SIK2 FUNCTIONS AS A NOVEL TUMOR SUPPRESSOR IN THE BREAST TUMORIGENESIS

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To my mother...

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ABSTRACT

SIK2 FUNCTIONS AS A NOVEL TUMOR SUPPRESSOR IN THE BREAST TUMORIGENESIS

Breast cancer has highly malignant phenotype and is the leading cause of cancer death in women. The heterogenous character of the disease points to the importance of novel target identification for the development of effective therapies. Perturbation in receptor tyrosine kinase (RTK) pathway elements are frequently implicated in cancer. Our group has shown that SIK2 functions downstream of FGFR signaling, and control proliferation and survival. Given the importance of strict control of RTK pathways in cancer, in this study, we aimed to investigate the potential role of SIK2 in the development of tumorigenicity. In this context, screening of a cDNA array indicated downregulation of SIK2 transcript levels in breast cancer. A query into Oncomine database validated frequent reduction of SIK2 expression and loss of copy number in dataset covering a large breast cancer patient cohort. Immunohistochemical studies localized SIK2 in ductal epithelia of breast tissue and showed that SIK2 levels decline in all triple negative breast cancer (TNBC) cases and in half of the hormone positive tumors. These studies also indicated that inverse correlation exists between SIK2 and Ki67 indicating a negative effect of SIK2 on mitotic potential. Reduced SIK2 expression in breast tumor cell lines was also evident. Modulation of SIK2 expression in MDA-MB-231 and MCF12A breast lines suggested that SIK2 negatively regulate proliferation and survival by preventing ERK and Akt activation. In this context, its kinase activity appears to be required. In vitro studies also indicate that SIK2 may also be involved in the regulation of cellular adhesion and invasion. Xenografting experiments provided strong evidence that SIK2 hampers tumor growth through downregulation of proliferation and survival. In the light of these findings, we propose SIK2 as a novel tumor suppressor, loss of its expression/activity promote breast tumorigenesis.

ÖZET

SIK2 MEME TÜMÖROGENEZİNDE BİR TÜMOR BASKILAYICI GEN OLARAK FONKSİYON GÖSTERMEKTEDİR

Meme kanseri oldukça malin bir fenotipe sahiptir ve kadınlarda kanserden kaynaklı ölümler arasında ön sırada yer almaktadır. Hastalığın heterojen karakteri efektif terapi yaklaşımlarının geliştirilmesi için yeni hedeflerin tanımlanmasının öneminin vurgulamaktadır. Reseptör tirozin kinaz (RTK) yolak elemanlarına ait pertürbasyonlar kanser olgularında sıklıkla görülmektedir. Grubumuz SIK2'nin FGFR sinyal yolağında işlev gördüğünü ve hücre çoğalması ile sağkalım cevaplarını kontrol ettiğini göstermiştir. RTK yolaklarının sıkı kontrolünün kanserdeki önemi gözönüne alınarak, bu çalışmada SIK2'nin tümorigenezdeki olası rolünün araştırılması amaçlanmıştır. Bu bağlamda, SIK2 transkript düzeyleri çeşitli doku orijinli tümörlerde irdelenmiş ve meme tümorlerinde anlatımın düşük olduğu görülmüştür. Oncomine veri tabanı taramaları genin anlatımında azalma ve gen kopya kaybı varlığını doğrulamıştır. Immünohistokimyasal çalışmalarımız, SIK2 anlatımının meme dokusunda duktal epitelde bulunduğunu ve tüm üçlü negatif meme kanseri (TNBC) olgularında ve ER/PR pozitif tümörlerin yarısında anlatım azalması olduğunu gösermiştir. Çalışmalarda gözlemlenen SIK ve Ki67 arasındaki negatif korelasyon, SIK2'nin proliferasyonu inhibe edici etkisini göstermektedir. In vitro çalışmalarımız SIK2'nin anlatımının kanserli hücre hatlarında normal hücrelere göre düşük olduğunu göstermiştir. SIK2 anlatımının MDA-MB-231 ve MCF12A meme hatlarında modülasyonları, genin ERK ve Akt aktivasyonlarını engelleyerek hücre çoğalması ve sağkalım süreçlerini baskıladığını göstermektedir. Çalışmalarımız ayrıca SIK2'nin hücresel adhezyon ve invazyonun regülasyonuna katkısı olabileceğine işaret etmektedir. Zenograft deneyleri genin tümör büyümesinin baskılanması üzerindeki etkisini proliferasyon apoptozu baskılayarak gerçekleştirme olasılığını desteklemektedir. Tüm bu bulgular ışığında, SIK2'nin yeni bir tümör baskılayıcı gen olduğunu, ve anlatım/aktivitesindeki düşüşlerin meme tümorü oluşumuna katkıda bulunduğunu önermekteyiz.

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LIST OF ACRONYMS/ABBREVIATIONS

AI	Aromatase Inhibitor
AMPK	AMP-activated kinase
AURKA	Aurora Kinase A
BCA	Bicinchoninic acid
Bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumine
cAMP	Cyclic adenosine 5'-monophosphate
CBP	CREB binding protein
Cbl	Casitas B-lineage Lymphoma
cDNA	Complementary DNA
chREB	Carbohydrate responsive element-binding protein
c-Nap1	Chromosome condensation-related SMC-associated protein 1
CREB	CRE binding protein
CO_2	Carbondioxide
CTL	Cytotoxic T lymphocytes
DAPI	Diaminophenylindolamine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epithelial growth factor receptor
EMT	Epithelial Mesenchymal Transition
ER	Eostrogen Receptor
ERK	Extracellular Regulated Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast growth factor receptor

FITC	Fluorescein isothiocyanate
Fl	Full length
FLRT	Fibronectin Leucine Rich Transmembrane
FRS2	Fibroblast growth factor receptor substrate
Gab1	Grb2-associated binder 1
GFP	Green Fluorescent Protein
Grb2	Growth factor receptor-bound protein
HER2/neu/ERBB2	Human Epidermal Growth Factor Receptor2
H_2O	Water
HGFR	Hepatocyte growth factor receptor
HRP	Horse radish peroxidase
IGF-IR	Insulin-like growth factor-insulin receptor
IgG	Immunoglobulin G
IR	Insulin receptor
IRS	Insulin receptor substrate
Kb	Kilobase
KD	Kinase domain
kDa	Kilodalton
KI	Kinase inactive
LB	Luria Bertani Broth
LKB1	Liver kinase B 1
MAPK	Mitogen-activated protein kinase
Mg	Miligram
min.	Minutes
ml	Mililiter
mm	Millimeter
mM	Milimolar
mRNA	Messenger ribonucleic acid
NaCl	Sodium Chloride
NKC	Natural Killer Cells
Nm	Nanometer
NMDAR	N-methyl-D-aspartate receptor
Ng	Nanogram

PAGE	PolyAcylamide Gel Electrophoresis
pAkt	PhosphoAkt
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PDGFR	Platelet Derived Growth Factor Receptor
pERK	Phospho Extracellular Regulated Kinase
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein Kinase A
PLCγ	Phospholipase C-gamma
PP2A	Protein phosphatase 2A
PR/PgR	Progesterone Receptor
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RK-rich	Arginine-lysine-rich
RNA	Ribonucleic acid
Rpm	Rotations per minute
RTK	Receptor tyrosine kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dedocyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
Sef	Similar expression to fgf genes
Ser	Serine
shRNA	Small hairpin RNA
SIK2	Salt inducible kinase 2
Sos1	Son of sevenless1
Spry	Sprouty
TAE	Tris-acetate-EDTA
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TEMED	Tetramethylethylenediamine

TGFβ	Transforming Growth Factor-Beta
Thr	Threonine
TNBC	Triple Negative Breast Cancer
TORC2	Transducer of regulated CREB activity
TSC	Tuberous sclerosis complex
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UBA	Ubiquitin-associated
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WB	Western Blot
WT	Wild-Type
Xg	Times Gravity

LIST OF SYMBOLS

^{o}C	Centigrade degree
S	Serine
V	Volt
H	Hour
v	Volume
w	Weight
β	Beta
µg	Microgram
µl	Microliter
µM	Micromolar
M	Molar

1. INTRODUCTION

1.1. Cancer

Cancer is characterized by genetic and epigenetic alterations which lead to loss of control in critical cellular events (Osborne *et al*., 2004; Ferguson *et al*., 2015; Benada and Macurek *et al*., 2015; Yadav *et al*., 2015). Through accumulation of such alterations, cancer cells gain ability for sustained proliferation, resistance to cell death, escape from the control of growth suppressors, enhanced migration/invasion capacity. Induction of neo-angiogenesis, reprogramming of energy metabolism and evasion of immune system are also part of the neoplastic transformations (Figure 1.1) (Hanahan and Weinberg, 2011).

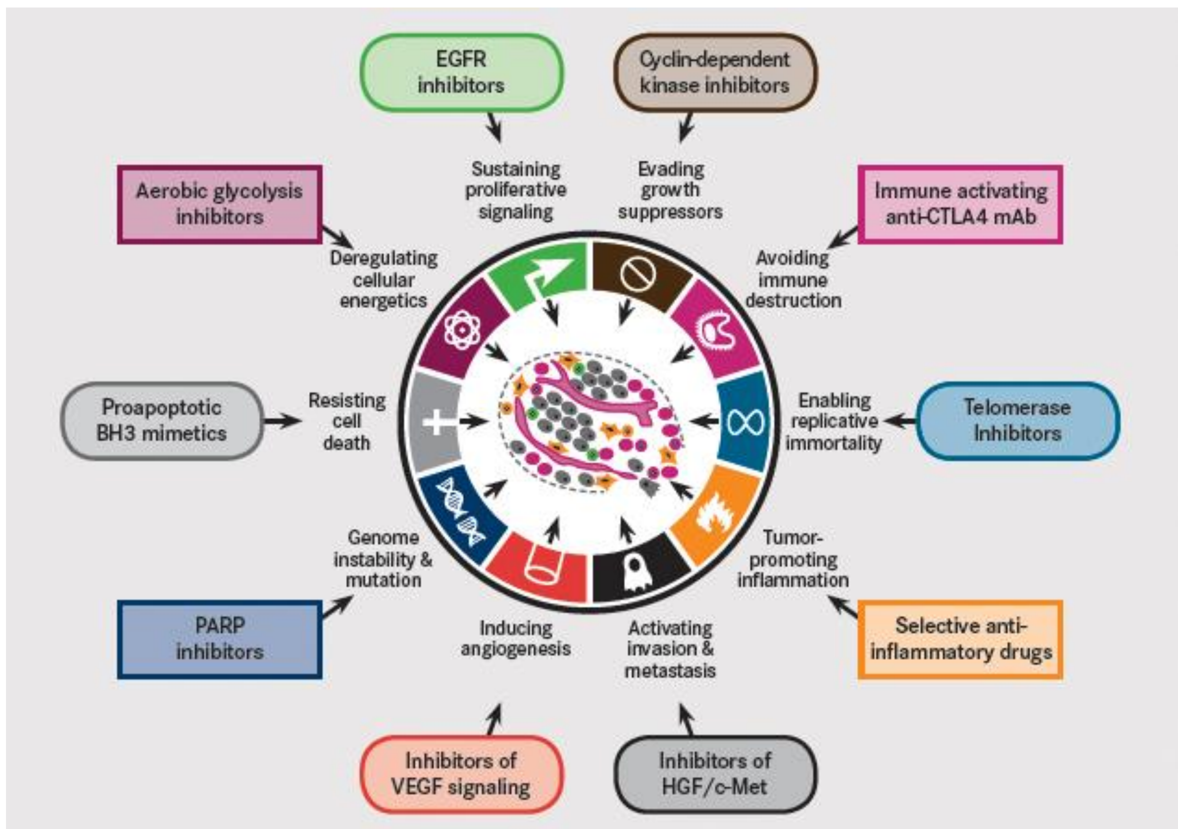

Figure 1.1. Cancer Promoting Mechanisms (Hanahan and Weinberg, 2011).

1.1.1. Cell Cycle Control

In metazoans cell cycle entry is mainly controlled through paracrine action of growth factors, and cell cycle progression is coordinated by cyclins and CDKs. Activation of the downstream growth factor signaling pathways result in transcriptional activation of cell cycle regulators such as cylin D1 (Figure 1.4). Cyclin D1 binds and activates its partner CDK4/6 to phosphorylate Rb protein, which promotes the release of Rb from E2F/DP1/Rb complex. E2F then facilitates transcriptional activation of various genes including cyclin E, cyclin A, DNA polymerase, thymidine kinase. CyclinE-CDK2 complex promotes entry into S phase and DNA replication process progress (Lim and Kaldis, 2013; Duronia and Xiang, 2013).

The progression of cell cycle is tightly controlled by diverse cell cycle checkpoint modulators. In the activation of CDKs, Cdc25 phosphatases function by dephosphorylating Wee1 and Myt1 kinases. To trigger checkpoint arrest, Chk1 and Chk2 kinases function to inhibit Cdc25A/B/C phosphatases (Lim and Kaldis, 2013; Gopinathan *et al*., 2011).

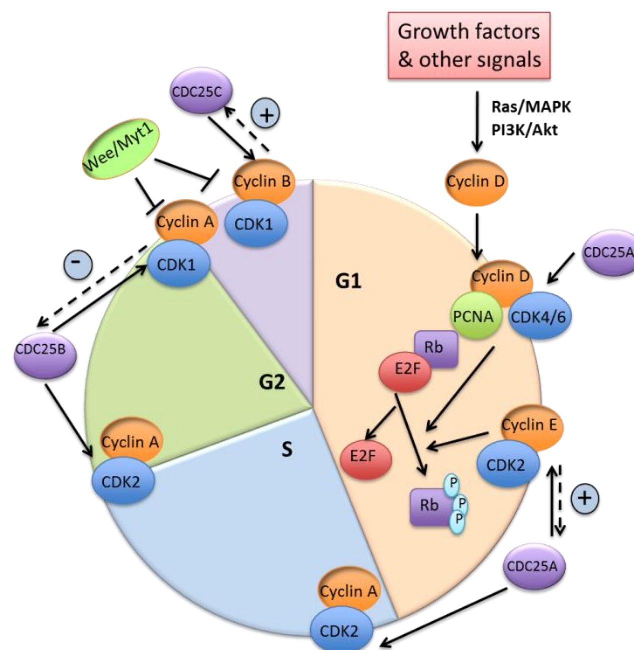

Figure 1.2. Cell Cycle Regulation (modified from Lyon *et al*., 2002).

In response to genotoxic stress, cells stop dividing via activation of cell cycle checkpoint regulators. CDK interacting proteins such as p21, p27 and p57 and INK4a/ARF family including $p16^{INK4a}$ arrest cell cycle in G1. Following DNA damage cell cycle progression is also interrupted via the activation of G1/S cell cycle regulator p53 tumor suppressor which promotes transcriptional activation of several genes. ATM/ATR, Chk1/Chk2, and p38 as DNA damage sensors and cell cycle checkpoint kinases have a potential to phosphorylate and stabilize p53 to induce cell cycle arrest. If DNA repair system successfully corrects the replication errors, checkpoint signals is attenuated and the cell cycle continues. When DNA damage is not properly repaired by the system, cells are reprogrammed for the permanent senescence or they go apoptosis. However, DNA damage checkpoints are turned down by cancer cells to gain infinite proliferative ability (Esposito *et al*., 2013; Benada and Macurek, 2015; Wang *et al*., 2015; Nath *et al*., 2015).

1.1.2. Apoptosis

Caspase dependent cell death occurs through activation of signaling pathways, and at the end cellular destruction occurs in an orderly manner without induction of inflammation (Figure 1.3) (MacKenzie and Clark, 2012; Vriz *et al*., 2014) The cell death response regulators are categorized into two groups. In one group the extrinsic signal responsive regulators such as Fas ligand/receptors and TNF receptors receive and process extracellular signals. These receptors oligomerize upon ligand binding and undergo conformational changes allowing the assembly of a multi-protein complex known as Death Initiation Signalling Complex (DISC) (Yang, 2015). Subsequently caspase cascade is activated. In the second group the intrinsic cell death modulators are responsive to cellular stress including DNA damage, loss of growth factor signaling, and protein misfolding trigger mitochondrial outer membrane permeabilization. This in turn results in loss of mitochondrial membrane potential and release of a number of proteins, such as cytochrome c, to form apoptosome complex that will contribute to caspase activation. (Cullen and Martin, 2015). The Bcl-2 family proteins are essential components of this pathway. The pro-apoptotic members include Bax, Bak, Bcl-xs, Bok, Bad, Bik, Bid, Bim, Noxa, Puma, and they mediate apoptosis by disrupting mitochondrial membrane integrity. The anti-apoptotic members Bcl-2, Bcl-xl, Bcl-w and Mcl-1 interfere with pro-apoptotic member

aggregation (Chen and Wang, 2002; Galluzzi *et al*., 2014; Hensley *et al*., 2013; Yu *et al*., 2013, Hassan *et al*., 2014; Benada and Macurek, 2015).

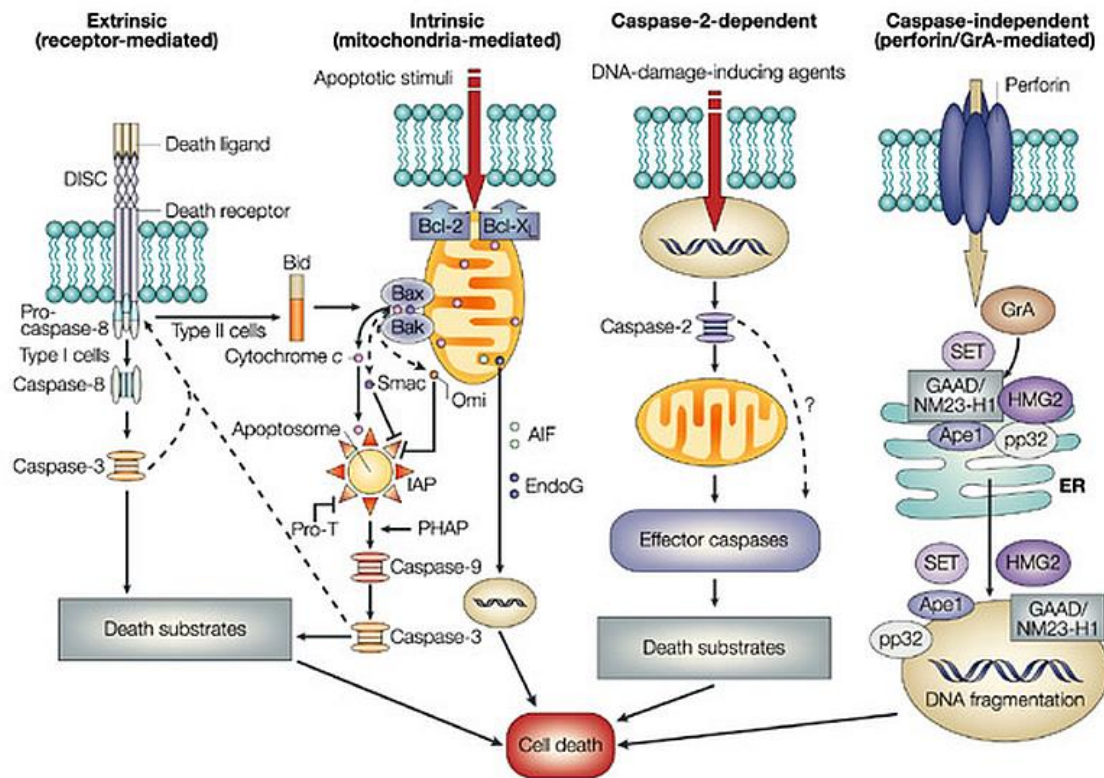

Figure 1.3. Apoptotic Cell Death Mechanism (Orrenius *et al*., 2003).

Cancer cells generally escape from controlled cell death processes to gain resistance against apoptosis. The controlled cell death mechanisms may be partially or totally overwhelmed (Hanahan and Weinberg, 2011). Mechanisms which block the activation of apoptotic machinery are the elimination of critical DNA damage sensors such as p53 and ATM, elevation of antiapoptotic gene expression including Bcl-2 and Bcl-Xl, activation of survival machinery via inducing Igf1/2 expression, silencing of proapoptotic signaling (Bax and Bim) and interrupting autophagy (Sperka *et al*., 2012; Mirzayans *et al*., 2012; Hassan *et a*l., 2014; Mohammad *et al*., 2015).

1.1.3. Motility

Perturbations in the multistep migration and invasion processes (Figure 1.4) are are accepted as one of the major characteristics of cancer cell behaviour (Talmadge and Fiddler, 2010). The inhibition of cell-cell and cells-ECM adherence junction formations through the downregulation of critical genes such as e-cadherin initiates the metastatic cascades. Epithelial mesenchymal transition (EMT) has been implicated in transformation (Cavallaro and Christofori, 2004; Berx, 2009). Some transcription factors such as Snail, Slug, Twist, Zeb1/2 have been shown to induce invasion process. Matrix degrading enzymes including MMP family members have been indicated in promotion of motility and acquiring resistance against apoptosis by activating PI3K/Akt pathway. Aberrant induction of TGF-beta, Wnt and RTK pathway trigger complicated downstream pathways leading to metastatic processes. Tumor microenvironment is also reorganized by cancer cells, interaction with neighbouring tumor associated stromal cells are modulated (Clark and Vignjevic; 2015; Krakhmal *et al*., 2015).

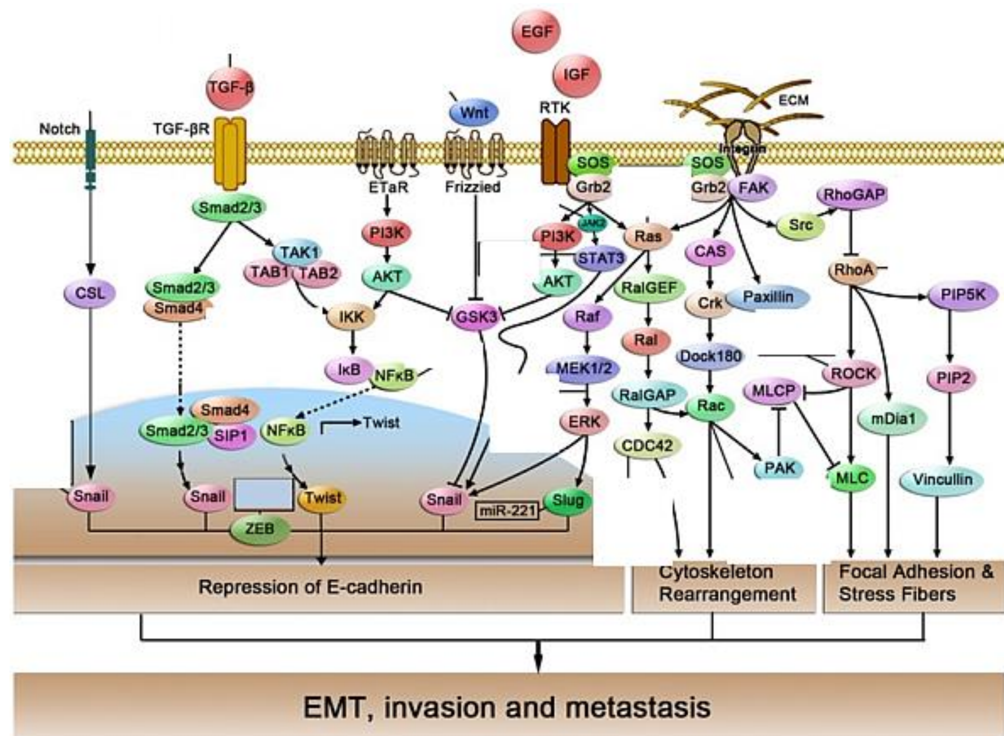

Figure 1.4. Mechanisms of EMT, invasion and metastasis (Jiang and Qiu, 2013).

1.2. Genetic Alterations and Tumor Heterogeneity

Several acquired genetic alterations should be accumulated in the critical genes relevant to the above mentioned cellular processes to establish fully transformed cell. Specific genes show gain of function mutations through copy number amplifications, point mutations and chromosomal rearrangements that lead to overexpression or constitutive activation of the gene products (Shen *et al*., 2015, Langie *et al*., 2015, Giam and Rancati, 2015, Di Lonardo *et al*., 2015, Morris *et al*., 2015). Such genes promote cell cycle entry/progression, survival, motility, neo-angiogenesis and classified as oncogenes. On the other hand, the negative regulators of such processes are grouped as tumor suppressors (Osborne *et al*., 2004, Morris *et al*., 2015). Down regulation of expression by promoter methylations, deletions, point mutations resulting in loss of function or elevation in proteosomal degradation of these genes promote tumorigenesis (Osborne *et al*., 2004). Mutations in oncogenes or tumor suppressors which provide selective advantage to the cells for initiating cancer formation and induce progression are called as driver mutations. Tumor cells also accumulate large number of mutations that do not have any direct contribution to neoplastic transformation events which are determined as passenger mutations. (Greaves *et al*., 2015; Wills and Mead 2015; Pagliarini *et a*l., 2015; Morris *et al*., 2015; Sever and Brugge 2015; Di Lonardo *et al*., 2015).

All these mutations which accumulate in neoplastic formations vary between tumors of the same origin and the cells of the same tumor. This extensive inter- and intra-tumor heterogeneity represents a great challenge in understanding the biology of tumors and developing treatment options (Parker and Perou, 2015).

Two models have been proposed to explain origins of tumor heterogeneity. The first model, the clonal evolution theory (Figure 1.5) suggests that in a similar way to natural selection, cells in the tumor mass compete for nutrients, oxygen and space. The ones that gain survival advantage over the others by stochastically acquired mutations clonally expand (Greaves and Maley, 2012).

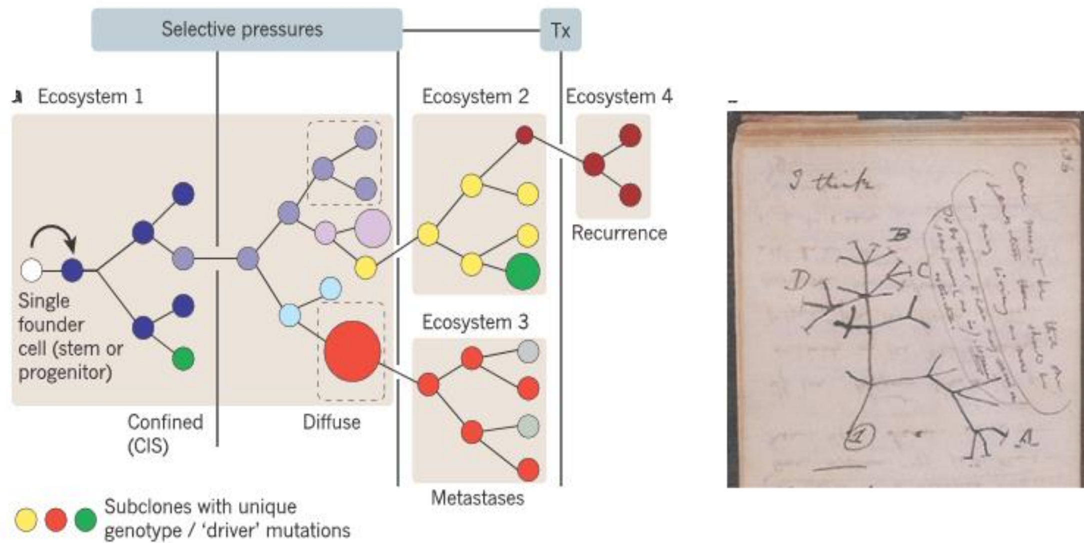

Figure 1.5. Clonal evolution theory in cancer (Greaves and Maley, 2012).

The second model, the cancer stem cell hypothesis (Figure 1.6) proposes that each tumor mass has its own stem cell population. The variation in their stemness or degree of differentiation generates tumor heterogeneity (Shackleton *et al*., 2009; Bapat, 2007, Dalerba *et al*., 2007).

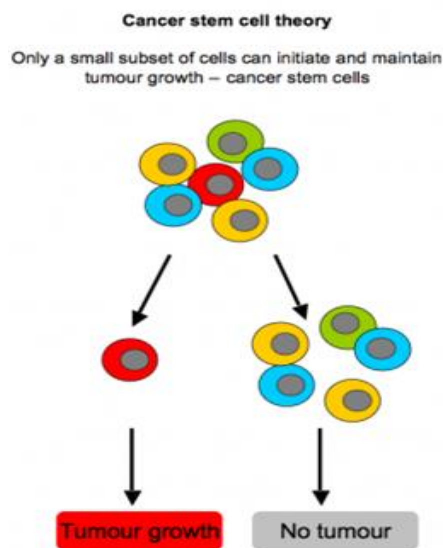

Figure 1.6. Cancer stem cell hyphothesis (Wang and Dick, 2005).

1.3. Receptor Tyrosine Kinases and Cancer

1.3.1. Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) transduce signals from wide range of growth factors, thus function in the regulation of several essential cellular events such as cell growth, proliferation, survival, apoptosis, differentiation, morphogenesis and motility. Perturbations in all of these processes as mentioned before are directly involved in the development of tumorigenesis (McDonell *et al*., 2015).

RTK superfamily consists of 20 subfamilies with 58 members including EGFR, VEGFR, HGFR, PDGFR and FGFR families. They all share common structure (Figure 1.7), including an extracellular ligand binding domain, a single pass transmembrane domain and intracellular tyrosine kinase domains (Choura and Rebai, 2011).

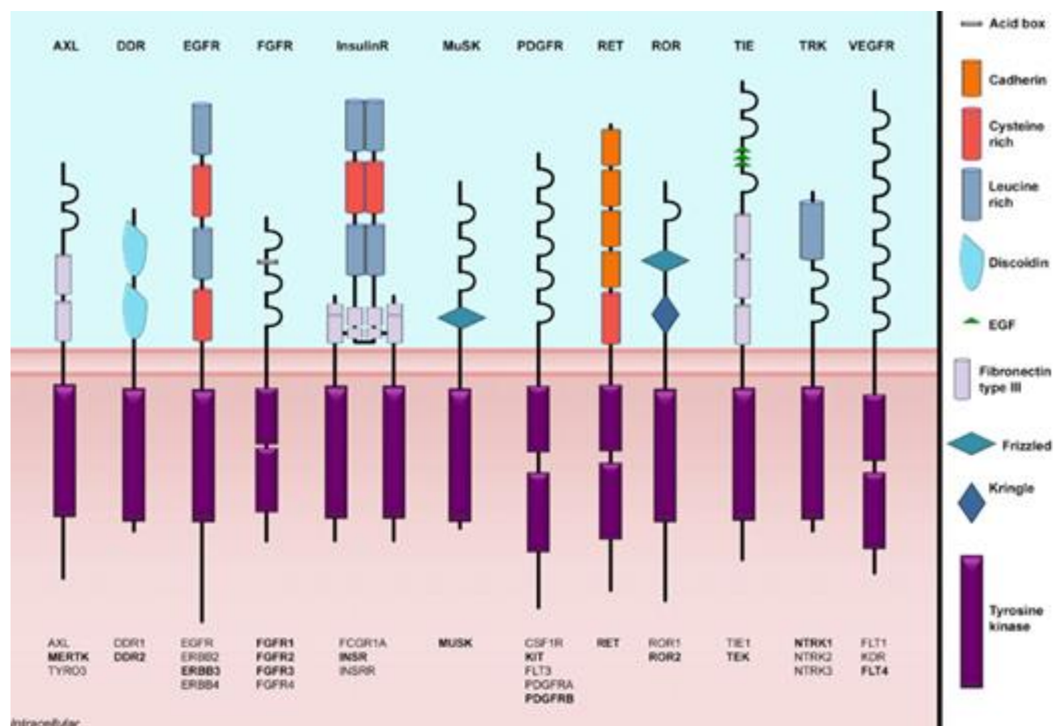

Figure 1.7. Structure of RTK receptors (McDonell *et al*., 2015).

Typically, specific ligand binding promotes RTK activation through induction of dimerization, conformational changes and trans-autophosphorylation of tyrosine residues

on the receptor. These phoshorylated residues facilitate recruitment of SH2 or PTB domain containing downstream proteins including the docking molecules Gab1, IRS1 and FRS2. These proteins have large number of phosphorylation sites targeted by the receptor, which allows for the recruitment of various effectors. While Gab1 is known to interact with multiple RTKs, IRS1 and FRS2 bind to restricted number of receptor types. Although it is still ill defined, the selective recruitment of effectors by the docking proteins contribute to activation of alternative downstream pathways and coordinate cross-talk between them (Schlessinger, 2014; Yamanashi *et al*., 2012; Takeuchi and Ito, 2011). RTK signaling is tightly regulated via various mechanisms including positive and negative feedback regulations, post-translational modifications of the signaling pathway elements, downregulation of receptors and the signaling components (Volinsky and Kholodenko, 2013).

The best-studied signaling axes activated by RTKs are Ras/MAPK, PI3K/Akt and PLCγ/Ca^{2+} pathways (McDonell *et al*., 2015). In the next section Ras/MAPK and PI3K/Akt pathways, and their regulation will be discussed in the context of FGFR signaling.

1.3.2. FGFR Signaling

FGFR signaling plays critical roles in development and adult physiology, and also contributes to neoplastic transformations through regulation of cellular processes such as proliferation, apoptosis, differentiation, EMT, invasion and angiogenesis (Gong, 2014; Wesche *et al,*. 2011). Similar to most other RTKs, FGFRs as single pass transmembrane proteins are activated by ligand dependent dimerization and autophosphorylation. The phosphorylated tyrosine residues act as docking platform for the assembly of the transducer proteins via recruiting FRS2 and Gab1 to activate the downstream pathways, most prominent ones being Ras/MAPK/ERK and PI3K/Akt cascades (Katoh and Nakagama, 2013; Turner and Grose, 2010).

1.3.2.1. Ras/MAPK/ERK Pathway. Subsequent to the recruitment and activation of FRS2 by tyrosine phosphorylation Grb2 and Gab1 join to form a ternary complex near the receptor. Next Ras-specific guanine nucleotide exchange factor (GEF) Sos is recruited to

the complex for the activation of Ras. Ras in turn initiates sequential activation of serine-threonine kinases Raf, MEK and ERK (Kouhara *et al.,* 1997). ERK, upon activation, phosphorylates target transcription factors such as Elk-1 and c-Myc to induce the expression of multiple genes required for proliferation (Yang *et al*., 2004). Ras/MAPK is implicated also in survival and cell migration events in different contexts. Gab1 has been shown to contribute to sustained and/or enhanced ERK activation by recruiting phosphatase SHP2, thereby down-regulate RasGAP, negative regulator of Ras activation (Figure 1.8) (Araki *et al*., 2003; Cunnick *et al*., 2000).

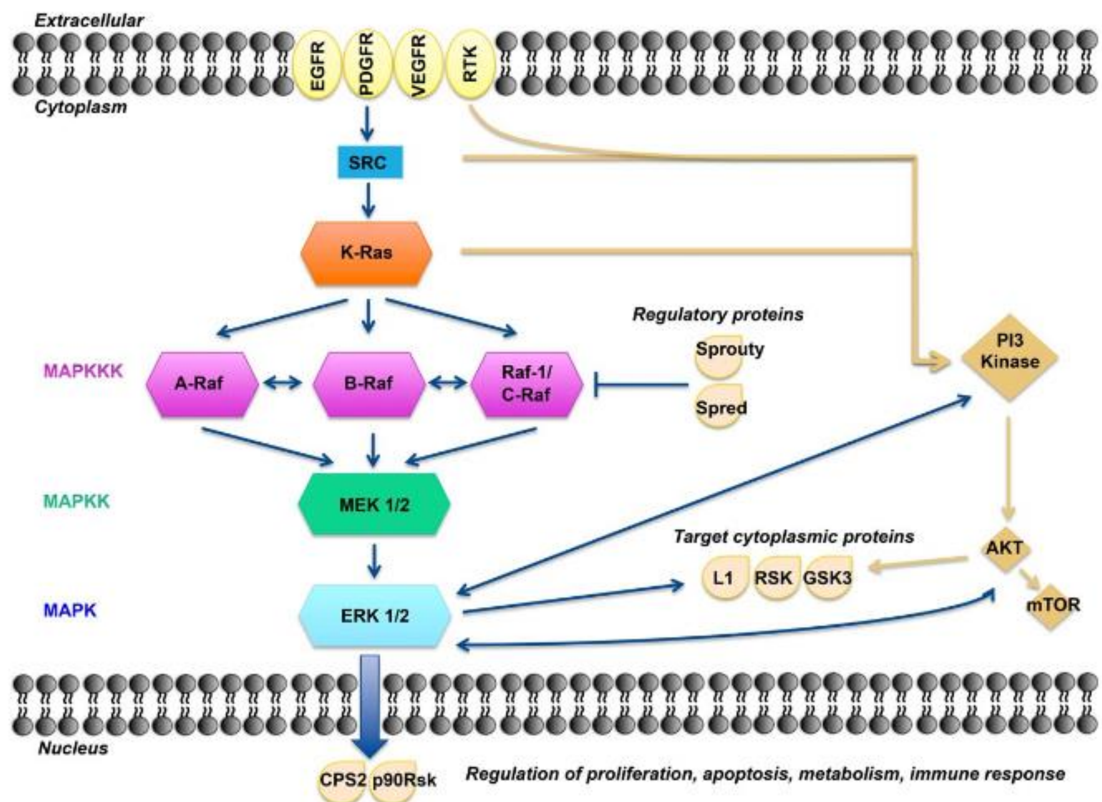

Figure 1.8. Activation of MAPK/ERK Pathway (Burotto *et al.*, 2014).

Sprouty (Spry), Similar Expression to FGF (SEF), MAPK phosphatase 1 (MKP1) and MAPK phosphatase 3 (MKP3) proteins negatively regulate the pathway and their expressions are activated via ERK signaling (Tsang and Dawid, 2004). Spry interferes with binding of Raf to MEK and also competitively binds to Sos1 (Yusoff *et al*., 2002; Lao *et al*., 2006). Sef inhibits phosphorylation of ERK by MEK (Kovalenko *et al.,* 2003). MKP1

anchor ERK protein in the nucleus and MKP3 locks it in the cytoplasm via dephosphorylation (Pouyssegur *et al*., 2003). ERK itself is involved in a negative feedback mechanism by phosphorylation on Frs2 and Gab1 (Lax *et al*., 2002; Gotoh, 2008). FGFR, Frs2, Gab1 are ubiquitinated and subsequently degraded by binding of ubiquitin ligase Casitas B-lineage Lymphoma (Cbl) (Figure 1.9) (Wong *et al*., 2002). Our group has recently showed that SIK2 negatively regulates FGF2 dependent Erk activation via phosphorylating docking protein Gab1 (Kuser Abali et al., unpublished data, Figure 1.11).

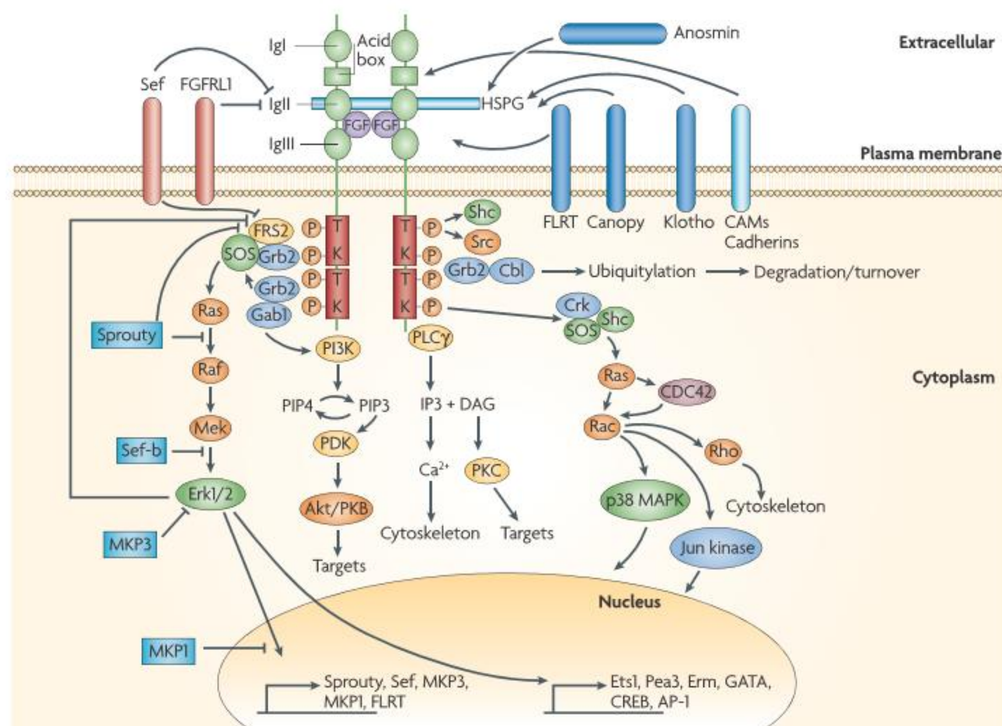

Figure 1.9. Regulation mechanisms of FGF signaling (Mason, 2007).

<u>1.3.2.2. PI3K/ Akt Pathway.</u> PI3K/Akt pathway regulates many biological functions and cellular processes including cell proliferation, growth, survival, motility and metabolism. It functions as the main inhibitory pathway for apoptosis. Signaling from this highly conserved pathway starts by receptor proximal recruitment of p85, the regulatory subunit of PI3K, which then associates with the catalytic subunit p110. Though many RTKs directly interact with p85, in case of FGFR its recruitment is mediated by Gab1 (Wohrle *et al*., 2009). The holoenzyme catalyses phosphatidylinositol(3,4)-bisphosphates (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphates (PIP_3). PIP_3 recruits Akt to the plasma

membrane, allowing its phosphorylation PDK1. Akt has a large number of cytosolic substrates, thus mediates multitude of cellular processes. It promotes cell survival by inhibiting pro-apoptotic Bcl-2 family members and by increase transcription of several DNA damage sensors, anti-apoptotic and pro-survival genes (Kim *et al*., 2002; Perego *et al*., 2010; Roos and Kaina, 2013). Akt also, via phosphorylation of Mdm2, downregulates p53-mediated apoptosis and hampers transcription of cell death promoting proteins (Abraham and O'Neill, 2014). Akt targets tuberous sclerosis complex protein 2 (TSC2) (Araki *et al*., 2003; Krejci *et al*., 2007; Cai *et al*., 2011) and proline-rich Akt substrate of 40 kDa (PRAS40) resulting up-regulation of protein synthesis by activation of mTOR pathway (Hetchman *et al*., 2015). Another target of Akt is glycogen synthase kinase 3 (GSK3), whose phosphorylation induces Myc and cyclin D1 activity with subsequent increased proliferation (Esfnadiari *et a*l., 2012; Kim *et al*., 2013). Dephosphorylation of Akt by PP2A (Andjelković *et al*. 1996) and PHLPP1/2 (Brognard *et al*. 2007) downregulate the pathway. The conversion of PIP_3 to PIP_2 by lipid phosphatase PTEN (Stambolic *et al.* 1998) inhibit PI3K signaling (Figure 1.10) (Nicholson *et al.*, 2002; Andjelkovic *et al.*, 1997; Balendran *et al.*, 1999; Lynch *et al.*, 1999; Toker *et al.*, 2000; Feng *et al.*, 2004; Sarbassov *et al.*, 2004).

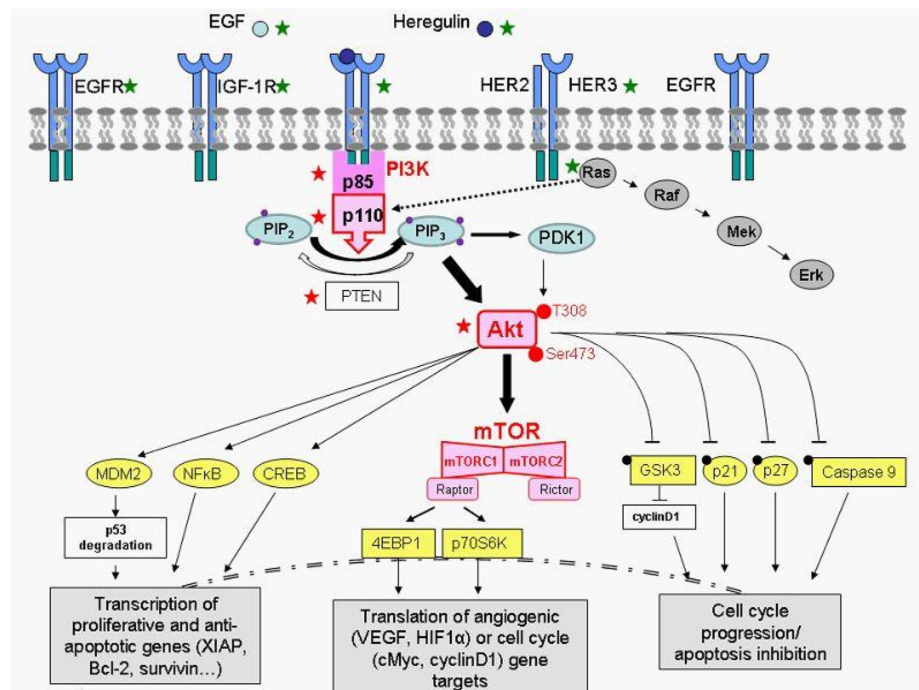

Figure 1.10. PI3K/Akt/mTOR pathway (Cheaib et al., 2015).

1.3.3. Deregulation of FGF Signaling and Cancer

Gain or loss of function mutations resulting in aberrations in temporal and spatial expression of growth factors and downstream elements of their signaling pathways as well as changes in duration and strength of signaling are frequently involved in neoplastic transformation of human tumors (Turner and Grose, 2010).

FGF signaling pathway can be deregulated via four independent mechanisms. The first one is the point mutations which generate constitutively active receptors with no dependency to their ligands. The second mechanism is the gene amplifications and abnormal transcriptional regulation of the pathway elements. The third one is the chromosomal translocation which can lead to the formation of fusion FGFR proteins and overactive signaling. The last one is the isoform switching which can result in the development of alternative ligand binding and sensitizes the cells to different FGF/FGFR signaling pathway responses (Turner and Grose, 2010).

To date, different kinds of genetic alterations in FGF signaling have been identified in various cancers. FGF-1 overexpression has been reported in ovarian cancer with poor survival rate. Up-regulation of FGF-2, -6 and -8 have been associated with prostate cancer (Corn *et al*., 2013). FGF18 up-regulation has been linked to colorectal cancer (Sonvilla *et al*., 2008). FGFR1 gene amplification has been observed in lung cancer cases (Katoh and Nakagama, 2014), FGFR2 gene amplification is seen in diffuse-type gastric cancer (Hong *et al*., 2013). The chromosomal translocation defects are seen in FGFR1 and FGFR3 in different cancer cases including multiple myeloma, myeloproliferative syndrome and peripheral T-cell lymphoma (Still *et al*., 1999; Hu *et al*., 2011; Kim *et al*., 2014). In Glioblastoma multiform FGFR1 and FGFR3 fusion proteins have been detected (Singh *et al*., 2012). Missense mutations have been demonstrated for FGFR2 gene in endometrial and melanoma cancers, (Gartside *et al*., 2009; Gatius *et al*., 2011) for FGFR3 gene in invasive bladder tumors (Guancial *et al*., 2014) and for FGFR4 gene in rhabdomyosarcoma (Tan *et al*., 2014 and Katoh and Nakagama, 2014).

In the breast tissue context, FGF signaling is highly active during ductal morphogenesis at the developmental stage coinciding with high proliferation rate (Zhang *et*

al., 2014; Brisken and Ataca, 2015). FGF/FGFRs upregulations have been identified in different breast cancer subtypes. Approximately 10% of human breast cancers include 8p-11-12 amplification where FGFR1 is located and this is associated with poor prognosis. While this gene amplification has been associated with ER positive breast cancers, FGFR2 gene amplification is also seen in TNBCs cases (Turner *et al*., 2010). *In vitro* cell line and *in vivo* mouse model approaches have demonstrated that activated FGFR1 promotes cell proliferation, survival and invasion of breast cells. Upregulation in FGFR4 was observed in breast cancer cases which is associated with resistance to chemotherapy. FGFR2 constitutive activation has been identified in the cell lines derived from TNBCs (Turner et al., 2010). Although FGFR2 amplification is rarely seen in breast cancer subtypes, 4% of TNBC is also identified with FGFR2 amplification (Burstein *et al*., 2015).In node negative breast carcinoma, FGFR3 amplification exists which is correlated with elevated invasion potential (Chaffer *et al.*, 2007). Genome wide association studies identified certain SNPs in FGFR1 implicated in elevated in cancer (Agarwal *et al*., 2012). Alternative splicing specifically affecting FGFR2 gene is another important mechanism which deregulate the FGF signaling in breast cancer (Moffa *et al*., 2004).

FGF ligands overexpression has also been shown in breast cancers. The ectopic expression in FGF8 gene has been reported in human breast tumors. *In vivo* nude mice studies have shown that FGF8b overexpression result in tumor growth and promotes angiogenesis (Daphna-Iken,1998). Breast cancer cells overexpressing FGF10 induce malignant tumors formations in mice (Katoh, 2008).

In addition to FGFRs and FGFs,genetic deregulations in downstream pathway elements were also shown in different studies (Katoh and Katoh, 2009, Ghayad and Cohen, 2010). Scanning of 40 breast cancer cell lines for the components of ERK and PI3K pathways in showed that approximately 25% of all mutations were found in KRAS, HRAS, NRAS, and BRAF; several mutations in the PI3K pathway elements such as such as PTEN and PIK3CA were also detected in this scan (Medarde and Santos, 2011).

1.4. Breast Cancer

Breast cancer is one of the most common cancer types and ranks second cause of cancer death among women after lung adenocarcinoma. Approximately 1.7 million women are diagnosed with this disease each year and more than 450,000 death are seen per year. (Siegel *et al*., 2013; Dubey *et al*., 2015; Anampa *et al*., 2015).

Heterogeneous character of the disease, like all other cancers, complicates classification of breast tumors (Parker and Perou., 2015). There are various criteria used in the classification of these tumors.

According to cells of origin, breast carcinomas are categorized into two different classes. Approximately 85% of breast cancers originate from ductal tissue, hence called ductal adenocarcinomas and the remaining represents the lobular carcinomas (Ogunbiyi *et al*., 2015).

The second classification is done according to analysis of histological grade, lymph node status, proliferation rates, metastatic potential and responses to therapeutic agents. In the estimation of disease, relapse risk, expression of proliferation marker Ki-67 and apoptotic marker p21 are also considered in clinical evaluations (Holliday *et al*., 2011; Rosa *et al*., 2015).

A more recent classification approach depends on gene expression profiling. According to the molecular expression composition of the tumors, breast cancer is subcategorized to three groups including estrogen receptor (ER)/progesterone receptor (PR) positive hormone dependent, human epidermal growth factor receptor 2 (HER2) overexpressed/amplified and basal like ones (Denkert *et al*., 2015; Polyak, 2011; Prat *et al*., 2015; Ellsworth *et al*., 2015; Badve *et al*., 2015; Parker and Perou., 2015).

1.4.1 Hormone Positive/Luminal Breast Tumors

Hormone positive tumors are identified according to the presence of ER/PR receptor expression and subcategorized to Luminal A and B types. These tumors originate

from luminal lineage committed progenitors. Luminal A and B subcategories have different characteristics basically relying on cell cycle regulation and proliferation related pathways and also luminal/hormone dependent mechanisms (Johnston *et al*., 2015). Both luminal subtypes have similar ER expression level. Although Luminal B subtype has been indicated more aggressive in comparison to Luminal A tumors, both of the the subtypes are responsive to ER/PR targeted hormonal therapies at different levels (Prat *et al*., 2015, Zelnak *et al*., 2015).

1.4.2. HER2 Positive Breast Tumors

HER-2 gene encoding a specific receptor tyrosine kinase is frequently amplified and/or overexpressed in breast carcinomas. Her2 positive tumors similar to hormone dependent ones are formed by luminal lineage committed progenitors. *HER2* amplification/overexpression is seen in 30 % of breast cancer cases (D'Amato *et al*., 2015). Up-regulation of *HER-2* levels is generally accepted as early signature of neoplastic transformation of breast tissue. Elevated *HER-2* levels lead to several tumor promoting events including proliferation and survival of primary tumor and its metastasis to distant sites (Prat *et al*., 2015). In addition, Her-2/neu RTK pathway and its downstream signaling have a potential to induce motility, reduce apoptosis and enhance several signaling networks by promoting related protein expression in the tumor microenvironment. Trasuszumab therapies are used in clinic in the treatment of *HER-2* positive breast cancer (Yamamoto-Ibusuki *et al*., 2015, Wu *et al*., 2015).

1.4.3. Basal Like Breast Tumors (TNBC)

The basal like subtype is generally called as triple negative because there is no ER, PR and *HER2* expression/amplification seen in these tumors. TNBCs account for the 10-15% of all breast cancers (Prat *et al*., 2015) and represents the most aggressive phenotype with poor prognosis and early recurrence risk (Polyak *et al*., 2011; Lehmann *et al*., 2015). Basal like tumors originate from less differentiated stem like cells (Lehmann and Pietenpol., 2014; Leidy *et al*., 2014). When it is compared to other subtypes of breast cancer, overall survival rates and disease free survival after therapy is dramatically low because of high proliferation rates. It has highly heterogeneous character as in all cancers

including several oncogenic gene defects but no individual focal gene amplification is seen at high frequency. The recently identified subgroup of TNBC, claudin-low group, is described with lower claudin-3 and claudin-4 expression, down-regulated Ki67 proliferative marker, high EMT and mammary stem cell marker expressions (Lehmann and Pietenpol., 2014; Tomao *et al.*, 2015).

1.4.4. Frequently Deregulated Genes in Breast Cancer

In 20% of primary breast cancer cases, *HER-2* gene amplification leads to aggressive clinical behavior especially in ductal tumors (Slamon *et al.*, 1987; Van de Vijnet *et al.*, 1988). HER-1 has been also linked to progression of breast carcinoma in some patients (Downward *et al.*, 2003, Witton *et al.*, 2003; Arteaga *et al.*, 2011; Savas *et al.*, 2014). Although cell cycle regulator *cyclinE* has been identified to be amplified in only 2% of breast cancers (Keyomarsi *et al.*, 2002), other cell cycle chekpoint regulator , *cyclin D1,* has been found to be overexpressed in 40-50% of all invasive breast cancers and amplified in 10-20% of HER-2 amplified breast cancer cases (Grose *et al.*, 2007). c-Myc oncogene has also been found to be amplified and overexpressed in 15-25% of breast cancer cases with more aggressive clinical outcomes (Nass *et al.*, 1997). Ras mutations have been shown in some breast carcinoma cases (Osborne *et al.*, 2004).

In luminal A tumors, *PIK3CA* and *MAP3K1* mutations are more frequent compared to luminal B tumors. On the other hand, luminal B tumors have higher mitogenic rates with elevated *Ki67* and *AURKA* gene expressions and less luminal proteins such as FOXA1 and PR receptor (Prat *et al.*, 2015). HER2 subtype has the highest number of gene mutations. Luminal related proteins such as *ESR1* and *PGR* are moderately expressed but basal like related gene expressions including keratin 5 and *FOXC1* are relatively low in this subtype. *HER2*(+) tumors with 39% ratio also carry *PIK3CA* mutations. In TNBC tumors, *HER2* related genes are moderately expressed, and luminal related gene expression is very low. *BRCA-1* gene mutation is specifically associated with basal like phenotype. Some overexpressed genes including *BUB*, *RAB20*, *PKN1* and *NOTCH3* and *FGFR2* have been linked to TNBC subtype. The constitutive activation of *FGFR2* was also found in two TNBC cell lines together with the overexpression of the gene. This gene amplification which leads to the activation of PI3K/Akt signaling to promote survival has been reported

only for TNBC cell lines, but not demonstrated in other subtypes of breast cancer. Upon treatment with FGFR inhibitors or gene silencing via RNAi approach, the increase in sensitivity to apoptotic responses has been shown to be induced in TNBC cells with *FGFR2* gene defects (Johnston *et al*., 2015; Prat *et al*., 2015; Zelnak *et al*., 2015).

p53 mutations have been found in 20-30% of breast cancers. Overexpression of p53 and/or mutations in the gene have been associated with ER negativity and increases recurrence risk While Luminal A tumors show infrequently mutation/copy number changes in chromosomal level for *p53*, 72% of HER-2 amplified tumors have *p53* mutation. In 80% of all basal like tumors, *p53* mutations are also evident (Hollstein *et al*., 1991; Thor *et al*., 1992; Ioakim-Liossi *et al*., 2001).

Defects in other negative regulators of cell cycle progression such as *p27* and *Skp-2* have been linked to progression of breast cancer (Porter *et al*., 1997; Catzavelos *et al*., 1997; Osborne *et al*., 2004). *P27* with reduced expression in some breast tumors has been linked to reduced overall survival rates and reduced time to metastatic progression through declining (Tan *et al*., 1997). Reduced expression of *Skp-2*, S-phase associated protein, has also been indicated in basal like breast tumors (Signoretti *et al*., 2002).

BRCA-1 and *BRCA-2* genes with familial mutations are also important in the progression of breast carcinomas (Osborne *et al*., 2004). In 80% of tumors with higher grade basal like phenotype carrying myc oncogene amplification, truncation of BRCA-1 DNA repair enzyme is seen (Couch *et al*., 1997). *BRCA-2* gene with the similar structural pattern to *BRCA-1* shows premature truncation abnormality in breast cancers but it is not strongly associated with the aggressive phenotype (Friedman *et al*., 1997; Thorlacius *et al*., 1997). *PTEN* (Nelen *et al*., 1997; Mills *et al*., 2001), *CHK2* (Bell *et al*., 1999; Vahteristo *et al*., 2002) and *ATM*, (Shiloh *et al*., 2003) have also been reported as the important tumor suppressors in breast tumorigenesis. Loss of copy number in *Rb* tumor suppressor or loss function mutations in the gene have been shown in 30% of breast cancer cases and associated with increased rate of progression (Andersen *et al.*, 1992).

1.4.5. Therapy Approaches in Breast Carcinoma

Treatment strategies in breast cancer are determined according to the presence/absence of previously applied treatments, disease-free interval, tumor burden, existence of specific marker expressions, clinical trial availability and patient preference (Howard and Bland, 2012).

Chemotherapy regimens are generally given after surgery started with alkylating agents such as thiotepa, L-phenylalinine mustard. Then, adjuvant polychemotherapy "CMF" which has been applied after excision of tumors reduce reccurence risk and improve survival rates (Fisher *et al*., 1975, Bonadonna *et al*., 1976). In the following period, adjuvant chemotherapy with combined cytotoxic reagents (Tan et al., 1973; Brambilla *et al*., 1978; Sparano *et al*., 1998) and taxanes provide additional benefit for overall survival ratios (Wani *et al*., 1971; Schiff *et al*., 1979; Horwitz *et al*., 2004).

Recent clinical treatment regimens have focused on targeted therapy approaches where the basic aim is to achieve selective, efficient and safe treatment for both early and late stage cancer patients. To reduce recurrence and metastasis risk, systemic adjuvant therapies such as endocrine therapy, anti-HER2 therapy are frequently used in clinics (Nienhuis *et al*., 2015).

In endocrine therapy based approaches, recurrence risk was initially inhibited by adjuvant tamoxifen (Baum *et al*., 1983). The duration of this therapy was initially identified as 1 year, but then it has prolonged up to 10 years to obtain more effective treatment outcomes. In the latest endocrine therapy approaches, aromatase inhibitors in combination with sequential tamoxifen treatment have been widely preferred (Howell *et al*., 2005; Gos *et al*., 2003; Davies *et al*., 2013).

In anti-HER2 based therapy approaches, in addition to adjuvant chemotherapy, trastuzumab is given simultenously or sequentially and has been shown to reduce relapse of the disease in patients with *HER2* overexpressing node positive or high-risk node-negative. The duration of trastuzumab treatment is determined as 2 years, but additional tyrosine kinase inhibitors do not support the outcome (Romond *et al*., 2005; Gradishar *et*

al., 2015). In more recent clinical regimens for HER2 subgroup, anti-EGFR therapeutics gefitinib and erlotinib, anti-EGFR monoclonal antibodies cetuximab or panitumumab for breast tumors with EGFR alterations or HER2 small-molecule inhibitor lapatinib are the mostly preferred drugs (Lluch *et al*., 2014; Li *et al*., 2013; Guiu *et al*., 2010; Modjtahedi and Essapen, 2009).

Since (PI3K) pathway frequently altered in tumorigenic cases, it is explored for a broad range of cancer types including breast carcinoma as a targeted therapeutic approach (Giuliano *et al*., 2011). First generation non-isoform-selective panPI3K inhibitors did not give statisfying outcomes (Dienstmann *et al*., 2011; Bauer *et al.*, 2015; Yap *et al.*, 2015). Then, the inhibitors against downstream elements of the pathway including mammalian target of rapamycin (mTOR) or AKT were started to be explored as potential anticancer therapeutics (Johnston, 2015; Vanacker *et al*., 2015). Recent publications has emphasized the importance of more detailed understanding in the roles of downstream modulators in several growth factor signaling pathways for the development of novel therapeutics and to provide effective management of the disease (Miyoshi *et al*., 2008; Kobayashi *et al*., 2008; Lehmann and Pietenpol., 2014).

1.5. Salt-Inducible Kinase Family

Salt-inducible kinase family which are serine threonine kinases, are the members of AMP-dependent protein kinase (AMPK) superfamily (Bright *et al*., 2009). Although all SIKs show structural similarity with AMPK-related family, they are not sensitive to AMP and function as single subunit enzymes (Lizcano *et al*., 2004). This family is comprised of 3 members (SIK1-3) that are conserved from *D. Melanogaster* to humans. SIK1 was identified in myocardium (Ruiz *et al*., 1994) and cloned from adrenal glands of rats fed with high salt diet (Wang *et al*., 1999). The other members, SIK2 and SIK3, were identified according to their sequence similarity to SIK1 (Katoh *et al*., 2004). While *SIK1* is localized on chromose 21, both *SIK2* and *SIK3* genes are in chromosome 11 (Katoh *et al*., 2004). The N-terminal kinase domain with flexible activation loop and A-loop are localized near the substrate binding pocket of SIKs. *LKB1* tumor suppressor phosphorylates all the members on a specific Thr residue in the catalytic region. The phosphorylation by upstream kinase LKB1 results in structural change in catalytic site and

leads to increased kinase activity of SIK proteins (Lizcano *et al*., 2004). SIKs also have ubiquitin-associated (UBA) domain close to the catalytic site and this region is thought to be critical for phosphorylation by LKB1 (Jaleel *et al*., 2006). In the C-terminal regions of all SIKs, PKA phosphorylation sites exist adjacent to arginine-lysine-rich (RK-rich) region (Katoh *et al*., 2004). PKA is an upstream inhibitory kinase for all SIKs and phosphorylates SIK1, SIK2 and SIK3 at S577, S587 and S493, respectively at the C-terminal regions (Katoh *et al*., 2004).

SIK family members have also been associated to various cancer formations including, lung, ovarian, gastric, prostate and leukemia. Lack of SIK1 in combination with p53 loss has been shown to inhibit anoikis and distant metastasis has been promoted in the breast tissue (Cheng *et al*., 2009). SIK1 has been proposed to have a key regulatory function in E-cadherin expression (Eneling *et al*., 2012) and reduced level of SIK1 was associated with invasive phenotype in breast and ovarian cancer cells (Cheng *et al*., 2009). LKB-1 as the common upstream kinase of all SIK family members has been demonstrated to regulate E-cadherin level and cell polarity as well as survival of the cells (Roy *et al*., 2010). Drosophila studies have indicated that LKB-1 might regulate polarity through the regulation of downstream effectors SIK1, NUAK or Par-1 instead of AMPKs (Amin *et al*., 2009). LKB1 and SIKs were proposed as new therapeutic targets in T-cell leukemia where they were shown to inhibit Tax-mediated LTR activation and HTLV-1 transcription via regulating CRTCs and CREB (Tang *et al*., 2013). SIK1 was also implicated in gastrin induced migration events in pancreatic and gastric adenocarcinoma cells (Selvik *et al*., 2014). SIK3 has been suggested as a novel tumor antigen in ovarian cancer to promote cell cycle progression via regulating PI3K activation and downregulating p21 (Charoenfuprasert *et al.,* 2011). Activated SIK2 and SIK3 by LKB-1 have been shown to negatively regulate HDAC4/5/7/9 expressions which are essential for MEF-2 dependent transcription and this may lead to the oncogenic transformation events (Walkinshaw *et al*., 2013).

1.5.1. SIK2

SIK2 was initially identified in mouse adipose tissue (Horike *et al*., 2003). Since the expression of the protein was induced in 3T3-L1 preadipoctes at the onset of

differentiation, the protein was suggested to be involved in the early phase of adipocyte differentiation (Yeh *et al*., 1995). The rat IRS1, an important regulator of insulin signaling, pathway was identified as the first substrate of SIK2 (Horike *et al*., 2003). Although the activation of downstream elements of insuling signaling pathway is initiated by Tyr phosphorylation of IRS1, Ser 789 phosphorylation of the protein inhibits insulin signaling either by increasing IRS-1 degradation, preventing downstream effectors or disrupting its interaction with the insulin receptor at the plasma membrane (Gual *et al*., 2005). S789 phosphorylation of IRS1 is found to be elevated in insulin resistant rats (Qiao *et al*., 2002). During insulin resistance conditions, SIK2 expression and activity were induced markedly in the white adipose tissue of diabetic mice suggesting SIK2 involvement in the development of type 2 diabetes (Horike *et al*., 2003). SIK2 is involved in liver gluconeogenesis through phosphorylation of TORC2 in glucagon dependent manner (Dentin *et al*., 2007). SIK2 has been shown to inhibit lipogenic gene expression downstream of ChREBP in liver by phosphorylating p300 from the inhibitory S89 residue (Bricambert *et al*., 2010). Recently SIK2 was shown to be involved in glucose-induced insulin secretion from pancreatic β-islet cells (Sakamaki *et al*., 2014).

In addition to metabolic functions, SIK2 is also involved in diverse biological processes. It was shown that upon UV damage, decrease in SIK2 level promotes transcriptional activation of melanogenic program through de-phosphorylation of TORC1 (Horike *et al*., 2010). SIK2 degradation was reported to increase under cerebral ischemia through the activation of NMDA receptors, and leads to the CREB dependent transcriptional activation of neuronal survival genes (Sasaki *et al*., 2011).The posttranslational regulation of SIK2 kinase activity via acetylation was proposed to play a role in autophagosome maturation (Yang *et al*., 2013). A recent study by our group showed that SIK2 inhibits insulin mediated survival of Müller cells through the regulation of Akt phosphorylation in normal and in chronic hyperglycemia conditions. Since SIK2 expression also exists in retinal ganglion cells, it has been speculated that Akt mediated-survival of these neurons should be modulated by SIK2 gene (Kuser-Abali et al., 2013).

SIK2 were shown to take part in cell cycle progression in ovarian and prostate cancer cases. In ovarian cancer cells, SIK2 function in the pro-metaphase centrosome splitting thorugh the phosphorylation of c-Nap-1 centrosome seperator and contributes to

G1/S cycle progression during mitosis. Therefore, it was suggested that inhibiton of SIK2 might help to blocking of drug resistance occuring after mitotic inhibitor treatments in ovarian cancer patients (Ahmed *et al*., 2010). SIK2 gene was also demonstrated in the cell cycle progression and transcription factor activation in prostate cancer cells through the regulation of CREB-1 dependent gene expression (Bon *et al*., 2015). In lung adenocarcinoma patients, whole genome sequence analysis suggested that SIK2 exon duplication exists in the kinase domain (Imielinski *et al*., 2012).

Activated SIK2 and SIK3 kinases have been suggested to promote tissue overgrowth in *Drosophila* through the negative regulation of Hippo signaling pathway (Wehr *et al*., 2013). SIK2 serine threonine kinase is also identified by our group in the negative regulation of FGF signaling pathway by inhibiting Erk and Akt activations in retinal glial cells (Figure 1.11) (Kuser-Abali *et al.,* unpublished data). Silencing of the gene in these cells promoted an increase in FGF dependent proliferation and migration/invasion ability and inhibited the controlled cell death (unpublished data). This data highlighted the possibility of SIK2 as a tumor suppressor in tumorigenic events and its contribution to cancer progression.

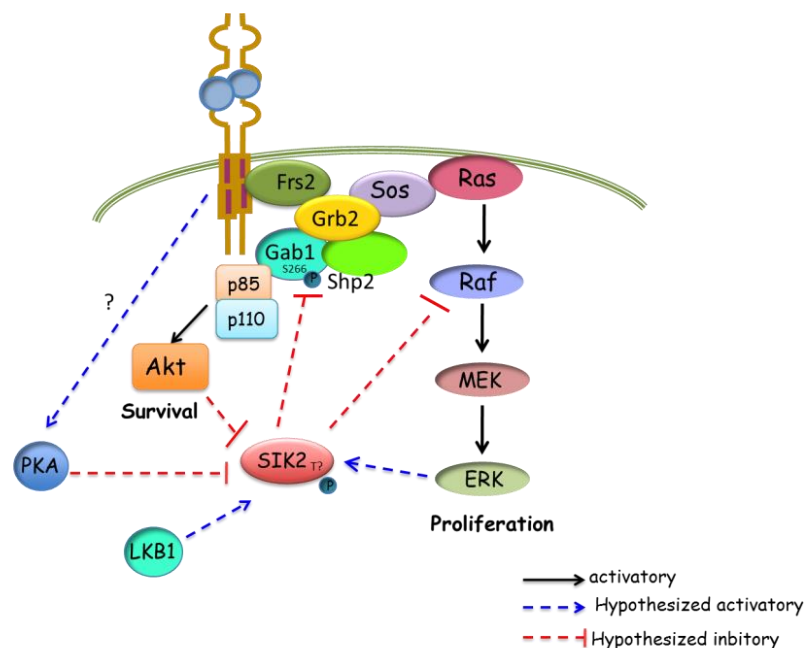

Figure 1.11. Our proposed model depicting the negative-feedback modulation of FGF signaling pathway by SIK2 (Kuser-Abali, unpublished data).

2. PURPOSE

Our previous data has demonstrated that SIK2 functions in FGF signaling to downregulate proliferation and survival responses of cells via inhibiting ERK and Akt activation. Enhanced mitotic potential and resistance to cell death are the basic hallmarks for neoplastic transformation of the cells. Therefore, in the context of this thesis study, we aimed to identify a potential contribution of SIK2 in the development of tumorigenicity and to gain insight into the underlying mechanism in this control.

Towards this aim, the study was conducted in 2 parts:

In primary tissue samples, we investigated;

(i) the modulation of SIK2 expression in various cancer types using cDNA array

(ii) the expression pattern of SIK2 in normal breast tissue and tumor samples and whether a correlation exists between SIK2 levels and proliferation

were analyzed.

In non-transformed and transformed breast cell lines we studied;

The effect of modulation of SIK2 expression

(i) on the proliferative potential and ERK activation

(ii) the apoptosis and Akt activation profiles

(iii) on the migration and invasion capacity of MCF12A non-transformed cells via analyzing EMT marker e-cadherin and invasion marker MMP-2 profiles.

(iv) on the tumorigenicity by xenogafting

(v) characterization of the xenograft tumors for SIK2 levels and proliferation/survival.

3. MATERIALS

3.1 Cell Lines and Primary Tumor Samples

Non-transformed and transformed human breast cell lines (MCF12A, MDA-MB-231, MDA-MB-453, MDA-MB-157, MDA-MB-361, SKBR-3, MCF-7, T47D) were kindly provided by Dr. Isik Yulug from Bilkent, Department of Moleculary Biology and Genetics, Ankara. Primary tumor samples were obtained from surgical operations accomplished in Florance Nightingale hospital with the approval of the Bogazici University and Florance Nightingale hospital ethics committee. The patients were informed about the usage of the tissues in the research study.

3.2. SCID Animals in Xenografting

Female SCID mice 4-6 weeks of age were obtained from Boğaziçi University Vivarium. The animals were maintained under pathogen free conditions and handled according to the university guidelines and with the approval of the local ethics committe BUHADYEK.

3.3. Chemicals, Plastic and Glassware

All chemicals used in this study were purchased from Sigma Aldrich Ltd. (USA) or Merck (Germany) unless otherwise stated in the text. All solutions, plastic and glassware were sterilized by autoclaving at 121^0C for 20 minutes when possible.

3.4. Real-time PCR Primers

Table 3.1. Real-time PCR primers used in this study.

Real-Time Fragments	Name of Primers	Primer sequence (5'-3')	Melting Temp. (^{0}C)	bp
SIK2	SIK2-Fw SIK2-Rw	TTGCTGAACAAACAGTTGCC TCAAGCAGACAGCCATTCAC	59,5	106
GAPDH	GAPDH-Fw GAPDH-Rw	AAGATCAAGATCATTGCTCCTC GGGTGTAACGCAACTAAGTC	55	138

3.5. Plasmids

Table 3.2. Vectors used in this thesis.

Vector Name	Company
pEGFP-C3	Clontech, USA
pEGFP-C3-fl-SIK2	Clontech, USA, Redesigned Kuser
pEGFP-C3-KI-SIK2	Clontech, USA, Redesigned Kuser
sh-RNA Lentiviral particles (sh-SIK2 and sh-control)	Santa Cruz, USA

3.6. Antibodies

Table 3.3. Antibodies used in immunostaining and Western blotting studies.

Antibody	Brand	Application	Antibody dilution	M_w (kDa)
SIK2	Cell Signal.	WB	1/1000	130
pERK	Santa Cruz	WB	1/1000	42-44

Table 3.4. Antibodies used in immunostaining and Western blotting studies (cont.).

ERK	Santa Cruz	WB	1/1000	42-44
pAkt (S473)	Cell Signal.	WB	1/2000	60
panAKT	Cell Signal.	WB	1/1000	60
β-Actin	Santa Cruz	WB	1/1000	42
GAPDH	Cell Signal.	WB	1/1000	34
E-cadherin	Cell Signal.	WB	1/1000	135
MMP-2	Cell Signal.	WB	1/1000	64
SIK2	Novus Biologicals	IF	1/250	
Ki67	BD Pharmingen	IF	1/50-1/100	
Active Caspase-3	Cell Signal.	IF	1/100	
Rabbit/Mouse/ Alexafluor 555/488 conjugated Ab.	Thermo Life Technologies	IF	1/1000	
Rabbit/Mouse/ Horseradish peroxidase(HRP) conjugated IgG	SantaCruz	WB	1/5000	

3.7. Kits

Table 3.4. Kits used in this thesis.

Kits	Supplier
QIAprep Spin Miniprep kit	Qiagen, Germany

Table 3.4. Kits used in this thesis (cont.).

Bicinchoninic acid (BCA) assay kit	Thermo, USA
RNAeasy Minikit	Qiagen, Germany
cDNA Tissue Array kit	Origene, USA
X-treme Gene Transfection Reagent	Roche, Germany
In Situ cell death detection kit, fluorescent	Roche, Germany
In situ cell proliferation kit, fluorescent	Roche, Germany
Transwell Invasion Assay Kit	BioRAD/Corning, USA

3.8. Equipments

Table 3.5. Equipments used in this thesis.

Equipments	Models
Autoclave	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	Electronic Balance VA 124, Gec Avery,USA DTBH 210, Sartorius, GERMANY
Carbon dioxide tank	2091, Habaş, TURKEY
CCD camera	CCD Camera, JAI Corporation, JAPAN
Centrifuges	ProFuge, 10K, Strategene, USA Mini Centrifuge 17307-05, Cole Parmer, USA Genofuge 16M, Techne, UK Centurion K40R, UK Beckman J2-21, USA Centrifuge B5,B.Braun B. Int. (GERMANY) Centrifuge 5415R, Microfuge tube, USA J2-MC Centrifuge, Beckman Coulter, USA J2-21 Centrifuge, Beckman Coulter, USA

Table 3.5. Equipments used in this thesis (cont.)

Cold room	Birikim Elektrik Soğutma, Turkey
Cryostat	LEICA CM 3050S GERMANY
Deep Freezers	2021D (-20^{0}C), Arçelik, Turkey -70^{0}C Freezer, Harris, UK -86^{0}C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio Doc, ITALY Stella, Raytest, Germany
Electrophoretic Equipments	Mini-PROTEAN 3 Cell, BIO-RAD, USA Easy-cast system, Hybaid, UK
Heat blocks	DRI-Block DB-2A, Techne, UK StableTemp Dry Bath Incubator, Cole Parmer, (USA)
Hemocytometer	Improved Neubauer, Weber Scientific International LTD, UK
Homogenizer	Pellet Pestles Tissue Grinder, Kimble Kontes USA
Hybridization Oven	Shake'n'Stack, Hybaid, UK
Ice Machine	Scotsman Inc., AF20, ITALY
Incubator	Forma Series II Water Jacket CO_2 Incubator Thermo Scientific USA
Laminar flow cabinet	Class II A Tezsan, TURKEY Class II B Tezsan, TURKEY
Magnetic Stirrer	M221 Elekro-mag, TURKEY Clifton Hotplate Magnetic Stirrer, HS31, UK
Micropipettes	Gilson, FRANCE
Microscopes	LEICA DM 6000 CS Confocal Microscope, Germany Zeiss, Axio Observer Z1 Inverted Mic., USA Olympus X2-SL CKX41 Light Microscope
pH meter	WTW, GERMANY

Table 3.5. Equipments used in this thesis (cont.)

Power Supplies	EC135-90, Thermo Electron Corporation Power Pac Universal, BIO-RAD, USA LEICA CTR 6500 Power Supply Confocal M.
Western Blotting Membrane Visualization	Stella, Raytest, Germany
Refrigerators	2082C, Arçelik, TURKEY 4030T, Arçelik, TURKEY Sonya Ultra Low Refrigerator, Japan
Sealer	Vacuplus FS400A, Electric Petra, GERMANY
Shakers	VIB Orbital Shaker, InterMed, DENMARK Lab-Line Universal Oscillating Shaker, USA Adjustable Rocker, Cole Parmer, USA
Software	Metasystems, GERMANY Quantity One, Bio-Rad, ITALY
Spectrophotometer	CE5502, Cecil, UK NanoDrop ND-1000, Thermo, USA
Vortex	Vortexmixer VM20,Chiltern Scientific, UK
Water Bath	TE-10A, Techne, UK
Water Purification	UTES, TURKEY

4. METHODS

4.1. qRT-PCR for cDNA Arrays of Primary Human Samples

For quantitative PCR studies, Origene cDNA tissue array kit was used. Reaction mixtures containing the reference h-GAPDH primers and SIK2 specific primers at final concentration of (10 um each) were prepared in SYBR Premix Ex Taq (Takara, Otsu, Japan). Quantitative-PCR mix were added to the cDNA samples and incubated on ice for 15 minutes. Following 5 minutes initial denaturation step at 95°C for 10 seconds, samples were subjected to 40 cycles of PCR amplification. Each cycle included a denaturation step at 95°C for 10 seconds, an annealing step at 59.5°C for 10 seconds and an extension step at 72°C for 15 seconds. Subsequently melting curve analysis was performed to see whether any undesired product is amplified during the reaction. This analysis was completed in 61 cycles, 10 seconds each. Data was analyzed by using Bio-RAD IQ5 software program.

4.2. Screening of Oncomine Database

Oncomine database (http://www.oncomine.org) was screened for SIK2 transcript levels and cDNA copy number variation. All independent breast cancer sample datasets (14) which are included in database were analyzed. The selected filtering criteria was defined as P-value ≤0.05, in all fold changes and in all gene ranks.

4.3. Cryosectioning and Immunofluorescence Staining

Tumor tissues from SCID mice and normal and tumor samples from patients were fixed in 4%PFA immediately after their excision and freezed in -80°C until use. For cryosectioning, they were removed from the freezer and equilibrated at -20°C for approximately 15 minutes and 10-12 um sections were taken on positively charged slides. For immunohistostaining, slides with sections were incubated in permeabilization buffer containing 0.1% BSA and 0.1% Tween-20 in PBS. Tissues were blocked within blocking solution (0.1% Glycine/1% BSA/10% normal donkey serum in PBS) for 1 hr at room

temperature. Subsequently the sections were incubated overnight at 4°C with anti-Ki67, anti-SIK2 and anti-cleaved caspase-3 at dilutions indicated in Table 3.6. Following washing in PBS containing 0.1% Tween-20 three times for 10 minutes each, sections were incubated with the appropriate secondary antibodies conjugated with Alexa Fluor 555 or Alexa Fluor 488 at 1:1000 dilution. Diaminophenylindolamine (DAPI) was used to stain nuclei. After extensive washing, sections were mounted with coverslips and examined using a Zeiss Inverted Fluorescent Microscope (USA) and Leica Confocal (Germany) microscope. Images were optimized for color, brightness and contrast. Double-labeled images overlaid by using Adobe Photoshop 6.0 (Adobe Systems, Inc., New York, NY).

4.4. Maintenance of Breast Cell Lines

MCF-12A normal breast line was maintained in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Invitrogen, Inc.), 20 ng/ml epidermal growth factor, 1% non-essential aminoacid (Bichrom, Germany), 0.01 mg/ml human insulin, 500 ng/ml hydrocortisone, 1% penicillin/streptomycin and 10% fetal bovine serum. MCF-7, MDA-MB-231, MDA-MB-453, MDA-MB-157 and MDA-MB-361 cell lines were cultured in Dulbecco's modified Eagle's medium (Life Technologies, USA) supplemented with 10% fetal bovine serum, 1% NEAA and 1% penicillin/streptomycin; T47D were grown in RPMI 1640 (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin- streptomycin and 1% non-essential aminoacid (PAN, USA). SKBR3 cell line was maintained in Mc'Coys (Sigma Aldrich, USA) medium supplemented with % 10 fetal bovine serum, 1% non-essential aminoacid and 1% penicillin/streptomycin. All cells were incubated at 37 °C under 5% CO_2. The medium was refreshed every 3 days and the cells were divided into three plates when they are reached to confluency.

4.5. Plasmid DNA Isolation

Stocks of bacteria carrying plasmids EGFP-C3 or EGFP-C3 with full length SIK2 (fl-SIK2) or kinase inactive SIK2 (KI-SIK2) were grown overnight in LB medium containing appropriate antibiotic at 37^0C with shaking at 225 rpm and plasmids were isolated with MidiPrep kit (Qiagen, USA). Briefly bacterial cells were lysed and chromosomal DNA

was denatured under strong alkaline conditions (pH 13.0), cell debris and chromosomal DNA were removed by centrifugation at 1000 g for 10 minutes. Supernatant was applied to QIA-prep spin columns where DNA binds to the silica gel membrane in the presence of high salt. Impurities were removed by washing with buffer containing absolute ethanol and plasmid DNA was eluted with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA).

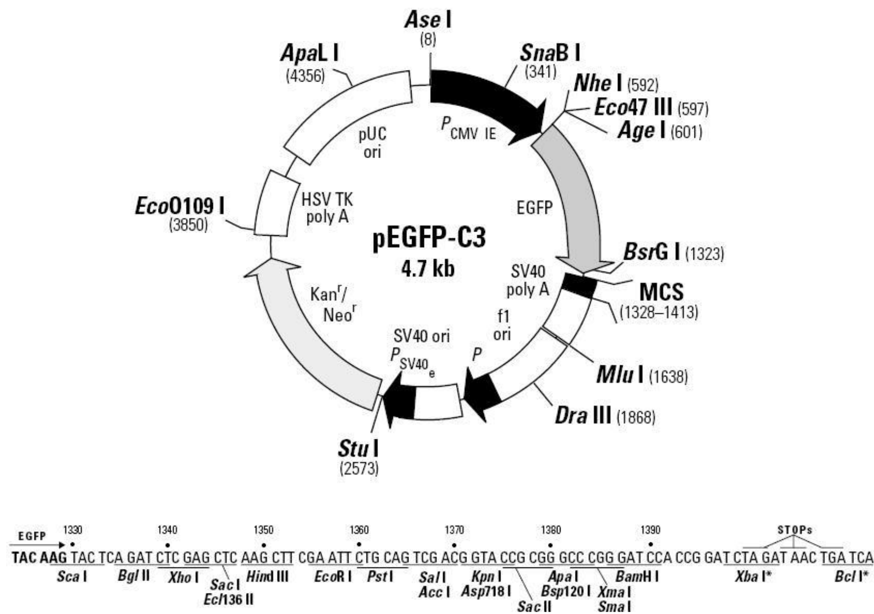

Figure 4.1. Map of the pEGFP-C3 vector.

4.6. Transfection of pEGFP, pEGFP-SIK2, pEGFP-KI-SIK2 Vectors to MDA-MB-231 Cells

One day before the transfection, MDA-MB-231 cell density was determined, 250.000 cells were seeded on 6-well plates, and grown in DMEM containing 10% FBS and 0.1% streptomycin/penicillin. At the day of transfection, 2 ug p-EGFP-C3-fl-SIK2, p-EGFP-C3-KI-SIK2 and empty pEGFP-C3 plasmids were diluted in 200 ul DMEM solution and 6 ul X-treme Gene transfection reagent (Roche, Germany) was added into this mixture. Following 30 min incubation at room temperature, prepared mixture was added onto the cells in a drop-wise manner. The plate contents were swirled on a rotating platform shaker for 30 seconds and plates were placed into the incubator for 8 hours. Then transfection medium containing culture medium was decanted and cells were washed three times with

PBS and medium was refreshed. At the next day cells were taken into antibiotic G418 (Sigma, USA) including fresh media and maintained for 3 weeks with media refreshment at every 2 days.

4.7. SIK2 Gene Silencing in MCF12A Cells via shSIK2

Lentiviral particles carrying human SIK2 shRNA and the corresponding scrambled shRNA were purchased from Santa Cruz (USA). This is a pool of concentrated, transduction-ready viral particle mixture containing three target-specific constructs that encode 19-25 nucleotides (plus hairpin) shRNA designed to knock down specific gene expression. MCF12A cells were subjected to lentiviral infection as instructed by the manufacturer and propagated in the presence of puromycin (1 mg/mL; Sigma). To select stable shSIK2 or scrambled shRNA containing colonies, cells were passaged into three plates and after 48 hours, they were transferred into culture medium containing 10 µg/ml puromycin. Every 3 days, medium was replenished. After 3 weeks, mixed colonies were collected from at least 4 independent 6-well plates and the ones having SIK2 down-regulation were stored at 156°C until further use. In order to investigate SIK2 gene silencing, collected samples of cells were analyzed with Western blotting using anti-SIK2 antibodies.

4.8. Bicinchoninic Acid (BCA) Assay

For determination of the concentration of proteins, BCA Assay kit was used. Experimental samples and bovine serum albumin (BSA) dilutions ranging from 0.025 to 2 mg/ml were mixed with 170 µl of 50:1 diluted BCA Working Solution. After 30 minutes at 37^0C incubation, absorbance measured at 595 nm. Protein concentrations of the samples were extrapolated from the standard curve.

4.9. SDS-PAGE and Western Blot

Cell lysates were fractionated on 8/10/12,5% polyacrylamide gels (8% acrylamide:bisacrylamide (29:1), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% N,N,N',N'-tetramethylethylenediamine) with 5% stacking gel (5% acrylamide:bisacrylamide (29:1), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% N,N,N',N'-tetramethylethylenediamine). Samples were boiled in 6X sample buffer (300 mM Tris-Cl, pH 6.8, 600 mM DTT, 10% SDS, 0.6% bromophenol blue, 50% glycerol) at 95^0C for 5 minutes before loading. The gel was run in 25 mM Tris-HCl, 250 mM glycine and 0.2% SDS buffer at 100-120 V.

For Western blotting, the samples fractionated on polyacrylamide gels were electroblotted to PVDF membranes (Roche, Germany) in transfer buffer (200 mM glycine, 25 mM Tris.HCl, 15% methanol) at 100 V for 1-1.5 hours. To equilibrate, the membrane was washed in tris buffered saline-tween (TBST) solution (150 mM NaCl, 20 mM Tris.HCl, pH 8.0, 0.1 % Tween 20) three times for total of 30 minutes. The membranes were incubated in blocking solution (1% -5% BSA or 1% - 5% skimmed milk powder in TBST) for 1 hour at room temperature with gentle shaking. The membrane was left overnight in the blocking solution containing appropriate antibodies at 4^0C Table 3.2. Subsequently, the membrane was washed with TBST three times for 5 minutes each to remove unbound antibody and incubated in Lumi-light Western blotting substrate (Santa Cruz, USA) or WestFemto High Sensitive substrate (Thermo Scientific, USA) for 6 minute and images were captured using Stella gel imaging system (Raytest, Germany) for 15/30 seconds intervals.

4.10. Cell Proliferation Assay

100.000 cells were seeded on 6-well plate culture dishes (Greiner, USA) and were allowed to grow to sub-confluency. The cells were washed with PBS and starved in DMEM and 0.1% penicillin/streptomycin overnight. Subsequently, they were treated with 1 ng/ml FGF2 (R&D Systems, USA) and 10 µg/ml heparin for 24 hour. At the end of the treatment periods, cells were immediately washed with PBS. Then, Brd-U assay was

performed using In Situ Cell Proliferation assay kit (Roche), as per manufacturer's instructions.

Briefly, after washing with PBS, the cells were incubated in a medium containing 10 µM Brd-U for 5 hours. To detect Brd-U incorporation, the cells were fixed with the solution containing absolute ethanol and 50 mM, pH:2.0 glycine for 45 minutes, washed twice with PBS to rinse the fixative solution, and treated with 4 M HCl for 15 minutes at room temperature for denaturation. Following an extensive washing in PBS, cells were left in blocking solution for 10 minutes and incubated with FITC-conjugated anti-BrdU antibody for 1 hour at 37°C in dark at humidified conditions. To visualize the cells, DAPI (0.1ug/ml) staining was carried out for 10 minutes. After washing three times with PBS, the photos were taken under 20X magnification of Zeiss Axio-vision inverted fluorescent microscope. In randomly selected areas total of 150 cells were counted.

4.11. TUNEL Assay

Apoptotic cell death was examined by TUNEL assay using in situ cell death kit, (Roche, Germany). Cultured MCF12A or MDA-MB-231 cells seeded on 6-well culture well dishes with a density of $100x10^3$cells/well and grown overnight. At the end of the period, cells were starved for 16 hours in serum starvation conditions for providing the synchronization of the cell cycles. Next morning, cells were fixed with 4% paraformaldehyde for 45 minutes and then permeabilized with the sodium citrate buffer containing 0.1% Triton X-100. After washing with PBS three times, cells were incubated in TUNEL labeling solution for one hour in the dark at 37^0C. To visualize the nuclei cells were incubated with DAPI for 5 minutes. After washing twice with PBS, the cells were observed under fluorescent microscope and 150 cells were counted in randomly selected areas.

The next day, the monolayer was wounded using a plastic pipette tip (P200) and rinsed with PBS. The bottom of the wells were marked to indicate where the initial pictures of the wound area were taken. The closure of the wound was observed in the following 8 and 24 hours by taking the photos of the same areas at 10X magnification and recorded using an Axio-vision inverted fluorescent microscope (Zeiss Inverted Microscope).

4.12. Wound Healing Assay

To perform wound healing assay, 250x10^3 cells seeded in a 6-well plate until confluenCY, then serum starved overnight for cell cycle syncronization. The next day, the monolayer was wounded using a plastic pipette tip (P200) and rinsed with PBS. The bottom of the wells were marked to indicate where the initial pictures of the wound area were taken. The closure of the wound was observed in the following 8 and 24 hours by taking the photos of the same areas at 10X magnification and recorded using an Axio-vision inverted fluorescent microscope (Zeiss).

4.13. Transwell Invasion (Boyden Chamber) Assay

Trans-well invasion assay was performed according to the protocol from BD Biosciences (Durham, USA). Briefly, after an overnight starvation, 250x10^3 cells were layered the top chamber (8µm pore size) in a medium devoid of serum. The chamber insert was coated with ECM matrigel matrix (Corning, USA) at 0.3 mg/ml final concentration, and chambers were set into 6-well plates containing medium supplemented with 10% bovine serum albumin. Cells were overlaid on top the inserts coated with ECM matrigel matrix and 2 ml medium without fetal bovine serum was added onto the cells. Plates were swirled then cells were settled in the incubator for 48 hours to allow the migration to the underside of the insert. Then they were fixed with 4% PFA for 10 minutes washed with PBS and incubated with methanol for 30 minutes. After Giemsa staining for 1 hour, cells remain in the upper face of the membrane were gently removed, the images of the cells attached to the bottom side of the inserts were taken and the cells were counted.

4.14. Generation of SCID Xenografts

SCID animals were injected with 1x10^6 MDA-MB-231 cells subcutaneosly at their flank regions. While right flanks of animals injected with control cells, parental MDA-MB-231 or p-eGFP-C3 empty vector transfected ones, left flanks of the same animals were injected with p-eGFP-fl-SIK2 transfected MDA-MB-231 experimental cells in each case.

An independent set of 3 SCID animals received p-eGFP-C3-KI-SIK2 transfected MDA-MB-231 cells into the left flanks p-eGFP-C3-fl-SIK2 transfected MDA-MB-231 cells at their right flanks. Animals were sacrificed 5 weeks post-injection by cervical dislocation. Tumors formed in the flank regions were excised and photographed. The tumors were weighed and the sizes measured by a caliper. Tumor volume was calculated according to the following formula: V = (L*W2*π)/6, V, volume (mm3); L, biggest diameter (mm); W, smallest diameter (mm). Data were presented as both tumor volume (mean 6 S.D.) and tumor weight (mean 6 S.D.). Statistical analysis was performed using the Student's *t* test.Half of the dissected tumor tissues were rapidly put into the freshly prepared 4% PFA for further cryosectioning and immunoflorescence staining experimentS and the remaining partS were stored -80^0C for immunoblotting experiments.

4.15. Statistical Analysis

Experimental groups were compared statistically using the Mann-Whitney test (one-tailed) or one-way ANOVA. Means with $P \leq 0.05$ and $p \leq 0.001$ were considered statistically significant.

5. RESULTS

5.1. Studies with Human Primary Tumor Samples

5.1.1. SIK2 Expression Is Modulated Differentially in Tumors of Diverse Tissue Origin

As a first step to probe into a possible involvement of SIK2 in tumorigenesis, we investigated whether its expression is modulated in primary human tumors from 19 different tissues using an Origene cDNA array (USA). This screen indicated that SIK2 transcript levels were frequently increased in some tumors (3/19) such as adrenal and colon cancer cases (Figure 5.1 top panels), but no significant change was evident in others (10/19) such as ovary and lung cancers (Figure 5.1 bottom panels). Yet in another group (6/19) including glial (Figure 5.2) and breast tumors, a marked decrease in SIK2 transcript levels was evident (Figure 5.3).

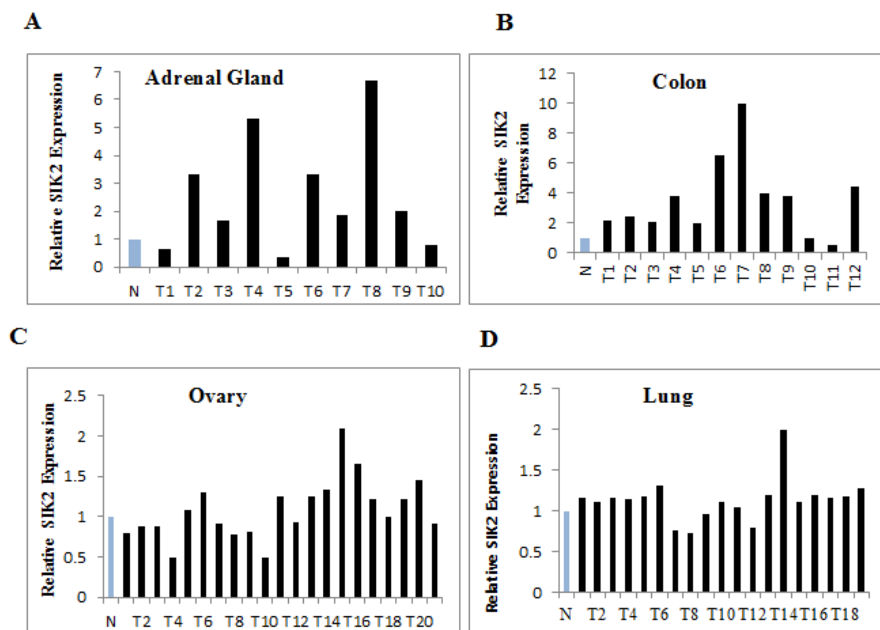

Figure 5.1. Relative SIK2 expression in Primary Human Tissue Samples. N represents the mean value of SIK2 transcript levels from normal tissues which includes 5 samples in A, 7 samples in B, 3 samples in C and 4 samples in D. T represents normalized SIK2 transcript levels of individual tumor samples to that of N. GAPDH was used as internal control.

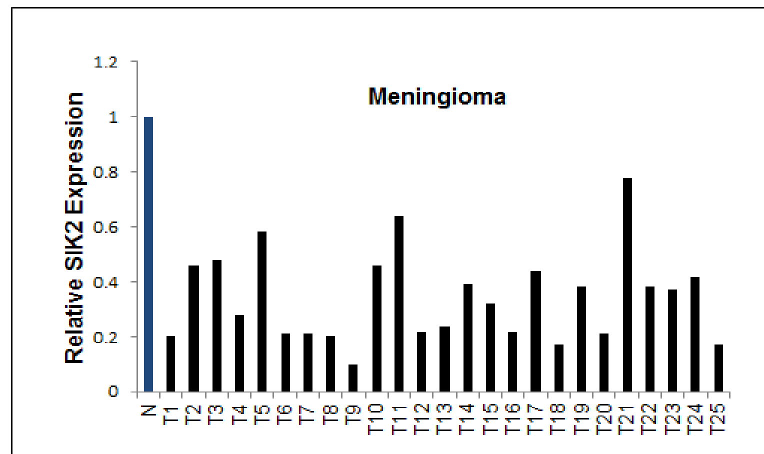

Figure 5.2. Relative SIK2 expression in primary meningioma samples. N represents the mean value of SIK2 transcript levels in two normal brain tissues. T represents normalized SIK2 transcript levels of individual tumor samples to that of N. GAPDH was used as internal control.

The breast array data demonstrated that a reduced SIK2 level was evident in 23 breast cancer samples compared to the mean value of two independent normal tissues. There is one patient sample (T13) which has a 3' fold increase in SIK2 expression in this cohort (Figure 5.3).

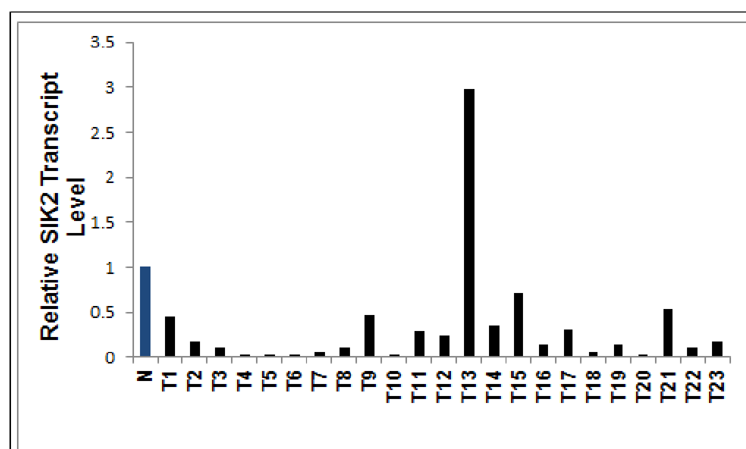

Figure 5.3. Relative SIK2 expression in primary breast tumors. N represents the mean value of SIK2 transcript levels in two normal breast tissue samples. T represents normalized SIK2 transcript levels of individual tumor samples to that of N. GAPDH was used as internal control.

We next screened the Oncomine database (http://www.oncomine.org) that includes 14 independent datasets generated from normal tissue samples and different types of breast cancer samples. In 9 datasets statistically significant downregulation in SIK2 transcript level was indicated using P ≤0.05 as treshold in all fold changes and in all gene ranks. No significant change was evident in the remaining 4 reports. In addition, in one data set, SIK2 gene copy number loss was reported (Figure 5.1.4). There was no significant correlation between SIK2 expression and ER/PR or HER2 status and/or of tumor grade in these datasets were evident.

This data gave us support for the hypothesis on the possible tumor suppressor role of SIK2 in the transformation events of certain tissues and breast tumor malignancies were chosen for further studies.

Table 5.1. Summary of oncomine database screening. In this screening, threshold values were applied as ≤0.05 p-value, in all fold changes and in all gene ranks within all measured genes.

Data Sample/ Publication/ Total Genes Measured	**Total Samples Normal/ Tumors Verified**	**Transcript Level Change**	**Fold Change Interval**	**Gain/Loss Significance (P-Value < 0.05)**
TCGA Breast/ No Ass. P. 2011/09/02/ 20423 genes	61/532	Decrease	(-2.5) - (-3.0)	sign. change
Sorlie Breast/ Proc Natl Acad Sci U S A 2001/09/11/6304 genes	4/79	Decrease	(-1,6) - (-3.1)	sign. change

Table 5.2. Summary of oncomine database screening. In this screening, threshold values were applied as ≤0.05 p-value, in all fold changes and in all gene ranks within all measured genes (cont.)

Data Sample/ Publication/ Total Genes Measured	Total Samples Normal/ Tumors Verified	Transcript Level Change	Fold Change Interval	Gain/Loss Significance (P-Value < 0.05)
Curtis Breast/Nature/ 2012/04/08 19.273 genes	144/1992	Decrease	(-1,5) - (-1.6)	sign. change
Perou Breast/ Nature 2000/08/17/ 6625 genes	4/59	Decrease	(-2.0) - (-2.5)	sign. change
Sorlie Breast/ Proc Natl Acad Sci U S A 2003/07/08/ 6,197 genes	3/138	Decrease	(-1.7) - (-3.1)	sign. change
Finak Breast/ Nat Med 2008/05/01/ 19,189 genes	6/53	Decrease	-8	sign. change
Richardson Breast/ Cancer Cell 2006/02/01/ 19,574 genes	7/40	Decrease	-2.3	sign. change
Gluck Breast/ Breast Cancer Res Treat 2011/03/04/ 17.862 genes	4/154	Decrease	-2.8	sign. change
Ma Breast 4/ Breast Cancer Res 2009/02/02/ 19,139 genes	28/38	Decrease	(-1.2)-(-2.9)	sign. change (Epithelial) n.s. change (Stroma)

Table 5.1. Summary of oncomine database screening. In this screening, threshold values were applied as ≤0.05 p-value, in all fold changes and in all gene ranks within all measured genes (cont.)

Data Sample/ Publication/ Total Genes Measured	Total Samples Normal/ Tumors Verified	Transcript Level Change	Fold Change Interval	Gain/Loss Significance (P-Value < 0.05)
Karnoub Breast/ Nature 2007/10/04/ 19.574 genes	15/7	Decrease	-1.5	n.s. change
Zhao Breast/ Mol Biol Cell 2004/06/01/ 12,482 genes	3/58	Decrease	(-1.7) - (-2.1)	n.s. change
Radvanyi Breast/ Proc Natl Acad Sci U S A 2005/08/02/ 16,775 genes	5/21	Decrease	(-1.5) - (-1.9)	n.s. change
Turashvili Breast/ BMC Cancer 2007/03/27/ 19.574 genes	20/30	No Decrease		
Data Sample/ Publication/ Total Genes Measured	**Total Samples Normal/ Tumor Verified**	**DNA Copy Number**	**Fold Change Interval**	**Gain/Loss Significance (P-Value < 0.05)**
TCGA Breast 2/ No Associated Paper 2012/02/29/ 18,823 genes	813/759	Decrease	(1.061) – (-1.299)	sign. change

5.2. SIK2 in Normal and Cancerous Breast Tissue

For this study 23 mixed grade ER/PR positive or TNBC tumor samples and their normal counterparts were collected during therapeutic operations of breast cancer patients and used in immunostaining studies.

Immunohistochemical stainings showed that SIK2 was specifically expressed in ductal epithelial cells of normal breast tissue (Figure 5.4a), while the other areas were devoid of SIK2 staining. The specificity of the staining was verified by preincubation of anti-SIK2 antibody with a SIK2 peptide (Figure 5.4b). SIK2 staining in ductal epithelial cells of normal breast tissue was also verified via demonstration in bright field images (Figure 5.4c) and by performing Hematoxylin and Eosin (H&E) staining (Figure 5.4d).

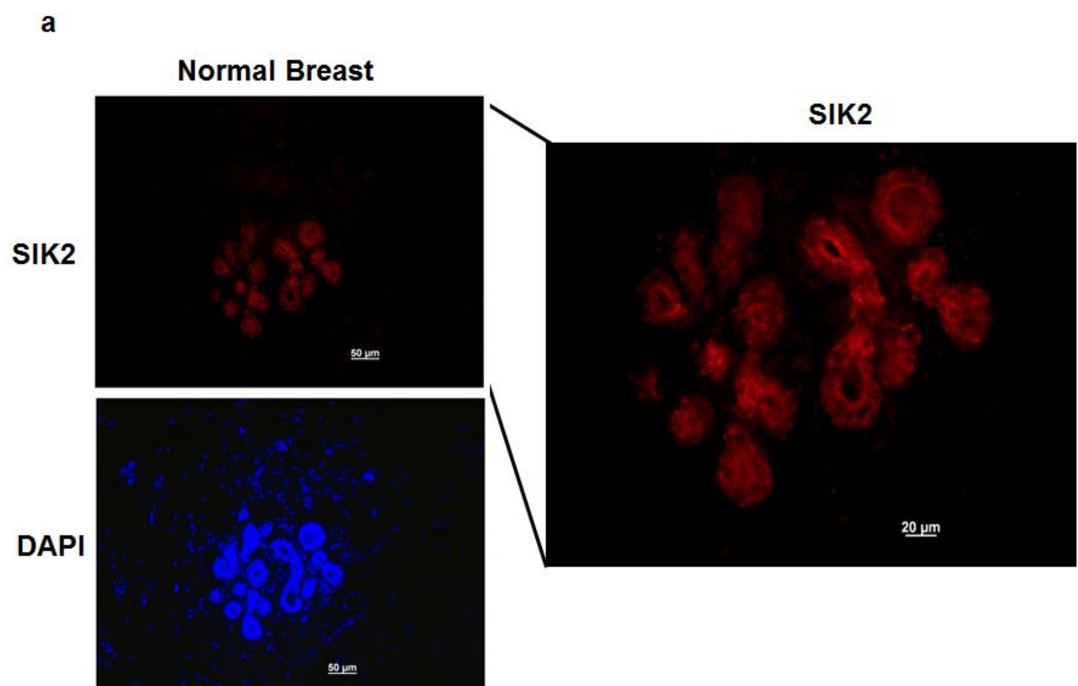

Figure 5.4. SIK2 characterization in normal breast tissue. Sections of normal breast tissue samples from the patients were stained with anti-SIK2 (red) antibody, the nuclei were visualized with DAPI. a. In the left panel SIK2 staining is shown in the normal tissue sample with 20X magnification. In the right panel 40X magnification of the same for SIK2 staining is represented. Sample. Scale bar: 50 µm for all panels

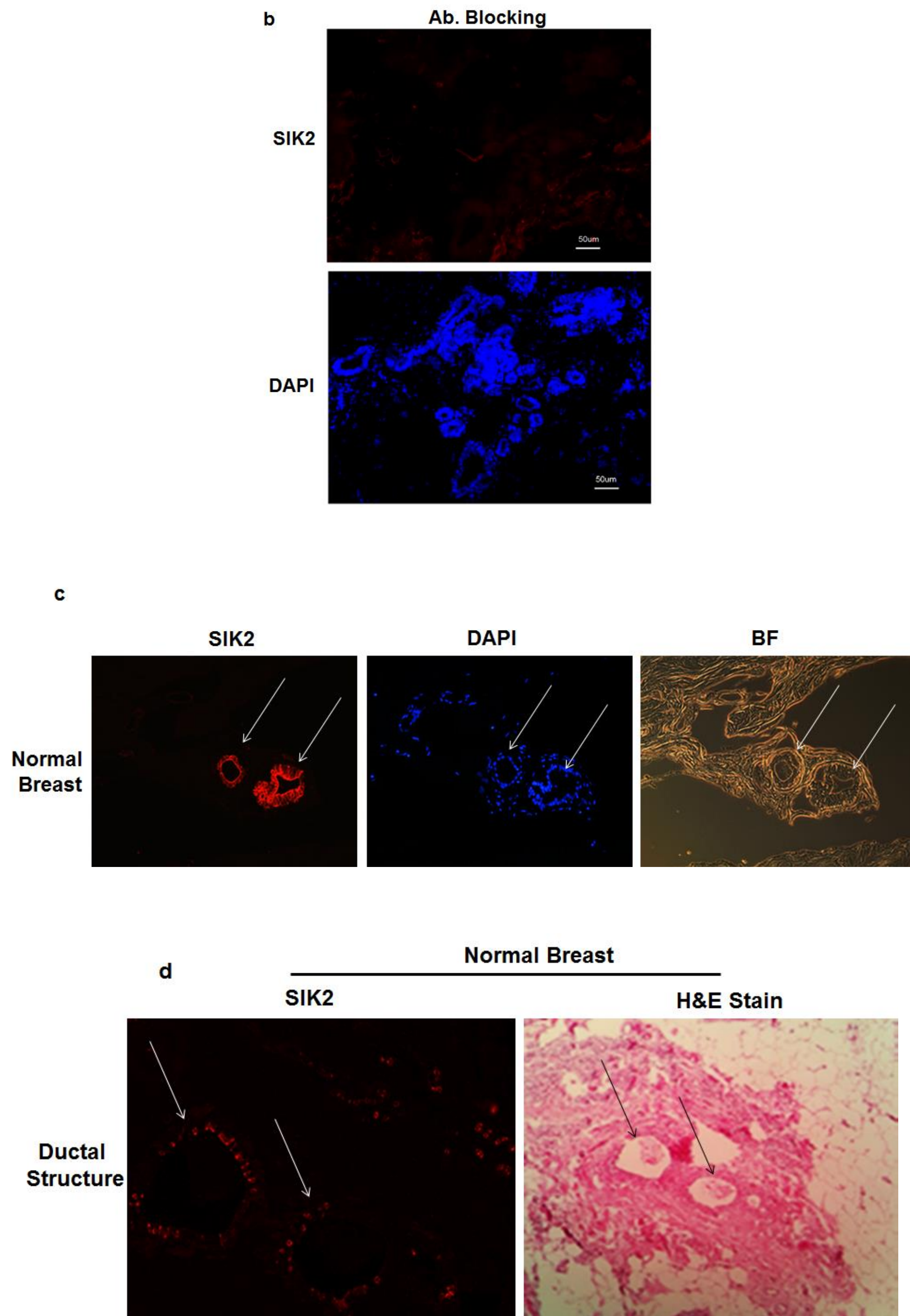

Figure 5.4. (cont.). SIK2 characterization in normal breast tissue b. The primary antibody was pre-incubated with affinity purified recombinant SIK2 protein. c. Bright field image of normal breast tissue section stained with anti-SIK2 (red) antibody was shown. All the nuclei were visualized with DAPI. Scale bar: 50 μm for all panels d.SIK2 staining in ductal epithelial cells of breast tissue was verified via H&E staining.

Subsequently, immunostaining studies were performed using primary tumor samples collected from patients and compared to normal breast tissue counterparts. The results showed that all tumor samples that were classified as TNBC had reduced SIK2 levels compared to non-transformed regions adjacent to the tumor areas (Figure 5.5).

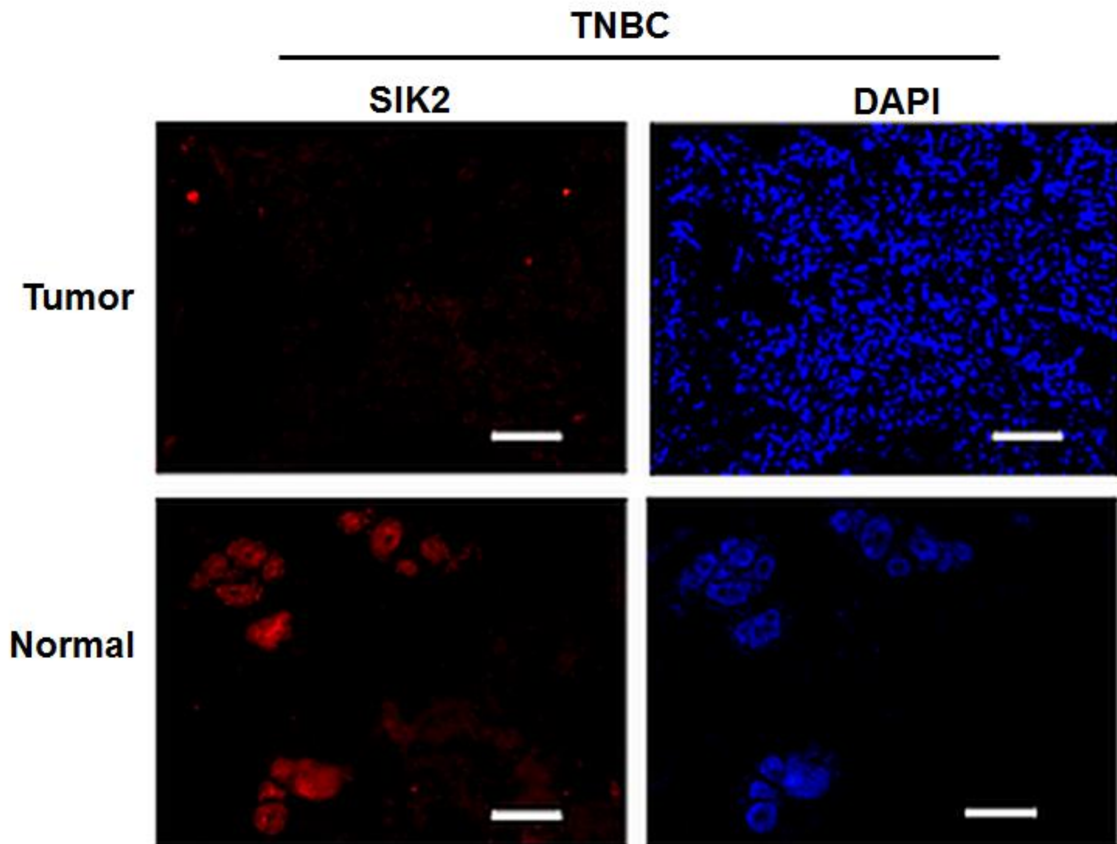

Figure 5.5. SIK2 immunostaining in TNBC tumors. The upper panels show a representative image of SIK2 staining (red) in TNBC cases. The cell nuclei were visualized with DAPI (blue). The lower panel represents the same staining in a normal tissue sample obtained from the same individual. Scale bar: 50 µm for all panels.

In 6/14 (43%) hormone positive cancer samples, reduced SIK2 expression has been observed (Figure 5.6a). In the remaining ER/PR positive breast tissue samples, the strength of the SIK2 signal was equal in normal and tumor areas (Figure 5.6b).

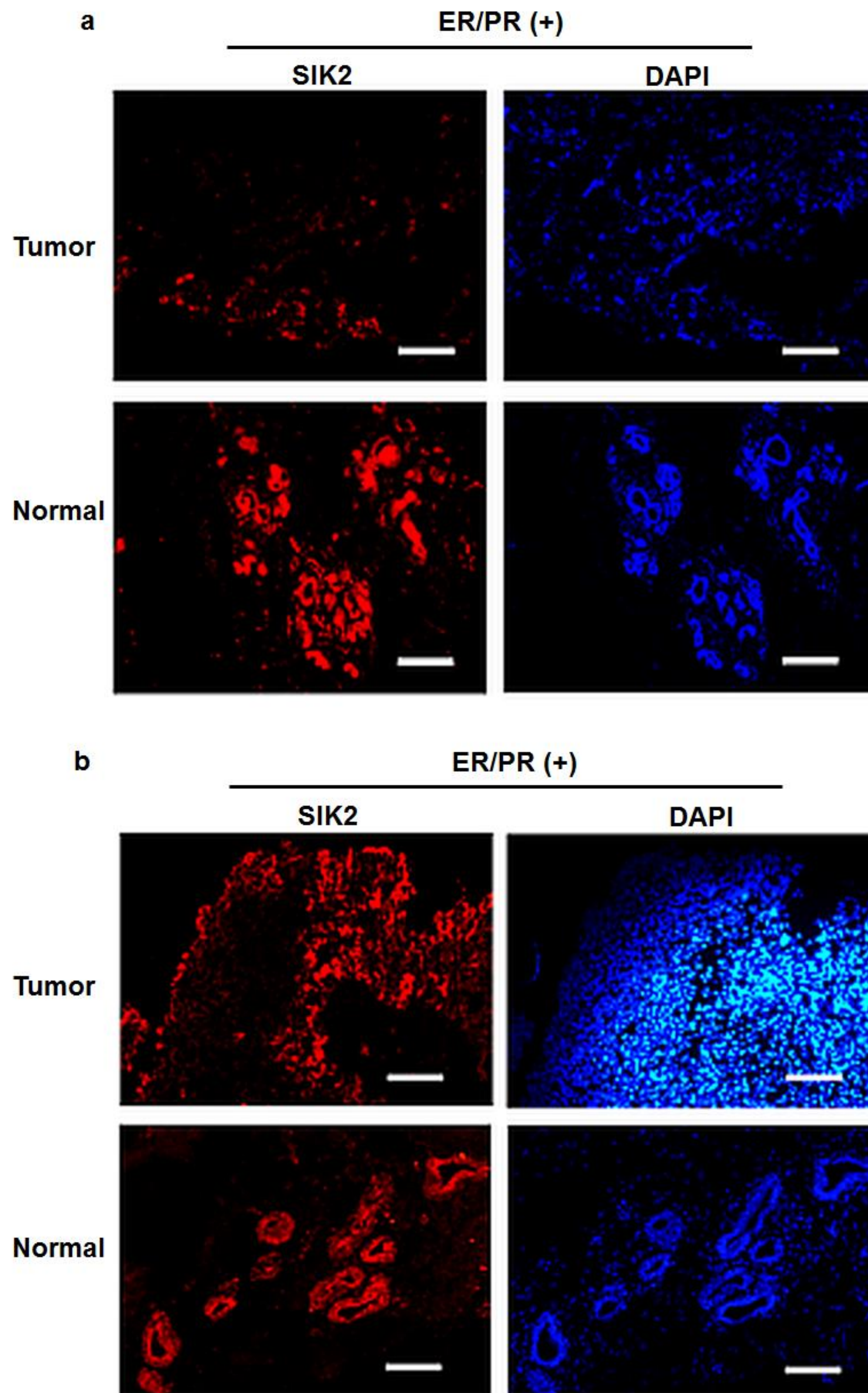

Figure 5.6. SIK2 Expression in ER/PR(+) Primary Breast Cancer Samples. Tissue sections obtained from ER/PR(+) breast cancer patients were stained with anti-SIK2 (red) and the nuclei were visualized with DAPI (blue). a. ER/PR (+) tumors with reduced SIK2 levels b. ER/PR (+) tumors with equal SIK2 strength in transformed regions compared to normal counterparts. Scale bar: 50 µm for all panels.

5.2.1. SIK2 Expression Levels Are Inversely Correlated with Proliferation

To question whether SIK2 expression has any correlation with proliferation rate in tumor samples, we performed co-immunostaining experiments using SIK2/Ki67 antibodies in the ER/PR(+) and TNBC tumor samples. As indicated above, all of the TNBC samples in our cohort showed highly reduced SIK2 levels. This group of tumors are known to have a high proliferation rate. In agreement with this, we observed a strong and widespread Ki67 signal (Figure 5.7, lower row). In ER/PR positive tumor samples with no evident loss of SIK2 expression, the Ki67 signal was undetectable (Figure 5.7, upper row). However, in ER/PR- positive tumor samples where we observed significantly reduced SIK2 expression, highly elevated Ki67 signal was evident (Figure 5.7, middle row). Taken together, these data indicated that SIK2 expression is frequently downregulated in breast tumors and there exists an inverse correlation between SIK2 expression and the mitotic activity of the tumor cells.

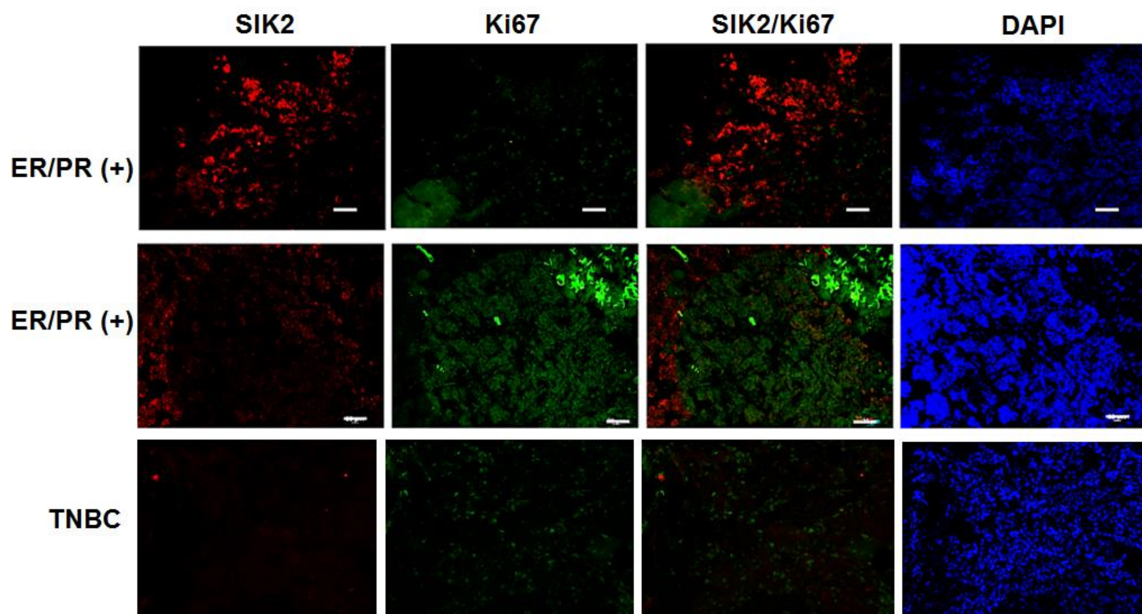

Figure 5.7. Co-immunostaining of breast tumors with SIK2 and Ki67 antibodies. Tissue sections were stained with anti-SIK2 (red), anti-Ki67 (green) and the nuclei were visualized with DAPI (blue). Merged images of SIK2/Ki67 staining are also shown. Scale bar: 50 µm for all panels.

5.3. Studies with Breast Cell Lines

5.3.1. SIK2 Is Differentially Expressed in Transformed and Untransformed Cell Lines.

To further investigate the possible involvement of SIK2 in the development of breast tumorigenesis, we performed *in vitro* studies using a set of breast cell lines. We first investigated the expression of SIK2 protein level in transformed lines compared to non-tumorigenic MCF12A cell line. Western Blot analysis indicated that SIK2 was significantly lower in breast cancer lines, T47D (52%), MDA-MB-361 (64%), MDA-MB-157 (52%), MCF-7 (53%), MDA-MB-231 (59%), and SKBR-3 (23%) compared to MCF12A non-transformed mammary epithelial cell line (Figure 5.8).

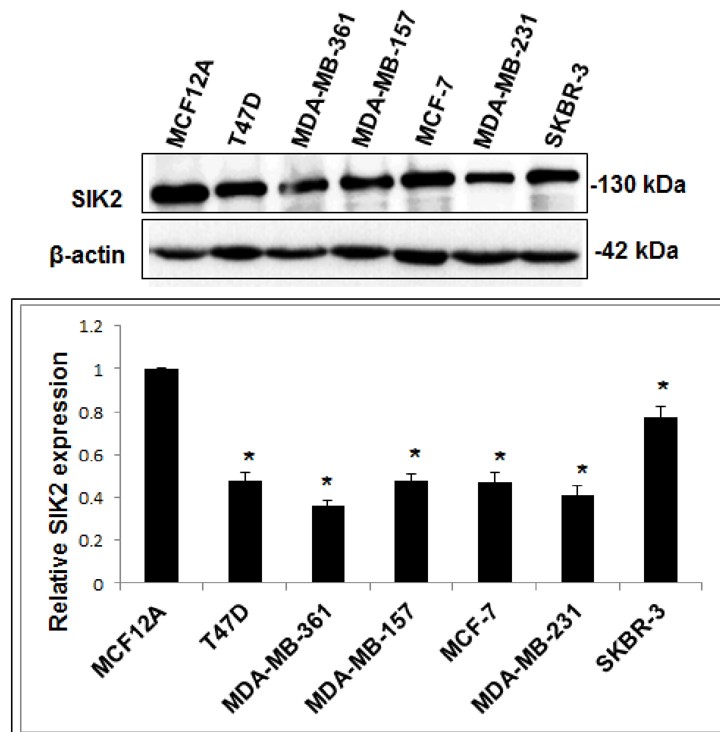

Figure 5.8. The expression profile of SIK2 in breast cell lines. Western Blot analysis was performed using the cell lysates prepared from breast cell lines with anti-SIK2 antibody; β-actin was used as loading control. The histograms represent the mean value of five independent biological samples after normalization of band intensities of SIK2 to β-actin in the same samples. The data are expressed as mean ± SD, * $P< 0.001$.

These results indicate that the reduced SIK2 levels evident in primary tumors is also reflected in cell lines derived from breast tumors.

5.3.2. Generation of SIK2 Modulated Breast Cell Lines

To reveal the possible involvement of SIK2 in the progression of breast tumorigenesis, we aimed to modulate the expression level of the gene in the breast cell lines and test the effect of these modulations in their tumorigenic characteristics. For these studies, we selected the non-tumorigenic epithelial cell line MCF12A and the highly tumorigenic MDA-MB-231 TNBC cancer cell line. MDA-MB-231 shows nearly 60% reduced SIK2 expression compared to MCF12A cells. This line was reported to have elevated FGFR1/2 expression and showed increased migration upon FGF2 treatment (Nurcombe *et al.*, 2000). Since our previous results implicated SIK2 in FGFR signaling, we reasoned that MDA-MB-231 will provide a better context to reveal phenotypic changes upon SIK2 perturbations.

We upregulated SIK2 in MDA-MB-231 cells by transfecting with pEGFP-SIK2 or with pEGFP-KI-SIK2. As shown in Figure 5.9, independent colony mixes of pEGFP-SIK2 transfected MDA-MB-231 cells have significantly increased level of SIK2 expression compared to parental cells or the ones carrying empty vector.

Reciprocally, in the MCF12A non-tumorigenic breast cell line SIK2 silencing was accomplished by infection with lentiviral vectors carrying SIK2 specific shRNAs. The scrambled shRNA transduced cells constituted the controls. Western blot analysis indicated that SIK2 expression was reduced to 75% of the parental or the scrambled shRNA infected MCF12A cells (Figure 5.10).

5.3.3. Modulation of SIK2 Expression Effects on Proliferation and ERK Activation

The effect of changes in SIK2 levels on the proliferation profile of MDA-MB-231 or MCF12A cells was investigated by a BrdU incorporation assay. As proliferation is often associated with the activation of the ERK pathway, we also evaluated the changes in pERK levels by Western blotting. The results demonstrated that the ratios of proliferating cells

were comparable in parental, mock, and KI-SIK2 transfected MDA-MB-231 cells (Figure5.11). In cells with upregulated of wildtype SIK2 expression, the fraction of proliferating cells reached 30% of the controls.

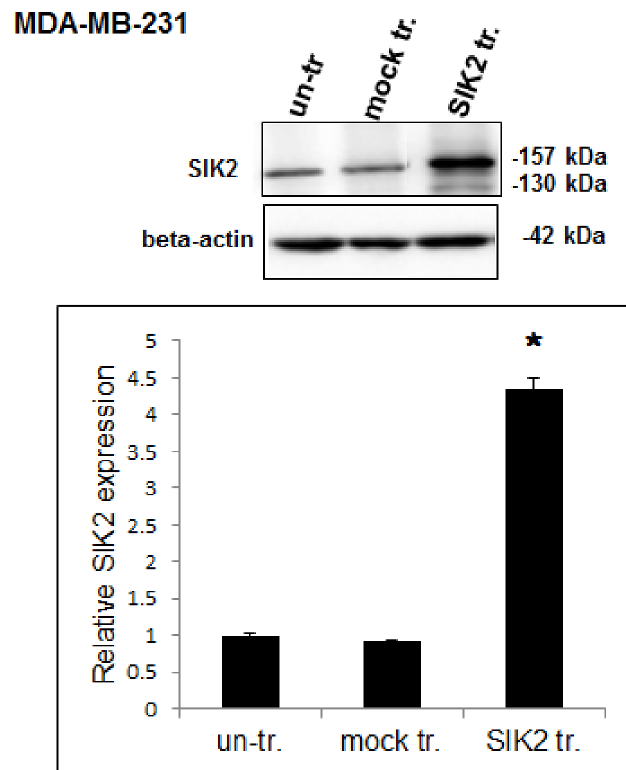

Figure 5.9. Stable up-regulation of SIK2 gene expression in MDA-MB-231 cells. Lysates from MDA-MB-231 cells transfected either with full-length SIK2 or the empty vector were subjected to Immunoblotting using antibodies against SIK2 and beta-actin. The histograms represent the mean value of five independent biological samples after normalization of band intensities of SIK2 to that of b-actin. **P* is <0.001 compared to controls.

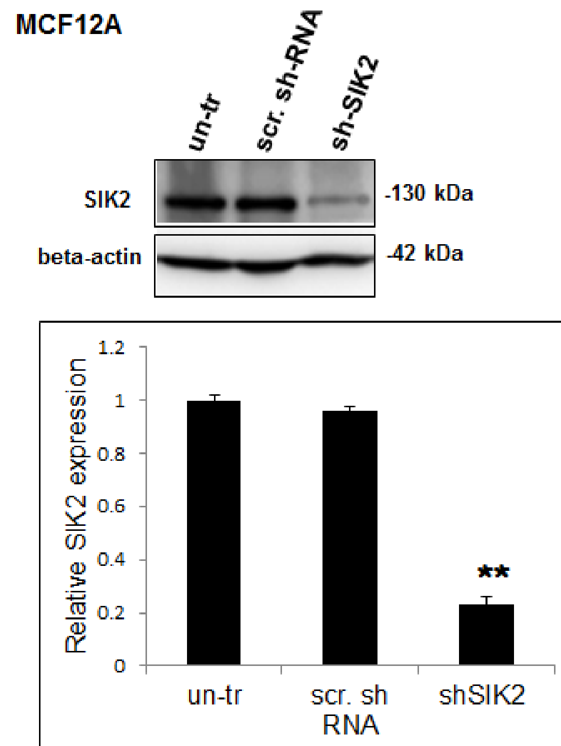

Figure 5.10. SIK2 silencing in the MCF12A cell line. Cells were infected with either sh-SIK2 or scrambled sh-RNA. SIK2 down-regulation was verified by Western Blotting using anti-SIK2 antibody; beta-actin was used as loading control. The data are expressed as mean ± SD (n=3), ** $P < 0.05$ compared to the control samples.

In parallel to the reduced proliferation, MDA-MB-231 cells with enhanced SIK2 showed significantly lower pERK levels than the parental line. The decrease appears to be correlated with SIK2 levels, as seen in Figure 5.12. Two independent transfections where clone 2 that has an approximately 2 fold higher SIK2 expression compared to clone 1, exhibited about 2-fold decrease in p-ERK levels.

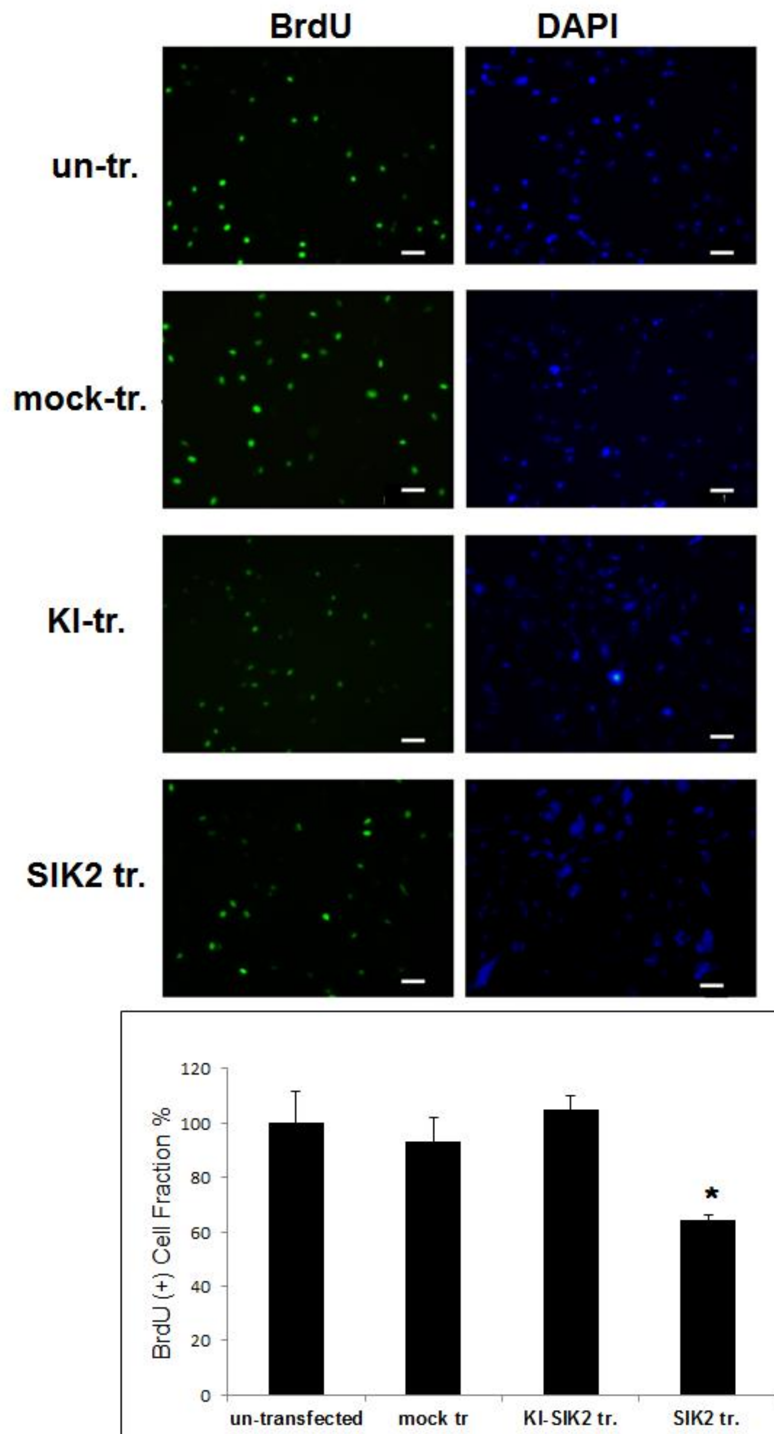

Figure 5.11. Effect of SIK2 up-regulation on FGF2-induced proliferation. (A). In MDA-MB-231 cells overexpressing the SIK2, KI-SIK2 or carrying the vector alone, proliferative cell fraction was assessed by BrdU incorporation assay. (b) Mean values of proliferating cells in triplicates were graphically represented. At least 150 cells were counted for each triplicate sample. Scale bar=50µm. * $P<0.001$ compared to control samples.

Reciprocally, silencing of SIK2 in MCF12A cells resulted in a 2.6 fold increase in the fraction of sh-SIK2- infected proliferative cells compared to the scrambled shRNA infected and parental MCF12A cells (Figure 5.13). While cells carrying scrambled shRNA showed similar ERK activation to uninfected cells, depletion of SIK2 expression remarkably enhanced the phosphorylated ERK level (Figure 5.14).

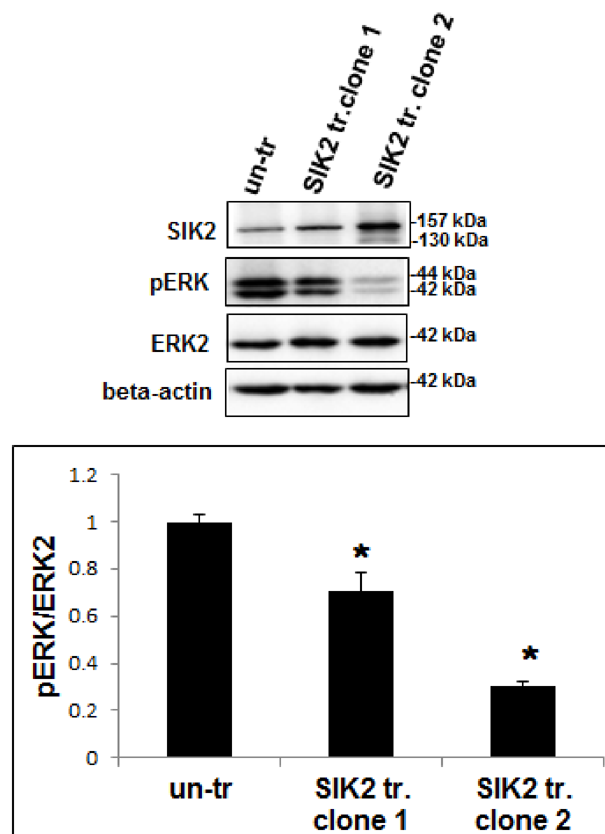

Figure 5.12. Effect of SIK2 up-regulation on ERK activation in MDA-MB-231 cells. (A) Cells were transfected with SIK2 and the pERK profile was evaluated by Western blotting in comparison to basal ERK level in the same lysates (B) Graphic representation of pERK band intensities normalized to that of ERK levels in the same samples. The data are expressed as mean ± SE (n=3) * $P< 0.001$ compared to un-transfected sample.

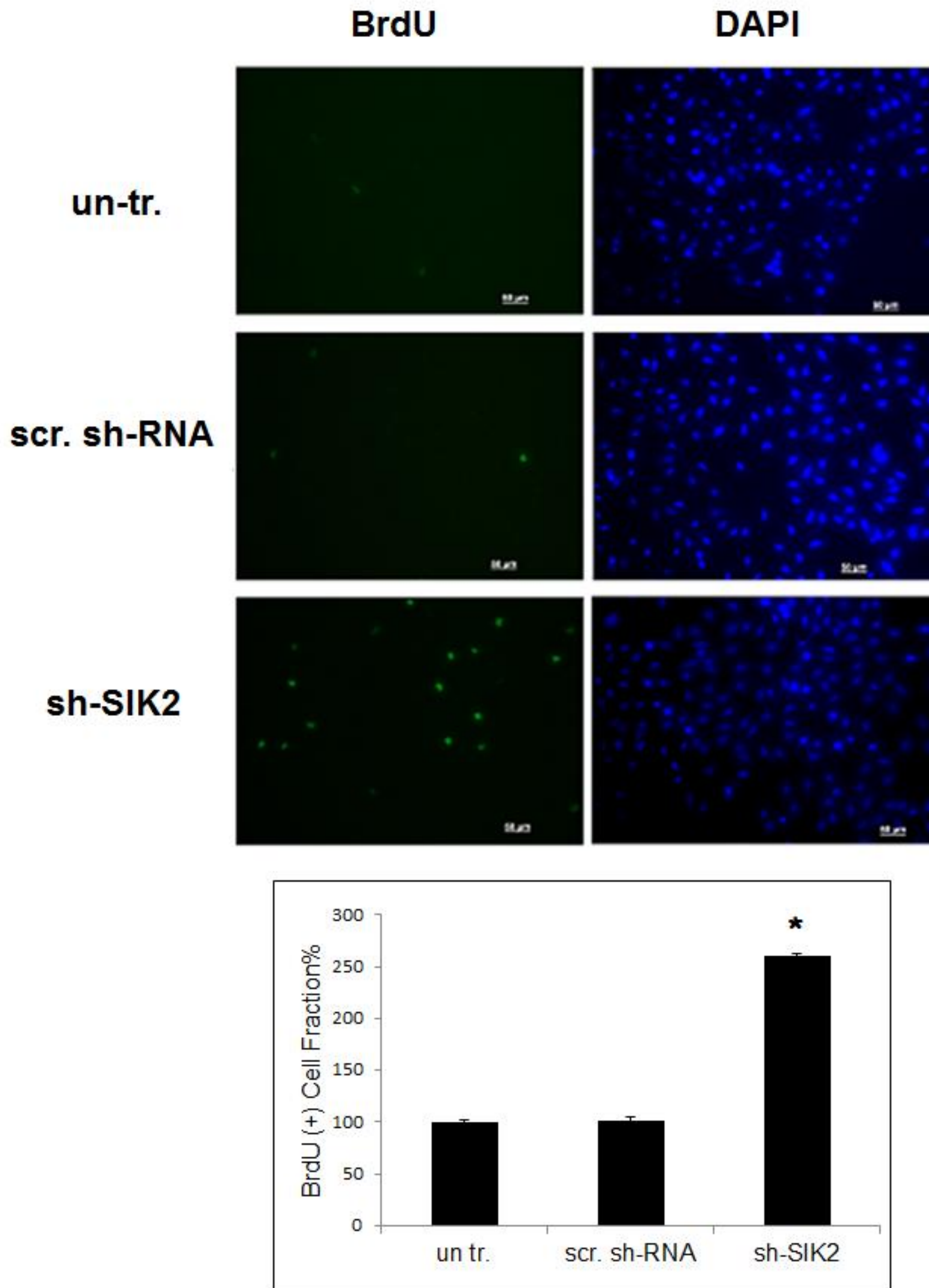

Figure 5.13. Effect of SIK2 silencing on FGF2-induced proliferation. (A). In MCF12A cells infected with the sh-SIK2, scrambled sh-RNA particles or in parental cells, proliferative cell fraction was assessed by BrdU incorporation assay. (b) Mean values of proliferating cells in triplicates were graphically represented. At least 150 cells we count for each triplicate sample. Scale bar=50µm. *p<0.001 compared to controls.

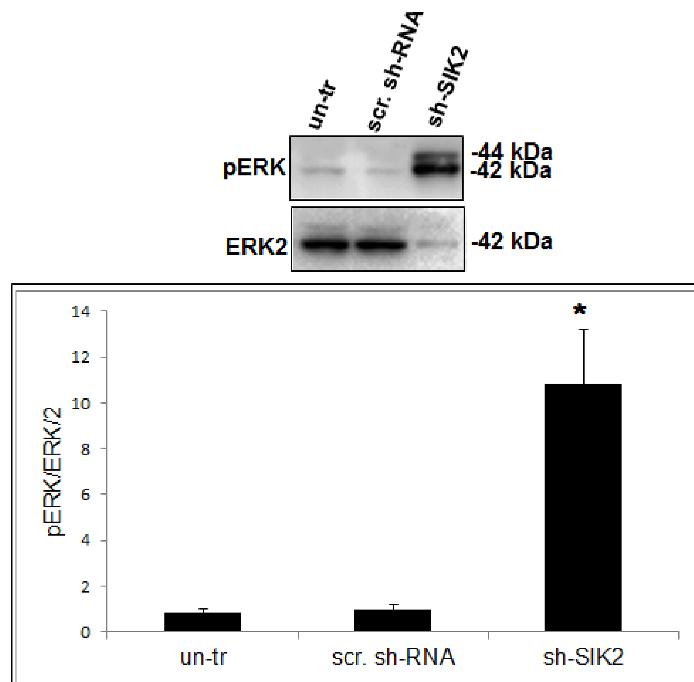

Figure 5.14. Effect of SIK2 up-regulation on ERK activation in MCF12A cells. (A) Cells were transfected with SIK2 and the pERK profile was evaluated by Western blotting in comparison to basal ERK level in the same lysates (B) Graphic representation of pERK band intensities normalized to that of ERK levels in the same samples. The data are expressed as mean ± SE (n=3) * $P< 0.001$ compared to control samples.

5.3.4. Modulation of SIK2 Expression Effects on Survival and Akt Activation

To further probe into SIK2 involvement in breast tumorigenesis as a candidate tumor suppressor, potential changes in the fraction of cells undergoing apoptosis was assessed using a TUNEL assay and Akt activation was evaluated by Western blotting.

Transfection of MDA-MB-231 cells either with empty vector or KI-SIK2 did not result in a statistically significant difference in the number of TUNEL positive cells. With enhanced SIK2 expression, the fraction of apoptotic cells was doubled in comparison to the parental line (Figure 5.15).

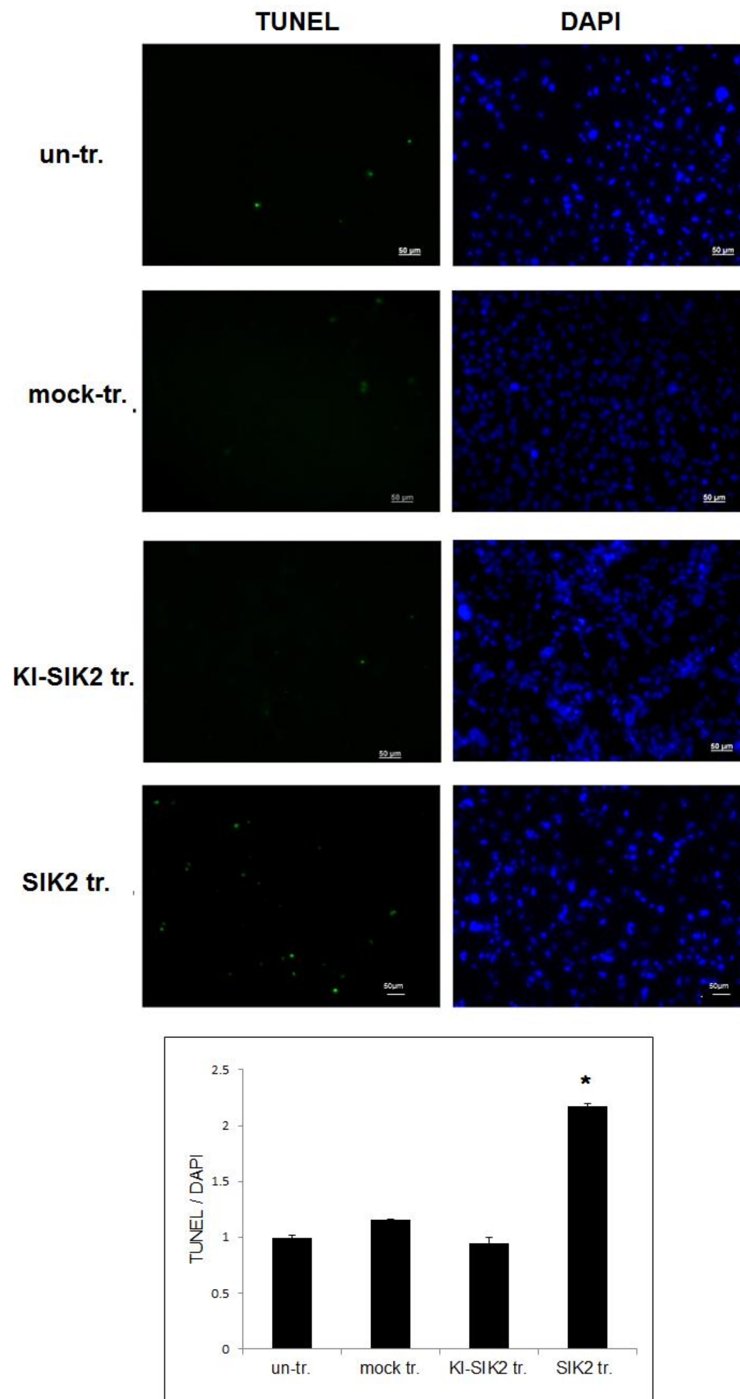

Figure 5.15. Effect of SIK2 up-regulation on apoptosis of MDA-MB-231 cells. Tunel assay was performed in MDA-MB-231 cells transfected with wildtype SIK2, KI-SIK2 or the empty vector. Nuclei were stained with DAPI. In each case at least 150 cells were counted and the mean values of TUNEL positive cells in three independent experiments were graphically represented (± SD). *$P < 0.001$. Scale bar=50 µm.

In agreement with the increase in apoptotic cell numbers, expression of exogenous SIK2 resulted in a decrease of active Akt by 67% as compared to un-transfected MDA-MB-231 cells (Figure 5.16).

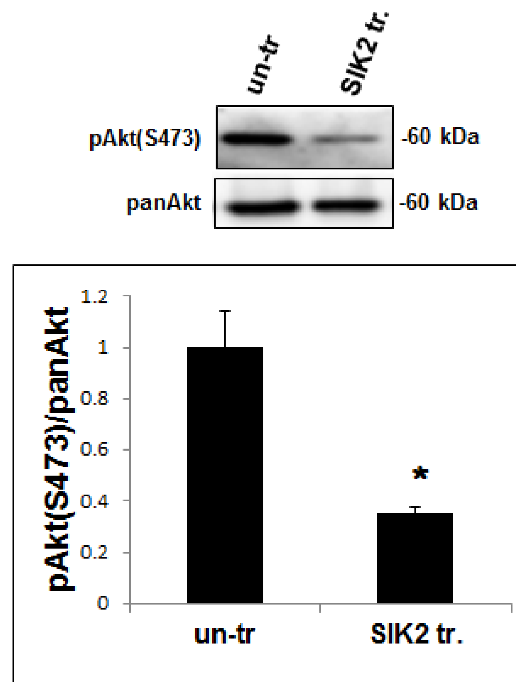

Figure 5.16. Effect of SIK2 up-regulation on Akt activation of MDA-MB-231 cells. Phosphorylation of Akt was evaluated by Western blotting. Membranes were probed with anti-pAkt and anti-panAkt antibodies; mean values of five independent experiments after p-Akt band intensities normalized to that of pan-Akt in the same samples were represented in the histograms (± SD). *$P < 0.001$.

Parallel experiments indicated that depletion of SIK2 in MCF12A cells led to a 64% reduction in the apoptotic cell population (Figure 5.17). Akt activation was enhanced 6.6-fold in these cells compared to coresponding controls (Figure 5.18).

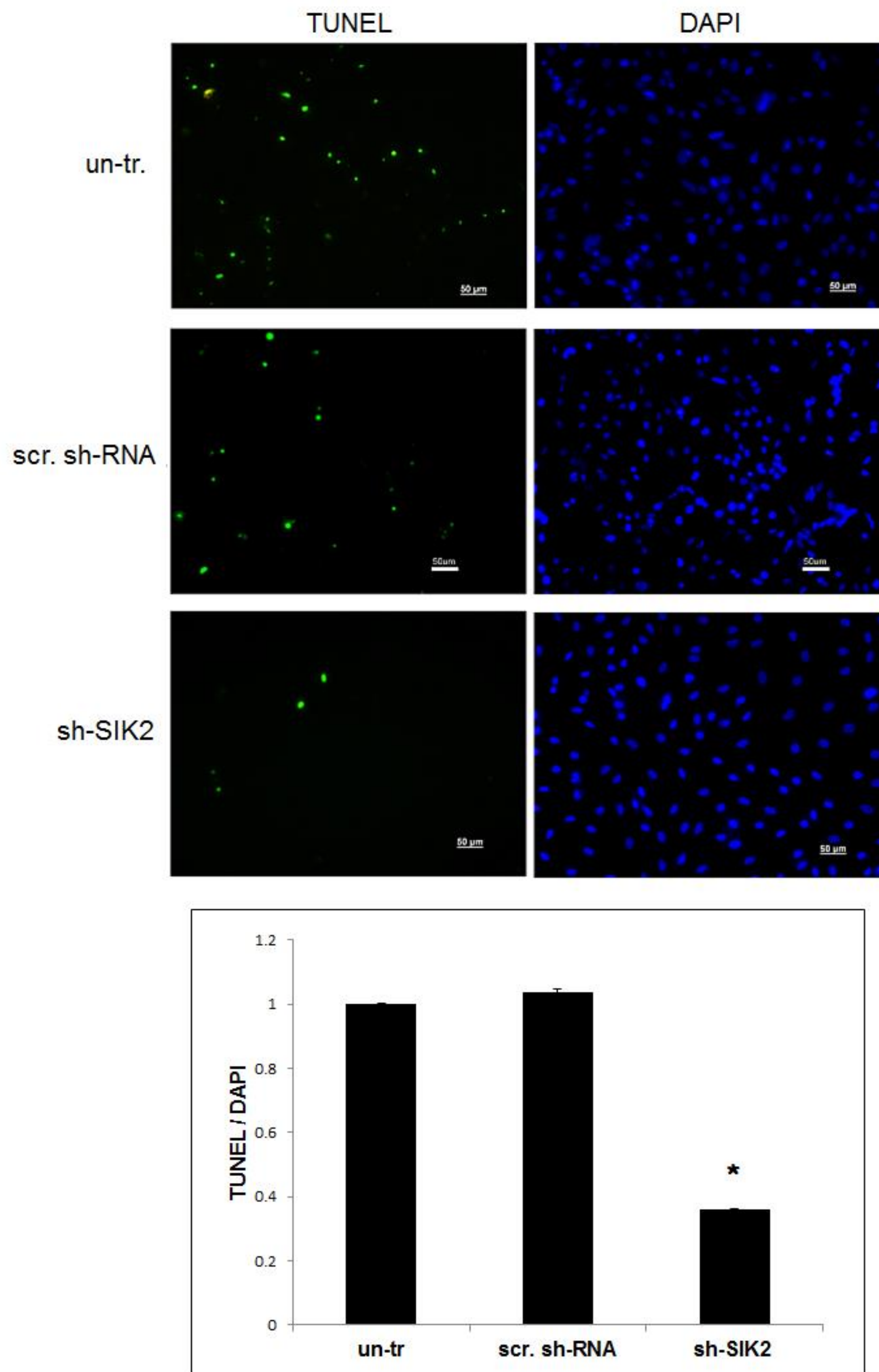

Figure 5.17. Effect of SIK2 down-regulation on apoptotic responses of MCF12A cells. Tunel assay was performed in parental cells, the cells infected with scrambled sh RNA and SIK2 specific shRNAs. Nuclei were stained with DAPI. In each sample at least 150 cells were counted and the mean values of TUNEL positive cells in three independent experiments were graphically represented (± SD). $*P < 0.001$. Scale bar=50 µm.

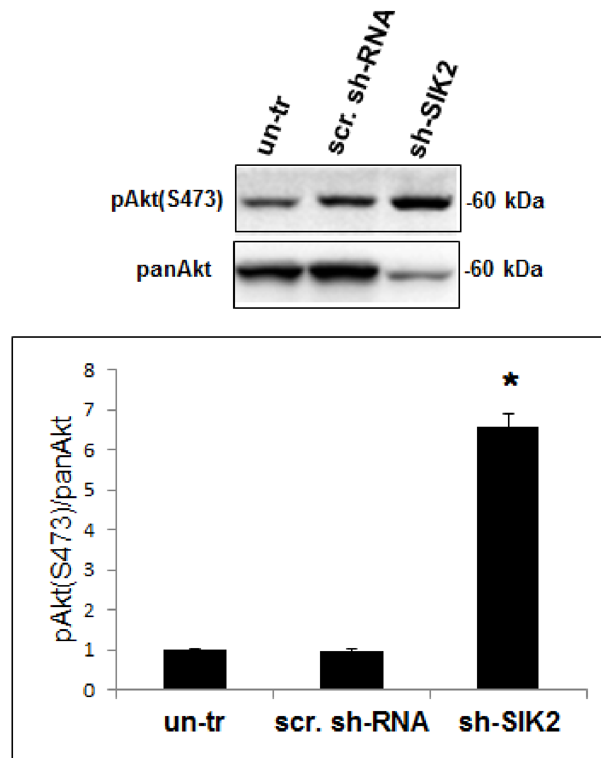

Figure 5.18. Effect of SIK2 down-regulation on Akt activation of MCF12A cells. In the cells, Akt phosphorylation profiles were analyzed for by Western blotting. Membranes were probed with anti-pAkt(S473) and anti-panAkt antibodies; Histograms represent the mean values of three independent experiments after p-Akt band intensities normalized to that of pan-Akt in the same samples (± SD). * $P < 0.001$ compared to controls.

Taken together, the data strongly suggest that SIK2 has a function in the regulation of proliferation and survival responses of breast cells by affecting the activities of ERK and Akt, which are important downstream modulators of RAS/MAPK and PI3K/Akt pathways, respectively. Maintenance of proliferative signaling and evasion from cell death are the main mechanisms to promote tumorigenic transformations and thus our data suggest that SIK2 functions as a potential tumor suppressor in breast tumorigenesis.

5.3.5. SIK2 Silencing Increases Motility and Invasion Characteristics of Non-transformed MCF12A Cells

Motility and invasiveness are accepted as essential signatures of tumorigenic transformation (Hanahan and Weinberg, 2015). In the following steps, we investigated whether downregulation of SIK2 expression has any effect on the migration and invasion abilities of non-transformed breast cells using wound healing and trans-well assays.

We observed that 8 hours after the initial wound formation in cells in which SIK2 was downregulated the number of cells migrating into the wound area were increased. The mean increase was 38% and 56% over the scrambled-sh RNA infected cells and wild type controls, respectively. At the end of 24 hours, the number of migrating cells elevated by 59% and 53% in comparison to scrambled-sh RNA infected cells and parental cells, respectively. Although scrambled sh-RNAs infected control cells seem to be more motile compared to wild type controls at the very beginning of the migration event, at the end of 24 hours, there was no significant difference between these cell populations (Figure 5.19). These data indicate that wound closure is more readily achieved by SIK2 silenced cells.

A trans-well invasion assay was performed to analyze the changes in invasive characteristics of the cells upon SIK2 silencing. The results demonstrated that the percentage of invasive cells significantly increased (~3.4 fold) upon down-regulation of SIK2 at the end of 48 hours. While MCF12A and control sh-RNA infected cells stay in the starvation medium at the top chamber of the trans-wells, the SIK2 silenced cell population migrated to the bottom of the filter to reach the fresh medium (Figure 5.20). These results suggest that reduced SIK2 gene expression provides higher motility and invasion potential.

For further evidence that loss of SIK2 expression affects the motility/invasiveness of breast cells, we explored the modulation in expression of E-cadherin and MMP-2, two proteins considered critical in these processes, in SIK2 downregulated MCF12A cells. The significant reduction in E-cadherin expression (69% as compared to controls) suggests that modulation of SIK2 may contribute to EMT (Figure 5.21., Left Panel). The increased matrix metalloproteinase MMP-2 level (41.7% higher than the parental line) is in agreement with the observed increase in invasiveness (Figure 5.21., Right Panel).

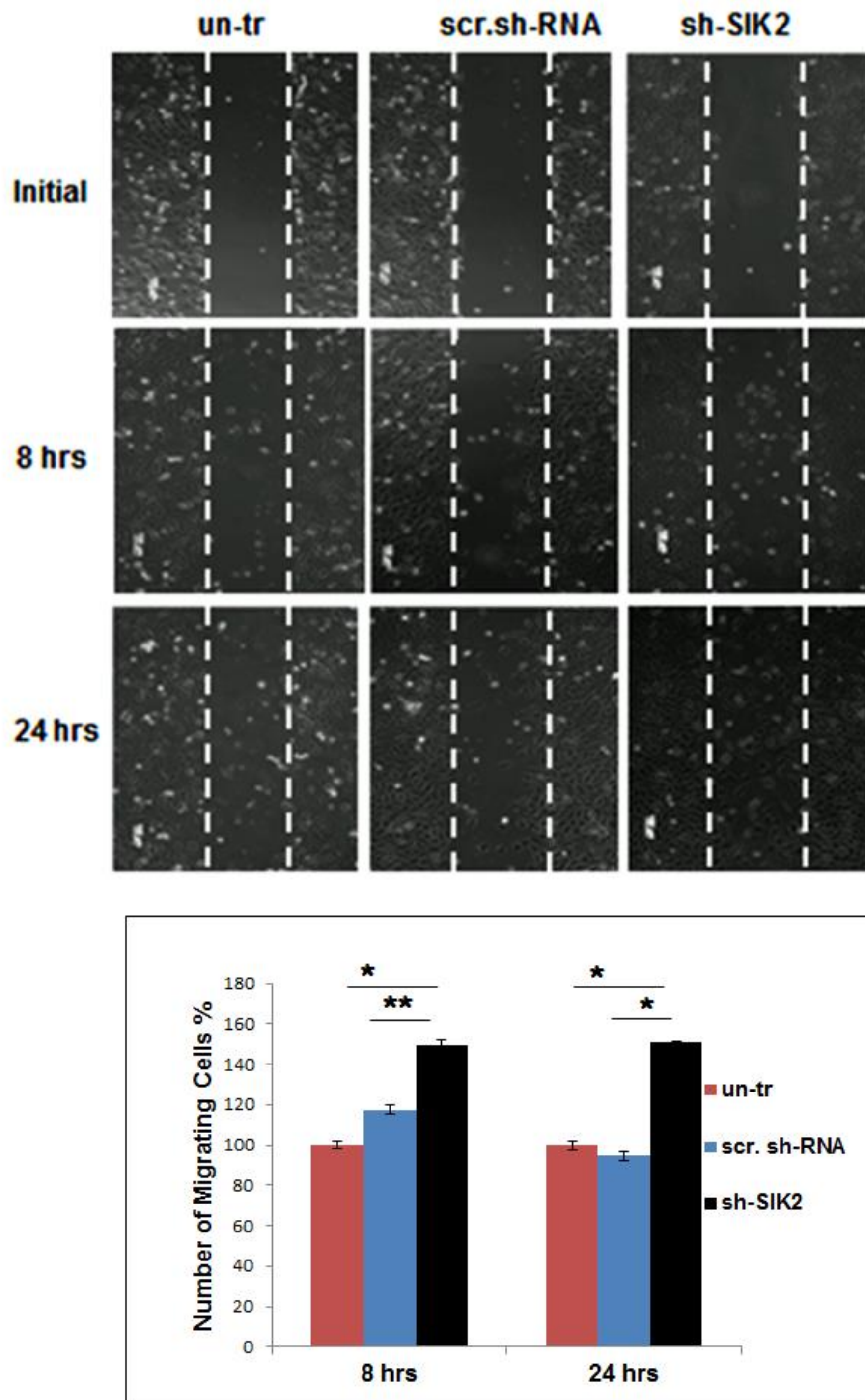

Figure 5.19. Effect of SIK2 silencing on migration capacity of MCF12A cells. Motility of SIK2 silenced MCF12A cells were evaluated in comparison to the uninfected and scrambled sh-RNA harboring cells by wound healing assay. The mean values of number of cells closing the wounded area at the indicated time points were graphically represented (± SD). Three independent experiments were carried out. * $P < 0.001$, ** $P < 0.05$.

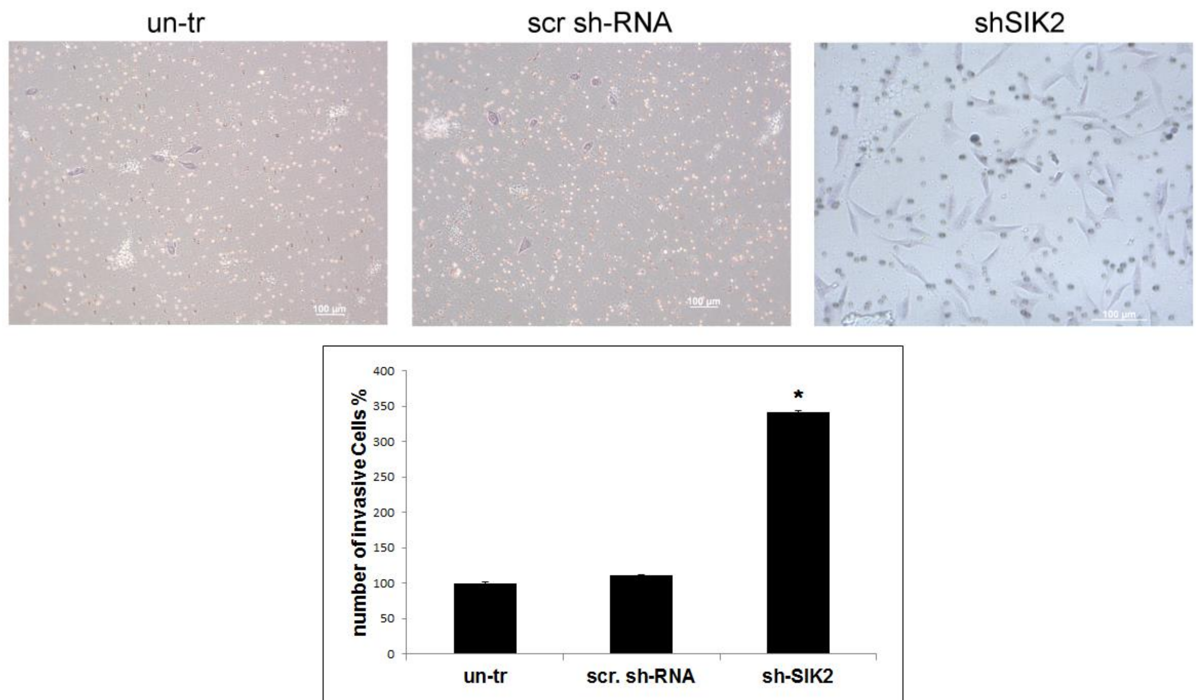

Figure 5.20. Effect of SIK2 silencing on invasion potential of MCF12A cells. Potential changes in invasive characteristics of the MCF12A cells were measured by Boyden Chamber assay. Filters were collected at 48 hours after the initial seeding, cells were counted after Giemsa staining. Mean values of invasive cells in three independent experiments were graphically represented (± SD). * $P< 0.001$. Scale bar:100 µm

The results indicate that the increase in motility and invasion abilities of non-transformed mammary epithelial cells upon SIK2 down-regulation stems from the alterations in critical proteins in the migration/invasion processes and are not consequences of increase in proliferation or survival of these cells. These data indicate that SIK2 may play a negative role in the development of breast tumorigenesis through multiple mechanisms.

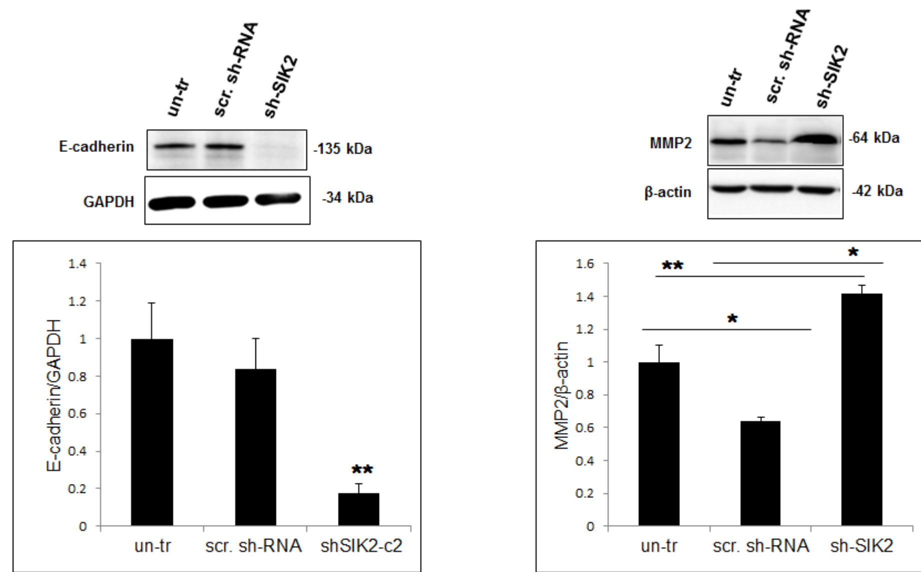

Figure 5.21. Effect of SIK2 down-regulation on E-cadherin and MMP-2 profiles. E-cadherin and MMP-2 levels were studied by Western blot analysis. Anti-GAPDH or anti-β-actin were used as loading controls. Three independent experiments were done in each case. Mean values of relative E-cadherin and MMP-2 band intensities were normalized to that of the loading controls in the same samples (± SD). * $P < 0.001$ and ** $P < 0.05$.

5.4. Xenografting Studies

5.4.1. SIK2 Suppressed Tumor Growth *In Vivo*

To examine whether SIK2 affects tumor growth *in vivo*, MDA-MB-231 cells with elevated SIK2 levels and the control lines were injected subcutaneously into the flanks of SCID mice and tumor growth was assessed 5 weeks post injection. At the end of this time frame a parental MDA-MB-231 line and mock transfected cells formed significant tumors in weight and volume. On the other hand, MDA-MB-231 cells with enhanced SIK2 expression consistently formed more than 2 fold smaller tumors in weight and size compared to the control cells (Figure 5.22).

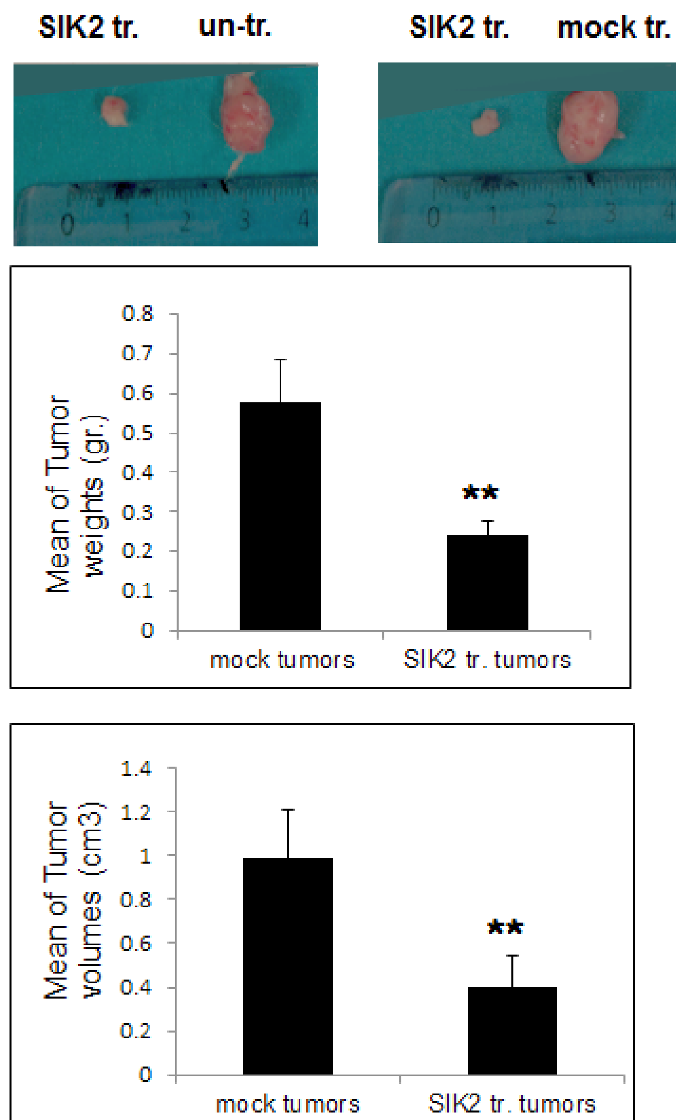

Figure 5.22. Effect of SIK2 upregulation on *in-vivo* tumor growth. $1x10^6$ MBA-MD-231 cells were subcutaneously injected to the flanks of SCID mice (n=6). Tumors were excised 5 weeks post injection. Representative tumor images were obtained as shown in the top panel. Histograms represent mean values of the tumor weights and volumes of six independent experiments (± SD) ** *P*<0.05.

We have also generated tumor xenografts with KI-SIK2-transfected MDAMB231 cells to see whether SIK2 activity has an impact on tumor growth. The mean size of the tumors originating from cells harboring kinase inactive SIK2 were significantly larger than

the ones derived from cells harboring wild-type protein (Figure 5.23) pointing to the importance of its enzymatic activity in inhibition of tumor growth *in vivo*.

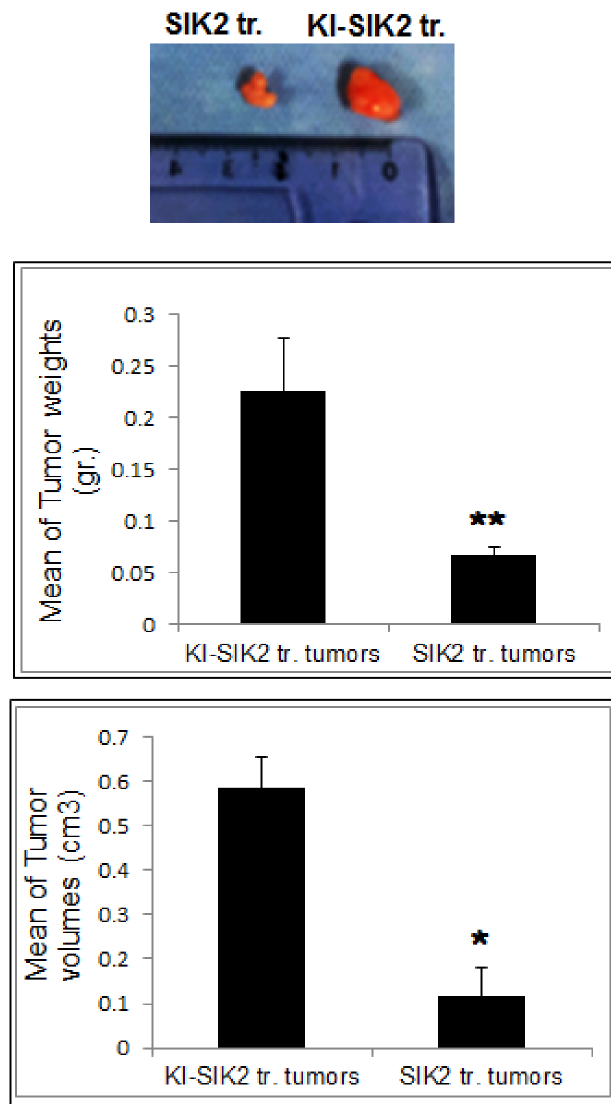

Figure 5.23. Effect of KI-SIK2 upregulation on *in-vivo* tumor growth. $1x10^6$ MBA-MD-231 cells were subcutaneously injected to the flanks of SCID mice (n=3). Tumors were excised 5 weeks post injection. Representative tumor images were obtained as shown in the top panel. Histograms represent mean values of the tumor weights and volumes of three independent experiments (± SD) * $P< 0.001$ and ** $P<0.05$.

5.4.2. Characterization of the Tumors Generated in SCID Mice

In this part of the study we compared the tumors derived from MDA-MB-231 cells with upregulated SIK2 and the ones derived from mock transfected cells in terms of SIK2 expression, proliferation, and cell death profiles, as well as Erk and Akt activation status.

Western blot analysis verified that the elevated levels of SIK2 were maintained in all the tumors derived from SIK2 upregulated MDA-MB-231 cells compared to tumors of mock transfected cells (Figure 5.24).

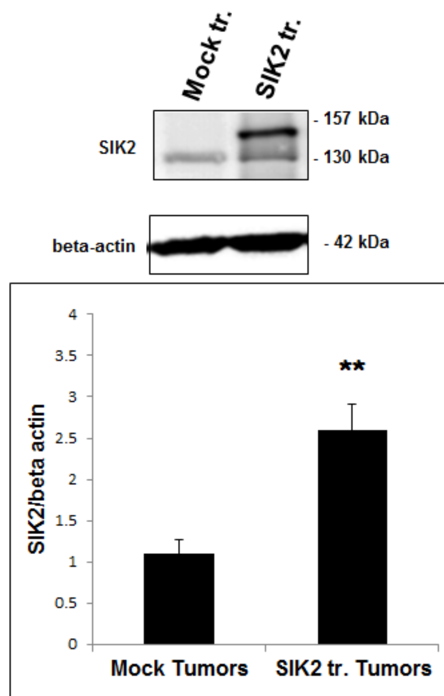

Figure 5.24. Confirmation of SIK2 expression in tumors excised from SCID xenografts. Lysates from excised tumors originating from MDA-MD-231 cells transfected with SIK2 or mock transfected ones were subjected to Western blotting. Band intensities of SIK2 were normalized to that of β-actin, mean values of three independent experiments are presented in histograms (± SD). ** $P< 0.05$.

Activation of ERK (Figure 5.25) and the fraction of Ki67 positive cells (Figure 5.26) were significantly downregulated in the tumors derived from cells with enhanced SIK2 expression. In these cells active Akt levels were lower (Figure 5.27) and active caspase-3-positive cell numbes were significantly higher (Figure 5.28) compared to the control cells.

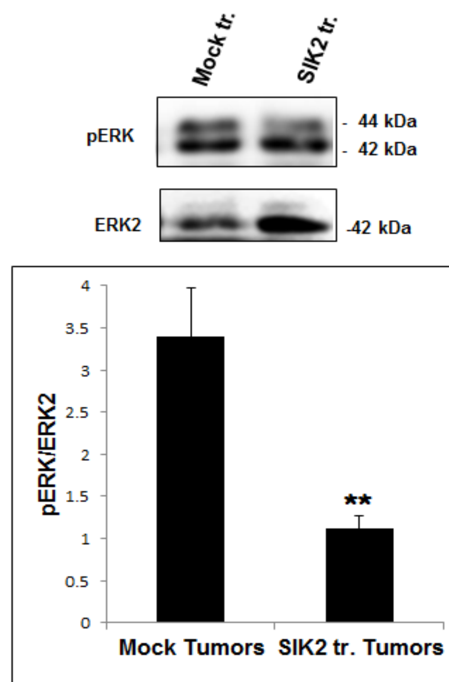

Figure 5.25. ERK phosphorylation levels in the tumors excised from SCID mice. Western blot analysis was performed using anti-p-ERK and anti-ERK2 antibodies. The graph represents the p-ERK band intensities normalized to that of ERK levels in the same samples. n=3; error bars represent mean ± SD; * $P< 0.05$ compared to control samples.

All the *in vivo* data agree with the findings of the primary tumor and cell line studies, and support the notion that SIK2 contributes to the suppression of tumor development through regulation of proliferation as well as survival processes via modulating the ERK and Akt activation status of breast cells. The results from the tumors

derived from MDA-MB-231 cells expressing the kinase inactive form of SIK2 underlines the importance of catalytic activity of the protein in tumorigenesis.

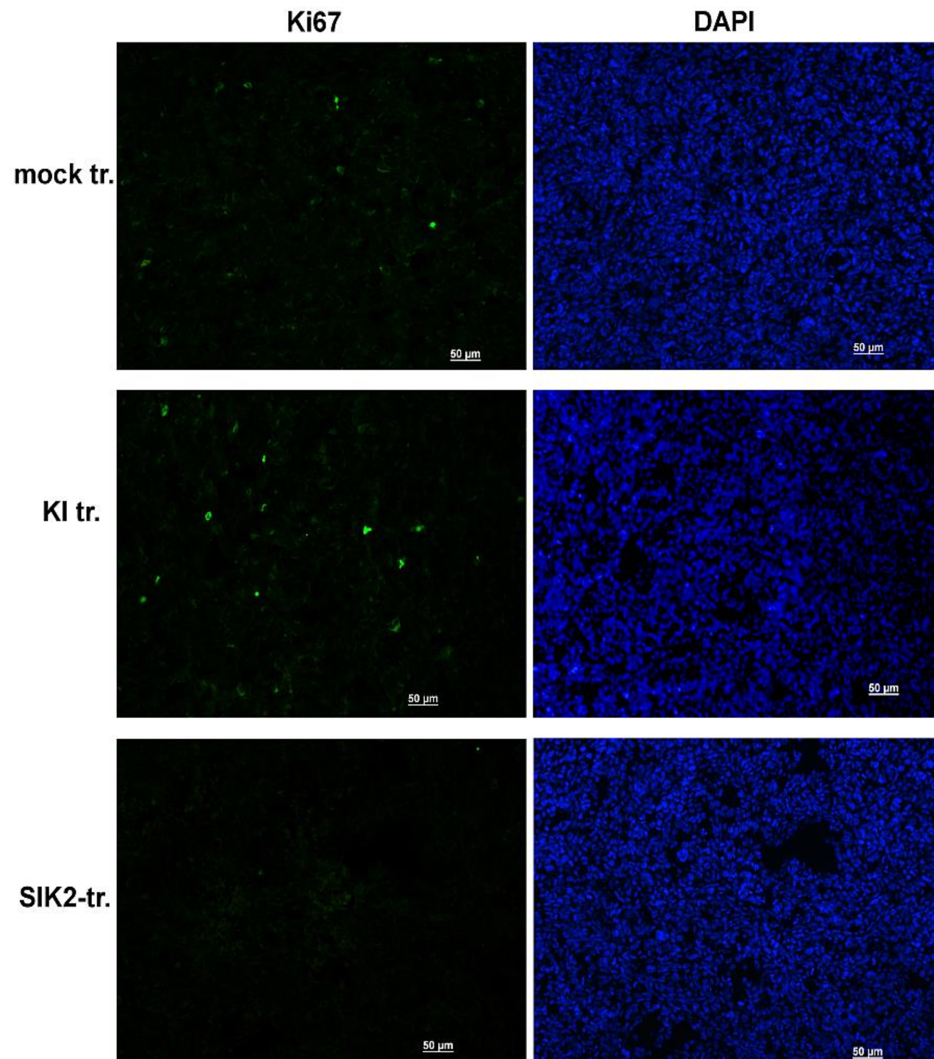

Figure 5.26. Proliferation profiles of tumors excised from SCID mice. Sections of tumors obtained from SCID animals were stained with anti-Ki67 (green) antibody. Nuclei were stained with DAPI. Scale bar: 50 µm in all panels.

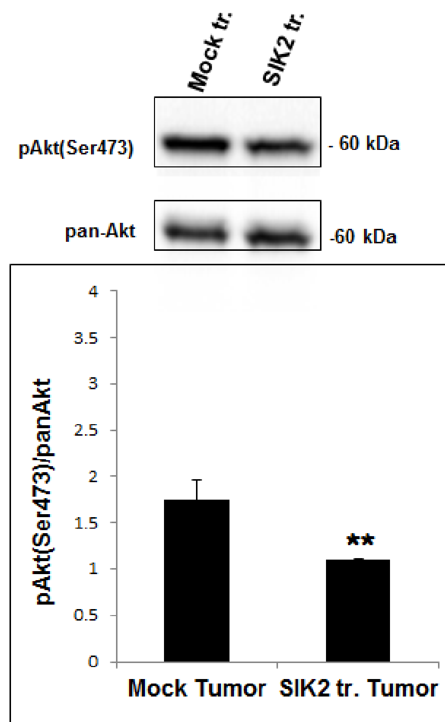

Figure 5.27. Akt phosphorylation profiles of the tumors excised from SCID mice. Lysates from excised tumors were subjected to Western blotting. Blots were probed with anti-pAkt and anti-panAkt. Band intensities of pAkt were normalized to that of pan-Akt; mean values of three independent experiments are presented in histograms (± SD). ** P< 0.05.

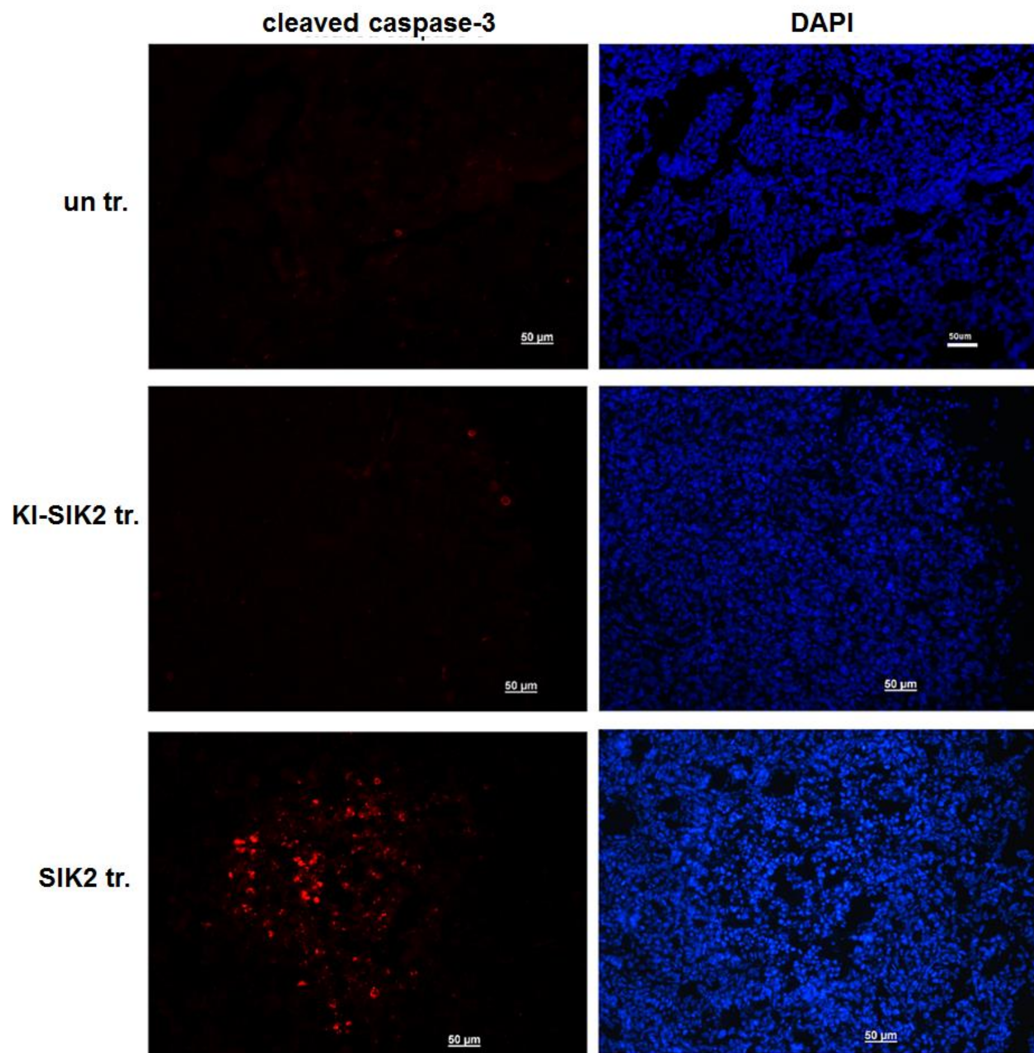

Figure 5.28. Cell death in tumors excised from SCID mice. Sections of tumors obtained from SCID animals were stained with anti-cleaved-caspase-3 (red) antibody, nuclei were stained with DAPI. Scale bar: 50 µm in all panels

6. DISCUSSION

Breast cancer is the leading cause of cancer death among women and has a highly heterogeneous phenotype with inter/intra tumor diversity. Although recently developed prognostic methods contribute to the efficient therapeutic outcomes, recurrence risk and metastasis still remain important for most of the patients. Therefore, improving our understanding about the underlying genetic and molecular background of the disease and revealing the defects leading to the initiation and progression of cancer is important for the development of novel targeted therapies. Several kinases are modulated under the control of growth factor signaling pathways and function in the regulation of various critical cellular events including survival, proliferation, cell cycle progression and invasion to promote neoplastic transformations (Weigelt *et al.*, 2005; Ghayad and Cohen, 2009; Dey *et al.*, 2010; Jemal *et al.*, 2010; Polyak, 2010; Dubey *et al.*, 2015; Prat *et al.*, 2015; Rosa, 2015; Parker and Perou, 2015; Roskoski, 2015).

Based on our previous data indicating SIK2 negative regulatory function in FGF-dependent proliferation and survival of retinal glial cells, in this study we aimed to investigate the possible involvement of this gene in the development of tumorigenesis as a candidate tumor suppressor.

Towards this aim, we initially surveyed the transcript level of the gene using a commercial cDNA tissue array including tumor samples originating from 19 different tissues. The data showed reduced SIK2 levels in breast and glial tumors. Our query into the Oncomine database revealed that SIK2 transcript levels were frequently downregulated in breast tumors and in one report, copy number loss was reported. Thus, these analyses lend support to the notion that loss of SIK2 may be part of tumor development in specific tissue contexts.

For further studies, we focused on breast cancer and initially carried out immunohistochemical studies using primary tissue samples from a small patient cohort. The analysis indicated that SIK2 is expressed specifically in ductal epithelial cells of normal breast tissue. Its expression levels were reduced in all TNBC cases and

approximately in half of the ER/PR+ tumors examined. TNBC tumors are known for their high proliferation rates and in agreement, we observed strong and widespread signal for the proliferation marker Ki67 in our cohort of TNBC cases. In some ER/PR+ tumor samples, we observed that SIK2 expression existed in some localized regions with no concurrent Ki67 signal. Interestingly, a remarkable loss of SIK2 was evident in the regions where proliferative marker staining was highly abundant. This inverse correlation with SIK2 expression and mitotic activity suggests that SIK2 may have a potential contribution to breast tumorigenicity via downregulating the proliferative capacity of tumor cells.

To further investigate a prospective role of SIK2 in tumorigenicity, we analyzed SIK2 expression in breast cell lines. This study showed significant reduction in SIK2 levels in a set of transformed breast lines compared to non-transformed MCF12A cells. Silencing of the gene in this non-transformed cell line resulted in an increase in the fraction of the proliferative cell population and elevation in phosphorylated ERK levels compared to control cells. In these cells, decrease in apoptosis with concomitant increase in active Akt levels was also evident. Reciprocally, when SIK2 levels were upregulated in the highly tumorigenic TNBC cell line MDA-MB-231, reduced proliferation and survival capacities are correlated with a decline in active ERK and Akt levels, respectively. Overall, these data suggest that SIK2 has a negative regulatory function in proliferation and survival of breast cells through fine-tuning of ERK and Akt activation, similar to Müller glia (Kuser-Abali, unpublished data).

Furthermore MDA-MB-231 cells with enhanced SIK2 level generated significantly smaller xenograft tumors in SCID mice compared to the parental and mock transfected cells. Thus, we concluded that SIK2 loss in breast tumors may lead to the disruption of required balances in proliferation and survival to promote tumor growth. Since transfection of a kinase dead SIK2 mutant to these cells did not result in reduction of tumor size, we suggest that the kinase activity is essential for the proposed tumor suppressor role of the gene in development of breast tumors.

The MDA-MB-231 cell line was reported to have high FGFR1/2 expression (Nurcombe *et al*., 2000). Though we did not directly address SIK2 modulations in the FGF signaling context in this study, the reduced SIK2 levels as a candidate tumor suppressor

might be amplifying the oncogenic activity of the receptor, thus contributing to the aggressive phenotype of the MDA-MB-231 cancer cell line. In the same vein, it is plausible that abnormal/overactive FGFR signaling concomitant with SIK2 loss might be an aggravating factor in breast and other malignancies.

In a different perspective, we also investigated a potential contribution of SIK2 in the metastatic characteristics of breast cells. Our data showed that SIK2 silencing in the MCF12A nontransformed cell line resulted in a significant increase in motility and invasiveness of these cells. Reduced E-cadherin and enhanced MMP-2 levels via downregulation of SIK2 indicated that the observed changes in motility and invasiveness are not the indirect result of increased cell numbers due to upregulation of mitotic activity and downregulation of apoptotic processes. Activated PI3K/Akt pathway is thought to have a critical role in the modulation of tumor growth via the regulation of MMP pathway activation (Bae *et al*., 2006; Hwang *et al*., 2009). Although it wasn't investigated in this study, it is possible that reduced SIK2 expression/activity may upregulate MMP-2 levels through enhanced Akt activation. Several studies report that transition from epithelial to mesenchymal phenotype is the direct consequence of reduced E-cadherin levels. This decline has also been associated with sustained activation of MAPK/ERK and PI3K/Akt pathways (Lau *et al*., 2013). Thus, it is conceivable that loss of SIK2 expression/activity may play a part in promoting metastatic behaviour of breast cells through perturbations in both of these cascades.

In the FGF signaling context, the negative regulation appears to be orchestrated by serine phosphorylation of Gab1 by SIK2, interfering with the formation of a signalosome complex to promote both Erk and Akt activation (Kuser-Abali et al., unpublished data). Our earlier data also indicate that in the same cellular context, SIK2 downregulate insulin signaling by targeting IRS1, the critical regulator of the pathway (Küser-Abali et al., 2013). Since both of these docking proteins are key factors in various RTK pathways, SIK2 may have a regulatory function in diverse growth factor signaling pathways as a common regulatory component. Therefore one can further speculate that reduction in SIK2 levels/activity could result in perturbations in RTK pathways such as EGFR and IR that are implicated in development of breast malignancies (Holgado *et al*., 1996; Rocchi *et al*., 1998; Rodrigues *et al*., 2000; Buck *et al*., 2010 and Aasrum *et al*., 2013).

More recently SIK2 was implicated in the regulation of survival and growth of glioblastoma cells under glucose depletion via S6K, an effector of the mTOR pathway (Li *et al*., 2015). The mTOR pathway is important in integration of growth factor, nutrient signals and the energy status of cells, and regulation of translation (Laplante and Sabatini, 2012). Akt and ERK1/2 are upstream activating kinases of this pathway (Laplante and Sabatini, 2012; Copps and White, 2012; Guo, 2014). Given the proposed role of SIK2 in the regulation of their growth factor-dependent activation by our studies and its reported function in metabolic processes, SIK2 may also be taking part in the reprogramming of energy metabolism and regulating protein biosynthesis. As such loss of SIK2 might be important for cancer cells to acquire survival advantages.

In contrast to our findings, in a recent study SIK2 was reported to regulate centrosome separation during mitosis of ovarian cancer cells and proposed to act as an oncogene by promoting cell cycle progression (Ahmed *et al*., 2010). In another study, SIK2 was suggested to suppress cell cycle arrest and apoptosis in prostate cancer cells via the regulation of CREB dependent gene expression (Bon *et al*., 2014). The underlying reason of the difference between our results and these reports are not clear at this time. Such apparent contradictions are not rare. Binary functions for several critical genes including p53 and Notch in different cellular contexts can be found in the literature. So, it is conceivable that abberations in SIK2 expression/function may result in divergent phenotypes due to differences in pathways involved, variations in repertoire of interacting partners or to the existing mutational signatures in specific cellular contexts.

In summary, in this study SIK2 expression was identified in ductal epithelia of breast tissue for the first time. Significantly reduced SIK2 levels in tumor tissues compared to normal tissue counterparts were demonstrated using primary human samples. We provided strong evidence that SIK2 negatively regulates mitotic as well as apoptotic responses of breast cells via suppressing ERK and Akt activities. Our data also suggest that loss of SIK2 expression/activity may enhance metastatic potential of breast cells through the modulation of expression of critical genes. However, mechanisms underlying the reduced SIK2 expression remain to be elucidated. It will be important to investigate possible genetic and epigenetic modifications in the SIK2 gene. The identification of

signaling mechanisms which regulate its function or its interaction partners specifically in the breast tissue context will provide a much clearer understanding of its involvement in the development of breast cancer.

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