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Identification of Sphingosine 1-Phosphate Receptors in Exosomes Released from Breast Cancer Cells

A thesis presented

By

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DECLARATION

‘I declare that, except where specifically indicated, all the work presented in this report is my own and I am the sole author of all parts.’

Signed: _____

Date: _____

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ABBREVIATIONS

A-SMase, Acid sphingomyelinase; AP-2, Adaptin-2; AD, Alzheimer's disease; Aβ, Amyloid β protein; APAF1, Apoptotic protease activating factor 1; AT1R, Angiotensin II type I receptor; APC, Antigen presenting cells; ATP, Adenosine tri phosphate; ABC transporters, ATP-binding cassette transporters; BACE1, beta-site amyloid precursor protein-cleaving enzyme 1; BAK, Bcl-2 homologous antagonist/killer; BCRP, Breast cancer resistant protein; CCV, Clathrin coated vesicles; CERK, Ceramide kinase; CHO, Chinese Hamster Ovary; CL, Cell lysate; C1P, Ceramide-1- phosphate; C1PP, Ceramide 1-phosphate phosphatase; CerS6, Ceramide synthase 6; 231CM, MDA-MB-231 cells cultured conditioned medium; 453 CM, MDA-MB-453 cells cultured conditioned medium; DAG, Diacylglycerol; DC, Dentric cells; DMSO, Dimethyl sulphoxide; E2, 17 β-estradiol; EBV, Epstein-Bar virus; ECs, Endothelial cells; ECM, extracellular matrix; EGF, epidermal growth factor; ER, estrogen receptor; ERM, Ezrin, radixin and moesin; ERK-1/2, extracellular regulated kinase; ESCRT, Endosomal sorting complex required for transport; EVs, Extracellular vesicles; EXO, Exosomes; FCS, Fetal calf serum; GPCR, G-protein coupled receptor; HDAC1, Histone deacetylase 1; HDAC2, Histone deacetylase 2; HEK293, Human embryonic kidney 293 cells; HER2, Human epidermal growth factor receptor-2; Hsp70, Heat shock protein 70; hTERT, Human telomerase reverse transcriptase; hr, hour; HUVEC, Human umbilical vein endothelial cells; KO, Knockout; JNK, c-Jun N-terminal kinase; IP_3, inositol triphosphate; MHC, Major histo-compatibility complex; MβCD, Methyl-β cyclodextrin; MAP, Mitogen activated kinase; MBV, Multi-vesicular bodies; MCF-7, Michigan Cancer Foundation-7; min, minute; GM3,

monosialodihexosylganglioside,; MEF, Mouse embryonic fibroblast; NCM, Non-Conditioned Medium; MMPs, Matrix Metalloproteinases; NKs, Natural Killer cells; RNA, Ribonucleic acid; RTK, Receptor tyrosine kinase; PAK, p21-activated kinase; (PA), Phosphatidic acid; PBS, Phosphate buffer saline; PDGF, Platelet derived growth factor; PLC, Phospholipase C; PKC, Protein kinase C; PMNs, Polymorph nuclear leukocytes; PR, Progestin receptor; PP2A, Protein phosphatase 2A; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; SM, Sphingomyeline; SMase, Sphingomyelinase; S1P, Sphingosine-1- phosphate; SKs, Sphingosine kinases; SK1, Sphingosine kinase 1; SK2, sphingosine kinase-2; SPP1, Sphingosine phosphate phosphatase-1; SPP2, Sphingosine phosphate phosphatase-2; SMS, Sphingomyelin synthase; TAFs, Tumour associated fibroblasts; TGF-β, Transforming growth factor beta; TNFα, Tumour necrosis factor-α; Spns2, Spinster homologue 2; VEGF, Vascular endothelial growth factor.

LIST OF PUBLICATIONS

ELBuri, A., Adams, D., Smith, D., Tate, R., Mullin, M., Pyne, S. and Pyne, N. (2018). The sphingosine 1-phosphate receptor 2 is shed in exosomes from breast cancer cells and is N-terminally processed to a short constitutively active form that promotes extracellular signal regulated kinase activation and DNA synthesis in fibroblasts. Oncotarget, 9(50).

Pyne, NJ, El Buri, A, Adams, DR & Pyne, S 2018, 'Sphingosine 1-phosphate and cancer' Advances in Biological Regulation, (68):97-106

Obeid, M., Elburi, A., Young, L., Mullen, A., Tate, R. and Ferro, V. (2017). Formulation of Nonionic Surfactant Vesicles (NISV) Prepared by Microfluidics for Therapeutic Delivery of siRNA into Cancer Cells. Molecular Pharmaceutics, 14(7):2450-2458.

ABSTRACT

Sphingosine-1-phosphate (S1P) is a bioactive lipid that promotes cell survival, proliferation, migration, angiogenesis and immune response, which are critical processes in cancer progression. Although some essential roles of intracellular S1P have recently been revealed, the majority of its biological effects are recognised to be mediated through activation of its cognate $S1P_{1-5}$ receptors. This work demonstrates for the first time that the sphingosine 1-phosphate receptor 2 ($S1P_2$, Mr = 42kDa) is shed in Hsp70 and CD63 containing exosomes from MDA-MB-231 breast cancer cells. These exosomes can be taken up by fibroblasts where exosomal $S1P_2$ receptor is proteolysed to a constitutively active shorter form of $S1P_2$ ($S1P_2$, Mr=38 kDa) which activates ERK-1/2 and stimulates DNA synthesis in fibroblasts. Therefore, conditioned medium and an exosomal preparation containing shed $S1P_2$ and isolated from MDA-MB-231 cells was able to stimulate the ERK-1/2 pathway and DNA synthesis in fibroblasts. Moreover, $S1P_2$ siRNA knockdown in MDA-MB-231 cells reduced the ability of conditioned medium and exosomal preparations containing $S1P_2$ to induce ERK-1/2 activation in fibroblasts. This finding links the constitutively active shorter form of $S1P_2$ released from cancer cells with the proliferation of fibroblasts required for metastatic spread. In addition, it was demonstrated that $S1P_4$ is released from MDA-MB-453 cells breast cancer cells. However, conditioned medium containing $S1P_4$ failed to activate ERK-1/2 in fibroblasts, suggesting that ERK-1/2 signalling is specifically regulated by the short form $S1P_2$. Future work requires *in vivo* study of the truncated version of $S1P_2$ receptor in an orthotopic graft mouse model to examine its impact on cancer metastasis.

In addition, S1P and inhibitors of sphingosine kinase (SK) increase the release of $S1P_4$ from MDA-MB-453 cells. These findings, suggest that exogenous S1P might enhance $S1P_4$ internalisation and its subsequent release into CM. SK inhibitors might increase intracellular ceramide, which has been demonstrated to be enriched in lipid raft microdomains and regulate MVBs formation and cargo sorting into ILVs (exosomes).

S1P and SK inhibitors had no effect on $S1P_2$ release, suggesting that the effect on $S1P_4$ is cell type-specific. In addition, it was demonstrated that the combination of the ceramide kinase (CERK) inhibitor, NVP-231 and the SK1 inhibitor, PF-543 produced less than additive effect on DNA synthesis, suggesting a functional interaction or overlapping regulation of CERK and SK1 in MDA-MB-453 and MDA-MB-231 breast cancer cells survival. Therefore, modulating the sphingolipid rheostat using CERK and SK1 inhibitor failed to sensitise breast cancer cells to cell death. Further investigation is required to examine the effect of CERK inhibitor alone and in combination with SK1 inhibitors on $S1P_4$ release.Taken together; the findings of this study, suggest a novel regulation of cancer progression by the S1P signalling pathway.

CHAPTER I

GENERAL INTRODUCTION

CHAPTER 1: General Introduction

1.1 Sphingolipid Biosynthesis

All eukaryotic cells have a lipid bilayer in their surrounding membranes which is composed mainly from three key groups of lipid, which include glycerolipid, sphingolipid and sterol. Sphingolipids were originally believed solely to regulate the membrane fluidity and rigidity (Lahiri and Futerman, 2007; Takabe *et al*., 2008). More than two decades since revealing the pleiotropic role of the bioactive lipid sphingosine on protein kinases and other cellular proteins. Sphingosine and several of its associated metabolites have been shown to have a role in signalling, endocytosis, cytoskeletonal rearrangment, cell cycle, differentiation, apoptosis, lipid raft integrity and function (Hannun and Obeid, 2008). Sphingolipid *de novo* biosynthesis begins at the cytoplasmic leaflet of the endoplasmic reticulum regulated by a group of four enzymes that coordinately generate ceramide with different acyl chain lengths (Obeid and Hannun, 2010). In the endoplasmic reticulum, 3-ketodihydrosphingosine is formed by a condensation of cytosolic serine and palmitoyl CoA catalysed by serine palmitoyl-CoA transferase, which followed by a coordinated actions of 3-ketodihydrosphingosine reductase, dihydroceramide synthases and dihydroceramide desaturase to generate ceramide (Obeid and Hannun, 2010). In addition, ceramide is also generated by the hydrolysis of sphingomyelin by sphingomyelinase. Ceramide can be phosphorylated by ceramide kinase (CERK) to generate ceramide 1-phosphate (C1P) or used for the synthesis of sphingomyelin or glycosphingolipids or hydrolysed by acid ceramidase to form sphingosine (Chatelut *et al*., 1998; Chalfant and Spiegel, 2005). Several studies have demonstrated the

importance of sphingolipid metabolites in the regulation of several biological processes in health and disease. An active lipid sphingosine-1-phosphate (S1P) is produced from phosphorylation of sphingosine by two sphingosine kinase isoforms, sphingosine kinase 1 (SK1) which is located in the cytoplasm and sphingosine kinase 2 (SK2) which localised mainly in the nucleus, and can also be transported into the cytoplasm. S1P degradation is controlled by two enzymes, namely S1P lyase and S1P phosphatases (figure 1.1). Therefore, the level of S1P is controlled by the balance between its synthesis, recycling and degradation (Maceyka *et al*., 2012). Ceramide, sphingosine and sphingosine 1-phosphate (S1P) are the most studied sphingolipids. In this regards, ceramide and sphingosine are generally associated with growth arrest and apoptosis. In contrast, S1P has been implicated as a pro-survival molecule (Pitson, 2011). Indeed, the balance between S1P and ceramide, which is termed the sphingolipid 'rheostat' is proposed to contribute to cell fate (Pyne *et al*. 1996; Cuvillier *et al*., 1996). Therefore, targeting sphingolipid metabolising enzymes and S1P in order to enhance the role of ceramide and accordingly cell death has gained immense focus in research especially in relation to potential anti-cancer treatment. However, the sphingolipid 'rheostat' is now recognised to be more complex as new signalling proteins regulating sphingolipid metabolism are discovered. Recent studies proposed that ceramide with different fatty acids chain size might promote proliferation as opposed to stimulating apoptosis. For example, C18 ceramide, which is produced by ceramide synthase 1 (CerS1) has been shown to act as a pro-apoptotic, whereas C16 ceramide, which is produced by ceramide synthase 6 (CerS6) acts as pro-survival (Senkal *et al*., 2010). Also, the cellular role of S1P in the sphingolipid 'rheostat' model has modified to

include autocrine and paracrine S1P actions via its receptors termed “inside-out” signalling (Newton *et al*. 2015).

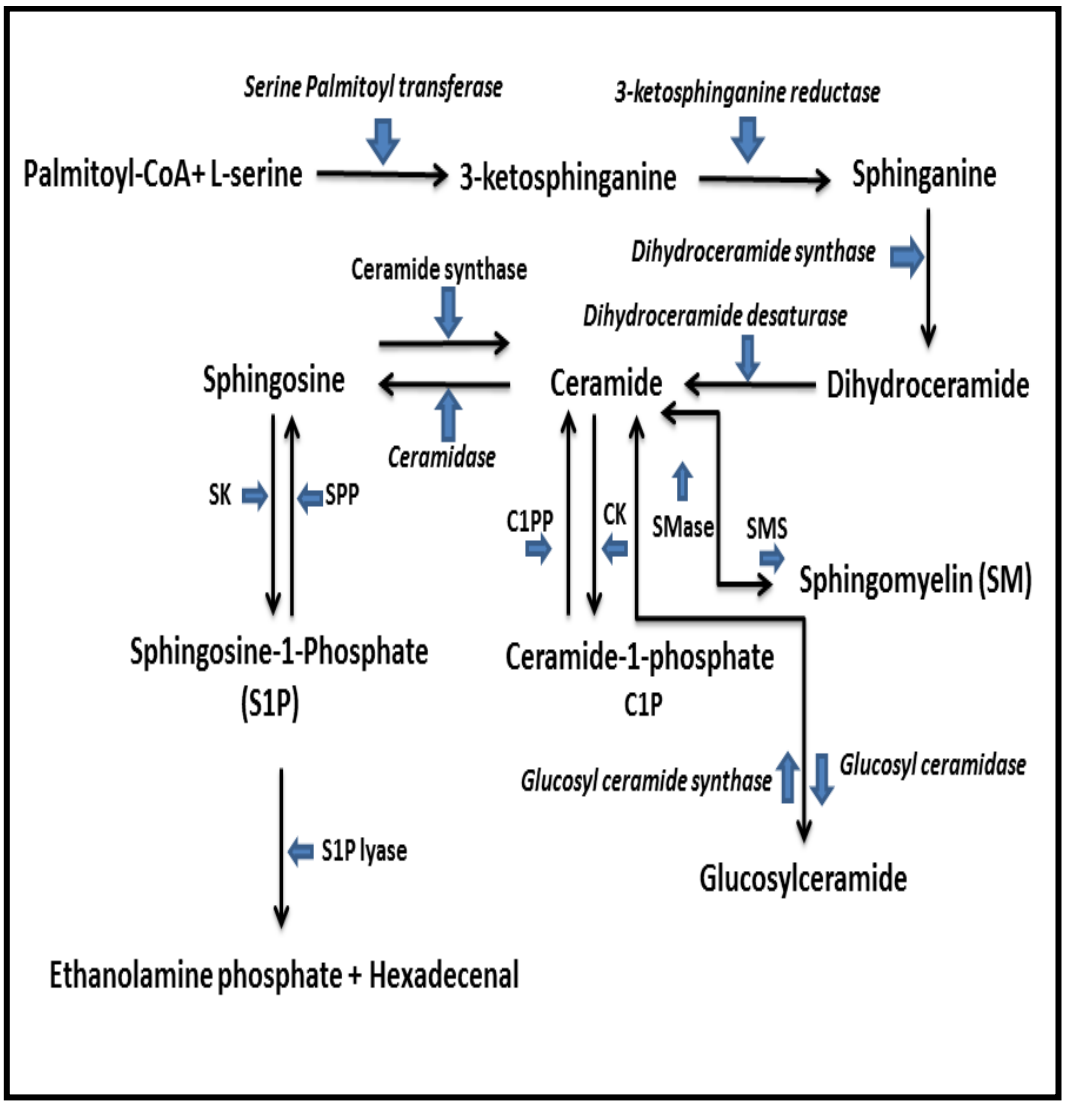


Figure 1.1 ***The synthesis of ceramide and its interconversion to sphingosine and S1P.***

CK, Ceramide Kinase; C1PP, Ceramide 1-Phosphate Phosphatase; SK, Sphingosine Kinase; SPP, Sphingosine Phosphate Phosphatase; SMS, Sphingomyelin Synthase; SMase, phingomyelinase.

1.2 Sphingosine Kinases

Sphingosine kinases are members of the diacyglycerol (DAG) kinase family (Wattenberg. 2006), which include SK1 and SK2. These enzymes are produced from two distinct genes located on chromosome 17 (17q25.2) and 19 (19q13.2)

respectively. SKs are considerably different in amino acid sequence and composition, with SK1 containing 384 amino acids and SK2 containing 618 amino acids (Liu *et al*., 2000). All known eukaryotic SKs share five highly conserved regions in their amino acid sequence, termed C1-C5, which are necessary for ATP and sphingosine binding and catalysis (Leclercq and Pitson, 2006), and they do not share sequence homology with other lipid kinases such as phosphatidylinositol-3 kinase (PI3K) (Kohama *et al*., 1998). SK1 and SK2 are phosphorylating sphingosine to generate S1P. Each enzyme appears able to compensate for the absence of the other, since double knockout mice of *Sk1* and *Sk2* genes have been shown to completely eliminate S1P and cause embryonic lethal consequences due to the disturbance of angiogenesis and severe haemorrhage. However, *Sk1* or *Sk2* knockout mice are viable and healthy (Mizugishi *et al*., 2005), suggesting some redundancy in function and regulation of overlapping signalling pathways.

SK1 was first purified as a 49 kDa protein from rat kidney (Olivera *et al*., 1998). Subsequently, the two SK1 isoforms, SK1a and SK1b were cloned and identified in mice (Kohama *et al*., 1998). There are in fact three splice variants of SK1 existing, termed SK1a (Mr=42 kDa, NM_001142601), SK1b (Mr=51 kDa, NM_182965) and SK1c (Mr=43.5 kDa, NM_021972), the molecular mass and Genbank respectively (Hatoum *et al*., 2017; Haddadi *et al*., 2017).

In comparison with SK1a, SK1b possesses an additional 86 amino acids at its N-terminus, while SK1c has 14 amino acids (Pitson, 2011). SK1b has been shown to be more resistant to removal from cells by proteasome degradation compared with SK1a. For instance, treatment of androgen-sensitive prostate cancer (LNCaP-AS)

cells, human pulmonary artery smooth muscle and MCF-7 breast cancer cells with a catalytic inhibitor of SK1, SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole) (French *et al*., 2003), induced the proteasomal degradation of SK1a and SK1b, which is likely due to ceramide-induced apoptosis (Loveridge *et al*., 2010), while in androgen-insensitive prostate cancer (LNCaP-AI) cells, SKb has been shown to be less sensitive to SKi-induced proteasomal degradation and apoptosis. These observations demonstrate different functional role of SK1a and SK1b and confers an important role of SK1b in chemotherapeutic resistance in cancers (Lim *et al*., 2012; Loveridge *et al*., 2010). In addition, SK1a and SK1b can form complexes with different proteins to potentially affect unique cell biology. For example, SK1a can interact with protein phosphatase 2A (PP2A), while SK1b was found to interact with allograft inflammatory factor 1-like protein, the latent-transforming growth factor β-binding protein and dipeptidyl peptidase 2 (Yagoub *et al*., 2014). SK1a has many important conserved regions and crucial amino acids such as Aspartate 81 (act as a nucleophilic base), which is essential for catalysis and aspartate 178 which is necessary for sphingosine binding via the 3-OH group (Adamas *et al*., 2016; Wang *et al*., 2013). Moreover, SK1 has been shown to include residues that bind phosphatidylserine and other acidic phospholipids, thereby regulating the cytosolic localization of the enzyme SK1 (Stahelin *et al*., 2005).

Activation of SK1 commence by phosphorylation of its Ser225 residue mediated by the extracellular signal-regulated kinase (ERK-1/2), and subsequent SK1 translocation to the plasma membrane (Pitson *et al*., 2003). However, the phosphorylation process is reversible and transient due to dephosphorylation of SK1 at Ser225 by PP2A (Barr *et al*., 2008). SK1 association with plasma membranes may

occur by the transient opening of the lipid binding loop-1 (LBL-1), and the rearrangement of head group interacting residues in a way that facilitates substrate recognition at the membrane interface. This is followed by lipid extraction and closure of the LBL-1 to encapsulate the lipid substrate (Adams *et al*., 2016). Moreover, the acidic phospholipid phosphatidylserine (PS) has been shown to induce dose response increase in sphingosine kinase activity (Olivera *et al*., 1996), while other acidic phospholipids such as phosphatidic acid, phosphatidylinositol, phosphatidylinositol bisphosphate and cardiolipin stimulate sphingosine kinase activity to a lesser extent than PS (Olivera *et al*., 1996). In addition, Thr54 and Asn89 residues in SK1 were reported as contributory residues to a PS-binding site, as both were shown to be important for SK1 binding to PS-derived vesicles and plasma membranes (Adams *et al*., 2016). In addition, SK1 contains a Ca^{2+}/calmodulin binding site (Sutherland *et al*., 2006). In this regard, the calcium and integrin-binding protein 1 (C1B1) has been shown to regulate SK1 translocation process in a Ca^{2+}-dependent manner and regulated by Ca^{2+}/calmodulin site. However, knockdown of the C1B1 protein with siRNA oligonucleotide prevents agonist mediated SK1 activation and subsequent translocation to the plasma membrane (Jarman *et al*., 2009). In contrast, C1B2 has been shown to play an opposite role to C1B1 in SK1 signalling (Zhu *et al*., 2017).

The CIB2 can bind to SK1 on the same site as CIB1, but it lacks the Ca^{2+} myristoyl switch function which impedes SK1 translocation to the plasma membrane and its subsequent signalling. In addition, CIB2 downregulation in ovarian cancer has been shown to be associated with poor prognosis (Zhu *et al*., 2017). SK1 activity has also been shown to be increased by EGF, PDGF, TNFα, IgE and GPCR agonists such as

histamine possibly by ERK-1/2 catalysed phosphorylation and $G\alpha_q$ activation (Taha *et al*., 2006; Alvarez *et al*., 2007). For example, SK1 translocation to the plasma membrane has been shown to be mediated by M3 receptor-induced G_q activation (Braak *et al*., 2009). Indeed, constitutively active $G\alpha_q$ mutants promote SK1 translocation to the plasma membrane (Braak *et al*., 2009). Moreover, SK1 has been shown to shuttle between cytosol and nucleus (Inagaki *et al*., 2003). Interestingly, SK1 can be exported to the extracellular milieu (Ancellin *et al*., 2002), and a catalytically active form of SK1 has been shown to be released in vesicles derived from conditioned medium of human hepatocarcinoma Sk-Hep1 cells (Rigogliuso *et al*., 2010). In addition, sphingosine was also detected in the shed vesicles (Rigogliuso *et al*., 2010). Therefore, the shed vesicles can be a site for S1P production following vesicles endocytosis. In addition, export of SK1 to the extracellular milieu might contribute to the formation of the vascular S1P gradient (Venkataraman *et al*., 2006).

SK2 has an extended N-terminus and an additional central proline-rich region, which is not found in SK1 (Pitson, 2011). There are two SK isoforms, which are ubiquitously expressed in all human tissues with some differential expression; SK1 is highly expressed in the spleen, leukocyte and lung, while highest SK2 expression was detected in liver and kidney (Neubauer and Pitson, 2013). Several experimental studies have shown contrasting roles of SK1 and SK2 in both normal and disease conditions. For example, specific targeting of SK1 has been shown to reduce the incidence and acuteness of the murine collagen-induced arthritis model. In contrast, siRNA knockdown of SK2 induced more aggressive form of the disease (Lai *et al*., 2009). Also, *Sk2* ablation in A498, Caki-1 and MDA-MB-231 cells has been shown to increase mRNA, expression and the activity of SK1 and subsequently S1P levels,

while *Sk1* ablation did not affect mRNA or expression levels of SK2, but it reduces S1P levels (Gao and Smith, 2011). Selective ablation of SKs has been shown to differentially affect sphingolipid signalling such as proliferation, migration and invasion (Gao and Smith, 2011). For example, SK1 selective ablation reduces the cytosolic pool of S1P and elevates ceramide, which might reduce the 'inside-out' growth stimulatory signalling through S1P receptors. In contrast, selective depletion of SK2 might reduce the nuclear pool of S1P, which is quantitatively smaller than S1P-derived from SK1 pool. (Gao and Smith, 2011). Moreover, siRNA knockdown of SK1 in human mast cells (hMCs) has been shown to completely abrogate FcεRI-induced degranulation of hMCs and reduces CCL2 production (Oskeritzian *et al*., 2008). In comparison, a 50% reduction in SK2 by siRNA knockdown had no significant effects on FcεRI-induced degranulation or production of CCL2 (Oskeritzian *et al*., 2008). Thus, these studies reveal a distinct SK2 signalling and partial overlapping with SK1 signalling.

SK1/2 isoforms are differing in their substrate specificity. Thus, while both enzymes use sphingosine, SK1 preferred substrate is D-*erythro*-dihydrosphingosine, while SK2 prefers phytosphingosine, DL-*threo*-dihydrosphingosine and FTY720 (fingolimod) (Liu *et al*., 2000; Billich *et al*., 2003).

SK2 appears to have a more complex role, which may be opposed to SK1. However, *in vivo* studies revealed some overlapping functions. For example, *Sk1* knockout (KO) mice or *Sk2* KO mice have been shown to develop normally, while double *Sk1/Sk2* KO mice displayembryonic lethality due to defective angiogenesis and neurogenesis associated with neuronal apoptosis (Mizugishi *et al*., 2005). Although,

SK1 commonly promotes cell survival and growth, a number of studies have shown that overexpression of SK2 suppresses cell growth and induces apoptosis (Liu *et al*., 2003). For example, apoptosis induced by tumour necrosis factor (TNFα) or serum withdrawal was shown to be abrogated by siRNA knockdown of SK2 in HEK293 cells. Moreover, Mesangial cells taken from *Sk2*$^{-/-}$ mice displayed greater resistance to staurosporine-induced apoptosis than wildtype or *Sk1*$^{-/-}$ cells (Hofmann *et al*., 2008). In contrast, SK2 has been shown to support survival and growth (Neubauer and Pitson, 2013), which was indicated when siRNA knockdown of SK2 increased apoptosis in a number of tumour cells (Maceyka *et al*., 2005; Okada *et al*., 2005; Van Brocklyn *et al*., 2005; Sankala *et al*., 2007; Nemoto *et al*. 2009). Therefore, these studies indicate that SK2 can have an opposite role to SK1 and demonstrated SK2 cell-type specific signalling.

SK2 localisation in the mitochondria has been shown to contribute in apoptosis by mediating the activation of BAK (member of Bcl2 family proteins), which subsequently enhances mitochondrial permeability and cytochrome c release (Chipuk *et al*., 2012). In addition, nuclear S1P which is produced by SK2 inhibits histone deacetylase 1 and 2 (HDAC1 and HDAC2) activity and increases histone acetylation and transcription at promoters of the gene encoding the cyclin-dependent kinase inhibitor p21 and the transcriptional regulator c-fos (Hait *et al*., 2010). Moreover, intracellular S1P binds to TRAF2 at the N-terminus of RING domain and stimulates its E3 ligase activity, which resulted in TRAF2-catalysed Lys-63-ubiquitination of receptor-interacting protein1 (RIP1). This is emphasizing that TRAF2 is a novel intracellular target of S1P (Alvarez *et al*., 2010).

In addition, human telomerase reverse transcriptase (hTERT) in human fibroblasts has been shown to be stabilized by S1P generated from SK2 phosphorylation (Panneer Selvam *et al*., 2015), and inhibition or ablation of SK2 leads to a decreased hTERT stability and enhances senescence and loss of telomere integrity (Panneer Selvam *et al*., 2015). In addition, S1P produced from SK2 has been shown to bind with high affinity and specificity to a highly conserved protein Prohibitin 2 (PHB2), which regulates mitochondrial assembly and function, and SK2 depletion leads to a dysfunction in mitochondrial respiration (Strub *et al*., 2018). In addition, treatment of mouse neuroblastoma (N2a) cells by SK2 inhibitor or siRNA knockdown of SK2 or overexpression of S1P degrading enzymes has been shown to decrease β-site APP cleaving enzyme-1 (BACE1) activity. Thus, S1P increases Aβ production by activation of β-site APP cleaving enzyme-1 (BACE1) (Tomita *et al*., 2011). Therefore, SK2/S1P in the brain might be a novel molecular target for AD therapeutics.

1.2.1 SK1 Activation and Agonist-Stimulated S1P Production

The activation of SK1 is initiated by the phosphorylation of its Ser225 residue by extracellular signal-regulated kinase (ERK-1/2), which induces a conformational change in the enzyme to facilitate its catalytic efficiency and subsequent SK1 translocation to the plasma membrane (Pitson *et al*., 2003). The phosphorylation process is transient and reversed by the action of PP2A (Adams *et al*., 2016). SK1 translocation to the plasma membrane after being phosphorylated by ERK-1/2 appears to be a common aspect of SK1 activation by many stimuli, which lead to

S1P formation, which can be exported outside the cells to activate its receptors through "inside-out" signalling (Hait *et al*., 2007), see section (1.4). Agonists such as growth factors, for example platelet-derived growth factor (PDGF) in the airways and smooth muscle cells, epidermal growth factor (EGF) in MCF-7 breast-cancer cells (Sarkar *et al*., 2005), and nerve growth factor (NGF) in PC12 cells (Toman *et al*., 2004), have been shown to activate SK1 (Hait *et al*., 2006). Moreover, ligands for GPCRs such as acetylcholine, lysophosphatidic acid and S1P itself have been shown to activate SK1 (Koppen *et al*., 2001; Delon *et al*. 2004; Heringdorf *et al*., 2001; Long *et al*., 2010).

SK1 has also been shown to translocate to plasma membrane compartments enriched in phosphatidic acid (PA) through direct SK1-PA interaction (Delon *et al*., 2004). Thus, SK1 represents a novel regulator to PA effector. Also, S1P has been shown to induce SK1 re-translocation into the membrane ruffles/ lamellipodia in MCF-7 Neo cells. In addition, tumour necrosis factor (TNFα) has been shown to induce SK1 phosphorylation and activation in HUVAC and HEK293 cells (Xia *et al*., 2002; Toman *et al*., 2004). EGF has been shown to regulate SK2 in HEK-293 and MDA-MB-453 breast cancer cells (Hait *et al*., 2005). Furthermore, SK2 has been shown to interact directly with the cytoplasmic part of the IL-12 receptor, indicated by suppressed IL-12-stimulated production of interferon-γ following expression of dominant negative SK2 (Yoshimoto *et al*., 2003).

1.3 Ceramide Kinase and C1P

Ceramide is one of the important components that regulates or mediates cell apoptosis in response to different stimuli and stress (Sugiura *et al*., 2002).

Ceramide kinase (CERK) is a member of the diacylglycerol kinase enzymes and can convert ceramide into ceramide -1 phosphate (C1P) (Bajjalieh *et al*., 1989).

CERK was identified in brain synaptic vesicles (Bajjalieh *et al*., 1989), and subsequently in human leukemia (HL-60) cells (Kolesnick and Hemer, 1990). C1P has been shown to be involved in apoptosis and inflammatory responses (Gómez-Muñoz, 2004). Moreover, CERK and C1P were reported necessary for mast cell degranulation (Mitsutake *et al*., 2004), and this process is calmodulin-dependent (Mitsutake and Igarashi, 2005). Human CERK (hCERK) is a protein of 537 amino acids (Sugiura *et al*., 2002), and found to share sequence homology with SKs although it differs significantly at its N-terminus which is myristoylated (Carré *et al*., 2004). The N-terminus contains a pleckstrin homology (PH) domain (amino acids 32–121), which is only found in CERK and not SK1 or SK2 and found important for the CERK activity (Sugiura *et al*., 2002; Kim *et al*. 2005). Moreover, the PH domain of CERK has high affinity for phosphatidylinositol 4, 5-bisphosphate, which regulates CERK-PH plasma membrane binding (Kim *et al*., 2006). Initial studies on CERK subcellular localisation demonstrated that CERK is enriched in three major compartments; these are the Golgi appartus, the plasma membrane and the cytoplasmic vesicles (Lamour *et al*., 2007; Bornancin, 2011). C1P is reported to stimulate DNA synthesis and cell division in murine bone marrow derived macrophages (BMDMs). This occurs by phosphorylation of MAPKs including ERK-1/2 and JNK (Gangoiti *et al*., 2008). In addition, exogenous C1P has been shown to induce migration of RAW 264.7 macrophages mediated by coupling to $G\alpha_i$ protein (Granado *et al*., 2009); suggesting that C1P may act through a receptor that is different from S1P receptors to stimulate cells migration. This is a result of the

activation of ERK-1/2 and Akt, and the latter is linked with PI3-kinase and PIP_3 signalling (Granado *et al*., 2009). Therefore, C1P promotes cell survival and growth in response to extracellular stimuli.

1.3.1 C1P and Cell Survival

The short-chain C1P has been shown to stimulate DNA synthesis in fibroblasts (Gomez-Muñoz *et al*., 1995), and to induce ERK-1/2 catalysed phosphorylation in human osteoblastic cells (Carpio *et al*., 1999). C1P has also been shown to induce Ca^{2+} mobilisation in calf pulmonary artery endothelial cells (Gijsbers *et al*., 1999), thyroid FRTL-5 cells (Hogback *et al*., 2003) and Jurkat T-cells (Colina *et al*., 2005). In contrast, the short-chain C1P did not induce Ca^{2+} mobilisation in fibroblasts (Gomez-Muñoz *et al*., 1997) and neutrophils (Rile *et al*., 2003). Therefore, the effect of C1P on Ca^{2+} homeostasis is cell type-specific.

Exogenous C1P has been shown to block DNA fragmentation in BMDMs after removal of macrophage colony-stimulating factor (M-CSF). However, removal of M-CSF induces apoptosis in these cells, and results in the activation of acid sphingomyelinase which increases ceramide levels and decreases intracellular C1P. Moreover, exogenous C1P prevents the activation of caspase-9/caspase-3 and subsequent DNA fragmentation and promotes cell survival due to inhibition of apoptosis (Gomez-Munoz *et al*., 2003).

1.3.2 The Role of C1P in Cancer

Microarray data collected from 1681 breast cancer tumour samples demonstrated high CERK expression in ER^- compared to ER^+ breast cancer cells (Ruckhäberle *et al*., 2009). Moreover, C1P has been shown to induce pancreatic cancer cell migration and invasion, and treatment of these cells with selective inhibitor of PI3K or Akt1 or gene silencing of these kinases with siRNA causes a potent inhibition of C1P-induced cell migration and invasion (Rivera *et al*., 2016). Moreover, S1P and C1P have been shown to increase *in vitro* motility and adhesion of human rhabdomyosarcoma (RMS) cells (Schneider *et al*., 2013). High expression of S1P and C1P were reported in several organs after γ-irradiation or chemotherapy, which implies an adaptation of cancer cells that might result in a pro-metastatic environment (Schneider *et al*., 2013). In addition, inhibition of CERK using the potent pharmacological inhibitor NVP-231 has been shown to reduce cell viability, DNA synthesis and colony formation in MCF-7 and NCI-H358 cells by inducing growth arrest of these cells in the M phase of the cell cycle, which subsequently results in apoptosis of these cells (Pastukhov *et al*., 2014).

1.4 S1P Signalling

S1P is a bioactive pleiotropic lipid involved in several biological processes including proliferation, migration, survival and differentiation via a family of five GPCRs termed $S1PR_{1-5}$. These receptors are differentially coupled to heterotrimeric G proteins and Rac or Rho to control various effectors (Pyne and Pyne, 2010). For example, the $S1P_2$ receptor has been shown to be expressed in all gastric cancer cell lines and $S1P_3$ in only four cell lines (Yamashita *et al*., 2006), and S1P has been shown to induce MKN1 and HCG-27 gastric cancer cells migration mediated by $S1P_3$, and inhibited migration of AZ-521 gastric cancer cells that express $S1P_2$ (Yamashita *et al*., 2006). In addition, high cytoplasmic $S1P_1$ and $S1P_3$ expression in the ER^+ breast cancer was associated with reduced disease-specific survival and development of tamoxifen resistance (Watson *et al*., 2010).

Also, there is a linked cooperation between S1P signalling components and oncogenes. For example, SK1 activity and foci formation are increased in NIH-3T3 fibroblasts overexpressing a mutant H-RAS (V12-Ras), which can be inhibited by overexpression of $SK1^{GD82}$ dominant-negative mutant (Xia *et al*., 2000). Thus, SK1 and H-Ras are connected in a sequential signalling pathway. In addition, a truncated isoform of eukaryotic elongation factor 1A (eEF1A1) also termed prostate tumour inducer-1 (PTI-1), which is an oncogene that lacks the GDP/GTP binding domain of eEF1A1 has been shown to activate SK1. This regulatory mechanism regulating SK1 activity appears to be essential for PTI-1-induced neoplastic transformation (Leclercq *et al*., 2011). Moreover, S1P has been shown to promote the differentiation of human lung fibroblasts to myofibroblasts (Urata *et al*., 2005).

S1P has physiological and pathophysiological effects in the cardiovascular, immune system and central nervous system. Although its role in different biological processes in normal physiological state is essential, the SK1-S1P axis has an oncogenic role in the case of cancer due to its involvement in cancer migration, proliferation and cancer resistance to chemotherapy (Verzijl *et al*., 2010; Kihara *et al*., 2014; Pyne and Pyne, 2010). Moreover, S1P has been shown to regulate several physiological responses such as T and B cells egress from the lymph nodes (Matloubian *et al*., 2004) and embryonic neurogenesis (Mizugishi *et al*., 2005). Also, S1P and its receptors are reported to regulate the heart rate, coronary artery blood flow, endothelial integrity and blood pressure (Forrest *et al*., 2004; Karliner, 2004). Furthermore, S1P has been shown to play a central role in blood vessel formation as neutralisation of S1P with anti-S1P monoclonal antibody reduces VEGF-induced blood vessel formation (Visentin *et al*., 2006).

The action of the extracellular S1P is mediated through specific cognate GPCRs termed $S1PR_{1-5}$. Indeed, S1P is exported outside cells such as platelets, vascular endothelial cells and erythrocytes to regulate different actions by its binding to $S1PR_{1-5}$ (Ancellin *et al*., 2002; Ito *et al*., 2007). This process is known as ‘‘inside-out’’ signalling and it is considered very important for S1P to mediate its diverse actions in an autocrine and paracrine manner (Takabe *et al*., 2008). However, both SK1 and SK2 have been shown to translocate to other cellular compartments, suggesting that S1P may be produced locally in these compartments to act on specific intracellular targets (Strub *et al*., 2010). For example, S1P has been shown to act as a cofactor to confer E3 ligase activity on the TNF receptor associated factor 2 (TRAF2) (Xia *et al*. 2002). Also, S1P has been shown to increase the proteolytic

activity of the BACE1, which is the rate-limiting enzyme for amyloid-β peptide formation (Takasugi *et al*., 2011). Moreover, S1P produced in the mitochondria by SK2 has been shown to bind with high affinity to the prohibitin 2 (PHB2), a highly conserved protein that regulates mitochondrial assembly and function (Strub *et al*., 2011). Moreover, the transcription factor peroxisome proliferator-activated receptor (PPAR-γ) and S1P have been reported to form a complex regulating neo-vascularisation in endothelial cells (Parham *et al*., 2015).

1.4.1 Export of S1P through ATP-Binding Cassette (ABC) Transporters and Spinster 2 (Spns2) Transporters are Important for Maintaining a S1P Gradient

Intracellularly produced S1P is incapable of crossing the hydrophobic mammalian plasma membrane since it retains a polar head group. Therefore, there must be a mechanism, which allows S1P to move out of cells and induce its actions at its $S1P_{1-5}$ receptors. Also, S1P level in blood is higher compared with that in tissue since endothelial cells, RBCs, platelets and erythrocytes are reported to release stored S1P (Yang *et al*., 1999; Kim *et al*., 2009).

ATP-binding cassette (ABC) proteins are transmembrane proteins consuming ATP to deliver various substrates across membranes including drugs, sterols, peptides and lipids. Several of these have been reported to be involved in the export of S1P from cells. For example, ABCC1, ABCA1 and ABCA7 transporters have been shown to export S1P from mast cell, astrocytes and red blood cells respectively (Mitra *et al*., 2006; Sato *et al*. 2007; Kobayashi *et al*., 2009).

However, genetic knockout of *ABCC1, ABCA1* and *ABCA7* in mice did not affect S1P concentrations in blood and tissues when compared with wild type; this might suggest the existence of other transporters or compensatory mechanisms to maintain the S1P gradient (Lee *et al*., 2007). Indeed, the human breast cancer resistance proteins ABCC1/ABCG2 have been shown to facilitate S1P transport out of MCF-7 breast cancer cells (Nagahashi *et al*., 2014).

Another protein which has been identified as a S1P transporter is the spinster homolog 2 (Spns2) transporter, which is a member of the major facilitator superfamily (MFS) that does not have a typical ATP binding motif (Nagahashi *et al*., 2014).

The Spns2 transporter was identified in zebrafish and has been shown to be essential for the migration of heart cell precursors during embryogenesis (Osborne *et al*., 2008). Moreover, Spns2 expression in Chinese hamster ovary (CHO) cells has been shown to facilitate S1P release (Hisano *et al*., 2011). Spns2 has also been shown to export di-hydro S1P and other sphingosine analogues such as phosphorylated FTY720 (Hisano *et al*., 2011). In addition, *Spns2* gene deletion in mice resulted in a 50% reduction in the concentration of S1P in plasma (Hisano *et al*., 2012). Additionally, a similar result has been obtained with endothelial cell-specific *Spns2* knockout, which supports two concepts. First, Spns2 is responsible for S1P secretion from endothelial cells and second, S1P released from endothelial cells is important in maintaining S1P concentration in blood (Fukuhara *et al*., 2012), (figure 1.2). Recently, a new S1P transporter, the major facilitator superfamily transporter 2b (Mfsd2b) has been identified (Vu *et al*., 2017). The Mfsd2b is an orphan transporter

essential for the S1P export from red blood cells and platelets (Vu *et al*., 2017). This is indicated by a dramatic S1P accumulation in the *Mfsd2d* knockout red blood cells and platelets compared with wild type. Also, plasma S1P level has been shown to be decreased in the *Mfsd2d* knockout mice compared with wild type, indicating that Mfsd2d pathway contributes to half of the plasma S1P pool (Vu *et al*., 2017). Therefore, targeting these transporters might be a good therapeutic target in conditions where S1P signalling is involved in its pathogenicity.

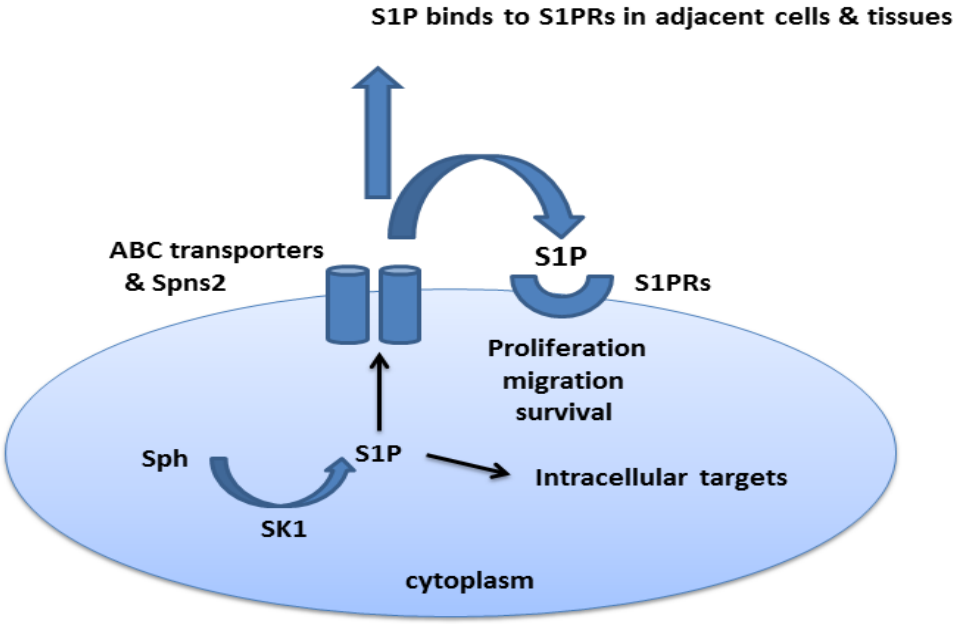


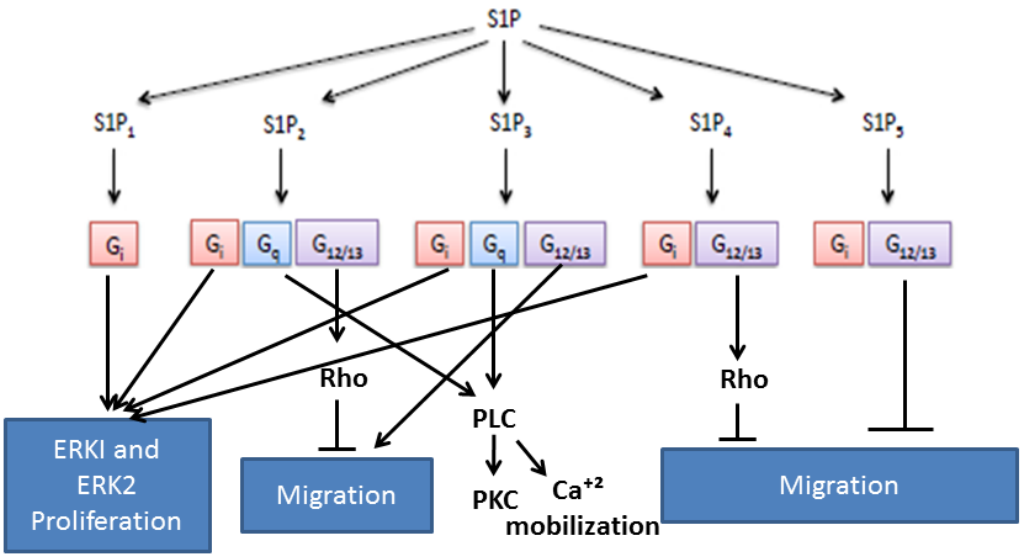
Figure 1.2 ***The "Inside-out" signalling of sphingosine-1-phosphate (S1P).***

1.4.2 S1P Receptors Expression and Functions

Before the discovery of S1P receptors, it was thought that S1P acts as an intracellular mediator (Zhang *et al*., 1991). This is based on the fact that intracellular S1P is increased upon stimulation by growth factors such as PDGF and FCS (Olivera and Spiegel, 1993), cytokines such as TNFα (Xia *et al*., 1999) and hormones such as 17 β-estradiol (E2) (Sukocheva *et al*., 2003). The expression patterns of the S1PRs differ between tissues, and also during development and ageing. The $S1P_1$, $S1P_2$ and $S1P_3$ receptors are essentially ubiquitously expressed, although $S1P_4$ and $S1P_5$ receptors expression is highly restricted to distinct cell types (Blaho and Hla, 2014). Each S1P receptor subtype signals through a distinctive set of G proteins that leads to several downstream cellular effects. There are four essential families of heterotrimeric G proteins, which are involved in S1P signalling through its receptors $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_{12/13.}$ S1P-$S1PR_{1\text{-}5}$ coupling leads to the activation of different cellular responses according to which G protein binds. For examples, S1P-$S1P_1$ couples exclusively to $G\alpha_i$, which can activate its downstream targets such as Ras/ERK-1/2 and Rac/PAK (Van Brocklyn *et al*., 2000). S1P-$S1P_2$ and S1P-$S1P_3$ both couple to $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13.}$ S1P-$S1P_3$ has a similar potency to S1P-$S1P_1$ in activating ERK-1/2, but higher than S1P-$S1P_2$ (Okamoto *et al*., 1999). Also, both are capable of activating PLC to induce IP3 formation and Ca^{2+} mobilization (Kon *et al*., 1999; Van Brocklyn *et al*., 2000; Young and Van Brocklyn, 2006). In addition, $S1P_2$ couples strongly to $G\alpha_{12/13}$, which can activate Rho small GTPase, stress fiber formation and cell rounding (Gonda *et al*., 1999; Van Brocklyn *et al*., 1999). $S1P_4$ couples to $G\alpha_i$ and $G\alpha_{12/13}$, but not $G\alpha_q$ (Gräler *et al*., 2003). $S1P_4$ coupling to

$G\alpha_i$ can activate PLC, ERK-1/2 and the Rho family small GTPase Cdc42 (Yamazaki *et al*., 2000; Van Brocklyn *et al*., 2000; Kohno *et al*., 2003). In addition, Cdc42 activation by $S1P_4$ can regulate cellular motility (Kohno *et al*., 2003), while $S1P_4$ coupling to $G\alpha_{12/13}$ leads to Rho activation, formation of peripheral stress fibers and cell rounding (Gräler *et al*., 2003).

S1P-$S1P_5$ couples to both $G\alpha_i$ and $G\alpha_{12/13}$, but not $G\alpha_q$ (Malek *et al*., 2000). $G\alpha_i$ activation leads to the inhibition of cAMP in both CHO and mice hepatoma RH7777 cells (Im *et al*., 2000; Malek *et al*., 2000). Surprisingly, $S1P_5$ can cause a decrease in ERK-1/2 phosphorylation and activity (Malek *et al*., 2000).



Taken and reproduced from Shimizu and Furuya, (2013).

Figure 1.3 ***A schematic representation of S1PRs and their different coupled G proteins.***

1.4.2.1 $S1P_1$

The $S1P_1$ receptor was the first S1P receptors to be functionally cloned and identified, and is the most well studied S1P receptors (Lee *et al*., 1998). The gene encoding $S1P_1$ is located on chromosome 1 (1p21) and encodes a 382-amino acid, and has a molecular mass of 43 kDa. The human $S1P_1$ receptor has 94% sequence similarity with the murine receptor, and has been shown to efficiently couple to G_i protein (Kihara *et al*., 2014), (figure 1.3).

The $S1P_1$ receptor has been shown to regulate trafficking of haematopoietic cells such as T and B lymphocytes, NK cells, dendritic cells, neutrophils, macrophages, mast cells, haematopoietic progenitors and osteoclasts (Cyster and Schwab, 2012). It also plays a role in the vascular development and integrity (Liu *et al*., 2000). $S1P_1$ participates in vascular barrier homeostasis and prevents extravasation of plasma during infections, sepsis and anaphylactic shock, which can be life threatening (Camerer *et al*., 2009; Wang and Dudek, 2010). In addition, circulating S1P activates endothelial $S1P_1$ to stabilize blood vessel development and homeostasis (Jung *et al*., 2012).

Furthermore, mice with endothelium-specific deletion of *$S1P_1$* develop severe pathology in a model of renal ischemia/reperfusion injury (Ham *et al*., 2014). Moreover, the high molecular weight of hyaluronan (HA) induces CD44s-mediated transactivation of AKT-dependent threonine phosphorylation of the $S1P_1$ receptor, which results in Rac1 signalling that lead to cortical actin thickening and barrier development in human pulmonary endothelial cells. In contrast, silencing of either $S1P_1$ receptor or AKT1 or Rac1 by siRNA oligonucleotide reverses the barrier-

protective effect of high molecular weight HA (Singleton *et al*., 2006). Moreover, using the active chiral $S1P_1$ receptor antagonist *in vivo* has been shown to induce loss of the capillary integrity in mice (Sanna *et al*., 2006). Therefore, these studies indicate the importance $S1P_1$ in maintaining vascular integrity and haemostasis.

1.4.2.2 $S1P_2$

The gene encoding the human $S1P_2$ receptor is located on chromosomal locus 19p13 with highly conserved sequence among species. Human $S1P_2$ receptor contains 353 amino acids, and has a molecular mass of 42 kDa (Kihara *et al*., 2014).The $S1P_2$ receptor is expressed in different tissues and organs, and can couple to multiple G-proteins including $G\alpha_i$, $G\alpha_q$ with preferential coupling towards $G\alpha_{12/13}$.The $S1P_2$ receptor has been shown to mediate an inhibitory effect of S1P on Rac, Akt and cell migration by activation of $G_{\alpha 12/13}$ and Rho-dependent signalling (Du *et al*., 2010), (figure 1.3).

The $G\alpha_{12/13}$ activation occurs through Rho GTPase nucleotide exchange factors (RhoGEFs). There are four mammalian RhoGEFs regulated by $G\alpha_{12/13}$ proteins, which include p115-RhoGEF, PSD-95/Disc-large/ZO-1 homology-RhoGEF, leukemia-associated RhoGEF and lymphoid blast crisis-RhoGEF. These proteins link GPCRs to the activation of the small monomeric GTPase RhoA and other downstream effectors (Siehler, 2009). In addition, $S1P_2$ receptor overexpression has been shown to suppress migration. However, inhibition of $S1P_2$ expression using siRNA knockdown of $S1P_2$ reversed this inhibitory effect (Lepley *et al*., 2005). Although constitutive *S1P2* gene deletion studies have shown that the $S1P_2$ receptor is not important for normal development, the $S1P_2$ receptor is reported to have a

cooperative function with $S1P_1$ and $S1P_3$ receptors in the stabilisation and maturation of the vascular system during embryonic development (Kono *et al*., 2004). This is indictated by *S1P1*, *S1P2* and *S1P3* gene deletion studies in embryos, as *S1P1*, *S1P2* and *S1P3* triple null embryos developed more severe condition than that of the *S1P1* and *S1P2* double null embryos (Kono *et al*., 2004). Also, $S1P_2$ and $S1P_1$ receptors have been shown to induce cell proliferation and transcriptional regulation (Kihara *et al*., 2014). Moreover, several reports demonstrated that $S1P_2$ receptor can activate the ERK-1/2 pathway, which is involved in regulating both migration and proliferation. For instance, $S1P_2$ receptor expression in C6 glioma and HTC4 hepatoma cells leads to ERK-1/2 phosphorylation and immediate early generation of c-Jun and c-Fos proto-oncogenes in response to S1P (An *et al*., 2000; Sato *et al*., 1999). In mice which are null of both $S1P_2$ and $S1P_3$ receptors, they were shown to develop consistently progressive cochlear and vestibular defects with hair cell loss and incomplete deafness by 4 weeks of age (Herr *et al*., 2007). In B16 cells, S1P was also demonstrated to induce inhibition and activation of Rac and RhoA respectively. However, these effects were abrogated by $S1P_2$ antagonist JTE-013 (Arikawa *et al*., 2003). *S1P2* receptor knockout mice have been reported to develop diffuse large B-cell lymphoma at advanced ages (Cattoretti *et al*., 2009). More recently, EGF has been shown to induce the Ezrin, radixin and moesin (ERM) proteins phosphorylation through activation of $S1P_2$ receptor. The ERM are proteins, which known to play an important role in coordinating cellular events that involve cytoskeletal rearrangement and migration (Gandy *et al*., 2013). Thus, these studies, suggest that $S1P_2$ receptor acts as anti-tumourigenic. In contrast, $S1P_2$ receptor has been shown to be implicated in promoting metastasis. For example, genetic loss of SK1 has been shown to

activate breast carcinoma metastasis suppressor 1 gene (BRMS1) (Ponnusamy *et al*., 2012). The BRMS1 is a protein that has the ability to reduce the metastatic capacity of breast cancer cells (Kodura and Souchelnytskyi, 2015). Nevertheless, inhibition of $S1P_2$ receptor using either $S1P_2$ pharmacologic antagonists JTE-013 or $S1P_2$ siRNA knockdown induced an increase in BRMS1 expression in MB49 cells (Ponnusamy *et al*., 2012). Moreover, Bi *et al* (2014) demonstrated that matrix metalloproteinase-9 (MMP9) was up-regulated in pancreatic stellate cells (PSCs), which are classified as myofibroblast-like cells (Allam *et al*., 2017). In addition, S1P has been shown to mediate conditioned medium stimulation of pancreatic tumour cell migration (Bi *et al*., 2014). This action has been shown to be mediated by the $S1P_2$ and not the $S1P_1$ receptor, determined by a decreased MMP9 luciferase reporter activity following inhibition of the $S1P_2$ receptor by either $S1P_2$ siRNA knockdown or $S1P_2$ antagonist JTE-013, and not by inhibition of the $S1P_1$ receptor by $S1P_1$ siRNA knockdown or $S1P_1$ antagonist W146 (Bi *et al*., 2014). In addition, the conditioned medium from siRNA $S1P_2$ treated PSCs and stimulated with S1P has been shown to reduce tumour migraton and invasion compared with conditioned medium from control PSCs (Bi *et al*., 2014). Therefore, $S1P_2$ on PSCs mediates the stimulating effect of S1P treated PSCs conditioned medium on tumour cell migration and invasion. However, this controversial effect of the $S1P_2$ receptor remains unresolved and need further investigation.

1.4.2.3 $S1P_3$

The $S1P_3$ receptor is ubiquitously expressed by the immune cells, heart cells, lung, spleen, kidney, intestine, testis and skeletal muscle (Yamaguchi *et al*. 1996; Ishii *et*

al., 2001). The gene for Human $S1P_3$ receptor is located on chromosome 9q22.1-q22.2 and encodes a 378-amino acid, and has a molecular mass of 42 kDa. Human $S1P_3$ shares 87% similarity with murine $S1P_3$ receptor (Kihara *et al*., 2014). Similar to $S1P_2$, the $S1P_3$ receptor can couple to multiple G-proteins including $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ (Chun *et al*., 2010), (figure 1.3). However, $S1P_3$ receptor coupling to G_q resulted in the production of IP_3 and DAG followed by calcium mobilisation and activation of PKC respectively (Kihara *et al*., 2014), (figure 1.3). Although $S1P_3$ receptors are widely expressed, their deletion in mice did not reveal an obvious phenotype or developmental defects. However, S1P has a variety of important effects on the cardiovascular system such as negative chronotropic, hypertensive effects, constriction of the basilar artery and endothelium-dependent vasodilation. Nevertheless, deletion of *$S1P_3$* in mice has abrogated these effects (Peters and Alewijnse, 2007; Forrest *et al*, 2004; Takuwa, 2008). Moreover, *$S1P_3$* knockout in mice has been shown to significantly decrease the intracellular Ca^{2+} and Akt activation; these effects were also associated with nitric oxide mediated vasodilation (Nofer *et al*., 2004; Theilmeier *et al*., 2006). In addition, $S1P_2$ and $S1P_3$ double knockdown have also been shown to reduce fertility (Ishii *et al*., 2002). $S1P_3$ receptors have been shown to play a fundamental role in atherosclerosis by stimulating inflammatory monocyte and / or macrophage recruitment and changing smooth muscle cell behaviour (Keul *et al*., 2011).

In breast cancer models, SK1 has been shown to regulate cell migration by modulating $S1P_3$ receptor expression (Pyne *et al*., 2012). In lung adenocarcinoma, $S1P_3$ signalling has been shown to induce EGFR expression through the Rho kinase pathway (Hsu *et al*., 2012). In addition, E2 has been shown to induce SK1 activation,

followed by EGFR transactivation mediated by S1P-$S1P_3$ signalling in a matrix metalloprotease-dependent manner (Sukocheva *et al*., 2006).

In ER^+ breast cancer patients, high expression of $S1P_3$ receptors was shown to be correlated with a reduced disease-free survival times (Watson *et al*., 2010). Importantly, a newly developed $S1P_3$ receptor monoclonal antibody 7H9 has been shown to act as a functional antagonist to block the growth of breast cancer tumours in a xenograft model (Harris *et al*., 2012). Moreover, $S1P_1$ and $S1P_3$ receptors have been shown to promote the motility of hepatic stellate cells, their differentiation into hepatic myofibroblasts and liver angiogenesis and fibrosis (Liu *et al*., 2011; Yang *et al*., 2013). Therefore, targeting $S1P_3$ receptors in breast cancer appears effective and beneficial, and could improve a patient's survival rate.

1.4.2.4 $S1P_4$

The $S1P_4$ receptor was first cloned from differentiated human dendritic cells (Gräler *et al*., 1998). The human $S1P_4$ gene is localised on human chromosome 19p13.3 and encodes a 384-amino acid protein, and has a molecular mass of 42 kDa (Kluk and Hla, 2002). The human $S1P_4$ shares 82% sequence similarity with the murine $S1P_4$ receptor (Brinkmann, 2007; Chun *et al*., 2010). $S1P_4$ receptor has a more restricted expression compared with $S1P_1$, $S1P_2$ and $S1P_3$ receptors. $S1P_4$ receptor is predominantly expressed in the lymphoid tissue, immune compartments and airway smooth muscle cells. $S1P_4$ receptor has been shown to couple to $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12/13}$ (Gräler *et al*., 2003; Kohno *et al*., 2003), (figure1.3). Moreover, $S1P_4$ receptor has been shown to mediate the immunosuppressive effect of S1P by affecting T cell proliferation and T cell cytokine production (Wang *et al*., 2005). In a $S1P_4^{-/-}$ mouse

model, the lymphoid organ structure and lymphocyte numbers in blood were not affected by $S1P_4$ deficiency. In contrast, $S1P_4$ deficiency markedly affects dendritic cell function leading to reduced Th17 function and differentiation (Schulze *et al*., 2011). Moreover, *S1P4* deletion decreased the neutrophil recruitment and inflammation in S1P lyase-deficient mice, implicating the role of $S1P_4$ receptor signalling in the phenotype (Allende *et al*., 2010). An example of the role of $S1P_4$ in a disease is its expression in ER^- and $HER2^+$ breast cancer patient tumours, where high expression of $S1P_4$ in tumours has been shown to be associated with poor prognosis in patients (Ohotski *et al*., 2012). Moreover, S1P has been reported to stimulate ERK-1/2 signalling through a mechanism involving $S1P_4$ and HER2 receptors in MDA-MB-453 breast cancer cells (Long *et al*., 2010).

1.4.2.5 $S1P_5$

$S1P_5$ is expressed mainly in the brain, especially in white matter tracts. The human $S1P_5$ receptor gene is located on chromosomal locus 19p13.2 and encodes a highly conserved 398 amino acid, and has a molecular mass of 39 kDa (Im *et al*., 2000). $S1P_5$ couples to multiple G-proteins, but exhibits favour toward $G\alpha_{12/13}$. For example, $S1P_5$ coupling to $G\alpha_{12/13}$ mediates an anti-migratory effect in oligodendrocyte cells, while coupling to $G\alpha_i$ induces mature oligodendrocyte cell survival (Novgorodov *et al*., 2007). Moreover, mice with $S1P_5$ receptor knocked down are viable and fertile. Interestingly, these mice have decreased numbers of NK cells (Walzer *et al*., 2007). Moreover, S1P binding to the $S1P_5$ receptor has been shown to promote the egress of NK cells from bone marrow and lymph nodes into blood, which is essential for the recruitment of NK cells to the site of inflammation (Walzer *et al*. 2007). Increasing

the S1P level has been shown to promote the mitotic progression and subsequently lead to chromosome segregation defects. Indeed, $S1P_5$ siRNA knockdown was shown to prevent an S1P-induced spindle defect phenotype *(*Andrieu *et al*., 2017). Also, $S1P_5$ receptor has been shown in centrosomes of mammalian cells, suggesting its role in regulating mitosis by the G-protein-dependent regulation of tubulin polymerisation/de-polymerisation dynamics, and the microtubule formation required for mitosis (Gillies *et al*., 2009). In addition, $S1P_5$ receptors have been shown to enhance the blood-brain barrier integrity, to reduce the transendothelial migration of monocytes and also to maintain immune quiescence of the barrier endothelium (Van Doorn *et al*., 2012). Therefore, $S1P_5$ receptors contribute to the blood brain function by maintaining the immuno quiescent state of ECs of the brain with low expression levels of leukocyte, adhesion molecules, inflammatory chemokines and cytokines (Van Doorn *et al*., 2012).

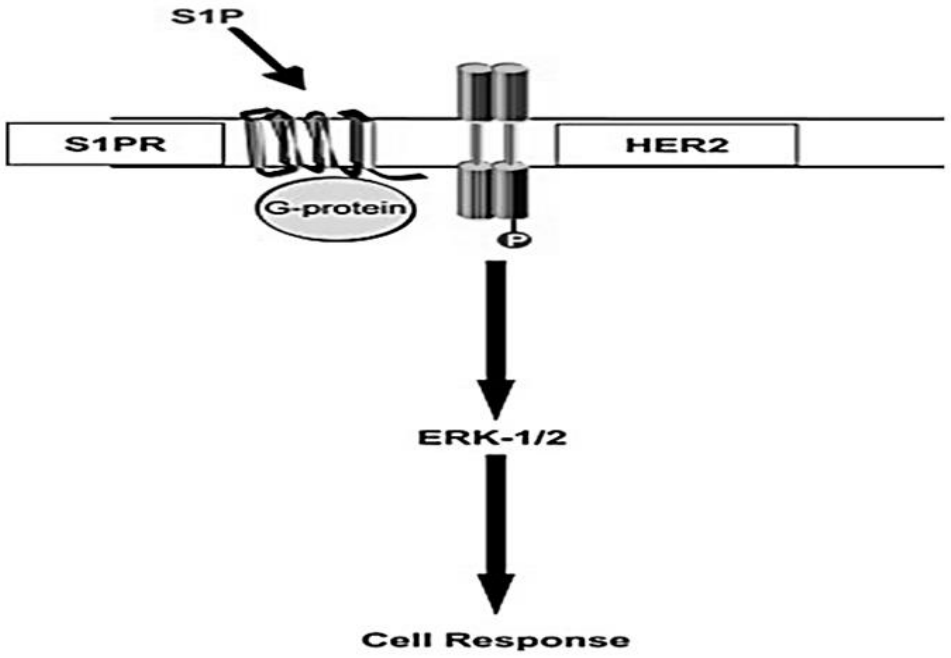
1.4.3 Receptor Tyrosine Kinase-S1P Receptor Signalling Interaction

There are broad interactions and cross-talk between GPCRs signalling and the RTK signalling pathway that were reported (Spiegel and Weinstein, 2004; Reya and Clevers, 2005; Krause *et al*., 2005). For example, GPCRs agonists such as endothelin-1, lysophosphatidic acid and thrombin have been shown to transactivate EGFR signalling (Daub *et al*., 1996). Moreover, the receptor tyrosine kinase inhibitors are demonstrated to block GPCR stimulated growth factor receptor transactivation (Daub *et al*., 1996). S1P receptors and growth factor receptors can work in concert to regulate different biological processes such as proliferation,

migration and angiogenesis (Pyne and Pyne. 2010). For example, SK1 and/or SK2 expression has been shown to be stimulated by EGF to promote migration in cell-specific fashion (Le Stunff *et al*., 2004). Moreover, SK1 has been shown to promote EGF-induced proliferation and migration in MCF-7 cells; proposing that S1P may boost EGF effects (Sarkar *et al*., 2005). Furthermore, S1P has been reported to induce EGFR expression in vascular smooth muscle cells mediated by the activation of ERK-1/2 (Hsieh *et al*., 2008). Also, S1P induces processing and release of EGF through its binding to the $S1P_2$ receptor in a metalloproteinase-dependent mechanism, which results in EGFR transactivation (Shida *et al*., 2004). Moreover, inhibition of $S1P_3$ has been shown to prevent E2-induced activation of Cdc42 in MCF-7 breast cancer cells (Sukocheva *et al*., 2012). Importantly, Long *et al* (2010) have extended these findings to include HER2 growth factor receptors, which belong to the EGFR family (Long *et al*., 2010), (figure 1.4).

In this regard, S1P is demonstrated to increase the tyrosine phosphorylation of HER2 in MDA-MB-453 cells (Long *et al*., 2010). In addition, S1P and EGF were demonstrated to promote the nuclear translocation of activated ERK-1/2 in MCF-7 cells lacking HER2. However, in cells overexpressing HER2, these growth factors activate a pool of ERK-1/2 that is retained in the cytoplasm (Long *et al*., 2010). An additional model whereby RTK might use the G-protein activated by GPCR involves signal integration through GPCR-RTK complexes in the absence of transactivation. For example, Waters *et al* (2003) demonstrated that endogenous PDGFRβ and $S1P_1$ receptors form a functional complex in airway smooth muscle cells (Waters *et al*., 2003), and in mouse embryonic fibroblast cells where

constitutively active $S1P_1$ receptors enhance PDGF stimulated cells migration (Long *et al*., 2006).



Taken from Pyne *et al*, (2009).

Figure 1.4 ***A schematic representation of functional interaction between S1P receptors and HER2 which allows spatial regulation of ERK-1/2 pathway*****.** S1P can increase tyrosine phosphorylation of HER2 in MDA-MB-453 brest cancer cells that lead to ERK-1/2 activation.

1.4.4 Sphingosine Kinases and S1P in Cancer

Sphingolipids metabolism has been shown to be dysregulated in cancer (Ogretmen, 2018). Indeed, SK1 overexpression was reported in many cancers such as in the breast (Ruckhäberle *et al*., 2007), lung (Johnson *et al*., 2005), ovary (Sutphen *et al*., 2004) brain (Van Brocklyn *et al*., 2005) and colon (Kawamori *et al*., 2006) compared with their healthy tissue. Furthermore, several studies demonstrated that S1P stimulates the growth and survival of leukaemia and lymphoma cells (Stevenson *et al*., 2011). In addition, S1P has been shown to promote oestrogen-dependent tumorigenesis of MCF-7 human breast cancer cells (Hait *et al*., 2006), and to regulate MMP9-induced invasiveness in MCF10A breast cancer cells (Kim *et al*., 2011). SK1 overexpression is also known to enhance the proliferation and survival of glioma and breast cancer cells (Van Brocklyn *et al*., 2002; Nava *et al*., 2002). SK1 transfection was also shown to increase fibroblast transformation into fibrosarcoma cells (Pitson *et al*., 2005). In addition, SK1 and SK2 are involved in EGF activation and migration of MDA-MB-453 breast cancer cells (Hait *et al*., 2005). Moreover, high expression of $S1P_1$ and $S1P_3$ receptors has been shown to be associated with a poor prognosis in ER^+ breast cancer patients (Watson *et al*., 2010). Inhibition of SK1 using siRNA, also decreases the $S1P_3$ receptor expression and ERK-1/2 activation in response to S1P, indicating that SK1 and $S1P_3$ functionally regulate ER^+ breast cancer progression and development (Long *et al*., 2010). SK2 is suggested to be associated with apoptosis (Igarashi *et al*., 2003), however there are several reports that support a role for SK2 in cancer cell growth. For instance, downregulation of SK2 with siRNA in MCF-7 breast cancer and HCT116 colon cancer cells decreases G2/M phase transition and significantly enhances apoptosis induced by doxorubicin,

thereby contributing to the maintainence of cell survival (Sankala *et al*., 2007).In addition siRNA knockdown of SK2 in glioblastoma has been shown to inhibit cells proliferation (Brocklyn *et al*., 2005). Moreover, tumour-associated macrophages from SK2 deficient tumours, demonstrated an obvious anti-tumour phenotype (Weigert *et al*., 2009). Also, in A498 kidney adenocarcinoma, Caki-1 kidney carcinoma and MDA-MB-231 breast adenocarcinoma cells, the expression and phosphorylation of pro-survival signalling proteins such as STAT3, AKT and ERK-1/2 were markedly blocked by SK2 inhibition by the selective SK2 inhibitor ABC294640 or siRNA knockdown of SK2, which also disrupted the cell cycle with arrest in G1 and reduced the expression of p53 and p21 (Gao and Smith, 2011).

1.4.5 The Role of Sphingosine Kinase 1 and S1P in Therapeutics Resistance

SK1 overexpression was reported to be associated with a chemotherapeutics resistance (Sordillo *et al*., 2016), thus SK1/S1P balance disruption may improve or restore the sensitivity to chemotherapeutics. For instance, S1P/ceramide ratio has been shown to be high in M221 and Mel-2a resistant melanoma cells compared with sensitive A-375 and M186 melanoma cells (Bektas *et al*., 2005). Moreover, high SK1 expression in pancreatic cancer and chronic myeloid leukaemia cells has been shown to be associated with resistance to gemcitabine and imatinib respectively (Guillermet-Guibert *et al*., 2009). In addition, inhibition of SK1 sensitizes cancer cells to chemotherapeutics, suggesting increased ceramide levels induce apoptosis in these cells (Guillermet-Guibert *et al*., 2009; Baran *et al*., 2007; Bonhoure *et al*., 2008). Moreover, SK1 and $S1P_{1/3/5}$ receptors have been shown to be upregulated in

primary, secondary and recurrent glioblastoma cells compared with normal non-tumourous brain tissue, which is associated with low survival rate (Quint *et al*., 2014). Additionally, SK1, $S1P_1$ and $S1P_3$ receptors expression has been shown to be increased in camptothecin resistant prostate cancer cells PC3 (Akao *et al*., 2006).Furthermore, siRNA knockdown of SK1 or inhibition of S1P receptors signalling by pertussis toxin resulted in significant inhibition of PC3 cells growth (Akao *et al*., 2006). Moreover, high SK1 activity has been shown to be associated with daunorubicin resistance in leukemia cells (Sobue *et al*., 2008), cisplatin resistance in lung cancer cells (Min *et al*., 2005), oxaliplatin resistance in colon cancer cells (Nemoto *et al*., 2009) and N-(4-hydroxyphenyl) retinamide resistance in ovarian carcinoma cells (Illuzzi *et al*., 2010). In addition, high SK1 expression has been shown to be associated with docetaxel, doxorubicin and tamoxifen resistance in breast cancer cells (Gao *et al*., 2015).

1.4.6 Breast Cancer Overview

Breast cancer is the most common occurring cancer in women and the second highest common cause of cancer-associated mortality in this gender wide world. It accounted for 14% of total cancer mortality in 2008 (Ferlay *et al*., 2010). According to Cancer Research UK statistics around 53,700 new cases were diagnosed with breast cancer in 2013, accounting for 15% of all new cases of cancer in UK. Moreover, in 2014 the number of people who died in UK from breast cancer was 11,400. Furthermore, about 65% of women diagnosed with breast cancer are surviving for twenty years or more in England and Wales (Cancer Research UK, 2017).

Breast cancer incidence varies according to geographical region. For example, 19.3 per 100, 000 women are diagnosed with breast cancer in Eastern Africa, while 89.7 per 100, 000 women are diagnosed in Western Europe. Indeed, higher incidence of breast cancer is evident in developed countries except Japan (Ferlay *et al*., 2010). However, breast cancer incidence is increasing in low economic developing countries as a result of increased urbanisation and adoption of Western lifestyles (Kanavos, 2006). Although, there is no well-defined aetiology of breast cancer there are several risk factors, which have been identified and well-studied. These include age, race, genetic factors, hormonal factors, diet, alcohol and environmental and occupational exposure (Cypel and Coughlin, 2013).

The earlier breast cancer is diagnosed, the better chances of successful treatment. The mammogram is the main recommended tool for breast cancer screening by Cancer Research UK (Cancer Research UK, 2017) and the American Cancer Society (Cancer.org, 2017), together with magnetic resonance imaging (MRI). In case of detection, further tests are recommended to check the size of the tumour and whether metastasis to the lymph nodes has occurred, and a tumour biopsy to determine its status with regard to hormone receptors HER2/neu, progesterone receptor and oestrogen receptor. Breast cancer treatment is based on cancer stage grade and genotypic background, and includes surgery, chemotherapy, radiotherapy, hormonal therapy and biological therapy (e.g. Herceptin).

1.4.6.1 Molecular Classification of Breast Cancer

Breast cancer classification has been developed over the years as a result of development of molecular profiling using DNA microarray technology (Holliday and

Speirs, 2011). DNA microarray technology has provided information on breast cancer cells heterogeneity as demonstrated by gene expression profiling and the immuno-histochemical expression of ER, PR and HER2. Using these biomarkers, breast cancer is classified into four subtypes, which include luminal A, luminal B, HER2 and basal (Perou *et al*., 2000).

1.4.6.1.1 Luminal A Breast Cancer

Luminal A breast cancer is the most common subtype and constitutes 50-60% of all breast cancer (Yersal and Barutca, 2014). Luminal A tumours are characterised by low mitotic activity, histological grade and degree of nuclear pleomorphism. Luminal A breast cancer is also characterised by higher levels of ER and low proliferative index. Patients with luminal A breast cancer have good prognosis with low relapse rates (Yersal and Barutca, 2014).

1.4.6.1.2 Luminal B Breast Cancer

Luminal B tumours represent 15-20% of breast cancer and are characterised by higher histological grade, proliferative index and a poorer prognosis (Creighton, 2012). Additionally, this subtype of breast cancer is characterised by high recurrence rate and low survival rates after relapse when compared with luminal A tumours (Ellis *et al*., 2008).

1.4.6.1.3 Basal-Like

Basal-like subtype comprises 7-37% of breast cancer and is associated with high histological and nuclear grade. Most of these tumours are infiltrating ductal tumours

with solid growth arrangement and with high metastatic spread to the brain and lung (Heitz *et al.*, 2009). These types of tumours express high level of basal myoepithelial markers such as cytokeratins CK 17, CK 14 and CK 5 and laminin. Basal-like cancers do not express ER, PR and HER2 and are thus, termed triple-negative (Heitz *et al*., 2009).

Herschkowitz *et al* (2007) described claudin-low breast cancer as one of the triple negative subtypes (Herschkowitz *et al*., 2007). This subtype expresses low transmembrane protein claudin, which is involved in formation of tight junctions and low expression of this protein is correlated with high invasion and poor prognosis in breast cancer cell patients (Morohashi *et al*., 2007).

1.4.6.1.4 HER2 Positive Breast Cancer

HER2 positive breast cancer comprises 15-20% of breast cancer subtypes. This subtype is characterised by high HER2 expression. The cancer is characterised by high proliferative index, histological and nuclear grade (Yersal and Barutca, 2014). Three subtypes of HER2 positive breast cancer were identified by Staaf *et al* (2010) using HER2 derived prognostic predictor (HDPP), which is a strong independent prognostic factor in HER2-positive breast cancer. One subtype is characterised by poor prognosis with 12% survival compared with the other two subtypes where survival rate reached 50-55%. HDPP analysis was not based on HER2 gene expression but, directly related to tumour invasion, metastasis and immune response (Staaf *et al*., 2010).

Table 1-1 Molecular categorisation of breast cancer

Taken and reproduced from Holliday and Speirs, (2011).

Classafication	Immuno-profile	Cell line
Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	MCF-7, SUM185
Luminal B	ER^+, $PR^{+/-}$, $HER2^+$	BT474, ZR-75
Basal	ER^-, PR^-, $HER2^-$	MDA-MB-468, SUM190
Claudin-low	ER^-, PR^-, $HER2^-$	BT549, MDA-MB-231
HER2	ER^-, PR^-, $HER2^+$	SKBR3, MDA-MB-453

This table summarises the major breast cancer cell subtypes according to its oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expressions.

1.4.6.2 Sphingosine Kinase and S1P Signalling in Breast Cancer

Several reports implicated the role of SK1 in breast cancer where it functions to promote tumourigenesis and metastasis (Geffken and Spiegel, 2017). For example: MCF-7 cells that ectopically overexpress SK1 have been shown to form larger tumours in nude mice compared with vector transfected MCF-7 cells (Nava *et al*., 2002). Also, poor prognosis and low survival rate have been reported to be correlated with high tumour SK1 expression in patients with ER^+ breast cancer (Long *et al*., 2010; Watson *et al*., 2010; Pyne *et al*., 2011). Moreover, S1P has been shown to enhance the induction of MMP9 *in vivo* and *in vitro* from MCF10A human breast epithelial cells (Kim *et al*, 2011), which provides information on the molecular basis for the role of S1P in cancer migration and invasion. Furthermore, S1P has been shown to stimulate breast cancer growth through activation of the serum response element and by intensifying insulin-like growth factor (IGF) II production and function (Goetzl *et al*., 1999). In addition, the expression of $S1P_1$, $S1P_3$ and ERK-1/2

has been shown to be elevated in ER^+ breast cancer patients, which is associated with shorter recurrence times (Watson *et al*., 2010). Amorim *et al* (2014) demonstrated the presence of $S1P_3$ and $S1P_4$ receptors in EVs released from mammary luminal epithelial C5.2 cells, which are characterised by high HER2 expression, indicated by proteomic analysis of 20K and 100K sets of EVs (Amorim *et al*., 2014). In addition, Kajimoto *et al* (2013) demonstrated that S1P is involved in ESCRT-independent protein sorting into intralumenal vesicles (ILVs). Moreover, G_i-coupled S1P receptors such as $S1P_1$ and $S1P_3$ are involved in maturation of multivesicular endosomes (MVEs). Furthermore, siRNA knockdown of SK2 or $S1P_1$ receptor impairs the formation of CD63 and CD81 positive ILVs (Kajimoto *et al*., 2013). Thus, these findings raised the question of whether extracellular vesicles released from breast cancer cells expressing S1P receptors are contained S1P receptors and whether released receptors enable cancer/fibroblasts cells communication, critical for metastasis.

1.5 Role of the Extracellular Vesicles in Cancer

1.5.1 Extracellular Vesicles

EVs are membrane vesicles of endosomal and plasma membrane origin that are released by almost all type of cells into the extracellular milieu (Thery *et al*., 2006). In recent years EVs have gained attention in different biological and medical fields, because they function as a mean of intercellular communication. EVs can communicate between adjacent and distant cells and organs in normal, and also in pathological conditions such as cancer metastasis, spread of viral infectivity and synaptic spreadof amyloid-β-derived peptides in Alzheimer's disease and α-synuclein

in Parkinson's disease. EVs are classified according to their biogenesis and size into exosomes, micro-vesicles and apoptotic bodies (Yáñez-Mó *et al*., 2015; Zaborowski *et al*., 2015; EL Andaloussi *et al*., 2013).

Exosomes are plasma membrane vesicles of endocytic origin and are also known as intraluminal vesicles (ILVs) with 30-100 nm size. They are enriched in proteins, lipids and nucleic acids and have been shown to participate in cancer progression and metastasis, where they can modify or alter the phenotype of recipient cells (Green *et al*., 2015). This is acheived by transferring different proteins and oncogenes from cancer cells to tumour surrounding cells leading to the development of the pre-metastatic niche (Yang and Robbins, 2011; Kobayashi *et al*., 2015). Exosomes are formed through invagination of the plasma membrane into endosomes, which is followed by the formation of multi-vesicular endosomes or bodies (MVBs). MVBs are then released into the extracellular milieu or can be directed toward lysosomal degradation (Mathivanan *et al*., 2010).

Most of exosomeare homogenous in size with cup-shaped when visualised by the electron microscope (Mathivanan *et al*., 2010; Thery *et al*., 2009; Vlassov *et al*., 2012; Simons and Raposo, 2009). Tram and colleagues (1981) were the first to use the term 'exosomes' to describe small vesicles released into the extracellular milieu from reticulocytes (Tram *et al*., 1981). Subsequently, small vesicles were demonstrated to be released from rat reticulocytes (Harding *et al*., 1983), followed by identification of 50 nm bodies released from sheep erythrocytes (Pan *et al*., 1985). Exosomes have been shown to float on a sucrose gradient in density range 1.13 to 1.19 g/ml. As a consequence of their endosomal origin, nearly all exosomes,

independent from which cell type they originate, are enclosing proteins involved in the membrane transport and fusion such as Rab GTPases, annexins and flotillin; MVBs biogenesis proteins such as Alix and Tsg101; proteins associated with integrins and tetraspanins such as CD63, CD9, CD81 and CD82; heat shock proteins such as hsp70 and 90, which are also present as markers (Simons and Raposo, 2009; Urbanelli *et al*., 2013), (figure 1.5). Exosomes are also enriched with lipid rafts containing cholesterol, sphingolipids, ceramide and glycerophospolipids with long and saturated fatty acyl chains (Subra *et al*., 2007).

1.5.2 Exosome Biogenesis

Exosomes are derived from the late endosomal system, where late endosomes which are also known as MVBs bud off parts of their membrane into their lumen to form ILVs, which fuse with the cell membrane surface in an exocytic manner (Urbanelli *et al*., 2013). Following maturation, these vesicles are either integrated with the lysosome where their cargo ingredients undergo lysosomal degradation or with the plasma membrane where their contents are released into the extracellular milieu. However, the determinants that regulate the final destination of these vesicles are not well defined (Frydrychowicz *et al*., 2014).

1.5.2.1 The Role of the Endosomal Sorting Complex Required for Transport (ESCRT) Machinery in Exosomes Formation

The formation of MVBs is synchronised by the endosomal sorting complex required for transport termed ESCRT, which is composed of approximately thirty proteins that assemble into four complexes ESCRT-0, -I, -II and –III with associated proteins VPS4, VTA1, ALIX also called PDCD6IP conserved from yeast to mammals (Hanson and Cashikar, 2012). ESCRT proteins complex has been shown to engage ubiquitinated cargo at the endosome and to mediate sorting into MVBs (Katzmann *et al*., 2001). This complex is usually recruited to the cytosolic side of the endosomal membrane to enable sorting proteins into MVBs (Urbanelli *et al*., 2013). The process of plasma membrane receptors sorting into inner vesicles of MVBs requires ubiquitination of the cytosolic tail of endocytosed receptors (Katzmann *et al*., 2002). Thereafter, Tsg101 which belongs to the ESCRT-I complex is recruited to ubiquitinated cargo proteins and subsequently, activates the ESCRT-II complex, which in turn, initiates oligomerisation and formation of the ESCRT-III complex (Raposo and Stoorvogel, 2013; Urbanelli *et al*., 2013). Despite the fact that ESCRT machinery is involved in MVBs formation and sorting cargo content, there are studies which have shown that mammalian cells can form MVBs in the absence of ESCRT complex. For instance, human epithelial types 2 (HEp-2) cells can still form MVEs with late endosomal marker CD63 even after depletion of four subunits of the ESCRT complex (Stuffers *et al*., 2009). Moreover, the sphingolipid metabolite ceramide has been shown to regulate ESCRT-independent ILVs biogenesis and to

induce the formation of internal vesicles in liposomes *in vitro* (Trajkovic *et al*., 2008). This was evidenced by reduced exosomes release upon inhibition of sphingomylinase, the enzyme that catalyses the formation of ceramide (Trajkovic *et al*., 2008). These observations led to a conclusion that ESCRT-independent pathways can be used to form MVBs and can be driven by the presence of certain lipids, such as lysophosphatidic acid and ceramide (Babst, 2011). Recently, G_i proteins have been found to be constitutively active on MVEs, suggesting involvement of the receptor-mediated regulation of MVEs maturation and proteins sorting into ILVs in ESCRT- independent manner (Kajimoto *et al*., 2013). Importantly, this continuous activation of G_i proteins has been shown to be mediated by a constant S1P supply and binding to $S1P_1$ and/or $S1P_3$ receptors as evidenced by the co-localisation of these receptors in CD63 positive vesicles. These findings support a role of S1P and its receptors in the formation of exosomes and possibly S1P receptors present in exosomes released from cells overexpressing specific S1P receptor.

1.5.3 Exosome Contents

Using advanced tools such as high resolution electron microscopy and advanced proteomic analysis of exosomes from different origin in disease and health conditions have increased our understanding of the structure and composition of exosomes. Exosome composition is diverse and depends on the cell type of origin. A comparative proteomics analysis study performed by Mathivanan *et al* (2010) demonstrated that colorectal cancer cell (LIM1215)-derived exosomes, human urine-derived exosomes and murine mast cell-derived exosomes contain both common and

unique proteins that reflects their cellular origin and possible physiological role (Mathivanan *et al*., 2010).

1.5.3.1 Exosome Protein Contents

According to an exosome database ExoCarta which provides information on exosome content in multiple organisms, there are more than 2399 proteins that have been identified in exosomes from 64 independent studies (Mathivanan and Simpson, 2009). Moreover, exosomes can also carry proteins that are involved in specific cell function, such as major histo-compatibility complex (MHC) class II molecules, which are released from antigen-presenting cells (APCs) (Théry *et al*., 2002). In addition, exosomes have been shown to contain proteins involved in cell signalling pathways, such as Wnt-β-catenin, the Notch ligand Delta-like 4 and interleukins (Azmi *et al*., 2013; Mathivanan *et al*., 2012). In addition, exosomes carry some cell-specific proteins such as CD86, which is released from APCs. Similarly, milk fat globule EGF/factor VIII (MFG-E8) also termed lactadherin is present only in exosomes derived from immature dendritic cells (DCs) (Schorey and Bhatnagar, 2008; Véron *et al*., 2005). Moreover, exosomes are reported to carry functional GPCRs. For example, the angiotensin II type 1 receptor also termed AT1R has been detected in exosomes released from mice cardiomyocytes exposed to cardiac pressure (Pironiti *et al*., 2015). In addition, exogenous administered AT1R-enriched exosomes can confer pressure responsiveness to angiotensin II infusion in cardiomyocytes, skeletal myocytes and mesenteric resistance vessels in *AT1R* knockout mice (Pironti *et al*., 2015).

Moreover, in a proteomic analysis study of EVs released from C5.2 cells, $S1P_3$ and $S1P_4$ receptors were indicated to be present in EVs. However, no functional role was attributed to these receptors (Amorim *et al*., 2014).

1.5.3.2 Exosomes Nucleic Acid Contents

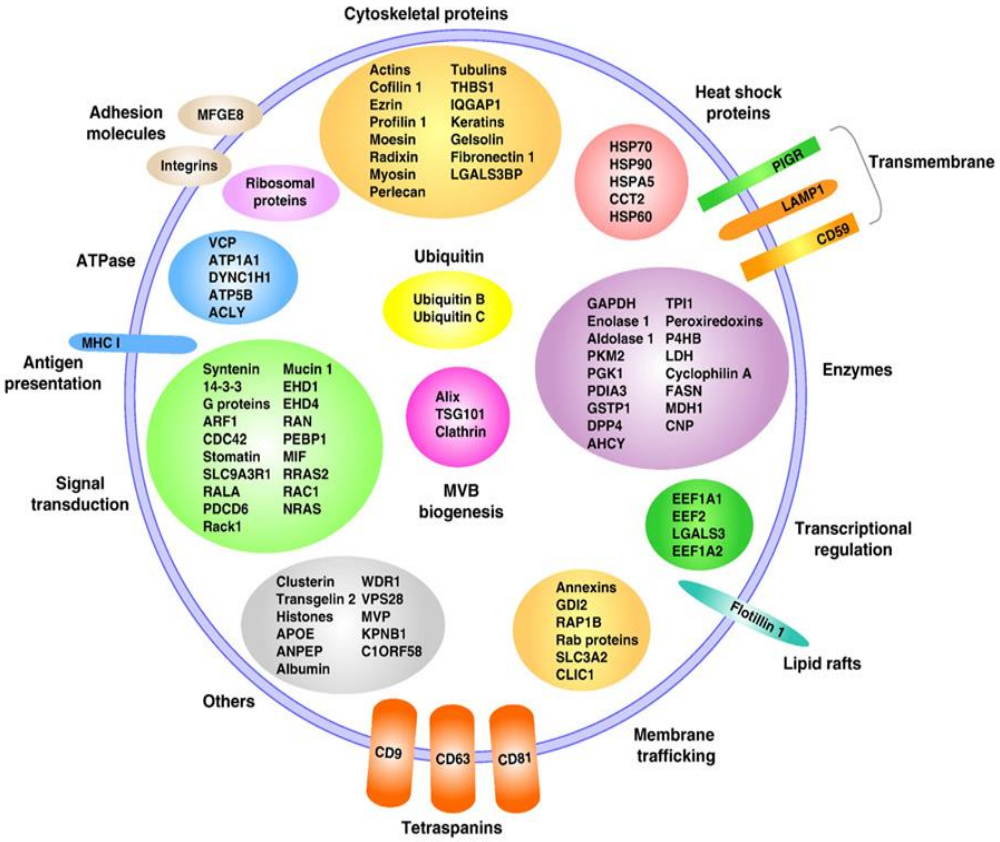
Exosomes have been found to contain nucleic acid such as DNA, mRNA, microRNAs (miRNAs), mitochondrial DNA (mtDNA), long non-coding RNAs (lncRNAs), ribosomal RNAs (rRNAs), small-nuclear RNAs (snRNAs) and transfer RNAs (tRNAs) (Roma-Rodrigues *et al*., 2014). Indeed, exosomal mRNAs can be transferred to recipient cells, where they translate into proteins and/or modifying recipient cells phenotype (Beach *et al*., 2014). The amount of RNA in exosomes varies depending on the origin of cells. For instance, cancer-derived exosomes have been shown to contain more total RNA compared with exosomes released from normal cells (van der Vos *et al*., 2011). In addition, exosomal miRNA might function as either a tumour suppressor or an oncogene upon transfer to recipient cells (Roma-Rodrigues *et al*., 2014). For instance, miRNA-21 and miRNA-155 are also well characterised oncomiRNAs that promote both tumour growth and metastasis by targeting numerous mRNAs. In contrast, miRNA-15a, miRNA-16-1, miRNA-34a, and the let-7 family of miRNAs have been shown to suppress tumour growth and metastasis by inducing apoptosis, cell cycle arrest and senescence (Taylor and Schiemann, 2014).

T cells have been shown to transfer miRNAs to APCs in CD63 positive exosomes, and these miRNAs are able to modulate gene expression in APCs (Mittelbrunn *et al*., 2011). Furthermore, mature EBV-encoded miRNAs have been shown to be released

by EBV-infected B cells in exosomes (Pegtel *et al*., 2010). These EBV-miRNAs are functional and can be taken up by primary immature monocyte-derived DC, resulting in a dose dependent miRNA-mediated repression of confirmed EBV target gene such as CXCL11/ ITAC (Pegtel *et al*., 2010). In addition, incubation of exosomal miRNA-342–3p and miRNA-1246 released from highly metastatic human oral cancer (HOC313-LM) cells with poorly metastatic cancer (HOC313-P) cells, resulted in an increased motility and invasiveness of HOC313-P cells (Sakha *et al*., 2016). Moreover, tumour-associated macrophages also termed TAMs have been shown to regulate the invasiveness of breast cancer cells by releasing 'oncogenic' miRNAs, such as miRNA-223 in exosomes into a tumour microenvirment evidenced by decreased breast cancer invasivness upon treating IL-4-activated macrophages with a miRNA- 223 antisense oligonucleotides (Yang *et al*., 2011).

1.5.3.3 Exosome Lipid Contents

Compared with the plasma membrane of parental cells, exosomes are enriched in cholesterol, sphingolipid including sphingomyelin and ceramide, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol (PI), phosphatidylserine (PS) and polyglycerophospholipids (Villarroya-Beltri *et al*., 2014). This high ratio of exosomal lipids compared to plasma membrane lipids of parental cells might account for the role of these lipids in exosomes biogenesis. Also, these lipids provide wall rigidity and fluidity to exosomes and might also facilitate exosomes fusion with plasma membrane, thereby enabling release of their contents into the extracellular milieu and subsequent uptake by recipient cells (Record *et al*. 2014; Subra *et al*., 2007).



Taken from Mathivanan *et al*, (2010).

Figure 1.5 ***Exosome protein content.***

Schematic representations of the major exosome protein compositions which have been identified from proteomic studies on exosomes provided by ExoCarta

1.5.4 Exosome Release Mechanisms

The factorsthat determine MVBs fate are not yet defined. The MVBs docking and fusion with the cell membrane require the involvement of cytoskeleton proteins such as actin, microtubular machinery molecules such as kinesin and myosin, molecular switches such as small GTPases and the fusion machinery (Raposo and Stoorvogel, 2013; Villarroya-Beltri *et al*., 2014). A screening of Rab GTPase-activating proteins identified the TBC1D10-C (Hsu et al., 2010). Importantly, TBC1D10-C and Rab35 can regulte exosomes secretion in a catalytic activity-dependent manner in oligodendroglial cells (Hsu *et al*., 2010), and inhibition of Rab35 function leads to intracellular accumulation of endosomal vesicles and impairs exosome secretion (Hsu *et al*., 2010). In addition, Rab protein and soluble NSF attachment protein receptor also termed SNARE have been shown to regulate exosome fusion with cell membranes (Jahn and Scheller, 2006). Importantly, sphingosine has been shown to activate synaptobrevin interaction with the SNARE complex assembly in synaptic vesicles, thereby facilitating synaptic fusion and transmission. This is important for high spatial and temporal precision of synaptic vesicle exocytosis (Darios *et al*., 2009). Sphingosine has been shown to relieve the inhibition resulting from the association of the amphipathic cytoplasmic part of synaptobrevin with the phospholipid membrane, which is followed by synaptobrevin engagement of syntaxin/SNAP25, resulting in SNARE assembly and an augmented pool of readily-releasable vesicles (Darios *et al*., 2009). In addition, exosomes secretion has been shown to be influenced by the intracellular level of Ca^{2+} (Savina *et al*., 2003), which is evidenced by reduced exosome release after DMA, a blocker of the Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchanger (Merendino *et al*., 2010). Furthermore, the intracellular and

intercellular pH has been shown to affect exosome release. For example, the low pH in the tumour microenvironment, which resulted fom limited vascularisation, nutrient and oxygen supply increases exosomes release and uptake by recipient cells. Parolini *et al* (2009) used exosomes derived from a metastatic melanoma as a model to study the effect of different pH on exosome release and fusion with cancer cells. They have found that an increased exosome release and uptake at low pH compared with a buffered condition. Also, pre-treated exosomes with a proton pump inhibitor has been shown to reduce their uptake by melanoma cells (Parolini *et al*., 2009). These findings, suggest that high sphingomyelin and monosialodihexosylganglioside lipids content in acidic exosomes positively affect their fusion ability.

1.5.5 Exosome Uptake

Although the process of exosome uptake by recipient cells remains uncertain, it may be explained by two mechanisms. These are receptor-mediated uptake and direct fusion of released exosomes with the plasma membrane of recipient cells (Figure 1.6).

1.5.5.1 Receptor Mediated Uptake

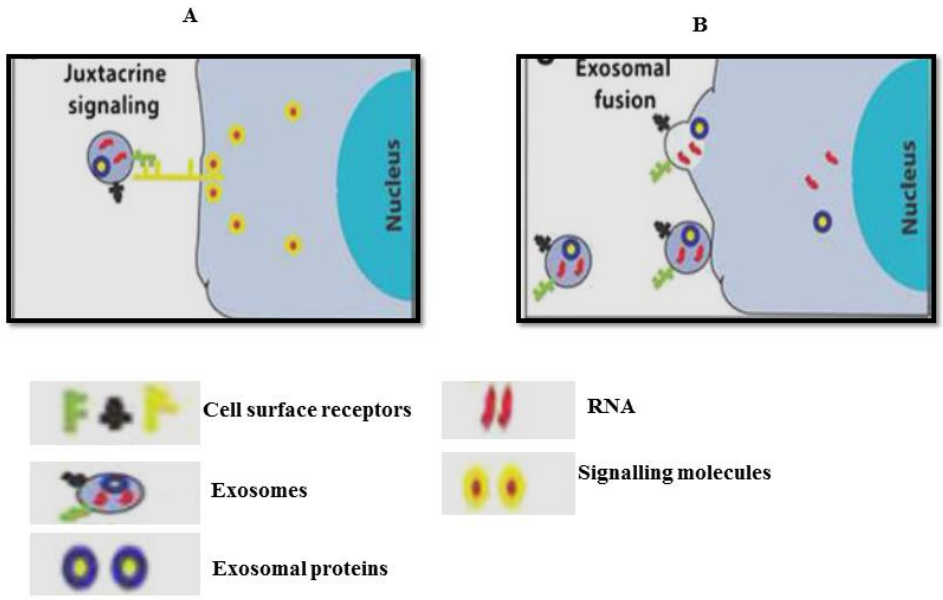
Exosome membranes are enriched in phospholipids, especially phosphatidylserine and proteins, which can act as anchors for binding exosomes to the plasma membrane of recipient cells. The T-cell immunoglobulin and mucin domain containing molecule Tim1 and Tim4 have been shown to bind tightly to the exosomal phosphatidylserine, suggesting that they attach exosomes to recipient cells. For example, Tim4 or Tim1 expressing Ba/F3 B cells were found to bind to the

exosomal phosphatidylserine (Miyanishi *et al*., 2007). Moreover, the intercellular adhesion molecule 1 also termed ICAM-1 was found to enhance the ability of APCs to capture exosomes, and to facilitate the binding of APCs to T-cells (Kharaziha *et al*., 2012).

Also, tetraspanins have been shown to be involved in a number of processes, including the vesicular and cellular fusion (Hemelr, 2005), and targeting of the CD81 or CD9 with antibodies has been shown to reduce the uptake of EVs by DCs (Morelli *et al*., 2004). In addition, the integrin CD49d and Tspan8 have been shown to contribute to exosomes binding to ECs (Nazarenko *et al*., 2010). Importantly, ECs uptake of the Tspan8 and CD49d containing exosomes was associated with enhanced ECs proliferation, migration, sprouting and maturation of ECs progenitors (Nazarenko *et al*., 2010), (figure 1.6 A).

1.5.5.2 Direct Fusion with Plasma Membrane

The second mechanism by which exosomes can deliver their contents to recipient cells is through a fusion of exosomes with the plasma membrane. Melanoma cells were observed to take up exosomes by a fusion process, which was enhanced by a low pH in the tumour microenvironment (Parolini *et al*., 2009). Indeed, Montecalvo and colleagues (2012) demonstrated that addition of exosomes labeled with self-quenching concentrations of R18 to BMDCs, resulted in a time-dependent fluorescence increase, indicative of a fusion/hemifusion of the exosomes with the BMDCs (Montecalvo *et al*., 2012), (Figure 1.6 B).



Taken and reproduced from Kahlert and Kalluri, (2013).

Figure 1.6 ***Mechanisms involved in exosome uptake by recipient cells.***

(A) Receptor mediated uptake which is an example of juxtacrine signalling. (B) Direct fusion with the cell membrane.

1.6 Roles of Sphingosine Kinases, S1P and S1P Receptors in Exosome Formation, Maturation and Signalling

The first study to show the involvement of sphingosine and S1P in the endocytic process was performed by Rosa *et al* (2010). These authors demonstrated that sphingosine and S1P have a Ca^{2+}-dependent permissive role in the initiation of an endocytic process (Rosa *et al*., 2010). Genetic studies in yeast indicated that sphingolipids are essential in endocytosis (Hannich *et al*., 2011). In addition, sphingosine and S1P have been shown to regulate the endosomal trafficking of G protein-coupled receptors in *Drosophila* (Collinet *et al*., 2010). Moreover, SK1 is involved in an early endocytic process evidenced by the accumulation of SK1 on the tubular structures following cholesterol extraction by methyl-β–cyclodextrin (MβCD) (Shen *et al*., 2014). Moreover, an increase in the membrane trafficking is initiated by the recruitment of SK1 to the plasma membrane and the formation of S1P (Young *et al*., 2016). Also, S1P has been shown to facilitate glutamate secretion in the hippocampus, therefore it might regulate synaptic vesicle transmission and recycling in neurons (Kajimoto *et al*., 2007). Moreover, in HeLa and HUVEC cells, inhibition of SK2 expression using siRNA has been shown to reduce the formation of CD63 positive MVBs, suggesting SK2 is responsible for the production of S1P on these MVEs (Kajimoto *et al*., 2013). In addition, $S1P_1$ and $S1P_3$ receptors are continuously activated on CD63-positive MVEs, suggesting the involvement of these receptors in exosome formation. In this regard, S1P can regulate CD63 sorting into ILVs via ESCRT-independent mechanism (Kajimoto *et al*., 2013). Therefore, there is a role for S1P and its receptors in the sorting and maturation of MVBs. In addition, these findings raise the question as to whether the presence or absence of S1P and its

receptors in exosomes derived from cancer cells have a role in cancer dissemination and metastasis.

1.7 GPCRs Sorting to Endosomes

GPCRs constitute the largest family of cell surface signalling receptors encoded in the human genome with ~900 members. Upon activation, GPCRs undergo conformational changes that enable activation of heterotrimeric G-proteins and signalling at the plasma membrane (Marchese *et al*., 2010). Agonist activation of GPCRs not only results in signalling through G-proteins, but also leads to other molecular interaction such as feedback regulation of G-protein coupling, receptor endocytosis and independent G-protein signalling (Ferguson, 2001). Despite the fact that GPCRs internalisation is considered to be an agonist-mediated process, some evidence suggests that GPCRs can be endocytosed in the absence of an agonist. This process or phenomena called a constitutive internalisation. Constitutive GPCRs internalisation is exemplified by the cannabinoid CB1 receptor in neurons (Leterrier *et al*., 2004). In this case, constitutive internalisation has been shown to be responsible for the redistribution of CB1 receptors from the somatodentritic membrane to the axonal membrane (McDonald *et al*., 2007; Stanasila *et al*., 2008; Bohn, 2007). Desensitisation of GPCRs is a multistep process. A well-studied example is a GPCR prototype β2-AR, where the phosphorylation of activated β2-AR by G protein-coupled receptor kinases also termed GPKs, facilitates β-arrestin recruitment to the active phosphorylated receptor, thereby uncoupling the GPCR from its associated heterotrimeric G-proteins and facilitating association of the receptor with clathrin and AP-2 of endocytic machineries (Pyne and Pyne, 2017;

Moore *et al*., 2007; Lefkowitz, 2005; Hirsch *et al*., 1999; Goodman *et al*., 1996; Laporte *et al*., 1999; Shenoy and Lefkowitz, 2003). Arrestins also function as ligand-regulated scaffolds, recruiting catalytically active proteins into a receptor based multiprotein "signalsome" complexes. Arrestin binding thus marks the transition from a transient G protein-coupled state on the plasma membrane to a persistent arrestin-coupled state that continues to signal as the receptor internalises (Strungs and Luttrell, 2014). An example of arrestin-dependent signaling pathways include regulation of Src family non-receptor tyrosine kinases and the ERK-1/2 mitogen-activated kinase (Thomsen *et al*., 2016; Strungs and Luttrell, 2014). According to the binding pattern of β-arrestin, GPCRs are classified into two classes. Class A GPCRs exhibit weak binding to β-arrestin, and this binding appears to be transient and regulate rapid recycling of the receptor from endosomes back to the plasma membrane. Examples of these receptors are the β_2-AR, μ-opioid receptor, endothelin ET_A receptor and dopamine D_{1A} receptor (Oakley *et al*., 2000). Class B GPCRs exhibit stable binding to β-arrestin, which results in continued internalisation and signalling. Examples of these receptors include V2R, AT1R, thyrotropin-releasing hormone receptor, neurotensin 1 receptor, neurokinin NK1 receptor and $S1P_1$ receptor (Oakley *et al*., 2000; Feinstein *et al*., 2013; Ferrandon *et al*., 2009; Pyne and Pyne. 2017). Moreover, class B GPCRs has been shown to induce sustained signalling in endosomes, commencing non-classical signals such as mitogen-activated protein kinase (Calebiro *et al*., 2009). Moreover, β-arrestin has been shown to co-internalise with protease activated receptor-2 also termed PAR2, a type of GPCRs that does not recycle back to the cell surface and promote continuous ERK-1/2 signalling independent of G-protein activation (DeFea *et al*., 2000;

Stalheim *et al*., 2005). Therefore, internalised GPCRs with arrestin into endosomes and the ability of these endosomes to signal from the cytoplasm raise the question of whether these proteins might be packed into MVBs and released outside cells in exosomes.

1.8 Roles of Exosomes in Different Physiological and Pathophysiological Aspects

Exosomes are secreted by several cell types such as hematopoietic (B cells, T cells, dendritic cells, mast cells, platelets), intestinal epithelial cells, Schwann cells, adipocytes, neuronal cells, fibroblasts (NIH3T3), numerous tumour cell lines and stem cells. Therefore, they are suggested to be involved in various physiological processes such as lactation, immune response and neuronal function and in pathological disease such as cancer (Urbanelli *et al*., 2013; Vlassov *et al*., 2012; Van Niel *et al*., 2006; DB *et al*., 2017). Moreover, exosomes are indicated to be a mean of removal of unnecessary cell membrane proteins and RNA during the cell maturation process, and also an effective mean to remove cell contents in the absence of effective degradation pathways (Vlassov *et al*., 2012).

1.8.1 Role of Exosomes in Immune System

Exosomes are shown to play an essential role in the regulation of both adaptive and innate immune responses. Primarily, there are several lines of evidence, which demonstate that exosomes are implicated in antigen presentation in MHC-peptide complexes (Urbanelli *et al*., 2013). B cell-derived exosomes have been shown to induce antigen-specific MHC class II that regulates T cell responses *in vitro* (Denzer *et al*., 2000). Moreover, B cell-derived exosomes have been shown to express

functional integrins, which are capable of mediating anchorage to the extracellular matrix and cell-surface adhesion molecules (Clayton *et al*., 2004). Moreover, exosome-containing MHC–peptide complexes can directly bind to their cognate T-cell receptor and activate primed $CD4^+$ and $CD8^+$ in T cells (Admyre *et al*., 2006). Exosomes also play a role in immune tolerance, which is a complex series of mechanisms that impair the immune system to mount responses against self-antigens (Romagnani, 2006). It was observed that introducing exosomes to a patient from a donor prior to transplant surgery enabled the recipient to have a longer transplant acceptance time by the immune system (Février and Raposo, 2004). Also, exosomes derived from acute myelogenous leukaemia have been shown to promote down-regulation of CD3ζ and JAK3 expression in primary activated T-cells, and to mediate Fas/FasL driven apoptosis of $CD8^+$ T-cells as well as reducing the proliferation of NK cells to enable cancer cells to evade the immune system (Whiteside, 2013). Additionally, FasL has been found in the membrane of tumour-derived exosomes and was shown to induce T cell apoptosis, thereby suppressing immune response (Abusamra *et al.*, 2005). Also, exosomes can produce immunosuppressive effect via inhibition of anti-tumour response elicited by immune cells. For example, exosomes derived MDA-MB-231 breast cancer cells have been shown to inhibit the differentiation of human $CD14^+$ monocytes into mature APCs (Yu *et al*., 2007). Similarly, mouse adenocarcinoma TS/A cells-derived exosomes have been shown to block the differentiation of murine myeloid precursor cells into DCs *in vitro*, suggesting these exosomes induce the production of IL-6, which block cells differentiation (Yu *et al*., 2007). Thus, blocking the differentiation might be

underlying the escape of tumours from a host immune response, which represents a major obstacle to successful cancer immunotherapy.

In contrast, other studies have demonstrated that exosomes can produce anti-tumour response. For example, NK cells release exosomes that transport CD56, perforin and granzyme B (Lugini et al., 2012), and these proteins have been shown to retard tumour growth (Lugini *et al*., 2012).

1.8.2 Role of Exosomes in Inflammation

Cell derived microparticles like exosomes have been shown to induce the production of tissue factor (TF) and pro-inflammatory cytokines in recipient cells. For example, derived vesicles from N-formyl-Met-Leu-Phe-stimulated human polymorph nuclear leukocytes also termed PMLs, can activate endothelial cells by increasing tyrosine phosphorylation of the 46kDa c-Jun NH2-terminal kinase (JNK1). This leads to expression and production of pro-inflammatory TF and interleukin-6 (Pol and Bo, 2012). In contrast, PMLs derived microvesicles have also been shown to contain the anti-inflammatory protein annexin-1 (Dalli *et al*., 2008).

Exosomes are implicated in the pathogenesis of Alzheimer's disease (AD), through the transport of Amyloid β (Aβ) (a protein which causes progressive loss of neurons leading to the AD) to the extracellular environment (Rajendran *et al*., 2006). In this regard, exosomal proteins such as Alix and flotillin-1 accumulate around amyloid plaques in the brain of AD patients, suggesting a role of exosomes in AD progression (Rajendran *et al*., 2006).

1.8.3 Role of Exosomes in Cancer

Cancer metastasis to other tissues and remote organs from the primary site within the body is the major cause of cancer mortality (Tickner *et al*., 2014). The hypothesis of the ‘‘Seed and Soil’’ (where the metastatic cell is the seed and the microenvironment is the soil), in addition to metastatic niche formation at secondary sites are both well recognised (Kharaziha *et al*., 2012). It is suggested that exosomes play a role in tumour dissemination by influencing and modulating the tumour microenvironment and stromal cells to receive oncogenic and proangiogenic signalling, which generates a pre-metastatic niche in remote tissues and organs (Zhang *et al*., 2015; Shkarina *et al*., 2014; Kahlert and Kalluri, 2013; Greening *et al*., 2015; Yang and Robbins, 2011). Differentiation of fibroblasts into myofibroblasts of many solid cancers has been shown to be linked with exosomal signalling (Webber *et al*., 2010). These myofibroblasts are able to support tumour growth, vascularisation and metastasis (De Wever *et al*., 2008). In this regard, Hood *et al* (2011) demonstrated that melanoma-derived exosomes located in sentinel lymph nodes are able to recruit melanoma cells, thereby facilitating tumour cell metastasis to lymph nodes.

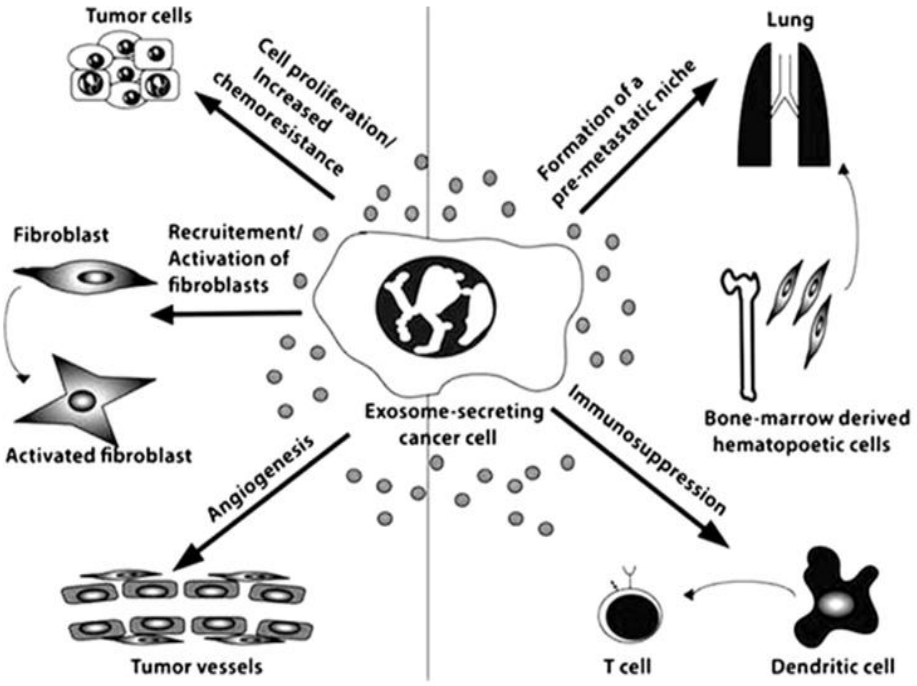
Moreover, exosomes derived from $CD105^{+}$ renal cancer stem cells, but not from $CD105^{+}$ tumour cells, are able to induce lung metastasis disposition when injected into mice (Grange *et al*., 2011); thus, exosomes are implicated in the initiation of cancer and its spread. Mesenchymal stem cells derived exosomes also regulate Wnt signalling pathway, which contributes to breast cancer cell MCF-7 migration (Zhao *et al*., 2013). Importantly, Wnt signaling has been shown to be activated in over 50% of breast cancer patients, which is associated with short overall survival (Lin *et al*.,

2000). Furthermore, exosomes derived from a panel of cancer cells including prostate cancer (DU145, LNCAP and PC3), colorectal cancer (CaCo2) and bladder carcinoma (HT1376) were shown to induce the differentiation of primary fibroblasts AG02262 into myofibroblast phenotypes (Webber *et al*., 2010). Thus, myofibroblasts can produce a variety of growth factors and cytokines that promote tumour progression, which include TGFβ1, HGF, EGF, VEGF, stroma-derived factor-1, basic FGF and the pro-inflammatory cytokines CXCL14, IL-1, IL-6 and IL-8 (Räsänen and Vaheri, 2010; Joyce and Pollard, 2008;). Moreover, myofibroblasts contribute to ECM remodeling and promote cancer cell invasion by producing MMPs such as MMP1, MMP2, MMP3, MMP9, MMP13 and MMP14 (Lu *et al*., 2011). In addition, fibroblast-derived exosomes have been shown to induce Wnt-pathway signalling, leading to increased motility and migration of breast cancer cells in an orthotopic mouse model of breast cancer (Luga *et al*., 2012). This is revealing an intercellular communication pathway whereby fibroblast exosomes activate autocrine Wnt signaling to drive breast cancer invasion.

One of the approaches of communicating between cells is the transfer or the exchange of genetic materials especially mRNAs and miRNAs. For example, glioblastoma-derived exosomes transport mRNA, which is functionally able to translate into protein in recipient cells (Skog *et al*., 2012). Furthermore, exosomes derived from the serum of glioblastoma patients have been shown to contain EGFRvIII mRNA that can induce the proliferation of human glioma cells in autocrine manner (Al-Nedawi *et al*., 2008). In addition, macrophage-derived exosomes carry miRNAs such as miRNA-223, which can be taken up by breast cancer cells and induce breast cancer cell invasion (Yang *et al*., 2011). Furthermore,

exosomes derived from nasopharyngeal carcinoma contain the EBV latent membrane protein 1 (LMP1), and these exosomes are able to induce activation of ERK-1/2 and AKT signalling pathways in the epithelial cells, endothelial cells and fibroblasts (Meckes *et al*., 2010).

Notably, a recent study conducted by Lin *et al* (2016) demonstrated that tumour cells-derived exosomes confer a tumour phenotype to bone marrow-derived Mesenchymal stromal cells (BM-MSCs). In addition, tumour cells-derived exosomes enhance the ability of MSCs to promote macrophage infiltration into B16-F0 melanoma or EL-4 lymphoma (Lin *et al*., 2016). However, ablation of macrophages reversed the tumour promoting effect of MSCs educated by tumour cells-derived exosomes on tumour growth (Lin *et al*., 2016). Figure 1.7 summarises the major effects of tumour-derived exosomes on the tumour microenvironment and distant organs.



Taken from (Kahlert and Kalluri, 2013).

Figure 1.7 ***A Schematic representation to illustrate different roles of cancer cell secreted exosomes on the microenvironment and distant organs.***

1.9 Cancer Fibroblasts Cross Talk

Fibroblasts are derived from a mesenchymal lineage origin and are generally located in the interstitial space or sporadically near capilliaries lacking any connection with a basement membrane, but inserted within fibrillar ECM of interstitium (Kalluri, 2016). In addition, fibroblasts in normal tissue are considered indolent (Kalluri, 2016). Activated fibroblasts (myofibroblasts) were first demonstrated to play a role in the wound healing process (Gabbiani *et al*., 1971), and later in acute and chronic inflammatory conditions (Micallef *et al*., 2012). Carcinoma epithelial cells are in close proximity with fibroblasts and myofibroblasts, which constitute the major components of stroma within the tumour microenvironment (Orimo and Weinberg, 2006). These cells are involved extensively in tumour progression and development (Orimo and Weinberg, 2006). For example, myofibroblasts are involved in tumour angiogenesis because of their ability to modulate the extracellular matrix by production of matrix metalloproteinase (MMPs) such as MMP1, MMP2, MMP3, MMP9, MMP13 and MMP14 (Vong and Kalluri, 2011; Lu *et al*., 2011). Moreover, CXCL14 and CXCL12 chemokines have been shown to be overexpressed and released from myofibroblast and myoepithelial cells respectively into the tumour microenviroment. These chemokines can signal in an autocrine and paracrine manners to regulate normal epithelial and breast carcinoma epithelial cells, leading to their proliferation, migration and invasion (Allinen *et al*., 2004). Moreover, TAFs have been shown to acquire the ability to express alpha-smooth muscle actin (α-SMA), secrete growth factors and MMPs, which can modify the ECM to promote tumour invasion and metastasis (Cirri and Chiarugi, 2012). Moreover, Duda *et al* (2010) demonstrated that metastatic cancer cells can carry activated fibroblasts from

the primary site to the lung. However, partial depletion of associated activated fibroblasts considerably decreases cancer metastases and prolongs survival after primary tumour resection (Duda *et al*., 2010). Several reports demonstrated that the tumour microenviroment is enriched in EVs, particularly exosomes shed from cancer cells, and these exosomes can modulate intercellular communication (Janowska-Wieczorek *et al*., 2006). For example, the interaction between fibroblasts and EVs derived from Lewis lung carcinoma (LL-2) cells signals feedback to tumour cells to activate STAT3, AKT and ERK-1/2 in LL-2 cells (Wyssoczynski and Ratajczak, 2009). Importantly, the melanoma cell-fibroblast interaction is SK1/S1P-dependent signalling, as co-culture of SK1 expressing melanoma cells with fibroblasts induced its transformation to myofibroblasts. Moreover, melanoma cells growth and metastasis were enhanced by co-injection with wild type skin fibroblasts (Albinet *et al*., 2013). However, co-injection with *Sk1*$^{-/-}$ skin fibroblasts showed less growth (Albinet *et al*., 2013), indicating a key role of SK1 in fibroblast transformation and tumour growth.

1.10 Role of Exosomes in Breast Cancer Therapeutic Resistance

Acquired resistance to chemotherapy, radiation and targeted therapy is the major concern and obstacle in treatment of breast cancer (Meads *et al*., 2009). There are three mechanisms of exosome-mediated drug resistance. These are (i) drug export via exosomes (ii) neutralisation of antibody-based drugs, and (iii) exosome mediated miRNAs transfer (Giallombardo *et al*., 2016). In terms of drug export, a number of ABC transporters such as P-glycoprotein (P-gp/ ABCB1), breast cancer resistant protein (BCRP / ABCG2) and multiple drug-resistant protein 1 (MDR1 / MRP1 /

ABCC1) have been shown to be implicated in chemotherapeutic resistance, thereby increasing the efflux of drugs from cancer cells (Sun *et al*., 2012). For example, P-gp expression in MCF-7 breast cancer cells sensitive to docetaxel chemotherapy was increased after incubation with exosomes-derived from MCF-7 resistant cells. This finding suggests that exosomes can transfer P-gp protein or factors that induce its expression in recipient cells and chemoresistance (Lv *et al*., 2014). In terms of antibody resistance, $HER2^{+}$ exosomes released from breast carcinoma were shown to neutralise the activity of Trastuzumab (a monoclonal antibody targeting HER2 used in early and advanced stages of breast cancer) by direct binding to Trastuzumab. This effectively reduces the efficacy of Trastuzumab (Ciravolo *et al*., 2012). In terms of miRNA transfer, exosomes carrying several miRNAs such as miRNA-100, miRNA-222, miRNA-30a and miRNA-17 and derived from doxorubicin-resistant MCF-7 cells have been shown to partially induce chemoresistance in non-resistant MCF-7 cells (Chen *et al*., 2014). Therefore, exosomes can convey resistance to chemotherapeutics by mean of their ability to induce expulsion of drugs taken up by cancer cells and also through delivering proteins that can suppress apoptosis. For example, exosomes released from cancer-associated adipocytes (CAAs) and CAFs express higher levels of miRNA-21 than do ovarian cancer cells, and the exosomal transfer of miRNA-21 from CAAs or CAFs to cancer cells can increase the chemoresistance of cancer cells to paclitaxel through the downregulation of apoptotic protease activating factor 1 (APAF1), which is a known downstream target of miRNA-21 (Au Yeung *et al*., 2016). Notably, APAF1 protein is reported to be associated with apoptosis (Campioni *et al*., 2005).

1.11 The Aims and Objectives

I. To identify whether MDA-MB-231 and MDA-MB-453 breast cancer cells release S1P receptors in exosomes.

II. To determine if exosomes containing S1P receptors released from cancer cells can modulate the function of fibroblasts.

III. To define the specificity of the exosomes carrying S1P receptors and the functional outcome in fibroblasts by comparing S1P receptor sub-types released.

IV. To determine the role of SK1 in regulating the formation of exosomes containing S1P receptors.

V. To determine if there is a functional interaction between SK1 and ceramide kinase in relation to regulating the survival of cancer cells. This objective was 2-fold. First, to establish whether there is a functional interaction between CERK and SK, and if so then the second fold, is to examine whether this might affect exosomes release from breast cancer cells.

CHAPTER II

MATERIALS AND METHODS

CHAPTER 2: Materials and Methods

2.1 Materials

2.1.1 General Reagents

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Company Ltd (UK) unless otherwise stated.

Fisher Scientific (U.K.)

Scintillation cocktail (ScintiSafe 3); Trichloroacetic acid.

GE Healthcare Ltd (Buckinghamshire, U.K.)

Amersham ^{TM} Hybond-ECL Nitrocellulose membrane.

Agfa Healthcare NV (Belgium)

CEA Films (medical x-ray film)

PerkinElmer (U.K.)

Thymidine, [Methyl-^{3}H] (25 Ci/mmol) (lot #NET027L001MC).

2.1.2 Antibodies

AURION, Binnenhaven 56709 PD WAGENINGEN, THE NETHERLANDS

Immunogold goat anti-rabbit of particle size 10 nm, product code 810.011; immunogold goat anti-mouse of particle size 15 nm, product code 815.022.

BD Transduction Laboratories™

Mouse anti-ERK-2 antibody (#610104).

Cayman Chemical (U.K.)

Rabbit polyclonal $S1P_4$ antibody (#13489).

Fisher Scientific (U.K.)

$S1P_2$ Rabbit polyclonal antibody PA5-23208; anti-CD63 (#10628D).

Santa Cruz Biotechnology (California, USA)

Rabbit anti-GAPDH antibody (FL-335): sc-25778; Monoclonal anti-phosphorylated ERK-1/2 (E-4) antibody (#sc-7383); mouse monoclonal HA-probe (#sc-7392); green fluorescent protein GFP (B-2) (sc-9996).

Cell Signalling Technology (USA)

HER2/ErbB2 polyclonal antibody (#2242).

Sigma-Aldrich (Poole, Dorset, U.K.)

Rabbit Polyclonal anti-actin antibody (#A2066); Anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in rabbit (A9044); Anti-Rabbit IgG (whole molecule)–Peroxidase antibody (A0545); Anti-Rabbit IgG (whole molecule)-TRITC produced in goat (#T6778); Anti-Mouse IgG (whole molecule)-TRITC antibody produced in goat (T 5393); Anti-Rabbit IgG (whole molecule)-FITC antibody produced in goat (#F9887); Anti-Mouse IgG (whole molecule)-FITC produced in goat (#F0257).

Vector laboratories (Peterborough, U.K.)

Texas Red® Goat Anti-Rabbit IgG Antibody TI-1000.

2.1.3 Agonist, Antagonist and Inhibitors

Avanti Polar Lipids (Alabaster, USA)

D-*erythro*-sphingosine-1-phosphate (#860641).

Calbiochem (U.K.)

Selective Ceramide Kinase Inhibitor NVP-231 (#219493); Sphingosine Kinase 1 Inhibitor PF543 (#567741).

Cayman Chemical (U.K.)

$S1P_2$ receptor agonist CYM-5520 (#17638), $S1P_4$ agonist CYM 50308 (#14667),

$S1P_2$ and $S1P_4$ antagonist JTE-013 (#10009458).

Enzo (U.K.)

Matrix metalloproteinase (MMP) inhibitor GM-6001.

MERCK (U.K.)

Matrix metalloproteinase (MMP) inhibitor 1, 10-Phenanthroline CAT no 8414910005.

Gifted chemical

Selective SK2 inhibitor (R)-FTY720 methyl ether (ROMe, is a gift from Professor Robert Bittman of City University of New York

2.1.4 Molecular Biology

Addgene (U.K)

pEGFP hsp70 plasmid (#15215); pEF6.mCherry-tsg101 plasmid (#38318).

Becton Dickinson (U.K.)

Bacto-yeast extract, Bacto-tryptone, Bacto-agar.

Dharmacon (U.K.)

Dharma FECT^{TM} 4 Transfection Reagent; Dharma FECT^{TM} 2 Transfection Reagent; ON-TARGET plus SMART pool® S1PR2 siRNA, (Table 2-1).

Table 2-1 $S1P_2$ siRNA nucleotide targeting sequence

S1P2 SMART pool siRNA Targeting sequence
5' UUGCCAAGGUCAAGCUGUA 3'
5' CCAACAAGGUCCAGGAACA 3'
5' GACAAGAGCUGCCGCAUGC 3'
5' GUGACCAUCUUCUCCAUCA 3'

Invitrogen (Paisley, UK)

Lipofectamine ^{TM} 2000.

QIAGEN (Manchester, UK)

Scrambled siRNA; EndoFree Plasmid Maxi Kit Cat No ID: 12362.

UMR cDNA Resource Centre (Bloomsburg University, USA)

Endothelial differentiation, G-protein couples receptor $S1P_2$ 3x-HAtagged (N-terminus) (#EDG050TN00), Endothelial differentiation G-protein coupled receptor $S1P_4$ 3xHA-tagged (N-terminus) (#EDG060TN00).

Life Technologies, Paisley

Empty DNA constructs (pcDNA3.1).

2.1.5 Cell Culture Reagents

Invitrogen (Paisley, UK)

Dulbecco's modified Eagle's medium (DMEM 1 x glutamax); Opti-MEM® low serum media; Penicillin (10,000 units/ml) and Streptomycin (10,000µg/ml) (Pen-Strep); L-glutamine and trypsin/EDTA solution.

Seralab (West Sussex, United Kingdom)

European foetal calf serum (EFCS).

American Type Culture Collection (ATCC)

MDA-MB-453 breast cancer cells; MDA-MB-231 breast cancer cells.

Gifted cells

Mouse embryonic fibroblast (MEF) was a gift from Professor Gabor Tigyi, University of Tennessee.

2.2 Methods

2.2.1 Cell Culture

All cell culture work was performed in a cell culture hood under aseptic conditions. All cells were grown in 75 cm^2 flasks, unless otherwise stated. All cells were routinely maintained in humidified conditions at 37 °C in 95% air and 5% CO_2.

2.2.1.1 Maintenance of MDA-MB-453, MDA-MB-231 and Mouse Embryonic Fibroblasts (MEF) Cells

Cells were grown in high glucose Dulbecco's modified Eagle's media (DMEM) 1 x glutamax containing 10 % v/v European foetal calf serum (EFCS) and 1% v/v Penicillin and streptomycin (Pen-Strep) (complete media). When cells reached 90% confluency, they were washed once with serum free medium then, detached from the flask surface by incubating with 1 ml trypsin/EDTA solution for 2-3 mins. 8 ml of complete medium containing 10% v/v (EFCS) was added to the cell/trypsin mixture and the cell suspension was transferred to a new flask containing complete medium in a ratio (1:9) for MDA-MB-231; (2:8) for MDA-MB-453 and (1:9) for MEF cells.

2.2.1.2 Cell Culture for Experiment

Cells were grown in either 12 wells plate (1 ml/well) or 6 wells plate (2 ml/well) or 24 wells plate (0.5 ml/well) or 75 cm^2 flasks (10 ml/flask) depending on experimental nature and plan and the details of the experiment is fully described either in experiment explanation or in the legend of experiment.

2.2.2 Cell Transfection

2.2.2.1 Maxi-Prep DNA Plasmid Preparation

Three colonies of either HA-$S1P_2$ or HA-$S1P_4$ or pEGFP-hsp70 or pEF6 mCherry-tsg101 plasmid DNA were selected from LB agar plate (up to 3 weeks old if stored at 4°C) using a sterile loop under aseptic conditions. These colonies were added to a 30 ml sterile universal tube with 10 ml of LB Broth containing 10 µl of (100 mg/ml) Ampicillin for HA-$S1P_2$, HA$S1P_4$ and mCherry-tsg101 or 10 µl of (100 mg/ml) Kanamycin for EGFP-hsp70. The tube was placed horizontally in a shaking incubator at 37°C overnight at 250 revolutions per minute (rpm). After that, the incubated tube was transferred into 500 ml conical flask containing 100 ml of autoclaved LB broth including either 100 µl of Ampicillin (100 mg/ml) or 100 µl of Kanamycin according to the plasmid selected. The flask was placed vertically in a shaking incubator at 37°C overnight. The next day, 0.8 ml of this overnight bacterial culture was added to 0.2 ml of sterile glycerol into a sterile micro-centrifuge tube. The tube was vortexed and stored immediately at -80°C for long-term storage. The remaining bacterial culture was divided into two sterile 50 ml tubes and centrifuged at 3000 g for 10 mins at 4 °C. The supernatant was discarded and the air-dried pellets

were used for DNA extraction and purification or stored at -20 °C for future work. Purification of DNA from bacterial pellets was performed using Qiagen Endo Free Plasmid Maxi Kit containing 10 QIAGEN filter and cap. The idea of extraction and purification of DNA from pellets using this kit is based on the selectivity of QIAGEN resin to allow anion exchange between DNA and resin. Pellets containing DNA were first exposed to alkaline lysis buffer followed by binding DNA to resin under low salt and pH conditions. Other protein impurities like RNA and low molecular weight impurities were eluted with medium salt wash, while plasmid DNA was eluted in high salt buffer then concentrated, desalted and precipitated using sterile isopropanol. The resulting isopropanol precipitate was dissolved in 70% sterile ethanol to replace isopropanol (ethanol more volatile) then pellets were air-dried before being dissolved in 250 µl of endotoxin-free alkaline TE buffer.

2.2.2.2 Quantification of Nucleic Acids

The DNA concentration and purity was measured by using spectrophotometer (Nano Drop 1000 Thermo Scientific) according to the manufacturer's protocol. Briefly, DNA samples 2 µl were loaded on platform of the spectrophotometer and absorbance reading taken at 260 nm, which was used to calculate DNA concentration of DNA samples which found 2013 ng/µl for HA-S1P_2, 1954 ng/µl for HA-S1P_4, 3098 ng/µl for mCherry-tsg101and 2682.6 ng/µl for GFP-hsp70. The ratio between absorbance at 260 nm and 280 nm reflects the purity of nucleic acid and all ratios of indicated concentration for plasmid DNA were above 1.8, which reflects good DNA purity.

2.2.2.3 Transient Transfection of Plasmid DNA

Either MDA-MB-231 or MDA-MB-453 breast cancer cells were seeded in 75 cm^2 flasks at a density of 3 x 10^6 cells/ml in complete medium. Cells were incubated until 70% confluence, attained on the day of transfection. For each flask of the cells to be transfected, 10μg of desired DNA plasmid and 10 μl of Lipofectamine ^{TM} 2000 were diluted independently in 500 μl of Opti-MEM media and incubated for 5 mins at room temperature before being mixed and incubated for further 20 mins to allow the formation of the DNA–transfection reagent complex. Whilst waiting for complex formation, medium was aspirated and replaced with 9 ml Opti-MEM media. This was completed to 10 ml by slow addition of 1 ml transfection complex mix, and the flask was gently tilted to ensure a uniform distribution of the complex mix. Cells were incubated for 24 hrs. After that, Opti-MEM/transfection medium was aspirated, and cells were washed twice with 10 ml of fresh Opti-MEM, which was wash aspirated and replaced with 10 ml of fresh Opti-MEM medium for 24 hrs before the addition of agonist/antagonist or enzyme inhibitors for a further 24 hrs. Thereafter, cells were incubated with the final 10 ml of Opti-MEM for 48 hrs (the time required to prepare conditioned media see section (2.5.1). Conditioned media (CM) was used for either protein precipitation using trichloroacetic acid (2.5.2) or exosome isolation and purification (2.6.1). Cells producing conditioned media were centrifuged and lysed in SDS-PAGE lysis buffer, for details see section (2.5.1) and stored at -20 °C for future Western blot analysis (2.3).

2.2.2.4 Transfection of Small Inhibitory RNA (siRNA)

2.2.2.4.1 Transfection of MEF Cells with siRNA

(MEF) cells were grown on 12 wells plates in complete medium until 60-70% confluence was attained at day of transfection. For each well, either scrambled siRNA and/or S1P2 siRNA oligonucleotide targeting 4 sequence sites (table 2-1) was diluted in 100 µl of serum and antibiotic free medium and mixed gently (final concentration of siRNA when added to cells was 200 nM). In the same time 3.5 µl of the transfection reagent, DharmaFECT 2 was diluted to final volume 50 µl with serum and antibiotic free medium and mixed gently. The diluted scrambled siRNA and S1P2 siRNA were incubated for 5 mins at room temperature before being combined with the DharmaFECT 2 andmixed gently and the combined mixture was incubated for further 20 mins at room temperature. During this time, media was removed from the well to be transfected and replaced with 850 µl/ well of fresh DMEM medium containing 10% EFCS but without antibiotics. After completion of 20 mins incubation the mix was added gently to the cells, as required, and carefully mixed. Cells were incubated between 15-24 hrs then transfection mixture was removed and replaced with fresh quiescent media (OptiMEM) for 24 hrs before cells being treated with either agonist or antagonist or non-conditioned medium (NCM) or CM according to experiment plan. Ultimately, cells were harvested, lysed in SDS-PAGE lysis buffer and successful knockdown was assessed by Western blot analysis.

2.2.2.4.2 Transfection of MDA-MB-231 Cells with siRNA

For MDA-MB-231 cells, at least three 75 cm^2 flasks were prepared for each experiment. Cells at 60-70 % confluence were attained at the day of transfection. Either scrambled siRNA or $S1P_2$ siRNA oligonucleotide targeting sequences sites (table 2-1) was diluted in 150 µl of DMEM media free from EFCS and antibiotics (final concentration of siRNA when added to the cells was 200 nM). In the same time 5 µl of transfection reagent DharmaFECT 4 was diluted with DMEM media free from EFCS and antibiotics to final volume 50 µl. The diluted samples were kept at room temperature for 5 mins before being combined and mixed gently. The scrambled siRNA and $S1P_2$ siRNA mix were left for further 20 mins at room temperature to ensure formation of transfection reagent/ siRNA complex. During waiting for complex formation MDA-MB-231 medium was replaced with DMEM media containing 10% v/v EFCS without antibiotics. After, completion of 20 mins the siRNA/ transfection reagent complex was added slowly to the MDA-MB-231 cells (final concentration is 200 nM of either scrambled siRNA or S1P2 siRNA), and cells were incubated for 18-24 hrs. After that the medium was removed and replaced with fresh OpiMEM media (2 x 10 ml wash) and incubated with a further 10 ml OptiMEM, for 48 hrs before CM see (2.5.1). Cells that produced CM were washed with phosphate buffer saline (PBS) 2 x 5 ml wash, scraped into 10 ml PBS, centrifuged, the supernatant discarded and cell pellets were re-suspended in 400 µl of SDS-PAGE sample buffer (see 2.3.1), homogenized using 23-gauge syringe and 1 ml syringe (10 times at least). The cell lysate was stored at -20 °C prior to western blot analysis.

2.2.3 Immunofluorescence Confocal Microscopy

Either MEF or MDA-MB-231 cells were seeded at density of 50 $x10^4$ cells/ ml with complete medium in 12 wells plate that contained autoclaved 13 mm coverslips. Cells were incubated until approximately 60-70% confluence was achieved. For MEF cells, they were quiescent for 24 hrs before been treated with either NCM or MDA-MB-231 cells cultured CM (231CM) or cultured CM from MDA-MB-231 cells that had been transiently transfected with GFP-hsp70 plasmid (an exosomal marker) construct or treated with CYM-5520 ($S1P_2$ selective agonist) according to the experiment plan (see figure legends). Cells were washed with cold PBS then fixed with 3.7% (v/v) formaldehyde in PBS at room temperature for 10 mins. MDA-MB-231 cells were grown on autoclaved coverslips until approximately 60-70% confluence was achieved and then processed for immunofluorescence confocal microscopy.

2.2.3.1 Preparation of Adhered cells on Coverslips to Immunofluorescence

Cells were fixed after 10 mins incubation with 3.7% formaldehyde in PBS solution (1 ml/well). The fixed cells were washed twice with cold PBS and, then permeabilised with 0.1% Triton X-100 in PBS solution (1 ml/well) for 2 mins followed by washes with cold PBS. Non-specific binding of antibodies was blocked using blocking solution (2.5 ml EFCS, 0.5 g BSA and 47.5 ml PBS) for 30 mins. During this time, the primary antibody solution was prepared by diluting it in a ratio of 1:100 with blocking solution. 50 µl of primary antibody solution per coverslip was

dropped on clean paraffin film and coverslips were inverted (cells facing down) onto this solution, then left to incubate for 1 hr at room temperature. Whilst waiting for primary antibody conjugation, rabbit or mouse secondary antibody conjugated to either Tetramethylrhodamine (TRITC) with orange fluorescence or Fluorescein isothiocyanate (FITC) with green fluorescence or Texas red with red fluorescence was prepared by dilution with blocking solution in ratio (1:100). After completion of 1 hr incubation with primary antibody, each coverslip was washed three times with 4°C PBS then incubated with 50 µl of secondary antibody solution in the same way. Coverslip were kept away from direct light as TRITC, FITC, GFP and Texas Red are photosensitive. After completion of secondary antibody incubation, each coverslip was washed three times with 4°C PBS and excess PBS was removed by carefully dabbing the edge of coverslip on absorbent tissue. Each coverslip was inverted (cells facing down) onto 10 µl of VECTASHIELD® Hard Set™ Mounting Medium with DAPI on the labelled glass slide. Glass slides were stored at 4 °C or directly examined using an oil immersion lens of a confocal microscope (Leica TCS SP5).

2.3 SDS-PAGE and Western Blotting Analysis

2.3.1 Cell Lysate Preparation

Cells were lysed in sodium dodecylsulphate-polyacrylamide gel electrophoresis SDS-PAGE sample buffer (Laemmli buffer) consisting of sodium dodecyl sulfate (0.5% w/v) as a detergent in buffer containing tris (hydroxymethyl) aminomethane (125 mM; pH 6.7), ethylenediaminetetraacetic acid (1.25 mM), sodium pyrophosphate (0.5 mM), dithiothreitol (50 mM), bromophenol blue (0.06% w/v) and glycerol (1.25% v/v).

2.3.2 Preparation of Polyacrylamide Gels

Every polyacrylamide gel used for resolving proteins by electrophoresis consists of two layers, the separating gel or resolving gel (lower layer), in which the resolution of proteins by electrophoresis take place, and the stacking gel (upper layer), which holds the samples and allows the proteins to concentrate before crossing into the separating layer. The separating gel was made by 10% (v/v) acrylamide, 0.375 M Tris-Base (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.025% (w/v) tetramethylethylenediamine (TEMED), whilst, the stacking gel was made by 4.5% (v/v) acrylamide-bis-acrylamide, 0.125 M Tris-Base (pH 6.7), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.1% (v/v) of TEMED.

2.3.3 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out using the Bio-Rad Mini-Protean II electrophoresis kit. Samples were loaded into the gel using a Hamilton syringe, for example, 50 µl of protein precipitate was loaded onto 1.5 mm thick separating gel using 10 well combs, 20 µl of cells lysate loaded onto 1 mm separating gel using 10 well combs. Pre-stained molecular weight markers, whose molecular weight is known, table 2-2, were also loaded into the gel in order to identify the band corresponding to the protein of interest. Electrophoresis was carried out using a buffer containing 25 mM Tris-Base, 0.21 M glycine and 0.1% (w/v) SDS, at a voltage of 120 V and a current limit of 1.0 mA for about 2 hrs.

2.3.4 Proteins Transfer into Nitrocellulose Membranes

The electrophoretic transfer of the resolved proteins from the gel into a nitrocellulose membrane was carried out using a Bio-Rad Mini Trans-Blot kit filled with a buffer containing 25 mM Tris-Base, 0.21 M glycine and 20% (v/v) methanol. The transfer occurs by applying a voltage of 100 V with a current limit of 0.6 mA for 1 hr.

2.3.5 Western Blotting

Before the nitrocellulose membranes were incubated with the antibody specific for the protein of interest, membranes were incubated for 1 hr at room temperature in a blocking solution, in order to reduce the antibody binding to non-specific sites. The blocking solution consisted of 3% (w/v) non-fat dry milk in Tris buffered saline with Tween-20 (TBST) buffer [10 mM Tris-Base, 100 mM NaCl, 0.1% (v/v) Tween-20; pH 7.4] or 3 % (w/v) bovine serum albumin (BSA) in TBST buffer, depending on the primary antibody to be used. After blocking, membranes were incubated overnight with gentle agitation at 4°C with the primary antibody specific for the detection of the protein of interest, diluted in 1% (w/v) (BSA) in TBST buffer.

Incubation with primary antibody was followed by 3 washes (5 mins each) of the membranes in TBST buffer to remove any unbound antibody followed by incubation for 1 hr at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG or anti-rabbit IgG, depending on the primary antibody origin) diluted 1: 80,000 in 1% (w/v) non-fat dry milk in TBST buffer. Excess antibody was then removed by 3 washes in TBST buffer (5 mins each), before incubating the membranes in the enhanced chemi-luminescence (ECL)

reagent, which provides the substrate for the peroxidase, thus allowing the detection of immunoreactive proteins. The ECL reagent was freshly prepared by mixing equal volumes of ECL-solution 1 (containing 0.04% (w/v) luminol, 0.1 M Tris-Base (pH 8.5) and 0.016% (w/v) *p*-coumaric acid) and ECL-solution 2 (containing 2% (v/v) hydrogen peroxide (H_2O_2) and 0.1 M Tris-Base (pH 8.5). After 2 minutes incubation at room temperature, membranes were dried from the excess of ECL reagent, placed between two transparent plastic sheets in an autoradiography cassette and exposed to an X-ray film, which was then developed by passing it through an X-Omat machine. The exposure time was varied depending on the intensity of the chemiluminescence signal. Immunoreactive proteins appeared on the film as dark bands.

2.3.6 Determination of Molecular Weight of Proteins from SDS-PAGE

The molecular weight of immunoreactive proteins was assessed by comparing their movement on SDS-PAGE to pre-stained SDS-PAGE molecular weight marker standards.

Table 2-2 Pre-stained SDS-PAGE molecular weight standards

Standard	Source	Molecular weight
α2-macroglobulin	Equine serum	180
B-galactosidase	E.coli	116
Lactoferrin	Human milk	90
Pyruvate kinase	Rabbit muscle	58
Fumarase	Porcine heart	48.5
Lactic dehydrogenase	Rabbit muscle	36.5
Triosephosphate isomerase	Rabbit muscle	26.6

2.3.7 Stripping and Re-probing of Nitrocellulose Membrane

Membranes can be stripped to remove bound antibodies before being re-probed with different antibodies, in order to detect other proteins. Stripping of the membranes was carried out by incubating them in a buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% (w/v) SDS and 100 mM β-mercaptoethanol for 1 hr at 70°C with gentle agitation. After that, membranes were rinsed with distilled water and washed with TBST buffer (3 washes, 10 mins each) with mild agitation before incubating them overnight with the primary antibody specific for the protein of interest.

2.3.8 Densitometry Analysis

In order to quantity protein intensities of specific bands of protein of interest, densitiometric analysis was performed using Image J software (Schneider *et al*. 2012) and the data obtained was exported to either Excel or Graph Prism software to

perform statistical analysis, generating curves or bar graphs according to the nature of the experiment.

2.4 [^{3}H] Thymidine Incorporation Assay

Cells were seeded into 24 wells plate in complete medium and incubated until they reached 75-80% confluence. After that, the medium was aspirated and replaced by DMEM media containing 0.1% EFCS (quiescing medium), and cells incubated for a further 24 hrs. Subsequently, cells were treated with either agonist or antagonist or inhibitor (as indicated in the figure legends) and incubated for 24 hrs. [^{3}H] thymidine (0.25 uCi = 9.25 kBq) for each well was added 5 hrs before the termination of 24 hrs. Incubations were terminated by aspirating the medium; each well was washed with 1 ml of ice cold PBS once, followed by the addition of 1 ml ice cold 10% (w/v) trichloroacetic acid (TCA) to precipitate nuclear contents for 10 mins. Then the 10% TCA was aspirated, and this step was repeated three times. After that, 0.25 ml of 0.1% (w/v) SDS and 0.3 M NaOH mixture was added to each well in order to dissolve the nuclear content of cells. Samples were transferred to separate beta vials followed by addition of scintillation liquid (2 ml each) and mixed thoroughly until clear. Radioactivity incorporated into newly synthesised DNA was quantified using a scintillation counter. Values of disintegrations per minute (dpm) were used to calculate (mean+/- SEM) for each sample and were subjected to statistical analysis, which indicated in the figure legends of experiment.

2.5 Protein Detection in Conditioned Medium

2.5.1 Preparing Conditioned Medium

Either MDA-MB-231 or MDA-MB-453 breast cancer cells were seeded in a 75 cm^2 flask at density of 3 $x10^6$ /ml/flask, and cells were incubated in complete medium until 75% confluence was reached. Then the medium was aspirated and the monolayer adhered cells were gently washed twice with 10 ml of fresh Opti-MEM media, which was replaced finally with 10 ml of fresh Opti-MEM media per flask, and cells were incubated for a further 48 hrs before collection of the medium.

For experiments including treatments with agonist/antagonist or enzyme inhibitors, treatments were performed in the final 24 hrs before media collection. After that cultured conditioned media was collected and centrifuged at 1000 rpm for 5 mins to pellet any dead cells and fragments. The supernatant was collected carefully and filtered through 0.22 μm size filter membrane (Merck Millipore). The filtrate represents clear unturbid (CM).

The monolayer adhered cells that produce the CM of either MDA-MB-231 or MDA-MB-453 cells were washed twice with sterile cold PBS, scraped in 10 ml ice cold PBS, centrifuged and cell pellets were re-suspended in SDS-PAGE lysis buffer without DTT to form homogenous cell lysate (CL) and stored in -20 °C for future Western blot analysis.

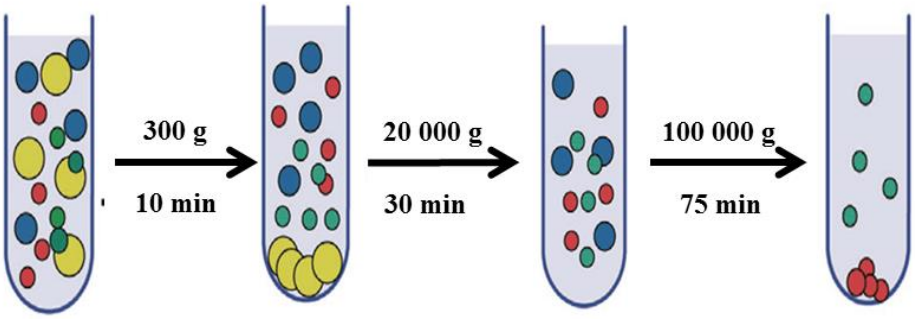
2.5.2 Protein Precipitation by Trichloroacetic Acid Method

CM prepared from step (2.5.1) was incubated with 6 M trichloroacetic acid (TCA) in ratio of 125 μl of (TCA) per 1 ml of CM; the mixture was shaken well and incubated

on ice for a minimum of 2 hrs. Following incubation, the mixture was transferred into clean (ultra-clear) centrifuge tubes (Beckman, Z50901SCA) and centrifuged at 11300 rpm (16125g), 4 °C for 10 mins using the SW40 Ti rotor in a Beckman-Coulter ultracentrifuge XL-100K. The supernatant was discarded and pellets (PPT) were re-suspended in 40 µl of 9 M urea and 50mM Tris (pH 7.5) mixture.5 µl of SDS-PAGE lysis buffer was added to the mixture and 2-5 µl of 2 M Tris base solution was added to neutralise excess acid until the blue colour of SDS-PAGE lysis buffer sustained. Finally, samples were stored at -20 °C for future Western blot analysis.

2.6 Exosome Isolation and Purification by Differential Centrifugation

Differential centrifugation is a commonly employed technique and is the most common method to isolate extracellular vesicles (EVs), and is widely used to isolate them from body fluids and conditioned media. Differential centrifugation comprises sequential centrifugation steps with increasing centrifugation force or speed aimed to isolate smaller particles from heterogeneous mixture of different sized particles (figure 2.1).



Taken and modified from Momen-Heravi *et al*, (2013).

Figure 2.1 ***A Schematic representation of general sequential centrifugation steps involved in exosomes isolation from body fluids and conditioned media***

Red spheres represent exosomes and other spheres represent different EVs.

2.6.1 Exosomes Isolation from MDA-MB-231 Cells Conditioned Medium

The conditioned medium filtrate prepared from a minimum of three 75 cm^2 flasks (section 2.5.1) was transferred into clean ultracentrifuge tubes and centrifuged at 20.000g (12585 rpm) using the SW40 Ti rotor in a Beckman-Coulter ultracentrifuge XL-100K at 4°C for 30 mins to remove remaining cell debris.

The supernatant was transferred into clean ultracentrifuge tubes and centrifuged at 100.000g (24000 rpm) at 4 °C for 75 mins usingthe SW40 Ti rotor in a Beckman-Coulter ultracentrifuge XL-100K. The supernatant was discarded and the exosome pellet from each tube re-suspended in1 ml sterile 4 °C PBS before being transferred into one clean ultracentrifuge tube. Another round of ultracentrifugation at 100.000g (24000 rpm) at 4 °C for 75 mins using the SW40 Ti rotor in a Beckman-Coulter ultracentrifuge XL-100K was performed to further purify the exosomes and ensure

removal of all contaminants and unwanted larger particles. Finally, the pellet was re-suspended in 250 µl sterile filtered PBS and stored at -80 °C for future work.

2.6.2 Analysis of Exosomes by Immunoblotting

Isolated exosome pellets from MDA-MB-231 cells were re-suspended in 4x non-reducing SDS-PAGE sample buffer, (i.e., without dithiothreitol (DTT) 50 mM) in appropriate ratio (1:3) (i.e. one volume of 4 x non-reducing sample buffer + 3 volumes of PBS exosome pellets). MDA-MB-231 cells in which the exosomes were produced were also harvested and lysed in SDS-PAGE lysis buffer containing no DTT (DTT affect CD63 detection by Western blot)

Both samples were loaded onto 10% polyacrylamide gel and subjected to electrophoresis, protein transfer into nitrocellulose membrane followed by Western blot analysis and immunoblot with specific exosomal marker antibodies.

2.6.3 Transmission Electron Microscope Analysis of Exosomes

2.6.3.1 Characterisation of Exosome proteins by Immuno-gold Labelling

Concentrated frozen exosome pellets isolated from MDA-MB-231 cells were re-suspended in equal volume of 4% paraformaldehyde/PBS in a 1:1 ratio to generate exosomes/2% formaldehyde solution, which can be stored at 4 °C up to one week. However, 5 µl droplets of 2% exosomes/PFA solution were placed onto Carbon coated/Poly-lysine coated Nickle mesh grids, which were previously Glow

Discharged (this makes surfaces hydrophilic so samples spread evenly) for 30minutes making sure they did not dry out. Grids with attached exosomes were passed sample side down over PBS droplets 6 times for 1 min each to remove Paraformaldehyde fixative, followed by 3 times for 5 mins washes on droplets of 0.05 M Glycine/PBS (blocks Aldehyde groups). Grids were then passed over droplets of 3% BSA/PBS blocking solution 3 times for 5 mins then onto Primary Antibodies as follows: 2 grids on anti-CD63 antibody (diluted in ratio 1/100), 2 grids on anti-SIP_2 antibody (diluted in ratio 1/100) and 2 grids without antibody (only blocking solution). The samples were left for 1 hr at room temperature. After that, sample grids were washed by passing over droplets of 3% BSA/PBS blocking solution to remove excess antibody 6 times for 5 mins each then placed onto droplets of secondary gold-labelled antibodies. CD63 grids were processed with gold (15 nm in size) labelled goat anti-mouse IgG (diluted in ratio 1/20), SIP_2 grids were processed using /goat (10 nm in size) labelled anti-rabbit IgG (diluted in ratio 1/20) and blocking buffer only grids were processed with both gold reagents (both anti-mouse and anti-rabbit). All the grids were left for 1 hr at room temperature. After 1 hr incubation, the grids were washed 6 times for 5 minutes with droplets of blocking solution (3% BSA/PBS) followed by 6 times wash with PBS for 5 mins each then grids were transferred to be fixed on droplets of 1% Glutaraldehyde/PBS solution for 5 mins followed by 8 times washes for 5 mins each with distilled water. The contrasting and embedding of the exosome samples followed the methods of Thery *et al*. (2006). Grids were placed onto droplets of Uranyl-Oxalate solution pH 7 for 10 mins at room temperature then transferred onto droplets of Methyl Cellulose- Uranyl Acetate (on ice) for 10 mins. After that, grids were picked up from droplets with

platinum loops and excess methyl cellulose stain gently removed with filter paper (edge of grid) to leave a thin embedding layer. Then, grids were left overnight to dry and picked off loop the next day, storing in grid box until ready to view on the transmission electron microscope (TEM). Grids were examined by FEI Tecnai T20 TEM running at 200Kv and images were captured using Gatan Multiscan 794 camera.

2.7 Statistical Analysis

Experiments were repeated at least three times with consistent results and all data is stated as mean +/- standard error of the mean (SEM). The difference between groups were analysed using one-way ANOVA test and t-test and the difference was considered significance when $^{*}p < 0.05$.

CHAPTER III

IDENTIFICATION OF ENDOGENOUS $S1P_2$ RECEPTOR IN EXOSOMES DERIVED FROM MDA-MB-231 BREAST CANCER CELLS

CHAPTER 3: Identification of Endogenous $S1P_2$ Receptor in Exosomes Derived from MDA-MB-231 Breast Cancer Cells

3.1 Introduction

Sphingosine kinases SK1/2 are upregulated in ER^+ breast cancer cells and this is associated with increased growth, proliferation and poor prognosis in ER^+ breast cancer patients (Sukocheva *et al*., 2009; Ruckhäberle *et al*., 2008). S1P is generated and released from cells via specific transporters in the plasma membrane in close proximity to a family of GPCRs termed $S1PR_{1-5}$, commencing different cellular activity such as proliferation and migration (Pyne and Pyne, 2010). The $S1P_2$ receptor is reported to couple to multiple G-proteins such as $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ proteins that regulate phospholipase C, Rho, Rho-dependent kinase and extracellular signal-regulated kinase (ERK-1/2) (Skoura and Hla, 2009; Adada *et al*., 2013). However, the role of $S1P_2$ in cancer is controversial; for example, the $S1P_2$ receptor has been shown to promote tumourigenesis indicated by increased proliferation and inhibition of apoptosis upon co-transfection of $S1P_2$ and $S1P_3$ in hepatoma and Jurkat cells (An *et al*., 2000). Moreover, S1P has been shown to bind to $S1P_2$ and prevents induction of the metastasis suppressor brms1 (breast carcinoma metastasis suppressor1) (Ponnusamy *et al*., 2012). In contrast, others have demonstrated that $S1P_2$ has an anti-migratory effect mediated through $G\alpha_{12/13}$ and Rho-dependent kinase inhibitory of S1P effect on Akt, Rac and cell migration, thereby negatively regulating tumour angiogenesis and tumour growth. For instance, Lewis lung carcinoma or B16

melanoma cells implanted in $S1P_2$-deficient ($S1P_2^{-/-}$) mice exhibited enhanced tumour growth and angiogenesis (Du *et al*., 2010; Lepley *et al*., 2005; Van Brocklyn, 2010).

Moreover, MDA-MB-231 cells have been shown to express $S1P_2$ and $S1P_4$ and using SK2 inhibitor (*R*)-FTY720 methyl ether (ROMe) or siRNA knockdown of SK2 or $S1P_4$ antagonist or siRNA knockdown of $S1P_4$ receptor has been shown to induce $S1P_2$ translocation to the nucleus promoting MDA-MB-231 cells arrest (Ohotski *et al*., 2014).

Exosomes are nanosized (30-150 nm) vesicles of an endocytic origin and have been shown to participate in cancer proliferation, growth and metastasis (Green *et al*., 2015). In this regard, exosomes have emerged as a mean of intercellular communication carrying active protein molecules and receptors such as GPCRs, DNA and RNA, which can mediate phenotypic change and oncogenic transformation of recipient cells in the tumour microenvironment. In addition, exosomes have been shown to confer therapeutic drug resistance in cancer cells (Zhang *et al*., 2015; Green *et al*., 2015; Villagrasa *et al*., 2014). Genetic studies in yeast have revealed the importance of sphingolipid and enzymes involved in sphingolipid metabolites in endocytosis (Hannich *et al*., 2011), and mutation in neural ceramidase and sphingosine kinase resulted in a synaptic vesicle recycling defect at the neuromuscular junction in *Drosophila melanogaster* (Rohrbough *et al*., 2004). Also, Amorim *et al* (2014) demonstrated through proteomic study that S1P receptors are releasd in EVs from C5.2 cells. For instance, $S1P_3$ receptors were found in a 20 K (20,000 × g = 20K) set of EVs, while $S1P_4$ receptors in 100 K (100,000 × g =100K)

set of EVs which exhibit similar features of exosomes morphology (Amorim *et al*., 2014). Moreover, SK1-GFP was found enriched on Rab5-positive early endosomes and early endocytic intermediates, suggesting a role of sphingolipid phosphorylation in initiation of endocytosis (Shen *et al*., 2014).

Furthermore, Kajimoto *et al* (2013) demonstrated that the inhibitory G-proteins (G_i) coupled to S1P receptors are involved in MVBs maturation and protein sorting into ILVs, mediated by a continuous supply of S1P produced by SKs. Therefore, the possibility of the presence of S1P receptors in exosomes is potential.

The aim of this chapter is to investigate the presence or absence of S1P receptors in CM derived from MDA-MB-231 and MDA-MB-453 breast cancer cells and whether they are released in EVs.

3.2 Results

3.2.1 The Shedding of $S1P_2$ Receptor from MDA-MB-231 and $S1P_4$ Receptor from MDA-MB-453 Breast Cancer Cells

It has previously been shown that MDA-MB-231 cells express $S1P_2$ receptors and MDA-MB-453 cells express $S1P_4$ receptors (Long *et al*., 2010). Therefore, these cell types were used to investigate whether these receptors are released in exosomes. The conditioned medium (CM) from MDA-MB-231 cells was found to contain the $S1P_2$ receptor, which was detected as two immunoreactive proteins using anti-$S1P_2$ antibody with a molecular mass of (~ 42kDa). The $S1P_4$ receptor was also detected as a 42 kDa protein using anti-$S1P_4$ antibody in CM isolated from MDA-MB-453

cells. The $S1P_4$ receptor was detected as two proteins with a molecular mass of 42-44 kDa in MDA-MB-453 cell lysates (figure 3.1), suggesting that only one of these species is released into CM. These findings, suggests that these receptors are shed in exosomes/ extracellular vesicles into CM.

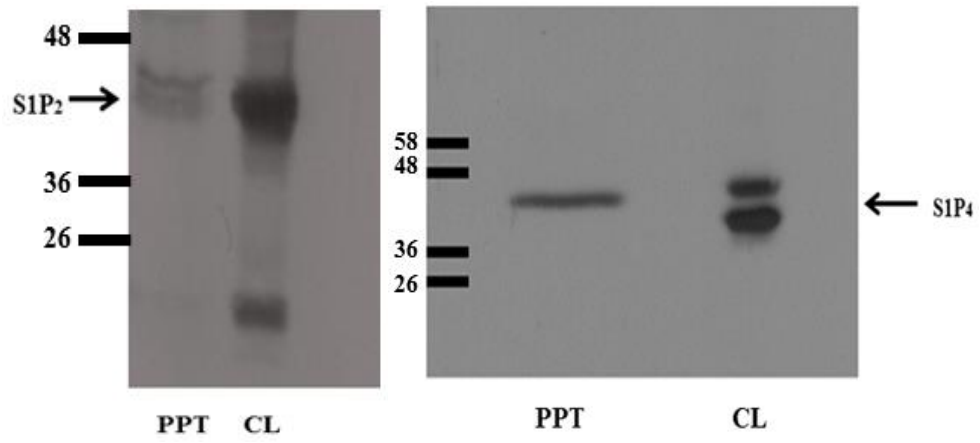


Figure 3.1 ***Shedding of endogenous $S1P_2$ receptor from MDA-MB-231 and $S1P_4$ receptor from MDA-MB-453 breast cancer cells to conditioned medium***.

The conditioned medium (CM) of MDA-MB-231 breast cancer and MDA-MB-453 was collected after 48 hrs incubation. CM from 3 T75 flasks was precipitated and the entire protein content loaded onto SDS gels. For cell lysate, each T75 flask was lysed in 600 ul lysis buffer and 20 ul loaded onto the SDS gel. Proteins in CM were acid precipitated (PPT) and mixed with Laemmli buffer. Cells lysates were also produced in Laemmli buffer (CL). PPT and CL were analysed by Western blot. $S1P_2$ receptor was assessed by Western blot analysis using anti-$S1P_2$ and anti-$S1P_4$ antibodies. Results are typical of three independent experiments.

3.2.2 Transient Transfection of MDA-MB-231 Breast Cancer Cells with HA Tagged $S1P_2$, GFP-hsp70 and mCherry-tsg101 Plasmids Constructs

Tsg101 and hsp70 have been identified in exosomes from different cell types and are markers of exosomes (van Niel *et al*., 2006; Mathivanan *et al*., 2010). Therefore, in order to establish whether these markers are present in CM from MDA-MB-231 and MDA-MB-453 cells, these cells were transiently transfected with plasmid constructs encoding GFP-hsp70 or mCherry-tsg101 or HA-$S1P_2$. Cells were also transfected with empty vector as a control. HA-$S1P_2$ was also detected in CL and CM from MDA-MB-231 cells over-expressing HA-$S1P_2$ receptor (figure 3.2). The exosome marker proteins were also detected in lysate (figure 3.3) and, CM (figure 3.4) from MDA-MB-231 and MDA-MB-453 cells.

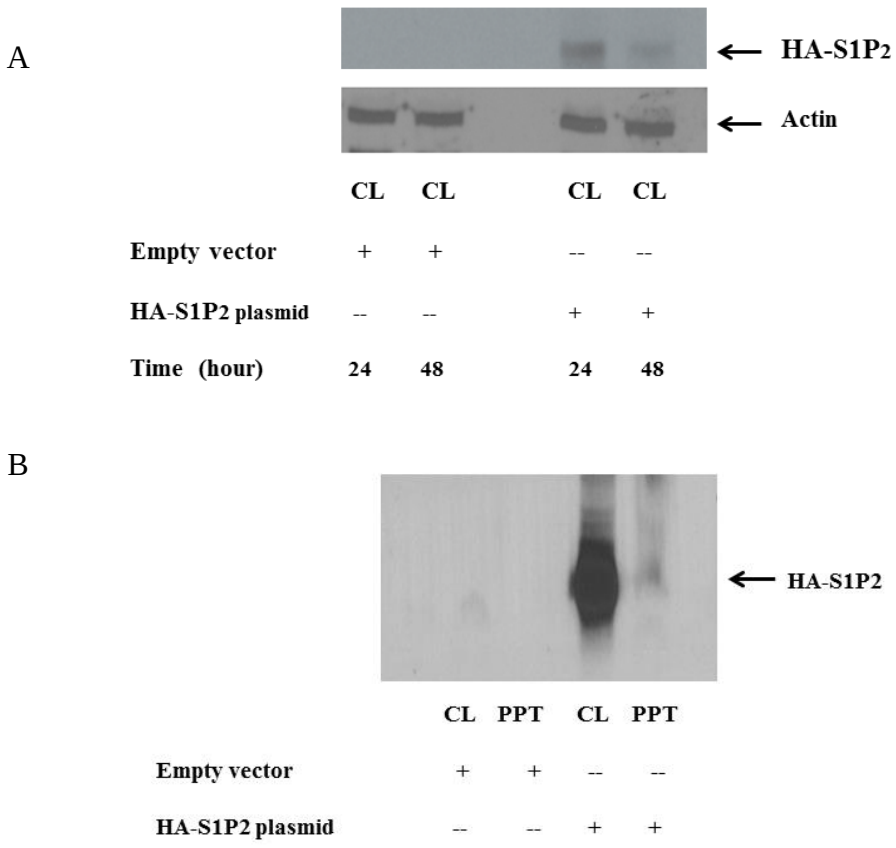


Figure 3.2 ***Transient transfection of MDA-MB-231 cells with HA-S1P$_2$ plasmid and detection in conditioned medium.***

(A) MDA-MB-231 cells were transfected with either HA-S1P$_2$ encoded plasmid or empty vectorfor 24 or 48 hrs as indicated in the figure. Cells were harvested in laemmli buffer and analysed by Western blotting using anti-HA antibody. The membrane was stripped and re-probed with actin to ensure equal protein loading. (B) HA-S1P$_2$ receptor was detected in CM of MDA-MB-231 cells overexpressing HA-S1P$_2$. Results are typical of three independent experiments.

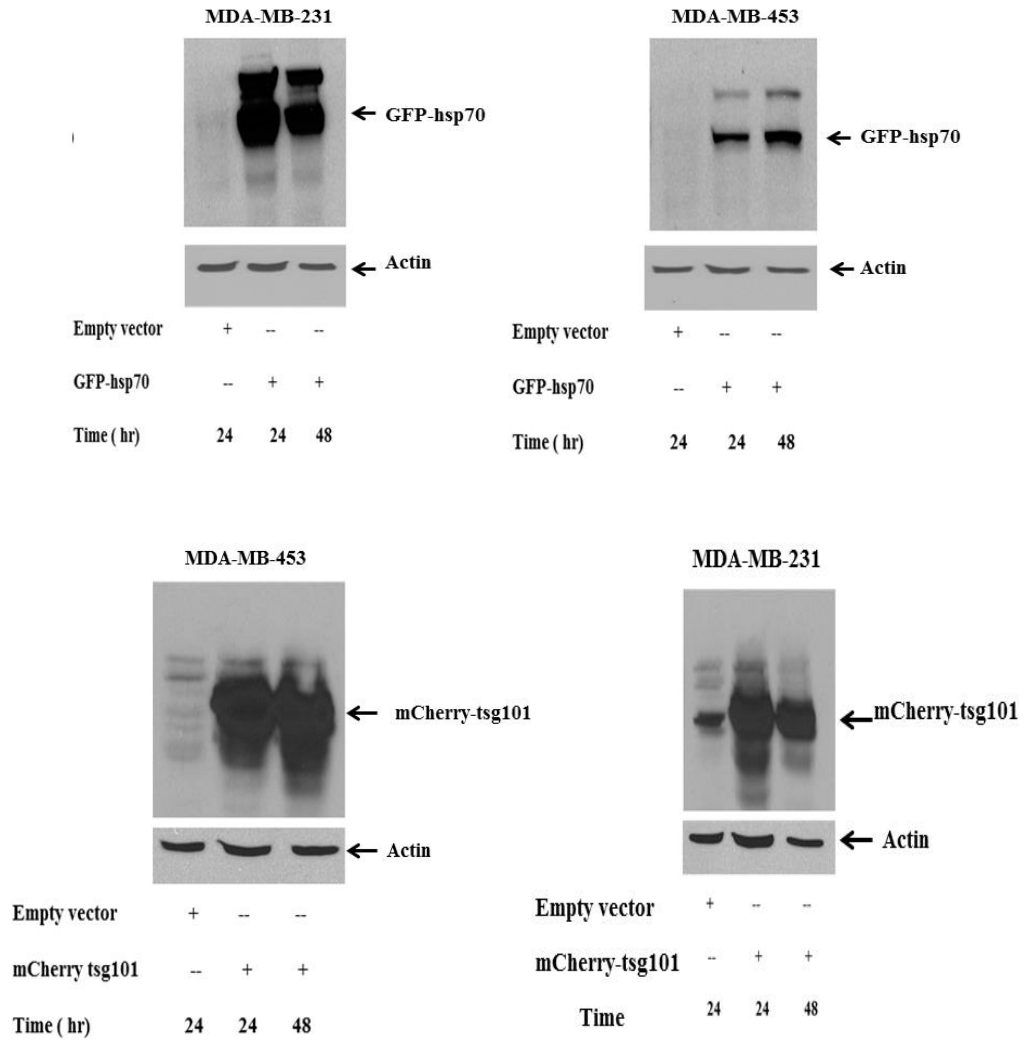


Figure 3.3 ***Transient transfection of MDA-MB-231 and MDA-MB-453 cells with GFP-hsp70 and mCherry-tsg101plasmids.***

MDA-MB-231and MDA-MB-453 cells were transiently transfected with either GFP-hsp70 or mCherry-tsg101 plasmids or vector for 24 or 48 hrs as indicated. Cells were harvested in laemmli buffer and analysed by Western blotting using anti-GFP antibody to detect GFP-hsp70 and anti-mCherry antibody to detect mCherry-tsg101. The membrane was stripped and re-probed with anti-actin to ensure equal protein loading. Results are typical of three independent experiments.

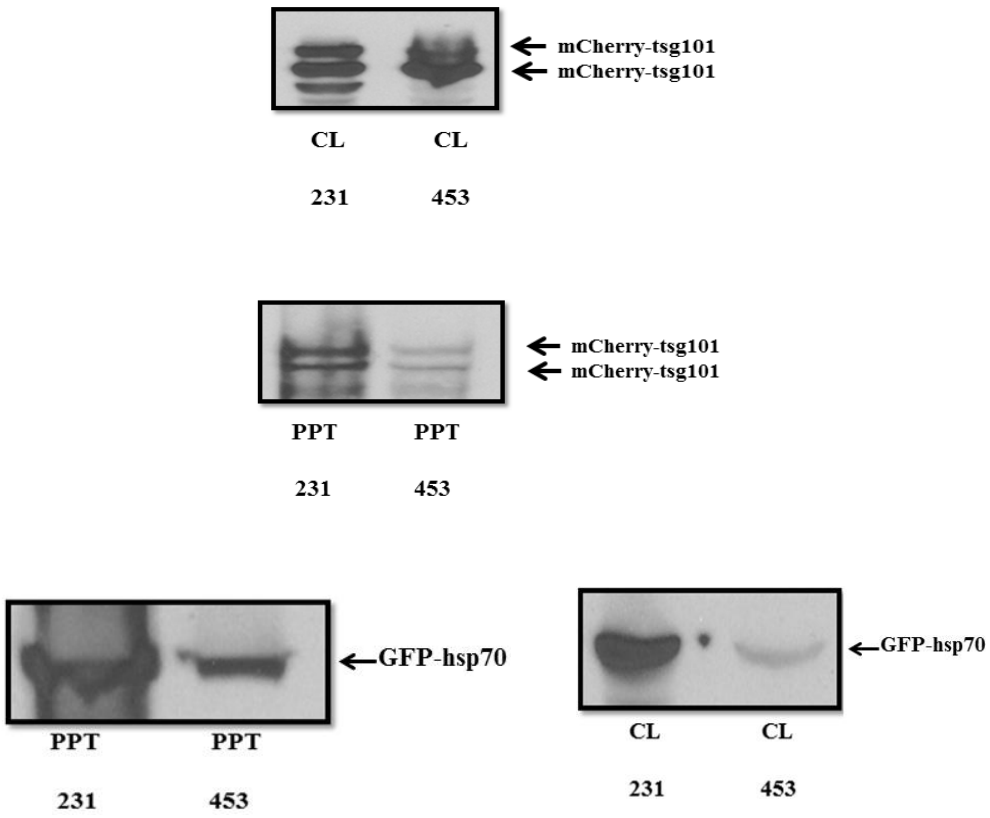


Figure 3.4 ***Identification of mCherry-tsg101 and GFP-hsp70 in MDA-MB-231 and MDA-MB-453 cell lysate and Conditioned Medium.***

Protein precipitate (PPT) of conditioned medium (CM) derived from either MDA-MB-231 cells or MDA-MB-453 cells transiently transfected with either mCherry-tsg101 or GFP-hsp70 plasmids were analysed by Western blotting using anti-mCherry and anti-GFP antibodies. 231 denotes MDA-MB-231 cells and 453 denotes MDA-MB-453 cells. Results are typical of three independent experiments.

3.2.3 Characterisation of Exosomal Markers GFP-hsp70 and CD63 and $S1P_2$ Receptors in Exosomes by Western Blotting

The CM from MDA-MB-231 cells was subjected to centrifugation to isolate exosomes. Analysis of enriched exosomes revealed the presence of exosomal markers GFP-hsp70 (Figure 3.5 A) and MVBs marker CD63 (Figure 3.5 B). Endogenous $S1P_2$ receptor with a molecular mass of ~ 42 kDa was detected in exosomes purified from CM of MDA-MB-231cells (Figure 3.5 C).

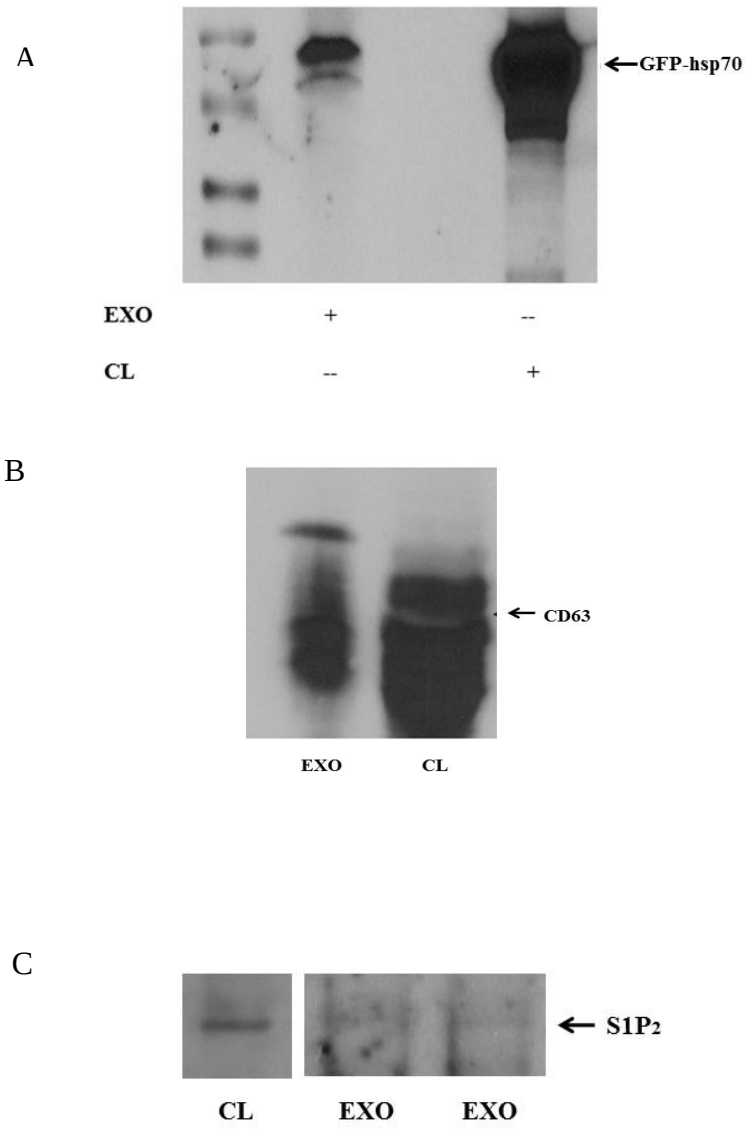


Figure 3.5 ***Identification of GFP-hsp70, endogenous CD63 and endogenous S1P$_2$ receptors in exosomes derived from MDA-MB-231 cells.***

Exosomes isolated from CM of MDA-MB-231 cells were analysed by Western blotting using anti-GFP antibody (A), anti-CD63 antibody (B) and anti-S1P$_2$ antibody (C). Results are typical of three independent experiments.

3.2.4 Visualisation of MDA-MB-231 Derived Exosomes and Characterisation of Exosomal Marker CD63 and $S1P_2$ Receptors by Immuno-gold Labelling Using Transmission Electron Microscope

Isolated exosomes from MDA-MB-231 cells were visualised and identified by transmission electron microscope (TEM). The exosomes exhibit a typical shape and morphology indicated by cup-shaped vesicles with size ranged from 30-150 nm and contained the exosomal marker CD63 detected by anti-CD63 antibody and secondary gold conjugated antibody. These exosomes also contained $S1P_2$ receptor detected by anti-$S1P_2$ antibody and secondary gold conjugate antibody (figure 3.6-3.8).

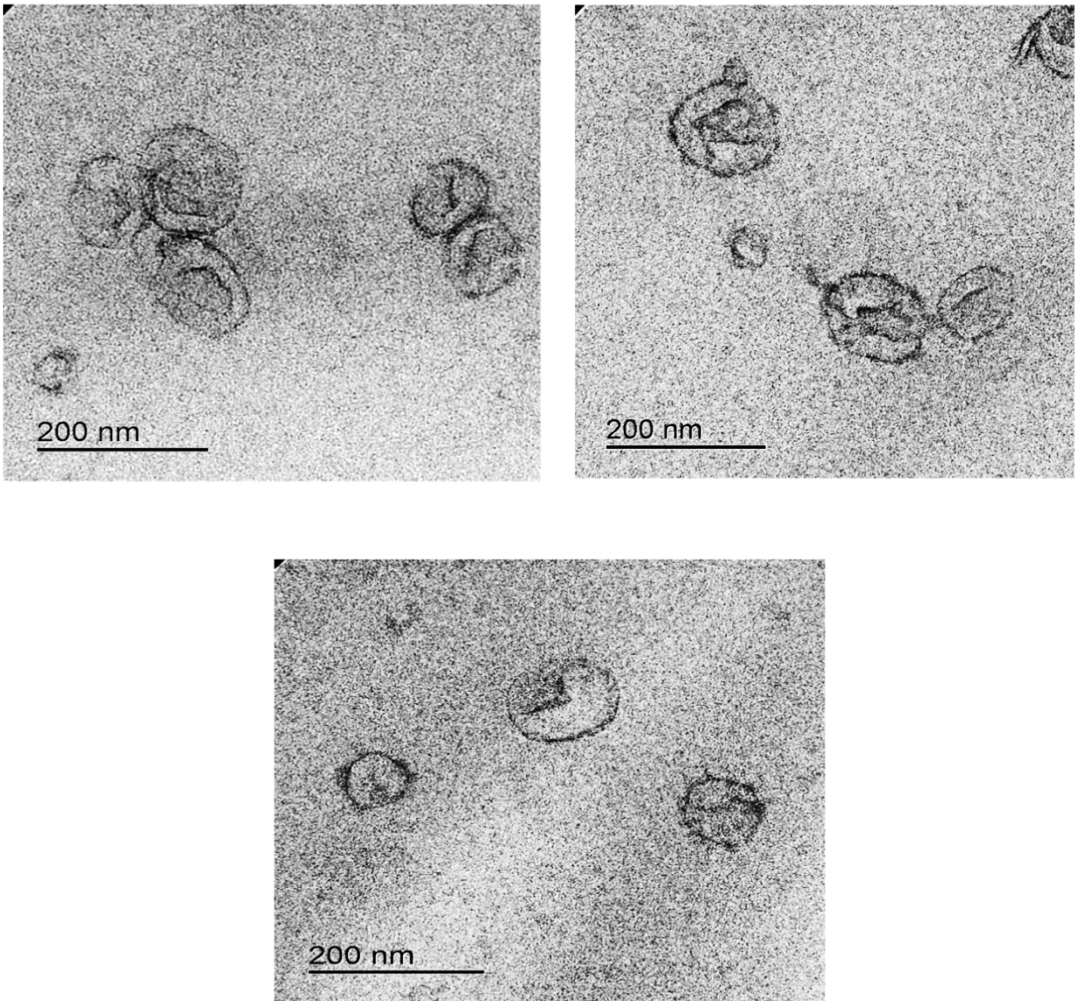


Figure 3.6 ***Imaging of exosomes with mouse and rabbit immuno-gold secondary antibodies.***

Exosomes isolated from MDA-MB-231 cells conditioned medium were incubated with mouse and rabbit immuno-gold secondary antibodies and images were taken using transmission electron microscope. Exosomes display typical structure and morphology with cup-shaped exosomes and size ranges (40-150 nm). Results are representative of three independent experiments. Distance scale is 200 nm.

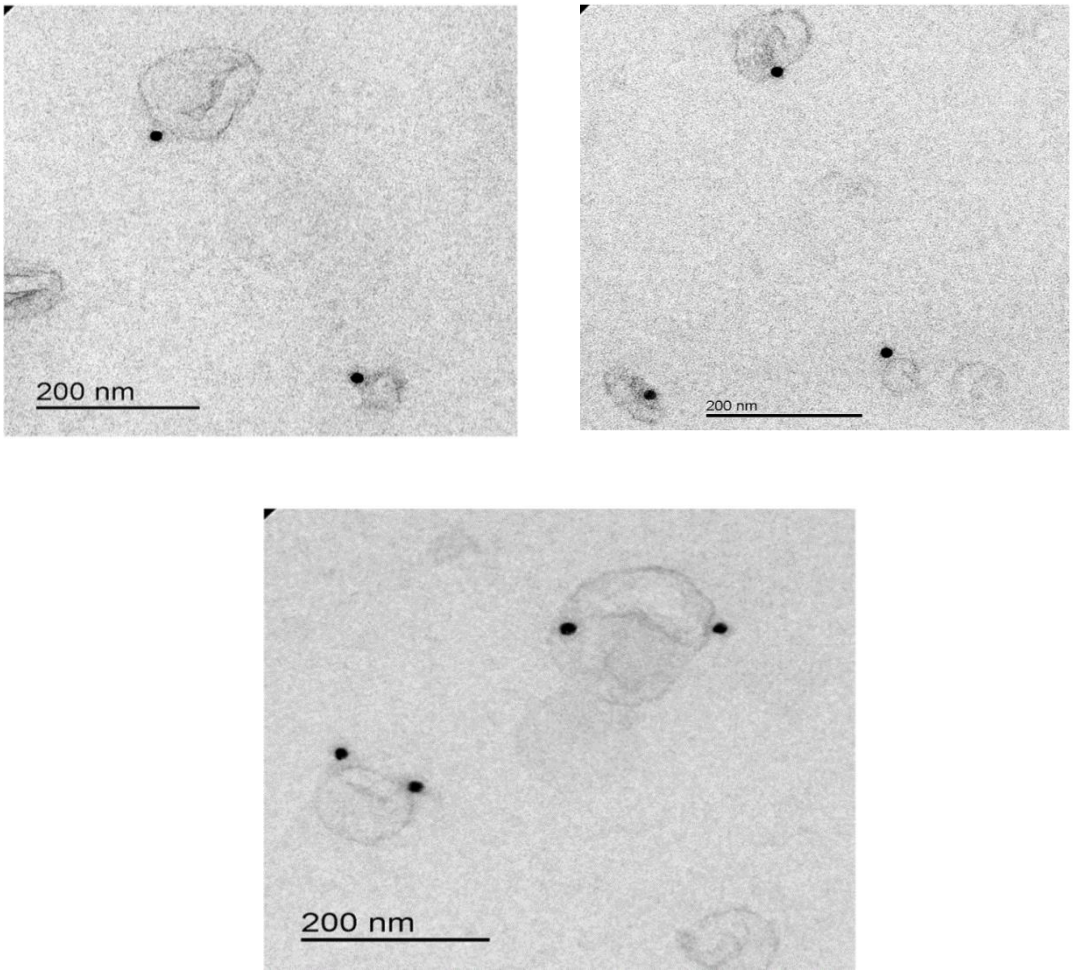


Figure 3.7 ***Imaging of exosomes with anti-CD63 antibody.***

Typical morphology and structure of exosomes containing CD63 exosomal marker detected with anti-CD63 antibody and gold conjugated secondary mouse antibody (particle size = 15nm). Results are representative of three independent experiments. Distance scale is 200 nm.

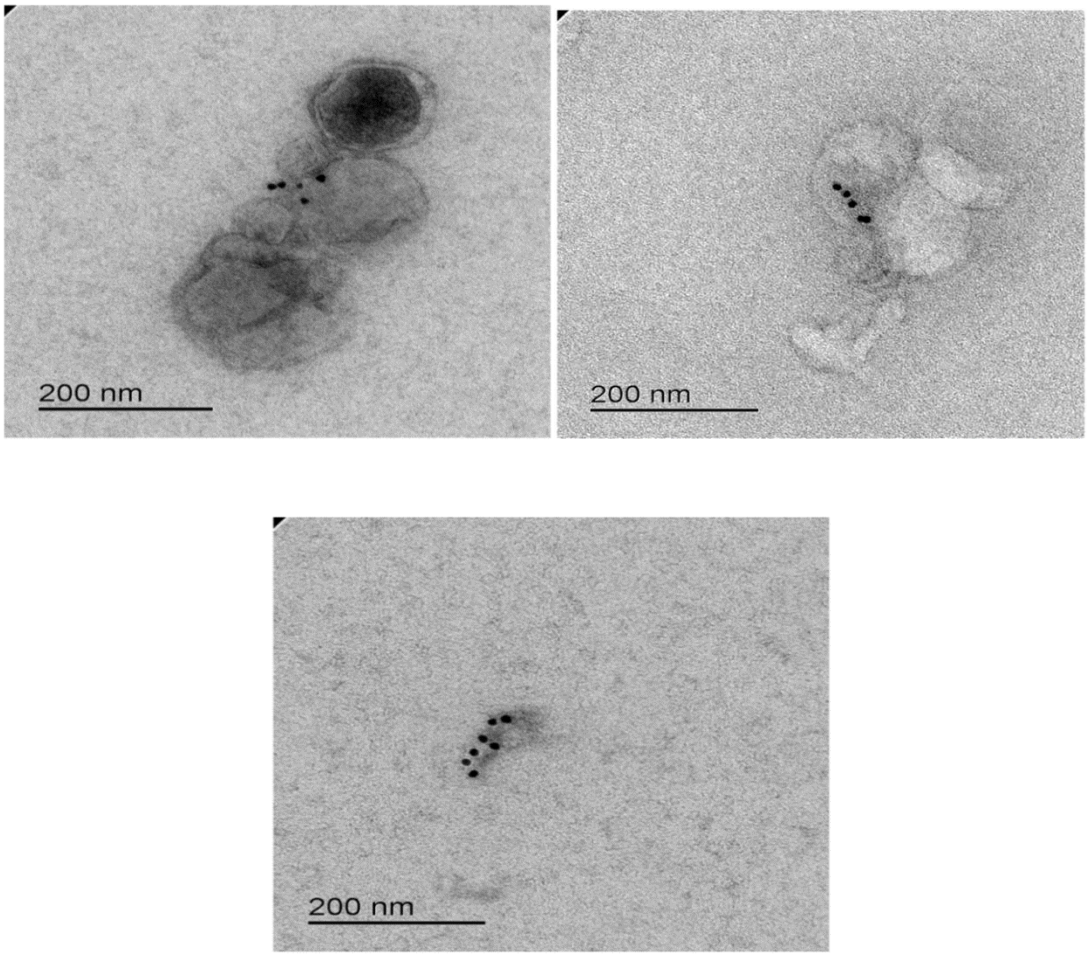


Figure 3.8 ***Imaging of exosomes with anti-S1P$_2$ antibody.***

Typical morphology and structure containing $S1P_2$ receptors detected with anti-$S1P_2$ antibody and gold conjugated secondary rabbit antibody (particle size = 10 nm). Results are representative of three independent experiments. Distance scale is 200 nm.

3.2.5 Immunofluorescence Characterisation of $S1P_2$ and CD63 in MDA-MB-231 Cells Using Confocal Laser Microscope.

In order to identify whether $S1P_2$ and CD63 are co-localised in MVBs as a prelude to their release in exosomes, MDA-MB-231 cells were immuno-stained with anti-$S1P_2$ and anti-CD63 antibody. CD63 was detected by anti-CD63 antibody and anti-mouse antibody conjugated to TRITC (red stain); this co-localised with $S1P_2$ detected by anti-$S1P_2$ antibody and anti-rabbit conjugated to FITC (green stain) in large vesicles similar to MVBs (figure 3.9). The co-localisation is detected as yellow spots as a result of integration of red and green colours and indicated by white arrows in (figure 3.9).

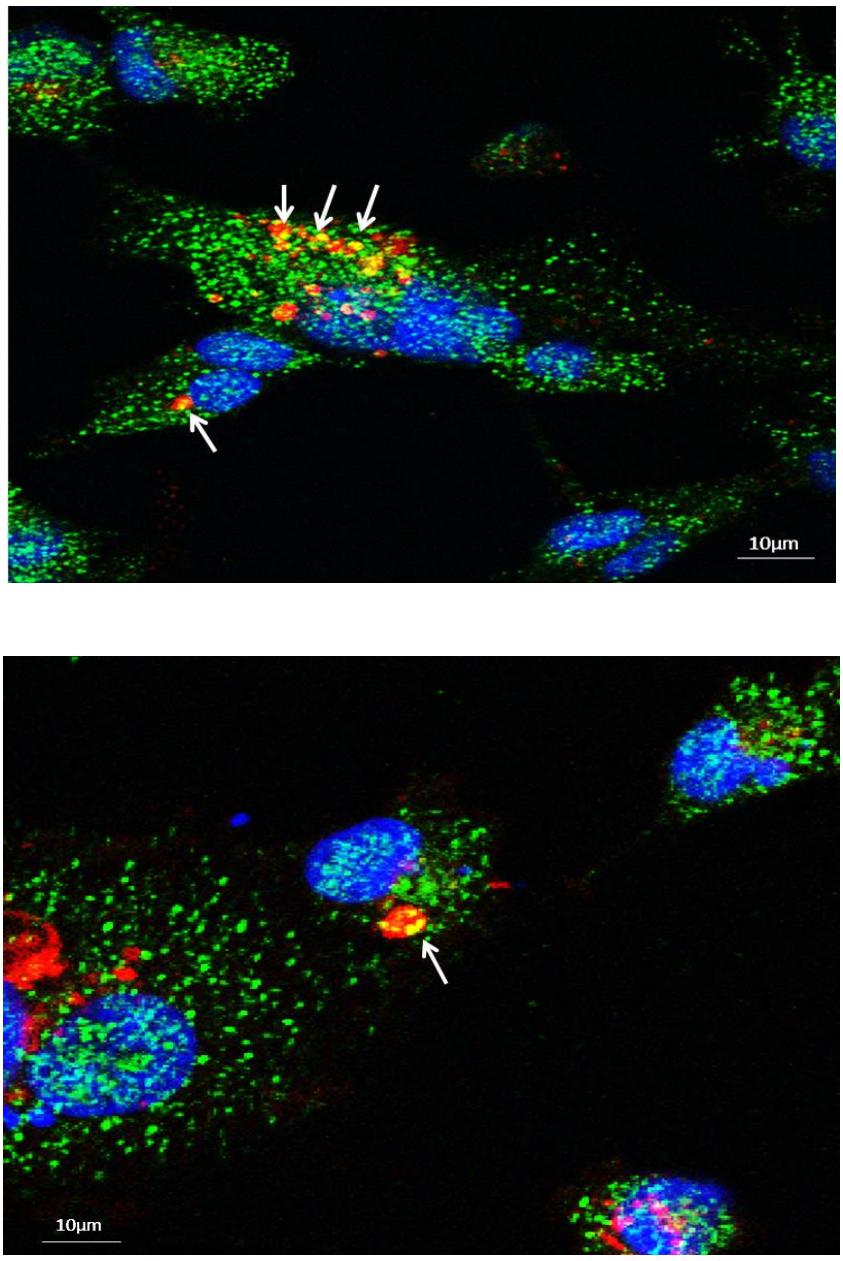


Figure 3.9 ***Characterization of S1P$_2$ and CD63 location in MDA-MB231 cells using confocal laser microscope.***

CD63 was stained with anti-CD63 antibody and anti-mouse secondary antibody conjugated to TRITC (red stain), and S1P$_2$ was stained with anti-S1P$_2$ antibody and anti-rabbit secondary antibody conjugated to FITC (green stain). Co-localisation of CD63 and S1P$_2$ receptor was seen as yellow punctate bodies indicated by white arrows. Results are representative of three independent experiments Scale bar is 10 µm.

3.3 Discussion

Exosomes are lipid bilayer membrane vesicles with nano-size range between 30-150 nm and which have been shown to be released by most mammalian cells. Exosomes can be considered as intercellular signalling moieties that can carry nucleic acid molecules such as mRNA, miRNA and DNA and functional proteins that can alter phenotypic properties in recipient cells. Cancer cells are reported to release more exosomes than normal cells (Whiteside, 2016), and these exosomes are able to transfer oncogenes that mediate carcinogenesis and malignant transformation in recipient cells.

The major finding of the current study is the first-time detection of the GPRC $S1P_2$ receptor in exosomes purified from conditioned medium of MDA-MB-231 cells. Initial work using Western blotting analysis of CM isolated from MDA-MB-231 cells indicate the presence of $S1P_2$ receptors with an Mr ~42 kDa, suggesting that it is released in its native form in extracellular vesicles (exosomes) from MDA-MB-231 cells. This is based upon several findings. First, the exosomal markers GFP-hsp70 and mCherry-tsg101 along with HA-$S1P_2$ receptors were detected in protein precipitate of CM from MDA-MB-231 cells by Western blot analysis. These findings strongly indicate the release of exosomes containing $S1P_2$ receptor into CM. Second, immunostaining of MDA-MB-231 cells with anti-CD63 antibody and anti-$S1P_2$ antibody demonstrated their co-localisation in vesicles resembling MVBs, suggesting that these proteins are packaged from endosomes into intraluminal vesicles (ILVs) in MVBs. Notably, ILVs display endocytosed transmembrane proteins such as GPCRs

in the same alignment as at the plasma membrane with the N-terminal domain facing the extracellular space (Thery *et al*., 2002), see (figure 3.10) for illustration.

Third, the electron microscope analysis of enriched exosomes from CM of MDA-MB-231 cells, and immuno-gold labelling of exosomes with anti-CD63 and anti-$S1P_2$ receptor antibodies indicate the presence of CD63 and $S1P_2$ in exosomes. Fourth, Western blot analysis of exosomes lysate detected the presence of CD63 with anti-CD63 antibody and $S1P_2$ receptor with anti-$S1P_2$ antibody. Collectively, all these findings indicate the presence of $S1P_2$ receptor in exosomes. The first study to demonstrate that S1P receptors can be released from cells in exosomes was by Amorim *et al* (2014). These workers used proteomic analysis of exosomes isolated from immortalised human mammary luminal epithelial HB4a and C5.2 cells, and they detected $S1P_3$ and $S1P_4$. However, no functional role was attributed to these receptors in this study (Amorim *et al*., 2014). In the current study, $S1P_4$ receptors were demonstrated to be released into CM from MDA-MB-453 cells along with exosomal markers GFP-hsp70 and mCherrytsg-tsg101. These findings show for the first time the release of $S1P_2$ and $S1P_4$ receptors into CM from MDA-MB-231 and MDA-MB-453 cells respectively. The CM and exosomes preparation from MDA-MB-231 cells was demonstrated to exhibit functional activity indicated by stimulation of extracellular signal-regulated kinase (ERK-1/2) and DNA synthesis in MEFs. However, CM containing $S1P_4$ receptors from MDA-MB-453 was unable to induce ERK-1/2 activation in MEFs; for details see section (4.2.1) and (figure 4.1) chapter (IV). This demonstrates functional specificity for $S1P_2$ in MEFs. Importantly, exosomes derived from cancer cells have been shown to contain functional protein molecules such as TGF-β that can induce stromal cell differentiation into

myofibroblasts, which play important role in cancer metastasis (Cho *et al*., 2012). In addition, the inhibitor apoptosis protein survivin that blocks cell death, is detected in exosomes derived from Hela cells and its level in exosomes were increased after exposure of Hela cells to proton irradiation when compared with exosomes isolated from non-irradiated cells (Khan *et al*., 2010). Metastasis-Associated Protein 1 (MTA1) is also detected in exosomes released from breast cancer and has been shown to regulate hypoxic-dependent signalling via exosome-transfer between breast cancer and endothelial cells (Hannafon *et al*., 2017).

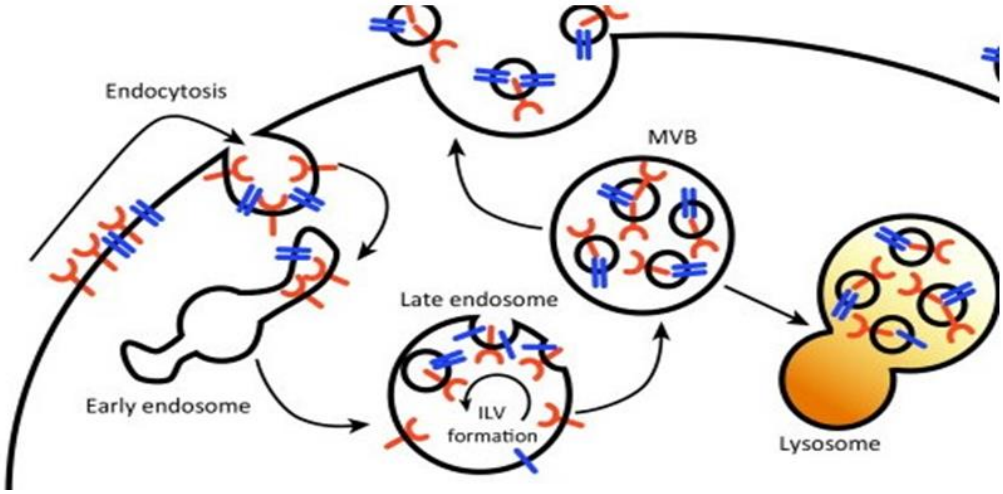
Moreover, the presence of GPCRs in exosomes was studied by Guescini *et al* (2012) who demonstrated that HA tagged Adenosine 2A and Dopamine D2 GPCRs are present in Tsg101 and Rab5 positive exosomes released from HEK293T and COS-7 cells transfected with plasmids encoding these proteins (Guescini *et al*., 2012). In addition, COS-7 or HEK293T cells co-cultured with exosomes isolated from either HEK293T or COS-7 cells over expressing HA-Adenosine 2A displayed increased cAMP level in response to the potent adenosine A2A receptor agonist CGS-21680 compared with cells co-cultured with exosomes isolated from non-transfected cells. These findings indicate that exosomes containing the adenosine A2A receptor exhibit functional ability to transduce signalling in recipient cells (Guescini *et al*., 2012). Furthermore, the AT1R containing exosomes were isolated from mice serum after cardiac pressure overload and these receptors demonstrated a functional ability to confer blood pressure responsiveness to angiotensin II infusion in *AT1R*$^{-/-}$ knockout mice (Pironti *et al*., 2015). Moreover, Delta-like 4 (Dll4) which is a Notch ligand has been shown to be present in exosomes released from endothelial cells and also from cancer cells overexpressing DII4 (Sheldon *et al*.,

2010). In addition, DII4-containing exosomes have been shown to be taken by endothelial cells and were found to affect tube formation, increasing branching and overall vessel density in an *in vitro* tube assay, DII4 also increased vessel branching in *in vivo* tumour xenograft model (Sheldon *et al*., 2010). The future work should focus on investigating other proteins that present in MDA-MB-231 cells and not released into CM rather than ERK proteins (figure 4.3) chapter IV. This is important to distinguish CM/exosomes preparation from impurity from MDA-MB-231 cells.

In addition, an electron microscope analysis should be done on exosomes isolated from $S1P_2$ siRNA knockeddown MDA-MB-231 cells. This is important to show the specificity of $S1P_2$ antibody used during experiment and to exclude any suggestion of non-specific binding. Also, it is important in the future work to perform 3D live fluorescence imaging for a better understanding of CD63 and $S1P_2$ co-localization in endosomes like vesicles and to exclude that $S1P_2$ membranes in close proximity to CD36 membranes rather than the proteins being present on the same membrane compartment.

Collectively, all thiese scientific evidence from current and previous studies demonstrate that exosomes are signalling compartments that convey functional proteins that are able to induce different cellular responses in recipient cells. The current study is the first to demonstrate that $S1P_2$ can be released in exosomes from breast cancer cells, consistent with the known ability of exosomes to transfer GPCRs to recipient cells. The next step in this study is to investigate whether $S1P_2$ receptors in exosomes released in CM from MDA-MB-231 cells or $S1P_4$ detected in CM from

MDA-MB-453 cells are able to confer a functional response in mouse embryonic fibroblasts (MEFs).



Taken from Cocucci and Meldolesi, (2015).

Figure 3.10 ***Exosome biogenesis.***

Transmembrane proteins (red and blue) are endocytosed and trafficked to early endosomes. Subsequent inward budding of late endosomes resulted in formation of ILVs which displaying transmembrane protein in the same architecture as the plasma membrane alignments. The multivesicular bodies (MVBs) containing ILVs can either follow a degradation pathway fusing with lysosomes or proceed to release the ILVs as exosomes to the extracellular space**.**

CHAPTER IV

PROCESSING OF THE EXOSOMAL $S1P_2$ BY MOUSE EMBRYONIC FIBROBLASTS

CHAPTER 4: Processing of the Exosomal $S1P_2$ by Mouse Embryonic Fibroblasts

4.1 Introduction

Several reports have demonstrated an interaction between tumour microenvironment which involves non-neoplastic stromal cells such as lymphocytes, macrophages, dendritic cells (DC), fibroblasts, mast cells (MC) and endothelial cells with neighbouring cancer cells. This bidirectional interaction or communication is believed to happen through miRNAs, soluble factors or signalling proteins including internalised functional receptors in EVs (Kosaka, 2016). For example, glioblastoma cells have been shown to release EVs containing an oncogenic mutant form of the epidermal growth factor receptor (EGFRvIII), which is taken up by adjacent cancer cells causing activation of the MAPK/Akt cascade, thereby promoting cancer cells proliferation (Al-Nedawi *et al*., 2008).

There are two recogised interactive pathways involved in the interplay between cancer and stromal cells. The first is the "efferent" pathway, where cancer cells induce a reactive response in the stroma. For instance, TGF-β and PDGF produced from cancer cells induce differentiation and activation of fibroblasts, thereby converting these cells into myofibroblasts (Postlethwaite *et al*., 1987). The second is the "afferent" pathway where the activated fibroblasts produce several cytokines, chemokines and inflammatory mediators, which influence cancer invasion and malignancy (Orimo *et al*. 2001; Powell, 2000). In this regard, S1P and SK1 have been shown to regulate the interaction between cancer cells and tumour associated

fibroblasts. Thus, SK1 expressing fibroblasts have been demonstrated to increase melanoma cell migration, while fibroblasts from *Sk1*$^{-/-}$ mice were less able to induce this effect (Albinet *et al*., 2013).

Therefore, this chapter is concerned with the investigation of the effect of conditioned medium (CM) and isolated exosomes containing $S1P_2$ from MDA-MB-231 breast cancer cells on the activation of mouse embryonic fibroblasts. This has involved establishing the effect of CM and exosomes on the extracellular signal-regulated kinase-1/2 pathway and DNA synthesis in fibroblasts.

4.2 Results

4.2.1 Characterisation of the Effect of Conditioned Medium from MDA-MB-231 and MDA-MB-453 Cells on Extracellular Signal-regulated Kinase Signalling in Mouse Embryonic Fibroblasts (MEFs)

The first investigation sought to establish the functional role of $S1P_2$ released in exosomes from MDA-MB-231 cells. In this regard, the effect of CM on extracellular signal-regulated kinases (ERK-1/2), which are members of a large family of mitogen activated protein kinase (MAPK) that are involved in different biological processes such as proliferation, cell cycle progression, differentiation, migration, senescence and cell death (Ramos, 2008) was examined. CM from MDA-MB-231 cells, which contains $S1P_2$ is able to induce ERK-1/2 activation in MEFs ($p < 0.05$ *versus* NCM) (figure 4.1 A, B). In contrast, CM from MDA-MB-453 cells, which contains $S1P_4$ was unable to induce ERK-1/2 activation (figure 4.1 A, B).

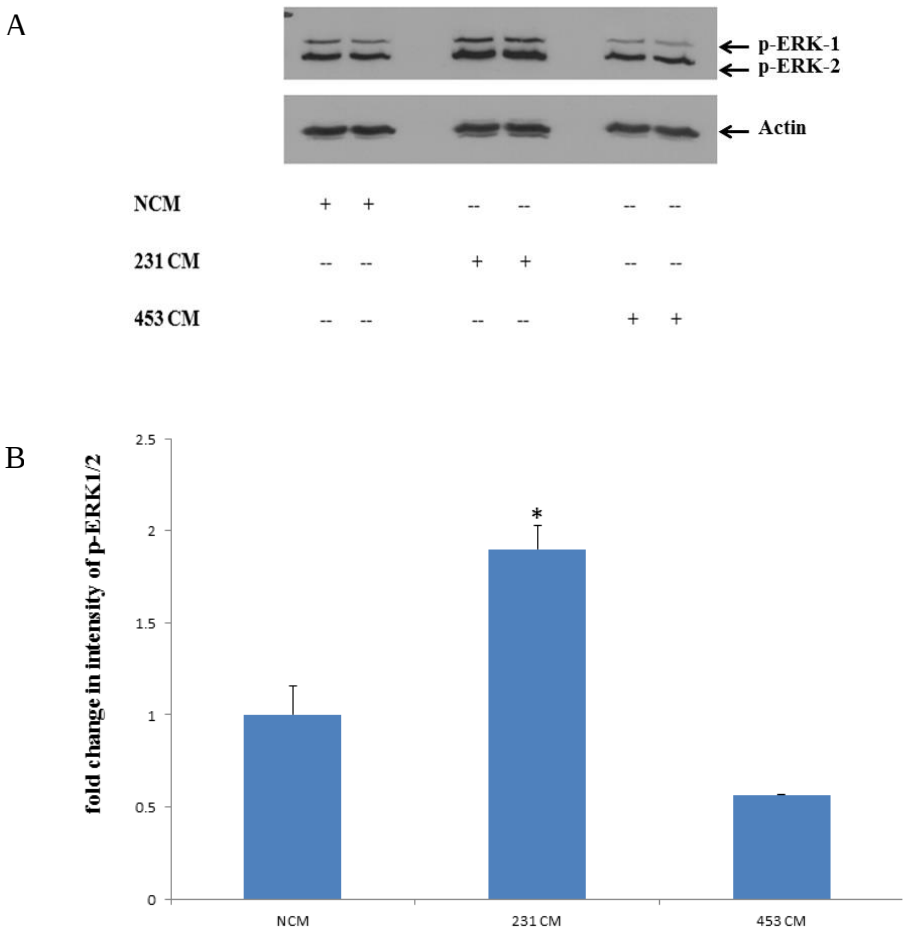


Figure 4.1 ***The effect of Non Conditioned Medium and Conditioned Medium from MDA-MB-231 and MDA-MB-453 cells on MEF extracellular signal-regulated kinase-1/2 in MEFs***.

MEFs were grown on 12 wells plate in complete medium until 70% confluence attained. MEFs were then quiescent for 24 hrs before being incubated with either NCM or CM from MDA-MB-231 cells (231 CM) or CM from MDA-MB-453 cells (453 CM) for 10 mins. (A) MEFs were then harvested in Laemmli buffer and analysed by Western blotting using anti-p-ERK1/2 antibody. The membrane was stripped and re-probed for anti-actin antibody to ensure equal protein loading. Results are typical of three independent experiments. (B) Densitometric quantification expressed as mean of optical density of p-ERK-1/2/actin ratio expressed as a fold change in the intensity of p-ERK1/2 +/- SEM. Unpaired *t*-test was performed using Graph Pad Prism program and bar graph generated using Excel. Difference was considered significant at $^{*}p < 0.05$ *versus* NCM, (n=3).

4.2.2 Characterisation of the Effect of $S1P_{2/4}$ Antagonist JTE-013 on Conditioned Medium Stimulated ERK-1/2 in MEFs

The 231 CM was found to significantly induce MEF extracellular signal-regulated kinase (ERK-1/2) activation compared with NCM.The next step was to establish whether the activation of ERK-1/2 by 231 CM involved $S1P_2$. For this purpose, the $S1P_{2/4}$ antagonist JTE-013 (final concentration, 10 μM) was used. Pre-treating MEFs with JTE-013 for 15 mins partially reduced basal ERK-1/2 levels, but with no obvious greater inhibitory effect of JTE-013 in cells treated with CM. These findings, suggest that the activation of ERK-1/2 by CM from MDA-MB-231 cells is not blocked by JTE-013 (figure 4.2 A, B). In addition, no ERK-1/2 is released into CM from MDA-MB-231cells, thereby excluding the possibility that ERK-1/2 from breast cancer cells is contributing to the observed activated ERK-1/2 levels in MEFs (figure 4.3).

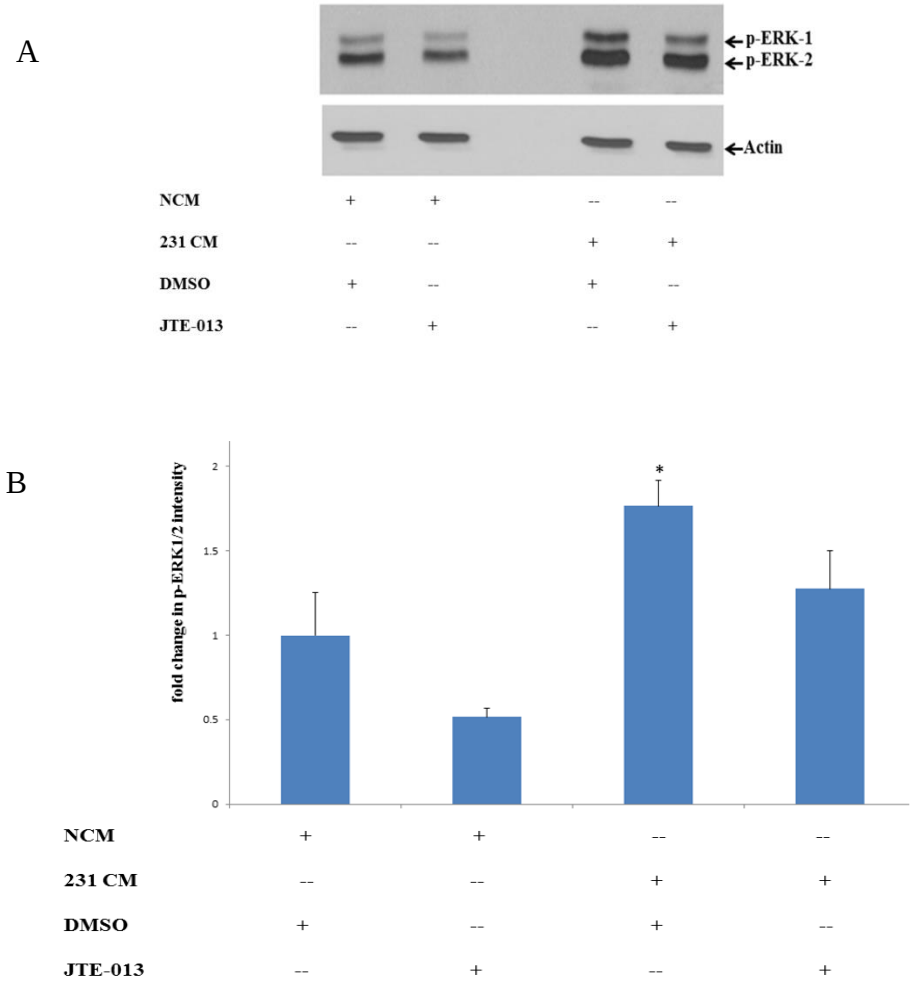


Figure 4.2 ***The effect of JTE-013 on Conditioned Medium stimulated ERK-1/2 activation in MEFs.***

MEFs were grown on 12 wells plate in complete medium until 70% confluence attained. MEFs were quiescent for 24 hrs prior to treatment with either NCM or CM for 10 mins. In some cases, MEFs were pre-treated with JTE-013 (10 µM) or 0.1% v/v DMSO for 15 mins. MEFs lysate in Laemmli buffer were analysed by Western blotting and ERK-1/2 activation was measured using anti-p-ERK-1/2 antibody. The membrane was stripped and re-probed with anti-actin to ensure equal protein loading. Results are typical of three independent experiments. (B) Densitometric quantification expressed as mean +/- SEM fold change in thep-ERK-1/2/actin ratio. Unpaired t-test was performed using Graph Pad Prism program and bar graph generated using Excel. Difference was considered significant at $*p < 0.05$ *versus* NCM, (n=3).

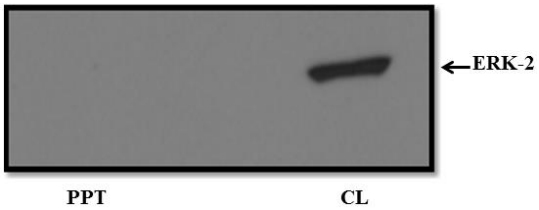


Figure 4.3 ***Characterisation of endogenous ERK-1/2 presences in Conditioned Medium from MDA-MB-231 cells*****.**

Acid precipitate of CM from MDA-MB-231 was analysed by Western blotting and total ERK-2 was measured by anti-ERK-2 antibody. MDA-MB-231 cells lysate was used as positive control.

4.2.3 Characterisation of the Effect of Conditioned Medium Derived from MDA-MB-231 cells on MEFs DNA Synthesis

As ERK-1/2 is linked with the regulation of DNA synthesis and proliferation (Meloche and Pouysségur, 2007), the next step was to investigate the effect of CM on [^{3}H] thymidine incorporation in MEFs, as a measure of DNA synthesis. Treatment of MEFs with CM significantly induced DNA synthesis in MEFs, indicated by increased thymidine incorporation (*$p < 0.05$ *versus* NCM) (figure 4.4). In contrast, the treatment of MEFs with $S1P_2$ agonist CYM-5520 failed to increase DNA synthesis ($p > 0.05$ *versus* NCM), suggesting that endogenous $S1P_2$ in MEFs is not linked with the regulation of DNA synthesis. However, JTE-013 reduced DNA synthesis below its basal level (figure 4.4). Taken together with the lack of effect of CYM-5520, these findings, suggest that the effect of JTE-013 on basal DNA synthesis might not be related to endogenous $S1P_2$ in MEFs.

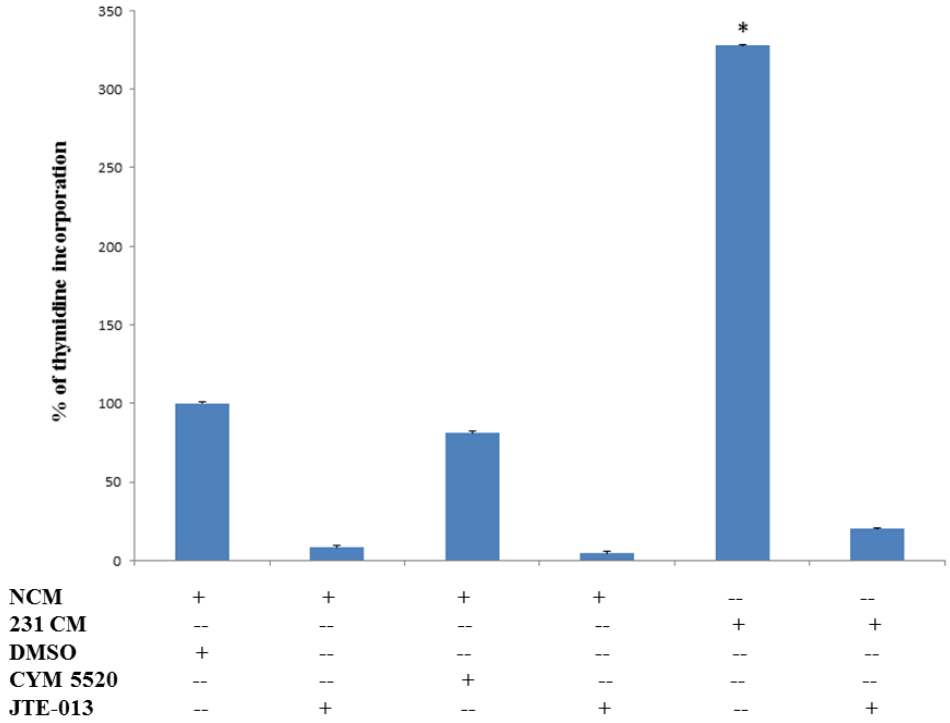


Figure 4.4 ***Characterization the effect of Conditioned Medium from MDA-MB-231 cells on DNA synthesis in MEFs.***

MEF cells were grown on 12 wells plate in complete medium until 70% confluence attained. MEFs were made quiescent for 24 hrs before being treated with NCM containing 0.1% v/v DMSO or CM from MDA-MB-231 cells or CYM-5520 (10 µM) or JTE-013 (10 µM) and incubated for 24 hrs. [^{3}H] thymidine incorporation was performed as described in section 2.4. MEFs treated with CM from MDA-MB-231 cells showed a significant increase in DNA synthesis ($^{*}p < 0.05$ *versus* NCM) and thymidine incorporation. Data are the pooled mean +/- SEM from three separate experiments, each containing triplicate determinations and was presented as bar graph. A *t*-test statistical analysis between group was performed and result considered significant when $^{*}p<0.05$.

4.2.4 Characterisation of the Effect of Addition of Exosomes Isolated from MDA-MB-231 Conditioned Medium on MEF Extracellular Signal-regulated Kinase

Varying amounts of exosomes isolated from CM of MDA-MB-231 cells were then added to MEFs to establish the effect on ERK-1/2 activation. Indeed, isolated exosomes containing $S1P_2$ were able to induce activation of ERK-1/2 in MEFs $^{*}p < 0.05$ *versus* phosphate buffer saline (PBS) (figure 4.5 A, B).

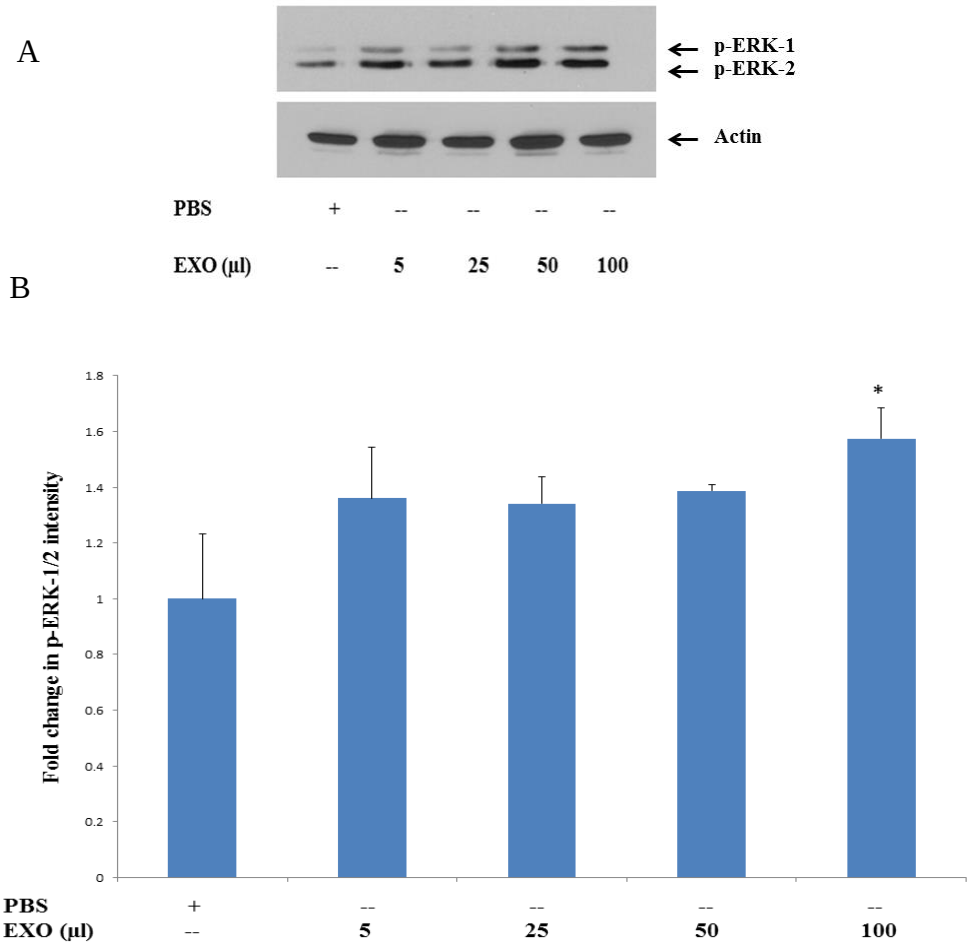


Figure 4.5 Effect *of variant amounts of exosomes on extracellular signal regulated kinase in MEFs*.

MEF cells were grown on 12 wells plate in complete medium until 70% confluence attained. Cells were quiescent for 24 hrs before been treated with different volumes of exosomes (EXO) as indicated for 10 mins. (A) MEFs were harvested in Laemmli buffer and cells lysate were analysed by Western blotting. ERK-1/2 activation was measured using anti-p-ERK-1/2 antibody and the membrane was stripped and re-probed with anti-actin to ensure equal protein loading. This result represents the repeats of three experiments. (B) Densitometric quantification of the effect of different amounts of exosomes (EXO) on ERK-1/2 activation. Results were expressed as mean +/- SEM fold change in p-ERK-1/2/actin ratio for three independent experiments. Statistical unpaired *t*-test was performed between group and the increase was considered significant * when $p < 0.05$ *versus* PBS, (n=3).

4.2.5 Characterisation of the Effect of Conditioned Medium Derived from MDA-MB-231 Cells Transfected with $S1P_2$ siRNA on ERK-1/2 in MEFs

In order to establish whether $S1P_2$ in CM is responsible for the activation of ERK-1/2 in MEFs, it was necessary to establish the effect of conditioned medium isolated from MDA-MB-231 cells in which $S1P_2$ had been previously eliminated. Treatment of MDA-MB-231 cells with $S1P_2$ siRNA significantly reduced the expression of $S1P_2$ in these cells (figure 4.6 A). In addition, CM derived from MDA-MB-231 treated with either $S1P_2$ siRNA 100 nM or 200 nM failed to induce the activation of ERK-1/2 in MEFs $*p < 0.05$ (figure 4.6 B, C). These findings provide compelling evidence that $S1P_2$ receptors from cancer cells mediate the stimulatory effect of CM on ERK-1/2 activation in MEFs.

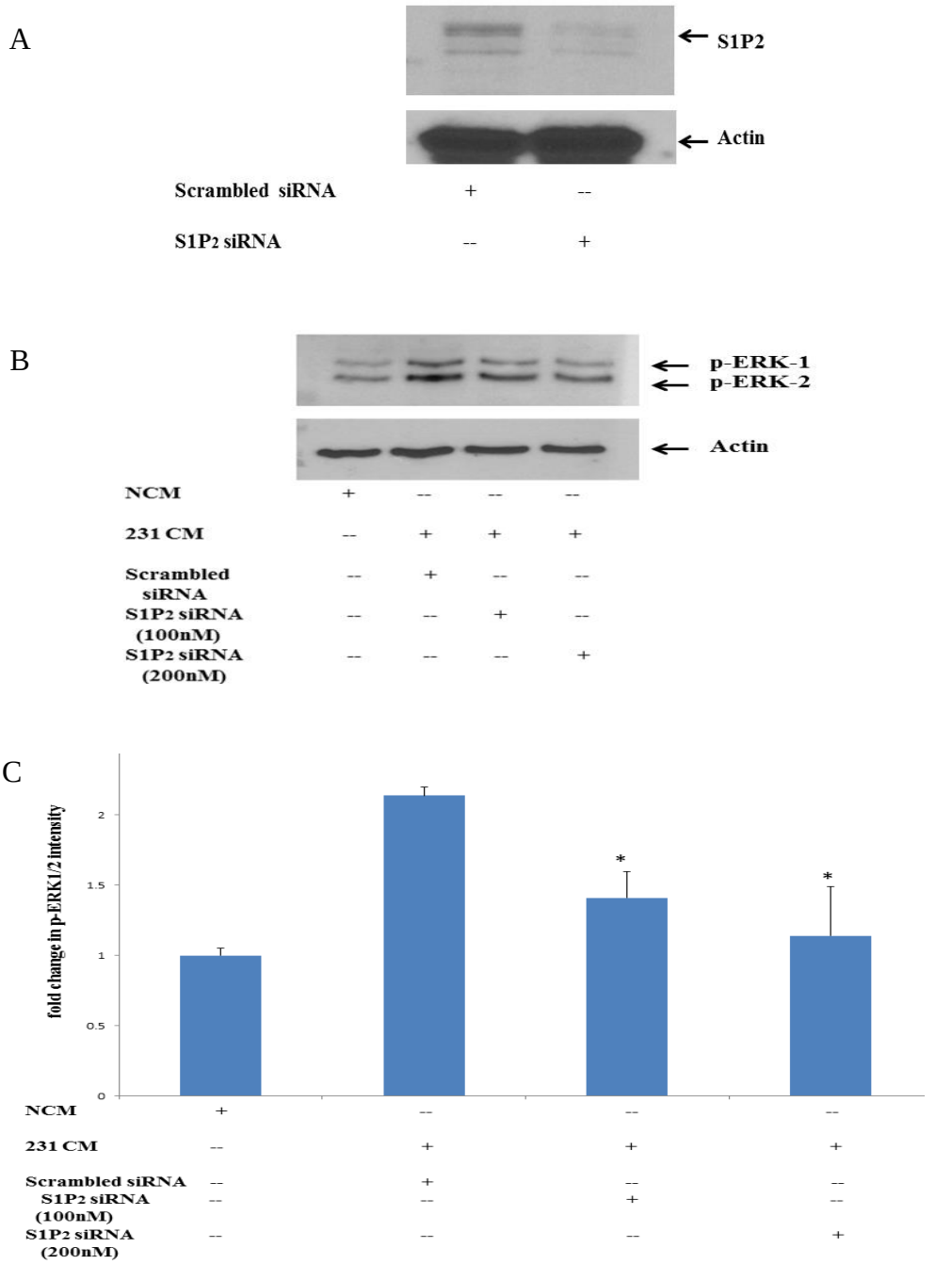


Figure 4.6 ***Characterszation the effect of Conditioned Medium derived from S1P$_2$ siRNA treated MDA-MB-231 cells on ERK-1/2 activation in MEFs.***

MDA-MB-231 cells were treated with $S1P_2$ siRNA (100 and 200 nM) to knockdown $S1P_2$ for 24 hrs and then CM was collected and added to MEFs for 10 mins. MEFs were harvested in Laemmli buffer and analysed by Western blotting. ERK-1/2 activation in MEFs was measured using anti-p-ERK-1/2 antibody (B). $S1P_2$ in MDA-MB-231 cells was assessed using anti-$S1P_2$ antibody (A). The membrane was stripped and reprobed with anti-actin to ensure equal protein loading. Densitometric quantification of optical density and expressed as mean +/- SEM fold change in p-ERK-1/2/actin/ratio for three independent experiments (C). Unpaired *t*-test statistical analysis was performed and reduction in p-ERK-1/2 fold intensity was considered significant at $^{*}p < 0.05$ *versus* CM treated with scrambled siRNA, (n=3).

4.2.6 Charcterisation of the Effect of Exosomes Isolated from Conditioned Medium Derived from MDA-MB-231 Cells Treated with $S1P_2$ siRNA

Exosomes isolated from CM of MDA-MB-231 cells treated with $S1P_2$ siRNA also had no stimulatory effect on ERK-1/2 in MEFs regardless of exosome volume, indicating that $S1P_2$ from cancer cells is mediating the stimulatory effect of exosomes on ERK-1/2 in MEFs (figure 4.7 A, B).

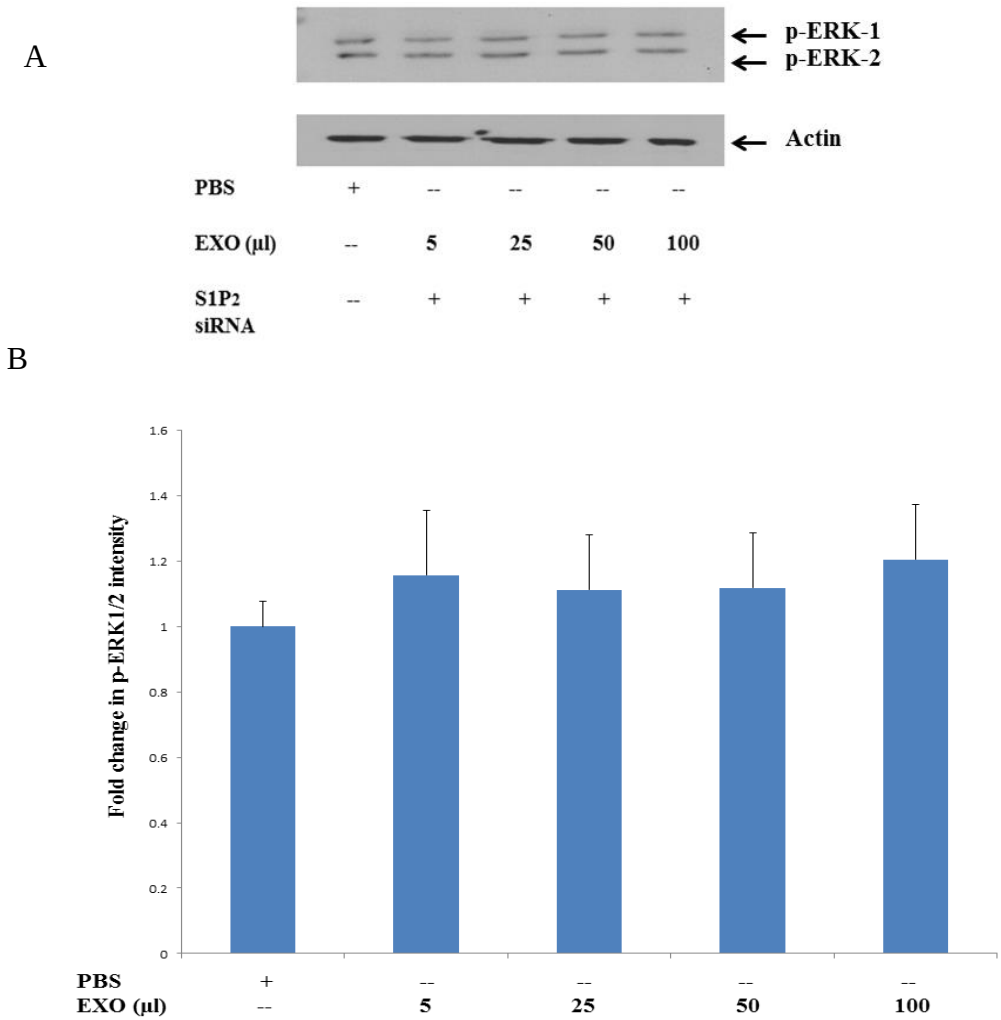


Figure 4.7 ***Characterisation of the effect of exosomes derived from S1P$_2$ siRNA transfected MDA-MB-231 cells on ERK-1/2 activation in MEFs.***

MEFs were grown on 12 wells plate until 70% confluence attained. The MEFs were made quisecent for 24 hrs before being incubated with different amounts of exosomes (EXO) derived from S1P$_2$ siRNA treated MDA-MB-231 cells for 10 mins as indicated. (A) MEFs were then harvested in Laemmli buffer and analysed by Western blotting using anti-p-ERK-1/2 antibody. The membrane was stripped and re-probed with anti-actin to ensure equal protein loading. Results are representative of three independent experiments. (B) Densitomertic quantification expressed as mean +/- SEM fold change in p-ERK-1/2 actin/ratio for three independent experiments. Statistical analysis *t*-test was performed and there was no significant increase in intensity of p-ERK-1/2 $p > 0.05$ upon addition of different amounts of exosomes.

4.2.7 Characterisation of the Effect of Non Conditioned Medium, CYM-5520 and Conditioned Medium from MDA-MB-231 Cells on MEFs Morphology

Although $S1P_2$ receptors are linked with anti-migratory effect in cancer cells (Yamamura *et al*., 2006), it has also been reported to be involved in cancer invasion and progression. For instance, glioma cells invasiveness through adhesion is modulated by $S1P_2$ receptors, which increase the matricellular protein CCN1/Cyr61 expression to promote tumour cells adhesion and invasion (Young, N. and Van Brocklyn, J.R. 2007). In addition, $S1P_2$ has been shown to mediate pancreatic cancer cells growth and invasion through modulating MMP9 transcription in pancreatic stellate cells (PSCs), which reside in tumour microenvironment and promote cancer growth and invasion. This is indicated by less cancer progression when $S1P_2$ is knocked down in PSCs co-implanted with cancer cells (Bi *et al*., 2014). Therefore, the effect of CM on formation of a migratory phenotype in fibroblasts was investigated. For this purpose, cells were stained for actin, a component of the cytoskeleton and which undergoes rearrangement in cells that exhibit a migratory phenotype.

MEFs have been previously shown to express endogenous $S1P_2$ (Goparaju *et al*., 2005). MEFs were immune-stained with anti-$S1P_2$ (Green) and anti-actin (red) antibodies. In MEFs treated with NCM containing 0.1% v/v DMSO, the actin was localised into lamellipodia extrusions, while $S1P_2$ receptor was localised to punctuate bodies in the cytoplasm (figure 4.8), suggesting that these receptors might be constitutively internalised in endosomes. The localisation of actin into lamellipodia is

typical of a migratory phenotype. Treatment of MEFs with the $S1P_2$ agonist CYM-5520 (10 μM) or CM induced rounding with loss of actin enriched lamellipodia protrusions (figure 4.8), indicating that cells lose their migratory phenotype. Therefore, agonism of endogenous $S1P_2$ receptors in MEFs or addition of CM containing exosomal $S1P_2$ induces loss of the migratory phenotype. This is consistent with the ability of CM to stimulate DNA synthesis, especially as these cells would need to be stationary in order to undergo mitosis. In addition, CM or CYM-5520 appears to induce accumulation of $S1P_2$ in punctuate bodies in the nucleus (figure 4.8), although it is unclear whether this is endogenous and/or exosomal derived $S1P_2$.

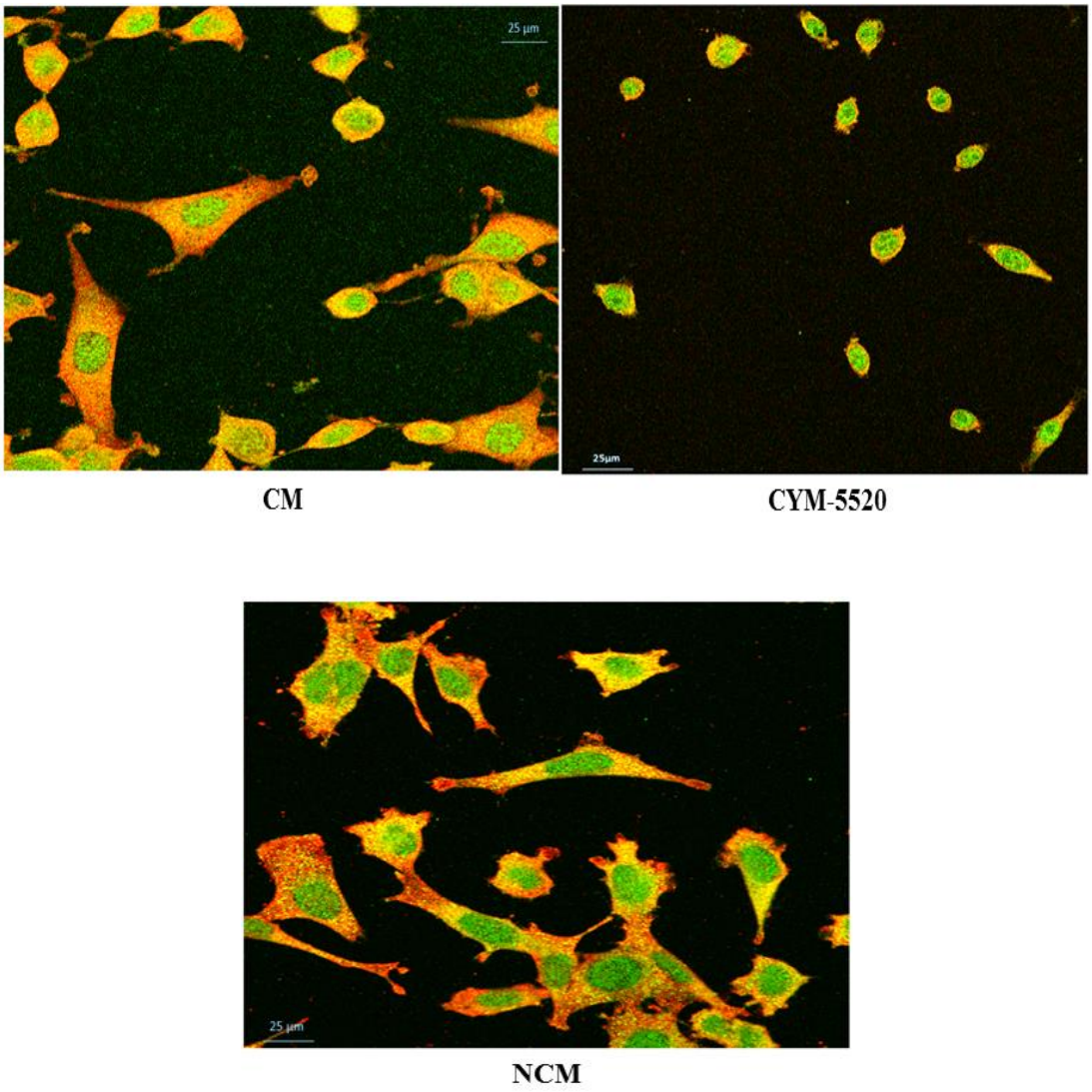


Figure 4.8 ***Characterization of the effect of Non-Conditioned Medium, CYM-5520 and Conditioned Medium from MDA-MB-231 cells on morphology, actin rearrangement and S1P$_2$ receptor distribution in MEFs.***

MEFs were immune-stained with anti-S1P$_2$ antibody or anti-actin antibody with secondrary antibody conjugated to FITC (green stain) or secondary antibody conjugated to TRITC (red stain) respectively. Images were taken using Leica TCS SP5 laser confocal microsope and lense used were Leica oil immersion lens. Results are representitive of three independent experiments. Scale bar is 25 µm.

4.2.8 Characterisation of the Effect of $S1P_2$ Selective Agonist CYM-5520 and Conditioned Medium from MDA-MB-231 Cells on ERK-1/2 Signalling in MEFs

In order to rule out the possibility that the CM might contain a factor (e.g. S1P) that stimulates the $S1P_2$ receptor to induce activation of ERK-1/2, $S1P_2$ receptor characterisation in MEFs was performed. First, treatment of MEFs with $S1P_2$ agonist CYM-5520 failed to induce ERK-1/2 activation, suggesting that endogenous $S1P_2$ receptors in MEFs are not involved in ERK-1/2 signalling (figure 4.9). Second, the lack of effect of JTE-013 on CM-induced activation of ERK-1/2, suggests that the exosomal $S1P_2$ receptor might not be modulated by JTE-013. JTE-013 is a competitive antagonist of $S1P_2$ with S1P (Blankenbach *et al*., 2016). However, there is a low-level expression of the shorter form of $S1P_2$ that is derived from endogenous full length $S1P_2$ in MEFs (figure 4.9 A). Interestingly, the amount of this shorter form is abolished when cells are treated with JTE-013 (figure 4.9 A), suggesting that endogenous S1P might cause activation of a small fraction of $S1P_2$, and that this involves proteolysis of the receptor. Indeed, this might account for why JTE-103 reduces the basal activation state of ERK-1/2. Therefore, the lack of effect of JTE-013 on the ability of CM containing $S1P_2$ to stimulate ERK-1/2 activation in MEFs, suggests that the factor is not S1P released from MDA-MB-231 cells. Alternatively, these results could be accounted for by constitutively active form of $S1P_2$ released from MDA-MB-231 cells. This would potentially render the $S1P_2$ insensitive to S1P and therefore also JTE-013. To establish whether the exosomal $S1P_2$ receptor derived from breast cancer cells might be modified to a constitutively active form by MEFs,

CM containing $S1P_2$ (42 kDa) isolated from MDA-MB-231 cells was added to MEFs and then the subsequently obtained MEFs lysates with anti-$S1P_2$ antibody were Western blotted. MEFs expressed a 42 kDa form of $S1P_2$ (figure 4.9). However, an additional ~38 kDa protein was detected with anti-$S1P_2$ antibody and this appeared only in MEFs treated with CM and this was not prevented by JTE-013 (figure 4.9 A, B). These findings, suggest that $S1P_2$ taken up by MEFs from CM and processed to a shorter form, which is likely constitutively active. Since the antibody used to detect $S1P_2$ is raised to the C-terminal tail of $S1P_2$, the generation of the shorter form is likely due to proteolysis at the N-terminus of the protein.

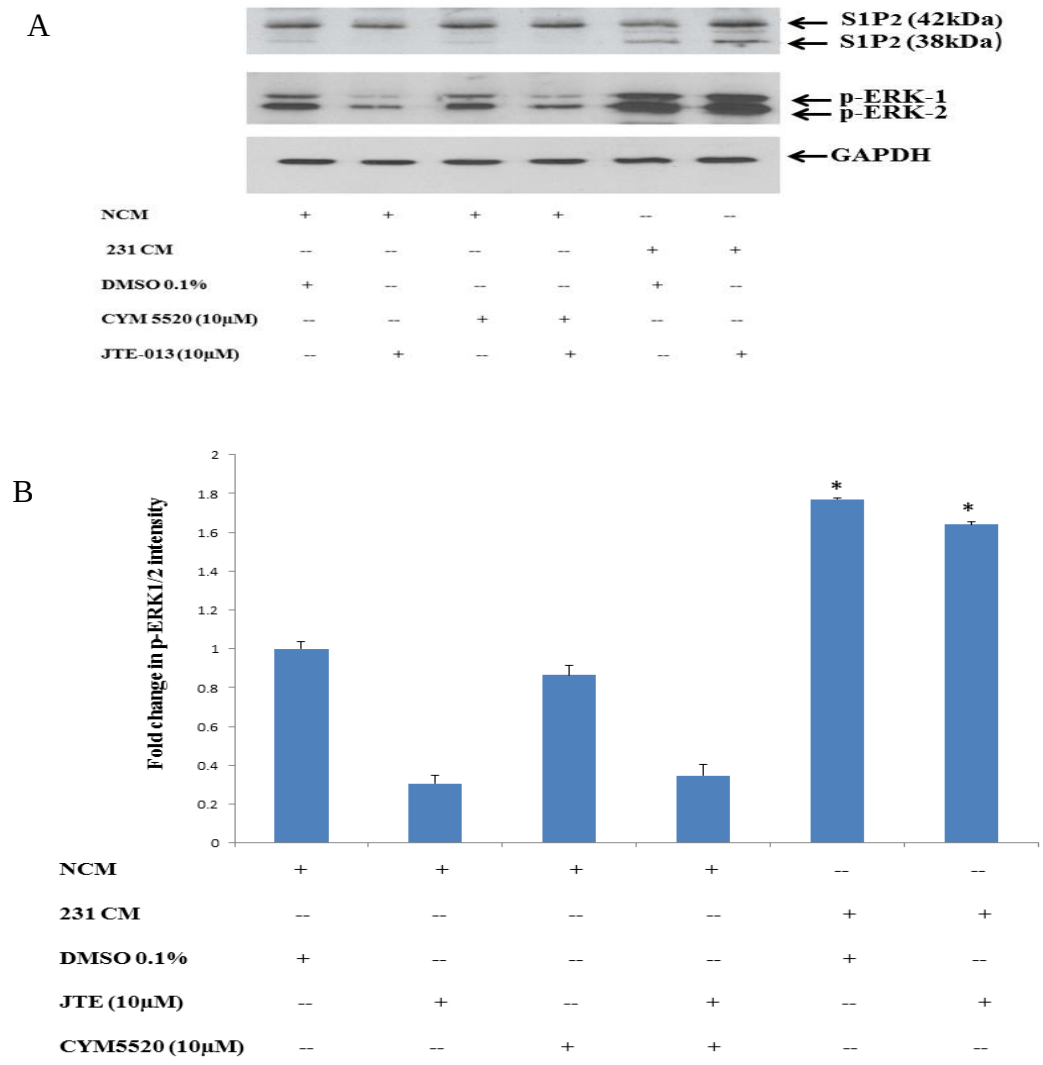


Figure 4.9 ***Characterisation of the effect of S1P$_2$ selective agonist CYM-5520 and Conditioned Medium from MDA-MB-231 cells on ERK-1/2 in MEFs.***

MEFs were grown on 12 wells plate until 70% confluence attained, then cells were quiescent for 24 hrs before being treated with either NCM containing 0.1% v/v DMSO or JTE-013 (10 µM) or CYM-5520 (10 µM) or CM for 25 mins. (A) MEFs were then harvested in Laemmli buffer and analysed by Western blotting with anti-S1P$_2$ and anti-p-ERK-1/2 antibodies. The membrane was stripped and re-probed with anti-GAPDH antibody to ensure equal protein loading. Results are typical of three independent experiments. (B) Densitometric quantification produced from immunoblots was presented as a mean +/- SEM fold change in p-ERK-1/2/GAPDH ratio for three independent experiments. The increase in fold change of p-ERK-1/2 was considered significance at $^{*}p < 0.05$ *versus* NCM (n=3).

4.2.9 Identification of Processing of the $S1P_2$ Receptor in Exosomes by MEFs

To further investigate whether the $S1P_2$ receptor in isolated exosomal preparations is similarly processed by MEFs to a shorter form, isolated exosomes were incubated with MEFs and the $S1P_2$ receptor analysed by Western blotting. A 38 kDa short form was detected in lysates but this was markedly increased in MEFs treated with exosomes containing the large $S1P_2$ receptor (Mr = 42 kDa) (figure 4.10).

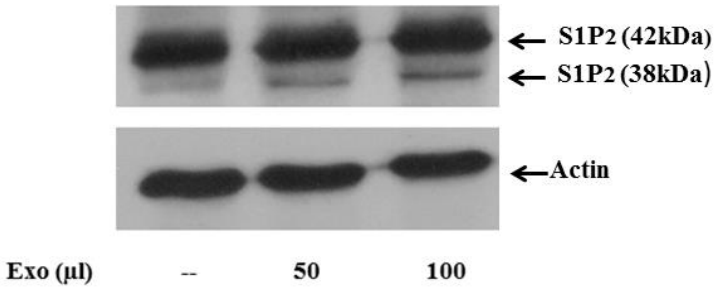


Figure 4.10 ***Identification of the short form of $S1P_2$ receptor in MEFs after incubation with exosomes isolated from Conditioned Medium derived from MDA-MB-231 cells.***

MEFs were grown on 12 wells plate until 70% confluence attained in complete medium. MEFs were made quiescent for 24 hrs before being treated with different amounts of exosomes as indicated for 10 mins. (A) MEFs were then harvested in Laemmli buffer and analysed by Western blotting using anti-$S1P_2$ antibody. The membrane was stripped and re-probed with anti-actin to ensure equal protein loading.

4.2.10 Detection of Exosomal marker GFP-hsp70 in MEFs Using Immuno-staining

In order to establish that MEFs can take up exosomal content, the exosomal marker GFP-hsp70 was used to establish whether it is also taken up by MEFs. GFP-hsp70 containing vesicles (green stain) were taken present in MEFs after incubation with CM from MDA-MB-231 cells over-expressing GFP-hsp70 protein (figure 4.11). Therefore, these findings, suggest that MEFs do take up proteins from exosomes preparation and that $S1P_2$ is processed by MEFs to the short form.

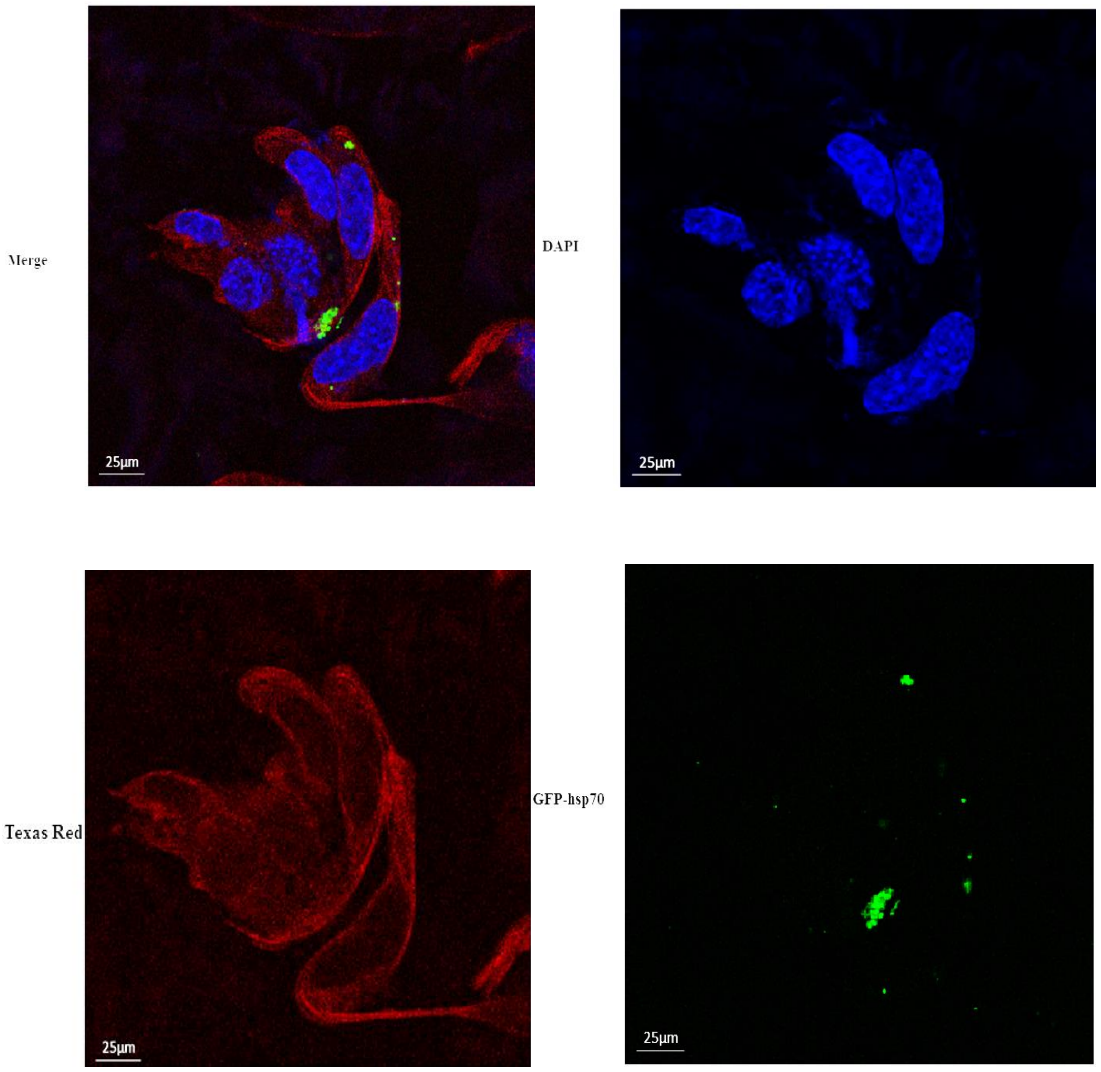


Figure 4.11 ***Immunofluorescence characterisations of GFP-hsp70 containing vesicles taken up by MEFs.***

GFP-hsp70 s taken up from exosomes in CM indicated by green fluorescence located within MEFs. Actin filaments are indicated by (red stain, Texas red) and nucleus indicated by (DAPI, blue stain). Results are representative of three independent experiments. Scale bar is 25 µm**.**

4.2.11 Characterisation ofthe Effect of Protease Enzyme Inhibitors on MDA-MB-231 Conditioned Medium Induced MEF Extracellular Signal-regulated Kinase

Matrix metalloproteinases (MMP) and a disintegrin and metalloproteinases (ADAM) are members of the metzincins superfamily of zinc-based proteinases. These proteinases are capable of digesting the ECM, macromolecules and non-ECM molecules including receptors, growth factors, cytokines and chemokines, all of which are elements of the tissue microenvironment (Shiomi *et al*., 2010). For instance, ADAM 10 and ADAM 17 are abundant transmembrane proteases, which proteolytically cleave, or shed the extracellular regions of other transmembrane proteins (Matthews *et al*., 2016). Thus, it is important to investigate the effect of protease inhibitors on CM induced MEFs ERK-1/2 activation. The 1, 10 phenanthroline and GM6001 were unable to inhibit CM induced ERK-1/2 activation (figure 4.12) and (figure 4.13), and this is might be attributed to the existence of large number of proteinases, which make difficult to target all of them by 1, 10 phenanthroline or GM6001.

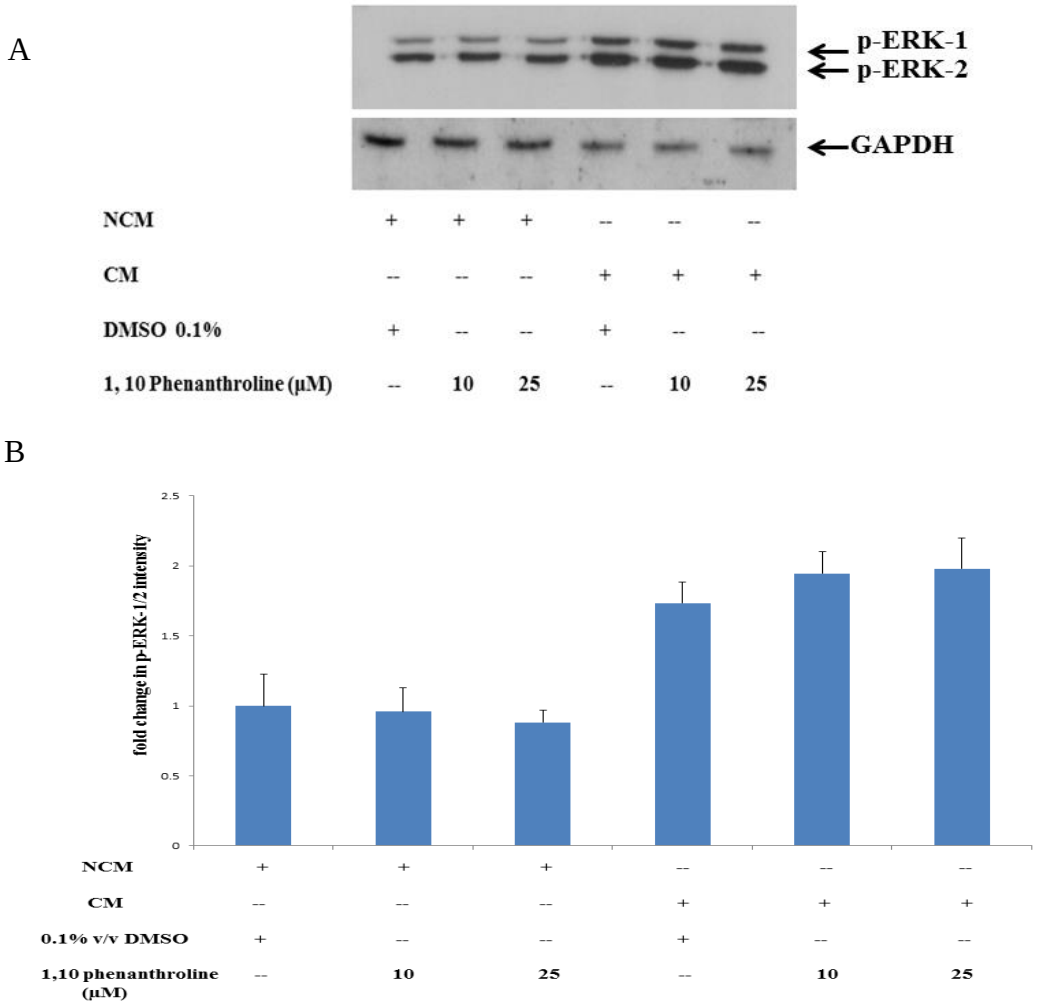


Figure 4.12 ***Characterisation of the effect of 1, 10 phenanthroline on MDA-MB-231 Conditioned Medium induced MEF extracellular signal-regulated kinase.***

MEFs were grown on 12 wells plate until 70% confluence attained. MEFs were quiescent for 24 hrs before being pretreated with either 0.1% v/v DMSO or 1, 10 phenanthroline (10-25 µM) for 15 mins and incubated for 10 mins with either NCM or CM. (A) MEFs were then harvested in Laemmli buffer and analysed by Western blotting using anti-p-ERK-1/2 antibody. The membrane was stripped and re-probed with GAPDH to ensure equal protein loading. Results are typical of three independent experiments**.** (B) Densitometric quantification produced from immunoblots was presented as a mean +/- SEM fold change in p-ERK-1/2/GAPDH ratio for three independent experiments. Statistical analysis *t*-test was performed and there was no significant reduction in intensity of p-ERK-1/2 $p > 0.05$ *versus* NCM, (n=3).

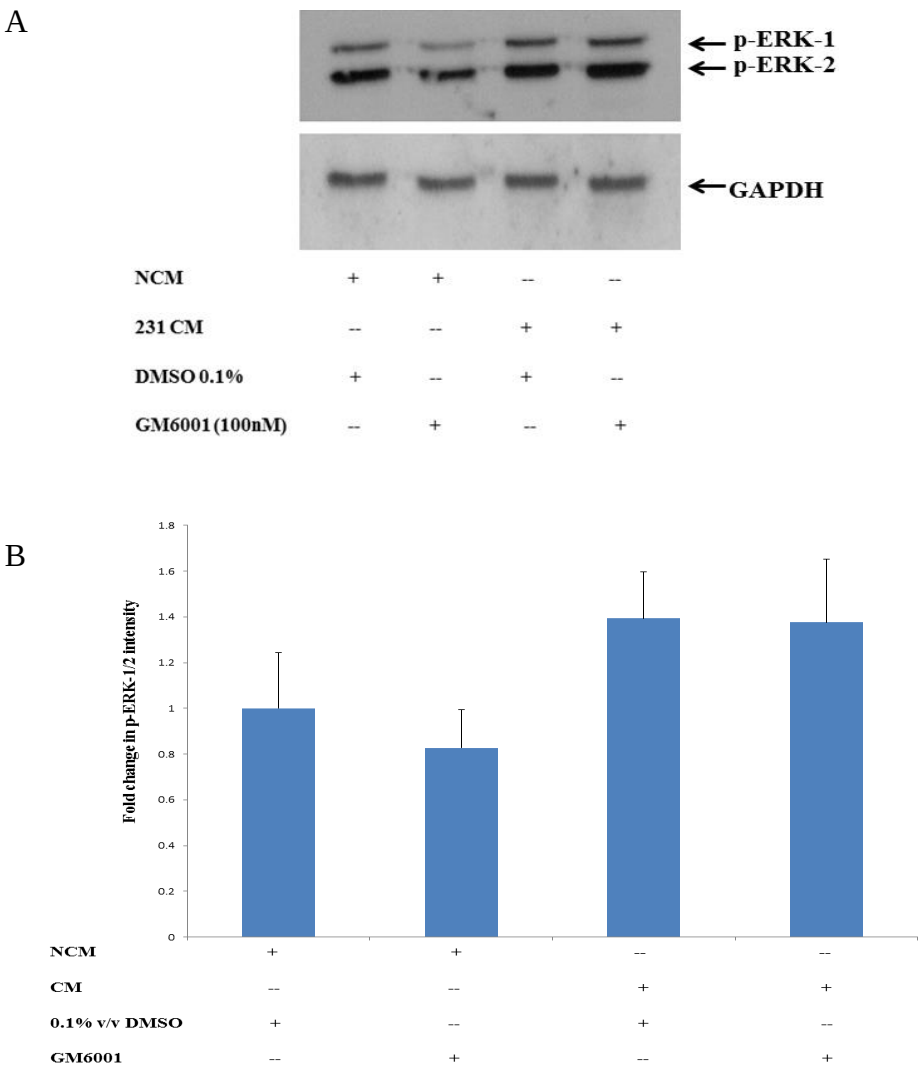


Figure 4.13 ***Characterisation of the effect of GM6001 on MDA-MB-231 Conditioned Medium induced MEF extracellular signal-regulated kinase.***

MEFs were grown on 12 wells plate, until 75% confluence attained. MEFs were made quiescent for 24 hrs before being pretreated with 0.1% v/v DMSO or GM6001 (100 nM) for 15 mins and incubated for 10 mins with either NCM or CM. (A) MEFs were then harvested in Laemmli buffer and analysed by Western blotting using anti-p-ERK-1/2 antibody. The membrane was stripped and re-probed with GAPDH to ensure equal protein loading. Results are typical of three independent experiments. (B) Densitometric quantification produced from immunoblots was presented as a mean +/- SEM fold change in p-ERK-1/2/GAPDH ratio for three independent experiments. Statistical analysis *t*-test was performed and there was no significant reduction in intensity of p-ERK-1/2 $p > 0.05$ *versus* NCM, (n=3).

4.3 Discussion

First, it is necessary to give a brief overview on the role of cancer associated fibroblasts (CAF) or activated fibroblasts in tumour stroma. Fibroblasts are multi-functional and are present in connective tissue depositing ECM, regulating differentiation in associated epithelial cells, modulating immune responses and mediating homeostasis (Tomasek *et al*., 2002; Kalluri and Zeisberg, 2006). The recruitment of stromal fibroblasts to the tumour is largely directed by growth factors released from cancer cells such as TGFβ, PDGF and fibroblast growth factor 2 (FGF2) (Elenbaas and Weinberg, 2001). In several co-culture experiments, CAFs have been shown to increase the tumourigenesis of cancer cells when compared with normal activated fibroblasts (NAFs) (Orimo *et al*., 2005) for instance, normal prostate epithelial cells develop into intraepithelial neoplasia in mice when co-injected with CAFs compared with normal fibroblasts (Olumi *et al*., 2000). Upon accumulation in the tumour microenvironment, CAFs are activated by growth factors and cytokines that are present in the close environment such as TGF-β, PDGF, monocyte chemotactic protein (MCP1), FGF and proteases (Marsh, Pietras and McAllister, 2013; Kalluri and Zeisberg, 2006). Importantly, Yes-associated protein 1 (YAP) has been shown to be upregulated in CAFs and is responsible for remodelling of the ECM and modulation of the cytoskeleton and matrix stiffness that lead to an increase in cancer invasion (Calvo *et al*., 2013). Activated fibroblasts produce ECM degrading protease such as MMP3 that cleaves E-cadherin, prompting epithelial to mesenchymal transition (EMT) and enhancing cancer invasiveness (Lochter *et al*., 1997). In addition, activated fibroblasts produce growth factors, which enhance tumourigenesis, including VEGF, which induces enhanced vascular permeability and

promotes angiogenesis. Moreover, CAFs produce pro-inflammatory factors that activate NFκB signalling in cancer cells to promote tumour progression (Fukumura *et al*., 1998; Erez *et al*., 2010).

Therefore, all these studies reveal the complexity of the interaction between cancer cells and CAFs and demonstrate their importance within the tumour microenvironment in modulating cancer invasion and metastasis.

In this study $S1P_2$ and $S1P_4$ receptors were shown to be released into CM from MDA-MB-231 and MDA-MB-453 cells respectively. $S1P_2$ are unequivocally demonstrated to be present in exosomes (chapter III). Moreover, CM from MDA-MB-231 cells containing $S1P_2$ receptors was demonstrated to induce ERK-1/2 activation of MEFs, whereas CM from MDA-MB-453 cells containing $S1P_4$ was unable to induce such activation of this kinase pathway, thereby demonstrating specificity for $S1P_2$ in fibroblasts. Also, addition of the $S1P_2$ competitive antagonist JTE-013 to MEFs failed to reduce ERK-1/2 activation induced by CM from MDA-MB-231 cells. These preliminary observations raise the possibility that the released $S1P_2$ in CM from MDA-MB-231 cells might be constitutively active, since it was insensitive to the competitive antagonist JTE-013 in terms of its ability to induce ERK-1/2 activation in MEFs. These findings, therefore also exclude the possibility that the effect on ERK-1/2 activation in MEFs are mediated by S1P itself.

The major finding in this study is that $S1P_2$ receptor released into CM from MDA-MB-231 cells is present in exosomes. Moreover, exosomal $S1P_2$ appears to be taken up and processed by MEFs to produce the shorter form of $S1P_2$ with a (Mr ~38 kDa) (figure 4.15). Therefore, the shorter form of $S1P_2$ might be constitutively active and

able to induce ligand independent signalling to ERK-1/2 to promote DNA synthesis in MEFs. Validation that the activation of ERK-1/2 in MEFs was mediated by exosomal $S1P_2$ was provided by the demonstration that CM from MDA-MB-231 cells in which $S1P_2$ expression had been reduced by siRNA knockdown failed to stimulate the ERK-1/2 pathway in MEFs. In addition, $S1P_2$ competitive antagonist JTE-013 failed to block the CM-stimulated activation of ERK-1/2, suggesting that $S1P_2$ receptors that are taken up and processed by MEFs are constitutively active, and are therefore not modulated by $S1P_2$ competitive antagonist JTE-013.

Previously, the release of S1P receptors subtype $S1P_3$ and $S1P_4$ in exosomes was demonstrated by Amorim *et al* (2014) using proteomic analysis. Nevertheless, the functional role of $S1P_{3/4}$ receptors in exosomes was not examined in this study (Amorim *et al*., 2014), and thus the current study is the first to evaluate the role of exosomal S1P receptors from cancer cells on fibroblast cell activation. Importantly, CM derived from MDA-MB-453 breast cancer cells containing $S1P_4$ receptors had no effect on ERK-1/2 signalling in MEFs, suggesting that $S1P_4$ receptor might be linked to other signalling pathway in MEFs which have not been examined.

It is necessary herein to discuss the role of Ser/Thr cluster for example, SSS, SXSS and SSXS in C-terminal of GPCR in formation of stable GPCR-β-arrestin complex (Pyne and Pyne, 2017). According to NCBI reference sequence $S1P_2$, $S1P_4$ and V2R have Ser/Thr cluster in its C-terminal (Ncbi.nlm.nih.gov, 2017) (figure 4.14).

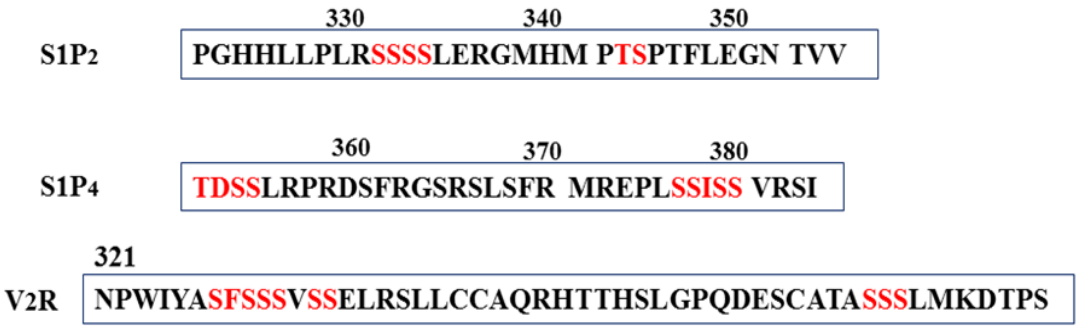


Figure 4.14 ***Amino acids sequences of the C-terminus for $S1P_2$, $S1P_4$ and V2R with Serine/Thr amino acid cluster potentially involved in β-arrestin binding in red.***

Therefore, $S1P_2$ and $S1P_4$ belong to class B GPCRs similar to V2R and, hence can exhibit strong C-terminal binding to β-arrestin. Moreover, this strong interaction between class B GPCRs C-tail and β-arrestin has been shown to induce sustained internalisation and continuous G-protein signalling in endosomes (Oakley *et al*., 2001; Calebiro *et al*., 2009; Irannejad *et al*., 2013). This strong association between β-arrestin and receptors might confer ability of these receptors to be packaged in exosomes and released. Importantly, $G_{\beta\gamma}$ subunit of $G\alpha_i$ protein has been shown to be associated with activation of Rho family GTPases on MVBs (Kajimoto *et al*., 2017). Thus, $S1P_2$ might be involved in exosomes formation and subsequent release by activating Rho kinase signalling through G_i. Importantly, $S1P_2$ has been shown to couple to multiple G-proteins including $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ (Adada *et al*., 2013; Blaho and Hla, 2014).

Class A GPCRs which lack Ser/Thr clusters exhibit weak binding between C-tail and β-arrestin and therefore inefficient interaction with G-proteins. In addition, these

receptors utilise G-protein or β-arrestin separately to regulate ERK-1/2 signalling (Thomsen *et al*., 2016; Shenoy and Lefkowitz, 2011).

Endogenous $S1P_2$ receptors in MEFs are not efficiently coupled to ERK-1/2 evidenced by lack of the effect of $S1P_2$ agonist CYM-5520; although this agonist promotes loss of actin enriched lamellipodia indicating that the endogenous $S1P_2$ receptor is functionally active in these cells. Therefore, this finding differentiates between endogenous $S1P_2$ in MEFs from the shorter constitutively active form derived from breast cancer cells, which can activate ERK-1/2 in MEFs. Indeed, $S1P_2$ has been shown to mediate S1P inhibitory effect on Akt, Rac and migration, thereby negatively regulating tumour cells angiogenesis and proliferation through coupling to $G_{12/13}$ Rho-dependent signalling (Du *et al*., 2010). Thus, a postulation was made that in order to metastasise, MDA-MB-231 cancer cells release $S1P_2$ in exosomes and not S1P, attempting to reduce its inhibitory effect on cancer migration and invasion. In turn, MEFs can take up exosomal $S1P_2$ released from cancer cells and process them to a constitutively active form that can promote MEFs proliferation.

Subsequently, the increased growth of MEFs means more growth factors; metalloproteinase and cytokines might be potentially released from MEFs, and which reciprocally interact with cancer cells facilitating MDA-MB-231 breast cancer cells growth and metastasis. Notably, the N-terminus of GPCRs is demonstrated to regulate ligand binding, for instance, parathyroid hormone with its cognate receptor (Vilardaga *et al*., 2011). In addition, the extracellular domain of GPCR has been shown to affect ligand recognition, ligand "escorting" into the binding pocket and ligand binding kinetics (Wittmann, 2011). Importantly, the $S1P_2$ antibody used

during this work is engineered to bind the C-terminus of the $S1P_2$ receptor, suggesting that $S1P_2$ receptor proteolysis might occur at the N-terminus as only 4 kDa is missing. In this regard, constitutive activation of $S1P_2$ might occur as a consequence of proteolysis of the $S1P_2$ receptor at the N-terminus, which might normally exert an inhibitory influence on $S1P_2$ signalling. Therefore, removal of N-terminal tail by proteolysis would relieve this inhibitory influence and lead to constitutive activation of $S1P_2$. This observation was supported by previous work by Zhang *et al* (2002) who demonstrated that deletion of 98% of the N-terminus of thyroid stimulating hormone receptor (TSHR) resulted in 4-7 fold increase in basal activity of the receptor compared with TSHR wild type (Zhang *et al*., 2002).

In addition, truncation of GPR56 (an orphan adhesion GPCR characterized by long N-terminus extracellular domain) resulted in constitutive activation of the receptor as evidenced by significant increase in Rho signalling activity compared with full length wild type of the receptor. Moreover, the N-terminus truncated version of GPR56 demonstrated enhanced binding to β-arrestin compared with the wild type GPR56 full-length receptor (Paavola *et al*., 2011).

Also, brain-specific angiogenesis inhibitor 2 (BAI2) is a member of the adhesion GPCRs that are highly expressed in the brain. The functionality of these receptors has been shown to be regulated by proteolysis cleavage of the N-terminus of a GPCR proteolysis site (GPS), demonstrated by a tenfold increase in the nuclear factor of activated T-cells (NFAT) signalling compared with full length receptor (Okajima *et al*., 2010). An additional example where N-terminal proteolysis of GPCRs can modulate signalling is protease activated receptors (PARs), where thrombin binds to

PAR1 and cleaves the N-terminus, generating a tether ligand that binds intra-molecularly to transmembrane domains and triggers receptor activation and signal transduction (Soh *et al*., 2010).

The N-terminus removal or proteolysis is also reported to inactivate GPCRs. For example, GPR37L1 is an orphan GPCR without a validated ligand and abundant in the central nervous system. The full length GPR37L1 is constitutively coupled to $G_{\alpha s}$ and genetic deletion of the N-terminus creates a functionally inactive receptor (Leng *et al*., 1999; Coleman *et al*., 2016). In addition, the ERK-1/2 activation by full endothelin receptor type B (ETB) involves an early phase followed by a second slow phase, which is EGFR-dependent. In contrast, the truncated version of ETB receptor (Δ2-64-ETB) is able to stimulate the early phase of ERK-1/2 activation in response to ET-1, but the second phase is absent (Grantcharova *et al*., 2006).

Preliminary work with protease enzyme inhibitors 1, 10 phenanthroline and GM6001 in MEFs failed to reduce CM induced ERK-1/2 stimulation. However, there are many protease family members with multi-functions and require further investigation in the future. For example, MMP 11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP17, MMP24, MMP26, MMP27 and MMP28 (Sagi *et al*., 2016).

CM isolated from MDA-MB-231 cells also increased DNA synthesis in MEFs, suggesting a possible linkage with the effect of short form $S1P_2$ on ERK-1/2 activation, as ERK-1/2 signalling can regulate cell proliferation (Mebratu and Tesfaigzi, 2009). Moreover, ERK-1/2 activation and translocation into the nucleus is required for gene expression and ensuring G1 to S phase progression (Yamamoto *et al*., 2006).

Therefore, these findings underline the potential and significant role of the N-terminus in regulating $S1P_2$ signalling, and present a role for processing at the N-terminus in enabling intercellular communication between cancer cells and fibroblasts.

In addition, these findings describe a different model from that reported by Ponnusamy *et al*. (2012) who demonstrated that S1P formed by host SK1 binds to $S1P_2$ in breast cancer cells and inhibits the induction of the metastasis suppressor, Brms1 (breast carcinoma metastasis suppressor 1), thus promoting cancer metastasis (Ponnusamy *et al*., 2012).

Future studies will require characterisation of the signalling properties of the truncated $S1P_2$ receptor (figure 4.16) and whether this form can constitutively promote metastatic spread. This can be explored by over-expression of the truncated short form of $S1P_2$ with a Mr ~38 kDa in fibroblasts *in vivo* in order to establish the effect on metastatic spread in breast cancer orthotopic mouse models. Nevertheless, this finding establishes a potentially novel paradigm for the role of $S1P_2$ in fibroblast-cancer cells interactions.

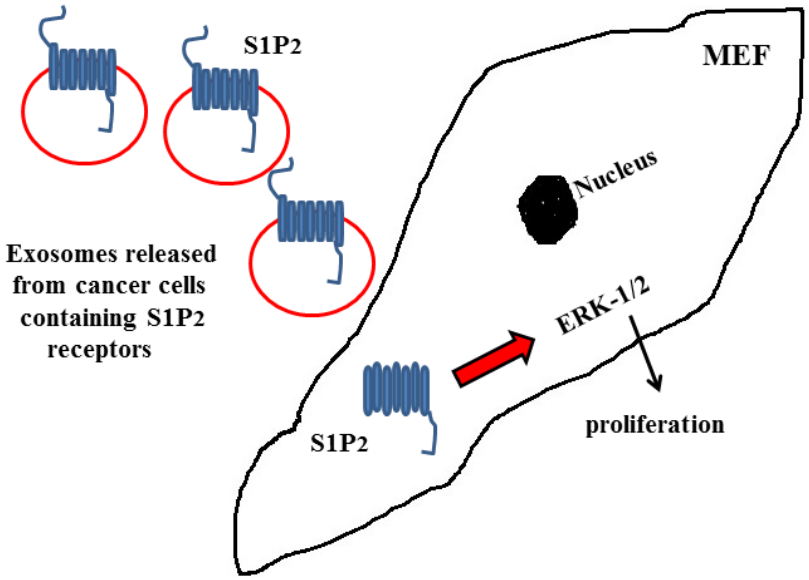


Figure 4.15 ***Basic diagram illustrating exosomes fibroblasts interaction.***

Exosomes released from MDA-MB-231 breast cancer cells and containing $S1P_2$ receptor taken up by MEFs and processed (N-terminus proteolysis) to constitutively active shorter form, which is able to induce ERK-1/2 activation in MEFs, thereby inducing MEFs proliferation. Exosomes represented in red and GPCR represented in blue.

Figure 4.16 ***Amino acid sequence of S1P$_2$ with proposed site of cleavage.***

Amino acid sequence of human S1P$_2$ receptor, the amino acids in red represent the truncated N-terminus (~36 amino acids = 4 kDa) of S1P$_2$ receptor and arrow refer to the possible site of proteolysis.

CHAPTER V

CHARACTERISATION OF THE ROLE OF S1P AND SPHINGOSINE KINASES ON $S1P_2$ AND $S1P_4$ RELEASE

CHAPTER 5: Characterisation of the Role of S1P and Sphingosine Kinases on $S1P_2$ and $S1P_4$ Release

5.1 Introduction

Exosomes are endocytic compartments in nature derived from endosomal vesicles MBVs, which are formed from internalisation of plasma membrane components, macromolecules, fluids and other proteins; this process is generally termed endocytosis (Huotari and Helenius, 2011; Théry, 2011). The first study that reported the involvement of sphingosine and S1P in endocytic process was performed by Rosa *et al* (2010). The team demonstrated that sphingosine and S1P have a Ca^{2+}-dependent permissive role in initiation endocytosis process (Rosa *et al*., 2010). Moreover, SK1 has been shown to be involved in the early endocytic process based on evidence demonstrating that depletion of SK1 in Hela cells using small interference RNA resulted in an increase of internalised transferrin (Collinet *et al*., 2010). In addition, disturbing the balance between cholesterol and sphingomyelin using methyl-β–cyclodextrin (MβCD), resulted in the formation of collections of narrow endocytic tubular invaginations enriched with SK1 and Bin/Amphiphysin/Rvs (N-BAR) proteins; the latter is involved in the membrane remodelling that determines the curvature state of the membrane (Shen *et al*., 2014; Mim *et al*., 2012).

In recent years, Kajimot *et al* (2013) demonstrated that a constant supply of S1P generated by SK2 in Hela cells enables S1PRs to be constitutively active on MVBs, regulating cargo sorting into ILVs (exosomes) and maturation of MVBs. In addition,

depletion of $S1P_1$ and SK2 expression resulted in a decrease of mCherry-CD63 positive MVBs detected by fluorescense resonance energy transfer (FRET). These findings, suggest that activation of S1PRs by S1P is involved in the ESCRT independent maturation of MVBs (Kajimoto *et al*., 2013).

Moreover, EVs have been shown to be released from HB4a, a mammary luminal epithelial cell line and HER2 over-expressing C5.2 cell line, with higher HER2 level in EVs derived from C5.2 cell line (Amorim *et al*., 2014). In addition, $S1P_3$ and $S1P_4$ receptors were detected in EVs released from C5.2 cell line (Amorim *et al*., 2014). Notably, Long *et al* (2010) have demonstrated a functional link between $S1P_4$ and HER2 regulating ERK-1/2 signalling in MDA-MB-453 breast cancer cells (Long *et al*., 2010).

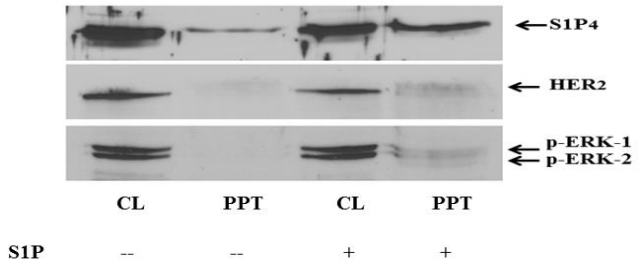
Therefore, it is important to investigate whether exogenous S1P or SKs inhibitors or $S1P_{2/4}$ agonists might mediate any change in the level of $S1P_2$ or $S1P_4$ in CM and thereby their release into CM.

5.2 Results

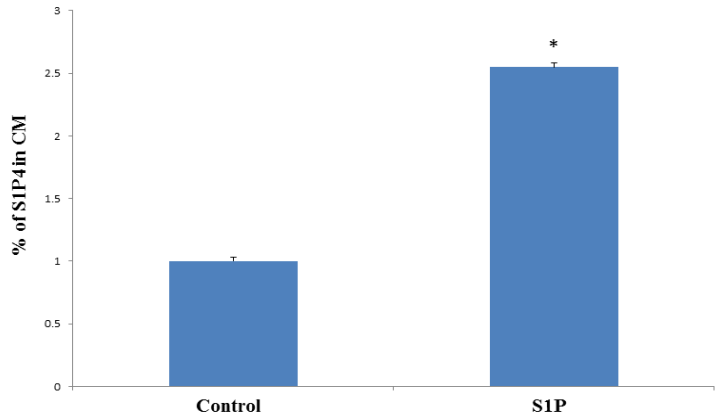
5.2.1 The Effect of Exogenous S1P on $S1P_4$ Release into Conditioned Medium from MDA-MB-453 Cells

Most of GPCRs undergo receptor internalisation upon binding to an agonist or endogenous ligand. Therefore, it is important to examine the effect of S1P on $S1P_4$ release into CM. In this regard, added S1P (5 µM) induced an increase in the amount of $S1P_4$ receptor (Mr 42 kDa) released into CM from MDA-MB-453 cells detected by Western blotting using anti-$S1P_4$ antibody (figure 5.1 A, B). Previous studies have shown that $S1P_4$ functionally interacts with HER2 to regulate activation of ERK-1/2 in MDA-MB-453 cells (Long *et al.*, 2010). Moreover, Amorium *et al* (2014) demonstrated that HER2 and $S1P_4$ are present in exosomes released from mammary cells. Therefore, CM from MDA-MB-453 cells was analysed for HER2 and ERK-1/2 content and significant amount of HER2 (Mr180 kDa) and ERK-1/2 were also present in CM from S1P treated MDA-MB-453 cells (figure 5.1 A-D).

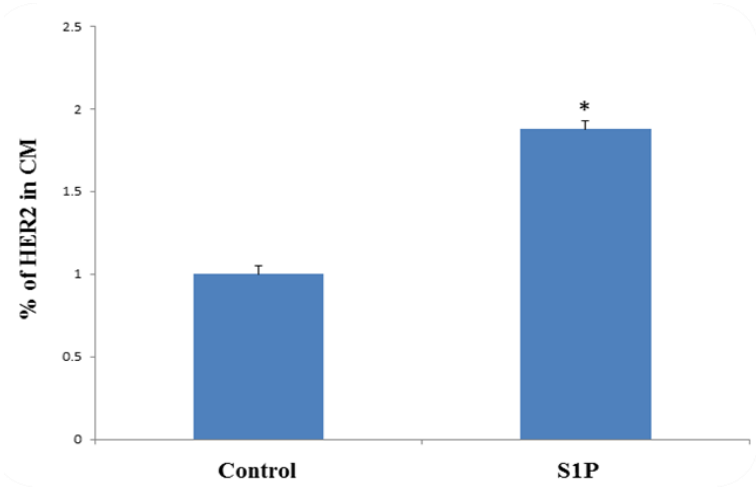
A


S1P4
HER2
p-ERK-1
p-ERK-2
CL
PPT
CL
PPT
S1P
--
--
+
+

B


% of S1P4 in CM
3
2.5
2
1.5
1
0.5
0
*
Control
S1P

C


% of HER2 in CM
2.5
2
1.5
1
0.5
0
*
Control
S1P

D

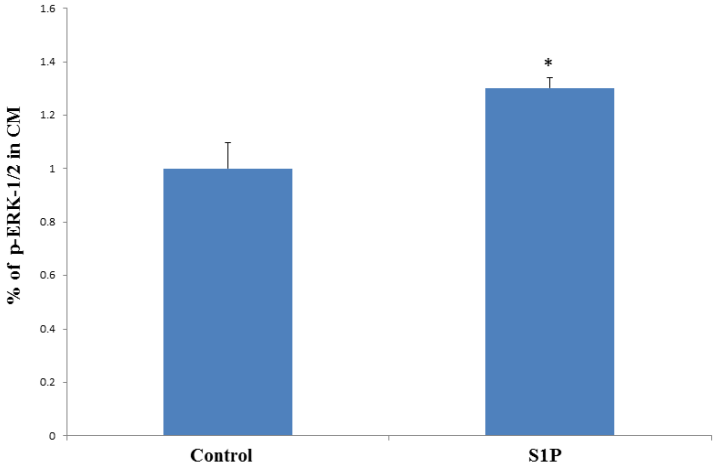

% of p-ERK-1/2 in CM
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
S1P

Figure 5.1 ***The Effect of addition of exogenous S1P on $S1P_4$, HER2 and extracellular signal-regulated kinase release into Conditioned Medium from MDA-MB-453 cells.***

Cells were treated with either 0.1% v/v DMSO (vehicle) or exogenous S1P (5 μM) for 1 hr prior to collection of CM. CM was collected and its protein contents were acid precipitated (PPT) and Laemmli sample buffer added. (A) Cells lysate (CL) were also harvested in Laemmli buffer. These samples were analysed by Western blotting and $S1P_4$ was assessed using anti-$S1P_4$ antibody. Membrane was stripped and reprobed with anti-HER2 and anti-p-ERK-1/2 antibodies. Results are typical of three independent experiments. (B, C and D) Densitometric quantification expressed as mean +/- SEM for the percentage of $S1P_4$, HER2 and p-ERK-1/2 in CM for three independent experiments. Statistical analysis was completed by Graph Pad Prism program using statistical *t*-test and difference was considered significance when * $p < 0.05$ *versus* control (vehicle).

5.2.2 The Effect of Exogenous S1P on $S1P_2$ Release into Conditioned Medium from MDA-MB-231 Cells

In order to examine S1P effect on $S1P_2$ release into CM, exogenous S1P (5 μM) or 0.1% v/v DMSO was added to MDA-MB-231 cells for 1 hr. CM was collected and its protein contents were acid precipitated (PPT). PPT was harvested in Laemlli buffer and analysed by Western blotting and $S1P_2$ was assessed using anti-$S1P_2$ antibody. A small shorter form ~38 kDa of $S1P_2$ detected in CM of S1P (5 μM) treated sample (figure 5.2), suggesting $S1P_2$ might be degraded by proteolysis in manner to limit S1P-$S1P_2$ anti-migratory signalling, which considered anti-tumourigenic.

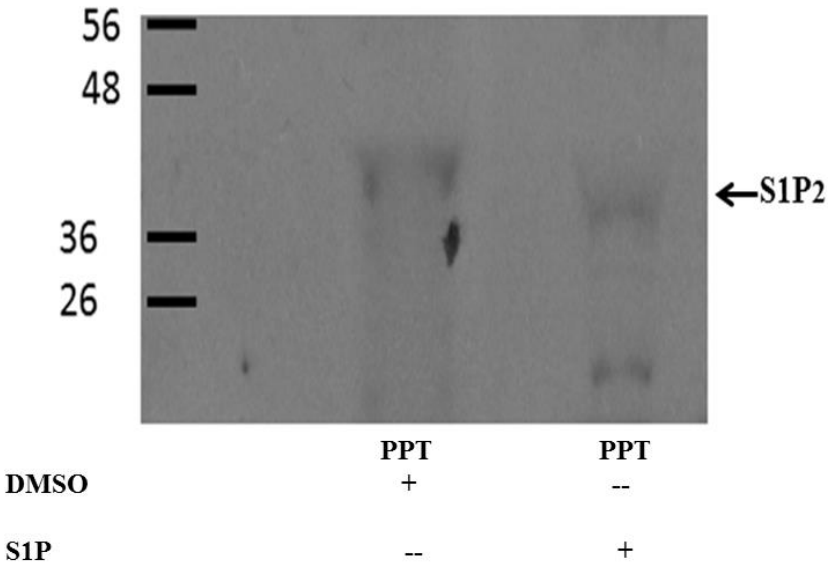


Figure 5.2 ***The effect of addition of exogenous S1P on MDA-MB-231 cells.***

MDA-MB-231 cells were treated with either 0.1% v/v DMSO (vehicle) or exogenous S1P (5 μM) for 1 hr prior to collection of CM. CM was collected and its protein contents were acid precipitated (PPT). (PPT) was mixed with Laemmli sample buffer and analysed by Western blotting and $S1P_2$ was assessed using anti-$S1P_2$ antibody. Results are typical of three independent experiments.

5.2.3 Characterisation of the Effects of $S1P_2$ Agonist CYM-5520 and $S1P_2/_4$ Antagonist JTE-013 on $S1P_2$ Release from MDA-MB-231 Cells

Treatment of MDA-MB-231 cells with exogenous S1P (5 µM, 1h) induced the release of a smaller form of $S1P_2$ with a low molecular mass of 38 kDa into CM (figure 5.2). It was important to establish whether modulators of $S1P_2$ can also cause release of the short form of $S1P_2$. In order to investigate this question, a synthetic $S1P_2$ agonist CYM-5520 (Satsu *et al*., 2013), and the $S1P_{2/4}$ antagonist, JTE-013 (Long *et al*., 2010) were used. MDA-MB-231 cells were treated with either 0.1% v/v DMSO (vehicle) or $S1P_2$ agonist CYM-5520 or the $S1P_{2/4}$ antagonist JTE-013 for 24 hrs prior to collection of CM. Notably, this experiment is separate from S1P effect as on MDA-MB-231 cells and therefore these are not comparable. Treatment of these cells with either CYM-5520 or JTE-013 (each 10 µM) had no effect on the release of $S1P_2$ into CM (figure 5.3 A, B), suggesting $S1P_2$ release into CM is independent of S1P.

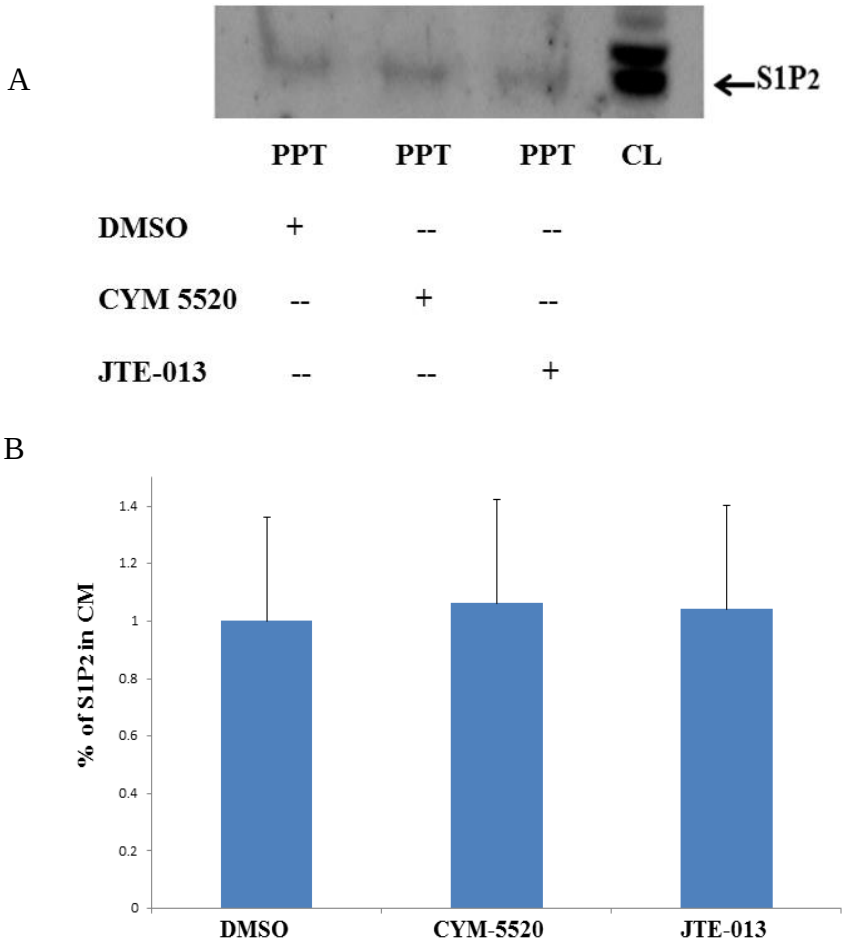


Figure 5.3 ***Effects of $S1P_2$ agonist CYM-5520 and $S1P_2/_4$ antagonist JTE-013 on $S1P_2$ release from MDA-MB-231 cells into Conditioned Medium.***

MDA-MB-231 cells were treated with either 0.1% v/v DMSO (vehicle) or CYM-5520 (10 µM) or JTE-013 (10 µM) for 24 hrs prior to collection of CM. (A) CM was collected and its protein contents were acid precipitated (PPT) and Laemmli buffer was added. Cell lysate (CL) were also harvested in Laemmli buffer. These samples were analysed by Western blotting and $S1P_2$ was assessed using anti-$S1P_2$ antibody. Results are representative of three independent experiments. (B) Densitometric quantification expressed as mean +/- SEM for the percentage of $S1P_2$ in CM for three independent experiments. Statistical analysis was completed by Graph Pad Prism program using statistical *t*-test and difference was considered significance when * $p < 0.05$ *versus* vehicle.

5.2.4 Characterisation of the Effects of Addition of $S1P_4$ Agonist CYM-50308 and $S1P_{2/4}$ Antagonist JTE-013 on $S1P_4$ Basal Level in MDA-MB-453 Cells

To establish whether the synthetic $S1P_4$ agonist CYM-50308 might modulate $S1P_4$ release into CM. MDA-MB-453 cells were treated with either 0.1% v/v DMSO (vehicle) or $S1P_4$ agonist CYM-50308 (10 μM) (Urbano *et al*., 2011) or $S1P_{2/4}$ antagonist JTE-013 for 24 hrs prior to collection of CM. Treatment of cells with CYM-50308 (10 μM) had no effect on the release of $S1P_4$ into CM. However, treatment of these cells with JTE-013 (10 μM) has significantly $p < 0.05$ reduced the amount of $S1P_4$ released into CM (figure 5.4 A, B), suggesting that JTE-013 might block the effect of endogenous S1P on the release of $S1P_4$. Alternatively, JTE-013 might act as an inverse agonist on $S1P_4$ to block the release of this receptor from MDA-MB-453 cells.

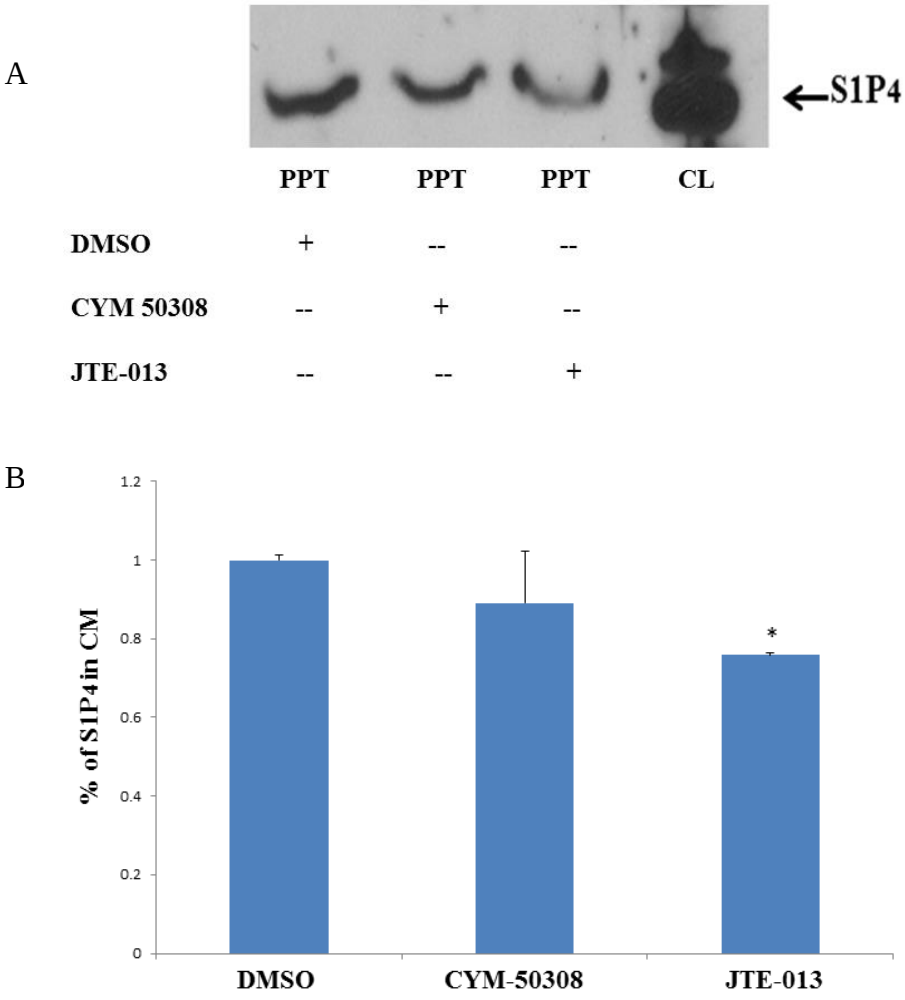


Figure 5.4 ***Effects of $S1P_4$ agonist CYM-50308 and $S1P_{2/4}$ antagonist JTE-013 on $S1P_4$ level in Conditioned Medium from MDA-MB-453 cells.***

MDA-MB-453 cells were treated with either 0.1% v/v DMSO (vehicle) or CYM-50308 (10 µM) or JTE-013 (10 µ M) for 24 hrs prior to collection of CM. CM was collected and its protein contents were acid precipitated (PPT) and Laemmli buffer added. Cell lysate (CL) were also harvested in Laemmli buffer. (A) These samples were analysed by Western blotting and $S1P_4$ was assessed using anti-$S1P_4$ antibody. Results are representative of three independent experiments. (B) Densitometric quantification expressed as mean +/- SEM for the percentage of $S1P_4$ in CM for three independent experiments. Statistical analysis was completed by Graph Pad Prism program using statistical *t*-test and difference was considered significant when * $p < 0.05$ *versus* vehicle.

5.2.5 The Effects of Addition of Exogenous S1P, $S1P_2$ Agonist CYM-5520 and $S1P_4$ Agonist CYM-50308 on $S1P_2$ and $S1P_4$ Receptors Expression Level in MDA-MB-231 and MDA-MB-453 Cells.

It is important to investigate whether some of the effects observed on S1P receptor release are related to changes in the expression of these receptors in MDA-MB-231 and MDA-MB-453 cells. In this regard, S1P (5 µM) and CYM-50308 (10 µM) had no effect on $S1P_4$ receptor expression levels in MDA-MB-453 cells (figure 5.5 A, B). Similarly, S1P (5 µM) and CYM-5520 (10 µM) had no effect on $S1P_2$ expression levels in MDA-MB-231 cells (figure 5.6 A, B).

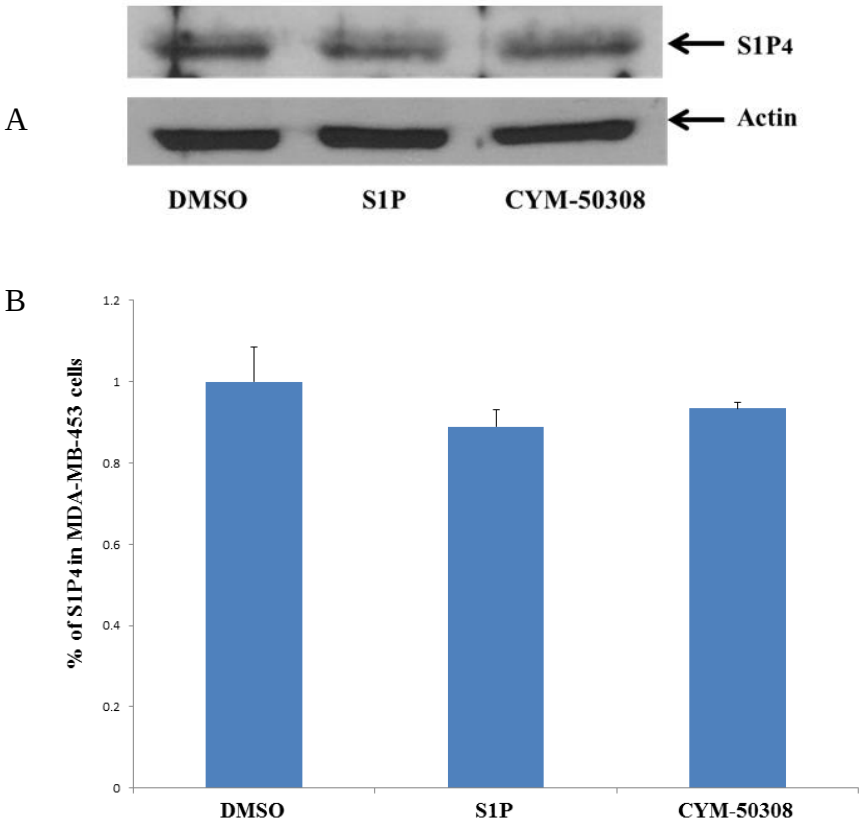


Figure 5.5 ***Effects of exogenous S1P and S1P$_4$ agonist CYM 50308 on S1P$_4$ receptor expression level in MDA-MB*-453 cells.**

Cells were treated with either 0.1% v/v DMSO (vehicle) or exogenous S1P (5 μM) or CYM-50308 (10 μM) for 24 hrs. (A) Cells lysate (CL) were harvested in Laemmli sample buffer and analysed by Western blotting and $S1P_4$ was assessed using anti-$S1P_4$ antibody. The membrane was stripped and re-probed with anti-actin antibody to ensure equal protein loading. Results are representative of three independent experiments. (B) Densitometric quantification expressed as mean +/- SEM for the percentage of $S1P_4$ in MDA-MB-453 cells for three independent experiments. Statistical analysis was completed by Graph Pad Prism program using statistical *t*-test and difference was considered significant when * $p < 0.05$ *versus* vehicle.

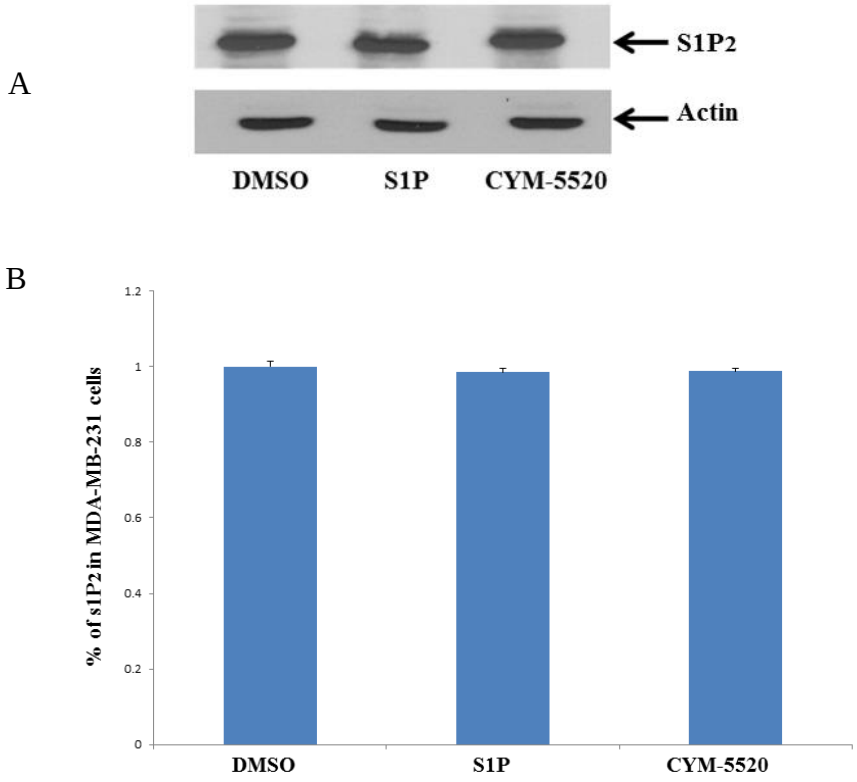


Figure 5.6 ***Effects of exogenous S1P and S1P$_2$ agonist CYM-5520 on S1P$_2$ receptor expression level in MDA-MB*-231 cells.**

Cells were treated with either 0.1% v/v DMSO (vehicle) or exogenous S1P (5 μM) or CYM-5520 (10 μM) for 24 hrs. (A) Cells lysate (CL) were harvested in Laemmli buffer and analysed by Western blotting and $S1P_2$ was assessed using anti-$S1P_2$ antibody. The membrane was stripped and re-probed with anti-actin antibody to ensure equal protein loading. Results are representative of three independent experiments. (B) Densitometric quantification expressed as mean +/- SEM for the percentage of $S1P_2$ in MDA-MB-231 cells for three independent experiments. Statistical analysis was completed by Graph Pad Prism program using statistical t-test and difference was considered significance when * $p < 0.05$ *versus* vehicle.

5.2.6 The Effect of the SK1 Inhibitor PF-543 and SK2 Inhibitor (*R*)-FTY720 Methyl Ether (ROMe) on $S1P_4$ Release into Conditioned Medium from MDA-MB-453 Cells

Sphingomyelin and cholesterol are enriched in the cell membrane raft and/or microdomains (Brown and London, 2000), and the balance between these lipids is essential to maintain plasma membrane homeostasis and sustain endocytosis (Breslow and Weissman, 2010). The defective SK1 function is shown to induce aberrant endocytic recycling, thereby suggesting a role of S1P, sphingosine and ceamide in regulating endocytosis (Shen *et al*., 2014). Moreover, perturbation of cholesterol and sphingomyelin balance induces entrapment of SK1 to early endocytic intermediates (Shen *et al*., 2014). Exosomes are derived from an endocytic origin (Kowal *et al*., 2014). Therefore, the role of SK1 in exosome formation and thus, $S1P_4$ release was investigated. This was established using SK1 and SK2, selective inhibitors. Treatment of MDA-MB-453 cells with PF-543 (0.1 µM) and (*R*)-FTY720 methyl ether (10 µM) resulted in enhanced release of $S1P_4$ into CM ($p < 0.05$) (figure 5.7 A, B). These findings, suggest the involvement of SK1 in the release of $S1P_4$ from MDA-MB-453 cells into CM, thereby modulating $S1P_4$ sorting into endosomal compartments.

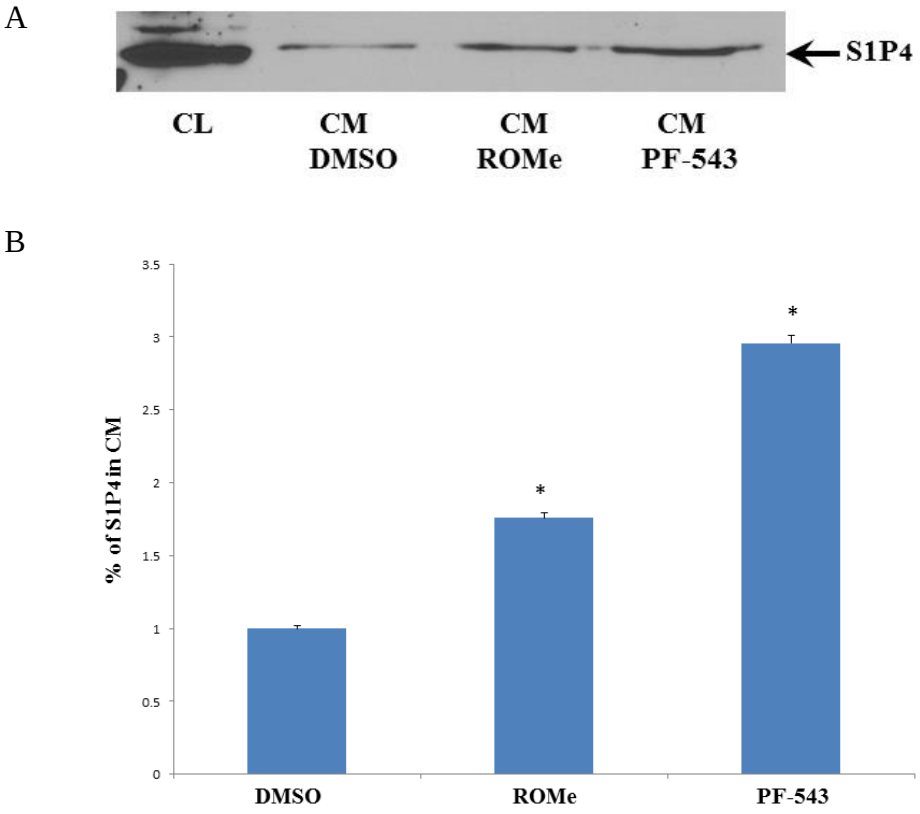


Figure 5.7 ***Effects of addition of SK1 inhibitor PF-543 and SK2 inhibitor (R)-FTY720 methyl ether to MDA-MB-453 on S1P$_4$ in Conditioned Medium.***

Cells were treated with either 0.1% v/v DMSO (vehicle) or PF-543 (0.1 μM) or (*R*)-FTY720 methyl ether (10 μM) for 24 hrs prior to collection of CM. CM was collected and its protein contents were acid precipitated (PPT) and Laemmli buffer added. Cell lysate (CL) were also harvested in Laemmli buffer. (A) These samples were analysed by Western blotting ans $S1P_4$ was assessed using anti-$S1P_4$ antibody. Results are representative of three independent experiments. (B) Densitometric quantification expressed as mean +/- SEM for the percentage of $S1P_4$ in CM for three independent experiments. Statistical analysis was completed by Graph Pad Prism program using statistical t-test and difference was considered significance when * $p < 0.05$ *versus* vehicle.

5.3 Discussion

Exosomes are of endocytic origin initiated from in-budding of plasma membrane proteins into endosomes, with further inward budding of endosome membranes to form ILVs in MVBs (Hurley, 2008; Williams and Urbé, 2007). Endocytosis is a highly controlled membrane remodelling process that depends on several endocytic factors. Cancer is usually accompanied by abnormal expression of proteins, especially plasma membrane proteins such as transmembrane receptors, and these changes in plasma membrane proteins and constant phenotypic flux require intensified plasma membrane remodelling through endocytosis to maintain homeostasis (Milane *et al*., 2015). The balance between sterol and sphingolipids within the plasma membrane is important for normal endocytic membrane traffic, which is supported by much evidence. For example, endocytic pathways are perturbed by pharmacological disturbance of cholesterol extraction with methyl-β cyclodextrin (MβCD), which strongly reduced clathrin mediated endocytosis of transferrin and EGF receptors in HEp-2 cells (Sigismund *et al*., 2008; Rodal *et al*., 1999). Moreover, conversion of sphingosine into S1P by SK1 has been shown to be involved in synaptic vesicle recycling and neurotransmitter release in *C. elegans* (Chan, Hu and Sieburth, 2012). Furthermore, SK1 is the only enzyme in sphingolipid metabolism that is present in endocytic tubules induced by MβCD or sphingomyelinase treatment, where it is co-localised with endophilin 2-Ruby protein that contains curvature-generating/sensing properties, and that contributes in clathrin-dependent endocytosis of synaptic vesicle membranes (Shen *et al*., 2014; Milosevic *et al*., 2011).

In addition, SK1 has been shown to be recruited to early endosomes under normal physiological condition; evidenced by enrichment of Rab5-positive early endosomes with GFP tagged SK1 (Shen *et al*., 2014).

S1P has been shown to induce migration associated with increased endocytosis of the Fluorescein Isothiocyanate-Dextran (FITC-dextran) in mature but not immature bone marrow dendritic cells (BMDC), and this mediated by $S1P_3$ receptor signalling (Maeda *et al*., 2007). In contrast, switch associated protein 70 knockout mature BMDC (*Swap*-70$^{-/-}$ BMDCs), failed completely to endocytose FITC-dextran, suggesting an indirect link between S1P and switch associated protein 70 in the regulation of endocytosis through Rho-A signalling (Ocana-Morgner *et al*., 2011).

Moreover, $G\alpha_i$ proteins-coupled S1P receptors on endosomes have been shown to display continuous activation in response to S1P, and this is critical for MVBs maturation and cargo sorting into ILVs in ESCRT-independent mechanism (Kajimoto *et al*., 2013).

The key finding in this chapter is the release of $S1P_4$ into CM from MDA-MB-453 cells and modulation of this release by S1P. $S1P_4$ receptors are released into CM under control conditions, suggesting that this might be regulated by intracellular S1P released through "inside-out" signalling. The subsequently-released S1P might bind to $S1P_4$ to induce endocytosis and exosomal release via MVBs formation. The other possibility is that $S1P_4$ might be released through a mechanism involving initially its constitutive endocytosis. However, treatment of MDA-MB-453 cells with S1P does increase the amount of $S1P_4$ in CM. This might indicate a possible role of S1P in internalisation and release of $S1P_4$ receptors from MDA-MB-453 cells via initial

endocytosis of $S1P_4$ and packaging in MVBs. It is possible that added S1P might activate SK1 by its phosphorylation at serine 225 by ERK-1/2 catalysed phosphorylation (Pitson *et al*., 2003) and subsequent SK1 translocation to the plasma membrane in close proximity to its substrate (Long *et al*., 2010).

The subsequently-formed intracellular S1P then acts in autocrine and paracrine manner through its $S1PR_{1-5}$. Notably, privileged access to S1P receptors has been shown to occur with subsequently formed intracellular S1P. For instance, S1P binding to $S1P_3$ induced vasorelaxation in rat coronary arteries (Mair *et al*., 2010).

It is known that binding of S1P to S1P receptors such as $S1P_4$ can induce the recruitment of β-arrestin to the activated receptor (Marchese *et al*., 2008). In this context, β-arrestin serves as adaptor to link the GPCRs with the endocytic machineries, which includes clathrin and its adaptor protein 2 (AP-2), thereby allowing receptors to internalise in endosomes. Stable β-arrestin complexes with GPCRs appear to confer persistent signalling from endosomes. This is achieved by binding of β-arrestin to Serine/Threonine cluster in the C-tails of class-B GPCRs such as S1PRs, V2R and AT1R (Ferguson *et al*., 1996; Goodman *et al*., 1996; Oakley *et al*., 2001). Therefore, there is a possibility that the stable binding of β-arrestin with $S1P_4$ might promote receptor traffic and internalisation to endosomes with subsequent MVB formation and exosomal release of the receptor. Thus, stable β-arrestin-GPCR complexes might specify which type of GPCR can be released in exosomes. For instance, AT1R internalisation to endosomes and packaging into exosomes was impaired by silencing β-arrestin1 and 2 in AT1R-YFP stable expressing cells (Pironti *et al*., 2015). The fate of $S1P_4$ in endosomes might follow

three possible routes these are (i) $S1P_4$ dissociates from the β-arrestin complex and is recycled back to the plasma membrane or (ii) the endocytosed $S1P_4$ undergoes lysosomal degradation or (iii) the $S1P_4$ is packaged in MVBs within endosomes which ultimately fuse with the plasma membrane and are released from cells as ILV (exosomes). Indeed, others have demonstrated that exogenous S1P causes $S1P_4$ receptor internalisation in Jurkat T cells (Gräler *et al*., 2003). Moreover, SK1 is associated with early endocytic membrane and has been shown to affect trafficking of the GPCRs rhodopsin photoreceptors in *Drosophila melanogaster* model and the light-sensitive transient receptor potential (TRP) channel by modulating the levels of dihydrosphingosine 1 phosphate and S1P (Yonamine *et al*., 2011). Also, the addition of $S1P_2$ antagonist JTE-013 reduced the amount of $S1P_4$ in CM from MDA-MB-453 cells under control conditions, which indicate that S1P released from these cells might mediate $S1P_4$ internalisation and release into CM and that this can be blocked in a competitive manner by JTE-013. Alternatively, if JTE-013 acted as an inverse agonist at $S1P_4$ it might block endocytosis of constitutively active $S1P_4$. Although JTE-013 is $S1P_2$ selective antagonist, it has been shown to reduce S1P binding to $S1P_4$ receptor in MDA-MB-453 cells (Long *et al*. 2006), but it is not known whether this is due to competitive antagonism or inverse agonism. In addition, treatment of MDA-MB-453 cells with the $S1P_4$ selective agonist CYM-50308 had no effect on $S1P_4$ release into CM, suggesting that synthetic $S1P_4$ agonist is unable to induce $S1P_4$ receptor internalisation to endosomes. In addition, S1P and $S1P_4$ agonist CYM-50308 had no effect on $S1P_4$ levels in MDA-MB-453 cells, suggesting these agents do not alter the transcriptional regulation of $S1P_4$.

Interestingly, HER2 and phosphorylated ERK-1/2 were found to be released into CM from S1P treated MDA-MB-453 cells. Notably, S1P-dependent activation of ERK-1/2 mediated by $S1P_4$ has been shown to be blocked by siRNA knockdown of HER2 (Long *et al*., 2010). Also, β-arrestin-GPCRs activated complex has been shown to recruit ERK-1/2 with most class-B GPCRs containing Serine/Threonine cluster at their C-tails (Tohgo *et al*., 2002). Therefore, there is a possibility that the $S1P_4$-β-arrestin complex with HER2 and recruited ERK-1/2 are present in endosomes and then packaged in MVBs for release. Importantly, treatment of MDA-MB-453 cells with SK1 inhibitor PF-543 or the SK2 inhibitor (*R*)-FTY720 methyl ether (ROMe) increased the release of $S1P_4$ into CM. This is somewhat surprising as SK inhibition is expected to reduce intracellular S1P levels and therefore the amount of $S1P_4$ that can be released. However, in both cases SK1 and SK2 inhibition could increase ceramide levels that could induce exosomes release and that this mechanism predominates or is more effective than S1P in promoting exosome release. Indeed, Trajkovic *et al* (2008) demonstrated that ceramide is essential for exosome formation and release independent of the ESCRT machinery, and involves raft-based lipid microdomains. These microdomains are enriched in ceramide and can induce lateral segregation of cargo within the endosomal membrane. Moreover, inhibition of neutral sphingomyelinases, the enzyme that regulate ceramide synthesis, reduced exosome formation and release (Trajkovic *et al*., 2008). In addition, ceramide is enriched in lipid raft microdomains, which forms a cone-like structure that can induce the merging of small lipid microdomains into larger domains through hydrogen bonding, which promotes domain-induced budding (Gulbins and Kolesnick, 2003).

Therefore, disrupting the balance between sterol and sphingolipid with either SK1 inhibitor PF-543 or SK2 inhibitor ROMe might lead to accumulation of ceramide, which in turn promotes lipid raft and plasma membrane budding and release. Further evidence supporting a role of ceramide in exosome formation is demonstrated by several studies. For instance, inhibition of neutral sphingomyelinase by chemical inhibitor GW4869 or specific siRNA targeting neutral sphingomyelinase attenuated secretion of miRNA such as endogenous miRNA-16 and exogenous miRNA-146a from HEK293 cells (Kosaka *et al*., 2010).

Additionally, exogenous C6-ceramide induces endocytic vesicle formation and triggering production of large late endosomes in two mouse embryonic fibroblast cell lines, 3T3-L1 and 3T3-F442A (Li *et al*., 1999). Thus, from all above evidence it appears that ceramide accumulation might cause the release of $S1P_4$ into CM.

Importantly, an accumulation of sphingosine by SK1 like lipid inhibitors has also been shown to participate in vacuole and vesicle formation, emphasising the fusogenic role of sphingosine. Indeed, Young *et al* (2016) demonstrated that inhibition of SK1 by sphingosine like inhibitor such as N-dimethyl-sphingosine (DMS) and FTY-720 overloads endocytic membranes with sphingosine-like lipids to stimulate SK1-dependent membrane fusion and vacuole formation (Young *et al*., 2016). Also, the SK2 inhibitor (R)-FTY720 methyl ether (ROMe), which is a sphingosine like structure increased $S1P_4$ release into CM, possibly emphasising the role of sphingosine in regulating $S1P_4$ release and providing an alternative mechanism from the inhibition of SK2 by this compound. Notably, ROMe has also been shown to bind $S1P_1$ to reduce its surface expression on CCL39 cells (Barbour

et al., 2016). Therefore, ROMe might also bind to $S1P_4$ in MDA-MB-453 cells to promote $S1P_4$ internalisation and subsequent release into CM. Consistently, FTY-720 phosphate fails to induce vacuole formation, thus emphasising that vacuole formation might occur independently on S1P receptor with this compound (Young *et al*., 2016). Notably, FTY-720 phosphate binds to all S1P receptor except $S1P_2$ (Chun and Hartung, 2010). In addition, catalytically inactive $SK1a^{G82D}$ has been shown to enhance the fusion of Lysosomal-associated membrane protein 1 (Lamp1) positive vacuoles induced by SK1 like sphingosine inhibitor or sphingosine, suggesting that SK1 may stimulate membrane fusion independently of kinase catalytic activity. Therefore, SK1 might facilitate membrane fusion independent of kinase activity involving direct SK1 binding to the plasma membrane. In this regard, SK1 dimers contain a concave positively charged groove, which separates the two Lipid binding loop-1 (LBL-1) in the plasma membrane and which can dock with negatively charged convex membranes that direct LBL-1 motifs to the plasma membrane for substrate extraction (Adams *et al*., 2016).

Notably, LBL-1 has been shown to participate in the curvature-sensitive recruitment of SK1 to negatively charged membranes during endocytosis (Shen *et al*., 2014). Moreover, sphingosine has been shown to regulate synaptic vesicles exocytosis or fusion by facilitating synaptobrevin accumulation in synaptic vesicles to form the SNARE complex assembly involved in membrane fusion (Darios *et al*., 2009).

In MDA-MB-231 cells, S1P appears to induce the proteolysis and release of $S1P_2$ receptors indicated by the presence of a lower molecular mass ~38 kDa form in CM. This finding, suggests that $S1P_2$ receptors might be removed from plasma membrane

by proteolysis with the subsequently proteolysed form being released from cells in response to S1P. However, failure of JTE-013 to prevent formation of constitutively active shorter form ~38 kDa excludes the possible involvement of S1P (for details see chapter IV). Nevertheless, these findings do suggest regulation of GPCRs by proteolysis and this is further supported by previous studies by Kojro and Fahrenholz (1995). The team demonstrated that the activity of vasopressin receptor type 2 (V2R) is regulated by plasma membrane metalloproteinase (Kojro and Fahrenholz, 1995). Addition of $S1P_2$ agonist CYM-5520 and the $S1P_2$ competitive antagonist JTE-013 had no effect on $S1P_2$ release into CM as there was no change in the amount of $S1P_2$ in CM. These observations, suggest that $S1P_2$ receptors might be internalised and endocytosed in agonist independent manner. This process is known as constitutive internalisation and there are previous observations support this finding. For instance, redistribution of cannabinoid receptor subtype-1 (CB1) from the somatodendritic membrane to axonal membrane is by constitutive endocytosis (McDonald *et al*. 2007).Moreover, melanocortin-4 receptors (MC4R) have been shown to traffic constitutively as indicated by receptor distribution on the plasma membrane and intracellular compartment in the absence of its endogenous ligand α-melanocyte-stimulating hormone (α-MSH) (Mohammad *et al*., 2007). Therefore, there is a possibility that $S1P_2$ receptors are constitutively released from MDA-MB-231 cells, and this is supported by the fact that the intact receptor is released rather than a proteolysed version that is observed upon treatment of cells with S1P.

Future studies in this context need to address the factors that induce $S1P_2$ internalisation and release in exosomes and its regulations. Although, $S1P_2$ receptors were shown in exosomes and can be taken up by MEFs and processed to generate a

constitutively active short form $S1P_2$ (Mr ~38 kDa) receptor that can couple and activate ERK-1/2 signalling in MEFs, further *in vivo* studies are required to elucidate its action in modulating pre-metastatic niche formation.

CHAPTER VI

CHARACTERISATION OF CERAMIDE KINASE ACTIVITY IN BREAST CANCER CELLS

CHAPTER 6: Characterisation of Ceramide Kinase Activity in Breast Cancer Cells

6.1 Introduction

Ceramide 1 phosphate (C1P) is produced from the phosphorylation of ceramide by ceramide kinase (CERK) and is reported to be involved in several cellular processes such as cell growth, proliferation, inflammation and macrophage migration (Arana *et al*., 2010). The first biological report concerning the role of C1P in proliferation was by Gomez-Munoz and team in 1995. They demonstrated that C1P-induced DNA synthesis in Rat-1 fibroblasts (Gomez-Muñozt *et al*., 1995). Subsequent studies demonstrated that C1P increases DNA synthesis and induces proliferating cell nuclear antigen (PCNA) expression in T17 fibroblasts (Gomez-Muñoz *et al*., 1997). Moreover, Gangoiti *et al* (2008) demonstrated that C1P induces macrophage proliferation via ERK-1/2 and JNK-dependent pathways. Notably, the proliferation and migration effects mediated by exogenous C1P might involve cell surface receptors. For instance, exogenous C1P has been shown to induce RAW 264.7 macrophages migration via a pertussis toxin-sensitive mechanism (Granado *et al*., 2009) Moreover, exogenous C1P has been shown to induce migration in an extracellular manner through surface receptors, which is blocked by pertussis toxin and increased GTPγS binding to macrophages membranes (Levi *et al*., 2010). Moreover, pre-treatment of pancreatic cancer cells PANC-1 and MIA PaCa-2 with pertussis toxin abrogated C1P-induced cells migration (Rivera *et al*., 2016). In addition, C1P formed by CERK is also likely to exert "inside-out" signalling. For instance, exogenous C1P has been shown to increase human pancreatic cancer cells

invasion and migration (Rivera *et al*., 2016), and this effect is potently blocked by CERK inhibitor NVP-231 or siRNA used to knockdown CERK expression in pancreatic cancer (Rivera *et al*., 2016).

In addition, Pastukhov *et al* (2014) have demonstrated that the CERK inhibitor NVP-231 with nanomolar potency (Graf *et al*., 2008) induces cell cycle arrest in MCF-7 breast cancer cells and NCI-H358 lung cancer cells, and sensitises these cells to apoptosis (Pastukhov *et al*., 2014).

The term sphingolipid 'rheostat' was first proposed in 1996 to explain how extracellular ligands can alter the interconversion of ceramide, sphingosine and S1P to differentially regulate cell growth and survival (Pyne *et al*., 1996; Cuvillier *et al*., 1996; Zhang, 1991; Obeid *et al*., 1993). This is based on evidence demonstrating that ceramide is a pro-apoptotic and induces growth arrest, whereas S1P is a pro-survival molecule that regulates cell proliferation and suppresses ceramide mediated apoptosis (Newton *et al*., 2015; Cuvillier *et al*., 1996). Thus, the sphingolipid 'rheostat' is a sensing mechanism regulating cells fate. Discovery of C1P as a molecule involved in many cellular processes such as release of synaptic vesicles, phagocytosis, degranulation of mast cells, inflammation and proliferation raises the question that C1P might be another counter molecule to ceramide in the sphingolipid 'rheostat'. Therefore, it was proposed that C1P (pro-survival) might compensate low S1P (pro-survival) level induced by SK1 inhibition. This would therefore, counteract the accumulation of ceramide (pro-apoptotic), thereby maintaining cells in a proliferative and survival state (homeostasis). CERK might also play a role in acquisition resistance of cancer cells to sphingosine kinases inhibitors, (figure 6.1).

Therefore, the effectiveness of SK1 inhibitors might be compromised by CERK which could convert ceramide that is generated into C1P, which can promote cancer cells proliferation (figure 6.1 for illustration). Therefore, the aim of the chapter was to (i) investigate the effect of a potent ceramide kinase inhibitor NVP-231 on DNA synthesis in MDA-MB-231 and MDA-MB-453 breast cancer cells. (ii) examine the hypothesis or the notion that elevation of intracellular ceramide (pro-apoptotic) level by concomitant inhibition of SK1 and CERK can sensitise breast cancer cells to cell death signalling (figure 6.1), thereby reducing DNA synthesis.

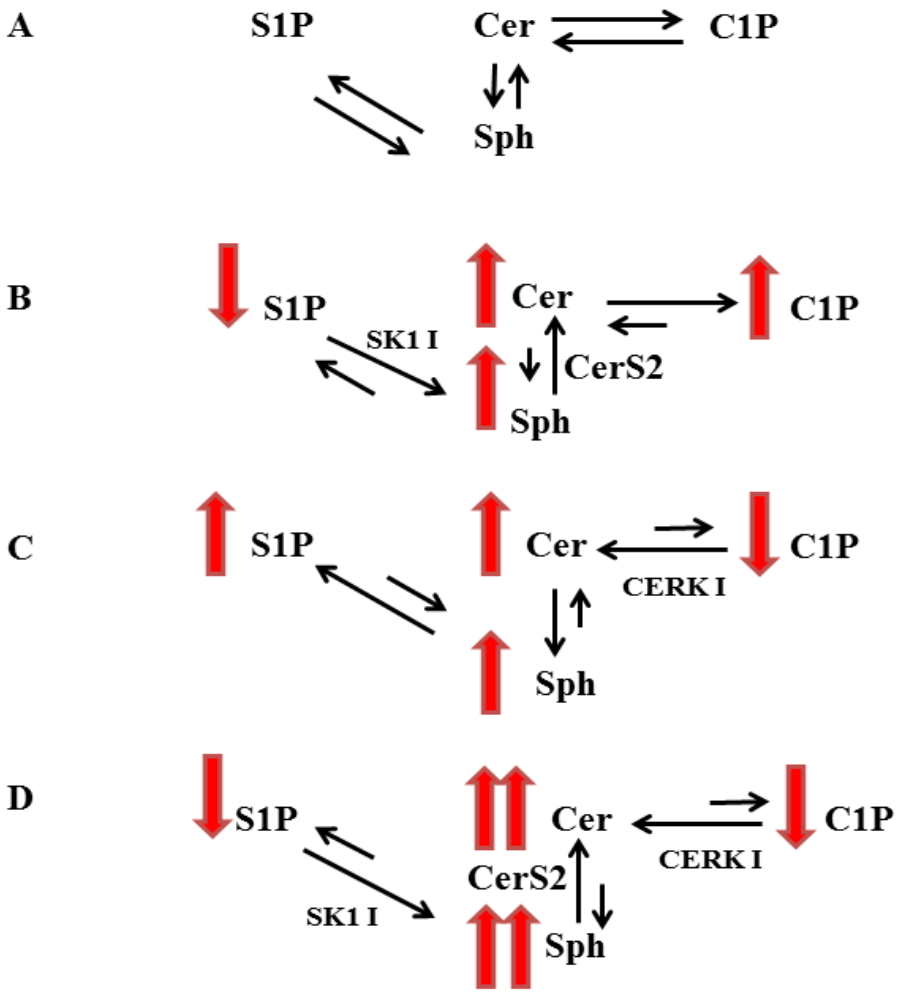


Figure 6.1 ***A Schematic representation explaining the postulated sphingolipid 'rheostat' situation with CERK inhibitor or SK1 inhibitor or combination of both inhibitors.***

Sphingosine 1 phosphate, (S1P); Sphingosine, (Sph); Ceramide, (Cer); Ceramide 1 phosphate, (C1P); Ceramide synthase 2, (Cer2), Sphingosine kinase 1 inhibitor (SK1I) and Ceramide kinase inhibitor (CERK I).

(A) Normal sphingolipid ‘rheostat’ where sphingolipid metabolites S1P, sphingosine (Sph), ceramide (Cer) and C1P are all in a steady state. (B) Perturbation state generated from inhibition of SK1 and subsequent low production of S1P (pro-survival), which resulted in increased Sph that might be converted to ceramide by ceramide synthase 2 (CerS2), which allosterically inhibited by S1P (Laviad *et al*., 2007). Accordingly, increased intracellular ceramide (pro-apoptotic) promotes phosphorylation of ceramide to C1P (pro-survival) to counteract ceramide-induced apoptosis, therefore restoring sphingolipid steady state (homeostasis). (C) Perturbation state generated from CERK inhibition by ceramide kinase inhibitor resulting in accumulation of intracellular ceramide (pro-apoptotic).

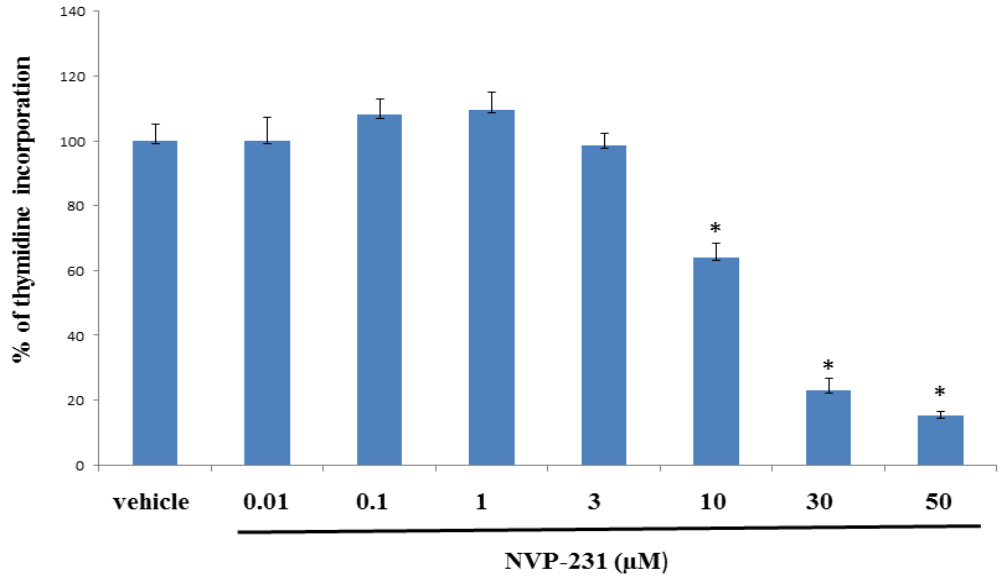
Increased intracellular ceramide can be converted to sphingosine by ceramidase followed by phosphorylation of sphingosine to S1P (pro-survival) by SK1, therefore restoring the sphingolipid ‘rheostat’ steady state (homeostasis). (D) Perturbation state generated from inhibition of SK1 and CERK lead to enhanced accumulation intracellular ceramide (pro-apoptotic) over and above that generated by each inhibitor alone; predicted to result in enhanced inhibition of DNA synthesis and promoted apoptosis compared with each inhibitor alone.

6.2 Results

6.2.1 The Effect of NVP-231on MDA-MB-231 Breast Cancer Cells DNA Synthesis

Ceramide, S1P, sphingosine and C1P all are sphingolipids metabolites that are enzymatically interconverted in the sphingolipid 'rheostat'. C1P is known to induce migration and invasion (Rivera *et al*., 2016), and also it is a potent stimulant of DNA synthesis (Gomez *et al*., 1997). Therefore, it is important to elucidate the effect of CERK on DNA synthesis using potent, specific, and reversible CERK inhibitor NVP-231 that competitively inhibits binding of ceramide to CERK (Graf et al., 2008). Treatment of MDA-MB-231 breast cancer cells with NVP-231 (10-50 µM) induced significant ($^{*}p < 0.05$ *versus* 0.1 % v/v DMSO) dose-dependent reduction in [^{3}H] thymidine incorporation into DNA synthesis of MDA-MB-231 cells (figure 6.2 A) with a calculated IC50 of ~13 µM, (figure 6.2 B).

A



B

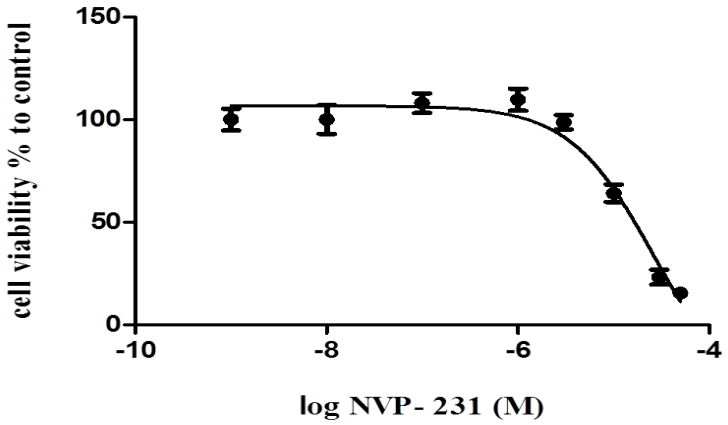


Figure 6.2 ***NVP-231 dose response curve in MDA-MB-231 breast cancer cells.***

(A) MDA-MB-231 cells were seeded in 24 wells plate at density 50,000/well and incubated in complete medium until 80% confluence attained. Cells were quiescent for 24 hrs prior to addition of either NVP-231 or 0.1 % v/v DMSO (vehicle) as indicated and incubated for further 24 hrs. [^{3}H] thymidine was added in the last 5 hrs and to the wells and assay was performed as described in section (2.4). Statistical analysis one way ANOVA was performed by Graph prism software and observation was considered significant when $*p < 0.05$ *versus* 0.1% v/v DMSO (vehicle). The experiment was performed in triplicate and the results were expressed as mean +/- SEM. (B) The IC50 was calculated using Graph Prism software and IC50 value obtained is ~ 13 µM.

6.2.2 The Effect of SK1 Inhibitor PF-543 on DNA Synthesis in MDA-MB-231 Breast Cancer Cells

SK1 is shown to be over expressed in MDA-MB-231 triple negative breast cancer cells and this high expression is associated with low response to doxorubicin-based treatment and subsequent poor prognosis (Datta *et al*., 2014). In addition, inhibition of SK1 using siRNA specific oligonucleotide has been shown to induce apoptosis and inhibit breast cancer cells proliferation (Datta *et al*., 2014). PF-543 is an SK1 inhibitor with nanomolar potency (*K*i 3.6 nM), and is competitive with sphingosine (Schnute *et al*., 2012). Thus, elucidation of the effect of PF-543 on DNA synthesis in MDA-MB-231 breast cancer cells was performed. Indeed, treatment of MDA-MB-231 breast cancer cells with PF-543 (1-10 µM) induced significant ($^{*}p < 0.05$ *versus* 0.1% v/v DMSO) dose dependent reduction in [^{3}H] thymidine incorporation into DNA (figure 6.3 A) with a calculated IC50 of ~ 5 µM, (figure 6.3 B).

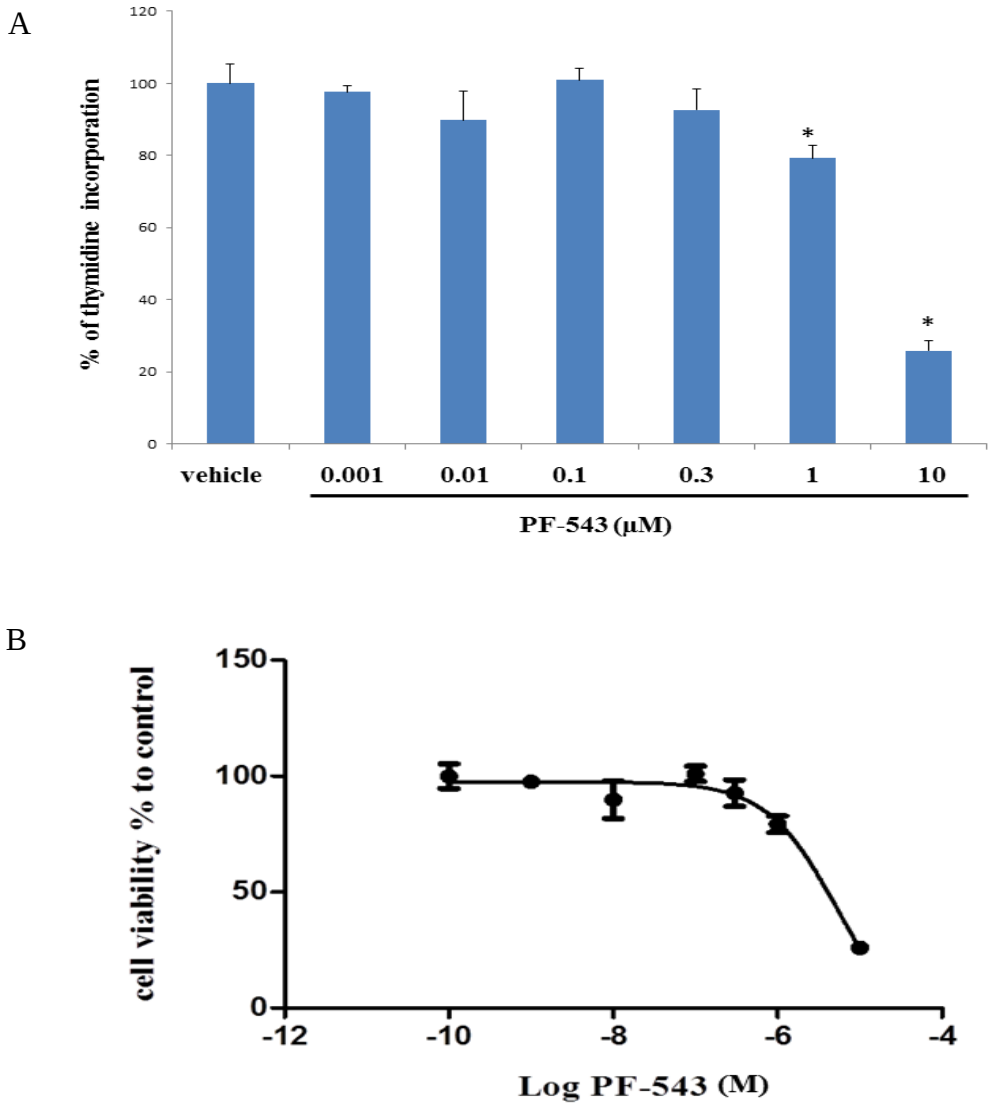


Figure 6.3 ***PF-543 dose response curve in MDA-MB- 231 cells.***

(A) MDA-MB-231 cells were seeded in 24 wells plate at density 50,000/well and incubated in complete medium until 80% confluence attained. Cells were quiescent for 24 hrs prior to addition of either PF-543 or 0.1 % v/v DMSO (vehicle) as indicated and incubated for further 24 hrs. [^{3}H] thymidine was added in the last 5 hrs and to the wells and assay was performed as described in section (2.4). Statistical analysis one way ANOVA was performed by Graph prism software and observation was considered significant when $*p < 0.05$ *versus* 0.1% v/v DMSO (vehicle). The experiment was performed in triplicate and the results were expressed as mean +/- SEM. (B) The IC50 was calculated using GraphPrism software and IC50 obtained is ~ 5 µM.

6.2.3 The Combination Effect of NVP-231 with PF-543 on DNA Synthesis in MDA-MB-231 Breast Cancer Cells

A possible synergism effect of combined treatment of cells with NVP-231 and PF-543 on DNA synthesis in MDA-MB-231 cells was investigated. However, the combination of NVP-231 (3 μM) and PF-543 (1-10 μM) produced less than additive effect (figure 6.4). Notably, additive effect means the effect of combination can equal the sum of the expected effects of the two drugs (Toews, 2005).

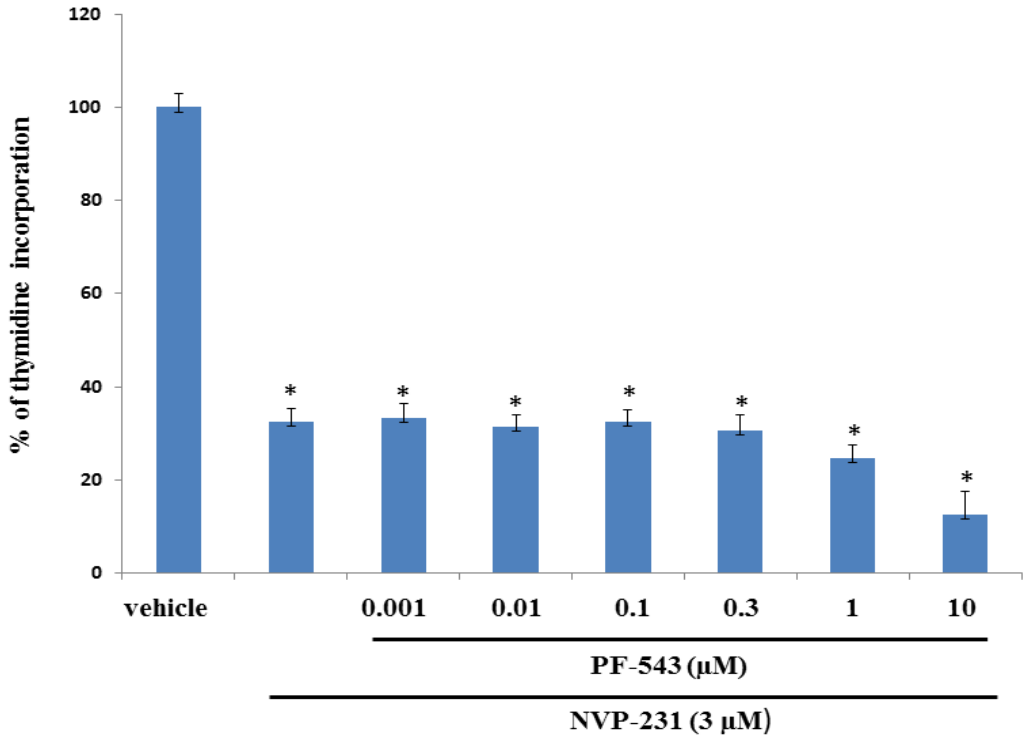


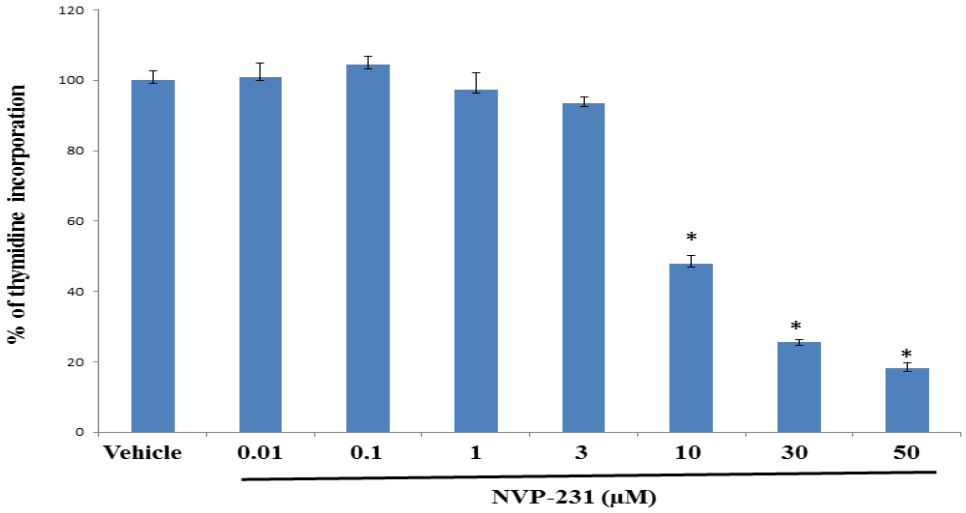
Figure 6.4 ***Effect of combination of NVP-231 and PF-543 in MDA-MB-231 breast cancer cells.***

MDA-MB-231 cells were seeded in 24 wells plate at density 50,000/well and incubated in complete medium until 80% confluence attained. Cells were quiescent for 24 hrs prior to addition of either 0.1 % v/v DMSO (vehicle) or NVP-231 (3 µM) with different concentrations of PF-543 as indicated and incubated for further 24 hrs. [^{3}H] thymidine was added in the last 5 hrs and to the wells and assay was performed as described in section (2.4). Statistical analysis one way ANOVA was performed by Graph prism software and observation was considered significant when *$p < 0.05$ *versus* 0.1% v/v DMSO (vehicle). The experiment was performed in triplicate and results were expressed as mean +/- SEM.

6.2.4 The Effect of NVP-231 in MDA-MB-453 Cells

CERK high expression in ER^- $HER2^+$ breast cancer has been shown to be associated with significant poor prognosis (Ruckhäberle *et al*., 2009). Thus, ER^- and $HER2^+$ MDA-MB-453 breast cancer cells were tested with NVP-231. Treatment of MDA-MB-453 cells with NVP-231 induces significant ($^*p < 0.05$ *versus* 0.1% DMSO) dose response (10-50 µM) reduction in [^{3}H] thymidine incorporation into DNA in MDA-MB-453 cells (figure 6.5 A) with a calculated IC50 of ~ 10 µM, (figure 6.5 B).

A



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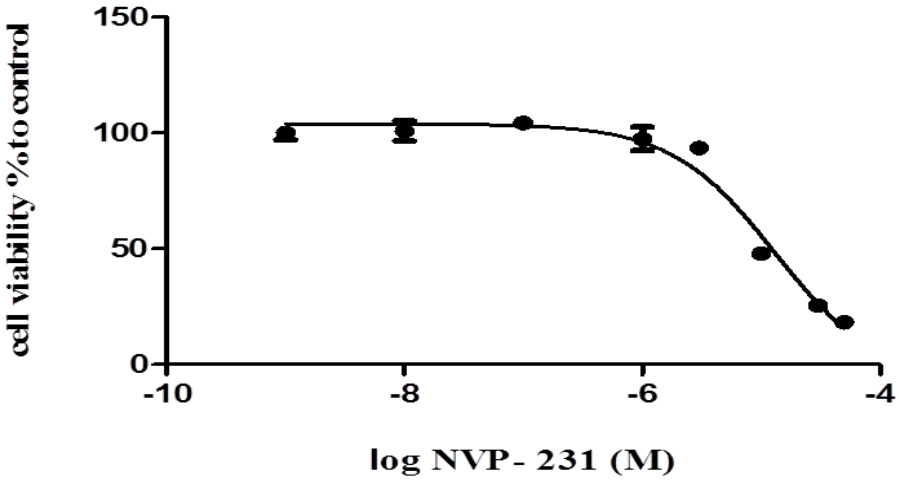


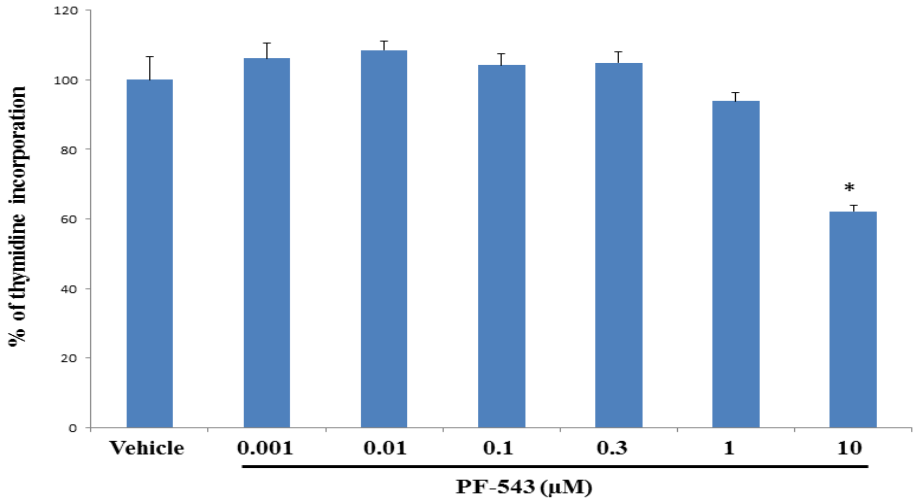
Figure 6.5 ***NVP-231 dose response in MDA-MB-453 cells.***

(A) MDA-MB-453 cells were seeded in 24 wells plate at density 50,000/well and incubated in complete medium until 80% confluence attained. Cells were quiescent for 24 hrs prior to addition of either NVP-231 or 0.1 % v/v DMSO as indicated and incubated for further 24 hrs. [^{3}H] thymidine was added in the last 5 hrs and to the wells and assay was performed as described in section (2.4). Statistical analysis one way ANOVA was performed by Graph prism version analysis 5 software and observation was considered significant when $*p < 0.05$ *versus* 0.1% v/v DMSO (vehicle). The experiment was performed in triplicate and the results were expressed as mean +/- SEM. (B) The IC50 was calculated using Graph Prism software and value obtained is ~ 10 µM.

6.2.5 The Effect of SK1 Inhibitor PF-543 on DNA Synthesis in MDA-MB-453 Breast Cancer Cells

$S1P_4$ and HER2 are reported to regulate ERK-1/2 signalling in MDA-MB-453 breast cancer cells in functional manner, thereby regulating breast cancer cells proliferation (Long *et al*., 2010). In addition, SK1 high expression is reported in $HER2^+$ breast cancer tumours and is associated with shorter disease-specific survival (Ohotski *et al*., 2012). Therefore, it is important to examine the effect of PF-543 on DNA synthesis as a measure of cells proliferation. Treatment of MDA-MB-453 cells with 10 µM PF-543 induced significant ($^*p < 0.05$ *versus* 0.1% v/v DMSO) reduction in [^{3}H] thymidine incorporation into DNA (figure 6.6 A) with a calculated IC50 of ~15 µM, (figure 6.6 B).

A



B

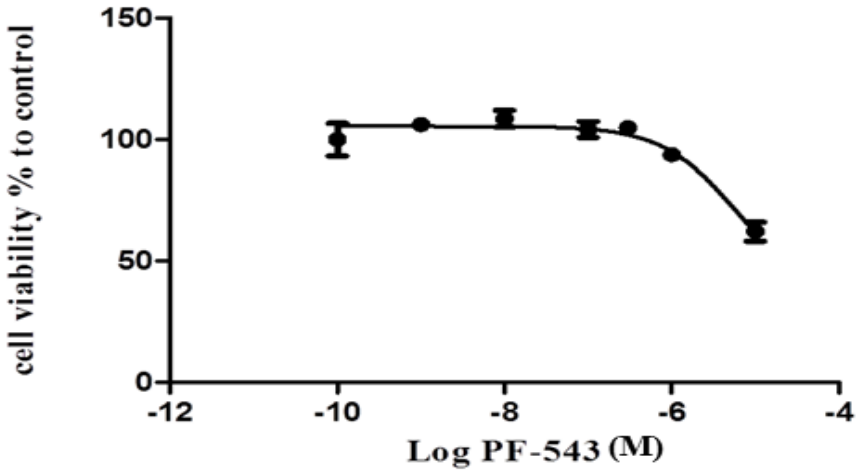


Figure 6.6 ***PF-543 dose response curve in MDA-MB-453 cells*****.**

(A) MDA-MB-453 cells were seeded in 24 wells plate at density 50,000/well and incubated in complete medium until 80% confluence attained. Cells were quiescent for 24 hrs prior to addition of either PF-543 or 0.1 % v/v DMSO as indicated and incubated for further 24 hrs. [^{3}H] thymidine was added in the last 5 hrs and to the wells and assay was performed as described in section (2.4). Statistical analysis one way ANOVA was performed by Graph prism software and observation was considered significant when $*p < 0.05$ *versus* 0.1% v/v DMSO (vehicle). The experiment was performed in triplicate and the results were expressed as mean +/- SEM. (B) The IC50 was calculated using Graph Prism software and value obtained is ~ 15 µM.

6.2.6 The Effect of Combination of NVP-231 with PF-543 on DNA Synthesis in MDA-MB-453 Breast Cancer Cells.

In order to investigate whether inhibitory synergism effect can be produced from combined inhibition of SK1 and CERK on DNA synthesis in MDA-MB-453 cells. Treatment of MDA-MB-453 cells with PF-543 (10 μM)) along with NVP-231 (3 μM) induced less than additive effect (figure 6.7).

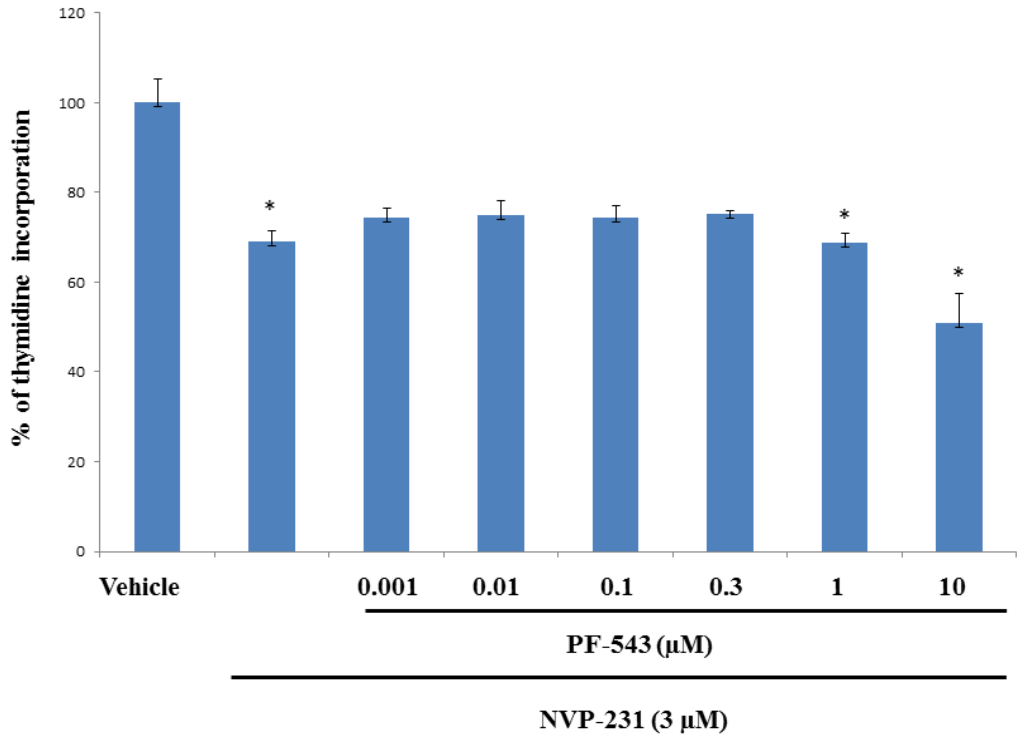


Figure 6.7 ***The effect of combination of NVP-231 and PF-543 on DNA synthesis in MDA-MB-453 breast cancer cells*.**

MDA-MB-453 cells were seeded in 24 wells plate at density 50,000/well and incubated in complete medium until 80% confluence attained. Cells were quiescent for 24 hrs prior to addition of either 0.1 % v/v DMSO or NVP-231 (3 µM) with different concentrations of PF-543 as indicated and incubated for further 24 hrs. [^{3}H] thymidine was added in the last 5 hrs and to the wells and assay was performed as described in section (2.4). Statistical analysis one way ANOVA was performed by Graph prism software and observation was considered significant when *$p < 0.05$ *versus* 0.1% v/v DMSO (vehicle). The experiment was performed in triplicate and results were expressed as mean +/- SEM.

6.3 Discussion

IC50 values for inhibition of DNA synthesis in breast cancer cells are much higher than the concentration required for inhibiting CERK and SK1 activity. Notably, the (*K*i) for NVP-231 for CERK is 7.4 nM (Graf *et al*., 2008), and the (*K*i) for PF-543 for SK1 is 3.6 nM (Schnute *et al*., 2012). This might suggest that in order to inhibit DNA synthesis a threshold level of inhibition of the kinases is required; this is indicated by rather steep inhibition curve response for DNA synthesis. Such a threshold effect can be rationalised by the fact that relatively low levels of S1P and C1P might be required to sustain DNA synthesis, and therefore, substantial inhibition at a threshold e.g. > 95% inhibition of each enzyme might be required to lower C1P and S1P levels below a threshold, whereupon inhibition of DNA synthesis occurs. Also, this can be explained by excessive enzyme activity, which also might be correlated with high enzyme concentration (Savir, Tu and Springer, 2015).

NVP-231 demonstrated similar potency on DNA synthesis in MDA-MB-231 and MDA-MB-453 breast cancer cells indicated by similar dose response effect and approximate IC50. However, PF-543 appeared more potent in MDA-MB-231 cells with IC50 ~ 5 µM, while IC50 is ~15 µM in MDA-MB-453 cells. Importantly, triple negative breast cancer tumours were reported to exhibit the highest SK1 expression level among other breast cancer types (Geffken and Spiegel, 2017). Therefore, threshold high IC50 dose of PF-543 is required to eliminate or even reduce enzyme activity by 50%. S1P has been shown to negatively regulate CerS2 activity therefore, low S1P level might enhance CerS2 activity to form a pro-apoptotic C20-C26 ceramide (Laviad *et al*., 2007). Notably, S1P is a potent mitogenic and can

synergistically stimulate DNA synthesis in mouse Swiss 3T3 fibroblasts and in mouse JB6 epidermal cells (Kiss and Mukherjee, 1997). Thus, inhibition of SK1 by PF-543 has been shown to reduce S1P level in 1483 cells (Schnute *et al*. 2012). Moreover, several chemotherapeutic agents have been shown to exert their effect by increasing ceramide formation (Dimanche-Boitreln *et al*., 2011). For instance, bortezomib has been shown to induce apoptosis in cultured pancreatic cancer cells by increasing cellular ceramide. Also, ceramide synthase inhibitor fumonisin B1 (F-B1) reduced bortezomib-induced ceramide production, and therefore apoptosis (Gong *et al*., 2013). In addition, N-(4-hydroxphenyl) retinamide has been shown to induce dose-dependent reduction in MCF-7 cells growth associated with a 10-fold increase in ceramide level (Rehman *et al*., 2004). Furthermore, the cyclosporine A analogue and multidrug resistance modulator SDZ PSC 833 have been shown to increase ceramide level, which associated with reduced KB-V-1 MDR human epidermoid carcinoma cells survival (Cabot *et al*., 1998). Ceramide has been shown to exhibit complex divergent apoptosis signalling. For instance, ceramide has been shown to accumulate in mitochondria in response to chemotherapeutic drugs and TNFα, which in turn leads to release of apoptotic mediators (Ohanian and Ohanian, 2001; Siskind, 2005). Moreover, ceramide has been reported to bind the aspartic protease cathepsin D, which is associated in TNFα, Fas and chemotherapy-induced apoptosis (Pettus *et al*., 2002). Furthermore, ceramide is reported to directly recruit and activate PP2A, and through this mechanism inactivates Akt leading to stimulation of pro-apoptotic Bad (Pettus *et al*., 2002; Ruvolo, 2003).

Moreover, blocking of VEGF receptor-2 (VEGFR-2), which is the main receptor for VEGF with pharmacological inhibitor DMH4 or with specific VEGFR-2 siRNA, significantly inhibited C1P-stimulated cell growth (Ouro *et al*., 2017). Notably, at 100 nM NVP-231 has been shown to be more than 80% efficient for CERK inhibition in cell-based assays. However, off-targets to explain anti-proliferative effects may occur at this concentration (Graf *et al*., 2008; Graf *et al*., 2009).

The combination study revealed an observation that might be important and might demonstrate a functional interaction between NVP-231 and PF-543 or CERK and SK1, indicated by less than additive effect of both inhibitors when used in combined treatment of breast cancer cells. This is possibly explained as NVP-231 might reduce the activity of PF-543 or vice versa or there is overlapping between CERK and SK1, which need further investigation. Also, further investigation is required in context of exosome formation and release following NVP-231 treatment alone and in combination with PF-543 in breast cancer cells. Nevertheless, future work requires examining CERK expression in both cell types, using specific siRNA targeting CERK to exclude the possibility of off-target effect of NVP-231 and define the mechanism of inhibition of proliferative signalling.

CHAPTER VII

GENERAL DISCUSSION

CHAPTER 7: General Discussion

Bioactive sphingolipids including ceramide, sphingosine, C1P and S1P have an important role in regulating many cellular processes including cell proliferation, apoptosis, migration, angiogenesis and cellular transformation. The bioactive lipid S1P mediates its actions through its cognate $S1PR_{1-5}$ although; S1P can also bind to several intracellular targets, which are not all fully characterised. S1P and SKs are implicated in several pathological diseases such as inflammation, autoimmune disease and cancer (Pyne *et al*., 2016). SK1 over-expression is reported in colon, lung, brain, prostate, stomach and breast cancers (Zhang *et al*., 2014). In addition, high expression of SK1 in breast cancer grade 4 is associated with poor clinical prognosis (Watson *et al*., 2010). Exosomes are endocytic membrane vesicles in origin of 30-100 nm in size, and implicated in intercellular communication by delivering functional protein molecules to recipient cells (Harding *et al*., 2013). In addition, exosomes released from cancer cells can modulate tumour microenvironments for cancer metastasis and progression (Kahlert and Kalluri, 2013; Rodrigues *et al*., 2014). This study is the first to show the presence of $S1P_2$ receptors in exosomes in CM derived from MDA-MB-231 breast cancer cells. Indeed, Amorim *et al* (2014) demonstrated $S1P_3$ and $S1P_4$ presence in EVs released from HB4a and C5.2 cells. However, this study did not examine their signalling impact on other cells. Also, $S1P_4$ receptor was detected in CM derived from MDA-MB-453 breast cancer cells. Preliminary co-culture work of CM from both cell types with MEFs demonstrated that CM from MDA-MB-231 breast cancer cells containing $S1P_2$ is able to significantly activate ERK-1/2 signalling in MEFs, while CM from MDA-

MB-453 breast cancer cells had no effect. Immunostaining of MDA-MB-231 cells with a primary anti-CD63 antibody with secondary anti-mouse conjugated to TRITC antibody (red), and with primary anti-$S1P_2$ antibody with secondary anti-rabbit FITC antibody (green), indicated co-localisation of $S1P_2$ and CD63 in vesicles that are likely to represent MVBs.

Importantly, $S1P_2$ activation has been shown to be associated with anti-migratory effect in different cell types such as endothelial cells and cancer cells such as glioblastoma U118 and U138 cell lines. This process is mediated by Rho kinase activation through coupling to $G\alpha_{12/13}$ (Lepley *et al*., 2005; Du *et al*., 2010). Moreover, enhanced growth and angiogenesis were observed when Lewis lung carcinoma or B16 melanoma cells are implanted in $S1P_2$ deficient (*S1P2*$^{-/-}$) mice (Du *et al*., 2010). Therefore, $S1P_2$ has a protective effect in limiting cancer growth and metastasis, and its shedding into the extracellular milieu might relieve this inhibitory constraint on the cancer cell.

In addition, immunogold labelling indicated the presence of $S1P_2$ receptor in CD63 positive exosomes isolated from CM of MDA-MB-231 cells. The presence of $S1P_2$ receptor in exosomes might indicate the involvement of $S1P_2$ receptor in formation and maturation of exosomes, thereby activating G-proteins, which have been shown to be involved in the fission/ fusion processes in vesicular trafficking (Kajimoto *et al*., 2013). Also, sequestration of $G_{i\beta\gamma}$ protein in intact cells led to the inhibition of endocytosis (Lin *et al*., 1998). Moreover, inhibitory G-protein (G_i) coupled to $S1P_1$ receptor has been shown to regulate MVBs maturation and cargo sorting and is active on MVBs as a result of continuous supply of S1P (Kajimoto *et al*., 2013). In

very recent study Kajimoto *et al* (2017) have further demonstrated that Rho family GTPases, including Cdc42 and Rac1 are constitutively active on MVEs and are regulated by $G_{i\beta\gamma}$ subunits coupled S1P receptor signalling (Kajimoto *et al*., 2017). Therefore, $S1P_2$ might be coupled to $G\alpha_i$-protein on MVBs and released in exosomes into CM from MDA-MB-231 cells. Notably, $S1P_2$ receptor can couple to multiple G-proteins including $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$.

The full length $S1P_2$ receptor with Mr 42 kDa released into CM in exosomes is taken up by MEFs and processed to a shorter form with Mr ~38 kDa. This shorter form appears to be constitutively active and able to significantly activate ERK-1/2 signalling in MEFs. This constitutive activation is suggested as a result of proteolysis of the N-terminus of $S1P_2$ evidenced by failure of $S1P_2$ competitive antagonist JTE-013 to inhibit CM-induced ERK-1/2 activation in MEFs. Importantly, the N-terminus in GPCRs is involved in ligand recognition and binding (Wittmann, 2011).

Moreover, the N-terminus of GPCRs has been shown to exert an inhibitory effect on receptor activity, and deletion of the N-terminus might prevent this inhibitory effect. For example, deletion of 98% of the N-terminus of thyroid stimulating hormone receptor (TSHR) resulted in 4-7 fold increase in basal activity of the receptor compared with wild type TSHR (Zhang *et al*., 2002).

In contrast, deletion or removal of the N-terminus can inactivate GPCRs. For example, genetic deletion of the N-terminus of GPR37L1 receptor generated a functionally inactive receptor (Leng *et al*., 1999; Coleman *et al*., 2016). In order to examine whether CM-induced ERK-1/2 activation in MEFs is modulated by $S1P_2$ receptors from cancer cells released in CM, incubation of MEFs with CM derived

from MDA-MB-231 cells pre-treated with specific siRNA targeting $S1P_2$ prevented ERK-1/2 activation in response to CM or exosomes. Endogenous $S1P_2$ receptors in MEFs demonstrate a functional activity indicated by cytoskeleton rearrangement, reduced actin density and rounding of MEFs upon treatment with $S1P_2$ agonist CYM-5520. However, CYM-5520 had no effect on basal ERK-1/2 activity, suggesting that endogenous $S1P_2$ is not coupled to the ERK-1/2 pathway, and thereby differentiating it from $S1P_2$ released from cancer cells and taken up by MEFs. Pre-treatment of MEFs with CM containing $S1P_2$ in exosomes derived from MDA-MB-231 cells induced significant DNA synthesis. This finding is in accordance with Guo *et al* (2017), who demonstrated that CM derived from MDA-MB-231 cells significantly enhanced MCF10A cell proliferation upon co-culture for 24, 48 and 72 hrs (Guo *et al*., 2017). Also, this finding reflects the impact of CM containing exosomes and other proteins including $S1P_2$ released from MDA-MB-231 cells on MEFs proliferation. Enhanced proliferation of fibroblasts therefore likely increases the release of growth factors, MMPs, cytokines and others factors that could potentially facilitate cancer metastasis, spread and secondary expansion.

Preliminary work using GM6001 and 1,10 phenanthroline protease inhibitors in MEFs showed no effect on CM induced ERK-1/2 signalling, suggesting that a protease that is not inhibited by these compounds could be involved in the proteolysis of $S1P_2$. These include MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP17, MMP24, MMP26, MMP27 and MMP28 (Sagi *et al*., 2016). This requires further investigations. Collectively, these findings provide evidence for an entirely new paradigm of communication between cancer-derived exosomes and fibroblasts involving proteolysis and constitutive activation of exosomal $S1P_2$

receptors in fibroblasts, which is independent of S1P. Future work requires *in vivo* study by making DNA constructs encoding the $S1P_2$ short form to establish the effect of over-expressing this form on tumour associated fibroblast and cancer metastasis.

$S1P_4$ receptors were found to be released in CM derived from MDA-MB-453 breast cancer cells (in addition to HER2 and phosphorylated ERK). Indeed, Long *et al* (2010) demonstrated a functional interaction between $S1P_4$ and HER2 regulating ERK-1/2 signalling in MDA-MB-453 cells. Thus, blocking HER2 using ErbB2 inhibitor II or HER2 siRNA completely abolished S1P-induced ERK activation (Long *et al*., 2010). Therefore, the possibility of transactivation of HER2 by S1P mediated by $S1P_4$ followed by co-internalisation and release of both receptors into CM is a possibility that requires further study. The pathophysiological effect of $S1P_4$ in exosomes is unknown and requires further characterisation.

Interestingly, $S1P_4$ receptors internalise in an agonist-dependent manner. This might be important in terms of the current findings where the $S1P_2$/$S1P_4$ antagonist JTE-013 was shown to reduce S1P induced $S1P_4$ receptor release into CM. However, S1P and JTE-013 had no effect on $S1P_2$ receptors internalisation and release into CM, suggesting $S1P_2$ is internalised through constitutive endocytosis and that the mode of exosomal release of the two receptors is somewhat different.

$S1P_2$ and $S1P_4$ receptors contain Serine/Threonine (S/T) cluster in its C-tail (Gene Bethesda (MD) (2018a)), which is known to form stable β-arrestin-GPCRs complex and sustained internalisation to endosome as exemplified by the V2 vasopressin receptor (V2R) (Oakleys *et al*., 2001). Thus, this strong complex might facilitate $S1P_2$ and $S1P_4$ internalisation, packaging and release of these receptors in exosomes.

Importantly, β-arrestin has been shown to act as scaffolding for other proteins that can lead to activation of MAPK signalling. Moreover, β-arrestin can interact with three different kinases in two MAPK signalling the ERK-1/2/MAPK signalling and the c- Jun-amino terminal kinase 3 (JNK3) (Pierce *et al*., 2002). Therefore, the $S1P_2$ short form might induce ERK-1/2 signalling via coupling to endogenous β-arrestin in MEFs. This requires further investigation.

Endocytosis is highly controlled process that depends on several factors. One important factor is the balance between sphingomyelin and cholesterol and perturbing this balance reported to induce formation of clusters of narrow endocytic tubular invaginations positive for N-BAR proteins and SK1 (Shen *et al*., 2014), and deletion of RVS161 and RVS167, which belong to BAR domains has been shown to cause defective endocytosis in *Candida albicans* (Douglas *et al*., 2009). However, there is a functional link between sphingolipid metabolism and the BAR domains RVS161 and RVS167 (Morgan *et al*., 2009; Youn *et al*., 2010).

Moreover, ceramide, which is the central sphingolipid metabolite has been shown to induce budding formation and exosomes release; in addition, it is involved in cargo sorting into endosomes lumen independent of endosomal sorting complex required for transport (ESCRT machinery) (Trajkovic *et al*., 2008). The SK1 inhibitor PF-543 significantly increased $S1P_4$ release into CM from MDA-MB-453 cells, suggesting that blocking SK1 increases sphingosine that is in turn converted to ceramide and which might mediate increased $S1P_4$ release into CM. Also, there is a possibility that sphingosine itself can increase ILVs (exosomes) fusion with plasma membrane (Young *et al*., 2016), and thus $S1P_4$ release.

Targeting sphingolipid metabolising enzymes such as SK1 is one of the approaches in the development of anti-cancer drugs (Pyne *et al*., 2012). High SK1 expression in cancer patients is correlated with poor prognosis and low survival rate in breast cancer patients (Long *et al*., 2010; Watson *et al*., 2010). In addition, SK1 can confer chemotherapeutics resistance, for example, tamoxifen responsiveness can be restored after siRNA knockdown of SK1 in MCF-7 cells (Sukocheva *et al.*, 2009). The sphingolipid 'rheostat' is considered as a sensor mechanism for regulating cell fate. Ceramide kinase (CERK) belongs to diacylglycerol kinase family and can convert ceramide (pro-apoptotic) to C1P (pro-survival) (Bornancin, 2011). Therefore, ceramide kinase can be considered as another component of sphingolipid metabolites regulating cell fate. Targeting SK1 and CERK might be useful as a combination, therapy aimed at enhancing cellular ceramide (pro-apoptotic) increase in cancer treatment compared with either treatment alone.

The effect of NVP-231 alone or in combination with PF-543 on $S1P_4$ release in MDA-MB-453 breast cancer cells was not examined, but is certainly worthy of further investigation in future studies in order to provide a functional link between the sphingolipids 'rheostat' and exosomal release of $S1P_4$.

NVP-231 exhibited a threshold dose response in MDA-MB-231 and MDA-MB-453 breast cancer cells indicated by steep fall or reduction in thymidine incorporation into DNA synthesis. This is might be due to high CERK concentration or expression, therefore, high NVP-231 is required to override CERK activity. Also, MDA-MB-231 and MDA-MB-453 cells might need a small concentration of CERK to proliferate

and survive assuming 10% of the total cellular CERK concentration; therefore, NVP-231 must occupy more than 90 % of CERK to start to induce anti-proliferative effect.

PF-543 is a SK1 potent competitive inhibitor with high selectivity for SK1 over SK2 (Schnute *et al*., 2012). PF-543 reduced thymidine incorporation into DNA synthesis in a threshold dose response manner. However, PF-543 appears more potent in MDA-MB-231 breast cancer cells. Notably, triple negative breast cancer has the highest expression of SK1 among other types of breast cancer (Geffken and Spiegel, 2017). Combination of NVP-231 and PF-543 resulted in less than additive effect in both types of cells with enhanced reduction in thymidine incorporation in MDA-MB-231 cells compared with MDA-MB-453 breast cancer cells. Also, SK1 inhibition by PF-543 reduced NVP-231 effectiveness in combination work, suggesting that increased sphingosine might enhance CERK activity therefore; high NVP-231 dose is required to override CERK activity.

Collectively, this study demonstrated multiple significant observations, which can be summarised as follows.

(i) $S1P_2$ and $S1P_4$ receptors are released into CM from MDA-MB-231 and MDA-MB-453 breast cancer cells respectively.

(ii) The study demonstrates for the first time the presence of the full length 42 kDa $S1P_2$ in exosomes released from MDA-MB-231 cells

(iii) Processing of exosomal $S1P_2$ receptor possibly by metalloproteinase (proteolysis) in MEFs produces a 38 kDa constitutively active form of $S1P_2$

(iv) The short form of $S1P_2$ induces activation of ERK-1/2 in MEFs and this is distinctive from endogenous $S1P_2$ in MEFs, which does not couple to ERK-1/2. The finding demonstrates the importance of the $S1P_2$ N-terminus proteolysis in modifying $S1P_2$ receptor signalling, thereby unmasking $S1P_2$ constitutive activity, which promotes ERK-1/2 signalling in MEFs.

(v) S1P stimulates $S1P_4$ release from MDA-MB-453 breast cancer cells into CM.

(vi) SK1 inhibition can modulate $S1P_4$ release indicating ceramide might have a role in regulating $S1P_4$ release possibly by regulating MVB maturation and loading with cargo

(vii) S1P and SK1 inhibition had no effect on $S1P_2$ receptor release into CM indicating cancer cell specific effects on $S1P_4$.

(viii) Attempts to alter or modulate the sphingolipid 'rheostat' using the CERK inhibitor, NVP-231 and SK1 inhibitor, PF-543 combination demonstrated less than additive effect, which suggests a possible functional interaction between CERK and SK1 that requires further investigation.

7.1 Future Work

Future work requires *in-vivo* study by preparation of DNA plasmid construct encoding the short form 38 kDa $S1P_2$ receptor tagged with suitable fluorophore, and over expressing them in tumour associated fibroblast, and injecting these cells into breast cancer orthotopic graft mouse model, and examining the impact of truncated version of $S1P_2$ on cancer metastasis.

CHAPTER VIII

REFERENCES

CHAPTER 8: References

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