SIK2 IS A POTENTIAL MEDIATOR OF CROSS-TALK BETWEEN

FGF AND PKA PATHWAYS

by

Demet Candaş

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To my beloved uncle, Şenol Candaş;

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ABSTRACT

SIK2 IS A POTENTIAL MEDIATOR OF CROSS-TALK BETWEEN FGF AND PKA PATHWAYS

FGF signaling pathway is initiated upon FGF binding to RTKs and the signal transduction is achieved by a series of phosphorylation/dephosphorylation events mediated by several kinase and phosphatases. The pathway is tightly controlled through negative feedback mechanisms adopted by the activated effector proteins in the pathway as well as via interacting pathways. Recent studies suggested interplay between Ras/ERK pathway and PKA pathways. Results obtained in our laboratory raise the possibility that serine/threonine kinase SIK2 may be part of this cross-talk as PKA modulates FGF9-dependent ERK activation and nuclear localization of SIK2.

In order to gain further insight into the involvement of SIK2 in FGF signal transduction and in the cross-talk between FGF and PKA pathways, we focused on the prototypic member of the family, FGF2. The results show that SIK2 is both serine and threonine phosphorylated in response to FGF2 stimulation and PKA activation. Upon FGF2 induction, Ras/ERK pathway is transiently activated, resulting in the proliferation of MIO-M1 cells. The inhibition of PKA activity prior to FGF2 treatment leads to an increase in pERK levels and a retardation in signal attenuation. The proliferation data indicates prior PKA activation leads to no significant changes, while inhibition of PKA activity leads to an increased FGF2-dependent proliferation of MIO-M1 cells. Immunocytochemical studies reveal that 10 minutes or longer durations of FGF2 stimulus exported SIK2 out of nuclei; and that inhibition of PKA activity results in retardation of this translocation. We also demonstrated that the expression profile of SIK2 changes with PKA activity and upon FGF2 induction indicating a feedback mechanism. These results firmly places SIK2 in FGF signal transduction cascade, and provides evidence that this protein might be an important component of cross-talk machinery between growth factor and metabolic pathways.

ÖZET

SIK2, FGF ve PKA SİNYAL İLETİM YOLAKLARININ ENTEGRASYONUNDA OLASI BİR ARACIDIR

Fibroblast Büyüme Faktörü (FGF) sinyal iletim yolağı, FGF reseptör tirozin kinazlara bağlanmalarıyla başlar ve sinyal iletimi yolaktaki çeşitli kinaz ve fosfatazlarca gerçekleştirilen fosfor bağlanma/koparılma olaylarıyla sağlanır. Yolak, içerdiği aktif efektör proteinlerce benimsenmiş negatif geribesleme mekanizmalarıyla ve etkileşen diğer sinyal iletim yolaklarıyla sıkı bir kontrol altında tutulmaktadır. Yakın zamanda yapılmış çalışmalar, Ras/ERK sinyal iletim yolağıyla Protein Kinaz A (PKA) iletim yolaklarının etkileşimini göstermiştir. Laboratuvarımızda bu iki yolağın etkileşiminde, bir serin/treonin kinaz olan Tuz İndüklenebilir Kinaz (SIK2)'ın olası varlığı tespit edilmiştir. Çünkü, PKA'nın FGF9'a dayalı ERK aktivasyonunda ve SIK2'nin nükleer lokalizasyonunda modülatör etkileri olduğu gözlemlenmiştir.

SIK2'nin FGF sinyal iletim yolağındaki ve PKA ile FGF yolaklarının etkileşimindeki varlığının daha iyi anlaşılması amacıyla, çalışmalarımıza FGF2 ile devam ettık. Sonuçlar, SIK2'nin, FGF2 uyarımına ve PKA aktivasyonuna cevaben serin ve treonin aminoasitlerinden fosforlandığını göstermiştir. MIO-M1 hücrelerinde, Ras/ERK yolağının FGF2 ile indüklendiğinde geçici olarak aktive olduğu ve bu aktivasyonun hücrelerde çoğalma cevabı oluşturduğu görülmüştür. FGF2 uyarımından önce PKA'nın inhibe edilmesi, fosfoERK seviyelerinde bir artışa ve de sinyalin bastırılmasında gecikmeye yol açmaktadır. FGF2 uyarımının hemen öncesinde PKA'nın, aktivasyonu hücrelerin çoğalmasında önemli bir değişikliğe yol açmazken, inhibisyonu hücrelerin FGF'e dayalı çoğalmalarını önemli ölçüde artırmaktadır. İmmünositokimyasal çalışmalar, 10 dakika veya daha uzun süreli FGF2 uyarımının, SIK2'nin çekirdekten atılmasına;

uyarım öncesinde PKA aktivitesinin engellenmesinin ise bu translokasyonda gecikmeye yol açtığını göstermiştir. Ayrıca, SIK2'nin gen anlatımının PKA aktivitesiyle ve FGF2 uyarımıyla değiştiği gösterilmiş ve bu noktada bir geri besleme mekanizmasının varlığı düşünülmüştür. Bütün bu sonuçlar, SIK2'nin FGF sinyal iletim yolağında yer aldığını ve bu proteinin, büyüme faktörü sinyal yolakları ile metabolik sinyal yolakların etkileşiminde önemli bir öğe olabileceğini göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

S	Serine
T	Threonine
Y	Tyrosine
8BrcAMP	8 Bromo cylicadenosinemonophosphate
ACTH	Adenocorticotrophic hormone
AKAP	A-kinase anchoring protein
AMPK	AMP-activated protein kinase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
bp	Base pair
BSA	Bovine serum albumine
cAMP	Cyclic adenosine 5’-monophosphate
cDNA	Complementary deoxyribonucleic acid
CFR	Cysteine-rich fibroblast growth factor receptor
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CRE	cAMP-responsive element
CREB	CRE binding protein

DAG	Diacylglycerol
DAPI	4’-6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosidetriphosphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FHF	FGF homology factor
FIRE	FGF induced response element
FITC	Fluorescein Isothiocyanate
Frs2	Fibroblast growth factor receptor substrate
GAF	Glia activating factor
GCL	Ganglion cell layer
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein

GTC	Guanidium isothiocyanate
HLGAG	Heparin - like glycosaminoglycan
HRP	Horse radish peroxidase
HSPG	Heparan sulfate proteoglycans
Ig	Immunoglobulin
INL	Inner nuclear layer
IRS1	Insulin receptor substrate 1
KD	Kinase Domain
kDa	Kilo dalton
KGF	Keratinocyte growth factor
MAPK	Mitogen-activated protein kinase
MIO-M1	Moorfields/Institute of Ophthalmology-Muller 1
MMLV	Moloney-murine- leukaemia-virus
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
OD	Optical density
ONL	Outer nuclear layer
PAGE	PolyAcrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma
PCR	Polymerase chain reaction
PH	Pleckstrin homology

PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase C-gamma
PTB	Phosphotyrosine binding
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-Time PCR
RGC	Retina ganglion cells
RNA	Ribonucleic acid
RNasin	RNase inhibitor
RPE	Retina pigmented epithelium
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription coupled polymerase chain reaction
SDS	Sodium Dodecyl Sulfate
Ser	Serine
SH	Src homology domain
SHP2	SH2-domain containing phosphatase 2
SIK	Salt-inducible kinase
SNF	Sucrose nonfermenting
SNH	Sucrose nonfermenting homology
Sos	Son of sevenless

TAE	Tris-acetate/EDTA
TBST	Tris-Buffered Saline Tween-20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming growth factor
TORC2	Transducer of regulated CREB activity
TR	Texas red

1. INTRODUCTION

1.1. Retina

Retina is located in the back of the eye and is responsible for the conversion of light energy into chemical signals and relaying them to brain. The neural retina is highly organized in layers and comprises six neural cell types- ganglion, amacrine, horizontal, bipolar, rod photoreceptor and cone photoreceptor cells- and a single type of glial cell which is Müller cell (Figure 1.1). The outer segments of photoreceptors, rods and cones, are in contact with retinal pigment epithelium (RPE) cells whereas their cell bodies forms the outer nuclear layer (ONL). The photoreceptors convert the light energy into neuronal signals and pass the signal to the bipolar cells located in the inner nuclear layer (INL). The amacrine and horizontal interneurons together with Muller glia cell bodies are also in INL. The connection between those interneurons and bipolar cells integrates the signal coming from photoreceptor cells. The ganglion cell layer (GCL) contains ganglion cells whose axons combine to form the optic nerve that relays the signal to its eventual destinations in the brain (Newman and Reichenbach, 1996; Hatakeyama and Kageyama, 2004).

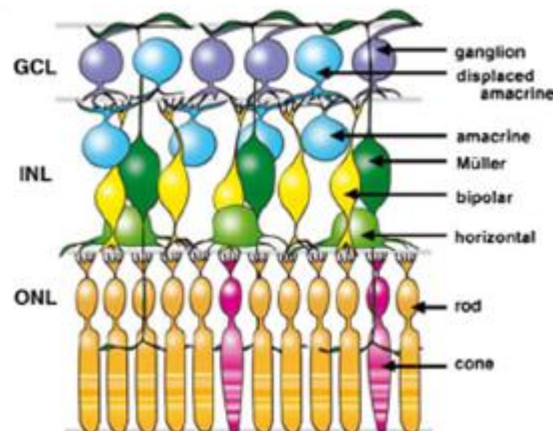


Figure 1.1. Schematic structure of the neural retina

1.2. Fibroblast Growth Factors

Fibroblast growth factor (FGFs) family has at least 23 members (Yamashita *et al.*, 2000; Ornitz and Itoh, 2001). They are structurally related glycosylated polypeptides with molecular masses around 17-34 kilo Dalton (kDa). Between vertebrate species, FGFs are highly conserved in both gene structure and amino acid sequence (Ornitz and Itoh, 2001). FGF family members have been characterized as having strong affinity for heparin and heparan-like glycosaminoglycans (HLGAGs) of the extracellular matrix (ECM). The central core of 120 amino acids (Figure 1.2) is highly homologous between different family members (Burgess and Maciag, 1989; Ago *et al.*, 1991; Zhang *et al.*, 1991; Ornitz and Itoh 2001)

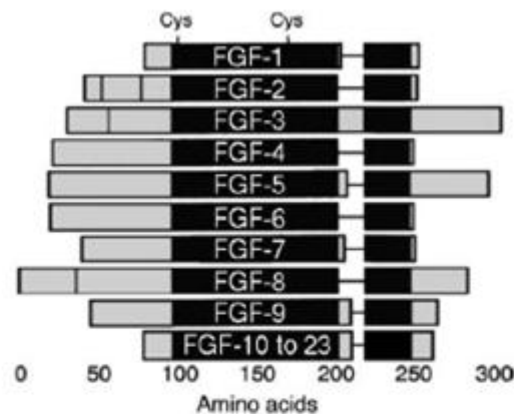


Figure 1.2. Schematic domain alignment of FGFs (Reuss *et al.,* 2003)

FGF1 is also known as “acidic fibroblast growth factor”, whereas FGF2 is known as “basic fibroblast growth factor”. FGF11-14 are called FGF homology factors (FHFs) that share between 58% and 71% amino acid identity between themselves, but show less than 30% amino acid identity with other members. While most of the FGFs possess classical signal peptide, FGF1, 2, 9, 16 and 20 lack this signal sequence. FHFs all have nuclear localization signals that and are proposed to have roles in intracellular processes. FGF16-19 were identified recently and less is known about them (Powers *et al.*, 2000).

FGFs are widely expressed in developing and adult tissues and have various functions including embryonic development, angiogenesis, cell proliferation,

differentiation, survival, motility as well as tumor development and progression (Mason, 1994; Powers *et al*., 2000; Presta *et al*., 2005). FGF1 and FGF2 have been shown to promote differentiation and survival of central nervous system (CNS) neurons (Morrison *et al*., 1986; Unsicker *et al*., 1987). FGF2, 3, 4, 8, 17 and 18 have been implicated in establishing the anteroposterior body axis, chondrogenesis and osteogenesis (Ford-Perriss *et al*., 2001; Dono, 2003). Although these factors have been known to control patterning of several regions of the CNS, FGF signaling is required to be attenuated for neuronal differentiation (Diez del Corral, 2002). FGF4, 5 and 6 are also expressed during embryonic development (Powers *et al*., 2000). FGF7, also known as keratinocyte growth factor (KGF), and FGF10 are mitogenic for epithelial cells and function in limb, lung and kidney development (Min *et al*., 1998; Sekine *et al*., 1999). FGF22 has been recently shown to be crucial in presynaptic differentiation (Umemori *et al*., 2004).

A number of FGF family members are expressed in the developing eye and in adult retina. FGF1 has been shown to be important in ganglion cell differentiation early in development (Guillemot and Cepko, 1992; McCabe *et al*., 1999). FGF1 and 2 are mainly expressed late in retinal maturation indicating that may act as survival factors, especially for photoreceptors (Bugra et al., 1994; Bugra and Hicks, 1997). FGF3 and 8 are expressed in the optic stalk in several species (Wilkinson *et al*., 1989; Reifers *et al*., 2000; Vogel-Hopker *et al*., 2000; Walshe and Mason, 2003). FGF15 has been shown to be expressed in neuronal progenitors differentiating into ganglion and amacrine cells, whereas its human ortholog FGF19 is expressed in horizontal cells of developing chicken retina (Kurose *et al*., 2004). FGF1, 2, 5 and 9 were shown to be expressed in mature retina (Baird *et al*., 1985; Bugra *et al*., 1994; Kitaoka *et al*., 1995; Colvin *et al*., 1999; Cinaroglu *et al*., 2005). Although the function of FGFs in mature retina remains to be understood, the response generated in cultured Muller glia to FGF1, 2 and 9 is proliferation (Cinaroglu *et al*., 2005).

1.3. Fibroblast Growth Factor Receptors

FGFs mediate their cellular responses by binding to and activating fibroblast growth factor receptor (FGFR) molecules, FGFR1-4. They also bind to heparin or heparan sulfate proteoglycans (HSPG) that assist in FGF binding to high affinity receptors and regulate the activation of receptors. FGFRs belong to the receptor tyrosine kinase (RTKs) family (Lee

et al., 1989; Givol and Yayon, 1992; Jaye *et al*., 1992). Like all RTKs, FGFR1-4 are composed of an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain that bears the catalytic protein tyrosine kinase core and some regulatory sequences (Schlessinger, 2000). There are three immunoglobulin (Ig) like domains in the extracellular region of FGFR. Ig like domain 2 contains a conserved positively charged region that serves as a binding site for heparin (Schlessinger *et al*., 2000). There is a region called "acid box" in the linker between Ig like domain 1 and 2 that comprises a stretch of seven to eight acidic residues (Figure 1.3).

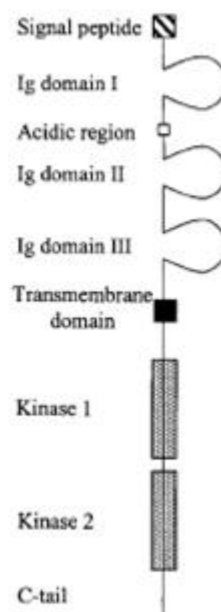


Figure 1.3. Schematic representation of FGFR structure (Powers *et al*., 2000).

Alternative splicing gives rise to distinct receptor isoforms with either two or three Ig-like domains (Figure 1.4). Additionally, by altering the C-terminal half of the third Ig-like domain, splicing creates IIIa which is the secreted form of receptor that lacks transmembrane region, IIIb and IIIc isoforms in FGFR1-3 (Johnson and Williams, 1993; Eswarakumar *et al*., 2005). Alternative splicing variants differ significantly in their ligand-binding specificities (Table 1.1).

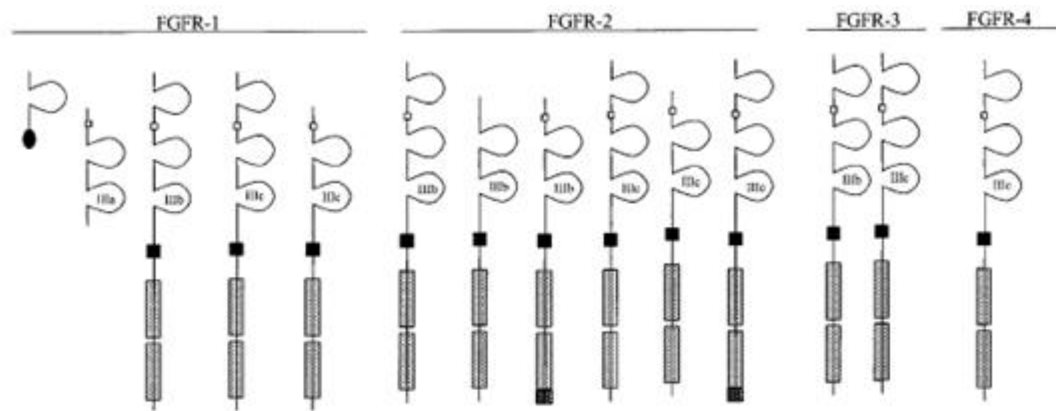


Figure 1.4. FGFR isoforms generated by alternative splicing (Johnson and Williams, 1993)

Table 1.1. Ligand binding specificities of FGFRs (modified from Eswarakumar *et al*., 2000)

FGFR Isoform	Ligand Specificity
FGFR1IIIb	FGF1, 2, 3 and 10
FGFR1IIIc	FGF1, 2, 4, 5 and 6
FGFR2IIIb	FGF1, 3, 7, 10 and 22
FGFR2IIIc	FGF1, 2, 4, 6, 9, 17 and 18
FGFR3IIIb	FGF1 and 9
FGFR3IIIc	FGF1, 2, 4, 8, 9, 17, 18 and 23
FGFR4	FGF1, 2, 4, 6, 8, 9, 16, 17, 18 and 19

Upon ligand binding, FGFRs are known to dimerize and be activated by tyrosine phosphorylation (Schlessinger, 1988; Lemmon and Schlessinger, 1994; Jiang and Hunter, 1999). Crystal structures of ligand-binding domains of FGFR1 and FGFR2 in complex with FGF1 or FGF2 and crystal structure of FGF-heparin-FGFR complex revealed how the dimerization and activation is induced by ligand binding (Plotnikov *et al*., 1999; Plotnikov *et al*., 2000; Schlessinger *et al*., 2000). FGF signaling complex at the cell surface is shown to be a ternary complex formed between two identical FGF ligands, two identical FGFR molecules and heparin sulfate proteoglycans. FGFs are unable to activate FGFRs without the help of heparin sulfate chains (Spivak-Kroizman *et al*., 1994). Heparin binds to a

positively charged crevice generated by exposed Lys and Arg residues extending across D2 domains of the two FGFR molecules in the dimer and two bound FGF molecules. D1 domain and acid box are not involved in ligand binding. Deletion of D1 and acid box enhances binding of receptor to FGF and heparin suggesting that these regions have an autoinhibitory function (Wang *et al.*, 1995; Plotnikov *et al.*, 1999). The cytoplasmic domain of FGFRs contains several regulatory sequences and a conserved protein tyrosine kinase (PTK) core which is stimulated upon ligand binding and receptor dimerization. PTK domain is responsible for trans-phosphorylation of receptors at several tyrosine residues in kinase domains. Phosphorylatable tyrosine residues in FGFR1 are known to be Y463, Y583, Y585, Y653, Y654, Y730 and Y766 (Mohammadi *et al.*, 1996). Tyrosine auto-phosphorylation sites recruit and activate numerous intracellular signaling proteins that generates cellular responses (Kouhara *et al.*, 1997; Eswarakumar *et al.*, 2005)

1.4. Cell Signaling via FGF Receptors

Signaling via FGFR is mediated by the recruitment of signaling proteins directly to those tyrosine auto-phosphorylation sites on active receptor or by docking proteins that are tyrosine phosphorylated upon FGF stimulation. Docking proteins form complexes with other signaling proteins to convey the signal (Lemmon and Schlessinger, 1994; Pawson, 1995; Eswarakumar *et al.*, 2005).

Downstream signaling proteins interact with activated receptor or with mediator proteins via their src homology 2 (SH2) domains, which specifically bind to tyrosine phosphorylated RTKs and cytoplasmic proteins (Moran *et al.*, 1990). SH3 domains that preferentially bind to proline rich regions also mediate receptor binding (Ren *et al.*, 1993). A third important motif in receptor interaction is the phosphotyrosine binding (PTB) domains (Pawson, 2004).

There are three main pathways that are activated by FGFRs (Figure 1.5): phosphoinositide-3 (Pl3) kinase/Akt (protein kinase B) pathway, Phospholipase C (PLC) γ/Ca^{2+} pathway and Ras/ mitogen-activated protein kinase (MAPK) pathway (Böttcher and Niehrs, 2003; Eswarakumar *et al.*, 2005).

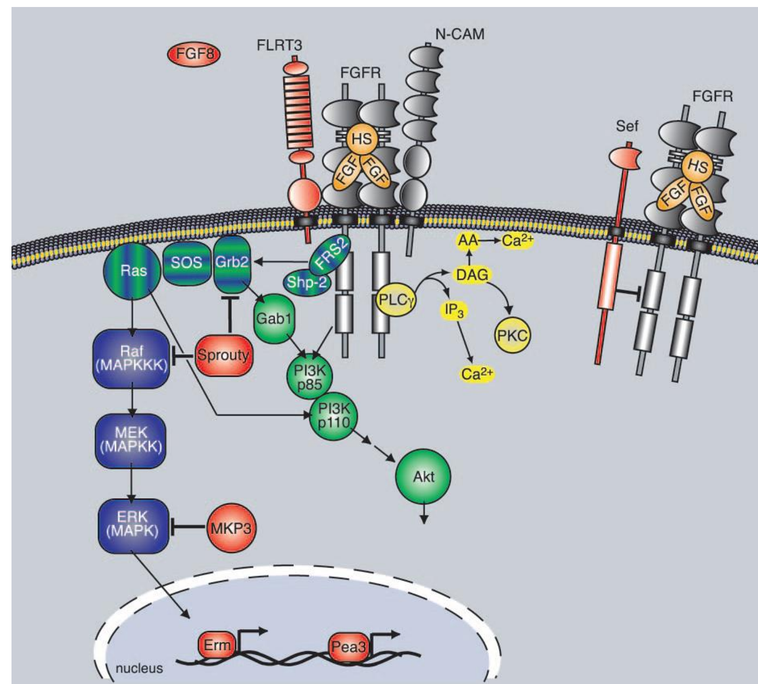


Figure 1.5. Signaling pathways downstream of FGFR. Blue and blue/green molecules belong to Ras/MAPK pathway; green and blue/green elements belong to PI3K/Akt pathway; Yellow molecules belong to PLCγ/Ca^{2+} pathway (Böttcher and Niehrs, 2003)

1.4.1. PI3 kinase/Akt Pathway

FGF stimulation of this pathway is implicated in cell survival. PI3 kinase is recruited to the plasma membrane via its SH2 domain, that directs the kinase to the phosphotyrosine residues on Gab1 in growth factor receptor bound protein (Grb2)-fibroblast growth factor receptor substrate 2 (FRS2)-Gab1 assembly (Rodriguez-Viciana *et al*., 1994; Eswarakumar *et al*., 2005). Akt is then recruited to the plasma membrane via its pleckstrin homology (PH) domain and activated directly by PI3 kinase. Akt may also be phosphorylated indirectly at its T308 and S473 residues by PDK1/PRK-2 complex (Hadari *et al*., 2001). Upon activation, Akt returns to cytoplasm and inhibits either the activity of pro-apoptotic proteins or the transcription of the genes that encode them (Schlessinger, 2000).

1.4.2. PLCγ/Ca^{2+} Pathway

PLCγ is recruited to the Y766 tyrosine phosphorylated receptor via its SH2 domain and is activated by phosphorylation. Active PLCγ stimulates the hydrolysis of phosphatidylinositol-4,5-diphosphate (PI) and results in the generation of two second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (Eswarakumar *et al*., 2005). In turn, DAG activates protein kinase C (PKC) whereas inositol-1,4,5-triphosphate stimulates the release of Ca^{2+} from intracellular compartments and activates calcium/calmodulin dependent kinases (Mohammadi *et al.,* 1991). Phosphorylation status of Raf is modified by PLCγ activated PKC, and this leads to the activation of Ras/MAPK pathway (Huang *et al*., 1995).

1.4.3. Ras/MAPK Pathway

Activation of Ras/MAPK pathway by FGFs results in the generation of proliferation or differentiation responses. The main modulator of the pathway is thought to be FRS2 (Kouhara *et al*., 1997) and it is known to interact with juxtamembrane region of FGFRs and phosphorylated on several tyrosine residues. Upon activation, FRS2 recruits Grb2/son of sevenless (Sos) adaptor protein complex in addition to recruiting a tyrosine phosphatase, SH2-domain containing phosphatase 2 (Shp2) (Kouhara *et al*., 1997; Ong *et al*., 2000). However, FGF2 induced MAPK-dependent threonine phosphorylation of FRS2 is accompanied with reduced tyrosine phosphorylation of the docking protein and decreased recruitment of Grb2, eventually resulting in attenuation of the MAPK response (Powers *et al.,* 2000). Alternatively, FGF stimulation leads to phosphorylation of Shp2 which results in complex formation with additional Grb2 molecules. Sos, being a guanine nucleotide exchange factor facilitates guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange upon recruitment to close proximity of small G-protein Ras on membrane, resulting in Ras activation. Activated Ras recruits Raf, a serine/threonine kinase, to the membrane. Phosphorylation of Raf on several tyrosine residues activates the protein, which in turn phosphorylates MAPK-kinase (MEK) on two serine residues. Activated MEK phosphorylates MAPK, extracellular signal-regulated kinase (ERK), on tyrosine and threonine residues. Activated ERK in turn phosphorylates a variety of downstream proteins, including other kinases, as well as gene regulatory proteins in the nucleus (Yang

et al., 2004). The duration that MAP-kinases remain active can influence the nature of the response. MAP-kinases are usually activated only transiently in response to extracellular signals and their transient activation leads to cell proliferation. However, the activity of MAP-kinases can remain high for longer periods of time and this sustained activation is thought to direct cellular response from proliferation towards differentiation (Yamada and Yoshimura, 2002).

1.5. Fibroblast Growth Factor 2

FGF2 was first purified from bovine pituitary gland as a protein capable of inducing proliferation and phenotypic transformation of Balb/c3T3 fibroblast cell line (Gospodarowicz, 1975). The protein was also named basic fibroblast growth factor (bFGF) because of its basic isoelectric point of 9.6. It was first identified as a 15 kDa protein which was later found to be a proteolytic product of an 18 kDa form (Bikfalvi *et al*., 1997). Isoforms arising from alternate CUG-translation start site usage are larger such as 22, 22.5, 24 and 34 kDa. These large isoforms possess an aminoterminal nuclear-localizing sequence (NLS). Although the 18 kDa isoform is cytosolic, its nuclear presence has been reported (Okada-Ban *et al*., 2000). FGF2 has a very high sequence homology (> 90%) among a wide range of species (Nugent and Iozzo, 2000; Chen *et al*., 2004). FGF2 lacks a terminal signal peptide (Shibata *et al*., 1991), it may be released from cells by an exocytotic mechanism different from endoplasmic reticulum-golgi pathway (Mignatti *et al*., 1992). FGF2 binds to low affinity heparin that stabilizes FGF2 and facilitates its binding to high affinity FGF receptors in order to exert its affect (Yayon *et al*., 1991; Ornitz *et al.*, 1992; Ornitz and Itoh, 2001).

Besides its mitogenic role, FGF2 was found to be involved in tissue remodeling and regeneration (Gospodarowicz, 1987). In endothelial and neural cells, FGF2 was shown to be a survival factor which blocks apoptosis. Additionally, FGF2 was found to stimulate the growth of capillaries by acting as an angiogenic factor (Schweigerer *et al*., 1987). Formation of new capillaries may initate tumor growth and promotes metastasis in cancerous tissues (Folkman, 2002). Apart from stimulating blood vessel growth, FGF2 is known to be an important player in wound healing. It stimulates fibroblasts to proliferate and give rise to granulation tissue that fills up a wound space early in healing process (Lee

and Kay, 2006). It has also been shown that FGF2 is linked to many developmental processes including antero-posterior patterning, neural induction, limb formation and axon extension (Arese and Chen, 1999). Loss of FGF2 function results in a range of developmental defects (Böttcher and Niehrs, 2004).

In the context of embryonic retina, FGF2 was implicated in cell fate specification. Overexpression of FGF2 in retinal progenitor cells resulted in a 35 per cent increase of RGCs and a 50 per cent increase of Muller cells. Although the proportion of photoreceptor cells among total cells remained constant, the ratio of rod versus cone photoreceptors was shown to be affected by overexpression of FGF2 (Yang, 2004). In embryonic chick addition of ectopic FGF2, RPE starts proliferating and gives rise to a neuroepithelium which will then differentiate into all subtypes of cells of retina in embryonic chick. FGF2 stimulates the RPE transdifferentiation through FGFR/Ras/ERK signaling (Spence *et al.,* 2007).

It was also shown that FGF2 mediates axon regeneration in the mature, injured visual system through Ras/ERK pathway (Sapieha *et al*., 2006). FGF2 expression was found to be increased in retina but decreased in RPE of diabetic rats. However, insulin produced a dose-dependent increase in FGF2 in RPE cells grown in high glucose. It is therefore suggested that reduced systemic insulin as well as high glucose levels contibute to decreased FGF2 expression in RPE (Layton et al., 2006).

1.6. Protein Kinase A

Protein kinase A, also called cyclic- cyclic adenosine monophosphate (cAMP)-dependent protein kinase, is the effector of cAMP in most animal cells. The enzyme catalyzes the transfer of a terminal phosphate group from adenosine triphosphate (ATP) to specific serine or threonine residues of target proteins and regulates their activity. Since PKA has a variety of substrates in different cell types, the effects of cAMP vary greatly depending on cell context. PKA is known to affect stimulation of insulin secretion, sperm motility, modulation of ion channels, glycogen and triacylglycerol breakdown (Krebs, 1989; Lacroix and Hontela, 2001; Langfort *et al*., 2003). PKA also regulates gene expression by directly phosphorylating a gene regulatory protein called cAMP-responsive

element binding protein (CREB) or by indirectly phosphorylating other proteins that affect CREB activity (Rosenberg *et al*., 2002; Screaton *et al*., 2004).

PKA consists of two catalytic subunits and two regulatory subunits in its inactive form. The conformational change generated in PKA upon binding of cAMP to the regulatory subunits results in dissociation of these subunits from the complex. The catalytic subunits are thereby activated and phosphorylate specific substrates. Special PKA anchoring proteins belonging to a family of A-kinase anchoring protein (AKAP), locates the enzyme complex to a particular subcellular compartment by binding both to the regulatory subunits of PKA and to a membrane or a component of cytoskeleton or act as a scaffold to recruit other proteins including kinases and phosphatases (Scott, 1991; Colledge and Scott, 1999).

Recent studies suggest an important role for PKA in the regulation of growth factor signaling. Depending on the cell line and the stimulus used, the effect of PKA may be stimulatory or inhibitory. It was suggested in a recent study that PKA has a crucial role in balancing growth factor induced signaling through the Ras/ERK pathway. Raf-1 and B-Raf serine/threonine kinases were identified as candidate mediators of this regulation (Pursiheimo *et al*., 2002a). In the same study, it was shown that inhibition of PKA activity prior to FGF2 induction causes an increase in the signal flow through Ras/ERK pathway. Additionally, PKA was implied in regulation of the growth factor activated transcription. FGF2 induced FGF-inducible response element (FiRE) activation requires cooperation of Ras/ERK and PKA pathways (Pursiheimo *et al*., 2002b).

1.7. Salt-Inducible Kinase Family

Salt-inducible kinases constitute a family of three serine/threonine kinases. The first member of the family, SIK1, was isolated from adrenocortical tissue of high salt fed rats (Wang *et al*., 1999). The search for similar genes in human and mouse genome database revealed two related genes, SIK2 and SIK3 (Okamoto *et al*., 2004).

1.7.1. SIK1

SIK1 enzyme was shown to be expressed in adrenal tissue, brain, pituatory, ovary, lung, heart and testis (Lin *et al*., 2001; Horike *et al*., 2003). SIK1 is predicted to be a 776 amino acid protein that contains an N-terminal serine-threonine kinase domain (KD), a sucrose nonfermenting-1 (SNF-1) kinase homology (SNH) domain and a potential PKA phosphorylation residue containing C-terminal domain (Figure 1.6).

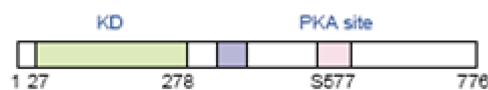


Figure 1.6. Schematic representation of SIK1 structure (Okamoto *et al*., 2004)

SIK1 was found to be transiently induced by adrenocorticotropin (ACTH) treatment, and upon induction its rapid translocation from nucleus to the cytoplasm was reported (Lin *et al*., 2001). Since SIK1 represses the transcription of a steroidogenic enzymes by inhibiting the activity on CRE in the promoter, its phosphorylation and resulting translocation to cytoplasm allows gene transcription from CRE containing promoter (Figure 1.7). Therefore, it was suggested that SIK1 has a fine tuning role on steroidogenic enzyme production (Okamoto *et al*., 2004). SIK1 was shown to be able to return to nucleus after it was dephosphorylated in the cytoplasm (Takemori *et al*., 2002).

In addition to PKA, LKB1 was shown to phosphorylate SIK1 at T182 in the activation loop and the resulting conformational change stabilizes SIK1 in active form. LKB1 phosphorylated SIK1 recruits 14-3-3 protein resulting in SIK1 translocation from nucleus to the cytosol (Lizcano *et al.,* 2004; Al-Hakim *et al.,* 2005). SIK1 has an autophosphorylation activity but the target site is still unknown (Wang *et a*l., 1999).

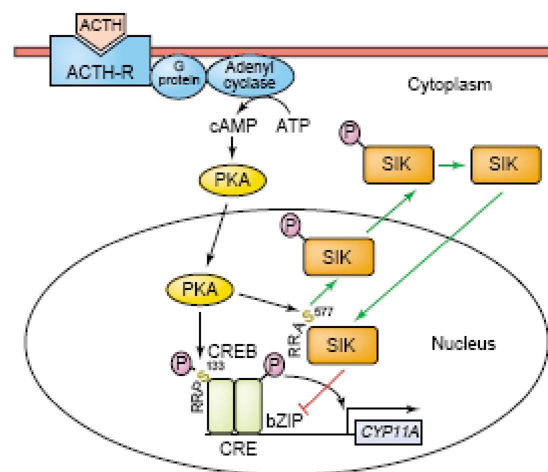


Figure 1.7. SIK1 activity on steroidogenic gene expression (Okamoto *et al*., 2004)

1.7.2. SIK2

SIK2 is a 931 amino acid protein (Figure 1.8) that has a 78 per cent amino acid similarity with SIK1 in the KD, 70 per cent in the SNH domain and 73 per cent in the phosphorylation domain (Katoh *et al.,* 2004).

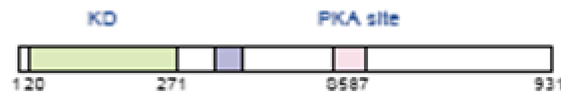


Figure 1.8. Schematic representation of SIK2 structure (Okamoto *et al*., 2004)

SIK2 is highly expressed in mature adipocytes and was shown to phosphorylate a specific serine residue in insulin receptor substrate-1 (IRS-1) both in 3T3-L1 adipocytes and COS-7 cells (Horike *et al*., 2003). It is known that insulin dependent tyrosine phosphorylation of IRS-1 by insulin receptor initiates signaling cascade downstream (Figure 1.9), however, phosphorylation of S789 of IRS1 leads to attenuation of insulin signaling either by preventing docking of downstream effectors or by enhancing IRS-1 degradation (Gual *et al*., 2005). Phosphorylation of S789 of IRS1 is increased in insulin resistant rats and interestingly, SIK2 expression and activity is shown to be elevated in the white adipose tissues of diabetic mice suggesting involvement of SIK2 in development of type-2 diabetes (Horike *et al*., 2003).

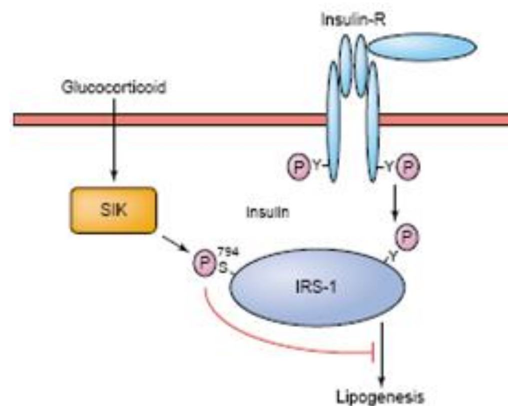


Figure 1.9. SIK2 involvement in insulin signaling (Okamoto *et al*., 2004)

Another substrate of SIK2 identified to date is TORC2 in pancreatic islet cells (Screaton *et al.,* 2004). Under resting conditions, TORC2 is known to be sequestered in the cytoplasm via a phosphorylation-dependent interaction with 14-3-3 proteins. The dephosphorylation of TORC2, a CREB coactivator, and its subsequent translocation into nucleus upregulates CREB-dependent transcription. SIK2 was proposed to phosphorylate TORC2 at S171 and cause its sequestering in cytoplasm (Figure 1. 10) and thereby inhibiting CREB-dependent gene transcription (Screaton *et al*., 2004). In the proposed model, SIK2 was suggested to be inhibited by phosphorylation via PKA.

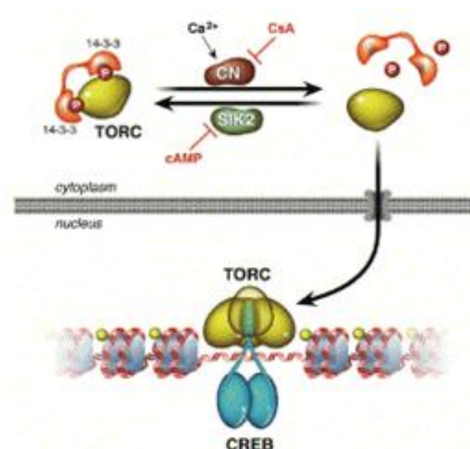


Figure 1.10. SIK2 involvement in modulation of TORC2 activity (Screaton *et al*., 2004)

LKB1 was shown to phosphorylate SIK2 at T172 residue in the activation loop and suggested to increase its activity 30 fold in vitro (Lizcano *et al.,* 2004). PKA

phosphorylation domain lies between amino acid residues 577-623 and S587 phosphorylation was shown to mark SIK2 for nuclear export (Horike *et al*., 2003).

Rat SIK2, cloned from retina (Uysal, 2005; Özcan, 2003), shows 94 per cent overall identity to mouse and 89.3 per cent to human orthologs. The kinase domain was found to be 98 per cent conserved. SIK2 gene consists of 16 exons and alternative splicing within the last exon gives rise to 3 transcripts encoding 2 proteins varying in their C-terminal regions (Uysal, 2005). The protein has putative SH2-, SH3-binding sites in addition to serine, threonine and tyrosine phosphorylation sites. Moreover, SIK2 was shown to be modulated by phosphorylation and nuclear translocation in response to both PKA and FGF9. Possible involvement of SIK2 as a modulator of Ras/ERK signaling was also suggested (Küser 2006; Özmen, 2006). Gab1 and A-Raf, which have major roles in Ras/ERK pathway, such as defining the level and duration of ERK activity, were found to be phosphorylated by SIK2 in vitro (Küser, 2006).

1.7.3. SIK3

SIK3 is a 1263 amino acid protein which has 68 per cent amino acid similarity with SIK1 in kinase domain, 37 per cent in SNH domain and 47 per cent in phosphorylation domain (Figure 1.11). It is ubiquitously expressed in humans, mice and rats but no functional data is available (Okamoto *et al*., 2004).

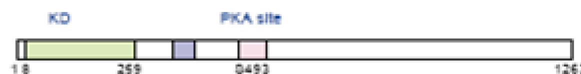


Figure 1.11. Schematic representation of SIK3 structure (Okamoto *et al.,* 2004)

2. AIM

An interplay between PKA and FGF pathways has been suggested before (Özmen, 2006; Pursiheimo *et al*., 2002a and 2002b), but little was known about the components of this integration. This study presented here aims to explore the possibility of SIK2 as a key mediator of the integration process. In this context; we

- analyzed FGF2 dependent serine/threonine phosphorylation of SIK2 and the effect of PKA in this event.
- studied the effect of PKA on Ras/ERK pathway stimulated by FGF2 in MIO-M1 cells.
- studied the modulation of FGF2 dependent proliferation of MIO-M1 cells by PKA,
- investigated the intracellular localization of SIK2 in response to FGF2 induction and its modulation upon PKA activation/inhibition.
- investigated the expression profile of SIK2 in response to FGF2 and PKA activation/inhibition.

3. MATERIALS

3.1. Chemicals

All chemicals used in this study were obtained from Sigma Aldrich (USA) or Merck (Germany) unless stated otherwise in the text. All solutions, plastic and glassware were sterilized by autoclaving at $121^{0}C$ for 20 minutes when possible. Solutions used in RNA protocols, when possible, were diethylpyrocarbonate (DEPC) treated for 1 hour before autoclaving and glassware was baked at $150^{0}C$ for 4 hours. Buffer compositions are given in Table 3.1 through Table 3.3.

Table 3.1. Buffers and solutions for cell culture and assays

Complete Medium for MIO-M1 Cells	Dulbeco's Modified Eagle Medium (DMEM) with glutamax (Invitrogen, USA) supplemented with 10% Fetal bovine serum (FBS) (Biochrom, Germany) 0.1% Penicillin/streptomycin
H89 Solution	10 µM H89 in DMEM
8BrcAmp Solution	500 µM 8BrcAmp in DMEM
FGF2 Medium	DMEM supplemented with 1 ng/ml FGF2 10 µg/ml heparin
Trypsin Solution	0.05% Trypsin in phosphate buffered saline (PBS)
Chemically Defined Medium (CDM)	DMEM (Invitrogen, USA) supplemented with 1X Insulin-transferrin-sodium selenite mix 1 mM Sodium pyruvate 0.1% Penicillin/streptomycin
Fixative Solution	30% 50 mM Glycine (pH 2.0) 70% Absolute ethanol
Blocking Buffer	0.5% Bovine serum albumin (BSA) 0.1% Tween 20 in phosphate buffered saline (PBS)

Table 3.2. Buffers and solutions for immunocytochemistry

PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
Blocking Solution	1% BSA 0.1% Tween 20 in PBS

Table 3.3. Buffers and solutions for Western blot analysis

8% SDS-polyacrylamide gel (running gel)	8% Acrylamide:bisacrylamide (29:1) 375 mM Tris.HCl (pH 8.8) 0.1% Sodium dodecyl sulfate (SDS) 0.1% Ammonium persulfate (APS) 0.1% N,N,N',N',-tetramethylethylenediamine (TEMED)
12.5% SDS- polyacrylamide gel (running gel)	12.5% Acrylamide:bisacrylamide (29:1) 375 mM Tris.HCl (pH 8.8) 0.1% SDS 0.1% APS 0.1% TEMED
5% SDS- polyacrylamide gel (stacking gel)	5% Acrylamide:bisacrylamide (29:1) 125 mM Tris.HCl (pH 6.8) 0.1% SDS 0.1% APS 0.1% TEMED
Protein Sample Buffer	2% SDS 80 mM Tris.HCl (pH 6.8) 20% Glycerol 10% β-mercaptoethanol 0.005% Bromophenol blue

Table 3.3. Buffers and solutions for Western blot analysis (continued)

Running Buffer	25 mM Tris.HCl 250 mM Glycine 0.2% SDS
Transfer Buffer	200 mM Glycine 25 mM Tris.HCl 15% Methanol
Coomassie Blue Solution	50% Methanol 0.05% Coomassie R250 10% Acetic acid
Fixing Solution	50% Methanol 10% Acetic acid
Destaining Solution	5% Methanol 7% Acetic acid
Tris Buffered Saline with Tween 20 (TBST)	150 mM NaCl 20 mM Tris.HCl (pH 8.0) 0.1% Tween 20
Blocking Solution	1% BSA in 0.1% TBST
Stripping Solution	62.5 mM Tris.HCl, pH 6.8 2% SDS 0.7% β-mercaptoethanol

Table 3.4. Buffers and solutions for Reverse Transcriptase coupled Polymerase Chain Reaction (RT-PCR)

Reverse Transcriptase (RT) Buffer	50 mM Tris.HCl (pH 8.3) 75 mM KCl 3 mM $MgCl_2$ 10 mM Dithiothreitol (DTT)
Taq Polymerase Buffer	10 mM Tris.HCl (pH 9.5) 50 mM KCl 0.1% TritonX-100
6X Loading Buffer	30% Glycerol 0.005% Bromophenol blue
TAE Buffer	40 mM Tris.HCl 1 mM Ethylenediaminetetraacetic acid (EDTA) 0.1% Acetic acid

Table 3.5. Buffers and solutions for immunoprecipitation

NP-40 Cell Lysis Buffer	50 mM Tris-HCl (pH 8.0) 150 mM NaCl 1% NP-40

Table 3.6. Antibodies used in this study. G: Gift of Dr. Hiroshi Takemori

Epitope	Supplier	Origin-modification	Application	Dilution
Phospho-T182 (pT182)	G	Rabbit	Western blotting	1:1000
Phosphoserine (pSer)	Zymed, USA	Rabbit	Western blotting	1:1000
SIK2	Novagen, Taiwan	Rabbit	Western blotting	1:1000
			Immunocytochemistry	1:250
			Immunoprecipitation	1:125

Table 3.6. Antibodies used in this study (continued).

5-bromo-2-deoxyuridine (BrdU)	Roche, Germany	Mouse – flourescein isothiocyanate (FITC) conjugated	Cell proliferation assay	1:500
ERK	Santa Cruz, USA	Rabbit	Western blotting	1:1000
pERK	Santa Cruz, USA	Mouse	Western blotting	1:1000
Flourescent secondary	Santa Cruz, USA	Rabbit – Texas Red (TR) conjugated	Immunocytochemistry	1:5000
Secondary	Santa Cruz, USA	Rabbit – Horseradish peroxidase (HRP) conjugated	Western blotting	1:5000
		Mouse – HRP conjugated		

3.2. Cell Lines

Spontaneously immortalized human Muller glia cell line (MIO-M1) was kindly provided by Dr. A. Limb (Moorfields Institute of Ophthalmology, London).

3.3. Kits

Total RNA was isolated from cell lines with RNeasy Mini Kit (Qiagen; Hilden, Germany). For quantitative Real Time PCR (qRT-PCR), Light Cycler Fast Start DNA Master SYBR Green I kit was purchased from Roche (Mannheim, Germany). For cell proliferation assay, In Situ Cell Proliferation Assay, FLUOS was used (Roche; Mannheim, Germany).

3.4. Equipment

Autoclave	Midas 55, Prior Clave, UK
Balances	DTBH 210, Sartorius, GERMANY
	Electronic Balance VA 124, Gec Avery, UK
Blotting apparatus	Mini Trans-Blot Cell, Bio-Rad, ITALY
Carbon dioxide tank	2091, Habaş, TURKEY
CCD camera	CCD Camera, JAI Corporation, JAPAN
CO_2 incubator	WTB Binder, GERMANY
Centrifuges	ProFuge 10K, Stratagene, USA
	Mini Centrifuge 17307-05, Cole Parmer, USA
	Genofuge 16M, Techne, UK
	Centurion K40R, UK
	Centrifuge B5, B. Braun Biotech International, GERMANY
	Centrifuge 5415R, Eppendorf, USA
Deep freezers	-20°C, Arçelik, TURKEY
	-70°C Freezer, Harris, UK
	-86°C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio-Doc, ITALY
Electrophoresis Systems	Easi-cast system, Hybaid, UK
	Mini-Protean III Cell, Bio-Rad, ITALY
Filters	DAPI Chroma 11000, GERMANY
	FITC Chroma 41001, GERMANY
	Texas Red Chroma 41004, GERMANY
Hand tally counter	Milky Way Counter, TAIWAN
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
Magnetic Stirrers	M221 Elektro-mag, TURKEY
	Clifton Hotplate Magnetic Stirrer, HS31, UK

Micropipettes	Gilson, FRANCE
Microscopes	B3000, Prior, UK
	CM110 Inverted Microscope, Prior, UK
	VE-3 Dissecting Microscope, Olympus, JAPAN
	Zeiss Axioscope, GERMANY
Microwave oven	M1733N, Samsung, MALAYSIA
pH meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY
Power Supply	PS250, Hybaid, UK
	PowerPac Basic, Bio-Rad, ITALY
Real Time PCR	LightCycler 1.5, Roche, 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
Software	Isis digital FISH imaging system, Metasystems, GERMANY
	Quantity One, Bio-Rad, ITALY
	Light Cycler 4.0 Analysis Software, Roche, GERMANY
	ImageJ, Image Analysis Software, (http://rsb.info.nih.gov/ij/)
Spectrophotometer	CE5502, Cecil, UK
	NanoDrop 1000, USA
Thermocyclers	MyCycler, Bio-Rad, ITALY
	PTC-200, MJ Research, USA
	Gene Amp. PCR System 2400, Perkin Elmer, USA
Vacuum pump	KNF Neuberger, USA
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water baths	TE-10A, Techne, UK
X-Ray film cassettes	24x30 IMS, ITALY
	24x30 DIA-X, GERMANY

4. METHODS

4.1. Cell Cultures

4.1.1. Maintenance of MIO-M1 Cells

Spontaneously immortalized MIO-M1 Muller glia cells were maintained in DMEM with glutamine supplemented with 10 per cent FBS and 0.1 per cent penicillin/streptomycin. When the plates reached confluence, the cells were washed with PBS, treated with 0.05 per cent trypsin solution for 3 minutes and scraped. The cells were pelleted by centrifugation at 2000 x g for 5 minutes and after resuspension in complete medium they were divided into three plates once a week.

4.1.2. Treatment of MIO-M1 Cells

Cells were seeded on 10 cm tissue culture dishes and were allowed to grow to sub-confluency. The cells were washed with PBS and starved in DMEM (Invitrogen, USA) and 0.1 per cent penicillin/streptomycin overnight. Subsequently, they were treated with 1 ng/ml FGF2 and 10 μg/ml heparin for 0 minutes (negative control), 5, 10, 30 minutes and 1 hour, they were immediately washed with ice-cold PBS with 1 mM sodium orthovanadate (Na_3VO_4), a phosphatase inhibitor. The cells were then scraped and collected into microfuge tubes, pelleted with centrifugation and stored at -70°C until used. In the studies involving PKA activation/ inhibition, the cells were incubated with 8BrcAMP or with H89 30 minutes prior to the addition of FGF2 and heparin. The final concentration of 8BrcAMP was 500μM and that of H89 was 10μM.

4.1.3. Immunocytochemistry

Cultured MIO-M1 cells were seeded on round coverslips in a 24-well tissue culture dish and were grown to near-confluency. The cells were washed with PBS, fixed in 4 per cent paraformaldehyde (pH7.2) for 15 minutes and permeabilized with 0.1 per cent Triton X-100 in PBS for 5 minutes. After washing with PBS, the cells were incubated in blocking

solution for 15 minutes. Primary antibody incubation was done overnight at 4°C with 1:250 dilutions. Cells were then washed with PBS and incubated with TR-conjugated secondary antibody diluted 1:1000 in the blocking solution for 1 hour at room temperature in the dark and for the visualization of nuclei 4'-6-diamidino-2-phenylindole (DAPI) was added to the secondary antibody solution at a dilution of 1:5000. Antibody dilutions are given in Table 3.6. Observations were done under fluorescent microscope.

4.2. Reverse Transcription Coupled Quantitative Real-Time PCR (qRT-PCR)

4.2.1. RNA Isolation

Total RNA was isolated from MIO-M1 cells with RNeasy Mini Kit. Briefly, 10^7 cells were homogenized using 350 µl of the lysis buffer provided in the kit and vortexed. After the addition of equal volumes of 70 per cent ethanol to the tubes, the samples were applied to the RNeasy mini spin columns. Columns were washed with high salt buffers included in the kit before the RNAs were eluted from columns using 60 µl of RNAse free, DEPC treated water. The concentrations of RNA samples were measured using Nanodrop spectrophotometer. The integrity of RNAs were determined by agarose gel electrophoresis.

4.2.2. cDNA Synthesis

cDNA was synthesized from 2 µg of total RNA in a reaction mixture containing 500 ng of random hexamer primers, 750 nmol of deoxyribonucleotide triphosphate (dNTP), 12 units of RNasin Plus RNase inhibitor (Promega, USA) in reverse transcription buffer. The mixture was heated to 70°C for 5 minutes for denaturation and immediately chilled on ice. Subsequently, 200 units of M-MLV reverse transcriptase (Promega, USA) and 20 units of RNasin Plus RNase inhibitor was added and the volume was brought up to 30 µl with DEPC treated water. The mixture was first incubated at 25°C for 10 minutes to anneal random hexamer primers (Promega, USA) to RNA and the synthesis was carried out at 37°C for 2 hours. The enzyme was inactivated by heating the reaction mixture to 95°C for 10 minutes, the volume was brought up to 360µl in order to have a 1:12 final dilution. The samples were stored at - 70°C until used.

4.2.3. PCR

cDNAs were checked by PCR using human β-actin primers. Amplifications were carried out with 6 μl of cDNA, synthesized and diluted as above, 0.2 mM of dNTPs, 0.2 μM of each primer, 3 mM of $MgCl_2$ and 0.6 units of Taq DNA polymerase (Fermentas, Lithuania) in Taq polymerase reaction buffer in a total volume of 25 μl. The reaction program consisted of an initial denaturation at 95°C for 2 minutes, 30 amplification cycles and a final extension at 72°C for 5 minutes. Each amplification cycle contains a denaturation step at 95°C for 30 seconds, an annealing step at 55°C for 30 seconds and an extension step at 72°C for 30 seconds. Amplification products were checked on 2 per cent agarose gel. The list of primers and their sequences are given in Table 4.1.

Table 4.1. Primers used in the study

β-actin	Upper	AAGATCAAGATCATTGCTCCTC
	Lower	GGGTGTAACGCAACTAAGTC
SIK2	Upper	TTGCTGAACAAACAGTTGCC
	Lower	TCAAGCAGACAGCCATTCAC

4.2.4. qRT-PCR

Reaction mixtures of target and reference genes were prepared using 10 μl of 2x SYBR Premix Ex Taq (Takara, Japan), 1 μl of each primers (5 μM each), 2 μl of PCR grade dH_2O and 6 μl of cDNA prepared as indicated above. For negative controls, cDNA was replaced with PCR grade dH_2O. The reaction program contained an initiation step at 50°C for 2 minutes, followed by an initial denaturation step at 95°C for 10 seconds, 35 amplification cycles, a melting curve construction step and a cooling step at 40°C for 30 seconds. Each amplification cycle consists of a denaturation step at 95°C for 5 seconds, an annealing step at 55°C for 10 seconds and an extension step at 72°C for 10 seconds. Data were analyzed by LinRegPCR software 7.2 (Ramakers et al., 2003).

4.2.5. Agarose Gel Electrophoresis

Isolated RNAs were mixed with 6x loading buffer and loaded onto 2 per cent agarose gel containing 0.5 µg/ml ethidium bromide. The gel was run in 1X TAE buffer at 90 V. The images were documented with BioRad XR system.

Amplification products were processed same as described above except the gels were run at 120 V.

4.3. Immunoprecipitation

After growth factor treatments, approximately 10^7 cells were harvested. The cell pellet was resuspended in 1 ml of cold lysis buffer containing 1X Protease Inhibitor Cocktail (Roche, Germany. The cells were kept on ice for 30 minutes with occasional mixing, spinned at 10000 xg for 15 minutes at 4°C, and the supernatant was collected.

A 50 µl aliquot of Protein A agarose beads (Roche, Germany) was equilibrated with washing the beads with 450 µl cold lysis buffer and spinned at 10000 xg for 30 seconds. The washing was repeated, the beads were resuspended in 50 µl of cold lysis buffer. This Protein A slurry and 500 µl of cell lysate were mixed and incubated on ice for 1 hour. The mixture was then spinned at 10000 xg for 10 minutes at 4°C and the supernatant was transfered carefully to a fresh tube. Ten µg of antibody was added and the tube was incubated at 4°C for 1 hour. Subsequently, 50 µl of Protein A slurry, prepared as before, was added and incubation was carried out for 1 hour at 4°C on a rocking platform. The mixture was spinned at 10000 xg for 30 seconds at 4°C to collect the beads. After washing the beads four times with 500 µl of lysis buffer, 1X protein sample buffer was added. Beads were vortexed, boiled at 95°C for 10 minutes and spinned at 10000 xg for 5 minutes. The supernatant was collected and loaded on 8 per cent SDS-polyacrylamide gel.

4.4. SDS-PAGE and Western Blotting

Protein extracts were obtained by sonicating cells in protein sample buffer. Cell debris was pelleted by centrifugation at 12000 xg and the supernatant was boiled for 5

minutes before loading onto polyacrylamide gels. The gels were run in running buffer at 130 V. For Western blotting, the samples resolved on polyacrylamide gels were electroblotted to polyvinyl difluoride (PVDF) membranes (Roche, Germany) in transfer buffer at 100 V for 1.5-2 hours depending on the gel concentration. The membranes were washed in TBST solution for 30 minutes and incubated in blocking solution for 1 hour at room temperature in order to mask nonspecific binding. The membranes were incubated overnight in the blocking solution containing the appropriate primary antibodies, the antibody dilutions were given in Table 3.6. Subsequently, the membranes were washed with TBST solution for 30 minutes and incubated for 1 hour in blocking solution containing HRP-conjugated secondary antibodies at final dilution of 1:5000. The washed membranes were incubated in Lumi-light Western blotting substrate (Roche, Germany) for 5 minutes before exposure to chemiluminescence detection film (Roche, Germany) for varying times. The bands were visualized by immersing the film sequentially in the developer solution (Kodak, USA) for 30 seconds-3 minutes, tap water and the fixer solution (Kodak, USA) for 3 minutes.

In order to reuse the membranes, bound antibodies were removed by incubating the blot in stripping solution for 30 minutes at 70°C. Next, the membranes were washed with TBST and incubated in Lumi-light Western blotting substrate and exposed to chemiluminescence detection film to confirm the removal of antibodies. The membranes were washed with TBST and reprobed.

4.5. Bradford Assay

Dye reagent concentrate (BioRad, Italy) was diluted 1:5 and filtered through a Whatman paper. Dilutions of BSA used as a protein standard was prepared ranging from 0.2 mg/ml to 1.2 mg/ml with 0.2 mg intervals. Twenty µl of each standard and sample solution were added into tubes containing 1 ml dye reagent and vortexed. After incubating for 5 minutes at room temperature, the absorbance at 595 nm were measured.

4.6. Cell Proliferation Assay

Cultured MIO-M1 cells were seeded on round coverslips in a 24-well tissue culture dish with a density of 1.5 x 10^3 cells/mm^2 and were grown for 2 days. After washing with PBS, the cells were incubated in CDM containing 10 μM BrdU for 1 hour. The cells were treated with 1 ng/ml FGF2, 500 μM 8BrcAMP or 10 μM H89 with 10 μg/ml heparin in CDM for two days. When the cells were treated with both PKA activator/inhibitor and FGFs, activator/inhibitor treatments were done 30 minutes prior to the addition of FGF2 and heparin.

To detect BrdU incorporation, the cells were fixed and washed twice with PBS. They were permeabilized with trypsin solution for 8 minutes at 37°C and treated with 4 M HCl for 10 minutes at room temperature for denaturation. The cells were washed several times with PBS to brought pH of the medium above 6.5. After incubation with blocking solution for 10 minutes, the cells were incubated in FITC-conjugated anti-BrdU antibody for 1 hour at room temperature in the dark. To visualize the nuclei cells were incubated with DAPI for 10 minutes. After washing twice with PBS, the cells were observed under fluorescent microscope and counted. Antibody dilutions are given in Table 3.6.

5. RESULTS

5.1. Phosphorylation of SIK2 in Response to FGF2 Induction and PKA Activation/Inhibition

Analysis of phosphorylation status of SIK2 with the available phospho-specific antisera necessitated immunoprecipitation of the protein prior to Western bloting. The blots prepared with FGF2 treated cells for 10 min and probed with the same antibody used in immunoprecipitations revealed four different bands at 120 kDa, 100 kDa, 72 kDa and 55 kDa (Figure 5.1A), in agreement with our earlier results (Küser, unpublished data). Predicted size of SIK2 from the cDNA clones is 120 kDa, the lower bands are possibly due to degradation or specific proteolytic cleavage. Antibody specific for threonine phosphorylated SIK2 reveals the same bands (Figure 5.1A). However, in later experiments, only a 55 kDa band was detected (Figure 5.1B), probably due to epitope masking or inefficient transfer of large proteins. This 55 kDa band was also detected by another anti-SIK2 antibody raised against a different epitope on SIK2.

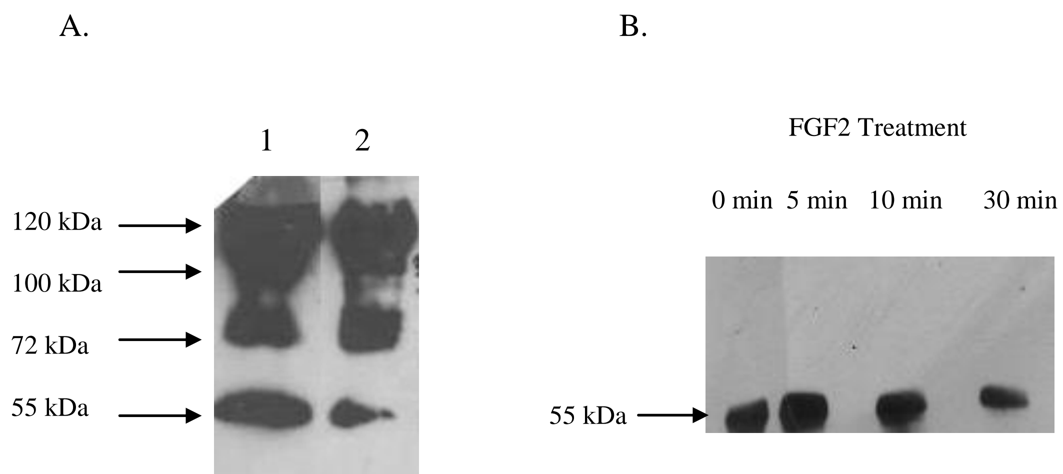


Figure 5.1. Western blot analysis of immunoprecipitated SIK2. A: Anti-SIK2 antibody immunoprecipitated material was subjected to Western blot analysis and probed with the same antibody (first lane) or with anti-pThr antibody (second lane), B: 55 kDa band obtained using anti-SIK2 antibody.

To observe changes in SIK2 threonine/serine phosphorylation levels as a result of stimuli SIK2 was immunoprecipitated from FGF2 and/or PKA activator treated MIO-M1 cell lysates using anti-SIK2 antibody and analyzed by Western blotting using anti-pT182 or anti-pSer primary antibodies. Subsequent to the chemiluminescent detections, the blots were stripped and probed with anti-SIK2 antibody. The densitometric readings from phospho antibodies were normalized to that of internal SIK2 levels.

The experiments indicate that the level of threonine phosphorylation was dropped at 5 minutes of FGF2 induction, then doubled at 10 minutes and subsequently a gradual return to basal levels was observed (Figure 5.2). When the same samples were probed with anti-phosphoserine antibody, we observed an initial modest increase. This was followed by a decrease in serine phosphorylated SIK2 levels, at 30 minute treatments the levels are at the lowest, then within 60 minutes return to that of untreated cells (Figure 5.3).

When MIO-M1 cells were treated with PKA activator, 8BrcAMP, 30 minutes prior to induction with FGF2, phosphoserine levels of SIK2 are increased until 30 minutes and then maintained until one hour. Activation of PKA in MIO-M1 cells seems to increase serine phosphorylation of SIK2 (Figure 5.4).

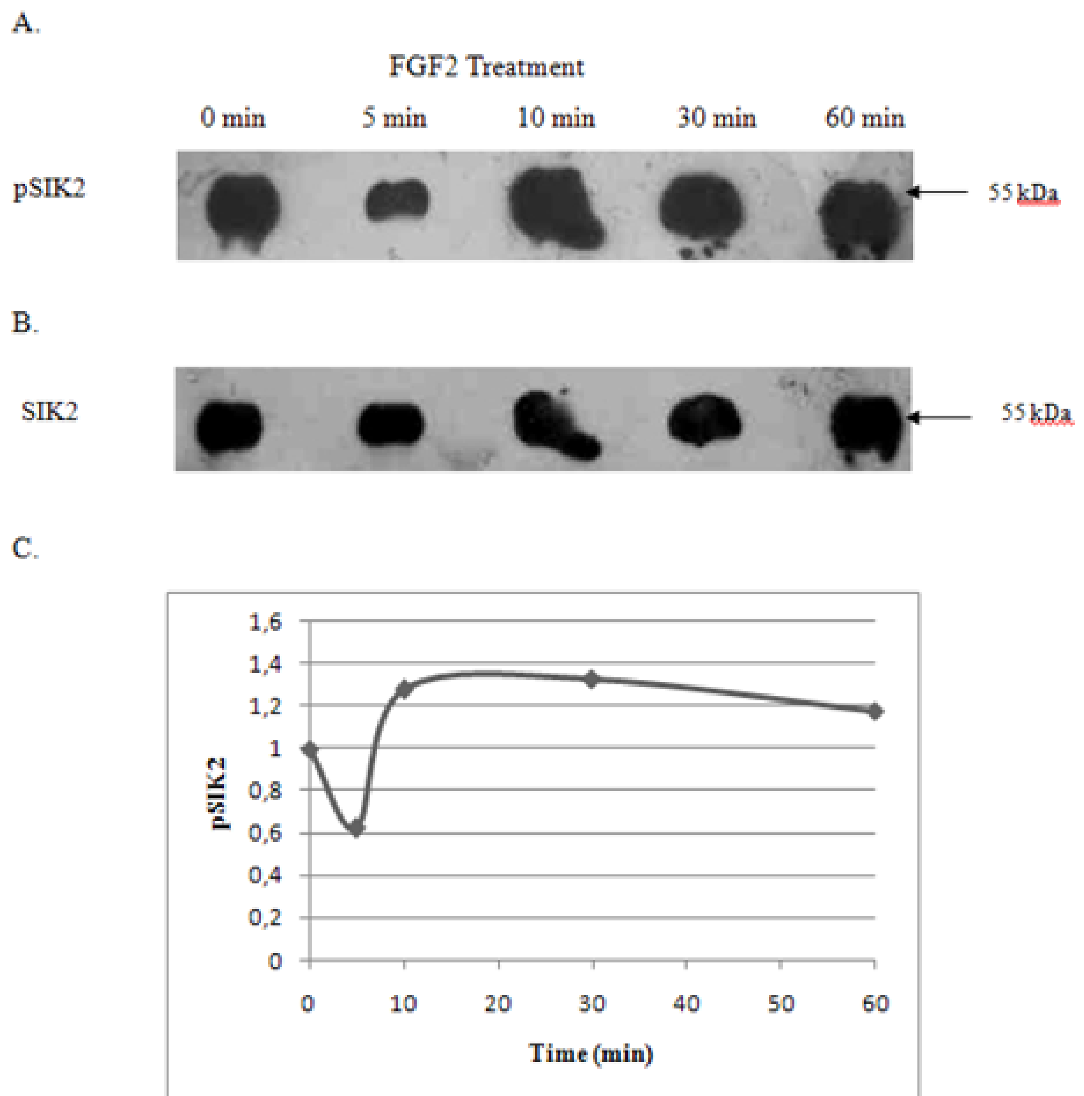


Figure 5.2. Phosphorylation of SIK2 at T182 residue in FGF2 induced MIO-M1 cells. The cells were treated with FGF2 for 5-60 minute durations, lysates were subjected to immunoprecipitation with anti-SIK2 antibody and the Western blots were probed with anti-pT182 antibody (A), following the chemiluminescent detection, stripped membranes were probed with anti-SIK2 antibody (B). Panel C shows the normalized phosphoprotein levels in arbitrary units.

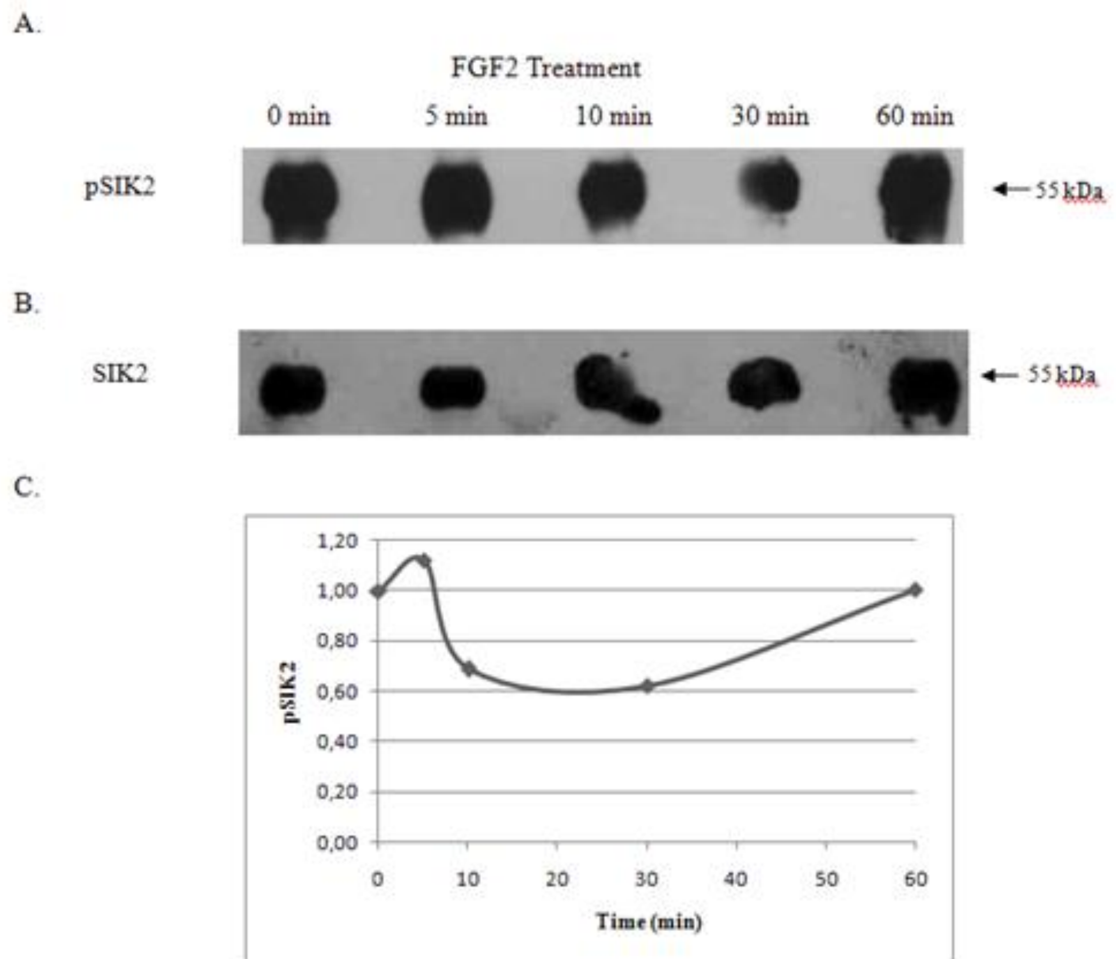


Figure 5.3. Serine phosphorylation of SIK2 in FGF2 induced MIO-M1 cells. The cells were treated with FGF2 5-60 min durations, lysates were subjected to immunoprecipitation with anti-SIK2 antibody and the Western blots were probed with anti-phosphoserine antibody (A), the stripped membranes were probed with anti-SIK2 antibody (B). Panel C shows the normalized phosphoprotein levels in arbitrary units.

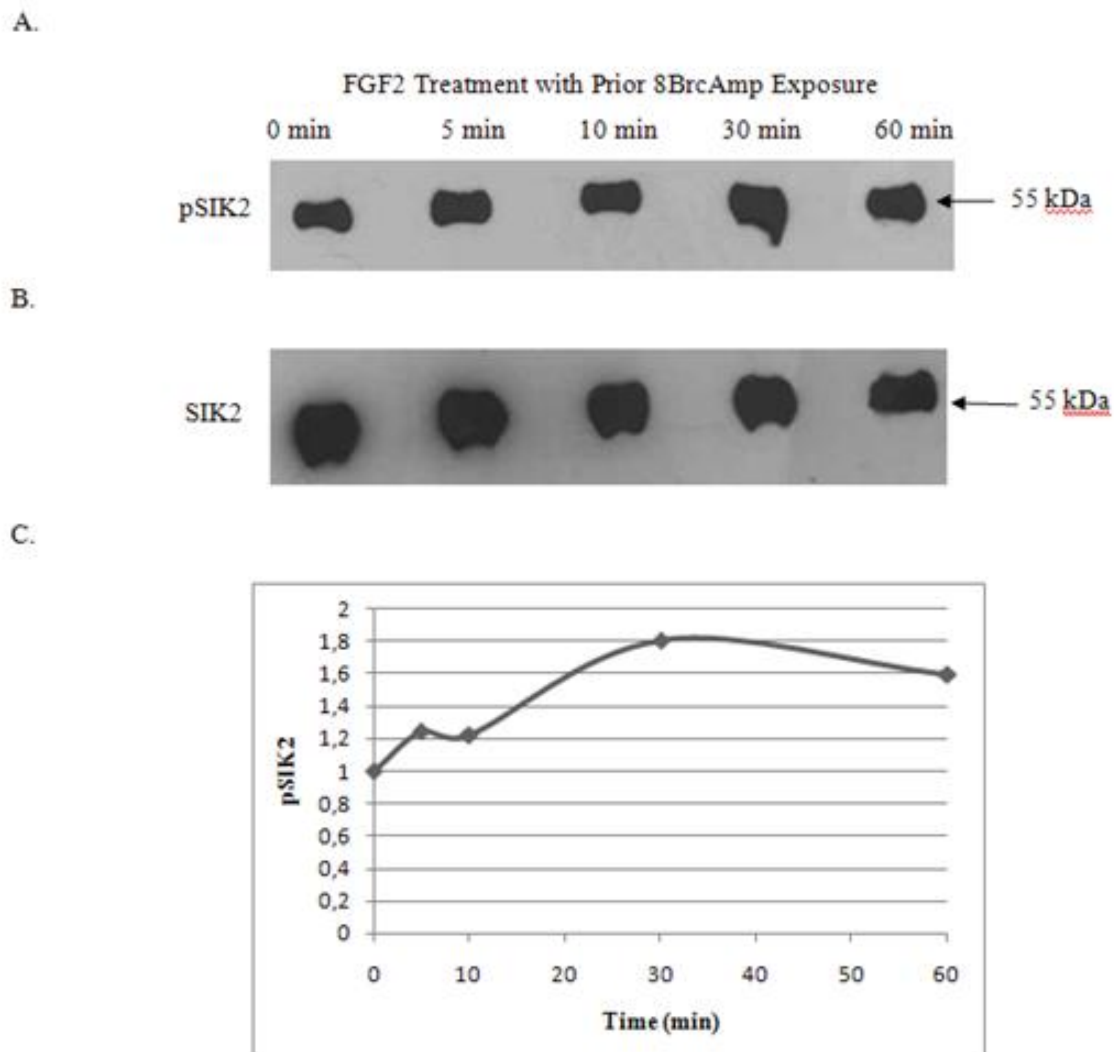


Figure 5.4. Phosphorylation of SIK2 in 8BrcAmp treated and FGF2 induced MIO-M1 cells. Lysates from ells treated for 30 min with the PKA activator prior to FGF2 induction were immunoprecipitated using anti-SIK2 antibody and Western blots were probed with anti-phosphoserine antibody (A), subsequently the stripped membranes were incubated with anti-SIK2 antibody (B). Panel C shows the normalized phosphoprotein levels in arbitrary units.

5.2. Phosphorylation of ERK in Response to FGF2 Induction and PKA Activation/Inhibition

The effect of FGF2 on MIO-M1 cells is mainly mediated by transient activation of ERK pathway (Çınaroğlu *et al*., 2005). Therefore the activation kinetics of ERK was analyzed by Western blotting of lysates from FGF2 and/or PKA activator/inhibitor treated MIO-M1 cells with anti-phosphoERK antibody in order to observe changes in ERK phosphorylation levels as a result of FGF2 induction, where the results were normalized to that of internal ERK levels.

Upon FGF2 induction of MIO-M1 cells, pERK levels were increased at around fifty per cent at 10 minutes and then the level was dropped to basal levels within 30 minutes and maintained at that level thereafter (Figure 5.5).

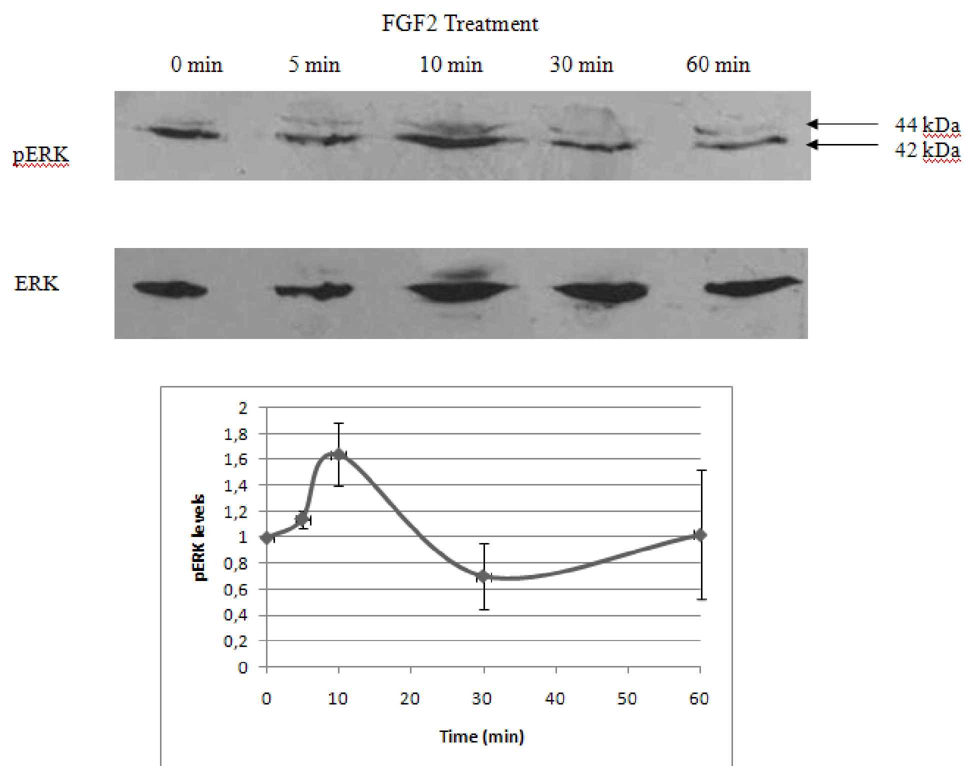


Figure 5.5. Phosphorylation of ERK in FGF2 induced MIO-M1 cells

The depletion of PKA activity by H89 prior to FGF2 treatment resulted in an increase in pERK levels and signal attenuation was appeared to be retarded (Figure 5.6). However, when PKA was activated by 8BrcAmp 30 minutes prior to FGF2 induction, the phosphorylation pattern of ERK with respect to FGF2 induced cells was not altered (Figure 5.7). The data indicated that PKA modulates signal flow through Ras/ERK pathway.

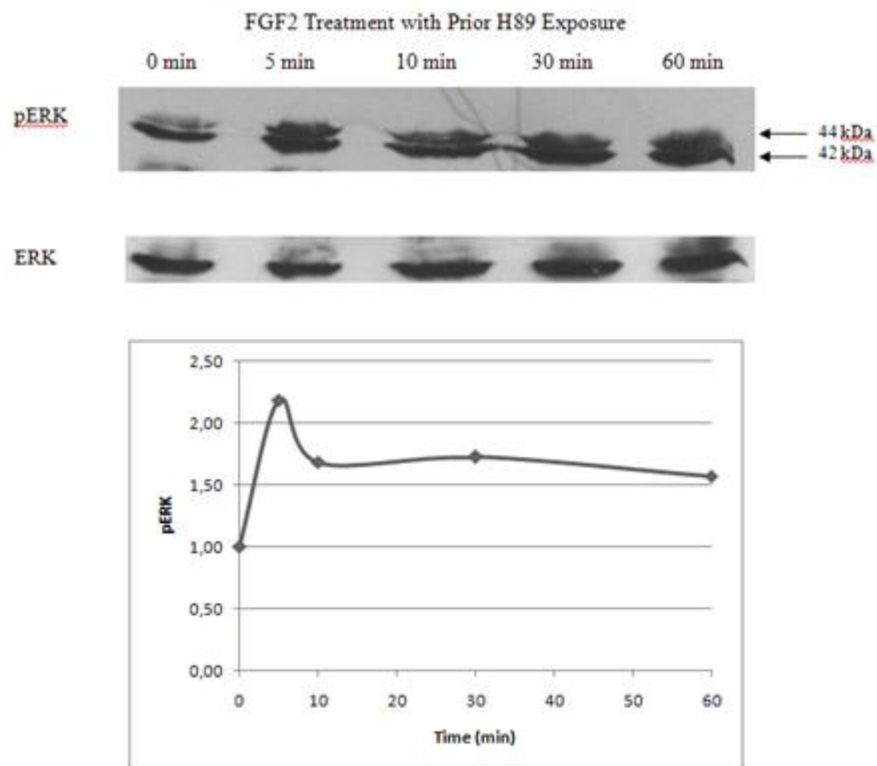


Figure 5.6. Phosphorylation of ERK in H89 pretreated and FGF2 induced MIO-M1 cells

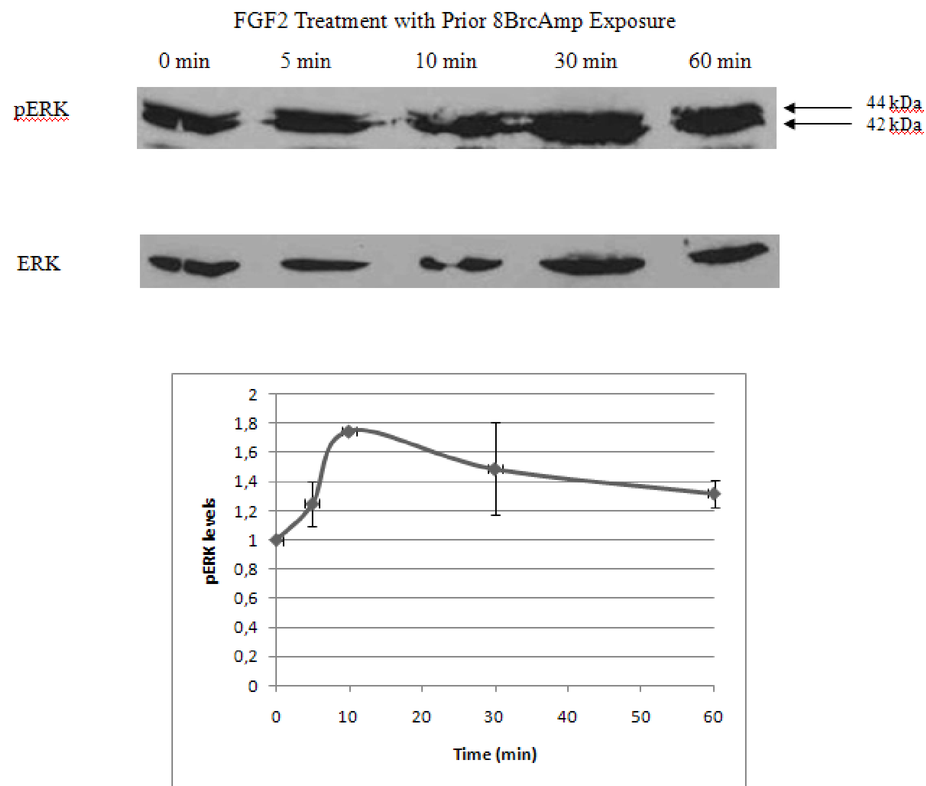


Figure 5.7. Phosphorylation of ERK in 8BrcAmp pretreated and FGF2 induced MIO-M1 cells

5.3. Proliferation of MIO-M1 Cells in Response to FGF2 Induction and PKA Activation/Inhibition

As FGF2 is known to be a potent mitogen for Müller cells, potential modifying effect of PKA on the FGF2 dependent proliferation of MIO-M1 cells were tested using BrdU incorporation assay.

In agreement with earlier findings (Çınaroğlu, 2005), upon FGF2 induction the number of cells were nearly doubled compared to the resting cells (Figure 5.8). Inhibition of PKA activity led to an increase in the proliferation of cells, whereas the activation of PKA did not altered the proliferation significantly. However, when PKA was activated prior to FGF2 induction, the proliferation of cells was decreased although it was still higher than that of control cells (Figure 5.8). Prior inhibition of PKA did not significantly

alter the proliferation of MIO-M1 cells with respect to FGF2 induced ones. Proliferation data were analyzed statistically by Analysis Of Variance (ANOVA), using Bonferroni as a post-hoc test. $p < 0.05$ was considered as statistically significant. The ANOVA gave an F value of 14.56. Bonferroni's post hoc test revealed that the changes in proliferation profile of MIO-M1 cells in response to FGF2 induction alone or with prior H89 treatment and H89 alone treatments with respect to untreated cells were significant. In contrast, proliferation profile of cells upon 8BrcAmp treatment was not changed significantly with respect to untreated cells according to the test. However, 8BrcAmp treatment prior to FGF2 induction significantly altered the proliferation of cells with respect to FGF2 induced ones.

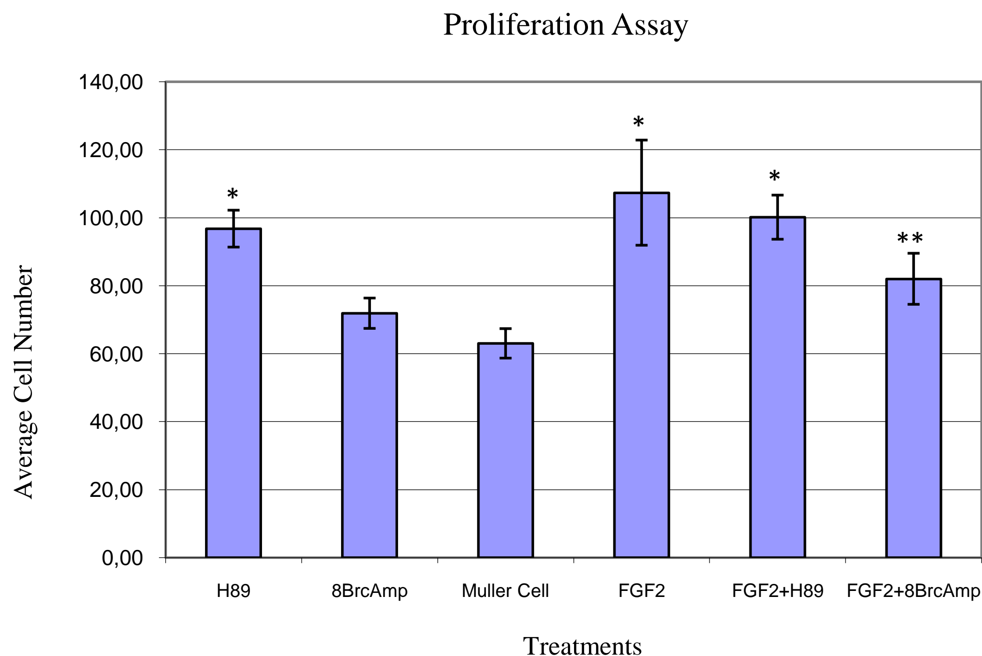


Figure 5.8. Proliferation of Muller cells treated with FGF2, PKA activator or PKA inhibitor.*$p \leq 0.001$, F= 14.56 (ANOVA test), **$p \leq 0.05$

5.4. Cellular Localization of SIK2 in Response to FGF2 Induction and PKA Activation/Inhibition

In order to investigate the potential changes in FGF2 or PKA dependent cellular localization of SIK2, sub-confluent cultures of MIO-M1 cells starved in serum-free medium, treated with 1 ng/ml FGF2 and 10 mg/ml heparin. In experiments to test the PKA

involvement, cells were exposed to PKA activator or inhibitor for 30 minutes prior to the addition of FGF2. After different durations of FGF2 induction MIO-M1 cells were analyzed by immunocytochemical stainings using anti-SIK2, where the secondary antibody was conjugated with Texas Red.

In resting cells SIK2 was observed both in cytoplasm and nucleus (Figure 5.9B). When cells were treated with PKA activator, 8BrcAmp, it was observed that SIK2 moves out of nuclei (Figure 5.9E). However, when cells were treated with PKA inhibitor, H89, cellular localization of SIK2 was similar to that in resting cells (Figure 5.9H).

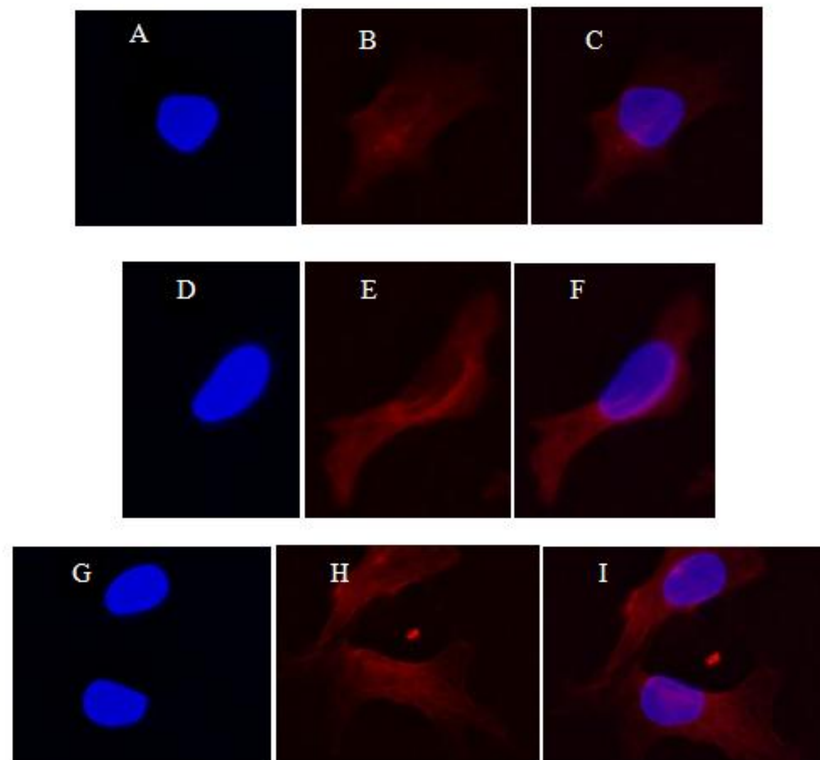


Figure 5.9. PKA dependent cellular localization of SIK2 in MIO-M1 cells. A-C: untreated cells; D-F: 8BrcAmp treated cells; G-I: H89 treated cells. The first panels show nuclei stained with DAPI; the second panels show SIK2 staining; and the third panels represent the merged images.

When cells were induced with FGF2 for 5 minutes, cellular distribution of SIK2 was similar to that of resting cells (Figure 5.10B). However, upon 10 minutes or longer FGF2 induction, SIK2 was observed to exit the nuclei (Figure 5.10E, H, K). The data suggest that both PKA and FGF2 alter nuclear localization pattern of SIK2 within MIO-M1 cells, which might be phosphorylation dependent.

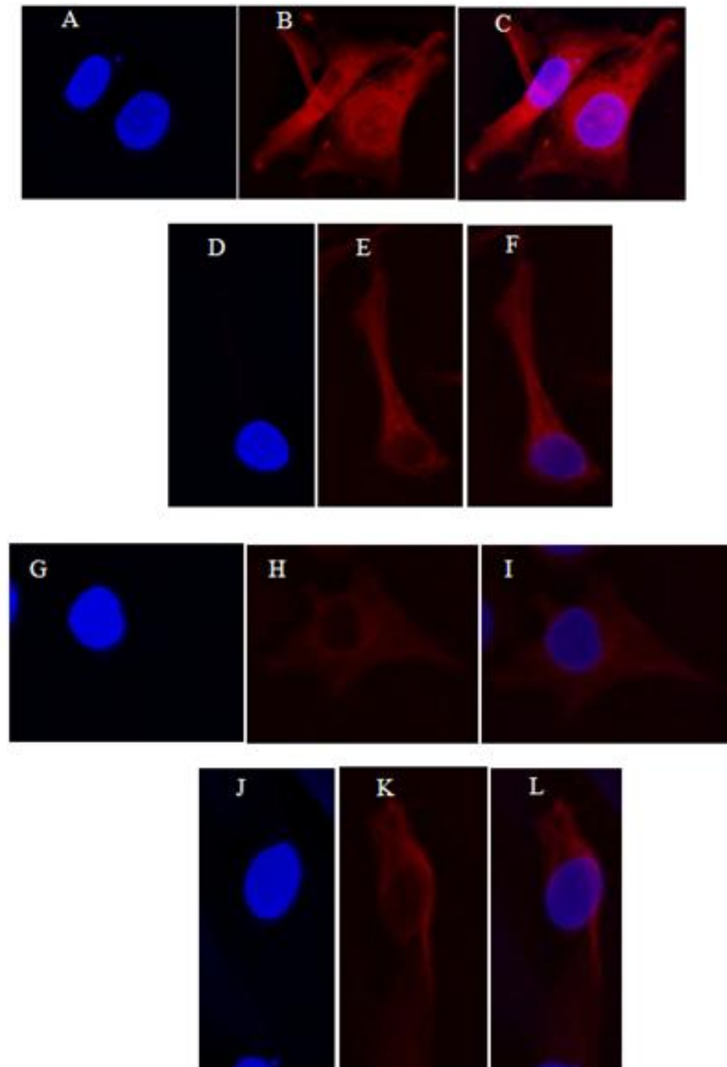


Figure 5.10. Cellular localization of SIK2 in MIO-M1 cells induced with FGF2. Cells were treated with FGF2 for 5 min (A-C), 10 min (D-F), 30 min (G-I) or 60 min (J-L). First panels show DAPI staining of nuclei; second panels show SIK2 staining and third panels represent the merged images.

In the next set of experiments, cellular localization of SIK2 was investigated upon induction of cells with FGF2 for different durations, 5-60 minutes, with prior 8BrcAmp or H89 treatments. When the cells were induced with FGF2 for 5 minutes with prior 8BrcAmp treatment, SIK2 was observed both in the nucleus and in cytoplasm (Figure 5.11B). However, SIK2 appeared to translocate to cytoplasm in the cells that were treated with FGF2 for 10 minutes or longer with prior 8BrcAmp treatment (Figure 5.11E, H, and K).

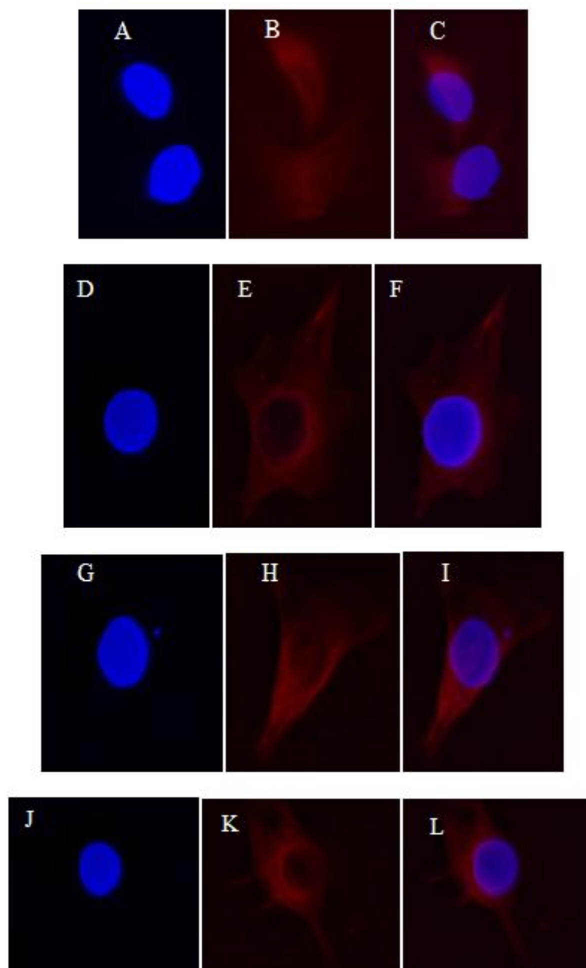


Figure 5.11. Cellular localization of SIK2 in MIO-M1 cells treated with 8BrcAmp prior to induction with FGF2. Cells were treated with 8BrcAmp for 30 minutes, subsequently induced with FGF2 for 5 minutes (A-C); for 10 minutes (D-F); for 30 minutes (G-I) and for 60 minutes (J-L). First panels show DAPI stained nuclei; second panels show SIK2 staining and third panels show the merged images.

In the cells treated with H89 before induction with FGF2 for 5 minutes or 10 minutes the cellular localization of SIK2 was shown to be similar to that in resting cells (Figure 5.12B, E). When exposure to FGF was extended to 30 minutes, SIK2 was mainly seen in cytoplasm although there are some in nucleus, as well (Figure 5.12H). However, upon 60 minutes FGF induction, SIK2 was translocated into cytoplasm in most cells but it was observed in the nucleus in a few others (Figure 5.12K). These results indicate that PKA activity can modulate FGF2 dependent cellular distribution of SIK2 in a time dependent manner.

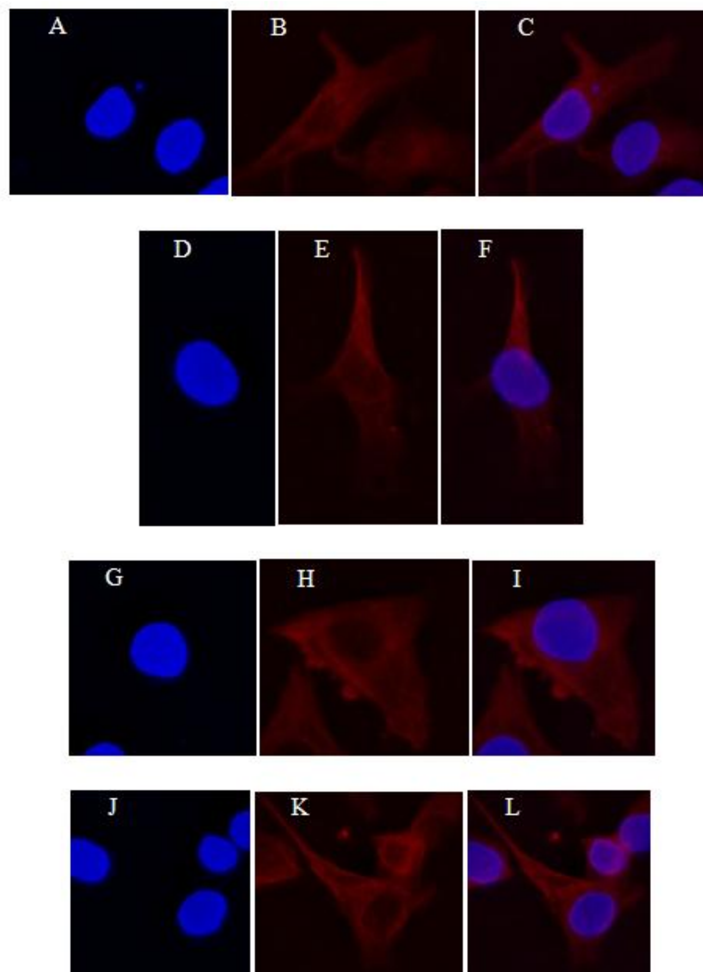


Figure 5.12. Cellular localization of SIK2 in MIO-M1 cells treated with H89 prior to induction with FGF2. Cells were treated with H89 for 30 minutes , subsequently induced with FGF2 for 5 minutes (A-C); for 10 minutes (D-F); for 30 minutes (G-I) and for 60 minutes (J-L). First panels show DAPI stained nuclei; second panels show SIK2 staining and third panels show the merged images.

5.5. Expression Profile of SIK2 in Response to FGF2 induction and PKA activation/inhibition in MIO-M1 Cells

In order to investigate potential modulation of steady state levels of SIK2 transcripts by FGF2 and possible involvement of PKA, cells were treated as described earlier, and transcript levels were assayed by qRT-PCR. The qualities of starting RNAs are shown in Figure 5.13A, where no degradation is evident and quantities in different samples are comparable. cDNAs were checked by conventional PCR with β-actin primers and an expected 185 bp band was amplified from each cDNA (Figure 5.13B). Panel C in 5.5 represents an agarose gel profile of amplification with SIK2 specific primers where a single fragment of expected 210 bp amplification product is seen. No amplification products were detectable in controls where reverse transcription step was omitted (data not shown).

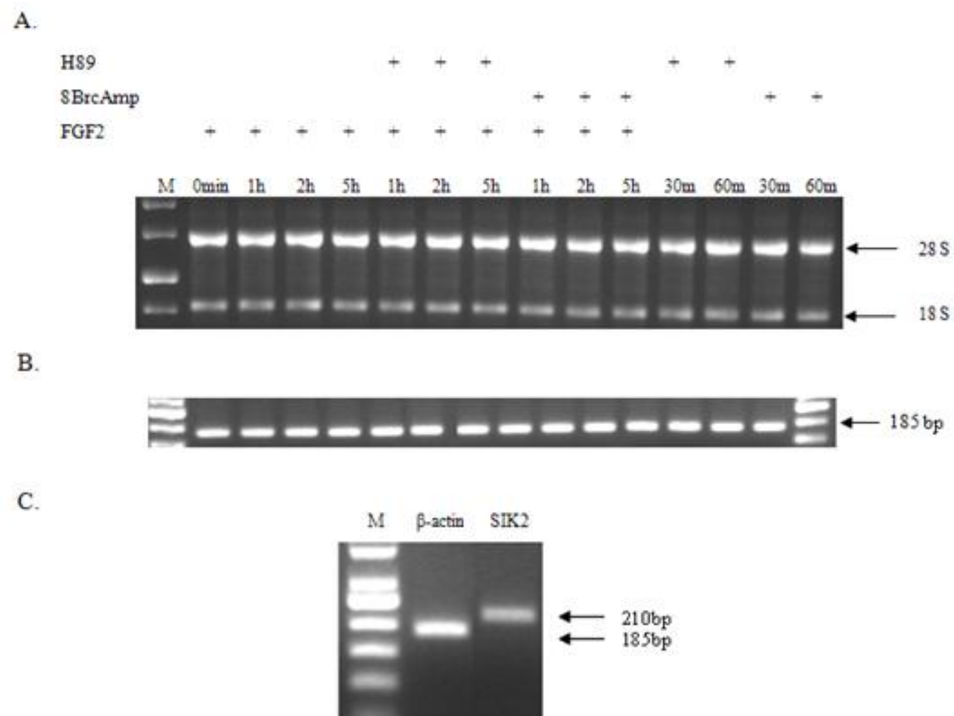
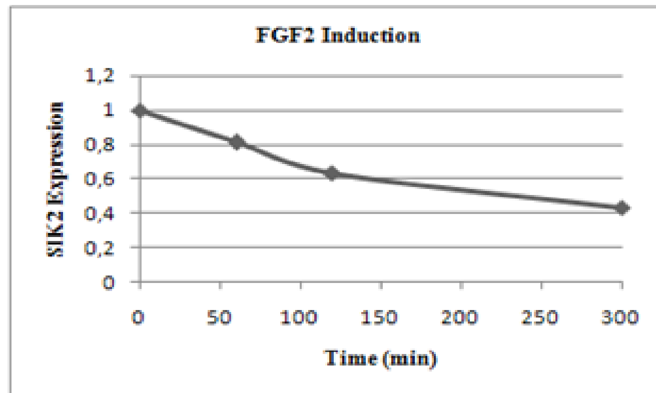


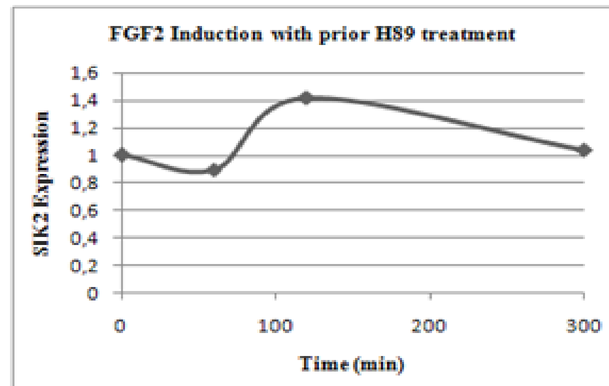
Figure 5.13. RNA quality and amplification product of SIK2. Panel A: total RNA samples isolated from the MIO-M1 after the indicated treatments run on agarose gels; B: PCR products of cDNAs amplified with β-actin primers; C: RT-PCR product obtained with the SIK2 specific primers.

For comparative analysis in the same samples actin was also assayed in real time PCR and its levels were used to normalize the SIK2 transcript levels. Normalized readings indicate that upon FGF2 treatment, the expression of SIK2 decreased steadily with time (Figure 5.14A). However, when the cells were treated with PKA inhibitor, H89, prior to FGF2 induction, expression of SIK2 was seemed to increase within 2 hours and then drops to basal levels at 5 hours (Figure 5.14B). In contrast, SIK2 expression was dropped, reaching minimum levels at 2 hours when the cells were treated with PKA activator, 8BrcAmp, prior to induction with FGF2; and then recovered at 5 hours (Figure 5.14C). The results indicate that the SIK2 expression can be downregulated by FGF2 as well as by PKA, which may represent a feedback mechanism.

A.



B.



C.

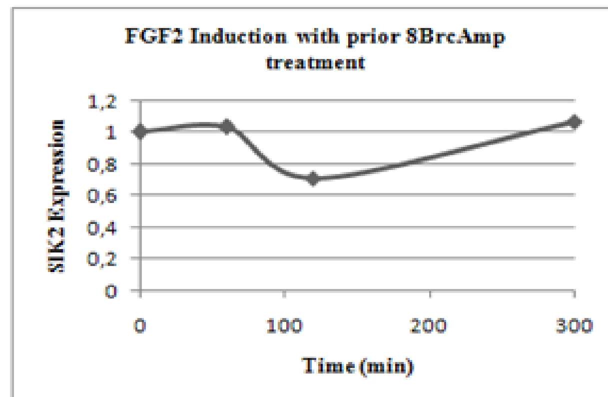


Figure 5.14. Steady state levels of SIK2 transcripts upon FGF2 induction and PKA activation/inhibition. Starved MIO-M1 cells were exposed to PKA activator or inhibitor for 30 minutes, subsequently induced with FGF2 for various durations. Panel A shows modulations in SIK2 transcript levels with FGF2 induction alone; panel B shows the expression levels of SIK2 in FGF2 induction with prior H89 treatment; panel C shows FGF2 induction with prior 8BrcAmp treatment.

6. DISCUSSION

Prototypic member of FGF gene family FGF2 is the first neurotrophic factor described in the retinal context and since has been shown to invoke proliferative response and act as differentiation or survival factor for retinal cells. It exerts its effects through transmembrane FGFRs, and the Ras/ERK pathway is the best known FGF2 signal transduction pathway. The signal is relayed by three different general mechanisms; membrane translocation, tyrosine phosphorylation and conformational changes resulted from phosphorylation that leads to release of autoinhibition and stimulation of enzymatic activity. Signal duration and amplitude as well as cellular compartmentalization of proteins involved in the pathways seems to be important components of the final cellular response. It is also becoming evident that signaling pathways activated by RTKs are interconnected with other signaling pathways via protein networks that are tightly controlled by multiple stimulatory and inhibitory effectors as well as positive and negative feedback mechanisms (Schlessinger, 2000). Recent studies suggested interplay between Ras/ERK pathway and PKA pathways through Raf-1 and B-Raf serine/threonine kinases (Yao *et al.*, 1995; Barbier *et al.*, 1999; Pursiheimo *et al.*, 2002a). Findings in our laboratory raise the possibility that serine/threonine kinase SIK2 may be part of this cross-talk (Özmen, 2006) based on modulations exerted by PKA both on FGF9-dependent ERK activation and nuclear localization of SIK2 (Özmen, 2006). FGF signal pathway elements Gab1 and A-Raf were suggested as novel SIK2 substrates based on their phosphorylations by SIK2 *in vitro* (Küser, 2006).

In order to gain further insight into the involvement of SIK2 in FGF signal transduction and in the cross-talk between FGF and PKA pathways we focused on FGF2, since it is the best studied member of the family. We examined the kinetics of FGF2 dependent ERK activation, SIK2 phosphorylation, changes in intracellular localization of SIK2 and the modulations exerted on these profiles by PKA activation or inhibition using an immortal Müller cell line. Since FGF2 is a mitogen for these cells, we also explored if PKA had any modulatory effect on this proliferative response. And finally expression profile of SIK2 in response to FGF2 and PKA activation/inhibition was investigated.

Threonine and serine phosphorylation status of SIK2 in the presence of FGF2 stimulus was studied with Western blotting of immunoprecipitated MIO-M1 cell lysates. The results indicated a decrease in the level of threonine phosphorylation within five minutes of FGF2 induction, then a rise at ten minutes and subsequently a gradual return to basal levels was observed. We observed an initial modest increase followed by a decrease in serine phosphorylated SIK2 levels. The levels are at the lowest at 30 minutes and returned to basal levels within 60 minutes. The data demonstrates that FGF2 induction modulates serine/threonine phosphorylation of SIK2, thus strongly implicates this kinase as a part of FGF signaling machinery. At this time, we are not able to identify specific serine residues involved, but as T182 is the epitope for the antibody used in Western blot analysis, this residue is the presumably phosphorylated threonine. The significance of the data emerges from the fact that threonine/serine phosphorylations are crucial components of regulation of signaling pathways downstream from RTKs. LKB1 was shown to phosphorylate SIK2 at T182 residue in the activation loop and suggested to increase its activity in vitro (Lizcano *et al.,* 2004) and PKA has been suggested to inhibit SIK2 by serine phosphorylation (Screaton *et al*., 2004). To investigate whether PKA activation increases serine phosphorylation of SIK2 or not, we have treated MIO-M1 cells with PKA activator 30 minutes prior to FGF2 stimulation. Phosphoserine levels of SIK2 increases steadily for 30 minutes and then maintained. Whether this profile is correlated with inhibition of SIK2 activity remains to be investigated.

The effect of FGF2 on MIO-M1 cells found to be mediated mainly by transient activation of ERK pathway leading to proliferation (Çinaroğlu, 2005). Since PKA is proposed to modulate signal flow through Ras/ERK pathway, we investigated the effect PKA activation/inhibition on FGF2-dependent ERK phosphorylation and MIO-M1 cell proliferation. The inhibition of PKA activity by H89 prior to FGF2 treatment results in an increase in pERK levels and a retardation in signal attenuation. When PKA is activated by 8BrcAmp 30 minutes prior to FGF2 induction, the phosphorylation pattern of ERK is similar to that of FGF2 induced cells. The data are in agreement with the previous results obtained in the context of FGF9 induction (Özmen, 2006) and PKA is, therefore, proposed to contribute to the downregulation of the pathway. FGF pathway elements Gab1 and A-Raf, has been proposed as potential SIK2 substrates based on in vitro phosphorylation data (Küser, 2006). Thus it is conceivable that the attenuation of ERK activity via SIK2 may be

mediated by these proteins.The proliferation data indicates PKA activation leads to no significant changes. But inhibition of PKA activity leads to an increased proliferation of MIO-M1 cells. The data contributes to the conclusion that PKA acts to balance growth factor induced response.

Immunocytochemical studies indicated that PKA and FGF2 signaling results in changes of cellular distribution of SIK2. In resting cells, SIK2 is observed both in nucleus and in cytoplasm. FGF2 induction for five minutes did not altered cellular distribution of SIK2; but, ten minutes or longer durations of FGF2 stimulus led to export of SIK from nuclei. PKA inhibition did not seemed to change cellular localization of SIK2 with respect to resting cells. Similarly FGF2 induction subsequent to inhibition of PKA activity for 5 and 10 minutes did not show any changes in intracellular distribution of SIK2. But, when the FGF2 signal was extended to 30 minutes, SIK2 started to leave the nuclei which become devoid of SIK2 by 60 minutes of induction. Thus, we can conclude that inhibition of PKA activity results in retardation of FGF2 response. PKA activation alone also leads to the movement of SIK2 out of the nucleus. FGF2 induction for five minutes following PKA activation initiates return of SIK2 to nucleus. However, prolonged FGF2 stimulus results in translocation of SIK2 to cytosol which is the same behavior seen in cells that are induced with FGF2 alone. In the case of SIK1, LKB1 phosphorylation at T182 in the activation loop marks it for translocation from nucleus to the cytosol (Lizcano *et al.*, 2004). Similarly, one would expect to see SIK2 in the nucleus at the time point where T182 phosphorylation is dropped which corresponds to five minutes for this particular case. The importance of these findings is more apperent when we consider the fact that serine/threonine phosphorylation dependent activation/deactivation loops are crucial components of RTK signal transduction. It must be noted that results obtained from combined treatment of cells with FGF9 and PKA activator/inhibitor were not straightforward, making comparisons difficult. Ten minutes induction with FGF9 leads to export of SIK2 out of nucleus which is similar in FGF2 induction (Özmen, 2006). However, in contrast to our findings upon 30 minutes of FGF9 induction, SIK2 returned to nucleus. The differences in this profile generated by stimulation with two members of same growth factor family with same cellular effect requires further research.

The specificity of antibodies in immunocytochemical analysis is a source of concern, especially when there are closely related proteins expressed. There is no formal proof that the antibody used in this study does not cross react with other members of salt-inducible kinases in MIO-M1 cells. Preliminary data from our laboratory indicate that SIK1 is not expressed in our cells, but SIK3 transcript can be detected. Using GFP-fused SIK2 proteins in localization studies should provide definitive data.

In the last part of this study, we investigated if the steady state levels of SIK2 transcripts is modulated upon FGF2 induction, and if PKA is involved. From untreated, FGF2 and/or PKA activator/inhibitor treated MIO-M1 cells; we isolated RNAs and assayed transcript levels by qRT-PCR. FGF2 induction leads to a gradual decrease in the expression of SIK2. However, when the cells were treated with PKA inhibitor, H89, prior to FGF2 induction, expression of SIK2 was seemed to increase within two hours and then drops to basal levels at five hours. In contrast, SIK2 expression was dropped at two hours when the cells were treated with PKA activator, 8BrcAmp, prior to induction with FGF2 and then recovered at five hours. The results indicate that the SIK2 expression can be downregulated by FGF2 as well as by PKA. Some FGF target genes are known to contain FiRE element in their promoter regions (Pursiheimo *et al*., 2002). Our search in human genomic and promoter databases failed to identify the presence of this enhancer element on SIK2 promoter. Similarly, the search for the presence of CRE element on SIK2 promoter was negative.

The study discussed here illustrated that FGF and PKA signaling pathways are interconnected. SIK2, a PKA substrate and having its own substrates such as Gab1 and A-Raf to be main modulators of Ras/ERK pathway, may be a key regulator in the integration of two pathways. In global terms, these results will contribute to our efforts to construct models towards understanding of FGFR signal transduction mechanism, and the cross-talk between RTK and G-protein coupled pathways.

In the specific context of retina, this work will contribute to elucidate the mechanisms involved in Muller glia proliferation and modulations exerted by metabolic pathways on the process. In case of several neuronal damage paradigms, Muller cells undergo proliferation, process known as reactive gliosis proposed to be a neuroprotective.

If the reactive gliosis cannot be controled, it might lead to retinal detachment resulting in blindness. Thus, understanding the molecular mechanisms of Müller cell proliferation have importance for therapeutic reasons (Dyer and Cepko, 2000). SIK2 has been implicated in the development of insulin resistance associated with type 2 diabetes through IRS1 phosphorylation (Gual *et al.*, 2005). In this work, we have shown that FGF2 modulates SIK2 phosphorylation status. It will be important to study whether this modulation activates or inhibits SIK2 activity, and contributes to the development of diabetic retinopathies.

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