Transcriptional Regulation of Soluble Guanylyl Cyclase

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Transcriptional Regulation of Soluble Guanylyl Cyclase

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in Biomedical Science
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at the Brown Institute, University of Texas, Houston, Texas.
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Abstract

Each year cardiovascular disease causes over 4.3 million deaths in Europe and is the cause of nearly one in three deaths in the US. Nitric oxide (NO) is a toxic atmospheric gas which exists in tissues as a biological product of mammalian cells. It has been used to manage cardiovascular disease for over a century and to this day remains an important treatment option in cardiovascular medicine. NO is produced by many cells in the body including vascular endothelial cells. Because of its importance in vascular function, abnormal production of NO, which occurs in different disease states, can adversely affect blood flow and other vascular functions.

NO binds to the haem moiety of the enzyme soluble guanylyl cyclase (sGC) which is found in vascular smooth muscle cells and most other cells of the body. NO diffuses from vascular endothelial cells into the vascular smooth muscle cells adjacent to the endothelium where it binds to and activates sGC. sGC is a heterodimer composed of two different subunits: α and β. The structure of sGC consists of a haem-containing regulatory domain, formed by N-terminal portions of both subunits, and a catalytic domain formed by the C-terminal half of both subunits. The N-terminal regions of α and β subunits are often referred to as the regulatory domain of the enzyme. Several isoforms of these subunits have been described: α1, α2 and β1, β2 and β3. The heterodimer α1β1 is practically present in all tissues studied.

In response to NO binding, sGC catalyses the conversion of guanosine-5′-triphosphate (GTP) to cGMP. cGMP is an intracellular messenger and acts on several cGMP-regulated receptor proteins, such as cGMP-stimulated protein kinases, cGMP-regulated phosphodiesterases (PDEs), and cGMP-gated ion channels which subsequently leads to vasorelaxation. Expression levels of sGC have been found altered in several models of cardiovascular disease however the mechanisms behind these alterations remain largely unknown. Several extracellular regulators such as inflammatory cytokines have been shown to affect sGC steady state levels. The regulation of sGC expression is unquestionably important to support cardiovascular function.

The main objectives of this project were to study the effects of selected inflammatory cytokine stimulation on levels of sGC in human endothelial and smooth
muscle cells. Primary human aortic vascular endothelial (HAOECs) and human aortic smooth muscle cells (HAOSMCs) were cultured and treated with the inflammatory cytokines GM-CSF and IL-4, to determine the effects they would have on sGC mRNA and protein expression. The mRNA level was determined using QPCR and the protein was analysed using Western blotting. QPCR results showed that cytokine treatment of HAOSMCs increase both of the sGC subunits. The opposite effect was seen in HAOECs, with a decrease in subunit mRNA expression being observed. Western blot analysis revealed that the level of sGC expression in primary cells is too low to be detected by this method, with very little antibody binding to either subunit.

It has been suggested that alternative splicing of sGC may be an important mechanism in its regulation. A307838 is a recently discovered sGC splice form of the β1 subunit. Based on observations of the importance of sGC alternative splicing, a further objective of this project was to clone this splice form into an expression vector for future expression and testing in viral vectors. A307838 was PCR amplified as two separate fragments (1 and 2), which were transformed into separate vectors and ligated together in one plasmid vector. The ligation of fragment 1 and 2 was successful, however the orientation of fragment 1 was not correct by the time this study had finished. Work still continues on this splice form.

The results from this study identify the effects of GM-CSF and IL-4 on sGC regulation in primary cells. Our findings point further to a role of cytokines in the regulation and activity of sGC. sGC regulation may be finely tuned, and this regulation may play an important part in the understanding of the development of many cardiovascular disorders. Considering the importance of sGC function, and reports concerning the functional significance of its splice forms, the study of A307838 should help us to gain further insights into the regulation of sGC.
**Declaration**

I certify that this thesis which I now submit for examination for the award of ___________________, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Signature ______________________________ Date ________________

**Candidate**
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And as usual the last thanks have to go to my parents. Thanks for all your support over the past year, for coming to visit me and for always believing in me.
List of Abbreviations

NO - Nitric Oxide

EDRF - Endothelium-Derived Relaxing Factor

cGMP - Cyclic Guanosine Monophosphate

sGC – Soluble Guanylyl Cyclase

PKG – Protein Kinase G

CNG - Cyclic Nucleotide Gated Channels

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NO⁺ - Nitrosonium Cation

NO⁻ - Nitrite

CVS – Cardiovascular System

CA²⁺ - Calcium

IL-1 – Interleukin 1

TNF-β - Tumour Necrosis Factor Beta

ECs – Endothelial Cells

vSMCs – Vascular Smooth Muscle Cells

nNOS/NOSI - Neuronal Nitric Oxide Synthase

eNOS/NOSIII – Endothelial Nitric Oxide Synthase
CVD – Cardiovascular Disease
IRI - Ischaemia Reperfusion Injury
GTP - Guanosine Triphosphate
pGC - Particulate Soluble Guanylyl Cyclase
Fe^{2+} - Iron
HAOSMC - Human Aortic Smooth Muscle Cells
mRNA – Messenger Ribonucleic acid
NCBI - National Center for Biotechnology Information
ORF – Open Reading frame
RT-PCR – Real-Time Polymerase Chain Reaction
PCR – Polymerase Chain Reaction
LPS – Lipopolysaccharide
IL-1β - Interleukin-1β
TNF-α - Tumour Necrosis Factor-α
ILs – Interleukins
IL4 – Interleukin 4
CD4 – Cluster of Differentiation 4
GM-CSF - Granulocyte Macrophage Colony-Stimulating Factor
CSF – Colony-Stimulating Factor
AP-1 – Activator Protein 1

NF-kB - NF-kappaB

NFAT – Nuclear factor of activated T-cells

IL-2 – Interleukin 2

IL-5 – Interleukin 5

IL-13 – Interleukin 13

kDa – Kilodalton

HAOEC – Human Aortic Endothelial Cells
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1.0. Introduction

Heart disease remains the first leading cause of death for both men and women in the United States and together with stroke, accounts for almost 40% of all deaths annually. Nitric oxide donors in the form of nitrovasodilators have been used to manage cardiovascular disease for over a century (Lauder Brunton, 1867) even before the mechanism of their action was established. To this day, NO donors remain an important treatment option in cardiovascular medicine (Murad, 2006). The primary physiological target for NO is soluble guanylyl cyclase (sGC) which is expressed in many tissues (Waldman Murad, 1987). In addition to direct activation by NO, sGC function may be modulated by changes in expression levels. Expression levels of sGC have been found altered in several models of cardiovascular disease, and decreased activity and expression has been proposed as one of the mechanisms underlying the development of endothelial dysfunction (Melichar et al., 2004; Ruetten et al., 1999; Mulsh et al., 2001). The molecular mechanisms responsible for regulation of sGC expression remain largely unknown. Several extracellular regulators including cytokines affect sGC levels and these changes have been associated with cardiovascular disorders (Filippov et al., 1997; Papapetropoulos et al., 1996; Ujiie et al., 1994). Knowing sGC levels are affected by cytokines, we aimed to take a closer look at these effects.

1.1 NO discovery

Nitric oxide, a toxic atmospheric gas exists in tissues as a biologic product of mammalian cells. Its actions are as diverse as the cells which produce it (Furchgott & Zawadzki, 1980). It is an uncharged free radical compound (Krumenacker et al., 2004) and a diffusible messenger which functions as an intercellular signalling molecule in most tissues (Roy & Garthwaite, 2006).

NO was largely viewed as a pollutant released from smokestacks and present in automobile exhaust and cigarette smoke (Murad, 2006). It was long believed that the biosynthesis of nitrogen oxides was an exclusive function of microorganisms, which are
well known for their oxidative or reductive pathways. As early as 1916, it was noted that mammals secrete more nitrate in their urine than was present in their diets and in the early 1980s, nitrogen oxides were established as normal by-products of mammalian metabolism (Dawson & Snyder, 1994).

The uncovering of nitric oxide (NO) and the L-arginine-citrulline metabolic pathway by which it is formed, represented remarkable and unexpected discoveries in biological science. It had been known for years that endothelial cells release a highly labile factor involved in the relaxation of the underlying vascular smooth muscle when stimulated by a variety of vasodilator substances such as acetylcholine or bradykinin (Furchgott & Zawadzki, 1980). This factor was termed endothelium derived relaxing factor (EDRF), of which its structure was not known. Early studies indicated that EDRF could be viewed as the endogenous equivalent of the nitrovasodilators or the “endogenous nitrate”. It appeared that activation of sGC also mediated the effects of EDRF. EDRF was later shown to share a common mechanism of action with NO and organic nitrates i.e. by activating sGC and elevating cGMP levels in the vascular smooth muscle (Papapetropoulos et al., 1996). A number of groups became convinced that EDRF was NO and in 1987 irrefutably published data by Salvador Moncada demonstrated that EDRF/NO was generated from the oxidation of the terminal guanidine nitrogen atom(s) of the amino acid (Scott-Burden, 1999).

1.2 NO pathway

The effects of NO can be summarized into two categorical mechanisms of action: cGMP-dependent, which involves the production of cGMP following NO activation of sGC, and cGMP-independent, which are mediated by reactive nitrogen species that are produced as a result of the interaction of NO with oxygen or superoxide radicals (Krumenacker et al., 2004). Why Nature selected such an unstable and highly reactive diatomic molecule to serve such critically important signalling functions is still in question (Poulos, 2006).

NO acts via a well-characterised NO-cGMP pathway (Steinert et al., 2008), and its actions are mostly due to cGMP formation. (Marcondes et al., 2006). The prominent
receptor for NO known to date is sGC. Stimulation by NO results in the production of the secondary messenger cGMP, which exerts its effects via cGMP-dependent kinases, channels, or phosphodiesterases (Friebe et al., 2007). It has been only within the last 20 years that the complete outline of the NO/cGMP signalling pathway has been understood. After the discovery of cAMP in 1958 and the initial elucidation of its role in physiology the search for other physiologically relevant cyclic nucleotides began. In 1963, cGMP was detected in the urine of rats, several years later guanylate cyclase was found in mammalian tissues (Denninger & Marletta, 1999).

The physiological role of NO depends on its local concentrations as well as availability. At low nanomolar concentrations, activation of sGC is the major event initiated by NO. The resulting elevation in the intracellular cGMP levels serves as signals for regulating diverse cellular and physiological processes. cGMP directly regulates the activities of its downstream effectors such as Protein Kinase G (PKG), cyclic nucleotide gated channels (CNG) and cyclic nucleotide phosphodiesterases (Fig. 1.2.1). Each of these downstream effectors then transmits the signals to an array of intracellular signalling molecules (Madhusoodanan & Murad, 2007). These NO-dependent signal molecules are associated with a number of important physiological processes including smooth muscle relaxation, platelet aggregation, neurotransmission and cellular differentiation (Sharina, 2000).
Figure 1.2.1 Synthesis of cGMP, downstream intracellular signalling targets modulated by cGMP and the role of phosphodiesterases (PDEs) in cGMP breakdown. This pathway mediates relaxation of vascular smooth muscle relaxation. Smooth muscle relaxation is in part mediated via protein kinase G (PKG) activation, subsequent potassium channel opening and reductions in intracellular calcium levels (Ghofrani et al., 2006).
1.3. Nitric oxide synthase isoforms

Production and release of NO represents one paracrine/autocrine mechanism coordinating energy supply and demand in tissues (Stewart, 2004). NO has been shown to play a key role in vascular homeostasis by contributing to the regulation of vascular tone, the inhibition of smooth muscle proliferation and the antithrombogenicity of the vascular wall (Papapetropoulos, 1996). Much has been discovered about the biology of NO through the characterisation of the enzyme that is responsible for its formation in living tissues, called nitric oxide synthase (NOS). NOS is a highly regulated enzyme which catalyzes the five electron oxidation of L-arginine to citrulline and nitric oxide (NO) (White & Marletta, 1992). Three isoforms have been identified in a variety of tissues (Friebe, 2007). The classification of the nitric oxide synthases has depended on the finding that the enzymes in the brain and vascular endothelium are constitutive and are activated by calcium and calmodulin, whereas the enzyme in macrophages is inducible and calcium-independent. One of the major functional differences between the isoforms of NOS is their need for calcium (Ca\(^{2+}\)).

Isoform I is constitutively expressed in brain and was first purified from rat and porcine cerebellum (Bredt et al., 1990; Schmidt et al., 1990). It is a Ca\(^{2+}\) and calmodulin-dependent enzyme that is inactive at 100nmol/L Ca\(^{2+}\) and fully active at 500nmol/L (Forstermann et al., 1991; Schmidt et al., 1991). This represents typical change in intracellular Ca\(^{2+}\) concentrations upon receptor stimulation of excitatory cells such as neurons. The enzyme is phosphorylated by Ca\(^{2+}\)-calmodulin-dependent protein kinase II, protein kinase C, and cyclic AMP-dependent protein kinase (Bredt et al., 1990). It is constitutively expressed in neurones and has a molecular weight of 155kDa (Papapetropoulos et al., 1996).

Endothelial cells constitutively express isoform III (endothelial isoform), which is shorter (135kDa) than type I (Papapetropoulos et al 1996). It is also found in other cell types, is Ca\(^{2+}\)-dependent and is called eNOS. eNOS may be phosphorylated in response to various forms of cellular stimulation (Michel et al., 1993; Garcia-Cardena et al., 1996).
Isoform II of NO synthase is usually not constitutively expressed but can be induced in macrophages and many other cells with bacterial lipopolysaccharide and/or cytokines including smooth muscle cells (Steuhr et al., 1991; Hevel et al., 1991, Stewart et al. 2004). The enzyme is also referred to as iNOS or mac-NOS, and in mouse macrophages, calmodulin is found stoichiometrically associated with the enzyme (Steuhr et al., 1991).

1.4. Blood Vessel Physiology

All arteries have a common pattern of organisation and are made up of similar materials, though the proportions vary in different parts of the circulation. The arterial walls are well-organised connective tissue structures composed of cells and matrix fibres arranged in three tunicae: the intima, the media and the adventitia (Levy & Tedgui, 1999) (Fig. 1.4.1). The intima is the innermost layer and the surface is covered with a single layer of endothelial cells (ECs), which rest on the basement membrane of subendothelial microfibrils. The media contains mainly circularly arranged smooth muscle cells and collagenous fibrils. The adventitia is composed of collagen fibres and fibroblasts that protect the blood vessel and anchor it to surrounding structures (Kemball-Cook et al., 2005).
Figure 1.4.1 Diagram showing a blood vessel and the location of vascular smooth muscle cells and endothelial cells. Anatomy overview of a human artery made for PhD project (Stijn, 2005).

Blood vessels are therefore composed of two interacting cell types, endothelial cells and smooth muscle cells. Endothelial cells form the inner lining of the vessel wall and vascular smooth muscle cells envelop the surface of the vascular tube (Bergers & Song, 2005). The endothelium is a confluent monolayer of thin, flattened, rhomboid-shaped cells lining the intimal surface of all blood vessels. Due to its strategic position, it is important in the mediation and modulation of smooth muscle activity and inflammatory responses within the vessel.

The endothelial cells regulate smooth muscle cell growth by producing both growth promoting factors (oxygen free radicals, angiotensin II) and growth inhibiting factors (NO, TGF-β, Prostaglandin I₂) (Davies & Hagen, 1993). Endothelial cells have
receptors for the cytokines IL-1 and tumour necrosis factor beta (TNF-β). Endothelial cells, as well as smooth muscle cells produce cytokines, such as IL-1, IL-6 and IL-8. These cells may differentially produce and interact with cytokines, thereby contributing to the regulation of inflammatory processes in the vessel wall (Schonbeck, 1995).

Smooth muscle cells possess a cell body with a prominent nucleus and a small amount of cytoplasm with several long processes embracing the endothelium wall. They communicate with endothelial cells by direct physical contact and paracrine signalling pathways. Gap junctions provide direct connections between them and enable the exchange of ions and small molecules (Bergers & Song, 2005; Yamamoto & Suzuki, 2005).

Endothelial NO diffuses to the smooth muscle cells, where it combines with the haem moiety of the sGC enzyme (Ignarro, 1987). This guanylate cyclase is activated by NO and increases the intracellular concentration of cyclic guanosine monophosphate (cGMP). The resulting rise in cGMP from activation of sGC by NO induces vascular smooth muscle relaxation by lowering the intracellular Ca²⁺ concentration (Nimmegeers et al, 2007). NO regulates many vascular smooth muscle cell functions, including vascular tone, as well as cellular proliferation, apoptosis, migration, relaxation and synthesis of extracellular matrix (Takata, 2001).

1.5. NO normal function

Studies have implicated NO as a mediator, messenger, or regulator of cell function in physiologic states that include vascular tone, platelet function, short- and long-term memory, hepatocyte respiratory function, septic shock, and penile erectile function (Kuo & Schroeder, 1995). NO functions as a vasodilator and is an important mediator of homeostatic processes and host defense mechanisms (Moncada et al., 1991; Moncada, 1992). The NO signalling cascade regulates many physiological functions in the cardiovascular, neuronal, and gastrointestinal systems (Nimmegeers et al., 2007). NO has also emerged as a central participant in the immune response. The potential
involvement of NO in both the vascular and cellular process of inflammation was suggested from its involvement as an effector molecule of macrophage activity (Wagner et al., 1983).

The continuous release of basal NO keeps the cardiovascular system in a state of constant active vasodilation and plays a substantial role in regulating blood flow and blood pressure (Nimmegeers et al., 2007). In the gastrointestinal system, evidence suggests that NO regulates mucosal blood flow, mucosal protection, hemodynamic responses to liver disease, hepatocyte synthetic function, and relaxation of the muscularis (Kuo & Schroeder, 1995).

In the vascular system, NO has been shown to mediate smooth muscle relaxation and inhibition of platelet aggregation (Friebe et al., 2007). It does this through the secondary messenger cGMP.

1.6. Nobel Prize in Physiology or Medicine 1998

On October 12th, 1998, the Nobel Assembly awarded the Nobel Prize in Medicine and Physiology to scientists Robert Furchgott, Louis Ignarro and Ferid Murad for their discoveries concerning NO as a signalling molecule in the cardiovascular system. NO was shown to be produced by the NO synthases with the neuronal (nNOS or NOSI) and endothelial NOS (eNOS or NOSIII) being of major importance for the production of NO as a signalling molecule (Mergia et al., 2008).

The idea that a simple gas like NO should, at low concentrations, have very specific biological effects was initially hard to accept as it gives rise to nitric acid, and at high concentrations this would be lethal to cells. On December 2nd 1976 Murad’s laboratory managed to generate pure nitric oxide and ventilate it into guanylyl cyclase incubations (Murad, 2004). In 1979 Murad showed that guanylyl cyclase is one of the enzymes on which NO acts in the nanomolar range. For Murad to show conclusively such a small amount of NO exerts so many biological effects was not easy. The difficulty was in showing that NO was in fact the active compound, and not some other vasodilatory
molecule such as nitroglycerin or nitroprusside. Work then began on the identification of EDRF’s target.

Establishing the link between cyclic guanosine 3’;5’-monophosphate (GMP), organic nitrates (such as nitroglycerin), and NO would bring it all together. It was here that Dr. Murad made his important contribution to medicine and physiology. Murad and colleagues focused on cGMP and the cyclase enzymes responsible for its production from guanosine triphosphate (GTP). His early experiments were carried out on guanylate cyclase activation by vasodilators and later on the relaxation of smooth muscle by virtue of NO generation. Murad studied the numerous pathways of signal transduction wherein the NO-cGMP axis plays a role (Scott-Burden, 1999).

1.7. Derangement of NO levels in vascular disease

The importance of NO/cGMP signalling for the maintenance of blood pressure is well established (Rees et al, 1989). NO is thought to play a role in the pathway of several inflammatory disease states such as arthritis, myocarditis, colitis and other pathological conditions such as cancer and diabetes (Krumenacker et al, 2004). Dysfunction of the endothelial NO/cGMP signalling pathway contributes to the pathophysiology of a variety of cardiovascular disorders (CVD) including hypertension, thrombosis, atherosclerosis, myocardial infarction and angina pectoris (Nimmegeers et al., 2007). CVD is associated with progressive changes in the production of free radicals and radical-derived reactive species. NO and superoxide (O$_2^-$) are the most relevant free radicals in biology. Evidence suggests that increased NO and O$_2^-$ production make a significant contribution to the progression of CVD (Turko & Murad, 2002).

Normally, endothelial-derived NO is one of the key protective mechanisms in the vasculature (Melichar et al., 2004). In the vascular and cardiac tissue, NO is constitutively produced in modest amounts from L-arginine by eNOS or nNOS (Muller et al., 2000). eNOS is activated by mechanical stress such as blood shear-stress and stimulation with agonists such as bradykinin. The action of NO as EDRF is important for maintaining vascular homeostasis (Kawashima & Yokoyama, 2004). The reversible
activation of sGC probably accounts for most of the effect of NO on vascular contraction. The activation of sGC and subsequent cGMP accumulation is an important mechanism underlying the effect of NO in heart and blood vessels (Muller et al., 2000).

Endothelial dysfunction is characterised by a decreased responsiveness to endothelium dependent vasodilators. This may result from impaired signalling downstream from NO in the vascular smooth muscle (Klob et al, 2000). Patients who have hypertension, diabetes or atherosclerosis often exhibit endothelial dysfunction because their blood vessels produce insufficient amounts of NO (Murad, 2006).

Changes in the mRNA expression levels of sGC subunits have been reported in several disease models. In aortic tissue from spontaneously hypertensive rats, the vasodilator response to an NO donor was markedly attenuated compared with normotensive rats. The detailed analysis of hypertensive animals revealed that α1- and β1-sGC mRNA levels, as well as β1-sGC protein levels, were reduced significantly, indicating that vasodilator dysfunction is related to the sGC gene expression (Jiang, 2006).

Evidence suggests that chronic exposure to NO decreases sGC enzyme activity by decreasing the stability of the mRNAs encoding its subunits and that destabilization of sGC subunit mRNAs is dependent on gene transcription and protein synthesis (Filippov et al, 1997).

CVD is the leading causes of death in Western societies. Arterial hypertension is one of the most important public health problems in the developed world. In arterial hypertension models, endothelium-dependent vasodilation is impaired as a result, at least in part, by oxidative stress and mRNA and protein levels of both the α1 and β1 subunits of sGC and sGC activity being reduced (Evgenov, 2006). Adaptation to hypertension promotes morphological changes in the aorta characterised by vessel wall thickening. Medial hypertrophy and enhanced levels of various growth factors seem to account for this adaptive morphological response (Klob et al, 2000). Atherosclerosis is a major risk factor to CVDs such as heart disease and stroke. The pathophysiology of atherosclerosis is highly complex, multifactorial and yet to be fully understood, but involves endothelial injury (Melichar, 2004; Evgenov, 2006). Atherosclerosis is
associated with alterations in NO/cGMP signalling. In early stages of the disease, inflammatory and possibly other cells produce reactive oxygen species that scavenge vasoprotective NO. In addition to the oxidative stress, expression and activity of enzymes downstream to NO formation may also be affected (Melichar, 2004). An impairment of endothelium-dependent relaxations is present in atherosclerotic vessels even before structural changes occur. All major risk factors for atherosclerosis such as hyperlipidemia, diabetes, hypertension and smoking are associated with impaired endothelium dependent relaxations. Although the underlying mechanisms of reduced endothelium dependent relaxation are multifactorial, its most important cause is a derangement of the eNOS/NO pathway, which include reduced activity and expression of eNOS. It seems that eNOS normal function inhibits atherogenesis by producing NO (Kawashima & Yokoyama, 2004). Vascular dysfunction in atherosclerosis is partly attributed to reduced bioavailability of endothelium-derived NO, which is supported by the reduced activity and expression of eNOS (Melichar, 2004).

1.8. Role of subunits of sGC and their functions

sGC is a member of the guanylyl cyclase family of proteins, which respond to various ligands by converting GTP into cGMP. It stands apart from other members of its family by the nature of its activating ligand. sGC is activated several hundred fold upon exposure to NO produced by nitric-oxide synthase. sGC is the key component of the NO/cGMO pathway and is crucial in mediating various physiological effects of NO mentioned earlier such as blood vessel relaxation, inhibition of platelet aggregation and neurotransmission (Martin et al., 2006). In the absence of NO, sGC exhibits very low basal activity. Conformational changes following the binding of NO to haem result in marked activation of the enzyme (Madhusoodanan & Murad, 2007). sGC was first purified (from lung) in the early 1980s (Poulos, 2006). Since its discovery 30 years ago, the biochemical properties of this enzyme have been relatively well characterised (Sharina et al., 2003).
The human genome encodes two sGC α-subunit and two sGC β-subunit genes (Fitzpatrick DA et al., 2006). The α1 and β1 sGC genes have been localized to the same chromosome in rat and human (Sharina et al., 2000).

It is known that the conversion of guanosine triphosphate (GTP) to cyclic GMP is catalyzed by at least two isoenzyme forms of guanylate cyclase, of which the kinetic, physiochemical and antigenic properties are quite different (Friebe et al., 2007). pGC (particulate soluble guanylyl cyclase) serves as a receptor for atrial natriuretic peptides and sGC (soluble guanylyl cyclase) serves as a receptor for gaseous ligands, namely NO and carbon monoxide (Evgenov et al., 2006). There are six or more particulate isoforms of GC. These are receptor cyclases that are transmembrane proteins with an extracellular peptide receptor domain, a short transmembrane domain, and intracellular kinase-like and catalytic domains (Murad, 2006). The relative abundance of the soluble and particulate enzyme is variable in different tissues and species. Intestinal mucosa and retina possess predominantly the particulate isoenzyme, and platelets contain the soluble isoenzyme. Tissues such as vascular smooth muscle have both isoenzymes (Murad, 1986). sGC has been accepted as the most important mediator of NO effects, with the two most important being aortic relaxation and platelet inhibition (Friebe, 2007).

sGC is a heterodimer made up of two subunits, α and β (Nimmegeers et al., 2007) which are encoded by separated genes (Figure 1.8.1) (Sharina et al., 2007). Two isoforms are known to exist, α1β1 and α2β1, in which the β1 subunit acts as the dimerising partner for either α subunit. α subunits in the absence of the β1 subunit do not form dimers and are not catalytically active. Theoretically, the association of α and β subunits could give rise to at least four different isoforms, but only the α2β1 and α1β1 isoforms are reported to be active (Nimmegeers et al., 2007) with α1β1 being the most highly and ubiquitously expressed. Human vascular smooth muscle, vascular endothelial cells, cerebral cortex, and cerebellum express predominantly α1β1 subunits (Sharina et al., 2003).
Figure 1.8.1 sGC is an obligatory heterodimer composed of $\alpha_1$ and $\beta_1$ subunits. The $\beta_1$ subunit binds the prosthetic haem which is the target for NO.
The structure of sGC consists of a haem-containing regulatory domain, formed by N-terminal portions of both subunits, and a catalytic domain formed by the C-terminal half of both subunits which convert GTP into cGMP (Martin et al., 2003). Because of the crucial role in binding haem and providing regulation by NO, the N-terminal regions of α and β subunits are often referred to as the regulatory domain of the enzyme. In the absence of any activators, the enzyme displays a relatively small activity with a low turnover number (Martin et al., 2005). sGC contains only one ferrous pentacoordinated haem with the His-105 residue of the β subunit acting as the axial ligand. Binding of NO to haem Fe²⁺ and the formation of nitrosyl haem result in the disruption of the axial coordinating bond and displacement of iron from the protoporphyrin plane (Martin et al., 2003). The bond between the haem and a nearby histidine residue breaks, causing a conformational change that propagates to the catalytic domain, greatly speeding sGC activity and the conversion of GTP into cGMP several hundred fold (Roy & Garthwaite, 2006).

1.9. Pathophysiological effects on sGC regulation

Findings on the activity of putative promoter regions demonstrate different transcriptional activity for both subunits, suggesting the potential for finely tuned regulation. The ratio of expression levels for both subunits varies in a tissue-dependent manner, suggesting that the regulation of expression for these subunits is not tightly coordinated. (Sharina et al., 2000).

The mechanisms by which the soluble or membrane forms of guanylyl cyclase are regulated have been explored at both the protein and the gene level (Behrends et al., 1995). Emerging data indicates that regulation of sGC expression could be an important factor in the modulation of sGC activity (Sharina et al., 2003). Evidence suggesting that sGC activity is regulated at the protein and mRNA levels has begun to emerge (Sharina et al., 2000). Expressional regulation of human sGC α₁ and β₁ subunit expression is likely to play an important role in the pathophysiological regulation of enzyme activity. (Marro et al., 2008). The identification and characterisation of human α₁ and β₁ promoters suggest
that these subunits may be targets for regulation by a number of transcription factors that play critical roles in a variety of cellular processes (Madhusoodanan & Murad, 2007). Exposure of cells to pro-inflammatory cytokines or NO is known to induce changes in sGC expression For example, lipopolysaccharide (LPS), interleukin 1β, and NO donors cause reduced sGC β1 mRNA expression in pulmonary artery smooth muscle cells (Marro et al., 2008).

The vasculature, when exposed to inflammatory mediators may be able to adapt to elevated NO levels by decreasing sGC. Cytokine-mediated changes in the function of other components of the NO-cGMP signal transduction system, such as phosphodiesterases and cGMP-dependent protein kinases, may also have important roles in regulating vascular responsiveness to NO. Cytokine-induced desensitization of vascular responsiveness to NO may represent a homeostatic mechanism preventing excessive signaling via cGMP and may serve to limit the vasodilation associated with sepsis (Masao et al., 2001). It has been demonstrated that in vivo 17β-Estradiol administration differentially affects sGC subunits, increasing α1 levels and reducing β1. sGC activity was also dramatically reduced by 17β-Estradiol (Cabilla et al., 2006). It has also been observed that exposure of rat pulmonary artery smooth muscle cells to the cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), induced NOS2 gene expression and decreased sGC α1- and β1-subunit mRNA levels. Decreased sGC subunit mRNA levels were accompanied by decreased α1- and β1-subunit protein concentrations and decreased NO-stimulated sGC enzyme activity. These observations suggest that cytokines may impair sGC enzyme-specific activity as well as decrease sGC subunit protein levels (Masao et al., 2001).

A family of glycoprotein molecules, termed colony stimulating factors (CSF), are known to control proliferation, maturation and functional activities of granulocytes, macrophages and their precursors (Bussolino et al., 1991). GM-CSF is a 23-kDa glycoprotein cytokine first characterised for its ability to stimulate progenitor haemopoietic cells to proliferate and differentiate into mature granulocytes and macrophages. It has been shown to have multiple effects in immune activation and to influence mitogenesis, acting in concert with other members of the CSF family as a key mediator in inflammation and host defence (Weissen-Plenz et al., 2008). GM-CSF is
among the cytokines that modulate the functional status of endothelial cells. It has been shown that human endothelial cells derived from umbilical veins have high affinity receptors for GM-CSF. After binding to its receptor on human endothelial cells, GM-CSF starts sequential events, including a rise of intracellular pH and expression of the c-fos proto-oncogene, followed by cell migration and proliferation (Bussolino et al., 1991).

Evidence implicating GM-CSF as a critical player in vascular processes associated with structural remodelling such as arteriogenesis and atherogenesis has been steadily mounting. In atherogenesis, multiple processes are affected by GM-CSF. GM-CSF lowers plasma cholesterol levels in humans and animals. Characteristic expression and distribution patterns of GM-CSF arise in human coronary arteries during atherogenesis. Recent studies point to a key role of GM-CSF in the cytokine network regulating the vascular extracellular matrix. In vitro studies on cultured vascular smooth muscle cells revealed that GM-CSF modulates the transcription of type VIII collagen, which is a key structural component of the vascular (Weissen-Plenz et al., 2008; MacBeath et al., 1996).

Much of the regulatory function of T cells is mediated by the secretion of a set of potent polypeptides often designated as interleukins (ILs). IL-4 is made in response to immunologic recognition, principally, although not exclusively, by CD4+ T lymphocytes. It mediates much of its action in short range interactions between target cells and IL-4 producing T cells, and it has a wide range of functions. In vitro analysis has demonstrated a wide range of functions of IL-4 on B cells, on T cells, on macrophages, on haematopoietic precursor cells, and on stromal cells. The extent of these functions remains to be determined. IL-4 mediates its functions by binding to receptors expressed on target cells (Paul, 1991).

The 0.3- and 0.5kb regions upstream of the transcription start sites of sGC are optimal for α1 and β1 sGC promoter activity (Marro et al., 2008). Figure 1.9.1 shows the sGC α1β1 promoter regions. Activator Protein 1 (AP-1) is a ubiquitous transcription factor and a pleiotropic regulator of the inducible expression of many genes that encode proteins involved in the modulation of inflammatory and host defense processes. AP-1 has been shown to alter gene expression in response to growth factors and cytokines...
Previous studies have revealed that Nuclear factor of activated T-cells (NFAT) is one of the principal transcription factors regulating basal sGC expression (Marro et al., 2008). Cooperation between NFAT and AP-1 transcription factors has been demonstrated in the promoter/enhancer region of several genes in immune cells (Macian et al., 2001).

![Figure 1.9.1 sGC α₁β₁ promoter regions on chromosome 4q32](image)

The sGC promoter region contains factors which are known to regulate the inducible expression of many genes that encode proteins involved in the modulation of inflammatory and host defense processes. We know from the literature that α₁ and β₁ sGC levels and activity are affected by a diverse array of inflammatory conditions, and that cytokines play a part in this process. In light of this information, one of the aims of this project was to determine the effects two such cytokines (GM-CSF and IL-4) would have on sGC.
1.10. Splice Variants of sGC and previous work carried out

Very little information is available regarding the role of transcription regulation by modulators affecting sGC steady-state mRNA, or protein levels, and this mechanism of regulation of sGC remains largely unexplored (Gerassimou et al., 2007). There is increasing evidence, supporting the existence of sGC splice forms for $\alpha_1$, $\alpha_2$, $\beta_1$ and $\beta_2$ genes (Sharina et al., 2008). With the strong indications that sGC genes undergo an alternative splicing which could potentially have a role in regulating its activity in healthy and diseased vascular tissue, we set about to clone one of these splice forms into an expression vector to study it further.

Alternative splicing frequently occurs in eukaryotic genes and provides an important mechanism for tissue-specific and developmental regulation of gene expression (Sharina et al., 2008). Differential RNA splicing can serve to switch an active protein to an inactive protein (Behrends et al., 1995). Recently, splicing started to emerge as a mechanism essential for cardiovascular function and development. A better understanding of the mechanisms modulating intracellular cGMP levels, such as regulation of sGC expression, are of high importance (Kalsotra et al., 2008). It is conceivable that a given cell modulates its degree of NO responsiveness by the expression of differing amounts of alternative subunits of sGC (Behrends et al., 1995).

In mammals, the alternative splicing for the $\alpha_2$-subunit of sGC generates a dominant-negative variant. Splice forms for $\alpha_1$ and $\alpha_2$ subunits have been also demonstrated. Recently, a shortened $\alpha_1$ sGC transcript has been found, and its expression was correlated with lower sGC activity in several cell lines (Sharina et al., 2008). Several transcription factors important for housekeeping $\alpha_1$ and $\beta_1$ sGC transcription have been identified. Recent studies have demonstrated that the exposure of rat aortic smooth muscle cells to hydrogen peroxide inhibits $\alpha_1$ sGC promoter activity and $\alpha_1$ steady-state mRNA levels (Gerassimou et al., 2007).
The wild type transcript of β₁ sGC has several Accession Numbers in the NCBI database including: NM00857, BC047620, AK315999. AK307838 is a novel β₁ sGC splice form given the name SH-β₁. SH-β₁ sGC AK307838 is a 592 amino acid β₁ sGC protein which was isolated focusing on alternative splicing from the human brain (Isogai & Yamamoto, Takao Isogani Reverse Proteomics Research Institute, 2008). Figure 1.10.1 shows the comparative genomic organization for human β₁ and the SH-β₁ sGC splice form. Blue bars indicate conservative exons. Red bars indicate alternative pieces inserted by splicing. As can be seen from the diagram, exon 2‘ is specific for SH-β₁ and exon 10’ has an additional 5’ fragment. The 2‘ exon introduces a premature stop codon. The SH-β₁ open reading frame (ORF) starts with a second Methionine (Met) and is restored at the 3’ end of exon 2‘. Intron 9, which precedes Intron 10’, has an alternative splice acceptor at its 3’ end which is upstream of the canonical one. It introduces an additional 125 base pairs at the 5’ end of the exon, which makes exon 10’ longer. This additional sequence is an in-frame insertion and encodes 43 unique amino acids in SH-β₁.

Figure 1.10.1. A comparative genomic organization of wild type and splice form transcript of β₁ sGC
A global alignment of protein sequences encoded by wild type mRNA and SH-β₁ mRNA is shown in Figure 1.10.2. The alignment searches for identical sequences and differences between the two proteins. If both sequences encode identical proteins, no change in protein function is expected. As can be seen from the diagram, there are two major differences between canonical β₁ sGC and SH-β₁ sGC protein. SH-β₁ has a deletion of 68 amino acids at its N-terminal part of the protein (highlighted in green), and a 43 amino acid insertion in the middle of the protein (highlighted in red).
Figure 1.10.2. Global alignment of the accepted β₁ and SH-β₁ sGC proteins. The sequence highlighted in green shows a deletion of 68 amino acids at the N-terminal part of the protein. The sequence highlighted in red shows a 43 amino acid insertion in the middle of the protein (Diagram prepared by and included with permission by Dr. Ira Sharina, Dr. Ferid Murad’s laboratory, Unpublished).

Figure 1.10.3 shows the β₁ sGC domain structure and compares it with the protein encoded by SH-β₁. The N-terminal deletion is located close to the haem-binding domain. This indicates that domain function may be affected, and could change NO sensitivity of sGC heterodimers composed of the SH-β₁ splice form. The 43 amino acid insertion is positioned very close to the catalytic domain, which suggests that catalysis might be
affected also. Based on this analysis, the laboratory hypothesised that regulation of expression of SH-β₁ by splicing is a new regulatory mechanism to modulate sGC function. It was previously shown that splicing does affect enzymatic properties of sGC (Martin et al., 2003).

Figure 1.10.3. Comparative domain organisation of β₁ and SH-β₁ sGC proteins. Wild type β₁ sGC is showed on top with the haem-binding domain, dimerisation domain and the catalytic domain. Underneath is the SH-β₁ sGC splice form which is 25 amino acids shorter than the wild type. Wild type domain structure adapted from Zhou et al., 2004; Wagner et al., 2005.

Recently, one of the focuses of the Murad laboratory has been to isolate and functionally characterise the SH-β₁ sGC splice form and to prove its biological significance. The initial part of the project was to demonstrate that the splice form was indeed expressed in human tissue and cancer lines. Twenty normal human tissue samples were screened for
SH-β₁ transcript detection. RT-PCR screening was performed on cDNA from the twenty human samples. SH-β₁ fragment was isolated and its identity was confirmed by sequencing. cDNA from twelve human cancer cell lines were also used for analysis, which SH-β₁ was also isolated from. The primers used were localised in a unique sequence of SH-β₁ exon 10’. The analysis showed that splice form was differentially expressed in normal tissues and cancer lines. This confirmed the hypothesis that expression of this variant might be regulated to modulate sGC function, possibly in a tissue specific manner. mRNA from cancer lines expressing high levels of the variant were used for SH-β₁ subcloning.

The next step was to compare the functional properties of the α₁/β₁ wild type heterodimers and α₁/SH-β₁ heterodimer. To do this the laboratory planned to over-express both heterodimers in the Sf9 insect line (as they do not contain endogenous sGC) and to use them to determine kinetic constants (Vmax, Km) for wild type and splice form by measuring cGMP accumulation. To achieve recombinant expression, the aim was to create a Baculovirus containing an ORF of SH-β₁. In order to achieve this, two SH-β₁ ORF fragments were to be subcloned into the commercial Baculovirus exchange vector, pBacPak8.

The subcloning strategy is described in figure 1.10.4. The ORF had to be separated into two fragments because the wild type and the splice form have identical sequences surrounding the start and stop codons, which makes it difficult to PCR amplify SH-β₁ ORF from start to finish without amplifying the wild type at the same time. The primers Sh491 (fr1 3’) and Sh492 (fr2 5’) are located in a unique sequence insertion of exon 10’, and will be used to prevent wild type amplification. Stu1 sites will be used as linkers to join the two fragments together and to restore SH-β₁ ORF, as the Stu1 site is located uniquely in the SH-β₁ insertion.
Figure 1.10.4. Procedure to insert fragments 1 and 2 into pBacpak8. Human SH-β₁ was broken into two separate fragments (fragment 1 and fragment 2) using specific primers and each fragment was then cloned separately into pBacPak8.
2.0. Aims

Very little is known about the regulation of the expression of sGC. In this study the aim was to learn more about this regulation by analysing the effects of cytokine treatment on the mRNA and protein levels of sGC. In order to achieve this, we aimed to:

- Culture smooth muscle and endothelial primary cells successfully and treat them with a selection of cytokines.
- The mRNA levels were to be analysed using QPCR. \( \alpha_1 \) and \( \beta_1 \) sGC plasmid standard curves had to be set up to allow us to determine the transcript numbers of sGC in cytokine-treated mRNA samples.
- QPCR was then to be carried out to detect \( \alpha_1 \) and \( \beta_1 \) sGC levels in unstimulated and stimulated HAOECs and HAOSMCs.
- Protein levels of samples were to be tested using Western blot analyses. A panel of antibodies for \( \alpha_1 \) and \( \beta_1 \) sGC was to be evaluated first on BE2 and Sf9 cell lysates for use in Western blotting on primary cell protein samples. \( \alpha_1 \) and \( \beta_1 \) sGC protein levels from HAOECs and HAOSMCs were to be analysed using Western blotting.

In addition to looking at the effects of cytokine treatment on sGC, we wanted to successfully incorporate the sGC splice form A307838 into the plasmid vector pBacPak8 for future enzymatic and functional assays using viral vectors. To achieve this we aimed to:

- Ligate fragment 1 of A307838 into the plasmid vector pCR-Blunt.
- In addition to this we aimed to re-isolate fragment 2 from pBacPak8 plasmid vector to which it had been previously cloned and to restrict and join fragments 1 and 2.
3.0. Materials and Methods

3.1. Materials

3.1.1. Cell Culture Reagents

- HAOEC grown in Endothelial Cell Growth Medium (Cell Applications Inc, Cat No. 211-500 Lot 555, stored at 4°C, Exp 04/09)
- HAOSMC grown in Smooth Muscle Cell Growth Medium (Cell Applications Inc, Cat No. 311-500 Lot 552, stored at 4°C, Exp 04/09)
- Human Aortic Endothelial Cells (HAOEC) and Human Aortic Smooth Muscle Cells (HAOSMC) (Cell Applications, Inc, 5820 Oberlin Drive, Suite 101, San Diego, CA 92121)
- Trypsin (Cellgro, Mediatech Inc, Manassas, VA 20109. Trypsin EDTA, IX 0.255 Trypsin/2.21mM EDTA in HBSS without sodium bicarbonate, calcium and magnesium. Porcine Parvovirus Tested, Cat No. 25-053-C1, Lot No. 25053195, Exp 07/09, Stored at -5º to -20ºC)
- Trypan Blue dye (Sigma)
- Lysis buffer (50mM TrisHCL, 150mM NaCL, 1 mM EDTA, 1% Triton, PI cocktail from Sigma, 50µl/1.5ml)
- Recombinant Human Granulocyte Macrophage Colony Stimulating factor (GM-CSF) (5µg eBioscience, Cat No. 14-8339, 10ng/µl, diluted in 500µl PBS+BSA 1%)
- Recombinant Human Interleukin 4 (IL-4) (2µg, eBioscience, Cat No. 14-8049, 10ng/µl, diluted in 500µl PBS+BSA 1%)
3.1.2. RNA extraction materials

- RNAqueous Phenol-free total RNA Isolation Kit. Ambion Inc-The RNA Company, Cat No. AM1912, Lot No. 0804010, 2130 Woodward St, Austen, TX 78744.
- Agarose (Sigma A5093, 100g, Batch #038K00011, EC 232-731-8, 1g in 100mls)
- Quick-Load 2-Log DNA Ladder (New England Biolabs, #N0469S, 100µg/ml, 1.25mls, Lot 0060812, Assay 12/08, Exp 12/10)
- Gel Loading Dye 6X (New England Biolabs, #B7021S, 1.5mls, Lot 0010811, Exp 11/11).

3.1.3. QPCR materials

RT-PCR Reagents

- 10X RT Buffer (4319981)
- 25X dNTP (100mM, Lot No. 0810098, Part No. 362271)
- 10X RT Random Primers (Part No. 4319979, Lot No. 0810140)
- RT Enzyme (multi-scribe, Part No. 4319983, Lot No. 0811074)
- DEPC H2O

Ligation

- 10X Buffer for T4 DNA Ligase with 10mM ATP (#B02025, NE Biolabs, Lot 0010806)
- T4 DNA Ligase (M02025, NE Biolabs, 0.05mls, 400,000 U/ml, Lot 0920805, Exo 05/10)
- 1.2M NaCl

Transformation

- SOC Medium (Invitrogen, Lot No. 313531, Cat No. 15544-034)
- Competent Cells (E Coli, Invitrogen)
- Kanamycin plates (1000X)

Colony Selection
- LB Broth, Buncen Burner
- Conical Flask, Kanamycin (1000X)
- Forceps
- filtered tips
- Culture Test tubes.

Miniprep
- QIAprep Spin Miniprep Kit (250), Cat. No. 27106, Lot No. 42477737

Restriction Digest
- EcoR1 (New England Biolabs, R0101S, Lot 0320808, Exp. 08/10, 20,000 U/ml)
- EcoR1 Buffer (New England Biolabs, B0101015, Lot. 0010808, Exp. 08/11)
- DEPC H20

QPCR
- GUCYB3 (Applied Biosystems, Amplicon Length=82, Lot#625260, 250µl 20X Mix, Exp 07/2013, 20X)
- GUCY1B3 (Applied Biosystems, Amplicon Length=100, Lot#487918, Exp 04/2012, 20X)
- GAPDH (Lot#642641, Exp 09/2013, 20X)
- PCR mix (2X, Applied BioSystems)
- DEPC H20
LB broth/agar

- 20 LB tablets added to 1000mls double distilled deionised water and placed on a magnetic stirrer until dissolved. 15g of agar added per litre. Autoclaved.

- 1.5g of Agar was added per 100mls LB broth.

Making a molecular weight marker

- 10µl Hind III, 10µl PhiX (New England Biolabs, 500µg/ml, Exp 9/09) 40µl loading buffer, 40µl water. Heat at 70 degrees for 5 minutes.

3.1.4. Western blotting materials

- BE2 cells (kept on ice)
- Protein Inhibitor A (1000X, Inhibits water soluble inhibitors)
- Protein Inhibitor B (1000X, Inhibits LMW compounds which are DMSO soluble)
- BSA stock (5mg/ml)
- TEA 40mM pH 7.4.
- Blotto Non-Fat Dry Milk, Santa Cruz Biotechnology SC-2325 Lot No. G1408, 250g, Santa Cruz Biotechnology, Lot #G1408)
- Sigma N, N, N’, N’-Tetramethyethyl-enediamine, 99%, T9281-100ml Batch No. 114K06101
- Kaleidoscope Prestained Standards, Bio-Rad (Catalog No. 161-0324)
• 10X TBST+0.1% Tween (1ml tween per 1000mls 10X TBST, 10X TBST made up using 100mls 100X TBST+900mls deionised water)
Western Buffers

- 10X Transfer Buffer (0.25 M Tris, 1.92 M Glycine)
- Transfer Buffer (100mls 10X Transfer buffer, 200mls methanol, 700mls H2O)
- 10X TBS Buffer (pH 7.6, 12.5g Trizma Base, 60.5g NaCL, 500mls)
- 1x TBST (1X TBS, 0.1% Tween 20, 50mL NaCL (1.5g), 500mls, 50mls 10X TBS, 0.5mls Tween, 450mls H2O.)
- Blocking Buffer (100mls 1X TBST, 5g Blotto Milk, microwave, spin, cool on ice)

SDS/PAGE Gels

- Cell Lysate Buffer (40mM TEA, 0.742g Triethanolamine, HCL (185.52 FW, 100mls H20)
- 4X Lameli Loading Buffer (5ml Upper buffer, 0.8ml SDS, 4ml glycerol, Bromophenol Blue, 1ml 2 beta mecaptoethanol).
- 4X Lower Buffer (1.5M Tris, ),4% SDS, pH 8.8, 91g Trizma Base, 0.4g SDS, 500mls)
- 4X Upper Buffer (0.5M Tris, 0.4% SDS, pH 6.8, 30g Trizma Base, 2g SDS, 500mls).
- 10X Running Buffer (0.25M Tris, 1.92 Glycine, 1%SDS, 1L, 30g Trizma Base, 144g Glycine, 10g SDS (added last), pH not adjusted)
- 8% Upper Gel (6.6mls H20, 3mls 4X Lower Tris Buffer, 2.4mls 29:1 Acrylamide Bis (40%), 120µl 10% APS (100%), 12µl TEMED (added last),
- 12% Lower Gel (5.4mls H20, 3mls 4X Lower Tris Buffer, 3.6mls 29:1 Acrylamide:Bis (40%), 240µl 10% APS (100%), 28µl TEMED, layer of water saturated with 1-n-butanol on top of polymerized lower gel).
- Costar Stripettes, 5mls (4487), 10mls (4488) and 25mls (4489), Corning Incorporated, Non-Pyrogenic Serological Pipets, NY 14831)
3.1.5. Splice variant materials

PCR Amplification
- Pfu (house purified)
- Pfu 10x Buffer (Stratagen)
- Primers, 20 uM stocks, custom order (IDT, Integrated DNA Technologies, Coralville, IA) sequences designed to cover ORF of SH-b1 sGC splice form and based on A307838 sequence

Ligation
- 10X Buffer for T4 DNA Ligase with 10mM ATP (#B02025, NE Biolabs, Lot 0010806)
- T4 DNA Ligase (M02025, NE Biolabs, 0.05mls, 400,000 U/ml, Lot 0920805, Exp 05/10)
- 1.2M NaCL

Transformation
- SOC Medium (Invitrogen, Lot No. 313531, Cat No. 15544-034)
- Competent Cells (E Coli, Invitrogen)
- Kanamycin plates

Colony Selection
- LB Broth, Bunsen Burner
- Conical Flask,
- Kanamycin (1000X)
- Forceps
- filtered tips
- Culture Test tube
DNA Extraction
- QIAPrep Spin Miniprep Kit (250), Cat. No. 27106, Lot No. 42477737
- Pure Yield Plasmid Miniprep System (Promega)
- NucleoSpin Plasmid Kit (Cat No. 74058810, Lot No. 806/004) (Macherey-Nagel)

Dephosphorylation
- Antarctic Phosphastase (M02895, 5,000U/ml, Exp 01/09, Lot 014080L, 0.2mls, NE Biolabs).
- NEBuffer Antarctic Phosphatase (B02895, 10X, 1.5mls, Lot 0707, NE Biolabs).

Band Purification
- Qiagen QIAquick Gel Extraction Kit (50)

DPN1 Treatment
- DPN1, New England Biolabs, 20,000U/ml

DNA Precipitation
- Sodium Acetate (3M)
- 100% v ethanol
- 80% ethanol
- Nuclease-free water

Ligation Reagents
- 10X Buffer for T4 DNA Ligase with 10mM ATP, #B02025, New England BioLabs, 0.5ml, Lot 0010806, Exp. 06/11
- T4 DNA Ligase, #M02025, New England BioLabs, 400,000 U/ml, 0.05ml, Lot 0920805, Assay 05/08, Exp. 05/10
Dephosphorylation reagents

- New England BioLabs, NE Buffer Antarctic Phosphatase, B)2895, 10X, concentration 1.5ml, Lot 0707
- New England Biolabs Antarctic Phosphatase, M02895, 5,000 U/ml, Lot 0140801, 0.2ml, Exp 01/09

3.1.6. Equipment

- Thermo Electron Corporation PX2 Thermal Cycler, Labnet Rocker 25
- Thermo Electron Corporation Forma 3LG 4500R Centrifuge, Multi-Blok Heater No. 2097-1, Lab-Line Instruments, Inc. Designers and Manufacturers, Melrose Park, ILL.
- AquaBath-Barnstead Lab-Line, Cecil CE9500, 9000 Series-Super Aquarius, Sharp Carousel
- Thermolyne-Maxi Mix Plus
- Thermo Electron Corporation, Forma Series II, Water Jaceted CO₂ Incubator, HEPA Class 100
- Thermo Electron Corporation, KeyWrite-D, Jouan, CR3i Multifunction Centrifuge
- Magni Whirl Constant Temperature Bath, Blue M Electronic Company, Blue Island, Illinois, USA
- MicroStar AO Scientific Instruments, CRC Precision Electronics, 8704 Haverstock Drive, Houston Texas, 77031)
- SterilchemGARD III Advance°
- Falcon Pipet-Aid, Sarstedt-Scre Cap Tube Conical 15ml, 17x120mm
- Falcon Pipet-Aid Lot 8122088 Exp 2010 05, Sarstedt-Scre Cap Tube Conical 50ml, 28x114mm Lot 8217087 Exp 2010 08 Sarstedt, Inc. Newton. NC 28658-0468
- Posi-Click Tube by Denville, Lot 080912-229
- Cecil CE9500, 9000 Series, Super Aquarius
• Molecular Devices-Emax precision microplate reader
• Fisher Scientific Isotemp Incubator
• Sonics Vibra Cell Sonicator
• Applied Biosystems ABI Prism 7900 HT Sequence Detection System, 200582
3.2. Methods

3.2.1. Culture of endothelial and human vascular smooth muscle cells

Cell Culture
Human Aortic Endothelial Cells (HAOEC) and Human Aortic Smooth Muscle Cells (HAOSMC) (Cell Applications) were each received in two T25 flasks, both at passage three and approximately 60% confluent. HAOEC were grown in Endothelial Cell Growth Medium, and HAOSMC were grown in Smooth Muscle Cell Growth Medium according to the manufacturer’s recommendations (37°C, 5% CO₂ humidified).

Passaging Cells
Cells were split when they reached 80-90% confluency. The medium was aspirated and 3mls of trypsin at room temperature was added to each flask and left for up to four minutes, checking cells under the microscope after each minute and gently tapping the flask until they began to lift from the base. 3mls of appropriate medium was then added to each flask to dilute the trypsin and halt the trypsinisation process. This was followed by washing with a further 2mls of medium to ensure all cells had been harvested. Flasks were checked microscopically to determine if most of the cells had been removed, and rinsed in an additional 2mls of media. Cells were collected into 50ml Universal tubes (Sarstedt Inc, Newton NC) and centrifuged at 1100rpm at 4°C for 5 minutes. The media was aspirated, taking care not to remove the pellet which was resuspended in 2mls of media. For determination of the total cell count, a 10µl aliquot of the 2ml suspension of cells was added to 10µl of Trypan Blue dye and mixed well. A haemocytometer was then used to count the total number of cells. The total cell count was then used to determine the suspension volume to add to each well.

HAOSMCs were seeded at 6,000/cm², and HAOECs were seeded at 5,000/cm² as recommended by the manufacturer.
Freezing Cells

100µl of room temperature DMSO was aliquoted into 4 cryovials. Splitting of cells was carried out as described. The cell pellet was resuspended in 3600µl serum-free media (SFM) and 900µl was added to each cryovial. Cryovials were kept at -70ºC overnight, and then transferred to liquid nitrogen the following day.

3.2.2. Stimulation of cells with cytokines

Human recombinant cytokines were received as follows: GM-CSF (5µg/50µl) and IL-4 (5µg/50µl) (eBiosciences) and were diluted using 1% BSA in PBS (w/v) to a final treatment concentration of 10ng/ml and 50ng/ml for GM-CSF and 100ng/ml for IL4. Leftover stocks were kept and frozen at -80ºC. HAOSMC and HAOEC cells at passages four and five were treated for nine hours with GM-CSF/IL4. Treatment was as follows:

<table>
<thead>
<tr>
<th>Treatment in 6 well plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x 6 wellplate - control (untreated)</td>
</tr>
<tr>
<td>1x 6 wellplate - 10ng/ml GM-added (3µl of 10ng/µl stock)</td>
</tr>
<tr>
<td>1x 6 wellplate - 50ng/ml GM-added (15µl of 10ng/µl stock)</td>
</tr>
<tr>
<td>1x 6 wellplate - 50ng/ml GM (15µl of stock) + 100ng/ml for IL4 (30µl of stock).</td>
</tr>
</tbody>
</table>

3.2.3. Harvesting of cells and extraction of RNA and protein

Three of the wells on each six well plate were harvested for protein lysate analysis and the other half for RNA purification. For protein lysates, 200µl of lysis buffer (50mM TrisHCL, 150mM NaCL, 1 mM EDTA, 1% Triton, PI cocktail from Sigma, 50µl/1.5ml) was added into the top three wells of the six well plates, incubated on ice for 10 minutes and scraped by rubber policiemen. An additional wash of 50µl of cold lysis buffer was carried out for some wells. 400µl of lysis-binding buffer (RNAqueous, Ambion) was added to the other three wells containing cells to be used for RNA purification. Samples were stored at -20ºC.
3.2.4. RNA Purification

RNA was purified using the RNAqueous Phenol-free total RNA Isolation kit (Ambion Inc, Cat #AM1912). Samples had been previously suspended in 400µl lysis solution and were thawed on ice. 400µl of ethanol were added and samples were vortexed. The suspensions were then gently pipetted into transfer columns, taking care not to pipette directly onto the filter. Columns were then spun for 30 seconds at 10,000rpm. The flow-through was discarded, 700µl of Wash Solution 1 was added to the filter cartridge and samples were spun as before. The wash step was repeated using 500µl Wash Solutions 2 and 3. An additional spin was carried out for 10-30 seconds to ensure the filter was dry before it was transferred quickly into a fresh collection tube. 1.5mls of the Elution Solution was pre-heated to 70-80ºC and 50µl was added to columns, taking care not to touch the filter. Columns were spun for 30 seconds and the elution was repeated using 20µl of elution solution. 70µl RNA was eluted in total for each sample.

The concentration and purity of the RNA was determined using a spectrophotometer and a 1% agarose gel. 5µl of RNA was diluted by a factor of 200 and the absorbance measured on the spectrophotometer. 5µl of RNA sample was mixed with 5µl loading buffer containing formaldehyde and heated for 2-5 minutes at 70ºC. The samples were then run on the gel.

3.2.5. Reverse Transcription PCR

RNA collected from HAOEC and HAOSMC was used for reverse transcription PCR. One microgram of total RNA from each sample was reverse transcribed. 10µl of RNA was added to 10µl RT-PCR master mix containing:
For one sample | µl
---|---
10X RT-Buffer | 2.00
25X dNTP (100mM) | 0.8
10X RT Random Primers | 2.00
RT Enzyme (multi-scribe) | 1.00
DEPC H20 | 4.2
Total | 10µl

Reverse Transcription-PCR reaction for one sample

48 samples in total were reverse transcribed in two batches, with 4 non-amplified controls (NAC) in each.

NAC Controls | µl
---|---
10X RT-Buffer | 2.00
25X dNTP (100mM) | 0.8
10X RT Random Primers | 2.0
RT Enzyme (multi-scribe) | -
DEPC H20 | 5.2
Total | 10.0

Reverse Transcription-PCR reaction for NAC controls

The Reverse Transcription-PCR conditions were as follows:

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>37°C</td>
<td>85°C</td>
<td>4°C</td>
<td>Time</td>
</tr>
<tr>
<td>10 minutes</td>
<td>120 minutes</td>
<td>5 seconds</td>
<td>∞</td>
<td>time</td>
</tr>
</tbody>
</table>
3.3. QPCR on HAOECs and HAOSMCs

3.3.1. Preparation of plasmid standard curve

It was necessary to set up a standard curve in order to determine the copy number of sGC in each of our samples. pCR-Blunt plasmids containing either alpha or beta target genes were diluted such that a standard curve containing a range of copy numbers could be prepared.

3.3.2. Ligation

In order to prepare plasmids containing our target genes, our target genes first had to be ligated into plasmids. pCR-Blunt was the plasmid used. Previously PCR-amplified $\alpha_1$ and $\beta_1$ sGC were used in the ligations. One ligation was carried out for $\alpha_1$ sGC and one for $\beta_1$ sGC. 4µl of PCR product was added to 1µl pCR-Blunt along with 1µl NaCl (1.2M) and left on ice for 5 minutes.

3.3.3. Transformation

All solutions were kept on ice. 3µl-5µl of ligation product ($\alpha_1$ sGC and $\beta_1$ SGC) was added to 50µl of competent cells E. Coli. and gently mixed. The transformation was left for 20 minutes on ice then heat-shocked for 30 seconds at 42°C. Cells were immediately transferred to ice and incubated with 250µl of SOC medium at 37°C for one hour. Two different volumes of cells were spread on pre-warmed kanamycin plates and incubated overnight at 37°C. Once grown, 10 colonies from the $\alpha_1$ transformation and 10 colonies from the $\beta_1$ transformation were used to inoculate 20 tubes containing 10ml of LB broth with kanamycin. Tubes were then incubated overnight at 37°C.

3.3.4. Miniprep (DNA Extraction)

A miniprep on pCR-Blunt plasmids was carried out using the Qiagen QIAprep Spin Miniprep Kit. Overnight cultures were spun at 20 degrees Celsius at 6000 rpm for three
minutes. LB supernatant was discarded and pellets were resuspended in 250µl of Buffer P1 and transferred to a micro-centrifuge tube. 250µl of Buffer P2 was then added and samples were mixed thoroughly 4-6 times. After mixing 350µl of Buffer N3 was added and tubes were inverted 4-6 times. Samples were then spun for 10 minutes at 13,000rpm. A compact white pellet formed. Supernatants were applied to QIAprep spin columns by pipetting then centrifuged for one minute. The flow-through was then discarded. Columns were washed twice, the first wash with 500µl Buffer PB and the second with 750µl Buffer PE with one minute spins in between each step. DNA was then eluted using 50µl water.

3.3.5. Restriction Digests
A master-mix containing nuclease-free water, EcoR1 Buffer (10X) and EcoR1 enzyme was made up. EcoR1 was added last. 10µl of the mastermix was added to 10µl of sample and incubated for 1.5 hours at 37°C. Following incubation, a 5µl aliquot of restriction product was run on a 1% agarose gel to ensure both plasmids contained alpha 1 sGC and beta 1 sGC. 6X loading dye was added to samples. The gel was then analysed under UV light using GelDoc apparatus.

The restriction digest was carried out as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>For one sample</th>
<th>X5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>10µl</td>
<td>-</td>
</tr>
<tr>
<td>EcoR1 Enzyme</td>
<td>1µl</td>
<td>5µl</td>
</tr>
<tr>
<td>EcoR1 Buffer</td>
<td>2µl</td>
<td>10µl</td>
</tr>
<tr>
<td>H2O</td>
<td>7 µl</td>
<td>35µl</td>
</tr>
<tr>
<td>Total</td>
<td>20µl</td>
<td>50µl</td>
</tr>
</tbody>
</table>
3.3.6 Determination of Plasmid DNA Concentration

Once we had ensured that each plasmid contained $\alpha_1$ sGC or $\beta_1$ sGC, their concentrations could be determined. The plasmids were then diluted to prepare two standard curves, one for $\alpha_1$ sGC and one for $\beta_1$ sGC containing known copy numbers of our target genes. Plasmids were diluted by a factor of 200 (2µl in 1000µls) measured on a spectrophotometer and their corresponding concentrations determined. Their concentrations were then used to set-up dilutions on QPCR plates.

The absorbance was multiplied by a factor of 50 to calculate the concentration of the DNA in µg/µl. These results were then plotted against the volume and the equation of the line determined. The concentration of each plasmid per 1µl was calculated using a spectrophotometer. The $\alpha_1$ plasmid contained 5,570 basepairs, and the $\beta_1$ plasmid contained 5357 basepairs. The weight of one molecule of each plasmid was calculated by multiplying the number of base pairs by 660 Daltons to give the number of grams per each molecule. Next we calculated the weight of 300,000 molecules in 1µl. To know how much more concentrated our plasmids were their concentrations were divided by the weight of 300,000 molecules in 1µl. For pipetting convenience, we wanted our 300,000 molecules in 5µl, which meant we needed to further dilute our plasmids by five.

<table>
<thead>
<tr>
<th>Stock</th>
<th>Copy Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>300,000</td>
</tr>
<tr>
<td>B1</td>
<td>30,000</td>
</tr>
<tr>
<td>C1</td>
<td>3,000</td>
</tr>
<tr>
<td>D1</td>
<td>300</td>
</tr>
<tr>
<td>F1</td>
<td>30</td>
</tr>
</tbody>
</table>

Dilution of plasmid standards
3.3.7. QPCR on primary cell samples

After standards for alpha and beta had been set-up, QPCR could be performed on our samples. Each well of the 96 well QPCR plates contained:

<table>
<thead>
<tr>
<th>QPCR Mastermix</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes 20X</td>
<td>1</td>
</tr>
<tr>
<td>PCR Mix 2X</td>
<td>10</td>
</tr>
<tr>
<td>Samples</td>
<td>5</td>
</tr>
<tr>
<td>H2O</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20µl</strong></td>
</tr>
</tbody>
</table>

Three mastermixes were prepared. One for the alpha gene, one for the beta gene and one for GAPDH which was used as a loading control to ensure the same amount of DNA was loaded for each sample. All cDNA samples were analyzed in triplicate and reagent containing no sample was used as a background control. Plates were run on the QPCR analyzer (7900 HT Sequence Detection System). QPCR was performed on all 48 cDNA samples.
3.4. Western Blotting

Western blotting is a technique used to detect specific protein in a sample. It uses gel electrophoresis to separate denatured protein by the length of their polypeptides. The proteins are then transferred to a membrane (PVDF) where they are probed using specific antibodies to detect the target protein. The overall purpose of us performing Western blot experiments was to detect α₁ and β₁ sGC in protein samples from our primary cells.

3.4.1. Western blotting on BE2 and Sf9 cell lysates to determine the antibody dilution for sGC detection.

Westerns were to be performed on primary cell protein samples but as no ideal antibody to detect α₁ and β₁ forms of sGC had been determined, and primary cell samples were precious, several antibodies were tested on other proteins containing sGC. Some antibodies were new and others had been used previously. Western blots were carried out on BE2 cells and on Sf9 cells which contained recombinant alpha and beta sGC.

3.4.1.1. BE2 and Sf9 cell lysates

BE2 cells are a neuroblastoma cell line. They are known to express to high levels of sGC and are used as controls in Western Blot experiments for the identification of the correct band for α₁ and β₁ sGC. BE2 cells were grown in the laboratory and collected once they reached 80% confluency. They were then kept on ice and trypsinised, then washed with PBS and spun. The supernatant was aspirated and cells were frozen at -80°C. 250µl of lysis buffer (5mls, 5X) and 5µl Proteinase A and B (1000X, kept on ice) were added to BE2 cell pellets and transferred to labeled eppendorfs. Samples were sonicated using a medium-sized sonication tip and surrounded by ice to prevent denaturation. Sonication was set at 8 pulses every two seconds and 40% amplitude. Samples were then spun at 14,000g for one hour at 4°C. A ninety six well plate was prepared to determine the protein concentrations of samples before loading them onto a gel. BSA stock (5mg/ml)
was diluted with deionised water and used to set up a protein concentration standard curve.

Sf9 cells are derived from pupal ovarian tissue of the Fall Armyworm *Spodoptera frugiperda*. The Sf9 cell line is highly susceptible to infection and is commonly used to isolate and propagate recombinant baculoviral stocks and to produce recombinant proteins. Human recombinant $\alpha_1\beta_1$ sGC was expressed by using the Sf9/baculovirus system. Sf9 cells were previously co-infected with baculoviruses expressing independently $\alpha_1$ and $\beta_1$ subunits, and cells were harvested 3 days after infection. After harvesting they were then treated in the same manner as the BE2 cells as detailed above.
3.4.1.2. Antibodies used for detection of $\alpha_1$ and $\beta_1$ subunits in BE2 and Sf9 lysates

The following tables list the antibodies tested:

**Panel of anti $\alpha_1$ sGC Antibodies used**

<table>
<thead>
<tr>
<th>Antibody Description</th>
<th>Epitope</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>9908-1 (First Boost)</td>
<td>C-Terminal</td>
<td>1:1000</td>
</tr>
<tr>
<td>9908-2 (Second Boost)</td>
<td>C-Terminal</td>
<td>1:1000</td>
</tr>
<tr>
<td>9908-0 (Trial Sample)</td>
<td>C-Terminal</td>
<td>1:1000</td>
</tr>
<tr>
<td>9907-1 (First Boost)</td>
<td>C-Terminal</td>
<td>1:1000</td>
</tr>
<tr>
<td>9908 (C-\alpha new)</td>
<td>C-Terminal</td>
<td>1:10</td>
</tr>
<tr>
<td>1168-2 (Second Boost)</td>
<td>C-Terminal</td>
<td>1:2000</td>
</tr>
<tr>
<td>F1168 (company purified)</td>
<td>C-Terminal</td>
<td>1:10</td>
</tr>
<tr>
<td>1168-1</td>
<td>N-Terminal</td>
<td>1:2000</td>
</tr>
<tr>
<td>4096</td>
<td>C-Terminal</td>
<td>1:1000</td>
</tr>
<tr>
<td>4096</td>
<td>C-Terminal</td>
<td>1:500</td>
</tr>
<tr>
<td>5214' (4096)</td>
<td>C-Terminal</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Table 3.4.1.2.1. List of $\alpha_1$ sGC antibodies evaluated, detailing the epitope and the dilution used.
Panel of anti β1 sGC Antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 4843 (affinity purified)</td>
<td>C-Terminal</td>
<td>1:1000</td>
</tr>
<tr>
<td>2 0028-2</td>
<td>C-Terminal</td>
<td>1:2000</td>
</tr>
<tr>
<td>3 F0028 (company purified)</td>
<td>C-Terminal</td>
<td>1:2000</td>
</tr>
<tr>
<td>4 4843</td>
<td>C-Terminal</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 3.4.1.2.2. List of β1 sGC antibodies evaluated, detailing the epitope and the dilution used

3.4.1.3. Bradford assay on BE2 cells for protein determination
Samples were defrosted on ice. 5µl or 20µl of supernatant from each lysate was added to the plate and made up to 200µl using deionised water. Samples were then diluted 1:2 to a final volume of 100µl in each well. 25µl of Bio-Rad Protein Assay Solution was added last. The plate was tapped gently to ensure mixing and read on a spectrophotometer at 590nm to determine protein concentration.

3.4.1.4. Gel set-up, transfer and blocking

Gel set-up
The lower gel (12mls, 8%) was made using 3mls 4X Lower Tris Buffer, 2.4mls 40% acrylamide, 6.6mls H₂O, 120µl and 12µl TEMED. Once all reagents had been added, the gel was quickly poured into Bio-Rad glass plates. A layer of n-butanol was added over the lower gel and gently poured off once it set. Deionised water was used to rinse the top of the lower gel. The upper gel (5ml, 5%) was made up using 2mls 4X Upper Tris Buffer, 1ml 40% acrylamide, 5mls H₂O, 80µl APS, and 10µl TEMED and poured into the glass plates on top of the lower gel. A comb to create 50µl wells was added to the gel. Once both gels were set, glass plates were suspended in running buffer. BE2 lysates were
added to wells. Protein lysates were prepared by adding Lameli loading buffer (4X) and boiling for five minutes. Different volumes of protein were added depending on the results of the Bradford assay and pre-calculated volumes of samples were added to wells. 15ul of a molecular weight marker was added to the middle well of each gel. Gels were run for approximately 2 hours at 100V.

**Transfer**

After electrophoresis was complete, the gel was opened gently and the upper gel cut off. Transfer solution (kept on ice) was poured into a large Pyrex dish. The blotting apparatus was set up as follows: negative terminal of blotting cassette, two sponges, three sheets of filter paper, gel, membrane (cut to size and dipped briefly in methanol), two sheets of filter paper, two sponges, positive terminal of blotting cassette. The cassette was closed and placed in the transfer bucket (Bio-Rad Mini-Protean II) in the Pyrex dish which was packed with ice and a magnetic stirrer. Frozen water, in universal tubes was added to the bucket to maintain the temperature before it was setup in the cold room for transfer for 1.5 hours. After the transfer, the membrane was removed from the apparatus and was ready for the blocking step.

**Blocking**

Blocking was carried out using 5% dried milk in TBST (w/v, 200mls volume), heated for 30 seconds and cooled. Prepared blotting solution was poured on the membranes and left for 15-30 minutes on an automated rocker (LabNet Rocker 85).

3.4.1.5. Antibody addition and membrane development

A total of sixteen antibodies for both \( \alpha_1 \) and \( \beta_1 \) sGC were evaluated: Nine \( \alpha_1 \) antibodies and four \( \beta_1 \). The dilutions used ranged from 1:10 to 1:2000, however most antibodies were used at a dilution of 1:1000. Most were specific for the C-terminal of either the \( \alpha_1 \)
or β₁ sGC subunit. Many of the α₁ sGC antibodies came from the same rabbit, but were purified from different boosts, some being more specific than others. Several blots were performed for each antibody. All antibodies were diluted in milk and incubated in trays for two hours at room temperature on an automated cell rocker.

Membrane Development
After incubation, the antibody was poured off and the membrane washed three times in TBST (1% (v/v)). The secondary antibody (goat anti rabbit, IgG) was prepared in milk and diluted 1:6500 (dilution used as a standard in the laboratory) and incubated with washed membranes for forty five minutes. Membranes were washed again after incubation allowing 5 minutes in between each wash step.

The ECL Western Blotting Detection Reagents kit was used to develop membranes. 2mls of this solution was added to the membrane, and left to develop for five minutes. Excess reagent was removed and membranes were placed on plastic trays to be developed in the dark room. Blots were allowed to develop anywhere from a few seconds, to overnight exposure, depending on the protein concentration and the strength of the antibody being used.

3.4.2. Western blotting on HAOECs and HAOSMCs

3.4.2.1 Endothelial and smooth muscle cell preparation for Western blotting
Protein lysates from endothelial and smooth muscle cells previously stored at -80°C were defrosted on ice. Once defrosted, proteinase inhibitors A and B (1000X) were added to each sample. Samples were then sonicated twice using the smallest tip at a setting of two pulses every two seconds at 40% amplitude. Samples were then spun in the cold room for ten minutes at 10,000 rpms. The supernatant was removed and placed into 1.5ml eppendorf tube. 5µl and 20µl was taken from each sample and the protein concentration determined on a ninety six well plate prepared in the same way as for BE2 lysates and
recombinant cell proteins. The plate was read on a spectrophotometer to determine the protein concentration.

3.4.2.2. Performing Western blotting on primary cells
Now that the antibody evaluation had been carried out using BE2 and Sf9 lysates, our primary cell samples were run on Western blots for BE2 and recombinant cell proteins as described above.

Secondary Antibody Addition
Membranes were washed as before. The secondary antibody (goat anti rabbit, IgG) was prepared in milk, diluted 1:6500 (standard dilution used in the laboratory routinely) and incubated with washed membranes for forty five minutes. Membranes were washed three times after the incubation. All TBST was drained from washed membranes before the addition of ECL, as described previously.
3.5. Splice variant work

The human sGC splice form A307838 has recently been discovered in brain tissue. In this project, we aimed to incorporate the full splice form into the plasmid vector pBacPak8 so it could then be used in further experiments using viral vectors. A307838 had been broken into two fragments: fragment 1 and fragment 2. Fragment 2 had previously been ligated to pBacPak8 and needed to be re-joined to fragment 1. To achieve this aim we first had to PCR amplify fragment 1 cDNA and ligate it to pCR-Blunt. It was then transformed into competent cells, the DNA was extracted and the fragment was re-isolated using restriction digest. The next step was to ligate it to fragment 2 in pBakPak8 and to transform it into competent cells. A brief overview of this procedure is shown in Figure 3.5.1 below.

![Figure 3.5.1 Flow-through of procedure to incorporate A307838 in pBacPak8.](image-url)
3.5.1. **Fragment 1**

**PCR Amplification.**

Fragment 1 was PCR amplified from cDNA sources including human brain, gastric cancer samples and BE2 cells. Several PCRs were carried out using different primers which had been designed previously to amplify the fragment 1 section of human β1 sGC A307838 splice form. The PCR procedure was set up as follows:

```
cDNA 2µl
Pfu 2µl
Primers 2.5µl (of each) (20 uM)
10X buffer 5µl
dNTPs (2.5mM) 4µl
H₂O 32µl
```

Primers used were: Sh498, Sh499, Sh491 and Sh494. See table 3.5.1 below.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sh 494</td>
<td>5' AGGATCCATGTGTGGAAGATGTTTCGTC 3'</td>
<td>AK307838, Fragment 1, starts with Met2, contains BamH1 linker</td>
</tr>
<tr>
<td>Sh 491</td>
<td>5' ATAGGCTGGATCTACGACATCCTG 3'</td>
<td>Subcloning AK307838 ORF, Downstream fragment1, Stu I site included</td>
</tr>
<tr>
<td>Sh 498</td>
<td>5' AGGATTCATGGTTTGGAAGATGTTTCGTC 3'</td>
<td>AK307838, Fragment 1, replacement for 494, starts with Met2, contains BamH1 linker</td>
</tr>
<tr>
<td>Sh 499</td>
<td>5' ATAGGCTGGATCTACGACATCCTGTC 3'</td>
<td>Subcloning AK307838 ORF, replacement for 491, downstream fragment1, Stu I site included</td>
</tr>
</tbody>
</table>

Table 3.5.1.1. Primers used to PCR amplify fragment 1 of A307838. Sequences are given along with comments on each primer.
Ligation

Non-stick RNase-free 1.5ml Microfuge tubes were used for ligations. 4µl of isolated fragment 1 and 1µl of pCR-Blunt (Fig. 3.5.1.12) with activated toperisomerase (kept on ice) were added to tubes. 1µl NaCl (1.2M) was also added. Ligation reactions were carried out for 5 minutes at room temperature.

Figure 3.5.1.12. Diagram of pCR-Blunt plasmid vector (Invitrogen Life Technologies).
LB media preparation
LB media on which to grow chemically competent *E.Coli* bacteria was prepared in a 1 litre container filled with deionised water. 20 LB tablets were added and stirred until dissolved. To prepare LB agar plates, 15g of Bacto Agar was added before the flask was autoclaved.

Transformation
Transformation was carried out the same way as previously described. See section 3.3.3.

Colony Selection
The lid of the LB broth container was flamed before approximately 100mls was added to a conical flask. Kanamycin (1000X) was then added. 5mls was then dispensed into plastic Culture Test tubes in the presence of an open Bunsen flame. A kanamycin selective plate was labelled with a grid and numbered. Using a sterile forceps and a pipette tip, up to 20 colonies were selected, touched off the numbered grid plate, and then placed in a numbered Culture Test tube. Tubes were then incubated overnight in an automated shaker at 37˚C.

Miniprep (DNA Extraction)
DNA extraction was carried out as described in section 3.3.4. The amount of DNA extracted was determined using a 10µl aliquot of DNA samples run on a 1% agarose gel.

DNA Extraction Kit evaluation
The standard DNA extraction kit used in the lab was made by Qiagen. However during the course of this project, the DNA yield from this kit became very low. As a result, two new DNA extraction kits were evaluated: The Nucleospin Plasmid kit and the Promega Pure Yield Plasmid Miniprep System.
NucleoSpin Plasmid Kit (Cat No. 74058810, Macherey-Nagel, Clontech)

Overnight cultures were spun at 20 degrees Celsius at 6000 rpm for three minutes. LB supernatant was discarded and pellets were resuspended in 250µl Buffer A1 and samples were mixed thoroughly by gentle pipetting up and down. Samples were then moved to NucleoSpin Plasmid Columns, and 250µl of Buffer A2 was added. Tubes were inverted 6-8 times. Samples were then incubated at room temperature for up to five minutes, or until the lysate disappeared. 300µl of Buffer A3 was then added and samples were mixed thoroughly by inverting tubes 6-8 times. Tubes were spun for 5 minutes at 11,000 x g at room temperature until the supernatant became clear. Plasmid columns were then placed into collection tubes (2mls) and the supernatant was decanted into the column by pipetting. Tubes were then centrifuged for one minute at 11,000 x g and the flow-through was discarded. 600µl of Buffer A4 was added. Tubes were spun as before and the supernatant was discarded. The silica membrane in the column was dried by centrifuging for two minutes at 11,000 x g and the supernatant discarded. DNA was eluted by placing tubes in a clean tube and adding 50µl Buffer AE. Samples were incubated for one minute at room temperature and then spun for one minute at 11,000 x g.

PureYield™ Plasmid Miniprep System (Promega)

Overnight cultures were spun at 20 degrees Celsius at 6000 rpm for three minutes. LB supernatant was discarded and pellets were resuspended in 600µl of TE buffer. 100µl of Cell Lysis Buffer was added and tubes were mixed gently by inverting 6 times. The solution changed from opaque to clear blue indicating complete lysis. After no more than 2 minutes, 350µl of cold (4-8ºC) Neutralization Solution was added and mixed thoroughly by inverting the tubes. The solution turned yellow when neutralization was complete and tubes were inverted another 3 times. Tubes were centrifuged at 13,000 rpms for 3 minutes. The supernatant was transferred to a PureYield™ Minicolumn which was placed into a PureYield™ Collection Tube and centrifuged at 13,000 rpms for 15 seconds. The flow through was discarded and 200µl of Endotoxin Removal Wash was
added to the Minicolumn and samples were spun as before. 400µl of Column Wash solution was added and minicolumns were spun at 13,000rpm for 30seconds. They were then transferred to clean 1.5 ml microcentrifuge tubes and 30µl of Elution Buffer was added directly to the minicolumn matrix and let stand for one minute. Tubes were then spun as before for 15 seconds to elute the plasmid DNA.

Restriction Digests

Restriction digests were carried out to determine if fragment 1 had been successfully ligated to pCR-Blunt (Fig. 3.5.1.1), and to test for the presence of fragment 2 which had already been cloned into the plasmid vector pBacPak8. All restriction digests were carried out the same way, the only difference being the NEB buffer, addition of 10X BSA and the enzyme/enzymes used. Figure 3.5.1.2 shows the common restriction sites used to cut pCR-Blunt in order to detect fragment 1. The expected fragment size after various restrictions used are shown in figure 3.5.1.3.
Figure 3.5. Restriction sites on pCR-Blunt-fragment1

Restriction Enzymes used on Fragment 1 ligated to pCR-Blunt are shown in Figure 7. Figure 3.5.1.23 is a representation of the plasmid vector pCR-Blunt containing fragment 1. The plasmid is 3.5kb in size and fragment 1 is 1.0kb in size, resulting in a plasmid vector 4.5kb in length. Each restriction site that was used during the cloning process is shown, including those present inside the fragment.
Fig. 3.5. Length of restriction products of pCR-Blunt-fragment1 when cut with different restriction enzymes.

An example of one such restriction digest to test for the presence of the fragment 1 in pCR-Blunt was set up as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>For one sample</th>
<th>X5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>10µl</td>
<td>-</td>
</tr>
<tr>
<td>EcoR1 Enzyme</td>
<td>1µl</td>
<td>5µl</td>
</tr>
<tr>
<td>EcoR1 Buffer</td>
<td>2µl</td>
<td>10µl</td>
</tr>
<tr>
<td>H20</td>
<td>7 µl</td>
<td>35µl</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Total</td>
<td>20µl</td>
<td>50µl</td>
</tr>
</tbody>
</table>
A master-mix containing nuclease-free water, EcoR1 Buffer (10X) and EcoR1 enzyme was made up. The enzyme was added last. 10µl of the mastermix was added to 10µl of sample and incubated for 1.5 hours at 37°C. Following incubation, a 5µl aliquot of restriction product was run on a 1% agarose gel. 6X loading dye was added to samples. The gel was then analysed under UV light using GelDoc apparatus.

Several restriction digests were carried out on the fragment 1-pCR Blunt product using a variety of different restriction enzymes to test for the presence of fragment 1. These enzymes were: EcoR1, Spe1, Xho1, BamH1, HindIII, Stu1, Kpn1, Nsi1.

**Band Isolation**

Selected bands were cut from 1% agarose gels in the darkroom using UV light. As much gel as possible was cut from the fragment, which was placed in an eppendorf tube. The QIAquick Gel Extraction Kit was used to purify bands. 500µl of buffer QX1 was added to the tube. 15µl of beads was then added and mixed well by vortexing. The solution became milky and was left for ten minutes at 50°C, giving it a quick vortex every two minutes. It was then spun for one minute at 13,000 rpm.

The supernatant was aspirated and discarded. The beads were resuspended in another 500µl of QX1 buffer and given a quick vortex, before it was spun again and the supernatant aspirated. 700µl of PE buffer was then added and the tube spun again and the supernatant aspirated as before. This step was repeated using 500µl of PE buffer.

The pellet at the bottom of the tube was allowed to dry completely after all the supernatant had been aspirated. 25µl of nuclease-free water was added to the tube, mixed well and incubated for one minute at 50°C. The tube was re-spun for one more minute and the supernatant transferred to a clean eppendorf. An aliquot was run on a 2% agarose gel to test for the presence of a band.

**Dpn 1 Treatment of Fragment 1**

Dpn 1 is a 4-base cutter with the recognition sequence 5’ GATC 3’. It is specific for methylated DNA. The purpose of Dpn 1 treatment is to digest away wild type methylated DNA, so that the only remaining plasmid is the PCR product. 1µl of DPN was added to
10µl of PCR product to purify the fragment and remove methyl groups present in plasmid DNA. It was incubated for 1 hour at 37°C. The DPN was destroyed by heating the sample to 80°C for 20 minutes.

DNA Precipitation
When low levels of DNA were extracted from our minipreps, DNA precipitation was performed to concentrate the DNA and measure it on the spectrophotometer. One tenth the DNA volume of sodium acetate (3M), and double the DNA volume of ethanol, was added to the DNA. It was then kept -80°C overnight. The next morning the tube was centrifuged at 14,000 rpms at 4°C. A white pellet formed at the bottom of the tube. The ethanol was decanted, and 80% ethanol (kept at -20°C) was added. The tube was then spun at 4°C for 15 minutes at 14,000 rpms. This wash step was repeated 3 times. The supernatant was discarded and 60µl of nuclease-free water was added. The DNA concentration was then measured on the spectrometer (µg/µl).

3.5.2. Fragment 2

Figure 3.5.2 shows pBacPak8, which was used to clone fragment 2 of the splice form A307838. All major restriction sites and the binding sites of the Bac1 and Bac2 primers used for sequencing of the plasmid vector are clearly marked.
Sequencing

Sequencing of samples was carried out by Lone Star Labs (7501 Fannin, Suite 740, Houston TX 77054). 10µl of sample was aliquoted and sent for sequencing.

Designing new Primers

New primers were designed to correct the Stu1 restriction site. Primers which were 15 basepairs upstream of Stu1 and 15 basepairs downstream of the Stu1 site were designed. When choosing the sequences for the new primers it was decided that they
should begin or end with a C or a G to add more stability. The following primers were used:

**Upstream Forward primer:** Sh503 (primer for mutagenesis to correct StuI site in pBacPak8-A307-Fr2-plasmid.
5’ CTGTTCGTAAAGGCCTAAAGTACAAACGAC 3’

**Downstream Reverse Primer:** Sh504
3’ GACAAAGCATTCCGAAATTTTCATGTTTGCTG 5’

**Mutagenesis**

A mutagenesis uses primers containing a desired mutation to incorporate a mutation into our plasmid DNA. The principle behind a mutagenesis is as follows:

Plasmid DNA is denatured and primers containing the desired mutation are annealed to the complementary strand. DNA polymerase then uses the plasmid DNA as a template to extend the primers. **PCR utilises thermostable DNA polymerases**, which enable the high temperature denaturation of DNA plasmids to create single-stranded templates without simultaneously denaturing the polymerase enzyme. In order to remove template DNA from the PCR product mixture, the mixture is then treated with *Dpn I* which selectively digests the parent template DNA, leaving only mutant DNA assembled in vitro. The nicked vector DNA (containing the desired mutation) is then transformed into our competent cells. The cell machinery repairs the nicked DNA, and the repaired mutant plasmid can be isolated by miniprep.

A mutagenesis on pBacPak8-fragment2 was performed using the new primers (made up to a concentration of 20µM).

It was carried out as follows:
DNA (pBacpak8-fr2) 3µl
Sh503 (forward primer) 2µl
Sh504 (reverse primer) 2µl
10X Pfu buffer 5µl
dNTPs (2.5mM) 4µl
Pfu (10X) 4µl
H2O 30µl
Total 50µl

After the initial set-up, the mutagenesis was PCR amplified on the following cycle:

**Denaturation**
95 °C - 2 minutes
94 °C – 2 minutes

**Amplification**
72 °C – 10 minutes
60 °C – 1 minute
Repeated seventeen times

**Elongation**
72 °C – 20 minutes
Reaction held at 4 °C

**Dpn 1 Treatment**
Dpn 1 treatment was carried out as previously described in section 3.5.
Transformation

DPN1-treated mutagenesis products (5µl and 2.5µl) were transformed into competent cells using the same transformation method used previously.
Dephosphorylation

pBacPak8-fragment2 needed to be dephosphorylated to minimize a self-ligation background, before it could be ligated to cut fragment 1. 5’ phosphate was removed from linearised pBacPak8-fragment2 so that when fragment 1 was added, pBacpak8–fragment2 would ligate to fragment 1 and not to itself. By removing the phosphates on pBacPak8, self ligation became impossible.

The procedure was carried out as follows:

| 10µl DNA |
| 2µl Antarctic Phosphatase |
| 2µl Antarctic Phosphatase Buffer |
| 6µl H2O |
| 20µl Total |

It was then left for one hour at 37°C.

RestrictionDigests

Many restriction digests were performed on pBacPak8-fragment2 to ensure that the fragment had been successfully cloned into the plasmid vector and that all restriction sites were correct. Figure 3.5.2.26 shows the restriction sites used to cut pBacPak8 to isolate fragment 2 and ensure its correct insertion into the plasmid vector. Different restrictases gave different sized fragments. A representation of some of the fragments and their sizes are shown in figure 3.5.2.32.
Restriction digests carried out on pBacPak8-fragment2 were:

1. Sph1, Kpn1
2. Stu1 (to linearise)
3. Kpn1 (to linearise)
4. Not1, Pst
5. Stu1, Eag1
pCR-Blunt-fragment1 and pBacPak8-fragment2 were re-transformed separately using sterile techniques previously described into competent *E Coli* cells and incubated at 37°C overnight to achieve as many colonies as possible, and a high level of DNA of each fragment. This DNA was then extracted and run on a 2% agarose gel. pCR-Blunt-fragment1 was restricted using Stu1 to completely cut fragment 1 from pCR-Blunt (Fig. 3.5.3.14). The restriction digest was carried out the same way as previous restrictions.

Figure 3.5.3.244 illustrates the expected fragment sizes when pCR-Blunt, containing fragment 1 was cut with Stu1. Assuming correct cutting was achieved, two fragments, one 1.0 kilobases in length and the other 3.5 kilobases in length should be present.
pBacPak8-fragment2 had to be linearised in order to ligate it to fragment 1. This was achieved by a restriction digest using Stu1, performed in the same manner as previous restriction digests. The linearised fragment was 5.5kb in length if it had been cut correctly and was ready to be ligated to fragment 1.
The Stu1 site in pBacPak8-fragment2 had been repaired. Fragment 1 was isolated and pBacPak8-fragment2 was linearised. An aliquot of fragment 1 and linearised pBacPak8-fragment2 were run on a 2% agarose gel to ensure that both fragments were present, had been cut and were the correct size. The next step was to ligate fragment 1 and fragment 2 together.

This ligation was made up of: 2µl of pBacPak8-fragment2 (vector), 1.5µl of 1.5 10X buffer for T4 DNA ligase, 2µl of T4 DNA ligase and 9.5µl of cut pCR-Blunt-fragment1 (insert). The total volume was 15µl. The reaction was left for 48 hours in the
4°C cold-room. A transformation was then carried out in the same way as previously described, and colonies grown up as before. Other ligations used 1µl of vector (diluted 1:5), 7µl of fragment 1, 1µl of T4 DNA ligase and 1µl of T4 DNA ligase buffer and the reaction kept at room temperature overnight.

3.6. Data analysis
Data are presented as means ± standard deviations. To test for statistical differences between mean values, paired t tests were used where appropriate. A value of p < 0.05 was accepted as statistically significant.
4.0. Results

In this study we aimed to learn more about the regulation of sGC, and the role cytokines may play. We carried this out by culturing primary HAOECs and HAOSMCs in a 37°C, 5% CO₂ humidified incubator. It was hoped that these primary cells would give a more true to life picture of sGC regulation than BE2 cells on which experiments had previously been carried out. Primary cells were treated with a selection of cytokines. The mRNA levels were analysed using QPCR. α₁ and β₁ sGC plasmid standard curves were set up to allow us to determine the transcript numbers of sGC in cytokine-treated mRNA samples. QPCR was then carried out to detect α₁ and β₁ sGC levels in unstimulated and stimulated HAOECs and HAOSMCs. Protein levels of samples were tested using Western blot analyses. A panel of antibodies for α₁ and β₁ sGC was evaluated first on BE2 and Sf9 cell lysates for use in Western blotting on primary cell protein samples. α₁ and β₁ sGC protein levels from HAOECs and HAOSMCs were then analysed using Western blotting.

4.1. Culture of primary cells

Cell culture proved successful. Cells remained uncontaminated and healthy. Smooth muscle cells grew faster than endothelial cells and were harvested at passages 4 and 5. Endothelial cells being slower growers were harvested at passages 5 and 6. The manufacturers recommended that primary cells should not be cultured past passage five due to cell aging, resulting in loss of phenotype. Fig 4.1.1 and 4.1.2 show images taken of endothelial cells and human vascular smooth muscle cells at passage 4. In the images, the endothelial cells are at approximately 50% confluency and the human vascular smooth muscle cells are at approximately 80-90% confluency.
Figure 4.1.1. Human endothelial cells, passage 4, approximately 50% confluency

Figure 4.1.2. Human vascular smooth muscle cells, passage 4, 80-90% confluency
4.2 RT-PCR

RNA was collected from primary cells to be used for RT-PCR. Tables 4.2.1 and 4.2.2 show the RNA concentration from each primary cell line, and the amount used to give a total of 1µg of RNA in each sample for reverse transcription. All samples were made up to a final volume of 10µl. HAOSMC samples had an average RNA concentration of 0.218µg/µl. The average RNA concentration was 0.2325 µg/µl in passage 4, and 0.2033 µg/µl in passage 5. On average, the RNA concentration was 12.6% lower in passage 5 compared to passage 4. HAOEC RNA concentrations were over 50% lower than HAOSMCs, with the average being 0.083µg/µl. The average RNA concentration in passage 5 was 28.33% higher than the average RNA concentration in passage 6 (0.0967 µg/µl in passage 5 and 0.0693 µg/µl in passage 6). There was a larger drop in RNA expression between passages in HAOECs, than in HAOSMCs (28.33% vs. 12.6%).
<table>
<thead>
<tr>
<th>Samples</th>
<th>Conc. µg/µl</th>
<th>RNA (µl)</th>
<th>H2O (µl)</th>
<th>Total Vol.(µls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMC passage 4 Control 1</td>
<td>0.28</td>
<td>3.57</td>
<td>6.43</td>
<td>10</td>
</tr>
<tr>
<td>SMC passage 4 Control 2</td>
<td>0.27</td>
<td>3.7</td>
<td>6.3</td>
<td>10</td>
</tr>
<tr>
<td>SMC passage 4 Control 3</td>
<td>0.22</td>
<td>4.46</td>
<td>5.54</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 1</td>
<td>0.21</td>
<td>4.81</td>
<td>5.19</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 2</td>
<td>0.25</td>
<td>4.03</td>
<td>5.97</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 3</td>
<td>0.22</td>
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<td>5.54</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 1+ IL4 100ng/ml</td>
<td>0.20</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 2+ IL4 100ng/ml</td>
<td>0.25</td>
<td>4.03</td>
<td>5.97</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 3+ IL4 100ng/ml</td>
<td>0.26</td>
<td>3.91</td>
<td>6.09</td>
<td>10</td>
</tr>
<tr>
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<td>3.91</td>
<td>6.09</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 2</td>
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<tr>
<td>SMC passage 5 control 1</td>
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<td>5</td>
<td>5</td>
<td>10</td>
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<tr>
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<tr>
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<td>5.21</td>
<td>4.79</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 1</td>
<td>0.192</td>
<td>5.21</td>
<td>4.79</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 2</td>
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<td>4.63</td>
<td>5.37</td>
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</tr>
<tr>
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</tr>
<tr>
<td>GM-CSF 50ng/ml 2</td>
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<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 3</td>
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<td>4.81</td>
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<td>10</td>
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</table>

Table 4.2.1 Dilutions of HAOSMCs RNA to prepare first 24 samples for RT-PCR
<table>
<thead>
<tr>
<th>Samples</th>
<th>Conc. µg/µl</th>
<th>RNA (µl)</th>
<th>H2O (µl)</th>
<th>Total Vol. (µls)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6.3</td>
<td>10</td>
</tr>
<tr>
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<td>5.2</td>
<td>10</td>
</tr>
<tr>
<td>EC P5 Control 3</td>
<td>0.096</td>
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<td>4.8</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 1</td>
<td>0.072</td>
<td>6.9</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 2</td>
<td>0.088</td>
<td>5.7</td>
<td>4.3</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 3</td>
<td>0.096</td>
<td>5.2</td>
<td>4.8</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 1+ IL4 100ng/ml</td>
<td>0.104</td>
<td>4.8</td>
<td>5.2</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 2+ IL4 100ng/ml</td>
<td>0.08</td>
<td>6.3</td>
<td>3.8</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 3+ IL4 100ng/ml</td>
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<td>5.7</td>
<td>4.3</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 1</td>
<td>0.104</td>
<td>4.8</td>
<td>5.2</td>
<td>10</td>
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<tr>
<td>GM-CSF 50ng/ml 2</td>
<td>0.096</td>
<td>5.2</td>
<td>4.8</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 3</td>
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<td>5.2</td>
<td>4.8</td>
<td>10</td>
</tr>
<tr>
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<td>3.8</td>
<td>10</td>
</tr>
<tr>
<td>EC P6 Control 2</td>
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<td>6.9</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td>EC P6 Control 3</td>
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<td>6.9</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 1</td>
<td>0.064</td>
<td>7.8</td>
<td>2.2</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 2</td>
<td>0.08</td>
<td>6.3</td>
<td>3.8</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 10ng/ml 3</td>
<td>0.072</td>
<td>6.9</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 1+ IL4 100ng/ml</td>
<td>0.072</td>
<td>6.6</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 2+ IL4 100ng/ml</td>
<td>0.08</td>
<td>6.3</td>
<td>3.8</td>
<td>10</td>
</tr>
<tr>
<td>GM-CSF 50ng/ml 3+ IL4 100ng/ml</td>
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<td>10</td>
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<td>10</td>
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<tr>
<td>GM-CSF 50ng/ml 3</td>
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<td>1.1</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 4.2.2 Dilutions of HAOECs RNA to prepare second 24 samples for RT-PCR
4.3. Plasmid standard curve results

In order to accurately detect sGC transcript numbers, a standard curve with known transcript numbers needed to be set up before our primary cell RNA samples could be QPCR analysed. To set up these standard curves previously PCR-amplified $\alpha_1$ and $\beta_1$ were ligated separately into the plasmid vector pCR-Blunt and transformed into competent cells. Plasmid DNA was extracted, measured on a spectrophotometer and electrophoresed on a 2% agarose gel. Plasmid standards underwent restriction digest to ensure $\alpha_1$ and $\beta_1$ sGC had been successfully ligated to pCR-Blunt.

4.3.1 Spectrophotometer results

Plasmid DNA was extracted and the DNA concentration was measured on a spectrophotometer (Table 4.3.1.1)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>µl added</th>
<th>Concentration (µg/µl)</th>
<th>Abs</th>
<th>Average Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR-Blunt- $\alpha_1$ sGC</td>
<td>2</td>
<td>0.075</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.0875</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.0833</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.075</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.07</td>
<td>0.014</td>
<td>0.0781</td>
</tr>
<tr>
<td>pCR-Blunt- $\beta_1$ sGC</td>
<td>2</td>
<td>0.05</td>
<td>0.002</td>
<td>0.0536</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.05</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.05</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.0625</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.055</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.05416</td>
<td>0.013</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3.1.1 $\alpha_1$ and $\beta_1$ plasmid absorbance and concentration to prepare standard curve. An increase in volume gave a corresponding increase in concentration and absorbance.

The absorbance was multiplied by a factor of 50 and the dilution factor to calculate the concentration of the DNA in µg/ml. Dividing by 1000 gave the concentration in µg/µl. These results were then plotted against the volume and the equation of the line
determined (Fig 4.3.1 and 4.3.1.2). The concentration of each plasmid in 1µl was calculated.

Figure 4.3.1.1 Graph showing α₁ sGC-pCR Blunt concentration (µg/µl) plotted against the volume (microlitres)
Figure 4.3.1.2 Graph showing $\beta_1$ sGC-pCR Blunt concentration ($\mu$g/$\mu$l) plotted against the volume (microlitres)
4.3.2 Plasmid standard electrophoresis and restriction digest

α₁ and β₁ sGC were successfully ligated and transformed into competent cells. The plasmid DNA was extracted and an aliquot run on a 2% agarose gel to evaluate the concentration extracted (Fig 4.3.2.1). CHECK NUMBERING OF FIGURES.

Figure 4.3.2.1 2% agarose gel of DNA extracted from α₁ and β₁ pCR-Blunt transformation. Lanes 1-5 contain α₁ sGC plasmid DNA, and lanes 6-10 contain β₁ sGC plasmid DNA.

Plasmids were restricted with EcoR1 to confirm the presence of α₁ and β₁ sGC inserts. The restriction digest on our plasmid standards was successful. EcoR1 cut on either side
of the α₁ sGC and β₁ sGC fragment. β₁ (2.2kb) being smaller in size than α₁ (2.6kb), ran slightly further on the gel (Fig. 4.3.2).

Figure 4.3.2-2% agarose gel of pCR-Blunt-alpha sGC/pCR-Blunt-beta sGC restricted with EcoR1.

4.3.3 QPCR of standard curves

We needed a way of determining the number of α₁ and β₁ sGC transcripts. In order to achieve this, we set up a standard curve for α₁ and β₁ using pCR-Blunt. This setup was successful. Figures 4.3.3.1 and 4.3.3.2 show the standard curve plots for our α₁ and β₁ standard curves. The Ct (threshold cycle) value on the y-axis is the number of PCR cycles that elapse before the threshold is reached and is a measure of the input DNA. The higher the Ct value, the smaller the initial amount of DNA in the sample. The x-axis shows the log of the dilution. The dilutions of each standard were very accurate, producing a straight line through the points. The R² value of the α₁ sGC standard curve was 0.996 and the R² value for the β₁ sGC standard curve was 0.999. A random sample was run with the standard curves to see where it would fall on the curve and to give an
idea of any dilutions which might be needed. Using the curve, it would be possible to
determine the number of transcripts in our primary cell samples

Tables 4.3.3.1 and 4.3.3.2 show the $\alpha_1$ and $\beta_1$ Ct values for each standard and the
corresponding number of DNA molecules present. Each sample was performed in
triplicate, with a high level of accuracy being seen between each one.

Figure 4.3.3.1- $\alpha_1$ sGC-pCR Blunt standard curve. Y-axis shows the Ct value which is the
number of PCR cycles elapsed and is a measure of the input DNA. The x-axis values are
the log of the dilutions of each standard. The blue squares depict the individual standards
and the small x’s are the results of a single random sample which underwent a serial
dilution to see where it would fall on the curve.
<table>
<thead>
<tr>
<th>Quantity (transcript number)</th>
<th>α₁ sGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>300000</td>
<td>17.414785</td>
</tr>
<tr>
<td>300000</td>
<td>17.558645</td>
</tr>
<tr>
<td>300000</td>
<td>17.530777</td>
</tr>
<tr>
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<td>21.611876</td>
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<td>3000</td>
<td>25.135029</td>
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<td>3000</td>
<td>24.59122</td>
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<td>31.853695</td>
</tr>
<tr>
<td>30</td>
<td>32.234535</td>
</tr>
</tbody>
</table>

Table 4.3.3.1 Ct values for each dilution of the α₁ sGC standard curve. Quantity depicts the transcript numbers of α₁ sGC in each sample. Each sample was performed in triplicate. The higher the Ct value, the smaller the initial amount of DNA in the sample.
Figure 4.3.3.2- β1 sGC-pCR-Blunt plasmid standard curve. Y-axis shows the Ct value which is the number of PCR cycles elapsed and is a measure of the input DNA. The x-axis values are the log of the dilutions of each standard. The blue squares depict the individual standards and the small x’s are the results of a single random sample which underwent a serial dilution to see where it would fall on the curve.
Table 4.3.3.2 Ct values for each dilution of the $\beta_1$ sGC standard curve. Quantity depicts the transcript numbers of $\beta_1$ sGC in each sample. Each sample was performed in triplicate. The higher the Ct value, the smaller the initial amount of DNA in the sample.

<table>
<thead>
<tr>
<th>Quantity (transcript number)</th>
<th>$\beta_1$ sGC</th>
<th>Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>300000</td>
<td></td>
<td>19.730127</td>
</tr>
<tr>
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</table>
Figures 4.3.3.3 and 4.3.3.4 show the amplification plots for GAPDH (loading control) and β1 sGC. Plots show ΔRn vs. cycle number. Rn represents the raw fluorescence signal over time. ΔRn values are corrected for the background signal which gives the correct fluorescence increase. The detection method is based on the changes in fluorescence, which are proportional to the increase of the target. Fluorescence is monitored during each PCR cycle, providing an amplification plot, allowing the reaction to be followed in real time.

Figure 4.3.3.3. Amplification plot of serial diluted GAPDH cDNA (loading control). ΔRn vs. cycle number. The detection method is based on the changes in fluorescence. The green line represents the threshold above background fluorescence. ΔRn represents the increase in fluorescence above the threshold which is monitored during each PCR cycle, providing an amplification plot.
Figure 4.3.3.4. Amplification plot of serial diluted β₁ sGC cDNA to prepare a standard curve. ΔRn vs. cycle number. The detection method is based on the changes in fluorescence. The green line represents the threshold above background fluorescence. ΔRn represents the increase in fluorescence above the threshold which is monitored during each PCR cycle, providing an amplification plot.

The setting up of our two standard curves was complete. α₁ and β₁ sGC had been successfully ligated to pCR-Blunt, the DNA isolated and restricted to ensure this was the case and the concentration determined. QPCR was then performed using our standards which were serially diluted. Our standard curve plots yielded straight lines which showed a high level of accuracy. Now that we had made working tested standards, we could go ahead with the QPCR of the mRNA previously isolated from our HAOSMCs and HAOECs to determine the effect cytokine treatment had on the expression of sGC.
4.4. QPCR on human vascular smooth muscle cells

We wanted to know the effects GM-CSF and IL-4 would have on mRNA levels of sGC. In order to examine this, QPCR was carried out on human vascular smooth muscle cells to determine if mRNA levels of $\alpha_1$ and $\beta_1$ sGC were affected. All results were normalised against GAPDH to ensure equal loading of samples.

Treatment of HAOSMCs with GM-CSF and IL-4 resulted in an increase of $\alpha_1$ and $\beta_1$ sGC levels. Figure 4.4.1 shows results obtained for $\alpha_1$ sGC on HAOSMCs at passage 4. Cells showed a significant increase in the transcript number of $\alpha_1$ sGC in cells treated with a combination of GM-CSF at 10ng/ml and IL-4 at 100ng/ml ($p$ value=0.037). This combination of cytokines resulted in an increase in the level of $\alpha_1$ sGC from a transcript number of approximately 75 for our control unstimulated cells to over 100 (Fig 4.4.1). Treatment using GM-CSF at 50ng/ml approached significance with a transcript number of approximately 85 ($p$ value=0.059). A lower dose of GM-CSF of 10ng/ml gave no significant result compared to control, and even caused a slight but negligible drop in transcript number.
Figure 4.4.1 QPCR results of $\alpha_1$sGC on Human Primary Vascular Smooth Muscle Cells at passage 4. Each QPCR graph depicts the transcript number normalised using our loading control GAPDH on the x-axis, and the treatment received by each sample on the y-axis. n=3 in all groups. *=significantly greater than control

In order to examine the effect of continued passaging of the cells, HAOSMCs were cultured to passage 5. Figure 4.4.2 shows the results for $\alpha_1$sGC obtained at passage 5. At passage five there was a small increase in the overall transcript number of $\alpha_1$sGC when compared to passage 4. This could be due to the smooth muscle cells growing better at passage 5 than at passage 4. There is a significant increase in $\alpha_1$sGC levels compared to the control (p value=0.023) when GM-CSF and IL-4 were used in combination (Fig 4.4.2). When the cytokines were used in combination, the transcript number doubled compared to the control cells. GM-CSF at 10ng/ml and at 50ng/ml did not affect the transcript number in any significant way.
Figure 4.4.2 QPCR results of $\alpha_1$ sGC on Human Primary Vascular Smooth Muscle Cells at passage 5. n=3 in all groups *=significantly greater than control.

$\beta_1$ sGC was examined under the same conditions. Figures 4.4.3 and 4.4.4 show results obtained for $\beta_1$ sGC in HAOSMCs at passage 4 and passage 5 respectively. The transcript number of $\alpha_1$ was approximately 75, compared with nearly 10,000 transcripts of $\beta_1$. There was a large increase in transcript number when the cells were treated with GM-CSF and IL-4 in combination. Levels nearly doubled compared to control cells after cytokine treatment. Unfortunately, one of the samples treated with the combination of cytokines was lost during QPCR analysis. As a result statistical analysis could not be performed to determine if there was any significance for this sample. GM-CSF on its own at both the lower and higher doses had no significant effect. 50ng/ml resulted in a decrease of $\beta_1$ sGC, but this decrease was only by a transcript number of approximately 2500. The transcript number of $\beta_1$ sGC for the control cells at passage 4 was over 130X greater than the $\alpha_1$ sGC control cells. (Fig. 4.4.3).
There was very little difference in transcript numbers of β₁ sGC levels between passage 4 and passage 5. Cytokine treatment did not cause a significant change in transcript numbers (p value=0.249), however higher levels were achieved when GM-CSF and IL-4 were used in combination (Fig 4.4.4). GM-CSF used on its own at both concentrations decreased β₁ sGC levels slightly, but this decrease was not significant. As was observed with passage 4, there was a very large difference in the levels of β₁ sGC compared with α₁ sGC. β₁ sGC levels were almost one hundred times greater than that seen with α₁ sGC.
4.5. QPCR data on endothelial cells

QPCR was carried out on HAOECs at passage 5 and passage 6. Endothelial cells were grown to passage 6 as they grew slower than smooth muscle cells. Opposite effects were seen in endothelial cells compared with smooth muscle cells, with transcript levels decreasing as a result of cytokine treatment.

Figure 4.5.1 shows $\alpha_1$ sGC levels at passage 5 in ECs. The levels of $\alpha_1$ sGC in ECs cells at passage 5 were almost 15 times higher than that seen in smooth muscle cells. Cytokine treatment resulted in a decrease in transcript levels, with the combination of cytokines achieving the greatest decrease (Fig. 4.5.1). This decrease was significantly different ($p$ value=0.037) from control values. $\alpha_1$ sGC levels following treatment with GM-CSF at 50ng/ml were lower but not significant from control levels ($p$ value=0.056).
Treatment with 10ng/ml GM-CSF led to lower levels of α₁ sGC when compared to control, but this was again not significant (p value=0.248).

Figure 4.5.1 QPCR results of α₁ sGC on Human Primary Endothelial Cells at passage 5. Treatment with the combination of cytokines produced a significant decrease in transcript numbers. n=3 in all groups. *=significantly lower than control.

Figure 4.5.2 shows α₁ sGC levels at passage 6. There was a large decrease in α₁ transcript numbers between passage 5 and passage 6. Levels dropped by approximately 50% and this was probably due to cell aging and a lower expression of sGC. Levels of α₁ sGC in cells treated with GM-CSF and IL-4 did not drop as much as the control cells and cells treated with 10ng/ml and 50ng/ml GM-CSF. Treatment with GM-CSF at 10ng/ml resulted in a slight increase in transcript numbers which was close to significant (p value=0.05519). In addition to this, treatment with 50ng/ml GM-CSF caused a reduction in transcript level which approached significance (p value=0.055). However, overall there was no significant difference between treated groups and control.
Figure 4.5.2 QPCR results of $\alpha_1$ sGC on Human Primary Endothelial Cells at passage 6. $n = 3$ in all groups. No significant difference between groups.

Figures 4.5.3 and 4.5.4 show results obtained for $\beta_1$ sGC. There was a significant decrease in transcript numbers of $\beta_1$ sGC when cytokines were used in combination ($p$ value=0.001138). This decrease reflected the same trend seen for $\alpha_1$ sGC. GM-CSF at 50ng/ml showed a slight decrease in transcript number. As with smooth muscle cells, $\beta_1$ levels were much higher than $\alpha_1$ levels, with the control levels of $\beta_1$ sGC at passage 5 nearly seven times higher than $\alpha_1$ sGC.
Fig 4.5.3. QPCR results of $\beta_1$ sGC on Human Primary Endothelial Cells at passage 5. Treatment with the combination of cytokines yielded a significant decrease in transcript number. n=3. * = significantly lower than control.

At passage 6, there was no significant drop in the levels of $\beta_1$ sGC at passage 6, however GM-CSF cells treated with 50ng/ml approached significance (p value=0.0531). As with $\alpha_1$ sGC, the transcript number of the control cells was lower in passage 6 compared with passage 5. However levels of $\beta_1$ sGC treated with GM-CSF and IL-4 did not drop as much as the other groups. $\beta_1$ sGC levels at passage 6 were around 4 times higher than $\alpha_1$ levels at passage 6.
Fig 4.5.4. QPCR results of β₁ sGC on Human Primary Endothelial Cells at passage 6. No significant difference between groups.

QPCR results on HAOSMCs for α₁ sGC showed that the combination of cytokines resulted in a significant increase in transcript numbers compared with the control among both passages. For β₁ sGC, in passage 4 the combination of cytokines resulted in a significant increase in transcript numbers compared with the control. In passage 5 cytokine combination causes an increase in transcript number but this increase was not significant.

HAOEC results showed that α₁ sGC levels in passage 5 decreased significantly compared with the control. β₁ sGC levels also had significant decreases when treated with the combination of cytokines. For both α₁ and β₁, no significant decreases were seen in passage 6.
At this stage in the project we had cultured our primary cells, cytokine-treated them, isolated RNA and protein, set up a QPCR standard curve and ran QPCR on all 48 of our primary cell RNA samples. Having achieved the results we had using our isolated mRNA, the next step was to look at the protein levels of sGC. We wanted to see if the protein levels reflected mRNA levels in our samples.
4.6 Antibody experiments to detect $\alpha_1$ and $\beta_1$ sGC in BE2 and Sf9 cell lysates

Now that our mRNA had been QPCR analysed, the next stage of the study looked at protein levels. In order to do this we had to optimise a selection of antibodies for use on our primary cell protein samples. The laboratory had several anti-$\alpha_1$ and $\beta_1$ sGC antibodies available for use, but at no stage had any tests been carried out to determine how specific these antibodies were, or if they worked at all. Our protein samples were precious and not to be wasted, therefore, BE2 and Sf9 cells expressing recombinant sGC were used to carry out this evaluation. In addition to determining the ideal antibody for $\alpha_1$ and $\beta_1$ sGC detection, the antibody dilution and exposure time also had to be optimised. For most of the antibodies, the dilutions used were 1:1000 or 1:2000, with higher dilutions being used for some (tables 4.6.1.1 and 4.6.2.1). Exposure times also varied from a few seconds to overnight depending on the strength of the signal of the membrane. Exposure was initially for one minute, and was increased or decreased depending on the signal strength. The success of the antibody evaluation on BE2 and Sf9 cell lysates varied.

4.6.1 Antibody experiments to detect $\alpha_1$ sGC in BE2 and Sf9 cell lysates

$\alpha_1$ sGC is approximately 81kDa. Therefore, in order for an antibody to detect correct $\alpha_1$ there must be a distinct and clear band in the 81kDa region. Figure 4.6.1.1 shows the results of anti $\alpha_1$ F9908-1 on BE2 lysates at increasing protein concentrations. A dilution factor of 1:1000 was used as this was a standard dilution for anti-$\alpha_1$ sGC antibodies. The antibody looks specific for a large band running lower than the 81kDa molecular weight marker. The higher the BE2 lysate concentration, the higher the signal. This large band runs too low on the gel to be $\alpha_1$ sGC. This $\alpha_1$ antibody is not specific for $\alpha_1$. The specificity it shows may due to an immune response to something else in the sample. This antibody was subsequently not used on our primary cell samples.
Figure. 4.6.1.1. Western blot carried on BE2 lysate (2.5µg/µl) using anti-α1 F9908-1 (serum) at a dilution of 1:1000. The secondary antibody was anti-rabbit.

The bands recognized by anti α1 sGC 9908-2 (Fig. 4.6.1.2) ran too low to be real α1. The antibody did appear to be specific for one band in particular, and increased in intensity with increasing protein concentration, however this band was not α1 sGC due to its size being too small. This antibody was therefore not used any further experiments.
Figure 4.6.1.2. Western blot carried out on BE2 lysates (5, 37.5µg) at different protein concentrations, and Sf9 cell lysates ($\alpha_1/\alpha_1$ and $\beta_1/\beta_1$). Anti-$\alpha_1$ sGC 9908-2 (serum) at a dilution of 1:1000 was used.

Another anti-$\alpha_1$ sGC antibody used was 1168-2 (Fig. 4.6.1.3). This antibody was specific for the N-terminal of $\alpha_1$ sGC. It reacted with several bands in the BE2 cells lysate, including a small band that ran slightly lower than real $\alpha_1$ sGC. It did not recognize any protein from the Sf9 cell lysates, including recombinant $\alpha_1$. As a result of this, it could not be said that this antibody was specific for $\alpha_1$ and it subsequently wasn’t used.
Figure. 4.6.3. Anti-α1 1168-2 (N-terminal), dilution 1:2000, used on BE2 (5µg, 35µg) and Sf9 cell lysates (β1/β1, α1/α1).

A successful antibody used was anti-α1 sGC 5214’ (Fig 4.6.1.4). This antibody recognized a clean, sharp band close to the 80kDa, which looked specific for true α1 sGC. The band increased in intensity with increasing protein concentration. The antibody also reacted strongly with a band at approximately 160kDa which increased with protein concentration. As mentioned before, this may be the sGC α1β1 dimer which remains undissociated in the denatured protein sample. This dilution for this antibody was very low, however we did go on to use this α1 antibody on our primary cell samples.
Anti-α1 sGC 5214’ 1:100

Figure. 4.6.1.4. Western blot carried out using BE2 cells lysates at: 5, 7.5, 25 and 50µg. Anti-α1 sGC 5214’ at a dilution of 1:100 was used.

Anti-α1 9908 (C-α new) was an antibody which showed specificity for α1 sGC (Fig 4.6.1.5). It recognized a clean, sharp band at approximately 80kDa which is the correct running position for true α1. The dilution of this antibody was very low as it had been company purified. It also showed specificity for a double band between the 216 and 146 molecular weight markers. This may be the α1β1 dimer of sGC, however work is ongoing in the laboratory to determine if some of the sGC dimer run on a western blot remains undissociated after denaturation (unpublished data).
Figure 4.6.1.5. Western blot carried out using BE2 cell lysates at 5, 7.5, 25 and 50µg. Anti-α1 sGC 9908 (C-α new) used at a dilution of 1:10.

A total of ten α1 sGC antibodies were evaluated (Table 4.6.1.1). Dilutions ranged from 1:10 to 1:2000, with four detecting the correct band. The antibody 5214’ which was re-boosted 4096 at a dilution of 1:100 performed the best and it was decided this antibody would be used on our isolated protein. It was surprising that only about one third of the antibodies evaluated recognised the α1 band. These antibodies were being used regularly, and in some cases, being bought from companies, so they were expected to perform better than they did.
### Panel of anti α1 sGC Antibodies used

<table>
<thead>
<tr>
<th></th>
<th>Antibody</th>
<th>Epitope</th>
<th>Dilution</th>
<th>Correct band identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9908-1 (First Boost)</td>
<td>C-Terminal</td>
<td>1:1000</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>9908-2 (Second Boost)</td>
<td>C-Terminal</td>
<td>1:1000</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>9908-0 (Trial Sample)</td>
<td>C-Terminal</td>
<td>1:1000</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>9907-1 (First Boost)</td>
<td>C-Terminal</td>
<td>1:1000</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>9908 (C-α new)</td>
<td>C-Terminal</td>
<td>1:10</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>1168-2 (Second Boost)</td>
<td>C-Terminal</td>
<td>1:2000</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>F1168 (company purified)</td>
<td>C-Terminal</td>
<td>1:10</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>1168-1</td>
<td>N-Terminal</td>
<td>1:2000</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>4096</td>
<td>C-Terminal</td>
<td>1:1000</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>4096</td>
<td>C-Terminal</td>
<td>1:500</td>
<td>Yes</td>
</tr>
<tr>
<td>11</td>
<td>4096</td>
<td>C-Terminal</td>
<td>1:50</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>5214' (4096)</td>
<td>C-Terminal</td>
<td>1:100</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 4.6.1.1 List of α1 sGC antibodies evaluated detailing the epitope, the dilution and if it detected the correct protein band

4.6.2. Antibody experiments to detect β1 sGC on BE2 and Sf9 cell lysates

The β1 sGC subunit is approximately 79kDa. The laboratory did not have a selection of anti-β1 sGC antibodies as large as it did for anti α1 sGC. The anti-β1 sGC antibody 4843 was tested first. This recognised β1 but the band was not very strong or sharp. The same result was seen in the affinity purified form of the antibody. Anti-β1 sGC 0028-2 (Fig. 4.6.2.1) recognised a band at approximately 79kDa in the BE2 lysates at 5µg and 37.5µg. It didn’t react with anything in the α1 recombinant Sf9 lysate, but we can see a large dark band on the β1 Sf9 lysate sample. This dark band is due to overloading of the sample and the strong reactivity of the antibody for β1 sGC. A swelling on the dark band around the 79kDa mark can just about be seen. This corresponds to the location of
the β₁ sGC subunit on the gel. This antibody performed well and showed good specificity. The company purified form of the antibody did not show any specificity. As a result of this, no further β₁ sGC antibodies were evaluated for β₁, and 0028-2 was subsequently used on primary cell samples. Instead, evaluations to find the optimal dilution for anti-β₁ sGC 0028-2 were performed. Table 4.6.2.1 shows the table of anti β₁ sGC antibodies evaluated, including their dilutions and the epitope they recognise.

![Western blot](image)

**Figure.** 4.6.2.1. Western blot carried out on Sf9 cell lysates (α₁/α₁, β₁/β₁) and BE2 cell lysates (5, 37.5 µg). Anti-β₁ sGC 0028-2 (serum) at a dilution of 1:2000 was used.
Panel of anti β1 sGC Antibodies used

<table>
<thead>
<tr>
<th></th>
<th>Antibody</th>
<th>Epitope</th>
<th>Dilution</th>
<th>Correct band identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4843 (affinity purified)</td>
<td>C-Terminal</td>
<td>1:1000</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>0028-2</td>
<td>C-Terminal</td>
<td>1:2000</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>F0028 (company purified)</td>
<td>C-Terminal</td>
<td>1:2000</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>4843</td>
<td>C-Terminal</td>
<td>1:1000</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 4.6.2.1. List of β1 sGC antibodies evaluated detailing the epitope, the dilution and whether or not it detected the correct protein band

A total of fourteen antibodies for both α1 and β1 sGC were evaluated: Ten α1 antibodies and four β1, with only one of each being selected for use on primary cell protein samples (Table 4.6.2.2). Based on the experiments above, one α1 antibody and one β1 antibody were chosen. The α1 antibody selected was anti-α1 5214’ and the β1 antibody selected was anti- β1 0028-2. Both of these antibodies showed a strong signal for bands at the appropriate molecular weight and their signal increased with higher protein concentrations of BE2 and Sf9 cell lysates. As a result of this evaluation, companies which provided some of the antibodies were contacted and were no longer considered when purchasing new antibodies.
Table 4.6.2.2 Final antibodies chosen and concentrations used on primary cell samples

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta 0028 C-Terminal</td>
<td>1:250, 1:1500, 1:1000</td>
</tr>
<tr>
<td>Beta Actin</td>
<td>1:5000, 1:10000</td>
</tr>
</tbody>
</table>

4.7. Bradford assays

After the antibody evaluations had been performed, Bradford assays were carried out on all samples to determine their protein concentration. This was done in order to ensure equal loading of samples in each lane. The final volume of samples in each well was 100µl.

Figure 4.7.1 shows an example of one of the results of the Bradford Assays performed on HAOECs at Passage 6. The absorbances read from the spectrophotometer decrease as the dilution increases. Lanes 1-2 are the standard curves. Lanes 3-12 show the results from 5 of the HAOEC cell samples.
Figure 4.7.1. Absorbance values for HAOECs Passage 6. Columns 1-2 are the standard curve results. 5µl and 10µl of each sample were used. As the dilution increases (A-H), the absorbances decrease as less protein is present.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ECP6 Sample 1</th>
<th>ECP6 Sample 2</th>
<th>ECP6 Sample 3</th>
<th>ECP6 Sample 4</th>
<th>ECP6 Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5µl</td>
<td>10µl</td>
<td>5µl</td>
<td>10µl</td>
<td>5µl</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>A</td>
<td>0.255</td>
<td>0.243</td>
<td>0.452</td>
<td>0.608</td>
<td>0.423</td>
<td>0.667</td>
</tr>
<tr>
<td>B</td>
<td>0.358</td>
<td>0.388</td>
<td>0.36</td>
<td>0.441</td>
<td>0.373</td>
<td>0.451</td>
</tr>
<tr>
<td>C</td>
<td>0.406</td>
<td>0.402</td>
<td>0.312</td>
<td>0.352</td>
<td>0.312</td>
<td>0.368</td>
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<tr>
<td>D</td>
<td>0.445</td>
<td>0.444</td>
<td>0.283</td>
<td>0.31</td>
<td>0.287</td>
<td>0.311</td>
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<td>E</td>
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<td>0.466</td>
<td>0.266</td>
<td>0.281</td>
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<tr>
<td>F</td>
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<td>0.267</td>
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<tr>
<td>G</td>
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<td>0.265</td>
<td>0.267</td>
<td>0.267</td>
<td>0.268</td>
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<tr>
<td>H</td>
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<td>0.082</td>
<td>0.258</td>
<td>0.262</td>
<td>0.263</td>
<td>0.27</td>
</tr>
</tbody>
</table>
The results of the Bradford assays are shown in tables 4.7.1 - 4.7.3 below. The protein concentration in µg/µl was determined for all 36 samples. Each table shows the protein concentration and the corresponding volume added to achieve this concentration. As previously mentioned, there were no protein samples left from HAOSMCs at passage five, as samples were depleted in the initial western blot optimisation experiments.

### Protein determination for HAOECs and HAOSMCs

<table>
<thead>
<tr>
<th>Protein µg/µl</th>
<th>Vol. to add per sample (µl)</th>
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<tbody>
<tr>
<td>0.6</td>
<td>21</td>
</tr>
<tr>
<td>0.36</td>
<td>35</td>
</tr>
<tr>
<td>0.56</td>
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</tr>
<tr>
<td>0.53</td>
<td>23.77</td>
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<tr>
<td>0.5</td>
<td>25.2</td>
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<tr>
<td>0.36</td>
<td>35</td>
</tr>
<tr>
<td>0.466</td>
<td>27</td>
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<tr>
<td>0.366</td>
<td>34.4</td>
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<tr>
<td>0.43</td>
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<tr>
<td>0.4</td>
<td>31.5</td>
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<tr>
<td>0.533</td>
<td>23.63</td>
</tr>
<tr>
<td>0.5</td>
<td>25.2</td>
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Table 4.7.1. HAOECs passage 5 Samples 1-12

<table>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>28.6</td>
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<tr>
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<tr>
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<td>23.62</td>
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<td>0.4</td>
<td>23.62</td>
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<tr>
<td>1.2</td>
<td>7.875</td>
</tr>
<tr>
<td>0.37</td>
<td>25.5</td>
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<tr>
<td>0.37</td>
<td>25.5</td>
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<tr>
<td>1.47</td>
<td>6.42</td>
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<tr>
<td>0.97</td>
<td>9.74</td>
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Table 4.7.2 HAOECs passage 6 Samples 1-12
Table 4.7.3 HAOSMCs passage 5 Samples 1-12

<table>
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<th>Vol. to add per sample (μl)</th>
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<tbody>
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</tr>
<tr>
<td>0.6</td>
<td>23.3</td>
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<tr>
<td>0.566</td>
<td>24.7</td>
</tr>
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<td>0.7</td>
<td>20</td>
</tr>
<tr>
<td>1.2</td>
<td>11.66</td>
</tr>
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<td>24.7</td>
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<td>22.11</td>
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<tr>
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<td>23.33</td>
</tr>
<tr>
<td>0.666</td>
<td>21</td>
</tr>
<tr>
<td>0.733</td>
<td>19</td>
</tr>
<tr>
<td>0.766</td>
<td>18.27</td>
</tr>
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Now that antibody dilutions and protein concentrations had been determined, Western blot analysis began on primary cells.

4.8. Western blotting results for endothelial cells.

**Passage 5**

Blots were carried out on endothelial cells at passage 5. All samples were carried out in triplicate. Each membrane was blotted with an anti α₁, an anti β₁ and an anti beta actin antibody. Some membranes were re-blotted with primary antibody at different dilutions, and exposed at different times depending on the result achieved in each blot.

Figure 4.8.1 shows a blot using anti-α₁ sGC 5214’ at a dilution of 1:160. In the antibody evaluation, a dilution of 1:100 was successful for BE2 cells, producing a strong band. This gave us an estimate of what dilution to use on our primary cells. Several blots using varying dilutions of 5214’ were carried to determine the optimum for our smooth muscle cells. Two distinct bands can be seen in each lane however both of these bands run too low to be α₁ sGC. The α₁ region of the membrane shows no antibody binding. There is also no difference in intensity between samples. The third sample of the GM-CSF at 50ng/ml
treated cells shows a weaker band than the others, but this is more than likely due to protein degradation.

Figure 4.8.1 Western blot performed on endothelial cells at passages 5 using anti-α1 sGC 5214’ at a dilution of 1:1500. Exposure was for 5 minutes.

The membrane was washed and re-blotted with anti-β1 sGC 0028 (Fig. 4.8.2). As with the previous blot, more than one dilution, including the one tested on BE2 cells (1:2000) were used to determine which gave the strongest signal. Similar to what was seen with α1 sGC, no specific binding was present. There was no beta band. Some non-specific binding was present, but overall there was very little antibody binding to the membrane.
Figure 4.8.2 Western blot performed on endothelial cells at passages 5 using anti-β1 sGC 0028 at a dilution of 1:1500. Exposure was for 2 minutes.

Beta actin was used as a loading control to ensure approximately the same amount of protein was added for each sample. Figure 4.8.3 shows the binding of anti beta actin to the blot. Binding is strong and specific for beta actin and shows a close specificity of loading of each sample.

Figure 4.8.3 Western blot performed on endothelial cells at passages 5 using anti-beta actin at a dilution of 1:6500. Exposure was for 5 seconds.
Passage 6

The same antibodies were used on endothelial cells at passage 6 and were also performed in triplicate. A smaller dilution factor was used for anti-α₁ sGC 5214’ than was used in passage 6 to try and achieve specific binding to α₁ sGC (Fig 4.8.4). Like in passage 5, no specific α₁ sGC band was present on the blot. The same two larger bands as seen in passage 5 were on the membrane. No specificity for α₁ was seen.

![Anti α₁ sGC 5214’ (1:100)](image)

Figure 4.8.4. Western blot performed on endothelial cells at passages 6 using anti-α₁ sGC 5214’ at a dilution of 1:100. Exposure was for 20 minutes.

The membrane was washed to ensure no anti-α₁ sGC antibodies were still bound. It was then incubated with anti β₁ sGC 0028 at a dilution of 1:1000 (Fig. 4.8.5). Other dilutions were used, but this one showed the strongest signal. As was seen in passage 5, no β₁ antibody bound to the membrane. The same non-specific binding that was observed when the α₁ antibody was used can be seen again. There was no band present in the region of the membrane where β₁ sGC should be located.
Figure 4.8.5 Western blot performed on endothelial cells at passages 6 using anti-β1 sGC 0028 at a dilution of 1:1000. Exposure was for 4 minutes.

As for passage 5, beta actin was used as a loading control. The loading for passage 6 was not as accurate as expected (Fig. 4.8.6). The band intensity was not as even as in passage 5. Bradford assays had been carried out before loading of protein samples to ensure equal loading. Protein denaturation is a possible reason for the differences between lanes. There were not enough passage 6 samples left to re-load and repeat this Western.

Figure 4.8.6 Western blot performed on endothelial cells at passages 6 using anti-beta actin at a dilution of 1:6500. Exposure was for 1 minute.

Despite our antibodies being evaluated before their use on our primary cells, and the determination of protein levels in each sample, in most blots, no α1 or β1 sGC was detected.
There was a lot of non-specific staining, but the correct bands were not identified. The strong anti beta actin antibody signal indicated that there was enough protein in the HAOEC samples to be detected using Western blot analysis, but it appeared from these experiments that sGC protein levels were expressed at a level too low to be detected in routine Western blotting.

Results of combined P5 and P6 samples:

As there was little or no antibody binding to protein cell samples, we combined the triplicate samples together in an attempt to load the highest amount of protein into each well. Figure 4.8.7 shows the results of a Western blot using combined passage 5 and passage 6 endothelial cells. Anti-α₁ sGC 5214’ at a dilution of 1:160 was used. As can be seen in the blot, there is a faint band at approximately 80kDa which could be α₁ sGC, however even if it is α₁, the band is too faint to see any change in protein levels between samples.

Anti α₁ sGC 5214’ (1:160)

BE2   Ctrl  10ng/ml GM/IL 50ng/ml Ctrl  10ng/ml GM/IL 50ng/ml

81kDa

Endothelial Cells Passage 5   Endothelial Cells Passage 6

Figure 4.8.7 Western blot performed on endothelial cells at passages 5 and 6 using anti-α₁ 5214’ at a dilution of 1:160. Exposure was for 15 minutes. Ctrl=Control cells, 10ng/ml=GM-CSF at 10ng/ml, GM/IL=GM-CSF at 10ng/ml+IL4 and 10ng/ml, 50ng/ml=GM-CSF at 50ng/ml.
In figure 4.8.8 anti-β1 sGC was used on the combined samples. BE2 was used as a control. No β1 sGC band can be seen on the blot. There is a faint band at approximately 80kDa but this appears to be the band picked up by anti-α1 sGC.

![Anti β1 sGC 0028 (1:1000)](image)

Figure 4.8.8 Western blot performed on endothelial cells at passages 5 and 6 using anti-β1 0028 at a dilution of 1:1000. Exposure was for 12 minutes. Ctrl=Control cells, 10ng/ml=GM-CSF at 10ng/ml, GM/IL=GM-CSF at 10ng/ml+IL4 and 10ng/ml, 50ng/ml=GM-CSF at 50ng/ml.

The purpose of performing Western blots on our primary cell protein samples was to see if protein levels of HAOEC α1 and β1 sGC were affected in the same way as mRNA levels. We found that protein levels of sGC subunits are too low, even when samples are combined, to be detected by our antibodies. Non-specific binding was seen in some blots, but no bands were detected that were the correct size. We know from the beta actin that there were high levels of protein in the samples but sGC protein levels were not as high. The next step was to see if we got the same results using the protein samples from our HAOSMCs.
4.9. Western blotting results- for smooth muscle cells

During the initial western blotting optimisation experiments, passage 4 smooth muscle cell protein samples were used up. This meant that Western blots were carried out on smooth muscle cells at passage 5. All samples were run in triplicate. In figure 4.9.1, there are three rows of bands around 80kDa. The top band is α₁ sGC, and the next lower one is most likely β₁ sGC. The antibody appears to be picking up both subunits.

![Western blot image](image)

Figure 4.9.1 Western blot performed on smooth muscle cells at passages 5 using anti-α₁ sGC 5214’ at a dilution of 1:160. Exposure was for 5 minutes.

Figure 4.9.2 shows a blot performed using anti-β₁ sGC. In this blot, the control samples and the sample treated with GM-CSF at 10ng/ml did not show bands in the β₁ sGC region. However, protein samples treated with GM-CSF at 50ng/ml showed distinct dark bands in the β₁ region. Protein samples from cells treated with GM-CSF and IL-4 showed dark bands which appeared to be β₁ sGC. One of these bands was not as strong as the other but this was more than likely due to protein degradation.
Figure 4.9.2 Western blot performed on smooth muscle cells at passages 5 using anti-β1 sGC 0028 at a dilution of 1:1500. Exposure was for 1 minute.

Once again beta actin was used as a loading control. Loading between samples was fairly even. The anti beta actin antibody reacted very strongly (Fig. 4.9.3).

Figure 4.9.3 Western blot performed on smooth muscle cells at passages 5 using anti beta actin at a dilution of 1:5000. Exposure was for 5 seconds.
Results of combined P5 and P6 samples:

As with the endothelial cells, smooth muscle cell protein samples performed in triplicate were also combined to maximise the amount of protein in each band. Anti-α1 sGC at a dilution of 1:160 was incubated for 15 minutes. As can be seen in figure 4.9.4, α1 sGC is present in the control BE2 cells, but no α1 sGC is present in our combined smooth muscle cell samples. This blot indicates that either none, or very low levels of α1 sGC are present in primary smooth muscle cells.

![Anti α1 sGC 5214' 1:160](image)

Figure 4.9.4 Western blot performed on smooth muscle cells at passages 5 using anti-α1 sGC at a dilution of 1:160. Exposure was for 15 minutes.

Anti-β1 sGC did not show any clear binding or specificity for β1 sGC on the blot. Some faint non-specific binding was present but this was not β1 (Fig. 4.9.5).

![Anti β1 sGC 0028 (1:1000)](image)

Figure 4.9.5 Western blot performed on smooth muscle cells at passages 5 using anti-β1 sGC at a dilution of 1:1000. Exposure was for 12 minutes.
Anti beta actin showed specificity for beta actin, and gave four clear, sharp bands (Fig 4.9.68). Once again, it was used as a control to ensure our antibodies were showing specificity.

Figure 4.9.69 Western blot performed on smooth muscle cells at passages 5 using anti beta actin at a dilution of 1:10000.

The antibody evaluation had been performed and was successful, and the best $\alpha_1$ and $\beta_1$ antibodies were selected to be used on the primary cell protein samples. It was not known before this study if primary cells were suitable to be used for the detection of sGC using Western blot analysis. These results show that HAOECS and HAOSMCs do not express either subunit of sGC at high enough levels to be detected by the chosen antibodies.
4.10. Splice variant work

Alternative pre-mRNA splicing is a central mode of genetic regulation in higher eukaryotes. Variability in splicing patterns is a major source of protein diversity from the genome (Black DL, 2003). It is one of the most important mechanisms to generate protein isoforms and changes the structure of transcripts and their encoded proteins. Alternative splicing determines the binding properties, intracellular localisation, enzymatic activity, protein stability and post-translational modifications of a large number of proteins. Evidence is now accumulating that alternative splicing coordinates physiologically meaningful changes in protein isoform expression and is a key mechanism to generate the complex proteome of multicellular organisms (Stamma S et al., 2005).

A307838 is a β1 sGC splice-form. Its function and significance is not known and in this study, we set about cloning it into a plasmid vector for future viral vector expression and enzymatic studies. We did this by separating it into 2 separate fragments: fragment 1 and fragment 2 (figure 4.10.1). Fragment 2 had previously been ligated to pBacPak8 (pBacPak8-fragment2) so work was mostly with fragment 1 and its incorporation into pCR-Blunt and then pBacpak8.

Refer to the figure 4.10.1 on next page.
Figure 4.10.1 Insert a legend here. Diagram showing the cloning procedure of A307838. cDNA was PCR amplified using specific primers to amplify it separately as 2 fragments (1 and 2). These were then ligated and amplified separately into two different vectors to be joined again in the plasmid vector pBacPak8.
4.10.1 Amplification of fragment 1

The results of a PCR to amplify fragment 1 are shown in figure 4.10.1. Fragment 1 was successfully amplified as can be seen from the sharp band between the 1.5kb and 2.0kb markers. This PCR product was then ligated to pCR-Blunt and transformed in competent cells.

![Image](image.png)

**Figure 4.10.1** 2% agarose gel of PCR product showing isolated fragment 1.

4.10.2 DNA Extraction using Miniprep

Plasmid DNA was extracted from our competent cells then underwent restriction digests to determine if they had successfully taken up our plasmid. The standard DNA extraction kit used in the laboratory was the Qiagen QIAprep Spin Miniprep Kit. However during the course of many minipreps, DNA yields began to get lower and lower. The quality of the kit came under question and two other DNA extraction kits were evaluated to see how they would compare.

The first kit tested was the NucleoSpin Plasmid kit. Figures 4.10.2.1 and Fig 4.10.2.2 show the results of this evaluation. The Qiagen kit was used on samples in lanes 1-10, and the NucleoSpin kit was used on samples in lanes 11-20. There was no DNA observed in the lanes where the NucleoSpin kit was used. The DNA yield from the Qiagen kit while not very high, still performed better than the NucleoSpin kit.
Figure 4.10.2.13. Gel showing DNA extraction from samples 1-12. 1-10 were extracted using the Qiagen kit. 11-12 were extracted using the new NucleoSpin Plasmid kit.

Figure 4.10.2.24. Gel of DNA from samples 13-20 using NucleoSpin Plasmid Kit.

The second kit evaluated was the Promega PureYield™ Plasmid Miniprep System. The results can be seen in figure 4.10.2.35. Samples 1-5 had practically no DNA present, showing that the Qiagen kit was no longer working. The Promega kit performed better with a much higher yield of DNA. A reagent in the Qiagen kit was most likely contaminated, and the Promega kit was used for all subsequent DNA extractions.
4.10.3. Restriction Digests

Many restriction digests were carried out in an attempt to determine if fragment 1 had been successfully ligated into pCR-Blunt. Fragment 1 should have been completely cut from pCR-Blunt when StuI was used, leaving two bands, one approximately 3.5kb and one at approximately 1.0kb.

Figure 4.10.3.16 shows the results of pCR-Blunt-fragment1 post restriction with EcoR1. Cut fragment 1 should be 1kb in size. The sample which ran in lane 2 had a band at 1.0kb, which is the correct size for fragment 1. The band was purified from the gel and an aliquot sent for sequencing. Sequencing results confirmed this showing a 98% match to fragment 1. None of the other nine samples showed the isolated fragment in the gel.
At this stage we had successfully PCR amplified fragment 1, ligated it to pCR-Blunt, transformed it, extracted the plasmid DNA and purified the fragment. The next step was to ligate it to pBacpak8-fragment2.

4.10.4. Fragment 2

Although fragment 2 had previously been cloned successfully into pBacPak8, attempts to re-isolate the fragment before it was ligated to fragment 1 using the restriction enzymes Stu1 and Kpn1 were unsuccessful.

Figure 4.10.4.12 shows 14 pBacPak8-fragment2 samples run on a 2% agarose gel. Samples were digested using Stu1 and Kpn1. The expected 5.5kb plasmid and 0.8kb fragment were not present in any of the samples on the gel. Either no cutting or non-specific cutting was observed. From the gel shown in figure 3.5.5, samples 8, 10, 11 and 14 were sent for sequencing as these bands appeared the sharpest on the gel.
The sequencing results revealed that samples showed slight discrepancies in their sequence. These could have been due to random errors or actual errors in the sequence. Before a mutagenesis could be performed to correct the error, we had to be certain that the pBacPak8-fragment2 sample being used showed no errors in its sequence.

Sample number 8 was re-sent for sequencing using different primers to take a closer look at it. The primers used were: Polyhedrin (PH) and Sh176 (diluted 1:10).

Sequencing results showed that fragment 2 was present in pBacPak8, however restriction digests were unsuccessful because primers supplied by the company were incorrect. The forward primer used when amplifying fragment 2 contained a Stu1 restriction site which was incorrect (Fig. 4.10), and resulted in pBacPak8-fragment2 being linearised instead of completely cut. The Stu1 site should have read: 5’...AGGCCT...3’. However, the sequence our plasmid read was: 5’...AGCCCT...3’

Figure 4.10. pBacPak8-fragment2 post Stu1, Kpn1 restriction digest
4.10.5. Mutagenesis to correct Stu1 site

Several mutageneses were performed in order to successfully correct the Stu1 restriction site in pBacPak8-fragment2. Figure 4.10.5 shows a miniprep carried out on a mutagenesis product. As can be seen from the gel, a high level of DNA was extracted.
Miniprep performed (Promega kit) on DPN1-treated mutagenesis colonies

Mutagenesis samples were then restricted using Stu1. Figure 4.10. shows the restriction results of this restriction. Lanes 2, 4 and 7 (blue arrow) contain fragment 2 (0.8kb) with the corrected Stu1 site, indicating that the mutagenesis was successful.

Restriction digest (Stu1, Kpn1) performed on mutagenesis/DPN1-treated pBacPak8-fragment2 products.
4.10.6. Joining of Fragments 1 and 2

Fragment 1 had been successfully cloned into pCR-Blunt, cut and isolated into its 1kb fragment. The Stu1 site in pBacPak8-fragment2 had been corrected, and the next step of restricting and joining the fragments could begin.

Figure 4.10.6.11 shows the results of a transformation to try to achieve the highest amount of DNA possible before ligation of the 2 fragments. Wells 1-5 contain pCR-Blunt-fragment1 DNA and wells 6-10 contain pBacPak8-fragment2 DNA.

Before the ligation was performed, Fragment 1 was isolated and pBacPak8-fragment2 was linearised using Stu1. An aliquot of fragment 1 and linearised pBacPak8-fragment2 was run on a 2% agarose gel to ensure that, both fragments were present, had been cut and were the correct size (Fig. 4.10.6.24). As can be seen from the gel, fragment 1 is present at the correct size (blue arrow), and linearised pBacPak8-fragment2 is present in lane 2.
Figure 4.10.6. Lane one shows fragment 1 cut with Stu1. The fragment can be seen at the 1kb mark (blue arrow). Lane 2 contains pBacPak8-fragment 2 cut and linearised using Stu1. As there is a lot more vector present than fragment 1, the vector was diluted 1:5 before the ligation was carried out.

4.10.7. Restriction digest of pCR-Blunt-fragment1 and pBacpak8-fragment2

Fig 4.10.7 shows the restriction digest of pCR-Blunt-fragment1 and pBacpak8-fragment2 using Stu1. pBacPak8-fragment2 should become linearised (lane 2) and pCR-Blunt-fragment1 should be completely cut leaving pCR-Blunt and fragment 1 (lane 1). A small band at approximately 1kb can be seen in the gel (lane 1), indicating cutting did take place. The gel also shows cut and uncut pCR-Blunt.
The results of the restriction digest of fragment 1 and pBacPak8-fragment2 should yield a fragment approximately 1.8kb in size, and another fragment 5.5kb in size. This is what was expected in Fig. 4.10. Seventeen colonies which in theory contained the pBacPak-A307 splice form, were selected and underwent a restriction digest using the enzymes EcoRV and Not1. Samples 6, 8, 9, and 13 showed a small fragment, but the size appeared to be nearer the 1kb mark, rather than 1.8kb. An aliquot of each was sent for sequencing to make sure.
Results of sequencing showed that our fragment had not been ligated successfully and the ligation was repeated. Twenty colonies were once again selected and this time, clone number 13 was confirmed by sequence to contain fragment 1 in a reverse orientation. To correct this, N13 was cut with Stu1 to isolate fragment 1 and it was then re-ligated to linearised pBacPak8-fragment2. Obtained colonies were then analysed using a BglII/EcoR1 digest (Fig.4.10.7.345). In colonies containing pBacPak8 with fragment 1 in the proper orientation, the restriction produced a 1.5kb fragment as BglII cuts close to the 5’ end of fragment 1 and EcoR1 in the vector after the 3’ of fragment 2. The correct clone was identified and the laboratory is in the process of confirming SH-β1 ORF incorporated into pBacPak8 by sequencing.
Figure 4.10. Restriction digest performed on pBacPak8 containing fragment 1 and fragment 2 using the enzymes BgIII and EcoR1. A correct alignment of fragment 1 yields a fragment 1.5kbs in length. A reverse alignment of fragment 1 yields a fragment 0.8kbs in length.
5.0. Discussion

Evidence that sGC activity is regulated both at the protein and mRNA level has begun to emerge over the past few years. However the transcriptional regulation of the expression of sGC has not been previously examined. Relatively little is known about the genes or the promoter region of mammalian sGC (Sharina et al., 2000). In our study, transcript numbers of α₁ and β₁ subunits of sGC in primary cells were affected by treatment with pro-inflammatory cytokines GM-CSF and IL-4. Changes in the mRNA expression levels of sGC subunits have been reported in several disease models. In aortic tissue from spontaneously hypertensive rats, the vasodilator response to an NO donor was markedly attenuated compared with normotensive rats. The detailed analysis of hypertensive animals revealed that α₁ and β₁ sGC mRNA levels, as well as β₁ sGC protein levels, were reduced significantly, indicating that vasodilator dysfunction is related to the sGC gene expression (Jiang et al., 2006).

We hypothesised that primary endothelial and smooth muscle cells would respond to treatment with cytokines which would have an effect on sGC production. Vascular cells are both a target for and a source of cytokines (Mantovani et al., 1992). It has been suggested that the vasculature, when exposed to inflammatory mediators can adapt to elevated NO levels by decreasing the NO receptor sGC. Cytokine-mediated changes in the function of other components of the NO-cGMP signal transduction system, such as phosphodiesterases and cGMP-dependent protein kinase, may also have important roles in regulating vascular responsiveness to NO. Relatively little is known about the effects of cytokines on sGC, but it has been demonstrated that α₁ and β₁ sGC mRNA levels are regulated by inflammatory cytokines (Papapetropoulos et al., 1996). Cytokine-induced desensitisation of vascular responsiveness to NO may represent a homeostatic mechanism preventing excessive signalling via cGMP and may serve to limit vasodilation (Takata et al., 2001). Previous work in our lab had shown that BE2 cells responded to cytokine treatment using GM-CSF and IL-4. The cytokine treatment caused a decrease in sGC subunit transcript levels. It was observed that these cytokines, used in combination, had the strongest effect on transcript levels of both α₁ and β₁ sGC (Fig. 5.1).
The effect of IL-4 & GM-CSF treatment on mRNA levels of α1 & β1 sGC subunits in human Neuroblastoma (BE2) cells

![Graph showing mRNA levels of α1-sGC and β1-sGC subunits in control, IL-4, GM-CSF, and GM+IL4 treatments.]

Figure 5.1. Q-PCR analysis of α1 and β1 subunit mRNA steady state levels in BE2 cells. Transcript levels vs cytokine treatment (Joanna McCarthy, MPhil thesis, unpublished work)

We wanted to know if this same effect on sGC transcript level would be observed using primary cells which would reflect a more in vivo-like response. The promoter regions of sGC contain elements which are known to respond to certain cytokines, therefore it was hypothesised that cytokines may play a role in the regulation of sGC subunit expression.

HAOSMCs. Cytokines play a role in the regulation of smooth muscle cells. HAOSMCs results showed that GM-CSF and IL-4, when used in combination increase the transcript number of α1-sGC significantly in both passages when compared with the control. The same results were observed in the β1-sGC transcript levels. Although passage 5 was not significant, transcript levels were still higher than untreated samples. These observations indicate that cytokine treatment increased both subunits of the sGC heterodimer in HAOSMCs. This is the opposite to what has been reported in the literature (Papapetropoulos et al., 1996; Masao et al., 2001). It has been reported previously that the exposure of rat SMCs to IL-1β decreases α1 mRNA levels and corresponding sGC activity (Papapetropoulos et al., 2006). Tumour
necrosis factor-α (TNF-α), alongside IL-1β, was demonstrated to decrease sGC α1 and β1 mRNA, protein levels and activity in rat pulmonary artery SMCs via NO-independent mechanisms (Masao et al., 2001). 17β-estradiol decreased β1 sGC in rat anterior pituitary gland, but increased α1 mRNA and protein levels, which leads to the inhibition of sGC activity (Cabilla et al., 2006). Most of these studies used different cytokines to the ones used in our study, and this may be a possible explanation as to why their results differed to ours. It makes sense that cytokines would affect cells differently in order to regulate their function. Our data demonstrates that α1 and β1 sGC mRNA levels from SMCs are affected by inflammatory cytokines, and QPCR experiments analysed the mRNA transcript levels of α1 and β1 sGC levels to determine if similar effects were seen when the cytokines GM-CSF and IL-4 were used.

**Transcript levels of α1 vs β1.** It was observed that the transcript number of both α1 sGC and β1 sGC increased in passage 5, compared with passage 4. This indicated that HAOSMCs reach an optimum level of protein expression at passage 5. It is known that primary cells in early passages express more proteins than repetitively passaged cultures (Cornwell et al., 1989). It may be that smooth muscle cells reach their highest level of sGC expression at passage five. Among the sGC subunit isoforms, β1 seems to be the most functionally significant. It is able to heterodimerise with both α1 and α2 and in the absence of it, no sGC activity is observed (Wagner et al., 2005). β1 has been found expressed ubiquitously in all tissues and so far only sGC heterodimers containing β1 have been shown to have catalytic activity in vivo. Decreases in β1 expression have been associated with aging, changes in vasodilatory potency, and salt-sensitivity in rats. Increases in β1 subunit level have been associated with altered vasodilator response in aortic rings from rats after myocardial infarction. Expression of the β1 subunit was also shown to be developmentally regulated in fish embryos and rat lung and brain (Sharina et al, 2003). In both passages, HAOSMCs β1 sGC transcript levels were approximately 100 times higher than α1 sGC transcript levels. This could indicate the importance of β1 sGC but may also suggest that it has more of a function in the cell than once thought.

**HAOECs.** It has been demonstrated that murine lung vascular endothelial cells can be activated in vitro by incubation with IFN-γ and TNF-α (Li et al., 1991). IL-1 and TNF have an array of effects on ECs, and their description occupies a large portion of the literature in the area. Recently it was found that ECs express receptors for GM-CSF and respond to these
cytokines with migration and proliferation. GM-CSF has been reported to induce expression of adhesion structures such as ICAM-1 weakly, and to have angiogenic activity. IL-4 has emerged recently as a modulator of EC function, favouring the selective recruitment and activation of lymphocytic and monocytic elements crucial for inflammation and immune reactions (Mantovani et al., 1992). Unlike appropriate reference cytokines (IL-1, TNF-α, IFN-γ), GM-CSF is not known to modulate endothelial cell functions related to haemostasis, thrombosis or inflammation. Various cell types, including activated immunocompetent cells, fibroblasts, and endothelial cells themselves, can produce GM-CSF. Thus, locally produced GM-CSF could be part of the regulatory network of neovascularisation and, in bone marrow, contribute to the maintenance of the hematopoietic microenvironment, of which endothelial cells are one important component (Bussolino et al., 1991).

Treatment of HAOECs with pro-inflammatory cytokines yielded results which were the opposite to that seen in HAOSMCs. In passage 5, the combination of GM-CSF and IL-4 resulted in a significant decrease in both α1 and β1 sGC transcript levels compared with the controls. GM-CSF at 10ng/ml and 50ng/ml had no effect on transcript levels. Passage 6 results were not significantly different from control. This was more than likely due to the cells beginning to decrease their protein expression as they age. Transcript levels of both α1 and β1 in passage 6 dropped by over 50%. This result was not surprising but culturing to passage 6 was needed in order to ensure the cells were confluent enough before treatment. Interestingly, although overall passage 6 transcript levels dropped compared with passage 5, the samples treated with the combination of cytokines did not undergo the same drop as the control samples and those treated with just GM-CSF. Transcript levels remained similar to the levels observed in passage 5. The reason for this is unclear.

**HAOSMCs/HAOECs**

In our study, HAOECs expressed much higher levels of α1 sGC than HAOSMCs. Up to 30 times more. Both primary cell lines express more β1 sGC than α1 sGC. As one subunit of each combine to form the sGC heterodimer, this leaves a surplus of β1 sGC which remains as a single subunit. This could be a further indication that β1 sGC may have other functions in the cell.

The differences in transcript numbers between passages was much greater in HAOECs, compared with HAOSMCs Primary cultured aortic smooth muscle cells in later
passages (8-10) have been shown not to express enzymes such as cGMP-dependent protein kinase, which can be detected using Western Blot analysis in earlier cell passages (Cornwell TL et al., 1989). Similarly, it has been reported that myosin is lost in cultured smooth muscle cells as part of the process of phenotypic modulation (Rovner et al., 1986). Various disease states are thought to involve decreased expression of sGC. For example, models of hypertension, atherosclerosis, and Alzheimer’s disease all correlate with decreased levels of sGC (Krumenacker et al., 2005).

Previous QPCR experiments performed on BE2 cells involving GM-CSF and IL-4 treatment resulted in an increase in both α1 and β1 sGC transcript numbers. HAOECS showed the same response with cytokines treatment. As primary cells are difficult to culture successfully, and are prone to contamination, BE2 cells could possibly be used as a model to study the regulation of sGC mRNA levels in ECs.

Our laboratory had a large selection of anti α1 and anti β1 sGC antibodies. Some were bought commercially, some had been purified in the laboratory and others were different boosts of the same antibody. BE2 and Sf9 cells were routinely used in Western blot analysis as they express high levels of sGC. With the various antibodies available in the laboratory, an evaluation on the quality and specificity of each antibody had never been carried out. As it was planned to use an anti α1 and an anti β1 antibody from the laboratory selection on our primary cell sample, we first had to determine which antibody performed the best. The performance of most of the antibodies was disappointing, with very few showing any specificity and binding to random bands on the blots. One anti α1 and one anti β1 antibody were chosen for use on our primary cells. It was surprising how badly most of the antibodies performed considering they were purified to be specific for sGC. The results of this antibody evaluation meant that the laboratory no longer used some of the companies which provided these antibodies.

Western blots were performed on primary cell samples as a method to mirror the results obtained from the QPCR data. It was not known before this study if our primary cells would express sGC at adequate levels for detection by our chosen α1 and β1 sGC antibodies. As was determined from Western blots performed on these samples, overall protein sGC levels were too low to detect clear bands on the blots. QPCR results showed that in both cell types, β1 sGC transcript numbers were higher than α1 sGC transcript numbers. 50ng/ml GM-CSF and GM-CSF in combination with IL-4 affected protein levels of β2 sGC in SMCs.
passage 5, showing clear distinct bands on the blot. No band for α₁ sGC was detected. It appears that the sGC subunits are not expressed at a high enough level to be detected using Western blot analysis, however there is a small possibility that the β₁ sGC subunit can be detected using appropriate antibodies. This would require further investigation.

Alternative splicing frequently occurs in eukaryotic genes and provides an important mechanism for tissue-specific and developmental regulation of gene expression. Several reports have suggested that splicing may also be a method of sGC regulation (Sharina et al., 2000). RT-PCR of sGC mRNA levels have indicated that there is an abundance of splice forms in different human tissues (Sharina et al., 2007). The β₁ sGC splice variant A307838 has previously been discovered in the human brain.

In this project, we aimed to clone A307838 into the expression vector pBacpak8 so that eventually it could be expressed in a viral vector to functionally characterise it, and prove its biological significance. A307868 was separated into two fragments. Fragment 1 had previously been isolated and PCR amplified with StuI restriction sites added to both ends. Attempts were made to ligate fragment 1 to the vector pCR-Blunt and then transform it into competent E. Coli cells. This process took time to optimise and perfect. After several attempts, a newly ordered StuI enzyme and a newly evaluated DNA extraction kit, fragment 1 was successfully ligated to pCR-Blunt, and transformed into competent cells. Cultured cells then underwent restriction digests and fragment 1 was found to be present.

Initial attempts to ligate fragment 1 with fragment 2 (already ligated to pBacPak8) were unsuccessful due to it being discovered that company-produced primers had been synthesised incorrectly leading to inaccurate or no cutting of the enzyme StuI. New primers were designed in an attempt to correct this restriction site and several mutageneses were performed. Once pBacpak8-fragment2 had been corrected it was restricted and ligated to fragment 1. Initial attempts were unsuccessful, with isolated fragments being of an incorrect size. Eventually fragment 1 was successfully ligated to pBacPak8-fragment2, however, it was discovered that it had been ligated backwards. There was a 50% chance of this occurring, but it was hoped that enough colonies would be grown to ensure at least one was correct. Unfortunately this was not the case, and attempts continue in the laboratory to achieve the successful ligation and correct orientation of fragment 1 to fragment 2.

The function and biological significance of A307838 is still unknown. A functional β₁ sGC splice variant has never previously been identified, and it is hoped that with further
research and kinetic and enzymatic studies, A307838 will be found to play an important role in regulation and expression of sGC.
6.0. Conclusion

Considering the importance of the function of sGC, and the reports concerning the functional significance of the splice variants for both subunits, our results should help to gain insights into the genetic basis of regulation of this important enzyme.

From the QPCR results we can conclude that the effect of cytokine regulation of transcription is highly dependent on cell type. In HAOSMCs, the coordination of transcription levels between α₁ and β₁ genes is indicative of synchronised activity of the promoters. In HAOECs, gene expression changes with each passage. The response of HAOECs to cytokine treatment is a similar response in BE2 cells, which validates BE2 as a model to study the regulation of sGC mRNA levels in HAOEC but not HAOSMCs. QPCR results from both cells types indicate that β₁ sGC may have further functions in the cell in addition to dimerisation with α₁.

We can conclude from our Western results that in general, protein levels from HAOSMC and HAOECs are too low to yield usable blots. For our HAOSMCs at passage 4, both combination of GM-CSF and IL4 affect protein and mRNA levels of β₁ sGC. But high concentrations of GM-CSF affect only protein levels, implicating post-transcriptional regulation.

In summary, this study identifies the effects of GM-CSF and IL-4 on sGC expression in primary cells. Our findings point to a role of cytokines in the regulation and activity of sGC subunits. It suggests that regulation may be finely tuned and may have significant effects on nitric oxide and cyclic GMP signalling.
7.0. References


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