SIK3 INVOLVEMENT IN FGF2 SIGNALING IN MÜLLER CELLS

by

Yıldız Koca

B.S., Molecular Biology and Genetics, Bilkent University, 2010

Submitted to the Institute for Graduate Studies in

Science and Engineering in partial fulfillment of

the requirements for the degree of

Master of Science

Graduate Program in Molecular Biology and Genetics

Boğaziçi University

2013

To my family

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my thesis supervisor Prof. Kuyaş Buğra Bilge for her guidance, continuous support and encouragement throughout my studies.

I also would like to thank Prof. Esra Battaloğlu and Assoc. Prof. Ferruh Özcan for devoting their valuable time to evaluate my thesis.

I would like to express my special thanks to my lab members Gamze Küser, Funda Ejder, Neslihan Zöhrap, Aslı Uğurlu, Yeliz Yılmaz Sert and Özden Akay for helping me whenever I needed and for being great friends in and out of the lab. I also would like to thank our recently joined lab members Emirhan Taşöz and Burcu Ersoy. Many thanks go to the members of RECEP: Duygu Dağlıkoca, Burçak Özeş, Merve Sıvacı, Mahmut Can Hız, Balkan Canher and Harun Niron.

I cannot thank Alperen Erdoğan enough for being such an amazing friend and for standing right next to me unconditionally. I sincerely thank Kaya Akyüz for his indispensable friendship, constant help and support. My heartfelt thanks go to Selen Zülbahar for accompanying me throughout this journey with her warm and generous friendship. I also thank my beloved friends Merve Kılınç and Kerem Yıldırım for their continuous support.

I would also like to thank Emine Dindar, Ulduz Sobhi Afshar, Gönenç Çobanoğlu, Zeynep Özcan, Yetiş Gültekin, İpek Even and all other friends from the department for their encouragement.

I'm grateful to TÜBİTAK-BIDEB 2210 for supporting and awarding me during my master studies.

Last but not the least, I would like to express my infinite thanks to my family for their endless love and support in every moment of my life.

ABSTRACT

SIK3 INVOLVEMENT IN FGF2 SIGNALING IN MÜLLER CELLS

FGF2 stimulation of Müller cells regulates several processes including cell proliferation and survival by triggering the activation Ras/MAPK and PI3K/Akt pathways. Data from our lab indicate that serine/threonine kinase SIK2 regulates FGF2 signaling pathway. Regarding the high level of sequence and functional similarity between SIK family proteins, we aimed to investigate the possibility of SIK3, which is also a serine/threonine kinase, being involved in FGF2 signaling in Müller cells. To understand if SIK3 is involved in FGF2 signaling pathway, we examined FGF2-dependent changes in the phosphorylation status and expression pattern of SIK3 in Müller cells. Our results showed that SIK3 undergoes a transient increase in total serine phosphorylation levels, peaking in 10 mins of FGF2 stimulation, whereas total threonine phosphorylation levels show a transient decrease in 5 mins of FGF2 stimulation and then recover back to its basal levels. We observed no tyrosine phosphorylation at any point of FGF2 stimulation. SIK3 protein expression, on the other hand, significantly increased in 10 mins of FGF2 stimulation and decreased back to initial levels. These results suggest that SIK3 may have a regulatory role in FGF2 signaling pathway in Müller cells. Based on the presence of ERK phosphorylation motif on SIK3 and paralel profiles of FGF2-dependent ERK and SIK2 activation, in the second part, we explored the possibility of ERK being an upstream regulatory kinase of SIK3 in the context of FGF2 signaling in Müller cells. Our data revealed that the inhibition of ERK activity prior to FGF2 treatment results in reduced serine phosphorylation of SIK3, whereas threonine phosphorylation was enhanced compared to the controls. These results are consistent with the hypothesis that ERK regulates SIK3 phosphorylation status in Müller cells in an FGF2-dependent manner. Whether ERK directly or indirectly regulates SIK3 phosphorylation remains to be elucidated.

ÖZET

SIK3'ÜN MÜLLER HÜCRELERİNDE FGF2 SİNYAL İLETİMİNE KATILIMI

Retinal Müller hücrelerinin FGF2 tarafından stimülasyonu hücre proliferasyonu ve sağkalımı benzeri süreçleri Ras/MAPK ve PI3K/Akt yolaklarini aktifleştirerek düzenler. Bu yolakların temel düzenlenme yöntemleri arasında yolak elemanlarının fosforilasyon/defosforilasyon yoluyla post-translasyonel olarak modifikasyonu yer alır. Laboratuvarımızda elde edilen veriler bir serin/threonin kinaz olan SIK2'nin FGF2'ye bağlı bu düzenleyici sistemde rol aldığını önermektedir. SIK aile üyeleri arasında bulunan yüksek derecedeki dizin ve işlev benzerliğini göz önünde bulundurarak, bu çalışmada bir diğer serin/threonin kinaz olan SIK3'ün Müller hücrelerinde FGF2 sinyal iletimine katılım olasılığının irdelenmesi amaçlanmıştır. Bu çerçevede SIK3 proteinin fosforilasyon statüsünde ve anlatım profilinde FGF2'ye bağlı olarak meydana gelen değişimler Müller hücrelerinde irdelenmiştir. Verilerimiz FGF2 stimülasyonu ile SIK3'ün total serin fosforlanma düzeyinde 10. dakikada maksimuma ulaşan geçici bir artış olduğunu göstermektedir. Aynı koşullarda total threonin fosforlanmasında 5 dakika içinde düşüş gözlenmiş, tirozin fosforlanmasında ise bir değişiklik saptanmamıştır. Diğer yandan, SIK3'ün hücresel ifade seviyelerinde FGF2'ye bağlı olarak 10. dakikada maksimuma ulaşan geçici bir artış gözlendi. Bu sonuçlar Müller hücrelerinde SIK3'ün FGF2 sinyal yolağında düzenleyici bir rolü olabileceğini önermektedir. SIK3'ün ERK fosforlanma motifini içermesi ve bu iki kinazın FGF2'ye bağlı aktivasyon profillerindeki benzerlikten yola çıkarak çalışmanın ikinci bölümünde, Müller hücrelerinde ERK'in bu yolak bağlamında SIK3'ün bir üst kinazı olma olasılığını değerlendirdik. Verilerimiz FGF2 stimülasyonu öncesinde ERK aktivitesinin inhibe edilmesi ile SIK3 serin fosforlanma düzeylerinin düştüğünü, SIK3 treonin fosforlanmasında ise artış olduğunu göstermiştir. Bu sonuçlar Müller hücrelerinde ERK'in SIK3'ün fosforlanma statüsünü FGF2'ye bağlı olarak düzenlediğini önermektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS/ABBREVIATIONS

ACTH	Adenocorticotropic Hormone
AMPK	AMP-activated Kinase
APS	Amonium Persulfate
ATP	Adenosine Triphosphate
bp	Base Pair
BSA	Bovine Serum Albumine
cAMP	Cyclic Adenosine 5'-monophosphate
CRE	cAMP-responsive Element
CREB	CRE Binding Protein
CO_2	Carbondioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular Regulated Kinase
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FBS	Fetal Bovine Serum
Frs2	Fibroblast Growth Factor Receptor Substrate

g	Gram
GAB1	Grb2-associated Binder 1
GFP	Green Fluorescent Protein
Grb2	Growth Factor Receptor-bound Protein
H_2O	Water
HRP	Horseradish Peroxidase
HSPG	Heparan Sulfate Proteoglycans
IgG	Immunoglobulin G
IP	Immunoprecipitation
IRS	Insulin Receptor Substrate
kb	Kilobase
kDa	Kilodalton
L	Liter
LKB1	Liver Kinase B 1
MAPK	Mitogen-activated Protein Kinase
MIO-M1	Moorfields/Institute of Opthalmology-Müller 1
mg	Miligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Mililiter
mM	Milimolar
NaCl	Sodium Chloride

Nm	Nanometer
ng	Nanogram
PAGE	PolyAcylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
Pen/Strep	Penicillin/Streptomycin
PH	Plekstrin Homology
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase C-gamma
PTB	Phosphotyrosine Binding
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic Acid
rpm	Rotations Per Minute
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dedocyl Sulfate
sec	Seconds
Ser	Serine
SH	Src Homology Domain
SHP2	SH2-domain Containing Phosphatase 2
SIK3	Salt Inducible Kinase 3

SNF	Sucrose Nonfermenting
SNH	Sucrose Nonfermenting Homology
Sos	Son of Sevenless
Srebp	Sterol Regulatory Element Binding Proteins
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TEMED	Tetramethylethylenediamine
Thr	Threonine
TORC2	Transducer of Regulated CREB Activity
TWEEN	Polysorbate
WB	Western Blot
x g	Times Gravity

1. INTRODUCTION

1.1. The Retina

The retina, a complex neural circuit, is located at the back of the eye and it functions in the transduction of light to the brain as a pattern of electrical impulses. The neural retina has a multi-layered structure and it comprises six classes of neurons, including two types of light sensitive photoreceptor cells; cones and rods in mammalian species. Processing of photoreceptor signals are conducted by three types of interneurons which are horizontal cells, bipolar cells and amacrine cells. In addition, in the mammalian retina, three types of glial cells are found, these are microglial cells, astrocytes and Müller cells (Wassle *et al.*, 1991; Bringmann *et al.,* 2006).

1.2. Müller Cells of The Retina

Müller cells, the principal macroglial cells of the vetebrate retina, are specialized radial glial cells which span the entire thickness of the retina and contact all retinal neuronal somata and processes. Müller cells express a variety of ion channels, ligand receptors, transmembraneous transporter molecules, and enzymes giving them a broad range of functions (Bringmann *et al.,* 2006). During retinal development, they play an essential role in creating and maintaining the neuroretinal architecture and they support neuronal survival and information processing (Bringmann *et al.,* 2006). They function in retinal glucose metabolism, providing retinal neurons with nutrients such as lactate/pyruvate for their oxidative metabolism and removing metabolic waste products (Bringmann *et al.,* 2006). They modulate the retinal blood flow and contribute to the formation and maintenance of the blood-retinal barrier (Bringmann *et al.,* 2006). They also have role in the neuronal signaling processes, they perform rapid uptake and recycling of neurotransmitters and provide precursors of neurotransmitters to neurons (Bringmann *et al.,* 2006). Besides, they maintain the ion and water homeostasis of the retinal tissue and involved in uptake and recycling of neurotransmitters (Bringmann *et al.,* 2006).

1.3. Fibroblast Growth Factors

Fibroblast growth factors (FGFs) comprise a large family of polypeptide growth factors. In vertebrates, 22 members of the FGF family were identified and they range in molecular mass from 17 to 34 kDa (Ornitz *et al.*, 2001). Among vertebrate species, FGFs are well conserved in terms of gene structure and amino-acid sequence (Eriksson *et al.*, 1991; Plotnikov *et al.*, 1999; Zhu X *et al.*, 1991). FGFs are known to have a high affinity for heparan sulfate proteoglycans and upon binding to heparan sulfate they activate one of four cell surface FGF receptors. During embryonic development, FGFs play various roles in regulating cell proliferation, migration and differentiation. In the adult organism, FGFs act as homeostatic factors and function in tissue repair and response to injury (Ornitz *et al.*, 2001).

Most FGF family members contain an amino-terminal signal peptide, thus they are secreted by a classical pathway. FGF1, 2, 9 and 11-14 lack signal peptide and they are secreted by an alternative pathway, these ones also have nuclear localization motif (Powers *et al.*, 2000). Spatial and temporal expression of FGF family members varies in different tissues. Some FGFs (FGF3, 4, 8, 15, 17, and 19) are expressed only during embryonic development and others (FGF1, 2, 5-7, 9-14, 16, 18, and 20-23) are expressed in both embryonic and adult tissues (Ornitz *et al.*, 2001).

Many FGF family members are expressed in the developing eye and in the mature retina of vertebrates. FGF1 shows a high level of expression in the peripheral retina during the initial stages of chick retina development (McCabe *et al.*, 1999). FGF3, FGF8, and FGF17 expression was detected during eye development of various vertebrate models (Crossley and Martin, 1995; Reifers *et al.*, 1998; Vogel-Hopker *et al.*, 2000; Walshe and Mason, 2003). FGF2 regulates neural differentiation in chick embryonic retina (Pittack *et al.*, 1997). FGF19 expression in embryonic human retina was also identified (Xie *et al.*, 1999). It was demonstrated that FGF1, FGF2 and FGF9 are expressed in the neuronal cells of postnatal and adult retina (Buğra *et al.*, 1993; Buğra and Hicks, 1997; Çınaroğlu *et al.*, 2005). In mature chick retina, FGF2 signal leads to the proliferation and subsequent transdifferentiation of Müller glia into neurons by activating MAPK (Fischer *et al.*, 2002;

Fischer *et al.*, 2009). FGF1, FGF2 and FGF9 lead to the proliferation of Müller cells *in vitro* (Cinaroglu *et al.*, 2005).

1.4. Fibroblast Growth Factor Receptors

FGFs exert their cellular effects upon binding and activating fibroblast growth factor receptors (FGFRs), which belong to the superfamily of receptor tyrosine kinases (RTKs) (Acevedo *et al.*, 2009). Heparan sulfate proteoglycans (HSPGs) assist FGFs in binding and activating FGFRs (Kan *et al.*, 1999).

FGFRs have three immunoglobulin like domains (D1-D3) in their extracellular region, a single pass transmembrane domain, a cytoplasmic domain with tyrosine kinase activity and an "acid box" which is a contiguous stretch of acidic residues between D1 and D2. The D2-D3 fragment of FGFR ectodomain is involved in ligand binding and specificity, whereas D1 domain and acid box are involved in autoinhibition (Mohammadi *et al.*, 2005). FGFR family members, FGFR1-4, share 55% to 72% similarity in amino acid sequence (Johnson and Williams, 1993). Several isoforms of FGFRs arise from numerous alternative splicing events taking place in the extracellular and intracellular regions of the receptors (Mohammadi *et al.*, 2005). These isoforms have different ligand binding affinities and they trigger differential responses in the cell (Powers *et al.*, 2000).

All of the FGFR isoforms are expressed in the retina of vertebrates (Kinkl *et al.*, 2002). In Müller cells, FGFR1, FGFR2 and FGFR3 expression has been detected (Cınaroglu *et al.*, 2005).

1.5. FGF/FGFR Signal Transduction Pathways

Receptor dimerization following FGF ligand binding in the presence of HSPGs, leads to the autophosphorylation of several tyrosine residues in the cytoplasmic domain of FGFRs. This generates docking sites for the downstream proteins with Src homology 2 (SH2), Src homology 3 (SH3) and phosphotyrosine binding (PTB) domains such as FRS2, Shp2, and PLCγ (Pawson, 1995). Although the exact nature of signaling may differ

depending on the cell context via recruitment of different signaling elements, signaling through FGFRs leads to the activation of three main pathways (Figıre 1.1): the phospholipase C-gamma (PLCγ)/Ca2+ pathway, the Ras/Mitogen-activated protein kinase (Ras/MAPK) pathway and the phosphoinositide 3-kinase (PI3 kinase)/Akt pathway (Mason, 2007).

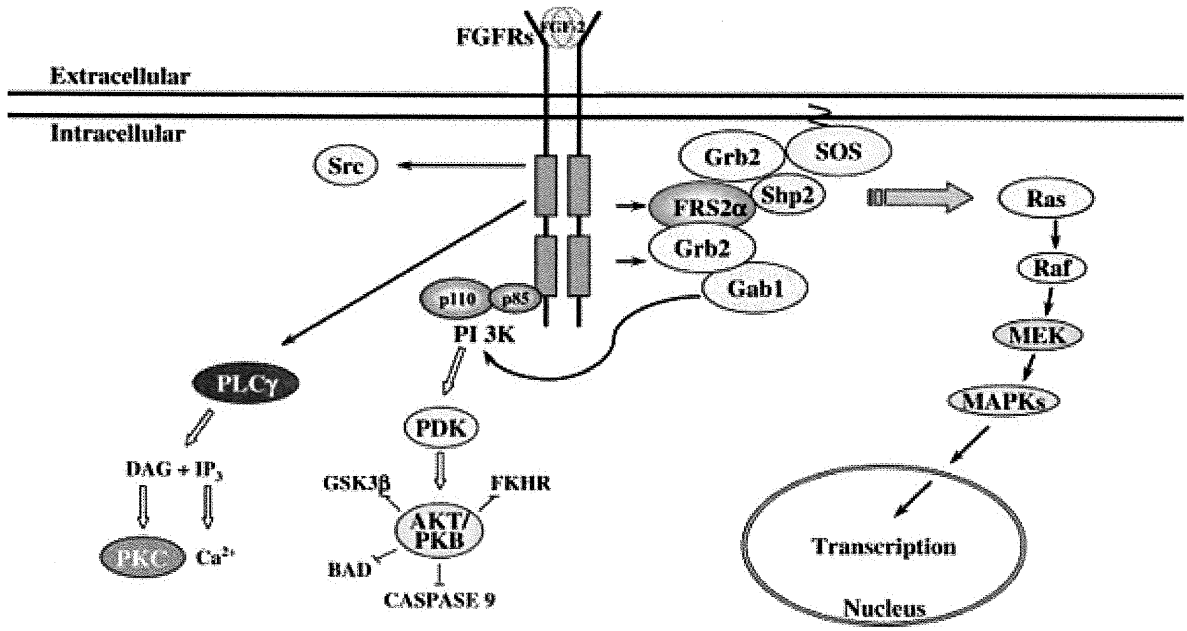


Figure 1.1. Three main pathways activated by signaling through FGFRs. They include (PLCγ)/Ca^{2+} pathway, Ras/MAPK and PI3 kinase/Akt pathway (Lee *et al.*, 2006).

1.5.1. PLCγ/Ca^{2+} Pathway

PLCγ, which is a substrate of all FGFRs, is recruited to autophosphorylated Tyr-766 of FGFR1 through the interaction of its SH2 domain with the receptor. Once activated, PLCγ hydrolyzes phosphotidylinositol-4,5-diphosphate (PIP2) yielding two secondary messengers: inositol-1,4,5-triphosphate and diacylglycerol (DAG). Subsequently, DAG leads to the activation of protein kinase C (PKC) and inositol-1,4,5-triphosphate triggers Ca^{2+} release from intracellular compartments (Mohammadi *et al.*, 1991). PKC, activated by DAG, alters the phosphorylation status of Raf and causes Ras/MAPK pathway activation (Huang *et. al.*, 1995).

1.5.2. Ras/MAPK Pathway

FGFs modulate proliferation and differentiation responses mainly through Ras/ERK pathway. FRS2 is thought to be regulating the pathway by interacting with the juxtamembrane region of FGFRs. Phosphorylated on several tyrosine residues, FRS2 creates docking sites for the Grb2-Sos complex and for Shp2 binding (Kouhara et al., 1997). Once recruited to the membrane, Sos, which is a guanine nucleotide exchange factor, activates small G-protein Ras by facilitating GDP/GTP exchange. Ras, in turn, recruits a serine/threonine kinase, Raf, to its close proximity and phosphorylates it on several tyrosine residues. Active Raf phosphorylates MEK on two serine residues, which then becomes active and phosphorylates MAPK, extracellular signal-regulated kinase (ERK), on tyrosine and threonine residues. Activated ERK, in turn, phosphorylates a number of downstream proteins including various kinases and transcription factors (Yang *et al.*, 2004). On the other hand, FGF stimulation triggers Shp2 phosphorylation, which allows its interaction with Grb2 (Powers *et al.*, 2000). Cellular responses to FGF can be influenced by the duration of MAPK activation. When MAPKs are activated transiently, cell proliferation is promoted whereas rolonged activation of MAPKs culminates in cell differentiation (Yamada and Yoshimura, 2002).

1.5.3. PI3K/ AKT Pathway

This branch of FGF signaling is involved in cell survival (Katoh, 2009). Once FGFR becomes activated, PI3 kinase is recruited to the membrane via the interaction of its SH2 domain with phosphotyrosine residues on Grb2-FRS2-Gab1 complex (Ong *et al.*, 2001). Subsequently, Akt is brought to the membrane via its PH domain, activated by phosphorylation by PI3 kinase. Alternatively, Akt may be phosphorylated on its T308 and S473 residues by PDK1/PRK-2 complex (Hadari *et al.*, 2001). Once becomes activated, Akt is released to the cytoplasm and negatively regulates the activity of pro-apoptotic proteins, Bad and Caspase 9. Besides, Akt phosporylates the forkhead transcription factors which then interacts with 14-3-3 proteins and are sequestered in the cytoplasm. These processes promote cell survival (Schlessinger, 2000).

1.6. Salt Inducible Kinase Family

Salt inducible kinase (SIK) family comprises three serine/threonine kinases (Figure 1.2). SIK1, the first member of the family, was initially cloned from the adrenal glands of high-salt fed rats, and this paved the way for subsequent cloning of the other members, SIK2 and SIK3 (Katoh *et al.*, 2004).

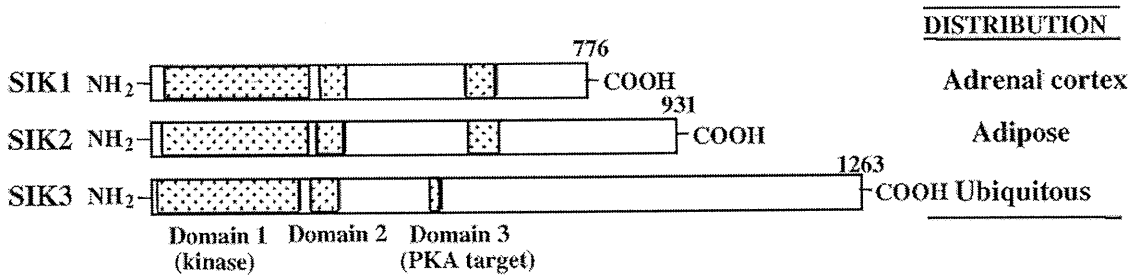


Figure 1.2. Three members of salt inducible kinase family (Katoh *et al.*, 2004).

1.6.1. SIK1

In mammals SIK1 mRNA is expressed abundantly in the adrenal gland, brain, testes and skeletal muscle, and to a lower extent in adipose, liver, and heart (Horike *et al.*, 2003). It encodes a 776-amino acid protein that contains a kinase domain, a sucrose-nonfermenting-1 protein kinase homology (SNH) domain and a nuclear localization domain (Katoh *et al.*, 2004; Katoh *et al.*, 2002).

SIK1 was shown to be involved in the regulation of cAMP responsive element-binding (CREB)-dependent gene expression (Figure 1.3). In response to adrenocorticotropin hormone (ACTH), SIK1 is phosphorylated at Ser-577 by PKA. This leads to the export of SIK1 from nucleus and initiates CRE-dependent gene transcription (Takemori *et al.*, 2002; Doi *et al.*, 2002).

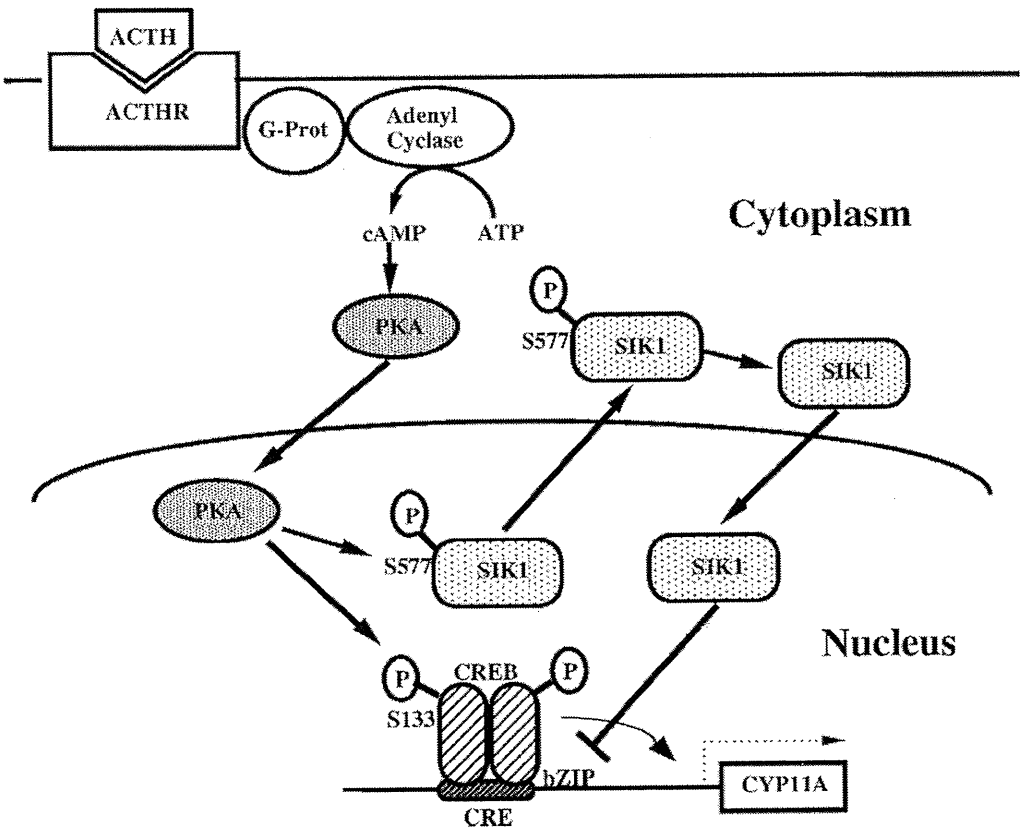


Figure 1.3. SIK1 involvement in repression of steroidogenic gene expression. The cascade is initiated in response to ACTH (Katoh *et al.*, 2004).

SIK1 also contains an LKB1 phosphorylation site at Thr-182, which is located in its activation loop. Phosphorylation generates a 14-3-3 protein-binding site on SIK1 and alters its structure to stabilize it in an active conformation. This binding also causes the translocation of SIK1 from nucleus to the cytosol (Al-Hakim *et al.*, 2005).

It was demonstrated that SIK2 negatively regulates lipogenesis by phosphorylating and inactivating nuclear sterol regulatory element-binding protein-1 (SREBP-1) in liver (Yoon *et al.*, 2009). SIK1 can also phosphorylate class IIa histone deacetylase-5 (HDAC-5), which targets it to nuclear export and inhibits its enzymatic activity (Cheng *et al.*, 2011). On the other hand, recent data implicates that SIK1 in modulating the intracellular sodium levels via multiple signaling networks (Bertorello *et al.*, 2009; Taub *et al.*, 2010).

1.6.2. SIK2

SIK2 is a 931-amino acid protein, which contains an N-terminal serine/threonine protein kinase domain, a central domain with a ubiquitin-associate motif, and a C-terminal PKA phosphorylation site. SIK2 expression was detected in all tissues examined, the highest expression being in white and brown adipose tissues (Katoh *et al.*, 2004; Horike *et al.*, 2003).

It was shown that SIK2 phosphorylates IRS1 on Ser794 and involved in the regulation of insulin signaling pathway. SIK2 activity was revealed to be increased in the adipocytes of diabetic rats suggesting that SIK2 promotes the development of insulin resistance (Horike *et al.,* 2003) in mice by Dentin *et al.* (2007) showed that SIK2 is an important regulator of gluconeogenesis in liver. In the presence of glucagon, cAMP levels increase and PKA becomes activated. In turn, PKA phosphorylates SIK2 and inhibits its kinase activity on Transducer of Regulated CREB Activity 2 (TORC2), which is able to remain in the nucleus when dephosphorylated and to activate gluconeogenic gene expression. Insulin stimulation activates SIK2 through AKT2-mediated phosphorylation, which in turn phosphorylates and leads to the cytoplasmic translocation of TORC2, inhibiting gluconeogenic gene expression (Figure 1.4.).

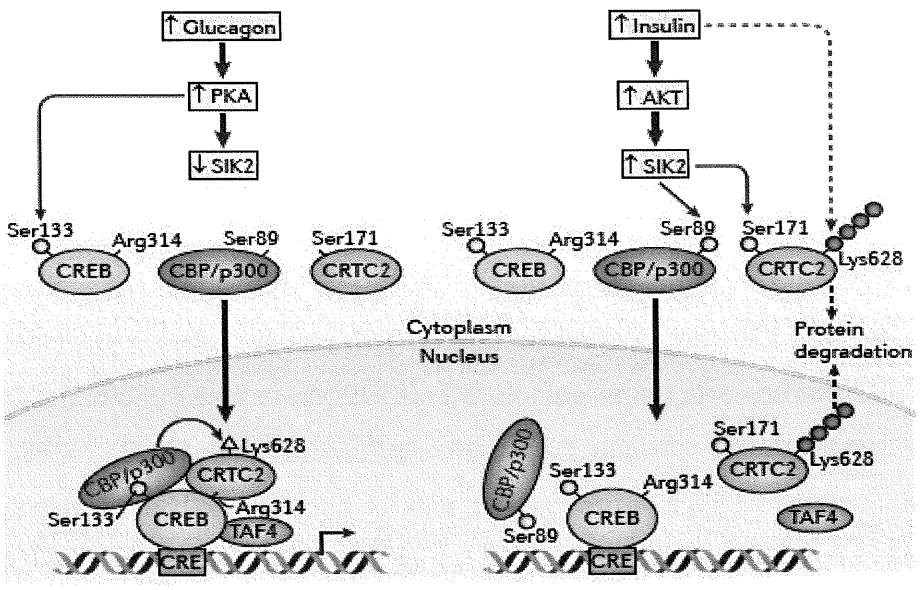


Figure 1.4. Modulation of gluconeogenesis via SIK2. It occurs through SIK2-dependent TORC regulation in response to glucagon or insulin horomones (Altarejos *et al.*, 2011).

Screaton *et al.* (2004) reported another regulatory role for SIK2 in the context of glucose and gut hormone signal integration in pancreatic islet cells. Under resting conditions, TORC2 is phosphorylated by SIK2 and sequestered in the cytoplasm via its interaction with 14-3-3 proteins. In response to glucose and gut hormones, TORC2 is dephosphorylated as a result of increased calcineurin activity and SIK2 inhibition, and translocates to nucleus to activate CREB target gene expression (Figure 1.5).

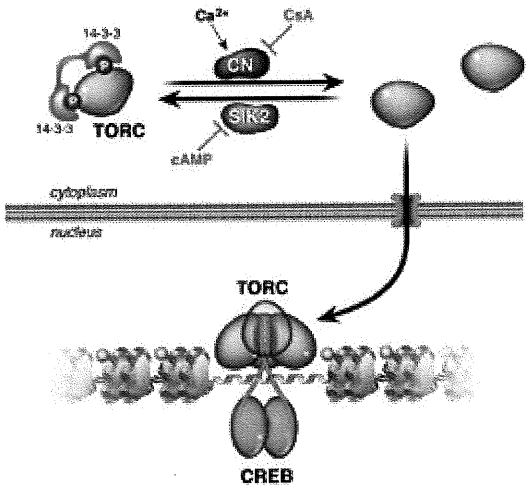


Figure 1.5. Regulation of TORC2 activity by Ca2+ and cAMP signals (Screaton *et al.*, 2004).

SIK2 was also shown to be involved in the regulation of lipogenesis in liver. Glucose stimulation induces the acetylation and activation of ChREBP by p300. When active, SIK2 phosphorylates and inhibits p300, thereby downregulating ChREBP promoted lipogenic gene expression (Bricambert *et al.*, 2010).

SIK2 expression was observed in all layers of the rat retina except the photoreceptor layer and three alternatively spliced isoforms of SIK2 were detected (Uysal, 2005; Özcan, 2003; Küser *et al.*, 2013). Developing retina also showed SIK2 expression (Özmen 2006). Particularly in Muller cells, SIK2 phosphorylation status, activity and cellular localization were reported to alter in response to FGF2 treatment (Kuser, 2011; Candas, 2004). Moreover, SIK2 was shown to phosphorylate Gab1 and A-Raf1, *in vitro*, which are known to be the components of FGF2 signaling pathway (Kuser, 2006; Sert, 2011). These findings suggest a regulatory role for SIK2 in the context of FGF2 signaling (Kuser, 2011).

1.6.3. SIK3

SIK3 is the largest member of SIK family with 1263-amino acids. Similar to the first two members, it also comprises a kinase domain (8-259), a ubiquitin associated domain (283-336) and a phosphorylation domain (486-518). It shows ubiquitous expression including Müller cells in human, mice and rats. (Katoh, 2004; Vural, 2009).

SIK3 is the least studied of SIK family member therefore its function is not well understood. Bettencourt-Dias *et al.* (2004) reported that knockdown of SIK3 homologous protein in Drosophila is associated with mitotic spindle malformations and chromosomal abnormalities. In addition, SIK3 was indicated to be a tumor associated antigen, its overexpression promoting cell proliferation and providing advantages to cancer cells to grow (Charoenfupraset, 2011). The data was supported by correlation of clinicopathological conditions of patients with ovarian cancer. SIK3 was also implicated in the promotion of chondrocyte hypertrophy throughout skeletal formation (Sasagawa *et al.*, 2012). These findings from different groups suggest that SIK3 is essential for several processes.

Aside from these findings, like the other members of SIK family, SIK3 was also demonstrated to be involved in the processes regulating glucose and lipid metabolism. It was revealed that SIK3 becomes activated in murine liver after fat, sucrose and cholesterol uptake to the body. Further studies with Sik3(-/-) mice showed that SIK3 deficiency results in abnormal metabolic phenotypes such as lipodystrophy, hypolipidemia, hypoglycemia, and hyper-insulin sensitivity (Uebi *et al.*, 2012). These phenotypes were accompanied by low expression levels of fatty acid synthesis pathway components in the liver. Furthermore, Berggreen *et al.* (2012) showed that upon forskolin stimulation, elevated levels of cAMP leads to SIK3 phosphorylation, accompanied by increased 14-3-3 protein binding and reduced SIK3 activity in primary adipocytes. In this pathway, PKA is thought to be the kinase regulating SIK3 activity. These findings suggest that SIK3 is involved in regulation of metabolic responses mediated by cAMP (Berggreen *et al.* 2012).

2. PURPOSE

Previous data from our lab suggested that SIK2 is a component of FGF2 signaling in MIO-M1 cells (Çınaroğlu et al., 2005; Özmen, 2006; Candaş, 2007). Considering the high level of sequence and functional similarity between SIK family proteins and the finding that SIK3 is also expressed in MIO-M1 cells, we wanted to investigate the possibility of SIK3 being a component of FGF2 signaling.

In order to understand this, we aimed to analyze in MIO-M1 cells

- the effect of FGF2 stimulation on serine, threonine and tyrosine phosphorylation status of SIK3
- the effect of FGF2 stimulation on the expression level of SIK3
- whether ERK is an upstream regulator of SIK3 in the context of FGF2 signaling

3. MATERIALS

3.1. Cell Lines

Spontaneously immortalized human Müller glia cell line (MIO-M1) was generously supplied by Prof. Astrid Limb from University College London, Institute of Ophthalmology, London.

3.2. Chemicals, Plastic and Glass Ware

All chemicals used were purchased from Merck (Germany) or Sigma Aldrich (USA), and all cell culture products were obtained from Invitrogen (USA) unless stated otherwise in the text. The sterilization of solutions, plastic and glassware was performed by autoclaving at 121^0 C for 20 minutes.

3.3. Buffers and Solutions

3.3.1. Cell Culture

Table 3.1. Cell Culture Materials.

Complete Medium for MIO-M1	DMEM with Glutamax supplemented with 10% Fetal bovine serum (FBS) 0,1% Penicillin/Streptomycin

Table 3.1. Cell Culture Materials (continued).

Freezing Medium	20% FBS 70% DMEM GlutaMAX 10% Dimethyl sulfoxide (DMSO)
FGF2 Medium	DMEM supplemented with 1 ng/ml FGF2 (R&D Systems, USA) 10 µg/ml heparin
10X Trypsin-EDTA Solution	2.5% Trypsin 7 mM Ethylenediaminetetraacetic acid (EDTA) 0.9% NaCl diluted with Phosphate Buffered Saline (PBS)
U0126 Solution	10 µM U0126 (Cell Signaling, USA) in DMEM

3.3.2. Protein Isolation

Table 3.2. Protein Isolation Materials.

Protease Inhibitor Cocktail	10 µg/ml leupeptin, 5µg/ml aprotinin (Roche, Germany)

Table 3.2. Protein Isolation Materials (continued).

Phosphatase Inhibitor Cocktail	1 mM Na_3VO_4, 1 mM phenylmethylsulphonylfluoride (PMSF), 20 mM NaF (Roche, Germany)

3.3.3. Western Blot

Table 3.3. Western Blot (WB) Materials.

Acrylamide:Bisacrylamide (37.5:1)	30 g acrylamide 0.8 g N`N`-bis-methylene- Acrylamide (TEMED) to 100 ml with dd H_2O
Ammonium Persulfate	Ammonium persulfate (APS) (w/v) in dd H_2O
Sodium Dodecyl Sulfate	10% Sodium dodecyl sulfate (SDS) (w/v) in dd H_2O
Blocking Solutions for Salt Inducible Kinase 3 (SIK3) B actin pThreonine, pSerine pTyrosine, pERK, ERK	5% non-fat milk powder in Tris Buffered Saline Tween (TBST) 1% non-fat milk powder in TBST 3% BSA in TBST 1% non-fat milk powder in TBST
Resolving Gel (10%)	10% Acryalmide:Bisacrylamide (37.5:1) 1.5 M Tris.HCl pH 8.8 0.1% SDS

Table 3.3. Western Blot (WB) Materials (continued).

	0.1% APS 0.1 % TEMED
Stacking Gel (5%)	5% Acrylamide: Bisacrylamide (37.5:1) 1 M Tris-HCl (pH 6.8) 0.1 % SDS 0.1% APS 0.1 % TEMED
6X Sample Buffer	1.2 g SDS 0.9 g Dithiothreitol (DTT) 6 mg bromophenol blue 4.7 mL glycerol 1.2 mL Tris 0.5 M pH 6.8 2.1 ml dd H_2O
Transfer Buffer (10X)	72 g Glycine 15,15 g Tris-Base 500 ml dd H2O
Transfer Buffer (1X)	70 ml 10X Transfer Buffer 105 ml Methanol (Sigma, USA) 525 ml dd H2O
Tris Buffered Saline (TBS) (10X)	24,2 g. Tris-Base 87,66 g. NaCl

Table 3.3. Western Blot (WB) Materials (continued).

	1000 ml dd H2O (pH 8.0)
Mild Stripping Solution	Buffer, 1 liter 15 g glycine 1 g SDS 10 ml Tween20 Adjust pH to 2.2 Bring volume up to 1 L with ultrapure water.
Strong Stripping Solution	62.5 mM Tris-HCl, pH 6.8 2% SDS 0.7% β-Mercaptoethanol

3.3.4. Immunoprecipitation

Table 3.4. Immunoprecipitation (IP) Materials.

Cell Signaling Lysis Buffer	20 mM Tris-Cl (pH: 7.5) 150 mM NaCl 1 mM EDTA 1 % Triton 2,5 mM Na pyrophosphate

3.4. Fine Chemicals

3.4.1. Antibodies

Table 3.5. Antibodies Used in Western Blot and Immunoprecipitation

Antibody	Source	Company	Dilution Used	Usage
Anti-Beta-actin, Horseradish Peroxidase (HRP) Conjugated	Mouse	Santa Cruz	1:2000	WB
Anti-rabbit Immunoglobulin G (IgG)	Goat	Santa Cruz	1:5000	WB
Anti-mouse Immunoglobulin G (IgG)	Goat	Santa Cruz	1:5000	WB
Anti-SIK3	Rabbit	Abcam	1:1000	WB, IP
Anti-phosphoTyrosine (pTyr)	Mouse	Santa Cruz	1:1000	WB
Anti-phosphoThreonine (pThr)	Rabbit	Invitrogen	1:1000	WB
Anti-phosphoSerine (pSer)	Rabbit	Invitrogen	1:1000	WB
Anti-phosphoERK	Mouse	Santa Cruz	1:1000	WB
Anti-ERK	Rabbit	Santacruz	1:1000	WB

3.5. Kits

3.5.1. BCA Assay Kit

BCA Protein Assay : Pierce, Thermo (USA)

3.6. Equipment

Table 3.6. Equipments Used.

Equipments	Models
Autoclave	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	Electronic Balance VA 124, Gec Avery,USA DTBH 210, Sartorius, GERMANY
Carbondioxide Tank	2091, Habaş, TURKEY
CCD Camera	CCD Camera, JAI Corporation, JAPAN
Centrifuges	ProFuge, 10K, Strategene, USA Mini Centrifuge 17307-05, Cole Parmer, USA Genofuge 16M, Techne, UK Centurion K40R, UK Centrifuge B5,B.Braun B. Int. (GERMANY)

Table 3.6. Equipments Used (continued).

	Centrifuge 5415R, Microfuge tube, USA J2-MC Centrifuge, Beckman Coulter, USA
Deep Freezers	2021D (-20^{0}C), Arçelik, Turkey -70^{0}C Freezer, Harris, UK -86^{0}C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio Doc, ITALY
Electrophoretic Equipments	Mini-PROTEAN 3 Cell, BIO-RAD, USA Easi-cast system, Hybaid, UK
Heat Blocks	DRI-Block DB-2A, Techne, UK StableTemp Dry Bath Incubator, Cole Parmer (USA)
Hemocytometer	Improved Neubauer, Weber Scientific International LTD, UK
Homogenizer	Pellet Pestles Tissue Grinder, Kimble Kontes USA
Hybridization Oven	Shake'n'Stack, Hybaid, UK
Ice Machine	Scotsman Inc., AF20, ITALY
Incubator	Hepa Class II Forma Series, Thermo Electron

Table 3.6. Equipments Used (continued).

Laminar Flow Cabinet	Class II A Tezsan, TURKEY Class II B Tezsan, TURKEY
Magnetic Stirrer	M221 Elekro-mag, TURKEY Clifton Hotplate Magnetic Stirrer, HS31, UK
Micropipettes	Gilson, FRANCE
Microscopes	CM110 Inverted Microscope, Prior, UK Zeiss, Axio Observer Z1 Inverted Mic., USA
pH Meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY
Power Supplies	EC135-90, Thermo Electron Corporation Power Pac Universal, BIO-RAD, USA
Protein Visualization	Stella, Raytest, Germany
Refrigerators	2082C, Arçelik, TURKEY 4030T, Arçelik, TURKEY
Sealer	Vacuplus FS400A, Electric Petra, GERMANY
Shakers	VIB Orbital Shaker, InterMed, DENMARK Lab-Line Universal Oscillating Shaker, USA Adjustable Rocker, Cole Parmer, USA
Software	Metasystems, GERMANY Quantity One, Bio-Rad, ITALY

Table 3.6. Equipments Used (continued).

Spectrophotometer	CE5502, Cecil, UK NanoDrop ND-1000, Thermo, USA
Vortex	Vortexmixer VM20,Chiltern Scientific, UK
Water Bath	TE-10A, Techne, UK
Water Purification	UTES, TURKEY

4. METHODS

4.1. MIO-M1 Cell Culture

4.1.1. Maintenance of MIO-M1 Cells

DMEM with Glutamax supplemented with 10% FBS and 0.1% penicillin/streptomycin was used to maintain MIO-M1 cells at 37°C, 5% CO_2. When cells grow 80-90% confluent, they were washed with PBS, incubated with 0.05% trypsin solution at 37°C for 5 mins, scraped and collected by centrifugation at 2000 x g for 5 mins and cell pellets were split into three plates after resuspended in the culture medium.

4.1.2. FGF2 Treatment of MIO-M1 Cells

Cells were seeded in 10 cm tissue culture plates and were grown to 80% confluency at 37oC, 5% CO_2. These cells were washed with PBS and incubated in DMEM supplemented with 0.1% penicillin/streptomycin for 16-18 hours under same conditions. Then, the cells were treated with DMEM containing 1 ng/ml FGF2 and 10 μg/ml heparin for 0, 5, 10, 20, 30 or 60 mins. Right after treatment, cells were washed with ice-cold PBS containing protease and phosphatase inhibitor cocktails, scraped and collected by centrifugation at 13200 rpm for 3 mins at 4oC. Pelleted cells can be stored at -70°C until next step. For ERK inhibition studies, cells were incubated with U0126 (10 μM) for 30 mins prior to FGF2 treatment.

4.2. BCA Assay

For the purpose of measuring total protein concentration, BCA kit was used. Bovine Serum Albumin (BSA) solutions ranging in concentration from 0.025 to 2 mg/ml were prepared in lysis buffer. 10 μl from cell lysates and BSA standards were added to 170 μl of 50:1 diluted BCA Working solution. Mixtures were incubated at 37°C for 30 mins

and BSA standard absorbances at 595 nm and their corresponding concentrations were integrated into a graph in nanodrop, to extrapolate total protein concentration of cell lysates.

4.3. SIK3 Immunoprecipitation

Cell pellets were resuspended in ice-cold 500 µl Cell Signaling Lysis Buffer containing protease and phosphatase inhibitors, pellets from duplicate plates were pooled for each time point. The pellets were incubated on ice for 1-2 hours followed by three times 3 secs of sonication at 90V. Subsequently, they were centrifuged at 13 200 rpm, 4°C for 25 mins and their supernatants were collected. Protein A agarose beads of 50 µl were washed three times with 1 ml lysis buffer and collected by centrifugation at 13 200 rpm for 3 mins. The supernatants collected from cell lysates were precleared with these beads by incubating them at 4°C for 35 mins. The samples were centrifuged at 13 200 rpm, 4°C for 2 mins, the supernatans were collected and incubated with 3-5 µg anti-SIK3 antibody at 4°C overnight. Then, the samples were incubated with 80 µl of pre-washed Protein A agarose beads for 5 hours, centrifuged at 13 200 rpm, 4°C for 3 mins and washed with lysis buffer with protease and phosphatase inhibitors three times. Equal volume of 2X protein sample buffer was added to the beads, they were boiled at 95 °C for 5 mins and centrifuged at 13 200 rpm for 10 mins. The supernatant was collected and 36 µl of it was loaded on 10 % SDS-polyacylamide gel.

4.4. SDS-PAGE and Western Blot

Cell lysates were boiled in 6X sample buffer at 95°C for 5 mins and spinned down for 10 sec before loading on 10% gel. Cell lysates or immunoprecipitation products were run on gel using Running Buffer; 80 V was applied until the loading dye passes the stacking gel and then 120 V was applied until the dye comes out of the gel.

In Western blotting, the samples run on the gel were electroblotted to Polyvinylidene fluoride (PDVF) membrane by using Transfer Buffer; 100 V was applied for 2 hours at 4°C. PVDF membranes were activated by methanol washing before use.

After transfer, the membrane was washed in TBST for 10 mins and incubated in corresponding blocking solution (Table 3.3) for 1 hour at room temperature on shaker. The membrane was incubated overnight in blocking solution containing appropriate concentration of the antibody at 4°C. Subsequently, the membrane was washed three times with TBST for 5 mins and incubated with blocking solution containing appropriate secondary antibody for 1 hour at room temperature. The membrane was washed three times with TBST and luminol reagent was applied onto the membrane for 10 mins. The chemoluminiscence was detected in Stella; IrfanView and Image J programs were used for the analysis of the protein bands.

For performing mild stripping, the membrane was incubated two times with Mild Stripping Solution for 10 minutes in room temperature. Then, it was incubated two times with PBS for 10 mins followed by incubation with TBST for 5 mins. The membrane was then ready for blocking stage. For performing harsh stripping, membranes were incubated by rolling at 50°C for 35 mins and then washed with TBST three times for 10 mins. The membrane was then ready for blocking stage.

4.5. Statistical Analysis

All statistical analyses were performed on Microsoft Office Excel 2007. Student's t-test was applied for triplicate data and statistical significance were analyzed at the level of $\alpha = 0.05$, error bars representing 1 standard error of the mean (SEM) in all charts.

5. RESULTS

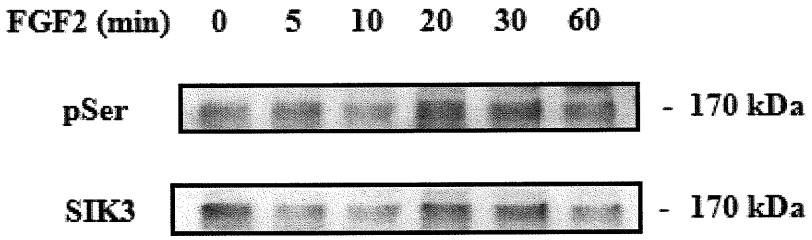
5.1. SIK3 Involvement in FGF2 Signaling

In this section, possibility of SIK3 involvement in FGF2 signaling was investigated. Towards this end FGF2-dependent changes in the phosphorylation pattern and expression level of SIK3 were examined.

5.1.1. Modulation of SIK3 Phosphorylation by FGF2

In order to understand if SIK3 phosphorylation pattern changes upon FGF2 stimulation, MIO-M1 cells were treated by 1 ng/ml FGF2 in the presence of 10 μg/ml heparin for 0-60 minutes. SIK3 was immunoprecipitated from cell lysates and SIK3 IP products were assayed for serine, threonine and tyrosine phosphorylations by Western blotting using antibodies against phosphoserine, phosphothreonine and phosphotyrosine residues. After chemoluminiscence detection, membranes were mildly stripped and reprobed by SIK3 antibody to normalize phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr) levels to SIK3 levels. Results showed that upon FGF2 stimulation of MIO-M1 cells, serine phosphorylation levels of SIK3 significantly increase (about 1.5-fold $p<0.05$) in 10 mins and then decrease back to its inital levels (Figure 5.1). On the other hand, threonine phosphorylation levels of SIK3 significantly decreased (about 50% $p<0.05$) in 5 minutes after FGF2 stimulation and then recover back to its initial levels (Figure 5.2,). SIK3 protein did not show any tyrosine phosphorylation at any time point examined (Figure 5.3).

(a)



(b)

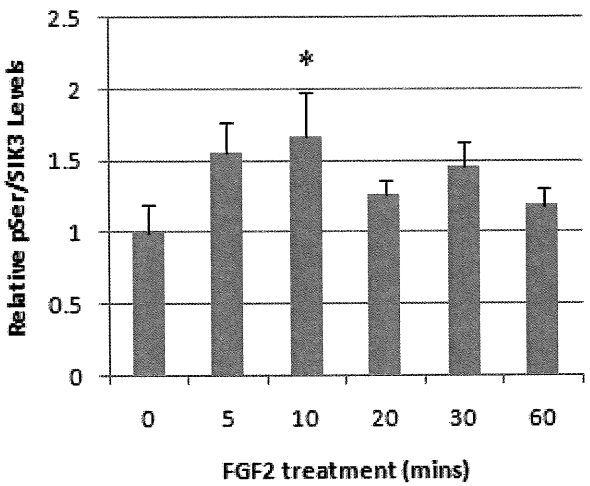
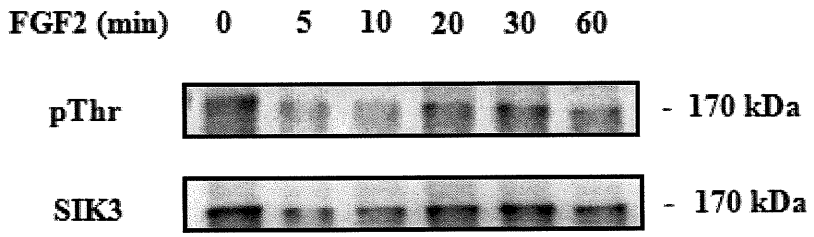


Figure 5.1. FGF2-dependent changes in SIK3 serine phosphorylation pattern . a) SIK3 IP products from FGF2-treated MIO-M1 cells were immunoblotted with antibodies against pSer or SIK3. b) The graph represents the average of relative pSer/SIK3 levels in three independent experiments. *p< 0. 05 compared to 0 min.

(a)



(b)

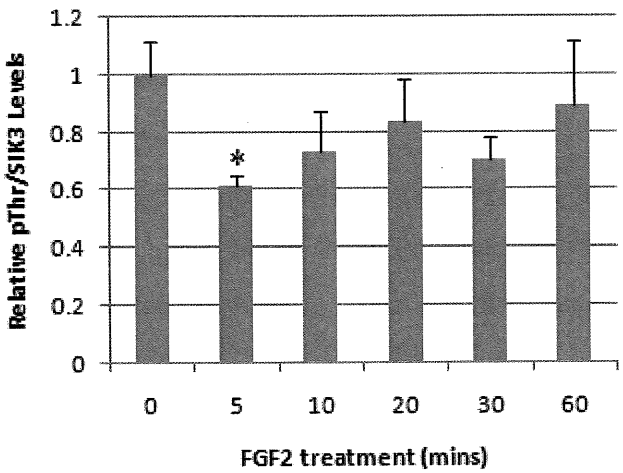


Figure 5.2. FGF2-dependent changes in SIK3 threonine phosphorylation pattern. a) SIK3 IP products from FGF2-treated MIO-M1 cells were immunoblotted with antibodies against pThr or SIK3. b) The graph represents the average of relative pThr/SIK3 levels in three independent experiments. *p< 0. 05 compared to 0 min.

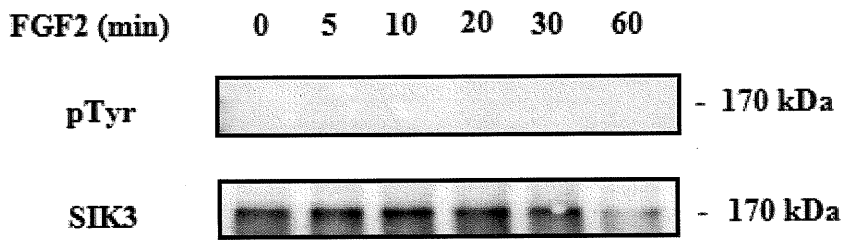
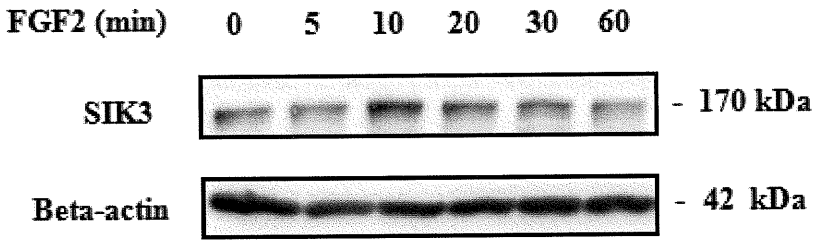


Figure 5.3. FGF2-dependent changes in SIK3 tyrosine phosphorylation pattern. SIK3 IP products from FGF2-treated MIO-M1 cells were immunoblotted with antibodies against pTyr or SIK3. The result was observed in three independent experiments.

5.1.2. Modulation of SIK3 Expression by FGF2

In order to understand if SIK3 expression levels change upon FGF2 stimulation, MIO-M1 cells were treated by 1 ng/ml FGF2 in the presence of 10 μg/ml heparin for 0-60 minutes. Cell lysates were Western blotted by using antibodies against SIK3 and β-actin. After chemoluminiscence detection, SIK3 levels were normalized to Beta-actin levels. Results showed that in response to FGF2 stimulation of MIO-M1 cells, SIK3 expression levels significantly increase (about 2-fold $p<0.05$) in 10 mins and then return gradually back to its initial levels (Figure 5.4).

(a)



(b)

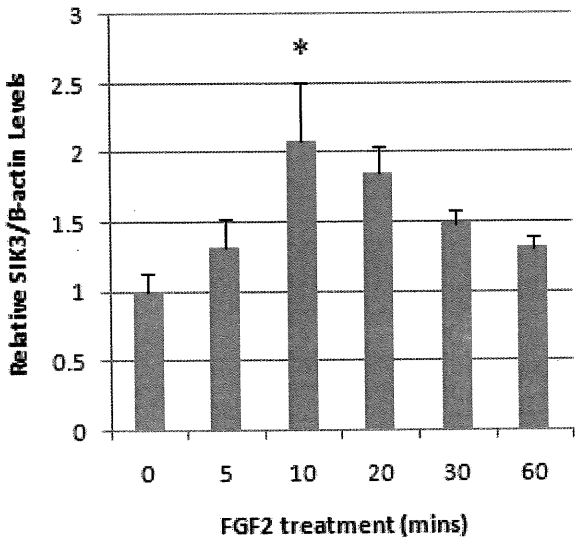


Figure 5.4. FGF2-dependent changes in SIK3 expression. a) Cell lysates from FGF2-treated MIO-M1 cells were immunoblotted using antibodies against SIK3 or β-actin. b) The graph respresents the average of relative SIK3/β-actin levels in three independent experiments. *p< 0. 05 compared to 0 min.

5.2. Modulation of FGF2-Dependent SIK3 Phosphorylation via ERK

In this section, the possibility that ERK acts as an upstream regulatory kinase of SIK3 was investigated. Towards this end, MIO-M1 cells were treated with MEK inhibitor (U0126) for 30 mins prior to FGF2 induction for 10 min. ERK inhibiton by U0126 was verified by Western blotting where signal obtained by pERK antibodies normalized to that of ERK antibodies (Figure 5.5). To follow changes in SIK3 phosphorylation levels in the

presence of the inhibitor SIK3 was immunoprecipitated from cell lysates and SIK3 IP products were evaluated for serine and threonine phosphorylations by Western blotting; subsequently the stripped blots were reprobed with SIK3 antibody to normalize serine and threonine levels. The control samples were treated only with FGF2.

The results indicated that inhibition of ERK activity prior to FGF2 treatment in MIO-M1 cells leads to a 2-fold decrease in serine phosphorylation levels (Figure 5.6) and to significantly higher levels of threonine phosphorylation levels of SIK3 (Figure 5.7, $p<0.05$) compared to FGF2 only treatment.

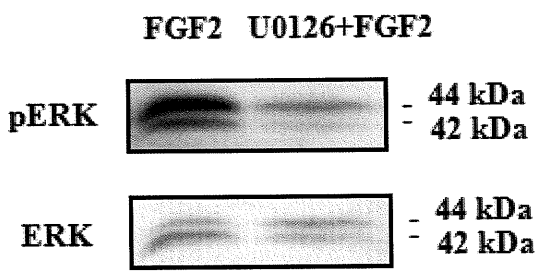


Figure 5.5. Effect of U0126 on ERK activation. Lysates from cells treated with FGF2 only or cells treated with MEK inhibitor (U0126) prior to FGF2 induction, were analyzed by western blotting using pERK or ERK antibodies.

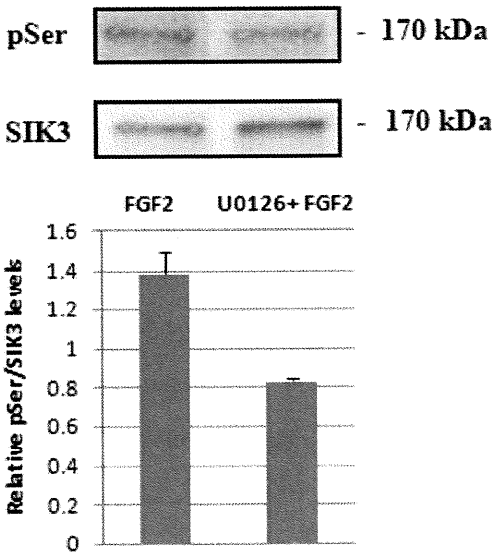


Figure 5.6. Changes in FGF2-dependent SIK3 serine phosphorylation with prior ERK inhibition. Western blots were carried out with SIK3 IP products from MIO-M1 cells treated with FGF2 only or U0126 followed by FGF2. SIK3 IP products were probed with antibodies against pSer or SIK3. The graph represents the average of relative pSer/SIK3 levels in the cells in two independent experiments.

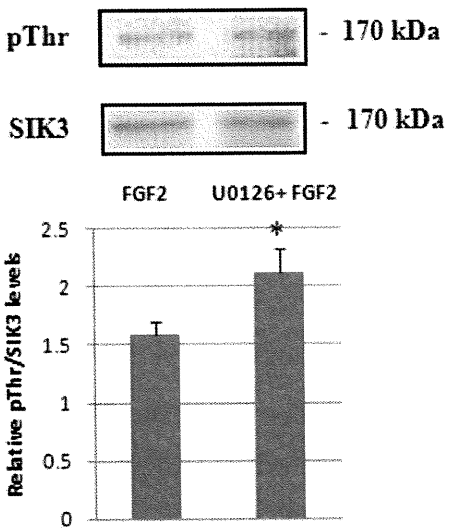


Figure 5.7. Changes in FGF2-dependent SIK3 serine phosphorylation with prior ERK inhibition. Western blots were carried out with SIK3 IP products from MIO-M1 cells treated with FGF2 only or U0126 followed by FGF2. SIK3 IP products were probed with antibodies against pThr or SIK3. The graph represents the average of relative pThr/SIK3 levels in the cells in three independent experiments. *p< 0. 05 compared to 0 min.

6. DISCUSSION

The members of SIK family (Figure 1.2) show extensive amino acid sequence similarity to each other particularly in their three domains; kinase domain (Domain 1), SNH domain (Domain 2) and phosphorylation domain (Domain 3) (Katoh *et al.*, 2004). Not surprisingly, all three proteins were shown to be involved in related processes. SIK1 and SIK2 were both indicated to be the regulators of CREB-dependent gene expression (Takemori *et al.*, 2002; Doi *et al.*, 2002; Dentin *et al.,* 2007). Furthermore, both proteins were demonstrated to be the substrates of PKA and LKB1 in the context of hormone signaling to regulate carbohdrate and fat metabolism (Lizcano *et al.*, 2004; Al-Hakim *et al.*, 2005; Takemori *et al.*, 2002; Doi *et al.*, 2002; Dentin *et al.,* 2007; Lizcano *et al.*, 2004). Their expression pattern in mouse suggests that they are critical regulators of carbohydrate and fat metabolism in different tissues. SIK1 is highly expressed in adrenal cortex, whereas SIK2 is abundant in adipocytes (Katoh *et al.*, 2004). Although SIK3 is the least studied of three members, recent data implicates also SIK3 in the regulation glucose and lipid mechanism in addition to its involvement in cancer (Uebi *et al.*, 2012; Berggreen *et al.* 2012).

Studies from our lab showed that SIK2 phosphorylation, activity and localization changes in MIO-M1 cells in response to FGF2 stimulation (Özmen, 2006; Candaş, 2007; Kuser, 2011). These findings suggest that SIK2 is a component of FGF2 signaling in MIO-M1 cells. Identification of two novel *in vitro* substrates of SIK2 – Gab1 and A-Raf-supports this finding because these two proteins are well known elements of FGF2 signaling (Küser, 2006; Sert, 2011). Moreover, data from our lab showed that ERK, which is a central component of FGF2 signaling pathway, phosphorylates SIK2 *in vitro*, suggesting that SIK2 activity might be regulated through ERK in the context of FGF2 signaling (Ejder, 2011). In MIO-M1 cells, both ERK and SIK2 activities peak in 10 mins of FGF stimulation and gradually decreases back to initial levels (Kuser, 2011). Based on these findings, our lab proposes a model where SIK2 is involved in a negative feedback mechanism, which serves to fine-tuning of FGF2-dependent ERK activity in MIO-M1 cells. According to this model, FGF2 stimulation leads to the activation of ERK in 5 mins, which in turn phosphorylates and activates SIK2. Once SIK2 is active, it phosphorylates

Gab1 and this phosphorylation reduces Gab1 interaction with Grb2 and Shp2, resulting in the downregulation of ERK signaling after 10 mins of FGF2 stimulation. Due to the high level of sequence and functional similarity between SIK family proteins and the finding that SIK3 is also expressed in MIO-M1 cells, we aimed to investigate the possibility that SIK3 is also a component of FGF2 signaling (Kuser, 2011).

In order to undestand if SIK3 phosphorylation status is altered upon FGF2 stimulation, we treated MIO-M1 cells with FGF2 for 0, 5, 10, 20, 30 and 60 minutes, immunoprecipitated SIK3 from these cells and followed their serine, threonine and tyrosine phosphorylation levels. The normalized phosphorylation signal to total SIK3 signal, indicates that serine phosphorylated SIK3 levels increase 1.5-fold within 10 mins of FGF2 stimulation and then decrease back to the initial levels (Figure 5.1). Threonine phosphorylation levels, on the other hand, decrease 2-fold in 5 mins of FGF2 stimulation, and then start to return to basal levels (Figure 5.2). In the literature, there are examples of proteins that show sustained phosphorylation as well as transient phosphorylation in response to FGF2 stimulation in various cell contexts. p42/44MAPK and p90rsk are among the proteins that undergo sustained phosphorylation in response to FGF2 signal in rat mammary fibroblasts, whereas FGF2 leads to a rapid transient phosphorylation of ERK in endothelial cells (Delehedde *et al.*, 2000; Sulpice *et al.*, 2002). Data from our lab also showed that FGF2 treatment of MIO-M1 cells leads to a transient phosphorylation of ERK, peaking at 10 minutes of stimulation (Kuser, 2011). Hence, the transient increase in serine phosphorylation levels of SIK3 in response to FGF2 treatment in MIO-M1 cells suggests that SIK3 is likely to be a component of FGF2 signaling pathway. Although there is not a well-known example of a protein that undergoes transient dephosphorylation in response to FGF2 signal, FGF2 was shown to regulate the activity of its downstream components via the involvement of phosphatases. For instance, activation of MAPK3/1 and AKT1 by FGF2 signaling is dependent on the activity of protein phosphatase 3 in endothelial cells, suggesting that this phosphatase is regulated by FGF2 signal (Wang *et al.*, 2008). So, the transient decrease in threonine phosphorylation levels of SIK3 in response to FGF2 stimulation could be explained with the involvement of certain phosphatases in the process. Because total serine and threonine phosphorylation levels were examined here, it might be the case that more than one serine/threonine residue undergo phosphorylation/dephosphorylation. To address this question, putative phosphorylation

sites on SIK3 could be mutated via site-directed mutagenesis method and the change in total serine or threonine phosphorylation levels in response to FGF2 treatment could be re-examined. It is also possible that more than one kinase/phosphatase is involved in the process. To investigate this possibility, a bioinformatic analysis could be performed on SIK3 by integrating the information about kinase/phosphatase binding motives of proteins that are known to be FGF2-dependent. The effect of individual kinases/phosphatases could be examined by inhibiton of these enzymes and evaluating the resulting SIK3 phosphorylation status.

FGFs exert their cellular effects upon binding and activating FGFRs, which are receptor tyrosine kinases on the cell membrane (Acevedo *et al.*, 2009). In the presence of FGF ligand and HSPGs, receptor dimerization causes autophosphorylation of several tyrosine residues in the cytoplasmic domain generating docking sites for the recruitment of downstream proteins such as PLCγ, FRS2 and PI3K. Some of these proteins undergo tyrosine phosphorylation at this stage, which either makes them active or creates further docking sites for the recruitment of other protein (Mohammadi *et al.*, 1991; Kouhara et al., 1997; Ong *et al.*, 2001). According to our results, SIK3 undergoes no tyrosine phosphorylation at any point of FGF2 stimulation (Figure 5.3) suggesting that SIK3 is not a substrate for FGFRs.

To understand whether SIK3 expression is regulated by FGF2 signaling, cells from different time points of FGF2 stimulation were checked for SIK3 levels in Western blotting and B-actin levels were used for normalization. Our results showed that upon FGF2 stimulation SIK3 expression levels significantly increase, peaking at the 10th min of stimulation up to ~2-fold and then decreases back to control levels (Figure 5.4). This relatively rapid change is unlikely to be arising from transcriptional regulation. One possibility is that SIK3 protein stability is enhanced by FGF signaling so that its levels are higher in MIO-M1 cells treated with FGF2. FGF2 stimulation results in ERK-mediated stabilization of certain proteins including Runx2 and Hsf4b gene products (Park *et al.*, 2010; Hu *et al.*, 2012). At this time, we have no information whether SIK3 activity is regulated by FGF2 signaling. SIK2 activity, however, was shown to increase upon FGF2 stimulation where it peaks in 10 mins of stimulation and then returns back to the basal levels gradually (Kuser, 2011). Regarding the changes in the phosphorylation status of

SIK3 upon FGF2 stimulation in MIO-M1 cells, SIK3 activity might also be regulated by FGF2 signaling,

ERK is a central component of FGF2 signaling pathway and data from our lab showed that ERK activity peaks in 10 mins of FGF2 stimulation in MIO-M1 cells (Kuser, 2011). Furthermore, SIK2 is suggested to be a downstream substrate of ERK in the context of FGF2 signaling in MIO-M1 cells (Ejder, 2011). The finding that both ERK activity and SIK3 serine phosphorylation levels peaked in 10 mins of FGF2 stimulation and the fact that SIK3 protein sequence contains several ERK phosphorylation motifs made us think that ERK could be the kinase involved in the process of SIK3 regulation. Once activated, ERK is able to phosphorylate numerous substrates having various functions. Being a serine/threonine kinase, it usually phosphorylates these residues followed by a proline, designated by (S/T)P. The presence of ERK docking sites on its substrates further determines which S/T residue will be phosphorylated by ERK. Two of well-characterized ERK docking sites are the D-domain and FXTP sequence. However, there are ERK substrates that do not contain any of these docking sites raising the possibility that ERK interacts also with yet-to-be-identified docking sites (Douglas *et al.*, 2001). SIK3 does not contain D-domain or FXTP sequence; however, it interestingly contains 17 ERK phosphorylation motifs including a serine residue. A proline at -1 position of consensus sequence is unfavorable, excluding 3 of them being potential ERK phosphorylation sites (Douglas *et al.*, 2001). So, potential ERK phosphorylation sites could be ser 167, 562, 673, 729, 778, 844, 849, 857, 885, 916, 1018, 1066 and 1124. Among these, ser 167, 562 and 1124 residues are conserved between fruit fly and human, and all these residues except ser 857 is conserved between mouse and human (NCBI, Blast). To understand if ERK is actually the upstream kinase of SIK3 in FGF2 signaling context, we treated MIO-M1 cells with MEK inhibitor (U0126) for 30 mins prior to FGF2 induction for 10 mins and compared SIK3 serine phosphorylation levels with uninhibited cells treated with FGF2 for 10 mins. Inhibition of ERK activity prior to FGF2 treatment results in lower levels of serine phosphorylation (Figure 5.6) compared to FGF2 only treatment. This suggests that ERK acts upstream of SIK3 and regulates its phosphorylation status. It is possible that ERK directly phosphorylates SIK3 on its specific serine residue(s) and this should be investigated by performing further experiments such as *in vitro* kinase assay or co-immunoprecipitation. Another possibility is that ERK indirectly regulates SIK3 by

activating another kinase that phosphorylates SIK3. We also checked SIK3 threonine phosphorylation levels and we observed that inhibition of ERK activity prior to FGF2 treatment results in higher levels of theronine phosphorylation (Figure 5.7) compared to FGF2 only treatment. In addition to activating protein kinases, ERK is also known to activate protein phosphatases (Yoon *et al.*, 2006). This raises the possibility that ERK activates a phosphatase that acts on threonine residues of SIK3. It is important to note that the change in serine and threonine phosphorylation levels upon ERK inhibition is quite substantial (~50%). This suggests that ERK could be the major kinase that regulates SIK3 phosphorylation upon FGF2 signal; nevertheless, additional kinases might also be involved in the process.

In summary, our results suggest that SIK3 expression and phosphorylation status change upon FGF2 stimulation in MIO-M1 cells and SIK3 phosphorylation is regulated via ERK activation, suggesting that SIK3 is a component of FGF2 signaling pathway. However, how the changes in the phosphorylation status of SIK3 translate into SIK3 activity is not yet elucidated. To understand how SIK3 activity changes with FGF2 stimulation, further experiments (e.g. *in vitro* kinase assay) should be performed. As noted earlier, SIK3 is not a well-studied protein and not much is known about its function. Yet, regarding the finding that SIK3 is a tumor associated antigen and promotes proliferation (Charoenfupraset, 2011), one may speculate that SIK3 is likely to be involved in the regulation of Müller cell proliferation by FGF2 signaling.

Müller cells are involved in sustaining the retinal homeostasis and they provide support for neuronal and vascular functioning. Therefore, they were implicated in several retinal disorders, usually undergoing abnormal proliferation in these states (Bringmann *et al.*, 2006; Bringmann and Reichenbach, 2001). The molecular mechanisms that leads to this state, so-called Müller cell gliosis, is not well understood. A number of studies suggest that FGF2 may be involved in the process of Müller cell gliosis development (Geller *et al.*, 2001). Hence, elucidating the details of FGF signaling pathway is important and a better understanding of the process would provide novel approaches into Müller cell gliosis that can be manipulated for future therapeutic applications.

REFERENCES

Acevedo, V.D., M. Ittmann, and D.M. Spencer, 2009, "Paths of FGFR-Driven Tumorigenesis", *Cell Cycle*, Vol. 8, No. 4, pp. 580-588.

Al-Hakim, A.K., O. Goransson, M. Deak, R. Toth, D.G. Campbell, N.A. Morrice, A.R. Prescott, and D.R. Alessi, 2005, "14-3-3 Cooperates with LKB1 to Regulate the Activity and Localization of QSK and SIK", *Journal of Cell Science*, Vol. 118, No. 23, pp. 5661-5673.

Altarejos, J.Y., and M. Montminy, 2011, "CREB and the CRTC Co-Activators: Sensors for Hormonal and Metabolic Signals", *Nature Reviews Molecular Cell Biology*, Vol. 12, No. 3, pp. 141-151.

Berggreen, C., E. Henriksson, H.A. Jones, N. Morrice, and O. Goransson, 2012, "cAMP-Elevation Mediated by Beta-Adrenergic Stimulation Inhibits Salt-Inducible Kinase (SIK) 3 Activity in Adipocytes", *Cell Signaling*, Vol. 24, No. 9, pp. 1863-1871.

Bertorello, A.M., and J.K. Zhu, 2009, "SIK1/SOS2 Networks: Decoding Sodium Signals via Calcium-Responsive Protein Kinase Pathways", *Pflugers Archiv*, Vol. 458, No. 3, pp. 613-619.

Bettencourt-Dias, M., R. Giet, R. Sinka, A. Mazumdar, W.G. Lock, F. Balloux, P.J. Zafiropoulos, S. Yamaguchi, S. Winter, R.W. Carthew, M. Cooper, D. Jones, L. Frenz, and D.M. Glover, 2004, "Genome-Wide Survey of Protein Kinases Required for Cell Cycle Progression", Nature, Vol. 432, No. 7020, pp. 980-987.

Bricambert, J., J. Miranda, F. Benhamed, J. Girard, C. Postic, and R. Dentin, 2010, "Salt-Inducible Kinase 2 Links Transcriptional Coactivator p300 Phosphorylation to the Prevention of ChREBP-Dependent Hepatic Steatosis in Mice", *Journal of Clinical Investigation*, Vol. 120, No. 12, pp. 4316-4331.

Bringmann, A., T. Pannicke, J. Grosche, M. Francke, P. Wiedemann, S.N. Skatchkov, N.N. Osborne, and A. Reichenbach, 2006, "Muller Cells in the Healthy and Diseased Retina". *Progress in Retinal and Eye Research*, Vol. 25, No. 4, pp. 397-424.

Bugra, K., and D. Hicks, 1997, "Acidic and Basic Fibroblast Growth Factor Messenger RNA and Protein Show Increased Expression in Adult Compared to Developing Normal and Dystrophic Rat Retina", *Journal of Molecular Neuroscience*, Vol. 9, No. 1, pp. 13-25.

Bugra, K., L. Oliver, E. Jacquemin, M. Laurent, Y. Courtois, and D. Hicks, 1993, "Acidic Fibroblast Growth Factor Is Expressed Abundantly by Photoreceptors within the Developing and Mature Rat Retina", *European Journal of Neuroscience*, Vol. 5, No. 12, pp. 1586-1595.

Charoenfuprasert, S., Y.Y. Yang, Y.C. Lee, K.C. Chao, P.Y. Chu, C.R. Lai, K.F. Hsu, K.C. Chang, Y.C. Chen, L.T. Chen, J.Y. Chang, S.J. Leu, and N.Y. Shih, 2011, "Identification of Salt-Inducible Kinase 3 as a Novel Tumor Antigen Associated with Tumorigenesis of Ovarian Cancer", *Oncogene*, Vol. 30, No. 33, pp. 3570-3584.

Cinaroglu, A., Y. Ozmen, A. Ozdemir, F. Ozcan, C. Ergorul, P. Cayirlioglu, D. Hicks, and K. Bugra, 2005, "Expression and Possible Function of Fibroblast Growth Factor 9 (FGF9) and Its Cognate Receptors FGFR2 and FGFR3 in Postnatal and Adult Retina", *Journal of Neuroscience Research*, Vol. 79, No. 3, pp. 329-339.

Delehedde, M., M. Seve, N. Sergeant, I. Wartelle, M. Lyon, P.S. Rudland, and D.G. Fernig, 2000, "Fibroblast Growth Factor-2 Stimulation of p42/44MAPK Phosphorylation and IkappaB Degradation is Regulated by Heparan Sulfate/Heparin in Rat Mammary Fibroblasts", *Journal of Biological Chemistry*, Vol. 275, No. 43, pp. 33905-33910.

Dentin, R., Y. Liu, S.H. Koo, S. Hedrick, T. Vargas, J. Heredia, J. Yates, and M. Montminy, 2007, "Insulin Modulates Gluconeogenesis by Inhibition of the Coactivator TORC2", *Nature*, Vol. 449, No. 7160, pp. 366-369.

Eriksson, A.E., L.S. Cousens, L.H. Weaver, and B.W. Matthews, 1991, "Three-Dimensional Structure of Human Basic Fibroblast Growth Factor", *Proceedings of the National Academy of Sciences USA*, Vol. 88, No. 8, pp. 3441-3445.

Fischer, A.J., C.R. McGuire, B.D. Dierks, and T.A. Reh, 2002, "Insulin and Fibroblast Growth Factor 2 Activate a Neurogenic Program in Muller Glia of the Chicken Retina", *Journal of Neuroscience*, Vol. 22, No. 21, pp. 9387-9398.

Fischer, A.J., M.A. Scott, and W. Tuten, 2009, "Mitogen-Activated Protein Kinase-Signaling Stimulates Muller Glia to Proliferate in Acutely Damaged Chicken Retina", *Glia*, Vol 57, No. 2, pp. 166-181.

Geller, S.F., G.P. Lewis, and S.K. Fisher, 2001, "FGFR1, Signaling, and AP-1 Expression after Retinal Detachment: Reactive Muller and RPE Cells", *Investigative Ophthalmology and Visual Science*, Vol. 42, No. 6, pp. 1363-1369.

Hicks, D., and Y. Courtois, 1992, "Fibroblast Growth Factor Stimulates Photoreceptor Differentiation *in vitro*", *Journal of Neuroscience*, Vol.12, No. 6, pp. 2022-2033.

Horike, N., H. Takemori, Y. Katoh, J. Doi, L. Min, T. Asano, X.J. Sun, H. Yamamoto, S. Kasayama, M. Muraoka, Y. Nonaka, and M. Okamoto, 2003, "Adipose-Specific Expression, Phosphorylation of Ser794 in Insulin Receptor Substrate-1, and Activation in Diabetic Animals of Salt-Inducible Kinase-2", *Journal of Biological Chemistry*, Vol. 278, No. 20, pp. 18440-18447.

Huang, J., M. Mohammadi, G.A. Rodrigues, and J. Schlessinger, 1995, "Reduced Activation of RAF-1 and MAP Kinase by a Fibroblast Growth Factor Receptor Mutant Deficient in Stimulation of Phosphatidylinositol Hydrolysis", *Journal of Biological Chemistry*, Vol. 270, No. 10, pp. 5065-5072.

Johnson, D.E., and L.T. Williams, 1993, "Structural and Functional Diversity in the FGF Receptor Multigene Family", *Advances in Cancer Research*, Vol. 60, pp. 1-41.

Kan, M., X. Wu, F. Wang, and W.L. McKeehan, 1999, "Specificity for Fibroblast Growth Factors Determined by Heparan Sulfate in a Binary Complex with the Receptor Kinase", *Journal of Biological Chemistry*, Vol. 274, No. 22, pp. 15947-15952.

Katoh, M., 2009, "FGFR2 Abnormalities Underlie a Spectrum of Bone, Skin, and Cancer Pathologies", *Journal of Investigative Dermatology*, Vol. 129, No. 8, pp. 1861-1867.

Katoh, Y., H. Takemori, J. Doi, and M. Okamoto, 2002, "Identification of the Nuclear Localization Domain of Salt-Inducible Kinase", *Endocrine Research*, Vol. 28, No. 4, pp. 315-318.

Katoh, Y., H. Takemori, N. Horike, J. Doi, M. Muraoka, L. Min, and M. Okamoto, 2004, "Salt-Inducible Kinase (SIK) Isoforms: Their Involvement in Steroidogenesis and Adipogenesis", *Molecular and Cellular Endocrinology*, Vol. 217, No. 1-2, pp. 109-112.

Kinkl, N., G.S. Hageman, J.A. Sahel, and D. Hicks, 2002, "Fibroblast Growth Factor Receptor (FGFR) and Candidate Signaling Molecule Distribution within Rat and Human Retina", *Molecular Vision*, Vol. 8, pp. 149-160.

Kouhara, H., Y.R. Hadari, T. Spivak-Kroizman, J. Schilling, D. Bar-Sagi, I. Lax, and J. Schlessinger, 1997, "A Lipid-Anchored Grb2-Binding Protein that Links FGF-Receptor Activation to the Ras/MAPK Signaling Pathway", *Cell*, Vol. 89, No. 5, pp. 693-702.

Lee, J.G., and E.P. Kay, 2006, "FGF-2-Mediated Signal Transduction During Endothelial Mesenchymal Transformation in Corneal Endothelial Cells", *Experimental Eye Research*, Vol. 83, No. 6, pp. 1309-1316.

Lizcano, J.M., O. Goransson, R. Toth, M. Deak, N.A. Morrice, J. Boudeau, S.A. Hawley, L. Udd, T.P. Makela, D.G. Hardie, D.R. Alessi, 2004, "LKB1 Is a Master Kinase that Activates 13 Kinases of the AMPK Subfamily, Including MARK/PAR-1", *EMBO Journal*, Vol. 23, No. 4, pp. 833-843.

Mason, I., 2007, "Initiation to End Point: the Multiple Roles of Fibroblast Growth Factors in Neural Development", *Nature Reviews Neuroscience*, Vol. 8, No. 8, pp. 583-596.

McCabe, K.L., E.C. Gunther, and T.A. Reh, 1999, "The Development of the Pattern of Retinal Ganglion Cells in the Chick Retina: Mechanisms that Control Differentiation", *Development*, Vol. 126, No. 24, pp. 5713-5724.

Mendillo, M.L., S. Santagata, M. Koeva, G.W. Bell, R. Hu, R.M. Tamimi, E. Fraenkel, T.A. Ince, L. Whitesell, and S. Lindquist, 2012, "HSF1 Drives a Transcriptional Program Distinct from Heat Shock to Support Highly Malignant Human Cancers", *Cell,* Vol. 150, No. 3, pp. 549-562.

Mohammadi, M., A.M. Honegger, D. Rotin, R. Fischer, F. Bellot, W. Li, C.A. Dionne, M. Jaye, M. Rubinstein, and J. Schlessinger, 1991, "A Tyrosine-Phosphorylated Carboxy-Terminal Peptide of the Fibroblast Growth Factor Receptor (Flg) Is a Binding Site for the SH2 Domain of Phospholipase C-Gamma 1", Molecular Cellular Biology, Vol. 11, No. 10, pp. 5068-5078.

Mohammadi, M., S.K. Olsen, and O.A. Ibrahimi, 2005, "Structural Basis for Fibroblast Growth Factor Receptor Activation", *Cytokine & Growth Factor Reviews,* Vol. 16, No. 2, pp. 107-137.

Ong, S.H., Y.R. Hadari, N. Gotoh, G.R. Guy, J. Schlessinger, and I. Lax, 2001, "Stimulation of Phosphatidylinositol 3-Kinase by Fibroblast Growth Factor Receptors Is Mediated by Coordinated Recruitment of Multiple Docking Proteins", *Proceedings of the National Academy of Sciences USA*, Vol. 98, No. 11, pp. 6074-6079.

Lizcano, J.M., O. Goransson, R. Toth, M. Deak, N.A. Morrice, J. Boudeau, S.A. Hawley, L. Udd, T.P. Makela, D.G. Hardie, D.R. Alessi, 2004, "LKB1 Is a Master Kinase that Activates 13 Kinases of the AMPK Subfamily, Including MARK/PAR-1", *EMBO Journal*, Vol. 23, No. 4, pp. 833-843.

Mason, I., 2007, "Initiation to End Point: the Multiple Roles of Fibroblast Growth Factors in Neural Development", *Nature Reviews Neuroscience*, Vol. 8, No. 8, pp. 583-596.

McCabe, K.L., E.C. Gunther, and T.A. Reh, 1999, "The Development of the Pattern of Retinal Ganglion Cells in the Chick Retina: Mechanisms that Control Differentiation", *Development*, Vol. 126, No. 24, pp. 5713-5724.

Mendillo, M.L., S. Santagata, M. Koeva, G.W. Bell, R. Hu, R.M. Tamimi, E. Fraenkel, T.A. Ince, L. Whitesell, and S. Lindquist, 2012, "HSF1 Drives a Transcriptional Program Distinct from Heat Shock to Support Highly Malignant Human Cancers", *Cell,* Vol. 150, No. 3, pp. 549-562.

Mohammadi, M., A.M. Honegger, D. Rotin, R. Fischer, F. Bellot, W. Li, C.A. Dionne, M. Jaye, M. Rubinstein, and J. Schlessinger, 1991, "A Tyrosine-Phosphorylated Carboxy-Terminal Peptide of the Fibroblast Growth Factor Receptor (Flg) Is a Binding Site for the SH2 Domain of Phospholipase C-Gamma 1", Molecular Cellular Biology, Vol. 11, No. 10, pp. 5068-5078.

Mohammadi, M., S.K. Olsen, and O.A. Ibrahimi, 2005, "Structural Basis for Fibroblast Growth Factor Receptor Activation", *Cytokine & Growth Factor Reviews,* Vol. 16, No. 2, pp. 107-137.

Ong, S.H., Y.R. Hadari, N. Gotoh, G.R. Guy, J. Schlessinger, and I. Lax, 2001, "Stimulation of Phosphatidylinositol 3-Kinase by Fibroblast Growth Factor Receptors Is Mediated by Coordinated Recruitment of Multiple Docking Proteins", *Proceedings of the National Academy of Sciences USA*, Vol. 98, No. 11, pp. 6074-6079.

Ornitz, D.M., 2000, "FGFs, Heparan Sulfate and FGFRs: Complex Interactions Essential for Development", *Bioessays*, Vol. 22, No.2, pp. 108-112.

Ornitz, D.M., and N. Itoh, 2001, "Fibroblast Growth Factors", *Genome Biology*, Vol. 2, No. 3, pp. REVIEWS3005.

Park, O.J., H.J. Kim, K.M. Woo, J.H. Baek, and H.M. Ryoo, 2010, "FGF2-Activated ERK Mitogen-Activated Protein Kinase Enhances Runx2 Acetylation and Stabilization", *Journal of Biological Chemistry*, Vol. 285, No. 6, pp. 3568-3574.

Pawson, T., 1995, "Protein Modules and Signalling Networks", *Nature*, Vol. 373, No. 6515, pp. 573-580.

Plotnikov, A.N., J. Schlessinger, S.R. Hubbard, and M. Mohammadi, 1999, "Structural Basis for FGF Receptor Dimerization and Activation", *Cell*, Vol. 98, No. 5, pp. 641-650.

Powers, C.J., S.W. McLeskey, and A. Wellstein, 2000, "Fibroblast Growth Factors, Their Receptors and Signaling", *Endocrine Related Cancer*, Vol. 7, No. 3, pp. 165-197.

Rodriguez-Viciana, P., P.H. Warne, R. Dhand, B. Vanhaesebroeck, I. Gout, M.J. Fry, M.D. Waterfield, and J. Downward, 1994, "Phosphatidylinositol-3-OH Kinase as a Direct Target of Ras", *Nature*, Vol. 370, 6490, pp. 527-532.

Sasagawa, S., H. Takemori, T. Uebi, D. Ikegami, K. Hiramatsu, S. Ikegawa, H. Yoshikawa, and N. Tsumaki, 2012, "SIK3 is Essential for Chondrocyte Hypertrophy During Skeletal Development in Mice", *Development*, Vol. 139, No. 6, pp. 1153-1163.

Schlessinger, J., 2000, "Cell Signaling by Receptor Tyrosine Kinases", *Cell*, Vol. 103, No. 2, pp. 211-225.

Screaton, R.A., M.D. Conkright, Y. Katoh, J.L. Best, G. Canettieri, S. Jeffries, E. Guzman, S. Niessen, J.R. Yates, H. Takemori, M. Okamoto, and M. Montminy, 2004, "The CREB Coactivator TORC2 Functions as a Calcium- and cAMP-Sensitive Coincidence Detector", *Cell*, Vol. 119, No.1, pp. 61-74.

Sulpice, E., M. Bryckaert, J. Lacour, J.O. Contreres, and G. Tobelem, 2002, "Platelet Factor 4 Inhibits FGF2-Induced Endothelial Cell Proliferation via the Extracellular Signal-Regulated Kinase Pathway but Not by the Phosphatidylinositol 3-Kinase Pathway", *Blood*, Vol. 100, No. 9, pp. 3087-3094.

Taub, M., J.E. Springate, and F. Cutuli, 2010, "Targeting of Renal Proximal Tubule Na,K-ATPase by Salt-Inducible Kinase", *Biochemical and Biophysical Research Communications*, Vol. 393, No. 3, pp. 339-344.

Uebi, T., Y., Itoh, O., Hatano, A., Kumagai, M., Sanosaka, T., Sasaki, S., Sasagawa, J., Doi, K., Tatsumi, K., Mitamura, E., Morii, K., Aozasa, T., Kawamura, M., Okumura, J., Nakae, H. Takikawa, T. Fukusato, M. Koura, M. Nish, A. Hamsten, A. Silveira, A.M. Bortorello, K. Kitagawa, Y. Nagaoka, H. Kawahara, T. Tomonaga, T. Naka, S. Ikegawa, N. Tsumaki, J. Matsuda, and H. Takemori, 2012, "Involvement of SIK3 in Glucose and Lipid Homeostasis in Mice", *PLoS One*, Vol. 7, No 5., pp. e37803.

Wang, K., Y. Song, D.B. Chen, and J. Zheng, 2008, "Protein Phosphatase 3 Differentially Modulates Vascular Endothelial Growth Factor- and Fibroblast Growth Factor 2-Stimulated Cell Proliferation and Signaling in Ovine Fetoplacental Artery Endothelial Cells", Biology of Reproduction, Vol. 79, No.4, pp. 704-710.

Wassle, H., and B.B. Boycott, 1991, "Functional Architecture of the Mammalian Retina", *Physiological Reviews*, Vol. 71, No. 2, pp. 447-480.

Xie, M.H., I. Holcomb, B. Deuel, P. Dowd, A. Huang, A. Vagts, J. Foster, J. Liang, J. Brush, Q. Gu, K. Hillian, A. Goddard, and A.L. Gurney, 1999, "FGF-19, a Novel

Fibroblast Growth Factor with Unique Specificity for FGFR4", *Cytokine*, Vol. 11, No. 10, pp. 729-735.

Yang, X.J., 2004, "Roles of Cell-Extrinsic Growth Factors in Vertebrate Eye Pattern Formation and Retinogenesis", *Seminars in Cell & Developmental Biology*, Vol. 15, No. 1, pp. 91-103.

Yoon, Y.S., W.Y. Seo, M.W. Lee, S.T. Kim, and S.H. Koo, 2009, "Salt-Inducible Kinase Regulates Hepatic Lipogenesis by Controlling SREBP-1c Phosphorylation", *Journal of Biological Chemistry*, Vol. 284, No. 16, pp. 10446-10452.

Zhu, X., H. Komiya, A. Chirino, S. Faham, G.M. Fox, T. Arakawa, B.T. Hsu, and D.C. Rees, 1991, "Three-Dimensional Structures of Acidic and Basic Fibroblast Growth Factors", *Science*, Vol. 251, No. 4989, pp. 90-93.