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Identification of novel biomarkers for clinical prognosis in breast cancer

by

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Abstract

Sphingosine kinase/sphingosine 1-phosphate (SK/S1P) signalling interacts with major cellular pathways controlling cell proliferation, migration, survival, and resistance to chemotherapeutics. Moreover, extensive research has shown that the SK/S1P signalling is up-regulated in numerous human cancers (e.g. stomach cancer, colon, rectal, glioblastoma, ovarian, renal, lung and breast) making S1P signalling an important candidate as a biomarker and a key player in promoting cancer progression. Several inhibitors of SK/S1P signalling pathway have been identified and have shown to inhibit cancer cell survival and resistance to chemo- and radio-therapies.

In this study, human breast cancer tissue microarrays at various tissue histological grades of ERα negative breast tumours were analysed for the expression of S1P signalling proteins (e.g. SKs and $S1P_{1-5}$ receptors) to identify the impact of expression level of these proteins on clinical outcomes. High SK1 and $S1P_4$ receptor tumour expression is associated with poor cancer prognosis in ERα negative breast cancer patients. Moreover, high SK1 and $S1P_4$ receptor expression in these tumours was also associated with cancer recurrence and this was dependent on the HER2 receptor expression. Indeed, the SK1/2 dual inhibitor SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) abrogated the S1P-stimulated ERK-1/2 activation in $ER\alpha^-/HER2^+$ MDA-MB-453 cells suggesting that SK1 activity is required for the $S1P_4$/HER2-mediated ERK-1/2 activation that is known to promote cancer progression.

In addition, a diverse array of kinases and transcription factors e.g. c-RAF-1, ERK-1/2, AKT, LYN, SRC family kinases (SFKs) and NFκB (p50 RelA) were analysed in combination with SK1 and S1P receptors to discover novel prognostic interactions that drive cancer progression in ERα positive breast cancer patients. High tumour SK1 expression in combination with high expression of either $S1P_1$ receptor or $S1P_3$ receptor or phosphorylated ERK-1/2 or phosphorylated AKT or phosphorylated NFκB or phosphorylated RAF-1 or Y416 phosphorylated SFK or LYN is associated with shorter disease-specific patient survival and disease-free cancer recurrence. Similarly, high $S1P_1$ receptor tumour expression in combination with high expression of either Y216

phosphorylated SRC or c-RAF-1 or ERK-1/2 or AKT kinase is associated with shorter disease-specific patient survival and disease-free cancer recurrence. High $S1P_2$ receptor tumour expression is associated with prolonged patient survival and this is enhanced in combination with high expression of c-SRC and Y416 phosphorylated SFK in ERα positive breast cancer tumours. Finally, high tumour $S1P_3$ receptor expression in combination with high expression of LYN or c-RAF-1 kinases is associated with shorter disease-specific patient survival and disease-free cancer recurrence.

Lastly, a new signalling pathway involving SK2, Y416 phosphorylated c-SRC, $S1P_4$ receptor and $S1P_2$ receptor was identified using pharmacological agents/gene silencing in ERα negative MDA-MB-231 breast cancer cells. In this pathway, SK2 possibly through 'inside out' S1P signalling activates the $S1P_4$ receptor that promotes cellular growth by preventing the nuclear accumulation of $S1P_2$ receptor. Moreover, SK2 activity also prevents the accumulation of Y416 phosphorylated c-SRC into the nucleus that might be crucial for tumour growth.

Thus, this study shows that the high tumour expression of S1P signalling proteins is associated with poor disease prognosis in both ERα positive and ERα negative breast cancer patients. However, cancer progression is mediated by distinct set of S1P signalling proteins in different types of breast cancer. Hence, different treatment regiments including SK inhibitors and S1P receptor antagonist must be employed in treatment of ERα positive and ERα negative breast cancer patients.

List of publications

1. Ohotski J, Long JS, Orange C, Elsberger B, Mallon E, Doughty J, Pyne S, Pyne NJ, Edwards J. Expression of sphingosine 1-phosphate receptor 4 and sphingosine kinase 1 is associated with outcome in oestrogen receptor-negative breast cancer. *Br J Cancer* 2012;**106**:1453-1459.

2. Ohotski J, Edwards J, Elsberger B, Watson C, Orange C, Mallon E, Pyne S, Pyne NJ. Identification of novel functional and spatial associations between sphingosine kinase 1, sphingosine 1-phosphate receptors and other signalling proteins that affect prognostic outcome in estrogen receptor-positive breast cancer. *Int J Cancer* 2013;**132**:605-616.

3. Ohotski J, Rosen H, Bittman R, Pyne S, Pyne NJ. Sphingosine kinase 2 prevents the nuclear translocation of sphingosine 1-phosphate receptor-2 and tyrosine 416 phosphorylated c-Src and increases estrogen receptor negative MDA-MB-231 breast cancer cell growth: The role of sphingosine 1-phosphate receptor-4. *Cell Signal* 2014;**26**:1040-1047.

4. Pyne NJ, Ohotski J, Bittman R, Pyne S. The role of sphingosine 1-phosphate in inflammation and cancer. *Adv Biol Regul* 2014;**54**:121-129.

5. Pyne S, Edwards J, Ohotski J, Pyne NJ. Sphingosine 1-phosphate receptors and sphingosine kinase 1: novel biomarkers for clinical prognosis in breast, prostate, and hematological cancers. *Front Oncol* 2012;**2**:168.

CHAPTER I

Introduction

1.1. The sphingolipid metabolism

Sphingolipids are one of the major components of both eukaryotic and prokaryotic cell membranes. Ceramide, sphingomyelin, glycosphingolipids and gangliosides are the most abundant plasma membrane sphingolipids. Besides their structural role involved in maintaining the integrity of cell membrane, these lipids have shown to be involved in cell signalling. Among all sphingolipids, sphingosine has the simplest molecular structure possessing a 2-aminooctadec-4-ene-1,3-diol (sphingosine) backbone (Claus *et al.,* 2009) (**Figure 1.1**).

Collective research has shown that sphingolipids are bioactive compounds involved in cellular processes such as cell proliferation, differentiation, migration, survival and apoptosis, that are major processes controlling cancer progression, neovasculogenesis, angiogenesis, metastasis, inflammation and drug resistance (Obeid *et al.,* 1993, Mathias *et al.,* 1998, Hannun *et al.,* 2008, Weigert *et al.,* 2009). Ceramide, sphingosine and sphingosine 1-phosphate (S1P) are the most prominently experimented sphingolipids, possessing opposing effects on biological systems (Hannun *et al.,* 2008).

1.1.1. The synthesis of sphingolipids

Upon cell activation, sphingolipid synthesis is mediated by two distinct pathways. *De novo* synthesis of sphingolipids starts in the endoplasmic reticulum (ER) with condensation of serine and palmitoyl-CoA by serine palmitoyl transferase creating 3-keto-dihydrosphingosine (Claus *et al.,* 2009, Hannun *et al.,* 2002). This product is then converted into sphinganine by 3-ketosphinganine reductase. Subsequently, sphinganine is acylated to dihydroceramide by ceramide synthase (also called dihydroceramide synthase) and eventually into ceramide by the enzyme dihydroceramide desaturase (**Figure 1.2**). Six different ceramide synthases (CerS1-6) have been characterised each selectively binding to acyl chains with varying length (C_{16}-C_{26}) linked to coenzyme A required for N-acylation of the sphinganine base (Venkataraman *et al.,* 2002). Ceramide is transferred from ER to the Golgi apparatus by the ceramide transfer protein (CERT) for the synthesis of sphingomyelin. Sphingomyelin is synthesised and translocated into cell membrane (Claus

et al., 2009, Fukasawa *et al.,* 1999). Thus ceramide is further converted into sphingomyelin via sphingomyelin synthases and it is incorporated into target membranes. There are two isoforms of sphingomyelin synthases which differ in intracellular localisation: SMS1 localises in the Golgi and SMS2 localises in both Golgi and plasma membrane (Yamaoka *et al.,* 2004). Upon stimulus, sphingomyelin is hydrolysed back to ceramide via sphingomyelinases (Dai *et al.,* 2004). Sphingomyelinases form a vast class of enzymes that are divided into various categories according to optimal pH for maximum catalytic activity and required cofactors for their catalytic activity (Goni and Alonso, 2002). Ceramide is phosphorylated or glycosylated to form ceramide 1-phosphate or glucosylceramide respectively (Liu *et al.,* 2001) (**Figure 1.3**). Alternatively, ceramide is used as a substrate

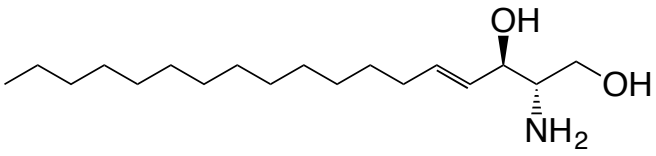

Figure 1.1. The chemical structure of sphingosine

by ceramidase to synthesise sphingosine by hydrolysing the N-acyl fatty acid linked to sphingosine backbone. Following the activation of sphingosine kinases (SK1 and SK2), sphingosine is phosphorylated at the primary hydroxyl group to form S1P.

1.1.2. The biological activity of ceramide and sphingosine

Chemotherapeutic agents such as Paclitaxel (Taxol™) induce apoptosis by promoting ceramide generation in breast and prostate cancer cells (Charles *et al.,* 2001, Gouaze *et al.,* 2005, Sumitomo *et al.,* 2002). In addition, exposure to radiation or treatment with death ligands, such as tumour necrosis factor-alpha (TNFα) activates the enzyme sphingomyelinase, which hydrolyses sphingomyelin to form ceramide. Consequently, the increase in the level of intracellular ceramide initiates apoptosis (Birbes *et al.,* 2002). Ceramide has been reported to bind and activate protein kinase C zeta (PKC**ζ**) which activates stress-activated protein kinases, such as p38 mitogen-activated kinase (MAPK) and c-Jun N-terminal kinase (JNK) (Modrak *et al.,* 2006). Ceramide also induces activation of protein phosphatases, such as PP2A and PP1 that inactivate AKT kinase

thereby inhibiting cell proliferation and survival (Wolff *et al.,* 1994 and Dobrowsky *et al.,* 1993). Recently, p38 MAPK and JNK activation has been shown to promote BAX translocation to the mitochondria. This process is synergistic with ceramide accumulation leading to formation of channels at the mitochondrial membrane that result in the release of pro-apoptotic proteins, such as cytochrome C and pro-caspases (Colombini *et al.,* 2010, Martinez-Abundis *et al.,* 2009, Kong *et al.,* 2005, Saelens *et al.,* 2004). Furthermore, ceramide-mediated activation of PP2A and glycogen synthase kinase 3β leads to activation of cathepsin D that activates caspase-2 and caspase-8. Sphingosine also possesses pro-apoptotic properties, inducing cell death. Indeed, sphingosine has been shown to activate BAX via PKCδ and activate caspase-3/9 to initiate apoptosis in various cell lines (Kanno *et al.,* 2012, Phillips *et al.,* 2007). Additionally, TNF-induced apoptosis is mediated through sphingosine accumulation on the lysosomal membrane that permeabilizes and releases lysosomal cathepsins in rat HTC hepatoma cells (Ullio *et al.,* 2012).

Cancer cells use various strategies to overcome ceramide/sphingosine-mediated apoptosis. These include down-regulation of ceramide synthesis via the *de novo* and sphingomyelin pathways and/or over-expression of enzymes that convert ceramide into non-apoptotic sphingolipids (**Figure 1.3**). Sphingomyelinase and serine palmitoyltransferase have been shown to be down-regulated in human colon cancer cell lines (Duan *et al.,* 2005, Duan *et al.,* 2009). In contrast, ceramide kinase, acid ceramidase, glucosylceramide synthase and sphingosine kinase 1 have been shown to be over-expressed in various cancer cell lines maintaining cell survival, proliferation and drug resistance (Malavaud *et al.,* 2010, Ruckhaberle *et al.,* 2008, Mitra *et al.,* 2007, Morales *et al.,* 2007).

1.1.3. The activation of sphingosine kinases

In contrast to the biological activity of ceramide and sphingosine, S1P inhibits apoptosis, and promotes survival, proliferation and migration of cells. For example, NGF protects cells from apoptosis via activation of SK1 upon serum withdrawal (Edsall *et al.,* 2001). Indeed, over-expression of SK1 induces cell survival and chemoresistance in prostate, pancreatic and leukaemia cancer cell lines. Moreover, knock-down of SK1 expression was shown to induce apoptosis in these cell lines (Akao *et al.,* 2006, Guillermet-Guibert *et al.,*

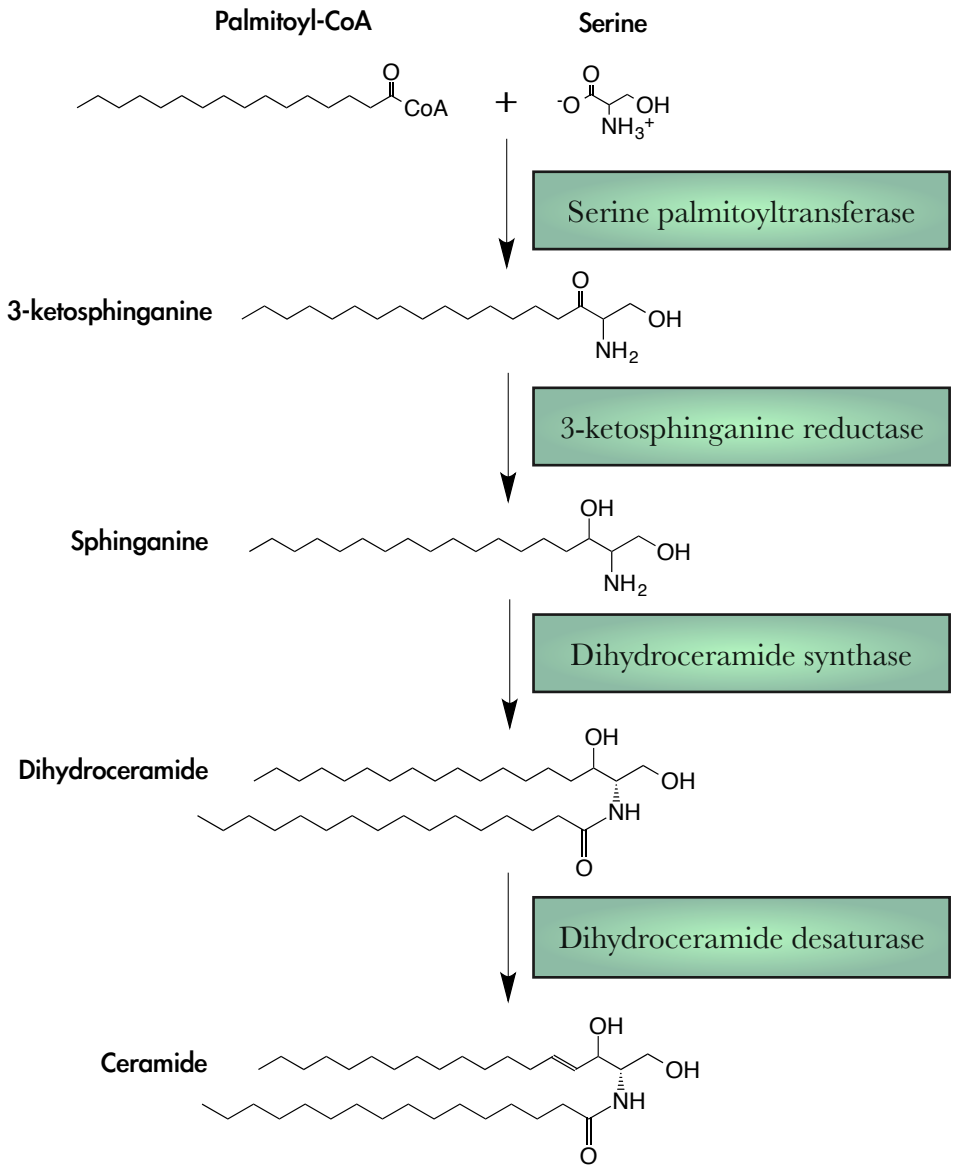

Figure 1.2. The *De novo* sphingolipid synthesis. Following the reaction of palmitoyl-CoA with serine leads to the synthesis of ceramide catalysed by various enzymes. Subsequently, the pro-apoptotic ceramide can be processed into ceramide 1-phosphate, glucosylceramide, sphingomyelin and sphingosine.

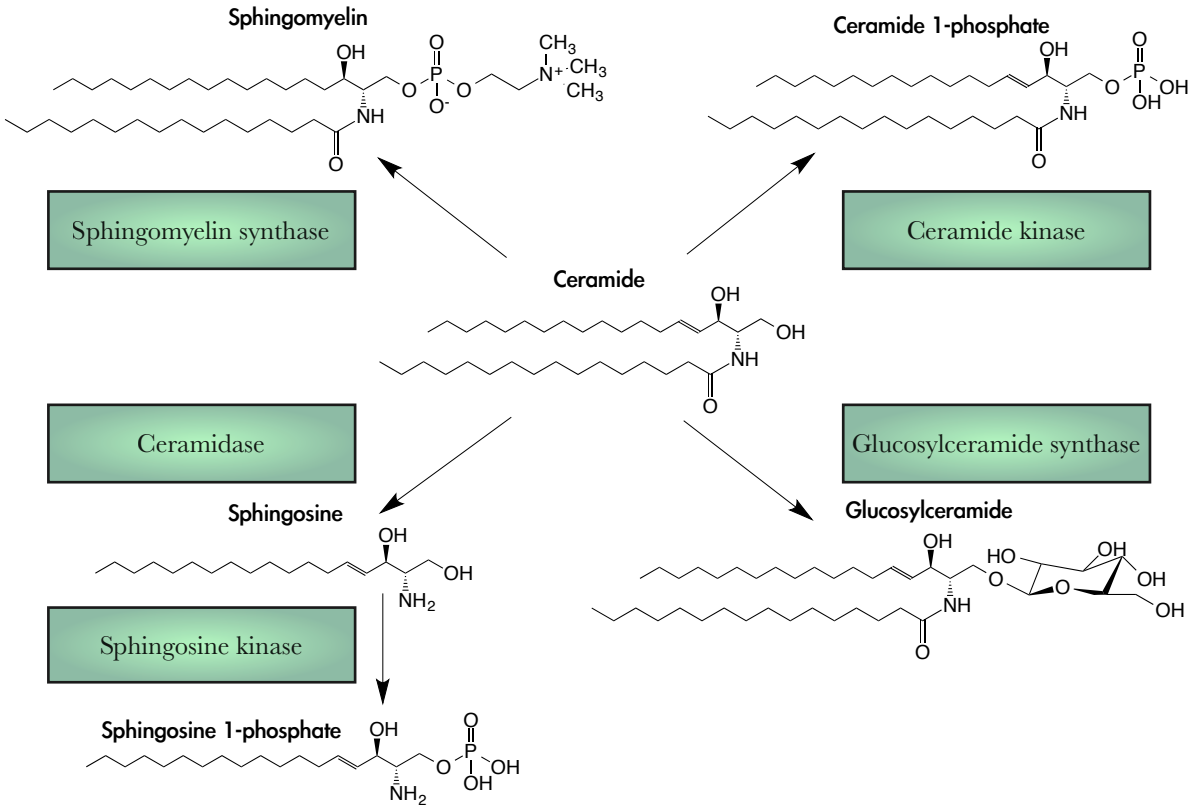

Figure 1.3. The conversion of pro-apoptotic ceramide into non-apoptotic lipids. Sphingomyelin synthase converts the ceramide into sphingomyelin which serves as a reservoir in production of bioactive lipids. The ceramidase converts ceramide into sphingosine that leads to the formation of S1P catalysed by sphingosine kinase. The glucosylceramide synthase catalyses the conversion of ceramide into glucosyl ceramide. The ceramide kinase catalyses the conversion of ceramide into ceramide 1-phosphate. These enzymes except sphingomyelin synthase are over-expressed in various cancers and inhibit apoptosis while maintaining tumour growth, while also conferring resistance to chemotherapeutics.

2009, Baran *et al.,* 2007). SK1 activity inhibits caspases and possibly JNK protein kinase, thereby inhibiting stress-induced apoptosis (Edsall *et al.,* 2001). Therefore, opposing cellular effects of ceramide and S1P suggests a strict regulation of enzymes involved in metabolism of these two lipids.

In mammalian cells, SK1 and SK2 are encoded by two genes *SPHK1* and *SPHK2* respectively. These two proteins share 80% similarity in protein sequence (Liu *et al.,* 2000). Three splice isoforms of SK1 (termed SK1a, SK1b and SK1c) and SK2 (SK2a, SK2b and SK2c) are expressed in cells, each possessing distinct N-terminal sequences (Pitson *et al.,* 2011). Genetic studies have shown that SK1 or SK2 homozygous mutant mice have regular longevity lacking any physical and physiological abnormality (Mizugishi *et al.,* 2005). However, homologous deletion of both SK1 and SK2 was lethal and embryos die between days 11.5 and 13.5 due to undeveloped vasculature leading to haemorrhage. The ability of SK1 and SK2 to supplement each other functionally suggest that in development these two kinases exhibit overlapping functions. For example, SK1 and SK2 modulate cell migration induced by epidermal growth factor (EGF) and transforming growth factor (TGF)-β in breast cancer and oesophageal cancer cells respectively (Hait *et al.,* 2005, Miller *et al.,* 2008). In addition, stimulation of IgE receptor leads to translocation of both SK1 and SK2 to the plasma membrane (Olivera *et al.,* 2006, Olivera *et al.,* 2007). However, Gao and Smith have shown that SK1 and SK2 have different downstream targets as siRNA-mediated knockdown of SK1 or SK2 have different effects on p53, pAKT, pERK1, pERK2, and p21 in A498 kidney carcinoma cells (Gao and Smith, 2011). Moreover, although both SK1 and SK2 isoforms synthesise S1P upon activation in ATP- and Mg^{++}-dependent manner, various functional differences have also been determined. Initially, the two isoforms have been shown to possess different substrates preferences. SK1 has high affinity for D-erythrosphingosine, while SK2 has preference for D-erythrodihydrosphingosine, which paradoxically is an inhibitor of SK1 (Liu *et al.,* 2000 and Kohama *et al.,* 1998). SK1 and SK2 also have diverse tissue-specific expression profiles and different subcellular localisation suggesting that each isoform have different cellular functions (Lai *et al.,* 2009 and Oskeritzian *et al.*, 2008). In this regard, over-expression of SK1 has been shown to induce proliferation and chemotherapeutic resistance against apoptosis, and its inhibition induced apoptosis and increased sensitivity of cells to chemotherapeutics (Pitman and Pitson, 2010). Over-expression of SK1 prevents

apoptosis while over-expression of SK2 enhances apoptosis upon serum withdrawal in NIH 3T3 cells (Liu *et al.,* 2003). SK2 over-expression inhibits DNA synthesis and causes cell cycle arrest at G_1/S phase in HeLa and NIH 3T3 cells respectively (Igarashi *et al.,* 2003). Moreover, Liu and coworkers have shown that SK2-mediated apoptosis involves the release of cytochrome *c* from mitochondria and the activation of caspase 3 (Liu *et al.,* 2003). In addition, SK2 has been reported to contain a BH3 domain that sequesters Bcl-x_L, an anti-apoptotic protein, thereby inducing apoptosis (Liu *et al.,* 2003). SK2 is also required for TNF-α induced apoptosis in mouse embryonic fibroblasts and HEK 293 cells, and siRNA-mediated down-regulation of SK2 inhibits apoptosis (Okada *et al.,* 2005).

Inhibition of SK2 expression induces apoptosis in various cancer cell lines. For example, siRNA-mediated knock-down of SK2 induces apoptosis in U-87 MG glioblastoma, A498 kidney adenocarcinoma, Caki-1 kidney carcinoma and MDA-MB-231 breast adenocarcinoma cells (Gao and Smith, 2011, Van Brocklyn *et al.,* 2005). Indeed, SK2 is also a preferable target over SK1 as induction of apoptosis via SK2-specific siRNA is more effective compared to inhibition of SK1. siRNA mediated knock-down or pharmaceutical inhibition delays the growth of MCF-7 human breast carcinoma, Bxpc-3 human pancreatic adenocarcinoma, A498 kidney carcinoma and chemoresistant MCF-7TN-R cells in mice xenograft model (Beljanski *et al.,* 2011, Antoon *et al.,* 2011, Weigert *et al.,* 2009). In addition, SK2 promotes cell survival and resistance to doxorubicin-induced apoptosis in breast and colon cancer cells and down-regulation of SK2 recovered sensitivity to doxorubicin in these cell lines (Sankala *et al.,* 2007). Thus, multiple studies have shown that SK2 can mediates apoptosis as well as cell proliferation.

SK1 is activated by various stimuli, including growth factors such as platelet-derived growth factor (PDGF) (Pyne *et al.,* 1996, Olivera *et al.,* 1993), epidermal growth factor (EGF) (Meyer zu Heringdorf *et al.,* 1999), vascular endothelial growth factor (VEGF) (Shu *et al.,* 2002) and nerve growth factor (NGF) (Rius *et al.,* 1997), cytokines, and immunoglobulin receptors (Spiegel and Milstien, 2003, Wang *et al.,* 2010). SK1 is activated by phosphorylation at Ser^{225} by ERK-1/2, which results in its translocation to plasma membrane from the cytoplasm (Johnson *et al.,* 2002, Pitson *et al.,* 2003, Jolly *et al.,* 2005, Olivera *et al.,* 2006). At the plasma membrane, SK1 binds phosphatidylserine through Thr^{54} and Asn^{89} residues, and phosphatidic acid in Golgi apparatus (**Figure 1.4**)

(Stahelin *et al.,* 2005, Delon *et al.,* 2004). Recently, calcium and integrin binding protein 1 (CIB1) has been shown to mediate the translocation of SK1 to plasma membrane via calcium-dependent mechanism involving interaction with calmodulin-binding site of SK1 (Jarman *et al.,* 2010). SK1 has also shown to possess protein kinase C (PKC) and protein kinase A (PKA) phosphorylation sites (Maceyka *et al.,* 2002). Additionally, SK1 is acetylated at Lys^{27} and Lys^{29} via p300 and CBP acetyltransferases that inhibits ubiquitin-mediated degradation of SK1 (Yu *et al.,* 2012, Loveridge *et al.,* 2010).

SK2 is located in the plasma membrane, cytoplasm, ER and nucleus in a cell context-dependent manner (Maceyka *et al.,* 2005, Igarashi *et al.,* 2003). Plasma membrane localisation is mediated by the N-terminal region of SK2 that binds either glycolipid sulfatide (3-O-sulfogalactosylceramide) or phosphatidylinositol monophosphates (e.g. phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-phosphate and phosphatidic acid). Binding of glycolipid sulfatide has been shown to inhibit the activity of SK2, whereas binding of phosphatidylinositol 4,5-bisphosphate activates catalytic activity of SK2 (Don and Rosen, 2009). In addition, SK2 is activated by various kinases downstream of IgE and EGF receptor (Olivera *et al.,* 2006 and Hait *et al.,* 2005). SK2 is phosphorylated at Ser^{351} and/or Thr^{578} by ERK-1/2 (Hait *et al.,* 2007). Protein kinase D (PKD) has also been shown to phosphorylate SK2 at Ser^{383} and Ser^{385}, leading to its export from the nucleus and promoting growth arrest (**Figure 1.4**) (Ding *et al.,* 2007). Phosphorylation of SK2 by PKC is proposed to promote interaction of SK2 with histone deacetylase 1/2 (HDAC1/2) and histone 3 (H3). S1P formed by SK2 inhibits HDAC1/2 and induce transcription of specific genes (Hait *et al.,* 2009). SK2 has also been shown to interact with various proteins such as FHL-2, eEF1A and Lyn/Fyn kinases (Leclercq *et al.,* 2008, Olivera *et al.,* 2006, Sun *et al.,* 2006).

1.1.4. The inactivation of sphingosine kinases

Inactivation of SK1 leads to accumulation of ceramide and sphingosine that induces cell cycle arrest and apoptosis (Taha *et al.,* 2006). SK1 has been shown to be inactivated through dephosphorylation at Ser^{225} by protein phosphatase 2A (PP2A) (Barr *et al.,* 2008). PP2A is a tumour suppressor that belongs to serine/threonine phosphatases that mediates

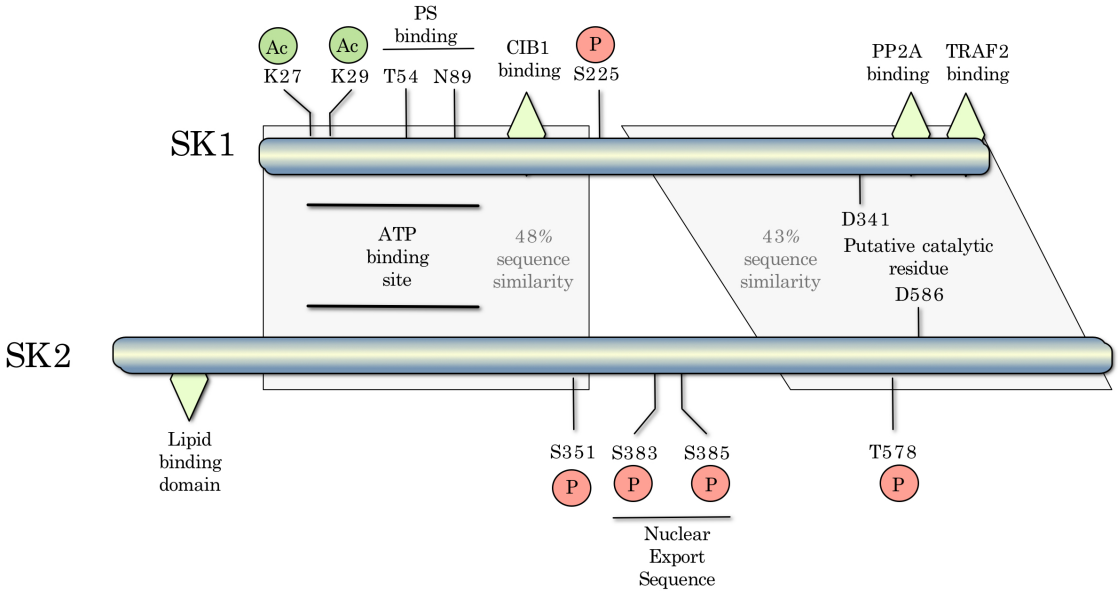

Figure 1.4. The sequence homology of SK1 and SK2. In comparison to SK1, SK2 has two additional structural domains, an N-terminal region and a central region. SK1 is activated either by phosphorylation or through protein-protein interaction. ERK-1/2 was shown to phosphorylate SK1 at Ser^{225} and SK2 at Ser^{351} and/or Thr^{578} (Stahelin *et al.,* 2005, Jolly *et al.,* 2005, Delon *et al.,* 2004, Pitson *et al.,* 2003, Johnson *et al.,* 2002). SK1 also possesses binding sites for phosphatidylserine, CIB1, PP2A and TRAF2 (Pitman *et al.,* 2011, Alvarez *et al.,* 2010, Jarman *et al.,* 2010, Barr *et al.,* 2008, Stahelin *et al.,* 2005). In addition, SK1 has two acetylation sites at its N-terminal. The current knowledge on SK2 is more restricted compared to SK1. PKD catalyses the phosphorylation of SK2 at Ser^{383} and Ser^{385} leading to export from the nucleus (Ding *et al.,* 2007). SK2 has a nuclear export sequence at its central region and a lipid binding site at its N-terminal (Maceyka *et al.,* 2005). Both kinases possess an ATP binding site and a catalytic residue that mediate the phosphorylation of sphingosine (Wang *et al.,* 2013) (Ac: Acetylation site, P: Phosphorylation site).

inactivation of pAKT, β-catenin, pERK-1/2, BAD and Bcl-2 inhibiting cellular proliferation and survival (Kuo *et al.,* 2008, Li *et al.,* 2001). PP2A activity is controlled by protein-protein interaction and post-transcriptional modification through methylation and phosphorylation. Type 2 A interacting protein (TIP), adenomatous polyposis coli (APC) protein and Rac1 have been shown to activate PP2A whereas phosphoprotein SET and cancerous inhibitor of PP2A (CIP2A) have been shown to inhibit PP2A activity (Li *et al.,* 2012, Junttila *et al.,* 2007, McConnell *et al.,* 2007, Neviani *et al.,* 2005, Seeling *et al.,* 1999).

Various compounds have been shown to activate PP2A. For example, sphingosine analog FTY720 (Fingolimod™, Novartis) which is an $S1P_1$ receptor functional antagonist activates PP2A resulting in cell death in various cancer models (Yang *et al.,* 2012, Saddoughi *et al.,* 2012, Roberts *et al.,* 2010). Activation of PP2A was induced through down-regulation of PP2A inhibitor SET in acute myeloid leukemia (AML) cells (Roberts *et al.,* 2010). Moreover, FTY720 has been shown to bind to SET disrupting its interaction with PP2A mimicking the action of ceramide that results in activation of PP2A (Oaks *et al.,* 2013, Saddoughi *et al.,* 2012).

Inhibition of SK1 activity is also mediated through interaction with inhibitory proteins such as SKIP protein. SKIP share 75-80% similarity in amino acid sequence with protein kinase A anchoring protein 110 (AKAP110). Protein kinase A anchoring proteins (AKAPs) are involved in localisation of proteins at specific sites within the cell, mediating the activation of signalling pathways at distinct cellular compartments. AKAPs interact with kinases, protein phosphatases and phosphodiesterases (e.g. protein kinase A and protein phosphatase 2B) to modulate cell proliferation, cell migration and membrane trafficking (Dell'Acqua *et al.,* 2002, Colledge *et al.,* 2000) Lacana and coworkers have shown that SKIP over-expression inhibits SK1 catalytic activity negating SK1-mediated cell proliferation and sensitising NIH 3T3 cells to apoptosis upon serum withdrawal (Lacana *et al.,* 2002). Thus, SKIP might act as a direct inhibitor or a scaffolding protein mediating the interaction of SK1 with other inhibitory proteins such as protein phosphatases (e.g. PP2A).

SK1 is degraded upon stimulation with TNF-α, disruption of DNA integrity or microtubule functioning (Taha *et al.,* 2004, Pchejetski *et al.,* 2005, Taha *et al.,* 2005). TNF-α induces

apoptosis through activation of TNFR1 that activates caspase and cathepsin family of proteases (e.g. caspase-8/9, cathepsin B/D) (Foghsgaard *et al.,* 2001). These proteases induce the release of cytochrome *c* from mitochondria through activation of BH3-only protein Bid that activates executioner caspases (e.g. caspase-3/7) (Stoka *et al.,* 2001). Executioner caspases inactivate DNA repair mechanisms through cleavage of poly(ADP-ribose) polymerase (PARP) and DNA-PK while inducing DNA degradation through activation of caspase-activated deoxyribonuclease (CAD) (Fan *et al.,* 2005). Caspases also induce decomposition of nuclear and cytoplasmic membranes through cleavage of lamin A and α-fodrin (Cowling *et al.,* 2002). Taha and coworkers have shown that the degradation of SK1 was mediated by cathepsin B in MCF-7 breast cancer cells upon stimulation with TNF-α (Taha *et al.,* 2005). In addition, genotoxic stress-mediated downregulation of SK1 is promoted by tumour suppressor protein p53. Upon DNA damage, p53 is activated which acts as a transcription factor promoting the expression of genes involved in DNA repair (e.g. PARP-1), cell cycle arrest (e.g. cdk1/2) and apoptosis (Riley *et al.,* 2008). Taha and coworkers have reported that p53-mediated degradation of SK1 is through cathepsin B in human T-cell Molt-4 leukemia cells (Taha *et al.,* 2006a, Taha *et al.,* 2004). Pharmacological inhibition of SK1 also induces its degradation through various mechanisms. Ren and coworkers have reported that the degradation of SK1 is mediated by lysosomal pathway involving activation of cathepsin B in podocytes (Ren *et al.,* 2010). In addition, Loveridge and coworkers have shown that the SKi-mediated SK1 degradation is insensitive to cathepsin B inhibitor and involves the ubiquitin/proteasomal pathway in human pulmonary artery smooth muscle (hPASMC), human prostate cancer (LNCaP) and human breast cancer (MCF7) cell lines (Loveridge *et al.,* 2010). SKi treatment decreases intracellular SK1 expression while increasing ceramide (C22:0) levels and activating caspase-3 in LNCaP cells. The proteasomal degradation of SK1 is dependent on ceramide accumulation as inhibition of ceramide synthase using fumonisin B1 reduces the down-regulation of SK1 expression in these cells. In addition, treatment of MCF-7 cells and astrocytes with ceramide activates the proteasome (Loveridge *et al.,* 2010, Catalyud *et al.,* 2005). In contrast, SKi-induced caspase-3 activation is not required for degradation of SK1 but is involved in promoting apoptosis of LNCaP cells (Loveridge *et al.,* 2010).

1.1.5. The degradation of sphingosine 1-phosphate

Besides the inactivation of sphingosine kinases, biological activity of S1P is inhibited through conversion of S1P back to sphingomyelin or through its degradation. S1P is degraded by two membrane-bound enzymes denoted as sphingosine 1-phosphate lyase (S1PL) and sphingosine 1-phosphate phosphatase (S1PP) (Pyne and Pyne, 2000). S1PL is encoded in *SGPL1* gene and its transcription is controlled by Sp1 and GATA-4 transcription factors. S1PL is an endoplasmic reticulum- and mitochondrial associated membrane (MAM)-localised enzyme that catalyses the irreversible cleavage of S1P at C_2-C_3 carbon-carbon bond to produce phosphoethanolamine and fatty acid aldehyde (hexadecenal) (Ikeda *et al.,* 2004, Oskouian *et al.,* 2006, Van Veldhoven *et al.,* 1993). The over-expression of S1PL results in increased sensitivity to apoptosis upon serum withdrawal, chemotherapy and irradiation (Oskouian *et al.,* 2006, Min *et al.,* 2005, Reiss *et al.,* 2004). Oskouian and coworkers have shown that S1PL mediates apoptosis through activation of p38, p53, p53-inducible death domain protein (PIDD) and caspase-2 upon DNA damage in HEK293 cells (Oskouian *et al.,* 2006). In addition, the over-expression of S1PL increases ceramide levels while decreasing S1P levels that enhances sensitivity to chemotherapy and radiotherapy in human C4-2B and PC-3 prostate cancer cell lines (Brizuela *et al.,* 2012). The S1PL catalytic activity was also required for the S1PL-mediated sensitisation of HEK293 cells to apoptosis. S1PL induces the activation of p38, a tumour suppressor protein that belongs to MAP kinase family responsible for activation of p53 and caspase-2 upon DNA damage (Olson *et al.,* 2004). In addition, Brizuela and coworkers have shown that silencing S1PL expression induces SK1 expression accompanied by increase in the intracellular S1P levels that transforms murine embryonic fibroblasts into tumourigenic phenotype (Brizuela *et al.,* 2012).

The S1PL catalytic activity does not only prevent the accumulation of S1P but also other lipids such as sphingosine, ceramide, sphingomyelin, diacylglycerol and cholesterol in serum and in liver indicating its role in lipid metabolism (Bektas *et al.,* 2010). In addition to the accumulation of lipids in liver, mice lacking the S1PL expression have reduced mass of adipose tissue. Microarray analysis of mRNA expression have revealed that the peroxisome proliferator-activated receptor (PPAR)γ expression was up-regulated in the liver of mice lacking S1PL expression. PPAR family of nuclear receptors are transcription

factors that control the expression of proteins involved in glucose and lipid metabolism (e.g. GLUT4, Adiponectin) and adipocyte differentiation (Maeda *et al.,* 2001, Wu *et al.,* 1998). PPARs are activated by fatty acid and cholesterol metabolites that results in heterodimerization with retinoid X receptors (RXRs) allowing the interaction with specific DNA response elements (Kliewer *et al.,* 1997). PPARγ has a central role in diseases such as diabetes and liver steatosis. For example, PPARγ activation stimulates the glucose uptake and metabolism in adipose tissue and liver (Lemberger *et al.,* 1996). However, the over-expression of PPARγ results in liver steatosis (Gavrilova *et al.,* 2003). Moreover, PPARγ activation induces cell cycle arrest and apoptosis through inhibition of E2F/DP transcription factors and up-regulation of BAX and BAD proapoptotic proteins in NIH 3T3 mouse embryonic fibroblasts and C6 glioma cells respectively (Zander *et al.,* 2002, Altiok *et al.,* 1997). In addition, Brockman and coworkers have shown that the PPARγ activation inhibits the transition from G1 to S phase in various colorectal cancer cell lines (Brockman *et al.,* 1998).

Bektas and coworkers have also reported that the expression of *SPTLC1* mRNA encoding the serine palmitoyltransferase (SPT) was down-regulated in the liver of mice lacking the S1PL expression (Bektas *et al.,* 2010). SPT belongs to α-oxoamine synthases that catalyses the formation of 3-ketodihydrosphingosine from L-serine and acyl-coenzyme A which is the initial step in *de novo* sphingolipid synthesis. The imatinib-mediated inhibition of BCR-ABL catalytic activity promotes the SPT activation that induces apoptosis in K562 and LAMA-84 human myeloid leukemia cell lines (Taouji *et al.,* 2013). In addition, the myroicin-mediated inhibition of SPT enhances the expression of p53 and p21 that inhibits cancer cell proliferation in melanoma mice. The inhibition of SPT activity was accompanied by the decrease in levels of ceramide, sphingomyelin and sphingosine-1-phosphate in serum, liver and tumour tissue in melanoma mice (Lee *et al.,* 2012, Lee *et al.,* 2011). Thus, the outcome of SPT activation on cell survival also depends on the expression and the activation of other proteins involved in sphingolipid metabolism.

By the action of S1PP, S1P can be utilised to resynthesise the sphingolipid metabolites, such as sphingosine and ceramide thereby affecting the ceramide/S1P rheostat. There are two types of S1P-specific phosphatases that belong to the family of type 2 lipid phosphate phosphohydrolases. The S1P-specific S1PPs was shown to localise at the endoplasmic

reticulum, and the general lipid phosphatases (LPP) was shown to localise to the plasma membrane and the endoplasmic reticulum (Mandala *et al.,* 2000, Alderton *et al.,* 2001). The down-regulation of S1PP1 was shown to enhance the EGF-stimulated migration in HEK 293 cells, whereas the over-expression of S1PP1 inhibits the migratory response generated by the EGF through degradation of S1P required for the activation of S1P receptor (Stunff *et al.,* 2004). Moreover, Johnson and coworkers have reported that the down-regulation of S1PP1 expression increases the intracellular and the extracellular S1P levels while decreasing the sphingosine levels. In addition, the down-regulation of S1PP1 impairs the apoptotic response to TNF-α and daunorubicin in MCF-7 breast cancer cells (Johnson *et al.,* 2003).

In addition to the role of S1PP1 in cell migration and proliferation, S1PP2 is involved in pro-inflammatory signalling. Mechtcheriakova and coworkers have shown that inflammatory stimuli (e.g. TNF-α and LPS) induces the expression and the activation of S1PP2 in neutrophils and HUVEC cells (Mechtcheriakova *et al.,* 2007). Indeed, the promoter region of S1PP2 gene contains NFκB binding site and the inhibition of expression of RelA subunit of NF*κ*B inhibits the expression of S1PP2 upon stimulation with TNF-α. In addition, siRNA mediated silencing of S1PP2 expression inhibits the TNF-α-induced expression of IL-1β and IL-8 in HUVEC cells (Mechtcheriakova *et al.,* 2007).

1.2. The extracellular receptors of sphingosine 1-phosphate

The S1P is present in human plasma at a concentration of ~200 nM (Yatomi *et al.,* 1997). Approximately 65% of the S1P in plasma is associated with lipoproteins (e.g. LDL, VLDL and HDL) and ~54% of the S1P in plasma is associated with high-density lipoproteins (Murata *et al.,* 2000). Various studies have shown that erythrocytes synthesise and release S1P in the blood stream contributing to 90% of total S1P in plasma (Pappu *et al.,* 2007, Hanel *et al.,* 2007). Moreover, SK1 is secreted into the blood stream by endothelial cells and airway smooth muscle cells (SMCs) (Venkataraman *et al.,* 2006, Ancellin *et al.,* 2002, Waters *et al.,* 2003). In addition, the intracellular S1P formed by SK1 is transported by specific transporters (e.g. ABCC1 and SPNS2) to the extracellular milieu, where it is in close vicinity to S1P receptors thereby maintaining so-called 'inside-out' signalling (Hisano *et al.,* 2012, Takabe et al., 2010, Kawahara *et al.,* 2009). Extracellular targets for S1P are five G protein-coupled receptors (GPCRs) denoted as $S1P_{1-5}$ (Spiegel and Milstien, 2003). Gene symbols of SKs and S1P receptors are summarised in **Table 1.1.** The S1P receptors couple to various G-proteins such as G_i, $G_{12/13}$ and G_q thereby inducing the activation of diverse intracellular signalling pathways (**Figure 1.5**) (Watterson *et al.,* 2007, Pyne & Pyne, 2000). Although, these receptors bind S1P with comparable affinities, the final effect caused by S1P depends on cell-type specific expression of the S1P receptors and the specificity of G protein-coupling of these receptors.

The knockout studies have shown that the $S1P_1$ receptor is required for the maturation of blood vessels during embryonic development (Liu *et al.,* 2000). In addition, the $S1P_1$

Table 1.1. Protein names and corresponding gene symbols.

Protein	Gene symbol
SK1	SPHK1
SK2	SPHK2
S1P1	S1PR1
S1P2	S1PR2
S1P3	S1PR3
S1P4	S1PR4
S1P5	S1PR5

receptor expression was crucial for neo-angiogenesis that mediates the extension of blood vasculature supporting tumour growth (Chae *et al.,* 2004). The $S1P_1$ receptor also mediates the migration of HT-1080 human fibrosarcoma cell *in vitro* (Fisher et al., 2006) and the migration of T- and B-lymphocytes through lymphoid tissue *in vivo* (Matloubian *et al.,* 2004). The $S1P_1$ receptor was shown to couple to G_i resulting in the activation of Ras/ extracellular signal-regulated kinase (ERK) and the phosphatidylinositol-3 kinase (PI3K)/ AKT pathways to promote cell survival and proliferation. In this regard, S1P was shown to protect intestinal epithelial cells and adult cardiac mouse myocytes from apoptosis via the $S1P_1$ receptor/PI3K/AKT signalling pathway (Zhang *et al.,* 2007 and Greenspon *et al.,* 2009). Indeed, the PI3K/AKT signalling pathway was shown to up-regulate the expression of anti-apoptotic proteins such as Bcl-2 and Mcl-1, and down-regulating pro-apoptotic proteins such as BAD and BAX (Limaye *et al.,* 2005, Sauer *et al.,* 2005, Li *et al.,* 2008 and Avery *et al.,* 2008). The PI3K/AKT signalling pathway is also involved in the activation of Rac1; a small molecular mass G protein involved in regulation of actin polymerisation and lamellipodia formation at the leading edge of cells that are migrating (Sanchez *et al.,* 2005). The $S1P_1$ receptor also activates JAK2 tyrosine kinase that maintains the persistent activation of STAT3 to promote tumourigenesis, cancer progression and resistance to therapeutics through up-regulation of anti-apoptotic proteins (e.g. Bcl-x_L, Survivin) and proteins involved in proliferation (e.g. Myc, cyclin D1/D2) (Catlett-Falcone *et al.,* 1999, Gao *et al.,* 2007, Hedvat *et al.,* 2009).

In contrast to the function of $S1P_1$ in promoting cell migration, the $S1P_2$ receptor expression is associated with reduced migratory phenotype in mouse melanoma cells, human glioblastoma cells, mast cells, endothelial cells and osteoblasts (Roelofsen *et al.,* 2008, Kono *et al.,* 2007, Lepley *et al.,* 2005, Arikawa *et al.,* 2003). Okamoto and coworkers have shown that the insulin-like growth factor I (IGF-I)-mediated cell migration was inhibited through activation of GTPase-activating protein (GAP) that reduces the Rac activation in $S1P_2$ over-expressing CHO cells (Okamoto *et al.,* 2000). The $S1P_2$ receptor also activates RhoA via $G_{12/13}$ that induces the focal adhesion assembly and the stress fiber formation required for cell anchorage (Sanchez *et al.,* 2005). Furthermore, $G_{12/13}$ signalling activates phosphatase and tensin homologue (PTEN) that inhibits the activation of Rac1, thereby preventing cell migration (Sanchez *et al.,* 2005). In addition, the $S1P_2$ receptor expression reduced the proliferation of Wilms tumour cancer cells through RhoA/Rho-

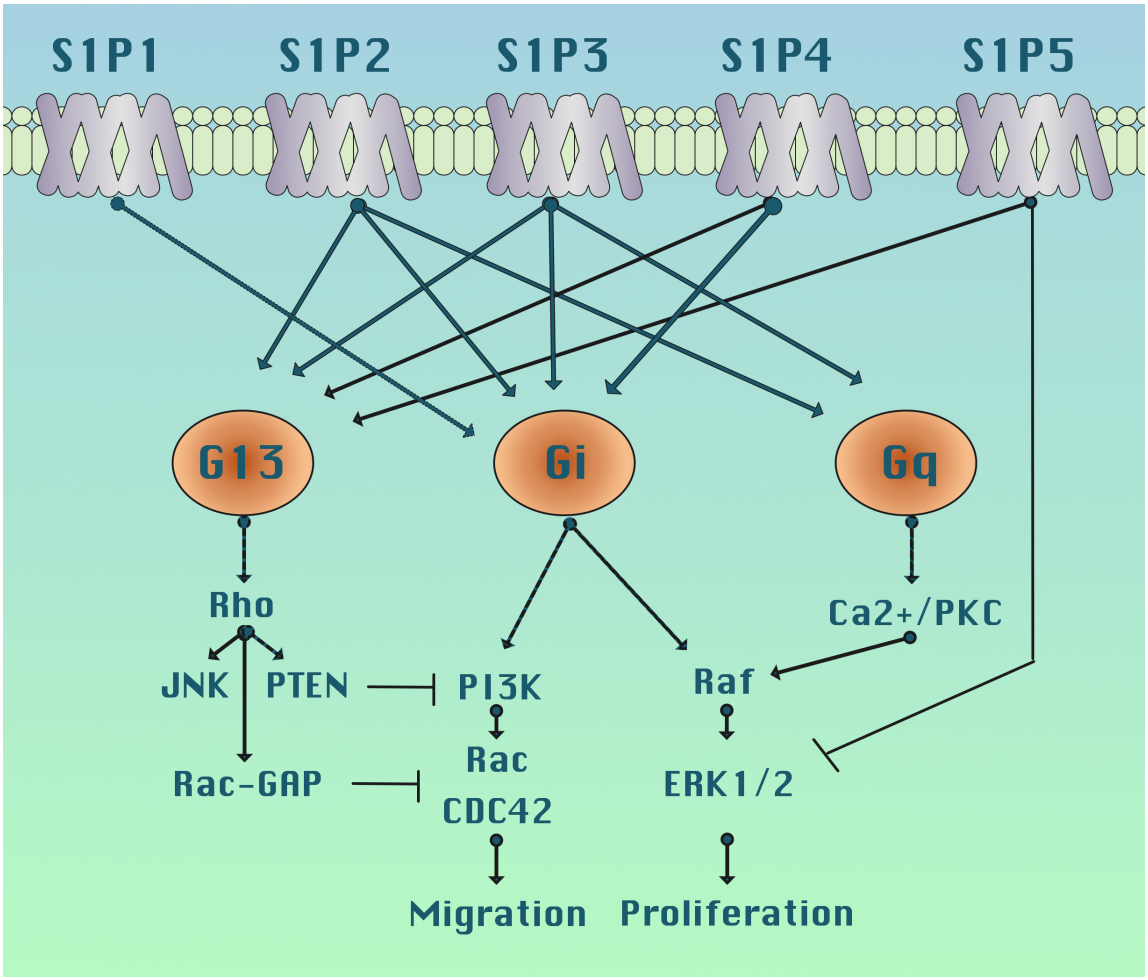

Figure 1.5. The G-protein coupling profile of S1P receptors. The $S1P_{2-5}$ receptors couple G_{13} that activates JNK, Rac-GAP and PTEN through Rho kinase. JNK activates the transcription factor AP1 that stimulates cell proliferation. PTEN and Rac-GAP inhibits the PI3K/AKT-mediated migratory response. $S1P_{1-4}$ receptors couple to G_i. G_i stimulates the activation of PI3K/AKT that in turn activates Rac and CDC42 inducing cellular migration. G_i also stimulates the activation of Raf kinase inducing cellular proliferation through ERK-1/2 kinase. $S1P_{2-3}$ receptors also couple to G_q that activates the PKC through PLC that stimulates the Raf kinase signalling. $S1P_5$ receptor inhibits the ERK-1/2 activation however the mechanism is currently unknown.

associated kinase (ROCK)/PTEN and c-Jun NH_2-terminal kinase pathways (Li *et al.,* 2008). Similarly, binding of S1P to the $S1P_2$ receptor inhibits keratinocyte proliferation by inducing the dephosphorylation of AKT kinase (Schuppel *et al.,* 2008). However, the $S1P_2$ receptor was also shown to inactivate protein phosphatase 2A (PP2A) and to down-regulate breast carcinoma metastasis suppressor 1 (brms1) protein that enhances tumour growth and metastasis in various cancer models (Ponnusamy *et al.,* 2012, Salas *et al.,* 2011). Thus, in addition to $G_{12/13,}$ the $S1P_2$ receptor was shown to couple G_i and G_q that activates JNK, p38 MAPK, Rho, PLC and ERK (Taha *et al.,* 2004).

The $S1P_3$ receptor is implicated in tumourigenesis and cancer progression. Yamashita and coworkers have demonstrated that the S1P-mediated cell migration was controlled by the $S1P_3$ receptor expression in gastric cancer cell lines (Yamashita *et al.,* 2006). Hsu and coworkers have shown that the $S1P_3$ receptor increased the expression of epidermal growth factor receptor (EGFR) through Rho-associated kinase (ROCK) thereby maintaining the tumour growth in lung adenocarcinoma cells (Hsu *et al.,* 2012). Furthermore, the oestrogen-mediated activation of SK1 was shown to activate the $S1P_3$ receptor that in turn activates metalloproteinase (MMP). This induces the release of membrane-bound EGF, leading to the activation of EGFR in MCF-7 breast cancer cells (Sukocheva *et al.,* 2006). Additionally, the $S1P_3$ receptor enhances the survival of cardiomyocytes in myocardial ischemia/reperfusion model in mice (Theilmeier *et al.,* 2006). The $S1P_3$ receptor is also required for the neutrophil infiltration in mice zymosan-induced acute inflammation model (Roviezzo *et al.,* 2011). Similar to $S1P_2$ receptor, the $S1P_3$ receptor couples to G_i, G_q and $G_{12/13}$, and activates Rho, PLC and ERK (Taha *et al.,* 2004, Sugimoto *et al.,* 2003).

The $S1P_4$ receptor expression is limited to hematopoietic cells (Mayol *et al.,* 2011). In murine knockout studies, the deletion of the $S1P_4$ receptor results in impaired dendritic cell migration and cytokine secretion. Recently, the S1P-stimulated activation of ERK-1/2 was shown to be mediated by an $S1P_4$-HER2 receptor functional complex in ERα negative MDA-MB-453 breast cancer cells (Long *et al.,* 2010b). Thus, the $S1P_4$ receptor might be involved in *other* pathophysiological processes. The $S1P_4$ receptor was shown to couple G_i and $G_{12/13}$ activating PLC, ERK, Cdc42, Rho and AC (Taha *et al.,* 2004).

Similar to $S1P_4$ receptor, the $S1P_5$ receptor expression is limited to hematopoietic cells (e.g. natural killer (NK) cells). The $S1P_5$ receptor mediates the egress of natural killer (NK) cells from lymphoid tissue (Mayol *et al.,* 2011). The $S1P_5$ receptor expression is also implicated in the functioning of blood-brain barrier. Its expression was shown to decrease the permeability of blood-brain barrier and restrain the transverse of leukocytes (Van Doorn *et al.,* 2012). Furthermore, the expression of $S1P_5$ receptor was shown to inhibit the S1P-stimulated proliferation and migration in human oesophageal squamous cell carcinoma cell line (Hu *et al.,* 2010). The $S1P_5$ receptor was shown to localise in centrosome together with SK1 and SK2 and might be involved in cell division. In addition, S1P-stimulation of $S1P_5$ receptor inhibits its localisation to the centrosomes (Gillies *et al.,* 2009). The $S1P_5$ receptor was shown to couple G_i and $G_{12/13}$ activating PLC, JNK, Cdc42, Rho while inhibiting ERK and AC (Taha *et al.,* 2004).

Three orphan GPCRs; GPR3, GPR6 and GPR12 were also shown to bind S1P. These receptors couple to G_s and G_i signalling pathways (Uhlenbrock *et al.,* 2002). Recently, S1P was shown to bind to cannabinoid receptor CB2 coupled to G_i and induce vasorelaxation (Mair *et al.,* 2010).

1.3. The intracellular targets of SK1 and sphingosine 1-phosphate

1.3.1. The interaction of SK1 and S1P with the intracellular proteins

The S1P receptor-independent regulation of intracellular calcium level provided the evidence for the existence of intracellular targets of S1P (Choi *et al.,* 1996). For instance, components of sphingolipid signalling actively interact with oncoproteins and stimulate both the expression and the activation of these oncoproteins. Thus, the S1P synthesised by SK1 was shown to bind to TRAF2, an E3 ubiquitin ligase involved in polyubiquitination of receptor interacting protein 1 (RIP1) that masks the kinase binding domain on TNF receptor (Alvarez *et al.,* 2010). The TNF receptor is activated upon proinflammatory signals, such as cytokines and pathogen/danger-associated molecular patterns (Kitamura *et al.,* 2000). Upon activation of the TNF receptor by TNF-α, TRAF2/SK1 complex acts as a scaffold that enables the binding and the phosphorylation of IκB kinase (Lee *et al.,* 2004). The IκB kinase inhibits the nuclear translocation of NF-κB where it acts as a transcription factor regulating the expression of proteins involved in immune response and cell survival (Mercurio *et al.,* 1997, Muller *et al.,* 1995). The phosphorylation of IκB kinase results in polyubiquitination and degradation of IκB kinase that activates NF-κB (Huang *et al.,* 2000).

Delta-catenin/NPRAP (neural plakophilin-related armadillo repeat protein) is an oncoprotein that interacts with SK1. Delta-catenin has a well characterised role in cancer. For example, the over-expression of delta-catenin is associated with enhanced tumour growth in mouse xenografts, anchorage-independent growth in soft agar and migratory response (Zeng *et al.,* 2009, Lu *et al.,* 1999). Recently, Kim and coworkers have shown that PS-1/γ-secretase-mediated E-cadherin cleavage was dependent on delta-catenin (Kim *et al.,* 2012). E-cadherin is a cell adhesion molecule that maintains the epithelial tissue integrity through formation of adherens junctions. The proteolytic fragments of E-cadherin activate diverse signalling pathways. For example, cleaved cytoplasmic domain of E-cadherin in complex with p120 catenin interacts with DNA to induce the transcription of specific genes (Ferber *et al.,* 2008). Moreover, cleaved extracellular domain of E-cadherin binds and activates the ErbB receptors (HER2 and HER3) that results in the activation of ERK-1/2 signalling pathway mediating E-cadherin-dependent cell migration and cell

proliferation (Najy *et al.,* 2008). In addition to role of delta-catenin in E-cadherin-mediated cell migration and cell proliferation, the activation of SK1 was also required for delta-catenin-mediated cell migration in Madin–Darby canine kidney (MDCK) cells. Moreover, the inhibition of SK1 activation reduces the delta-catenin-mediated cell migration. Using yeast two-hybrid screening, Fujita and coworkers have shown that delta-catenin binds to and activates SK1 in MDCK cells (Fujita *et al.,* 2004). Furthermore, the activation of SK1/S1P/S1PR signalling pathway was crucial in delta-catenin-mediated cell migration in MDCK cells.

SK1 also interacts with RPK118 (RSK-like protein) recently renamed as RPS6KC1 (ribosomal protein S6 kinase delta-1) (GeneID:26750). Ribosomal protein S6 kinases are serine/threonine kinases activated via various signalling pathways such as PI3K/AKT/mTOR signalling pathway (Choo *et al.,* 2008). Upon activation, ribosomal protein S6 kinases phosphorylate and activate ribosomal protein S6 that result in the up-regulation of translation of genes involved in ribosomal biogenesis which drives cell proliferation (Chauvin *et al.,* 2013, Pende *et al.,* 2004, Shima *et al.,* 1998). These genes encode ribosomal RNAs and proteins involved in pre-rRNA synthesis and processing, rDNA transcription, assembly of ribosome and rRNA methylation (De Marchis *et al.,* 2005, Dosil *et al.,* 2004, Liu *et al.,* 2001, Gautier *et al.,* 1997). The expression of RPS6KB1 was associated with increased tumour size and differentiation in hepatocellular carcinomas. Additionally, the patients with tumours expressing high levels of RPS6KB1 had significantly shorter disease-free survival (Li et al., 2012). The interaction between RPS6KC1 and SK1 does not alter the catalytic activity of SK1 in COS-7 monkey fibroblast-like cells. In addition, Hayashi and coworkers have shown that RPS6KC1 does not phosphorylate SK1. However, RPS6KC1 have also a microtubule interacting and trafficking (MIT) domain that is common to proteins involved in protein trafficking and sorting (e.g. sorting nexin 15). Moreover, RPS6KC1 possess a Phox (PX) homology domain that binds phosphoinositide 3-phosphate crucial for vesicular trafficking (Cantrell *et al.,* 2001). Hayashi and coworkers have shown that RPS6KC1 and SK1 are co-localised in early endosomes and the expression of RPS6KC1 was required for the localisation of SK1 in early endosomes (Hayashi *et al.,* 2002). Thus, RPS6KC1 might act both as an adaptor protein and a ribosome protein S6 kinase.

SK1 also interacts with proteins involved in protein synthesis. Using two-hybrid screening, Leclercq and coworkers have shown that the eukaryotic elongation factor 1A1 (eEF1A1) directly binds to and activates SK1 and SK2 (Leclercq *et al.,* 2008). The eEF1A1 is involved in protein translation carrying the aminoacyl-tRNA required for the elongation of the peptide chain in the ribosome (Moldave *et al.,* 1985). The eEF1A is activated by ribosomal protein S6 kinase via phosphorylation upon insulin stimulation in 3T3-L1 mouse embryonic fibroblasts (Chang *et al.,* 1997). Moreover, eEF1A is oncogenic as its over-expression in NIH3T3 rodent fibroblast cells increases the proliferation rate and supports anchorage-independent growth (Anand *et al.,* 2002). The eEF1A2 gene is also amplified in breast and ovarian cancers, resulting in poor prognosis (Diebold *et al.,* 2000, Tanner *et al.,* 1995). The interaction of eEF1A1 with SK1 or SK2 increases the catalytic activity of these enzymes through direct interaction. The phosphorylation of eEF1A1 and SK1/2 is not required for changes in activity.

The mast cell activation occurs upon antigen (IgE) binding to high-affinity receptor for immunoglobulin E (FcεRI)-typo resulting in the degranulation that releases the mediators of allergic response (e.g. histamines and cytokines). The FcεRI-typo activation enhances the catalytic activity of both SK1 and SK2 resulting in generation of S1P (Olivera *et al.,* 2005, Urtz *et al.,* 2004). Subsequently, the S1P transported to extracellular milieu acts in autocrine manner to stimulate the chemotaxis towards antigen via an $S1P_1$ receptor-dependent mechanism (Jolly *et al.,* 2004). The FcεRI-typo activation stimulates the interaction of Lyn and Fyn tyrosine kinases with SK1 that enhances the catalytic activity of SK1 at the plasma membrane. The activation of SK1 is independent of tyrosine kinase activity. The SK2 also interacts with Lyn and Fyn kinases and this is required for the translocation of SK2 to the plasma membrane upon FcεRI-typo activation in bone marrow-derived mast cells. In contrast to SK1, the interaction of SK2 with Lyn and Fyn kinases does not alter its catalytic activity (Olivera *et al.,* 2006, Urtz *et al.,* 2004).

The SKs regulate gene expression through interaction with proteins involved in epigenetic modifications. At the epigenetic level, the gene expression is controlled through posttranscriptional modification of histones (H2A, H2B, H3 and H4) that assemble into multimeric complexes with DNA forming nucleosomes (Khorasanizadeh *et al.,* 2004). The acetylation of histones at C-terminal lysine residues reduces the positive charge on the

histones that loosens its interaction with DNA allowing transcription of genes (Haberland *et al.,* 2009). In contrast, the deacetylation of histones enhances the interaction between histones and DNA which represses gene expression. The regulation of gene expression through acetylation is directed by two classes of enzymes named as histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Bannister and Kouzarides, 2011). Hait and coworkers have reported that S1P synthesized by SK2 binds and inhibits HDAC1/2 activity in MCF-7 breast cancer cells (Hait *et al.,* 2009). Upon phosphorylation of SK2 by PKC (activation induced by phorbol 12-myristate 13-acetate), SK2 was reported to form a complex with HDAC1/2. The S1P formed by SK2 inhibits the HDAC1/2 activity, which inhibits the deacetylation of lysine residues on histones H2B/H3/H4. This enhances the transcription of tumour suppressors and protooncogenes such as p21 and c-fos respectively (Hait *et al.,* 2009).

Recently, prohibitin (PHB) 2, a tumour suppressor protein was shown to bind S1P. PHBs are associated with various processes and their function is controlled through phosphorylation and compartmental-specific localisation (Thuaud *et al.,* 2013). Monomeric and dephosphorylated PHB1 and PHB2 was shown to inhibit transcription through direct interaction with HDAC1/2, transcription factor E2F and retinoblastoma protein (RB) (Wang *et al.,* 1999a, Wang *et al.,* 1999b). For example, the monomeric PHB2 inhibits the transcription and activity of oestrogen receptor alpha (ERα) through direct interaction with oestrogen-regulated pS2 promoter and ERα in MCF-7 breast cancer cell (He *et al.,* 2007). Furthermore, the inhibition of nuclear translocation of PHB2 fails to reduce the ERα-mediated transcriptional activity (Kim *et al.,* 2009). Hence, the nuclear export of PHB1 and/or PHB2 was shown to promote cell survival and proliferation. Moreover, the phosphorylation of PHB1 and/or PHB2 was also associated with cell proliferation and survival. Phosphorylated PHB1 displaces the inhibitor protein 14-3-3 of c-Raf, thereby mediating the c-Raf activation and consequent stimulation of the ERK-1/2 signalling pathway (Rajalingam *et al.,* 2005). Mishra and coworkers have reported that PHB1-PHB2 complex also acts as a chaperon, stabilising OPA-1 protein to maintain mitochondrial integrity (Mishra *et al.,* 2010). PHB2 was also shown to mediate the assembly of cytochrome-c oxidase by stabilising the interaction between subunits I and IV. Furthermore, the PHB2-mediated cytochrome-c assembly is dependent on the mitochondrial inner membrane associated SK2. In addition, Strub and coworkers have

shown that the S1P synthesised by SK2 binds to PHB2 and that is required for the correct assembly of the cytochrome-c oxidase complex. Thus, the down-regulation of expression of either SK2 or PHB2 results in partial depolarisation of mitochondrial membrane (Strub *et al.,* 2011).

1.3.2. The interaction of SK1 and S1P receptors with the tyrosine kinase receptors

The cross-talk between S1P receptors and oncogenic membrane receptors provides another avenue by which cancer cells increase their survival, growth and metastatic potential. Indeed, various receptor tyrosine kinases were shown to interact with S1P receptors. For example, functional complexes formed between $S1P_1$ receptor and PDGFβ receptor were reported in human embryonic kidney 293 cells, airway smooth muscle cells and mouse embryonic fibroblasts (Long *et al.,* 2005, Waters *et al.,* 2003). The PDGF receptor has a crucial role in regulating the vasculogenesis, organogenesis, and the growth/migration of mesenchymal cells (e.g. fibroblasts and vascular smooth muscle cells) (Kohler *et al.,* 1974, Ross *et al.,* 1974). PDGF and S1P activates a preformed $S1P_1$-PDGFRβ complex to induce the phosphorylation of G_i, leading to stimulation of ERK-1/2 pathway that mediates the cellular migration (Long *et al.,* 2005, Waters *et al.,* 2003, Alderton *et al.,* 2001). In this signalling platform, the S1P stimulation does not induce phosphorylation of the PDGFRβ receptor. However, the $S1P_1$ receptor over-expression enhances the PDGF-induced ERK-1/2 phosphorylation indicating that PDGFRβ uses G_i coupled to $S1P_1$ receptor to stimulate the activation of the ERK pathway and cell migration (Waters *et al.,* 2006, Waters *et al.,* 2003).

The VEGF also regulates vasculogenesis and lymphangiogenesis. VEGF induces the proliferation and the migration of endothelial cells crucial for the formation, extension and maturation of vasculature in embryonic and post-embryonic development. The VEGF receptors interact with the S1P receptors to stimulate cell proliferation and migration. Indeed, the S1P induced activation of VEGFR-2 is required for the PI3K/AKT-and CrkII-dependent cell migration in human umbilical vein endothelial cells. Tanimoto and coworkers have also shown that the S1P-mediated activation of the PI3K/AKT signalling pathway is sensitive to pertussis toxin and VEGFR tyrosine kinase inhibitor (Tanimoto *et*

al., 2002). Moreover, Endo and coworkers have shown that the S1P-induced activation of VEGFR involves the G_{β} subunit of G_i and induces cell migration through activation of CrkII-DOCK180-Rac mediated signalling pathway (Endo *et al.,* 2002). Interaction between VEGFR and $S1P_1$ receptor is significant in cancer metastasis. Indeed, VEGF and S1P gradients are crucial for haptotaxis of follicular thyroid carcinoma cell line ML-1 (Bergelin *et al.,* 2010). Bergelin and coworkers have shown that S1P or VEGF-mediated activation of ERK-1/2 was sensitive to pertussis toxin and VEGFR tyrosine kinase inhibitor in FTC-133 human follicular thyroid carcinoma cells (Bergelin *et al.,* 2010). Furthermore, $S1P_1$ receptor forms a functional complex with VEGFR-2 to promote S1P- and VEGF-A-mediated ERK-1/2 activation. This activation was in part dependent on SK1 and its inhibition attenuated both S1P- and VEGF-A-induced ERK-1/2 activation (Bergelin *et al.,* 2010). In addition, S1P and VEGF signalling pathways function in positive feedback mechanism where S1P stimulation induces VEGF-A secretion and VEGFR-2 activation induces $S1P_1$ expression in ML-1 cells (Bergelin *et al.,* 2008).

The S1P signalling pathway also functionally interacts with EGF signalling pathway. The EGFR is a member of the ErbB receptor tyrosine kinase family and upon activation forms homo- or hetero-dimers that induce the autophosphorylation of c-terminal tail. These phosphorylated tyrosine residues are recognised by adapter proteins such as Src homology 2 domain containing protein (SHC) and growth factor receptor-bound protein 2 (GRB2) inducing the activation of downstream signalling pathways (e.g. Ras/ERK-1/2, PI3K/ AKT). The EGFR deletion results in hemorrhagic vasculature and underdeveloped intestines and lungs in mice (Miettinen *et al*., 1995). Moreover, Marino and coworkers have shown that the EGFR expression was crucial for the development of lymphatic vessels in skin using *in vivo* mice model (Marino *et al*., 2013).

Sukocheva and coworkers have shown that the 17β-estradiol-induced ERK-1/2 activation was dependent on the activation of SK1, $S1P_3$ receptor and EGFR in MCF-7 beast cancer cells (Sukocheva *et al.,* 2006, Sukocheva *et al.,* 2003).Oestrogen was shown to increase the SK1 activity through activation of ERα and G-protein coupled E2 receptors (GPR30). Furthermore, SK1 activates $S1P_3$ receptor via 'inside-out' signalling that is required for the oestrogen induced EGFR activation. The activation of $S1P_3$ receptor mediates the release

of membrane-bound EGF via matrix metalloproteases (MMP) that binds to EGFR and activates downstream effectors (e.g. ERK-1/2) (Sukocheva *et al.,* 2006).

The S1P receptors also interact with other ErbB receptor family members. The HER2 receptor is an oncoprotein that is commonly mutated or hyper-activated through gene amplification in various cancers resulting in decreased patient survival. This occurs through induction of tumour growth and metastasis (Ross *et al.,* 1998). The HER2 receptor functionally interacts with the $S1P_4$ receptor to stimulate ERK-1/2 signalling pathway in MDA-MB-453 breast cancer cells (Long *et al.,* 2010b). Long and coworkers have shown that the S1P-induced tyrosine phosphorylation of HER2 receptor was dependent on the $S1P_4$ receptor activation. Additionally, the inhibition of HER2 receptor expression or activation via HER2 receptor ATP binding kinase inhibitor (ErB2 inhibitor II) inhibits the S1P-mediated ERK-1/2 activation. The HER2 receptor is an orphan receptor lacking an identified ligand. However, the ligand-mediated activation of other ErbB receptor family members induces their heterodimerisation with HER2 receptor (Klapper *et al.,* 1999). The EGFR is a well characterised dimerisation partner of HER2 receptor. However, the $S1P_4$-HER2 receptor signalling does not require the EGF-mediated activation of EGFR. Thus, the activation of $S1P_4$-HER2 signalling platform might depend on the MMPs-mediated release of *other* ErbB receptor ligands or the $S1P_4$ receptor-induced phosphorylation of HER2 receptor through coupled G protein (Eguchi *et al.,* 1999, Luttrell *et al.,* 1997).

The SK1/S1PR signalling pathway also interacts with HER3-HER4 heterodimer to direct cellular migration. Maceyka and coworkers have reported that heregulin-induced HER3 (ErbB3)-HER4 (ErbB4) dimerisation orchestrates the cell movement though activation of SK1 in melanoma cells (Maceyka *et al.,* 2008). Moreover, the S1P synthesized by SK1 binds and activates serine/threonine kinase p21-activated kinase 1 (PAK1). PAK1 is anchored by Filamin A, which forms a complex with SK1 and $S1P_1$ receptor in the membrane ruffles to promote cell migration. PAK1 is an oncogenic protein over-expressed in various cancers (Bostner *et al.,* 2007, Carter *et al.,* 2004, Ong *et al.,* 2011, Davidson *et al.,* 2008). Upon activation by Rho-family GTPases (e.g. Rac1 and Cdc42), PAK1 promotes cell proliferation and survival through activation of Ras/Raf/MEK/ERK-1/2 and NFκB signalling pathways (Dadke *et al.,* 2003). PAK1 also promotes the cell migration

through activation of LIM kinase and ARP2/3 that promotes the formation and the extension of actin filaments through inhibition of cofilin (Edwards *et al.,* 1999, Kiosses *et al.,* 1999). PAK1 is also associated with HER2 receptor-induced breast cancer progression through phosphorylation of β-catenin. This results in the activation of the Wnt/β-catenin signalling pathway (Arias-Romero *et al.,* 2013).

1.4. The expression of SK1 and S1P receptors in human cancers

Extensive studies have shown that the SK1 expression is up-regulated both at the mRNA and/or protein level in various cancers and that this is associated with poor clinical prognosis (French *et al.,* 2003, Ruckhaberle *et al.,* 2008, Erez-Roman *et al.,* 2010, Watson *et al.,* 2010). The analysis of SK1 expression in breast cancer tissue samples have shown that the SK1 expression was higher in ERα negative tumours compared to ERα positive tumours. In addition, high SK1 expression significantly reduced the patient survival from 72.0% to 65.7% in 5 year disease-free survival (Ruckhaberle *et al.,* 2008). In another study, high tumour expression of SK1 was associated with the reduced patients survival time and the development of tamoxifen resistance in ERα positive breast cancer patients (Long *et al.,* 2010). However, the HER2 receptor positive tumours expressing high levels of SK1 in the tumour had significantly prolonged patient survival time compared to patients with tumours expressing low levels of SK1. Similarly, the expression of SK1 at high levels delayed the development of tamoxifen resistance in patients with HER2 receptor positive tumours. Van Brocklyn and coworkers have assessed the prognostic outcome of SK1 expression in astrocytoma patients. The mean patient survival time was reduced from 12 months to 4 months in patients with tumours expressing high levels of SK1 compared to patients with tumours expressing low levels of SK1 (Van Brocklyn *et al.,* 2005). In gastric cancer patients, cumulative 5-year survival was 49.6% in patients with tumours expressing low SK1 expression group, whereas it was reduced to 23.8% in the high SK1 expression group (Li *et al.,* 2009). In this cohort, the SK1 expression was higher in advanced tumours and in metastatic tumours. The prognostic significance of SK2 expression has also been analysed. Wang and coworkers have shown that SK2 expression was higher in advanced tumours and in metastatic tumours in non-small-cell lung carcinoma (NSCLC) patients. The patient survival was decreased from 45.3% to 27.1% in patients with tumours expressing high SK2 compared to patients with tumours expressing low levels of SK2 in 5-year disease-free survival. Moreover, SK2 expression was an independent prognostic marker both in univariate and multivariate analysis (Wang *et al.,* 2013). In addition to prognostic significance of SK1 and SK2 expression, Watson and coworkers have shown that the high expression of $S1P_1$ and $S1P_3$ receptors were associated with poor prognosis in ERα positive breast cancer patients (Watson *et al.,* 2010). The mean

disease-specific survival was reduced from 18.4 years to 10.3 years for patients with tumours expressing high $S1P_1$ receptor. Moreover, the mean disease-specific survival was reduced from 17.5 to 14.7 years for patients with tumours expressing high level of $S1P_3$ receptor. The expression of $S1P_1$ and $S1P_3$ receptors was also higher in advanced breast tumours.

The sphingolipid signalling is also involved in development of chemotherapeutic resistance in cancer cells. For example, the treatment of prostate cancer cells with camptothecin induces an increase in the expression of SK1 and S1P receptors that impairs the sensitivity of these cells to camptothecin (Pchejetski *et al.,* 2005). In support of these findings, the SK1 over-expression prevents apoptosis induced by chemotherapeutics (e.g. camptothecin and docetaxel) in LNCaP and PC-3 prostate cancer cells (Pchejetski *et al.,* 2005). Moreover, prostate cancer cells sensitive to camptothecin exhibit a high ceramide/ S1P ratio compared with their drug resistant counterparts (Pchejetski *et al.,* 2005, Guillermet-Guibert *et al.,* 2009). Thus, sensitivity to chemotherapy is determined by the inhibition of SK1 and subsequent increase in ceramide level that triggers apoptosis.

In leukemia cell line, doxorubicin or etoposide-induced apoptosis was also dependent on the SK1 inhibition and subsequent generation of ceramide. In addition, the over-expression of SK1 renders HL-60 cells resistant to doxorubicin or etoposide (Bonhoure *et al.,* 2006). Song and coworkers have shown that the SK1 over-expression also confers resistance to docetaxel and doxorubicin in 95D and A549 non-small cell lung cancer (NSCLC) cells. Moreover, the SK1-mediated resistance to apoptosis was dependent on the activation of NF-κB via the PI3K/AKT signalling pathway resulting in up-regulation of anti-apoptotic proteins such as Bcl-x_L, c-IAP1 and c-IAP2 (Song *et al.,* 2011).

The over-expression of SK1 is also associated with the development of tamoxifen resistance in MCF-7 breast cancer cells (Sukocheva *et al.,* 2009). Sukocheva and coworkers have shown that the SK1 expression and the activity is increased in tamoxifen-resistant MCF-7 cells. Moreover, the inhibition of SK1 activation or the expression restored the sensitivity to tamoxifen. In addition to NF-κB-mediated inhibition of apoptosis, the SK1 activation might induce the PAK1-catalysed phosphorylation of Ser^{305} and the ERK-catalysed phosphorylation of Ser^{118} on ERα, resulting in resistance to

tamoxifen through ligand-independent activation (Kok *et al.,* 2009, Maceyka *et al.,* 2008, Rayala *et al.,* 2007).

1.5. The pharmacological inhibitors of sphingosine kinases and S1P receptors

1.5.1. The inhibitors of SK1 and SK2

Various inhibitors of SK1 and SK2 have been characterised. The SKi also referred as SKI-II (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) is a non-lipid inhibitor of SK1 and SK2 that was identified through screening a library of synthetic compounds (**Figure 1.6)** (French *et al.,* 2003). SKi is a mixed inhibitor of SK1 that can occupy the sphingosine and ATP binding sites (Lim *et al.,* 2011, Wang *et al.,* 2013). Treatment of various cancer cells lines with micromolar concentration of SKi reduces SK1 activity and subsequently the S1P levels and inhibits cell proliferation and induces apoptosis (IC_{50}=0.5 μM) (Chatzakos *et al.,* 2012, Lim *et al.,* 2011, Loveridge *et al.,* 2010, French *et al.,* 2003). Furthermore, SKi induces proteasomal and lysosomal degradation of SK1 that is discussed in Section 1.4.

SK1-I also called BML-258 ((*2R,3S,4E*)-N-methyl-5-(40-pentylphenyl)-2-aminopent-4-ene-1,3-diol) is a sphingosine analogue that is a competitive inhibitor of SK1 (**Figure 1.6)** (Paugh *et al.,* 2008). The micromolar concentrations of SK1-I inhibits the cell growth of various leukemia and glioblastoma cell lines (e.g. U937, HL60, Molt-4, K566, LN229 and U373) (Paugh *et al.,* 2008, Kapitonov *et al.,* 2009). SK1-I also inhibits the cellular growth of U937 and LN229 cell xenografts in SCID/beige mice without any weight loss and muscular atrophy.

Various natural inhibitors of SK1 were isolated from a variety of organisms. B-5354 series of benzoic acid derivatives with unsaturated alcohol chains were isolated from marine bacterium that belongs to genus *Ruegeria* (Kono *et al.,* 2000a). F-12509A (2'-*O*-methyl hyatellaquinone) a hydroquinone derivative from Discomycetes fungi *Trchopezizella barbata* was characterised by Kono and coworkers (Kono *et al.,* 2000b). S-15183 series of compounds are bicyclic azaphilones that was isolated from fungal strain of *Zopfiella inermis*. These compounds were shown to inhibit SK activity at micromolar concentrations *in vitro* (Kono *et al.,*2001).

The FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) is a structural analogue of myroicin synthesised by Adachi and coworkers (**Figure 1.6)** (Adachi

et al., 1995). FTY720 is phosphorylated to (*S*)-FTY720 phosphate by SK2 which is an antagonist of $S1P_{1,3-5}$ receptors and a functional antagonist of $S1P_1$ receptor (Brinkman *et al.,* 2002, Mandala *et al.,* 2002). FTY720 acts as a immunosuppressant through down-regulation of S1P receptors on T-lymphocytes required for egress from the lymphoid tissue (Mandala *et al.,* 2002, Yanagawa *et al.,* 1998, Chiba *et al.,* 1998). Indeed, FTY720 has been approved by Food and Drug Administration (FDA) in treatment of relapsing remitting multiple sclerosis. In addition to the S1P receptors, FTY720 has intracellular targets. For example, FTY720 inhibits the activity of SK1 (and induces proteasomal degradation), the ceramide synthases and the S1PL *in vitro* (Lim *et al.,* 2011, Berdyshev *et al.,* 2009, Vessey *et al.,* 2007, Bandhuvula *et al.,* 2005).

Moreover, FTY720 induces cell cycle arrest, apoptosis and/or necrosis in various cancer cell lines (e.g. breast, ovarian, prostate, leukemia, hepatoma and glioma cell lines) (Zheng *et al.,* 2010, Lui et al., 2008, Lee *et al.,* 2004, Azuma *et al.,* 2002, Permpongkosol *et al.,* 2002, Sonoda *et al.,* 2001, Matsuda *et al.,* 1998, Shinomiya *et al.,* 1997). Zheng and coworkers have shown that FTY720 induces caspase-dependent apoptosis through up-regulation of PTEN and Bcl-2 family of proteins that inhibits the activation of AKT and MDM2 resulting in the accumulation of p53 in AGS and MGC803 gastric cancer cell lines (Zheng *et al.,* 2010). FTY720 also induces the production of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) that induces the activation of PKCgamma initiating caspase-dependent apoptosis in HuH-7 hepatoma cells (Hung *et al.,* 2008).

Baek and coworkers have synthesised various FTY720 analogs. RB-005 (1-(4-octylphenethyl)piperidin-4-ol) is a selective SK1 inhibitor (**Figure 1.6)** (IC_{50}=3.6 μM) synthesised through piperidination of terminal nitrile in FTY720. RB-005 also induces the proteasomal degradation of SK1 in PASMC cells (Baek *et al.,* 2013).

Various sphingoid based compounds have been shown to inhibit sphingosine kinases. L-*threo*-dihydrosphingosine ((2S,3S)-2-amino-1,3-octadecanediol), also known as Safingol is a competitive inhibitor of SK1 (Ki: 5 μM) (**Figure 1.6)** (Olivera *et al.,* 1998, Vessey *et al.,* 2007). Phase I trials revealed that Safingol prevents cancer progression in patients with advanced solid tumours that was accompanied with significant reduction in S1P levels in

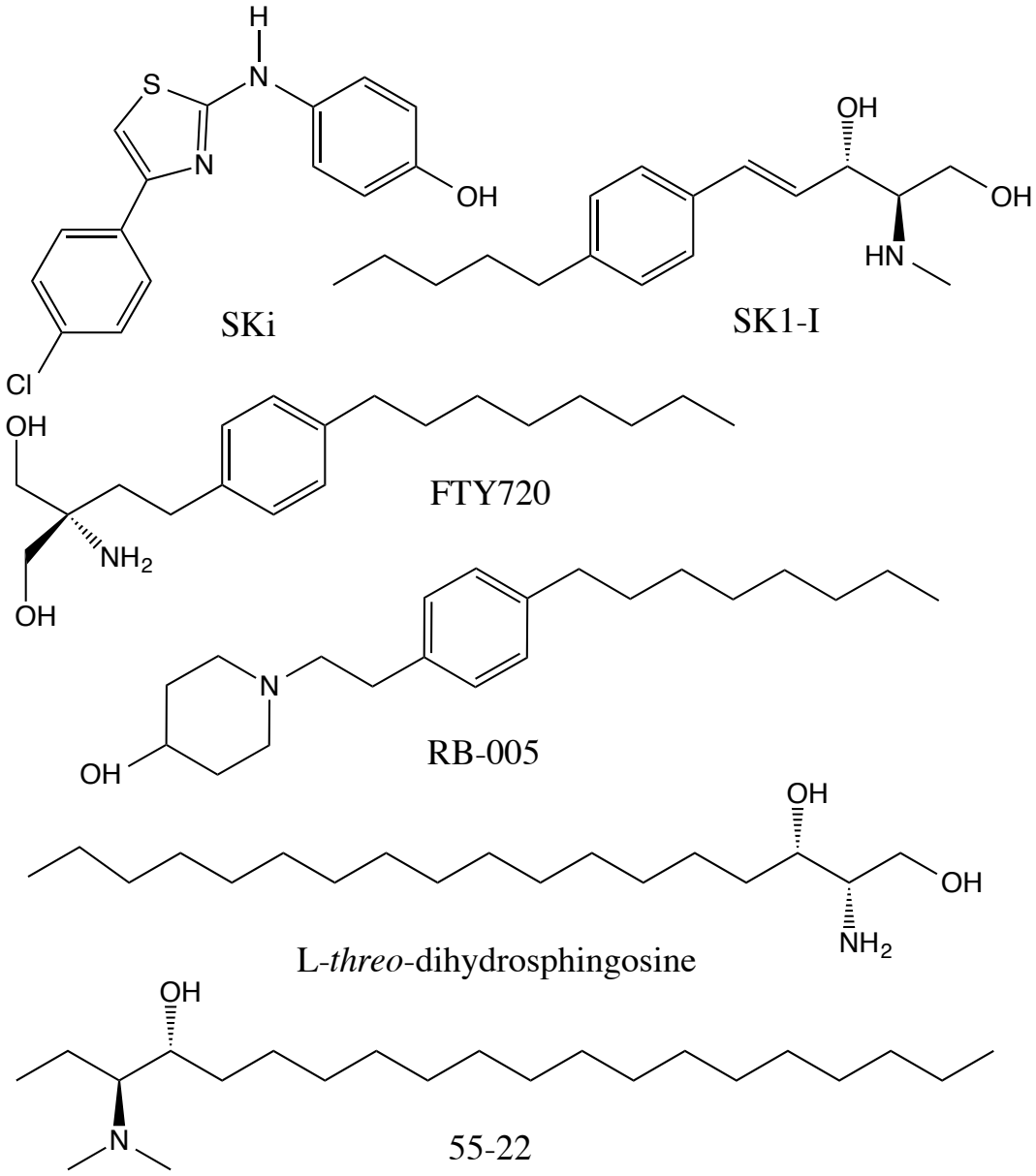

Figure 1.6. The structures of SKi, SK1-I, FTY720, RB-005, L-threo-dihydrosphingosine and compound 55-22.

blood stream within 4 hours after intravenous infusion (Dickson *et al.,* 2011). Similarly, Byun and coworkers have shown that sphinganine analogues 55-21 (*2S,3R*)-2-aminooctadecan-3-ol) and 55-22 (*2S,3R*)-2-(dimethylamino)octadecan-3-ol) are SK1 specific inhibitors (**Figure 1.6)**. Similar to other SK1 inhibitors, treatment of PASMC cells with 55-21 induces the proteasomal degradation of SK1 and inhibits DNA synthesis (Byun *et al.,* 2013).

Various SK2 selective inhibitors have also been characterised. Lim and coworkers have synthesised and tested various FTY720 analogues *in vitro*. (*R*)-FTY720 methyl ether ((*2R*)-2-Amino-3-(O-methyl)-(2-(4′-n-octylphenyl)ethyl)propanol) is competitive inhibitor of SK2 (Ki: 16.5 μM) where a hydroxyl group is replaced by a methoxy group (**Figure 1.7)**. The treatment of HEK 293 cells with (*R*)-FTY720 methyl ether reduces the SK2 protein level that induces PARP cleavage and apoptosis of HEK 293 cells (Lim *et al.,* 2011). (*R*)-FTY720 methyl ether also inhibits the S1P-stimulated assembly of filamentous actin in MCF-7 cells (Lim *et al.,* 2011). Thus, inhibiting the formation of lamellipodia required for cell migration. Moreover, the treatment of LNCaP cells with (R)-FTY720 methyl ether decreases the S1P levels while increasing the sphingosine levels but does not alter the C22:0 ceramide levels. Thus, (R)-FTY720 methyl ether does not induce apoptosis in these cells (Watson *et al.,* 2013).

ABC294640 (3-(4-chlorophenyl)- adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide) is an adamantane derivative that is characterised as a competitive inhibitor of SK2 (Ki: 9.8 μM) (**Figure 1.7)**. The treatments of MDA-MB-231 cells with ABC294640 increases the levels of ceramide species while decreasing the S1P levels (French *et al.,* 2010). The inhibition of SK2 activity with ABC294640 also reduces the proliferation of various cell lines and migration of A-498 metastatic human kidney carcinoma cells. In addition, the ABC294640 treatment increases G-actin while decreasing F-actin resulting in reduction in number of stress fibers and lamellipodia generated by A-498 cells. Additionally, the intraperitoneal injection of ABC294640 (35 mg/kg) in mice with JC mammary adenocarcimona reduces tumour volume (French *et al.,* 2010).

ABC294640 induces autophagy that results in cell death through destruction of cellular organelles in A-498, PC-3 and MDA-MB-231 cells (Beljanski *et al.,* 2010). Indeed,

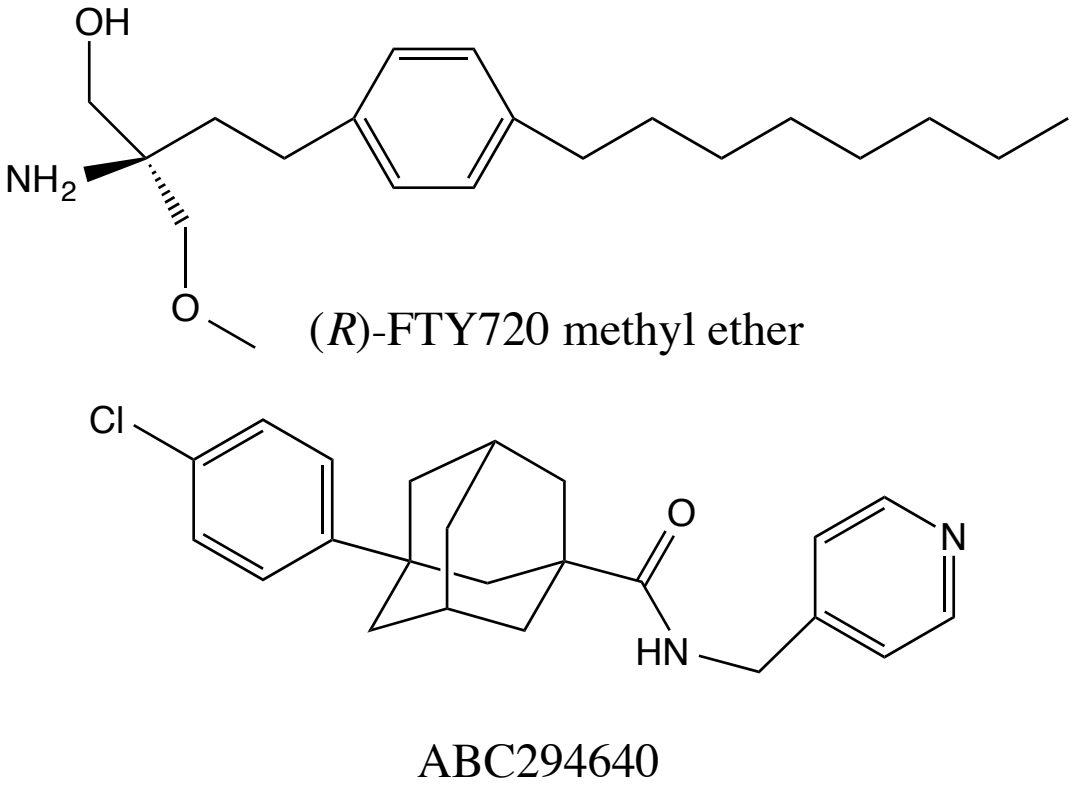

Figure 1.7. The structures of (R)-FTY720 methyl ether and ABC294640.

ABC294640 induces formation of autophagosomes in A-498 cells. In support of these findings, ABC294640 (50 mg/kg) delays tumour growth of A-498 cells in mouse xenograft model. The inhibition of SK2 activity with ABC294640 also up-regulates the tumour suppressor beclin-1 and induces the dephosphorylation of ERK and AKT kinases that inhibits cell proliferation. Moreover, ABC294640 induces apoptosis upon inhibition of autophagy using 3-methyladenine in A-498 cells (Beljanski *et al.,* 2010).

1.5.2. The antagonists of S1P receptors

VPC23019 (2-Amino-N-(3-octylphenyl)-3-(phosphonooxy)-propanamaide) is an S1P derivate containing aryl amide group and has been characterised as an $S1P_1$ (Ki ≤ 50 nM) and $S1P_3$ (Ki ≤ 100 nM) receptor antagonist (**Figure 1.8)**. VPC23019 is an agonist of $S1P_4$ and $S1P_5$ receptors (Davis *et al.,* 2005). Zhou and coworkers have shown that the $S1P_1$-mediated ERK-1/2 activation was abrogated by VPC23019 in cultured hippocampal neurons (Zhou *et al.,* 2013). In addition, the injection of VPC 20319 (0.5 mg/kg) prior to sevoflurane also abrogated the neuro-protective effects of $S1P_1$ receptor in a sevoflurane-induced neurotoxicity model in mice (Zhou *et al.,* 2013). VPC23019 also inhibits the oxidative stress-induced activation of AKT and ERK-1/2 that is mediated by $S1P_1$ and $S1P_3$ receptors in cultured human luteinized granulosa cells (Nakahara *et al.,* 2012).

JTE-013 (N-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1H-pyrazolo[3, 4-b]pyridin-6-yl]-hydrazinecarboxamide) is a pyrazolopyridine derivative characterised as an $S1P_2$ specific antagonist by Central Pharmaceutical Research Institute in Japan (**Figure 1.8)**. Osada and coworkers have reported that JTE-013 reverses the S1P-mediated inhibition of human coronary smooth muscle cell migration (Osada *et al.,* 2002). Moreover, the activation of RhoA and the inhibition of Rac was required for the $S1P_2$ receptor-mediated inhibition of B16 melanoma cell migration (Arikawa *et al.,* 2003). JTE-013 also abrogates anti-proliferative effects mediated by $S1P_2$-Rho signalling in rat hepatocytes (Ikeda *et al.,* 2003). *In vivo,* JTE-013 protects islet β-cells from $S1P_2$ receptor-mediated apoptosis in mice streptozotocin-induced diabetes model (Imasawa *et al.,* 2010). JTE-013 is also an $S1P_4$ antagonist. Treatment of HTC4 cells over-expressing $S1P_4$ receptor with JTE-013 (10 μM) reduces the calcium mobilisation from intercellular stores

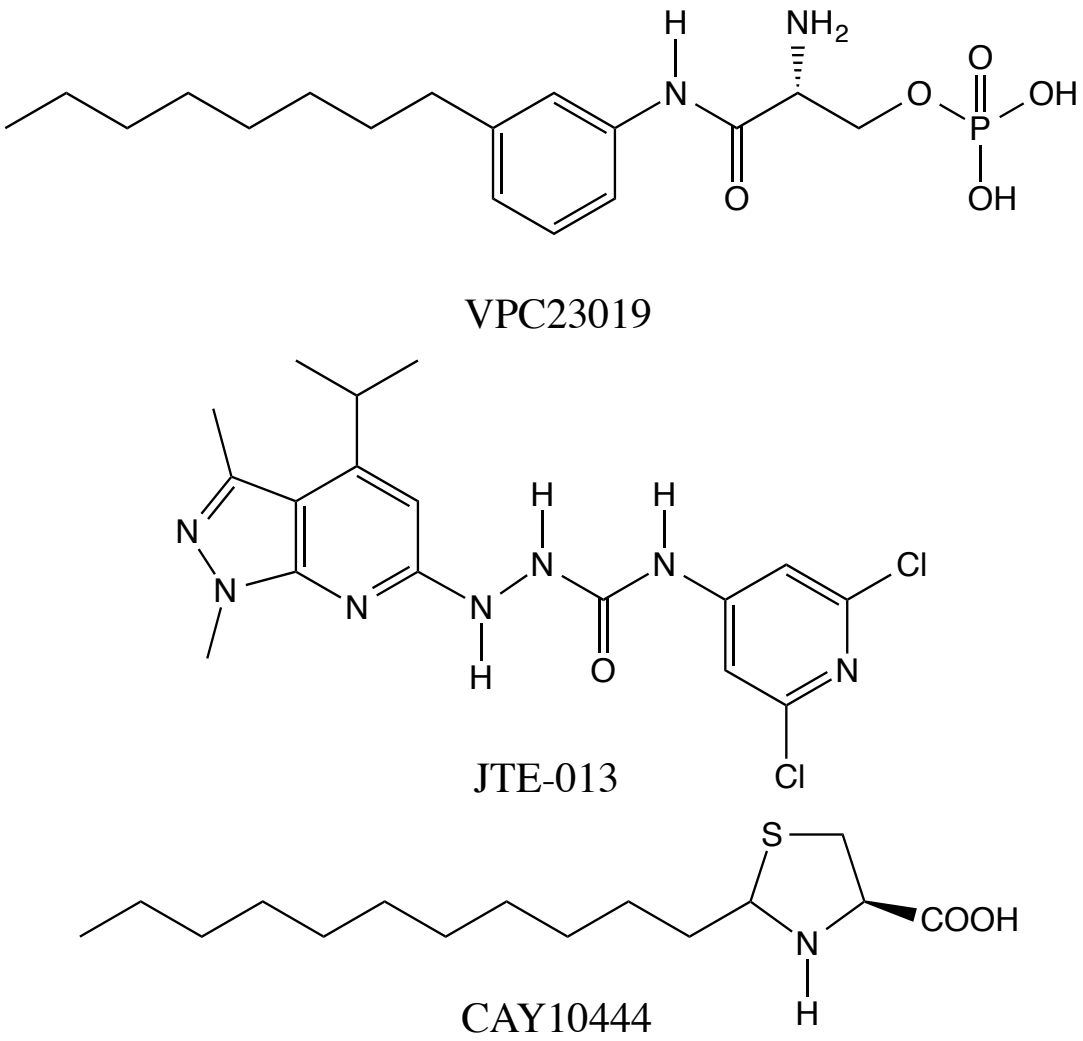

Figure 1.8. The structures of VPC23019, JTE-013 and CAY10444.

upon S1P stimulation (Long *et al.,* 2010b).

CAY10444 (BML-241) (2-undecyl-thiazolidine-4-carboxylic acid) is a thiozolidine derivative characterised as $S1P_3$ receptor specific antagonist (**Figure 1.8)** (Koide *et al.,* 2002). Long and coworkers have shown that CAY10444 inhibited the S1P-stimulated increase in intracellular calcium levels in $S1P_3$ over-expressing HTC4 cells (Long *et al.,* 2010a). Similarly, CAY10444 also inhibited the S1P-induced calcium increase in isolated human keratinocytes (Lichte *et al.,* 2008). Moreover, the $S1P_3$ receptor mediated ERK-1/2 activation is also abrogated by both CAY10444 and $S1P_3$ specific siRNA in MCF-7 cells (Long *et al.,* 2010a). *In vivo*, CAY1044 (1 mg/kg) treatment inhibited zymosan-induced neutrophil recruitment in mouse air pouch (Roviezzo *et al.,* 2011).

1.6. Breast cancer

Breast cancer is the most common cancer among women in the United Kingdom with approximately 55,000 women diagnosed solely in 2010. Identification of independent biomarkers have advanced the treatment of breast cancer such that 2 out of 3 women that has been diagnosed with breast cancer survive beyond 20 years in UK. Despite of the successful treatments, breast cancer is the second leading cause of cancer-related death among women in UK after lung cancer. This is due to the vast heterogeneity of breast cancers. Hence, better understanding of the nature of different types of breast cancer can lead to development of sub type-specific treatment regimens.

1.6.1. The classification of breast cancers

World Health Organization recognises 18 distinct groups of breast cancer that are classified according to histopathological and genetic features of the tumour. Breast cancers are classified according to two features: the location of the tumour and the modality of carcinogenesis. The most common type of breast cancer is ductal carcinoma that originates from epithelium of the lactiferous ducts within the breast. The second most common type of breast cancer is lobular carcinoma that originates from lobular epithelium of the breast. These two types comprise 83% and 9% of the breast cancer cases determined between 2008 and 2010 in the UK respectively. These groups are further sub-divided into two groups according to invasiveness beyond the basement membrane named as carcinoma *in situ* or invasive. The remaining 10% of the breast cancer cases comprise cancer types such as medullary and phylloides tumours that have distinct histopathological representation.

Breast cancers are also classified according to molecular profile of the cancer cells. These classes are listed in **Table 1.2.** The expression of specific biomarkers such as estrogen receptor alpha (ERα), progesterone receptor (PgR), HER2 receptor, epidermal growth factor receptor (EGFR), Ki67, E-cadherin, cytokeratin 5/6, claudin-3 and claudinin-4/7 are used to divide the breast cancers into different classes (Perou *et al.,* 2000). These classes determine the prognosis and responsiveness of the tumour to chemo- and endocrine

Table 1.2. The classification of breast cancer according to the molecular profile of the cancer cells (Cancer Genome Atlas Network, 2012).

Classification	Molecular Profile	
	Receptors	*Other*
Laminal A	ERα^{+}, PgR$^{+/-}$, HER2^{-}	Ki67 low
Laminal B	ERα^{+}, PgR$^{+/-}$, HER2^{+}	Ki67 high
Basal A	ERα^{-}, PgR^{-}, HER2^{-}	Ki67 high, EGFR^{+} and/or cytokeratin 5/6^{+}
Basal B	ERα^{-}, PgR^{-}, HER2^{-}	Ki67 low, E-cadherin, claudin/ claudinin low
HER2	ERα^{-}, PgR^{-}, HER2^{+}	-

therapies that in turn allow the establishment of cancer-type specific treatment regimens (Perou *et al.,* 2000).

1.6.2. The role of ERα, PgR and HER2 receptor in breast cancer

The expression of steroid hormone receptors such as ERα and PgR is common in breast cancers and these receptors promote cell proliferation and metastasis (Li *et al.,* 2010, Harrington *et al.,* 2006). ERα is activated by ovarian oestrogen hormone leading to its translocation from cytoplasm to nucleus where it acts as a transcription factor, inducing transcription of *other* transcription factors (ZNF9, BTF3), proteins involved in translation (EIF5A, EEF1A1) and cell cycle progression proteins (cyclin D1, RAN kinase, calmodulin 2) (Carpentier *et al.,* 2000). Cavalier and coworkers have shown that the administration of exogenous oestrogen (17β-estradiol) induced tumour formation in mice after oophorectomy. These findings provide evidence of the role of oestrogen hormone in breast carcinogenesis (Cavalieri *et al.,* 2006). The ERα expression is strictly controlled in normal human breast throughout the menstrual cycle and it is expressed only in ~7% of the luminal cells in non-lactating breast (Clarke *et al.,*1997, Markopoulos *et al.,* 1988, Petersen *et al.,* 1987). In contrast to normal breast epithelial cells, breast cancer cells can express ERα independently of menstrual cycle (Markopoulos *et al.,* 1988). Moreover, mutations and/or exon deletions have been detected in gene encoding ERα which result in constitutive activation and elevated expression of ERα (Fuqua *et al.,* 2000, Leygue *et al.,* 1996). Thus, therapeutics such as tamoxifen, fulvestrant and letrozole are commonly used

in treatment of ERα positive breast cancers, targeting oestrogen/ERα signalling. However, the effectiveness of endocrine therapy is also determined by the expression of PgR receptor (Cui *et al.,* 2005). PgR is a hormone receptor activated by progesterone hormone secreted by the ovaries. The expression of PgR is strictly controlled by ERα through an oestrogen response element (ERE) located in the promoter of the *PGR* gene (Wang *et al.,* 1990). Upon activation, PgR translocates into the nucleus and activates the transcription of cyclin D1, WNT4 and RANKL (receptor activator of nuclear factor-κB ligand) that promotes cell proliferation (Beleut *et al.,* 2010, Brisken *et al.,* 2000). ERα and PgR are determined to be independent prognostic markers of patient survival in various studies (Bardou *et al.,* 2003, Lamy *et al.,* 2002, Ferno *et al.,* 2000). In fact, patients with $ERα^{+}/PgR^{+}$ tumours have higher overall survival rates compared to $ERα^{+}/PgR^{-}$ tumours after tamoxifen treatment (Coi *et al.,* 2005, Baum *et al.,* 2003). Thus, the PgR expression is utilised to assess the effectiveness of endocrine therapy in treatment of ERα positive breast cancers.

Besides ERα and PgR, the expression of oncogene HER2/ErbB2 in breast tumours has a prognostic value and determines the responsiveness to specific therapies. HER2 receptor is a member of epidermal growth factor receptor family of tyrosine kinases. HER2 receptor forms dimers with HER1/3 and HER4 receptors in response to agents such as heregulin resulting in activation of PI3K/AKT/mTOR and Ras/Raf/MEK/ERK-1/2 signalling pathways that stimulate cell proliferation and migration (Roberts *et al.,* 2012, She *et al.,* 2008, Kurokawa *et al.,* 2012). HER2 receptor amplification comprises ~20% of breast cancers and this correlates with tumour size and lymph node metastasis that results in poor prognosis in breast cancer (Ross *et al.,* 1998). In addition, HER2 receptor expression is associated with poor endocrine response in $ERα^{+}/PgR^{+/-}$ tumours due to down-regulation of ERα (Creighton *et al.,* 2006, Borg *et al.,* 1994)). In contrast, breast tumours with HER2 receptor amplification/over-expression are more responsive to chemotherapy (Horitz *et al.,* 2011, Gennari *et al.,* 2008, Konecny *et al.,* 2004). Thus, therapeutics such as herceptin, pertuzumab and lapatinib targeting HER2 receptor signalling are commonly used in addition to anthracyclines in treatment of HER2 receptor positive breast tumours.

In contrast to tumours that express steroid and/or HER2 receptors, triple negative breast cancers (TNBC) lack the expression of these receptors. This group comprises ~15% of all breast cancers and characterised as highly aggressive with the median patient survival of

13 months (Oakman *et al.,* 2010, Dent *et al.,* 2007). Currently, conventional chemotherapy is the only modality of treatment for these patients. These therapies include; nucleoside analogs (gemcitabine, capecitabine), microtubule interactors (docetaxel, vinorelbine, ixabepilone) and DNA interactors (cisplatin, carboplatin) (Andre *et al.,* 2012). However, solely ~30% of tumours respond to combination therapies formed using these therapeutics. Moreover, these therapies represent adverse side effects due to un-specific nature of these therapeutics. Thus, the development of tumour-specific therapeutics for the treatment of TNBC is an unmet challenge.

Development of novel therapeutics requires the identification of novel biomarkers. Thus, in this study, human breast cancer tissue microarrays at various tissue histological grades of ERα positive and ERα negative breast tumours were analysed for the expression of S1P signalling proteins (e.g. SKs and $S1P_{1-5}$ receptors) to identify the impact of expression of these proteins on clinical outcomes. Moreover, the expression of these proteins were stratified against the expression of diverse array of kinases and transcription factors e.g. c-RAF-1, ERK-1/2, AKT, LYN, SRC family kinases (SFKs) and NFκB (p50 RelA) to discover novel associations that might be crucial for cancer progression in breast cancer patients. In deed, therapeutic potential of these findings was further assessed through characterisation of signalling pathway between SKs, c-SRC and $S1P_2$ receptor. In addition, therapeutic potential of various SK1/2 inhibitors and S1P receptors was assessed in triple negative MDA-MB-231 breast cancer cells. Thus, this study provides insight into understanding the diversity of breast cancers and therapeutic potential of targeting sphingolipid signaling in breast cancer patients.

CHAPTER II

Materials and Methods

2.1. Materials

All biochemicals were from Sigma-Aldrich (Poole, UK). Antibodies used in western blotting were anti-phosphorylated ERK-1/2 antibody, Santa Cruz (California, USA); anti-ERK-2 antibody, BD Transduction Laboratories (Oxford, UK); anti-$S1P_2$ antibody, Sigma (Poole, UK); anti-HA antibody, Santa Cruz (California, USA); anti-GAPDH antibody, Santa Cruz (California, USA); anti-PARP antibody, Cell Signaling Technology (Massachusetts, USA); anti-RAF-1 antibody, Santa Cruz (California, USA); anti-phosphorylated-RAF-1 antibody, Cell Signaling Technology (Massachusetts, USA); anti-Lamin A/C antibody, Cell Signaling Technology (Massachusetts, USA); anti-phosphorylated-Src Family (Tyr416), Cell Signaling Technology (Massachusetts, USA); anti-SRC antibody, Santa Cruz (California, USA); anti-Actin antibody and conjugated anti-IgG secondary antibodies, Sigma (Poole, UK). S1P was from Avanti Polar Lipids (Alabaster, USA). SKi (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole) was from Merck Biosciences (Nottingham, UK). CAY10444 was from Cayman Chemical Company (Michigan, USA). JTE 013 was from Tocris Bioscience (Abingdon, UK). ROMe, RB-005 and 55-22 were gifts from R. Bittman.

2.2. Cell culture

Cell lines MDA-MB-453, MDA-MB-231 and HEK 293 were purchased from ATCC. Cells were grown in high glucose Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) containing European fetal calf serum (10 % v/v) (Seralab, West Sussex, UK), penicillin G sodium (100 U/ml) and streptomycin sulfate (10 μg/ml) (Pen-Strep, Gibco, Paisley, UK). Cultures were grown at 37 degrees Celsius in 95% air and 5% CO_2.

2.3. siRNA treatment

The expression of SK1, SK2, $S1P_3$ receptor and $S1P_4$ receptor was down-regulated using sequence-specific single interfering RNAs (L-004172-00-0005 ON-TARGETplus SPHK1 siRNA, L-004831-00-0005 ON-TARGETplus SPHK2, L-005208-00-0005 ON-TARGETplus $S1P_3$ siRNA, L-005484-00-0005 ON-TARGETplus $S1P_4$ siRNA).

Scrambled siRNA with no intracellular target was used as a control (D-001810-01-05 ON-TARGETplus Non-targeting siRNA). Cells were grown up to 50% confluence in T-25 flasks. DharmaFECT 2 reagent (Invitrogen, Paisley, UK) was used to transfect the cells. The cells were transfected for 24 hours and then serum starved for 24 hours prior to treatment with compounds.

2.4. Transfection

Cells were grown up to 80% confluence prior to experiment. Cells were transiently transfected with either HA (N-terminus)-tagged $S1P_2$ receptor (purchased from UMR cDNA Resource Center, University of Missouri-Rolla, Montana, USA) or empty plasmid (pcDNA3.1, Life Technologies, Paisley, UK) constructs. Cells were transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Cell were transfected for 24 hours in serum-free high glucose Dulbecco's modified Eagle's medium, and treated with compounds as described previously.

2.5. Cell lysate preparation

Cells were lysed in the presence 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). This buffer is referred as SDS-PAGE sample buffer.

2.6. Nuclear isolation

Cell were washed with phosphate buffered saline (PBS). Cells were scraped and pelleted at 700×g for 5 minutes. FractionPREP Cell Fractionation kit (BioVision, California, USA) was used to isolate cellular nucleus. Proteins were denatured in SDS-PAGE sample buffer described in section 2.5 and processed for western blotting.

2.7. Western blotting

Protein expression was analysed using sodium dodecyl sulfate containing polyacrylamide gel electrophoresis under reducing conditions. Subsequently, proteins from the gel were blotted onto nitrocellulose membrane (GE Healthcare, Buchinghamshire, UK). Non-specific sites on the membrane were blocked using 2% (w/v) bovine serum albumin (BSA) (Thermo Fisher Scientific, Massachusetts, USA) in TBST buffer (0.1% v/v Tween 20, 48 mM NaCl and 20 mM tris(hydroxymethyl) aminomethane (pH 7.5)) for 1 h at room temperature. Primary antibody was diluted in buffer specified by the manufacturer and the membrane was incubated at 4 degrees celsius overnight. HRP-conjugated anti-mouse or anti-rabbit antibodies (Sigma-Aldrich, Poole, UK) were used to detect the primary antibody. These antibodies were diluted in 5% w/v bovine serum albumin in TBST buffer and the membrane was incubated for an hour at room temperature. Luminol-based enhanced chemiluminescence method was used to visualise the proteins.

2.8. Densitometry

ScanImage (Scion Corporation, MD, USA) was used to quantify the signal generated on western blots. Measured signal was normalised according to α-actin for whole cell lysates or lamin A/B for nuclear extracts. The statistical significance between different treatment groups was assessed using Unpaired Student's t-Test.

2.9. RT-PCR

The expression of SK1, SK2, $S1P_3$ and $S1P_4$ mRNA was assessed using reverse transcription polymerase chain reaction (PCR). Total RNA was extracted from MDA-MB-231 cells using ISOLATE II RNA mini kit (Bioline Reagents, London, UK). mRNA was transcribed into cDNA using BioScript (Bioline Reagents, London, UK). The synthesised cDNA was used in PCR amplification. The following primers were used: GAPDH (forward: TGAAGGTCGGAGTCAACGGATTTGGC, reverse: CATGTGGGCC ATGAGGTCCACCAC), SK1 (forward: CTGTCACCCATGAACCTGCTGTC, reverse: C ATGGCCAGGAA GAGGCGCAGCA), SK2 (forward: GCCTACTTCTGCATCTACACC

TACC, reverse: GA GGTTGAAGGACAGCCCAGCTTC), $S1P_3$ (forward: GACTGCTCT ACCATCCTGCCC, reverse: GTAGATGACCGGGTTCATGGC) and $S1P_4$ (forward: GGC ACAGCCGGCTCA TTGTT, reverse: AAGCTGAGCACGGCTCTGCACA). The amplification reaction was performed using the ABI Model 7300 PCR machine (Applied Biosciences, CA, USA).

2.10. xCELLigence cell viability assay

The viability of MDA-MB-231 cells were monitored using xCELLigence system (Roche Diagnostics Limited, West Sussex, UK). Cells were plated in E-plates (Acea Biosciences, CA, USA) and incubated for 24 hours at 37 degrees celsius. Cells were transfected as described previously. Cells were treated with compounds (10 μM) for 4 hours then stimulated with S1P (5 μM) for an hour. Cell index was monitored by xCELLigence software (Acea Biosciences, CA, USA).

2.11. *In situ* apoptosis detection assay

Apoptotic cells in paraffin-embedded tissue samples were determined using ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, USA) TMAs were hydrated through progressive incubation in xylene and ethanol (Fisher Scientific, Leicestershire, UK). Proteinase K (20 μg/ml) (Millipore, Billerica, USA) solution in phosphate buffer saline (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) (Sigma-Aldrich, St. Louis, MO) was used for antigen retrieval. Endogenous peroxidases were inactivated using 3% H_2O_2 (Fisher Scientific, Leicestershire, UK). As a positive control, a TMA was treated with 0.1 U/μl of RQ1 RNase-Free DNase (Promega, WI, USA) for 30 minutes at 37 degrees Celsius. Further steps were followed as described by the supplier. Apoptotic cells were visualised using 3,3'-diaminobenzidine (DAB) peroxidase substrate detection kit (Vector Laboratories, Burlingame, CA). The sections were counterstained in Mayer's hematoxylin, dehydrated in ethanol and xylene, and mounted. Apoptotic index represents the percentage of apoptotic cells to non-apoptotic cells.

2.12. Patient information and tissue microarray (TMA) construction

The ERα negative cohort was formed from 140 patients diagnosed with operable invasive breast carcinoma between 1995 and 1998 in the Greater Glasgow area. Patients have received diverse treatment regimens; 36 patients (25.7%) have received chemotherapy, 17 patents (12.1%) have received radiotherapy and 61 patients (43.6%) have received both chemotherapy and radiotherapy.

The ERα positive cohort was formed from 304 patients diagnosed with operable invasive breast carcinoma between 1980 and 1999 in the Greater Glasgow area. Patients have received either single or combination of treatments; 155 patients have received tamoxifen (52%), 48 patients have received tamoxifen plus radiotherapy (16.1%), 49 patients have received tamoxifen plus chemotherapy (16.4%), 46 patients have received tamoxifen plus radiotherapy plus chemotherapy (15.4%).

Formalin-fixed paraffin-embedded breast cancer tissue samples were used to construct the TMA blocks. 0.6mm^2 cores were removed from the samples and inserted into a new paraffin block. All TMA blocks were constructed in triplicate containing three individual tumour cores taken from the same embedded tissue sample.

2.13. Immunohistochemistry

TMAs were stained for SK1 (Abgent, Oxfordshire, UK), $S1P_1$ (Exalpha, Shirley, MA), $S1P_2$ (Sigma Life Sciences, St. Louis, MO), $S1P_3$ (Exalpha, Shirley, MA) and $S1P_4$ (Exalpha, Shirley, MA). All antibodies were polyclonal except monoclonal $S1P_2$ antibody. Sections were dewaxed in xylene (Fisher Scientific, Leicestershire, UK) and rehydrated in ethanol (Fisher Scientific, Leicestershire, UK) and water. Antigen retrieval was performed by microwaving under pressure for 5-7 minutes in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase was inactivated by incubating the sections in 3% H_2O_2 (Fisher Scientific, Leicestershire, UK) for 20 minutes. Blocking was performed by incubating slides in 1:10 casein (Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes. Sections were incubated with primary antibody in 1:75 (SK1), 1:300 ($S1P_1$), 1:75

($S1P_2$), 1:25 ($S1P_3$), 1:200 ($S1P_4$), 1:300 ($S1P_5$) dilution at 4 degrees Celsius for overnight. Concentration of these antibodies were determined experimentally. EnVision-HRP conjugate (Dako, Cambridgeshire, UK) was utilised to amplify the signal. Signal was visualised using 3,3'-diaminobenzidine (DAB) chromagen (Vector Laboratories, Burlingame, CA). The sections were counterstained in Mayer's hematoxylin, dehydrated in ethanol and xylene, and mounted. Formalin-fixed paraffin-embedded HEK 293 cell pellets over-expressing $S1P_2$ protein were used to confirm the specificity of $S1P_2$ antibody. Specificity of SK1, $S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$, phosphorylated ERK-1/2, ERK-1/2, phosphorylated AKT, phosphorylated RAF-1, LYN, Y216 phosphorylated c-SRC and Y416 phosphorylated SFK antibodies have been previously reported for the ERα positive and ERα negative breast cohorts. These studies are listed in **Table 2.1**.

2.14. Scoring

The protein expression was quantified by two independent observers using the weighted histoscore method determined by Tovey *et al.,* 2004. The consistency of scoring between the observers was verified utilising Interclass correlation analysis (ICC) (Kirkegaard *et al.,* 2006). Quantification method first grades the intensity of staining from 0 (negative) to 3 (strong) and then multiplies the grade by the percentage of tumour cells within each category. The final histoscore is the average for the triplicate samples.

2.15. Statistical analyses

All statistical analyses were performed in SPSS 19.0 (Chicago, IL). Mann-Whitney test or Kruskal-Wallis One-way ANOVA test was utilised to analyse the relationship between clinical parameters and the specific marker ($P<0.05$). Kaplan-Meier method was used to obtain disease-specific survival or disease recurrence curves. The significance of disease-specific survival or disease recurrence between different subgroups were assessed using Log-rank test. Cox regression model was utilised to investigate the relationship between disease-specific survival or cancer recurrence and expression of specific markers. A

Table 2.1. Studies confirming the specificity of antibodies used to determine the levels of expression of specified proteins in TMAs.

Proteins	Specificity confirmed
SK1	Long et al., 2010a
S1P1	Watson et al., 2010
S1P2	Ohotski et al., 2012
S1P3	Watson et al., 2010
S1P4	Long et al., 2010b
ERK-1/2	McGlynn et al., 2009
pERK-1/2	McGlynn et al., 2009
pAKT	Kirkegaard et al., 2010
pRAF-1	McGlynn et al., 2009
LYN	Elsberger et al., 2010
Y219 c-SRC	Elsberger et al., 2009
Y416 c-SRC	Elsberger et al., 2009
c-SRC	Elsberger et al., 2009

multivariate cox regression model was used to determine independent prognostic markers ($P<0.05$).

2.16. Patient Approval

Ethical approval was obtained from the local ethics committee. Patient consent was not required for this study.

CHAPTER III

The expression of SK1 and S1P receptors in ERα positive breast cancer patients

3.1. Introduction

ERα positive breast cancer is the most common type of breast cancer comprising ~75% of cases (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2011). Endocrine therapy is a common treatment regimen used in patients with ERα positive tumours at all stages. Endocrine therapy consists of therapeutics targeting either the synthesis of oestrogen (aromatase inhibitors e.g. Anastrozole, Exemestane and Letrozole) or the oestrogen receptor signalling (selective oestrogen receptor modulators (SERMs) e.g. Tamoxifen and Fulvestrant). The SERMs in combination with the aromatase inhibitors are commonly used in the treatment of both early and advanced breast tumours (Johnston, 2010). Despite the proven efficacy, the aromatase inhibitors cannot be used in the treatment of premenopausal women, thus the SERMs are the sole selective therapeutics available for these patients (Johnston, 2010). Moreover, single treatment with SERMs such as tamoxifen has poor efficacy, reducing the patient mortality by only 28% (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2011). In addition, cancer recurrence during the endocrine therapy is a common phenomenon. Thus, discovery of novel therapeutic targets are required for the treatment of ERα positive breast tumours.

The SK/S1P signalling interacts with oncogene proteins to maintain cell proliferation, survival, and resistance to chemotherapeutics (Pyne and Pyne, 2010). For example, H-RAS induced tumourigenic transformation is mediated by the SK1 catalytic activity in NIH 3T3 mouse embryonic fibroblasts (Xia *et al.*, 2000). Furthermore, the inhibition of SK1 catalytic activity by SK1 inhibitors or the over-expression of an SK1 mutant devoid of catalytic activity (G82D) abolishes H-RAS-dependent tumourigenesis. SK1 also interacts with the oncogenic eukaryotic elongation factor 1A1 (eEF1A1) required for protein translation (Leclercq *et al.,* 2008). The eEF1A over-expression was shown to increase the proliferation rate and anchorage-independent growth in NIH3T3 rodent fibroblast cells (Anand *et al.,* 2002). Moreover, the SK1 catalytic activity is required for the eEF1A1-mediated tumourigenesis (Leclercq *et al.,* 2011). In addition to SK1, the S1P receptors also interact with oncogene proteins. For example, the $S1P_4$ receptor functionally interacts with HER2 receptor to stimulate the ERK-1/2 signalling pathway in MDA-MB-453 breast cancer cells (Long *et al.,* 2010b). Furthermore, the S1P-induced ERK-1/2 activation was dependent on the activation of both HER2 and $S1P_4$ receptor.

The SK/S1P signalling is up-regulated in various human cancers (e.g. colon, rectal, glioblastoma, ovarian, lung and breast) making the S1P signalling an important candidate as a therapeutic target (Liu *et al.,* 2010, Van Brocklyn *et al.,* 2005, Johnson *et al.,* 2005, French *et al.,* 2003, Watson *et al.,* 2010). Various studies have assessed the outcome of expression of proteins involved in sphingolipid signalling on breast cancer patient. French and coworkers have shown that the SK1 mRNA expression was up-regulated in breast cancer tissue samples compared to the normal breast tissue (French *et al.,* 2003). Moreover, SK1 mRNA expression was gradually enhanced from early tumours to advanced tumours, and associated with poor prognosis in ERα positive breast cancer patients (Ruchaberle *et al.,* 2008, Ling *et al.,* 2011). Similarly, Watson and coworkers have shown that the patients with ERα positive breast tumours expressing high levels of SK1 had shorter disease-specific survival and cancer recurrence (Watson *et al.,* 2010).

In this chapter, tissue microarrays composed of ERα positive breast tumours were used to determine the impact of SK1/$S1P_{1-3}$ receptor expression in combination with *other* signalling proteins (e.g. NFκB (p50 RelA), c-RAF-1, ERK-1/2, AKT, SRC family kinases (SFKs) and LYN kinase) on clinical outcome. This systematic analysis has allowed the determination of possible functional interactions between signalling pathways and SK1/ $S1P_{1-3}$ receptors related to clinical prognosis.

3.2. Results

3.2.1. The clinicopathological details of ERα positive breast cancer cohort

A total of 304 ERα positive patients were involved in the study. The median age of the patients was 62 with inter-quartile range from 53 to 70. The tumours were classified as ductal (82.9%), lobular (6.9%) and medullary (6.6%) breast carcinomas. The pathological grading have shown that 63 (20.7%) cases were 1st grade, 139 (45.7%) cases were 2nd grade and 79 (26.0%) cases were 3rd grade tumour. The median tumour size was 20 mm with inter-quartile range from 15 mm to 30 mm. The examination of lymph nodes have shown that 135 patients (44.4%) had metastasis in comparison to 139 patients (45.7%) that had no metastasis in the lymph nodes. According to last patient follow-up date, 167 (54.9%) patients were alive, 79 (26.0%) patients died of breast cancer and 57 (18.8%) patients died of another cause.

3.2.2. The expression of SK1 and S1P receptors correlates with the clinical parameters in ERα positive breast cancer cohort

The expression of SK1 and $S1P_{1-3}$ receptors was analysed in ERα positive breast cancer patients. Images of tissues stained for SK1 and $S1P_{1-3}$ receptors are presented in **Figure 3.1**. Patients were divided into low and high expressing subgroups using a technique described by Ruckhaberle and coworkers (Ruckhaberle *et al.,* 2007). The Mann-Whitney test or Kruskal-Wallis One-way ANOVA was used to analyse the correlation between protein expression and clinicopathological parameters **(Table 3.1)**. In ERα positive tumours, the tumour type was correlated with the cytoplasmic and nuclear expression levels of $S1P_2$ receptors. The analysis of distribution of nuclear $S1P_2$ receptor expression within the patients with different cancer types have shown that the tumours originated from mammary lobular epithelium expressed the highest levels compared with ductal and medullary carcinoma **(Figure 3.2A)**. The cytoplasmic $S1P_2$ receptor expression showed a pattern similar to the nuclear $S1P_2$ receptor expression within different cancer types. The nuclear expression of the $S1P_2$ receptor was also correlated with tumour grade **(Table 3.1)**. The analysis of distribution of the nuclear $S1P_2$ receptor expression within tumours with

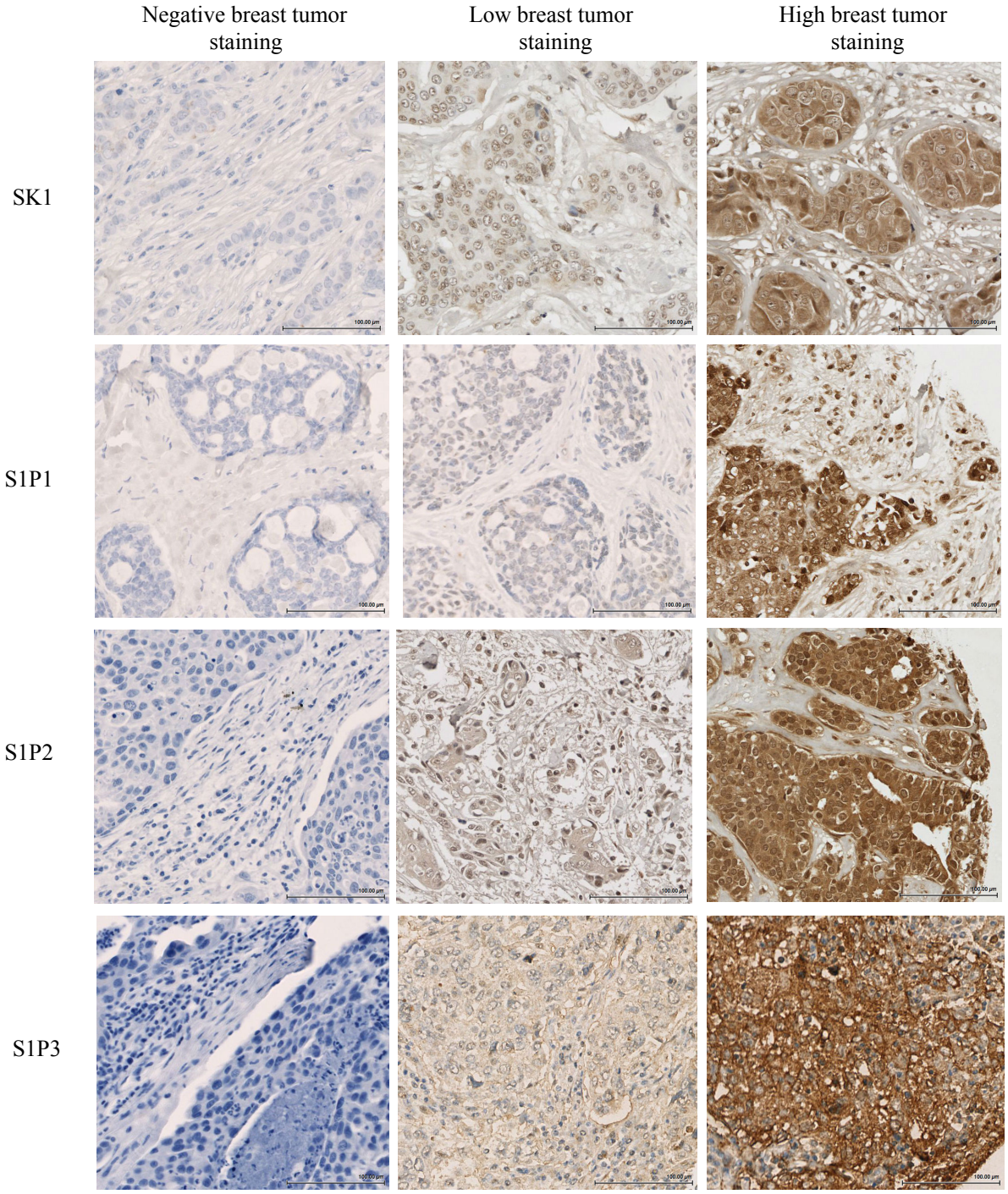

Figure 3.1. Images of ERα positive breast cancer tissues stained for SK1 and $S1P_{1-3}$ receptors. Panels from left to right present tissue samples with negative staining (left panel), low staining (middle panel) and high staining (right panel).

Table 3.1. The Mann-Whitney test results showing the differences in protein expression within different subgroups in ERα positive cohort (yrs: years; Dc: ductal carcinoma; Lc: lobular carcinoma; Mc: medullary carcinoma; P: positive; N: negative; L: low; H: high; mem: plasma membrane; cyto: cytoplasm; nuc: nucleus).

		Mann-Whitney P values											
Variables	Numbers	SK1 mem	SK1 cyto	SK1 nuc	S1P1 mem	S1P1 cyt	S1P1 nuc	S1P2 mem	S1P2 cyto	S1P2 nuc	S1P3 mem	S1P3 cyto	S1P3 nuc
Age (<50yrs/ >50yrs)	62/242	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Tumour type (D/L/ M)	252/21/20	0.012	NS	NS	NS	NS	NS	NS	0.04	0.007	NS	NS	NS
Tumour grade (1/2/3)	63/139/79	NS	NS	NS	NS	NS	NS	NS	NS	0.03	NS	NS	NS
Tumour size (0/1/2)	144/130/12	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.02
Lymph nodes (Y/N)	135/139	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PR status (P/N)	171/118	NS	NS	0.046	0.002	NS	NS	NS	NS	0.05	0.001	0.007	NS
Her2 status (P/N)	26/276	NS	NS	NS	NS	0.002	0.001	NS	NS	NS	NS	NS	NS
SK1 mem (L/H)	254/15	-	NS	NS	0.027	NS	NS	NS	NS	0.001	0.022	NS	NS
SK1 cyto (L/H)	249/20	0.004	-	0.015	0.02	0.023	NS	NS	NS	NS	NS	NS	NS
SK1 nuc (L/H)	174/95	NS	0	-	0	NS	NS	NS	NS	NS	NS	NS	0.024
S1P1 mem (L/H)	224/58	NS	NS	NS	-	0	0.001	0.001	NS	0.017	NS	NS	0.05
S1P1 cyto (L/H)	243/39	NS	NS	NS	NS	-	0	0.009	0.004	0	NS	0	NS
S1P1 nuc (L/H)	227/55	NS	NS	NS	0.022	0	-	0.031	0.008	0	NS	0	0.001
S1P2 mem (L/H)	195/86	NS	NS	NS	0	NS	NS	-	0	NS	NS	NS	0.009
S1P2 cyto (L/H)	198/82	NS	0.002	NS	NS	NS	NS	0	-	0.002	NS	NS	NS
S1P2 nuc (L/H)	226/54	0.036	NS	0.002	0	0	0	0.031	0	-	NS	0	NS
S1P3 mem (L/H)	247/37	NS	NS	NS	0.024	0.028	NS	NS	NS	NS	-	0	0.001
S1P3 cyto (L/H)	175/109	NS	NS	NS	NS	0	0	NS	0.001	0	0	-	0
S1P3 nuc (L/H)	221/63	NS	NS	NS	NS	NS	0.017	NS	NS	0.037	0	0	-

different grades have shown that the $S1P_2$ receptor expression levels were comparably similar in 1st and 2nd grade tumour, however the expression was significantly reduced in the 3rd grade tumours **(Figure 3.2B)**. The tumour size was correlated with the expression of nuclear $S1P_3$ receptor. The tumours were grouped into three categories; the tumours smaller than 20 mm, the tumours bigger than 21 but smaller than 50 mm, and the tumours bigger than 50 mm. The analysis of distribution of the $S1P_3$ receptor expression within the tumours with different sizes has shown that the expression of $S1P_3$ receptor increased with tumour progression **(Figure 3.2C)**. The lymph node metastasis was not correlated with the expression of proteins included in the analysis. The PgR and HER2 receptor expression was known for the tumours included in the cohort. The expression of plasma membrane-bound $S1P_1$ and $S1P_3$ receptors, cytoplasmic $S1P_3$ receptor and nuclear $S1P_2$ receptor

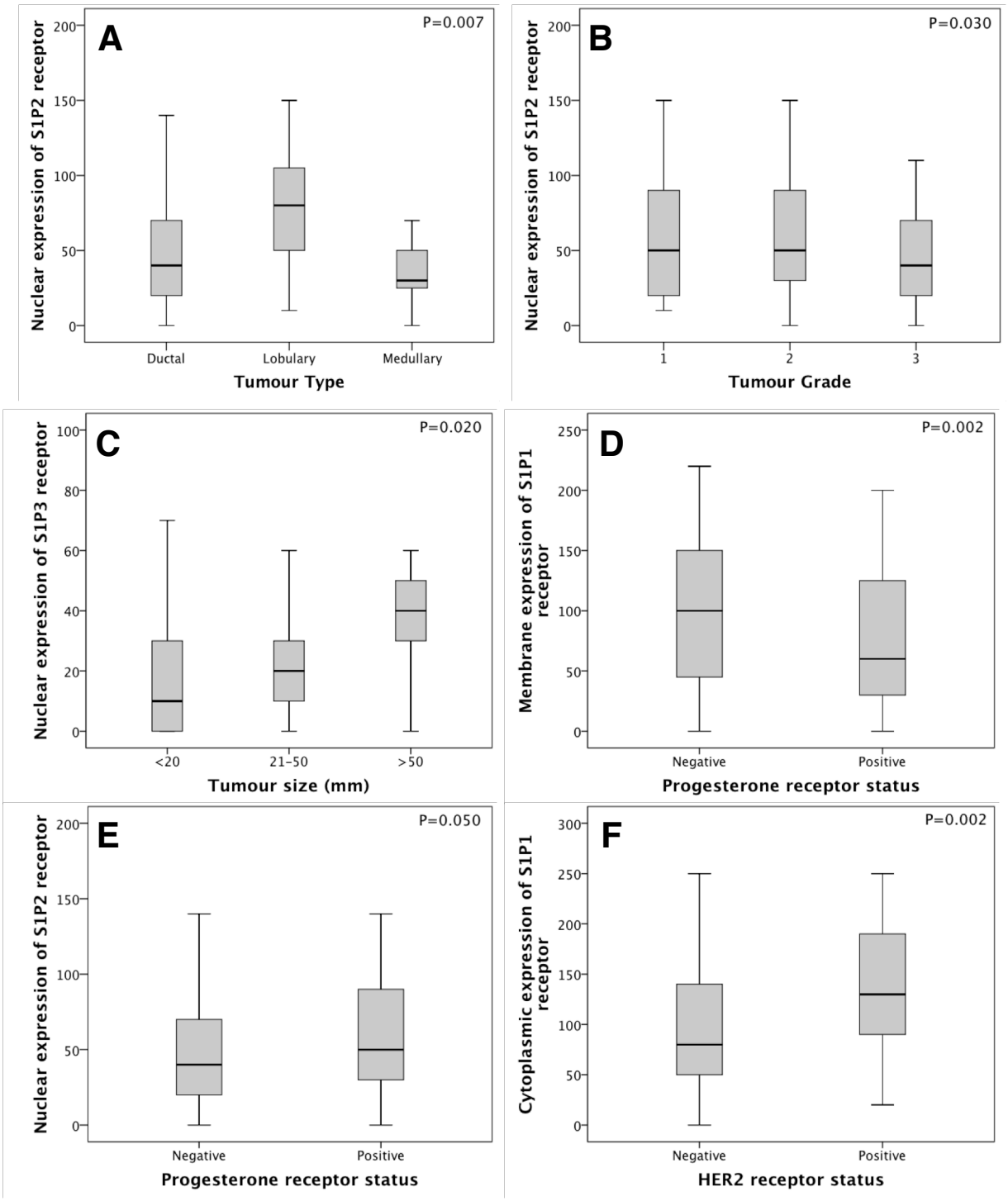

Figure 3.2. The expression of SK1 and $S1P_{1-3}$ receptors in different subgroups of clinicopathological parameters in ERα positive cohort. **A** shows the nuclear expression of $S1P_2$ receptor in different tumour types; **B** shows the nuclear expression of $S1P_2$ receptor in different tumour grades; **C** shows the nuclear expression of $S1P_3$ receptor in tumours with different sizes; **D** shows the membrane expression of $S1P_1$ receptor in progesterone receptor negative or positive tumours; **E** shows the nuclear expression of $S1P_2$ receptor in progesterone receptor negative or positive tumours; **F** shows the cytoplasmic expression of $S1P_1$ receptor in HER2 receptor negative or positive tumours.

expression was correlated with PgR expression. The analysis of plasma membrane-bound $S1P_1$ receptor expression within the PR positive or negative tumours has shown that the PgR positive tumours expressed lower levels of the plasma membrane-bound $S1P_1$ compared to the PgR negative tumours **(Figure 3.2D)**. Similarly, the plasma membrane-bound and cytoplasmic expression of $S1P_3$ receptor was significantly lower in PgR positive tumours compared to PgR negative tumours. However, the analysis of nuclear $S1P_2$ receptor expression has revealed that the PgR positive tumours expressed higher levels of nuclear $S1P_2$ receptor compared to the PgR negative tumours **(Figure 3.2E)**. The HER2 receptor expression was correlated with the cytoplasmic and nuclear expression levels of $S1P_1$ receptor. Further analysis has shown that the HER2 receptor positive tumours expressed higher levels of cytoplasmic and nuclear $S1P_1$ receptor **(Figure 3.2F)**.

The expression of all the proteins was positively correlated within different cellular compartments. For instance, the plasma membrane-bound SK1 expression was positively correlated with the cytoplasmic and nuclear SK1 expression (**Table 3.1**). Furthermore, the plasma membrane-bound SK1 expression was positively correlated with the expression of $S1P_1$ and $S1P_3$ receptors at the plasma membrane (**Figure 3.3A, data not shown for $S1P_3$ receptor)**, and negatively correlated with the nuclear $S1P_2$ receptor (**Figure 3.3B**). The cytoplasmic SK1 expression was correlated positively with the $S1P_1$ receptor expression at the plasma membrane and in the cytoplasm (**Table 3.1**). The nuclear expression of SK1 was positively correlated with the expression of membrane $S1P_1$ and nuclear $S1P_3$ receptor (**Figure 3.3C**). The expression of $S1P_1$ receptor at the plasma membrane was positively correlated with the expression of $S1P_2$ receptor at the plasma membrane (**Figure 3.3D**) and $S1P_3$ receptor in the nucleus (**Table 3.1**). Moreover, there was a negative correlation with the nuclear $S1P_2$ receptor expression (**Figure 3.3E**). The cytoplasmic expression of $S1P_1$ receptor was positively correlated with the expression of $S1P_2$ receptor in the cytoplasm and nucleus (**Figure 3.4A**), and the cytoplasmic $S1P_3$ receptor (**Figure 3.4B**). However, there was a negative correlation with the $S1P_2$ receptor expression at the plasma membrane (**Figure 3.3F**). The nuclear expression of $S1P_1$ receptor positively correlated with the expression of $S1P_2$ and $S1P_3$ receptors in the cytoplasm and nucleus (**Figure 3.4C** and **Figure 3.4D**). However, the $S1P_2$ receptor expression at the plasma membrane was negatively correlated with the nuclear expression of $S1P_1$ receptor. The expression of $S1P_2$ receptor at the plasma membrane was positively correlated with the $S1P_1$ receptor

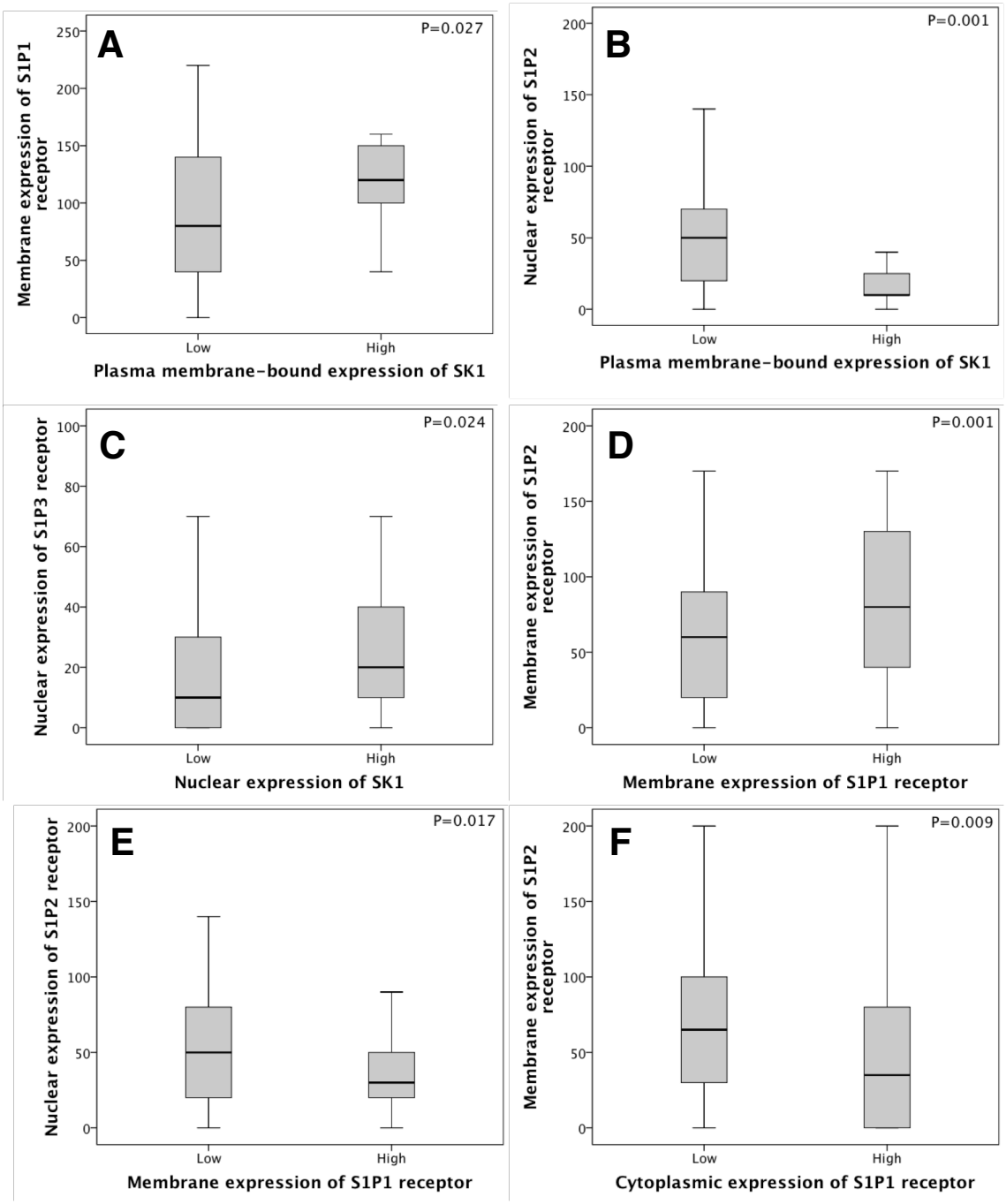

Figure 3.3. The expression of SK1 and $S1P_{1-3}$ receptors in tissues expressing SK1 or $S1P_{1-3}$ receptors in ERα positive cohort. **A** shows the plasma membrane expression of $S1P_1$ receptor within low or high SK1 expressing tumours at the plasma membrane; **B** shows the nuclear expression of $S1P_2$ receptor within low or high SK1 expressing tumours at the plasma membrane; **C** shows the nuclear expression of $S1P_3$ receptor within low or high SK1 expressing tumours in the nucleus; **D** shows the plasma membrane expression of $S1P_2$ receptor within low or high $S1P_1$ receptor expressing tumours at the plasma membrane; **E** shows the nuclear expression of $S1P_2$ receptor within low or high $S1P_1$ receptor expressing tumours at the plasma membrane; **F** shows the plasma membrane expression of $S1P_2$ receptor within low or high $S1P_1$ receptor expressing tumours in the cytoplasm.

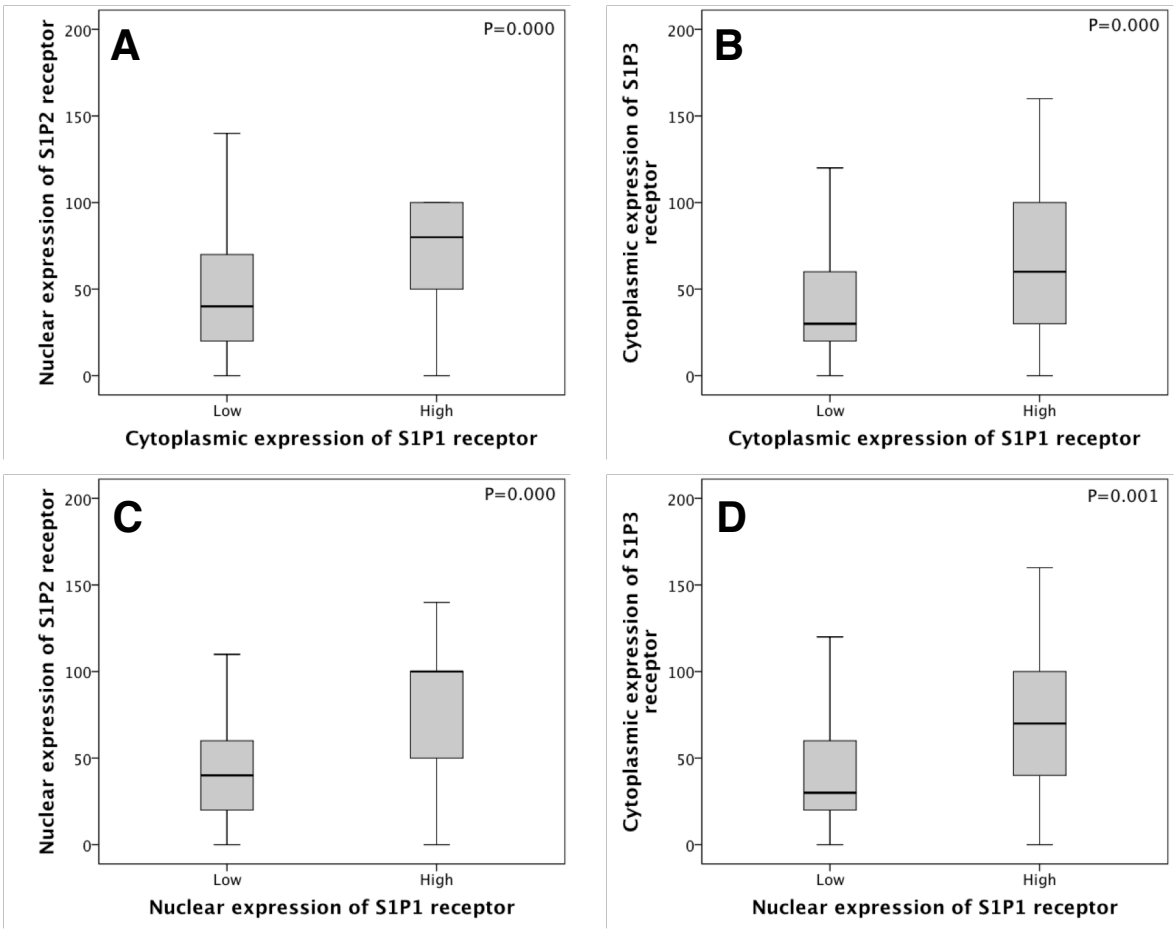

Figure 3.4. The expression of $S1P_{1-3}$ receptors in tissues expressing other $S1P_{1-3}$ receptors in ERα positive cohort. **A** shows the nuclear expression of $S1P_2$ receptor within low or high $S1P_1$ receptor expressing tumours in the cytoplasm; **B** shows the cytoplasmic expression of $S1P_3$ receptor within low or high $S1P_1$ receptor expressing tumours in the cytoplasm; **C** shows the nuclear expression of $S1P_2$ receptor within low or high $S1P_1$ receptor expressing tumours in the nucleus; **D** shows the cytoplasmic expression of $S1P_3$ receptor within low or high $S1P_1$ receptor expressing tumours in the nucleus.

expression at the plasma membrane (**Figure 3.5A)** and $S1P_3$ receptor expression in the nucleus **(Figure 3.5B)**. The cytoplasmic expression of $S1P_2$ receptor was positively correlated with the cytoplasmic SK1 expression. The nuclear expression of $S1P_2$ receptor was negatively correlated with the nuclear SK1 **(Figure 3.5C)** and the plasma membrane $S1P_1$ expression. However, the nuclear expression of $S1P_2$ receptor was positively correlated with the expression of $S1P_1$ receptor at the cytoplasm and nucleus **(Figure 3.5D)**, and $S1P_3$ receptor in the cytoplasm. The expression of $S1P_3$ receptor at the plasma membrane was positively correlated with the expression of $S1P_1$ receptor at the plasma membrane and cytoplasm. The cytoplasmic expression of $S1P_3$ receptor was positively correlated with the cytoplasmic and nuclear expression of both $S1P_1$ and $S1P_2$ receptors **(Figure 3.4E)**. The nuclear expression of $S1P_3$ receptor was positively correlated with the expression of both nuclear $S1P_1$ and $S1P_2$ receptors **(Figure 3.5F)**.

3.2.3. The effect of SK1 expression on disease-specific survival and disease-free cancer recurrence in ERα positive breast tumours

Watson and coworkers have shown that the expression of SK1, $S1P_1$ and $S1P_3$ receptors was associated with reduced survival in ERα positive breast cancer patients (Watson *et al.,* 2010). Extending these findings, the effect of SK1 and S1P receptors on patient survival and cancer recurrence was further analysed by subdividing patients into sub groups according to the expression of specific proteins. This approach allowed further characterisation of the SK1- and $S1P_{1-3}$ receptor-mediated effects in relation to expression and sub-cellular distribution of the S1P receptors.

Watson and coworkers have shown that the high expression of SK1 in the nucleus or cytoplasm was associated with shorter disease-specific patient survival in ERα positive breast cancer (Watson *et al.,* 2010). In the current analysis, low or high nuclear SK1 expressing tumours were further classified according to the expression of plasma membrane-bound $S1P_1$ receptor creating four different classes; the tumours expressing both SK1 and $S1P_1$ receptor at high levels, the tumours expressing low levels of SK1 but high levels of $S1P_1$ receptor, the tumours expressing high SK1 but low levels of $S1P_1$ receptor and the tumours expressing both SK1 and $S1P_1$ receptor at low levels. High

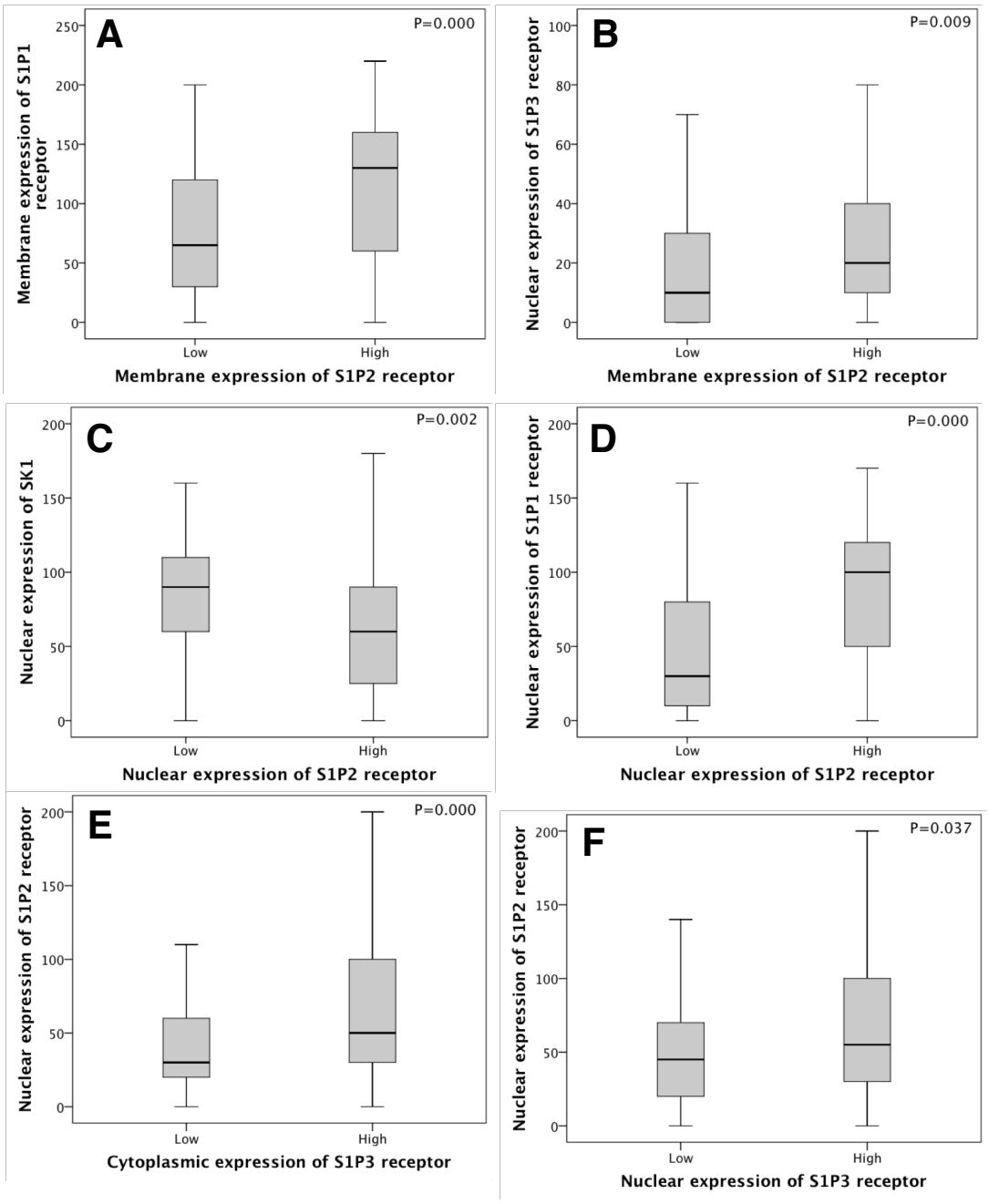

Figure 3.5. The expression of SK1 and $S1P_{1-3}$ receptors in tissues expressing other $S1P_{1-3}$ receptors in ERα positive cohort. **A** shows the membrane expression of $S1P_1$ receptor within low or high $S1P_2$ receptor expressing tumours on the membrane; **B** shows the nuclear expression of $S1P_3$ receptor within low or high $S1P_2$ receptor expressing tumours on the membrane; **C** shows the nuclear expression of SK1 within low or high $S1P_2$ receptor expressing tumours in the nucleus; **D** shows the nuclear expression of $S1P_1$ receptor within low or high $S1P_2$ receptor expressing tumours in the nucleus; **E** shows the nuclear expression of $S1P_2$ receptor within low or high $S1P_3$ receptor expressing tumours in the cytoplasm; **F** shows the nuclear expression of $S1P_2$ receptor within low or high $S1P_3$ receptor expressing tumours in the nucleus.

expression of both nuclear SK1 and plasma membrane $S1P_1$ receptor in the same tumour was associated with shorter disease-specific survival (**Figure 3.6A**). The mean disease-specific survival time for these patients was 6.2 years compared with 8.3 years for patients with tumours expressing low levels of both nuclear SK1 and plasma membrane-bound $S1P_1$ receptor (P=0.019). The disease-specific survival time for the patients with tumours that contained high expression levels of nuclear SK1 and low plasma membrane-bound $S1P_1$ receptor or high plasma membrane-bound $S1P_1$ receptor and low nuclear SK1 expression were not significantly reduced compared to the patients whose tumours express low levels of both nuclear SK1 and plasma membrane-bound $S1P_1$ receptor (**Figure 3.6A**). Thus, the expression of both SK1 and $S1P_1$ receptor was required in the same tumour for observed shorter disease-specific survival of patients with ERα positive tumours. The effect of cytoplasmic SK1 on disease-specific survival could not be analysed due to small sample number in each sub group ($n<10$).

The impact of $S1P_3$ receptor expression on the association of nuclear SK1 with shorter disease-specific survival in the ERα positive patients was also analysed. Similarly, low or high nuclear SK1 expressing tumours were classified according to the expression of cytoplasmic $S1P_3$ receptor creating four different sub groups. The mean disease-specific survival time for the patients with tumours expressing high levels of both nuclear SK1 and cytoplasmic $S1P_3$ receptor was 6.1 years compared to 8.2 years for the patients with tumours expressing low levels of both nuclear SK1 and cytoplasmic $S1P_3$ receptor (P=0.005) (**Figure 3.6B**). The disease-specific survival time for the patients with tumours expressing high levels of nuclear SK1 and low levels of cytoplasmic $S1P_3$ receptor, or high levels of cytoplasmic $S1P_3$ receptor and low levels of nuclear SK1 expression was not significantly reduced compared to the patients whose tumours expressed low levels of both nuclear SK1 and cytoplasmic $S1P_3$ receptor (**Figure 3.6B**). Thus, the expression of both cytoplasmic $S1P_3$ receptor and nuclear SK1 at high levels in the same tumour was required for shorter disease-specific patient survival.

The effect of expression of various signalling proteins such as NFκB (p50 RelA), c-RAF-1, ERK-1/2, AKT, SRC family kinases (SFKs) and LYN on cancer prognosis and cancer recurrence in relation to the expression of SK1 or S1P receptors was assessed in ERα positive patients. The immunohistochemical staining of these proteins were available for

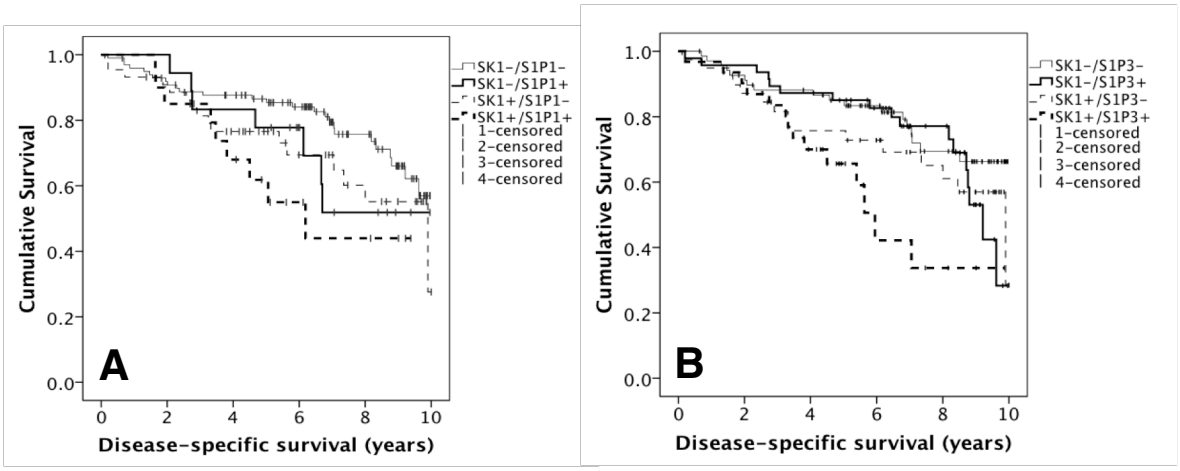

Figure 3.6. The Kaplan-Meier survival graphs showing the effect of $S1P_1$ or $S1P_3$ receptor expression on SK1 associated disease-specific patient survival. The tumours with low expression of SK1 and $S1P_1$/$S1P_3$ receptors were used as a control to calculate a *P* value for the other subgroups. **A** shows the effect of plasma membrane-bound $S1P_1$ receptor and nuclear SK1 expression on disease-specific survival (SK1-/$S1P_1$ +: n=18, P=0.235; SK1+/ $S1P_1$-: n=44, P=0.167; SK1+/$S1P_1$ +: n=20, P=0.019); **B** shows the effect of cytoplasmic $S1P_3$ receptor and nuclear SK1 expression on disease-specific survival (SK1-/$S1P_3$ +: n=47, P=0.342; SK1+/ $S1P_3$-: n=39, P=0.232; SK1+/$S1P_3$ +: n=31, P=0.005).

this cohort. In this analysis, the patients with tumours expressing low or high nuclear SK1 was further classified into distinct groups according to the expression of signalling protein of interest.

The expression of phosphorylated NFκB (p-NFκB) and SK1 in ERα positive tumours was associated with shorter disease-specific patient survival and disease-free cancer recurrence **(Figure 3.7A, Figure 3.8A)**. The patients with tumours expressing high levels of both nuclear phosphorylated NFκB and nuclear SK1 had the mean disease-specific survival time of 6.3 years compared to 8.1 years for the patients with tumours expressing low levels of both nuclear phosphorylated NFκB and nuclear SK1 (P=0.015) **(Figure 3.7A)**. Similarly, the patients with tumours expressing high levels of both cytoplasmic phosphorylated NFκB and nuclear SK1 had the mean disease-free cancer recurrence time of 3.8 years compared to 6.8 years for the patients with tumours expressing low levels of both nuclear SK1 and cytoplasmic phosphorylated NFκB (P=0.023) **(Figure 3.8A)**. The loss of high expression of either phosphorylated NFκB or SK1 annulled the effects on patient survival and cancer recurrence. Therefore, NFκB and SK1 might function together in the ERα positive tumours and this might account for shorter disease-specific survival and disease-free cancer recurrence in patients.

The expression of phosphorylated c-RAF-1 (p-RAF) and SK1 was associated with shorter disease-specific patient survival and disease-free cancer recurrence. The patients with tumours expressing high levels of both cytoplasmic phosphorylated c-RAF-1 and nuclear SK1 had the mean disease-specific survival time of 6.3 years compared to 8.2 years for the patients with tumours expressing low levels of both cytoplasmic phosphorylated c-RAF-1 and nuclear SK1 (P=0.009) **(Figure 3.7B)**. In terms of disease-free cancer recurrence, the patients with tumours expressing high levels of both cytoplasmic phosphorylated c-RAF-1 and nuclear SK1 had the mean cancer recurrence time of 4.6 years compared to 7.2 years for the patients with tumours expressing low level of both cytoplasmic phosphorylated c-RAF-1 and nuclear SK1 (P=0.008) **(Figure 3.8B)**. High expression of both cytoplasmic phosphorylated c-RAF-1 and nuclear SK1 was linked with shorter disease-specific patient survival and disease-free cancer recurrence. Thus, the loss of expression of either cytoplasmic phosphorylated c-RAF-1 or nuclear SK1 annulled these effects.

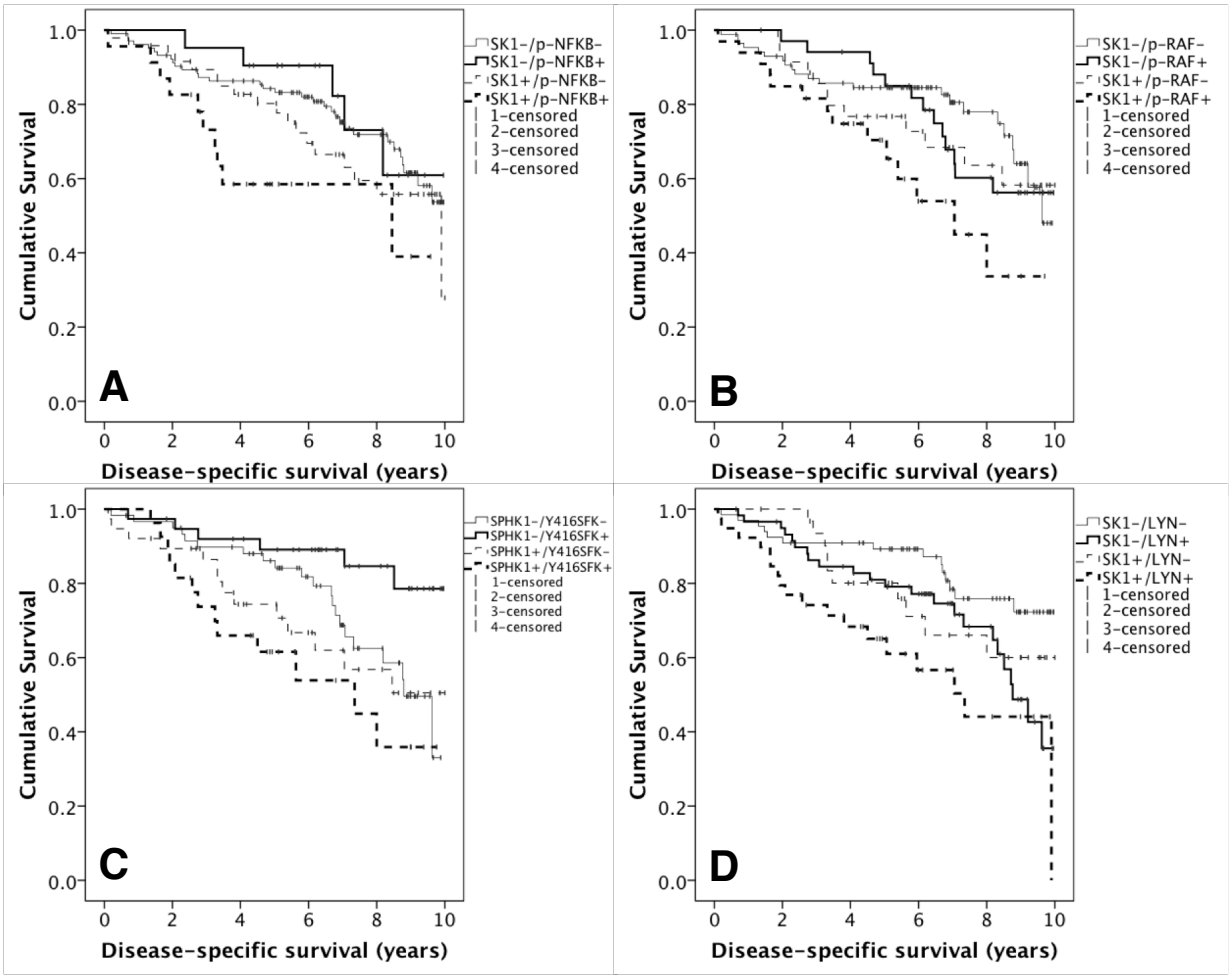

Figure 3.7. The Kaplan-Meier survival graphs showing the effect of nuclear p-NFKB, cytoplasmic p-RAF-1, nuclear ERK-1/2 or cytoplasmic p-AKT expression on SK1 associated disease-specific patient survival. The tumours expressing low levels of both proteins in each individual analysis were used as a control to calculate a P value for the other subgroups. **A** shows the effect of nuclear SK1 and nuclear phosphorylated NFκB expression on disease-specific survival (SK1-/p-NFκB+: n=21, P=0.740; SK1+/p-NFκB-: n=48, P=0.353; SK1+/p-NFκB+: n=23, P=0.015); **B** shows the effect of nuclear SK1 and cytoplasmic phosphorylated RAF-1 expression on disease-specific survival (SK1-/p-RAF-1+: n=34, P=0.617; SK1+/p-RAF-1-: n=36, P=0.561; SK1+/p-RAF-1+: n=33, P=0.009); **C** shows the effect of cytoplasmic Y416SRC kinase and nuclear SK1 expression on disease-specific survival (SK1-/Y416SRC+: n=38, P=0.023; SK1+/Y416SRC-: n=38, P=0.520; SK1+/Y416SRC+: n=26, P=0.067); **D** shows the effect of cytoplasmic LYN and nuclear SK1 expression on disease-specific survival (SK1-/LYN+: n=60, P=0.030; SK1+/LYN-: n=32, P=0.184; SK1+/LYN+: n=39, P=0.001).

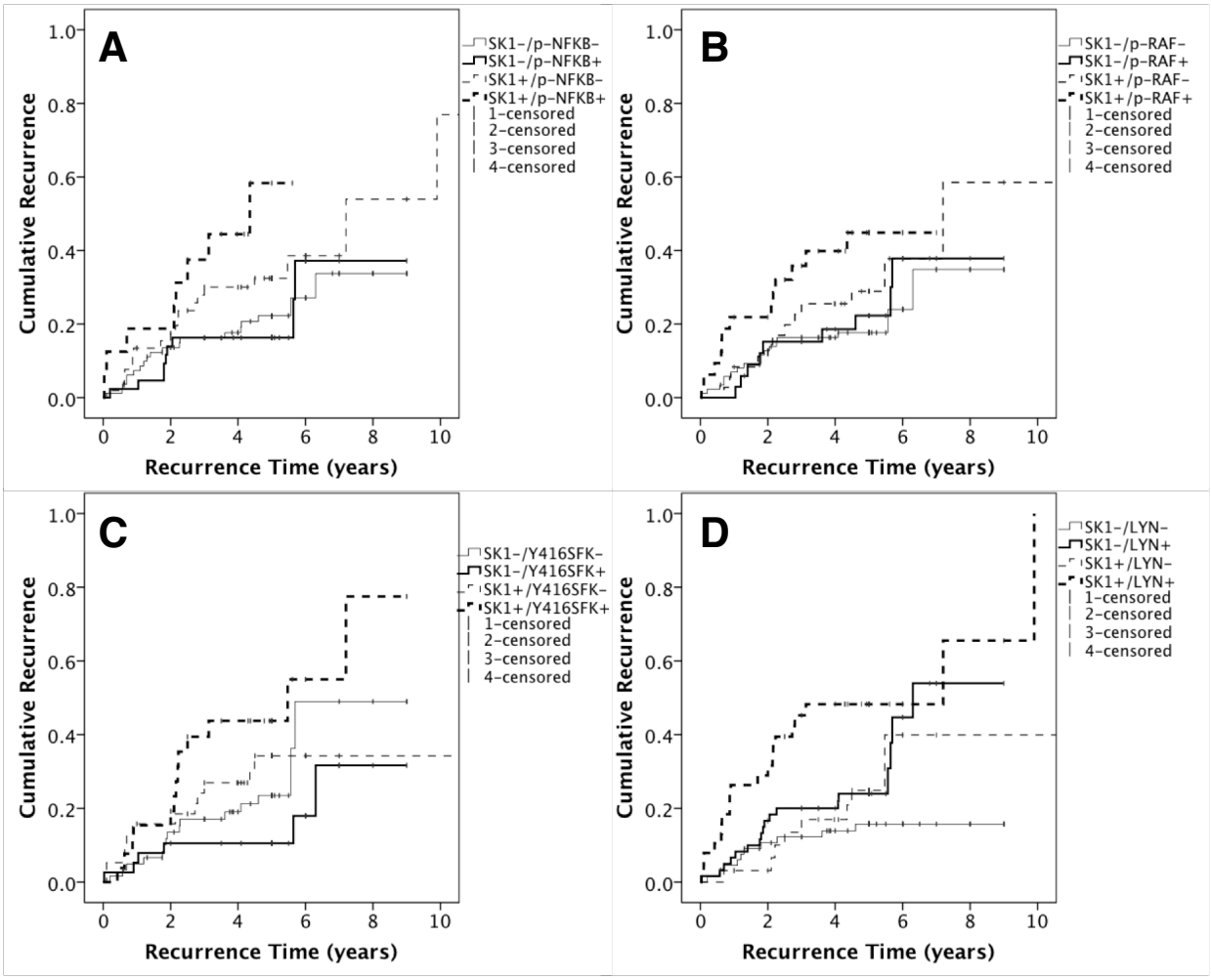

Figure 3.8. The Kaplan-Meier survival graphs showing the effect of cytoplasmic p-NFKB, cytoplasmic p-RAF-1, nuclear Y416SRC kinase or cytoplasmic LYN expression on SK1 associated disease-free cancer recurrence. The tumours expressing low levels of both proteins in each individual analysis were used as a control to calculate a P value for the other subgroups. **A** shows the effect of nuclear SK1 and cytoplasmic phosphorylated NFκB expression on disease-free recurrence (SK1-/p-NFκB+: n=43, P=0.741; SK1+/p-NFκB-: n=52, P=0.153; SK1+/p-NFκB+: n=16, P=0.009); **B** shows the effect of nuclear SK1 and cytoplasmic phosphorylated RAF-1 expression on disease-free recurrence (SK1-/p-RAF-1+: n=34, P=0.626; SK1+/p-RAF-1-: n=36, P=0.180; SK1+/p-RAF-1+: n=32, P=0.008); **C** shows the effect of nuclear Y416SRC kinase and nuclear SK1 expression on disease-free recurrence on (SK1-/Y416SRC+: n=38, P=0.560; SK1+/Y416SRC-: n=38, P=0.028; SK1+/Y416SRC+: n=26, P=0.245); **D** shows the effect of cytoplasmic LYN and nuclear SK1 expression disease-free recurrence (SK1-/LYN+: n=61, P=0.055; SK1+/LYN-: n=32, P=0.232; SK1+/LYN+: n=38, P=0.0001).

The effect of expression of SRC family kinases phosphorylated at Y416 (Y416SFK) in relation to SK1 associated shorter disease-specific patient survival and disease-free cancer recurrence was also assessed in ERα positive tumours. High cytoplasmic Y416 phosphorylated SFK expression prolongs the patient survival in tumours expressing low levels of nuclear SK1 **(Figure 3.7C)**. The mean patient survival time was 8.9 years for the patients with tumours expressing high cytoplasmic Y416 phosphorylated SFK and low nuclear SK1 compared to 7.8 years for the patients with tumours expressing low cytoplasmic Y416 phosphorylated SFK and low nuclear SK1 (P=0.023). Moreover, the nuclear expression of SK1 shortens the mean survival from 8.9 years to 6.3 years reducing the effect of Y416 phosphorylated SFK (P=0.001). In disease-free cancer recurrence, high nuclear Y416 phosphorylated SFK expression inhibits the SK1-mediated cancer recurrence **(Figure 3.8C)**. The mean cancer recurrence time for the patients with tumours expressing low levels of nuclear Y416 phosphorylated SFK and high levels of nuclear SK1 was 5.5 years compared to 7.1 years for the patients with tumours expressing low levels of nuclear Y416 phosphorylated SFK and low levels of nuclear SK1 (P=0.028). However, the mean cancer recurrence time for the patients with tumours expressing high levels of both nuclear Y416 phosphorylated SFK and nuclear SK1 was 6.1 years compared to 7.1 years for the patients with tumours expressing low levels of both nuclear Y416 phosphorylated SFK and nuclear SK1 (P=0.245), thus the expression of nuclear Y416 phosphorylated SFK inhibited the cancer recurrence mediated by SK1.

In disease-specific patient survival, high expression of phosphorylated LYN kinase in tumours expressing low nuclear SK1 was associated with reduced survival in ERα positive breast cancer patients **(Figure 3.7D)**. The mean patient survival time was 7.7 years for the patients expressing high cytoplasmic phosphorylated LYN and low nuclear SK1 expressing tumours compared to 8.6 years for the patients expressing low cytoplasmic phosphorylated LYN and low nuclear SK1 (P=0.030). High expression of nuclear SK1 in tumours expressing low levels of cytoplasmic phosphorylated LYN did not reduce the patient survival (P=0.184) however, the expression of both cytoplasmic phosphorylated LYN and nuclear SK1 reduced the patient survival to 6.4 years compared to 7.7 years for the patients with tumours expressing high cytoplasmic phosphorylated LYN and low nuclear SK1 (P=0.001). In disease-free cancer recurrence, the expression of both phosphorylated LYN

kinase and nuclear SK1 was associated with shorter cancer recurrence time **(Figure 3.8D)**. The mean cancer recurrence time was 5.3 years for the patients with tumours expressing high cytoplasmic phosphorylated LYN and high nuclear SK1 compared to 7.8 years for the patients expressing low cytoplasmic phosphorylated LYN and low nuclear SK1 (P=0.0001). The loss of expression of either the cytoplasmic phosphorylated LYN or the nuclear SK1 annulled the effects of LYN and SK1 on disease prognosis.

The effect of ERK-1/2 and phosphorylated AKT expression on SK1 associated disease-specific patient survival and disease-free cancer recurrence was assessed in ERα positive tumours. The patients with tumours expressing high levels of both nuclear SK1 and nuclear ERK-1/2 had the mean disease-specific survival of 3.9 years compared to 7.3 years for the patients with tumours expressing low levels of both nuclear SK1 and nuclear ERK-1/2 (P=0.0001) (**Figure 3.9A**). Moreover, the patients with tumours expressing high levels of nuclear SK1 and nuclear ERK-1/2 had the mean disease-free cancer recurrence time of 5.9 years compared to 8.2 years for the patients with tumours expressing low levels of both nuclear SK1 and nuclear ERK-1/2 (P=0.001) (**Figure 3.10A).** Moreover, the loss of expression of either the SK1 or the ERK-1/2 annulled the effects on shorter disease-specific patient survival and disease-free cancer recurrence. In case of phosphorylated AKT, the patients with tumours expressing high levels of nuclear SK1 and cytoplasmic phosphorylated AKT had the mean disease-specific survival time of 6.7 years compared to 8.7 years for the patients with tumours expressing low levels of nuclear SK1 and cytoplasmic phosphorylated AKT (P=0.003) (**Figure 3.9B**). In addition, the mean survival time for the patients with tumours expressing high cytoplasmic AKT and low nuclear SK1 was significantly shorter (P=0.046) compared to the patients with tumours expressing low levels of nuclear SK1 and cytoplasmic phosphorylated AKT, however additional expression of SK1 significantly reduced the mean patient survival time (**Figure 3.9B**). In disease-free cancer recurrence, the cancer recurrence time for the patients with tumours expressing high levels of nuclear SK1 and low levels of cytoplasmic phosphorylated AKT was 5.2 years compared to 7.7 years for the patients with tumours expressing low levels of both nuclear SK1 and cytoplasmic phosphorylated AKT (P=0.019) (**Figure 3.10B)**. In addition, high expression of cytoplasmic phosphorylated AKT in combination with nuclear SK1 did not further reduce the mean disease-free cancer recurrence time. However, the mean disease-free recurrence time for patients with tumours expressing high levels of both

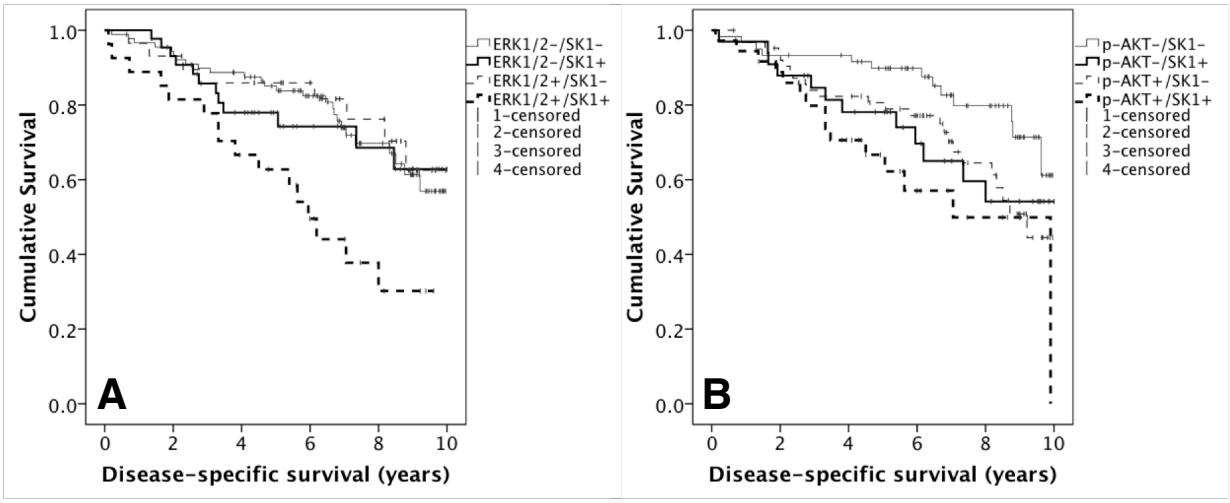

Figure 3.9. The Kaplan-Meier survival graphs showing the effect of nuclear ERK-1/2 or cytoplasmic phosphorylated AKT expression on SK1 associated disease-free cancer recurrence. The tumours expressing low levels of both proteins in each individual analysis were used as a control to calculate a P value for the other subgroups. **A** shows the effect of nuclear ERK-1/2 and nuclear SK1 expression on disease-specific survival (ERK-/SK1+: n=44, P=0.745; ERK+/SK1-: n=29, P=0.746; ERK+/SK1+: n=27, P=0.001); **B** shows the effect of cytoplasmic phosphorylated AKT and nuclear SK1 expression on disease-specific survival (p-AKT-/SK1+: n=33, P=0.091; p-AKT+/SK1-: n=64, P=0.046; p-AKT+/SK1+: n=36, P=0.003).

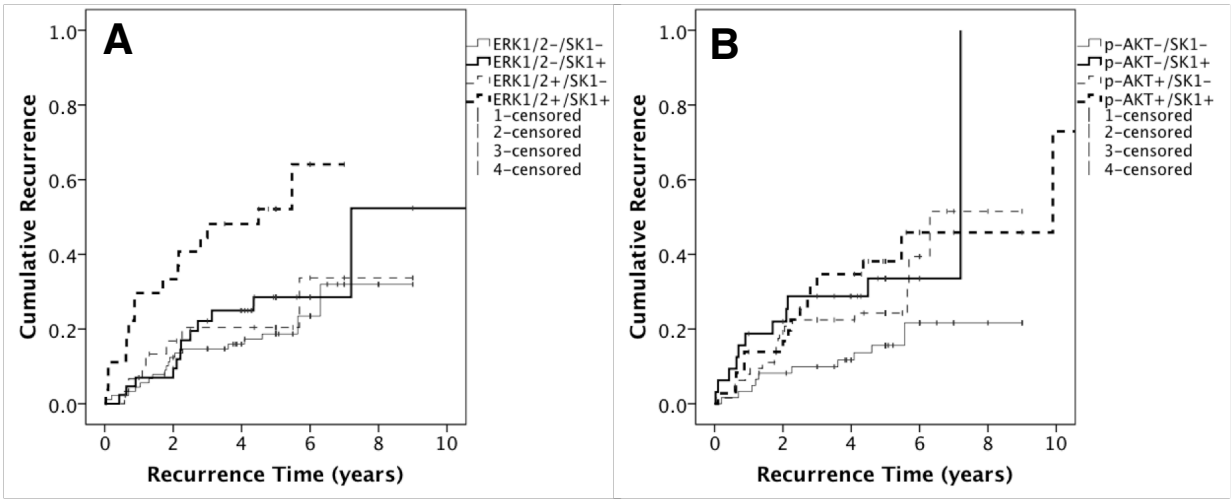

Figure 3.10. The Kaplan-Meier survival graphs showing the effect of nuclear ERK-1/2 or cytoplasmic phosphorylated AKT expression on SK1 associated disease-free cancer recurrence. The tumours expressing low levels of both proteins in each individual analysis were used as a control to calculate a P value for the other subgroups. **A** shows the effect of nuclear ERK-1/2 and nuclear SK1 expression on disease-free recurrence (ERK-/SK1+: n=43, P=0.286; ERK+/SK1-: n=30, P=0.710; ERK+/SK1+: n=27, P=0.0001); **B** shows the effect of cytoplasmic phosphorylated AKT and nuclear SK1 expression on disease-free recurrence (p-AKT-/SK1+: n=32, P=0.019; p-AKT+/SK1-: n=64, P=0.086; p-AKT+/SK1+: n=36, P=0.010).

nuclear SK1 and cytoplasmic phosphorylated AKT was 5.1 years compared to 7.7 years for the patients with tumours expressing low levels of both nuclear SK1 and cytoplasmic phosphorylated AKT (P=0.010) (**Figure 3.10B)**.

3.2.4. The effect of $S1P_1$ receptor expression on disease-specific survival and disease-free cancer recurrence in ERα positive breast tumours

Watson and coworkers have shown that the cytoplasmic $S1P_1$ receptor expression was associated with shorter disease-specific patient survival in ERα positive breast cancer patients (Watson *et al.,* 2010). The effect of expression of various signalling proteins such as SK1, c-RAF-1, ERK-1/2 and AKT and c-SRC on cancer prognosis and cancer recurrence in relation to the expression of $S1P_1$ receptor was assessed in ERα positive patients. In this analysis, the patients with tumours expressing low or high $S1P_1$ receptor was further classified into distinct groups according to the expression of signalling protein of interest.

The expression of phosphorylated c-RAF-1 (p-RAF) and $S1P_1$ receptor was associated with shorter disease-specific patient survival and disease-free cancer recurrence. The mean disease-specific survival time was 6.5 years for the patients with tumours expressing high levels of both plasma membrane $S1P_1$ receptor and cytoplasmic phosphorylated c-RAF-1 compared to 8.3 years for the patients with tumours expressing low level of both plasma membrane $S1P_1$ receptor and cytoplasmic phosphorylated c-RAF-1 (P=0.021) (**Figure 3.11A)**. The loss of expression of either the plasma membrane $S1P_1$ receptor or the cytoplasmic phosphorylated c-RAF-1 annulled the effect on disease-specific patient survival. Diseases-free cancer recurrence time was 6.8 years for the patients with tumours expressing low levels of both plasma membrane $S1P_1$ receptor and cytoplasmic phosphorylated c-RAF-1. This rate was reduced to 4.5 years in the patients with tumours expressing high levels of both plasma membrane $S1P_1$ receptor and cytoplasmic phosphorylated c-RAF-1 (P=0.003) (**Figure 3.12B).** The loss of expression of either the plasma membrane $S1P_1$ receptor or the cytoplasmic phosphorylated c-RAF-1 annulled the effects on disease-free cancer recurrence.

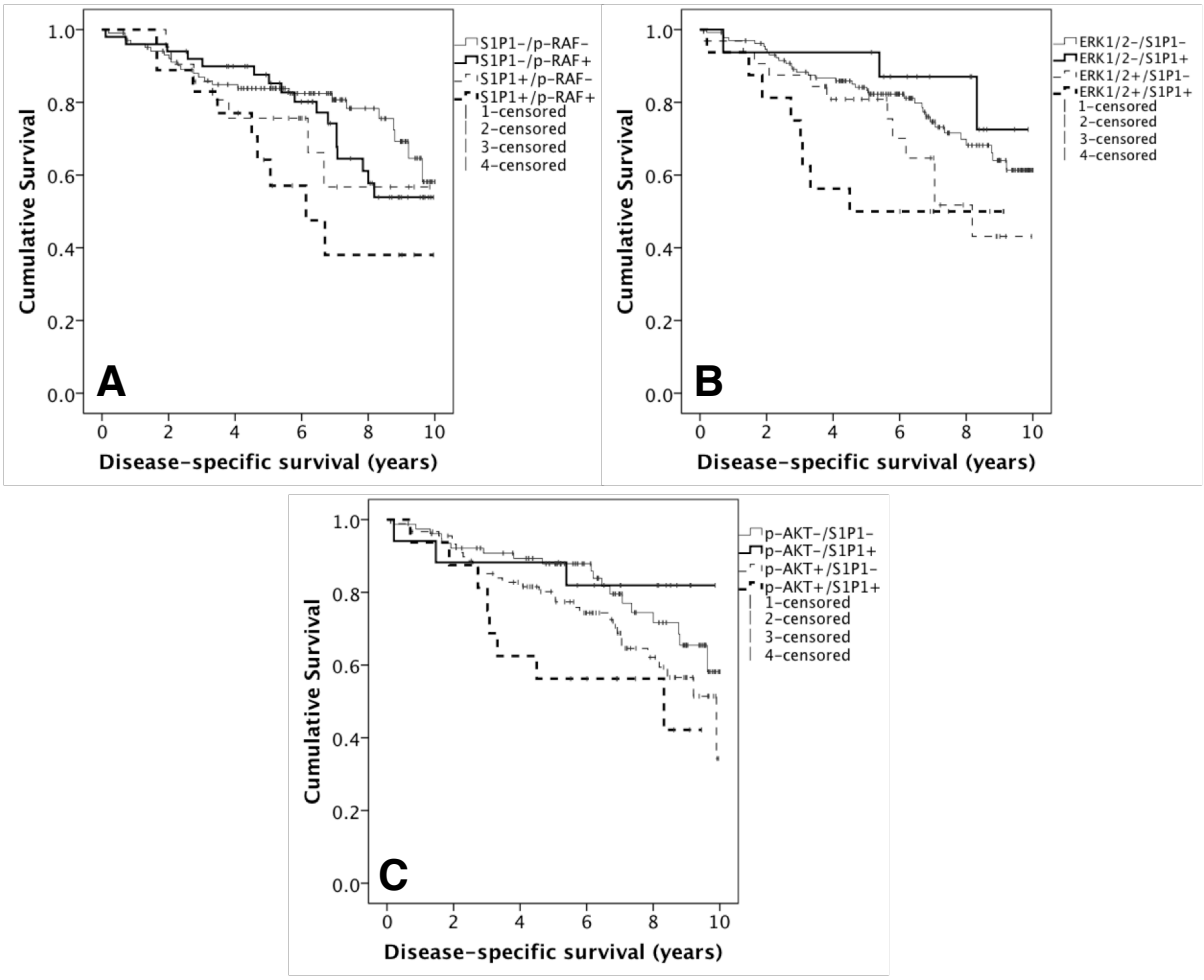

Figure 3.11. The Kaplan-Meier survival graphs showing the effect of P-RAF-1, ERK-1/2, P-AKT expression on $S1P_1$ receptor associated disease-specific patient survival. The tumours expressing low levels of both proteins in each individual analysis were used as a control to calculate a P value for the other subgroups. **A** shows the effect of cytoplasmic phosphorylated RAF-1 and plasma membrane $S1P_1$ receptor expression on disease-specific survival ($S1P_1$-/p-RAF+: n=50, P=0.338; $S1P_1$ +/p-RAF-: n=21, P=0.310; $S1P_1$ +/p-RAF+: n=18, P=0.021); **B** shows the effect of cytoplasmic ERK-1/2 and cytoplasmic $S1P_1$ receptor expression on disease-specific survival (ERK-/$S1P_1$ +: n=16, P=0.436; ERK+/$S1P_1$-: n=32, P=0.127; ERK+/ $S1P_1$ +: n=16, P=0.025); **C** shows the effect of cytoplasmic phosphorylated AKT kinase and cytoplasmic $S1P_1$ receptor on disease-specific survival (p-AKT-/$S1P_1$ +: n=17, P=0.565; p-AKT+/$S1P_1$-: n=91, P=0.126; p-AKT+/$S1P_1$ +: n=16, P=0.022).

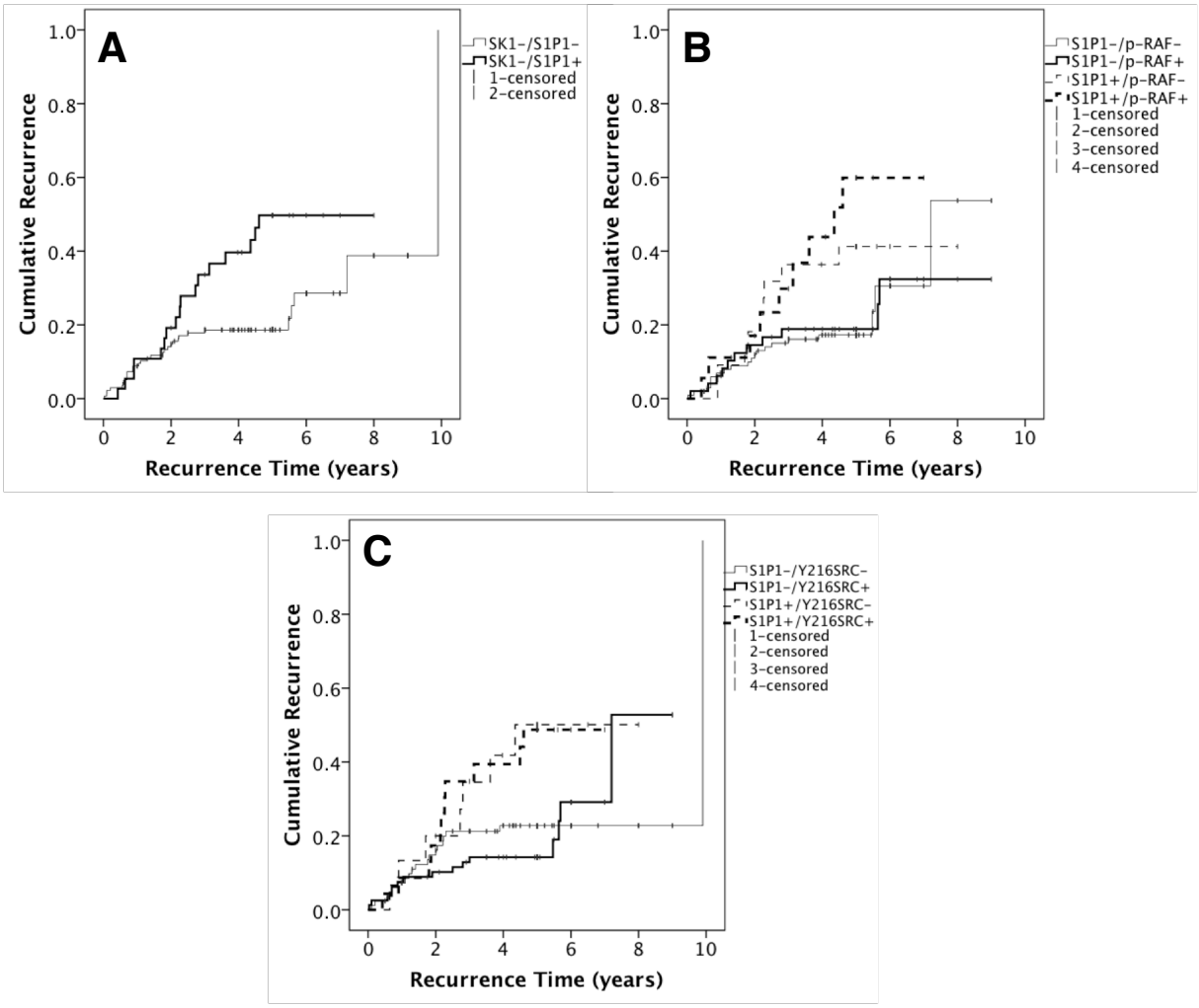

Figure 3.12. The Kaplan-Meier survival graphs showing the effect of SK1, P-RAF-1, Y216SRC expression on $S1P_1$ receptor associated disease-free cancer recurrence. The tumours expressing low levels of both proteins in each individual analysis were used as a control to calculate a P value for the other subgroups. **A** shows the effect of membrane SK1 and plasma membrane $S1P_1$ receptor expression on disease-free cancer recurrence (SK1-/$S1P_1$-: n=137, SK1-/$S1P_1$+: n=37; P=0.004); **B** shows the effect of cytoplasmic phosphorylated RAF-1 and plasma membrane $S1P_1$ receptor expression on disease-free cancer recurrence ($S1P_1$-/p-RAF+: n=49, *P*=0.948; $S1P_1$+/p-RAF-: n=22, *P*=0.060; $S1P_1$+/p-RAF+: n=18, *P*=0.003); **C** shows the effect of cytoplasmic Y216 phosphorylated c-SRC and plasma membrane $S1P_1$ receptor expression on disease-free cancer recurrence ($S1P_1$-/Y216SRC+: n=79, *P*=0.540; $S1P_1$+/ Y216SRC-: n=15, *P*=0.070; $S1P_1$+/Y216SRC+: n=23, *P*=0.037).

The effect of ERK-1/2 and AKT expression on $S1P_1$ receptor associated shorter disease-specific patient survival was also analysed. High expression of cytoplasmic ERK-1/2 and cytoplasmic $S1P_1$ receptor in tumours reduced the mean disease-specific patient survival to 5.8 years from 8.2 years for the patients with tumours expressing low levels of both cytoplasmic $S1P_1$ receptor and cytoplasmic ERK-1/2 (P=0.025) (**Figure 3.11B)**. The expression of both cytoplasmic ERK-1/2 and cytoplasmic $S1P_1$ receptor was required for shorter survival time as the loss of expression of either one of these proteins annulled the effects on disease-specific survival. Moreover, high expression of both cytoplasmic $S1P_1$ receptor and cytoplasmic phosphorylated AKT reduced the mean disease-specific patient survival time to 6.4 years from 8.4 years for the patients with tumours expressing low levels of both cytoplasmic $S1P_1$ receptor and cytoplasmic phosphorylated AKT (P=0.022) (**Figure 3.11C)**. The loss of expression of either the cytoplasmic $S1P_1$ receptor or the cytoplasmic phosphorylated AKT annulled the effect on disease-specific patient survival.

Watson and coworkers have also shown that high expression of plasma membrane $S1P_1$ receptor was associated with shorter disease-free cancer recurrence (Watson *et al.,* 2010). The effect of $S1P_1$ receptor on disease-free cancer recurrence was independent of SK1 expression in ERα positive patients (**Figure 3.12A**). High expression of $S1P_1$ receptor in tumours reduced the mean cancer recurrence time to 5.3 years from 7.6 years for the patients with tumours expressing low levels of $S1P_1$ receptor (P=0.004). Subsequently, the effect of phosphorylated RAF-1 or Y216 phosphorylated c-SRC expression on the ability of $S1P_1$ receptor to affect the disease-free cancer recurrence was analysed. High expression of both cytoplasmic phosphorylated RAF-1 and plasma membrane $S1P_1$ receptor in tumours reduced the mean cancer recurrence time to 4.5 years form 6.8 years for the patients with tumours expressing low levels of both cytoplasmic phosphorylated RAF-1 and plasma membrane-bound $S1P_1$ receptor (P=0.003) (**Figure 3.12B**). The expression of Y216 phosphorylated c-SRC was also required for the effect of $S1P_1$ receptor on cancer recurrence. High expression of both cytoplasmic Y216 phosphorylated c-SRC and plasma membrane-bound $S1P_1$ receptor in tumours reduced the mean cancer recurrence time to 4.8 years from 7.9 years for the patients with tumours expressing low levels of both cytoplasmic Y216 phosphorylated c-SRC and plasma membrane-bound $S1P_1$ receptor (P=0.037) (**Figure 3.12C**). The loss of expression of either the cytoplasmic phosphorylated

RAF-1 or the cytoplasmic Y216 phosphorylated c-SRC annulled the effect of $S1P_1$ receptor on cancer recurrence.

3.2.5. The effect of $S1P_2$ receptor expression on disease-specific patient survival and disease-free cancer recurrence in ERα positive breast tumours

Watson and coworkers have previously reported that the $S1P_2$ receptor expression was not associated with either disease-specific patient survival or disease-free cancer recurrence in ERα positive patients (Watson *et al.,* 2010). In the current analysis, the effect of $S1P_2$ receptor expression on the ability of SK1 to affect disease-specific patient survival was analysed (**Figure 3.13A**). The patients with tumours expressing low levels of plasma membrane $S1P_2$ receptor and high levels of nuclear SK1 had the mean disease-specific survival time of 6.7 years (P=0.011). Moreover, the expression of both nuclear SK1 and $S1P_2$ receptor at the plasma membrane prolonged the mean patient survival time to 7.7 years thus annulling the effect of SK1 on disease prognosis in ERα positive patients (P=0.477).

In addition to ameliorated prognosis mediated by the $S1P_2$ receptor, the impact of $S1P_2$ receptor expression on the expression of other proteins was analysed. High expression levels of $S1P_2$ receptor in the nucleus was correlated with low expression levels of SK1 in the nucleus (P=0.002) (**Figure 3.14A**). In contrast, the expression of SK1 in the nucleus was not correlated with the expression of $S1P_2$ receptor in the nucleus (P=0.922) (**Figure 3.14B**). High expression levels of $S1P_2$ receptor in the nucleus was also correlated with high expression levels of $S1P_1$ receptor in the nucleus (P=0.0001) (**Figure 3.14C**). However, the expression levels of $S1P_3$ receptor was not effected (P=0.080) (**Figure 3.14D**). In contrast, the expression of SK1 in the nucleus was not correlated with the $S1P_1$ receptor expression in the nucleus (P=0.602) (**Figure 3.15A**). However, the expression of $S1P_3$ receptor was significantly increased (P=0.024) (**Figure 3.15B**).

In addition, high expression of both nuclear $S1P_2$ receptor and c-SRC in tumours improved the disease prognosis (**Figure 3.13B**). The expression of nuclear $S1P_2$ receptor and nuclear c-SRC prolonged the mean disease-specific patient survival time from 5.3 to 6.5 years in ERα positive breast cancer patients (P=0.0038). Moreover, the loss of expression of either

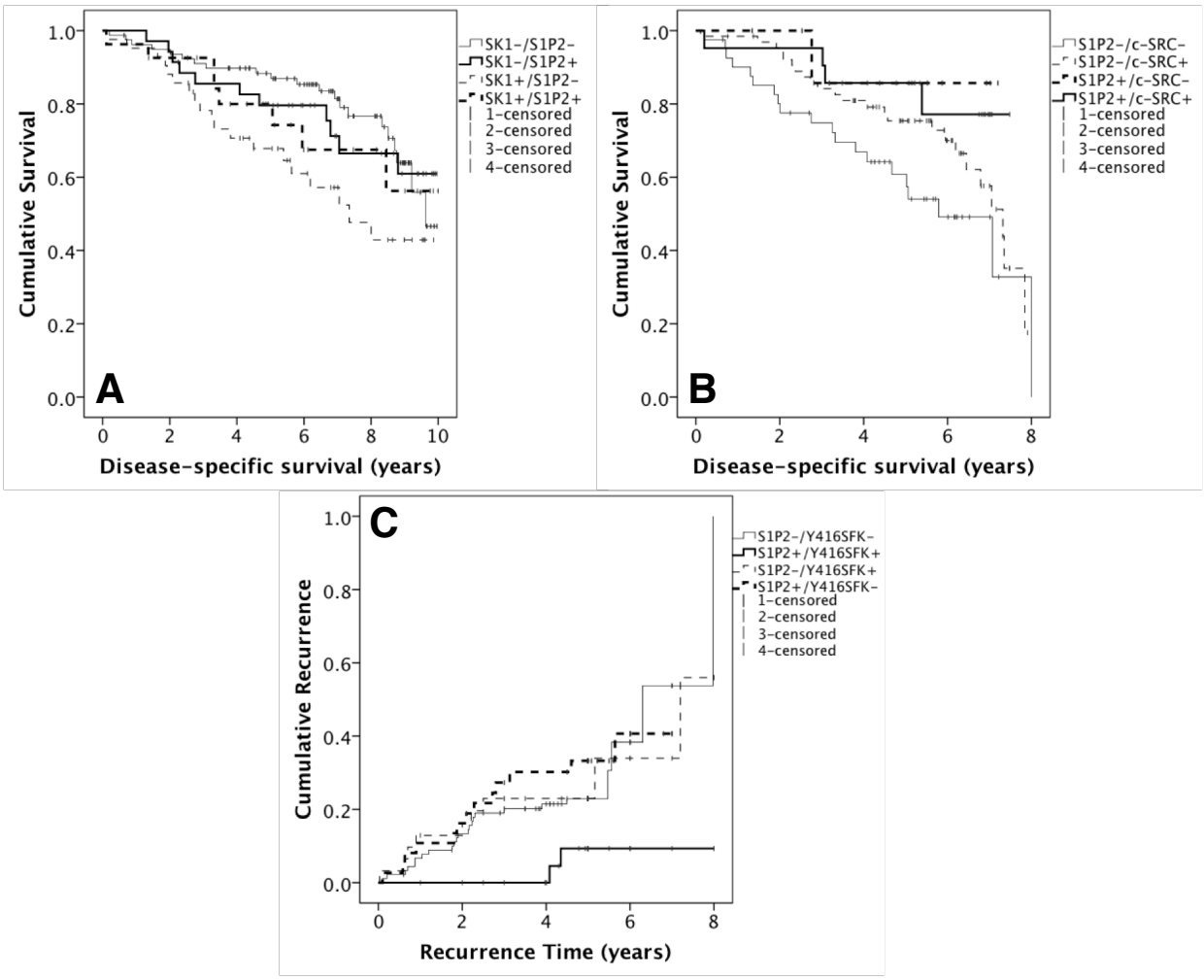

Figure 3.13. The Kaplan-Meier survival graph showing the impact of nuclear Y416 phosphorylated SFK, nuclear c-SRC and nuclear SK1 expression in combination with the $S1P_2$ receptor expression on disease-specific patient survival and disease-free cancer recurrence. The tumours expressing low levels of both plasma membrane/nuclear $S1P_2$ and nuclear Y416 phosphorylated SFK/c-SRC/SK1 were used as a control to calculate a P value for the other subgroups. **A** shows the effect of plasma membrane $S1P_2$ receptor and nuclear SK1 expression on disease-specific patient survival (SK1-/$S1P_2$+: n=35, P=0.825; SK1+/ $S1P_2$-: n=42, P=0.011; SK1+/$S1P_2$+: n=27, P=0.477); **B** shows the effect of nuclear c-SRC and nuclear $S1P_2$ receptor expression on disease-specific patient survival ($S1P_2$-/c-SRC+: n=65, P=0.099; $S1P_2$+/c-SRC-: n=9, P=0.099; $S1P_2$+/c-SRC+: n=21, P=0.038); **C** shows the effect of nuclear Y416 phosphorylated SFK and membrane $S1P_2$ receptor expression on disease-free cancer recurrence ($S1P_2$-/Y416SFK+: n=31, P=0.098; $S1P_2$+/Y416SFK-: n=37, P=0.472; $S1P_2$ +/Y416SFK+: n=29, P=0.013).

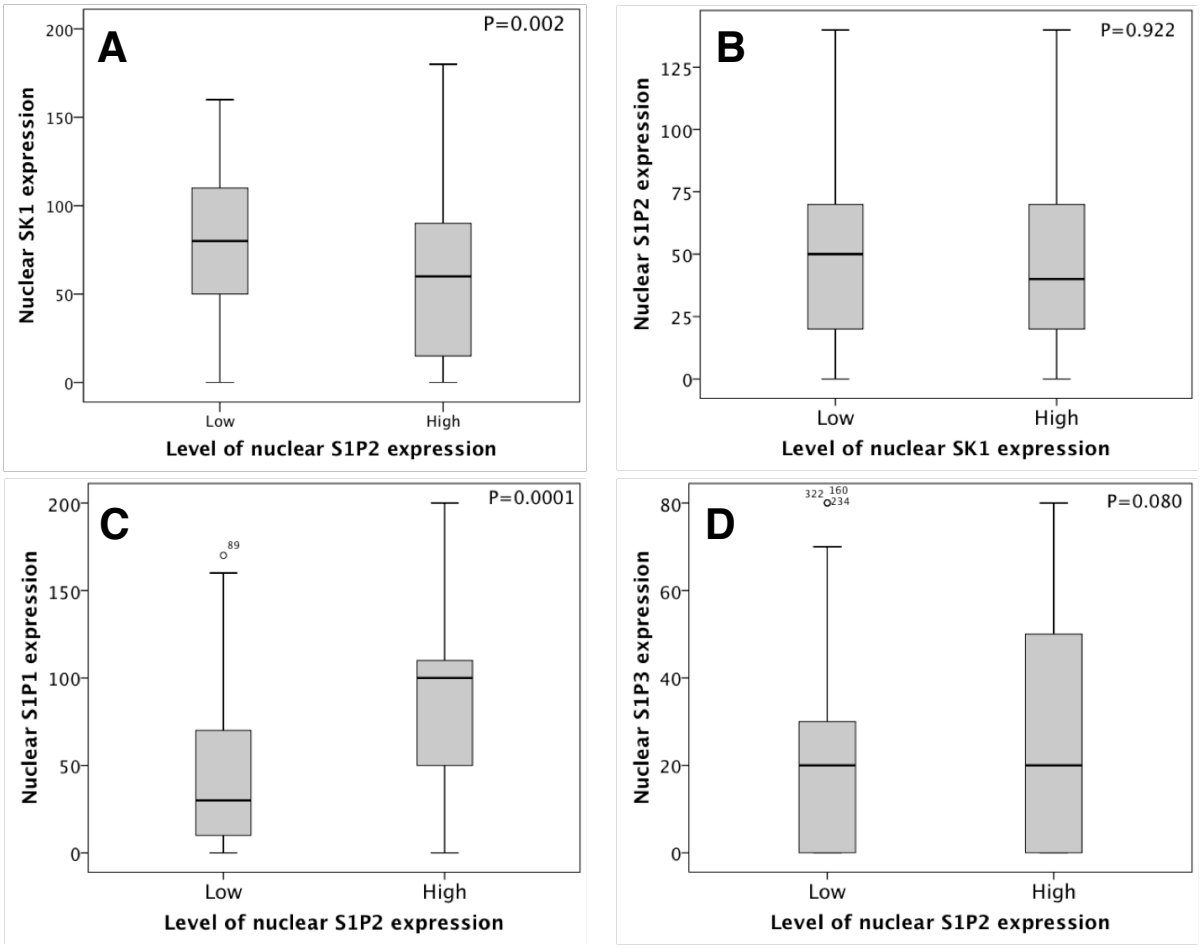

Figure 3.14. The box plots showing the expression of SK1 and $S1P_{1-3}$ receptors in tumours expressing low or high levels of SK1 or $S1P_2$ receptor in ERα positive tumours. **A** shows the expression of nuclear SK1 in tumours expressing low or high levels of nuclear $S1P_2$ receptor; **B** shows the expression of nuclear $S1P_2$ receptor in tumours expressing low or high levels of nuclear SK1; **C** shows the expression of nuclear $S1P_1$ receptor in tumours expressing low or high levels of nuclear $S1P_2$ receptor; **D** shows the expression of nuclear $S1P_3$ receptor in tumours expressing low or high levels of nuclear $S1P_2$ receptor.

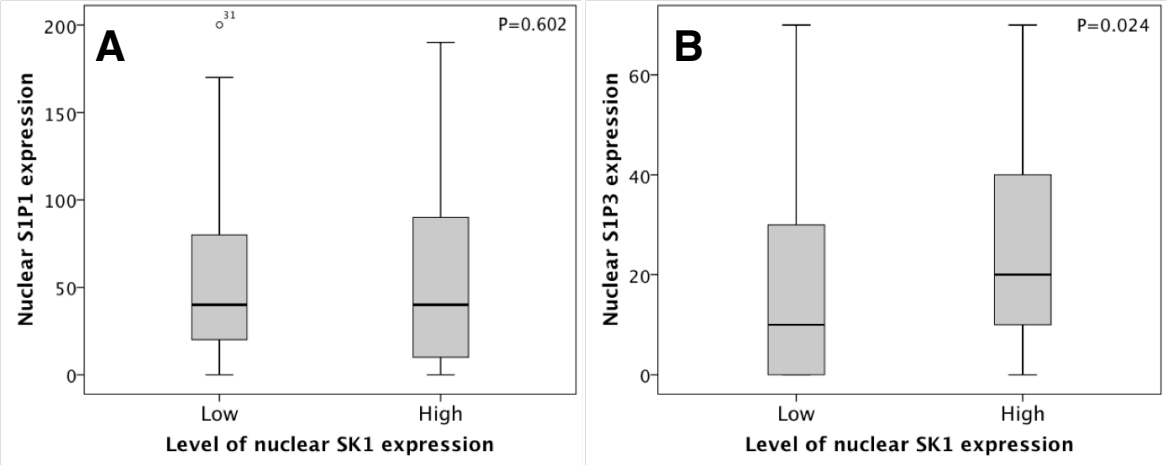

Figure 3.15. The box plots showing the expression of $S1P_1$ and $S1P_3$ receptors in tumours expressing low or high levels of SK1. **A** shows the expression of nuclear $S1P_1$ receptor in tumours expressing low or high levels of nuclear SK1; **B** shows the expression of nuclear $S1P_3$ receptor in tumours expressing low or high levels of nuclear SK1.

the $S1P_2$ receptor or the c-SRC annulled the effects on patient survival. In support of these findings, high expression of $S1P_2$ receptor in the nucleus was associated with increased levels of c-SRC expression in the nucleus (P=0.0001) (**Figure 3.16A**). Similarly, high expression of c-SRC in the nucleus was associated with increased levels of $S1P_2$ receptor expression in the nucleus (P=0.001) (**Figure 3.16B**). In contrast, the expression of c-SRC was not associated with SK1 expression (P=0.877, P=0.978) (**Figure 3.16C, Figure 3.16D**).

The expression of membrane $S1P_2$ receptor and nuclear Y416 phosphorylated SFK also prolonged the mean disease-free cancer recurrence time from 5.9 years to 7.6 years in ERα positive breast cancer patients (P=0.013) (**Figure 3.13C**). Moreover, the loss of expression of either the $S1P_2$ receptor or the Y416 phosphorylated SFK annulled the effects on disease-free cancer recurrence. Further analysis revealed that the expression of $S1P_2$ receptor was not correlated with the expression of Y416 phosphorylated SFK in ERα positive breast cancer patients (**Data not shown)**. However, the expression of both $S1P_2$ receptor and Y416 phosphorylated SFK in the nucleus was associated with higher apoptotic index in ERα positive breast cancer patients (**Figure 3.17A, Figure 3.17B**) (P=0.023, P=0.001).

3.2.6. The effect of $S1P_3$ receptor on disease-specific patient survival and disease-free cancer recurrence in ERα positive breast tumours

Watson and coworkers have shown that the $S1P_3$ receptor expression was associated with disease-specific patient survival in ERα positive breast cancer patients (Watson *et al.,* 2010). In the current analysis, the effect of expression of SK1, LYN and RAF-1 and $S1P_3$ receptor on disease-specific patient survival and disease-free cancer recurrence was analysed. High expression of both nuclear $S1P_3$ receptor and nuclear SK1 was associated with shorter disease-free cancer recurrence (**Figure 3.18A**). The expression of nuclear $S1P_3$ receptor and nuclear SK1 reduced the mean cancer recurrence time to 4.6 years from 6.9 years for the patients with tumours expressing low levels of both nuclear $S1P_3$ receptor and nuclear SK1 (P=0.026). The loss of expression of either the nuclear $S1P_3$ receptor or the nuclear SK1 annulled the effects on cancer recurrence.

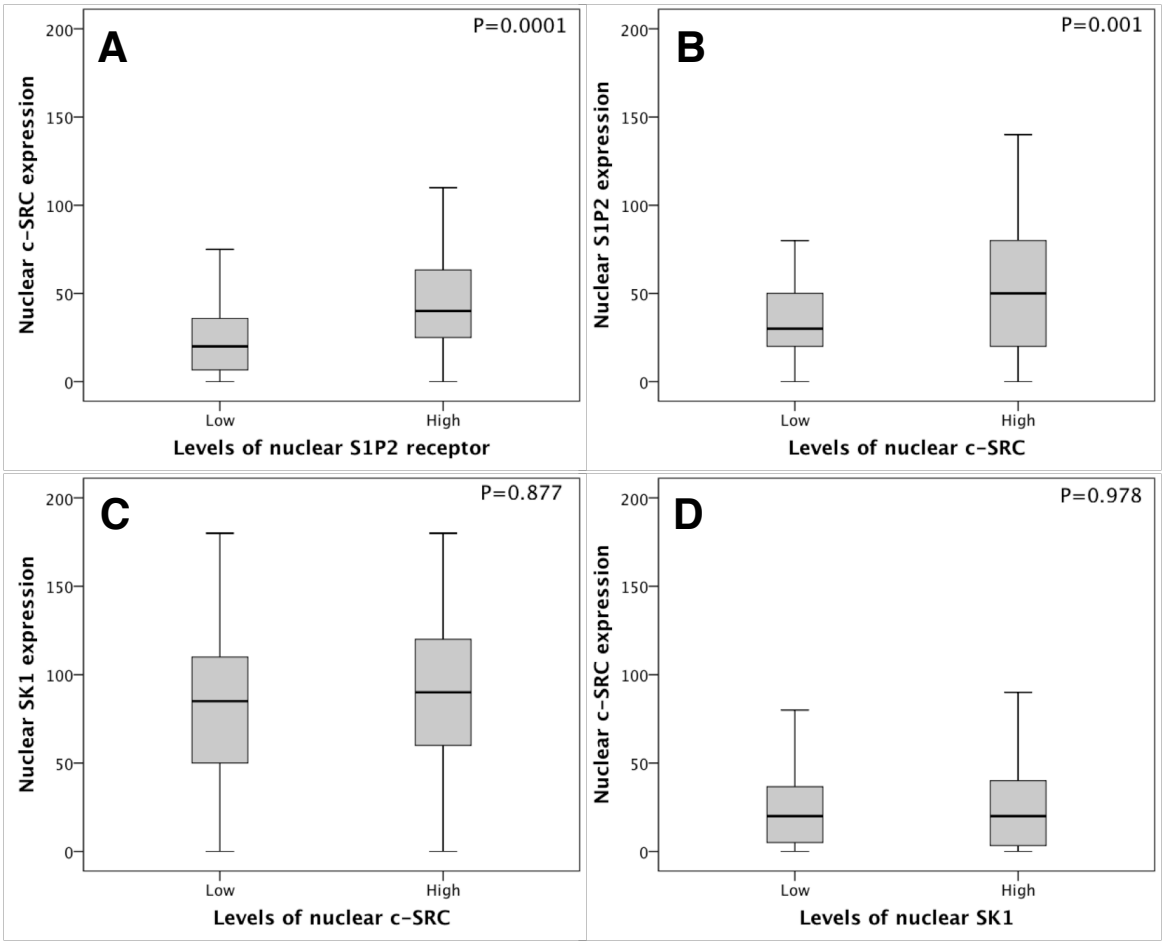

Figure 3.16 The box plots showing the expression of c-SRC, $S1P_2$ and SK1 in tumours expressing low or high levels of $S1P_2$/c-SRC/SK1. **A** shows the expression of nuclear c-SRC in tumours expressing low or high levels of nuclear $S1P_2$ receptor; **B** shows the expression of nuclear $S1P_2$ receptor in tumours expressing low or high levels of nuclear c-SRC; **C** shows the expression of nuclear SK1 in tumours expressing low or high levels of nuclear c-SRC; **D** shows the expression of nuclear c-SRC in tumours expressing low or high levels of nuclear SK1.

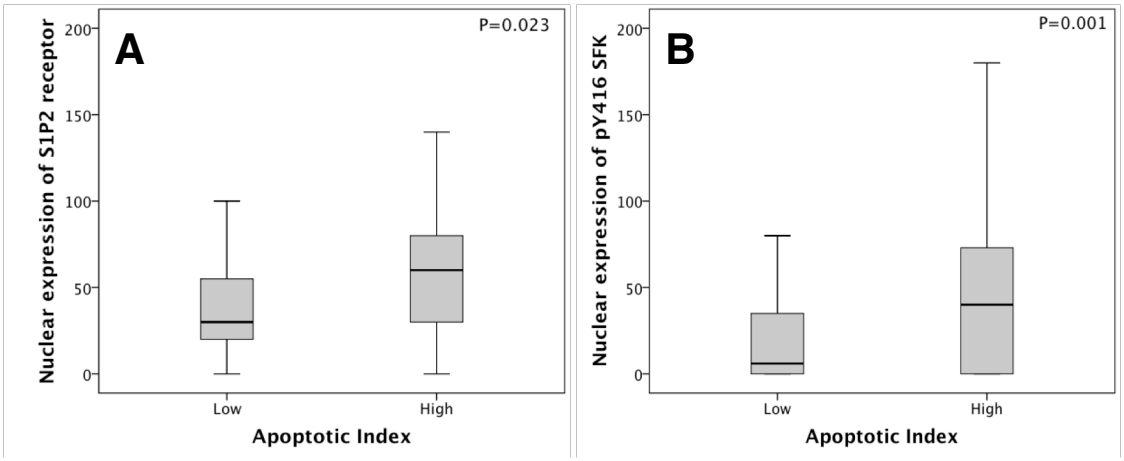

Figure 3.17. The expression of $S1P_2$ and pY416 SFK in low or high levels of apoptotic index. **A** shows the expression of nuclear $S1P_2$ receptor in low or high levels of apoptotic index; **B** shows the expression of nuclear pY416 SFK in low or high levels of apoptotic index.

The expression of LYN kinase and $S1P_3$ receptor was associated with shorter disease-free cancer recurrence (**Figure 3.18B**). The expression of both cytoplasmic $S1P_3$ receptor and cytoplasmic LYN reduced the mean cancer recurrence time to 5.1 years from 6.8 years for the patients with tumours expressing low levels of both cytoplasmic $S1P_3$ receptor and cytoplasmic LYN (P=0.015). High expression of either cytoplasmic $S1P_3$ receptor or cytoplasmic LYN was not associated with disease-free cancer recurrence.

The $S1P_3$ receptor associated shorter disease-specific patient survival was dependent on the expression of LYN kinase and phosphorylated RAF-1 (**Figure 3.18C, Figure 3.18D**). The patients with tumours expressing high levels of both cytoplasmic $S1P_3$ receptor and cytoplasmic LYN had the mean survival time of 7.2 years compared to 8.6 years for the patients with tumours expressing low levels of both cytoplasmic LYN and cytoplasmic SK1 (P=0.005). The loss of expression of either the cytoplasmic $S1P_3$ receptor or the cytoplasmic LYN annulled the effects on disease-specific survival. Similarly, the patients expressing high levels of both nuclear $S1P_3$ receptor and nuclear phosphorylated RAF-1 had the mean survival time of 6.8 years compared to 8.1 years for the patients with tumours expressing low levels of both nuclear $S1P_3$ receptor and nuclear phosphorylated c-RAF-1 (P=0.043). Moreover, the loss of expression of either the nuclear $S1P_3$ receptor or the nuclear phosphorylated c-RAF-1 annulled the effects on disease-specific patient survival.

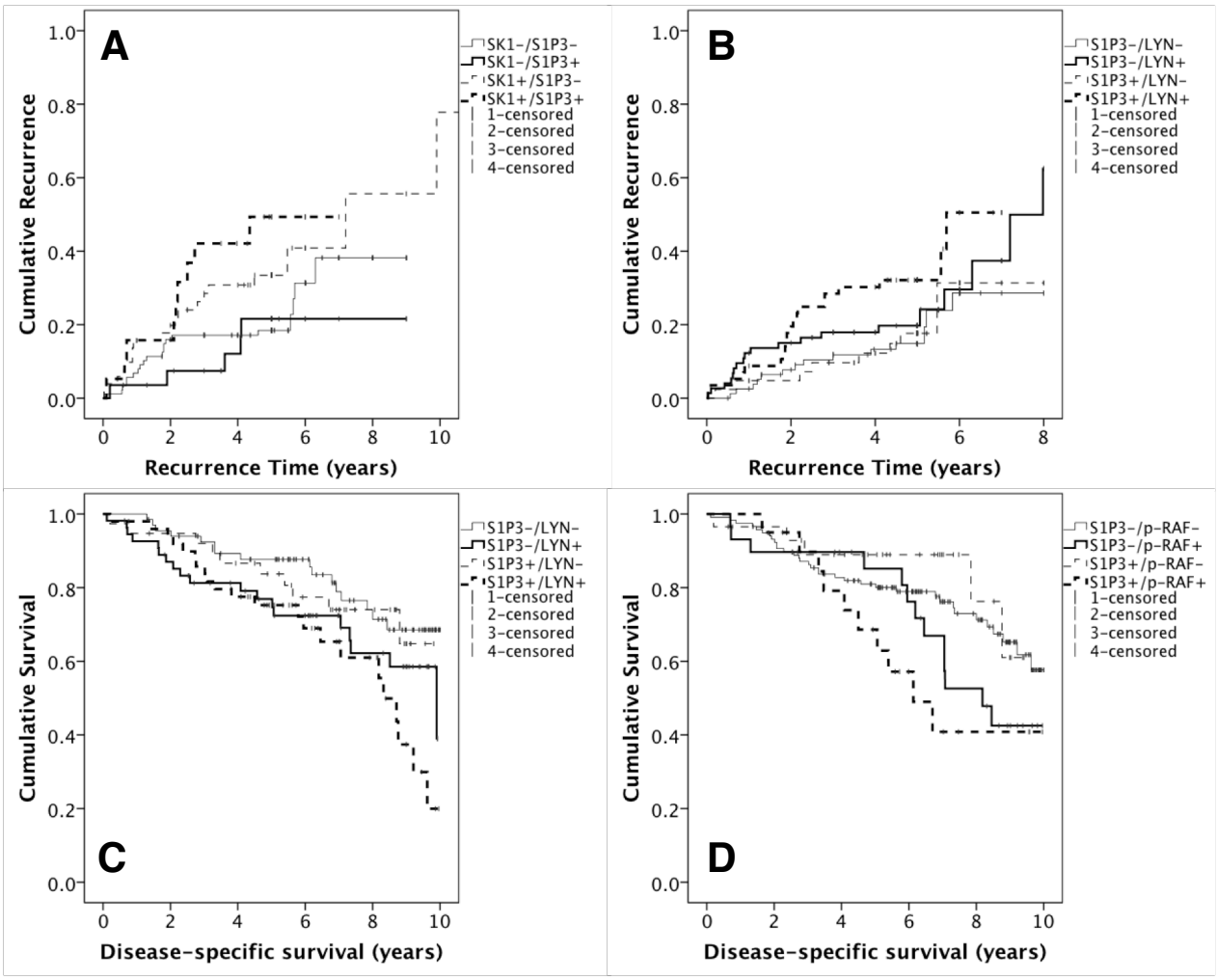

Figure 3.18. The Kaplan-Meier survival graphs showing the effect of expression of SK1, LYN, phosphorylated RAF-1 on cytoplasmic/nuclear $S1P_3$ receptor associated disease-specific patient survival and disease-free cancer recurrence. The tumours expressing low levels of both cytoplasmic $S1P_3$ receptor and cytoplasmic LYN/nuclear phosphorylated RAF-1 were used as controls to calculate a P value for the rest of the subgroups. **A** shows the effect of nuclear SK1 and nuclear $S1P_3$ receptor expression on disease-free cancer recurrence (SK1-/$S1P_3$+: n=28, P=0.672; SK1+/$S1P_3$-: n=51, P=0.078; SK1+/$S1P_3$+: n=19, P=0.026); **B** shows the effect of cytoplasmic LYN and cytoplasmic $S1P_3$ receptor expression on disease-free cancer recurrence ($S1P_3$-/LYN+: n=74, P=0.257; $S1P_3$+/LYN-: n=42, P=0.807; $S1P_3$+/LYN+: n=57, P=0.015); **C** shows the effect of cytoplasmic LYN and cytoplasmic $S1P_3$ receptor expression on disease-specific patient survival ($S1P_3$-/LYN+: n=55, P=0.118; $S1P_3$+/LYN-: n=38, P=0.710; $S1P_3$ +/ LYN+: n=50, P=0.005); **D** shows the effect of nuclear phosphorylated RAF-1 and nuclear $S1P_3$ receptor expression on disease-specific patient survival ($S1P_3$-/p-RAF+: n=29, P=0.154; $S1P_3$ +/p-RAF-: n=29, P=0.466; $S1P_3$+/p-RAF+: n=20, P=0.043).

3.3. Discussion

High expression of SK1, $S1P_1$ and $S1P_3$ receptors in ERα positive tumours was shown to reduce the patient survival (Watson *et al.,* 2010). In this chapter, the effects of expression of proteins involved in sphingolipid signalling on patient survival and cancer recurrence was assessed through systematic classification of tumours regarding to the expression of NFκB (p50 RelA), c-RAF-1, ERK-1/2, AKT, SRC family kinases (SFKs) and LYN kinase. This methodology has allowed the determination of novel functional interactions or associations between diverse signalling pathways that leads to poor disease prognosis in breast cancer patients.

3.3.1. The expression of $S1P_1$, $S1P_3$, NFkB, RAF-1 and SRC family kinases are required for SK1 associated shorter disease-specific patient survival in ERα positive breast tumours

The effect of SK1 on shorter disease-specific patient survival was dependent on the expression of $S1P_1$ and/or $S1P_3$ receptors. Indeed, the loss of expression of either the $S1P_1$ receptor or the $S1P_3$ receptor annuls the correlation between SK1 expression and patient survival. In support of these data, SK1 was shown to activate mitogenic (e.g. ERK-1/2) signalling pathways through the S1P receptors upon VEGF/TGFβ stimulation in various cancer cell lines (Miller *et al.,* 2008, Wu *et al.*, 2003). Thus, the expression of S1P receptors might be crucial for the SK1-driven cancer progression in ERα positive breast cancer tumours.

The effect of SK1 on disease prognosis was also dependent on its nuclear localisation. Pitson and coworkers have reported that the ERK-1/2-mediated activation of SK1 induced the translocation of SK1 from cytoplasm to the plasma membrane (Pitson *et al.,* 2003). Indeed, simultaneous analysis of cytoplasmic phosphorylated c-RAF-1 and nuclear ERK-1/2 expression with the nuclear SK1 expression has shown that the effect of SK1 on shorter disease-specific patient survival and disease-free cancer recurrence was dependent on the expression of both c-RAF-1 and ERK-1/2. In support of these data, phosphorylated ERK-1/2 was shown to accumulate into the nucleus upon S1P stimulation in MCF7 breast cancer cells (Long *et al.,* 2010a). Thus, c-RAF-1/ERK-1/2 signalling might be involved in

nuclear translocation of SK1 that drives the disease progression in breast cancer patients. The role of $S1P_1$ and $S1P_3$ receptors in breast cancer prognosis will be discussed later in this chapter.

The effect of SK1 on shorter disease-specific survival and cancer recurrence was enhanced in tumours expressing LYN, Y416 phosphorylated SFK and AKT kinases in ERα positive breast cancer patients. In support of these data, SK1 was shown to interact with these proteins *in vitro*. For example, the mast cell activation upon antigen (IgE) binding to high-affinity receptor for immunoglobulin E (FcεRI) was shown to stimulate the formation of complex between LYN kinase and SK1 that activates SK1 at the plasma membrane (Olivera *et al.,* 2005, Urtz *et al.,* 2004). Subsequently, the S1P transported to extracellular milieu acts in autocrine manner to stimulate the chemotaxis towards antigen via an $S1P_1$ receptor-dependent mechanism (Jolly *et al.,* 2004). In cancer cells, LYN kinase activation was shown to promote cell proliferation and chemotherapeutic resistance in prostate and colon cancer cell lines respectively (Goldenberg-Furmanov *et al.,* 2004, Bates *et al.,* 2001). Moreover, LYN kinase expression was also associated with poor prognosis in breast cancer patients (Choi *et al.,* 2010). Thus, the interaction between SK1 and LYN kinase might be crucial for the survival and the development of chemotherapeutic resistance in breast cancer cells.

The expression of Y416 phosphorylated SRC family kinases (SFK) and AKT kinases were also required for the effect of SK1 on disease-specific patient survival and disease-free cancer recurrence. The interaction between c-SRC and PI3K/AKT signalling pathways is well documented in various studies (Liu *et al.,* 1998, Datta *et al.,* 1996). In addition, SK1 was shown to activate the PI3K/AKT signalling pathway. Indeed, Guan and coworkers have shown that the survival of glioma cells was dependent on SK1-induced activation of the PI3K/AKT/FOXO3a/Bim pathway (Guan *et al.,* 2011). Moreover, SK1 was shown to mediate the survival of non-small cell lung cancer cells through activation of the PI3K/AKT/NF-κB pathway (Song *et al.,* 2011). The SK1-mediated PI3K/AKT activation might also involve the interaction with LYN kinase. In support of these findings, various studies have shown that the activation of PI3K/AKT pathway was mediated by LYN kinase in cancer cell lines (Iqbal *et al.,* 2010, Bates *et al.,* 2001). Thus, the signalling between SK1,

LYN and Y416 phosphorylated SRC family kinases (SFK)/AKT kinases might be crucial for the survival of ERα positive breast cancer tumours.

The effect of SK1 on shorter disease-specific patient survival and cancer recurrence was also enhanced in tumours expressing NFκB (p50 RelA) in ERα positive breast cancer tumours. NFκB family members are transcription factors that control the expression of anti-apoptotic proteins such as p53, Bcl-2, Bcl-xL and JNK (Shen *et al.,* 2009). SK1 was shown to mediate the TNF-α-induced IκBα-degradation that activates p65 RelA in A549 human epithelial lung carcinoma cells (Billich *et al.,* 2005). Indeed, SK1 was shown to interact with tumour necrosis factor receptor-associated factors (TRAF2), an E3 ubiquitin-protein ligase that induces the IκBα-degradation in HEK 293T cells (Xia *et al.,* 2002). Moreover, the over-expression of SK1 was shown to induce chemotherapeutic resistance through activation of the PI3K/AKT/NFκB pathway in non-small cell lung cancer cells (Song *et al.,* 2011). Thus, SK1 might interact with the NFκB signalling pathway at multiple levels to mediate cancer cell survival.

3.3.3. The expression of RAF-1, ERK-1/2, AKT and SRC kinases are required for $S1P_1$ associated shorter disease-specific patient survival and disease-free cancer recurrence in ERα positive breast tumours

The association between high tumour expression of $S1P_1$ receptor and breast cancer prognosis was reported previously (Watson *et al.,* 2010). Indeed, the effect of $S1P_1$ receptor on disease-specific patient survival and disease-free cancer recurrence was dependent on the expression of Y216 phosphorylated SRC, c-RAF-1, ERK-1/2 and AKT kinases in ERα positive breast cancer patients. The S1P receptors were shown to activate diverse array of signalling pathways through interaction with small G proteins. The $S1P_1$ receptor coupled to G_i protein was shown to activate the c-RAS/c-RAF-1/ERK1/2 signalling pathway (Khleif *et al.,* 1999, Okamoto *et al.,* 1998). Moreover, the c-RAS-mediated activation of c-RAF-1 requires the activation of c-SRC that in turn activates the AKT signalling pathway (Rexer *et al.,* 2009, Tran *et al.,* 2003, Bakin *et al.,* 2003). In support of these findings, the effects of $S1P_1$ receptor on patient survival was annulled upon loss of expression of these kinases suggesting that the $S1P_1$ receptor might mediate

the cell survival and proliferation through activation of c-RAS and c-SRC in ERα positive breast cancer tumours.

3.3.4. The expression of $S1P_2$ receptor was associated with longer disease-specific patient survival and disease-free cancer recurrence in ERα positive breast tumours

In contrast to $S1P_1$ and $S1P_3$ receptors, the high tumour expression of $S1P_2$ receptor is associated with prolonged patient survival in ERα positive breast cancer tumours. Moreover, the expression of c-SRC and Y416 phosphorylated SFK was required for longer disease-specific and recurrence-free survival respectively. The nuclear localisation of these proteins might be crucial as neither plasma membrane-bound nor cytoplasmic localisation have prolonged the patient survival. Supportingly, various research groups have shown that GPCRs such as $S1P_1$ and LPA_1 were localised into the nucleus (Liao *et al.,* 2007, Gobeil *et al.,* 2006).

The SRC family kinases are involved in numerous cellular processes such as cell proliferation, survival and migration (Martin *et al.,* 2001). Indeed, c-SRC is a proto-oncogene up-regulated in breast, colon, pancreatic, leukemia and lymphoma cancer cells (Egan *et al.,* 1999, Lutz *et al.,* 1998, Nowak *et al.,* 2007, Termuhlen *et al.,* 1993). c-SRC activates various signalling pathways (e.g. ERK-1/2, PI3K/AKT and STAT3) that induces the cancer cell growth and metastasis (Martin *et al.,* 2001). However, Webb and coworkers have shown that the expression of v-SRC induced apoptosis upon inhibition of RAS and PI3K signalling pathways in Rat-2 fibroblasts. Similarly, c-Myc oncogene was shown to induce apoptosis upon serum withdrawal in lymphoma cell line derived from CD2–MycER transgenic mice (Pelengaris *et al.,* 2000). Indeed, cells expressing nuclear $S1P_2$ receptor had significantly reduced levels of SK1 in the nucleus suggesting that SK1 and $S1P_2$ receptor might have opposing effects in the cell nucleus. Thus, the down-regulation of SK1 in cell nucleus might be crucial for the activation of $S1P_2$ receptor and SRC kinases. Moreover, the expression of $S1P_2$ receptor and Y416 phosphorylated SFK was associated with higher apoptotic index. This observation suggests that $S1P_2$ receptor and SRC kinases might induce apoptosis of cancer cells thus prolonging the patient survival.

3.3.5. The expression of SK1, LYN and RAF-1 kinases are required for $S1P_3$ receptor associated shorter disease-specific patient survival and disease-free cancer recurrence in ERα positive breast tumours

The high tumour expression of $S1P_3$ receptor was associated with shorter disease-specific patient survival and cancer recurrence in ERα positive breast cancer tumours. Indeed, the effect of $S1P_3$ receptor on cancer prognosis was dependent on the expression of SK1. These findings suggest that the activation of $S1P_3$ receptor in an SK1/S1P-mediated autocrine effect might be crucial for breast tumourigenesis. Moreover, the expression of LYN and c-RAF-1 kinases was essential for the effect of $S1P_3$ receptor on disease-specific patient survival and cancer recurrence. Although $S1P_3$ receptor-mediated activation of these kinases was not reported before, various cell surface receptors were shown to activate LYN and c-RAF-1 kinases. For example, the intercellular adhesion molecule 1 (ICAM-1) cross-linking was shown to induce the activation of both LYN and c-RAF-1 in B lymphoma cell line A20 (Holland *et al.,* 1997). Moreover, interleukin 5 (IL-5) was shown to activate the c-RAS/c-RAF-1/ERK-1/2 signalling pathway through LYN kinase that induces the eosinophil survival (Pazdrak *et al.,* 1998, Alam *et al.,* 1995). Thus, $S1P_3$ receptor might mediate the activation of LYN and c-RAF-1 kinase that induces cancer cell growth and survival in ERα positive breast cancer tumours.

In summary, a number of potential functional interactions and/or associations between SK1/S1P receptor and *other* signalling proteins were identified that affect the disease-specific patient survival and cancer recurrence in ERα positive breast cancer tumours. These signalling pathways involve NFκB, c-SRC/LYN, c-RAF-1, ERK-1/2 and AKT kinases. In addition, novel role for $S1P_2$ receptor was identified being associated with favourable prognosis of breast cancer patients.

CHAPTER IV

The expression of SK1 and S1P receptors in ERα negative breast tumours

4.1. Introduction

Cancer research UK statistics estimates that breast cancer is the most common cancer type comprising 30% of all cancer cases diagnosed in women. Breast cancers are categorised according to the expression of ERα, PgR and HER2 receptors. ERα positive breast cancer is the most common type of breast cancer comprising ~75% of cases. These patients are subjected to endocrine therapy that inhibits the oestrogen-induced tumour growth. In addition, anti-HER2 treatment is an option for these patients determined by the HER2 receptor expression in these tumours. Triple negative breast cancers have the poorest disease prognosis compared to other types of breast cancer. These tumours comprise 15-20% of all breast cancer cases that are devoid of expression of ERα, PgR and HER2 receptors. Current treatment regimens for this group of patients are limited to classical chemotherapy that includes therapeutics such as doxorubicin, paclitaxel and capecitabine (Brouckaert *et al.,* 2012). However, these therapeutic approaches are not tumour selective and thus, have severe side effects on patients. Thus, understanding the nature of triple negative breast cancers might lead to development of cancer cell specific therapeutics.

There is extensive evidence of oncogenic SK1/S1P signalling being involved in the formation and development of tumours. Indeed, the SK1/S1P signalling interacts with vast numbers of oncogenes (e.g. TRAF2, Delta-catenin/NPRAR, RPK118 and eEF1A1) to maintain tumour growth (Alvarez *et al.,* 2010, Leclercq *et al.,* 2008, Fujita *et al.,* 2004, Hayashi *et al.,* 2002). In support of these findings, the SK1 expression was up-regulated both at the mRNA and/or protein level in various cancers that was associated with poor clinical prognosis (Ohotski *et al.,* 2013, Ohotski *et al.,* 2012, Watson *et al.,* 2010, Erez-Roman *et al.,* 2010, Ruckhaberle *et al.,* 2008, French *et al.,* 2003). In addition, the SK1 expression was associated with the development of tamoxifen resistance in ERα positive breast cancer patients (Long *et al.,* 2010a).

The high tumour expression of $S1P_1$ and $S1P_3$ receptors were also associated with poor prognosis in ERα positive breast cancer patients (Watson *et al.,* 2010). Indeed, S1P receptors activate downstream signalling pathways (e.g. ERK1/2 and AKT) through coupling to small G-proteins that induce the tumour growth and survival. The S1P receptors also interact with *other* plasma membrane-bound receptors. For example, HER2

receptor was shown to form a functional complex with the $S1P_4$ receptor in ERα negative MDA-MB-453 breast cancer cells. Moreover, EGF or S1P-mediated activation of ERK1/2 signalling pathway was dependent on the activation of both HER2 and $S1P_4$ receptors (Long *et al.,* 2010b). Thus, the interaction between $S1P_4$ and HER2 receptors might be crucial in formation and development of breast tumours.

In this chapter, tissue microarrays composed of ERα negative breast tumours were used to determine the associations between protein expression levels of SK1/S1P receptors and clinicopathological parameters. The expression of both SK1 and $S1P_4$ receptor was associated with poor disease prognosis and cancer recurrence. Moreover, the effect of SK1 and $S1P_4$ receptor on recurrence-free survival was associated with the expression of HER2 receptor. In addition, SK1/2 dual inhibitor SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) inhibits the S1P-induced ERK-1/2 activation in MDA-MB-453 cells suggesting that the $S1P_4$/HER2 signalling is mediated by SK1/2. In addition, the predominant action of the SK1/2 inhibitor appears to be associated with proteasomal degradation of SK1. Thus, SK1 inhibitors and $S1P_4$ receptor antagonists might represent novel therapeutics to be used in treatment of patients with ERα negative breast tumours.

4.2. Results

4.2.1. The clinicopathological details of ERα negative breast cancer cohort

A total of 140 patients with ERα negative tumours were involved in the study. The median age of the patients was 55 with inter-quartile range from 45 to 64. The tumours were classified into three groups as ductal (129, 90.7%), lobular (2, 2.9%) and medullary (9, 6.3%) breast cancer. The pathological grading indicates that 4 (2.9%) cases were 1st grade, 25 (22.9%) cases were 2nd grade and 107 (71.4%) cases were 3rd grade tumour. The median tumour size was 22 mm with inter-quartile range from 15 mm to 30 mm. The analysis of lymph nodes indicates that 70 patients (44.3%) had metastasis in comparison to 67 patients (55.0%) that had no metastasis in the lymph nodes. Regarding to the last follow-up date of patients, 75 (57.1%) patients were alive, 41 (27.1%) patients were died of breast cancer and 18 (12.9%) patients died of another cause.

4.2.2. The specificity of anti-$S1P_2$ receptor antibody

The specificity of monoclonal anti-$S1P_2$ antibody was determined using formalin-fixed paraffin-embedded pellets of HEK 293 cell pellets over-expressing $S1P_2$ receptor (**Figure 4.1**). IHC staining of HEK 293 cells over-expressing $S1P_2$ receptor is stronger compared to vector transfected HEK 293 cells. This demonstrates that anti-$S1P_2$ antibody is specific for the $S1P_2$ receptor. The specificity of anti-SK1, anti-$S1P_1$ and anti-$S1P_4$ antibodies were confirmed previously (Long *et al.,* 2010a, Long *et al.,* 2010b, Watson *et al.,* 2010).

4.2.3. The expression of SK1 and S1P receptors correlates with the clinical parameters in ERα negative breast cancer cohort

The expression of SK1, $S1P_1$, $S1P_2$ and $S1P_4$ receptor was analysed in ERα negative breast tumours. The protein levels in plasma membrane, cytoplasm and nucleus were individually quantified. Patients were divided into low and high expressing subgroups using a technique described by Ruckhaberle and coworkers (Ruckhaberle *et al.,* 2007). The Mann-Whitney or Kruskal-Wallis One-way ANOVA tests was used to analyse the correlation between

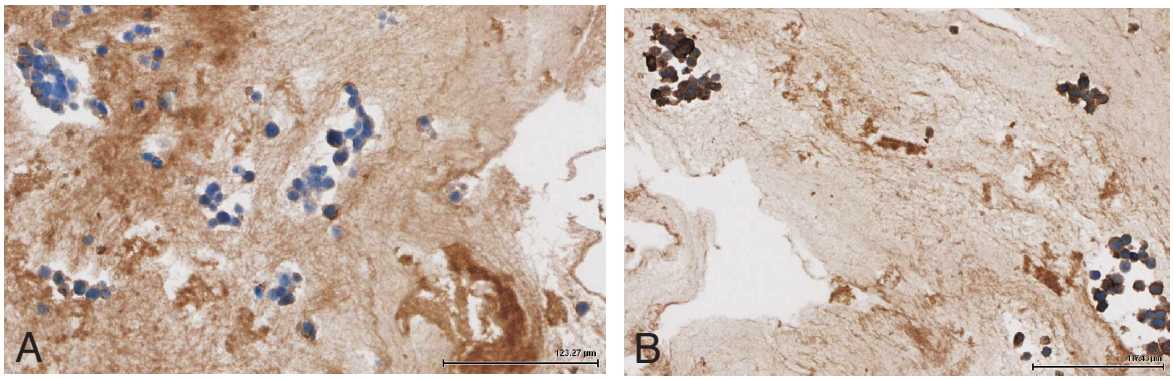

Figure 4.1. The specificity of anti-$S1P_2$ receptor antibody tested on formalin-fixed paraffin-embedded HEK-293 cells. **A** shows the IHC staining for HEK-293 cells transfected with empty vector; **B** shows the IHC staining for HEK-293 cells transfected with $S1P_2$ plasmid construct.

protein expression and clinicopathological parameters (**Table 4.1**). Results indicate a correlation between the age of the patients, and the SK1 and $S1P_4$ receptor expression. The analysis of distribution of SK1 and $S1P_4$ receptor expression within patients that are younger (48 patients) or older than 50 years old (92 patients) demonstrated that the patients younger than 50 years old have higher levels of expression of SK1 and $S1P_4$ receptor in all three compartments compared with older patients (**Figure 4.2A, data not shown for $S1P_4$ receptor**). The expression of $S1P_4$ receptor was also diverse within different types of breast tumours. The analysed patient cohort included three different types of breast tumour named as ductal, lobular and medullary. The analysis of $S1P_4$ receptor expression in three different cellular compartments have shown that the $S1P_4$ receptor expression was the highest in ductal carcinoma, followed by medullary carcinoma and lowest in lobular carcinoma (**Figure 4.2B**). In addition, the $S1P_4$ receptor expression levels between these tumour subtypes were significantly different (**Table 4.1).** However, it must be noted that the number of lobular and medullary carcinomas included in the cohort are 2 and 9 respectively, thus these results must be interpreted carefully. The $S1P_4$ receptor expression at the plasma membrane was also correlated with tumour grade. The analysis of distribution of $S1P_4$ receptor expression within the tumours at different grades have shown that the first grade tumours had the highest expression which was reduced in the second grade tumours and increased slightly in the third grade tumours (**Figure 4.2C**). The metastasis into lymph nodes in relation to the expression of SK1 and S1P receptors was also analysed. The cytoplasmic $S1P_2$ receptor expression was significantly lower in metastatic tumours (73 samples) compared to non-metastatic tumours (67 samples) (**Figure 4.2D**). The expression of PgR and HER2 receptors was also known for the tumours included in the cohort. The PgR expression was correlated with the nuclear SK1 expression ($P=0.033$). The distribution analysis have revealed that the PgR positive tumours (5 samples) expressed significantly higher levels of nuclear SK1 compared with PgR negative tumours (142 samples) (**Figure 4.2E**). However, the number of PgR positive samples is limited to 5 samples thus the significance of this correlation must be interpreted carefully. The HER2 receptor expression was correlated with the cytoplasmic $S1P_1$ receptor expression. The analysis of distribution of $S1P_1$ receptor expression between the HER2 receptor positive (39 samples) and HER2 receptor negative (100 samples) have shown that the expression of cytoplasmic $S1P_1$ receptor was significantly higher in tumours expressing HER2 compared with HER2 negative tumours (**Figure 4.2F**).

Table 4.1. Mann-Whitney test results showing the differences in protein expression within different subgroups of clinicopathological parameters in ERα negative cohort (yrs: years; Dc: ductal carcinoma; Lc: lobular carcinoma; Mc: medullary carcinoma; P: positive; N: negative; L: low; H: high; mem: plasma membrane; cyto: cytoplasm; nuc: nucleus).

		P values											
Variables	**Numbers**	**SK1 mem**	**SK1 cyto**	**SK1 nuc**	**S1P$_1$ mem**	**S1P$_1$ cyt**	**S1P$_1$ nuc**	**S1P$_2$ mem**	**S1P$_2$ cyto**	**S1P$_2$ nuc**	**S1P$_4$ mem**	**S1P$_4$ cyto**	**S1P$_4$ nuc**
Age (<50yrs/ >50yrs)	48/92	0.042	0.035	0.003	NS	NS	NS	NS	NS	NS	0.029	0.029	0.036
Tumour type (Dc/Lc/ Mc)	129/2/9	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.023	0.005	0.017
Tumour grade (1/2/3)	4/25/107	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.022	NS	NS
Tumour size (0/1/2)	64/69/2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Lymph nodes (P/N)	70/67	NS	NS	NS	NS	NS	NS	NS	0.033	NS	NS	NS	NS
PR status (P/N)	5/142	NS	NS	0.032	NS	NS	NS	NS	NS	NS	NS	NS	NS
Her2 status (P/N)	39/100	NS	NS	NS	NS	0.024	NS	NS	NS	NS	NS	NS	NS
SK1 mem (L/H)	128/12	-	0.002	0.016	NS	NS	NS	NS	NS	NS	NS	0.006	NS
SK1 cyto (L/H)	116/24	NS	-	0.001	NS	0	0	NS	NS	NS	NS	0.005	NS
SK1 nuc (L/H)	110/30	NS	0.001	-	0.008	0.002	0.001	NS	NS	0.021	NS	NS	NS
S1P$_1$ mem (L/H)	118/32	NS	NS	0.004	-	0	0	NS	NS	NS	NS	0.005	NS
S1P$_1$ cyto (L/H)	121/28	NS	0.004	0.001	0	-	0	NS	NS	NS	NS	0.015	NS
S1P$_1$ nuc (L/H)	108/42	NS	0.001	0.003	0	0	-	0.009	0.031	NS	NS	0.008	NS
S1P$_2$ mem (L/H)	77/70	NS	NS	NS	NS	NS	NS	-	0	0	NS	NS	NS
S1P$_2$ cyto (L/H)	75/71	NS	0	NS	0.012	0.001	0.003	0	-	0	0.003	0	0.009
S1P$_2$ nuc (L/H)	88/59	NS	NS	NS	NS	NS	NS	0	0	-	NS	NS	NS
S1P$_4$ mem (L/H)	114/26	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	0	0
S1P$_4$ cyto (L/H)	120/20	0.012	0.014	NS	NS	NS	NS	NS	NS	NS	0	-	0
S1P$_4$ nuc (L/H)	118/22	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	0	-

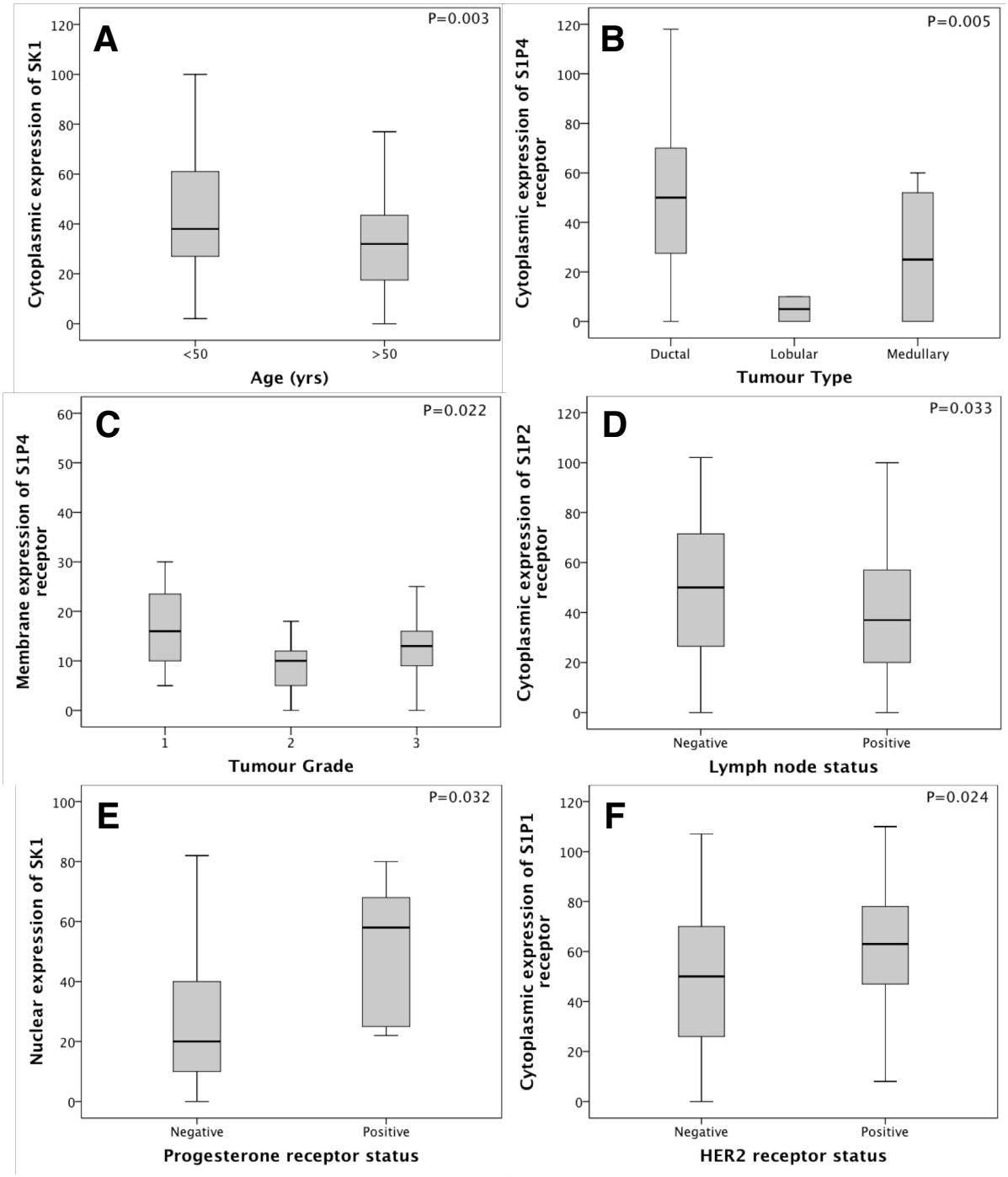

Figure 4.2. The expression of SK1 and S1P receptors in different subgroups of clinicopathological parameters in ERα negative cohort. **A** shows the cytoplasmic expression of SK1 in patients younger or older than 50 years; **B** shows the cytoplasmic expression of $S1P_4$ receptor in different tumour types; **C** shows the plasma membrane-bound expression of $S1P_4$ receptor in different tumour grades; **D** shows the cytoplasmic expression of $S1P_2$ receptor in lymph node negative or positive tumours; **E** shows the nuclear expression of SK1 in progesterone receptor negative or positive tumours; **F** shows the cytoplasmic expression of $S1P_1$ receptor in HER2 receptor negative or positive tumours.

The expression level of SK1 was correlated between different cellular compartments (**Table 4.1**). For example, the tumours expressing high plasma membrane-bound SK1 expressed high levels of cytoplasmic SK1 (P=0.002). Similarly, the tumours expressing high levels of cytoplasmic SK1 expressed high levels of SK1 in the nucleus (P=0.001). Similarly, the expression levels of $S1P_1$, $S1P_2$ or $S1P_4$ receptor were correlated between different cellular compartments (**Table 4.1**). The expression of SK1, $S1P_1$, $S1P_2$ and $S1P_4$ receptors were also analysed in combination in each cellular compartment. The high expression levels of plasma membrane-bound SK1 was correlated with high levels of cytoplasmic $S1P_4$ receptor expression (P=0.006). Additionally, the tumours expressing high levels of SK1 in cytoplasm expressed high levels of $S1P_1$ receptor (P=0.000, **Figure 4.3A**) and $S1P_4$ receptors (P=0.005, **Figure 4.3B**) in the cytoplasm. Similarly, the tumours expressing high levels of $S1P_1$ receptor in cytoplasm expressed high levels of SK1 (P=0.004, **Figure 4.3E**) and $S1P_4$ receptor in the cytoplasm (P=0.015, **Figure 4.3F**). Moreover, the high expression levels of $S1P_4$ receptor in the cytoplasm was correlated with the high expression levels of cytoplasmic SK1 (P=0.014). Furthermore, the tumours expressing high levels of SK1 in the nucleus expressed high levels of $S1P_1$ receptor (P=0.001, **Figure 4.3C**), but expressed low levels of $S1P_2$ receptor in the nucleus (P=0.021, **Figure 4.3D**). Although, the nuclear $S1P_2$ receptor expression was not correlated with the SK1 expression in any compartment, the expression of cytoplasmic $S1P_2$ receptor was correlated with the cytoplasmic expression of SK1, $S1P_1$ and $S1P_4$ receptors. According to the distribution analysis, tumours expressing high levels of $S1P_2$ receptor in the cytoplasm expressed high levels of SK1, $S1P_1$ and $S1P_4$ receptors in the cytoplasm (P=0.000, P=0.001 and P=0.000 respectively).

4.2.4. The SK1 and $S1P_4$ receptor expression correlates with poor prognosis and cancer recurrence in ERα negative breast cancer

The effect of SK1, $S1P_1$, $S1P_2$ and $S1P_4$ receptor expression on disease-specific patient survival and disease-free cancer recurrence was analysed and quantified using Log-rank and Cox-regression analysis respectively. Images of tissues stained for SK1 and $S1P_4$ receptors are presented in **Figure 4.5**. On univariate analysis, the patients with tumours expressing high levels of plasma membrane-bound SK1 had shorter disease-free cancer

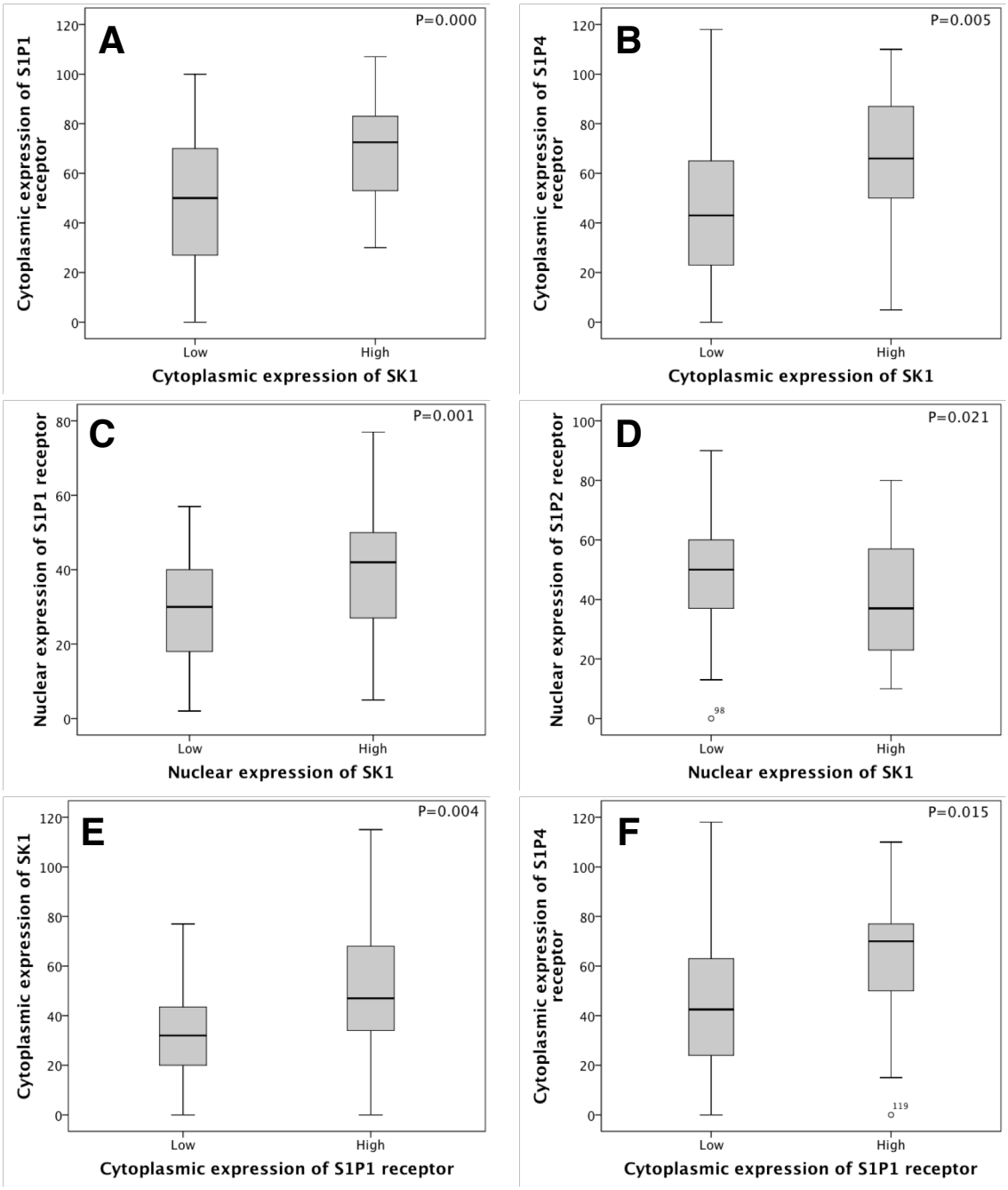

Figure 4.3. The expression of SK1 and S1P receptors within different clinicopathological parameters in ERα negative cohort. **A** shows the cytoplasmic expression of $S1P_1$ receptor within the low or high cytoplasmic SK1 expressing tumours; **B** shows the cytoplasmic expression of $S1P_4$ receptor within the low or high cytoplasmic SK1 expressing tumours; **C** shows the nuclear expression of $S1P_1$ receptor within the low or high nuclear SK1 expressing tumours; **D** shows the nuclear expression of $S1P_2$ receptor within the low or high nuclear SK1 expressing tumours; **E** shows the cytoplasmic expression of SK1 within the low or high cytoplasmic $S1P_1$ receptor expressing tumours; **F** shows the cytoplasmic expression of $S1P_4$ receptor within the low or high cytoplasmic $S1P_1$ receptor expressing tumours.

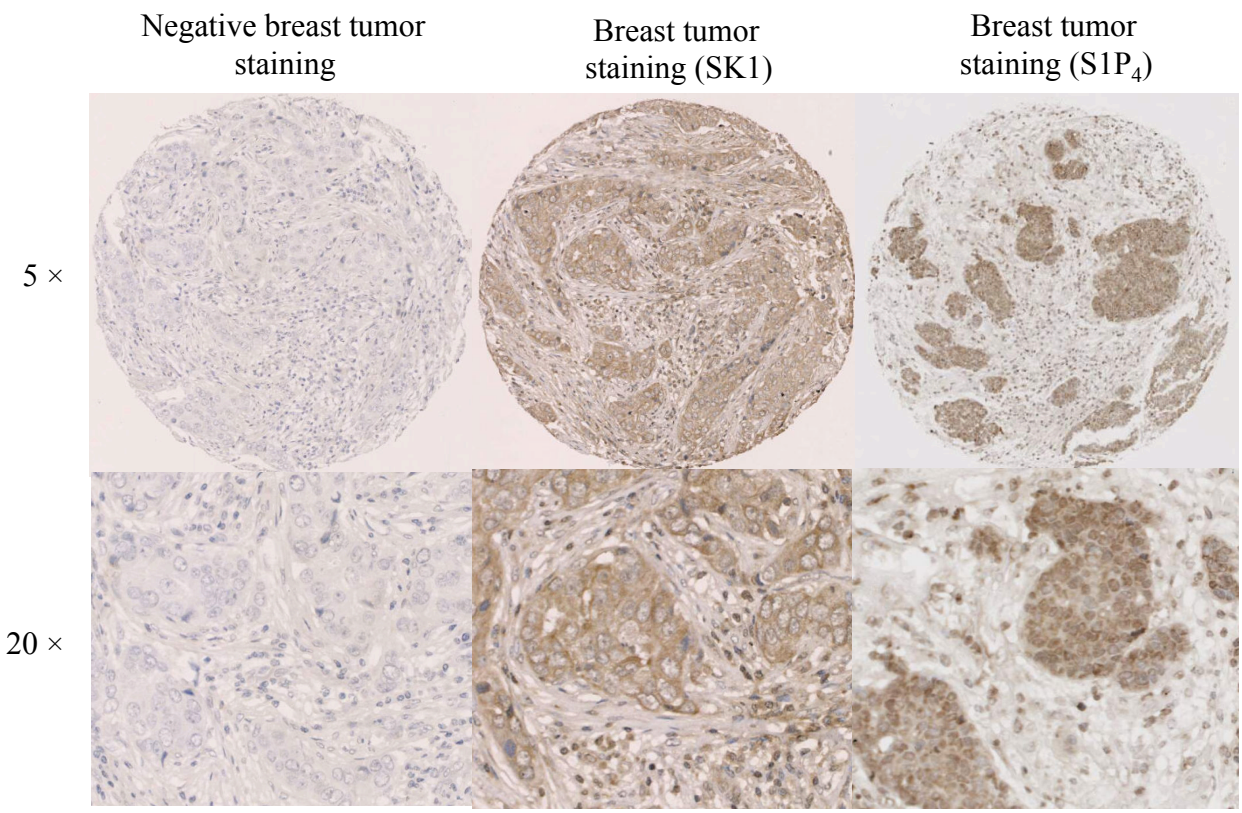

Figure 4.4. Images of ERα negative breast cancer tissues stained for SK1 and $S1P_4$ receptors. Panels from left to right present tissue samples with negative staining (left panel), tissue samples with positive SK1 staining (middle panel) and tissue samples with positive $S1P_4$ receptor staining (right panel).

recurrence time compared to the patients with tumours expressing low levels of plasma membrane-bound SK1 (P=0.005). The mean disease-free cancer recurrence time for patients with tumours expressing high levels of plasma membrane-bound SK1 was 4.0 years (IQR 2.5-5.5) compared to 6.8 years (IQR 6.3-7.3) for the patients with tumours expressing low levels of plasma membrane-bound SK1 (**Figure 4.5C**). The expression of membrane-bound SK1 was not associated with patient survival (P=0.052, **Figure 4.5A**). However, increasing the patient number in this cohort might reduce the *P* value resulting in a significant value. On multivariate analysis, the effect of plasma membrane-bound SK1 expression on disease-free cancer recurrence was not significant, indicating that SK1 is dependent on other prognostic markers included in the analysis.

The expression of cytoplasmic $S1P_4$ receptor was associated with shorter disease-specific patient survival and disease-free cancer recurrence. On univariate analysis, the patients with tumours expressing high levels of cytoplasmic $S1P_4$ receptor had shorter disease-specific survival and disease-free cancer recurrence compared to patients with tumours expressing low levels of cytoplasmic $S1P_4$ receptor (P=0.004 and P=0.010). The mean disease-specific survival time for the patients with tumours expressing high levels of cytoplasmic $S1P_4$ receptor was 8.0 years (IQR 5.5-10.4) compared to 11.7 years (IQR 10.6-12.6) for the patients with tumours expressing low levels of cytoplasmic $S1P_4$ receptor (**Table 4.2, Figure 4.5B**). The mean disease-free cancer recurrence time for the patients with tumours expressing high levels of cytoplasmic $S1P_4$ receptor was 5.0 years (IQR 3.5-6.6) compared to 6.5 years (IQR 6.0-7.0) for the patients with tumours expressing low levels of cytoplasmic $S1P_4$ receptor (**Table 4.3, Figure 4.5D**). Multivariate analysis revealed that the effect of $S1P_4$ receptor expression was independent of other prognostic markers.

On univariate and multivariate analysis, the metastasis into lymph nodes was associated with shorter disease-specific patient survival and disease-free cancer recurrence (P=0.001 and P=0.005 respectively). The expression of $S1P_1$ and $S1P_2$ receptors was not associated with patient survival or cancer recurrence (**Table 4.2** and **Table 4.3**).

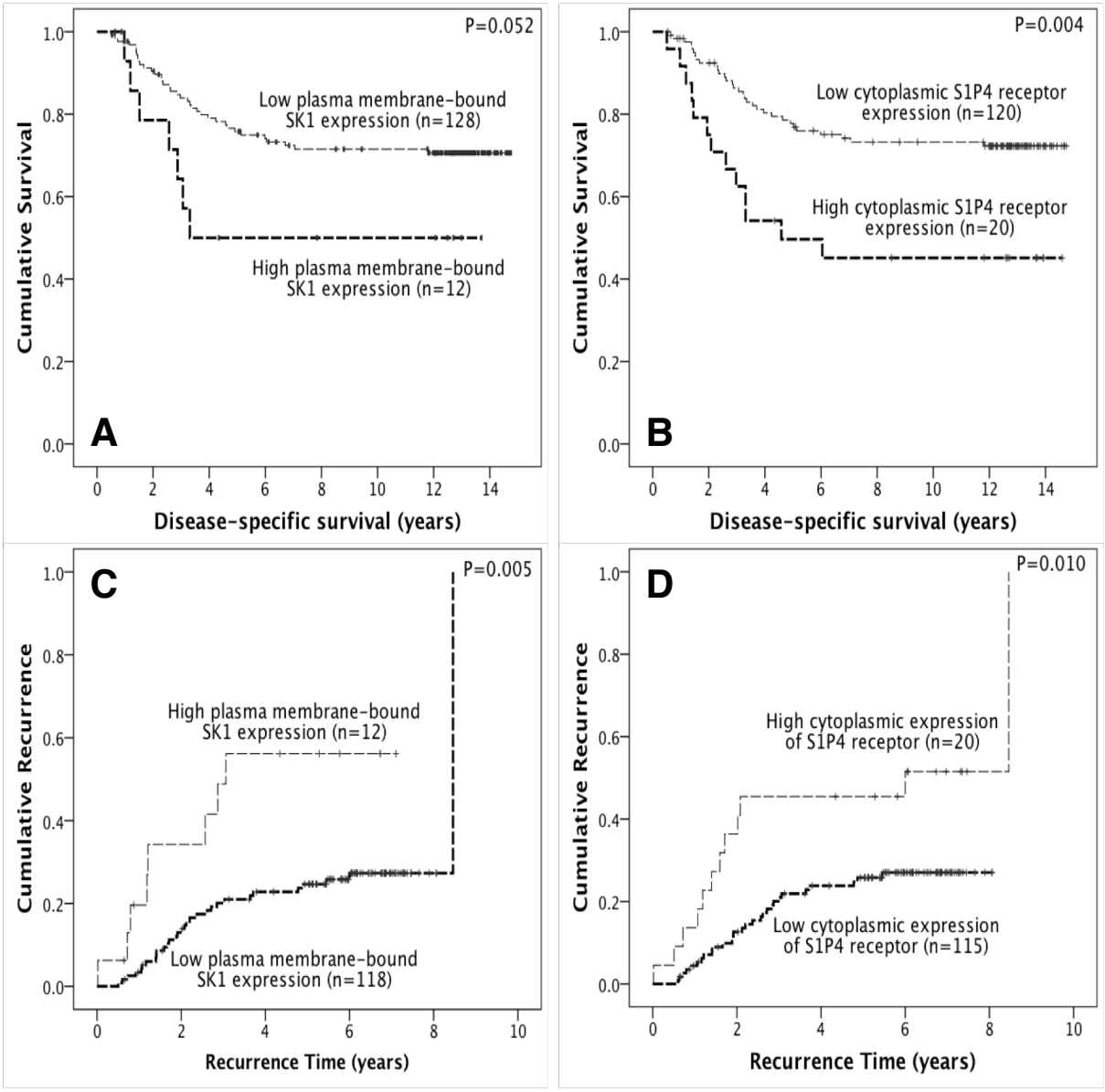

Figure 4.5. The Kaplan-Meier graphs showing the effect of SK1 and $S1P_4$ receptor expression on disease-specific patient survival and disease-free cancer recurrence. **A** shows the impact of plasma-membrane bound SK1 expression on disease-specific patient survival; **B** shows the impact of cytoplasmic $S1P_4$ receptor expression on disease-specific patient survival; **C** shows the impact of plasma-membrane bound SK1 expression on disease-free cancer recurrence; **D** shows the impact of cytoplasmic expression of $S1P_4$ receptor on disease-free cancer recurrence.

Table 4.2. The influence of clinicopathological parameters and SK1/S1P receptor expression on disease-specific patient survival in ERα negative cohort (yrs: years; P: positive; N: negative; L: low; H: high; mem: plasma membrane; cyto: cytoplasm; nuc: nucleus).

Disease-specific survival							
		Univariate analysis			***Multivariate analysis***		
	Numbers	**P value**	**HR**	**IQR**	**P value**	**HR**	**IQR**
Age (<50yrs/ >50yrs)	48/92	0.941	0.899	0.495-1.632			
Tumour grade (1/2/3)	4/25/107	0.444	1.482	0.721-3.047			
Tumour size (0/1/2)	64/69/2	0.088	1.615	0.913-2.854			
Lymph nodes (P/N)	70/67	*0.001*	3.790	1.918-7.489	*0.002*	3.000	1.500-6.000
PR status (P/N)	5/142	0.352	0.047	0.000-29.793			
Her2 status (P/N)	39/100	0.806	1.122	0.585-2.152			
SK1 mem (L/H)	128/12	0.118	2.193	0.974-4.939			
SK1 cyto (L/H)	116/24	0.559	1.338	0.644-2.778			
SK1 nuc (L/H)	110/30	0.563	0.916	0.439-1.910			
S1P1 mem (L/H)	110/30	0.652	0.845	0.407-1.755			
S1P1 cyto (L/H)	121/28	0.551	0.782	0.349-1.755			
S1P1 nuc (L/H)	108/42	0.470	0.778	0.394-1.536			
S1P2 mem (L/H)	77/70	0.110	0.604	0.325-1.121			
S1P2 cyto (L/H)	75/71	0.409	0.773	0.419-1.425			
S1P2 nuc (L/H)	88/59	0.510	0.810	0.432-1.517			
S1P4 mem (L/H)	114/26	0.539	1.135	0.547-2.357			
S1P4 cyto (L/H)	120/20	*0.004*	2.617	1.371-4.993	*0.031*	2.270	1.100-4.500
S1P4 nuc (L/H)	118/22	0.392	0.651	0.257-1.649			

Table 4.3. The influence of clinicopathological parameters and SK1/S1P receptor expression on disease-free cancer recurrence in ERα negative breast cancer patients (yrs: years; P: positive; N: negative; L: low; H: high; mem: plasma membrane; cyto: cytoplasm; nuc: nucleus).

Recurrence-free survival							
		Univariate analysis			***Multivariate analysis***		
	Numbers	**P value**	**HR**	**IQR**	**P value**	**HR**	**IQR**
Age (<50yrs/ >50yrs)	48/92	0.290	0.714	0.383-1.333			
Tumour grade (1/2/3)	4/25/107	0.339	1.424	0.690-2.938			
Tumour size (0/1/2)	64/69/2	0.056	1.818	0.985-3.353			
Lymph nodes (P/N)	70/67	*0.005*	2.623	1.347-5.109	*0.002*	3.450	1.568-7.589
PR status (P/N)	5/142	0.363	0.046	0.000-34.647			
Her2 status (P/N)	39/100	0.274	1.454	0.743-2.843			
SK1 mem (L/H)	128/12	*0.005*	2.940	1.344-6.429			
SK1 cyto (L/H)	116/24	0.296	1.487	0.707-3.125			
SK1 nuc (L/H)	110/30	0.745	0.879	0.404-1.912			
S1P1 mem (L/H)	110/30	0.764	0.888	0.409-1.928			
S1P1 cyto (L/H)	121/28	0.435	0.708	0.297-1.686			
S1P1 nuc (L/H)	108/42	0.318	0.685	0.326-1.440			
S1P2 mem (L/H)	77/70	*0.049*	0.510	0.261-0.998			
S1P2 cyto (L/H)	75/71	0.866	0.946	0.496-1.804			
S1P2 nuc (L/H)	88/59	0.631	0.851	0.440-1.645			
S1P4 mem (L/H)	114/26	0.803	1.110	0.489-2.516			
S1P4 cyto (L/H)	120/20	*0.010*	2.435	1.215-4.879	*0.004*	3.627	1.520-8.652
S1P4 nuc (L/H)	118/22	0.181	0.447	0.138-1.452			

4.2.5. HER2 receptor expression is required for SK1 and $S1P_4$ receptor associated shorter disease-free cancer recurrence in ERα negative breast cancer

The effect of SK1 and $S1P_4$ receptor expression on patient survival and cancer recurrence was further analysed by stratifying the data for the SK1, $S1P_4$ and HER2 receptor expression. High plasma membrane-bound SK1 expression was associated with shorter disease-specific patient survival (**Figure 4.6A**, P=0.001) and disease-free cancer recurrence (**Figure 4.6B**, P=0.006) in tumours expressing low levels of cytoplasmic $S1P_4$ receptor. The mean disease-specific survival time for the patients with tumours expressing high levels of membrane-bound SK1 was 6.0 years (IQR 3.0-9.0) compared to 11.6 years (IQR 10.7-12.7) for the patients with tumours expressing low levels of membrane-bound SK1 (**Figure 4.6A**). The mean disease-free cancer recurrence time for the patients with tumours expressing high levels of membrane-bound SK1 was 4.0 years (IQR 2.3-5.8) compared to 6.8 years (IQR 6.3-7.3) for the patients with tumours expressing low levels of membrane-bound SK1 (**Figure 4.6B**). Similarly, high cytoplasmic $S1P_4$ receptor expression was associated with shorter disease-specific patient survival (**Figure 4.6C**, P=0.001) and disease-free cancer recurrence in tumours expressing low levels of plasma membrane-bound SK1 (**Figure 4.6D**, P=0.005). The mean disease-free cancer recurrence time for the patients with tumours expressing high levels of cytoplasmic $S1P_4$ receptor was 5.0 years (IQR 3.2-6.8) compared to 6.8 years (IQR 6.3-7.3) for the patients with tumours expressing low levels of cytoplasmic $S1P_4$ receptor (**Figure 4.6C**). Furthermore, high cytoplasmic $S1P_4$ receptor expression was associated with shorter disease-free cancer recurrence in tumours expressing low levels of cytoplasmic SK1 (P=0.030). The mean disease-free cancer recurrence time for the patients with tumours expressing high levels of cytoplasmic $S1P_4$ receptor was 5.1 years (IQR 3.2-7.1) compared to 6.6 years (IQR 6.1-7.1) for the patients with tumours expressing low levels of cytoplasmic $S1P_4$ receptor (**Figure 4.6D**).

The effect of HER2 receptor expression on patient survival and cancer recurrence was also analysed. High expression of plasma membrane-bound SK1 was associated with shorter cancer recurrence solely in HER2 receptor positive tumours (**Figure 4.7A**). High expression of both plasma membrane-bound SK1 and HER2 receptor significantly reduced the mean recurrence-free survival rate from 6.8 years (IQR 6.2-7.4) to 2.6 years (IQR

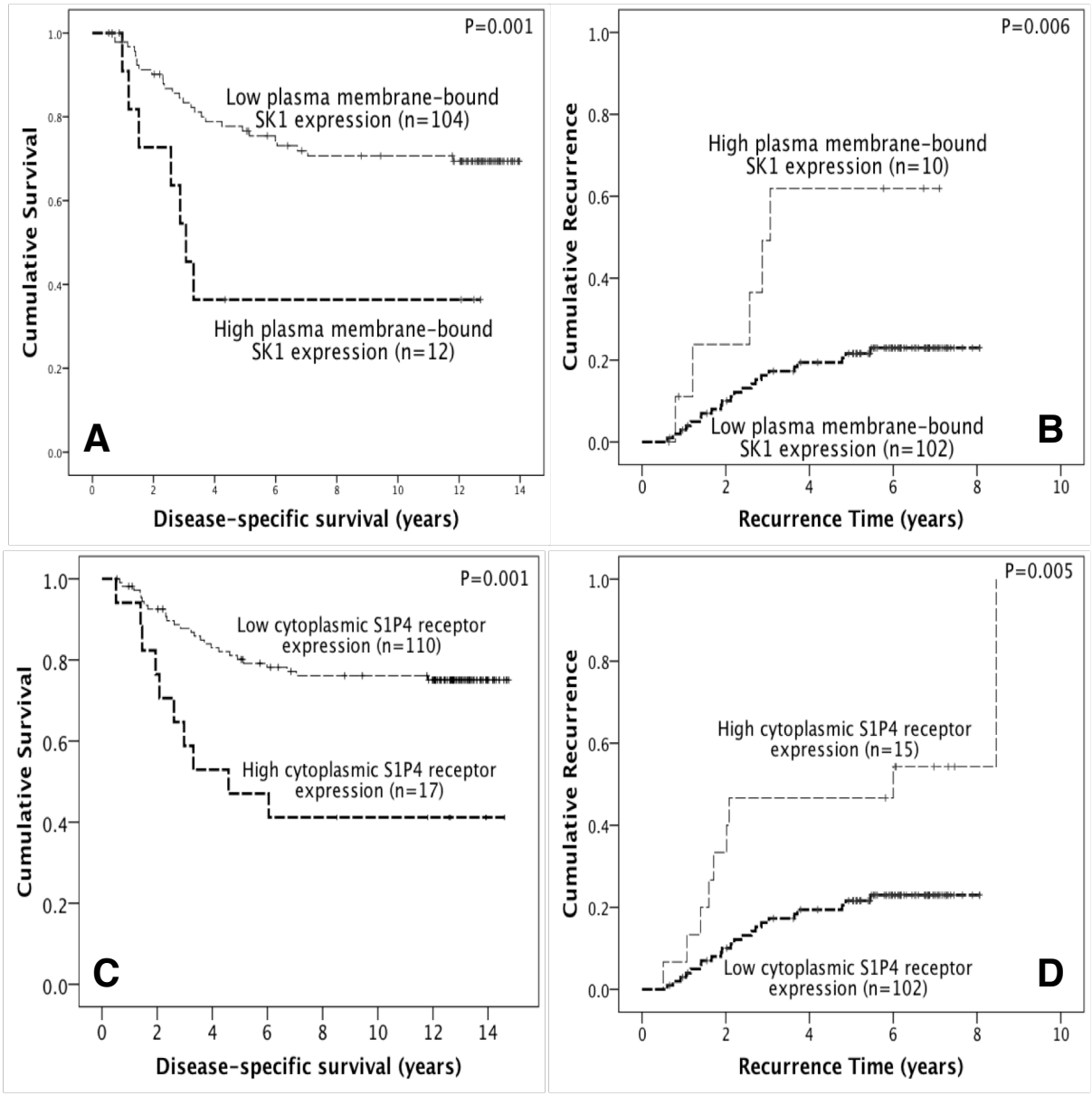

Figure 4.6. The Kaplan-Meier graphs showing the effect of SK1/S1P$_4$ receptor expression on SK1 and S1P$_4$ receptor associated disease-specific patient survival and disease-free cancer recurrence in ERα negative breast cancer patients. **A** high plasma membrane-bound SK1 expression in a low cytoplasmic S1P$_4$ receptor expression background is associated with shorter disease-specific patient survival; **B** high plasma membrane-bound SK1 expression in a low cytoplasmic S1P$_4$ receptor expression background is associated with shorter disease-free cancer recurrence; **C** high cytoplasmic S1P$_4$ receptor expression in a low plasma membrane-bound SK1 expression background is associated with shorter disease-specific patient survival; **D** high cytoplasmic S1P$_4$ receptor expression in a low membrane-bound SK1 expression background is associated with shorter disease-free cancer recurrence.

0.9-4.2) (P=0.0001). Moreover, the loss of HER2 receptor expression annulled the association between membrane-bound SK1 expression and disease-free cancer recurrence (**Figure 4.7A**).

Similarly, high expression of $S1P_4$ receptor at the plasma membrane was associated with shorter disease-free cancer recurrence time solely in HER2 receptor positive tumours (**Figure 4.7B**). High expression of both $S1P_4$ receptor and HER2 receptor significantly reduced the mean disease-free cancer recurrence time from 6.4 years (IQR 5.8-7.1) to 3.5 years (IQR 1.0-5.9) (P=0.022). Moreover, the loss of HER2 receptor expression annulled the association between $S1P_4$ receptor expression and disease-free cancer recurrence (**Figure 4.7B**). These findings suggest that HER2 receptor might functionally interact with SK1 and $S1P_4$ receptor to mediate cancer recurrence in ERα negative breast cancer.

4.2.6. SK1 is required for $S1P_4$ receptor/HER2-mediated ERK-1/2 activation in ERα negative breast cancer

Long and coworkers have demonstrated that the S1P-induced ERK-1/2 activation was mediated by $S1P_4$ and HER2 receptors in MDA-MB-453 cells (Long *et al.,* 2010b). However, the involvement of SK1 in S1P-mediated ERK1/2 activation was not assessed. The treatment of MDA-MB-453 cells with SK1 inhibitor SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole)) reduced the S1P-stimulated ERK-1/2 phosphorylation and activation (**Figure 4.8**). Indeed, Long and coworkers have shown that the siRNA-mediated down-regulation of SK1 expression reduced the S1P-stimulated ERK-1/2 activation in MCF-7 breast cancer cells (Long *et al.,* 2010a). Thus, the S1P stimulated ERK-1/2 activation was also mediated by SK1 in MDA-MB-453 cells. Furthermore, the SKi treatment induced a reduction in SK1 expression as well as basal ERK-1/2 activation in MDA-MB-453 cells. In support of this observation, Long and coworkers have shown that the basal ERK-1/2 phosphorylation was dependent on HER2 receptor but independent of $S1P_4$ receptor suggesting that SK1 is involved in HER2-mediated regulation of basal ERK-1/2 phosphorylation (Long *et al.,* 2010b) (**Figure 4.8**).

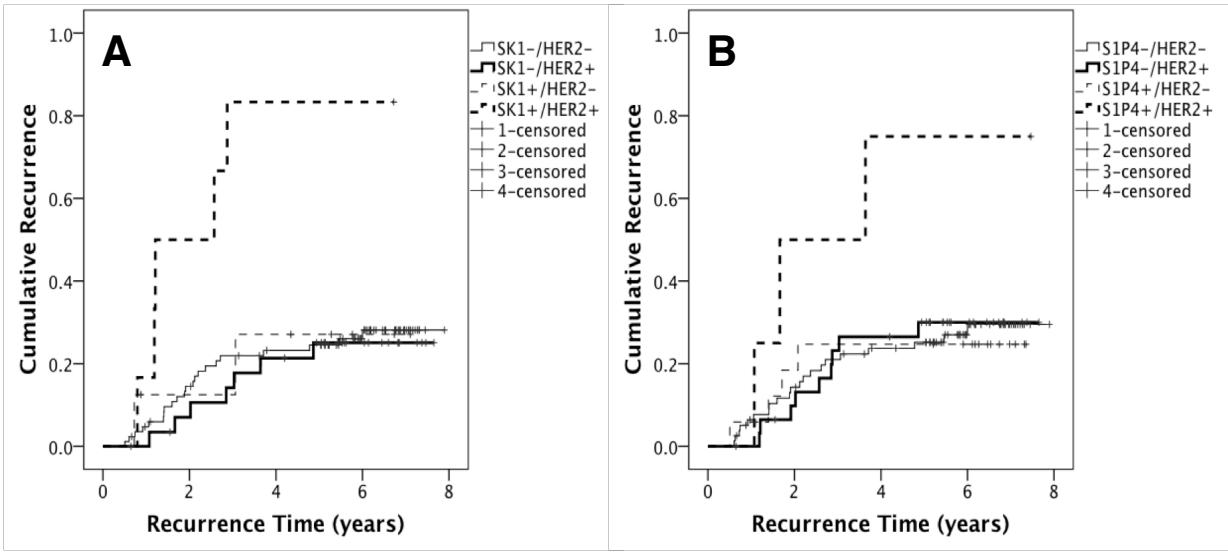

Figure 4.7. The Kaplan-Meier graphs showing the effect of HER2 receptor expression on SK1 and $S1P_4$ receptor associated disease-free cancer recurrence in ERα negative breast cancer patients. **A** high plasma membrane-bound SK1 expression in HER2 positive tumours is associated with shorter disease-free cancer recurrence; **B** high membrane expression of $S1P_4$ receptor in HER2 positive tumours is associated with shorter disease-free cancer recurrence.

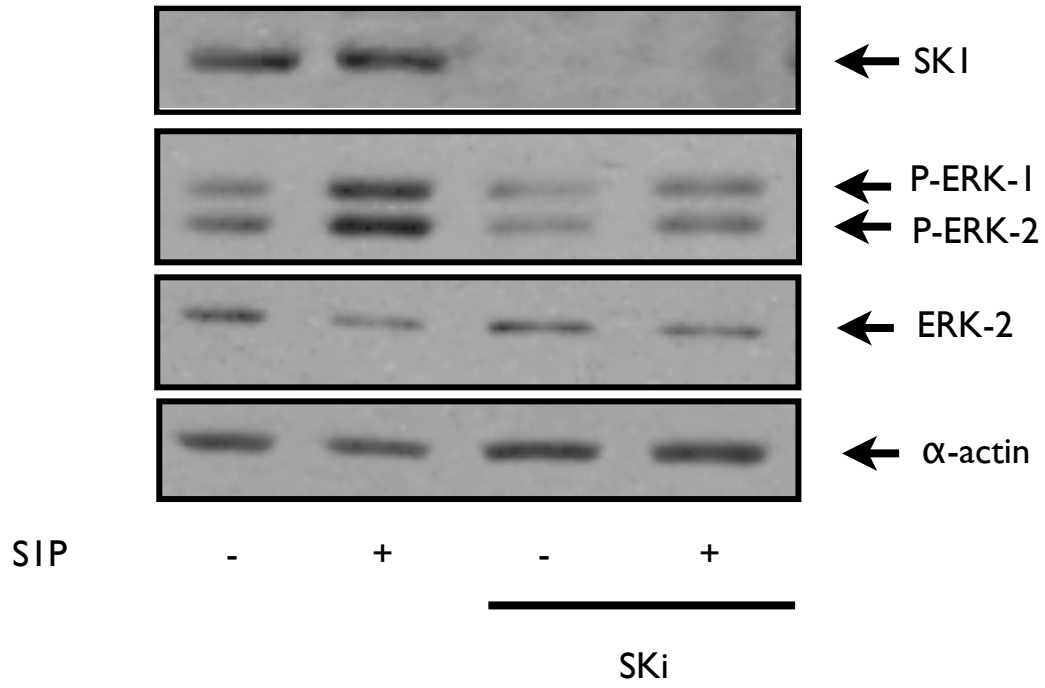

Figure 4.8. The western blots showing the effect of SKi treatment on S1P-stimulated ERK-1/2 activation in MDA-MB-453 cells. Cells were treated with SKi (10 μM) for 24h and stimulated with S1P (5 μM, 10 min). The SKi treatment reduces both basal and S1P-stimulated ERK-1/2 activation. α-actin was used to determine uniform protein loading. Results are representative of three independent experiments.

4.3. Discussion

The expression of SK1, $S1P_1$ receptor, $S1P_2$ receptor and $S1P_4$ receptor was analysed in ERα negative breast cancer tissue samples. The expression of neither $S1P_1$ receptor nor $S1P_2$ receptor was associated with cancer prognosis in ERα negative breast cancer patients. However, $S1P_2$ receptor expression was significantly reduced in metastatic tumours compared to non-metastatic tumours. Moreover, SK1 expression reduced the $S1P_2$ receptor expression levels in the nuclear compartment of the cells. The role of $S1P_2$ receptor expression in these tumours could not be analysed due to low number of patients. However, the role of $S1P_2$ receptor signalling in MDA-MB-231 cells was further investigated in chapter 5. The association between hormone/growth factor receptor and the SK1/S1P receptors was also analysed. The PgR and HER2 receptor expression significantly enhanced the expression levels of SK1 and $S1P_1$ receptor in ERα negative breast tumours respectively. Indeed, progesterone administration was shown to up-regulate the SK1 mRNA expression in rat endometrium (Jeng *et al.,* 2007). Moreover, the up-regulation of SK1 was associated with elevated levels of cyclin D1 expression required for the growth of Eker rat myometrial cell line (ELT3). Thus, the expression of SK1 and $S1P_1$ receptor might be crucial for PgR/HER2-mediated breast tumourigenesis and cancer progression.

The expression of $S1P_4$ receptor and metastasis into lymph nodes were independent prognostic markers that predict disease outcome in ERα negative breast cancer patients. Indeed, $S1P_4$ receptor expression was associated with shorter disease-specific patient survival and disease-free cancer recurrence in ERα negative breast cancer. In addition to the role of $S1P_4$ receptor in disease progression, first grade tumours expressed the highest levels of $S1P_4$ receptor indicating that $S1P_4$ receptor might be involved in tumourigenesis of breast tissue.

The expression of SK1 was associated with shorter disease-free cancer recurrence in ERα negative breast cancer patients. Moreover, the effect of SK1 expression on patient survival was more pronounced in patients with tumours expressing low levels of $S1P_4$ receptor. This suggests that the SK1 and $S1P_4$ receptor activation might be partially independent in development of breast cancer. Supportingly, Long and coworkers have shown that the

ERK-1/2 activation stimulated by S1P was dependent on the activation of both HER2 and $S1P_4$ receptors in MDA-MB-453 cells (Long *et al.,* 2010b). Indeed, the loss of HER2 receptor expression abrogated the effect of SK1 and $S1P_4$ receptor on cancer recurrence in ERα negative breast cancer patients. Furthermore, the $S1P_4$ receptor expression was correlated exclusively with the expression levels of SK1 in ERα negative breast tumours suggesting that the $S1P_4$ receptor activation might be mediated by SK1 through 'inside-out' signalling. Indeed, SK1/2 dual inhibitor SKi abrogated S1P-stimulated ERK-1/2 activation in MDA-MB-453 cells suggesting that SK1 activity was required for the $S1P_4$/HER2-mediated ERK-1/2 activation. Thus, SK1 inhibitors and $S1P_4$ receptor antagonists might represent novel therapeutics for the treatment of patients with HER2 positive/ERα negative breast cancer.

CHAPTER V

The role of $S1P_2$ receptor in triple negative breast cancer

5.1. Introduction

S1P activates diverse array of signalling pathways through S1P receptors. Moreover, cell type-specific expression of the S1P receptors determines the effect of S1P on cell function. For example, the S1P-induced migration of KM-H2 and SUP-HD1 Hodgkin lymphoma cell lines was mediated through an $S1P_1$ receptor/Gi/PI3K signalling pathway (Kluk *et al.,* 2013). In contrast, the activation of $S1P_2$ receptor signalling inhibited the migration of these cells. Indeed, Yamashita and coworkers have shown that the S1P-induced chemotaxis was determined by the expression of S1P receptor subtypes (Yamashita *et al.,* 2006). For example, S1P stimulation of MKN1 and HCG-27 gastric cancer cell lines expressing exclusively $S1P_3$ receptor had an enhanced migratory phenotype. However, the S1P stimulation of AZ-521 gastric cancer cells expressing exclusively $S1P_2$ receptor inhibited the migration of these cells (Yamashita *et al.,* 2006). In support of these findings, the $S1P_2$ receptor signalling was shown to inhibit cell migration through activation of Rho kinase that prevents the Rac kinase activation required for migration of B16 melanoma cells (Arikawa *et al.,* 2003).

In contrast, the $S1P_2$ receptor activation enhanced the invasiveness of glioblastoma multiforme cell lines through up-regulation of CCN1/Cyr61 expression, a matricellular protein crucial for cell adhesion (Young *et al.,* 2007). Moreover, Ponnusamy and coworkers have shown that the activation of $S1P_2$ receptor inhibits the expression of Brms1 (breast carcinoma metastasis suppressor 1), a suppressor of metastasis in MB49 murine bladder cancer cells (Ponnusamy *et al.,* 2012). In addition, $S1P_2$ receptor signalling induces the activation of ezrin, radixin and moesin (ERM) proteins required for cancer metastasis through formation of filopodia upon S1P stimulation in HeLa cells (Gandy *et al.,* 2013).

In addition, the $S1P_2$ receptor signalling regulates cell survival and proliferation. For example, the S1P-mediated activation of $S1P_2$ receptor inhibited the proliferation of WiT49 Wilms tumour cells through induction of Connective Tissue Growth Factor (CTGF) expression (Li *et al.,* 2008). CTGF expression was regulated by Rho/ROCK and JNK signalling pathways in these cells. Moreover, CTGF expression was down-regulated in Wilms tumour tissue samples indicating that the suppression of $S1P_2$ receptor signalling

might be crucial for cancer progression. In contrast, the $S1P_2$ receptor signalling was shown to stabilise the Bcr-Abl1 tyrosine kinase through inhibition of PP2A in human chronic myeloid leukemia cell lines. The dephosphorylation of Bcr-Abl1 induces its proteasomal degradation (Salas *et al.,* 2011). In addition, the inhibition of Bcr-Abl1 degradation leads to development of imatinib resistance in chronic myeloid leukemia cell lines (Salas *et al.,* 2011). Thus, the role of $S1P_2$ receptor in cancer is not fully understood.

Triple negative breast cancer comprises 15-20% of breast cancer cases. These tumours are devoid of ERα, PgR and HER2 receptor expression, thus the treatment of these tumours comprise solely classical chemotherapy. Moreover, triple negative breast cancers have the worst survival outcome when compared to other types of breast cancer (Rouzier *et al.,* 2005). Classical chemotherapy comprised of anthracyclines (doxorubicin, epirubicin), taxanes (paclitaxel, docetaxel), nucleoside analogs (capecitabine, gemcitabine) and epothilone analogs are commonly used in treatment of triple negative breast cancer (Brouckaert *et al.,* 2012). However, these cytotoxic agents are not tumour-selective, hence pharmaceuticals selective for tumours are in demand.

In this chapter, the role of $S1P_2$ receptor signalling in MDA-MB-231 cells was investigated using pharmacological SK1 and SK2 selective inhibitors (SKi, ROMe, RB-005 and compound 55-22), S1P receptor antagonists (JTE-013, CAY10444, and CYM50367) and siRNA approach. MDA-MB-231 cells are triple negative human breast cancer cell line isolated from 56 years old female caucasian (Cailleau *et al.,* 1974). The transient expression of HA-tagged $S1P_2$ receptor in MDA-MB-231 cells did not inhibit cell proliferation determined using xCELLigence assay. However, inhibition of either SK2 and/ or $S1P_4$ signalling induced the accumulation of $S1P_2$ receptor into the nucleus and this was associated with inhibition of cellular growth. Thus, SK2 inhibitors and $S1P_4$ receptor antagonists represent novel therapeutics for the treatment of triple negative breast cancer patients.

5.2. Results

5.2.1. The effect of the SK1/SK2 dual inhibitor SKi on the nuclear accumulation of the HA-S1P$_2$ receptor

MDA-MB-231 cells were transfected with HA-S1P$_2$ receptor plasmid construct and nuclei was isolated (**Figure 5.1A**). The S1P$_2$ receptor was detected using anti-HA antibody. The HA-S1P$_2$ receptor was present in the nucleus of untreated cells (**Figure 5.1B)**. Moreover, the level of HA-S1P$_2$ receptor in the nucleus was significantly enhanced upon SKi treatment, a dual inhibitor of SK1 and SK2 activity (**Figure 5.1B/C)**. Furthermore, SKi treatment also increased the levels of c-SRC and Y416 phosphorylated c-SRC in the nucleus (**Figure 5.1B/D/E)**. SKi treatment of cells transfected with empty vector also significantly increased c-SRC levels in the nucleus. However, the Y416 phosphorylation state of nuclear c-SRC was not altered (**Figure 5.2A/B/C**). Thus, the phosphorylation of c-SRC might be dependent on the expression of S1P$_2$ receptor in these cells. In addition, HA-S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC levels were not altered upon SKi treatment in total cell lysates suggesting that the observed increase in the levels of these proteins in the nucleus was not due to cellular up-regulation of expression (**Figure 5.3A/B**).

The S1P stimulation prevents the effect of SKi on nuclear accumulation of HA-S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC in cells over-expressing HA-S1P$_2$ receptor (**Figure 5.1B/C/D/E)**. Indeed, the pharmacological inhibition or siRNA-mediated down-regulation of S1P$_4$ receptor induces the accumulation of HA-S1P$_2$ receptor into the nucleus (**Figure 5.10B/F/G**). Thus, the S1P$_4$ receptor activation by S1P might inhibit the nuclear accumulation of HA-S1P$_2$ receptor. However, the inhibition of S1P$_4$ receptor activation did not induce the nuclear accumulation of c-SRC or Y416 phosphorylated c-SRC. This suggests that the nuclear accumulation of these proteins were independent of S1P$_4$ receptor. Thus, the nuclear accumulation of c-SRC and Y416 phosphorylated c-SRC in response to inhibition of SK1/2 might involve the alteration in activity of an intracellular target that binds S1P. The role of S1P receptors in nuclear accumulation of the S1P$_2$ receptor is further discussed in Section 5.2.4 and Section 5.2.5.

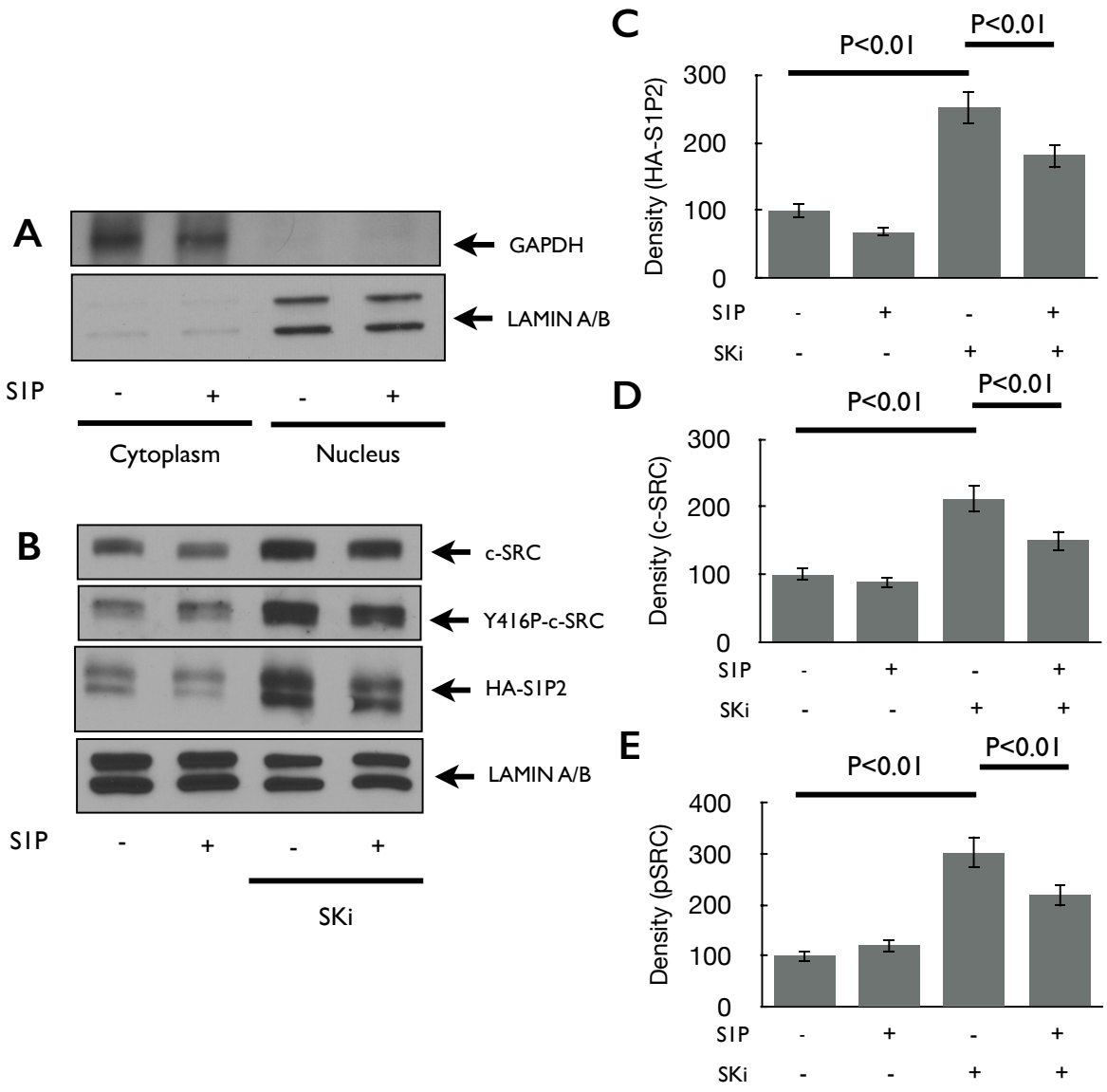

Figure 5.1. The western blots and densitometric quantifications showing the effect of SKi treatment on the nuclear accumulation of HA-S1P$_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in HA-S1P$_2$ transfected MDA-MB-231 cells. MDA-MB-231 cells were treated with SKi (10 μM) for 4 hours before stimulation with or without S1P (5 μM, 1 hour). **A** The western blots showing the purity of cytoplasmic and nuclear fractions probed with anti-GAPDH and anti-Lamin A/B antibodies; **B** The western blots showing the change in the nuclear levels of c-SRC, Y416 phosphorylated c-SRC and HA-S1P$_2$ upon treatment with SKi and/or S1P; **C** Densitometric quantification of blot probed with anti-HA TAG antibody; **D** The densitometric quantifications of blot probed with anti-c-SRC antibody; **E** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation. Unpaired Student's t-Test was used to assess the significance between different subgroups.

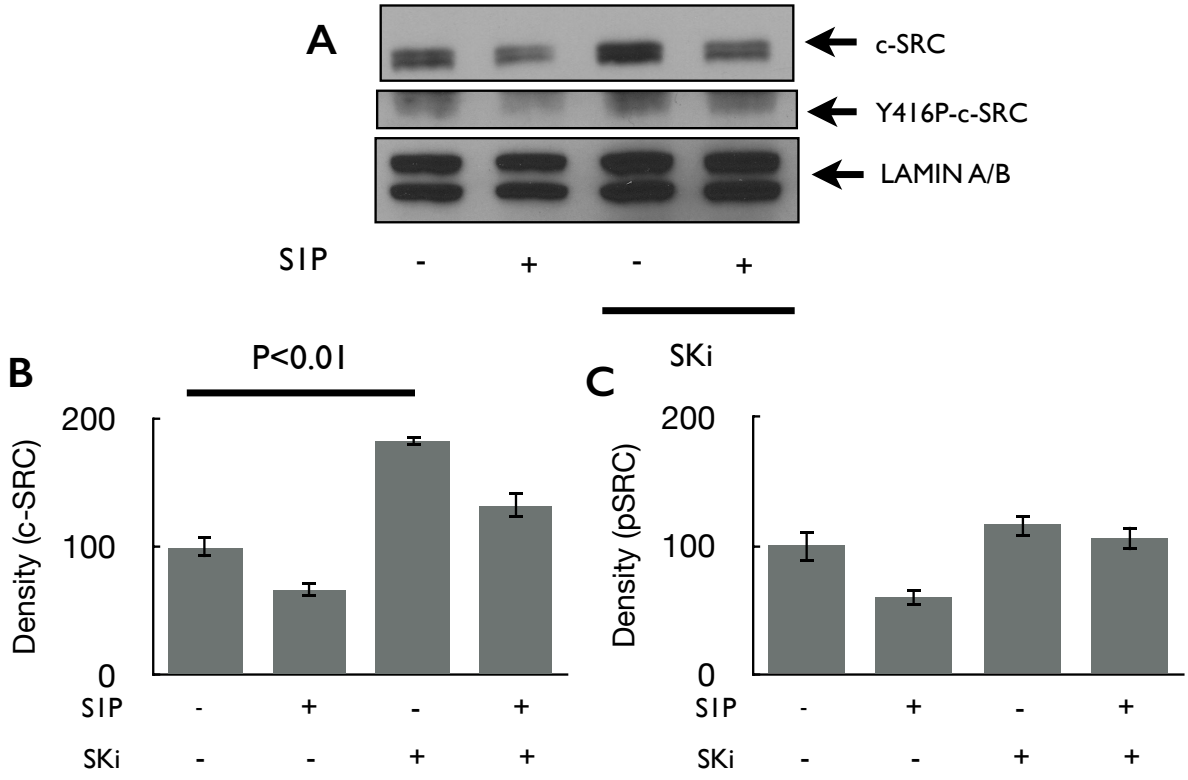

Figure 5.2. The western blots and densitometric quantifications showing the effect of SKi treatment on the nuclear accumulation of HA-S1P$_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector transfected MDA-MB-231 cells. MDA-MB-231 cells were treated with SKi (10 μM) for 4 hours before stimulation with or without S1P (5 μM, 1 hour). **A** The western blots showing the change in the nuclear levels of c-SRC and Y416 phosphorylated c-SRC upon treatment with SKi and/or S1P; **B** The densitometric quantification of blot probed with anti-c-SRC antibody; **C** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation. Unpaired Student's t-Test was used to assess the significance between different subgroups.

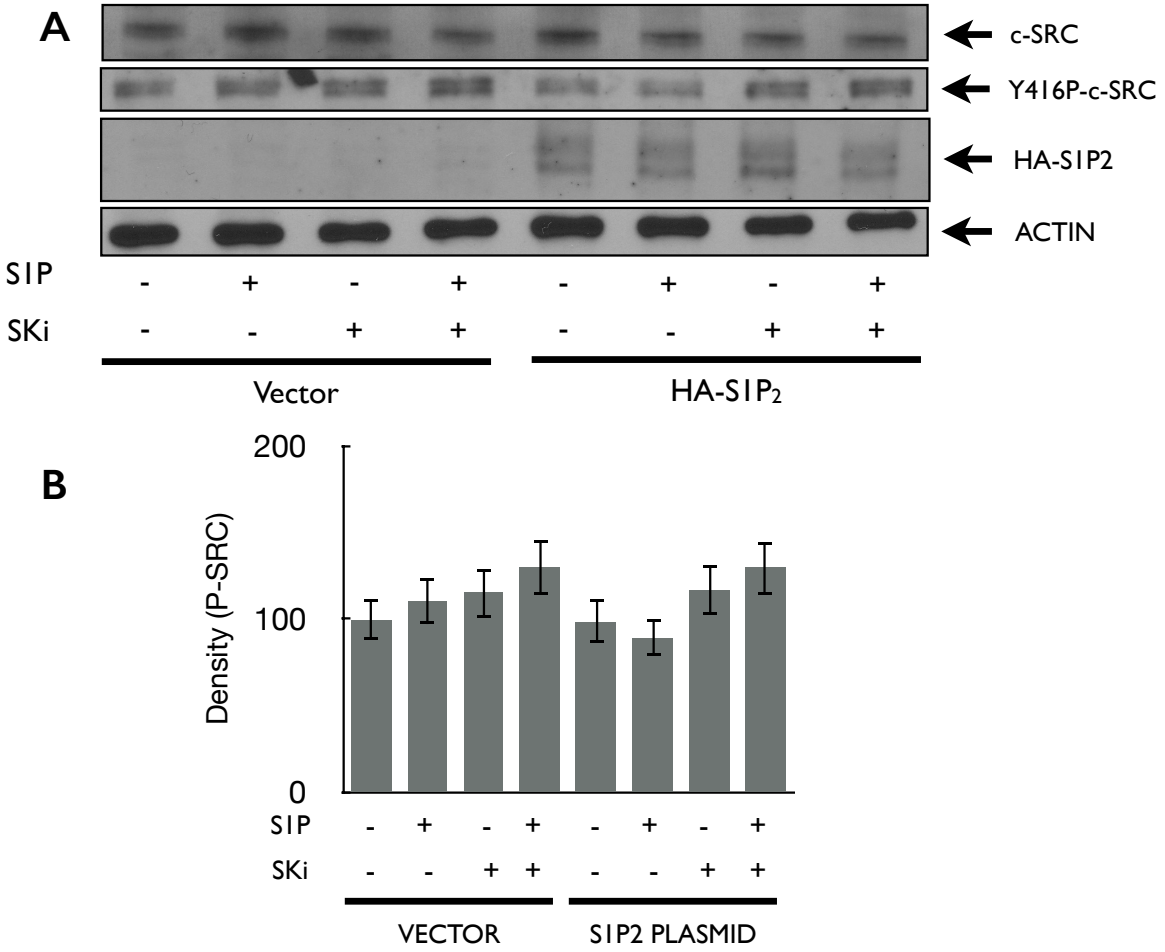

Figure 5.3. The western blots and densitometric quantifications showing the effect of SKi treatment on the cellular expression levels of HA-S1P$_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector or HA-S1P$_2$ transfected MDA-MB-231 cells. MDA-MB-231 cells were treated with SKi (10 μM) for 4 hours before stimulation with or without S1P (5 μM, 1 hour). **A** The western blots showing the change in cellular levels of HA-S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon treatment with SKi and/or S1P; **B** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against actin expression and expressed in percentages relative to control +/- relative standard deviation.

5.2.2. The effect of the SK1 selective inhibitors on the nuclear accumulation of the HA-S1P$_2$ receptor

The effect of SK1 and SK2 selective inhibitors was also tested on the nuclear accumulation of S1P$_2$ receptor. SK1 selective inhibitors RB-005 (Baek *et al.,* 2013) and compound 55-22 (Byun *et al.,* 2013) did not induce the nuclear accumulation of S1P$_2$ receptor (**Figure 5.4A/B**). Moreover, these compounds did not induce the nuclear accumulation of c-SRC or Y416 phosphorylated c-SRC in cells transfected with empty vector or HA-S1P$_2$ receptor plasmid construct. In support of these findings, SK1 siRNA mediated down-regulation of SK1 did not induce the nuclear accumulation of HA-S1P$_2$ receptor or Y416 phosphorylated c-SRC (**Figure 5.5A/C/D**). In addition, the levels of c-SRC and Y416 phosphorylated c-SRC were not altered in cells transfected with empty vector or HA-S1P$_2$ receptor plasmid construct. SK1 mRNA levels were successfully down-regulated in these cells using SK1 siRNA compared to scrambled siRNA (**Figure 5.5B)**.

5.2.3. The effect of the SK2 selective inhibitors on the nuclear accumulation of the HA-S1P$_2$ receptor

The treatment of MDA-MB-231 cells with the SK2 selective inhibitor ROMe induced the nuclear accumulation of both HA-S1P$_2$ receptor and Y416 phosphorylated c-SRC (**Figure 5.6A/D/E)**. Moreover, S1P stimulation prevents the nuclear accumulation of HA-S1P$_2$ receptor in ROMe treated cells. In support of these findings, SK2 siRNA mediated down-regulation of SK2 expression induced the nuclear accumulation of both HA-S1P$_2$ receptor and Y416 phosphorylated c-SRC (**Figure 5.6B/F/G)**. However, S1P stimulation did not prevent this effect. The treatment of cells transfected with empty vector with ROMe or SK2 siRNA did not promote the nuclear accumulation of Y416 phosphorylated c-SRC (**Figure 5.6A/B)**. In addition, the expression levels of HA-S1P$_2$ receptor or Y416 phosphorylated c-SRC in total lysate were not altered in cells treated with ROMe (**Figure 5.7A/B/C)**. Thus, the increase in levels of these proteins in the nucleus was not due to up-regulation of protein expression. In addition, HA-S1P$_2$ receptor levels were not altered upon S1P stimulation in total cell lysates. The SK2 expression was successfully down-regulated in MDA-MB-231 cells treated with SK2 siRNA compared to scrambled siRNA (**Figure 5.6C)**.

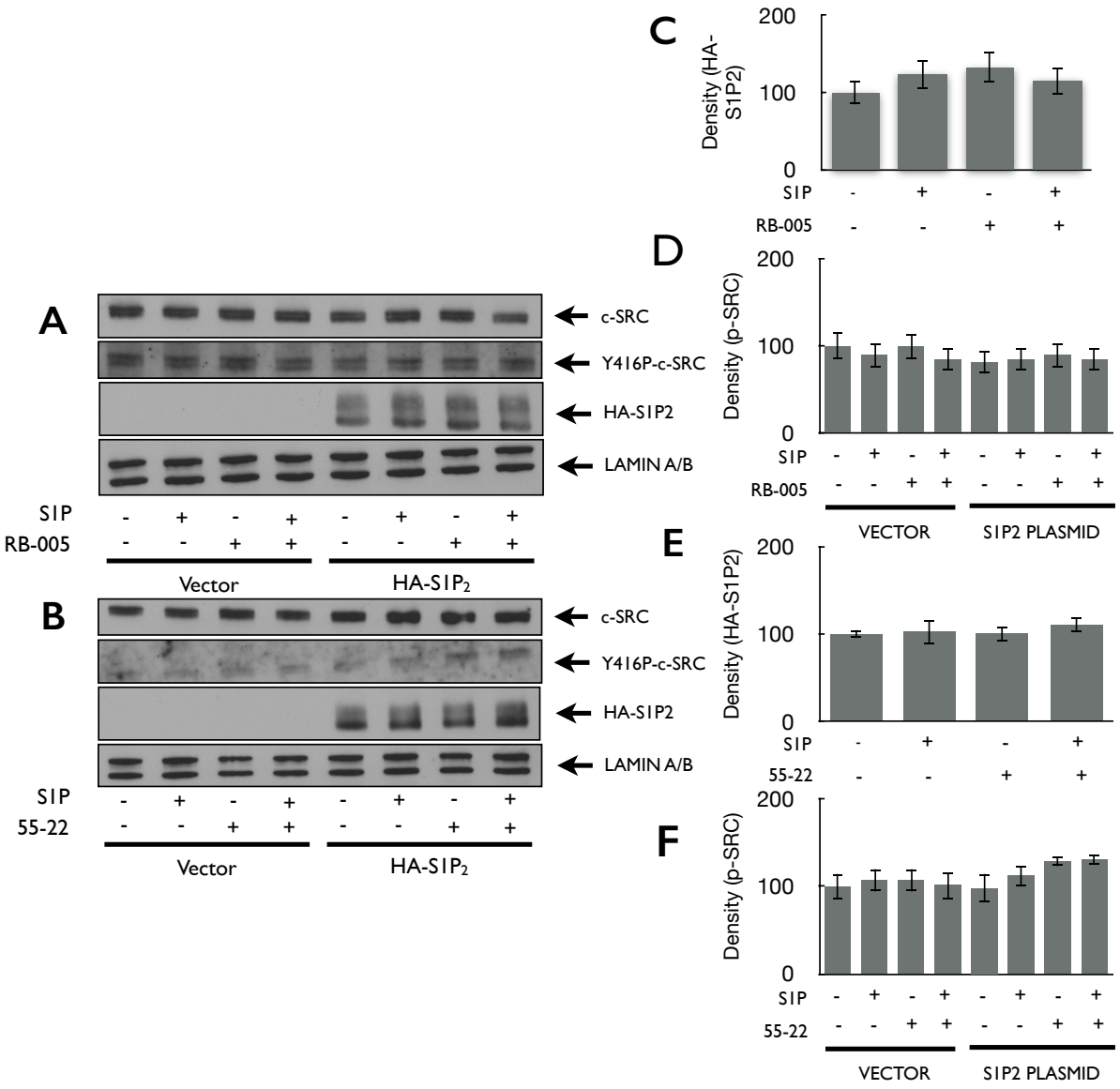

Figure 5.4. The western blots and densitometric quantifications showing the effect of SK1 inhibitors on the nuclear levels of HA-S1P$_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector or HA-S1P$_2$ transfected MDA-MB-231 cells. MDA-MB-231 cells were treated with inhibitors (10 μM) for 4 hours before stimulation with or without S1P (5 μM, 1 hour). **A** The western blots showing the change in nuclear levels of HA-S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon treatment with RB-005 and/or S1P; **B** The western blots showing the change in nuclear levels of HA-S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon treatment with 55-22 and/or S1P; **C, E** The densitometric quantification of blot probed with anti-HA TAG antibody; **D, F** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation.

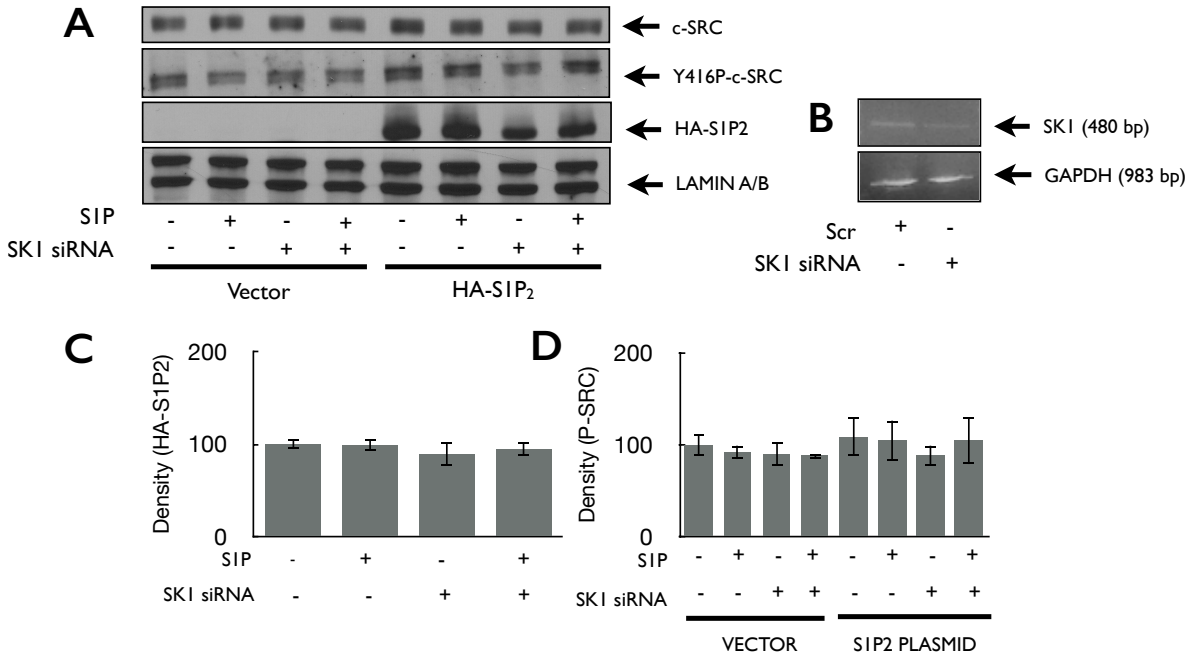

Figure 5.5. The western blots and densitometric quantifications showing the effect of SK1 down-regulation on the nuclear levels of HA-S1P$_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector or HA-S1P$_2$ transfected MDA-MB-231 cells. **A** The western blots showing the change in nuclear levels of S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon SK1 down-regulation and/or stimulated with/without S1P; **B** The RT-PCR mediated analysis of SK1 expression upon treatment with either scrambled (Scr) or SK1-selective siRNA; **C, D** The densitometric quantification of blot probed with anti-HA TAG antibody and anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation.

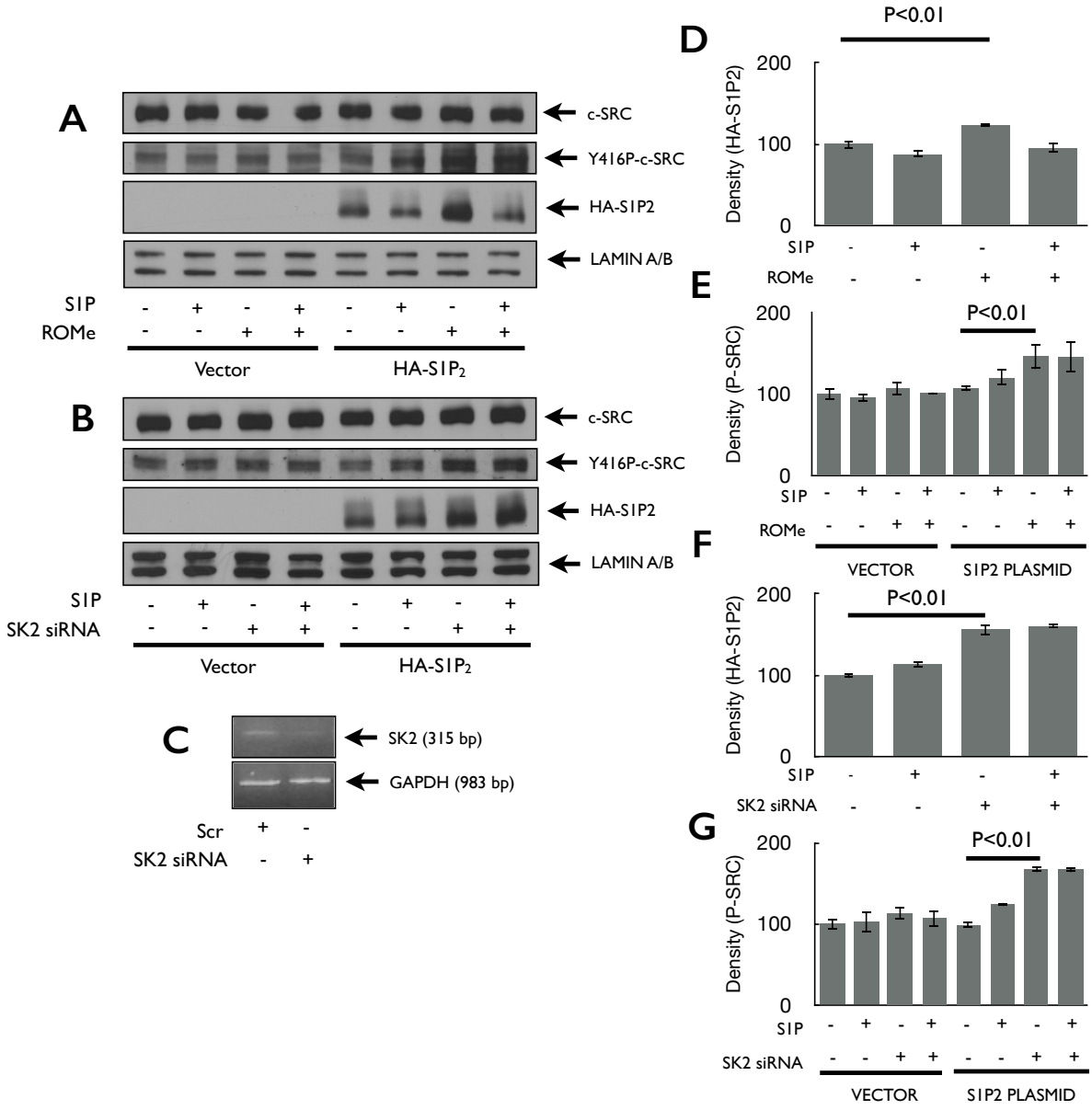

Figure 5.6. The western blots and densitometric quantifications showing the effect of inhibition or SK2 siRNA mediated down-regulation of SK2 on the nuclear levels of HA-S1P$_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector or HA-S1P$_2$ transfected MDA-MB-231 cells. **A** The western blots showing the change in nuclear levels of HA-S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon treatment with SK2 inhibitor and/or S1P; **B** The western blots showing the change in nuclear levels of HA-S1P$_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon SK2 down-regulation and/or stimulation with S1P; **C** The RT-PCR mediated analysis of SK2 expression upon treatment with either scrambled (Scr) or SK2-selective siRNA; **D, F** The densitometric quantification of blot probed with anti-HA TAG antibody. **E, G** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation.

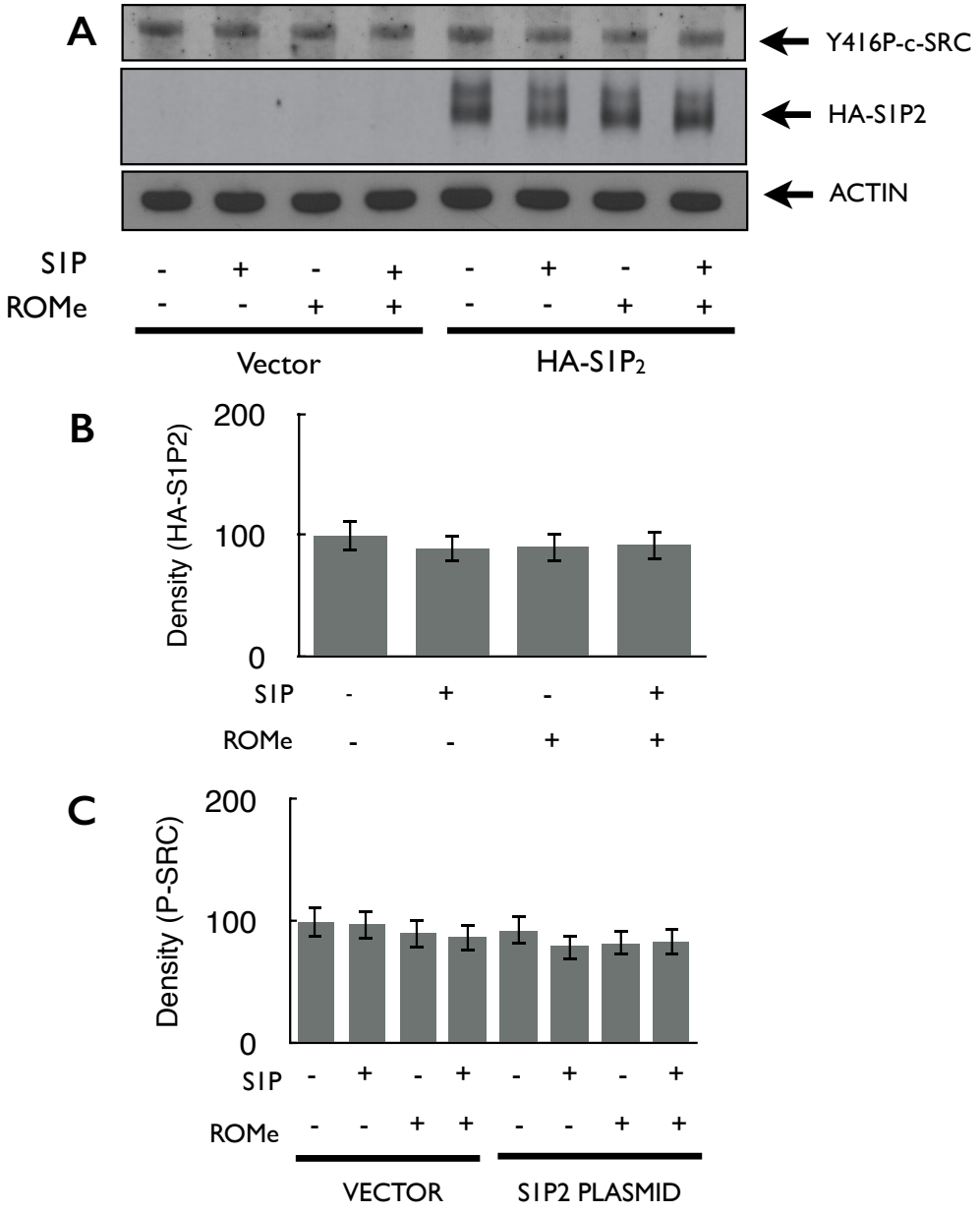

Figure 5.7. The western blots and densitometric quantifications showing the effect of ROMe on the cellular expression levels of HA-S1P$_2$ receptor and Y416 phosphorylated c-SRC in empty vector or HA-S1P$_2$ transfected MDA-MB-231 cells. **A** The western blots showing the change in cellular levels of HA-S1P$_2$ receptor and Y416 phosphorylated c-SRC upon treatment with SK2 inhibitor and/or S1P; **B** The densitometric quantification of blot probed with anti-HA TAG antibody; **C** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against actin expression and expressed in percentages relative to control +/- relative standard deviation.

5.2.4. The effect of the S1P receptor antagonists on the nuclear accumulation of the HA-S1P$_2$ receptor

The S1P stimulation prevents the nuclear accumulation of HA-S1P$_2$ receptor upon treatment with SKi or ROMe. Thus, the role of *other* S1P receptors in nuclear accumulation of HA-S1P$_2$ receptor was tested. The treatment of MDA-MB-231 cells with JTE-013, an antagonist of S1P$_2$ and S1P$_4$ receptors, did not induce the nuclear accumulation of HA-S1P$_2$ receptor or Y416 phosphorylated c-SRC (**Figure 5.8A/C/D)**. Similarly, the treatment with CAY10444, an antagonist of S1P$_3$ receptor did not induce the nuclear accumulation of HA-S1P$_2$ receptor or Y416 phosphorylated c-SRC (**Figure 5.8B/ E/F)**. In support of these data, the treatment of cells with S1P$_3$ siRNA did not induce the nuclear accumulation of HA-S1P$_2$ receptor or Y416 phosphorylated c-SRC (**Figure 5.9A/ C/D)**. The S1P$_3$ receptor expression was successfully down-regulated in MDA-MB-231 cells treated with S1P$_3$ siRNA compared to scrambled siRNA (**Figure 5.9B)**.

The treatment of MDA-MB-231 cells with S1P$_4$ receptor antagonist CYM50367 induced the nuclear accumulation of HA-S1P$_2$ receptor. However, the expression levels of Y416 phosphorylated c-SRC in the nucleus was not altered (**Figure 5.10A/D/E)**. Moreover, the S1P stimulation prevented the nuclear accumulation of HA-S1P$_2$ receptor. Similarly, S1P$_4$ siRNA induced the nuclear accumulation of HA-S1P$_2$ receptor (**Figure 5.10B/F/G)**. Interestingly, the S1P stimulation did not prevent the nuclear accumulation of HA-S1P$_2$ receptor suggesting that the nuclear accumulation of HA-S1P$_2$ receptor is normally restrained by S1P binding to S1P$_4$ receptor. The expression levels of HA-S1P$_2$ receptor was not altered upon down-regulation of S1P$_4$ receptor in total cell lysates indicating that the increase in nuclear levels of HA-S1P$_2$ was not due to up-regulation of HA-S1P$_2$ receptor expression (**Figure 5.11A/B/C)**. Moreover, the levels of HA-S1P$_2$ receptor were not altered upon S1P stimulation in total cell lysates. The S1P$_4$ receptor expression was successfully down-regulated in MDA-MB-231 cells treated with S1P$_4$ siRNA compared to scrambled siRNA (**Figure 5.10C)**.

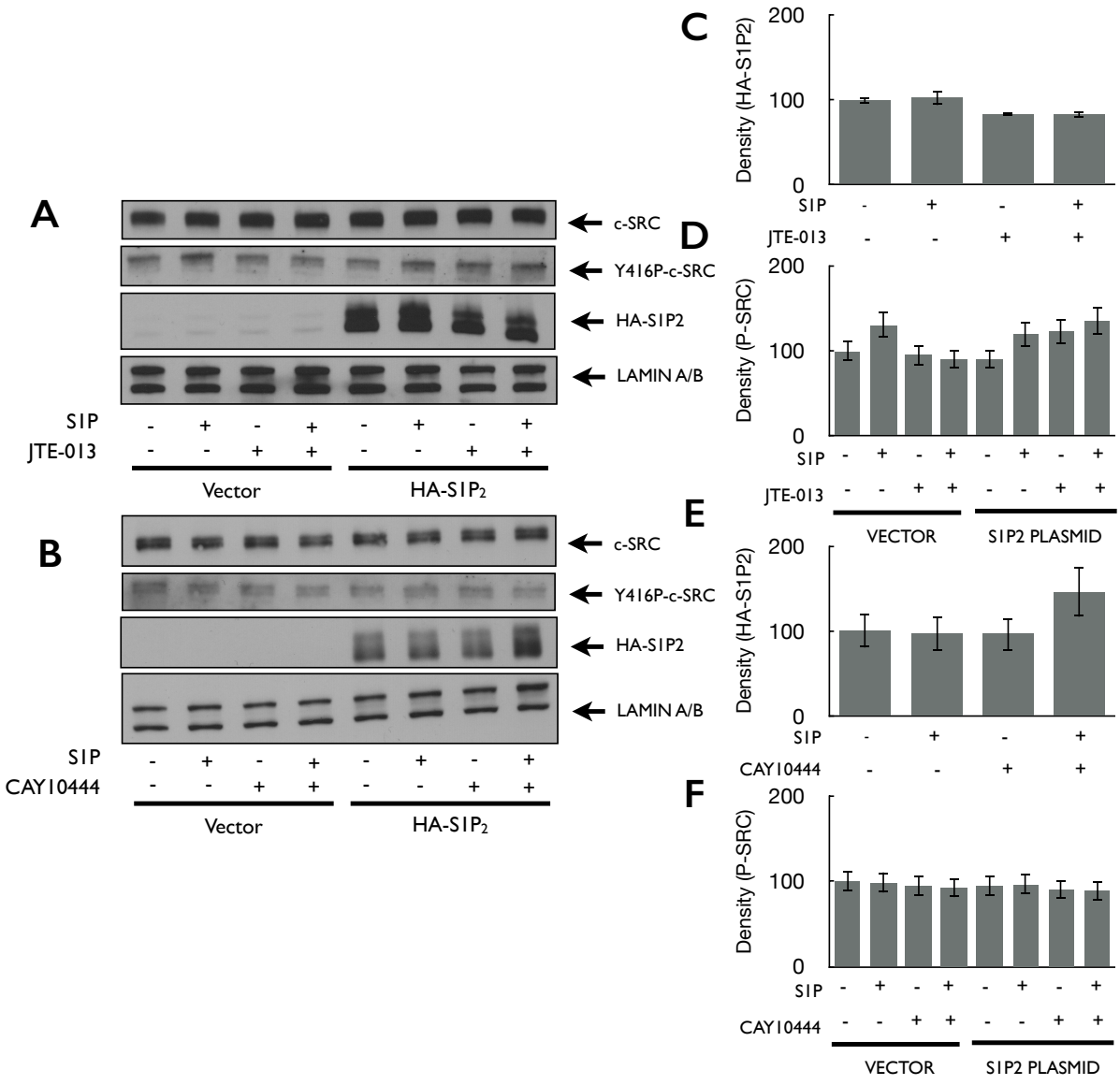

Figure 5.8. The western blots and densitometric quantifications showing the effect of $S1P_2$ receptor antagonist or $S1P_3$ receptor antagonist on the nuclear levels of HA-$S1P_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector or HA-$S1P_2$ transfected MDA-MB-231 cells. MDA-MB-231 cells were treated with antagonists (10 μM) for 4 hours before stimulation with or without S1P (5 μM, 1 hour). **A** The western blots showing the change in nuclear levels of HA-$S1P_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon treatment with JTE-013 and/or S1P; **B** The western blots showing the change in nuclear levels of HA-$S1P_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon treatment with CAY10444 and/or S1P; **C, E** The densitometric quantification of blot probed with anti-HA TAG antibody; **D, F** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation.

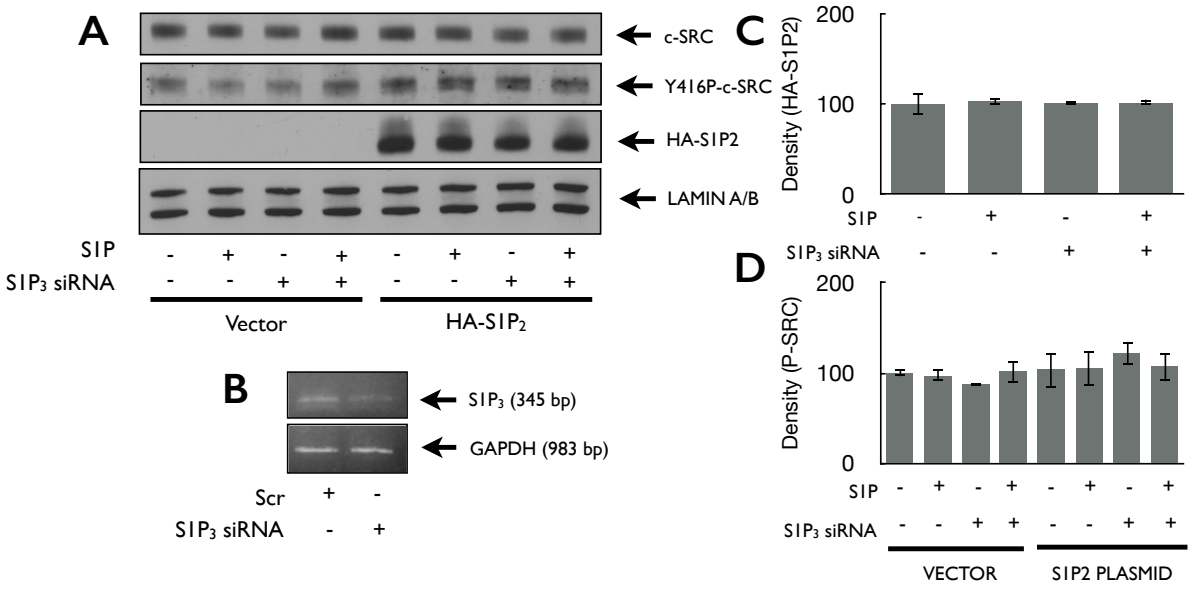

Figure 5.9. The western blots and densitometric quantifications showing the effect of $S1P_3$ receptor down-regulation on the nuclear levels of HA-$S1P_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector or HA-$S1P_2$ transfected MDA-MB-231 cells. **A** The western blots showing the change in nuclear levels of HA-$S1P_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon $S1P_3$ receptor down-regulation and/or stimulation with S1P; **B** The RT-PCR mediated analysis of $S1P_3$ receptor expression upon treatment with either scrambled (Scr) or $S1P_3$ receptor-selective siRNA; **C** The densitometric quantification of blot probed with anti-HA TAG antibody; **D** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation.

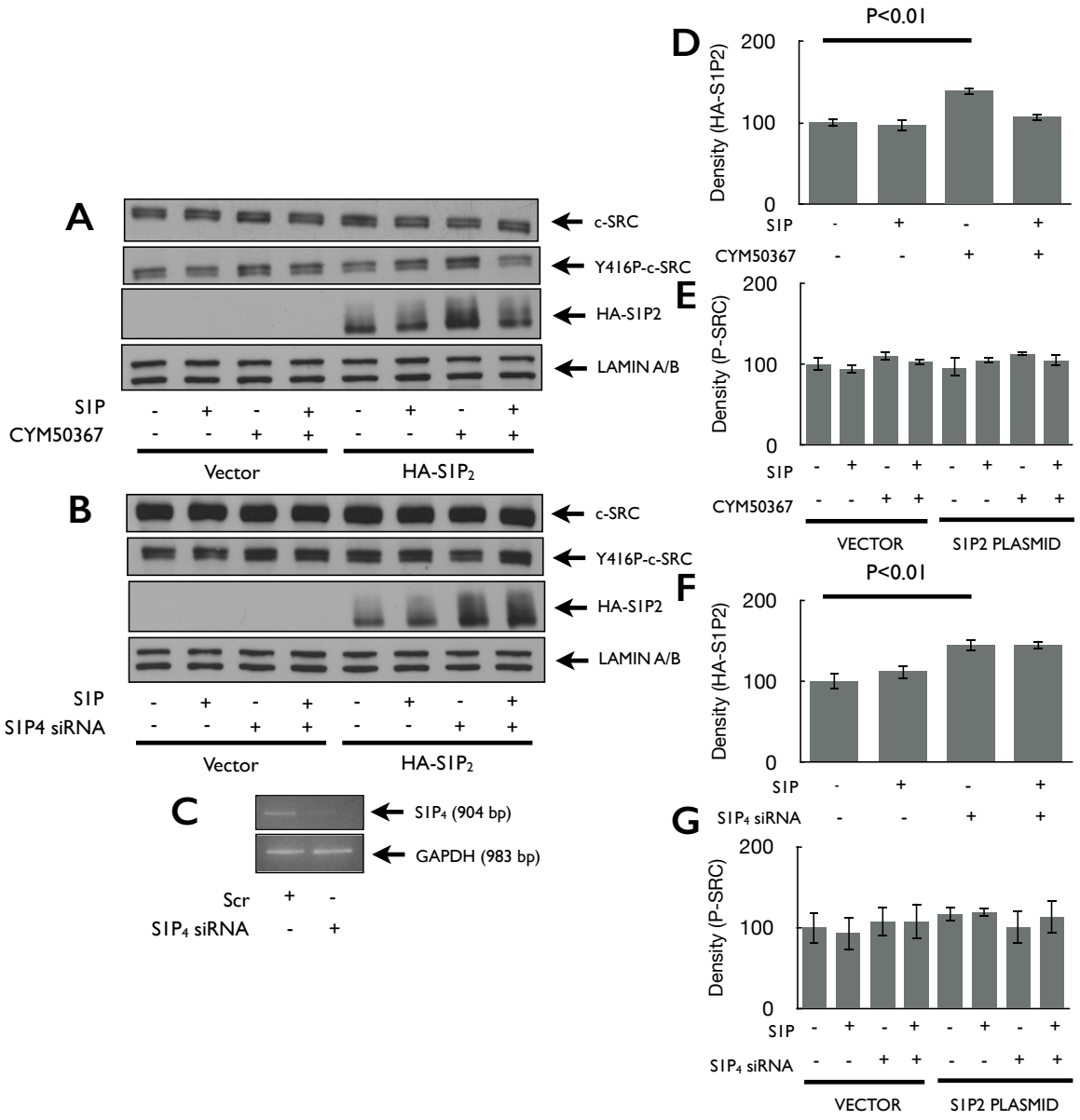

Figure 5.10. The western blots and densitometric quantifications showing the effect of $S1P_4$ receptor inhibition or down-regulation on the nuclear levels of HA-$S1P_2$ receptor, Y416 phosphorylated c-SRC and c-SRC in empty vector or HA-$S1P_2$ transfected MDA-MB-231 cells. **A** The western blots showing the change in nuclear levels of HA-$S1P_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon treatment with $S1P_4$ receptor antagonist and/or S1P; **B** The western blots showing the change in nuclear levels of HA-$S1P_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon $S1P_4$ receptor down-regulation and/or stimulation with S1P; **C** The RT-PCR mediated analysis of $S1P_4$ receptor expression upon treatment with either scrambled (Scr) or $S1P_4$ receptor-selective siRNA; **D, F** The densitometric quantification of blot probed with anti-HA TAG antibody; **E, G** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against lamin A/B expression and expressed in percentages relative to control +/- relative standard deviation.

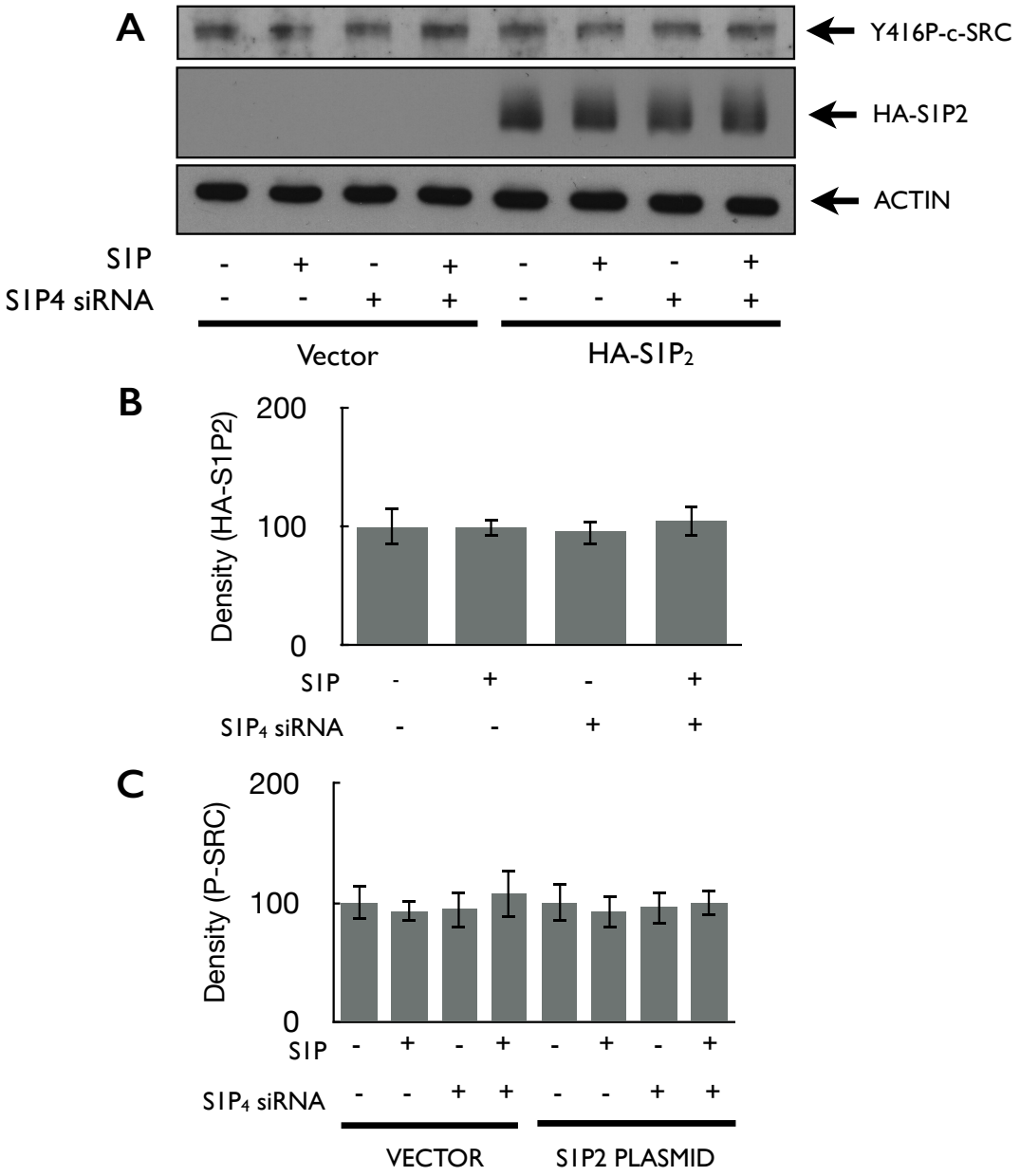

Figure 5.11. The western blots and densitometric quantifications showing the effect of $S1P_4$ receptor down-regulation on the cellular expression levels of HA-$S1P_2$ receptor, and Y416 phosphorylated c-SRC in empty vector or HA-$S1P_2$ transfected MDA-MB-231 cells. **A** The western blots showing the change in cellular levels of HA-$S1P_2$ receptor, c-SRC and Y416 phosphorylated c-SRC upon $S1P_4$ receptor down-regulation and/or stimulation with S1P; **B** The densitometric quantification of blot probed with anti-HA TAG antibody; **C** The densitometric quantification of blot probed with anti-Y416 phosphorylated c-SRC antibody. Results from three separate experiments were standardised against actin expression and expressed in percentages relative to control +/- relative standard deviation.

5.2.5. The effect of nuclear accumulation of HA-S1P$_2$ receptor and Y416 phosphorylated c-SRC on cell growth

The effect of nuclear accumulation of HA-S1P$_2$ receptor and Y416 phosphorylated c-SRC on cellular growth was measured using xCELLigence system. MDA-MB-231 cells transfected with either HA-S1P$_2$ receptor plasmid construct or empty vector was separately treated with SKi, RB-005, compound 55-22, ROMe, CAY10444, JTE-013, CYM50367 or siRNA targeting SK1 or SK2. The expression of HA-S1P$_2$ receptor had no effects on cellular growth compared to cells transfected with empty vector (**Figure 5.12)**. In all cases, the growth of MDA-MB-231 cells was reduced compared to cells transfected with either empty vector or HA-S1P$_2$ receptor plasmid construct. Moreover, the treatment of MDA-MB-231 cells expressing HA-S1P$_2$ receptor with SKi, ROMe, CYM50367 or SK2 siRNA exhibited a more significant reduction in cellular growth compared to vector transfected counterparts treated with these agents. The reduction in cellular growth upon treatment with RB-005, compound 55-22, CAY10444, JTE-013 or siRNA SK1 siRNA was comparable between HA-S1P$_2$ receptor and empty vector transfected MDA-MB-231 cells.

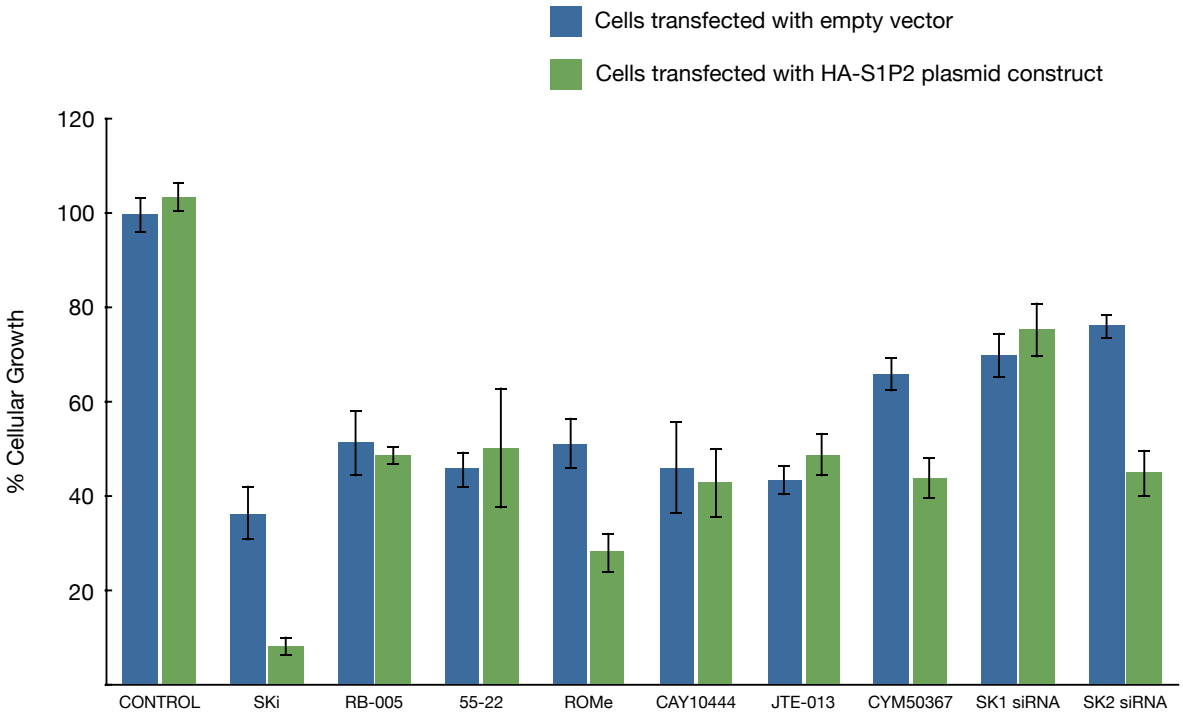

Figure 5.12. The effect of SKi, RB-005, compound 55-22, ROMe, CAY10444, JTE-003, CYM50367, FTY720, SK1 siRNA and SK2 siRNA treatment on the growth of MDA-MB-231 cells transfected with vector or HA-S1P$_2$ plasmid construct. Cells were treated with the inhibitors/receptor antagonist (10 uM) or SK1/SK2-specific siRNA for 4 hours. Cellular growth was monitored by xCELLigence system software. Results were expressed in percentages relative to control +/- relative standard deviation. Each treatment was prepared in triplicates (n=3).

5.3. Discussion

The treatment of MDA-MB-231 cells transiently over-expressing HA-$S1P_2$ receptor with dual SK1/2 inhibitor SKi or SK2 selective inhibitor ROMe or siRNA-mediated down-regulation of SK2 expression induced the nuclear accumulation of HA-$S1P_2$ receptor and this was associated with reduced cellular growth. Indeed, the nuclear accumulation of GPCRs were previously reported in various studies e.g. $S1P_1$, AT1 and LPA_1 (Liao et al., 2007, Waters et a., 2006, Lee et al., 2004). Moreover, SK2 inhibitor ROMe was more effective in inhibiting cell growth compared to SK1 selective inhibitors in cells over-expressing HA-$S1P_2$ receptor. Supporting these findings, Antoon and coworkers have shown that the inhibition of SK2 activity reduced the proliferation and viability of MDA-MB-231 cells (Antoon *et al.,* 2011). Similarly, Gao and Smith (2011) have shown that the siRNA-mediated down-regulation of SK2 expression was more effective in inhibiting cell proliferation compared to down-regulation of SK1 expression in MDA-MB-231 cells. In the same study, the inhibition of SK2 expression reduced the AKT and ERK2 kinase activation suggesting that the SK2/S1P-induced cell proliferation was mediated by these kinases (Gao and Smith, 2011).

The nuclear accumulation of HA-$S1P_2$ receptor was also regulated by *other* S1P receptors. Indeed, S1P stimulation prevented the SKi/ROMe-induced nuclear accumulation of HA-$S1P_2$ receptor. The S1P receptors involved in nuclear accumulation of HA-$S1P_2$ receptor was identified using pharmacological inhibitors and siRNA-mediated down-regulation of receptor expression. The treatment of MDA-MB-231 cells with $S1P_4$ receptor antagonist CYM50367 induced the nuclear accumulation of HA-$S1P_2$ receptor. Moreover, S1P stimulation prevented the nuclear accumulation of HA-$S1P_2$ receptor induced by the $S1P_4$ receptor antagonist. Similarly, siRNA-mediated down-regulation of $S1P_4$ receptor expression also induced the nuclear accumulation of HA-$S1P_2$ receptor. The S1P stimulation of the cells transfected with $S1P_4$ siRNA did not prevent the nuclear accumulation of HA-$S1P_2$ receptor. Similar to SKi and ROMe, the inhibition of $S1P_4$ receptor activation was more effective in inhibiting cell growth in HA-$S1P_2$ receptor over-expressing cells compared to cells transfected with the empty vector.

These findings suggest a possible functional interaction between SK2, $S1P_4$ and $S1P_2$ receptor. Indeed, S1P synthesised by SK2 might activate the $S1P_4$ receptor through 'inside-out' signalling that might induce its heterodimerisation with the $S1P_2$ receptor to prevent the nuclear accumulation of $S1P_2$ receptor into the nucleus. Interestingly, S1P stimulation did not prevent the nuclear accumulation of $S1P_2$ receptor in cells transfected with SK2 siRNA suggesting that the down-regulation of SK2 expression might affect the expression of $S1P_4$ receptors required for the nuclear export of $S1P_2$ receptor. Although this hypothesis requires further investigation, the down-regulation of SK1 expression was previously shown to diminish the expression of $S1P_3$ receptor in MCF-7 breast cancer cells (Long *et al.,* 2010a).

The treatment of MDA-MB-231 cells transiently over-expressing HA-$S1P_2$ receptor with SKi or ROMe also induced the nuclear accumulation of Y416 phosphorylated c-SCR. Indeed, siRNA mediated down-regulation of SK2 expression also enhanced the nuclear accumulation of Y416 phosphorylated c-SCR in these cells. In contrast, the treatment of cells transfected with empty vector with SKi induced the nuclear accumulation of c-SRC. However the levels of Y416 phosphorylated c-SRC was not altered suggesting that the phosphorylation of c-SRC was mediated by over-expressed $S1P_2$ receptor. The nuclear accumulation of Y416 phosphorylated c-SRC upon ROMe treatment might be crucial in inhibition of cell growth. Indeed, various studies have shown that apoptosis induced by oestrogen or anticancer drug taxotere was mediated by c-SRC in MCF-7:5C breast cancer cells and HAG-1 human gallbladder cancer cells respectively (Fan *et al.,* 2013, Boudny and Nakano, 2002).

The S1P stimulation of MDA-MB-231 cells expressing HA-$S1P_2$ receptor prevented the SKi-induced nuclear accumulation of Y416 phosphorylated c-SRC. However, S1P stimulation did not prevent the nuclear accumulation of Y416 phosphorylated c-SRC in ROMe-treated cells suggesting that the SKi-induced nuclear accumulation of Y416 phosphorylated c-SRC might involve *other* cellular mechanisms. Moreover, the nuclear accumulation of Y416 phosphorylated c-SRC was independent of $S1P_4$ receptor.

In summary, a new signalling pathway between SK2, $S1P_4$, $S1P_2$ and Y416 phosphorylated c-SRC was identified in MDA-MB-231 cells. In these pathways, S1P synthesised by SK2

might activate the $S1P_4$ receptor to promote cellular growth by preventing the nuclear accumulation of $S1P_2$ receptor. However, the loss of SK2 catalytic activity impedes the $S1P_4$ receptor signalling that allows the accumulation of $S1P_2$ receptor into the nucleus. Moreover, the loss of SK2 catalytic activity allows the accumulation of Y416 phosphorylated c-SRC into the nucleus. However, the role of Y416 phosphorylated c-SRC accumulation requires further investigation. Supporting these findings, the expression of $S1P_4$ receptor was associated with poor prognosis in patients with ERα negative breast tumours (Ohotski *et al.,* 2012). Moreover, the $S1P_4$ receptor expression was correlated with cancer metastasis into lymph nodes (Pyne *et al.,* 2012). In addition, the nuclear $S1P_2$ receptor expression was associated with prolonged patient survival in ERα positive breast cancer tumours (Ohotski *et al.,* 2012). Thus, SK2 and $S1P_4$ receptor might represent novel therapeutic targets for the treatment of patients with ERα negative tumours.

CHAPTER VI

General Discussion

Breast cancer is the most common type of cancer diagnosed in women in UK and is responsible for the highest death rate after lung cancer (CRUK cancer incidence statistics 2011). Despite the therapies developed over the last decade, understanding the nature of different breast cancer subtypes is critical for development of successful treatment regimens. For example, the expression of ERα, PgR and HER2 receptors is a valuable prognostic marker in breast cancer. Thus, targeting the activation of these receptors is the primary strategy for treatment of hormone-dependent breast cancers. However, acquired resistance to endocrine therapy is a common phenomenon upon long-term treatment. Moreover, the classical chemotherapy is the sole option for these patients despite the severe side effects. Thus, novel therapeutics selectively targeting tumour cells are in demand.

SK1 and S1P-binding proteins are involved in various cellular processes such as cell proliferation, survival, migration and chemotherapeutic resistance (Pyne and Pyne, 2010). Moreover, research over the last two decades have shown that SK/S1P signalling is up-regulated in various human cancers (e.g. colon, rectal, glioblastoma, ovarian, lung and breast) making S1P signalling an important candidate as a therapeutic target (Liu *et al.,* 2010, Van Brocklyn *et al.,* 2005, Johnson *et al.,* 2005, French *et al.,* 2003, Watson *et al.,* 2010). In this study, the expression of SK1 and S1P-related proteins was analysed in ERα negative and ERα positive tumours in order to enhance our understanding on the mechanism of action of these proteins in breast carcinogenesis and progression that is crucial for the development of potential therapeutics.

The expression of SK1 was associated with the expression of S1P receptors in both ERα positive and negative cohorts. For example, SK1 expression was associated with the expression of $S1P_1$ and $S1P_3$ receptors in ERα positive cohort. Similarly, SK1 expression was associated with the expression of $S1P_1$ and $S1P_4$ receptors in ERα negative cohort. Indeed, SK1 was shown to maintain the activation of S1P receptors through 'inside-out' signalling (Hisano *et al.,* 2012, Takabe *et al.,* 2010, Kawahara *et al.,* 2009). The functional interaction between SK1 and S1P receptors was also identified using patient survival analysis. Watson and coworkers have reported that the expression of SK1, $S1P_1$ and $S1P_3$ receptors was associated with poor cancer prognosis in ERα positive breast cancer patients (Watson *et al.,* 2010). Moreover, the current study shows that the association between SK1

and cancer prognosis was dependent on the expression of $S1P_1$ and $S1P_3$ receptors. Similarly, the expression of SK1 and $S1P_4$ receptor was associated with poor cancer prognosis in ERα negative breast cancer patients. However, the association between SK1 and cancer prognosis was not dependent on the expression of $S1P_4$ receptor suggesting that *other* SK1-dependent signalling pathways might also be involved. Indeed, HER2 receptor expression was necessary to maintain the association between SK1/$S1P_4$ receptor and ERα negative cancer prognosis. In support of these findings, Long and coworkers have shown that the HER2 receptor-mediated ERK1/2 activation was dependent on the $S1P_4$ receptor activation in MDA-MB-453 breast cancer cells (Long *et al.,* 2010b). Moreover, the current study shows that the S1P/$S1P_4$ receptor-mediated ERK1/2 is also dependent on the SK1 activation in MDA-MB-453 breast cancer cells. Thus, SK1 functionally interacts with the HER2/$S1P_4$ receptor signalling pathway in ERα negative breast cancer cells. These findings show that the ERα positive breast cancer cells utilise different set of S1P receptors compared to ERα negative breast cancer cells to maintain tumour growth. Thus, distinct set of S1P receptors must be targeted depending on the molecular profile of the tumour for successful treatment of breast cancer patients using S1P receptor antagonists.

The ERK-1/2-dependent phosphorylation and activation of SK1 induces its localisation to the plasma membrane (Johnson *et al.,* 2002, Pitson *et al.,* 2003). The current study shows that the membrane-bound SK1 expression is negatively correlated with the $S1P_2$ receptor expression in the nucleus of the cells in ERα positive cohort. Moreover, the nuclear expression of $S1P_2$ receptor annuls the association between SK1 and poor cancer prognosis. Thus, the nuclear expression of $S1P_2$ receptor prolongs the survival of ERα positive breast cancer patients. The current study also shows that the association between $S1P_2$ receptor and cancer prognosis is dependent on the expression of c-SRC/Y416 phosphorylated SFK. However, the sole expression of c-SRC/Y416 phosphorylated SFK in these tumours does not prolong the patient survival suggesting that the expression of both $S1P_2$ receptor and c-SRC/Y416 phosphorylated SFK is required. Indeed, v-SRC was shown to induce apoptosis upon inhibition of ERK-1/2 and PI3K/AKT signalling pathways in Rat-2 fibroblasts (Webb *et al.,* 2000). Similarly, the down-regulation of SK1 expression might be required for the activation of $S1P_2$ receptor. Moreover, the expression of $S1P_2$ receptor and Y416 phosphorylated SFK is associated with higher apoptotic index suggesting that the $S1P_2$ receptor and SRC kinases might induce apoptosis of cancer cells

hence prolong the patient survival. In addition to these findings, 3rd grade tumours express significantly lower expression levels of $S1P_2$ receptor compared to 1st and 2nd grade tumours suggesting that the down-regulation of $S1P_2$ receptor might allow the rapid growth of the ERα positive cancer cells. Similar to ERα positive cohort, the nuclear SK1 expression negatively correlates with the $S1P_2$ receptor expression in ERα negative cohort. Moreover, metastatic tumours express lower levels of $S1P_2$ receptor compared to non-metastatic tumours suggesting that the $S1P_2$ receptor might inhibit the growth and metastasis of breast cancer cells.

Further investigation of the role of interaction between SKs and $S1P_2$ receptor have revealed that the SK2 signalling through the $S1P_4$ receptor prevents the nuclear localisation of $S1P_2$ receptor in ERα negative MDA-MB-231 breast cancer cells. Moreover, the accumulation of $S1P_2$ receptor in the nucleus inhibits cellular growth. Thus, SK2 and $S1P_4$ receptor signalling promotes cellular growth by maintaining the exclusion of $S1P_2$ receptor from the nucleus. In support of these findings, Gao and coworkers have shown that the down-regulation of SK2 expression inhibited the growth and migration/invasion of various cancer cell lines including MDA-MB-231, A498 and Caki-1 (Gao *et al.,* 2011). Moreover, the down-regulation of SK2 expression reduced the levels of phosphorylated AKT and phosphorylated ERK-2 and this might provide part of the mechanism of action for inhibition of cellular growth in A498 kidney carcinoma cells (Gao *et al.,* 2011). Similarly, the $S1P_2$ receptor was shown to inhibit the PDGF-induced cell proliferation in mouse embryonic fibroblasts (Goparaju *et al.,* 2005). In the current study, the siRNA-mediated down-regulation of SK2 expression or inhibition of SK2 activity (using SK2-selective inhibitor ROMe) induces the nuclear accumulation of Y416 phosphorylated c-SRC. However, the treatment with the $S1P_4$ receptor antagonist does not induce the nuclear accumulation of Y416 phosphorylated c-SRC indicating that different SK2-mediated mechanisms were regulating the nuclear accumulation of Y416 phosphorylated c-SRC and $S1P_2$ receptor. Thus, the role of c-SRC accumulation into cell nucleus requires further investigation. Moreover, the analysis of SK2 expression in breast cancer cohorts might provide further insight into understanding SK2/$S1P_4$ signalling. In conclusion, SK2 inhibitors and $S1P_4$ receptor antagonists might represent novel therapeutics for the treatment of ERα negative breast cancer patients.

PgR is a nuclear hormone receptor that is a ligand-dependent transcription factor functioning by binding to progesterone response elements (Mangelsdorf *et al.,* 1995, Kastner *et al.,* 1990). Various downstream elements of the PgR signalling includes Wnt4 and receptor activator of NFκB ligand (RANKL), the latter being a secreted cytokine binding TNFα and activating NFκB (Gonzalez-Suarez 2011, Mulac-Jericevic *et al.,* 2003, Brisken *et al.,* 2000). Anderson and coworkers have shown that progesterone, a ligand of PgR is a risk factor for breast cancer (Anderson *et al.,* 2002, Clarke *et al.,* 2006). However, the involvement of PgR in breast carcinogenesis and tumour progression is not fully understood. Studies on MDA-MB-231 breast cancer cells that lack the expression of both ERα and PgR expression have shown that the PgR expression down-regulates the expression of genes required for cell proliferation and metastasis (Leo *et al.,* 2005). Furthermore, Skildum and coworkers have shown that the progesterone induced cell proliferation in a transient way that was followed by induction of cell cycle inhibitors in $ERα^+/PgR^+$ T47D breast cancer cells (Skildum *et al.,* 2005). Furthermore, Bardou and coworkers have shown that the expression of PgR in ERα positive breast tumours was associated with improved patient survival and responsiveness to endocrine therapy (Bardou *et al.,* 2003). In support of these findings, the current study shows that the expression of PgR is associated with reduced $S1P_1$ and $S1P_3$ receptor expression in ERα positive breast tumours. Moreover, the PgR expression enhances the nuclear $S1P_2$ receptor expression in these tumours. Indeed, Purdie and coworkers have shown that the loss of PgR expression was associated with poor prognosis in ERα positive breast tumours (Purdie *et al.,* 2013). Thus, the PgR expression might prevent breast cancer progression through down-regulation of the $S1P_{1/3}$ receptors and up-regulation of the $S1P_2$ receptor expression. In contrast, the PgR expression was associated with increased SK1 expression in ERα negative breast tumours. Thus, the expression of PgR might have diverse effects on pathophysiology of breast cancer that requires further investigation.

The over-expression of HER2 receptor was shown to induce tumour growth and tamoxifen resistance in breast cancer (Benz *et al.,* 1992). Moreover, the HER2 receptor signalling interacts with sphingolipid signalling. For example, Maceyka and coworkers have shown that the $S1P_1$ receptor activity was required for the heregulin-induced migration in melanoma cell lines (Maceyka *et al.,* 2008). In support of these findings, the current study shows that the HER2 receptor expression correlates with enhanced $S1P_1$ receptor

expression in both ERα positive and ERα negative breast tumours. Moreover, the expression of $S1P_1$ receptor is associated with poor cancer prognosis in ERα positive breast tumours. Thus, the role of HER2/$S1P_1$ receptor signalling requires further investigation.

In addition to interaction of SK1/S1P receptor with PgR and HER2 receptors, a diverse array of kinases and transcription factors e.g. c-RAF-1, ERK-1/2, AKT, LYN, SRC family kinases (SFKs) and NFκB (p50 RelA) were evaluated with SK1 and S1P receptors to discover novel interactions that drive cancer progression in ERα positive breast cancer patients. These associations are summarised in **Table 6.1**. For example, the SK1 expression correlates with the expression of $S1P_1$ receptor, $S1P_3$ receptor, phosphorylated ERK-1/2, phosphorylated AKT, phosphorylated NFκB, phosphorylated RAF-1, Y416 phosphorylated SFK and LYN in shorter disease-specific patient survival and disease-free cancer recurrence. Indeed, the loss of expression of either SK1 or any of these kinases abolishes the association with disease-specific patient survival and disease-free cancer recurrence.

Table 6.1. The summary of clinically relevant associations between SK1/S1P receptors and signalling proteins.

Sphingolipid signaling	Associated proteins
SK1	$S1P_1$, $S1P_3$, p-ERK-1/2, p-AKT, p-NFκB, p-RAF-1, Y416 SFK, LYN
$S1P_1$	SK1, p-ERK-1/2, p-AKT, p-RAF-1, Y216 c-SRC, p-RAF-1
$S1P_2$	Y416 SFK, c-SRC
$S1P_3$	SK1, p-RAF-1, LYN

Thus, these proteins might be interacting at multiple levels to maintain tumour cell growth, survival and migration.

There are significant functional interactions between the proteins studied. For instance, the LYN kinase activation via cell surface receptors such as intercellular adhesion molecule 1 (ICAM-1) or interleukin 5 (IL-5) activates the c-RAS/c-RAF/ERK-1/2 signalling pathway (Holland *et al.,* 1997, Pazdrak *et al.,* 1998). Similarly, LYN kinase and c-SRC was shown to activate the PI3K/AKT/NFκB signalling pathway (Iqbal *et al.,* 2010, Bates *et al.,* 2001, Liu *et al.,* 1998, Datta *et al.,* 1996). Moreover, LYN kinase also activates the SK1 (Olivera *et al.,* 2005, Pitson *et al.,* 2003). Interestingly, SK1 is linked to these proteins through 'inside-out' signalling involving the activation of S1P receptors (Guan *et al.,* 2011, Song *et al.,* 2011). Indeed, $S1P_1$ receptor coupled to G_i protein was shown to activate the c-RAS/c-RAF-1/ERK1/2 signalling pathway (Khleif *et al.,* 1999, Okamoto *et al.,* 1998). Furthermore, c-RAS-mediated activation of c-RAF-1 requires the activation of c-SRC that in turn activates the AKT signalling pathway (Rexer *et al.,* 2009, Tran *et al.,* 2003, Bakin *et al.,* 2003). Similarly, $S1P_2$ and $S1P_3$ receptor signalling was shown to activate the AKT signalling pathway (Means *et al.,* 2007, Baudhuin *et al.,* 2004). Thus, the functional interaction of these receptors with LYN, c-SRC and p-RAF-1 kinases are possibilities. The functional interaction of SK1 and $S1P_{1-3}$ receptors with these signalling pathways is summarised in **Figure 6.1**.

In conclusion, the high tumour expression of S1P signalling proteins is associated with poor disease prognosis in both ERα positive and ERα negative breast cancer patients. However, disease progression is governed by distinct set of proteins in different types of breast cancer. Indeed, targeting the right set of proteins might exhibit more selectivity towards cancer cells. For example, SK1 inhibitors and $S1P_{1/3}$ receptor antagonist might improve the survival of ERα positive breast cancer patients, while SK1/2 inhibitors and $S1P_4$ receptor antagonists might improve the survival of ERα negative breast cancer patients. Indeed, $S1P_1$ receptor functional antagonist Fingolimod is approved by US Food and Drug Administration (FDA) in 2010 for the treatment of patients with relapsing–remitting multiple sclerosis. Similarly, the SK1 inhibitor Safingol was shown to enhance the anti-tumour activity of cisplatin in advanced solid tumours in Phase I clinical trial

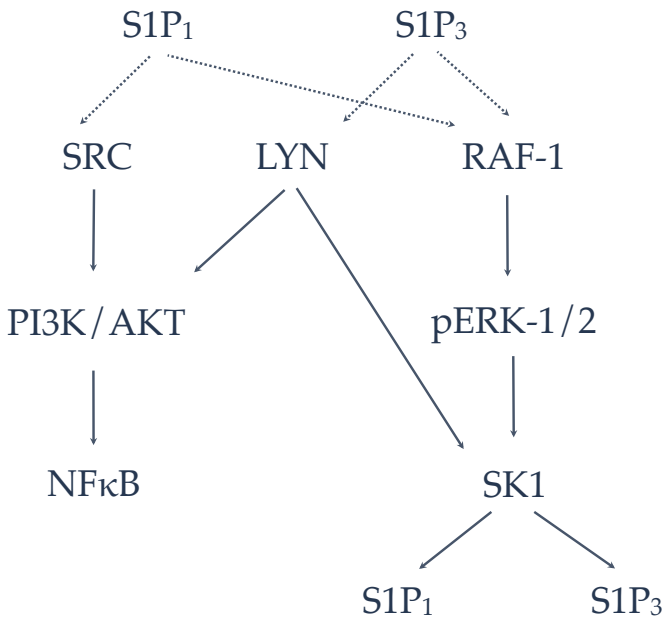

Figure 6.1. The interaction of SK1 and $S1P_{1/3}$ receptors with *other* signalling proteins.

(NCT00084812) (Dickson *et al.,* 2011). Thus, SK inhibitors and S1P receptor antagonists might present novel therapeutics for the treatment of breast cancer patients.

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