Gene Regulation by Sphingosine kinase

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ABBREVIATIONS

ABC;	ATP binding cassette
AC;	Adenylate cyclase
AD;	Alzheimer disease
ADP;	Adenosine-5'-diphosphate
ApoE;	Apolipoprotein E
ApoM;	Apolipoprotien M
AREs;	AU-rich mRNA destabilizing elements
ATP;	Adenosine-5'-triphosphate
AML;	Acute myeloid leukaemia
BAL;	Bronchoalveolar lavage
BH3;	Bcl-2 homology 3
BrdU;	5-bromo-2-deoxyuridine
BSA;	Bovine serum albumin
Cdc42;	Cell division cycle 42
CerS;	Ceramide synthase
CIB1;	Calcium and integrin binding protein 1
C1P;	Ceramide-1-phosphate
CML;	Chronic myeloid leukaemia
CNS;	Central nervous system
COX-2;	Cyclooxygenase 2
DAPI;	4',6-diamidino-2-phenylindole
DEPC;	Diethylpyrocarbonate
DMS;	*N,N*-dimethylsphingosine
Dox;	Doxycycline
DTT;	Dithiothreitol
EAE;	Encephalomyelitis
EC;	Endothelial cell
eEF1A;	Eukaryotic elongation factor 1A
EGF;	Epidermall growth factor
EGFP;	Enhanced green fluorescent protein

eNOS;	Endothelial nitric oxide synthase
ER;	Endoplasmic reticulum
ERK1/2;	Extracellular signal regulated kinase 1/ 2
eYFP;	Enhanced yellow fluorescent protein
FACS;	Fluorescence associated cell sorter
FBS;	Foetal bovine serum
FcϵRI;	High-affinity receptor for IgE
FDR;	False discovery rate
FHL-2;	Four and a half LIM domains protein 2
FLNa;	Filamin A
GAP43;	Growth associated protein 43
GC;	Germinal centre
GDNF;	Glial cell line-derived neurotrophic factor
HDAC;	Histone deactetylases
HDL;	High density lipoproteins
HEK293;	Human embryonic kidney 293 cells
HRP;	Horseradish peroxidase
HSPs;	Heat shock proteins
IRES;	Internal ribosome entry site
IRPs;	Iron regulatory proteins
LDL;	Low density lipoproteins
LPA;	Lysophosphatidic acid
LPP;	Lipid phosphate phosphatase
LPS;	Lipopolysaccharide
MAPK;	Mitogen-activated protein kinase
MiRNA;	microRNA
MMP;	Matrix metalloproteinase
MS;	Multiple sclerosis
MTs;	Metallothioneins
NES;	Nuclear export signal
NGF;	Nerve growth factor

NO;	Nitric oxide
OVA;	Ovalbumin
Ox-LDL;	Oxidised LDL
PA;	Phosphatidic acid
PBS;	Phosphate buffered saline
PCR;	Polymerase chain reaction
PDGF;	Platelet-derived growth factor
PECAM-1;	Platelet endothelial cell adhesion molecule
PGE2;	prostaglandin E2
PHB2;	Prohibitin 2
PI3K;	Phosphatidylinositol 3-kinase
PLC;	Phospholipase C
PMA;	Phorbol 12-myristate 13-acetate
PS;	Phosphatidylserine
PS2;	Presenilin 2
qPCR;	Quantitative real-time PCR
rtTA;	Reverse tetracycline-responsive transcriptional activator
siRNA;	Small interfering RNA
SDS;	Sodium dodecyl sulfate
SDS-PAGE;	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SKs;	Sphingosine kinases
SKIP;	SK1-interacting protein
SMP;	Skim milk powder
S1P;	Sphingosine 1-phosphate
$S1PR_{1-5}$;	Sphingosine 1-phosphate receptors 1-5
SPP1/2;	S1P phosphatases 1/ 2
SPT;	Serine palmitoyltransferase
SREBP;	Sterol regulatory element binding protein
SSC;	Saline-sodium citrate
Tet;	Tetracycline
TetR;	Tet-repressor

Tf;	Transferrin
TFR1;	Transferrin receptor 1
TGFβ;	Transforming growth factor-β
TIMP;	Tissue inhibitor of metalloproteinase
TNF-α;	Tumour necrosis factor-α
TRAF2;	TNF receptor-associated factor 2
TRE;	Tet-responsive element
VEGF;	Vascular endothelial growth factor
VCAM;	Vascular cell adhesion molecule
VSMC;	Vascular smooth muscle cells

ABSTRACT

Sphingosine kinases (SKs) are lipid kinases that catalyse the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P), a bioactive phospholipid that plays important roles in a wide variety of cellular processes, including calcium mobilisation, proliferation, apoptosis, angiogenesis, inflammatory responses and cytoskeletal rearrangement. Two SK isoforms exist in mammals, termed SK1 and SK2, which originate from different genes, but possess a high degree of sequence similarity. Although the two enzymes utilise the same substrate, sphingosine, to generate S1P, surprisingly, studies have suggested that SK1 and SK2 may have opposing cellular functions, with SK1 inducing cell survival and SK2 appearing to promote apoptosis. However, the molecular mechanisms mediating these apparently divergent roles for the two SKs have not been extensively examined at present. Furthermore, mouse knockout studies have suggested the two enzymes may have at least some overlapping functions.

There is strong evidence implicating SK1 in crucial role(s) in the development and progression of tumourigenesis. However, the mechanism whereby this enzyme induces tumourigenic processes is less clear and remains an important question to be answered in the field. Although high levels of intracellular S1P appears to have a role in regulation of cell proliferation and survival, various observations also suggest a role for extracellular S1P in cell surface G protein-coupled receptor-mediated cell proliferation and survival. However, the specific downstream pathways mediating this oncogenic signalling by SK1 are still poorly defined.

In attempts to answer these questions, studies to date have mainly focused on elucidating the cellular signalling pathways that are transiently modulated following SK1 activation. Considerable evidence suggests that SK1 is transcriptionally upregulated in many human cancers and also that its product, S1P, can induce activation of various transcription factors to regulate transcription of other genes. While this type of cellular regulation by SK1 is likely to play an important role in tumourigenesis, no studies have yet been published that systematically examined the molecular mechanisms whereby enhanced SK1 levels lead to oncogenesis. Thus, the main aim of the studies outlined in this thesis was to elucidate the genes regulated by increased cellular SK activity that may be important for normal and pathological cellular regulation.

In order to do this, we generated cell lines with tight doxycycline-inducible expression of SK1 and SK2 via a novel approach that involves the incorporation of AU-rich mRNA destabilizing elements (AREs) into the 3' untranslated regions of the tetracycline-inducible constructs. Use of these tightly controlled SK inducible systems allowed us to perform DNA microarrays and microRNA arrays to elucidate genes and microRNAs regulated soon after a moderate increase in cellular SK levels (approximately 10- and 6-fold overexpression of SK1 and SK2, respectively). This was done to maximise the likelihood of observing direct downstream effects of physiologically relevant increased SK expression that may have been missed by very high constitutive SK expression. While no microRNA regulation was observed following SK1 expression, screening of the Compugen human 19,000-oligonucleotide library, lead to the identification of various genes that were regulated by either SK1 or SK2 or by both enzymes. Of the various SK-regulated genes identified, transferrin receptor 1 (TFR1) was examined in greater detail in this study since its upregulation has been reported in various human cancers, and implicated in tumourigenic progression.

Here, we demonstrate a novel mechanism whereby SK1 regulates cell survival, proliferation and neoplastic transformation through upregulation of TFR1 expression. We show that elevated levels of SK1 enhanced total as well as cell-surface TFR1 expression resulting in increased transferrin (Tf) uptake into the cells. We also found that SK1 phosphorylation and/or translocation to the plasma membrane, which have been shown previously to be critical for SK1-mediated oncogenic effects, are necessary for regulation of TFR1 expression. Furthermore, we also demonstrated that S1P receptor 2 ($S1P_2$) is essential for SK1-induced TFR1 expression through the use of a $S1P_2$-specific inhibitor and siRNA knock-down of $S1P_2$. Finally, we show that blocking TFR1 function with a neutralizing antibody attenuated SK1-induced cell proliferation, survival and transformation. Together, these findings suggest that TFR1 plays an important role in oncogenesis mediated by SK1.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Duyen Hong Pham and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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* **Pham, D. H.**, Moretti, P. A., Goodall, G. J. & Pitson, S. M. (2008) Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3' AU-rich mRNA destabilizing elements. *Biotechniques,* 45**,** 155-160.

* Pitman MR, Jarman KE, Leclerq TM, **Pham DH** and Pitson SM (2011) Sphingosine kinases: biochemistry, regulation and role. In: *Lysophospholipid Receptors: Signaling and Biochemistry*. Chun J, Hla T, Spiegel S and Moolenaar W (Eds). John Wiley & Sons, Hoboken, New Jersey, USA. in press

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Chapter 1 Introduction

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See **Appendix 1** for author contributions

1 CHAPTER 1: INTRODUCTION

Sphingosine kinases (SKs) are enzymes that catalyse the formation of sphingosine 1-phosphate (S1P) (Figure 1.1), a bioactive lipid that regulates many cellular processes, including cell proliferation, survival, differentiation, motility, and cytoskeletal rearrangement [reviewed in (Takabe *et al.*, 2008)].

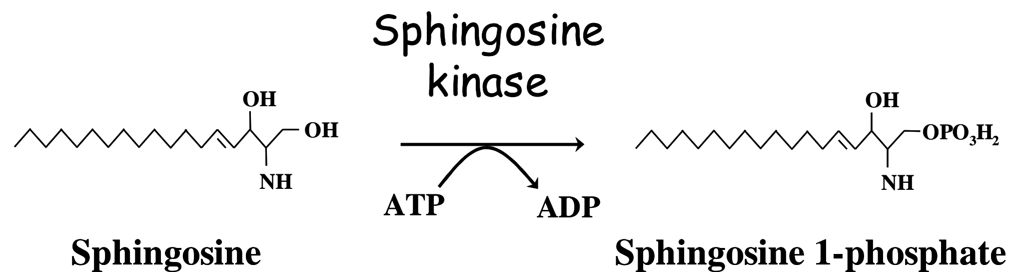


Figure 1.1 SK phosphorylates sphingosine to form S1P. SK uses ATP as a phosphate donor to catalyse the phosphorylation of the lipid sphingosine to produce S1P.

There is now considerable evidence implicating elevated levels of SK/S1P in the development and progression of tumourigenesis (Leong & Saba, 2010; Pyne & Pyne, 2010). Although high levels of intracellular S1P appears to have a role in regulation of cell proliferation and survival (Olivera *et al.*, 1999a; Olivera *et al.*, 2003; Spiegel *et al.*, 1994; Alvarez *et al.*, 2010), various observations also suggest a role for extracellular S1P in the pro-proliferative and pro-survival effects of this phospholipid (Bonnaud *et al.*, 2010; Schuppel *et al.*, 2008; Harada *et al.*, 2004). However, the specific downstream pathways mediating this oncogenic signalling by SK are still not fully determined. A number of studies have reported that at least one of the SK isoforms, SK1, is transcriptionally upregulated in human cancers and that SK1/S1P can regulate gene transcription (Alvarez *et al.*, 2010; Yamanaka *et al.*, 2004; Xia *et al.*, 2002; Takeshita *et al.*, 2000). Therefore, this thesis examines the molecular mechanisms whereby increased cellular levels of SK lead to pathological conditions, particularly cancer.

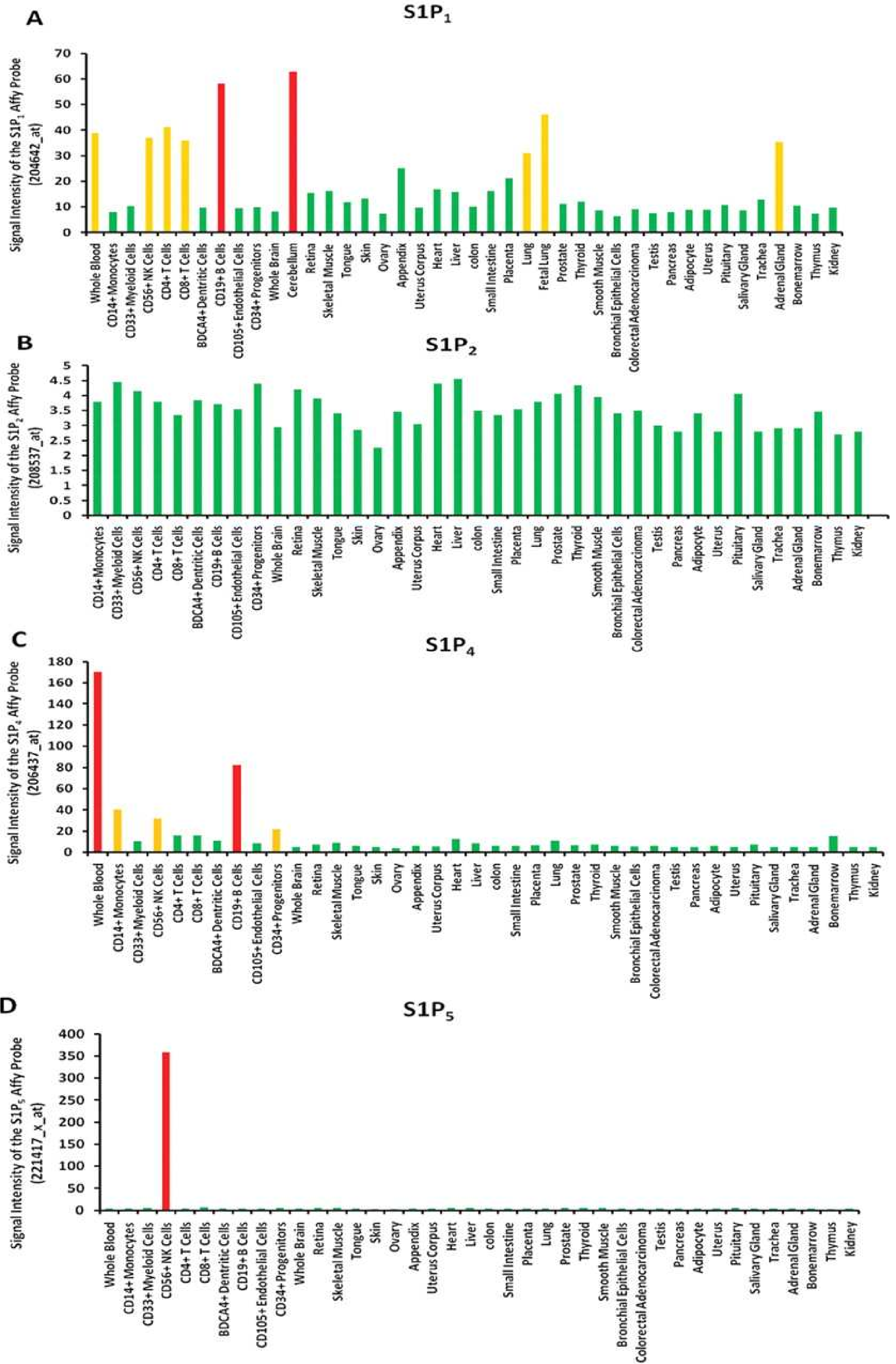
1.1 S1P as a signalling molecule

Sphingolipids are a class of lipids characterized by a serine head group with one or two fatty acyl tails [reviewed in (Fyrst & Saba, 2010)]. These molecules which are present in all eukaryotic cells are enriched in lipid rafts where they contribute to membrane biology and signalling events that control cellular behaviour and function (Furuya *et al.*, 2011). For a long time sphingolipids, including sphingomyelin and its metabolites were considered mainly as structural components of cell membranes or as metabolic intermediates. However, sphingolipid metabolites including ceramide, sphingosine, ceramide 1-phosphate (C1P) and S1P have emerged as bioactive signalling molecules that regulate a diverse range of cellular processes. Of these, S1P is of particular interest. In the last two decades, it has become increasing clear that S1P is an important signalling molecule that has the ability to function through either a family of specific G-protein coupled membrane receptors or via intracellular targets to regulate a diverse range of cellular processes including cell growth and proliferation, angiogenesis, cell motility and migration, and lymphocyte trafficking [reviewed in (Strub *et al.*, 2010)].

Although the intracellular functions of S1P are only beginning to emerge, its extracellular functions as a ligand for five S1P receptors (S1PR), named $S1P_{1\text{–}5}$ are well established

[reviewed in (Strub *et al.*, 2010)]. These receptors display tissue-specific expression patterns (Figure 1.2) and are coupled to various G proteins, enabling them to regulate a range of downstream signalling pathways, leading to the regulation of numerous physiological processes [reviewed in (Maceyka *et al.*, 2012)]. Dysregulation of S1P and its receptors have been implicated in numerous human pathophysiological processes such as cancer, asthma, inflammation, atherosclerosis and neurodegenerative diseases [reviewed in (Strub *et al.*, 2010; Aarthi *et al.*, 2011; Strub *et al.*, 2011)]. Therefore, targeting of the S1P/S1PR system by either genetic or pharmacologic means may have therapeutic potential for a range of diseases.

Figure 1.2 Tissue distribution of human $S1P_1$, $S1P_2$, $S1P_4$ and $S1P_5$ (A-D) based on Affymetrix gene expression analysis. The bar graph shows the average microarray intensity across the selected probe set (y-axis), representing the relative gene expression level in each cell line or tissue analysed (x-axis) [from (Aarthi *et al.*, 2011)].


A
S1P1
Signal Intensity of the S1P1 Affy Probe (204642_at)
B
S1P2
Signal Intensity of the S1P2 Affy Probe (208537_at)
C
S1P4
Signal Intensity of the S1P4 Affy Probe (206437_at)
D
S1P5
Signal Intensity of the S1P5 Affy Probe (221417_x_at)
Whole Blood
CD14+ Monocytes
CD33+ Myeloid Cells
CD56+ NK Cells
CD4+ T Cells
CD8+ T Cells
BDCA4+ Dentritic Cells
CD19+ B Cells
CD105+ Endothelial Cells
CD34+ Progenitors
Whole Brain
Cerebellum
Retina
Skeletal Muscle
Tongue
Skin
Ovary
Appendix
Uterus Corpus
Heart
Liver
colon
Small Intestine
Placenta
Lung
Fetal Lung
Prostate
Thyroid
Smooth Muscle
Bronchial Epithelial Cells
Colorectal Adenocarcinoma
Testis
Pancreas
Adipocyte
Uterus
Pituitary
Salivary Gland
Trachea
Adrenal Gland
Bonemarrow
Thymus
Kidney

1.2 Sphingolipids

Sphingolipids are ubiquitous components of the lipid bilayer of eukaryotic cells [reviewed in (Fyrst & Saba, 2010)]. Numerous agonists regulate sphingolipid metabolism to generate signalling molecules including ceramide (N-acyl sphingosine), sphingosine and S1P. S1P is an important cellular metabolite that is synthesized as part of the sphingomyelin cycle [reviewed in (Gault *et al.*, 2010; Fyrst & Saba, 2010)] (Figure 1.3). In this pathway membrane associated sphingomyelin is converted to ceramide. Ceramide is deacylated to remove one acyl chain via the activity of ceramidase to generate sphingosine which is phosphorylated by the SKs to yield S1P. S1P can then be irreversibly broken down into phosphoethanolamine and hexadecenal via S1P lyase or recycled back to sphingosine via phosphatases including two S1P-specific phosphatases, termed SPP1 and SPP2, as well as by a family of three broad-specificity lipid phosphatases, known as LPP1, LPP2 and LPP3. Ceramide can be produced by both *de novo* synthesis or from complex glycosphingolipids by sphingomyelinases which can be enhanced in response to many inducers of stress such as heat, UV radiation, hypoxia/reperfusion, cytokines or chemotherapeutic agents.

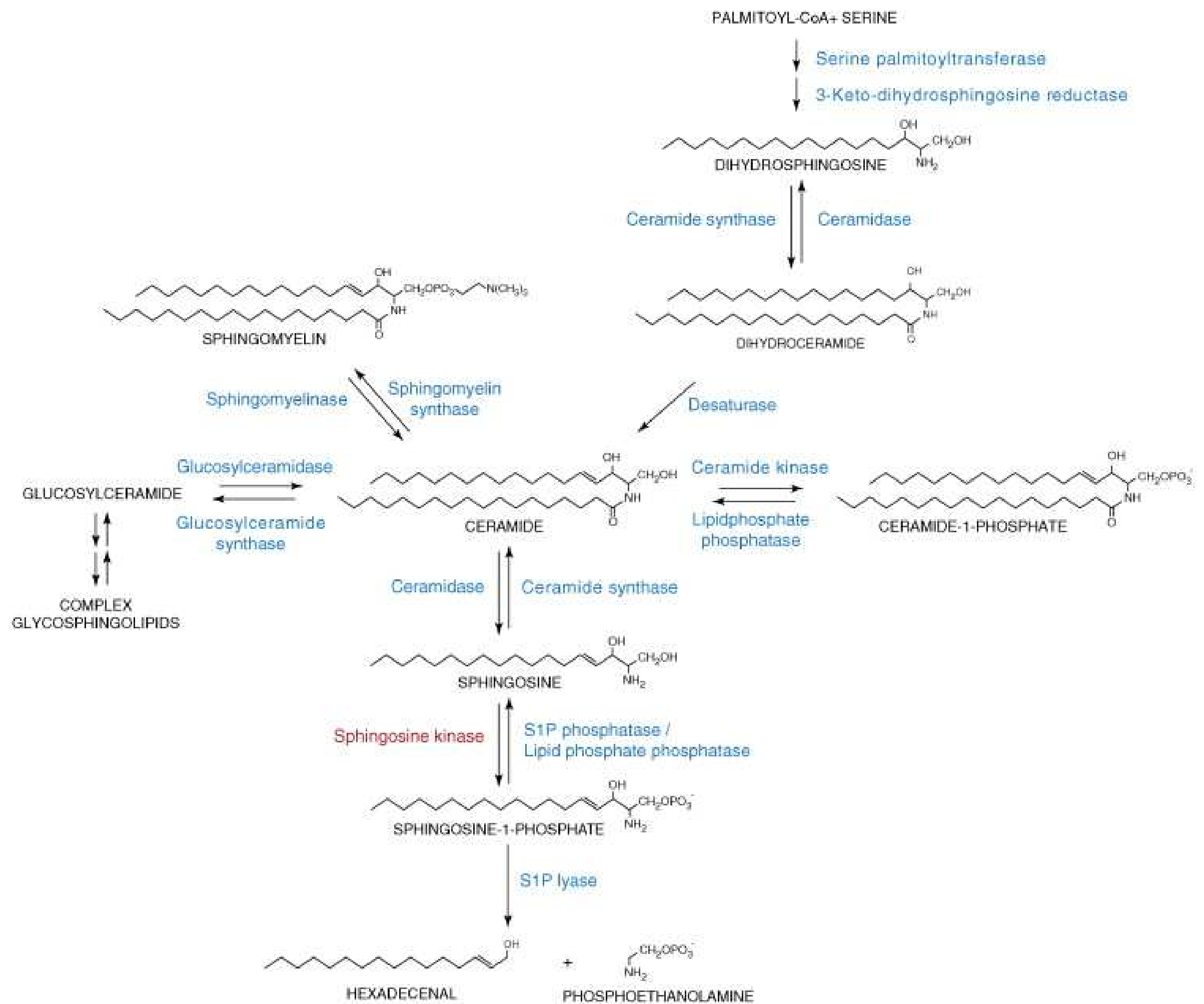


Figure 1.3 The sphingomyelin cycle showing the pathway involved in the regulation of S1P. Degradation of S1P can occur via two pathways; S1P phosphatases convert S1P back to sphingosine, while S1P lyases cleave S1P into hexadecenal and phosphoethanolamine which are reused for biosynthesis of phosphatidylethanolamine [from (Pitson, 2011)].

LPPs are integral membrane proteins that are localized at the plasma membrane as well as internal membranes with their catalytic centres facing the extracellular space and the luminal side of organelles, respectively. As a consequence they can regulate extracellular as well as intracellular levels of lipid phosphates including S1P (Fyrst & Saba, 2010). The two known SPPs and S1P lyase are also integral membrane proteins and are localized at the endoplasmic reticulum (ER). The catalytic centre of SPPs has been predicted to face the luminal side (Sigal *et al.*, 2005) whereas that of S1P lyase is directed toward the cytoplasm (Ikeda *et al.*, 2004). Both the SPPs and S1P lyase are proposed to regulate intracellular levels of S1P and contribute to the cellular balance of relative levels of S1P, sphingosine and ceramide (Fyrst & Saba, 2010).

Ceramide can also be generated *de novo* by condensation of serine and palmitoyl-CoA at the ER (Figure 1.3) (Gault *et al.*, 2010). This rate-limiting first step and is catalysed by serine palmitoyltransferase (SPT) to form 2-ketosphinganine. The 2-ketosphinganine formed is then reduced to dihydrosphingosine which is subsequently N-acylated by one of a family of six (dihydro) ceramide synthase (CerS) to form dihydroceramide, with the CerSs having differing but overlapping preferences for acyl chains from 16 to 26 carbons long. Finally, a 4-5 trans-double bound is introduced into the sphingoid base to produce ceramide. For sphingomyelin synthesis, ceramide is then trafficked from the ER to the Golgi via a ceramide transport protein known as CERT. Once in the Golgi apparatus, ceramide can be further metabolized to other sphingolipids, such as sphingomyelin and the complex glycosphingolipids. For example, ceramide may be subsequently phosphorylated by ceramide kinase to form ceramide-1-phosphate, or glycosylated by glucosylceramide synthase or galactosylceramide synthase. Additionally, it can be converted to sphingomyelin by the addition of a phosphorylcholine headgroup by sphingomyelin synthase. Finally, ceramide may be broken down by a ceramidase to form sphingosine. Sphingosine can then be re-utilised for complex sphingolipid biosynthesis or phosphorylated by SKs to form S1P.

1.3 Control of S1P levels

The sphingomyelin pathway was once considered purely a sphingolipid degradative pathway for relieving the cell of excess sphingomyelin. However, over the past 20 years, this pathway has been recognized as much more than a degradative pathway since various sphingolipid metabolites including ceramide, sphingosine and S1P have been identified as important bioactive mediators regulating an array of cellular processes [reviewed in (Oskouian & Saba, 2010)]. While elevated levels of S1P promotes cell survival and proliferation (Hengst *et al.*, 2009; Pitson *et al.*, 2005; Sarkar *et al.*, 2005; Safadi-Chamberlain *et al.*, 2005; Jarman *et al.*, 2010), its precursors, ceramide and sphingosine induce cell growth arrest and apoptosis (Woodcock *et al.*, 2010; Xu *et al.*, 2006; Taha *et al.*, 2006; Dagan *et al.*, 2003; Connor *et al.*, 2001). The cellular levels of S1P are primarily controlled by the activity of SK and to a lesser extent by its degradation by S1P lyase and S1P phosphatase activities (Fyrst & Saba, 2010) (Figure 1.3). Thus, this places SK at a critical step in maintaining a tight balance between the levels of these metabolites for normal cellular function. In the basal state this balance between S1P generation and degradation results in low levels of S1P in the cell (Aarthi *et al.*, 2011). However, when cells are exposed to specific growth factors and other agonists, S1P levels increase rapidly and transiently as a direct consequence of a rapid increase in SK1 activity in the cell. This agonist-induced increase in cellular S1P and its downstream consequences can be blocked by the addition of SK1 inhibitors or expression of a dominant-negative SK1 (Pitson *et al.*, 2000b), suggesting that SK1 plays an important role in these agonist-induced downstream effects. Notably, many of these external stimuli activate SK1 in a biphasic manner consisting of an ‘acute’ rapid and transient first phase of activation which is most likely via post-translational modifications that increase SK1 enzymatic activity and its translocation to the plasma membrane where its substrate resides (Hengst *et al.*, 2009; Pitson *et al.*, 2005; Sarkar *et al.*, 2005; Safadi-Chamberlain *et al.*, 2005; Jarman *et al.*, 2010), followed by a ‘chronic’ delayed activation that involves upregulation of transcription (Nakade *et al.*, 2003; Doll *et al.*, 2005; Doll *et al.*, 2007; Sobue *et al.*, 2005; Huwiler *et al.*, 2006) which will be discussed later.

1.4 Sphingosine kinases (SKs)

SKs are members of a highly conserved gene family that are expressed in many eukaryotic organisms ranging from yeasts to humans, with orthologues also expressed in worms and flies [reviewed in (Alemany *et al.*, 2007; Pitson, 2011; Leclercq & Pitson, 2006)]. All these SKs have five conserved regions in their amino acid sequences, designated C1-C5, which distinguishes them from all other known proteins (Wattenberg *et al.*, 2006; Pitson, 2011; Leclercq & Pitson, 2006). Following the purification of a rat SK (Olivera *et al.*, 1998), Spiegel and colleagues were successful in cloning the first two mammalian SK1 isoforms from mouse (Kohama *et al.*, 1998). The two isoforms found probably arose from alternate splicing and were termed mSK1a and mSK1b. Soon after, the human SK1 was cloned by multiple groups (Pitson *et al.*, 2000a; Melendez *et al.*, 2000; Nava *et al.*, 2000). Subsequently, a second human (and mouse) SK was cloned, SK2 (Liu *et al.*, 2000a).

In humans, at least three splice variants of SK1 [named SK1a, SK1b and SK1c] (Venkataraman *et al.*, 2006) and two variants of SK2 [named SK2-S and SK2-L or SK2a and SK2b] (Liu *et al.*, 2000a; Okada *et al.*, 2005) have been described that differ at their N termini (Figure 1.4). SK1a appears to be the major form of SK1 and is the most studied compared to the other two isoforms (SK1b and SK1c). The three SK1 isoforms appear to have similar enzymatic properties and are predominantly cytosolic, but have slightly different subcellular distributions, with SK1b and SK1c having greater plasma membrane localisation. Compared to SK1, much less is known about SK2 and its isoforms. The smaller, SK2-S, was the original cloned variant from mammalian cells based on its homology to SK1 (Liu *et al.*, 2000a). However the N-terminally extended SK2-L is believed to be the predominant SK2 mRNA in a range of human tissues and cell lines but not in mice (Okada *et al.*, 2005). While SK1 and SK2 are the only two SK isoforms cloned from mammalian cells, it should be noted that the existence of a third potential SK isoform has been proposed, which exhibits a different substrate specificity and subcellular localisation in some tissues (Fukuda *et al.*, 2003). The validity of this third SK isoform, however, awaits further experimentation.

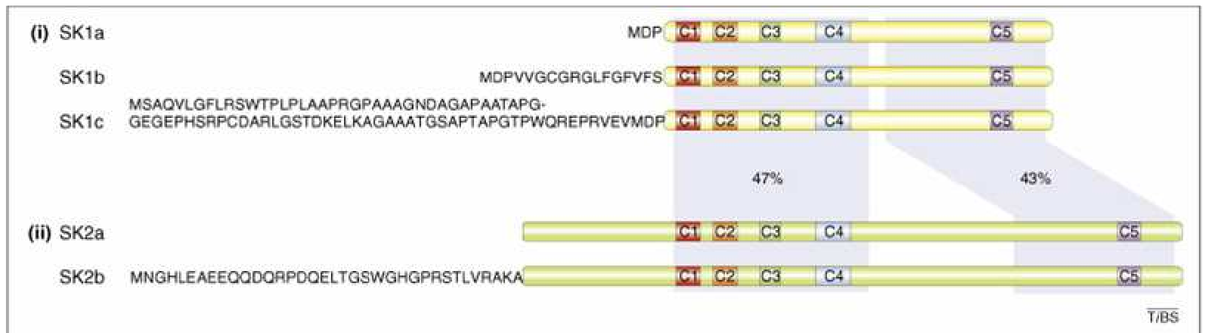


Figure 1.4 Human sphingosine kinases (hSK1 and hSK2). A schematic representation of human SK1 and SK2, highlighting the three splice variants [SK1a (RefSeq NM_001142601), SK1b (RefSeq NM_021972) and SK1c (RefSeq NM_182965)] for SK1 **(i)** and two splice variants (SK2a and SK2b) for SK2 **(ii)**. All human SK isoforms have five conserved regions (C1-C5). All SK catalytic domains contain the conserved ATP binding sequence, SGDGx$_{17\text{-}21}$K/R (Pitson *et al.*, 2000b; Pitson *et al.*, 2002). Human SK2 contains two additional polypeptide regions at its N-terminus and within the middle of its sequence that are quite distinct from SK1 [from (Pitson, 2011)].

Although mammalian SK1 and SK2 have regions of very similar amino acid sequence (80% amino acid sequence similarity), they originate from different genes [the *SPHK1* gene is located on chromosome 17 (17q25.2) while the *SPHK2* gene on chromosome 19 (19q13.2)]. In addition, SK2 is considerably larger than SK1 due to the presence of two additional polypeptide regions at its N-terminus and within the middle of its sequence that are quite distinct from SK1 (Alemany *et al.*, 2007; Pitson, 2011) (Figure 1.4). However, SK2 still contains all five of the regions (C1-C5) that are highly conserved between this family of enzymes, including a conserved catalytic domain and C-termini, which are related to the diacylglycerol kinase family (Pitson, 2011). At least part of the ATP-binding site in SK was shown to reside in the C2 domain of the enzyme within the consensus sequence $SGDGX_{17\text{-}21}K$ (Pitson *et al.*, 2002) and mutation of the glycine downstream of aspartic acid produces a catalytically inactive SK protein (Pitson *et al.*, 2000b).

In addition to these sequence differences, SK2 has lower specific activity than SK1 (Roberts *et al.*, 2004), and appears to be considerably less selective than SK1 in the substrates it can utilize (Liu *et al.*, 2000a; Pitson *et al.*, 2000a; Roberts *et al.*, 2004). Both SK1 and SK2 can efficiently use D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine. SK2, however, has much greater affinity towards phytosphingosine and an artificial substrate ω-biotinyl D-*erythro*-sphingosine compared to SK1 (Roberts *et al.*, 2004). Furthermore, L-*threo*-dihydrosphingosine, an inhibitor of SK1, is phosphorylated by SK2 (Kohama *et al.*, 1998; Liu *et al.*, 2000a). SK2 has also been reported to be the main enzyme responsible for phosphorylation and activation of the immunosuppressive pro-drug FTY720, with SK1 appearing to show little activity toward this agent and related analogs (Billich *et al.*, 2003; Paugh *et al.*, 2003; Don *et al.*, 2007). The physiological significance of these differences in substrate specificity between the two SKs still remains to be clarified.

SK1 and SK2 have differential tissue distribution and developmental expression. While SK1 is abundantly expressed in adult mouse heart, spleen, lung and brain (Melendez *et al.*, 2000), SK2 expression is highest in brain, kidney and liver (Liu *et al.*, 2000a). The expression pattern of the two SKs in humans are quite different from mice, with SK1 expression its highest in the bronchial epithelial cells and smooth muscles, whereas SK2 expression is similar across all the tissues analysed (Figure 1.5), suggesting that the two enzymes are involved in distinct cellular functions. This notion is further supported by the fact that SK1 and SK2 can play

contrasting roles in a number of disease conditions including murine collagen-induced arthritis (Lai *et al.*, 2009), LPS-induced lung injury (Wadgaonkar *et al.*, 2009) renal ischemia-reperfusion injury (Park *et al.*, 2011) and immune cell functions (Schroder *et al.*, 2011). A number of *in vitro* studies have also reported that SK1 and SK2 appear to play quite contrasting roles in the cell under different conditions. While SK1 promotes cellular survival and proliferation (Olivera *et al.*, 1999a; Xia *et al.*, 2000; Le Scolan *et al.*, 2005; Kohno *et al.*, 2006), overexpressed SK2 appears to have a pro-apoptotic effect in cells (Liu *et al.*, 2003; Igarashi *et al.*, 2003b; Maceyka *et al.*, 2005a; Okada *et al.*, 2005). Although the reason for these opposing functions remains to be investigated, the distinct cellular localisation of the two isoforms appears to be important in determining their function. SK1 is mainly cytoplasmic and upon activation, it translocates to the plasma membrane which appears to be integral for enhancing cell proliferation and survival (Pitson *et al.*, 2005). Whereas, SK2 localises predominantly to internal membranes, and the levels of SK2 at the ER are enhanced during serum starvation and this localisation of SK2 appears critical for its pro-apoptotic function (Igarashi *et al.*, 2003b; Maceyka *et al.*, 2005a).

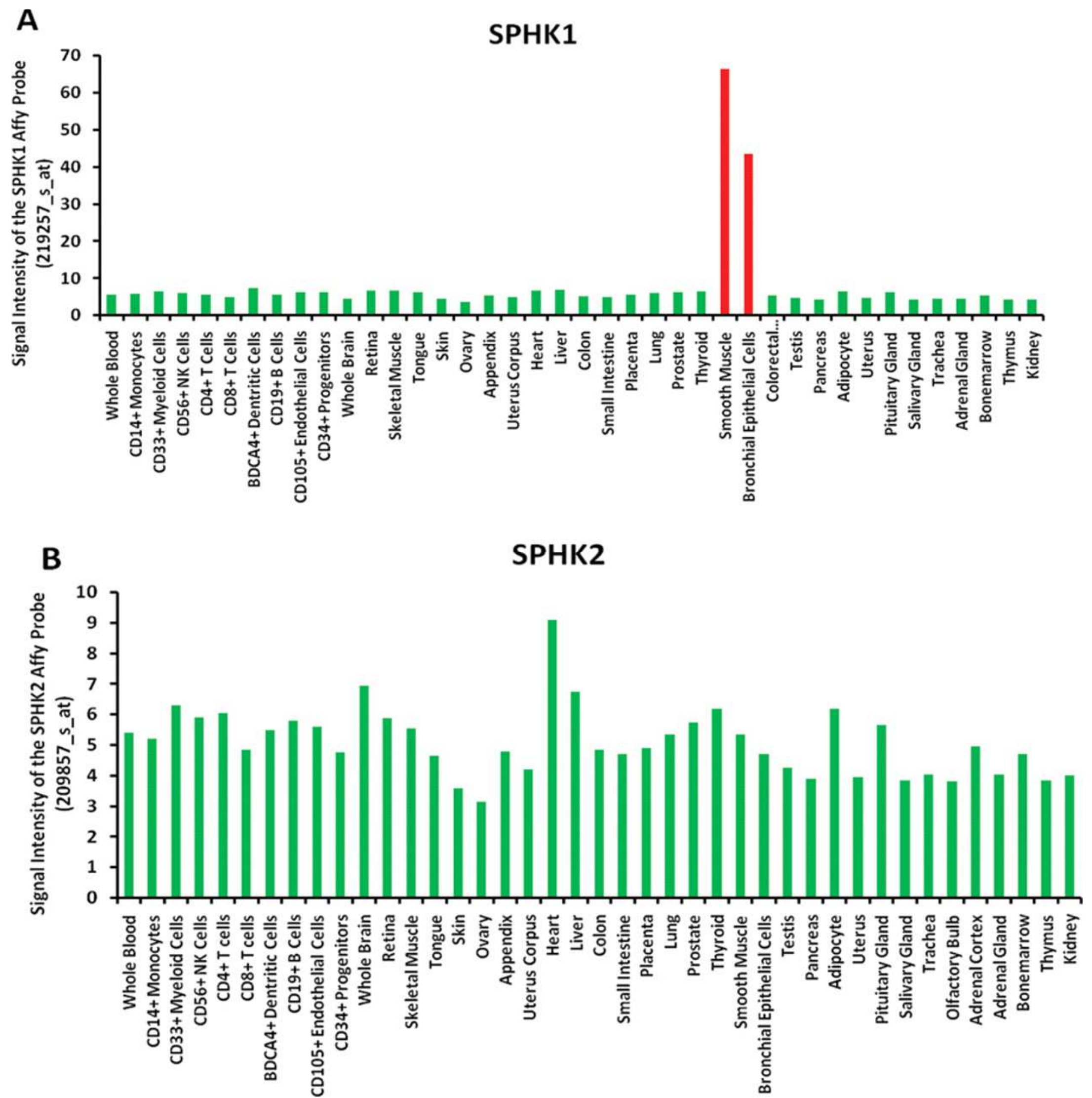


Figure 1.5 Tissue distribution of human SK1 (A) and SK2 (B) based on Affymetrix gene expression analysis. The bar graph shows the average microarray intensity across the selected probe set (y-axis), representing the relative gene expression level in each cell or tissue analysed (x-axis) [from (Aarthi *et al.*, 2011)].

Despite these divergent roles of SK1 and SK2, SK knockout mice have added further complexity to this area. Mice lacking either SK1 or SK2 are viable, fertile and lack any obvious abnormalities (Allende *et al.*, 2004; Mizugishi *et al.*, 2005). Simultaneous deletion of both enzymes, however, produces mice that die *in utero* due to severe defects in neurogenesis and angiogenesis (Mizugishi *et al.*, 2005). Although the single knockout mice have not been extensively examined, these findings suggest that despite SK1 and SK2 having some different cellular effects, these enzymes also have some functional redundancy in mice that enables each to compensate for the loss of the other (Mizugishi *et al.*, 2005).

Although SK1 is mainly in the cytoplasm, it is also seen to some extent in the plasma membrane and the nucleus (Inagaki *et al.*, 2003; Pitson *et al.*, 2003). SK1 activation-induced translocation from the cytosol to the plasma membrane appears to be crucial for its oncogenic effects (Pitson *et al.*, 2003; Pitson *et al.*, 2005; Jarman *et al.*, 2010). In addition, SK1 was shown to translocate from the cytosol to the nucleus upon platelet derived growth factor (PDGF) stimulation (Kleuser *et al.*, 2001). Subsequent to this, SK1 was reported to shuttle between the cytosol and the nucleus due to its nuclear export sequences since deletion or inhibition of these sequences caused accumulation of the enzyme in the nucleus (Inagaki *et al.*, 2003).

The localisation of SK2 appears more complex, the enzyme has been reported to localise to the cytosol, nucleus, plasma membrane and predominantly to internal membranes, dependent on cell type and also cell density (Igarashi *et al.*, 2003b; Maceyka *et al.*, 2005a). In particular, the localisation of SK2 is enhanced at the ER during serum starvation and this localisation appears critical for the enzyme's pro-apoptotic function (Maceyka *et al.*, 2005a). Localisation of SK2 in the nucleus appears to be due to the presence of a novel nuclear localisation signal sequence (Ding *et al.*, 2007). Consistent with this notion, more recently, SK2/S1P was shown to interact directly with histone deactetylases (HDAC1 and HDAC2) in the nucleus to influence gene expression (Hait *et al.*, 2009). Interestingly, a recent report has shown that SK1 and SK2 are localized in the centromere along with $S1P_5$ where it is proposed to participate in the regulation of mitosis (Gillies *et al.*, 2009). Taken together, these results indicate that SK1 and SK2 can be present in the nucleus, suggesting the possibility that they may regulate transcriptional processes.

1.5 S1P Signalling

All cells appear to be able to generate S1P during the normal physiologic metabolism of sphingomyelin. S1P is present at high concentrations in human serum ranging from 0.4 μM to 1 μM, mainly bound by albumin and other plasma proteins (Murata *et al.*, 2000a; Okajima, 2002). The high levels of S1P found in serum have long been thought to be derived from platelets (Yatomi *et al.*, 1997). Indeed, platelets store a considerable amount of S1P, and release it during platelet activation (Dindo *et al.*, 2006). More recently, however erythrocytes, have emerged as the major source of S1P in plasma under normal homeostatic conditions, (Hanel *et al.*, 2007; Bode *et al.*, 2010; Pappu *et al.*, 2007) whereas platelets and mast cells appear to contribute to the local synthesis of S1P during platelet activation and inflammation reactions, respectively (Ulrych *et al.*, 2011; Dahm *et al.*, 2006; Oskeritzian *et al.*, 2010). Other cells, such as vascular endothelium have also been shown to release a significant amount of S1P following SK1 activation (Venkataraman *et al.*, 2008).

Members of the ATP binding cassette (ABC) transporters have been implicated in cellular export of S1P in a range of different cell types including mast cells, platelets, endothelial cells, astrocytes and erythrocytes [reviewed in (Kim *et al.*, 2009)]. For example, the release of S1P from astrocytes and platelets appear to be dependent on ABCA1 transporter (Kobayashi *et al.*, 2006), while ABCC1 was important for the export of S1P from both human and rodent mast cells (Mitra *et al.*, 2006). More recently, however, ABCC1 and ABCG2 have been reported to be involved in estradiol-mediated transport of S1P out of MCF-7 cells (Takabe *et al.*, 2010). Interestingly, recent studies in zebrafish and human cells have also identified spinster homologue 2 (two of hearts) as a putative S1P transmembrane transporter (Osborne *et al.*, 2008; Kawahara *et al.*, 2009).

1.5.1 Extracellular actions of S1P

As mentioned above, it is well established that cellular S1P, produced by SK, can be secreted from cells to act in an autocrine or paracrine fashion, a process called inside-out signalling, to activate a family of specific cell surface G-protein coupled receptors [reviewed in (Kim *et al.*, 2009)]. To date, five S1P receptors have been identified: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$, which can specifically bind and be activated by only S1P and dihydro-S1P (Taha *et*

al., 2004; Aarthi *et al.*, 2011). These S1P receptors are coupled to a variety of distinct heterotrimeric G proteins, which are linked to different effector molecules with varying affinities, allowing them to trigger a wide range of cellular responses in a highly cell-specific manner (Taha *et al.*, 2004) (Figure 1.6).

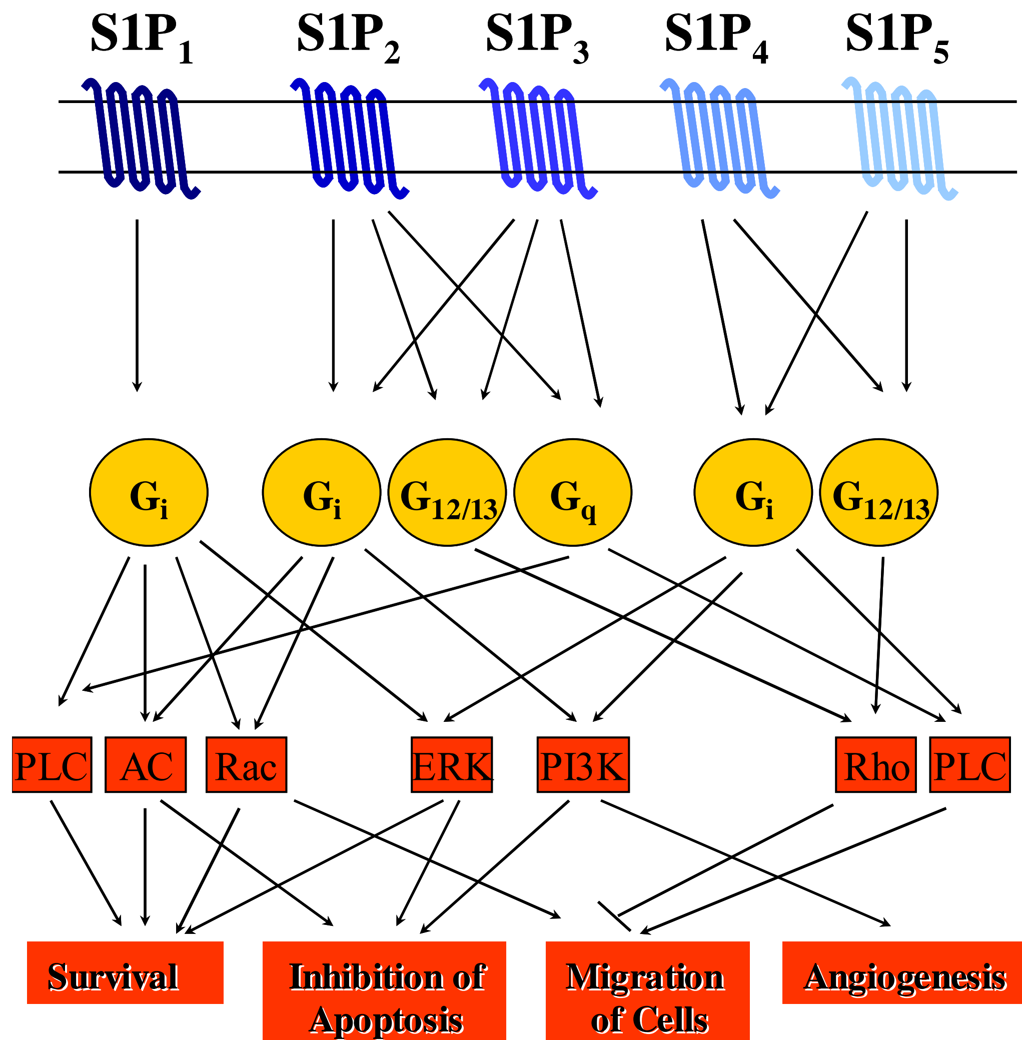


Figure 1.6 Major downstream biological processes regulated by S1P via the five S1P receptors. S1P binds S1P receptors and activates partially overlapping downstream via coupling to different G proteins to regulate numerous cellular and biological processes. Coupling of $S1P_1$ to G_i activates ERK, PI3K, AC and Rac to promote cell proliferation, migration and angiogenesis, respectively. $S1P_2$ couples to $G_{12/13}$ and activates Rho to inhibit cell migration, while, $S1P_3$ enhances cell migration and angiogenesis via Rac and PLC singalling through G_i and G_q, respectively [Adapted from (Aarthi *et al.*, 2011)].

1.5.1.1 $S1P_1$

$S1P_1$ was the first S1P receptor to be functionally identified as being coupled exclusively to G_i (Windh *et al.*, 1999; Okamoto *et al.*, 1998). This receptor subtype localises widely within the cell including the plasma membrane, caveolae, cytoplasmic vesicles and the nucleus [reviewed in (Aarthi *et al.*, 2011)]. Binding of S1P to $S1P_1$ leads to the inhibiton of adenylate cyclase (AC) and activation of phospholipase C (PLC), the Ras/ERK pathway, Rac, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Kihara *et al.*, 2007b). In particular, activation of ERK1/2 and PI3K signalling, stimulates cell proliferation and angiogenesis respectively (Kimura *et al.*, 2000; Wang *et al.*, 2010c). Activation of the small GTPase Rac by $S1P_1$ stimulates cell migration, actin cytoskeletal rearrangement and adherens junction assembly (Li *et al.*, 2009b). In addition, $S1P_1$ has been reported to play crucial role in neurogenesis, immune cell trafficking, endothelial barrier and vascular tone. Knockout of $S1P_1$ has been shown to be embryonic lethal mainly due to defects in the development of the vascular and central nervous system (Liu *et al.*, 2000b; Kono *et al.*, 2004).

1.5.1.2 $S1P_2$

Unlike $S1P_1$, $S1P_2$ receptor can be coupled to multiple G-proteins, including G_i, G_q, and $G_{12/13}$, but it couples most efficiently to $G_{12/13}$ protein (Windh *et al.*, 1999; Ancellin & Hla, 1999). Engagement of this receptor participates in cell proliferation, motility and transcriptional activation, generally acting in the opposite way to $S1P_1$ [reviewed in (Skoura & Hla, 2009)]. Coupling of $S1P_2$ to $G_{12/13}$ is known to activate the small GTPase, Rho that inhibits cell migration through inhibition of Rac (Lepley *et al.*, 2005; Malchinkhuu *et al.*, 2008). $S1P_2$ has also been reported to activate PLC leading to the release of Ca^{2+} in response to coupling to G_q protein. Although the $S1P_2$ receptor has not been shown to couple to G_s protein, it can activate AC and increase intracellular levels of cAMP, possibly through coupling to G_{13} protein (Jiang *et al.*, 2007).

$S1P_2$ is also able to activate ERK and mediate cell proliferation and survival through G_i (An *et al.*, 2000; Blom *et al.*, 2010). Indeed, expression of $S1P_2$ in various cancer cell lines has been shown to promote ERK phosphorylation and induction of c-Jun and c-Fos oncogenes (An

et al., 2000). Apart from ERK, $S1P_2$ has been reported to activate other members of the MAPK family, such as JNK and p38 MAPK and play a role in cell stress, inflammation and apoptosis (Goparaju *et al.*, 2005; Donati *et al.*, 2005; Michaud *et al.*, 2010). In addition to its pro-proliferative and pro-survival effects, $S1P_2$ has also been reported to inhibit cell proliferation in a different aspect of cellular physiology. Previously, $S1P_2$ was shown to inhibit hepatocyte proliferation through coupling to $G_{12/13}$ protein and activation of the small Rho GTPase (Ikeda *et al.*, 2003). This was consistent with a recent study which proposed that S1P/$S1P_2$ act through the RhoA/ROCK and JNK pathways to enhance connective tissue growth factor expression and appears to act as a tumor suppressor in Wilms tumor (Li *et al.*, 2008b). Recently, it was also reported that knock out of $S1P_2$ leads to a high incidence of clonal B-cell lymphoma formation in mice (Cattoretti *et al.*, 2009). These tumours displayed a uniform phenotype with characteristics of germinal centre (GC)-derived diffuse large B-cell lymphoma due to an increase in GC B-cells and CD69+ T-cells as well as an increased proliferation of spontaneous GCs, suggesting a role of $S1P_2$ in maintaining B-cell homeostasis. Notably, more recent studies suggest that the anti-tumour effect of $S1P_2$ appears to be due to inhibition of Akt signalling through $G_{12/13}$ and RhoA/ROCK, which prevent GC B-cell survival and migration towards the follicle center necessary for GC cell growth control. Thus, loss of this receptor in part could potentially disrupt this balance leading to the development of tumours (Green *et al.*, 2011).

$S1P_2$ knockout mice do not demonstrate any striking abnormalities, but do appear to develop epileptic seizures and deafness, indicating an important role of $S1P_2$ in the development of the auditory and vestibular systems (Herr *et al.*, 2007).

1.5.1.3 $S1P_3$

$S1P_3$ is localized mainly in the cell surface on the plasma membrane (Dolezalova *et al.*, 2003). Similarly to $S1P_2$, $S1P_3$ can also be coupled to G_i, G_q, and $G_{12/13}$ [reviewed in (Aarthi *et al.*, 2011)]. However, this receptor seems to couple most efficiently to G_q to stimulate PLC leading to the production of inositol 1,4,5-trisphosphate and subsequent release of Ca^{2+} from internal stores and activation of protein kinase C. In contrast to $S1P_2$, $S1P_3$ appears to activate Rac and enhance cell migration through G_i (Arikawa *et al.*, 2003; Becker *et al.*, 2011). Knockout of $S1P_3$ alone does not result in any obvious phenotype, but deletion of $S1P_{1\text{-}3}$ shows

vascular defects earlier than those of $S1P_1$ alone, suggesting that these three S1P receptors have redundant or cooperative functions for regular and mature vascular development during embryogenesis (Kono *et al.*, 2004).

1.5.1.4 $S1P_4$ and $S1P_5$

Unlike the other three S1P receptor subtypes, $S1P_4$ and $S1P_5$ have narrower patterns of expression, with $S1P_4$ expressed primary in lymphoid tissues and $S1P_5$ mainly distributed in the brain and the skin [reviewed in (Aarthi *et al.*, 2011)]. Although the G proteins coupled to $S1P_4$ and $S1P_5$ remain largely unclear at present, various studies have suggested that $S1P_4$ couples to G_i, and $G_{12/13}$ proteins (Van Brocklyn *et al.*, 2000; Yamazaki *et al.*, 2000), whereas the $S1P_5$ receptor couples to G_i, and G_{12} proteins (Malek *et al.*, 2001). Ligation of $S1P_4$ appears to activate PLC, ERK and the Rho-family GTPase Cdc42 through G_i [reviewed in (Kihara *et al.*, 2007a)]. In addition, activation of Rho by engagement of this receptor subtype has also been reported to induce cytoskeletal rearrangements and cell rounding (Anliker & Chun, 2004). Unlike the other S1PRs, ligation of $S1P_5$ decreases oligodendrocyte cell migration via the Rho/ROCK pathway and inhibits ERK activation leading to anti-proliferative effects (Novgorodov *et al.*, 2007). Consistent with this, recent studies also showed that binding of S1P to $S1P_5$ inhibits the proliferation and migration of esophageal cancer cells (Hu *et al.*, 2010).

In summary, the coupling of S1P receptors to diverse G proteins results in the activation of numerous downstream signalling pathways including AC, PLC, Ras/ERK1/2, PI3K/Akt, Rho, and several protein kinases, such as, JNK and p38 MAPK, enabling them to regulate a range of downstream signalling pathways, leading to regulation of numerous physiological processes. Many of these well known roles of extracellular S1P are associated with angiogenesis, differentiation, motility and cytoskeletal organization [reviewed in (Schuchardt *et al.*, 2011)]. However, S1P receptor engagement can also activate multiple pathways involved in enhancing cell proliferation and survival. The true importance of S1P receptors in S1P-mediated enhanced survival and proliferation, however, has been difficult to determine due to the apparent role of intracellular S1P in these similar processes (Van Brocklyn *et al.*, 1998; Olivera *et al.*, 2003; Kohno *et al.*, 2006).

1.5.2 S1P as an intracellular second messenger

In addition to its extracellular action, intracellular signalling of S1P also exists to mediate several cellular functions, such as proliferation, cell survival, and autophagy [reviewed in (Strub *et al.*, 2010)]. Various studies performed in systems lacking identifiable S1P receptors have shown that disrupting S1P metabolism can result in marked changes in calcium mobilisation, cell growth and survival, stress responses, endocytosis, tissue homeostasis, infectivity, viability and reproduction. These effects could be explained by the direct interaction of S1P with intracellular targets.

First of all, yeast do not posses S1P receptors, yet intracellular S1P regulates environmental stress responses and survival (Mandala *et al.*, 1998), in a manner reminiscent of the function of S1P in mammalian cells. This was further supported by later studies which showed plants lacking identifiable S1P receptors are able to regulate stomata closure and prevent water loss in drought conditions by increasing their S1P levels (Ng *et al.*, 2001). Consistent with this notion, the intracellular signalling of S1P has been proposed to be responsible for the enhanced cell proliferation and suppression of apoptosis following SK1 activation. For example, overexpression of SK1 promotes cell survival and protects the cell from apoptosis through inhibition of NF-κB and activation of Akt, an effect which cannot be reproduced by either exogenous S1P or dihydro-S1P (Suomalainen *et al.*, 2005; Limaye *et al.*, 2005). In addition, overexpression of SK1 in cells lacking functional S1P receptors was still able to promote cell growth and survival (Olivera *et al.*, 2003). Deletion of SK1 in mice reduces spontaneous tumour formation, whereas deletion of the S1P receptors does not, which further suggests an intracellular effect of S1P and/or sphingosine on the regulation of adenoma growth (Kohno *et al.*, 2006). Recently, a study has demonstrated that intracellularly generated S1P offers protection against LPS-induced lung injury and inflammation in a murine model of acute lung injury (Lin *et al.*, 2011). In addition, endothelial cell motility mediated by extracellular S1P was dependent on intracellular S1P production which was regulated by SK1 and S1P lyase (Berdyshev *et al.*, 2011). Furthermore, elevation of intracellular S1P either by depleting S1P phosphatase (S1PP) or overexpressing SK1 causes autophagy in MCF7 cells (Lepine *et al.*, 2011).

Further evidence for a second-messenger action of S1P is through activation of calcium channels (Young *et al.*, 2000; Hong *et al.*, 2008). There is strong evidence which implies that

S1P can activate thapsigargin-sensitive calcium channels in the ER, although the direct target for S1P in the ER is yet been identified (Mattie *et al.*, 1994). Indeed, intracellular S1P released through either direct micro-injection of S1P or by photolysis of caged S1P, which circumvents surface S1P receptors, results in mobilization of calcium (Blom *et al.*, 2005) and enhanced cell proliferation and survival (Van Brocklyn *et al.*, 1998; Morita *et al.*, 2000) independent of inositol trisphosphate formation and of S1P-receptor activation. This intracellular action of S1P seems to be cell-type specific, since microinjection of S1P had no effect on neuronal cells (Postma *et al.*, 1996) and it in fact reduced cell proliferation in keratinocytes (Kim *et al.*, 2004). Furthermore, chemical inhibition of SK demonstrated that endogenously generated S1P functions as a positive modulator of calcium entry via store operated channels, whereas exogenously administrated S1P initiated calcium release from the ER (similarly to histamine) and decreased endothelial cell (EC) permeability (Itagaki *et al.*, 2007). These results suggest that S1P targeting ECs from the extracellular space exerts it effects through different receptor and signalling mechanisms than those of S1P generated intracellularly (Zhi *et al.*, 2006). However, these studies could not completely exclude the fact that S1P generated intracellularly could be released from the cell, activating S1P receptors (or altering membrane function) and have other nonspecific effects on sphingolipids. Thus, the intracellular signalling function of S1P has remained a controversial issue.

Recently, some clarity has begun to emerge with the identification of several direct intracellular targets of S1P. This includes studies which show that S1P in the nucleus can directly interact with histone deacetylases (HDAC) to play a role in epigenetic gene regulation (Hait *et al.*, 2009). Nuclear S1P was shown to form complexes with HDAC and prevent deacylation of lysine residues within the histone tail, thereby affecting its DNA binding and resulting in upregulation of p21 and c-fos. S1P has also been identified to be a cofactor for the ubiquitin ligase activity of TNF receptor-associated factor 2 (TRAF2) (Alvarez *et al.*, 2010). Binding of TRAF2 to S1P increases its activity and leads to subsequent activation of the NF-κB transcription factor to mediate pro-survival signalling. Moreover, prohibitin 2 (PHB2), a highly conserved protein that regulates mitochondrial assembly and function, has just recently been reported to bind to S1P both *in vitro* and *in vivo* (Strub *et al.*, 2011). This interaction between S1P and PHB2 appears to play an important role in cytochrome-c oxidase assembly and mitochondrial respiration.

1.6 SK/S1P in diseases

Elevated levels of SK1/S1P have been implicated in a number of disease pathologies, including asthma, inflammation and autoimmune diseases, atherosclerosis, hypertension, and neurodegenerative diseases [reviewed in (Pyne *et al.*, 2009; Takabe *et al.*, 2008)]. However, the most studied patho-physiological role of SK1 is in cancer.

1.6.1 Cancer

There is considerable evidence implicating SK and S1P in tumourigenesis [reviewed in (Pyne & Pyne, 2010; Pyne *et al.*, 2012)]. One of the initial studies examining the oncogenic potential role of SK1 showed that its overexpression in NIH 3T3 fibroblasts enhanced cell survival and proliferation, and induced neoplastic cell transformation and the formation of tumours in NOD/SCID mice (Xia *et al.*, 2000). These initial observations have been supported by other studies that have shown that SK1 activation is important in the promotion of estrogen-dependent tumour cell growth in breast cancer cells (Nava *et al.*, 2002; Sukocheva *et al.*, 2003). More recently, various studies have provided further and more specific evidence in support of a role for SK1 deregulation in naturally occurring solid tumours (French *et al.*, 2003b; French *et al.*, 2006). Indeed, there is now considerable evidence linking SK1 up-regulation to the development and progression of several types of human cancers, including glioblastoma (Van Brocklyn *et al.*, 2005), intestinal adenoma (Kohno *et al.*, 2006), colon cancer (Kawamori *et al.*, 2006; Kawamori *et al.*, 2009), prostate (Malavaud *et al.*, 2010), head and neck (Shirai *et al.*, 2011), oral (Liu *et al.*, 2010), thyroid (Guan *et al.*, 2011a) and non-small cell lung cancers (Song *et al.*, 2011). This increased SK1 expression has been associated with a poor survival of patients with brain tumours (Van Brocklyn *et al.*, 2005), gastric (Li *et al.*, 2009c), oral (Liu *et al.*, 2010), breast (Watson *et al.*, 2010) and non-small cell lung cancers (Song *et al.*, 2011). Overexpression of SK1 also correlates with resistance of cancer cells to chemotherapeutic agents (Illuzzi *et al.*, 2010; Akao *et al.*, 2006; Watson *et al.*, 2010; Guillermet-Guibert *et al.*, 2009). However, the most compelling evidence supporting the role of SK1 in cancer comes from studies showing that down-regulation of SK1 either by genetic or pharmacological approaches significantly reduces tumour growth *in vivo* in mice (Kohno *et al.*, 2006; French *et al.*, 2006; Kawamori *et al.*, 2009; Fuereder *et al.*, 2011; Shirai *et al.*, 2011), induces apoptosis

in cancer cells (Bektas *et al.*, 2009; Taha *et al.*, 2006; Paugh *et al.*, 2008) and also sensitizes tumor cells to other chemotherapeutics (Bonhoure *et al.*, 2008; Pchejetski *et al.*, 2008; Sauer *et al.*, 2009) and radiation therapy (Pchejetski *et al.*, 2010; Sinha *et al.*, 2011). Notably, specific monoclonal antibodies against S1P have also been produced which reduced tumour progression in murine xenograft and allograft models and are in clinical trials in cancer (Visentin *et al.*, 2006; O'Brien *et al.*, 2009).

In addition to the solid tumours, deregulation of SK1 has also been found in both acute and chronic myeloid leukemia [reviewed in (Pitson *et al.*, 2011; Ekiz & Baran, 2011)]. This includes findings that elevated levels of SK1 occur in a variety of leukemic cell lines which correlates with resistance of these lines to chemotherapeutic agents (Paugh *et al.*, 2008; Bonhoure *et al.*, 2008; Marfe *et al.*, 2011), while high SK1 expression appears to be integral for erythroleukemic progression (Le Scolan *et al.*, 2005). In addition, SK1 expression is involved in BCR/ABL-induced upregulation of Mcl-1 expression which appears to be important for survival and chemotherapy resistance of chronic myeloid leukemia (CML) cells (Li *et al.*, 2007; Li *et al.*, 2011a). Notably, a recent report has shown that SK1/S1P enhances BCR/ABL protein stability, which appears to be signalled through the $S1P_2$ pathway via inhibition of PP2A (Salas *et al.*, 2011). Inhibition of the SK1/ S1P pathway via either genetic manipulation or pharmacogenic methods inhibits growth of acute myeloid leukemia xenografts in mice (Paugh *et al.*, 2008), and also reduces chemotherapeutic resistance in both AML (Bonhoure *et al.*, 2006; Cakir *et al.*, 2011; Park *et al.*, 2010) and CML cells (Li *et al.*, 2011a). Furthermore, due to the opposing effects of S1P and ceramide/sphingosine, which can be inter-convertible within cells, chemotherapeutic agents which enhance the generation/accumulation of ceramides have been shown to induce apoptosis in CML cells (Cakir *et al.*, 2011; Kartal *et al.*, 2011; Camgoz *et al.*, 2011).

1.6.2 Inflammation and immunity

There is considerable evidence linking SK and S1P with inflammation and immunity [reviewed in (Spiegel & Milstien, 2011)]. S1P and $S1P_1$ play important functions in lymphocyte homeostasis by regulating the trafficking of these cells between the blood and lymphoid tissues (Chiba *et al.*, 2006). Furthermore, S1P is released from platelets and contributes to the invasion

of tissues by inflammatory cells, including mast cells and eosinophils (Rivera *et al.*, 2008). Notably, SK1 is required for TNFα-induced adhesion molecule expression in vascular endothelial cells which is necessary for leukocyte recruitment during inflammatory responses (Xia *et al.*, 1998; Xia *et al.*, 1999a). SK1 mRNA levels have also been reported to increase in RAW macrophages during inflammatory responses (Hammad *et al.*, 2008), supporting the importance of the SK1/S1P pathway in the inflammatory response. This is consistent with findings that chronic overexpression of SK1 promotes a pro-inflammatory phenotype in endothelial cells (Limaye *et al.*, 2009). In addition, other studies have shown that SK1 activation contributes to induction of pro-inflammatory cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) release, and this could be further increased by knockdown of S1P-degrading enzymes (Pettus *et al.*, 2003).

In addition to its pro-inflammatory role, however, S1P has also been shown to inhibit the expression of adhesion molecules in endothelial cells and suppress the production of pro-inflammatory cytokines via activation of phosphatidylinositol 3-kinase (PI3-K) and endothelial nitric oxide synthase (eNOS) (Kimura *et al.*, 2006). Furthermore, negative cross-talk between S1P receptors and toll-like receptor 2 has been reported in murine macrophages, thus preventing inflammation (Duenas *et al.*, 2008). Moreover oxidized LDL immune complexes induced release of SK1 from monocytes and promoted monocyte survival which could contribute to anti-inflammatory receptor engagement (Smith *et al.*, 2010). The anti-inflammatory property of S1P on macrophages was further highlighted in a model of acute necrotising pancreatitis in rats (Liu *et al.*, 2008). In this model, S1P as well as the S1PR agonist FTY720, decreased acute pulmonary inflammation and injury by suppression of NF-κB activity in alveolar macrophages and thereby production of IL1β, IL6 and TNF. Recently, $S1P_2$ signalling has been shown to play an important role during inflammation by showing that $S1P_2$ knockout mice enhanced macrophage recruitment during peritonitis (Michaud *et al.*, 2010).

The proposed role(s) of SK1 in inflammation, however, has become more contentious with SK1 knockout mice and specific knockdown of SK1 in a collagen-induced arthritis model, producing contrasting results. While SK1 knockout mice showed normal responses in collagen-induced arthritis, thioglycolate-triggered acute inflammation and efficient clearing of bacterial infection (Michaud *et al.*, 2006), specific targeting of SK1 by siRNA reduced the incidence and severity of disease in a murine collagen-induced arthritis model (Lai *et al.*, 2009). Factors

which possibly lead to these conflicting results could be due to different strains of mice and the mode of knockdown of the SK1 enzyme used in these two studies. Furthermore, the different dose of collagen used to induce arthritis in the two studies (Michchaud *et al.* used 100ug collagen whereas, *Lai et al.* used 200ug to induce the disease), may have contributed to the different outcome of these studies. Further studies are needed to clarify the underlining mechanisms of the effects of SK1/S1P on inflammation.

1.6.3 Asthma

A considerable number of studies have implicated SK/S1P in the progression of allergic responses including asthma, which is characterised by constriction of the smooth muscle cells in the airway and influx of inflammatory cells into the lungs [reviewed in (Lai *et al.*, 2011)]. Activation of S1P receptors and downstream signalling is also crucial for the migration of mast cells toward antigen (Jolly *et al.*, 2004). This is demonstrated by crosslinking of the high-affinity receptor for IgE (FcϵRI) with antigen on mast cells then activating SK. This leads to the generation and secretion of S1P which activates receptors ($S1P_1$ and $S1P_2$) in mast cells to promote degranulation and release of histamine, cytokines and chemokines to mediate inflammatory allergic reactions (Jolly *et al.*, 2004). Inhibition of SK blocks FcϵRI-mediated internalization of these receptors and reduces degranulation and chemotaxis. Although SK activation and S1P production are clearly required for FcεRI-dependent allergic responses in mast cells, results from various studies have suggest that differential formation of S1P by SK1 and SK2 has quite distinct and conflicting roles in mast cell functions. For example, in one study SK2 has been demonstrated to be required for FcεRI-mediated mast cell function (Olivera *et al.*, 2007), while, in another study it was found that both $Sphk1^{-/-}$ and $Sphk2^{-/-}$ mice have normal mast cell responses (Pushparaj *et al.*, 2009). In contrast, results from specific targeting of SK1 have shown that this enzyme seems to have a dominant role in the generation of S1P in mast cells (Pushparaj *et al.*, 2009; Oskeritzian *et al.*, 2008). Clearly, more studies are needed to clarify the importance of the selective role of the SK isoenzyme in inflammatory allergic reactions mediated by mast cell functions.

SK1 expression is elevated around bronchial epithelial walls and inflammatory areas in an asthmatic mouse model and S1P induces airways smooth muscle contraction and can

influence the migration of inflammatory cells, such as eosinophils towards the site of inflammation (Lai *et al.*, 2008a; Rosenfeldt *et al.*, 2003; Nishiuma *et al.*, 2008). Elevated levels of S1P are detected in bronchoalveolar lavage (BAL) fluid from allergic patients challenged with allergen, compared to normal non-allergic patients (Nishiuma *et al.*, 2008). S1P sustains inflammation in systemic inflammatory responses and is elevated in BAL fluid from asthmatics. Strikingly, inhalation of SK inhibitors in an ovalbumin (OVA) asthma mouse model improved the disease severity by decreasing the S1P levels in BAL as well as reducing peroxidase activity and eosinophil migration (Nishiuma *et al.*, 2008; Lai *et al.*, 2008b; Chiba *et al.*, 2010). Similarly, treatment with the S1P receptor modulator FTY720 in a murine asthma model prior to allergen challenge, appears to reduce airways inflammation and bronchial hyperresponsiveness (Idzko *et al.*, 2006). Interestingly, a functional variant of the *S1PR1* gene has recently been associated with asthma susceptibility and severity (Sun *et al.*, 2010c). Together these data support a significant role of SK/S1P in allergic asthma.

1.6.4 Atherosclerosis

SK and S1P are also implicated in the pathogenesis of atherosclerosis which is a result of a complex interaction of inflammatory signals in the vessel wall [reviewed in (Ipatova *et al.*, 2006; Weber *et al.*, 2008)]. Their involvement arises from studies showing that S1P in plasma is tightly associated with lipoproteins, HDL and LDL [reviewed in (Sato & Okajima, 2010; Nofer, 2008)]. LDL that accumulates in atherosclerotic lesions contains S1P at it is possibly produced locally in the atherosclerotic lesions by vascular smooth muscle cells (VSMC) activated by growth factors. Oxidised LDL (Ox-LDL) is a major risk factor for atherosclerosis as it can induce sphingomyelinase, ceramidase and SK in smooth muscle cells, resulting in S1P production and enhanced mitogenesis of these cells. The interaction between S1P and lipoproteins seems to reduce its bioactivity which may prevent full activation of S1P receptors in the vascular wall (Murata *et al.*, 2000b). In endothelial cells, SK activation is involved in TNFα induced ERK and NF-κB activities, as well as E-selectin and VCAM expression (Kimura *et al.*, 2006). HDL inhibits all these effects by preventing SK activation by TNFα, supporting an anti-atherogenic role of HDL via inhibition of intracellular SK activation and S1P production by pro-inflammatory cytokines (Xia *et al.*, 1999a). In fact, HDL-induced

vasorelaxation may be due to S1P which requires the $S1P_1$ receptor for its downstream Akt induction, eNOS activation and vasorelaxation (Keul *et al.*, 2007). These data implicate S1P as an anti-atherogenic, hypotensive and vasoprotective molecule. Nevertheless, the anti-atherogenic action of HDL is also suggested to be mediated by HDL-associated S1P bound solely to apolipoprotein M (ApoM), which is known to have anti-atherogeic effects (Christoffersen *et al.*, 2011).

In addition to the proposed anti-atherosclerotic role of S1P, a clinical trail has shown that S1P levels correlated with the severity of obstructive coronary artery disease (Sattler *et al.*, 2010). Treatment of endothelial cells with reconstituted HDL and *N*,*N*-dimethylsphingosine (DMS) suppressed TNFα-induced expression of adhesion molecules and apoptosis via inhibition of SK activity (Xia *et al.*, 1999a). In mouse models, it could be shown that treatment with FTY720, a pro-drug that forms an analogue of S1P, significantly reduced atherosclerotic plagues (Keul *et al.*, 2007). These data, again propose an active role of S1P in the atherosclerotic process. During vascular repair and thombosis after injury to an artery, thrombin activates platelets to release S1P that may promote vascular cell proliferation and platelet aggregation (Son *et al.*, 2008). This may benefit the repair process but can also contribute to the pathogenesis of atherosclerosis. Together, these studies point to an active but controversial role for SK and S1P in atherogenesis, with some studies implicating the lipid as a protector and other as a mediator of atherosclerosis.

Interestingly, $S1P_2$ signalling has been recently shown to be involved in atherosclerotic inflammation processes and hence inhibition of $S1P_2$ signalling by either genetic or pharmacological approaches attenuated atherosclerotic lesion formation in apolipoprotein E (ApoE) mice (Wang *et al.*, 2010a; Skoura *et al.*, 2011). $S1P_2$ seems to retain macrophages in atherosclerotic plaques and these macrophages from $ApoE^{-/-}$ /$S1P_2^{-/-}$ mice displayed reduced cytokine expression, ablated MCP-1 expression and elevated eNOS phosphorylation. Pharmacological inhibition of $S1P_2$ in wild type $ApoE^{-/-}$ /$S1P_2^{+/+}$ mice also reduced cytokine levels in plasma and decreased plaque size (Skoura *et al.*, 2011). Similarly, $S1P_3$ has been shown to mediate the chemotactic effect of S1P in macrophages both *in vitro* and *in vivo* (Keul *et al.*, 2011). Although there are no major differences in atherosclerotic lesions and lipid volume in the aorta of $ApoE^{-/-}$ /$S1P_3^{-/-}$ mice, these mice appear to have decreased monocyte and

macrophage recruitment to the atherosclerotic lesions, further suggesting a role of S1P and its receptors in the progression of atherosclerosis (Keul *et al.*, 2011).

1.6.5 Neurodegenerative diseases

In the central nervous system (CNS), S1PRs are expressed on neurons, astrocytes, oligodendrocytes and microglial cells [reviewed in (Pitson & Pebay, 2009; Soliven *et al.*, 2011)]. Both *in vivo* and *in vitro* studies suggest that S1P is important in a number of normal physiological functions in the CNS, such as maintenance of the integrity of the blood–brain barrier; migration of neuronal progenitor cells and astrocytes toward areas of damage; regulation of oligodendrocyte survival, function, and myelination following injury, and; regulation of microglial number and activation (Nayak *et al.*, 2010). Although the functions of SIP and its role in pathological processes involved in the CNS are still being unraveled, a number of studies have suggested an important role of the S1P receptor signalling pathway in neurodegenerative diseases including multiple sclerosis (MS) (Van Doorn *et al.*, 2010; Fischer *et al.*, 2011; Choi *et al.*, 2011) and alzheimer disease (AD) (He *et al.*, 2010b; Takasugi *et al.*, 2011). S1P is present in high levels in the blood and can enter the brain during CNS injury. In injured spinal cords, reactive astrocytes and microglia around the injury sites co-localise to regions with high levels of S1P, suggesting that the accumulation of reactive astrocytes and microglia is mediated through S1P receptors. In addition, stimulation of microglia with S1P induced the release of TNFα suggesting the potential involvement of S1P in CNS inflammation. Indeed, recent studies had demonstrated that S1P receptor levels were increased in encephalomyelitis (EAE), a mouse model for multiple sclerosis, and FTY720 appears to have protective function toward the disease (Chiba *et al.*, 2011; Choi *et al.*, 2011). Consistent with this, the level of S1P in cerebrospinal fluid correlates with increasing severity in patients with MS (Lee *et al.*, 2010; Wu *et al.*, 2008b; Nayak *et al.*, 2010).

Interestingly, SK2 activity appears upregulated in neuronal stress, such as ischemia. Furthermore, SK2 activity was increased in cells following exposure to β-amyloid fibrils, and was also found to be increased in the postmortem cerebral cortices of AD patients. This, together with the findings that chemical inhibitors of SK decreased β-amyloid levels in APP

transgenic mice (Takasugi *et al.*, 2011) suggest that SK2 and S1P are involved in the etiology of AD and are potential therapeutic targets for this disease.

1.7 SK regulation

1.7.1 Activation of SK1

SK1 has high intrinsic catalytic activity that is independent of post-translational modifications of the protein (Pitson *et al.*, 2000a) but is further activated by a number of external stimuli mentioned previously, such as various growth factors [platelet-derived growth factors (PDGF) (Olivera & Spiegel, 1993), epidermal growth factor (EGF) (Meyer zu Heringdorf *et al.*, 1999), hepatocyte growth factor (Duan *et al.*, 2004), vascular endothelial growth factor (VEGF) (Shu *et al.*, 2002), nerve growth factor (NGF) (Edsall *et al.*, 1997), and transforming growth factor beta (TGFβ) (Yamanaka *et al.*, 2004)], cytokines [tumour necrosis factor α (TNFα) (Xia *et al.*, 1999b) and interleukins (Mastrandrea *et al.*, 2005)], hormones [estradiol (Takabe *et al.*, 2010) and prolactin (Doll *et al.*, 2007)], and GPCR ligands [acetylcholine (van Koppen *et al.*, 2001), lysophosphatidic acid (Delon *et al.*, 2004) and S1P itself (Meyer zu Heringdorf *et al.*, 2001)]. While activation of SK1 by these agonists generally results in a modest enhancement of SK activity (approximately 2- to 3-fold) over basal levels, this is sufficient to increase cellular S1P levels to trigger downstream effects (Pitson *et al.*, 2000b; Pitson *et al.*, 2003).

1.7.1.1 Activation of SK1 by phosphorylation

A number of studies have examined the molecular mechanisms whereby SK1 is activated. Most studies to date have mainly focused on the post-translational regulation of SK1, in particular the cellular signalling pathways that are transiently modulated following SK1 activation (Pitson *et al.*, 2000b; Pitson *et al.*, 2003). This activation can occur through phosphorylation of SK1 at serine 225 by ERK1/2 (Pitson *et al.*, 2003). This results directly in enhanced catalytic activity of the enzyme (approximately 14-fold increase in V_{max}), but also its translocation to the plasma membrane (Pitson *et al.*, 2003) which is critical for agonist-induced

S1P generation and the pro-proliferative, pro-survival and oncogenic effects of SK1 (Pitson *et al.*, 2005). Mutation of this phosphorylation site (serine 225 to alanine) in human SK1 ($SK1^{S225A}$) ablates the ability for agonists to stimulate SK1 and abolishes the stimulatory effects of this enzyme on cell growth, resulting in attenuated survival and reduced colony growth in soft agar, demonstrating the necessity of this phosphorylation for the pro-survival, pro-proliferative and oncogenic signalling of SK1 (Pitson *et al.*, 2005). Interestingly, overexpression of $SK1^{S225A}$ inhibits Ras-dependent transformation of NIH3T3 fibroblasts despite exhibiting full basal SK1 activity (Pitson *et al.*, 2005). Moreover, artificial targeting of the $SK1^{S225A}$ mutant to the plasma membrane (by attaching a myristoylation and palmitoylation motif of Lck tyrosine kinase) rescued the ability of this SK1 variant to promote cellular transformation (Pitson *et al.*, 2005). Taken together, these data suggest that phosphorylation-induced localisation of SK1 to the plasma membrane and the localized production of S1P at the plasma membrane rather than the overall enhancement in SK1 activity is critical for agonist-induced S1P generation and the pro-proliferative, pro-survival and oncogenic effects of SK1.

1.7.1.2 Activation of SK1 by translocation to the plasma membrane

SK1 resides mainly in the cytoplasm, but translocates to the plasma membrane once activated by various stimuli including PDGF (Rosenfeldt *et al.*, 2001), NGF (Toman *et al.*, 2004), EGF (Sarkar *et al.*, 2005), TNFα (Pitson *et al.*, 2005), phorbol 12-myristate 13-acetate (PMA) (Johnson *et al.*, 2002), lipopolysaccharides (LPS) (Wu *et al.*, 2004), lysophosphatidic acid (Delon *et al.*, 2004) and calcium (Young *et al.*, 2003). As noted above this relocalisation of SK1 appears critical for the oncogenic effect of SK1. The mechanism of how SK1 is targeted to this location has only recently been determined. Early studies have suggested a mechanism for membrane targeting of SK1 which involves interaction with plasma membrane-associated phosphatidic acid (Delon *et al.*, 2004) or phosphatidylserine (Stahelin *et al.*, 2005). In particular, the phosphorylated state of SK1 appears to be essential for its interaction with phosphatidylserine at the membrane (Stahelin *et al.*, 2005). Indeed, phosphorylation of SK1 at Ser225 by ERK2 was proposed to expose threonine-54 and asparagine-89 and/or other phosphatidylserine-binding residues which are crucial for enhancing plasma membrane selectivity of SK1 (Stahelin *et al.*, 2005). Not all SK1 translocation depends on phosphorylation

of SK1 at Ser225, however, as SK1 robustly translocates to the phagosome membrane in macrophages in response to uptake of killed mycobacterium or latex beads (Thompson *et al.*, 2005). Clearly, the translocation mechanism of SK1 still required further clarification. Recently, the role of calcium and integrin binding protein 1 (CIB1) in SK1 translocation has been established (Jarman *et al.*, 2010). CIB1 has been shown to interact with SK1 as a calcium-myristoyl switch protein enabling SK1 translocation to the plasma membrane and hence siRNA knockdown of C1B1 inhibits SK1 translocation.

1.7.1.3 Activation of SK1 by protein-protein interactions

To date, several other SK1-interacting proteins have been shown to modulate the function of SK1 in cells. The first interaction with SK1 identified was with the adaptor molecule TNF receptor-associated factor 2 (TRAF2) (Xia *et al.*, 2002). Activation of SK1 by TNFα requires its interaction with TRAF2 which also mediates S1P-induced activation of NF-κB and subsequent anti-apoptotic effects. A number of other proteins have since been identified to interact with SK1 and modulate its activity. These include: SK1-interacting protein (SKIP) (Lacana *et al.*, 2002), δ-catenin (Fujita *et al.*, 2004), aminoacylase 1 (Maceyka *et al.*, 2004), four and a half LIM domains protein 2 (FHL-2) (Sun *et al.*, 2006) and platelet endothelial cell adhesion molecule (PECAM-1) (Fukuda *et al.*, 2004). While δ-catenin has been shown interact with SK1 and directly enhance its activity, SKIP, PECAM-1, FHL-2, and aminoacylase 1 have been shown to reduce the activity of SK1 *in vitro*. SK1-protein interactions also occur at the membrane level. Activation of mast cells induces interaction of SK1 with Lyn kinase, which brings the lipid kinase into close proximity with FcRI receptor within lipid raft domains (Urtz *et al.*, 2004). This interaction enhanced the enzymatic activities of both SK1 and Lyn kinase, although SK1 was not phosphorylated by Lyn. In addition Fyn, another Src-family protein tyrosine kinase, is also essential for SK1 and SK2 activation, since mast cells from Fyn deficient mice exhibit impaired SK1 and SK2 enzyme activity and S1P production (Olivera *et al.*, 2006). Recently, protein elongation factor 1A (eEF1A) was also shown to directly interact with both SK1 and SK2 and enhance their catalytic activity (Leclercq *et al.*, 2008).

1.7.2 Activation of SK2

Very few studies have examined the mechanism of regulation of SK2. Similar to SK1, the activity of SK2 can also be rapidly increased following cell exposure to TNFα (Mastrandrea *et al.*, 2005), IL-1β (Yoshimoto *et al.*, 2003), EGF (Hait *et al.*, 2007), and crosslinking of the IgE receptor FcεRI (Olivera *et al.*, 2006). In addition, SK2 can also be activated through phosphorylation by ERK1/2 (Hait *et al.*, 2007). Although the exact phosphorylation sites of SK2 still require further clarification, recent studies have demonstrated that activation of SK2 by EGF results in ERK1-mediated phosphorylation of SK2 at Ser351 and Thr578 thereby increasing its enzymatic activity which is crucial for EGF-induced cell migration of MDA-MB-453cells (Hait *et al.*, 2005; Hait *et al.*, 2007).

1.7.2.1 Regulation of SK2 localisation by phosphorylation

As mentioned earlier, SK2 is predominantly localised in the nucleus and the ER (Igarashi *et al.*, 2003b; Maceyka *et al.*, 2005a). Overexpression of SK2 has been reported to suppress cell growth and induce cell cycle arrest, with these effects correlating with its nuclear localisation (Igarashi *et al.*, 2003b). However, phosphorylation of SK2 by protein kinase D (PKD) at either Ser383 or Ser385 within the nuclear export signal (NES) results in its export from the nucleus to the cytoplasm (Ding *et al.*, 2007), suggesting that phosphorylation-induced cytoplasmic localisation of SK2 by this agonist may modulate its pro-apoptotic effects. In addition, it was also shown that upon phorbol ester stimulation of cells, Ser419 and Ser421 in the NES of SK2 were phosphorylated and stimulated the export of the enzyme from the nucleus to the cytosol (Ding *et al.*, 2007). Mutation of these two serine residues within this NES abolished SK2 export. Again, these findings suggest that phosphorylation of SK2 not only has a direct effect on the enzyme catalytic activity but may also alter its cellular localisation, which appears to play a critical role in its cellular function. This is consistent with a recent report which proposed the function of nuclear SK2 in epigenetic gene regulation since at this location it can associate with HDACs, inhibit histone deacetylation and enhance transcription of p21 and c-fos (Hait *et al.*, 2009).

1.7.2.2 Regulation of SK2 by protein-protein interactions

Interaction of SK2 with other proteins has also been described. This includes studies suggesting that the pro-apoptotic effect of SK2 is due to its ability to behave like a BH3-only protein and associate with Bcl-XL to block pro-survival singalling by this protein (Liu *et al.*, 2003). Since these findings were only shown with overexpressed SK2, the physiological significance is still unclear. SK2 also directly interacts with the cytoplasmic region of the IL-12 receptor subunit, IL-12B1, and expression of a dominant negative SK2 suppressed IL-2 induced production of interferon-γ (Yoshimoto *et al.*, 2003). In addition, like SK1, SK2 can directly interact with Fyn kinase which is required for SK2 activation by FcεRI crosslinking, since mast cells from Fyn deficient mice exhibit impaired SK1 and SK2 enzyme activity and S1P production (Olivera *et al.*, 2006). Similarly, as mentioned earlier in section 1.7.1.3 SK2 has also been shown to interact with eEF1A to enhance its catalytic activity *in vitro* (Leclercq *et al.*, 2008).

1.8 Transcriptional Regulation of SKs

1.8.1 Transcriptional regulation of SK1

While some detail of the post-translational regulation of SK1 has been elucidated, comparatively little is known of its transcription regulation despite the fact that SK1 is upregulated in many human cancers (Facchinetti *et al.*, 2010; Li *et al.*, 2009c; Shida *et al.*, 2008; Bayerl *et al.*, 2008; French *et al.*, 2003b; Kawamori *et al.*, 2006).

A number of agonists, including PDGF (Kleuser *et al.*, 2001), estrogen (Sukocheva *et al.*, 2003), phorbol esters (Nakade *et al.*, 2003), NGF (Sobue *et al.*, 2005), EGF (Doll *et al.*, 2005), histamine (Huwiler *et al.*, 2006), prolactin (Doll *et al.*, 2007), extracellular nucleotides (Klawitter *et al.*, 2007) and IL-1 (Paugh *et al.*, 2009) induce biphasic increases in cellular SK1 activity; an initial and transient activation that occurs within minutes, followed by a delayed but more sustained elevation in SK1 activity that appears due to increased SK1 transcription. While some information is now known of the signalling pathways linking these agonists to transcriptional regulation of SK1, which appear to differ between agonists, the direct

transcription factors involved remain unclear. A range of transcription factor binding sites have been identified in the *SPHK1* promoter, including those for Sp1, AP-1 and AP-2, all of which, depending on the agonist, appear to be involved in *SPHK1* transcription. For example, the Sp1-binding site appears to be important in expression of SK1 in rat pheochromocytoma PC12 cells in response to NGF (Sobue *et al.*, 2005), as well as in the human leukaemia MEG-01 cell line following exposure to phorbol esters (Nakade *et al.*, 2003), and in human neuroblastoma cells stimulated with glial cell line-derived neurotrophic factor (GDNF) (Murakami *et al.*, 2007). The latter two studies, however, suggest that Ap-2 binding motifs are also necessary to mediate *SPHK1* transcription induced by these agents. In contrast, other studies have implicated an AP-1 binding element in enhancing *SPHK1* transcription in glioblastoma cells in response to IL-1 (Paugh *et al.*, 2009). Upregulation of *SPHK1* transcription was required for SK1-mediated cell survival and invasiveness (Paugh *et al.*, 2009), suggesting SK1 transcriptional regulation may play important role(s) in tumourigenesis.

In addition to growth factors and other cell agonists, hypoxia has also been shown to induce SK1 expression in a range of cell types in a process dependent on HIF2α and hypoxia response elements in the *SPHK1* promoter (Ahmad *et al.*, 2006; Schwalm *et al.*, 2008; Anelli *et al.*, 2008).

Interestingly, recent studies suggest that in addition to transcriptional regulation, SK1 expression can also be regulated via its mRNA stability (Sobue *et al.*, 2008a). It was reported that the stability of the SK1 mRNA is regulated by the AU-rich element-binding proteins AUF1 and HuR, and that v-Src oncogene induces SK1 expression via modulation of these proteins and subsequent enhancement of SK1 mRNA stability, rather than by increasing *SPHK1* transcription (Sobue *et al.*, 2008a).

1.8.2 Transcriptional regulation of SK2

Unlike SK1, there is very little published data on the transcriptional regulation of SK2. Despite this, however, there are observation(s) suggesting that SK2 is also elevated in various solid tumours (French *et al.*, 2010; Beljanski *et al.*, 2010) and under hypoxic conditions (Schnitzer *et al.*, 2009).

1.9 Transcriptional regulation by SK/S1P

A number of studies have suggested the involvement of SK1 and S1P in regulating transcription of other genes. Studies showing SK1 is essential for TNFα-induced activation of the pro-survival transcription factor NF-κB provided some of the first evidence in this area (Xia *et al.*, 2002; Alvarez *et al.*, 2010). This was further confirmed by Bu *et al.* (2005) where they found that overexpression of SK1 up-regulated MMP1 protein, mRNA and its promoter activity via activation of the ERK1/2-Ets1 and NF-κB pathways (Bu *et al.*, 2006). SK1 has also been suggested to play a crucial role in the regulation of inflammatory genes such as MCP-1, VCAM-1 and E-selectin in response to inflammatory cytokines in endothelial cells (Chen *et al.*, 2004). Overexpression of SK1 induced tissue inhibitor of metalloproteinase 1 (TIMP-1) upregulation through the Ap-1 response element of the TIMP-1 promoter (Yamanaka *et al.*, 2004). Moreover, overexpression of SK1 increased growth associated protein 43 (GAP43) expression (Murakami *et al.*, 2011). It appears that SK1/S1P stimulates GAP43 transcription may be due to increased binding of C/EBPβ transcription factor to the C/EBP binding site located at the 5'-promoter of GAP43 (Murakami *et al.*, 2011). Besides the observations suggesting a role for SK1 in gene regulation, several studies have also acknowledged that S1P can activate gene transcription. This includes the role of S1P in induction of CYP17 transcription by stimulating the binding of sterol regulatory element binding protein (SREBP) 1 to its promoter following dibutyryl cyclic AMP stimulation (Ozbay *et al.*, 2006). Recently, S1P has also been shown to upregulate MMP2 and VEGF expression via activation of the transcription factor, ZNF580, which appears to be important for human endothelial cell migration and proliferation (Sun *et al.*, 2010a). Together, these studies show that some transcription factors are regulated by SK and S1P. Thus, in addition to the activation of numerous cell signalling pathways that can enhance cell survival and proliferation through post-translational mechanisms, evidence of transcriptional regulation by SK and S1P is also emerging. The transcriptional profile induced by the SKs, however, has not been defined.

1.10 Hypothesis:

Although transcriptional regulation by SK and S1P appears to play an important role in a number of pathophysiological processes, no studies have yet been published that systematically examined gene regulation by SK. Thus, we hypothesize that gene regulation induced by increased cellular SK activity and activation is important for normal and pathological cellular regulation.

1.11 Aims

1) To characterize gene and miRNA regulation induced in response to low, physiologically relevant overexpression of SK1 and SK2
2) To determine gene regulation induced specifically in response to SK activity and or SK1 activation
3) To determine the role of SK-responsive gene products in oncogenic and other signalling by the SKs

Chapter 2
Generation and characterisation of cell lines with tightly regulated inducible expression of sphingosine kinase 1 and 2

The majority of this Chapter has been published as:

Duyen H. Pham, Paul A.B. Moretti, Gregory J. Goodall, and Stuart M. Pitson (2008) Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3' AU-rich mRNA destabilizing elements. *Biotechniques* 45**,** 155-160.

See **Appendix 2** for author contributions and reprint

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3. CHAPTER 3: GENE AND MICRORNA REGULATION BY SPHINGOSINE KINASES

3.1 ABSTRACT

Sphingosine kinases (SKs) are highly conserved signalling enzymes that phosphorylate sphingosine to form sphingosine-1-phosphate (S1P). S1P plays important roles in a wide variety of cellular processes, including calcium mobilisation, mitogenesis, apoptosis, atherosclerosis, inflammatory responses and cytoskeletal rearrangement. Two SK isoforms exist (SK1 and SK2), which originate from different genes, termed *SPHK1* and *SPHK2*. Although the two enzymes show high sequence similarity, and generate the same product, S1P, some studies have suggested that SK1 and SK2 can have opposing cellular functions. Indeed, while SK1 appears to almost universally enhance cell survival, in at least some situations SK2 promotes apoptosis. Despite this, recent mouse knockout studies have suggested the two enzymes may have overlapping functions in vascular development and neurogenesis. The molecular mechanism(s) mediating the roles of the SKs and their product S1P have been extensively examined, but much is still unknown. Several observations suggest that transcriptional regulation by the SKs may play important role(s) in their cellular functions. Thus, using tightly controlled inducible expression of the SKs we have performed microarray analyses to elucidate genes and microRNAs regulated by the enhanced expression of the SKs. Notably, from these studies, we have identified a number of genes that were regulated by both SK1 and SK2 as well as genes that are differentially regulated by these two enzymes. We also found that catalytically dead SK1 and SK2 could regulate gene expression suggesting that these enzymes might have regulatory properties that are independent of catalytic activity. These findings may lead to new insights into the mechanism(s) whereby SK1 and SK2 mediate their cellular effects.

3.2 INTRODUCTION

There is considerable evidence indicating that elevated levels of sphingosine kinase 1 (SK1) and sphingosine 1-phosphate (S1P) in cells play an important role in generating many pathological processes through control of cell survival, proliferation, differentiation, migration and immune responses (Pitson, 2011; Pyne & Pyne, 2010; Leong & Saba, 2010). In particular, SK1-induced enhancement of cell survival and proliferation and promotion of neoplastic transformation (Xia *et al.*, 2000; Pitson *et al.*, 2005) has lead to SK1 receiving considerable attention as a target for cancer therapy (Pitman & Pitson, 2010; Gangoiti *et al.*, 2010). Indeed, numerous studies have shown that SK1 expression is elevated in a number of solid tumours (French *et al.*, 2003a; Kawamori *et al.*, 2006; Johnson *et al.*, 2005b; Kawamori *et al.*, 2009; Bayerl *et al.*, 2008) and leukaemias (Sobue *et al.*, 2008b; Paugh *et al.*, 2008; Bonhoure *et al.*, 2008; Li *et al.*, 2007; Bonhoure *et al.*, 2006; Le Scolan *et al.*, 2005). High SK1 expression has been correlated with poor survival of brain, gastric and breast cancer patients and others (Li *et al.*, 2009c; Ruckhaberle *et al.*, 2008; Van Brocklyn *et al.*, 2005). However, the most striking evidence for a role of SK1 in cancer includes findings that targeting SK by chemical or genetic means reduced tumour growth in mice and sensitized tumour cells to other chemotherapeutics (Guillermet-Guibert *et al.*, 2009; Pchejetski *et al.*, 2005a; Baran *et al.*, 2007; Bonhoure *et al.*, 2006; Bonhoure *et al.*, 2008; Sauer *et al.*, 2009).

Although it is becoming increasingly clear that SK1 can play a role in cancer development and progression, the molecular mechanisms whereby SK1 promotes tumourigenesis have not been clarified. Most studies to date have focused mainly on the cellular signalling pathways that are transiently modulated following SK1 activation, such as, Ras/ERK and PI3K/Akt pathways that appear to be mediated through the S1P G protein-coupled receptors (El-Shewy *et al.*, 2006; Radeff-Huang *et al.*, 2007; Kim *et al.*, 2010). While activation of these signalling pathways may directly contribute to the oncogenic effects of SK1, several lines of evidence suggest that SK1 and/or S1P can also activate various transcription factors to regulate gene transcription via these and other pathways. This includes findings that SK1 can mediate TNF-α induced activation of the NF-κB transcription factor (Xia *et al.*, 2002; Alvarez *et al.*, 2010), and enhance the expression of various inflammatory genes in endothelial cells (Chen *et al.*, 2004). SK1 overexpression has also been shown to result in the up-regulation of the matrix metalloproteinase (MMP) 1 via activation of the ERK1/2-Ets1 and NF-κB

pathways (Bu *et al.*, 2006). Moreover, overexpression of SK1 induced tissue inhibitor of metalloproteinase (TIMP) 1 upregulation via an Ap-1 response element in the TIMP1 promoter (Yamanaka *et al.*, 2004). Other evidence has indicated a role for S1P in gene regulation. For example, S1P stimulates the transcriptional activity of AP-1 (Takeshita *et al.*, 2000), upregulates MMP2 production via activation of ZNF580 transcription factor (Sun *et al.*, 2010a), and induces CYP17 transcription by stimulating the binding of sterol regulatory element binding protein (SREBP) 1 to its promoter following dibutyryl cyclic AMP stimulation (Ozbay *et al.*, 2006).

In contrast to SK1, the role and regulation of SK2 is less well defined. While a number of *in vitro* studies have suggested that under certain conditions SK2 can promote apoptosis (Igarashi *et al.*, 2003a; Liu *et al.*, 2003; Maceyka *et al.*, 2005b; Okada *et al.*, 2005; Hofmann *et al.*, 2008), the mechanism for these pro-apoptotic effects of SK2 remains to be determined. Knockdown of SK2 in various cancer cell lines, however, induced apoptosis and enhanced the sensitivity of these cancer cells to chemotherapeutics (Van Brocklyn *et al.*, 2005; Sankala *et al.*, 2007; Nemoto *et al.*, 2009; Schnitzer *et al.*, 2009) and SK2 inhibitors blocked tumour growth (French *et al.*, 2010; Beljanski *et al.*, 2011), suggesting a pro-survival role for this enzyme.

Recent studies have shown that SK2 and S1P in the nucleus interact with histones deacetylases (HDACs) and inhibit HDAC activity and appear to be involved in epigenetic changes in breast cancer cells (Hait *et al.*, 2009). Furthermore, SK1 translocates to the plasma membrane upon activation by various agonists and this activation-induced translocation appears to be integral for the oncogenic effects of this enzyme (Pitson *et al.*, 2005). Taken together, these data demonstrate the importance of subcellular localised SK/S1P in the regulation of cancer cell growth and/or apoptosis. The exact mechanism(s) whereby the SKs elicit these effects, however, awaits further examination.

Systematic studies to investigate gene regulation by the SKs have not yet been reported. Here, using microarray and microRNA array technology, together with human cell lines with inducible expression of SK1 and SK2, we have begun to elucidate the SK/S1P-regulated gene transcription profile to assist in correlating this with normal and pathological cellular function.

3.3 MATERIALS AND METHODS

3.3.1 Construction of expression plasmids

The catalytically inactive human SK1 (SK1^{G82D}) (Pitson *et al.*, 2000b) was sub-cloned into pcDNA5/FRT/TO-SK1(FLAG)-AU following digestion with BamHI and NotI. The construct for expression of catalytically inactive human SK2 (SK2^{G212D}) was produced by sub-cloning SK2^{G212D} cDNA containing a C-terminal FLAG epitope tag (Moretti & Pitson, unpublished) into pcDNA5/FRT/TO-AU vector following digestion with BamHI and NotI by replacing the wild-type SK1 with the mutant BamHI/NotI SK2^{G212D} cassettes/fragments.

3.3.2 RNA preparation, and DNA microarray and microRNA array analysis

Microarray studies were performed on cells that were either induced to express recombinant SK1 proteins with 0.5 ng/ml doxycycline or the same cells not exposed to the inducing agent. For mRNA arrays, RNA was extracted at various time points (t=6, 12 and 24 h) after induction of SK expression by part TRIzol extraction (Invitrogen) followed by Qiagen RNAeasy column prep (Qiagen, Valencia, CA) according to the manufacturers specifications. RNA samples were run on Bioanalyser (Agilent) before committing to arrays to ensure high-quality RNA integrity.

Compugen human 19,000-oligonucleotide library microarrays were performed at the Adelaide Microarray Facility. Briefly, cDNA probes were prepared by incubating 20 µg of RNA with 4 µg of anchored polyT(V)N at 70 °C for 10 min followed by incubation on ice. Samples were mixed with 400 U/µl Superscript II and and aminoallyl (aa) dNTP mix (25 mM dATP, 25 mM dGTP, 25 mM dCTP, 10mM dTTP and 15 mM aa dUTP) in 5X Superscript II buffer containing 0.1 M dithiothreitol and incubated at 42 °C for 2.5 hours. RNAs were then hydrolysed by incubating with 0.25 M NaOH and 0.5 M EDTA (pH 7.5) at 65 °C for 15 min with immediate neutralization into 0.2 M acetic acid to terminate the reaction. cDNAs were purified using QIAquick PCR purification kit according to the manufacture's protocol, dried under reduced pressure, dissolved in 0.1 M $NaHCO_3$ (pH 9.0) and incubated with Cy3 or Cy5 dyes (Amersham) at room temperature (in the dark) for 60 min. The labeled cDNAs were diluted in Milli Q water and again purified using a QIAquick PCR purification kit. Purified cDNAs were mixed with hybridization buffer (16 µg yeast tRNA, 8 µg poly A and 20 µg Cot-1

DNA), dried, dissolved in 6.25X saline-sodium citrate (SSC) containing formamide, heated at 100 °C for 3 min followed by immediate incubation on ice with addition of 10 % sodium dodecyl sulfate (SDS) and then hybridized to arrays at 42 °C in a humidified chamber overnight. The following day, arrays were washed with 0.5X SSC containing 0.01 % SDS for 5 min followed by another 5 min wash with 0.5 X SSC and then 3 min with 0.2 X SSC. Slides were dried by centrifugation at 750 rpm for 5 min and scanned with a Genepix 4000B Scanner (Axon). Gene expression patterns in these cells were examined at various time points following induction of SK1 or SK2 expression and compared to zero time point just prior to induction. All arrays were performed in dual colour mode using two biological replicates for each time point, analysed by microarray in duplicate, with dye-swap replicates also performed for each comparison. The zero time (T_0) point was used as a reference to obtain relative expression levels following SK induction.

For miRNA arrays, total RNA was extracted using the complete Invitrogen TRIzol procedure with isopropanol precipitation to preserve the small RNA fraction. miRNAs were labeled by incubating 5 µg of RNAs with labelling mix containing 500 ng Cy3 or Cy5 dinucleotide (Dharmacon), 10 X Igloi buffer (1 mM ATP, 500 mM HEPES pH 7.8, 35 mM DTT, 200 mM $MgCl_2$ and 100 mg/ml BSA), DMSO, 20 (U) RNA ligase (NEB) and DEPC water in a total volume of 10 µl on ice in the dark for 2 h. Reactions were terminated by addition of Diethylpyrocarbonate (DEPC) water containing 3 M sodium acetate (pH 5.2) and 20 µg glycogen followed by precipitation with 100 % ethanol. Pellets were dried, resuspended in Exiqon hybridization buffer and incubated at 95°C for 3 min. Labelled miRNAs were hybridized to spotted microarrays printed using the Exiqon library, v8.1 in the dark at 56°C overnight. Following hybridization, slides were washed for 2 min in DEPC water containing 2 X Exiqon salt buffer and 0.2 % Exiqon detergent solution, followed by another 2 min in DEPC water containing 1 X Exiqon salt buffer and then 2 min with DEPC containing 0.2 X Exiqon salt buffer. Slides were dried and scanned with a Genepix 4000B Scanner (Axon). MiRNA expression patterns in these cells were examined at t=6 h following induction of SK1 expression and compared to T_0. All arrays were performed in dual colour mode using two biological replicates, with dye-swap performed for each array comparison.

3.3.3 Data analysis

For each channel (Cy3, Cy5), the foreground and background median pixel intensity values were extracted from the scanned images using the Spot v3 plugin (CSIRO, Australia) for R (www.r-project.org). After background subtraction, the foreground intensities were log2 transformed and a single ratio (Cy5/Cy3) value was obtained for each probe. For each array the probe ratio values were normalized with the Limma plugin for R, using the printtip-loess normalization option (Smyth & Speed, 2003). The five replicate arrays were normalized to each other to give similar ranges of mRNA expression values. For each probe across the arrays, a linear model was fitted to determine the final expression value for each mRNA and associated statistics (Smyth, 2004). An empirical Bayes method was used to generate statistics which enabled the ranking of mRNA expression, from the most likely to be differentially expressed to the least between the 2 populations. For miRNA arrays, similar approaches were used to analyze the data, except, global loess normalization method was used to identify differentially expressed miRNAs (Smyth & Speed, 2003).

To examine the distribution of differentially expressed genes/miRNAs for a given comparison of interest, a volcano plot was constructed, where the $\log_2$ (fold change) was on the x-axis and $-\log_{10}$ [false discovery rate (FDR)-adjusted P value] on the y-axis. This generates a volcano shaped chart, which is useful as it takes into account both the fold-change of the genes/miRNAs as well as the reproducibility of the results. Genes identified from this analysis were categorised into groups according to their functions (i.e. unknown function versus various cellular functions). In particular, we focused on genes that are known to be involved in cellular proliferation and survival. Genes that are differentially regulated by SK expression at early times were preferred to enhance the chances of identifying primary effects of SK overexpression.

3.3.4 Quantitative real-time PCR (qPCR)

QPCR analyses were performed using RNA preparations from the samples used in the microarray experiments or RNA samples prepared from subsequent independent experiments. Briefly, cDNA was synthesized from 2µg of total RNA in a 20µl reaction volume by using Omniscript RT kit (Qiagen, Valencia, CA) and oligo$(dT)_{17}$-adaptor primers (Frohman *et al.*,

1988) for amplification. QPCR analyses were performed using 2x Quantitect SYBR Green PCR Master mix reagent (Qiagen, Valencia, CA) with various primers designed for each gene candidates (Table 1) (Geneworks, Adelaide, Australia) using the following profiles: 50 °C for 2 min, 95 °C for 15 min, 45 cycles at 95 °C for 15 s and 55 °C for 20 s to give rise to 100 bp products. Data were acquired using the Rotor-Gene™ 6000 software 1.7 (Corbett Research) and expression of mRNA was normalized against the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')	Product size (bp)
RASD1	AGGGTGACCGCGACTTCTA	GCAGGTCTGGGCTCATCTC	180
SFPQ	GTTACAGCCGAATGGGCTACA	TTCTGGCCTCCTGAACCATAG	103
TFR1	ACCCATTCGTGGTGATCAAT	CGTTTCCAACTGCCCTATGA	127
HSPA5	TGGATCCCAACACCAAACTC	GTATTGGGCTTGGCCTGAG	119
FUS	ACAAACAAGAAAACGGGACAGC	GTGGGTCATCAAAAGAGACCG	97
PPP1R10P	TTCACCCTGTCAACCACCTC	CTGCACAAAACCCAACCAAT	121
CLK1	CCAACCATGTGATGTCTGGA	TCCATCATTGCTAAATGCTCCT	119
EIF4B	GCCACCCTACACTGCTTTTCT	TCTCTGGATTGCTGGGTTCAC	125
PCGF2	CTATGCAGCGTACCCCCTG	TCCCTGGCACCTTCGTAGAA	138
PCTK3	TATGAATCCAAGAGTCGCATGTC	GAAGGCCAAGCCTCGGTAG	159
ZNF6	ATGTGAGCATTGTCCCCAAG	GCCGCTTAAGGTCACTTGAAT	111
ARMET	CGGACCGATTTGTAGTCTGC	AGGAAAGCTCCAGGCTTCAC	149
HSPA8	CCCCGAGGTGTTCCTCAGAT	CAAACGGCCCTTGTCATTAGT	129
IRS4	GTGAGAATGGATTTTGCCAGAC	TAGACTGTAGCGCATCGAATCA	123
TNFRSF10D	TCAGAGGCCTTCCTTGAAGA	GCCCGGGTATAAAGCAAAAC	147
TSC23D3	CTCCCCAAGCATCATCTCAC	CTCTTGTCAGGGGTCTGTCG	114

Table 1: Primer sequences used in qPCR

3.4 RESULTS AND DISCUSSION

3.4.1 Expression profiling of gene regulation by SK1

In an attempt to identify gene regulation in response to cellular SK1 expression, we conducted a large-scale gene expression profile using microarray technology. For the purpose of SK1 gene regulation studies, tightly controlled SK1 inducible cell lines were generated using a novel approach by incorporating the AREs into the 3' UTR of the Tet-inducible constructs (Pham *et al.*, 2008).

The major advantage of using inducible expression of SK1 over cells with constitutive overexpression of SK1 is the stringent control of the level and timing of SK1 overexpression. Thus, using this system, we were able to examine immediate early genes regulated in response to a subtle increased cellular SK1 overexpression which would be more likely to resemble the *in vivo* level of SK1 previously detected in human cancers. We reasoned this approach would enhance the likelihood of identifying direct and physiologically relevant target genes by SK1 and reduce the possibility of non-specific secondary effects due to high and long term overexpression.

SK1 overexpression was induced in Flp-In™ T-Rex™ HEK293 cells to around 10-fold higher than endogenous levels, RNA was isolated and then a number of arrays were performed using a Compugen human 19,000-oligonucleotide library (Adelaide Microarray Centre) comparing the gene expression pattern in cells induced to express SK1 for 6 h, 12 h and 24 h, to cells that were untreated. Results from volcano plots (Figures 3.1A, B and C) showed genes differentially regulated by SK1 overexpression following 6 h, 12 h and 24 h, respectively and the top 50 genes that were differentially regulated over time following induction of SK1 overexpression are shown in Figure 3.2. Remarkably, even though SK1 overexpression was only moderately higher than endogenous levels, this was sufficient to generate a considerable number of genes that showed statistically significant regulation. Since robust gene regulation was observed after 6 h, this early time point was selected for further studies to optimize the likelihood of identifying direct effects of gene regulation by SK1. To eliminate SK1 protein effects that were not dependent on its catalytic activity, we performed additional microarrays using cells induced to overexpress the catalytically inactive variant of SK1, $SK1^{G82D}$. Somewhat surprisingly, low overexpression of $SK1^{G82D}$ to a similar level to that of wild-type

SK1 (Figure 3.3) also resulted in the regulation of a number of genes at 6 h, suggesting at least some non-catalytic effects of the SK1 protein on gene regulation in this system. Gene expression profiles were then compared between arrays to identify genes differentially expressed in response to low regulated overexpression of wild-type SK1, but not $SK1^{G82D}$ (Figure 3.4).

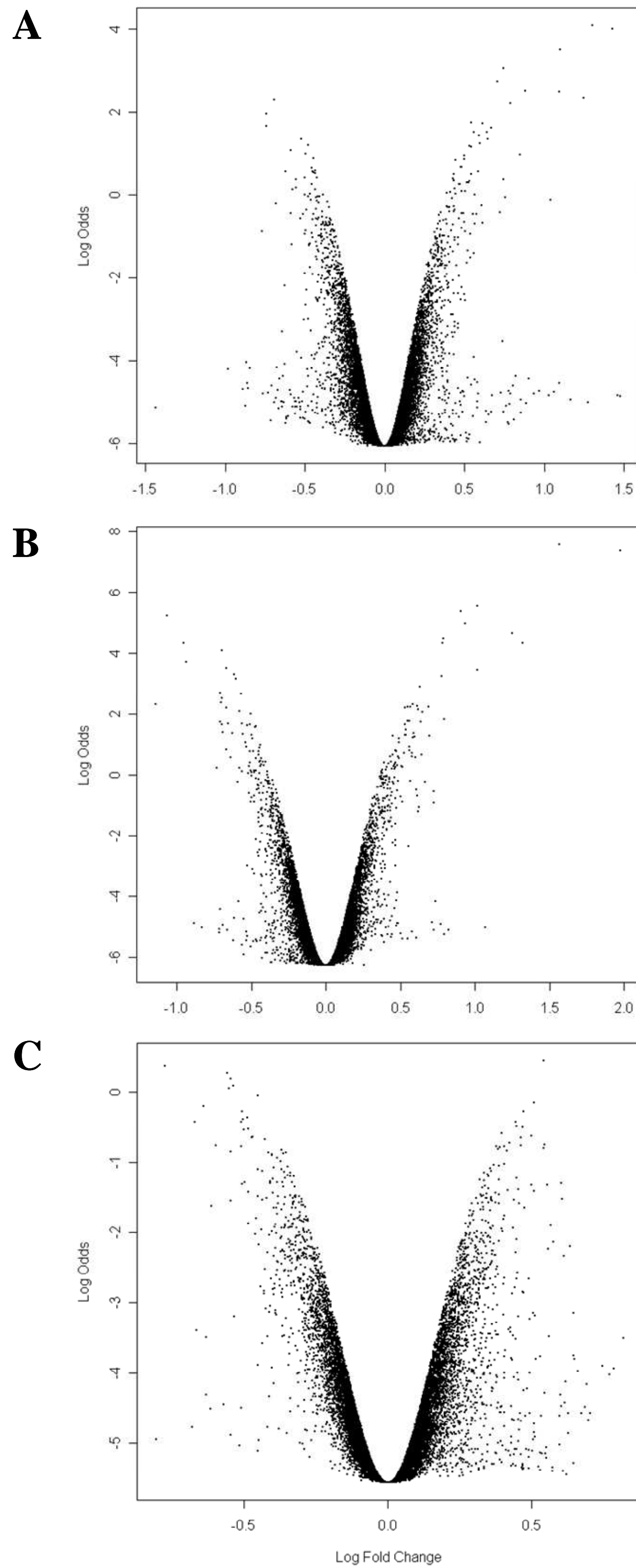
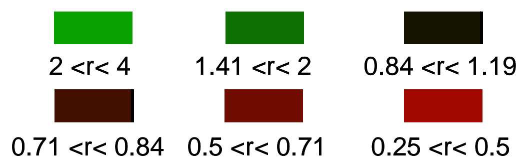


Figure 3.1 Differential genes expression mediated by cellular SK1. Volcano plots showing gene modulation following induction of SK1 at 6 h **(A)**, 12 h **(B)** and 24 h **(C).**

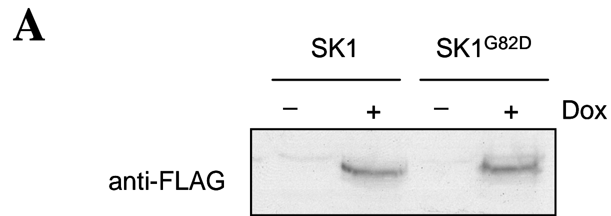
Figure 3.2 Heat plot of differentially expressed genes altered by inducible expression of SK1. Heat plot of genes that showed differential regulation by cellular expression of SK1 following 6 h, 12 h and 24 h post induction. Genes shown in green were up-regulated, and those in red were down-regulated, while those that did not show any change with respect to a normalized matched control are shown in black (see color scale).

Gene ID	Gene Abbr.	Gene Name
NM_006597	HSPA8	Heat shock 70kDa protein 8
AF216292	HSPA5	Heat shock 70kDa protein 5
Y11162	U68	U68 small nucleolar RNA
NM_006010	MANF	Mesencephalic astrocyte-derived neurotrophic factor
NM_005346	HSPA1B	Heat shock 70kDa protein 1B
NM_004960	FUS	Fused in sarcoma
NM_003234	TFRC	Transferrin receptor
NM_018371	CSGALNACT′	Chondroitin sulfate N-acetylgalactosaminyltransferase 1
NM_003840	TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d
U12210	E1b	Small nucleolar RNA (E1b)
NM_018307	RHOT1	Ras homolog gene family, member T1
NM_004089	TSC22D3	TSC22 domain family, member 3
NM_005066	SFPQ	Splicing factor proline/glutamine-rich
AL161977	PCTK3	PCTAIRE protein kinase 3
NM_004396	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
K01564	RoScR	Ro small cytoplasmic ribonucleoproteins
NM_005323	HIST1H1T	Histone cluster 1, H1t
AF070552	CDT1	Chromatin licensing and DNA replication factor 1
NM_003746	DYNLL1	Dynein, light chain, LC8-type 1
NM_005962	MXI1	MAX interactor 1
NM_006644	HSPH1	Heat shock 105kDa/110kDa protein 1
AK025862	HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1
V00589	5SrRNA	5S ribosomal RNA
K03191	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
AF116620	SLC38A2	Neutral amino acid transporter 2
NM_004071	CLK1	CDC-like kinase 1
D28449	ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (13.0 kD)
NM_000319	PEX5	Peroxisomal biogenesis factor 5
NM_003513	HIST1H2AB	Histone cluster 1, H2ab
NM_005911	MAT2A	Methionine adenosyltransferase II, alpha
AF071569	CAMK2D	calmodulin-dependent protein kinase II (56.4 kD)
AL110141	SNORD81	Putative protein (5.5 kD) (GAS5_)
NM_007144	PCGF2	Polycomb group ring finger 2
NM_004634	BRPF1	Bromodomain and PHD finger containing, 1
NM_020143	PNO1	Partner of NOB1 homolog (S. cerevisiae)
AF283645	SLC25A32	Solute carrier family 25, member 32
NM_001417	EIF4B	Eukaryotic translation initiation factor 4B
NM_003299	HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1
NM_002131	HMGA1	High mobility group AT-hook 1
NM_012484	HMMR	Hyaluronan-mediated motility receptor (RHAMM)
NM_003017	SRSF3	Serine/arginine-rich splicing factor 3
NM_003075	SMARCC2	SWI/SNF related, matrix associated, subfamily c, member 2
NM_000466	PEX1	Peroxisome biogenesis factor 1
AF110265	EPS15L1	Epidermal growth factor receptor substrate 15-like 1 family member (94.3 kD)
NM_014175	MRPL15	Mitochondrial ribosomal protein L15
NM_018445	SELS	Selenoprotein S
NM_014303	PES1	Pescadillo homolog 1, containing BRCT domain (zebrafish)
NM_018118	MCM3APAS	MCM3AP antisense RNA (non-protein coding)
NM_016448	DTL	Denticleless homolog (Drosophila)
NM_002913	RFC1	Replication factor C (activator 1) 1, 145kDa

6 h 12 h 24 h



A



B

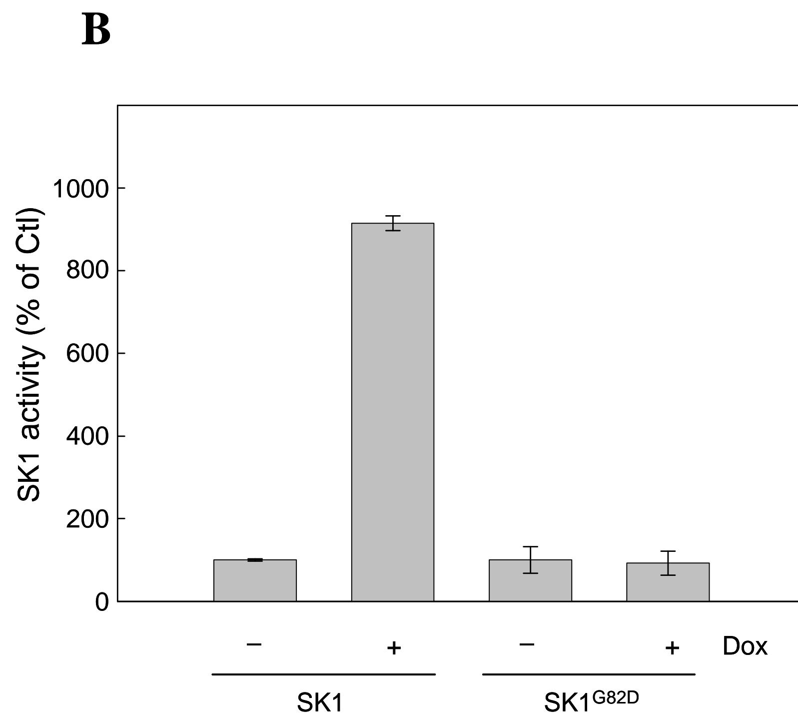
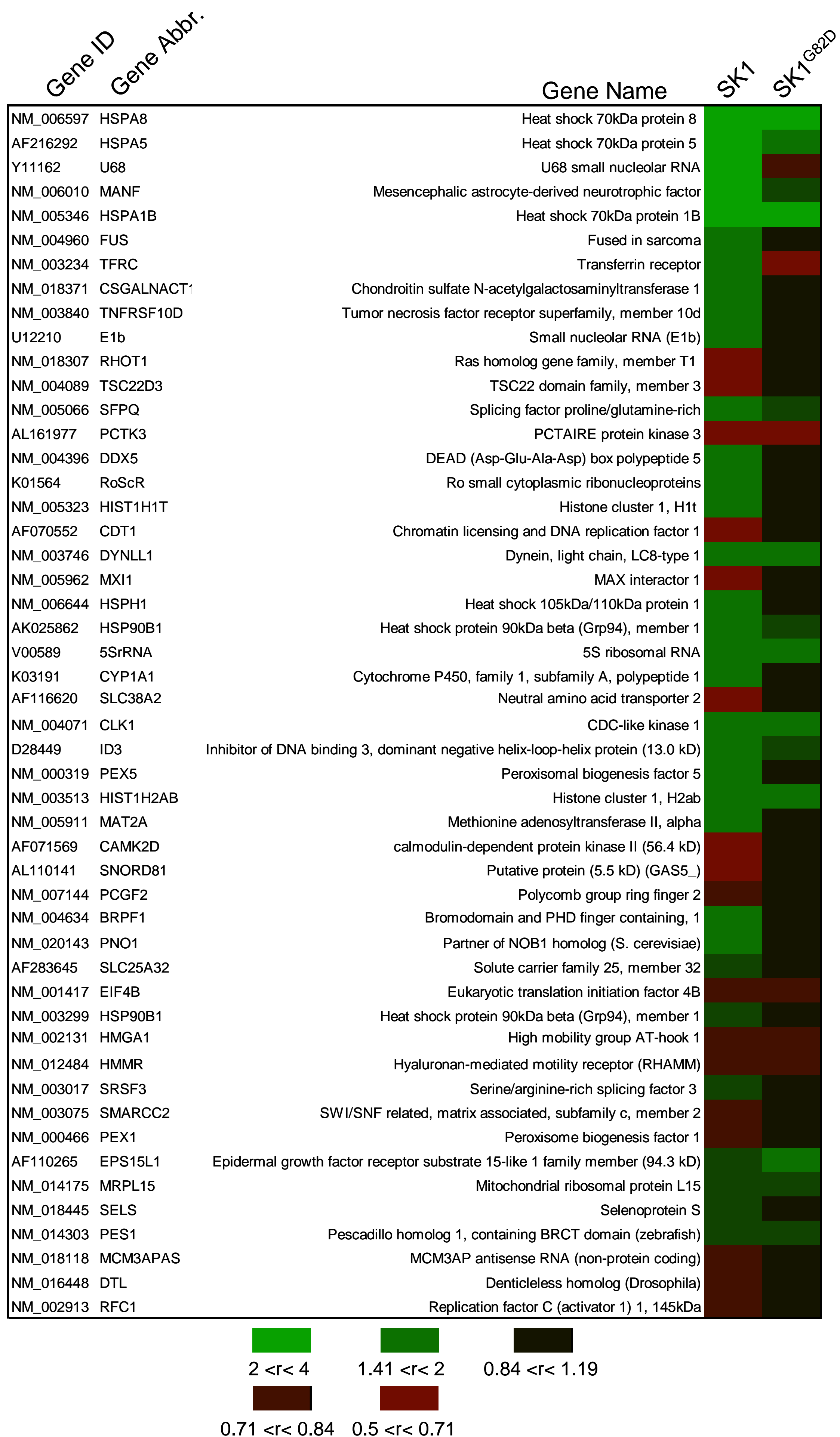


Figure 3.3 Low SK1 and SK1^{G82D} expression level used in arrays. SK1 or SK1^{G82D} Flp-In T-Rex HEK293 cells were cultured in medium for 6 h either in the absence or the presence of 0.5 ng/ml doxycycline. The expression of SK1 and SK1^{G82D} was then examined in cell lysates by immunoblot analysis using an anti-FLAG antibody (**A**) and SK activity assays (**B**).

Figure 3.4 Heat plot comparing differentially expressed genes by SK1 versus SK1^{G82D}. Hierarchical clustering of the genes that were differentially regulated by changes in cellular level of SK1 versus SK1^{G82D} following 6 h induction. Genes shown in green were up-regulated, and those in red were down-regulated, while those that did not show any change with respect to a normalized matched control are shown in black (see color scale).


Gene ID
Gene Abbr.
Gene Name
SK1
SK1G82D
NM_006597 HSPA8 Heat shock 70kDa protein 8
AF216292 HSPA5 Heat shock 70kDa protein 5
Y11162 U68 U68 small nucleolar RNA
NM_006010 MANF Mesencephalic astrocyte-derived neurotrophic factor
NM_005346 HSPA1B Heat shock 70kDa protein 1B
NM_004960 FUS Fused in sarcoma
NM_003234 TFRC Transferrin receptor
NM_018371 CSGALNACT1 Chondroitin sulfate N-acetylgalactosaminyltransferase 1
NM_003840 TNFRSF10D Tumor necrosis factor receptor superfamily, member 10d
U12210 E1b Small nucleolar RNA (E1b)
NM_018307 RHOT1 Ras homolog gene family, member T1
NM_004089 TSC22D3 TSC22 domain family, member 3
NM_005066 SFPQ Splicing factor proline/glutamine-rich
AL161977 PCTK3 PCTAIRE protein kinase 3
NM_004396 DDX5 DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
K01564 RoScR Ro small cytoplasmic ribonucleoproteins
NM_005323 HIST1H1T Histone cluster 1, H1t
AF070552 CDT1 Chromatin licensing and DNA replication factor 1
NM_003746 DYNLL1 Dynein, light chain, LC8-type 1
NM_005962 MXI1 MAX interactor 1
NM_006644 HSPH1 Heat shock 105kDa/110kDa protein 1
AK025862 HSP90B1 Heat shock protein 90kDa beta (Grp94), member 1
V00589 5SrRNA 5S ribosomal RNA
K03191 CYP1A1 Cytochrome P450, family 1, subfamily A, polypeptide 1
AF116620 SLC38A2 Neutral amino acid transporter 2
NM_004071 CLK1 CDC-like kinase 1
D28449 ID3 Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (13.0 kD)
NM_000319 PEX5 Peroxisomal biogenesis factor 5
NM_003513 HIST1H2AB Histone cluster 1, H2ab
NM_005911 MAT2A Methionine adenosyltransferase II, alpha
AF071569 CAMK2D calmodulin-dependent protein kinase II (56.4 kD)
AL110141 SNORD81 Putative protein (5.5 kD) (GAS5_)
NM_007144 PCGF2 Polycomb group ring finger 2
NM_004634 BRPF1 Bromodomain and PHD finger containing, 1
NM_020143 PNO1 Partner of NOB1 homolog (S. cerevisiae)
AF283645 SLC25A32 Solute carrier family 25, member 32
NM_001417 EIF4B Eukaryotic translation initiation factor 4B
NM_003299 HSP90B1 Heat shock protein 90kDa beta (Grp94), member 1
NM_002131 HMGA1 High mobility group AT-hook 1
NM_012484 HMMR Hyaluronan-mediated motility receptor (RHAMM)
NM_003017 SRSF3 Serine/arginine-rich splicing factor 3
NM_003075 SMARCC2 SWI/SNF related, matrix associated, subfamily c, member 2
NM_000466 PEX1 Peroxisome biogenesis factor 1
AF110265 EPS15L1 Epidermal growth factor receptor substrate 15-like 1 family member (94.3 kD)
NM_014175 MRPL15 Mitochondrial ribosomal protein L15
NM_018445 SELS Selenoprotein S
NM_014303 PES1 Pescadillo homolog 1, containing BRCT domain (zebrafish)
NM_018118 MCM3APAS MCM3AP antisense RNA (non-protein coding)
NM_016448 DTL Denticleless homolog (Drosophila)
NM_002913 RFC1 Replication factor C (activator 1) 1, 145kDa
2 <r< 4
1.41 <r< 2
0.84 <r< 1.19
0.71 <r< 0.84
0.5 <r< 0.71

From the expression profiles, 22 genes were shown to be modulated by wild-type SK1 but had either no major change or opposite changes in their mRNA expression by $SK1^{G82D}$, suggesting that these genes that were specifically regulated by SK1 activity. Among these genes, 13 genes were upregulated and 9 genes were downregulated specifically by SK1. The genes that were upregulated were involved in cell proliferation and survival [*TFRC* (also known as p90 and CD71) (O'Donnell *et al.*, 2006; Habashy *et al.*, 2010), *DDX5* (Wortham *et al.*, 2009), *MAT2A* (Lu & Mato, 2008)], cell cycle and transcriptional regulation [*FUS*, (Buratti & Baralle, 2010), *SFPQ* (also known as polypyrimidine tract binding protein associated splicing factor) (Tapia-Paez *et al.*, 2008), *HIST1H1T* (Grimes *et al.*, 2003), *BRPF1* (Ullah *et al.*, 2008)], transportation and biochemical pathways [*B4GALNT1* (Dobrovic *et al.*, 2011), *CYP1A1* (Sergentanis & Economopoulos, 2010), *SLC25A32* (Haitina *et al.*, 2006), *SELS* (Olsson *et al.*, 2011)] and other, had unknown functions [*U68*, *E1b* (is also known as *SNORA73A*), *RoScR*]. On the other hand, genes associated with tumor-suppressor functions [*MXI1* (Manni *et al.*, 2002), *PCGF2* (is also known as *MEL18*) (Guo *et al.*, 2007; Zhang *et al.*, 2010)], cell cycle and DNA replication [*CDT1* (Nishitani & Lygerou, 2002), *DTL* (Sansam *et al.*, 2006), *RFC1* (Ryu *et al.*, 2006)], and transportation/lipid biosynthesis [*RHOT1* (Fransson *et al.*, 2006), *SLC38A2* (Zhang *et al.*, 2011), *PEX1* (Wanders, 2004)] were significantly down-regulated.

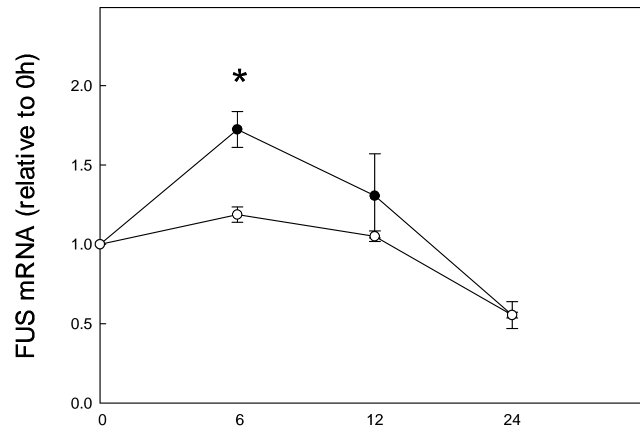
Notably, it appears that the mRNA expression of *U68* and *TFRC* were enhanced by wild-type SK1, but repressed by $SK1^{G82D}$, suggesting that these two genes were regulated by SK1 activation. *TFRC* encodes for transferrin receptor 1 (TFR1) and is a cell membrane-associated glycoprotein involved in regulation of cell growth (Daniels *et al.*, 2006). *U68* encodes for SNORA68, an H/ACA box class of small nucleolar RNAs (snoRNAs), and is involved in cellular processes, such as protein synthesis, mRNA splicing and telomerase function (Mallardo *et al.*, 2008). Interestingly, both genes have been implicated in cancers. For example, increased expression of TFR1 was detected in various human cancers (Helpman *et al.*, 2009; Wirth *et al.*, 2006; Ha *et al.*, 2009) and overexpression of TFR1 correlated with enhanced resistance of breast cancer to tamoxifen (Habashy *et al.*, 2010). Although snoRNAs have not been widely studied like TFR1, dysregulation of various snoRNAs has also recently been proposed to contribute to carcinogenesis (Liao *et al.*, 2010). Thus, it is likely that these two genes will have potential functional significance in SK1-mediated oncogenesis. SK1-induced expression of TFR1 is examined in more detail in Chapter 4 of this thesis.

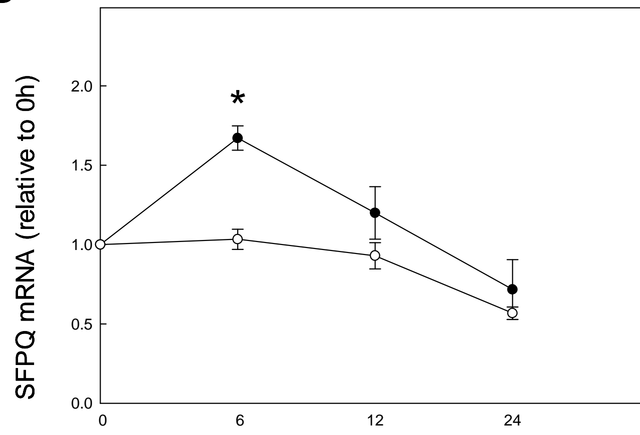
In addition to the potential genes regulated by SK1 activity, surprisingly, a considerable number of genes encoding for heat shock proteins (HSPs), such as, *HSPA8*, *HSPA5* (also known as *GRP78*), *HSPA1B*, *HSPH1*, and *HSP90B1* were elevated by both wild-type SK1 and SK1^{G82D}, suggesting that these genes could be regulated by SK1 protein independent of its catalytic activity. HSPs are known to function as chaperones to assist in protein folding, assembly, degradation and translocation (Li & Srivastava, 2004; Zhao & Houry, 2005; Jose-Eneriz *et al.*, 2008; Mellati, 2006). Interestingly, SK1 has been shown to be inherently unstable (Pitson *et al.*, 2000a), which may contribute to the large numbers of HSPs upregulated in the SK1 arrays. However, recent studies have indicated that various high molecular weight HSPs, for example, HSP70, HSP90 and HSP105, which were also identified in our arrays, are overexpressed in a wide variety of human tumours (Onda *et al.*, 2004; Park *et al.*, 2009; Jose-Eneriz *et al.*, 2008; Muchemwa *et al.*, 2008). Furthermore, high expression of several HSPs has been shown to promote cancer cell proliferation and survival (Muchemwa *et al.*, 2006; Huang *et al.*, 2009) and inhibition of HSPs by either genetic or chemotherapeutic approaches has been shown to induce apoptosis in various cancer cells and reduce tumour growth in various xenograft mouse models (Breinig *et al.*, 2009; Tran *et al.*, 2010; Sun *et al.*, 2010b; Hosaka *et al.*, 2006; Ohba *et al.*, 2010; Schwock *et al.*, 2008; Cheung *et al.*, 2010). Thus, it is possible that enhanced HSPs induced by SK1 may be a true biological response of SK1 and not simply a result of the presence of unfolded SK1 protein.

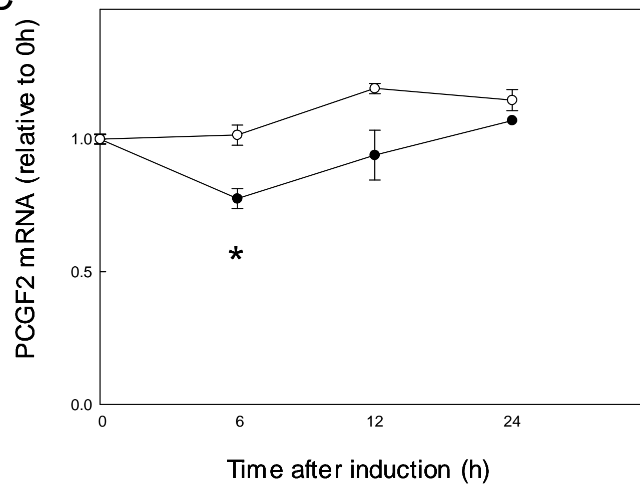
Since some of the main biological effects of elevated cellular SK1 levels are enhanced cell survival and proliferation and induction of neoplastic transformation, various genes which are known to be involved in cellular proliferation, survival and/or tumourigenesis were selected for further confirmation studies. From this validation we identified five genes that were differentially regulated by SK1, and not SK1^{G82D}, suggesting that they are regulated by elevated cellular SK1 activity. These were *TFRC*, *RASD1*, *SFPQ*, *FUS* and *PCGF2*. In agreement with our microarray data, results from qPCR showed that FUS (Figure 3.5A) and SFPQ (Figure 3.5B) mRNA were significantly up-regulated, and PCGF2 (Figure 3.5C) mRNA was significantly down-regulated at 6h following overexpression of SK1, but not SK1^{G82D}. RASD1 mRNA was shown to be upregulated in SK2 arrays (see below), was also enhanced by SK1 (Figure 3.6A) although it was not one of the top 50 genes identified from the arrays. Notably,

we also demonstrated that TFR1 mRNA expression (Figure 3.6B) was up-regulated by wild-type SK1 and down-regulated by $SK1^{G82D}$ consistent with the results from the microarray.

Figure 3.5 Validation of FUS, SFPQ and PCGF2 mRNA expression by SK1 versus SK1^{G82D}. QPCR analysis of **(A)** FUS [Fusion (involved in t(12;16) in malignant liposarcoma)], **(B)** SFPQ [splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)] and **(C)** PCGF2 (polycomb group ring finger 2) in SK1 (●) and SK1^{G82D} (○) inducible cell lines at various time points following induction. The expression of mRNA was normalized against the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). Data represent the mean (± range) from two independent experiments, each analysed in triplicate (*$P < 0.05$ compared with SK1^{G82D}). Statistical significance was calculated by using an unpaired *t* test.


A
FUS mRNA (relative to 0h)
*
2.0
1.5
1.0
0.5
0.0
0
6
12
24


B
SFPQ mRNA (relative to 0h)
*
2.0
1.5
1.0
0.5
0.0
0
6
12
24


C
PCGF2 mRNA (relative to 0h)
1.0
*
0.5
0.0
0
6
12
24
Time after induction (h)

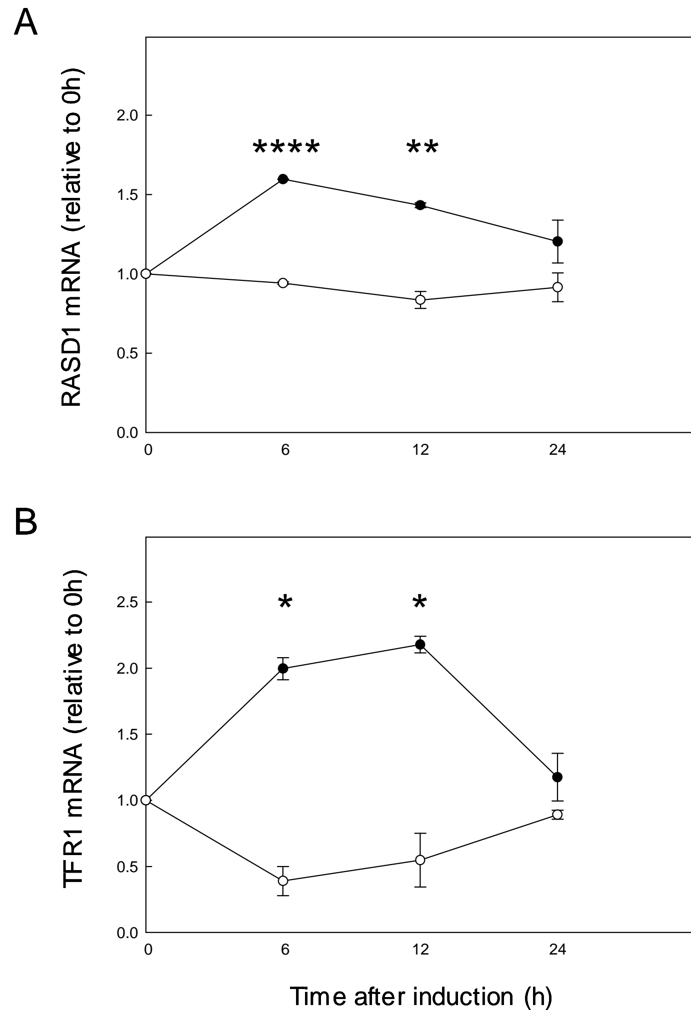


Figure 3.6 Validation of RASD1 and TFR1 mRNA expression in SK1 versus SK1^{G82D}. QPCR analysis of **(A)** RASD1 (RAS, dexamethasone-induced 1) and **(B)** TFR1 (transferrin receptor 1) in SK1 (●) and SK1^{G82D} (○) inducible cell lines at various times following induction. The expression of mRNA was normalized against the housekeeping gene, GAPDH. Data represent the mean (± range) from two independent experiments, each analysed in triplicate (*P < 0.05 compared with SK1^{G82D}, **P < 0.01 compared with SK1^{G82D} and ****P < 0.001 compared with SK1^{G82D}). Statistical significance was calculated by using an unpaired *t* test.

SFPQ encodes for PSF (polypyrimidine tract-binding protein-associated splicing factor), a nuclear protein implicated in transcription, DNA binding, unwinding, repair and pre-mRNA splicing and RNA editing (Shav-Tal & Zipori, 2002). Similarly, the gene product of *FUS* is also mainly a nuclear protein which was initially identified as a component of a fusion pro-oncogene resulting from a chromosomal translocation seen in liposarcoma (Crozat *et al.*, 1993; Kanoe *et al.*, 1999; Spitzer *et al.*, 2011). Like SFPQ, FUS is involved in pre-mRNA splicing and mRNA transport from nucleus to the cytoplasm (Buratti & Baralle, 2010; Yang *et al.*, 2010). Interestingly, both of these genes (*FUS* and *SFPQ*) have been reported to be involved in several chromosomal translocation-mediated cancers (Tanaka *et al.*, 2009; Hidalgo-Curtis *et al.*, 2008; Berg *et al.*, 2009). TFR1 (encoded by *TFRC*) is overexpressed in a number of solid tumours and leukemias (Habashy *et al.*, 2010; Kukulj *et al.*, 2010; Takahashi *et al.*, 2008; Boult *et al.*, 2008; Singh *et al.*, 2007; Brookes *et al.*, 2006; Hogemann-Savellano *et al.*, 2003; Whitney *et al.*, 1995; Ryschich *et al.*, 2004), and *PCGF2* has been shown to act as a tumour suppressor gene (Guo *et al.*, 2007). Thus, it is possible that up-regulation of *FUS*, *SFPQ* or *TFRC* and down-regulation of *PCGF2* by elevated SK1 expression may have functional importance in SK1-induced tumourigenesis. The role of TFR1 in SK1-induced oncogenesis will be further explored in Chapter 4 of this thesis. *RASD1* encodes for a Ras family member and was originally identified as a dexamethasone-inducible gene. Interestingly, although not involved in chromosomal rearrangements, loss of *RASD1* hetorozygosity is frequently detected in human tumours (Vaidyanathan *et al.*, 2004; Furuta *et al.*, 2006; de Souza Rocha Simonini *et al.*, 2010), and epigenetic inactivation of RASD1 has been reported to promote tumour cell growth and resistance of multiple melanoma cells to dexamethasone (Nojima *et al.*, 2009). Future studies may be to investigate whether *SFPQ*, *FUS*, *PCGF2* and *RASD1* play a role in SK/S1P-mediated tumourigenesis.

Several lines of evidence also suggest a role of FUS in neurodegenerative diseases (Lagier-Tourenne & Cleveland, 2009). Mutations of *FUS* have been found in amyotrophic lateral sclerosis (ALS) which is a progressive neurodegenerative disease that affects nerve cells in the brain and the spinal cord (Vance *et al.*, 2009; Kwiatkowski *et al.*, 2009; Ling *et al.*, 2010). FUS mainly localised to the nucleus due to its nuclear localisation signal (Dormann *et al.*, 2010), however abnormal FUS cytoplasmic redistribution forming inclusions consisting of FUS, GRP78/BiP, p62 and ubiquitin in neurons and glial cells were normally observed in

patients with FUS mutations (Dormann *et al.*, 2010). Interestingly, SK1/S1P appear to promote glial cell proliferation, astrogliosis and the development of neurodegenerative diseases (Wu *et al.*, 2008a; Nayak *et al.*, 2010; Lee *et al.*, 2010). As SK1 is mainly cytoplasmic (Kohama *et al.*, 1998; Pitson *et al.*, 2005), it is tempting to speculate that SK1/S1P could play a role in the accumulation of FUS in the cytoplasm resulting in the formation of cytosolic aggregates in neurons contributing to the pathological processes of neurodegenerative diseases. Therefore, it would be interesting to examine any potential role of SK1/S1P in FUS-mediated neurodegenerative diseases.

Recently, an involvement of SK/S1P in the pathogenesis of asthma has also been suggested (Oskeritzian *et al.*, 2007; Lai *et al.*, 2011). For example, S1P levels are elevated in the airways of asthma patients after allergen challenge and SK1 plays important roles in the regulation of IL-13 induced MUC5A production via ERK1/2 signalling pathway, independent of STAT6 phosphorylation (Kono *et al.*, 2010). Asthma is characterized by increased STAT6 activity and IgE gene expression that are associated with an increase in histone acetyltransferase and reduction in HDAC activities in the bronchi and alveolar macrophages (Barnes *et al.*, 2005). Intriguingly, a recent study suggest that SFPQ may have a protective role in allergic diseases based on the fact that this protein can function as a repressor of STAT6-mediated transcription through recruitment of HDAC complex, which resulted in reduction of H3 acetylation at the promoter regions of IgE and inhibition of STAT6-mediated transcription (Dong *et al.*, 2011). Thus, future studies should examine the biological function of SFPQ in SK/S1P mediated allergic diseases and whether this protein has any potential therapeutic implications for SK-mediated allergic diseases.

Furthermore, expression of genes such as, *HSPA5* (Figure 3.7A), *CLK1* (Figure 3.7B), *IRS4* (Figure 3.7C), *HSPA8* (Figure 3.7D), *MANF* (Figure 3.7E) and *TNFRSF10D* (Figure 3.7F) were enhanced while, genes like *ZNF711* (Figure 3.8A) and *TSC23D3* (Figure 3.8B) were repressed by both wild-type SK1 and $SK1^{G82D}$, demonstrating that these genes may be regulated by SK1 independently of its catalytic activity. These observations may be due to non-specific effects of protein overexpression, despite the use of low level overexpression. Previous studies using similar inducible systems have shown that doxycycline (at 1 µg/ml) had no appreciable effect on gene expression (Grigo *et al.*, 2008; Tian *et al.*, 2002; Thomas *et al.*, 2004; Darby *et al.*, 2008). So, coupled with the fact that a very low concentration of

doxycycline (0.5ng/ml) was used to induce SK1 in the array experiments, this suggests that the results are more likely to arise from non-catalytic effects of SK1 protein rather than non-specific effects due to doxycycline. Other genes like *PPP1R10* (Figure 3.9A), *PCTK3* (Figure 3.9B) and *EIF4B* (Figure 3.9C) only had either modest changes or no major change by both SK1 and $SK1^{G82D}$.

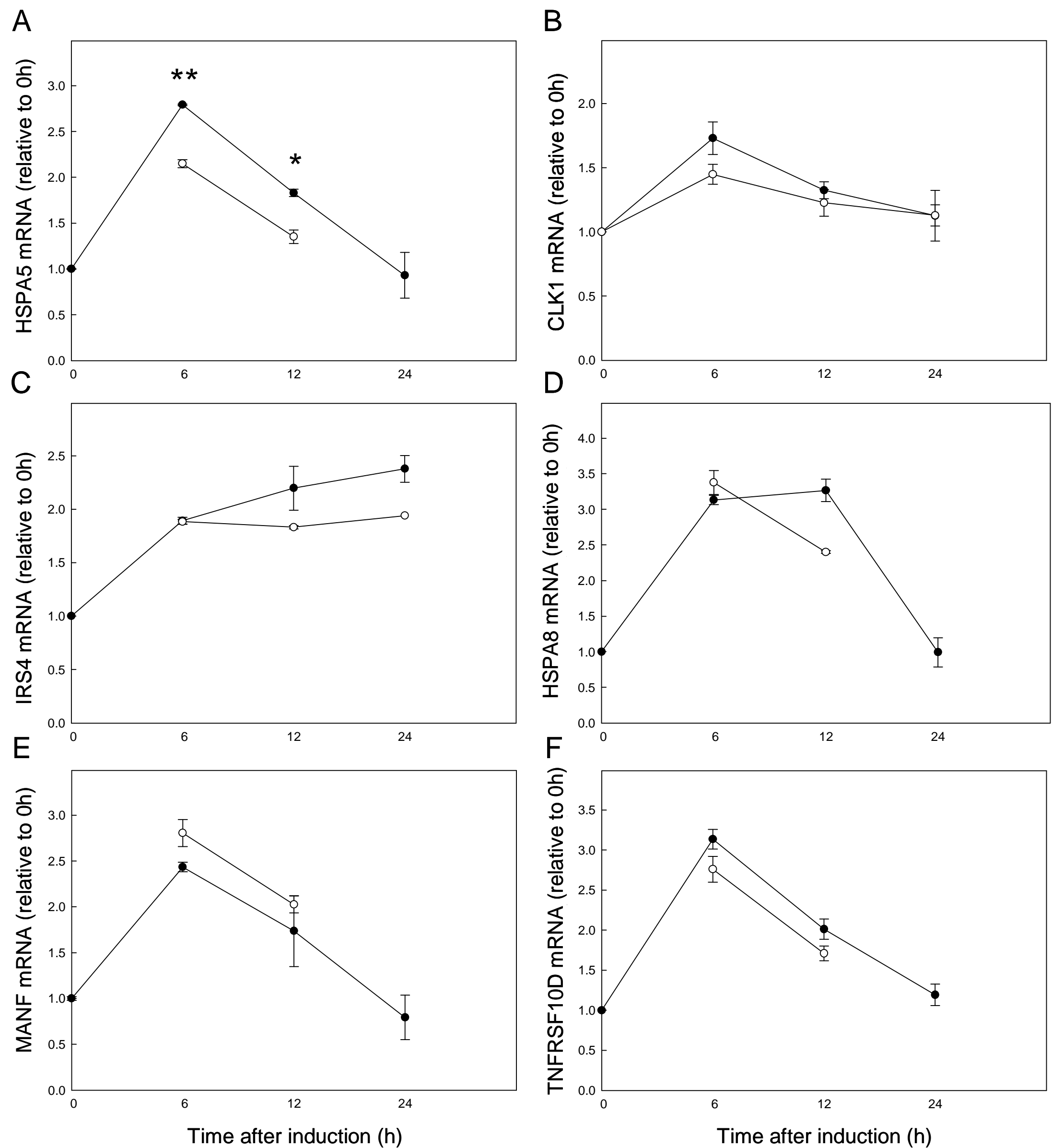


Figure 3.7 Validation of HSPA5, CLK1, IRS4, HSPA8, MANF and TNFRSF10D mRNA expression in SK1 versus SK1^{G82D}. QPCR analysis of **(A)** HSPA5 [Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)], **(B)** CLK1 (CDC-like kinase 1), **(C)** IRS4 (insulin receptor substrate 4), **(D)** HSPA8 (heat shock 70kDa protein 8), **(E)** MANF (mesencephalic astrocyte-derived neurotrophic factor) and **(F)** TNFRSF10D (tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain) in SK1 (●) and SK1^{G82D} (○) inducible cell lines at various time points following. The expression of mRNA was normalized against the housekeeping gene, GAPDH. Data represent the mean (± range) from two independent experiments, each analysed in triplicate (*P < 0.05 compared with SK1^{G82D} and **P < 0.01 compared with SK1^{G82D}). Statistical significance was calculated by using an unpaired *t* test.

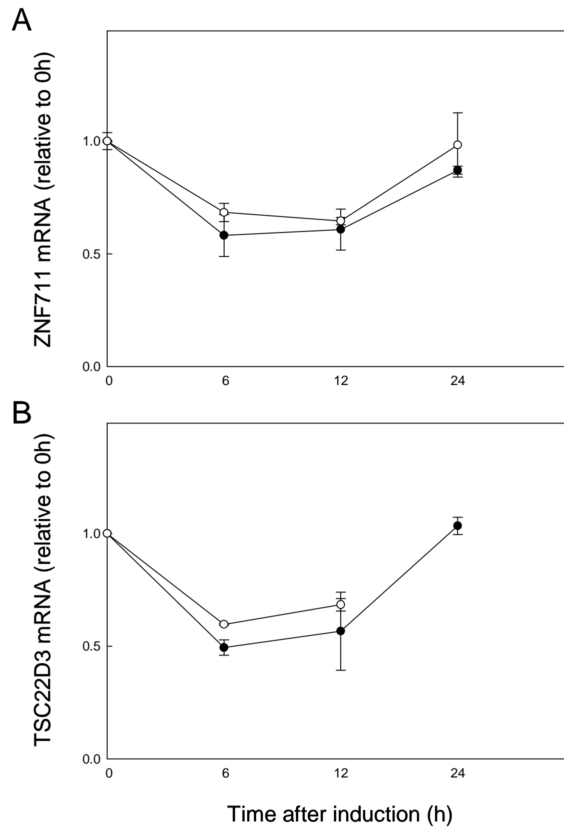


Figure 3.8 Validation of ZNF711 and TSC23D3 mRNA expression in SK1 versus SK1^{G82D}. QPCR analysis of **(A)** ZNF711 (Finger protein 711) and **(B)** TSC23D3 (TSC22 domain family, member 3) in SK1 (●) and SK1^{G82D} (○) inducible cell lines at various time points following induction. The expression of mRNA was normalized against the housekeeping gene, GAPDH. Data represent the mean (± range) from two independent experiments, each analysed in triplicate.

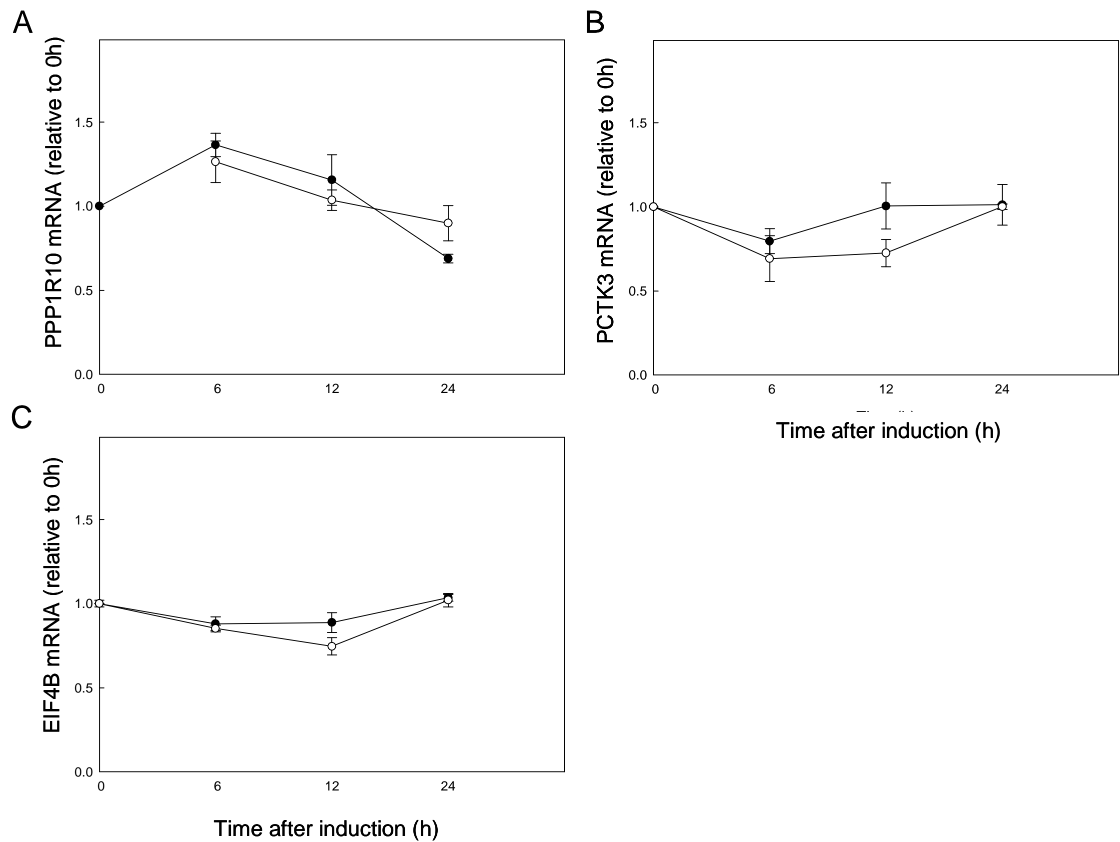


Figure 3.9 Validation of PPP1R10, PCTK3 and EIF4B mRNA expression in SK1 versus SK1^{G82D}. QPCR analysis of **(A)** PPP1R10 (protein phosphatase 1 regulatory (inhibitor) subunit 10), **(B)** PCTK3 (PCTAIRE protein kinase 3) and **(C)** EIF4B (eukaryotic translation initiation factor 4B) in SK1 (●) and SK1^{G82D} (○) inducible cell lines at various time points following induction. The expression of mRNA was normalized against the housekeeping gene, GAPDH. Data represent the mean (± range) from two independent experiments, each analysed in triplicate.

3.4.2 Expression profiling of gene regulation by SK2

DNA microarrays using cells overexpressing SK2 were also performed to assess the gene transcription profile regulated by this enzyme. Flp-In™ T-Rex™ HEK293 with doxycyclin-inducible expression of SK2 were treated to induce approximately 6-fold higher SK2 activity over endogenous levels. The cells were then harvested 6 h later, RNA extracted and the gene expression profile compared to uninduced cells by DNA microarray. Figure 3.10 shows the volcano plot of genes that were differentially regulated following SK2 induction. Somewhat surprisingly, the level of gene regulation identified in the SK2 array appears to be generally higher than those observed in SK1 array. Like for SK1 analysis, additional microarray experiments were performed using cells expressing comparable levels of a catalytically inactive version of SK2, $SK2^{G212D}$ (Figure 3.11), in an attempt to eliminate non-specific (or specific) protein effects of SK2 and identify gene regulation specifically induced by elevated cellular SK2 activity. It appears that only a small number of genes were predominantly regulated by active SK2 compared to the $SK2^{G212D}$. These include *UBE2D4* (encodes for a ubiquitin-conjugating enzyme), *RASD1*, and genes involved in transport and metabolism [*SLC16A9* (Illig *et al.*, 2010), *CYP1A1* (Sergentanis & Economopoulos, 2010)], and transcriptional regulation [*WDR7* (Gu *et al.*, 2011) and *BRPF3* (Mishima *et al.*, 2011)]. These genes were all upregulated by wild-type SK2 but largely unaltered by expression of $SK2^{G212D}$, suggesting that they were regulated predominantly by SK2 activity. Interestingly, many of these genes appear to be involved in nuclear processes (Mishima et al., 2011; Gu et al., 2011) which would be expected as SK2 has been reported to localise in the nucleus and so this data further supports it potential role(s) in the nucleus.

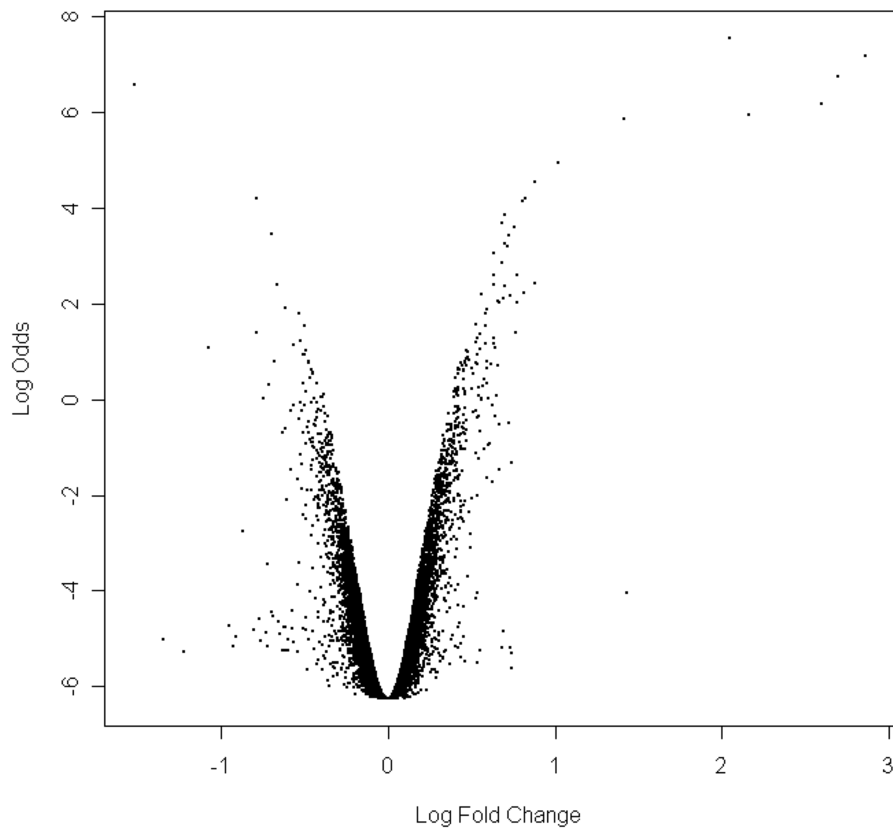


Figure 3.10 Differential expression of genes modulated by increased cellular SK2. Volcano plot showing genes regulation by SK2 expression at 6 h subsequent to induction.

Figure 3.11 Heat plot of differentially expressed genes by increased cellular SK2 versus SK2^{G212D}. Heat plot of genes that showed differential regulation by cellular expression of SK2 versus SK2^{G212D} following 6 h induction. Genes shown in green showed up-regulation, and those in red were down-regulated, while those that did not show any change with respect to a normalized matched control are shown in black (see color scale).

Gene ID	Gene Abbr.	Gene Name	SK2	SK2G212D
S68954	MT1G	Metallothionein 1G		
X97261	MT1R	Metallothionein 1R		
NM_005953	MT2A	Metallothionein 2A		
NM_002450	MT1L	Metallothionein 1L (gene/pseudogene)		
NM_003746	DYNLL1	Dynein, light chain, LC8-type 1		
AB033091	SLC39A10	Solute carrier family 39 (zinc transporter), member 10		
NM_012068	ATF5	Activating transcription factor 5		
NM_006597	HSPA8	Heat shock 70kDa protein 8		
NM_004071	CLK1	CDC-like kinase 1		
NM_005066	SFPQ	Splicing factor proline/glutamine-rich		
NM_006441	MTHFS	5,10-methenyltetrahydrofolate synthetase		
AK022581	WDR75	WD repeat domain 75 family member		
U69645	ZNF32	Zinc finger protein 32		
X56465	ZNF711	Finger protein 711		
AK001102	HEMBB	CDNA FLJ10240 fis, clone HEMBB1000591		
NM_016539	SIRT6	Sirtuin 6		
NM_015983	UBE2D4	Ubiquitin-conjugating enzyme E2D 4		
NM_005762	TRIM28	Tripartite motif-containing 28		
AB046857	NCOA5	Nuclear receptor coactivator 5		
NM_016084	RASD1	RAS, dexamethasone-induced 1		
AL122071	SLC16A9	solute carrier family 16, member 9		
AF216292	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)		
NM_001539	DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1		
NM_003544	HIST1H4B	Histone cluster 1, H4bm		
NM_003529	HIST1H3A	Histone cluster 1, H3a		
AB046781	UACA	Uveal autoantigen like family member		
NM_012437	SNAPIN	SNAP-associated protein		
NM_003418	CNBP	CCHC-type zinc finger, nucleic acid binding protein		
NM_014175	MRPL15	Mitochondrial ribosomal protein L15		
AK026208	C2orf47	Chromosome 2 open reading frame 47		
AB033118	ZDHHC8	Zinc finger, DHHC-type containing 8		
K03191	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1		
AK022860	WDR77	cDNA FLJ12798 fis, clone NT2RP2002076		
NM_001983	ERCC1	Excision repair cross-complementing, group 1		
D31885	RPS15A	ADP-ribosylation factor-like protein 1		
NM_016274	PLEKHO1	Pleckstrin homology domain containing, family O member 1		
NM_017503	SURF2	Surfeit 2		
AF113007	METTL7A	Methyltransferase like 7A		
NM_013293	TRA2A	Transformer-2 alpha (TRA2A)		
AB033112	BRPF3	Bromodomain and PHD finger containing, 3		
AK026783	N/A	CDNA: FLJ23130 fis, clone LNG08419		
AB011099	SUSD5	Sushi domain containing 5		
NM_006325	RAN	RAN, member RAS oncogene family		
NM_001383	DPH1	DPH1 homolog (S. cerevisiae)		
D63487	TTLL12	Tubulin tyrosine ligase-like family, member 12		
NM_032727	LNG08419	Internexin neuronal intermediate filament protein, alpha		
NM_006601	PTGES3	Prostaglandin E synthase 3		
NM_002266	KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)		
NM_005903	SMAD5	SMAD family member 5		
AB040887	SCAPER	Zinc finger protein 291 CRA c family member		

3.4.3 Analysis of differential gene regulation by SK1 and SK2

When comparing of genes differentially regulated by SK2 versus SK1 (Figure 3.12), various genes particularly those encoded for metallothioneins (MTs) [*MT1G*, *MT1R*, *MT2A* and *MT1L*] were upregulated specifically by both active and inactive SK2. MTs are low-molecular weight metal-binding proteins that bind excess heavy metal ions (in particular copper and zinc) and thus play an important role in protection against toxic heavy metals and to maintain homeostasis of essential trace metals (Cherian *et al.*, 2003; Thirumoorthy *et al.*, 2007; Nielsen *et al.*, 2007). Interestingly, like SK2, MT appears to have two faces in tumourigenesis which will be further discussed in Chapter 5.2. Interestingly, when SK1 was compared against SK2 (Figure 3.13), genes encoding for SNORA68, TFR1, SNORA73A, 5S ribosomal RNA, and Ro small cytoplasmic ribonucleoproteins appeared to be upregulated exclusively by active SK1. Furthermore, gene candidates like *HSPA8*, *CLK1*, *SFPQ*, *RASD1*, *HSPA5*, *FUS*, *ZNF711*, *TSC23D3*, and *HIST1H2AB*, previously shown to be differentially regulated by SK1, were also regulated in the SK2 arrays. From the microarray validation using qPCR, two of the identified gene candidates appear to be moderately up-regulated mainly by SK2 activity. These genes were *RASD1* (Figure 3.14A) and *FUS* (Figure 3.14B). Other genes including *SFPQ* (Figure 3.14.C), *CLK1* (Figure 3.15A) and *HSPA8* (Figure 3.15B), which were up-regulated by overexpression of both active and in-active SK1 were also up-regulated by both active and inactive SK2. Although it appears that the two SK isoforms have different subcellular localisations (Pitson *et al.*, 2005; Igarashi *et al.*, 2003a; Maceyka *et al.*, 2005b; Ding *et al.*, 2007), they generate the same product. Therefore, it is not surprising that various genes appear to be regulated specifically by either SK1 or SK2 or by both enzymes. Future studies aim to examine the potential role(s) of these regulated genes in mediating the biological functions of these two SK isoforms.

Figure 3.12 Heat plot of differentially expressed genes by cellular SK2 versus SK1, SK2^{G212D} and SK1^{G82D}. Heat plot of genes that showed differential expression by SK2 versus SK1, SK2^{G212D} and SK1^{G82D} following 6 h induction. Genes shown in green showed up-regulation, and those in red were down-regulated, while those that did not show any change with respect to a normalized matched control are shown in black (see color scale).

Gene ID	Gene Abbr.	Gene Name
S68954	MT1G	Metallothionein MT-1g isoform
X97261	MT1R	Metallothionein 1R
NM_005953	MT2A	Metallothionein 2A (MT2A)
NM_002450	MT1L	Metallothionein 1L (MT1L)
NM_003746	DYNLL1	Dynein, light chain, LC8-type 1 (DYNLL1)
AB033091	SLC39A10	Solute carrier family 39 (zinc transporter), member 10 (SLC39A10)
NM_012068	ATF5	Activating transcription factor 5
NM_006597	HSPA8	Heat shock 70kDa protein 8 (HSPA8)
NM_004071	CLK1	CDC-like kinase 1
NM_005066	SFPQ	Splicing factor proline/glutamine-rich (SFPQ)
NM_006441	MTHFS	MTHFS
AK022581	WDR75	WD repeat domain 75
U69645	ZNF32	Zinc finger protein 32 (ZNF32)
X56465	ZNF711	Zinc finger transcription factor (Znf6)
AK001102	HEMBB	CDNA FLJ10240 fis, clone HEMBB1000591
NM_016539	SIRT6	Sirtuin 6 (SIRT6)
NM_015983	UBE2D4	Ubiquitin-conjugating enzyme E2D 4 (UBE2D4)
NM_005762	TRIM28	Tripartite motif-containing 28 (TRIM28)
AB046857	NCOA5	Nuclear receptor coactivator 5 (NCOA5)
NM_016084	RASD1	RAS, dexamethasone-induced 1(RASD1)
AL122071	SLC16A9	Solute carrier family 16, member 9 (SLC16A9)
AF216292	HSPA5	Heat shock 70kDa protein 5 (HSPA5)
NM_001539	DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1 (DNAJA1)
NM_003544	HIST1H4B	Histone cluster 1, H4bm (HIST1H4B)
NM_003529	HIST1H3A	Histone cluster 1, H3a (HIST1H3A)
AB046781	UACA	UACA
NM_012437	SNAPIN	SNAP-associated protein (SNAPIN)
NM_003418	CNBP	CCHC-type zinc finger, nucleic acid binding protein (CNBP)
NM_014175	MRPL15	Mitochondrial ribosomal protein L15 (MRPL15)
AK026208	C2orf47	Chromosome 2 open reading frame 47 (C2orf47)
AB033118	ZDHHC8	Zinc finger, DHHC-type containing 8 (ZDHHC8)
K03191	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1)
AK022860	WDR77	WD repeat domain 77 (WDR77)
NM_001983	ERCC1	ERCC1
D31885	RPS15A	ADP-ribosylation factor-like 6 interacting protein 1 (ARL6IP1)
NM_016274	PLEKHO1	PLEKHO1
NM_017503	SURF2	Surfeit 2 (SURF2)
AF113007	METTL7A	Methyltransferase like 7A (METTL7A)
NM_013293	TRA2A	Transformer-2 alpha (TRA2A)
AB033112	BRPF3	Bromodomain and PHD finger containing, 3 (BRPF3)
AK026783	LNG08419	CDNA: FLJ23130 fis, clone LNG08419
AB011099	SUSD5	Sushi domain containing 5 (SUSD5)
NM_006325	RAN	RAN, member RAS oncogene family (RAN)
NM_001383	DPH1	DPH1 homolog (DPH1)
D63487	TTLL12	Tubulin tyrosine ligase-like family, member 12 (TTLL12)
NM_004692	INA	Internexin neuronal intermediate filament protein, alpha (INA)
NM_006601	PTGES3	Prostaglandin E synthase 3 (PTGES3)
NM_002266	KPNA2	Karyopherin alpha 2 (KPNA2)
NM_005903	SMAD5	SMAD family member 5 (SMAD5)
AB040887	SCAPER	S phase cyclin A-associated protein in the ER (SCAPER)

SK2^{G212D} SK2 SK1 SK1^{G82D}

Figure 3.13 Heat plot of differentially expressed genes by SK1 versus SK2, SK1^{G82D} and SK2^{G212D}. Heat plot of differentially expressed genes by cellular SK1 versus SK2, SK1^{G82D} and SK2^{G212D} following 6 h induction. Genes shown in green showed up-regulation, and those in red were down-regulated, while those that did not show any change with respect to a normalized matched control are shown in black (see color scale).

Gene ID	Gene Abbr.	Gene Name	$SK1^{G82D}$	SK1	SK2	$SK2^{G212D}$
NM_006597	HSPA8	Heat shock 70kDa protein 8				
AF216292	HSPA5	Heat shock 70kDa protein 5				
Y11162	U68	U68 small nucleolar RNA				
NM_006010	MANF	Mesencephalic astrocyte-derived neurotrophic factor				
NM_005346	HSPA1B	Heat shock 70kDa protein 1B				
NM_004960	FUS	Fused in sarcoma				
NM_003234	TFRC	Transferrin receptor				
NM_018371	CSGALNACT1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1				
NM_003840	TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d				
U12210	E1b	Small nucleolar RNA (E1b)				
NM_018307	RHOT1	Ras homolog gene family, member T1				
NM_004089	TSC22D3	TSC22 domain family, member 3				
NM_005066	SFPQ	Splicing factor proline/glutamine-rich				
AL161977	PCTK3	PCTAIRE protein kinase 3				
NM_004396	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5				
K01564	RoScR	Ro small cytoplasmic ribonucleoproteins				
NM_005323	HIST1H1T	Histone cluster 1, H1t				
AF070552	CDT1	Chromatin licensing and DNA replication factor 1				
NM_003746	DYNLL1	Dynein, light chain, LC8-type 1				
NM_005962	MXI1	MAX interactor 1				
NM_006644	HSPH1	Heat shock 105kDa/110kDa protein 1				
AK025862	HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1				
V00589	5SrRNA	5S ribosomal RNA				
K03191	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1				
AF116620	SLC38A2	Neutral amino acid transporter 2				
NM_004071	CLK1	CDC-like kinase 1				
D28449	ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (13.0 kD)				
NM_000319	PEX5	Peroxisomal biogenesis factor 5				
NM_003513	HIST1H2AB	Histone cluster 1, H2ab				
NM_005911	MAT2A	Methionine adenosyltransferase II, alpha				
AF071569	CAMK2D	calmodulin-dependent protein kinase II (56.4 kD)				
AL110141	SNORD81	Putative protein (5.5 kD) (GAS5_)				
NM_007144	PCGF2	Polycomb group ring finger 2				
NM_004634	BRPF1	Bromodomain and PHD finger containing, 1				
NM_020143	PNO1	Partner of NOB1 homolog (S. cerevisiae)				
AF283645	SLC25A32	Solute carrier family 25, member 32				
NM_001417	EIF4B	Eukaryotic translation initiation factor 4B				
NM_003299	HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1				
NM_002131	HMGA1	High mobility group AT-hook 1				
NM_012484	HMMR	Hyaluronan-mediated motility receptor (RHAMM)				
NM_003017	SRSF3	Serine/arginine-rich splicing factor 3				
NM_003075	SMARCC2	SWI/SNF related, matrix associated, subfamily c, member 2				
NM_000466	PEX1	Peroxisome biogenesis factor 1				
AF110265	EPS15L1	Epidermal growth factor receptor substrate 15-like 1 family member (94.3 kD)				
NM_014175	MRPL15	Mitochondrial ribosomal protein L15				
NM_018445	SELS	Selenoprotein S				
NM_014303	PES1	Pescadillo homolog 1, containing BRCT domain (zebrafish)				
NM_018118	MCM3APAS	MCM3AP antisense RNA (non-protein coding)				
NM_016448	DTL	Denticleless homolog (Drosophila)				
NM_002913	RFC1	Replication factor C (activator 1) 1, 145kDa				

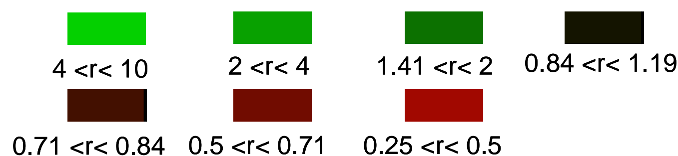
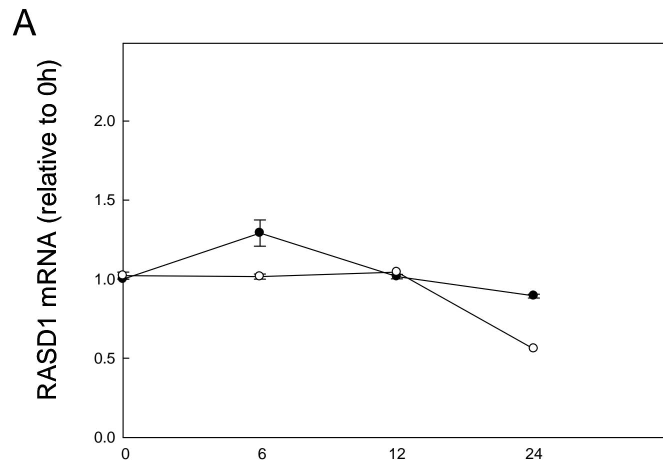
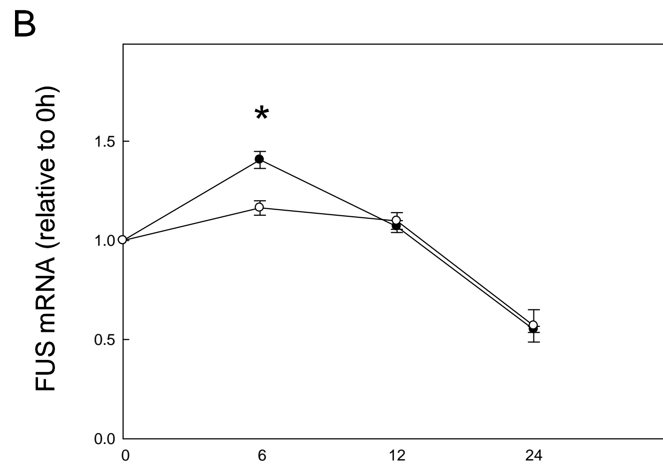
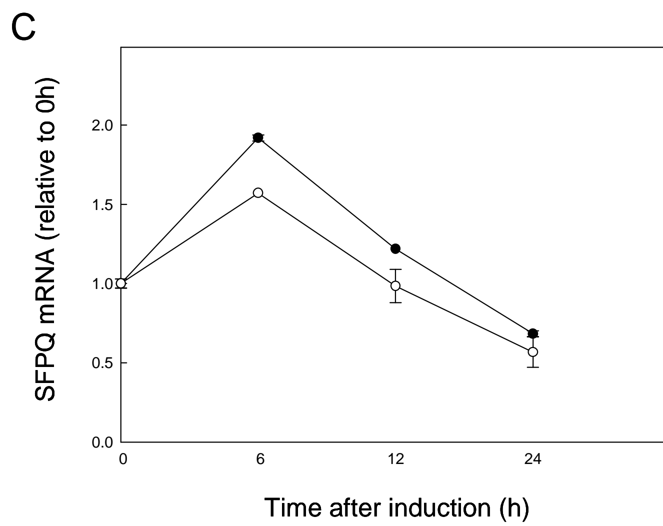


Figure 3.14 Validation of RASD1, FUS and SFPQ mRNA expression in SK2 versus $\mathbf{SK2^{G212D}}$. QPCR analysis of (**A**) RASD1, (**B**) FUS and (**C**) SFPQ in cells induced to express SK2 and $SK2^{G212D}$ inducible cell lines at various time points following induction. The expression of mRNA was normalized against the housekeeping gene, GAPDH. Data represent the mean (± range) from two independent experiments, each analysed in triplicate (*$P < 0.05$ compared with $SK1^{G82D}$). Statistical significance was calculated by using an unpaired *t* test.


A
RASD1 mRNA (relative to 0h)
2.0
1.5
1.0
0.5
0.0
0
6
12
24


B
FUS mRNA (relative to 0h)
*
1.5
1.0
0.5
0.0
0
6
12
24


C
SFPQ mRNA (relative to 0h)
2.0
1.5
1.0
0.5
0.0
0
6
12
24
Time after induction (h)

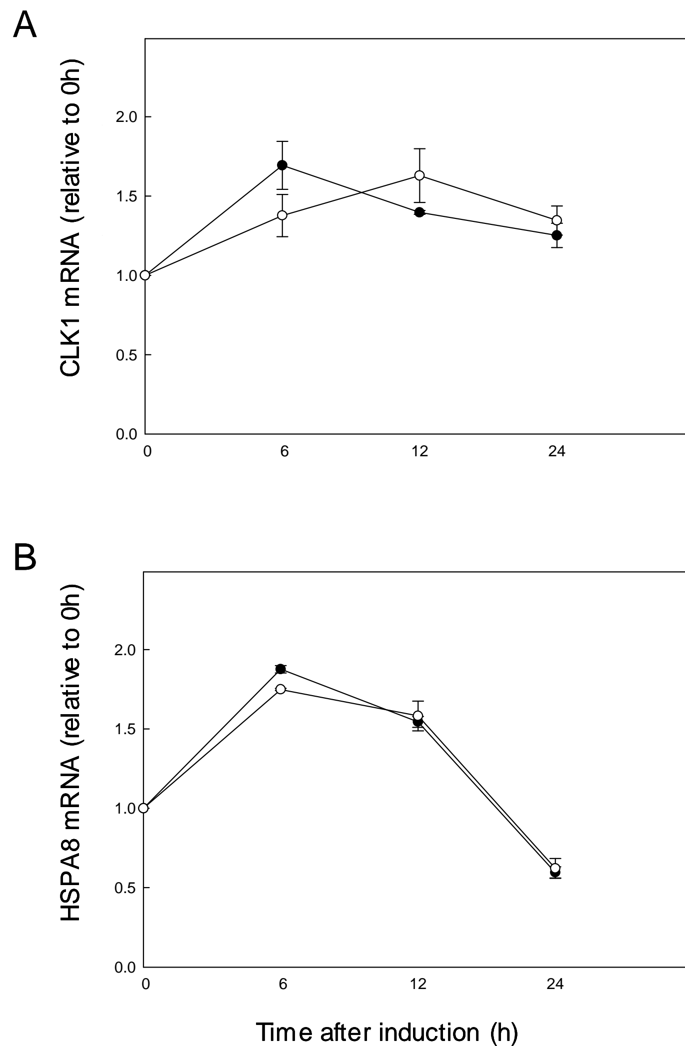


Figure 3.15 Validation of CLK1 and HSPA8 mRNA expression in SK2 versus SK1^{G212D}. QPCR analysis of (**A**) CLK1 and (**B**) HSPA8 in SK2 and SK2^{G212D} inducible cell lines at various times following induction. The expression of mRNA was normalized against the housekeeping gene, GAPDH. Data represent the mean (± range) from two independent experiments, each analysed in triplicate.

3.4.4 MiRNA regulation by SK1

MicroRNAs are small, non-coding, regulatory RNAs of about 21-25 nucleotides in length that are expressed in animals, plants and viruses (Huang *et al.*, 2011; Zhang *et al.*, 2007; Meltzer, 2005; Pfeffer & Voinnet, 2006; Anglicheau *et al.*, 2010). MiRNAs are expressed as long precursor RNAs that are processed by a cellular nuclease, Drosha, before being transported by an Exportin-5-dependent mechanism into the cytoplasm. Once in the cytoplasm, miRNAs are cleaved further by the enzyme DICER and the resulting 21-25 nucleotide miRNA can post-transcriptionally regulate gene expression by either inhibiting mRNA translation or inducing mRNA degradation, and participate in a wide variety of physiological and pathological cellular processes, such as, cell differentiation, development, proliferation and progression of human diseases (Zeng, 2006; O'Connell *et al.*, 2010; Pauley *et al.*, 2009; Carissimi *et al.*, 2009). While altered expression of miRNAs has been implicated in a number of different diseases including cardiovascular diseases (Chen *et al.*, 2011; Fukushima *et al.*, 2011; Huang *et al.*, 2010), diabetes (Zampetaki *et al.*, 2010; Donnem *et al.*, 2011), neurodegenerative diseases (Haramati *et al.*, 2010; Wang *et al.*, 2010b), autoimmune diseases (Ha, 2011; Thamilarasan *et al.*, 2012; Leng *et al.*, 2011) and viral infection (Roberts *et al.*, 2011; Lu *et al.*, 2010; Carpio *et al.*, 2010), the most studied role of miRNA is in tumourigenesis. Considerable evidence now exists, supporting a role for dysregulation of miRNAs in the initiation and progression of a wide range of human cancers (Wu *et al.*, 2011; Tsuchiya *et al.*, 2011; Ueda *et al.*, 2010; Sarver *et al.*, 2010; Li *et al.*, 2011b; Gregory *et al.*, 2008). Thus, in addition to gene regulation, it was also of interest to examine if SK1 was involved in regulating miRNA expression.

Analysis of miRNA regulation in response to cellular SK1 expression was performed using miRNA array technology. Again, SK1 expression was induced for 6 h in Flp-In™ T-Rex™ HEK293 cells to achieve around 10-fold higher SK1 activity than endogenous levels, RNA isolated, and then miRNA arrays were performed and compared to the miRNA expression pattern of uninduced cells. Unfortunately, from the studies we were unable to identify any miRNA which was significantly regulated by SK1 expression. While this may suggest that SK1 does not play a role in miRNA regulation, it should be noted that these initial miRNA arrays were performed using only one condition, where SK1 was overexpressed at a moderate level slightly higher than that of endogenous levels for 6 h. Notably, recent findings from other

miRNA array studies using similar doxycycline-inducible expression systems showed that high overexpression of the transcription factor, E2F1, for 24h post induction led to consistent up-regulation of miR-449a and miR-449b in both Saos2 and U2OS cells (Lize *et al.*, 2010; He *et al.*, 2010a; Yang *et al.*, 2009). Based on our array data and other miRNA array studies (Lize *et al.*, 2010; He *et al.*, 2010a; Yang *et al.*, 2009), it appears that higher and longer overexpression of SK1 may be necessary to observe any potential miRNA regulation. Thus, future miRNA array studies should be performed using inducible cell lines with higher and longer overexpression of SK1 in order to thoroughly identify potential miRNA(s) involved in SK1-mediated oncogenic effects.

3.5 CONCLUSIONS

In summary, we have elucidated a number of genes that were differentially regulated by moderately increased SK expression via DNA microarray-based approaches. The fact that several of the genes identified from the arrays have previously been shown to be dysregulated in cell growth and survival further substantiates the success of our approach in identifying down-stream targets mediated by SK-induced oncogenesis. As expected, we have identified various genes that were regulated by either SK1 or SK2 or both enzymes. However, the most significant finding was the identification of a number of gene candidates (TFR1, SFPQ, FUS, PCGF2, and RASD1), which were regulated specifically by SK1 or SK2 activity. It is unclear whether the molecular mechanism(s) driving the oncogenic pathways from each SK are the same or different. From our initial data analysis, it appears that the expression levels of genes, such as, RASD1 and FUS were up-regulated by both active SK enzymes suggesting that these two enzymes could activate similar down-stream pathway(s) leading to oncogenesis. Future experiments should include functional studies to further clarify the molecular mechanisms of these gene candidates in oncogenic signalling by the two enzymes. Although no significant miRNA regulation was observed from our arrays, it is still inconclusive whether miRNAs could be regulated by SK since we only performed miRNA arrays with SK1 expression under one condition. Thus, additional miRNA arrays should be performed in the future using optimized conditions to gain a more accurate picture of miRNA regulation by SK.

4. CHAPTER 4: ACTIVATED SPHINGOSINE KINASE 1 INDUCES TRANSFERRIN RECEPTOR 1 EXPRESSION TO PROMOTE CELL PROLIFERATION, SURVIVAL AND NEOPLASTIC TRANSFORMATION

4.1 ABSTRACT

Sphingosine kinase 1 (SK1) is a lipid kinase that catalyses the formation of sphingosine 1-phosphate (S1P). There is convincing evidence implicating elevated cellular SK1 in tumour development and progression; notably the enhancement of cellular proliferation and survival, induction of neoplastic transformation, and stimulation of angiogenesis. However, the specific downstream pathways mediating this oncogenic signalling by SK1 are still poorly defined. Here, we have demonstrated a novel mechanism whereby SK1 regulates cell survival and proliferation through enhancing expression of transferrin receptor 1 (TFR1). We show that elevated levels of SK1 enhanced total as well as cell-surface TFR1 expression resulting in increased transferrin uptake into the cells. Notably, we also found that SK1 activation and relocalisation to the plasma membrane, which are critical for its oncogenic effects, are necessary for regulation of TFR1 expression specifically through engagement of the S1P G-protein coupled receptor, $S1P_2$. Furthermore, we show that blocking TFR1 function with a neutralizing antibody inhibits SK1-induced cell proliferation, survival and neoplastic transformation of NIH3T3 fibroblasts, suggesting that TFR1 plays an important role in SK1-mediated oncogenesis.

4.2 INTRODUCTION

Sphingosine kinase 1 (SK1) catalyses the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P), a bioactive phospholipid that plays important roles in a wide variety of cellular processes, including calcium mobilisation, cell proliferation, apoptosis, angiogenesis, inflammatory responses and cytoskeletal rearrangement (Takabe *et al.*, 2008). Elevated levels of SK1/S1P have been shown to enhance cell survival and proliferation, and there is now substantial evidence implicating an important role of SK1 in tumour development and progression. This includes findings that SK1 expression is elevated in a variety of human tumours (French *et al.*, 2003b; Kawamori *et al.*, 2006; Kawamori *et al.*, 2009; Johnson *et al.*, 2005a; Bayerl *et al.*, 2008; Facchinetti *et al.*, 2010), high SK1 expression correlates with poor survival of patients with a range of solid tumours (Van Brocklyn *et al.*, 2005; Li *et al.*, 2008a; Ruckhaberle *et al.*, 2008; Malavaud *et al.*, 2010; Shirai *et al.*, 2011; Liu *et al.*, 2010), dysregulation of SK1 plays a important role in both acute and chronic myeloid leukemia(Sobue *et al.*, 2008b; Ricci *et al.*, 2009; Paugh *et al.*, 2008; Bonhoure *et al.*, 2008; Bonhoure *et al.*, 2006; Li *et al.*, 2007; Le Scolan *et al.*, 2005; Pitson *et al.*, 2011), and overexpression of SK1 in NIH 3T3 fibroblasts induces full neoplastic cell transformation (Xia *et al.*, 2000). Furthermore, targeting SK1 by genetic or pharmacological approaches has been shown to significantly reduce tumour growth in mice (Kohno *et al.*, 2006; Kawamori *et al.*, 2009; Sinha *et al.*, 2011) and also sensitize tumour cells to radiation (Pchejetski *et al.*, 2010; Sinha *et al.*, 2011) and other chemotherapeutics (Guillermet-Guibert *et al.*, 2009; Pchejetski *et al.*, 2005b; Baran *et al.*, 2007; Bonhoure *et al.*, 2006; Bonhoure *et al.*, 2008; Sauer *et al.*, 2009).

Oncogenic signalling by SK1 has been previously shown to be dependent on both its activation and translocation from the cytosol to the plasma membrane (Pitson *et al.*, 2003; Pitson *et al.*, 2005). This localisation of SK1 to the plasma membrane appears to place the enzyme into close proximity with its substrate and also enables localised production of S1P to be either released from the cell to act on cell surface S1P receptors (S1PRs) (Johnson *et al.*, 2002; Pitson *et al.*, 2005) or to regulate intracellular signalling targets (Strub *et al.*, 2010; Hait *et al.*, 2009; Alvarez *et al.*, 2010). However, the molecular mechanism(s) by which SK1 exerts its oncogenic effects are yet to be elucidated. While early evidence suggested SK1 may enhance cell survival and proliferation via mechanisms independent of S1PRs (Olivera *et al.*, 2003), considerable evidence now supports the notion of pro-survival and pro-proliferative

signalling by S1PRs (Leong & Saba, 2010; Radeff-Huang *et al.*, 2004; Saddoughi *et al.*, 2008). These effects appear to be mediated primarily through G_i-mediated PI3K/Akt and ERK1/2 (Bonnaud *et al.*, 2010; Kim *et al.*, 2010; Schuppel *et al.*, 2008; Harada *et al.*, 2004; Yamada *et al.*, 2004) , although the specific downstream pathways which lead to SK1-mediated oncogenic signalling are still poorly defined.

In this study, we have elucidated a novel mechanism whereby SK1 regulates cell survival and proliferation through control of transferrin receptor 1 (TFR1) expression and function. We show that elevated levels of SK1 enhanced both total and functional TFR1 expression resulting in enhanced rates of transferrin (Tf) uptake into the cells. We also demonstrate that it is specifically the phosphorylated, plasma membrane-localised, oncogenic form of SK1 that mediates these effects on TFR1 expression via engagement of the $S1P_2$ receptor. Furthermore, we show that blocking TFR1 function with a neutralizing antibody inhibits SK1-induced cell proliferation, survival and neoplastic transformation, suggesting that TFR1 plays an important role in SK1-induced oncogenesis.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Anti-human TFR1 antibodies for immunoblot analysis were from ZYMED® Laboratories, neutralizing anti-human TFR1 [RVS10] and control antibodies were from Abcam (Cambridge, MA), while LEAF™ purified anti-mouse TFR1 and isotype control antibodies were purchased from Biolegend (San Diego, CA). Monoclonal α-tubulin [DM1A] and M2 anti-FLAG antibodies were from Abcam (Cambridge, UK) and Sigma, respectively. Alexa-488 conjugated anti-mouse IgG, alexa-594 conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Thermo Scientific (Rockford, CA). RNeasy columns, Omniscript RT kit, and 2x Quantitect SYBR Green PCR Master Mix reagent were from Qiagen (Valencia, CA). S1P, VPC-23019 and JTE-013 were purchased from Cayman Chemical (Ann Arbor, MI). Tf-alexa 568 was from Molecular Probes (Eugene, OR). Poly-L-lysine was from Sigma (St. Louis, MO).

4.3.2 Generation of expression constructs

Cell lines with tight doxycycline-inducible expression of SK1, SK2, SK1^{G82D} and SK1^{G212D} were generated as previously described in Section 2.3.2 and 3.2.1. To generate inducible cell lines for the non phosphorylatable human SK1 (SK1^{S225A}), the cDNA of SK1^{S225A} with *C*-terminal FLAG epitope tag (Pitson *et al.*, 2000a; Pitson *et al.*, 2003) was cloned into pcDNA5/FRT/TO-SK1-AU (Pham *et al.*, 2008) by digestion with BamHI and NotI. The expression construct for the constitutively plasma membrane-localised, non-phosphorylatable SK1 (SK1$^{pm\text{-}S225A}$; containing the *N*-terminal ten amino acids of the Lck tyrosine kinase that results in myristoylation and dual palmitoylation) was produced by digesting human SK1$^{pm\text{-}S225A}$ in pcDNA3 (Pitson *et al.*, 2005) with HindIII and NotI, blunted with Pfu, and then cloned into pcDNA5/FRT/TO-AU plasmid following digestion with EcoRV and NotI. Restriction analyses were performed to verify the integrity of all cDNAs.

4.3.3 Cell culture and generation of stably transfected inducible HEK293 cell lines

Flp-In T-Rex HEK293 cells (Invitrogen) were cultured, transfected, selected to generate stable cell lines, induced with doxycycline, and harvested as previously described in Section 2.3.3.

4.3.4 SK enzyme activity

SK activity specific for the two SK enzymes was measured as described previously in Section 2.3.7. Assays were performed using sphingosine-solublised with Triton X-100 under conditions that are largely selective for SK1, while assays for SK2 were performed with sphingosine-solublised with fatty acid free BSA in assay buffer containing 1 M KCl specific for SK2 activity (Pitman *et al.*, 2012). One unit (U) of activity is defined as 1 pmol of S1P formed per minute per mg of protein.

4.3.5 Western blot analysis

Samples containing equal protein concentrations were separated by SDS-PAGE using 12% acrylamide gels and transferred onto nitrocellulose membranes. Membranes were generally blocked with PBS containing 5% (w/v) skim milk powder (SMP) and 0.1% (v/v) Triton X-100. SK1, SK1^{S225A}, SK1$^{pm\text{-}S225A}$ were detected via their FLAG-epitope tags with the

monoclonal M2 anti-FLAG antibody as described in Section 2.3.6. To detect TFR1, membranes were blocked with PBS containing 5% (w/v) SMP and 0.2% (v/v) Tween-20 then incubated with monoclonal anti-TFR1 antibody at 1:2500 in the same buffer for 1 hr at room temperature. In most cases blots were washed and then re-probed with monoclonal anti-tubulin antibody at 1:5000 in PBS containing 0.1 % Tween-20 for 1 hr at room temperature for loading controls. The immunocomplexes were detected with HRP-conjugated anti-mouse IgG using an enhanced chemiluminescence kit (ECL, GE Healthcare) and LAS 4000 imager.

4.3.6 siRNA knock-down of $S1P_2$

Cells were transfected with a pool of siRNA duplexes targeting human $S1P_2$ (siGENOME SMARTpool siRNA D-003952-05; Dharmacon, Lafayette, CO) or control siRNA (Dharmacon) using Lipofectamine^{TM} RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. The cells were incubated for 48 h to achieve maximum silencing effects then subjected to 16 h induction of SK1 prior to harvesting for total RNA and protein. The levels of SK1 activity were then determined by SK1 activity assays, TFR1 protein was examined by Western blotting, and the expression of $S1P_2$ was analysed by qPCR (see below).

4.3.7 Quantitative real-time PCR (qPCR)

QPCR analysis of human TFR1 was performed using RNA preparations from either samples used in the microarray experiments or RNA samples prepared from subsequent independent experiments using the same RNA extraction and qPCR procedure as detailed earlier (Section 3.3.4). QPCR of $S1P_2$ was performed under the same conditions using the primers 5'-ACCATCGTGCTAGGCGTCT-3' and 5'-GTGGGCTTTGTAGAGGATCGG-3' (Geneworks, Adelaide, Australia).

4.3.8 Immunofluorescence

For immunofluorescence analysis of cell surface TFR1 and total SK1 expression, cells were plated onto poly-L-lysine coated cover slips, incubated for 24 h and then induced with doxycycline for a further 16 h. The cells were then fixed for 15 min with 4% paraformaldehyde, incubated in PBS with 2% BSA for 10 min to block any non-specific binding of antibodies in later steps and then incubated with 2 ng/ml anti-TFR1 antibody [RVS10] in PBS with 2% BSA

for 1 h at room temperature. The cells were then washed thoroughly to remove any residual TFR1 antibody, permeabilised with 0.1% TritonX-100 in PBS for 15 min, and then incubated with affinity purified polyclonal anti-SK1 antibodies (from Section 2.3.4) at 1:1000 in PBS containing 2% BSA and 0.1% Triton X-100 for 1 h at room temperature. The immunocomplexes were detected with alexa-488 conjugated anti-mouse IgG (for TFR1) or alexa-594 conjugated anti-rabbit IgG (for SK1). Cover slips were mounted with Dako Fluorescent Mounting Medium. Membrane TFR1 and total SK1 were visualized using a 60X water-immersion objective on an Olympus IX81 inverted microscope (Solent Scientific, Segensworth, UK). Fluorescence intensity was quantified using AnalySIS Five Life Sciences software (Olympus).

4.3.9 Transferrin (Tf) alexa 568 uptake assay

Tf uptake was determined with cells plated onto poly-L-lysine coated cover slips and incubated for 24 h and then induced with doxycycline for a further 16 h. After induction, cells were serum starved for 1 h, then incubated at 4 °C for 5 min to block any non-specific internalization of Tf, and then incubated with 50 ng/ml of Tf alexa-568 for 30 min at 4 °C. Unbound Tf was then removed by washing with cold serum free media and then cells at 0 min point were immediately fixed for 15 min with 4% paraformaldehyde. The remaining cells were chased in serum free media at 37 °C for 10 min, washed with cold serum free media and then fixed in paraformaldehyde. Cover slips were mounted with Dako Fluorescent Mounting Medium. Tf uptake was visualized via Fluorescence microscopy as described above (section 4.3.9).

4.3.10 Cell proliferation and apoptosis assays

Cell proliferation was determined with BrdU incorporation and cell death was measured with DAPI staining as detailed earlier (Section 2.3.9).

4.3.11 Focus formation assays

NIH3T3 fibroblasts were cultured in DMEM with 10% FBS, 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, 1.2 mg/ml penicillin and 1.6 mg/ml streptomycin. For generation of pooled stable cell lines, the cells were made with pcDNA3/IRES/EGFP or

pcDNA3/IRES/SK1(FLAG)/EGFP constructs which express EGFP alone or SK1 from the CMV promoter and EGFP via an IRES, respectively. These constructs were previously generated (Moretti & Pitson, unpublished) and transfected into cells using Lipofectamine™ 2000 reagent (Invitrogen) as described by the manufacturer. One day after transfection, the cells were passaged and the medium was replaced with a selective medium containing 0.8 mg/ml neomycin (G418) (Invitrogen) for 7 days with media and selection changed every 2-3 days. Given that both of our constructs contained the enhanced green fluorescence protein (EGFP) cDNA, cells expressing EGFP should, in theory, also express SK1. Thus, after a week of selection the cells were sorted for EGFP expression using fluorescence associated cell sorter (FACS), in an attempt to increase the likelihood of having pools of stable cells that would all express SK1. For focus formation assays, selection was removed, and cells were seeded into 12-well plates, and then remaining cells were harvested for Western analysis to confirm the expression of SK1. Cells were cultured for 3 weeks, with media replaced every 3-4 days in the presence or either 10 µg/ml TFR1 neutralizing antibody or the corresponding isotype control antibody. Foci were visualized after fixing with methanol and staining with 0.01% methyl violet.

4.4 RESULTS

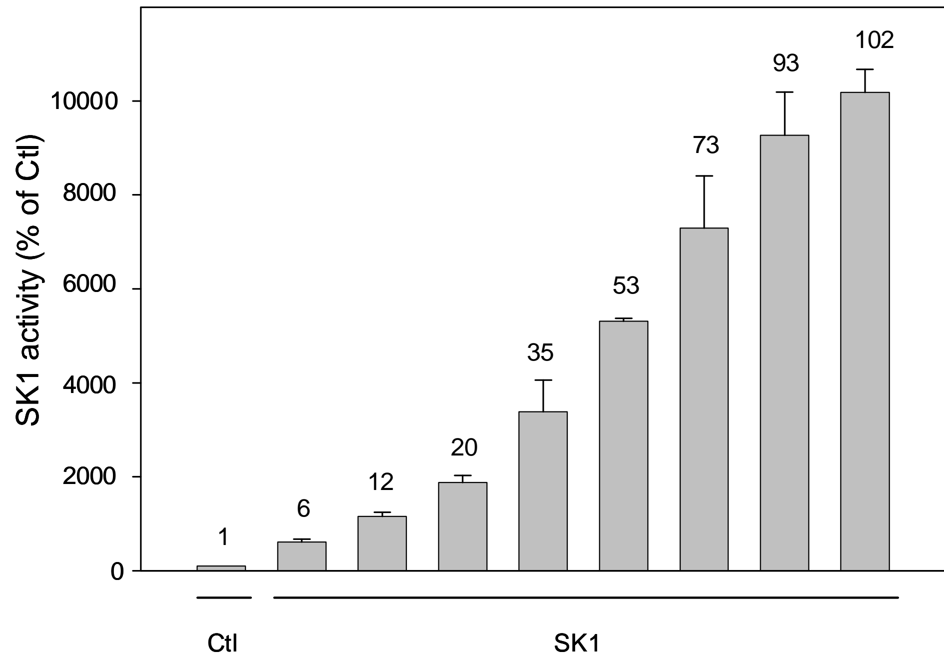
4.4.1 SK1-mediated changes in TFR1 mRNA and protein

Our initial microarray and subsequent validation studies demonstrated that low level SK1 overexpression up-regulates TFR1 mRNA expression (Figure 3.6). We next investigated whether SK1 regulates TFR1 expression in a dose-dependent manner. Cells were induced to overexpress various levels of SK1 (Figure 4.1A) and then TFR1 mRNA expression was measured 12 h later by qPCR. Interestingly, the results show that TFR1 expression varied depending on the level of SK1 overexpression (Figure 4.1B). Increases in cellular SK1 activity of around 12 to 20-fold over endogenous levels resulted in robust increases of TFR1 mRNA expression. In contrast, however, TFR1 mRNA levels appeared unchanged at 35-fold overexpression of SK1 and higher.

To further confirm that SK1 regulates TFR1 expression, we induced cells to express around 20-fold higher SK1 activity over endogenous levels (Figure 4.2A), and then assessed total cellular TFR1 protein levels by Western blotting, along with TFR1 mRNA by qPCR. The results showed that enhanced SK1 activity resulted in a 2-fold increase in both TFR1 mRNA (Figure 4.2B) and protein (Figure 4.2C) compared to the uninduced control cells.

Figure 4.1 SK1 increases TFR1 mRNA in a dose-responsive manner. SK1-AU (SK1) inducible cells were cultured for 12 h in various concentrations of doxycycline. Following induction, cells were harvested to isolate both (**A**) cell lysates for SK1 activity assays and total RNA for TFR1 qPCR analysis (**B**). Data are mean (± range) of duplicate determinations from a single experiment, and are representative of results obtained from two independent experiments.

A



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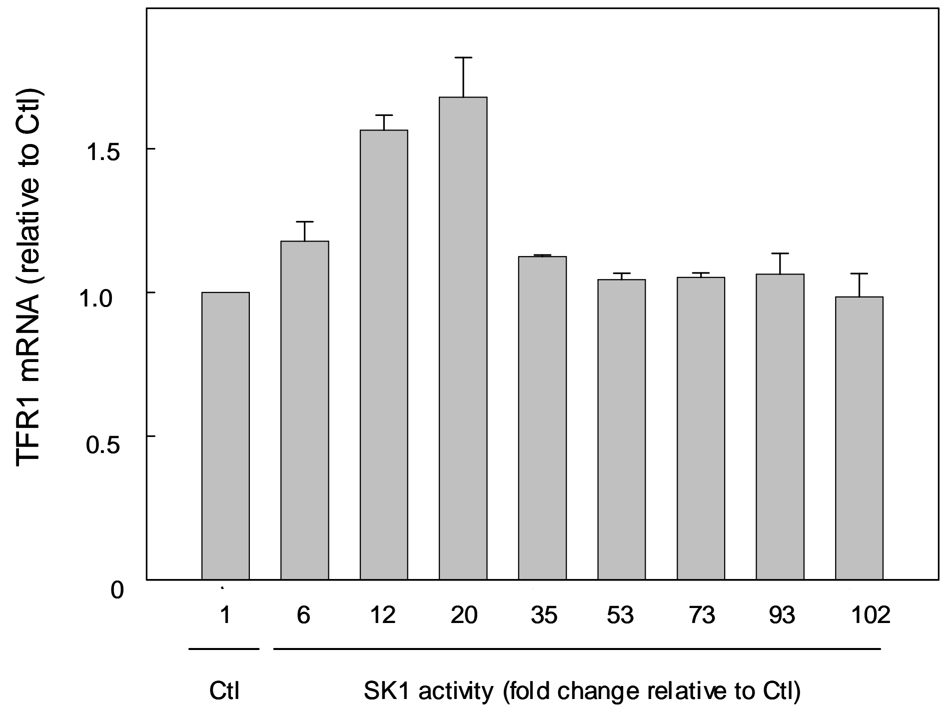


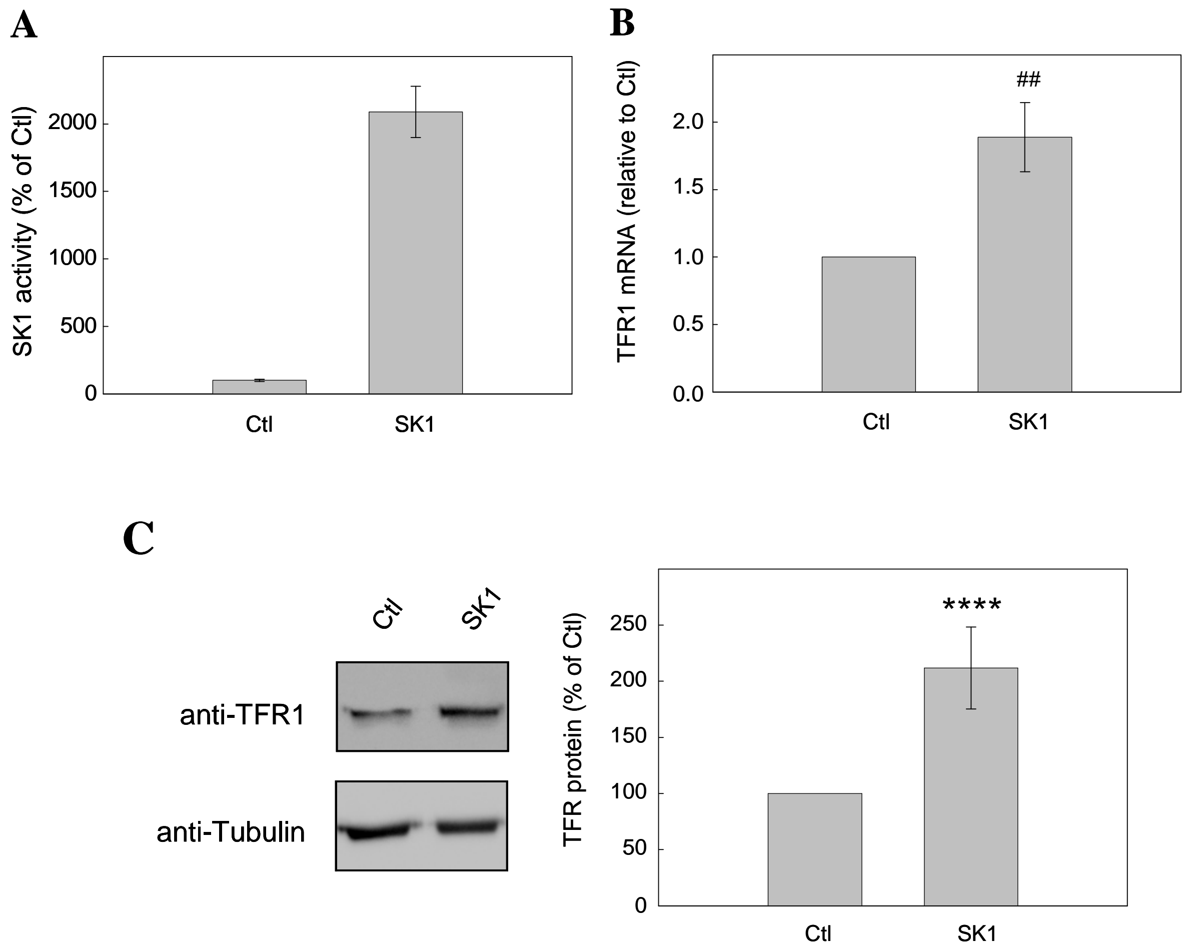


Figure 4.2 SK1 mediates increased TFR mRNA and protein expression. SK1 inducible cells were cultured for 16 h either in the absence or the presence of doxycycline to induce to approximately 20-fold SK1 activity over endogenous levels. After induction, the cells were harvested, and then cell lysates were prepared for SK1 activity assays (**A**), total RNA isolated for TFR1 qPCR analysis (**B**) and Western analysis with anti-TFR1 antibody (**C**). Data represent the mean ± SEM from six experiments. Statistical significance was calculated by an unpaired t-test, (##$P < 0.0001$ compared with Ctl cells and ****$P < 0.001$ compared with Ctl cells).

4.4.2 SK1 induces cell-membrane TFR1 expression and mediates Tf uptake

TFR1 is a type II membrane protein that mediates iron uptake into cells (Wang & Pantopoulos, 2011; Macedo & de Sousa, 2008; Daniels *et al.*, 2006; Kuhn, 1989). Although, we showed that SK1 enhanced total cellular levels of TFR1 expression, the presentation of this receptor at the cell surface is necessary for its binding and uptake of iron bound-Tf into cells (Wang & Pantopoulos, 2011; Klausner *et al.*, 1984; Cavanaugh *et al.*, 1999; Trowbridge & Shackelford, 1986). Therefore, we next examined the functional effects of SK1 on cell surface presentation of TFR1 via fluorescence microscopy. Control (uninduced) cells showed very low cell surface presentation of endogenous TFR1. Following induction of SK1 expression, however, a clear increase in the levels of TFR1 at the cell surface was observed (Figure 4.3). This effect was further confirmed by examining the level of Tf uptake in these cells as a direct measure of functional TFR1. Consistent with an increase in total and cell surface TFR1 expression, there was also an approximate 2-fold increase in Tf uptake into the cells expressing SK1 compared to control cells (Figure 4.4). Together these results firmly indicate that SK1 overexpression enhances total TFR1 expression which results in subsequent functional TFR1 presentation on the cell surface.

4.4.3 SK1 phosphorylation and localisation to the plasma membrane is critical for its effects on TFR1 regulation

Since SK1 phosphorylation at Ser225 and its subsequent translocation to the plasma membrane is crucial for oncogenic signalling by this enzyme (Pitson *et al.*, 2005), and we had already shown SK1 becomes phosphorylated following low overexpression (Figure 2.14), we further examined if TFR1 expression is dependent on this type of SK1 regulation. To do this we first generated HEK293 cell lines with inducible expression of SK1^{S225A} (the non phosphorylatable human SK1) (Figure 4.5) and SK1$^{pm\text{-}S225A}$ (the constitutively plasma membrane-localised, non-phosphorylatable SK1) (Figure 4.6). Following establishment of these cell lines, we induced expression of SK1^{S225A} and SK1$^{pm\text{-}S225A}$ to approximately 20-fold higher levels than endogenous SK1, as determined by cellular SK1 activity (Figure 4.7A), and then performed TFR1 qPCR and Western blot analysis to assess the effect on TFR1 expression (Figure 4.7B & C) . In contrast to the effects of wild-type SK1, TFR1 mRNA and protein expression were unaltered following induction of SK1^{S225A} expression. However, we found that

expression of the plasma membrane version of this SK1 variant, $SK1^{pm\text{-}S225A}$, resulted in a 2-fold increase in TFR1 mRNA as well as protein expression to a similar extent to that seen with wild-type SK1. This data suggests that the phosphorylation and subsequent plasma membrane localisation of SK1 are critical for SK1-induced regulation of TFR1 expression.

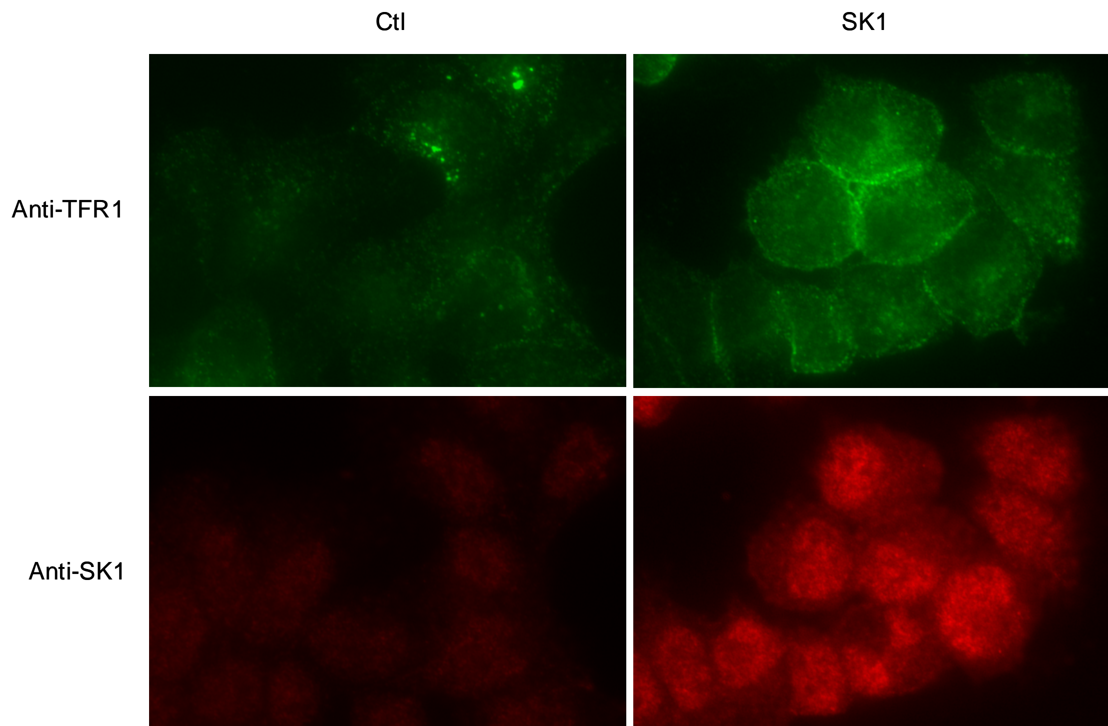
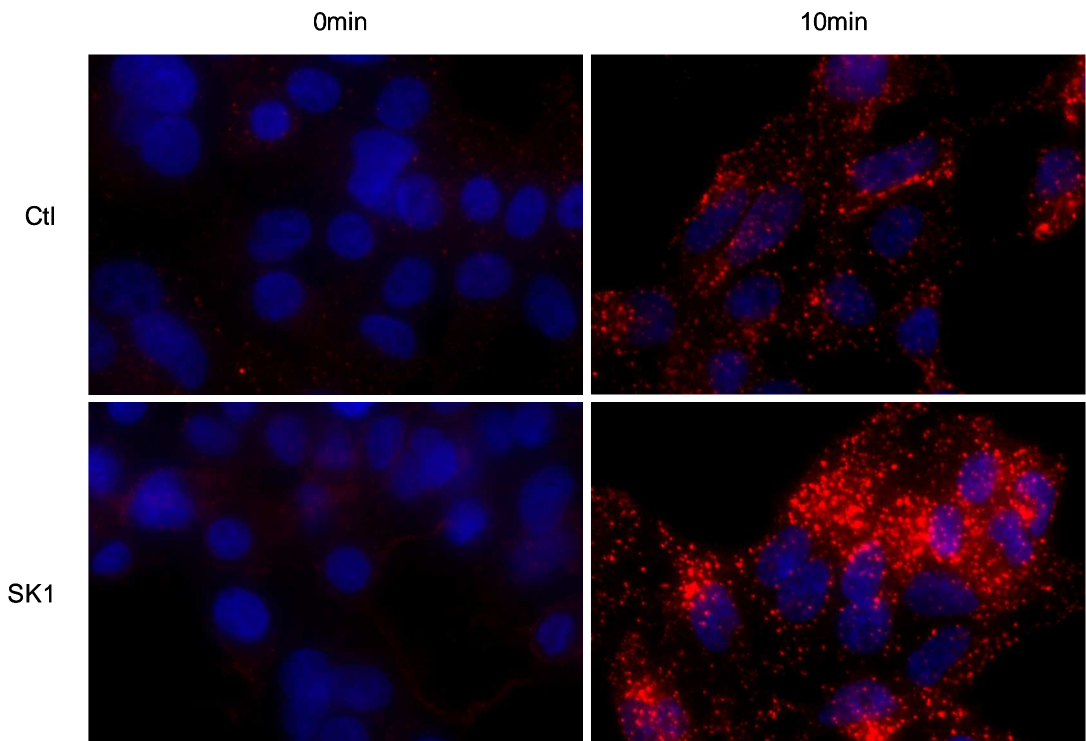


Figure 4.3 SK1 mediates enhanced cell-surface TFR1 expression. SK1 inducible cells were either uninduced or induced with 4 ng/ml doxycycline for 16 h to approximately 20-fold over endogenous level. Cell-surface TFR1 expression was detected in unpermeabilised cells using anti-TFR1 antibody and then cells were subsequently permeabilised and expression of total SK1 in cells was confirmed using anti-SK1 antibodies. Cells were visualized with a 60X water-immersion objective on an Olympus IX81 inverted microscope. Images are representative of more than 300 cells examined.

Figure 4.4 SK1 mediates increase Tf uptake into cells. SK1 inducible cells were either uninduced or induced with doxycycline for 16 h. Cells were incubated with alexa 568-labelled Tf for 30 min at 4 ^{o}C. Following that, unbound Tf was removed and cells were incubated at 37 ^{o}C for 10 min and the internalization of Tf (red) was observed by a 60X water-immersion objective on an Olympus IX81 inverted microscope (**A**). Cell nuclei were visualized by staining with DAPI (blue). Tf uptake into cells at 10 min was quantitated using Image Quant (**B**). Data represent the mean ± SEM from four independent experiments, with statistical significance calculated by an unpaired t-test, (***P < 0.005 compared with Ctl cells).

A



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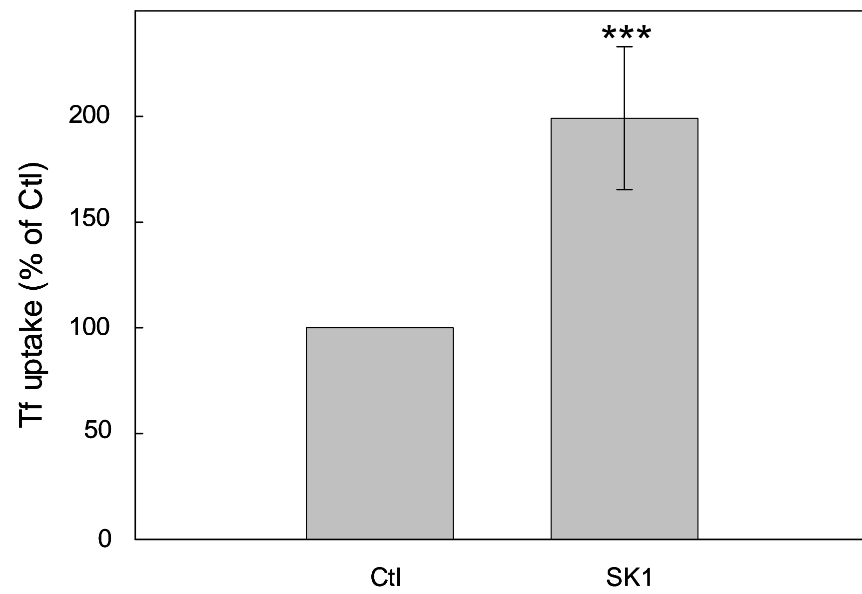

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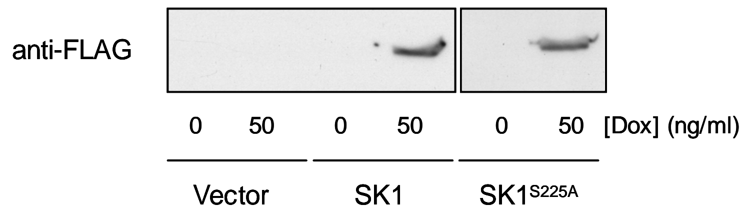

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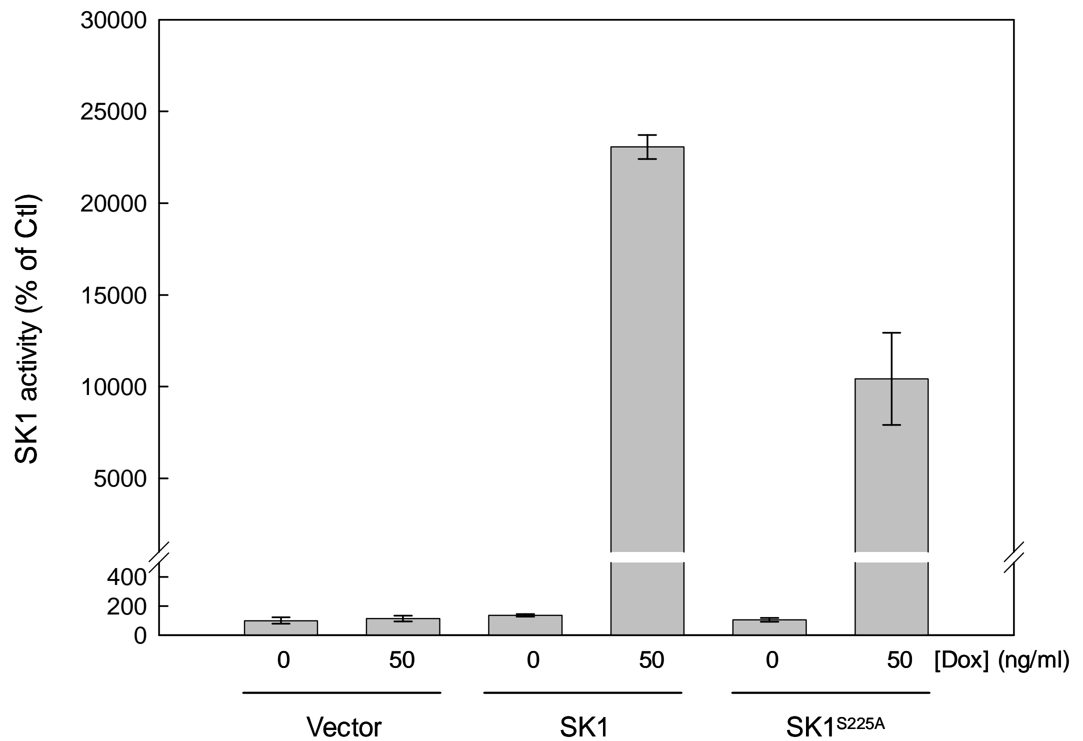
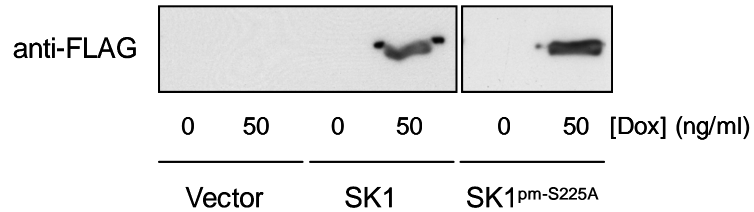


Figure 4.5 Characterisation of non-phosphorylatable SK1 ($SK1^{S225A}$) in doxycycline-inducible system. Flp-In T-Rex HEK293 cells containing inducible SK1, or FLAG-epitope tagged $SK1^{S225A}$-AU ($SK1^{S225A}$) or vector cells were cultured for 24 h either in the absence or the presence of 50 ng/ml doxycycline. The expression of SK1 and $SK1^{S225A}$ were examined in cell lysates by immunoblot analysis with M2 anti-FLAG (**A**) and SK1 activity assays (**B**). Data represent the mean ± SEM from three independent experiments.

A



B

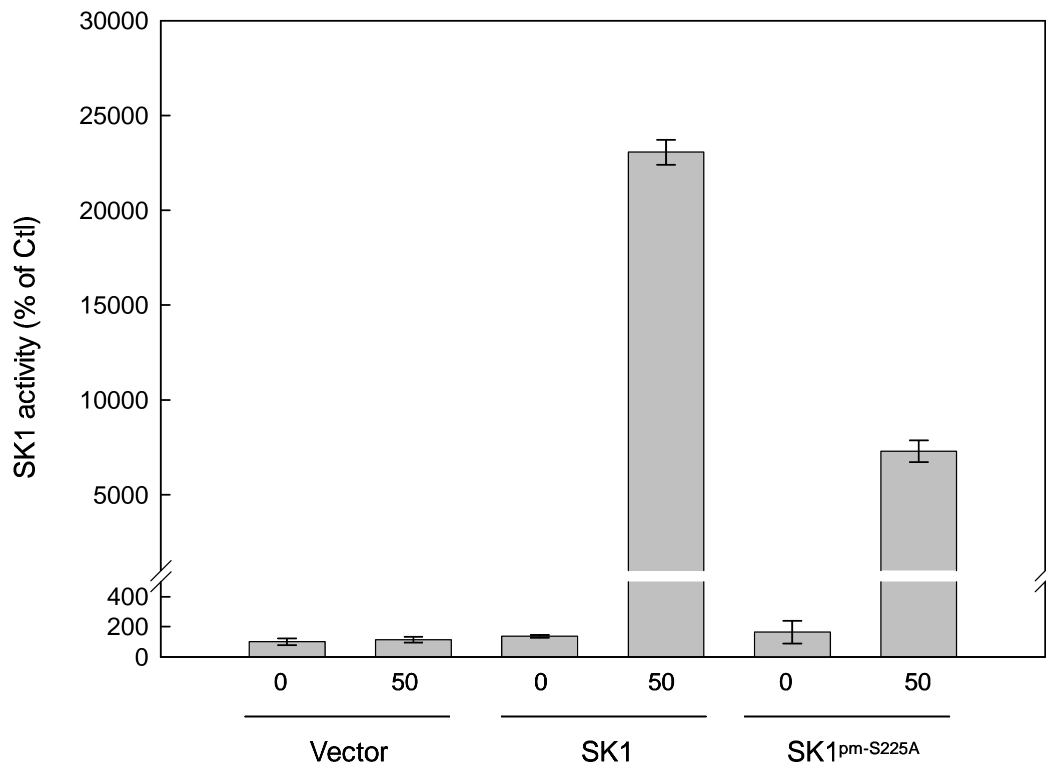
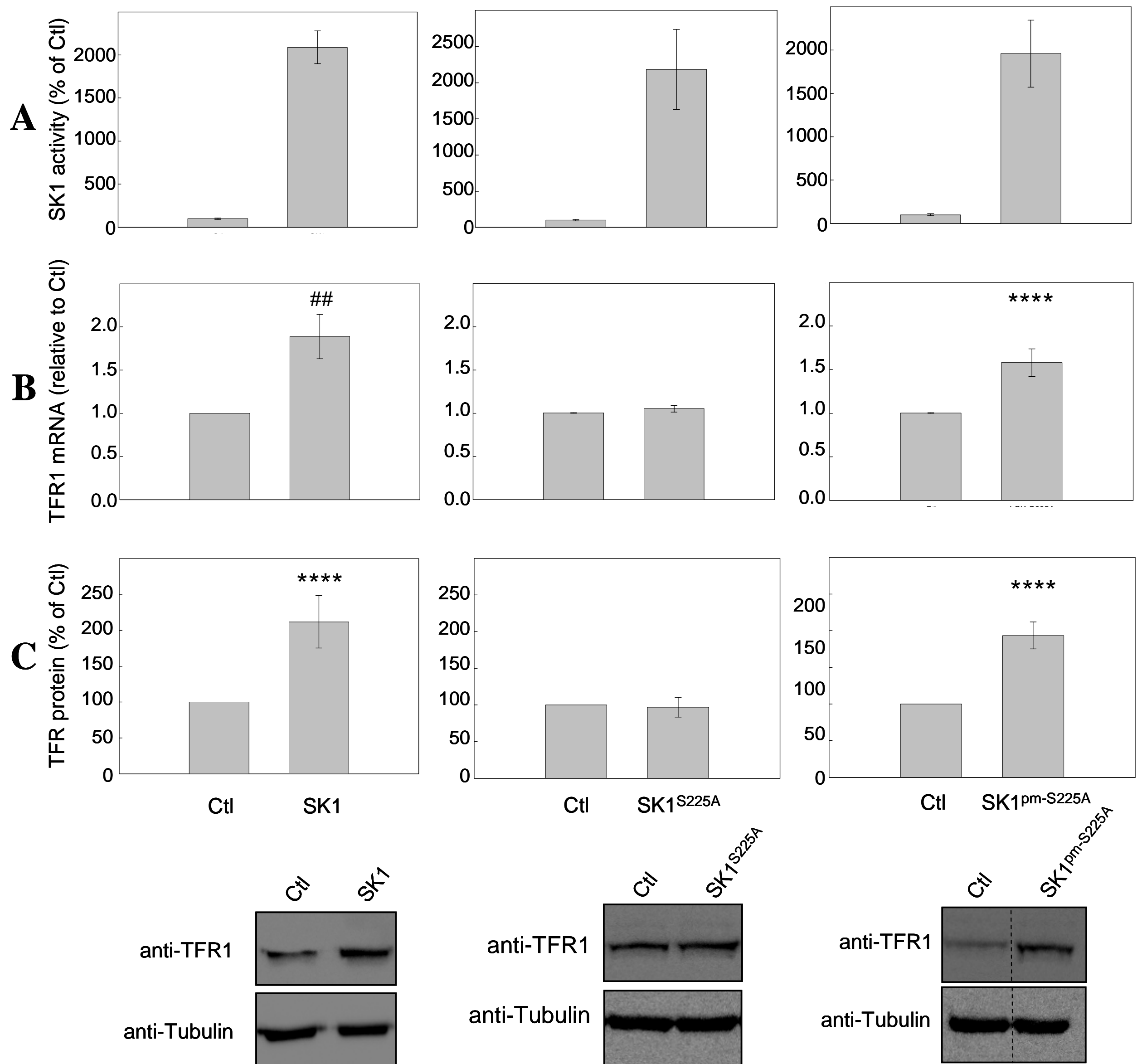


Figure 4.6 Characterisation of a non-phosphorylatable SK1 mutant that constitutively localises to the plasma membrane (SK1$^{pm\text{-}S225A}$) in a doxycycline-inducible system. Flp-In T-Rex HEK293 cells containing inducible SK1, or FLAG-epitope tagged SK1$^{pm\text{-}S225A}$-AU (SK1$^{pm\text{-}S225A}$) or vector cells were cultured for 24 h either in the absence or the presence of 50 ng/ml doxycycline. The expression of SK1 and SK1$^{pm\text{-}S225A}$ were examined in cell lysates by immunoblot analysis with M2 anti-FLAG (**A**) and SK1 activity assays (**B**). Data represent the mean ± SEM from three independent experiments.

Figure 4.7 SK1$^{pm\text{-}S225A}$ but not SK1^{S225A} mediates increased TFR1 mRNA and protein expression. SK1$^{pm\text{-}S225A}$ and SK1^{S225A} inducible cells were cultured for 16 h either in the absence or the presence of 5 ng/ml doxycycline to induce approximately 20-fold SK1 activity over endogenous levels. Cells were harvested, and lysates were prepared for SK1 activity assays (**A**), total RNA isolated for TFR1 qPCR assays (**B**), and Western analysis performed with anti-TFR1 antibody (**C**). Data represent the mean ± SEM from three independent experiments, with statistical significance calculated by an unpaired t-test, (##$P < 0.0001$ compared with Ctl cells and ****$P < 0.001$ compared with Ctl cells). The dividing lines indicate where lanes from the same immunoblot have been spliced to simplify viewing. Data from Figure 4.2 has been shown again in Figure 4.7 to enable easy direct comparison of TFR1 expression level between SK1, SK1^{S225A} and SK1$^{LCK\text{-}S225A}$.


A
B
C
SK1 activity (% of Ctl)
TFR1 mRNA (relative to Ctl)
TFR protein (% of Ctl)
##
****
Ctl
SK1
SK1S225A
SK1pm-S225A
anti-TFR1
anti-Tubulin

4.4.4 Addition of exogenous S1P regulates TFR1 expression

We previously showed that activation and subsequent translocation of human SK1 to the plasma membrane results in both increases in intracellular S1P and enhanced release of S1P into the extracellular environment (Pitson *et al.*, 2003; Pitson *et al.*, 2005). Therefore, to assess the roles of intra- and extracellular S1P we next examined the effect on TFR1 expression following addition of exogenous S1P to cells. The results (Figure 4.8) show a dose-dependent increase in TFR1 protein expression in cells treated with S1P. Notably, this increase in TFR1 protein expression was detectable at very low concentrations of S1P; as low as 10 nM (Figure 4.8), suggesting the involvement of S1P cell surface receptor(s) in this process. Interestingly, Figure 4.1 and Figure 4.8 show quite different dose-response curves with high levels of SK1 not inducing TFR1 expression while high levels of exogenous S1P did. While the reason(s) for this difference remains unclear it may be a result of the different nature of the two experiments. The studies shown in Figure 4.1 examined the effect of SK1 activity on TFR1 expression at the mRNA level, while those described in Figure 4.8 examined the effect of exogenous S1P on TFR1 expression at the protein level. Alternatively, SK1 regulation of TFR1 expression occurs through the cell surface S1P receptor(s). Thus, S1P produced by SK1 intracellularly must be released from the cells to enhance TFR1 expression. Since high levels of SK1 activity result in high levels of S1P within the cells, it could be possible that this might trigger other, as yet unknown signaling pathways that suppress TFR1 expression under these conditions.

4.4.5 SK1 regulates TFR1 expression via $S1P_2$

To directly examine whether SK1 regulates TFR1 by acting through the S1P cell-surface receptor(s), we next employed isoform-selective S1P receptor inhibitors. Cells were induced to express SK1 and treated with various S1P receptor inhibitors and the effects on TFR1 expression examined. The results showed that VPC-23019, an inhibitor of $S1P_1$ and $S1P_3$ [reviewed in (Im, 2010)], had no effect on SK1-induced TFR1 protein expression (Figure 4.9). In contrast, however, treatment of cells with JTE-013, an inhibitor of $S1P_2$ (Pyne & Pyne, 2011), effectively blocked TFR1 expression induced by SK1 (Figure 4.10A). As expected, JTE-013 had no effect on SK1 activity (Figure 4.10B). Since JTE-013 has been suggested to also be an antagonist of $S1P_4$ (Long *et al.*, 2010) and possibly have other off-target effects (Salomone & Waeber, 2011), we further examined the involvement of $S1P_2$ by utilising siRNA knock-

down of $S1P_2$ in cells expressing SK1 and then examined its effect on TFR1 mRNA and protein expression. The results again, show that TFR1 expression induced by SK1 was blocked in cells following knock-down of $S1P_2$ (Figure 4.11B & C). Taken together, these results indicate that SK1 regulates TFR1 expression via the $S1P_2$ receptor.

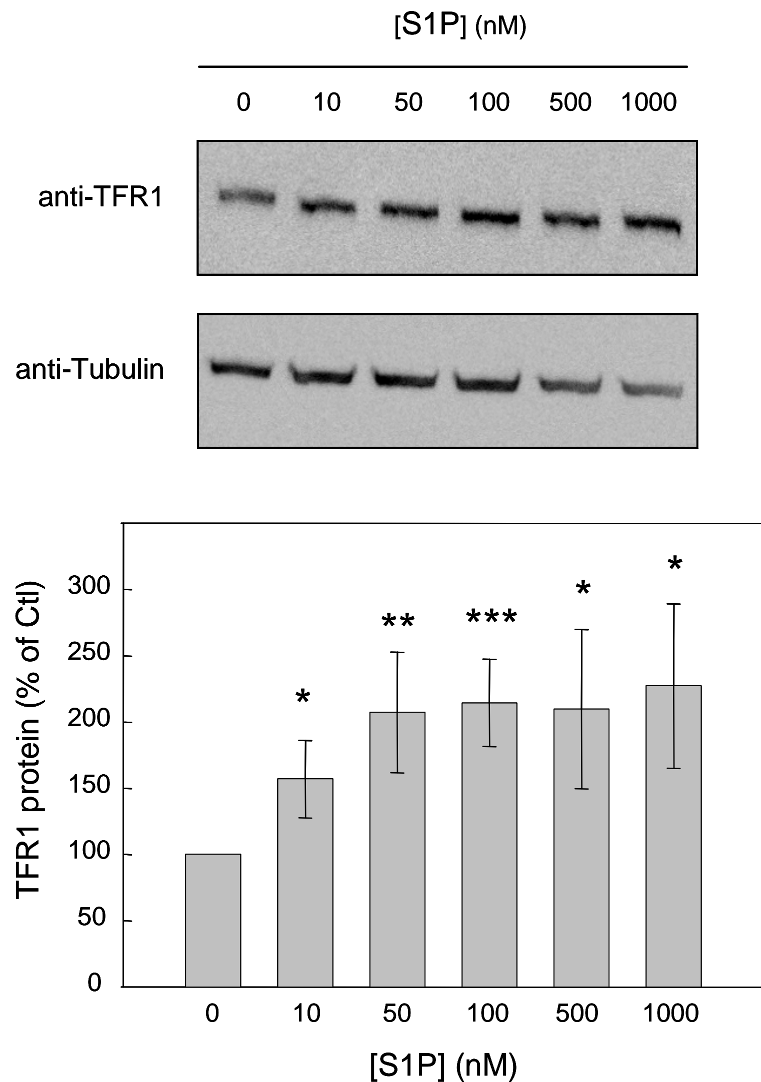


Figure 4.8 S1P increases TFR1 protein expression in a dose-responsive manner. Vector cells were cultured in the absence or the presence of increasing concentrations of S1P for 16 h. TFR1 protein expression was examined in cell lysates by immunoblot analysis with anti-TFR1 antibody. Data represent the mean ± SEM from three independent experiments, with statistical significance calculated by an unpaired t-test, (*P < 0.05, **P < 0.01, and ***P < 0.005, compared with Ctl cells).

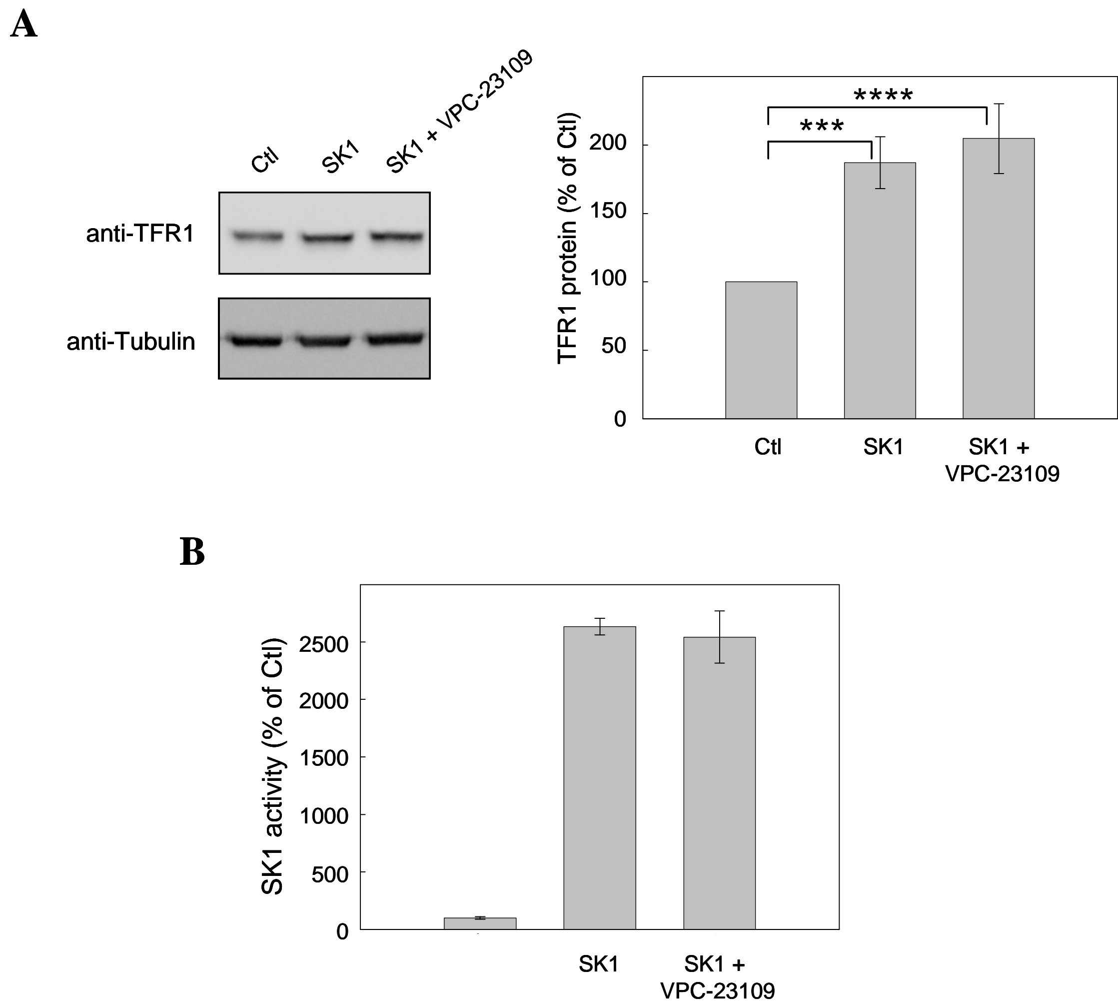
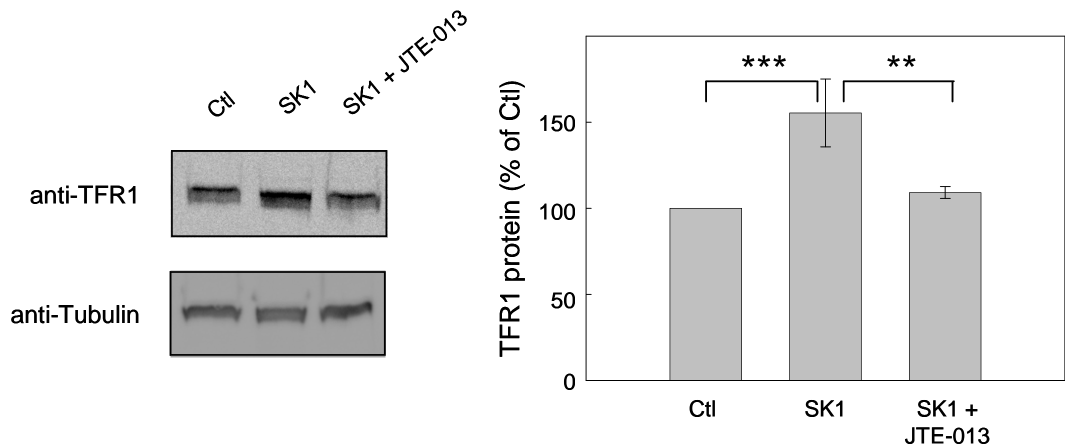


Figure 4.9 SK1 does not mediate increased TFR1 expression via $S1P_1$ or $S1P_3$ receptors. SK1 inducible cells were cultured for 16 h either in the absence or the presence of doxycycline and untreated or treated with 10μM VPC-23019 (antagonist of $S1P_1/S1P_3$). Western blot using anti-TFR1 antibody shows that treatment of cells with VPC-23019 had no effect on TFR1 expression (**A**). As expected, VPC-23019 had no effect on SK1 activity (**B**). Data represent the mean ± SEM from three independent experiments, with statistical significance calculated by an unpaired t-test, (***P < 0.005 and ****P < 0.001, compared with Ctl cells).

A



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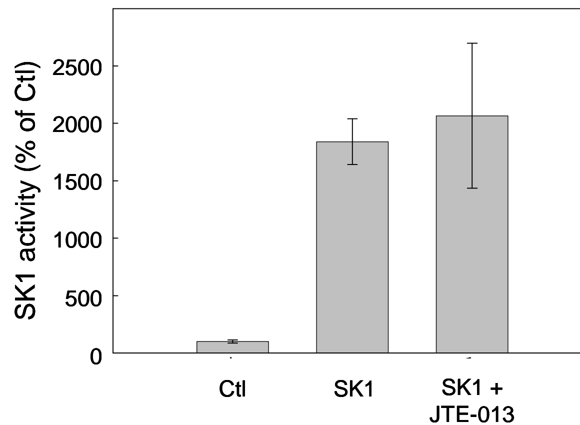


Figure 4.10 SK1 mediates increased TFR1 expression via $S1P_2$ receptor. SK1 inducible cells were cultured for 16 h either in the absence or the presence of doxycycline and untreated or treated with JTE-013 (antagonist of $S1P_2$). Western blots using anti-TFR1 antibody showed that treatment of cells with JTE-013 significantly prevented the SK1-mediated enhanced TFR1 protein expression (**A**). As expected, JTE-013 had no effect on SK1 activity (**B**). Data represent the mean ± SEM from four independent experiments, with statistical significance calculated by an unpaired t-test, (**P < 0.01 and ***P < 0.005, compared with Ctl cells).

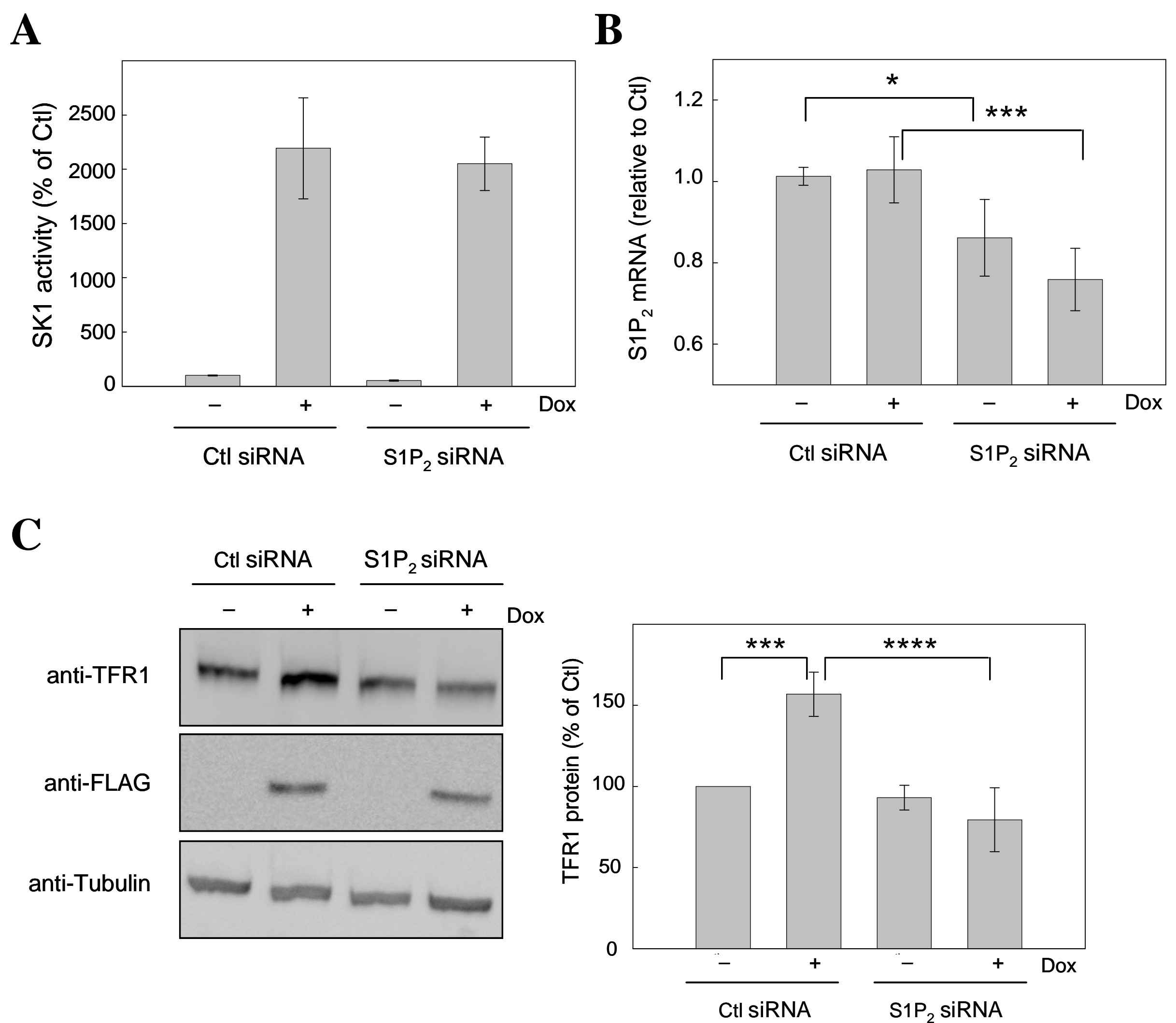


Figure 4.11 $S1P_2$ receptor knock-down inhibits SK1-enhanced TFR1 expression. SK1 inducible cells were transfected with either control or $S1P_2$ siRNA for 48h and then subsequently uninduced or induced with doxycycline for 16 h. After induction, the cells were harvested, cell lysates were generated for SK1 activity assays (**A**), total RNA isolated for qPCR assays of $S1P_2$ expression (**B**), and Western analysis with anti-TFR1 antibody (C). Data represent the mean ± SEM from quantitations of six independent experiments, with statistical significance was calculated by an unpaired t-test, (*$P < 0.05$, ***$P < 0.005$, and ****$P < 0.001$, compared with Ctl cells).

4.4.6 Inhibition of cell-surface TFR1 ablates SK1-induced cell proliferation, survival and neoplastic transformation.

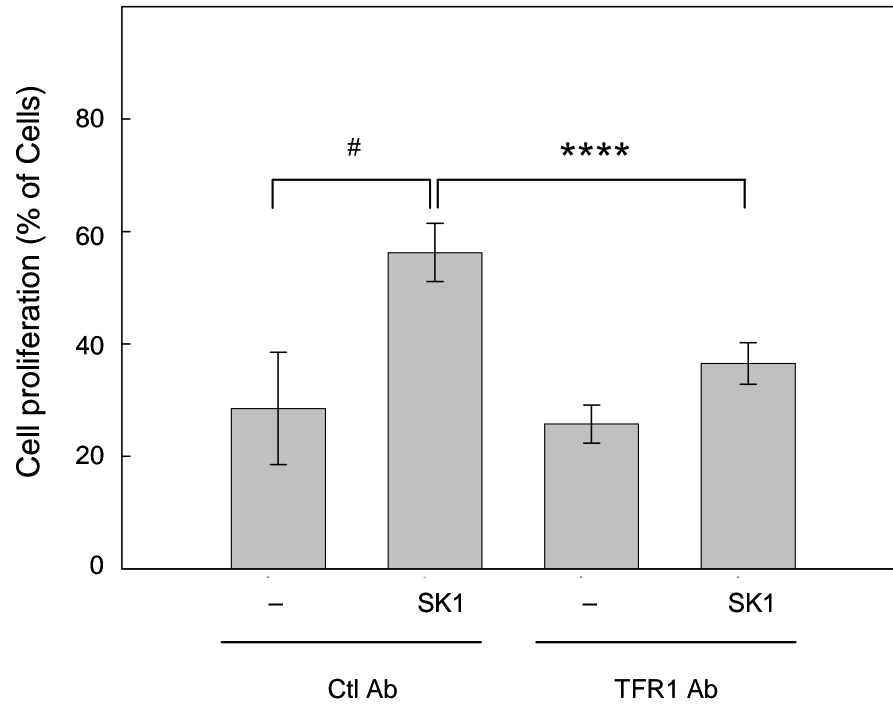
Overexpression of SK1 has been shown to enhance cell proliferation and survival, and induce neoplastic transformation (Olivera *et al.*, 1999a; Olivera *et al.*, 2003; Xia *et al.*, 2000; Pitson *et al.*, 2005). Notably, TFR1 is also associated with cancer. For example, increased expression of TFR1 promoted cell growth, and enhanced c-myc-mediated tumour formation in mice (O'Donnell *et al.*, 2006; Habashy *et al.*, 2010). Therefore, we investigated if TFR1 plays a role in oncogenic signalling by SK1 through the use of a TFR1 neutralizing antibody. This antibody is commercially available and has been utilised in various studies to block the biological functions of TFR1, both in cultured cells and *in vivo* (Efferth *et al.*, 2004; Lee *et al.*, 2004; Trowbridge & Lopez, 1982; Trowbridge *et al.*, 1982; Grisendi *et al.*, 2005). Our results showed that SK1 overexpression enhanced cell proliferation (Figure 4.12A) and protected cells from serum-deprivation-induced apoptosis (Figure 4.12B), consistent with previous studies (Pitson *et al.*, 2005; Le Scolan *et al.*, 2005; Xia *et al.*, 2000). However, strikingly, this SK1-induced cell proliferation and survival was blocked in the presence of TFR1 neutralising antibody. Notably, the TFR1 antibody showed little effect on cell proliferation and survival in the absence of SK1 overexpression, suggesting its effects were specific for SK1-mediated signalling.

We next examined the role of TFR1 in SK1-induced neoplastic transformation by performing focus formation assays using NIH3T3 cells ectopically expressing SK1 in the presence of either TFR1 neutralising antibody or isotype control antibody (Figure 4.13A). Consistent with previous studies, our results showed that SK1 overexpression in NIH3T3 cells induced the formation of numerous foci. Remarkably, however, the number of SK1-induced foci was significantly reduced in the presence of the TFR1 neutralising antibody (Figure 4.13B). Interestingly, neoplastic transformation of NIH3T3 cells has been previously shown to be induced by high SK1 overexpression (approximately 200-fold over endogenous level) (Pitson *et al.*, 2005). In contrast, our current data show that only moderate and not high levels of SK1 activity in HEK293 cells appear to regulate TFR1 expression. A possible explanation for this might be due to the differences in the two cell types and expression systems used. Previously, neoplastic transformation was observed in pools of NIH3T3 stables consisting of cells with different levels (moderate to very high) of SK1 activity, which when assayed together

gave ‘high’ level overexpression. In contrast, our current results were generated from doxycycline-inducible HEK293 system which produces a clonal population of cells with a similar level of SK1 activity. Therefore, it is possible that the previously observed neoplastic transformation were selectively formed only from a sub-population of cells from the pool with moderate SK1 activity. Alternatively, there may simply be differences in the response to SK1 expression between human and mouse cells.

Figure 4.12 TFR1 neutralizing antibodies ablate SK1-induced cell proliferation and survival. SK1 inducible cells were grown on coverslips overnight. After 24h, cells were cultured in serum-free medium for 24 h either in the absence or the presence of doxycycline and untreated or treated with anti-TFR1 neutralizing antibody. (**A**) Cell proliferation of these cells as measured by BrdU incorporation into nacent DNA. (**B**) Serum deprivation-induced apoptosis of these cells as measured by nuclear condensation and fragmentation. Data represent the mean ± SEM from four independent experiments, with statistical significance calculated by an unpaired t-test, (***P < 0.005, ****P < 0.001, and #P < 0.0005, compared with Ctl cells).

A



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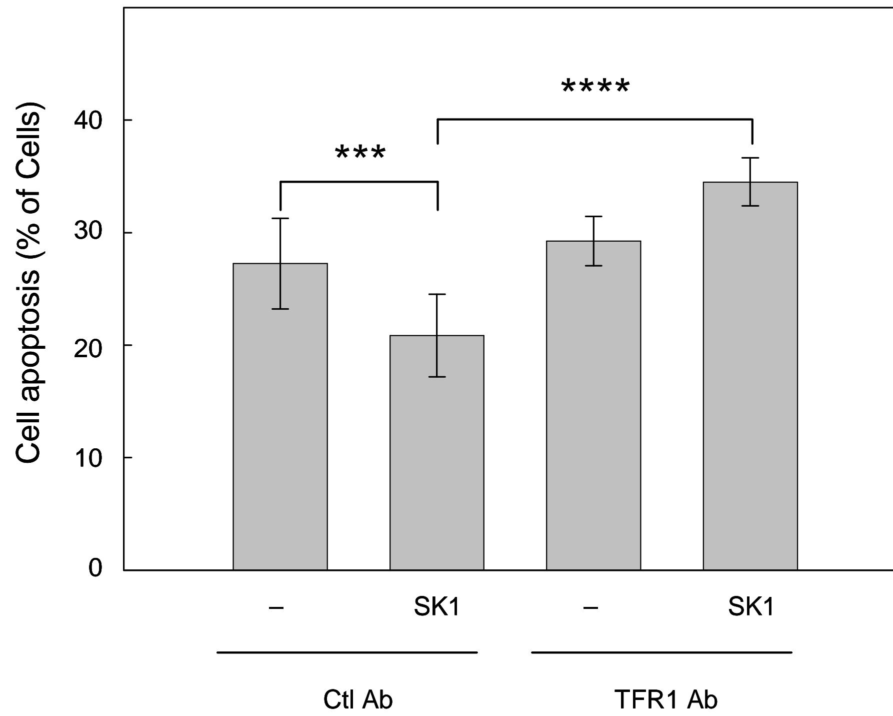

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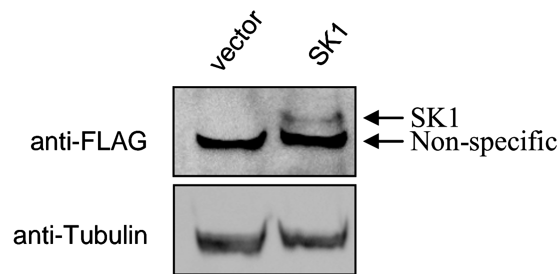

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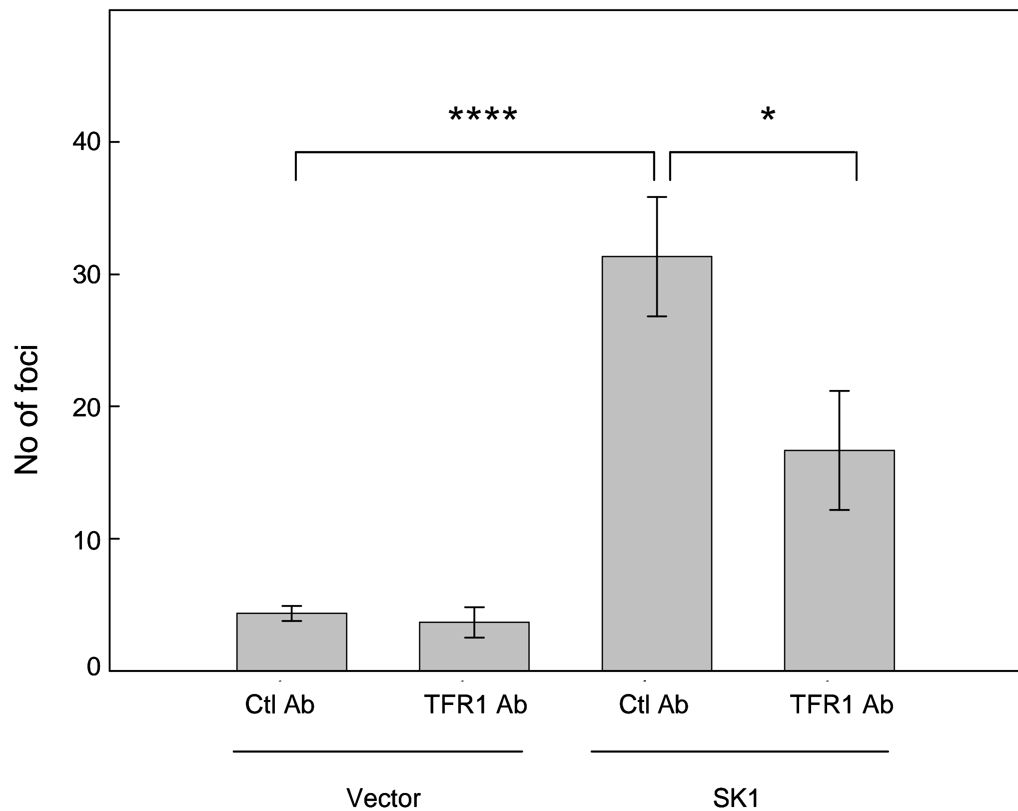

Figure 4.13 Inhibition of TFR1 expression reduces SK1-induced neoplastic transformation. NIH3T3 cells stably expressing the empty pcDNA3/IRES/EGFP vector (vector) or pcDNA3/IRES/SK1/EGFP (SK1) (Moretti & Pitson, unpublished data) were sorted for SK1-posittive cells according to their high GFP expression using FACS. GFP-positive cells were cultured in 3 wells, with media replaced every 3-4 days in the presence or absence of 10 µg anti-TFR1 neutralizing antibody. Foci were scored after fixing with methanol and staining with methyl violent. Data are the mean ± SEM from three independent experiments, with statistical significance calculated by an unpaired t-test, (*P < 0.05 and ****P < 0.001 compared with Ctl cells).

4.5 DISCUSSION

Significant steps towards understanding the molecular mechanisms of cellular regulation by SK1 have been made in the past few years through the identification that SK1 activation and translocation from the cytosol to the plasma membrane is crucial for oncogenesis mediated by this enzyme (Pitson *et al.*, 2003; Pitson *et al.*, 2005; Jarman *et al.*, 2010). Indeed, localisation of SK1 to the plasma membrane where its substrate, sphingosine, is enriched, appears critical for agonist-induced S1P generation and the pro-proliferative, pro-survival and oncogenic effects of SK1 (Hengst *et al.*, 2009; Pitson *et al.*, 2005; Sarkar *et al.*, 2005; Safadi-Chamberlain *et al.*, 2005; Jarman *et al.*, 2010). The downstream mechanisms whereby SK1 leads to tumourigenesis, however, are still being elucidated. In attempts to understand these downstream targets of SK1, we have examined genes that are differentially regulated by SK1 using DNA microarray technology. One of the genes identified in this study that was up-regulated by SK1 expression was *TFRC*. Notably, elevated TFR1 (the protein product of *TFRC*) has been widely reported in a variety of human tumours (Habashy *et al.*, 2010; Kukulj *et al.*, 2010; Takahashi *et al.*, 2008; Boult *et al.*, 2008; Singh *et al.*, 2007; Brookes *et al.*, 2006; Hogemann-Savellano *et al.*, 2003; Whitney *et al.*, 1995; Ryschich *et al.*, 2004), high TFR1 expression enhances cell growth and tumour formation in mice (O'Donnell *et al.*, 2006; Habashy *et al.*, 2010), and inhibition of TFR1 by either genetic, pharmacological or antibody-neutralization approaches has been shown to reduce cancer cell proliferation, tumour growth and metastases in mice (Yang *et al.*, 2001; Zhou *et al.*, 2008; Schaar *et al.*, 2009; Peng *et al.*, 2007; Shen *et al.*, 2008; Callens *et al.*, 2008; Jiang *et al.*, 2010). Together, these findings raised the possibility that TFR1 may play a role in SK1-mediated oncogenesis.

4.5.1 SK1 enhances TFR1 expression and subsequent Tf uptake into cells

Previous studies have used constitutive overexpression systems to investigate gene regulation by SK1 (Bu *et al.*, 2006; Yamanaka *et al.*, 2004). These have limitations because high and long-term overexpression of proteins could potentially lead to non-physiologic effects. Here, we have for the first time, examined the immediate effects of moderate (close to physiological) inducible expression of SK1 on gene regulation. One of a number of apparent

SK1-regulated genes identified by this approach was *TFRC*, which we examined in more detail here.

We demonstrated that SK1 expression could upregulate TFR1 mRNA and protein levels via $S1P_2$. The mechanism(s) mediating this upregulation of TFR1 by SK1 and $S1P_2$, however, awaits further examination. Numerous studies have examined the regulation of TFR1 transcription. In most tissues, TFR1 expression is also controlled by iron availability at the post-transcriptional level in a manner resembling feedback inhibition: fewer receptors are expressed when iron is abundant and more receptors are expressed when iron is scarce (Daniels *et al.*, 2006). The regulation of TFR1 expression is post-transcriptionally controlled by means of the well characterized interaction between iron regulatory proteins (IRPs) and the iron-responsive elements in the 3' untranslated regions of iron-related TFR1 mRNAs (Figure 4.14). Interestingly, TFR1 expression is known to be also regulated by the transcription factor AP-1, which appears to be activated by S1P (Takeshita *et al.*, 2000). C-Myc has also been reported to activate TFR1 transcription during tumourigenesis (O'Donnell *et al.*, 2006; Okazaki *et al.*, 2009), and notably, c-Myc has been shown to be downregulated at both the mRNA and protein levels in the mice that lack the *Sphk1* gene (Kohno *et al.*, 2006). Therefore, it is tempting to speculate that SK1/S1P could enhance TFR1 transcription through the activation of one of these transcription factors. TFR1 expression, however, also is up-regulated by other mechanisms, including the hypoxia-inducible factor 1α (HIF1α) (Bianchi *et al.*, 1999), which is typically activated under hypoxic conditions but can also respond to a number of non-hypoxic stimuli, including inflammatory signals such as nitric oxide (NO) and lipopolysaccharide (LPS) (Pantopoulos & Hentze, 1995; Tacchini *et al.*, 2008). Interestingly, SK1 was recently show to enhance HIF1α stability and hence increase its function (Ader *et al.*, 2008; Ader *et al.*, 2009). Notably, this is consistent with another study which demonstrated that S1P increases HIF-1α protein stability through activation of the $S1P_2$ receptor (Michaud *et al.*, 2009). Therefore, it is possible that SK1/S1P increases TFR1 transcription through the accumulation of HIF-1α and its transcriptional activity. To test this hypothesis, it would be interesting to knockdown HIF-1α by RNA interference in cells expressing SK1 and then examine the effect of SK1 on TFR1 regulation via HIF-1α.

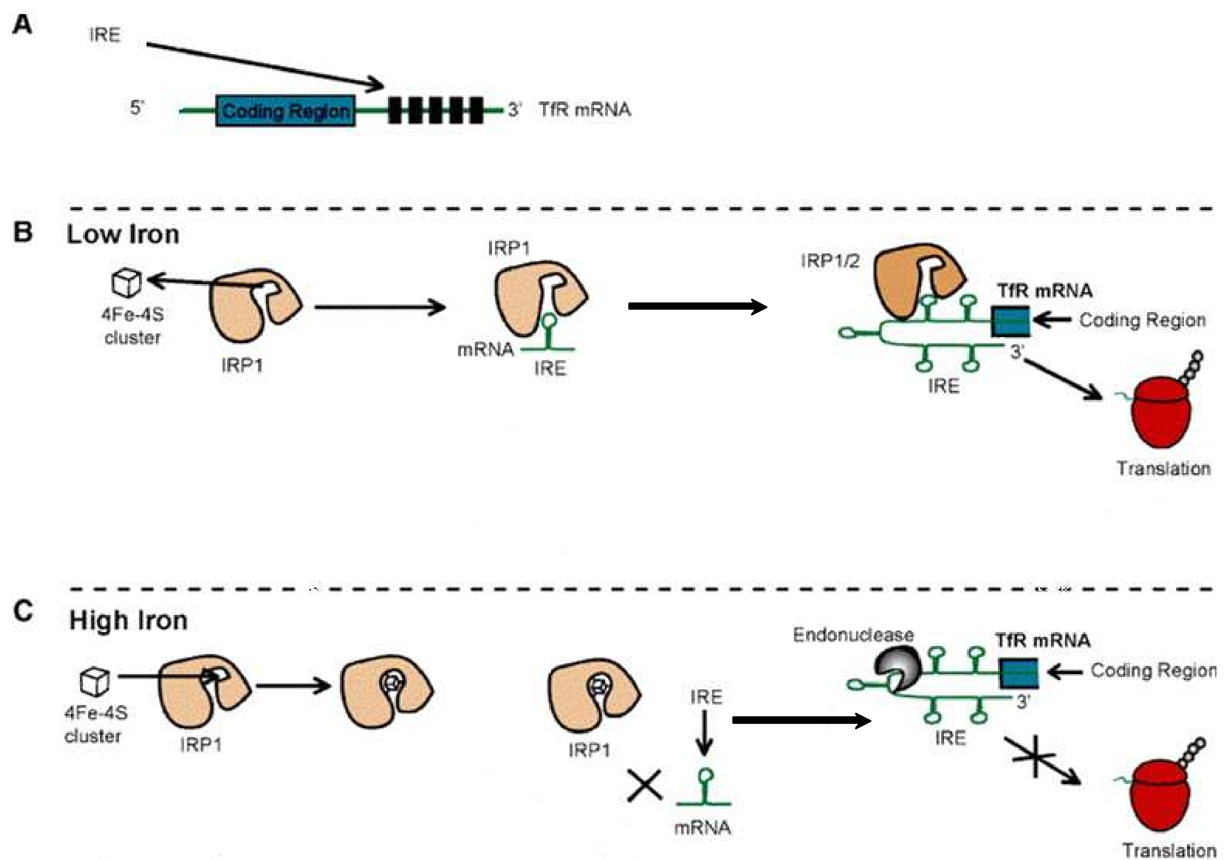

Figure 4.14. Regulation of TFR1 (TfR) expression in response to cellular iron levels. (**A**) Expression of TFR1 is post-transcriptionally regulated by the binding of iron regulatory proteins (IRP) to iron response elements (IRE) in 3′ untranslated regions (3′ UTR) of the TFR1 mRNA. (**B**) Low iron conditions cause the dissociation of 4Fe-4S clusters from IRP1 as well as the de novo synthesis of IRP2. This allows binding of both IRP1 and IRP2 to the multiple IREs in the 3′ UTR of the TFR1 mRNA. Binding of IRPs to IRE sites in the 3′ UTR of TFR1 stabilizes its mRNA and promotes its translation. (**C**) High iron conditions allow the association of 4Fe-4S clusters to cysteine residues within the IRE-binding site of IRP1 as well as the ubiquitination and proteosomal degradation of IRP2. High iron conditions prevent IRP binding to TFR1 mRNA promoting degradation of TfR1 mRNA through endonucleosis of its 3′ UTR [Adapted from (Daniels *et al.*, 2006)].

TFR1 is expressed on the cell surface and is involved in the uptake of iron into cells through the binding and internalization of Tf (Daniels *et al.*, 2006). TFR1 binds Tf, which binds to two ferric ions, and is endocytosed in clathrin-coated pits. It then moves to the sorting endosomes, where the ferric ions are released due to the lower pH of the endosomes, to the endocytic recycling compartment and back to the plasma membrane (Maxfield & McGraw, 2004). Cancer cells are known to express higher TFR1 levels than normal cells (Daniels *et al.*, 2006). Generally, low amounts of TFR1 are detectable on the surface of resting cells, but this level is markedly increased upon antigen (Baumgartner *et al.*, 2005) or mitogen (Davis *et al.*, 1986) stimulation, presumably to support the iron needs of rapidly proliferating cells.

Our findings demonstrate that SK1 not only enhances TFR1 expression, but that this also results in enhanced cell surface presentation of TFR1 and subsequent Tf uptake. At present it is unclear, however, if this enhanced cell surface presentation of TFR1 is simply a result of the overall increase in TFR1 expression levels, or whether it is a result of another level of SK1-mediated signalling through altering TFR1 trafficking. Since TFR1 has to pass sequentially through early endosomes and recycling endosomes before returning to the cell surface (Maxfield & McGraw, 2004), an additional layer of regulation of TFR1 cell surface expression and iron uptake is controlled by components of the vesicular trafficking machinery which appear to be regulated by clathrin/dynamin-coated vesicles and phosphatidylinositol-3 kinase (PI3K) (van Dam *et al.*, 2002). Interestingly, treatment of adipocytes with either insulin or rosiglitazone (a widely prescribed anti-diabetic drug) increases cell surface levels of TFR1 (Davis *et al.*, 1986; Martinez *et al.*, 2010). Intracellular TFR1 is present exclusively in endosomes and is constitutively recycled towards the plasma membrane. Its recycling kinetics, however, are increased upon acute insulin stimulation, which appears to be through activation of class I PI3K activity (Millar *et al.*, 2000). Notably, SK1 has been shown to activate PI3K/Akt pathway (Song *et al.*, 2011; Guan *et al.*, 2011b), and the PI3K activity seems to regulate both the overall amount of observed TFR1 and presentation of the receptor at the cell surface (Kanayasu-Toyoda *et al.*, 2002; Barata *et al.*, 2004; Habashy *et al.*, 2010). Thus, it is possible that the SK1-induced effects on TFR1 may be mediated via PI3K activation which will be further discussed in chapter 5.3.

4.5.2 SK1 activation and plasma membrane localisation are necessary for enhanced TFR1 expression

While the catalytically inactive SK1 (SK1^{G82D}) resulted in downregulation of TFR1 expression, SK1 enhanced TFR1 expression. This is important, since firstly it demonstrates that elevated TFR1 expression is a specific result of increased SK1 catalytic activity in the cell, rather than other potential non-catalytic effects of this protein. Secondly, this finding also suggested that SK1 activation may be important in the process of TFR1 expression since SK1^{G82D} has been previously shown to act like a dominant negative to block activation of endogenous SK1 (Pitson *et al.*, 2000b).

We have previously shown that activation of SK1 through phosphorylation and subsequent translocation from the cytoplasm to the plasma membrane is required for oncogenic signalling of this enzyme (Pitson *et al.*, 2003; Pitson *et al.*, 2005). Notably, here we showed that the non-phosphorylatable SK1 (SK1^{S225A}) which does not induce neoplastic cell transformation (Pitson *et al.*, 2005) does not upregulate TFR1. In contrast, the oncogenic plasma membrane localised non-phosphorylatable SK1 (SK1$^{pm\text{-}S225A}$) did enhance TFR1 expression. This is a clear demonstration that plasma membrane localisation of SK1, which normally occurs after phosphorylation-mediated activation (Pitson *et al.*, 2005), is necessary to regulate TFR1 expression.

4.5.3 S1P$_2$ is necessary for TFR1-mediated SK1 oncogenesis

Upon activation and translocation of SK1 to the plasma membrane, cellular S1P levels increase (Pitson *et al.*, 2003; Pitson *et al.*, 2005; Jarman *et al.*, 2010). This elevated level of cellular S1P regulates a wide range of biological processes mediated through either intracellular targets (Hait *et al.*, 2009; Alvarez *et al.*, 2010) or the S1P G-protein coupled receptors present on the cell surface (Young & Van Brocklyn, 2006; Rosen *et al.*, 2009). Although intracellular S1P has been shown to enhance cell proliferation and survival (Olivera *et al.*, 2003; Illuzzi *et al.*, 2010), there is growing evidence which suggests that extracellular S1P mediated signalling via the S1P-receptors also promotes cell survival and proliferation (Harada *et al.*, 2004; Yoshida *et al.*, 2010b; Yoshida *et al.*, 2010a).

Indeed, we found that SK1-induced TFR1 expression is mediated via the cell surface receptor S1P$_2$. This finding was somewhat surprising since, in contrast to the positive effects of

$S1P_1$ and $S1P_3$ receptors on cell survival and proliferation (Yamada *et al.*, 2004; Kimura *et al.*, 2000; Harada *et al.*, 2004; Osawa *et al.*, 2001; Wang *et al.*, 2010c; Bonnaud *et al.*, 2010; Ushitora *et al.*, 2009), $S1P_2$ has been implicated in decreased cell proliferation and migration in a number of cell types (Ikeda *et al.*, 2003; Goparaju *et al.*, 2005; Lepley *et al.*, 2005; Du *et al.*, 2010; Takashima *et al.*, 2008). Furthermore, approximately half of $S1P_2$ knock-out mice develop B-cell lymphomas at old age (1.5 to 2 years) (Cattoretti *et al.*, 2009), which would point away from the oncogenic role for this receptor. These mice, however, do not develop any other neoplasms. The anti-tumour effect of $S1P_2$ in B-cell lymphomas appears to be due to inhibition of Akt signalling through $G_{12/13}$ and RhoA/ROCK, which prevent germinal centre (GC) B-cell survival and migration towards the follicle centre, necessary for GC cell growth control (Green *et al.*, 2011). In contrast to the above findings, the contribution of $S1P_2$ to cancer is not new. $S1P_2$ mRNA was found to be elevated in Wilms tumour, and furthermore, S1P induced cyclooxygenase-2 (Cox-2) expression in Wilms tumour via $S1P_2$ (Li *et al.*, 2009a). Since Cox-2 has been implicated in regulation of cell growth, apoptosis and the development of several human neoplasms, particularly Wilms tumour (Fridman *et al.*, 2006; Giordano *et al.*, 2008), $S1P_2$ appears to play an oncogenic role in this neoplasm. More recently SK1/S1P was shown to enhance Bcr-Abl1 protein stability via $S1P_2$-mediated increases in Bcr-Abl phosphorylation (Salas *et al.*, 2011). This contributed to resistance to the Abl kinase inhibitor imatinib, in chronic myeloid leukemia (CML). Inhibition of the SK1/$S1P_2$ pathway however, restored PP2A-induced Bcr-Abl1 dephosphorylation leading to enhanced imatinib-induced apoptosis in drug resistant CML cells.

Interestingly, the downstream signalling effects of S1P have been proposed to be largely influenced by differential expression of the S1P receptor subtypes and the downstream effector proteins in specific cell types(Young & Van Brocklyn, 2006) (Young & Van Brocklyn, 2006; Rosen *et al.*, 2009). HEK293 cells used in this study have been reported to express three of the S1P receptor subtypes $S1P_1$, $S1P_2$, and $S1P_3$ (El-Shewy *et al.*, 2006; Meyer zu Heringdorf *et al.*, 2001). The pro-proliferative, pro-survival effects of $S1P_1$ and $S1P_3$ have been shown to be predominantly mediated through G_i proteins (Osinde *et al.*, 2007; Tao *et al.*, 2009). However, $S1P_2$ appears to act primarily through $G_{12/13}$ to inhibit cell proliferation and migration, but can also signal via G_i or G_q pathways [reviewed in (Skoura & Hla, 2009)]. Since signalling through G_i can promote cell proliferation and survival (Bonnaud *et al.*, 2010; Kim *et al.*, 2010; Schuppel

et al., 2008; Harada *et al.*, 2004; Yamada *et al.*, 2004), it is tempting to speculate that elevated SK1 expression in these cells may preferentially enable $S1P_2$ to act predominantly via G_i to elicit TFR1 expression and its subsequent oncogenic effects (Figure 4.15).

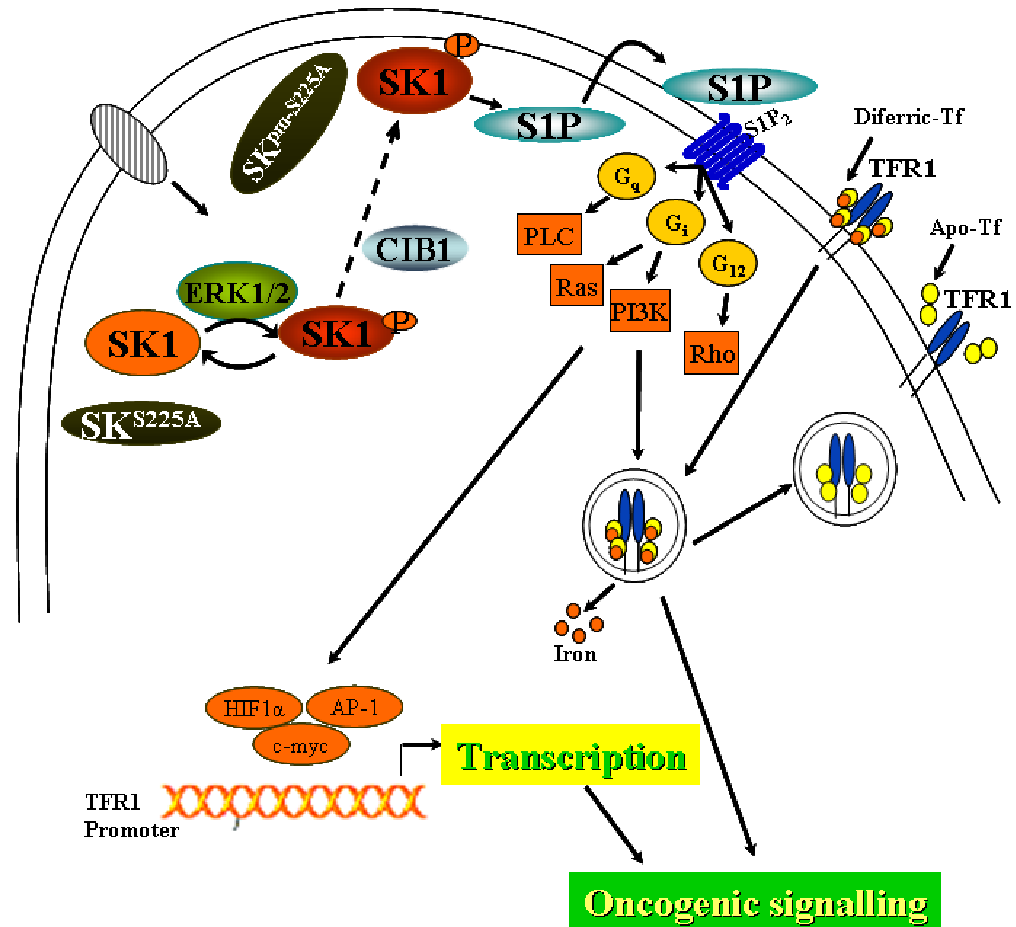

Figure 4.15 Proposed pathways for the regulation of TFR1 by SK1. Elevated cellular levels of SK1, following agonist stimulation, induce phosphorylation-mediated activation/translocation of SK1 to the plasma membrane and the generation of S1P at this location appears to regulate TFR1 expression. SK1/S1P enhanced TFR1 expression and uptake appears to be through engagement of the $S1P_2$ receptor which may be mediated through G_i via activation of PI3K by increasing TFR1 recycling pathways. In addition to this, it is possible that SK1/S1P enhances TFR1 expression by promoting its transcription through activation of transcription factors, such as AP-1, c-Myc and HIF1α.

4.5.4 Conclusions and implications to this study

In summary, we have identified TFR1 as a novel downstream target which appears to be important in oncogenic signalling by SK1. The observation that blocking TFR1 function prevented SK1-mediated cellular proliferation, survival and neoplastic transformation suggests that TFR1 may represent a downstream target for SK1-mediated tumourigenesis. Our results provide new insight into the mechanisms of regulation by which SK1/S1P exerts its oncogenic effects by enhancing TFR1 expression via signalling through the $S1P_2$ receptor.

5. CHAPTER 5: GENERAL DISCUSSION

Elevated levels of SK/S1P have been implicated in a number of different diseases including asthma (Nishiuma *et al.*, 2008; Kono *et al.*, 2010), inflammation (Baker *et al.*, 2010; Lai *et al.*, 2008b), atherosclerosis (Pacheco *et al.*, 2003; Keul *et al.*, 2011), neurodegenerative diseases (Wu *et al.*, 2008a; Nayak *et al.*, 2010) and infection (Puneet *et al.*, 2010). By far the most studied pathological role for SK, however, is in cancer (Pitman & Pitson, 2010; Pyne & Pyne, 2010; Fuereder *et al.*, 2011). Cancer in humans is a multi-step process which is derived from the accumulation of clonal cells that have lost their ability to control normal cell proliferation, survival and homeostasis (Hanahan & Weinberg, 2011). This is in concurrence to the theory of 'acquired capabilities of cancer', which have proposed that; the majority of human cancer is a manifestation of six essential alterations in cell physiology that collectively results in uncontrollable growth (Hanahan & Weinberg, 2011; Hanahan & Weinberg, 2000). Thus, we hypothesize that pathological conditions in particular cancers mediated by SK/S1P are not mediated through a single mechanism but may be via a coordinated number of mechanisms.

5.1 Advantages of using tight-inducible system to study SK cellular functions

Two human SK isoforms exist which appear to have both conserved and divergent cellular functions (Allende *et al.*, 2004; Kharel *et al.*, 2005; Lai *et al.*, 2009; Wadgaonkar *et al.*, 2009; Jo *et al.*, 2009). SK1 appears to almost universally enhance cell proliferation and survival (Pyne & Pyne, 2010; Pitson, 2011). However, the cellular effects of SK2 are more controversial with some studies proposing that SK2 promotes cell apoptosis (Liu *et al.*, 2003; Igarashi *et al.*, 2003a; Maceyka *et al.*, 2005b), and various other studies suggesting that SK2 has pro-survival and pro-proliferative roles (Weigert *et al.*, 2009; French *et al.*, 2010). However, the exact mechanism(s) mediating these distinct and overlapping roles have yet to be fully elucidated and still remains an important question to be answered in the area. Unlike the majority of *in vitro* studies which use constitutive overexpression systems (Liu *et al.*, 2003; Igarashi *et al.*, 2003a; Maceyka *et al.*, 2005b; Pitson *et al.*, 2005), we used tight doxycycline-inducible SK1 and SK2 cell lines in attempts to examine the cellular effects mediated by these two enzymes in a more physiological manner.

Consistent with previous studies, 'high' SK1 overexpression promotes cellular proliferation and survival (Olivera *et al.*, 1999b; Xia *et al.*, 2000; Kohno *et al.*, 2006; Le Scolan *et al.*, 2005; Pitson *et al.*, 2005), but more remarkably is the identification that 'low' levels in SK1 expression could also induce the same cellular effects. Whether these two overexpression levels of SK1 mediated cellular survival and proliferation effects are through the same or distinct mechanism(s), however, still needed to be clarified. Interestingly, we showed that although both 'low' and 'high' levels of SK1 overexpression promote cell survival and proliferation, surprisingly, only 'moderately' enhanced cellular levels of SK1 (but not 'high' overexpression levels) appear to regulate TFR1 expression which is necessary for the observed SK1-induced cellular proliferation, survival and neoplastic transformation. These findings suggest that although different overexpression levels of SK1 in cells result in overlapping cellular effects, its cellular overexpression levels appear to direct SK1 to activate differential downstream signalling pathways. This will be further discussed below in section 5.3.

In contrast to SK1, enhanced expression of SK2 at 'low' and 'high' levels results in differential cellular functions, with 'high' SK2 inducing apoptosis, while 'low' SK2 promotes cell proliferation and survival. Again, clearly, this further supports the idea that the expression levels of the enzyme in the cell appears to be crucial in determining its downstream pathways and potentially different cellular functions. This therefore raises important issue(s) with the use of strong constitutive overexpression systems to study the biological functions of SK2 and further indicates that inducible expression system(s) may be a more appropriate approach to accurately study the cellular effects of this protein.

The localisation of the two SKs appears to be crucial for determining their downstream cellular effects (Pitson *et al.*, 2005; Maceyka *et al.*, 2005b). Since SK1 is present mainly in the cytosol (Kohama *et al.*, 1998; Pitson *et al.*, 2005), it is tempting to speculate that SK1 remains predominantly cytoplasmic whether it is overexpressed at either 'low' or 'high' levels. Here it can bind to the calcium and integrin-binding protein 1 (CIB1) which normally resides in the cytoplasm (Jarman *et al.*, 2010) and thereby facilities active translocation of SK1 to the plasma membrane bringing it in to close proximity to its substrate to generate S1P necessary for the above SK1-mediated pro-proliferative and pro-survival effects. In contrast, SK2 appears to localise to the ER and the nucleus (Igarashi *et al.*, 2003a; Maceyka *et al.*, 2005b; Ding *et al.*, 2007), where it is thought to elicit its pro-apoptotic effects (Liu *et al.*, 2003; Igarashi *et al.*,

2003a; Maceyka *et al.*, 2005b). Therefore, the different overexpression levels of the enzyme in the cells could preferentially direct SK2 to localise to a particular subcellular site to innovate distinct downstream target(s) triggering different cellular effects. The physiological significance of 'low' and 'high' overexpression of the two SK enzymes still requires further clarification. Considerable evidence has suggested that the balance between pro-apoptotic sphingosine, and ceramide, and pro-proliferative, pro-survival S1P is crucial in determining cellular effects (Pyne & Pyne, 2010; Leong & Saba, 2010; Hait *et al.*, 2006). Therefore, future studies should focus on determining the level of these different sphingolipid species in cells produced by 'low' or 'high' expression levels of SK1 and SK2, to tease out how the different expression levels of SK result in similar and differential cellular functions.

5.2 Array studies

In recent years increasing effort has focused on elucidating the oncogenic mechanisms induced by SK1 using different approaches (Pitson, 2011; Pyne & Pyne, 2010). Prior to my studies there was no global gene expression analysis available in the field examining genes regulated by enhanced cellular SK1 expression and in particular its activation/translocation to the plasma membrane which appears to be essential for SK1-mediated oncogenesis. As discussed earlier in the thesis, we found a considerable number of genes that were differentially regulated by enhanced cellular expression of SK1 and SK2. As expected, these genes are involved in a diverse range of cellular functions including, nuclear processes, cell proliferation and survival, cell signalling, cytoskeleton and extracellular matrix and cell metabolism pathways.

Using a tightly inducible expression system allowed us to directly examine genes regulated by subtle increases in SK expression. Surprisingly, however, a large number of these genes appear to be regulated by the SKs independent of their catalytic activity, despite the fact that the SK protein levels were only slightly enhanced over endogenous levels. Interestingly, when we compared our study with previous approaches using constitutive overexpression systems to identify genes regulated by SK1 and or S1P (Xia *et al.*, 2002; Yamanaka *et al.*, 2004; Takeshita *et al.*, 2000; Ozbay *et al.*, 2006; Sun *et al.*, 2010a), our system appears more

physiologically relevant as the system allowed us to examine immediate early genes regulated by ‘low’ SK close to physiological levels. The genes which have been previously reported to be regulated by SK1 and or S1P were mainly identified through high and/or long-term overexpression (Xia *et al.*, 2002; Yamanaka *et al.*, 2004). In light of our findings it is possible some of these may be due to non-catalytic effects resulting from other downstream targets independent of the S1P pathway.

As illustrated earlier in chapter 3, genes encoded for HSPs, particularly the Hsp70 and Hsp90 were most noticeable among the genes which appear to be upregulated by the SK proteins. Hsp70 and Hsp90 are molecular chaperones that can interact with newly synthesized unfolded protein polypeptides preventing premature self-associations in the nascent chain, or retain already matured proteins in an inactive state, respectively, which participate in multi-protein complexes known as ‘chaperosomes’ (Li & Srivastava, 2004; Zhao & Houry, 2005; Lanneau *et al.*, 2010). Notably, Hsp70 has been shown to form a complex with other proteins and can also bind to intracellular lipids (Harada *et al.*, 2007; Smith *et al.*, 2010). The initial clues for a potential role of HSPs in the regulation of sphingolipids came from studies whereby overexpression of Hsp70 appeared to inhibit ceramide-induced apoptosis (Mosser *et al.*, 1997; Ahn *et al.*, 1999; Kondo *et al.*, 2000; Niimura *et al.*, 2010). Consistent with this, our group has recently showed that the chaperonin CCT/TRiC interacts with newly synthesized SK1 and appear to be essential for folding and maturation of the SK1 protein (Zebol *et al.*, 2009). Notably, CCT/TRiC cooperates with numerous other chaperones, including Hsp70 and Hsp90 in its role in folding and re-folding proteins (Siegers *et al.*, 2003; Melville *et al.*, 2003; Cuellar *et al.*, 2008; Gong *et al.*, 2009). Therefore, we hypothesize that at least one or more of these HSPs identified from the arrays is involved in the regulation of SK or the sphingolipid rheostat. Further studies will include examining whether these HSPs interact directly with SK or associate with SK in a complex, using immunoprecipitation assays both *in vitro* and in cells. In addition to this, it may also be useful to examine whether these HSPs are involved in the maturation or stability of SK.

Recently, however, the HSPs, in particular the Hsp90, have emerged as a potential therapeutic target for cancer therapy since it has been implicated to play a crucial role in the folding, maturation and maintenance of a number of oncogenic proteins including Bcr-Abl, mutated p53, ErbB2, Akt, B-Raf and Wilms tumor 1 (Porter *et al.*, 2010; Trepel *et al.*, 2010;

Gimenez Ortiz & Montalar Salcedo, 2010; Banerji, 2009). More strikingly is that inhibition of Hsp90 resulted in ubiquitination and subsequent proteasome-dependent degradation of these oncogenes and its downstream targets significantly reduced cancer cell growth and tumour formation in mice (Cheung *et al.*, 2010; Jang *et al.*, 2010; Karkoulis *et al.*, 2010; Tran *et al.*, 2010; Sun *et al.*, 2010b). Interestingly, it has been reported Hsp90 can be secreted into the extracellular environment by cancer cells and associates with proteins on the plasma membrane (Whitesell & Lindquist, 2005; Lagarrigue *et al.*, 2010), but the mechanism of its recruitment is not understood. Therefore, it is tempting to speculate that Hsp90 might play a role in the folding and stabilization of SK1 to facilitate the interaction of SK1 with other proteins which could be crucial for active translocation and or retention of SK1 to the plasma membrane where it can generate S1P at this location necessary for its oncogenic signalling. Thus, future studies will look at co-localisation of SK1 and Hsp90 at the plasma membrane via immunofluoresence studies. Furthermore, we will also examine the effect of the HSPs on SK-mediated cell proliferation, survival and neoplastic transformation by performing cell proliferation, apoptosis and focus formation assays in cells ectopically expressing SK1 or SK2 in the presence of either Hsp90 inhibitors or siRNA knock-down of Hsp90.

Potentially adding further prospects to our array work is the identification of various genes including, *SFPQ*, *FUS*, *PCGF2*, *RASD1* and *TFRC* that appear to be differentially regulated by SK catalytic activity. Remarkably, however, as discussed earlier in section 3.4.1, we found that like SK, these genes have been implicated in a number of different pathological conditions, such as, asthma, cardiovascular dieases, neurodegenerative diseases and cancers. Taken together, these data suggests that at least one of these gene candidates could possibly play important role(s) in mediating the involvement of the SKs in various disease conditions and future studies will investigate if there are any potential role(s) of some of these genes in SK-mediated diseases.

As noted earlier in chapter 3, it appears that genes encoding for SNORA68, SNORA73A, 5S ribosomal RNA, and Ro small cytoplasmic ribonucleoproteins were upregulated predominantly by active SK1. Interestingly, these genes were mainly associated with either the nucleolus or ribosomes (Boisvert *et al.*, 2007; Montanaro *et al.*, 2008). The nucleolus is the organelle of the interphase cell nucleus where the biogenesis of ribosomes takes place (Hernandez-Verdun, 2006; Montanaro *et al.*, 2008). Increasing evidence has suggested that the

morphological and functional changes in the nucleolus that have been widely observed in cancer tissues are a consequence of both the increased demand for ribosome biosynthesis which is characterized by proliferating cells and the changes in the mechanisms controlling cell proliferation (Boisvert *et al.*, 2007; Montanaro *et al.*, 2008). For example, the c-Myc oncoprotein promotes cell growth and tumourigenesis through controlling the transcription of several nucleolar proteins necessary for ribosome biosynthesis (Schlosser *et al.*, 2003; van Riggelen *et al.*, 2010). Therefore, taken together, it is tempting to hypothesize that overexpression of SK1 upregulates various nucleolar and ribosomal proteins due to the increase in nucleolar function and ribosome biosynthesis necessary for SK1-mediated tumourigenic processes. Although it is thought that changes in the morphology of the nucleolus and ribosomes is a consequence of neoplastic transformation, various evidence exists suggesting that it is changes in the organelles, both quantitatively and qualitatively, that may promote tumourigenesis (Montanaro *et al.*, 2008). Therefore, to clarify this future studies should examine the effect of overexpression of SK1 on the morphology of both the nucleolus and the ribosomes using contrast-phase light microscopy.

Unlike the SK1 arrays, only a few genes appeared to be specifically upregulated by SK2 activity. Interestingly, some of these genes, including *WDR77* and *BRPF3* have been reported to be involved in nuclear functions including transcriptional regulation and histone modifications (Furuno *et al.*, 2006; Slama & Geman, 2010; Ullah *et al.*, 2008). Notably, a recent report showed that SK2 and its product, S1P, are found in association with HDACs and inhibit their activity (Hait *et al.*, 2009). These findings further support the notion that SK2 and or S1P appear to play a role in epigenetic regulation, possibly through upregulation of various nuclear proteins which may form part of a large multiprotein complex important for initiation of transcription, DNA replication and DNA repair. Therefore, alteration in the expression of various nuclear proteins by SK2 and or S1P could involve methylation of specific genes which could contribute to pathological conditions such as cancer and neurodegenerative diseases.

Although we failed to identify any miRNA with significant differential regulation by SK1 expression, it is worth noting that from the DNA microarrays we have identified various small nucleolar RNAs, including the *SNORA68*, *SNORA73A* that appear to be upregulated by active SK1. Small nucleolar RNAs and miRNAs are two classes of small non-coding regulatory RNAs (Brameier *et al.*, 2011). Interestingly, recent studies have suggested that various

miRNAs are evolved from snoRNAs, and thus it is believed that they may share the same biogenesis and processing machinery (Brameier *et al.*, 2011). Although they have different cellular functions, members from these two types of small RNAs display numerous genomic similarities and a small number of snoRNAs have been shown to encode miRNAs in several organisms (Scott *et al.*, 2009; Ono *et al.*, 2011). Notably, FUS and RASD1 discussed earlier appear to play a role in miRNA synthesis and/or regulation (de Souza Rocha Simonini *et al.*, 2010; Buratti & Baralle, 2010). This includes miR-375, that appears to have a pro-proliferative role in ERα-positive breast cancer cells by targeting RASD1 (de Souza Rocha Simonini *et al.*, 2010) and FUS was recently found in the large Drosha microprocessor complex and hence has been proposed to play a role the primary processing of miRNA (Buratti & Baralle, 2010). Taken together, we postulate that miRNA(s) may be likely to be differentially regulated by SK1 and possibly by SK2. As discussed earlier, there were various factors which may have contributed to our unsuccessful attempt to examine miRNAs regulation by SK1, such as not performing arrays with optimized conditions and/or the limited 400-miRNA library used which could possibly result in potential regulated miRNAs being missed. Thus, future experiments will be to examine miRNA(s) regulated by both SK1 and SK2 using optimized conditions together with the most current miRNA library available.

5.3 Transferrin receptor 1 (TFR1)

There is clear evidence supporting a role of SK1 in tumourigenesis (Paugh *et al.*, 2008; Kawamori *et al.*, 2009; Facchinetti *et al.*, 2010; Malavaud *et al.*, 2010; Liu *et al.*, 2010; Sinha *et al.*, 2011), however, the mechanism through which SK1 drives tumourigenesis is still not fully understood. In agreement with the literature (Xia *et al.*, 2000; Sukocheva *et al.*, 2003; Pitson *et al.*, 2005), our study showed that SK1 appears to be universally mitogenic since both ‘moderate’ or ‘high’ overexpression levels of SK1 promote cell proliferation and survival. As discussed earlier in the thesis (section 1.5.1), it has been difficult to differentiate between the pro-survival, pro-proliferative cellular effects mediated by either engagement of S1P receptors or intracellular signalling since both pathways appear to elicit similar effects. Strikingly, our results demonstrated that only ‘moderate’ levels of SK1 overexpression appear to upregulate

TFR1 expression. Therefore, it is possible that the different overexpression levels of SK1 in the cell are what determines whether it activates pathways involving either intracellular or extracellular signalling to enhance cell survival and proliferation. Presumably, the pro-proliferative and pro-survival effect induced by 'moderate' levels of SK1 expression is mediated through S1P cell-surface receptors since only 'moderately' enhanced cellular levels of SK1 (but not 'high' overexpression levels) appear to regulate TFR1 expression through the $S1P_2$ receptor. 'High' level overexpression of SK1 therefore appears to enhance cell proliferation and survival through a distinct mechanism(s), possibly via intracellular targets.

As noted above, TFR1 is a membrane protein that mediates iron uptake through endocytosis (Daniels *et al.*, 2006; Macedo & de Sousa, 2008; Wang & Pantopoulos, 2011). However, receptor-induced endocytosis is mediated by two different mechanisms; clathrin-dependent and clathrin-independent (Le Roy & Wrana, 2005). TFR1 is internalised by clathrin-dependent endocytosis which is dependent on the formation of clathrin-coated pits, whereas, clathrin-independent endocytosis mainly involves lipid rafts (Le Roy & Wrana, 2005). Interestingly, recent findings have shown that phosphorylated SK1 translocates to lipid rafts and this appears to be essential for the enzyme induced mitogenic effects (Hengst *et al.*, 2009). In line with this, studies have suggested that $SK1^{pm\text{-}S225A}$; containing the *N*-terminal ten amino acids of the Lck tyrosine kinase that results in myristoylation and dual palmitoylation drives the protein to lipid rafts, facilitating pro-survival, pro-proliferative and oncogenic signalling, while $SK1^{c\text{-}Src}$; containing a single myristoylation site that would likely drive SK1 to a membrane microdomain distinct from lipid rafts, inhibits cell proliferation but can still protect the cell against serum deprivation-induced apoptosis (Pitson *et al.*, 2005; Brown, 2006; Safadi-Chamberlain *et al.*, 2005). Together, these findings suggest that targeting SK1 to different compartments of the plasma membrane may result in different cellular effects. However, since clathrin-coated pits and lipid-rafts are localised in proximity (Frankel *et al.*, 2006), it is not clear whether these effects involve clathrin or lipid rafts due to the lack of tools to clearly separate these two membrane microdomains. Therefore, to further clarify this, future experiments will be to examine whether $SK1^{c\text{-}Src}$ (SK1 with Src N-terminal attached) could enhance TFR1 expression.

Ser225 phosphorylation-induced translocation of SK1 to the plasma membrane is essential for the enzyme to mediate oncogenic signalling (Pitson *et al.*, 2005). As

phosphorylated SK1 appears to be retained at the plasma membrane by binding to phosphatidylserine and other plasma membrane-associated lipids (Delon *et al.*, 2004; Stahelin *et al.*, 2005), it could be postulated that different levels of overexpression of SK1 in cells produces different amounts of phosphorylated SK1 that could target the enzyme to different locations on the plasma membrane. Therefore, although both 'moderate' and 'high' overexpression levels of SK1 lead to mitogenic effects, they could activate distinct downstream pathways. Future studies will be to examine the localisation of 'moderate' and 'high' overexpression of SK1, $SK1^{S225A}$ and $SK1^{pm\text{-}S225A}$ in membrane microdomains by using electron microscopy and fluorescence resonance energy transfer (FRET) (Loura *et al.*, 2009; Loura *et al.*, 2010). Interestingly, like wild-type SK1, 'moderate' overexpression of $SK1^{pm\text{-}S225A}$ also appeared to upregulate TFR1 which again supports the notion that plasma membrane-localised SK1 is essential for the oncogenic effect mediated through enhanced TFR1 expression (Pitson *et al.*, 2005). Thus, future studies will be to tease out whether SK1 activation-induced-generation of S1P at the plasma membrane or localized production of S1P at the plasma membrane site alone is required for enhanced TFR1 expression. This will be carried out by looking at the localisation of phosphorylated SK1 and $SK1^{pm\text{-}S225A}$ to examine whether the two co-localise to similar or distinct membrane microdomains.

The exact mechanism(s) regulating TFR1 expression by SK1/ $S1P_2$ still remain to be clarified, we proposed earlier in Chapter 4 that SK1 enhanced TFR1 expression may not be due to a single mechanism, but possibly through various mechanisms involving both transcription and the endosomal recycling pathways. Therefore, future studies should examine whether SK1/S1P increases TFR1 transcription through activation of transcription factors, such as AP-1, c-Myc and HIF1α, employing genetic or pharmacological approaches to inhibit the above transcription factors in cells expressing SK1.

As discussed earlier, intracellular TFR1 is present in endosomes and is recycled back to the plasma membrane through recycling endosomes (Maxfield & McGraw, 2004). The molecular mechanism of the TFR1 recycling pathway has already been widely investigated, and several members of the small GTPase Rab proteins have been shown to regulate distinct steps in the TFR1 recycling pathway (Vanlandingham & Ceresa, 2009; Hirota *et al.*, 2007; McCaffrey *et al.*, 2001; Mohrmann & van der Sluijs, 1999; Matsui *et al.*, 2011). Thus, it will be of interest to examine whether members of Rab-type GTPases are involved in SK1 enhanced

TFR1 expression, and if so, which specific Rab is involved. Furthermore, the recycling of TFR1 appears to be dependent on PI3K activation (Millar *et al.*, 2000). Therefore, in future studies, to examine whether SK1 regulates TFR1 by acting through the PI3K pathway, cells induced to express SK1 will be treated with LY294002 (a specific PI3K inhibitor) and the subsequent effects of SK1 on TFR1 expression examined.

Because coupling of $S1P_2$ to G_i proteins appears to enhance cell proliferation and survival (Bonnaud *et al.*, 2010; Kim *et al.*, 2010; Schuppel *et al.*, 2008; Harada *et al.*, 2004; Yamada *et al.*, 2004), it would be necessary to determine whether $S1P_2$-induced TFR1 expression is mediated through G_i proteins. This will be preformed by examining the effect of TFR1 on cells that have been simultaneously induced with SK1 and treated with pertussis toxin (PTx) to block G_i activation (Sunyer *et al.*, 1989).

5.4 Conclusions & future work

Overall, we have established a tight and reliable inducible system which allows us to examine the cellular functions of SK under more 'physiological' conditions than many other constitutive overexpression systems previously used in the past. Using this system enabled us to perform DNA microarray-based approaches to identify: (i) genes regulated by active SK1 and/or SK2; (ii) genes regulated specifically by SK1 activation/translocation important in SK1-mediated oncogenesis under more controlled conditions. From the microarrays, we identified various gene candidates that were either differentially regulated by SK1 or SK2 or both, suggesting that these two enzymes could target similar and/or distinct downstream target(s). Since some of these genes have been reported to be involved in various disease conditions, such as cancers, asthma and neurodegenerative diseases, we hypothesize that under 'physiological' conditions, SKs could play important role(s) in various pathological conditions through regulating at least some of these downstream target(s). Thus, functional studies to investigate the potential mechanism of potential gene candidates in these SK-mediated pathological conditions should be performed in the future.

Overexpression of SK1 at either 'moderate' or 'high' levels promotes cell proliferation and survival. Notably, only 'moderate' SK1 levels appear to regulate TFR1 expression, despite

the fact that both ‘moderate’ and ‘high’ overexpression levels mediate similar cellular effects. This suggests that there are at least two distinct mechanisms responsible for mediating SK1-enhanced cell proliferation, survival and tumourigenesis which is consistent with the literature that cancer is a disease of multiple processes and SK1 appears to play crucial roles in more than one of these tumourigenic processes. Future studies will focus on further clarifying these pathways involved in SK1-induced tumourigenesis.

Although significant progress has been made in the past few years towards understanding the molecular mechanisms controlling the two SK isoforms activation and subcellular localisation, our current studies have added further insights into the molecular mechanisms whereby SK is involved in gene transcription. Investigation into these genes regulated by SK appears to be important in order to gain a better understanding of the regulation of diseases mediated by SK. These insights will contribute to the identification and development of better and more specific therapeutics which are necessary for the diagnosis and treatment of SK-mediated diseases, such as cancers.

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APPENDIX 1

Sphingosine kinases: biochemistry, regulation and role

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Tamara M. Leclercq : Wrote aspects of the article.

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Duyen H. Pham (Candidate) : Wrote aspects of the article, specifically those relating to regulation of sphingosine kinase gene expression.

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Signed.... ....*Date*...4/5/12........

Stuart M. Pitson : Wrote aspects of the article, and compiled and edited contributions from the other authors.

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APPENDIX 2

Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3' AU-rich mRNA destabilizing elements

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Statement of Authorship

Duyen H. Pham, Paul A.B. Moretti, Gregory J. Goodall, and Stuart M. Pitson (2008) Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3' AU-rich mRNA destabilizing elements. *Biotechniques* 45, 155-160.

Duyen H. Pham (Candidate) : Designed and performed all of the experiments, interpreted most data and wrote the draft manuscript.

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Paul A.B. Moretti : Assisted in the molecular cloning to generate DNA constructs required for experiments.

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Gregory J. Goodall : Aided in data interpretation and preparation of the manuscript.

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Stuart M. Pitson : Supervised work, helped design experiments, helped with data analysis, and performed editing of the draft manuscript.

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Signed……… ……………………………………*Date*… 1/6/12……

Pham, D.H., Moretti, P.A.B., Goodall, G.J. & Pitson, S.M. (2008) Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3' AU-rich mRNA destabilizing elements.
Biotechniques, v. 45(2), pp. 155-160

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