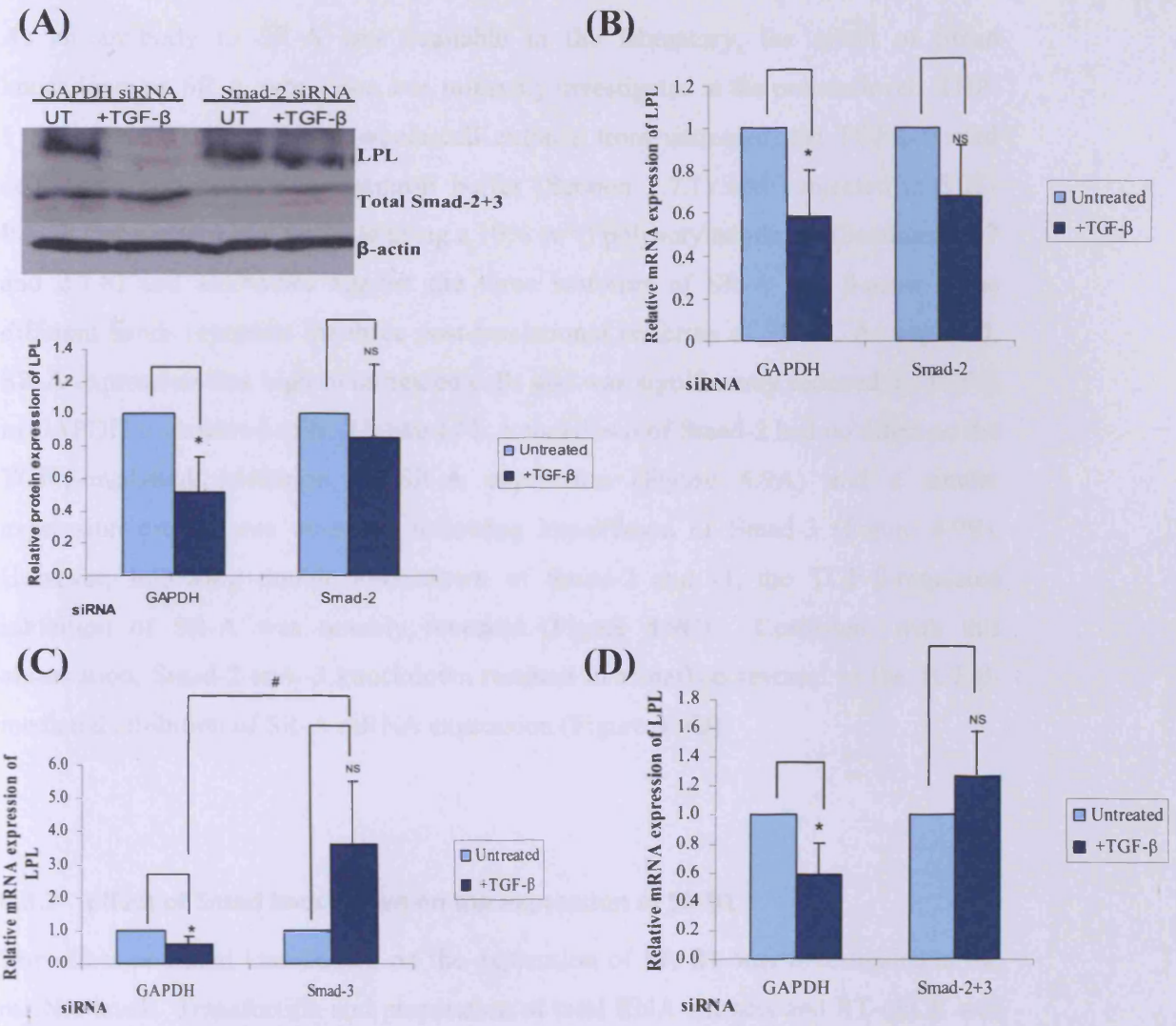

Figure 4.8 **Smad knockdown attenuates the TGF-β-mediated inhibition of LPL expression in THP-1 macrophages.** Whole-cell protein extracts (Panel A) or total RNA extracts (Panels B-D) were prepared from untreated or TGF-β-treated THP-1 macrophages transfected with GAPDH or validated Smad-2 (B), Smad-3 (C) or Smad-2+3 (D) siRNA. Western blotting was carried out using antibodies against LPL and β-actin. RT-qPCR was carried out using primers against LPL and 28SrRNA. Relative expression (mean± SD) of LPL, normalised to β-actin (for western blotting) or 28SrRNA (for RT-qPCR) levels (values from untreated samples arbitrarily assigned as 1), from three independent experiments is shown (*P<0.05, NS-not significant, # significant induction-P<0.05).

4.3.6 Effect of Smad knockdown on the expression of SR-A

As an antibody to SR-A was available in the laboratory, the effect of Smad knockdown on SR-A expression was primarily investigated at the protein level. THP-1 cells were transfected and whole-cell extracts from untreated and TGF-β-treated cells were prepared using Laemmli buffer (Section 2.7.1) and subjected to SDS-PAGE and western blot analysis using a 10% (v/v) polyacrylamide gel (Sections 2.7.7 and 2.7.8) and antibodies against the three isoforms of SR-A and β-actin. The different bands represent the three post-tanslational isoforms of SR-A. As expected, SR-A expression was high in untreated cells and was significantly reduced by TGF-β in GAPDH-transfected cells. Unlike LPL, knockdown of Smad-2 had no effect on the TGF-β-mediated inhibition of SR-A expression (Figure 4.9A) and a similar expression profile was observed following knockdown of Smad-3 (Figure 4.9B). However, following double knockdown of Smad-2 and -3, the TGF-β-regulated inhibition of SR-A was notably reversed (Figure 4.9C). Consistent with this observation, Smad-2 and -3 knockdown resulted in a marked reversal of the TGF-β-mediated inhibition of SR-A mRNA expression (Figure 4.9D).

4.3.7 Effect of Smad knockdown on the expression of SR-B1

The effect of Smad knockdown on the expression of SR-B1 was investigated at the mRNA level. Transfection and preparation of total RNA extracts and RT-qPCR was carried out as for LPL (Section 4.3.5). Expression of SR-B1 was significantly reduced by TGF-β in GAPDH-transfected cells. Following Smad-2 knockdown, the expression of SR-B1 was no longer significantly inhibited by TGF-β (Figure 4.10A). The TGF-β-regulated expression of SR-B1 was similarly attenuated by Smad-3 knockdown (Figure 4.10B) but to a larger extent than Smad-2. Double knockdown of Smad-2 and -3 also resulted in the attenuation of TGF-β-regulated SR-A expression (Figure 4.10C).

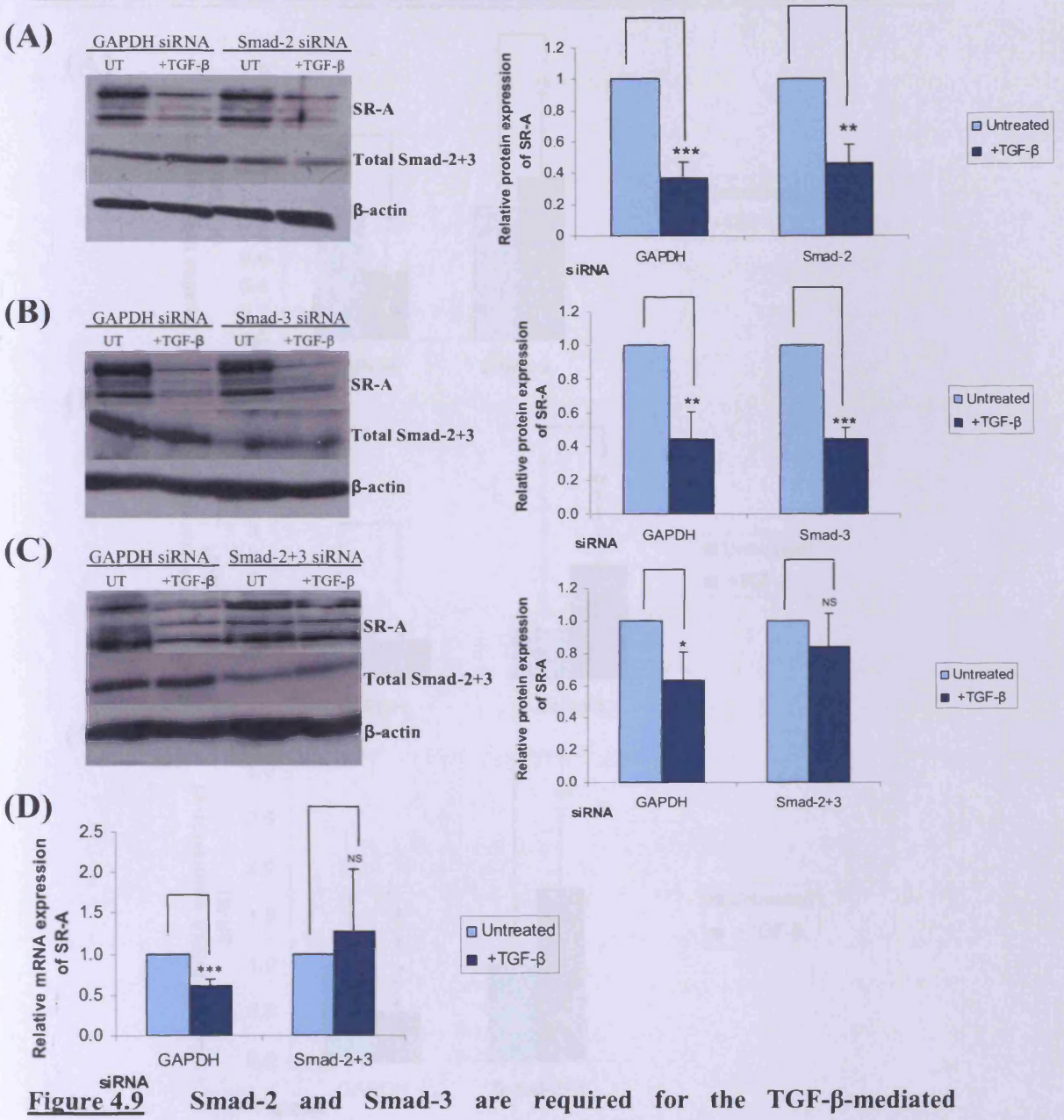

Figure 4.9 **Smad-2 and Smad-3 are required for the TGF-β-mediated inhibition of SR-A expression in THP-1 macrophages**. Whole-cell protein extracts from untreated and TGF-β-treated cells transfected with validated GAPDH or Smad-2 (Panel A), Smad-3 (Panel B) or Smad-2 and -3 (Panel C) siRNAs were subjected to SDS-PAGE and western blotting using antibodies against SR-A (all three isoforms) and β-actin. In panel D, total RNA was reverse-transcribed and subjected to RT-qPCR using primers specific for SR-A and 28SrRNA. Relative expression (mean ±SD) of SR-A normalised to β-actin (or 28SrRNA), with values from untreated samples arbitrarily assigned as 1, from three independent experiments is shown (**P*<0.05, ***P*<0.01, ****P*<0.001, NS-not significant).

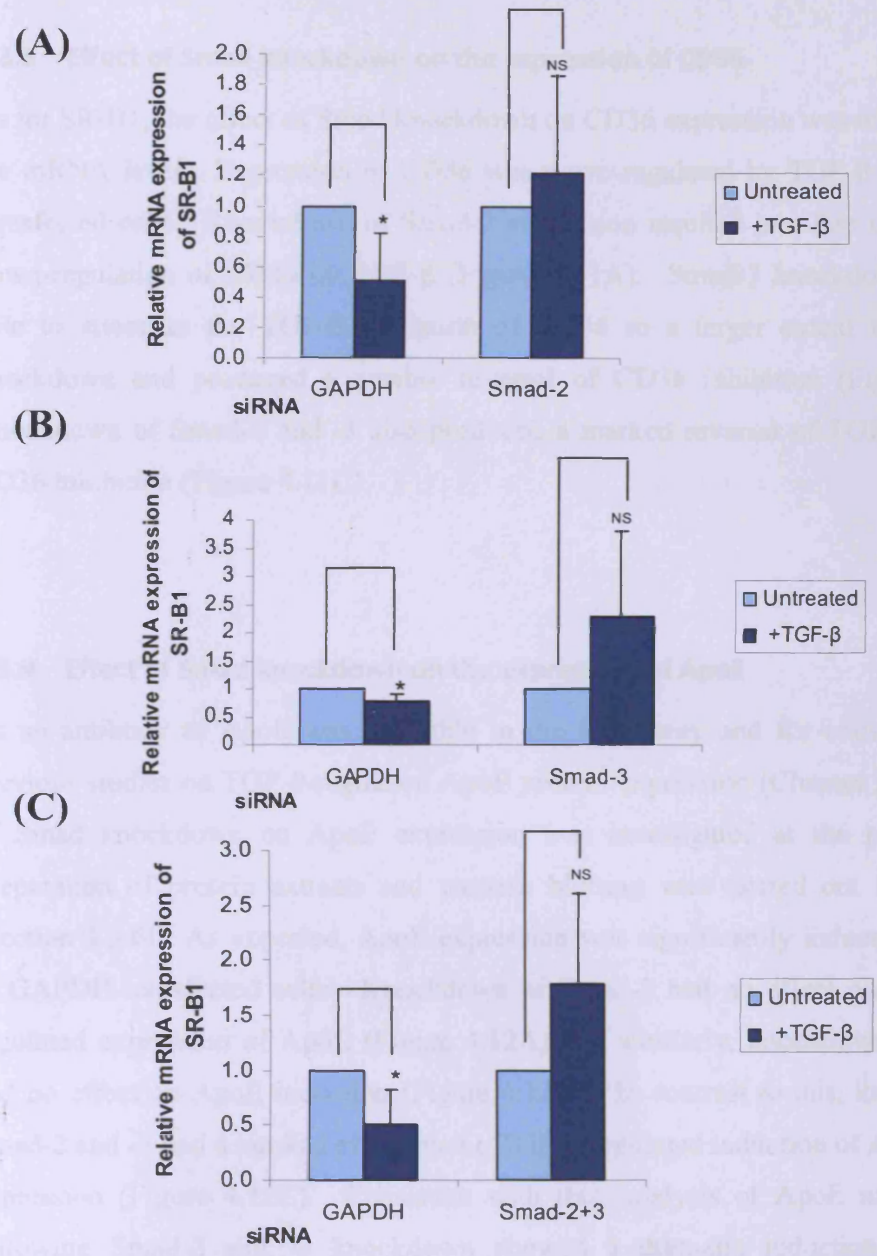

Figure 4.10 **Smad knockdown negates the TGF-β-mediated inhibition of SR-B1 expression in THP-1 macrophages.** Total RNA extracts from untreated and TGF-β-treated cells transfected with GAPDH or Smad-2 (Panel A), Smad-3 (Panel B) or Smad-2 and -3 (Panel C) were subjected to RT-qPCR using primers specific to SR-B1 and 28SrRNA. Relative expression (mean ±SD) of SR-B1 normalised to 28SrRNA from three independent experiments is shown, with values from untreated samples arbitrarily assigned as 1 (*P<0.05, NS-not significant).

4.3.8 Effect of Smad knockdown on the expression of CD36

As for SR-B1, the effect of Smad knockdown on CD36 expression was investigated at the mRNA level. Expression of CD36 was down-regulated by TGF-β in GAPDH-transfected cells. Knockdown of Smad-2 expression resulted in a loss of significant down-regulation of CD36 by TGF-β (Figure 4.11A). Smad-3 knockdown was also able to attenuate the TGF-β-regulation of CD36 to a larger extent than Smad-2 knockdown and produced a notable reversal of CD36 inhibition (Figure 4.11B). Knockdown of Smad-2 and -3 also produced a marked reversal of TGF-β-regulated CD36 inhibition (Figure 4.11C).

4.3.9 Effect of Smad knockdown on the expression of ApoE

As an antibody to ApoE was available in the laboratory and for consistency with previous studies on TGF-β-regulated ApoE protein expression (Chapter 3), the effect of Smad knockdown on ApoE expression was investigated at the protein level. Preparation of protein extracts and western blotting was carried out as for SR-A (Section 4.3.6). As expected, ApoE expression was significantly induced by TGF-β in GAPDH-transfected cells. Knockdown of Smad-2 had no effect on the TGF-β-regulated expression of ApoE (Figure 4.12A) and similarly, knockdown of Smad-3 had no effect on ApoE induction (Figure 4.12B). In contrast to this, knockdown of Smad-2 and -3 had a marked effect on the TGF-β-regulated induction of ApoE protein expression (Figure 4.12C). Consistent with this, analysis of ApoE mRNA levels following Smad-2 and -3 knockdown showed a dramatic reduction in TGF-β-stimulated ApoE induction (Figure 4.12D).

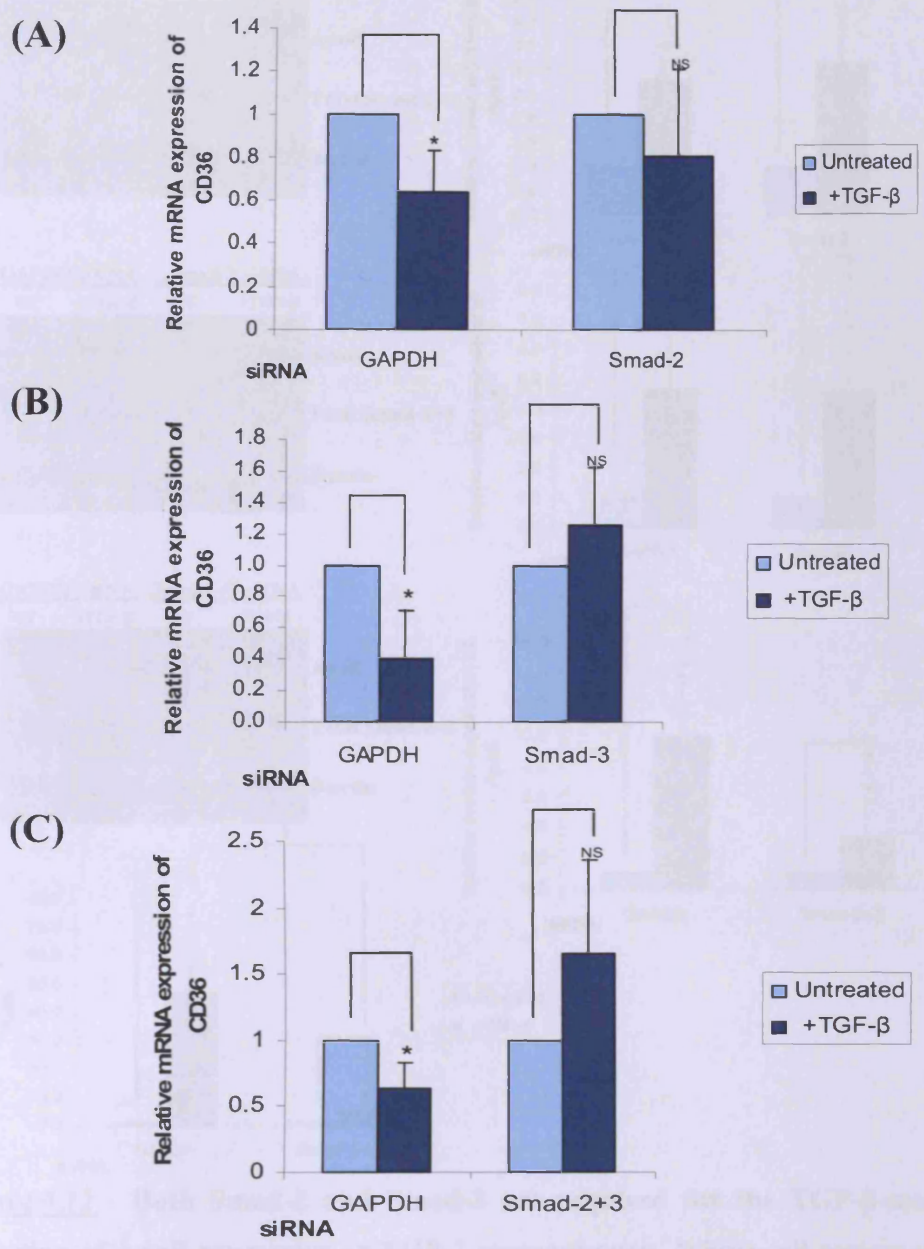

Figure 4.11 **Smad knockdown negates the TGF-β-mediated regulation of CD36 expression in THP-1 macrophages.** RT-qPCR was carried out using primers specific to CD36 and 28SrRNA using total RNA extracts from cells transfected with GAPDH or Smad-2 (Panel A), Smad-3 (Panel B) or Smad-2 and -3 (Panel C) siRNA. Relative expression (mean ±SD) of CD36, normalised to 28SrRNA is shown, with values from untreated samples arbitrarily assigned as 1 (*P<0.05, NS-not significant).

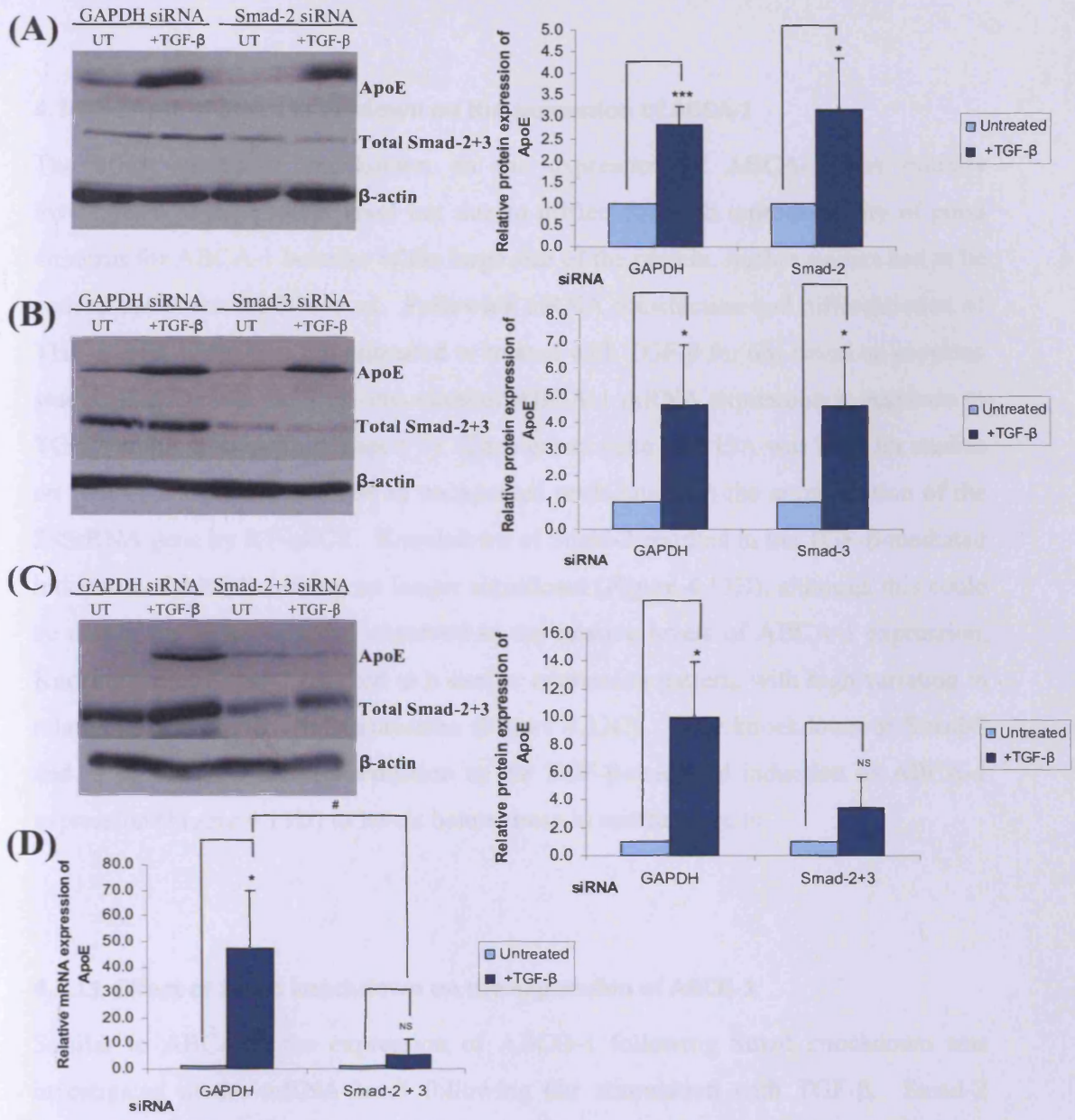

Figure 4.12 Both Smad-2 and Smad-3 are required for the TGF-β-mediated induction of ApoE expression in THP-1 macrophages. Whole-cell protein extracts from untreated and TGF-β-treated cells transfected with validated GAPDH or Smad-2 (Panel A), Smad-3 (Panel B) or Smad-2 and -3 (Panel C) siRNAs were subjected to SDS-PAGE and western blotting using antibodies against ApoE and β-actin. In panel D, total RNA was reverse-transcribed and subjected to RT-qPCR using primers specific for ApoE and 28SrRNA. Relative expression (mean ±SD) of ApoE normalised to β-actin (or 28SrRNA) from three independent experiments is shown, with values from untreated samples arbitrarily assigned as 1 (*P<0.05, ***P<0.001, NS-not significant, #-significant inhibition P<0.05).

4.3.10 Effect of Smad knockdown on the expression of ABCA-1

The effect of Smad knockdown on the expression of ABCA-1 was initially investigated at the protein level but due to difficulties with reproducibility of good westerns for ABCA-1 because of the large size of the protein, further studies had to be carried out at the mRNA level. Following siRNA transfection and differentiation of THP-1 cells, cells were left untreated or treated with TGF-β for 6hr based on previous studies that showed maximal induction of ABCA-1 mRNA expression in response to TGF-β at this time point (Chapter 3). The control gene RPL13A was used for studies on ABCA-1 and ABCG-1 due to unexpected problems with the amplification of the 28SrRNA gene by RT-qPCR. Knockdown of Smad-2 resulted in the TGF-β-mediated induction of ABCA-1 being no longer significant (Figure 4.13B), although this could be due to the large variation observed in the relative levels of ABCA-1 expression. Knockdown of Smad-3 resulted in a similar expression pattern, with high variation in relative levels of ABCA-1 expression (Figure 4.13C). Only knockdown of Smad-2 and -3 produced a marked reduction in the TGF-β-mediated induction of ABCA-1 expression (Figure 4.13D) to levels below those in untreated cells.

4.3.11 Effect of Smad knockdown on the expression of ABCG-1

Similar to ABCA-1, the expression of ABCG-1 following Smad knockdown was investigated at the mRNA level following 6hr stimulation with TGF-β. Smad-2 knockdown produced a notable reduction in the TGF-β-regulated levels of ABCG-1 to levels comparable with those in untreated cells (Figure 4.14A). Smad-3 knockdown resulted in a similar expression pattern (Figure 4.14B). Interestingly, knockdown of Smad-2 and -3 resulted in a reduction of TGF-β-mediated ABCG-1 induction that was similar to that observed following single Smad knockdown.

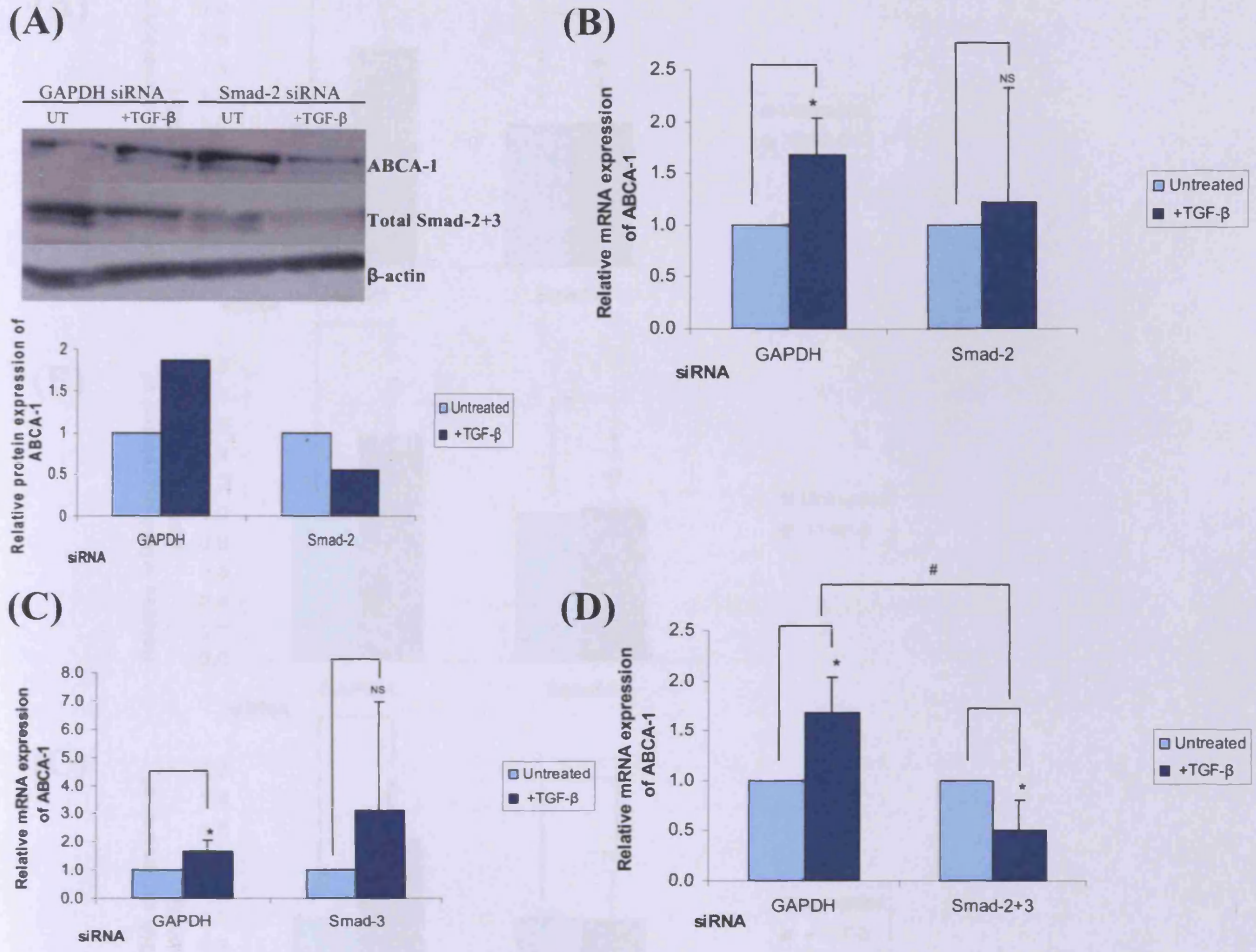

Figure 4.13 Knockdown of Smad-2 and Smad-3 attenuates the TGF-β-mediated induction of ABCA-1 expression in THP-1 macrophages. Total RNA extracts from untreated and TGF-β-treated cells transfected with validated GAPDH or Smad-2 (Panel B), Smad-3 (Panel C) or Smad-2 and -3 (Panel D) siRNAs were subjected RT-qPCR using primers specific to ABCA-1 and RPL13A. Relative expression (mean ±SD) of ABCA-1 normalised to RPL13A, with values from untreated samples arbitrarily assigned as 1, from three independent experiments is shown (*P<0.05, NS-not significant, #-significant inhibition P<0.05). In panel A, whole-cell protein extracts were subjected to SDS-PAGE and western blotting using antibodies against for ABCA-1 and β-actin. Mean relative expression of ABCA-1 normalised to β-actin, with values from untreated samples arbitrarily assigned as 1, from two independent experiments is shown.

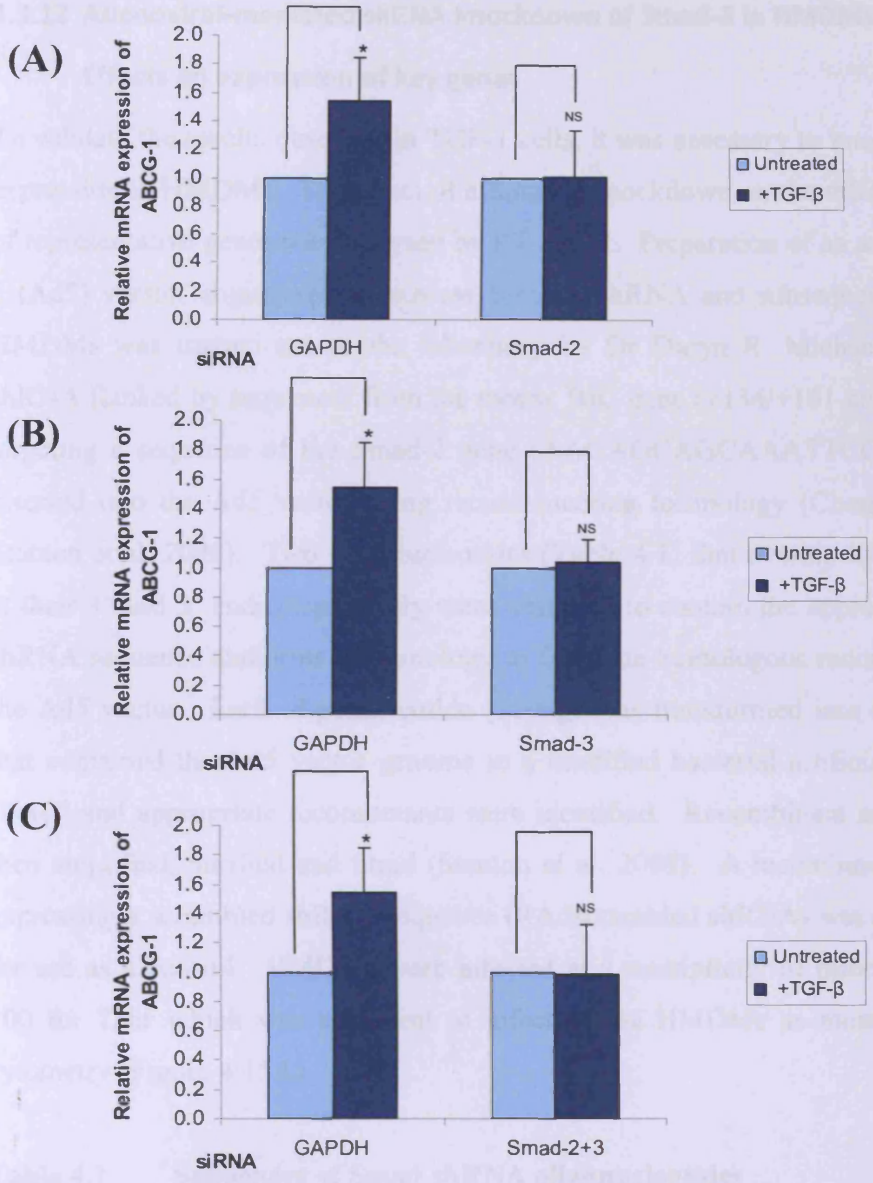

Figure 4.14 Smad knockdown attenuates the TGF-β-mediated induction of ABCG-1 expression in THP-1 macrophages. RT-qPCR was carried out using primers specific to ABCG-1 and RPL13A using total RNA extracts from cells transfected with GAPDH or Smad-2 (Panel A), Smad-3 (Panel B) or Smad-2 and -3 (Panel C) siRNA. Relative expression (mean ±SD) of ABCG-1, normalised to RPL13A with values from untreated samples arbitrarily assigned as 1, is shown (*P<0.05, NS-not significant).

4.3.12 Adenoviral-mediated shRNA knockdown of Smad-2 in HMDMs: Effects on expression of key genes

To validate the results observed in THP-1 cells, it was necessary to knockdown Smad expression in HMDMs. The effect of a Smad-2 knockdown on the mRNA expression of representative genes was analysed by RT-qPCR. Preparation of an adenovirus type 5 (Ad5) vector, engineered to express Smad-2 shRNA and subsequent infection of HMDMs was carried out in the laboratory by Dr Daryn R. Michael. A Smad-2 shRNA flanked by sequences from the mouse BIC gene (+134/+161 and +221/+265), targeting a sequence of the Smad-2 gene (AAGAGCAGCAAATTCCTGGTT) was inserted into the Ad5 vector using recombineering technology (Chung et al. 2006; Stanton et al. 2008). Two oligonucleotides (Table 4.1) that overlap by 25 base pairs at their 3' and 5' ends respectively were designed to contain the appropriate Smad-2 shRNA sequence and arms of homology to facilitate homologous recombination into the Ad5 vector. Each oligonucleotide (100ng) was transformed into SW102 *E-coli* that contained the Ad5 vector genome in a modified bacterial artificial chomosome (BAC) and appropriate recombinants were identified. Recombinant adenovirus was then amplified, purified and titred (Stanton et al. 2008). A recombinant adenovirus expressing a scrambled shRNA sequence (RAdscrambled shRNA) was also generated for use as a control. HMDMs were infected at a multiplicity of infection (MOI) of 100 for 72hr which was sufficient to infect >90% HMDMs as measured by flow cytometry (Figure 4.15A).

Table 4.1 **Sequences of Smad shRNA oligonucleotides**

shRNA oligonuculeotide	Sequence (5'-3')
Smad-2 Forward	CAGCCTGGATCCCTGGAGGCTTGCTGAAGGCTGTATGCTG AAGAGCAGCAAATTCCTGGTTGTTTTGGCCACTGACTGAC AAC
Smad-2 Reverse	TTTGTTCCATGTGAGTGCTAGTAACAGGCCTTGTGTCCTGA AGAGCAGCAATCCTGGTTGTCAGTCAGTGGCCAAAACAAC
Smad-3 Forward	CAGCCTGGATCCCTGGAGGCTTGCTGAAGGCTGTATGCTG TGCAGGTCCAAGTTATTATGTGTTTTGGCCACTGACTGACA CA
Smad-3 Reverse	ACACAAAACCGGTGACTGACTGTGTATTATAACCTGGACG TGTCCTGTGTTCCGGACAATGATCGTGAGTGTACCTTGTTT

Figure 4.15B shows that Smad-2 expression was significantly reduced in untreated and TGF-β treated HMDMs following transfection of RAd-Smad-2 shRNA into HMDMs. RT-qPCR analysis of gene expression (Figure 4.16) demonstrated that knockdown of Smad-2 in HMDMs reversed the TGF-β-mediated inhibition of CD36, consistent with observations in THP-1 macrophages (Section 4.3.8). Knockdown of Smad-2 reduced the TGF-β-mediated induction of ABCA-1, consistent with observations in THP-1 macrophages (Section 4.3.10). Interestingly, the TGF-β-mediated induction of ApoE expression was also reduced following Smad-2 knockdown whereas in THP-1 macrophages, only a double knockdown of Smad-2 and -3 was able to reduce ApoE induction by the cytokine (Section 4.3.9). This could potentially be due to the larger percentage knockdown of Smad-2 in Smad-2 shRNA infected HMDMs compared to the siRNA knockdown of Smad-2 achieved in THP-1 macrophages.

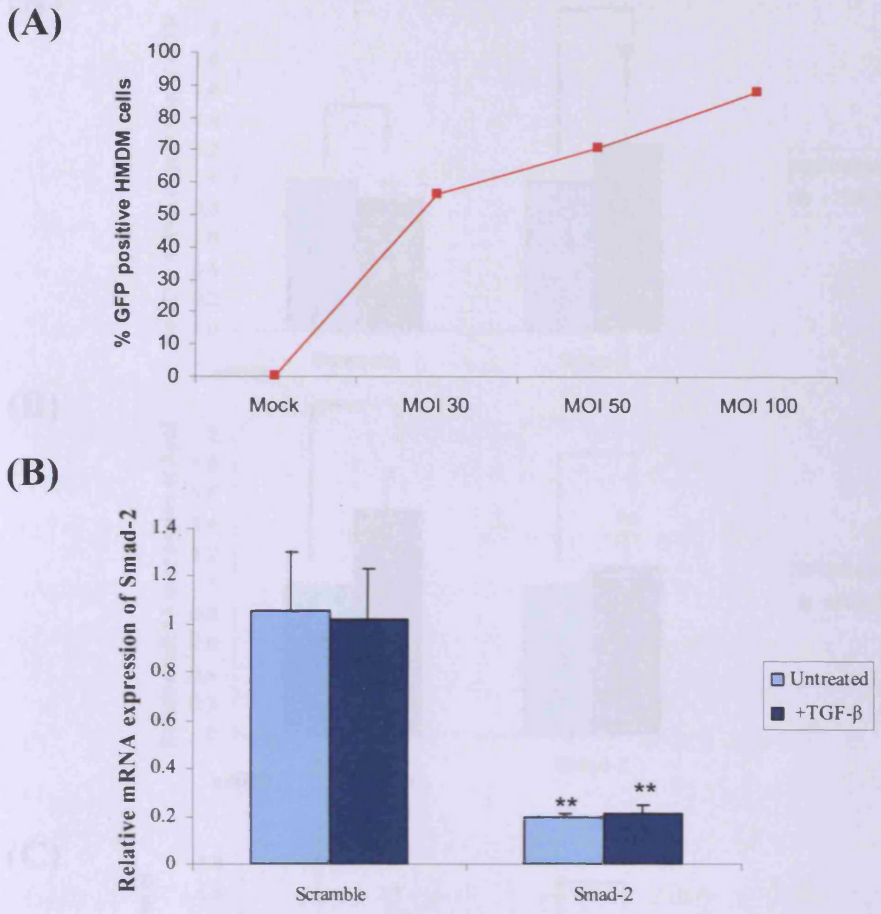

Figure 4.15 **Ad5-GFP infection of HMDMs.** HMDMs were infected with an adenovirus type 5 vector linked to green fluorescent protein (GFP) at a multiplicity of infection of 100 for 72hr. Percentage of GFP-positive cells was measured using flow cytometry (Panel A). In panel B, total RNA extracts from RAd-Scrambled shRNA or RAd-Smad-2 shRNA transfected cells were subjected to RT-qPCR using primers specific to Smad-2 and GAPDH. The relative expression (mean ±SD) of Smad-2 normalised to GAPDH from three experiments is shown, with values from RAd-scrambled shRNA transfected cells arbitrarily assigned as 1 (**P<0.01). This work was carried out by Dr Daryn R. Michael.

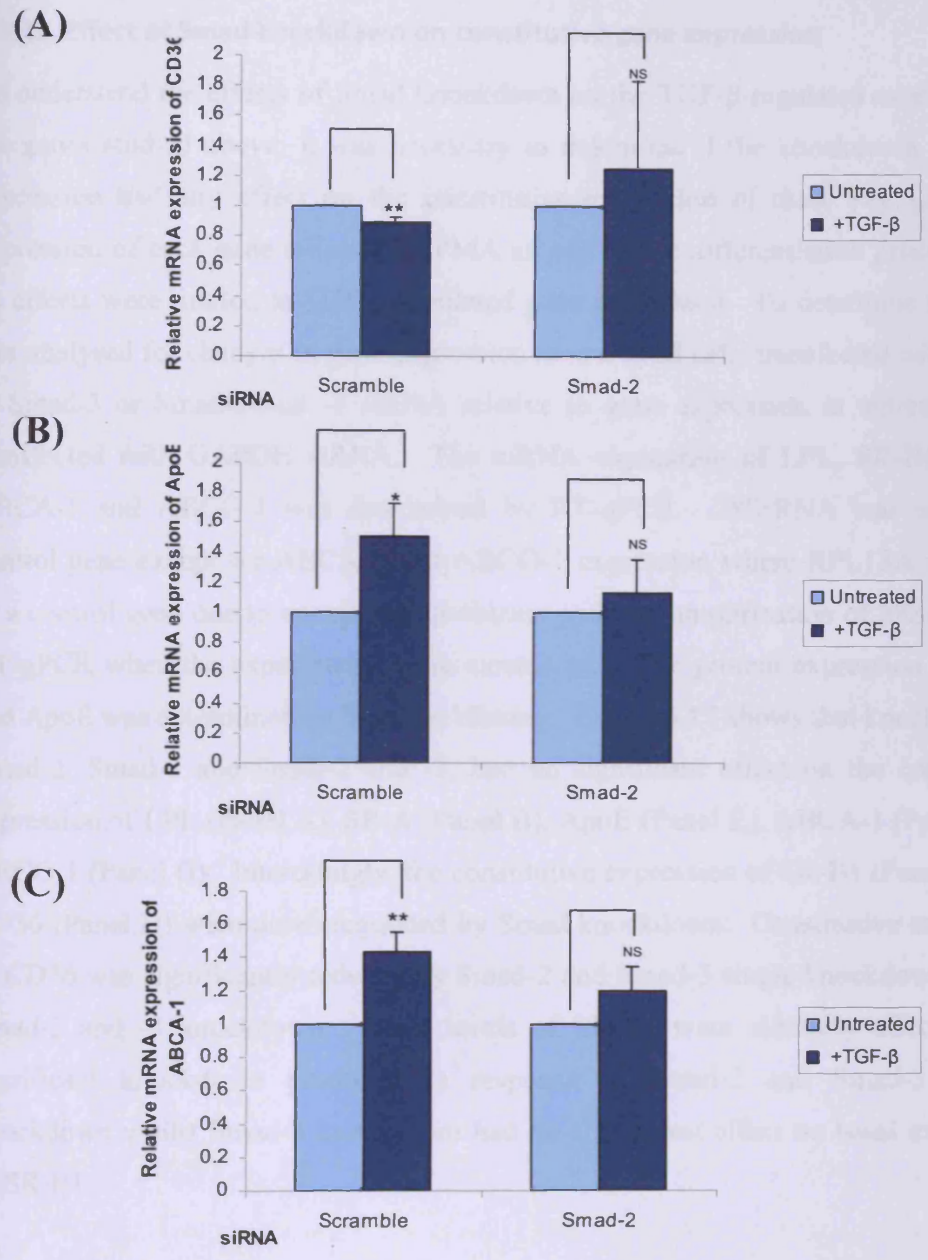

Figure 4.16 The effects of Smad-2 knockdown on TGF-β-regulated gene expression in HMDMs. Total RNA from RAd-Scrambled shRNA or RAd-Smad-2 shRNA transfected cells were subjected to RT-qPCR using primers specific to CD36 (Panel A), ApoE (Panel B), ABCA-1 (Panel C) and GAPDH. The relative expression (mean± SD) of each gene, normalised to GAPDH (mean ±SD) from three experiments is shown, with values from untreated cells arbitrarily assigned as 1 (*P<0.05, **P<0.01, NS-not significant).

4.3.13 Effect of Smad knockdown on constitutive gene expression

To understand the effects of Smad knockdown on the TGF-β-regulated expression of the genes studied above, it was necessary to determine if the knockdown of Smad expression had any effect on the constitutive expression of these key genes (the expression of each gene induced by PMA as part of the differentiation process) or if its effects were limited to TGF-β-regulated gene expression. To determine this, data was analysed for changes in gene expression in untreated cells transfected with Smad-2, Smad-3 or Smad-2 and -3 siRNA relative to gene expression in untreated cells transfected with GAPDH siRNA. The mRNA expression of LPL, SR-B1, CD36, ABCA-1 and ABCG-1 was determined by RT-qPCR. 28SrRNA was used as a control gene except for ABCA-1 and ABCG-1 expression where RPL13A was used as a control gene due to unexpected problems with the amplification of 28SrRNA by RT-qPCR when the experiments were carried out. The protein expression of SR-A and ApoE was determined by Western blotting. Figure 4.17 shows that knockdown of Smad-2, Smad-3 and Smad-2 and -3, had no significant effect on the constitutive expression of LPL (Panel A), SR-A (Panel B), ApoE (Panel E), ABCA-1 (Panel F) or ABCG-1 (Panel G). Interestingly, the constitutive expression of SR-B1 (Panel C) and CD36 (Panel D) were down-regulated by Smad knockdown. Constitutive expression of CD36 was significantly reduced by Smad-2 and Smad-3 single knockdown and by Smad-2 and -3 knockdown. Basal levels of SR-B1 were similarly affected with significant knockdown produced in response to Smad-2 and Smad-2 and -3 knockdown whilst Smad-3 knockdown had no significant effect on basal expression of SR-B1.

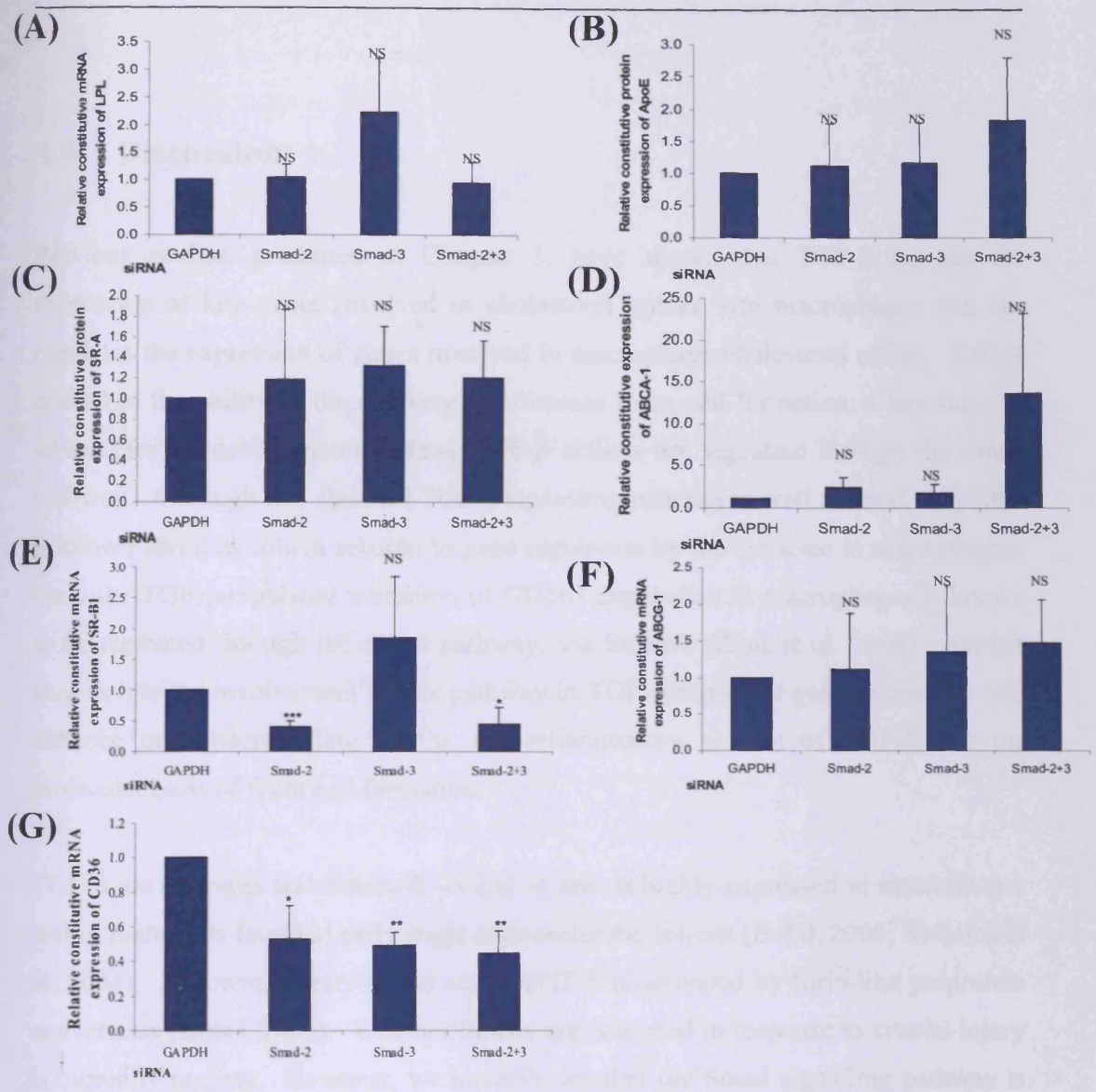

Figure 4.17 **Smad knockdown down-regulates the basal expression of SR-B1 and CD36 but has no effect on the constitutive expression of other genes in THP-1 macrophages.** RT-qPCR was carried out using primers specific to LPL (Panel A), SR-B1 (Panel C), CD36 (Panel D), ABCA-1 (Panel F), ABCG-1 (Panel G) using total RNA from cells transfected with GAPDH or Smad-2, Smad-3 or Smad-2 and -3 siRNA. Relative expression (mean ±SD) of each gene, normalised to 28SrRNA or RPL13A with GAPDH-transfected samples arbitrarily assigned as 1, is shown. For SR-A (Panel B) and ApoE (Panel E) expression, western blotting using antibodies against SR-A, ApoE and β-actin was carried out. Relative expression (mean ±SD) of SR-A or ApoE, normalised to β-actin is shown, with GAPDH-transfected samples arbitrarily assigned as 1 (*P<0.05, **P<0.01, NS-not significant).

4.4 Discussion

Previous studies, presented in Chapter 3, have shown that TGF-β inhibits the expression of key genes involved in cholesterol uptake into macrophages and up-regulates the expression of genes involved in macrophage cholesterol efflux. This is critical to the ability of the cytokine to attenuate foam cell formation, a key stage in atherosclerosis development. Many TGF-β actions are signalled through the Smad pathway. Although this classical TGF-β signalling pathway is well defined, very little is known about its role in relation to gene regulation by the cytokine in macrophages. Only the TGF-β-regulated inhibition of CD163 expression in macrophages is known to be mediated through the Smad pathway, via Smad-3 (Pioli et al. 2004). Further studies into the involvement of this pathway in TGF-β-regulated gene expression will enhance our understanding of the anti-inflammatory actions of TGF-β and the molecular basis of foam cell formation.

TGF-β, its receptors and Smads-2, -3 and -4 are all highly expressed in macrophages and in foam cells found in early stage atherosclerotic lesions (Bobik 2006; Kalinina et al. 2004). Following injury to the artery TGF-β is activated by furin-like proprotein convertases (Bobik 2006). Whether Smads are activated in response to arterial injury is currently unclear. However, we have shown that the Smad signalling pathway is activated by TGF-β in both THP-1 macrophages and HMDMs and that this activation has rapid kinetics (Figure 4.3 and Figure 4.4). This finding correlates well with previous studies which have shown that signalling through the Smad pathway is active in human atherosclerotic lesions (Bot et al. 2009) and can be enhanced in vascular smooth muscle cells (VSMCs) by statins (Rodríguez-Vita et al. 2008).

The role of the Smad pathway in the TGF-β regulation of gene expression was investigated using siRNA knockdown of Smad-2 and Smad-3. Knockdown of Smad expression was determined using western blotting. As antibodies to individual total Smad-2 and total Smad-3 were not available, an antibody that detected total levels of Smad-2 and -3 was used. One disadvantage of an antibody that detects both R-Smads

is that the expression levels of individual Smads following siRNA knockdown can not be determined accurately. It is possible that following a drop in levels of one Smad, levels of the 'other' Smad increase to compensate. However, studies using Smad-2 or Smad-3 null fibroblasts have suggested that this does not occur (Roberts et al. 2003). The use of validated siRNA sequences against each Smad suggests that any residual Smad expression observed following knockdown is due to the expression of the 'other' Smad.

GAPDH siRNA was used as a control for studies on siRNA knockdown. In RNA interference assays, the use of a siRNA against a housekeeping gene or against a random sequence of bases (Scramble siRNA) is used as a control. The use of Scramble siRNA has the obvious advantage of targeting random sequences instead of targeting a sequence coding for a functional molecule, ensuring that no adverse effects resulting from loss of expression are observed. One disadvantage is that due to the random base sequence, no gene is knocked down by a control Scramble siRNA. Although GAPDH siRNA targets a key housekeeping gene in the cell (GAPDH catalyses the breakdown of glyceraldehyde-3-phosphate, as part of glycolysis) GAPDH siRNA has been successfully used in a number of RNA interference studies (Ding et al. 2009; Zhang et al. 2005; Zhao et al. 2008). It also appeared to have no effect on the TGF-β-regulated expression of any of the genes studied in this chapter (Figure 4.6).

The results presented in this chapter demonstrate for the first time that the Smad signalling pathway plays a key role in the expression of key genes implicated in the regulation of foam cell formation by TGF-β. The use of siRNA knockdown assays using siRNA specific against Smad-2 and Smad-3 showed that the Smad pathway was required for the TGF-β-regulated inhibition of LPL, SR-A, SR-B1 and CD36 expression (Figures 4.8 to 4.11) and also for the TGF-β-regulated induction of ApoE, ABCA-1 and ABCG-1 (Figures 4.12 to 4.14) in THP-1 macrophages. Representative experiments in HMDMs (Figure 4.16) suggested that these changes would be conserved in primary macrophages. This is summarised in Figure 4.18. The contribution of each R-Smad to the transcriptional regulation of the genes studied was assessed using single Smad knockdown to take into account the possibility of differential regulation by each of the R-Smads. The effect of a Smad-2 and -3

knockdown was assessed to take into account the possibility of functional redundancy or competition between the R-Smads. As expected, knockdown of Smad-2 and -3 produced markedly more dramatic changes in all genes investigated as compared to single knockdown of either Smad-2 or Smad-3. Our findings suggested crucial roles for Smad-2 and Smad-3 in the regulation of genes implicated in macrophage cholesterol homeostasis suggesting that they may have key roles in foam cell formation.

Microarray analysis of human keratinocytes has shown that there are subsets of genes that depend on regulation by Smad-2, by Smad-3 and by both Smad-2 and -3 (Kretschmer et al. 2003). In our study, TGF-β-stimulated levels of ABCG1 showed similar reductions in response to Smad-2 or Smad-3 knockdown in addition to Smad-2 and -3 knockdown (Figure 4.14) while TGF-β-stimulated levels of ABCA-1 showed similar reductions in response to Smad-2 or Smad-3 knockdown but showed a more marked reduction following Smad-2 and -3 knockdown (Figure 4.13). This could suggest that either both Smads are required for this regulation or that regulation can be mediated by either Smad-2 or Smad-3 to the same effect.

Of notable interest in our study, TGF-β-inhibited levels of LPL, CD36 and SR-B1 showed greater changes in response to Smad-3 knockdown (Figures 4.8 to 4.11) that could suggest that this set of genes is regulated through Smad-3 although Smad-2 may also contribute to this action, particularly in the case of SR-B1. In support of this, the expression of CD36 has previously been shown to be regulated by a reduction in PPAR-γ activity mediated by TGF-β through Smad-3 and the transcription factor AP-1 (Fu et al. 2003).

This observation could suggest that Smad-3 is a key player in the inhibition of cholesterol uptake and therefore in foam cell formation. However, a number of factors could contribute to this effect including the larger knockdown of Smad-3 protein expression following siRNA-mediated transfection (Figure 4.7) and this cannot be ruled out as a contributor to this effect. Compensatory mechanisms between Smad-2 and Smad-3 also exist. The specificity of the siRNA sequences against individual Smad knockdown also needs to be verified before firm conclusions can be drawn on the involvement of individual Smads in gene expression.

One interesting observation is that of the genes studied; only SR-A and ApoE demonstrated a requirement for both Smads in their regulation by TGF-β (Figure 4.9 and Figure 4.12 respectively). Whether this is due to functional redundancy between Smads or a requirement for regulation by both Smad-2 and Smad-3 is unclear. Smad-2 and Smad-3 can be differentially regulated to mediate distinct TGF-β responses, although the relative importance and mechanism of regulation of each remains unclear. Interestingly, it has previously been shown that Smad-2 and Smad-3 null fibroblasts do not display any changes in the expression or phosphorylation of the 'other' Smad suggesting that they do not compete for receptor binding and may be phosphorylated by different receptor pools located in distinct endosomal compartments (Janknecht et al. 1998; Roberts et al. 2003). Association and availability of SARA may also control the phosphorylation ability of Smad-2 versus Smad-3 (Brown et al. 2007). Studies have shown that cellular context can result in differential roles for individual Smads. Interactions with different proteins may also contribute to specific Smad activation. For instance TRAP-1 like protein is able to activate Smad-2 responses whilst blocking the formation of Smad-3/4 complexes to inhibit Smad-3 dependent responses (Brown et al. 2007). In addition to this Smads can be differentially negatively regulated by E3 ubiquitin ligases such as PRAJA which targets Smad-3 and TG-interacting factor (TGIF)-interacting ubiquitin ligase which targets Smad-2 (Ross and Hill 2008).

Although the two regulatory Smad proteins are highly homologous, one of the key differences between the two is that Smad-3 to be able to bind directly to DNA through its MH1 domain whilst Smad-2 requires Smad-4 for DNA binding due to a 30 amino-acid insert in its MH1 domain (Brown et al. 2007). This difference may be important in the regulation of genes involved in macrophage cholesterol homeostasis and foam cell formation. It has previously been demonstrated that Smad-3 can directly activate early TGF-β target genes while Smad-2 regulates early and intermediate target genes through TGF-β and Smad-3 (Brown et al. 2007). The high frequency of Smad binding elements in promoters means that it is often necessary for Smads to interact with other transcription factors to facilitate transcription of target genes, this may also have a role in the differential actions of Smads (Ross and Hill 2008). The MH2 domain present in both Smads is involved with interactions with cofactors and other

transcription factors such as p300/CBP which is required for the transactivation of Smad-3 activated promoters such as PAI-1 (Janknecht et al. 1998). Protein: protein interactions and the regulation of transcription factors such as Sp1 in the case of LPL (Irvine et al. 2005) could mediate some of the effects of the Smads.

Whilst the mechanisms behind TGF-β regulation of gene activation and inhibition in relation to foam cell formation and atherosclerosis are not well characterised, several lines of evidence have suggested that Smad-3 is the key player in gene repression by TGF-β. Smad-3 has been implicated in the repression of class II MHC expression (Dong et al. 2001), the expression of c-myc and human telomerase reverse transcriptase, both implicated in cancer (Frederick et al. 2004; Li and Liu 2007). An involvement of Smad-3 in the repression of adipocyte differentiation and skeletal muscle differentiation has also been identified (Choy and Derynck 2003; Liu et al. 2001). Transfection of macrophages with a Smad-3 expression plasmid can reproduce the inhibitory effect of TGF-β on MMP-12 and iNOS, two markers of macrophage activation and this action requires the MH1 domain of Smad-3 (Werner et al. 2000). Smad-3-deficient macrophages also display altered chemotactic responses (Ashcroft et al. 1999). Targeted deletion of Smad-3 enhances neointimal hyperplasia suggesting a protective role in the vascular response to injury (Yokote et al. 2006). Further studies are needed to elucidate the roles of individual Smads in foam cell formation. However, these studies, together with our findings, suggest that Smads have a role in macrophages consistent with the protective, antiatherogenic action of TGF-β in atherosclerosis.

Interestingly, our study also revealed a role for the Smads in the constitutive expression of SR-B1 and CD36 in THP-1 macrophages (Figure 4.17). Constitutive expression of SR-B1 appeared to be dependent on Smad-2 whilst CD36 expression was reduced by both Smad-2 and Smad-3 knockdown. This suggests that Smad signalling is necessary for the constitutive expression of SR-B1 and CD36 following PMA-induced differentiation in addition to their regulation by TGF-β in THP-1 macrophages. The decrease in SR-B1 levels in response to Smad-2 and not Smad-3 could indicate that constitutive SR-B1 expression in THP-1 cells is dependent on Smad-2.

In conclusion, the studies presented in this chapter have identified a role for the Smad signalling pathway in the control of macrophage cholesterol homeostasis by TGF-β. This is summarised in Figure 4.18. The TGF-β-regulation of expression of genes implicated in macrophage cholesterol homeostasis is defined for key genes (see Chapter 3), but very little is known about the role of signalling pathways in this regulation. The Smad signalling pathway is the classical TGF-β signalling pathway and these studies suggest a critical role for this pathway in the TGF-β-regulation of LPL, SR-A, SR-B1, CD36, ApoE, ABCA-1 and ABCG-1 expression in THP-1 macrophages and primary macrophages. This suggests that the pathway is crucial for the attenuation of foam cell formation by TGF-β and therefore contributes to the anti-atherogenic role of TGF-β in atherosclerosis.

Further work will be needed to extend these findings at a cellular level and to further elucidate the exact molecular mechanisms behind the TGF-β regulation of genes implicated in macrophage cholesterol homeostasis. In particular, studies on the involvement of Smad-4 in the TGF-β-regulation of gene expression will be important to enhance understanding of the exact mechanisms behind the TGF-β-regulation of individual genes. Signalling through the Smad pathway involves the formation of complexes between either Smad-2 and Smad-4 or Smad-3 and Smad-4 to allow translocation of the Smads to the nucleus (Schmierer and Hill 2007). However, some studies have shown that Smad-4 has a variable role in TGF-β signalling (Liu et al. 1997; Sirard et al. 2000). Analysis of the promoter sequence of the genes investigated in this chapter for transcription factor binding sites will also be required to determine if Smad binding sites are present within the promoter regions of these genes.

TGF-β is also able to signal through non-classical pathways such as the MAPK and PI3 kinase pathways. These pathways have been well-characterised and their involvement in a large number of cellular processes and regulation of genes has been suggested by a range of studies. However, the involvement of MAPK pathways in TGF-β-regulated gene expression, in particular in relation to macrophages and foam cell formation has not been described. This is the focus of Chapter 5 of this thesis.

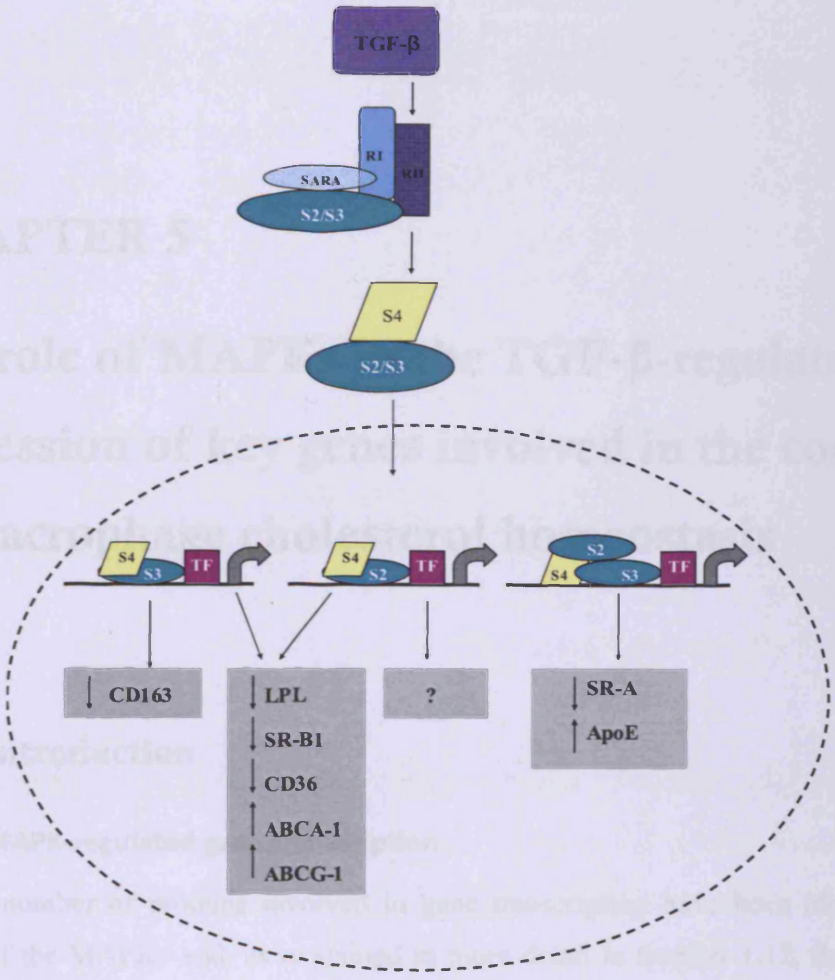

Figure 4.18 The involvement of the Smad signalling pathway in the TGF-β-regulated expression of key genes implicated in macrophage cholesterol homeostasis. Previously, the only macrophage-expressed gene known to be directly regulated through the TGF-β-Smad pathway (via Smad-3) was CD163 whose expression is down-regulated by the cytokine (Pioli et al. 2004). From the results presented in this chapter the TGF-β-regulated expression of LPL, SR-A, SR-B1, CD36, ApoE, ABCA-1 and ABCG-1 can be added to this list. Whilst some genes (SR-A and ApoE) showed a requirement for both Smads in their regulation, others showed similar responses to single Smad-2 and Smad-3 knockdown (ABCA-1 and ABCG-1). Of the genes studied that were implicated in cholesterol efflux, LPL, SR-B1 and CD36 showed greater responses to Smad-3 knockdown compared to Smad-2 (LPL, SR-B1, CD36) although single knockdown of Smad-2 did also effect their expression. The exact contribution of each to their TGF-β-regulated gene expression remains unclear.

CHAPTER 5

The role of MAPKs in the TGF-β-regulated expression of key genes involved in the control of macrophage cholesterol homeostasis

5.1 Introduction

5.1.1 MAPK-regulated gene transcription

A large number of proteins involved in gene transcription have been identified as targets of the MAPKs and, as explained in more detail in Section 1.12, the MAPKs are mediators of transcriptional responses to a number of external stimuli including stress, growth factors, hormones and cytokines. In addition to this, the MAPKs can also modulate the localisation and binding ability of other transcriptional regulators (Qureshi et al. 2005, Whitmarsh 2007). Often, the roles of MAPKs in gene regulation appear cell-type-, gene-, and MAPK-specific. For instance, the induction of iNOS expression by IFN-γ can be augmented by ERK but inhibited by p38 kinase in the murine RAW264.7 macrophage cell line (Chan and Riches 2001).

Activation of MAPKs by TGF-β and roles for these signalling pathways in TGF-β-regulated gene transcription has been demonstrated in a number of cell types. For example, TGF-β activates ERK and p38 kinase in human gingivial fibroblasts and induction of MMP-13 by the cytokine in these cells can be blocked using the p38 kinase specific inhibitor SB203580 or an adenovirally-expressed dominant-negative form of p38 kinase (Ravanti et al. 1999). Similarly, TGF-β-regulated induction of fibronectin mRNA and protein expression in mesangial cells is accompanied by an

increase in ERK activity and can be blocked using the ERK specific inhibitor, PD98059 (Inoki et al. 2000). The use of ERK specific inhibitors PD98059 and UO126 in chondrocytes has also implicated ERK in the TGF-β-regulated induction of mRNA and protein expression of the MMP and aggrecanase inhibitor, TIMP-3 (Qureshi et al. 2005). In VSMCs, TGF-β can induce the activity of p38 kinase and this activation is required for the upregulation of VSMC marker genes such as serum-response factor and the GATA transcription factor (Deaton et al. 2005).

Apoptotic mechanisms downstream of TGF-β have consistently been found to involve MAPKs with a number of studies implicating JNK in this regulation. In both hepatocytes and epithelial cells, an interaction between the TGF-β type II receptor and the pro-apoptotic adaptor protein Daxx has been demonstrated to lead to the activation of JNK signalling and induction of apoptosis (Moustakas and Heldin 2005).

5.1.2 MAPK-regulated gene transcription and inflammation and atherosclerosis

The action of MAPKs on the expression of genes implicated in inflammation appears to show some uniformity. NFκB is a central regulator of genes involved in inflammation and the immune response. A number of studies on the MAPK regulation of NFκB-regulated genes have shown that they augment NFκB-driven gene transcription (Sun et al. 2008, Xiao et al. 2002). For example, the induction of NFκB-mediated cytokine and chemokine gene expression in macrophages by inflammatory mediators including serum amyloid A (SAA) and Substance P has been shown to be augmented by MAPKs. SAA is an acute phase protein produced by the liver in response to pro-inflammatory cytokines and is a biomarker for inflammation with elevated levels in cardiovascular disease and in atherosclerotic plaques. In addition to this, SAA can up-regulate the expression of a number of genes in macrophages including IL-1β, IL-6, IL-8, MCP-1 and MIP-1 and the use of MAPK inhibitors has demonstrated that this induction is mediated through MAPK signalling pathways (Song et al. 2009). Substance P is another inflammatory mediator able to activate the MAPK pathways in macrophages. Substance P is able to enhance antigen presentation and phagocytosis by macrophages but can also induce the release of the chemokines MCP-1 and MIP-2 from macrophages. This is mediated through

activation of the ERK and p38 kinase pathways and can be blocked by inhibitors of these MAPKs (Sun et al. 2008).

In relation to atherosclerosis, roles for the MAPKs in the expression of genes induced by shear stress have been noted in endothelial cells and VSMCs. The expression of IL-8 in endothelial cells is induced under low shear stress and can be blocked by pharmacological inhibitors of ERK, JNK and p38 kinase (Cheng et al. 2008). In addition to this, ERK and p38 kinase have been implicated in the shear-stress induced expression of MMP-9 in endothelial cells (HUVECs) (Sun et al. 2007) and p38 kinase has been implicated in the mechanical stress-induced expression of IL-6 in smooth muscle cells (Zampetaki et al. 2005). Interestingly, the expression of MAPK phosphatase-1 (MKP-1), which negatively regulates ERK and JNK expression through de-phosphorylation and inactivation, is also activated in the arteries in response to shear stress and increases in blood pressure(Li and Xu 2000, Metzler et al. 1999). It is thought that a balance between MAPK activation and MKP-1 induction can modulate cellular responses to external stimuli (Li and Xu 2000, Metzler et al. 1999). Interestingly, LDL can stimulate the expression of MPK-1 in VSMCs which can lead to the inhibition of ERK and Elk-1-mediated transcription in these cells (Li and Xu 2000, Metzler et al. 1999).

A number of roles for MAPKs in monocyte/macrophage gene regulation have been identified. Induction of calveolin-1 expression in response to oxLDL is dependent on the phosphorylation and activation of all three MAPKs (Wu et al. 2009). Calveolin-1 is present in higher amounts in $ApoE^{-/-}$ mice and in oxLDL-treated murine RAW264.7 macrophages (Wu et al. 2009). Induction by oxLDL is associated with increased adherence of monocytes to endothelial cells and subsequent monocyte accumulation in the intima following hypercholesterolemia implicating the MAPKs in this process (Wu et al. 2009). Phosphorylation of p38 kinase can be modulated by angiotensin II in atherosclerotic regions of arteries and is associated with reduced collagen accumulation in $LDLR^{-/-}$ mice (Dandapat et al. 2008). In addition to this, the upregulation of macrophage ACAT-1 expression in response to angiotensin II can be blocked by the ERK inhibitor PD98059 (Kanome et al. 2008). A role for p38 kinase has been identified in the induction of chemokine receptor CXCR2 expression in human monocytes and U937 cells in response to oxLDL (Lei et al. 2002). Similarly,

the induction of phospholipase II by oxLDL is mediated by p38 kinase in THP-1 monocytes (Wang et al. 2009).

In addition to the regulation of target gene expression through MAPK activation, it appears that the expression of some MAPK target genes can activate the MAPKs. For instance, studies by Rahaman and colleagues (2006) have shown that oxLDL-induced activation of JNK in macrophages is dependent on CD36 expression (Rahaman et al. 2006). Co-immunoprecipitation and pull-down assays showed that macrophage CD36 associates with a complex containing the upstream MAPK regulator MEKK2 (Rahaman et al. 2006). In addition to this, oxLDL activated JNK in wildtype but not in CD36 null mice and inhibition of JNK significantly reduced CD36-dependent oxLDL uptake and foam cell formation *in vitro* and *in vivo* (Rahaman et al. 2006). The expression of ERK, JNK and p38 kinase expression can be induced by M-CSF in macrophages and by MCP-1 in endothelial cells (Werle et al. 2002, Zhu et al. 2008).

A role for the MAPKs in cytokine-regulated gene transcription in macrophages has previously been described for IFN-γ. IFN-γ can activate each of the MAPK pathways in macrophages (Baldassare et al. 1999, Valledor et al. 2008). The cytokine has the most potent action on the p38 kinase pathway which interestingly, appears to be involved in IFN-γ-regulated effects on inflammation. Pharmacological inhibitors and knockout models have demonstrated distinct roles for each of the MAPKs in IFN-γ-regulated gene transcription. While p38 kinase appears to have roles in the expression of pro-inflammatory cytokine genes involved in inflammatory responses, such as TNF-α and IL-1β, and chemokine genes involved in monocyte chemotaxis such as CXCL9, CXCL10 and the chemokine receptor CCR2, ERK appears to have more modest effects on the expression of pro-inflammatory genes while JNK is implicated in the IFN-γ-mediated regulation of genes involved in antigen presentation such as the transctivator CIITA (Baldassare et al. 1999, Valledor et al. 2008).

In contrast to IFN-γ, very little is currently known about the activation and role of the MAPKs in TGF-β-regulated effects in macrophages. One action of the cytokine that is known to be regulated through MAPK pathway activation is the inhibitory action of TGF-β on the production of pro-inflammatory cytokines by macrophages. This is crucial to producing an anti-inflammatory phenotype following phagocytosis of

apoptotic cells by macrophages (Xiao et al. 2002). This inhibition of pro-inflammatory cytokine production is mediated by ERK and p38 kinase pathways where the activation of ERK by TGF-β up-regulates MKP-1, resulting in the inactivation of p38 kinase and subsequent reduction in NFκB-driven transcription of pro-inflammatory cytokine genes. This is an example of crosstalk between different MAPK signalling pathways (Xiao et al. 2002).

5.2 Aims of experimental studies

The primary aim of the studies presented in this chapter was to investigate the role of the MAPK pathways in the TGF-β-regulated expression of key genes implicated in macrophage cholesterol homeostasis. The TGF-β-regulated expression of these genes (LPL, SR-A, SR-B1, CD36, ApoE, ABCA-1 and ABCG-1) has been characterised by previous studies, as described in Chapter 3 of this thesis. As the classical mediator of TGF-β responses, activation and role of the Smad pathway in TGF-β-mediated gene expression has also been investigated, as presented in Chapter 4 of this thesis. In addition to this however, the cytokine is known to activate other signalling cascades to regulate target genes. These include the three primary MAPK pathways, ERK, JNK and p38 kinase. The activation of these kinases in macrophages has not been investigated in detail so the initial aim of the studies presented in this chapter was to determine if each of the MAPK pathways were activated by TGF-β in THP-1 and primary macrophages and to investigate the kinetics of this activation. This was carried out using western blot analysis of the phosphorylated forms of ERK, JNK and p38 kinase. It was also important to determine if any increase in MAPK phosphorylation led to a functional increase in activity of these kinases-this was investigated using non-radioactive kinase activity assays. Figure 5.1 outlines the experimental strategy used during these studies.

Following this, the involvement of each of the MAPKs in TGF-β-regulated gene expression was investigated using siRNA-mediated knockdown as described in Section 4.1.2. The majority of studies on MAPK involvement in gene expression have relied on pharmacological inhibitors against the MAPKs or transfection of dominant-negative forms of upstream regulators. Although specific inhibitors to each

MAPK have been widely and successfully used in a large number of studies (Section 5.1.1) including studies on the roles of MAPKs in gene expression, there are a number of disadvantages of using pharmacological inhibitors in cell lines, not least with balancing specificity and concentration with unwanted off-target and possible toxic effects. For instance, the p38 kinase specific inhibitor PD169316 has been demonstrated to inhibit TGF-β signalling in ovarian cells through a reduction in phosphorylation of Smad-2 and -3 (Fu et al. 2003, Moustakas and Heldin 2005), whilst the p38 kinase inhibitor SB203580 activates the ERK and JNK pathways in human hepatoma cells (Henklova et al. 2008). Although these effects are highly likely to be cell-type specific, they do highlight the disadvantages of using pharmacological inhibitors. In contrast, a relatively small number of studies have used siRNA against MAPKs to investigate the role of these signalling pathways in gene expression. These studies are outlined in Table 5.1.

Data from siRNA knockdown studies in THP-1 cells was analysed for effects on basal and TGF-β regulated expression. Mostly, the effects on mRNA expression were analysed using RT-qPCR and initially, where antibodies to the protein were available, effects on protein expression were analysed with further studies carried out at the mRNA level. For ease of reading, studies are presented on a gene by gene basis.

Following on from this, and as previous work in the laboratory had implicated the JNK pathway in the TGF-β-mediated expression of LPL, further studies into the mechanism of its regulation by TGF-β were carried out. Previous work in U937 cells had shown an involvement for SEK-1, JNK and c-Jun in TGF-β-mediated LPL promoter activity (Section 5.3.15). Transient transfection assays were employed to assess the TGF-β-regulation of LPL promoter activity and the effects of dominant-negative forms of key components of the JNK pathway (SEK-1, JNK and c-Jun) on this regulation. Preliminary data obtained in the laboratory using transient transfection had also suggested a role for the SRC-1 coactivator in LPL expression (Section 5.3.16). The effect of SRC-1 siRNA on LPL expression was investigated in THP-1 macrophages to see if this would support previous data.

Table 5.1 **Recent studies using siRNA against MAPKs**

siRNA	Details of study	Reference
JNK-1 ERK-2	Resveratrol acts through JNK and ERK pathways to enhance the expression of perforin in natural killer cells.	Lu and Chen 2010
ERK-1/2 JNK-1	Basic fibroblast growth factor stimulates human dermal fibroblast proliferation through the ERK and JNK pathways.	Makino et al. 2009
ERK-2	The ERK pathway is involved in the formation of joint adhesion *in vivo* through increased collagen expression and fibroblast proliferation in response to TGF-β.	Li et al. 2009
JNK	In cultured endothelial cells, expression of kallistatin is negatively regulated by oxidative stress activated JNK pathway.	Shen et al. 2010
JNK	Prostaglandin-E2 inhibits the IL-1β induction of MMP-1 and MMP-13 by suppressing the JNK pathway in human articular chondrocytes.	Nishitani et al. 2009
JNK-1/2	The JNK pathway has a role in complement-mediated cell death.	Gancz et al. 2009
JNK-1/2	The TGF-β-regulated induction of connective tissue growth factor expression in human cornea stroma fibroblast cells is mediated by upregulation of the JNK pathway and can be inhibited by siRNA against JNK-1/2.	Chang and Wu 2009
p38 kinase	p38 kinase regulates pigmentation in B16 melanoma cells by proteosomal degradation of tyrosinase proteins.	Bellei et al. 2010
p38α kinase	Lysophosphatidic acid induction of IL-6 secretion from human aortic smooth muscle cells is p38α kinase dependent.	Hao et al. 2009
p38α kinase	siRNA against p38α kinase suppresses the proliferation of ER-negative breast cancer cells.	Chen et al. 2009
c-Jun	Thrombin induction of endothelial arginase-I involves the activation of the JNK and p38 kinase pathways which result in the activation of c-Jun (AP-1) transcription factors.	Zhu et al. 2009

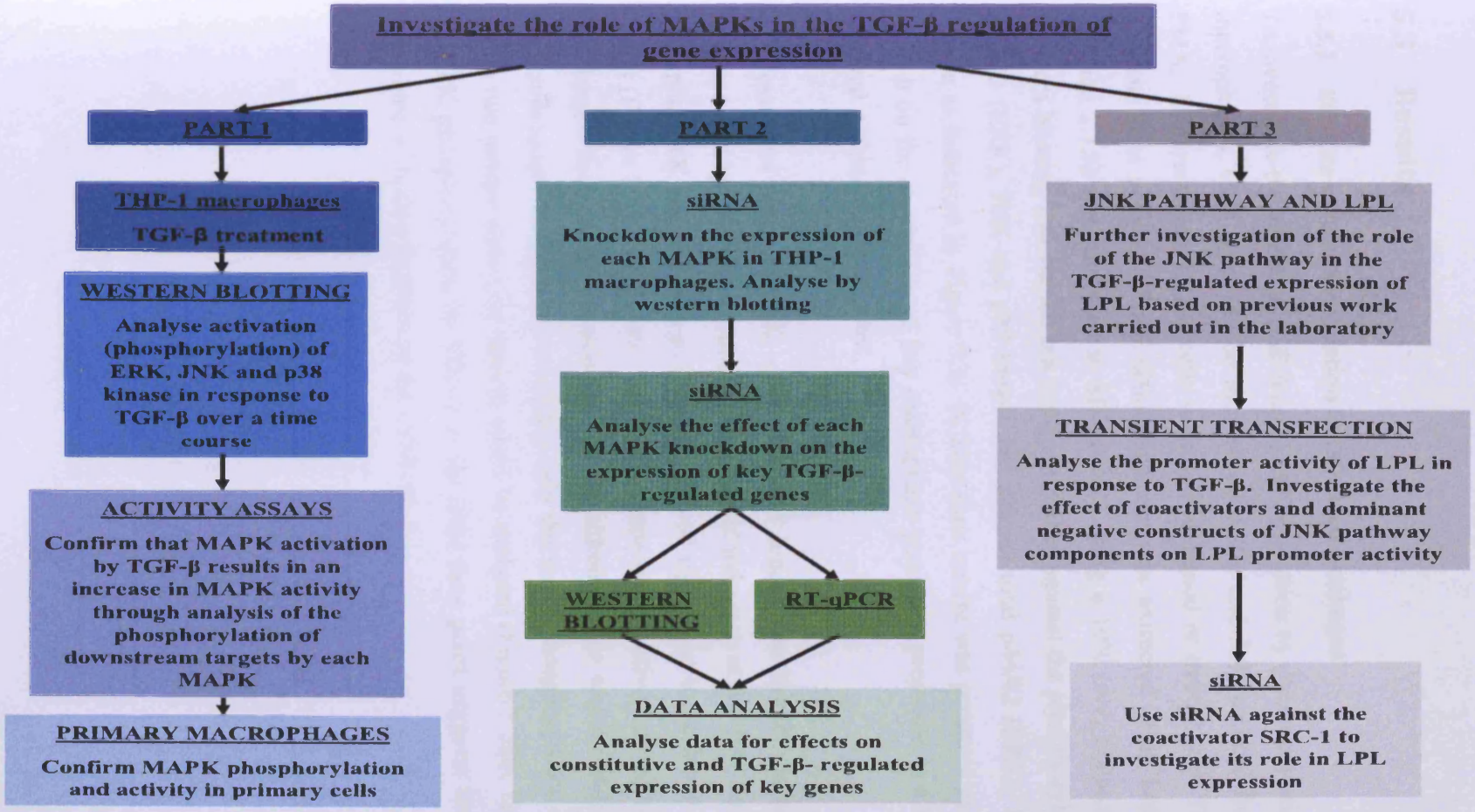

Figure 5.1 Experimental Strategy Diagram

5.3 Results

5.3.1 Kinetics of MAPK activation in THP-1 macrophages

To investigate the effect of TGF-β on MAPK activation by phosphorylation in THP-1 macrophages, cells were seeded into 6-well plates and differentiated for 24hr using PMA. Differentiated THP-1 cells were left untreated or treated with TGF-β for the requisite time periods. Total cellular protein was extracted using Laemmli buffer (Section 2.7.1) and subjected to SDS-PAGE using a 10% (v/v) polyacrylamide gel. Western blotting was carried out using antibodies against the phosphorylated forms of p44/42 (ERK), JNK and p38 kinase and against total p44/42 (ERK), JNK and p38 kinase as indicated in Figure 5.2. A 24hr time course was prepared as the action of TGF-β on the expression of key macrophage genes (as presented in Chapter 3) was optimal within this time frame.

Expression of phospho-ERK, -JNK and -p38 kinase was low in untreated cells and was induced by TGF-β treatment with maximal induction at the 24hr time point. Both phospho-ERK and phospho-p38 kinase showed significant induction at the 24hr time point (Figure 5.2A and Figure 5.2C). In comparison, levels of phospho-JNK showed the largest increase in response to TGF-β although, due to unanticipated problems withsubsequent western blot analysis, only the mean phosphorylation levels of JNK from two independent experiments could be analysed (Figure 5.2B). The induction of MAPK phosphorylation by TGF-β at the 24hr time point suggests that MAPKs are activated with slow kinetics by the cytokine.

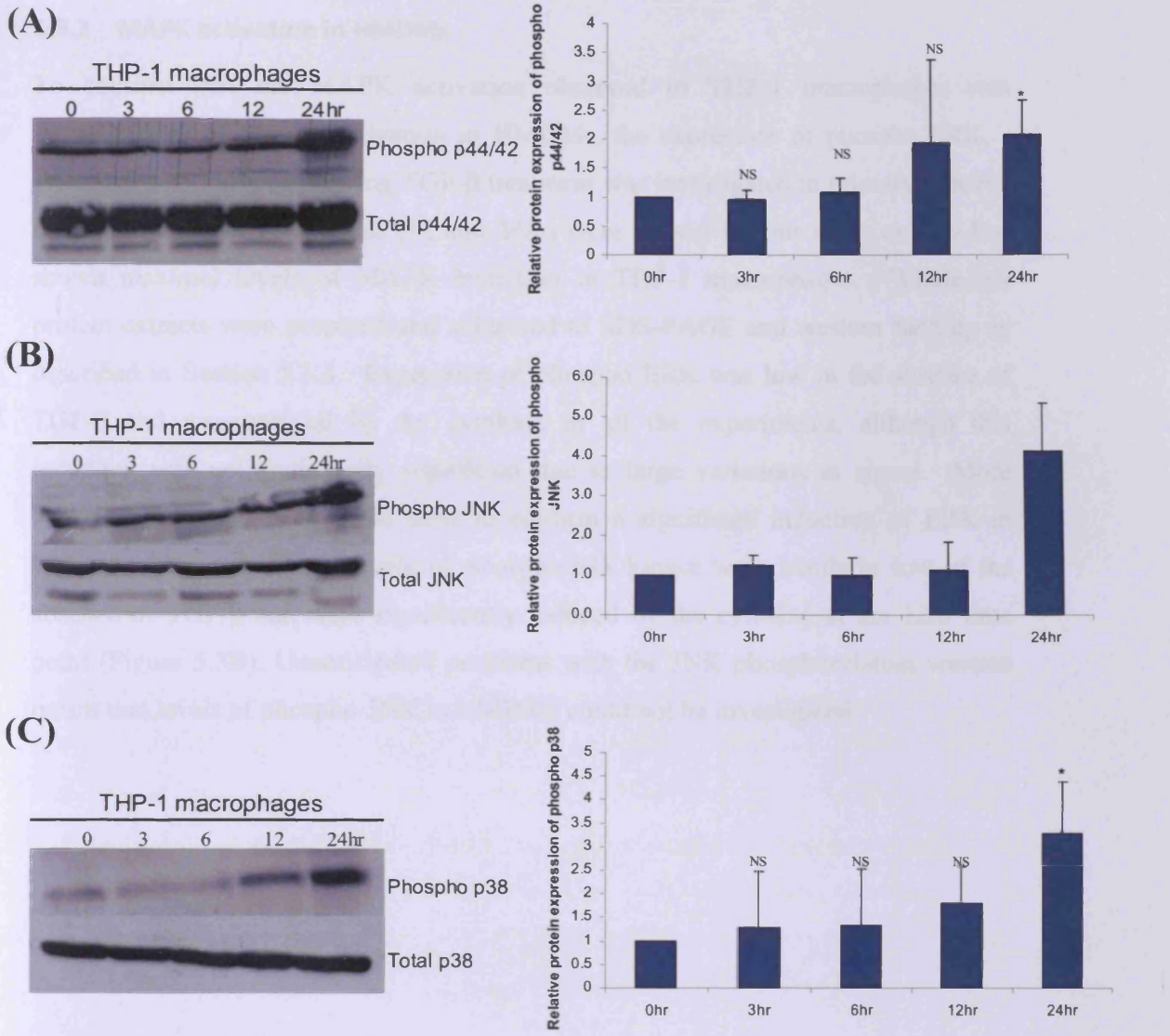

Figure 5.2 **TGF-β activates ERK, JNK and p38 kinase with slow kinetics in THP-1 macrophages.** THP-1 macrophages were left untreated (0hr) or treated with TGF-β for 3, 6, 12 or 24hr as indicated. Whole-cell protein extracts were subjected to SDS-PAGE and western blotting and levels of phospho-ERK (Panel A), -JNK (Panel B) and -p38 kinase (Panel C) were compared to total levels of these proteins. Protein size was determined by comparison against a standard molecular weight marker (Appendix I). The relative expression (mean± SD) of phospho-ERK, -JNK and -p38 kinase (untreated samples (0hr) arbitrarily assigned as 1), normalised to total ERK, JNK or p38 kinase expression respectively, from three independent experiments is shown (*P<0.05, NS-not significant).

5.3.2 MAPK activation in HMDMs

To confirm that the MAPK activation observed in THP-1 macrophages was representative of MAPK activation in HMDMs, the expression of phospho-ERK, -JNK and -p38 kinase following TGF-β treatment was investigated in primary cultures of HMDMs. Two time points (12 and 24hr) were chosen for this study as they had shown maximal levels of MAPK induction in THP-1 macrophages. Whole-cell protein extracts were prepared and subjected to SDS-PAGE and western blotting as described in Section 5.3.1. Expression of phospho-ERK was low in the absence of TGF-β and was induced by the cytokine in all the experiments, although this induction was not statistically significant due to large variations in signal. More experiments would need to be done to confirm a significant induction of ERK in HMDMs (Figure 5.3A). Levels of phospho-p38 kinase were similarly low in the absence of TGF-β and were significantly induced by the cytokine at the 12hr time point (Figure 5.3B). Unanticipated problems with the JNK phosphorylation western meant that levels of phospho-JNK in HMDMs could not be investigated.

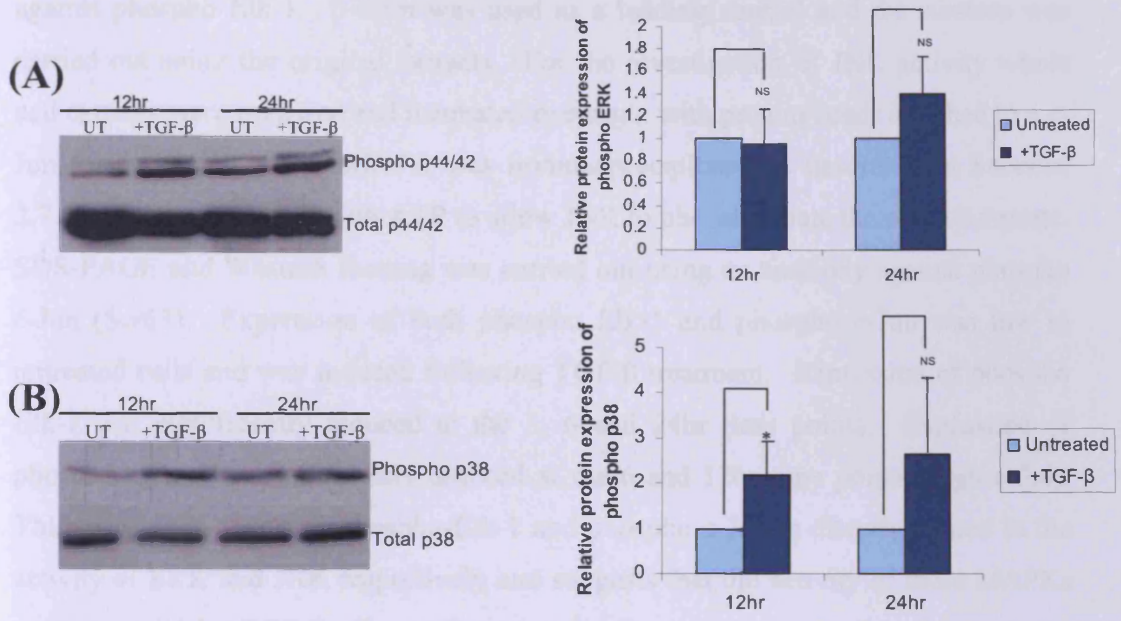

Figure 5.3 TGF-β augments ERK and p38 kinase phosphorylation in HMDMs. HMDMs were left untreated or treated with TGF-β for 12 or 24hr. Whole-cell protein extracts were subjected to SDS-PAGE and western blotting using antibodies against phospho-ERK (Panel A) or p38 kinase (Panel B) and total ERK or total p38 kinase. Relative expression (mean± SD) of phospho-ERK or phospho-p38 kinase, normalised to total levels of ERK or p38 kinase respectively (untreated samples arbitrarily assigned as 1) from three independent experiments is shown (*P<0.05, NS-not significant).

5.3.3 Assay of MAPK activity in THP-1 macrophages

To confirm that the induction of MAPK phosphorylation was indicative of an increase in MAPK activity, the kinase activity of ERK and JNK was investigated. The activity of p38 kinase could not be investigated as no activity assay kit was available in the laboratory. THP-1 macrophages were left untreated or stimulated with TGF-β for the requisite time periods. For the investigation of ERK activity whole cell extracts were prepared and incubated overnight with protein beads attached to an immobilised antibody against p44/42. The MAPK was immunoprecipitated as described in Section 2.7.6 and incubated with ATP and an Elk-1 substrate. SDS-PAGE and western

blotting was carried out using the immunoprecipitated extracts and an antibody against phospho Elk-1. β-actin was used as a loading control and the western was carried out using the original extracts. For the investigation of JNK activity whole cell extracts were prepared and incubated overnight with protein beads attached to a c-Jun fusion protein. The MAPK was immunoprecipitated as described in Sections 2.7.5 and then incubated with ATP to allow JNK to phosphorylate the c-Jun substrate. SDS-PAGE and Western blotting was carried out using an antibody against phospho c-Jun (Ser63). Expression of both phospho Elk-1 and phospho c-Jun was low in untreated cells and was induced following TGF-β treatment. Expression of phospho Elk-1 was significantly induced at the 3, 6 and 24hr time points. Expression of phospho c-Jun was significantly induced at the 6 and 12hr time points (Figure 5.4). This increase in levels of phospho-Elk-1 and phospho-c-Jun is directly related to the activity of ERK and JNK respectively and suggests that the activity of these MAPKs was increased by TGF-β. To confirm that the increase in expression observed was due to increased kinase activity in response to TGF-β and not to the status of the cells, a single time point was chosen and untreated and TGF-β-treated extracts from a single experiment were subjected to the same assay. An increase in phospho-Elk-1 and phospho-c-Jun expression indicated that this increase was solely due to TGF-β treatment.

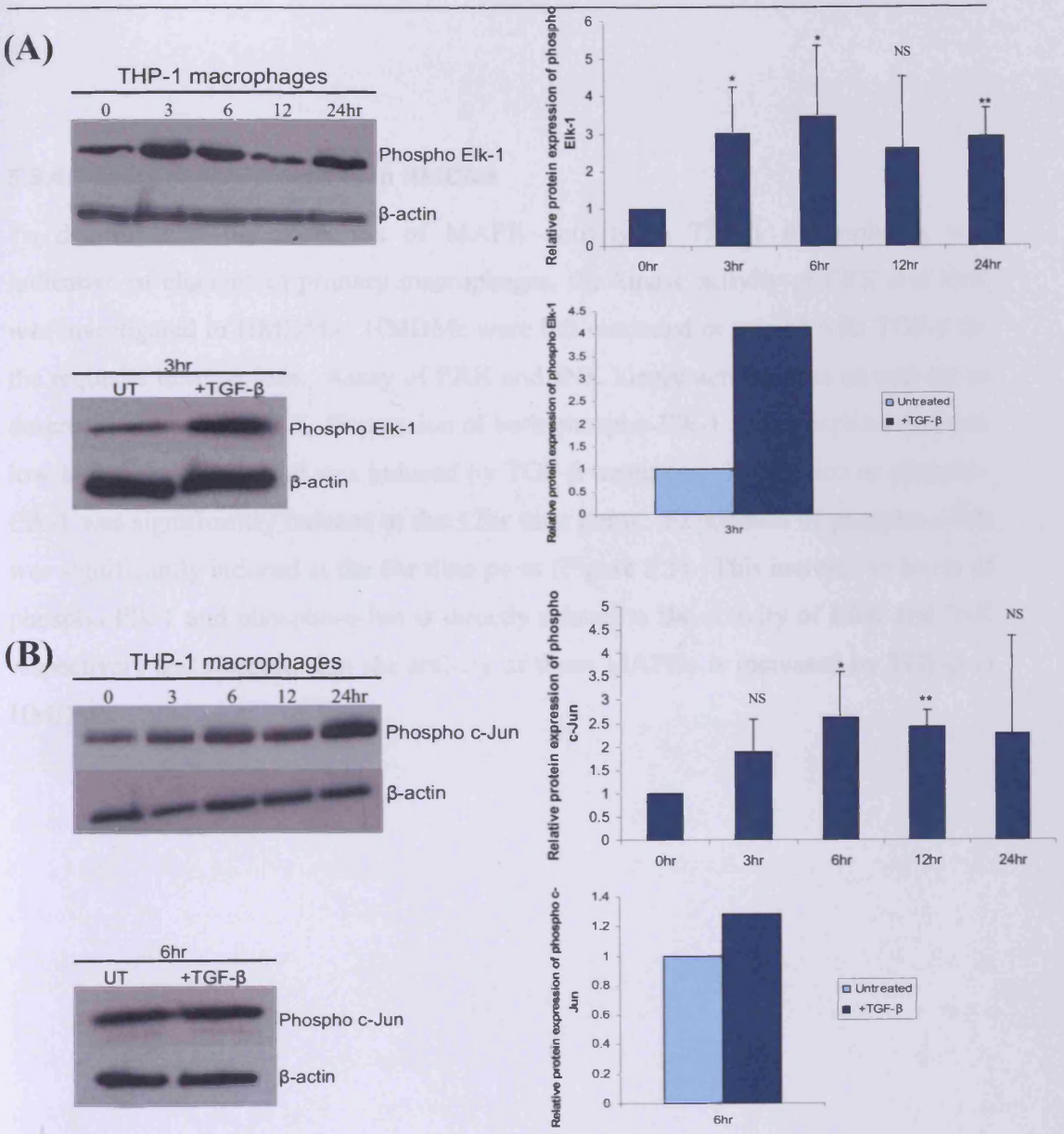

Figure 5.4 TGF-β induces the activity of ERK and JNK in THP-1 macrophages. THP-1 macrophages were left untreated (0hr) or treated with TGF-β for 3, 6, 12 or 24hr. ERK or JNK was immunoprecipitated as described in Sections 2.7.6 and 2.7.5 respectively and incubated with ATP to allow phosphorylation of an exogenous substrate. The kinase reaction with immunoprecipitated protein was subjected to SDS-PAGE and western blotting using antibodies against phospho-Elk-1 (Panel A) or phospho-c-Jun (Panel B). Whole-cell protein extracts were used for the β-actin western blot. Relative expression (mean± SD) of phospho-Elk-1 or phospho-c-Jun, normalised to levels of β-actin (untreated samples arbitrarily assigned as 1) from four independent experiments is shown (*P<0.05, **P<0.01, NS-not significant).

5.3.4 Assay of MAPK activity in HMDMs

To determine if the induction of MAPK activity in THP-1 macrophages was indicative of changes in primary macrophages, the kinase activity of ERK and JNK was investigated in HMDMs. HMDMs were left untreated or treated with TGF-β for the requisite time periods. Assay of ERK and JNK kinase activity was carried out as described in Section 5.3.3. Expression of both phospho-Elk-1 and phospho-c-Jun was low in untreated cells and was induced by TGF-β treatment. Expression of phospho-Elk-1 was significantly induced at the 12hr time point. Expression of phospho-c-Jun was significantly induced at the 6hr time point (Figure 5.5). This increase in levels of phospho-Elk-1 and phospho-c-Jun is directly related to the activity of ERK and JNK respectively and suggests that the activity of these MAPKs is increased by TGF-β in HMDMs.

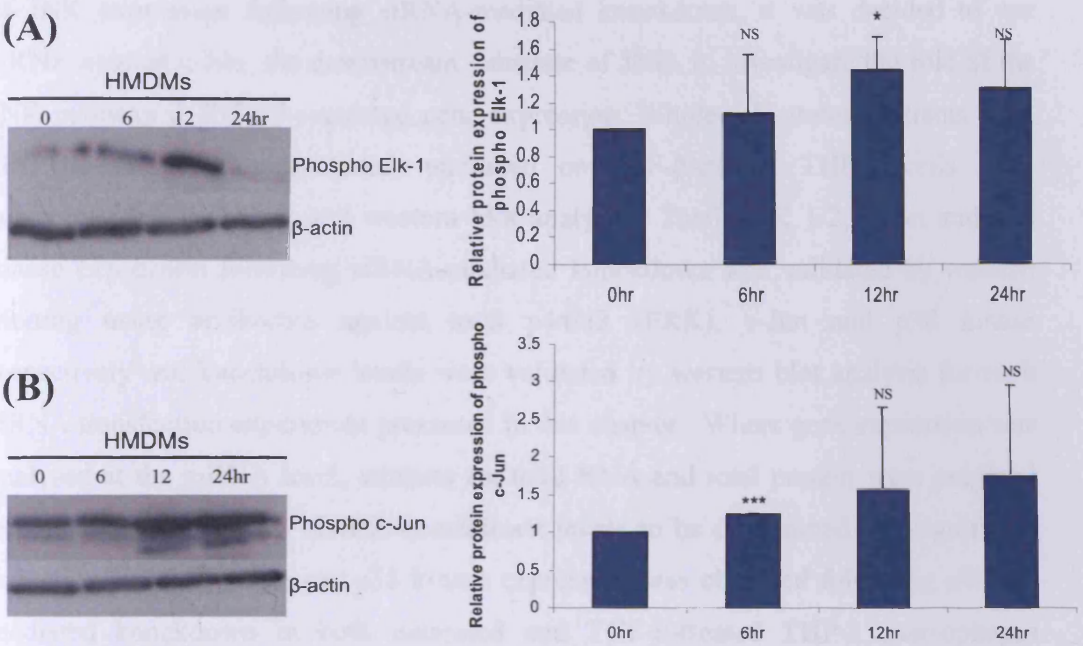

Figure 5.5 **TGF-β induces the activity of ERK and JNK in HMDMs.** HMDMs were left untreated (0hr) or treated with TGF-β for 6, 12 or 24hr. ERK or JNK was immunoprecipitated as described in Sections 2.7.6 and 2.7.5 respectively and incubated with ATP to allow phosphorylation of an exogenous substrate. The kinase reaction with immunoprecipitated protein was subjected to SDS-PAGE and western blotting using antibodies against phospho-Elk-1 (Panel A) or phospho-c-Jun (Panel B). Whole-cell protein extracts were used for the β-actin western blot. Relative expression (mean± SD) of phospho-Elk-1 or phospho-c-Jun, normalised to levels of β-actin (untreated samples arbitrarily assigned as 1) from three independent experiments is shown (*P<0.05, ***P<0.001, NS-not significant).

5.3.5 siRNA-mediated knockdown of MAPKs in THP-1 macrophages

Before the effects of MAPK knockdown on the TGF-β-regulated gene expression could be investigated, it was necessary to determine the level of MAPK knockdown following siRNA transfection in THP-1 macrophages. This was determined for each individual experiment. Individual knockdown of ERK 1/2, c-Jun and p38 kinase was investigated (Figures 5.6A, 5.6B and 5.6C). Knockdown of JNK was initially investigated but due to unexpected problems with producing a significant knockdown

of JNK expression following siRNA-mediated knockdown, it was decided to use siRNA against c-Jun, the downstream substrate of JNK, to investigate the role of the JNK pathway in TGF-β-regulated gene expression. Whole-cell protein extracts from GAPDH or MAPK-transfected, untreated or TGF-β-treated THP-1 cells were subjected to SDS-PAGE and western blot analysis. Total ERK 1/2, c-Jun and p38 kinase expression following siRNA-mediated knockdown was validated by western blotting using antibodies against total p44/42 (ERK), c-Jun and p38 kinase respectively and knockdown levels were validated by western blot analysis for each siRNA transfection experiment presented in this chapter. Where gene expression was analysed at the mRNA level, extracts for total RNA and total protein were prepared simultaneously allowing MAPK knockdown levels to be determined. A significant reduction in ERK, c-Jun and p38 kinase expression was observed following siRNA-mediated knockdown in both untreated and TGF-β-treated THP-1 macrophages (Figure 5.6).

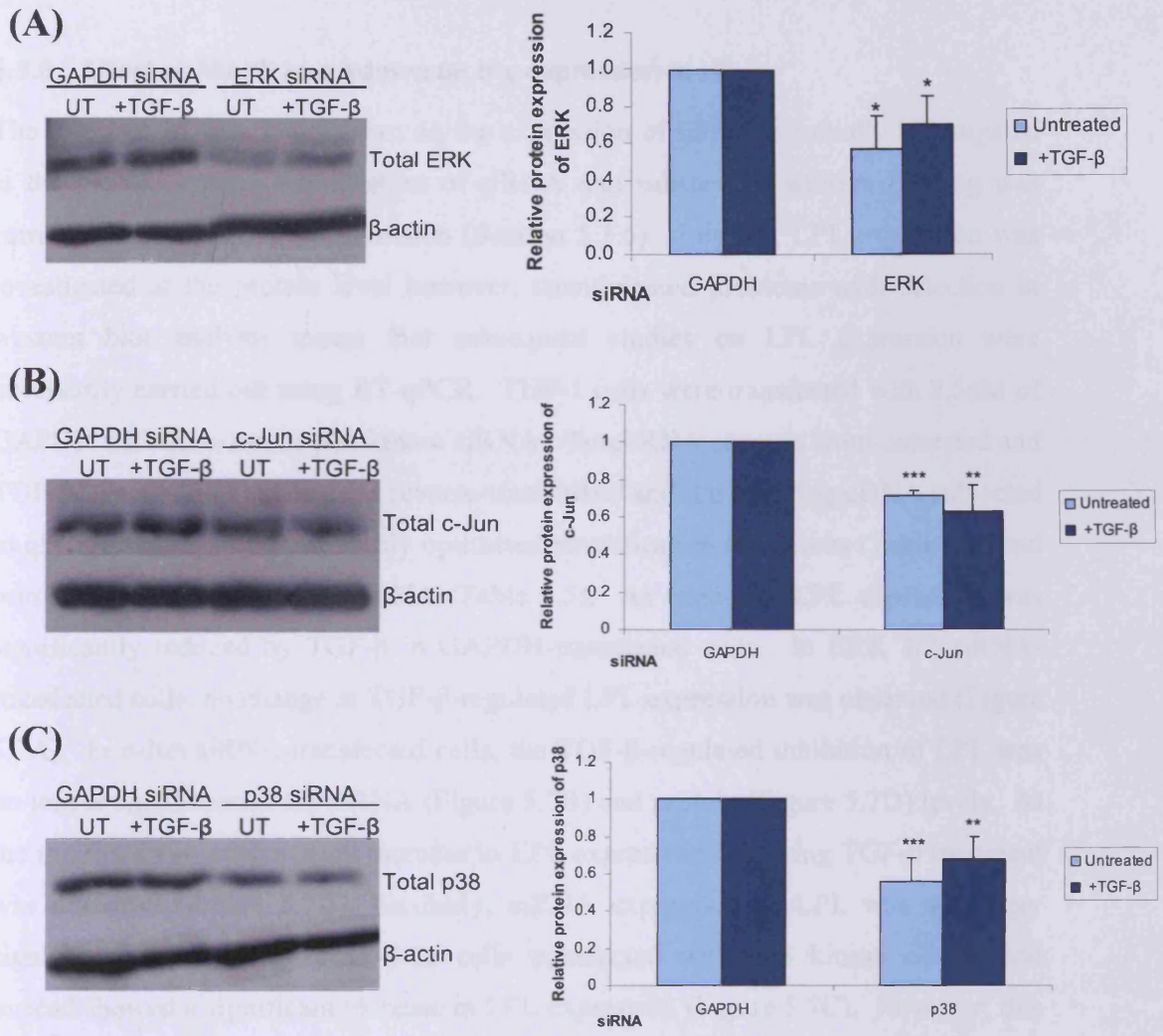

Figure 5.6 **siRNA-mediated knockdown of ERK 1/2, c-Jun and p38 kinase in THP-1 macrophages.** Whole-cell protein extracts prepared from untreated or TGF-β-treated THP-1 cells transfected with 7.5nM validated GAPDH siRNA or ERK 1/2 (Panel A), c-Jun (Panel B) or p38 kinase (Panel C) siRNA as described in Section 2.6.1. Extracts were subjected to SDS-PAGE and western blotting and levels of total ERK, c-Jun or p38 kinase were compared against levels of β-actin. Relative expression (mean± SD) of ERK, c-Jun or p38 kinase normalised to β-actin levels, with GAPDH-transfected cells arbitrarily assigned as 1, from three (Panel A) or four (Panels B and C) independent experiments is shown (*P<0.05, **P<0.01, ***P<0.001).

5.3.6 Effect of MAPK knockdown on the expression of LPL

The effect of MAPK knockdown on the expression of LPL was initially investigated at the protein level. Transfection of siRNA and subsequent western blotting was carried out as for MAPK expression (Section 5.3.5). Initially, LPL expression was investigated at the protein level however, unanticipated problems with detection in western blot analysis meant that subsequent studies on LPL expression were necessarily carried out using RT-qPCR. THP-1 cells were transfected with 7.5nM of GAPDH siRNA, c-Jun or p38 kinase siRNA. Total RNA extracts from untreated and TGF-β-treated (24hr) cells were reverse-transcribed and the resulting cDNA subjected to qPCR analysis using previously optimised amplification conditions (Table 2.6) and primers against LPL and 28SrRNA (Table 2.5). As expected, LPL expression was significantly reduced by TGF-β in GAPDH-transfected cells. In ERK 1/2 siRNA-transfected cells, no change in TGF-β-regulated LPL expression was observed (Figure 5.7A). In c-Jun siRNA-transfected cells, the TGF-β-regulated inhibition of LPL was no longer significant at the mRNA (Figure 5.7B) and protein (Figure 5.7D) levels. At the mRNA level, a significant increase in LPL expression following TGF-β treatment was observed (Figure 5.7B). Similarly, mRNA expression of LPL was no longer significantly reduced by TGF-β in cells transfected with p38 kinase siRNA and instead showed a significant increase in LPL expression (Figure 5.7C). However, this change was not observed at the protein level (Figure 5.7D). This may be due to the higher sensitivity of RT-qPCR in comparison to western blotting.

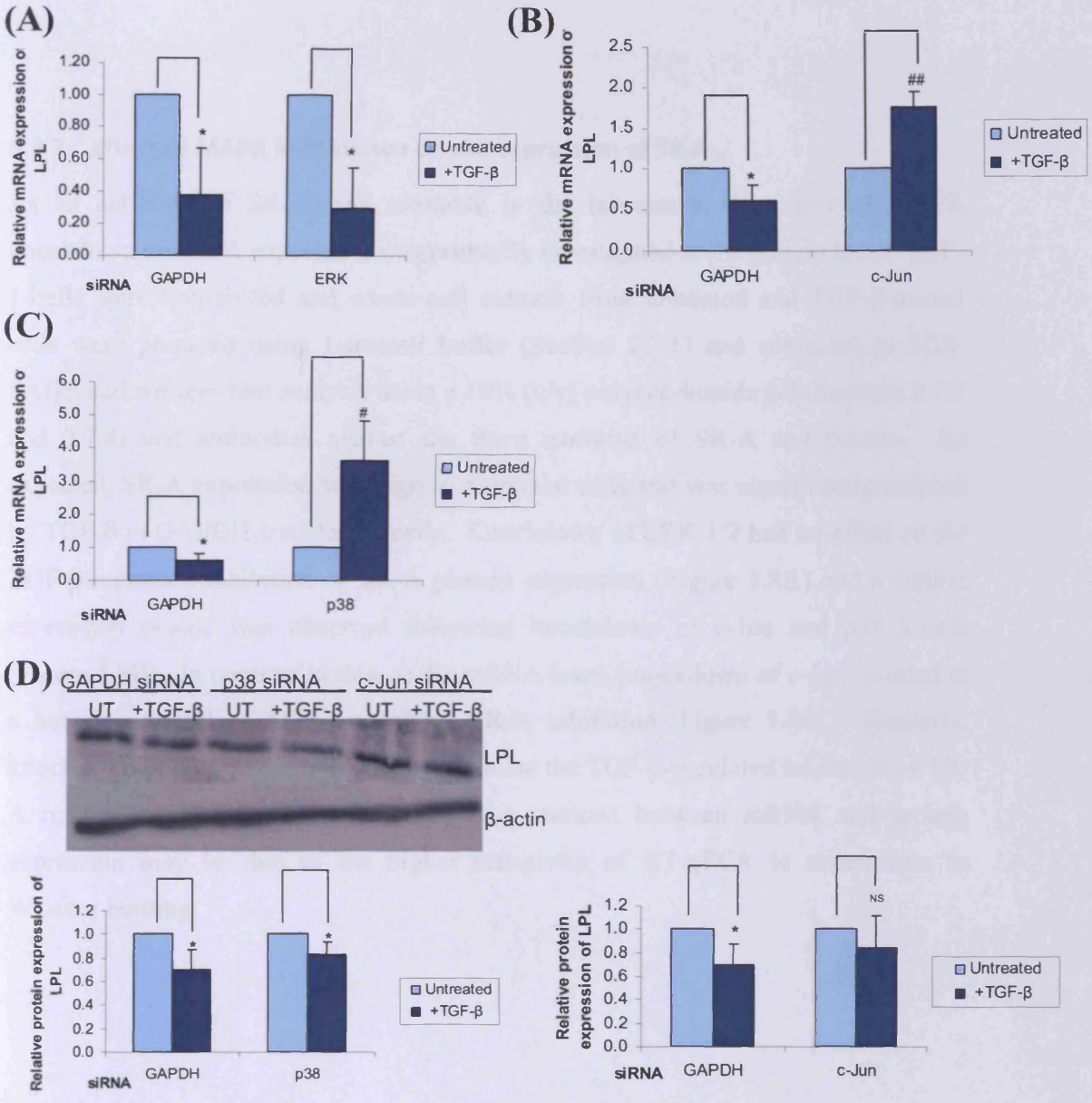

Figure 5.7 **Knockdown of p38 kinase and c-Jun attenuates the TGF-β-mediated inhibition of LPL expression in THP-1 macrophages.** Total RNA (Panels A-C) or whole-cell protein extracts (Panel D) were prepared from untreated or TGF-β-treated THP-1 macrophages transfected with GAPDH or validated ERK 1/2 (A), p38 kinase (B) or c-Jun (C) siRNA. Western blotting was carried out using antibodies against LPL and β-actin. RT-qPCR was carried out using primers against LPL and 28SrRNA. Relative expression (mean± SD) of LPL, normalised to β-actin (for western blotting) or 28SrRNA (for RT-qPCR) levels (untreated samples arbitrarily assigned as 1), from three independent experiments is shown (*P<0.05, **P<0.01, NS-not significant, #-significant induction P<0.05, ##-P<0.01).

5.3.7 Effect of MAPK knockdown on the expression of SR-A

As an antibody to SR-A was available in the laboratory, the effect of MAPK knockdown on SR-A expression was primarily investigated at the protein level. THP-1 cells were transfected and whole-cell extracts from untreated and TGF-β-treated cells were prepared using Laemmli buffer (Section 2.7.1) and subjected to SDS-PAGE and western blot analysis using a 10% (v/v) polyacrylamide gel (Sections 2.7.7 and 2.7.8) and antibodies against the three isoforms of SR-A and β-actin. As expected, SR-A expression was high in untreated cells and was significantly reduced by TGF-β in GAPDH-transfected cells. Knockdown of ERK 1/2 had no effect on the TGF-β-regulated inhibition of SR-A protein expression (Figure 5.8A) and a similar expression profile was observed following knockdown of c-Jun and p38 kinase (Figure 5.8B). In contrast to this, at the mRNA level, knockdown of c-Jun resulted in a notable reversal of TGF-β-regulated SR-A inhibition (Figure 5.8C). Similarly, knockdown of p38 kinase was able to attenuate the TGF-β-regulated inhibition of SR-A mRNA expression (Figure 5.8D). The contrast between mRNA and protein expression may be due to the higher sensitivity of RT-qPCR in comparison to Western blotting.

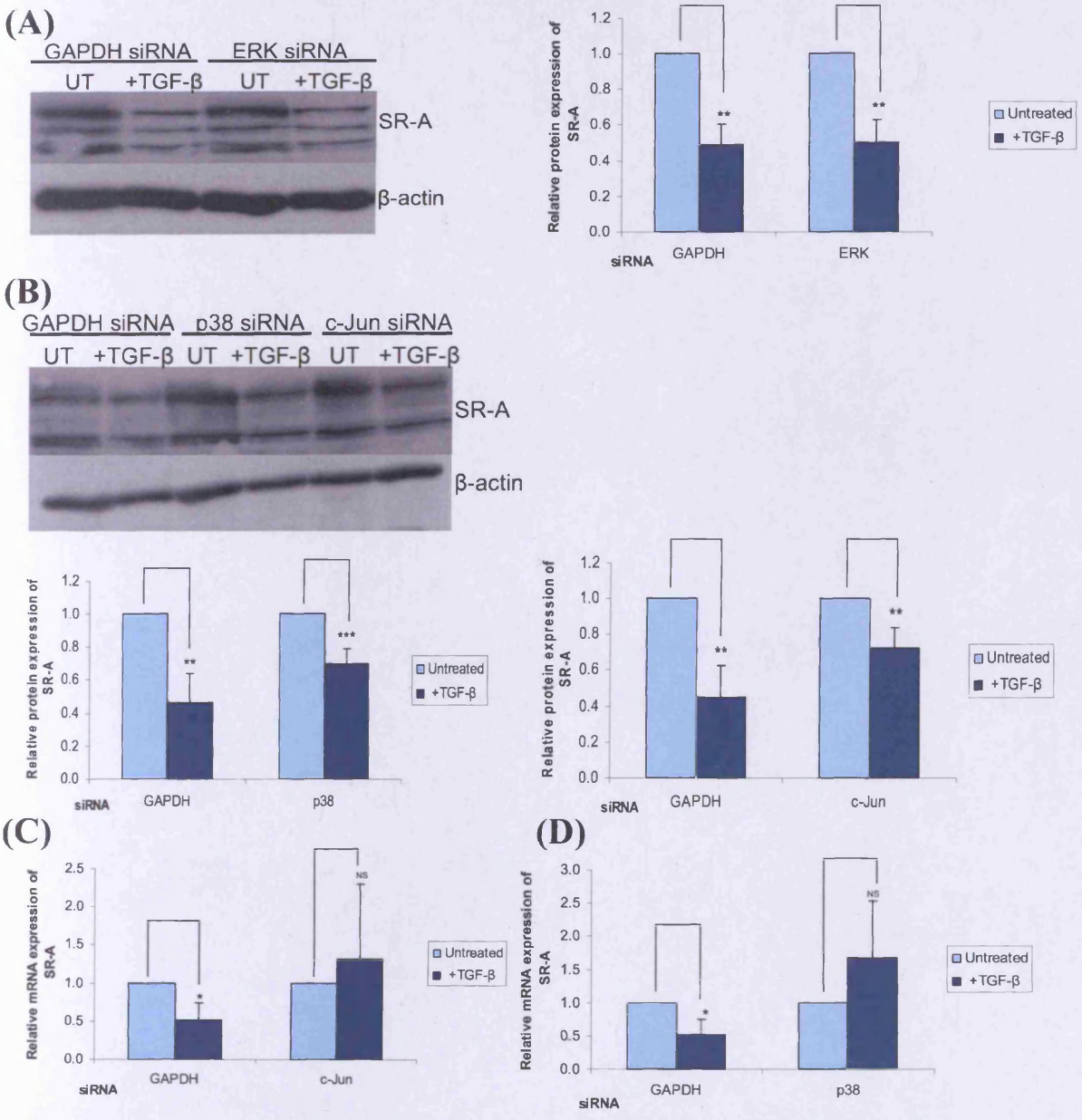

Figure 5.8 **c-Jun and p38 kinase are required for the TGF-β-mediated inhibition of SR-A in THP-1 macrophages**. Whole-cell protein extracts from untreated and TGF-β-treated cells transfected with validated GAPDH or ERK 1/2 (Panel A), c-Jun or p38 kinase (Panel B) siRNAs were subjected to SDS-PAGE and western blotting using antibodies against SR-A (recognises all three isoforms) and β-actin. In panels C and D, total RNA was reverse-transcribed and subjected to RT-qPCR using primers specific for SR-A and 28SrRNA. Relative expression (mean ±SD) of SR-A normalised to β-actin (or 28SrRNA), with untreated samples arbitrarily assigned as 1, from three independent experiments is shown (**P*<0.05, ***P*<0.01, ****P*<0.001, NS-not significant).

5.3.8 Effect of MAPK knockdown on the expression of SR-B1

The effect of MAPK knockdown on the expression of SR-B1 was investigated at the mRNA level. Transfection and preparation of total RNA and RT-qPCR was carried out as for LPL (Section 5.3.6). As expected, the expression of SR-B1 was significantly reduced by TGF-β in GAPDH-transfected cells. This regulation was not affected by knockdown of ERK 1/2 (Figure 5.9A). Following c-Jun knockdown, the expression of SR-B1 was no longer significantly inhibited by TGF-β (Figure 5.9B). The TGF-β-regulated expression of SR-B1 was similarly attenuated by p38 kinase knockdown (Figure 5.9C) but to a larger extent so that a significant induction in SR-B1 expression was observed.

5.3.9 Effect of MAPK knockdown on the expression of CD36

As for SR-B1, the effect of MAPK knockdown on CD36 expression was investigated at the mRNA level. Expression of CD36 was down-regulated by TGF-β in GAPDH-transfected cells. Knockdown of ERK 1/2 expression resulted in the loss of significant down-regulation of CD36 by TGF-β (Figure 5.10A). However, the mean expression levels of CD36 following ERK 1/2 knockdown are not significantly different to that following GAPDH knockdown and show a wide variation in expression, suggesting that the loss of significant down-regulation may be due to this variation as opposed to the knockdown of ERK 1/2 itself. Knockdown of c-Jun and p38 kinase were able to attenuate the TGF-β-regulation of CD36 and produced a notable reversal of CD36 inhibition (Figure 5.10B and Figure 5.10C).

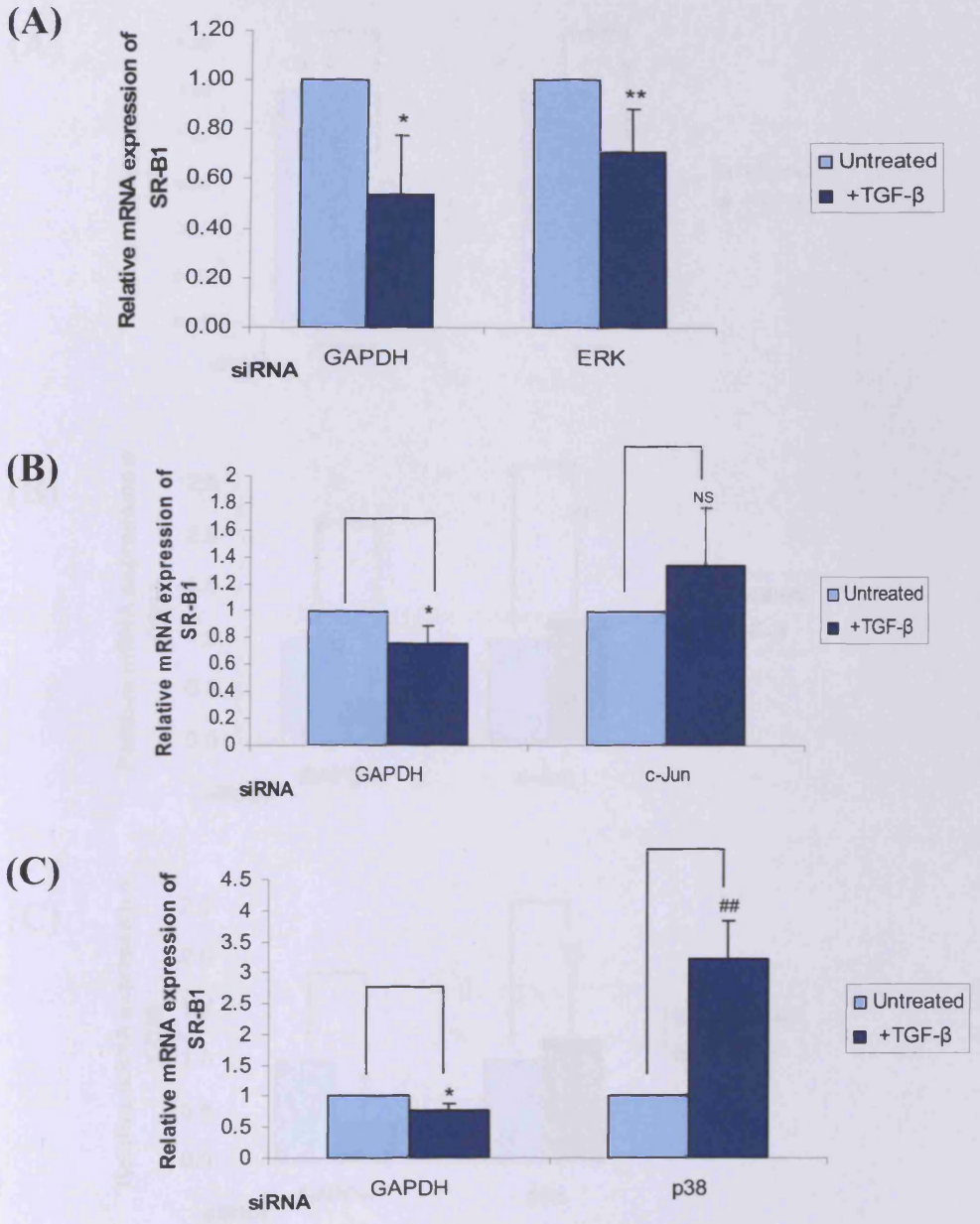

Figure 5.9 **Knockdown of p38 kinase or c-Jun negates the TGF-β-mediated inhibition of SR-B1 expression in THP-1 macrophages.** Total RNA from untreated and TGF-β-treated cells transfected with siRNAs against GAPDH, ERK 1/2 (Panel A), c-Jun (Panel B) or p38 kinase (Panel C) were subjected to RT-qPCR using primers specific to SR-B1 and 28SrRNA. Relative expression (mean ±SD) of SR-B1 normalised to 28SrRNA from three independent experiments, with untreated samples arbitrarily assigned as 1, is shown (*P<0.05, **P<0.01, ##-significant induction P<0.01, NS-not significant).

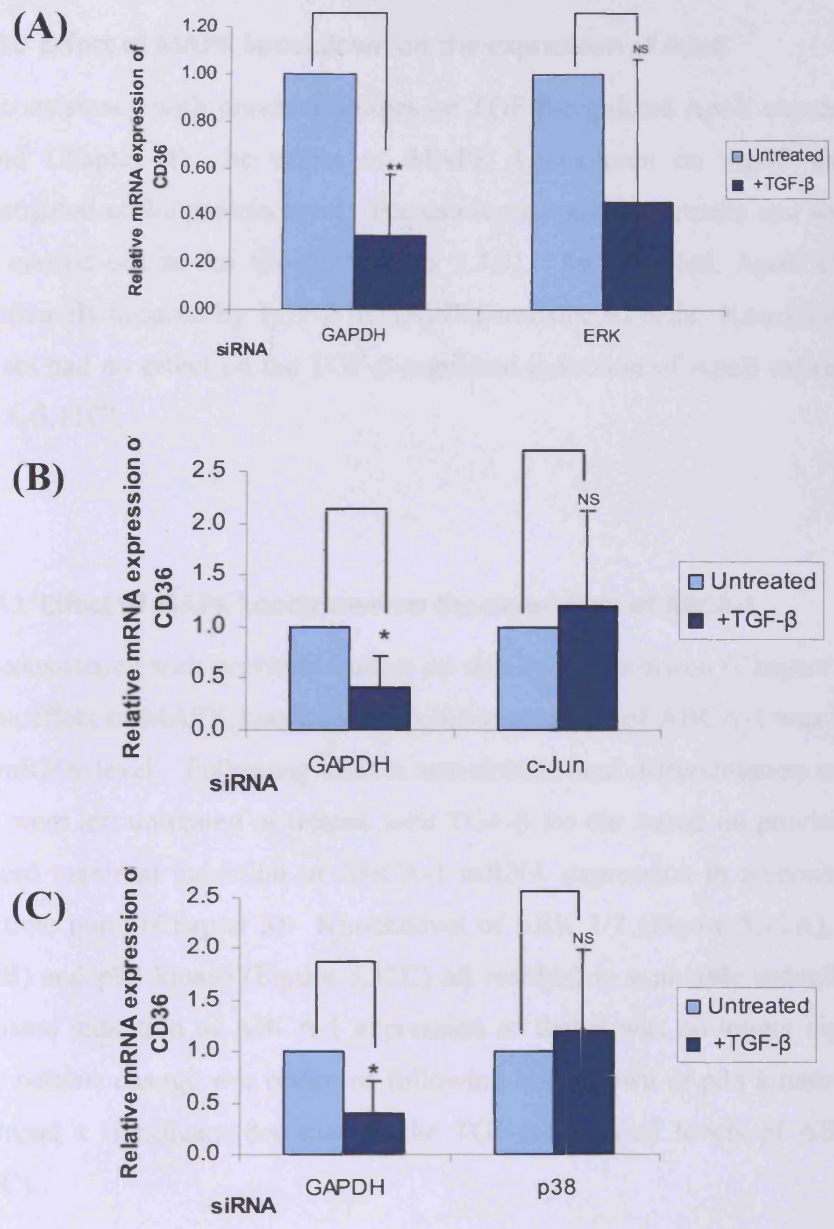

Figure 5.10 **Knockdown of c-Jun and p38 kinase negates the TGF-β-regulation of CD36 expression in THP-1 macrophages.** RT-qPCR was carried out using primers specific to CD36 and 28SrRNA using total RNA from cells transfected with siRNA against GAPDH or ERK 1/2 (Panel A), c-Jun (Panel B) or p38 kinase (Panel C). Relative expression (mean ±SD) of CD36, normalised to 28SrRNA, with untreated samples arbitrarily assigned as 1, is shown (*P<0.05, **P<0.01, NS-not significant).

5.3.10 Effect of MAPK knockdown on the expression of ApoE

For consistency with previous studies on TGF-β-regulated ApoE expression (Chapter 3 and Chapter 4), the effect of MAPK knockdown on ApoE expression was investigated at the protein level. Preparation of protein extracts and western blotting was carried out as for SR-A (Section 5.3.7). As expected, ApoE expression was significantly induced by TGF-β in GAPDH-transfected cells. Knockdown of all three kinases had no effect on the TGF-β-regulated induction of ApoE expression (Figures 5.11A-5.11C).

5.3.11 Effect of MAPK knockdown on the expression of ABCA-1

For consistency with previous studies on ABCA-1 expression (Chapter 3 and Chapter 4) the effect of MAPK knockdown on the expression of ABCA-1 was investigated at the mRNA level. Following siRNA transfection and differentiation of THP-1 cells, they were left untreated or treated with TGF-β for 6hr based on previous studies that showed maximal induction of ABCA-1 mRNA expression in response to TGF-β at this time point (Chapter 3). Knockdown of ERK 1/2 (Figure 5.12A), c-Jun (Figure 5.12B) and p38 kinase (Figure 5.12C) all resulted in a notable reduction of TGF-β-mediated induction of ABCA-1 expression so that it was no longer significant. The most notable change was observed following knockdown of p38 kinase which instead produced a significant decrease in the TGF-β-regulated levels of ABCA-1 (Figure 5.12C).

5.3.12 Effect of MAPK knockdown on the expression of ABCG-1

As with ABCA-1, the expression of ABCG-1 following MAPK knockdown was also investigated at the mRNA level following 6hr stimulation with TGF-β. Knockdown of ERK 1/2 (Figure 5.13A) resulted in a loss of significant TGF-β-mediated ABCG-1 induction. Knockdown of c-Jun (Figure 5.13B) and p38 kinase (Figure 5.13C) produced analogous changes in the TGF-β-mediated induction of ABCG-1.

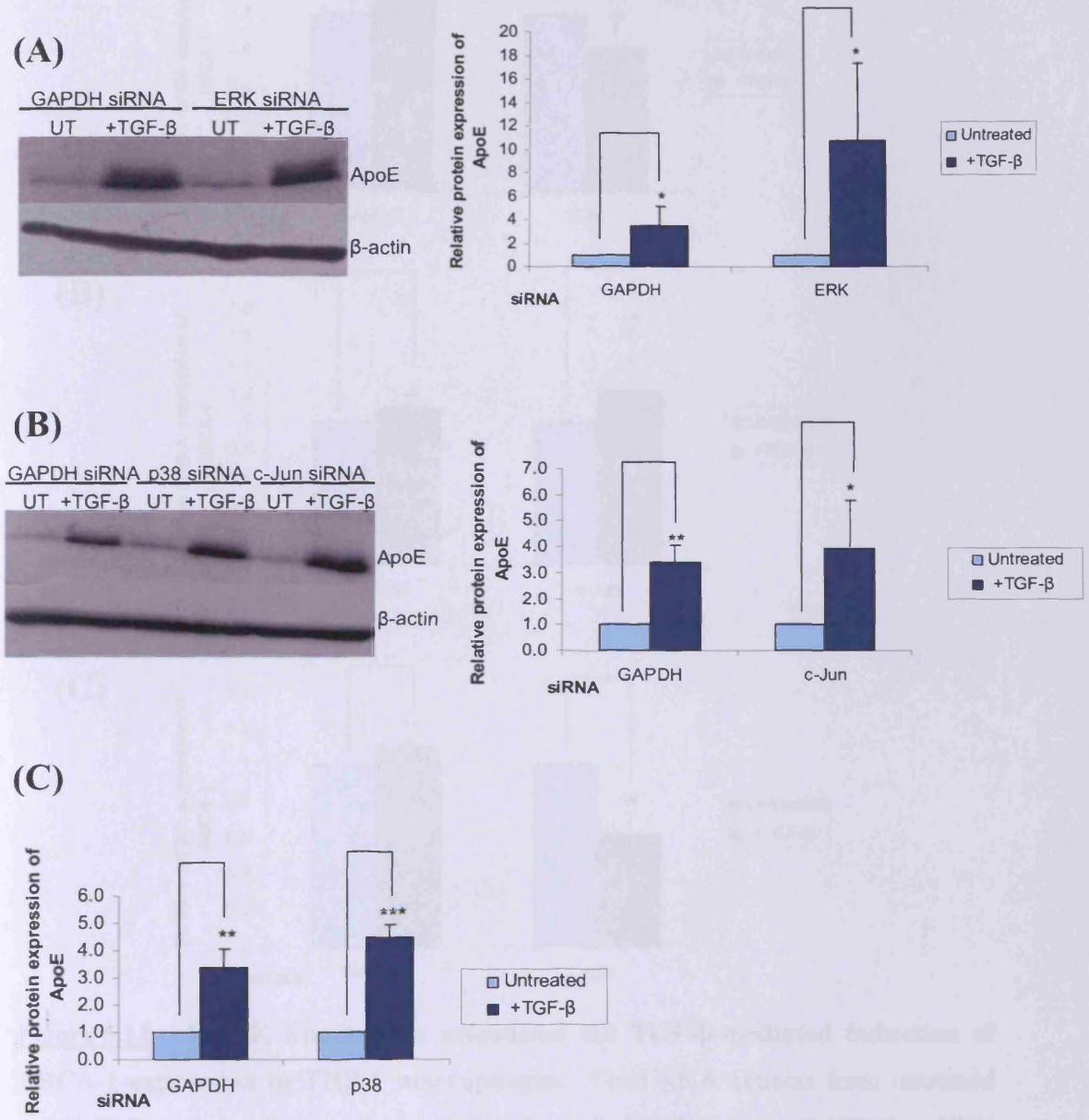

Figure 5.11 **Knockdown of individual MAPKs has no effect on the TGF-β-mediated induction of ApoE expression.** Whole-cell protein extracts from untreated and TGF-β-treated cells transfected with validated siRNA against GAPDH or ERK 1/2 (Panel A), c-Jun (Panel B) or p38 kinase (Panel C) were subjected to SDS-PAGE and western blotting using antibodies against ApoE and β-actin. Relative expression (mean ±SD) of ApoE normalised to β-actin, with untreated samples arbitrarily assigned as 1, from three independent experiments is shown (*P<0.05, **P<0.01, ***P<0.001).

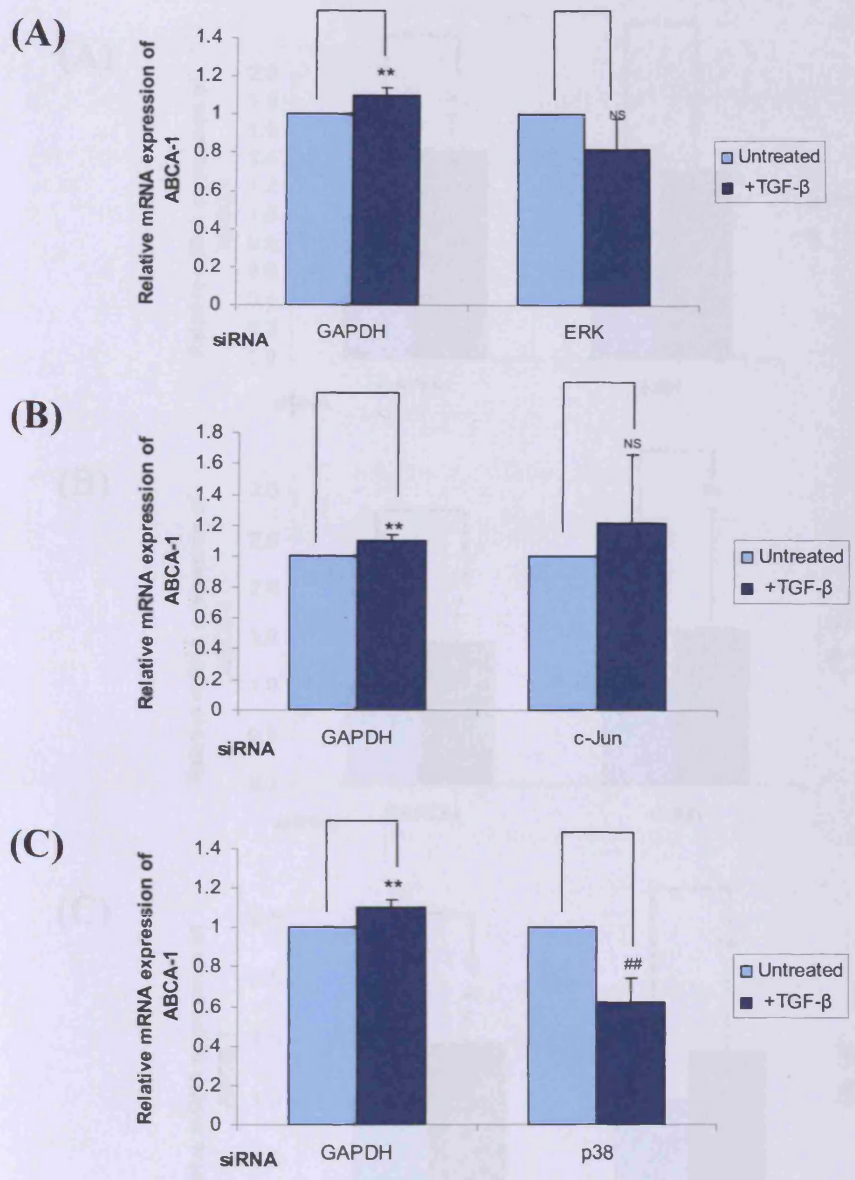

Figure 5.12 MAPK knockdown attenuates the TGF-β-mediated induction of ABCA-1 expression in THP-1 macrophages. Total RNA extracts from untreated and TGF-β-treated cells transfected with validated siRNAs against GAPDH or ERK 1/2 (Panel A), c-Jun (Panel B) or p38 kinase (Panel C) were subjected RT-qPCR using primers specific to ABCA-1 and RPL13A. RPL13A was used as a control for consistency with previous work on ABCA-1 and ABCG-1 expression as presented in Chapters 3 and 4 of this thesis. Relative expression (mean ±SD) of ABCA-1 normalised to RPL13A, with untreated samples arbitrarily assigned as 1, from three independent experiments is shown (**P<0.01, NS-not significant, ##-significant inhibition P<0.05).

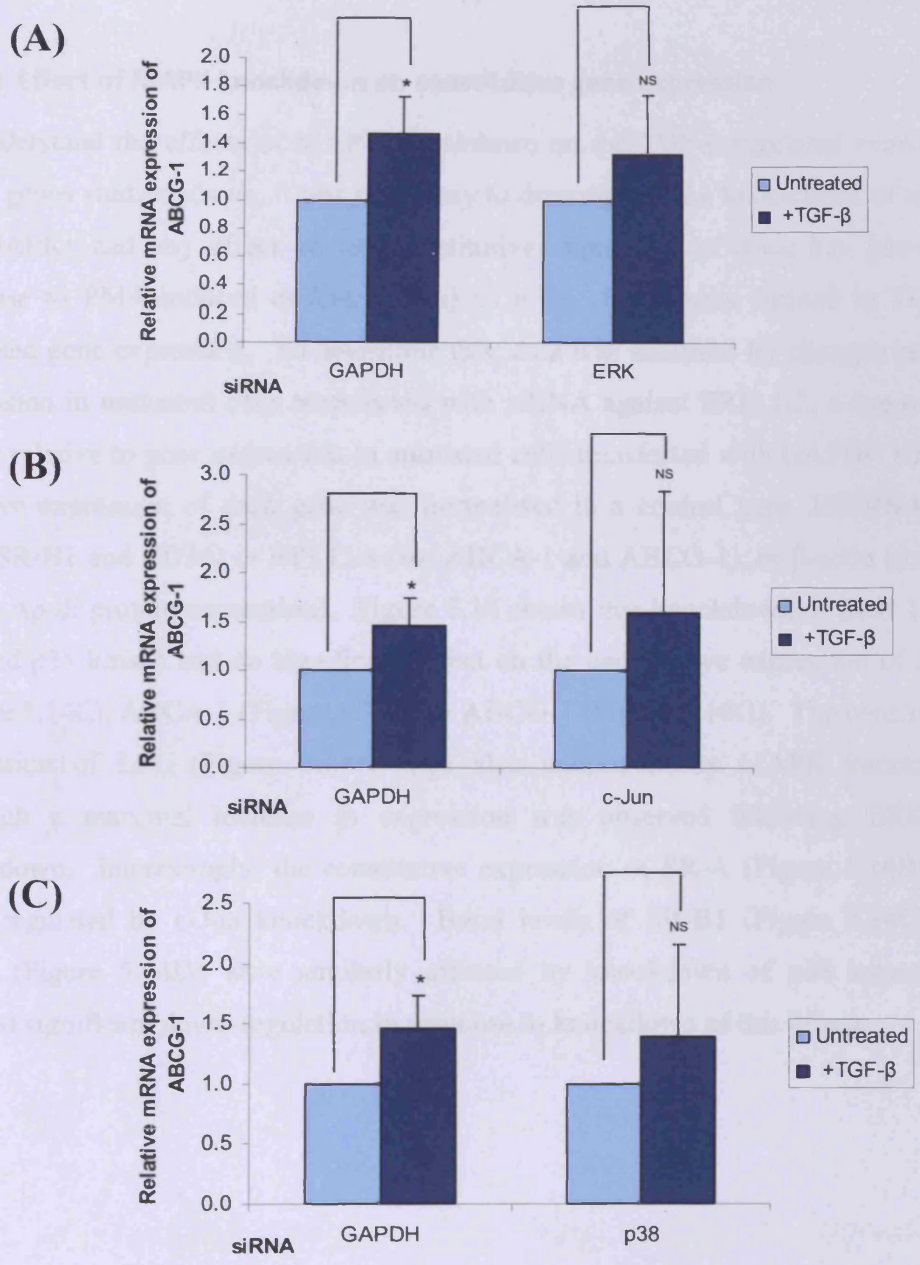

Figure 5.13 MAPK knockdown attenuates the TGF-β-mediated induction of ABCG-1 expression in THP-1 macrophages. RT-qPCR was carried out using primers specific to ABCG-1 and RPL13A using total RNA from cells transfected with siRNAs against GAPDH or ERK 1/2 (Panel A), c-Jun (Panel B) or p38 kinase (Panel C). Relative expression (mean ±SD) of ABCG-1, normalised to RPL13A with untreated samples arbitrarily assigned as 1, is shown (*P<0.05, NS-not significant).

5.3.13 Effect of MAPK knockdown on constitutive gene expression

To understand the effects of MAPK knockdown on the TGF-β-regulated expression of the genes studied above, it was necessary to determine if the knockdown of any of the MAPKs had any effect on the constitutive expression of these key genes (in response to PMA-induced differentiation) or if its effects were limited to TGF-β-regulated gene expression. To determine this, data was analysed for changes in gene expression in untreated cells transfected with siRNA against ERK 1/2, c-Jun or p38 kinase relative to gene expression in untreated cells transfected with GAPDH siRNA. Relative expression of each gene was normalised to a control gene 28SrRNA (for LPL, SR-B1 and CD36) or RPL13A (for ABCA-1 and ABCG-1), or β-actin (for SR-A and ApoE protein expression). Figure 5.14 shows that knockdown of ERK 1/2, c-Jun and p38 kinase had no significant effect on the constitutive expression of ApoE (Figure 5.14E), ABCA-1 (Figure 5.14F) or ABCG-1 (Figure 5.14G). The constitutive expression of LPL (Figure 5.14A) was also unaffected by MAPK knockdown although a marginal increase in expression was observed following ERK 1/2 knockdown. Interestingly, the constitutive expression of SR-A (Figure 5.14B) was down-regulated by c-Jun knockdown. Basal levels of SR-B1 (Figure 5.14C) and CD36 (Figure 5.14D) were similarly affected by knockdown of p38 kinase and showed significant down-regulation in response to knockdown of this kinase.

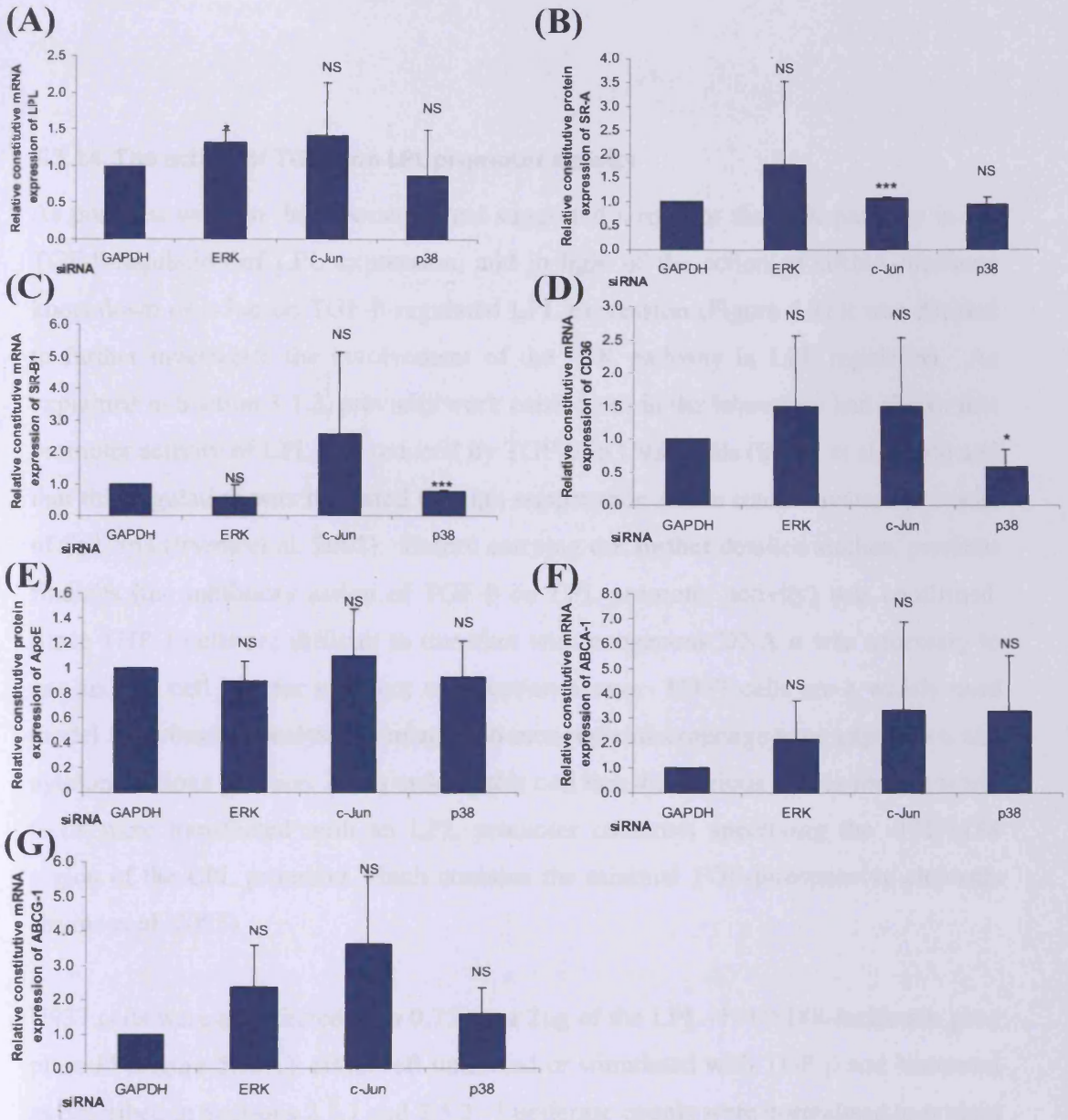

Figure 5.14 **The effect of MAPK knockdown on the constitutive expression of cholesterol uptake and efflux genes in THP-1 macrophages.** RT-qPCR was carried out using primers specific to LPL (Panel A), SR-B1 (Panel C), CD36 (Panel D), ABCA-1 (Panel F), ABCG-1 (Panel G) using total RNA from cells transfected with siRNAs against GAPDH or ERK 1/2, c-Jun or p38 kinase. For SR-A (Panel B) and ApoE (Panel E) whole cell extracts were subjected to SDS-PAGE and western blotting using antibodies against SR-A, ApoE and β-actin. Relative expression (mean ±SD) of each gene, normalised to 28SrRNA, RPL13A or β-actin with GAPDH-transfected samples arbitrarily assigned as 1, is shown (*P<0.05, ***P<0.001, NS-not significant).

5.3.14 The action of TGF-β on LPL promoter activity

As previous work in the laboratory had suggested a role for the JNK pathway in the TGF-β-regulation of LPL expression, and in light of the action of siRNA-mediated knockdown of c-Jun on TGF-β-regulated LPL expression (Figure 5.7) it was decided to further investigate the involvement of the JNK pathway in LPL regulation. As explained in Section 3.1.2, previous work carried out in the laboratory had shown that promoter activity of LPL was reduced by TGF-β in U937 cells (Irvine et al. 2005) and that this regulation was mediated through suppression of the transactivation potential of Sp1/Sp3 (Irvine et al. 2005). Before carrying out further detailed studies, previous findings (the inhibitory action of TGF-β on LPL promoter activity) was confirmed. Since THP-1 cells are difficult to transfect with exogenous DNA it was necessary to use another cell line for transient transfection assays. U937 cells are a widely used model for promoter analysis in relation to monocyte/macrophage gene expression and cytokine actions (Section 2.3.1) making this cell line the obvious choice for this work. Cells were transfected with an LPL promoter construct specifying the -101/+188 region of the LPL promoter which contains the minimal TGF-β-responsive elements (Irvine et al. 2005).

U937 cells were transfected with 0.75, 1 or 2μg of the LPL -101/+188-luciferase gene plasmid (Figure 5.15A), either left untreated or stimulated with TGF-β and harvested as described in Sections 2.5.1 and 2.5.2. Luciferase counts were normalised to protein concentration as many promoters of control plasmids are regulated by cytokines. This was determined using the BCA protein assay kit (Section 2.7.4). Initial experiments showed that 2μg of the LPL promoter-luciferase DNA plasmid produced the largest inhibition in response to TGF-β treatment. More experiments were therefore carried out using this concentration of the plasmid and Figure 5.15B shows that, as expected TGF-β was able to significantly inhibit the promoter activity of LPL in U937 cells.

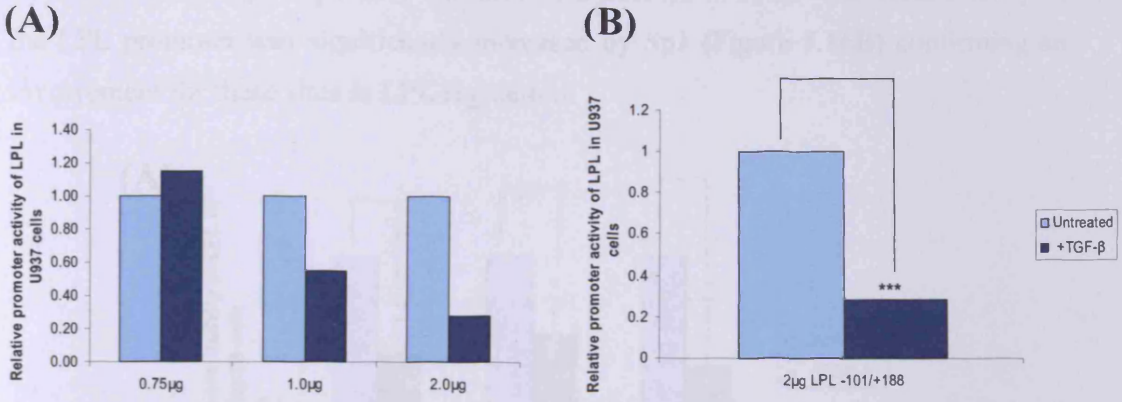

Figure 5.15 TGF-β inhibits the promoter activity of LPL in U937 cells. U937 cells were transfected with the -101/+188 LPL promoter-luciferase DNA construct using Superfect™ as described in Section 2.5.1. Cells were either left untreated or treated with TGF-β overnight. PMA was added immediately following transfection and cells were harvested using 1x passive lysis buffer (Promega) and measured for luciferase activity. Luciferase readings were normalised to protein concentration as determined by the BCA protein assay (Section 2.7.4). Data shown is the ratio of luciferase activity:protein concentration normalised to the untreated samples and expressed as the fold change from this. Data (excluding Panel A which represents a single experiment) is representative of three independent experiments (***P<0.001).

Unfortunately, differentiation of new U937 cells purchased from ECACC became defective, meaning that future work using this cell line became unfeasible. As adherent Hep3B cells were available in the laboratory, are easy to transfect and have previously been used for studies on the cytokine regulation of gene expression (Section 2.3.1) this cell line was used for further studies on the regulation of LPL promoter activity. Figure 5.16A shows the effect of TGF-β treatment on LPL promoter activity in the Hep3B cell line. Promoter activity is significantly reduced by the cytokine. Since it had been demonstrated that, in macrophages, the transactivation potential of Sp1 is suppressed by TGF-β to inhibit LPL promoter activity, it was reasoned that Sp1 must be required for basal LPL promoter activity. Hep3B cells were transfected with plasmids specifying for Sp3 and Sp1. The $pcDNA_3$ plasmid is

an empty vector used as a control for the Sp1 and Sp3 expression plasmids and an Sp1 multimer containing 4 Sp1 sites was used as a positive control. The basal activity of the LPL promoter was significantly increased by Sp1 (Figure 5.16B) confirming an involvement for these sites in LPL regulation.

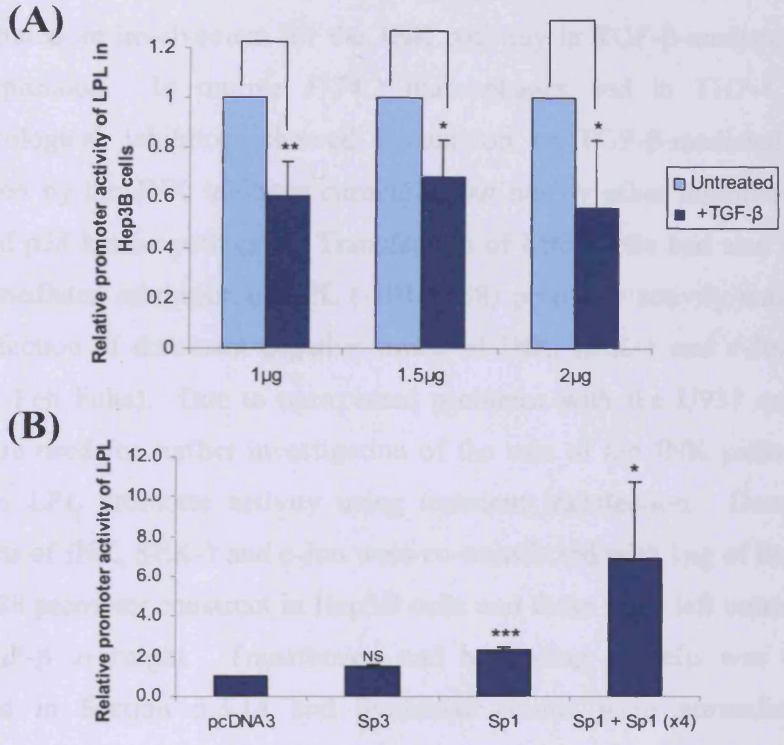

Figure 5.16 TGF-β inhibits the promoter activity of LPL in Hep3B cells. Hep3B cells were transfected with the -101/+188 LPL promoter-luciferase construct using Superfect™ as described in Section 2.5.1. Cells were either left untreated or treated with TGF-β overnight (Panel A). In Panel B, cells were co-transfected with the -101/+188 LPL promoter-luciferase construct with plasmids specifying for Sp3, Sp1, or the control vector pcDNA$_3$. For the positive control the Sp1 (x4) multimer was cotransfected with the Sp1 expression plasmid. Cells were harvested using 1x passive lysis buffer (Promega) and measured for luciferase activity. Luciferase readings were normalised to protein concentration as determined by the BCA protein assay (Section 2.7.4). Data shown is the ratio of luciferase activity:protein concentration normalised to the untreated samples and expressed as the fold change from this. Data is representative of three independent experiments (*P<0.05, **P<0.01***P<0.001, NS-not significant).

5.3.15 The role of the JNK pathway in TGF-β-regulated LPL promoter activity

Previous work carried out in the laboratory by Dr Peli Foka and Dr Scott Irvine had demonstrated an involvement for the JNK pathway in TGF-β-mediated inhibition of LPL expression. In murine J774.2 macrophages and in THP-1 cells, use of pharmacological inhibitors showed attenuation of TGF-β-mediated LPL mRNA expression by the JNK inhibitor curcumin, but not by other inhibitors targeting the ERK and p38 kinase pathways. Transfection of U937 cells had also shown that the TGF-β-mediated inhibition of LPL (-101/+188) promoter activity was attenuated by co-transfection of dominant-negative forms of JNK, SEK-1 and c-Jun (unpublished data, Dr Peli Foka). Due to unexpected problems with the U937 cell line, Hep3B cells were used for further investigation of the role of the JNK pathway in TGF-β-regulated LPL promoter activity using transient transfection. Dominant-negative constructs of JNK, SEK-1 and c-Jun were co-transfected with 1μg of the LPL -101/+188 promoter construct in Hep3B cells and these were left untreated or treated with TGF-β overnight. Transfection and harvesting of cells was carried out as described in Section 5.3.14 and luciferase counts were normalised to protein concentration. Figure 5.17 shows that transfection of dominant-negative JNK has little effect on TGF-β-regulated inhibition of LPL promoter activity. This contrasts with findings in U937 cells where reversal of LPL inhibition following transfection of dominant-negative JNK, c-Jun and SEK-1 was observed at several concentrations suggesting that this may be a cell-type specific response. However, transfection of dominant-negative c-Jun resulted in a loss of significant LPL inhibition at low (1μg) concentration whilst transfection of dominant-negative SEK-1 showed the most marked effect and resulted in a loss of significant LPL inhibition using higher (2 and 3μg) concentrations of construct. This suggests that differences are likely to be due to variation rather than any changes in the trend observed.

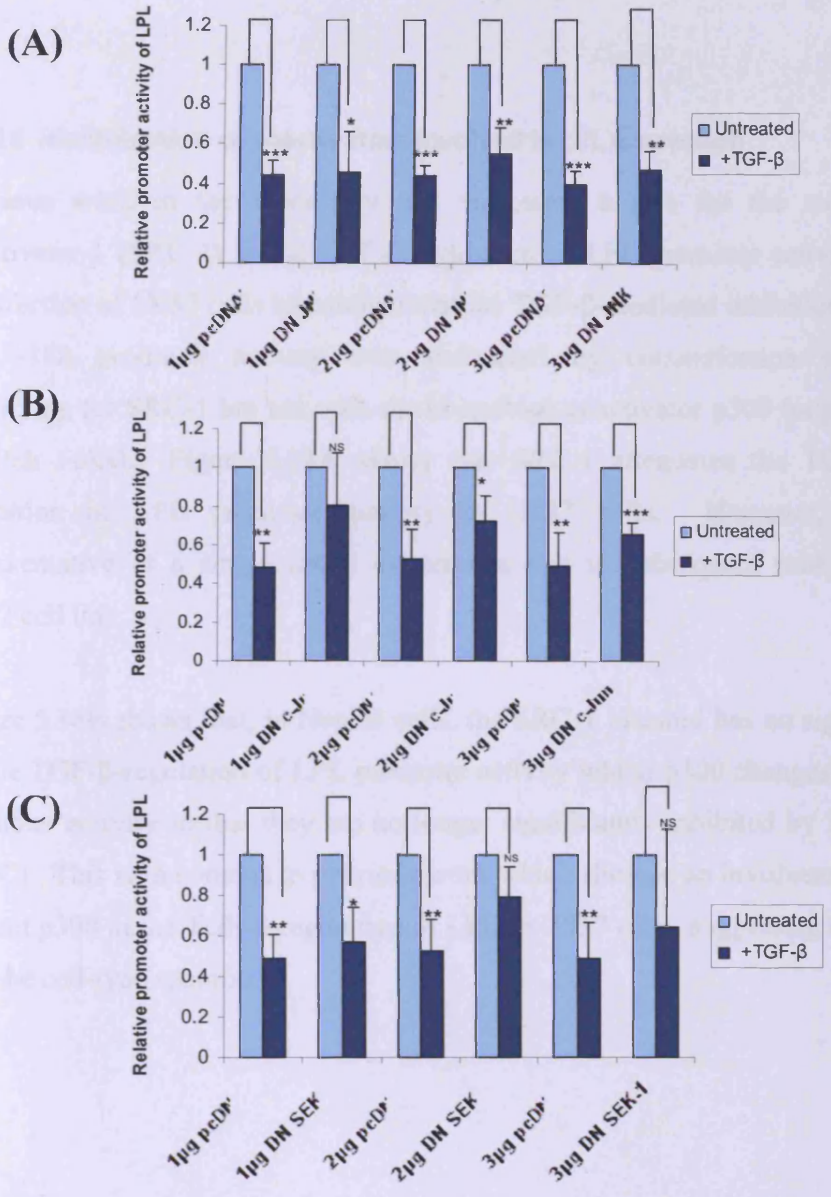

Figure 5.17 The role of the JNK pathway in the TGF-β-regulation of LPL in Hep3B cells. Hep3B cells were transfected with 1μg LPL -101/+188 promoter construct and 1, 2 or 3μg of $pcDNA_3$, DN JNK, DN c-Jun or DN SEK-1 as indicated, using Superfect™ as described in Section 2.5.1. Cells were either left untreated or treated with TGF-β overnight. Cells were harvested using 1x passive lysis buffer (Promega) as described in Section 2.5.2 and measured for luciferase activity. Data shown is the ratio of luciferase activity: protein concentration normalised to the untreated samples and expressed as the fold change from this. Data is representative of three independent experiments (*P<0.05, **P<0.01, ***P<0.001, NS-not significant).

5.3.16 Identification of coactivators involved in LPL expression

Previous work in the laboratory had suggested a role for the steroid receptor coactivator-1 (SRC-1) in the TGF-β-regulation of LPL promoter activity. Transient transfection of U937 cells had shown that the TGF-β-mediated inhibition of LPL -101/+188 promoter activity was attenuated by cotransfection with plasmids specifying for SRC-1 but not with the ubiquitous coactivator p300 (unpublished data, Dr Peli Foka). Figure 5.18A shows that SRC-1 attenuates the TGF-β-mediated inhibition of LPL promoter activity in U937 cells. However, this is only representative of a single initial experiment due to subsequent problems with the U937 cell line.

Figure 5.18B shows that, in Hep3B cells, the SRC-1 plasmid has no significant effect on the TGF-β-regulation of LPL promoter activity whilst p300 changes levels of LPL promoter activity so that they are no longer significantly inhibited by TGF-β (Figure 5.18C). This is in contrast to previous work which showed an involvement for SRC-1 but not p300 in the TGF-β-regulation of LPL in U937 cells, suggesting that this action may be cell-type specific.

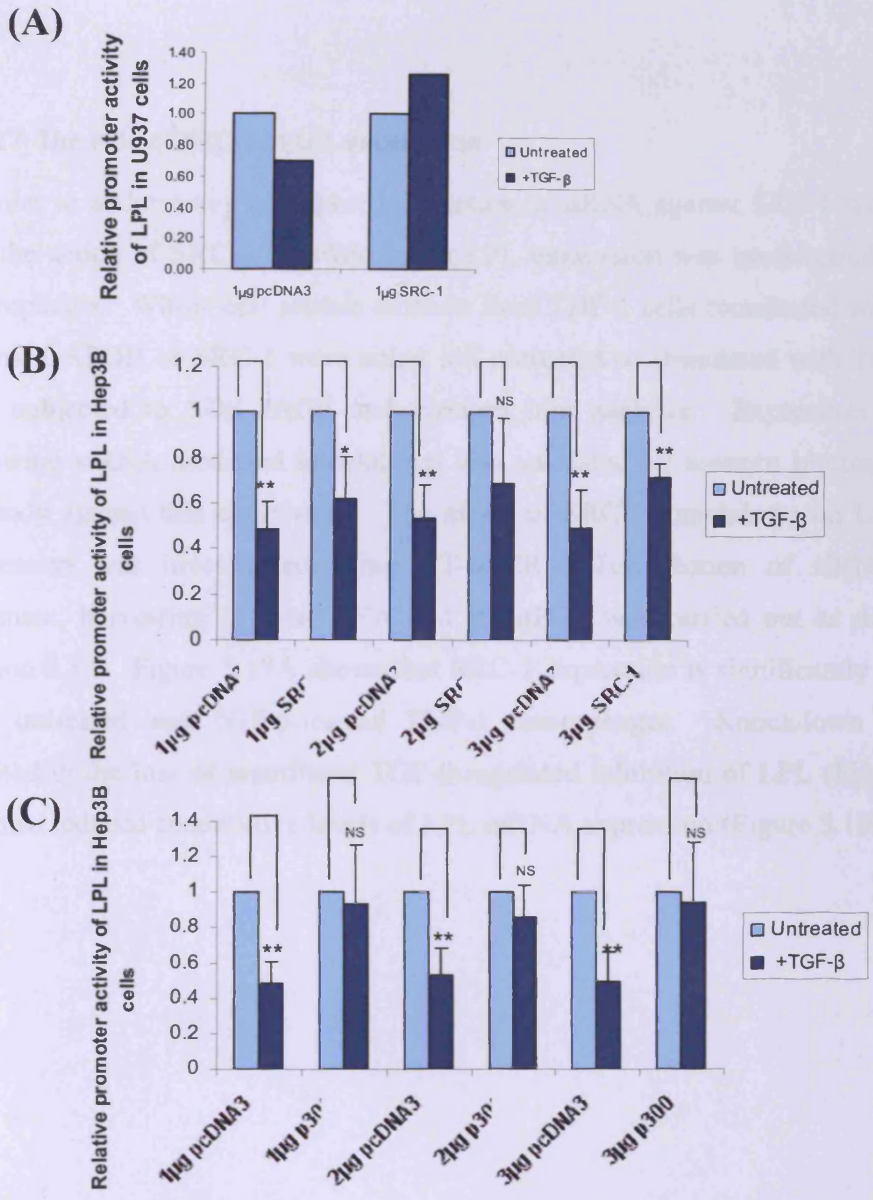

Figure 5.18 **The role of coactivators in the TGF-β-regulation of LPL promoter activity.** U937 (Panel A) or Hep3B cells (Panels B and C) were transfected with 1μg LPL -101/+188 promoter construct and 1, 2 or 3μg of $pcDNA_3$, p300 or SRC-1 as indicated, using Superfect™ as described in Section 2.5.1. Cells were either left untreated or treated with TGF-β overnight and then harvested using 1x passive lysis buffer. Luciferase activity readings were normalised to protein concentration. Data shown is the ratio of luciferase activity:protein concentration normalised to the untreated samples and expressed as fold change from this. With the exception of Panel A which is representative of a single experiment, data is representative of three independent experiments (*P<0.05, **P<0.01, ***P<0.001, NS-not significant).

5.3.17 The role of SRC-1 in LPL expression

In order to address any cell specificity issues, a siRNA against SRC-1 was obtained and the action of SRC-1 knockdown on LPL expression was investigated in THP-1 macrophages. Whole-cell protein extracts from THP-1 cells transfected with siRNAs against GAPDH or SRC-1 were either left untreated or stimulated with TGF-β, and then subjected to SDS-PAGE and western blot analysis. Expression of SRC-1 following siRNA-mediated knockdown was validated by western blotting using an antibody against this coactivator. The effect of SRC-1 knockdown on LPL mRNA expression was investigated using RT-qPCR. Transfection of siRNA, TGF-β treatment, harvesting of total RNA and RT-qPCR was carried out as described in Section 5.3.6. Figure 5.19A shows that SRC-1 expression is significantly reduced in both untreated and TGF-β-treated THP-1 macrophages. Knockdown of SRC-1 resulted in the loss of significant TGF-β-regulated inhibition of LPL (Figure 5.19B) and also reduced constitutive levels of LPL mRNA expression (Figure 5.19C).

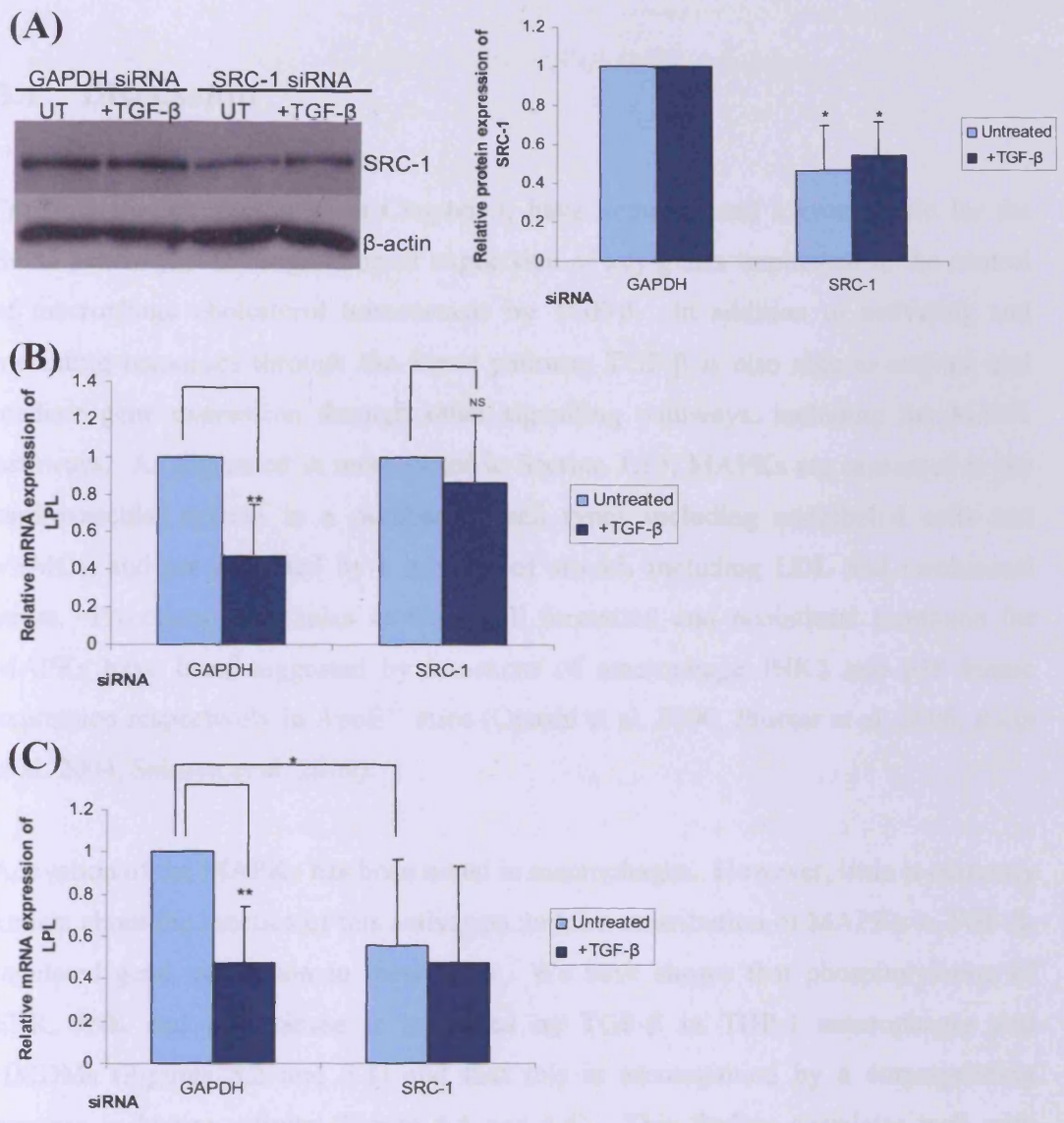

Figure 5.19 SRC-1 has a role in the constitutive and TGF-β-regulated expression of LPL in THP-1 macrophages. Whole cell extracts from untreated and TGF-β-treated cells transfected with validated GAPDH or SRC-1 siRNA were subjected to western blotting using antibodies against SRC-1 and β-actin. Relative expression (mean ±SD) of SRC-1 normalised to β-actin, with untreated samples arbitrarily assigned as 1, from three independent experiments is shown. In panel B and C, total RNA extracts were subjected to RT-qPCR using primers specific to LPL and RPL13A. Relative expression (mean ±SD) of LPL normalised to RPL13A, with untreated samples arbitrarily assigned as 1, from three independent experiments is shown (*P<0.05, **P<0.01).

5.4 Discussion

Previous studies, presented in Chapter 4, have demonstrated a crucial role for the Smad pathway in the regulation of expression of key genes implicated in the control of macrophage cholesterol homeostasis by TGF-β. In addition to activating and mediating responses through the Smad pathway TGF-β is also able to activate and mediate gene expression through other signalling pathways, including the MAPK pathways. As explained in more detail in Section 1.13, MAPKs are expressed in the cardiovascular system in a number of cell types including endothelial cells and VSMCs, and are activated by a number of stimuli including LDL and mechanical stress. Pro-atherogenic roles in foam cell formation and neointimal formation for MAPKs have been suggested by knockout of macrophage JNK2 and p38 kinase expression respectively in $ApoE^{-/-}$ mice (Ohashi et al. 2000, Proctor et al. 2008, Ricci et al. 2004, Seimon et al. 2009).

Activation of the MAPKs has been noted in macrophages. However, little is currently known about the kinetics of this activation and the contribution of MAPKs to TGF-β-regulated gene expression in these cells. We have shown that phosphorylation of ERK, JNK and p38 kinase is increased by TGF-β in THP-1 macrophages and HMDMs (Figures 5.2 and 5.3) and that this is accompanied by a corresponding increase in kinase activity (Figure 5.4 and 5.5). This finding correlates well with studies that have shown that MAPK signalling is active in macrophages and human atherosclerotic lesions (Cheng et al. 2008, Li and Xu 2000). Data from our laboratory has also shown that TGF-β activates the JNK (c-Jun) and p38 kinase pathways in THP-1 monocytes (Singh and Ramji, 2006). The MAPKs are activated with slow kinetics with maximal induction occurring between 12 and 24hr. Slow kinetics may be suggestive of MAPK activation following Smad pathway activation and possibly Smad-dependent transcriptional responses whilst fast kinetics would suggest Smad-independent activation of MAPKs by TGF-β.

The role of each of the MAPK pathways in the TGF-β regulation of gene expression was investigated using siRNA-mediated knockdown of ERK 1/2, c-Jun and p38

kinase. An siRNA against c-Jun, the downstream target of JNK was used in place of a siRNA against JNK as a significant siRNA-mediated knockdown of JNK expression could not be produced in the THP-1 cell line. Knockdown of ERK, c-Jun and p38 kinase expression was determined using western blotting and analysis demonstrated a significant reduction in protein levels of each of the MAPKs following siRNA-mediated knockdown (Figure 5.6).

The results presented in this chapter demonstrate for the first time that the MAPK signalling pathways have a key role in the expression of key genes implicated in the regulation of foam cell formation by TGF-β. The use of siRNA knockdown assays showed that the p38 kinase pathway was required for the TGF-β-regulated inhibition of LPL, SR-A, SR-B1 and CD36 mRNA expression (Figures 5.7 to 5.10) and also for the TGF-β-regulated induction of ABCA-1 and ABCG-1 expression (Figures 5.12 and 5.13). The most potent effects of p38 kinase siRNA-mediated knockdown on TGF-β-regulated gene expression were observed for LPL and SR-B1 whose expression was significantly induced and for ABCA-1 whose expression was significantly decreased by p38 kinase knockdown. This is consistent with a number of studies suggesting that p38 kinase is required for foam cell formation. Inhibition of the p38 kinase pathway using pharmacological inhibitors has demonstrated a requirement for the kinase in the 12/15 lipoxygenase-regulated reduction of ABCG-1 expression and also in the TNF-α-regulated reduction of scavenger receptor expression in murine macrophages (Hsu and Twu 2000, Nagelin et al. 2009). Blockade of the p38 kinase pathway in murine J774 macrophages has also demonstrated a requirement for p38 kinase in oxLDL induced CD36 expression and subsequent foam cell formation through the transactivation of PPARγ (Zhao et al. 2002).

Interestingly our study also revealed roles for p38 kinase in the constitutive expression of both SR-B1 and CD36. Knockdown of p38 kinase resulted in a significant reduction in the mRNA expression of SR-B1 and CD36 (Figure 5.14) suggesting that p38 kinase is required for the basal expression of these scavenger receptors following PMA differentiation of THP-1 cells.

Assays using siRNA specific against c-Jun demonstrated a requirement for c-Jun in the TGF-β-regulated expression of LPL, SR-A, SR-B1, CD36, ABCA-1 and ABCG-1

with the TGF-β-regulated expression of LPL appearing to be most potently affected by c-Jun knockdown. The involvement of p38 kinase and c-Jun in the TGF-β-regulated expression of such a large number of genes suggests a critical role for these MAPKs in the control of foam cell formation. In contrast to this, knockdown of ERK 1/2 had little effect on TGF-β-regulated gene expression. Only the TGF-β-regulated expression of CD36 (Figure 5.10), ABCA-1 and ABCG-1 (Figures 5.12 and 5.13) were reversed following ERK 1/2 knockdown. This finding however is consistent with studies that have demonstrated a role for ERK in the expression of CD36 (Han et al. 2000, Nicholson et al. 2001). As explained in more detail in Section 3.1.5, a requirement for ERK (p44/42) in the TGF-β-regulated expression of CD36 has been shown previously in macrophages where suppression of CD36 is thought to be mediated through an increase in ERK activity leading to inactivation of PPARγ (Han et al. 2000, Nicholson et al. 2001). An involvement for ERK in ABCA-1 and ABCG-1 expression was not supported by a recent study that showed that in murine RAW264.7 macrophages pharmacological and siRNA-mediated inhibition of ERK 1/2 activity resulted in increased ABCA-1 expression and increased cholesterol efflux to ApoAI whilst ERK 1/2 activation resulted in a reduction in both (Zhou et al. 2009). However, this may represent a species-specific difference.

One interesting finding in our study is that the knockdown of ERK 1/2, c-Jun or p38 kinase had no effect on the TGF-β-regulated expression of ApoE (Figure 5.11). This may be due to sensitivity issues with western blotting as compared to RT-qPCR. Changes in the protein expression of SR-A and LPL following p38 kinase knockdown were not consistent with those observed at the mRNA level suggesting that this may be the case. This finding contrasts with previous findings in our laboratory that have demonstrated, using pharmacological inhibitors, a requirement for the JNK and p38 kinase pathways in the TGF-β-mediated induction of ApoE mRNA and protein expression in THP-1 monocytes and macrophages (Singh and Ramji 2006). As described in Section 3.1.6, dominant-negative forms of JNK, c-Jun and p38 kinase blocked ApoE induction while binding of AP-1 was induced by the cytokine but blocked by inhibitors of JNK and p38 kinase (Singh and Ramji 2006). As with the action of the Smad signalling pathway on ApoE expression (Chapter 4), functional redundancy may exist between the MAPKs and this could partly explain the lack of response seen following individual MAPK knockdown. It is also possible that

residual kinase expression following knockdown may be sufficient for the response. Futher studies using siRNA-mediated knockdown of multiple pathway components would be needed to investigate this possibility.

Whilst the mechanisms behind TGF-β regulation of gene activation and inhibition in relation to foam cell formation and atherosclerosis are not well characterised, an involvement for the MAPKs (in particular p38 kinase and c-Jun) in this has been suggested by the results presented in this chapter. However, it is well established that TGF-β classically signals through the Smad pathway and a crucial role for this pathway in the regulation of expression of genes implicated in macrophage cholesterol homeostasis has been demonstrated in Chapter 4 of this thesis. Several lines of evidence have demonstrated an involvement for the Smad pathway and one or more of the MAPK pathways in TGF-β-regulated gene expression. For example, expression of the tumour-suppressor gene PTEN is inhibited at a post-transcriptional level by TGF-β in a number of cell lines and this can be blocked by inhibitors of p38 kinase and the TGF-β type I receptor, suggesting an involvement for both Smad and MAPK pathways in this regulation (Yang et al. 2009). TGF-β-induced expression of CTGF in osteoblasts is Smad-3 dependent but can be blocked by the ERK inhibitor PD98059 and by a dominant-negative ERK (Arnott et al. 2008) and induction of TIMP-3 by the cytokine in chondrocytes also shows a requirement for Smad and ERK pathways (Qureshi et al. 2008, Qureshi et al. 2005).

In light of this, it seems likely that both the Smad and MAPK pathways play critical roles in the TGF-β-regulated expression of key genes implicated in the control of macrophage cholesterol homeostasis and the possibility of crosstalk between the pathways cannot be dismissed. The kinetics of Smad and MAPK activation and the crossover observed in their regulation of the TGF-β-mediated expression of a number of the genes studied in Chapters 4 and 5 of this thesis certainly seem to point towards the regulation of gene expression through crosstalk between different TGF-β-activated signalling pathways. A number of studies on TGF-β-regulated gene expression have evidenced crosstalk between Smad and MAPK pathways although the exact mechanism of crosstalk is often unclear. In human gingivial fibroblasts, MMP-13 expression is upregulated by TGF-β (Leivonen et al. 2002). Adenoviral gene delivery of each of the Smads demonstrated an involvement for Smad-3 in this

regulation and a dominant negative Smad-3 containing adenovirus was able to block this induction (Leivonen et al. 2002). Inhibitors of the upstream regulators of p38 kinase- MKK3b and MKK6b, and the p38 kinase inhibitor SB203580 were able to block Smad-3 induced expression of MMP-13 whilst activation of p38α kinase induced Smad-3 nuclear translocation suggesting that p38α kinase activated Smad-3 (Leivonen et al. 2002). The MKK6-p38 kinase pathway has also been implicated in the activation of the ATF-2 transcription factor in response to TGF-β (Hanafusa et al. 1999). Induction of a TGF-β-inducible reporter construct by Smad-2 and -4 expression was modestly enhanced by MKK6 suggesting that p38 kinase and Smad pathways may act cooperatively, possibly through complex formation in the nucleus (Hanafusa et al. 1999).

Interaction between signalling pathways can often be cell-type and gene specific. For instance, cells of mesangial origin tend to show a synergy between ERK and Smad pathways whilst epithelial cells tend to show that this interaction inhibits downstream events (Hayashida et al. 2003). Stimulation of collagen I and fibronectin production by TGF-β in mesangial cells is mediated by the Smads but can be inhibited by blocking the expression of ERK. The activity of a TGF-β-responsive promoter can also be reduced by blocking the expression of ERK suggesting that maximal induction of Smad activity requires this kinase (Hayashida et al. 2003). Another example of where crosstalk between Smad and MAPK pathways enhances TGF-β-dependent responses in mesangial cells is the regulation of PAI-1 gene expression by the ERK and Smad pathways (Hong et al. 2006). The TGF-β-Smad pathway is stimulated by oxLDL to lead to an increase in the mRNA expression of PAI-1. However this is not a direct regulation and can be inhibited by the ERK inhibitors PD98059 or UO126. Instead, oxLDL causes an increase in ERK activation by TGF-β and this is associated with an increase in the nuclear expression of Smad-3 and subsequent formation of DNA:protein complexes at Smad binding sites within the PAI-1 promoter (Hong et al. 2006).

Interplay between Smad and MAPK pathways is increasingly being observed in relation to atherosclerosis. Overexpression of angiotensin II in $LDLR^{-/-}$ mice reduced collagen accumulation and the expression of MMPs (Dandapat et al. 2008). In addition, such overexpression can modulate p38 kinase phosphorylation in

atherosclerotic regions of arteries in these mice (Zhang et al. 2009). Stimulation of angiotensin II in human dermal fibroblasts activates Smad-2 and Smad-2-dependent transcription and this activation is dependent on p38 kinase activation suggesting that both pathways are active in the regulation of angiotensin II (Dandapat et al. 2008, Zhang et al. 2009). In VSMCs, the ERK and Smad pathways have been implicated in the TGF-β-mediated induction of PAI-1 expression. PAI-1 promotes ECM accumulation and fibrosis therefore impacting on vascular remodelling, neointimal growth and VSMC migration and proliferation and is used as a biomarker for cardiovascular disease-associated mortality (Samarakoon and Higgins 2008). The ERK pathway and Rho/ROCK pathways are required for PAI-1 induction by TGF-β and can modulate duration of Smad phosphorylation and nuclear accumulation (Samarakoon and Higgins 2008). Interplay between the p38 kinase and Smad pathways has also been reported in VSMCs (Dadlani et al. 2008). Both these pathways are involved in proteoglycan synthesis and the elongation of the GAG chains of proteoglycans. This has physiological relevance to cardiovascular disease as this elongation promotes the binding of proteoglycans to LDL that is a key part of the pathogenesis of atherosclerosis. Inhibitors against both pathways reduced chain elongation and proteoglycan binding to LDL (Dadlani et al. 2008).

Of particular interest in our study was the finding that all the genes studied (with the exception of ApoE) showed a requirement for p38 kinase and c-Jun in their TGF-β-regulated expression (Figures 5.7 to 5.10 and Figures 5.12 and 5.13). This involvement of multiple signalling pathways is likely to require convergence of signals at the transcriptional level, possibly through common transcription factors such as AP-1 (Zhang et al. 1998). This has been observed to be the case for the TGF-β-regulated expression of ApoE in THP-1 monocytes and macrophages where an involvement for the JNK and p38 kinase pathways has been suggested and AP-1 binding to the ApoE promoter is induced by the cytokine (Singh and Ramji 2006).

The interaction of the MAPKs and Smads with transcription factor substrates adds complexity to the interplay between different signalling pathways. MAPK-associated transcription factors can modulate the activity of Smads and conversely these transcription factors can be modulated by the Smad pathway (Javelaud and Mauviel 2005). This regulation can be positive or negative and often occurs in a cell-type-,

gene- and/or MAPK-specific manner (Javelaud and Mauviel 2005). ERK, JNK and p38 kinase are able to phosphorylate Smads within the linker region of the protein. Phosphorylation of Smads by MAPKs can affect their ability to move to the nucleus or thereby affect transcriptional activity. For instance, ERK phosphorylation of Smad-1, -2 and -3 reduces the nuclear translocation of Smads following TGF-β stimulation (Javelaud and Mauviel 2005). TGF-β activated JNK can also phosphorylate Smad-3 to induce its nuclear translocation and activity. MSK1 kinase, a substrate of p38 kinase, can modulate the transcriptional activity of Smad-3 by promoting association with the p300 coactivator (Moustakas and Heldin 2005).

The transcription factor AP-1 and Smads tend to cooperate to regulate AP-1-driven promoters while they antagonise each other on Smad-dependent transcription (Javelaud and Mauviel 2005). Smad-3 and c-Jun can interact at the transcriptional level through AP-1 sites. Interestingly, the c-Jun promoter itself contains both AP-1 and Smad3/4 binding sites and can be induced by TGF-β (Dennler et al. 2000). Repression of the Smad pathway by JNK activation in HepG2 cells is mediated by an interaction between Smad-3 and c-Jun (Dennler et al. 2000). Overexpression of constitutively active MKK4 and MEKK1 inhibited TGF-β-induced transcription and disrupts the c-Jun/Smad-3 interaction suggesting that this interaction impairs TGF-β-mediated transcription (Dennler et al. 2000).

Recent evidence has suggested that the inhibitory effect of c-Jun/Smad-3 interaction is a result of the ability of c-Jun to stabilise the interaction of Smad-3 with co-repressors (Javelaud and Mauviel 2005; Pessah et al. 2001). The TGF-β-regulated inhibition of forkhead activin signal transducer-1 (FAST-1) expression is mediated by an interaction between c-Jun and Smad-2 where JNK activation represses Smad-2 activity and enhances the association of c-Jun with the co-repressor TGIF (Pessah et al. 2001). This blocks the association between Smad-2 and coactivators such as CBP/p300 and stabilises interaction with TGIF (Pessah et al. 2001). Association of c-Jun with the Smad corepressor Ski has also been demonstrated. This association, in the absence of TGF-β signalling functions to keep Smad-2-regulated genes in a repressed state until TGF-β stimulation when the complex disassociates to allow gene transcription (Javelaud and Mauviel 2005, Pessah et al. 2002).

Of particular interest in our study, is the finding that the TGF-β-regulated inhibition of LPL expression is reversed by siRNA-mediated knockdown of c-Jun (Figure 5.7). Previous work carried out in the laboratory had suggested an involvement for the JNK pathway in the TGF-β-regulation of LPL expression in macrophages (Section 5.3.15). This was partly confirmed using transient transfection of dominant-negative forms of JNK, SEK-1 and c-Jun in Hep3B cells but cell-type-specific differences were observed (Figure 5.17).

Previous work carried out in the laboratory had also demonstrated a role for SRC-1 in LPL expression (Section 5.3.16). In addition to the transfection studies described in Section 5.3.16, plasmid constructs specifying for multiple copies of the Sp1(62/65) and Sp1(44) sites of the promoter were used to demonstrate a role for SRC-1 in the TGF-β-mediated inhibition of LPL, which was reversed following co-transfection with SRC-1 in U937 cells but not in Hep3B cells (Figure 5.18). This response is therefore likely to be cell-type specific. Indeed LPL is expressed in foetal liver but not adult liver (Semenkovich et al. 1989) and cancerous cell lines tend to have properties of foetal cells. In addition, the basal expression of LPL was increased following co-transfection with SRC-1, suggesting a role for the coactivator in constitutive LPL expression (unpublished data, Dr Peli Foka). We have confirmed these findings in THP-1 macrophages using a siRNA against SRC-1 where knockdown of SRC-1 resulted in a reversal of TGF-β-mediated inhibition of LPL mRNA expression and a reduction in its basal expression (Figure 5.19). This finding, coupled with the effect of c-Jun knockdown on LPL expression suggests that the TGF-β-regulated expression of LPL is mediated through the JNK pathway and involves interaction with SRC-1. Based on this it is possible that the mechanism of TGF-β-mediated LPL inhibition in THP-1 macrophages is mediated through activation of the JNK pathway. Subsequent activation of c-Jun/ AP-1 may serve to 'mop up' SRC-1 (the likely required coactivator for constitutive LPL expression) or inactivate the coactivator through dephosphorylation resulting in a reduction of LPL expression following TGF-β stimulation. As explained in more detail in Section 1.16.1, SRC-1 has previously been linked with TGF-β signalling. The coactivator has been shown to potentiate TGF-β signalling through Smad-3 by enhancing interaction between the Smad and p300/CBP, a coactivator associated with Smad-mediated transcription (Dennler et al. 2005). In addition, the coactivator contains consensus

sites for MAPK phosphorylation and has been shown to be phosphorylated by ERK-2 *in vitro* suggesting that SRC-1 is a regulatory target of the MAPK pathways (Rowan et al. 2000).

In conclusion, the studies presented in this chapter have identified a role for the MAPK signalling pathways in the control of macrophage cholesterol homeostasis by TGF-β. This is summarised in Figure 5.20. The TGF-β-regulation of expression of genes implicated in macrophage cholesterol homeostasis is defined for key genes (see Chapter 3), but very little is known about the role of non-classical signalling pathways in this regulation. These studies suggest a critical role for the MAPK pathways in the TGF-β-regulation of LPL, SR-A, SR-B1, CD36, ABCA-1 and ABCG-1 expression in THP-1 macrophages. This suggests that the JNK, p38 kinase and (to a lesser extent) ERK pathways are crucial for the attenuation of foam cell formation by TGF-β and therefore contribute to the anti-atherogenic role of TGF-β in atherosclerosis.

Further work will be needed to extend these findings at a cellular level and to further elucidate the exact molecular mechanisms behind the TGF-β regulation of expression of genes implicated in macrophage cholesterol homeostasis as has been partly carried out for LPL. In particular, studies on the how the involvement of multiple signalling pathways converges to regulate target gene expression will be important to enhance understanding about the exact mechanisms of gene regulation by TGF-β.

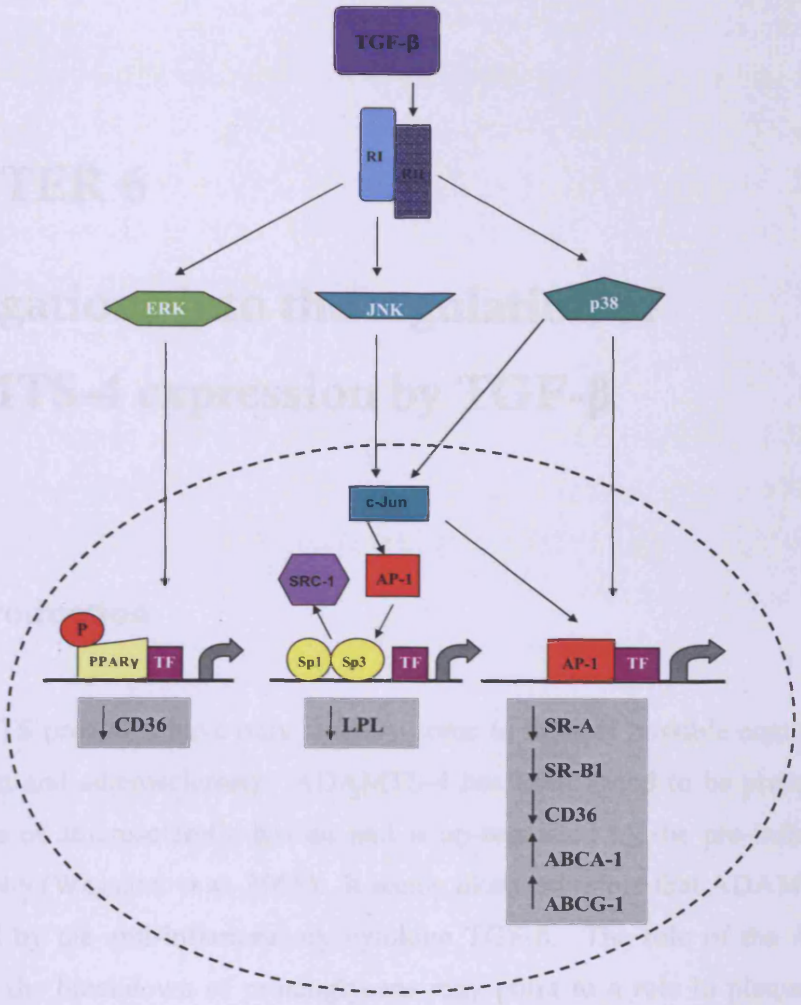

Figure 5.20 **The involvement of the MAPK signalling pathways in the TGF-β-regulated expression of key genes implicated in macrophage cholesterol homeostasis.** Previously, the only macrophage-expressed gene known to be regulated through a TGF-β-MAPK pathway was CD63 whose expression is down-regulated by the cytokine through activation of ERK and subsequent phosphorylation of PPARγ (Han et al. 2000). From the results presented in this chapter the TGF-β-regulated expression of LPL, SR-A, SR-B1, ABCA-1 and ABCG-1 can be added to this list. Whilst the mechanism behind LPL regulation has been partly characterised the exact mechanism of regulation through MAPK activation of the other genes studied is unclear. Interestingly, with the exception of CD36 and ApoE, all the genes studied showed some requirement for both c-Jun and p38 kinase pathways in their TGF-β-regulation. The exact contribution of these pathways to gene regulation and the mechanisms of crosstalk remain unclear.

CHAPTER 6

Investigations into the regulation of ADAMTS-4 expression by TGF-β

6.1 Introduction

The ADAMTS proteases have only recently come to light as possible contributors to inflammation and atherosclerosis. ADAMTS-4 has been found to be present within macrophages of atherosclerotic lesions and is up-regulated by the pro-inflammatory cytokine IFN-γ (Wågsäter et al. 2008). It seems likely therefore that ADAMTS-4 will be regulated by the anti-inflammatory cytokine TGF-β. The role of the ADAMTS proteases in the breakdown of proteoglycans may point to a role in plaque stability and the latter stages of atherosclerosis (Wight 2005). It was therefore of interest to study the regulation of ADAMTS-4 as a new TGF-β target gene in macrophages. This would increase our knowledge of TGF-β-mediated regulation of gene expression with respect to both foam cell formation and plaque stability. As the structure and function of ADAMTS proteases has not been discussed previously in this thesis, a review of the current knowledge regarding them has been included in this chapter.

6.1.1 The ADAMTS family of proteases

The ADAMTS family of proteases are a distinct family of 19 members, ADAMTS-1 to ADAMTS-19, that are structurally related to the ADAM and MMP families of proteinases, as detailed in Section 6.1.2 (Jones and Riley 2005; Porter et al. 2005). ADAMTS proteases are non-membrane bound enzymes that bind to components of the ECM such as pro-collagen, hyalectans and cartilage oligomeric matrix protein to

cause their degradation (Jones and Riley 2005; Wang et al. 2003). They are cleaved to become active through their pro-domain which contains at least one furin cleavage consensus motif (Tang 2001). Following secretion, mature ADAMTS proteases can undergo further processing at the C-terminal end. Cleavage occurs within the spacer region but while studies on ADAMTS-4 have shown that this can be autocatalytic, it is also possible that cleavage is mediated by matrix metalloproteinases (Jones and Riley 2005; Porter et al. 2005).

The ADAMTS family is usually subdivided into four classes based on structural and/or functional similarities. These are the aggrecanases (hyelectanases), the procollagen-n-peptidases, von Willibrand cleaving factor (ADAMTS-13) and the other ADAMTS proteins (Jones and Riley 2005). The mammalian ADAMTS proteases share between 20 and 40% homology with ADAMTS-13 being the most distinct (Porter et al. 2005; Tang 2001). Little is known about the function of a number of the ADAMTS members with most of the genes are only expressed at low levels. ADAMTS-1, -4 and -7 are the most abundant of the proteases (Tang 2001). ADAMTS members with unknown function include ADAMTS-6, -7, -10, -11, -12 and -16 to -19 (Jones and Riley 2005; Porter et al. 2005).

6.1.1.1 The aggrecanases

ADAMTS-1, -4, -5, -8, -9 and -15 are collectively known as the aggrecanases as they cleave the hyalectan, aggrecan. Aggrecan is a 1000-2000kDa protein with 3 globular domains, 2 in the N-terminal region connected by an interglobular domain and one in the C-terminus linked through a large GAG attachment region (Malfait et al. 2002; Yamanishi et al. 2002). It is present in large amounts in the articular cartilage and is important for withstanding compressive deformation during joint movement and for protecting collagen from degradation (Naito et al. 2007; Tang 2001). Aggrecan is cleaved by both MMPs and ADAMTS proteases at distinct sites and may itself contain a conserved recognition site for cleavage by the ADAMTS proteases (Miwa et al. 2009). ADAMTS proteases cleave aggrecan at the Glu^{373}-Ala^{374} bond within the interglobular region and also at four other sites (Glu^{1480}–Gly^{1481}, Glu^{1667}–Gly^{1668}, Glu^{1771}–Ala^{1772} and Glu^{1871}–Leu^{1872}) within the GAG region (Haddock et al. 2007; Hashimoto et al. 2001; Malfait et al. 2002; Porter et al. 2005).

ADAMTS-1 was the first of the ADAMTS proteases to be discovered in 1997 (Kuno et al. 1997) and was later identified as a potent inhibitor of angiogenesis (Vázquez et al. 1999) and then as an 'aggrecanase' (Kuno et al. 2000). A role for the ADAMTS proteases in ECM degradation was first suggested by knockout of ADAMTS-1 in mice where impaired formation of adipose tissue and accumulation of collagen within the skin tissue suggested that the processing of collagen and other ECM molecules was defective (Shindo et al. 2000).

ADAMTS-4 and -5 show the highest aggrecanase activity and have been implicated in osteoarthritis and rheumatoid arthritis (Bondeson et al. 2008; Huang and Wu 2008). Studies in knockout mice revealed that ADAMTS-5 was the primary protease responsible for aggrecan degradation in mice (Glasson et al. 2004; Glasson et al. 2005). Although ADAMTS-4 is predominantly expressed in human osteoarthritic cartilage it is currently unclear whether ADAMTS-4 or ADAMTS-5 is responsible for aggrecan degradation in humans (Huang and Wu 2008; Naito et al. 2007). In osteoarthritis aggrecan is cleaved by ADAMTS proteases, and fragments are released into the synovial fluid (Haddock et al. 2007; Hashimoto et al. 2001; Malfait et al. 2002; Porter et al. 2005). This cleavage may be caused by overproduction of cytokines by synovial macrophages. TNF-α and IL-1β have been identified as likely key players in this process. Studies using cultures of osteoarthritic synovial cells have shown that ADAMTS-4 expression can be induced by TNF-α and IL-1β and down-regulated in response to blocked TNF-α and IL-1β signals (Bondeson et al. 2006; Naito et al. 2007). Studies in mice have shown that knockout of ADAMTS-5 (and not ADAMTS-4) can prevent degradation of cartilage suggesting that ADAMTS-5 is the active cartilage proteinase in mice while ADAMTS-4 is active in human cartilage (Jones and Riley 2005; Naito et al. 2007).

In addition to aggrecan, the ADAMTS proteases have been shown to cleave additional proteoglycan substrates. ADAMTS-4 also cleaves brevican, one of the primary proteoglycans in the central nervous system (CNS). This action may be relevant to the pathophysiology of stroke (Haddock et al. 2007). ADAMTS-1, -4 and -9 are able to cleave versican, a proteoglycan structurally similar to aggrecan but found within the vasculature. Its expression is upregulated in all forms of vascular disease and it

accumulates in blood vessels susceptible to atherosclerosis (Porter et al. 2005; Wight and Merrilees 2004). Interestingly aggrecan, brevican and versican are all cleaved by the aggrecanases at specific Glu-Xaa bonds (Haddock et al. 2007).

6.1.1.2 The procollagen-n-proteinases

ADAMTS-2, -3 and -14 are termed procollagen-n-proteinases due to their involvement in the removal of N-terminal peptides from procollagen to form mature collagen. ADAMTS-2 is thought to play a role in the formation of skin and (similar to other collagen C-proteinases) its expression can be up-regulated by the cytokine TGF-β (Wang et al. 2003). ADAMTS-3 and -14 are thought to function primarily in cartilage and tendon respectively (Jones and Riley 2005).

6.1.1.3 Von Willibrand cleaving factor

ADAMTS-13 has been identified as the protease that cleaves the multimeric glycoprotein, von-Willibrand factor (VWF) (Fujikawa et al. 2001). This protein, present predominantly in the plasma, has a key role in coagulation. VWF is a carrier for clotting factor VIII and has a significant role in coagulation and thrombus formation as part of the thrombin pathway. In atherosclerosis, VWF binds to ECM molecules to promote platelet adhesion and aggregation at sites of damage to the vascular wall (Jones and Riley 2005; Porter et al. 2005). ADAMTS-13 has been demonstrated to colocalise with VWF in thrombi of human coronary arteries. Cleavage of VWF by ADAMTS-13 is thought to attenuate thrombus formation in atherosclerotic plaques and a role for the dinsintegrin domain of ADAMTS-13 in this process has been identified (Moriguchi-Goto et al. 2009).

6.1.2 Domain structure of ADAMTS proteases

The ADAMTS proteases have a domain structure, as shown in Figure 6.1, that is conserved throughout the family. ADAMTS proteases are synthesised as proproteins which are cleaved to become active through the removal of the pro-domain by a furin proprotein convertase within the trans-Golgi (Kuno et al. 1999; Wang et al. 2004). The catalytic region of ADAMTS proteases is comprised of a catalytic zinc coordinated by three histidine residues. This active site motif is followed by a highly conserved methionine residue, a pattern also observed in the ADAM and MMP

families (Jones and Riley 2005). The catalytic region is followed by a smaller, disintegrin-like domain. This region shares 25-45% homology with the snake venom disintegrins but does not interact with integrins. Following this region are the thrombospondin type 1-like repeats (TSR). These sequences are unique to the ADAMTS proteases amongst the matrix protease families and are homologous to the type-1 repeats found within thrombospondins 1 and 2. One important function of this domain is to bind to the ECM and to glycosaminoglycans such as heparin (Jones and Riley 2005; Porter et al. 2005; Tang 2001). The highly conserved cysteine-rich domain (CRD) follows the TSR region and contains 10 cysteine residues. The crystal structure of ADAMTS-1 revealed that this was stacked against the active site of the protease (Gerhardt et al. 2007). It is followed in by a spacer region that varies in length and sequence but usually contains several hydrophobic residues towards the N-terminal end. Between 1 and 14 TSRs follow the CRD and spacer in all ADAMTS proteases except ADAMTS-4 (Jones and Riley 2005; Tang 2001).

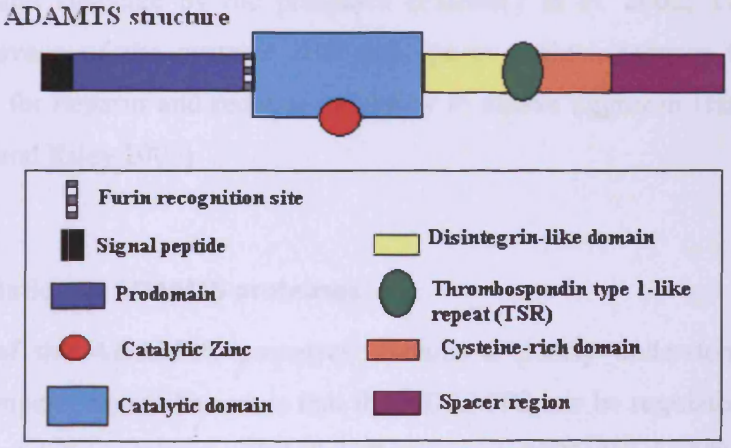

Figure 6.1 **The domain structure of the ADAMTS proteases.** The prodomain of the protein is cleaved by furin to form the mature form of the proteinase. ADAMTS proteases are zinc-metalloproteinases so their catalytic domain contains zinc coordinated by three histidine residues at the active site. The thrombospondin type 1-like repeats and the lack of a transmembrane domain distinguishes the ADAMTS from the ADAM proteases.

The ability of the ADAMTS proteases to bind to the extracellular matrix is one important property that distinguishes the ADAMTS family from the ADAM and MMP families (Kuno et al. 1999). The proteases bind to sulphated glycosminoglycans present in the ECM including heparin and aggrecan (Porter et al. 2005). Both the catalytic and the non-catalytic regions of the ADAMTS proteases are required for full proteolytic activity and substrate binding. This is achieved through post-translational processing (C-terminal truncation) of mature ADAMTS proteases within the spacer region and may be mediated by MMPs (Gao et al. 2002).

Studies using murine ADAMTS-1 domain-deletion constructs and C-terminally processed forms of human ADAMTS-4 identified the TSP type 1 motifs and the cysteine-rich and spacer regions in the C-terminal domain as crucial for ECM binding, with truncation of this region resulting in a loss of ECM binding (Kuno and Matsushima 1998; Porter et al. 2005). In addition, the spacer and cysteine rich regions together with the thrombospondin motifs are required for substrate recognition and cleavage by the proteases (Flannery et al. 2002; Tortorella et al. 2000). Cleavage of the cysteine rich and spacer regions reduces the affinity of ADAMTS-4 for heparin and reduces its ability to cleave aggrecan (Hashimoto et al. 2004; Jones and Riley 2005).

6.1.3 Regulation of ADAMTS proteases

Regulation of the ADAMTS proteases remains a poorly understood area. One regulatory property that is known is that the ADAMTS can be regulated by the tissue inhibitors of metalloproteinases (TIMPs) (Porter et al. 2005; Wight 2005). TIMPs -1, -2, -3 and -4 are endogenous inhibitors of the MMPs (Galis and Khatri 2002). However, TIMP-3 has been found to be an important specific inhibitor of ADAMTS-4 and -5. In addition TIMP-2 and TIMP-3 can inhibit ADAMTS-1 aggrecanase activity (Rodríguez-Manzaneque et al. 2002). Hashimoto et al. (2001) incubated ADAMTS-4 with TIMPs to demonstrate the inhibitory action of these molecules on the ability of ADAMTS-4 to cleave aggrecan. Analysis of the IC_{50} values revealed that the activity of TIMP-3 was 50-fold higher than that observed with TIMPs-1 and 2

and approximately 250-fold higher than the activity of TIMP-4, suggesting that this inhibitory action on ADAMTS-4 is specific to TIMP-3 (Hashimoto et al. 2001). Although the mechanism of inhibition has not been fully elucidated, a study by Kashiwagi et al. (2001) demonstrated that the N-terminal domain of TIMP-3 was able to inhibit the aggrecanase activity of ADAMTS-4 and -5 suggesting that the N-terminal end of TIMP-3 may interact with ADAMTS. A recent study has demonstrated that this interaction is enhanced by the C-terminal domain of ADAMTS-4 and ADAMTS-5. Binding of TIMP-3 may prevent interaction between the ADAMTS and aggrecan by altering the active site to favour interaction with TIMP-3 rather than the aggrecan substrate (Kashiwagi et al. 2001; Troeberg et al. 2009).

6.1.4 ADAMTS proteases – Links with inflammation and atherosclerosis

Changes in the degradation of ECM by MMPs and other proteases have been associated with a number of pathological inflammatory conditions including atherosclerosis and rheumatoid arthritis. This shift in balance of synthesis and degradation is often driven by inflammation, injury and oxidative stress (Galis and Khatri 2002; Galis et al. 1994; Malemud 2006; Wågsäter et al. 2008). In atherosclerosis, macrophages and monocytes are known to secrete proteolytic enzymes to influence the development of the lesion and/or the stability of the plaque (Worley et al. 2003; Wågsäter et al. 2008). Differentiation of monocytes to macrophages is also accompanied by an increase of matrix-degrading proteases (Whatling et al. 2004). Immunohistochemical analysis of human carotid lesions and advanced atherosclerotic plaques has shown that ADAMTS-1, -4, -5 and -8 are present within lesions (Wågsäter et al. 2008). Of these, ADAMTS-4, -5 and -8 colocalise with macrophages whilst ADAMTS-1 colocalises with endothelial and smooth muscle cells (Jönsson-Rylander et al. 2005; Wågsäter et al. 2008). ADAMTS-1 and ADAMTS-4 are the most abundant of the proteases and have consequently been the focus of studies on the link between ADAMTS proteases and atherosclerosis (Jones and Riley 2005).

The enhanced expression of ADAMTS-1 in the kidney and heart observed in response to *in vivo* lipopolysaccharide (LPS) administration first suggested that ADAMTS

proteases were associated with inflammatory processes. The observation that murine ADAMTS-1 mRNA expression could be up-regulated by IL-1 (a pro-inflammatory mediator) was also consistent with a proposed role for ADAMTS-1 in inflammation (Kuno and Matsushima 1998; Tang 2001; Wight 2005).

In endothelial cells ADAMTS-1 expression can be induced by the pro-inflammatory cytokines TNF-α and LPS and this up-regulation is significantly reduced in the presence of HDL (Norata et al. 2004). This demonstrates that ADAMTS-1 can be produced in response to inflammatory stimuli and may promote inflammation. This can be countered by HDL, possibly due to its protective role in endothelial cell function and atherosclerosis (Norata et al. 2004). It is possible that HDL may inhibit the expression of genes coding for proteolytic enzymes by endothelial cells as can be observed for the expression of several adhesion molecules by endothelial cells (Norata et al. 2004). ADAMTS-1 has also been shown to inhibit the proliferation of endothelial cells by sequestration of VEGF. This is achieved through direct binding of the protease to VEGF, thereby preventing its interaction with the cognate receptor (Luque et al. 2003; Norata et al. 2004). Conversely, the expression of ADAMTS-1 can be up-regulated by VEGF and this is protein kinase C-dependent, suggesting a feedback mechanism for the regulation of angiogenesis (Xu et al. 2006). The expression of both ADAMTS-1 and VEGF in endothelial cells can be induced by hypoxia (Hatipoglu et al. 2009).

ADAMTS-1 has been linked with atherosclerosis development through a role in promoting inflammation and VSMC migration (Jönsson-Rylander et al. 2005; Wågsäter et al. 2008). Immunohistochemistry has shown that ADAMTS-1 mRNA is present at high levels in the aorta and colocalises with endothelial cells and VSMCs in atherosclerotic lesions (Jönsson-Rylander et al. 2005; Wågsäter et al. 2008). Expression of ADAMTS-1 is higher in migrating and proliferating VSMCs. Mice overexpressing ADAMTS-1 crossed with ApoE-deficient mice show an increased thickening of the arterial intima suggesting that ADAMTS-1 could be involved with expansion of lesions (Wight 2005). ADAMTS-1 expression is also up-regulated by shear stress in endothelial cells (HUVECs) suggesting a role for the protease in the adaptations in the vessel structure during vascular events (Bongrazio et al. 2000).

In contrast with ADAMTS-1, ADAMTS-4 colocalises with macrophages of atherosclerotic lesions (Wågsäter et al. 2008). Studies using the LDL-receptor and ApoB 100/100 deficient, atherosclerotic mouse model, have shown that the levels of ADAMTS-4 mRNA are induced almost three-fold during lesion development (Wågsäter et al. 2008). *In vitro* experiments in THP-1 cells and primary monocytes have shown that the expression of ADAMTS-4 and -8 expression are increased following the differentiation of monocytes to macrophages whilst the expression of ADAMTS-1, -2, -5, -7 and -10 remains unchanged (Porter et al. 2005; Worley et al. 2003; Wågsäter et al. 2008). The induction of ADAMTS-4 and -8 expression is thought to be mediated through a secondary signal. IL-1β is a suggested candidate for this as it is induced by PMA in monocytes and has also been demonstrated to induce ADAMTS-4 expression in human chondrocytes (Worley et al. 2003) and decrease versican synthesis in arterial smooth muscle cells (Lemire et al. 2007). However, studies have shown that IL-1β does not induce ADAMTS-4 expression in differentiating THP-1 cells (Wågsäter et al. 2008) suggesting that this regulation may be cell-type specific.

6.1.5 Cleavage of versican by ADAMTS proteases – relevance to atherosclerosis

The ability of both ADAMTS-1 and -4 to cleave the proteoglycan versican is likely to be central to hypothesised roles for the proteases in atherosclerosis (Worley et al. 2003). Versican fragments are present in both arterial samples and in non-human primate vascular graft models, indicating that versican undergoes processing and this processing is likely to be carried out by the ADAMTS proteases (Kenagy et al. 2005).

6.1.5.1 Versican

Versican is a proteoglycan belonging to the same 'hyaluronan-binding' family as aggrecan and brevican and is similar in structure to aggrecan in particular in the N and C-terminal domains (Sandy et al. 2001). Versican is a key component of the ECM where it can interact with a number of molecules to regulate cellular processes including adhesion, proliferation and migration (Wight 2002).

Four isoforms of versican (V0, V1, V2 and V3) arise from alternative splicing of the same gene, located on chromosome 5q in humans (Rahmani et al. 2006; Wight 2002).

The four isoforms differ by the presence or absence of two glycosaminoglycan binding domains designated α-GAG and β-GAG. All versican isoforms are made up of an N-terminal globular domain which contains binding elements for interaction with ECM components such as hyaluronan, and a C-terminal globular domain containing two EGF-like repeats, a complement-regulatory protein-like repeat and a C-type lectin domain (Rahmani et al. 2006; Wight and Merrilees 2004). The glycosaminoglycan binding domains contain between 5 and 23 chondroitin sulphate attachment regions depending on versican isoform and tissue type and location (Rahmani et al. 2006). V0 contains both α-GAG and β-GAG binding domains, V1 contains α-GAG, V2 contains β-GAG and V3 has no glycosaminoglycan binding domains (Kenagy et al. 2006; Wight 2002).

6.1.5.2 Versican and atherosclerosis

The contribution of versican to atherosclerosis is not straightforward. In addition to being present within developing blood vessels, the expression of versican is upregulated in all forms of vascular disease and has also been found to accumulate in different arterial lesions including restenotic lesions and atherosclerotic plaques (Lemire et al. 2007; Wight 2005; Worley et al. 2003). Studies in non-human primates have shown that the proteoglycan localises with macrophages in developing and advanced atherosclerotic lesions but is the dominant proteoglycan in areas rich in VSMCs (Raines 2000; Worley et al. 2003). Versican is present within all layers of arteries and expression is increased in intimal thickenings (Wight 2002; Wight and Merrilees 2004). Of the different isoforms, the V0, V1 and V3 forms are detectable in the human aorta and expressed by VSMCs (Sandy et al. 2001). Versican is synthesised by arterial smooth muscle cells and is able to influence the phenotypes of arterial smooth muscle cells and endothelial cells (Lemire et al. 2007). In addition, versican expression has been detected at the plaque thrombus interface where it is thought to promote platelet adhesion and aggregation (Mazzucato et al. 2002).

In non-disease states versican is likely to provide structure to vasculature through its interaction with hyaluronan (Wight and Merrilees 2004). Formation of complexes between hyaluronan and versican are necessary for the migration and proliferation of VSMCs following wounding. This complex formation is rapid and occurs during the

detachment stage of SMC migration (Evanko et al. 1999) and can be up-regulated by PDGF in arterial SMCs (Evanko et al. 2001). In addition to binding to versican, hyaluronan can serve an attachment ligand for macrophages and lymphocytes suggesting that hyaluronan-versican complexes may also influence the retention and adhesion of inflammatory cells (Wight and Merrilees 2004). The chondroitin sulphate (CS) chains of versican can interact with a number of adhesion molecules and chemokines such as L-selectin and P-selectin that may influence the migration and recruitment of vascular cells including macrophages (Hirose et al. 2001; Kawashima et al. 2000).

In advanced atherosclerosis, the location of versican close to accumulated lipoproteins at the edge of lesions, suggests that versican and versican complexes may have a role in the retention of lipoproteins in addition to inflammatory cells (Wight and Merrilees 2004). Multiple LDL particles are able to bind to the CS chains of versican and other proteoglycans. Elongation of CS chains is observed in vascular injury and promotes binding to LDL (Wight and Merrilees 2004). Versican-LDL complex formation can modulate (increase) lipoprotein uptake in both SMCs and macrophages (Ismail et al. 1994; Srinivasan et al. 1995). Versican-LDL complexes are rapidly taken up by macrophages and can be taken up through the LDL receptor pathway in SMCs (Hurt-Camejo et al. 1992; Llorente-Cortés et al. 2002). Formation of these complexes is enhanced by LPL (Olin et al. 1999).

6.1.5.3 Versican cleavage and atherosclerosis

Sandy et al. (2001) first showed that in the human aorta, versican fragments could be generated by ADAMTS-1 and ADAMTS-4 digestion of human intact versican (Sandy et al. 2001). The production of a 70kDa fragment demonstrated that consensus motifs (Glu-X bonds) for ADAMTS cleavage were present within versican and other aggregating proteoglycans. The inability of other MMP enzymes to cleave at this site (despite their ability to degrade the protein) further supported the cleavage of versican by ADAMTS proteases (Sandy et al. 2001). It is now known that the ADAMTS-1 and -4 proteases cleave versican (isoformsV0 and V1) at the Glu441-Ala442/ Glu1428-Ala1429 bond (Jönsson-Rylander et al. 2005; Sandy et al. 2001). In the case of ADAMTS-1, the degradation of the primary proteoglycan component of the

vascular ECM could facilitate the migration of VSMCs (Jönsson-Rylander et al. 2005). Interestingly, ADAMTS-7 has been demonstrated to facilitate the migration of VSMCs and intimal thickening in a rat vascular balloon-injury model and this is thought to be mediated by the breakdown of cartilage oligomatrix protein (COMP) by the protease (Wang et al. 2009).

In addition to increased proliferation and migration of VSMCs, versican fragments have also been linked with changes in ECM volume of vascular lesions and therefore with intimal growth and regression (Kenagy et al. 2005; Sandy et al. 2001). The ECM plays a large role in neointimal thickening, contributing between 60 and 80% to the mass in vascular lesions. The high number of glycosaminoglycan chains present in versican contributes to the water content and volume of the intima making the removal/deposition of proteoglycans a highly effective way to control ECM volume (Kenagy et al. 2005). Using a graft repair model, it is possible to show that generation of versican fragments can be increased by high blood flow, indicating that the activity of ADAMTS proteases in blood vessels may be mediated by shear stress (Kenagy et al. 2005). Intimal tissue regression following balloon- or stent-mediated injury has been shown to be associated with increased blood flow and loss of versican. In a baboon vascular graft model high blood flow causes cell death and loss of ECM. This is accompanied by an increase of a versican cleavage product that can be generated by the ADAMTS proteases -1, -4, -5 and -9. Kenagy and colleagues measured the mRNA levels of ADAMTS proteases in this graft model and found that levels of ADAMTS-4 were significantly increased following a switch to high blood flow and that this was linked with tissue atrophy (Kenagy et al. 2005; Kenagy et al. 2009).

6.1.5.4 Regulation of versican cleavage by cytokines

Versican degradation by ADAMTS proteases may be regulated by cytokines. Little is currently known about cytokine regulation of ADAMTS proteases in relation to atherosclerosis although the expression of ADAMTS-1 and ADAMTS-4 are upregulated by pro-inflammatory cytokines (Wågsäter et al. 2008). In contrast, the regulation of versican expression is known to be regulated by a number of cytokines including PDGF, TGF-β and IL-1β (Schönherr et al. 1991; Lemire et al. 2007). Studies in monkey arterial smooth muscle cells showed that PDGF and TGF-β

upregulate the expression of versican mRNA (Schönherr et al. 1991). In addition, immunostaining of atherosclerotic lesions from hypercholesterolemic nonhuman primates have shown colocalisation of versican-hyaluronan complexes with TGF-β and PDGF positive cells in the ECM (Evanko et al. 1998). Further studies in arterial smooth muscle cells have shown that IL-1β reduces the mRNA stability of versican leading to decreased levels of synthesis (Lemire et al. 2007) and interestingly the cytokine is also able to upregulate decorin expression and induce aggrecan degradation through the upregulation of ADAMTS aggrecanase activity (Lemire et al. 2007). This is an area for further study.

6.2 Aims of experimental studies

A role for the ADAMTS proteases in atherosclerosis is only beginning to be investigated. Previous studies have shown that ADAMTS-1 and ADAMTS-4 are highly expressed in vascular cells and in atherosclerotic lesions. In particular, it has been shown that ADAMTS-4 colocalises with macrophages of lesions (Wågsäter et al. 2008). We therefore wanted to investigate the expression and regulation of ADAMTS-4 in the THP-1 cell line as this is widely accepted as a model for human monocyte-derived macrophages. As previous work in our laboratory has concentrated on the regulation of key genes implicated in atherosclerosis by cytokines, it was of particular interest to us to characterise the regulation of ADAMTS-4 by the anti-inflammatory cytokine TGF-β and to delineate the mechanisms underlying this regulation.

Very few studies have demonstrated the regulation of the ADAMTS proteases by growth factors, hormones and cytokines and many of the studies focus on cell lines relevant to osteoarthritis as a major role for ADAMTS-4 in this process has been defined. In the case of TGF-β, the cytokine has been demonstrated to induce ADAMTS-4 expression in a number of cell lines including human synoviates and articular chondrocytes (Moulharat et al. 2004; Yamanishi et al. 2002). In articular chondrocytes ADAMTS-4 is also up-regulated by the pro-inflammatory cytokines TNF-α and IL-1 but by different pathways to that of TGF-β (Thirunavukkarasu et al. 2006). This suggests that TGF-β may act in a pro-inflammatory manner in this context. However, TGF-β is known to stimulate the synthesis of proteoglycans, collagen and TIMPs in a number of cell types so enhancing the deposition of ECM during the early stages of osteoarthritis and antagonising the effects of pro-inflammatory mediators (Moulharat et al. 2004). In this way TGF-β may enhance cartilage turnover by stimulating both synthesis and degradation. It will be interesting to see if this is the case in relation to ECM turnover in atherosclerosis.

To our knowledge only one study has been conducted that investigated the expression of ADAMTS proteases by cytokines in THP-1 cells. It demonstrated that treatment

with pro-inflammatory cytokines TNF-α or IFN-γ up-regulated the expression of ADAMTS-4, -7, -8 and -9 but had no effect on ADAMTS-1 (Wågsäter et al. 2008). Initial studies in our laboratory had indicated that ADAMTS-4 expression was inhibited by TGF-β treatment in THP-1 cells (Arnaoutakis 2008). We therefore wanted to investigate this further as an initial step towards functional dissection of the ADAMTS-4 promoter. The aims of the studies presented in this chapter were to investigate the regulation of ADAMTS-4 by TGF-β. Initially this was carried out using RT-PCR and Western blot analysis with key results confirmed in primary cultures of HMDMs. siRNA technology was used to try and define which TGF-β signalling pathways were involved in the action of TGF-β on ADAMTS-4. Following this the aims were to characterise the action of TGF-β on the promoter activity of ADAMTS-4 using transfection studies and to identify the region of the promoter involved in this response. DNA:protein interactions were investigated by EMSA. The final aim of the studies was to confirm any results observed with EMSA by cloning fragments of the promoter region into a heterologous promoter followed by transfection studies to further investigate the response and attempt to characterise any transcription factors required for the action of TGF-β on ADAMTS-4 with the aim of characterising the mechanism behind TGF-β regulation of ADAMTS-4. Figure 6.2 outlines the experimental strategy used during these studies.

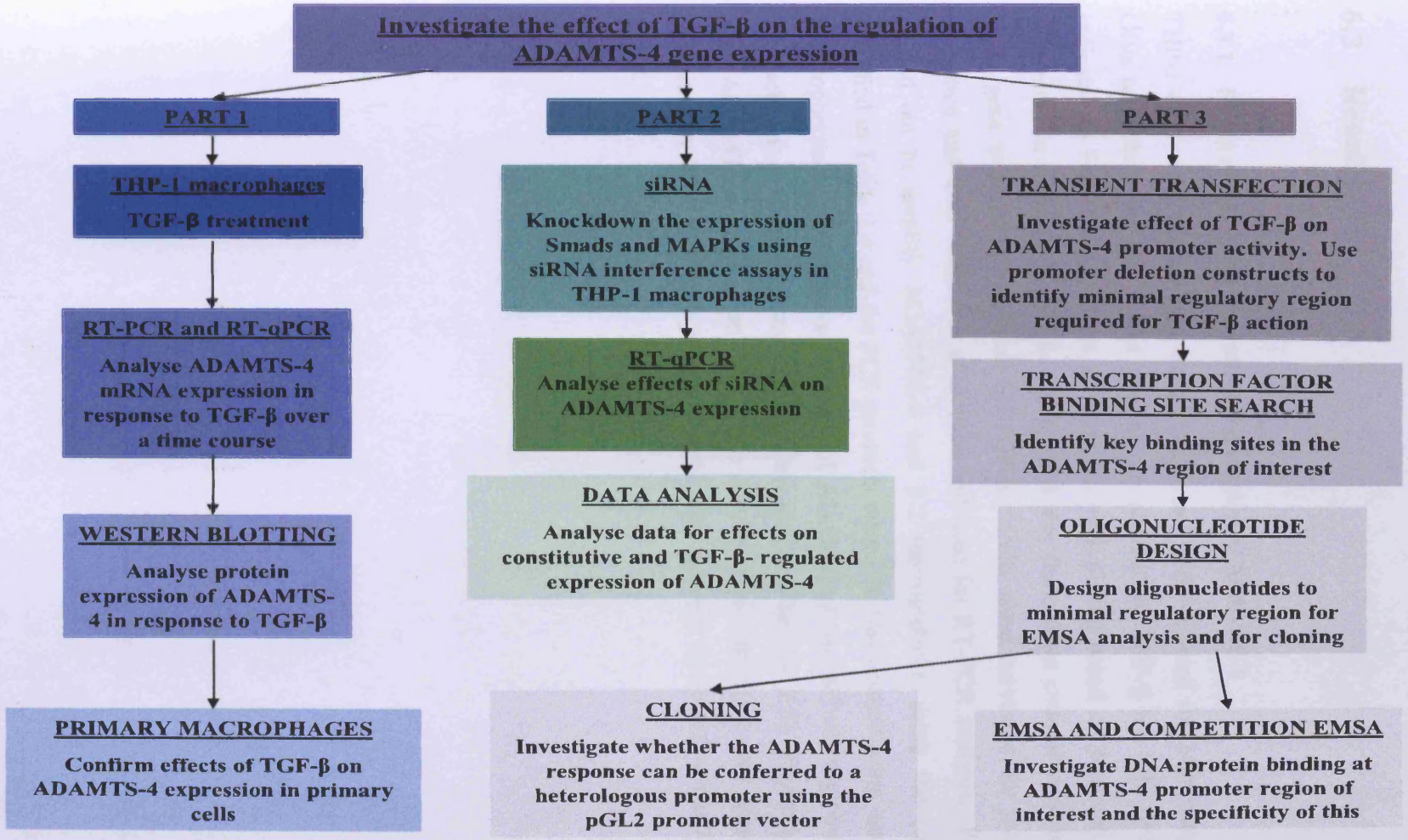

Figure 6.2 Experimental strategy diagram.

6.3 Results

6.3.1 Effect of TGF-β on the mRNA expression of ADAMTS-4

THP-1 cells were seeded into 6-well plates and differentiated for 24hr with PMA. Cells were then cultured in the presence or absence of TGF-β for 12hr or 24hr as indicated in Figure 6.3. These time points were picked based on previous studies (presented in Chapter 3) that had suggested that these were suitable time points to study gene expression regulated by TGF-β. Cells were harvested for total RNA extraction and total RNA was quantified and used for RT-PCR analysis. PCR was carried out to amplify ADAMTS-4 and β-2 microglobulin using the conditions specified in Table 2.4 and the PCR products were size-fractionated using agarose gel electrophoresis. The relative intensity of ADAMTS-4 and β-2 microglobulin PCR products was determined using densitometric analysis (Section 2.9). Figure 6.3 shows that ADAMTS-4 expression was high in untreated cells and was significantly inhibited by TGF-β at both 12hr and 24hr with optimal inhibition at the 24hr time point.

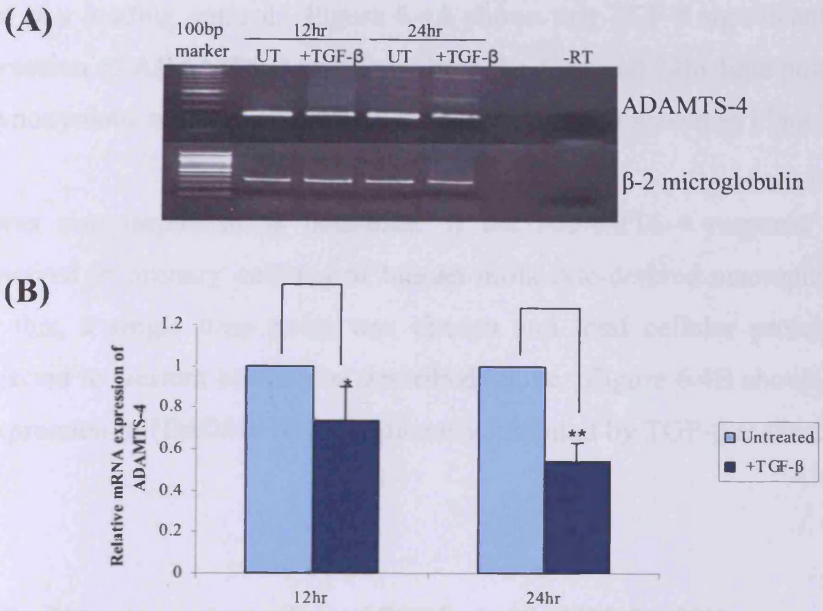

Figure 6.3 TGF-β down-regulates ADAMTS-4 mRNA expression in THP-1 macrophages. THP-1 cells were treated with TGF-β or left untreated for 12 or 24hr and harvested for total RNA extraction. Total RNA was subjected to RT-PCR analysis and the PCR amplification products for ADAMTS-4 and β-2 microglobulin were resolved by agarose gel electrophoresis (Panel A). The size of the product was determined by comparison with a standard DNA molecular weight marker (see Appendix I). –RT denotes a reaction in which no reverse transcriptase was included in cDNA preparation (untreated sample from the 24hr time point was used). Panel B shows the relative expression (mean± SD) of ADAMTS-4 normalised to β-2 microglobulin (untreated samples arbitrarily assigned as 1) from three independent experiments (*P<0.05, **P<0.01).

6.3.2 Effect of TGF-β on the protein expression of ADAMTS-4 in THP-1 and primary human macrophage cultures

To confirm that the inhibition of ADAMTS-4 mRNA by TGF-β resulted in a corresponding change in the expression of ADAMTS-4 protein, western blot analysis was carried out. Differentiated THP-1 cells were treated with TGF-β or left untreated for the indicated time periods. Total cellular protein was extracted using Laemmli

buffer, subjected to SDS-PAGE using an antibody against ADAMTS-4. β-actin was used as a loading control. Figure 6.4A shows that TGF-β significantly inhibited the expression of ADAMTS-4 protein at both the 12hr and 24hr time point. This change is synonymous with the changes in mRNA expression shown in Figure 6.3.

It was also important to determine if the ADAMTS-4 response to TGF-β was conserved in primary cultures of human monocyte-derived macrophages (HMDMs). For this, a single time point was chosen and total cellular protein extracts were subjected to western blotting as described above. Figure 6.4B shows that ADAMTS-4 expression in HMDMs was significantly inhibited by TGF-β at the 24hr time point.

6.3.3 Time dependent effect of TGF-β on ADAMTS-4 mRNA expression

In light of the results presented in Figures 6.3 and 6.4 it was important to determine how ADAMTS-4 was regulated by TGF-β over a time course. This would also determine an optimal time point for future studies on ADAMTS-4 expression. Untreated and TGF-β-treated THP-1 cells were harvested for total RNA and reverse transcribed. The resulting cDNA template was subjected to RT-qPCR analysis using specific primers against ADAMTS-4 and the control gene RPL13A as detailed in Table 2.5 and the amplification conditions set out in Table 2.6. RT-qPCR was used here as the technique had become available in the laboratory. Figure 6.5 shows that ADAMTS-4 expression was high in untreated cells and was significantly reduced by TGF-β, 1hr after treatment with the cytokine. Levels of ADAMTS-4 mRNA expression remained significantly inhibited by TGF-β over the 24hr time course.

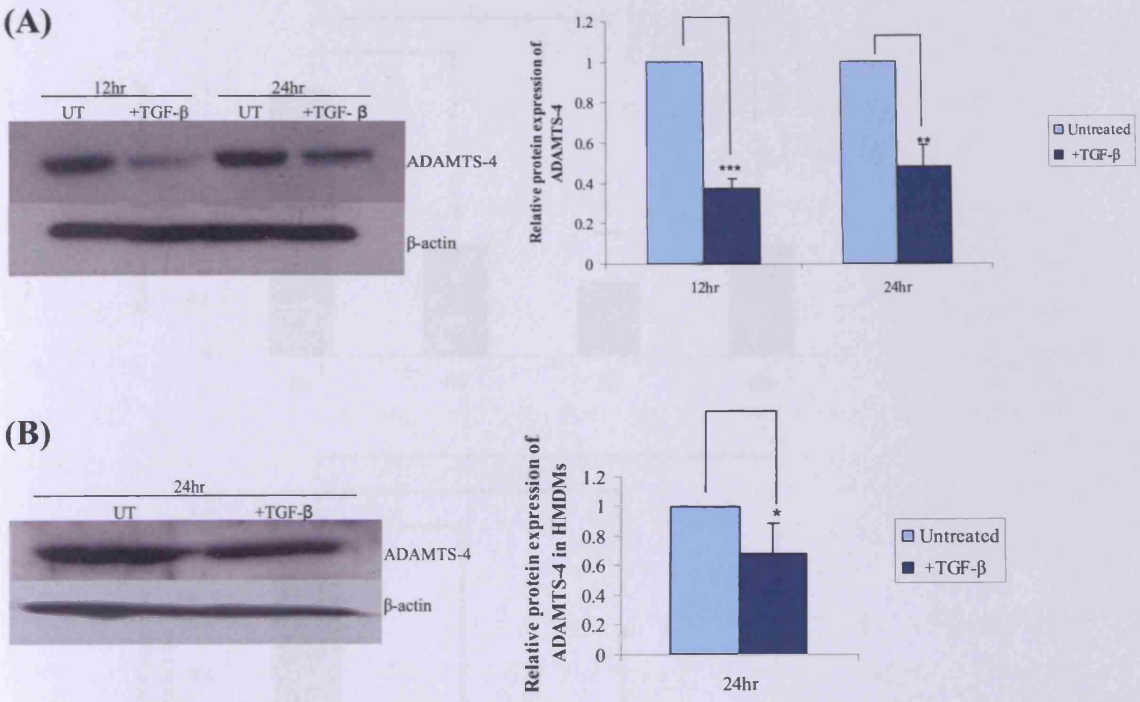

Figure 6.4 **TGF-β inhibits ADAMTS-4 protein expression in THP-1 and HMDMs**. THP-1 cells or HMDMs were treated with TGF-β or left untreated for the time points indicated. Whole cell-protein extracts were subjected to SDS-PAGE and western blotting using antibodies against ADAMTS-4 and β-actin. Protein size was determined by comparison against a standard protein molecular weight marker (see Appendix I). β-actin was used as a loading control. Panel A (THP-1) and Panel B (HMDMs) show the relative expression (mean ± SD) of ADAMTS-4 normalised to β-actin (values from untreated samples arbitrarily assigned as 1) from three independent experiments (**P<0.01, ***P<0.001).

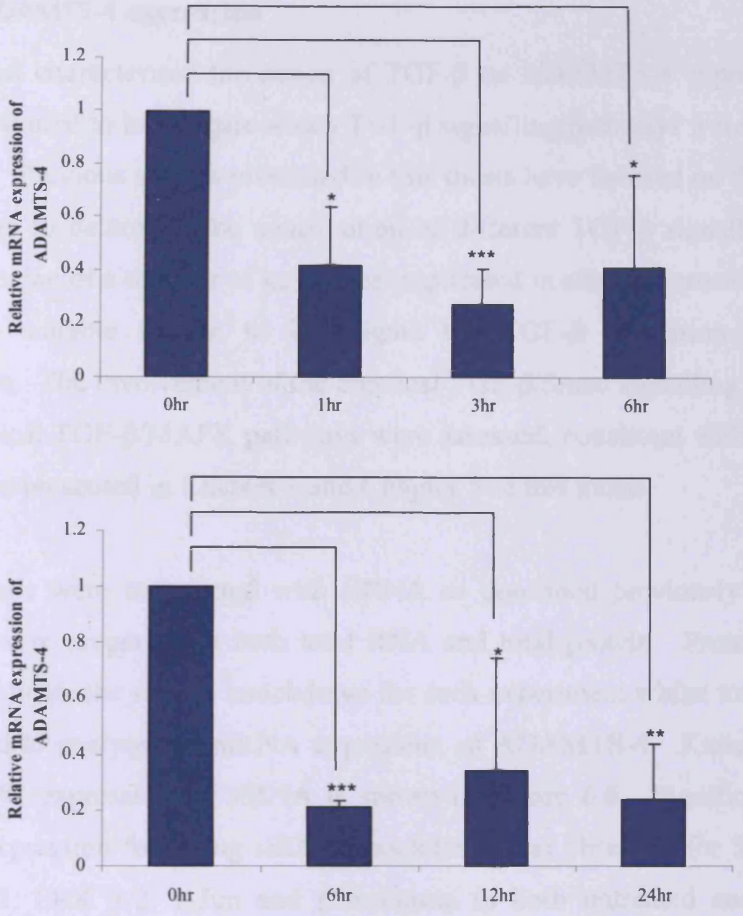

Figure 6.5 TGF-β inhibits ADAMTS-4 mRNA expression over a 24hr time course. THP-1 cells were left untreated (0hr) or treated with TGF-β for 1, 3, 6, 12 or 24hr before being harvested for total RNA extraction. Total RNA was reverse-transcribed and the resulting cDNA subjected to RT-qPCR analysis using primers specific to ADAMTS-4 and the control gene RPL13A. The relative expression of ADAMTS-4 (mean ± SD) normalised to β-actin from three independent experiments is shown, with values from untreated (0hr) samples arbitrarily assigned as 1 (*P<0.05, **P<0.01, ***P<0.001).

6.3.4 TGF-β signalling pathways that mediate the action of this cytokine on ADAMTS-4 expression

As we had characterised the action of TGF-β on ADAMTS-4 expression in THP-1 cells we wanted to investigate which TGF-β signalling pathways were involved in this response. Previous studies presented in this thesis have focused on the use of siRNA technology to determine the contribution of different TGF-β signalling pathways to the expression of a number of key genes implicated in atherosclerosis. This technique was also suitable to use to investigate the TGF-β regulation of ADAMTS-4 expression. The involvement of the classical TGF-β/Smad signalling pathway and the non-classical TGF-β/MAPK pathways were assessed, consistent with studies on gene expression presented in Chapter 4 and Chapter 5 of this thesis.

THP-1 cells were transfected with siRNA as described previously (Section 2.6.1). Extracts were prepared for both total RNA and total protein. Protein extracts were used to validate the siRNA knockdown for each experiment whilst total RNA extracts were used to analyse the mRNA expression of ADAMTS-4. Knockdown of Smad and MAPK expression by siRNA is shown in Figure 6.6. Significant reduction in protein expression following siRNA knockdown was observed for Smad-2, Smad-3, Smad-2+3, ERK 1/2, c-Jun and p38 kinase in both untreated and TGF-β-treated samples. Protein extracts were prepared with Laemmli buffer and subjected to SDS-PAGE and western blotting. Total RNA extracts were reverse-transcribed and the resulting cDNA analysed by RT-qPCR using specific primers against ADAMTS-4 and RPL13A. Figure 6.7 and Figure 6.8 show the effect of siRNA-mediated Smad knockdown and MAPK knockdown respectively on ADAMTS-4 mRNA expression.

The inhibition of ADAMTS-4 by TGF-β was no longer significant following knockdown of Smad-2 or Smad-3. Knockdown of Smad-3 showed the largest change in ADAMTS-4 expression with the TGF-β-regulated inhibition being reversed (Figure 6.7B). Knockdown of both Smad-2 and -3 also reversed ADAMTS-4 inhibition suggesting that the Smad pathway is involved in this response and that Smad-2 and Smad-3 may act in a functionally redundant manner. Knockdown of c-Jun and p38 kinase similarly resulted in a loss of significant ADAMTS-4 inhibition by TGF-β. This suggests that both c-Jun and p38 kinase may be involved in the action of TGF-β

on ADAMTS-4 in addition to Smad-2 and -3. One noteworthy observation was that knockdown of p38 kinase had a negative effect on the constitutive expression of ADAMTS-4 (PMA-induced expression during differentiation) as well as its TGF-β regulated expression (Figure 6.8C) suggesting a role for the kinase in the constitutive expression of ADAMTS-4. Knockdown of ERK 1/2 had no effect on ADAMTS-4 expression as shown in Figure 6.8A.

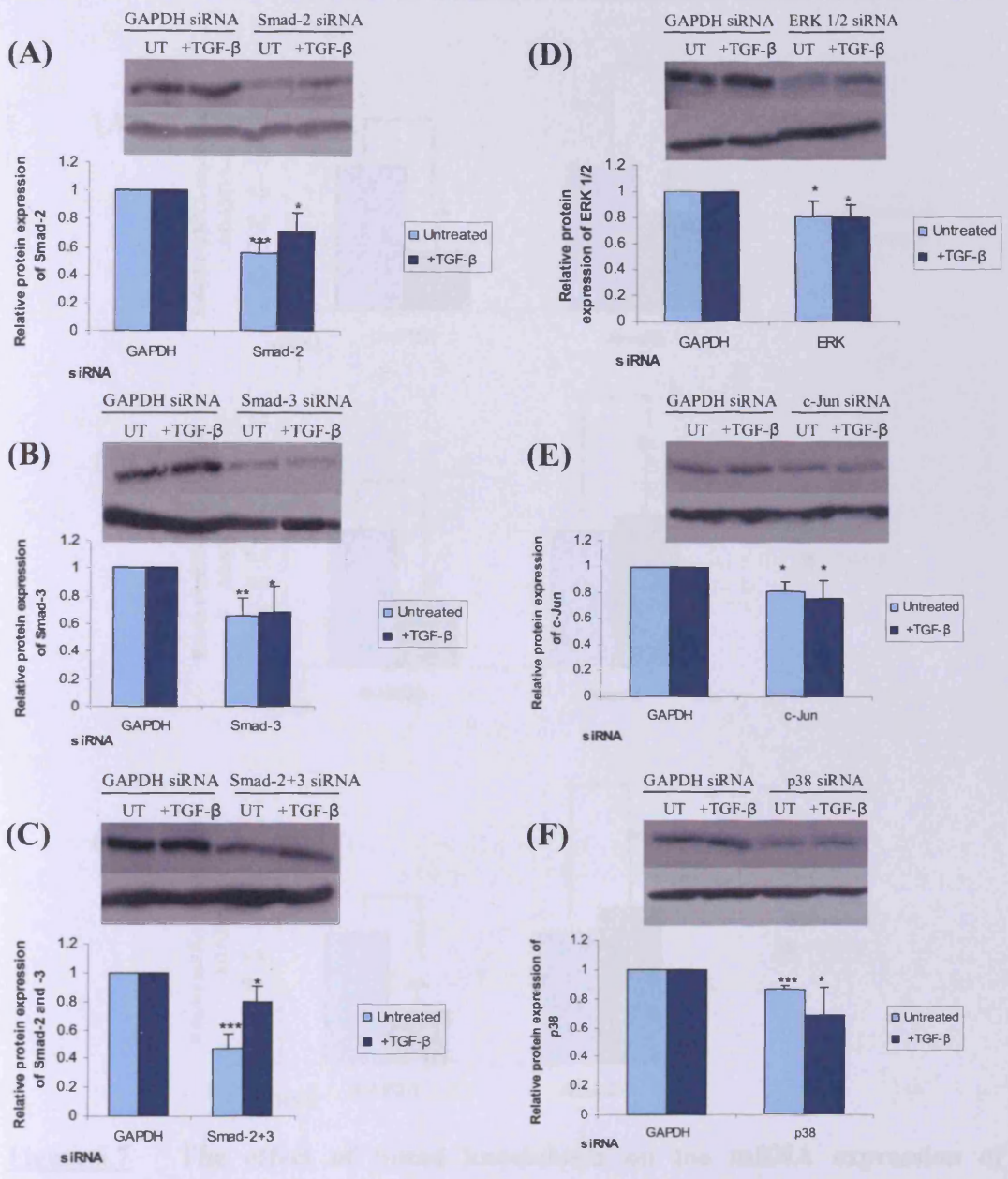

Figure 6.6 Smad and MAPK expression following siRNA-mediated knockdown in THP-1 macrophages. Whole-cell protein extracts were prepared from untreated or TGF-β-treated THP-1 cells transfected with validated GAPDH siRNA or Smad-2 (Panel A), Smad-3 (Panel B), Smad-2 and Smad-3 (Panel C), ERK 1/2 (Panel D), c-Jun (Panel E), or p38 (Panel F) siRNA as described in Section 2.6.1. Extracts were subjected to SDS-PAGE and western blotting and levels of expression were compared against levels of β-actin. Relative expression (mean± SD) of each protein normalised to β-actin levels, with values from GAPDH-transfected cells arbitrarily assigned as 1, from three independent experiments is shown (**P*<0.05, ***P*<0.01, ****P*<0.001).

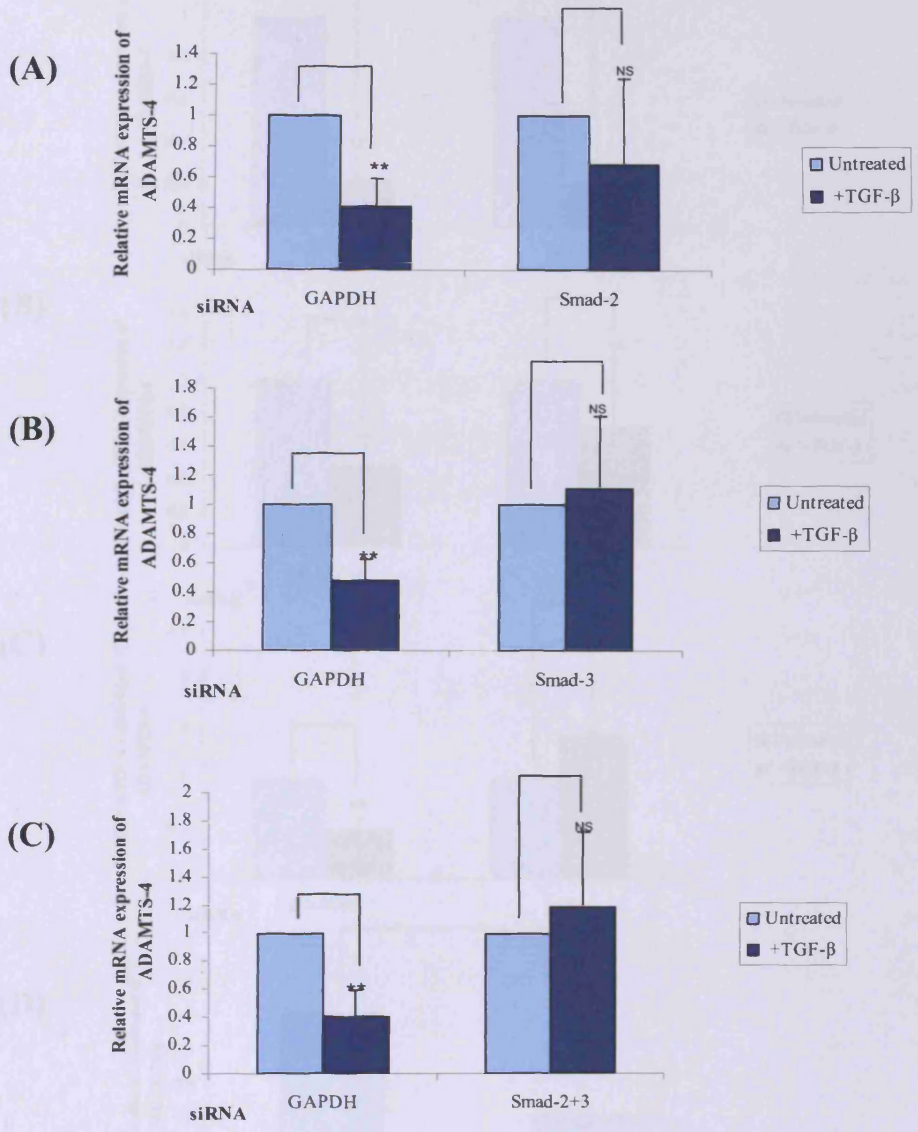

Figure 6.7 The effect of Smad knockdown on the mRNA expression of ADAMTS-4. THP-1 cells were transfected with 7.5nM siRNA using Interferin™ as described in Section 2.6.1. At 24hr following transfection cells were differentiated using 0.16μM PMA and subsequently treated with TGF-β or left untreated for 24hr. Cells were harvested for total RNA and subjected to RT-qPCR analysis using primers specific to ADAMTS-4 and the control gene RPL13A. The relative expression (mean± SD) of ADAMTS-4 normalised to RPL13A (values from untreated samples arbitrarily assigned as 1) from three independent experiments is shown (**P<0.01, NS-not significant). Panel A: Smad-2 knockdown; Panel B: Smad-3 knockdown; Panel C: Smad-2 and -3 knockdown.

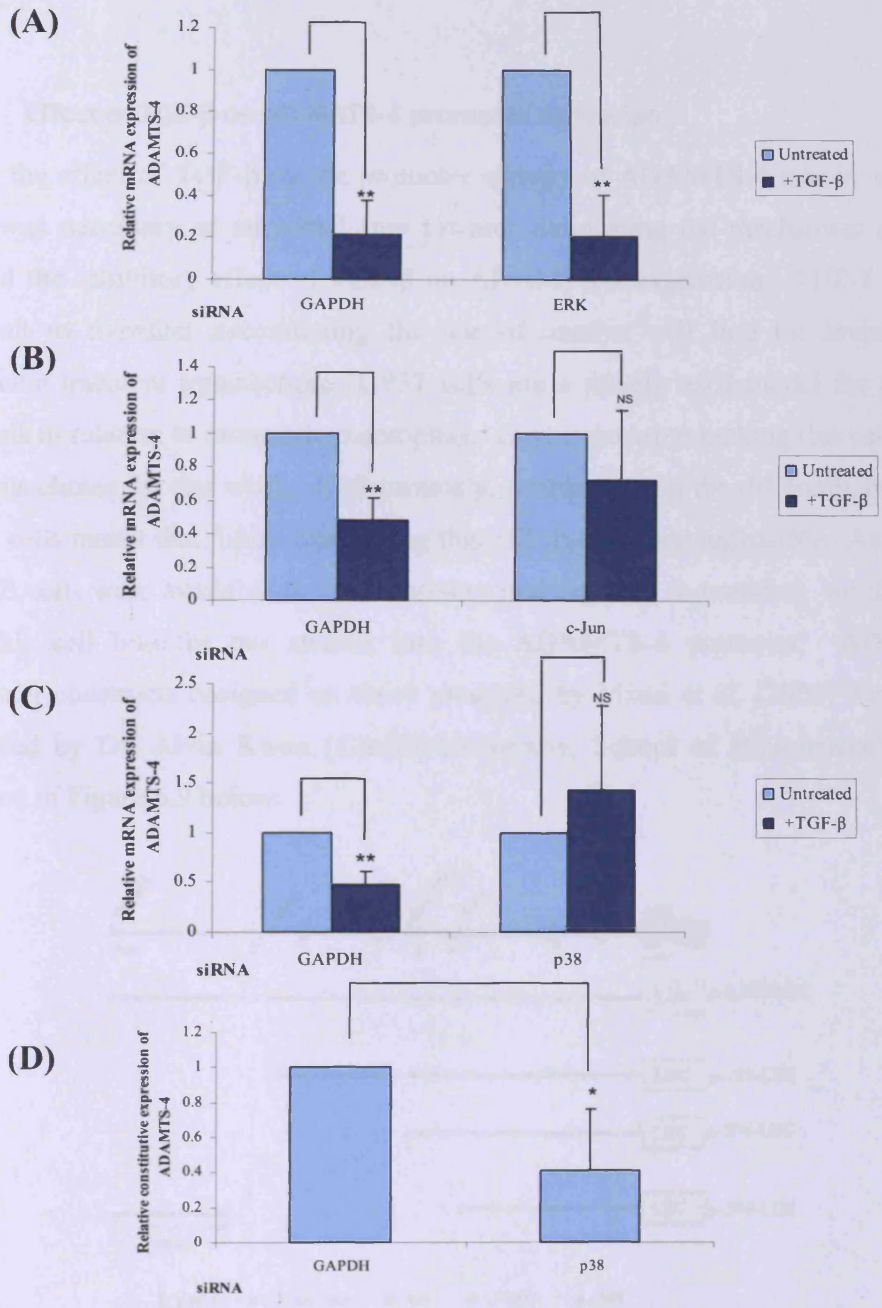

Figure 6.8 **The effect of MAPK knockdown on the mRNA expression of ADAMTS-4.** THP-1 cells were transfected as described in Figure 6.6. Following transfection, cells were differentiated using PMA and then treated with TGF-β or left untreated for 24hr. Cells were harvested for total RNA and subjected to RT-qPCR analysis. The relative expression (mean± SD) of ADAMTS-4 normalised to RPL13A (values from untreated samples arbitrarily assigned as 1) from three independent experiments is shown (**P<0.01, NS-not significant). Panel A: ERK 1/2 knockdown; Panel B: c-Jun knockdown; Panel C: p38 knockdown; Panel D: basal expression of ADAMTS-4 following p38 knockdown.

6.3.5 Effect of TGF-β on ADAMTS-4 promoter activation

Next, the effect of TGF-β on the promoter activity of ADAMTS-4 was investigated. This was necessary as an initial step towards delineating the mechanism of action behind the inhibitory effect of TGF-β on ADAMTS-4 expression. THP-1 cells are difficult to transfect necessitating the use of another cell line for investigations involving transient transfection. U937 cells are a widely used model for promoter analysis in relation to monocyte/macrophage gene expression making this cell line the obvious choice for this work. Unfortunately, problems with the differentiation of the U937 cells meant that future work using this cell line became unfeasible. As adherent Hep3B cells were available in the laboratory and are easy to transfect, we decided to use this cell line for our studies into the ADAMTS-4 promoter. ADAMTS-4 promoter constructs designed on those produced by Mizui et al. (2000) were kindly provided by Dr. Alvin Kwan (Cardiff University, School of Biosciences) and are detailed in Figure 6.9 below.

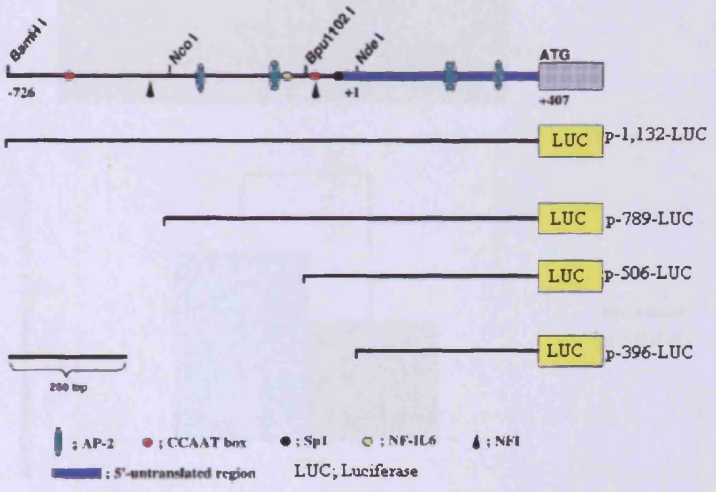

Figure 6.9 Constructs of the human ADAMTS-4 promoter. The ADAMTS-4 promoter (1,132bp) was inserted into a luciferase reporter plasmid upstream of the firefly luciferase gene. Deletion constructs (789bp, 506bp and 396bp) were constructed using restriction enzyme digestion as shown and inserted into the luciferase reporter plasmid. Numbers indicate positions relative to the transcription start site. Sites for the transcription factors AP-2, Sp1, NF-1, NF-IL6 and the CCAAT box are also indicated. Figure adapted from Mizui et al. (2000).

Before we could investigate the action of TGF-β on the ADAMTS-4 promoter it was necessary to determine that the effect of this cytokine on its expression was conserved in the Hep3B cell line. This was ascertained by western blot analysis. Hep3B cells were either treated with TGF-β or left untreated for 48hr. This time point was chosen following an initial time course that demonstrated inhibition at this time point. Total cellular protein was extracted using Laemmli buffer and the extracts subjected to SDS-PAGE and western blotting. Membranes were probed with an anti-ADAMTS-4 antibody and subsequently re-probed with an antibody against a β-actin control. Figure 6.10 shows that, as expected, TGF-β down-regulated the protein expression of ADAMTS-4 in Hep3B cells, verifying the use of this cell line as a feasible model for studying the action of TGF-β on the ADAMTS-4 promoter.

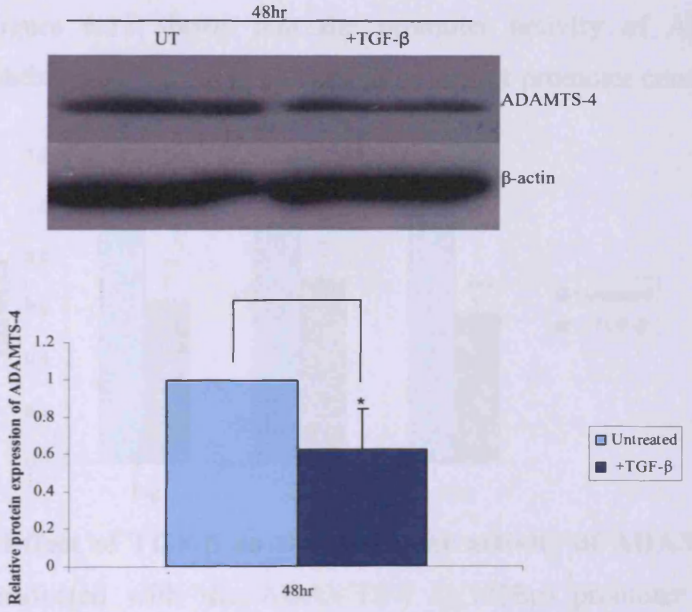

Figure 6.10 ADAMTS-4 protein expression is inhibited by TGF-β in Hep3B cells. Confluent Hep3B cells were treated with TGF-β or left untreated for 48hr and harvested for protein extraction. Extracts were subjected to SDS-PAGE and western blotting using antibodies specific to ADAMTS-4 and β-actin. The relative expression (mean± SD) of ADAMTS-4 normalised to RPL13A (values from untreated samples arbitrarily assigned as 1) from three independent experiments is shown (*P<0.05).

Initially, the action of TGF-β on the full length (1,132bp) ADAMTS-4 promoter was examined. Hep3B cells were cultured in 12-well plates until they reached approximately 70% confluency when transfection was carried out. Prior to transfection the medium was replaced with fresh complete DMEM. The transfection mix was prepared as described in Section 2.5.1 with the Superfect™ and DNA added in a ratio of 3:1. Varying amounts of the p-1,132-LUC construct were used to identify if the response would occur in a concentration-dependent manner. The complete transfection mix was added to Hep3B cells and cells were left untreated or treated with TGF-β, 30min after transfection. Cells were incubated overnight and then harvested using 1x passive lysis buffer as described in Section 2.5.2. Relative luciferase activity (RLA) readings were normalised to protein concentration rather than to another internal control plasmid (such as CMV-β-galactosidase) as previous studies have shown that many such control plasmids are themselves affected by cytokines. Figure 6.11 shows that the promoter activity of ADAMTS-4 was significantly inhibited by TGF-β at all concentrations of promoter construct used.

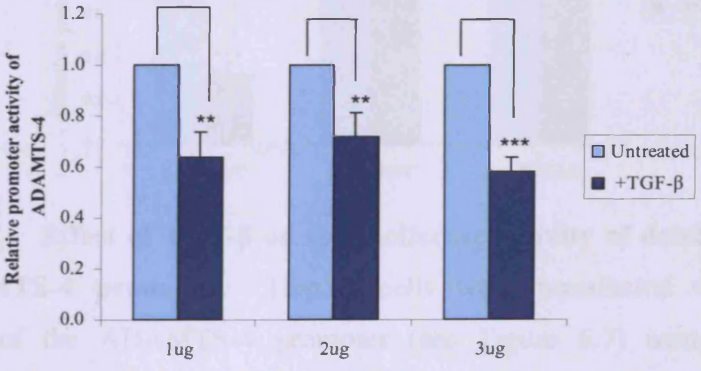

Figure 6.11 **Effect of TGF-β on the promoter activity of ADAMTS-4.** Hep3B cells were transfected with the ADAMTS-4 (1,132bp) promoter plasmid using Superfect™ as described in Section 2.6.2. Cells were either left untreated or treated with TGF-β overnight. Cells were harvested using 1x passive lysis buffer (Section 2.5.2) and measured for luciferase activity. Relative luciferase activity (RLA) readings were normalised to protein concentration as determined by the BCA protein assay (Section 2.7.4). The relative luciferase activity (mean ±SD) of ADAMTS-4 normalised to protein concentration (values from untreated samples arbitrarily assigned as 1) from three independent experiments is shown (**P<0.01, ***P<0.001).

6.3.6 Identifying the minimal promoter region of ADAMTS-4 that is necessary for TGF-β action

In order to try and identify the minimal region of the ADAMTS-4 promoter that contains the TGF-β response elements necessary for the action of TGF-β on ADAMTS-4, deletion constructs of the promoter were used as detailed in Figure 6.8 above. Transfection was carried out as described previously. Figure 6.12 shows that when cells were transfected with the p-789-LUC and the p-506-LUC deletion constructs, a significant reduction in the relative luciferase activity was observed. No significant reduction in relative luciferase activity occurred following transfection of the p-396-LUC deletion construct, suggesting that the minimal promoter region containing the necessary TGF-β response elements is between -396 and -506.

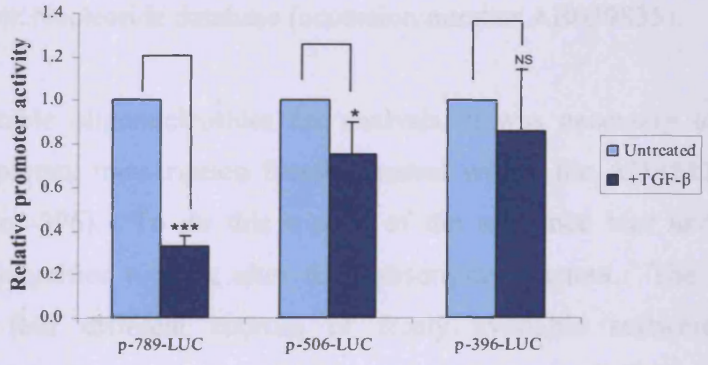

Figure 6.12 Effect of TGF-β on the luciferase activity of deletion constructs of the ADAMTS-4 promoter. Hep3B cells were transfected with the deletion constructs of the ADAMTS-4 promoter (see Figure 6.7) using Superfect™ as described in Section 2.6.2. Cells were either left untreated or treated with TGF-β overnight. Cells were harvested and measured for luciferase activity. Relative luciferase activity (RLA) readings were normalised to protein concentration as determined by the BCA protein assay. The relative luciferase activity (mean ±SD) of ADAMTS-4 normalised to protein concentration (values from untreated samples arbitrarily assigned as 1) from three independent experiments is shown (***P<0.001, *P<0.05, NS-not significant).

6.3.7 Analysis of DNA: protein interactions with the minimal ADAMTS-4 promoter region

Cytokine-regulated gene expression is mediated through the interaction of transcription factors with both regulatory parts of the promoter sequence and other transcription factors. It was therefore important to study the interaction of proteins with this DNA sequence using EMSA. Further studies could then focus on the region of the promoter that interacted with DNA-binding proteins, with particular emphasis on those whose binding was regulated by TGF-β, with a view to identifying any transcription factors involved with the TGF-β response on ADAMTS-4. This strategy had previously been successfully carried out in the laboratory for studies on the minimal regulatory regions of the LPL promoter required for the inhibitory action of IFN-γ and TGF-β (Hughes et al. 2002; Irvine et al. 2005). The sequence of the ADAMTS-4 promoter region was taken from Mizui et al. 2000 and identified using the NCBI Entrez Nucleotide database (accession number AB039835).

To design suitable oligonucleotides for analysis, it was necessary to identify any potentially important transcription factors present within the ADAMTS-4 promoter region (-506 to -396). To do this a scan of the sequence was undertaken using software that identifies binding sites for transcription factors. The sequence was submitted to four different sources of freely available software; SignalScan (Prestridge 1991), Transcription element search system (TESS) (Schug 2003), TFSearch (Heinemeyer et al. 1998) and MatInspector by Genomatix (Cartharius et al. 2005) and the results used to produce a map of transcription factor binding sites within the region (see Appendix IV). Key binding sites of interest were identified and oligonucleotides were designed so that these sites were not disrupted (Figure 6.13). The sequences of the four overlapping double-stranded oligonucleotides that spanned the -506/-396 region (-502/-463; -464/-439; -443/-423; -423/-390) are shown in Table 2.13. Oligonucleotides were designed with 5' overhangs containing a G residue to allow for radiolabelling using [α-^{32}P]-dCTP available in the School. EMSA was carried out to determine if any increase or decrease in DNA:protein binding was observed after incubation of the cells with TGF-β.

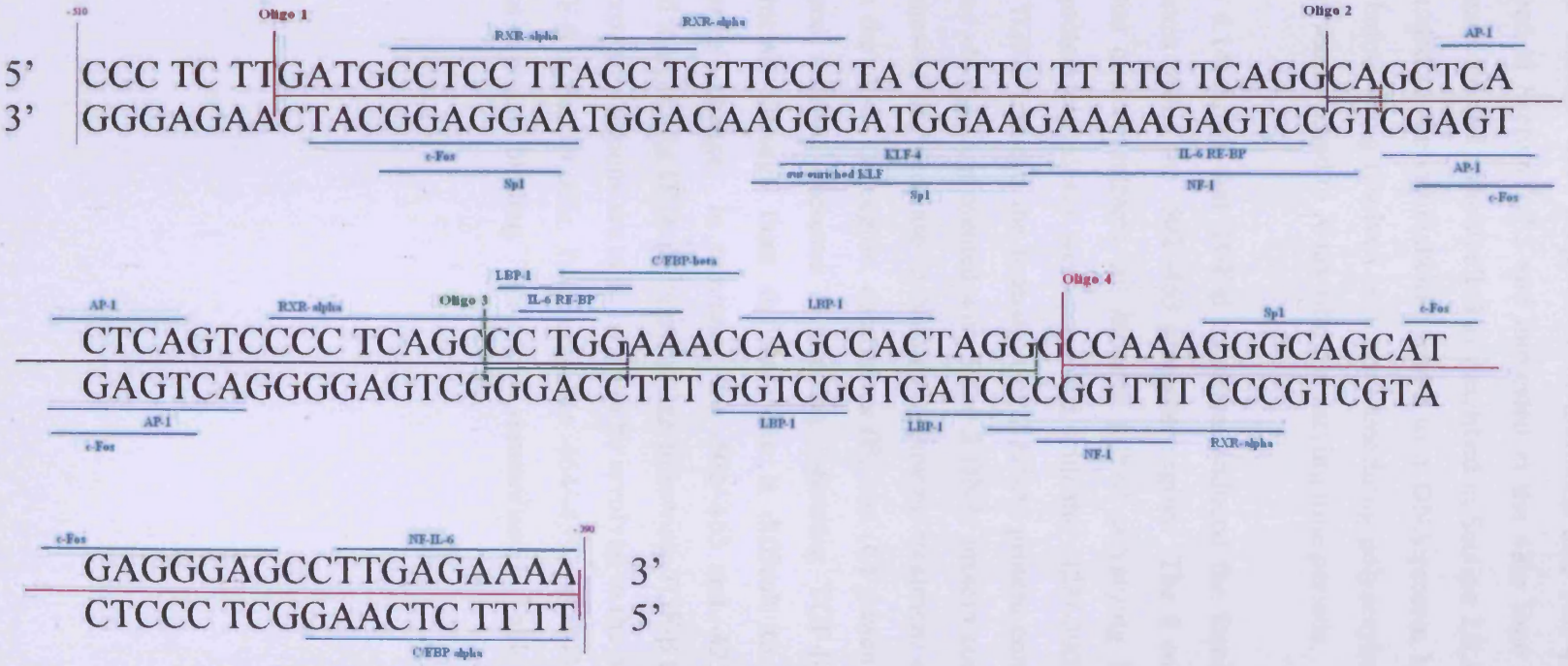

Figure 6.13 **The -510 to -390 region of the human ADAMTS-4 promoter.** Key transcription factor binding sites were identified using TESS and MatInspector software (see Appendix II). Binding sites of interest have been marked along with the oligonucleotides used for EMSA. Oligonucleotides were designed so that key TF binding sites were not disrupted and contained a 5'overhang containing at least one G residue to allow radiolabelling using [α-^{32}P]-dCTP.

Nuclear extracts were prepared from untreated and TGF-β treated Hep3B cells as described in Section 2.7.3 and harvested at the 48hr time point. Oligonucleotides were annealed and radiolabelled as described in Section 2.8.1. Nuclear extracts (2μg) were added to the radiolabelled probe in a DNA:protein binding reaction (Section 2.8.2) before being resolved on a non-denaturing polyacrylamide gel (Section 2.8.4). The gel was exposed to X-ray film for varying time periods.

Figure 6.14 shows that TGF-β treatment induced the formation of 6 DNA:protein complexes within the -502/-463 promoter region. The 6 complexes were present in untreated nuclear extracts but induced (albeit at varying levels) by TGF-β. Five DNA:protein complexes were produced with the -423/-390 promoter region and of these, TGF-β induced the formation of 2 DNA: protein complexes. Regions -464/-439, and -443/-423, presented with 5 and 3 DNA: protein complexes respectively and none showed any increase in binding following treatment of the cells with TGF-β. Within the -464/-439 region, complexes (B) and (C) presented in untreated samples displayed a small decrease in binding following TGF-β treatment in all three experiments although faint signals make it difficult to determine if this is a reproducible change. In summary, the -502/-463 and -423/-390 promoter regions showed increases in DNA:protein binding following TGF-β treatment suggesting that these promoter regions are most likely to be involved in the regulation of ADAMTS-4 by TGF-β in Hep3B cells. In contrast the -464/-439 and -443/-423 regions showed no changes in protein binding. This data is summarised in Table 6.1.

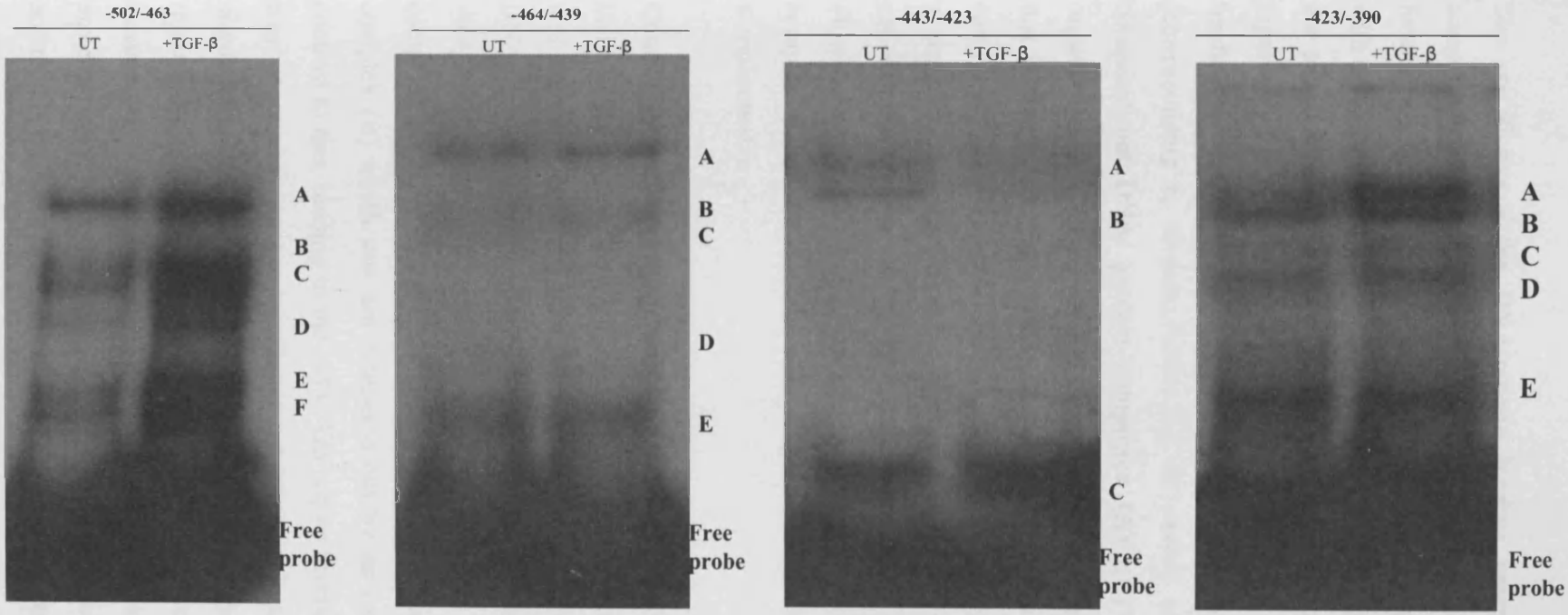

Figure 6.14 **Effect of TGF-β on protein binding to the minimal ADAMTS-4 promoter region.** Hep3B cells were treated with TGF-β or left untreated for 48hr. Nuclear extracts were used for EMSA. Nuclear extracts (2μg) were added to the radiolabelled oligonucleotide (Oligo 1, 2, 3 or 4) in a DNA: binding reaction before being resolved on a 6% non-denaturing polyacrylamide gel. Complexes were visualised by autoradiography. Images shown are representative of three independent experiments (-502/-463: Oligo 1; -464/-439: Oligo 2; -443/-423: Oligo 3; -423/-390: Oligo 4).

The specificity of the DNA:protein binding reaction was next investigated by competition EMSA analysis. This would also allow us to see whether common factors bound to different oligonucleotides. Nuclear extracts (2μg) were incubated with a 250-fold excess of an unlabelled competing oligonucleotide for 20min prior to the protein binding reaction. TGF-β treated extracts were used for the analysis. Figures 6.15 shows that an excess of oligonucleotides was able to compete out protein binding to the corresponding oligonucleotide sequence. For the -502/-463 region (corresponding to oligonucleotide 1), an excess of unlabelled oligonucleotide 1 competed out DNA: protein complexes (A), (C) and (D) while an excess of oligonucleotides 2 and 3 did not compete out binding at complex (A). This suggests that protein binding is specific. An excess of oligonucleotide 4 also competed out complexes (B), (C) and (D). Taking these findings together, this suggests that binding at complex (A) is specific and other binding to this promoter region can be classed as non-specific binding or that the same transcription factors bind to more than one oligonucleotide. Interestingly, an excess of oligonucleotide 2 increased the binding of complex (C) whilst an excess of oligonucleotide 3 did compete out one of the complexes (D).

Competition screens using the -464/-439 and -443/-423 promoter regions were included for comparative purposes (Figure 6.16). As no change in protein binding was observed following TGF-β treatment (Figure 6.14) it can be expected that any DNA:protein complexes formed will not be specific to the TGF-β response. With the -464/-439 region (corresponding to oligonucleotide 2) all visible complexes were competed out by excesses of all four oligonucleotides with the notable exception of complex (B) which was not competed out by an excess of oligonucleotide 2. In contrast to this, binding to the -443/-423 region (corresponding to oligonucleotide 3) was competed out by an excess of oligonucleotide 3 but not by the other oligonucleotides. This suggests that there is some specific binding to the -443/-423 region. As there was no visible change in DNA: protein binding following TGF-β treatment, it is possible that any DNA: protein complexes within this region are due to proteins involved in constitutive ADAMTS-4 expression whose trans-activation potential is inhibited by TGF-β or that are part of the transcriptional machinery.

For the -423/-390 region (corresponding to oligonucleotide 4), complexes (A), (B), (C) and (D) were all competed out by an excess of oligonucleotide 4. This was not seen with an excess of other oligonucleotides suggesting that binding in this region is specific. Interestingly, competition EMSA using oligonucleotide 1 and oligonucleotide 2 did decrease complexes (A) and (B) but competition with oligonucleotide 2 and oligonucleotide 3 resulted in an increase in complex (C). Taken together with the data presented in Figure 6.14, analysis by EMSA indicates that it is the -502 to -463 (Oligo 1) region and the -423 to -390 (Oligo 4) region that are most likely to be involved in the inhibitory action of TGF-β on ADAMTS-4. This data is summarised in Table 6.1.

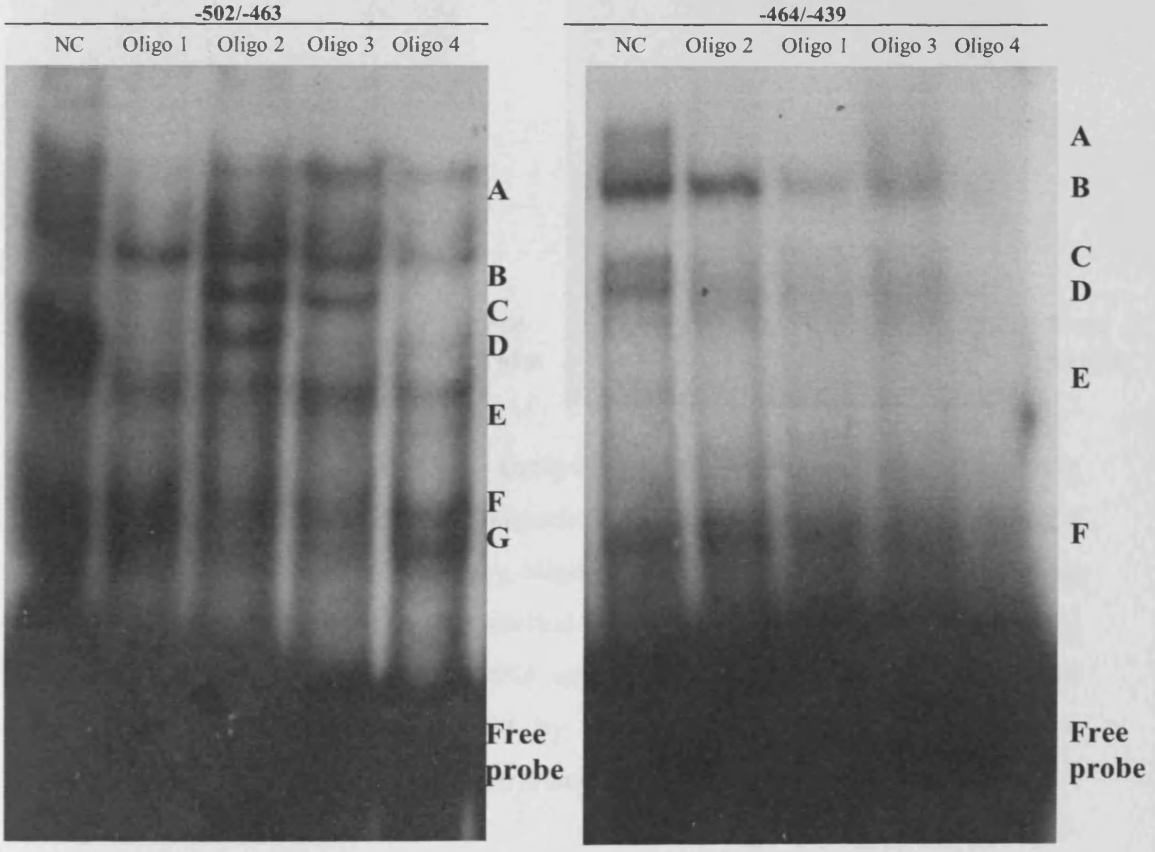

Figure 6.15 Competition EMSA analysis of the DNA:protein complexes produced in response to TGF-β. Competition EMSA was carried out by adding a 250-fold excess of unlabelled competing oligonucleotides to the DNA:protein binding reaction prior to addition of the radiolabelled probe for oligonucleotide 1 (-502/-463) or oligonucleotide 2 (-463/-439). EMSA analysis was carried out as described in Figure 6.14 and complexes visualised by autoradiography. Images shown are representative of three independent experiments.

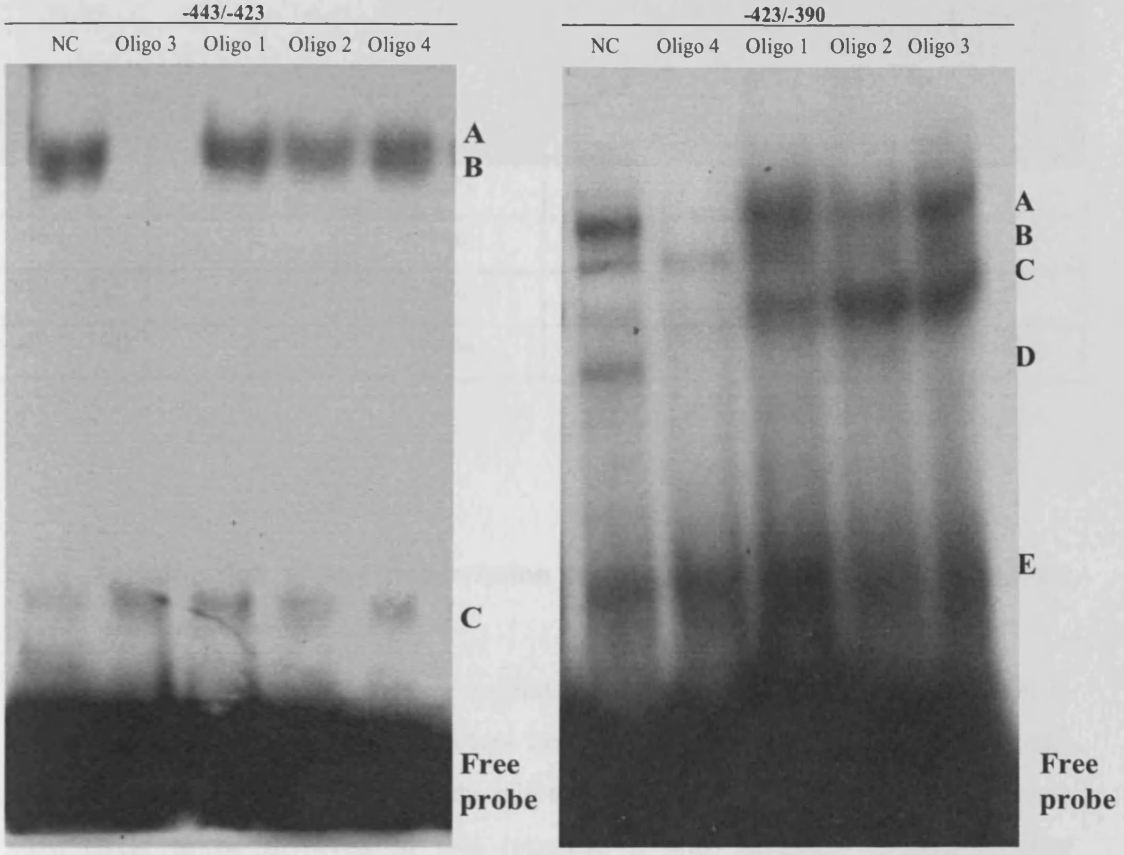

Figure 6.16 Competition EMSA analysis of the DNA:protein complexes produced in response to TGF-β. Competition EMSA was carried out by adding a 250-fold excess of unlabelled competing oligonucleotides to the DNA:protein binding reaction prior to addition of the radiolabelled probe for oligonucleotide 3 (-443/-423) or oligonucleotide 4 (-423/-390). EMSA analysis was carried out as described in Figure 6.14 and complexes visualised by autoradiography. Images shown are representative of three independent experiments.

Table 6.1 **Summary Table of EMSA data**

Promoter Region	Number of DNA:protein complexes present	Increase in protein binding following TGF-β treatment?	Number of DNA:protein complexes induced	Specific binding to promoter region?	Complexes induced following TGF-β treatment
-502/-463	6	Yes	6	Yes	A
-464/-439	5	No	-	No	-
-443/-423	3	No	-	Yes	A, B
-423/-390	5	Yes	2	Yes	C, D

6.3.8 Identification of key transcription factors present in minimal ADAMTS-4 promoter region

In order to decipher the mechanism behind the action of TGF-β, it was important to try and identify any transcription factors involved in the response. As previous data (Figures 6.14, 6.15 and 6.16) had indicated that regions -502/-463 and -423/-390 were most likely to be involved in this response it was necessary to identify key transcription factors present in these regions. A screen of competing oligonucleotides was carried out using oligonucleotides specifying for binding sequences of transcription factors that were available in the laboratory and shown previously to be involved in TGF-β responses. Competition EMSA was carried out as described previously. The results are shown in Figure 6.17. Specific oligonucleotides corresponding to the binding sequences of the transcription factors NF-1, AP1, Sp1 and C/EBPα were used. For the -502/-463 region competition with NF-1 had the most effect whilst for the -423/-390 region competition with AP1 had the most effect. However none of the competing oligonucleotides investigated were able to fully compete out all complexes. More work is therefore required to determine the involvement of transcription factors in the TGF-β response.

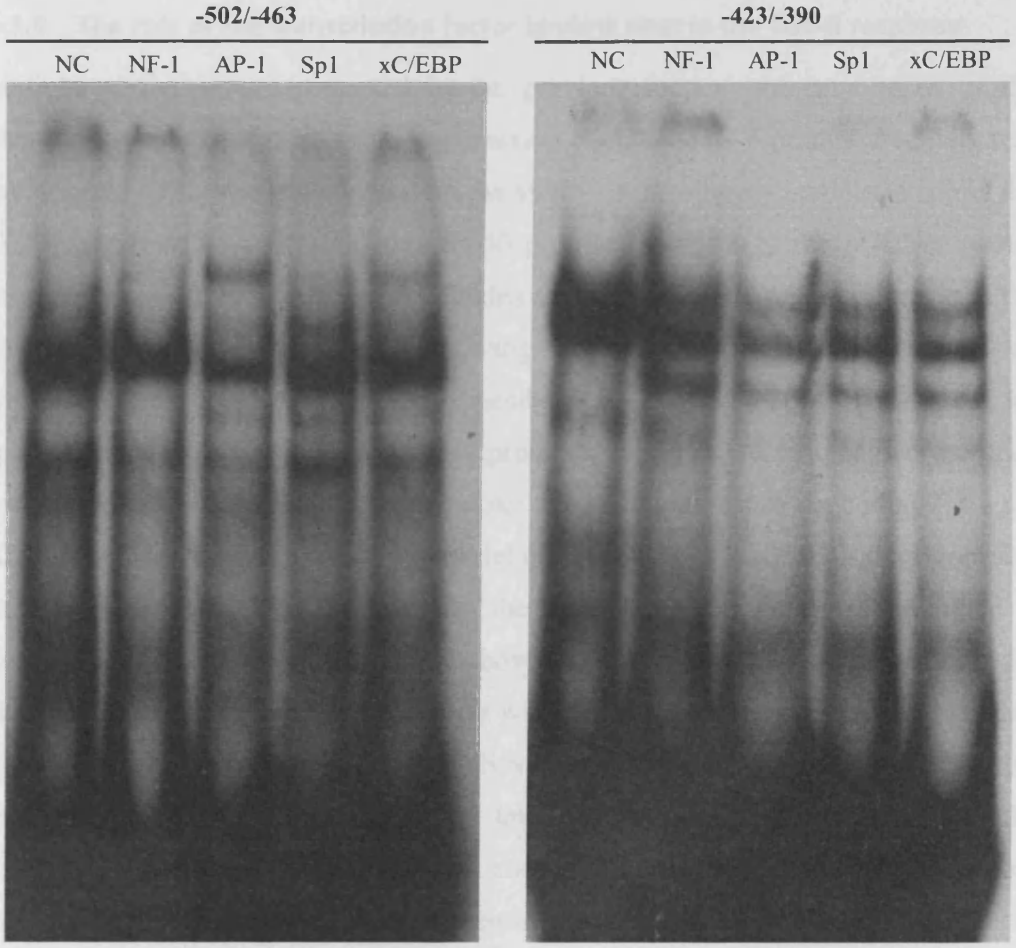

Figure 6.17 EMSA screen for competing Oligonucleotides for DNA:protein complexes produced in response to TGF-β. Competition EMSA analysis was carried out as described in Figure 6.13 but in place of competing ADAMTS-4 oligonucleotides, specific oligonucleotides coding for the transcription factors NF-1, AP-1, Sp1, and C/EBP were used in a 250-fold excess. Images shown are representative of two independent experiments.

6.3.9 The role of the transcription factor binding sites in the TGF-β response

In light of the results presented in the previous section, the importance of the identified promoter regions to the suppression of ADAMTS-4 promoter activity was investigated. This was done by testing the ability of the oligonucleotides to confer the TGF-β response to a heterologous SV40 promoter present in the pGL2 promoter vector. The pGL2 promoter vector contains a multiple cloning region upstream of the SV40 promoter and luciferase gene allowing the luciferase activity in cells transfected with the plasmid to be analysed (see Appendix II for plasmid map). Oligonucleotides spanning the -506 to -396 ADAMTS-4 promoter region were designed so that they contained part of the ADAMTS-4 promoter flanked by an overhang coding for a cut XmaI restriction site or a cut XhoI restriction site at the 5' end to produce cohesive ends to allow their direct cloning into the pGL2 promoter vector as described in Sections 2.4.10 and 2.4.11. Table 2.8 shows the oligonucleotide sequences used for cloning. Unfortunately due to difficulties with cloning and time restrictions this work was not able to be carried out in full. Several attempts were made but no positive clones were isolated. This will be an important area of future research into the regulation of ADAMTS-4 by TGF-β and will allow the identification of key transcription factors involved in this response.

6.4 Discussion

Macrophages are a major source of proteases in the atherosclerotic plaque. Plaques that are prone to rupture have high levels of macrophages, macrophage-derived foam cells and MMPs (Galis et al. 1995; Raines 2000). Activation of macrophages and subsequent release of proteases, such as MMPs and other proteases, including the ADAMTS proteases, is likely to lead to destabilisation of the plaque and subsequent acute coronary events (Loftus et al. 2002; Newby 2007). However, very little is known about the role or regulation of the ADAMTS proteases in relation to atherosclerosis. ADAMTS-4 has been shown to colocalise with macrophages in atherosclerotic lesions and cleaves the primary vascular proteoglycan versican (Porter et al.2005; Wågsäter et al. 2008). It was therefore of interest to study the expression of macrophage ADAMTS-4 as a protease recently identified to be expressed in atherosclerotic lesions. Studies into the cytokine regulation of ADAMTS-4 will help to further understanding about the role of the ADAMTS proteases in relation to inflammation and atherosclerosis and may lead to potential therapeutic targets for treatment of inflammatory diseases.

The studies presented in this chapter demonstrate that the expression of ADAMTS-4 (at both the mRNA and protein level) is down-regulated by TGF-β in the THP-1 cell line (Figure 6.3 and 6.4A). This is a novel finding as to our knowledge there are no published studies detailing the regulation of ADAMTS-4 by TGF-β in a macrophage-like cell line. A negative action of TGF-β on the expression of ADAMTS-4 in the THP-1 cell line correlates well with one of the few studies carried out on the regulation of ADAMTS proteases in THP-1 cells which showed that pro-inflammatory cytokines could up-regulate the expression of a number of ADAMTS proteases (Wågsäter et al. 2008). These authors studied the expression of ADAMTS-1, -4, -7, -8, and -9 in differentiated THP-1 cells and demonstrated that following treatment with IFN-γ or TNF-α, ADAMTS-4, -7, -8 and -9 showed enhanced mRNA expression. Induced levels were observed after 6hr treatment with IFN-γ for ADAMTS-4 and at later time points for the other proteases (Wågsäter et al. 2008). It is well established that TGF-β antagonises many of the actions of IFN-γ and often has opposing effects on gene expression (Greenow et al. 2005; McLaren and Ramji 2009;

Panousis et al. 2001). We have shown that the mRNA expression of ADAMTS-4 is significantly reduced within 1hr of TGF-β treatment and that this reduced level of expression is sustained over a 24hr time course (Figure 6.5). This is the first time that the TGF-β-regulated expression of ADAMTS-4 has been studied over an extensive time course in THP-1 cells.

Consistent with our observation that TGF-β inhibits ADAMTS-4 expression, the cytokine has also been shown to inhibit the expression of a number of MMPs including MMP-1 (White et al. 2000), MMP-9 (Ogawa et al. 2004) and MMP-3 (Kerr et al. 1990). The promoters of MMP-1, MMP-3 and MMP-9 contain a TGF-β inhibitory element (TIE). In MMP-3 this is able to bind to AP-1 while in MMP-9 deletion of the TIE has no effect on MMP-9 expression but deletion constructs of the promoter suggest that NFκB is essential for the TGF-β regulated expression of this metalloprotease (Kerr et al. 1990; Ogawa et al. 2004)

Interestingly, the study by Wågsäter et al. (2008) showed that the expression of ADAMTS-1 remained unchanged in response to IFNγ and TNFα treatment suggesting that ADAMTS proteases can be differentially regulated, perhaps in a cell-type specific manner. This may be the case for ADAMTS-4. In contrast to the down-regulation by TGF-β observed in macrophages, ADAMTS-4 expression can be up-regulated by TGF-β in articular chondrocytes, human synoviates and also in prostate cells (Cross et al. 2005; Moulharat et al. 2004; Yamanishi et al. 2002). It is also possible that functional redundancy exists between the ADAMTS proteases. A number of ADAMTS proteases have been shown to be present within atherosclerotic lesions. However, only ADAMTS-4 has been demonstrated to colocalise with macrophages of lesions (Wågsäter et al. 2008).

Differentiated THP-1 cells share many of the same characteristics and properties as human monocyte-derived macrophages. ADAMTS-4 and ADAMTS-8 are upregulated following differentiation of THP-1 monocytes and human monocyte-derived macrophages (Worley et al. 2003; Wågsäter et al. 2008). We show here that the regulatory action of TGF-β on ADAMTS-4 is conserved between THP-1 cells and primary human monocyte-derived macrophages (Figure 6.4B). Taken together with

the data of Wågsäter et al. (2008) these data suggest that ADAMTS-4 may play a pro-inflammatory role in relation to atherosclerosis.

The ability of the protease to cleave the proteoglycan versican is likely to be key to its actions in atherosclerosis. Versican is the primary proteoglycan component of the ECM that surrounds VSMCs. In normal physiological situations the ECM prevents migration of VSMCS into the arterial intima. In atherosclerosis however, degradation of the ECM by proteases results in the migration of VSMCs into the intima (Lusis et al. 2004; Raines 2000). It is possible that during the early stages of the disease the action of TGF-β on ADAMTS-4 that we have observed in THP-1 macrophages is anti-atherogenic as reduced levels of the ADAMTS-4 protease may prevent versican cleavage and subsequent ECM breakdown and VSMC migration into the arterial intima. ADAMTS-7 has been demonstrated to facilitate the migration of VSMCs and intimal thickening in a rat vascular balloon-injury model and this is thought to be mediated by the breakdown of cartilage oligo matrix protein (COMP) by the protease (Wang et al. 2009).

In the later stages of atherosclerosis the production of ECM by VSMCs is important for the formation of a stable plaque to prevent rupture (Lusis et al. 2004). TGF-β is known to promote a stable ECM-rich plaque phenotype and may achieve this partly through inhibition of ADAMTS-4 expression. The cytokine increases deposition of proteoglycans and collagen to promote a stable plaque and has been shown to stimulate the synthesis of collagens type I and III in human arterial SMCs and increase versican mRNA expression in monkey arterial SMCs (Schmidt et al. 2006). The synthesis and secretion of certain proteases including plasminogen activator and collagenase are inhibited by TGF-β (Kähäri et al. 1991) and the synthesis of TIMP-3 (an inhibitor of ADAMTS-4 expression) is induced by TGF-β in cartilage and also in rabbit aortic SMCs (Fabunmi et al. 1996; Qureshi et al. 2008).

The action of ADAMTS-4 on versican may also contribute to lesion regression or growth during atherosclerosis. Intimal tissue regression following balloon- or stent-mediated injury has been shown to be associated with increased blood flow and loss of versican (Kenagy et al. 2005). In a baboon vascular graft model high blood flow causes cell death and loss of ECM. This is accompanied by an increase of a versican

cleavage product that can be generated by the ADAMTS proteases -1, -4, -5 and -9 (Kenagy et al. 2005). Kenagy and colleagues measured the mRNA levels of ADAMTS proteases in this graft model and found that levels of ADAMTS-4 were significantly increased following a switch to high blood flow and that this was linked with tissue atrophy (Kenagy et al. 2005; Kenagy et al. 2009).

In addition to characterising the response of ADAMTS-4 to TGF-β we also used siRNA technology with the aim of determining which of the TGF-β signalling pathways played a role in mediating this response in THP-1 cells. The Smads are the classical transducers of the TGF-β signal and are expressed in macrophages and foam cells of atherosclerotic lesions (Kalinina et al. 2004). Data presented in Chapter 4 of this thesis suggests that this pathway plays a critical role in the regulation of expression of genes implicated in foam cell formation and atherosclerosis by TGF-β. We therefore wanted to see if this was the case with the regulation of ADAMTS-4. Our findings indicate that the Smad pathway may be involved in the action of TGF-β on ADAMTS-4. The Smad pathway has been demonstrated to be involved in the TGF-β-regulated induction of TIMP-3 (a negative regulator of ADAMTS-4 expression) in chondrocytes. Qureshi and colleagues showed that pharmacological and siRNA-mediated inhibition of Smad-2 and Smad-3 resulted in the inhibition of TIMP-3 induction by TGF-β (Qureshi et al. 2008).

Following siRNA-mediated knockdown of Smad-2 and Smad-3 the TGF-β-mediated inhibition of ADAMTS-4 mRNA expression was no longer significant (Figure 6.7A and 6.7B). It is interesting to note that knockdown of Smad-3 produces a more potent change on the ADAMTS-4 response than knockdown of Smad-2 although again, this is likely to be due to different levels of knockdown and is likely to be affected by the unspecific nature of Smad-2 versus Smad-3 knockdown using siRNA. Knockdown of both Smad-2 and -3 also showed a reversal of TGF-β-mediated inhibition that was akin to that seen with Smad-3 knockdown (Figure 6.7C). Smad-3 has been implicated in the repression of a number of genes including MCP-1 and the scavenger receptors CD36 and CD163 as discussed in more detail in Chapter 4 (Feinberg et al. 2004a; Fu et al. 2003; Pioli et al. 2004). An involvement of Smad-3 in the repression of VSMC activation and macrophage activation has also been identified (Feinberg et al. 2004b; Werner et al. 2000). This finding is also consistent with the data presented in Chapter

4 of this thesis that suggests that Smad-3 is the key player in the repression of LPL, CD36 and SR-B1 by TGF-β. It is possible that a conserved mechanism for negative regulation of gene expression by the cytokine may exist in THP-1 cells and that ADAMTS-4 is regulated in this manner.

As TGF-β can signal through Smad-independent pathways the effect of MAPK knockdown on the expression of ADAMTS-4 was also investigated. Both siRNA-mediated knockdown of c-Jun and p38 kinase resulted in increased levels of ADAMTS-4 expression in TGF-β treated cells as compared to knockdown of the control gene GAPDH (Figure 6.8A and 6.8B). This suggests that in addition to the Smad pathway, these pathways may also contribute to the TGF-β-regulated expression of ADAMTS-4. Whether these pathways act independently or in conjunction with the classical Smad pathway is unclear. The TGF-β-mediated induction of TIMP-1 expression and inhibition of MMP-1 expression has been shown to be dependent on the AP-1 sites found within their promoters (Hall et al. 2003). TIMP-1 induction requires JunD, c-fos and c-Jun but is Smad independent while the AP-1 site of MMP-1 is able to interact with Smads to mediate the TGF-β-regulated expression (Hall et al. 2003).

Various studies have suggested that crosstalk between the Smad pathway and the p38 MAPK pathway mediates the TGF-β regulation of MMP expression. For example, the induction of MMP-13 expression by TGF-β in human gingival fibroblasts is dependent on p38 kinase activity (Ravanti et al. 1999). Adenoviral-mediated gene delivery of Smads has shown that Smad-3 augments this induction and that delivery of a dominant-negative Smad-3 blocks the induction of MMP-13 by TGF-β and p38 kinase. Nuclear translocation of Smad-3 was also induced by p38 kinase activation suggesting that crosstalk between the two pathways is responsible for the TGF-β-mediated induction of MMP-13 (Leivonen et al. 2002). Interestingly sites for AP-1 are present within both the -502/-463 and -423/-390 promoter regions of ADAMTS-4 (Figure 6.13). No Smad binding elements were identified suggesting that Smads are likely to mediate the TGF-β response through interactions with other proteins or transcription factors.

Following the identification of TGF-β signalling pathways that are likely to be involved in the inhibition of ADAMTS-4 expression, we wanted to identify the regulatory region of the ADAMTS-4 promoter involved in this response. Although many of the ADAMTS proteases have now been cloned and characterised only the promoters of ADAMTS-4 and ADAMTS-5 have been investigated. A study by Thirunavukkarasu and colleagues found that the ADAMTS-4 promoter contains consensus binding sites for the nuclear factor of activated T-cells (NFAT) transcription factors that are identical to those found in the regulatory regions of cytokine genes (including IFNγ and TNFα) expressed by T-lymphocytes. Overexpression of NFAT can enhance ADAMTS-4 promoter activity suggesting that transactivation by NFAT can take place (Thirunavukkarasu et al. 2006). Other binding sites of interest identified in this study included PEA3 binding sites, also found in the promoters of MMPs, and a site for NF-IL6 (Mizui et al. 2000).

Mizui et al (2000) have identified the -383 to +10 region of the ADAMTS-4 promoter as the region required for full promoter activity. This region contains one site for Sp1 and three sites for AP2. Studies of the promoter activity of various deletion constructs by the group also suggested that the region between -726 and -384 is likely to contain silencer elements. The presence of an NF-1 site within this region suggests that this site may be involved in negative regulation of the ADAMTS-4 promoter; deletion of the NF-1 site did result in a recovery of promoter activity. NF-1 transcription factors have been shown to negatively regulate the transcription of a number of genes including the growth arrest and DNA damage inducible gene 153 (gadd153) in vascular smooth muscle cells and von-Willibrand factor in endothelial cells (Jahroudi et al. 1996; Nakamura et al. 2001).

We show here that ADAMTS-4 promoter activity is suppressed by TGF-β treatment (Figure 6.11). Transient transfection of ADAMTS-4 promoter deletion constructs demonstrates a potential involvement for the -506/-396 region in the negative regulation of the ADAMTS-4 promoter by TGF-β (Figure 6.12). This is consistent with the findings of Mizui et al. (2000) as these regions fall within the promoter region identified as likely to be involved in negative regulation of the ADAMTS-4 promoter. Further studies using EMSA analysis identified the regulatory regions involved in changes in DNA:protein interactions. DNA:protein binding increased in

response to TGF-β in the -502/-463 and -423/-390 regions of the promoter (Figure 6.14) and competition EMSA showed that this binding was specific (Figures 6.15 and 6.16).

Analysis of the ADAMTS-4 promoter -506/-396 region for putative Smad binding elements did not identify any Smad binding sites in this region of the promoter. However, the search did reveal putative binding sites for other transcription factors that could mediate the TGF-β response. Putative binding sites of interest present in both promoter regions identified by EMSA include sites for RXRα, Sp1, c-fos, NF-1 and Kruppel-like factor (KLF) (Figure 6.13). Of these, Sp1 and RXR have previously been shown to mediate TGF-β responses in THP-1 macrophages (Fu et al. 2003; Hughes et al. 2002; Irvine et al. 2005).

Sp1 is a zinc finger protein that binds to GC-rich sequences and is generally thought to act as a constitutive promoter element that supports basal transcription by recruiting the TATA binding protein (TBP). However, Sp1 sites have also been shown to be involved in tissue-specific gene expression and transcription controlled by a number of stimuli (Black et al. 2001). It is therefore possible that the ADAMTS-4 response is mediated by Sp1. As explained in more detail in Section 3.1.2, studies in our laboratory have shown that Sp1 is involved in both the IFN-γ- and TGF-β-mediated inhibition of LPL gene transcription in macrophages (Hughes et al. 2002; Irvine et al. 2005). Sp1 has also been shown to interact with Smads however; competition EMSA data (Figure 6.16) suggested that Sp1 is not involved in the ADAMTS-4 response.

Another possibility is the TGF-β response on ADAMTS-4 is mediated through RXRα. RXR forms heterodimers with PPARs to exert its effects on gene expression. As explained in more detail in Chapter 3, PPARs have been shown to be involved in the TGF-β-mediated regulation of expression of a number of genes. The induction of TIMP-3 and collagen by TGF-β has been shown to mediated by PPARδ in human aortic smooth muscle cells (Kim et al. 2009) and the inhibition of scavenger receptor CD36 expression is also mediated through PPAR and Smad-3 (Fu et al. 2003) as explained in Chapter 3. Interestingly Worley and colleagues have shown that the expression of ADAMTS-4 in THP-1 macrophages can be suppressed by both the PPARγ agonist GW9662 and the RXR ligand 9-cis retinoic acid both individually and

in combination (Worley et al. 2003). This suggests that regulation of constitutive expression of ADAMTS-4, mediated by PMA as part of the differentiation process in THP-1 macrophages, can be modulated by PPAR/RXR. Whether this effect extends to TGF-β-regulated expression of ADAMTS-4 has not been investigated.

Further work will be needed to elucidate the transcription factors involved in the TGF-β-mediated ADAMTS-4 response. In particular, analysis of the full-length ADAMTS-4 promoter could reveal binding sites for Smads or c-Jun that may participate in the inhibition of gene expression. Although these may not be contained within the minimal promoter region identified in this chapter they may become located close to the regulatory region following activation through folding and changes to the 3D structure of DNA within chromatin. Alternatively, involvement of Smad or p38 kinase/c-Jun may serve to enhance other transcription factors either indirectly or through protein:protein interactions.

In conclusion, we have characterised the regulation of ADAMTS-4 by TGF-β in THP-1 macrophages and identified the signalling pathways and minimal promoter regulatory region involved in this as summarised in Figure 6.18. Further studies will be needed to elucidate the exact mechanism of ADAMTS-4 regulation by TGF-β.

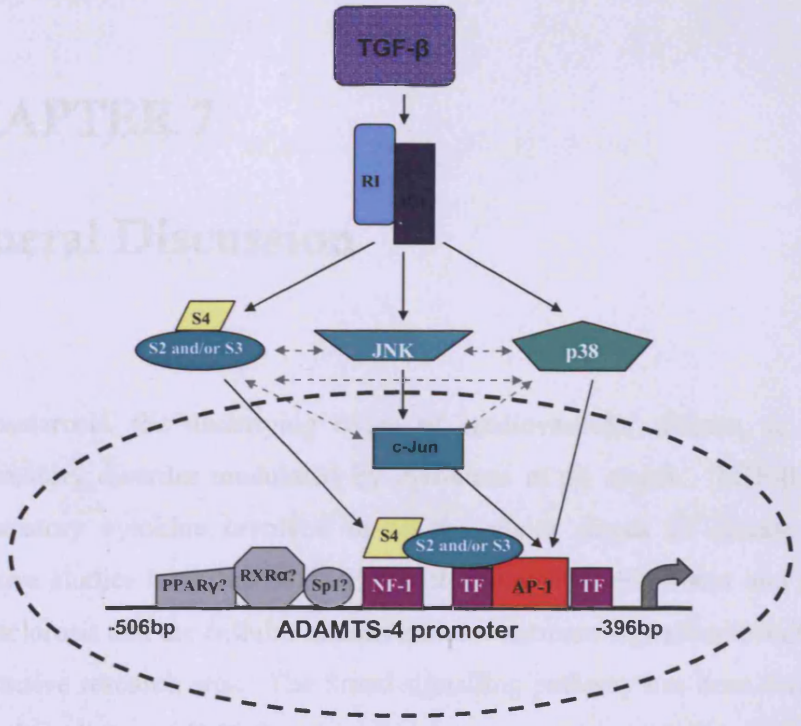

Figure 6.18 Regulation of ADAMTS-4 promoter activity by TGF-β in THP-1 macrophages. The Smad, JNK and p38 kinase pathways contribute to the inhibitory action of TGF-β on ADAMTS-4 as demonstrated by siRNA interference assays. Whether crosstalk between these pathways mediates this action is currently unclear (as indicated by dashed arrows). Transient transfection and EMSA analysis has identified the -506 to -396bp region of the ADAMTS-4 promoter as the minimal regulatory region for TGF-β action and other studies have shown that this region contains silencer elements such as the NF-1 transcription factor (Mizui et al, 2000). No Smad binding sites are present within this region suggesting that the Smads exert their effect through interaction with other transcription factors (TF) such as AP-1. Other transcription factors such as Sp1 and RXRα, binding sites which are found within this promoter region may be required for constitutive and/or TGF-β-regulated expression of ADAMTS-4.

CHAPTER 7

General Discussion

Atherosclerosis, the underlying cause of cardiovascular disease, is a progressive, inflammatory disorder modulated by cytokines at all stages. TGF-β is a key anti-inflammatory cytokine involved in all the major stages of disease development. Extensive studies have linked TGF-β with reduced development and progression of atherosclerosis and the cellular actions and downstream signalling events are therefore an attractive research area. The Smad signalling pathway has been established as the classical mediator of TGF-β action; however more recent studies have demonstrated activation of other signalling pathways by the cytokine. Despite this, many aspects of the molecular mechanisms underlying the regulation of gene expression by TGF-β remain unclear; in particular the actions of TGF-β on the expression of genes implicated in macrophage cholesterol homeostasis, warrants further study. The work detailed in this thesis has used several different approaches to delineate the molecular mechanisms underlying TGF-β-regulation of gene expression. The majority of this work has been carried out in macrophages as these cells have a central role in the pathology of atherosclerosis and are an important target for the actions of TGF-β. The aim of these studies was not only to enhance understanding about TGF-β signalling associated with atherosclerosis but potentially identify novel therapeutic avenues of investigation.

7.1 Mechanisms underlying the TGF-β regulation of gene expression

7.1.1 TGF-β-regulation of gene expression in macrophages

Experiments based on RT-qPCR demonstrated an inhibitory effect of TGF-β on the mRNA expression of genes implicated in cholesterol uptake and up-regulatory effect on genes implicated in cholesterol efflux (Chapter 3). Expression of LPL, SR-A, SR-B1 and CD36 was inhibited following TGF-β treatment of THP-1 macrophages and remained at low levels over a 24hr time course. Treatment with TGF-β resulted in the up-regulation of ApoE, ABCA-1 and ABCG-1 mRNA expression. Effects of the cytokine were substantiated at the protein level using Western blot analysis. An optimal time point for regulation by TGF-β in the THP-1 macrophage cell line was established by these studies to provide a foundation for further studies on the regulation of these chosen genes. In addition, these data demonstrated that the TGF-β-regulation of gene expression in THP-1 macrophages was consistent with literature on their regulation in murine macrophage cell lines and *in vivo* models as detailed in Chapter 3.

7.1.2 Roles of the Smad pathway in TGF-β-regulated gene expression

TGF-β markedly and rapidly induced the phosphorylation (and activation) of Smad-2 and Smad-3 in THP-1 macrophages (Chapter 4). This finding was extended to primary human monocyte-derived macrophages and suggested that the Smad signalling pathway was active in macrophages, consistent with a study showing that active Smad signalling is present in atherosclerotic lesions (Kalinina et al., 2004). The rapid kinetics of activation suggested that Smad phosphorylation is mediated through direct binding of TGF-β to its cognate receptors directly leading to Smad recruitment and activation.

Knockdown of Smad-2 and Smad-3 expression was achieved using siRNA transfection-based assays. Significant knockdown of Smad-2 and Smad-3 in THP-1 macrophages both singularly and together was demonstrated at the protein level using Western blotting. The use of siRNA combined with RT-qPCR and Western blotting revealed that the Smad pathway played a crucial role in the TGF-β regulation of

expression of LPL, SR-A, SR-B1 and CD36 and also in the TGF-β regulation of ApoE, ABCA-1 and ABCG-1 (Chapter 4). Knockdown of Smad-2 and -3 attenuated the TGF-β-induced expression of ApoE, ABCA-1 and ABCG-1 (with significant reductions in TGF-β-mediated expression levels observed for ApoE and ABCA-1). Smad-2 and -3 knockdown also produced a substantial attenuation of TGF-β-mediated inhibition of LPL, SR-A, SR-B1 and CD36 expression. It is difficult to determine the exact contribution of Smad-2 and Smad-3 to the regulation of gene expression for a number of reasons including differences in levels of knockdown using siRNA, and possible involvement of compensatory and regulatory mechanisms. However, the data undoubtedly demonstrates a crucial role for the Smad pathway in the TGF-β-regulation of gene expression in macrophages.

In addition, analysis of constitutive gene expression suggested roles for the Smads in the basal expression of SR-B1 and CD36 (Chapter 4). It is unclear how genes whose basal expression requires Smads are then differentially regulated by TGF-β through the Smad proteins, however, Smads may be a required part of the differentiation programme of THP-1 cells using PMA. Their expression is increased following monocyte-macrophage differentiation (Kalinina et al., 2004) and Smads are required for monocytic differentiation of myeloid leukemia cells by synthetic triterpenoids (Ji et al., 2006).

These studies were subsequently extended to primary macrophages using adenovirus-mediated delivery of Smad-2 shRNA (Chapter 4). Together, these studies suggest that TGF-β regulation of gene expression associated with macrophage cholesterol homeostasis is mediated through the Smad signalling pathway, specifically through phosphorylation and activation of Smad-2 and Smad-3 in macrophages. This is consistent with a study demonstrating direct involvement for Smad-3 in TGF-β-inhibited expression of the scavenger receptor CD163 (Pioli et al., 2004) which has been linked with foam cell formation in atherosclerosis. Our findings are also consistent with a number of studies that have suggested an involvement for Smads in TGF-β-regulated gene expression in macrophages (Al-Salleeh and Petro, 2008; Fu et al., 2003; Werner et al., 2000) as detailed in Chapter 4.

7.1.3 Roles of the MAPK pathways in TGF-β-regulated gene expression

TGF-β was also able to induce the phosphorylation of the MAPKs, ERK 1/2 (p44/42), JNK and p38 kinase in THP-1 macrophages (Chapter 5). This induction in phosphorylation translated to an increase in activity as measured by increased phosphorylation levels of downstream transcription factors targets Elk-1 and c-Jun through non-radioactive kinase activity assays. These findings were extended to primary human monocyte-derived macrophages and suggested that MAPKs are activated by TGF-β in macrophages. The slow kinetics of this activation suggest that although it is possible that the MAPKs can be activated by the cytokine through a direct route, activation is likely to be mediated by activation of other signalling mediators such as the Smad proteins or other signalling molecules or transcription factors. Although some downstream actions of TGF-β in macrophages have been shown to be mediated in part by the MAPKs (see Chapter 5), specific activation of the pathways had not previously been demonstrated in THP-1 macrophages.

Knockdown of individual MAPK expression (ERK 1/2, c-Jun, p38 kinase) using siRNA transfection of THP-1 macrophages revealed roles for c-Jun and p38 kinase in the TGF-β-regulated expression of LPL, SR-A, SR-B1, CD36, ABCA-1 and ABCG-1 (Chapter 5). TGF-β-mediated induction of ABCA-1 and ABCG-1 was reduced following knockdown of either p38 kinase or c-Jun. TGF-β-mediated inhibition of LPL, SR-A, SR-B1 and CD36 was attenuated following knockdown of either p38 kinase or c-Jun. A role for ERK 1/2 in the expression of ABCA-1, ABCG-1 and CD36 was also demonstrated. This is consistent with studies linking an increase in ERK activity with increased CD36 expression (Han et al., 2000) but contrasts with a study showing that siRNA against ERK 1/2 and activation of LXR increased ABCA-1 expression and activity in murine macrophages (Zhou et al., 2009). Contrasting data may reflect differences between murine and human macrophages, emphasising the necessity of data from human macrophages in addition to data from murine cell lines or *in vivo* disease models. No involvement of the MAPKs in the TGF-β-regulated expression of ApoE was demonstrated following siRNA knockdown, contrasting with previous studies by our laboratory using pharmacological inhibitors, and dominant-negative constructs in transfection assays, that had suggested a role for JNK and p38 kinase (Singh and Ramji, 2006). This may be due to functional redundancy between

the MAPKs/MAPK substrates. Another possibility is that the amount of kinase remaining following siRNA-mediated knockdown was sufficient for the response. This is one limitation of the siRNA knockdown technique.

Analysis of constitutive gene expression demonstrated a requirement for c-Jun in the expression of SR-A and a requirement for p38 kinase in the expression of SR-B1 and CD36 (Chapter 5). Taken together, these studies suggest that the MAPK pathways contribute to the TGF-β-regulation of genes associated with macrophage cholesterol homeostasis. Since a number of studies have demonstrated crosstalk between Smad and MAPK pathways in gene regulation this may be a potential mechanism of regulation of these genes (see Chapter 5). Documented interactions between Smad proteins and the AP-1 transcription factor complex (Dennler et al., 2000; Pessah et al., 2002; Pessah et al., 2001) may be an important part of the mechanism of gene regulation through these signalling pathways.

7.1.4 Mechanisms underlying TGF-β-regulation of LPL expression

The effect of c-Jun siRNA-mediated knockdown on the expression of LPL (Chapter 5) was consistent with unpublished data from our laboratory that suggested a role for the JNK pathway in the TGF-β-regulated inhibition of LPL expression. Transfection of dominant-negative constructs of JNK pathway components in the Hep3B cell line resulted in a small attenuation in the TGF-β-mediated inhibition of LPL promoter activity suggesting that the pathway had a role in this regulation, although some cell-type specific differences were observed between previous data from U937 cells and our data in Hep3B cells. Further studies focussed on identifying the co-factor involved in the TGF-β-mediated regulation of LPL. Transfection of siRNA targeting SRC-1 resulted in the attenuation of TGF-β-regulated LPL inhibition but also significantly reduced the constitutive expression of LPL (Chapter 5). This suggests that SRC-1 has a crucial role in both the constitutive and TGF-β-regulated expression of LPL. One potential mechanism of LPL regulation may involve activation of the JNK pathway (and subsequent activation of c-Jun/AP-1) by TGF-β which may then serve to mop up the limited amounts of SRC-1 co-activator required for basal LPL expression. Repression of LPL by TGF-β can then take place through suppression of Sp1/Sp3 transactivation potential (Irvine et al., 2005). The Smad pathway may also

contribute to this possibly through activation of JNK or through interaction with c-Jun/AP-1.

7.2 Characterisation of ADAMTS-4 regulation by TGF-β

The action of TGF-β on the expression of genes involved in the later stages of atherosclerosis is partially understood. The cytokine up-regulates the expression of genes encoding collagens and fibronectin and inhibits the expression of matrix metalloproteinases to promote stability and prevent rupture of the atherosclerotic plaque (Kähäri et al., 1991; Schmidt et al., 2006). The ADAMTS proteases are a group of secreted proteins that cleave components of the extracellular matrix, in particular the proteoglycans aggrecan, in cartilage, and versican, in the vasculature (Porter et al. 2005). A small number of studies suggest roles for ADAMTS-1 and -4 in atherosclerosis (see Chapter 6) and ADAMTS-4 has been shown to co-localise with macrophages in lesions (Wågsäter et al. 2008). Relatively little is understood about the expression and cytokine-regulation of ADAMTS-4 in macrophages and it is therefore a young and rapidly developing research area and may represent a novel avenue of therapeutic benefit.

7.2.1 TGF-β-regulation of ADAMTS-4 expression in macrophages

RT-qPCR demonstrated that treatment of THP-1 macrophages with TGF-β resulted in a significant decrease in ADAMTS-4 expression (Chapter 6). A time course demonstrated that inhibition of ADAMTS-4 had rapid kinetics. ADAMTS-4 expression remained low between 1 and 24hr following TGF-β treatment. Western blot analysis revealed that TGF-β also down-regulated levels of ADAMTS-4 protein expression. These findings were extended to human monocyte-derived macrophages and demonstrated that protein levels of ADAMTS-4 were down-regulated following TGF-β treatment of macrophages. Taken together, these data suggest that ADAMTS-4 expression is down-regulated by TGF-β in macrophages. This finding is consistent with the finding that IFN-γ, which antagonises the actions of TGF-β, up-regulates ADAMTS-4 expression in the THP-1 macrophage cell line (Wågsäter et al., 2008).

The use of siRNA against Smad-2 and Smad-3 attenuated the TGF-β-mediated inhibition of ADAMTS-4 mRNA expression (Chapter 6). Further studies using siRNA targeting p38 kinase and c-Jun showed that following knockdown of p38 kinase or c-Jun, the TGF-β-mediated expression of ADAMTS-4 was no longer significantly inhibited. Knockdown of p38 kinase also significantly reduced constitutive ADAMTS-4 expression indicative of a role for the kinase in the basal expression of ADAMTS-4. Together these studies revealed crucial roles for the Smad pathway in the TGF-β-mediated inhibition of ADAMTS-4 and, in addition, suggested roles for p38 kinase and c-Jun. Taken together, these studies suggest that TGF-β-mediated inhibition of ADAMTS-4 in macrophages is Smad-dependent but may also require p38 kinase and c-Jun. This is consistent with studies showing Smad involvement in the TGF-β-mediated induction of TIMP-3 (a negative regulator of ADAMTS-4) and the requirement of AP-1 sites within the promoter for TGF-β-regulated inhibition of MMPs (Hall et al., 2003; Qureshi et al., 2008). Further studies would be required to elucidate the exact mechanism underlying TGF-β-regulation of ADAMTS-4 expression in macrophages.

Transient transfection of a full-length promoter construct in Hep3B cells demonstrated that TGF-β down-regulated the promoter activity of ADAMTS-4 suggesting that TGF-β mediates ADAMTS-4 expression levels through effects at the gene promoter (Chapter 6). Further studies focussed on delineating the minimal sequence elements involved in the inhibitory effect of TGF-β on the activity of the ADAMTS-4 promoter. Transfection-based assays using deletion constructs of the ADAMTS-4 promoter located the minimal response elements for TGF-β action to the -506/-396 region. Subsequent EMSA analysis identified increased protein binding to the -502/-463 and -423/-390 regions of the promoter while competition EMSA showed that binding to these regions was specific. Due to limited availability of oligonucleotides, competition EMSA using oligonucleotides encoding known transcription factors failed to identify the transcription factors responsible for the increase in protein binding to the promoter. However, these data indicate that TGF-β down-regulates ADAMTS-4 expression through effects on promoter activity and the minimal response elements for this inhibitory action are located within the -506/-396 region of the ADAMTS-4 promoter. This is consistent with a study that suggested

that the -726/-384 region contained elements involved with negative regulation of the promoter (Mizui et al., 2000).

In summary, the work presented in this thesis has been successful in enhancing our understanding of the signalling pathways by which TGF-β regulates gene expression with relation to macrophage cholesterol homeostasis, in addition to the regulation of ADAMTS-4 by TGF-β in THP-1 macrophages. The main findings of this work are summarised below. The roles of Smads and MAPKs in individual gene expression are outlined in Table 7.1.

1. TGF-β down-regulates the expression of genes involved in macrophage cholesterol uptake (e.g. LPL, SR-A, SR-B1 and CD36) and up-regulates the expression of genes involved in macrophage cholesterol efflux (e.g. ApoE, ABCA-1 and ABCG-1) in THP-1 macrophages.

2. The Smad signalling pathway is activated by TGF-β with rapid (within 1hr) kinetics in THP-1 macrophages and primary human monocyte-derived macrophages.

3. Smad-2 and Smad-3 are crucial mediators in the regulation of gene expression by TGF-β in THP-1 macrophages and primary human monocyte-derived macrophages.

4. Smads are required for the constitutive expression of SR-B1 and CD36 in THP-1 macrophages.

5. ERK 1/2, JNK and p38 kinase are activated by TGF-β with slow (within 24hr) kinetics in THP-1 macrophages and primary human monocyte-derived macrophages.

6. Both p38 kinase and c-Jun are important mediators in the regulation of LPL, SR-A, SR-B1, CD36, ABCA-1 and ABCG-1 expression by TGF-β in THP-1 macrophages.

7. ERK1/2 is an important mediator of TGF-β-regulated expression of CD36 ABCA-1 and ABCG-1 in THP-1 macrophages.

8. c-Jun may be required for constitutive expression of SR-A and p38 kinase may be required for the constitutive expression of SR-B1 and CD36 in THP-1 macrophages.

9. TGF-β down-regulates the mRNA and protein expression of ADAMTS-4 in THP-1 macrophages with rapid kinetics (within 1hr) and expression remains inhibited over a 24hr time course.

10. Smad-2 and -3, p38 kinase and c-Jun are important mediators of the TGF-β-regulated expression of ADAMTS-4 and p38 kinase may be required for constitutive expression of ADAMTS-4 in THP-1 macrophages.

11. TGF-β down-regulates the promoter activity of ADAMTS-4 in Hep3B cells through minimal response elements within the -506/-396 region of the ADAMTS-4 promoter and specific protein binding to two regions within this promoter sequence (-502/-463 and -423/-390) is increased following TGF-β stimulation.

Table 7.1 **Involvement of Smads and MAPKs in constitutive and TGF-β-regulated gene expression in THP-1 macrophages**

Gene	Smad-2	Smad-3	Smad-2 and -3	ERK 1/2	c-Jun	p38 kinase
TGF-β-regulated gene expression						
LPL	✓	✓	✓	x	✓	✓
SR-A	x	x	✓	x	✓	✓
SR-B1	✓	✓	✓	x	✓	✓
CD36	✓	✓	✓	✓	✓	✓
ApoE	x	x	✓	x	x	x
ABCA-1	✓	✓	✓	✓	✓	✓
ABCG-1	✓	✓	✓	✓	✓	✓
ADAMTS-4	✓	✓	✓	x	✓	✓
Constitutive gene expression						
LPL	x	x	x	x	x	x
SR-A	x	x	x	x	✓	x
SR-B1	✓	x	✓	x	x	✓
CD36	✓	✓	✓	x	x	✓
ApoE	x	x	x	x	x	x
ABCA-1	x	x	x	x	x	x
ABCG-1	x	x	x	x	x	x
ADAMTS-4	x	x	x	x	x	✓

KEY: ✓ Role in expression of gene x No role in expression of gene

7.3 Future work

A number of avenues for further investigation have been highlighted by the results of this study. These can be split into investigations of Smads and MAPKs in the regulation of gene expression and cellular responses by TGF-β and the further characterisation of the ADAMTS-4 response to TGF-β.

7.3.1 Future investigations into the roles of the Smad and MAPK pathways in the regulation of gene expression by TGF-β

Although a key step in the Smad signalling pathway involves the formation of complexes between either Smad-2 and Smad-4 or Smad-3 and Smad-4 for translocation to the nucleus, some studies have noted a variable role for Smad-4 in TGF-β signalling (Liu et al., 1997; Sirard et al., 2000). Extending our studies to determine the role of Smad-4 is therefore important to fully understand the molecular mechanisms behind TGF-β-regulated gene expression and could be achieved using siRNA combined with RT-qPCR as for Smad-2 and -3. Studies in primary cultures of human monocyte-derived macrophages will be of great importance in extending the findings of our studies and determining their biological relevance. These studies could use adenovirus-mediated delivery of shRNA (as described in Chapter 4) against the Smads and MAPKs into primary macrophages. It would also be of interest to confirm some of our findings using cells derived from Smad-2/Smad-3/MAPK-deficient mice.

To investigate the roles of Smads and MAPKs in cellular responses, total cholesterol uptake and total cholesterol efflux from macrophages could be measured following siRNA-mediated knockdown of Smads/MAPKs. This would involve measuring uptake of radioactively labelled or fluorescent oxLDL/AcLDL, and/or efflux of radioactive cholesterol from foam cells to acceptors such as ApoAI (Geng and Hansson 1992; Kennedy et al. 2005). Additionally, lipid accumulation could be measured through staining of lipids within cells using a dye such as Oil Red O. Such studies would further understanding of the roles of TGF-β signalling pathways in cholesterol homeostasis and foam cell formation. Use of *in vivo* model systems may be helpful here to link changes in cellular response to pathophysiological changes such as changes in atherosclerotic lesion size or plaque stability. For example, an *in vivo* model of foam cell formation can be created by interperitoneal injection of J774 murine macrophage cells preloaded with radioactively labelled cholesterol into mice. This model is useful for analysing reverse cholesterol transport from macrophages into the plasma, liver and bile (Zhang et al., 2005; Zhang et al., 2003).

Crosstalk between signalling pathways is relatively common in the regulation of gene expression, as described in Chapter 5. To further investigate this possibility with relation to our genes of interest it would first be important to determine the effects of multiple knockdowns (Smads and individual MAPKs) on gene expression in THP-1 cells. Another method of investigating crosstalk between signalling pathways or pathway components could look at activation of a pathway/factor of interest in cells where key components of the other signalling pathways have been inhibited through pharmacological agents or siRNA-mediated knockdown. Interactions between Smads and MAPK-activated transcription factors such as AP-1 have been documented to regulate a number of genes (Chapter 5) and it would be important to investigate the activation of such transcription factors following Smad knockdown. It is also known that MAPKs can have effects on the phosphorylation status of Smads and vice versa. It would therefore be important to measure phosphorylated forms of the Smads and MAPKs following siRNA-mediated knockdown. These studies would greatly enhance our understanding of the molecular mechanisms of TGF-β-regulated gene expression. The use of pull-down assays and/or co-immunoprecipitation could be used here to determine if any complexes are formed between Smads and MAPKs/MAPK-activated transcription factors (Brown et al. 1999; Funaba et al. 2002; Quinn et al. 2001). An important consideration for these studies, particularly in terms of the requirement of Smads and/or MAPKs in constitutive gene expression, would be the levels of negative regulators of Smad and MAPK pathways such as inhibitory Smads, MAPK phosphatases and co-repressors and how these may be affected through activation of transcription factors by TGF-β signalling.

Of additional interest would be the investigation of promoter activity in cells with reduced or abolished Smad/MAPK expression. EMSA and promoter analysis could be used with the aim of identifying transcription factors/binding elements involved in TGF-β-regulated expression of each gene. Analysis of promoter sequences for transcription factor binding sites (see Chapter 6) would support these investigations and allow the exact mechanism by which TGF-β signalling regulates the expression of each gene.

One factor likely to play a role in TGF-β-regulated expression of our genes of interest either directly or indirectly is PPARγ. The factor has been linked with each of the

genes studied in this thesis, as explained in detail in Chapter 3. It would therefore be of significant importance to study its role in the TGF-β-regulation of these genes and its interactions/links with Smads and MAPKs using the techniques described above. Expression of PPARγ could be analysed using Western blot or RT-qPCR analysis following knockdown of Smads and/or MAPKs using pharmacological agents or siRNA while interactions with Smad/MAPK binding elements within the promoter of the gene of interest and with Smad and MAPK-activated transcription factors could also be an avenue for further investigation.

7.3.2 Future investigations into the regulation of ADAMTS-4 by TGF-β

Incomplete characterisation of TGF-β-mediated regulation of ADAMTS-4 promoter activity and expression necessitates further studies into this regulation. Expansion of competition EMSA data using oligonucleotides encoding the binding sequences of transcription factors of interest (i.e. those present within the minimal promoter response region) would be an initial step towards identifying the factors responsible for negative regulation of ADAMTS-4 promoter activity by TGF-β. Completion of studies designed to confer the response to a heterologous promoter would also provide a foundation for further studies using transient transfection such as analysis of the effects of mutation in these sites. Suggestions for further investigations into the exact roles of Smads and MAPKs as described in Section 7.3.1 are also applicable here. One important avenue for study is the investigation of what the down-regulation of ADAMTS-4 expression by TGF-β means in terms of ADAMTS-4 activity and cellular responses. Western blotting using an antibody targeted to cleaved versican fragments could be used to detect levels of ADAMTS-4 activity/versican cleavage. Alternatively, levels of radioactively labelled full length versican and cleaved versican fragments could be analysed using chromatography to separate molecules based on their molecular weights. Cells derived from ADAMTS-4 deficient mice and *in vivo* models would also be useful here to analyse effects on versican expression and cleavage, lesion development and plaque stability.

7.3.3 Future investigations into the role of TGF-β in macrophages

It has been shown that different subsets of monocytes and macrophages exist. These follow inflammatory pathways and are present in atherosclerotic lesions. In mice there are two primary monocyte phenotypes: $Ly6C^{high}$, $CCR2^{+}$, $CX3CR1^{low}$ which rapidly move into areas of inflammation in response to pro-inflammatory cytokines and chemokines (including CCL2), and $Ly6C^{low}$, $CCR2^{-}$, $CX3CR1^{high}$ which move into inflamed tissues at later stages of inflammation in a CX3CL1 dependent manner (Johnson and Newby, 2009; Shimada, 2009). Interestingly, high levels of CCL2 are observed in early plaques of ApoE null mice while CX3CL1 only increases in advanced and unstable plaques (Johnson and Newby, 2009). In humans, the abundant $CD14^{+}$, $CD16^{-}$, $CCR2^{+}$ monocytes are equivalent to the $Ly6^{high}$ mouse phenotype and appear to be anti-inflammatory as they release IL-10 in response to LPS. The less abundant $CD14^{-}$, $CD16^{+}$, $CCR2^{-}$ monocytes are equivalent to the $Ly6C^{low}$ mouse phenotype and appear to be pro-inflammatory as they produce pro-inflammatory cytokines and mediators in response to LPS (Johnson and Newby, 2009; Shimada, 2009).

Two distinct macrophage phenotypes exist. M1 macrophages have been classically activated, M2 macrophages have been alternatively activated (Charo, 2007). Th1 cytokines (IFNγ, Il-1β and LPS) induce so called classical activation (M1) and Th2 cytokines (Il-4 and IL-13) induce alternative activation (M2). Human monocytes differentiated with GM-CSF have M1 properties and those differentiated with M-CSF have M2 properties (Gordon and Martinez, 2010; Johnson and Newby, 2009; Mantovani et al., 2004). M1 macrophages are pro-inflammatory with roles in phagocytosis, killing pathogens and production of proteases and have high levels of IL-12 and IL-23. M2 macrophages are anti-inflammatory with roles in scavenging cell debris, promoting angiogenesis, tissue remodelling and repair. They release arginase-1 to counter production of iNOS and have high levels of IL-10 and low levels of IL-12 (Bouhel et al., 2007; Charo, 2007; Mantovani et al., 2004). Macrophages can switch between states in response to different signals (Bouhel et al., 2007; Shimada, 2009).

How TGF-β signalling and regulation of gene expression influences or is influenced by different subsets of macrophages is currently unclear, although TGF-β is associated with M2 macrophages by its anti-inflammatory properties. M1 macrophages can be switched to M2 macrophages in vitro by incubation with medium from M2 macrophages and this occurs through production of anti-inflammatory mediators including IL-10 and TGF-β (Bouhel et al 2007; Shimada 2009). The cytokine has a key role in differentiation of T-cell subsets, promoting the differentiation of $CD4^+$ T cells into Treg cells through induction of transcription of the Foxp3 gene, and also (with IL-6) promoting differentiation of $CD4^+$ T cells into Th17 cells. Knockout of T-cell Smad expression has demonstrated roles for Smad-3 and Smad-2 respectively in these processes (Malhotra et al., 2010). Further studies could investigate TGF-β signalling and regulation of key genes involved in foam cell formation in different subsets of macrophages.

PPARγ has been shown to be a crucial regulator of M1/M2 polarisation (Bouhel et al 2007; Duan et al 2009). Agonists of PPARγ can inhibit activation of the M1 phenotype and deletion of macrophage PPARγ shifts the balance of pro-inflammatory cytokines towards the M1 phenotype (Duan et al 2009). In contrast, IL-4-mediated monocyte differentiation into M2 macrophages can increase PPARγ expression and activity, and the expression of PPARγ correlates with the expression of other M2 markers including CD36, mannose receptor and IL-10 in human carotid lesions (Bouhel et al 2007). The inability of PPARγ to promote a switch of M1 or M0 (resting) macrophages to the M2 phenotype suggests that PPARγ promotes differentiation of monocytes to M2 macrophages (priming) and may not have any effect in fully differentiated macrophages. In terms of foam cell formation, deletion of PPARγ in macrophages can reduce IL-4-induction of LPL and CD36 expression and reduce the expression of ABCG-1, suggesting that PPARγ has roles in both uptake and efflux of cholesterol from macrophages Duan et al 2009; Odegaard et al 2007). Further studies into the role of PPARγ with relation to TGF-β-mediated regulation gene expression in different subsets of macrophages could help delineate the role of PPARγ in foam cell formation.

In addition to TGF-β, other anti-inflammatory (Th2) cytokines have been demonstrated to have atheroprotective roles in atherogenesis, disease development and foam cell formation. These include IL-10 and IL-33.

IL-10 is an anti-inflammatory cytokine and a key regulator of the immune response to viral, bacterial and fungal infections (Couper et al., 2008). The cytokine can regulate Th1 or Th2 responses (often to inhibit excessive cytokine production or immune cell function) to regulate immunopathology following infection (Couper et al., 2008). Although IL-10 can act directly on T cells to inhibit proliferation and cytokine production, many of the effects of IL-10 on T cell function are mediated though direct effects on monocytes/macrophages such as the inhibition of MHC class II molecules to limit production of pro-inflammatory cytokines and chemokines (Couper et al., 2008). A number of studies have suggested IL-10 has an anti-atherogenic role and overexpression of macrophage IL-10 can suppress atherosclerosis in $LDLR^{-/-}$ mice (Han et al., 2010). Interestingly, IL-10 can promote both cholesterol uptake and cholesterol efflux from macrophages through the up-regulation of CD36 and SR-A and the upregulation of ABCA-1 through the PPARγ-LXR pathway (Han et al., 2009). In addition, IL-10 can inhibit the down-regulation of ABCA-1 by TNFα (Mei et al., 2007). Foam cells treated with IL-10 display reduced apoptosis as well as reduced expression of pro-inflammatory mediators including TNFα and ICAM-1 (Han et al., 2009).

Our laboratory has recently demonstrated an anti-atherogenic role for IL-33 in foam cell formation (McLaren et al., 2010). $ApoE^{-/-}$ mice treated with IL-33 showed significant reductions in macrophage foam cell accumulation and treatment of THP-1 macrophages and primary HMDMs with IL-33 reduced uptake of oxLDL, total and esterified cholesterol content, and enhanced cholesterol efflux (McLaren et al., 2010). These changes were associated with down-regulation of genes involved in cholesterol uptake and up-regulation of genes involved in cholesterol efflux by the cytokine, similar to the effects of TGF-β treatment as presented in Chapter 3 of this thesis (McLaren et al., 2010). It would be interesting to investigate whether IL-10 or IL-33 are able to synergise with TGF-β to enhance anti-atherogenic effects on gene regulation and foam cell formation in atherosclerosis.

7.4 Therapeutic implications for atherosclerosis

There is a significant need for new therapeutic strategies or improvements to existing drugs to prevent the clinical symptoms of atherosclerosis. Currently, 70% of clinical events resulting from atherosclerosis cannot be prevented with existing drugs. Novel drugs to reduce cardiovascular inflammation must be designed not to interfere with the cardiovascular risk profiles such as lipid balance. Current strategies for the design of novel drugs to reduce atherosclerosis include the blockade of the pro-inflammatory cytokine TNF-α, the IL-1 receptor and leukotrienes (Klingenberg and Hansson, 2009). Antagonists of TNF-α reduce the expression of pro-inflammatory cytokines and chemokines in addition to TNF-α and also reduce the effects of TNF-α on MMP production. Blockade of this cytokine has been shown to be effective in reducing the incidence of cardiovascular events in rheumatoid arthritis patients (Klingenberg and Hansson, 2009).

The use of siRNA targeting pro-inflammatory or pro-atherogenic molecules such as adhesion molecules, chemokines or for selectively blocking inflammatory monocytes or T-cells could prove useful in reducing atherosclerosis development. The use of RNAi as a research tool for molecular biology applications such as functional gene analysis and the dissection of signalling pathways have become widespread. However, its use for therapeutic drug design has so far been limited (de Fougerolles et al., 2007; Hajeri and Singh, 2009). Outside of the laboratory there are many considerations that need to be taken into account when designing siRNAs and the carrier system that goes with them. Some of the issues with the design and potential for RNAi based drugs include drug specificity and stability. Although highly specific in nature, siRNAs can sometimes recognise and interfere with the expression of off-target genes *in vitro*. Many of these effects occur because of sequence similarity between the gene and the 5' end of the guide siRNA strand (de Fougerolles et al., 2007; Hajeri and Singh, 2009). siRNAs may also induce unwanted effects through activation of the immune system and immune receptors. Careful design of the carrier system is important to restrict these effects and some of these effects can be partially countered by the use of miRNA, as this occurs naturally within the human genome (de Fougerolles et al., 2007; Hajeri and Singh, 2009). Stability and half life of siRNAs is

often dependent on the carrier system and siRNAs for therapeutic use are often conjugated to molecules such as cholesterol or contained within liposomes. Many of the considerations for the design of RNAi based drugs are also cell type specific (de Fougerolles et al., 2007; Hajeri and Singh, 2009). Despite this, scope remains for the use of RNAi in the design of drugs to combat a number of critical diseases such as human immunodeficiency virus (HIV). RNAi based drugs have successfully gone through phase I trials for age-related macular degeneration and also for respiratory syncytial virus (de Fougerolles et al., 2007; Hajeri and Singh, 2009).

Statins are widely acknowledged as a hugely successful group of drugs for reducing the incidence of cardiovascular events and they are currently the best available drugs for primary prevention against atherosclerosis (Steinberg, 2006; Waters, 2010). Statins were discovered by Akira Endo in 1976 through identification of a fungal metabolite that inhibited 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG CoA reductase), a key rate-limiting enzyme in the cholesterol synthesis pathway (Buhaescu and Izzedine, 2007; Kapur and Musunuru, 2008). Statins lower serum LDL cholesterol levels by competitively inhibiting the HMG CoA reductase enzyme to reduce levels of cholesterol synthesis in the liver. They are also able to block the synthesis of isoprenoid intermediates of the cholesterol synthesis pathway (Steinberg, 2006; Waters, 2010). Examples of statins include lovastatin, pravastatin, atorvastatin and simvastatin (Kapur and Musunuru, 2008).

The first trial showing that statins reduced the incidence of cardiovascular events was published in 1994. Since then a large number of clinical trials have demonstrated that statins can reduce the incidence of cardiovascular events over 5 years by 20%. Statins have been shown to reduce cardiovascular events in patients irrespective of levels of LDL cholesterol and also in patients with no evidence of vascular disease but elevated levels of the biomarker C-reactive protein (Kapur and Musunuru, 2008; Steinberg, 2006; Waters, 2010). Increasing evidence suggests that statins exert their effects through mechanisms beyond lowering cholesterol. Statins also exert anti-inflammatory effects such as reducing levels of C-reactive protein, lowering levels of pro-inflammatory cytokines and inhibiting T-cell activation (Buhaescu and Izzedine, 2007).

Statins may provide an avenue to increase TGF-β through increasing TGF-β signalling pathway activation without non-specific and unwanted off-target side effects that could occur using overexpression of TGF-β or agonists for the TGF-β receptors and this may allow the development of therapies that induce and support anti-inflammatory and anti-atherogenic responses. A large number of cellular responses are mediated by statins through the blockade of isoprenoid production and intracellular signalling pathways including actions on transcription factors and the MAPK cascades (Redondo et al., 2007; Rodriguez-Vita et al., 2008). Interestingly, statins can increase circulating levels of TGF-β and synthesis of this cytokine by monocytes in atherosclerotic patients (Redondo et al., 2007; Rodriguez-Vita et al., 2008). Activation of the Smad pathway is increased by statins in cultured VSMCs and this activation mediates TGF-β-induced apoptosis and ECM production by VSMCs (Rodriguez-Vita et al., 2008).

A number of studies have suggested that PPARs may exert cardio-protective effects through its anti-inflammatory actions. This has led to the development of PPAR agonists. Many of the anti-inflammatory actions of PPARs are mediated through the inhibition of AP-1, NFκB and STAT transcription factors leading to transrepression of gene expression. Regulation of genes involved in lipid, glucose and cholesterol metabolism by PPARs makes them attractive targets for therapeutic agents to modulate their function (Shearer and Billin, 2007; Touyz and Schiffrin, 2006). Two classes of drugs, the fibrates and the glitazones are successful PPAR-targeting agents used in diabetes and metabolic syndrome. In rat models of vascular injury, PPAR activators limit intimal hyperplasia and in Angiotensin-II infused rats they prevent development of hypertension and reduce vascular inflammation. In LDL-deficient mice PPARγ activators reduce atherosclerotic lesion size and reduce inflammation. In addition, PPAR activators are able to modulate markers of vascular disease such as CRP, E-selectin and VwF (Shearer and Billin, 2007; Touyz and Schiffrin, 2006).

PPAR agonists have been shown to target the TGF-β pathway to block the pro-fibrotic actions of the cytokine. For example, PPAR agonists target and block the TGF-β pathway to attenuate renal interstitial fibrosis and inflammation linked with diabetic nephropathy and non-diabetic kidney diseases (Kawai et al., 2009; Wang et al., 2007). However, there is limited literature linking PPAR agonists with TGF-β-

mediated effects in atherosclerosis. Neointimal growth in hypercholesterolemic rabbits can be reduced by the PPARγ agonist Pioglitazone and this was linked with reduced release of TGF-β and MCP-1 (Joner et al., 2007). In addition to this, pioglitazone can induce apoptosis of human vascular smooth muscle cells from diabetic patients through activation of TGF-β and Smad-2 (Ruiz et al., 2007).

Despite some success therefore, PPAR activators have not been without dose-limiting adverse side effects, mainly through off-target transactivation. Mitigating these side effects is a major target of newly designed PPAR-targeting agents (Shearer and Billin, 2007; Touyz and Schiffrin, 2006).

The most important hurdle to overcome with the design of many drugs targeting atherosclerosis is the problem of specificity. The development of drugs/molecules that are tissue or cell-type specific is a therapeutic approach of vital importance to reduce unwanted side-effects of drugs designed to reduce or combat atherosclerosis.

7.5 Concluding remarks

From the work presented in this thesis it is clear that TGF-β regulates the expression of genes involved in macrophage cholesterol homeostasis through activation of several signalling cascades. The TGF-β signalling pathways and the genes studied have important functions in the pathogenesis of atherosclerosis. The studies presented in this thesis also characterised the regulation of ADAMTS-4 by TGF-β in macrophages. A complete understanding of the molecular mechanisms involved in gene regulation and signalling pathways will hopefully lead to the development of novel therapeutic strategies to combat atherosclerosis.

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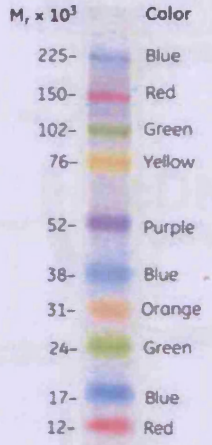
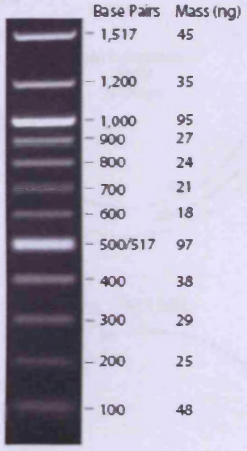
Appendix I



Full-range rainbow protein molecular weight marker
(GE Healthcare-RPN800E)



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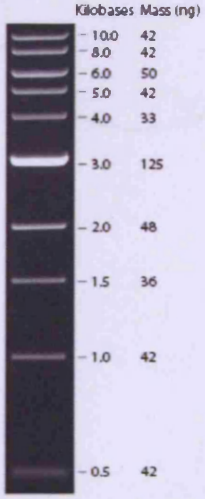
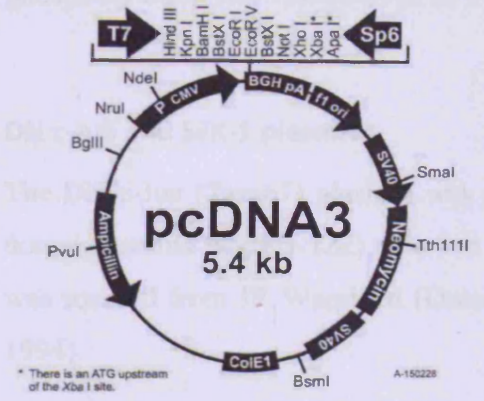
(New England Biolabs, UK)

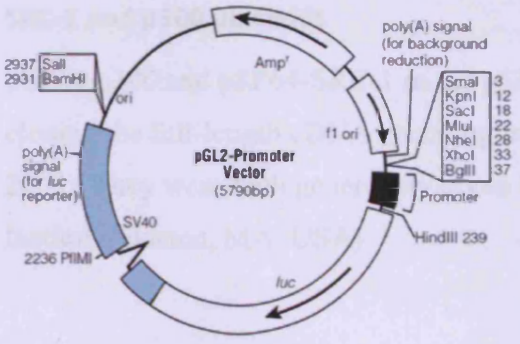


Quickload® 1Kb DNA ladder
(New England Biolabs, UK)

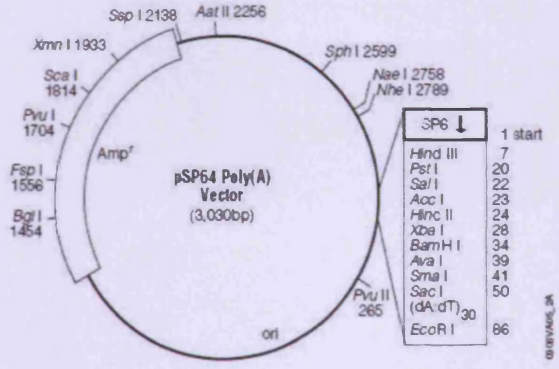
Appendix II



pcDNA3 vector



pGL2 promoter vector (Promega)



pSP64 (polyA) vector

DN JNK/SAPK plasmid

The DN JNK/SAPK plasmid, SAPKa-VPA was generated by changing the phosphorylation site Thr-Pro-Tyr to Val-Pro-Ala (Kawasaki et al. 1996).

DN c-Jun and SEK-1 plasmids

The DN c-Jun (Tam67) plasmid was generated by the removal of the transactivation domain (amino acids 3-122) of c-Jun (Brown et al. 1993). The DN SEK-1 plasmid was sourced from JR Woodgett (Ontario Cancer Laboratory, Canada) (Sánchez et al. 1994).

SRC-1 and p300 plasmids

pSP64-p300 and pSP64-SRC-1 in the pSP64(polyA) vector were constructed by cloning the full-length cDNA encoding human p300 or SRC-1 respectively (Li et al. 2000). They were both generously given by D M Livingston (Dana-Farber Cancer Institute, Boston, MA, USA).

Appendix III

Student's t test

Mean 1 = $\bar{x}_1$ Standard deviation 1 = σ_1

Mean 2 = x_2 Standard deviation 2 = σ_2

Variance = σd^2

$$\sigma_d{}^2 = \frac{\sigma_1{}^2}{n1} + \frac{\sigma_2{}^2}{n2}$$

$$t = \frac{\bar{x}1 - \bar{x}2}{\sigma d}$$

A difference between two means is significant (at the given probability level) if the calculated t value is greater than the value given in this table. A probability of p = 0.05 (95% probability of making a correct statement) is usually acceptable for biological work. When comparing two means, the number of degrees of freedom is (n1 + n2)-2, where n1 is the number of replicates of treatment 1, and n2 is the number of replicates of treatment 2 (adapted from: The Really Easy Statistics Site. Jim Deacon. Biology Teaching Organisation, University of Edinburgh, Edinburgh, Scotland, UK. URL (accessed on 03/05/10): http://www.biology.ed.ac.uk/research/groups/jdeacon/statistics/table1.html

Degrees of Freedom	t value			
P value	0.1	0.05	0.01	0.001
1	6.31	12.71	63.66	636.62
2	2.92	4.30	9.93	31.60
3	2.35	3.18	5.84	12.92
4	2.13	2.78	4.60	8.61
5	2.02	2.57	4.03	6.87
6	1.94	2.45	3.71	5.96
7	1.89	2.37	3.50	5.41
8	1.86	2.31	3.36	5.04
9	1.83	2.26	3.25	4.78
10	1.81	2.23	3.17	4.59

Appendix IV

Transcription factor binding site search results

SignalScan TFD-transcription factor database (Prestridge 1991)

Processed sequence:

```
1       CCCTCTTGATGCCTCCTTACCTGTTCCCTACCTTCTTTTCTCAGGCAGCT
51      CACTCAGTCCCCTCAGCCCTGGAAACCAGCCACTAGGGCCAAAGGGCAGC
101     ATGAGGGAGCCTTGAGAAAA
```

Links to BioBase at gene-regulation.com (Free, but registration/login required)

```
Input sequence contained 120 base pairs
Signal File(s): mammal
```

Factor or Site Name	Loc.(Str.)	Signal Sequence	SITE
input.seq: 120 base pairs			
polyoma.1	site 2 (-)	AGAGG	S00922
gamma-IRE_CS	site 5 (+)	CWKKANNY	S01622
T-Ag	fact 11 (-)	GAGGC	S00973

LVa	fact	21	(-)	GAACAG	S00038
LVa	fact	21	(-)	GAACAG	S00900
TCF-1	fact	21	(-)	MAMAG	S02023
TCF-1	fact	35	(-)	MAMAG	S02023
CAP-site	site	46	(+)	CANYYY	S00089
CAP-site	site	55	(+)	CANYYY	S00089
CAP-site	site	64	(+)	CANYYY	S00089
gamma-IRE_CS	site	69	(+)	CWKKANNY	S01622
H-APF-1	fact	69	(+)	CTGGRAA	S01971
NFI	fact	70	(+)	TGGNNNNNNGCCA	S00044
NF-I	fact	70	(+)	YGGMNNNNNGCCA	S01994
CP2	fact	78	(+)	AGCCACT	S00099
LBP-1	fact	82	(+)	WCTRG	S00487
LF-A1	fact	86	(-)	TGRMCC	S00250
element_II_rs-3	site	87	(-)	TTTGGCC	S01506
Ad-conserved-se	site	88	(+)	GCCAA	S01089
TCF-1	fact	90	(+)	MAMAG	S02023

Prestridge, D. 1991. SIGNAL SCAN: A computer program that scans DNA sequences for eukaryotic transcriptional elements. *CABIOS* 7, pp. 203-206.

Transcription element search system (TESS) (Schug 2003)

TESS Job M0399015305 : Summary Page

Home AnGEL CRM Searches Site Searches Query Databases Other Stuff
Guide Recall Search Combined Strings Filtered Strings

Need help? Check our FAQ page then please send questions and comments to TessMaster@cbil.upenn.edu.

Factor	Model	Beginning	Strand (N-normal, R-reverse complementary)	Length	Sequence

_00000 TCF-1	I00029 (TCF-1)	90	N	6	CAAAGG
_00000 FOXC1	J00032 (MA0032)	29	R	8	TACCTTCT
_00000 Ttk	I00261 (Ttk)	14	R	6	TCCTTA
_00000 SPI1	J00080 (MA0080)	23	R	6	GTTCCC
_00000 R1	I00042 (R1)	10	N	9	TGCCTCCTT
_00000 HAND1-TCF3	J00092 (MA0092)	73	R	10	AAACCAGCCA
_00000 zeste	I00263 (zeste)	47	R	6	AGCTCA
_00000 R1	I00042 (R1)	25	N	9	TCCCTACCT
_00000 PR	I00288 (PR)	115	R	6	AGAAAA
T00029 AP-1	Q00004 (-)	50	N	7	TCACTCA
_00000 Bapx1	J00122 (MA0122)	49	R	9	CTCACTCAG
T01498 IL-6 RE-BP T01499 IL-6 RE-BP	R01907 ()	69	N	6	CTGGAA
T02691 Dof3	M00354 (P$DOF3_01)	87	N	11	GGCCAAAGGGC
T00306 GATA-1	Q00069 (-)	114	N	7	GAGAAAA
T00479 LyF-1	M00141 (V$LYF1_01)	111	N	9	CTTGAGAAA
T02983 Pax-4a	M00378 (V$PAX4_03)	58	N	12	TCCCCTCAGCCC
_00000 AT-BP1/AT-BP2	I00141 (AT-BP1/AT-BP2)	56	R	8	AGTCCCCT
_00000 Dof2	J00020 (MA0020)	33	R	6	TTCTTT
T00913 Yi	R03154 ()	98	R	10	AGNNNGNGGG
T01427 p300	M00033 (V$P300_01)	51	R	14	CACTCAGTCCCCTC
_00000 ABI4	J00123 (MA0123)	55	N	10	CAGTCCCCTC
T01599 LCR-F1	M00285 (V$TCF11_01)	93	R	13	AGGGCAGCATGAG
_00000 LyF-1	I00084 (LyF-1)	111	N	9	CTTGAGAAA
_00000 BSAP	I00008 (BSAP)	61	R	11	CCTCAGCCCTG

_00000 H-2RIIBP	I00178 (H-2RIIBP)	57	N	6	GTCCCC
T00029 T00030 T00031 T00032 T01115 T01140 T01156 T00027 AP-1	M00174 (V$AP1_Q6)	48	R	11	GCTCACTCAGT
T00395 Hb	R03023 ()	110	R	10	CCTTGAaAAA
_00000 TFII-I	I00216 (TFII-I)	104	R	6	AGGGAG
_00000 RXR-alpha	I00038 (RXR-alpha)	12	N	11	CCTCCTTACCT
_00000 E74A	I00237 (E74A)	18	R	8	TACCTGTT
T00114 c-Ets-1_54 T00111 c-Ets-1	M00032 (V$CETS1P54_01)	68	N	10	CCTGGAAACC
T02841 FACB	R08603 ()	58	R	16	TCCNNNNNNNNNGGA
T00751 Sn	Q00157 (-)	19	R	7	ACCTGTT
_00000 MNF1	I00352 (MNF1)	92	N	7	AAGGGCA
_00000 DEP2	I00390 (DEP2)	18	N	8	TACCTGTT
_00000 IL-6.RE-BP	I00218 (IL-6.RE-BP)	38	R	7	TTCTCAG
T02450 GKLF	R05016 ()	26	R	14	CCYYtYYYTYNTTY
_00000 LBP-1	I00280 (LBP-1)	76	N	8	CCAGCCAC
_00000 NHP-1	I00285 (NHP-1)	97	N	8	CAGCATGA
T00114 c-Ets-1_54	M00074 (V$CETS1P54_02)	66	N	13	GCCCTGGAAACCA
_00000 CTF	I00123 (CTF)	70	N	14	TGGAAACCAGCCAC
T00108 C/EBPalpha	Q00024 (-)	111	R	10	CTTGAGAAAA
_00000 AP-2	I00151 (AP-2)	61	N	8	CCTCAGCC
_00000 GAGA	I00244 (GAGA)	49	R	7	CTCACTC
_00000 deltaEF1	J00103 (MA0103)	18	N	6	TACCTG
_00000 RXR-alpha	I00038 (RXR-alpha)	86	R	11	GGGCCAAAGGG
T00753 Sp1	R01540 ()	94	N	6	GGGCAG

_00000 c-ETS	J00098 (MA0098)	70	R	6	TGGAAA
T00759 Sp1	M00008 (V$SP1_01)	74	R	10	AACCAGCCAC
_00000 ID1	J00120 (MA0120)	81	R	12	CACTAGGGCCAA
_00000 RUSH1-alfa	J00109 (MA0109)	35	N	8	CTTTTCTC
_00000 NIP	I00061 (NIP)	93	R	8	AGGGCAGC
T00084 CBF (2) T00150 CP1 T00537 NF-1	R05057 ()	87	N	10	RRCCAAtSRG
_00000 LBP-1	I00191 (LBP-1)	76	R	5	CCAGC
_00000 SP1	J00079 (MA0079)	25	R	10	TCCCTACCTT
_00000 ID1	J00120 (MA0120)	22	N	12	TGTTCCCTACCT
_00000 ZNF42_1-4	J00056 (MA0056)	58	R	6	TCCCCT
_00000 NF-IL6	I00088 (NF-IL6)	112	N	9	TTGAGAAAA
_00000 ELK1	J00028 (MA0028)	66	N	10	GCCCTGGAAA
_00000 TFAP2A	J00003 (MA0003)	79	N	9	GCCACTAGG
T00305 GATA-1	Q00068 (-)	16	R	13	CTTACCTGTTCCC
T00368 HNF-1A T01950 HNF-1B T01951 HNF-1C	R03302 ()	76	R	7	CCAGCCA
_00000 deltaEF1	J00103 (MA0103)	29	N	6	TACCTT
_00000 myogenin	I00293 (myogenin)	43	N	9	AGGCAGCTC
T00152 CP2	R00444 ()	78	N	7	AGCCACT
T01470 Ik-2	M00087 (V$IK2_01)	21	R	12	CTGTTCCCTACC
T01944 NF-ATp T01948 NF-ATp	R05082 ()	106	N	6	GGAGCC
_00000 RXR-alpha	I00038 (RXR-alpha)	60	N	11	CCCTCAGCCCT
_00000 Sn	I00250 (Sn)	19	N	7	ACCTGTT
T02450 GKLF	M00286 (V$GKLF_01)	26	R	14	CCCTACCTTCTTTT

T00395 Hb _00000 Hunchback	J00049 (MA0049) M00022 (I$HB_01)	33	R	10	TTCTTTTCTC
_00000 Pap1	I00045 (Pap1)	49	N	8	CTCACTCA
T01591 P_(short_form) T01590 P_(long_form)	M00226 (P$P_01)	15	N	9	CCTTACCTG
T00697 PR	Q00145 (-)	51	N	12	CACTCAGTCCCC
T00759 Sp1	M00008 (V$SP1_01)	25	R	10	TCCCTACCTT
T00032 AP-1	Q00006 (-)	50	R	7	TCACTCA
T00123 c-Fos	Q00027 (-)	49	R	9	CTCACTCAG
_00000 GR/PR	I00104 (GR/PR)	22	N	6	TGTTCC
_00000 RUSH1-alfa	J00109 (MA0109)	16	N	8	CTTACCTG
_00000 T3R-alpha	I00033 (T3R-alpha)	93	R	6	AGGGCA
T00788 T-Ag	Q00168 (-)	11	R	5	GCCTC
_00000 fl-flI	I00222 (fl-flI)	31	N	8	CCTTCTTT
T00333 GR	Q00075 (-)	47	R	12	AGCTCACTCAGT
T00036 AP-4	M00175 (V$AP4_Q5)	44	N	10	GGCAGCTCAC
_00000 GR/PR	I00104 (GR/PR)	115	R	6	AGAAAA
_00000 F$STRE_01	M00154 (F$STRE_01)	58	R	8	TCCCCTCA
_00000 NF-X	I00067 (NF-X)	69	N	8	CTGGAAAC
_00000 LVa	I00193 (LVa)	21	R	6	CTGTTC
_00000 LBP-1	I00191 (LBP-1)	68	N	5	CCTGG
_00000 MafB	J00117 (MA0117)	42	R	8	CAGGCAGC
T00123 c-Fos	Q00027 (-)	49	N	9	CTCACTCAG
T00625 AREB6	M00415 (V$AREB6_04)	70	R	9	TGGAAACCA
T00529 MZF-1	M00083 (V$MZF1_01)	58	R	8	TCCCCTCA

_00000 MalT	I00377 (MalT)	61	R	10	CCTCAGCCCT
T01059 MNB1a	M00352 (P$DOF1_01)	32	R	11	CTTCTTTTCTC
T00035 AP-2	Q00008 (-)	59	R	11	CCCCTCAGCCC
_00000 Klf4	J00039 (MA0039)	27	R	10	CCTACCTTCT
T00306 GATA-1	Q00069 (-)	36	R	7	TTTTCTC
_00000 CP2	I00132 (CP2)	80	N	5	CCACT
T02855 CDC5	M00361 (P$CDC5_01)	60	N	11	CCCTCAGCCCT
_00000 GR/PR	I00104 (GR/PR)	36	N	6	TTTTCT
_00000 TEF	I00026 (TEF)	99	R	6	GCATGA
_00000 V$CAP_01	M00253 (V$CAP_01)	54	N	8	TCAGTCCC
T00997 T00996 SRY	M00148 (V$SRY_01)	32	R	7	CTTCTTT
_00000 zeste	I00263 (zeste)	51	R	6	CACTCA
_00000 HMG-1	J00044 (MA0044)	32	N	9	CTTCTTTTC
_00000 T3R-beta	I00031 (T3R-beta)	57	R	9	GTCCCCTCA
_00000 LBP-1	I00191 (LBP-1)	83	R	5	CTAGG
T00011 ADR1	M00048 (F$ADR1_01)	12	R	6	CCTCCT
_00000 E12	I00274 (E12)	61	R	7	CCTCAGC
T00372 HNF-4alpha1	M00411 (V$HNF4_01_B)	85	N	15	AGGGCCAAAGGGCAG
T00036 AP-4	Q00009 (-)	46	N	6	CAGCTC
_00000 Bapx1	J00122 (MA0122)	79	R	9	GCCACTAGG
T00479 LyF-1	M00141 (V$LYF1_01)	12	R	9	CCTCCTTAC
_00000 MNB1A	J00053 (MA0053)	34	R	5	TCTTT
_00000 TEF	I00026 (TEF)	41	N	6	TCAGGC
T00550 T01944 NF-ATx T01945 NF-ATc T01946 T01947 NF-ATc3 T01948 NFAT-1 T01949 NF-ATp	M00302 (V$NFAT_Q6)	67	N	12	CCCTGGAAACCA

T02462 NF-AT3 T00549 NF-AT					
T02690 Dof2	M00353 (P$DOF2_01)	32	R	11	CTTCTTTTCTC
T00385 HSF1	M00029 (F$HSF_01)	32	R	5	CTTCT
_00000 RXR-alpha	I00038 (RXR-alpha)	19	N	11	ACCTGTTCCCT
T00872 USF-1 T00874 T00875 T00876 USF1 T00877 T00870 USF	M00187 (V$USF_Q6)	79	N	10	GCCACTAGGG
_00000 ENKTF1	I00116 (ENKTF1)	85	R	7	AGGGCCA
_00000 V$CAAT_01	M00254 (V$CAAT_01)	75	N	12	ACCAGCCACTAG
_00000 DTF-1	I00235 (DTF-1)	96	R	8	GCAGCATG
_00000 V$CAP_01	M00253 (V$CAP_01)	63	N	8	TCAGCCCT
T02100 T00918 Zeste	M00283 (I$ZESTE_Q2)	46	R	16	CAGCTCACTCAGTCCC
T00386 HSTF	M00028 (I$HSF_01)	32	R	5	CTTCT
_00000 V$CAAT_01	M00254 (V$CAAT_01)	84	N	12	TAGGGCCAAAGG
T00278 delta factor T00915 YY1	R04142 ()	31	N	11	CCWTNTTNNNW
_00000 HNF4	J00114 (MA0114)	86	N	13	GGGCCAAAGGGCA
_00000 AP-1	I00224 (AP-1)	50	R	7	TCACTCA
_00000 C/EBP	I00034 (C/EBP)	111	R	10	CTTGAGAAAA
_00000 PEB1	I00052 (PEB1)	91	R	8	AAAGGGCA
_00000 AF-1	I00144 (AF-1)	90	R	8	CAAAGGGC
_00000 TCF-1	I00029 (TCF-1)	34	R	6	TCTTTT
T00386 HSTF	M00028 (I$HSF_01)	37	R	5	TTTCT
T01542 E2F-1	Q00230 (-)	84	R	10	TAGGGCCAAA
_00000 TFAP2A	J00003 (MA0003)	88	N	9	GCCAAAGGG
T01806 T00671 p53	M00272 (V$P53_02)	73	R	10	AAACCAGCCA
T01476 Abd-B	M00090 (I$ABDB_01)	3	N	14	CTCTTGATGCCTCC

T00123 c-Fos	Q00027 (-)	9	R	9	ATGCCTCCT
_00000 IUF-1	I00392 (IUF-1)	7	R	8	TGATGCCT
T00123 c-Fos	Q00027 (-)	101	N	9	ATGAGGGAG
T00036 AP-4	Q00009 (-)	46	R	6	CAGCTC
T02841 FACB	R08603 ()	58	N	16	TCCNNNNNNNNNGGA
_00000 MafB	J00117 (MA0117)	60	R	8	CCCTCAGC
_00000 AP-3	I00150 (AP-3)	36	R	8	TTTTCTCA
T00535 NF-1	Q00112 (-)	88	R	6	GCCAAA
_00000 Gata1	J00035 (MA0035)	7	N	6	TGATGC
_00000 Nuclear	J00127 (MF0004)	17	R	6	TTACCT
T00322 GCR1	M00046 (F$GCR1_01)	103	R	9	GAGGGAGCC
_00000 NFBK	I00208 (NFBK)	70	N	13	TGGAAACCAGCCA
T00305 GATA-1	M00075 (V$GATA1_01)	5	N	10	CTTGATGCCT
T00076 CAC-binding_protein	Q00018 (-)	75	R	7	ACCAGCC
_00000 R2	I00041 (R2)	1	R	7	CCCTCTT
_00000 Zen	I00262 (Zen)	46	N	8	CAGCTCAC
_00000 X2BP	I00015 (X2BP)	54	R	8	TCAGTCCC
T00625 AREB6	R09394 ()	16	N	9	CTTACCTGT
T01467 deltaEF1	M00073 (V$DELTAEF1_01)	15	N	11	CCTTACCTGTT
T00017 C/EBPbeta	R02170 ()	69	N	7	CTGGRAA
_00000 TTF-1	I00022 (TTF-1)	111	R	8	CTTGAGAA
T02691 Dof3	M00354 (P$DOF3_01)	32	R	11	CTTCTTTTCTC
T01591 P_(short_form) T01590 P_(long_form)	M00226 (P$P_01)	26	N	9	CCCTACCTT
T00373 HNF-4alpha2 T00372 HNF-	M00134 (V$HNF4_01)	83	N	19	CTAGGGCCAAAGGGCAGC

4alpha1					A
_00000 Dof3	J00021 (MA0021)	91	N	6	AAAGGG
T00385 HSF1	M00029 (F$HSF_01)	23	R	5	GTTCC
_00000 TCF-1	I00029 (TCF-1)	2	R	6	CCTCTT
_00000 RAR-gamma	I00404 (RAR-gamma)	58	N	7	TCCCCTC
_00000 EIIaE-A	I00143 (EIIaE-A)	104	R	7	AGGGAGC
T00045 ARP-1	Q00011 (-)	90	N	9	CAAAGGGCA
T00642 POU2F1a T00643 T00644 T00959 T01031 T01157 T01466 T00641 POU2F1	M00137 (V$OCT1_03)	11	R	13	GCCTCCTTACCTG
_00000 Klf4	J00039 (MA0039)	31	R	10	CCTTCTTTTC
_00000 Sp1	I00295 (Sp1)	12	R	6	CCTCCT
T00321 GCN4 T00918 Zeste T02100 Zeste	R00830 () R04938 () R04941 () R04954 ()	51	R	6	CACTCA
T02637 RAV1	M00344 (P$RAV1_02)	16	N	12	CTTACCTGTTCC
_00000 Eve	I00241 (Eve)	54	N	9	TCAGTCCCC
T02692 PBF	M00355 (P$PBF_01)	32	R	11	CTTCTTTTCTC
_00000 PHO2	I00324 (PHO2)	73	R	8	AAACCAGC
_00000 IL-6.RE-BP	I00218 (IL-6.RE-BP)	69	N	7	CTGGAAA
_00000 T-Ag	I00215 (T-Ag)	11	R	5	GCCTC
_00000 Nkx2-5	J00063 (MA0063)	3	R	7	CTCTTGA
_00000 TFAP2A	J00003 (MA0003)	81	R	9	CACTAGGGC
T00123 c-Fos	Q00027 (-)	9	N	9	ATGCCTCCT
T02450 GKLF	M00286 (V$GKLF_01)	27	R	14	CCTACCTTCTTTTC
_00000 YY1	I00012 (YY1)	68	R	9	CCTGGAAAC
_00000 cEBP	J00102 (MA0102)	108	R	12	AGCCTTGAGAAA

T00378 HOXA3	M00395 (V$HOXA3_01)	79	R	9	GCCACTAGG
T00386 HSTF	M00028 (I$HSF_01)	115	N	5	AGAAA
_00000 Ftz.2	I00243 (Ftz.2)	7	N	7	TGATGCC
T00918 Zeste T02100 Zeste	R01518 () R01519 () R04939 () R04942 () R04943 () R04944 () R04945 () R04953 () R04955 ()	51	N	6	CACTCA
_00000 AP-3	I00150 (AP-3)	113	N	8	TGAGAAAA
_00000 IUF-1	I00392 (IUF-1)	78	N	8	AGCCACTA
_00000 H-APF-1	I00175 (H-APF-1)	69	N	7	CTGGAAA
_00000 PR	I00288 (PR)	36	N	6	TTTTCT
T00385 HSF1	M00029 (F$HSF_01)	115	N	5	AGAAA
T00385 HSF1	M00029 (F$HSF_01)	37	R	5	TTTCT
_00000 USF	I00368 (USF)	99	R	7	GCATGAG
_00000 ABF-2	I00389 (ABF-2)	108	N	8	AGCCTTGA
_00000 R2	I00041 (R2)	28	R	7	CTACCTT
_00000 E12	I00274 (E12)	39	N	7	TCTCAGG
T00475 LVa	R01134 () R01135 ()	21	R	6	CTGTTC

Schug, J. 2003. *Current protocols in Bioinformatics*. J. Wiley and Sons.

TFSearch (Heinemeyer et al. 1998)

```
** TFSEARCH  ver.1.3 **          (c)1995 Yutaka Akiyama (Kyoto Univ.)

 This simple routine searches highly correlated sequence fragments
 versus TFMATRIX transcription factor binding site profile database
 by E.Wingender, R.Knueppel, P.Dietze, H.Karas (GBF-Braunschweig).

  <Warning> Scoring scheme is so straightforward in this version.
            score = 100.0 * ('weighted sum' - min) / (max - min)
            The score does not properly reflect statistical significance!

 Database:  TRANSFAC MATRIX TABLE, Rel.3.3 06-01-1998
 Query:     Rebecca Promoter_sequence1 (120 bases)
 Taxonomy:  Vertebrate
 Threshold: 70.0 point


 TFMATRIX entries with High-scoring:

   1 CCCTCTTGAT GCCTCCTTAC CTGTTCCCTA CCTTCTTTTC TCAGGCAGCT entry        score
        ---------->                                        M00075 GATA-1 86.5
                                                       --- M00173 AP-1   84.5
                                                        <- M00199 AP-1   84.1
      <------                                           M00240 Nkx-2. 83.7
                <-------------                          M00128 GATA-1 83.6
                                                       -- M00199 AP-1   83.4
                           <------------                M00087 Ik-2   82.9
        ------->                                        M00100 CdxA   82.1
                                      ------------>     M00131 HNF-3b 81.5
                                       <------          M00148 SRY    80.9
                                <--------               M00083 MZF1   80.9
              <-------------                            M00137 Oct-1  80.7
                                             <----------- M00173 AP-1   80.4
        ---------->                                     M00076 GATA-2 78.7
                                                 <------ M00237 AhR/Ar 78.3
                                <---------              M00077 GATA-3 78.1
       ---------->                                      M00106 CDP CR 77.6
                   ----------->                         M00073 deltaE 77.6
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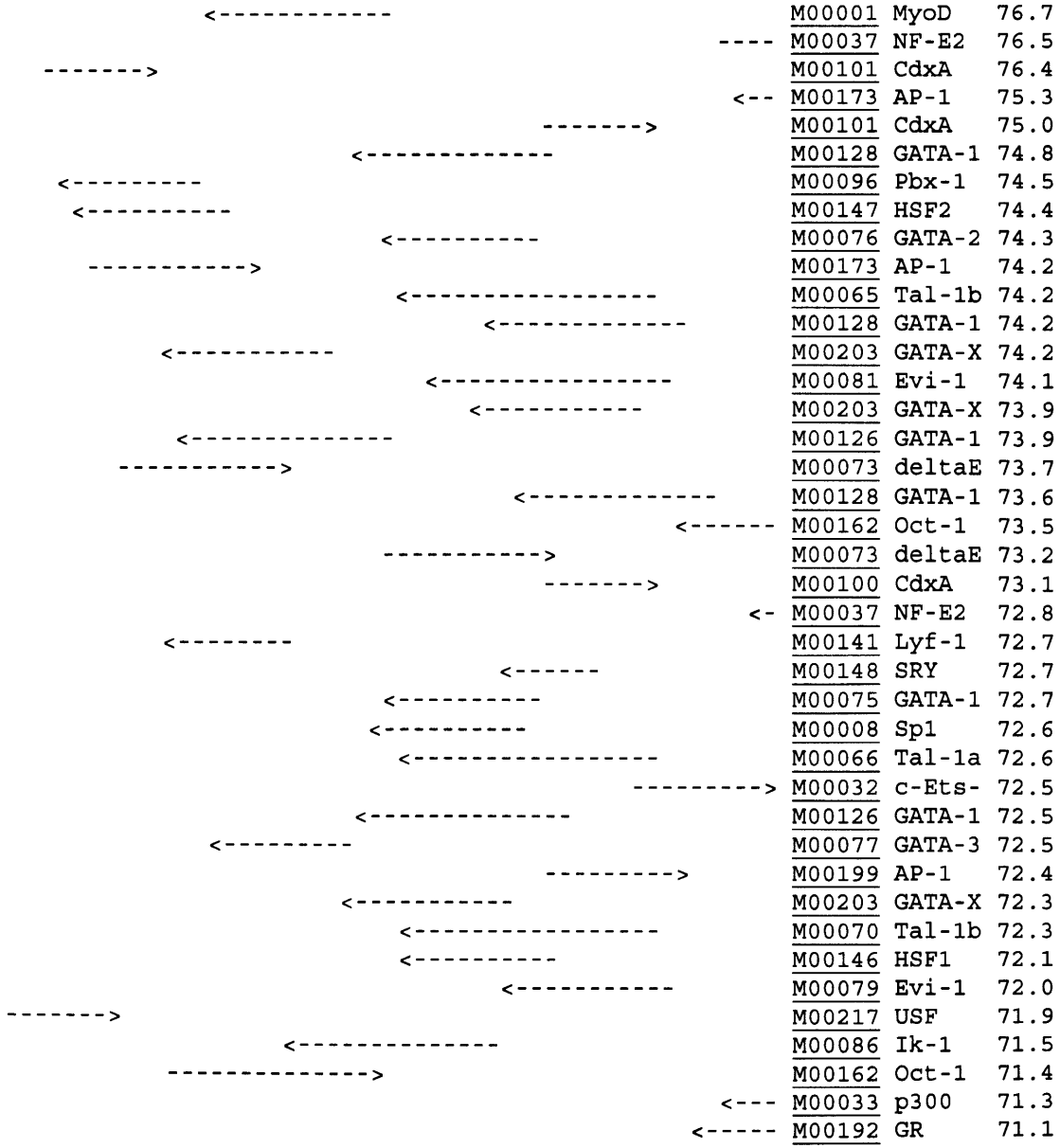
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M00101 CdxA 76.4
M00173 AP-1 75.3
M00101 CdxA 75.0
M00128 GATA-1 74.8
M00096 Pbx-1 74.5
M00147 HSF2 74.4
M00076 GATA-2 74.3
M00173 AP-1 74.2
M00065 Tal-1b 74.2
M00128 GATA-1 74.2
M00203 GATA-X 74.2
M00081 Evi-1 74.1
M00203 GATA-X 73.9
M00126 GATA-1 73.9
M00073 deltaE 73.7
M00128 GATA-1 73.6
M00162 Oct-1 73.5
M00073 deltaE 73.2
M00100 CdxA 73.1
M00037 NF-E2 72.8
M00141 Lyf-1 72.7
M00148 SRY 72.7
M00075 GATA-1 72.7
M00008 Sp1 72.6
M00066 Tal-1a 72.6
M00032 c-Ets- 72.5
M00126 GATA-1 72.5
M00077 GATA-3 72.5
M00199 AP-1 72.4
M00203 GATA-X 72.3
M00070 Tal-1b 72.3
M00146 HSF1 72.1
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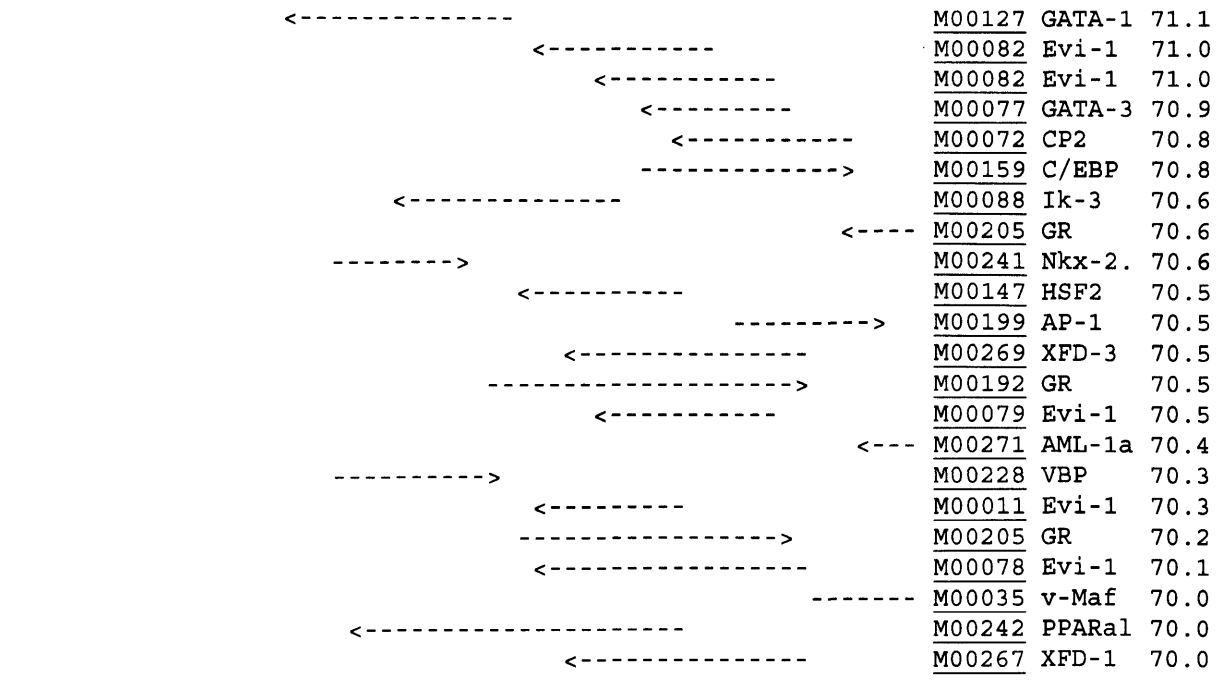

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M00159	C/EBP	70.8	
M00088	Ik-3	70.6	
M00205	GR	70.6	
M00241	Nkx-2.	70.6	
M00147	HSF2	70.5	
M00199	AP-1	70.5	
M00269	XFD-3	70.5	
M00192	GR	70.5	
M00079	Evi-1	70.5	
M00271	AML-1a	70.4	
M00228	VBP	70.3	
M00011	Evi-1	70.3	
M00205	GR	70.2	
M00078	Evi-1	70.1	
M00035	v-Maf	70.0	
M00242	PPARal	70.0	
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51 CACTCAGTCC CCTCAGCCCT GGAAACCAGC CACTAGGGCC AAAGGGCAGC

entry		score
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M00050	E2F	86.2
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M00199	AP-1	83.4
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M00173	AP-1	81.4
M00085	ZID	80.4
M00008	Sp1	79.5
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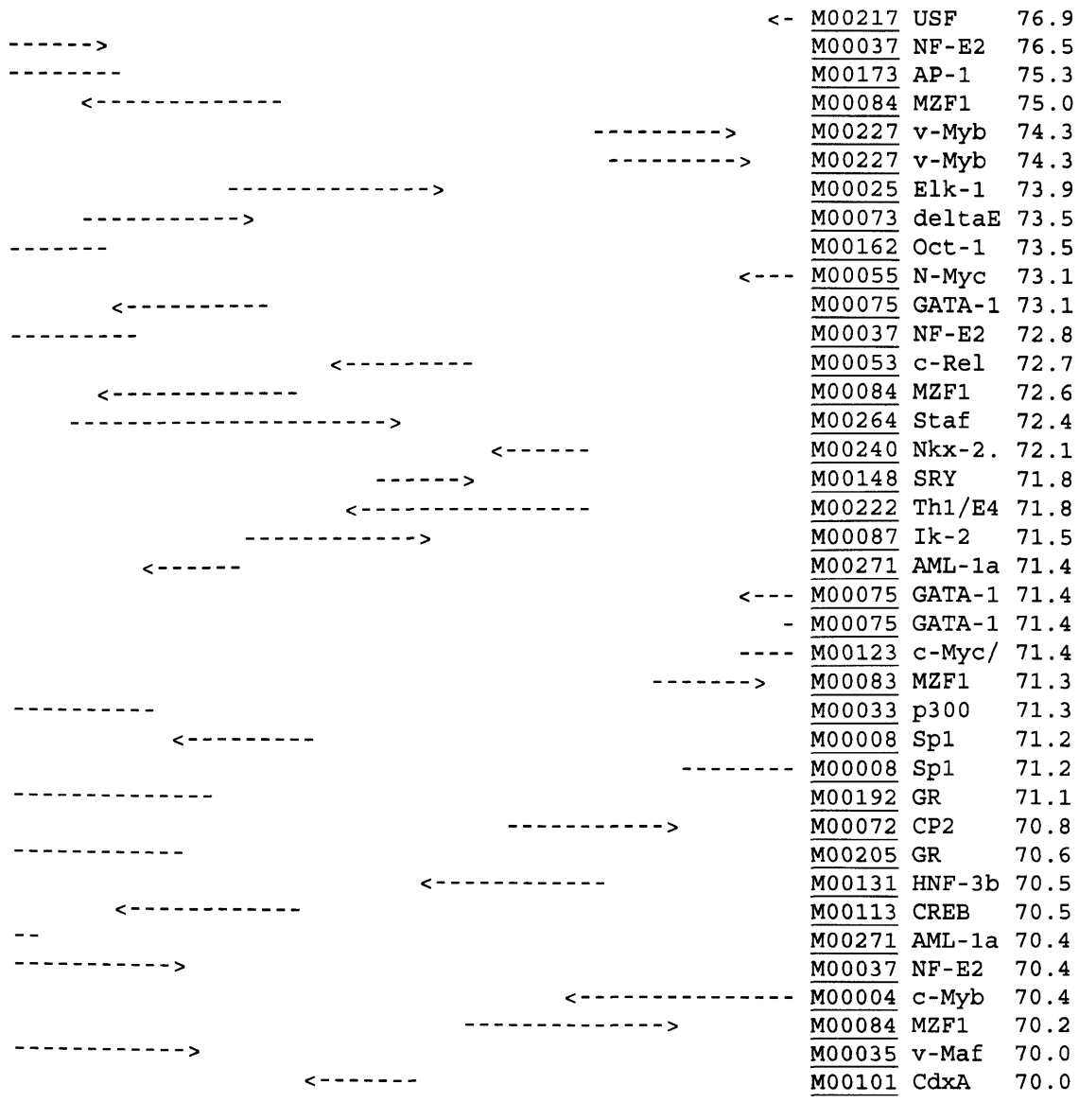
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M00227 v-Myb 74.3
M00025 Elk-1 73.9
M00073 deltaE 73.5
M00162 Oct-1 73.5
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M00075 GATA-1 73.1
M00037 NF-E2 72.8
M00053 c-Rel 72.7
M00084 MZF1 72.6
M00264 Staf 72.4
M00240 Nkx-2. 72.1
M00148 SRY 71.8
M00222 Th1/E4 71.8
M00087 Ik-2 71.5
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- M00075 GATA-1 71.4
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M00083 MZF1 71.3
M00033 p300 71.3
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M00008 Sp1 71.2
M00192 GR 71.1
M00072 CP2 70.8
M00205 GR 70.6
M00131 HNF-3b 70.5
M00113 CREB 70.5
M00271 AML-1a 70.4
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101 ATGAGGGAGC CTTGAGAAAA
entry score

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>                                                  M00134 HNF-4   88.0
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------                                             M00217 USF     76.9
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 -------->                                         M00141 Lyf-1   71.4
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->                                                 M00008 Sp1     71.2
---                                                M00004 c-Myb   70.4
------------>                                      M00087 Ik-2    70.2
Total 129 high-scoring sites found. Max score: 90.4 point, Min score: 70.0 point
```

Heinemeyer, T. et al. 1998. Databases on Transcriptional Regulation: TRANSFAC, TRRD, and COMPEL. *Nucleic Acids Research* 26, pp. 364-370.

MatInspector by Genomatix (Cartharius et al. 2005)

Inspecting sequence dbj|AB039835.1 [dbj|AB039835.1] (1 - 120):

[dbj|AB039835.1|:217-336 Homo sapiens ADAM-TS4 gene, promoter region]

Family	Detailed Information	Opt. thresh.	Start pos.	End pos.	Strand	Sequence
V$PAX6	PAX-4/PAX-6 paired domain binding sites	0.76	4	22	+	tcttgATGCctccttacct
V$NR2F	Nuclear receptor subfamily 2 factors	0.75	11	35	-	gaaggtAGGGaacaggtaaggaggc
V$RXRF	RXR heterodimer binding sites	0.78	13	37	-	aagaaggtagggaacAGGTaaggag
V$PERO	Peroxisome proliferator-activated receptor	0.76	14	36	-	agaaggtagggaacAGGTaagga

V$ZFHX	Two-handed zinc finger homeodomain transcription factors	0.93	14	26	+	tccttACCTgttc
V$GKLF	Gut-enriched Krueppel like binding factor	0.86	26	38	-	aaagaaggtAGGG
V$STAT	Signal transducer and activator of transcription	0.84	33	51	-	gagcTGCCtgagaaaagaa
V$AHRR	AHR-arnt heterodimers and AHR-related factors	0.77	41	65	-	tgaggggactGAGTgagctgcctga
V$AP1R	MAF and AP1 related factors	0.82	44	64	+	ggcaGCTCactcagtcccctc
V$SORY	SOX/SRY-sex/testis determinig and related HMG box factors	0.86	46	62	-	ggggactgAGTGagctg
V$AP1F	AP1, Activating protein 1	0.87	48	58	-	actGAGTgagc
V$NOLF	Neuron-specific-olfactory factor	0.88	52	74	+	actcagTCCCctcagccctggaa
V$MZF1	Myeloid zinc finger 1 factors	0.99	54	64	-	gaGGGGactga
V$GCMF	Chorion-specific transcription factors with a GCM DNA binding domain	0.85	58	68	+	tcCCCTcagcc
V$PAX6	PAX-4/PAX-6 paired domain binding sites	0.87	63	81	-	ggctggtttCCAGggctga
V$NF1F	Nuclear factor 1	0.82	66	86	-	ctaGTGGctggtttccagggc
V$RXRF	RXR heterodimer binding sites	0.78	78	102	+	agccactagggccaaAGGGcagcat
V$STAF	Selenocysteine tRNA activating factor	0.76	78	100	-	gctgCCCTttggccctagtggct
V$PERO	Peroxisome proliferator-activated receptor	0.76	79	101	+	gccactagggccaaAGGGcagca
V$NR2F	Nuclear receptor subfamily 2 factors	0.82	80	104	+	ccactagggcCAAAgggcagcatga
V$E2FF	E2F-myc activator/cell cycle regulator	0.84	81	97	+	cactagggcCAAAgggc
V$MOKF	Mouse Krueppel like factor	0.98	97	117	+	cagcatgagggagCCTTgaga
V$GCMF	Chorion-specific transcription factors with a GCM DNA binding domain	0.85	99	109	-	ctCCCTcatgc
V$CHRE	Carbohydrate response elements, consist of two E box motifs separated by 5 bp	0.82	100	116	+	CATGagggagccttgag

A total of 24 matches were found in 1 sequences. Sequences searched: 1 (120 bp).

Cartharius, K. et al. 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 21, pp. 2933-2942.