EFFECTS OF FYN-RELATED-KINASE ACTIVITY ON BREAST CANCER CELL PROLIFERATION, MIGRATION, INVASION AND COLONY FORMATION

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ABSTRACT

The human **F**yn-**r**elated **k**inase (**FRK)** is a member of subfamily of Src-related kinases family. FRK is 54 kDa non-receptor tyrosine kinase protein composed of 505 amino acids. FRK consists of three functional domains: Src homology 3 (SH3), SH2 and kinase domain, as well as a putative tyrosine kinase regulator at the C-terminus. FRK has a conserved auto-regulatory tyrosine residue within its kinase domain. It has been reported that FRK is repressed in about 30 % of human breast cancer samples. Over-expression of FRK in breast cancer cells of the mammary gland was shown to suppress cell growth by interacting, phosphorylating and stabilizing the tumor suppressor PTEN, thus inhibiting AKT/PI3K signaling. Although it has been suggested that FRK is a tumor suppressor gene, the effects of activated FRK on cell proliferation, migration and invasion are unclear. Likewise, the signaling pathways regulated by the activation of FRK have not been yet fully characterized. We hypothesize that the activation of FRK is essential for the regulation of its cellular functions. Mutation of the C-terminal auto-regulatory tyrosine 497 to phenylalanine (FRK-Y497F) resulted in the constitutive activation of FRK. We generated stable cell lines expressing either the FRK wild type (FRK-WT) or FRK-Y497F from triple negative breast cancer MDA-MB-231 cells. The introduction of FRK-Y497F in MDA-MB-231 cells significantly suppressed their proliferation, migration, invasiveness and colony formation as compared to cells that expressed the FRK-WT gene. Over-expression of either FRK-WT or FRK-Y497F in MDA-MB-231 cells inhibited the phosphorylation of AKT, STAT3, JNK and P38 MAPK as compared to either the MDA-MB-231 parental cells or those that were transfected with the empty vector. Our results suggested that FRK represses cell proliferation, migration, invasiveness and colony formation at least in part by the inhibiting the activation of AKT/PI3K, JAK-STAT and MAPK signaling pathways.

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LIST OF ABBREVIATIONS

Abbreviation	
ANOVA	one-way analysis of variance
APS	ammonium persulfate
AP-1	activator protein-1
ASK	apoptosis signal-regulating kinase
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
ATF2	activating transcription factor 2
BRK	breast tumor kinase
BSK	β-cell Src-homology tyrosine kinase
CCK-4	cholecystokinin tetrapeptide
CCK-8	cell counting kit-8
CDC2	cell division cycle 2
CDK	cyclin-dependent kinase
cDNA	complementary DNA
COX-2	cyclooxygenase 2
DLK	dual leucine zipper bearing kinase
DMEM	Dulbecco's modified eagle medium
ECL	enhanced chemiluminescence substrate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGFR-1	fibroblast growth factor receptor-1
FITS	4', 6-diamidino-2-phenylindole
FRK	Fyn-related tyrosine kinase
GAP	GTPase-activating protein
GDP	guanine diphosphate
GEF	guanine-nucleotide exchange factors
GPCR	G protein-coupled receptor
Grb2	growth factor receptor-bound protein 2
GTK	gastrointestinal tyrosine kinase
GTP	guanosine-5'-triphosphate
HER2	human epidermal growth factor receptor 2
IGF-1R	insulin-like growth factor-1 receptor
IL6	Interleukin 6
IRS-2	insulin receptor substrate-2
IYK	intestine tyrosine kinase
JIP1	JNK interacting protein 1
JNK	c-Jun N-terminal kinase
LB	lysogeny broth
LOH	loss of heterozygosity

LPA	lysophosphatidic acid
LZK	leucine zipper-bearing kinase
mTORC2	mammalian target of rapamycin complex 2
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAPKK/MEK	mitogen-activated protein kinases kinase
MAPKKK/MEKK	mitogen-activated protein kinases kinase kinase
NGF	nerve growth factor
NLS	nuclear localization signal
NRTK	non-receptor tyrosine kinase
pRb	retinoblastoma protein
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDK1	phosphoinositide-dependent kinase 1
PH	pleckstrin homology
PIAS	protein inhibitors of activated STAT
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog
PTK	protein tyrosine kinase
PTPase	protein tyrosine phosphatase
RAS	rat Sarcoma
Rheb	Ras-homolog enriched in brain
RTK	receptor tyrosine kinase
RT-PCR	real-time polymerase chain reaction
Sam68	a Src-Associated substrate in mitosis of 68 kDa
S. D.	Standard deviation
SFK	Src family kinase
SH	Src homology
SHB	Src homology 2 domain adapter protein
SIE	sis-inducible element
SOCS	suppressors of cytokine signaling
SOS	son of sevenless
SRMS	Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation site
STAT	signal transducer and activator of transcription
TAK1	TGF-β-activated kinase 1
TCF	ternary complex factor
TEMED	N,N,N',N'-Tetramethylethylenediamine
Thr	threonine
TNF-α	tumor necrosis factor-α SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophores
Tyr	tyrosine

VEGFR-2	vascular endothelial growth factor receptor-2
WST	water-soluble tetrazolium salts

1. OVERVIEW

Breast cancer is a global public health issue with over 1.5 million predicted diagnoses in women worldwide (Ginsburg and Love 2011). Breast cancer accounts for up to 23% of all cancers in women, and it causes 500,000 deaths worldwide each year (Ginsburg and Love 2011).

One of current breast cancer molecular classifications is based on gene expression profiles of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Rakha *et al.,* 2010). Based on the status of these receptors, breast cancer can be classified into 4 major molecular subtypes: Luminal A, Luminal B, triple negative and HER2 type (Perou *et al.,* 2000; Sorlie *et al.,* 2001; Prat and Perou 2011). HER2 type breast cancers are characterized by overexpression of the HER2 receptor and tend to be poorly differentiated and high-grade (Brenton *et al.,* 2005). Triple-negative type, lacking ER, PR or HER2 expression, is a relatively more malignant form of breast cancer with poor prognosis. 5-10% cancer patients have been diagnosed with triple-negative type (Carey *et al.,* 2010). Since triple-negative breast cancers lack the three receptors (ER, PR and HER2), common clinical drugs cannot be used towards treating such breast cancers (Carey *et al.,* 2010). Both luminal A and B cancers express ER/PR genes. Luminal B also contains HER2 and appears to be more aggressive (Perou *et al.,* 2000; Sorlie *et al.,* 2001; Prat and Perou 2011). Breast cancer cell lines also exhibit heterogeneity in the expression of several other cellular genes (Neve *et al.,* 2006). Tumor heterogeneity has complicated patient response to therapeutic strategies. Breast cancer heterogeneity in the context of genomic profile (gene mutation, gain or loss of gene copy number), expression signature of prognostic markers such as HER2 and ER, all contribute to the complexity of therapeutic intervention. The various interventions used in the treatment of breast cancer include surgery, radiation, chemotherapy and immunotherapy. Often, surgery is more effectively used in combination with chemotherapy and radiation. However, there are several limitations associated with the use of chemotherapeutic drugs, such as target inaccuracy. The inaccuracy can be compensated by the use of combination therapy by either using hormone blocking therapy or the use of monoclonal antibodies

specific to antigens overexpressed in cancerous cells such as tyrosine kinase inhibitors that target breast cancer cells overexpressing HER2/neu (Roskoski 2014). Mutation or overexpression of tyrosine kinases are known to contribute to the development of tumors (Morel *et al.,* 2014).

Protein kinases are classified into two sub-families based on their substrate specificity: serine/threonine kinases and tyrosine kinases (Hanks *et al.,* 1988). Protein tyrosine kinases (PTKs) catalyze the transfer of a phosphate group, derived from ATP, to specific tyrosine residues (Condorelli *et al.,* 2011). PTKs play a vital role in intracellular and extracellular signal transduction, and the regulation of cellular proliferation (Morel *et al.,* 2014). PTKs can be further sub-classified into two groups as receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs) based on their cellular locations (Chandrasekharan *et al.,* 2002).

Receptor tyrosine kinases (RTKs) consist of three key domains, a hydrophobic trans-membrane domain which is embedded in the cellular lipid membrane, an extracellular domain that is responsible for activating intracellular kinases via specific ligand binding, and a cytoplasmic domain which contains the recruitment sites for the downstream substrates (Blume-Jensen and Hunter 2001). Unlike RTKs, NRTKs mainly have a cytoplasmic localization and their downstream regulation is achieved by activating the tyrosine in the protein by transferring a phosphate group from a phosphate donor. The human genome encodes nearly 32 NRTKs. Src family kinases (SFKs) and and Fyn-related kinase (FRK) are examples of NRTKs (Robinson *et al.,* 2000). The hyperactivation of certain PTKs has been linked to the tumorigenesis of breast cancer (Nunes-Xavier *et al.,* 2013). For example, the overexpression of cholecystokinin tetrapeptide (CCK-4/PTK7) has been reported in different types of cancers including lung, colon and breast cancer (Mossie *et al.,* 1995; Speers *et al.,* 2009; Na *et al.,* 2012). Two PTK family proteins: the HER2 and SFKs have been shown to highly correlate with the development of breast cancer (Nunes-Xavier *et al.,* 2013).

c-Src is the prototypical member of the Src family kinases. Due to the sequence homology and structural similarities, SFKs have been separated into eight family members,

Src, Fyn, Yes, Blk, c-Fgr, Lyn, Lck and Hck (**Figure 2.5**) (Abram and Courtneidge 2000). v-Src was one of the first well-studied viral oncogenes (Smart *et al.,* 1981), while its ortholog, c-Src, was reported to exhibit protooncogenic properties, as studied in normal cells (Oppermann *et al.,* 1979). The presence of N-terminal myristoylated glycine residues in SFKs enable interaction with cellular membranes (Okada 2012).

The dephosphorylation of C-terminal tyrosine 530 relieves binding interaction with the Src homology 2 (SH2) domain. This results in a conformational change at the kinase domain that induces a hydrogen bond between glutamate 310 existing in αC-helix and lysine 295 (Okada 2012). As a result, tyrosine 416 which is located in the activation loop is auto-phosphorylated. c-Src is known to exhibit oncogenic properties (Oppermann *et al.,* 1979). Elevated expression and activation of c-Src has been commonly found in a variety of cancers, including human breast cancer (Verbeek *et al.,* 1996). For instance, the activity of c-Src has been found to be elevated in human brain and skin tumors (Homsi *et al.,* 2007; Ahluwalia *et al.,* 2010). In addition, elevated expression of c-Src has been reported in certain malignancies such as ovarian cancer (Wang *et al.,* 2014). Src activation has been also reported to associate with prostate cancer metastases (Vlaeminck-Guillem *et al.,* 2014).

Unlike c-Src, FRK appears to function as a tumor suppressor (reviewed by (Serfas and Tyner 2003; Brauer and Tyner 2009)). For instance, in a recent paper the mRNA and protein levels of FRK were found to inversely correlate with the grade of human glioma tumors. While normal brain tissue exhibited elevated FRK levels, high grade glioma tissues exhibited significantly reduced levels of FRK, suggesting a potential tumor suppressive role in human glioblastomas (Zhou *et al.,* 2012).

2. REVIEW OF LITERATURE

2.1 Cell signaling pathways in cancer

In cell signaling, receptors usually are activated by the ligand binding. Receptor activation leads to the transmission of intracellular signals which eventually elicits a cellular response. This entire process of cell response driven by signal transmission constitutes a cell signal transduction pathway (Dinasarapu *et al.*, 2011). A series of signaling pathways exist within cells, comprising several proteins that participate in signal transmission. These pathways are organized in complex networks with distinct pathways tending to cross-talk with other pathways within the cell. Before providing background information on Fyn-related kinase (FRK), which is the focus of the thesis, signaling pathways relevant to the cellular properties FRK and key signaling intermediates will be introduced briefly in the following sections.

2.1.1 The Mitogen-Activated Protein Kinase signaling pathway

The Mitogen-Activated Protein (MAP) kinases, protein serine/threonine kinases, are one of the major components of the signal transduction pathways regulating cellular processes in the nucleus, including cell proliferation, apoptosis, differentiation and activities of intracellular enzymes (Gao *et al.*, 2014). These kinases contain two domains, a highly helical C-terminal domain and an N-terminal domain. The former is composed of four β-strands which contain several catalysis-related residues and the latter is mainly constructed of β-sheets and 2 helices (αC and αL16) (Turjanski *et al.*, 2007).

The activation of MAP kinases (MAPKs) is modulated by three-kinase phosphorylation cascades (**Figure 2.1**). Mitogen-Activated Protein kinase kinases (MAPKKs) are responsible for phosphorylating the downstream target MAPKs, and its own activation relies on upstream protein kinases, Mitogen-Activated Protein kinase kinase kinases (MAPKKKs) (Pimienta and Pascual 2007). The phosphorylation of tyrosine and threonine are both required for activating MAPKs by MAPKKs. Moreover, the threonine phosphorylation is crucial for activation of tyrosine-phosphorylated proteins, thereby fully activating MAPKKs (Pimienta and Pascual 2007). Likewise, MAPKKKs

activate MAPKKs via a similar process, by the phosphorylation of either a tyrosine or threonine residue located in the activation loop (Alessi *et al.,* 1994; Zheng and Guan 1994). Consequently, activated MAPKs translocate from cytoplasm to the nucleus where they regulate gene transcription (Gonzalez *et al.,* 1993). In the nucleus, MAPKs interact with targeted transcription factors and initiate gene transcription (Pimienta and Pascual 2007).

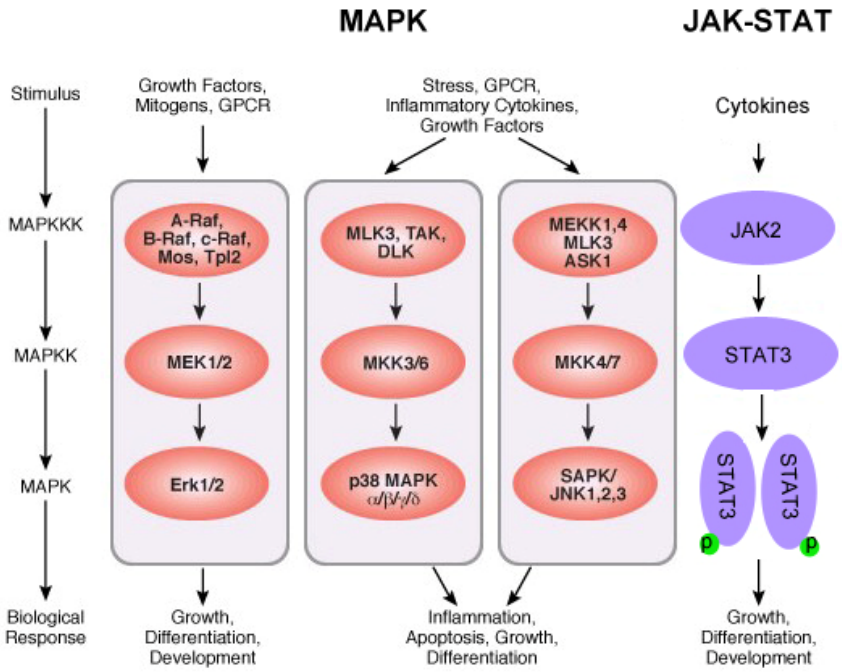


Figure 2.1. Simplified MAPK and JAK-STAT signaling pathway. Mitogenic-activated protein kinase (MAPK) regulates several cellular processes including cell proliferation, cell differentiation, cell migration and cell survival through the modulation of several signaling pathways. MAPKKK are activated by cell surface receptor stimulation by various growth factors. MAPKKK activates MAPKK, which in turn activates MAPK. JAKs (Janus kinase) and STATs (Signal Transducer and Activator of Transcription) are critical components of many cytokine receptor systems, regulating cell proliferation, cell differentiation and pathogen resistance. JAK2 receptor stimulation results in the phosphorylation of STAT3 at tyrosine 705, which induces dimerization, followed by nuclear translocation and activation of several target genes including those that modulate cell growth, differentiation and pathogen resistance (Turjanski *et al.,* 2007). Figure is modified from http://www.cellsignal.com/contents/science-cst-pathways/map-kinase-signaling-resources/science-pathways-mapk?Ntt=MAPK&fromPage=keywords.

According to sequence similarity, MAPKs can be categorized into 6 groups, including the extracellular signal-regulated protein kinases (ERK1 and ERK2), c-Jun N-terminal kinases (JNK1, JNK2, JNK3), p38s (p38α, p38β, p38γ, p38δ), ERK3s (ERK3, p97 MAPK, ERK4), ERK5 (ERK5), and ERK7 (ERK7, ERK8) (Schaeffer and Weber 1999), which will be discussed in detail in the following sections.

2.1.1.1 The ERK 1/2 pathway

ERK1 (extracellular signal-regulated kinase 1) and ERK2 are two isoforms, proteins of 42 and 44 kDa (also known as $p42^{MAPK}$ and $p44^{MAPK}$), respectively (Rossomando *et al.,* 1989). The activation loop of ERK contains a TEY motif (Thr-Glu-Tyr), in which theronine and tyrosine are essential for activating the kinase (Payne *et al.,* 1991). MEK1 and MEK2 are capable of fully activating ERK1 and ERK2 (Zheng and Guan 1993; Robinson *et al.,* 1996).

The mammalian ERK 1/2 pathway has several important biological functions, including cell survival, proliferation and development (Zhang and Dong 2007). The three-kinase module of MAPKs regulation can also be applied to describe the ERK 1/2 pathway. In epidermal growth factor receptor (EGFR) signaling, for example, interaction of its ligand epidermal growth factor (EGF) with EGFR results in receptor dimerization which consequently activates EGFR itself. Once the receptor is activated, tyrosine autophosphorylation creates tyrosine-specific binding sites which are crucial for phosphorylating a series of downstream substrates, including growth factor receptor-bound protein 2 (Grb2) (Ullrich and Schlessinger 1990; Schlessinger and Ullrich 1992; Buday and Downward 1993). Grb2 subsequently binds to SOS (Son of Sevenless, *drosophila* homolog 1), and induces the replacement of GDP (Guanine diphosphate) bound to Ras with GTP. Therefore, activated Ras can propogate the signaling to Raf1, MEK 1/2 and ERK 1/2, sequentially (Ullrich and Schlessinger 1990; Pearson *et al.,* 2001). MEK 1/2, then phosphorylates and activates ERK 1/2 (Prowse *et al.,* 2000) (**Figure 2.1**). The activation of ERK 1/2 triggers its nuclear translocation, and subsequent binding to certain transcription factors, thus regulating gene expression (Turjanski *et al.,* 2007). The activator protein-1 (AP-1) is a well-characterized example to illustrate the regulatory function of ERK 1/2 at the transcriptional and post-translational levels. The AP-1 complex, which is

required for *c-myc* expression, is formed by the Jun family of transcription factors (c-Jun, JunB and JunD) heterodimerizing with the Fos family proteins (c-Fos, FosB, Fra1 and Fra2) (Angel and Karin 1991). In addition, *c-fos* transcription is modulated by the binding of ternary complex factor (TCF, also known as Elk-1) to *c-fos* promoter region upon the activation of ERK 1/2 (Whitmarsh *et al.,* 1995) (**Figure 2.2**).

2.1.1.2 c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) pathway

JNKs (c-Jun N-terminal kinases), also known as stress-activated protein kinases, have three identified coding genes, including JNK1, JNK2 and JNK3 (Cargnello and Roux 2011). Like other MAPKs, the activation loop of JNK contains a specific Thr-Pro-Tyr motif (Cargnello and Roux 2011). JNK was discovered by its capability of serine-phosphorylating the transactivating domain, which is located at the N-terminus of c-Jun (Turjanski *et al.,* 2007).

JNK regulates several biological processes, including apoptosis, differentiation and proliferation (Briata *et al.,* 2005). Like MAPK signaling pathway, JNK initiates its kinase cascade by receiving extracellular stimuli, although the mechanism of the activation has not been fully investigated as that in ERK (**Figure 2.2**). Similar to the ERK 1/2 pathway, which is regulated by Ras, the activity of JNK was found to be regulated by two of the Rho family of GTPases (Rac1 and Cdc42) through switching on an independent pathway (Turjanski *et al.,* 2007). Substrates of GTPases involved in the JNK pathway include members of MAPKKK family (MEKK1-4), the apoptosis signal-regulating kinase (ASK) family (ASK1 and ASK2), the mixed-lineage protein kinase family (Zhu *et al.,* 2014), TPL2, and TGF-β-activated kinase 1 (TAK1) (Coffey 2014). In addition, two more kinases, MEK4 and MEK7, have been shown to regulate the function of JNK (Hsiao and Stapleton 2004). MEK4 activates JNK via tyrosine-phosphorylation at Tyrosine 185, whereas MEK7 prefers threonine phosohorylation at Threonine 183, although they both can activate JNK via dual phosphorylation (Hsiao and Stapleton 2004).

JNK and its upstream activators, MEK7 and MLK3, are recruited to the scaffolding protein JNK interacting protein 1 (JIP1), forming a complex which subsequently facilitates

the phosphorylation of JNKs induced by Rac (Turjanski *et al.*, 2007). Thus, the activated JNKs translocate from the cytoplasm to the nucleus to control the expression of several genes including the *c-jun* gene.

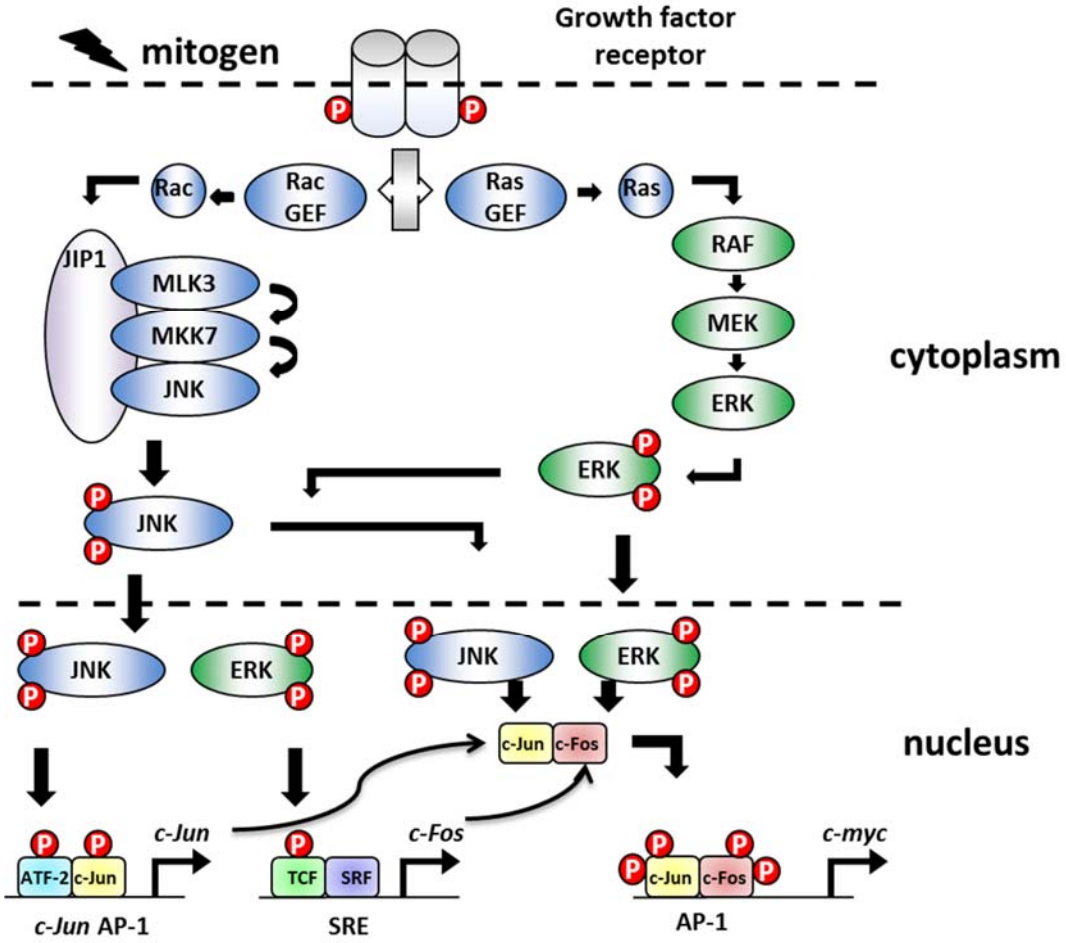


Figure 2.2. Spatiotemporal model of MAPK cascade. Stimulated by mitogens, certain growth factor receptors are phosphorylated and activated, thereby inducing the activation of GTPase of the Rho and Ras family via the guanine-nucleotide exchange factors (GEF). Rac and Ras then activate the corresponding downsteam kinase cascades. In one hand, Ras activates the Raf/MEK/ERK cascade. In the other hand, Rac activates another independent JNK interacting protein 1 (JIP1)-mediated signaling pathway. JNK is activated by forming a complex with its activators, MKK7 and MLK3, and the scaffolding protein JIP1. The phosphorylated JNK translocates to the nucleus to modulate the target gene expression. Activated JNK, ERK participate in the phosphorylation of activating transcription factor 2 (ATF2), Jun and T-cell factor (TCF) which are required for *c-Jun* and *c-Fos* gene expression. Figure is adapted from (Turjanski *et al.*, 2007).

2.1.1.3 The p38 pathway

The p38 family has four members, including p38α, p38β, p38γ and p38δ. p38α, the best known member in the family, has been identified as a tyrosine phosphoprotein in response to inflammatory cytokines (O'Callaghan *et al.,* 2014). Three other isoforms have also been identified, including p38β (p38-2), p38γ (ERK6 or SAPK3), and p38δ (SAPK4) (Jiang *et al.,* 1996; Lechner *et al.,* 1996; Li *et al.,* 1996; Goedert *et al.,* 1997; Jiang *et al.,* 1997; Kumar *et al.,* 1997; Stein *et al.,* 1997). All four p38 family members share a similar structure, which contains a dual phosphorylation motif (Thr-Gly-Tyr) located in the activation loop (Cargnello and Roux 2011). Therefore, these members are similar to one another in function to a degree. For example, p38α and p38β, not only have the similar downstream targets (MAPKAPK2 and ATF-2), but are both sensitive to pyridinyl imidazole which acts as a p38 kinase activity inhibitor competing with ATP (Hu *et al.,* 1999; Zarubin and Han 2005). However, these isoforms are also distinct in certain properties. Unlike p38α and p38β, the other two isoforms p38γ and p38δ, are resistant to pyridinyl imidazole (p38 MAPK inhibitor) and may even inhibit the activity of AP-1 (Goedert *et al.,* 1997; Kumar *et al.,* 1997; Pramanik *et al.,* 2003).

It has been proposed that the p38 family proteins are stimulated by a variety of factors, including exposure of cells to GPCRs, cytokines, osmotic and heat shock, cell stress signals (Conrad *et al.,* 1999) and hormones (Turjanski *et al.,* 2007). p38 has also been reported to respond to inflammation via the modulation of expression of Interleukin 1β (IL-1β), cyclooxygenase 2 (COX-2), Interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) (reviewed in (Zarubin and Han 2005)). MEK3 and MEK6 have been shown to contribute to the activation of p38s by phosphorylating the four isoforms (Carriere *et al.,* 2008). However, a preference of phosphorylation exists as MEK3 tends to phosphorylate p38α and p38β, while MEK6 prefers to initially activate p38γ and p38δ isoforms (O'Callaghan *et al.,* 2014). DNA-damage induced cell apoptosis, another important biological process, is also particularly regulated by p38s via serine-phosphorylation of tumor suppressor protein p53, inducing the chemosensitivity of cancer cells (Sanchez-Prieto *et al.,* 2000).

2.1.1.4 Other MAPK pathways

There are other kinases which are homologous to the MAPKs family, including ERK3 isoforms, ERK5, ERK7, NLK and MOK (Cargnello and Roux 2011). The activity of ERK5 is modulated by a series of stimulations, such as cell-stressing and proliferation-related agents. The activation pathways of ERK5 also depend on the cell types and extracellular stimuli (Nithianandarajah-Jones *et al.,* 2012). The proliferation-related ligands include EGF, serum, phorbol easer, lysophosphatidic acid (LPA) and nerve growth factor (NGF) (Kato *et al.,* 1997; Kato *et al.,* 1998; Kamakura *et al.,* 1999). The Ras-independent activation pathway has been indicated to be responsible for these proliferative stimuli in certain cell lines (Kato *et al.,* 1997; Kato *et al.,* 1998). On the other hand, the stressful conditions could be created by UV irradiation, ischemia, sorbitol, vascular shear stress and H_2O_2 (Abe *et al.,* 1996; Kato *et al.,* 1998; Kamakura *et al.,* 1999; Takeishi *et al.,* 1999; Yan *et al.,* 1999), and stress-induced activation prefers the Src-dependent pathway (Abe *et al.,* 1997). The signaling pathways of most members have not been fully investigated. For instance, ERK3 isoform, ERK3α, has been found localized to the nucleus, but only a few studies reveal its regulation pathways (Cheng *et al.,* 1996). ERK3α has been described to be activated via serine-phosphorylation at Ser 189 by an upstream regulator which was not able to stimulate other MAPKs; however, the molecular mechanism still remains unknown (Cheng *et al.,* 1996).

2.1.2 The PI3K-AKT/PKB signaling pathway

The phosphoinositide 3-kinase (PI3K) is an intracellular lipid heterodimeric enzyme composed of a catalytic and a regulatory subunit. PI3K family contains eight isoforms which are classified into three different categories (I, II and III) based on sequence homology and substrate preference (Cantley 2002; Vanhaesebroeck *et al.,* 2010; Vanhaesebroeck *et al.,* 2010). Class I, the most studied subfamily among the three classes, can be further divided into IA and IB (Zhao and Vogt 2008). The former, consisting of p85 regulatory subunit and p110 catalytic subunit, is activated by RTKs (Zhao and Vogt 2008). The latter contains p101 regulatory subunit and 110γ catalytic subunit (Zhao and Vogt 2008) and is stimulated by G-protein-coupled receptors (GPCRs) (Yap *et al.,* 2008).

Three isoforms were found in the p110 subunit (α, β and δ) and five isoforms in the p85 subunit (p85α, p55α, p50α, p85β and p85γ) (Okkenhaug and Vanhaesebroeck 2001; Ward *et al.,* 2011). There are SH2 and SH3 domains in the p85 subunit, and the SH2 domain tends to bind phosphorylated tyrosine usually within a (Y-X-X-M) motif (Songyang *et al.,* 1993; Yoakim *et al.,* 1994). The class I is responsible for the formation of phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol (3,4)-biosphosphate ($PI(3,4)P_2$) and phosphatidylinositol (3,4,5)-triphosphate ($PI(3,4,5)P_3$) (Okkenhaug 2013) which participates in numerous intracellular processes including cell proliferation, survival, reorganization of cytoskeleton, membrane transition, adhesion, mobility, angiogenesis and insulin action (Engelman 2009).

The conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$, the most important product *in vivo* (Cortot *et al.,* 2006), is achieved via the phosphorylation by PI3K on the membrane. $PI(3,4,5)P_3$ induces the recruitment of the pleckstrin homology (PH) domain-containing proteins to the cell membrane. Thus, the signal can be transduced upon the activation of PI3K (Engelman *et al.,* 2006).

AKT, also known as protein kinase B, a serine/threonine kinase, was first identified as an oncogene which is transduced by leukemia-inducing AKT-8 in mice (Staal 1987). There are three cancer-related isoforms in the AKT family: AKT1, AKT2 and AKT3 (Murthy *et al.,* 2000; Koseoglu *et al.,* 2007). AKT has been reported to be activated by various stimuli including EGFR and insulin-like growth factor-1 receptor (IGF-1R), as well as GPCRs (Hennessy *et al.,* 2005). AKT is an important central node in the PI3K signaling pathway, and the N-terminal PIP_3-binding PH domain of AKT is required for the anchorage of the plasma membrane (Andjelkovic *et al.,* 1997; Frech *et al.,* 1997). This results in a consequent conformational change of AKT which leads to the threonine-phosphorylation of its own activation loop by another phosphoinositide-binding protein, phosphoinositide-dependent kinase 1 (PDK1) at Thr308 (Alessi *et al.,* 1997; Engelman *et al.,* 2006). In order to be fully activated, the phosphorylation at C-terminal serine 473 of AKT is also required by the mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov *et al.,* 2004; Sarbassov *et al.,* 2005). Similar to MAPKs, fully active AKT then translocates to the nucleus to exert its gene regulatory function, involved in cell proliferation, survival, metabolism and glucose homeostasis (Manning and Cantley 2007; Yap *et al.,* 2008).

There are several AKT-regulated processes (Yap *et al.,* 2008). The anti-apoptotic property of the AKT signal pathway has been well characterized. AKT phosphorylates and inhibits the activation of potential apoptosis-inducing proteins, including MDM2, BAD and members of the Forkhead family (del Peso *et al.,* 1997; Brunet *et al.,* 1999; Mayo and Donner 2001). It has also been shown that AKT inhibits the activation of p27 (Fujita *et al.,* 2002), Myc and cyclin D1, which are activated by glycogen synthase kinase 3 (GSK3), thereby promoting cell growth (Vivanco and Sawyers 2002). AKT also participates in metabolism, proliferation and angiogenesis regulation. For instance, the inactivation of GSK3β via a phosphorylation by AKT (Cross *et al.,* 1995) blocks AS160 and triggers the glucose transporter Glut4 translocation to the cell membrane (Sano *et al.,* 2003), while the inactivation of FOXO induces the inhibition of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (Burgering 2008).

In addition, AKT also has been shown to act at transcriptional level via modulating the mTORC1 complex (**Figure 2.3**), consisting of mTOR and some interactors (Guertin and Sabatini 2007). The regulation of mTORC1 by AKT can be illustrated by two distinct pathways. One is through the phosphorylation and inactivation of GTPase-activating protein (GAP) TSC2 (Dan *et al.,* 2002), forming a complex with TSC1. In the other pathway, PRAS40 (proline-rich AKT substrate of 40 KDa) down-regulates the mTORC1 by competing with the GTPase Rheb (Ras-homolog enriched in brain) (Kovacina *et al.,* 2003; Sancak *et al.,* 2007).

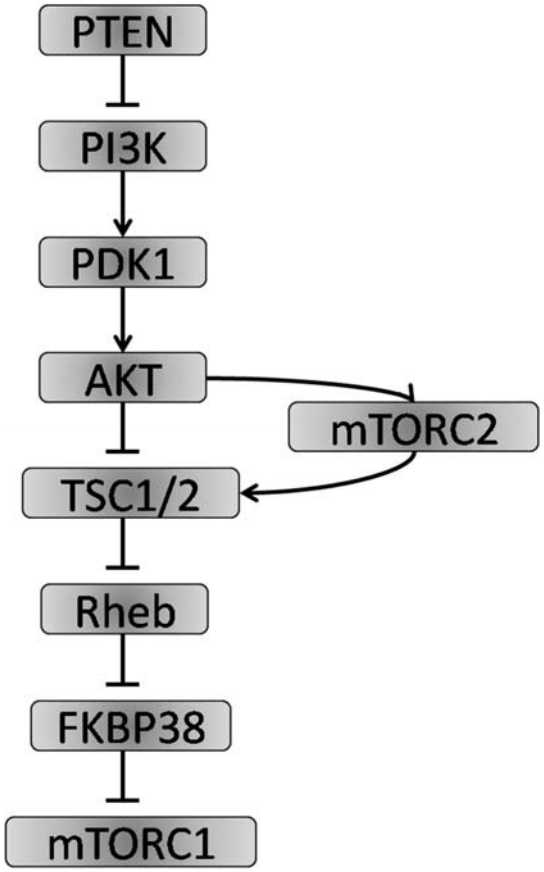


Figure 2.3. PI3K-AKT-mTORC1 pathway. The PI3K-AKT-mTORC1 signaling cascade is inhibited by the tumor suppressor PTEN. Figure is adapted from (Carracedo and Pandolfi 2008).

2.1.3 The JAK-STAT signaling pathway

Signal transducer and activator of transcription (STATs) have seven identified members in mammals that include STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Haricharan and Li 2014). STATs can directly interact with RTKs and NRTKs via phosphorylation (Aaronson and Horvath 2002).

The JAK-STAT system is responsible for transducing extracellular signal through the cell membrane into the cell nucleus, where the gene transcriptional activity is regulated via the binding of STATs and transcription factors. The JAK-STAT system consists of three components: a receptor, Janus kinase (JAK) and STAT proteins, all of which play key roles in the signal transduction cascades. There are four members in the JAK family:

JAK1, JAK2, JAK3, and TYK2 (O'Shea *et al.,* 2013). JAKs have tyrosine kinase activity, and are triggered by binding to cytokine receptors. Activated JAKs are capable of phosphorylating cytokine receptors and creating the recruitment sites for STATs which possess a phospho-tyrosine-binding SH2 domain (Qi and Yang 2014). The STATs are tyrosine-phosphorylated by JAKs after being recruited to the receptors (Qi and Yang 2014). Phosphorylated STATs dimerize and translocate into the nucleus, where they initiate the transcription of genes involved in the regulation of cell proliferation, migration and differentiation (Qi and Yang 2014).

There are three different signaling pathways in JAK-STAT depending on different extracellular stimuli, including type I IFN, type II IFN and interleukin 6 (IL-6). STAT3 regulation of gene transcription in response to IL-6 stimuli results in the modulation of diverse cellular and physiological process including cell proliferation, inflammatory responses and the development of early embryos (reviewed in (Akira 2000; Hirano *et al.,* 2000)). Being a member of the STAT family of transcription factors, STAT3 consists of a DNA binding domain, an SH2 domain which is required for its homo- or hetero-dimerization and a regulatory tyrosine 705 at the C-terminus controlled through phosphorylation by a tyrosine kinase (Qi and Yang 2014). Studies have indicated that the activation of STAT3 correlates with many types of cancer (Bollrath *et al.,* 2009; Grivennikov *et al.,* 2009). The tyrosine phosphorylation sites in intracellular domains of cytokine receptors and the binding of JAK1 and JAK2 proteins to receptors are two factors required for recruitment of STAT3 by providing a STAT3 phosphorylation docking site. The ensuing dimerization of phosphorylated STAT3, triggers its subsequent nuclear localization (**Figure 2.4**). STAT3 is responsible for initiating the target gene expression by specific binding to promoters, which contain a sis-inducible element (SIE) (also known as GAS element). STAT3 can be activated not only by cytokine, but also triggered by other routes, such as growth factors (EGF and PDGF) via specific tyrosine-phosphorylation (reviewed in (Aaronson and Horvath 2002)).

Activated STAT3 has also been reported to initiate the transcription of several genes involved in breast cancer development and progression, such as *cyclin D1*, *cMyc*, *Bcl-xL*, *Survivin*, *VEG-f* and *Klf-8* (reviewed by (Haricharan and Li 2014)).

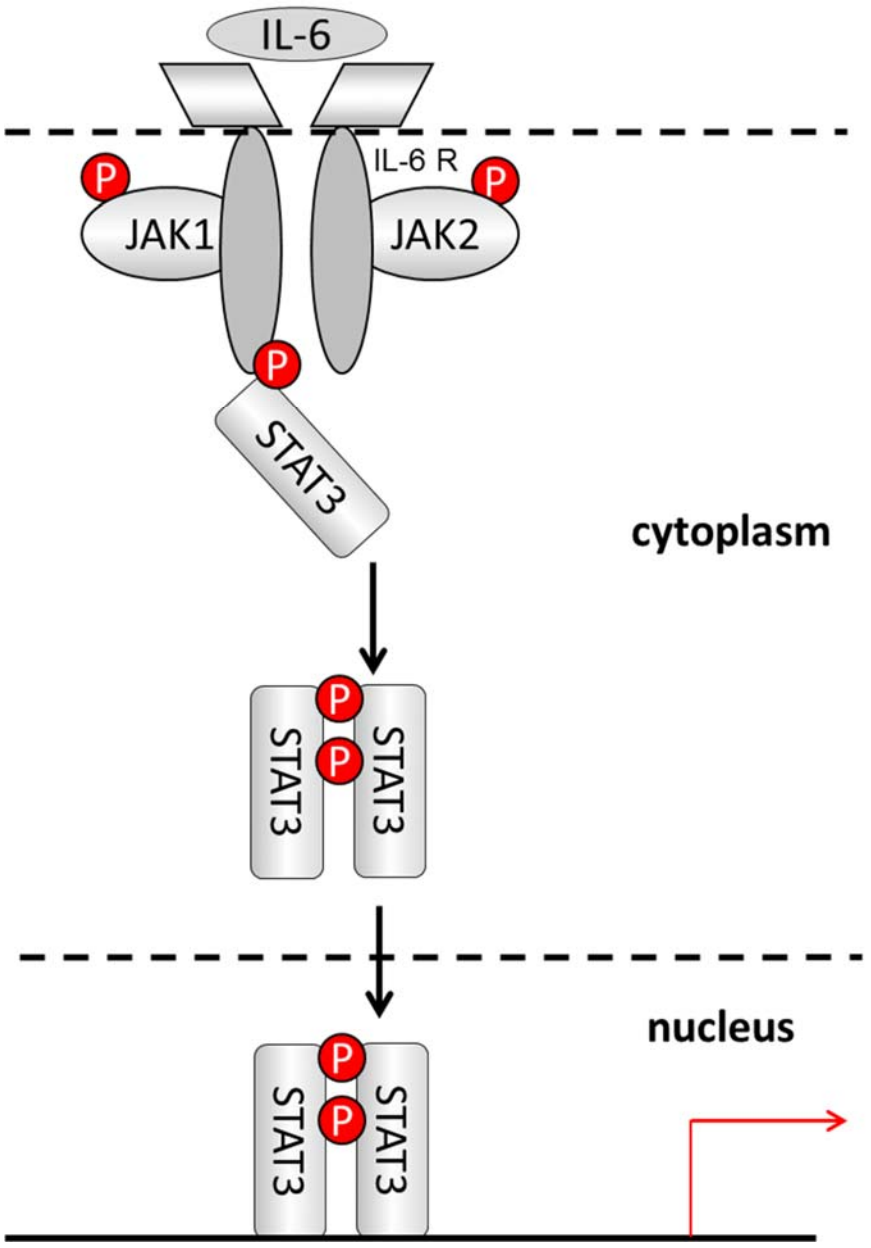


Figure 2.4. STAT3-mediated JAK-STAT signaling pathway. Upon IL-6 stimulation, JAK1 and JAK2 are phosphorylated and activated, creating a docking site for STAT3 and inducing its phosphorylation. Phosphorylated STAT3 dimerize and translocate to the nucleus and subsequently initiate the gene expression by binding to certain transcriptional promoters.

JAK-STAT signaling pathway has been proposed to be repressed at multiple levels which includes the inactivation of JAK1, JAK2 and STAT3 (Shuai 2000; Shuai and Liu 2003). Suppressors of Cytokine Signaling (SOCS) has been confirmed as an inhibitor of

cytokine signaling cascades through binding to JAKs and inhibiting its kinase activity (Krebs and Hilton 2001). JAK-STAT pathway can also be down-regulated by the protein tyrosine phosphatases (PTPase), keeping STATs and JAKs inactive. A typical example for this type of negative regulation involves TC45, a nuclear isoform of T-cell PTP (TC-PTP) which has been shown to dephosphorylate STAT1 both *in vitro* and in *vivo*, thereby impacting signaling via the STAT-JAK pathway (ten Hoeve *et al.,* 2002). Protein Inhibitors of Activated STAT (PIAS), another protein reported to regulate the JAK-STAT pathway, has been shown to interfere with STATs dimerization and inhibit the DNA-binding activity of the transcription factors (Shuai 2006). In addition, PIASs have been shown to suppress the transcription via recruitment of other co-repressors (Liu *et al.,* 2001; Arora *et al.,* 2003).

2.2 Human FRK and its murine orthologs

2.2.1 Human FRK

The Human Fyn-related Kinase (FRK, also known as Rak or protein tyrosine kinase 5, PTK5) is a non-receptor tyrosine kinase, composed of 505 amino acids with a molecular weight of 54 kDa (Lee *et al.,* 1994). As a distant member of Src family, FRK shares 51% similarity with the primary sequence of Fyn (Cance *et al.,* 1994). The FRK protein has so far been detected in the human hepatoma cell line Hep3B (Lee *et al.,* 1994), human melanocytes (Martinez *et al.,* 1987), human B-cell lymphoma cell line BL979, colon cancer cell line LS 180, breast cancer cell lines BT20 and 600 PEI and primary human breast cancer cells (Cance *et al.,* 1994). The expression of FRK also showed a tissue-specific pattern in normal cells. Both mRNA levels and expression levels of protein were shown to be predominantly in human epithelial tissues derived from the liver and kidneys (Cance *et al.,* 1994). A recent study also reported high mRNA levels of FRK in normal lung tissue (Chen *et al.,* 2013). Moreover, the expression level of FRK protein has also been observed in human normal breast epithelial tissues (Berclaz *et al.,* 2000). However, its mRNA expression was absent in cells derived from the mesenchymal tissue such as skeletal muscle (Cance *et al.,* 1994).

FRK belongs to a distinct family of NRTKs, known as the FRK family, that

includes protein tyrosine kinase 6 (PTK6, also called BRK) and SRMS (Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites) (Serfas and Tyner 2003). The FRK family kinases share a conserved gene structure that is distinct from that of the SFKs. From the protein level, the FRK family lacks palmitoylation and myristoylation signals which are responsible for membrane anchorage of the Src family.

Like SFKs, FRK consists of 3 functional domains, the SH3 domain, the SH2 domain and a kinase domain. The carboxy terminal region of FRK possesses an autoregulatory tyrosine 497, analogous to the Y530 of human c-Src. The kinase activity of FRK is inhibited when the regulatory tyrosine is phosphorylated (Pendergast 1996; Parsons and Parsons 2004). FRK has a conserved auto-regulatory tyrosine residue Y387 within its kinase domain. Overall, FRK displays a similar architecture to Src and has 30–40% sequence identity with SFKs (Serfas and Tyner 2003). The SH2 domain binds specifically to peptide sequences that contain a phosphorylated tyrosine (pTyr), while the SH3 domain tends to bind polyproline-containing ligands more commonly displaying a PXXP motif (Kaneko *et al.,* 2011).

In SFKs, the inactive conformation of the enzyme is stabilized by intermolecular interactions between the C-terminal phosphotyrosine (pY530 in human Src) and the SH2 domain, and between proline residues in the SH2-kinase linker and the SH3 domain (Parsons and Parsons 2004). Mutation of the C-terminal tyrosine in SFKs, as well as in PTK6-Y447F mutant and murine FRK-Y504F which is analogous to Y497 in the human sequence (**Figure 2.5)** have been shown to increase the catalytic activity of the enzymes (Oberg-Welsh *et al.,* 1998; Derry *et al.,* 2000; Roskoski 2004).

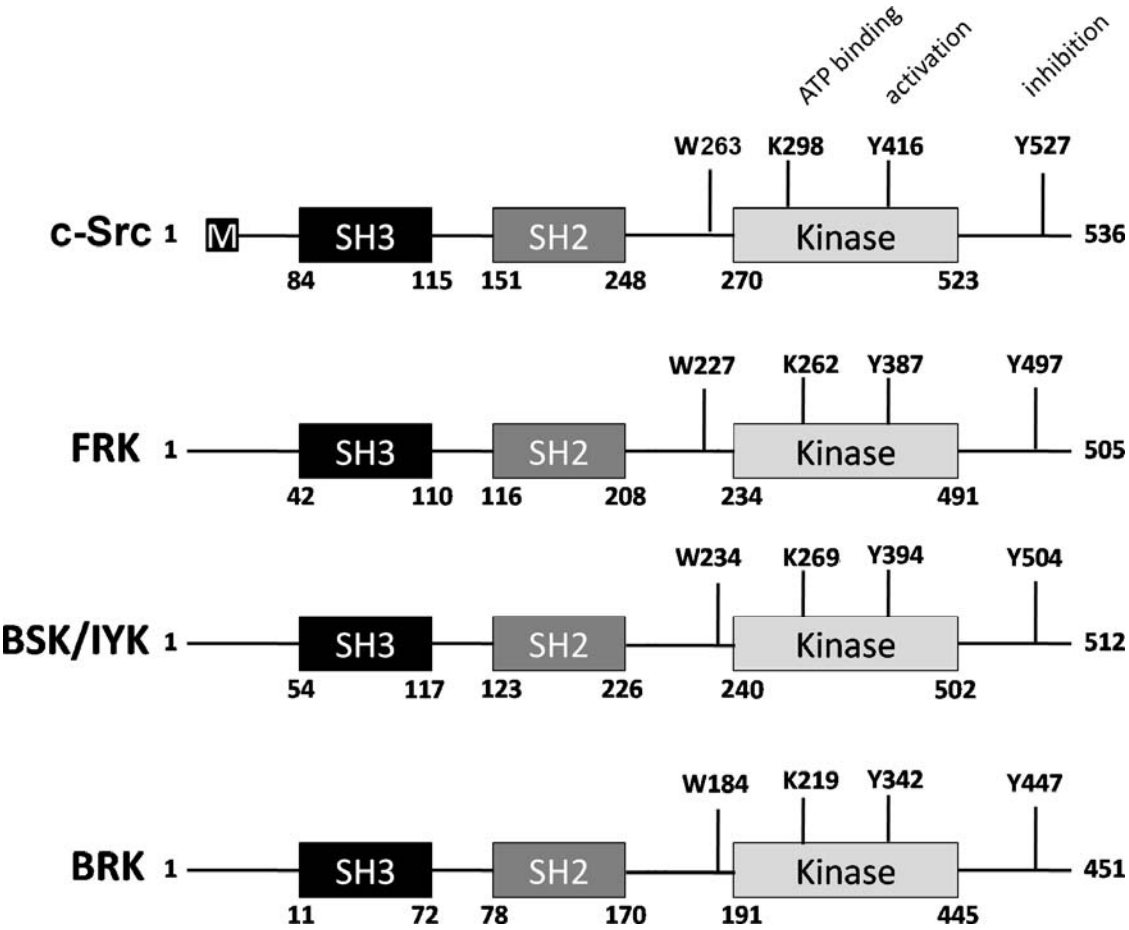


Figure 2.5. A schematic representation of the BRK, c-Src, FRK and its murine ortholog Bsk/Iyk protein structures showing the critical regulatory and functional elements. In the Src inactivation form, the SH2 domain binds to the C-terminal tyrosine 527 (FRK Y497, BSK Y504; BRK Y447), the SH2-catalytic linker which contains a key residue tryptophan 263 (FRK W227; BSK W234; BRK W184) binds to the SH3 domain and induces a conformational change in the catalytic domain. Thus, the tyrosine 416 (FRK Y387; BSK Y394; BRK Y342) located in the activation loop cannot be phosphorylated which is required for the activation of kinase activity (Ayrapetov *et al.*, 2006).

FRK differs significantly from SFKs in that it harbors a putative bipartite nuclear localization signal (NLS). The NLS comprises two clusters of basic amino acids, separated by a spacer of nine amino acids (KRLDEGGFFLTRRR motif) in the SH2 (Serfas and Tyner 2003) (**Figure 2.6**). The mouse and rat orthologs of FRK display myristoylation sequences that have been shown to potentiate membrane binding, indicating that the

intracellular targets and perhaps the cellular roles may differ across species (Sunitha and Avigan 1996; Sunitha *et al.,* 1999). The cellular and biochemical functions, and the physiological roles of FRK have not been fully characterized.

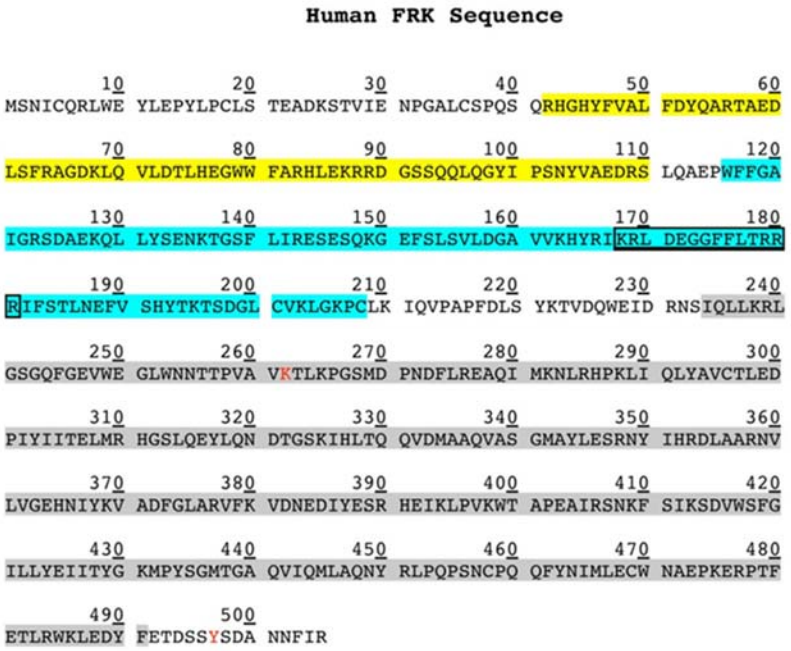


Figure 2.6. Amino acid sequence of human FRK (1-505). The SH3 domain (42-110) is in yellow; SH2 domain (116-208) in blue; kinase domain (234-491) in grey. The putative nuclear localization sequence (168-181) within the kinase domain is outlined. The regulatory C-terminal tyrosine (Y497) is in red.

2.2.2 Murine orthologs of FRK

Bsk (β-cell Src-homology tyrosine kinase), also known as Iyk (Intestine tyrosine kinase), is highly homologous to the human FRK gene (Thuveson *et al.,* 1995). Bsk was originally identified from insulinoma cells RINm5F by PCR. The cDNA was isolated from the insulin-producing cell line βTC-1 (Oberg-Welsh and Welsh 1995). The sequence comparison revealed 89% amino acid homology to FRK and 87% nucleotide identity (Oberg-Welsh and Welsh 1995), 49% to Src (Martinez *et al.,* 1987) and 49% to murine Lyn (Stanley *et al.,* 1991) (**Figure 2.5**). Bsk was also isolated from other cell lines, and

designated with another independent name Iyk. The cDNA of Iyk was also obtained by screening the primary mammary epithelial cell line 31E, using the PCR fragment amplifed from mature mouse resting mammary glands by reverse transcription PCR (RT-PCR) (Thuveson *et al.,* 1995). Another FRK homolog is Gtk (Gastrointestinal tyrosine kinase). It was cloned from rat small intestine by RNAse protection analysis (Sunitha and Avigan 1994).

The expression of Bsk was detected at low levels in normal mammary gland during the estrous cycle and pregnancy, and at undetectable levels in the end-differentiated, lactating gland (Thuveson *et al.,* 1995). Moreover, the expression of Bsk decreased with the embryonic development (Thuveson *et al.,* 1995). Bsk has been observed in intestine, especially the jejunum and ileum, with the highest expression levels, among mouse epithelial organs such as lung, liver (Oberg-Welsh and Welsh 1995), ovary, placenta, kidney and 13 day embryos (Thuveson *et al.,* 1995); while Bsk was absent in the brain, heart, spleen and testis (Oberg-Welsh and Welsh 1995). Gtk was found to be highly expressed in the small intestine and stomach, with lower expression seen in the lung, kidney, liver, spleen, brain, heart and leg muscle (Sunitha and Avigan 1994).

In normal breast epithelium, Bsk has been shown to localize to both nucleus and cytoplasm during the proliferating follicular phase and luteal phase of menstrual cycle. The localization became more nuclear at the luteal phase and predominantly nuclear after menopause, indicating that distribution of nuclear and cytoplastic localization depended on cell proliferation (Berclaz *et al.,* 2000). The linkage among the structure, localization and function have also been found. It has been elucidated that the C-terminal tyrosine residues Y497/504 of Bsk were responsible for regulation of the kinase activity and subcellular localization of Bsk, and the double mutation of Y497/504 both to phenylalanine at the C-terminus inhibited NIH3T3 cell growth (Oberg-Welsh *et al.,* 1998). This group also found the suppressive function of Y497F and Y497/504F of Bsk on the cell proliferaton of the insulin producing cell line, RINm5F cells (Anneren and Welsh 2000). It has been demonstrated that Gtk colocalized with hepatocyte growth factor (HGF) to the brush border membrane in the columnar gut epithelia cells, suggesting that Gtk might be involved in the HGF-linked pathway (Sunitha *et al.,* 1999).

Although the presence of Gtk lead to an increase in the β-cell mass, a negative regulation of islet cell growth by promoting the phosphorylation of ERK 1/2 and inhibiting the phosphorylation of p38 was also observed (Anneren and Welsh 2001). Further studies revealed that Gtk cannot only induce growth arrest following cytokine stimulation, but also promote β-cell replication after partial pancreatectomy and increases the susceptibility to streptozotocin, cooperating with SHB (SRC homology 2 domain adapter protein) (Anneren 2002). In addition, in rat pheochromocytoma cell line PC12, neurite outgrowth was induced in the presence of Gtk overexpression (Anneren *et al.,* 2000). Moreover, Gtk has also been detected to correlate with elevated phosphorylation levels of FAK (focal adhesion kinase) and SHB as well as an enhanced association between FAK and SHB, indicating that Gtk might play a role in cell differentiation and β-cell proliferation (Anneren *et al.,* 2000; Anneren *et al.,* 2003).

Chandrasekharan *et al.* generated the Iyk-deficient mice. In these mice models, no significant difference was detected in growth rate compared to wild-type models. They also examined the morphology of intestinal epithelium cells and suggested that Iyk was redundant for cell differentiation in intestine (Chandrasekharan *et al.,* 2002).

2.3 FRK-interacting proteins

Very few FRK interacting proteins have been identified. They include: phosphatase and tensin homolog (PTEN) (Yim *et al.,* 2009), Retinoblastoma protein (pRb) (Craven *et al.,* 1995), Src homology 2 domain-containing adapter protein B (SHB) (Anneren 2002) and epidermal growth factor receptor (EGFR) (Jin and Craven 2013). The following sections will describe PTEN, pRb, SHB and EGFR in details.

2.3.1 PTEN

PTEN has been recently shown to be a substrate of FRK (Yim *et al.,* 2009). The C2 domain of PTEN is reported to bind to the SH3 domain of FRK. FRK can stablize PTEN by phosphorylating the Tyr 336 in the C2 domain of PTEN, thereby protecting PTEN from ubiquitin-mediated degradation (Yim *et al.,* 2009).

PTEN plays an important role in negatively regulating the phosphoinositide 3-kinase (PI3K) signaling which is involved in the regulation of cell metabolism, proliferation and survival (Carracedo and Pandolfi 2008). PTEN can negatively regulate PI3K by converting the PIP_3 back to PIP_2 on the membrane (Makker *et al.,* 2014). As a lipid phosphatase, PTEN contains a phosphatase, C2 domain, and a lipid-binding domain which is responsible for anchoring the protein to the cell membrane, as well as several regulatory tyrosines which may modulate cell proliferation when phosphorylated (Carracedo and Pandolfi 2008).

PTEN has been identified as a frequently mutated gene on human chromosome 10q23, and its expression is lost in various cancers. Several studies have shown that the protein is a potential tumor suppressor (Li and Sun 1997; Li *et al.,* 1997; Steck *et al.,* 1997; Yim *et al.,* 2009). There are also some frequent mutation sites located in the catalytic domain, C2 domain and ubiquitinylation region, and these mutations have been found to be critically linked to the progression and development of tumors (Eng 2003; Trotman *et al.,* 2007). Mutations in PTEN have been shown to promote its membrane localization, thereby promoting the activity of PI3K signaling and impairing the tumor-suppressive function in glioblastoma (Walker *et al.,* 2004). The loss of *Pten* also contributed to the tumorigenesis in mouse models, and both lethal autoimmune disorders and various types of epithelial cancers were caused by the *Pten* allelic deletion (Di Cristofano *et al.,* 1998; Di Cristofano *et al.,* 1999).

It was recently reported that PTEN can be regulated at both transcriptional and post-translational levels (Salmena *et al.,* 2008). At the transcriptional level, several signaling pathways, including RAS and JNK, were demonstrated to be capable of regulating the transcription of PTEN, thereby modulating tumor progression (Beck and Carethers 2007; Vasudevan *et al.,* 2007; Xia *et al.,* 2007). It has been illustrated that the MEK-dependent (Mitogen-activated protein kinase kinase-dependent) pathway can also increase both c-Jun expression and the binding ability of transcription factor to the PTEN promoter (Hettinger *et al.,* 2007; Vasudevan *et al.,* 2007). At the post-translational level, the phosphorylation of several C-terminal tyrosine residues of PTEN was shown to correlate with increased protein stability (Georgescu *et al.,* 1999; Vazquez *et al.,* 2000; Torres and Pulido 2001),

while the downregulation of *Pten* stability was observed when the tyrosine residues at other sites were phosphorylated (Maccario *et al.,* 2007).

2.3.2 pRb

pRb, a known tumor suppressor, plays a role in regulating cell differentiation and cell cycle (Classon and Harlow 2002). Being a member of the pocket protein family, at least two protein-interacting pockets of pRb have been reported: the A/B pocket and a C-terminal C pocket. The former one is associated with E2Fs transcription factors (Weinberg 1995), and the latter is associated with c-Abl, which is a tyrosine kinase (Craven *et al.,* 1995).

pRb has also been reported to associate with FRK. The expression of FRK was detected at the highest level in the G1 and S phases, and at a low level during mitosis, indicating the important role of FRK in cell cycle control (Cance *et al.,* 1994; Pendergast 1996). Several studies have indicated that the SH3 domain of FRK is responsible for binding to the A/B pocket region of pRb, causing a breast cancer cell growth arrest during the G1 and S phases of cell cycle both *in vitro* and *in vivo* (Thuveson *et al.,* 1995; Chandrasekharan *et al.,* 2002; Meyer *et al.,* 2003). However, the enzymatic activity of FRK was not affected by the binding with FRK and pRb (Pendergast 1996). Hence, FRK-pRb binding may enhance the association of FRK with its substrates which may negatively regulate cell proliferation (Yim *et al.,* 2009).

2.3.3 SHB

SHB has been reported as a ubiquitously expressed protein (Welsh *et al.,* 1998). This protein contains N-terminal proline-rich motifs (Karlsson *et al.,* 1995), followed by a PhosphoTyrosine Binding (PTB) domain (Welsh *et al.,* 1998; Lindholm *et al.,* 2002), four tyrosine residues (Lu *et al.,* 2000; Lindholm *et al.,* 2002), and a C-terminal SH2 domain (Karlsson *et al.,* 1995). SHB has been previously demonstrated to regulate several receptors via SH2 domain binding, including fibroblast growth factor receptor-1 (FGFR-1) (Karlsson *et al.,* 1995; Cross *et al.,* 2002), T cell receptor (Lindholm *et al.,* 1999), Platelet-Derived Growth Factor (PDGF) receptor (Karlsson *et al.,* 1995), and Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) (Holmqvist *et al.,* 2004).

Furthermore, SHB participates in receptor tyrosine kinase signaling through forming the signaling complex upon extracellular stimuli, thereby regulating apoptosis, proliferation, differentiation and cytoskeletal physiology (Anneren *et al.,* 2003; Kriz *et al.,* 2003; Kriz *et al.,* 2006; Saldeen *et al.,* 2006). It has been reported that in SHB-overexpressing rat pheochromocytoma PC12 cells, phosphorylation levels of SHB increased upon the Nerve Growth Factor (NGF) stimulation, resulting in enhanced neurite outgrowth (Karlsson *et al.,* 1998). SHB has also been reported to associate with FRK. For example, increased phosphorylation levels of SHB were observed in cells overexpressing Gtk (Anneren *et al.,* 2003).

2.3.4 EGFR

EGFR plays an important role in the proliferation of certain types of tumor cells (Lemmon and Schlessinger 2010). The internalization of EGFR occurs via the ubiquitination and phosphorylation upon the extracellular stimuli, resulting in either degradation or reuse of EGFR (Sorkin and Goh 2008). FRK was recently reported to associate with the EGFR (Jin and Craven 2013). This work also revealed that the FRK-EGFR binding was directly through the SH2 and SH3 of FRK and the Y1173 located on EGFR which was required for ERK activation and EGFR internalization (Jin and Craven 2013). Moreover, Grb2 was also found to bind to EGFR and form a complex with EGFR subsequently, suggesting that FRK may associate with the EGFR-Grb2 complex (Jin and Craven 2013).

2.4 Intracellular localization of FRK

Unlike other Src-related kinases, human FRK has been identified as a nuclear protein (Chandrasekharan *et al.,* 2002). However, its intracellular localization has still been controversial. Human FRK has been reported to be expressed predominantly in the nuclei in COS7 monkey kidney cells (Cance *et al.,* 1994), while it has been shown to have a perinuclear localization in BT474 breast cancer cells and some other cell lines (Meyer *et al.,* 2003). It is worth mentioning that the structure of FRK provides a strong evidence for this perinuclear localization. SFKs have an N-terminal myristoylation motif MGXXXS/T required for plasma membrane-targeting. This motif is not conserved in human FRK

(Chandrasekharan *et al.,* 2002). FRK has a glutamine (Q) instead of a conserved serine (S) residue at position 6 of its N-terminal sequence (Chandrasekharan *et al.,* 2002), which may explain the absence of membrane-anchorage (Kaplan *et al.,* 1990; Brauer and Tyner 2009). Moreover, the SH2 domain of FRK has been found to contain a putative bipartite nuclear motif (KRXXXXXFFXXRRR), supporting the potential nuclear localization of FRK (Craven *et al.,* 1995).

Since human FRK has been detected in the nucleus as well as the cytoplasm in the breast cancer cell line BT-20, it is therefore possible that the localization of FRK may vary in different cell lines (Cance *et al.,* 1994). Additionally, a recent study has also shown that the intracellular localization of FRK was predominantly in the perinucleus and cytoplasm (Pilati *et al.,* 2014).

Unlike human FRK, the murine orthologs (Bsk and Gtk) possess both the consensus myristoylation motif and nuclear localization signal (Chandrasekharan *et al.,* 2002). Moreover, murine ortholog Bsk, has also been detected in both the nucleus and cytoplasm (Chandrasekharan *et al.,* 2002). Interestingly, although Gtk possesses two different localization signal motifs (the nucleus and membrane), it has been found to predominantly localize to the plasma membrane instead of nuclear localization in epithelial cells (Sunitha and Avigan 1996; Sunitha *et al.,* 1999).

2.5 Functional roles of FRK in cancer

Several lines of evidence illustrate that FRK has a potential inhibitory effect on the tumor progression. 30% of primary human breast carcinomas displayed loss of heterozygosity (LOH) at the long arm of chromosome 6 (6q 21-23), which is where the FRK gene locates (Sheng *et al.,* 1996). This FRK chromosomal region also displayed allelic loss in 40% of melanomas (Becher *et al.,* 1983; Millikin *et al.,* 1991). In addition, in certain ovarian cancers, genetic deletions in 6q have also been detected (Ehlen and Dubeau 1990). These pieces of evidence suggest the potential tumor suppressor function of FRK.

It has been reported that FRK overexpression in both epithelial and mesenchymal cells results in growth arrest, suggesting that FRK is a negative regulator of cell proliferation (Meyer *et al.,* 2003). In support of this finding, FRK depletion was shown to

induce transformation of non-tumorigenic MCF-10A epithelial cells (Yim *et al.,* 2009). Exogenous FRK has been shown to induce an inhibition in both colony formation and cell growth in NIH 3T3 fibroblasts (Craven *et al.,* 1995). Consistent with the previous results, the same inhibitory effect has also been shown in breast cancer cell lines BT474 and MCF-7 (Craven *et al.,* 1995). The anti-proliferative properties of FRK have also been studied in other types of cancer. Over-expression of FRK has been found to arrest the HeLa cells in the G1 phase of the cell cycle (Chandrasekharan *et al.,* 2002). In human glioma cell lines U251 and U87, FRK has also been shown to contribute to the suppression of cell migration and invasion. In the same study, mRNA comparison between non-tumorous and tumorous samples revealed that FRK is significantly highly expressed in non-tumor samples, suggesting FRK may possess a potential tumor suppressive function (Zhou *et al.,* 2012). As well, FRK has been shown to associate with Cdc2 and Cdk4, but not with Cdk5, Cdk6 or Cdk7 (Pendergast 1996), but the significance of the associations is unknown. These data all confirmed an inhibition to cell growth induced by overexpression of FRK (Craven et al., 1995).

Accumulating evidence also indicated this regulatory role of both human and rodent FRK in the process of cancer was through the regulation of signal transduction. A series of FRK-interacting proteins have been recently identified to be involved in cell growth regulation. Mechanistically, the overexpression of FRK has been found to inhibit cell proliferation by phosphorylating and preventing tumor suppressor PTEN from ubiquitination and degradation. The stabilization of PTEN after phosphorylation on Tyr 336 resulted in the inhibition of the PI3K/Akt signaling pathway, which suppressed downstream signaling responsible for the regulation of cell proliferation, migration and differentiation (Yim *et al.,* 2009). SHB has been confirmed to interact with FRK possibly via the SH3 domain of FRK which targeted proline-rich motifs and/or SHB phosphorylated tyrosine binding to the SH2 domain of FRK (Anneren *et al.,* 2003). The overexpression of rat FRK resulted in TRKA induction, followed by a subsequent increase in SHB phosphorylation and FAK expression (Anneren *et al.,* 2003). The interaction between SHB and FAK triggered the binding of CRKII-C3G complex to phosphorylated SHB via the SH2 domain of CRKII, thereby stimulating the neurite outgrowth through activating several RAP1-involved downstream signaling pathways (Anneren *et al.,* 2003). Rat FRK

orthologs also played an essential role in regulating the β-cell proliferation (Welsh *et al.,* 1999; Anneren and Welsh 2001). It has been reported that this regulation of cell growth may be through insulin receptor substrate-2 (IRS-2)-RAS-MAPK pathway (Anneren *et al.,* 2003). An elevated basal IRS-1/IRS-2 phosphorylation level (Anneren and Welsh 2002; Welsh *et al.,* 2002) and a consequent increase in MAPK activity (Anneren and Welsh 2001) were both detected due to the overexpression of both FRK and SHB in β-cell. FRK has been also reported to interact with the pRb, via binding to the A/B pocket domain of pRb in the human breast cancer cell lines BT474 and SK-BR-3 (Craven *et al.,* 1995). pRb acted as a tumor suppressor which regulated cell cycle during the G1 phase of the mitosis (Burkhart and Sage 2008). The interaction between tumor suppressor protein pRb and FRK was first detected in both G1 and S phases of the cell cycle in the SK-BR-3 breast cancer cell line, suggesting that FRK-Rb association may contribute to the regulation of the cell cycle (Yim *et al.,* 2009).

Human FRK has also been reported to be able to phosphorylate Y530 at the C-terminal region of Src which is responsible for negative regulation, indicating FRK may exert an important role in signaling (Cance *et al.,* 1994). A recent study revealed that FRK also regulated the signaling activity of receptor tyrosine kinases. It was demonstrated that FRK interacted with EGFR by directly binding to EGFR via the SH2 and SH3 domains of FRK, forming a complex upon EGF stimulation (Jin and Craven 2013). The EGFR expression on the cancer cell membrane was found to be suppressed, indicating the inhibitory function of FRK on EGFR signaling pathway in cancer (Jin and Craven 2013). Similar to EGFR signaling, cytokine-stimulated signaling has also been illustrated to be affected by FRK in human malignant glioma. It is suggested that FRK exerted a negative control in glioma cell migration and invasion through down-regulating the JNK/c-Jun signaling; a part of the MAPK pathway which is mainly required for cell survival, proliferation and differentiation. Therefore, JNK has been suggested to be one of the cellular targets of FRK (Zhou *et al.,* 2012). These studies all support the negative-regulatory properties of FRK in signal transduction although the entire complex mechanism of FRK regulation in the signaling has not been fully investigated. Furthermore, it is not known how the activation of FRK can potentiate its cellular function.

An oncogenic role of FRK has also been proposed in a few studies. The FRK gene has been previously found to fuse with ETV6, ETS transcription factor, endowing the fused protein with oncogenic properties in acute myelogenous leukemia (Hosoya *et al.,* 2005) although the mechanism of switching from tumorigenesis to tumor-suppressive function still remains unknown. More recently, high expression levels of FRK of protein and transcription have been detected in 4 human hepatocellular carcinoma (HCC) cell lines including HepG2, Hep3B, PLC/PRF/5 and Huh7 (Chen *et al.,* 2013). In that study, the authors found that the introduction of FRK induced an increase in cell proliferation, invasion and transformation in Hep3B cell lines. It was also recently demonstrated that FRK promoted the cell proliferation of HCC Hep3B cell line (Pilati *et al.,* 2014), suggesting FRK may be oncogenic depending on the tissue contexts.

2.6. Objectives and rationale

2.6.1 Rationale

FRK has been shown to localize to the nucleus and play key roles in the regulation of cellular function including cell survival, proliferation and migration. FRK is a candidate tumor suppressor and its overexpression in mammary gland cells was recently reported to inhibit cell growth via interacting, phosphorylating and stabilizing the tumor suppressor PTEN. However, the mechanism of FRK activation has not been fully explored. We hypothesized that like Src-family kinases, the activity of FRK is regulated by intermolecular interaction involving a C-terminal tyrosine residue. As well, much remains to be clarified about the functional differences between the proto-oncogenic Src kinase, for example, and the tumor suppressor activity of FRK. What is the expression pattern of FRK in the breast cancer cells and breast carcinomas? What is the intracellular localization pattern of FRK in breast cancer cells? Is stable overexpression of FRK in FRK-negative breast cancer cells sufficient to inhibit cell proliferation, migration and anchorage-independent growth? What signaling pathways are regulated by FRK? What is the role of FRK enzymatic activity in these processes?

A previous study demonstrated that the constitutive activation of FRK was achieved by mutating the C-terminal tyrosine 497 usually to phenylalanine (Y497F). We

hypothesize that the activity of FRK is essential in the regulation of its cellular function. The objective of this project is to investigate the effect of FRK activity on breast cancer cell proliferation and migration. We generated stable cell lines expressing the constitutively active FRK variant (Y497F) and examined the effect on cell proliferation, migration, invasion and colony formation. We also used kinome analysis to identify and validate FRK regulated signaling pathways and intermediates. We found that FRK-Y497F is significantly more active than FRK-WT and that the presence of FRK-Y497F resulted in a marked suppression of both cell proliferation and migration. Finally, we observed that both FRK-WT and FRK-Y497F inhibited the constitutive activation of several signaling molecules including STAT3.

2.6.2 Hypothesis and Objectives

2.6.2.1 Hypothesis

FRK activation suppresses cell proliferation, migration, invasion and cell formation via inhibition of specific signaling pathways in breast cancer cells.

2.6.2.2 Specific Objectives

1. To determine the expression and subcellular localization of FRK in breast cancer cells.
2. To clone and characterize constitutively active FRK mutants and generate stable cell lines.
3. To determine the effect of stable expression of FRK on the migration, proliferation and transformation of breast cancer cell lines.
4. To identify FRK-regulated signaling pathways.

3 MATERIALS AND METHODS

3.1 Reagents

All the reagents used in experiments are listed in **Table 3.1**. Names and locations of suppliers are listed in **Table 3.2**. The information regarding the primary antibodies used in the experiments is listed in **Table 3.3**.

Table 3.1. List of reagents and suppliers.

	Reagents	Suppliers
Inorganic and Organic Reagents	Acrylamide, 0341	AMRESCO
	Aprotinin, A6279	Sigma-Aldrich
	Ammonium persulfate (APS), A3678	Sigma-Aldrich
	Bisacrylamide, 0172	AMRESCO
	Crystal violet, 65092A-95	EMD
	4', 6-diamidino-2-phenylindole (FITS), D9542	Sigma-Aldrich
	Enhanced Chemiluminescence Substrate (ECL), NEL 104001EA	PerkinElmer
	Gelatin, G1890	Sigma-Aldrich
	Glycine, 0167	AMRESCO
	Laemmli sample buffer, S3401	Sigma-Aldrich
	MEM Vitamin Solution, 11120-052	Gibco
	Methanol, MX0485	EMD
	SDS, 151-21-3	Sigma-Aldrich
	Difco™ skim milk, 232100	BD
	Sodium azide, S8032	Sigma-Aldrich
	Sodium chloride (NaCl), 0241	AMRESCO
	Tris, 0826	AMRESCO
	Triton™ X-100, X-100	Sigma-Aldrich
	TWEEN® 20, 0777	AMRESCO
	Paraformaldehyde, PX0055-3	EMD
	Polyethylenimine, 25987-06-8	Sigma-Aldrich

	phenylmethylsulfonyl fluoride (PMSF), P7626	Sigma-Aldrich
	N,N,N',N'-Tetramethylethylenediamine (TEMED), 87689	Sigma-Aldrich
Bacterial Culture	Ampicillin, 0339	AMRESCO
	Trptone, 1.07213.1000	EMD
	Fermtech® Yeast Extract, 1.11926.1000	EMD
	Kanamycin sulfate, 0408	AMRESCO
	Lysogeny Broth (LB) agar, L2897	Sigma-Aldrich
Cell Culture	Dulbecco's Modified Eagle Medium (DMEM), SH30022.01	Thermo Fisher Scientific
	Fetal Bovine Serum (FBS), SH30397.03	Thermo Fisher Scientific
	Trypsin-EDTA, T4049	Sigma-Aldrich
Cloning	Agarose I™, 0710	AMRESCO
	Alkaline phosphatase, M0290S	NEB
	Adenosine Triphosphate (ATP), A1205	Teknova
	dNTP, D0056	GeneScript
	100bp DNA ladder, N3231S	NEB
	1kb DNA ladder, N3232S	NEB
	T4 DNA ligase, M0202S	NEB
	Taq Polymerase, M0273S	NEB

Table 3.2. List of names and Addresses of the Suppliers.

Suppliers	Locations
AMRESCO	North York, Ontario, Canada
BD	Mississauga, Ontario, Canada
Bio-Rad	Hercules, California, USA
Cell signaling	Whitby, Ontario, Canada
Corning	NY, USA
Dojindo	Rockville, Sunnyvale, USA
EMD	Madison, Wisconsin, USA
GeneScript	Piscataway Township, New Jersey, USA
Gibco	Burlington, Ontario, Canada

Invitrogen	Burlington, Ontario, Canada
New England Biolab (NEB)	Mississauga, Ontario, Canada
Olympus	Richmond Hill, Ontario, Canada
Pall Corporation	Washington, NY, USA
PerkinElmer	Boston, Massachusetts, USA
Qiagen	Mississauga, Ontario, Canada
Santa Cruz	Santa Cruz, California, USA
Sigma-Aldrich	Oakville, Ontario, Canada
Teknova	Hollister, California, USA
Thermo Fisher Scientific	Whatham, Massachusetts, USA
VWR	Mississauga, Ontario, Canada
Whatman	Piscataway, New Jersey, USA

Table 3.3. List of the antibodies and suppliers (R refers to rabbit; M refers to mouse).

Antibodies (Species) and working dilution	Suppliers
β-Tubulin (R) 1:1000	Santa-Cruz
FRK (m) 1:1000	Santa-Cruz
Phospho-tyrosine (pY20) (m) 1:1000	Santa-Cruz
BRK (m) 1:1000	Santa-Cruz
Sam68 (R) 1:1000	Santa-Cruz
GFP (R) 1:1000	Santa-Cruz
AKT (R) 1:1000	Cell signaling
Phospho-AKT (R) 1:1000	Cell signaling
MEK 1/2 (R) 1:1000	Cell signaling
Phospho-MEK 1/2 (R) 1:1000	Cell signaling
ERK 1/2 (m) 1:1000	Cell signaling
Phospho-ERK 1/2 (R) 1:1000	Cell signaling
P38 MAPK (R) 1:1000	Cell signaling
Phospho- P38 MAPK (R) 1:1000	Cell signaling
STAT3 (R) 1:1000	Cell signaling
Phospho-STAT3 (R) 1:1000	Cell signaling
JNK (m) 1:1000	Santa-Cruz
Phosphor-JNK (m) 1:1000	Santa-Cruz

3.2 Cell lines and Cell culture

Human embryonic kidney 293 (HEK293) cells and breast cancer cell lines (AU565, BT20, MDA-MB-231, MDA-MB-468, MDA-MB-435, SKBR3, T47D, HBL100, MCF7, Malme 3M) were purchased from ATCC (American Type Culture Collection). All the cells used in the experiment were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and incubated in a humidified CO_2 incubator (Thermo Fisher Scientific, HERAcell 150i) with 5% CO_2 at 37℃. Cells were detached by 0.25 % (w/v) Trypsin-EDTA and split into new culture plates when the confluence reached 85-95 %.

3.3 Cloning of human FRK mutants

3.3.1 Plasmids and cloning sites

FRK-WT (wild type) and FRK-K262M were obtained from Dr.Lukong's lab. FRK-Y497F was amplified by a polymerase chain reaction (PCR) using purified FRK-WT fragment as a template. For the GFP-FRK constructs (GFP-FRK-WT, GFP-FRK-K262M, and GFP-FRK-Y497F), purified PCR products (FRK-WT, FRK-K262M, and FRK-Y497F) were amplified using the corresponding primers, and sub-cloned into the *Xho*I and *Pst*I sites of the plasmid pEGFP-C1, respectively. Using the same strategy, the retroviral LPC-FRK-WT was constructed by inserting FRK into the *Xho*I site of the retroviral plasmid pLPC. LPC-FRK-Y497F was mutated by site-directed mutagenesis using LPC-FRK-WT as a template. These LPC-FRK mutants were used for further generation of stable cell lines (refer to **section 3.4**). Primers used in the experiments were purchased from Invitrogen. All the restriction enzymes and the corresponding enzyme buffers were purchased from New England Biolabs (NEB). Designed primers and restriction enzyme sites are listed in table 3.4. Plasmids pEGFP-C1 and pLPC were both generously provided by Dr. Stephane Richard (McGill University, Canada). The general cloning procedure is briefly described in **section 3.3.2**.

3.3.2 General cloning technique

3.3.2.1 PCR, digestion, purification and mutagenesis

Single PCR reaction was set up in a total volume of 50 µL, containing 1.25 units Taq polymerase with 10× Standard Taq reaction buffer, 10 mM dNTPs, 10 µM forward primer, 10 µM reverse primer, 50-100 µg template DNA, and nuclease-free water. The thermocycling conditions for PCR amplification consisted of 2 minutes initial denaturation at 95℃, followed by 30 cycles (30 seconds at 95℃, 30 seconds at annealing temperature, and 15 minutes at 68℃), and 5 minutes final extension at 68℃. Site-directed mutagenesis was performed by using KAPA HiFi™ HotStart DNA polymerase system (KAPABIOSYSYTEMS, KK2101) following the manufacturer's standard protocol. PCR was performed using the C1000™ Thermal Cycler (Bio-Rad, 185-1148). Vectors and inserts were both digested with restriction enzymes, and analysed by 1% (w/v) agarose gel electrophoresis. The inserts and linearized vectors were then extracted from the agarose gel. To prevent the re-circularization of vectors, 1-2 units of alkaline phosphatase were added to remove the 5' phosphate groups when the single digestion was applied before the purification step. Digested vectors were incubated with alkaline phosphatase and NEBuffer 3 in a total volume of 20 µL at 37℃ for 1 minute. The subsequent PCR and gel purification prior to ligation were conducted by using the QIAquick PCR Purification Kit (Qiagen, 28104) and the QIAquick Gel Extraction Kit (Qiagen, 28704) following the manufacturer's instructions.

Table 3.4. List of sequence and designed restriction enzyme sites of the primers.

Mutants	Sequences	Designed Restrict Enzyme
GFP-FRK-WT For	5'-AAACTCGAGGCCACCATGAGCAACATCTGT-3'	*Xho*I
GFP-FRK-WT Rev	5'-AATCTGCAGTCATCTTATGAAGTT-3'	*Pst*I
GFP-FRK-Y497F For	5'-AAACTCGAGGCCACCATGAGCAACATCTGT-3'	*Xho*I
GFP-FRK-Y497F Rev	5'-AATCTGCAGTCATCTTATGAAGTTATTTGCATCTGAAAATGAAGA-3'	*Pst*I
LPC-FRK-WT For	5'-AAACTCGAGGCCACCATGAGCAACATCTGT-3'	*Xho*I

LPC-FRK-WT Rev	5'-GCCCTCGAGTCATCTTATGAAGTTATTTGC-3'	*Xho*I
LPC-FRK-Y497F For (Mutagenesis)	5'-GAAACAGACTCTTCA**TTT**TCAGATGCAAATAACTTC-3'	N/A
LPC-FRK-Y497F Rev (Mutagenesis)	5'-GAAGTTA**TTT**GCATCTGAAGAGTCTGTTTC-3'	N/A

Note that the restriction sites are underlined, and mutation sites of mutagenesis are highlighted in bold.

3.3.2.2 Ligation

The purified vectors and restriction inserts were ligated by using 1× T4 DNA ligase reaction buffer, 1 μL T4 DNA ligase, 1 μL Adenosine Triphosphate (ATP), and nuclease-free water in a total reaction volume of 20 μL. The ligation mixture was incubated at 16℃ overnight. Ligated plasmids were transformed into *E.coli* DH5α competent cells the next day. Competent cells treated by calcium chloride were prepared using previous lab protocols.

3.3.2.3 Transformation of *E.coli*

The DNA of interest was transformed into *E.coli* DH5α cells which were used as a host. 5 μL ligation mixture was added into 50 μL competent cells once the cells were thawed, leaving it on ice for half an hour. Subsequently, 45 seconds exposure to a heat shock at 42℃ and 2 minutes cold shock were applied. 500 μL antibiotic-free LB Broth medium, containing Tryptone, Fermtech® Yeast Extract, NaCl and ddH_2O, was added into mixture, and the mixture was incubated in a shaker at 37℃ for 1 hour. Following centrifugation at 20,000 × g for 30 seconds, 450 μL cell supernatant was removed. The remaining cells were resuspended by a pulse vortex, and plated on LB agar plates containing the 10% appropriate antibiotic(s) (kanamycin for pLPC; ampicillin for pEGFP-C1). The selectable antibiotic resistance markers on plasmids facilitated the screening of the recombinants. The recombinants which the plasmid DNA was successfully transformed into bacterial was selected by picking up single colonies after 16-18 hours culture at 37℃. The plasmid DNA

was prepared and sent for sequencing to verify the mutations. Sequencing was performed by National Research Council Canada DNA sequencing facility (Saskatoon, SK).

3.4 Generation of stable cell lines

Two stable cell lines were generated by the transfection of FRK-WT and FRK-Y497F constructs into MDA-MB-231, respectively. Using a similar cloning strategy mentioned previously (refer to **section 3.3.2**), two FRK fragments (FRK-WT and FRK-Y497F) were subcloned into the *Xho* I site in pLPC retroviral vector with a puromycin selection marker, respectively. HEK 293-derived Phoenix packaging cells were transfected with the retroviral constructs, and the viral supernatant was used to infect MDA-MB-231 cells. pLPC-containing cells were selected with puromycin. All untransduced MDA-MB-231 cells died within 7 days of selection. The selected stable cell lines were cultured in puromycin for 4 weeks. Stable cells were obtained to measure the FRK expression level by Western blot analysis using a commercial FRK antibody generated from a C-terminal epitope (**Table 3.4**).

3.5 Cell assays

3.5.1 Cell transient transfection

Human FRK and its mutants were introduced into HEK 293 cell using transient transfection with polyethylenimine (PEI) (Polyscience, Warrington, PA, USA). PEI stock solution was prepared at a concentration of 1 mg/mL in water. Aliquots were stored at -20℃. A day prior to transfection, HEK 293 cells were seeded into 6-well plate (BD, 353046) in DMEM. Transfection was performed when cells were at 70% confluence. 5 μg DNA of interest (refer to **section 3.3.2**) was mixed with the 107.5 μL 0.15 M NaCl by a gentle vortex for 10 seconds. 15 μL 1% PEI was then added into the mixture followed by another pulse vortex for 10 seconds, and then the mixture was incubated at room temperature for 20 minutes. The medium in the 6-well plate was replaced with fresh pre-warm DMEM (1 mL/well) and the PEI mixture was added dropwise into each well and distributed evenly in the well. The efficiency of the transfection was monitored by GFP expression after 24-hour incubation if the DNA was cloned with GFP tag.

3.5.2 Cell lysate preparation

The whole procedure was conducted at 4℃ before adding the Laemmli sample buffer. Cells were scraped off from one 10 cm culture plate, transferred to pre-cooled micro-centrifuge tubes and washed with ice-cold PBS for two times by centrifuging at 750 × g for 5 minutes. Lysis buffer (10% Triton, 1.5 M NaCl, 1 M Tris [pH 7.4], aprotinin, 100 mM phenylmethylsulfonyl fluoride, and cold dH_2O) was used to lyse cells for 30 minutes on ice. Cell debris were removed by a full speed centrifugation for 15 minutes, and protein concentration was measured by the Lowry's assay. Then 2× Laemmli sample buffer was added to the lysates and the total lysates were boiled at 100℃ for 5 minutes. Prepared cell lysates were stored at -20℃.

3.5.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Proteins were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using vertical Mini-PROTEAN® Tetra cell electrophoresis system (Bio-Rad 165-8006). A gel of 1.5 mm thickness was clamped between a short plate and a spacer plate, and the casting frame was assembled on casting stands. Based on the molecular weight of target proteins, the 10% resolving gel was chosen for SDS-PAGE. The 10% resolving gel consists of dH_2O, 375 mM Tris-HCl (pH 8.8), 30% (w/v) acrylamide/bisacrylamide (29:1), 0.4% (w/v) SDS and 0.16% (w/v) ammonium persulfate, and 0.1% N,N,N',N'-Tetramethylethylenediamine. Stacking gel consisted of dH_2O, 0.5 M Tris-HCl (pH 6.8), 30% (w/v) acrylamide/bisacrylamide (29:1), 0.4% (w/v) SDS and 0.16% (w/v) ammonium persulfate, and 0.1% N,N,N',N'-Tetramethylethylenediamine. After the solidification of the resolving gel, appropriate well-forming combs (10-well and 15-well) were inserted in the stacking gel according to the loading volume of the samples. Preheated samples and ColorPlus prestained protein ladder (NEB, P7711S) were loaded when the polymerization of the gel was completed. SDS-PAGE was performed within 1× running buffer (0.1% SDS, 25mM Tris, 192 mM glycine, and dH_2O; pH 8.3) at a constant voltage of 125 V for 90 minutes at room temperature. Subsequently, the gel was analyzed by Western blot analysis (refer to **section 3.5.4**).

3.5.4 Western blot analysis

The transfer stack was prepared as follows: the SDS-PAGE gel was overlayed with the BioTrace™ nitrocellulose membrane (Pall Corporation, P/N 66485), and was sandwiched between 3 sheets of 3 MM filter paper (Whatman 3030-6189). The nitrocellulose membranes were pre-soaked in the transfer buffer (20% methanol, 25 mM Tris, 192 mM glycine, and dH_2O; pH 8.3) at 4℃ for 15 minutes. For transferring proteins to the nitrocellulose membrane, the prepared transfer stack was placed in Mini Trans-blot® electrophoretic transfer apparatus (Bio-Rad, 170-3930) within transfer buffer, and then a constant voltage of 100 V was applied for 80 minutes at 4℃.

The transferred nitrocellulose membrane was soaked in blocking solution (5% Difco™ Skim Milk, 0.05% sodium azide, and 1× TBST) at room temperature for 25 minutes, followed by a brief rinse with 1× TBST and incubation with primary antibody overnight at 4℃. The next day the membrane was washed with 1× TBST (TBS [10×], TWEEN® 20, and dH_2O) three times, 15 minutes each, and was then incubated with corresponding secondary antibody at room temperature for 40 minutes. Thereafter, three washes of TBST, 5 minutes each, were applied again. The western blot images were acquired using a signal development technique with enhanced chemiluminescence substrate according to the manufacturer's instruction. The density of the bands was analyzed by image J, and the numbers given under the bands were all normalized to their corresponding controls. This technique was used for analyzing all of the data obtained in experiments.

3.5.5 Subcellular fractionation

Subcellular fractionation was performed using the ProteoExtract Subcellular Proteosome Extraction Kit (EMD, 539790) following the company's recommendations. Briefly, cells cultured in 10 cm dishes were washed in 2 mL wash buffer (at 4℃ for 2 minutes) supplied with the kit. The cells were then subjected to a centrifugation at 750 × g for 5 minutes and the supernatant was discarded. The cells were washed to insure the quality before fractionation, as previously described.

The fractionation process was started by incubating the cells with 1 mL extraction buffer I and 5 µL protease inhibitor cocktail (supplied in the kit). Cells were then incubated at 4℃ for 10-minute. A gentle centrifugation was required for isolating the cytosolic part which was obtained in the supernatant. The remaining mixture was mixed with 1 mL extraction buffer II and 5 µL protease inhibitor cocktail (supplied in the kit) and were incubated at 4℃ for 30 minutes. The membrane fraction in the supernatant was isolated by a gentle centrifugation. To obtain the nuclear fractions, 500 µL extraction buffer III, 5 µL protease inhibitor cocktail and 1.5 µL Benzonase Nuclease (supplied in the kit) were all added into the rest of mixture. A 10-minute incubation at 4℃ and a subsequent gentle centrifugation were applied. The supernatant was collected as the nuclear fractions. 500 µL extraction buffer IV and 5 µL protease inhibitor cocktail (supplied in the kit) were then added into the remaining mixture. Lastly, the cytoskeletal matrix was isolated.

3.5.6 Immunofluorescence

This technique was performed based on the protocol described in a previous study (Miah *et al.,* 2012). Immunofluorescence was performed on cells fixed on the coverslip. The whole procedure was carried out at room temperature. Cells were incubated in 2% (w/v) paraformaldehyde diluted in PBS for fixation for 20 minutes, followed by permeabilization with 1% Triton™ X-100 for 20 minutes. After two PBS rinses, cells were incubated with primary antibody for 1 hour. Two PBS washes were applied again followed by 1.5-hour secondary antibody staining conjugated with fluorescein isothiocyanate, covered by a foil to avoid the light. 4', 6-diamidino-2-phenylindole (FITS) staining was used to indicate the nucleus. Immunofluorescence was examined using a fluorescence microscope (Olympus, 1X51). Images were photographed at 40X magnification.

3.5.7 *In vitro* proliferation assay

Cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8) (Dojindo, CK04-05) according to the manufacturer's protocol. CCK-8 contains water-soluble tetrazolium salts (WSTs) (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) which can be reduced by dehydrogenase and form an orange water-soluble

formazan dye, indicating cell mitochondrial viability. Exponentially growing stable cells were collected and seeded into 96-well plates (BD, 353077) in 100 μL culture medium (1000 cells/well). Control groups without cells were set up. The cell proliferation assay was designed for a period of 4 days. After 0, 24, 48, 72 and 96 hours, 10 μL CCK-8 solution was added into each well and then the cells were incubated for 2 hours. Following treatment with CCK-8, absorbance at 485 nm was measured using a POLARStar OPTIMA microplate reader (BMG Labtech, 413-1040). Each independent experiment was repeated six times.

3.5.8 Wound-healing assay

A confluent monolayer of cells was wounded by scratching a cross on a 10 cm cell culture plate with a 1000 μL sterile pipette tip. The old culture medium was then aspirated and replaced with new culture medium supplemented with 10% FBS. The center of wound was photographed by using an inverted microscope (Olympus, CK2) at time points of 0, 12, 18, 24 and 36 hours. The percentage of open area was analyzed and quantified by the TScratch software (Geback *et al.,* 2009). Each independent experiment was repeated at least three times.

3.5.9 Invasion assay

Cell invasion assay was performed by using transwell plates incorporating a polycarbonate membrane with 8.0 μm size pore (Corning, 3422) following the modified method which was originally described in a previous study (Miah *et al.,* 2012). Inserts were precoated with 100 μL 0.15% gelatin and incubated at 37 ℃ for 1 hour to produce a three-dimensional gel. Cells were counted and resuspended (1×10^5 cells/well) in serum-free culture medium and added into the inserts. Each insert was placed in the lower chamber containing 10% FBS culture medium. Following 4 hours incubation, culture medium in insert and chamber were removed and non-migrated cells were swabbed by Q-tips from the upper surface of the insert. The membrane was fixed with 20% methanol for 30 minutes at room temperature. Thirty minutes crystal violet staining on cells that attached to the polycarbonate membrane at the bottom of insert was used to evaluate the invasive ability. Each experiment was repeated three times.

3.5.10 Soft agar assay

0.61% (w/v) semisolid bottom agar containing 10% FBS culture medium was prepared and poured on 6 cm cell culture plates (Sigma-Aldrich, D8054). MEM Vitamin Solution (100×) was additionally added for boosting cell growth and viability. Stable cells were collected from exponential phase and were resuspended in 0.36% (w/v) top agar. Top and bottom agar contained the same components except for the percentage of agar. Low-melting top agar mix was then covered on the bottom agar. Plates were overlaid with fresh agar every 7 days to maintain the nutrient elements. Three parallel plates were set up for each cell line including the control. Colonies were counted after a 3-week incubation. Colonies larger than ~0.1 mm in diameter were considered as positive.

3.6 Kinome assay

The kinome microarray was performed by Dr. Scott Napper's lab. The HEK 293 cells transfected with FRK-WT and untransfected HEK293 as control were both cultured to confluency in 10 cm culture plates respectively. Cells were subsequently collected and lysed in 100 µL of prepared lysis buffer [Aprotinin (1 µg/ml), EDTA (1 mM), EGTA (1 mM), NaCl (150 mM), NaF (1 mM), Na_3VO_4 (1 mM), leupeptin (1 µg/ml), PMSF (1 mM), Tris-HCl (pH 7.5, 20 mM), Triton (1%) and sodium pyrophosphate (2.5 mM)]. The lysates were then mixed with 70µL of an activation mix [ATP (50 µM), Brij-35 (0.05% v/v), BSA (0.25 mg/ml), ^{32}P ATP (2 mCi/ml), Glycerol (50%), and $MgCl_2$ (60 mM)] and applied to the microarrays. These microarrays were incubated for 2 hours at 37°C and subsequently washed with 45mL of primary buffer [1% Triton X-100 in TBS buffer] and 45mL of secondary buffer [NaCl (2 M) and 1% Triton X-100 in TBS buffer]. Followed by air-drying, the microarrays were exposed under the phosphorimager screen for 1 week. Images were captured by using the TYPHOON® scanner (GE Healthcare) and processed by using the ImageQuant TL v2005 software. Signal reproducibility was analysed by normalizing the acquired data and correcting for background noise. The signal strength was calculated by using the GENESPRING® (Agilent Technologies) software.

3.7 Statistics

Statistical analyses were conducted using SPSS 16.0 (SPSS, USA). The normality of the data distribution was analyzed using Kolomogrov-Smirnov test. The homogeneity of variance of samples was tested by Levene's Test. One-way Analysis of Variance (ANOVA) followed by a Tukey *post-hoc* analysis was used for data comparisons. Data were represented by the mean ± S.D. (Standard Deviation) based on at least three independent experiments. A p value of less than 0.05 was considered significant ($*p < 0.05$, $**p < 0.005$).

4. RESULTS

4.1 Expression of FRK in human breast cancer cell lines

In the present study, using Western blots, the levels of FRK protein were determined in different breast cancer cell lines from various molecular subtypes including: HER2+ cells (AU565, SK-BR-3, HCC1419, HCC1569); ER+ cells (MDA-MB-361, MDA-MB-134-VI); ER+/HER2+ cells (BT483, MCF7); triple negative cells (MDA-MB-231, HCC1395, BT549, MDA-MB-157, HCC1806), triple positive cells BT474 and normal immortalized mammary epithelial cell line MCF10A. The relative levels of FRK was normalized to the expression of a house keeping gene, β-tubulin and then normalized to MCF10A which is a non-cancer cell line. The levels of FRK were found to be lowest in the triple negative cell lines MDA-MB-231 (0.31-fold) and HCC1395 (0.59-fold) compared to MCF10A (**Figure 4.1**). However, FRK was highest in the triple positive cell line BT474 (6.76-fold), triple negative cell line HCC1806 (6.61-fold), as well as ER/PR positive cell lines MCF7 (5.92-fold) and BT483 (5.12-fold) compared to MCF10A (**Figure 4.1**).

Our goal was to also identify a breast cancer cell line with low or undetectable FRK levels that could be used to generate an FRK stable cell line. Of the 13 cell lines examined, FRK was expressed lowest in the highly metastatic and tumorigenic cell line MDA-MB-231. These characteristics make MDA-MB-231 an ideal candidate for the stable expression of FRK for both *in vitro* and *in vivo* studies.

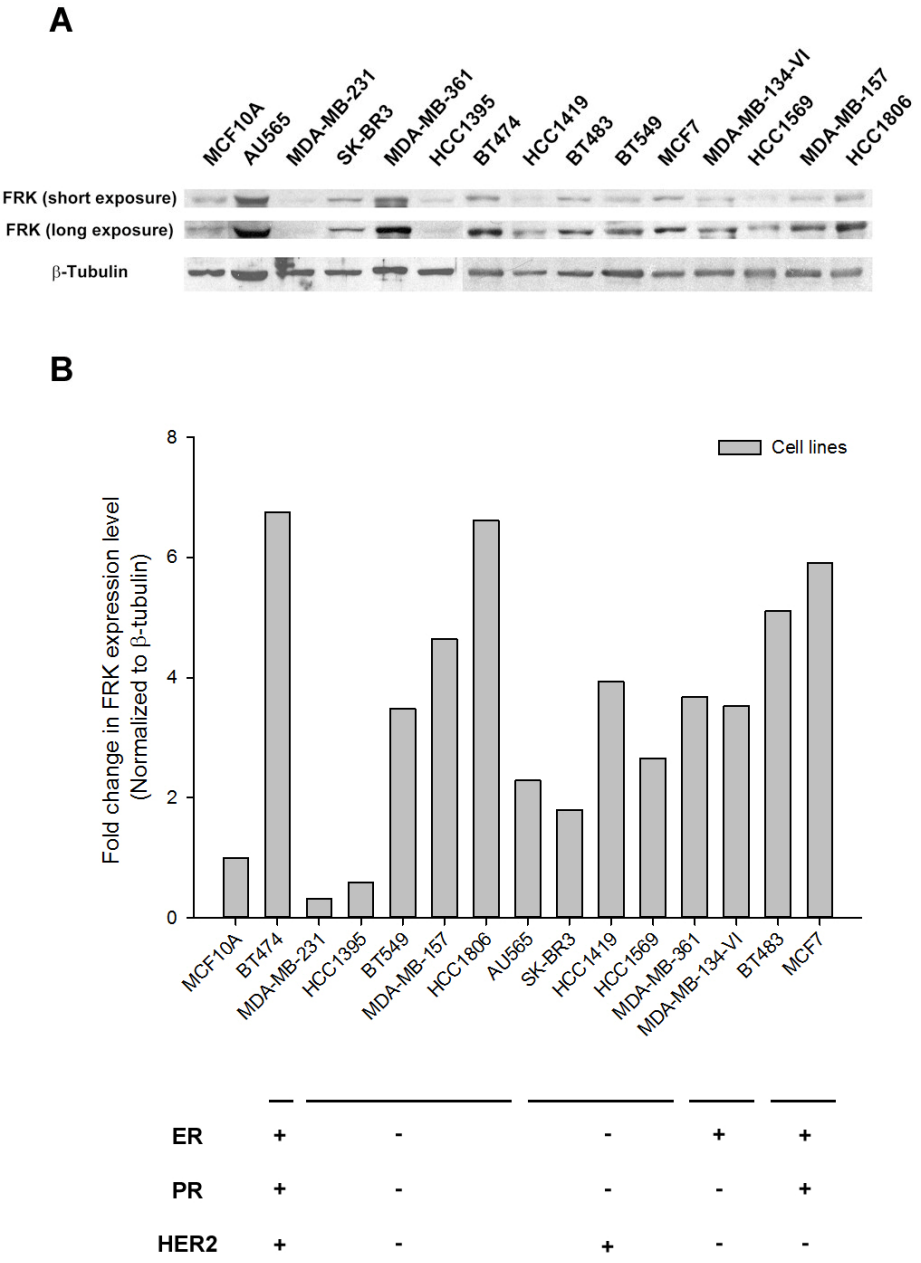


Figure 4.1. Levels of FRK protein in various human cell lines. (A) FRK is expressed in various human cell lines: triple positive (BT474), triple negative (MDA-MB-231, HCC1395, BT549, MDA-MB-157, and HCC1806), HER2 positive (AU565, SK-BR-3, HCC1419, and HCC1569), ER positive (MDA-MB-361, MDA-MB-134-VI), ER/PE positive (BT483, MCF7), normal immortalized mammary epithelial cell line MCF10A. (B) The FRK protein level in each cell line was normalized to its corresponding β-tubulin control and then normalized to MCF10A. The quantitative analysis was presented by the percentage of levels of FRK protein showing in the histogram.

4.2 Activity of GFP-FRK mutants

In previous studies, mutations of murine FRK gene at 497 from tyrosine (Y) to phenylalanine (F) was shown to increase its kinase activity (Oberg-Welsh *et al.,* 1998). Studies on BRK showed that GFP-BRK-Y447F is significantly more active than GFP-BRK-WT, and that kinase-defective BRK (GFP-BRK-KM) has no enzymatic activity (Miah *et al.,* 2012). As shown in **Figure 2.5**, FRK has a functional architecture similar to that of BRK and Src family kinases. Like Src kinases, BRK is negatively regulated by the phosphorylation of the C-terminal tyrosine 447, analogous to the regulatory Y530 of human Src and Y497 in human FRK (Derry *et al.,* 2000; Qiu and Miller 2002). The constitutive activation of the Src family of kinases is triggered by the mutation of the C-terminal tyrosine to phenylalanine.

In the present study, we aimed at determining the role of key conserved residue in human FRK activity. We also examined whether reintroduction of the expression of FRK in MDA-MB-231 cell line that were devoid of endogenous FRK expression, could alter its phenotype and oncogenic properties. Human FRK constructs: GFP-FRK wild-type (WT); kinase-defective GFP-FRK K262M (KM); GFP-FRK-ΔC (lacking C-terminal amino acids 497-505); and constitutive construct GFP-FRK-Y497F (Y497F) were generated and stably transfected in MDA-MB-231 cells. All of the human FRK mutants were cloned as recombinant proteins in pEGFP expression vector as previously described (**Section 3.3.1**).

As shown in **Figure 4.2**, using anti-phosphotyrosine antibody (pY20) to detect the level of tyrosine phosphorylation (a measure or indication of kinase activity) we observed that both GFP-FRK-Y497F (lane 2) and GFP-FRK-ΔC (lane 1) displayed a significantly higher kinase activity as compared to GFP-FRK-WT (lane 3). GFP-FRK-Y497F induced the phosphorylation of several potential substrates as compared to GFP-FRK-ΔC (**Figure 4.2, upper panel**). The enhanced kinase activity of GFP-FRK-Y497F indicated that the C-terminal tyrosine 497 is essential for the regulation of FRK activity. As expected, the kinase activity was significantly suppressed in the FRK-KM expressing cells compared to the control parental HEK 293 cells (lane 4 and 5 respective) (**Figure 4.2**).

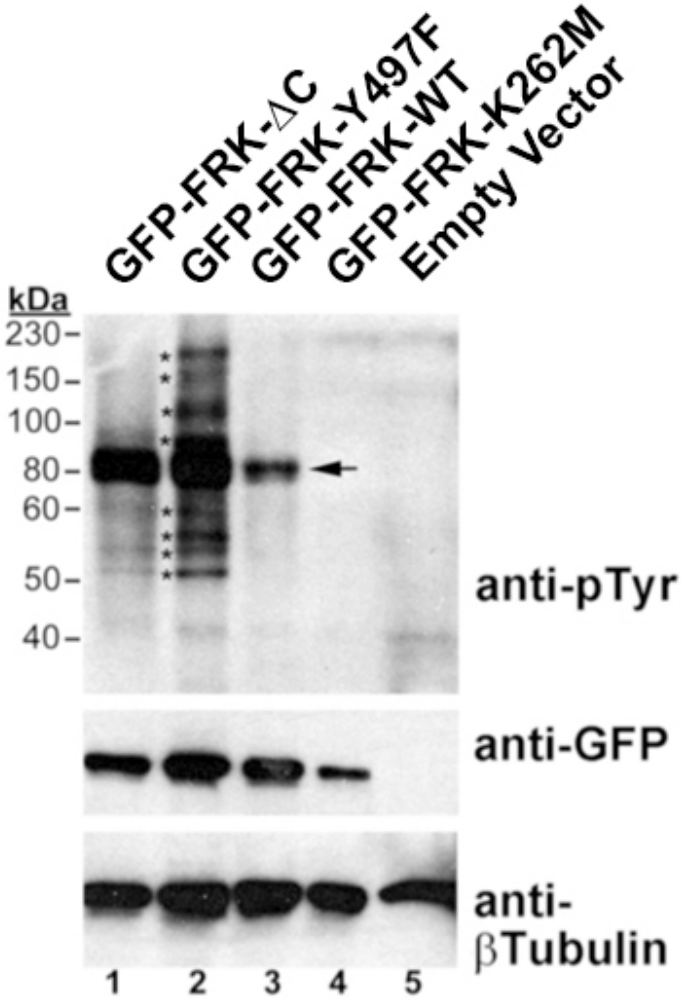


Figure 4.2. FRK-Y497F mutant is significantly more active than WT. HEK 293 cells were transfected with GFP-FRK constructs (WT, K262M, ΔC (497-505), Y497F). Cell lysates were immunoblotted using anti-phosphotyrosine antibody pY20. GFP-FRK expressions were detected by anti-GFP antibodies (middle panel). The asterisks in lane 2 (top panel) show the positions of potential FRK substrates. The arrow shows the potential auto-phosphorylation of FRK in lane 1, 2 and 3. Lane 4 contained GFP-FRK-K262M (kinase-dead) and reveals no tyrosine-phosphorylated substrates as expected. The experiment was repeated at least three times and the figure was the representation of the average result.

4.3 Subcellular localization of FRK in human cells

4.3.1 Subcellular localization of endogenous FRK in human breast cancer cells

Human FRK has a nuclear localization signal (KRLDEGGFFLTRRR motif) embedded in the SH2 domain (Serfas and Tyner 2003). Unlike the mouse FRK ortholog BSK/IYK, the human FRK lacks the myristoylated N-terminal consensus sequence (MGXXXS/T) that enables membrane anchorage (Serfas and Tyner 2003) (**Figure 2.6**). We hypothesized that human FRK localizes in the nucleus; however, using immunofluorescence on FRK-positive breast cancer cell lines AU565, SKBR3, MDA-MB-468, we observed that FRK was predominantly localized in the non-nucleus (**Figure 4.3**). In order to validate the localization of endogenous FRK determined by immunofluorescence, subcellular fractionation followed by Western blotting using specific antibodies directed against FRK, Sam68 (a Src-Associated substrate in Mitosis of 68 kDa) and PTEN was performed on AU565 and SKBR3 (**Table 4.4**). Sam68, a known nuclear RNA-binding protein served as a control for nuclear localization (Lukong and Richard 2007). Endogenous FRK was detected mainly in the cytosolic fraction; however, low levels of FRK were observed in the isolated membrane fractions (**Figure 4.4**).

Based on the literature, FRK was shown to promote the stability of PTEN in MCF7 cells (Yim *et al.,* 2009). We were also interested in the association between PTEN and FRK. We used subcellular fractionation and western blots to analyze the protein level of PTEN to examine if the localization of PTEN correlated with that of FRK. Interestingly, we found that the localization of PTEN did correlate with that of FRK since PTEN also localized predominantly in the cytosol and membrane fractions.

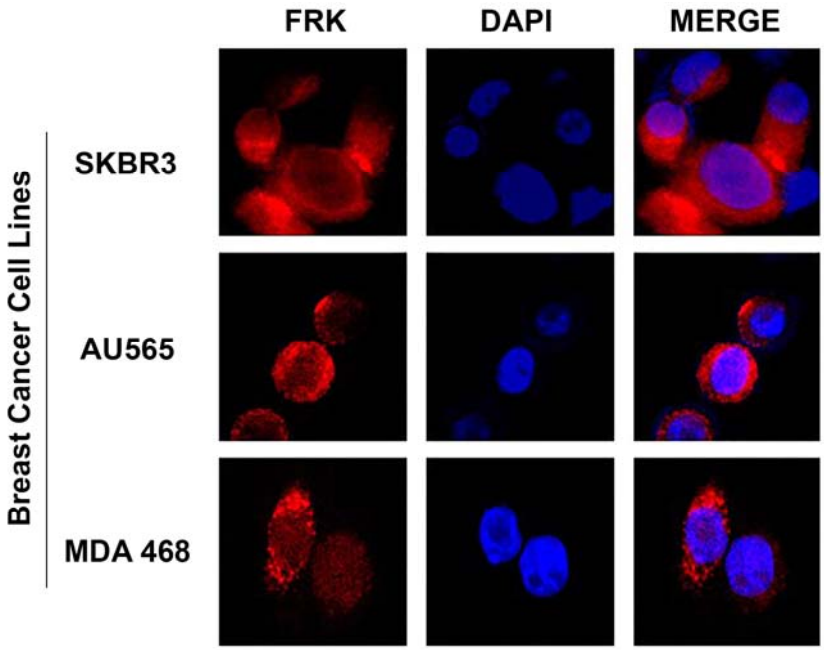


Figure 4.3. Endogenous FRK localizes in the cytoplasm in breast cancer cells. The indicated breast cancer cells were seeded onto coverslips in 6-well cell culture plates and immunofluorescence was performed using rabbit anti-FRK antibodies (red). The cells were also stained with DAPI to visualize the nucleus (blue).

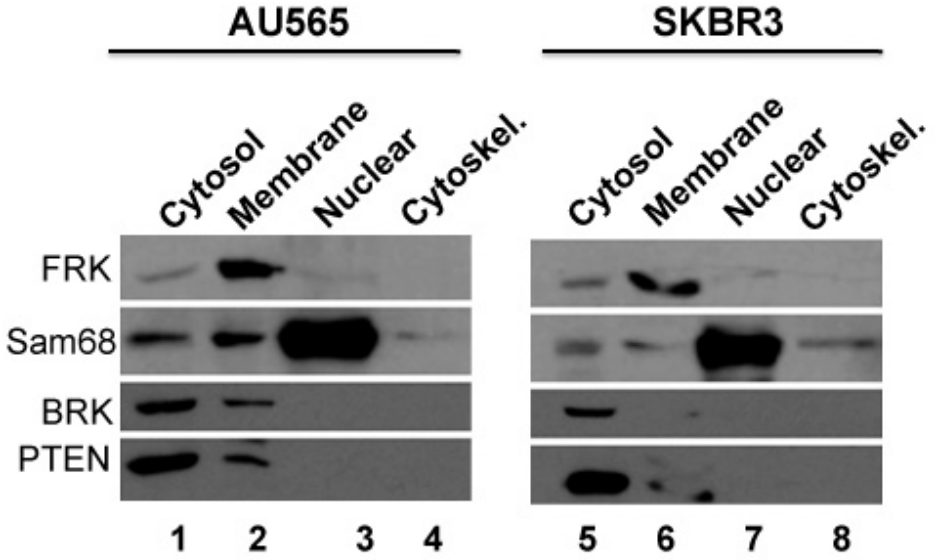


Figure 4.4. Endogenous FRK predominantly localizes in the membrane in breast cancer cells. Subcellular fractionation of breast cancer cells AU565 and SKBR3 was performed using the ProteoExtract Subcellular Proteosome Extraction Kit. Cell lysates of the various fractions were immunoblotted with anti-FRK, anti-Sam68, anti-BRK and anti-PTEN antibodies. Sam68 is a nuclear protein and was used as a control for nuclear localization.

4.3.2 Subcellular localization of exogenous FRK in human cells

In order to further substantiate the cytoplasmic or membrane cellular localization of FRK, HEK293 cells were transiently transfected with FRK (FRK-WT, FRK-KM, FRK-Y497F) using plasmid constructs that were tagged with GFP (to enable the evaluation of exogenous FRK). On examination of the GFP immunofluorescence, all FRK plasmid constructs exhibited a non-nuclear staining, however, the distribution of FRK-Y497F was punctate-like (**Figure 4.5A**). To ensure that GFP had no effect on the localization of FRK, FRK plasmids lacking a GFP insert (LPC-FRK-WT and LPC-FRK-Y497F) were transiently transfected in HEK293 cells. The LPC-FRK proteins encoded by the transiently transfected constructs displayed the same cytoplasmic localization pattern as seen with the FRK-GFP-tagged constructs (**Figure 4.5B**). We therefore concluded that both endogenously and exogenously expressed FRK localize in the cytoplasm but not in the nucleus as we hypothesized. Our previous hypothesis was based on the previously reported notion that FRK exerts a nuclear localization signal (Cance *et al.*, 1994).

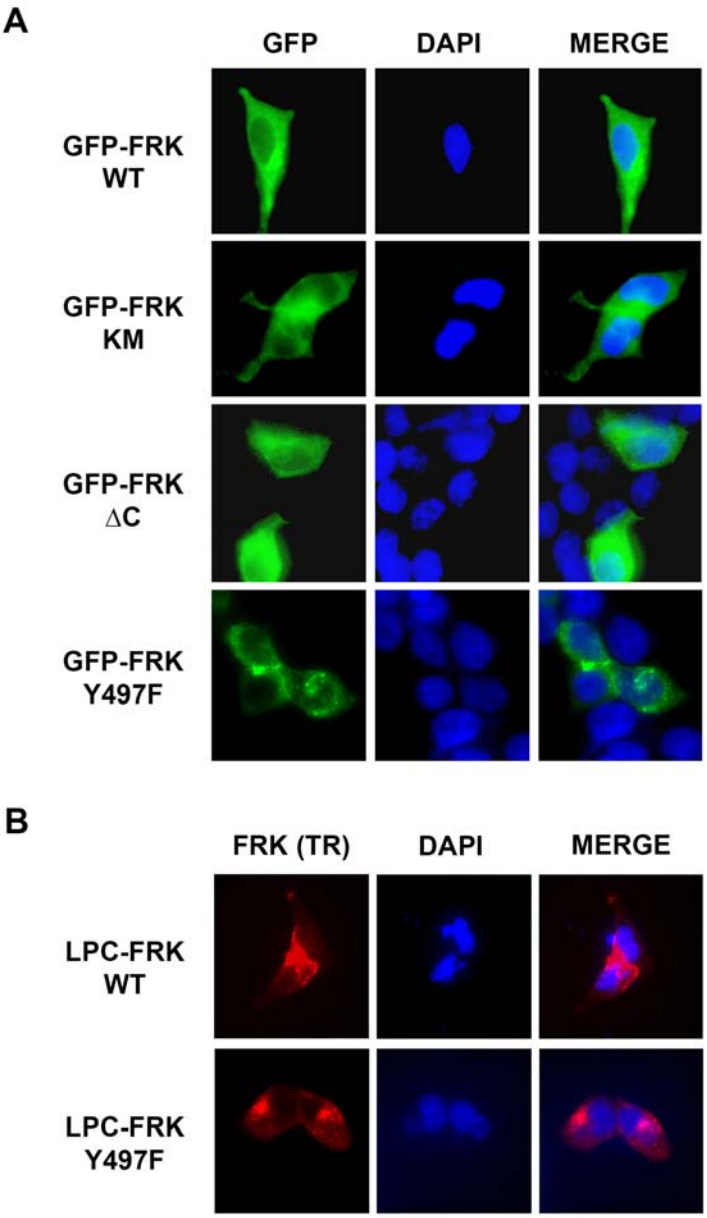


Figure 4.5. Exogenously expressed FRK variants localize in the cytoplasm in HEK 293 cells. (A) HEK 293 cells were transfected with GFP-FRK constructs (WT, K262M, ΔC (497-505), Y497F). GFP expression was visualized by immunofluorescence microscopy. The cells were also stained with DAPI to visualize the nucleus (blue). (B) HEK 293 cells were transfected with LPC-FRK constructs (WT, Y497F). FRK expression was reflected by using mouse anti-FRK antibody (red). The nucleus localization was shown by DAPI staining (blue).

4.4 Characterization of the FRK-WT-MDA-MB-231 and FRK-Y497F-MDA-MB-231 stable cell lines

Plasmid constructs encoding either the FRK wild type (WT) or mutant (Y497F) protein were stably transfected into MDA-MB-231 breast cancer cells. The MDA-MB-231 cell line was selected because they are highly tumorigenic and have low endogenous FRK expression levels (**Section 4.1**). The colonies of the MDA-MB-231-FRK cell lines (WT-1, 2 and 3; Y497F-1 and 2) that were selected for evaluation expressed higher levels of either the wild-type or mutant FRK protein compared to the endogenous levels in the parental MDA-MB-231 cells (Control). Clones WT3 and YF1 were selected for use in the subsequent experiments and were referred to as FRK-WT and YF (**Figure 4.6**).

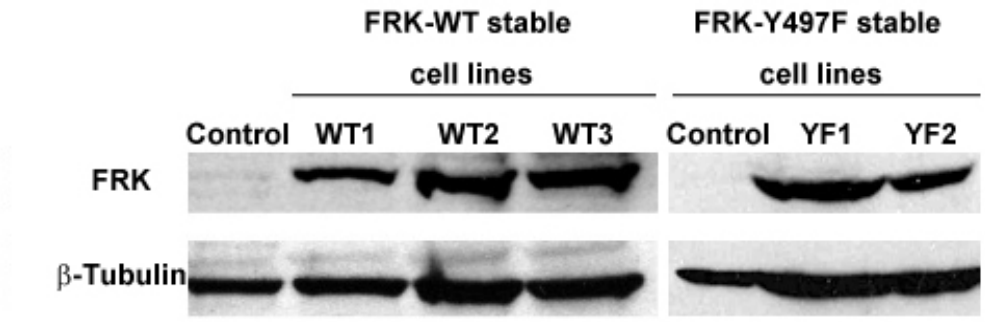


Figure 4.6. Both wild-type and constitutively active cell lines stably over-express FRK. FRK-WT and FRK-Y497F were both generated in MDA-MB-231 cell line. Western blot analysis was used to validate the expression of FRK (FRK-WT and FRK-Y497F). Cell lysates of the selected stable cell lines were immunoblotted using anti-FRK antibody. β-Tubulin was served as a loading control. Untransfected MDA-MB-231 cell line was used as control.

4.5 Morphology of MDA-MB-231 FRK stable cells

In a previous study, overexpression of FRK in the MCF7 breast cancer cells was reported to induce a cellular morphological change from a rounded to a stellate appearance (Yim *et al.,* 2009). In order to determine whether the introduction of FRK would affect MDA-MB-231 cell morphology, we examined MDA-MB-231-FRK stable cells expressing either WT

or Y497F proteins for morphological differences. Cells cultured in 10 cm culture dishes were photographed at 40× magnification. The MDA-MB-231 cells that stably express either the WT or Y497F FRK proteins appeared to be more rounded compared to the stellate (spindle shaped) control cells (**Figure 4.7**). Both MDA-MB-231 cells that express stable levels of either the FRK-WT or FRK-Y497F appeared larger in size as compared to the control parental MDA-MB-231 breast cancer cell lines (**Figure 4.7**).

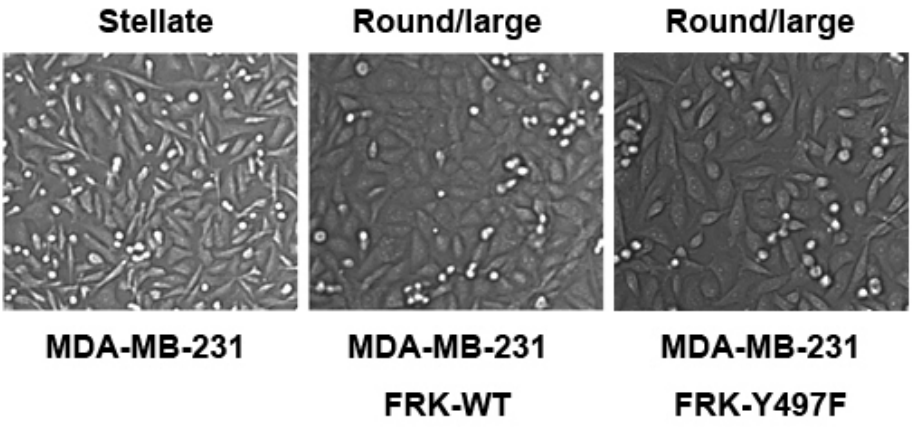


Figure 4.7. Morphologic difference between FRK-overexpressing MDA-MB-231 stable cells and control cells. FRK-negative MDA-MB-231 cells were transfected with the retroviral viruses expressing FRK-WT and FRK-Y497F. Cultures were photographed at inverted microscope with a 40X phase contrast objective.

4.6 Effects of FRK on MDA-MB-231 cell properties

FRK was previously reported as a candidate tumor suppressor gene in breast cancer (Yim *et al.,* 2009). Our goal was to evaluate the effects of FRK on the phenotypic characteristics of MDA-MB-231 breast cancer cells and also look at how constitutive activation of FRK induced by the mutation Y497F affected oncogenic processes such as cell proliferation, migration, invasion and colony formation.

4.6.1 Proliferation of FRK expressing MDA-MB-231 cells

The proliferation rate of the MDA-MB-231-FRK stable cell lines was measured using an MTT assay. The MTT assays are a measure of the mitochondrial activity, which is a direct

reflection of the number of viable cells and cell vitality (metabolic activity). Introduction of FRK (WT and Y497F) in MDA-MB-231 cells significantly diminished cell proliferation rate and metabolic activity as compared to the parental of MDA-MB-231 cells, 4 days after seeding the stable cell lines in a 96 wells plate ($P < 0.005$) (**Figure 4.8**). After 4 days in culture, the fold increase in MDA-MB-231, MDA-MB-231-FRK-WT and MDA-MB-231-Y497F was 11, 8, and 7-fold, respectively (4 days, $P < 0.005$). FRK-Y497F had a greater suppressive effect on cell proliferation than FRK-WT ($P < 0.005$).

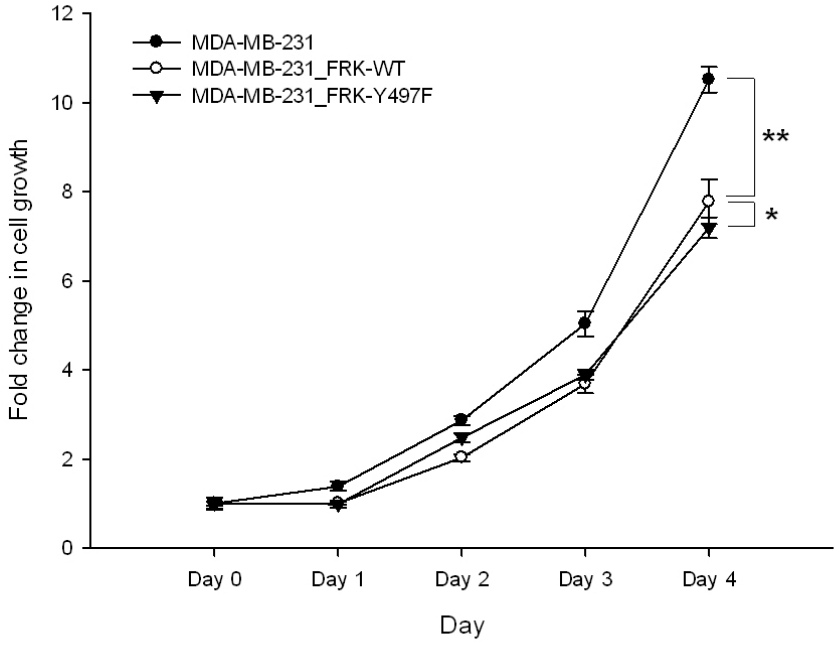


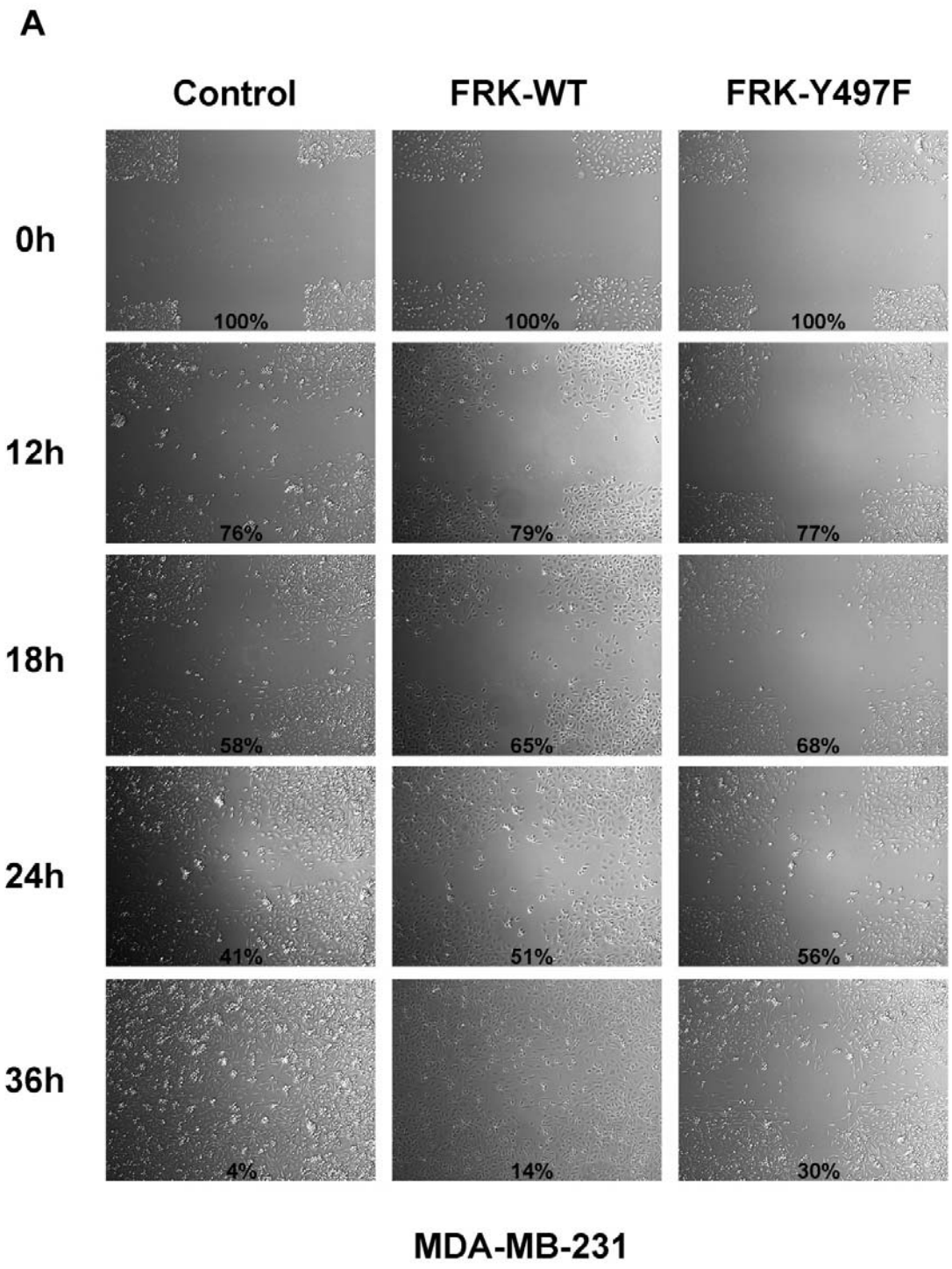
Figure 4.8. FRK-Y497F variant significantly decreases the proliferation of MDA-MB-231 breast cancer cells compared FRK-WT. An MTT assay was used to measure the proliferation rate of MDA-MB-231 cells that stably expressed either FRK-WT or FRK-Y497F; controls were devoid of FRK expression. Cell density at day 1, 2, 3, 4 after plating were measured by absorbance at 450 nm. For each cell line, fold change in growth was calculated by normalization to the corresponding absorbance at day 0. After 96 hours the cell proliferation was lower in cells that expressed FRK (WT or Y497F) as compared to the control cells. Data are represented as the mean ± S.D. and based on at least three independent experiments (n=6). * $P < 0.05$, ** $P < 0.005$.

4.6.2 Migration of FRK expressing MDA-MB-231 cells

A wound-healing assay was used to investigate the migratory effects of MDA-MB-231. When the different MDA-MB-231 cell lines attained confluency, two scratches that run perpendicular to each other across the diameter of a 10 cm well plate were introduced. As illustrated in **Figure 4.9A**, 36 hours after introduction of the perpendicular scratches in the confluent cells, the percentage of open area in the MDA-MB-231-FRK-WT and FRK-Y497F plated cells dropped to 14%, and 30%, respectively; while, that of the parental MDA-MB-231 was 4%. This implied that both MDA-MB-231-FRK stable cell lines had lower migration rates as compared to parental cell line ($P < 0.05$; **Figure 4.9B, 4.9C**). To ensure the GFP has no effect on cell migration, a cell line stably expressing GFP alone was generated, and the wound-healing assay was conducted. These was no difference in cell migration rates between the parental MDA-MB-231 cell line and the MDA-MB-231-GFP cell line (data not shown). The significant difference between the rates of migration of FRK-WT (65% open area) and FRK-Y497F (68% open area) stable cell lines first appeared 18 hours after wounding ($P < 0.05$; **Figure 4.9B, 4.9C**). This difference then became apparent 36 hours after wound introduction ($P < 0.005$; **Figure 4.9B, 4.9C**). We concluded that FRK-YF enhanced the suppression of cell migration compared to FRK-WT. The migration was closely related to the cellular invasiveness; as such we evaluated the effect of FRK activity on the invasive property of MDA-MB-231 cell line.

4.6.3 Invasiveness of FRK expressing MDA-MB-231 cells

The invasive characteristics of MDA-MB-231 cell lines were investigated using a transwell system that was precoated with gelatin. Three-dimensional gelatin was used as an intermediate for the invasion of the tumor cells from the surface to the inner gel. The gelatin matrix assay provides an *in vitro* system that mimics the metastatic behavior of cancer cells *in vivo*. Over expression of FRK- (WT and Y497F) in MDA-MB-231 significantly repressed the number of cells that traversed the gelatin matrix as compared to parental MDA-MB-231 cells ($P < 0.05$; **Figure 4.10**). The constitutively active FRK-Y497F exerted a greater repression of the MDA-MB-231 invasiveness characteristics than the FRK-WT ($P < 0.05$; **Figure 4.10**). Results from the invasion assay were in agreement with those of the proliferation and migration assay and further confirmed that the activity of FRK significantly repressed the invasive nature of MDA-MB-231 cells.


A
Control
FRK-WT
FRK-Y497F
0h
12h
18h
24h
36h
100%
100%
100%
76%
79%
77%
58%
65%
68%
41%
51%
56%
4%
14%
30%
MDA-MB-231

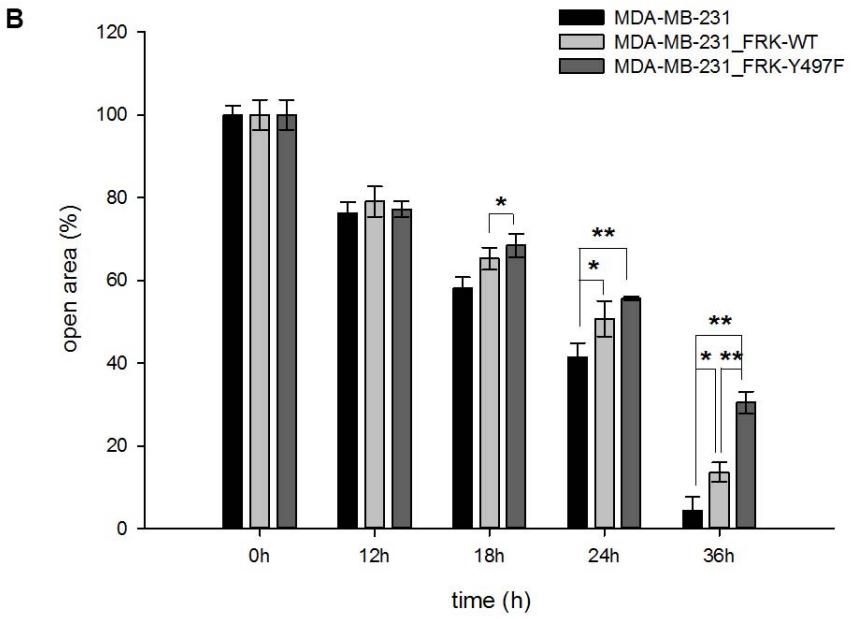


Figure 4.9. Stable expression of FRK-Y497F and FRK-WT suppresses migration of MDA-MB-231 breast cancer cells. (A) The migration of MDA-MB-231 cells that over-expressed FRK-WT and FRK-Y497F was analyzed by using a wound-healing assay. The percentage of wound showing at the bottom of each figure was measured by TScratch software. (B) Cells that expressed FRK (WT and Y497F) had a significantly greater wound area than control from 24 to 36 hours. The average of three independently performed wound-healing assays is shown as a bar chart and a line chart. Data are represented by the mean ± S.D. and based on at least three independent experiments. * $P < 0.05$, ** $P < 0.005$.

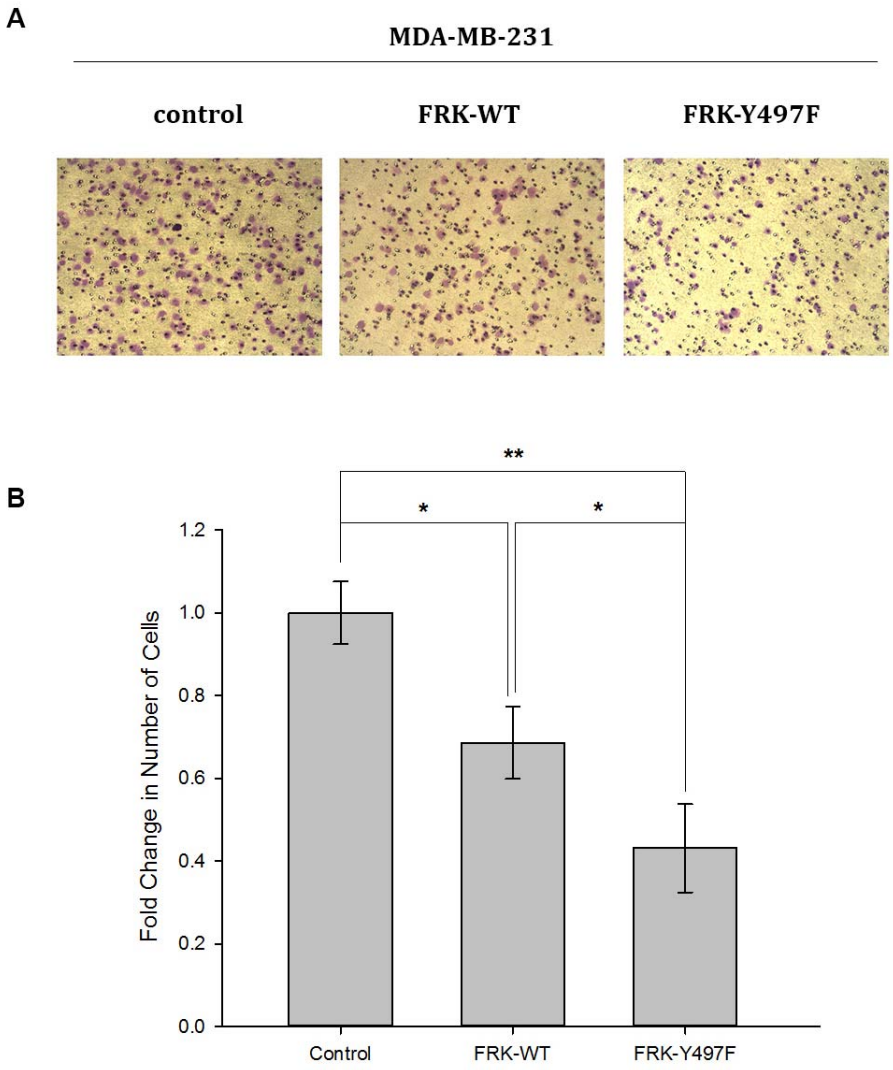


Figure 4.10. Over-expression of FRK-Y497F and FRK-WT significantly suppresses the invasive ability of MDA-MB-231 breast cancer cells. (A) The invasive property of MDA-MB-231 cells that over-expressed either FRK-WT or FRK-Y497F was determined using a transwell assay. Crystal violet staining was used to visualize the invasive cells. The number of cells that migrated across the transwell for each cell line that overexpressed either FRK-WT or FRK-Y497F was normalized to the controls (FRK negative). Cell invasion was significantly suppressed by the over expression of FRK (WT or Y497F). (B) The average of three independently performed transwell assays was shown as a bar chart. Data are represented by the mean ± S.D. and based on three independent experiments per panel. * $P < 0.05$, ** $P < 0.005$.

4.6.4 Colony formation of FRK expressing MDA-MB-231 cells

Anchorage-independent growth has been reported as one of the hallmarks of tumor cell transformation (Hanahan and Weinberg 2000). The effect of FRK activity on the anchorage-independent growth was measured using a soft agar colony formation assay. Exogenous expression of FRK-WT and FRK-Y497F significantly repressed the MDA-MB-231 cell growth in a soft agar gel as compared to that of the control parental MDA-MB-231 cells ($P < 0.005$; **Figure 4.11**). The suppressive function of FRK-Y497F (48 cells/field) was higher than that of FRK-WT ($P < 0.05$; **Figure 4.11**). Interestingly, the colony sizes of parental MDA-MB-231 cells was greater than that of both the MDA-MB-231-FRK-WT and MDA-MB-231-FRK-Y497F cells (**Figure 4.11**). We concluded that the introduction of FRK (WT and Y497F) repressed both cell proliferation and colony formation in the soft agar assays. As well, the constitutively active form FRK-Y497F had a greater repressive effect than FRK-WT on colony formation, consistent with its effect on cell proliferation, migration and invasiveness as reported in the literature.

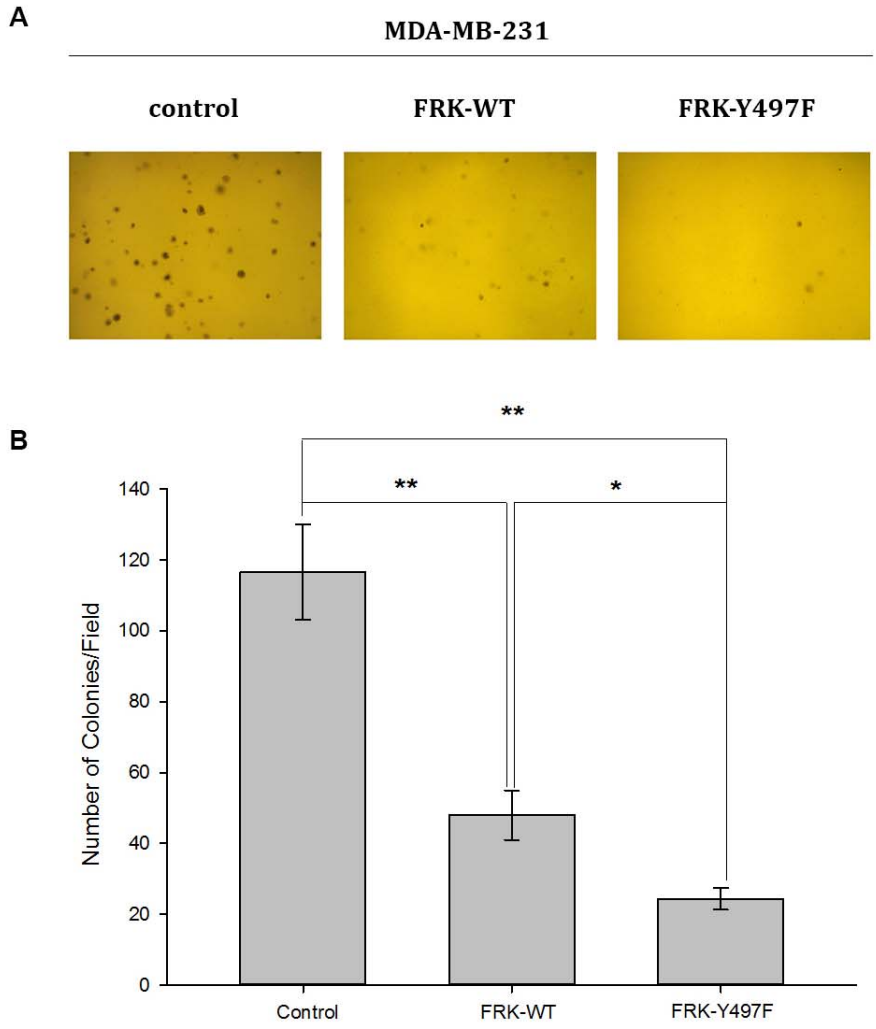


Figure 4.11. FRK significantly suppresses the anchorage-independent growth of MDA-MB-231 breast cancer cells. (A) A soft agar assay was conducted to investigate the role of FRK in the formation of cell colonies. The expression of FRK in MDA-MB-231 cells decreased the number of colonies formed after 21 days in that was evaluated in three randomly selected fields from each plate at magnification of 40X. (B) The average of three independently performed soft agar assay was shown as a bar chart. Data are represented by the mean ± S.D. and based on three independent experiments per panel. * $P < 0.05$, ** $P < 0.005$.

4.7 Identification of FRK-regulated signaling pathways

4.7.1 Kinome analysis

We have shown that FRK indeed has tumor suppressor activity. However, the growth inhibitory mechanism of action of FRK has not been fully defined. The goal of this section of the project was to use kinome analysis to identify the signaling pathways regulated by FRK in order to understand the mechanism by which FRK exerts its tumor suppressive effect on breast cancer cells. This part of the project was done in collaboration with Dr. Scott Napper (Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Canada). Since FRK was reported to be expressed in certain non-cancer cell tissues (Chen *et al.,* 2013), we are interested in the function of FRK in a non-cancer cell line. Besides, HEK 293 is a well-studied cell line and easy to be transfected with. Therefore, we decided to use HEK 293 cells. HEK 293 cells were transiently transfected with a plasmid that encoded the full length FRK wild-type protein (FRK-WT). Cell lysates of transfected and untransfected HEK 293 cells were harvested and used in a kinome array assay (refer to **Section 4.7**). An aliquot of the lysates was incubated on a kinome array composed of 300 peptides spotted in triplicates on slides. The majority of the peptides on the array were derived from signaling intermediates of various signaling pathways including the MAPK and the JAK/STAT cascades (**Figure 4.12**). Besides PTEN which is the only FRK substrate discovered so far, our data indicated that FRK can also potentially phosphorylate several cellular targets (**Figure 4.2; Table 4.1**). Our kinome data showed that phosphorylation levels of proteins involved in the JAK-STAT pathway that included JAK1, BCL2, JNK1, STAT1 and STAT3, were significantly suppressed in the HEK-293 cells transiently transfected with the FRK-WT full length protein as compared to the non-transfected HEK 293 cells (**Table 4.1**). This data was particularly interesting because it was previously shown that FRK inhibited JNK signaling in glioblastoma (Zhou *et al.,* 2012). Since SAPK/JNK belongs to the MAPK signaling cascades, we are also interested in the effects on other cascades, such as p38 MAPK, ERK1/2 (**Figure 2.1**).

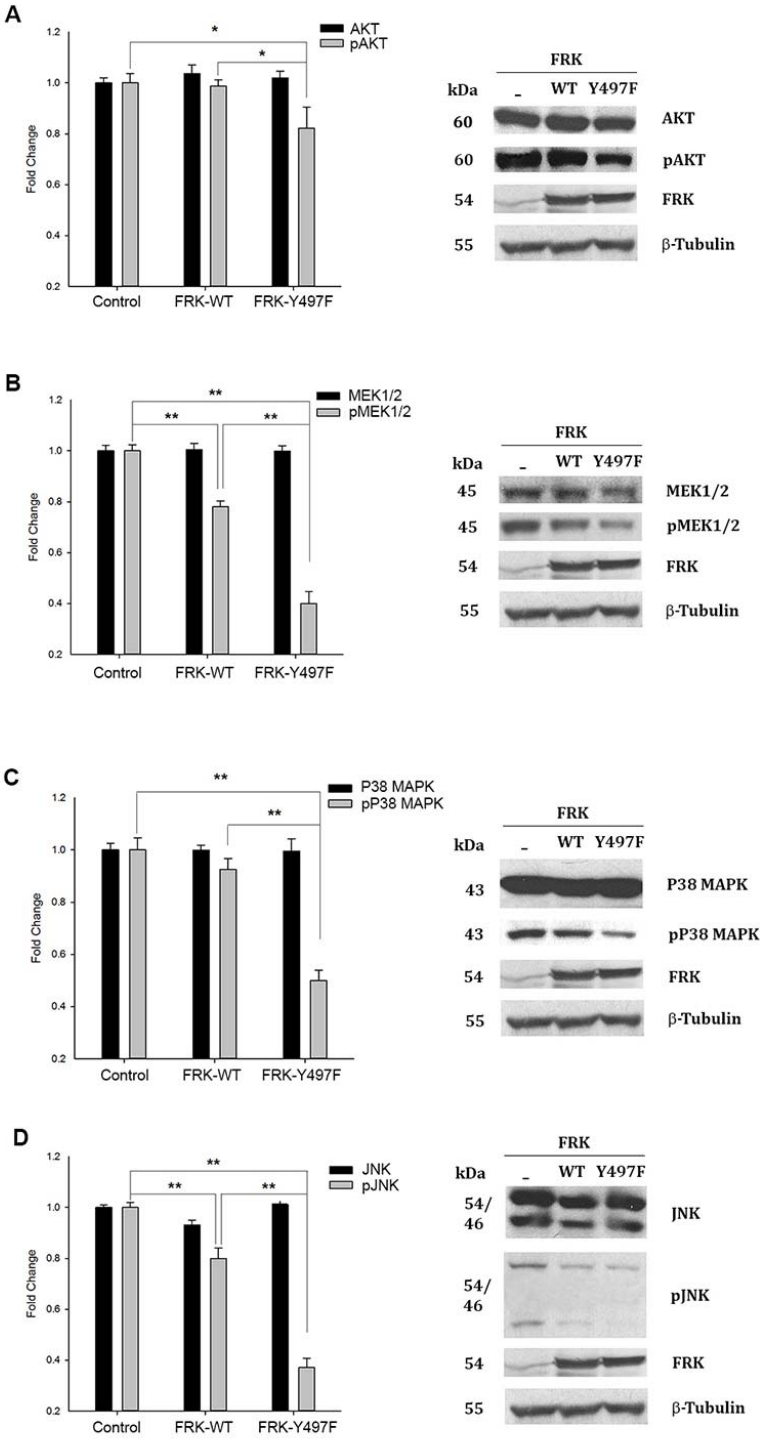
4.7.2 Validation of Kinome data

Using Western blot analysis, we evaluated signaling targets that were either significantly

phosphorylated or de-phosphorylated in the kinome assay following over-expression of FRK in HEK 293 cells ($P < 0.05$; **Table 4.1**). Cell lysates of FRK-WT and FRK-Y497F that were harvested from the MDA-MB-231 stable cell lines and untransduced MDA-MB-231 cells as control were immunoblotted with the antibodies against AKT, phospho-AKT, MEK 1/2, phospho-MEK 1/2, MAPK, phospho-MAPK, P38 MAPK, phospho-P38 MAPK, JNK, phospho-JNK, STAT3, phospho-STAT3, FRK and β-Tubulin as control. The exogenous expression of either the FRK-WT or the FRK-Y497F in the MDA-MB-231 cells repressed the phosphorylation of several signaling intermediates including STAT3 to p-STAT3, JNK to p-JNK, p38 MAPK to pp38 MAPK, AKT to p-AKT and MEK 1/2 to p-MEK 1/2 ($P < 0.005$; **Figure 4.13**). Expression of the FRK-Y497F in MDA-MB-231 cells resulted in a greater repression of the phosphorylation of STAT3, JNK, p38 MAPK, AKT and MEK1/2 as compared to the FRK-WT ($P < 0.05$; **Figure 4.13**). However, we observed a two fold increase in the activation of ERK1/2 following the introduction of FRK-WT and FRK-Y497F in MDA-MB-231 cells ($P < 0.05$; **Figure 4.13**). ERK 1/2 phosphorylation level was greater in MDA-MB-231 cells that expressed FRK-Y497F as compared to cells that expressed FRK-WT ($P < 0.005$; **Figure 4.13**). We concluded that the overexpression of either FRK-WT or Y497F repressed the phosphorylation of STAT3, JNK, AKT, p38 MAPK and MEK 1/2; while it up-regulated the phosphorylation of ERK 1/2.

Phosphorylated Peptides	Dephosphorylated Peptides
CXCR2	JAK1
IFNAR1	BCL2
IL6ST	JNK1
CRK	STAT1
GRB10	STAT3
IRS1	AKT1; CASP3;
SOCS3	CASP8; CHUK;
GRB2	IKBKG;IRAK1;
	MAP3K7; MYD88;
	PIK3R2

Table 4.1. Upregulated and downregulated proteins were detected by kinome array. The targets in red are intermediates in the JAK-STAT pathway. FRK-WT was transiently transfected into HEK 293 cells. Untransfected HEK 293 cells were used as a control. $p<0.05$ was considered as significant.


A
AKT
pAKT
Fold Change
Control
FRK-WT
FRK-Y497F
FRK
kDa
WT
Y497F
60
AKT
60
pAKT
54
FRK
55
β-Tubulin
B
MEK1/2
pMEK1/2
45
MEK1/2
45
pMEK1/2
C
P38 MAPK
pP38 MAPK
43
P38 MAPK
43
pP38 MAPK
D
JNK
pJNK
54/
46
JNK
pJNK

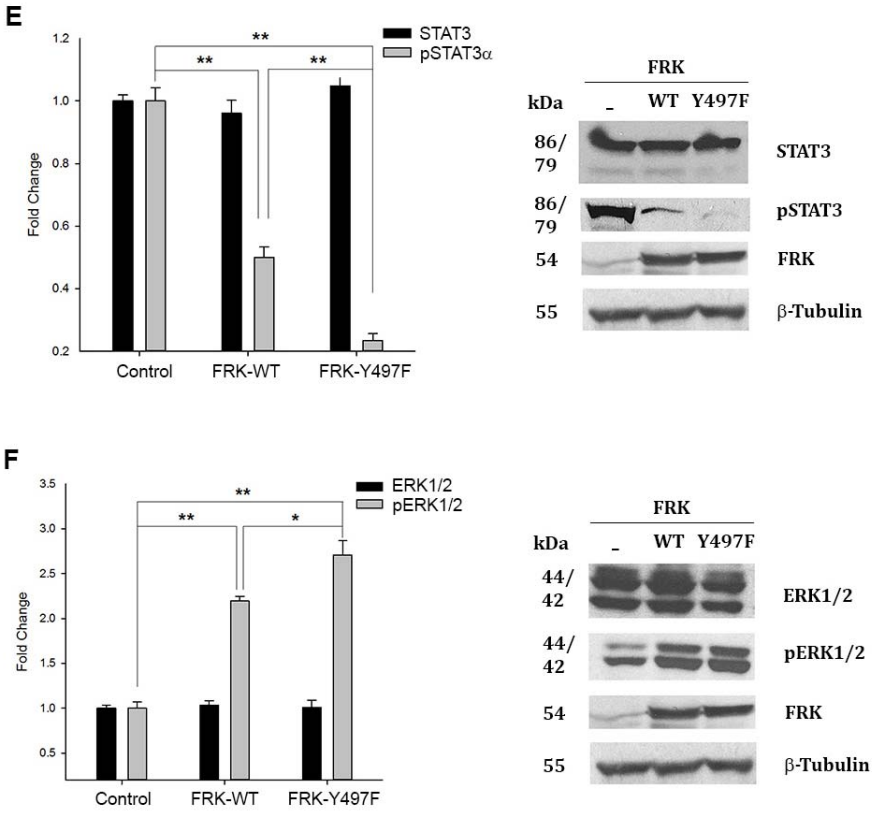


Figure 4.12. FRK suppresses the activation of STAT3. (A) - (F) FRK-negative MDA-MB-231 cells were transduced with the retroviral viruses expressing FRK-WT and FRK-Y497F. Lysates from FRK-WT and FRK-Y497F stable MDA-MB-231 cell lines and control cells were subjected to SDS-PAGE analysis followed by immunoblotting with antibodies against the indicated proteins. The over expression of FRK in MDA-MB-231 cells decreased the phosphorylation of AKT, JNK, MEK1/2, P38 MAPK, STAT-3, and increased the phosphorylation of ERK1/2. Quantification of western blot bands was analyzed by Image J software. Two isoforms were given by one number since their tendency of change was the same. Quantification of each pair of proteins is presented as a bar chart. Data are represented by the mean ± S.D. and based on at least three independent experiments. * $P < 0.05$, ** $P < 0.005$.

5. DISCUSSION AND FUTURE DIRECTIONS

5.1 Discussion and future direction

5.1.1 The expression pattern of FRK in human breast cancer cell lines

In the mammary gland and the human brain, FRK has been shown to be a tumor suppressor (Cance *et al.,* 1994; Yim *et al.,* 2009; Zhou *et al.,* 2012); however, in the colon it promoted tumorigenesis (Cance *et al.,* 1994). In humans, FRK is prevalent in approximately 30% of the tumor samples collected from breast and colon (Cance *et al.,* 1994). It is predominantly expressed in epithelial cells but not the mesenchymal and hematopoetic cells (Cance *et al.,* 1994). However, the profile of FRK expression in breast cancer epithelial cell lines is unknown. In a recent study the expression of FRK was linked to the severity of human brain cancer (Zhou *et al.,* 2012). In that study, it has been shown that the low expression level of FRK in glioblastomas was associated with aggressive tumors; while non-tumorous brain tissue had high FRK expression (Zhou *et al.,* 2012). A correlation between the expression level of FRK and the histological and molecular subtypes of breast cancer has not been yet examined or reported.

In our study, we examined the expression patterns of FRK in a cohort of breast cancer cells derived from the mammary gland epithelium that were categorized into 5 groups based on their molecular subtypes (ER+, ER/PR+, HER2+, triple negative and triple positive) (Rakha *et al.,* 2010). First, FRK was examined in 13 breast cancer cell lines; however, the expression was lowest in triple negative cell lines MDA-MB-231 and HCC1395 (**Figure 4.1**). The MDA-MB-231 cell line is derived from the mammary gland epithelia and was isolated from pleural effusion (Brinkley *et al.,* 1980). Due to its oncogenic properties, rapid growth, metastasis and low endogenous expression of FRK, MDA-MB-231 was selected as a candidate for cellular assays used in this project. Second, Iyk, the mouse FRK ortholog, has been suggested to differ in localization depending on the ratio of estrogen and progesterone levels (Berclaz *et al.,* 2000). We hypothesized that the expression of FRK might be correlated to these hormonal receptors as well. In our data, the expression levels of FRK were high in cell lines (BT474, BT483 and MCF7) that expressed both the estrogen and progesterone receptors (ER/PR positive and triple positive); while, the levels were lower in cell lines that expressed either ER or HER2 alone

(AU565, SK-BR-3, HCC1419, HCC1569, MDA-MB-361 and MDA-MB-134-VI). HER2 has been reported to be overexpressed in more aggressive breast cancer cell lines (Sotiriou and Pusztai 2009). FRK levels have also been shown to be low in high grade brain tumors (Zhou *et al.,* 2012). Compared to the HER2 negative groups (ER+ and ER/PR+), it is obvious that the average expression level of FRK was lower in HER2+ cell lines such as AU565, SK-BR-3 and HCC1569 (**Figure 4.1**), indicating the possible tumor-suppressive function of FRK which is consistent with the previous study. Although the sample size of cell lines was small, our preliminary data still suggested the potential correlation between expression pattern of FRK and the presence of certain receptors in breast cancer cell lines.

Recent data from Dr. Lukong's lab obtained during the preparation of this thesis shows FRK expression in 77.5% of breast cancer cell lines tested (data not shown). In the future study, larger sample size of cell lines and more categories of breast cancer cell lines will be used to acquire the complete FRK expression profile and the association between FRK expressing and molecular subtypes of breast cancer cell lines.

5.1.2 The cytoplasmic localization of FRK in human breast cancer cell lines

One of the goals of the present study was to examine the localization of FRK in FRK-positive breast cancer cells. We also examined the localization of exogenously expressed FRK wild-type and constitutively active variant FRK-Y497F. In our study, using immunofluorescence and subcellular fractionation, endogenous FRK was mostly detected in the cytoplasm and/or cell membrane rather than in the nucleus in breast cancer cell lines SKBR3, MDA468 and AU565 (**Figure 4.3; 4.4**). This is in contrast with Cance *et al.* and Meyer *et al.* studies on COS-7 monkey kidney cells and BT474 human breast cancer cells, where FRK was detected predominantly in the nucleus or perinucleus (Cance *et al.,* 1994; Meyer *et al.,* 2003). It is worth noting that in the first study by Cance *et al.* the resolution of immunofluorescence data was not high and a control for nuclear staining was not shown. FRK has been reported to possess a putative bipartite nuclear localization signal motif buried in the SH2 domain (Chandrasekharan *et al.,* 2002). Glycine 2 in the myristoylation motif which is responsible for the anchorage of the protein to the plasma membrane is absent in human FRK (Chandrasekharan *et al.,* 2002). The known tumor suppressor PTEN,

which has been confirmed as one of substrates of FRK and to be stabilized by FRK, also localized in the cytoplasm instead of nucleus (**Figure 4.4**). It is possible that FRK is sequestered to distinct cytoplasmic compartments by its interacting proteins that may explain why FRK and PTEN colocalize in the cytoplasm. Exogenously expressed FRK displayed the same localization pattern as well (**Figure 4.4**). It is worth mentioning that FRK has also been observed in the nucleus as well as in the cytoplasm in breast cancer cell line BT-20, supporting the possibility of various localization patterns across diverse cell lines (Cance *et al.,* 1994). Interestingly, we also found that the majority of FRK displayed a punctate localization pattern in the cytoplasm. In a recent study FRK was shown to have a punctate cytoplasmic localization in human hepatocellular carcinoma cell line Hep3B (Pilati *et al.,* 2014), and this data is consistent with our results. However, the reason for the punctate cytoplasmic distribution is still unknown.

The localization of the FRK mouse ortholog Iyk has been shown to depend on the hormonal status (Berclaz *et al.,* 2000). Another study on Bsk also reported that the tyrosine 497 and 504 altered the localization of the protein (Oberg-Welsh *et al.,* 1998). Our other goal was therefore to examine whether the activity of FRK affects the subcellular localization of the protein. Our data showed no difference in localization between wild type FRK and constitutively active mutant, suggesting that the enzymatic activity of FRK has no effect on protein localization (**Figure 4.5**).

How the localization of FRK affects its cellular function is still unknown. Studies on BRK have indicated that membrane targeted BRK has oncogenic properties while nuclear targeted BRK displayed tumor suppressive function (Derry *et al.,* 2003). It will be interesting in the future to target FRK to different cellular compartments in FRK-negative cells and examine the effect on cellular processes such as cell proliferation, migration, invasion and colonization.

5.1.3 The tumor suppressive role of FRK in human breast cancer

FRK has been proposed to function as a tumor suppressor in breast cancer via stabilizing the known tumor suppressor PTEN (Yim *et al.,* 2009), which triggered our interest to investigate how the constitutive enzymatic activity of FRK would affect its tumor

suppressive property. In our study, a series of cellular experiments showed that compared to the control group, both wild type and the constitutively active FRK significantly suppressed cell proliferation, migration, invasion and cell formation (**Figure 4.7-4.11**). These *in vitro* data also showed that constitutively active FRK-Y497F inhibited cell growth much more effectively compared to wild-type. Consistent with the role of FRK in inhibiting cell proliferation, the same trend was observed in cell migration, invasion and colony formation studies. FRK has been shown to induce a cell growth suppression by arresting cells in G1 phase in the breast cancer cell line BT474 and MCF7, and human glioma cell lines U251 and U87 (Meyer *et al.,* 2003; Hua *et al.,* 2014). Furthermore, in the U251 and U87 cell lines the authors also found that the G1 arrest was induced by the down-regulation of cyclin D1 accumulation and the up-regulation of pRb activation, decreasing the release of E2F which is responsible for entry into the S phase (Hua *et al.,* 2014). Future experiments examining the effect of FRK variants on cell cycle progression are necessary.

Interestingly, FRK has also been suggested to have oncogenic properties in a few studies. The tumorigenesis of FRK was first raised in 2005. FRK has been found to fuse to the ETS transcription factor ETV6, endowing the fused protein with oncogenic properties (Hosoya *et al.,* 2005). A recent study showed that endogenous FRK has been found expressed in normal lung tissue and liver tumor, suggesting that the cellular function of FRK could be organ-specific (Chen *et al.,* 2013). Interestingly, *Chen et al.* also found the increased cell growth and transformation promoted by FRK has only been found in the HCC cell line Hep3B, but not HepG2, indicating the oncogenic properties could depend on cellular context as well. The following year, this oncogenic function of FRK in the human HCC cell line was confirmed in another published paper (Pilati *et al.,* 2014). This study also showed that FRK was able to induce the tumor growth in human HCC Hep3B cell line (Pilati *et al.,* 2014), supporting the idea that the cellular function of FRK could be tissue-specific. The Hep3B and HepG2 are both derived from human hepatocellular carcinoma. A few speculations were made to address the varying oncogenic properties in these two cell lines upon FRK overexpression. Hep3B is generally a more malignant cell line compared to HepG2 which could imply that the Hep3B cell line is more prone to displaying an oncogenic potential (Chen *et al.,* 2013). A second reasoning may be attributed to the presence of Rb and p53 which are known tumor suppressors. These

proteins are not expressed in Hep3B but are expressed in HepG2 (Singer *et al.,* 2007; Chen *et al.,* 2013). Thus, it is possible that the proliferation-inducing potential of FRK in HepG2 could be suppressed by p53 and Rb. Therefore, in order to investigate the tissue-specific role of FRK, cells derived from different organs may be evaluated for FRK-mediated tumor-suppressive or pro-oncogenic function. Additionally, a correlation between the expression pattern of FRK and p53/pRb could be examined in these tissues/cells.

In addition to the effects on the cellular function, a morphological change from rounded to stellate shape due to the presence of FRK has also been observed in the MCF7 breast cancer cell line (Yim *et al.,* 2009). Parental MDA-MB-231 cells displayed a spindle-shaped morphology (Kenny *et al.,* 2007). We found that MDA-MB-231 cells stably expressing FRK-WT and FRK-Y497F were more rounded in shape. It is possible that this morphological change may reverse the epithelial-mesenchymal transition (EMT). EMT is a characteristic of tumor invasiveness in which epithelial cells acquire fibroblast-like properties, exhibit reduced cell-cell contacts and increased motility. EMT is essentially a tumor cell transformation from an epithelial to a mesenchymal-like condition in which a "cadherin switch" occurs (Steinestel *et al.,* 2014). This however has to be confirmed in future studies, by examining the expression of the mesenchymal markers such as N-cadherin, vimentin, fibronectin as well as the expression levels of "EMT master genes" such as *Snail*, *Slug* and *Twist* family (Thiery *et al.,* 2009; Wendt *et al.,* 2014), and epithelial markers such as E-cadherin, claudin and occludin in the FRK stable cell lines (Thiery *et al.,* 2009). IL-6 stimulation has been reported to upregulate the expression levels of twist and snail, which are EMT-related gene targets in human breast, neck and head tumors (Sullivan *et al.,* 2009; Yadav *et al.,* 2011). Furthermore, the IL-6-induced EMT has been found to be dependent on STAT3 through the JAK-STAT pathway (Yadav *et al.,* 2011). Therefore, the IL-6→STAT3→twist signaling axis has been postulated in breast cancer (Wendt *et al.,* 2014). During the preparation of the present thesis, our lab confirmed that IL-6-induced STAT3 activation is repressed by FRK, suggesting that the FRK-induced suppression of tumor cell proliferation via the JAK-STAT pathway may be one of its mechanisms of action. Thus, the IL-6→STAT3→twist/snail axis needs to be investigated in future studies, by examining the expression of Twist and Snail genes in the presence or absence of FRK.

5.1.4 The regulatory role of FRK in signaling transduction in human breast cancer

A variety of important biological processes including cell proliferation, migration and invasion in breast cancer have been tightly controlled by certain signaling pathways, such as MAPK and JAK-STAT kinase cascades. As one of the main pathways in the MAPK signaling transductions, nuclear signaling by JNKs have been involved in the regulation of cell death and differentiation (Chen 2012).

PTEN, a known tumor suppressor, has been identified as a substrate of FRK recently (Yim *et al.,* 2009). However, other substrates of FRK and the role of FRK tumor suppressor function have not been fully understood. One of the aims of my study was therefore to identify new FRK-regulated proteins using a Kinome assay.

In our study, we observed that the activation levels of several signaling intermediates including AKT, JNK, MEK 1/2, p38 MAPK and STAT3 were decreased when either FRK-WT or FRK-Y497F were overexpressed, compared to those in the FRK negative group (**Figure 4.13**). Our data suggested that FRK suppressed the cell growth potentially via the inhibition of MAPK, AKT and JAK/STAT signaling pathways. pSTAT3, a marker for activation of JAK/STAT signaling, was significantly repressed via JAK-STAT pathway. We consistently observed that elevated FRK activity enhanced its anti-cancer property possibly via the down-regulation of certain signaling pathways. Intriguingly, phosphorylation of ERK 1/2, a downstream target of MEK 1/2, was decreased in the presence of FRK-WT and FRK-Y497F. It is important to note that a similar observation was reported in a previous study in which murine FRK-transgenic mice demonstrated higher phosphorylation level of ERK 1/2 but lower level of phosphorylated p38 in islet cells compared to control islets (Anneren and Welsh 2001). This is in contradiction with the growth promoting role of pERK 1/2 (Cheng *et al.,* 2013). ERK 1/2 has also been shown to negatively regulate the activation of STAT3 in human hepatoma cell line HepG2 (Tian and An 2004). In that study, the suppression of pERK 1/2 allowed the increase of the phosphorylation level of STAT3 via serine 727 (Tian and An 2004). In the same cell line, serine 727 has also been reported to reduce the activity of STAT3 via dephosphorylating the tyrosine 705 of STAT3 (Wakahara *et al.,* 2012). Based on these findings, we hypothesized that FRK may play an important role in regulating the signaling

pathways through the repression of the activity of JNK, p38, AKT and STAT3 respectively, as well as through a bypass mechanism that promotes ERK 1/2 activation and consequently suppresses phospho-STAT3 (Tian and An 2004) (**Figure 5.1**). This may explain why the phosphorylation levels of STAT3 in FRK-WT (0.20-fold, $p < 0.005$) and FRK-Y497F (0.04-fold, $p < 0.005$) are both significantly less compared to the FRK negative control.

It is worth mentioning that although the activity of MEK 1/2 decreased, an unexpected increase in the phosphorylation level has been observed in one of its downstream substrates, ERK 1/2, suggesting that besides MEK 1/2, ERK 1/2 may be regulated by multiple upstream kinases simultaneously (**Figure 5.1**). It has been previously reported that ERK 1/2 can be upregulated by MEK 1/2, but downregulated by MEKK1 and p38 MAPK in neural systems (Lu and Xu 2006), indicating the decrease in the activity of p38 MAPK and other potential regulators could affect the phosphorylation level of ERK 1/2.

Focal adhesion and integrin clustering have also been demonstrated to induce certain regulatory signaling pathways including cell migration, survival and differentiation (Hynes 2002; Mitra *et al.,* 2005). Focal adhesion kinase (FAK) has been known to participate in the integrin-associated signaling. The activation of FAK at tyrosine 397 provides the docking site for Src, CSK, GRB7, SOCS and PI3K (Mitra *et al.,* 2005). A subsequent Src-induced tyrosine phosphorylation of FAK at Y925 creates a binding site for GRB2, linking the integrins to the MAPK signaling (Mitra *et al.,* 2005). It will be interesting to examine whether the integrin-signaling and activity of FAK are affected by the presence of FRK.

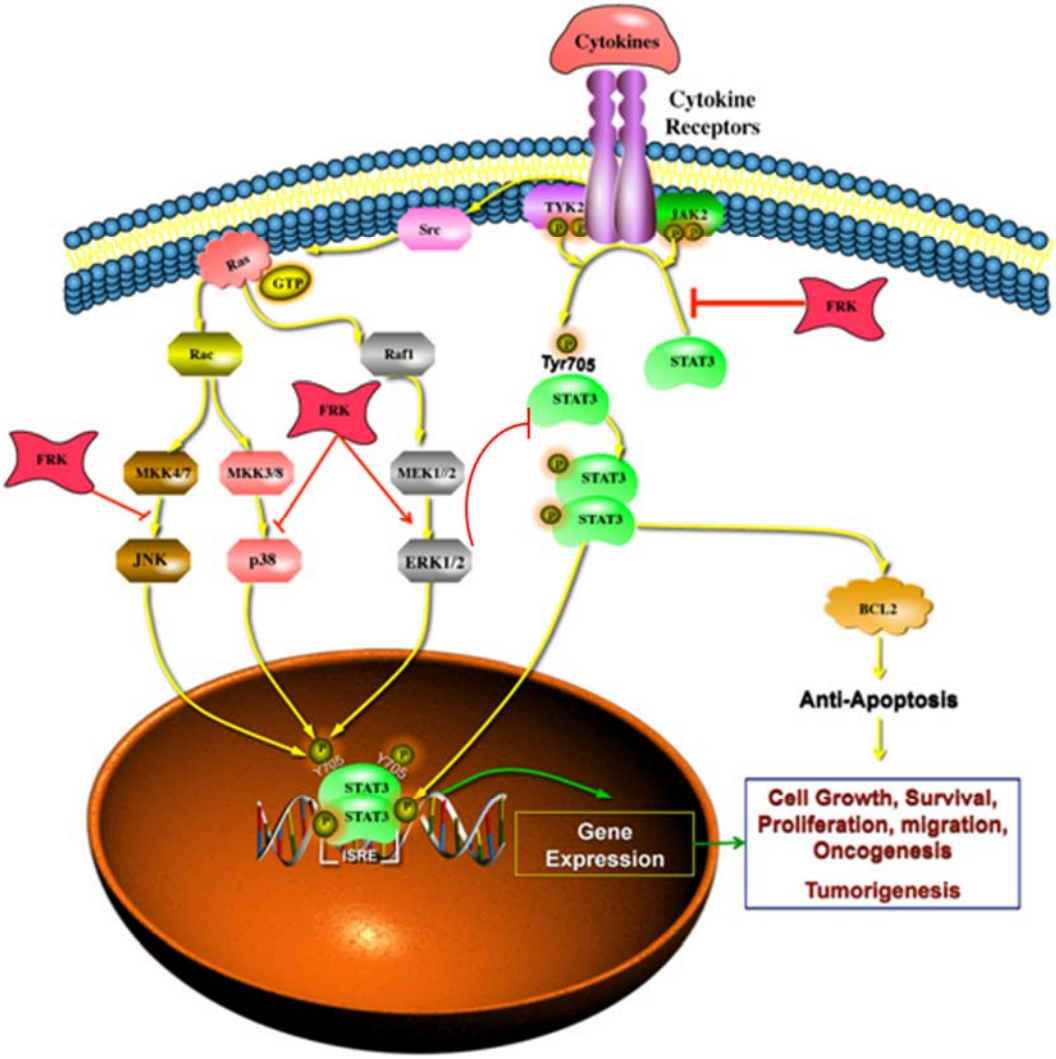


Figure 5.1. FRK inhibits the MAPK and JAK-STAT signaling pathway. Cytokine receptor stimulation results in the phosphorylation of STAT3 at tyrosine 705, which induces dimerization, followed by nuclear translocation and activation of specific target genes containing the ISRE (IFN-stimulated Response Element). Many STAT3 target genes are key components of the regulation of cell cycle progression from G1 to S phase. Other pathways such as MAPK (Mitogen Activated Protein Kinase), p38 MAPK, and MEK (MAPK/ERK Kinase) signaling cascades also lead to phosphorylation and activation of STAT3. We have shown here that FRK strongly inhibits activation of MAPKs (JNK and p38) and STAT3, but promotes activation of ERK 1/2. Figure is modified from http://www.sabiosciences.com/pathway.php?sn=STAT3_Pathway.

5.2 Conclusion and perspectives

FRK is a candidate tumor suppressor whose expression and localization pattern as well as its mechanisms of action in mammary epithelial cancer cells have not been fully understood. The present study was aimed at determining the expression and localization of FRK in breast cancer cells as well as the effects of stable overexpression of FRK on breast cancer cell proliferation, migration, invasion and colonization. We also aimed at identifying FRK-modulated signaling pathways via kinome analysis. A few significant conclusions can be draw from our data. First, both endogenous and exogenous FRK localize mainly in the cytoplasm although FRK has been reported as a nuclear tyrosine kinase in other studies. However, it is possible the subcellular localization may vary in different cell lines and FRK may be either an oncogene or tumor suppressor depending on tissue context. Second, compared to wild-type FRK, the constitutively active variant FRK-Y497F significantly enhances its tumor suppressive activity as determined by suppressed proliferation, migration, invasion, and transformation of MDA-MB-231 breast cancer cell line. Third, FRK may inhibit these biological processes via the regulation of several signaling intermediates including MEK 1/2, ERK 1/2, AKT, p38 MAPK, JNK, and STAT3. Finally, the JAK-STAT3 signaling, an important signaling pathway known to regulate cell proliferation, is significantly inhibited by the presence of FRK. Both Ras-Rac and Ras-Raf1 induced MAPK signaling pathways are affected by the activation of FRK as determined by its inhibition of the activation of their corresponding signaling intermediates. From our data we conclude that targeted over-expression of FRK could be a viable therapeutic strategy in breast cancer.

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