G protein coupled receptor transactivation of kinase receptors: the
new signalling frontier.

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy
(Biomedical Science)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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21 September 2016
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List of Abbreviations

ADAM - A disintegrin and A metalloprotease
ApoB - apolipoprotein B
APS - ammonium persulphate
B3GALT6 - β1,3-galactosyltransferase II
B4GALT7 - β1,4-galactosyltransferase-I
BCA - bincinchoninic acid assay
BSA - bovine serum albumin
cAMP - cyclic adenosine-3’, 5’-monophosphate
CASMCs - coronary artery smooth muscle cells
CDK - cyclin-dependent kinase
ChGn - chondroitin N-acetylgalactosamine transferase
CHPF - chondroitin polymerizing factor
CHST - chondroitin 4-O-sulfotransferase
CHST14 - dermatan 4-O-sulfotransferase
CHST3 - chondroitin 6-O-sulfotransferase
CHSY - chondroitin synthase
CPC - cetyl pyridinium chloride
CPM - counts per million
CS - chondroitin sulphate
CVD - cardiovascular disease
DAG - diacylglycerol
DEAE - diethylaminoethyl
DMEM- Dulbecco's Modified Eagle Medium (DMEM)
DMSO - dimethyl sulfoxide
DS - dermatan sulphate
ECM - extracellular matrix
EGF - epidermal growth factor
EGFR - epidermal growth factor receptor
ER - endoplasmic reticulum
Erk - Extracellular signal-regulated kinase
ERM - ezrin-radixin-moesin
ET-1 - endothelin-1
FBS - foetal bovine serum
GAG - glycosaminoglycan
Gal - galactose
GalNAc - N-acetyl-D-galactosamine
GDI - guanine nucleotide dissociation inhibitor
GDP - guanosine diphosphate
GEF - guanine nucleotide exchange factor
GlcA - glucuronic
GlcAT-I - β1,3-glucuronyltransferase I
GlcNAc - N-acetyl-D-glucosamine
GPCR - G protein coupled receptors
GSK3 - glycogen synthase kinase 3
GTP - guanosine triphosphate
HA - hyaluronan
HB-EGF - heparin binding EGF-like growth factor
HMG-CoA - 3-hydroxy-3-methylglutaryl coenzyme A
HS – heparin/heparin sulphate
IdoA - iduronic acid
IP3 - inositol-(1,4,5)-triphosphate
Jnk - c-Jun N-terminal kinase
KS - keratin sulphate
LDL - low density lipoproteins
LPA - lysophosphatidic acid
MAPK - mitogen-activated protein kinases
MH1 - mad-homology 1
MMP - matrix metalloproteinase
NADPH - nicotinamide adenine dinucleotide phosphate
PAI-1 - plasminogen activator inhibitor 1
PAR-1 - proteinase activated receptor-1
PBS - phosphate buffered saline
PDGF - platelet derived growth factor
PI3K - phosphatidylinositol 3-kinase
PIP2 - phosphatidylinositol biphosphate
PKA - protein kinase A
PKC - protein kinase C
PLC - phospholipase C
PTKR - protein tyrosine kinase receptor
PVDF - polyvinylidene fluoride
ROCK - Rho associated protein kinase
ROS - reactive oxygen species
RT-PCR - realtime-polymerase chain reaction
S/TKR - serine/threonine kinase receptor
SDS - sodium dodecyl sulphate
SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TGF - transforming growth factor
TGFBRI - transforming growth factor β receptor type 1
TRAP - thrombin receptor activator peptide
VSMCs - vascular smooth muscle cells
XXYLT-1 - xylosyltransferase
Xyl - xylose
Summary

G-protein coupled receptors (GPCRs) are the largest class of cell surface receptors. GPCR signalling is mediated through the classical signalling pathways, and non-classical signalling via utilisation of β-arrestin molecule and the transactivation of protein tyrosine kinase receptors (PTKR) and activation of serine/threonine kinase receptors (S/TKR), most notably the transforming growth factor (TGF)-β receptor (TGFBR1). Protease-activated receptors (PARs) are a family of GPCRs that are activated by the action of thrombin. Thrombin via its GPCR, PAR-1, can transactivate both PTKR in particular the epidermal growth factor receptor (EGFR) and the S/TKR TGFBR1. Thrombin signalling in human vascular smooth muscle cells (VSMCs) has been shown to contribute to atherosclerosis. Atherosclerosis commences by the trapping of lipoproteins in the vessel wall by modified proteoglycans specifically elongated glycosaminoglycan (GAG) chains. Thrombin through the transactivation of the EGFR and TGFBR1 leads to an increase of the GAG chain lengths allowing for an increase in low density lipoprotein retention. The two transactivation dependent pathways have distinct mechanisms. The PTKR mediated response involves matrix metalloproteinases and the phosphorylation of Erk. The S/TKR mediated response differs markedly and involves the phosphorylation of Smad2 carboxy terminus. The aim of this project was to expand on the mechanisms involved in transactivation dependent signalling and to find a common signalling intermediate which can inhibit both transactivation dependent signalling pathways leading to proteoglycan synthesis.

The in vitro model used human VSMCs. GAG synthesizing gene expression was measured and quantified by real time-PCR. Signalling
intermediate phospho-proteins were detected and quantified by western blotting. Proteoglycan synthesis was assessed by labelling of GAG chains with $^{35}$S-Sulphate and assessing incorporation into GAG chains via CPC precipitation assay. Proteoglycan GAG chain elongation was assessed by SDS-PAGE. Next generation RNA sequencing was conducted using the Ion proton sequencer. Reads were aligned to the human genome using TopHat software and differentially expressed genes were evaluated using the edgeR software. One-way ANOVA, followed by least significance difference post hoc analysis was used for statistical significance.

Thrombin treatment of VSMCs increased the mRNA expression of GAG synthesizing genes, CHST11, CHSY1 and CHST3. Thrombin via the transactivation of the PTKR, EGFR and S/TKR, TGFBR1 regulated the mRNA expression of CHST11, CHSY1, CHST3 and biglycan. The role of Gαq was investigated in this transactivation signalling pathway by utilizing Gαq antagonist UBO-QIC. Gαq played a role in thrombin transactivation of the EGFR and TGFBR1 and was involved in thrombin mediated proteoglycan synthesis, GAG elongation and regulation of the mRNA expression of CHST11 and CHSY1. Mechanistic studies revealed that thrombin transactivation of the TGFBR1 is mediated via Gαq which leads to Rho/ROCK signalling which activates cell surface integrin αVβ5 leading to the activation of the TGFBR1. siRNA knockdown of the Gαq and Gαq/11 revealed that thrombin mediated mRNA expression of CHST11 and CHSY1 occurs specifically via Gαq but not the Gαq/11 protein.

Thrombin treatment of VSMCs mediates phosphorylation of four serine/threonine residues of the Smad2 linker region. Thrombin mediated phosphorylation of the Smad2 linker region is via the transactivation of the
TGFBR1 and the EGFR. Phosphorylation of the linker region via transactivation of the EGFR occurs within 15 mins and is sustained for 4 hours. The phosphorylation of the Smad2 linker region via transactivation of the TGFBR1 is temporal initiating at 120 mins post thrombin treatment and sustained up to 4 hours. The individual serine/threonine Smad2 linker region residues were phosphorylated by either Erk, p38, PI3K or CDK, however Jnk was only involved in thrombin transactivation of the EGFR leading to the phosphorylation of the Thr220 residue. Erk, p38 PI3K and CDK are involved in regulating thrombin mediated mRNA expression of CHST11 and CHSY1.

RNA-Seq sequencing analysis revealed that in human VSMCs thrombin was involved in upregulating 293 genes. Thrombin treatment in the presence of EGFR antagonist and TGFBR1 antagonist inhibited the expression to 129 and 151 differentially expressed genes, respectively. Thrombin transactivation of the EGFR and TGFBR1 shared 140 genes. Thrombin transactivation of the EGFR regulated 512 biological processes and transactivation of the TGFBR1 regulated 428 biological processes. From the top 50 biological processes enriched by transactivation dependent signalling, 28 terms were found to be common between the two pathways. Many of the biological processes were associated with regulation of cell activity as well as vasculature development.

The findings demonstrate that thrombin via the transactivation of the EGFR and TGFBR1 regulates the mRNA expression of CHST11, CHSY1, CHST13 and biglycan. The transactivation pathways have distinct mechanisms and the Gαq protein plays as the central integrating point. The phosphorylation of the serine residues of the Smad2 linker region is most likely associated with GAG synthesizing genes involved in GAG chain elongation whereas the phosphorylation of the threonine residue of Smad2 linker region is not.
Transactivation dependent signalling contributes to 50% of PAR-1 mediated signalling, with the two transactivation pathways sharing in over 60% of common differentially expressed genes. Thus showing that a central integrating point in the transactivation dependent signalling pathways may not only be essential for proteoglycan synthesis and the initiation of atherosclerosis but may help regulate the biological processes associated with the 60% common differentially expressed genes.
Chapter-1. Introduction
1.1 Overview

Atherosclerosis is the main underlying aetiology of cardiovascular disease (CVD). The initiating event in atherosclerosis is described in the “response to retention hypothesis” (Williams and Tabas, 1995) in which low density lipoproteins (LDL) are trapped in the vessel wall by modified proteoglycans specifically the elongated and sulphated glycosaminoglycan (GAG) chains of biglycan. Proteoglycan synthesis and structure is regulated by vasoactive growth factors. G protein coupled receptors (GPCRs) are seven transmembrane receptors that are present on vascular smooth muscle cells (VSMCs) where they signal important actions such as vascular contraction, cellular migration and proliferation. GPCR agonist, thrombin, leads to proteoglycan synthesis with elongated GAG chains, which show a higher binding affinity to LDL (Ivey and Little, 2008).

The current paradigm of GPCR signalling covers three major pathways; firstly, the classic pathway in which agonist engagement causes G protein binding to the receptor with subsequent downstream signalling leading to functional responses; secondly the utilization of the β-arrestin signalling molecule that form scaffold complexes and lead to intracellular signalling and thirdly through a mechanism known as transactivation. Transactivation was first described by Ullrich in 1996 (Daub et al., 1996), GPCR agonists through their receptors can transactivate protein tyrosine kinase receptor (PTKR), such as epidermal growth factor receptor (EGFR), stimulating the immediate downstream product phosphorylated Erk1/2.
Transactivation has recently been defined as “the agonist occupancy of its cognate GPCR complex which leads in a relatively short time and in the absence of ‘de novo’ protein synthesis to the activation of and cytosolic generation of the immediate downstream product(s) of a second cell surface protein kinase receptor” (Kamato et al., 2013b). The activation of PTKR by a GPCR represents a major expansion in the cellular outcomes attributable to GPCR signalling.

The paradigm of GPCR mediated kinase receptor transactivation has been expanded to include GPCR mediated transactivation of serine/threonine kinase receptors (S/TKR) in particular the transforming growth factor (TGF)-β receptor type 1 (TGFBR1) (Burch et al., 2010). GPCR mediated transactivation of the TGFBR1 occurs via cytoskeletal rearrangement, which activated Rho associated protein kinase (ROCK) signalling, leading to the activation of cell surface integrins which bind to the latent TGF-β complex. This results in the TGF-β ligand binding to its receptor and activating its downstream signalling cascade. In VSMCs the transactivation of the EGFR and TGFBR1 results in the synthesis of biglycan molecules with elongated GAG chains. Little is known about the role of G proteins in GPCR transactivation of the PTKR and the S/TKRs. The role of G proteins is of great interest as it may play as the common point of signalling for these transactivation mediated pathways. This project will investigate the role of G protein, Gaq in proteinase activated receptor-1 (PAR-1) transactivation of the EGFR and TGFBR1 leading to GAG chain elongation.

1.2 Cardiovascular Disease

CVD is the largest cause of mortality in developed and developing countries (Deaton et al., 2011). CVD is a collective term to describe a group of diseases that affect the functioning of the heart, heart valves, blood and
vasculature of the body (Federation, 2014). The vasculature is a dynamic organ. Its functions include regulation of nutrients from blood to the surrounding tissues, maintenance of blood pressure and haemostasis. The major CVD condition that imposes a disease burden on the vascular wall and disrupts normal functioning is atherosclerosis (Frostegard, 2013, Weber and Noels, 2011). Atherosclerosis is a chronic inflammatory disorder of the blood vessels and is the main aetiology underlying most CVD, representing around 75% of associated deaths (Mendis et al., 2011). Atherosclerosis is a pathological condition characterised by the accumulation of lipid and inflammatory products in the arterial wall leading to the formation of atherosclerotic plaques (Weber and Noels, 2011).

Therapeutics currently available for atherosclerosis are largely restricted to targeting the risk factors such as alleviating hyperlipidaemia, hypertension and controlling homeostasis to prevent thrombotic complications (Weber and Noels, 2011). Lipid lowering drugs are the most effective pharmaceutical treatment for CVD and ongoing therapeutic improvements include more aggressive lowering of circulating lipoproteins. The most common drugs of this class is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins (Taylor et al., 2013). This therapy leads to the reduction of blood cholesterol levels, however they offer a less than optimal result in atherosclerosis prevention (Pignone, 2001, Pignone et al., 2000). Data collected from a cross sectional, retrospective study of 3 data sources: electronic medical records (2003-2010), administrative claims data (2003-2010) and National Health and Nutrition Examination Survey data (2007-2008) demonstrated that among patients undergoing statin therapy, approximately 25% of the high risk patients were able to reduce LDL cholesterol levels below
70 mg/dL (optimal level for patients high-risk of heart attack) (Jones et al., 2012). A meta-analysis using data from 8 randomised statin trials analysed the proportion of patients not reaching guideline recommended lipid levels on high-dose statin therapy (Boekholdt et al., 2014). More than 40% of patients treated with high-dose statin therapy did not reach a LDL target of less than 70 mg/dL (Boekholdt et al., 2014). In over thirty years since the discovery of statins, there has been no major new therapeutic agent arising for the existing strategy – targeting risk factors. Thus it has been proposed that therapies which target the vessel wall are required (Jankhande et al., 2014, Staels, 2002).

1.3 Atherosclerosis

Human arteries have three separate layers, the intima, the media and the adventitia (Figure 1.1) (Libby et al., 2002, Holzapfel and Ogden, 2010). The intima is a network of extracellular matrix (ECM) containing proteoglycans and collagen (Gasser et al., 2006, Rajendran et al., 2013). The intima is lined by a monolayer of endothelial cells that form a physical and functional barrier between flowing blood and the arterial wall surface, regulating a wide array of processes including thrombosis, vascular tone and leukocyte trafficking. Endothelial cells adhere on a basement membrane of extracellular matrix containing proteoglycans and collagen (Rajendran et al., 2013). In contrast to animal species used for atherosclerosis experiments, the human intima contains resident vascular smooth muscle cells (VSMC) (Allahverdian et al., 2014). The human arterial intimal thickening is made up of predominantly VSMCs (Allahverdian et al., 2014). The media is separated from the intima by an internal elastic lamina (Figure 1.1). The media is contractile and consists of interconnected smooth muscle cell layers that are held together by an ECM.
consisting largely of elastic fibres and collagen with a lesser content of proteoglycan. The layer furthest from the lumen is the adventitia, which is comprised of mostly connective tissue with populations of smooth muscle cells and fibroblasts (Figure 1.1) (Libby et al., 2002).

Atherogenesis refers to the development of atheromatous plaque in the inner lining of the arteries. Atherosclerotic plaque formation is slow and progressive and can be segmented into three phases, initiation, progression and plaque rupture (Figure 1.2). Initiation of an atherosclerotic lesion begins with lipid retention and inflammation. Under pro-atherosclerotic conditions, smooth muscle cells of the media migrate into the intima causing diffuse intimal thickening leading to the formation of a ‘neointima’ and secrete ECM molecules termed proteoglycans (Stary et al., 1992, Lusis, 2000). Proteoglycans show an increased affinity to bind LDL, subsequently resulting in LDL being retained in the neointima. The retained LDL shows an increased susceptibility to oxidation and other modifications such as lipolysis and proteolysis (Lusis, 2000). Oxidized LDL is taken up by macrophages. Minimally oxidised LDL has pro-inflammatory activities (Pentikainen et al., 2000) and can stimulate VSMCs and endothelial cells to express a range of inflammatory mediators. Additionally, VSMCs are capable of taking up aggregated LDL bound to proteoglycans (Llorente-Cortes et al., 2006). Macrophages and VSMCs that take up the oxidised LDL form large lipid laden ‘foam cells’ (Kaplan and Aviram, 2001, Tabas, 1999, Yutani et al., 1999, Mendez-Barbero et al., 2013, Badimon et al., 2012, Tarkin et al., 2016).
Figure 1.1: A cross sectional diagram of a non-diseased artery.

The arterial wall is composed of three distinct layers: the intima (I), the media (M) and the adventitia (A). Intima is the innermost layer closest to the lumen and lined by endothelial cells. Thickness of the subendothelial layer is influenced by age and disease state. Media layer is composed of smooth muscle cells and elastin and collagen fibrils which align circumferentially to provide mechanical integrity and contractility. The adventitia is the outer most layer surrounded by loose connective tissue. Image reproduced from (Gasser et al., 2006).
Lesions progress to an advanced complex lesion which is characterised by an underlying necrotic core with an overlying protective fibrous cap. The pathogenic consequences of an advance complex lesion occurs when the fibrous cap becomes vulnerable to rupture. As the fibrous cap's structural integrity weakens it is susceptible to fissure under haemodynamic pressure. This induces thrombus formation which can occlude the vessel at the site, or dislodge forming an embolus, leading to blockage, distal to the rupture site. Total obstruction of the blood vessel due to thrombus results in ischemia of surrounding tissue, leading to pathologies such as myocardial infarction, stroke and peripheral vascular disease resulting in death or disability (Sah et al., 2003, Crowther, 2005).

There are several hypotheses that have been formulated to explain atherogenesis. The most prominent and well-covered theories are the response to injury hypothesis (Ross and Glomset, 1976b, Ross and Glomset, 1976a), the oxidative modification hypothesis (Berliner et al., 1995) and the response to retention hypothesis (Williams and Tabas, 1995). Retention of lipid and the response of the body to this phenomenon forms the basis of the response to retention hypothesis and the basis for the work conducted in this project.

1.4 Response to retention hypothesis

The response to retention hypothesis was first postulated by Williams and Tabas in 1995 (Williams and Tabas, 1995) and describes the retention of LDL in the neointima of vessel walls as the key initiating event in lesion development. The hypothesis proposes that LDL, which normally diffuses through the blood vessel wall is trapped by intimal proteoglycans produced by VSMCs; and is consequently retained in the vessel wall (Tabas, 1999, Williams
and Tabas, 1995, Williams and Tabas, 1998). The apolipoprotein B (apoB) moiety of an LDL molecule contains a proteoglycan binding region (Boren et al., 1998, Camejo et al., 1988) that has an overall positive charge. The positively charged LDL interacts with the negatively charged constituents of the vessel wall ECM (Prydz and Dalen, 2000), of which the majority are sulphated proteoglycans. These proteoglycans have a high degree of negative charge owing to the sulphate and carboxyl groups on the GAG chains which are attached to the core protein. It is this ionic interaction which is hypothesized to be responsible for the entrapment of lipids within a vessel wall (Williams and Tabas, 1995) and hence a primary step in the formation of an atherosclerotic lesion (Figure 1.2). In human coronary artery, proteoglycans secreted by VSMCs include biglycan and decorin and to a much lesser extent versican and perlecan(Talusan et al., 2005). Histological studies in human arteries show strong co-localization between apoB lipoproteins and biglycan (Nakashima et al., 2007, O'Brien et al., 1998). The response to retention hypothesis proposes that subendothelial lipoprotein retention is the initiating event for atherosclerosis. It is estimated that more than 85% of subendothelial lipoprotein delivery is the consequence of transcytosis. The ECM of the subendothelium particularly proteoglycans are tightly associated with retention of atherogenic lipoproteins (Skalen et al., 2002).
Figure 1.2: Schematic representation of the initiation and progression of atherosclerotic plaque development.

The figure depicts the development of atherosclerosis inside the neointima of the arterial wall. (A) The pre-inflammatory phase involves initiation of atherosclerosis via the ‘response to retention hypothesis’. Normal LDL diffuse into the vessel wall where it is bound by modified proteoglycans and subsequently retained. Retained LDL is oxidised causing expression of adhesion molecules on endothelial cells resulting in invasion of circulating monocytes. (B) Inflammatory phase allows for monocytes to differentiate into macrophages and engulf the oxidised LDL to form a foam cell, leading to fatty streak formation. (C) Plaque rupture leads to the formation of a thrombus, which can occlude vessels, potentially resulting in complications such as myocardial infarction or stroke.
1.5 Proteoglycans

The ECM is a highly-ordered macromolecular structure with various physiological and pathophysiological roles. The cells which produce matrix molecules are important in determining matrix function and metabolism. (Alberts et al., 2002). Proteoglycans fill the majority of the extracellular interstitial space within the tissue in the form of a hydrated gel, synthesized primarily by VSMCs (Jarvelainen et al., 2009, Schaefer and Schaefer, 2010, Schwartz, 2000a, Schwartz, 2000b). Chondroitin sulphate and dermatan sulphate (CS/DS) proteoglycans are made up of a polypeptide backbone (core protein), with one or more covalently anchored GAG chains (Figure 1.3) (Schwartz, 2000a, Schwartz, 2000b). The proteoglycan core protein has a set molecular weight as encoded in its mRNA, this can range from as little as 10kDa to as large as 400-467kD. Proteoglycans contribute to both the maintenance of homeostasis, as well as the progression of many disease states including atherosclerosis (Wight and Hascall, 1983). Proteoglycans can be divided into different families (Table 1.1), based on the chemical composition of the GAG chains. The main proteoglycans concerned with the binding of LDL are biglycan (O'Brien et al., 2004) and decorin (Kovanen and Pentikainen, 1999) both of which contain a small leucine rich polypeptide core structure with adjoining CS and DS GAG chains.

1.5.1 Biosynthesis of glycosaminoglycan chains

GAG chains are polymers of non-branching repeating disaccharide units consisting of either N-acetyl-D-glucosamine (GlcNAc) or N-acetyl-D-galactosamine (GalNAc) and an uronic acid either glucuronic (GlcA) or iduronic acid (IdoA). Categorisation of proteoglycans is reliant upon the
constituents of their GAG chains. In mammals, there are five different subgroups of GAG chains hyaluronic acid or hyaluronan (HA), keratin sulphate (KS), CS, DS and heparin/heparan sulphate (HS) (Sugahara and Kitagawa, 2000, Caterson, 2012) (Table 1.1). CS and DS chains are classified as sulphated GAG chains made up of repeating disaccharide units. CS chains consists of alternating GlcNAc and GalNAc while DS chains are composed of alternating IdoA and GalNAc residues (Sugahara and Kitagawa, 2000). For CS synthesis, the chain elongates with the addition of GlcA and GalNAc residues by N-acetylgalactosaminyltransferase I and glucuronyltransferase (Uyama et al., 2002). This study will focus on understanding the signalling pathways that regulate CS chain modification due to its importance as a therapeutic target in atherosclerosis (Little et al., 2008a).
Figure 1.3: Diagrammatic representation of a proteoglycan with a hyperelongated GAG chain.

GAG chains are anchored to the core protein via the tetrasaccharide linkage region at specific serine residues along the core. The linkage region consists of a xylose (Xyl), two galactose (Gal) and a glucoronic acid (GlcA) residue. CS/DS GAG synthesizing enzymes work in a concerted manner to synthesize the linkage region and GAG chain. CS/DS GAG chains are made up of repeating disaccharide units consisting of GlcA and N-acetylglalactosamine (GalNAc). Under pathological conditions the GAG chains become hyperelongated, resulting in increased binding to LDL via an ionic interaction between the negative sulphate and the positive residues on the apoB moiety of LDL.
Table 1.1: Disaccharide composition of the 5 families of proteoglycans.

Dotted red boxes indicate the sulphation positions in the GAG chain. Adapted from Pyrdz (Prydz and Dalen, 2000).

<table>
<thead>
<tr>
<th>Family of proteoglycans</th>
<th>Proteoglycans from these families</th>
<th>Chemical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin sulphate</td>
<td>Versican</td>
<td>GlcA β(1→3) GalNAc β(1→4)</td>
</tr>
<tr>
<td>Dermatan sulphate</td>
<td>Biglycan, Decorin</td>
<td>IdoA β(1→3) GalNAc β(1→4)</td>
</tr>
<tr>
<td>Keratan sulphate</td>
<td>Lumican,</td>
<td>Gal β(1→4) GlcNAc β(1→3)</td>
</tr>
<tr>
<td>Heparan sulphate</td>
<td>Perlecan</td>
<td>GlcA β(1→4) GlcNAc α(1→4)</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>No core protein</td>
<td>GlcA β(1→3) GlcNAc β(1→4)</td>
</tr>
</tbody>
</table>
GAG chain formation on proteoglycans is an event that is sequential from the endoplasmic reticulum (ER) to the Golgi (Vertel et al., 1993). GAG chains are covalently attached to the proteoglycan core protein via a conserved linkage region. Disaccharide residues are added to the linkage region to form a chain of repeating units that are concomitantly sulphated (Ballinger et al., 2004). The initiation of the linkage region involves the action of glycosyltransferases. The four glycosyltransferases that are involved in the synthesis of the linkage region are xylosyltransferase (XXYLT-1), β1, 4-galactosyltransferase-I (B4GALT7), β1, 3-galactosyltransferase II (B3GALT6) and β1, 3-glucoronyltransferase I (B3GAT3). GAG chain synthesis begins in the cis compartment of the Golgi, where a pentose sugar, xylose, is covalently attached to a serine via a hydroxyl linkage catalysed by XXYLT-1 (Schwartz, 2000a). This initiating step is followed by the sequential addition of two GalNAc units by the two independent enzymes B4GALT7 and B3GALT6 (Figure 1.4).

Once the tetrasaccharide linker is completed the process of CS/DS chain assembly on proteoglycans is mediated by a number of membrane bound enzymes located in the ER/Golgi compartment (Silbert and Sugumaran, 2002, Mikami and Kitagawa, 2013, Izumikawa et al., 2011, Koike et al., 2014). The CS/DS GAG chain elongation occurs with the addition of GlcA and GalNAc residues through the actions of glycosyltransferases (Silbert and Sugumaran, 2002) chondroitin synthase (CHSY)-1, CHSY2, CHSY3, chondroitin polymerizing factor (CHPF), chondroitin N-acetylgalactosamine transferase (ChGn)-1 and ChGn-2 (Figure 1.4). Sulfotransferases catalyse the transfer of a sulpho group from a donor molecule to the acceptor (Kusche-Gullberg and Kjellen, 2003). Seven sulfotransferases are responsible for the sulphation of CS/DS including chondroitin 4-O-sulfotransferase (CHST11, 12 and 13),
dermatan 4-O-sulfotranferase (CHST14), chondroitin 6-O-sulfotransferase (CHST3), uronyl 2-O-sulfotransferase and GalNAc 4-sulfate 6-O-sulfotransferase (Table 1.2).
Table 1.2: Enzymes involved in CS/DS synthesis

Adapted from Mikami (Mikami and Kitagawa, 2013).

<table>
<thead>
<tr>
<th>Enzymes (activity)</th>
<th>Abbreviation</th>
<th>Gene symbols (synonym)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosyltransferases involved in the synthesis of the tetrasaccharide linkage region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylosyltransferase</td>
<td>XylT</td>
<td>XXYLTI-1</td>
</tr>
<tr>
<td>β1,4-Galactosyltransferase-I</td>
<td>GalT-I</td>
<td>B4GALT7</td>
</tr>
<tr>
<td>β1,3-Galactosyltransferase-II</td>
<td>GalT-II</td>
<td>B3GALT6</td>
</tr>
<tr>
<td>β1,3-Glucuronyltransferase-I</td>
<td>GlcAT-I</td>
<td>B3GAT3</td>
</tr>
<tr>
<td>Glycosyltransferases involved in the synthesis of the CS chains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondroitin synthase</td>
<td>ChSy-1</td>
<td>CHSY1</td>
</tr>
<tr>
<td></td>
<td>ChSy-2</td>
<td>CHSY2 (CSS3)</td>
</tr>
<tr>
<td></td>
<td>ChSy-3</td>
<td>CHSY3 (CHPF2)</td>
</tr>
<tr>
<td>Chondroitin polymerizing factor</td>
<td>ChPF</td>
<td>CHPF (CSS2)</td>
</tr>
<tr>
<td>Chondroitin GalNAc transferase</td>
<td>ChGn-1</td>
<td>CSGALNACT1</td>
</tr>
<tr>
<td></td>
<td>ChGn-2</td>
<td>CSGALNACT2</td>
</tr>
<tr>
<td>Sulfotransferases and epimerases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondroitin 4-O-sulfotransferase</td>
<td>C4ST-1</td>
<td>C4ST1 (CHST11)</td>
</tr>
<tr>
<td></td>
<td>C4ST-2</td>
<td>C4ST2 (CHST12)</td>
</tr>
<tr>
<td></td>
<td>C4ST-3</td>
<td>C4ST3 (CHST13)</td>
</tr>
<tr>
<td>Dermatan 4-O-sulfotransferase</td>
<td>D4ST-1</td>
<td>D4ST1 (CHST14)</td>
</tr>
<tr>
<td>Chondroitin 6-O-sulfotransferase</td>
<td>C6ST-1</td>
<td>C6ST1 (CHST3)</td>
</tr>
<tr>
<td>Uronyl 2-O-sulfotransferase</td>
<td>UST</td>
<td>UST</td>
</tr>
<tr>
<td>GalNAc 4-sulfate 6-O-sulfotransferase</td>
<td>GalNAc4S-6ST</td>
<td>GALNAC4S-6ST (CHST15)</td>
</tr>
</tbody>
</table>
As the chain is extended, chondroitin polymerization occurs with the action of glycosyltransferases CHSY1, CHSY2, CHSY3, CHPF, ChGn-1 and ChGn-2 (Izumikawa et al., 2007, Izumikawa et al., 2008, Kitagawa et al., 2003). When the growing structure enters the medial/trans Golgi it is subjected to post-translational modification. Sulphation is generally confined to the trans Golgi and may occur with the addition of sulphate at hydroxyl group positions 4’ and 6’ of the GalNAc residues and position 2 of the GlcA residue to create the CS backbone (Kusche-Gullberg and Kjellen, 2003). Sulphation of the 6’ position of the chondroitin sulphate GAG occurs in the medial/trans Golgi, whilst sulphation of the 4’ position occurs later in the trans Golgi (Silbert and Sugumaran, 2002). Sulfotransferase enzymes facilitate the elongation of GAG chains, specifically CHST11 in cooperation with ChGN-2 (Figure 1.4) (Izumikawa et al., 2011).

The regulatory mechanisms of glycosyltransferases and sulfotransferases involved in CS GAG synthesis are not well understood and require deeper investigations (Kitagawa et al., 2003, Koike et al., 2014, Sato et al., 2003, Gulberti et al., 2012, Izumikawa et al., 2011, Little et al., 2008b). There is considerable interest in understanding the development of atherosclerosis associated with CS GAG chain synthesis through the regulation of the GAG synthesizing enzymes. The focus of the current study is on CS GAG chain synthesis and structure because of its role in binding lipoproteins (Camejo et al., 1993, Sugahara and Kitagawa, 2000, Sugahara et al., 2003). Thus, changes in GAG chain structure are most likely to impact lipid binding and in the artery wall in early atherosclerosis.
Figure 1.4: Cell biology of CS/DS glycosaminoglycan chain synthesis.

An initial pentose sugar, xylose is covalently attached to a serine residue of the newly synthesized core protein, catalysed by the XXYLT-1. This occurs after the core protein exits the ER in a midway compartment between ER and the Golgi apparatus. This initiating step is followed by the sequential completion of the tetrasaccharide linkage region. This process takes place in the cis Golgi network. Synthesis of the repeated disaccharide GAG chain occurs and further modification by sulfotransferases proceeds in the trans Golgi region.
1.5.2 Role of proteoglycans in cardiovascular disease

There is a long history of evidence for the role of proteoglycans in the development and progression of atherosclerosis. Proteoglycans are present in the ECM of the vessel wall at all stages throughout life and the accumulation of proteoglycans is observed in early (Nievelstein et al., 1991) and late atherosclerosis (Wight, 1989). Biglycan, decorin and versican are most closely associated with lipoprotein binding in the vessel wall (Chang et al., 2000, Skalen et al., 2002). Biglycan is well known to be present in the intima of normal human saphenous veins and coronary arteries (Merrilees et al., 2001, Gutierrez et al., 1997). Biglycan is the major proteoglycan associated with atherosclerosis, particularly due to its importance in lipoprotein retention in human blood vessels (Williams and Tabas, 1995). This study will focus more on biglycan due to its significance in human atherosclerosis. A variety of factors such as hormones or growth factors can influence the ‘modification’ or hyperelongation of the GAG chains (Little et al., 2008a, Little et al., 2008b). Growth factors such as platelet derived growth factor (PDGF) and TGF-β lead to the stimulation of versican mRNA expression and GAG elongation in monkey aortic smooth muscle cells (Schonherr et al., 1993, Schonherr et al., 1991). However only TGF-β stimulated an increase in biglycan mRNA expression (Schonherr et al., 1993). The same trend is seen in human VSMCs where both TGF-β and PDGF invoke GAG elongation but only TGF-β results in increased expression of biglycan mRNA (Little et al., 2002, Dadlani et al., 2008, Burch et al., 2010, Getachew et al., 2010b). Several GPCR agonists also lead to GAG hyperelongation such as: angiotensin II in human aortic smooth muscle cells (Figueroa and Vijayagopal, 2002), and thrombin and endothelin-1 (ET-1) in human VSMCs (Ivey and Little, 2008, Ballinger et al., 2009, Little et al., 2010, Burch et al., 2010).
Immunohistochemistry studies of human arteries show evidence of strong co-localization between apo-B100 lipoproteins with biglycan (Nakashima et al., 2007, O'Brien et al., 1998, Camejo, 1982). Studies have shown that overexpression of biglycan by rat smooth muscle cells leads to the production of ECM with higher binding affinity to lipoproteins. The distribution of biglycan was shown to co-localize with lipids in the early phase of atherogenesis, which proposes that biglycan plays an essential role in initial lipid deposition in the arterial intima. The co-localization of biglycan with apo-B and apo-E is also found in early as well as advanced human and murine atherosclerotic lesions (Nakashima et al., 2007, O'Brien et al., 1998, Kunjathoor et al., 2002).

The concept of modified proteoglycans with hyperelongated GAG chains showing increased binding to LDL has recently been confirmed in an in vivo model of atherosclerosis. Anggraeni et al (Anggraeni et al., 2011) demonstrated that mice susceptible to atherosclerosis developed significant increase in aortic root plaque after 4 and 8 weeks on a Western diet. They detected increased biglycan core protein mRNA levels in the lesions after 8 weeks of feeding. Gel filtration of CS proteoglycans from the aortas revealed an increase in size as early as 2 weeks which continued as lesions progressed. Immunostaining revealed co-localization of biglycan and apo-B in the initial stages of fatty streak development in these mice. Importantly, it was observed that an increase in the mRNA expression and staining of the transferase enzymes CHST11 and ChGn-2 at 8 weeks of feeding coincided with biglycan at early stages of lesion development. Since these two enzymes may be involved in the retention of atherogenic lipoproteins by mediating CS chain elongation, information regarding the mechanisms of regulation of these synthesizing enzymes may provide a novel therapeutic target to prevent the initiation and
progression of atherosclerosis (Anggraeni et al., 2011). Another study in human cardiac fibroblasts found that XXYLT-1 mRNA expression was up-regulated by TGF-β in vitro and this increased level of XXYLT-1 activity corresponded to elevated GAG chain synthesis in cardiac tissues (Prante et al., 2007). Knockdown of the XXYLT-1 mRNA reduced GAG synthesis (Prante et al., 2007). This data provides a link not just between biglycan and LDL but also GAG hyperelongation and a potential mechanism involving GAG synthesizing enzymes in atherogenesis. GPCRs such as thrombin have been shown to be involved in proteoglycan synthesis and GAG hyperelongation (Burch et al., 2010, Burch et al., 2013, Ivey and Little, 2008), however the role of thrombin mediated GAG enzyme expression has not yet been investigated.

1.6 GPCRs

GPCRs are the most prolific and polyfunctional receptors in biology (Marinissen and Gutkind, 2001, Pierce et al., 2002). GPCRs control diverse functions including relaxation of blood vessels, acceleration of heart rate, transmission of sight impulses, biorhythms and olfaction (Spehr and Munger, 2009). The early finding that GPCR agonists activate adenylate cyclase, an increase in cyclic adenosine-3’, 5’-monophosphate (cAMP) and functional outcomes such as vasorelaxation established GPCR signalling as a major area of cell biology and the award of a Nobel prize in Physiology and Medicine in 1971 to Earl W. Sutherland (Robison et al., 1967). The G protein component of this signalling pathway elicited the Nobel Prize in Physiology or Medicine in 1994 to Martin Rodbell and Alfred Gilman (Gilman, 1995, Rodbell, 1995). More recently, three decades of outstanding work in GPCR biochemistry and cell biology and the seminal discovery of the commonality and generality of the
seven transmembrane G-protein-coupled structure (Dixon et al., 1986) was recognised by the award of a Nobel Prize in Chemistry in 2012 to Robert J. Lefkowtiz and his colleague Brian K. Kobilka (Lefkowitz, 2004, Kobilka, 2007).

GPCR signalling occurs due to the agonist induction of conformational changes in transmembrane receptor proteins (Chung et al., 2011, Neer, 1995, Gether et al., 1997, Rosenbaum et al., 2009). GPCR protein conformations can be active and inactive. Binding of the agonist favours the active conformation and this in turn increases the affinity for binding of the G protein on the cytoplasmic surface (Vogel and Siebert, 2001).

GPCRs are helical transmembrane receptors complemented by functional extracellular and intracellular loops (Wess, 1997). Within the GPCR superfamily, there have been five major families identified. They are the rhodopsin, secretin, glutamate, adhesion and frizzled/taste2 families (Fredriksson et al., 2003). Most GPCRs contain seven helices and three intracellular loops; however, some members of the rhodopsin family may have eight helices and four intracellular loops (Oldham and Hamm, 2008). GPCRs bind hormones, neurotransmitters or growth factors (Neer and Clapham, 1988), which initiate a plethora of cellular responses. GPCRs are generally ligand activated but they can also bind to Gα-subunits in the absence of a ligand, a phenomenon known as receptor pre-coupling. GPCRs interact with their respective G proteins only upon receptor activation known as the collision coupling model or in the absence of agonist known as the pre-coupled receptor model (Hein and Bunemann, 2009).

G proteins are classified into four families according to their α subunit: Gi/o, Gs, G12/13 and Gq (Figure 1.5). The Gs and Gi families regulate adenylyl
cyclase activity, while Gq activates phospholipase Cβ and G12/13 can activate small GTPase families (Neves et al., 2002). The Gq family consists of four members: Gq, G11, G14 and G15/16 (Strathmann and Simon, 1990, Wilkie et al., 1991) and their respective α subunits are thus Gαq, Gα11, Gα14 and Gα15/16.
Figure 1.5: Classification of G protein into four families and their intracellular targets in vascular smooth muscle cells.

The Gα subunit is made up of Gi, Gα12/13, Gαs and Gαq/11. The Gαq subunit is made up of four members, which include Gαq, Gαq/11, Gαq/14 and Gαq15/16. Different G proteins have different targets in vascular smooth muscle cells that allow a GPCR to activate a wide range of intracellular effects.
1.6.1 Thrombin receptors

Thrombin acts on its GPCR PAR-1. Thrombin is a trypsin-like serine protease of 39kDa. It was first identified by its role in the coagulation cascade in which it cleaves fibrinogen, liberating fibrin (Macfarlane et al., 2001) however, subsequent discoveries revealed additional receptor mediated actions of thrombin. Pro-thrombin is synthesized in the liver as a prepropeptide and undergoes a series of post translational modifications before its secretion (Goldsack et al., 1998). Thrombin is short lived in the circulation, with a half-life of 14 seconds (Goldsack et al., 1998) and it acts near the site of production (Coughlin, 2000). In 1991 the cloning of the thrombin receptor was first reported by Vu et al (Vu et al., 1991), this provided evidence for thrombin’s ability to contribute to cellular events in an agonist/receptor type mechanism. PAR-1 is activated when thrombin cleaves the N-terminal Arginine$^{41}$-Serine$^{42}$ bond (Figure 1.6). The N-terminal cleavage of PAR-1 receptor unmasks a sequence that takes the function of a ligand and is capable of activating the receptor. As a consequence, thrombin cleavage of PAR-1 liberates a “tethered ligand” and provides the receptor with its own intrinsic activation method (Coughlin, 2000). PAR-1 can also be activated synthetically by a peptide that mimics the tethered ligand with the sequence, SFLLRN, albeit at much higher concentrations than required by thrombin. Since the discovery of PAR-1 there have been 3 additional human PARs cloned these are PAR-2 (Nystedt et al., 1995), PAR-3 (Ishihara et al., 1997) and PAR-4 (Xu et al., 1998) however thrombin is only capable of activating PAR-1, 3 and 4. Table 1.3 outlines the expression of these receptors in human tissue and their agonists.
Table 1.3: PARs expressed in humans.

This table demonstrates the different PAR receptors found in humans and the cardiovascular tissues they are expressed in, their biological agonists and the amino acid sequence of the tethered ligand for each receptor. The table is adapted from (Adams et al., 2011)

<table>
<thead>
<tr>
<th>Protease activated receptor subtype</th>
<th>Cardiovascular tissue expression</th>
<th>Biological agonists</th>
<th>Tethered ligand sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>Platelets, endothelial cells, fibroblasts and smooth muscle cells</td>
<td>Thrombin, Granzyme A, Factor Xa, Plasmin, APC-EPCR, Trypsin IV, KLK1,4,5,6,14, MMP-1, Cathepsin G, Proatherocytin, Pen C 13</td>
<td>SFLLRN</td>
</tr>
<tr>
<td>PAR-2</td>
<td>Aortic, endothelial and smooth muscle cells</td>
<td>Trypsin, Mast cell tryptase, TF-Factor Xa, Factor Viia, Acprsom, Matriptase, HAT, Trypsin IV, Granzyme A, TMPRss2, Chitinase, KLK2,4,5,6,14, bacterial gingipains, Der P1,2&amp;3, Pen C 13</td>
<td>SLIGKV</td>
</tr>
<tr>
<td>PAR-3</td>
<td>Endothelial cells and smooth muscle cells</td>
<td>Thrombin</td>
<td>TFRGAP</td>
</tr>
<tr>
<td>PAR-4</td>
<td>Platelets and smooth muscle cells</td>
<td>Thrombin, Trypsin, Cathepsin G, plasmin and bacterial gingipains, KLK1,14, Factor Xa, MASP-1 and Trypsin IV</td>
<td>GYPGQV</td>
</tr>
</tbody>
</table>

.
Thrombin has also been implicated in many aspects of CVD. The main focus in anti-thrombin therapy has been on anti-platelet strategies for the treatment and prevention of atherothrombosis and subsequent ischaemia (Shah, 2009). Currently this therapy is showing promising results with the FDA approval of vorapaxar (formerly SCH530348), an antiplatelet agent that reduces the risk of cardiovascular related death and ischemic events in patients with atherosclerosis by 13% (Morrow et al., 2012), and atopaxar (formerly E5555) in clinical trials (Leonardi et al., 2010).

Thrombin and its receptor are important in other stages of atherosclerosis. Thrombin and prothrombin have both been detected in the neointima of pre-atherosclerotic arteries (Patterson et al., 2001, Nelken et al., 1992) and thrombin receptors are expressed in pre-lesion fatty streaks (Nelken et al., 1992). In addition, endogenous thrombin was found to be elevated in adults younger than 45 without clinically apparent atherosclerosis and this was associated with intima-media thickness as an index of subclinical atherosclerosis (Bernhard 2010). PAR-1 knockout mice show a decrease in neointima formation and increased medial area compared to wild type mice in an endothelial denudation model of vascular injury (Cheung 1999). Blockage of PAR-1 with the small molecule inhibitor, JNJ5551794, causes a dose dependent inhibition in neointima area and thickness (Andrade-Gordon 2001). As well as the initial and final stages of atherogenesis, thrombin plays a role in the early developing plaque by promoting inflammatory intermediates and lipid deposition in vessel wall due to modification of proteoglycans. Our group has published that thrombin leads to the elongation of GAG chains on proteoglycans synthesized by human VSMCs such that they show an increase in affinity for LDL and
hence may contribute to the deposition and retention of LDL in vessel wall (Ivey 2008).

Figure 1.6: Thrombin activation of the GPCR, PAR-1.

Thrombin is a serine protease, a feature it utilises when activating its receptor Protease Activated Receptor-1 (PAR-1). Thrombin cleaves an N-terminal arginine-serine bond to liberate an intrinsic tethered ligand which activates the receptor leading to downstream signalling.
1.7 Transactivation independent GPCR signalling

1.7.1 The adenylate cyclase/ cAMP pathway

The adenylate cyclase pathway is typically activated by the Gα subunit in particular Gαs (Ross and Yallampalli, 2007, Billington and Penn, 2003) and is inactivated by Gαi protein (Hsia et al., 1984). Adenylate cyclase converts ATP to cAMP. cAMP then activates protein kinase A (PKA), a serine/threonine kinase that further propagates signals to the transcriptional level. PKA once active, then phosphorylates a variety of intracellular target proteins, by transferring a phosphate group from ATP to a serine/threonine residue on the target protein. cAMP activates other intracellular signalling molecules, such as the cAMP and AMP regulated exchange factor for Rap1 and the cyclic nucleotide-gated channel (Neves et al., 2002). This signalling cascade can be terminated by the breakdown of cAMP by a family of phosphodiesterases which hydrolyse cAMP to AMP. Physiological effects of the activation of this pathway in vascular smooth muscle include relaxation, as PKA phosphorylates and inactivates myosin light chain kinase. cAMP metabolised to adenosine inhibits VSMCs growth, hence the cAMP pathway may contribute to the regulation of vascular biology (Dubey et al., 1996).

1.7.2 The phospholipase Cβ/ inositol phosphate system

Activation of this signalling pathway begins with the activation of the GPCR by ligand binding. This causes dissociation of the Gαq (Rhee, 2001) and Gβγ subunits (Blake et al., 2001, Boyer et al., 1992), which are able to activate the membrane bound enzyme phospholipase C (PLC)β. Once activated, PLCβ cleaves a membrane bound phospholipid, phosphatidylinositol biphosphate (PIP2) to form two second messengers, diacylglycerol (DAG) which remains associated with the plasma membrane and inositol-(1,4,5)-triophosphate (IP3)
which diffuses into the cytoplasm. DAG, with calcium and phosphatidylserine, activate protein kinase C (PKC), a family of serine/threonine kinases that translocate from the cytosol to the membrane where they become activated and phosphorylate intracellular proteins.

In VSMCs PKC mediates proliferation, an increase in contractile force and regulation of cell growth (Lee and Severson, 1994). IP$_3$ stimulates the release of calcium by activating receptors on the ER of cells. The ET-1 receptors have been shown to couple to G$_\alpha$q and activate this pathway in VSMCs (Eguchi et al., 1993) raising intracellular IP$_3$ and calcium levels (Griendling et al., 1989). In a similar manner the thrombin receptor, PAR-1, couples to G$_\alpha$q and causes an increase in intracellular calcium and the activation of PKC in VSMCs.

### 1.7.3 The Rho/Rho kinase pathway

In addition to the heterotrimeric G proteins that are bound to GPCRs, other low molecular weight GTPases are present in VSMCs and are able to activate their own signalling pathways. Members of the small GTPase family include Rac, Ras and Rho of which there are 3 isoforms termed RhoA, RhoB and RhoC. Like the G-proteins they are activated by the binding of guanosine triphosphate (GTP) which occurs as a result of the exchange of guanosine diphosphate (GDP) for GTP and this exchange is regulated by guanine nucleotide exchange factors (GEFs) (Seasholtz et al., 1999). The third class of Rho regulatory proteins are the guanine nucleotide dissociation inhibitors (GDIs). Under basal conditions the majority of cellular Rho is found bound to specific GDIs (Seasholtz et al., 1999). GDIs inhibit the GTPase cycling (Seasholtz et al., 1999).
1.8 Transactivation dependent GPCR signalling

The classic GPCR signalling pathway (Figure 1.7) is termed direct, from the agonist to the GPCR to the coupled G protein interaction with subsequent downstream signalling leading to the functional response(s). This well researched pathway has been the subject of many reviews (Smith and Luttrell, 2006) (Premont and Gainetdinov, 2007, Hill, 2006). Two decades ago, this linear pathway was complemented by the finding that GPCRs could directly activate other cell surface receptors of the tyrosine kinase type (e.g. EGFR) leading to the activation of the kinase receptor and its downstream signalling pathway (Daub et al., 1996, Prenzel et al., 1999). The activation of PTKRs by a GPCR represented a major expansion in the cellular outcomes attributable to GPCR signalling. An example in vascular biology is the acute activation of GPCR angiotensin II receptor where a classical linear response leads to very rapid mobilisation of intracellular calcium ions and vascular contraction (Lee et al., 2001, Wynne et al., 2009) whereas transactivation of PTKRs (EGFR) leads to a slow vascular remodelling due to cell proliferation and ECM synthesis (Lee et al., 2001).

In very recent times the paradigm of GPCR transactivation signalling has expanded to include the transactivation of cell surface receptors of the serine/threonine kinase type and notably the important receptor for TGF-β. The activation of TGFBR1 either directly by its agonist or via transactivation following activation of a thrombin (Burch et al., 2010) or ET-1 (Little et al., 2010) receptor has been implicated in the modification of the synthesis and structure of GAG chains. GAG chain elongation has been implicated in the early stages of atherosclerosis in what is termed the “response to retention” hypothesis (Williams and Tabas, 1995, Little et al., 2002, Yang et al., 2010).
Whereas PTKR and S/TKR have intrinsic catalytic activity, GPCRs do not have enzymatic activity but are linked to Gα proteins which are GTPases and mediate the signal transduction (Wedegaertner et al., 1995). G proteins of the α, β and γ families provide the specificity and functionality of GPCRs. The role of G proteins in GPCR signalling has not been as intensively investigated as other aspects of GPCR signalling possibly due to the limited availability of pharmacological tools. As GPCR signalling in physiology and pathophysiology continues to grow, the potential importance of G proteins increases both for the fundamental cell biology and as potential therapeutic targets.
GPCRs signal via 3 mechanisms

1. Transactivation-independent ("classic" etc.)
2. β-arrestin signalling
3. Transactivation-dependent (PTKR or S/TKR)

**Figure 1.7: G protein coupled receptor signalling mechanisms.**

GPCR signalling can occur via 3 main mechanisms, the first is through the intracellular activation of the G proteins which is referred to as transactivation independent signalling (1). The second is through the utilization of the β-arrestin molecule (2) and thirdly through a mechanism known as transactivation were the GPCR can lead to the activation of a PTKR or a S/TKR (3) this is referred to as transactivation dependent signalling.
1.8.1 Defining GPCR transactivation

The term transactivation (Figure 1.7) was applied when GPCR lead to the activation of either PTKR or S/TKR (Burch et al., 2010, Burch et al., 2013, Little et al., 2011). Earlier, several groups identified a similar process but did not apply the term transactivation (Sorescu, 2006, Leask, 2010, Xu et al., 2009, Jenkins et al., 2006). Berry and colleagues were the first to define transactivation as “the process whereby ligand stimulation of one receptor leads to activation of another, distinct receptor” (Berry et al., 2001). Wetzker and Bohmer (Wetzker and Bohmer, 2003) described transactivation as being when one receptor transactivates a heterologous receptor. In order to define GPCR transactivation of S/TKR and to conceptually and mechanistically relate the phenomena to GPCR transactivation of PTKR, Little and colleagues (Little et al., 2011) provided a definition of transactivation as “the agonist occupancy of its cognate GPCR complex which leads in a relatively short time and in the absence of ‘de novo’ protein synthesis to the activation and cytosolic generation of the immediate downstream product(s) of a second cell surface protein kinase receptor”. A key parameter is that the receptor to receptor pathways defined as transactivation do not involve gene transcription. Thus, there appear to be sufficient similarities between GPCR to PTKR and GPCR to S/TKR transactivation that is reasonable to apply the term transactivation to both responses. The term “transactivation dependent” will be used to describe GPCR transactivation signalling and the term “transactivation independent” will refer to all other form of signalling downstream of GPCRs (Figure 1.7).
1.8.2 GPCR transactivation of protein tyrosine kinase receptors

The phenomenon of PTKR transactivation by a GPCR was first described in 1996 by Axel Ullrich (Daub et al., 1996). In this ground-breaking paper, Ullrich and colleagues (Daub et al., 1996) reported that agonists of GPCRs could transactivate the PTKR, EGFR, in rat-1 fibroblasts. Rat-1 fibroblasts stimulated by agonists ET-1 and thrombin showed a temporal increase in phosphorylation of extracellular signal-regulated kinase (phospho-Erk)1/2 which was blocked by either the highly specific, and well characterised EGFR inhibitor, AG1478, or by transfection with a cytoplasmic truncated EGFR mutant. This indicated that the increase of phospho-Erk1/2 by these GPCR agonists is via the EGFR and is a direct readout of EGFR transactivation (Daub et al., 1996). This group expanded their work to show this novel signalling mechanism exists in multiple cell lines. They showed that thrombin and lysophosphatidic acid (LPA) are capable of transactivating EGFR in human HaCat keratinocytes and COS-7 cells (Daub et al., 1997). In COS-7 cells, the response is dependent on Gαq and Gαi coupled GPCRs, and provided the first mechanistic aspect of the GPCR mediated transactivation signalling (Daub et al., 1997). Transactivation of EGFR was shown to have important consequences in the vasculature. EGFR transactivation by Gαq- and Gαi-coupled to angiotensin II or LPA receptors respectively, stimulate protein synthesis in rat aortic smooth muscle cells (Voisin et al., 2002) indicating that the transactivation of PTKR may be a mechanism by which GPCR agonists regulate cell growth and division.

Since the original observation by Ullrich and colleagues (Daub et al., 1996) there have been almost 200 reports of GPCR transactivation of PTKR. There has been considerable work on the mechanisms of this transactivation and
several pathways are prominent – the “triple membrane bypass” pathway which involves the GPCR activating a membrane bound matrix metalloproteinase (MMP) or A Disintegrin and A Metalloprotease (ADAM) resulting in the cleavage and release of a PTKR ligand which subsequently activates its cognate receptor in an autocrine/paracrine manner (Prenzel et al., 1999, Gschwind et al., 2002). Another candidate for intracellular mediators of transactivation is the generation of reactive oxygen species (ROS) (Frank and Eguchi, 2003). Stimulation of angiotensin II causes the activation of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase and an increase in intracellular ROS production (Frank and Eguchi, 2003). ROS act as second messengers and activate a wide variety of serine/threonine and tyrosine kinases and may activate the EGFR by targeting the cysteine regions of the active sites of tyrosine phosphatases, which in turn activate tyrosine kinases (Shida et al., 2004). ROS stimulate the production of a heparin binding EGF-like growth factor (HB-EGF) via cleavage by an MMP which is able to activate the EGFR (Figure 1.8) (Kalmes et al., 2001). Evidence for the role of HB-EGF in EGFR transactivation is supported by the observation that neutralizing antibodies to HB-EGF block EGFR activation (Kalmes et al., 2001).

In addition, some drugs elicit activation of a GPCR and transactivation of the PTKR. Serotonin transactivates the PDGF-β receptor in pulmonary artery smooth muscle cells (Liu et al., 2007). Inhibition of PDGF receptor kinase with imatinib or AG1478 blocks serotonin mediated PDGF-β receptor phosphorylation. The selective serotonin reuptake inhibitor, fluoxetine blocks both the serotonin mediated binding and activation of PDGF-β receptor (Liu et al., 2007).
Figure 1.8: GPCR transactivation of protein tyrosine kinase receptors in vascular smooth muscle cells.

GPCRs can transactivate PTKRs in a ligand dependent manner, otherwise known as triple membrane bypass signalling. This involved the activation of cell surface matrix metalloproteinases (MMPs) that cleave and release membrane anchored EGFR ligands.
Many GPCRs and many PTKR partners participate in GPCR transactivation. GPCR transactivation of EGFR is the best characterised however transactivation of other PTKRs is well known and includes, Trk, fibroblast growth factor, insulin growth like factor 1 and PDGF receptors (Puehringer et al., 2013, Nagai et al., 2005, Zhao et al., 2011, Wang et al., 2010, Tsai et al., 2014). The Ullrich et al paper (Daub et al., 1996) described above is the first to utilize the description of ‘transactivation’ to describe the activation of EGFR by GPCRs, however it was not the first example of such mechanism. It was the PDGF receptor that first alluded to the potentially novel pathway as a link between the PDGF and angiotensin II receptors in VSMCs, although the authors did not directly mention transactivation, nor define it (Linseman et al., 1995). Subsequent research has revealed that angiotensin II can transactivate the PDGF receptor leading to phospho-Erk1/2 (Shida et al., 2004) as can LPA (Goppelt-Struebe et al., 2000, Herrlich et al., 1998).

1.8.3 Transactivation of serine/threonine kinase receptors

GPCR transactivation of PTKR has been extensively researched however it has appeared that this phenomenon is not restricted to these kinase receptors (Blume-Jensen and Hunter, 2001). Some GPCRs can activate S/TKRs specifically from the TGF-β receptor super family (Table 1.4) (Burch et al., 2013, Burch et al., 2012, Little et al., 2010, Chung et al., 2013).

It was initially observed that the PAR-1 mediated stimulation of proteoglycan synthesis in human VSMCs, was partially blocked by the specific TGFBR1 antagonist, SB431542 (Burch et al., 2010). Subsequently, it was observed that thrombin stimulated the accumulation of phospho-Smad2(465/467) in VSMCs and the response was blocked by SB431542 indicating the involvement of TGFBR1 (Burch et al., 2010). Burch et al. (Burch
et al., 2013) described the mechanism in more detail. The transactivation involves the GPCR, PAR-1, the cytoskeleton, integrin dependent signalling and ultimately the activation of the Large Latent Complex which holds TGF-β near the cell surface with the potential for rearrangement and activation of TGFBR1 (Burch et al., 2013). Recently almost identical data for the PAR-2 mediated transactivation of TGFBR1 and EGFR in human proximal tubular epithelial cells has been described (Chung et al., 2013). Chung et al (Chung et al., 2013) reported that activation of PAR-2 triggered the phosphorylation of Smad2 and Smad3, which was partially inhibited by SB431542. PAR-2 activation induced a robust phospho-Erk1/2 response which was completely abolished using the EGFR specific inhibitor, AGI478. An interesting point in comparison to our previous published work on PAR-1 transactivation of TGFBR1 (Burch et al., 2013) is that Chung et al. (Chung et al., 2013) showed that AG1478 diminished PAR-2 mediated phospho-Smad2 but it was not involved in PAR-1 mediated phospho-Smad2 in the carboxyl terminus.

The stimulation of proteoglycan synthesis by thrombin is blocked by 40-50% by the TGFBR1 receptor antagonist, SB431542, and 40-50% by the EGFR antagonist, AG1478 (Burch et al., 2013). This indicates that a partial effect of thrombin simulation of proteoglycan synthesis is mediated by transactivation of the TGFBR1 and a similar amount is due to the transactivation of the EGFR. Dual pharmacological inhibition of the TGFBR1 and the EGFR account for the total GAG elongation effect mediated by thrombin (Burch et al., 2013). Knowing that these effects are additive and yet share no common known signalling pathways can further broaden the current understanding of GPCR transactivation phenomenon.
The involvement of the cytoskeleton and integrin signalling in GPCR transactivation of S/TKR in VSMCs is consistent with that observed in other cell types (Jenkins et al., 2006, Xu et al., 2009, Belmadani et al., 2008). Thrombin mediated transactivation of TGFBR1 in mouse lung epithelial cells involves αVβ6 integrin signalling (Jenkins et al., 2006). In a vascular model using mouse microvessel VSMCs angiotensin II mediated TGFBR1 transactivation was abolished by an αVβ3 blocking antibody (Belmadani et al., 2008), indicating that the integrin isoforms that mediate transactivation of the S/TKR may be cell type and context specific. The thrombin transactivation of TGFBR1 requires an intact cytoskeleton and RGD binding integrins (Burch et al., 2013), however the specific integrins have not yet been determined (Figure 1.9).
Table 1.4: GPCR transactivation of serine/threonine kinase cell surface receptors.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>GPCR</th>
<th>S/TKR</th>
<th>Mechanism of action</th>
<th>Response</th>
<th>Cellular response</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>PAR-1</td>
<td>TGFBR1</td>
<td>No Data</td>
<td>pSmad2</td>
<td>Proteoglycan synthesis</td>
<td>Vascular smooth muscle cells</td>
<td>(Burch et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>PAR-1</td>
<td>TGFBR1</td>
<td>ROCK/integrin</td>
<td>pSmad2</td>
<td>Proteoglycan synthesis</td>
<td>Vascular smooth muscle cells</td>
<td>(Burch et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>PAR-1</td>
<td>TGFBR1</td>
<td>No Data</td>
<td>pSmad2</td>
<td>GAG synthesizing genes</td>
<td>Vascular smooth muscle cells</td>
<td>(Kamato et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>PAR-1</td>
<td>TGFBR1</td>
<td>RhoA αVβ6 ROCK/integrin</td>
<td>pSmad2</td>
<td>Oedema/permeability</td>
<td>Lung epithelium</td>
<td></td>
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<tr>
<td>2f-LIGRLO-NH₂</td>
<td>PAR-2</td>
<td>TGFBR1</td>
<td>MMPs</td>
<td>pSmad2</td>
<td>Renal fibrosis</td>
<td>Proximal tubular epithelial</td>
<td>(Chung et al., 2013)</td>
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<tr>
<td>Factor Xa</td>
<td>PAR-1</td>
<td>TGFBR1</td>
<td>Gαq/ROCK/Integrin αVβ5</td>
<td>pSmad2/3; α-SMA</td>
<td>Fibroblast differentiation</td>
<td>Lung fibroblasts</td>
<td>(Scotton et al., 2009)</td>
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<td>Endothelin-1</td>
<td>ETR</td>
<td>TGFBR1</td>
<td>No Data</td>
<td>pSmad2</td>
<td>Proteoglycan synthesis</td>
<td>Vascular smooth muscle cells</td>
<td>(Little et al., 2010)</td>
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<tr>
<td></td>
<td>ET₂R</td>
<td>TGFBR1</td>
<td>No Data</td>
<td>pSmad3</td>
<td>Epithelial mesenchymal transition</td>
<td>Lung alveolar epithelium</td>
<td>(Jain et al., 2007)</td>
</tr>
<tr>
<td>Lysophosphatidic acid</td>
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<td>TGFBR1</td>
<td>Gαq/ROCK/Integrin αVβ6</td>
<td>PAI-1</td>
<td>Fibrosis</td>
<td>Lung epithelium</td>
<td>(Xu et al., 2009)</td>
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<tr>
<td></td>
<td>LPA2R</td>
<td>TGFBR1</td>
<td>Integrin αVβ5</td>
<td>pSmad2; PAI-1</td>
<td>Airway remodelling</td>
<td>Airway smooth muscle</td>
<td>(Tatler et al., 2011)</td>
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<tr>
<td>Methacholine</td>
<td>Muscarinic R</td>
<td>TβRI</td>
<td>Integrin αvβ5</td>
<td>pSmad2; PAI-1</td>
<td>Airway remodelling</td>
<td>Airway smooth muscle</td>
<td>(Tatler et al., 2011)</td>
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<td>Parathyroid Hormone</td>
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<td>BMPR2</td>
<td>endocytosis</td>
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<td>MSC differentiation</td>
<td>Mesenchymal stem cells</td>
<td>(Yu et al., 2012)</td>
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</tbody>
</table>
Integrins are a family of ubiquitous cell surface class I transmembrane spanning receptors (Ruoslahti and Pierschbacher, 1987). Integrin signalling is an interesting biological phenomenon because it involves the transmission of signals from the outside to the inside of a cell, so-called ‘outside in signalling’, as well as ‘inside out signalling’ and this confers bidirectional signalling properties on integrins and places them at an important axis in cellular communication (Hynes, 2002). Several examples of integrins and GPCR transactivation of S/TKR have been described over the past decade (Jenkins et al., 2006, Xu et al., 2009, Tatler et al., 2011, Scotton et al., 2009). Latent TGF-β exists in a complex that is anchored to the extracellular plasma membrane. The latency associated protein constituent of the complex is known to contain a RGD motif, and thus is a candidate for integrin ligation (Worthington et al., 2011). Inside out integrin signalling has been associated with cytoskeletal rearrangement and ROCK signalling. Studies from our laboratory show that thrombin can transactivate TGFBR1 via cytoskeletal rearrangement which activates ROCK signalling leading to the activation of cell surface integrins (Burch et al., 2013). PAR-1 transactivation of PTKRs and the generation of phospho-Erk1/2 is not mediated by the same ROCK integrin signalling mechanism as for TGFBR1 but rather the well characterised ligand-dependent MMP mediated pathways. The first interaction between integrins and MMPs was identified in melanoma cells where it was demonstrated that the C-terminal domain of MMP-2 binds directly to αVβ3 (Brooks et al., 1996). MMP-8 and MMP-13 act in concert with integrin α5β1 to mediate bradykinin-induced transactivation of EGFR and phospho-Erk1/2 in kidney cells (Kramarenko et al., 2010).
We have shown that in VSMCs, MMPs are involved in thrombin generation of phospho-Erk1/2 and the stimulation of proteoglycan synthesis via transactivation of the EGFR. These MMP dependent pathways are not involved in thrombin transactivation of the TGFBR1 leading to phospho-Smad2(465/467) generation (Burch et al., 2013). In contrast Chung et al (Chung et al., 2013) examined the effect of broad spectrum MMP inhibitor, marmistat, on the phosphorylation of Smad2 linker region in human proximal tubular epithelial cells, presenting a partial role for MMPs in PAR-2 mediated transactivation of the TGFBR1. However as Smad linker region can be phosphorylated by a number of pathways it remains unclear that the response is due to PAR-2 transactivation of TGFBR1.
Figure 1.9: GPCR transactivation of serine/threonine kinase receptors in vascular smooth muscle cells.

S/TKR activation is achieved via cytoskeletal rearrangement which activates ROCK signalling, leading the activation of cell surface integrins which bind to the latent TGF-β complex. This allows for TGF-β to bind to its receptor leading to subsequent Smad signalling.
1.8.4 Role of G proteins in GPCR transactivation signalling

The response to GPCR agonists and the conformational changes in the GPCR that are induced by ligand binding are mediated by heterotrimeric G protein complexes. Consisting of three subunits; α, β and γ their role is to transduce external stimuli into intracellular signalling cascades. Daub et al. (Daub et al., 1997) investigated the role of G proteins in EGFR transactivation signalling using COS-7 cells and found that LPA induced EGFR transactivation was partially inhibited by treatment of cells with Gαi inhibitor, pertussis toxin. The response however was unaffected in thrombin stimulated cells indicating that LPA signals through Gαi and pertussis toxin-sensitive G proteins (Daub et al., 1997). Pertussis toxin catalyses the ADP-ribosylation of the Gαi proteins preventing the coupling with the GPCR. Cells transfected with Gaq-coupled GPCRs are unaffected by pertussis toxin while Gαi coupled receptors were blocked by pertussis toxin (Koch et al., 1994). Thus, EGFR transactivation may occur through both pertussis toxin sensitive and insensitive pathways. Similarly in rat-1 fibroblasts, Gαi coupled, but not Gaq/11 coupled receptors mediate tyrosine kinase dependent phospho-Erk1/2 (van Corven et al., 1989). The role of G proteins in transactivation dependent signalling is of considerable interest; however there has been a limited number of studies available in the area. The work in this thesis will focus on the role of the Gaq in GPCR mediated transactivation dependent signalling.

1.9 Gaq subunit

1.9.1 Structure of Gaq

Gaq and Gaq/11 are products from distinct genes on the same chromosome (Wilkie et al., 1991). These two proteins have a 90% similarity in the number of amino acids and are functionally almost identical. However, the
tissue distribution of the two isoforms is distinct (Wilkie et al., 1991). The Gαq/11 is generally expressed ubiquitously with the exception of platelets where only Gαq is expressed (Griewank et al., 2011). According to the gene expression microarray data from UCSC genome browser, the Gαq/11 gene is highly expressed in the heart however Gαq had a much lower expression level. Gαq is a 359 amino acid protein comprised of two domains: a helical domain and a GTPase binding domain (Figure 1.10). The GTPase domain is responsible for hydrolysing GTP to GDP (Figure 1.11), as well as binding the Gβγ subunits, GPCRs and other effectors. This domain is conserved between all members of the G protein superfamily (Oldham and Hamm, 2008). The GTPase domain contains three switch regions (Figure 1.10), which are flexible loops that change conformation when bound with GTP. The helical domain contains six α-helices which encapsulates nucleotides in the protein core by forming a lid over the nucleotide-binding pocket. Of all G protein families identified, members of the Gαq family share the most amino acid sequence homology. In humans, Gαq/11, Gαq/14 and Gαq/16 share 90%, 80% and 57% sequence similarities, respectively (Hubbard and Hepler, 2006).
Figure 1.10: Schema of Gαq functional domains.

The helical domain is represented in blue and the GTPase domain is shown in purple. The switch regions are as follows: Switch Region 1 (182-192) in orange, Switch region 2 (204-224) in red and switch region 3 (236-257) in yellow. Adapted from Mizuno and Itoh 2009 (Mizuno and Itoh, 2009).
1.9.2 Gaq/11 signalling

In an inactivated state, the α subunit binds GDP, however, upon ligand binding, GTPase activity is induced and promotes the exchange of bound GDP for GTP (Figure 1.11) to activate the GPCR. The α subunit and βγ complex then dissociate from one another and interacts with their associated effectors (Berman and Gilman, 1998). In the most common paradigm Gaq activates PLCβ which hydrolyses PIP2 releasing DAG and IP3. DAG activates a number of isoforms of PKC, whereas IP3 diffuses to the ER and binds to IP3 receptors on ligand-gated calcium channels leading to a release of calcium ions into the cytosol and subsequently in some cells (Little et al., 1992). The calcium cycle continues with the uptake of calcium back into the ER by calcium ATPases.

Activation of Rho mediated signalling pathways can be indirectly mediated by GPCRs, integrins or PTKRs. G proteins, Gaq/12 and Gaq/13, activate Rho by activation of a Rho GEF (Singh et al., 2007). It is only when RhoA is active that it can interact with and activate downstream effectors such as ROCK. Thrombin activation of PAR-1 involves both Gaq/11 and Gaq12/13, which cause RhoA activation signalling downstream to stimulate ROCK and PKC-related kinase. In human endothelial cells Gaq/11 involves intracellular release of calcium through the downstream activation of the two Rho-regulated protein kinases, which in turn regulate the contraction of actinomycin and the formation of focal adherence.

Overexpression of active Gaq/11 or stimulation of the m1 muscarinic acetylcholine receptor induces apoptosis in HeLa cells. Rho kinase and ROCK are stimulated due to the cleavage of activated caspase 3 during apoptosis. There have been several studies on the mechanisms involved in Gaq/11 induced apoptosis, which show that this phenomenon is cell and context dependent. In COS-7 and CHO cells, Gaq/11 induced apoptosis is dependent on PKC,
angiotensin II induced myocyte apoptosis is dependent on the release of intracellular calcium suggesting the involvement of PLC pathway. The molecular mechanism of Gaq/11 induced apoptosis leading to the activation of Rho/ROCK is not clearly understood, however some studies have shown that Gaq/11 signalling activated RhoA which inhibited insulin-stimulated Akt phosphorylation in HeLa cells. In CHO cells Gaq and Gaq/11 regulate actin cytoskeleton remodelling through the activation of ADP-ribosylation factor 6 (Boshans et al., 2000). Platelets stimulated with P2Y1 agonist leads to the activation of RhoA, this activation was inhibited by Gaq inhibitor YM-254890, indicating that RhoA activation downstream of P2Y1 receptors requires Gaq stimulation (Jin et al., 2009).
Figure 1.11: G-protein coupled receptor structure.

GPCRs are characterised by 7 membrane spanning domains. The N-terminal is located at the extracellular surface and generally provides the ligand docking site. The C terminus is located at the intracellular interface. Ligand engagement causes a conformational change in the GPCR resulting in activation of G protein via the exchange of GDP for GTP on the $\text{G}_\alpha$ unit and dissociation from the $\text{G}_\beta\gamma$ unit.
1.9.3 Functions of Gαq

Gαq protein plays a role in a number of cellular signalling pathways. In the vasculature studies utilising genetically modified mice suggest that receptors coupled to the Gαq play a role in the development of heart failure (Fan et al., 2005). Following treatment to activate Gαq in transgenic mice expressing a silent Gαq, the mice rapidly developed a dilated cardiomyopathy and heart failure. Transgenic mice expressing an inducible Gαq that cannot activate PLCβ do not develop heart failure. Thus, the activation of Gαq resulting in heart failure requires the activation of PLCβ (Fan et al., 2005). Gαq deficient mice compared to wild type dramatically increased bleeding time and resistance to thromboembolism (Offermanns et al., 1997). Angiotensin II dose-dependently increases cell proliferation in smooth muscle cells and this is inhibited by the Gαq antagonist, GP-2A (Tanski et al., 2004). Gαq is also implicated in insulin-stimulated glucose transport (Imamura et al., 1999). In 3T3-L1 adipocytes, Gαq is required for insulin-induced GLUT4 translocation and the stimulation of 2-deoxy-D-glucose uptake. Gαq/11 proteins are involved in HIV-1 envelope glycoprotein-dependent cell-cell fusion upstream of Rac-1 (Harmon and Ratner, 2008).

1.9.4 Role of Gαq in the GPCR transactivation of kinase receptors

There is a limited amount of studies on the role of Gαq in transactivation dependent signalling. In L6 myoblasts derived from rat skeletal muscle, ET-1 via the endothelin type A receptor mediated transactivation of PDGF receptor was inhibited in the presence of Gαq antagonist, YM-254890 (Harada et al., 2014). Shear stress induced phospho-Erk1/2 in CHO cells permanently transfected with wild type angiotensin II type 1 receptor was completely
inhibited in the presence of YM-254890 (Barauna et al., 2013). Shear stress activates PTKR (Jin et al., 2003) thus showing that the Gαq plays a role in angiotensin II mediated transactivation of PTKR. Purigenic receptor P2Y2R transactivation of the insulin-like growth factor receptor in keratinocytes was inhibited by Gaq/11 siRNA transfected cells and YM-254890 (Taboubi et al., 2010).

Moving onto the role of G proteins in transactivation of S/TKR. Xu and colleagues (Xu et al., 2009) showed that in mouse embryonic cells with Gaq and Gα12/13 knockout only that the Gaq is involved in LPA induced transactivation of the TGFBR1 (Xu et al., 2009). This was confirmed with a pharmacological approach using Gaq antagonist GP-2A (Tanski et al., 2004, Mukai et al., 1992). In primary epithelial cells LPA induced TGFBR1 activation was inhibited in the presence of GP-2A (Xu et al., 2009). PAR-1 induced transactivation of the TGFBR1 in idiopathic pulmonary fibrosis lung tissue occurred via the Gaq protein (Scotton et al., 2009). Moreover the attenuation of PAR-1 induced activation of the TGFBR1 with Rho kinase antagonist (Y-27632) firmly places the Rho signalling pathway downstream of this response (Scotton et al., 2009). Similarly in murine lung fibroblasts, thrombin mediated CCL2 production occurs via PAR-1 coupling to Gaq and the cooperation between Rho signalling pathways (Deng et al., 2008). It will be interesting to investigate the role of Gaq proteins in PTKR and S/TKR transactivation signalling as it has the potential to be a point of commonality in GPCR mediated transactivation dependent signalling.
Figure 1.12: Pharmacological agents to inhibit downstream signalling intermediates of Gαq.

Once GPCR is activated by its agonist, Gαq signalling activates PLCβ, which leads to the hydrolysis of PIP<sub>2</sub> and DAG. The former leads to initiate the release of IP<sub>3</sub> initiating calcium release, activating protein tyrosine kinase 2 (PYK2), which leads to proto-oncogene tyrosine protein kinase (Src) activating Ras guanine nucleotide exchange factor (RasGEF), which leads to the activation of MAPK signalling. MAPK signalling pathway can also be downstream of DAG that activates protein kinase C (PKC), which leads to the activation of MAPK signalling. Gαq signalling can also go indirect of PLCβ by activating RhoGEF leading to the activation of the Rho/ROCK signalling pathway.

Extracted from (Kamato et al., 2015)
1.9.5 Molecular and pharmacological approach for the study of G proteins

The reason for the restricted investigations on the role of Gaq in cell biology relates to the limited availability of the pharmacological agents. There is not a readily available small molecule, which interact with Gaq signalling. In the literature there have been some agents of natural products, however the availability has varied over the years, in the sections below the pharmacological (Figure 1.12) and molecular agents available to study the role of Gaq have been reviewed.

1.9.5.1 Pharmacology of Gaq inhibitor - YM-254890

The compound known as YM-254890, a cyclic depsipeptide isolated from the Chromobacterium sp. QS3666, is a specific Gaq inhibitor. YM-254890 was discovered and developed by Yamaguichi Pharmaceuticals Japan; Yamaguichi subsequently became the property of Astellas Pharmaceuticals Japan. YM-254890 was made available to researchers ten years ago and a small number of interesting studies were published. YM-254890 is an inhibitor of Gaq and as such blocks the cycling of GDP/GTP specifically blocking the release of GDP from the Ga protein (Takasaki et al., 2004, Taniguchi et al., 2003, Nishimura et al., 2010). YM-254890 has been shown to inhibit ADP-induced platelet aggregation which is mediated via GPCRs, P2Y1 and P2Y12 (Taniguchi et al., 2003). These receptors are associated with the Gaq and Gai signalling pathways respectively. YM-254890 has no effect on the P2Y12 signal transduction pathway, indicating that the compound has some specificity for Gaq. It was also shown to inhibit Gaq coupled GPCR signalling by inhibiting calcium mobilisation in P2Y2-expressing C6-15 cells but not cAMP accumulation (Takasaki et al., 2004). When bound to GDP, the non-polar side chains of YM-254890 form hydrogen bonds with the Switch I region, however,
this is a conformation that cannot be maintained when bound with GTP (Nishimura et al., 2010). Aside from antiplatelet activity, by electrically inducing carotid artery thrombosis in rodents, YM-254890 was also shown to have antithrombotic and thrombolytic effects (Kawasaki et al., 2003).

1.9.5.2 UBO-QIC/FR300359

FR300359, henceforth referred to as UBO-QIC is also, like YM-254890, a cyclic depsipeptide; it is isolated from the Ardisia crenata sims plant (Miyamae, 1989). UBO-QIC is structurally very similar to YM-254890 except UBO-QIC has one methyl group replaced by an ethyl group and another methyl group replaced by an isopropyl group (Kukkonen, 2016). UBO-QIC inhibits platelet aggregation in rabbits in vitro and causes dose-related hypotension in anaesthetised normotensive rodents which is consistent with the effect on blood pressure in Gaq knock down mice (Fujioka et al., 1988, Wirth et al., 2008). The blood pressure lowering effect was attributed to the ability of UBO-QIC to partially mediate nitric oxide release from endothelial cells and inhibit calcium migration caused by voltage-dependent and receptor operated channels (Zaima et al., 2013).

There have been limited studies with UBO-QIC since its discovery as a Gaq inhibitor. In HEK cells expressing TRPV4, PAR-2 mediated intracellular calcium release was abolished by UBO-QIC, however extracellular calcium influx through the TRPV4 ion channel was unaffected showing that PAR-2 coupling to TRPV4 is not mediated by Gaq signalling (Grace et al., 2014). L-Orn IP1 and calcium responses of transfected CHO cells with GPCR receptor 6 was mediated through Gaq activation, both these pathways were inhibited by UBO-QIC (Jacobsen et al., 2013). Muscarinic M3 receptor induced calcium signalling in both non-transfected and CHO cells transfected with Gβ5-RGS7 is
dependent on Gaq but not Gai signalling (Karpinsky-Semper et al., 2014). In CHO cells transfected with P2YP and cells expressing M1 muscarinic receptors, activation of PLC using ATP and oxotremorine-M was completely blocked in the presence of UBO-QIC (Kukkonen, 2016). PGE₂ stimulated adenylate cyclase production in CHO cells transfected with β₂-adrenoreceptors was not inhibited in the presence of UBO-QIC, thus showing UBO-QIC does not inhibit Gαs signalling (Kukkonen, 2016). PAR mediated platelet aggregation was inhibited in a dose dependent manner by UBO-QIC in human platelet cells (Inamdar et al., 2015). Furthermore, UBO-QIC had no effect on Akt phosphorylation downstream of Gai pathways or the phosphorylation of vasodilator-stimulated phosphoprotein downstream of Ga (Inamdar et al., 2015). UBO-QIC was found to be selective over other members of the the Gα subunit however it was demonstrated that it is not selective for Gaq as it is involved in inhibitin Gβγ mediated signalling events following Gai activation (Gao and Jacobson, 2016). There have been no studies directly comparing the activity of YM-254890 and UBO-QIC.

1.9.5.4 Gα gene knockdown using siRNA

Despite the very large number of GPCRs there are relatively few studies that have used the potential of Gaq/11 gene knockdown by siRNA to explore their roles in the signalling cascades. One of the first reported gene knockdown studies of Gα proteins was the knockdown of Gaq and Gaq/11 gene expression using siRNA in HeLa cells (Krumins and Gilman, 2006). This work demonstrated an absolute requirement of Gaq/11 to stimulate histamine mediated PLC activity. Silencing of Gaq or Gaq/11 caused indistinguishable phenotypes, loss of half of histamine-stimulated PLC activity, despite the fact that concentrations of Gaq/11 exceed those of Gaq by 10-fold. No
compensatory increases of either Gaq or Gaq/11 were observed following loss of either protein. Loss of Gaq or Gaq/11 did cause increased accumulation of Gaι (Krumins and Gilman, 2006). A study characterising the Ga subunits required for PAR-1 mediated endothelial cell permeability showed that both Gaq and Gaq/11 were necessary for thrombin to increase permeability while the need for Gaq12/13 was less. Both protein subunit families contributed significantly to RhoA activation by thrombin (Gavard and Gutkind, 2008). Knockdown of Gaq/11 in human pulmonary artery smooth muscle cells alters but does not prevent hypoxia-induced mitogenic factor-mediated calcium release demonstrating that Gaq/11 contributes to hypoxia-induced PLC signalling pathway (Fan et al., 2009). In HEK cells expressing high levels of thyrotropin-releasing hormone receptor 2, knockdown of Gaq/11 reduces persistent agonist-induced signalling by 82 percent and suggests that Gaq/11 is a required component of the activated receptor signalling pathway (Boutin et al., 2012). Clearly there is considerable scope to use siRNA technology more often as a very useful tool in delineating the importance of Ga proteins in GPCR signalling.

1.9.6 Potential of Gaq as a therapeutic target

Gaq as a protein has several functions which show its value as a therapeutic target. The binding of GDP and GTP (Figure 1.11) are potential targets in the same manner in which the ATP binding site is target of many drugs inhibiting kinases (Liu et al., 2013). The ligand activated GPCR acts as a GEF which stimulates the exchange of GDP for GTP on the Ga peptide and this could be targeted. Furthermore, the protein contains a switch mechanism and this can be targeted as it is the target of the YM class of inhibitors (Nishimura et
al., 2010). So it is both theoretically possible and has been demonstrated that 
Gαq can be exploited as a drug target.

The consequences of targeting signalling molecules have theoretical 
limitations based on the role of such targets in normal physiology but also 
conceptually there may be situations, in which the activity of Gαq is elevated or 
enhanced and presents itself as a target. Such situations are common in 
therapeutics but in most cases can only be established experimentally.

As discussed above, inhibition of Gαq/11 using YM-254890 has 
demonstrated anti-platelet aggregation, antithrombotic and thrombolytic 
properties in a rat model of carotid artery thrombosis (Kawasaki et al., 2003). Therefore, compounds that inhibit Gαq/11 could show enormous 
potential in the treatment of thrombotic conditions such as thrombotic stroke and 
myocardial infarction in humans. Additionally, a number of recent studies have 
also implicated a role for Gαq/11 in a range of metabolic conditions such as 
obesity and type 2 diabetes (Li et al., 2013, Kimple et al., 2014). Activation of 
Gαq results in pronounced increases in blood glucose levels in a mouse model 
(Li et al., 2013), thus, compounds that inhibit Gαq could also show promise as a 
future treatment option for type 2 diabetes.

In the important cardiovascular context of hypertension, Gαq knockout 
mice have reduced blood pressure (Wirth et al., 2008) and YM-254890 has 
demonstrated some anti-hypertensive properties (Kawasaki et al., 2003). 
Although there are many effective anti-hypertensive agents currently available, 
there are also many subjects with medication resistant hypertension which does 
require a niche for new therapies although it is unclear if a Gαq/11 inhibitor 
would suitable to consider for such a niche.
1.10 Biochemistry and signalling of Smad transcription factors

Burch et al (Burch et al., 2010) showed that there is a correlation between TGF-β mediated Smad2 linker region phosphorylation and proteoglycan synthesis. As covered in sections above thrombin through the transactivation of the EGFR and TGFBR1 leads to proteoglycan synthesis and GAG elongation. Hence Smads may play an important role in thrombin signalling leading to proteoglycan synthesis and GAG chain hyperelongation. Smad proteins are the intracellular mediators of TGF-β superfamily signalling which are transcription factors (Shi and Massague, 2003) that regulate gene expression (Verrecchia et al., 2006). Following the direct phosphorylation by TGFBR1 phosphorylated Smad complexes move to the nucleus where they have diverse effects on gene transcription. Smad activity is modulated through the recruitment of transcription co-activators and co-expressers (Wrighton et al., 2009). In the human genome, eight Smad proteins have been identified Smads 1-8. The Smad family can be divided into three functional groups, receptor regulated Smads (R-Smads) which are directly phosphorylated by the activated TGFBR1 on a conserved C-terminal motif and include Smad1, Smad2, Smad3, Smad5 and Smad8 (Massague et al., 2005), the common mediated Smad4 (co-Smad) that interacts with the R-Smads in cell signalling (Shi et al., 1997), and the inhibitory Smads (I-Smad) Smad6 and Smad7 which are responsible for blocking the activation of R-Smad and co-Smad and initiating receptor degradation (Itoh et al., 2001).

Smad proteins contain 400-500 amino acid residues and contain 3 distinct regions (Shi and Massague, 2003). A conserved N-terminal domain or
“Mad-homology 1” (MH1) domain which is involved in nuclear translocation (Massague, 1998, Derynck and Zhang, 2003). This region binds to DNA and interacts with DNA-binding cofactors (Massague et al., 2005). Smads contain a conserved C-terminal (MH2) domain and thirdly the non-conserved proline rich linker region that joins these two domains (Figure 1.13) (Derynck and Zhang, 2003, Kamato et al., 2013c, Rostam et al., 2015). Mammalian Smad2 and Smad3 have a similar amino acid sequence with a 66% and 96% identity in their MH1 and MH2 amino acid sequences, respectively (Brown et al., 2007). In contrast to the similarity in their amino acid sequences, there are two major differences between the overall structures of Smad2 and Smad3. Alignment of the MH1 domains of Smad2 and Smad3 reveals that there are two regions of amino acid sequences which are present in Smad2 but absent from Smad3. One of these is called GAG, a short N-terminal amino acid residue consisting of ten residues. The other is a 30-residue sequence referred to as TID (also known as exon3). The TID sequence is rich in serine and threonine residues and is shown to prevent Smad2 from direct binding to DNA (Yang et al., 2009). The stability and intracellular localization of Smad2 and Smad3 are regulated by distinct mechanisms resulting in functional and regulatory differences between these two proteins (Massague et al., 2005). In mouse astrocytes, TGF-β mediated proteoglycan deposition was Smad-dependent (Susarla et al., 2011). TGF-β mediated XXYLT-1 mRNA expression required both Smad2 and Smad3, however TGF-β mediated mRNA expression of CHSY1 was only dependent on Smad2 (Susarla et al., 2011). Thus showing that Smad2 and Smad3 play a distinct role in activation and transcription of genes. The structure of Smad2 and Smad3 containing residues which are phosphorylated and those that determine the properties and functions of the Smads are found in Figure 1.13.
Figure 1.13: Structure of human Smad2 and Smad3.

Amino acid sequence of part of the linker region of human Smad2 transcription factor containing the residues which can be phosphorylated in the Smad linker region. The sequence shows that three serine (S) residues and one threonine (T) residue which are available for phosphorylation. From Kamato et al. (Kamato et al., 2013c).
1.10.1 The canonical Smad signalling pathway

TGF-β signalling of Smads through TGFBR1 provides a rapid and specific signal transduction pathway. The R-Smads Smad2 and Smad3 are directly phosphorylated by TGFBR1 at the Ser-Ser-X-Ser (SSXS, X being any amino acid) motif in the C-terminal sequence. The three serine residues are located in positions 464, 465 and 467 of Smad2 and it has been shown that just two out of the three serine residues in the motif are phosphorylated (Ser465/467) and the other serine (Ser464) residue is not phosphorylated but is required for the phosphorylation of the other two sites (Abdollah et al., 1997).

Canonical TGF-β signalling refers to the receptor mediated carboxy terminal phosphorylation of R-Smads. A TGF-β ligand initiates signalling by binding to and bringing together type I and type II TGF-β receptors on the cell surface. This allows for TGFBR2 engagement leading to the phosphorylation of TGFBR1 kinase domain which then instigates the signalling through phosphorylation of the Smad proteins (Wang et al., 2009a). Phosphorylation of the C-terminal of Smad2 promotes the binding to Smad4 to form a heteromeric complex. The Smad complex enters and accumulates in the nucleus to regulate the transcription of numerous target genes (Figure 1.14) (Burch et al., 2011). TGF-β signalling is also known to activate a number of intracellular serine/threonine kinases in what is termed as non-Smad signalling (Derynck and Zhang, 2003; Zhang, 2009).

1.10.2 Smad linker region phosphorylation

In addition to the canonical TGF-β signalling involving C-terminal phosphorylation, experiments using linker region specific antibodies have shown that the linker region of Smad2/3 can also undergo phosphorylation and play an important role in TGF-β mediated biological activities (Hough et al.,
2012). This has led to the term Smad independent or non Smad-dependent cascades (Burch et al., 2011). The linker region of Smad2 has at least four important sites that can be phosphorylated. This includes threonine 220 and three serine residues in positions 245, 250 and 255 (Figure 1.13) (Wang et al., 2009a).

The linker region can undergo regulatory phosphorylation by multiple kinases, including mitogen-activated protein kinases (MAPKs) Erk, c-Jun N-terminal kinases (Jnk) and p38, PI3K, cyclin-dependent kinases (CDK), ROCK and glycogen synthase kinase (GSK3) (Kamato et al., 2013c). Among these kinases, MAPK and CDK are major groups of protein kinases showing preference for specific serine/threonine residues in the linker regions (Figure 1.14) (Malumbres and Barbacid, 2009, Feng et al., 2002). Other vasoactive agents can also activate MAPK potentially leading to linker region phosphorylation. Thus, linker region phosphorylation may be a central integrating point through which many pro-atherogenic agonists stimulate changes in proteoglycan synthesis and as such represent an attractive target for the development of a therapeutic target for atherosclerosis (Kamato et al., 2013c).

Previous work from our lab has shown that in VSMCs, regulation of proteoglycan synthesis and GAG hyperelongation was mediated by the phosphorylation of the Smad2 linker region (Burch et al., 2010). We know that thrombin treatment of VSMCs leads to proteoglycan synthesis and GAG hyperelongation, however the role of the Smad2 linker region has not yet been explored in this signalling pathway. This project will emphasize on the specific thrombin mediated signalling pathways that involve downstream Smad2 linker
region (Thr220, Ser245, 250 and 255) as well as the gene expression of specific GAG synthesizing genes for proteoglycan chain elongation in VSMCs.
Figure 1.14: TGF-β mediated signalling via the canonical and non-canonical pathway.

Smad2 carboxy terminus can be directly phosphorylated by activated TGF-β receptor. The Smad2 linker region is phosphorylated indirectly through the activation of serine/threonine kinases resulting in regulation of transcriptional activity.
1.11 Rationale for the study

Proteoglycans play a crucial role in atherogenesis through the entrapment of LDL as described by the response to retention hypothesis (Williams and Tabas, 1995, Skalen et al., 2002). Thrombin through its receptor, PAR-1, has been shown to transactivate both the EGFR and TGFBR1 which leads to an increase in GAG chain length of biglycan allowing for a binding affinity to LDL (Burch et al., 2010, Burch et al., 2013). GAG synthesizing genes are responsible for elongation of GAG chain on biglycan and in animal models their elevated expression level contributes to atherosclerotic development (Anggraeni et al., 2011). The regulation of GAG synthesizing genes in the development of atherosclerosis in humans has not been reported to date. The signalling pathway involving PAR-1 transactivation of the TGFBR1 and EGFR leading to the expression of these GAG synthesizing enzymes contributes to the identification of pathways which may be involved in atherosclerosis development. The ultimate aim is to find a universal target for the prevention of transactivation signalling which inhibits cellular responses such as proteoglycan synthesis which can act as a therapeutic target in the prevention of atherosclerosis caused by increased LDL binding to modified GAG chains.

1.12 Aims of the study

1. Identify specific GAG synthesizing gene expression that can be stimulated by thrombin treatment of VSMCs.
2. Characterise mechanisms of PAR-1 transactivation of PTKR and S/TKR that leads to the expression of GAG synthesizing genes.
3. Determine the contribution of G proteins in PAR-1 transactivation dependent signalling leading to GAG gene expression.

4. Investigate whether PAR-1 mediated transactivation of PTKR and S/TKR leads to Smad2 linker region phosphorylation.

5. Investigate which serine/threonine kinases are required for the regulation of PAR-1 mediated Smad2 linker region phosphorylation.

6. Determine the contribution of PAR-1 mediated transactivation of PTKR and S/TKR by studying the genes and pathways regulated by transactivation dependent signalling.
Chapter-2. Methods & Materials
2.1 Materials

Dulbecco’s Modified Eagle medium (DMEM) (0 and 25mM glucose), Trypsin-Versene, antibiotics (10,000 U/ml penicillin, 10,000 μg/ml streptomycin) were purchased from GIBCO, (Grand Island, USA). Foetal bovine serum (FBS) was from In Vitro Technologies, Pty. Ltd (VIC, Australia). RNase Away Reagent, chemiluminescent molecular weight marker (MagicMark XP) and chromogenic molecular weight marker (BenchMark) were obtained from Invitrogen Australia Pty Limited. Dulbecco’s phosphate buffered saline (PBS)(10X), SB431542, AG1478, GM6001, Hoechst, human epidermal growth factor (EGF), sodium dodecyl sulphate (SDS), diethylaminoethyl (DEAE)-Sephacel, chondroitin sulphate thrombin, Fura-2AM, Y217632, 2-mercaptopethanol, 2-propanol and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (MO,USA). Ethanol absolute was purchased from LabServ (VIC, Australia). Real-Time Polymerase chain reaction (RT-PCR) primers for GAG synthesizing enzymes Chondroitin 4-O-sulfotransferase-1 (CHST11), xylosyltransferase-1 (XXYLT-1), chondroitin polymerizing factor (CHPF) and Chondroitin sulphate synthase 1 (CHSY1) were based on their respective GenBank sequences were purchased from GeneWorks Pty Ltd (SA, Australia). Housekeeping gene 18S, biglycan gene primers, RNaseasy Mini Kit (containing sterile, RW1 buffer, RPE buffer, RPE buffer and RNase-free water), QuantiTect Reverse Transcription Kit (containing gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water), the RNase-Free DNase set (containing 1500kunits RNase-free DNase I, RNase free buffer RDD and RNase-free water), miRNeasy kit (containing RNaseasy mini spin columns, collection tubes, QIAzol lysis reagent, RNase-free reagents and buffers) and two-step RT-PCR kit (containing 2xRotor-Gene
SYBR Green PCR Master Mix and RNase-free water) were purchased from Qiagen (VIC, Australia). Human transforming growth factor beta-1, antibodies to, phospho-EGFR(Y845), phospho-Smad2(Ser465/467), phospho-Smad2(Ser245/250/255), phospho-Erk1/2(Thr202/Tyr204), phospho-Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558) and GAPDH were from Cell Signalling Technology (MA, USA). Carrier-free [³⁵S]-sulphate was obtained from MP Biomedicals (LA, USA). Cetyl pyridinium chloride (CPC) from Unilab Chemicals and Pharmaceuticals (India); Whatman 3MM chromatography paper from Biolab (Mulgrave, Australia); Instagel plus scintillation fluid from Perkin Elmer (MA, USA). PAR-1 inhibitor SCH79797 was from Invitro technologies (VIC, Australia). Chemiluminescence ECL detection kit was from GE Healthcare UK Ltd. (BKM, UK). Bicinchoninic acid (BCA) protein assay kit, SYBR® Gold Nucleic acid gel stain, goat anti-mouse IgG secondary antibody, Alexa Fluor® 488 and 594 were from Thermo scientific (IL, USA). Polyvinylidene fluoride (PVDF) membrane was from Millipore (MA, USA). TEMED, tris base and glycine were from Amresco (OH, USA). Tween 20, Poly-Prep columns, 30% acrylamide/Bis solution, Ammonium persulphate (APS), Trans-blot® Turbo™ RTA mini PVDF transfer kit (containing 40 mini-sized PVDF membranes, 80 transfer stacks, 1L 5×transfer buffer and 2 gel trays) and Quantity One imaging software were from BioRad Laboratories (CA,USA). Human coronary artery smooth muscle cells, AD1 4D-Nucleofector™ Y kit (including 24 well plate, electrode 24 well, buffer and vector), smooth muscle growth medium-2 bullet kit and Single Quot kit supplements and growth factors were from LONZA (VIC, Australia). SiGENOME Human GNA11 siRNA SMARTpool (target sequences: GCUUUGAGAAACGUGACAUC, GAGCAUCAGUACGUCAGUG,
GAACUCCUCGUCAUCCUC and CAAGAUCCUCUACAAGUAC). SiGENOME Human GNAQ siRNA SMARTpool (target sequences: CAAUAAGGCUCAUGCACAA, CAACAAGAUGUGCUUAGA, GCAAGGCUCUCUUAGAAC and UAGUAGCGCUUAGUGAAUA) and SiGENOME non targeting siRNA SMARTpool (target sequences: UAAGGCUAAGAGAGAAUAC, AUGUAUUGGCCUGUAAUAG, AUAACGUGAAUUUGCUCAA and UGGUUUACAGUCGACUAA) were purchased from Millennium Science (VIC, Australia). Mouse anti-human integrin αVβ3 (Vitronectin Receptor) monoclonal antibody and mouse anti-human integrin αVβ5 monoclonal antibody were purchased from MERCK Millipore (VIC, Australia). Dynabeads® mRNA DIRECT™ Micro kit (contains Dynabeads® Oligo, Lysis/binding buffer, Washing Buffer A, Washing buffer B and 10mM Tris HCl), Ion PI™ Template OT2 Supplies 200 kit v2 (contains Ion OneTouch™ Reagent Tubes, Ion OneTouch™ Recovery Routers, Ion PI™ Template OT2 Recovery tubes Ion OneTouch™ Sipper Tubes, Ion OneTouch™ 2 Amplification plates, Ion OneTouch™ ES supplies and Ion OneTouch™ 2 Cleaning adapters), Ion PI™ Template OT2 Reagents 200 Kit v2 (containing Ion PI™ Enzyme Mix TL, Ion PI™ Ion Sphere™ particles, Ion PI™ PCR Reagent X and Ion PI™ Reagent Mix TL), Ion PI™ Template OT2 Solutions 200 kit v2 (which contains Ion Proton™ OT2 oil, Ion Proton™ OT2 Reaction Oil, Ion PI™ PCR Reagent B, Nuclease free water, Ion PI™ OT2 Recovery solution, Ion OneTouch™ Wash solution, Ion PI™ MyOne™ Beads Capture solution and Ion PI™ ISP resuspension solution), Ion PI™ Sequencing Supplies 200 (contains Ion Proton™ Reagent tubes with labels, Ion Proton™ reagent tube caps, Ion Proton™ reagent tube sippers, Ion Proton™ wash 2 bottles with labels and Ion Proton™ wash bottle sippers), Ion PI™ Sequencing reagents 200
2.2 Methods:

2.2.1 Culturing human vascular smooth muscle cells

Primary cultures of human VSMCs were obtained from discarded sections of saphenous veins from patients undergoing coronary artery bypass grafting at the Alfred Hospital; the acquisition of the vessels was approved by the Alfred Hospital Ethics Committee. To isolate the VSMCs, the endothelial layer was removed and the vessel placed intima down under a glass cover-slip and cultured in 25mM DMEM containing 1% penicillin-streptomycin and 10% FBS (Neylon et al., 1990). The primary cultures were expanded in 5mM glucose DMEM (10% FBS and 1% penicillin-streptomycin), and stocks frozen and stored in liquid nitrogen (see Section 2.2.2). Human coronary artery smooth muscle cells (CASMCs) were purchased from Lonza, they were thawed out following the manufacturer’s instructions. Once adherent the CASMCs were cultured following the same protocols outlined.

Cells were grown and passaged from liquid nitrogen stocks for experiments between passages 5-17. Cells were grown in DMEM (5mM glucose, 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO₂) in 75cm²
Corning cell culture flasks with the medium replaced every 2-4 days until the cells reached approximately 90% confluency. To seed tissue culture dishes for experiments, cells were trypsinized with Trypsin-Versene and transferred into a Corning 50mL Falcon tube with approximately 25mL of DMEM. Cells were spun in an Eppendorf 5810R Centrifuge at room temperature for 3 min at 900 rpm. The supernatant was discarded and the pellet was resuspended in fresh DMEM. For experiments, the Z1 D/T Particle Counter (Beckman Coulter, NSW, Australia) was used to count cell densities. For each experiment cells were grown to confluency then rendered quiescent due to serum deprivation for 48 hours in DMEM (5mM glucose, 0.1% FBS and 1% penicillin-streptomycin) before treatment.

2.2.2 Freezing cells

Each freezing ampoule has a volume of 1.5mL to which 1mL of cell suspension was added. The “freezing medium” containing 1:10 ratio of DMSO to DMEM (5mM glucose 10%FBS) was prepared. Cells in 75cm² flasks were trypsinized, centrifuged at 900 rpm for 3 min and the total number of cells was calculated to determine amount of freeze medium needed (cells should be resuspended in freeze medium at 5×10⁶ to 2×10⁷ cells/ml). Cryogenic vials were labelled with date, name, and cell line type and passage number. The cells were then quickly resuspended in the freezing medium with 1mL of freeze suspension per cryogenic vial. Each vial was screwed tightly and placed in holders to store into −80° C overnight. Vials were then transferred liquid nitrogen for long term storage.
2.2.3 Fluorescent imaging of proteins

VSMCs were plated on 15mm glass coverslips, grown to near confluence and fixed in 2% paraformaldehyde in 1×PBS for 10 mins. The slides were then washed with 1×PBS for 5 mins with gentle rocking. To block non-specific binding of antibodies to cellular protein, the fixed cells were placed in a 10% horse serum solution for 45 mins at room temperature. The slides were then washed with 1×PBS and cells incubated with the primary antibody overnight at 4°C in a humified chamber (Table 1). A goat anti-mouse conjugated with fluorophore Alexa488 and Alexa594 was used as the secondary antibody. Coverslips were incubated with Hoechst stain (1:400) for 30 mins for the detection of the nucleus. Coverslips were then mounted on slides and were dried for 24 hours at 4°C. Imaging was with a Nixon C1 confocal microscope. The excitation wavelengths were 519 nm and 617nm.
Table 2.1: Primary antibodies used in fluorescent imaging of vascular smooth muscle cells.

The table details the antibodies, the dilution used and species they are derived from.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>1º Antibody</th>
<th>Dilution</th>
<th>2º Antibody</th>
<th>Dilution</th>
<th>Blocking Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actin</td>
<td>Mouse anti-alpha smooth muscle actin</td>
<td>1:100</td>
<td>Goat anti-mouse conjugated with flurophores Alexa594</td>
<td>1:500</td>
<td>10% horse serum</td>
</tr>
<tr>
<td>von Willebrand Factor</td>
<td>Mouse anti-Von Willebrand Factor</td>
<td>1:100</td>
<td>Goat anti-mouse conjugated with flurophores Alexa488</td>
<td>1:500</td>
<td>10% horse serum</td>
</tr>
<tr>
<td>αVβ3</td>
<td>Rabbit anti-αVβ3</td>
<td>1:500</td>
<td>Goat anti-mouse conjugated with flurophores Alexa594</td>
<td>1:500</td>
<td>10% horse serum</td>
</tr>
<tr>
<td>αVβ5</td>
<td>Rabbit anti-αVβ5</td>
<td>1:500</td>
<td>Goat anti-mouse conjugated with flurophores Alexa488</td>
<td>1:500</td>
<td>10% horse serum</td>
</tr>
</tbody>
</table>
2.2.4 Quantification of glycosaminoglycan biosynthetic enzyme gene expression

2.2.4.1 RNA Isolation

The level of the GAG synthesizing gene expression was measured by quantitative RT-PCR. Total RNA was isolated from treated cells grown to confluency in 6 well plates, and harvested in accordance with the column base method provided in the RNeasy mini kit (Qiagen). The addition of 2-mercaptoethanol (10μl to per 1mL of Buffer RLT) in 1.5mL microfuge tubes was performed in the fume hood. The medium was aspirated and cells were lysed directly with Buffer RLT (350μL) and 2-mercaptoethanol. Cells were gently scraped and collected in 1.5mL microfuge tubes. Cell samples were homogenised 10 times through a 20-gauge needle before the addition of 1 volume of 70% ethanol. Samples (700μL) were transferred to an RNeasy spin column, and centrifuged for 15s at 10,000 rpm at room temperature. The flow through, which contains cell debris, was discarded and 700μl of Buffer RW1 (to wash the spin column membrane) was added to the RNeasy spin column. Samples were centrifuged for 15 secs at 10,000 rpm at room temperature. The flow through was discarded and Buffer RPE (500μL) (to wash the spin column membrane) was added to the RNeasy spin column and samples were centrifuged for 15 secs at 10,000 rpm at room temperature. The flow through was discarded and 500μL of Buffer RPE (to wash the spin column membrane) was added to the RNeasy spin column were samples were centrifuged for 2 mins at 10,000 rpm at room temperature. The spin column membrane was placed in a new 1.5mL collection tube were 35μL of RNase-free water was added directly. Samples were centrifuged for 1 min at 10,000rpm at room temperature. The purity and concentration of total RNA extracted was measured by
spectrophotometry using a Nanodrop2000 spectrophotometer (Thermo Fisher Scientific). RNA samples were determined to be pure based on the 260/280nm wavelength ratios. All samples had pure nucleic acids yield ratio values that were between 1.8-2.1.

2.2.4.2 cDNA synthesis
First strand cDNA was synthesized from 500ng RNA by following the protocol provided by the Quantititect reverse transcriptase kit (Qiagen). This kit provides is a two-step process which comprises the elimination of genomic DNA (gDNA) and reverse transcription. The procedure for effective gDNA elimination was performed by incubating purified RNA samples with 2 μL of gDNA wipeout buffer for 2 mins at 42°C. DNase treated samples were then immediately kept on ice while reverse transcription master mix was prepared containing 1μL QuantiTect Reverse Transcriptase, 4μL QuantiScript RT buffer, and 1μL RT primer mix. DNase treated template RNA were added to reverse transcription master mix and incubated for 15 mins at 42°C. After completed cDNA synthesis, the samples were then incubated for 3 min at 95°C to inactivate the reverse transcription process by QuantiTect Reverse Transcriptase.

2.2.4.3 Quantitative real-time PCR
Quantitative real-time PCR was performed using a Rotor-Gene Q (Qiagen) and Quanti Fast SYBR Green PCR Master Mix kit (Qiagen). RT-PCR used commercial primers (Qiagen) and human specific primers based on GenBank database sequences. The data was normalised to 18S house-keeping gene (Qiagen). Each treatment sample was processed in duplicate. Each reaction tube contained 5μL of SYBR Green, 1μL of 10μM mix of forward and reverse
primers, 1µL of 500ng cDNA from Section 2.2.3.2 and 2 µL of RNA free water. The reactions were run in a Roto-Gene Q using the protocol outlined in Table 2. The Delta-Delta (ΔΔ) Ct method was used to analyse the fold change in gene expression from quantitative real-time PCR experiments.
Table 2.2: Cycling Conditions for quantitative real time PCR reactions

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR initial activation step (Hold)</td>
<td>5 min</td>
<td>95°C</td>
</tr>
<tr>
<td>Two-step cycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturing</td>
<td>15 sec</td>
<td>95°C</td>
</tr>
<tr>
<td>Combined/annealing/extension</td>
<td>30 sec</td>
<td>60°C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>
2.2.4.4 Quantitation using Comparative cycle threshold

Cycle threshold (Ct) values for all GAG enzyme gene expression samples were recorded and normalised against the endogenous ribosomal 18S as housekeeping gene. The formulas below were used to quantitate the results:

\[ \Delta Ct = Ct \text{ (gene interest)} - Ct \text{ (housekeeping gene)} \]

\[ \Delta\Delta Ct = \Delta Ct \text{ (treatment)} - \Delta Ct \text{ (control)} \]

Fold regulation = \(2^{\Delta\Delta Ct}\)

2.2.5 Proteoglycans

2.2.5.1 Culture of cells for proteoglycan experiments

VSMC were seeded on 24 well plates at a density of 50,000 cells per well. The media (DMEM with 5mM glucose, 10% FBS and 1% antibiotics) was replenished the next day and the cells were grown to approximately 90% confluency. Cells were allowed to quiesce in DMEM (5mM glucose 0.1%FBS and 1% antibiotics) for 48 hours. Quiescent cells were changed to fresh medium containing 25µC/mL of \[^{35}\text{S}\]-sulphate in the presence and absence of antagonists or growth factors as indicated in text for 24 hours.

2.2.5.2 Harvesting proteoglycans

VSMCs secreted proteoglycans were harvested by collecting the culture media and adding protease inhibitors (100mM 6-aminocaproic acid, 5mM benzamidine hydrochloride) to prevent degradation.

2.2.5.3 Quantitation of proteoglycans by cetylpyridinium chloride precipitation

To assess the quantity of radiolabel incorporation into proteoglycans a CPC precipitation assay was used.Duplicate aliquots of (50 µL) of harvest
media were spotted onto Whatman 3MM chromatography paper and air dried. The papers were then washed (5×30 mins) in CPC (1% CPC, 0.05M NaCl) to remove any unbound radiolabel [\(^{35}\)S]-sulphate and precipitate proteoglycans. This solution forms insoluble proteoglycan aggregates, precipitating them on to the paper, so that the unincorporated radiolabel is washed away. After air drying, papers were cut, scintillation cocktail was added and the papers were analysed for radioactivity [\(^{35}\)S]-sulphate using a liquid scintillation counter.

2.2.5.4 Isolation of proteoglycans for SDS-PAGE

Ion-exchange chromatography was used for the isolation of proteoglycans that were secreted into the culture medium. Harvested media containing secreted proteoglycans were applied to Poly-Prep columns containing 50%v/v DEAE-Sephacel (500µL) equilibrated in low salt buffer (8M urea, 0.25M NaCl, 2mM disodium EDTA, 0.5% Triton X-100). Samples were washed with low salt buffer (5×6mL) to remove any unbound radiolabel. Bound proteoglycans were eluted (2×0.3mL fractions per column) using high salt buffer (8M urea, 3M NaCl, 0.02M EDTA, Triton-X). An aliquot of (10µL) of each fraction was counted on a liquid scintillation counter and fractions containing the highest number of [\(^{35}\)S]-sulphate counts per million (cpm) were pooled.

2.2.5.5 Concentration of isolated proteoglycans

Equal counts of proteoglycans (20,000 cpm) were precipitated by ethanol solution (1.3% potassium acetate in 95% ethanol). Chondroitin sulphate (166µg/mL) was added to each sample to facilitate as a “cold carrier”, minimising sample loss through adhesion to the tube. Samples were precipitated overnight, then centrifuged at 14000 rpm for 10 mins at 4°C to obtain a pellet.
The supernatant was removed, the pellet re-suspended in water and re-precipitated in ethanol solution and centrifuged again. This procedure was repeated 1-2 times. The pellets of proteoglycans were air dried and resuspended in buffer (8M urea, 2mM disodium EDTA, pH7.5).

2.2.5.6 Assessing proteoglycan size by SDS-PAGE
The Laemmli system was used (Laemmli, 1970) to assess the electrophoretic mobility of the proteoglycans and glycosaminoglycan chains. A 3% stacking gel and a 4-13% gradient separating gel was used. Sample buffer (0.5M Tris-HCL pH 6.8, 10% SDS, 50% glycerol, 2-mercaptoethanol, and 0.1% bromophenol blue) was added to the samples prior to incubation at 95°C for 5 mins to denature the proteins. A molecular weight marker Rainbow™ [14C] was run simultaneously to indicate the relative molecular size of the proteoglycans. Gels were run overnight at 50V, then fixed (5% methanol, 7% acetic acid) for 1 hour, soaked in 20g/l polyethylene glycol, 12.5% methanol for 4 hours before being wrapped in cellophane and air dried at room temperature overnight. After air drying the gel was exposed to an imaging plate (Fujifilm BAS-MS 2040 imaging plate) for 3-5 days. Images were then developed using a phosphoimager (Perkin Elmer Cyclone Plus Storage Phosphor System) and viewed using imaging software (OptiQuant).

2.2.6 Western Blot analysis
As stated in Section 2.2.1, cells were cultured and treated with various inhibitors and agonists. Cells were then gently washed with cold 1×PBS and harvested by adding 55μL of lysis buffer (0.01% TritonX100, 150mM NaCl, 50mM Tris pH 7.5, 1mM EDTA, 1μM EGTA pH 9.0, 0.05% NP-40, 80μM PMSF, 100mM 6-amino caproic acid, 5mM benzamidine and 100mM sodium
orthovandate), followed by gently scraping and collecting the cells. The lysates were homogenised by 20 passes through a 19-gauge needle, centrifuged (1000rpm for 10 min at 4°C) the supernatant was collected and the protein content was determined using the BCA protein assay. Proteins (30-40μg) were separated by 10% SDS-PAGE and a 4% stacking gel for size comparison a chemiluminescent molecular weight maker as well as a chromogenic molecular weight marker were electrophoresed. Proteins were then transferred onto PVDF membrane.

2.2.6.1 Wet Transfer

PVDF membranes were activated by soaking in 100% methanol for 1 min. The membrane was then rinsed with water and soaked in transfer buffer (3.7% SDS, 20% methanol, 48mM Tris base, 39mM Glycine) together with filter paper and sponges at 4°C. The gel was gently removed from the glass plates and the cassette was assembled in the following order; clear plastic, sponge, filter paper, membrane, gel, filter paper, sponge and the black plastic. The transfer was set to 100V for 1 hour at 4°C.

2.2.6.2 Semi dry transfer (Turbo blot)

PVDF membranes were activated by immersing in 100% ethanol until membrane is translucent then immediately transferred into 1×transfer buffer (BIO-RAD). Transfer stacks were equilibrated with 1×transfer buffer. One wetted stack was then placed on the cassette serving as the bottom ion reservoir stack. The wetted membrane was then placed on top of the stack in the cassette. The gel was placed on top of the PVDF membrane. Bubbles were carefully removed using a roller this followed by the second wetted transfer stack which served as the top ion reservoir stack. The assembled sandwich was rolled on
with blot roller to expel trapped air bubbles. The cassette lid was closed and locked and inserted into the Trans-Blot Turbo Transfer system (BIO-RAD) to begin transferring. The protocol was set for mixed molecular weight.

### 2.2.7.3 Blocking and probing the membrane

Membranes were blocked with 5% bovine serum albumin (BSA) to limit non-specific binding of antibodies for 60 mins at room temperature with gentle rocking. The membrane underwent 3×5min washes with Tris-buffered saline with Tween-20 (TBST) (10mM Tris base, 0.15M NaCl, 1% Tween-20, pH7.6). The primary antibody was diluted (as detailed in text) in 5% BSA. Membranes were incubated overnight in primary antibody at 4°C with gentle rocking. Membranes were then washed for 3×5 min at room temperature with TBST and incubated with species specific secondary antibody diluted (as detailed in text) in 5% BSA for 1 hour at room temperature with gentle rocking. Membranes were washed again for 3×5 min at room temperature with TBST. The proteins were detected by chemiluminescence (ECL™ Western Blotting analysis system, Amersham™) using the BIO-RAD gel documentation system and then processed using ImageLab XRS® imaging software.

### 2.2.6.4 Image densitometry

Western blot images were opened in ImageLab XRS software and images rotated to align the bands for the protein of interest horizontally in the window. The rectangle tool was then used to select the band of interest. The width and height of the lane rectangle was drawn accurately to cover the band of interest without overlap with bands from adjacent lanes. The same rectangle size box was then copied and used to measure the background in the respective lane. The amount of background for each band was subtracted from the band of
interest. The density of the loading control (GAPDH) was measured using the same protocol described. The final intensity of the protein of interest was obtained after normalizing the measurement of the protein of interest with the respective gel loading protein.

2.2.7 Calcium Assay

VSMCs were seeded onto 96-well plates (15,000 cells/well) and cultured until 80% confluent and then serum deprived (5mM DMEM 0.1% FBS) for 48 hours. Cells were loaded with FURA-2AM ester (2µM, 30 min, 37°C) in the presence of 2µM probenecid and 0.01% pluronic F-127. Loading and experiments were performed in HANKS buffer (10mM HEPES, 5mM KCl, 140mM NaCl, 1mM MgCl₂, 2mM CaCl₂, 11mM Glucose). Cells were pre-incubated with antagonists for 15-30 min prior to stimulation with agonist. Fluorescence was measured (4s intervals) at 340nm and 380nm excitation and 510nm emission wavelengths using a Flexstation 3 plate reader (Molecular Devices, Sunnyvale, CA). The sustained calcium response was calculated as the difference between basal fluorescence and the peak fluorescence after thrombin treatment.

2.2.8 Next generation sequencing

2.2.8.1 RNA extraction of miRNA

CASMCs were seeded on 60 mm dishes at a density of 200,000 cells/plate with 3 plates being used per treatment. The media (DMEM with 5mM glucose, 10%FBS and 1% antibiotics) was replenished the next day and cells were grown to approximately 90% confluency. Cells were then made
quiescent in DMEM (5mM glucose, 0.1% FBS and 1% antibiotics) for 48 hours. VSMCs were pre-incubated with antagonists (SB431542, AG1478 or both for 30 mins prior to exposure with thrombin for 6 hours. Total RNA was isolated from treated VSMCs using the miRNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. The medium was aspirated and cells were lysed directly with QIAzol lysis reagent (350 µL/plate). Cells were gently scraped and plates from the same treatment were collected into a 1.5 mL microfuge tube. Samples were homogenised 10 times through a 19-gauge needle. The tubes containing the homogenate were incubated at room temperature for 5 mins. 140µL of chloroform was added to each tube and shook vigorously for 15 secs prior to 3 min incubation at room temperature. The samples were centrifuged for 15 mins at 10,000 rpm at 4°C. The upper aqueous phase (colourless) was transferred into a new microfuge tube and 525 µL of 100% ethanol was added and mixed by pipetting up and down. 700 µL of the sample was transferred to an RNeasy mini spin column and centrifuged for 15 secs at 10,000 rpm at room temperature, the flow-through was discarded and this was repeated with the remainder of the sample. Using the RNase-Free DNase Set (Qiagen) a DNase digest was conducted according to the manufacturer’s instructions. 350 µL of the RWT buffer was added to the RNeasy spin column and centrifuged for 15 secs at 10,000 rpm; the flow-through was discarded. For each sample 10 µL of DNase I stock solution and 70 µL of RDD buffer were gently mixed and added directly onto the RNeasy spin column membrane and incubated at room temperature for 15 mins. Buffer RWT (350 µL) was added to the RNeasy spin column where samples were centrifuged for 15 secs at 10,000 rpm at room temperature. The flow through was discarded and 500 µL of Buffer RPE was added to the RNeasy spin column and samples were centrifuged for 15 secs at
10,000 rpm at room temperature. The flow through was discarded and another 500 µL of Buffer RPE was added to the spin column and samples were centrifuged for 2 mins at 10,000 rpm at room temperature. The spin column was placed in a new 2mL collection tube and centrifuged for 1 min at full speed. The spin column was then transferred into a new 1.5 mL microfuge collection tube and 20µL of RNase-free water was added directly onto the spin column membrane. Samples were centrifuged for 1 min at 10,000 rpm at room temperature. RNA extracted was immediately placed on ice prior to the measurement of RNA quantity and integrity using a Nanodrop2000 spectrophotometer (260/280nm).

2.2.8.2 Purification and isolation of mRNA transcriptome using Poly-A enrichment

2.2.8.2.1 Preparation of Dynabeads®

200µL of Dynabeads were added to each microfuge tube. Tubes were placed on the DynaMag™-2 for 30 secs until the Dynabeads migrated to the tube wall. Supernatant was discarded and the tube removed from the magnet. 100µL of Binding buffer was added to calibrate the beads. Supernatant was discarded and 200µL of binding buffer was added to the Dynabeads.

2.2.8.2.2 Isolation of mRNA

RNA was heated to 65°C for 2 mins to disrupt the secondary structure then immediately placed on ice. 25µL of prepared Dynabeads/binding buffer suspension (from Section 2.2.8.2.1) and 25µL of RNA were gently mixed. The suspension was then placed on a mixer for 5 mins at room temperature to allow the mRNA to anneal to the oligo on the beads. The suspension was placed on
the magnet until the solution cleared; the supernatant was then discarded. Tube was removed from the magnet and the mRNA bead complex was washed twice with 60μL washing buffer B. The supernatant was removed between each washing step. The heat elution buffer was warmed up to 80°C for 2 mins. The dried beads were resuspended with 13μL of elution buffer by gentle mixing. The bead complex was placed on the magnet, the eluent was collected in a new RNase-free tube.

### 2.2.8.3 Preparation of whole transcriptome libraries

#### 2.2.8.3.1 Fragment the RNA using RNase III

A reaction containing (8-10μL of 100ng PolyA RNA, 1μL of 10V RNase III reaction buffer, 1μL RNase III) was prepared on ice. The reaction was briefly centrifuged to collect the liquid in the bottom of the tube. The reaction was incubated in a thermocycler for 2 mins at 37°C. 20μL of chilled nuclease free water was mixed with the fragmented RNA and was then kept on ice.

#### 2.2.8.3.2 Purify the fragmented RNA

Nucleic Acid Binding beads were gently vortexed to completely resuspend the magnetic beads, 5μL of resuspended beads was added to each tube with 90μL of binding solution concentrate. 30μL of fragmented RNA was transferred to bead-containing tube. Followed by 150μL of 100% ethanol to each tube. The suspension in each tube was thoroughly mixed by pipetting the suspension up and down 10 times. The samples were incubated at room temperature for 5 mins. Each tube was placed on the magnetic stand for 5-6 mins to separate the beads from the solution. Once the solution had cleared the supernatant was discarded. The tubes were kept on the magnetic stand, and
washed with 200µL of wash solution concentrate. The samples were incubated at room temperature for 30 secs, the supernatant and any residual liquid was discarded. The beads were air dried for 1-2 mins. Tubes were removed from the magnetic stand and mixed with 12µL of pre-warmed (37°C) nuclease free water. The samples were incubated at room temperature for 1 min then placed on magnetic stand for 1 min. The eluent for each of the samples was collected. The Qubit RNA Assay kit was used to assess the yield of the fragmented RNA.

**2.2.8.3.3 Hybridization and ligation of the RNA**

To hybridize and ligate the RNA 5µL of the hybridization master mix (2µL of Ion Adapto Mix v2, 3µL of Hybridization solution), was used per sample to 70ng of fragmented RNA (from Section 2.2.8.3.2). The sample containing the fragmented RNA was mixed gently, followed by brief centrifuging to collect reaction. The hybridization reaction was placed in thermal cycler (65°C for 10 mins, 30°C for 5 mins). On ice, 10µL of 2X ligation buffer and 2µL of ligation enzyme mix were added to each of the hybridization reactions. The ligation reactions were incubated in a thermal cycler at 30°C for 1 hour.

**2.2.8.3.4 Reverse transcription**

Reverse transcription master mix was prepared on ice for each sample (2µL of nuclease free water, 4µL 10X RT buffer, 2µL 2.5mM dNTP mix and 8µL of Ion RT Primer v2). 16µL of reverse transcription master mix was resuspended with the ligated RNA sample, the reaction was gently vortexed then briefly centrifuged. The reaction was incubated in a thermal cycler with a heated lid at 70°C for 10 mins, then snapped-cool on ice. 4uL of 10X SuperScript III
Enzyme mix was added to each ligated RNA sample, gently vortexed, then briefly centrifuged. The ligated RNA sample was incubated in a thermal cycler with a heated lid at 42°C for 30 mins.

2.2.8.3.5 Purification of the cDNA

To purify the cDNA Nucleic Acid Binding beads were gently vortexed to resuspend the beads. Each sample was designated a tube containing 5µL of beads and 120µL of binding solution concentrate. The reverse transcription reaction (from Section 2.2.8.3.4) was made up to 100µL by adding nuclease-free water then transferred to a bead containing tube. 125µL of 100% ethanol was added to each tube then mixed thoroughly by pipetting the suspension up and down. The samples were incubated for 5 mins at room temperature. The tubes containing the reactions were placed on the magnetic stand for 5-6 mins to separate the beads from the solution. Once the solution was clear, the supernatant was discarded. The tubes were left on the magnetic stand and washed with 200µL of wash solution concentrate. The samples were incubated at room temperature for 30 secs the supernatant and any residual liquid was then discarded. The beads were air dried for 1-2 mins. The tubes were removed from the magnetic stand and mixed with 12µL of pre-warmed (37°C) nuclease free water. The samples were incubated at room temperature for 1 min then placed on the magnetic stand for 1 min to separate the beads from the solution; the eluent was collected for each sample.

2.2.8.3.6 Amplification of the cDNA

For each cDNA sample a PCR reaction (45µL Platinum PCR SuperMix High Fidelity, 1µL Ion Xpress RNA 3’ Barcode primer) was prepared. 6µL of
cDNA sample was transferred to a new PCR tube, and mixed with 46µL of the PCR mix. 1µL of the selected Ion Xpress RNA-Seq Barcode primer was added to each PCR tube. The reaction was mixed well by gentle up and down pipetting, then briefly centrifuged. The PCR reaction was placed in a thermal cycler with the following conditions: Hold (94ºC for 2 mins), Cycle 2×(94ºC 30 secs, 50ºC 30 secs, 68ºC 30 secs), Cycle 16×(94ºC 30 secs, 62ºC 30 secs, 68ºC 3 secs), Hold (68ºC for 5 mins).

2.2.8.3.7 Purify the amplified cDNA

The Nucleic Acid Binding beads were gently vortexed to resuspend the magnetic beads. 5µL of beads was added to each tube and mixed with 180µL of binding solution concentrate. 53µL of cDNA was transferred to a bead containing tube. 130µL of 100% ethanol was added to each tube. The suspension was thoroughly mixed by pipetting up and down. The samples were incubated for 5 mins at room temperature. The tubes containing the cDNA were placed on the magnetic stand for 5-6 mins to separate the beads from the solution. Once the solution was clear, the supernatant was discarded. The tubes were left on the magnetic stand and washed with 200µL of wash solution concentrate. The sample was incubated at room temperature for 30 secs supernatant and any residual liquid was then discarded. The beads were air dried for 1-2 mins. Tubes containing the beads were removed from the magnetic stand, and mixed with 15µL of pre-warmed (37ºC) nuclease free water. The cDNA was incubated at room temperature for 1 min then placed on the magnetic stand for 1 min to separate the beads from the solution. The eluent for each sample was collected.
2.2.8.4 Template preparation

2.2.8.4.1 Preparation of template positive particles

The concentration of the RNA library from Section 2.2.8.3.7 was measured using a Bioanalyzer. The libraries were diluted to 1nM. In a 2.5mL Eppendorf LoBind tube the following solutions (160µL nuclease free water, 1200µL Ion PI reagent mix TL, 720µL Ion PI PCR reagent B, 120µL Ion PI Enzyme mix TL, 100µL Ion PI Ion Sphere Particles and 100µL diluted libraries) were mixed to make the amplification solution. The amplification solution was slowly pipetted through the sample port of the Ion PI plus reaction filter assembly by adding 800µL at a time. The Ion PI plus reaction filter was inserted into the Ion OneTouch 2 Instrument and the instrument ran overnight. The screen prompts were followed to centrifuge the sample for a final spin. All but 100µL of Ion PI OT2 recovery solution was removed from each of the Ion PI Template OT2 recovery tubes. The template-positive Ion Pi Ion Sphere particles in the remaining recovery solution in each tube were resuspended by pipetting the pellet up and down until the pellet was in solution. The suspension from each of the recovery tubes was transferred into a new Eppendorf LoBind tube. Each recovery tube was washed with 200µL of nuclease-free water two times to recover the residual beads, transferring the suspension into the same tube. The pellet was vortexed for 30 secs, then centrifuged for 8 mins at 15,500xg. All but 20µL of supernatant was removed, 80µL of Ion Pi ISP resuspension solution was then added and vortexed for 30 secs.

2.2.8.4.2 Enrichment of the template-positive particles

The Dynabeads MyOne Streptavidin C1 Beads were vortexed for 30 secs to resuspend the beads. 100 µL of the beads was placed in a new 1.5 mL
Eppendorf LoBind Tube and placed on the DynaMag magnet for 2 mins. The supernatant was removed and discarded without disturbing the pellet of beads. The beads were washed with 1 mL of Ion OneTouch wash solution and vortexed for 30 secs before placing on the magnet. The supernatant, was discarded and 130µL of Ion PI MyOne beads capture solution was added. The 8-well strip was filled according to Table 2.3. A new tip and an opened PCR tube were placed onto the Ion One Touch ES instrument. The 8-well strip was inserted and the program was left to run.

Table 2.3: Volume and description of reagents to be placed in the appropriate well of the 8-well strip.

<table>
<thead>
<tr>
<th>Well</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add 100µL of the ISP sample from previous step</td>
</tr>
<tr>
<td>2</td>
<td>130µL of Dyna beads from Section 2.2.8.4.2</td>
</tr>
<tr>
<td>3</td>
<td>300µL of OneTouch Wash solution</td>
</tr>
<tr>
<td>4</td>
<td>300µL of OneTouch Wash solution</td>
</tr>
<tr>
<td>5</td>
<td>300µL of OneTouch Wash solution</td>
</tr>
<tr>
<td>6</td>
<td>Empty</td>
</tr>
<tr>
<td>7</td>
<td>300µL of freshly prepared Melt off solution</td>
</tr>
<tr>
<td></td>
<td>(280µL of Tween solution and 40µL of 1M NaOH)</td>
</tr>
<tr>
<td>8</td>
<td>Empty</td>
</tr>
</tbody>
</table>
2.2.8.5 Sequencing

2.2.8.5.1 Chip preparation

Ion Proton chip adaptor was attached to the chip exit well. 200µL of 100% isopropanol was injected into the chip loading port; the expelled liquid was removed from the exit well. The isopropanol was aspirated from the chip for 5-10 secs to ensure that the chip is dry. 100µL of Ion PI chip preparation solution was injected into the chip loading port; the expelled liquid was removed from the exit well. The chip was placed on a 50°C heat block for 2 mins. 200µL of 100% isopropanol was injected into the chip loading port; the expelled liquid was removed from the exit well. This was repeated a total of two times. 200µL of nuclease free water was injected into the chip loading port; and the expelled liquid removed. 200µL of 0.1M NaOH was then injected into the chip loading port, and the expelled liquid was removed from the exit well. The chip was incubated at room temperature for 1 min then 200µL of nuclease free water was injected into the chip loading port. These steps were repeated a total of three times. 200µL of 100% isopropanol was injected into the chip loading port; the expelled liquid was removed from the exit well. 100µL of 100% isopropanol was pipetted onto the chip loading well, the liquid was then removed from the same well. The Ion Proton chip adapter was removed from the chip.

2.2.8.5.2 Loading of sample on the chip

To load the sample onto the chip, 55µL of the enriched template (from Section 2.2.8.4.2) was loaded onto the chip loading well. The chip was centrifuged for 10 mins using the Ion Chip Minifuge. 100µL of foam solution (50% Annealing buffer mixed with Triton X) was injected onto the loading well. The expelled liquid was removed from the opposite port. The chip was
centrifuged for 30 secs in the Ion Chip Minifuge followed by injection of 100µL of foaming solution and then centrifuged again for 30 secs. The chip was injected with 100µL of the flushing solution 2 times; the expelled solution was discarded after each injection. 100µL of 50% annealing buffer was injected into the chip loading port three times; the expelled liquid was removed between each injection. 65µL of polymerase solution (6µL Ion PI Sequencing polymerase with 60µL 50% annealing buffer) was injected; the expelled liquid was removed. The chip was left to incubate for 5 mins then secured in the Ion Proton sequencer.

2.2.9 Nucleofection using siRNA

CASMCS were seeded at 100,000 cells/well (5mM DMEM, 10% FBS, no antibiotics) in a 24 well plate (Nunc). The cells were incubated at 37°C in 5% CO₂ until they reached 70-80% confluency then serum deprived (5mM DMEM, 0.1% FBS, no antibiotics) for 24 hours prior to Nucleofection. Using the Lonza AD1 4D-Nucleofector Y kit, 1mL of the supplement was mixed with 4.5mL of AD1 4D-Nucleofector solution. 105pmol of siRNA was made up with 350µL Nucleofector solution. The media was carefully removed and 350µL of the siRNA substrate solution was carefully transferred into each well. The 24 well dripping electrode array was inserted into the 24 well plate. The Nucleofection process began when the plate was placed in 4D-Nucleofector core Y-unit using ER137 pulse. The 24 well dipping electrode array was carefully removed from 24 well plate. The siRNA substrate solution was removed from each well and immediately replaced with 1mL of pre-warmed medium (5mM DMEM, 0.1%FBS, no antibiotics). The pulsed cells were incubated for 36 hours (unless
stated otherwise) before they were treated with agonists to assess mRNA expression.

2.2.10 Statistical Analysis

Data was normalised and is shown as the mean ± standard error of the mean of three independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by least significant difference post-hoc analysis. Results were considered significant when the probability was less than 0.05 and 0.01.
Chapter-3. Proteinase activated receptor-1 mediated dual kinase receptor transactivation stimulates the expression of glycosaminoglycan synthesizing genes
3.1 Introduction

Seven transmembrane GPCRs are the largest group of cell surface receptors in biology. GPCR signalling involves the traditional cascade which will be referred to as transactivation-independent signalling (Kamato et al., 2014b) or GPCR mediated transactivation of cell surface PTKR. Two decades ago the initial finding of transactivation described GPCR activation of the PTKR, EGFR, stimulating the immediate downstream products phospho-Erk1/2 (Daub et al., 1996). This discovery greatly expanded the known functions of GPCRs.

The paradigm of GPCR mediated kinase receptor transactivation has been expanded to include GPCR mediated activation of S/TKR, in particular the TGFBR1 (Burch et al., 2010, Burch et al., 2013, Little et al., 2010, Ivey and Little, 2008). PAR-1 transactivation of the TGFBR1 is studied by the phosphorylation of the immediate downstream intermediate of TGFBR1, Smad2 in the carboxy terminal (phospho-Smad2C) (Burch et al., 2010, Little et al., 2010). Thrombin stimulation of human VSMCs leads to the synthesis of the ECM molecules proteoglycans. Recently our lab reported that thrombin treatment of VSMCs leads to an increase in radio sulphate incorporation into secreted proteoglycans a response which is approximately equally blocked by antagonists of the EGFR (AG1478) and the TGFBR1 (SB431542). The two inhibitors together completely block the response, this shows that all PAR-1 mediated proteoglycan synthesis signalling is via the transactivation of these two receptors (Burch et al., 2013). The involvement of the two kinase receptor signalling pathways are supported by the finding that thrombin mediated an increase in phospho-Erk1/2 and phospho-Smad2C both these responses are
blocked by the PAR-1 antagonist SCH79797 (Burch et al., 2010, Burch et al., 2013).

Previous work has shed light on the specific enzymes which mediate the synthesis of CS/DS GAG chains on proteoglycans (Sugahara and Kitagawa, 2000, Silbert and Sugumaran, 2002). The synthesis of CS/DS GAG chains on proteoglycans involves the concerted action of a number of GAG synthesizing genes (Mikami and Kitagawa, 2013). The growth factor and hormone signalling pathways that control the expression of these enzymes are important because GAG hyperelongation is a critical step in the early development of atherosclerosis (Ivey et al., 2008, Getachew et al., 2010a, Ivey and Little, 2008). During the progression of atherosclerosis, the elongation of CS chains in the arterial wall involves at least four GAG synthesizing genes specifically CHST11, CHSY1, CHPF, and chondroitin N-acetylgalactosaminoglycan-2 (Anggraeni et al., 2011) (Refer to Chapter 1 Section 1.3 for more details). Increased LDL binding in the 8 weeks of the development of atherosclerosis was associated with an elevated mRNA expression of the four genes. CHST11 and CHST3 are sulfotransferases whereas CHPF and CHSY1 are glycosyltransferases which are directly involved in the synthesis of the GAG chains thus the regulation of proteoglycan size (Izumikawa et al., 2011). As such, the signalling pathways leading to the expression of these GAG synthesizing enzymes represent potential therapeutic targets for the treatment of atherosclerosis.

Proteoglycans secreted by VSMCs in vivo are present in early atherosclerotic lesions (Nakashima et al., 2007). Thrombin treatment of VSMCs leads to the hyperelongation of GAG chains of proteoglycans such as biglycan (Ivey and Little, 2008). The involvement of transactivation pathways has been
described to be involved in this signalling cascade, however the downstream gene targets presumably the specific GAG synthesizing enzymes that are involved remain unknown. This chapter seeks explores the role of transactivation dependent signalling pathways that mediated GAG gene expression.

3.2 Materials and Methods

3.2.1 Materials

RT-PCR primers for GAG synthesizing enzymes CHST11, CHSY1, XXYLT-1, CHPF, CHST3 and biglycan primers were synthesized based on their respective GenBank sequences (see Table 3.1 for sequences) and were purchased from GeneWorks Pty Ltd (SA, Australia). Antibodies to phospho-Smad2(Ser245/250/255), phospho-Smad2(Ser465/467), GAPDH, phospho-EGFR(Tyr2068) and phospho-Erk1/2(Thr202/Tyr204) were from Cell Signalling Technology (MA, USA).

3.2.2 Cell Culture

Cells were grown in DMEM (5mM glucose, 10% FBS and 1% antibiotics at 37°C in 5% CO₂) VSMCs were seeded in 60 mm dishes and 6-well plates (described in more detail Chapter 2 Section 2.1). Cells were grown to confluence then rendered quiescent by serum deprivation for 48 hours. Inhibitors were pre-incubated for 30 mins prior treatment with agonists. The incubation and concentrations used are given in detail in the figure legends.
Table 3.1: Target gene and primer sequence

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Real-time PCR primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXYLT-1</td>
<td>Forward: GTGGATCCCGTCAATGTCATC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGTGTGAATTCCGCGGTGG</td>
</tr>
<tr>
<td>CHSY1</td>
<td>Forward: CCCGCCAGAAGAAGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCTCATAAAACCATTACTACTTGCCAA</td>
</tr>
<tr>
<td>CHPF</td>
<td>Forward: AGATCCAGGAGTTACAGTGAGGAGAT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCGGGCGGGATGGT</td>
</tr>
<tr>
<td>CHST11</td>
<td>Forward: GGCCCTGCAGCAGAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGGTGTGTGGTGCGATGAG</td>
</tr>
<tr>
<td>CHST3</td>
<td>Forward: GGATTCCACCTTTTTCCATCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGCCCTGCTGGTTGAAGAAC</td>
</tr>
<tr>
<td>Biglycan</td>
<td>Forward: CTCAACTACCTGCGCATCTCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GATGGGCCTGGATTTTGTTGTG</td>
</tr>
</tbody>
</table>
3.2.3 Quantitative RT-PCR

Quantitative RT-PCR was conducted as described in Chapter 2 Section 2.2.3. Data was normalised to the ribosomal 18S housekeeping gene to adjust for control variations between individual experiments. Experiments were performed at least three times and analysed in duplicate unless otherwise stated. Data are presented as mean ± standard error of the mean.

3.2.4 Western Blotting

The method of western blot analysis is described in detail in Chapter 2 Section 2.2.6. Whole cell lysates were resolved by SDS-PAGE and transferred onto PVDF. Membranes were blocked with 5% BSA and incubated with primary antibody targeting protein of interest followed by HRP-anti-rabbit IgG and ECL detection. The protein of interest was normalised with GAPDH to determine equal loading.

3.2.5 Statistical Analysis

Normalised data expressed as the mean ± standard error of the mean of three independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by Least Significant Difference post-hoc analysis. Results were considered significant when the probability was less than 0.05 (*p < 0.05) and 0.01 (**p < 0.01).
3.3 Results

3.3.1 Characterisation of vascular smooth muscle cells

VSMCs were phenotyped as smooth muscle cells. They exhibited a distinct “hill and valley” morphology in Figure 3.1 under phase contrast microscopy which is phenotypical of smooth muscle cells (Chamley-Campbell et al., 1979). Smooth muscle cells are a contractile cell type, a property that they utilise to mediate vascular tone, and as such contain the abundant amounts of the contractile protein, smooth muscle α-actin (Skalli et al., 1989). The cells were characterised using an antibody to smooth muscle α-actin and were visualised by confocal microscopy (Figure 3.1). Smooth muscle α-actin staining appears striations in the cells. There is a small possibility of endothelial cell contamination in VSMCs and can be detected by staining for endothelial cell specific von Willebrand factor cultures (Lin et al., 2000). Endothelial cells exhibit “cobblestone path” morphology in culture, which was not observed in our cells under microscopic examination. VSMCs were stained using primary antibody for von Willebrand factor (Figure 3.1). The VSMCs showed no staining for von Willebrand factor. Taken together these results demonstrate that the cells used in our in vitro model are VSMCs.
Figure 3.1: Characterisation of human VSMCs by immunofluorescence using confocal microscopy.

VSMCs were grown and fixed on coverslips. Cells were incubated with smooth muscle α-actin antibody (1:100) or von Willebrand (1:100) factor overnight followed by goat anti-mouse conjugated with fluorophores Alexa594 or Alexa 488 for 1 hour.
3.3.2 Thrombin stimulates the mRNA expression of GAG biosynthesizing genes CHST11 and CHSY1

We have previously demonstrated that the GPCR agonist thrombin can transactivate both EGFR and TGFBR1 to stimulate biglycan GAG chain elongation in human VSMCs (Burch et al., 2013). However, there have been no studies detailing the role of GAG gene expression in the PAR-1 mediated GAG chain elongation. To elucidate PAR-1 transactivation leading to GAG chain elongation, we investigated the mRNA expression of GAG biosynthesizing genes, specifically CHST11, CHSY1, XXYLTL-1, CHPF, CHST3 and biglycan in thrombin treated human VSMCs.

VSMCs were treated with thrombin (10 Units/ml) and mRNA expression of GAG synthesizing genes CHST11, CHSY1, XXYLTL-1 and CHPF was measured over a 24 time period at time points 0, 1, 2, 4, 6, 8, 16, 20, 22 and 24 hours as well as the expression of CHST3 and biglycan measured at 0, 2, 4, 6, 16, 20 and 24 hours (Figure 3.2). During the biosynthesis of GAG chains, GalNAc residues of the repeating disaccharide units are sulphated at C4 and C6 by CHST11 and CHST3 respectively. Thrombin generated a parabolic time dependent increase in the mRNA expression of CHST11 (Figure 3.2A). At 2 hours post thrombin treatment a 1.4-fold increase ($P<0.05$) in the mRNA expression of CHST11 was detected and reached a peak of 3-fold at 6-8 hours post stimulation ($P<0.01$) (Figure 3.2A). Thrombin treatment of human VSMCs also caused a rapid stimulation in the mRNA expression of CHST3 reaching a 2.5-fold ($P<0.01$) stimulation at 2 hours which was sustained up to 4 hours ($P<0.05$) post thrombin stimulation (Figure 3.2E). The addition of a monosaccharide to an acceptor oligosaccharide is referred to as chondroitin polymerization which occurs with the action of six glycosyltransferases:
CHSY1, CHSY2, CHSY3, CHPF, ChGn-1 and ChGn-2 (Mikami and Kitagawa, 2013). In this study, we investigated the thrombin mediated expression of CHSY1 and CHPF. Thrombin mediated mRNA expression of CHSY1 followed a similar response to the expression of CHST11 where it caused a parabolic time dependent increase in the mRNA expression (Figure 3.2B). Thrombin treated VSMCs caused a 2-fold increase ($P<0.05$) in the mRNA expression of CHSY1 at 4 hours and this was sustained reaching a peak of 3-fold ($P<0.01$) at 8 hours (Figure 3.2B). Thrombin had no effect on the mRNA expression of CHPF (Figure 3.2D).

To determine the role of VSMCs treatment by thrombin on the synthesis of glycosyltransferase XXYLT-1 mRNA expression a 24 hour time course was conducted. XXYLT-1 is an enzyme that attaches xylose, the first residue of the linkage region that links the CS GAG chain backbone to proteoglycan core protein. When looking at the mRNA expression of XXYLT-1 we see a slight increase of 1.8-fold ($P<0.01$) at 4 hours post treatment (Figure 3.2C). However we see that from 4 hours onwards thrombin has no effect on the mRNA expression of XXYLT-1. The role of thrombin on the mRNA expression of biglycan was also studied (Figure 3.2F). The 24 hour time course revealed that thrombin only slightly increased the mRNA expression of biglycan to 1.6-fold at 4 hours ($P<0.01$) (Figure 3.2F) however from 4 hours onwards thrombin has little to no effect on the mRNA expression of biglycan.

These results demonstrate that thrombin treated VSMCs lead to an increase in the mRNA expression of GAG synthesizing genes CHST11 and CHSY1 and to a much lesser effect mRNA expression of CHST3 and biglycan. The magnitude of thrombin stimulation of CHSY1 mRNA expression was higher compared to CHPF mRNA expression. These results suggest that
modification of GAG chain through sulphation by sulfotransferase CHST11 is
directly involved in thrombin mediated pathway associated with increased lipid
binding in VSMCs (Little et al., 2002). Hence the remainder of the study was
focused on the signalling pathways leading to the expression of these two genes
at 6 hours post thrombin stimulation.
Figure 3.2: Effects of thrombin treatment on the mRNA expression of five GAG synthesizing genes and biglycan.

VSMCs were treated with thrombin (10 Units/ml) to investigate the effect on mRNA expression of A. CHST11, B. CHSY1, C. XXYLT-1, D. CHPF, E.CHST3 and F. Biglycan. Total RNA was harvested, cDNA was synthesized and the mRNA expression of these genes was analysed using quantitative RT-PCR. 18S was used as the house keeping gene. Results are expressed as the mean ± standard error of the mean from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by the least significant difference post-hoc analysis, ** P<0.01 and * P<0.05 basal versus agonist.
3.3.3 Thrombin via PAR-1 activation leads to the mRNA expression of GAG biosynthesizing genes CHST11 and CHSY1

Our lab has published that thrombin mediated GAG chain elongation and the expression of biglycan core protein in VSMCs is elicited through PAR-1 (Ivey and Little, 2008, Burch et al., 2010). In this study we explore whether PAR-1 is involved in thrombin mediated mRNA expression of CHST11 and CHSY1 using the PAR-1 antagonist, SCH79797. Thrombin (10 Units/ml) at 6 hours increased the mRNA expression of CHST11 by 3-fold ($P<0.01$) (Figure 3.3A). Thrombin, in the presence of the PAR-1 antagonist SCH79797, showed a dose-dependent inhibition of CHST11 mRNA expression. At 10µM, the mRNA level was inhibited by 90% ($P<0.01$) (Figure 3.3A). We observed similar results for the effects of thrombin on the mRNA expression level of CHSY1 (Figure 3.2B). CHSY1 mRNA expression was increased to 1.8-fold ($P<0.05$) post thrombin stimulation (Figure 3.3B) and in the presence of SCH79797 was inhibited in a dose-dependent manner. The data indicates that PAR-1 mediates thrombin elicited CHST11 and CHSY1 mRNA expression in these cells.
Figure 3.3: The effect of a PAR-1 antagonist on thrombin stimulated GAG synthesizing gene mRNA expression.

VSMCs were pre-incubated with PAR-1 antagonist SCH79797 (1–30μM) for 30 mins and then treated with thrombin (10 Units/ml) for 6 hours to assess the effect on mRNA expression of CHST11 and CHSY1. SCH79797 (10 and 30μM) alone were used to assess any effects on basal expression. Total RNA was harvested and the mRNA expression of A. CHST11 and B. CHSY1 were analysed using quantitative RT-PCR. 18S was used as the housekeeping gene. Results are the mean ± standard error of the mean from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, p < 0.01 and #, p < 0.05 basal versus thrombin and **, p < 0.01 thrombin versus SCH79797.
3.3.4 Growth factors activating their respective receptors mediate mRNA expression of CHST11 and CHSY1

Our lab has previously shown that thrombin treated cells produce proteoglycans with increased GAG chain length (Ivey and Little, 2008). We have further shown that thrombin mediated GAG elongation is blocked by inhibition of both PTKR (EGFR) and S/TKR (TGFBR1) (Burch et al., 2010) using AG1478 and SB431542, respectively. To investigate the presence of the relevant pathways and responses, VSMCs were treated with growth factors thrombin, TGF-β and EGF in the presence and absence of their respective receptor antagonists SCH79797, SB431542 and AG1478 (Figure 3.4).

Thrombin treatment of VSMCs increased the mRNA expression of CHST11 by nearly 3-fold ($P<0.01$) (Figure 3.4A). In the presence of SCH79797 (30µM) this was completely abolished ($P<0.01$) (Figure 3.4A). A similar fold change in the mRNA expression of GAG genes was observed when VSMCs were exposed to TGF-β. CHST11 mRNA expression increased to 2.5-fold ($P<0.05$) when treated with TGF-β and to 2.7-fold ($P<0.01$) when treated with EGF (Figure 3.4A) and in the presence of their respective antagonists the response was completely blocked ($P<0.01$) (Figure 3.4A). The responses for the mRNA expression of CHSY1 were similar to the mRNA expression of CHST11 (Figure 3.4B). Thrombin treatment increased the mRNA expression of CHSY1 by almost 4-fold ($P<0.01$) (Figure 3.4B) and this response was inhibited by SCH79797 ($P<0.01$) (Figure 3.4B). The mRNA expression of CHSY1 in VSMCs treated with TGF-β increased by almost 7-fold ($P<0.01$) (Figure 3.4B) and EGF by 2.5-fold (Figure 3.4B). The mRNA expression of CHSY1 was inhibited by SB431542 ($P<0.01$) and AG1478 (Figure 3.4B). These results
show that thrombin, TGF-β and EGF activate their respective receptors to generate the mRNA expression of CHST11 and CHSY1.
Figure 3.4: Growth factors activating their respective receptors mediate mRNA expression of CHST11 and CHSY1.

VSMCs were pre-incubated with A-B. SCH79797 (30μM), SB431542 (3μM), AG1478 (5μM) or vehicle (-) for 30 min and then exposed to thrombin (Thr) (10 Units/ml), TGF-β (2ng/ml) or EGF (100nM) for 6 hours. Total RNA was harvested and the mRNA expression of A. CHST11 and B. CHSY1 were analysed using quantitative RT-PCR. 18S was used as the housekeeping gene. Results are the mean ± standard error from three separate experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, p < 0.01 and #, p < 0.05 basal versus agonist **, P<0.01 agonist versus antagonist.
3.3.5 Thrombin mediated mRNA expression of GAG biosynthesizing genes occurs through the transactivation of EGFR and TGFBR1

To investigate if thrombin employs transactivation of the S/TKR, TGFBR1, in mediating expression of CHST11 and CHSY1 mRNA levels, we used the TGFBR1 inhibitor, SB431542. Thrombin increased the mRNA expression of CHST11 by 2.5-fold ($P<0.01$) (Figure 3.5A) similar the results in Figure 3.4A. In the presence of SB431542, the thrombin mediated mRNA expression was inhibited dose-dependently ($P<0.01$) (Figure 3.5A). A similar result was seen for the mRNA expression of CHSY1, where thrombin elicited a 3-fold ($P<0.01$) (Figure 3.5B) increase and the response in the presence of SB431542, was dose-dependently inhibited. For CHSY1 the half maximal inhibitory effect was between 0.3-1μM and a maximal inhibitory effect of about 70-80% occurring at a SB431542 concentration of 3μM ($P<0.05$) (Figure 3.5B). These results suggest that in VSMCs, thrombin mediated mRNA expression of CHST11 and CHSY1 involves the PAR-1 mediated transactivation of the TGFBR1.

To evaluate the role of the PTKR, EGFR, in thrombin mediated CHST11 and CHSY1 mRNA expression, we employed the EGFR antagonist, AG1478 (Figure 3.5C and D). AG1478 had a dose dependent inhibitory effect on thrombin mediated mRNA expression of both CHST11 and CHSY1 (Figure 3.5 C and D). Thrombin stimulated a 2.5-fold ($P<0.01$) increase in the mRNA expression of CHST11 (Figure 3.5C). In the presence of AG1478 the inhibition was concentration dependent with half a maximal inhibitory effect at approximately 1μM and a maximal inhibitory effect of 100% occurred at 10μM ($P<0.01$) (Figure 3.5D). Similarly thrombin mediated mRNA expression of
CHSY1 showed a 2-fold increase (Figure 3.5D). In the presence of AG1478 there was a dose-dependent inhibition of the thrombin mediated mRNA expression. The half maximal inhibitory effect was approximately 0.5μM and a maximal inhibitory effect if 100% occurred at 10μM ($P<0.01$) (Figure 3.5D). These results indicate that thrombin can transactivate the EGFR to generate the mRNA expression of CHST11 and CHSY1.
Figure 3.5: Effect of EGFR and TGFBR1 antagonists on thrombin stimulated GAG gene mRNA expression.

VSMCs were pre-incubated with **A-B.** SB431542 (0.1–10μM) or **C-D.** AG1478 (0.5-10μM) or vehicle (-) for 30 mins and then exposed to thrombin (10 Units/ml) for 6 hours. SCH79797 (30μM) was used as a control. Total RNA was harvested and the mRNA expression of CHST11 and CHSY1 were analysed using quantitative RT-PCR. 18s was used as the housekeeping gene. Results are the mean ± standard error of the mean from three separate experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, p < 0.01 basal versus thrombin and **, p < 0.01 and *, p < 0.05 thrombin versus antagonists (SB431542, AG1478 and SCH79797).
3.3.6 The TGFBR1 and EGFR pathways combine in PAR-1 mediated expression of GAG biosynthesizing genes.

Burch et al. (Burch et al., 2013) showed that treatment of VSMCs with thrombin caused an increase in $[^{35}\text{S}]$-sulphate incorporation into secreted proteoglycans. This was inhibited by 84% in the presence of AG1478 and 68% in the presence of SB431542. In the presence of both AG1478 and SB431542, thrombin stimulation of $[^{35}\text{S}]$-sulphate incorporation was completely blocked. Here we investigated whether thrombin can transactivate EGFR and TGFBR1 to cooperate in generating increased mRNA expression of CHST11 and CHSY1. To address this aim, the EGFR and TGFBR1 inhibitors AG1478 and SB431542 respectively were used in VSMCS treated with thrombin. Thrombin treated VSMCs showed an increased mRNA expression of CHST11 to almost 3-fold ($P<0.01$) (Figure 3.6A). In the presence of AG1478 the mRNA expression was completely inhibited ($P<0.01$) (Figure 3.6A). SB431542 showed a 50% inhibition of thrombin mediated mRNA expression of CHST11 ($P<0.01$) (Figure 3.6A). In the presence of both of the receptor antagonists, AG1478 and SB431542, the mRNA expression was completely inhibited ($P<0.01$) (Figure 3.6A). Thrombin increased the mRNA expression of CHSY1 to 2-fold (Figure 3.6D). In the presence of AG1478 ($P<0.05$) and SB431542 ($P<0.01$) (Figure 3.6D) the mRNA expression for CHSY1 was completely inhibited. The combination of both receptor antagonists, AG1478 and SB431542 showed a total inhibitory effect ($P<0.01$) (Figure 3.6D).

To confirm our results we used thrombin receptor activator (TRAP), the synthetic peptide that specifically activates PAR-1 (Blackhart et al., 2000). TRAP treated VSMCs showed an increased mRNA expression of CHST11 to almost 2-fold ($P<0.01$) (Figure 3.6C). The mRNA expression of CHST11 was
inhibited by 80% in the presence of SB431542 ($P<0.01$) (Figure 3.6C). AG1478 had a 50% inhibition of TRAP mediated mRNA expression of CHST11 ($P<0.01$). In the presence of both receptor antagonists it showed a combined inhibitory effect ($P<0.01$). TRAP increased the mRNA expression of CHSY1 to 2.3-fold ($P<0.01$) (Figure 3.6F). In the presence of AG1478 ($P<0.01$) and SB431542 ($P<0.01$) the mRNA expression of CHSY1 was inhibited by 80%. In the presence of both of the receptor antagonists, the mRNA expression showed a combined inhibitory effect representing total inhibition of the response ($P<0.01$) (Figure 3.6F). This data demonstrates that PAR-1 signalling leading to GAG gene expression utilizes both the PTKR and the S/TKR transactivation pathways.

To investigate whether or not the PTKR and S/TKR signalling pathways independently combine in activating mRNA expression of CHST11 and CHSY1 we investigated the responses to the direct agonists EGF (100nM) and TGF-β (2ng/ml) both individually and in combination. EGF and TGF-β treatment individually increased the mRNA expression of CHST11 synthesizing enzyme to 2.3-($P<0.05$) and 2.1-fold ($P<0.05$), respectively (Figure 3.6B). When the two agonists were combined there was an additive effect in the mRNA expression of CHST11 to almost 5-fold ($P<0.01$) (Figure 3.6B). We observed a very similar pattern when looking at the mRNA expression of CHSY1. EGF and TGF-β increase the mRNA expression of CHSY1 to 1.5- and 3.1-fold ($P<0.01$) respectively (Figure 3.6E). The two agonists show an additive effect in the mRNA expression of CHSY1 to almost 5-fold ($P<0.01$) (Figure 3.6E). This data shows that both EGF and TGF-β pathways are involved in the increase expression of CHST11 and CHSY1. The combined effect of stimulating both
pathways produces an additive effect indicating that the pathways are at least to some extent independent.
Chapter 3. PAR-1 mediated GAG synthesizing gene expression
Figure 3.6: Analysis of agonists and antagonists, on the role on EGFR and TGFBR1 in mediating the expression of GAG synthesizing genes.

VSMCs were pre-incubated with AG1478 (5μM), SB431542 (3μM), both or vehicle (-) for 30 min and then exposed to A,D. thrombin (10 Units/ml) or C,F.TRAP (500μM) for 6 hours to determine the effects on the mRNA expression of A,C. CHST11 and D,F. CHSY1. VSMCs were treated with thrombin, EGF (100nM), TGF-β (2ng/ml) or both EGF and TGF-β for 6 hours to determine the combined activation for the mRNA expression of B.CHST11 and E.CHSY1. Total RNA was harvested and the mRNA expression of CHST11 and CHSY1 were analysed using quantitative RT-PCR. 18S was used as the housekeeping gene. Data are expressed as the mean ± standard error of the mean from three experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis A,C,D,F. ###, p < 0.01 basal versus thrombin and *, p < 0.05 and **, p < 0.01 thrombin versus antagonist (AG1478 and SB431542) B,E. **,P<0.01 basal versus agonist was used for statistical analysis.
3.3.7 The EGFR and TGFBR1 combine in thrombin mediated mRNA expression of CHST3 and biglycan

From our time course studies (Figure 3.2) we found that the mRNA expression of CHST3 and biglycan was increased by thrombin at 4 hours post treatment. Therefore the role of transactivation dependent signalling was studied on these two genes at this time point. Biglycan is the major proteoglycan synthesized and secreted by VSMCs (Shimizu-Hirota et al., 2004), and also the major proteoglycan that elongated in cells treated by thrombin (Burch et al., 2010). To study the transactivation pathways on thrombin stimulated mRNA expression of CHST3 and biglycan VSMCs were treated with thrombin for 4 hours in the presence and absences of TGFBR1 inhibitor, SB431542, or EGFR antagonist, AG1478.

Thrombin treated VMSCs showed an increased mRNA expression of CHST3 to 2-fold \( (P<0.01) \) (Figure 3.7A). In the presence of AG1478 and SB431542 the mRNA expression was completely inhibited \( (P<0.01) \) (Figure 3.7A). In the presence of both receptor antagonists, AG1478 and SB431542, the mRNA expression was completely inhibited \( (P<0.01) \) (Figure 3.7A). Thrombin treatment resulted in an approximately 1.2-fold increase \( (P<0.05) \) in the level of expression of biglycan mRNA (Figure 3.7B); this is a low level of expression but it is similar with earlier reports of TGF-β stimulation in VSMCs (Kamato et al., 2013a). In the presence of SB431542 \( (P<0.01) \) and AG1478 \( (P<0.05) \) the mRNA expression of biglycan was completely inhibited. When the two antagonists where used together this accounted for all the thrombin signalling leading to biglycan mRNA expression.
Figure 3.7: Thrombin mediated mRNA expression of CHST3 and biglycan is mediated by the cooperation of both EGFR and TGFBR1 signalling pathways.

VSMCs were pre-incubated with AG1478 (5µM), SB431542 (3µM), both or vehicle (-) for 30 min and then exposed to thrombin (10 Units/ml) for 4 hours to determine the inhibitory effect for the mRNA expression of A. CHST3 and B. biglycan. Total RNA was harvested and the mRNA expression was analysed using quantitative RT-PCR. 18S was used as the housekeeping gene. Data are expressed as the mean ± standard error of the mean from three experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, $P<0.01$ basal versus thrombin and *$P<0.01$ thrombin versus antagonist.
3.3.8 The combined roles of the TGFBR1 and EGFR pathways on the phosphorylation of Erk and the Smad2 linker region

The canonical TGF-β signalling pathway involves direct carboxy-terminal phosphorylation of the Smad2 by the TGFBR1. In addition to carboxy-terminal phosphorylation Smads can also be phosphorylated in the linker region (Burch et al., 2010, Burch et al., 2011, Kamato et al., 2013c, Kamato et al., 2014a, Rezaei et al., 2012, Matsuzaki, 2011, Matsuzaki et al., 2009). Smad linker region phosphorylation occurs indirectly via the phosphorylation of serine/threonine kinases including MAPKs (Erk, p38 and Jnk). In VSMCs, Smad2 linker region phosphorylation correlates with proteoglycan synthesis and GAG elongation. The data presented earlier in this chapter highlights that mRNA expression of GAG synthesizing genes CHST11 and CHSY1 correlates with previous published work on proteoglycan synthesis and GAG elongation (Burch et al., 2010, Burch et al., 2013). Hence, the signalling to Smad2 linker region could correlate with the results obtained on the regulation of mRNA of CHST11 an CHSY1.

Having established a role for thrombin in activation of GAG gene expression in VSMCs we wished to study each of the relevant signalling pathways. Thus, to investigate the roles of EGFR and TGFBR1 pathways on Erk1/2 (Figure 3.8A) and Smad2 linker region phosphorylation(Figure 3.8B) we used respective agonists EGF and TGF-β and compared the results to that of thrombin treated cells. Thrombin had a 2-fold increase of phospho-Erk1/2 (Figure 3.8A). EGF and TGF-β increased the phospho-Erk1/2 to 6- \( (P<0.01) \) and 2-fold respectively (Figure 3.8A). When the two agonists were combined phospho-Erk1/2 increased 6-fold \( (P<0.01) \) (Figure 3.8A). We observed a very
similar pattern with the Smad2 linker region, thrombin caused a 2-fold ($P<0.05$) increase in phospho-Smad2(245/250/255) (Figure 3.8B). EGF and TGF-β resulted in a 3.7- ($P<0.01$) and 2-fold ($P<0.05$) increase in the phospho-Smad2(245/250/255), respectively (Figure 3.8B). The signalling EGFR and TGFBR1 pathways appear to be additive as the action of the combined agonists shows a 3.7-fold ($P<0.01$) increase in phospho-Smad2(245/250/255). Thus, there is a correlation between the signalling response pattern of Smad2 linker region phosphorylation and the gene expression results.
Figure A: EGF+ mediated pSmad2(245/250/255) expression with GAPDH normalization. Treatment conditions: - Thr, EGF, TGFβ, TGFβ.

Figure B: EGF+ mediated pErk1/2(202/204) expression with GAPDH normalization. Treatment conditions: - Thr, EGF, TGFβ, TGFβ.
Figure 3.8: Direct stimulation of EGFR and TGFBR1 leads to an increase in the phosphorylation of Erk1/2 and Smad2 linker region.

VSMCs were treated with thrombin (10 Units/ml) A. 15 min B. 4 hours, EGF (100nM) A. 15 mins B. 60 mins, TGF-β (2ng/ml) A. 4 hours B. 30 min or both EGF and TGF-β A. 15 mins and 4 hours B. 60 mins and 30 mins respectively. Cell lysates were collected and proteins (40µg) were resolved on 10% SDS-PAGE then transferred to a PVDF membrane. The membrane was incubated with A. anti-phospho-Erk1/2(Thr202/Tyr204) (1:1000) or B. anti-phospho-Smad2(Ser245/250/255) (1:1000) followed by peroxidase-labelled rabbit IgG secondary antibody. The membranes were reprobed with GAPDH HRP-conjugated antibody (1:4000) to determine equal loading. Histograms represent band density expressed as fold per basal from three independent experiments (*P<0.05 vs. basal) (**P<0.01 vs. basal) using a one-way ANOVA followed by least significant difference post-hoc analysis.
3.3.9 Thrombin mediated Smad2 linker region phosphorylation is partially dependent on MMP shedding however phosphorylation of Smad2 in the carboxy terminal is not.

We have previously shown that the PAR-1 mediated EGFR transactivation in VSMCs follows the well characterised MMP mediated triple-membrane bypass system of activation (Prenzel et al., 1999) however the TGFBR1 transactivation is not dependent on MMPs (Burch et al., 2013). The MMP mediated involvement in the transactivation of TGFBR1 by PAR-2 was demonstrated by Chung et al (Chung et al., 2013). Chung et al (Chung et al., 2013) used an antibody directed to the Smad2 linker region to study PAR-2 transactivation of the TGFBR1 which produces an unclear outcome (see details in Chapter 5). Here we examined the role of MMPs in PAR-1 to TGFBR1 transactivation using antibodies which target both phosphorylated linker and carboxy terminal sites on Smad2. To determine if thrombin mediated phosphorylation of the Smad linker region requires the activation of MMPs, we used the broad-spectrum MMP inhibitor, GM6001 (Santiskulvong and Rozengurt, 2003). Thrombin treated cells had a 3- and 3.5-fold stimulation of phospho-Smad2(245/250/255) at 2 and 4 hours respectively ($P<0.05$, $P<0.01$) (Figure 3.9A). GM6001 markedly inhibited the thrombin mediated stimulation of phospho-Smad2(245/250/255) at both 2 and 4 hour stimulation to 1.7- and 2-fold ($P<0.01$)(Figure 3.9A) which is consistent with the results of Chung et al (Chung et al., 2013). We then showed that GM6001 has no effect on the ability of TGF-β mediated stimulation of phospho-Smad2(245/250/255) (Figure 3.9A). VSMCs treated with thrombin showed an increase in the carboxy terminal phosphorylation of Smad2(Ser465/467) and GM6001 had no effect the same results as previous published data (Burch et al., 2013). To confirm that PAR-1
mediated EGFR transactivation occurs through MMP activation in this cell model phospho-EGFR antibody was utilised. Thrombin stimulated a 5-fold increase in phospho-EGFR at 30 min ($P<0.01$) (Figure 3.9B). This was completely abolished in the presence of GM6001 ($P<0.01$) (Figure 3.9B). As a control we also show that GM6001 has no effect on the direct EGFR pathway as it fails to inhibit EGF stimulation of phospho-EGFR at 5 min (Figure 3.9B). These results show that MMPs are involved in PAR-1 to EGFR transactivation but as we have previously reported MMPs are not involved in PAR-1 mediated transactivation of TGFBR1.
Chapter 3. PAR-1 mediated GAG synthesizing gene expression

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**Graphs:**

- **A:**
  - pSmad2(245/250/255)
  - pSmad2(465/467)
  - GAPDH
  - Fold Change
  - *p < 0.05, **p < 0.01*

- **B:**
  - phoshoEGFR(Tyr1068)
  - GAPDH
  - Fold Change
  - **p < 0.01, ##p < 0.001**
Figure 3.9: Smad2 linker region phosphorylation is partially dependent on MMP shedding however phosphorylation of Smad2C is not.

A. VSMCs were pre-incubated with GM6001 (10μM), SCH79797 (10μM) or vehicle for 30 min and then exposed to thrombin (10 Units/ml) for 120 and 240 mins. VSMCs stimulated with TGF-β (2 ng/ml) for 60 min in the presence and absence of GM6001 or SB431542 (3μM). B. VSMCs were pre-incubated with GM6001 or vehicle for 30 min and then exposed to thrombin or EGF (100nM) for 5 min. Cell lysates were collected and proteins (40μg) were resolved over 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated with A. anti-phospho-Smad2(245/250/255) (1:1000) or B. anti-phospho-EGFR(Tyr1068) (1:1000). The membranes were reprobed with anti-phospho-Smad2(465/467) (1:1000) and with GAPDH HRP-conjugated antibody. Histograms represent band density expressed as fold per basal from three independent experiments (*P<0.05 versus basal) (##P<0.01 agonist versus antagonist) using a one-way ANOVA followed by least significant difference post-hoc analysis.
3.4 Summary of results of Chapter 3

The key findings from this chapter are:

- Thrombin treatment of VSMCs leads to the mRNA expression of GAG biosynthesizing genes CHST11, CHSY1, XXYLT-1, CHST3 and the mRNA expression of biglycan.

- Thrombin acts through its receptor PAR-1 to mediate the mRNA expression of GAG biosynthesizing genes CHST11 and CHSY1.

- Thrombin mediated mRNA expression CHST11, CHSY1, CHST3 and biglycan are inhibited by TGFBR1 antagonist, SB431542 and EGFR antagonist, AG1478.

- Agonists to the TGFBR1 and EGFR pathways lead to the mRNA expression of GAG synthesizing genes.

- Thrombin leads to the phosphorylation of the Smad2 linker region.

- Agonists to TGFBR1 and EGFR pathways are involved in the phosphorylation of the Smad2 linker region.

- Thrombin mediated Smad2 linker region phosphorylation is partially inhibited by MMP inhibitor GM6001. The phosphorylation of Smad2 carboxy terminal is unaffected.

3.5 Discussion

The signalling pathways that control the processes that regulate the length and sulphation pattern of GAG chains on the proteoglycan, biglycan, represent a potential therapeutic target for the prevention of atherosclerosis (Little et al., 2002). Thrombin is expressed in atherosclerotic plaques and is therefore a potential contributor to CVD (Stoop et al., 2000). Our lab has shown that all of
the signalling for GAG hyperelongation response occurred through PAR-1 mediated transactivation of the EGFR and TGFBR1 (Burch et al., 2010, Burch et al., 2013). In this chapter the main aim was to investigate whether thrombin regulates the mRNA expression of GAG synthesizing genes potentially associated with the expression of the enzymes which mediate GAG elongation. These genes which are responsible for CS GAG chain synthesis where chosen as they had previously been shown to play a critical role in the development of early atherosclerosis (Anggraeni et al., 2011).

The lipid binding properties of CS proteoglycans specifically biglycan are dependent on the polyanionic GAG chains. In human VSMCs, selected GAG synthesizing gene mRNA expression was regulated through PAR-1 signalling pathway namely, CHST11, CHSY1 and CHST3 as well as biglycan mRNA expression. The increased expression of these genes correlates with the GAG elongation effect of thrombin (Burch et al., 2013).

The expression of CHST11 and CHSY1 was increased up to at least 8 hours with a peak increase of 3-4 fold. In a high fat diet mouse study CHSY1 was reported to be the most highly expressed gene in atherosclerotic plaque development (Anggraeni et al., 2011) suggesting that these genes mediate GAG elongation and hyperelongation \textit{in vivo}. In addition, \textit{in vivo} studies show that in a CHSY1 knockout mouse model, the CS GAG synthesis was reduced (Wilson et al., 2012). CHSY1 plays an important role by its capability of producing dual transferase activities as both B3GALT6 and B4GALT7 activity which enables the synthesis of repeating disaccharide units of CS and GAG chain (Izumikawa et al., 2008, Izumikawa et al., 2007, Kitagawa et al., 2001). Therefore we speculate that in the VSMC model thrombin mediated GAG chain hyperelongation requires CHSY1 regulation. The CHST11 enzyme is crucially
as important in CS GAG chain synthesis as it catalyses the transfer of sulphate to position 4 of the GalNAc residue of chondroitin. A CHST11 deficient mouse model showed a 90% decrease in GalNAc structure and CS amount (Izumikawa et al., 2011). Through its sulphation actions, CHST11 induces GAG chain hyperelongation while producing sulphated disaccharides. Therefore CHST11 is directly involved in thrombin mediated GAG chain hyperelongation associated with an increase in LDL binding in VSMCs (Ivey and Little, 2008).

We then investigated the cellular signalling pathways underlying the action of thrombin on CHST11 and CHSY1 gene expression. The thrombin stimulated expression of CHST11 and CHSY1 was blocked in a concentration dependent manner and to a total inhibition of 100 per cent by the PAR-1 antagonist, SCH79797, strongly suggesting that the response is mediated via PAR-1. We then investigated the contribution of GPCR to kinase receptor transactivation pathways in this response. Thrombin mediated expression of CHST11 and CHSY1 was blocked in a concentration dependent manner by the TGFBR1 and EGFR antagonists. This data shows that thrombin mediated GAG gene regulation is via transactivation of the TGFBR1 and EGFR consistent with our previous work showing that thrombin mediated proteoglycan synthesis occurs via the activation of these two receptors (Burch et al., 2010). The expression of CHST11 and CHSY1 are both increased when treated with EGF and TGF-β confirming the presence of these receptors and active pathways in these cells. When the antagonists SB431542 and AG1478 were concomitantly present all of the thrombin mediated increase in CHST11, CHSY1, CHST3 and biglycan mRNA expression was blocked strongly indicating that all of the signalling is through the (dual) kinase receptor transactivation pathways.
Chung et al. (Chung et al., 2013) recently demonstrated PAR-2 transactivation of TGFBR1, and they reported identical mechanisms to that occurring in their renal fibrosis model as in the VSMC model with the notable exception that they reported the process of transactivation of TGFBR1 to be dependent on MMPs whereas it was previously reported that the process does not involve MMPs (Burch et al., 2013). Smad linker region phosphorylation can occur via the TGF-β mediated activation of a plethora of serine/threonine kinases (or alternatively many other agonist-activated serine/threonine kinases) (Kamato et al., 2013c) whereas the response of carboxy terminal phosphorylation of Smad occurs only as a direct result of the kinase activity of TGFBR1 directed to Smad2 or Smad3 (Kamato et al., 2013c, Kamato et al., 2014a). To address the role of MMPs in PAR-1 mediated transactivation of S/TKR the phosphorylation of Smad2 in both the linker region and the carboxy terminal was assessed. Thrombin stimulated the phosphorylation of Smad2 in both the linker region and the carboxy terminal domains but only linker region phosphorylation was blocked by the broad spectrum MMP inhibitor, GM6001, therefore indicating the role of MMPs in linker region but not carboxy terminal phosphorylation. There are many pathways leading to linker region phosphorylation but based on the current literature only one pathway to carboxy terminal phosphorylation. These results thus explain the findings of Chung et al. (Chung et al., 2013) and confirms that MMPs are not involved in GPCR transactivation of TGFBR1.

The results observed in these studies in VSMCs of thrombin stimulated mRNA expression for two of the genes associated with GAG hyperelongation are consistent with those observed when studying functional or structural readout of the actual size of the biglycan molecules determined by SDS-PAGE
(Burch et al., 2013). In the studies based on biglycan bands we observed a partial role for each of the EGFR and TGFBR1 transactivation pathways and based on studies with pharmacological agents it was concluded that all of the signalling was mediated via the two transactivation pathways (Burch et al., 2013). In the present studies examining the expression of the mRNA of two genes associated with GAG hyperelongation we observed that each of the individual pathways, when assessed by the use of the relevant pharmacological antagonists appeared to mediate almost 100 per cent of response. Consistent with earlier data both pathways accounted for all of signalling which reinforces the importance of the transactivation pathways in this response. We do not at the moment have an understanding of why the individual pathways of EGFR and TGFBR1 mediate such a high proportion of the gene expression responses to thrombin. Importantly, our lab has previously reported that SB431542 does not block EGFR signalling and that AG1478 does not block TGFBR1 signalling (Burch et al., 2010) so there is no spillover of inhibitory activity from one pathway to another which could explain these results.

There is considerable evidence from in vitro, animal and human studies that hyperelongation of GAG chains on biglycan is associated with the early stages of atherosclerosis (Nakashima et al., 2007, Ballinger et al., 2010). Ultimately, to establish causation, one would need to analyse the size of biglycan molecules or their GAG chains an atherosclerotic model(Anggraeni et al., 2011, Skalen et al., 2002). It is sometimes more practical to use a surrogate measure of a potential therapeutic target, in our case GAG hyperelongation. Thus, we have commenced studies of the expression of the genes which might represent the multitude of enzymes which mediate the synthesis of CS/DS GAG chains (Silbert and Sugumaran, 2002, Kitagawa et al., 2001, Gotoh et al., 2002, Uyama et al., 2002,
Uyama et al., 2003, Yada et al., 2003, Sato et al., 2003). The mechanism of CS/DS GAG synthesis has not been fully elucidated but as many as 12 enzymes are involved being transferases to add monosaccharides moieties to the growing chains, as well as sulfotransferases which add sulphate residues to the disaccharide units in the growing chain but may also be involved in the control of GAG length. We have been able to deduce by correlation that thrombin increases GAG hyperelongation and also increases the expression of three of the genes of interest, CHST11, CHST3 and CHSY1, but clearly further studies are required to establish the specific role of these enzymes in thrombin mediated GAG hyperelongation. However, on the assumption that these agents are important in GAG synthesis and GAG hyperelongation, and noting that Anggraeni and colleagues (Anggraeni et al., 2011) have reported an increase in the expression of CHST11 in the vessels of a murine model of atherosclerosis, then we can speculate that these genes can be used as targets to assess the role of up regulation of these genes in atherosclerosis. Also our specific interest is the potential to inhibit the expression of these genes thus preventing GAG hyperelongation, lipid deposition and atherosclerosis an outcome we have previously reported for studies with imatinib (Ballinger et al., 2010).

The signalling consequences of these studies are that the role of GPCR mediated transactivation of TGFBRI1 extends from the general response of GAG hyperelongation to the specific responses of the expression of two individual genes and that concurrently the transactivation of the EGFR along with TGFBRI1 explains all of the signalling in this model. Our data showing that MMPs are not involved in PAR-1 to TGFBRI1 transactivation indicates the mechanisms of PAR-1 transactivation of the EGFR and the TGFBRI1 are mechanistically completely distinct (Kamato et al., 2014b, Burch et al., 2013).
Chapter-4. Gq is the central regulator of PAR-1 mediated transactivation of multiple protein kinase receptors.
4.1 Introduction

The major proximal effectors of GPCR signalling are the GTP-binding, GTPase active, G proteins. G proteins are a point of convergence in GPCR signalling. G proteins are classified into four families according to their α subunit: Gi, Gs, G12/13 and Gq. The Gs and Gi families regulate adenylyl cyclase activity, while Gq activates PLCβ and G12/13 can activate small GTPase families (Neves et al., 2002). The Gq family consists of four members: Gq, G11, G14 and G15/16 (Strathmann and Simon, 1990, Wilkie et al., 1991) and their respective α subunits thus are: Gαq, Gαq/11, Gαq/14 and Gαq/15/16. The Gαq, and Gαq/11, isoforms share in 88% homology at the protein level (Hubbard and Hepler, 2006), these two isoforms are the most prominent and ubiquitously expressed and will be the focus of this chapter.

In an inactivated state, in the absence of a cognate ligand, the G protein α subunit is bound to GDP, however upon ligand engagement with the GPCR, the receptor undergoes conformational changes promoting the exchange of bound GDP for GTP (described in (Tuteja, 2009)). The α subunit and βγ complex then dissociate from one another and interact with their associated effectors (Berman and Gilman, 1998). In the most common signalling pathways, Gαq activates PLCβ, which hydrolyzes PIP2 releasing DAG and IP3. DAG activates several isoforms of PKC, whereas IP3 diffuses to the ER and binds to IP3 receptors on ligand-gated calcium channels on the surface of the ER leading to a massive release of calcium ions into the cytosol and subsequently in some cells, the opening of cell surface calcium channels leading to the influx of extracellular calcium (Little et al., 1992, Rochdi and Parent, 2003, McCudden et al., 2005).
One of the major and expanding areas of GPCR signalling is transactivation dependent signalling (Kamato et al., 2014b) in which GPCRs transactivate PTKRs such as EGFR or S/TKR such as TGFBR1. In Chapter 3 the paradigm of GPCR signalling in which PAR-1 transactivates both the TGFBR1 and the EGFR leading to an increase in the mRNA expression of GAG genes CHST11 and CHSY1 was presented. Mimicking the increase in intracellular calcium with ionomycin does not stimulate proteoglycan synthesis and GAG elongation (Survase et al., 2005). Calcium channel blockers slightly inhibit growth factor stimulated GAG hyperelongation (Vijayagopal et al., 1980) but the mechanism does not depend on the blocking of calcium channels (Survase et al., 2005). Yang et al. (Yang et al., 2006) studied Gaq coupled glutamate receptor responses in cultured neurones found that the stimulation of the phosphorylation of the MAPK, Jnk, occurred via transactivation of the EGFR and did not involve the traditional PLC, PKC and IP₃ pathways. These results suggested that the PLC pathway and the pathway to transactivation of the EGFR are occurring in parallel.

The role of G proteins in GPCR signalling has not been as intensively investigated as other aspects of GPCR signalling due to the limited availability of pharmacological tools. Two natural products YM-254890 from the bacteria Chromobacterium sp. QS3666 (Taniguchi et al., 2003) and UBO-QIC also known as FR300359 from the Ardisia crenata sims plant (Miyamae, 1989) are potent inhibitors of Gaq. These two compounds have been investigated in the therapeutic context of inhibitors of the thrombin receptor, PAR-1 and the inhibition of platelet aggregation (Kawasaki et al., 2003, Taniguchi et al., 2003, Grace et al., 2014, Kamato et al., 2015, Schrage et al., 2015). The YM-254890 has only recently become commercially available and there are no other suitable
alternatives hence the difficulty in studying the role of Gαq in cell biology. For this Chapter we had a very limited amount of UBO-QIC gifted to us, which was used to study the role of Gαq in GPCR mediated transactivation signalling.

The inhibitory profile of the UBO-QIC compound has recently been characterised (Kukkonen, 2016). Endogenously expressed receptor utilizing Gαi, Gαs and Gαq protein were assessed in CHO, UBO-QIC was an effective inhibitor of the Gαq mediated responses, but was inactive at Gαi and Gαs mediated responses. In human erythroleukemia cells, UBO-QIC inhibited responses downstream of the Gαq/11 and the Gαq/16 (Kukkonen, 2016). Within the same time period, Schrage and colleagues (Schrage et al., 2015) published a major study on the characterisation of UBO-QIC. Inhibition of Gαq, Gαq/11 and Gαq/14 signalling by UBO-QIC was observed whereas, signalling via Gαq/16 was completely unaffected. These results in transfected HEK cells describe UBO-QIC to be an inhibitor of Gαq, Gαq/11 and Gαq/14. Thus showing a difference between the two studies, which may be cell and context dependent. A very recent paper confirms that UBO-QIC inhibits Gαq mediated signalling and had no effect on Gαi and Gαs mediated signalling (Gao and Jacobson, 2016). However UBO-QIC was also observed to interact with Gβγ mediated signalling following the activation of the Gαi- coupled receptor (Gao and Jacobson, 2016). Thus we acknowledge that UBO-QIC may not be specific for Gαq mediated signalling, hence siRNA will be used to validate whether this signalling is Gαq dependent. However for the purpose of this thesis we have referred to UBO-QIC as a broad Gαq antagonist.
4.2 Materials and Methods

4.2.1 Materials

RT-PCR primers for GAG synthesizing genes; CHST11 and CHSY1 were based on their respective GenBank sequences and were purchased from GeneWorks Pty Ltd (SA, Australia)(Table 3.1). AD1 4D-Nucleofector Y kit was purchased from Lonza. siGENOME Human GNA11, GNAQ and non targeting siRNA SMARTpool, were purchased from Millennium Science. Mouse human integrin αVβ3 and αVβ5 monoclonal antibodies were purchased from MERCK Millipore. GNAQ antibody (ab75825) and GNA11 antibody (ab192507) were purchased from Abcam.

4.2.2 Cell culture

Cells were grown in DMEM (5mM glucose, 10% FBS and 1% antibiotics at 37°C in 5% CO₂). VSMCs were seeded in 96-, 24-, 12- and 6-well plates or on cover slips (described in more detail in Chapter 2 Section 2.1). Cells were grown to confluence then rendered quiescent by serum deprivation for either 24 or 48 hours. Inhibitors were pre-incubated for 30 mins prior treatment with agonists. The incubation times and concentration are given in detail in the figure legends.

4.2.3 Quantification of intracellular calcium release

Quiescent VSMCs seeded into 96-well plates were loaded with FURA-2AM ester. The cells were pre-incubated with antagonists for 30 mins prior to stimulation with thrombin. Fluorescence was measured at 340nm and 380nm excitation and 510nm emission wavelengths using a Flexstation 3 plate reader. The sustained calcium response was calculated as the difference between basal fluorescence and the peak fluorescence after thrombin stimulation.
4.2.3.1 YM-280193

YM-254890 has 5 naturally occurring analogues (Taniguchi et al., 2004). Professor Margaret Brimble (University of Auckland) provided us with one of the analogues, YM-280193, in two forms, cyclic and linear. The chemical structures of UBO-QIC and the YM-280193 peptide presented in Figure 4.1D show that the main difference between the two chemical structures is the additional side chain hydroxyleucine residue outlined by the circle.

![Chemical structure of UBO-QIC and YM-280193.](image)

**Figure 4.1: Chemical structure of UBO-QIC and YM-280193.**

4.2.4 Western blotting

The method of western blot analysis is described in detail in Chapter 2 Section 2.2.6. Whole cell lysates were resolved by SDS-PAGE and transferred onto PVDF. Membranes were blocked with 5% BSA and incubated with primary antibody targeting protein of interest followed by HRP-anti-rabbit IgG and ECL detection. The protein of interest was normalised with GAPDH to adjust for control variations.
4.2.5 Quantitative RT-PCR
Quantitative RT-PCR was conducted as described in Chapter 2 Section 2.2.3. Data was normalised to the ribosomal 18S housekeeping gene to adjust for control variations between individual experiments. Experiments were performed at least three times and analysed in duplicate.

4.2.6 Quantitation of proteoglycan synthesis and analysis of proteoglycan size
Quiescent cells were changed to fresh medium containing 50μCi/ml [35S]-sulphate in the presence or absence of agonists or antagonists for 24 hours. Media from the cell cultures was harvested and protease inhibitors were added to prevent degradation. Incorporation of the radiolabel into proteoglycans was measured by CPC precipitation assay (described in more detail Chapter 2 Section 2.2.4). Proteoglycans labelled with [35S]-sulphate were prepared for SDS-PAGE by isolation through DEAE-Sephacel anionic exchange mini columns. Fractions containing the highest number of [35S]-sulphate cpm were pooled. Radiolabeled proteoglycans were separated on 4-13% acrylamide gels with a 3% stacking gel at 50V overnight. Gels were then fixed and dried and exposed to phospho imaging plate (Fujifilm BAS-MS 2040 imaging plate) for 3-5 days. Images were then developed using a phosphoimager (Perkin Elmer Cyclone Plus Storage Phosphor System) and viewed using imaging software (OptiQuant).

4.2.7 siRNA Knockdown of G proteins
Quiescent VSMCs were seeded in 24 well plates, Lonza AD1 4D-Nucleofector Y kit was utilized according to manufacturers’ instructions (see Chapter 2 Section 2.2.11 for more detail). siRNA to GNAQ and GNA11 at
105pmol was utilized. Culture was continued for 36 hours post transfection prior to RNA extraction or treatment with thrombin.

**4.2.8 Confocal imaging of G protein and integrins**

Cells were grown and treated on glass coverslips then fixed with 2% paraformaldehyde in 1×PBS. The coverslips were then washed with 1×PBS and blocked by 10% horse serum for 30 mins. Cells were incubated with mouse anti-human αVβ3, αVβ5, anti-rabbit GNAQ and GNA11 overnight in a humidified chamber followed by a goat anti-mouse or anti-rabbit conjugated with fluophores Alexa594 and Alexa488 for 1 hour. Coverslips were incubated with Hoechst stain for 30 mins and then mounted on slides and dried overnight at 4°C. Cells were imaged using a Nixon C1 confocal microscope.

**4.2.9 Statistical analysis**

Data was normalised and is shown as the mean ± standard error of the mean of three independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by least significant difference *post-hoc* analysis. Results were considered significant when the probability was less than 0.05 and 0.01.

**4.3 Results**

**4.3.1 UBO-QIC inhibits thrombin mediated intracellular release of calcium**

YM-254890 has 5 naturally occurring analogues (Taniguchi et al., 2004). Professor Margaret Brimble (University of Auckland) provided us with one of the analogues YM-280193 in two forms, cyclic and linear. The hypothesis was that in our model UBO-QIC and the 2 forms of the YM-280193 peptide would
act as Gαq inhibitors. To address our aim GPCR mediated intracellular calcium release was used to study the Gαq signalling pathway (Masuda et al., 1998). VSMCs treated with thrombin showed a substantial increase in the intracellular release of calcium \((P<0.01)\) (Figure 4.2A). In the presence of UBO-QIC (1-1000nM) we observed a dose-dependent inhibition with 50% inhibition seen at 30nM and a complete block of thrombin mediated intracellular calcium release at 100-1000nM \((P<0.01)\). This data shows that UBO-QIC in VSMCs works as a Gαq antagonist.

Closely related analogue to the Gαq/11 antagonist YM-254890, YM-280193 (Kaur et al., 2015), was evaluated for its effects as a Gαq inhibitor. VSMCs treated with thrombin stimulated \((P<0.01)\) the intracellular release of calcium, YM-280193-linear (1-1000nM) (Figure 4.2B) had no effect on the thrombin mediated intracellular calcium levels, however UBO-QIC used as a positive control almost completely inhibited thrombin mediated intracellular calcium release. Thrombin mediated intracellular calcium release was unaffected by YM-280193-cyclic (1-1000nM) (Figure 4.2C), however as expected UBO-QIC inhibited the thrombin mediated intracellular calcium release. These results show that both the linear and cyclic YM-280193 peptide is ineffective at inhibiting GPCR mediated intracellular calcium release, thus unlike its analogue YM-254890, YM-280193 is not a Gαq antagonist. The chemical structures of UBO-QIC and the YM-280193 peptide presented in Figure 4.1 show that the main difference between the two chemical structures is the additional side chain hydroxyleucine residue outlined by the circle. From this we postulate that this hydroxyleucine side chain plays an important role in the binding to the Gαq protein to inhibit Gαq signalling.
Thrombin

UBO-QIC(nM)

Fluorescence ratio ($F_{340}/F_{380}$)

0.0
0.2
0.4
0.6
0.8

Thrombin

YM-280193 Cyclic (nM)

Fluorescence ratio ($F_{340}/F_{380}$)

0.0
0.5
1.0
1.5

**B**
Figure 4.2: UBO-QIC dose dependently inhibits thrombin mediated intracellular calcium release.

VSMCs loaded with FURA-2A ester (2µM) for 30 min and pre-incubated by A. UBO-QIC (1-1000nM), B. YM-280193 cyclic (1-1000nM) and C. YM-280193-linear (1-1000nM) for 15 min prior stimulation with thrombin (1 Units/ml). The peak fluorescence ratio (340/380nm) minus the baseline was used to express the relative data. The results are the mean and standard error from three separate experiments repeated in triplicate. Statistical significance was determined by one-way ANOVA, followed by least significant difference post hoc analysis ##, $P<0.01$ basal versus thrombin and **, $P<0.01$ thrombin versus antagonist was used.
4.3.2 The Gaq protein is involved in PAR-1 mediated transactivation of the TGFBR1 and EGFR

We have previously demonstrated that GPCR agonist thrombin transactivates the TGFBR1 leading to the generation of phosphorylated Smad2 in the carboxy terminal. Thrombin has also been shown to transactivate the EGFR leading to the phospho-Erk1/2. The mechanisms which define and characterise the two transactivation pathways have been extensively reviewed (Kamato et al., 2014b, Burch et al., 2012, Cattaneo et al., 2014, Wang, 2016). The literature as well as our findings (Burch et al., 2013, Kamato et al., 2014b, Prenzel et al., 1999, Daub et al., 1997, Gschwind et al., 2002, Frank and Eguchi, 2003, Jenkins et al., 2006, Xu et al., 2009) indicate that the distal components of these two transactivation pathways are mechanistically distinct. From the point of view of discovery of a potential therapeutic target, the ultimate aim is to find a common mechanism or target which can mediate the transactivation dependent signalling. We hypothesize that G proteins such as the Gaq family members may play a role in GPCR mediated transactivation of both the EGFR and TGFBR1 therefore Gaq could be common hence we sought to test this hypothesis experimentally.

VSMCs treated with thrombin showed a 2.8-fold increase ($P<0.01$) in the phospho-Smad2(465/467) (Figure 4.3A). In the presence of UBO-QIC thrombin mediated phospho-Smad2(465/467) was inhibited by 75% to 1.4-fold ($P<0.01$) when compared to thrombin stimulated cells (Figure 4.3A). In the presence of TGFBR1 antagonist SB431542 the thrombin mediated phospho-Smad2(465/467) was abolished ($P<0.01$) (Figure 4.3A). To evaluate any possible direct effects on TGFBR1 signalling, VSMCs were treated with TGF-β in the presence and absence of UBO-QIC and SB431542. The results
show that TGF-β treated cells had a 30-fold increase in the phospho-Smad2(465/467) which as expected was unaffected by UBO-QIC (Figure 4.2A). As expected TGFBR1 antagonist, SB431542, inhibited TGF-β mediated signalling by approximately 70% (Figure 4.2A).

Similarly, the role of Gαq was investigated on thrombin mediated transactivation of the EGFR. Thrombin treatment showed a 2-fold increase ($P<0.01$) of phospho-Erk1/2 (Figure 4.3B). Thrombin mediated phospho-Erk1/2 was inhibited in the presence of UBO-QIC by 95% ($P<0.01$)(Figure 4.3B). EGFR antagonist, AG1478, completely inhibited ($<0.01$) thrombin mediated phospho-Erk1/2. To study whether UBO-QIC had any effect on direct EGFR signalling, VSMCs were treated with EGF in the presence and absence of UBO-QIC and AG1478. As expected EGF showed a substantial 5-fold increase in phospho-Erk1/2 (Figure 4.3B) and this was unaffected by UBO-QIC but completely abolished by AG1478 (Figure 4.3B). These results demonstrate that thrombin mediated transactivation of the EGFR and TGFBR1 involved UBO-QIC sensitive Gαq signalling. Gαq may act as the common integrating point for all transactivation dependent signalling.
A

Thrombin  | TGF-β
---|---
- | -
UBO | SB

pSmad2(Ser465/467)

GAPDH

pSmad2(Ser465/467) (Fold Change)

##

##

**

**

B

Thrombin  | EGF
---|---
- | -
UBO | AG

pErk1/2(Thr202/Tyr204)

GAPDH

pErk1/2(Thr202/Tyr204) (Fold Change)

##

##

**

**

**
Figure 4.3: Goq is involved in PAR-1 mediated transactivation of the TGF-β and EGF receptors.

VSMCs were pre-incubated with A. UBO-QIC (100nM), SB431542 (3µM) or vehicle for 30 mins then exposed to thrombin (10 Units/ml) for 240 mins or TGF-β (2ng/ml) for 10 mins B. UBO-QIC (100nM), AG1478 (5µM) or vehicle for 30 mins then exposed to thrombin (10 Units/ml) for 15 mins or EGF (100nM) for 5 mins. Cell lysates were collected and proteins (40µg) were resolved over 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was then incubated with A. anti-phospho-Smad2(Ser465/467) (1:1000) or B. anti-phospho-Erk1/2(Thr202/Tyr204) (1:1000) followed by peroxidase-labelled rabbit IgG secondary antibody. Membranes were reprobed with GAPDH HRP-conjugated antibody. Histograms represent band density expressed as fold per basal from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post hoc analysis ##,P<0.01 basal versus agonist and **,P<0.01 agonist versus antagonist.
4.3.3 Thrombin signalling via Gαq requires the activation of ROCK signalling.

Thrombin mediated transactivation of the TGFBR1 is achieved through cytoskeletal rearrangement, which involves ROCK signalling activating cell surface integrins which bind to the latent TGF-β complex (Burch et al., 2013). To investigate whether thrombin mediated transactivation of the TGFBR1 via the Gαq follows this signalling cascade, we investigated the effects of thrombin mediated Gαq signalling against the immediate downstream product of ROCK, the group of homologous proteins ERM. Thrombin caused a small increase up to 1.4-fold in the phosphorylation of ERM, in the presence of UBO-QIC, thrombin mediated phosphorylation of ERM was completely inhibited (Figure 4.4). Y-27632 a small molecule inhibitor of ROCK completely abolished thrombin mediated phosphorylation of ERM. These results show that thrombin mediated transactivation of the TGFBR1 involves the Gαq protein signalling to activate ROCK signalling pathways which will lead to integrin activation allowing for the TGF-β ligand to bind to its receptor.
Figure 4.4: Thrombin signalling via Gαq requires the activation of ROCK signalling.

VSMCs were pre-incubated with UBO-QIC (100nM), Y-27632 (10μM) or vehicle for 30 min in the presence and absence of thrombin (10 Units/ml) for 5 mins. Cell lysates were collected and proteins (40μg) were resolved over 10% SDS-PAGE and then transferred to a PVDF membrane. The membrane was then incubated with anti-phospho-Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558) antibody (1:4000) followed by peroxidase-labelled rabbit IgG secondary antibody. Membranes were reprobed with GAPDH HRP-conjugated antibody. Histograms represent band density expressed as fold per basal from two independent experiments.
4.3.4 *Gaq* and Rho/ROCK are involved in PAR-1 mediated mRNA expression of GAG enzymes CHST11 and CHSY1.

Previously our lab has published that thrombin mediated proteoglycan synthesis and GAG chain elongation occurs via the transactivation of the EGFR and TGFBR1 (Burch et al., 2010, Burch et al., 2013). Chapter 3 shows that thrombin leads to an increase in the mRNA expression of GAG synthesizing genes CHST11 and CHSY1. Thrombin mediated mRNA expression of these two GAG synthesizing genes occurred via the transactivation of both the EGFR and TGFBR1. The CHSY1 enzyme is directly involved in addition of repeated disaccharide units on GAG chains, the CHST11 is involved in the sulphation of the GAG chain. Based on the results of the signalling data presented in Chapter 4 Section 4.3.1 and 4.3.2 we hypothesize that the Gaq protein will be involved in thrombin mediated proteoglycan synthesis and the mRNA expression of GAG synthesizing genes CHST11 and CHSY1.

We assessed the role of thrombin mediated Gaq signalling in proteoglycan synthesis using UBO-QIC. VSMCs treated with thrombin showed a 3-fold (*P*<0.01) increase $[^{35}S]$-sulphate incorporation into secreted proteoglycans compared to untreated controls (Figure 4.5A). Thrombin treated VSMCs in the presence of UBO-QIC had a 30% inhibition to 2-fold of $[^{35}S]$-sulphate incorporation into proteoglycans. VSMCs were treated with TGF-β or EGF alone which increased the incorporation of $[^{35}S]$-sulphate into secreted proteoglycans to approximately 2-fold (*P*<0.01). The $[^{35}S]$-sulphate incorporation was unaffected in the presence of UBO-QIC (Figure 4.5A).

Separation of proteoglycans by SDS-PAGE showed a decrease in electrophoretic mobility of biglycan in the presence of thrombin compared to
untreated controls (Figure 4.5B). In the presence of 30nM and 100nM UBO-QIC, relative to thrombin treatment alone, there was an increase in biglycan electrophoretic mobility (Figure 4.5B). TGF-β and EGF showed a decrease in electrophoretic mobility of biglycan which was unaffected in the presence of UBO-QIC. These results demonstrate that the increase in the size of biglycan molecules in thrombin stimulated VSMCs is inhibited by UBO-QIC.

To investigate whether Gαq or Rho/ROCK pathways are involved in PAR-1 mediated mRNA expression of GAG synthesizing genes, VSMCs were treated with thrombin in the presence and absence of UBO-QIC, Rho/ROCK antagonist Y-27632 and PAR-1 antagonist SCH79797 and the expression of both CHST11 and CHSY1 was studied. Thrombin showed a 2.2-fold increase ($P<0.01$) in the mRNA expression of CHST11 (Figure 4.5C). In the presence of UBO-QIC and Y-27632 the thrombin mediated mRNA expression of C4ST-1 was abolished ($P<0.01$) (Figure 4.5C). In the presence of SCH79797 thrombin mediated mRNA expression of CHST11 was inhibited by 50% to 1.2-fold ($P<0.01$) (Figure 4.5C). A similar trend was seen when looking at the mRNA expression of CHSY1. Thrombin showed a 2.5-fold increase in the mRNA expression of CHSY1 ($P<0.01$) (Figure 4.5D). Thrombin mediated mRNA expression of CHSY1 was completely inhibited ($P<0.01$) in the presence of UBO-QIC. Rho/ROCK inhibitor Y-27362 and PAR-1 antagonist SCH79797 partially inhibited the thrombin mediated mRNA expression of CHSY1 to 2- and 1.2-fold respectively ($P<0.01$) (Figure 4.5D). The results presented show that thrombin mediated GAG synthesizing gene mRNA expression involves Gαq protein.


A

\[
\begin{array}{cccc}
\text{Thrombin} & \text{TGF-\(\beta\)} & \text{EGF} \\
- & 30 & 100 & - \\
- & 100 & - & 100 \\
- & 100 & - & UBO-QIC(nM) \\
\end{array}
\]

\(35^S\)S\textsubscript{4} Incorporation (cpm/well) \times 1000

B

\[
\begin{array}{cccc}
\text{Thrombin} & \text{TGF-\(\beta\)} & \text{EGF} \\
- & 30 & 100 & - \\
- & 100 & - & 100 \\
- & 100 & - & UBO-QIC(nM) \\
\end{array}
\]

CHST11 mRNA expression (Fold Change)

C

\[
\begin{array}{cccc}
\text{Thrombin} \\
- & - & - & - \\
- & - & - & + \\
- & - & - & - \\
- & - & - & + \\
- & + & + & + \\
\end{array}
\]

UBO-QIC

D

\[
\begin{array}{cccc}
\text{CHSY1 mRNA expression} \\
- & - & - & - \\
- & - & - & + \\
- & - & - & - \\
- & - & - & + \\
- & + & + & + \\
\end{array}
\]

UBO-QIC

Chapter-4. Role of Gaq
**Figure 4.5:** Gaq is involved in PAR-1 mediated proteoglycan synthesis, GAG chain elongation and the regulation of mRNA expression CHST11 and CHSY1.

A,B. VSMCs were pre-incubated with UBO-QIC (100nM) in the presence and absence of thrombin (10 Units/mL), TGF-β (2ng/mL) or EGF (100nM) for 24 hours with [35S]-sulphate (50μCi/mL). A. Medium containing secreted proteoglycans harvested and spotted onto 3MM paper and were quantitated by CPC precipitation to measure radiolabel incorporation. The histogram shows the fold-change compared to basal. B. Secreted proteoglycans were isolated using ion exchange chromatography and electrophoresed on a 4-13% SDS-PAGE.

C,D. VSMCs were pre-incubated with UBO-QIC, Y-27632 (10µM) or SCH79797 (10µM) for 30 min and then exposed to thrombin for 6 hours. Total RNA was harvested and the mRNA expression of C. CHST11 and D. CHSY1 were analysed using quantitative RT-PCR. Results are the mean and standard error from three separate experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis ##, P<0.01 basal versus thrombin and **, P<0.01 thrombin versus antagonists.
4.3.5 siRNA knockdown validation of GNAQ and GNA11 in human coronary artery smooth muscle cells

In the experiments presented earlier UBO-QIC was used as a Gaq antagonist. UBO-QIC has been shown to inhibit both Gaq signalling as well as Gaq/11 signalling (Schrage et al., 2015). Since these two Gaq isoforms share similarities and have approximately 90% homology at the protein level (Wilkie et al., 1991) we wanted to find whether the Gaq mediated transactivation leading to gene expression is isoform dependent. To address this we used siRNA specific to the GNAQ and GNA11.

The GNAQ and GNA11 mRNA expression was measured 48 hours post electroporation. VSMCs were pulsed in the presence of siRNA specific to either GNAQ or GNA11. The GNAQ siRNA caused a 90% knockdown in the mRNA expression of GNAQ (Figure 4.6A). The siRNA specific to GNA11 had no effect on the mRNA expression of GNAQ (Figure 4.6A). To study the specificity of the siRNA, CASMCs were transfected with siRNA to GNAQ and GNA11. GNA11 siRNA caused an 80% knockdown in the mRNA expression of GNA11, the GNAQ siRNA had no effect (Figure 4.6C). These results show that although the Gaq and Gaq/11 share approximately 90% homology at the protein level there is no crossover in the knockdown targets of the siRNA regarding the two Gaq isoforms.

To ensure that the knockdown of the mRNA expression results in knockdown at the protein level, the protein expression was measured as detailed in Section 4.2.8. siRNA which targets the GNAQ had a 50% knockdown of the protein expression of Gaq at 24 hours post transfection and an 80% knockdown at 48 hours (Figure 4.6B). siRNA specific to the GNA11 isoform lead to a 50%
knock down of Gaq/11 protein expression 24 hours post transfection and only a 65% knockdown at 48 hours (Figure 4.6D). These results show that the knockdown of the mRNA expression results in a knockdown at the protein level.
Figure 4.6: Validation of siRNA knock own of GNAQ and GNA11 on mRNA and protein expression in human VSMCs.

VSMCs were loaded with 4D-Nucleofector Y-kit solution and siRNA to GNAQ, GNAQ11 or non-targeting at 105pmol. A.C. total RNA was harvested 48 hours post electroporation and the mRNA expression A.GNAQ and C. GNA11 were analysed using RT-PCR. 18S was used as the house keeping gene. Data are expressed as the mean and standard error of the mean from two independent experiments. B.D. Cells were fixed with 2% paraformaldehyde 24 or 36 hours post electroporation. Cells were incubated with B. anti-Gαq or D. anti Gαq/11 overnight followed by goat anti-rabbit conjugated with Alexa488 or Alexa594. Images are representative of two independent experiments. Histograms represent mean intensity of cells presented in 5 field views. Data expressed as mean ± standard error of the mean.
To distinguish which of the Gaq proteins is involved isoform specific siRNA was used to knockdown the levels of GNAQ and GNA11 (described in detail in Chapter 4 Section 4.3.5). VSMCs were treated with thrombin in the presence and absence of the siRNA and the mRNA expression of CHST11 and CHSY1 was measured. Thrombin treatment resulted in a 2.7-fold ($P<0.01$) increase in the mRNA expression of CHST11. The knockdown of GNAQ had a 50% inhibition in thrombin mediated mRNA expression of CHST11 to 1.6-fold ($P<0.01$) (Figure 4.7A). The knockdown of GNA11 had no effect on thrombin mediated mRNA expression of CHST11. To determine the effects on basal cell activity a non-targeting siRNA was used and this had no effect on thrombin mediated mRNA expression of CHST11. A similar pattern was observed when investigating thrombin mediated mRNA expression of CHSY1. Thrombin treatment of VSMCs mediated a 1.8-fold ($P<0.01$) (Figure 4.7B) increase in the mRNA expression of CHST11. Knockdown of GNAQ completely inhibited ($P<0.01$) (Figure 4.7B) thrombin mediated mRNA expression of CHSY1. In contrast thrombin mediated mRNA expression of CHSY1 was unaffected by GNA11 knockdown. As expected the non-targeting siRNA had no effect (Figure 4.7B). These results demonstrate that thrombin mediated mRNA expression of CHST11 and CHSY1 occurs via of the Gaq but not the Gaq/11 protein.
Chapter 4. Role of Gaq

**A**

Thrombin

CHST11 mRNA expression (Fold Change)

- -

GNAQ **

GNA11

NT

**B**

Thrombin

CHSY1 mRNA expression (Fold change)

- -

GNAQ **

GNA11

NT

**

##
Figure 4.7: Specific knockdown of each of the two Gαq isoforms – Gaq and Gαq/11 on thrombin mediated mRNA expression of the GAG synthesizing genes CHST11 and CHSY1.

VSMCs were loaded with 4D-Nucleofector Y kit solution and siRNA to GNAQ, GNAQ11 or non targeting (NT) at 105pmol followed by thrombin stimulation for 6 hours. Total RNA was harvested and the mRNA expression of A. CHST11 and B. CHSY1 were analysed using RT-PCR. 18S was used as a house keeping gene. Results are the mean and standard error from three separate experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, $P<0.01$ basal versus thrombin and **,$P<0.01$ thrombin versus antagonists.
4.3.7 Thrombin mediated transactivation of the TGFBR1 occurs via the activation of the αVβ5 but not the αVβ3 integrin

PAR-1 transactivation of EGFR and TGFBR1 have distinct mechanisms, the Goq plays as the central integrating point for transactivation dependent signalling. In VSMCs thrombin mediated transactivation of the TGFBR1 occurs via cytoskeletal rearrangement which activates ROCK signalling (Burch et al., 2013). Earlier in this Chapter (Section 4.3.4) the role of ROCK signalling being downstream of the Goq was outlined. Inside out integrin signalling has been associated with cytoskeletal rearrangement and ROCK signalling (Jenkins et al., 2006). Various members of the integrin family including all αV integrins (αVβ3 and αVβ5), αIIβ3, α5β1 and α8β1 of cell surface molecules activate the latent TGF-β complex (Munger et al., 1999, Asano et al., 2005a, Asano et al., 2005b). Taking into account the relationship of integrin and the cytoskeleton/ROCK signalling, integrins make a likely candidate for a key player in the transactivation of the TGFBR1. Integrins contain an RGD binding motif, using an RGD peptide which prevents them from binding to further RGD sites, the role of integrins was studied in thrombin mediated transactivation of the TGFBR1 in VSMCs (Burch et al., 2013). Integrins play an important role in the transactivation of the TGFBR1, however it remains unknown which specific integrin is involved in the transactivation signalling cascade in VSMCs. In these experiments we endeavoured to uncover the specific integrins involved in this signalling cascade.

To evaluate whether the αVβ3 and αVβ5 integrins are present in the cell surface of the VSMCs, immunofluorescence experiments were conducted (as per Section 4.2.5). The immuno-fluorescent images shows that the αVβ3 integrin shown by the red fluorescence is present on the cell surface of the VSMCs (Figure 4.8A).
The αVβ5 integrin represented by the green fluorescence (Figure 4.8B) also shows to be present on the cell surface of the VSMCs. These results demonstrate that both the αVβ3 and αVβ5 integrins are present on the cell surface of VSMCs.

To evaluate the role of αVβ3 in this study, VSMCs were pre-treated with an αVβ3 specific blocking antibody, in the presence and absence of thrombin. Thrombin lead to a 2.5-fold increase in phospho-Smad2(465/467) at four hours (Figure 4.9A) in the presence of αVβ3 neutralizing antibody there was no effect on thrombin mediated phospho-Smad2(465/467). Utilisation of antibody which blocks the αVβ5 integrin, thrombin mediated phospho-Smad2(465/467) at one and four hours ($P<0.01$)(Figure 4.9B) was completely inhibited ($P<0.01$). These results demonstrate that thrombin mediated transactivation of the TGFBR1 occurs specifically via αVβ5 but not the αVβ3 integrin.
**Figure 4.8: Human VSMCs express cell surface αVβ3 and αVβ5.**

VSMCs were grown on coverslips for 24 hours. Cells were fixed with 2% paraformaldehyde and incubated with **A.** anti-αVβ3 or **B.** anti-αVβ5 overnight followed by goat anti-mouse conjugated with flurophores Alexa594 or Alexa488. Confocal images are representative of three independent experiments.
Figure 4.9: Thrombin mediated stimulation of phospho-Smad2(Ser465/467) is dependent on the αVβ5 but not αVβ3 integrin in human VSMCs.

VSMCs were pre-incubated with A. anti-αVβ5 or B. anti-αVβ3 for 30 minutes and then treated with thrombin (10 units/mL) for 1 and 4 hours. Cell lysates were collected and proteins (40μg) were resolved over 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was then incubated with anti-phospho-Smad2(Ser465/467) (1:1000) followed by peroxidase labelled rabbit IgG secondary antibody. Membranes were reprobed with GAPDH HRP-conjugated antibody. Histograms represent band density as fold over basal from three separate experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post hoc analysis ##,P<0.01 basal versus thrombin and **,P<0.01 thrombin versus integrin.
4.4 Summary of results

The results presented in this chapter demonstrate that thrombin mediated transactivation of the EGFR and TGFR1 is via the Gαq protein which leads to GAG chain elongation evaluated by an increase in the mRNA expression of CHST11 and CHSY1 in VSMCs *in vitro*. The specific findings from these experiments are:

- Thrombin mediated intracellular release of calcium is dose dependently inhibited in the presence of Gαq antagonist UBO-QIC.
- Thrombin mediated phospho-Smad2(465/467) and phospho-Erk1/2 were both inhibited in the presence of, UBO-QIC.
- Thrombin mediated phosphorylation of ERM, the immediate downstream products of ROCK were inhibited in the presence of UBO-QIC.
- UBO-QIC, partially inhibits thrombin mediated proteoglycan synthesis and GAG elongation.
- Thrombin mediated mRNA expression of CHST11 and CHSY1 was completely inhibited by UBO-QIC.
- Gαq and Gαq/11 were both present in CASMCs (mRNA and protein). siRNA targeting each isoform successfully knockdown both mRNA and protein expression but to different extents.
- Thrombin mediated mRNA expression of CHST11 and CHSY1 was inhibited in Gαq knockout cells and unaffected in Gαq/11 knockout cells.
- Thrombin mediated phospho-Smad2(465/467) was inhibited in the presence of αVβ5 blocking antibody but remains unaffected in the presence of αVβ3 blocking antibody although both integrins were present on the cells surface of VSMCs.
4.5 Discussion

The studies in this chapter show that PAR-1 signals through the Gαq protein which leads to the transactivation of the EGFR and the TGFBR1 leading to proteoglycan synthesis and GAG chain elongation. PAR-1 mediated transactivation of the TGFBR1 via the Gαq leads to ROCK activation which triggers cell surface integrin αVβ5. Thrombin mediated mRNA expression of the two GAG synthesizing genes CHST11 and CHSY1 occurs via Gαq activation but not the Gαq/11 isoform.

Limited availability of antagonists to the Gαq proteins has made it very difficult to study their role in GPCR signalling. YM-254890 and the UBO-QIC are potent inhibitors of Gαq protein (Inamdar et al., 2015, Kukkonen, 2016, Gao and Jacobson, 2016, Uemura et al., 2006a, Uemura et al., 2006b, Kawasaki et al., 2005, Taniguchi et al., 2003). YM-254890 was discovered and developed by Yamaguichi Pharmaceuticals, Japan, and was made available for researchers for a limited time before being taken off the market (Kawasaki et al., 2006)(In the time that this thesis was being prepared (2016) YM-254890 has been made available again). UBO-QIC has a chemical structure very similar to YM-254890, a cyclic depsipeptide. UBO-QIC was available for purchase from University of Bonn in Germany for a brief time before it was withdrawn. Hence there is a need for agents which can inhibit downstream signalling pathways of Gαq. Taniguchi et al (Taniguchi et al., 2004) isolated three analogues of the YM-254890; YM-254891, YM-254892 and YM-280193. The YM-280193 had the same cyclic core of the YM-254890 minus the hydroxyleucine residue. The first report of the synthesis of the YM-280193 was by our collaborator Brimble and colleagues (Kaur et al., 2015). The biological studies of the YM-280193 presented in this chapter (conducted in our lab) showed that unlike its analogue YM-254890, this cyclic depsipeptide was not a Gαq antagonist as it did not
inhibit thrombin mediated intracellular calcium release. The YM-254890 was co-crystalized with a Gαq-Gβγ complex and it shows that YM-254890 inhibits Gaq by binding to a pocket in the Gaq that prevents the activation of the Gaq by a cognate GPCR (Nishimura et al., 2010). A major study by Schrage and colleagues (Schrage et al., 2015) compared the binding energies and mode of action of UBO-QIC and YM-254890. The binding capacity was very similar and the results showed that UBO-QIC and YM-254890 share the same mode of action (Schrage et al., 2015). The YM-254890 and the UBO-QIC share a similar chemical structure however the YM-280193 lacks the hydroxyleucine residue, hence we postulate that this residue plays an important role in the cyclic depsipeptide inhibiting Gaq activation.

PAR-1 mediated transactivation of the EGFR and TGFBR1 leads to proteoglycan synthesis and GAG elongation (Burch et al., 2010). The signalling through each of the transactivation pathways has a distinct mechanism (Burch et al., 2013). Finding a common target could account for all the thrombin mediated GAG elongation thus this target could be potentially therapeutically beneficial as it can inhibit the pre-inflammatory phase of atherosclerosis (Little, 2013). Thrombin mediated transactivation of the EGFR and TGFBR1 is regulated by the Gaq protein (Figure 4.3). Thrombin mediated mRNA expression of the GAG synthesizing genes leading to proteoglycan synthesis and GAG chain elongation is regulated by Gaq (Figure 4.4). This shows that Gaq plays a central integrating point in GPCR mediated GAG elongation. UBO-QIC has no selectivity for the Gaq isoforms (Schrage et al., 2015) hence, using siRNA knockdown, the results obtained show that only the Gaq is involved in thrombin mediated GAG elongation evaluated by the mRNA expression of CHST11 and CHSY1. Although the role of the Gaq and Gaq/11 was not studied on the downstream intermediate of EGFR (phospho-Erk1/2) and TGFBR1 (phospho-
Smad2(465/467), we postulate that only the Gaq is involved in GPCR mediated transactivation in VSMCs. Future studies require the use of siRNA to knockdown the Gaq and Gaq/11 to study their role on thrombin mediated phospho-Smad2(465/467) and the phospho-Erk1/2. Apart from the results in this Chapter there have been no studies which compare these two isoforms in any human model.

The results in this chapter show that Gaq plays a central integrating point in GPCR mediated proteoglycan synthesis (Burch et al., 2010, Burch et al., 2013, Little et al., 2010). There have been a number of studies showing the importance of Gaq signalling in the vasculature (Adams et al., 1998, D'Angelo et al., 1997). The Gaq signalling was pharmacologically studied using the YM-254890. The Gaq played an important role in platelet function and in vitro thrombus formation under high shear stress in cynomolgus monkeys (Uemura et al., 2006a). YM-254890 inhibited phenylephrine, serotonin and ET-1 induced contractions in the rat aorta improving blood flow (Uemura et al., 2006b). Thus YM-254890 was useful in treating peripheral arterial disease in a rat model. YM-254890 compound also had antithrombotic effects in an electrically induced arterial thrombosis in a rat model (Kawasaki et al., 2003). Shortly after the same group showed that oral administration of YM-254890 inhibited neointima formation 3 weeks after vascular injury (Kawasaki et al., 2005). However YM-254890 caused hypotensive effects at doses three times higher than those that produced inhibitory effects on neointima formation (Kawasaki et al., 2005). Hence the systemic use of a Gaq antagonist may be limited, owing to the narrow therapeutic window of YM-254890. There is a need for a small chemical entity that targets GPCR mediated Gaq signalling which can be used to inhibit the pre-inflammatory stage of atherosclerosis by inhibiting GAG chain elongation.
There are a limited amount of studies on the role of Gαq in GPCR transactivation of PTKR. In L6 myoblasts derived from rat skeletal muscle, ET-1 via the endothelin type A receptor mediated transactivation of PDGF receptor was inhibited in the presence of YM-254890 (Harada et al., 2014). Shear stress induced phospho-Erk1/2 in CHO cells permanently transfected with wild type angiotensin II type 1 receptor was completely inhibited in the presence of YM-254890 (Barauna et al., 2013). Shear stress activates PTKRs (Jin et al., 2003) thus showing that the Gαq plays a role in angiotensin II mediated transactivation of PTKR. Purigenic receptor P2Y2R transactivation of the insulin-like growth factor receptor in keratinocytes was inhibited by Gaq/11 siRNA transfected cells and YM-254890 (Taboubi et al., 2010). Consistent with the literature the data presented in this Chapter shows that the Gαq plays a role in transactivation of the PTKR in this case the EGFR. In keratinocytes transactivation dependent signalling was observed to occur via the Gaq/11, however in VSMCs transactivation dependent signalling leading to GAG enzyme expression occurs via the Gαq.

VSMC data presented in Figure 4.3A shows the Gαq plays a role in thrombin mediated transactivation of the TGFBR1. The GPCR mediated transactivation of the TGFBR1 mechanisms downstream of Gαq, shows that PAR-1 activation of ROCK activates cell surface integrin αVβ5. The exact mechanism of how integrin signalling activated TGFBR1 is unknown. It has been proposed that the pathway is restricted to the extracellular surface where a GPCR activates ROCK signalling via cytoskeletal rearrangement, leading to integrin activation. Activated integrins can bind to the large latent complex leading to conformational changes, exposing the TGF-β ligand (Lawrence et al., 1984). The ligand is then free to engage the TGFBR1 and initiate downstream signalling (Munger et al., 1999, Wipff and Hinz, 2008). There are 24
known integrins however the RGD motif of fibronectin can recognise and be activated by eight of the αV integrins (Margadant and Sonnenberg, 2010, Jenkins et al., 2006). Three different ligands including thrombin were used to show PAR-1 transactivation of the TGFBR1 via αVβ6 dependent mechanisms in mouse lung epithelial cells (Jenkins et al., 2006). The same group has also shown that LPA can induce αVβ6 mediated TGFBR1 activation in human epithelial cells via Gαq mediated activation pathways (Xu et al., 2009). In mouse VSMCs isolated from mouse arteries angiotensin II induced transactivation of the TGFBR1 was inhibited in the presence of αVβ3-integrin antagonist, SB22345 (Belmadani et al., 2008). LPA and methacholine induced TGFBR1 transactivation in human airway smooth muscle cells was inhibited in the presence of αVβ5 neutralizing antibody (Tatler et al., 2011). TGFBR1 signalling in dermal fibroblasts is also dependent on the αVβ5 integrin (Asano et al., 2006a, Asano et al., 2006b, Boo and Dagnino, 2013, Vi et al., 2011).

Taking into account the results presented in this Chapter and the literature, integrin activation of the TGFBR1 is context dependent. In the mouse VSMCs the αVβ3 integrin is involved in angiotensin II transactivation of the TGFBR1 (Belmadani et al., 2008) however in human VSMCs and idiopathic pulmonary fibrosis lung tissue (Scotton et al., 2009) the αVβ5 integrin is involved in PAR-1 transactivation of the TGFBR1. Thus showing that the integrins involved in transactivation may be dependent to the GPCR agonist or the difference in isoform expressed in different cell types.

Studies on role of Gαq in GPCR mediated transactivation of the S/TKR are limited. Xu and colleagues (Xu et al., 2009) studied the role of G proteins in LPA mediated transactivation of the TGFBR1. Mouse embryonic cells with Gαq and Gα12/13 knockout showed only that the Gαq is involved in LPA induced
transactivation of the TGFBR1 (Xu et al., 2009). This was confirmed with a pharmacological approach using Gaq antagonist GP-2A (Tanski et al., 2004, Mukai et al., 1992). In primary epithelial cells LPA induced TGFBR1 activation was inhibited in the presence of GP-2A (Xu et al., 2009). In idiopathic pulmonary fibrosis lung tissue, PAR-1 induced transactivation of the TGFBR1 occurred via the Gaq protein (Scotton et al., 2009). Moreover the attenuation of PAR-1 induced activation of the TGFBR1 with Rho kinase antagonist (Y-27632) firmly places the Rho signalling pathway downstream of this response (Scotton et al., 2009). Similarly in murine lung fibroblasts, thrombin mediated CCL2 production occurs via PAR-1 coupling to Gaq and the cooperation between Rho signalling pathways (Deng et al., 2008). The results from the literature outline that GPCR transactivation of the TGFBR1 occurs via Gaq mediated signalling which activates the Rho kinase. The results observed in this Chapter are consistent with what is currently known of this cascade. With the novel finding that of the two highly expressed Gaq isoforms, the transactivation dependent signalling may be specific to only the Gaq.

In summary, this Chapter has identified that PAR-1 transactivation of the TGFBR1 and the EGFR is via the Gaq protein. Thrombin mediated mRNA expression of CHST11 and CHSY1 leading to proteoglycan synthesis and GAG elongation is regulated by Gaq. Thus Gaq is a central target for transactivation dependent GAG elongation. Gaq isoforms Gaq and Gaq/11 are the most highly and ubiquitously expressed isoforms. In VSMCs thrombin mediated mRNA expression of CHST11 and CHSY1 occurs via the activation of Gaq but not Gaq/11 isoform. Thrombin transactivation of the TGFBR1 is via Gaq which activates ROCK signalling, leading to the activation of the αVβ5 integrin but not the αVβ3 integrin. These findings show specific mechanisms in thrombin transactivation of the
TGFBR1. The results of this Chapter show that Gαq mediates all transactivation signalling and is a potential target to prevent pathophysiology arising from PAR-1 signalling.
Chapter-5. GPCR mediated Smad2 linker region phosphorylation is dependent on transactivation of EGFR and TGFBR1
5.1 Introduction

From the data presented in Chapter 3 it was concluded that thrombin stimulated mRNA expression of CHST11 and CHSY1 is through the transactivation of both the TGFBR1 and EGFR. TGFBR1 signals downstream via Smad transcription factors (Derynck and Zhang, 2003). The canonical TGF-β signalling pathway involves direct carboxy-terminal phosphorylation of Smad2 by TGFBR1. Two serine residues 465 and 467 are phosphorylated then the product complexes with co-Smad4 for translocation to the nucleus and the regulation of gene expression. In addition to the carboxy-terminal phosphorylation, Smads can be phosphorylated in the linker region (Massague et al., 2005, Hough et al., 2012, Burch et al., 2010, Burch et al., 2011, Kamato et al., 2013c, Kamato et al., 2014a, Rezaei et al., 2012, Matsuzaki, 2011, Matsuzaki et al., 2009). Smad linker region has been shown to be phosphorylated by multiple kinases including MAPK (Erk, Jnk and p38), phosphatidylinositol 3-kinase (PI3K) and CDK (Kamato et al., 2013c). The role of serine/threonine kinases in the Smad linker region phosphorylation has been reviewed in the literature (Kamato et al., 2013c, Rostam et al., 2015, Rezaei et al., 2012). The linker region has been shown to play an important role in TGF-β mediated biological activity. Initial observations indicated that linker region phosphorylation prevented nuclear translocation and thus inhibited TGF-β signalling (Kretzschmar et al., 1999, Matsuzaki, 2011) however, subsequent studies have shown that the phosphorylation of this region can result in a range of complex responses (Kamato et al., 2014a, Hough et al., 2012, Burch et al., 2010).

There are multiple sites in the linker region of Smads that are potential phosphorylation sites and these include Thr220, Ser245, Ser250 and Ser255 (in
Smad2) (Burch et al., 2010, Kamato et al., 2013c, Kamato et al., 2014a, Matsuzaki, 2011, Matsuzaki et al., 2009, Liu and Feng, 2010, Wrighton et al., 2009). Previous work from our lab used phosphorylation site-specific antibodies to these four residues in vascular endothelial cells to explore the signalling pathways with the ultimate aim of understanding the role of TGF-β in endothelial dysfunction (Kamato et al., 2014a). The pathways of phosphorylation by different serine/threonine kinases were highly specific for the four target residues. The TGF-β mediated regulation of PAI-1 mRNA expression is via the activation of different serine/threonine kinases, thus demonstrating the complex nature of non-Smad signalling.

In the context of ECM synthesis and proteoglycan modification, the stimulation of biglycan expression as well as the hyperelongation of biglycan GAG chains are dependent on Smad2 linker region phosphorylation (Burch et al., 2010). Using a Smad2 linker region antibody which targets the three phosphorylated serine residues, it was found that Erk and p38 mediated Smad2 linker region phosphorylation and these kinases also stimulated proteoglycan synthesis. Smad2 linker region phosphorylation is an important avenue for the integration of multiple signalling networks to influence the outcome of proteoglycan synthesis in this model. As previously described, thrombin is a well characterised agonist which drives the pathways that result in proteoglycan modifications (Burch et al., 2010, Ivey and Little, 2008). In human VSMCs, thrombin mediated proteoglycan synthesis occurs via the transactivation of PTKR and S/TKR, EGFR and TGFBR1, respectively (Burch et al., 2013). The data in Chapter 3 shows that PAR-1 mediated GAG synthesizing gene mRNA expression also requires the activation of these two signalling pathways. Hence in this Chapter we will investigate the hypothesis that thrombin will signal via
the transactivation of both the TGFBR1 and the EGFR leading to the activation of serine/threonine kinases which will phosphorylate the individual Smad2 linker region residues. Furthermore, these results will provide mechanistic insight into thrombin mediated mRNA expression of CHST11 and CHSY1 in response to the same serine/threonine kinases.

5.2 Materials and Methods

5.2.1 Materials

Flavopiridol, SP600125, SB202190 and LY294002 were purchased from Sigma-Aldrich (St Louis, USA). UO126 was from Promega (WI, USA). Anti-phospho-Smad2(Thr220), anti-phospho-Smad2(Ser245), anti-phospho-Smad2(Ser250) and anti-phospho-Smad2(Ser255) rabbit IgG polyclonal were a gift from Professor Koichi Matsuzaki (Kansai Medical University, Osaka, Japan)

5.2.2 Cell culture

VSMCs were grown in DMEM (5mM glucose, 10% FBS and 1% antibiotics at 37°C in 5% CO2). VSMCs were seeded in 60 mm dishes and 6 well-plates (described in detail Chapter 2 Section 2.1). Cells were grown to confluence then rendered quiescent by serum deprivation for 48 hours. Inhibitors were pre-incubated for 30 mins prior to treatment with agonists. Incubation times and concentrations are given in detail in the figure legends.

5.2.3 Western blotting

The method of western blot analysis is described in detail in Chapter 2 Section 2.2.6. Whole cell lysates were resolved by SDS-PAGE and transferred
onto PVDF. Membranes were blocked with 5% BSA and incubated with primary antibody targeting protein of interest followed by HRP-anti-rabbit IgG and ECL detection. The protein of interest was normalised with GAPDH to determine equal loading.

5.2.4 Measurement of mRNA gene expression

Quantitative RT-PCR was conducted as described in Chapter 2 Section 2.2.3. Data was normalised to the ribosomal 18S housekeeping gene to adjust for control variations between individual experiments.

5.2.5 Statistical analysis

Normalised data is expressed as the mean ± standard error of the mean of three independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by least significant difference post-hoc analysis. Results were considered significant when the probability was less than 0.05 and 0.01.

5.3 Results

5.3.1 Thrombin stimulates the phosphorylation of individual Smad2 linker region residues in VSMCs

There are at least four important phosphorylation sites on the human Smad2 linker region at Thr220, Ser245, Ser250 and Ser255 (Kamato et al., 2013c). To determine whether site-specific linker region phosphorylation is mediated by thrombin signalling in VSMCs a time course study was preformed utilising separate antibodies specific for the individual phosphorylated linker region residues (Thr220, Ser245, Ser250 and Ser255) (Sekimoto et al., 2007).
Thrombin treated VSMCs showed a rapid increase in phospho-Smad2(Thr220) residue of the Smad2 linker region. Thrombin mediated phosphorylation peaked at 15 mins with a 2.5-fold change ($P<0.01$) when compared to basal (Figure 5.1A). Thrombin mediated phospho-Smad2(Thr220) was sustained although there was a gradual decline over the 240 min treatment. There was a rapid increase in phospho-Smad2(Ser245) residue reaching a peak of 1.9-fold at 15 mins ($P<0.01$) when compared to basal (Figure 5.1B). The thrombin mediated phosphorylation was sustained for the 240 min treatment. Similarly there was a rapid increase of phospho-Smad2(Ser250) in the presence of thrombin with a peak phosphorylation of 2.5-fold ($P<0.01$) at 15 mins (Figure 5.1C). The phosphorylation of this residue was sustained for 60 mins. Thrombin causes a rapid increase in phospho-Smad2(Ser255) with a peak at 15 mins of 2.5-fold ($P<0.01$) when compared to basal and this gradually declined over the 120 mins.

These results demonstrate that the phosphorylation of Thr220, Ser245, Ser250 and Ser255 residues on the Smad2 linker region are mediated by thrombin signalling pathway. Thrombin mediated phosphorylation of each of the individual residues showed a rapid response with the maximum phosphorylation for all residues at 15 mins, the earliest time point evaluated. Thr220, Ser250 and Ser255 showed maximal phosphorylation with 2.5-fold increase at 15 mins (Figure 5.1). Ser245 showed the least amount of phosphorylation when VSMCs were treated with thrombin. The four residues show similar temporal characteristics but the question is the signalling pathways and kinases that are involved in mediating their phosphorylation.
C

Thrombin

-  15'  30'  60'  120'  240'

pSmad2(Ser250)
Smad2
GAPDH

phospho-Smad2(Ser250)
(Fold Change)

D

Thrombin

-  15'  30'  60'  120'  240'

pSmad2(Ser255)
Smad2
GAPDH

phospho-Smad2(Ser255)
(Fold Change)
**Figure 5.1: Time course of thrombin mediated phosphorylation of Smad2 linker region residues.**

VSMCs treated with thrombin (10 Units/ml) for up to 240 min. Cells lysates were collected and proteins were resolved over 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated overnight with A anti-phospho-Smad2/3(Thr220/179) B anti-phospho-Smad2(Ser245) C anti-phospho-Smad2(Ser250) and D anti-phospho-Smad2(Ser255) (1:1000). Followed by peroxidase-labelled rabbit IgG secondary antibody. Membranes were reprobed with Smad2 monoclonal antibody and GAPDH HRP-conjugated antibody. Histograms represent band density expressed as fold over basal from three independent experiments (*P<0.05 vs basal) (**P<0.01 vs basal) using one-way ANOVA followed by least significant difference post-hoc analysis.
5.3.2 Thrombin mediated Smad2 linker region phosphorylation occurs via transactivation signalling

Burch et al (Burch et al., 2010) showed a correlation between TGF-β mediated Smad2 linker region phosphorylation and proteoglycan synthesis. Thrombin mediated proteoglycan synthesis occurs due to the transactivation of both EGFR and TGFBR1 (Burch et al., 2013). Therefore to study whether thrombin mediated Smad2 linker region phosphorylation occurs via the transactivation pathways we utilized the EGFR antagonist, AG1478 (Zhu et al., 2001) and the TGFBR1 antagonist, SB431542 (Inman et al., 2002).

To have an initial look at whether thrombin mediated phosphorylation of the Smad2 linker region was dependent on transactivation of the EGFR and TGFBR1, the pan Smad2 linker antibody targeting the three serine residues was used. VSMCs stimulated with thrombin for 30 mins showed a 4.5-fold ($P<0.01$) (Figure 5.2) increase in the phospho-Smad2(Ser245/250/255). SB431542 had a slight inhibitory effect on thrombin mediated linker region phosphorylation however was not statistically significant. AG1478 inhibited thrombin mediated phospho-Smad2(Ser245/250/255) by approximately 60% to 2.5-fold ($P<0.01$)(Figure 5.2). To show that these distinct pathway are activated and the antagonists are inhibiting their respective pathways, VSMCs were treated with TGF-β and EGF in the presence and absence of their antagonists. TGF-β had a 3-fold ($P<0.01$) (Figure 5.2) increase in phospho-Smad2(Ser245/250/255), in the presence of SB431542 the TGF-β mediated phospho-Smad2(Ser245/250/255) was inhibited to 1.5-fold ($P<0.01$). EGF stimulated VSMCs caused a 2.5-fold ($P<0.01$) increase in the phospho-Smad2(Ser245/250/255), this was abolished in the presence of AG1478 (Figure 5.2). These results demonstrate that at 30 mins thrombin mediated Smad2 linker
region phosphorylation occurs via the transactivation of the EGFR but not the TGFBR1. The results presented in this experiment show that direct stimulation of the TGFBR1 and the EGFR result in Smad2 linker region phosphorylation. Hence in the following sections the role concentration and time dependence of the TGFBR1 in thrombin mediated Smad2 linker region phosphorylation was determined.
Figure 5.2: Thrombin mediated phosphorylation of Smad2 linker region is mediated through transactivation of the EGFR.

VSMCs treated with thrombin (10 Units/ml), TGF-β (2 ng/ml) or EGF (100 nM) at 30 mins in the presence and absence of SB431542 (3µM) AG1478 (5µM). Cells lysates were collected and proteins were resolved over 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated overnight with anti-phospho-Smad2(Ser245/250/255) (1:1000). Followed by peroxidase-labelled rabbit IgG secondary antibody. The membranes were reprobed with GAPDH HRP-conjugated antibody. Histograms represent band density expressed as fold per basal from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis **, $P<0.01$ versus basal and ##, $P<0.01$ agonist versus antagonist.
5.3.3 Thrombin mediated Smad2 linker region phosphorylation occurs through the transactivation of the EGFR

The results presented in Figure 5.2 show that thrombin mediated Smad2 linker region phosphorylation at 30 mins is only via transactivation of the EGFR. The results presented in Figure 5.2 as well as published data (Burch et al., 2010) show that TGF-β stimulated VSMCs leads to Smad2 linker region phosphorylation at the same time point. Our previous published data also shows that TGF-β mediated phosphorylation of the Smad2 linker region correlates with proteoglycan synthesis and GAG chain elongation (Burch et al., 2010). Thrombin mediated proteoglycan synthesis and GAG elongation occurs via the transactivation of both the EGFR and TGFBR1 (Burch et al., 2010). The hypothesis is that thrombin mediated Smad2 linker region phosphorylation occurs via transactivation of both EGFR and TGFBR1. TGF-β mediated phosphorylation of the Smad2 linker region was studied in bovine aortic endothelial cells, a 10μM concentration of SB431542 was used to effectively inhibit signalling downstream of TGFBR1 (Kamato et al., 2014a). This concentration is consistent with the published study characterising SB431542 as a TGFBR1 antagonist in which 10μM of the antagonist was optimal to inhibit TGFBR1 signalling (Inman et al., 2002). The concentration of SB431542 used in Figure 5.2 was 3μM therefore we propose that the response may be concentration dependent. To address this hypothesis a dose-dependent study was conducted using EGFR antagonist, AG1478 (0.5-10μM) and TGFBR1 antagonist, SB431542 (0.3-30μM), in the presence of thrombin for 30 mins. VSMCs were also treated with agonists to the EGF and TGF-β pathways in the presence and absence of their respective inhibitors to ensure that the activation of these pathways leads to the phosphorylation of the Smad2 linker region.
Thrombin increased phospho-Smad2(Thr220) to 2.7 fold \( (P<0.01) \) when compared to basal (Figure 5.3A). In the presence of AG1478, thrombin mediated phospho-Smad2(Thr220) was dose dependently inhibited showing significant inhibition at concentrations of 0.5-10µM \( (P<0.01) \) (Figure 5.3A). AG1478 at 1µM completely inhibited thrombin mediated phospho-Smad2(Thr220). EGF used as a positive control had a 2.5-fold \( (P<0.01) \) increase in phospho-Smad2(Thr220) and as expected this response was abolished in the presence of AG1478. Thrombin mediated phosphorylation of the serine residues of the Smad2 linker region had a similar response to the phosphorylation of the Thr220 residue. Thrombin treated VSMCs showed a 7.7-fold \( (P<0.01) \) increase in phospho-Smad2(Ser245/250/255) (Figure 5.3B). In the presence of AG1478 a partial inhibition of approximately 4-fold \( (P<0.01) \) (0.1-5µM) was seen (Figure 5.3B). EGF treated VSMCs showed a 5-fold \( (P<0.01) \) increase in the phospho-Smad2(Ser245/250/255) and as expected this was completely abolished in the presence of AG1478. The data presented demonstrates that thrombin transactivates the EGFR to phosphorylate the serine and Thr220 residues of the Smad2 linker region. Thrombin mediated phospho-Smad2(Thr220) at 30 mins is completely due to the transactivation of the EGFR. However thrombin mediated phospho-Smad2(Ser245/250/255) at 30 mins is only partially due to transactivation as AG1478 does not completely inhibit this response.

Thrombin treated VSMCs showed a 5-fold \( (P<0.01) \) increase in phospho-Smad2(Thr220) (Figure 5.3C). This response was unaffected in the presence of SB431542 from 0.3-30µM. VSMCs stimulated with TGF-β as a positive control resulted in a 10-fold \( (P<0.01) \) increase in phospho-Smad2(Thr220) residue and this was abolished in the presence of SB431542.
(P<0.01) (Figure 5.3C). Thrombin treated VSMCs showed a 12-fold (P<0.01) increase in phospho-Smad2(Ser245/250/255). This response was maintained in the presence of SB431542 (0.3-30µM) (Figure 5.3D). However, as expected TGF-β increased phospho-Smad2(Ser245/250/255) and this was inhibited by SB431542 (P<0.01) (Figure 5.3D). These results indicate that at 30 mins thrombin mediated Smad2 linker region phosphorylation is occurring only through the transactivation of the EGFR and not the TGFBR1.
Chapter 5. Smad2 linker region phosphorylation

A

**Thrombin**
- 0.5 1 5 10
**EGF**
- 5 AG1478 (μM)

pSmad2/3 (Thr179/220)

GAPDH

Fold Change

B

**Thrombin**
- 0.5 1 5 10
**EGF**
- 5 AG1478 (μM)

pSmad2 (Ser245/250/255)

GAPDH

Fold Change
Figure 5.3: Thrombin mediated linker region phosphorylation is through the transactivation EGFR

VSMCs treated with thrombin (10 Units/ml) or TGF-β (2 ng/ml) for 30 mins EGF (100 nM) for 15 mins, in the presence and absence of A-B AG1478 (0.5-10µM) or C-D SB431542 (0.3-30µM). Cell lysates were collected and proteins were resolved on 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with A,C. anti-phospho-Smad2/3(Thr220/179) (1:1000) or B,C. anti-phospho-Smad2(Ser245/250/255) (1:1000). Followed by peroxidase-labelled rabbit IgG secondary antibody. The membranes were reprobed with GAPDH HRP-conjugated antibody. Histograms represent band density expressed as fold per basal from three independent experiments Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, P<0.01 versus basal and **, P<0.01 agonist versus antagonist.
5.3.4 Thrombin mediated transactivation of the TGFBR1 leading to phosphorylation of the Smad2 linker region is time dependent.

The results presented in Figures 5.2 and 5.3C and D show that at 30 mins thrombin mediated phosphorylation of the Smad2 linker region does not occur via the transactivation of the TGFBR1. In bovine aortic endothelial cells TGF-β mediated phosphorylation of the Smad2 linker region residues peaked at 60 mins (Kamato et al., 2014a). Similarly in VSMCs, TGF-β mediated phosphorylation of the Smad2 linker region was not studied until the 60 min time point (Burch et al., 2010). The hypothesis is thrombin transactivation of the TGFBR1 leading to Smad2 linker region phosphorylation has a different temporal response when compared to thrombin transactivation of the EGFR. To address this hypothesis VSMCs were treated with thrombin (10 Units/ml) at 60, 120 and 240 mins in the presence and absence of TGFBR1 antagonist SB431542. TGF-β for 60 mins in the presence and absence of SB431542 was used as a positive control.

Thrombin treatment of VSMCs at 60 mins caused a 2.2-fold increase in phosho-Smad2(Thr220), this was unaffected in the presence of SB431542. At 120 min post thrombin stimulation there was a 4.2-fold \( (P<0.01) \) increase in phosho-Smad2(Thr220), this was inhibited to 1.8 fold \( (P<0.01) \) in the presence of SB431542 (Figure 5.4A). Similarly at 240 mins a 3.8-fold \( (P<0.01) \) increase in phosho-Smad2(Thr220) was seen and this was inhibited by 50% in the presence of SB431542 \( (P<0.01) \) (Figure 5.4A). TGF-β caused a large increase of 4-fold \( (P<0.01) \) in phosho-Smad2(Thr220) and this was abolished by SB431542.
The thrombin mediated phospho-Smad2(Ser245/250/255) followed a similar pattern where at 60 mins post thrombin treatment there was a 2.2-fold increase that was unaffected in the presence of SB431542 (Figure 5.4B). Thrombin treatment of 120 mins leads to a 3.8-fold ($P<0.01$) increase in phospho-Smad2(Ser245/250/255), this was inhibited to 1.8-fold ($P<0.01$) in the presence of SB431542 (Figure 5.4B). VSMCs stimulated with thrombin for 240 mins had a 4-fold ($P<0.01$) increase in phospho-Smad2(Ser245/250/255) and this was inhibited by 50% to 1.8-fold ($P<0.01$) in the presence of SB431542. TGF-β treated VSMCs caused a large increase of 6.2-fold ($P<0.01$) in phospho-Smad2(Ser245/250/255) which was abolished in the presence of the receptor antagonist, SB431542 ($P<0.01$). These results indicated that in VSMCs thrombin mediated phosphorylation of the Smad2 linker region via the transactivation of the TGFBR1 is time dependent. The same pathway occurs for phosphorylation of both the serine and Thr220 residues. In summary the thrombin transactivation of the EGFR occurs in 30 mins post thrombin stimulation however the transactivation of the TGFBR1 is activated at 120 mins and is sustained for 240 mins.
Figure 5.4: Thrombin mediated Smad2 linker region phosphorylation via the TGFBR1 is time dependent.

VSMCs treated with thrombin (10 Units/ml) over 240 mins time course or TGF-β (2ng/ml) for 60 mins in the presence and absence of SB431542 (10µM). Cell lysates were collected and proteins were resolved on 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with A. anti-phospho-Smad2/3(Thr220/179) (1:1000) or B. anti-phospho-Smad2(Ser245/250/255) (1:1000). Followed by peroxidase-labelled rabbit IgG secondary antibody. The membranes were reprobed with Smad2 antibody and GAPDH HRP conjugated antibody. Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, $P<0.01$ versus basal and **, $P<0.01$ agonist versus antagonist.
5.3.5 Thrombin mediated transactivation of the EGFR leading to the phosphorylation of the Smad2 linker region is sustained for 4 hours.

We have shown that in VSMCs thrombin mediated transactivation of the TGFBR1 leading to Smad2 linker region phosphorylation is time dependent. We know that in VSMCs thrombin mediated transactivation of the EGFR leading to linker region phosphorylation occurs rapidly at 30 mins however we wanted to investigate whether this response was sustained for 240 mins. To address this question VSMCs were treated with thrombin in a time dependent manner in the presence and absence of EGFR antagonist AG1478. EGF stimulation at 15 mins in the presence of AG1478 was used as a positive control.

Thrombin stimulated VSMCs at 60 mins showed a 6-fold ($P<0.01$) increase in phospho-Smad2(Thr220), this was inhibited by 75% to 2-fold ($P<0.01$) in the presence of AG1478 (Figure 5.5A). Thrombin treated VSMCs at 120 mins lead to a 4.5-fold ($P<0.01$) increase in the phospho-Smad2(Thr220) and at 240 mins a 4-fold ($P<0.01$) increase (Figure 5.5A). When VSMCs where treated with thrombin in the presence of AG1478 the phosphorylation of both these time points was inhibited by 50% to 2- ($P<0.01$) and 1.8-fold ($P<0.01$) respectively. EGF treated VSMCs caused a large increase in phospho-Smad2(Thr220) and this response was abolished in the presence of AG1478 ($P<0.01$). Thus showing that the phosphorylation of the Thr220 residue occurs via thrombin transactivation of the EGFR and is sustained for the 4 hours of this study.
Thrombin stimulation of VSMCs at 60 mins had a 2.5-fold ($P<0.01$) increase in phospho-Smad2(Ser245). In the presence of AG1478 at 60 mins there was no change in the phosphorylation of this residue. Thrombin treated VSMCs at 120 mins leads to a 2-fold ($P<0.01$) increase in phospho-Smad2(Ser245) and this was partially inhibited to 1.8-fold ($P<0.01$) in the presence of EGFR antagonist AG1478 (Figure 5.5B). At 240 mins post thrombin stimulation VSMCs had a 2-fold ($P<0.01$) increase in phospho-Smad2(Ser245), this response was partially inhibited to 1.5-fold ($P<0.05$) in the presence of AG1478. EGF treatment caused a 2-fold ($P<0.01$) increase in phospho-Smad2(Ser245), which was abolished ($P<0.01$) in the presence of AG1478 (Figure 5.5B).

Thrombin stimulated VSMCs at 60 mins leads to a 2.2-fold ($P<0.01$) increase in phospho-Smad2(Ser250) (Figure 5.5C) which is completely inhibited ($P<0.01$) in the presence of AG1478. The pattern was the same for this residue at the 120 and 240 min time points, with a 2- and 2.5-fold ($P<0.01$) increase in phospho-Smad2(Ser250) that was inhibited to 1- and 1.5-fold ($P<0.01$), respectively, in the presence of AG1478. EGF treatment caused a 2.2-fold ($P<0.01$) increase in phospho-Smad2(Ser250) and in the presence of AG1478 the response was abolished ($P<0.01$)(Figure 5.5C). The Smad2 linker region residue Ser255 followed the same signalling pathway as described with the previous residues (Figure 5.5D). Thrombin treated VSMCs caused a 2-fold increase in phospho-Smad2(Ser255) at 60, 120 and 240 min ($P<0.01$). In the presence of AG1478, the response was inhibited by approximately 90-95% ($P<0.01$) for the 3 time points (Figure 5.5D).

These results demonstrate that thrombin transactivation of the EGFR phosphorylates Thr220, Ser245, Ser250 and Ser255 residues of the Smad2
linker region. The EGFR transactivation pathway leading to the phosphorylation of the Smad2 linker region residues is activated from 30 to 240 mins in the residues investigated, with the exception of the Ser245 residue. The phosphorylation of Smad2 linker region residue Thr220, Ser250 and Ser255 is inhibited by 80-90% in the presence of AG1478 at the three time points studied, thus showing that almost all the phosphorylation of these three residues is occurring through the transactivation of the EGFR. However when looking at the Ser245 residue we see that there is no inhibition with AG1478 at 60 min and only a 20-30% inhibition at 120 and 240 mins, thus showing that not all the thrombin mediated signalling is through EGFR transactivation for this residue.
Chapter 5. Smad2 linker region phosphorylation

A

Thrombin

EGF

60' 120' 240' 15'

- - + - + + +

AG1478 (5\(\mu\)M)

pSmad2/3(Thr179/220)

GAPDH

phospho-Smad2(Thr220)

(Fold Change)

B

Thrombin

EGF

60' 120' 240' 15'

- - + - + + +

AG1478 (5\(\mu\)M)

pSmad2(Ser245)

GAPDH

phospho-Smad2(Ser245)

(Fold Change)
Figure 5.5: Thrombin mediated Smad2 linker region phosphorylation via the EGFR is sustained for four hours.

VSMCs were treated with thrombin (10 Units/ml) over 240 min time course or EGF (100nM) for 15 mins in the presence and absence AG1478 (5µM). Cell lysates were collected and proteins were resolved on 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated overnight A. anti-phospho-Smad2/3(Thr220/179) B. anti-phospho-Smad2(Ser245) C. anti-phospho-Smad2(Ser250) and D. anti-phospho-Smad2(Ser255) (1:1000). Followed by peroxidase-labelled rabbit IgG secondary antibody. The membranes were reprobed with GAPDH HRP conjugated antibody. Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##,P<0.01 versus basal and **, P<0.01 agonist versus antagonist.
5.3.6 The involvement of serine/threonine kinases in thrombin mediated phosphorylation of Smad2 linker region residues via the transactivation of the EGFR in VSMCs.

Smad2 linker region phosphorylation also termed ‘non Smad signalling’ (Zhang, 2009) or ‘Smad independent signalling’ (Derynck and Zhang, 2003) is activated by intracellular serine/threonine kinases (Burch et al., 2010, Kamato et al., 2014a, Kamato et al., 2013c, Rezaei et al., 2012). The results presented above show that thrombin mediated phosphorylation of the Smad2 linker region is via the transactivation of both the EGFR and the TGFBR1 however the specific intermediate kinases which lead to the phosphorylation of the individual linker region residue is yet to be determined.

GAG chain hyperelongation on biglycan involves the phosphorylation of Smad2 linker region by serine/threonine kinases (Burch et al., 2010). We have shown that at 30 mins thrombin mediated phosphorylation of the Smad2 linker region is solely through the transactivation of the EGFR. This section will focus on the kinases which mediate thrombin transactivation of the EGFR leading to the phosphorylation of the individual residues on the Smad2 linker region. The following serine/threonine kinase were examined using these inhibitors; MEK 1/2 inhibitor, UO126, to inhibit its downstream kinase Erk, SB202190 as a p38 inhibitor, Jnk inhibitor SP600125, PI3K inhibitor, LY294002 and flavopirodol as the CDK inhibitor. Concentrations of the inhibitors were based on a previous study from our laboratory which examined the phosphorylation of the individual residues on the Smad2 linker region in bovine aortic endothelial cells (Kamato et al., 2014a).
**Smad2 linker region residue - Thr220**

VSMCs treated with thrombin for 30 mins showed a 2.7-fold \( (P<0.01) \) increase in phospho-Smad2(Thr220) (Figure 5.6A). The presence of the Erk and CDK inhibitor, UO126 and flavopiridol respectively they had no effect on the thrombin mediated phospho-Smad2(Thr220). The presence of p38 and Jnk inhibitors, SB202190 and SP600124 partially inhibited thrombin mediated phospho-Smad2(Thr220) to 1.8-fold \( (P<0.01) \) (Figure 5.6A). PI3K inhibitor, LY294002, completely inhibited the thrombin mediated phospho-Smad2(Thr220). These results demonstrate that the phosphorylation of the Thr220 residue of the Smad2 linker region via thrombin tranactivation of the EGFR involves p38, Jnk and PI3K pathways but not Erk and CDK.

**Smad2 linker region residue – Ser245**

Consistent with earlier results (Figure 5.1), thrombin treatment of VSMCs caused an increase in phospho-Smad2(Ser245) to 2-fold \( (P<0.01) \) at 30 mins (Figure 5.6B). The presence of inhibitors to Erk, p38 and Jnk had no effect on the thrombin mediated phospho-Smad2(Ser245). PI3K inhibitor, LY294002, partially inhibited \( (P<0.01) \) thrombin mediated phospho-Smad2(Ser245). The presence of CDK inhibitor, flavopiridol, completely inhibited thrombin mediated phospho-Smad2(Ser245). Thus, thrombin mediated transactivation of the EGFR stimulated Ser245 phosphorylation through the activation of PI3K and CDK pathways.

**Smad2 linker region residue – Ser 250**

Cells treated with thrombin, increased the phospho-Smad2(Ser250) to 3-fold \( (P<0.01) \) (Figures 5.6C). The presence of Erk inhibitor, UO126, partially
inhibits phospho-Smad2(Ser250) to 2-fold ($P<0.05$). In the presence of inhibitors to p38, Jnk, PI3K and CDK the thrombin mediated phospho-Smad2(Ser250) is unaffected (Figure 5.6C). Thus showing that Erk is the only kinase involved in thrombin mediated transactivation of the EGFR leading to the phosphorylation of Smad2 linker region residue Ser250.

**Smad2 linker region residue – Ser 255**

Consistent with the results in Figure 5.1D VSMCs treated with thrombin showed a 3-fold ($P<0.01$) increase in the phospho-Smad2(Ser255) (Figure 5.6D). In the presence of U0126 the thrombin mediated response is completely inhibited ($P<0.01$). Treatment with p38 inhibitor, SB202190, caused a partial inhibition to 2-fold ($P<0.01$) of thrombin mediated phospho-Smad2(Ser255). Jnk inhibitor had no effect on this response. PI3K and CDK antagonists inhibit phospho-Smad2(Ser255) to 1.5- and 1.2-fold ($P<0.01$), respectively (Figure 5.6D). Thus showing that thrombin mediated transactivation of the EGFR leading to the phospho-Smad2(Ser255) occurs via the activation of the Erk, p38, PI3K and CDK pathways. Taken together the results of these experiments show that the individual Smad2 linker region residues are phosphorylated by different serine/threonine kinases, with the PI3K being a common kinase for Thr220, Ser245 and Ser255 (see Figure 5.9A).
Figure 5.6: Effects of inhibitors to MAPK (Erk, P38 and Jnk), CDK and PI3K on thrombin mediated Smad2 linker phosphorylation via the transactivation of the EGFR.

VSMCs were treated with thrombin (10Units/mL) in the presence and absence of each of the following serine/threonine kinase inhibitors: UO126, SB202190, SP600125, LY294002 and flavopiridol for 15 mins. Cell lysates were collected and protein (30µg) were resolved on 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated overnight

A. anti-phospho-Smad2/3(Thr220/179)  B. anti-phospho-Smad2(Ser245)
C. anti-phospho-Smad2(Ser250) and D. anti-phospho-Smad2(Ser255) (1:1000).

Followed by peroxidase-labelled rabbit IgG secondary antibody. The membranes were reprobed with GAPDH HRP conjugated antibody. Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis

##, P<0.01 versus basal and **, P<0.01 agonist versus antagonist.
5.3.7 The involvement of serine/threonine kinases in thrombin mediated phosphorylation of Smad 2 linker region residues via the transactivation of the TGFBR1.

The data presented so far shows that thrombin mediated phosphorylation of the Smad2 linker region at 4 hours is via the transactivation of both the EGFR and TGFBR1. This section will focus on the serine/threonine kinases involved in thrombin mediated phosphorylation of the Smad2 linker region residues via the transactivation of the TGFBR1. To address this aim the serine/threonine kinases inhibitors were utilised in the presence of the EGFR antagonist AG1478, to eliminate the contribution of the EGFR and to show that the kinases involved in the TGFBR1 transactivation signalling pathway only.

**Smad2 linker region residue - Thr220**

Thrombin treated VSMCs showed a 2.4-fold ($P<0.01$) increase phospho-Smad2(Thr220) at 4 hours compared to the non-treated cells (Figure 5.7A). The presence of EGFR antagonist, AG1478, blocked the thrombin mediated phospho-Smad2(Thr220) to 1.2-fold ($P<0.01$). In the presence of both AG1478 and the MAPK inhibitors, there is a further reduction in phospho-Smad2(Thr220) when compared to thrombin treatment in the presence of AG1478. UO126 and SB202190 inhibited thrombin mediated phospho-Smad2(Thr220) via transactivation of TGFBR1 to 0.6-fold ($P<0.05$) (Figure 5.7A). SP600125 had a slight inhibitory effect on thrombin mediated phospho-Smad2(Thr220) via transactivation of TGFBR1 however this effect did not reach statistical significance. LY294002 and flavopiridol in the presence of AG1478 inhibited phospho-Smad2(Thr220) to 0.4- ($P<0.01$) and 0.8-fold when compared to VSMCs treated with thrombin in the presence of AG1478. These results demonstrated that thrombin mediated phospho-Smad2(Thr220) is mostly
via the transactivation of the EGFR through p38, Jnk and CDK. The thrombin mediated transactivation of the TGFBR1 leading to phospho-Smad2(Thr220) appears to be predominantly through the activation of Erk, p38 and PI3K.

**Smad2 linker region residue – Ser245**

Thrombin treatment of VSMCs caused an increase in phospho-Smad2(Ser245) to 2-fold \( (P<0.01) \)(Figure 5.7B). In the presence of AG1478, there is a partial inhibition in the thrombin mediated phospho-Smad2(Ser245) to 1.6-fold \( (P<0.05) \). MAPKs inhibitor UO126 \( (P<0.01) \) and SP600125 had a slight inhibition on phospho-Smad2(Ser245) via thrombin transactivation of TGFBR1. However the SB202190, LY294002 and flavopiridol had no effect (Figure 5.7B). These results show that thrombin mediated phospho-Smad2(Ser245) is via the transactivation of the TGFBR1 and it requires the activation of Erk.

**Smad2 linker region residue – Ser250**

Cells treated with thrombin at 4 hours stimulated phospho-Smad2(Ser250) to 2.1-fold \( (P<0.01) \)(Figure 5.7C). AG1478 blocked the thrombin mediated phospho-Smad2(Ser250) to 1.4-fold \( (P<0.01) \)(Figure 5.7C). AG1478 in the presence UO126, had a slight inhibition of the phospho-Smad2(Ser250) to 0.9-fold \( (P<0.05) \) when compared to thrombin in the presence of AG1478. When AG1478 was in the presence of SB202190, SP600125, LY294002 and flavopiridol there was no change to phospho-Smad2(Ser250) (Figure 5.7C). These results demonstrate that Erk but not p38, Jnk, PI3K and CDK are mediators of thrombin transactivation of TGFBR1 stimulated phosphorylation of Ser250 (see Figure 5.9B).

**Smad2 linker region residue – Ser255**
Phosphorylation of Ser255 was stimulated to 1.5-fold ($P<0.01$)(Figure 5.7D) when VSMCs were treated with thrombin for 4 hours. AG1478 slightly inhibited thrombin mediated phospho-Smad2(Ser255) to 1.25-fold ($P<0.05$). UO126 and SP600125 in the presence of AG1478 had no effect on thrombin mediated phospho-Smad2(Ser255) via the TGFBR1. In the presence of AG1478 with either SB202190, LY294002 or flavopiridol the thrombin mediated phosphorylation of Ser255 was inhibited ($P<0.01$) when compared to thrombin mediated phospho-Smad2(Ser255) in the presence of AG1478. These results indicated that p38, PI3K and CDK are involved in the thrombin mediated phospho-Smad2(Ser255) via the transactivation of TGFBR1. Taken together with our earlier findings, this implies that thrombin mediated phosphorylation of the Smad2 linker region occurs via the transactivation of both the EGFR and the TGFBR1 to induce the mRNA expression of CHST11 and CHSY1 in human VSMCs. The signalling intermediates involved in thrombin mediated phosphorylation of the Smad2 linker region via the transactivation of the EGFR differ to those involved in the transactivation of the TGFBR1. A summary of the findings of the kinases involved in the two transactivation pathways is presented in Table 5.1.
Chapter-5. Smad2 linker region phosphorylation
Chapter 5. Smad2 linker region phosphorylation

C

Thrombin
- - + + + + + +
- - - - - - - -
AG1478 (5 μM)
UO126 (3 μM)
SB202190 (3 μM)
SP600125 (1 μM)
LY294002 (20 μM)
Flavopiridol (500 nM)

pSmad2(Ser250)
PbGAPDH

phospho-Smad2(Ser250)
(Fold Change)

D

Thrombin
- - + + + + + +
- - - - - - - -
AG1478 (5 μM)
UO126 (3 μM)
SB202190 (3 μM)
SP600125 (1 μM)
LY294002 (20 μM)
Flavopiridol (500 nM)

pSmad2(Ser255)
PbGAPDH

phospho-Smad2(Ser255)
(Fold Change)
Figure 5.7: Effects of inhibitors to MAPK (Erk, P38 and Jnk), CDK and PI3K on thrombin mediated Smad2 linker phosphorylation via the transactivation of the TGFBR1 in VSMCs.

VSMCs were treated with thrombin (10Units/mL) in the presence and absence of each of the following serine/threonine kinase inhibitors: UO126, SB202190, SP600125, LY294002 and flavopiridol for 4 hours. Cell lysates were collected and protein were resolved on 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated overnight with

A. anti-phospho-Smad2/3(Thr220/179)  B. anti-phospho-Smad2(Ser245)
C. anti-phospho-Smad2(Ser250)  and  D. anti-phospho-Smad2(Ser255) (1:1000).

Followed by peroxidase-labelled rabbit IgG secondary antibody. The membranes were reprobed with GAPDH-HRP conjugated antibody. Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis ##, P<0.01 versus basal, **, P<0.01 agonist versus AG1478 and ^^, P<0.01 AG1478 versus serine/threonine kinase inhibitors.
5.3.8 Serine/threonine kinases differentially regulate thrombin mediated mRNA expression of GAG synthesizing genes CHST11 and CHSY1.

Chapter 3 shows that thrombin mediated mRNA expression of GAG synthesizing enzymes CHST11 and CHSY1 correlate to our published data on proteoglycan synthesis and GAG elongation (Burch et al., 2013). The data shows that thrombin mediated GAG gene mRNA expression and GAG elongation signal via the transactivation of both the EGFR and TGFBR1 (Chapter 3 Section 3.3.5). Proteoglycan synthesis and GAG chain elongation have previously been shown to correlate with the phosphorylation of the Smad2 linker region (Burch et al., 2010). Smad2 linker region phosphorylation occurs indirectly via serine/threonine kinases (Matsuzaki, 2011). Hence, the serine/threonine kinases which are involved in thrombin mediated phosphorylation of the Smad2 linker region residues (Sections 5.3.6 and 5.3.7) will differentially regulate thrombin mediated mRNA expression of CHST11 and CHSY1.

In VSMCs, TGF-β mediated GAG chain hyperelongation requires Erk and p38 activity (Burch et al., 2010). To study the role of these serine/threonine kinases on the mRNA expression of CHST11 and CHSY1, VSMCs were treated with thrombin for 6 hours in the presence and absence of inhibitors towards each of the following kinases; MEK 1/2 (UO126), p38 (SB202190), Jnk (SP600125), PI3K (LY294002) and CDK (flavopiridol). The concentration of the inhibitors used was identical to the previous experiments in this Chapter.

VSMCs treated with thrombin showed a 2.9-fold ($P<0.01$)(Figure 5.8 A) increase in the mRNA expression of CHST11 compared to non-treated cells.
(Figure 5.8A). In the presence of inhibitors to Erk and p38; UO126 and SB202190 thrombin stimulated mRNA expression of CHST11 was almost completely inhibited to 1.2- and 1.1-fold ($P<0.01$), respectively. The presence of Jnk inhibitor SP600125 showed no inhibition of CHST11 mRNA expression stimulated by thrombin. Inhibitors to PI3K and CDK, LY294002 and flavopiridol, completely inhibited thrombin mediated mRNA expression of CHST11. Thus data correlates to that observed with TGF-β signalling of GAG hyperelongation in which Erk and p38 are involved but not Jnk. This data shows that Erk, p38, PI3K and CDK pathways are involved in thrombin stimulation of CHST11 mRNA expression.

The kinases involved in thrombin mediated mRNA expression of GAG synthesizing gene, CHSY1, also examined. Thrombin treatment of VSMCs showed a 2-fold increase ($P<0.01$) of CHSY1 mRNA expression (Figures 5.8B) compared to untreated cells. VSMCs treated with thrombin in the presence of UO126 showed an inhibition of CHSY1 mRNA expression to 1.5-fold ($P<0.05$) when compared to thrombin treatment alone (Figure 5.8 B). The presence of SB202190 resulted in a total inhibition ($P<0.01$) of thrombin mediated mRNA expression of CHSY1. The CHSY1 mRNA expression was not blocked by SP600125. However cells treated with thrombin in the presence of LY294002 and flavopiridol showed an inhibition of CHSY1 mRNA expression to 1.2- and 0.8-fold ($P<0.01$), respectively. These data demonstrated that thrombin signalling to CHSY1 mRNA expression involved MAPK Erk and p38 but not Jnk, as well as PI3K and CDK. The serine/threonine kinases involved in thrombin mediated mRNA expression of GAG enzymes CHST11 and CHSY are the same for both genes (see Figure 5.9).
Figure 5.8: Serine/threonine kinases differentially regulate thrombin mediated mRNA expression of GAG synthesizing enzymes CHST11 and CHSY1.

VSMCs were pre-incubated with UO126 (3μM), SB202190 (3μM), SP600125 (1μM), LY294002 (20μM) or flavopiridol (500nM) for 30 mins prior to thrombin (10 Units/ml) exposure for 6 hours to determine the effects on the mRNA expression of A. CHST11 and B. CHSY1. Total RNA was harvested and the mRNA expression of CHST11 and CHSY1 were analysed using RT-PCR. 18S was used as a house keeping gene. Data are expressed as the mean ± standard error from three experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference post-hoc analysis. ##,P<0.01 basal versus thrombin and *,P<0.01 and **,P<0.01 thrombin versus antagonist was used for statistical analysis.
Chapter 5. Smad2 linker region phosphorylation

---

A

EGFR → PAR-1

→ Gα Gβγ

↓

Jnk p38 Erk PI3K CDK

↓

Thr220 Thr220 Ser255 Ser255 Thr220 Ser245 Ser255 Ser245 Ser255

↓

CHST11

CHSY1

Possible downstream activation
Downstream activation

Ser/Thr Kinases
Smad linker region residues
GAG gene expression

B

PAR-1 → TGFB1

↓

Gα Gβγ

↓

p38 Erk PI3K CDK

↓

Thr220 Thr220 Ser255 Ser255 Thr220 Ser245 Ser255 Ser245 Ser255

↓

CHST11

CHSY1

Possible downstream activation
Downstream activation

Ser/Thr Kinases
Smad linker region residues
GAG gene expression
Figure 5.9: Schematic diagram illustrating the proposed thrombin mediated transactivation leading to Smad2 linker region phosphorylation. Transactivation of A. EGFR or B. TGFBR1 involving serine/threonine kinases and individual Smad2 linker region residues to stimulate CHST11 and CHSY1 mRNA expression.
5.4 Summary of results

The results presented in this chapter demonstrate that thrombin through PAR-1 mediates transactivation of the EGFR and TGFBR1 to phosphorylate the individual residues of the Smad2 linker region by serine/threonine kinase intermediates as well as the signalling to GAG synthesizing enzyme mRNA expression in VSMCs *in vitro*. The specific findings from these experiments are:

- Thrombin mediated phosphorylation of the Smad2 linker region residues had a rapid increase response at 15 min, the phosphorylation of the residues gradually declined however was sustained for the 4 hours.
- Thrombin mediated phosphorylation of the Smad2 linker region occurs via the transactivation of the EGFR within 15 mins and is sustained for up to 4 hours.
- Thrombin mediated phosphorylation of the Smad2 linker region also occurs via transactivation of the TGFBR1, this signalling pathway is delayed with an activation period from 2-4 hours.
- Thrombin mediated transactivation of the EGFR leads to the phosphorylation of the Smad2 linker Thr220 residue through the activation of p38, Jnk and PI3K and not through Erk and CDK. Phosphorylation of Ser245 is mediated by PI3K and CDK. Ser250 phosphorylation occurs through Erk whereas Ser255 phosphorylation involved Erk, p38 and CDK (see Table 5.1).
- Thrombin mediated transactivation of the TGFBR1 leads to the phosphorylation of the Thr220 residue through the activation of Erk, p38 and PI3K. Phosphorylation of Ser245 occurs through Erk activation. The
Ser250 phosphorylation is mediated through Erk whereas p38, PI3K and CDK lead to the phosphorylation of Ser255 (see Table 5.1).

- Thrombin mediated mRNA expression of CHST11 and CHSY1 involves Erk, p38, PI3K and CDK but not Jnk (see Table 5.1).
Table 5.1: A summary of thrombin signalling via the transactivation of the EGFR and TGFBR1 pathways.

Transactivation dependent signalling pathways involving serine/threonine kinases, individual Smad2 linker region residues and the mRNA expression of CHST11 and CHSY1.

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<td>Ser255</td>
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<td><strong>TGFBR1</strong></td>
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<td>Ser245</td>
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<td><strong>GAG genes</strong></td>
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5.5 Discussion

The studies in this chapter show that in VSMCs, thrombin mediated Smad2 linker region phosphorylation occurs via the EGFR and TGFBR1. The phosphorylation of the individual Smad2 linker region residues is mediated by a number of serine/threonine kinases. Data outlined in Chapter 3 demonstrates that thrombin mediated proteoglycan modification correlates with mRNA expression of GAG synthesizing genes. These results show that thrombin signals through the transactivation of both the EGFR and TGFBR1, which leads to an increase in biglycan size and an increase in the expression of CHST11 and CHSY1 mRNA expression. Earlier studies from our lab had shown that Erk and p38 are associated with the increase in size of GAG chain on biglycan through Smad2 linker region phosphorylation. Here we aimed to characterise whether or not Smad2 linker region phosphorylation occurs via thrombin transactivation of the EGFR and TGFBR1. Phosphorylation of Smad2 at the carboxy terminal occurs directly through the activation of the TGFBR1. Smad2 linker region phosphorylation occurs indirectly through the activation of serine/threonine kinases which in turn phosphorylate the Smad2 linker region residues (Matsuzaki, 2011). Here we aimed to identify the serine/threonine kinases involved in thrombin transactivation of the EGFR compared to the kinases involved in thrombin transactivation of the TGFBR1 and whether these kinases are involved in regulating the mRNA expression of GAG synthesizing genes responsible for GAG elongation.

There are at least four important phosphorylation sites in the Smad2 linker region. In humans, the Smad2 linker region residues are Thr220, Ser245, Ser250 and Ser255. This study has focused on the Smad2 transcription factor as in VSMCs it has been shown to play an important role in proteoglycan
synthesis. The TGF-β stimulated CHSY1 protein was inhibited in nucleus pulposus cells of intervertebral discs with Smad2 siRNA but not Smad3 siRNA, indicating that Smad2, but not Smad3 was involved in the regulation of CHSY1 expression (Hu et al., 2015). Thrombin treatment of VSMCs has a rapid increase in the phosphorylation of the individual Smad2 linker region residues with a peak phosphorylation at 15 min. The Smad2 linker region phosphorylation varies between the individual residues. Thrombin had a substantial increase in the phosphorylation of Thr220, Ser250 and Ser255 compared to the phosphorylation of Ser245. TGF-β mediated phosphorylation of the Ser245 residue in bovine aortic endothelial cells (Kamato et al., 2014a) and VSMCs (Rostam et al., 2016) was less then two fold in both studies whereas the other residues were phosphorylated to a much higher fold change. In mouse embryo fibroblasts infected with Smad2 mutant, TGF-β mediated phospho-Smad2(Ser245) was considerably less than the phosphorylation of the three other residues of the linker region (Matsuzaki et al., 2009). These results indicate that the Ser245 residue of the Smad2 linker region may not be as heavily implicated in signalling via the TGFBR1 as compared to the other residues.

The rapid increase in the phosphorylation of the Smad2 linker region residues is due to thrombin transactivation of the EGFR. GPCR agonists such as LPA (Brusevold et al., 2014, Jeong et al., 2013, Daub et al., 1997), thyrotropin releasing hormone (Ohmichi et al., 1994), ET-1 (Cazaubon et al., 1994, Daub et al., 1996) and thrombin (Burch et al., 2013, Huang et al., 2012, Smiljanic et al., 2014) rapidly induce the transactivation of PTKR, EGFR. GPCR mediated transactivation of the EGFR occurs via the activation of a membrane bound MMPs resulting in the cleavage and release of the EGF ligand which
subsequently activated its cognate receptor in an autocrine manner (Prenzel et al., 1999, Fischer et al., 2003, Gschwind et al., 2002). The results presented in this chapter reveal that thrombin transactivation of the TGFBR1 leading to the phosphorylation of the Smad2 linker region is time dependent and is delayed when compared to EGFR transactivation. GPCR mediated transactivation of the TGFBR1 is time dependent in various conditions. In mouse embryonic fibroblast transfected with β6 integrin, LPA mediated transactivation of the TGFBR1 studied by the phosphorylation of the downstream intermediate Smad2 was evident at 2-4 hours post treatment (Xu et al., 2009). In human airway smooth muscle cells, LPA and methacholine induced cells resulted in a delayed increase in the phosphorylation of the Smad2 protein signifying a delay in GPCR mediated transactivation of the TGFBR1 (Tatler et al., 2011). In VSMCs, the protracted time course in thrombin mediated transactivation of the TGFBR1 is not due to new protein synthesis. Inhibitor of translation and transcription cycloheximide had no effect on thrombin mediated phosphorylation of the Smad2 (Burch et al., 2013). GPCR transactivation of the TGFBR1 occurs via cytoskeletal rearrangement which activates ROCK signalling, preceding to the activation of cell surface integrins which bind to the latent TGF-β complex. This results in the exposure of the TGF-β ligand to the receptor (Munger et al., 1999). The cell surface integrin activation which are involved in the transactivation of the TGFBR1 are predicted to be the reason this transactivation cascade is delayed. Integrins signal via inside out signalling, which allow for the talin and kindlin to bind to the cytoplasmic end of the β subunit (Munger et al., 1999). Signalling via transactivation of TGFBR1 leading to Smad2 linker region phosphorylation as compared to GPCR transactivation of EGFR is delayed due
to the cell surface mechanisms which are involved in transactivation dependent signalling.

Interestingly, thrombin mediated phosphorylation of Smad2 linker region via the EGFR was sustained for the 4 hour duration of the study, coinciding with the TGFBR1 mediated transactivation pathway. The cross over in the transactivation pathways makes it difficult to distinguish whether there is a difference in the kinases which lead to the phosphorylation of the Smad2 linker region residues. To study the kinases involved in thrombin mediated phosphorylation of the Smad2 linker region residues via the transactivation of the TGFBR1, EGFR antagonist was utilised to block out EGFR signalling. The use of the EGFR inhibitor blocks most of the thrombin mediated phosphorylation of Thr220, Ser250 and Ser255 therefore not allowing for a true representation of thrombin transactivation of the TGFBR1. To distinguish the signalling occurring via the TGFBR1 leading to the phosphorylation of Smad2 linker region, EGFR signalling will need to be neutralised. An experimental approach that could address this issue is by using siRNA specific to the EGFR can be used to knock down EGFR signalling in VSMCs. Once the knockdown has been validated the transfected cells will need to be treated in the presence of thrombin and the serine/threonine kinases outlined above to determine specifically which kinases are involved in thrombin transactivation of TGFBR1 leading to phosphorylation of individual residues of Smad2 linker region. A genetically modified cell line containing a frame shift mutation in a coding exon of EGFR may be another way to studying signalling pathways of thrombin transactivation of the TGFBR1. This cell line may be purchased or can be customised using clustered regularly interspaced short palindromic repeat
(CRISPR) technology. The cell line can be used as a model to signify how the signalling may occur in human VSMCs.

Phosphorylation of Thr220 via thrombin transactivation of the EGFR is through the activation of MAPKs, p38 and Jnk and through the activation of PI3K but not through Erk and CDK. Transactivation of the TGFBR1 leading to the phosphorylation of the Thr220 did not require Jnk activity, however was through the stimulation of Erk, p38 and PI3K. Studies published by our lab show that in VSMCs, TGF-β mediated Smad2 linker region phosphorylation of the serine residues occurs through the activation of Erk and p38 but not Jnk, thus showing that the transactivation pathway going through the TGFBR1 signal via the same kinases to phosphorylate the Thr220 residue. Interestingly, Thr220 phosphorylation via the transactivation of the EGFR required the activation of Jnk. There has been a number of studies showing that PTKR agonists have activated the Jnk pathway leading to the phosphorylation of the Smad2/3 linker region. Hepatocyte growth factor treated RGM-1 cells activated the Jnk pathway, inducing phosphorylation of Smad2 carboxy terminal and Smad2 linker region (Mori et al., 2004). PDGF treated rat hepatic stellate cells caused a rapid increase in the phosphorylation of the Smad2/3 threonine residue that was dose dependently inhibited in the presence of Jnk inhibitor (Yoshida et al., 2005). Interestingly, Jnk, was not involved in the phosphorylation of any of the other Smad2 linker region residues in thrombin mediated transactivation of both EGFR and TGFBR in VSMCs. However in a rat model of liver injury activation of Jnk was accompanied by phosphorylation of Smad2 Ser249 and 254 (Yoshida 2005). Thus showing that Jnk activation of the threonine residue of the Smad2 linker region may be specific for PTKR signalling in VSMCs.
Erk was shown to regulate thrombin mediated transactivation of the EGFR leading to the phosphorylation of Ser250 and Ser255 (Figure 5.9A). In thrombin transactivation of the TGFBR1 Erk was shown to activate the Thr220, Ser245 and Ser250 residues of the Smad2 linker region (Figure 5.9B). In VSMCs, TGF-β signalling of Smad2 linker region leading to proteoglycan synthesis and GAG hyperelongation occurred via Erk activation (Burch et al., 2010). However due to the use of the pan linker antibody, the individual residues involved were not identified. In this study antibodies targeting the individual residues of the Smad2 linker region where utilised. The data presented in this chapter demonstrates that Smad2 linker region phosphorylation varies between the individual residues. Another recent study by our lab showed that in bovine aortic endothelial cells, TGF-β activation of Erk leads to the phosphorylation of Ser245, Ser250 and Ser255 (Kamato et al., 2014a). In TGF-β mediated phosphorylation of the Smad2 linker region in human VSMCs, Erk was involved in the phosphorylation of Ser245, Ser250 and Ser255 residues but not the phosphorylation of the Thr220 (Rostam et al., 2016). Interestingly the Thr220 residue was not phosphorylated by TGF-β mediated Erk phosphorylation in both bovine aortic endothelial cells and VSMCs. Hence we could postulate that signalling of TGFBR1 via transactivation could activate different signalling intermediates when compared to direct stimulation of the TGFBR1. To address this, a knock out model of the EGFR as outlined above could be used to study the signalling specifically via the transactivation of the TGFBR1. The cell surface mechanisms involved in the transactivation dependent signalling may also justify why there is a difference between direct TGFBR1 signalling and transactivation dependent signalling on the Smad2 linker region. To address this the role of the cell surface proteins involved in the
transactivation dependent signalling will need to be investigated on thrombin mediated phosphorylation of the individual serine/threonine kinases involved. Thus allowing to interpret whether the difference between the direct TGFBR1 signalling and transactivation dependent signalling is due to the cell surface mechanisms.

CDK activation is involved in thrombin transactivation of the EGFR and the TGFBR1 leading to the phosphorylation of Smad2 linker region serine residues. Thrombin mediated transactivation of the EGFR via CDK phosphorylates the Ser245 and Ser255 residues, however transactivation via the TGFBR1, CDK only phosphorylates the Ser255 residue (Figure 5.9). In both signalling pathways the activation of CDK does not phosphorylate the Thr220 and the Ser250 residue. In bovine aortic endothelial cells, TGF-β activation of CDK lead to the phosphorylation of Ser245 and Ser255 but not Ser250 (Kamato et al., 2014a). Thus showing that in these two cell models CDK does not activate the Ser250 residue of the Smad2 linker region leading to PAI-1 expression in endothelial cells (Kamato et al., 2014a) or the expression of CHST11 and CHSY1 in VSMCs. Consistent with the results presented in this chapter flavopiridol is not involved in the phosphorylation of the Smad2 linker region residue Thr220 in a melanoma model (Cohen-Solal et al., 2011). Direct TGFBR1 signalling in VSMCs reveals that CDK is only involved in the phosphorylation of the Thr220 residue of the Smad2 linker region (Rostam et al., 2016). Interestingly, unlike what we see in our model the literature shows that TGF-β stimulated CDK family phosphorylation has a greater preference over the Thr220 than the serine residues (Yumoto et al., 2013, Matsuura et al., 2004, Matsuzaki et al., 2009). The use of specific Smad3 linker phosphorylated antibodies shows that the four sites (Thr179, Ser204, Ser208 and Ser213) were
phosphorylated by CDK4 and CDK2 (Matsuura et al., 2004). CDK 8 and CDK9 were able to phosphorylate the linker region of Smad3 at Thr179, Ser208 and Ser213 (Alarcon et al., 2009). The CDK mediated phosphorylation events create variable Smad2/3 phospho-isoforms that can differentially interact with Smad4 and either translocate or be blocked from entering the nucleus to initiate transcription of target genes. Based on these studies, we found that there may be a number of CDK isoforms that may be responsible for the phosphorylation of the Smad2 linker region. This study used the CDK inhibitor flavopiridol, which targets at least six CDK isoforms and limits the conclusions about specific CDK isoforms. Future investigations should endeavour to identify which of the many CDK isoforms are activated by thrombin in VSMCs. In the event that more than one is activated, the use of specific CDK inhibitors or isoforms knockdown can be used to identify which CDK isoforms are responsible for the phosphorylation of the individual Smad2 linker residues. In Mv1Lu mink lung epithelial cells, the Smad3 linker region is phosphorylated by CDK 2 and 4 acting as mediators in the anti-proliferative effects of TGF-β (Matsuura et al., 2004). CDK activity is involved in cell cycle progression (Malumbres and Barbacid, 2009), hence is increased in the proliferative diseases such as cancer (Massague, 2004). The progression of atherosclerosis involves cellular proliferation and migration of VSMCs and there is evidence to suggest it is regulated by CDK driven cell cycle (Wessely, 2010, Ross et al., 2001). In aortas of cholesterol-fed rabbits, elevated expression and activity of CDK1, CDK2 and CDK4 was reported after 8 weeks of modest plaque development (Zettler et al., 2010). The results presented here support the involvement of CDK in the development of atherosclerosis highlighting a novel role of CDK which causes an increased GAG chain.
elongation represented by the change in expression levels of GAG genes, CHST11 and CHSY1.

PDGF and thrombin are known to activate PI3K via Akt (Osman et al., 2011). In VSMCs, PI3K participates in PDGF stimulated synthesis of proteoglycan core protein (Getachew et al., 2010a, Osman et al., 2014). Unpublished data from our lab shows that PI3K is partially involved in thrombin mediated proteoglycan synthesis. The role of Akt in thrombin mediated Smad2 linker region was studied at the 1-hour time point (Osman et al., 2011). VSMCs treated with thrombin increased the phosphorylation of the serine residues of Smad2 linker region, this was unaffected by Akt inhibitor SN30978 (Osman et al., 2011). PAR-1 stimulation has been shown to promote intracellular signalling though the activation of Erk, PKC and PI3K cascades which are involved in the regulation of the cell cycle regulator cyclin D (Parrales et al., 2011). PI3K in this study was involved in thrombin mediated phosphorylation of Smad2 linker region through the transactivation of both the EGFR and TGFBR1. PI3K was also shown to participate in thrombin mediated mRNA expression of CHST11 and CHSY1. Studies have shown that PI3K could act as upstream mediators for CDK activation (Major et al., 2004). PI3K/PDK1 (phosphoinistide dependent kinase-1) signalling is reported to activate the CDK-cyclin complexes required for ForkheadBoxM1B dependent transcriptional activity (Major et al., 2004). In the data presented in this chapter, thrombin mediated transactivation of the EGFR leads to the phosphorylation of the Smad2 linker region residues Ser245 and Ser255 is via PI3K and CDK. Similarly both CDK and PI3K are involved in thrombin mediated transactivation of TGFBR1 leading to the phosphorylation of Smad2 linker region residue Ser255. These results demonstrate that in VSMCs thrombin regulation of the Smad2 linker serine residues does not involve the
PI3K/Akt signalling but instead the PI3K/CDK pathway. Thus correlating with the literature that thrombin mediated linker region phosphorylation does not involve Akt (Osman et al., 2011).

In fibroblasts, TGF-β induced replication can be regulated through PI3K activated Erk signalling pathway and lead to the phosphorylation of Smad linker region phosphorylation and gene expression (Hough et al., 2012). PI3K acts upstream of Erk in TGF-β mediated phosphorylation of Smad2 linker residues Ser245, Ser250 and Ser255 in bovine aortic endothelial cells (Kamato et al., 2014a). In this study the PI3K/Erk pathway is involved in thrombin mediated phosphorylation of Ser255 residue via transactivation of the EGFR and thrombin mediated phosphorylation of Thr220 via transactivation of TGFBR1. Thus showing that PI3K signalling cascade can be cell or receptor dependent. TGF-β mediated phosphorylation of Thr220 in bovine aortic endothelial cells occurs via the PI3K/CDK signalling cascade. In this present study, we demonstrate that thrombin mediated transactivation of the EGFR leads to the phosphorylation of Thr220 through PI3K activity however CDK was not involved. The PI3K/Erk pathway was also ruled out as Erk inhibitor had no effect on thrombin mediated phosphorylation of Thr220 via transactivation of the EGFR. We postulate that phosphorylation of the Thr220 in this model occurs via the PI3K/Akt pathway as published data in VSMCs shows that the three serine residues are unaffected by PI3K.

Thrombin mediated mRNA expression of CHST11 and CHSY1 is via the transactivation of the TGFBR1 and the EGFR (see Chapter 3). The mRNA expression of the GAG synthesizing genes correlates with proteoglycan synthesis and GAG elongation (Burch et al., 2011). TGF-β mediated GAG chain elongation has been shown to correlate with the phosphorylation of the Smad2
linker region. Our results show that thrombin can activate Erk, p38, PI3K and CDK to stimulate an increase in the mRNA expression of CHST11 and CHSY1. Among the MAPK involved in TGF-β signalling in VSMCs, Erk and p38 but not Jnk have been identified to modify GAG chains. In nucleus pulposus cells of invertebral discs, Erk is shown to participate in TGF-β regulation of CHSY1 expression (Hu et al., 2015). The data presented in this chapter is consistent with these two models. Interestingly the TGF-β mediated GAG elongation does not require Jnk activation, similarly thrombin signalling to GAG gene mRNA expression does not require Jnk. This study shows that Jnk activity is not required in the phosphorylation of the serine residues in thrombin transactivation of the EGFR or TGFBRI however thrombin mediated transactivation of the EGFR leading to the phosphorylation of Thr220 required Jnk activity. From this we can postulate that the Thr220 residue may not be involved in the GAG elongation process but may be involved in other processes such as the initiation of the linkage region. To study whether thrombin mediated transactivation of EGFR leading to the phosphorylation of Thr220 via Jnk is involved in the initiation of the linkage region of the GAG chains, VSMCs should be treated with thrombin in the presence and absence of Jnk inhibitor and the mRNA expression of XXYLT-1 should be measure. The XXYLT-1 enzyme is responsible for the transfer of a xylose to the proteoglycan core protein to initiate the biosynthesis of the CS GAG chain.

The use of phospho-specific antibodies highlights the unique role of each responsible kinase that phosphorylates different individual serine/threonine residues despite mediating similar outcomes of increased GAG synthesizing enzyme CHSY1 and CHST11 mRNA expression. The exact mechanism(s) by which the specificity of individual Smad2 linker region phosphorylation regulate
Smad2 signalling remains to be clearly elucidated. Linker region phosphorylation may allosterically regulate intramolecular interactions between the MH1 and MH2 domains, and intermolecular interactions between Smads and other molecules (e.g. cytoplasmic anchors or cofactors) (Ross and Hill, 2008). Differential localization of protein kinases that can phosphorylate Smad2/3 whether in the cytoplasm or nucleus also affects multiple signalling responses that determine the functional cell outcome (Matsuzaki, 2013). Apart from investigations on the upstream signalling events at the protein kinase level, it is also essential to elucidate the involvement of co-activators or co-repressors that modulate the promoter sequences of each individual GAG enzyme genes that ultimately affects the transcriptional gene regulation (Matsuzaki, 2013). Smad2/3-Smad4 complex must interact with other DNA-binding cofactors to achieve high affinity and selectivity for specific subsets of target genes (Massague et al., 2005). The activator protein-1 knockout cells was shown to prevent the TGF-β-mediated CHSY1 promoter activity indicating that this protein is required for CHSY1 promoter activity to occur (Hu et al., 2015). It remains to be shown whether CHST11 promoter is activated by similar combinations of co-activators as to CHSY1, which could provide explanation behind the same serine/threonine kinase mediators involved in CHSY1 and CHST11 regulation.

In summary thrombin transactivates both the EGFR and TGFBR1 which leads to the phosphorylation of the Smad2 linker region residues. The two transactivation pathways activate different serine/threonine kinases which leads to the phosphorylation of Smad2 linker region residues. Thrombin mediated transactivation of the EGFR activates Jnk which phosphorylates the Thr220. Of the serine/threonine kinases studied Jnk was the only kinase which was not
involved in thrombin mediated mRNA expression of GAG synthesizing enzymes CHST11 and CHSY1. Therefore we postulate that phosphorylation of the serine residues are most likely to induce GAG synthesizing genes that are closely associated with GAG chain elongation whereas the Thr220 phosphorylation may be associated with enzymes which determine the level of GAG chain synthesis. These findings highlights the complex nature but also the specificity of signalling pathways contributing to GAG chain hyperelongation.
Chapter-6. The contribution of transactivation to GPCR signalling
6.1 Introduction

We wished to address the question as to the overall contribution of transactivation dependent signalling to total GPCR (PAR-1) signalling to try to put transactivation signalling into context. To determine the extent of transactivation dependent GPCR signalling a combined genome-wide gene expression and differential pharmacology study can help identify genes and biological processes regulated by PAR-1 transactivation of the TGFBR1 or EGFR. Whole genome RNA sequencing allows for the comprehensive study of the mRNAs in the individual treatment groups relating to each of the transactivation signalling pathways which can be isolated by the use of our well-characterised pharmacological tools (described below). This new platform has produced high throughput sequencing in the study of various diseases such as bone dysplasia, schizophrenia, rheumatic disease and cancers such as lymphoid cancer and melanoma (Giannopoulou et al., 2015, Xu et al., 2012, Ceol et al., 2011, Steidl et al., 2011, Briggs et al., 2011). RNA-Seq sequencing has been a superior tool in profiling complex diseases (Costa et al., 2013, Byron et al., 2016, Robert and Watson, 2015), but has not been implicated in studies of specific signalling pathways. Champlovier (Champelovier et al., 2010) adapted a pharmacological approach to study the role of Jnk in gene modulation. Jnk inhibitor, SP600125, was used to determine differentially expressed genes and signalling pathways as a potential therapeutic in treatment of leukaemia cells.

Hannan (George et al., 2013) using a functional siRNA screen of the human genome to identify the underlying biology in transactivation of the EGFR. The role of 720 kinases was investigated in Angiotensin II mediated transactivation of the EGFR (George et al., 2013). In the present study, RNA sequencing analysis was employed to examine the changes induced by PAR-1
transactivation dependent signalling in global and selective pathway gene expression in human VSMCs.

6.2 Materials and Methods

6.2.1 Materials

RNAse-Free DNase set and miRNeasy kit were purchased from Qiagen. Dynabeads® mRNA DIRECT™ Micro kit, Ion PI™ Template OT2 Supplies 200 kit v2, Ion PI™ Template OT2 Reagents 200 Kit v2, Ion PI™ Template OT2 Solutions 200 kit v2, Ion PI™ Sequencing Supplies 200, Ion PI™ Sequencing reagents 200, Ion PI™ Sequencing solutions 200 v2, Ion PI™ Chip preparation solution and Ion Proton™ Chip Adapter where purchased from Life Technologies.

6.2.2 Cell culture

Cells were grown in DMEM (5mM glucose, 10% FBS and 1% antibiotics at 37°C in 5% CO₂) CASMCs were seeded in 60mm dishes (described in more detail Chapter 2 Section 2.2.1). Cells were grown to confluence then rendered quiescent by serum deprivation for 48 hours. Inhibitors were pre-incubated for 30 mins prior to treatment with thrombin for 6 hours. The RNA extraction and library preparation are described in detail in Chapter 2 Section 2.2.9.

6.2.3 Quality control and read alignment

Venkata Vijayanand Bhaskarala from the Department of Biotechnology and Environmental Biology at RMIT University conducted the RNA-sequencing analysis. QUADtrim was used to remove the adapter from the RNA-sequencing
reads and to check the quality of the raw reads. The reads were mapped to the human reference genome (hg19) using TopHat v2.1.1. Parameters of TopHat were set to allow only unique alignment to the reference genome. HTSeq was used to generate the counts.

6.2.4 Differential gene expression and functional analysis

The genes differentially expressed between the different treatments were identified using the Bioconductor edgeR software (v3.0.7) (Robinson et al., 2010). This method uses a Poisson distribution to model genic read counts following normalization based on size factors and variance, therefore this software allows normalization of RNA-sequence data based on sequencing depth GC content and gene length for analysis of differentially expressed genes. Differentially expressed genes were defined with log2FC of >0.7 or <-0.7 significant at p value <0.01. STRING (v10.0) (http://string-db.org), a publically available online database of functional interaction (Szklarczyk et al., 2015) was used to identify gene ontology analysis and networks, the differentially expressed genes were analysed. The Venn diagrams were depicted using JVenn an interactive tool to view Venn diagrams and respective lists (Bardou et al., 2014).

6.3 Results and Discussion

6.3.1 Gene expression profiles of treatments involved in PAR-1 mediated transactivation of the EGFR and the TGFBR1

A genome wide RNA-Seq sequencing study was conducted to determine the portion of GPCR signalling which is occurring via transactivation dependent signalling in human CASMCs (Figure 6.1). The individual treatment groups
(Basal, Thrombin, Thrombin+AG1478, Thrombin+SB431542 or Thrombin+AG1478+SB431542) contained 2-4 individual samples pooled together for each of the groups to identify the differentially expressed genes. A total of 3 pooled samples from RNA isolated from untreated CASMCs (Basal, Figure 6.1A), averaged 8 million reads and 82.7% of the total reads were mapped to the human reference genome (hg19). The RNA was harvested from human CASMCs treated with thrombin for 6 hours (Figure 6.1B Thrombin), consisted of 4 pooled samples with an average of 12 million reads. Among the total number of reads 83.3% of the reads were mapped to the human reference genome (hg19). To study the differentially expressed genes occurring via the transactivation of the EGFR VSMCs were treated with thrombin in the presence of EGFR antagonist, AG1478. For this treatment, there was a total of 2 pooled samples (Figure 6.1C) with an average of 13.5 million reads, 82.3% of these reads were mapped to the human reference genome (hg19). To determine the number of differentially expressed genes occurring via the transactivation of the TGFBR1, VSMCs were treated with thrombin in the presence of TGFBR1 antagonist, SB431542. For this treatment group there was a total of 4 samples pooled together with an average read of 13.1 million reads (Figure 6.1D). Among the total number of reads 81.2% of the reads were mapped to the human reference genome (hg19). The treatment showing the genes regulated via dual transactivation dependent signalling pathways were treated with thrombin in the presence of EGFR and TGFBR1 antagonists AG1478 and SB431542, respectively (Figure 6.1E). A total of 3 individual samples were pooled to give an average of 10.9 million reads. Among the total reads 82.2% of the reads were aligned to the human genome (hg19).
The small sample size in our RNA-Seq study is consistent with other RNA-Seq sequencing studies published which are based on sample sizes of equal to or less than 3 (Xu et al., 2012). In human samples greater than 80% of the read pairs can be expected to be mapped to the reference genome (Djebali et al., 2012). Consistent with the literature (Wang et al., 2009b) the output from our RNA-Seq study shows that over 80% of the reads are mapped to the human genome. Thus validating that the RNA used to identify the differentially expressed genes is of high quality.
Figure 6.1: Summary of RNA sequencing data.

Average mapping statistics for each of individual treatments using aligned using TopHatv2.1.1. RNA was extracted from human VSMCs which were A. untreated, B. treated with thrombin (10 unit/ml), pre-incubated for 30 mins with C. EGFR antagonist, AG1478 (5µM), D. TGFBR1 antagonist, SB431542 (3µM), or E. both receptor antagonists, followed by thrombin treatment for 6 hours. The results show that over 80% of the reads are aligned to the human genome in the 5 treatment conditions.
6.3.2 Number of genes regulated by thrombin transactivation dependent and independent signalling in coronary artery smooth muscle cells.

To study the role of transactivation dependent pathways in GPCR signalling, RNA sequencing was conducted to assess the number of genes regulated by each of the transactivation dependent signalling pathways. To study the role of PTKR, the EGFR antagonist, AG1478, was used and to study S/TKR transactivation the TGFBR1 antagonist, SB431542, was utilized. In the present study the differential expression analysis was carried out using edgeR which required multiple comparison analysis. Each of the treated samples were initially compared to the basal sample. The samples treated with thrombin in the presence of the receptor antagonists were further compared to the CASMCs treated with thrombin alone. The results from this multiple comparison are presented in Figure 6.2.

VSMCs treated with thrombin (6hours) resulted in 293 differentially expressed genes increasing in expression as compared to basal. A total of 209 genes are differentially regulated when CASMCs were treated with thrombin in the presence of AG1478. The presence of the EGFR antagonist, AG1478, resulted in an increase in the expression of 80 genes when compared to thrombin treatment alone. The expression of 129 genes was down regulated in the presence of AG1478 as compared to thrombin alone (Figure 6.2). A total of 177 genes are differentially expressed when CASMCs are treated with thrombin in the presence of SB431542. The expression of 26 genes increase in the presence of SB431542 as compared to thrombin treatment alone and 151 genes down regulated in the presence of the antagonist (Figure 6.2). When the two antagonists are combined a total of 212 genes are differentially expressed, 166
genes were down regulated as compared to thrombin and the expression of 46 genes were increased as compared to thrombin treatment alone (Figure 6.2). Thus these results show that transactivation dependent signalling plays an important role in GPCR signalling with 209 genes regulated via the transactivation of the EGFR, 177 genes regulated via thrombin transactivation of the TGFBR1 and 212 genes regulated via dual transactivation of the EGFR and TGFBR1.

Over the past two decades there have been studies expanding on GPCR mediated transactivation signalling of PTKR specifically the EGFR (Smiljanic et al., 2014, George et al., 2013, Wang, 2016). GPCR mediated transactivation dependent signalling also spans out to include activation of the TGFBR1. Although this pathway was first identified a decade ago (Jenkins et al., 2006) there has been a limited amount of studies investigating this transactivation pathway as compared to the EGFR transactivation dependent pathway. Interestingly the results of this RNA sequencing data show that transactivation dependent signalling via the TGFBR1 is equally as important as transactivation of the EGFR as there is a large number of genes regulated when CASMCs are treated with the respective receptor antagonists in the presence of thrombin.

Of the total number of genes differentially regulated by the individual treatments a Venn diagram was composed to show commonly regulated genes between the individual transactivation dependent pathways (Figure 6.3). Of the 209 genes differentially expressed in thrombin transactivation of the EGFR, there are 14 genes common to transactivation of TGFBR1 and 19 genes were found to be common in thrombin transactivation of the EGFR and in transactivation of both EGFR and TGFBR1. The genes differentially expressed by thrombin transactivation of the EGFR have 50 genes, which are not common
with either of the other two treatments (Figure 6.3). 177 genes that are differentially expressed via thrombin transactivation of the TGFBR1, 20 of these genes are in common with thrombin transactivation of both the EGFR and TGFBR1, and 17 genes are specific to transactivation of the TGFBR1. The three individual lists of differentially expressed genes from each of the transactivation pathways share 126 genes which are common. This shows that over 60% of the differentially expressed genes are common between the transactivation dependent signalling pathways. These results indicate that although these two transactivation dependent pathways are independent of each other and their activation are mechanistically distinct, they regulate over 60% of the same genes which may indicate that they may be involved in regulating common biological processes.

There are a number of common downstream signalling intermediates which are common to both EGFR and TGFBR1 signalling. MAPKs such as Erk, p38 and Jnk are amongst the common signalling intermediates between the two pathways. MAPKs play a role in transmitting extracellular signals from cell surface receptors to intracellular target and are activated by both TGF-β (Yu et al., 2002, Gui et al., 2012, Yamaguchi et al., 1995, Zhang, 2009) and EGF. (Roberts and Der, 2007, Scaltriti and Baselga, 2006, Hong et al., 2014). Similarly other serine/threonine kinases such as PI3K and glycogen synthase kinase 3 are common downstream intermediates to both these signalling pathways (Scaltriti and Baselga, 2006, Moustakas and Heldin, 2005, Yi et al., 2005, Osman et al., 2011, Guo et al., 2008, Mills et al., 2011). Thus common downstream signalling intermediates may allow for convergence; allowing for over 60% of differentially expressed genes to be common between the two pathways.
Chapter 5 of this thesis showed that phosphorylation of the linker region of transcription factor Smad2 was common to both transactivation signalling pathways (Figures 5.4 and 5.5). The phosphorylation of the Smad2 linker region has been shown to be activated by multiple serine/threonine kinases (Figure 5.9) (Rostam et al., 2016, Kamato et al., 2014a, Burch et al., 2010, Alarcon et al., 2009, Matsuura et al., 2004). The work from our lab (Burch et al., 2010, Rostam et al., 2016) shows that the phosphorylation of the Smad2 linker region correlates with stimulation of proteoglycan synthesis and GAG chain elongation. Smad2 linker region phosphorylation has been associated with a diverse range of pathophysiological conditions. The Smad2 linker region has been associated with cancer (Kretzschmar et al., 1999, Matsuzaki et al., 2009, Suzuki et al., 2015), renal fibrosis (Sun et al., 2014), endothelial dysfunction (Kamato et al., 2014a), esophagitis mucosae (Takahashi et al., 2016) and inflammatory related diseases (Yoon et al., 2015). Thus showing that the Smad2 linker region can also play as a common downstream convergence point for regulating various differentially expressed genes mediated via transactivation dependent signalling.

The differential expression data was divided into the up and down regulated genes of each of the transactivation pathways. The Venn diagram in Figure 6.4 illustrates that from the 80 genes up regulated in thrombin transactivation of EGFR, 19 genes are also commonly up regulated in thrombin transactivation of the TGFBR1. There are 129 genes that are down regulated via thrombin transactivation of the EGFR and of this gene list 108 are commonly down regulated in thrombin mediated transactivation of the TGFBR1. Interestingly there are 13 genes which are up regulated in thrombin transactivation of the EGFR and down regulated in transactivation of the
TGFB1 (Figure 6.4). These results show that over 80% of the genes down regulated by either pathway are common with only 33% of EGFR mediated transactivation dependent signalling to be specific to EGFR and 20% specific to only TGFB1 transactivation.
Figure 6.2: Number of genes regulated by thrombin transactivation of the EGFR and TGFBR1 or both.

Thrombin compared to basal treatment has a total of 293 genes up regulated.

Thrombin transactivation of the EGFR denoted by thrombin in the presence of EGFR antagonist AG1478 has a total of 209 genes differentially regulated (80 up regulated and 129 down regulated). Thrombin transactivation of TGFBR1 denoted by thrombin in the presence of TGFBR1 antagonist SB431542 has a total of 177 genes differentially regulated (26 up regulated and 151 down regulated). Thrombin transactivation of both EGFR and TGFBR1 results in 212 genes differentially regulated (46 up regulated and 166 down regulated).
Figure 6.3: Venn diagram showing the differentially expressed genes of each of the treatments.

Thrombin transactivation of the EGFR (209 genes), thrombin transactivation of the TGFBR1 (177 genes) and thrombin transactivation of both EGFR and TGFBR1 (212 genes).
Figure 6.4: Venn diagram showing the genes up and down regulated by each of the transactivation dependent signalling pathways.

209 genes are differentially expressed via thrombin transactivation of the EGFR (80 upregulated and 129 down regulated) and 177 genes are differentially expressed via thrombin transactivation of the TGFBR1 (26 upregulated and 141 down regulated).
6.3.3 Genes most up and down regulated by the individual transactivation dependent pathways.

Overall, there were 209 genes differentially regulated by thrombin transactivation of the EGFR (Figure 6.2). The top 10 up regulated genes are involved in molecular functions such as cytokine activity which controls cell growth, survival and differentiation as well as genes which are involved in transcription factor activity (Table 6.1). The top most down regulated genes via EGFR transactivation are involved in receptor protein activity and protein kinase binding (Table 6.1).

There were a total of 177 genes regulated by thrombin transactivation of the TGFBR1 (Table 6.2). The top 10 most upregulated genes include genes which were associated with GTP-binding including TUBB2A, TUBB4B and TUBA1C (Table 6.2). Also included in the top most up regulated genes by thrombin transactivation of the TGFBR1 were genes associated with phospholipid binding and phosphatase activity. The majority of the genes down regulated by this transactivation dependent pathway include genes which were associated with transcription factor activity (Table 6.2).

Treatment with the two receptor antagonists to account for all the transactivation dependent signalling resulted in 212 genes differentially expressed. The top 10 most upregulated genes of this transactivation pathway were associated with transcription factor activity, cytokine activity and receptor binding activity (Table 6.3). From the list of differentially expressed genes occurring via the transactivation of both receptors the most down regulated genes are associated with receptor protein and cytokine activity.
Interestingly many of the top 10 up and down regulated genes are common between the three treatments. NR4A1, CCL20 and IL6 are amongst the top 10 most up regulated genes in thrombin transactivation of the EGFR (Table 6.1) and the genes regulated via dual transactivation dependent pathways (Table 6.3). These genes are involved in regulating transcription factor, cytokine and growth factor activity. The SERPINB2 and the EGR1 are common in the top up regulated genes in thrombin transactivation of EGFR (Table 6.1) and TGFBR1 (Table 6.2). Interestingly although these two genes are in the top upregulated genes of each of the transactivation dependent pathways when the two receptor antagonists were used together these genes did not appear to be in the top 10 most upregulated genes.

In the top most down regulated genes of each of the treatments there were more than 70% commonly expressed genes. FGL2, PGCC, IL11, MALL, LRRc38 and EDN1 are common in the top 10 most down regulated genes dependent on transactivation of the EGFR, TGFBR1 or both (Tables 6.1, 6.2 and 6.3). These genes are commonly associated with cytokine activity and receptor binding. The NR4A2 and NR4A3 genes are common in the top 10 most down regulated genes in thrombin transactivation of TGFBR1 (Table 6.2) and transactivation of the EGFR (Table 6.1), interestingly when the two receptor antagonists were applied together (Table 6.3) these two genes are amongst the top most up regulated genes. The NR4A1 gene found in the most up regulated genes in thrombin transactivation of the EGFR (Table 6.1) and dual transactivation (Table 6.3) is amongst the top most down regulated gene via thrombin transactivation of the TGFBR1 (Table 6.1). Similarly, the PDGFB and the AREG genes associated with protein hetrodimerization and EGFR binding respectively were found in the top 10 most down regulated genes in the dual
transactivation dependent signalling (Table 6.3) and in the top 10 most up regulated genes in thrombin transactivation of the EGFR alone (Table 6.1). These results show that while genes may be up/down regulated when the dual transactivation pathways are activated, the genes may act opposing in the presence of a single receptor antagonist thus showing the complex nature of transactivation dependent signalling.

In ovarian cancer cells the ET-1 receptor transactivates the EGFR (Vacca et al., 2000, Rosano et al., 2007) and the vascular endothelial growth factor receptor (Spinella et al., 2013) leading to the expression of EDN1. The expression of EDN1 has also been shown to be involved in colon and prostate cancer cell lines (Kim et al., 2005, Sun et al., 2006). EDN1 is one of the most down regulated genes in thrombin mediated transactivation of the TGFBR1 (Table 6.2) and is highly down regulated in the presence of receptor antagonists to both transactivation receptor pathways (Table 6.3). The role of EDN1 in GPCR mediated transactivation of S/TKR is unknown however EDN1 has a binding site for TGF-β activated Smad transcription factor (Stow et al., 2011). In palate development TGF-β treatment increased the expression of EDN1 (Zhu et al., 2012), thus consistent with our results which show that thrombin can transactivate the TGFBR1 which is involved in the regulation of EDN1. Thus showing that thrombin transactivation of the TGFBR1 and EGFR has the potential to be involved in growth related diseases such as cancer and palate development.
Table 6.1: Genes most up regulated and down regulated by thrombin transactivation of the EGFR.

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Table 6.2: Genes most up regulated and down regulated by thrombin transactivation of the TGFBR1.

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Table 6.3: Genes most up regulated and down regulated by thrombin transactivation of the TGFBR1 and EGFR in vascular smooth muscle cells.

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6.3.4 Biological processes regulated by the transactivation dependent pathways

One of the objectives of this RNA-sequencing work was to determine the biological terms enriched by the transactivation dependent pathways. In order to examine the biological processes influenced by each of the transactivation pathways, genes differentially expressed by the different treatments were subjected to functional enrichment analysis based on their gene ontology (GO) annotations. From the 209 differentially expressed genes by thrombin transactivation of the EGFR there was 512 GO terms with a false discovery rate (FDR) value of less than 0.05. The top 50 biological terms occurring via thrombin transactivation of the EGFR are listed in Table 6.4. There was a total of 177 genes differentially regulated by thrombin transactivation of the TGFBR1, from this gene list 428 GO terms with an FDR value of less than 0.05. The top 50 biological terms from the 428 genes associated with thrombin transactivation of the TGFBR1 listed in Table 6.5. Treatment with two receptor antagonists to account for all the transactivation dependent signalling resulted in 212 differentially expressed genes. From this list of genes 440 terms were enriched by dual transactivation dependent signalling. The top 50 GO terms respective to this signalling cascade are listed in Table 6.6.

From the lists of the top 50 processes most significantly enriched by transactivation dependent signalling, 28 terms were found to be common between the three lists (Table 6.4, 6.5 and 6.6). Many of these GO terms are associated with regulation of cell activity as well as terms which are associated with vascular development. This includes development of the cardiovascular system, circulatory system, vasculature and blood vessel. From the list of 50 terms associated with dual transactivation dependent signalling (Table 6.6) five
enriched terms were common to thrombin transactivation of the TGFBR1 (Table 6.5). The common terms include biological processes which are involved in cell growth, cell death and tissue development. Similarly there are five GO terms which are common to dual transactivation dependent signalling (Table 6.6) and thrombin transactivation of the EGFR (Table 6.4). Terms common to these two lists include biological processes associated with the regulation of angiogenesis, vasculature development and blood vessel morphogenesis as well as terms involved in cellular activity. When comparing the terms regulated by transactivation of the TGFBR1 (Table 6.5) and the EGFR (Table 6.4) there are 5 GO terms which are common to these two pathways which were not found in the top 50 list of terms via dual transactivation (Table 6.6). These 5 terms are associated with regulation of metabolic processes and signal transduction. Furthermore, network analysis was conducted using String DB, Figure 6.5, 6.6 and 6.7 show the networks over represented in the gene list of the respective transactivation dependent pathways.

The results above show that thrombin transactivation of the EGFR, TGFBR1 or both receptors are involved in regulating a large number of biological processes 512, 428 and 440 terms, respectively. With the two transactivation dependent pathways being mechanistically distinct (Burch et al., 2013), the results of these experiments show that over 55% of the top 50 terms regulated are common between the three treatments. Over 60% of the differentially expressed genes described in section 6.3.2 were common to both thrombin mediated transactivation of the TGFBR1 and transactivation of the EGFR. The common differentially expressed genes resulted in over 55% biological terms, which are common between both transactivation pathways. The commonality between the two individual pathways is due the EGFR
pathway sharing common downstream signalling intermediates with the TGFBR1 downstream pathway. Serine/threonine kinases such as MAPKs (Yu et al., 2002, Gui et al., 2012, Yamaguchi et al., 1995, Zhang, 2009) are one of many common downstream intermediates to both the transactivation dependent pathways and may play a role in the convergence of the two signalling pathways to regulate the same biological processes.

Our lab has focused on GAG elongation because of its associated with atherosclerosis but also because signalling pathways for GAG elongation are distinct from there signalling common responses such as protein signalling and cell proliferation (Little et al., 2010, Burch et al., 2010, Burch et al., 2013, Ghiselli and Maccarana, 2016). Never the less GAG elongation has also proceeded to be an excellent response to characterise transactivation dependent signalling and in that context our work has shown how transactivation dependent signalling makes a major contribution to PAR-1 signalling.
Table 6.4: Gene ontology terms in the analysis of genes expressed in thrombin transactivation of the EGFR

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Chapter-6. Transactivation dependent RNA Sequencing
**Figure 6.5: Network represents the protein-protein interactions involved in thrombin transactivation of the EGFR.**

The functions of this network include biological processes, molecular functions and cellular component. The colour of the nodes indicates the query protein and its first shell of interactions. Protein nodes which are enlarged indicate the availability of 3D protein structure information. The coloured lines between the nodes indicate the query protein-protein interactions. The known interactions use a light blue (from curated database) or pink (experimentally determined) lines between the proteins. The predicted interactions use green (gene neighbourhood), red (gene fusions) and dark blue (gene co-occurrence). The yellow represents text mining, the black represents co-expression and the light purple lines represent protein homology.
Table 6.5: The top 50 Gene ontology terms in the analysis of genes expressed in thrombin transactivation of the TGFBR1.

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Chapter-6. Transactivation dependent RNA Sequencing
Figure 6.6: Network represents protein-protein interactions involved in thrombin transactivation of the TGFBR1.

The colour of the nodes indicates the query protein and its first shell of interactions. Protein nodes which are enlarged indicate the availability of 3D protein structure information. The coloured lines between the nodes indicate the query protein-protein interactions. The known interactions us a light blue (from curated database) or pink (experimentally determined) lines between the proteins. The predicted interactions use green (gene neighbourhood), red (gene fusions) and dark blue (gene co-occurrence). The yellow represents text mining, the black represents co-expression and the light purple lines represent protein homology.
Table 6.6: Top 50 Gene ontology terms in the analysis of genes expressed in thrombin transactivation of both the EGFR and TGFBR1.

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Chapter 6. Transactivation dependent RNA Sequencing
Figure 6.7: Network represents protein-protein interactions involved in thrombin transactivation of both the EGFR and TGFBR1.

The colour of the nodes indicates the query protein and its first shell of interactions. Protein nodes which are enlarged indicate the availability of 3D protein structure information. The coloured lines between the nodes indicate the query protein-protein interactions. The known interactions use a light blue (from curated database) or pink (experimentally determined) lines between the proteins. The predicted interactions use green (gene neighbourhood), red (gene fusions) and dark blue (gene co-occurrence). The yellow represents text mining, the black represents co-expression and the light purple lines represent protein homology.

6.3.5 Transactivation dependent signalling is associated with genes regulating vasculature development.

In order to more clearly assess the processes influenced by transactivation dependent signalling, the differentially expressed genes were subjected to functional enrichment analysis based on their GO annotations as described in Section 6.3.4. The processes most commonly enriched by the three treatments were linked with vascular development. There were 29 genes associated with cardiovascular system development in thrombin transactivation of the EGFR (Table 6.4); 28 genes in thrombin transactivation of the TGFBR1 (Table 6.5) and 31 genes in thrombin transactivation of both the TGFBR1 and EGFR (Table 6.6). Table 6.7 shows gene expression data for significantly regulated genes categorized as cardiovascular system development (GO.0072358). All the genes were up regulated by thrombin when compared to basal and down regulated in the presence of AG1478 with the exception of EGR1, HES1, NR4A1, PDGFB, RHOB, SPHK1 and TIPARP (Table 6.7). In
the presence of thrombin and TGFBR1 antagonist, SB431542, most of the genes associated with cardiovascular system development were down regulated with the exception of EGR1 and TIPARP (Table 6.7). When looking at dual transactivation dependent signalling associated with cardiovascular system development all genes apart from EGR1, HES1 ID1, KLFS, NR4A1, PTGS2 and RHOB were down regulated as compared to thrombin stimulation alone (Table 6.7).

The EGR1 (early growth response 1) gene associated with cardiovascular system development was regulated by the three treatments when compared to thrombin (Table 6.7). EGR1 is involved in the development of atherosclerotic lesions and has been shown to play a role in regulating neointimal thickening in response to vascular injury (Blaschke et al., 2004, Harja et al., 2004, Khachigian et al., 1996, McCaffrey et al., 2000). The transactivation dependent signalling discussed in Chapter 4 in which treatment with antagonists to the EGFR and TGFBR1 inhibit thrombin mediated GAG elongation evaluated by the mRNA expression of CHST11 and CHSY1. However the RNA-Sequencing results show that with treatment of the receptor antagonists in the presence of the thrombin there is an increase in the expression of the EGR1 gene, this may be due to work in earlier chapters looking at the pre-inflammatory phase of atherosclerosis. The EGR1 gene is commonly involved in the neointimal thickening which is in the inflammatory phase of atherogenesis.

The ANKRD1 (Ankyrin Repeat Domain 1) gene belongs to the conserved muscle Ankyrin repeat family and is induced in response to inflammation, injury and response to cell stress (Miller et al., 2003, Jeyaseelana et al., 1997). The ANKRD1 gene is amongst the differentially expressed genes
associated with vascular development. The ANKRD1 gene was involved in the transactivation of the EGFR and the TGFBR1 (Table 6.7). ANKRD1 is directly mediated via TGF-β mediated signalling as the ANKRD1 promotor region contains a Smad binding motif (Kanai et al., 2001, Labbe et al., 2007). The ANKRD1 gene is associated with the actin cytoskeleton signalling network (Ponsuksili et al., 2009), in Chapter 4 (Sections 4.3.3 and 4.3.4) of this thesis thrombin mediated transactivation of the TGFBR1 activates Rho/ROCK signalling via cytoskeletal rearrangement leading to the activation of cell surface integrins. Thus demonstrating a potential signalling pathway for the activation of the ANKRD1 gene via thrombin transactivation of the TGFBR1. The role of ANKRD1 downstream of the EGFR in unknown however ANKRD1 is induced by stress activated kinases (Zhong et al., 2015, Liang and Sheikh, 2016, Song et al., 2012) hence we speculate that thrombin mediated transactivation of the EGFR induces the expression of ANKRD1 via the activation of downstream stress activated MAPKs (Erk, p38 and Jnk). In Chapter 5 (refer to Figure 5.9) we show that thrombin mediated phosphorylation of the Smad2 linker region occurs via the transactivation of the EGFR and the TGFBR1 which activate downstream serine/threonine kinases including Erk, p38 and Jnk. Thus providing a potential signalling pathway in GPCR mediated activation of ANKRD1.
Table 6.7 List of genes and respective fold change of expression associated with gene ontology term cardiovascular development.

The differentially expressed genes with no fold change presented in the table did not reach the statistical significance cut off ($P<0.01$ and Log2FC>0.7)

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6.4 Conclusions

GPCRs are one of the most successful class of drug targets on the human genome for the treatment of a variety of diseases (Fang et al., 2015, Stockert and Devi, 2015). Hence the role of GPCR signalling is of great interest to medical research. GPCRs signal in three main mechanisms, which includes signalling via G proteins to secondary messenger intermediates and via utilization of the signalling pathway and via the utilization of a β-arrestin molecule both which have been extensively reviewed (Tuteja, 2009, Ferguson et al., 1996). The third GPCR signalling pathway is via transactivation of PTKR and via transactivation of S/TKR. We subsequently described S/TKR transactivation but again the contribution to overall GPCR signalling was unknown. Hence in this present study, the aim was to determine the contribution of dual transactivation of GPCR mediated signalling by utilizing RNA sequencing to facilitate the identification of the genes regulated by these two transactivation pathways. Using this approach, we have uncovered that thrombin mediated signalling results in 293 differentially expressed genes of these genes approximately 50% of the signalling is occurring via transactivation dependent signalling. Using this approach we have also uncovered that transactivation of the TGFBR1 is as equally important as the transactivation of the EGFR noting that the TGFBR1 transactivation pathway has not yet been as extensively researched.

Hannan et al. (George et al., 2013) using a siRNA approach studied 720 kinase genes associated with Angiotensin II transactivation of the EGFR. Knockdown of 3 genes, TRIO, BMX and CHKA revealed that their activity is required for Angiotensin II mediated transactivation of the EGFR in human mammary epithelial cells (George et al., 2013). This RNA-Seq sequencing study we explored all the genes that were involved in GPCR mediated transactivation
of the EGFR and TGFBR1 in human VSMCs. Although the two transactivation signalling pathways have been reported as to be mechanistically distinct (Burch et al., 2010, Burch et al., 2013) this study shows that these two pathways share in over 65% of the differentially expressed genes. The transactivation dependent signalling pathways shared in over 55% of its top 50 enriched terms, with a majority of these GO terms associated with cardiovascular system development.

The two transactivation dependent signalling pathway share in over 65% of common differentially expressed genes. The top most regulated genes of these transactivation mediated pathways are involved in molecular functions such as cytokine activity which controls cell growth survival and differentiation. Cytokine activity is involved in different aspects of cancer, development, treatment and prognosis (Bower et al., 2002, Chen et al., 2013, Esquivel-Velazquez et al., 2015). The EGFR (Normanno et al., 2006a, Normanno et al., 2006b, Seshacharyulu et al., 2012) and the TGFBR1 (Pasche et al., 2014, Principe et al., 2014, Moore-Smith and Pasche, 2011) signalling pathways alone play a role in cancer development. Both the EGFR and TGFBR1 signalling pathways share a common transcription factor, Smad2 linker region. The Smad2 linker region plays a role in cancer development (Kretzschmar et al., 1999, Matsuzaki et al., 2009, Suzuki et al., 2015). The Smad2 linker region is also associated with other pathophysiological conditions which include but are not limited to proteoglycan synthesis and GAG chain elongation associated with atherosclerosis development (Chapter 5) (Rostam et al., 2016, Burch et al., 2010). Thus showing that the linker region of the Smad2 may be a transcription factor of interest for to study different biological process occurring via thrombin mediated transactivation of both the EGFR and TGFBR1.
In summary by providing high throughput RNA-Seq data, we have identified that GPCR signalling via transactivation of either of the EGFR or TGFBR1 account for 50% of GPCR signalling. We have identified that these two pathways share in regulation of over 65% of genes of which a majority are involved in the development of the vasculature. This work provides the platform for investigating the molecular basis for transactivation dependent signalling and provides proof that genome wide studies can offer powerful insights into this process.
Chapter-7. General Discussion
The current paradigm of GPCR signalling includes transactivation independent signalling, β-arrestin signalling and transactivation dependent signalling. The work in this thesis has focused on thrombin receptor, PAR-1, mediated transactivation of the TGFBR1 and EGFR in human VSMCs. Transactivation has been defined as “the agonist occupancy of its cognate GPCR complex which leads in a relatively short time and in the absences of ‘de novo’ protein synthesis to the activation of and cytosolic generation of the immediate downstream products of a second cell surface protein kinase receptor” (Little et al., 2011). Our group has shown that PAR-1 is involved in the transactivation of EGFR and the TGFBR1 leading to proteoglycan synthesis and GAG elongation (Burch et al., 2010). Proteoglycan synthesis and GAG elongation, are implicated in the initiation of atherosclerosis as modified proteoglycans show a higher binding affinity to LDL (Ivey and Little, 2008). Variation in the structure of proteoglycans either by an increase in chain length or a greater degree of sulphation is the main contributor that influences the binding towards lipids (Chait and Wight, 2000, Little et al., 2002). Therefore, preventing the alteration in proteoglycan synthesis and structure is proposed to be a potential therapeutic target for the prevention of atherosclerosis (Ballinger et al., 2004). Findings from this current study revealed that increased GAG synthesizing enzyme expression led to GAG chain hyperelongation on biglycan. Therefore, it is postulated that elevated GAG synthesizing enzyme expression is associated with an increased capacity of lipid binding by biglycan ultimately contributing to the development of atherosclerosis. For that reason, identifying the specific target and rate limiting enzymes and the signalling pathways that control this process is of utmost importance for the future treatment of atherosclerosis.
The first aim of this study was to identify whether PAR-1 mediated transactivation of the EGFR and TGFBR1 is involved in the regulating the expression of GAG synthesizing genes relevant to GAG chain hyperelongation and is addressed in Chapter 3. In human VSMCs, thrombin an agonist of the GPCR PAR-1, stimulates GAG chain hyperelongation via transactivation of the EGFR and TGFBR1 (Burch et al., 2013). Findings from this study showed that in VSMCs, thrombin stimulates the mRNA expression of CHST11, CHSY1 and CHST3 (Figure 3.2) via the transactivation of EGFR and TGFBR1 (Figure 3.5). In vivo studies show that in a CHSY1 knockout mouse model, the CS GAG chain synthesis was reduced (Wilson et al., 2012). In a CHST11 deficient mouse model there was a 90% decrease in GalNAc structure and CS amount (Izumikawa et al., 2011). Thus in the human VSMCs model thrombin mediated GAG hyperelongation requires CHSY1 regulation and the CHST11 is most likely the enzyme regulating the sulphation on the GAG chains. Hence thrombin mediated GAG chain hyperelongation associated with an increase in LDL binding would most likely require direct involvement of both genes. On the assumption that these agents are important in GAG synthesis and hyperelongation, and noting that Emoto and colleagues (Anggraeni et al., 2011) have reported an increase in the expression of CHST11 in the vessels of murine model of atherosclerosis, then we can speculate that the inhibition of the expression of these genes can prevent GAG hyperelongation, lipid deposition and atherosclerosis. It is important to note that we are focusing on preventing GAG chain hyperelongation by inhibiting the signalling pathways that cause the hyperelongation process rather than the fundamental processes of GAG elongation, a target which would most likely interfere with normal physiological processes. The two transactivation pathways signal via distinct mechanisms and
both pathways contribute to proteoglycan synthesis and GAG elongation hence lead to the second aim of this thesis to find a common signalling point to inhibit all transactivation dependent signalling.

Limited availability of antagonists to the GPCR Gaq proteins has restricted the study of their role in GPCR signalling. The Gaq family consists of four subunits, however the Gaq and the Gaq/11 are the most prominent and ubiquitously expressed in VSMCs and hence were the focus of this study. With an identical number of amino acids and an 88% homology at the protein level, Gaq and Gaq/11 would be expected to have similar if not identical functions. However in Chapter 4 we showed that PAR-1 mediated transactivation of EGFR and TGFBR1 leading to GAG elongation is regulated exclusively via Gaq. Although both isoforms were found to be present in human VSMCs at a similar expression level only knockdown of the Gaq, not the Gaq/11 inhibited transactivation dependent signalling. GPCR transactivation of both PTKR (Harada et al., 2014, Barauna et al., 2013, Jin et al., 2009, Taboubi et al., 2010) and S/TKR (Xu et al., 2009, Scotton et al., 2009, Deng et al., 2008) is dependent on Gaq signalling. The current knowledge in human VSMCs is that Gaq mediates all transactivation dependent signalling hence Gaq can play as a potential therapeutic target to prevent GAG hyperelongation which (Figure 7.1), under the “response to retention hypothesis” of atherosclerosis, is the initiating step in atherogenesis. Although of lesser importance than Gaq in PAR-1 signalling it may be of interest to investigate the role of other G proteins such as the Gai and Gas in this model.

Gaq signalling plays an important role in the vasculature (D'Angelo et al., 1997, Adams et al., 1998). The role of Gaq signalling has been shown to be important in platelet function (Uemura et al., 2006a), vascular contractions
(Uemura et al., 2006a, D'Angelo et al., 1997), arterial thrombosis (Kawasaki et al., 2005, Kawasaki et al., 2003), neointimal formation (Kawasaki et al., 2005) and cardiac hypertrophy (Dorn and Force, 2005, Keys et al., 2002, Adams et al., 1998). The findings from this current study support the involvement of the Gαq protein in mediating the progression of atherosclerosis through the role of Gαq in GAG chain hyperelongation. This work outlines that Gαq is the central integrating point of transactivation dependent signalling and a potential therapeutic target during atherogenesis (Figure 7.1). Hence, there is a need for the development of a small chemical entity that targets GPCR mediated Gαq signalling.

Our group has shown that PDGF inhibitor, imatinib, when administered to ApoE knockout mice fed high fat diet, reduced lesion size in the aortic arch of the mice (Ballinger et al., 2010). Imatinib treatment also inhibited GAG chain synthesis and reduced LDL binding to proteoglycans \textit{in vitro} (Ballinger et al., 2010). Hence a small chemical entity, which inhibits GPCR mediated Gαq signalling should be studied \textit{in vivo} in a high fat fed atherosclerotic mouse model to establish the role of Gαq in early atherosclerotic development occurring via transactivation dependent signalling. Supporting this future study is the work by Soto (Soto et al., 2012) and colleagues, which showed that targeting proteoglycans is efficacious in preventing lesion progression \textit{in vivo}. A chimeric mouse/human IgG1 monoclonal antibody that recognizes pro-atherogenic CS GAG chains was shown to dose dependently interfere with LDL binding to CS GAGs \textit{in vitro} (Soto et al., 2012). This is an alternative approach to ours demonstrating the validity of our overall approach. The antibody also affected lesion state \textit{in vivo}. 
Proteoglycan synthesis and GAG chain elongation are dependent on Smad2 linker region phosphorylation in human VSMCs (Burch et al., 2010). Erk and p38 are involved in TGF-β mediated phosphorylation of the 3 serine residues of the Smad2 linker region. The Erk and p38 kinases are also shown to be involved in TGF-β mediated proteoglycan synthesis and GAG chain elongation (Burch et al., 2010). Thrombin is a well characterized agonist which drives the pathways that result in proteoglycan modification (Ivey and Little, 2008, Burch et al., 2010). In human VSMCs thrombin mediated proteoglycan synthesis and GAG chain elongation is via the transactivation of the EGFR and the TGFBR1 (Chapter 3)(Burch et al., 2013). In human VSMCs, Smad2 linker region phosphorylation mediates proteoglycan synthesis and GAG chain elongation hence the Smad2 linker region can play as an important integrating point that can allow cross talk between various receptor-signalling pathways.

The signalling pathway of thrombin mediated up regulation of CHSY1 and CHST11 mRNA expression involved Erk, p38, PI3K and CDK but not Jnk (Figure 5.3 Section 5.3.8). These kinases are important regulators of atherosclerosis (Rivard and Andres, 2000, Senokuchi et al., 2004, Elkhawad et al., 2012, Hu et al., 2000, Fougerat et al., 2009). Of these kinases Erk, p38, Jnk and PI3K have been shown to be downstream of Gαq signalling (Cramer et al., 2001, Esposito et al., 2001, Taboubi et al., 2010, Yamauchi et al., 1997). There have been no studies to support whether CDKs are involved in Gαq signalling however in bone epithelial cells the PI3K signalling cascade can activate CDK (Major et al., 2004), hence there is a possibility that the PI3K downstream of Gαq (Taboubi et al., 2010) can direct its signalling to activate CDK.
Figure 7.1: Gαq as the central integrating point of PAR-1 mediated transactivation of the EGFR and TGFBR1 leading to GAG chain elongation.

The GPCR, PAR-1 via its Gαq leads to the transactivation of the EGFR as studied by the phosphorylation of downstream intermediate Erk. PAR-1 via Gαq leads to the activation of ROCK signalling which activates cell surface integrins, in particular the αVβ5, which leads to the activation of the TGFBR1 as denoted by the phosphorylation of downstream intermediate Smad2 carboxy terminal. Both these transactivation pathways lead to the regulation of GAG genes and GAG chain hyperelongation in VSMCs.
Thrombin mediated signalling to four important phosphorylation sites of the Smad2 linker region were studied in Chapter 5. The transactivation of the EGFR (Figure 5.6) and the TGFBR1 (Figure 5.7) activate different serine/threonine kinases which lead to the phosphorylation of Smad2 linker region residues. The two transactivation dependent pathways at the 2-4 hour time points are both involved in the phosphorylation of the Smad2 linker region. The crossover in signalling times proposes difficulties in studying the signalling pathways involved specifically in Smad2 linker region phosphorylation occurring via the transactivation of the TGFBR1. In this study the EGFR antagonist was utilized to block EGFR dependent signalling allowing for the study of signalling pathways involved in Smad2 linker region phosphorylation occurring via the TGFBR1 transactivation. However as the signalling occurring via the EGFR transactivation was not completely blocked using a pharmacological approach a molecular approach should be considered. To address this siRNA specific to the EGFR can be used to knock down EGFR signalling in VSMCs. The findings from these future experiments should give insight into whether there is a common signalling cascade in thrombin mediated phosphorylation of Smad2 linker region residues occurring via transactivation of EGFR and TGFBR1.

Among the MAPK involved in TGF-β signalling in VSMCs, signalling is via Erk and p38 but not Jnk has been identified to modify GAG chains (Burch et al., 2010 ). In nucleus pulposus cells of invertebral discs, Erk participates in TGF-β regulation of CHSY1 expression (Hu et al., 2015). The data presented in this thesis (Chapter 5) is consistent with these two models. Thrombin transactivation of the TGFBR1 leading to GAG gene mRNA expression does not involve Jnk, however phosphorylation of the Thr220 via the transactivation
of the EGFR required Jnk. Interestingly Jnk was not involved in the phosphorylation of either of the three serine residues downstream of either of the two transactivation pathways supporting our earlier work (Burch et al., 2010). In human VSMCs, TGF-β mediated phosphorylation of the Thr220 occurred via CDKs and only the phosphorylation of Thr220 was associated with the regulation of XT-1 mRNA expression, a critical enzyme associated in chain initiation (Rostam et al., 2016). From this we can speculate that the serine residues of the Smad2 linker region are involved in thrombin mediated GAG elongation, however the Thr220 residue may be involved in other processes such as the initiation of the linkage region.

Of the serine/threonine kinases investigated in this study, there was no common kinase that phosphorylated all three serine residues. ROCK is a member of intracellular serine/threonine kinases that is involved in the regulation of Smad activity via the linker region (Chen et al., 2006, Kamaraju and Roberts, 2005). The ROCK signalling pathways is downstream of the EGFR (Nakashima et al., 2011, Ramis et al., 2012) and our group amongst others has shown the ROCK pathways in involved in GPCR transactivation of TGFBR1 (Burch et al., 2013, Jenkins et al., 2006, Scotton et al., 2009, Xu et al., 2009). Hence further experiments are warranted to study ROCK in thrombin mediated phosphorylation of the serine residues of the Smad2 linker region. Chapter 4 of this thesis outlines the role of ROCK in thrombin mediated mRNA expression of CHSY1 and CHST11 (Figure 4.4) hence the role of this kinase in GAG elongation is established we postulate that ROCK should play a role in the phosphorylation of Smad2 linker region.

Thrombin mediated Smad2 linker region phosphorylation occurs via the transactivation of both TGFBR1 and EGFR (Figure 5.9). The results presented
in this thesis reveal that thrombin mediated transactivation of the TGFBR1 leading to the phosphorylation of the Smad2 linker region is time dependent and delayed when compared to the transactivation of the EGFR (Figures 5.4 and 5.5). This is consistent with what has previously been described about these two transactivation pathways. In human VSMCs and in mouse epithelial cells thrombin and LPA mediated transactivation of the TGFBR1 did not involve ‘de novo’ protein synthesis (Jenkins et al., 2006, Xu et al., 2009, Burch et al., 2013). As discussed in this thesis, TGFBR1 transactivation is mediated by a dynamic cytoskeletal, ROCK, integrin interplay whereas transactivation mediated via EGFR involves MMP mediated ligand-dependent mechanisms. In human VSMCs, thrombin and ET-1 mediated transactivation of the TGFBR1 occurred 2 hours post treatment (Burch et al., 2010, Little et al., 2010). LPA induced MEF cells had a peak phosphorylation of Smad2 for 1-4 hours post treatment (Jenkins et al., 2006). Moreover, agonists of various GPCRs within minutes induced the phosphorylation of endogenous EGFR in keratinocytes, COS7 cells, VSMCs and proximal tubular epithelial cells (Daub et al., 1997, Burch et al., 2010, Chung et al., 2013). Taking into account these previous studies, we expected thrombin mediated Smad2 linker region phosphorylation via transactivation of EGFR to occur much earlier than transactivation of the TGFBR1. We postulate that the difference in the transactivation dependent signalling pathways is due to the cell surface mechanisms involved. Although there is a difference in the time it takes to transactivate the individual receptors, the aim of this work was to find a common point that will inhibit transactivation dependent GAG elongation.

We then addressed the question as to how important transactivation signalling was to overall GPCR (PAR-1) signalling. High throughput RNA-Seq
data (Chapter 6) showed that thrombin mediated transactivation of the EGFR and TGFBR1 accounted for 50% of all thrombin mediated signalling assessed as gene expression at 6 hours. The two transactivation dependent signalling pathways share in over 65% of common differentially expressed genes. PAR-1 mediated transactivation of the EGFR and TGFBR1 common transcription factor, Smad2 in the linker region (Chapter 5). The work from our group shows that the phosphorylation of the Smad2 linker region is associated with proteoglycan synthesis and GAG elongation (Burch et al., 2010, Rostam et al., 2016). The Smad2 linker region has been associated with cancer (Kretzschmar et al., 1999, Matsuzaki et al., 2009, Suzuki et al., 2015), renal fibrosis (Sun et al., 2014), endothelial dysfunction (Kamato et al., 2014a), esophagitis mucosae (Takahashi et al., 2016) and inflammatory related diseases (Yoon et al., 2015). Thus showing that the Smad2 linker region may be a transcription factor of interest not only for its role in transactivation dependent GAG chain elongation but it for its role in advancing the signalling pathways for the common differentially expressed genes from the two transactivation pathways. This work provides proof the genome wide studies can offer powerful insights into transactivation dependent signalling pathways.

Earlier work had shown that GAG chains were elongated but in the current work it has been shown that the expression of several genes, which my implication are rate limited, are activated as a result of PAR-1 mediated transactivation signalling. In conclusion, the current study has identified that thrombin via PAR-1 mediated the transactivation of the EGFR and TGFBR1 regulates the mRNA expression of GAG synthesizing genes CHST11, CHSY1 and CHST13 in human VSMCs. Thrombin mediated transactivation of the TGFBR1 is mediated by PAR-1 and is dependent on ROCK mediated
cytoskeletal contraction leading to the activation of cell surface integrin αVβ5, which activate the latent TGF-β complex. In addition, thrombin utilizes transactivation of the TGFBR1, cooperatively with the well-characterized transactivation of the EGFR, to stimulate GAG hyperelongation in human VSMCs. The Gaq mediates all transactivation dependent signalling and plays as a central target for transactivation dependent GAG elongation. A complex and highly specific signalling pathway from PAR-1 to Smad2 linker region phosphorylation exists in these cells. Serine/threonine kinases Erk, p38 PI3K and CDK were found to be key mediators in this pathway. Individual Smad2 linker region sites are selectively phosphorylated. CHSY1 and CHST11 mRNA expression, closely associated with GAG chain elongation are induced by the phosphorylation of the serine residues of the Smad2 linker region. However the phosphorylation of the Thr220 residue may be associated with expression of another GAG gene such as XXYL-T-1. Thrombin mediated transactivation of the EGFR and TGFBR1 in human CASMCs accounts for 65% of thrombin mediated signalling thus showing the importance of transactivation dependent signalling to overall PAR-1 signalling. Together these findings demonstrate the complex nature of transactivation dependent signalling and the complexity of the signalling pathways that contribute to GAG hyperelongation but the identification of Gaq as a central mediator may facilitate the identification of a specific therapeutic target in early development of atherosclerosis.

The most interesting finding from this work has been the identification of the very specific role of Gaq in both pathways (PTKR and S/TKR) of transactivation dependent signalling. As we have identified that transactivation dependent signalling makes a major contribution to PAR-1 mediated signalling then it is likely that all of the vasoactive peptides in a diseased vessel ET-1,
angiotensin II and thrombin might mediate transactivation dependent signalling and this $\alpha_q$ might be a highly efficacious target to prevent the deleterious pathophysiological effects of multiple GPCR agonists. G proteins such as $\alpha_q$ may have important physiological roles apart from the findings presented in this thesis but the extent to which therapeutically targeting $\alpha_q$ proteins in multiple diseases and in particular in the role of proteoglycans in atherosclerosis, cannot be known for certain until the appropriate experiments in reputable models are undertaken.
Chapter-8. References


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