SIK2 EXPRESSION IN RETINAL CELLS AND ITS POSSIBLE INVOLVEMENT ALONG WITH PKA IN FGF9 SIGNAL TRANSDUCTION

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ABSTRACT

SIK2 EXPRESSION IN RETINAL CELLS AND ITS POSSIBLE INVOLVEMENT ALONG WITH PKA IN FGF9 SIGNAL TRANSDUCTION

FGF signal transduction pathway activates a cascade of kinase and phosphatase dependent phosphorylation and dephosphorylation events. The pathway is subject to tight control mechanisms through negative feedback exerted by the the activated effector molecules, action of kinases and phosphatases. Besides the cellular context and interplay between the regulators, integration of heterologous signaling is also critical in shaping the cellular responses.

In this study, we have shown FGF9 receptors FGFR2, FGFR3 and a putative modulator SIK2 is widely expressed in neuronal cells and Muller glia, FGF9 is detected in neuronal cell layers but not in Muller cells. Our data, when compared with the primary Muller cells, indicate that MIO-M1 cells are equipped with the components of signal transduction. Using these cells as model system we had shown the activation of FGF9 pathway and that the pathway is modulated by PKA activity. We had also shown that SIK2 is modulated both in response to PKA and FGF9 by phosphorylation and nuclear translocation. Regulation of SIK2 translocation correlates with levels of ERK phosphorylation. When both stimuli are present SIK2 tends to act in accordance with FGF9 but not PKA.

ÖZET

RETİNA HÜCRELERİNDE SIK2 GEN ANLATIMI VE FGF9 SİNYAL YOLAĞINDA PKA İLE BİRLİKTE OLASI VARLIĞI

FGF sinyal iletim yolağı kinaz ve fosfataz zincirinin aktivasyonunu içeren fosforilasyon ve defosforilasyon olgularını içermektedir. Yolak etkinliği aktive efektörlerin negatif geri bildirimi, kinaz ve fosfatazlar aracılığı ile kontrol edilmektedir. Hücre repertuvarları ve regülatorlerin yanısıra paralel sinyallerin entegrasyonu hücre cevaplarının şekillenmesinde kritik rol oynamaktadır.

Bu çalışmada FGF9 reseptörleri FGFR2 ve FGFR3 ile potansiyel modülator SIK2'nin sıçan retinasında nöronal ve glial hücrelerde yaygın anlatımı olduğu, FGF9'un ise tüm retinal katmanlarda bulunmasına karşılık Müller glia hücrelerinde gen anlatımının bulunmadığı belirlenmiştir. Verilerimiz, primer Müller hücreleri ile karşılaştırıldığında, MIO-M1 hücrelerinin FGF9 sinyal iletiminde yer alan elemanları taşıdığına işaret etmektedir. Bu çalışma kapsamında, MIO-M1 hücreleri model sistem olarak kullanılarak FGF9 yolağı aktivasyonunun ERK fosforilasyonu yoluyla gerçekleştiği, ve sinyal iletiminin PKA aracılığı ile modüle edildiği gösterilmiştir. Ayrıca SIK2'nin PKA ve FGF9 uyarımı ile fosforlandığı, ve çekirdeğe geçişinin etkilendiği bulunmuştur; bu geçiş fosfo-ERK düzeyi ile paralellik göstermektedir. Her iki stimulusun bir arada bulunması halinde, SIK2, PKA değil FGF9 bağlamında davranmaktadır.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iii
ABSTRACT v
ÖZET vi
LIST OF FIGURES ix
LIST OF TABLES xi
LIST OF ABBREVIATIONS xii
1. INTRODUCTION 1
1.1. Retina 1
1.2. Fibroblast Growth Factors 3
1.3. Fibroblast Growth Factor Receptors 4
1.4. FGF Signal Transduction 6
1.4.1. PLCγ 8
1.4.2. PI3 Kinase/Akt Pathway 8
1.4.3. RAS/ERK Pathway 9
1.5. Fibroblast Growth Factor 9 (FGF9) 10
1.6. Protein Kinase A 12
1.7. Salt-Inducible Kinase Family 13
1.7.1. SIK1 13
1.7.2. SIK2 14
1.7.3. SIK3 16
2. AIM 18
3. MATERIALS AND METHODS 19
3.1. Chemicals 19
3.2. Animals 19
3.3. Cell Lines 19
3.4. RT-PCR Reaction 20
3.4.1. RNA Preparation 20
3.4.2. Reverse Transcription 20
3.4.3. PCR Reaction 21
3.4.4. DNA Gel Electrophoresis 21

3.4.5. RNA Gel Electrophoresis 22
3.5. *In Situ* Hybridization 22
3.6. Western Blot Analysis 23
3.7. Immunological Staining 24
3.8. Treatment of MIO-M1 Cells 25
3.9. Phosphoprotein Purification 25
4. RESULTS 26
4.1. Distribution of FGF9 in Postnatal Rat Retina 26
4.2. Immunological Detection of FGFR2 and FGFR3 in Rat Retina and MIO-M1 .. 28
4.3. Expression Profile and Cellular Distribution of SIK2 in Postnatal Rat Retina and MIO-M1 Cells 30
4.4. Phosphorylation of ERK in Response to FGF9 Induction and PKA Activation/Inhibition 33
4.5. Phosphorylation of SIK2 in Response to FGF9 Induction and PKA Activation 37
4.6. Cellular Localization of SIK2 in Response to FGF9 Induction and PKA Activation/Inhibition 38
5. DISCUSSION 45
REFERENCES 52

LIST OF FIGURES

Figure 1.1. Organization of retinal cells 2

Figure 1.2. Schematic representation of FGFs 4

Figure 1.3. Schematic representation of FGFR structure 5

Figure 1.4. Schematic representation of different forms of FGFRs 5

Figure 1.5. Schematic representation of FGFR activation..... 7

Figure 1.6. FGF signaling pathway 10

Figure 1.7. Schematic representation of SIK1 13

Figure 1.8. SIK1 in stereoidogenic gene expression 14

Figure 1.9. Schematic representation of SIK2 15

Figure 1.10. SIK2 in insulin signaling..... 15

Figure 1.11. TORC2 regulation of CREB-dependent gene transcription..... 16

Figure 1.12. Schematic representation of SIK3 17

Figure 4.1. Immunohistochemical labeling of FGF9 in developing rat retina 27

Figure 4.2. In situ hybridization of DIG-labeled FGF9 transcripts in adult rat retina.... 28

Figure 4.3. Immunocytochemistry of FGF9 in MIO-M1 28

Figure 4.4. Immunohistochemical labeling of FGFR2 and FGFR3 in rat retina..... 29

Figure 4.5. Immunocytochemical staining of FGFR2 and FGFR3 in MIO-M1 cells ... 30

Figure 4.6. Expression of SIK2 in developing rat retina 31

Figure 4.7. Immunohistochemical labeling of SIK2 in developing rat retina 32

Figure 4.8. SIK2 immunocytochemistry in MIO-M1 cells 33

Figure 4.9. Western blot analysis of SIK2 from MIO-M1 cells 33

Figure 4.10. Phosphorylation of ERK in FGF9 induced MIO-M1 cells 35

Figure 4.11. Phosphorylation of ERK in H89 treated and FGF9 induced MIO-M1 cells 36

Figure 4.12. Phosphorylation of ERK in 8BrcAmp treated and FGF9 induced MIO-M1 37

Figure 4.13. Phosphorylation of ERK in treated MIO-M1 cells 38

Figure 4.14. Phosphorylation of SIK2 with FGF9 and PKA induction..... 39

Figure 4.15. Cellular localization of SIK2 in MIO-M1 cells induced with PKA..... 41

Figure 4.16. Cellular localization of SIK2 in MIO-M1 cells induced with FGF9 42

Figure 4.17. Cellular localization of SIK2 in MIO-M1 cells treated with FGF9 and PKA activator or inhibitor 43

Figure 4.18. Cellular localization of SIK2 in MIO-M1 cells treated with FGF9 and PKA activator or inhibitor 44

Figure 5.1. Schematic representation of the proposed model..... 51

LIST OF TABLES

Table 1.1. Specificity of different FGFs for different receptor isoforms 12

Table 3.1. List of primers used in this study..... 22

Table 3.2. Optimal amplification conditions and the product lengths..... 22

Table 3.3. List of antibodies used in this study 24

LIST OF ABBREVIATIONS

8BrcAMP	8 bromo cylic adenosine monophosphate
ACTH	Adenocorticotrophic hormone
AKAP	A-kinase anchoring protein
AMPK	AMP-activated protein kinase
APS	Ammonium persulfate
ARVO	Association for research in vision and ophthalmology
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix loop helix
Bp	Base pair
BSA	Bovine serum albumine
Ca^{2+}	Calcium
cAMP	Cyclic adenosine 5'-monophosphate
cDNA	Complementary deoxyribonucleic acid
CFR	Cysteine-rich fibroblast growth factor receptor
CNS	Central nervous system
CO_2	Carbon dioxide
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CREB	CAMP-responsive element binding
DAG	Diacylglycerol
DAPI	4', 6-Diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosidetriphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acedic acid
ERK	Extracellular-signal-regulated kinase
FAK	Focal adhesion kinase

FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FRS2	Fibroblast growth factor receptor substrate 2
GAF	Glia activating factor
GC	Ganglion cell
GCL	Ganglion cell layer
GPCR	G protein-coupled receptor
GFP	Green fluorescent protein
GRB2	Growth factor receptor bound protein
GTC	Guanidium isothiocyanate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HLGAG	Heparin - like glycosaminoglycan
HSPG	Heparin sulfate proteoglycan
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IGF	Insulin-like growth factor
INL	Inner nuclear layer
IP3	Inositol-1,4,5-triphosphate
IPL	Inner plexiform layer
IRS1	Insulin receptor substrate
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LB	Luria's broth
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium Chloride
MIO-M1	Moorfields/Institute of Ophthalmology-Muller 1
MMLV	Moloney-murine- leukaemia-virus
MOPS	Morpholinopropane sulfonic acid
mRNA	Messenger ribonucleic acid
Na_2HPO_4	Disodium hydrogen phosphate

Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaOAc	Sodium Acetate
NBT/BCIP	Nitro blue tetrazolium/5-bromo-4-chloro-3-. indolyl phosphate
NGF	Nerve growth factor
NT-3	Neutrophin-3
NZ	Neuroblastic zone
O-2A	Oligodendrocyte-type 2 astrocyte
OD	Optical density
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC12	Pheochromocytoma
PCR	Polymerase chain reaction
PI3	Phosphoinositide 3
PIP2	Phosphotidylinositol-4,5-diphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase C-gamma
PN	Post natal
PR	Photo receptor
PTB	Phosphotyrosine binding domain
PVDF	Polyvinylidene fluoride
REF	MAPK phosphatase
RNA	Ribonucleic acid
RNasin	Rnase inhibitor
RPE	Retinal pigment epithelium
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription coupled polymerase chain reaction
S	Serine
SDS	Sodium dodecyl sulfate
SH2	Src homology domain

SHH	Sonic hedgehog
SHP2	SH2-domain containing phosphatase 2
SIK	Salt-inducible kinase
SNF-1	Sucrose nonfermenting 1
SOS	Son of sevenless
SSC	Sodium chloride/sodium citrate
T	Threonine
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
Y	Tyrosine

1. INTRODUCTION

Fibroblast growth factors (FGF) have been shown to play important roles in fate decisions in retina. Activation of FGF signal transduction leads to a cascade of phosphorylation and dephosphorylation events. G-protein coupled receptors (GPCR) have been shown to be important in modulation of FGF signal. Salt inducible kinase 2 (SIK2), a novel serine/threonine kinase that posses SH2, SH3 binding domains was shown to be expressed in retina in our laboratory. In this study a downstream kinase in GPCR signaling, protein kinase A (PKA), and SIK2, have been shown to be involved in FGF9 signal transduction.

1.1. Retina

Retina has a highly ordered and relatively simple structure (Figure 1.1). In the adult tissue there are six neuronal, and one glial cell type organized in a laminar fashion. Photoreceptor cells (PR), rods and cones, capable of converting photon signal to chemical signal, are located at the outer most layer of the neural retina (outer nuclear layer, ONL) and in a close contact with the retinal pigment epithelium (RPE) cells. The PRs make synaptic contacts, at the outer plexiform layer (OPL), with the bipolar cells of the inner nuclear layer (INL). INL also contains horizontal and amacrine interneurons. These cells are interconnected, they process/integrate the information coming from the PRs. The cell bodies of non-neuronal Muller cells are also located in this stratum. The integrated information is relayed to the ganglion cells at the inner plexiform layer (IPL), and the axons of the ganglion cells (GC) fasciculate to form optic nerve (Newman and Reichenbach, 1996).

In vertebrates, all cell types of the neural retina derive from progenitor cells of the inner optic cup with a distinct temporal sequence. Progenitor cells undergo several mitotic divisions and commit to one of the possible fates during or shortly after the final division, migrate into an appropriate location, acquire the required phenotype and finally make correct synaptic connections. Ganglion cells are the first born cells in several species, are

followed by horizontal cells, cones, and amacrines. Their differentiation is finished before birth; rod, bipolar and Muller cell birth is postnatal (Young, 1985; Barnstable *et al*., 1985).

Cell lineage studies indicate that the progenitors are multipotent (Turner and Cepko, 1987; Turner *et al*., 1990) and fate determination is thought to be regulated by a combination of extrinsic and intrinsic influences (Cepko *et al*., 1996; Harris, 1997; Edlund and Jessell, 1999). A number of growth factors have been shown to be expressed in retina during development and in the adult tissue and shape the tissue (Yang, 2004). These include FGF family, nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived growth factor (BDNF), neurotrophin-3 (NT-3), insulin-like growth factor (IGF), transforming growth factor (TGF) α and TGFβ, sonic hedgehog (SHH) and WNT family members. The specific roles played by these factors are mostly unknown.

The connection between the growth factors and the transcription factors instrumental in paterning and the physiology of the tissue are not evident at this time. However, basic helix loop helix (bHLH), homeobox, leucine zipper motif containing transcription factors appear to be important in cell fate determination during retinogenesis. Pax6 and its homologue can induce ectopic eyes when misexpressed in *Xenopus* and *Drosophila*, respectively (Halder *et al*., 1995; Chow *et al*., 1999). RPF-1, ASH, Otx2, Chx10, Crx are implicated in neural retina development (Harris, 1997). Math5, Mash1, Hes1 and Hes5 determine neuronal subtypes within specified layers (Hatakeyama and Kageyama, 2004).

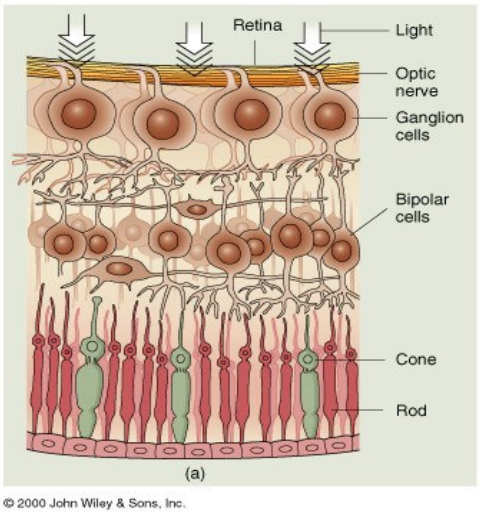

Figure 1.1. Organization of retinal cells

1.2. Fibroblast Growth Factors

FGFs are a large family of polypeptides containing at least 23 separate members encoded by different genes thought to be generated by gene duplication and translocation (Itoh and Ornitz, 2004). FGF15 appears to be the rodent ortholog of FGF19 of human, zebrafish and chicken (Reuss *et al*., 2003). FGFs are found in all multicellular organisms and a number of them exist in multiple isoforms owing to differential splicing or initiation from alternate start codons (Ornitz and Itoh, 2001). Defining features of the FGF family are their strong affinity for heparin sulfate and heparin sulfate proteoglycans (HSPGs), as well as a central core of 120 amino acids that show 30-70 per cent homology (Figure 1.2), (Burgess and Maciag, 1989; Ago *et al*., 1991; Zhang *et al*., 1991).

FGFs appear to play essential roles in embryonic processes ranging from formation of primary body axis, patterning of various organs (Kimelman and Kirschner, 1987; Launay *et al*., 1996; Hongo *et al*., 1999; Ford-Perriss *et al*., 2001; Dono, 2003), and to development of central nervous system (CNS) (Diez del Corral, 2002). In adult organisms they take part in angiogenesis, wound healing, repair of tissues, control of nervous system (Mason, 1994; Presta *et al*., 2005) and implicated to have a role in learning and memory (Calamandrei and Alleva, 1995). *In vitro*, FGFs are potent mitogens for a variety of cell types of ectodermal origin, lead to cell-cycle arrest, promote survival of neurons, induce differentiation in neuronal and endothelial cells, but inhibit osteoblast differentiation (Lin *et al*., 1998; Szebenyi and Fallon, 1999; Mansukhani *et al*., 2000; Powers *et al*, 2000; Dailey *et al*., 2003, Dono, 2003

A number of FGF family members have been detected in the developing eye and in adult retina. FGF1 is proposed to be important in GC differentiation early in retinogenesis (Guillemot and Cepko, 1992; McCabe *et al*., 1999). FGF2 was shown to be important in neural differentiation in early optic cup and it appears to be critical for maintaining retinal stem cells (Yang, 2004). FGF3 and FGF8 are expressed in the optic stalk (Wilkinson *et al*., 1989; Reifers *et al*., 2000; Vogel-Hopker *et al*., 2000; Walshe and Mason, 2003). FGF19 is expressed in horizontal cells of the developing chicken retina and lens primordia; its mouse ortolog FGF15 is detected in the optic vesicle, a subset of neuronal progenitors, differentiating ganglion and amacrine cells (Kurose *et al*., 2004). In mature retina four

members of the FGF family; FGF1, FGF2, FGF5, FGF9 were shown to be expressed (Baird *et al*., 1985; Buğra *et al*., 1994; Kitaoka *et al*., 1995; Buğra and Hicks, 1997; Colvin *et al*., 1999; Çınaroğlu *et al*., 2005). *In vitro* experiments indicated that FGF1 and FGF2 can induce neurite outgrowth, and opsin expression in photoreceptor cells (Hicks and Courtois, 1988), and mediate survival of neurons (Bahr *et al*., 1989). Muller glia in culture respond to FGF1, 2 and 9 by proliferation (Çınaroğlu *et al*., 2005).

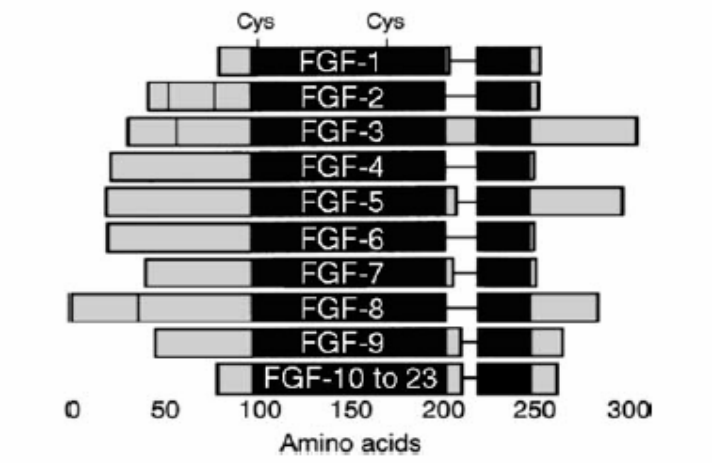

Figure 1.2. Schematic representation of FGFs (Reuss *et al*., 2003)

1.3. Fibroblast Growth Factor Receptors

FGFs exert their biological effects through four distinct high affinity receptors, FGFR1-FGFR4, that belong to receptor tyrosine kinase (RTK) superfamily (Lee *et al*., 1989; Dionne *et al*., 1990; Keegan *et al*., 1991; Partanen *et al*., 1991). These receptors share between 55% to 72% homology at the protein level (Johnson and Williams, 1993), and in order to be fully activated, they operate in conjunction with HSPG (Klint and Claesson-Welsh, 1999; Powers *et al*., 2000). An additional FGFR, termed the cysteine-rich FGFR (CFR) has been cloned in the chicken. CFR is an integral membrane protein with an extracellular domain comprising 16 cysteine-rich repeated units and an intracellular domain of 13 amino acids. Although distinct from other FGFRs its ability to bind FGF1, FGF2, and FGF4 suggested that it might play an important role in FGF action (Burrus *et al*., 1992).

FGFRs are transmembrane proteins (Figure 1.3) containing three extracellular immunoglobulin (Ig)-like domains (designated IgI, IgII, IgIII), an acidic region between IgI and IgII, a transmembrane domain and an intracellular split tyrosine kinase domain (Johnson *et al*., 1990). Differential exon usage generate receptor isoforms (Figure 1.4) with either two or three Ig-like domains, secreted forms that lack the transmembrane region, and IgIII domain variants (Johnson and Williams, 1993). Alternative splicing variants of IgIII domain, IgIIIb and IgIIIc, differ in their C terminus half of the domain and they display different ligand binding specificities (Miki *et al*., 1992; Yayon *et al*., 1992). The acid box and the Ig-like domain I of FGFRs compete to bind to the heparin and ligand binding sites on the receptor, thus appear to have an autoinhibitory function (Plotnikov *et al*., 1999). Ig-like domains II and III, together with the linker between the two domains interact with FGFs.

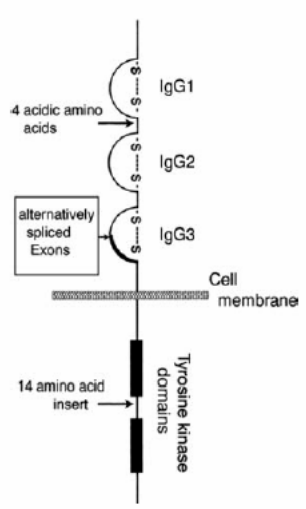

Figure 1.3. Schematic representation of FGFR structure (Reuss *et al*., 2003)

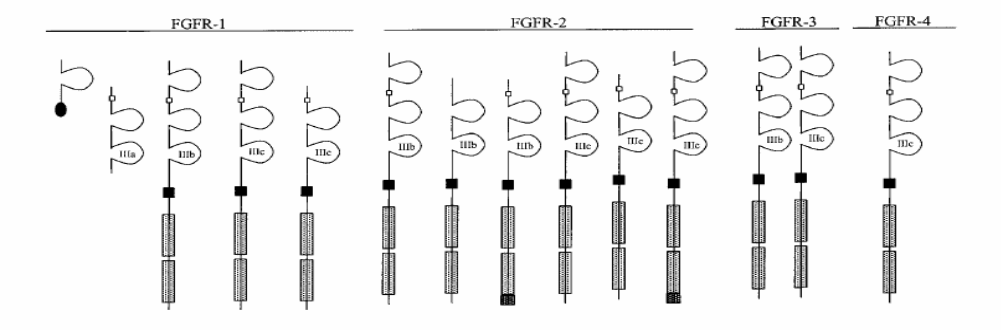

Figure 1.4. Schematic representation of different forms of FGFRs (Johnson and Williams, 1993)

FGFRs are widely expressed. In the CNS, FGFR1 expression was shown to be predominantly on neurons, whereas FGFR2 and FGFR3 were found on glial cells (Asai *et al*., 1993; Miyake *et al*., 1996). FGFR4 is expressed only during development and apart from a small region is not detected in adult CNS (Fuhrmann *et al*., 1999).

Targeted disruption of FGFR1 and FGFR2 are lethal in embryonic or perinatal stages, FGFR3 knockouts are viable with bone defects and that of FGFR4 results in no obvious changes in phenotype (Eswarakumar *et al*., 2005).

In the vertebrate retina, expression of all FGFR isoforms have been detected. FGFR1, FGFR2 and FGFR4 are expressed in photoreceptors, whereas FGFR3 is preferentially found in ganglion cells (Kinkl *et al*., 2002). Muller glia show strong expression of FGFR1, FGFR2 and FGFR3, but FGFR4 was not detected (Çınaroğlu *et al*., 2005). Expression of truncated FGFR1 and FGFR2 in photoreceptors under opsin promoter results in progressive retinal degeneration (Campochiaro *et al*., 1996). Transfection of truncated FGFR1 into Xenopus embryos leads to photoreceptor and amacrine cell loss in favor of Muller glia, underlining the importance of FGFs in cell fate determination (McFarlane *et al*., 1998).

1.4. FGF Signal Transduction

Following ligand binding and dimerization the receptors become capable of phosphorylating specific tyrosine (Y) residues on their own and each other's cytoplasmic tails (Figure 1.5) (Lemmon and Schlessinger, 1994). Through phosphorylated tyrosine residues, downstream signaling molecules are recruited and modifed by the activated receptors by phosphorylation and they propagate the signal through a number of possible transduction pathways (Pawson, 1995).

Most studies of FGFR-mediated signal transduction have been carried out using FGFR1 as the prototypical FGFR. Tyrosine residues that can be phosphorylated in FGFR1 are; Y463, Y583, Y585, Y653, Y654, Y730, and Y766 (Mohammadi *et al*., 1996) which are conserved in all members.

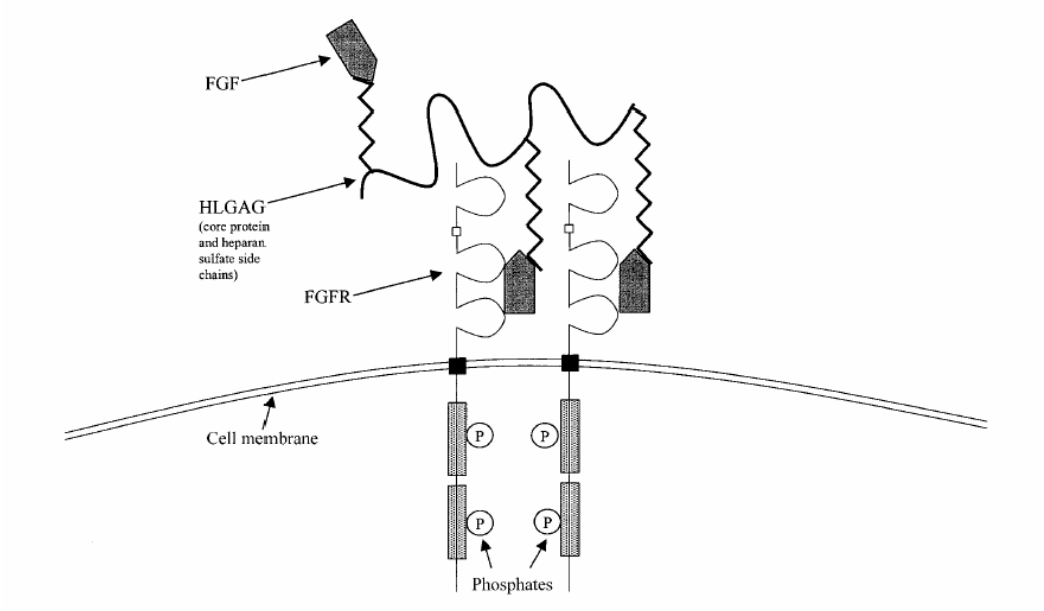

Figure 1.5. Schematic representation of FGFR activation (Powers *et al*., 2000)

Target proteins may be localized through interaction between their Src-homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Formation of signaling complexes by recruitment of adaptors, docking proteins or enzymes, results in activation of a cascade of phosphorylation events (Figure 1.6). Initiation of transduction of FGF signal involve mainly two independent interactions: first one is SH2-linked recruitment of phospholipase C-gamma (PLCγ) and activation of PLCγ/Ca^{2+} pathway; second one is PTB-linked docking of fibroblast growth factor receptor substrate 2 (FRS2) which is analogous to insulin receptor and insulin receptor substrate (IRS1) pathway (Yenush and White, 1997). FRS2 activation by phosphorylation on several tyrosine residues is important for induction of Ras/extracellular-signal-regulated kinase (ERK) and phosphoinositide 3 (PI3) kinase pathways.

One mechanism of signal down regulation is through dephosphorylation events on receptors as well as mediators by phosphotyrosine phosphatases such as SEF and mitogen-activated protein kinase (MAPK) phosphatases (Tsang and Dawid, 2004; Thisse and Thisse, 2005). RTKs also activate sprouty proteins which in turn inhibit growth factor receptor bound protein-son of sevenless (GRB-SOS) recruitment to FRS2 leading to signal attenuation (Hanafusa *et al*., 2002). FRS2 assembles negative signaling complexes by binding to CBL, a ubiquitin ligase, which leads to degradation of FRS2 as well as FGFR

(Wong *et al*., 2002). A negative feedback loop mediated by MAPK threonine phosphorylation of FRS2 results in decreased GRB2 recruitment (Lax *et al*., 2002).

Besides the cellular context and the interplay between the positive/negative regulators, the interpathway cross-talk, and the integration of heterologous signaling is also critical in defining the choice between proliferation, differentiation or survival responses (Schlessinger, 2000). It is becoming increasingly clear that FGF pathways are modulated by GPCR agonists, and themselves influence cellular responses to WNT and integrin signals (Liebmann, 2001; Stork and Schmitt, 2002; Dailey *et al*., 2005).

1.4.1. PLCγ

PLCγ, recruited to activated receptor via its SH2 domain, is activated by tyrosine phosphorylation. Active PLCγ hydrolyzes phosphotidylinositol-4,5-diphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), whereas IP_3 stimulates Ca^{2+} release from intracellular compartments and activation of calcium/calmodulin dependent kinases (Mohammadi *et al.,* 1991). Loss of this pathway doesn't affect proliferative response (Mohammadi *et al*., 1992). PKC activated by PLCγ modifies the phosphorylation status of RAF and consequently the RAS/MAPK pathway is activated (Huang *et al.,* 1995). It was also shown that FRS2 can link FGFR pathway to PKC which might play a role in mitogenesis or chemotaxis (Lim *et al*., 1999).

1.4.2. PI3 Kinase/AKT Pathway

This is the main growth factor dependent anti-apoptotic pathway (Eswarakumar *et al*., 2005). PI3 kinase can be recruited to the membrane either by binding to specific phosphotyrosines on FGFRs or GAB1-FRS2-GRB2 complex assembled on the receptors (Rodriguez-Viciana *et al*., 1994). GAB1 appears to be indispensible for the activation of this cascade. Tyrosine phosphorylation of GAB1 generate docking sites for PI3 kinase. The active PI3 kinase can activate AKT directly or via PDK1/PRK2 complex (Hadari *et al*., 2001). In turn AKT, inhibits the activity of pro-apoptotic proteins, or their expression by mediating transcription factor sequestering in the cytoplasm (Schlessinger, 2000).

1.4.3. RAS/ERK Pathway

Activation of RAS/ERK pathway is associated with both proliferation and differentiation responses. The key component of the pathway is the docking protein FRS2 (Eswarakumar *et al*., 2005). It binds to juxtamembrane domain of the receptor though its PTB. FRS2 is activated by phosphorylation on several tyrosine residues that creates binding sites for GRB2 and SH2-domain containing phosphatase 2 (SHP2) (Ong *et al*., 2000). The activated FRS2 forms a platform for the assembly of GRB2/SOS or SHP2-GRB2/SOS complexes, which activate RAS by SOS facilitated guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange. Alternatively GRB2-GAB1-SHP2 complex is formed on FRS2 which contributes to RAS activation (Hadari *et al*., 2001). Activated RAS recruits RAF to the membrane, RAF is activated by phosphorylation on several tyrosine residues and that in turn phosphorylates MAPK kinase 1/2 (MEK 1/2) on two serine (S) residues. Activated MEK phosphorylates now MAPK, ERK 1/2, on threonine (T) and tyrosine (Y) residues. Activated ERK phosphorylates transcription factors such as ELK1 and changes the catalytic activities of certain enzymes (Yang *et al*., 2004). It has been suggested that sustained MAPK activity may be responsible for directing cellular response towards differentiation, and its transient activation would lead to proliferation (Marshall, 1995).

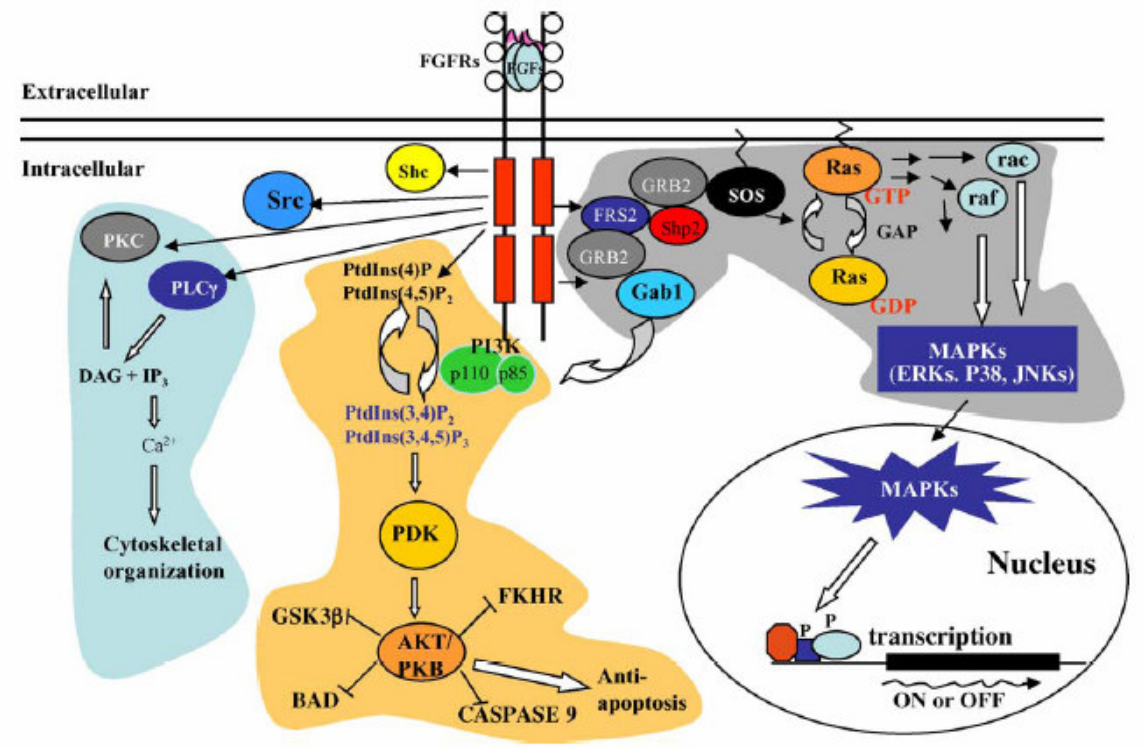

Figure 1.6. FGF signaling pathway (Szebenyi and Fallon, 1999)

1.5. Fibroblast Growth Factor 9 (FGF9)

FGF9 was originally purified from the conditioned medium of the glial cell line NMC-G1 and based on its mitogenic activity named glia activating factor (GAF) (Miyamoto *et al*., 1993). Later when it was shown to have about 30 per cent sequence homology with the known members of the FGF family, its name was changed to FGF9. It is a 208 amino acid protein with three molecular forms of 30, 29, 25 kDa due to differential glycosylation. Although FGF9 does not contain signal peptide sequence, it has been shown to be efficiently secreted (Miyamoto *et al*., 1993).

In the CNS, FGF9 was shown to be expressed exclusively in neurons of the cerebral cortex, hippocampus, thalamus, hypothalamus, midbrain, brainstem and the cerebellum (Tagashira *et al*., 1995; Todo *et al*., 1998). Low levels of the factor appear to be present in a subset of astrocytes and oligodendrocytes in the CNS (Nakamura *et al*., 1999). Physiological function of FGF9 in CNS is still unclear. In vitro FGF9 shows trophic effect on glial cells; it stimulates proliferation of rat primary cortical astrocytes and

oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells (Naruo, 1993), and it promotes survival of spinal motor neurons (Kanda, 1997) and pheochromocytoma (PC12) cells (Naruo, 1993). FGF9 has growth promoting activities on human brain tumors and teratocarcinoma cells (Todo *et al*., 1998; Granerus and Engstrom, 2003).

Targeted disruption of FGF9 in mice resulted in lung hypoplasia, early postnatal death, and male to female sex reversal (Colvin *et al*., 2001a, 2001b). FGF9 was also shown to stimulate thrombopoiesis in mice (Matsumoto-Yoshitomi *et al*., 1995). It is mitogenic for cultured dental mesenchymes (Kettunen *et al*., 1998), prostatic epithelial and stromal cells (Giri *et al*., 1999), as well as endometrial stromal cells (Tsai *et al*., 2002).

FGF9 is expressed in embryonic mouse retina (Colvin *et al*., 1999) starting at E10, as the optic cup is forming (Zhao *et al*., 2001). In postnatal development, FGF9 mRNA levels found to be highest in the newborn and in mature rat retina, however a gradual decrease detected starting at day 4 then a rise again around day 15 (Çayırlıoğlu, 1996; Ergörül, 1998). The physiological significance of this unique biphasic pattern is not known, however the drop coincides with synaps formation and high apoptotic period (Çınaroğlu *et al*., 2005).

Ectopic expression of FGF9 in early embryonic retinal pigment epithelium (RPE) results in the formation of a second layer of retina in reverse orientation by switching the differentiation of RPE into a neuronal fate (Zhao and Overbeek, 1999). This transdifferentiation of embryonic RPE is reminiscent of FGF2 induced fate choice switch in the chick and rat retina (Park and Hollenberg, 1989; Park and Hollenberg, 1991; Zhao *et al*., 1995). Mouse embryos lacking FGF9 exhibit abnormal differentiation of the anterior retina, indicating a role in defining boundary between neural retina and RPE (Zhao *et al*., 2001).

In vitro experiments indicate that FGF9 promotes survival of ganglion cells (Kinkl *et al*., 2003) and elicits proliferative response of Muller glia (Çınaroğlu *et al*., 2005).

FGF9 shows greatest affinity to the IIIb and IIIc splice forms of FGFR3 and the IIIc splice form of FGFR2 (Table 1.1), (Hecht *et al*., 1995, Santos Ocampo, 1996).

Table 1.1. Specificity of different FGFs for different receptor isoforms (Powers *et al*., 2000)

FGFR	FGF-1	FGF-2	FGF-3	FGF-4	FGF-5	FGF-6	FGF-7	FGF-8	FGF-9
1, IIIb	100	60	34	16	4	5	6	4	4
1, IIIc	100	**104**	0	102	59	55	**0**	1	21
2, IIIb	100	9	45	15	5	5	**81**	4	7
2, IIIc	100	64	4	94	25	61	2.5	16	89
3, IIIb	100	1	2	1	1	1	1	1	42
3, IIIc	100	107	1	69	12	9	1	41	96
4	100	113	6	108	7	79	2	76	75

Modified from Ornitz *et al*. (1996).

1.6. Protein Kinase A

PKA, a cyclic adenosine 5'-monophosphate (cAMP)-dependent serine/threonine kinase is an important effector of hormone and neurotransmitter signaling. PKA activation is stimulated by increase in cAMP levels mediated by GPCR agonists, and leads to diverse cellular responses depending on the cell type. Glycogen breakdown, triacylglycerol breakdown, stimulation of cortisol and insulin secretion, modulation of ion channels, sperm motility (Krebs, 1989, Langfort *et al*., 2003, Lacroix and Hontela, 2001) are few processes regulated by PKA. It also affects gene expression by directly phosphorylating a gene regulatory protein cAMP-responsive element binding (CREB) protein or by regulating its activity indirectly by phosphorylating other proteins (Rosenberg *et al*., 2002, Screaton *et al*., 2004). The enzyme is found as a tetramer, two cAMP binding regulatory and two catalytic subunits. Differential use of alternative subunit forms generate PKA isoforms, some of which is directed to specific intracellular locations via A-kinase anchoring protein (AKAP) family of anchoring proteins (Scott, 1991; Colledge and Scott, 1999).

Recently role of PKA signal transduction in the control of activation and duration RAS/ERK pathway and implication of this effect in crosstalk between growth factor signaling and hormones is appreciated (Stork and Schmitt, 2002). Although the interaction between PKA pathway and growth factor receptor signaling pathways were suggested several times, little is known about its role on growth factor induced signaling (Yao *et al*., 1995, Barbier *et al*., 1999). In one of the studies it was shown that inhibition of PKA activity prior to FGF2 stimulus increased the signal flow through RAS/ERK pathway (Pursiheimo et. al., 2002a). In another study, it was shown that FGF2 induced FGF

inducible response element (Fire) activation required cooperational function of RAS/ERK and PKA pathways (Pursiheimo *et al*., 2002b). Recently it was suggested that integration of growth factor signaling and PKA might include mediators such as RAF1 and B-RAF serine/threonine kinases (Pursiheimo *et al*., 2002b). These data lead to the suggestions that PKA may act to balance the activity level of RAS/ERK pathway which might function in a mechanism that ensures the proper responses to the growth factor signaling in cells.

1.7. Salt-Inducible Kinase Family

Salt-inducible kinase (SIK) was first isolated as a novel cDNA clone from adrenocortical tissue of high salt diet-treated rats and according to its sequence characteristics it was placed in AMP-activated protein kinase (AMPK) family of serine/threonine kinases (Wang *et al*., 1999). Based on the search for cDNAs in human and mouse genome databases that have similar structure to SIK1, two isoforms SIK2 and SIK3 were identified (Okamoto *et al*., 2004).

1.7.1. SIK1

SIK1 enzyme is a tripartite kinase having an N-terminal serine-threonine kinase domain, a sucrose nonfermenting 1 (SNF-1) kinase homology domain and a potential PKA target serine residue containing domain at its C-terminal (Figure 1.7) (Okamoto *et al*., 2004). Altough the site is yet to be determined, it was shown that SIK1 has an autophosphorylation activity (Wang *et al*., 1999). Kinase assays using synthetic peptide substrates suggested that (Hy)[(B)X or X(B)]XX(S)XXX(Hy) is the phosphorylation motif of SIK1 (Horike et. al., 2003).

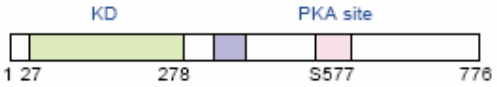

Figure 1.7. Schematic representation of SIK1 (Okamoto *et al*., 2004)

SIK1 has a suggested regulatory role in the early phase of adenocorticotrophic hormone (ACTH) signalling in the adrenal cortex (Katoh *et al*., 2004). It was demonstrated that PKA dependent phosphorylation of SIK1 at Ser-577 during ACTH stimulation it exits

nucleus, elevating SIK1 repression on CRE-dependent transcription of CYP11A gene, SIK1 is dephosphorylated in the cytoplasm and can reenter the nucleus (Takemori *et al*., 2002). This shuttling is suggested to be an important factor on steroidogenic gene expression at the early stage of ACTH signalling (Figure 1.8).

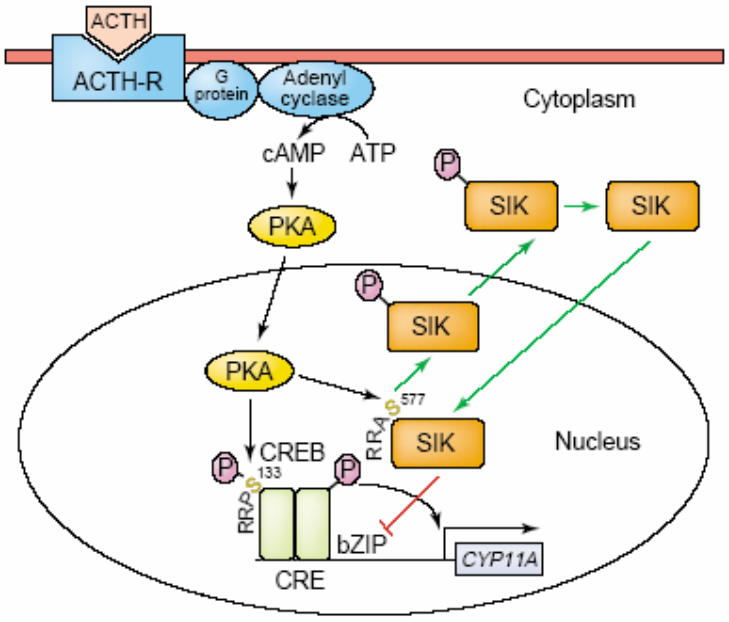

Figure 1.8. SIK1 in stereoidogenic gene expression (Okamoto *et al.*, 2004)

1.7.2. SIK2

SIK2, originally identified as an adipose specific isoform of the family, as predicted to be a 931 amino acid protein with a kinase domain, residues 20-271, a SNH domain, residues 293-346, and a phosphorylation domain, residues 577-623. Amino acid similarity of SIK2 with SIK1 is 78% in the kinase domain, 70 % in the SNH domain and 73% in the phosphorylation domain (Figure 1.9) (Katoh *et al*., 2004).

SIK2 was suggested to be part of the early phase of adipocyte differentiation and insulin signalling in mature adipocytes. Insulin receptor substrate 1 (IRS1) was found to contain the consensus peptide sequence which might be phosphorylated by SIK2, and it was later shown that Ser-789 was indeed phosphorylated by the enzyme (Horike *et al*., 2003). These findings suggested that serine phosphorylation of IRS1 by SIK2 would

modulate the efficiency of insulin signalling cascade (Figure 1.10) and lead to insulin resistance in adipose tissue (Qiao *et al*., 2002, Yenush and White, 1997).

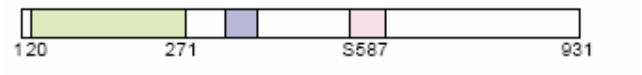

Figure 1.9. Schematic representation of SIK2 (Okamoto *et al*., 2004)

Another substrate for SIK2 phosphorylation was shown to be TORC2 based on in vitro phosphorylation studies where S 171 appears to be the target residue. This phosphorylation by SIK2, promote association of TORC2 with 14-3-3 proteins and cytoplasmic sequestration of TORC2 which leads to inhibition of CREB activity (Figure 1.11) in the basal state (Screaton *et al*., 2004). Also in this work it was shown that cAMP has a negative role on SIK2 acitivity via PKA- mediated phosphorylation of S 587.

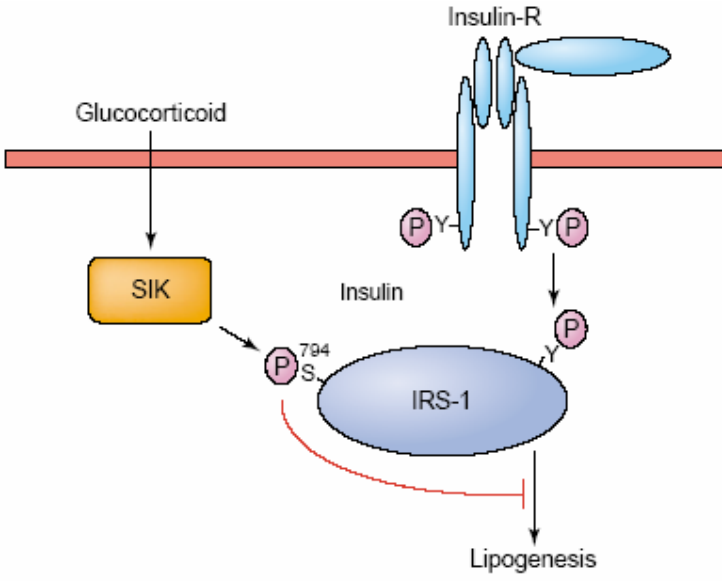

Figure 1.10. SIK2 in insulin signaling (Okamoto *et al.*, 2004)

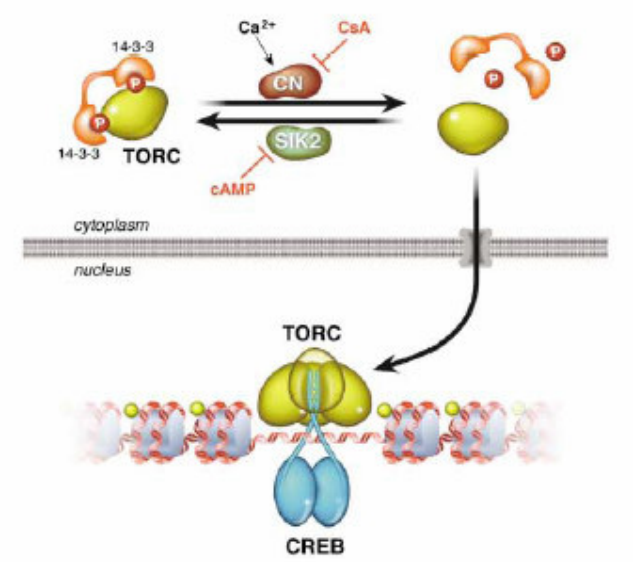

Figure 1.11. TORC2 regulation of CREB-dependent gene transcription (Screaton *et al.*, 2004)

Rat SIK2, cloned from retinal tissue (Özcan, 2003; Uysal, 2005), shows 94 per cent overall identity to mouse and 89.3 per cent to human ortologs, and 98 per cent conservation in kinase domains. It appears that the rat SIK2 gene spans a 100 kb region consisting of 16 exons. In retina alternative splicing within the last exon generates 3 transcripts encoding 2 proteins that differ at the very C-terminal region (Uysal, 2005). In the cognate proteins, in addition to kinase and ubiquitin-associated domains, putative SH2-, SH3-binding sites, as well as serine, threonine and tyrosine phosphorylation sites are present. In the adult rat, RT-PCR experiments show that SIK2 expressed in all retinal cell layers (Özcan, 2003).

1.7.3. SIK3

SIK3 is predicted to be a 1263 amino acid protein. It exhibits amino acid similarity of 68% in kinase domain, 37% in SNH domain, 47% in phosphorylation domain with SIK1 (Figure 1.12). Its expression seems ubiquitous but there is no information available as to its physiological role.

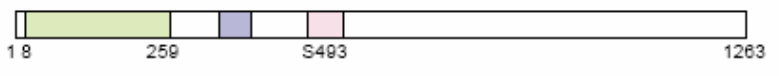

Figure 1.12. Schematic representation of SIK3 (Okamoto *et al*., 2004)

2. AIM

This study discussed here aims to show possible involvement of SIK2 in PKA and FGF9 signaling pathways and the integration of these two pathways. In order to analyze FGF9 signaling, distribution of FGF9, FGFR2, FGFR3, and SIK2 in postnatal rat retina and spontaneously immortalized MIO-M1 human Muller glia cell line will be studied. These results will confirm that MIO-M1 cells are a convenient tool for us to study FGF9 signaling. Response of MIO-M1 cells to FGF9 treatment will be compared to that of primary Muller glia cells and PKA modulation of FGF9 signal will be analyzed. In order to show possible involvement of SIK2 in PKA and FGF9 pathways, its phosphorylation and translocation in response to these signals alone or in combination will be studied.

3. MATERIALS AND METHODS

3.1. Chemicals

All chemicals were obtained from Merck (Germany) or Sigma (USA) unless stated otherwise. When possible all solutions, plastic and glassware were sterilized by autoclaving at 121^0C for 20 minutes. Solutions for ribonucleic acid (RNA) studies were treated with 0.1 per cent diethylpyrocarbonate (DEPC) for 1 hour prior to sterilization, glassware were baked at 150^0C for 4 hours.

3.2. Animals

Wistar rats from the colony at Boğaziçi University Psychology Department were used. Handling of the animals conformed to the Boğaziçi University ethical guidelines. Animals were kept on 12 hr light/dark cycle, and whenever necessary ether anesthetized rats killed by cervical dislocation.

For RNA and protein extraction retinas dissected from enucleated eyes were immediately frozen and stored at -70^0C until used. Eyecups for immunohistochemical studies were fixed in 4 per cent paraformaldehyde and embeded into parafin blocks.

3.3. Cell Lines

Spontaneously immortalized Moorfields Institute of Ophthalmology Muller 1 (MIO-M1) human Muller glia cell line was obtained from Moorfields Institute of Ophthalmology, London. Routinely they were maintained under Dulbecco's modified eagle medium (DMEM) with glutamine (Invitrogen, USA) supplemented with 10 per cent fetal bovine serum (Biochrom, Germany) and 0.1 per cent penicillin and streptomycin. Cells were treated with 0.05 per cent trypsin in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) scraped and divided into 3 once a week when the plates reached confluency.

3.4. RT-PCR Reaction

3.4.1. RNA Preparation

Total RNA was extracted from dissected retinas by the method of Chomczynski and Sacchi (1987) with minor modifications (Buğra et al., 1994). Retinal tissue was homogenized in guanidium thicyanate (GTC) solution (12.5 g guanidine thiocyanate, 0.625 ml 1 M sodium citrate, pH 7.0, 125 g sodium N-laurylsarcosine in 25 ml H_2O) using a hand-held tissue grinder. To the samples, 0.1 volume of 2 M sodium acetate (pH 4), equal volume of phenol, and 0.2 volume of chloroform were added sequentially, vortexed and left on ice for 20 minutes to fractionate RNA free from other cellular macromolecules. The samples were centrifuged at 4^0C for 10 minutes at 10,000 xg and the aqueous phase containing the RNA was transfered into clean tubes. RNA was allowed to precipitate under an equal volume of isopropanol at -20^0C at least 3 hours. The pellets were collected by centrifugation at 10,000 xg at 4^0C, and washed with 75 per cent ethanol. The pellets were resuspended in H_2O_{DT}. Residual GTC was removed from the samples with two cycles of ethanol precipitations, achieved by adding 0.1 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol, and incubating at -20^0C at least for 2 hours. Finally the pellets were ethanol washed, air-dried and resuspended in H_2O_{DT}.

The concentrations were determined by absorption at 260nm where OD_{260} of 1 is equal to 40 µg/ml of RNA and samples with 1.8-2.0 ratio for OD_{260}/OD_{280} were considered pure. The integrity of the RNAs were checked by running in formaldehyde agarose gel.

3.4.2. Reverse Transcription

One µg of total RNA was used for reverse transcription reaction catalysed by Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, USA). The reverse transcription reaction mixtures, each containing 1µg of total RNA, 1µl of hexanucleotide random primers (500 µg/ml), 1.2 µl of 100 mM dNTP, 12 units of RNasin, 6 µl of 5X reverse transcription buffer (50 mM Tris.HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol) were incubated at 95^0C for 1 minute for the denaturation of

secondary structures in RNA. Subsequently, 400 units of enzyme and 30 units of RNasin were added, and the volume was adjusted to 30 µl with H_2O_{DT}. The samples were incubated at 37^0C for 2 hours, the enzyme was heat inactivated at 95^0C for 10 minutes. The total volume of the samples was brought up to 100 µl with H_2O_{DT} and the cDNAs were stored at -70^0C until used. Quality of cDNAs were tested by β-actin amplification.

3.4.3. PCR Reaction

Amplifications were carried out using 1.5 µl of cDNA, 15-60 nmols of dNTP mixture, 10-80 ng of each primer, 1-7 mM of $MgCl_2$, 0.5 units of Taq DNA polymerase (Fermentas, Lithuania) in 1X Taq polymerase buffer (10 mM Tris.HCl, pH 9.5, 50 mM KCl, 0.1 per cent Triton X-100) in a total volume of 15 µl. Amplification cycles consisted of denaturation at 95^0C for 30 seconds, annealing at the optimized temperature for 30 seconds and extension at 72^0C for 30 seconds. In order to compare transcript levels at different stages of development, PCR amplifications were done in the exponential phase of the reactions and the product levels were normalized to that of an internal control; housekeeping gene β-actin, results represent data from 3 different RNA samples and 3 reverse transcription (RT) reactions from each. The number of amplification cycles and the concentration of primers in co-amplification reactions were determined experimentally. Primers for the PCR reactions were synthesized at Iontek, Turkey. The list of primers, their sequences, and the optimal conditions, expected product lengths are given in Table 3.1 and 3.2, respectively.

3.4.4. DNA Gel Electrophoresis

Amplification products were mixed with 6X loading buffer (30 per cent glycerol, 0.005 per cent bromophenol blue) and loaded onto 1.5-2.5 per cent agarose gels with 0.5 µg/ml ethidium bromide. The gels were run at 100 V in 1X tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (TAE) containing 40 mM Tris.HCl, 1 mM EDTA, 0.1 per cent acetic acid. The images were documented with BioRad XR system under UV.

Table 3.1. List of primers used in this study

β-actin	Upper	5'-TCATCAAGTGTGACGTTGACATCCGT-3'
	Lower	5'-CCTAGAAGCATTTGCGGTGCACGATG-3'
SSIKF3-R1	Upper	5'-TTGCTGAACAAACAGTTGCC-3'
	Lower	5'-TCAAGCAGACAGCCATTCAC-3'

Table 3.2. Optimal amplification conditions and the product lengths

Gene	$[Mg2^{+}]$	dNTP	Annealing temperature	Product length
β-actin	1.5 mM	1 mM	53°C	285 bp
SSIKF3-R1	3 mM	1 mM	54°C	208bp

3.4.5. RNA Gel Electrophoresis

In order to prepare one per cent formaldehyde agarose gel, 1.5 g agarose was boiled in 127 ml H_2O, 14 ml 10X MOPS buffer (200 mM Morpholinopropane sulfonic acid (pH 7.0), 80 mM sodium acetate, 10 mM EDTA), 8 ml 37 per cent formaldehyde was added after it was cooled to about 55^0C. The RNA samples were denatured at 65^0C for 2 minutes, and immediately chilled on ice and loaded with 2 volumes of RNA loading buffer (250 µl deionized formamide, 83 µl 37 per cent formaldehyde, 50 µl 10X MOPS, 50 µl RNase-free glycerol, 10 µl 2.5 per cent bromophenol blue, 57 µl DEPC-treated water, 0.25 µg/ml ethidium bromide). The gel was run at 50 V. The bands were visualized under UV light and images were documented with BioRad XR system.

3.5. *In Situ* Hybridization

For *in situ* hybridization, eyecups dissected from adult rats were frozen in tissue-freezing medium (OCT; Polysciences Inc.,Warrington, PA) and sectioned in a cryostat (section hickness of 10 µm). Sections were collected on sterile Vectabond-coated slides (Vector Labs, Burlingame, CA) and stored at -70^0C until used. Hybridizations were carried out from sections obtained from three different animals and in triplicate. Before hybridization, tissue sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Hybridizations were carried out overnight at 45^0C in 50% formamide, 5X sodium chloride/sodium citrate (SSC), 5X Denhardt's solution, 0.4 mg/ml torula RNA, 0.1

mg/ml tRNA, and 3 ng/ml digoxigenin (DIG) labeled probe. Slides were washed at room temperature sequentially in 5X SSC, in 2X SSC containing 50% formamide, and finally in 0.2X SSC at 55^0C. Signal detection was carried out using a detection kit from Boehringer Mannheim (Mannheim, Germany). Briefly, sections were incubated with blocking solution for 1.5 hr, and subsequently with alkaline phosphatase-conjugated anti-DIG antibodies for 30 min. Washed sections were treated with levamisole in reaction buffer (0.1 M Tris, 0.1 M NaCl, and 1 mM $MgCl_2$, pH 9.5), air dried, and incubated with chromogenic substrates nitro blue tetrazolium/5-bromo-4-chloro-3-. indolyl phosphate (NBT/BCIP) in the dark for 3–24 hr. Color development was stopped by washing in PBS.

3.6. Western Blot Analysis

Protein extracts were obtained by sonicating cells in lysis buffer (2 per cent SDS, 80 mM Tris.HCl, pH 6.8, 2 per cent glycerol, 10 per cent β-mercaptoethanol, 0.005 per cent bromophenol blue). After the cell debris was pelleted by centrifugation at 12 000 xg and the supernatant boiled for two minutes, the samples were resolved by sodium dodecyl sulfate (SDS) containing polyacrylamide gels, 10 per cent acrylamide:bisacrylamide (29:1), 375 mM Tris.HCl, pH 8.8, 0.1 per cent SDS, 0.1 per cent ammonium persulfate (APS), 0.1per cent N,N,N',N',-tetramethylethylenediamine (TEMED), and blotted on a polyvinylidene fluoride (PVDF) membrane (Roche, Germany) in transfer buffer (200 mM glycine, 25 mM Tris.HCl, 15 per cent methanol) at 100 V for 1-1.5 hours. Membranes were blocked with 1 per cent bovine serum albumin (BSA) and 0.5 per cent milk powder in tris-sodium chloride-tween (TBST) buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.1 per cent tween 20) for 1 hour. Subsequently, membranes were incubated overnight in TBST containing the appropriate antibodies at dilutions given in Table 4.3. The membranes were washed with TBST, and incubated for 1 hour with horseradish peroxidase (HRP) conjugated secondary antibodies. After washing with TBST again the membrane was incubated in Lumi-light Western Blotting substrate for 5 minutes and visualised by exposure to chemiluminescent detecting film (Amersham). Results presented here represent outcome of 3 different experiments.

Table 3.3. List of antibodies used in this study

Antigen	Supplier	Origin-modification	Dilution	
			Western	Immunology
FGF9	Santa Cruz	Rabbit		1:20
	Santa Cruz	Mouse		1:200
SIK2	Gift from Dr. Akira Miyauchi	Rabbit	1:500	1:250
	Abnova	Mouse	1:1000	1:500
Phospho-Erk1/2	Santa Cruz	Mouse	1:1000	
Erk1/2	Santa Cruz	Rabbit	1:1000	
FGFR2	Santa Cruz	Rabbit		1:200
FGFR3	Santa Cruz	Rabbit		1:250
IgG	Sigma	Mouse, Rabbit HRP conj.	1:5000	
IgG	Gift from Dr. David Hicks	Mouse, Rabbit Fluorescence conj.		1:1000
DIG	Roche	Sheep AP conj.		1:5000

3.7. Immunological Staining

When stainings done with the cell line, Muller cells seeded on round coverslips were washed with PBS, fixed in 4 per cent paraformaldehyde (pH 7.2) and permeabilized with 0.1 per cent Triton X-100 in PBS. For blocking 1 per cent BSA, 0.1 per cent tween 20 in PBS was used. Primary antibody incubation was done overnight at 4^0C with 1:250 dilutions. Cells were then washed with PBS, and incubated for 1 hour at 4^0C in the fluorescein isothiocyanate (FITC)or texas red (TR) conjugated secondary antibody, and 4'-6-diamidino-2-phenyindole (DAPI) diluted 1:5000, for the visualization of nuclei, in the blocking solution. Antibody dilutions are given in Table 4.3. Observations were done under fluorescent microscope.

When tissue samples were stained, retinal sections were deparaffinized by soaking in xylol and rehydrated by sequential immersion in 100 per cent, 90 per cent, 70 per cent ethanol and finally in water. The sections were boiled in 10 mM citrate buffer (pH 6.0) for antigen retrieval, allowed to cool down to room temperature and washed with water. For permeabilization, sections were incubated in 0.1 per cent Triton X-100 in PBS. Blocking was done in 5 per cent BSA in PBS for 1 hour. Antibody incubations at the dilutions indicated on the table was done overnight at 4^0C. After PBS washes, the sections were incubated at fluorescent conjugated secondary antibody solution for 1 hour and visualized under fluorescent microscope.

3.8. Treatment of MIO-M1 Cells

Muller cells were grown to sub-confluency washed with PBS and starved in DMEM containing 0.1 per cent streptomycin and penicillin overnight. After treatment with 10 ng/ml FGF9 and 10 µg/ml heparin for 0 minutes (negative control), 10, 30 minutes, 1, 2, 6 hours they were washed with ice-cold PBS containing 1 mM Na_3VO_4, a phosphatase inhibitor. Cells were scraped, collected in microfuge tubes, and pelleted with centrifugation and stored at -70^0C until used. For the treatment of cells with 8BrcAMP and H89, PKA activator and inhibitor, respectively, Muller cells were cultured as described above, except the cells were treated with PKA activator/inhibitor 30 minutes prior to the addition of FGF9 and heparin. The final concentration of PKA activator was 500 µM and PKA inhibitor was 10 µM.

3.9. Phosphoprotein Purification

Phosphoprotein purification from non-treated, FGF9 and PKA induced MIO-M1 cells were done with phosphoprotein purification kit from Qiagen, USA according to the manufacturer's manual. Briefly, 10^7 cells were lysed in 5ml of phosphoprotein lysis buffer and incubated at 4^0C for 30 minutes. After incubation cell lysate was centrifuged at 10 000 xg for 30 minutes at 4^0C. Collected supernatant was then passed through the phosphoprotein purification column allowing binding of phosphorylated proteins to the affinity column. After the column is washed, phosphoproteins were eluted with the phosphoprotein elution buffer. Eluates were then used for western blot analysis.

4. RESULTS

4.1. Distribution of FGF9 in Postnatal Rat Retina

Immunohistochemical labeling of postnatal day 0 (PN0) rat retina sections with anti-FGF9 antibody revealed immunoreactivity predominantly in the IPL, with fainter labeling also detectable in the cytoplasm of cells within the NZ, GCL (Fig. 4.1A, E). At PN8, with the formation of the three cellular layers virtually complete, significant labeling was visible only in the IPL, with no significant staining of any other structure (Fig. 4.1B, F). At PN16, FGF9 immunoreactivity was noteworthy in the IPL as well as the OPL, and faint expression was also evident in neuronal cell bodies in all three nuclear layers (Fig. 4.1C, G). Retina is essentially developed fully at this age, and moderate FGF9 labeling of photoreceptor inner segments was also evident. FGF9 expression was similar in adult retina, with relatively more intense immunoreactivity within the OPL (Fig. 4.1D, H). Similar results were obtained using either the monoclonal antibody raised against human full-length FGF9 or the polyclonal antibody directed against the N-terminus. We observed no staining in control experiments where the primary antibodies were preadsorbed with the immunizing peptide, indicating the specificity of the immunolabeling (Fig. 4.1I).

In situ hybridization on sections of adult retina was carried out using DIG-labeled antisense FGF9 or sense transcripts generated *in vitro*. The antisense probe produced a sparse signal mostly overlying the INL, with fainter staining overlying the ONL (Fig. 4.2B), agreeing with the immunological stainings. Sense probe on the other hand revealed no hybridization signal, indicating the specificity of the labeling (Fig. 4.2A).

Immunocytochemical stainings using anti-FGF9 antibodies failed to reveal detectable staining in cultured MIO-M1 cells (Figure 4.3A). This result is in agreement with the former data obtained by RT-PCR, clearly indicating that cultured Muller glia lack FGF9 expression (Çınaroğlu *et al*., 2005).

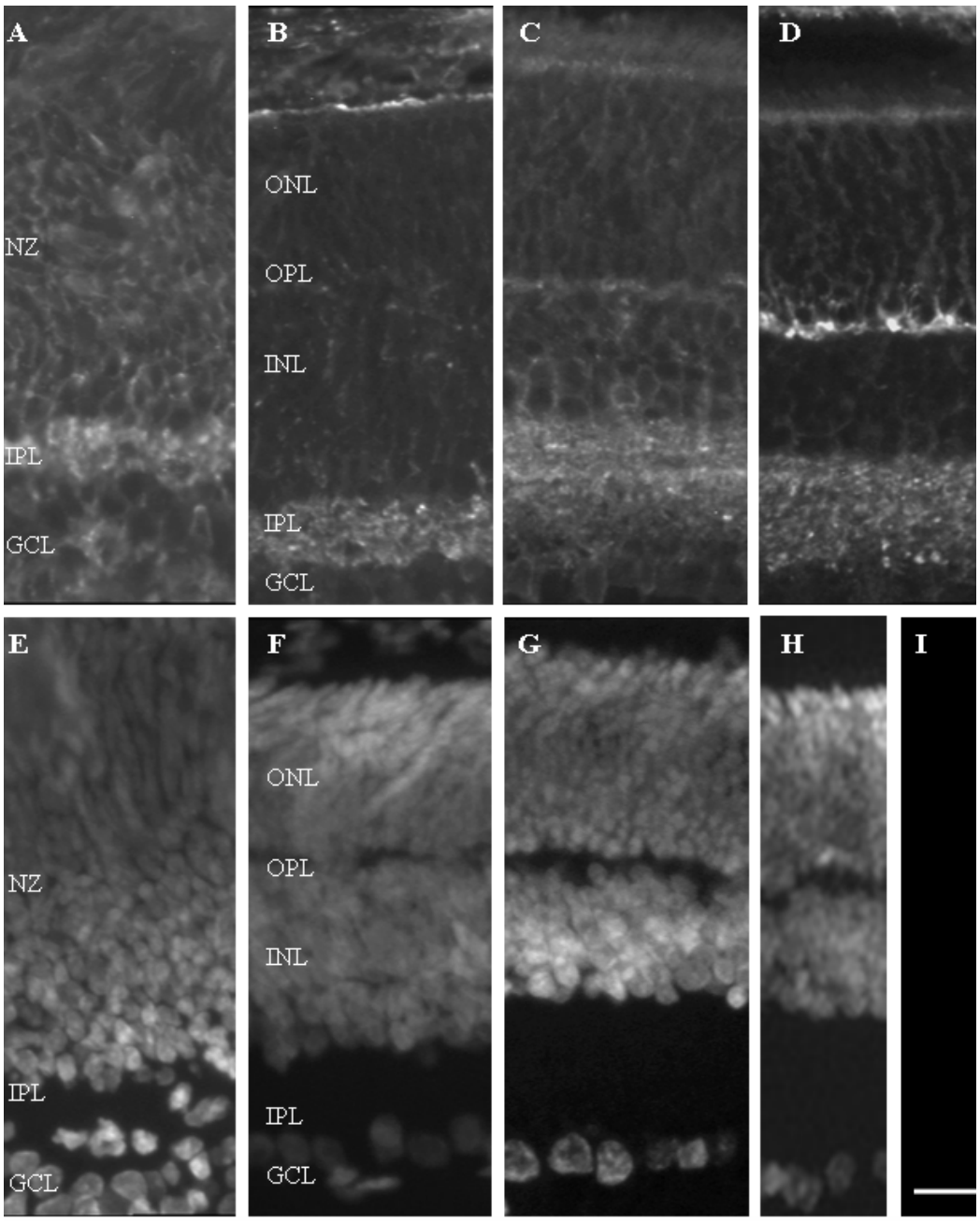

Figure 4.1. Immunohistochemical labeling of FGF9 in developing rat retina. Paraffin embedded sections from PN0 (A), PN8 (B), PN16 (C), and adult (D) were stained with anti-FGF9 and FITC conjugated secondary antibody. Panels E-H show the correspondingsections stained with DAPI. Panel I represents negative control where antibody preadsorbed with the immunizing peptide

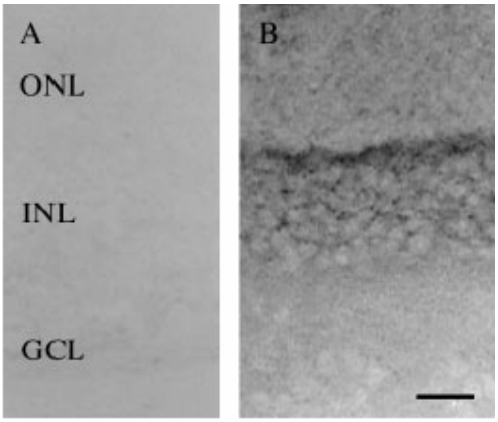

Figure 4.2. In situ hybridization of DIG-labeled FGF9 transcripts in adult rat retina A: Retinal sections hybridized with sense probe, B: Retinal sections hybridized with antisense probe

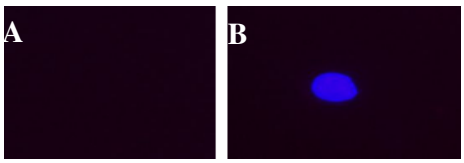

Figure 4.3. Immunocytochemistry of FGF9 in MIO-M1 A: Cells were stained with anti-FGF9 and anti-FITC conjugated secondary antibodies, B: DAPI staining to visualize nucleus

4.2. Immunological Detection of FGFR2 and FGFR3 in Rat Retina and MIO-M1

Immunolabeling patterns in sections using anti-FGFR2 or R3 antisera were largely similar with slight differences in intensity between layers. At PN0, staining was observed at the level of the newly differentiated GCL and IPL (Fig. 4.4A, D). By PN8, the staining had also spread through the INL and ONL (Fig. 4.4B, E). At PN16 and adult, labeling was essentially similar, although more intense throughout the inner layers (Fig. 4.4C, D; F, G). FGFR2 and R3 immunoreactivity was also observed in the RPE at all ages. Control sections in which antibody was preadsorbed with excess peptide were devoid of staining (Figure 4.4I).

Immunocytochemical staining of FGFR2 and FGFR3 in MIO-M1 cells reveal the expression of both receptors in these cells (Figure 4.5B,E). Stainings were observed in addition to diffuse staining across the cell surface with intense labelling within the nucleus.

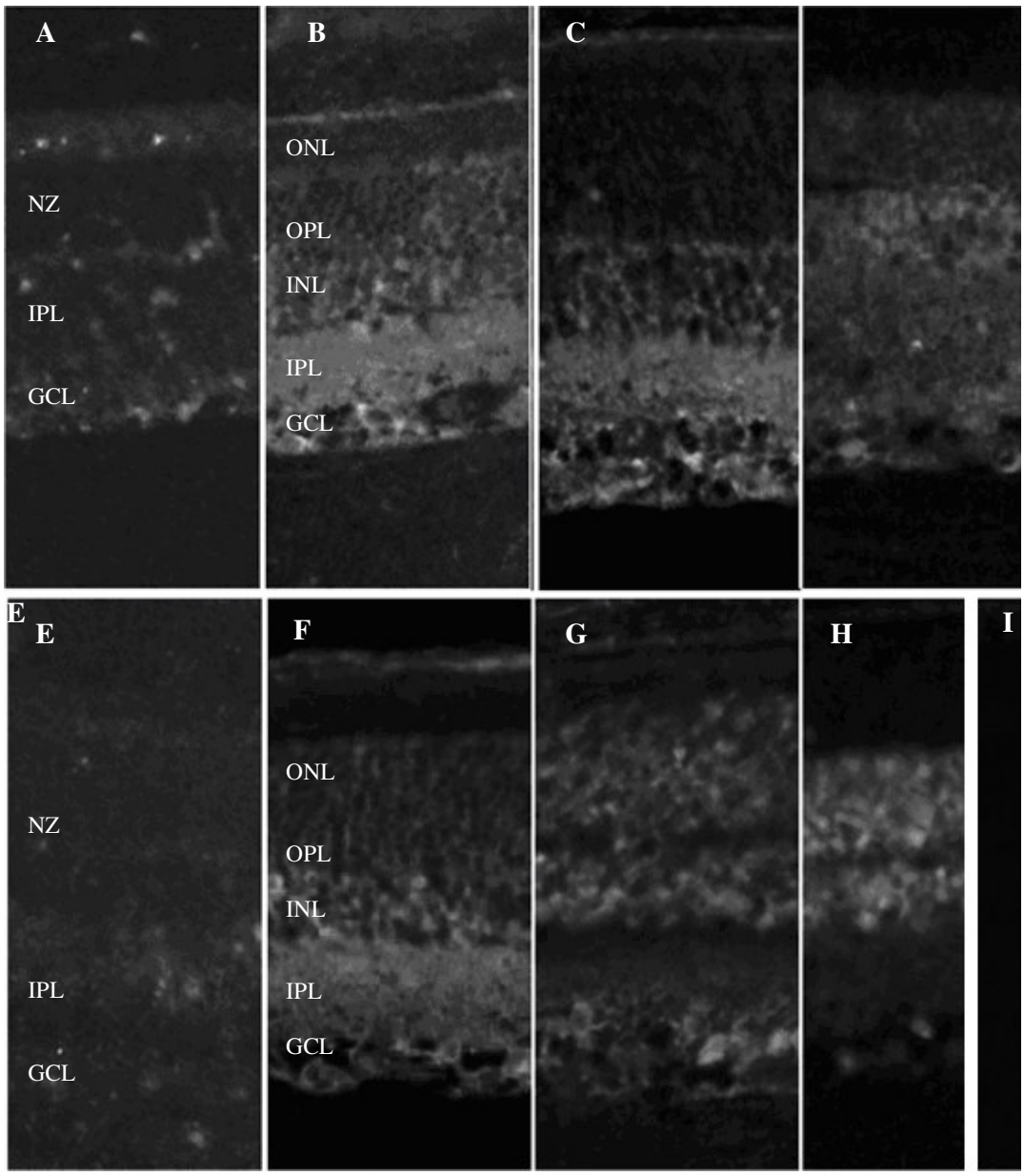

Figure 4.4. Immunohistochemical labeling of FGFR2 and FGFR3 in rat retina. Retinal sections of PN0 (A, E), PN8 (B, F), PN16 (C, G), and Ad (D, H) were stained with anti-FGFR2 (upper panel) or anti-FGFR3 (lower panel) and FITC conjugated secondary antibodies. A-D: FGFR2 immunohostochemistry, E-H: FGFR3 immunohistochemistry, I: Negative control

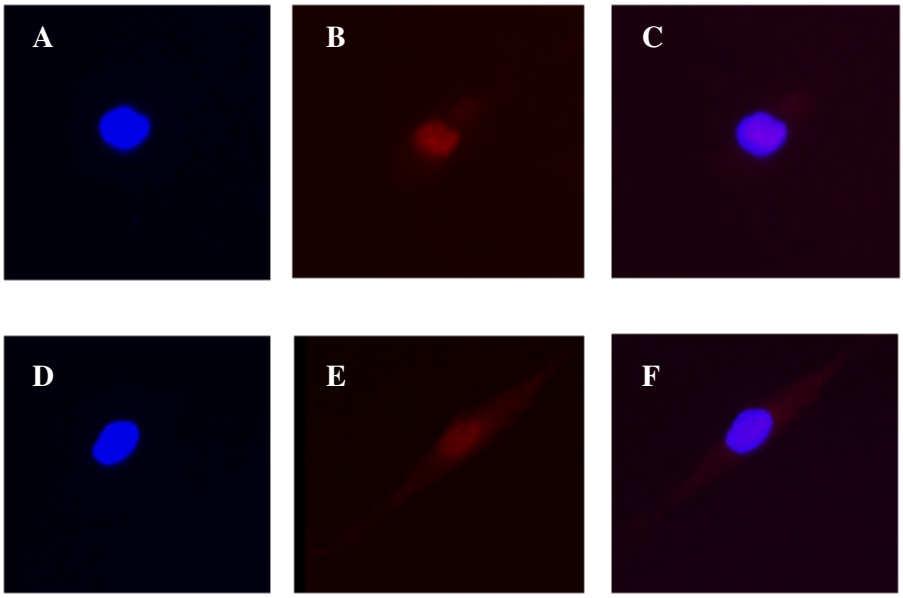

Figure 4.5. Immunocytochemical staining of FGFR2 and FGFR3 in MIO-M1 cells. Cells were stained with anti-FGFR2 (B) or anti-FGFR3 (E) and Texas red conjugated secondary antibody. Same samples were DAPI stained to visualize nucleus (A and D), the merged images are shown in C and F

4.3. Expression Profile and Cellular Distribution of SIK2 in Postnatal Rat Retina and MIO-M1 Cells

The relative steady-state levels of SIK2 transcripts were analyzed by RT-PCR in samples from PN0, PN4, PN6, PN10, PN14, PN20 and adult rat retinas, and the amplifications were done at the exponential phase. The quality of the starting RNA samples are shown in Figure 4.6. In samples of all ages the expected 208 bp band was obtained. When densitometric readings were normalized with that of internal control β-actin, the results indicate that SIK2 steady-state transcript levels increase gradually from PN0 to adult with a slight decrease at adult (Fig. 4.6). No amplification products were detectable in controls where reverse transcription step was omitted (data not shown).

Immunological detection of SIK2 in postnatal rat retina and MIO-M1 cells was done using two commercially available antibodies that are directed against overlapping but different C-terminal peptides of SIK2. Both antibodies revealed same immunological staining pattern, the data presented here represents both.

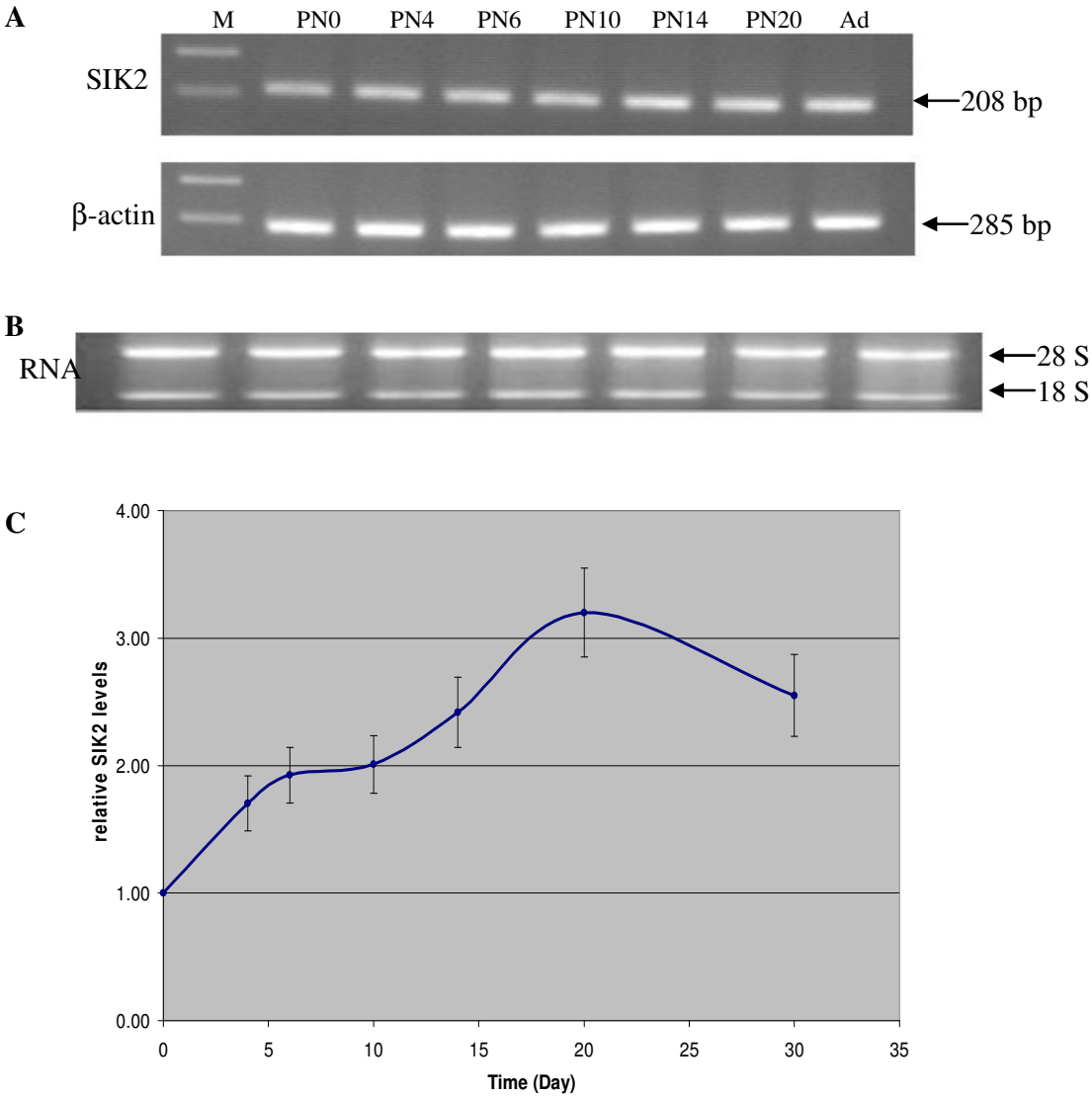

Figure 4.6. Expression of SIK2 in developing rat retina. A: Steady state transcript levels of SIK2 and β-actin in retina of different postnatal ages assesed by RT-PCR, B: Starting RNA levels in each sample, C: Relative transcript levels of SIK2 normalized to that of β-actin is represented in arbitrary units. M: DNA ladder

Immunohistochemical staining of SIK2 at PN0 was seen predominantly in IPL, with fainter labeling also detectable in the cytoplasm of cells within the NZ and GCL (Fig. 4.7A). At PN8, significant labeling was visible in the IPL, OPL, and both nuclear layers (Fig. 4.7B). At PN16, where retina is essentially developed fully, SIK2 immunostaining at both plexiform layers and moderate staining at both nuclear layers was observed (Fig.

4.7C). SIK2 expression was similar in adult retina, with relatively more intense immunoreactivity of photoreceptor inner segments (Fig. 4.7D).

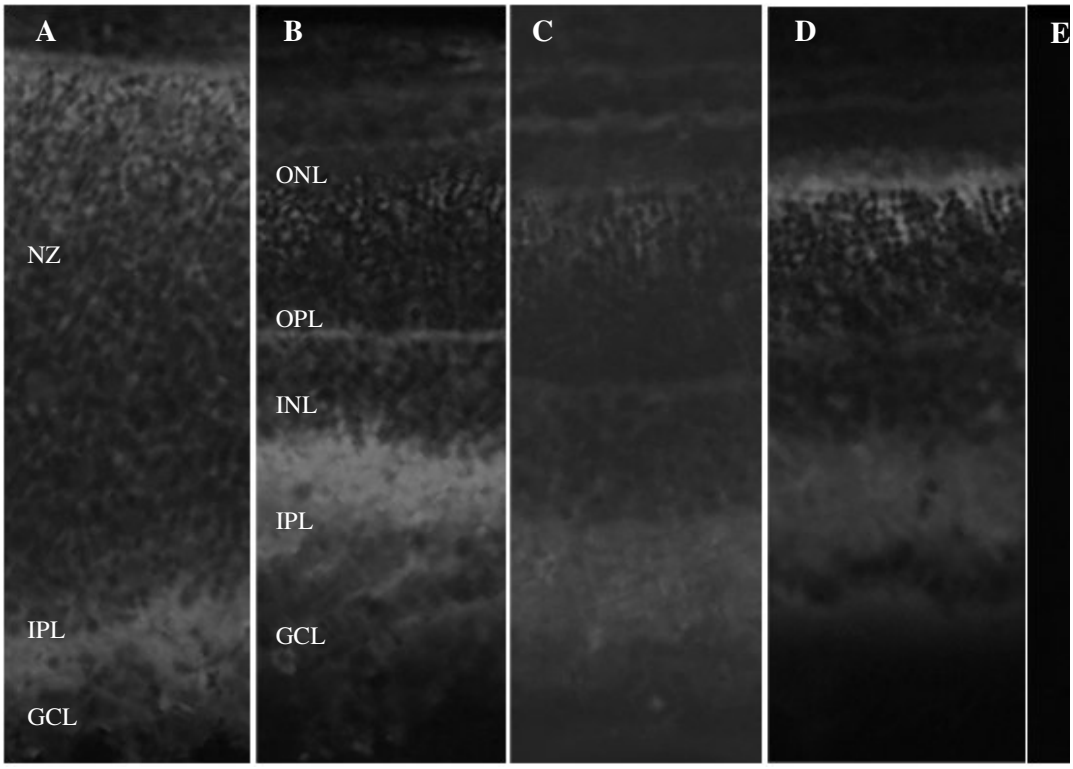

Figure 4.7. Immunohistochemical labeling of SIK2 in developing rat retina. Retinal sections were treated with anti-SIK2 and FITC conjugated secondary antibodies A: PN0, B: PN8, C: PN16, D: Adult, E: Negative control where primary antibody was omitted

Immunocytochemical detection of SIK2 in MIO-M1 cells were observed both in cytoplasm and in the nucleus (Fig. 4.8B). The merged image (Fig. 4.8C) clearly indicates nuclear localization of the protein.

Western blot analysis of SIK2 from MIO-M1 cell lysates revealed a predominant band around 50 kDa (Fig. 4.9). We also detect minor bands higher molecular weight as well as smaller ones.

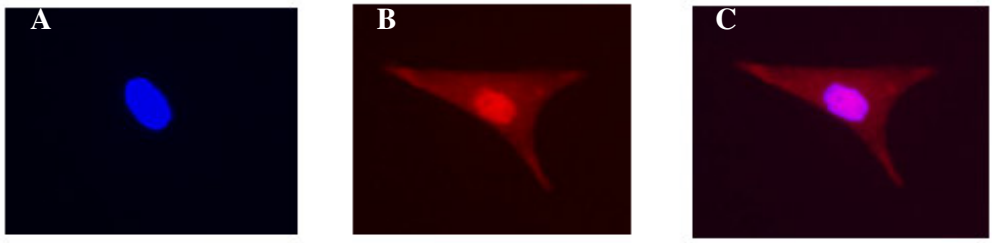

Figure 4.8. SIK2 immunocytochemistry in MIO-M1 cells. Panel A shows DAPI staining of cell nucleus, Panel B represents cells stained with anti-SIK2 and Texas red conjugated secondary antiboy. In Panel C the merged image is shown

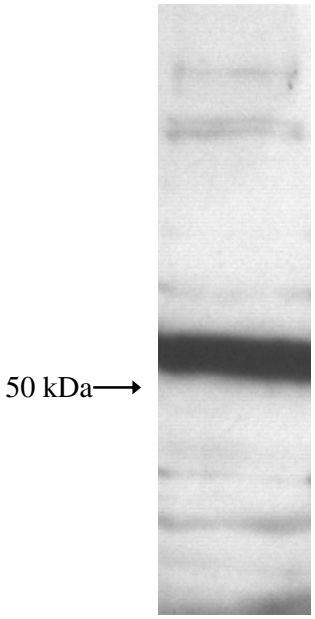

Figure 4.9. Western blot analysis of SIK2 from MIO-M1 cells. Protein extracts of MIO-M1 cells were fractionated in 10% polyacrylamide gells, and blotted. Detection was done using anti-SIK2 and HRP conjugated secondary antibodies

4.4. Phosphorylation of ERK in Response to FGF9 Induction and PKA Activation/Inhibition

Western blot analysis of lysates from FGF9 and/or PKA activator/inhibitor treated MIO-M1 cells were carried out using anti-pERK antibody to observe changes in phosphorylated ERK levels as a result of the stimuli, and results were normalized to the internal ERK levels.

Upon FGF9 induction of MIO-M1 cells, pERK levels doubled at 10 min induction and the signal was seen to return to basal levels at 30 min and maintained at that level then after (Figure 4.10).

Cells were treated with the PKA inhibitor, H89, prior to FGF9 induction and blotted with anti-pERK and anti-ERK antibodies to deplete PKA activity. The treatment resulted in an increase of pERK levels and signal attenuation evident at 30 min in cells subjected to FGF9 induction alone, is no longer observed (Figure 4.11).

We next activated PKA prior to FGF9 induction with the use of 8BrcAmp under the same conditions as above. Activation of PKA seem to decrease overall pERK levels, whereas the phosphorylation pattern with respect to FGF9 treated cells did not seem to be altered (Figure 4.12).

In the following set of experiments pERK levels of cells treated with FGF9 alone for 30 min were compared to that of cells exposed to H89 or 8BrcAmp prior to FGF9 induction for 30 min. With FGF9 alone and in combination with 8BrcAmp the signal seems to be downregulated to near basal levels as described above. In contrast, inhibition of PKA prior to FGF9 induction resulted in an increased ERK phosphorylation at 30 min (Figure 4.13).

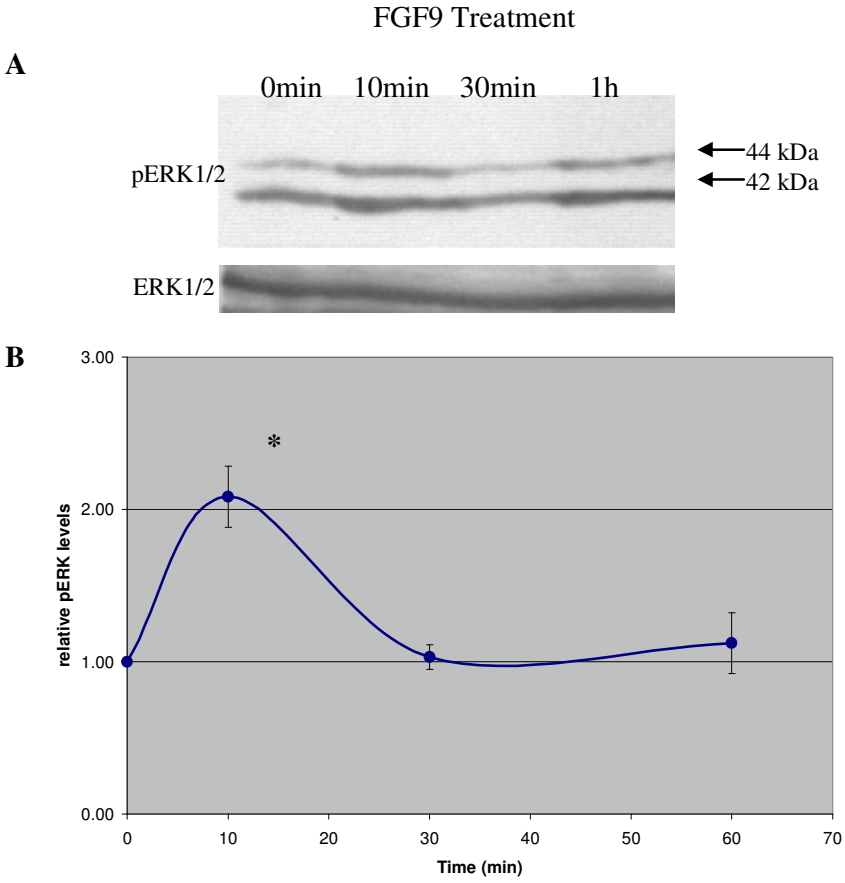

Figure 4.10. Phosphorylation of ERK in FGF9 induced MIO-M1 cells. A: Cells were treated with FGF9 for the indicated durations. Cell extracts were used in immunoblots probed with anti-pERK1/2 or anti-ERK1/2 and followed by HRP conjugated secondary antibodies, B: Relative pERK levels normalized to that of total ERK levels and presented in arbitrary units
* $p<0.05$, $F=5.898$ (anova test)

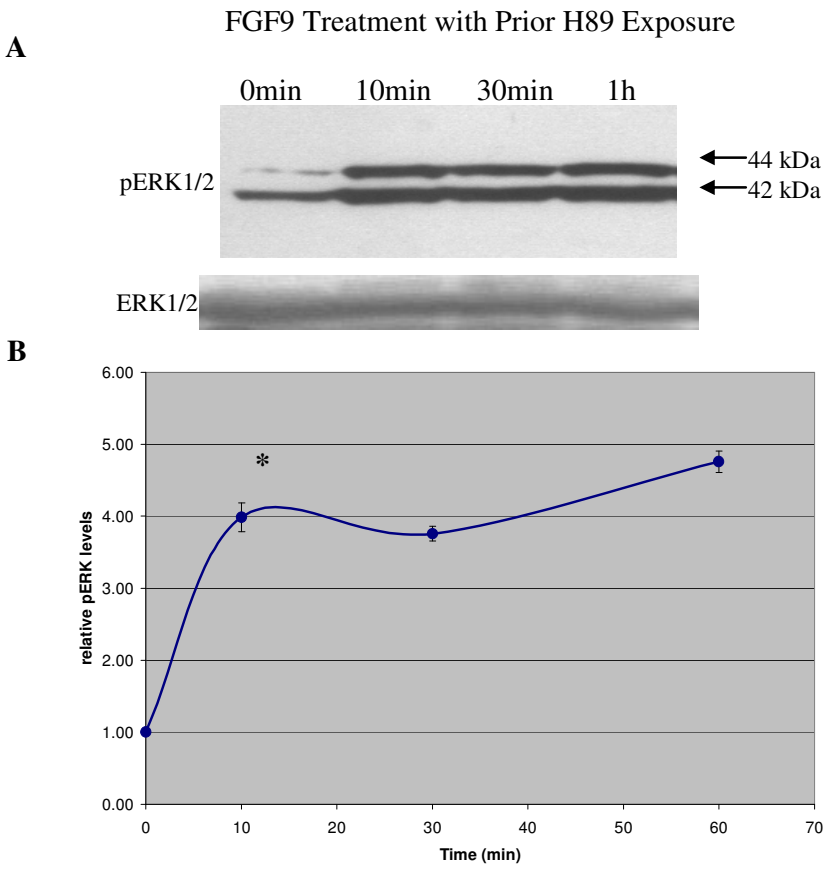

Figure 4.11. Phosphorylation of ERK in MIO-M1 cells treated H89 prior to FGF9 induction. A: MIO-M1 cells were FGF9 treated with prior H89 exposure and immunobloted with anti pERK1/2 or anti-ERK1/2 followed by HRP conjugated secondary antibody, B: Relative pERK levels normalized to that of total ERK and presented in arbitrary units

* $p<0.05$, $F=11.746$ (anova test)

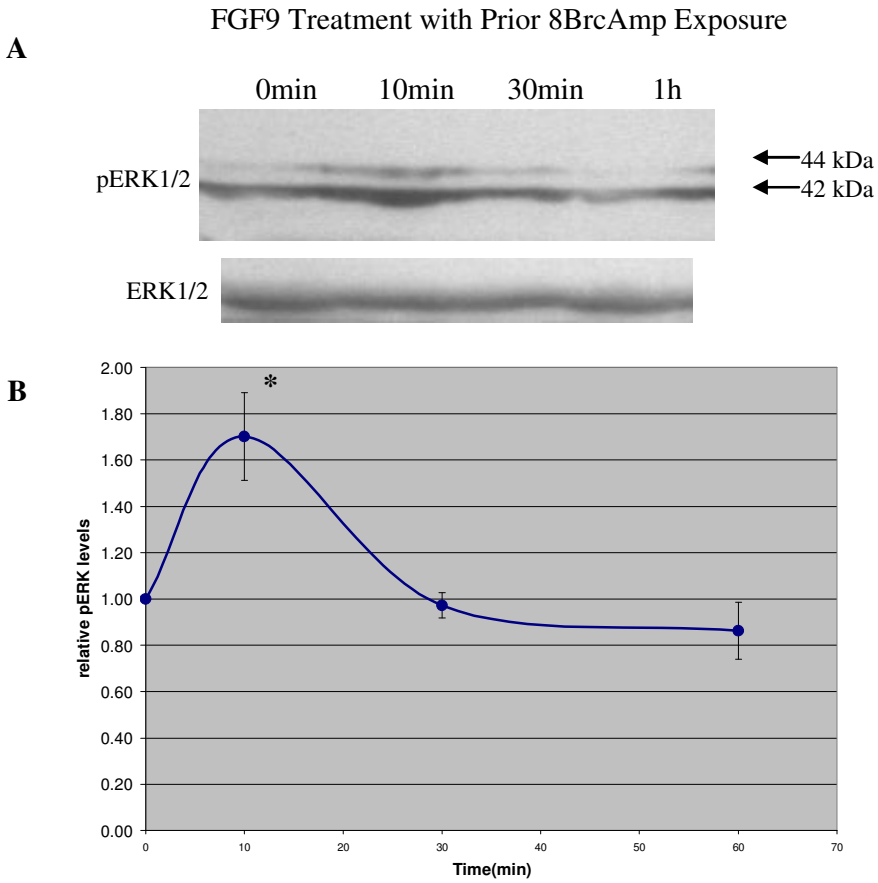

Figure 4.12. Phosphorylation of ERK in MIO-M1 cells treated with 8BrcAmp prior to FGF9 induction. A: MIO-M1 cells were exposed 8BrcAmp for 30 min and subsequently treated with FGF9 for the indicated durations. Lysates were immunobloted with anti-pERK1/2 or anti-ERK1/2, and HRP conjugated secondary antibodies. B: Relative pERK levels normalized to that of total ERK and presented in arbitrary units

* $p<0.001$, $F=33.018$ (anova test)

4.5. Phosphorylation of SIK2 in Response to FGF9 Induction and PKA Activation

In order to observe phosphorylation status of SIK2 in response to FGF9 induction and PKA activation, individual subconfluent plates of MIO-M1 cells were treated either with FGF9 or PKA activator, 8BrcAmp for 30 min, or left untreated. Cell lysates were passed through phosphoprotein purification columns and half of the eluate was subjected to Western blot analysis using anti-SIK2 antibody. Level of phosphorylated SIK2 appeared

to increase both in FGF9 and 8BrcAmp treated cells compared to that of untreated cells (Figure 4.14).

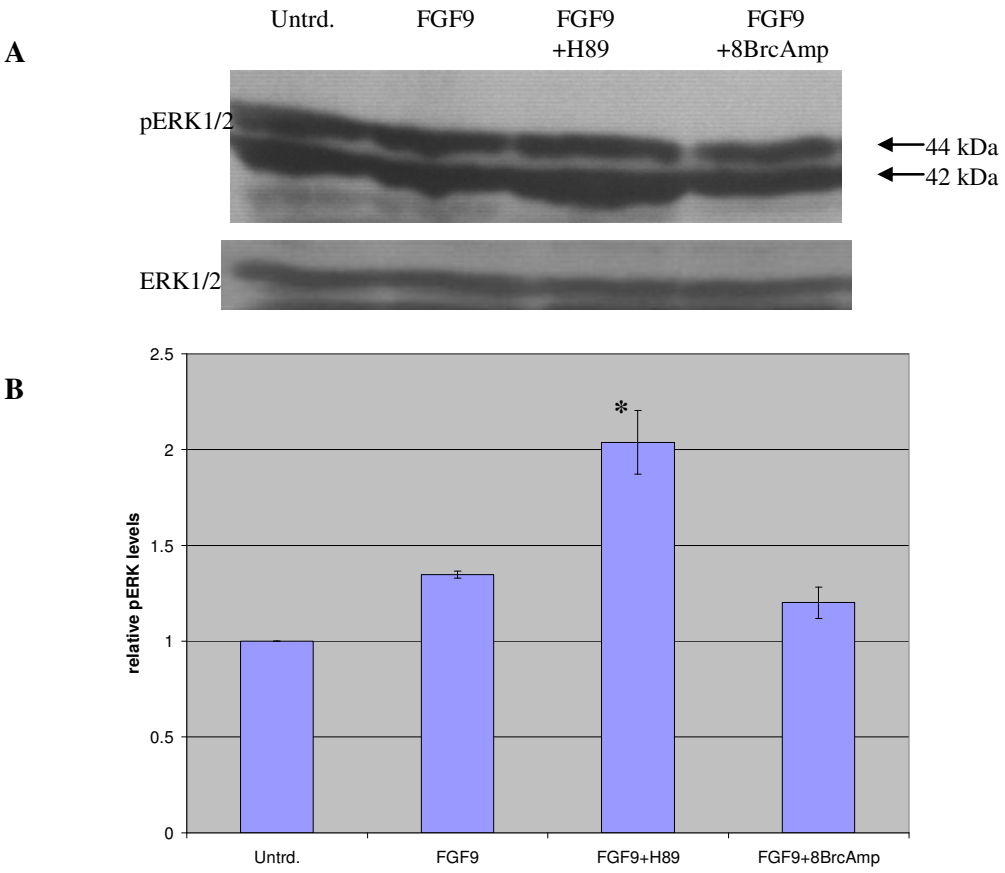

Figure 4.13. Phosphorylation of ERK in treated MIO-M1 cells. A: Extracts from untreated MIO-M1 cells, or the ones induced with FGF9 alone or with prior exposure to H89 or 8BrcAmp were immunoblotted using anti-pERK1/2, anti-ERK1/2, and HRP conjugated secondary antibodies. B: Relative pERK levels normalized to that of total ERK levels and presented in arbitrary units

* $p<0.001$, $F=71.127$ (anova test)

4.6. Cellular Localization of SIK2 in Response to FGF9 Induction and PKA Activation/Inhibition

In resting cells SIK2 was observed both in cytoplasm and nucleus (Figure 4.15B). However when cells were treated with PKA activator, 8BrcAmp, it was observed that SIK2 exited nuclei (Figure 4.15E). And when cells were incubated with PKA inhibitor, H89, SIK2 localization showed no difference with respect to resting cells (Figure 4.15H).

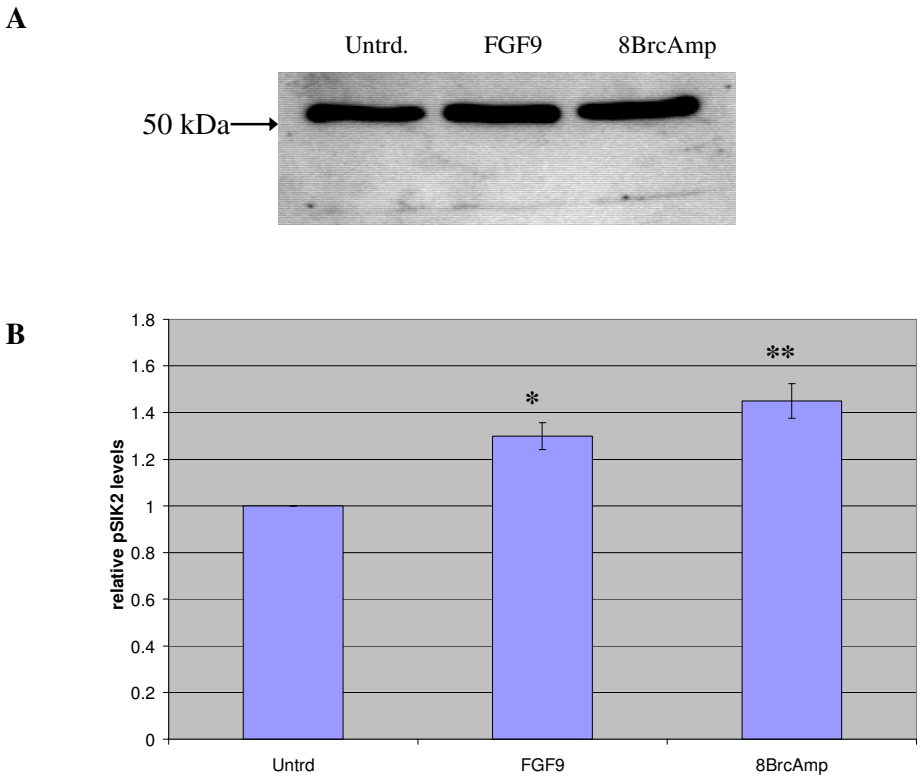

Figure 4.14. Phosphorylation of SIK2 with FGF9 and PKA induction. A: Lysates of MIO-M1 cells either untreated or treated with FGF9 or 8BrcAmp were purified using phosphoprotein purification columns and immunoblotted with anti-SIK2 and HRP conjugated secondary antibodies. B: Relative pSIK2 levels presented in arbitrary units
* $p<0.05$, ** $p<0.001$; $F=51.573$ (anova test)

When cells were induced with FGF9 for 10 min, again it was observed that SIK2 translocates to cytoplasm of the cells (Figure 4.16B). In contrast, upon 30 min FGF9 induction of cells resulted in distribution of SIK2 similar to the resting cells (Figure 4.16E).

Altogether these data indicated that both PKA and FGF9 phosphorylated SIK2 and leads to a change in its cellular localization.

We wanted to observe the combinatorial affect of FGF induction and PKA activity on SIK2 localization in MIO-M1 cells. When cells were treated with 8BrcAmp prior to 10 min FGF9 induction, intracellular SIK2 localization pattern did not conform to any of the

previously observed uniform distribution; SIK2 was seen in nuclei in some of the cells but exclusively cytoplasmic in others (Figure 4.17. B). When cells were treated with H89 prior to 10 min FGF9 induction the localization pattern was the same as 10 min FGF9 induction alone, no SIK2 was detected in nuclei (Figure 4.17. E). When PKA was activated prior to FGF9 induction for 30 min, SIK2 localization was observed in nuclei of the cells similar to the pattern seen in cells induced with FGF9 alone for 30 min (Figure 4.18. B). But when PKA was inhibited prior to FGF9 induction, localization of SIK2 in the majority of the cells did not represent either of the situations (Figure 4.18. E).

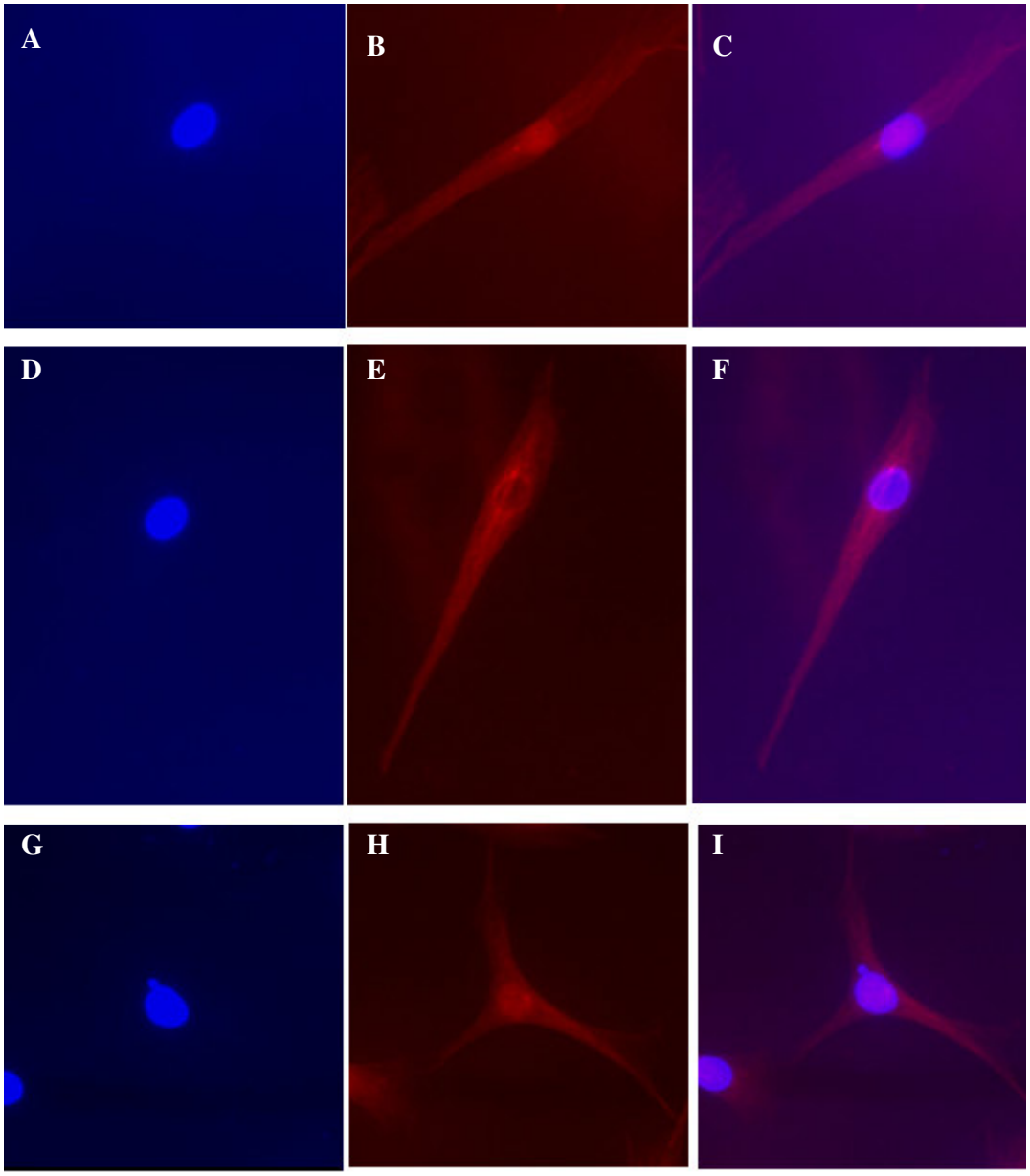

Figure 4.15. Cellular localization of SIK2. Immunocytochemical staining was carried out using nanti-SIK2 and Texas red conjugated secondary antibodies. A-C: untreated cells, D-F: 8BrcAmp treated cells, G-I: H89 treated cells. The nuclei were visualized with DAPI stainings (A, D and G), SIK2 stainings are given in panels B, E and H and the merged images are presented in panels C, F and I

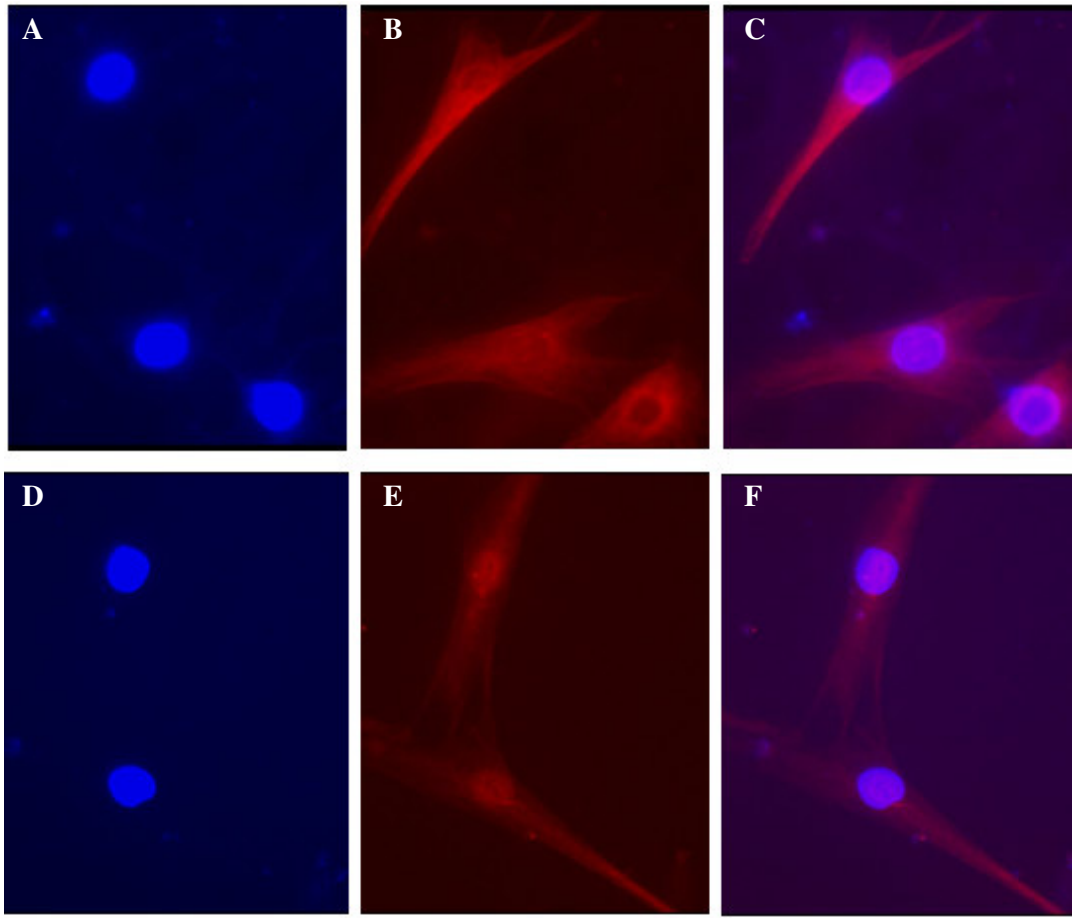

Figure 4.16. Cellular localization of SIK2 in MIO-M1 cells induced with FGF9. Immunocytochemical staining was done with anti-SIK2 and Texas red conjugated secondary antibodies. A-C: cells treated with FGF9 for 10 min, D-F: cells treated with FGF9 for 30 min. DAPI stainings are given in panels A and D; SIK2 stainings presented in panels B and E; merged images are shown in panels C and F

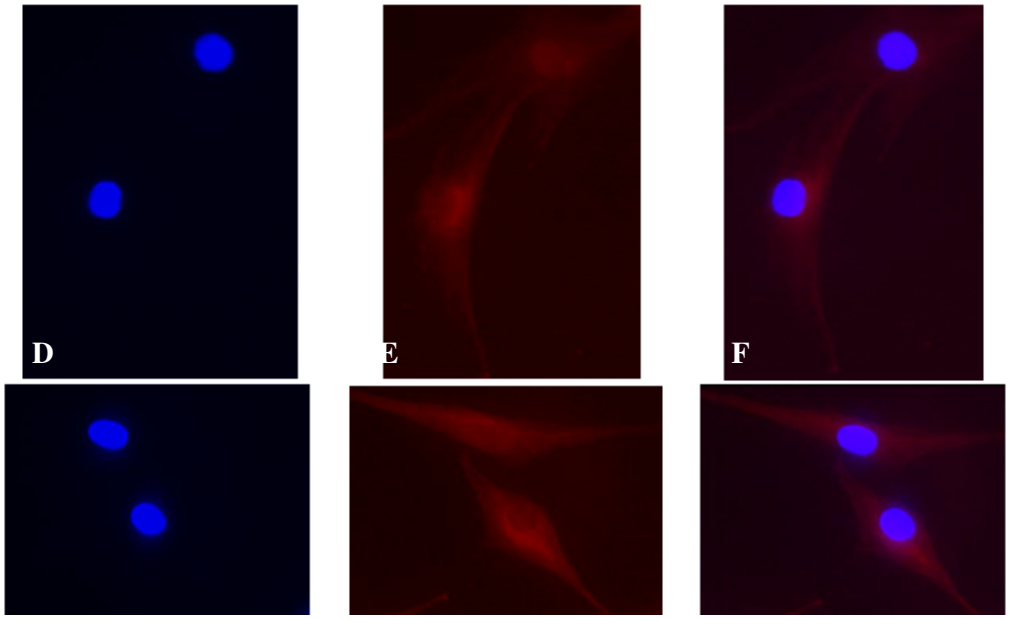

Figure 4.17. Cellular localization of SIK2 in MIO-M1 cells treated with FGF9 and PKA activator or inhibitor. A-C: Cells treated with 8BrcAmp and subsequently exposed to FGF9 for 10 min, D-F: Cells treated with H89 and subsequently exposed to FGF9 for 10 min. DAPI stainings are shown in panels A and D, anti-SIK2 stainings are presented in panels B and E; the merged images are given in panels C and F

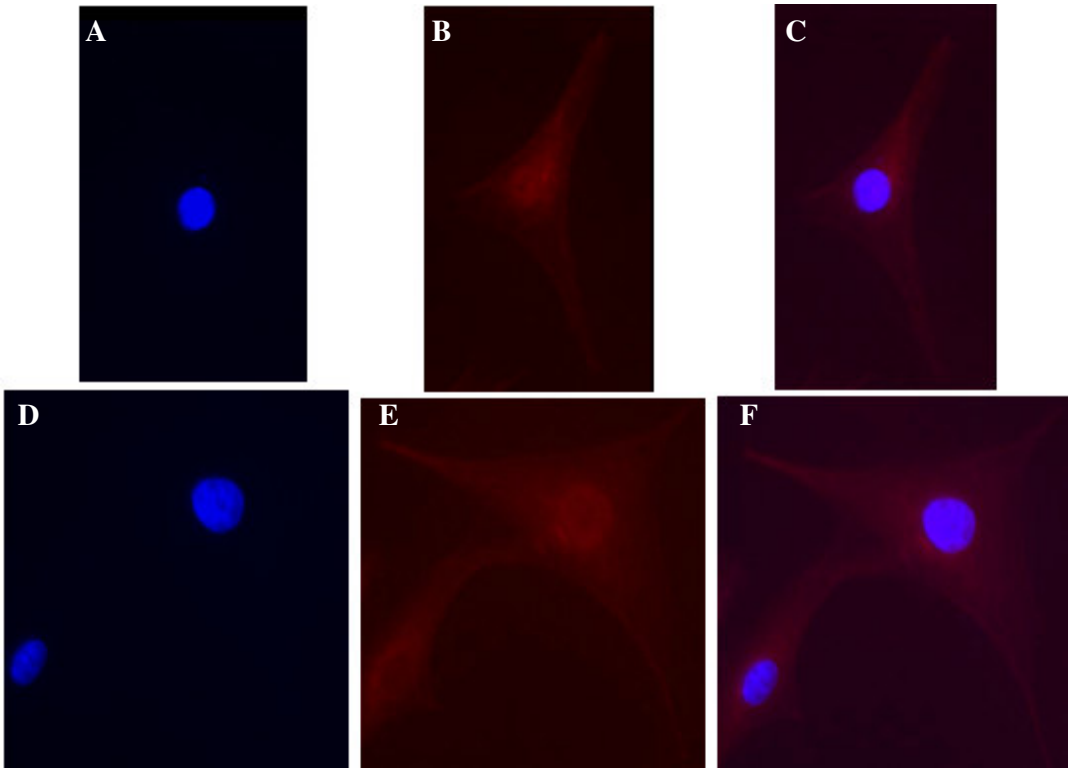

Figure 4.18. Cellular localization of SIK2 in MIO-M1 cells treated with FGF9 and PKA activator or inhibitor. A-C: cells treated with 8BrcAmp and and subsequently exposed to FGF9 for 30 min, D-F: cells treated with H89 and subsequently exposed to FGF9 for 30 min. DAPI stainings are shown in panels A and D, anti-SIK2 stainings are presented in panels B and E; the merged images are given in panels C and F

5. DISCUSSION

FGF9 is expressed in embryonic mouse retina (Colvin *et al*., 1999) starting at E10, as the optic cup is forming (Zhao *et al*., 2001). In postnatal development FGF9 mRNA levels found to be highest in the newborn and in mature rat retina, however a gradual decrease was detected starting at day 4 then rise again around day 15 (Çayırlıoğlu, 1996; Ergörül, 1998). In vitro experiments indicate that FGF9 promotes survival of ganglion cells (Kinkl *et al*, 2003) and elicits proliferative response of Müller glia (Çınaroğlu *et al*., 2005). Of the four distinct high affinity FGF receptors, FGFR1-FGFR4, FGF9 shows greatest affinity to the IIIb and IIIc splice forms of FGFR3 and the IIIc splice form of FGFR2 (Hecht, 1995, Santos Ocampo, 1996). In vertebrate retina FGFR1, FGFR2 and FGFR4 are expressed in photoreceptors, FGFR3 preferentially found in ganglion cells (Kinkl *et al*., 2002). In primary cultures Müller glia show strong expression of FGFR1, FGFR2 and FGFR3, but FGFR4 was not detected (Çınaroğlu *et al*., 2005).

FGF signal transduction pathway activates a cascade of tyrosine, serine/threonine kinase and phosphatase dependent phosphorylation and dephosphorylation events. The pathway is subject to tight control mechanisms. Control mechanisms are either through the activated effector molecules of the pathway as a feedback system and possibly through integration of input from independent signalling cascades. So, besides the cellular context and interplay between the positive/negative regulators, interpathway cross-talk, integration of heterologous signaling is also critical in defining the choice between proliferation, differentiation or survival responses, shaping the cellular responses (Schlessinger, 2000).

Although the interaction between PKA pathway and growth factor receptor signaling pathways were suggested several times, little is known how this cross-talk come about (Yao *et al*., 1995, Barbier *et al*., 1999) In one of the studies it was shown that inhibition of PKA activity prior to growth factor (FGF2) stimulus increased the signal flow through Ras/ERK pathway (Pursiheimo *et al*., 2002). And in another study it was shown that FGF2 induced Fire activation required cooperational function of Ras/ERK and PKA pathways (Pursiheimo *et al*., 2002). Recently it was suggested that integration of growth factor signaling and PKA might include mediators such as Raf1 and B-Raf

serine/threonine kinases (Pursiheimo, 2002a). These data lead to the suggestions that PKA may act to balance the kinetics of Ras/ERK pathway which might be instrumental in shaping cellular responses to growth factor signaling.

SIK2 is a serine/threonine kinase and has been shown to participate in two different pathways. In one case, serine phosphorylation of IRS at Ser-789 (Horike *et al*., 2003) by SIK2 was shown to modulate the efficiency of insulin signalling cascade and proposed to lead insulin resistance in adipose tissue (Qiao *et al*., 2002, White, 2002). In the second case phosphorylation of TORC2 at ser171 was shown to promote association of TORC2 with 14-3-3 proteins, and cytoplasmic sequestration of TORC2 which leads to inhibition of CREB activity at the basal state (Screaton *et al*., 2004). Also in this work it was shown that cAMP lead to the phosphorylation of Ser587 of SIK2 via PKA.

It was shown in our studies that FGF9 and its high affinity receptors; FGFR2 and, FGFR3 are expressed in rat retina. The expression levels and the expression patterns during postnatal development of the retina suggested that FGF9 is involved in regulation of developmental decisions in retina. To understand how FGF9 signals down from the receptors, a yeast two hybrid screen was established using the FGFR2 cytoplasmic domain as bait. This screen revealed a candidate clone which shows 90% homology to the 3' end of Mus musculus SIK2 mRNA sequence in cDNA database, and 99% homology to LOC315649 SSIK2 mRNA sequence in draft rat genome database (Özcan, 2003). Following the cloning of full length rat SIK2 (Uysal, 2005) this receptor SIK2 interaction is concluded to be artifactual, but given the widespread expression of SIK2 in retina confirmed by RT-PCR analysis (Özcan, 2003), activity of SIK2 as serine/threonine kinase, and presence of SH2/SH3 binding motifs and importance of serine/threonine phoshorylation in FGF pathways, our studies on SIK2 continued.

Based on earlier data from our laboratory this research focuses on the possible involvement of SIK2 in FGF signaling pathway in rat retina. We might postulate that; being a serine/threonine kinase, it might be involved in activation/deactivation of the signaling cascade down FGFR2 directly or indirectly by phosphorylation.

In the initial part of this study FGF9 distribution in postnatal rat retina by immunohistochemical and in situ hybridization methods was examined. Immunohistochemical staining of FGF9 was observed mainly in inner retina during development and in outer plexiform layer and nuclear layers in adult (Figure 4.1). In situ hybridization results also show a signal in inner retina with a fainter signal in ONL (Figure 4.2).

It was shown in our laboratory by RT-PCR and immunocytochemical staining that primary cultures of Muller glia from rat retina were deprived of FGF9 (Çınaroğlu *et al*., 2005). In this study it was also confirmed that the MIO-M1 cell line, which was shown to retain the characteristics of primary cells of Muller glial culture (Limb *et al*., 2002), did not show any signal from immunocytochemical staining with anti-FGF9 antibody (Figure 4.3). These results altogether suggested that FGF9 originating from neurons act in paracrine fashion on Muller glia.

Immunological stainings using anti-FGFR2 and anti-FGFR3 were carried out both in retinal sections and MIO-M1 cells. Immunohistochemical staining of both receptors had shown a widespread distribution in retinal layers, FGFR2 being more intense in IPL and INL and FGFR3 being more intense in nuclear layers (Figure 4.4). These receptors were both expressed in MIO-M1 cells. FGFR2 expression in nuclei of the cells was more prominent than FGFR3 nuclear expression (Figure 4.5).

SIK2 expression in retina and Muller glia cells with RT-PCR was shown previously in our lab (Özcan, 2003). In this study expression levels of SIK2 from birth to adult was analyzed with semiquantitative RT-PCR analysis in MIO-M1 cells. It was observed that SIK2 expression shows a gradual increase from birth onward (Figure 4.6) which might suggest that SIK2 is more likely to be implicated in molecular mechanisms regulating survival and maintenance but not differentiation.

Immunological staining of retinal sections and MIO-M1 cells were also carried out using anti-SIK2 antibody. SIK2 staining was observed in IPL and outer parts of NZ at birth and as the layers form the staining becomes intense at IPL and outer parts of photoreceptors, also OPL and the nuclear layers are not fully deprived of SIK2 expression

(Figure 4.7). MIO-M1 cells had shown immunoreactivity of SIK2 in both nucleus and cytoplasm (Figure 4.8). Western blot analysis of SIK2 in MIO-M1 cells revealed a dominant band of >50 kDa, which was rather unexpected since the predicted molecular weight of SIK2 is ~100 kDa. But it was reported that in human brain a 25 kDa band was seen with a 120 kDa band in western blots (unpublished data). This implies that SIK2 have cell type specific isoforms which might likely to occur as a result of post-translational modifications. Altogether these results correlate well with the primary culture data and makes MIO-M1 a convenient tool for the study of proliferative FGF mechanisms.

The second part of this study focuses on possible integration of FGF9 and PKA signal transduction pathways in MIO-M1 cells and investigate involvement of SIK2 in this integration.

It was shown that FGF9 pathway affect ERK phosphorylation in primary Muller glia cells (Çınaroğlu *et al*., 2005). The FGF9 treatment of MIO-M1 ERK phosphorylation was observed and was in parallel. ERK phosphorylation pattern of MIO-M1 cells when treated with FGF9 for 0, 10, 30 mins, 1h. ERK phosphorylation is maximum when cells were exposed to FGF9 for 10 mins and level drops to basal levels by 30 mins and maintained (Figure 4.11). We postulated that PKA may be involved in this downregulation of the signal after 10 minutes. Therefore affects of PKA activation and PKA inhibition in FGF9 treated cells were analyzed. In agreement with our proposal, inhibition of PKA with H89 treatment of cells prior to FGF9 induction abolished the decrease of ERK phosphorylation in 10 min, its maximal point (Figure 4.12). Activation of PKA before FGF9 induction however did not effect the trend of ERK phosphorylation but a general decrease of maximal phoshorylation level was observed (Figure 4.13). We also compared level of ERK phosphorylations at 30 min FGF9 induction, basal level, in presence of H89 and 8BrcAmp. Inhibition of PKA activity prior to 30 min FGF9 induction pERK levels were increased compared to that of basal and FGF9 induced levels. These results that are consistent with our postulation, suggest that PKA activity is functional in regulatory control of FGF9 signal transduction downregulation in MIO-M1 cells.

We next observed that FGF9 induction of MIO-M1 cells results in phosphorylation of SIK2. From untreated, FGF9 and PKA activator treated cells we purified

phosphoproteins and identified SIK2 in this population. Both FGF9 treated and PKA activator exposed cells show increase in phosphorylated SIK2 protein level compared to that of untreated cells (Figure 4.14). In this work we indicate that PKA is upstream kinase of SIK2 in retinal context, and observed increase in phosphorylated SIK2 from FGF9 treated cells show a possible involvement of SIK2 in FGF9 signal transduction pathway.

Our immunocytochemical studies indicated that FGF9 and PKA signals result in changes of cellular distribution of SIK2. In untreated cells SIK2 protein is detected both in cytoplasm and nucleus in MIO-M1, however when cells were treated with PKA activator nucleus becomes devoid of SIK2 staining. On the other hand PKA inhibitor treatment did not have any affect on localisation of SIK2 (Figure 4.15). These data suggest that phosphorylation of SIK2 might result in cellular relocalization of the protein. Thus we examined whether FGF dependent phosphorylation of SIK2 results in similar relocalization. When cells were induced with FGF9 for 10 min, time of maximum ERK activation, we see the same tendency of SIK2 distribution as in PKA activation. But when cells were induced with FGF9 for 30 min, SIK2 was seen to be translocated to nucleus as observed in untreated cells (Figure 4.16). This pattern together with SIK2 phosphorylation data indicate that SIK2 could be modulated in FGF9 signal transduction pathway and might be dependent on the strength of FGF9 signal.

Integration of PKA and FGF9 signaling on cellular distribution of SIK2 is tested by inhibition or activation of PKA prior to FGF9 induction and observing the localization of SIK2 in MIO-M1 cells. When PKA was activated in cells prior to 10 min FGF9 activation localization of SIK2 in cells were not uniform; in some cells it is in nuclei but in others excluded from nuclei. Inhibition of PKA activity prior to 10 min FGF9 treatment however, have shown a uniform exclusion from nuclei in all cells (Figure 4.17). We also checked the localization of SIK2 in presence of PKA activator prior to 30 min FGF9 induction, in which uniform nuclear localization was observed. However when PKA activity was inhibited in these experiments, localization of SIK2 in cells was not uniform reminiscent of 10 min FGF9 and PKA activator exposure (Figure 4.18).

Changes in localization of SIK1 was shown as a result of phosphorylation by PKA in ACTH signaling (Okamoto *et al*., 2004) as well. Here we show that SIK2 phosphorylation

also leads to cellular relocalization both in response to PKA and FGF9 independently. Results obtained from concurrent treatment of cells with PKA and FGF9 agents is not straightforward. As SIK2 is out of nuclei in the presence of PKA or 10 min FGF9 treatment alone, the simple expectation is in presence of both, SIK2 should be out of nuclei. Likewise one would expect to see SIK2 in nuclei in the presence of PKA inhibitor and 30 min FGF9 treatment, as alone both of these treatments had shown a uniform pattern. These two data are consistent, since these combination of PKA and FGF9 treatments had shown modulation of the eventual ERK phosphorylation (Figure 4.12; 4.13).

When two pathways are activated the outcome is more than a superimposed affect from both pathways. A highly speculative and simplistic model suggest that FGF9 and PKA activation singly may lead to the phosphorylation and relocalization of SIK2 and feedback signals lead to nuclear back localization of dephosphorylated SIK2. In our model we propose that feedback signal for dephosphorylation is upstream from PKA in the case of GPCR signaling and downstream of Raf in the case of FGF9 signaling. Thus in our experiments since PKA is not activated from the receptor, when two signals are integrated dephosphorylation is through the feedback from FGF9 signal transdcution. Schematic representation of the model is seen in Figure 5.1. We do not suggest a positive or negative role for the phosphorylation and localization of SIK2 as a result of FGF9 and PKA induction. Future experiments using constitutively active and kinase dead mutants of SIK2 will reveal direct involvement of SIK2 in these pathways and provide evidence for its role.

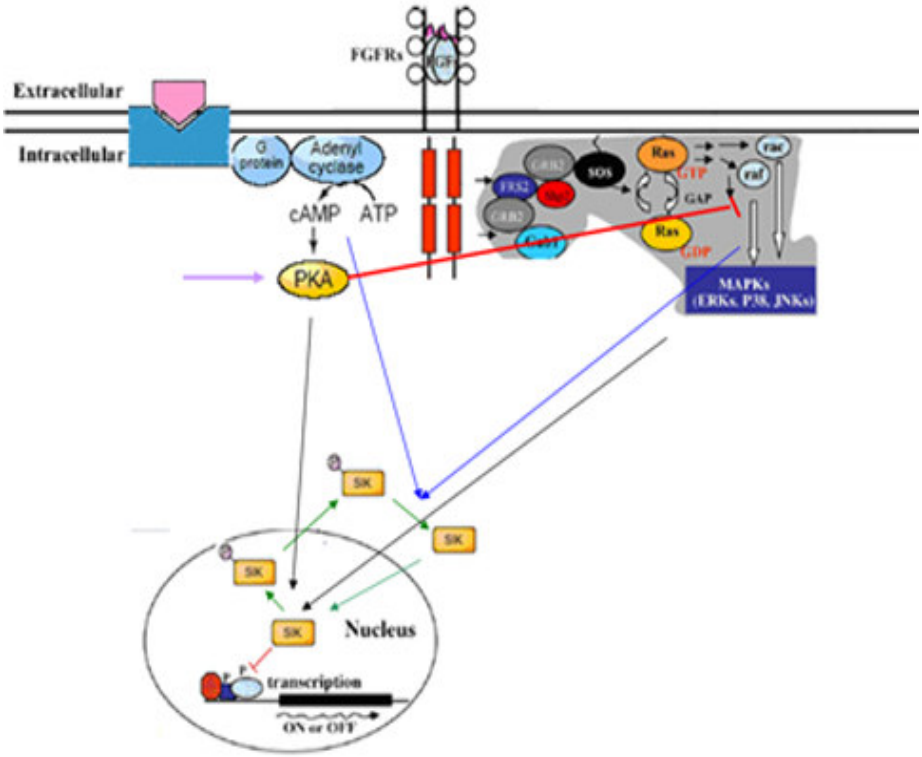

Figure 5.1. Schematic representation of the proposed model

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