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Regulation of p42/p44 Mitogen Activated Protein Kinase by Sphingosine-1-Phosphate in Embryonic Stem Cells

by
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ABBREVIATIONS

ABC	ATP binding cassette
ASM	airway smooth muscle
BMP	bone morphogenetic protein
DAG	diacyl glycerol
Dh-S1P	dihydro-S1P
DMEM	Dulbecco's modified eagles medium
DMS	dimethylsphingosine
DMSO	dimethyl sulphoxide
EB	embryoid bodies
EDG	endothelial differentiation gene family
EDTA	ethylenediaminetetraacetic acid
EFCS	European foetal calf serum
EGF	epidermal growth factor
ES cells	embryonic stem cells
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
Grb2	growth factor receptor bound 2
GPCR	G protein coupled receptor
HEK	human embryonic kidney
hES	human embryonic stem cells
HRP	horseradish peroxidise
Id gene	Inhibition of differentiation gene
IP_3	inositol triphosphate
JNK	c-Jun N-terminal kinase
KO-DMEM	knock out Dulbecco's modified eagles medium
KO-SR	knock out serum replacement
LIF	leukaemia inhibitory factor
LPP	lipid phosphate phosphatase
MAPK	mitogen activated protein kinase
MEK1	MAPK/extracellular-signal-regulated kinase kinase 1

NEAA	non essential amino acids
NGF	nerve growth factor
NLS	nuclear localisation sequence
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PH	plekstrin homology
Ph-S1P	phyto-S1P
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PTB	phospho tyrosine binding
PTX	pertussis toxin
OCT-4	octamer binding protein 4
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2/3	*src*-homology type-2/3
Shc	Src-homology-2-containing
siRNA	small interfering RNA
SNL	STO cells with neomycin resistant LIF expression vector
Sos	Son of sevenless
SphK	sphingosine kinase
STAT3	Signal transducer and activator 3
STO cells	SIM; 6-Thioguanine resistant; Ouabain resistant cells
S1P	sphingosine-1-phosphate
S1PP	sphingosine 1 phosphate phosphatase
TBS/T	tris buffered saline with Tween20
TGF-β	transforming growth factor beta
TNFα	tissue necrosing factor alpha

VEGF	vascular endothelial growth factor
αMHC	myosin heavy chain alpha
βMHC	myosin heavy chain beta

PUBLICATIONS

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ABSTRACT

Recent evidence suggests that sphingosine 1-phosphate (S1P) regulates self-renewal of human embryonic stem (ES) cells and differentiation of mouse embryoid bodies (derived from mouse ES cells) to cardiomyocytes. This study investigated the role of S1P in regulating p42/p44 MAPK signaling in mouse ES cells. In this regard, it demonstrates that both mouse ES-D3 and CGR8 cells express $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$ but lack $S1P_4$. The treatment of ES cells with S1P induced the activation of p42/p44 MAPK via a mechanism that was not mediated by $S1P_1$, $S1P_2$, or $S1P_3$. This was based on: (i) the failure of $S1P_1$, $S1P_2$, or $S1P_3$ antagonists to inhibit S1P-stimulated p42/p44 MAPK activation and (ii) the failure of SEW 2871 ($S1P_1$ receptor agonist) to stimulate p42/p44 MAPK activation. The treatment of ES cells with phytosphingosine 1-phosphate (phyto-S1P), which is shown to be an agonist of the $S1P_5$ receptor, stimulated p42/p44 MAPK activation. These findings therefore suggest that $S1P_5$ may mediate the effects of S1P in terms of regulating p42/p44 MAPK signaling in ES cells. The S1P-dependent activation of p42/p44 MAPK was sensitive to inhibition by pertussis toxin (uncouples the G-protein, G_i from GPCR), bisindolylmaleimide I (PKC inhibitor), and PP2 (c-Src inhibitor), but was not reduced by LY29004 (PI3K inhibitor) suggesting that S1P uses G_i-, PKC-, and c-Src-dependent mechanisms to activate the p42/p44 pathway in mES cells.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

1.1 Signal Transduction: an overview

In biology, intracellular and intercellular communication in multi-cellular organisms is fundamental for growth and differentiation. Cell communication is achieved via cell secreted signalling molecules which interact with cell surface receptors to initiate signal transduction across the membrane. Signal transduction is the process by which a cell converts one kind of signal or stimulus into another. A signal transduction pathway initiated by an external stimulus binding to a cell surface receptor, in turn activates an ordered sequence of biochemical reactions within a cell involving a diverse number of intracellular molecules (second messengers), which ultimately regulate the activation of genes in the cell nucleus. Signal transduction pathways can be rapid and transient, lasting seconds or minutes or more prolonged, lasting hours or even days. It is often compared to a cascade, where the number of second messengers participating in the transduction increases as the signal moves away from the initial stimulus. The more complex the organism, the more complex the repertoire of signal transduction pathways that co-ordinate the behaviour of individuals cells to support the function of the whole organism. This complexity is achieved by convergence and interaction between pathways. Due to the complexity of signalling pathways, malfunction is a major problem and many disease processes have been linked to defective signalling pathways and therefore, current research focuses on unravelling intracellular signalling to aid understanding and develop better therapies. Signal transduction pathways can be initiated by a variety of molecules including growth factors, hormones, peptides, neurotransmitters and lipids. Sphingolipids, as well as phospholipids and cholesterol, are vital components of cell membrane lipid bilayers but also play a crucial role in signal transduction by acting as ligands for cell surface receptors or as intracellular signalling mediators. The major sphingolipid involved in signal transduction is sphingomyelin (SM). SM is enriched in membrane structures known as calveolae, smooth vesicular inpocketings of the plasma membrane (Hla, 2001). Several enzymatic pathways metabolise SM into the sphingolipid mediators ceramide, sphingosine and

sphingosine-1-phosphate (S1P) which been linked to diverse cellular processes including growth, survival, differentiation and apoptosis. Specifically, ceramide and sphingosine have been linked to apoptosis and cell death, whereas S1P promotes cell growth proliferation and differentiation. Intracellular levels of these sphingolipid mediators are tightly controlled by enzymes which catalyse their synthesis and degradation. One particular pathway important for regulating proliferation, differentiation and migration is the p42/p44 mitogen activated protein kinase (MAPK) pathway.

1.2 p42/p44 mitogen activated protein kinase (p42/p44 MAPK) signalling pathway

It is known that many GPCR and receptor tyrosine kinases (RTK) regulate MAPK cascades and induce cellular processes including proliferation, migration and differentiation. Transmission of an extracellular signal to intracellular targets and subsequent regulation of cellular processes is mediated by a network of sequentially activated interacting cytoplasmic proteins, commonly known as a signalling pathway. The MAP kinases are a family of cytoplasmic proteins that control the regulation of various cellular processes including gene expression, proliferation, and differentiation (Seger & Krebs, 1995). MAPK are serine-threonine kinases that are activated by diverse stimuli ranging from cytokines, growth factors, hormones, neurotransmitters, irradiation, lipids and cellular stress (Widmann *et al*., 1999). There are at least six subfamilies of MAPK present in mammalian cells, which are: MAPK, also known as extracellular regulated kinase (ERK), the c-Jun N-terminal kinases (JNK1, JNK2 and JNK3) (or stress activated protein kinases (SAPK)) and the p38 MAPK (p38α, p38β, p38γ, p38δ) (Sugden & Clerk, 1997; Widmann *et al*., 1999) all of which are potently activated by G protein coupled receptors (GPCR). In addition, three more groups of MAPK have recently been characterised. These are big mitogen-activated protein kinase (BMK1)/ERK5, ERK3/4 and ERK 7/8 (Kasler *et al*., 2000; Krens *et al*., 2006). ERK5 is activated by growth factors and stress stimuli and participates in cell proliferation. ERK3 and ERK4 are structurally related

atypical MAPKs. ERK3 is a ubiquitously active MAPK and its activity is regulated by protein stability, whereas the mechanisms of ERK4 are still unclear. ERK 7 is also constitutively active but is not regulated by extracellular stimuli and ERK 8 is thought to be in sustained, long term signalling. p42/p44 MAPK is mostly connected to activation by GPCR and growth factors and regulation of growth and differentiation whereas both JNK and p38 MAPK are mostly concerned with stress and environmental responses. The same pattern of MAPK signalling is used by all three subfamilies described above, which is in its most basic form, a three component module, consisting of a linear sequence of three protein kinases, which has been strongly conserved through evolution and it present from yeast to human (Widmann *et al*., 1999). The components of the standard module, described diagrammatically in **Figure 1.1**, are three kinases that establish a sequential activation pathway. They are MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. For the purpose of this study, this review will focus on the p42/p44 MAPK module. The first kinase of the p42/p44 MAPK module (MAPKKK) can be A-Raf, B-Raf or Raf1, which can be phosphorylated by a MAPK kinase kinase kinase kinase or by interaction with the small GTPases Ras or Rho. The activated A-Raf, B-Raf or Raf-1 can then activate the next part of the module, the MAPKKs (MEK1, MEK2), by phosphorylating specific serine and threonine (Ser/Thr) residues on the enzyme, resulting in activation. Phosphorylation of p42/p44 MAPK by MEK1/2 occurs on a conserved dual threonine/tyrosine (Thr/Tyr) phosphorylation domain, leading to activation of the enzyme. p42 MAPK and p44 MAPK are the last kinases in the module and phosphorylate substrates on serine and threonine residues. p42/p44 MAPK can phosphorylate numerous cytoplasmic proteins or translocate into the nucleus to activate various downstream targets. Upon mitogenic stimulation, p42/p44 MAPK is transported into the nucleus where regulation of growth specific gene expression can result in cell cycle progression. Various transcriptional factors (Elk-1, c-myc, c-Fos and c-jun) can be phosphorylated by p42/p44 MAPK to enhance their transcriptional activity, resulting in expression of genes involved in growth, differentiation and migration. Brunet *et al*., (1999) demonstrated that prevention of p42/p44 MAPK nuclear translocation strongly inhibited Elk-1 dependent gene transcription and the ability of cells to initiate DNA replication in response to growth

factors (Brunet *et al.*, 1999). While many GPCR can activate the p42/p44 MAPK pathway, only sustained activation as opposed to transient activation of p42/p44 MAPK, results in progression of quiescent cells into G_1/S phase and initiation of DNA synthesis (Brunet *et al*., 1999).

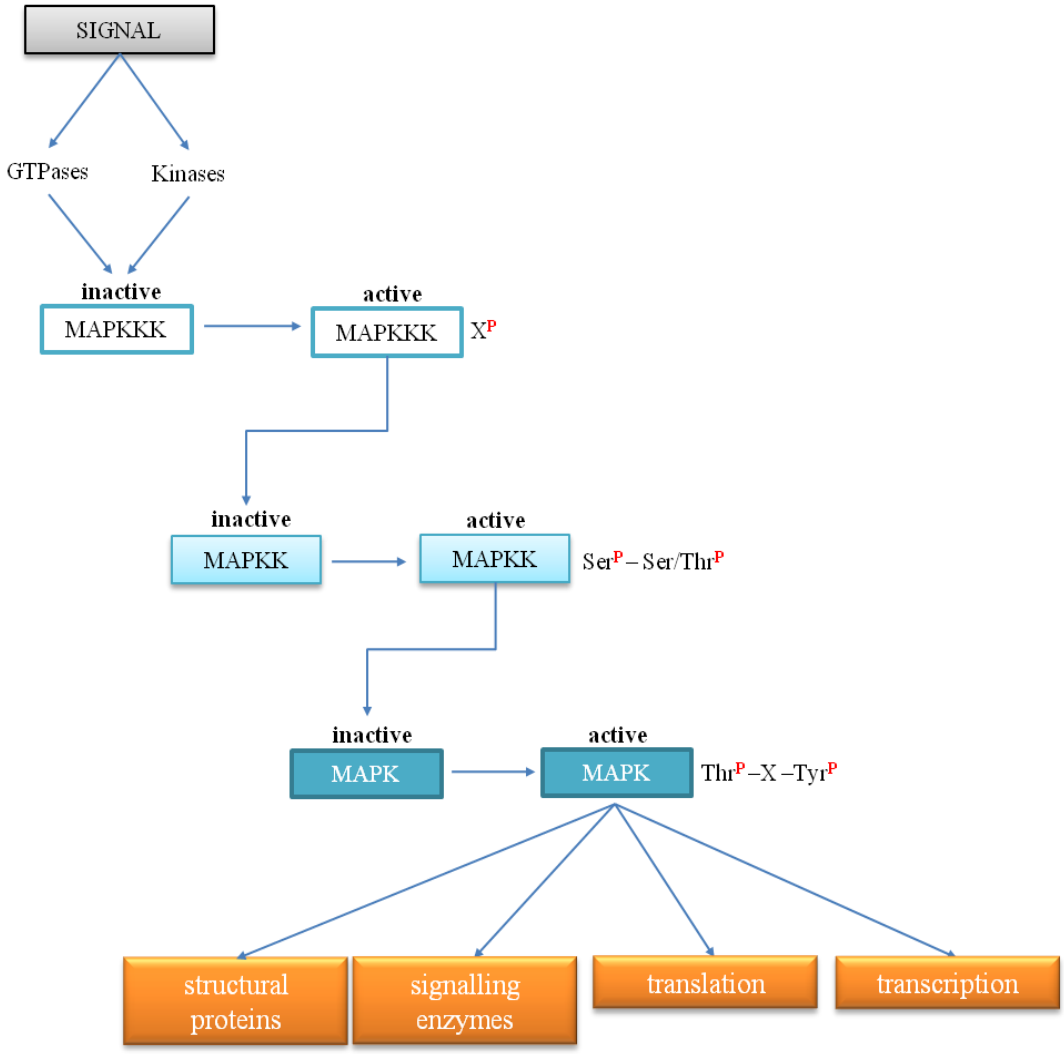

Figure 1.1 Activation of the three component MAPK module and its effects (Adapted from Sugden & Clerk, 1997; Krens *et al*., 2006)

1.2.1 **G protein coupled receptor (GPCR) activation of p42/p44 MAPK**

An important characteristic of S1P receptor signalling is the ability to activate the p42/p44 MAPK signalling pathway. This section will describe the signalling components of the pathway and the mechanism of activation by GPCRs.

The S1P receptors signal through heterotrimeric GTP binding regulatory proteins, called G proteins, which are located at the carboxyl-terminal of the receptor in the cytosol. GPCRs represent the largest family of membrane receptor, with more than 800 genes encoding several hundred GPCRs (Cotton & Claing, 2008). GPCRs consist of a single polypeptide chain, consisting of seven membrane spanning alpha helices, an extracellular N terminal domain of varying length, and an intracellular C-terminal domain (Rang, 1998). These receptors are activated by diverse stimuli ranging from small molecules such as neurotransmitters, chemokines, ions, photons and phospholipids to larger hormones and peptides (Dupre *et al*., 2009). GPCRs are linked to heterotrimeric G proteins, consisting of α, β and γ subunits that serve to transduce a signal initiated by binding of ligand to the receptor to an intracellular effector molecule (Heldin, 1998). They are located at the cytoplasmic surface of membranes, attached to the long third cytoplasmic loop of the GPCR by a fatty acid chain and in close proximity to wide variety of effectors such as enzymes or ion channels. The heterotrimer can dissociate into α subunits and βγ complexes to regulate intracellular effectors. The enzymatically active α subunits are the largest (ranging from 36-46 kDa) and contain a binding site for guanine nucleotides, catalysing the conversion of guanine triphosphate (GTP) to guanine diphosphate (GDP). The β (35-36 kDa) and γ (~8kDa) complexes are linked by stable non-covalent complexes tightly associated with membranes (Heldin, 1998; Rang *et al*., 2003). βγ complexes do not have a catalytic site but regulate G protein signalling through protein-protein interactions. More than twenty G protein-α subunits exist grouped into four major families. These are G_s (G_s, G_{olf}), G_i (G_{i1}, G_{i2}, G_{o3}, G_{o1}, G_{o2}, G_z, G_t, G_{gus}) G_q (G_q, G_{11}, G_{14}, $G_{15/16}$) and G_{12} (G_{12}, G_{13}). Five G protein-β subunits and eleven G protein-γ subunits also exist. Certain GPCRs will only interact with a

specific subset of G proteins whereas other receptors can activate more than one G protein subtype. The latter is true for S1P receptors.

In an inactive state, the G protein exists as a αβγ trimer and the α-subunit is bound to a molecule of GDP. Binding of agonist activates its specific receptor and the affinity of the α subunit for GDP is decreased. GDP is released in exchange for GTP, present in the cytosol. The GDP-GTP exchange causes a conformational change in the α subunit allowing it to dissociate from the βγ subunit. The released, activated α-GTP and βγ subunits diffuse in the membrane and associate with various effector enzymes, ion channels or receptors before hydrolysis of the bound GTP converts the α subunit back to the inactive form and re-associates with the βγ subunits (Heldin, 1998; Rang *et al*., 2003). Both the α-GTP and βγ subunits can initiate signalling responses through association with cytoplasmic targets [**Figure 1.2**]. Targets of specific G proteins are adenylyl cyclase (AC) for G_s and G_i, phosphoinositide 3-kinase (PI3K) for G_i, phosphoinositide-specific phospholipases C (PLC) for G_i and G_q. Rho specific guanine nucleotide exchange factors are targets for G_{12} and G_{13} and sodium, potassium and calcium channels are activated by G_s and G_i (Neer, 1995).

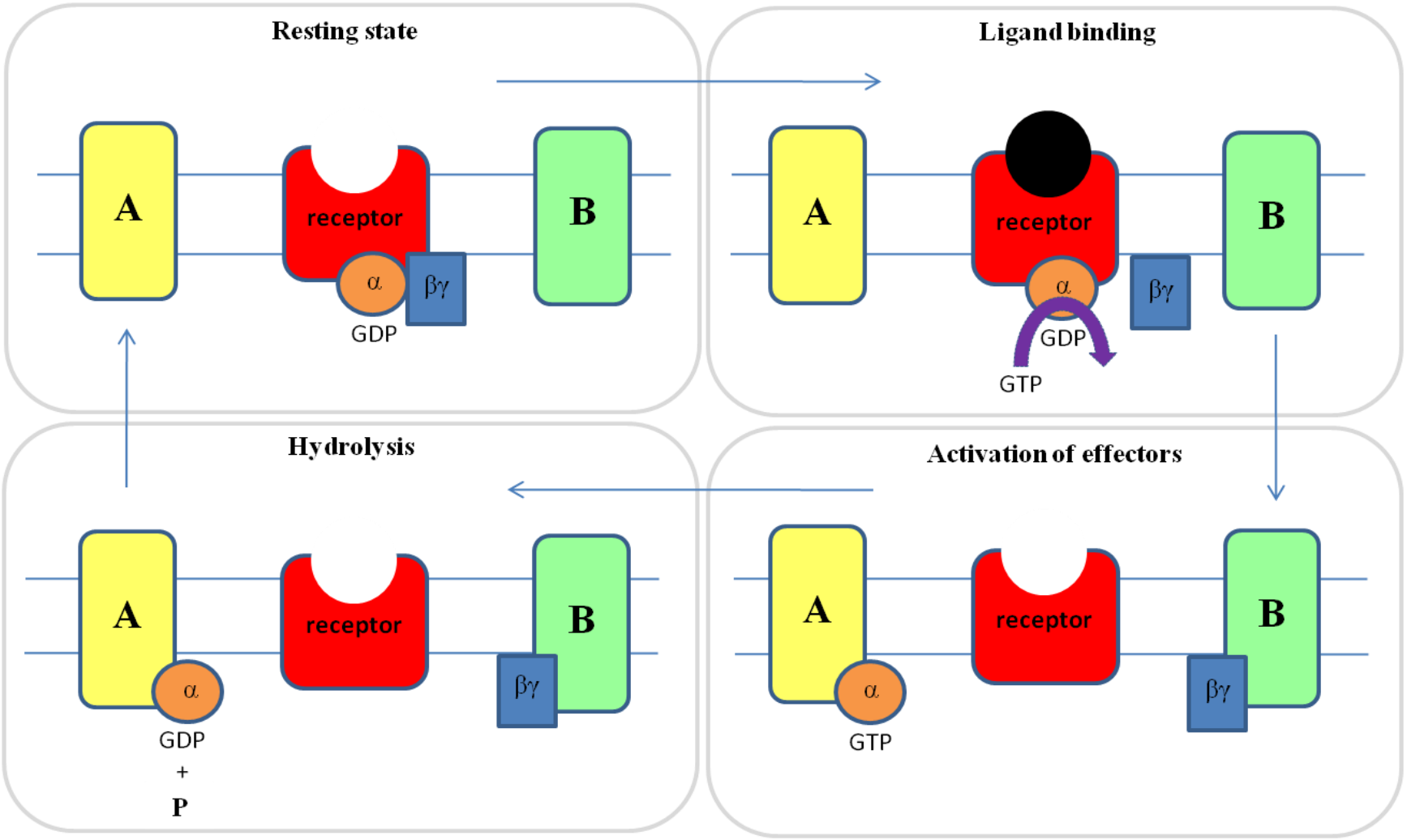

Figure 1.2 Activation of G proteins by GPCRs; A: effector protein A, B: effector protein B (adapted from Rang *et al.*, 2003).

The discovery that the bacterial toxin, pertussis toxin (PTX), catalyses ADP-ribosylation of a specific cysteine residue at position −4 from the C-terminus of the α subunits, and thus blocks downstream pathways by preventing receptor coupling to the G protein has greatly aided research in this area. It is a widely used tool for dissecting the essential role of $G\alpha_i$ proteins in a variety of physiological effects.

Many GPCRs can potently activate the p42/p44 MAPK pathway to regulate a plethora of cellular processes. GPCRs are linked to the activation of the p42/p44 MAPK cascade via two mechanisms as both G protein-α subunits and G protein-βγ subunits can directly or indirectly activate p42/p44 MAPK. Firstly, treatment of G_i coupled receptors with PTX results in complete or partial inhibition of p42/p44 MAPK activation. However, it is thought that when G_i coupled receptors are involved, p42/p44 MAPK activation is primarily mediated by the βγ subunit (Widmann *et al*., 1999). Originally, the βγ complex was thought to be necessary for the activation of Gα subunits to facilitate reassociation with the receptor. Its existence was thought to prevent spontaneous activation of Gα in absence of agonists and therefore act as a negative regulator of Gα (Neer, 1995). Many studies in recent years have shown that βγ subunits can directly interact and modulate effectors that are also regulated by Gα subunits (Dupre *et al*., 2009).

Studies have shown that the βγ subunit of G_i mediates p42/p44 MAPK signalling via PI3K (Hawes *et al*., 1996; Lopez-Ilasaca *et al*., 1997; Kranenburg *et al*., 2001). In COS-7 cells, the PI3K inhibitors LY294002 and wortmannin abolished G_i coupled receptor- and Gβγ-dependent p42/p44 MAPK activation and Ras activation. This was also the case when cells were treated with a dominant negative mutant of the p85 subunit of PI3K (Hawes *et al*., 1996). Lopez-Ilasaca *et al*., (1997) also demonstrated that in COS-7 cells co-expressing the m2 muscarinic receptor, wortmannin inhibited p42/p44 MAPK expression induced by transient expression of βγ-subunits or by co-expression of βγ-subunits and the guanine nucleotide exchange factor Sos. The dominant negative mutant N17-Ras also inhibited p42/p44 MAPK activation induced by PI3K, but the dominant negative mutants of the GTPases RhoA, Rac, and Cdc42 did not (Lopez-Ilasaca *et al*., 1997). A dominant negative mutant of Raf or Sos also

inhibited p42/p44 MAPK activation by PI3K. These data suggest a critical role for Ras, Raf and Sos in Gβγ-dependent PI3K-dependent p42/p44 MAPK activation, with PI3K placed upstream of Ras. Further studies have highlighted a role for c-Src in the Gβγ subunit mediated activation of p42/p44 MAPK. The release of βγ subunits activates c-Src, which leads to Ras activation and activation of the p42/p44 MAPK cascade (Luttrell *et al*., 1996).

The p42/p44 MAPK pathway can also be activated by the α-subunit of the G_o protein in a novel PKC dependent, Ras independent mechanism (Van Bisen *et al*., 1996). G_o-mediated p42/p44 MAPK activation was sensitive to pertussis toxin and completely blocked by cellular depletion of PKC but insensitive to inhibition by a Gβγ-sequestering peptide (βARK1ct) and a dominant negative mutant of Ras (Van Bisen *et al*., 1996). The p42/p44 MAPK pathway can also be regulated by $G\alpha_q$ proteins. Regulation of p42/p44 MAPK by $G\alpha_q$ proteins is mediated by two different mechanisms. The first links $G\alpha_q$ to phospholipase C-β (PLC-β), $[Ca^{2+}]_i$ and PKC. PLC-β catalyses the conversion of phosphatidylinositol into diacylglycerol (DAG) and inositol triphosphate (IP_3), leading to the activation of PKC. PKC then activates p42/p44 MAPK indirectly via Raf-1 (Kranenburg *et al*., 2001). Secondly, IP_3 binds to receptors on the endoplasmic reticulum, resulting in the mobilisation of intracellular Ca^{2+} which activates the proline rich tyrosine kinase (Pyk2). This in turn results in Ras activation via activation of Shc and recruitment of Grb2/Sos and finally p42/p44 MAPK activation (Widmann *et al*., 1999).

1.2.2 **Growth factor activation of p42/p44 MAPK**

Growth factor receptors, also known as receptor tyrosine kinases differ significantly in structure and function from GPCRs. Growth factor receptors mediate the effects of a wide variety of protein mediators including growth factors, cytokines and hormones (Rang, 2003). Upon engagement with a specific ligand, the receptors trigger a kinase cascade. The kinase cascade is initiated by ligand induced dimerisation of pairs of receptors, which allows autophosphorylation of tyrosine residues to occur. Phosphorylated tyrosine residues serve as high affinity binding

sites for intracellular proteins which can further perpetuate the signal by interacting with downstream intracellular signalling molecules. Growth factor receptor stimulation of the p42/p44 MAPK cascade is well defined. The cascade is initiated by SH2 (Src homology 2) adaptor proteins binding to specific phosphorylated tyrosine residues on the receptor. Binding of the SH2 domain protein, Src to the receptor permits tyrosine phosphorylation of Src. This results in recuitment of the Grb2-Sos1 complex. Grb2 is also a SH2 domain protein which allows binding to Src. This initiates activation of Ras GDP/GTP exchange where Ras is converted from the inactive GDP-bound form to the active GTP-bound form. Ras-GTP subsequently activates the first component of the kinase cascade, Raf, leading to the phosphorylation of MEK and then p42/p44 MAPK (Heldin & Westermark, 1999).

1.3 Sphingosine-1-phosphate: generation and metabolism

Sphingolipid signalling has been the subject of intense research over the last decade. In particular, the bioactive lipid, S1P, has emerged as an important signalling molecule that can regulate a variety of cellular processes including cell proliferation, survival, migration and differentiation in many cell types (Spiegel *et al*., 2002). The role of S1P in regulating these vital cellular processes has also been associated with several disease states including cancer. Given the role of S1P in regulating a variety of crucial cellular processes, an emerging role for S1P in embryonic stem cell regulation has emerged and been a focus of interest in the sphingolipid field over the last few years. This section will focus on the structure, biosynthesis and metabolism of S1P and introduce S1P signalling as an intracellular intermediate signalling molecule and as an extracellular ligand for a family of GPCRs.

1.3.1 **S1P structure and synthesis**

S1P (1-phospho-2-amino-4-*trans*-octadene-1,3-diol) is a polar lysophospholipid, consisting of a sphingosine backbone, comprising a long hydrocarbon tail attached to a polar amino group and a phosphate group [**Figure 1.3**] which is stored (~0.2μM) and released from platelets (Yatomi *et al*., 1997). Activation by thrombin, ADP or collagen induces S1P production and promotes extracellular release. S1P is present in blood plasma and high concentrations are detected in human serum (0.5μM), usually bound to albumin (Yatomi *et al*, 1997). Biosynthesis of S1P from SM is mediated by a series of enzymatic reactions [**Figure 1.4**]. Ceramide is a key precursor to S1P formation and can be generated *de novo* or by the agonist dependent (growth factors, cytokines, arachidonic acid) activation of sphingomyelinases, promoting conversion of sphingomyelin to ceramide. Ceramide can be formed *de novo* from the action of serine palmitoyl transferase on palmitoyl CoA and serine, forming 3-ketosphinganine which is then reduced to dihydrosphingosine. The enzyme ceramide synthase catalyses the conversion of dihydrosphingosine into dihydroceramide. A *trans* double bond is then introduced by ceramide desaturase, forming ceramide (Maceyka *et al*., 2002). Ceramide is deacylated by ceramidases to yield the sphingoid base, sphingosine. Both dihydrosphingosine and sphingosine can be phosphorylated on the 1-OH by sphingosine kinases (SphK), producing dihydro-sphingosine-1-phosphate (Dh-S1P) and S1P (Maceyka *et al*., 2002).

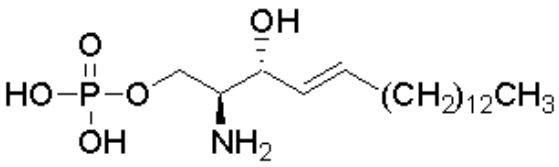

Figure 1.3 Chemical structure of S1P. S1P is comprised of a long hydrocarbon sphingoid backbone attached to a polar amino group and a phosphate group at C1.

However, dihydrosphingosine and sphingosine cannot directly be interconverted, which allows the concentrations of each to be differentially regulated. These reactions can be reversed by ceramide synthase and sphingomyelin synthase, converting sphingosine back to ceramide and SM. Therefore sphingosine, ceramide and S1P can easily and quickly be interconverted. Sphingosine/ceramide and S1P have opposing effects on cell survival. Sphingosine and ceramide are important regulatory components of stress responses and programmed cell death, apoptosis. Cell stress induced by serum starvation, increase sphingosine and ceramide levels leading to inhibition of cellular growth and apoptosis. In contrast, S1P promotes cell proliferation and inhibits apoptosis. S1P also protects against ceramide mediated apoptosis (Cuvillier *et al*., 1996). This 'sphingolipid rheostat' proposes that conversion of sphingosine and ceramide to S1P removes pro-apoptotic signals and promotes survival signals, with the relative amounts of each determining cell fate. The levels of ceramide, sphingosine and S1P are regulated by the enzymes which interconvert these metabolites. The levels of these enzymes can be increased or decreased to favour either ceramide or S1P production. Several factors have been shown to increase the sphingomyelinase and SphK activity and therefore dictate the fate of a cell. The next subsections focus on the enzymes that control S1P levels in the cell.

1.3.1.1 **Sphingosine kinase**

Intracellular levels of S1P are low and tightly controlled by the enzymes that catalyse its synthesis and degradation. Sphingosine kinases catalyse the ATP dependent phosphorylation of sphingosine to S1P (Pyne & Pyne, 2002). Two isoforms of human SphK have been identified, SphK1 (Nava *et al*., 2000) and SphK2 (Liu *et al*., 2000a) and are proteins of 384 (43 kDa) and 618 (65 kDa) amino acids respectively.

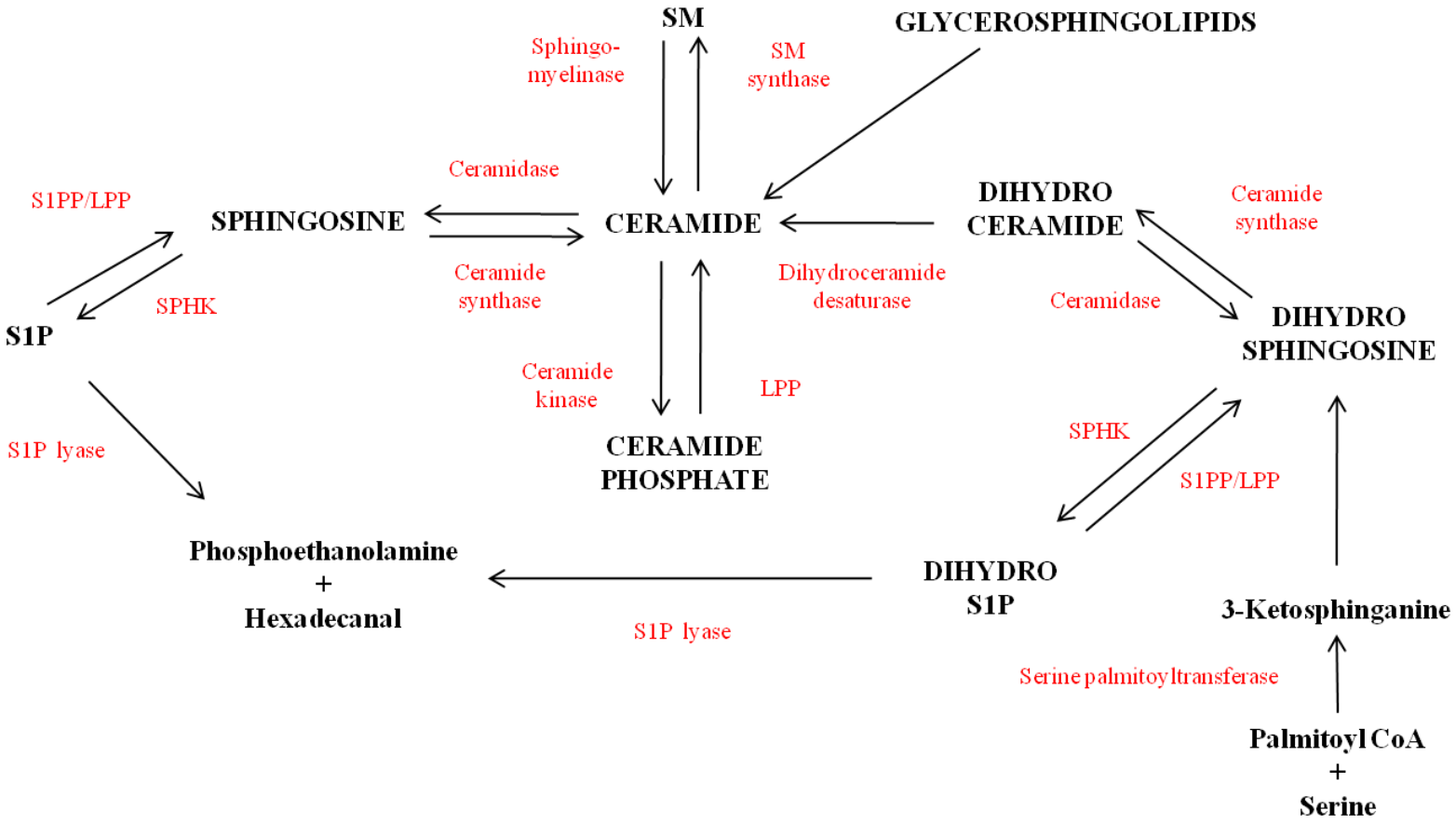

Figure 1.4 The biosynthesis and metabolism of S1P (adapted from Pyne & Pyne, 2000)**.**

The SphK isozymes have five conserved domains (C1-C5) with the catalytic domain located within C1 to C3, and the ATP-binding site within C2. Although both isoforms phosphorylate sphingosine, they differ slightly in enzymatic properties, cellular localisation, tissue distribution and biological functions. Both are located predominately in the cytosol although SphK2 has a novel nuclear localisation sequence for localisation in the nucleus (Igarashi *et al*., 2003). SphK1 has been associated with the endoplasmic reticulum and bound to membrane in smooth muscle cells (Ghosh *et al*., 1994). SphK1 is widely expressed in adult tissue with highest levels expressed in liver, kidney, heart and skeletal muscle (Nava *et al*., 2000). Sphk2 is highly expressed in kidney, liver and brain but barely detectable in skeletal muscle (Liu *et al*., 2000a). Sphingosine is a substrate for both SphK isoforms. However, SphK1 prefers sphingosine over dihydrosphingosine and does not recognise phytosphingosine as a substrate, whereas SphK2 prefers dihydrosphingosine over sphingosine and is able to phosphorylate phytosphingosine (Liu *et al*., 2000a). Both isoforms are inhibited by dimethylsphingosine (DMS), which inhibits SphK1 in a competitive manner and SphK2 in a non competitive manner.

S1P levels can be altered by stimuli that affect activity or expression of SphK enzymes. Activation of SphK occurs in response to a variety of biological stimuli, predominately growth and survival factors, resulting in an intracellular increase in S1P. Activators of SphK include platelet derived growth factor (PDGF), epidermal growth factor (EGF) and nerve growth factor (NGF) (Olivera & Spiegel, 1993; Meyer zu Heringdorf *et al*., 1999; Edsall *et al*., 1997). The aforementioned growth factors and TNF-α (Xia *et al*., 1999) induce rapid activation and translocation of SphK to the plasma membrane. Other stimuli include serum (Olivera & Spiegel, 1993), the protein kinase C (PKC) activator PMA (Cuvillier *et al*., 1996) and S1P (Meyer zu Heringdorf *et al*., 2001). SphK1 can also be directly activated by p42/p44 MAPK at serine 225 (Pitson *et al*., 2003). Phosphorylation of hSphK1 at this site increases catalytic activity 14-fold and is necessary for translocation to the plasma membrane, where the SphK1 substrate sphingosine resides (Pitson *et al*. 2005). Another study demonstrated that SphK2 is phosphorylated by p42 MAPK at Serine-

351 and Threonine-578 and downregulation of p42 MAPK blocked EGF-induced phosphorylation of hSphK2 and migration of MDA-MB-453 cells (Hait *et al.*, 2007).

SphK has also been detected extracellularly, in mouse and human plasma and found to be secreted by vascular endothelial cells, smooth muscle cells and mast cells. Export of S1P from mast cells is mediated by ATP bindng cassette (ABC) transporters (Mitra *et al*., 2006). Export of SphK has been implicated in localised generation of S1P available for cell surface S1P receptor binding (Venkataraman *et al*., 2006).

1.3.2 **S1P metabolism**

Two alternative pathways have been identified for S1P metabolism. S1P can be metabolised back to sphingosine by the enzyme, S1P phosphatase, which catalyses the dephosphorylation of S1P to produce sphingosine. Alternatively, the enzyme S1P lyase irreversibly cleaves S1P at the C2-C3 bond to produce phosphoethanolamine and palmitaldehyde.

1.3.2.2 **S1P phosphatase**

The dephosphorylation of S1P to produce sphingosine is catalysed by membrane bound S1P phosphatases (S1PP), which are specific for S1P, or lipid phosphate phosphatases (LPP), which have a broader specificity (Pyne & Pyne, 2002). Three mammalian LPP isoforms have been identified, known as LPP1, LPP2 and LPP3. They are six transmembrane spanning proteins located to the plasma membrane and endoplasmic reticulum. In addition to S1P, the three LPP isozymes dephosphorylate phosphatidic acid, lysophosphatidic acid (LPA) ad ceramide-1-phosphate to generate sphingosine, diacylglyerol, monoacylglycerol and ceramide, respectively (Pyne *et al*., 2005). Two known isozymes of S1PP exist which are structurally related to LPP. They are termed S1PP1 and S1PP2 and belong to the family of magnesium dependent, *N*-ethylmaleimide-insensitive type 2 lipid phosphate phosphatases that

reside in the endoplasmic reticulum (ER) (Le Stunff *et al*., 2002). S1PP1 and S1PP2 are proteins with a predicted molecular mass of 46 and 49kDa respectively, located to the endoplasmic reticulum. S1PP1 possesses specific enzymatic activity against S1P, dihydroS1P and phytoS1P. S1PP1 was detected in most tissues including human brain, lung, heart and skeletal muscle with highest levels detected in the highly vascularised tissue of the kidney and placenta (Johnson *et al*., 2003). S1PP2 exhibits high phosphohydrolase activity to S1P and dihyrdoS1P and is also located to the endoplasmic reticulum (Ogawa *et al*., 2003). Although similar in location and enzymatic properties to S1PP1, S1PP2 is more tissue specific with expression detected in brain, heart, colon, kidney, small intestine, and lung but absent in skeletal muscle, thymus, spleen, liver, placenta, and peripheral blood leukocyte (Ogawa *et al*., 2003).

1.3.2.3 **S1P lyase**

Alternatively, S1P can be irreversibly degraded by a pyridoxal phosphate-dependent S1P lyase. S1P lyase cleaves S1P at the C_2-C_3 bond to produce hexadecanal and phosphoethanolamine (Pyne & Pyne, 2002). S1P lyase is ubiquitously expressed in mammalian tissues including high activity in the liver, intestine and kidney and lower activity in the brain and heart (Van Veldhoven & Mannaerts, 1993). However, S1P lyase is completely absent from platelets, which results in the accumulation of S1P in these cells (Yatomi *et al*., 1997). S1P lyase is found in the endoplasmic reticulum where it the active site of the enzyme is exposed to the cytoplasm, ensuring easy access to S1P produced there. In contrast, the active site of S1PP is orientated towards the lumen of the ER or on the extracellular surface of the plasma membrane. Access to S1P would therefore require transcellular movement through membrane (Wattenberg *et al*., 2006).

1.4 Biological actions of S1P

The importance of the bioactive sphingolipid S1P as a regulator of a diverse range of cellular functions has been recognised within the last decade. Since the importance of S1P in cell growth was first demonstrated by Zhang *et al*. in 1991, the polar lysophospholipid has emerged as a critical regulator of a wide range of biological activities in various cell types including proliferation, migration, survival motility and differentiation. S1P is present intra-and extracellularly. Initially S1P was thought to be an intracellular signalling molecule acting as a second messenger based on observations that extracellular growth factors (PDGF, NGF, and TNFα) could activate SphK, induce S1P production and act on specific intracellular targets to induce a biological response (Olivera & Spiegel, 1993). However, intracellular targets mediating these actions have not yet been identified. The discovery in 1998 that S1P is a ligand for cell surface G protein coupled receptors (GPCRs) has accelerated studies in this area. S1P can act as both an intracellular second messenger and as an extracellular agonist for a family of GPCRs.

1.4.1 Intracellular actions of S1P

Several studies have implicated S1P as a second messenger by altering the activity of specific intracellular target proteins. The first indication that S1P may function as a second messenger, mobilising calcium independently of inositol triphosphate, arose when sphingosine was shown to release calcium from internal stores in permeabilised hamster smooth muscle cells (Ghosh *et al*., 1990). It was proposed that S1P generated intracellularly from sphingosine was responsible for this effect. This was confirmed by studies using fluorescent imaging in Swiss 3T3 fibroblasts (Zhang *et al*., 1991). S1P caused a rapid rise in cytosolic free calcium in the absence of intracellular calcium suggesting that S1P-dependent Ca^{2+} increases were from intracellular calcium stores. Furthermore, Ghosh *et al*., (1994) showed that S1P generated at the ER membrane by SphK induced intracellular calcium release independent of inositol triphosphate. Direct microinjection of S1P into HEK 293

cells or Swiss 3T3 fibroblasts resulted in rapid and transient Ca^{2+} mobilisation and increased DNA synthesis, respectively (Meyer zu Heringdorf *et al*., 1998; Van Brocklyn *et al*,. 1998; Himmel *et al*., 1998). Using a method whereby caged S1P is introduced into cells by photolysis, therefore avoiding damage of the plasma membrane, S1P induced intracellular calcium mobilisation in HEK 293, SKNMC and HepG2 cells (Meyer zu Heringdorf *et al*., 2003), suggesting a true intracellular action of S1P, although to date, the intracellular targets and mechanism of action is unknown.

Other evidence for an intracellular role for S1P has come from the ability to manipulate intracellular S1P levels, achieved by the cloning of SphK. Exogenous expression of SphK increased S1P levels in NIH 3T3 fibroblasts and HEK293 cells, but no detectable secretion of SPP into the medium was measured. Furthermore, over-expression of SphK promoted growth in low serum media, enhanced G_1/S cell cycle transition and increased cell number and DNA synthesis (Olivera *et al*., 1999). Increased SphK levels also protected against apoptosis induced by serum deprivation or ceramide elevation, which was blocked by the SphK inhibitor, DMS.

1.4.2 **Extracellular actions of S1P**

The extracellular effects of S1P are attributed to specific cell surface receptors, known as S1P receptors. Receptors for S1P belong to a group of G protein coupled receptors of the Endothelial Differentiation Gene (EDG) family so called because the first member of the EDG family (EDG1) was originally described as an inducible transcript that was highly expressed during the *in vitro* differentiation of human umbilical vein (HUVEC) cells (Hla, 1990). Originally described as EDG-1, EDG-5/AGR16/H218, EDG-3, EDG-6 and EDG-8/NRG-1, the five GPCRs for S1P have been renamed according to the International Union of Pharmacology (IUPHAR) guidelines (Chun *et al*., 2002). The receptors are now known by the abbreviation of the ligand with the highest potency followed by a number indicating the order in which the receptor was described and characterised: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and

$S1P_5$ (Chun *et al.*, 2002). S1P binds to these closely related proteins with high affinity. The S1P receptors are G protein coupled receptors (refer to section 1.2.1), consisting of seven hydrophobic transmembrane alpha helices linked to various heterotrimeric G proteins, and display ~50% homogeneity (Pyne & Pyne, 2000). As S1P is linked to various G proteins it has the capacity to influence different biological processes depending on cell and tissue specific expression of S1P receptors and coupling to different G proteins (summarised in **Table 1**).

Receptor	G-protein coupling	Intracellular effector	Tissue Expression	Cellular/Physiological Effect	Knockout Studies
$S1P_1$/EDG-1	G_i	↓AC ↑p42/p44MAPK ↑$PLC/IP_3/Ca^{2+}$ ↑PI3K/Akt ↑Rac ↑Rho	Wide distribution including brain, heart, lung, spleen liver, thymus, kidney	Stimulates cell proliferation/survival, cell migration , blood vessel maturation, neurogenesis, lymphocyte trafficking	Embryonic lethal, incomplete blood vessel maturation
$S1P_2$/EDG-5	G_i,G_q,$G_{12/13}$	↑AC ↑p42/p44MAPK ↑$PLC/IP_3/Ca^{2+}$ ↓Rho ↓Rac ↑JNK ↑p38	Wide distribution including brain, heart, lung, spleen liver, thymus, kidney and smooth muscle cells (SMC)	Decrease cell migration, increase cell proliferation, survival, inhibit membrane ruffling and stimulate stress fibre formation, differentiation of SMC	Cardia bifida in zebrafish, neuronal dysfunction in mice
$S1P_3$/EDG-3	G_i,G_q,$G_{12/13}$	↑↓AC ↑p42/p44MAPK ↑$PLC/IP_3/Ca^{2+}$ ↑Rac ↑Rho	Wide distribution including spleen, heart, lung, brain, kidney, intestine and skeletal muscle	Stimulate cell migration and stress fibre formation, increase cell proliferation/survival, cytoskeletal reorganisation, vasorelaxation, heart rate	KO mice exhibit no abnormal phenotype ($S1P_2$/$S1P_3$ KO mice die within 3 postnatal weeks)
$S1P_4$/EDG-6	G_i, $G_{12/13}$	↑AC ↑p42/p44MAPK ↑$PLC/IP_3/Ca^{2+}$ ↑Cdc42/Rho	Expression limited to haematopoetic cells and tissues including thymus, spleen, peripheral leukocytes	cell migration, proliferation, cytoskeletal changes, cytokine secretion in T cells	Unknown

Receptor	G-protein coupling	Intracellular effector	Tissue Expression	Cellular/Physiological Effect	Knockout Studies
$S1P_5$/EDG-8	G_i, $G_{12/13}$	↓AC ↓p42/p44MAPK ↑$[Ca^{2+}]$ ↑JNK	Mainly expressed in brain/ oligodendrocytes but also in spleen, lymphocytes	Inhibits cell proliferation, cell rounding, promotes cell survival in mature oligodendrocytes	Unknown

Table 1 Summary of S1P receptor signalling pathways (adapted from Kluk & Hla, 2002). Abbreviations: AC-adenylyl cyclase, MAPK-mitogen activated protein kinase, PLC-phospholipase C, IP3-inositol 1,4,5-trisphosphate, JNK- c-JUN N-terminal kinase, p38-p38 mitogen activated protein kinase.

1.4.3 **S1P receptors**

The first S1P receptor was identified and cloned in 1990 by Hla & McCaig and named EDG1 (now $S1P_1$). It was originally isolated as an immediate early gene, induced by PMA in human umbilical vein endothelial cells (HUVEC) cells, with a potential role in endothelial cell differentiation (Hla & McCaig, 1990). Later, a high-affinity ligand for $S1P_1$ was reported to be the bioactive lipid, S1P (Lee *et al*., 1998). $S1P_2$ was isolated as an orphan GPCR from rat cardiovascular and nervous system (Okazaki *et al*., 1993; MacLennan *et al*., 1994) before being characterised as a high affinity S1P receptor (Gonda *et al*., 1999; An *et al*., 1997). Similarly, degenerate PCR cloning from a human genomic DNA library isolated an orphan GPCR later identified as $S1P_3$ (Yamaguchi *et al*., 1996; An *et al*., 1997). In 1998, $S1P_4$ was isolated from in vitro differentiated human and murine dendritic cells and later described as the fourth high-affinity receptor for S1P (Graler *et al*., 1998; Van Brocklyn *et al*., 2000), although it differs from the others in that its ligand appears to be phytosphingosine-1-phosphate (Ph-S1P) (Candelore *et al*., 2002). $S1P_5$ was the most recent S1P receptor to be described. It was cloned from rat pheochromocytoma 12 (PC12) cells and originally called nerve growth factor-regulated gene-1 (NRG-1) as nerve growth factor repressed expression of this gene in PC12 cells (Im *et al*., 2000). Subsequent studies have revealed that S1P receptors couple to multiple types of G proteins to activate a range of downstream signaling effectors which are summarised in [**Figure 1.5**] and described in the following sections.

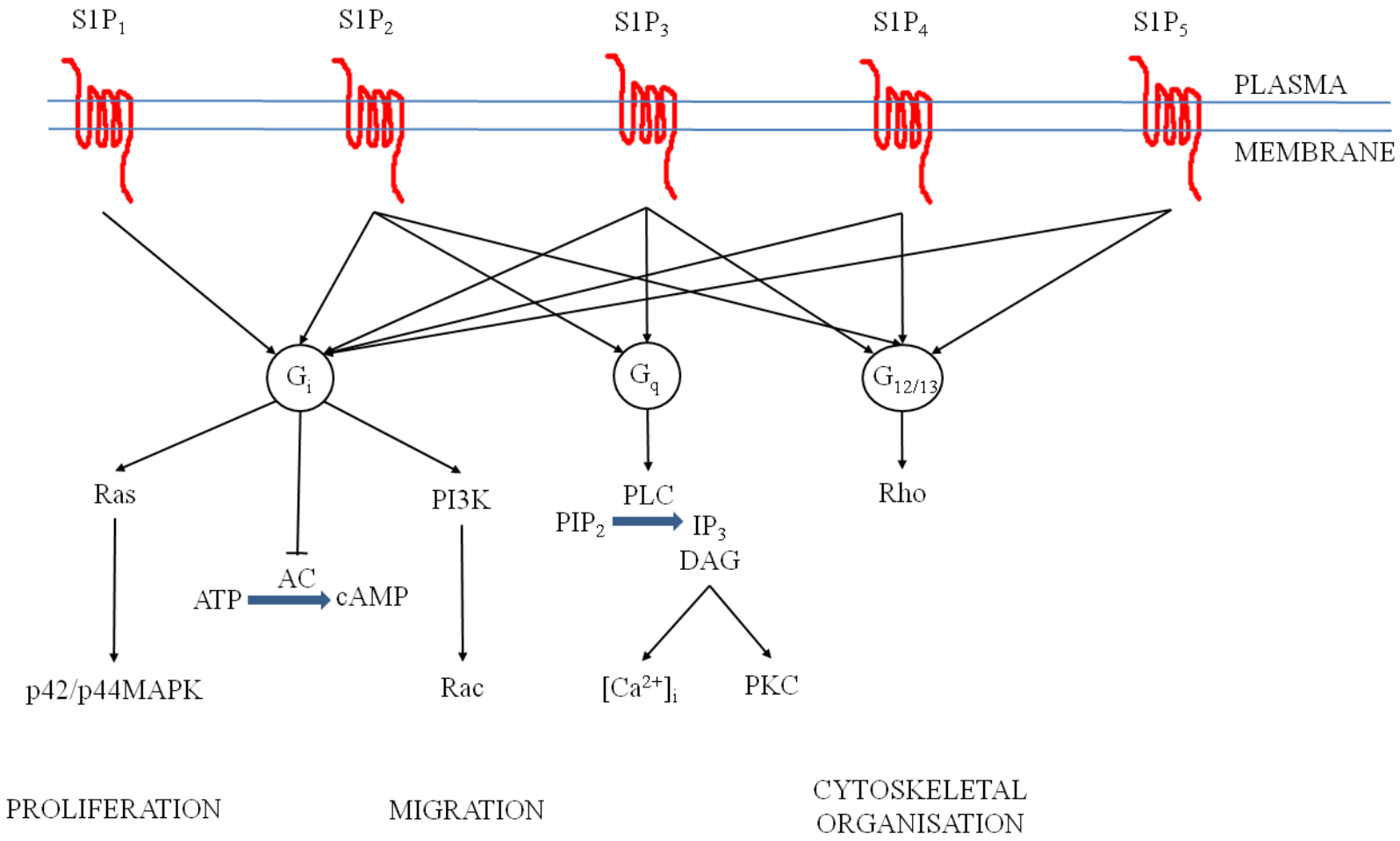

Figure 1.5 Summary of the G protein coupled receptor signalling pathways activated by S1P receptors (adapted from Spiegel & Milstein, 2002).

1.4.3.4 **The $S1P_1$ receptor**

$S1P_1$ (formerly EDG-1) was the first member of the S1P receptor family to be cloned (Hla, 1990). The *edg-1* gene encoded an inducible GPCR homologue that was induced *in vitro* during the phorbol ester-differentiation of human umbilical vein endothelial cells. The gene encoded a 380 amino acid protein containing seven membrane spanning domains with an apparent molecular mass of ~43kDa (Lee *et al*., 1998). Physical interaction of $S1P_1$ with G_i proteins was demonstrated when $S1P_1$ co-precipitated with $G\alpha_i$ polypeptides in human embryonic kidney 293 (HEK293) cells (Lee *et al*., 1998). Also, in transfected HEK293 cells, all four PTX sensitive Gα subunits, α_{i1}, α_{i2}, α_{i3}, and α_o, associate with the third intracellular loop of $S1P_1$ in a GTPγS-sensitive manner (Lee *et al*., 1998). $S1P_1$ induced activation of p42/p44 MAPK in a pertussis toxin (PTX) sensitive manner confirming that $S1P_1$ couples to the G protein G_i (Lee *et al*., 1998). PTX catalyses the ADP ribosylation of G protein α subunits (G_i, G_o, G_t and G_{gus}). This causes the G protein to uncouple from the receptor. Only G_i is expressed in every mammalian cell so it is accepted that activation of p42/p44 MAPK that is blocked by PTX is through G_i in most cell lines.

Later studies revealed that the ligand for $S1P_1$ and the factor that caused HEK293 cells overexpressing $S1P_1$ to differentiate was a lipid present in the lipid fraction of foetal bovine serum. A number of serum-borne lipids were tested including sphingosine, sphingomyelin, ceramide, ceramide-1–phosphate, lysophosphatidylserine, lysophosphatidyl ethanolamine, lysophosphatidylinositol, lysophosphatidylcholine, leukotriene B4 and C4, platelet activating factor, anandamide, 12-hydroxyeicosatetraenoic acid (HETE), 15-HETE, and 13-hydroxydodecanoic acid, at concentrations as high as 50mM but were ineffective at inducing morphogenesis in $S1P_1$ transfected HEK293 cells or displacing [^{32}P]S1P binding to $S1P_1$ in radioligand binding assays (Lee *et al*., 1998). However, S1P induced morphogenesis at doses between 1μM and 20μM and showed to be a high affinity ligand for $S1P_1$, binding to $S1P_1$ with a dissociation constant of ~8 nM (Lee *et al*, 1998). Dihydro-sphingosine-1-phosphate also successfully displaced radiolabelled S1P in dose dependent manner but with lower affinity (Van Brocklyn

et al., 1998). Further studies have excluded other structurally related lipids as S1P ligands. Lipids such as sphingosine, the nonhydrolyzable analogue, SPP-phosphonate, a short chain SPP analogue (C8-SPP), *N,N*-dimethylsphingosine, C2-ceramide, sphingosylphosphorylcholine (SPC), and *N*-octanoyl ceramide-1-phosphate (C8-cer-1-P), did not compete with S1P for binding to $S1P_1$ (Van Brocklyn *et al*., 1998).

Studies have shown that S1P via $S1P_1$ can activate various intracellular effectors including p42/p44 MAPK, a critical pathway in regulating DNA synthesis and cell proliferation. Lee *et al*., (1998) transfected HEK293 cells with $S1P_1$ which resulted in an increased and sustained p42/p44 MAPK activity. Treatment of cells with PTX blocked the *edg-1* dependent p42/p44 MAPK activation suggesting a G_i dependent effect (Wu *et al*., 1995; Rahkit *et al*., 1999). In NIH3T3 cells, p42/p44 MAPK activity was also increased in response to $S1P_1$ transient transfection, as was phospholipase A_2, an intracellular effector known to be phosphorlyated by p42/p44 MAPK (Lee *et al*., 1996). Zondag *et al*., (1998) also reported an S1P induced G_i dependent, increase in p42/p44 MAPK in COS-7 cells. In CHO cells transiently transfected with an expression vector for $S1P_1$, S1P induced a 25-fold increase in p42/p44 MAPK activity which was abrogated by pre-treatment with PTX (Okamoto *et al*., 1998). Addition of genistein, a tyrosine kinase inhibitor, and a dominant negative form of Ras also reduced p42/p44 MAPK activity (Okamoto *et al*., 1998). Also, in $S1P_1$ transfected CHO cells, S1P induced an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) as a result of mobilization of Ca^{2+} from both intracellular and extracellular pools. Similarly, in human erythroleukaemia (HEL) cells stably expressing $S1P_1$, S1P induced $[Ca^{2+}]_i$ was PTX sensitive. Overexpression of $G\alpha_t$, a $\beta\gamma$ scavenger, resulted in reduced $[Ca^{2+}]$ mobilization suggesting that $[Ca^{2+}]_i$ increases are mediated by $G\beta\gamma$ (Okamoto *et al*., 1998). Furthermore, in CHO cells stably expressing $S1P_1$, S1P induced an increase in IP_3 production mediated by phospholipase C (PLC) and inhibited forskolin induced cAMP production, in a PTX toxin, G_i dependent manner (Okamoto *et al*., 1998; Van Brocklyn *et al*., 1998). S1P-induced p42/p44 MAPK activation was resistant to down regulation of PKC by prolonged treatment with the phorbol-ester, PMA, and also the PKC inhibitor, GF109203X. It has been proposed that the effects of $S1P_1$ can also be mediated

through G_i independent pathways. This is due to the fact that S1P induced morphogenetic differentiation and increased P-cadherin production in $S1P_1$ transfected HEK293 cells, which was resistant to PTX and the Rho GTPase inhibitor, C3 exotoxin, suggesting Rho independence (Lee *et al*., 1998).

Ligand binding to a GPCR can induce receptor internalisation. COS-1 cells transfected with $S1P_1$ fused with a COOH-terminal green fluorescent protein ($S1P_1$-GFP) were treated with S1P which resulted in translocation of the receptor from the plasma membrane into intracellular vesicles (Lee *et al*., 1998). The $S1P_1$ containing intracellular vesicles are distinct from mitochondria but colocalise with endocytic vesicles and lysosomes and are recycled back to the plasma membrane ($t_{1/2}$~30minutes). Trafficking of the $S1P_1$ receptor in response to S1P involves the C-terminal of the receptor (Liu *et al*., 1999).

$S1P_1$ also induces activation of the PI3K/Akt pathway, needed for the activation of Rac which is critical in mediating cortical actin assembly and chemotaxis (Lee *et al*., 2001). Another study showed that S1P-dependent activation of p42/p44 MAPK via $S1P_1$ was inhibited by the PI3K inhibitors, LY294002 and wortmannin (Rahkit *et al*., 1999). In response to S1P, Ras followed by p42/p44 MAPK and phospholipase C (PLC) are also activated via the $\beta\gamma$ subunit of G_i. The α subunit of G_i mediates the inhibition of adenylyl cyclase (Taha *et al*, 2004). *In vitro* binding studies have failed to detect the interaction of $S1P_1$ with other G proteins including G_s, G_q, or $G_{12/13}$ (Windh *et al*., 1999).

The binding site for S1P in the $S1P_1$ receptor is highly specific as structurally similar lipids such as sphingosine, ceramide-1-phosphate and LPA demonstrate low binding towards $S1P_1$ (Van Brocklyn *et al*., 1998). Ligand binding studies have reported that the basic amino acids Arg^{120} and Arg^{292} interact with the phosphate group of S1P whereas the basic amino acid Glu^{121} pairs with the ammonium ion in the S1P backbone (Parrill *et al*., 2000).

$S1P_1$ is widely expressed. mRNA transcript for S1P has been detected in mouse and rat heart, brain, lung, thymus, kidney, spleen, skin, uterus, testes and liver (Chae *et*

al., 2004) . During embryonic development, $S1P_1$ is expressed in the forebrain and heart and high expression is detected in the dorsal aorta, intersomitic arteries and capillaries (Chae *et al*., 2004). In mouse embryonic brain, $S1P_1$ was localised adjacent to ventricles and $S1P_{1-3}$ co-localised with vascular endothelial markers suggesting it influences angiogenesis in the developing brain (McGiffert *et al*., 2002).

The $S1P_1$ receptor is physiologically essential as $S1P_1$ knock out models ($S1P^{(-/-)}$) die *in utero* (E12.5-E14.5) due to extensive intraembryonic haemorrhage. Although angiogenesis and vasculogenesis are normal, null mice lack blood vessel maturation as vascular smooth muscle cells and pericytes fail to migrate towards and reinforce vessel walls (Liu *et al*., 2000). These results implicate $S1P_1$ is required for blood vessel formation and further analysis of endothelial specific $S1P_1$ receptor knock out mice show the $S1P_1$ receptor functions within endothelial cells to regulate vascular smooth muscle cell coverage and correlate with expression of $S1P_1$ in the vascular system (Allende *et al*., 2003). MEFs derived from $S1P^{-/-}$ embryos, exhibit a decreased migratory response to S1P and do not activate Rac (Anliker & Chun, 2004; Yang *et al*., 2002).

Abnormal limb development was also observed in $S1P_1$ null mice and in endothelial specific null mice. Regulation of limb development by $S1P_1$ may occur via the hypoxia/vascular endothelial growth factor (VEGF) axis (Yang *et al*., 2002). Deletion of $S1P_1$ in T cells, has demonstrated that $S1P_1$ also has a role in lymphocyte trafficking and recirculation (Anliker & Chun, 2004; Kono *et al*., 2004). In adult tissues, $S1P_1$ is expressed in the endothelium of the lungs, heart (cardiomyocytes) and liver (hepatocytes), with strong expression detected in the pulmonary smooth muscle cell layers (Chae *et al*., 2004). Most capillaries and endothelial cells in the pulmonary artery and veins also express the receptor where it may have a role in maintenance of the vascular system. In addition, high levels of $S1P_1$ were detected in the adult brain (Chae *et al*., 2004). In the immune system, $S1P_1$ has been shown to be involved in lymphocyte trafficking (Allende *et al*., 2000). Deletion of $S1P_1$ from T cells showed that $S1P_1$is crucial in regulating mature T cell egress from the thymus to the blood and peripheral lymphoid organs (Choi *et al*., 2008).

1.4.3.5 **The $S1P_2$ and $S1P_3$ receptors**

$S1P_2$ (or H218/AGR16, names given independently to the same rat gene) was isolated from rat aortic smooth muscle by degenerate PCR (polymerase chain reaction) and proposed to be a growth factor receptor (Okazaki *et al.*, 1993; MacLennan *et al.*, 1994). $S1P_2$ is encoded on a single exon and contains 352 amino acids, with a molecular weight of ~39 kDa. S1P binds to $S1P_2$ specifically and with high affinity (Gonda *et al.*, 1999). In radiolabelled ligand binding assays, other lipids such as sphingosine, sphingomyelin, ceramide, lysophosphatidic acid, lysophosphatidylinositol and phosphatidic acid failed to compete for specific binding of [^{32}P]S1P to $S1P_2$ receptor. Only the structurally related sphingolipid, sphingosylphosphorylcholine (SPC) displaced [^{32}P]S1P but with lower affinity (Gonda *et al.*, 1999).

Studies have shown $S1P_2$ expression is widespread and present in heart, lung, brain, liver, kidney and spleen (Ishii *et al.*, 2004). Analysis of $S1P_3$ RNA by Northern blot has shown it is highly expressed in the heart, liver, placenta and kidney (Yamaguchi *et al*., 1996).

Functional data for $S1P_2$ indicates receptor coupling to G_i due to the fact that S1P-induced activation of p42/p44 MAPK in $S1P_2$ transfected CHO cells (CHO-$S1P_2$) was abolished by PTX (Gonda *et al.*, 1999). The expression of a dominant negative Ras in CHO-$S1P_2$ cells, inhibited p42/p44 MAPK activity by more than 80% indicating S1P-induced p42/p44 MAPK is Ras dependent (Gonda *et al*., 1999). However, downregulation of PKC by prolonged PMA treatment only minimally inhibited p42/p44 MAPK activation. In addition, S1P caused an increase in AC, JNK and p38, both of which were PTX insensitive (Gonda *et al*., 1999). $S1P_2$ also communicates via G_q and $G_{12/13}$ (Windh *et al*., 1999). $S1P_2$ couples to G_q resulting in PTX-insensitive increases in intracellular calcium in TAg-Jurkat cells and HTC4 hepatoma cells (An *et al*., 1999). Treatment of cells with the phospholipase C inhibitor, U73122, resulted in inositol phosphate formation and complete inhibition of Ca^{2+} formation suggests that $S1P_2$ mediated Ca^{2+} increases are PLC dependent (An *et al.*, 1999).

$S1P_2$ couples to $G_{12/13}$ to induce serum response element (SRE) in $S1P_2$ transfected TAg-Jurkat cells, which was sensitive to the Rho GTPase inhibitor and partially sensitive to PTX (An *et al.*, 2000). In CHO cells overexpressing $S1P_2$, $S1P_2$ stimulated the small GTPase Rho and Rho dependent stress fibre formation via $G_{12/13}$ (Okamoto *et al.*, 2000).

Interestingly, $S1P_2$ is unable to activate Rac in response to S1P treatment. Okamoto *et al.*, 2000, reported that stimulation of $S1P_2$ by S1P inhibited the IGF-induced activation of Rac resulting in inhibition of migration. $S1P_2$ mediated inhibition of Rac activity involved stimulation of Rac-GAP activity (Okamoto *et al.*, 2000). Rac-GAP activity results in switching of Rac from the active, GTP-bound from to the inactive GDP-bound form.

In addition, studies of the S1P receptors in human glioblastoma cell lines showed that the $S1P_2$ receptor inhibits glioblastoma cell migration (Lepley *et al.*, 2005). The study showed that cell migration was not inhibited using an S1P analogue that activated all S1P receptors except $S1P_2$ but overexpression of $S1P_2$ suppressed migration and knock down of $S1P_2$ by siRNA reverses the inhibitory effect. Further experiments showed this is mediated via a Rho kinase dependant pathway (Lepley *et al.*, 2005).

In zebra fish, studies on the mutant homolog of *s1p*$_2$, miles apart (the *mil* gene) revealed a role for $S1P_2$ in cardiac development due to formation of a bilateral heart on either side of the midline. Normally, cardiac muscle progenitor cells migrate from bilateral positions toward the dorsal midline and fuse to form a single heart tube. However, in the Mil mutant model, the myocardial precursor cells failed to migrate due to a mutation in the second intracellular loop of the receptor resulting in cardia bifida. The mutant receptors did not respond to S1P and failed to activate p42/p44MAPK (Kupperman *et al.*, 2000). This suggests $S1P_2$ is critical in cell migration.

$S1P_3$ was cloned by PCR using degenerate primers from cannabinoid type-1 receptor sequences and a full-length clone identified by human genomic DNA library screening (Yamaguchi *et al.*, 1996). The $S1P_3$ gene is encoded on a single exon and

both mouse and human $S1P_3$ are composed of 378 amino acid residues with a molecular weight of ~42kDa. The $S1P_2$ and $S1P_3$ genes are ~44% similar to each other and ~50% homologous to $S1P_1$. Like $S1P_2$, expression of $S1P_3$ is widespread, present in spleen, heart, lung, kidney, brain, liver and skeletal muscle (Yamaguchi *et al*., 1996). S1P displays high affinity for $S1P_3$ (K_d = 27nM). Other sphingolipids and lysolipids such as SPC, LPA, ceramide-1-phosphate, ceramide and sphingosine only poorly displaced [^{32}P]S1P. Only dihydro-S1P successfully displaced [^{32}P]S1P (Van Brocklyn *et al*., 1999).

The G protein coupling and biological activity of $S1P_3$ is similar to that of $S1P_2$. $S1P_3$ communicates through G_i, G_q and $G_{12/13}$ (Ancellin & Hla, 1999). Coupling of $S1P_3$ to G_i activates PI3K, and Ras followed by p42/p44 MAPK (Okamoto *et al*., 1999; An *et al*., 1999). For $S1P_3$, G_q activates PLC, although a G_i component has also been implicated because it can be blocked by PTX, and induces inositol triphosphate (IP_3) turnover and increases intracellular Ca^{2+} (Kon *et al*., 1999; Ancellin & Hla, 1999). Unlike $S1P_2$, $S1P_3$ can couple to the small GTPase Rac (Okamoto *et al*., 2000). Exogenous expression of $S1P_3$ in CHO cells, induced Rac activation and p65 PAK (upstream of Rac), in response to S1P. $S1P_3$ also activates Rho through $G_{12/13,}$ leading to migration in response to S1P (Okamoto *et al*., 2000). siRNA knockdown of $S1P_3$ expression caused inhibition of stress fibre assembly and focal contact formation, which are processes regulated by the small GTPase Rho. Therefore, multiple cellular functions regulated by $S1P_3$ include migration, cytoskeletal organisation, adherens junction assembly and morphogenic differentiation of HUVEC cells (Lee *et al*., 1999). Both $S1P_2$ and $S1P_3$ mediate S1P stimulation of Rho. However, $S1P_2$ but not $S1P_3$ mediates downregulation of Rac activation, membrane ruffling and cell migration in response to chemoattractants (Okamoto *et al*., 2000). Recent studies suggest that the balance of counteracting signals from the G_i- and the $G_{12/13}$-Rho pathways directs either positive or negative regulation of Rac and cell migration (Sugimoto *et al*., 2003).

Assessment of $S1P_2$ and $S1P_3$ *in vivo* has come from the generation of knock out mouse models (Kono *et al*., 2004). Both $S1P_2$-null, S1P3-null and $S1P_2$/$S1P_3$ double null models have been generated (Ishii *et al*., 2001; Ishii *et al*., 2002). Neither the

$S1P_2$ nor $S1P_3$ mutation is embryonic lethal or have any abnormalities in appearance, gross anatomy or nervous system development (Ishii *et al.*, 2002). A slight but significant decrease in litter size was observed which was more prominent in $S1P_2$/$S1P_3$ double knockouts. Some $S1P_2$ nulls had seizures between 3 and 7 weeks, which were sometimes lethal and some showed neuronal hyperexcitability. In $S1P_2^{-/-}$ MEFs, there was a decrease of S1P induced Rho activation but no effect on PLC activation, Ca^{2+} mobilisation and AC inhibition.

The $S1P_3$ null mice had no major phenotype but $S1P_3$ MEFs showed decreased PLC activation and slightly decreased adenylyl cyclase inhibition after S1P stimulation (Ishii *et al.*, 2001). Multiple knockouts revealed that the $S1P_1$ and $S1P_2$ double null and $S1P_1$, $S1P_2$ and $S1P_3$ triple null embryos had a more severe vascular phenotype compared to the $S1P_1$ null embryos. Partial embryonic lethality and vascular abnormalities were also seen in a $S1P_2$, $S1P_3$ double knockout model (Kono *et al.*, 2004) and a complete loss of Rho activation by S1P. This suggests that $S1P_{1-3}$ receptors have redundant or cooperative functions during embryonic angiogenesis.

1.4.3.6 **The $S1P_4$ receptor**

The $S1P_4$ receptor (previously EDG6) was first isolated and characterised in human and mouse dendritic cells and found to be predominately expressed in lymphoid and haematopoetic tissue including the spleen, bone marrow, thymus, lymph node, appendix, lung and peripheral leucocytes (Grahler *et al.*, 1998). $S1P_4$ has been shown to be a receptor for S1P (Van Brocklyn *et al.*, 2000). A variety of S1P binding affinities have been reported for $S1P_4$, ranging from 10-20nM (Yamazaki *et al.*, 2000), 50-70nM (Van Brocklyn *et al.*, 2000) and 10-150nM (Mandala *et al.*, 2002). In [^{32}P]S1P radioligand binding assays, SPC competed for binding to $S1P_4$ but only at high concentrations (Van Brocklyn *et al.*, 2000). Two natural S1P analogues, dihydrosphingosine-1-phosphate (Dh-S1P) and phytosphingosine-1-phosphate (Ph-S1P) are also recognised as high affinity $S1P_4$ receptor ligands. Ph-S1P is generated by the phosphorylation of phytosphingosine which is found abundantly in fungi, plants and man (Dickson, 1998). It is structurally similar to S1P although it has

hydroxyl group at C4 of the sphingoid long chain base whereas S1P has a *trans*-double bond between C4 and C5. Again, dihydro-S1P is structurally similar to S1P but lacks the *trans* double bond of S1P [**Figure 1.6**]. Ph-S1P has been reported to be the highest affinity ligand for $S1P_4$ (Candelore *et al*., 2002) although a more recent study has reported that Dh-S1P binds to $S1P_4$ with slightly higher affinity than Ph-S1P (Fossetta *et al*., 2004). It is clear however that both Dh-S1P and Ph-S1P bind $S1P_4$ with higher affinity then S1P, a noticeable difference from other S1P receptors. This may be due to differences in the binding pocket shape and electrostatic distributions between $S1P_4$ and other S1P receptors. In regard, the $S1P_4$ receptor lacks a cationic residue in the seventh transmembrane domain but has additional interactions with the S1P polar head group not observed in the $S1P_1$ receptor (Inagaki *et al*., 2005).

$S1P_4$ is again encoded on a single exon and contains 384-386 amino acids with a molecular mass of ~42kDa (Graler *et al*., 1998). $S1P_4$ couples to G_i and $G_{12/13}$ to initiate downstream signalling pathways, but not to G_q (Graler *et al*., 2003). $S1P_4$ activated p42/p44 MAPK in both HEK293 and CHO cells transfected with $S1P_4$, which was sensitive to PTX treatment indicating a G_i dependent response. Activation of p42/p44 MAPK resulted in activation of the transcription factor Elk1 (Van Brocklyn *et al*., 2000). CHO cells ectopically expressing $S1P_4$, activated the small GTPase Rho, induced cytoskeletal rearrangements, stress fibre formation and cell rounding in response to S1P (Graler *et al*., 2003). Overexpression of $S1P_4$ in Jurkat and CHO cells induced S1P-dependent cell motility which was suppressed by PTX, suggesting a G_i mediated response (Graler *et al*., 2003; Kohno *et al*., 2003). In mouse fibroblasts, Ph-S1P activates p42/p44MAPK and p38 kinase via G_i and stimulates an intracellular calcium release via PLC (Kim *et al*., 2007). Binding of S1P to $S1P_4$ induced internalisation of the receptor. S1P also induced transient activation of Cdc42 but not Rac, in $S1P_4$ transfected CHO cells, in a PTX sensitive manner. Activation of Cdc42 was required for S1P-induced migration of $S1P_4$ expressing CHO cells, as demonstrated by suppressed migration with a dominant-negative Cdc42 (Kohno *et al*., 2003). It has also been reported that S1P induces cell migration of mouse T cells which is inhibited by PTX which involves activation of both Rac

and Cdc42 (Matsuyuki *et al.*, 2006). In $S1P_4$ transfected CHO cells, S1P caused an increase in cytosolic Ca^{2+}, associated with a significant accumulation of inositol phosphate, reflecting activation of PLC. This effect was suppressed by PTX treatment suggesting involvement of G_i (Yamazaki *et al.*, 2000).

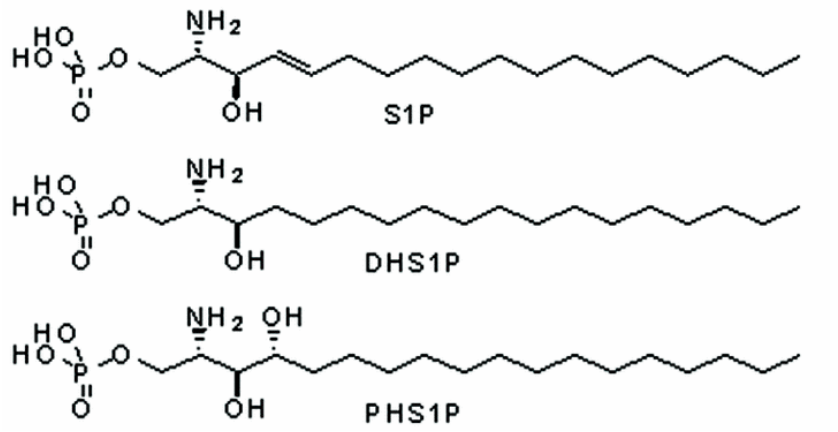

Figure 1.6 Structure of S1P, Dh-S1P and Ph-S1P.

1.4.3.7 **The $S1P_5$ receptor**

$S1P_5$, the most recent member of the S1P receptor family to be cloned, was originally isolated as an orphan GPCR from rat pheochromocytoma cells and termed *nrg-1* as its mRNA was downregulated by nerve growth factor (Glickman *et al.*, 1999). The nrg-1 cDNA encoded a protein of 398 amino acids with a molecular mass of 42kDa displaying a restricted expression profile, predominately expressed in brain, (Glickman *et al.*, 1999). In contrast to the other S1P receptors, $S1P_5$ transcript was not detected in heart, liver, kidney or intestine (Im *et al.*, 2000). High expression of $S1P_5$ was detected in both immature and mature oligodendrocytes, which are the myelin forming cells of the central nervous system (Jaillard *et al.*, 2005). Northern blot analysis has revealed that human $S1P_5$, as opposed to rat $S1P_5$ is an alternatively spliced gene, with a 5.4kb transcript that is primarily expressed in peripheral tissues, and a 2.4kb transcript, expressed in brain, spleen and peripheral blood leukocytes (Niedernberg *et al.*, 2002). $S1P_5$ is also highly expressed in human natural killer (NK) cells suggesting that signalling mediated by this receptor has a role in the immune system (Walzer *et al.*, 2007).

$S1P_5$ is a high affinity S1P receptor. S1P (and Dh-S1P) was shown to bind to $S1P_5$ with an EC_{50} of 90nM (Im *et al.*, 2000). In addition, SPC may function as a weak agonist for $S1P_5$ (Im *et al.*, 2000; Malek *et al.*, 2001). The $S1P_5$ receptor is linked to G_i and $G_{12/13}$, but not G_q or G_s (Im *et al.*, 2000; Malek *et al.*, 2001). Co-injection of Xenopus oocytes with $S1P_5$ RNA together with chimaeric α_{qi} or α_{qo}, but not with α_{qs} or α_q RNA, induced intracellular calcium transients (Im *et al.*, 2000), leading to the conclusion that $S1P_5$ couples to G_i/G_o but not to G_q or G_s. In CHO cells overexpressing $S1P_5$, S1P inhibited forskolin induced cAMP and p42/p44 MAPK signalling but activated c-Jun NH_2-terminal kinase (JNK). PTX toxin inhibited cAMP accumulation but had no effect on JNK activation or p42/p44 MAPK inhibition, suggesting lack of G_i signalling for the latter (Malek *et al.*, 2001). In these cells, S1P had no effect on phosphoinositide turnover, consistent with the lack of G_q signaling. However, the S1P-dependent inhibition of p42/p44 MAPK via $S1P_5$ was diminished by a tyrosine phosphatase inhibitor suggesting phosphatases may be downstream of $S1P_5$. S1P also inhibited proliferation in $S1P_5$-overexpressing cells,

which was also PTX insensitive but abolished by a tyrosine phosphatase inhibitor (Malek *et al*., 2001). $S1P_5$ is constitutively active and internalised in response to S1P (Niedernberg *et al*., 2003). Transfection of various cell lines with $S1P_5$ resulted in cell rounding which was accentuated in the presence of S1P (Niedernberg *et al*., 2003).

$S1P_5$-null mice developed normally and were fertile (Jaillard *et al*., 2005). They had no apparent behavioural deficit and analysis of $S1P_5$-null brain showed no evidence of myelin deficiency (Jaillard *et al*., 2005; Choi *et al*., 2008). S1P activation of $S1P_5$ in pre-oligodendrocytes induced process retraction via a Rho kinase signalling pathway, which was not observed in pre-oligodendrocytes derived from $S1P_5^{(-/-)}$ mice (Jaillard *et al*., 2005). However in mature oligodendrocytes, $S1P_5$ promoted cell survival mediated by a PTX sensitive, Akt dependent mechanism (Jaillard *et al*., 2005).

Human $S1P_5$ also contains a nuclear localisation sequence (NLS) in its third cytoplasmic loop, suggesting it could be localised to the nucleus. It has recently found to be localised in the centrosome of HEK293, PC12 and MEF cells, alongside SphK1 and SphK2, where it possibly has a role in G protein dependent spindle fibre formation and mitosis, by functioning as a guanine-nucleotide exchange factor (GEF) (Gillies *et al*., 2009).

1.5 Platelet derived growth factor (PDGF) receptor

Recently, there has been much interest in cross talk between receptor tyrosine kinases and GPCRs. Of interest to the current study is the recently discovered crosstalk between S1P and platelet derived growth factor (PDGF) receptors. In view of this, a concise review of the PDGF receptor and S1P-PDGF receptor crosstalk is presented.

Platelet derived growth factor (PDGF) is a potent serum mitogen, identified over thirty years ago as a growth promoting substance in human platelets (Kohler &

Lipton, 1974). PDGF is synthesised by a variety of cells types including fibroblasts, keratinocytes, kidney mesangial cells and smooth muscle cells and has broad specificity. The cellular effects of PDGF include mitogenesis and cell migration in cells from mesenchyml origin, including fibroblasts and smooth muscle cells (Heldin & Westermark, 1999). The PDGF family of growth factors consists of four polypeptide chains termed PDGF-A, PDGF-B, PDGF-C and PDGF-D, which are the products of four different genes (Fredricksson *et al*., 2004). PDGF-A and PDGF-B have been studied since the late 1970's whereas PDGF-C (Li *et al*., 2000) and PDGF-D (Bergsten *et al*., 2001) were identified recently. PDGF exists as a homo- or hetero- dimeric molecule consisting of two polypeptide chains linked by disulphide bonds. Five different dimeric isoforms have been described termed PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. The A and B chains of PDGF exist as precursor molecules that are modified in the NH_2 terminus and the COOH terminus (B chain only) by proteolysis resulting in activation (Heldin & Westermark, 1999). For PDGF-C and PDGF-D, the NH_2 terminus contains a distinct hydrophobic domain with a presumed peptidase cleavage site (Li *et al*., 2000; Bergsten *et al*., 2001). PDGF-A exists as polypeptides of 196 and 201 amino acid residues, due to alternative splicing, whereas PDGF-B is a longer 241 amino acid polypeptide. PDGF-C and PDGF-D are polypeptides containing 345 and 370 residues, respectively. Common to all PDGF isoforms is the growth factor domain, comprising 100 amino acids and involved in dimerisation of two subunits, receptor binding and activation (Claesson-Welsh, 1994).

The PDGF isoforms exert their effects on target cells by activating two structurally related protein tyrosine kinase receptors known as PDGF α- and β- receptors [**Figure 1.7**]. The PDGF receptor isoforms consist of a single transmembrane domain located near the middle of the polypeptide. A split tyrosine kinase domain is located in the intracellular portion of the receptor, consisting of two parts separated by a hydrophilic, non catalytic 100 amino acid residue portion (Claesson-Welsh, 1994). The extracellular section contains five immunoglobulin-like domains. The α-receptor isoform binds both PDGF-A and PDGF-B chains with high affinity, whereas the β-

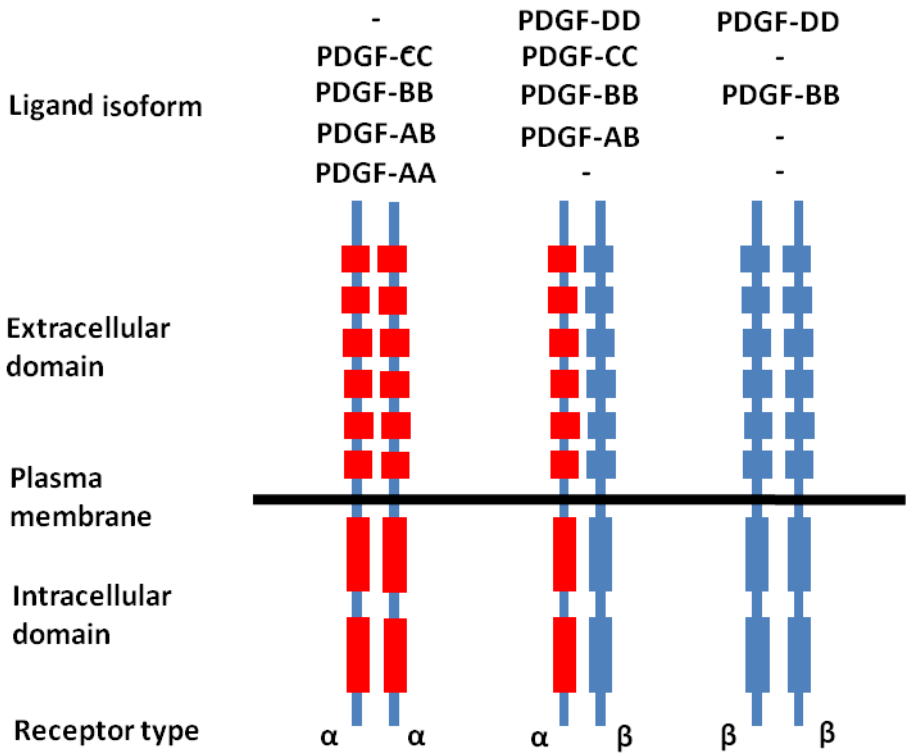

Figure 1.7 Structure of PDGF receptors-αα, -αβ and -ββ and PDGF isoform binding (adapted from Andrae *et al*., 2008; Claesson-Welsh, 1994).

receptor only binds the PDGF-B chain with high affinity (Fretto *et al.*, 1993). Binding of PDGF induces receptor dimerisation. Therefore, PDGF-AA induces PDGFR-αα homodimers, PDGF-AB induces PDGFR-αα homodimers or PDGFR-αβ heterodimers and PDGF-BB induces all three combinations (Fretto *et al.*, 1993). PDGF-CC resembles PDGF-AB in that it interacts with PDGFR-αα and PDGFR-αβ but not with PDGFR- ββ. PDGF-DD induces both PDGFR-αβ and PDGFR-ββ dimmers.

Binding of dimeric ligand induces homo- or hetero-dimerisation of PDGF receptor isoforms, thought to be essential for receptor activation (Heldin *et al.*, 1996). Dimerisation juxtaposes the intracellular domains of the receptor dimers, allowing transphosphorylation of tyrosine residues between the receptor dimers. This is known as autophosphorylation. Autophosphorylation of tyrosine residues outside the kinase domain and subsequent conformational changes within the receptor creates docking sites for signal transduction molecules containing *src*-homology type-2 (SH2) domains.

The SH2 domain is a phosphorylated tyrosine residue binding domain, containing a conserved motif of ~100 residues. It serves to mediate interactions between different components in signalling pathways. Other domains exist on signalling molecules that perform the same function. Phosphotyrosine binding (PTB) domains also recognise phosphorylated tyrosine residues but differs from SH2 in structure and mechanism, whereas *src*-homology type-3 (SH3) domains recognise proline rich sequences. Pleckstrin homology (PH) domains bind phosphoinositides with high affinity and PDZ domains only COOH-terminal valine residues (Heldin & Purton, 1998). Proteins containing SH2 domains can either be enzymes or adaptor or linking proteins that posses no enzymatic activity. Enzymes that contain SH2 domains include PI3 kinase, PLC-γ, the Src tyrosine kinase and its related family members, the tyrosine phosphatase SHP-2 and Ras GTPase (Heldin & Purton, 1998). Other intracellular signalling proteins recruited to the PDGF receptors via SH2 domains include Shc (Src-homology-2-containing) protein, Sos (Son of sevenless), Grb (growth factor receptor bound 2), Grb7 (growth factor receptor bound 7), NCK1 (NCK adaptor protein 1) and Crk (Heldin & Westermark, 1999). These proteins have

no enzymatic activity but function as adaptors to link PDGF receptors to downstream effectors. Binding of an SH2 domain protein to the PDGF receptor initiates a signal transduction pathway [**Figure 1.8**]. Binding of Src to the PDGF receptor activates Src, which consequently activates Ras, Raf, MEK1 and p42/p44 MAPK and is thought to be involved in the mitogenic response of PDGF (Funa & Uramoto, 2003). Several isoforms of PI3-kinase interact with and are activated by PDGF receptors and activate several downstream molecules including Akt/PKB, PKC family and Rho GTPase (Heldin *et al*., 1998). The two forms of PLC-γ (PLC-γ1 and PLC-γ2), containing two SH2 and one SH3 domain are activated by PDGF, catalysing the production of IP_3, $[Ca^{2+}]_i$ and DAG resulting in PKC activation. Grb2, an adaptor molecule with one SH2 and two SH3 domains binds to Sos, a nucleotide exchange factor that activates Ras. Grb2 can bind to PDGF receptors directly or indirectly via another adaptor protein, Shc. PDGF activation of Ras via Grb2 and Shc activates the p42/p4 MAPK signalling cascade controlling downstream cellular responses such as stimulation of cell migration, migration and differentiation (Heldin & Westermark, 1999).

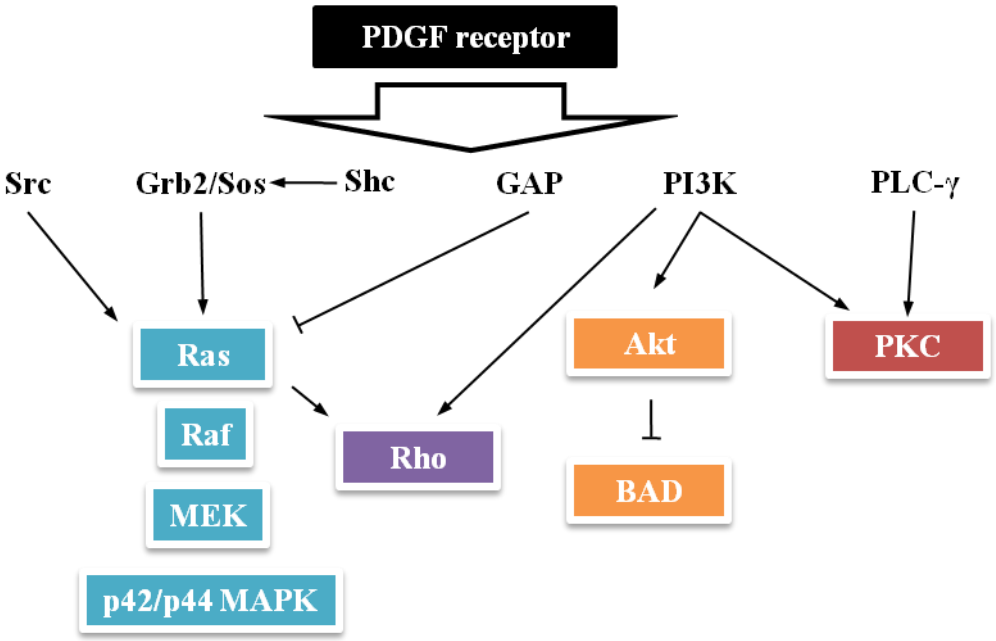

Figure 1.8 SH2 domain proteins that bind to PDGF receptors and initiation of downstream signalling components (adapted from Heldin *et al*., 1998).

1.6 Integrated signalling between S1P and PDGF receptors

An emerging concept of S1P receptor biology is cross talk or integrative signalling with tyrosine kinase receptors, which functions to enhance growth factor signalling (Waters *et al*., 2004). Integrative signalling between the $S1P_1$ receptor and the PDGFβ has been recently characterised (Pyne & Pyne, 2008). An indication of possible cross talk between these receptors was highlighted from gene knockout studies. As described previously, deletion of the *s1p1* gene in mice caused embryonic lethality due to incomplete vascular maturation (Liu *et al*., 2000b). Disruption of the *pdgfβ* gene in mice resulted in a similar phenotype, which suggested cross talk between these two receptors in regulating smooth muscle migration and vascular maturation (Lindhal *et al*., 1997). Experiments by Hobson *et al*., (2001) using HEK293 cells and mouse embryonic fibroblasts, demonstrated a connection between the $S1P_1$ receptor and PDGF by showing that cell motility in response to PDGF was dependent on the $S1P_1$ receptor. They demonstrated that PDGF promotes production and release of S1P, which binds to $S1P_1$ receptors in an autocrine or paracrine manner, inducing downstream activation of Rac and cell motility [**Figure 1.9**]. In this model, the activation of $S1P_1$ receptors is termed 'sequential' or 'inside-out' signalling (Hobson *et al*., 2001).

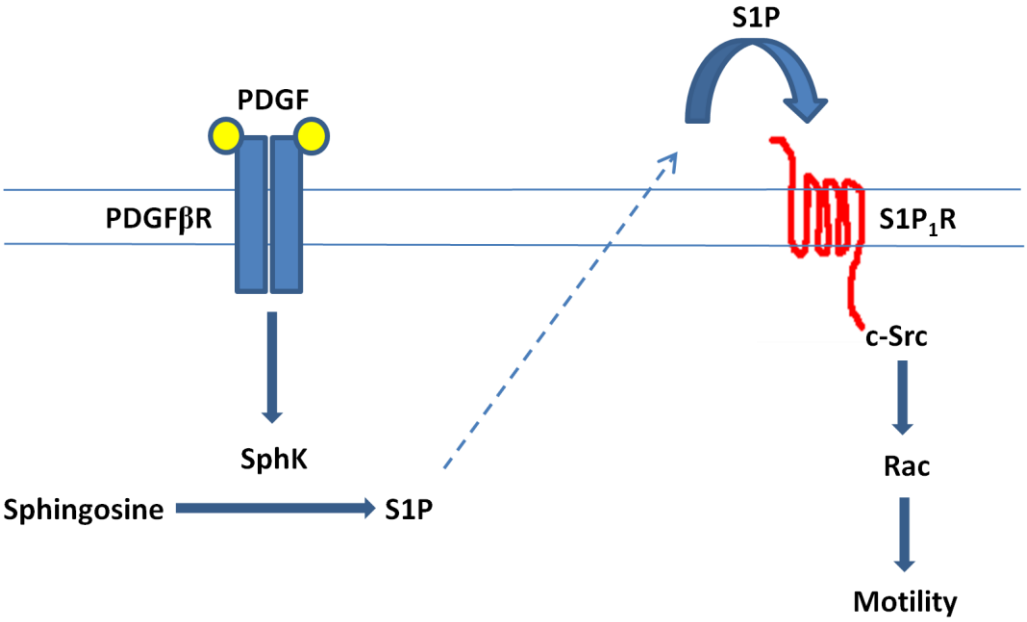

Figure 1.9 Sequential activation of $S1P_1$ receptor by PDGF-stimulated release of S1P (adapted from Hobson *et al*., 2001).

In the same year, a study by Alderton *et al*., revealed the relationship between the PDGFβ receptor and $S1P_1$ receptor and their downstream signals is more complex (Alderton *et al*., 2001; Pyne *et al*., 2003). Co-immunoprecipitation experiments revealed that there is a close interaction between the PDGFβR and $S1P_1$ receptor. The PDGFβ receptor could be co-immunoprecipitated with myc-tagged $S1P_1$ receptor using anti-PDGFβR- or anti-myc-tag antibodies in HEK 293 and airway smooth muscle (ASM) cells (Alderton *et al*., 2001; Waters *et al*., 2003). PDGF-dependent activation of p42/p44 MAPK was enhanced by co-expression of recombinant $S1P_1$ and PDGFβR compared to PDGFβ expression alone, suggesting the receptor tyrosine kinase and GPCR signals are integrated to produce more efficient p42/p44 MAPK stimulation. Overexpression of $S1P_1$ increased p42/p44 MAPK activation by PDGF and overexpression of PDGFβR increased stimulation of the p42/p44 MAPK pathway by S1P (Alderton *et al*., 2001). Further experiments revealed that in fact the GPCR-associated G proteins are downstream signalling intermediates of the PDGFβR based on the fact that (i) pre-treatment of $S1P_1$ and PDGFβ co-transfected HEK 293 cells with PTX, reduced the PDGF stimulation of p42/p44 MAPK (ii) transfection of cells with $G\alpha_i$ increased PDGF-dependent p42/p44 MAPK activation and (iii) PDGF stimulated the tyrosine phosphorylation of $G\alpha_i$, an effect blocked by the PDGF kinase inhibitor, AG1296 (Alderton *et al*., 2001). The clathrin adaptor protein, β-arrestin, was also found to be associated with the $S1P_1$-PDGFβR complex in HEK 293 cells. β-arrestin is recruited to ligand bound GPCRs that have been phosphorylated by the Ser/Thr protein kinase, GRK, whereby it initiates internalisation of receptor complexes containing Raf-1-MEK-1 and promotes p42/p44 MAPK activation. Subsequent experiments using immunofluorescence in ASM cells revealed that exogenous S1P and/or PDGF promoted endocytosis of $S1P_1$-PDGFβR complex into endocytic vesicles (Waters *et al*., 2003) [**Figure 1.10**]. The $S1P_1$-PDGFR complex was co-internalised by as a functional signalling unit, which co-localised with and regulated p42/p44 MAPK (Waters *et al*., 2003). Internalisation of the receptor complex, involves c-Src, regulated by Gβγ subunits (Waters *et al*., 2005). The endocytosis of the $S1P_1$-PDGFR complex, stimulation of MAPK and cell migration is reduced by a novel inverse

agonist of the $S1P_1$ receptor (SB649146), which blocks constitutive activity of $S1P_1$ and the function of the complex (Waters *et al.*, 2005a).

Other examples of crosstalk between S1P receptors and growth factor receptors include transactivation of the vascular endothelial growth factor 2/Flk-1/KDR receptor in endothelial cells by S1P. This induces endothelial nitric oxide synthase activation and cell motility (Tanimoto *et al.*, 2002). A recent study has reported cross communication between $S1P_2$ and PDGFR that negatively regulates PDGF induced migration, proliferation and SphK1 expression. Deletion of the $S1P_2$ receptor increases migration of MEFs and was dependant on expression of $S1P_1$ and sphK1. $S1P_2$ deletion prolonged PDGF dependent p38 phosphorylation and enhanced Rac activation (Goparaju *et al.*, 2005).

There is also cross talk between S1P and PDGFR that is mediated by $S1P_3$ and leads to Akt and p42/p44 MAPK activation in MEFs. In HEY cells, Akt and p42/p44 MAPK activation by S1P requires PDGFR to induce a G_i dependent tyrosine phosphorylation of PDGFR in these cells. These findings were also observed in other cell lines expressing $S1P_3$ but not in cell lines that do not express the receptor (Baudhuin *et al.*, 2004).

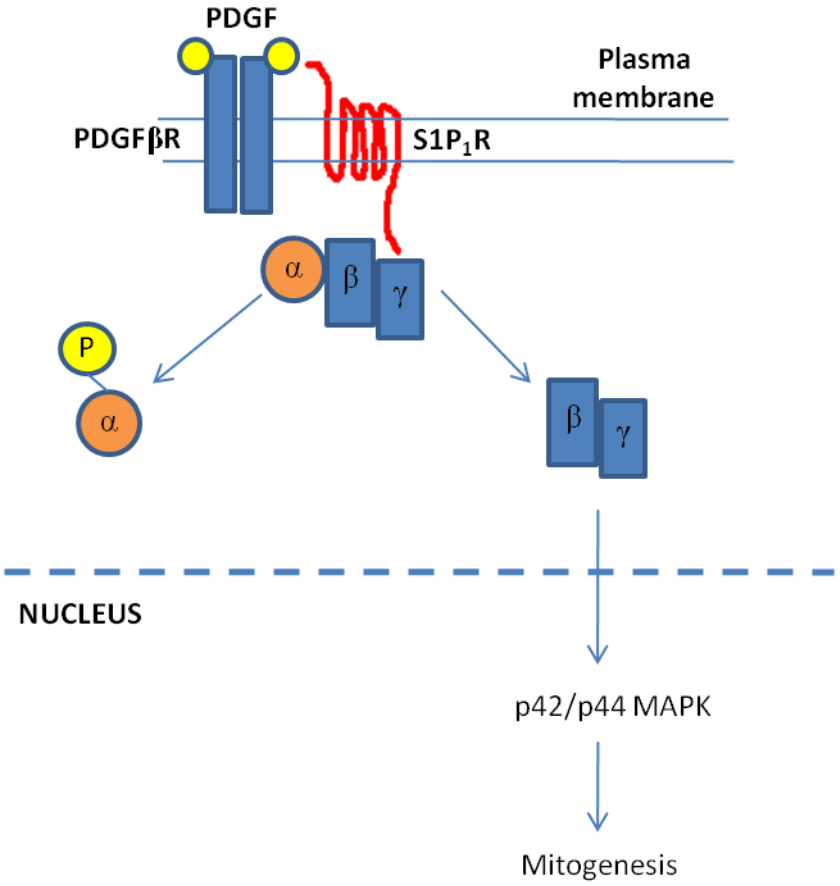

Figure 1.10 Integrative signalling between $S1P_1$ and PDGFβ receptor (adapted from Pyne & Pyne, 2008).

In rat aortic vascular smooth muscle cells (VSMC), S1P causes tyrosine phosphorylation of PDGFβR and EGFR mediated via $S1P_1$ receptors. This is essential for activation of PI3-kinase, p42/p44 MAPK and vascular smooth muscle cell proliferation, demonstrated using specific PDGFβR and EGFR tyrosine kinase inhibitors. S1P activated a PI3-kinase pathway that was blocked by PDGFβR and EGFR tyrosine kinase inhibitors. Activation of p42/p44 MAPK by S1P was only blocked by the EGFR tyrosine kinase inhibitor. In transfected CHO cells, S1P activated Akt and p42/p44 MAPK, a response that was enhanced by coexpression of PDGFβR or EGFR. VSMC migration by S1P was suppressed by both inhibitors (Tanimoto *et al.*, 2004).

1.7 Embryonic Stem (ES) Cells

1.7.1 Introduction

Embryonic stem (ES) cells are cells derived from the inner cell mass (ICM) of the preimplantation embryo or blastocyst. The blastocyst is a hollow ball of cells formed four days after fertilisation containing a population of undifferentiated cells, surrounded by an outer layer of cells (Martin, 1981). ES cells have been isolated from pre-implantation stage embryos of mouse (Martin, 1981), rabbit (Graves *et al.*, 1993), primate (Thomson *et al.*, 1995), pig (Li *et al.*, 2003) and human (Thomson *et al.*, 1998). Mouse ES (mES) cells were first isolated in 1981 (Martin *et al.*, 1981) and seventeen years later, human embryonic (hES) cells were first isolated by Thomson *et al.*, 1998. Cells isolated from the ICM of human embryos were cultured on inactivated murine embryonic fibroblasts (MEFs) and serially subcultured to generate the first human ES (hES) cell line. To date, 100 independent hES cell lines are known, seventeen of which have the possibility to be scaled up, differentiated and possibly used therapeutically. *In vitro,* they are self-renewing, pluripotent cells that proliferate extensively under specific culture conditions and maintain a normal euploid karyotype over extended culture (Martin *et al.*, 1981). ES cells are unique in that they can propagate symmetrically and asymmetrically and that they retain the ability to differentiate into derivatives of all three germ layers (endoderm, ectoderm and mesoderm) and under certain conditions differentiate into cells of different

lineages including cardiomyocytes, neural cells, hepatocytes and pancreatic islet cells (Doss *et al*., 2004). This has made them the focus of recent interest as they may offer a treatment for many diseases including heart disease, Parkinson's disease and type I diabetes (Klug *et al*., 1996; Kim *et al*., 2002; Lumelsky *et al*., 2001). mES cells express specific cell surface markers like stage-specific embryonic antigen 1 (SSEA1) and membrane bound receptors such as the cytokine receptor, gp130. hES cells display the surface proteoglycans, TRA-1-60, TRA-1-81, GCTM-2, and stage-specific embryonic antigens 3 and 4 (SSEA3, SSEA4) that are not expressed on mES cells (Wei *et al*., 2005). Although ES cell research began more than 20 years ago, still little is known about the mechanisms that allow them to self-renew indefinitely while maintaining pluripotency. Simultaneously, the signals required for cardiomyocyte differentiation are complex and the mechanisms which prompt differentiation of ES cells to cardiomyocytes are unclear.

Extrinsic factors have been reported to promote ES cell pluripotency, which are thought to eventually lead to the regulation of genes involved in pluripotency. Leukaemia inhibitory factor (LIF), acts through a heterodimeric receptor complex to maintain pluripotency of ES cells *in vitro* (Williams *et al*., 1988). The receptor complex consists of a low affinity LIF receptor (LIFRβ) and gp130 receptor which activates the signal transducer Signal Transducer and Activator 3 (STAT3) (Niwa *et al*., 1998; Burdon *et al.*, 2002). ES cells have been shown to differentiate into a mixed population of cells when LIF is removed from culture or STAT3 is directly inhibited (Pan & Thomson, 2007). Furthermore, if STAT3 is activated by a method other than LIF, then LIF can be removed from culture and ES cells remain undifferentiated (Matsuda *et al*., 1999). Other factors identified to promote pluripotency are the bone morphogenetic proteins (BMP), BMP2 and BMP4. In the presence of LIF they enhance ES cell pluripotency by activation of Smad 4 and members of the inhibition of differentiation (Id) gene family (Ying *et al*., 2003). The best characterised gene involved in pluripotency of ES cells is the POU domain transcription factor, octamer binding protein-4 (OCT4). OCT4 is expressed by undifferentiated mouse and human embryonic stem cells and in all pluripotent cells during mouse embryogenesis. OCT4 plays a critical role in maintaining pluripotency

in vivo and *in vitro*. OCT4 null mice do not progress beyond the blastocyst stage (Nichols *et al*., 1998). The more recently described gene, *Nanog*, also plays a critical role in pluripotency. Nanog was identified by expression cloning and discovered to be able to maintain ES cell pluripotency independently of LIF (Chambers *et al*., 2003).

1.7.2 **ES cell culture**

Mouse ES cells are cultured *in vitro* in the presence of leukaemia inhibitory factor (LIF) or on MEFs, enabling the cells to divide in an undifferentiated state. hES cells are also cultured on MEFs, but in serum supplemented with basic fibroblast growth factor (bFGF) as they do not remain undifferentiated in the presence of LIF alone (Doss *et al*., 2004). hES cells grow more slowly compared to mES cells with population doubling times being 8-15 hour and 25-30 hours respectively and they proliferate in compact colonies with high nucleus to cytoplasm ratio (Ulloya-Montoya *et al*., 2005). mES cells are subcultured every two to three days using trypsin to enzymatically dissociate the cells into single cells whereas hES cells can be passaged as small clumps of cells (Ulloya-Montoya *et al*., 2005). When LIF is removed from the culture medium, and a cell suspension is produced, ES cells spontaneously differentiate into structures that resemble early post implantation embryos, called embryoid bodies (EB). EB are responsive to growth factors, which via discrete signal transduction processes induce differentiation programmes. Formation of EB promotes differentiation into the three embryonic germ layers. Depending on the culture conditions, cells from the ectoderm can potentially be differentiated to skin cells, central and peripheral nerve cells and adrenal cells, whereas cells from the mesoderm differentiate into heart, muscle, endothelium, blood, bone and cartilage and those from the endoderm into gut, liver and pancreatic cells (Doss *et al*., 2004). Mouse ES cells have been differentiated into cardiomyocytes, haematopoietic progenitors, yolk sac, skeletal myocytes, smooth muscle cells, adipocytes, hepatocytes, chondrocytes, endothelial cells, melanocytes, neurons, glia and pancreatic islet cells. Furthermore, neural cells, cardiomyocytes, β

cells, osteoblasts, hepatocytes and haemopoietic progenitor cells have also been derived from hES cells (Vats *et al*., 2005). The differentiation process is regulated by extracellular signals that mediate cellular responses through cell surface receptors and addition of specific factors can promote differentiation to a specific cell type.

1.7.3 **ES cell differentiation**

The differentiation of pluripotent embryonic stem cells to a specific cell type is thought to be regulated by extrinsic factors which through cell surface receptors, initiate signalling molecules and activation of signalling pathways to regulate gene expression. The protocols to generate cardiomyocytes from ES cells *in vitro* are well established. Differentiation protocols start with the formation of structures that resemble the early embryo, called embryoid bodies (EB), (Lumelsky *et al*., 2001). Signalling events within the EB leads to the induction of developmental cues that promote differentiation. After culture of EB in suspension for 2-3 days followed by plating, beating cardiomyocytes can be identified as areas of contraction within the EB. The cardiomyocytes are located between an outer epithelial layer and a layer of mesenchymal cells (Wei *et al*., 2005). Typically, EB begin to contract up to 4 days after plating and can continue contracting for up to one month. ES cell derived cardiomyocytes show characteristics of cardiomyocytes found in the primitive heart tube and early myocardium (Boheler *et al*., 2002). Mouse ES cell derived cardiomyocytes have characteristics of pacemaker, Purkinje, atrial and ventricular cells (Passier *et al*., 2002; Winkler *et al*., 2005). Furthermore, cells isolated from beating areas of the EB express cardiac genes including alpha myosin heavy chain (αMHC), myosin light chains, cardiac troponin I and T, and atrial naturetic peptide and also the cardiac specific transcription factors GATA-4 and Nkx-2.5 (Winkler *et al*., 2005; Boheler *et al*., 2002; Maltsev *et al*., 1994).

The differentiation of pluripotent embryonic stem cells to a specific cell type is regulated by signals that mediate cellular responses through cell surface receptors. However, the signals required for cardiomyocyte differentiation are complex and the

mechanisms which prompt differentiation of ES cells to cardiomyocytes are unclear. Numerous studies have shown that cardiomyogenesis can be enhanced by addition of supplements to the culture media including bone morphogenic protein (BMP) (Bin *et al*., 2006), transforming growth factor β (TGF-β) (Kumar *et al*., 2005), retinoic acid (Wobus *et al*., 1997), vascular endothelial growth factor (VEGF) (Chen *et al*., 2006) and DMSO (Lumelsky *et al*., 2001).

1.7.4 **Regulation of ES cells by lysophospholipids**

Recently there has been emerging literature for lysophospholipids in the regulation of both embryonic stem cells and adult stem cells/progenitor cells, which are multipotent cells with a more restricted differentiation ability. Control of stem cells in regard to proliferation, pluripotency and differentiation is integral to their function and manipulation of these cells *in vitro* to generate stem cell therapies. Recently, some of these control mechanisms are beginning to emerge but much is still unknown. Recent evidence has implicated S1P in regulating stem cell proliferation, survival and differentiation.

S1P receptors have been identified in both mouse and human embryonic stem cells. In the mouse ES cell line R1, mRNA expression of $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ was detected by RT-PCR (Kleger *et al*., 2007). S1P receptor expression differs in human ES cells. RT-PCR and Western Blot detected $S1P_1$, $S1P_2$ and $S1P_3$ but not $S1P_4$ or $S1P_5$ in hES cell lines (Pebay *et al*., 2005).

The first study to highlight a role for S1P in embryonic stem cell regulation demonstrated that cardiomyogenesis was induced by S1P in embryoid bodies derived from mES cells, an effect also observed with PDGF (Sachinidis *et al*., 2003). Using a defined serum replacement protocol to identify specific growth factors promoting cardiac development, stimulation of EB with either S1P or PDGF-BB resulted in a 2.6-fold enhancement of cardiomyogenesis compared with controls. Cardiomyogenesis was quantified by cardiac specific myosin heavy chain alpha and

beta (MHCα/β) and beating activity. S1P treatment of EB resulted in a marked increase in cardiac MHCα/β and an increase in the number of beating EB from 40% to 80%. Similarly, PDGF-BB treatment of EB significantly increased cardiac MHCα/β and a parallel increase in beating EB (Sachinidis *et al*., 2003).

Recently, the lysophospholipid, sphingosylphosphocholine (SPC) was also shown to induce both neuronal and cardiac differentiation of mES cells (Kleger *et al*., 2007). SPC is known to be a ligand for the G protein coupled receptors ORG1, GPR4, GPR12 and G2A but also engages $S1P_{1-5}$ with low affinity. Experiments showed that mES cells, R1, expressed mRNA for $S1P_{1-5}$ and GPR4, but not ORG1 during different stages of differentiation. Levels of mRNA transcript for these receptors were consistent throughout the differentiation process, from undifferentiated ES cells to 20 day old EB outgrowths. Exogenous SPC promoted early stages of neuronal differentiation cardiac differentiation as measured by increased cardiac MHCα/β. Whether these receptors mediate the effects of SPC on mES differentiation remains to be established.

Although hES cells also express S1P receptors ($S1P_{1-3}$), in addition to PDGFR-α and PDGFRβ, exogenous S1P and PDGF have been shown to maintain the undifferentiated state of hES cells (Pebay *et al*., 2005). hES cell lines, HES-2, HES-3 and HES-4 were cultured in the presence of inactivated mouse embryonic fibroblasts (MEFs) in the absence of serum. Addition of exogenous S1P or PDGF increased levels of the pluripotent stem cell marker, GCMT-2, after long term culture suggesting these agonists prevent spontaneous differentiation. A greater increase in GMCT-2 was observed when cells were co-incubated with both S1P and PDGF, an effect that was sensitive to PTX treatment and the MEK inhibitor U0126, suggesting involvement of $G\alpha_i$ and the p42/p44 MAPK cascade. In addition, PDGF activated SphK in hES cells, increasing formation of intracellular S1P. Treatment with the SphK inhibitor (DMS) blocked the effect of S1P and PDGF suggesting a critical role for SphK (Pebay *et al*., 2005L; Avery *et al*., 2006).

A more recent study further elucidated the role of S1P and PDGF in maintenance of hES cell pluripotency. Using a terminal transferase dUTP nick end labelling (TUNEL) assay, Wong *et al*., (2007) demonstrated that S1P and PDGF significantly reversed apoptosis as a result of serum deprivation. This effect of S1P and PDGF was reduced by the MEK inhibitor, U0126 and inhibition of the PI3-kinase/Akt pathway the specific PI3-kinase inhibitor, LY294002. Both PI3-kinase and p42/p44 MAPK are important mediators of cell survival. Western blot analysis revealed that p42/p44 MAPK is activated by S1P or PDGF whereas Akt is activated in response to PDGF but not S1P. In addition, the authors show that p42/p44 MAPK and Akt signalling are independent of each other suggesting that PI3-kinase and p42/p44 MAPK regulate hES cell survival via independent pathways. Moreover, neither PDGF nor S1P had any effect on the transcription factors Smad2/3 or mTOR (Wong *et al*., 2007). These data are corroborated by another study which also reported a role for S1P in hES cell survival (Innis & Moore, 2006). In hES cells cultured in the presence of bFGF, S1P (20μM) significantly decreased levels of apoptosis and necrosis while increasing proliferation.

Avery *et al*., (2008) investigated downstream targets activated by S1P-dependent p42/p44 MAPK activation in hES cells, ribosomal s6 kinase-1 (RSK-1), c-myc, Bcl-2 associated death promoter (BAD) and Bcl-2 associated X protein (Bax), using RT-PCR. S1P resulted in upregulation of both RSK-1 and c-myc whereas there was a downregulation of pro-apoptotic transcription factors, Bad and Bax, suggesting a reduction of apoptosis with S1P treatment (Avery *et al*., 2008). Comparing gene expression profiles post S1P treatment using microarray, revealed 1049 genes were differentially expressed by a 2-fold increase or decrease with many differentially expressed genes involved in apoptosis, cell cycle control, proliferation and pluripotency [**Figure 1.11**].

	Gene	Symbol	Fold change
Apoptosis	Bcl-2 associated X protein	BAX	4.3↓
	BH3 interacting domain death agonist	BID	2.4↓
	Apoptosis inhibitor 5	API5	2.2↑
Proliferation	Growth arrest and DNA damage inducible	GADD45G	2.4↓
	Cyclin dependent kinase 6	CDK6	3.1↑
	Cyclin D2	CCND2	2.5↑
	G1 to S phase transition 1	GSPT1	2.7↑
Adhesion	Cadherin 11	CDH11	2.9 ↑
	Integrin alpha V	ITGAV	9.3 ↑
	Fibronectin 1	FN1	4.5 ↑
Pluripotency	Nanog homeobox	NANOG	2.2↓
	POU domain, class 5, transcription factor 1	OCT-4	4.0↓
Cell signalling	Leukemia inhibitory factor receptor	LIFR	4.4 ↑
	Bone morphogenic protein 2	BMP2	3.2 ↑
	Phosphoinositide-3-kinase, regulatory subunit 1	PIK3R1	2.5 ↑

Figure 1.11 Microarray analysis of gene expression in response to S1P in hES cells (adapted from Avery *et al.*, 2008).

1.8 Project Aim

Emerging evidence demonstrating a role for S1P in the regulation of embryonic stem cells has generated a great deal of research interest. Control of stem cell proliferation, pluripotency and differentiation is integral to their function and to the manipulation of these cells *in vitro* in order to generate viable stem cell therapies. Recently, some of these control mechanisms are beginning to be defined but much is still unknown. Recent evidence has implicated S1P in regulating stem cell proliferation, survival and differentiation. Some of the effects of S1P in embryonic stem cells can be enhanced by PDGF and may involve the p42/p44 MAPK signalling cascade. However, the receptor mechanism and intracellular signalling intermediates involved remain to be characterised. In this regard, using the mES cell lines ES-D3 and CGR8, the aims of this project include:

I. To characterise the S1P receptor expression profile in mES cell lines
II. To identify the S1P receptor subtypes involved in p42/p44 MAPK activation
III. To investigate the presence/absence of a functional $S1P_1$-PDGFβ receptor signalling complex in mES cells
IV. To examine the role of p42/p44 MAPK in stem cell self renewal
V. To characterise the G proteins and signalling intermediates involved in p42/p44 MAPK signalling in ES cell renewal

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 General Reagents

All biochemical reagents were of the highest grade commercially available and were purchased from Sigma (U.K) unless otherwise stated.

COMPANY	REAGENT
Anachem (U.K)	30% (w/v) acrylamide: bis-acrylamide (29:1)
GE Healthcare (U.K)	Hybond™, ECL™ Nitrocellulose membrane, Kodak X-ray films (X-Omat LS, X-Omat-AR5)
H.A West (U.K)	Kodak LX24 developer, Kodak Industrex fixer
ICN Flow (U.K)	Luminol

2.1.2 Cell culture

All cell culture supplies were from Invitrogen (U.K). ESD3 cells (#CRL-1934) were purchased from American Type Culture Collection (ATCC). CGR8 cells (#95011018) and Chinese Hamster Ovary (CHO) cells were purchased from the European Collection of Cell Cultures (ECACC). STO cells with Neomycin resistant LIF expression vector (SNL) cells were a kind gift from Dr. Nadire N. Ali (Imperial College London, U.K).

COMPANY	REAGENT
BD Bioscience	Gelatin: 0.1-0.2% (w/v) in phosphate buffered saline (PBS)
Millipore	Leukaemia inhibitory factor (LIF)
Merck	Geneticin

2.1.3 **Antibodies**

COMPANY	**REAGENT**
BD Transduction Lab. (U.K)	Mouse monoclonal anti-p42 MAPK (#610104)
Millipore (U.K)	Rabbit polyclonal anti-PDGFβ receptor (#06-498)
Santa Cruz (U.S.A)	Goat polyclonal anti-$S1P_1$ (#sc-16070) Rabbit polyclonal anti-$S1P_5$ (#sc-25493) Mouse monoclonal anti-HA-probe (#sc-7392) Mouse monoclonal anti-phospho-p42/p44 MAPK (#sc7383)
Sigma (U.K)	Reporter horseradish peroxidise-anti-mouse IgG (#A9169) Reporter horseradish peroxidise-anti-rabbit IgG (#A0545)

2.1.4 **Radioisotopes**

[*Methyl*-^{3}H] thymidine (37MBq/ml) was from G.E Healthcare (U.K).

2.1.5 **Agonists, Inverse agonists and Inhibitors**

Phytosphingosine-1-phosphate (Ph-S1P) and its stereoisomers DM95 and DM97 were synthesised and validated structurally by Dr David Mormeneo Julian, Dept Organic Chemistry, Faculty of Chemistry, University of Barcelona, Spain.

COMPANY	**AGONIST/INHIBITOR**
Avanti Polar Lipids (U.S.A)	Sphingosine-1-phosphate (S1P) [a] Phytosphingosine-1-phosphate (PhS1P) [a]
Alexis Corporation (U.K)	LY294002 [a] (PI3K inhibitor)
Calbiochem (U.K)	Platelet derived growth factor AB (PDGF-AB) [b] Platelet derived growth factor BB (PDGF-BB) [c] SEW 2781 [a] ($S1P_1$ selective agonist) JTE013 [a] ($S1P_2$ competitive antagonist)
Cayman Chemical (U.S.A)	CAY10444 [d] ($S1P_3$ competitive antagonist)
Glaxo Smith Kline (P.A)	SB649146 [a] ($S1P_1$ inverse agonist)
Merck	PP2 [a] (c-Src inhibitor)
Sigma-Aldrich	GF109203X [a] (PKC inhibitor)

[a] reconstituted in dimethyl sulfoxide (DMSO)

[b] reconstituted in 0.1% BSA in 4mM HCl

[c] reconstituted in 0.1% BSA in 0.1M acetic acid

[d] reconstituted in dimethylformamide (DMF):PBS (1:3)

2.1.6 **Molecular Biology**

COMPANY	REAGENT
ABgene (U.K)	Nucleospin RNA extraction kit
Becton Dickinson (U.K)	Bacto-yeast extract Bacto-tryptone Bacto-agar
Biomers (Germany)	PDGFβR primers OCT-4 primers (see **Table 2**)
CRUK (U.K)	*Taq* polymerase
GE Healthcare (U.K)	GFX™ Micro Plasid Prep kit
Invitrogen (U.K)	Superscript II reverse transcriptase DNA polymerisation mix 100bp DNA ladder DNase I LipofectAMINE™ 2000 Ultra pure agarose Primers for: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and α-MHC (see **Table 2**)
UMR cDNA resource centre (U.S.A)	pcDNA3.1-$S1P_4$-HA pcDNA3.1 -$S1P_5$-HA

2.2 Cell Culture

2.2.1 **Maintenance of ES-D3 cells**

ES-D3 cells were cultured on 0.1% (w/v) gelatin/PBS without feeder cells in Dulbecco's Modified Eagle Medium (DMEM) (high glucose, 4.5g/L)) supplemented with 15% (v/v) european foetal calf serum (EFCS), 2mM non-essential amino acids (NEAA), 100U/ml penicillin, 100μg/ml streptomycin, 0.1mM β-mercaptoethanol and 1000U/ml Leukaemia Inhibitory Factor (LIF) in a humidified atmosphere containing

5%CO_2/air at 37ºC. Cells were passaged every 2 days or when they had grown to ~80% comfluency and media refreshed every other day. To passage cells, media was removed and cells washed with serum free media containing supplements. The cells were detatched from the flask by incubating with 1ml of trypsin for 2-3 minutes at room temperature. The cells were then resuspended in 9mls of complete media and centrifuged for 5 minutes at 270g. The resulting cell pellet was resuspended gently in 1ml of complete media using a 1ml pipette followed by dilution using a further 9ml of complete media. For further culturing, 2ml of cell suspension was transferred to a new 0.1% gelatin coated 75cm^2 flask containing 13ml of complete media and placed at 37°C in a humidified atmosphere containing 5% CO_2. For experiments, the cell suspension was diluted in complete media (1:12) and 1ml or 0.5ml plated out in either 12- or 24-well plates respectively and grown to ~80% confluency. Cells were maintained in DMEM containing 1% (v/v) foetal calf serum, 2mM non-essential amino acids (NEAA), 100U/ml penicillin, 100µg/ml streptomycin, 0.1mM β-mercaptoethanol and 1000U/ml Leukaemia Inhibitory Factor (LIF) for 24 hours prior to agonist stimulation.

2.2.2 **Maintenance of CGR8 cells**

CGR8 cells were cultured on 0.2% (w/v) gelatin/PBS without feeder cells in Glasgow Minimum Essential Medium (GMEM) supplemented with 10% (v/v) EFCS, 2mM NEAA, 100U/ml penicillin, 100µg/ml streptomycin, 0.5mM β-mercaptoethanol and 100U/ml LIF in a humidified atmosphere containing 5%CO_2/air at 37ºC. Cells were passaged every 2 days or when ~80% confluent and media refreshed every other day. Passaging and plating of CGR8 cells for experimentation followed the same protocol as described for ES-D3 cells.

2.2.2.1 **Evaluation of ES cell pluripotency**

ES cells were cultured appropriately to maintain ES cell pluripotency and prevent spontaneous differentiation. Specifically, cells were checked daily for subconfluence.

Cells were plated at optimum density (no less than a 1:10 dilution), subcultured every 2 days or when ~70% confluent and media refreshed daily. Cells were examined daily for undifferentiated morphology, typically small colonies with a defined boundary. Spontaneous differentiation of ES cells results in morphological changes such as the loss of the discreet ES cell colony border, formation of 'cobblestone-like' cells and ES cells differentiating into fibroblast-like cells which extend outwards from the colony. Cells suspected of undergoing spontaneous differentiation were discarded.

2.2.2.2 **Preparation of ES cell stocks**

After initial revival of ES cells, cells were expanded and a single stock of cells was prepared from which all samples used in the experiments were drawn. Cells were stored in freezing medium (10% DMSO; ES culture medium) in liquid nitrogen.

2.2.3 **Maintenance of SNL cells**

For differentiation experiments, embryonic stem cells of the mouse line ES-D3 were cultured on a layer of mitotically inactive SNL (STO cells, stably transfected with a Neomycin resistance gene (neo^r) and a Leukaemia inhibitory factor (LIF) expression vector) feeder fibroblasts (Ali *et al*., 2004). SNL cells carrying the LIF expression vector were selected by culturing in the presence of G418 (400μg/ml) DMEM supplemented with 10% (v/v) EFCS for 14 days followed by a least 2 passages in the absence of G418. SNL cells were mitotically inactivated by incubation with mitomycin C (0.01mg/ml) in DMEM supplemented with 10% (v/v) EFCS and 100U/ml penicillin, 100μg/ml streptomycin at 37°C for 3 hours, followed by three washes in phosphate buffered saline (PBS). The now inactivated feeder cells (iSNLs) were then counted and either seeded ($1.5x10^6$ cells per $25cm^2$ flask) or frozen down ($1.5x10^6$ cells/ml/vial) for future use. Seeded cells were used after 24 hours for the revival of ES-D3 cells and subsequent differentiation experiments.

2.2.4 **Induction of cardiomyogenesis**

ES-D3 cells were cultured on iSNL cells in Knock Out Dulbecco's Modified Eagle Medium (KO-DMEM) supplemented with 15% knockout serum replacement (KO-SR), 2mM GlutaMAX-I, 2mM NEAA, 100U/ml penicillin, 100µg/ml streptomycin, 0.1mM β-mercaptoethanol and 1000U/ml Leukaemia Inhibitory Factor for three passages, then for 2 further passages on 0.1% gelatin coated flasks in the absence of iSNL cells.

To induce differentiation to cardiomyocytes, an established protocol was followed (Boheler *et al.*, 2002). At day 0, cells were trypinised, counted using a haemocytometer and a suspended solution prepared (20 cells/µl) in differentiation media consisting of DMEM supplemented with 15% (w/v) batch-tested FCS (#06Q9641F) (Ali *et al*., 2004), 2mM NEAA, 100U/ml penicillin, 100µg/ml streptomycin, 0.1mM β-mercaptoethanol, without LIF. 20µl drops were placed in a 96- conical well shaped plate then a further 100µl of differentiation media added and placed in humidified air with 5%CO_2 at 37ºC. The next day (day 1), differentiation media was replaced with KO-SR/KO-DMEM and were treated with 15% (w/v) batch-tested FCS, 5µM S1P, 10ng/ml PDGF or both S1P and PDGF. Media was replaced every other day and further stimulation as required, repeated. Treatments were changed every other day and EB examined daily. The time of development of beating and number of beating EB was recorded.

2.2.5 **Maintenance of CHO cells**

Chinese Hamster Ovary (CHO) cells were cultured in DMEM supplemented with 10% (w/v) EFCS, 100U/ml penicillin and 100µg/ml streptomycin in a humidified atmosphere containing 5%CO_2 at 37ºC and subcultured when confluent. To subculture, cells were washed in serum free media prior to addition of 1ml of trypsin for 2-3 minutes at 37ºC. Cells were resuspended in 9ml of complete media. For further subculture, 1ml of the cell suspension was transferred to a new 75cm^2 flask containing 9ml of complete media and placed in humidified air with 5% CO_2 at

37ºC. CHO cells were grown on 12 or 24 well plates for experiments and quiesced in serum free medium for 24 hours prior to stimulation as required.

2.3 Cell stimulation and sample preparation

Cells that had been quiesced as described previously were stimulated with agonists as described in each figure legend, as appropriate. Cell lysates were prepared by removing the media and adding an appropriate volume of boiling sample buffer containing 125mM Tris-Base (pH6.7), 0.5mM $Na_4P_2O_7$, 1.25mM EDTA, 1.25% (v/v) glycerol, 0.5% (w/v) SDS, 25mM 1.4-Dithiothreitol (DTT) and 0.02% (w/v) bromophenol blue. Cells were harvested by scraping the base of the wells with a 1ml pipette tip, followed by repeatedly (5x) passing the lysate through a 23g needle. Samples not immediately resolved by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were stored at -20°C.

2.4 SDS-PAGE and Western Blotting

2.4.1 Polyacrylamide gels

Polyacrylamide gels were prepared by layering a stacking gel on top of a separating gel. Polyacrylamide gels were prepared from acrylamide:bisacrylamide stock (30% (w/v)-acrylamide and 0.8% (w/v)-N,N,-bis-methylacrylamide). The separating gel contained 10% (v/v) acrylamide:bis-methylacrylamide, 0.375M Tris-Base (pH8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.025% (w/v) tetramethylethylenediamine (TEMED). Once polymerised, a 10 or 15 well stacking gel was layered onto the separating gel and contained 4.5% (v/v) acylamide:bisacrylamide, 0.125M Tris Base (pH6.7), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.1% (v/v) of TEMED.

2.4.2 **Gel electrophoresis**

Gel electrophoresis was carried out on the BIORAD Mini-Protean III electrophoresis apparatus. The apparatus was filled with buffer (containing 25mM Tris-Base, 0.21M glycine and 0.1% (w/v) SDS) and samples loaded into each well of the stacking gel using a Hamilton syringe (25µl/well in a 10-well gel, 15µl/well in a 15-well gel). The samples were run alongside 5µl of pre-stained SDS-PAGE molecular weight marker as a molecular weight reference. Electrophoresis was performed at 150V until the bromophenol blue marker in each sample reached the bottom of the gel (typically 45-60 minutes).

2.4.3 **Transfer to nitrocellulose**

The separated proteins in the gel were transferred onto nitrocellulose membrane using a Biorad Mini-blot transfer kit. The apparatus was filled with buffer (containing 25mM Tris-Base and 0.21M glycine in 20% (v/v) methanol) and samples transferred at 100V for 60 minutes.

2.4.4 **Immunoblotting**

The nitrocellulose sheets containing the separated proteins were submerged in blocking buffer containing 3% (w/v) bovine serum albumin (BSA) or 3% (w/v) milk powder in TBST (10mM Tris-Base (pH7.4), 0.1M NaCl and 0.1% (v/v) Tween-20) for 60 minutes at room temperature, with continual agitation, to prevent non-specific antibody binding. Nitrocellulose blots were then incubated overnight at 4°C with mild agitation with primary antibodies (diluted in 1% (w/v) BSA or 1% (w/v) milk powder in TBST) specific for detection of phospho-p42/p44 MAPK (1:1000), p42 MAPK (1:1000), $S1P_5$ receptor (1:1000), PDGFβ receptor (1:1000) or HA tagged proteins (1:500). Nitrocellulose blots were washed (3 × 7 minutes) with TBST at room temperature, followed by incubation with horse radish peroxidise (HRP)-linked

anti-mouse or anti-rabbit (as appropriate) IgG secondary antibody at a 1:80,000 dilution in 1% (w/v) BSA (or 1% (w/v) milk powder) in TBST for 1 hour at room temperature. The nitrocellulose blots were washed (3 × 7 minutes) in TBST prior to visualisation of immunoreactive bands. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection where nitrocellulose blots were incubated with equal volumes of ECL reagent 1 (containing 0.04% (w/v) luminol, 0.1M Tris-Base (pH 8.5) and 0.016% (w/v) *p*-coumaric acid) and ECL reagent 2 (containing 2% (w/v) H_2O_2 and 0.1M Tris-Base (pH 8.5)) for 3 minutes at room temperature. The blots were carefully placed between two sheets of transparent film in a metal cassette and exposed to X-ray film for 30 seconds. Films were developed by passing through an X-Omat machine and proteins visualised a dark bands on the film. Exposure time was adjusted depending on the intensity of the bands.

2.4.5 **Determination of protein molecular weight**

Proteins were identified by comparing their predicted molecular weight to that of pre-stained SDS-PAGE molecular weight markers.

Standard	**Source**	**Molecular Weight (kDa)**
α-macroglobulin	human placenta	180
B-galactosidase	*E.coli*	116
fructose-6-phosphate kinase	rabbit muscle	84
pyruvate kinase	chicken muscle	58
fumarase	porcine heart	48.5
lactic dehydrogenase	rabbit muscle	26.6

2.4.6 **Stripping and reprobing**

To detect a different protein in the same sample, blots were stripped and re-probed with a different primary antibody. Blots were stripped by incubation with stripping buffer (containing 62mM Tris-Base (pH6.7), 2% (w/v) SDS and 100mM β-mercaptoethanol) for 1 hour at 70°C with mild, continual agitation. After stripping, blots were then rinsed with distilled water and thoroughly washed with TBST (3 x 7 minutes) before incubation with primary antibody (see 2.4.4).

2.4.7 **Quantification**

Densitometric quantification was achieved using Scion Image software. Phosphorylated p42 MAPK/total p42 MAPK were calculated and expressed relative to unstimulated control cell (100%).

2.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

2.5.1 RNA extraction

RNA was extracted from proliferating CHO, ES-D3 and CGR8 cells and beating and non-beating EB. Proliferating CHO, ES-D3 and CGR8 cells were grown to ~80% confluency in 25cm^2 flasks (gelatin coated as required) for RNA extraction. EB were pooled into beating and non-beating and RNA was extracted using the Nucleospin II RNA Extraction kit, according to the manufacturer's instructions. Proliferating cells were washed three times with 5ml of filter sterile PBS. Using a sterile cell scraper, cells were scraped into 350µl of buffer RA1 supplemented with 3.5µl of β-mercaptoethanol and passed through a syringe fitted with a 21-gauge needle six times. For EB, media was removed from beating and non-beating EB which were then washed with 200µl of PBS. Beating EB (pooled) and non-beating EB (pooled) were scraped into 350µl of buffer RA1 supplemented with 3.5µl of β-mercaptoethanol. To reduce viscosity, the cell lysate was transferred to a filter column and filtered by centrifugation through a Nucleospin Filter unit at 11,000 ×g for 1 min. The flow-through was used for the isolation procedure. 350µl of 70% ethanol was added to the flow-through and mixed well by pipetting. The lysate and any precipitate was applied to a Nucleospin RNA II column, placed in a 2ml collection tube and centrifuged at 8,000 ×g for 30 seconds. 350µl of membrane desalting buffer (MDB) was applied to the column and centrifuged at 11,000 ×g for 1 min to dry the membrane. DNase I reaction mixture was prepared by adding 10µl of reconstituted DNase I to 90µl of DNase reaction buffer, and applied directly onto the silica membrane of the column, for 15 min at room temperature. The membrane was washed by adding 200µl of buffer RA2 to the column and centrifuged at 8,000 ×g for 30 seconds. This was followed by another wash with 600µl of buffer RA3 and centrifuged at 8,000 ×g for 30 seconds. A final wash was carried out by adding 250µl of buffer RA3 to the column which was centrifuged for 2 minutes at 11000 ×g to dry the membrane. The column was transferred to an RNase/DNase free 1.5ml collection tube and 50µl of RNase/DNase free water applied directly to the membrane. RNA

was eluted by centrifugation at 11000 ×g for 1 min. The eluate was applied once more to the column and re-eluted by centrifugation at 11000 ×g for 1 min. Total RNA concentration and $OD_{260/280}$ was measured using a Genequant II RNA/DNA calculator. Typical recovery of RNA was 1.0-5.0µg/µl. Only RNA with an $OD_{260/280}$ between 1.8 and 2 (indicating RNA devoid of contaminants) was used in further reactions.

2.6 DNase I treatment

To eliminate any remaining residual genomic DNA, total RNA was subjected to further DNase I treatment. To 1µg total RNA, 1µl of 10× DNase buffer and 1µl of DNase I was added in a total volume of 10µl and incubated at room temperature for 15 minutes. The reaction was terminated by adding 1µl of 25mM EDTA and incubated at 65°C for 10 minutes, followed by 1 minute on ice. Total RNA was stored at -70°C.

2.7 First strand cDNA synthesis

Reverse transcription was performed according to manufacturer's instruction (Superscript II, Invitrogen Ltd., U.K) with slight modification. 5µg of DNase I treated total RNA was combined with 0.5µg oligo$(dT)_{12\text{-}18}$, 0.5µl of 20mM dNTP mixture (containing dATP, dGTP, dCTP, dTTP) and RNAse/DNase free water to 12µl final volume, then heated to 65°C for 5 minutes. Following a quick chill on ice, 4µl of 5× First Strand buffer (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$), 2µl of 0.1M DTT was added and incubated at 42°C for 2 minutes. After that, 1µl of Superscript II RT (200 units) was added and the reaction incubated at 42°C for 50 minutes, followed by 15 minutes at 70°C, to inactivate the reaction. For each first strand reaction, a separate reaction excluding Superscript II RT was performed to ensure there was no genomic DNA contamination. For these reactions (-RT), Superscript II RT was replaced by 1µl of distilled water (RNase/Dnase free). The

cDNA produced was stored at -20°C for use as a DNA template in polymerase chain reaction (PCR) (Section 2.8).

2.8 Polymerase Chain Reaction

PCR was performed using *Taq* DNA polymerase in a 50µl reaction containing 5µl of 10× reaction buffer (containing 200mM Tris-HCl (pH 8.4) and 500mM KCl), 1.5µl of 50mM $MgCl_2$, 1µl of 20mM dNTP mix, 1µl each of forward and reverse primer (50pmol/ml), 2µl of template DNA, 38.6µl RNAse/DNase free water and 0.4µl *Taq* polymerase (5U/µl). For each PCR reaction, a negative control reaction (blank) was included where cDNA template was omitted. Reactions were performed in a Phoenix thermal cycler using conditions optimised for each primer pair. A typical reaction was: initial denaturation of the template at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds (denaturation), 30 seconds at specific annealing temperature for each primer set, 72°C for 1 minute (extension), followed by final primer extension at 72°C for 10 minutes. Amplification products were analysed by agarose gel electrophoresis (Section 2.9).

Primers	Nucleotide sequence	Predicted product size (bp)
$S1P_1$	5’- GAT ATC ATC GTC CGG CAT TAC -3’ (forward) 5’- ACC CTT CCC AGT GCA TTG TTC -3’ (reverse)	1265
$S1P_2$	5’- CAC TCA GCA ATG TAC CTG TTC C -3’ (forward) 5’- CAC CCA GTA CGA TGG TGA CC -3’ (reverse)	528
$S1P_3$	5’- GAC TGC TCT ACC ATC CTG CCC -3’ (forward) 5’- GTA GAT GAC CGG GTT CAT TGT CC -3’ (reverse)	330
$S1P_4$	5’- CCA CAG CCT CCT CAT TGT CC -3’ (forward) 5’- AGC GCG CTG CAC CTC ACG -3’ (reverse)	872
PDGFβ-receptor	5’- CACCTTCGTTCTGACCTGCT -3’ (forward) 5’- TGGATGACACCTGGAGTCTG -3’ (reverse)	481
α-MHC	5’- GGA AGA GTG AGC GGC GCA TCA -3’ (forward) 5’- AGG CTG CTG GAG AGG TTA TTC CTC G -3’ (reverse)	301
OCT-4	5’- TGTGGACCTCAGGTTGGACT-3’ (forward) 5’- CTTCTGCAGGGCTTTCATGT-3’ (reverse)	201
GAPDH	3’ - TGA AGG TCG GTG TCA ACG GAT TTG GC -5’ (forward) 3’ - CAT GTAGGC CAT GAG GTC CAC CAC - 5’(reverse)	996

Table 2 Nucleotide sequences of forward and reverse primers used in RT-PCR reactions.

2.9 Agarose gel electrophoresis

The PCR products were sized by electrophoresis on a 1% agarose gel, stained with ethidium bromide. Agarose gels were prepared by heating 0.5g of agarose in 50ml of 1× TBE buffer (containing 89mM Tris-Base (pH 8.3), 89mM boric acid, 2.2mM EDTA) in a microwave at maximum power for 2 minutes. 2.5µl of ethidium bromide was added to the molten agarose and mixed. The molton agarose was then poured into the electrophoresis apparatus and left for ~30 minutes. Once set, the gel was immersed in 1× TBE buffer. 10µl of loading dye (containing 0.25% (w/v) bromophemol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in sterile distilled water) was added to the 50µl PCR product and 15µl loaded into each well of the agarose gel. 7µl of a 100bp DNA ladder was run in parallel with the samples at 65V for ~30-45 minutes, until the boromophenol blue dye was approximately one third from the bottom of the gel. PCR products were visualised as bright bands under a UV transilluminator and photographed using a Kodak Digital Science EDAS120 camera.

2.10 GFX band purification

PCR products of the predicted size were excised from the gel and purified for sequencing using the GFX PCR DNA and Gel Band Purification Kit, according to the manufacturer's instruction. Briefly, 300µl of capture buffer was added to the gel slice containing the band of interest and vortexed vigorously. The sample was heated to 60°C until the gel had completely dissolved (~ 10 minutes) then transferred to a GFX column and incubated at room temperature for 1 minute. The column was centrifuged for 30 seconds at 16000 ×g and the flow through discarded. 500µl of wash buffer was added to the column which was centrifuged for 2 minutes at 16000 ×g and the flow through discarded. The column was transferred to an RNAse/DNAse free eppendorf and 50µl of RNAse/DNAse free water was added directly to the

column and incubated for 1 minute at room temperature. The purified DNA was eluted by centrifugation for 1 minute at 16000 ×g.

2.11 Sequencing

The purified DNA was sequenced using an Applied Biosytems 373 DNA sequencer using a BigDye Dye terminator cycle sequencing kit. PCR products were sequenced in both directions with the corresponding primers used in the PCR reactions by Dr. Roth J. Tate (Molecular Biology Facility, University of Strathclyde). Sequence data was analysed using BLAST (Altshcul *et al*., 1997) and aligned using ClustalW and BOXSHADE (Labarga *et al*., 2007).

2.12 [^{3}H] Thymidine uptake proliferation assay

ES-D3 cells and CGR8 cells were seeded onto 24 well plates in 0.5ml of complete media and grown to 60-70% confluency. The media was replaced with media supplemented with 1% (v/v) EFCS, 2mM NEAA, penicillin, streptomycin and LIF (as described in 2.2.1 and 2.2.2) for 24 hours. Cells were pre-incubated with antagonists, as required, for 30 minutes prior to addition of agonists for either 8 hours or 15 hours. For experiments involving 8 hour treatments, [^{3}H]thymidine (37KBq per well) was added for a further 60 minutes (9 hours total). For experiments involving 15 hour agonist treatment, [^{3}H]thymidine (9.25KBq per well) was added for a further 5 hours (20 hours total) In both cases, the medium was then aspirated and cells washed 3 times for 10 minutes with 1ml of 10% (w/v) ice-cold trichloroacetic acid (TCA). Nuclear material was dissolved by addition of 0.25ml of 0.1% SDS/0.3M NaOH and transferred to scintillation vials with 2ml of scintillant. Incorporation of [^{3}H]thymidine into newly synthesised DNA was quantified by liquid scintillation counting.

2.13 Statistical analysis

Experiments were repeated at least three times. All data is expressed as mean ± standard error of the mean (SEM), unless otherwise stated, using GraphPad Prism software. The difference between groups was analysed using the student t-test and the value of $p<0.05$ was considered significant.

2.14 Amplification and purification of plasmid contructs encoding HA-$S1P_4$ and HA-$S1P_5$

2.14.1 *Escherichia coli* transformation

Transformation of chemically competent *E. coli* cells (TOP10, Invitrogen) with cDNA plasmid (500ng) was carried out by mixing and then incubaion on ice for 30 minutes. The cells were heat shocked at 42ºC for exactly 30 seconds then immediately placed on ice for 2 minutes. 250µl of pre-warmed SOC (containing 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) media was added to the cells, and shaken at 37ºC for 1 hour. The cells were plated out on Luria-Bertani (LB) agar plates (containing 100µg/ml ampicillin) and incubated overnight at 37ºC. Colonies were used to innoculate 3 starter cultures (minipreps). Each miniprep contained 3ml of LB broth supplemented with 100 µg/ml of ampicillin. Minipreps were incubated for 12-15 hours 37ºC at 200rpm prior to plasmid purification.

Glycerol stocks were also prepared from miniprep cultures for future amplification. 700µl of culture was mixed with 300µl of 50% (v/v) sterile glycerol and stored at -80˚C.

2.14.2 **Plasmid purification**

1ml of miniprep culture was added to 100ml of LB medium containing 5mg of ampicillin in conical flasks and grown at 37˚C with vigorous shaking for 12-15 hours. The bacteria were pelleted by centrifugation at 6000 ×*g* for 15 minutes at 4ºC. Plasmid constructs were purified using the PureLink™ HiPure Plasmid Filter Purification Kit (Invitrogen, U.K). The bacterial pellet was resuspended in 10ml of chilled Buffer R3 (containing 50mM Tris-HCl (pH 8), 10mM EDTA and 100µg/ml RNase A) and mixed before the addition of 10ml of lysis Buffer L7 (containing 0.2M NaOH and 1% (w/v) SDS). The suspension was mixed and incubated at room temperature for 5 minutes. Next, 10ml of Buffer N3 (containing 3M potassium acetate, pH 5.5) was added to the lysate and mixed by inversion. The lysate was carefully poured into the HiPure anion-exchange filter column (previously equilibrated with 30ml of equilibration buffer) and allowed to drain through the column. This was followed by washing with 50ml of buffer W8 (containing 0.1M sodium acetate, pH5.0, 825mM NaCl). The DNA was eluted into an endotoxin-free tube with 15ml of E4 buffer (containing 100mM Tris-HCl, pH8.5, 1.25M NaCl) then precipitated with 10.5ml of room temperature isopropanol. This was immediately centrifuged at 16000 ×g at 4˚C for 30 minutes. The supernatant was carefully removed and the pellet washed with 5ml of endotoxin-free, room-temperature 70% (v/v) ethanol then centrifuged again at 16000 ×g for 10 minutes. The supernatant was decanted and the pellet air-dried for 5-10 minutes. Finally, the pellet was resuspended in 400µl of Buffer TE (containing 10mM Tris-HCl, pH 8, 1mM EDTA). The DNA yield was quantified by UV spectrophotometry using the GeneQuant RNA/DNA calculator (G.E Healthcare, U.K). Typical yield was 0.5-1.5µg/µl. The average $OD_{260/280}$ value was between 1.8 and 2.0.

2.14.3 **Transient cell transfection**

CHO cells were seeded onto 12 well plates in 1ml of their complete medium and grown to 50-60% confluency before undergoing transfection. The cells were

transiently transfected using LipofectAMINE™ 2000 transfection reagent. For each well, 1µg of HA-tagged $S1P_4$ plasmid construct or 1µg of HA-tagged $S1P_5$ plasmid construct (or mock transfected with pcDNA3.1 vector) was incubated with 1.5µl of LipofectAMINE™ 2000 diluted in 200µl serum free DMEM (without antibiotic) for exactly 25 minutes. The complexes were mixed gently by inversion. Meanwhile, the media in the plates was replaced with 800µl of DMEM supplemented with 1% (w/v) EFCS (for HA-tagged $S1P_4$) or 800µl of Optimem supplemented with 1% (w/v) EFCS (for HA-tagged $S1P_5$). Cells were transfected by applying 200µl of the prepared complexes in a drop-wise fashion directly into the wells with fresh media, as above, and mixed gently. Cells were maintained at 37°C in humidified air with 5% CO_2. For HA-tagged $S1P_4$ transient transfections, complexes were removed after 24 hours and cells were quiesced by addition of 1ml of serum-free medium for a further 24 hours. For HA-tagged $S1P_5$ transient transfections, complexes were removed after 18 hours and replaced with 1ml of complete medium for a further 8 hours. These cells were then quiesced by replacing the media with serum-free DMEM for 24 hours prior to experimentation. Cells were analysed for expression of the HA-epitope tag by SDS-PAGE and Western Blotting.

CHAPTER 3

CHARACTERISATION OF S1P AND PDGF REGULATION OF p42/p44 MAPK AND PROLIFERATION IN mES CELLS

CHAPTER 3: Characterisation of S1P and PDGF regulation of p42/p44 MAPK and proliferation in mES cells

3.1 Introduction

The mechanisms of embryonic stem (ES) cell proliferation and differentiation are still not fully understood and a great deal of interest has been placed on investigating factors and cell signalling pathways which influence these processes in order to successfully develop embryonic stem cell replacement therapies.

Embryonic stem cells proliferate by the process of symmetrical self-renewal, defined by the ability to produce two identical daughter stem cells whilst suppressing differentiation (Burdon *et al*., 2002). The fate of ES cells, i.e. self-renewal or differentiation, is regulated by various extracellular stimuli. Extracellular stimuli bind to cell surface receptors to initiate intracellular signalling cascades which promote either self-renewal or differentiation. Intracellular signalling events influence the expression of transcription factors involved in cell fate. In order to generate cells for cell replacement therapy, it is important to identify extracellular factors, signalling cascades and transcription factors that control ES cell self-renewal and differentiation.

Recently, a role for sphingosine-1-phosphate (S1P) in the regulation of both ES cells and adult stem cells has emerged. In both mouse and human ES cells, S1P receptors have been identified (Kleger *et al*., 2007; Sachinidis *et al*., 2003; Pebay *et al*., 2005) and these have been implicated in directing mouse ES cell differentiation and human ES cell self-renewal. Of interest to the current study, S1P, a bioactive lysophospholipid, and PDGF, a potent mitogen, have been found to promote the differentiation of mouse ES cells to cardiomyocytes (Sachinidis *et al*., 2003). Using a defined serum replacement protocol to identify specific growth factors promoting cardiac development, Sachinidis *et al*., reported that stimulation of EB with either S1P or PDGF-BB resulted in a 2.6-fold enhancement of cardiomyogenesis compared with controls as assessed by increased levels of cardiac specific myosin heavy chain

alpha and beta (αβMHC) and beating activity. More recently, another lysophospholipid, sphingosylphosphorylcholine (SPC), which engages S1P receptors with low affinity, was shown to induce cardiac and neuronal differentiation of mouse ES cells and was dependent on p42/p44 MAPK activation (Kleger *et al*., 2007). In contrast, S1P and PDGF have been shown to promote pluripotency in human embryonic stem cells (Pebay *et al*., 2005). Culture of human ES cells in the presence of S1P or PDGF maintained the undifferentiated state in the absence of serum. Human ES cells cultured in the presence of S1P and PDGF alone, reduced spontaneous differentiation compared with controls. This effect was reduced when cells were treated with pertussis toxin or a mitogen activated protein kinase kinase (MEK) inhibitor, U0126, suggesting that inhibition of differentiation is mediated via a G protein coupled receptor and involves activation of p42/p44 MAPK. More recently, Wong *et al*., reported that S1P and PDGF had an anti-apoptotic effect in human embryonic stem cells mediated by the p42/p44 MAPK and PI3K signalling pathways (Wong *et al*., 2007). To date, the effect of S1P (and PDGF) on undifferentiated embryonic stem cells remains relatively unknown.

It is well known that many GPCR and receptor tyrosine kinases regulate mitogen activated protein kinase (MAPK) cascades and induce cellular processes such as proliferation. The p42/p44 MAPK pathway is activated both by GPCR agonists, such as S1P and also by growth factor agonists such as PGDF. An emerging concept of S1P receptor biology is cross talk between GPCR and tyrosine kinase receptors (RTK). Of particular relevance to this study, the $S1P_1$ receptor and PDGFβ receptor were found to exist in a complex in various mammalian cell types including airway smooth muscle (ASM) cells and PDGF receptor transfected human embryonic kidney (HEK) 293 cells (Alderton *et al*., 2001; Waters *et al*., 2003), demonstrated by co-immunoprecipitation from cell lysates using either anti-$S1P_1$ or anti-PDGFβR antibodies. Furthermore, overexpression of $S1P_1$ increases p42/p44 MAPK activation by PDGF and overexpression of PDGFβR increases stimulation of the p42/p44 MAPK pathway by S1P (Alderton *et al*., 2001). This complex is a novel platform where the PDGFβ receptor uses the G protein subunits, $G\alpha_i$ and βγ, dissociated by the constitutively active $S1P_1$ receptor, to enhance stimulation of the p42/p44 MAPK

cascade by PDGF. This is due to the PDGFβ receptor kinase, which catalyses the tyrosine phosphorylation of the $G\alpha_i$ subunit, upon stimulation by S1P. S1P and PDGF promote internalisation of the $S1P_1$-PDGFβR complex as a functional signalling unit into endocytic vesicles, to which p42/p44 MAPK becomes co-localised and activated (Waters *et al*., 2003). Internalisation of the receptor complex involves c-Src, regulated by Gβγ subunits which can interact with β-arrestin (Waters *et al.,* 2005). The endocytosis of the $S1P_1$-PDGFβR complex, activation of p42/p44 MAPK and cell migration is reduced by a novel inverse agonist of the $S1P_1$ receptor (SB649146)' which blocks the function of the complex (Waters *et al*., 2005). As recent evidence clearly indicates a role for S1P and PDGF in ES cell biology, the possible presence of the $S1P_1$-PDGFβ receptor in ES cells may play an important role in physiological functions within these cells such as differentiation and proliferation.

In an attempt to investigate and elucidate the role of S1P and PDGF in undifferentiated ES cells, this section of the study focuses on examining the effect of S1P and PDGF in undifferentiated mouse ES cells in terms of p42/p44 MAPK activation, cell proliferation and pluripotency. As S1P and PDGF have been shown to promote differentiation of mouse ES cells to cardiomyocytes, and the $S1P_1$ and PDGFβ receptor are known to reside in a functional complex in various mammalian cells to promote more efficient p42/p44 MAPK activation, this study explores the possible existence and physiological role of this receptor complex in undifferentiated mouse ES cells.

3.2 Results

3.2.1 S1P receptor mRNA transcript expression in ES cells

Proliferating, undifferentiated ES-D3 and CGR8 cells, derived from mouse embryos, were investigated for mRNA transcript expression of S1P receptor subtypes using gene specific primers (**Table 2**).

3.2.1.1 $S1P_1$ receptor

RT-PCR analysis using gene specific $S1P_1$ primers amplified a single product of 1265bp in both ES-D3 cells [**Figure 3.1(a)**] and CGR8 cells [**Figure 3.1(b)**]. Bands from both cell lines were purified and subjected to nucleotide sequence analysis. For ES-D3 cells, [**Figure 3.2**] the nucleotides sequenced using either the forward primer or reverse primer showed 100% homology with mouse $S1P_1$.

For CGR8 cells, of the 1,214 nucleotides sequenced, all nucleotides showed 100% homology with mouse $S1P_1$ confirming CGR8 cells express mRNA transcript for $S1P_1$ [**Figure 3.3**].

3.2.1.2 $S1P_2$ receptor

$S1P_2$ gene specific primers amplified a single gene product of 528bp from ES-D3 cells [**Figure 3.4(a)**] and CGR8 cells [**Figure 3.4(b)**]. The product was purified and subjected to nucleotide sequence analysis. For ES-D3 cells, of the 485 nucleotides sequenced, all showed 100% homology with mouse $S1P_2$ [**Figure 3.5**].

For CGR8 cells, the amplicon showed 100% homology with the mouse $S1P_2$ sequence [**Figure 3.6**].

3.2.1.3 $S1P_3$ receptor

RT-PCR analysis using $S1P_3$ receptor primers amplified a PCR product of 344bp in ES-D3 cells [**Figure 3.7 (a)**], which was purified and sequenced. The 344

nucleotides sequenced matched the mouse $S1P_3$ Genebank™ sequence confirming the presence of mRNA transcript for $S1P_3$ in ES-D3 cells [**Figure 3.8**].
For CGR8 cells, $S1P_3$ primers also amplified a 344bp product [**Figure 3.7(b)**], which was purified and sequenced. The resulting sequence matched the nucleotide sequence of mouse $S1P_3$ [**Figure 3.9**].

3.2.1.4 **$S1P_4$ receptor**

For ES-D3 cells, RT-PCR analysis using gene specific $S1P_4$ receptor primers amplified a product of approximately 600bp and a trace product of approximately 1kb [**Figure 3.10(a**)]. In CGR8 cells, $S1P_4$ primers amplified a single product of approximately 600bp [**Figure 3.10(b)**]. The predicted size of the PCR product from $S1P_4$ primers was calculated to be 872bp. As a positive control, RT-PCR using $S1P_4$ primers, was carried out with pcDNA3.1-HA-$S1P_4$ plasmid in place of cDNA which amplified a product of approximately 872bp, the predicted product size, and two (non-specific) minor products of approximately 2072bp and 1400bp [**Figure 3.10(b)**]. For ES-D3 cells, the 600bp product was purified and sequenced. Alignment of the sequenced PCR product did not match the Genebank™ mouse $S1P_4$ sequence. Similarly, the 600bp product amplified in CGR8 cells was also purified and sequenced but failed to match with the mouse $S1P_4$ sequence suggesting that mRNA transcript for $S1P_4$ is not expressed in either ES-D3 or CGR8 cells (data not shown).

3.2.1.5 **$S1P_5$ receptor**

An RT-PCR approach was attempted for detection of $S1P_5$ in ES-D3 and CGR8 cells but was unsuccessful and therefore Western Blotting was used instead.

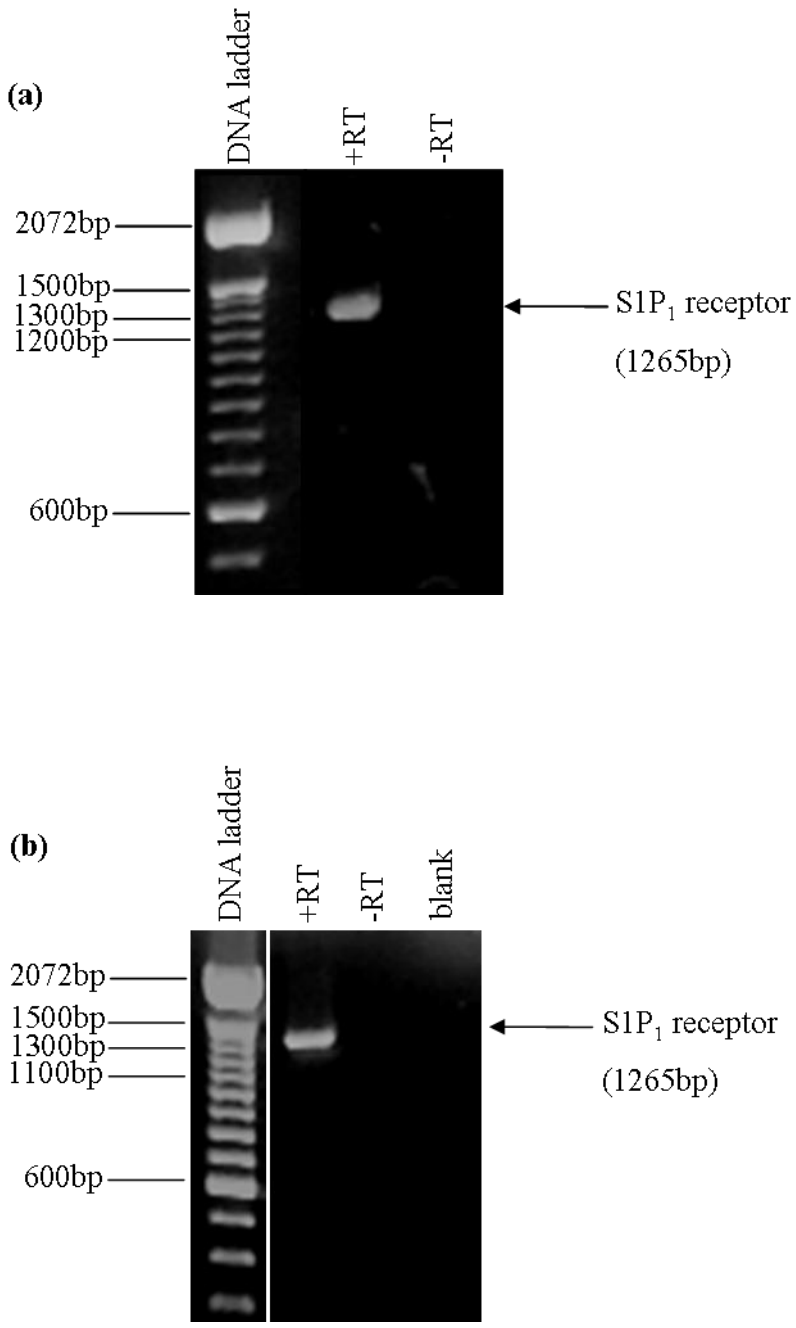

Figure 3.1 RT-PCR analysis of $S1P_1$ receptor mRNA transcript in (a) ESD3 cells and (b) CGR8 cells. RT-PCR analysis amplified a single 1265bp product using $S1P_1$ receptor primers. A negative control with omission of reverse transcriptase (-RT) was included. Blank is the negative control without cDNA.

```
ESD3_S1P1     1 ------------------------------------------------------------
Mouse_S1P1  421 CCCAGGCTCCTGGGGACACAATTAGCAGCTATGGTGTCCACTAGCATCCCGGAGGTTAAA

ESD3_S1P1     1 ------------------------------------------------------------
Mouse_S1P1  481 GCTCTCCGCAGCTCAGTCTCTGACTATGGGAACTATGATATCATAGTCCGGCATTACAAC

ESD3_S1P1     1 --------------------TCGGGGCGGAGAAGGACCATGGCATTAAACTGACTTCAGTG
Mouse_S1P1  541 TACACAGGCAAGTTGAACATCGGGGCGGAGAAGGACCATGGCATTAAACTGACTTCAGTG

ESD3_S1P1    42 GTGTTCATTCTCATCTGCTGCTTCATCATCCTAGAGAATATATTTGTCTTGCTAACTATT
Mouse_S1P1  601 GTGTTCATTCTCATCTGCTGCTTCATCATCCTAGAGAATATATTTGTCTTGCTAACTATT

ESD3_S1P1   102 TGGAAAACCAAGAAGTTCCACCGGCCCATGTACTATTTCATAGGCAACCTAGCCCTCTCG
Mouse_S1P1  661 TGGAAAACCAAGAAGTTCCACCGGCCCATGTACTATTTCATAGGCAACCTAGCCCTCTCG

ESD3_S1P1   162 GACCTATTAGCAGGCGTGGCTTACACAGCTAACCTGCTGTTGTCTGGGGCCACCACTTAC
Mouse_S1P1  721 GACCTATTAGCAGGCGTGGCTTACACAGCTAACCTGCTGTTGTCTGGGGCCACCACTTAC

ESD3_S1P1   222 AAGCTCACACCTGCCCAGTGGTTTCTGCGGGAAGGGAGTATGTTTGTGGCTCTCTCTGCA
Mouse_S1P1  781 AAGCTCACACCTGCCCAGTGGTTTCTGCGGGAAGGGAGTATGTTTGTGGCTCTCTCTGCA

ESD3_S1P1   282 TCAGTCTTCAGCCTCCTTGCCATCGCCATTGAGCGCTACATCACCATGCTGAAGATGAAA
Mouse_S1P1  841 TCAGTCTTCAGCCTCCTTGCCATCGCCATTGAGCGCTACATCACCATGCTGAAGATGAAA

ESD3_S1P1   342 CTACACAACGGGAGCAACAGCTCGCGCTCCTTTCTGCTGATCAGCGCCTGCTGGGTCATC
Mouse_S1P1  901 CTACACAACGGGAGCAACAGCTCGCGCTCCTTTCTGCTGATCAGCGCCTGCTGGGTCATC

ESD3_S1P1   402 TCCCTCATCCTGGGGGGCCTGCCCATCATGGGCTGGAACTGCATCAGCTCGCTGTCTAGC
Mouse_S1P1  961 TCCCTCATCCTGGGGGGCCTGCCCATCATGGGCTGGAACTGCATCAGCTCGCTGTCTAGC

ESD3_S1P1   462 TGCTCCACCGTGCTCCCGCTCTACCACAAGCACTATATTCTCTTCTGCACCACCGTCTTC
Mouse_S1P1 1021 TGCTCCACCGTGCTCCCGCTCTACCACAAGCACTATATTCTCTTCTGCACCACCGTCTTC

ESD3_S1P1   522 ACTCTGCTCCTGCTTTCCATCGTCATCCTCTACTGCAGGATCTACTCCTTGGTCAGGACT
Mouse_S1P1 1081 ACTCTGCTCCTGCTTTCCATCGTCATCCTCTACTGCAGGATCTACTCCTTGGTCAGGACT

ESD3 S1P1   582 CGAAGCCGCCGCCTGACCTTCCGCAAGAACATCTCCAA----------------------
Mouse_S1P1 1141 CGAAGCCGCCGCCTGACCTTCCGCAAGAACATCTCCAAGGCCAGTCGCAGTTCTGAGAAG

ESD3_S1P1   642 ------------------------------------------------------------
Mouse_S1P1 1201 TCTCTGGCCTTGCTGAAGACGGTGATCATTGTCCTGAGTGTCTTCATTGCCTGCTGGGCC

ESD3 S1P1   702 --------------------TGTTAGATGTGGGCTGCAAGGCGAAGACCTGTGACATCCTG
Mouse_S1P1 1261 CCTCTCTTCATCCTACTACTGTTAGATGTGGGCTGCAAGGCGAAGACCTGTGACATCCTG

ESD3 S1P1   762 TACAAAGCAGAGTACTTCCTGGTTCTGGCTGTGCTGAACTCAGGTACCAACCCCATCATC
Mouse_S1P1 1321 TACAAAGCAGAGTACTTCCTGGTTCTGGCTGTGCTGAACTCAGGTACCAACCCCATCATC

ESD3_S1P1   822 TACACTCTGACCAACAAGGAGATGCGCCGGGCCTTCATCCGGATCGTATCTTGTTGCAAA
Mouse_S1P1 1381 TACACTCTGACCAACAAGGAGATGCGCCGGGCCTTCATCCGGATCGTATCTTGTTGCAAA

ESD3 S1P1   882 TGCCCCAACGGAGACTCTGCTGGCAAATTCAAGAGGCCCATCATCCCAGGCATGGAATTT
Mouse_S1P1 1441 TGCCCCAACGGAGACTCTGCTGGCAAATTCAAGAGGCCCATCATCCCAGGCATGGAATTT
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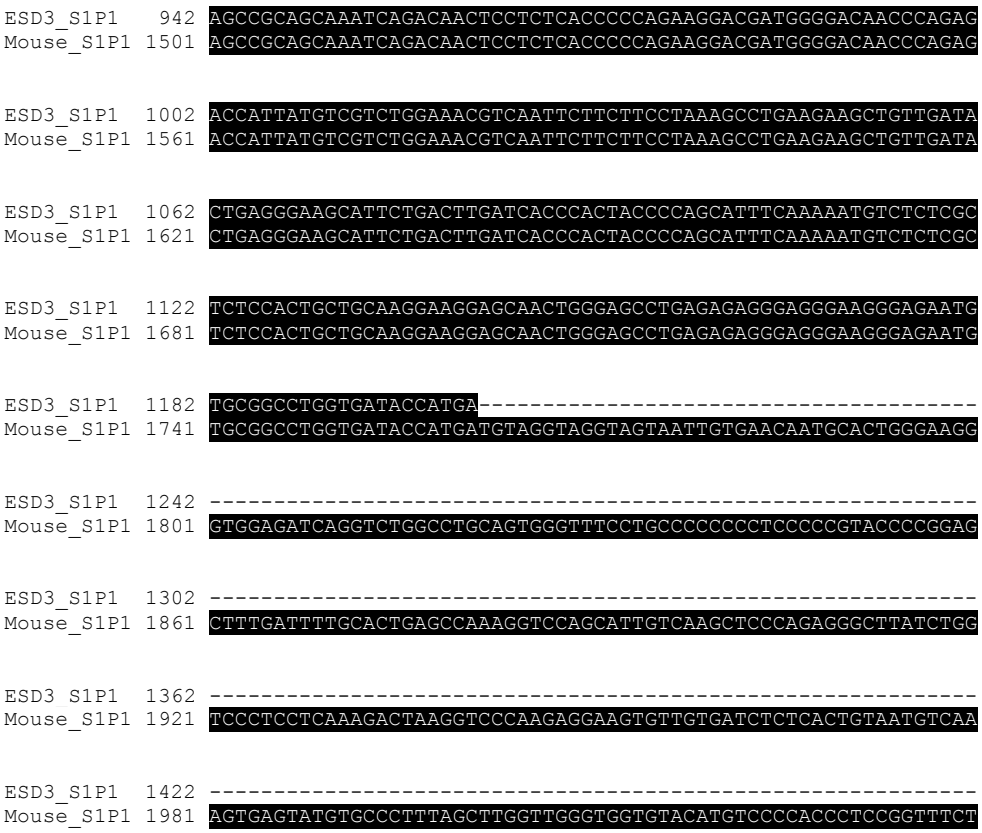
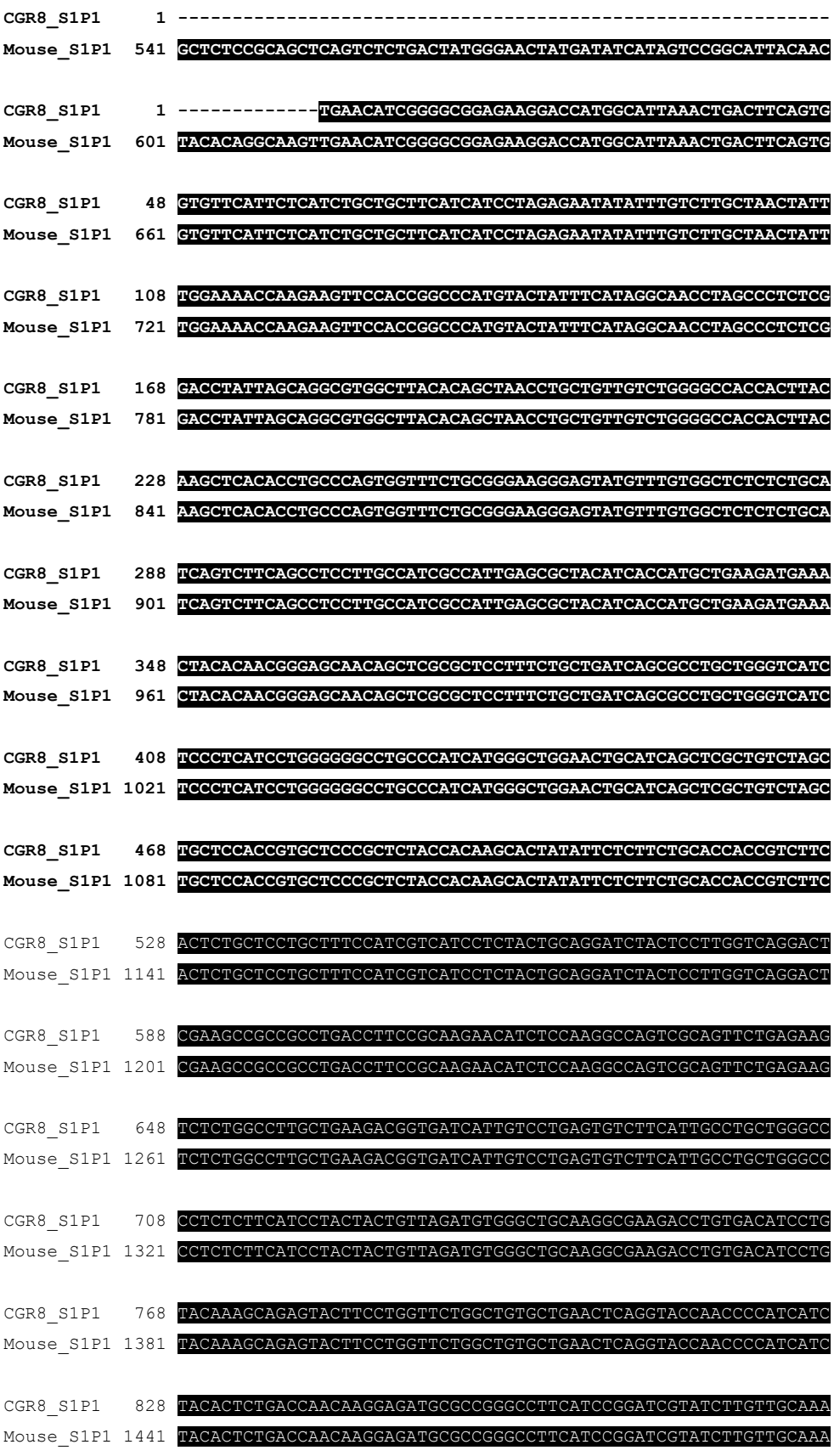


Figure 3.2 Sequence alignment of ES-D3 $S1P_1$ PCR product and mouse $S1P_1$ (Genebank™ accession number NM_007901.4). The 1256bp PCR product amplified by $S1P_1$ primers was purified and sequenced. The nucleotides sequenced using the forward primer or the reverse primer showed 100% identity with that of mouse $S1P_1$.


CGR8_S1P1 1 ----------
Mouse_S1P1 541 GCTCTCCGCAGCTCAGTCTCTGACTATGGGAACTATGATATCATAGTCCGGCATTACAAC
CGR8_S1P1 1 ----------TGAACATCGGGGCGGAGAAGGACCATGGCATTAAACTGACTTCAGTG
Mouse_S1P1 601 TACACAGGCAAGTTGAACATCGGGGCGGAGAAGGACCATGGCATTAAACTGACTTCAGTG
CGR8_S1P1 48 GTGTTCATTCTCATCTGCTGCTTCATCATCCTAGAGAATATATTTGTCTTGCTAACTATT
Mouse_S1P1 661 GTGTTCATTCTCATCTGCTGCTTCATCATCCTAGAGAATATATTTGTCTTGCTAACTATT
CGR8_S1P1 108 TGGAAAACCAAGAAGTTCCACCGGCCCATGTACTATTTCATAGGCAACCTAGCCCTCTCG
Mouse_S1P1 721 TGGAAAACCAAGAAGTTCCACCGGCCCATGTACTATTTCATAGGCAACCTAGCCCTCTCG
CGR8_S1P1 168 GACCTATTAGCAGGCGTGGCTTACACAGCTAACCTGCTGTTGTCTGGGGCCACCACTTAC
Mouse_S1P1 781 GACCTATTAGCAGGCGTGGCTTACACAGCTAACCTGCTGTTGTCTGGGGCCACCACTTAC
CGR8_S1P1 228 AAGCTCACACCTGCCCAGTGGTTTCTGCGGGAAGGGAGTATGTTTGTGGCTCTCTCTGCA
Mouse_S1P1 841 AAGCTCACACCTGCCCAGTGGTTTCTGCGGGAAGGGAGTATGTTTGTGGCTCTCTCTGCA
CGR8_S1P1 288 TCAGTCTTCAGCCTCCTTGCCATCGCCATTGAGCGCTACATCACCATGCTGAAGATGAAA
Mouse_S1P1 901 TCAGTCTTCAGCCTCCTTGCCATCGCCATTGAGCGCTACATCACCATGCTGAAGATGAAA
CGR8_S1P1 348 CTACACAACGGGAGCAACAGCTCGCGCTCCTTTCTGCTGATCAGCGCCTGCTGGGTCATC
Mouse_S1P1 961 CTACACAACGGGAGCAACAGCTCGCGCTCCTTTCTGCTGATCAGCGCCTGCTGGGTCATC
CGR8_S1P1 408 TCCCTCATCCTGGGGGGCCTGCCCATCATGGGCTGGAACTGCATCAGCTCGCTGTCTAGC
Mouse_S1P1 1021 TCCCTCATCCTGGGGGGCCTGCCCATCATGGGCTGGAACTGCATCAGCTCGCTGTCTAGC
CGR8_S1P1 468 TGCTCCACCGTGCTCCCGCTCTACCACAAGCACTATATTCTCTTCTGCACCACCGTCTTC
Mouse_S1P1 1081 TGCTCCACCGTGCTCCCGCTCTACCACAAGCACTATATTCTCTTCTGCACCACCGTCTTC
CGR8_S1P1 528 ACTCTGCTCCTGCTTTCCATCGTCATCCTCTACTGCAGGATCTACTCCTTGGTCAGGACT
Mouse_S1P1 1141 ACTCTGCTCCTGCTTTCCATCGTCATCCTCTACTGCAGGATCTACTCCTTGGTCAGGACT
CGR8_S1P1 588 CGAAGCCGCCGCCTGACCTTCCGCAAGAACATCTCCAAGGCCAGTCGCAGTTCTGAGAAG
Mouse_S1P1 1201 CGAAGCCGCCGCCTGACCTTCCGCAAGAACATCTCCAAGGCCAGTCGCAGTTCTGAGAAG
CGR8_S1P1 648 TCTCTGGCCTTGCTGAAGACGGTGATCATTGTCCTGAGTGTCTTCATTGCCTGCTGGGCC
Mouse_S1P1 1261 TCTCTGGCCTTGCTGAAGACGGTGATCATTGTCCTGAGTGTCTTCATTGCCTGCTGGGCC
CGR8_S1P1 708 CCTCTCTTCATCCTACTACTGTTAGATGTGGGCTGCAAGGCGAAGACCTGTGACATCCTG
Mouse_S1P1 1321 CCTCTCTTCATCCTACTACTGTTAGATGTGGGCTGCAAGGCGAAGACCTGTGACATCCTG
CGR8_S1P1 768 TACAAAGCAGAGTACTTCCTGGTTCTGGCTGTGCTGAACTCAGGTACCAACCCCATCATC
Mouse_S1P1 1381 TACAAAGCAGAGTACTTCCTGGTTCTGGCTGTGCTGAACTCAGGTACCAACCCCATCATC
CGR8_S1P1 828 TACACTCTGACCAACAAGGAGATGCGCCGGGCCTTCATCCGGATCGTATCTTGTTGCAAA
Mouse_S1P1 1441 TACACTCTGACCAACAAGGAGATGCGCCGGGCCTTCATCCGGATCGTATCTTGTTGCAAA

```
CGR8_S1P1    888 TGCCCCAACGGAGACTCTGCTGGCAAATTCAAGAGGCCCATCATCCCAGGCATGGAATTT
Mouse_S1P1  1501 TGCCCCAACGGAGACTCTGCTGGCAAATTCAAGAGGCCCATCATCCCAGGCATGGAATTT

CGR8_S1P1    948 AGCCGCAGCAAATCAGACAACTCCTCTCACCCCCAGAAGGACGATGGGGACAACCCAGAG
Mouse_S1P1  1561 AGCCGCAGCAAATCAGACAACTCCTCTCACCCCCAGAAGGACGATGGGGACAACCCAGAG

CGR8_S1P1   1008 ACCATTATGTCGTCTGGAAACGTCAATTCTTCTTCCTAAAGCCTGAAGAAGCTGTTGATA
Mouse_S1P1  1621 ACCATTATGTCGTCTGGAAACGTCAATTCTTCTTCCTAAAGCCTGAAGAAGCTGTTGATA

CGR8_S1P1   1068 CTGAGGGAAGCATTCTGACTTGATCACCCACTACCCCAGCATTTCAAAAATGTCTCTCGC
Mouse_S1P1  1681 CTGAGGGAAGCATTCTGACTTGATCACCCACTACCCCAGCATTTCAAAAATGTCTCTCGC

CGR8_S1P1   1128 TCTCCACTGCTGCAAGGAAGGAGCAACTGGGAGCCTGAGAGAGGGAGGGAAGGGAGAATG
Mouse_S1P1  1741 TCTCCACTGCTGCAAGGAAGGAGCAACTGGGAGCCTGAGAGAGGGAGGGAAGGGAGAATG

CGR8_S1P1   1188 TGCGGCCTGGTGATACCATGATGTAGG--------------------------------
Mouse_S1P1  1801 TGCGGCCTGGTGATACCATGATGTAGGTAGGTAGTAATTGTGAACAATGCACTGGGAAGG
```

Figure 3.3 Sequence alignment of CGR8 $S1P_1$ PCR product and mouse $S1P_1$ (Genebank™ accession number NM_007901.4). The 1256bp PCR product amplified by $S1P_1$ primers was purified and sequenced. The 1214 nucleotides sequenced showed 100% identity with that of mouse $S1P_1$.

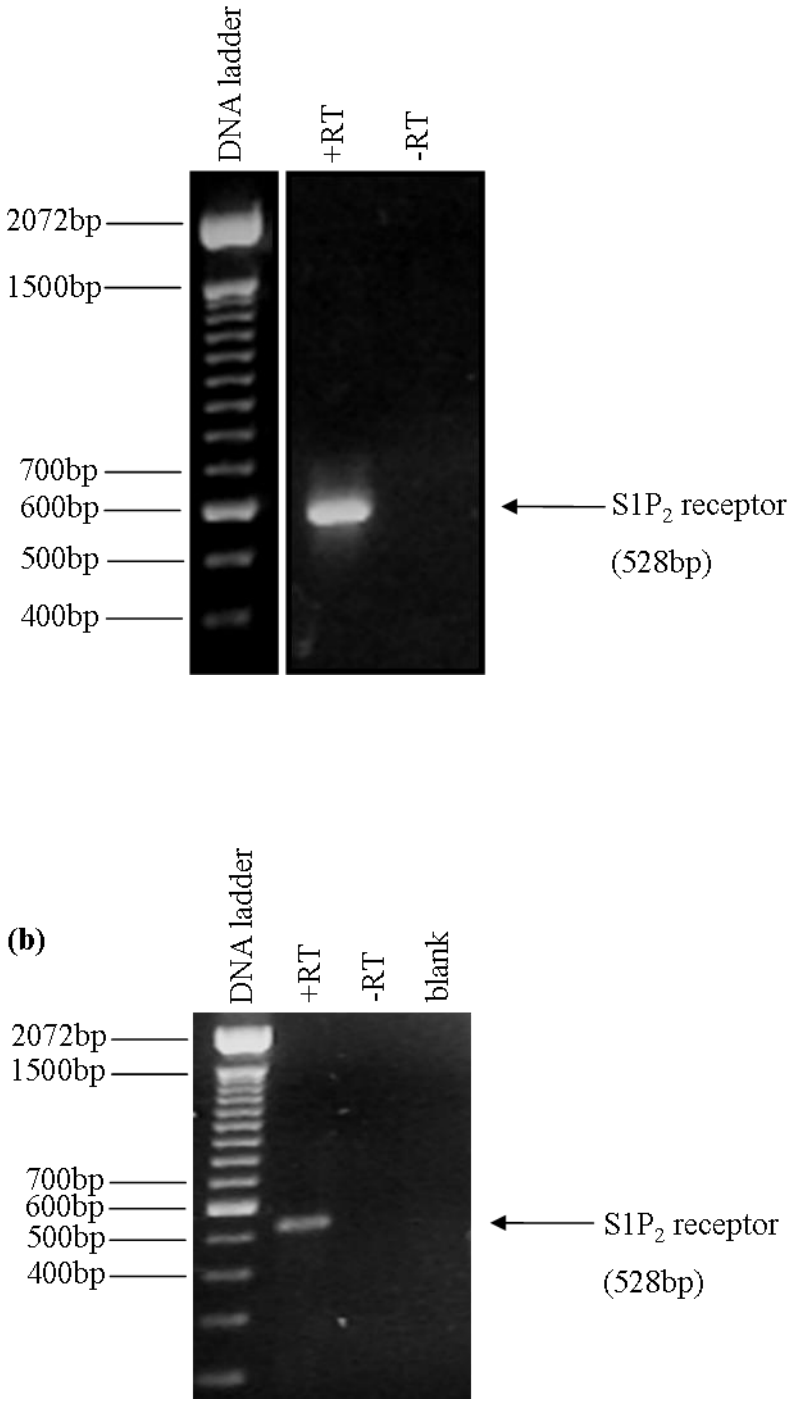


Figure 3.4 RT-PCR analysis of $S1P_2$ receptor transcript in (a) ESD3 cells and (b) CGR8 cells. RT-PCR analysis showed amplification of a 528bp product using $S1P_2$ receptor primers in both cell lines. A negative control with omission of reverse transcriptase (-RT) was included. Blank is the negative control without cDNA.

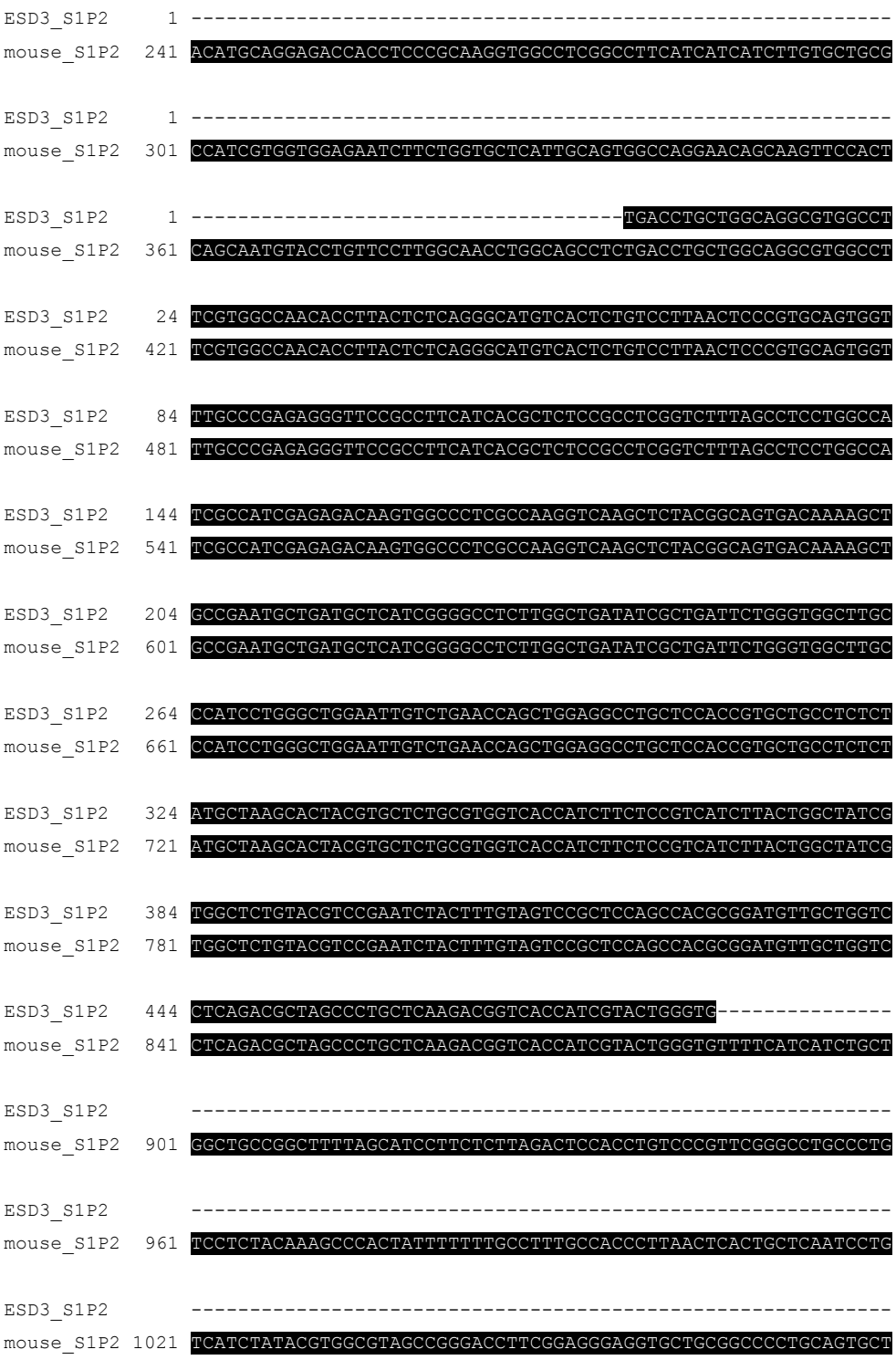


Figure 3.5 Sequence alignment of ES-D3 $S1P_2$ with PCR product and mouse $S1P_2$ (Genebank™ accession number NM_010333.3). The 528bp PCR product amplified using $S1P_2$ primers was purified and sequenced. The 485 nucleotides sequenced, showed 100% sequence identity with that of mouse $S1P_2$.

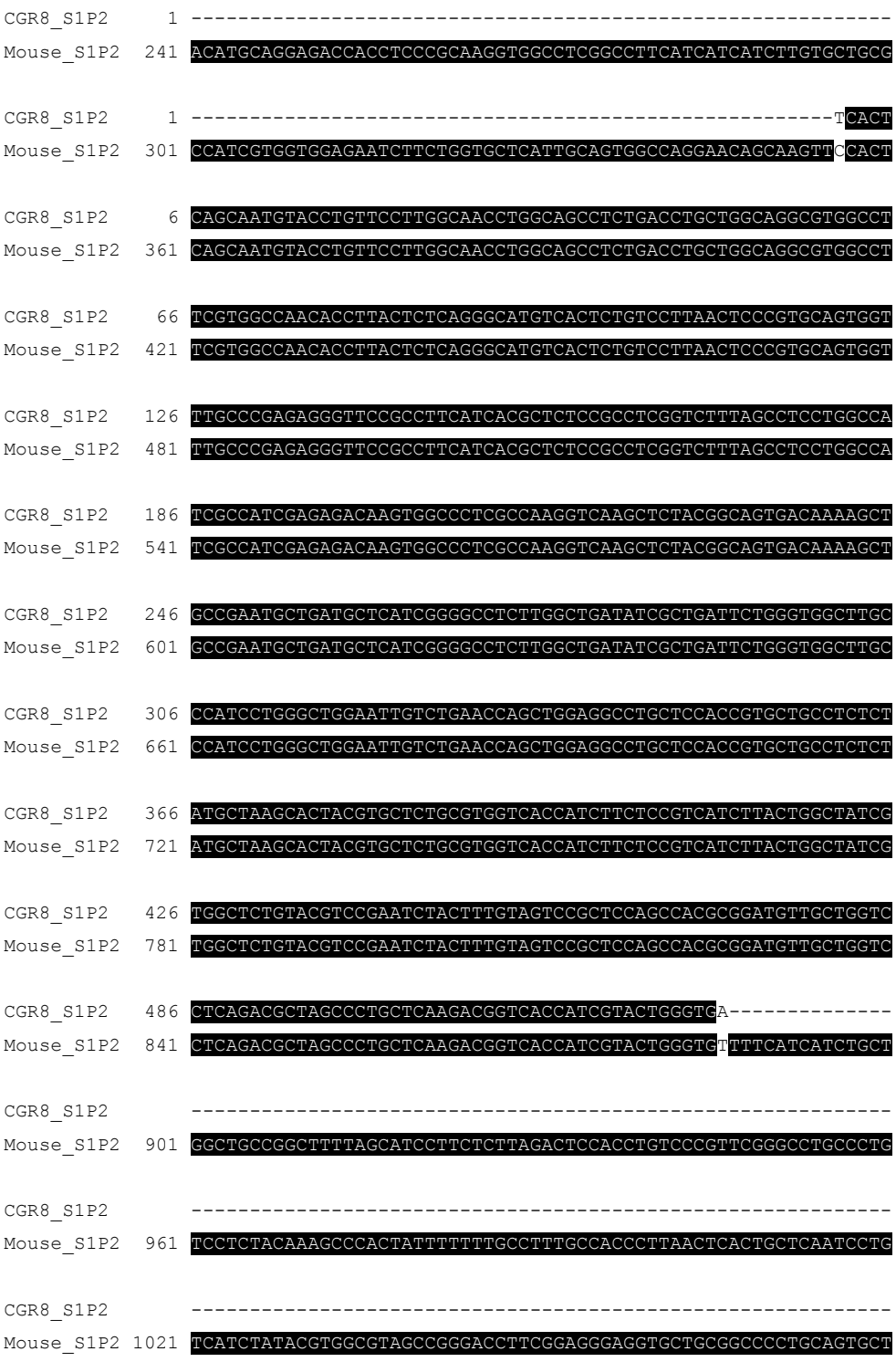


Figure 3.6 Sequence alignment of CGR8 $S1P_2$ PCR product and mouse $S1P_2$ (Genebank™ accession number NM_010333.3). The 528bp PCR product amplified using $S1P_2$ was purified and sequenced. The nucleotides sequenced showed 100% identity with that of mouse $S1P_2$.

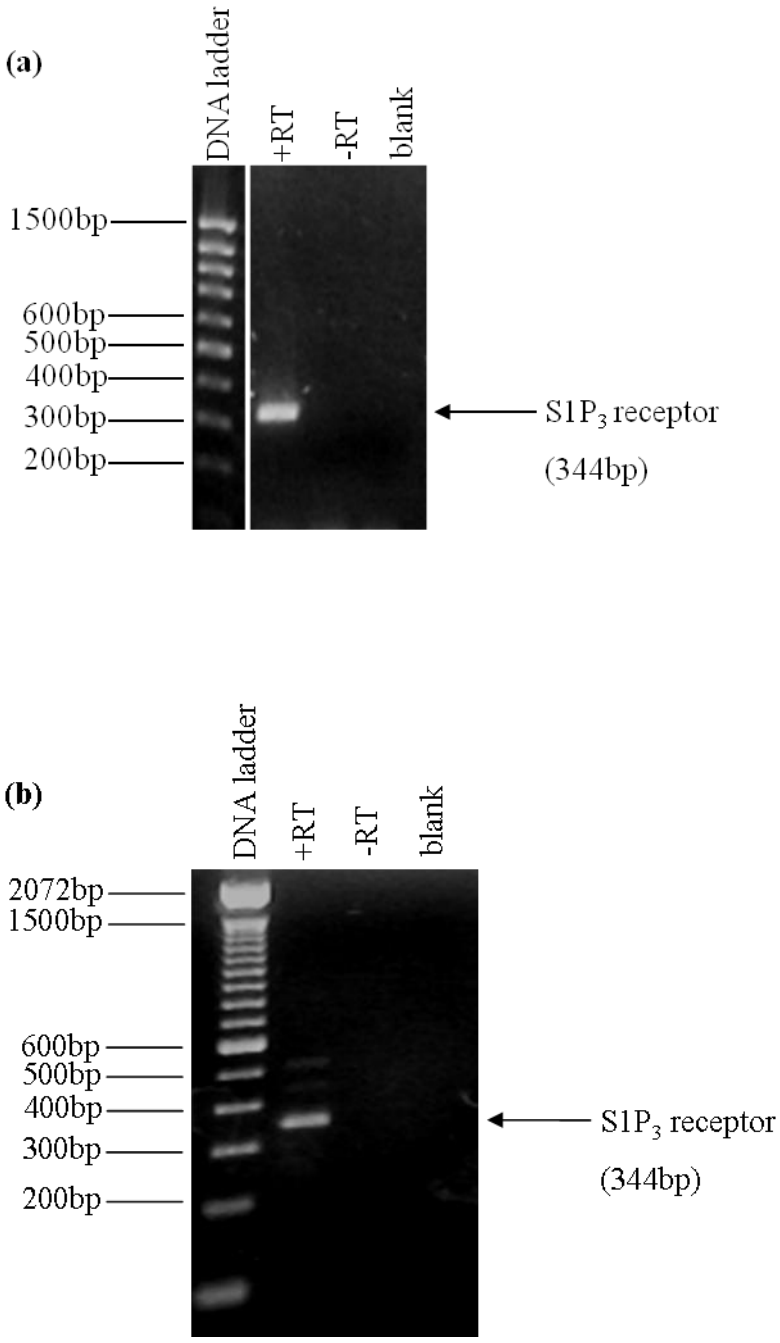


Figure 3.7 RT-PCR analysis of $S1P_3$ receptor mRNA transcript in (a) ESD3 cells and (b) CGR8 cells. RT-PCR amplified a 344bp product using $S1P_3$ receptor primers. A negative control with omission of reverse transcriptase (-RT) was included. Blank is the negative control without cDNA.

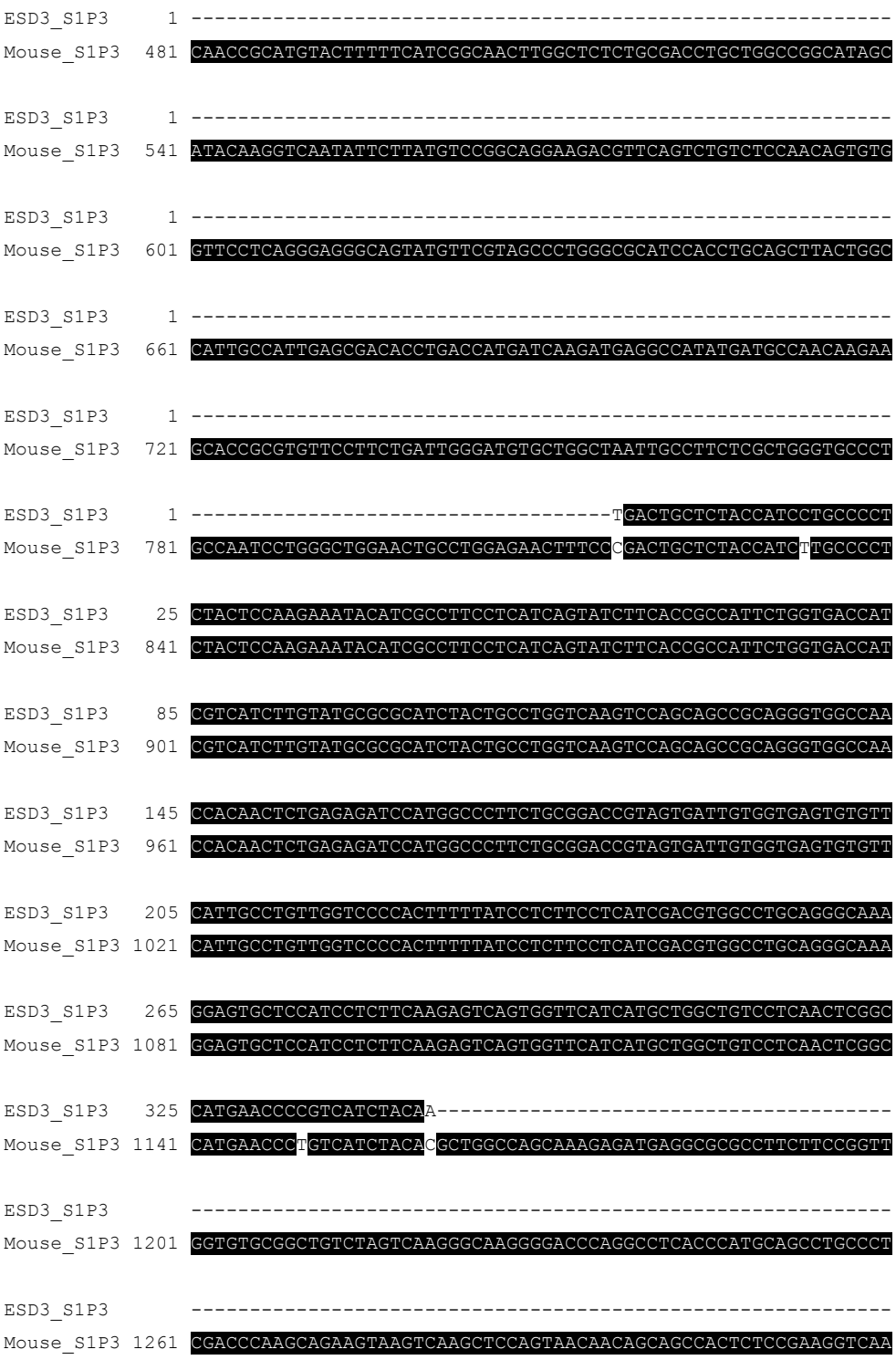


Figure 3.8 Nucelotide sequence alignment of ES-D3 $S1P_3$ against mouse $S1P_3$ (Genebank accession number NM_010101.3). The 344bp PCR product amplified by $S1P_3$ primers was purified and sequenced. The nucleotides sequenced showed 100% identity with that of mouse $S1P_3$.

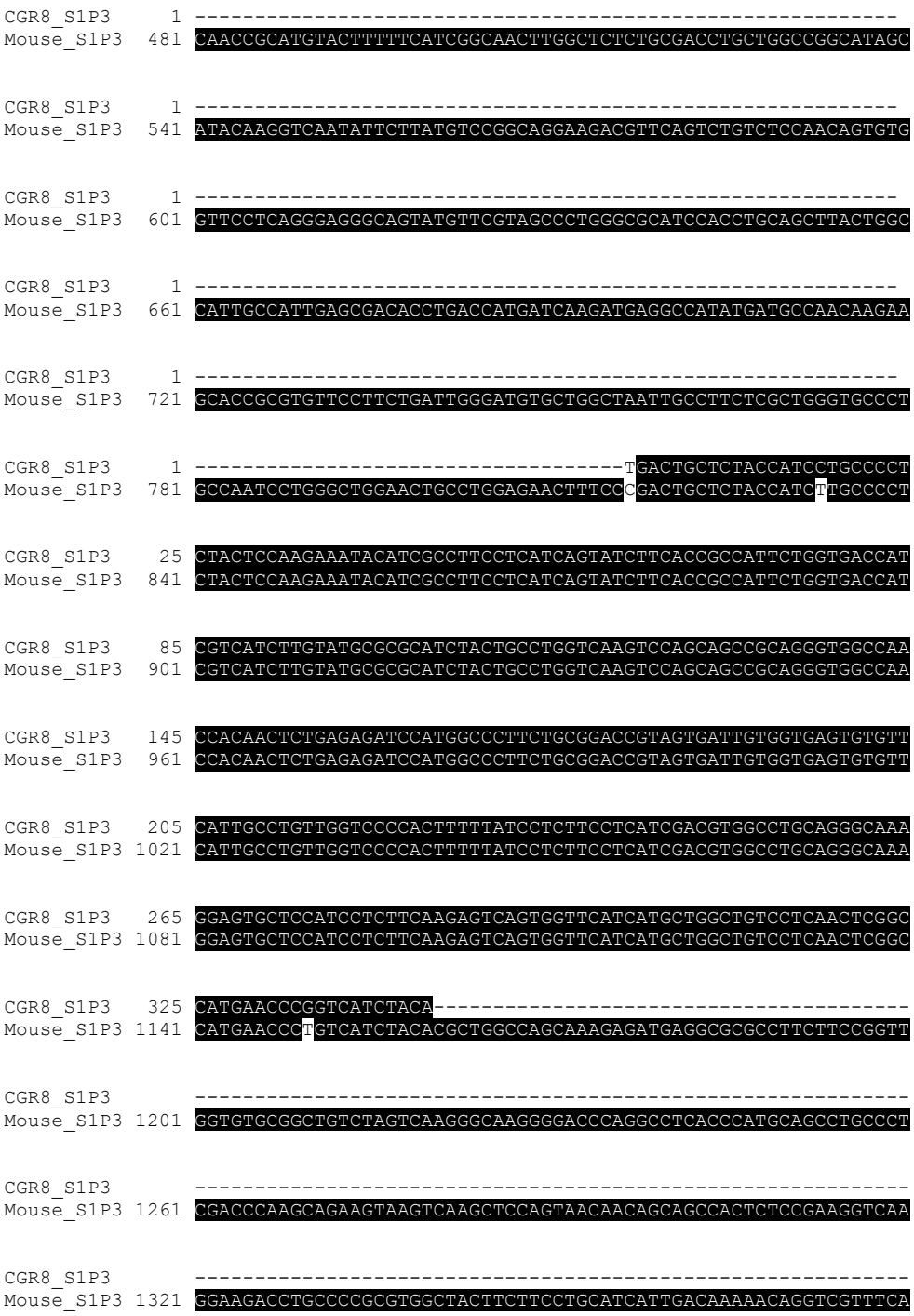


Figure 3.9 Sequence alignment of CGR8 $S1P_3$ with mouse $S1P_3$ (Genebank accession number NM_010101.3). The 344bp PCR product amplified by $S1P_3$ primers was purified and sequenced. The sequenced nucleotides showed 100% identity with that of mouse $S1P_3$.

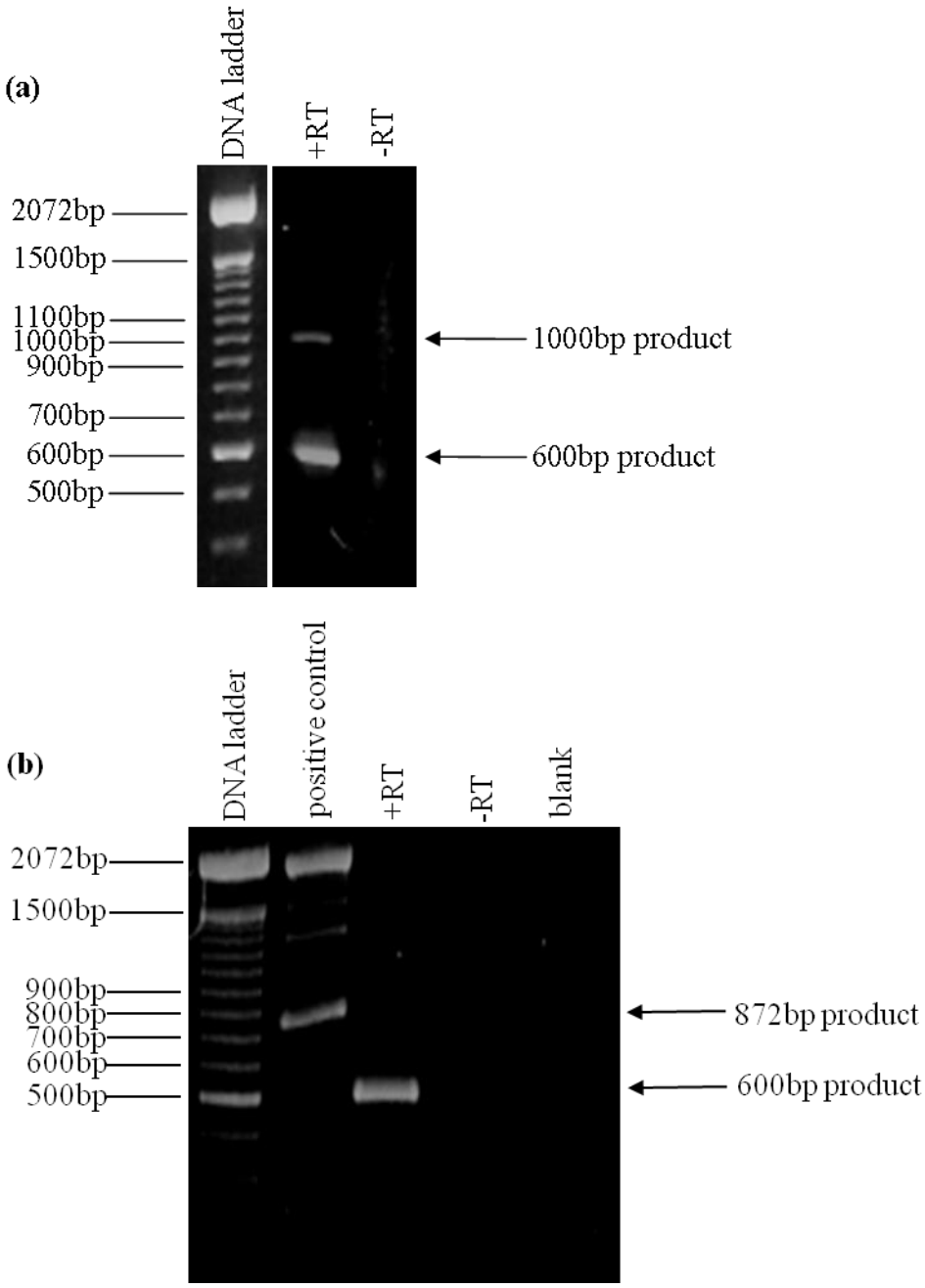


Figure 3.10 RT-PCR analysis of $S1P_4$ receptor mRNA transcript in (a) ES-D3 cells and (b) CGR8 cells. (a) RT-PCR amplified a ~600bp and ~1000bp product from ES-D3 cells using $S1P_4$ receptor primers. (b) RT-PCR amplified a ~600bp product from CGR8 cells. A positive control (pcDNA3.1-HA-$S1P_4$) was included which amplified a product of the predicted size, 872bp. A negative control with omission of reverse transcriptase (-RT) was included. Blank is the negative control without cDNA.

3.2.2 **PDGFβ receptor mRNA transcript expression**

PDGFβ receptor primers (**Table 2**) amplified a 481bp PCR product in both ES-D3 [**Figure 3.11(a)**] and CGR8 cells [**Figure 3.11(b)**]. Nucleotide sequencing of the purified band from ES-D3 cells and subsequent alignment showed that the 481 nucleotides sequenced completely matched that of mouse PDGFβ receptor sequence [**Figure 3.12**].

3.2.3 **OCT-4 mRNA transcript expression**

The undifferentiated state of the mouse ES cells used in this study was confirmed by examining the expression of the undifferentiated stem cell marker OCT-4. RT-PCR was used to analyse OCT-4 gene expression. OCT-4 primers (**Table 2**) amplified a 201bp product from ES-D3 at passage 3, passage 6 and passage 9 [**Figure 3.13(a)**]. RT-PCR analysis also amplified a 201bp product from CGR8 cells at passage 2 and passage 5 [**Figure 3.13(b)**]. The band from both ES-D3 cells and CGR8 cells was purified and sequenced. Nucleotide sequencing revealed that all 201 nucleotides matched the mouse OCT-4 sequence in both ES-D3 [**Figure 3.14**] and CGR8 cells [**Figure 3.15**] confirming the presence of the pluripotent transcription factor OCT-4 at all passages tested.

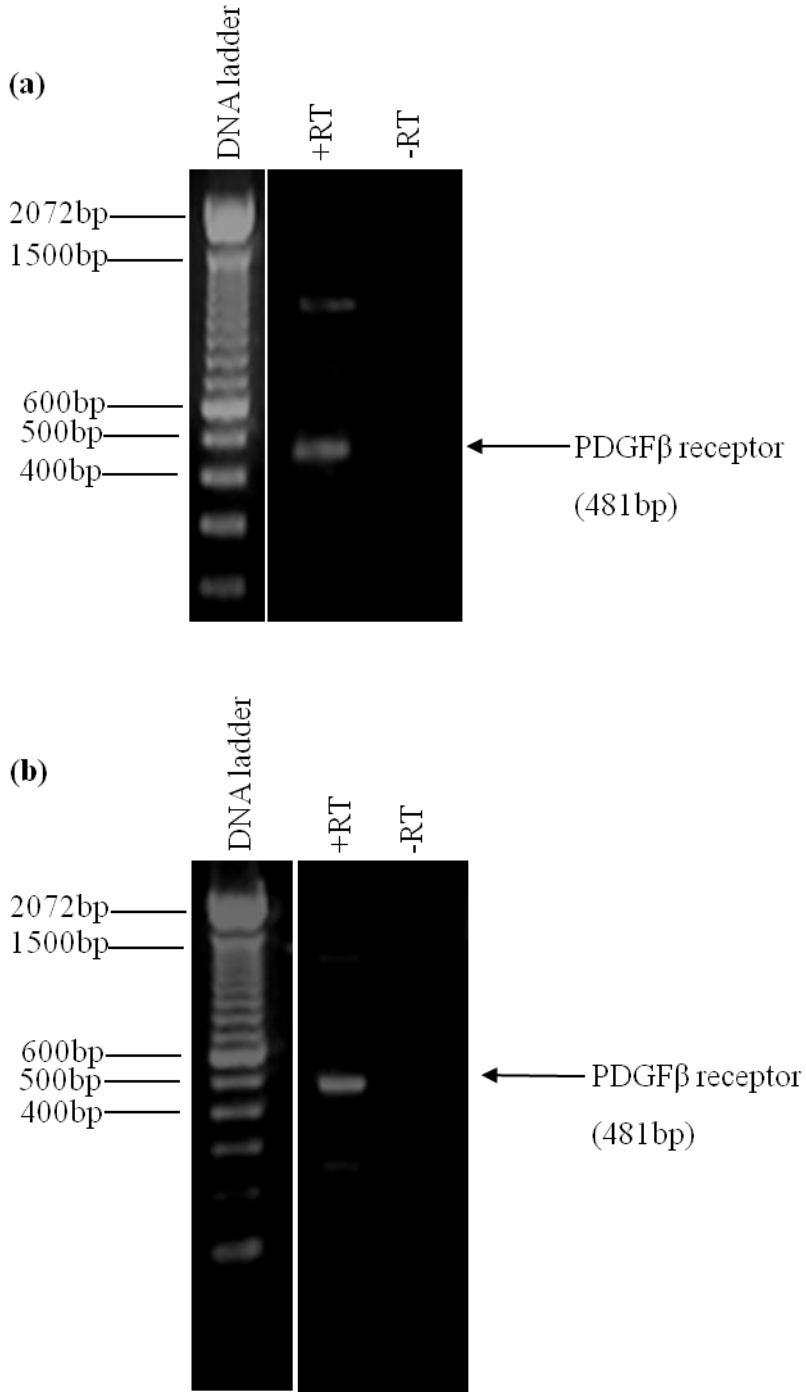


Figure 3.11 RT-PCR analysis of PDGFβ receptor mRNA transcript expression in (a) ES-D3 and (b) CGR8 cells. RT-PCR with PDGFβ receptor primers amplified a 481bp product. A negative control with the omission of reverse transcriptase was included (-RT). Blank is the negative control without cDNA.

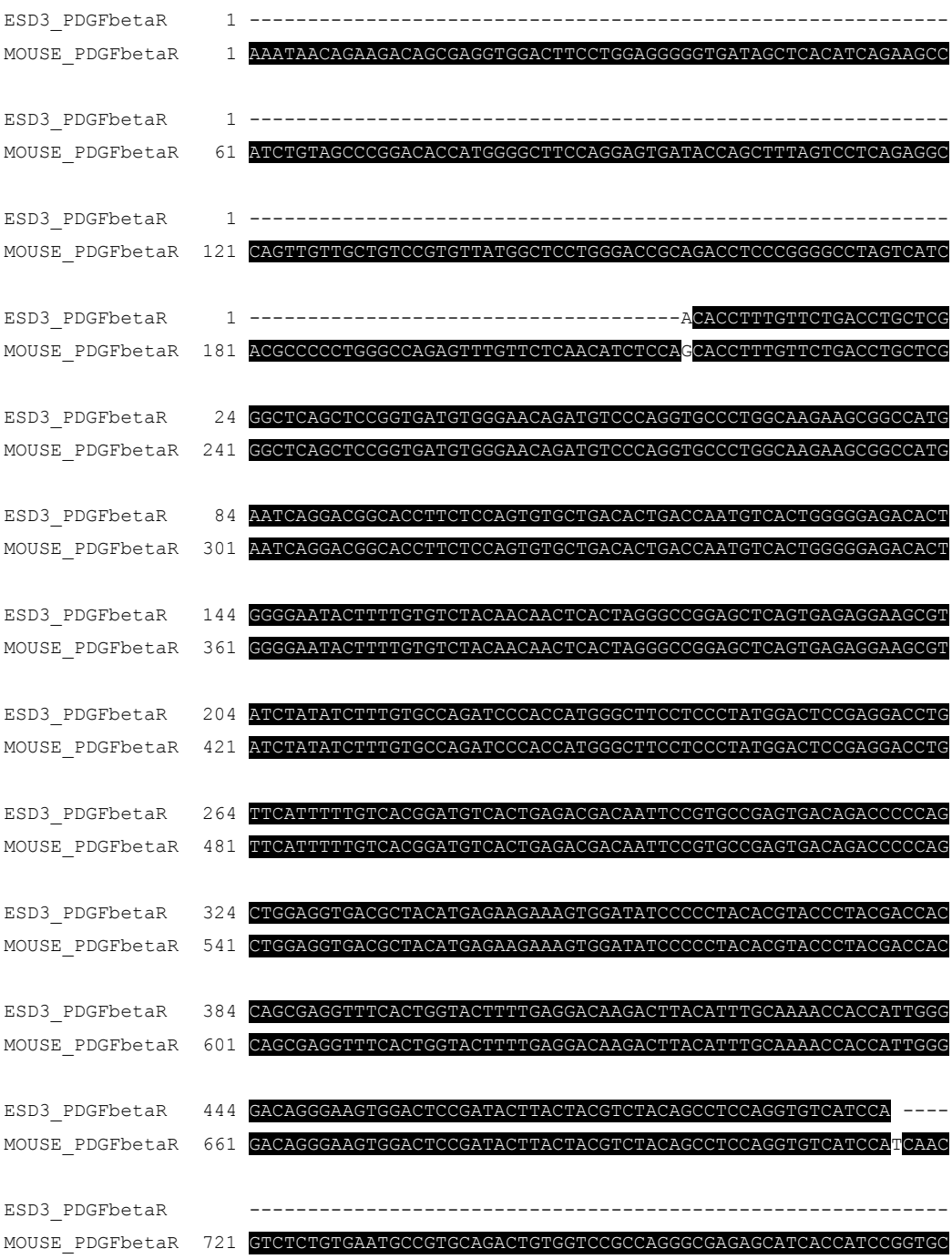


Figure 3.12 Sequence alignment of ES-D3 PDGFβ receptor PCR sequence with mouse PDGFβ receptor (Genebank™ accession number NM_008809.1). The 481bp PCR product amplified using PDGFβ receptor primers was purified and sequenced. The nucleotides sequenced showed 100% identity with that of mouse PDGFβ receptor.

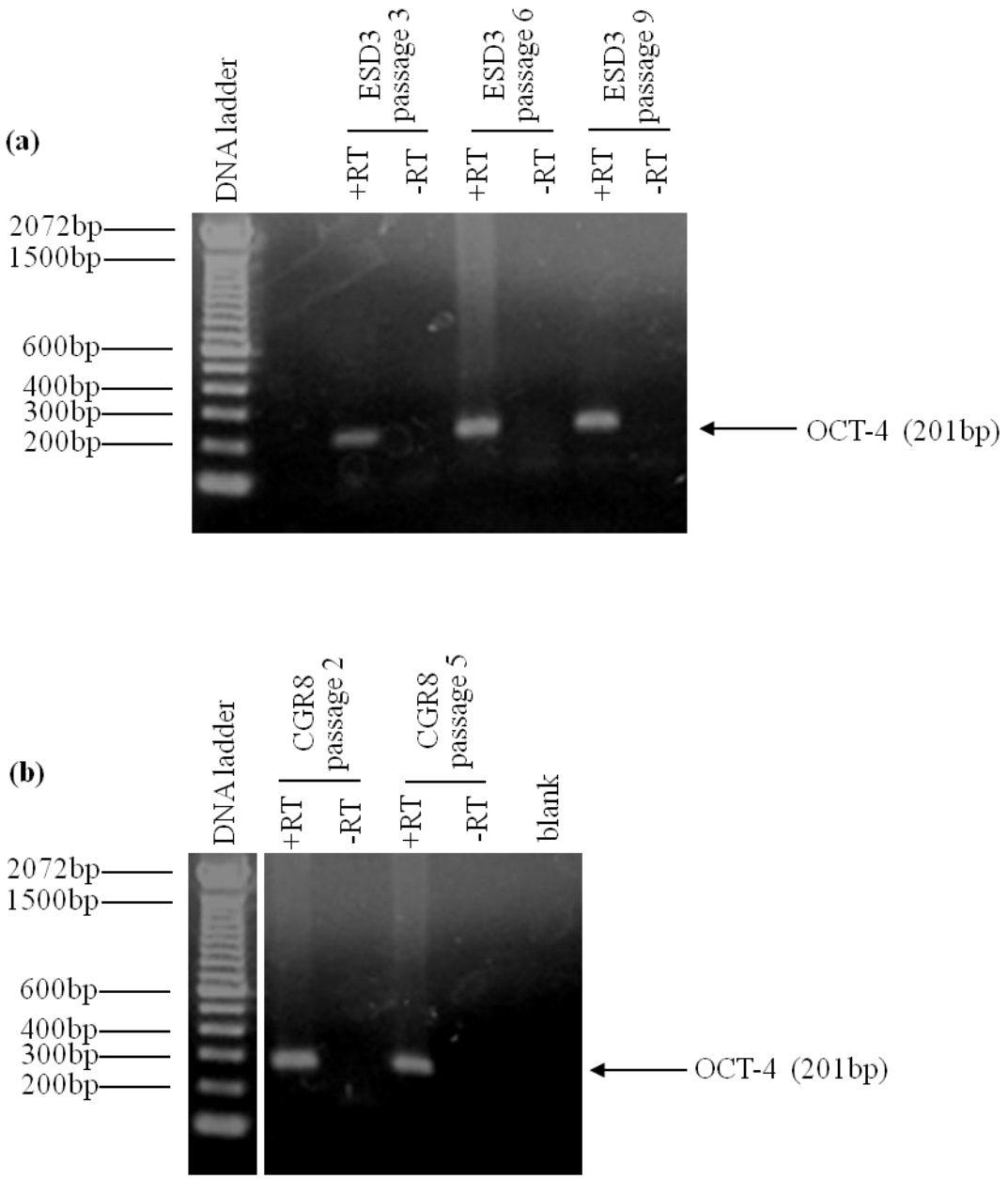


Figure 3.13 RT-PCR analysis of OCT-4 mRNA transcript expression in (a) ES-D3 and (b) CGR8 cells over a number of passages. RT-PCR with OCT-4 primers amplified a 201bp product. A negative control with the omission of reverse transcriptase was included (-RT). Blank is the negative control without cDNA.

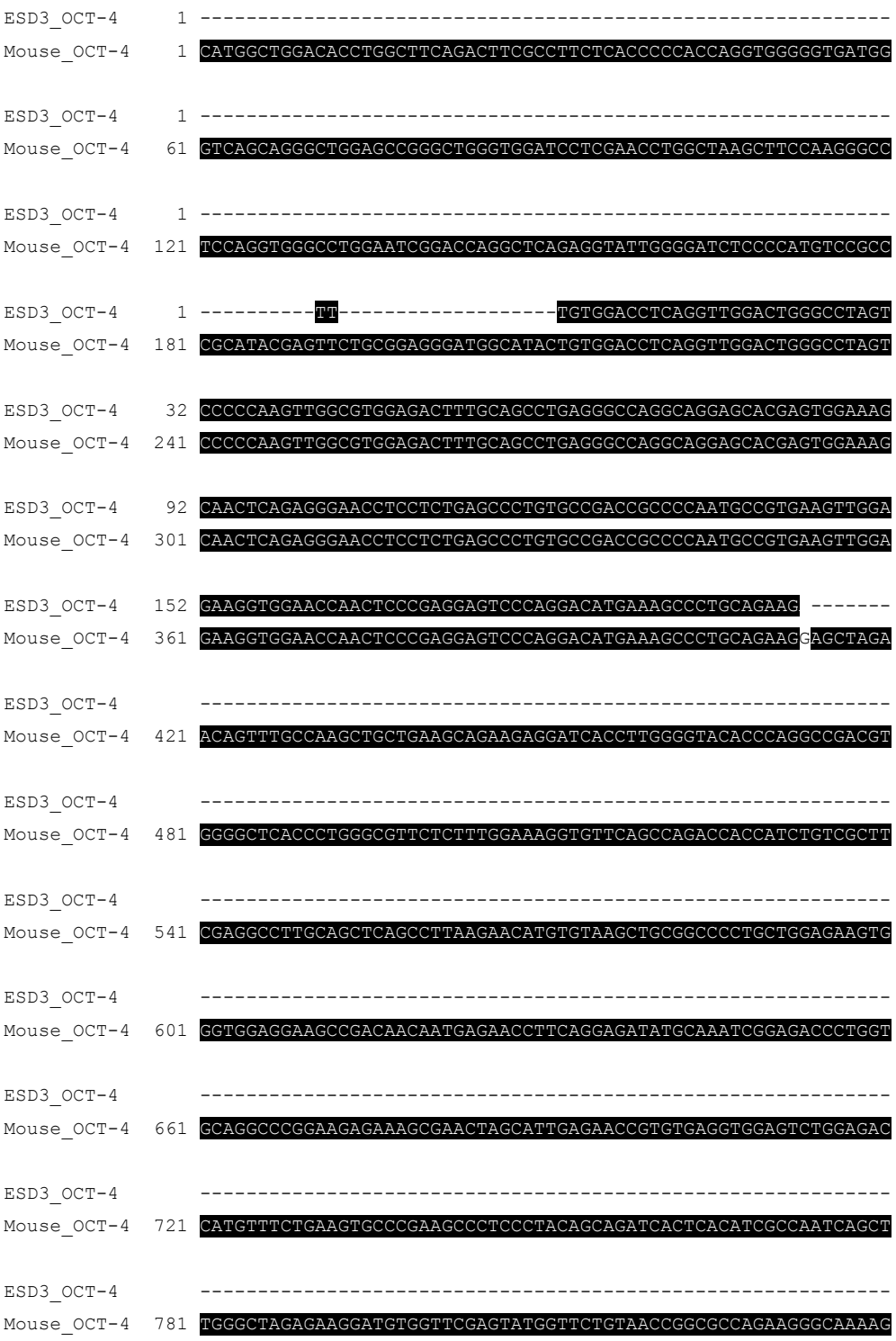


Figure 3.14 Nucleotide sequence alignment of ES-D3 OCT-4 against mouse OCT-4 (Genebank™ accession number NM_013633.2). The 201bp PCR product amplified by OCT-4 primers was purified and sequenced. The nucleotides sequenced showed 100% sequence homology with that of mouse OCT-4.

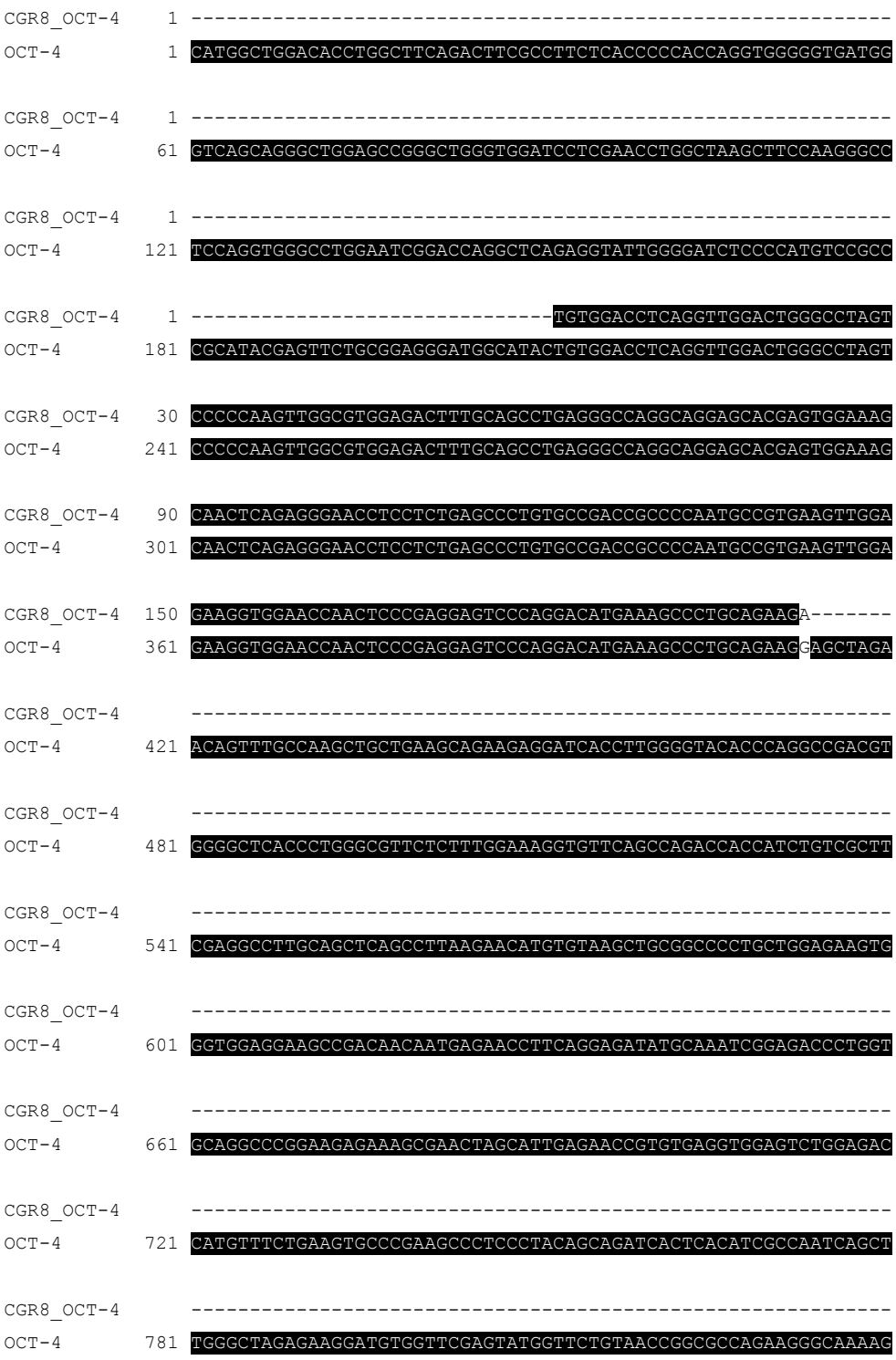


Figure 3.15 Nucleotide sequence alignment of CGR8 OCT-4 against mouse OCT-4 (Genebank™ accession number NM_013633.2). The 201bp PCR product amplified by OCT-4 primers was purified and sequenced. The nucleotides sequenced showed 100% sequence homology with that of mouse OCT-4.

3.2.4 **Protein expression**

3.2.4.6 **$S1P_5$ receptor protein expression**

$S1P_5$ receptor protein expression in ESD3 and CGR8 cells was studied by Western blot analysis as outlined in section 2.4. Western blot analysis of ES-D3 lysates with anti-$S1P_5$ (EDG-8) antibody detected a major band at approximately 42kDa [**Figure 3.16(a)**]. Western blotting with anti-$S1P_5$ antibody detected several protein bands in CGR8 cell lysates (31-58 kDa) [**Figure 3.16(b)**]. The major band was 42 kDa, with minor bands detected at ~32 kDa, 50 kDa and 58 kDa.

3.2.4.7 **PDGFβ receptor**

Western blot analysis of ES-D3 cell lysates using anti-PDGFβ receptor antibody was carried out to investigate protein expression of PDGFβ receptor in ES-D3 and CGR8 cells. Cell lysate from ES-D3 cells were run alongside lysates from HEK cells transiently transfected with PDGFβ receptor plasmid (positive control). The anti-PDGFβ receptor antibody detected a prominent band of ~190 kDa in the positive control and a weaker band of ~190 kDa in ES-D3 cell lysates [**Figure 3.17(a)**], suggesting expression of PDGFβ receptor in these cells. The anti-PDGFβ receptor antibody detected non-specific weak bands of approximately 70 kDa and 42 kDa in CGR8 lysates [**Figure 3.17(b)]**, which suggests that these cells do not express PDGFβ receptor.

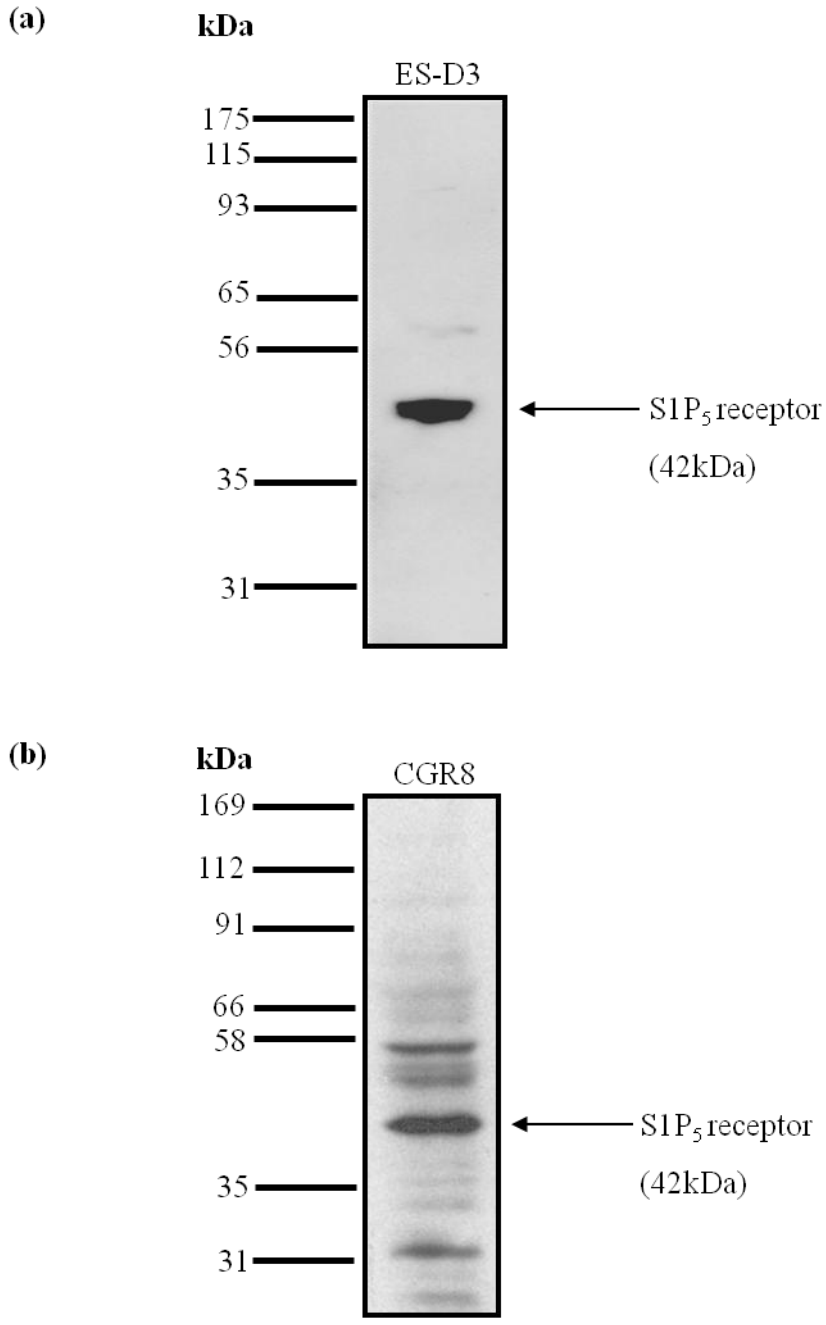


Figure 3.16 Western blot analysis of $S1P_5$ protein expression in ES-D3 (a) and CGR8 (b) cells. (a) Anti-$S1P_5$ receptor antibody detected a major 42 kDa band in ES-D3 cells. **(b)** In addition to a major 42 kDa detected in CGR8 cells, minor bands of ~32 kDa, 50 kDa and 58 kDa were also detected.

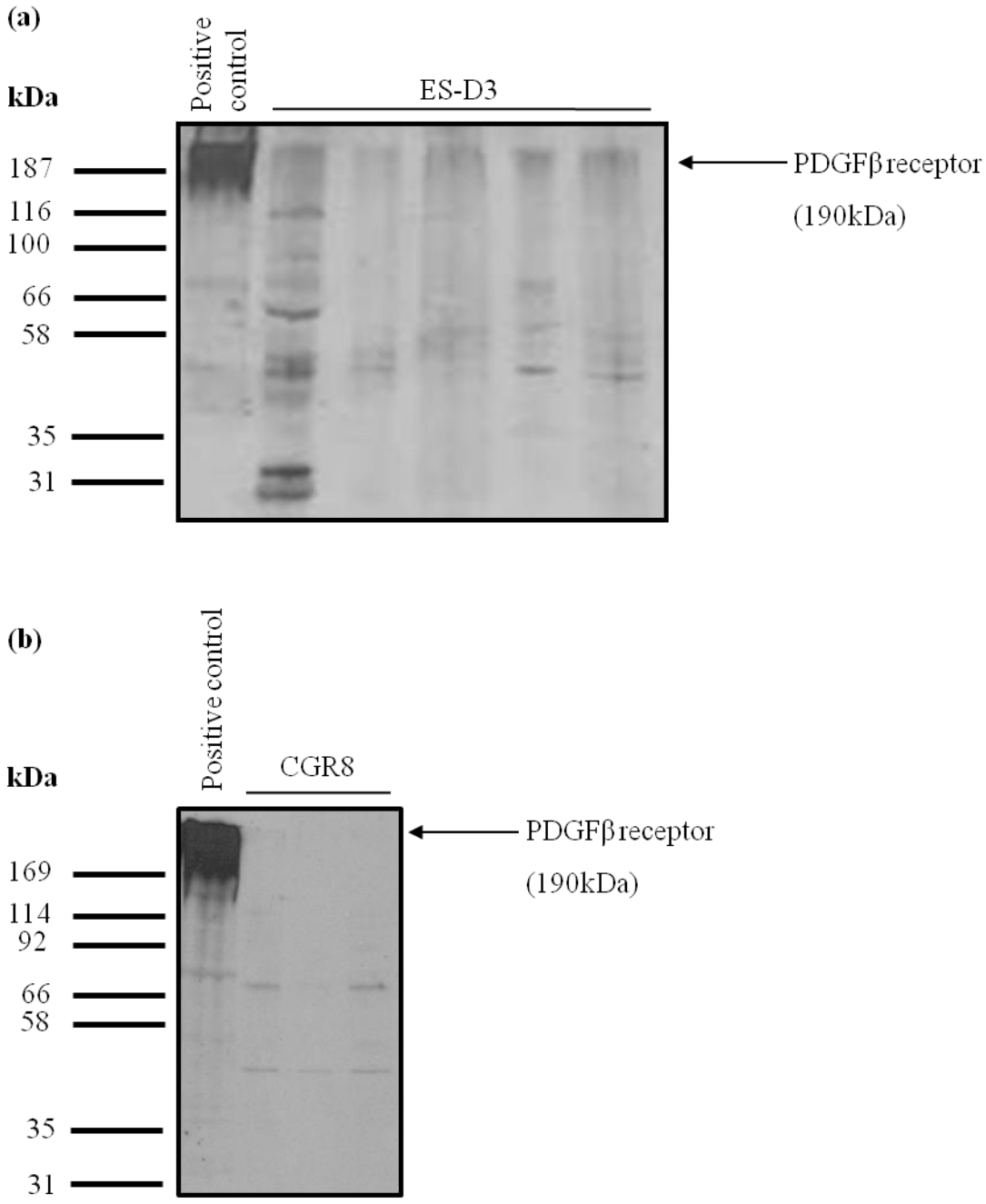


Figure 3.17 Western blot analysis of PDGFβ receptor protein expression in ES-D3 (a) and CGR8 cell lysates (b). Anti-PDGFβ receptor antibody detected weak immunoreactive bands of ~190 kDa in ES-D3 cells (a) corresponding to the predicted size of PDGFβ receptor and the positive control (PDGFβ receptor oveexpressed in HEk293 cells). Immunoreactive bands of ~70 kDa and 45kDa were detected by the anti-PDGFβ receptor antibody in CGR8 cells (b).

3.3 Characterisation of p42/p44 MAPK stimulation by S1P and PDGF in ES-D3 cells

The responsiveness of ES-D3 cells and CGR8 cells to S1P and PDGF was initially investigated. It was confirmed in the current study that S1P invoked a p42/p44 MAPK response in undifferentiated, proliferating ES-D3 cells [**Figure 3.18(a)**] and CGR8 cells [**Figure 3.18(b)**] compared to vehicle (DMSO) alone. In addition, stimulation of ES-D3 cells with 10% FCS and PMA (1μM) for 10 minutes invoked p42/p44 MAPK activation over control conditions. [**Figure 3.18(c**)]. In both cell types, S1P induced dose-dependent p42/p44 MAPK activation [**Figure 3.19(a)** and **Figure 3.19(b)**]. From these experiments, it was decided that the concentration of S1P that demonstrated the optimum p42/p44 MAPK activation (i.e. 5μM) should be used in subsequent experiments. ES-D3 and CGR8 cells were unresponsive to two different PDGF isoforms in terms of p42/p44 MAPK activation. **Figure 3.20(a)** and **Figure 3.21(a)** show that ES-D3 cells were unresponsive to both PDGF-AB and PDGF-BB respectively, in that no detectable activation of p42/p44 MAPK was observed after 10 minute treatments with each agonist. Similar results were also observed in CGR8 cells [**Figure 3.20 (b) and Figure 3.21(b)**] suggesting a lack of PDGF receptor expression in these cells.

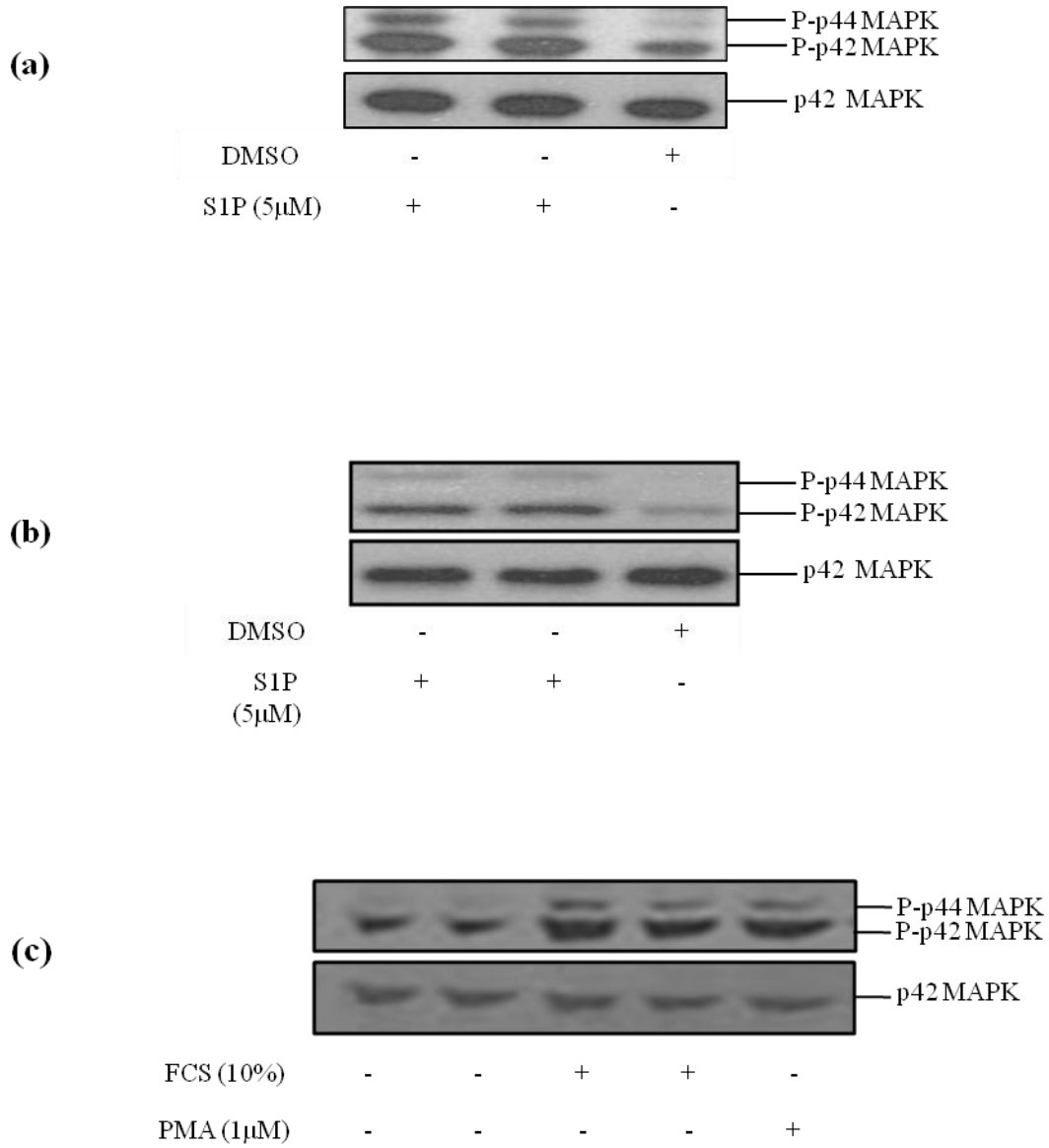


Figure 3.18 Stimulation of p42/p44 MAPK in ES-D3 and CGR8 cells by S1P. Quiesced ES-D3 cells (a) or CGR8 cells (b) were treated with S1P (5μM) or vehicle (DMSO) for 10 minutes. (c) Quiesced ES-D3 cells were unstimulated or treated with FCS (10%) or PMA (1μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panel). Blots were stripped and reprobed with antibody specific for p42 MAPK to establish equal protein loading (bottom panel). Results are representative of an experiment performed three times.

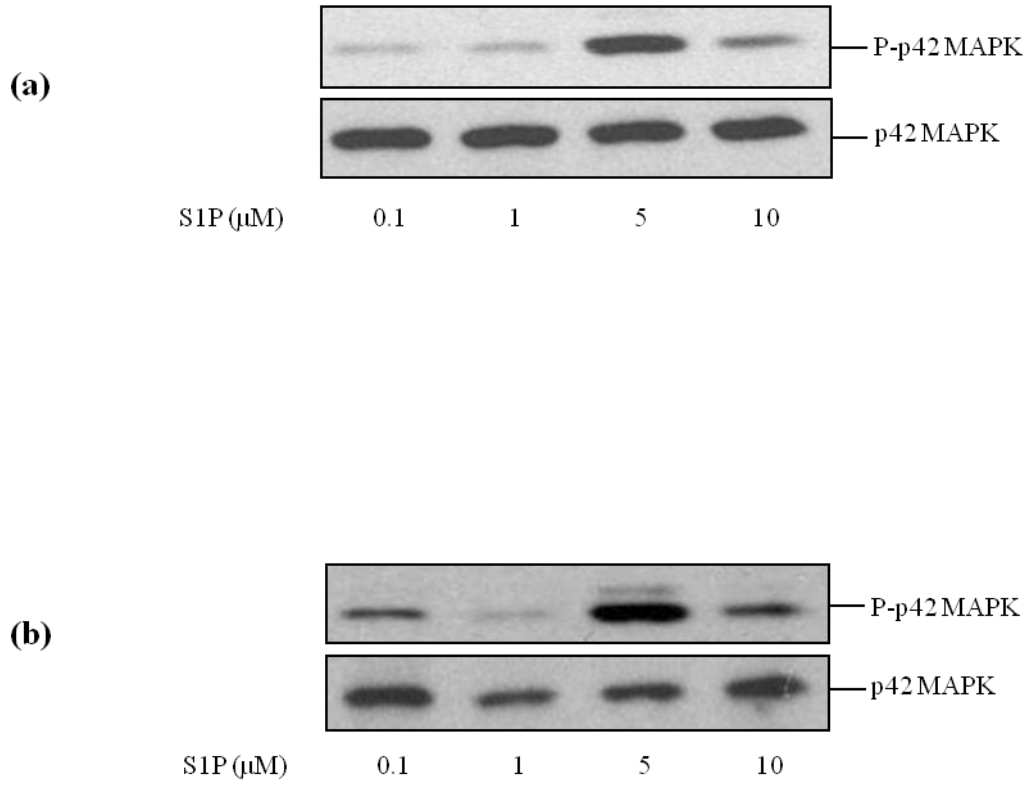


Figure 3.19 Stimulation of p42/p44 MAPK in ES-D3 and CGR8 cells by S1P.
In a separate experiment, ES-D3 cells (a) and CGR8 (b) cells were treated with increasing concentrations of S1P (0.1-10μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panel). Blots were stripped and reprobed with antibody specific for p42 MAPK to establish equal protein loading (bottom panel). Results are representative of an experiment performed three times.

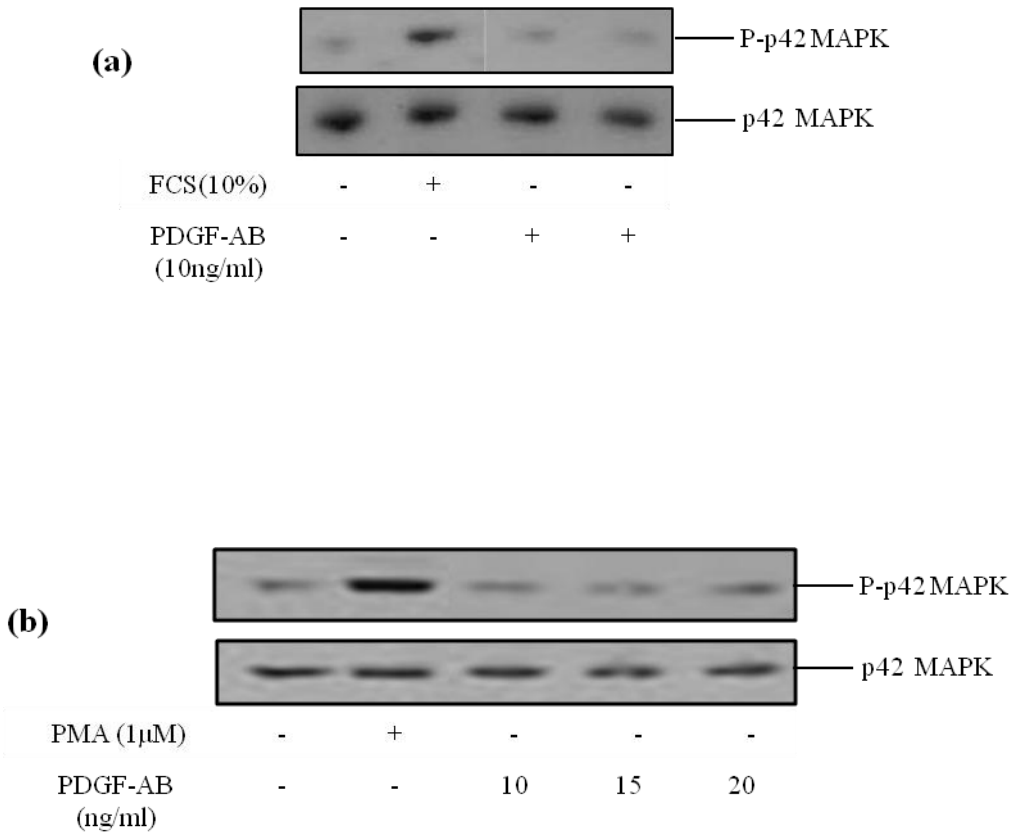


Figure 3.20 The effect of PDGF-AB on p42/p44 MAPK in ES-D3 and CGR8 cells

(a) ES-D3 cells were cultured in reduced serum medium before being treated with FCS (10%) or PDGF-AB (10ng/ml) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panel). Blots were stripped and reprobed with antibody specific for p42 MAPK to establish equal protein loading (bottom panel). Results are representative of an experiment performed three times.

(b) CGR8 cells were cultured in reduced serum medium before being treated with PMA (1μM) or PDGF-AB (10-20 ng/ml) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panel). Blots were stripped and reprobed with antibody specific for p42 MAPK to establish equal protein loading (bottom panel). Results are representative of an experiment performed three times.

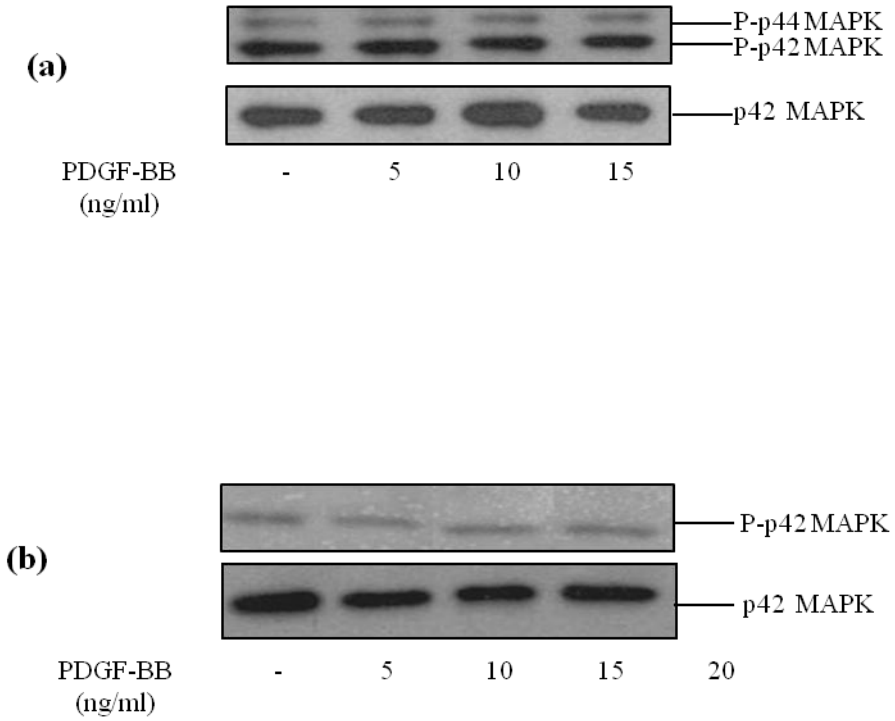


Figure 3.21 The effect of PDGF-BB on p42/p44 MAPK in ES-D3 and CGR8 cells.

ES-D3 (a) and CGR8 (b) cells were cultured in reduced serum medium for 24 hours then treated with PDGF-BB (5-15 ng/ml) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panel). Blots were stripped and reprobed with antibody specific for p42 MAPK to establish equal protein loading (bottom panel). Results are representative of an experiment performed three times.

3.4 Characterisation of agonists on embryonic stem cell proliferation

3.4.1 Serum starvation

To investigate the effect of serum starvation on ES cell proliferation, [^{3}H] thymidine uptake into DNA was measured. Zhang *et al*., reported that most ES cells could arrest in the G_0/G_1 phase using a serum deprivation culture. Upon readmission of serum, the synchronised ES cells could successfully re-enter a normal cell cycle (Zhang *et al*., 2005). It was observed that maintaining ES-D3 and CGR8 cells in media without FCS or in the presence of 0.2% FCS resulted in cell detachment. To overcome this, ES-D3 and CGR8 were incubated in media containing 1% FCS. In ES-D3 cells, reduced serum (1% FCS; 394648 DPM ± 10169) significantly inhibited proliferation over control conditions (15% FCS; 435888 DPM ± 6554) by ~10% [**Figure 3.22(a)**]. Similar observations in CGR8 cells showed that reduced serum (1% FCS; 286968 DPM ± 7009) significantly inhibited proliferation by ~30% over control conditions (15% FCS; 401944 DPM ± 8012) [**Figure 3.22(b)**]. RT-PCR was used to analyse the effect of reduced serum on OCT-4 gene expression to evaluate the effect on pluripotency. Both ES-D3 and CGR8 cells under normal and reduced serum conditions, expressed the pluriopotent stem cell marker OCT-4 [**Figure 3.23 (a)** and **(b)**]. Moreover, ES cells in reduced serum media expressed OCT-4 mRNA transcript equivalent to that of cells maintained in media supplemented with 15% FCS.

3.4.2 Sphingosine-1-phosphate

Next, the effect of S1P on ES cell proliferation was examined by treating ES-D3 and CGR8 cells (after 24hr in reduced serum media (1% FCS)) with S1P for 20 hours. **Figure 3.24(a)** shows that S1P (5μM) treatment for 20 hours significantly inhibited proliferation of ES-D3 cells (S1P (5μM); 231952 DPM ± 11982) compared to control cells (control; 258957 DPM ± 10681). For CGR8 cells, S1P (5μM) treatment for 20 hours also significantly inhibited cell proliferation (S1P (5μM); 35446 DPM ±

8005) compared to control conditions (control; 96727 DPM ± 9030) [**Figure 3.24(b)**].

The pluripotent state of both ES-D3 and CGR8 cells after treatment was S1P was examined using RT-PCR for OCT-4. In the presence and absence of S1P, both ES-D3 [**Figure 3.25**] and CGR8 [**Figure 3.26**] cells expressed mRNA for OCT-4. No difference in the gene expression levels of OCT-4 was observed in the presence or absence of S1P for 24 hours, suggesting that S1P does not affect the pluripotent state of ES cells. RT-PCR for the housekeeping gene GAPDH was performed to validate PCR reactions and verify equal quantities of cDNA.

3.4.3 **Platelet-derived growth factor**

Although PDGF was poorly effective in terms of p42/p44 MAPK activation, it was interesting to examine the effect of PDGF on mouse embryonic stem cell proliferation, both as an agonist alone and as a co-stimulator with S1P. In ES-D3 cells, treatment with PDGF had no significant effect on cell proliferation, as did co-stimulation with both S1P and PDGF [**Figure 3.27 (a)**]. The same trend was observed for CGR8 cells, where PDGF alone and co-stimulation with S1P had no significant effects on CGR8 cell proliferation over control conditions [**Figure 3.27 (b)**].

To investigate whether PDGF had any effect on the pluripotency of ES cells, OCT-4 mRNA expression was examined by RT-PCR after treatment with PDGF-AB or vehicle for 24 hours. **Figure 3.28** shows that treatment of ES-D3 [**Figure 3.28(a)]** and CGR8 **[Figure 3.28(b)]** cells with PDGF for 24 hours had no effect on the expression of OCT-4 mRNA, suggesting that PDGF has no effect in regulation of pluripotency in these cells.

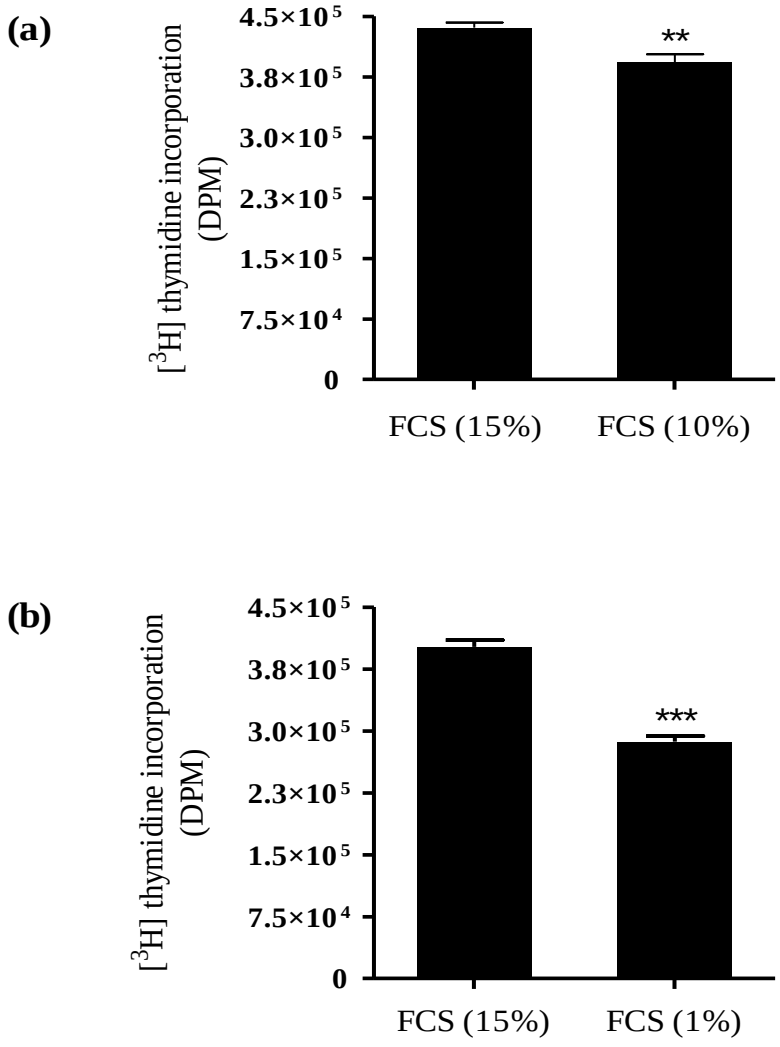


Figure 3.22 Effect of reduced serum on ES-D3 and CGR8 cell proliferation

ES-D3 (a) and CGR8 cells (b) in 24 well plates, were cultured in complete medium containing 15% FCS until ~70% confluent. Culture medium was either replaced with fresh complete medium (15% FCS) or changed to reduced serum medium (1% FCS) for 24 hours. After this time, medium was refreshed and cells incubated for a further 15 hours. [^{3}H] thymidine (9.25kBq per well) was added for a further 5 hours after which nuclear material was precipitated with 1ml of 10% (w/v) ice cold TCA and then dissolved in 0.1% SDS/0.3M NaOH. [^{3}H] thymidine uptake was quantified by liquid scintillation counting. Data shown represents mean ± S.E.M, n=12, **p<0.01, ***p<0.001.

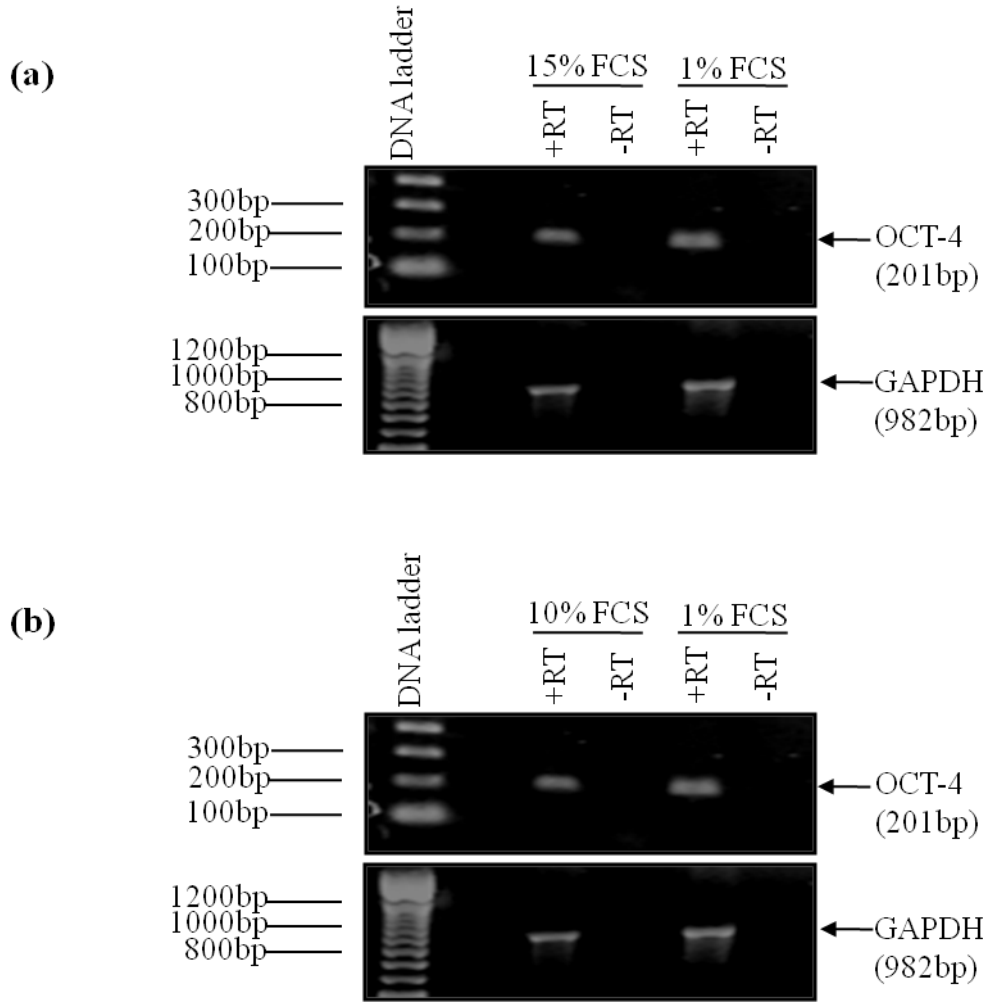


Figure 3.23 Gene expression levels of OCT-4 in complete and reduced serum culture medium.

ES-D3 (a) and CGR8 (b) cells were cultured in 25cm^2 flasks in complete medium until ~70% confluent. Cells were maintained in complete medium or incubated in medium containing 1% FCS for 24 hours. RT-PCR for OCT-4 was performed on cDNA synthesised from extracted RNA (+RT). Negative control reactions with omission of reverse transcritpase were also included (-RT). RT-PCR for the house keeping gene GAPDH was also performed on the cDNA.

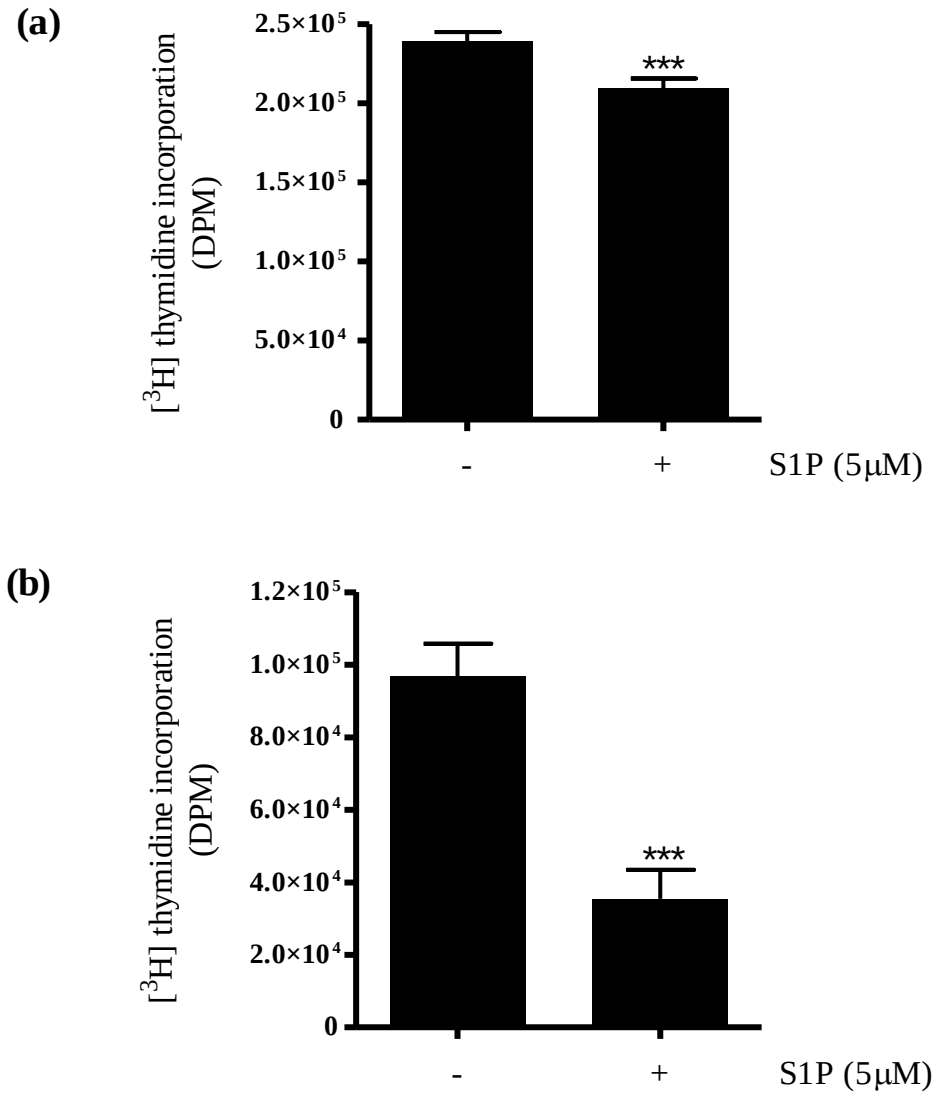


Figure 3.24 The effect of S1P on ES-D3 cell and CGR8 cell proliferation.

ES-D3 cells (a) and CGR8 cells (b) were cultured in complete medium containing 15% FCS plus supplements until ~70% confluent. Cells were cultured in reduced serum medium (1% FCS) for 24 hours then treated with S1P (5μM) for a further 20 hours. [^{3}H] thymidine was added for the last 5 hours then nuclear material was precipitated with 1ml of 10% (w/v) ice cold TCA and then dissolved in 0.1% SDS/0.3M NaOH. [^{3}H] thymidine uptake was quantified by liquid scintillation counting. Data shown represents mean DPM (disintegrations per minute) ± S.E.M, n=20, ***$p<0.0001$.

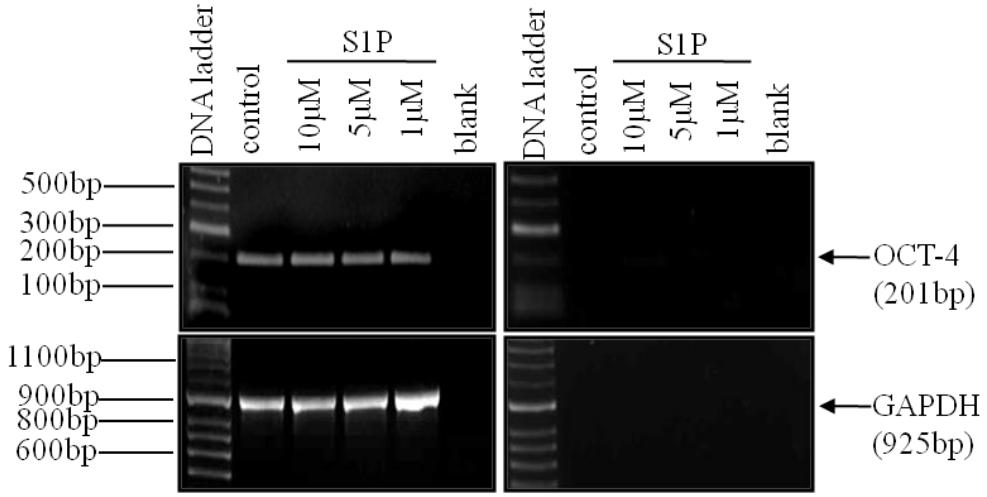


Figure 3.25 RT-PCR analysis of OCT-4 mRNA transcript expression in ES-D3 cells after treatment with S1P.

ES-D3 cells were cultured in 25cm^2 flasks in complete medium containing 15% FCS until ~70% confluent. Cells were cultured in reduced serum medium (1% FCS) for 24 hours then incubated in reduced serum media supplemented with either DMSO (control) or increasing concentrations of S1P (1-10μM) for 20 hours. RT-PCR for OCT-4 was performed on cDNA synthesised from extracted RNA (+RT; top left panel). Negative control reactions with omission of reverse transcritpase were also included (-RT; top right panel). RT-PCR for the house keeping gene GAPDH was also performed on the cDNA (+RT; bottom left) with inclusion of negative control reaction (-RT; bottom right). Blank is the PCR control without cDNA.

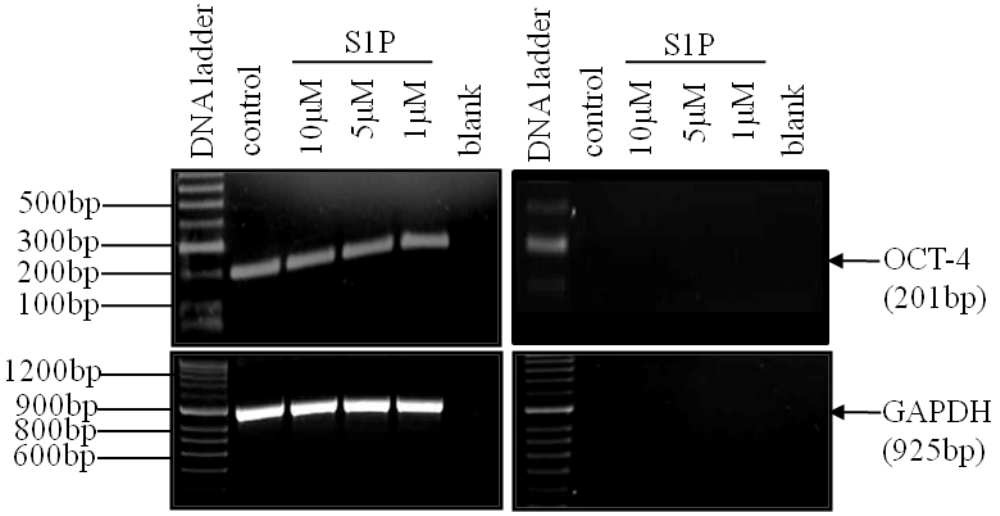


Figure 3.26 RT-PCR analysis of OCT-4 mRNA transcript expression in CGR8 cells after treatment with S1P.

CGR8 cells were cultured in 25cm^2 flasks in normal culture medium until ~70% confluent. Cells were quiesced for 24 hours then incubated in quiescent media supplemented with either vehicle (DMSO) or increasing concentrations of S1P (1-10μM) for 20 hours. RT-PCR for OCT-4 was performed on cDNA synthesised after cells were harvested (+RT; top left panel). Negative control reactions with omission of reverse transcritpase were also included (-RT; top right panel). RT-PCR for the house keeping gene GAPDH was also performed on cDNA from harvested cells (+RT; bottom left) with inclusion of negative control reaction (-RT; bottom right). Blank is the PCR control without cDNA.

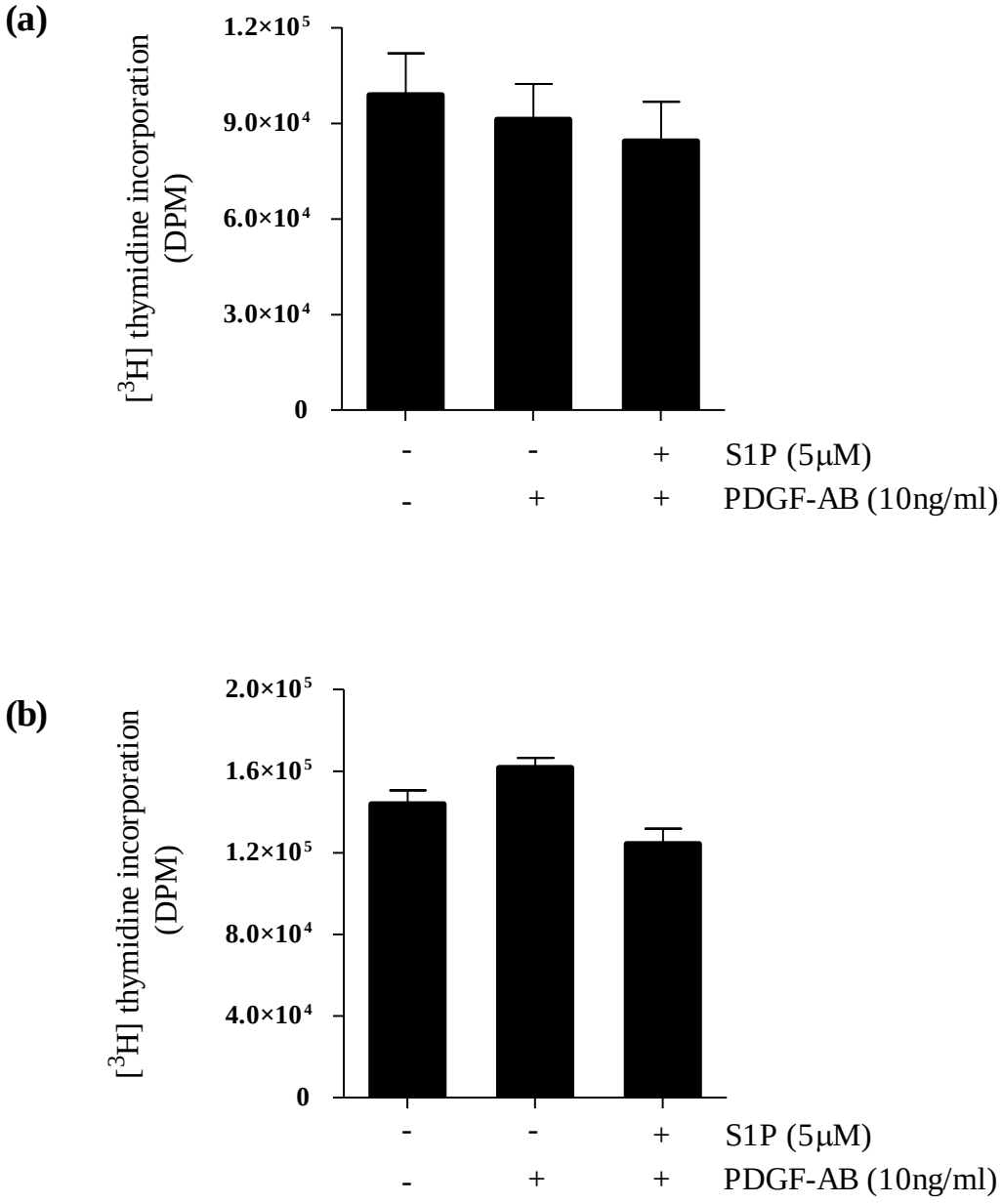


Figure 3.27 PDGF-AB alone and co-stimulation with S1P had no effect on ES-D3 and CGR8 cell proliferation.

ES-D3 (a) and CGR8 (b) cells were cultured in reduced serum media for 24 hours then treated with either S1P (5μM) or PDGF-AB (10ng/ml) alone or co-stimulated with both S1P (5μM) and PDGF-AB (10ng/ml) for 20 hours. [^{3}H] thymidine was added for the last 5 hours and [^{3}H] thymidine uptake was quantified by liquid scintillation counting. Data shown represents mean DPM (disintegrations per minute) ± S.E.M, n=8. Statistical tests were performed but found not to be significant.

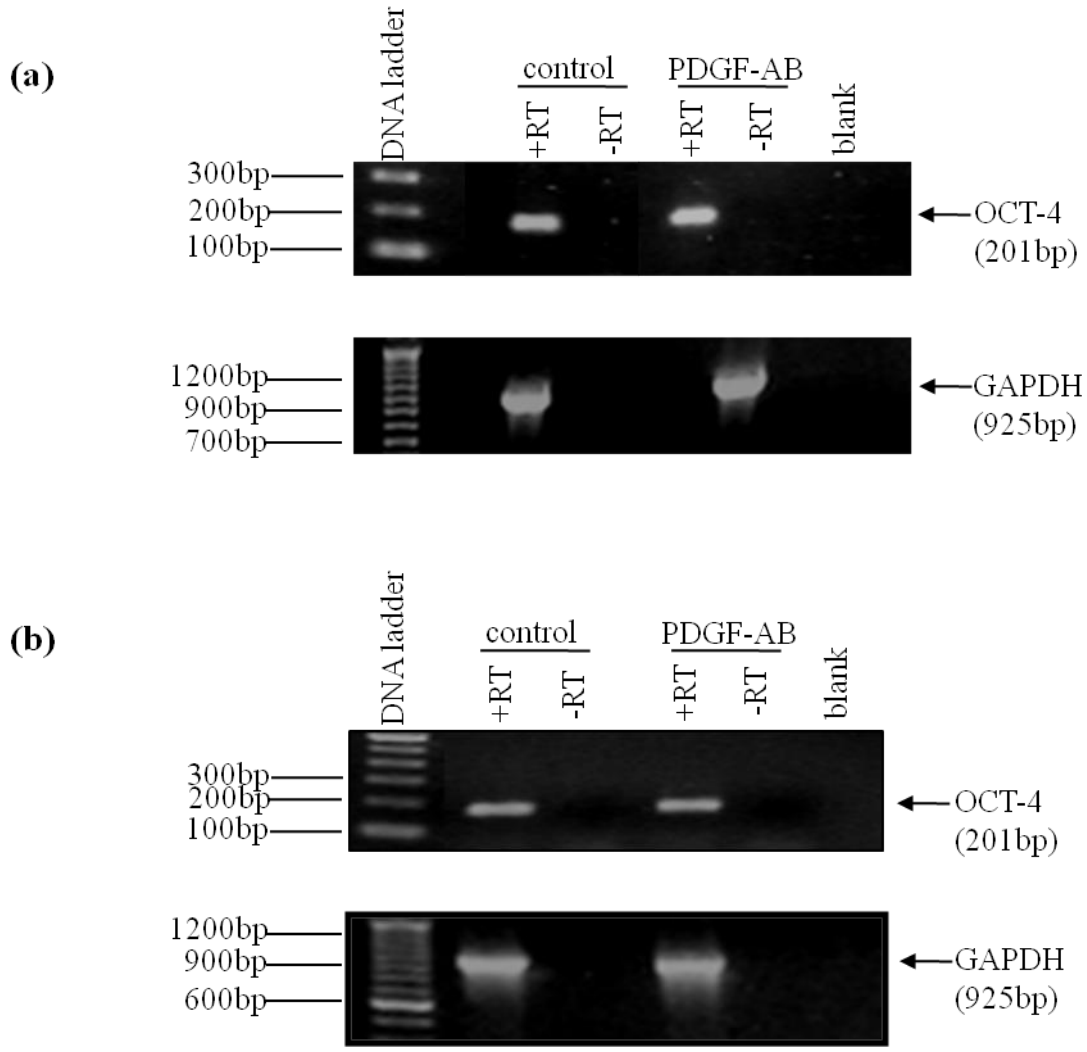


Figure 3.28 OCT-4 gene expression in ES-D3 and CGR8 cells after incubation with PDGF-AB.

ES-D3 (a) and CGR8 (b) cells were cultured in 25cm^2 flasks in normal culture medium until ~70% confluent. Cells were quiesced (1% EFCS) for 24 hours then incubated in quiescent media supplemented with either vehicle (dH_20) or PDGF-AB (10ng/ml) for 20 hours. RT-PCR for OCT-4 was performed on cDNA synthesised after cells were harvested (+RT). Negative control reactions with omission of reverse transcritpase were also included (-RT). RT-PCR for the house keeping gene GAPDH was also performed on cDNA from harvested cells. Blank is the PCR control without cDNA.

3.5 The effect of S1P on ES cell differentiation

This chapter demonstrated that S1P inhibited proliferation of ES-D3 and CGR8 cells, measured by reduced [^{3}H] thymidine incorporation into newly synthesised DNA in cells treated with S1P compared to untreated cells. ES cells have the unique capability to proliferate extensively *in vitro* while maintaining a pluripotent phenotype or to follow a differentiation pathway to a specific cell lineage which would be preceded by a period of growth arrest. The inhibition of proliferation observed by S1P in this study may be growth arrest of cells prior to initiation of a differentiation pathway. Therefore, we investigated whether we could repeat the findings of Sachinidis *et al*., (2005) who showed that S1P promoted differentiation of ES cells to cardiomyocytes.

To do this, embryoid bodies (EB) were formed as hanging drops in conical wells from ES-D3 cells. EB were cultured in the presence of 15% FCS (control), 5µM S1P, 10ng/ml PDGF-AB or both S1P and PDGF-AB, from day 1. S1P treatment resulted in the highest percentage of spontaneously beating EB where 18.7% of EB treated with S1P were beating [**Figure 3.29**]. The next highest proportion of beating EB was those treated with S1P and PDGF (8.3%) and finally treatment with PDGF resulted in 4.3% beating EB. These data suggest that S1P may promote differentiation of ES cells to cardiomyocytes. The group of EB cultured in 15% FCS failed to exhibit any spontaneous contraction. It is possible that the FCS used here contained factors which inhibited differentiation.

3.5.1 Detection of α-MHC in beating embryoid bodies

Beating and non-beating EB were pooled together for RNA extraction and subsequent analysis of mRNA for cardiac specific α-myosin heavy chain (α-MHC). RT-PCR analysis using gene specific α-MHC primers (**Table 2**) amplified a single product of 301bp in beating EB [**Figure 3.30**]. RT-PCR analysis of non-beating EB

also detected α-MHC mRNA transcript suggesting that differentiation had occurred but EB failed to spontaneously contract [**Figure 3.30**].

(a)

Treatment	Total no. of embryoid bodies	No. of beating embryoid bodies	% beating EBs
Control	21	0	0
S1P (5μM)	16	3	18.7
PDGF (10ng/ml)	21	1	4.3
S1P/PDGF	12	1	8.3

(b)

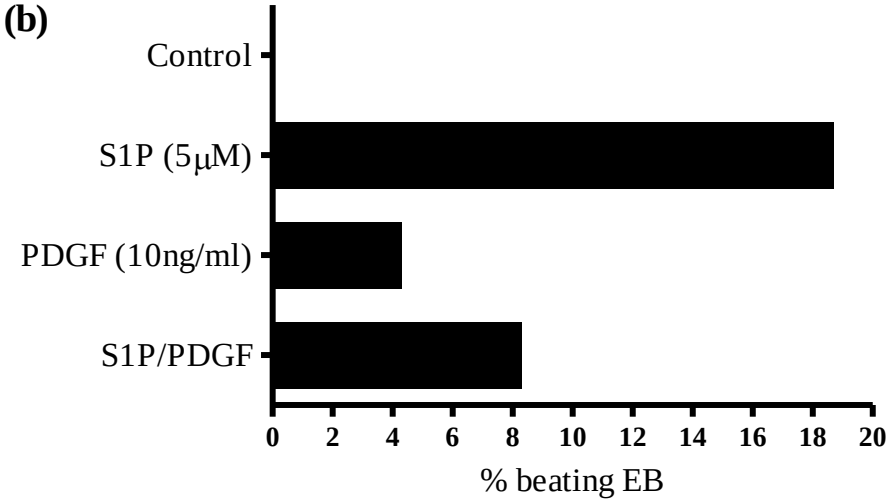


Figure 3.29 The effect of S1P and PDGF on differentiation of ES cells to cardiomyocytes.

EB created in conical wells from ES-D3 cells were treated with S1P (5μM), PDGF (10ng/ml) or both from day1. (a) Table shows the percentage of beating EB resulting from each treatment. (b) Data from table represented graphically.

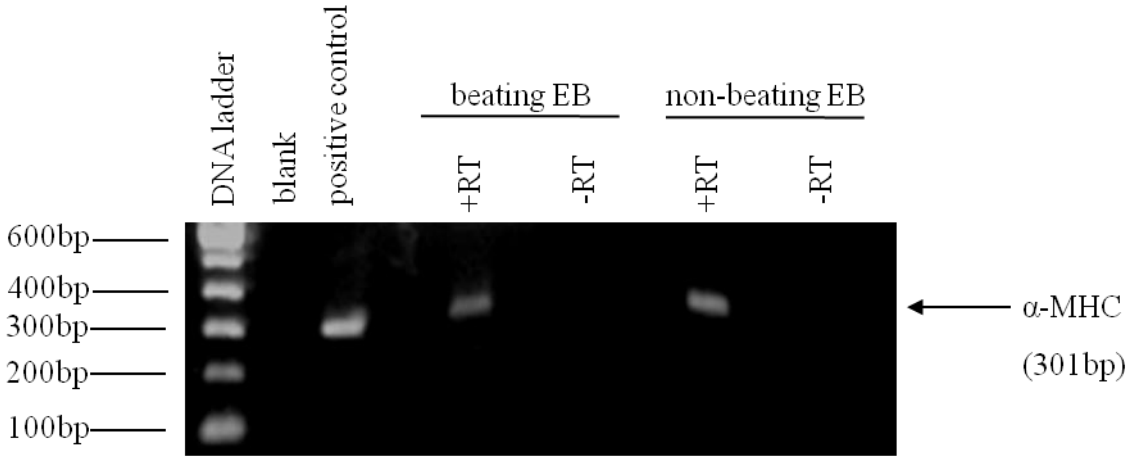


Figure 3.30 RT-PCR analysis of α-MHC in beating and non-beating EB from ES-D3 cells.

RT-PCR was carried out on cDNA synthesised from extracted RNA from pooled beating and pooled non-beating EB using α-MHC gene specific primers alongside a positive control (cDNA synthesised from mouse myocardial RNA). RT-PCR detected a product of 301bp in both beating and non-beating EB. A negative control with the omission of reverse transcriptase was included (-RT). Blank is the negative control without cDNA.

3.6 Discussion

Undifferentiated ES cells typically grow in spherical colonies with a defined boundary. A problem with ES cell culture is spontaneous differentiation into cells representing all three germ layers; mesoderm, endoderm and ectoderm. Embryonic stem cells have to be specifically cultured in such a way that prevents spontaneous differentiation into a mixed population of cells. This includes culture in the presence of LIF, plating of cells at an optimum density, subculture when no more than 80% confluent, and changing the culture media every day. Spontaneous differentiation results in morphological changes in embryonic stem cell colonies, with loss of spherical colonies possessing a defined boundary. ES-D3 and CGR8 cells were utilised in this study as they can be cultured without the need of a feeder layer. To determine the pluripotent state of the ES-D3 and CGR8 cells used in this study, RT-PCR using gene specific primers for the pluripotent ES cell transcription factor OCT-4 was carried out. OCT-4, a POU homeo-domain trasncrition factor is one of the most studied transcription factors involved in embryonic stem cell pluripotency (Pesce *et al*., 2001). OCT-4 is commonly used as a marker of embryonic stem cell pluripotency due to the fact that stem cell lines derived from the inner cell mass of the blastocyst only express OCT-4 if undifferentiated. OCT-4 mRNA was detected in ES-D3 and CGR8 cells cultured in complete medium (15% FCS plus supplements) including LIF. This was observed over a number of passages confirming the undifferentiated state of the cells used in this study.

Given that S1P regulates a diverse range of mammalian cell processes and S1P receptors are expressed in a wide variety of cells and tissues contributing to a variety of cellular responses including proliferation, survival, migration and differentiation it is not surprising S1P has an emerging role in embryonic stem cell biology. Complete elucidation of the role of S1P in self-renewal or differentiation of embryonic stem cells will contribute to producing stem cells viable for stem cell therapy. As S1P is a ligand to five S1P receptors, this part of the study characterised the expression of S1P receptors present in each cell line. This study demonstrated by RT-PCR and subsequent nucleotide sequencing, that the murine embryonic stem cell lines ES-D3

and CGR8 express mRNA transcript for the S1P receptor subtypes $S1P_1$, $S1P_2$ and $S1P_3$. $S1P_1$, $S1P_2$ and $S1P_3$ are ~42kDa proteins ubiquitously expressed in adult tissue (Kluk *et al*., 2002; Yamaguchi *et al*., 1996). $S1P_4$ mRNA was not detected in either ES-D3 or CGR8 cells using gene specific primers for $S1P_4$. The $S1P_4$-specific primers generated an amplicon of the predicted size from a $S1P_4$ plasmid construct, used as a positive control. No amplicon of this size was amplified from ES-D3 or CGR8 cells, indicating the absence of $S1P_4$ mRNA transcript in these cells. A non-specific amplicon (~600 bp) was generated from ES-D3 and CGR8 cells however, nucleotide sequencing identified this to be nucleophosmin 1 (data not shown).

Western blotting with a specific anti-$S1P_5$ antibody confirmed that ES-D3 and CGR8 cells also express $S1P_5$ receptor protein. $S1P_5$, the most recently described S1P receptor is predominately expressed in the central nervous system white matter particularly on oligodendrocyte precursor cells and rat oligodendrocytes in culture (Jaillard *et al*., 2005). Our findings are consistent with the S1P receptor profile in some human embryonic stem cells. Pebay *et al*. (2005), reported that the human embryonic stem cell lines HES-2, HES-3 and HES-4 express $S1P_1$, $S1P_2$ and $S1P_3$. However, these findings contrast with studies from Kleger and colleagues (Kleger *et al*., 2007), who demonstrated the presence of all five S1P receptor subtypes in R1 mouse ES cells. These cells differ from ES-D3 and CGR8 ES cells in that each is derived from different mice strains (R1 is from 129X1 × 129S1 crossed mice; ES-D3 from 129S2/SvPas mice and CGR8 from 129 mice) and suggests different phenotypic expression profiles.

These results show the expression profile of the S1P receptors in proliferating, undifferentiated ES-D3 and CGR8 cells. The expression of S1P receptors described in this study by RT-PCR and Western blotting is strengthened by the fact that addition of exogenous S1P to ES-D3 and CGR8 cells induces p42/p44 MAPK activation in a dose dependent manner with optimum p42/p44 MAPK response observed with 5μM of S1P. This suggests exogenous S1P activates p42/p44 MAPK by binding to one or more S1P receptors and activating downstream signalling events ultimately leading to p42/p44 MAPK activation. These findings are consistent with

activation of p42/p44 MAPK by S1P in human ES cells (Wong *et al*., 2007; Avery *et al*., 2008). Therefore, this study has demonstrated the presence of $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ in the ES-D3 and CGR8 cell lines and therefore activation of p42/p44 MAPK by S1P may be mediated via any one of these S1P receptors. In other cell types, $S1P_1$, $S1P_2$ and $S1P_3$ have been shown to have a positive effect on p42/p44 MAPK by inducing activation of p42/p44 MAPK via G_i in a pertussis toxin sensitive manner (Lee *et al*., 1996; Okamoto *et al*., 1999). However, $S1P_5$ has been shown to inhibit p42/p44 MAPK and cell proliferation in $S1P_5$ over expressing CHO cells, in a G_i independent manner (Malek *et al*., 2001). The role of S1P receptors in ES cells is further investigated in chapters 4 and 5.

RT-PCR identified the presence of PDGFβ receptor mRNA transcript in both ES-D3 and CGR8 cells. However, investigation of PDGFβ receptor protein by Western blotting using a polyclonal PDGFβ receptor antibody detected low protein expression of PDGFβ receptor protein in cell lysates of ES-D3 cells which was absent in CGR8 cells. It is possible that the PDGFβ receptor mRNA is not subsequently translated into protein or that the PDGFβ receptor protein is not correctly processed or targeted for degradation before it reaches the cell membrane. It may also be possible that PDGFβ receptor expression is untraceable by Western blotting, possibly due to the antibody. There are five known PDGF isoforms (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD), which exert their effects on target cells by activating two structurally related protein tyrosine kinase receptors (RTK) known as α- and β-receptors. The α-receptor binds both PDGF-A and PDGF-B chains with high affinity, whereas the β-receptor only binds the PDGF-B chain with high affinity (Heldin & Westermark, 1999). Binding of PDGF induces receptor dimerisation and hence, PDGF-AA induces PDGFR-αα homodimers, PDGF-AB induces PDGFR-αα homodimers or PDGFR-αβ heterodimers and PDGF-BB induces PDGFR-αα homodimers, PDGFR-αβ heterodimers and PDGF-ββ homodimers. The lack of effect of PDGF-AB on p42/p44 MAPK activation would suggest that ES-D3 and CGR8 cells do not express PDGFR-αα homodimers or PDGFR-αβ heterodimers although the presence or absence of PDGFα receptor would have to be confirmed by RT-PCR and Western blotting. Also the lack of effect of PDGF-BB on p42/p44 MAPK, even

at a particularly high concentration (20ng/ml) corroborates with the finding that low levels of PDGFβ receptor protein are expressed in ES-D3 cells and PDGFβ receptor protein is absent in CGR8 cells.

Studies of ES cell proliferation revealed that addition of exogenous S1P to both ES-D3 and CGR8 cells, significantly inhibited cell proliferation. PDGF-AB on the other hand had no effect on proliferation. Again this confirms the finding that that low levels of PDGF receptors are expressed in ES cells. However, the finding with S1P contrasts with studies in human ES cells, which have revealed that S1P reduced apoptosis (Wong *et al*., 2007) and increased proliferation in human ES cells (Inniss & Moore, 2006). The contrasting effect of S1P on proliferation in mouse and human ES cells may be due to the well documented phenotypic differences between cell types or possibly due to the concentration of S1P used in each study. The reduction of apoptosis and increase in proliferation in hES cells (Inniss and Moore, 2006) was observed after treatment of cells with 20μM of S1P, four times the concentration used in this study (5μM). Further experiments to investigate the effect of higher concentrations of S1P on mES cell proliferation are needed.

Although S1P significantly inhibited proliferation of mES cells, the undifferentiated state of both ES-D3 and CGR8 cells as measured by OCT-4 mRNA expression levels was unaffected by S1P treatment. The ES cell specific factor, OCT-4 is only expressed in undifferentiated ES cells and is quickly down-regulated upon induced or spontaneous differentiation. The differentiation of mammalian cells is preceded by growth arrest in the G1 phase of the cell cycle and is a critical stage in the differentiation process. The inhibition of proliferation of ES-D3 cells by S1P may be indicative of growth arrest in the G1 phase. Several studies link p42/p44 MAPK activation in ES cells with differentiation. In adipocyte differentiation of ES cells, Bost *et al*., (2005) reported a critical role for p42/p44 MAPK activation in ES cell commitment to an adipocyte lineage. Inhibition of p42/p44 MAPK by retinoic acid in the early stages of adipocyte differentiation resulted in a significant inhibition of adipocyte differentiation (Bost *et al*., 2002; Bost *et al*., 2005). Furthermore, in human ES cells, p42/p44MAPK activation by fibroblast growth factor stimulates ES cells to

exit self-renewal and proceed to lineage commitment (Kunath *et al*., 2007). However in this study, treatment of ES-D3 and CGR8 cells with S1P for 24 hours did not result in a decrease in OCT-4 mRNA suggesting S1P does not promote the differentiation of mouse embryonic stem cells. This contrasts with the findings of Sachinidis and colleagues (2003) who reported that S1P promoted differentiation of ES cell to cardiomyocytes. This study therefore investigated the effect of S1P on differentiation to cardiomyocytes by subjecting ES-D3 cells to the differentiation protocol where cells were treated with S1P or PDGF or both agonists. Cells treated with S1P had the highest proportion of beating embryoid bodies suggesting that in fact it may promote cardiomyocyte differentiation. However, these results could not be reproduced and would therefore need further investigation.

The current study demonstrates the expression of $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ in ES-D3 and CGR8 cells. In both cell lines, exogenous S1P induces p42/p44 MAPK activation and results in the inhibition of cell proliferation but does not alter the pluripotent state of these cells. The relevance of S1P receptor subtype, G protein involvement, and pathways downstream of S1P receptors in S1P, p42/p44 MAPK-induced inhibition of proliferation were pharmacologically investigated (Chapter 4).

CHAPTER 4

CHARACTERISATION OF S1P RECEPTOR SUBTYPES INVOLVED IN p42/p44 MAPK REGULATION IN mES CELLS

CHAPTER 4: Characterisation of S1P receptor subtypes involved in p42/p44 MAPK regulation in mES cells

4.1 Introduction

It is now well established that S1P regulates a diverse range of mammalian cell processes, largely through activation of a family of five G protein coupled receptors (GPCR) specific for S1P ($S1P_{1-5}$), that are differentially coupled to the heterotrimeric G proteins. Numerous studies have shown that S1P can regulate various cellular processes in many different cells types by engaging GPCR and initiating intracellular protein phosphorylation and second messenger systems such as p42/p44 MAPK and cAMP. These signals are translated to cellular long term changes including proliferation, migration and differentiation. As the S1P receptors are differentially expressed and coupled to multiple G proteins, S1P can activate a variety of cellular responses. Recently, a role for S1P in the regulation of embryonic stem cells in terms of proliferation and differentiation has emerged and the current study aims to evaluate this. In the previous chapter, results showed that mouse ES cells of the lines ES-D3 and CGR8 express $S1P_1$, $S1P_2$ and $S1P_3$ and $S1P_5$. It was also demonstrated that S1P induces p42/p44 MAPK activation in these cells and has an inhibitory effect on ES cell proliferation. This chapter utilises a pharmacological approach to investigate S1P receptor subtype involvement in activation of p42/p44 MAPK in ES cells, using synthetic agonists, antagonists and inverse agonists of S1P receptors. These pharmacological compounds were also used to investigate the role of S1P subtypes in the inhibition of proliferation by S1P in ES cells.

Synthetic agonists (SEW2871) and antagonists (SB649146, JTE013 and CAY10444) for S1P receptors have recently been generated and have been utilised *in vivo* and *in vitro* to elucidate the function and physiology of this GPCR family (Jo *et al*., 2005; Waters *et al*., 2006; Ohmori *et al*., 2003; Koide *et al*., 2002). The tetraromatic compound, SEW2871, was originally identified by high throughput screening of commercial chemical libraries (Sanna *et al*., 2004) and although structurally

unrelated to S1P binds specifically to $S1P_1$ receptors (Jo *et al*., 2005). SEW2871 has been shown to be an $S1P_1$ full selective agonist with respect to GTPγS binding, calcium flux and cell migration. Like S1P, SEW2871 activates p42/p44 MAPK, Akt and Rac signalling pathways and promotes receptor internalisation and recycling (Jo *et al*., 2005). SB649146 was originally generated and described as an $S1P_1$ receptor antagonist but further studies have shown it to act as a protean agonist (Waters *et al*., 2006) which is the term used to describe a compound that can act either as a partial agonist or as an inverse agonist at the same GPCR and is dependent on the conformation state of the GPCR (Kenakin, 2001). SB649146 demonstrates specificity for the $S1P_1$ receptor. In ASM cells, SB649146 acted as an inverse agonist by reducing constitutive basal $S1P_1$ receptor stimulated GTPγS binding and also partial agonism by weakly activating the p42/p44 MAPK pathway (Waters *et al*., 2006).

Previous studies in ES cells have highlighted a role for S1P and PDGF in stem cell function. Sachinidis and colleagues (2003) were first to provide evidence for a significant role for S1P and PDGF in ES cell differentiation to cardiomyocytes. The study demonstrated that both S1P (5μg/ml) and PDGF-BB (50 ng/ml) added to EB in serum replacement medium, promoted cardiomyocyte differentiation of mouse ES cells quantified by a 3 fold increase in α/β myosin heavy chain (MHC) expression accompanied by a parallel increase in number of beating EB (Sachinidis *et al*., 2003). More recently, and contrary to the previous study, S1P and PDGF were identified to promote pluripotency in human ES cells (Pebay *et al*., 2005). Culture of human embryonic stem (hES) cells in the presence of S1P or PDGF reduced spontaneous differentiation compared with controls. Furthermore, co-incubation of cells with S1P and PDGF resulted in greater inhibition of spontaneous differentiation. This effect was reduced when cells were treated with pertussis toxin (PTX) or the mitogen activated protein kinase kinase (MEK) inhibitor, U0126, suggesting that inhibition of differentiation is mediated via a G protein coupled receptor coupled to G_i and involves p42/p44 MAPK signalling (Pebay *et al*., 2005). More recently, Wong *et al*., reported that both S1P and PDGF have an anti-apoptotic effect on human ES cells and this effect is contingent on p42/p44 MAPK signalling (Wong *et al*., 2007). The

evidence implicating S1P and PDGF in ES cell function and evidence of novel signalling aspects relating to these agonists in somatic cells (see section 1.6), prompted the investigation of a functional role for the $S1P_1$-PDGFβ receptor complex in ES cells in this chapter.

S1P receptors can activate p42/p44 MAPK via various mechanisms. Previous studies in somatic cells have shown involvement of c-Src, PKC and PI3K upstream of p42/p44 MAPK (Rakhit *et al*., 1999). This chapter investigates the mechanism by which S1P activates p42/p44 MAPK in ES cells, using inhibitors of intracellular signalling molecules upstream from p42/p44 MAPK.

4.2 Pharmacological characterisation of S1P-dependent activation of p42/p44 MAPK by S1P receptors

4.2.1 S1P-dependent activation of p42/p44 MAPK is S1P receptor mediated

Having established that S1P can activate the p42/p44 MAPK pathway in ES-D3 and CGR8 cells, we next investigated whether this response was S1P-receptor mediated. Cells were treated with the G_i uncoupler, pertussis toxin (PTX), which functions to catalyse ADP-ribosylation of C-terminal cysteine in $G_i\alpha$ and thereby uncouples heterotrimeric G_i from the GPCR. An S1P receptor mediated response is implicated in both ES-D3 and CGR8 cells. For ES-D3 cells, pre-treatment with PTX (0.5μg/ml) for 18 hours, reduced the S1P dependent activation of p42/p44 MAPK by approximately 36% [**Figure 4.1(a), Figure 4.1(b)**], whereas pre-treatment with 0.1μg/ml PTX for 18 hours had no significant effect on S1P- dependent p42/p44 MAPK activation [**Figure 4.1(a)**]. The same effect was observed in CGR8 cells where pre-treatment of cells with PTX (0.5μg/ml) reduced the S1P-dependent activation of p42/p44 MAPK by approximately 20% [**Figure 4.2(b)**] implicating an S1P-receptor mediated response in both cell types. This suggests the S1P receptor involved in S1P-dependent p42/p44 MAPK activation appears to be coupled to the G protein G_i.

4.2.2 The role of S1P receptor subtypes in S1P-dependent p42/p44MAPK activation

Having established that the S1P-dependent activation of p42/p44 MAPK in ES cells appears to be mediated through an S1P receptor coupled to G_i, the next step was to investigate the identity of the S1P receptor involved. We have shown that ES-D3 and CGR8 cells express mRNA transcript for $S1P_1$, $S1P_2$ and $S1P_3$ and protein for $S1P_5$. A pharmacological approach was employed using compounds that are selective for

different S1P receptors functioning as inverse agonists or competitive antagonists. To investigate a functional role for $S1P_1$ in S1P-dependent p42/p44 MAPK, cells were pre-treated with the $S1P_1$ receptor protean agonist, SB649146, prior to S1P stimulation and analysed for p42/p44 MAPK activation by Western blot. Pre-treatment of ES-D3 cells with SB649146 (1 or 10μM) for 15 minutes, had no effect on the S1P induced activation of p42/p44 MAPK [**Figure 4.3(a)**, **Figure 4.3(b)**]. SB649146 has been previously shown to act as a partial agonist at $S1P_1$ receptors in HEK 293 and ASM cells. SB649146 treatment alone did not increase p42/p44 MAPK activation in ES-D3 cells. Similar observations were made in CGR8 cells where pre-treatment with SB649146 (1 or 10μM) for 15 minutes had no inhibitory effect on S1P induced p42/p44 MAPK activation, neither did it function as a partial agonist alone [**Figure 4.4(a)**, **Figure 4.4(b)**]. These results suggest the $S1P_1$ receptor is not involved in regulating p42/p44 MAPK in these cells.

To further investigate a minor role for $S1P_1$ in S1P-dependent p42/p44 MAPK, the effect of co-stimulation with S1P and PDGF on p42/p44 MAPK in ES cells was investigated. Its previously been shown that in cells expressing the $S1P_1$-PDGFβ receptor tethered complex, co-stimulation with S1P and PDGF results in a synergistic increase in p42/p44 MAPK activation over S1P alone (Rakhit *et al*., 1999). In ES-D3 cells, treatment with PDGF-AB or PDGF-BB alone had no effect on p42/p44 MAPK activation [**Figure 4.7(a), Figure 4.8(a)**]. This was also observed in CGR8 cells [**Figure 4.7(b), Figure 4.8(b)**]. Furthermore, both ES-D3 and CGR8 cells did not demonstrate synergistic activation in terms of p42/p44 MAPK activation when co-stimulated with S1P or the $S1P_1$ agonist, SEW2871, and PDGF [**Figure 4.6 (a)**]. Secondly, stimulation of ES-D3 [**Figure 4.6(a)]** and CGR8 **[Figure 4.6(b)]** cells with the synthetic selective $S1P_1$ full agonist, SEW2871, had no effect on p42/p44 MAPK activation [**Figure 4.6(b)**] further excluding a functional role for $S1P_1$ in regulating p42/p44 MAPK signalling in ES cells.

The role of the $S1P_2$ receptor in mediating the S1P-dependent p42/p44 MAPK activation in ES cells was investigated pharmacologically using a compound that is a specific competitive antagonist for the $S1P_2$ receptor, JTE013 (Ohmori *et al*., 2003;

Yokoo *et al*., 2004). JTE013 has been shown to inhibit specific binding of radio-labelled S1P to cell membranes of human $S1P_2$ and rat $S1P_2$ stably transfected CHO cells (Yokoo *et al*., 2004). Pre-treatment with JTE013 (1 or 10μM) for 15 minutes prior to stimulation with S1P (5μM), had no effect on the S1P-dependent p42/p44 MAPK activation in either ES-D3 [**Figure 4.9 (a), Figure 4.9(b)**] or CGR8 cells [**Figure 4.10(a), Figure 4.10(b)**] suggesting that S1P-induced p42/p44 MAPK activation is not $S1P_2$ receptor mediated.

Lastly, to explore the involvement of the $S1P_3$ receptor in the S1P-induced p42/p44 MAPK activation in ES cells, the specific $S1P_3$ antagonist, CAY10444 (Koide *et al*., 2002) was used. **Figure 4.11** and **Figure 4.12** show that pre-treatment of ES-D3 and CGR8 cells with CAY10444 (1 or 10 μM) for 15 minutes prior to S1P was without effect on S1P-dependent p42/p44 MAPK activation in both ES-D3 and CGR8 indicating that the $S1P_3$ receptor is not involved in mediating p42/44 MAPK by S1P.

Despite evidence to suggest a lack of a role for $S1P_{1-3}$ (and absence of $S1P_4$) in these cells, the PTX sensitivity of S1P signalling to p42/p44 MAPK implicates the involvement of a GPCR, therefore indicating a possible role for $S1P_5$ in regulation of p42/p44 MAPK in ES cells (investigated in chapter 5).

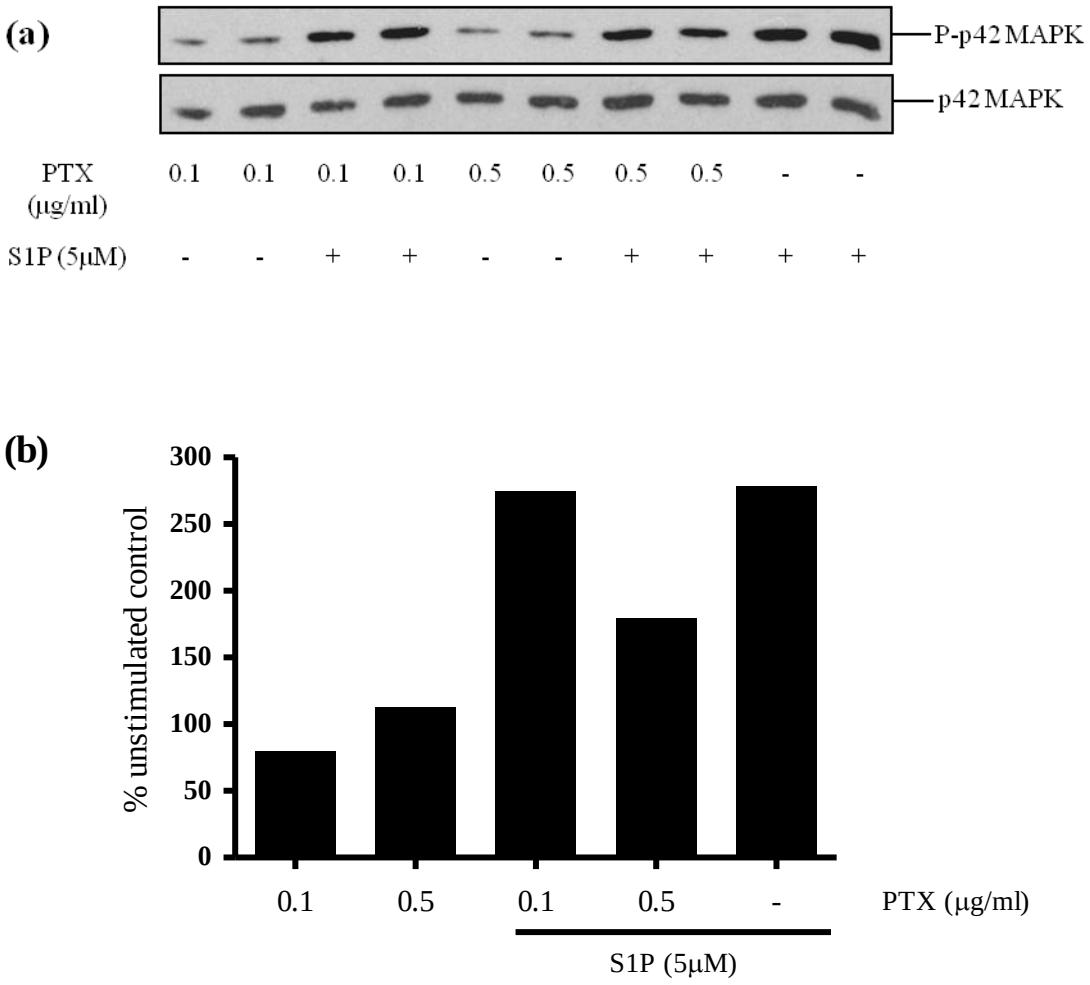


Figure 4.1 The effect of PTX on S1P-dependent p42/p44 MAPK activation in ES-D3 cells.

ES-D3 (a) cells were cultured in reduced serum medium for 24 hours prior to treatment with PTX (0.1μg/ml-0.5μg/ml) for 18 hours. Cells were then treated with S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and blotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK for n=2 experiments expressed relative to unstimulated control cells (100%).

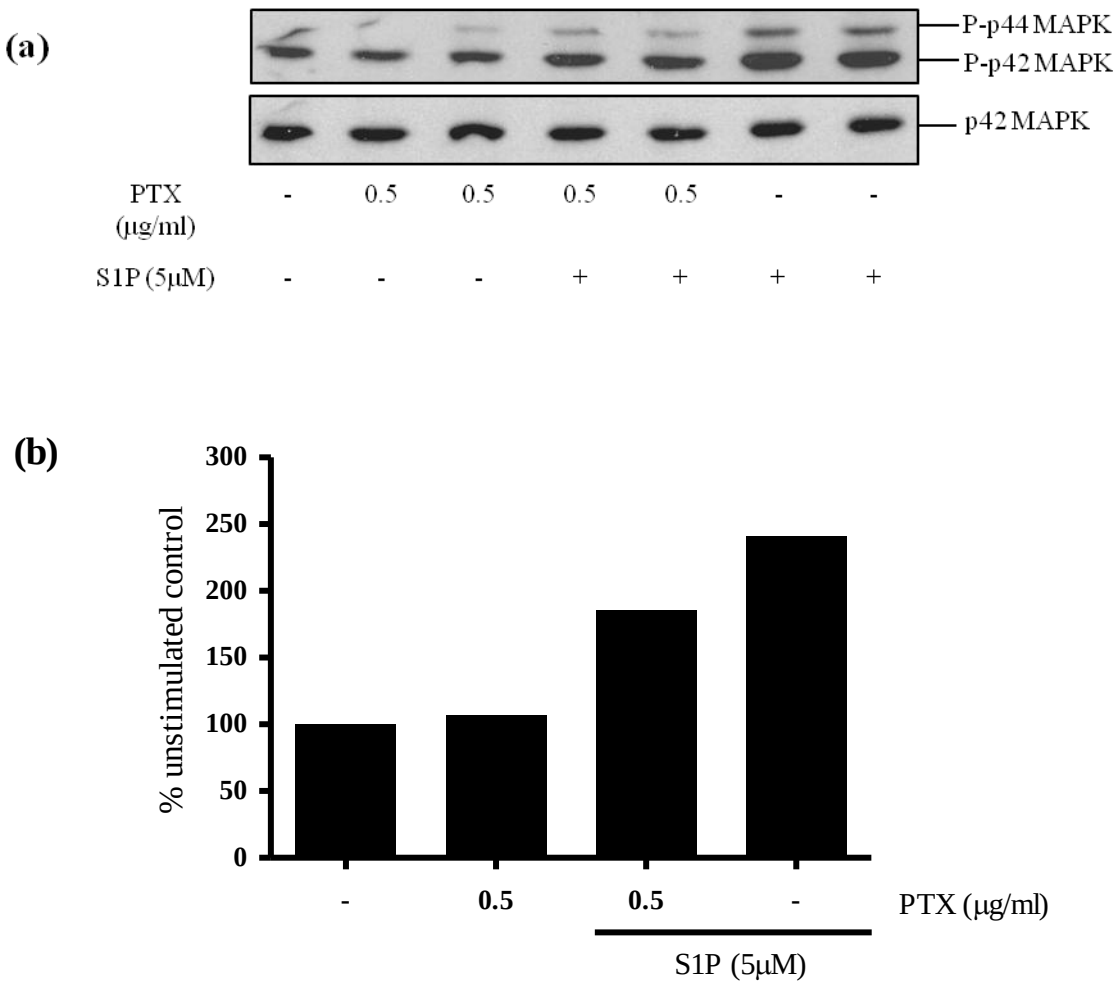


Figure 4.2 The effect of PTX on S1P-dependent p42/p44 MAPK activation in CGR8 cells

CGR8 (a) cells were cultured in reduced serum medium for 24 hours prior to treatment with PTX (0.5μg/ml) for 18 hours. Cells were then treated with S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and blotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK for n=2 experiments expressed relative to unstimulated control cells (100%).

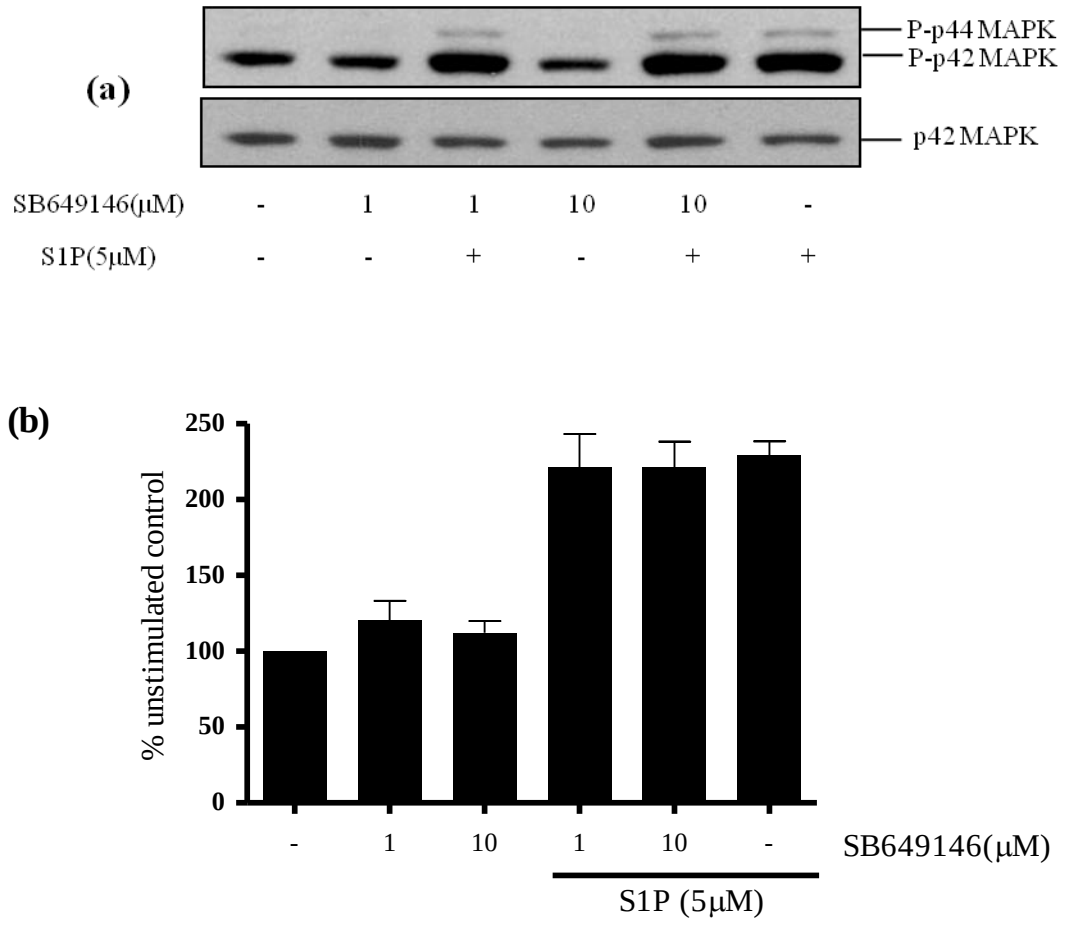


Figure 4.3 The effect of SB649146 on the S1P-dependent p42/p44 MAPK activation in ES-D3 cells.

(a) ES-D3 cells were cultured in reduced serum medium for 24 hours, pre-treated with SB649146 (1μM or 10μM) for 15 minutes then treated with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK for n=4 experiments expressed relative to unstimulated control cells (100%). Statistical tests were performed but found not to be significant.

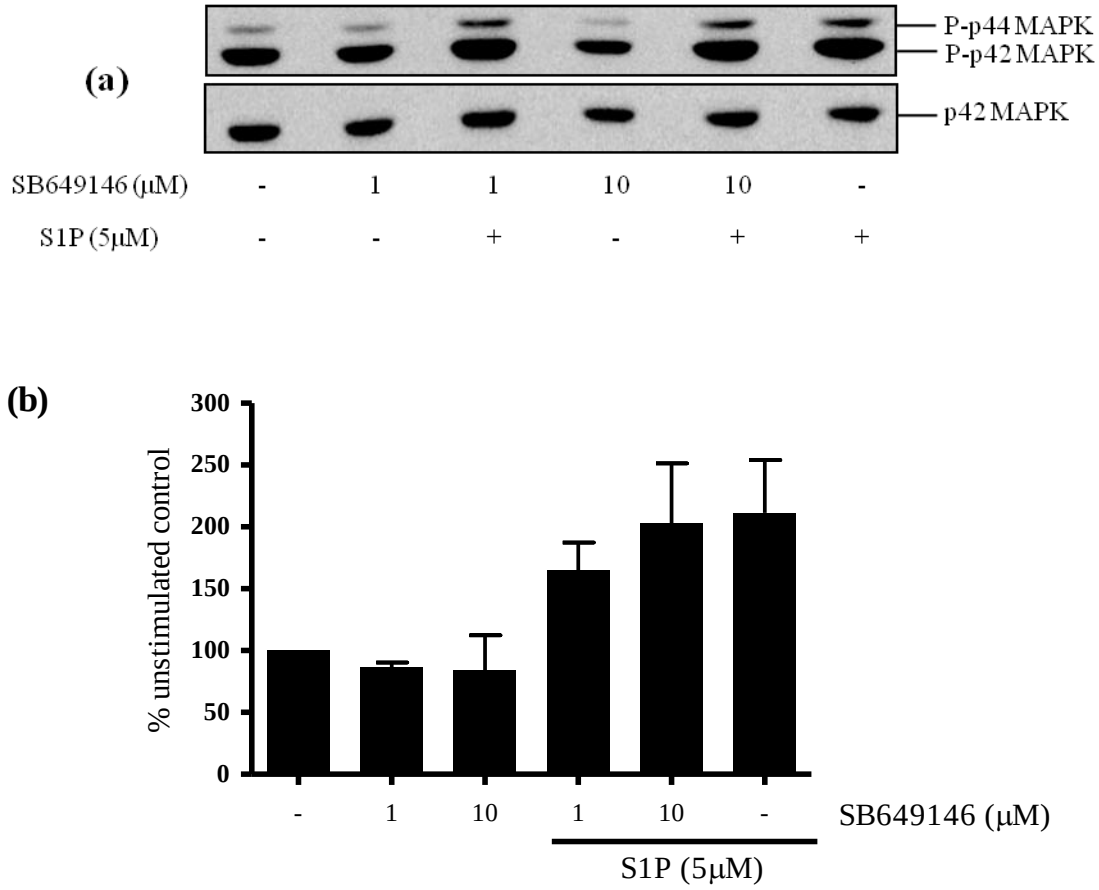


Figure 4.4 The effect of SB649146 on the S1P-dependent p42/p44 MAPK activation in CGR8 cells.

(a) CGR8 cells were cultured in reduced serum medium for 24 hours, pre-treated with SB649146 (1μM or 10μM) for 15 minutes then treated with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=3 experiments expressed relative to unstimulated control cells (100%). Statistical tests were performed but found not to be significant.

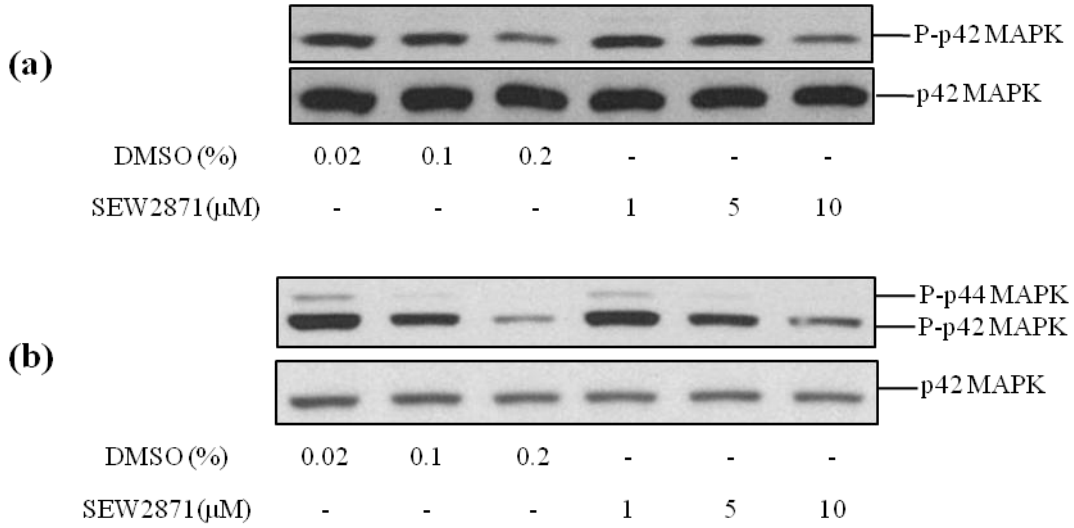


Figure 4.5 The effect of the $S1P_1$ agonist, SEW2871, on p42/p44 MAPK activation in ES cells.

ES-D3 (a) and CGR8 (b) cells were cultured in reduced serum medium for 24 hours then treated with either vehicle or SEW2871 (1-10μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels).

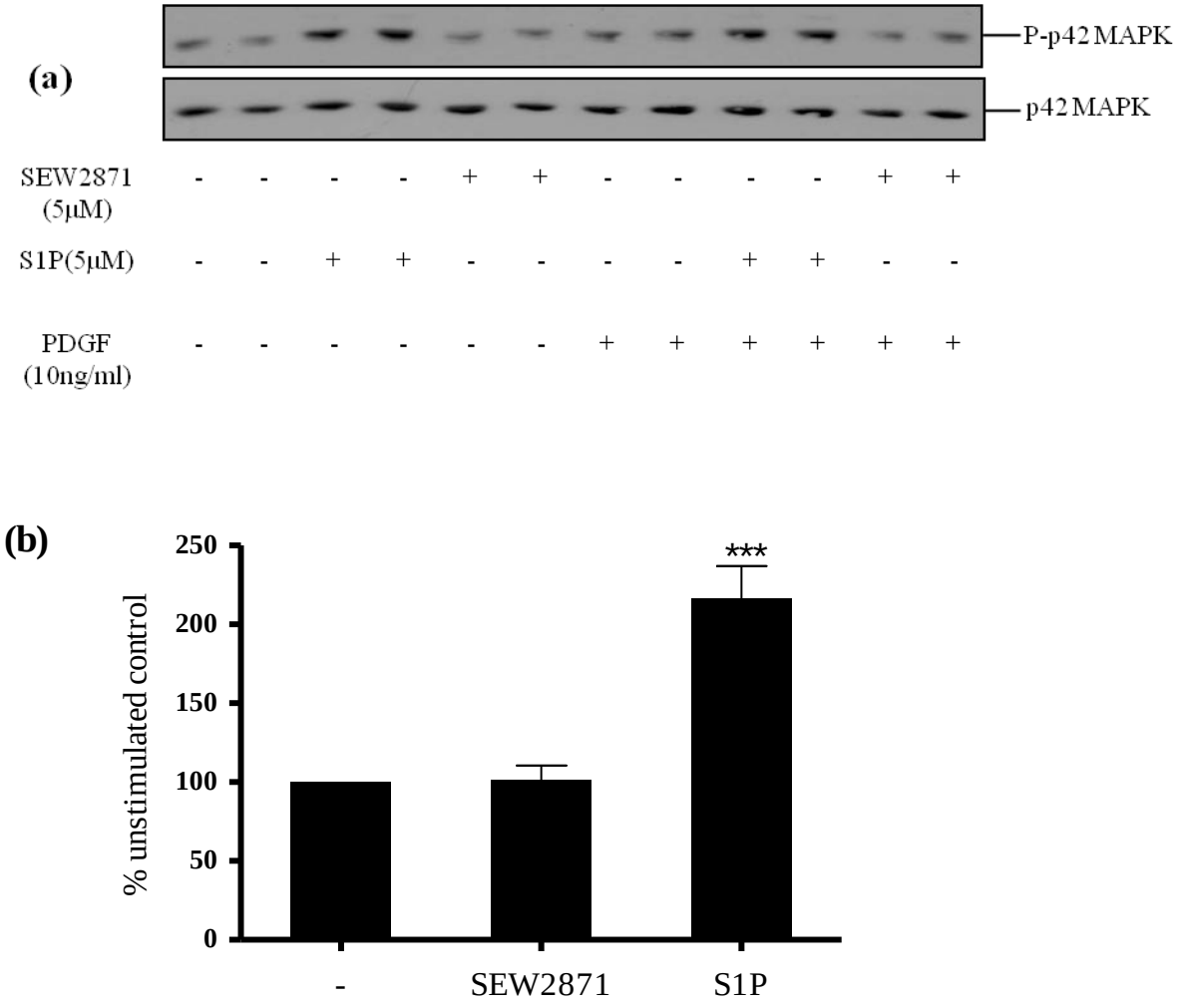


Figure 4.6 The effect of SEW2871 and PDGF co-stimulation on p42/p44 MAPK activation in ES-D3 cells.

(a) ES-D3 cells were cultured in reduced serum medium for 24 hours then treated with either S1P (5μM) or SEW2871 (5μM) alone or with PDGF (10ng/ml) for 10 minutes.

(b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=4 experiments expressed relative to unstimulated control cell (100%) and shows the lack of effect of the $S1P_1$ receptor agonist SEW2871 on p42/p44 MAPK activation.

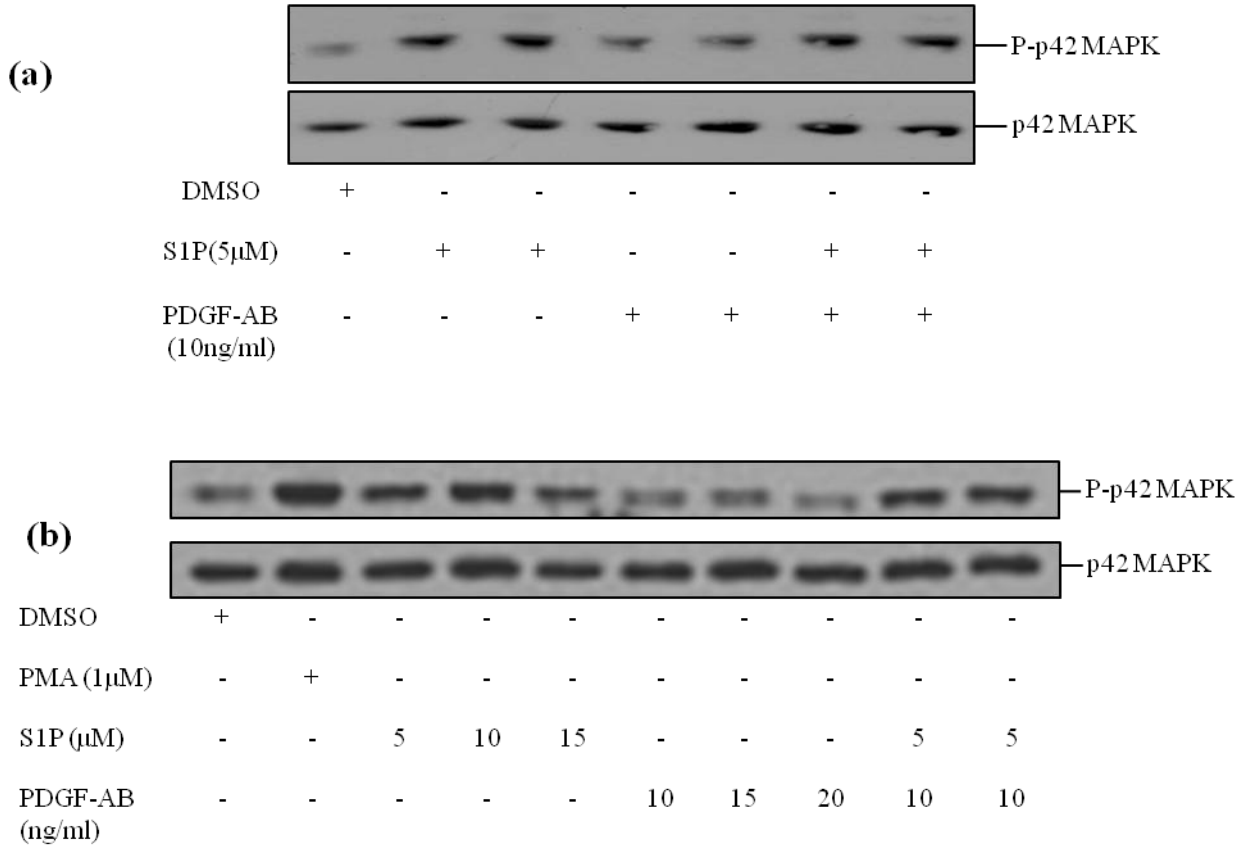


Figure 4.7 The effect of PDGF-AB alone and co-stimulation with S1P in ES-D3 and CGR8 cells.

ES-D3 (a) and CGR8 (b) cells were cultured in reduced serum medium for 24 hours then treated with S1P (5μM) alone or co-treated with PDGF-AB (10ng/ml) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels).

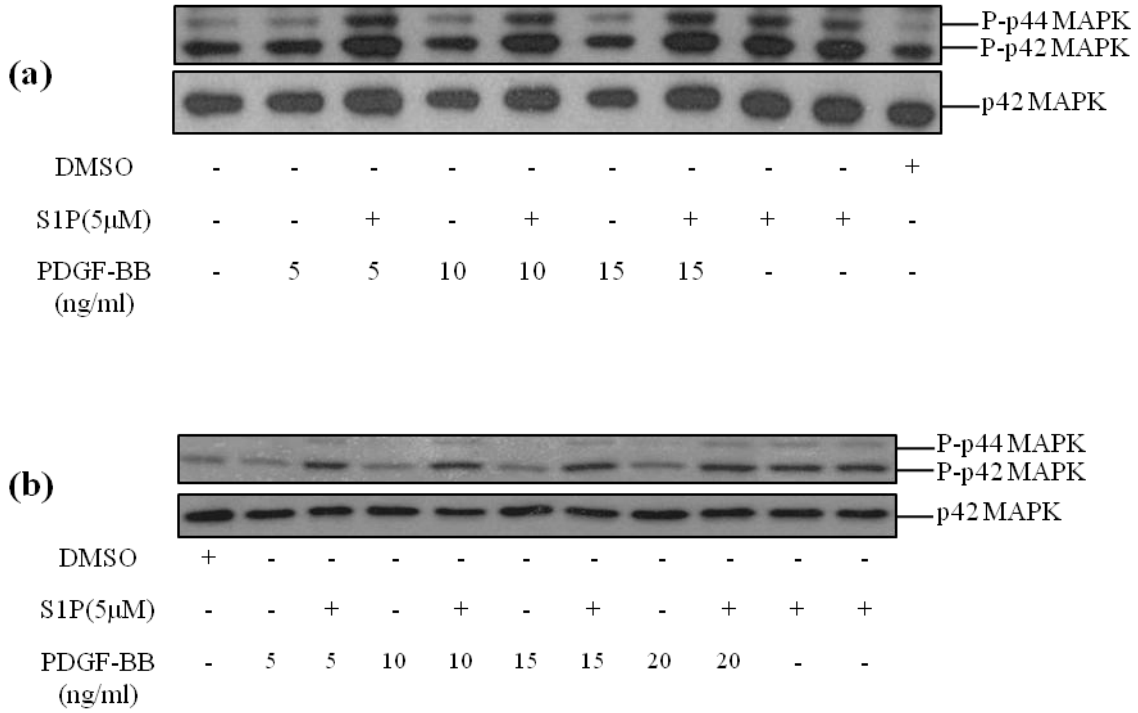


Figure 4.8 The effect of PDGF-BB alone and co-stimulation with S1P in ESD3 and CGR8 cells.

ES-D3 (a) and CGR8 (b) cells were cultured in reduced serum medium for 24 hours then treated with S1P (5μM) alone or co-treated with PDGF-BB (10ng/ml) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels).

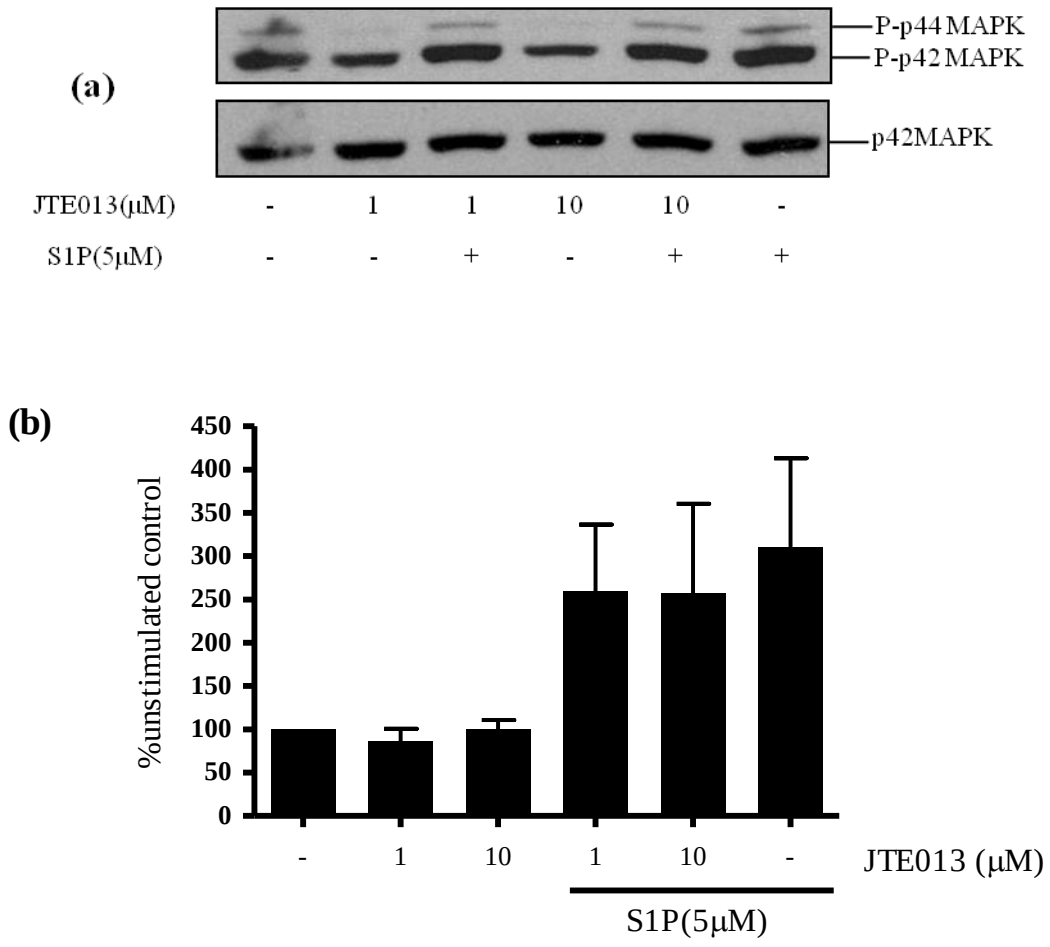


Figure 4.9 The effect of JTE013 on the S1P-dependent p42/p44 MAPK activation in ES-D3 cells.

(a) ES-D3 cells were cultured in reduced serum medium for 24 hours, pre-treated with JTE013 (1μM or 10μM) for 15 minutes then treated with or with out S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=4 experiments expressed relative to unstimulated control cells (100%). Statistical tests were performed but found not to be significant.

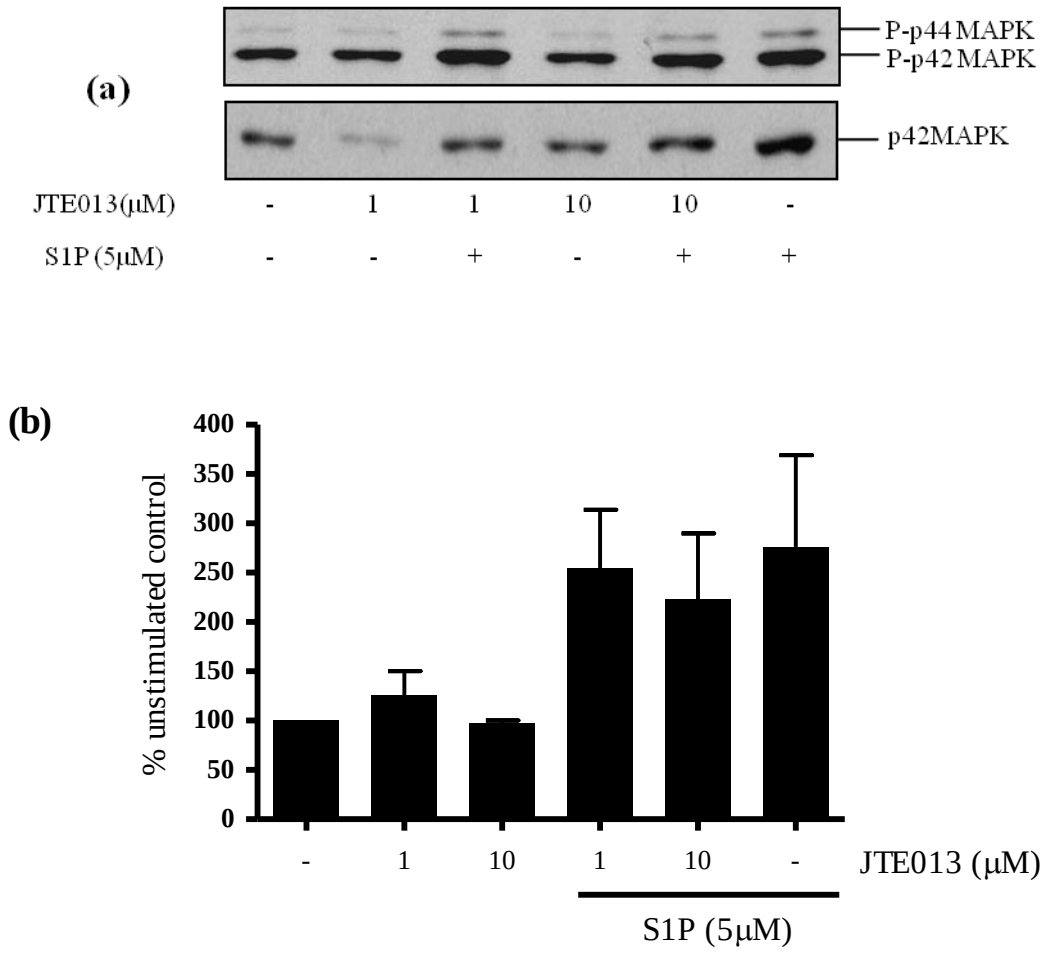


Figure 4.10 The effect of JTE013 on the S1P-dependent p42/p44 MAPK activation in CGR8 cells.

(a) CGR8 cells were cultured in reduced serum medium for 24 hours, pre-treated with JTE013 (1μM or 10μM) for 15 minutes then treated with or with out S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=4 experiments expressed relative to unstimulated control cells (100%). Statistical tests were performed but found not to be significant.

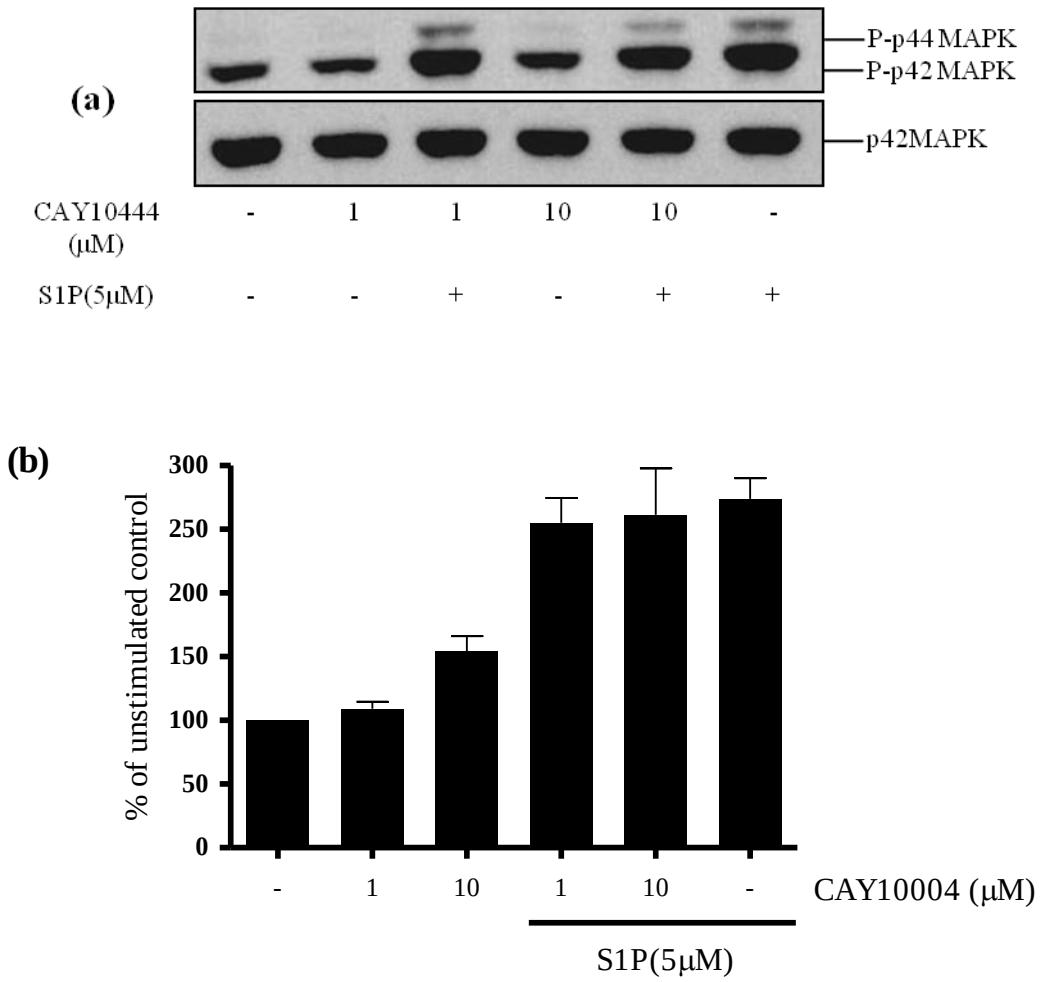


Figure 4.11 The effect of CAY10444 on the S1P-dependent p42/p44 MAPK activation in ES-D3 cells.

(a) ES-D3 were cultured in reduced serum medium for 24 hours, pre-treated with CAY10444 (1μM or 10μM) for 15 minutes then treated with or with out S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=4 experiments expressed relative to unstimulated control cells (100%). Statistical tests were performed but found not to be significant.

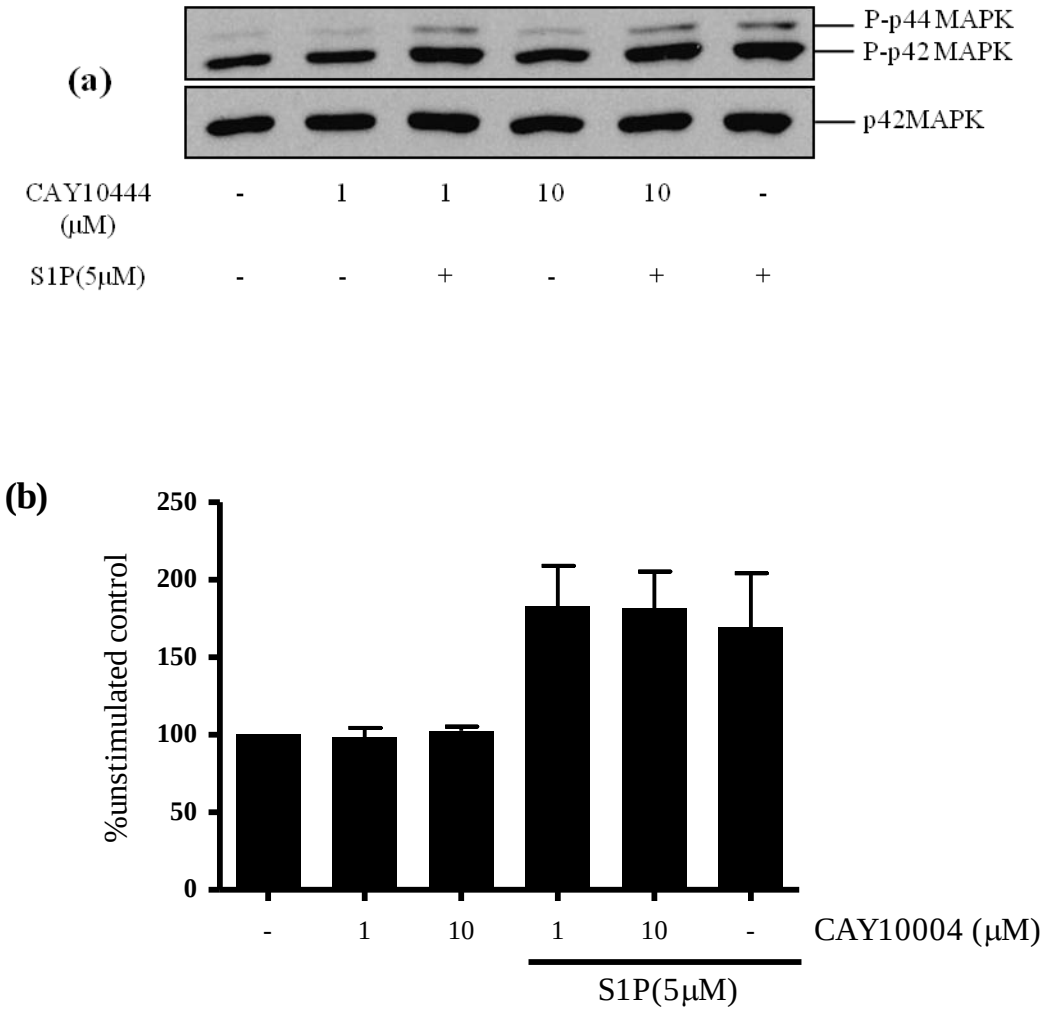


Figure 4.12 The effect of CAY10444 on the S1P-dependent p42/p44 MAPK activation in CGR8 cells.

(a) CGR8 cells were cultured in reduced serum medium for 24 hours, pre-treated with CAY10444 (1μM or 10μM) for 15 minutes then treated with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=3 experiments expressed relative to unstimulated control cells (100%). Statistical tests were performed but found not to be significant.

4.3 Pharmacological characterisation of S1P receptors involved in ES cell proliferation

The previous chapter has shown that S1P activates p42/p44 MAPK in ES cells. In other cell types, activation of p42/p44 MAPK by S1P mediated by S1P receptors can govern cell proliferation, differentiation and migration. Proliferation of ES cells involves the process of self-renewal where the pluripotent capacity of ES cells is maintained by the generation of identical daughter cells. Self-renewal is controlled by many extracellular factors and ultimately involves expression of transcription factors including OCT-4 and NANOG among others. This section aims to investigate the role of S1P receptors in regulating ES cell proliferation/self-renewal.

4.3.1 Effect of S1P-dependent p42/p44 MAPK activation on ES cell proliferation

In both ES-D3 and CGR8 cells, S1P induces p42/p44 MAPK activation and inhibition of proliferation. To assess whether the S1P-dependent p42/p44 MAPK activation has a role in inhibiting the proliferation of ES cells, cells were treated with the potent and selective cell permeable inhibitor of mitogen activated protein kinase kinase (MEK), PD98059 (Alessi *et al*., 1995) and proliferation measured by quantification of DNA synthesis. In ES-D3 cells, pre-treatment with PD98059 (10μM) did not reverse the effect of S1P on cell proliferation [**Figure 4.13**]. However, treatment of cells with PD98059 alone significantly reduced basal proliferation ($p=0.002$). Similar observations were made in CGR8 cells, where although PD98059 had no effect on inhibition of proliferation by S1P, it significantly reduced basal proliferation in these cells [**Figure 4.14**] suggesting that p42/p44 MAPK signalling has a role in basal proliferation of ES cells but that p42/p44 MAPK activation is not involved in the S1P-dependent inhibition of proliferation of these cells.

4.3.2 **S1P receptor regulation of basal proliferation**

To establish whether $S1P_1$, $S1P_2$ or $S1P_3$ are involved in regulating basal DNA synthesis and therefore proliferation of ES cells, the effect of S1P receptor antagonists on ES-D3 and CGR8 cell basal proliferation was investigated. Cells were treated with SB649146, JTE013 or CAY10444 (1 or 10μM) for 20 hours and synthesis of new DNA measured by [^{3}H] thymidine incorporation. In ES-D3 cells, SB649146 (1μM and 10μM) significantly inhibited cell proliferation by approximately 50% ($p<0.05$, control *vs* SB649146 1μM; control *vs* SB649146 10μM). JTE013 also inhibited proliferation of ES-D3 cells. JTE013 (10μM) reduced cell proliferation by approximately 70% ($p<0.05$, control *vs* JTE013 10μM) whereas basal proliferation of ES-D3 cells was not affected by CAY10444 (1 or 10μM) [**Figure 4.15**]. S1P receptor antagonists had similar effects on cell proliferation in CGR8 cells. SB649146 (1μM and 10μM) reduced basal proliferation by approximately 60% ($p<0.05$, control *vs* SB649146 1μM; control *vs* 10μM), JTE013 (10μM) by approximately 40% ($p<0.05$, control vs JTE013 10μm) whereas CAY10444 did not effect basal proliferation [**Figure 4.16**]. These data suggest that $S1P_1$ and $S1P_2$ but not $S1P_3$ may be involved in regulating basal proliferation of ES cells.

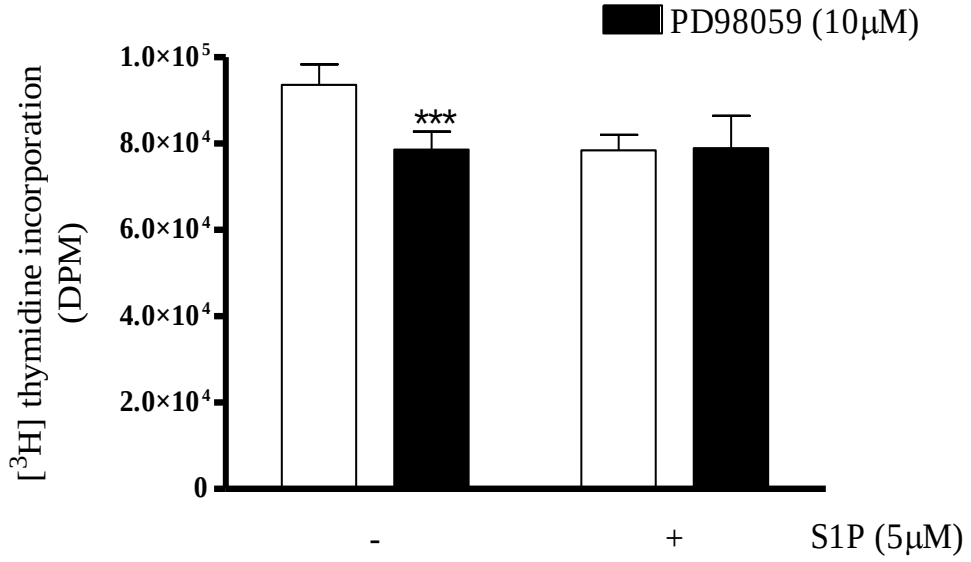


Figure 4.13 The effect of PD98059 on ES-D3 cell proliferation.

ES-D3 cells were cultured in 24-well plates in culture medium containing 15% EFCS plus supplements until ~70% confluent. Cells were cultured in reduced serum medium for 24 hours then treated with either vehicle (DMSO) or PD98059 (10μM) for 30 minutes then with S1P for 9 hours. [^{3}H] thymidine was added for the last hour and [^{3}H] thymidine uptake was quantified by liquid scintillation counting. Data shown represents mean DPM (disintegrations per minute) ± S.E.M, n=6. Statistical significance was assessed by Student's unpaired *t* test (***p=0.002, control *vs* PD98059 10μM).

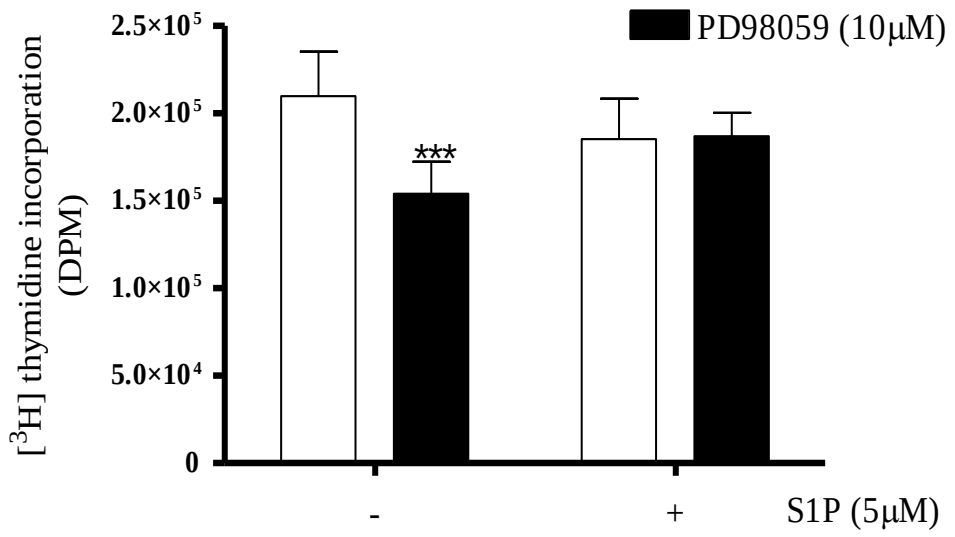


Figure 4.14 The effect of PD98059 on CGR8 cell proliferation.

CGR8 cells were cultured in 24-well plates in culture medium containing 10% EFCS plus supplements until ~70% confluent. Cells were cultured in reduced serum medium for 24 hours then treated with either vehicle (DMSO) or PD98059 (10μM) for 30 minutes then with S1P for 9 hours. [^{3}H] thymidine was added for the last hour and [^{3}H] thymidine uptake was quantified by liquid scintillation counting. Data shown represents mean DPM (disintegrations per minute) ± S.E.M, n=8. Statistical significance was assessed by Student's unpaired *t* test (***p=0.002, control *vs* PD98059 10μM).

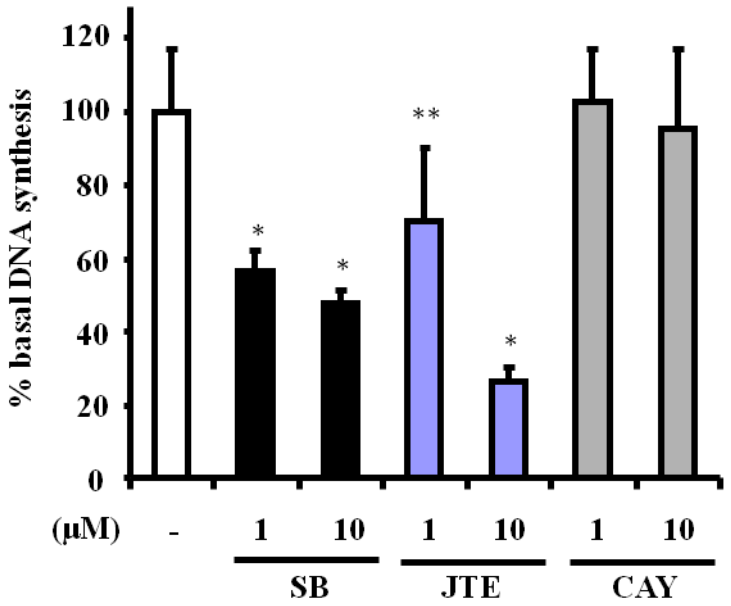


Figure 4.15 The effect of S1P receptor antagonists on basal cell proliferation in ES-D3 cells.

ES-D3 cells were cultured in reduced serum medium for 24 hours then treated with vehicle (-), SB649146 (SB, 1 or 10μM), JTE013 (JTE, 1 or 10μM) or CAY10444 (CAY, 1 or 10μM) for 9 hours. [^{3}H] thymidine was added for the last hour and [^{3}H] thymidine uptake was quantified by liquid scintillation counting. Data shown represents [^{3}H] thymidine incorporation (% inhibition of basal DNA synthesis, n=4-12). Statistical significance was assessed by Student's unpaired *t* test (*p<0.05, control *vs* SB649146 1μM, SB649146 10μM, JTE 10μM; **p<0.01, control *vs* JTE 10μM).

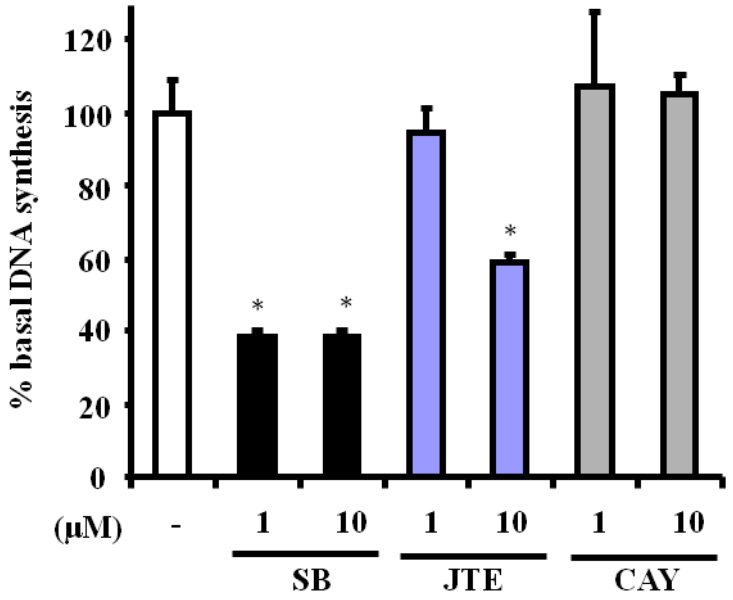


Figure 4.16 The effect of S1P receptor antagonists on basal cell proliferation in CGR8 cells.

CGR8 cells were cultured in reduced serum medium for 24 hours then treated with vehicle (-), SB649146 (SB, 1 or 10μM), JTE013 (JTE, 1 or 10μM) or CAY10444 (CAY, 1 or 10μM) for 9 hours. [^{3}H] thymidine was added for the last hour and [^{3}H] thymidine uptake was quantified by liquid scintillation counting. Data shown represents [^{3}H] thymidine incorporation (% inhibition of basal DNA synthesis, n=4-12). Statistical significance was assessed by Student's unpaired *t* test (*$p<0.05$, control *vs* SB649146 1μM, SB649146 10μM, JTE 10μM).

4.4 Pharmacological characterisation of S1P regulation of p42/p44 MAPK signalling in ES cells

The mechanism by which S1P activates p42/p44 MAPK in ES-D3 and CGR8 cells was next investigated pharmacologically. Previous studies have demonstrated the involvement of c-Src, PKC and PI3K in S1P receptor signalling in somatic cells (Rakhit *et al*., 1999). Therefore, we used inhibitors of the above intracellular signalling molecules to investigate their involvement in S1P-dependent p42/p44 MAPK responses in ES-D3 and CGR8 cells.

The non receptor tyrosine kinase, c-Src has been shown to have an essential role in the regulation of p42/p44 MAPK by G_i coupled GPCRs in that c-Src tyrosine kinases function as intermediates between G_i coupled receptors and the Ras-p42/p44 MAPK pathway mediated by $G_i\beta\gamma$ subunits (Luttrell *et al*., 1996). To investigate a role for c-Src in the S1P-dependent p42/p44 MAPK activation in ES cells, ES-D3 and CGR8 cells were treated with the potent and selective inhibitor of c-Src, PP2 (1μM or 10μM) prior to stimulation with S1P (5μM, 10 mins) and the effect on p42/p44 MAPK activation investigated by Western Blot. In ES-D3 cells, pre-treatment of cells with 10μM of PP2 for 15 minutes, completely abolished the S1P-dependent p42/p44 MAPK activation by S1P (p=0.059, PP2 (10μM) + S1P *vs* S1P), whereas 1μM of PP2 had no effect. In CGR8 cells, pre-treatment of cells with both 1μM (p=0.001, PP2 1μM + S1P *vs* S1P) and 10μM (p=0.0012, PP2 10μM *vs* S1P) of PP2 abolished activation of p42/p44 MAPK by S1P [**Figure 4.18(a) and (b)**]. This provides evidence for a critical role for c-Src in p42/p44 MAPK activation by S1P.

Next, to investigate whether S1P-dependent p42/p44 MAPK activation in ES cells may also involve PKC, ES-D3 and CGR8 cells were pre-treated with the potent and selective PKC inhibitor, GF109203X. GF109203X is a bisindolylmaleimide which inhibits PKC by interacting with the catalytic subunit (Toullec *et al*., 1991). In ES-D3 cells, pre-treatment with 1μM (p=0.0011, GFX109203X 1μM + S1P *vs* S1P) or 10μM (p=0.0167, GFX109203X 10μM + S1P *vs* S1P) of GFX109203X for 15 minutes, significantly inhibited p42/p44 MAPK activation by S1P by approximatley

50% [**Figure 4.19(a) and (b)**]. Pre-treatment of CGR8 cells with 1μM (GFX109203X 1μM + S1P *vs* S1P, not significant) or 10μM (p=0.0372, GFX109203X 10μM + S1P *vs* S1P) GFX109203X also inhibited the S1P-induced p42/p44 MAPK activation [**Figure 4.20(a) and (b)**] suggesting PKC is involved in activation of p42/p44 MAPK by S1P in ES cells.

Finally, the function of phosphatidylinositol-3 kinase (PI3K) in activation of p42/p44 MAPK by S1P was investigated using the potent, highly selective PI3K inhibitor, LY294002 (Vlahos *et al*., 1994). For ES-D3 cells, pre-treatment of cells with 1μM or 10μM of LY294002 for 15 minutes had no inhibitory effect on S1P-dependent p42/p44 MAPK activation and on its own (10μM) stimulated p42/p44 MAPK ($p<0.001$, control *vs* LY294002 10μM) [**Figure 4.21(a) and (b)**]. Interestingly, there was significantly greater activation of p42/p44 MAPK with S1P plus LY294002 *versus* S1P or LY294002 alone (p=0.0317, LY294002 + S1P *vs* S1P) [**Figure 4.21(a) and (b)**]. This effect of LY294002 was also observed in CGR8 cells, where LY294002 did not inhibit S1P-dependent p42/p44 MAPK activation [**Figure 4.22(a) and (b)**] but 10μM of LY294002 alone appeared to activate p42/p44 MAPK. Again, there was greater activation of p42/p44 MAPK by S1P when in the presence of LY294004. As LY294002 had no inhibitory effect on S1P p42/p44 MAPK activation by S1P, S1P regulation of p42/p44 MAPK in ES cells appears not to be dependent on PI3K.

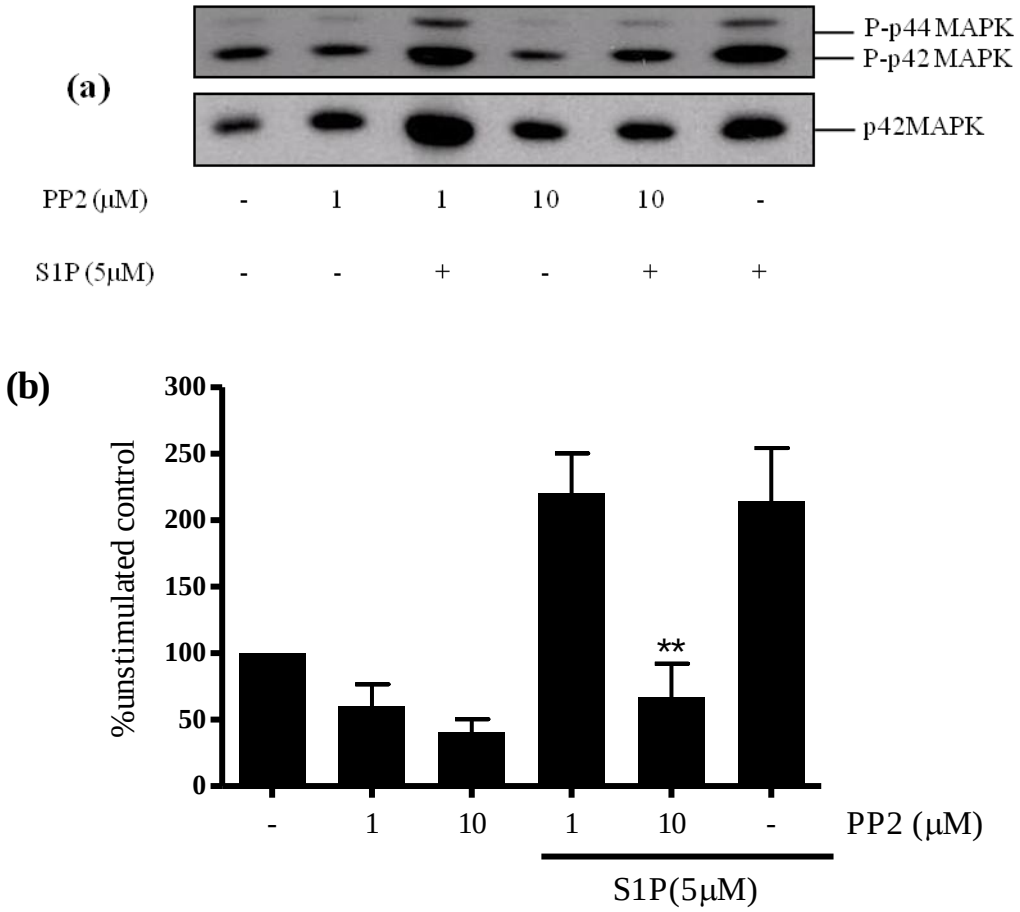


Figure 4.17 The effect of PP2 on S1P-dependent p42/p44 MAPK activation in ES-D3 cells.

(a) ES-D3 cells were cultured in reduced serum medium for 24 hours, pre-treated with PP2 (1 or 10μM) for 15 minutes prior to stimulation with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=3 experiments expressed relative to unstimulated control cells (100%). Statistical significance was assessed by Student's unpaired *t* test (**p<0.01, PP2 10μM + S1P *vs* S1P).

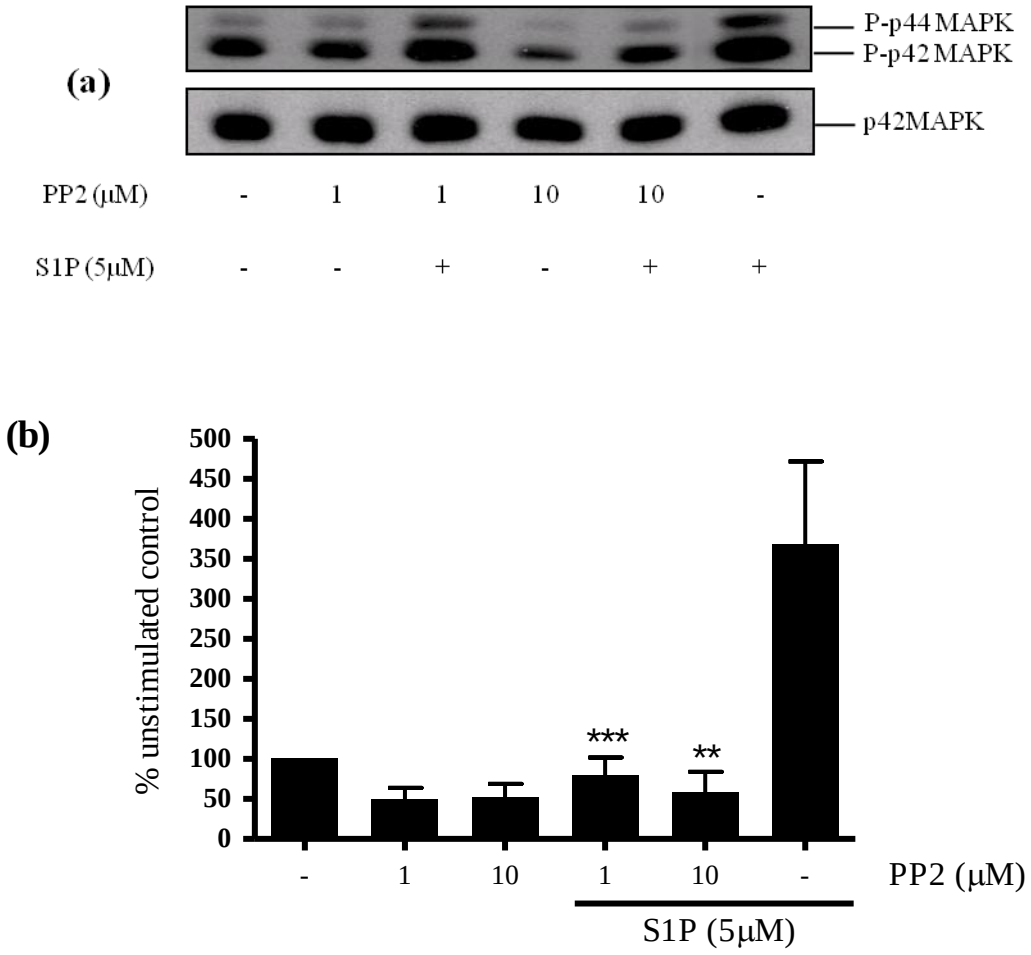


Figure 4.18 The effect of PP2 on S1P-dependent p42/p44 MAPK activation in CGR8 cells.

(a) CGR8 cells were cultured in reduced serum media for 24 hours, pre-treated with PP2 (1 or 10μM) for 15 minutes prior to stimulation with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=4 experiments expressed relative to unstimulated control cells (100%). Statistical significance was assessed by Student's unpaired *t* test (**$p<0.01$, PP2 10μM + S1P *vs* S1P; $p<0.001$, PP2 1μM + S1P *vs* S1P).

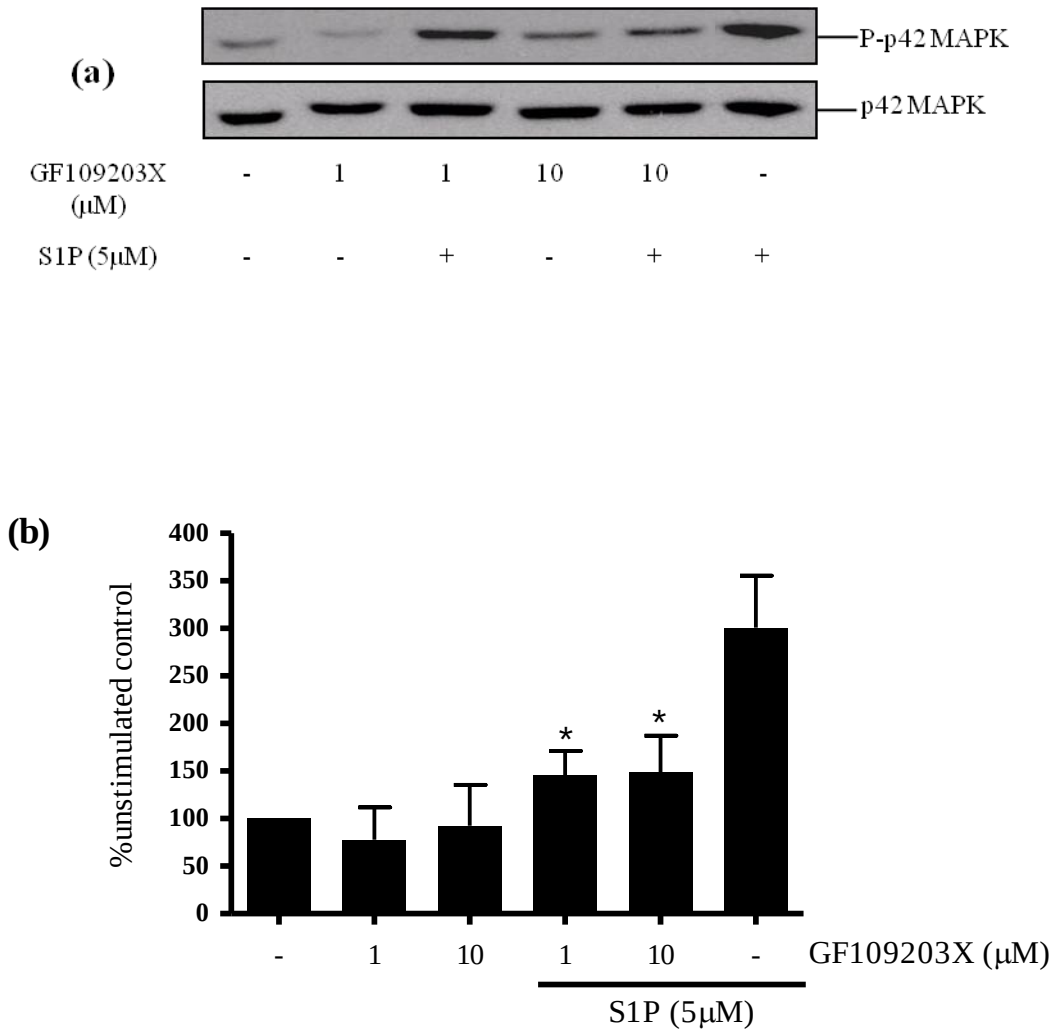


Figure 4.19 The effect of GF109203X on S1P-dependent p42/p44 MAPK activation in ES-D3 cells.

(a) ES-D3 cells were treated with GF109203X (1 or 10μM) for 15 minutes prior to stimulation with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=3 experiments expressed relative to unstimulated control cells (100%). Statistical significance was assessed by Student's unpaired *t* test (*$p<0.05$, GFX109203X 10μM + S1P *vs* S1P; GFX109203X 1μM + S1P *vs* S1P).

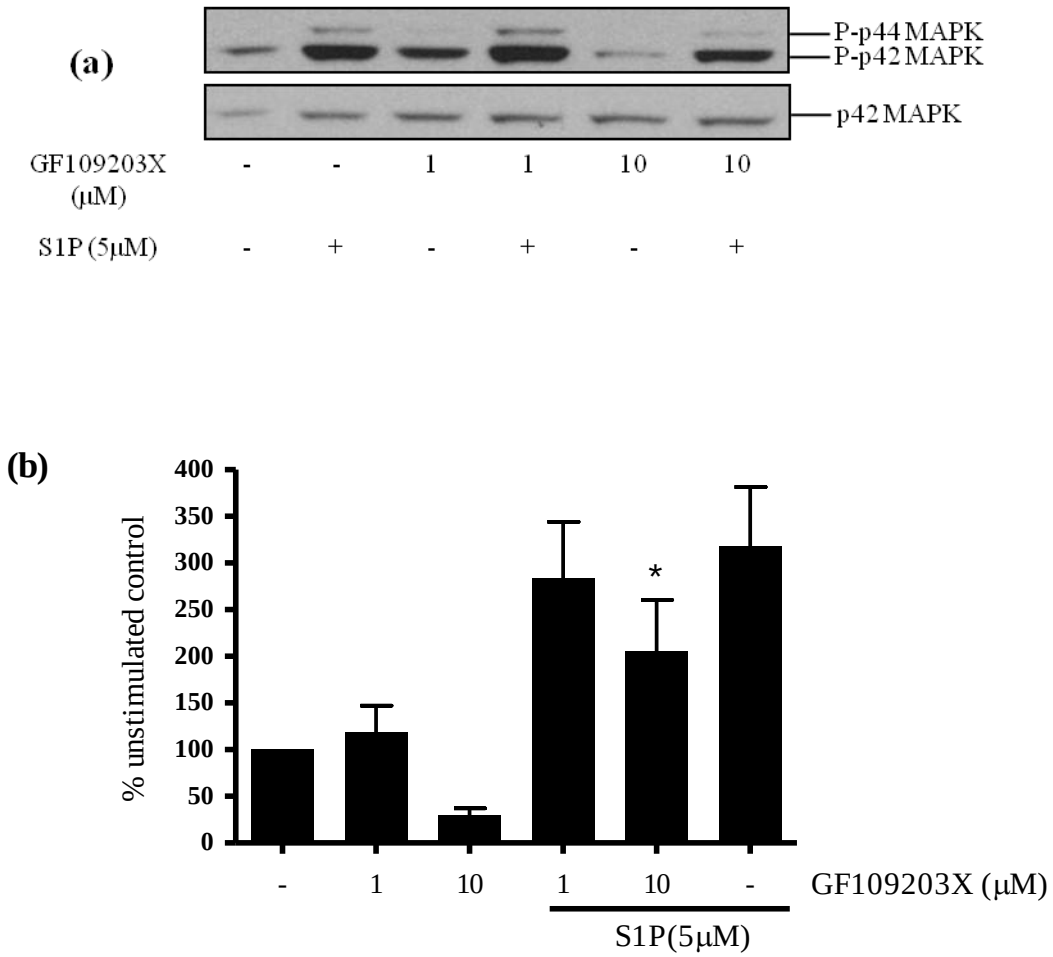


Figure 4.20 The effect of GF109203X on S1P-dependent p42/p44 MAPK in CGR8 cells.

(a) CGR8 cells were treated with GF109203X (1 or 10μM) for 15 minutes prior to stimulation with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK ratios for n=4 experiments expressed relative to unstimulated control cells (100%). Statistical significance was assessed by Student's unpaired *t* test (*$p<0.05$, GFX109203X 10μM + S1P *vs* S1P).

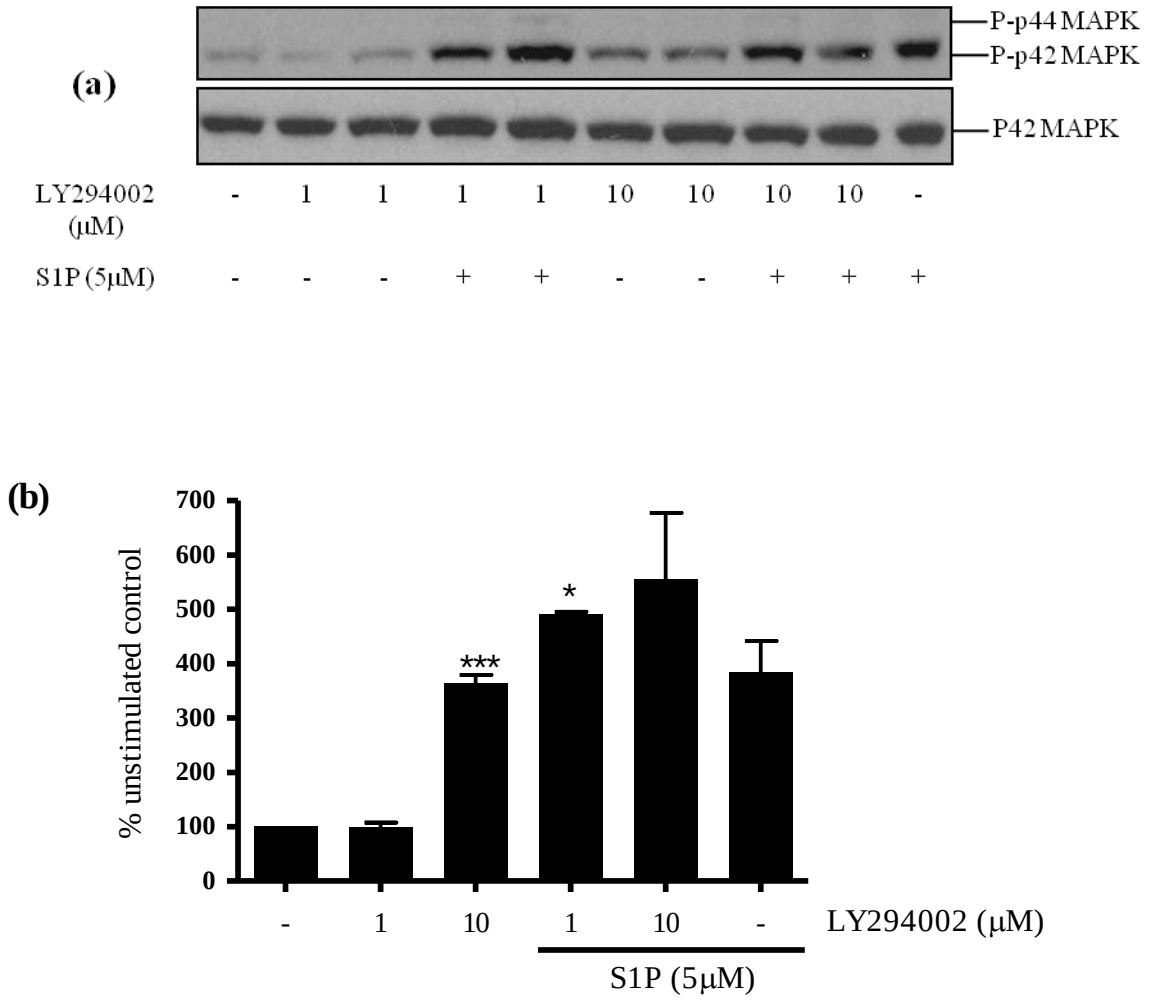


Figure 4.21 The effect of LY294002 on S1P-dependent p42/p44 MAPK activation in ES-D3 cells.

(a) ES-D3 cells were cultured in reduced serum medium then pre-treated with LY294002 (1 or 10μM) for 15 minutes prior to stimulation with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK for n=4 experiments.

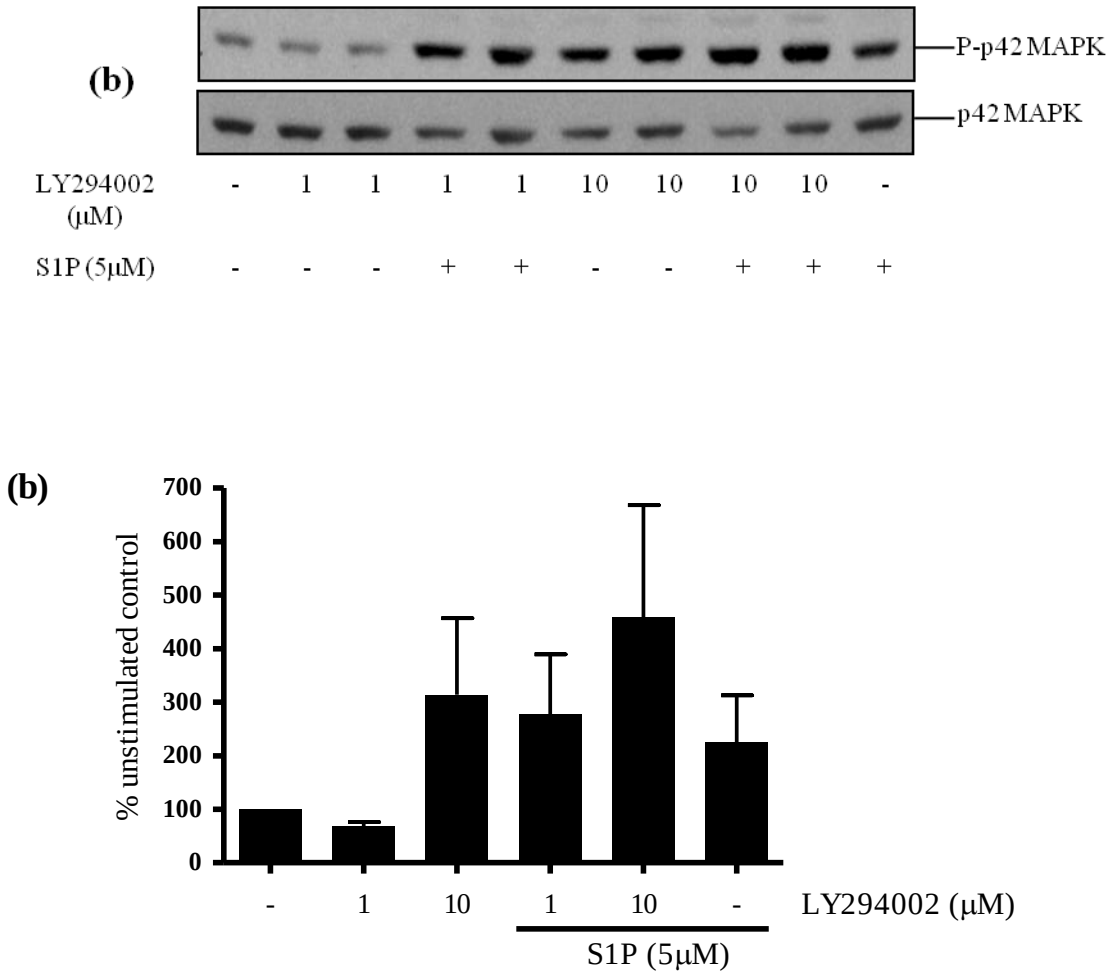


Figure 4.22 The effect of LY294002 on the S1P-dependent activation of p42/p44 MAPK in CGR8 cells.

(a) CGR8 cells were cultured in reduced serum medium then pre-treated with LY294002 (1 or 10μM) for 15 minutes prior to stimulation with or without S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels). (b) The histogram represents densitometric quantification of P-p42 MAPK/p42 MAPK for n=4 experiments. Statistical tests were performed but found not to be significant.

4.5 Discussion

The role and physiological function of S1P in somatic cells has been studied extensively over the last decade and studies have shown it has important role in many physiological and pathophysiological processes. More recently, a role for S1P in the regulation of both adult and embryonic stem cells has emerged and many studies are underway to elucidate the role of S1P in stem cell function which will result in a better understanding of factors that govern self-renewal and differentiation with the ultimate aim to generate viable ES cell derived cell types for use in replacement therapies.

This study has identified a role for S1P in the regulation of p42/p44 MAPK in ES-D3 and CGR8 cells. In cell expression studies (Chapter 3), S1P receptor subtypes $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ were identified in both ES-D3 and CGR8 cells. In this chapter, the functional relevance of these receptor subtypes in terms of p42/p44 MAPK activation and proliferation of ES cells was investigated pharmacologically using available S1P receptor antagonists ($S1P_{1-3}$). This study revealed that S1P-mediated p42/p44 MAPK activation in ES cells is S1P receptor mediated, based on evidence from experiments using pertussis toxin (PTX). Incubating ES-D3 and CGR8 cells with PTX, which functions to catalyse ADP-ribosylation of C-terminal cysteine from $G_i\alpha$ and uncouples heterotrimeric G_i (including $G_i\beta\gamma$) from the GPCR partially abolished p42/p44 MAPK activation in response to S1P stimulation. Activation of p42/p44 MAPK was reduced by approximately 20-36% suggesting p42/p44 MAPK activation by S1P is mediated by an S1P receptor and the S1P receptor involved appears to be coupled to G_i. It is known that the S1P receptors $S1P_1$, $S1P_2$ and $S1P_3$ are all coupled to the G protein G_i. In addition to G_i coupling, $S1P_2$ and $S1P_3$ are also linked to G_q and $G_{12/13}$. As PTX did not completely abolish p42/p44 MAPK activation by S1P, this could suggest that the S1P-dependent p42/p44 MAPK response is only partially mediated by a G_i coupled S1P receptor. As the $S1P_1$ receptor is exclusively coupled to G_i, and pertussis toxin did not completely abolish p42/p44 MAPK activation, it is unlikely that $S1P_1$ is exclusively responsible for the S1P-dependent p42/p44 MAPK activation in ES cells. This was confirmed by the

lack of effect of the $S1P_1$ specific inverse agonist, SB649146 on S1P-dependent p42/p44 MAPK activation. Two additional lines of evidence support this finding that $S1P_1$ has no involvement in regulating p42/p44 MAPK in ES cells. Firstly, the $S1P_1$ receptor has been shown to functionally interact with the PDGFβ receptor to regulate p42/p44 MAPK signalling and cell migration in various somatic cell lines (Alderton *et al*., 2001; Waters *et al*., 2003; Hobson *et al*., 2001). In this model, the PDGFβ receptor and S1P receptor exist in a tethered complex where the PDGFβ receptor tyrosine phosphorylates $G\alpha_i$ from the $S1P_1$ receptor to induce internalisation of the $S1P_1$-PDGFβR complex and enhance p42/p44 MAPK activation (Waters *et al*., 2006). Co-stimulation with S1P and PDGF resulted in a synergistic increase in p42/p44 MAPK activation in ASM cells which express endogenous $S1P_1$ and PDGFβ receptors. In this study of ES cells, in addition to finding that PDGF alone has no effect on p42/p44 MAPK activation, co-stimulation with S1P and PDGF also did not synergistically increase p42/p44 MAPK activation compared to S1P alone. Secondly, stimulation with the specific $S1P_1$ agonist, SEW2871, was without effect on p42/p44 MAPK providing further evidence supporting a lack of a role for $S1P_1$ in p42/p44 MAPK regulation in mES cells.

Further experiments to elucidate the S1P receptor involved in p42/p44 MAPK also exclude a role for $S1P_2$ and $S1P_3$ in S1P-dependent p42/p44 MAPK activation in ES cells based on evidence that the $S1P_2$ and $S1P_3$ receptor antagonists, JTE013 and CAY10444, were without effect on S1P induced p42/p44 MAPK activation. Despite evidence showing a lack of a role for $S1P_{1-3}$ in regulating the p42/p44 MAPK pathway in ES cells and the lack of $S1P_4$ receptor, the PTX sensitivity of S1P signalling to p42/p44 MAPK implicates the involvement of a G_i coupled GPCR, implicating a possible role for the $S1P_5$ receptor which is further investigated in Chapter 5.

The finding that PDGF was without effect on p42/p44 MAPK in ES cells corresponds to expression studies which showed that although ES-D3 and CGR8 cells express mRNA for PDGFβ receptor (section 3.2.2), analysis of PDGFβ receptor protein expression by Western blot was weakly detected in ES-D3 cells but not

CGR8 cells (section 3.2.4.7). Although the expression of the other PDGF receptor isoforms (PDGFαα and PDGFαβ) in ES-D3 and CGR8 cells remains unknown, the lack of effect of both PDGF-AB and PDGF-BB, which together can activate all three PDGF receptor isoforms also implicate low expression levels of all three receptor isoforms in both ES cell lines.

The lack of synergism on p42/p44 MAPK by S1P and PDGF also suggests there is a lack of cross regulation between S1P and PDGF in ES cells that has been well documented in other cell types (Alderton *et al*., 2001; Waters *et al*., 2003; Hobson *et al*., 2001). This also contrasts with the findings of Sachinidis and colleagues who reported synergism between S1P and PDGF in terms of promoting differentiation to cardiomyocytes from mouse ES-D3 cells (Sachinidis *et al*., 2003). The synergistic effect of S1P and PDGF observed by Sachinidis and colleagues resulted from mouse ES cells of the ES-D3 line that had formed into embryoid bodies, whereas this study focused on pluripotent self renewing mouse ES cells. It is possible that the complement of S1P receptor and PDGF receptors expressed in pluripotent self-renewing ES cells may differ from those expressed in the embryoid body. The complement of S1P receptors and PDGFβ receptor may be upregulated in embryoid bodies where formation of the $S1P_1$ and PDGFβ receptor signalling complex may occur, hence the reason for the synergistic effect of S1P and PDGF on cardiomyocyte differentiation and this would need further investigation.

This finding also contrasts with the study where S1P and PDGF exerted a synergistic anti-apoptotic effect on hES cells compared to either alone in maintaining human ES cells in an undifferentiated state which was found to be contingent on p42/p44 MAPK activation (Pebay *et al*., 2005; Wong *et al*., 2007). It has yet to be determined whether this is true for mouse ES cells however findings from this study excluding involvement of $S1P_1$, $S1P_2$ and $S1P_3$ in S1P-dependent p42/p44 MAPK activation in mES cells suggest that these receptors may not be involved in this effect of S1P in hES cells but would require further investigation as there are fundamental differences between mES and hES cells.

Although S1P has been shown to regulate a diverse range of mammalian cell processes, one of the most prominent biological functions of S1P is regulation of cell proliferation. In Swiss 3T3 fibroblasts, S1P induced Ca^{2+} resulting in increased DNA synthesis, cell division and induction of morphological changes where S1P treated cell appeared longer and flatter (Zhang *et al*., 1991). S1P also has pro-proliferative and pro-survivial effects on HTC4 hepatoma cells transfected with $S1P_2$ and $S1P_3$ (An *et al*., 2000). Furthermore, in human ES cells, S1P has been shown to promote survival by reducing apoptosis and increasing proliferation (Inniss & Moore, 2006). In contrast, in some cell types S1P has been shown to inhibit cell proliferation and promote differentiation. In myoblasts, S1P inhibited proliferation and induced differentiation, mediated by $S1P_2$, p42/p44 MAPK and p38 MAPK activation (Donati *et al*., 2005). The previous chapter demonstrated that S1P reduced proliferation of ES cells, measured by [^{3}H] thymidine incorporation into newly synthesised DNA. In this chapter, the MEK inhibitor, PD98059 revealed that S1P-dependent inhibition of proliferation in ES cells is independent of p42/p44 MAPK activation as PD98059 had no effect on the inhibition of proliferation induced by S1P. Therefore, S1P induced p42/p44 MAPK and had an inhibitory effect on ES cell proliferation, however, this inhibition of proliferation is p42/p44 MAPK independent and suggests that the S1P-dependent inhibition of proliferation is mediated by a signalling mechanism not dependent on p42/p44 MAPK.

However, basal proliferation of ES cells may be regulated by S1P receptors based upon findings that the $S1P_1$ inverse agonist, SB649146 and the $S1P_2$ antagonist, JTE013 significantly reduced incorporation of [^{3}H] thymidine compared to control cells, but the $S1P_3$ antagonist CAY10444 was without effect, implicates a role for $S1P_1$ and $S1P_2$ in regulating basal proliferation that is independent of p42/p44 MAPK signaling due to the fact that treatment with SB649146 and JTE013 had no effect on S1P-dependent p42/p44 MAPK activation. As the $S1P_1$ and $S1P_2$ antagonists had no effect on basal p42/p44 MAPK or S1P-dependent activation of p42/p44 MAPK, the mechanism of $S1P_1$ and $S1P_2$ in regulating proliferation of ES cells is most likely independent of p42/p44 MAPK signaling. Further studies would aim to confirm p42/p44 MAPK independence using the MEK inhibitor, PD98059

and investigate a role for p38 MAPK. The role of $S1P_1$ and $S1P_2$ in regulation of proliferation can also be examined by siRNA (small interfering RNA) to specifically knock down $S1P_1$ and $S1P_2$ expression and the effect on proliferation.

Pharmacological assessment of S1P regulation of p42/p44 MAPK in ES cells was continued by investigation of the mechanism by which S1P activates the p42/p44 MAPK signalling module. In somatic cells, intracellular signalling downstream of S1P receptors has been extensively studied and have revealed c-Src, PKC and PI3K, among others are involved in signal transmission from S1P receptor to p42/p44 MAPK. To assess the role of the aforementioned signalling intermediates in the S1P- dependent p42/p44 MAPK activation, inhibitors of these p42/p44 MAPK signalling intermediates were employed. This study is the first to provide evidence showing that the S1P-dependent activation of p42/p44 MAPK was reduced by GF109203X (PKC inhibitor) and PP2 (c-Src inhibitor) suggesting involvement of both PKC and c-Src in p42/p44 MAPK activation. The non receptor tyrosine kinase, c-Src has previously been shown to have an essential role in the regulation of p42/p44 MAPK by G_i coupled GPCRs in that c-Src tyrosine kinases function as intermediates between G_i coupled receptors and the Ras-p42/p44 MAPK pathway mediated by $G_i\beta\gamma$ subunits (Luttrell *et al*., 1996). This finding is in line with the PTX sensitivity of p42/p44 MAPK inhibition by S1P. As c-Src can be activated by $G_i\beta\gamma$ subunits, inhibition of $G_i\beta\gamma$ by PTX blocks activation of c-Src and therefore p42/p44 MAPK. Involvement of PI3K can be excluded in the S1P-dependent activation of p42/p44 MAPK activation in ES cells as the PI3K inhibitor, LY294002, did not reduce p42/p44 MAPK activation in response to S1P. Interestingly, treatment of both ES-D3 and CGR8 cells with LY294002 (10μM) alone resulted in p42/p44 MAPK activation compared to control conditions. LY294002 is a commonly used PI3K inhibitor, acting on the binding site of the PI3K enzyme and inhibiting the downstream mediator of PI3K signalling, Akt/PKB. However, LY294002 has been reported to have effects other than inhibition of PI3K. These include blockage of K(V) currents via a PI3K-independent mechanism in rat pancreatic β cells (El-Kholey *et al*., 2003) and induction of H_2O_2 generation in tumour cells also independent of PI3K (Poh & Pervaiz, 2005). The activation of p42/44 MAPK in

response to LY294002 in mES may be due to a PI3K independent effect of LY294002. One possibility is that in mES cells, LY294002 may induce stimulation of phosphorylation or activation of an upstream activator of p42/p44 MAPK such as Ras, Raf or MEK or by activating a different set of cellular targets. The effect of LY294002 on Ras, Raf and MEK could be further investigated by measuring activation of these p42/p44 MAPK upstream activators in response to LY294002.

In summary, pharmacological studies using S1P receptor antagonists excluded involvement of $S1P_1$, $S1P_2$ and $S1P_3$ in the S1P-dependent p42/p44 MAPK activation in ES cells. The S1P-dependent activation of p42/p44 MAPK was sensitive to inhibition by PTX, GFX109203X (PKC inhibitor) and PP2 (c-Src inhibitor), but was not reduced by LY29004 (PI3K inhibitor) suggesting that S1P uses G_i-, PKC-, and c-Src-dependent mechanisms to activate the p42/p44 MAPK pathway in ES cells. PTX sensitivity of S1P-dependent p42/p44 MAPK activation, suggests involvement of a GPCR and as $S1P_5$ is expressed in both ES-D3 and CGR8 cells, this receptor may be involved (Chapter 5). Finally, basal proliferation of mES cells may be regulated by $S1P_1$ and $S1P_2$.

CHAPTER 5

THE ROLE OF $S1P_5$ RECEPTOR IN p42/p44 MAPK REGULATION IN mES CELLS

CHAPTER 5 : The role of $S1P_5$ receptor in p42/p44 MAPK regulation in mES cells

5.1 Introduction

$S1P_5$ was originally identified by EST expression profiling from a rat PC12 cDNA library. Found almost exclusively in spleen and brain tissue (Glickman *et al*., 1999), it was subsequently shown to bind to S1P (and dihydroS1P) with an EC_{50} of 90nM (Im *et al*., 2000). Studies examining the signalling pathways resulting from S1P binding to $S1P_5$ have revealed activation of G_i and G_{12} but not G_s or $G_q/_{11}$ (Im *et al*., 2000). In transiently transfected CHO cells, $rS1P_5$ has been shown to inhibit forskolin-driven cAMP accumulation in a PTX toxin sensitive manner (i.e G_i mediated) and to inhibit p42/p44 MAPK-a novel effect of an S1P receptor-that was PTX insensitive, implicating a non-G_i mediated response (Malek *et al.*, 2001). In contrast to other S1P receptors, $rS1P_5$ inhibited proliferation when overexpressed in CHO cells (Malek *et al*., 2001) and HEK293 cells, which like p42/p44 MAPK inhibition was PTX insensitive (Malek *et al*., 2001). For human $S1P_5$, two spice variants have been reported (Niedemberg *et al*., 2002); a 5.4kb-transcript that is predominately expressed in muscle, heart and kidney and a 2.4-kb transcript that is expressed in brain, spleen and leukocytes. Interestingly, the anti-proliferative effect of $S1P_5$ was not observed in CHO cells over-expressing $hS1P_5$ (Niedemberg *et al*., 2002). In regard to the physiological functions of $S1P_5$, this receptor is predominately expressed in brain, particularly in oligodendrocytes and oligodendrocyte precursor cells (OPCs) where S1P binding to $S1P_5$ blocked migration of OPCs, an effect that was prevented by siRNA knock down of $S1P_5$ (Novgorodov *et al*., 2007). Inhibition of migration by $S1P_5$ in these cells was proposed to be via a mechanism involving $G\alpha_{12/13}$ coupled to the Rho, ROCK signaling pathway (Novgorodov *et al*., 2007).

Studies of $S1P_5$ and assignment of specific physiological functions for this receptor have been hampered by the lack of specific agonists and antagonists for this receptor.

Studies to date have identified S1P and dihydroS1P as ligands for the $S1P_5$ receptor (Im *et al*., 2000) but these ligands activate all members of the S1P receptor family and so give little insight into the specific function of the $S1P_5$ receptor. One study tested a host of bioactive lipids (including prostaglandins, thromboxanes, anandamides, sphingoids, platelet activating factors and LPA) for agonist activity at the $S1P_5$ receptor by measuring release of intracellular calcium (Niedemberg *et al*., 2002). Using a system where CHO-K1 cells were transiently transfected with cDNA for $hS1P_5$ or $rS1P_5$ and the chimeric G protein, $G\alpha_{qi5}$, which confers onto G_i coupled receptors the ability to stimulate the G_q pathway and therefore promote coupling to intracellular calcium, none of the lipids tested displayed any activity. However, this study did reveal the known $S1P_3$ antagonist, suramin, and its analogue NF023 to be antagonistic at both $hS1P_5$ and $rS1P_5$ but with different IC_{50} values.

The present study identifies the functional S1P receptor types in CHO cells. CHO cells were then used to explore the potential agonistic properties of phytosphingosine-1- phosphate (Ph-S1P) and two diastereomers, DM95 and DM97 upon transient transfection with $S1P_5$. The transfection protocols for CHO cells are well established and these cells are the most widely used mammalian cell line for transfection and protein expression as they provide stable and accurate glycosylation resulting in a more accurate replica of the natural protein.

Additionally, a possible role for the $S1P_5$ receptor, which is expressed in both ES cell types, was investigated. In these cells, expression studies (chapter 3) revealed the expression of $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ in mES cells and pharmacological studies (chapter 4) provided evidence to suggest a lack of a role for $S1P_{1–3}$ in regulating p42/p44 MAPK in these cells. However, the PTX sensitivity of S1P signaling to p42/p44 MAPK implicates the involvement of a GPCR. To investigate this, compounds that could be potential agonists at the $S1P_5$ receptor were studied.

Ph-S1P and diastereomeric DM95 and DM97 were obtained by selective phosphorylation of the corresponding *N*-Boc precursors (Mormeneo *et al*., unpublished) [**Figure 5.1**]. Ph-S1P is a natural S1P analogue that can be generated

by the phosphorylation of phytosphingosine, a naturally occurring sphingoid base, found it abundantly in fungi, plants and mammals. It is present in mammals as a minor component of cell membranes and as a bioactive lipid agonist at GPCRs. Ph-S1P is structurally similar to S1P but lacks the *trans*-double bond at between C-4 and C-5 and has a hydroxyl group at C-4 of the sphingoid long chain. Ph-S1P has been identified as an agonist for the $S1P_4$ receptor, with higher affinity than S1P for this receptor (Candelore *et al*., 2002). Using radiolabeled S1P ($S1^{33}P$), the affinity of Ph-S1P for the $S1P_4$ receptor is 1.6 nM, while that of S1P is nearly 50-fold lower (119 ± 20 nM) (Candelore *et al*., 2002). Ph-S1P has reportedly no activity at $S1P_1$, $S1P_2$ or $S1P_3$ receptors (Candelore *et al*., 2002; Kim *et al*., 2007; Inagaki *et al*., 2005). To date, agonist activity of Ph-S1P for $S1P_5$ is unknown. DM95 and DM97 are diastereomers of Ph-S1P. Although not naturally occuring compounds, the effect of the Ph-S1P diasteriomers, DM95 and DM97 at $S1P_5$ was tested to determine whether the stereochemical nature of the ligand was important.

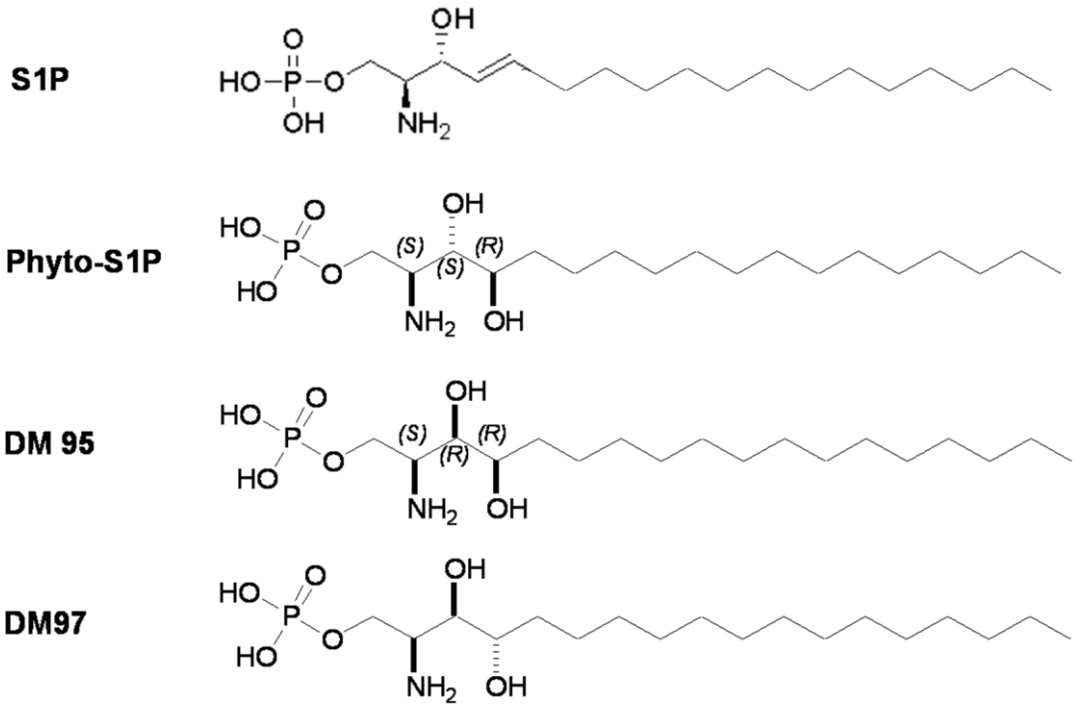


Figure 5.1 Structure of S1P, Ph-S1P and two diasteromeric compounds, DM95 and DM97.

5.2 Results

5.2.1 S1P receptor expression in CHO cells

Chinese hamster ovary cells were investigated for mRNA transcript expression of S1P receptor subtypes using gene specific primers and RT-PCR (**Table 2**).

5.2.1.1 $S1P_1$ receptor expression

RT-PCR using gene specific primers for $S1P_1$ did not detect mRNA transcript for $S1P_1$ from cDNA (+RT) from CHO cells [**Figure 5.2**]. cDNA from ES-D3 cells, shown previously to express $S1P_1$ mRNA transcript (section 3.2.1.1) was used as a positive control to validate the PCR reaction.

5.2.1.2 $S1P_2$ receptor expression

RT-PCR using gene specific primers for $S1P_2$ amplified a single product of 528bp from CHO cells [**Figure 5.3**] and a positive control sample (pcDNA3.1-myc-$S1P_2$), which was included to validate the PCR reaction. The CHO cell product was purified and subjected to nucleotide sequence analysis and confirmed to be $S1P_2$.

5.2.1.3 $S1P_3$ receptor expression

RT-PCR using gene specific primers for $S1P_3$ failed to amplify a product from CHO cells indicating that CHO cells do not express mRNA for $S1P_3$. The RT-PCR reaction was validated by including a positive control containing cDNA from ES-D3 cells (previously shown to express mRNA transcript for $S1P_3$: see 3.2.1.3), which amplified a 344bp product [**Figure 5.4**].

5.2.1.4 **$S1P_4$ receptor expression**

In CHO cells, RT-PCR using $S1P_4$ primers amplified a product of ~1000bp and a trace product of ~750bp [**Figure 5.5**]. A positive control reaction was included, using pcDNA3.1-h$S1P_4$-HA, which amplified a product corresponding to the predicted size (872bp). These data suggest CHO cells do not express $S1P_4$ mRNA. The 1000bp product amplified form CHO cells may be due to a mis-priming event.

5.2.1.5 **$S1P_5$ receptor expression**

An RT-PCR approach was attempted for detection of $S1P_5$ in CHO cells but was unsuccessful and therefore Western Blotting was used instead.

$S1P_5$ receptor protein expression in CHO cells was studied by Western blot analysis as outlined in section 2.4. Western blot analysis of CHO cell lysates with anti-$S1P_5$ (EDG-8) antibody detected a single band at approximately 42kDa, the predicted size for $S1P_5$, suggesting the presence of endogenous $S1P_5$ [**Figure 5.6**].

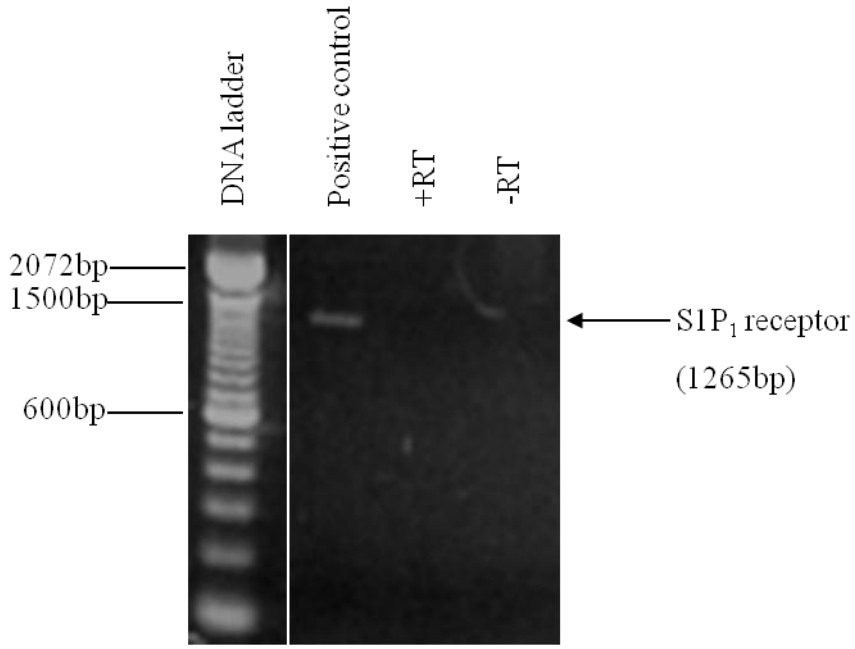


Figure 5.2 RT-PCR using gene specific $S1P_1$ primers did not detect $S1P_1$ mRNA transcript expression.

RT-PCR analysis using gene specific primers amplified a single 1265bp product by $S1P_1$ receptor primers in the positive control sample (cDNA from ES-D3 cells). mRNA for $S1P_1$ was not detected in cDNA (+RT) from CHO cells. A negative control with omission of reverse transcriptase (-RT) was included.

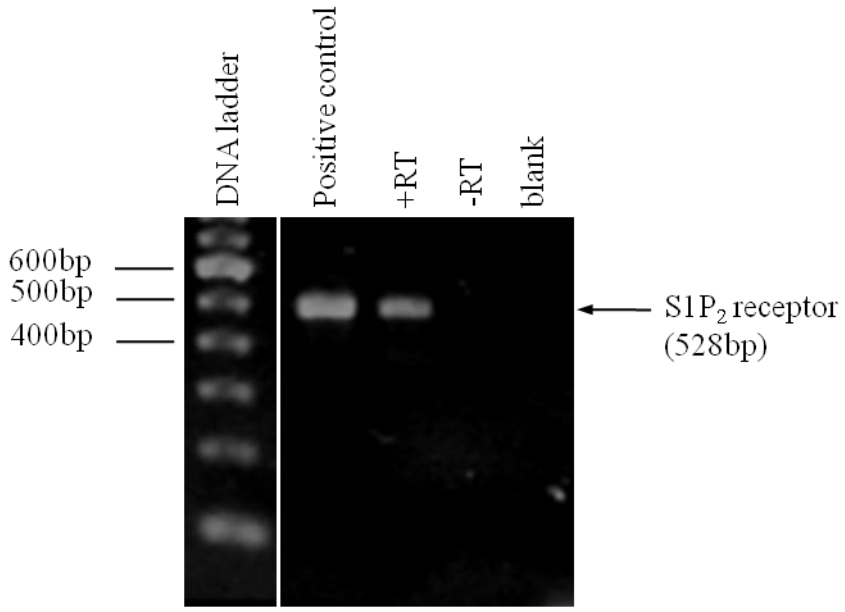


Figure 5.3 RT-PCR analysis of $S1P_2$ receptor mRNA transcript expression in CHO cells.

RT-PCR analysis using gene specific primers for $S1P_2$ amplified a single product of 528bp in positive control (pcDNA3.1-myc-$S1P_2$) and cDNA (+RT) from CHO cells. A negative control with omission of reverse transcriptase (-RT) was included. Blank is the negative control without cDNA.

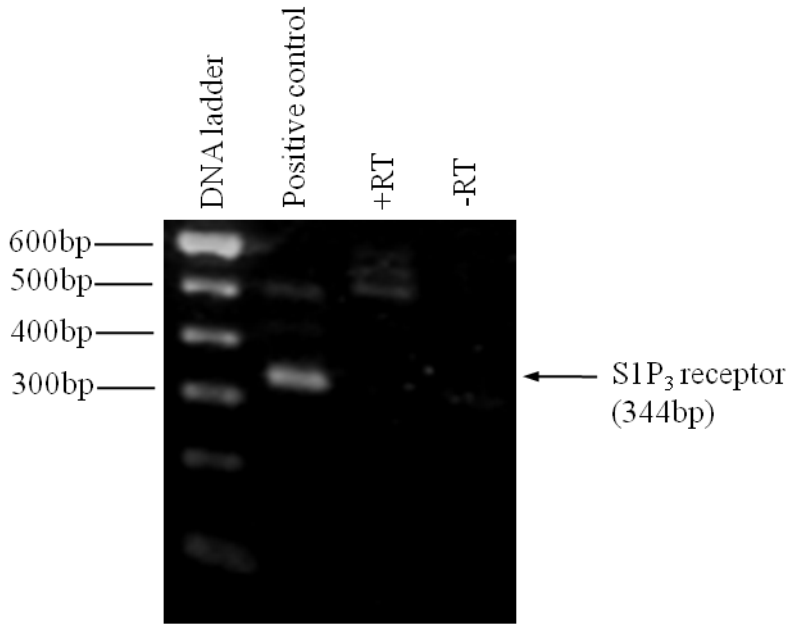


Figure 5.4 RT-PCR analysis of $S1P_3$ receptor mRNA transcript expression in CHO cells.

RT-PCR using gene specific primers amplified a single product of the predicted size (344bp) in the postive control sample (cDNA from ES-D3 cells) in addition to a 500bp non-specific product, but did not detect $S1P_3$ mRNA transcript expression from CHO cDNA (+RT). A negative control with omission of reverse transcriptase (-RT) was included.

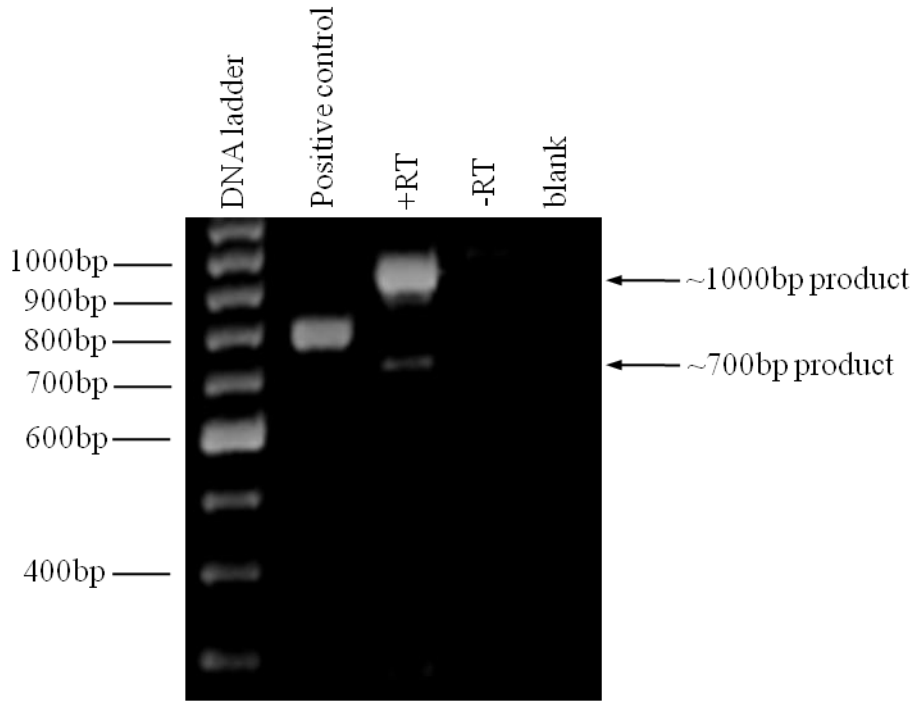


Figure 5.5 RT-PCR analysis of $S1P_4$ receptor mRNA transcript expression in CHO cells.

RT-PCR using $S1P_4$ gene specific primers amplified a product of the predicted size (872bp) from the positive control reaction (pcDNA3.1-$S1P_4$-HA), a product of ~1000bp and a trace product of ~700bp from CHO cells. A negative control with omission of reverse transcriptase (-RT) was included. Blank is the negative control without cDNA.

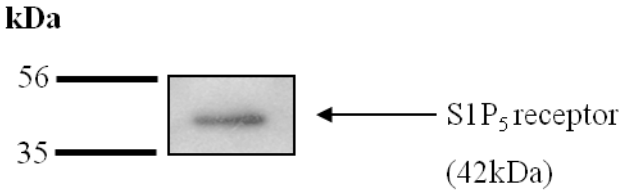


Figure 5.6 Western blot analysis of $S1P_5$ protein expression in CHO cells. Anti-$S1P_5$ receptor antibody detected a single band corresponding to the predicted molecular weight of $S1P_5$, ~42 kDa, in CHO cells.

5.2.2 The effect of $S1P_2$ antagonist (JTE013) and $S1P_3$ antagonist (CAY10444) on S1P-dependent p42/p44 MAPK activation in CHO cells

The previous section revealed expression of $S1P_2$ [**Figure 5.3**] and $S1P_5$ [**Figure 5.6**] in CHO cells by RT-PCR and Western blotting, respectively. Expression of functional $S1P_2$ receptors in CHO cells was tested using the competitive antagonist for the $S1P_2$ receptor, JTE013. **Figure 5.7(a)** shows that addition of exogenous S1P (5μM) induced activation of p42/p44 MAPK in CHO cells. Pre-treatment of CHO cells with the $S1P_2$ antagonist, JTE013 (1 or 10μM), had no effect on p42/p44 MAPK activation in response to S1P [**Figure 5.7(a)**]. This suggests a lack of functional $S1P_2$ receptor expression in CHO cells [**Figure 5.3**]. Unsurprisingly, the $S1P_3$ antagonist, CAY10444, had no effect on the S1P-induced p42/p44 MAPK activation in CHO cells [**Figure 5.7(b)**]. This coincides with lack of expression of $S1P_3$ receptor mRNA from RT-PCR experiments and confirms lack of $S1P_3$ receptor expression in CHO cells. As the $S1P_2$ antagonist, JTE013 had no effect on p42/p44 MAPK activation in response to S1P, and CHO cells do not express mRNA for $S1P_1$, $S1P_3$ or $S1P_4$, the S1P-dependent p42/p44 MAPK activation is likely be mediated by $S1P_5$.

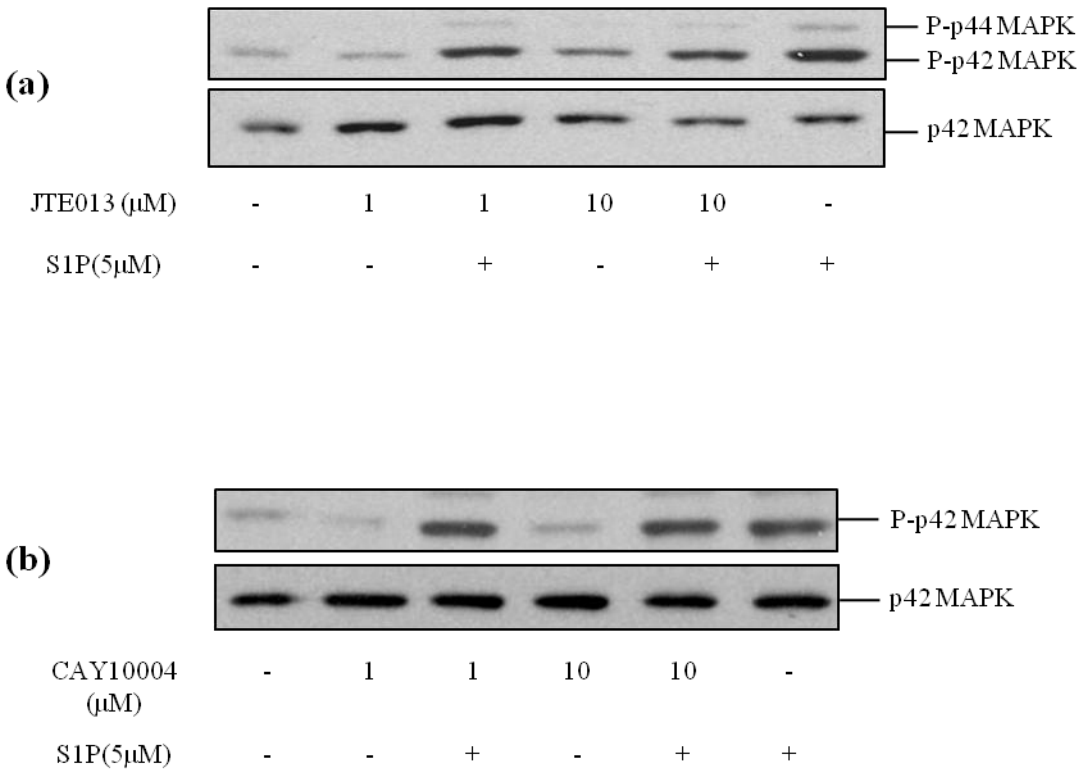


Figure 5.7 The $S1P_2$ antagonist (JTE013) and the $S1P_3$ antagonist (CAY10444) have no effect on S1P-dependent p42/p44 MAPK activation.

CHO cells were quiesced in serum free media for 24 hours prior to treatment with (a) JTE013 (1μM or 10μM) or (b) CAY10444 for 15 minutes and then with or with out S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and re-probed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels).

5.2.3 **HA-$S1P_5$ transient transfection into CHO cells**

To investigate the effects of S1P, Ph-S1P and its diastereomers, DM95 and DM97 on $S1P_5$, the receptor was over-expressed in CHO cells by transient transfection. Firstly, transfection conditions were optimised. Transfection of CHO cells with HA- tagged $S1P_5$ plasmid construct was carried out as described in section 2.14.3. Briefly, CHO cells were grown to ~50-60% confluency in 12-well plates then incubated with transfection mix (0.5-, 1- or 2µg of HA-$S1P_5$-pcDNA3.1 or pcDNA3.1 and Lipofectamine^{TM}2000 in Optimem) for 18 hours. The transfection mix was replaced with complete medium and incubated for a further 8 hours. Ectopic expression of HA-$S1P_5$ was established by Western blotting with an antibody for the HA epitope tag. **Figure 5.8** shows bands of approximately 42kDa corresponding to HA-$S1P_5$ were detected in pcDNA3.1-$S1P_5$-HA transfected cells and not in cells transfected with empty vector (pcDNA3.1). Transient transfection of HA-$S1P_5$ into CHO cells was optimal using 1µg of pcDNA3.1-$S1P_5$-HA, hence these conditions were used in further experiments.

5.2.4 **The effect of Ph-S1P, DM95 and DM97 on p42/p44 MAPK in CHO cell over-expressing $S1P_5$**

CHO cells were transiently transfected with HA-tagged $S1P_5$ receptor plasmid construct, as above, then treated with S1P, Ph-S1P, DM95 or DM97 and assessed for p42/p44 MAPK activation. Ph-S1P, DM95, DM97 and S1P (all 5µM) induced activation of p42/p44 MAPK in CHO cells over-expressing recombinant $S1P_5$ and in cells without $S1P_5$ overexpression [**Figure 5.9(a)]**. Densitometric analysis revealed that S1P (5µM) induced activation of p42/p44 MAPK was significantly enhanced in CHO cells over-expressing $S1P_5$ ($p=0.0068$, $S1P_5$ transfected *vs* mock transfected) [**Figure 5.9(b)**]. This was also the case for Ph-S1P. Ph-S1P (5µM) induced p42/p44 MAPK activation in CHO cells over-expressing $S1P_5$ was enhanced by more than 3-fold ($p=0.02$, $S1P_5$ transfected *vs* mock-transfected) [**Figure 5.9(b)**]. Ph-S1P has previously been shown to be an agonist at $S1P_4$ receptors but not $S1P_1$, $S1P_2$ and

$S1P_3$. Activation of p42/p44 MAPK by Ph-S1P in CHO cells overexpressing $S1P_5$ indicates Ph-S1P may also be an agonist for $S1P_5$. DM95 (5μM) induced p42/p44 MAPK activation was enhanced by 2-fold in $S1P_5$-transfected CHO cell compared to mock-transfected cells (not significant). DM97 induced p42/p44 MAPK activation was also significantly enhanced, by approximately 2-fold, in CHO cells over-expressing $S1P_5$ compared to mock-transfected cells (p=0.032, $S1P_5$-transfected *versus* mock-transfected) [**Figure 5.9(b)**]. Therefore, over-expression of recombinant $S1P_5$ in CHO cells increased Ph-S1P-, DM95- and DM97-induced p42/p44 MAPK activation suggesting these compounds have agonist activity at the $S1P_5$ receptor.

5.2.5 The effect of Ph-S1P, DM95 and DM97 on p42/p44 MAPK in ES cells

Having established that Ph-S1P, DM95 and DM97 have agonist activity at $S1P_5$ receptors, the next step was to utilise these compounds to investigate the function of $S1P_5$ in ES cells. To achieve this, ES-D3 and CGR8 cells were treated with Ph-S1P, DM95, or DM97 and p42/p44 MAPK activation assessed by Western Blotting. **Figure 5.10 (a)** shows that Ph-S1P, DM95 and DM97 activated p42/p44 MAPK in ES-D3 cells. Densitometric analysis showed that Ph-S1P (5μM) increased p42/p44 MAPK activation by approximately 2-fold, which was statistically significant (p=0.0028, Ph-S1P *vs* vehicle control) [**Figure 5.10(b)**]. DM95 and DM97 also significantly induced p42/p44 MAPK activation approximately 2-fold over vehicle alone (p=0.0032, DM95 *vs* vehicle; p=0.0041, DM97 *vs* vehicle). In CGR8 cells, a similar pattern was observed [**Figure 5.11(a)**]. Ph-S1P, DM95 and DM97 all induced activation of p42/p44 MAPK over vehicle alone (not statistically significant) [**Figure 5.11(b)**]. These data indicate that endogenous $S1P_5$ may mediate the effects of S1P in terms of p42/p44 MAPK in ES cells, which is a novel role for this receptor.

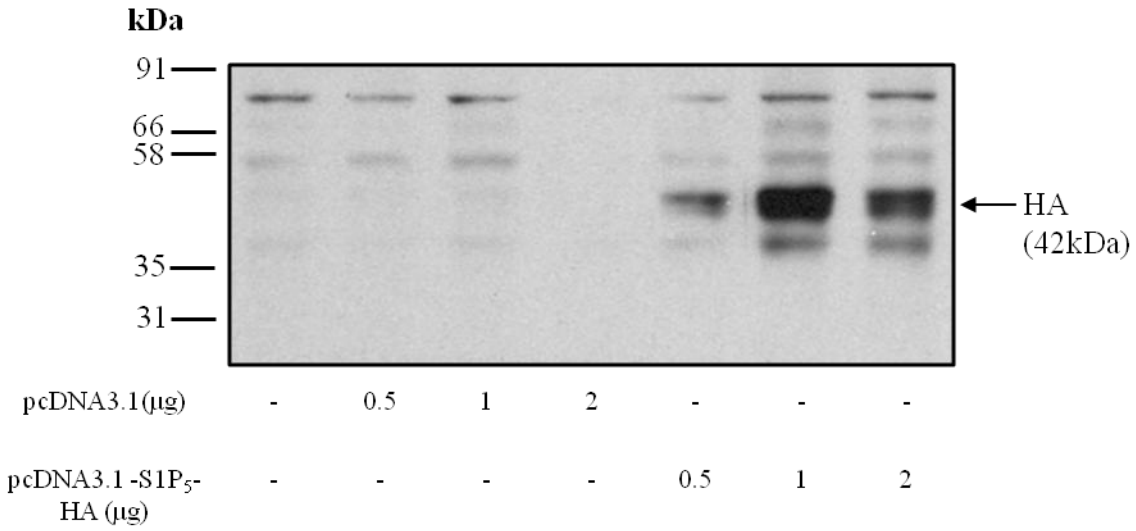


Figure 5.8 Optimisation of pcDNA3.1-S1P$_5$-HA plasmid transfection in CHO cells.

CHO cells transiently transfected with increasing concentrations of empty vector (pcDNA3.1) or pcDNA3.1-S1P$_5$-HA (0.5-2μg) for 18 hours were lysed and analysed by Western Blotting using anti-HA tag antibody. The ectopic expression of S1P$_5$ was indicated by the detection of 42kDa immunoreactive bands in pcDNA3.1-S1P$_5$-HA transfected cells compared to negative controls (mock transfected).

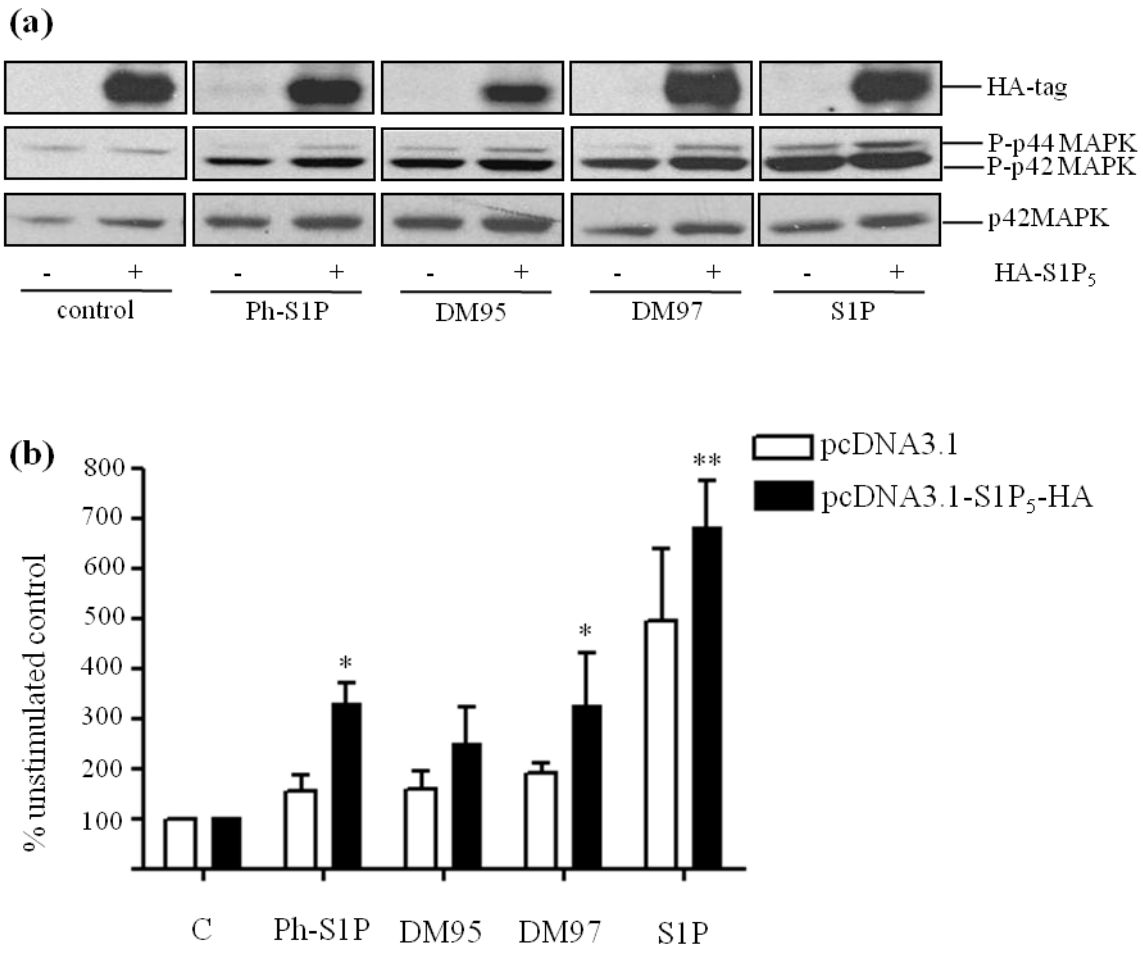


Figure 5.9 The effect of Ph-S1P, DM95 and DM97 on p42/p44 MAPK in CHO cells over-expressing $S1P_5$.

(a) CHO cells were transiently transfected with pcDNA3.1 (denoted by -) or pcDNA3.1-$S1P_5$-HA (denoted by +) then treated with DMSO (control), Ph-S1P (5μM), DM95 (5μM) or DM97 (5μM) or S1P (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and Western Blot. Ectopic expression of recombinant $S1P_5$ was detected by an antibody specific for HA tag (top panel). Lysates were aso blotted with an antibody specific for phosphorylated p42/p44 MAPK (middle panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels).

(b) The histogram represents densitometric quantification of phosphorylated p42 MAPK/p42 MAPK ratios for n=3 experiments showing a significant effect of Ph-S1P and DM97 on p42/p44 MAPK activation (*$p<0.05$, **$p<0.01$, $S1P_5$-transfected *versus* mock-transfected CHO cells).

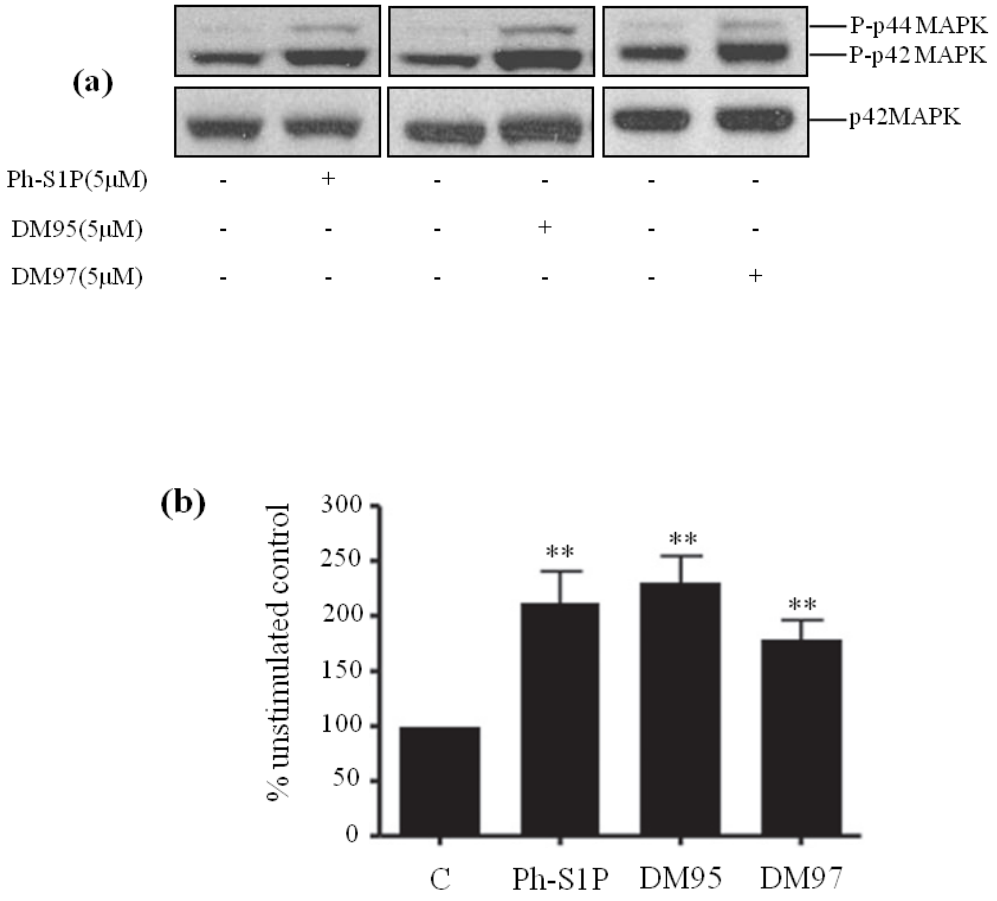


Figure 5.10 The effect of Ph-S1P, DM95 and DM97 on p42/p44 MAPK in ES-D3 cells.

(a) ES-D3 cells were cultured in reduced serum medium for 24 hours then treated with Ph-S1P (5μM), DM95 (5μM) or DM97 (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels).

(b) The histogram represents densitometric quantification of phosphorylated p42 MAPK/p42 MAPK ratios for n=3 experiments showing a significant effect of Ph-S1P, DM95 and DM97 on p42/p44 MAPK activation in ES-D3 cells (**$p<0.01$; agonist stimulation versus control).

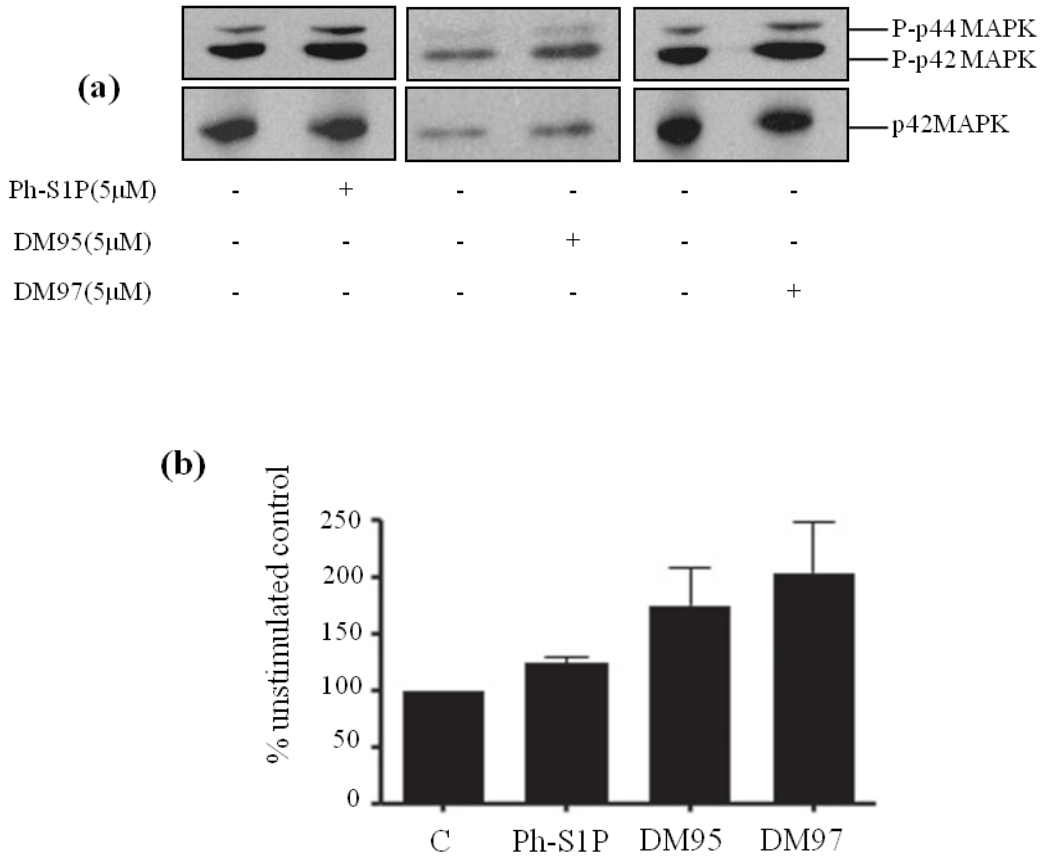


Figure 5.11 The effect of Ph-S1P, DM95 and DM97 on p42/p44 MAPK in CGR8 cells.

(a) CGR8 cells were cultured in reduced serum medium for 24 hours then treated with Ph-S1P (5μM), DM95 (5μM) or DM97 (5μM) for 10 minutes. Cell lysates were analysed by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p42/p44 MAPK (top panels). Blots were stripped and reprobed with an antibody specific for p42 MAPK to establish equal protein loading (bottom panels).

(b) The histogram represents densitometric quantification of phosphorylated p42 MAPK/p42 MAPK ratios for n=3 experiments showing a significant effect of Ph-S1P, DM95 and DM97 on p42/p44 MAPK activation in CGR8 cells ($p<0.01$; agonist stimulation versus control).

5.3 Discussion

The over-expression of $S1P_5$ in CHO cells was used in the current study to investigate the effects of Ph-S1P, a known high affinity agonist for $S1P_4$, and two diastereomers, on $S1P_5$ mediated p42/p44 MAPK activation. Having established these compounds to be agonists for this receptor, they were used to investigate the possibility of regulation of p42/p44 MAPK in ES cells by $S1P_5$.

Firstly, CHO cells were investigated for S1P receptor expression, by RT-PCR and Western Blot, which revealed endogenous expression of $S1P_2$ and $S1P_5$. Previously, Northern blot analysis demonstrated very low expression of $S1P_2$, but not $S1P_1$ or $S1P_3$ in CHO cells (Okamoto *et al*., 1998) and is consistent with the present study. As Ph-S1P has been shown to be a high affinity ligand for $S1P_4$ receptor, the endogenous expression of this receptor was investigated by RT-PCR. Gene specific primers for $S1P_4$ failed to amplify a product from CHO cells, therefore excluding endogenous expression of $S1P_4$ in this cell line. The expression of functional $S1P_2$ in CHO cells was investigated using the $S1P_2$ antagonist, JTE013. JTE013 had no effect on S1P-dependent p42/p44 MAPK activation in CHO cells, indicating a lack of functional $S1P_2$ receptor. As $S1P_5$ receptor is the only other S1P receptor endogenously expressed in this cell line, then the p42/p44 MAPK activation by S1P is likely to be mediated by $S1P_5$.

To test the efficacy of Ph-S1P, DM95 and DM97 on $S1P_5$, HA tagged $S1P_5$ receptor plasmid construct was transiently transfected into CHO cells in order to increase expression of this receptor. Successful ectopic expression of $S1P_5$ was detected by an antibody specific for the HA tag. Ph-S1P, DM95 and DM97 activated p42/p44 MAPK at least 2-fold in CHO cells over-expressing ectopic $S1P_5$ compared to mock transfected CHO cells, which suggests that these compounds induce p42/p44 MAPK mediated by $S1P_5$. These compounds also induced p42/p44 MAPK activation in mES cells. Chapter 3 demonstrated that activation of p42/p44 MAPK by S1P is not mediated by $S1P_1$, $S1P_2$ or $S1P_3$ and the ES cells in this study do not express $S1P_4$. Therefore, p42/p44 MAPK activation in ES cells may be mediated by $S1P_5$ due to the fact that the known $S1P_4$ receptor agonist, Ph-S1P and two diastereomers have

agonist activity at $S1P_5$ in CHO cells over-expressing $S1P_5$ and also induce p42/p44 MAPK activation in ES cells. This contrasts with previous studies which have reported $S1P_5$ stimulation by S1P to inhibit p42/p44 MAPK activation, in a PTX sensitive manner (Malek *et al.*, 2001). The inhibition of p42/p44 MAPK is an unusual effect for an S1P receptor as CHO cells transfected with $S1P_1$, $S1P_2$ and $S1P_3$ results in p42/p44 MAPK activation upon stimulation with S1P (Lee *et al*., 1998; Okamoto *et al*., 1998; Gonda *et al*., 1999; Okamoto *et al*, 1999). The current study would benefit from further investigation of the PTX sensitivity of p42/p44 MAPK activation by Ph-S1P, DM95 and DM97. $S1P_5$ is reported to couple to the G proteins G_i and G_{12} (Im *et al*., 2000). PTX sensitivity would indicate whether the effects of Ph-S1P, DM95 and DM97 are G_i mediated. In contrast to other S1P receptors, S1P inhibited proliferation in $rS1P_5$ over-expressing CHO cells which, like p42/p44 MAPK inhibition was PTX insensitive (Malek *et al*., 2001). In the current study, S1P also inhibited proliferation of ES cells. The next step would be to investigate the functional role of $S1P_5$ in ES cells in terms of proliferation. This would be done by measuring [^{3}H]-thymidine incorporation in ES-D3 cells after stimulation with Ph-S1P, DM95 and DM97.

In human ES cells, p42/p44 MAPK activation by S1P protects cells against apoptosis (Wong *et al*., 2007). It would be valuable to establish if this is also true for mES cells and if so, whether p42/p44 MAPK activation mediated by $S1P_5$ may contribute to an anti-apoptotic/cell survival program.

This study could be further enhanced by investigating binding of Ph-S1P, DM95 and DM97 to the $S1P_5$ receptor using a ligand binding study such as a [^{35}S]GTPγS assay. The [^{35}S]GTPγS assay measures the level of G protein activation following agonist occupation of a GPCR. Normally after activation of G protein by GPCR, GDP is dissociated from the Gαβγ heterotrimer, followed by binding of GTP to Gα and initiation of downstream signalling events. The [^{35}S]GTPγS assay uses the non-hydrolysable analogue [^{35}S]GTPγS, which blocks the subsequent reformation of the Gαβγ heterotrimer by GTPase activity (Harrison & Taynor, 2003).

Investigating the role of $S1P_5$ in ES cells is inhibited by the fact that chemical antagonists for the $S1P_5$ receptor are not currently commercially available, and therefore pharmacological inhibition of this receptor cannot be employed. This study could be enhanced by siRNA knockdown of $S1P_5$. This would serve to confirm the involvement of $S1P_5$ in p42/p44 MAPK activation. $S1P_5$ siRNA could also be used to demonstrate the involvement of $S1P_5$ in DNA synthesis (proliferation) and differentiation of ES cells and therefore demonstrate more directly the functional role of $S1P_5$ in ES cells.

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6: GENERAL DISCUSSION

Since the propagation of the first embryonic stem cell line *in vitro* nearly three decades ago, they have been an immensely powerful tool for studying early differentiation lineages *in vitro*. mES cells have also been used extensively as a vehicle to introduce genes into the early embryo and have been pivotal in the development of transgenic technology. Since the isolation of hES cells in 1998, these cells have held much promise as a clinical treatment for a range of degenerative diseases. However, a limiting factor in the manipulation of ES cells for cellular replacement therapy is that, *in vitro*, methods to direct differentiation towards a specific cell type are limited. Orientation of ES cells to a specific cell lineage is limited to a small population of cells in what is typically a mixture of different specialised cells. Control of stem cell survival, proliferation, differentiation and migration are integral to their function. Some of the mechanisms regulating these processes are beginning to emerge, but much is still unknown. Therefore, understanding the molecular mechanisms that govern these processes is an ongoing challenge in this field.

Over the last decade, S1P has emerged as a vital lipid mediator functioning both intracellularly and as an extracellular ligand for specific cell surface receptors. The discovery that S1P regulates cell growth and stimulates proliferation (Zhang *et al*., 1991) triggered great interest in this molecule and resulted in thousands of research articles linking S1P to many different cellular processes including cell migration, differentiation and apoptosis. It is now established that S1P plays an important role in immune, cardiovascular, nervous and respiratory systems as well as in cancer (Takabe *et al*., 2008). It is therefore not surprising then that S1P also has an emerging role in regulating many aspects of embryonic stem cell biology.

The p42/p44 MAPK pathway regulates many critical cellular events, including cell cycle progression, proliferation, cell survival and differentiation. p42/p44 MAPK signalling has been studied in both human and mouse ES cells. Studies suggest a role for p42/p44 MAPK in the maintenance of hES cells pluripotency (Pebay *et al*., 2005;

Wong *et al.*, 2007) while in mES cells it has been suggested to disrupt self-renewal and promote differentiation (Burdon *et al.*, 1999).

6.1 S1P receptor regulation of p42/p44 MAPK in mES cells

This study presented the first characterisation of S1P receptor subtype regulation of p42/p44 MAPK in undifferentiated mES cells. Evidence was obtained to support a role for the $S1P_5$ receptor in regulation of p42/p44 MAPK in mES cells. The mES cell lines used in this study (ES-D3 and CGR8) were found to express $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ but not $S1P_4$ as discussed in Chapter 3. Treatment of mES cells with S1P induced p42/p44 MAPK activation via a mechanism that did not involve $S1P_1$, $S1P_2$ or $S1P_3$. This was due to the fact that $S1P_1$, $S1P_2$ and $S1P_3$ antagonists failed to disrupt S1P-mediated p42/p44 MAPK activation. However, the PTX sensitivity of the p42/p44 MAPK response in regard to S1P highlights the involvement of a G_i coupled S1P receptor. The findings in this study, using Ph-S1P, suggest a role for the $S1P_5$ receptor in terms of regulating p42/p44 MAPK. In view of this, treatment of these mES cells with Ph-S1P and its diasteriomers, which have agonist properties at the $S1P_5$ receptor (this study in CHO cells) induced p42/p44 MAPK activation. As specific chemical $S1P_5$ receptor antagonists are currently unavailable, we could not specifically block the $S1P_5$ receptor in mES cells and therefore rely on evidence of a lack of effect of $S1P_1$, $S1P_2$ and $S1P_3$ antagonists on p42/p44 MAPK activation. The study would benefit from specific agonists and antagonists for $S1P_5$ to investigate $S1P_5$ signalling and function in mES cells in more detail. Specific agonists for $S1P_5$ are currently unavailable. However suramin, an antagonist at the $S1P_3$ and $S1P_5$ receptor could be used to evaluate the role of $S1P_5$ in S1P-dependent p42/p44 MAPK activation. The study could also be significantly enhanced by the inclusion of $S1P_5$ siRNA knockdown experiments to further verify the involvement of $S1P_5$ in the self-renewal or differentiation of mES cells.

The finding that S1P may induce p42/p44 MAPK activation via $S1P_5$ highlights a novel role for this receptor although little is known about the different signalling

cascades initiated by $S1P_5$ receptor activation. Previously, $S1P_5$ has been reported to bind S1P and dihydroS1P with high affinity, couple to G_i and G_{12} but not G_s or G_{11} and inhibit cAMP and p42/p44 MAPK activation (Im *et al.*, 2000; Malek *et al.*, 2001). To date, the possibility of Ph-S1P as an agonist at the S1P receptor has not been investigated. This study provides the first evidence to suggest that Ph-S1P induces p42/p44 MAPK activation via $S1P_5$, based on the ability of Ph-S1P to induce p42/p44 MAPK activation in both mock- and $S1P_5$-transfected CHO cells, a mechanism which does not involve $S1P_2$. To further evaluate Ph-S1P, DM95 and DM97 as agonists at the $S1P_5$ receptor, their ability to stimulate guanine nucleotide exchange in $S1P_5$ transfected cells using a GTPγS binding study should be investigated.

6.2 The role of S1P in mES cell proliferation

This study demonstrated that S1P activated the p42/p44 MAPK pathway in mES cells, via a mechanism that was mediated by a G_i coupled GPCR (based on evidence that PTX reduced S1P-dependent p42/p44 MAPK activation). Activation of the p42/p44 MAPK pathway is typically connected to promotion of growth, proliferation and survival in many cell types. Furthermore, S1P-dependent activation of p42/p44 MAPK has been shown to promote proliferation in different cell types (An *et al.*, 2000; Wu *et al.*, 1995; Zhang *et al.*, 1991). This would suggest that S1P-dependent p42/p44 MAPK activation may promote proliferation in mES cells. However in this study, S1P inhibited proliferation in mES cells, indicating that p42/p44 MAPK is not involved in the S1P-dependent inhibition of proliferation. The fact that the PD98059 compound, a potent inhibitor of MEK activation by upstream activators, which prevents downstream activation of p42/p44 MAPK had no effect on inhibition of proliferation by S1P in mES cells provides further evidence that this pathway is not involved. In mES cells, S1P promoted p42/p44 MAPK activation, possibly via the $S1P_5$ receptor but in contrast inhibited proliferation of mES cells, a possible indication of growth arrest/apoptosis/differentiation. Therefore, there could be two mechanisms involved in the response of mES cells to S1P. These are:

a) S1P activates p42/p44 MAPK via the $S1P_5$ receptor and therefore $S1P_5$ may be a 'proliferative receptor' in mES cells. In times where proliferation needs to be promoted, S1P may be specifically generated in cells by SphK1/2 and released in close proximity to $S1P_5$ receptors, allowing easy accessibility and activation of p42/p44 MAPK. Our data suggests that activation of p42/p44 MAPK by $S1P_5$ may be mediated by the non receptor tyrosine kinase, c-Src which could link $G_i\beta\gamma$ subunits of $S1P_5$ to Ras and ultimately to p42/p44 MAPK. Furthermore, we propose p42/p44 MAPK activation by $S1P_5$ can also be mediated by PKC based on the fact that a potent PKC inhibitor reduced p42/p44 MAPK activation by S1P. PKC enzymes are known to regulate cell cycle transitions such as cell cycle entry and exit.

(b) On the other hand, S1P reduces proliferation and this is not mediated by the $S1P_5$ receptor or p42/p44 MAPK activation. One possibility is that the effect of S1P in mES cells may be mediated by another lipid receptor ('growth arrest receptor') which binds S1P and inhibits proliferation of mES cells. The receptor(s) is most likely not $S1P_1$ or $S1P_2$ as these may be involved in stimulation of basal proliferation, as evidenced by the finding that inverse agonists of these receptors, which reduce their constitutive activation, reduced basal DNA synthesis. We demonstrated that under basal conditions, the $S1P_1$ receptor inverse agonist, SB649146, and the $S1P_2$ receptor antagonist, JTE013, reduced basal DNA synthesis. Alternatively, mES cells may synthesise low levels of S1P and release it into the extracellular environment, where it can bind to and activate cell surface S1P receptors in an autocrine manner consistent with the 'inside-out' signalling model (Hobson *et al*., 2001).

The 'growth arrest' receptor may mediate inhibition of proliferation by S1P via activation of the JNK pathway or PI3K/Akt but this remains to be investigated-especially as PI3K inhibitors actually increased p42/p44 MAPK activation, and therefore PI3K might function to suppress p42/p44 MAPK signalling.
S1P might also use small GTPases Rho, Rac and p21 activated kinase 1 (PAK-1) to regulate DNA synthesis. The $S1P_5$ receptor has been shown to activate Rho kinase via $G_{12/13}$ G protein coupling in oligodendrocytes where the Rho/ROCK signaling pathway was involved in migration (Novgorodov *et al*., 2007). Activation of Rho by

$S1P_5$ may promote self-renewal in undifferentiated mES cells. However, initiation of differentiation pathways may cause a switch in S1P receptor subtypes expressed in mES cells. This would result in a decrease in $S1P_5$ receptors and a subsequent decrease in Rho activation, leading to promotion of differentiation. It may be the case that among a class of receptors, expression of one particular subtype over another defines differentiation to a particular lineage. All of these possibilities could be tested experimentally in the future.

6.3 Summary and Conclusion

The current study has extended the knowledge of the role of S1P in mES cells, revealing that S1P regulation of the p42/p44 MAPK pathway in these cells may be mediated by the $S1P_5$ receptor, highlighting a novel role for this receptor. In human ES cells, p42/p44 MAPK appears to protect against apoptosis. It remains to be determined whether p42/p44 MAPK has a similar role in mouse ES cells. However, if this is the case, then our data might suggest that $S1P_5$-mediated activation of p42/p44 MAPK represents an antiapoptotic/cell survival signaling program.

The current study indicates that Ph-S1P may be an agonist at the $S1P_5$ receptor and therefore a useful pharmacological reagent for probing $S1P_5$ function in ES or somatic cells that lack $S1P_4$.

The results of this study will contribute significantly to both S1P research and ES cell research and may aid in understanding the molecular mechanisms of ES cell biology and ultimately help to overcome the challenges faced in generating ES cells of a specific lineage for stem cell replacement therapies.

CHAPTER 7

REFERENCES

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